AMELIORATION OF SALT TOLERANCE IN SOYBEAN (*Glycine max.* L) BY PLANT-GROWTH PROMOTING ENDOPHYTIC BACTERIA PRODUCE 1-AMINOCYCLOPROPANE-1-CARBOXYLASE DEAMINASE

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Abstract

Salinity is a major abiotic stress that can induce ethylene synthesis beyond the normal limits as plants response to stress and hence reduces crop productivity. The 1-aminocyclopropane-1-carboxylase deaminase (ACCD)-producing bacteria can reduce excessive ethylene synthesis by taking ACC (ethylene precursor) as a nitrogen source. This study showed the possibility of using endophytic bacteria in order to reduce the undesirable effects of salinity. Strain *Pseudomonas putida* PIR3C and *Roultella terrigena* PCM8 exhibited promising performance for promoting the growth of plant under salinity stress conditions. The results showed that bacterial inoculation was effective even in the presence of higher salinity levels. Strain *P. putida* PIR3C was the most efficient strain compared to the other strains and significantly increased shoot length, root length, dry weight, germination percentage, and reduced stem diameter. The role of ACCD in reducing ethylene production under salinity stress conditions was also studied by measuring the evolution of ethylene in vitro by soybean seeds treated with some ACCD bacterial strain. The maximum ethylene lowering capacity was observed in *R. terrigena* PCM8, the strain reduced ethylene production from 622.81 nmol.g^{-1}(control) to 352.78 nmol.g^{-1} (43% reduction). The production of α-ketobutyrate, chlorophyll content and germination percentage from *P. putida* PIR3C was higher than other strains. The results suggested that strain *P. putida* PIR3C and *R. terrigena* PCM8 can be employed for salinity tolerance in soybean seedlings and may have better prospects for an amelioration of stress condition.

Keywords: Salinity, ACC-deaminase, Ethylene, α-ketobutyrate, Soybean.

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Introduction

Soil salinity is a major abiotic stress and an enormous problem for irrigated agriculture because salt turns agronomically useful lands into unproductive areas. The deleterious consequences of high salt concentration affect plant health including the increasing of root ethylene synthesis, increasing the negative osmotic water pressure on plant cells, reducing the availability of nutrients in the soil, and imbalance in nutrient uptake (Mayak *et al*., 2004).

Plants respond to abiotic and biotic stressors by modulating the level of various hormones which in turn induce expression of stress-related proteins required for protection from the deleterious e□ects of stressors. Ethylene is one of the most common plant hormones that mediate the response to the stressors (Glick *et al.*, 2007).

Ethylene is an important growth hormone produced by almost all the plants, which mediates a wide range of plant responses (Arshad & Frankenberger, 2002; Belimov *et al*., 2002). The production of ethylene is tightly regulated by internal signals during development and in response to environmental stimuli from biotic and abiotic stresses. It has a profound influence on the

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growth and development of plants. Ethylene plays a significant role in seed germination, tissue differentiation, flowering initiation, fruit ripening, lowering senescence, root initiation, stem and root growth, and leaf and fruit abscission (Abeles *et al*., 1992).

Although ethylene plays a prominent role in the normal growth of plants, ethylene can have a negative impact on seed germination, shoot and root growth, and other growth parameters when it is produced more than its threshold level (Mattoo and Suttle, 1991). E□ect of stress ethylene in plants can be reduced by certain plant-associated bacteria that possess an enzyme ACCD (Glick, 2007).

Microbial such as plant growth promoting bacteria (PGPB) that secrete 1-aminocyclopropane 1-carboxylase deaminase (ACCD) restrict ethylene biosynthesis in plants. The enzyme converts ACC, the precursor of ethylene to ammonia and α-ketobutyrate. This means that more ACC has drawn away from the ethylene synthesis pathway, and that quantities of ACC become lower to convert into ethylene (Glick, 2014).

Many studies have shown enhanced stress tolerance and growth promotion in plants conferred by bacteria producing ACCD (Glick *et al*., 2007). Recently, the use of endophytes producing ACCD has become a promising alternative to alleviate plant stress caused salinity (Jha *et al.*, 2012; Rashid *et al.*, 2012). For example, the inoculation of cucumber and canola with *Pseudomonas putida* UW4 has been found to enhance plant growth under saline conditions (Cheng *et al.*, 2007; Gamalero *et al.*, 2010).

Endophytic bacteria are species that inhabit within plant tissues for at least one period of their life cycle without showing disease symptoms to its host and survive by forming a symbiotic relationship with the host plant (Strobel and Daisy, 2003). Endophytic bacteria are important for the growth and the developmental processes of plants, as well as when plants experience biotic and abiotic stresses including salinity. Under saline conditions, some endophytic microorganisms ameliorate the stress in plants by synthesizing osmoprotectant molecules such as proline and/or trehalose, quaternary ammonium compounds in the cytoplasm, volatile organic molecules, exopolysaccharides (Yaish *et al.*, 2015), and producing ACCD. Production of ACCD is likely an important and efficient way for endophytes to manipulate their plants' host. Little information is available about the potential of endophyte salt-tolerant bacteria producing ACCD that are isolated from the root tissue of *Oryza sativa*, *Solanum tubesorum* L. and *Theobroma cacao* L. under stressed conditions. We initiated studies to identify their potential at amelioration the growth of salt-stressed soybean (*Glycine max.* L).

In our earlier study, we have reported the isolation and identification of the ACCD endophytic bacteria from the root tissue of *O. sativa*, *S. tubesorum* L. and *T. cacao* L.. In addition, the present study is conducted to identify some of the mechanisms that endophytic bacteria producing ACCD may use in facilitating soybean (*Glycine max* L.) growth under salt-stressed conditions.

Soybean is important fodder crops worldwide, but their production has been severely reduced in recent decades due to soil salinity because this grain legume is highly sensitive to salinity (Shereen and Ansari, 2001). In addition, Soybean is considered to be a model legume plant for molecular research, similar to *Lotus japonicus* as a model plant for legume family (Dam *et al.*, 2009). For this reason, soybean was chosen for the current study into the impact of soil salinity on endophytes community richness in soybean.

Materials and Methods

Bacterial Strains

All of the bacterial strains that used in this research are ACCD producing bacteria isolated from Special Region of Yogyakarta. The bacteria are *Pseudomonas putida* PIR 3C*, Pseudomonas monteilii* KS 12, *Raoultella terrigena* PCM 8, and *Pantoea agglomerans* KD6.2. We use *Pseudomonas putida* UW4 as positive control strain (source: Glick *et al.*). The bacteria are sub cultured on Nutrient Agar medium.

Bacterial Culture for Inoculant Preparation

Bacterial strains are grown in *erlenmeyer* flask containing 100ml TSB (Tryptic Soy Broth) and then incubated at 28 ± 1°C for 24

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h under shaking (100 $r.min^{-1}$) conditions. After incubation, optical density is measured, moreover, the uniform population (OD_{600} = 0.5; 10^7-10^8 $cfu.mL^{-1}$) has been achieved by dilution prior to seed inoculation.

In Vitro Assay on Phytotron

One hundred seeds of soybean var. Gema was surface sterilized with 96% ethanol for 1 minute, NaOCl for 3 minutes and finally rinses with sterile distilled water ten times. The sterilized seeds were treated with *P. putida* PIR3C, *P. monteilii* KS12, *R. terrigena* PCM8, *P. agglomerans* KD6.2, and *P. putida* UW4 inoculation which were imbibed in bacterial suspension ($1x10^8$ $CFU.seed^{-1}$) for 2 h, and an excess suspension was drained. The bacterized seeds were placed in the pre-sterilized petridish containing a piece of filter paper Whatman no. 40 with an addition of 10 ml of sterile deionized water. After 4 days, the bacterized sprouts with the same size were placed in the pre-sterilized 25 ml flasks containing a piece of filter paper with the addition of 2 ml of sterile sodium chloride (NaCl) solution with concentrations 0; 58; 122 mM respectively. Three bacterized sprouts were placed on each flask with three replications of each treatment of bacterial inoculation. Seeds that were imbibed in deionized water and were not treated with bacterial inoculation and NaCl solution served as a negative control (KO), and the flasks were closed tightly with a parafilm seal to prevent the escape of the ethylene. Flasks were incubated in the growth chamber at 28°C, and 12 h of light alternated with 12 h of darkness (Govindasamy *et al*., 2009).

Gas Chromatographic Measurement of Plant Ethylene Production

After 48 h of addition of NaCl, the ethylene in the headspace was sampled and measured by gas chromatography against a reference compound of ethylene. The columns are alumina (1 m x 3 mm) with the temperature at 100□C, injector temperature 200-220□C, and the carrier is nitrogen (N_2) gas, pressure 0.5 kg. cm^{-2} and a detector are FID (Flame Ionization Detector). Ethylene produced was expressed as *nmoles* per gram dry weight of seedlings (Govindasamy *et al.*, 2009).

Alpha-Ketobutyrate of Soybean Sprout Assay

The soybean sprouts that harvested from phytotron are washed with sterile distilled water (48 h after addition of NaCl). The sprouts are grinded using a mortar and the products are extracted by ethanol as a solvent. Moreover, the crude extract is placed in the new 1.5ml tube and centrifuge 8000 rpm for 5 minutes. Two hundred and twenty microliters supernatant have been transferred into the test tube and incubated at 30°C for 15 minutes. The pellet cell is collected and dried in the oven at 60°C until the weight is stable (dried weight). As the first incubation, the supernatant is added with 1 ml HCl 0.56 M, vortex, and centrifuge 13.000 rpm for 5 minutes. One milliliters supernatant is added with 800 µl HCl 0.56 M and 300 µl dNPH (0.2% in 2M HCl), vortex and incubated at 30°C for 30 minutes. After conducting the second incubation, the supernatant is added with 2 ml NaOH 2N. The absorbance of the solution by adding of NaOH measured with spectrometer λ540 nm (Glick and Penrose, 2003).

Germinating Seed Bioassay

For evaluating the effect of ACCD producing bacteria on the germinating of soybean seeds in the presence of different salinity levels, we first selected uniformly-sized soybean seeds showing no signs of damage. The bacterial isolates were grown in Tryptic Soy Agar (TSA) medium at ± 28°C for 24h. The inoculants for treating seeds were prepared by suspending cells from agar plates in a TSB medium. The sterilized seeds were imbibed in bacterial suspension ($1x10^8$ $CFU.seed^{-1}$) for 2 h, and an excess suspension was drained. Seeds both imbibing in medium and were not treated with bacterial inoculation were served as a control (KO) and sown under the same conditions as the inoculated seeds. The bacterized seeds and control were placed in the pre-sterilized *petridish* containing a piece of filter paper with the addition of 10 ml of sterile deionized water. One hundred pre-germinated seeds for each treatment were used and incubated in the growth chamber at ±28°C, and 12 h of light alternated with 12 h of darkness. Each treatment of bacterial strain inoculation performed with three replications. The seed was considered germinated when the

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radicle emerged through the seed coat. Germination count was taken daily for 14 days (Nadeem *et al.,* 2013). Germination percentage was calculated according to the formula described by Maguire (1962):

$$Germination\ percentage = \frac{number\ of\ germinated\ seeds}{number\ of\ seeds\ used\ in\ the\ assay}\ x\ 100$$

Classical Triple Response Bioassay

Classical triple response bioassay is a useful marker to study the concentration-dependent effect of stress-induced ethylene on seedling growth (Shaharona *et al*., 2006). This bioassay was used to study the effect of microbial inoculation for reduction of the inhibitory effect of ethylene on seedling growth.

In order to study classical triple response, three bacterized soybean sprouts grown in the pre-sterilized 25 ml flasks containing a piece of filter paper were exposed to 2 ml of 0; 58; and 122 mM sterile NaCl solution as salinity levels, and placed at ±28°C, initially for 12 h followed by light and dark cycle in a growth chamber. Each treatment of bacterial strain inoculation performed with three replications. Data regarding shoot length, root length, dry weight, and stem diameter were recorded after 6 days since the bacterized sprouts placed in a flask (Shaharona *et al*., 2006).

Pigment Extraction

The leaf neither inoculated by selecting the bacteria nor inoculated it in salinity conditions is collected from the same age (6 weeks after planting). For pigment extraction, 50 mg of the fresh material from the middle part of fully developed leaves of each plant was ground using a pestle and extracted with 0.5 ml 90% acetone and 10 µM KOH. Mix the extract very well and incubate on the ice for 20 min. The resulting suspension was centrifuged at 4^{o}C at 13.000 rpm for 10 min. Chlorophyll and carotenoids contents were determined in acetone supernatants according to the method of Lichtenthaler (1987). The absorbance values were determined with a Shimadzu UV-1601 spectrophotometer at 470; 645; and 663 nm which was then used to determine total leaf chlorophyll expressed as mgL^{-1} (El Sabagh *et al.*, 2015).

Statistical Analysis

The data were analyzed by analysis of variance (ANOVA) and if there were significant different the analyses were continued by Duncan's multiple-range test (DMRT) at 95% confidence interval (α = 0.05).

Results

In the previous study, thirteen endophytic bacteria had been isolated from the root tissue of *O. sativa*, *S. tubesorum* L. and *T. cacao* L. grown in stress condition fields. The ACCD activity of these bacteria were assayed. The results showed that the strains varied in their ACCD activity and another growth promotion activity such as phosphate solubilization, production of IAA, and nitrogen fixation.

The selected bacterial strains *P. putida* PIR3C, *P. monteilii* KS12, and *R. terrigena* PCM8 that used in this experiment exhibited the high ACCD activity are as follows: 1461,44; 1290,29; 331,26 nmol α ketobutyrate/mg/h, respectively. Among of them, *R. terrigena* PCM8 showed a high production of IAA (10,33 ppm/h), and *P. monteilii* KS12 showed a high nitrogenase activity (467,84 nmol/g/h). The selected bacterial strains *P. agglomerans* KD6.2 showed a high phosphate solubilization activity (phosphate solubilizing index=3,04). All of four selected bacterial strains were used in this research.

Effect of ACCD Producing Bacterial Strains on Plant Ethylene Production under Salinity Stress

In accordance with this current study, soybean sprouts were planted at 3 NaCl concentrations (0; 58 and 122 mM) and treated with bacterial inoculation.

Ethylene production by total seedling ranged from 401 to 673 nmol.g^{-1} among the treatments were negatively correlated with ACCD activity value of each bacterium. The results showed that ethylene production on the plant can decrease if the plant was inoculated with ACCD producing bacteria isolates especially strain *R. terrigena* PCM8. The maximum ethylene lowering capacity was observed in *R. terrigena* PCM 8; the isolates showed ~43% reduction (from the negative

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control) which was statistically comparable with the reference strain *P. putida* UW 4 as a positive control. *P. putida* PIR 3C showed ~32% reduction in ethylene production while *P. agglomerans* KD 6.2 showed a minimum reduction (~17%) in ethylene production (Table 1).

Table 1. Effect of selected bacterial isolates on ethylene production of soybean seedlings under salinity stress

No.	Bacterial strain	Concentration of NaCl (mM)	Ethylene production (nmol.g^{-1} dw of total seedling	Reduction in ethylene production (%)
1	KO	0	397.03 ± 11.8^{abc}	0
		58	622.81 ± 19.75^{ab}	0
		122	673.22 ± 7.82^{a}	0
2	UW 4	58	402.98 ± 26.24^{g}	35.21 ± 5.51^{bc}
		122	401.15 ± 20.48^{g}	40.41 ± 3.0^{ab}
3	PIR 3C	58	419.61 ± 41.54^{fg}	32.59 ± 6.92^{bcd}
		122	455.94 ± 32.35^{ef}	32.31 ± 4.05^{bcd}
4	KS 12	58	467.04 ± 8.7^{e}	24.93 ± 3.67^{de}
		122	476.53 ± 33.42^{de}	29.2 ± 5.25^{cd}
5	PCM 8	58	352.78 ± 18.92^{h}	43.36 ± 2.29^{a}
		122	455.4 ± 30.42^{ef}	32.32 ± 5.33^{bcd}
6	KD 6.2	58	514.5 ± 25.68^{cd}	17.35 ± 3.14^{e}
		122	550.66 ± 55.24^{c}	18.23 ± 3.3^{e}

Note: Values are the means of three replications ± standard deviation. Values with the same superscripts within column indicate no significant difference with P ≥ 0.05. (KO = un-inoculated bacteria, UW 4 = inoculated with *P. putida* UW4, PIR3C = *P. putida* PIR3C, PCM8 = *R. terrigena* PCM8, KD6.2 = *P. agglomerans* KD 6.2)

The ethylene production increased with the increasing of NaCl concentration or salinity level (Table.1). It was revealed that the salinity stress affects the increase of ethylene plant production. Treatment with 122 mM NaCl concentration gave the significant ethylene production in all bacterial inoculation treatment except *R. terrigena* PCM8.

The levels of ethylene produced by un-inoculated seedlings (KO/negative control) in no salinity stress (0 mM NaCl) was lower than in salinity stress condition (58 and 122 mM), therefore, the reduction of ethylene levels was due to the salinity treatment in un-inoculated seedlings. On the other hand, the level of ethylene produced by seedlings inoculated with a specific bacterial strain in salinity stress was lower than un-inoculated seedlings (KO/negative control). It reveals that the reduction of ethylene levels was due to the bacterial inoculation.

The Role of ACCD Producing Bacteria on The α-Ketobutyrate Level of Salinity Stressed Soybean Seedlings

In this study, α-ketobutyrate production correlated with the reduction of ethylene production. The seedlings treated with bacterial strains *R. terrigena* PCM8, *P. putida* PIR3C, and *P. monteilii* KS12 had a high reduction in ethylene production which were similar to α-ketobutyrate production (Fig 1).

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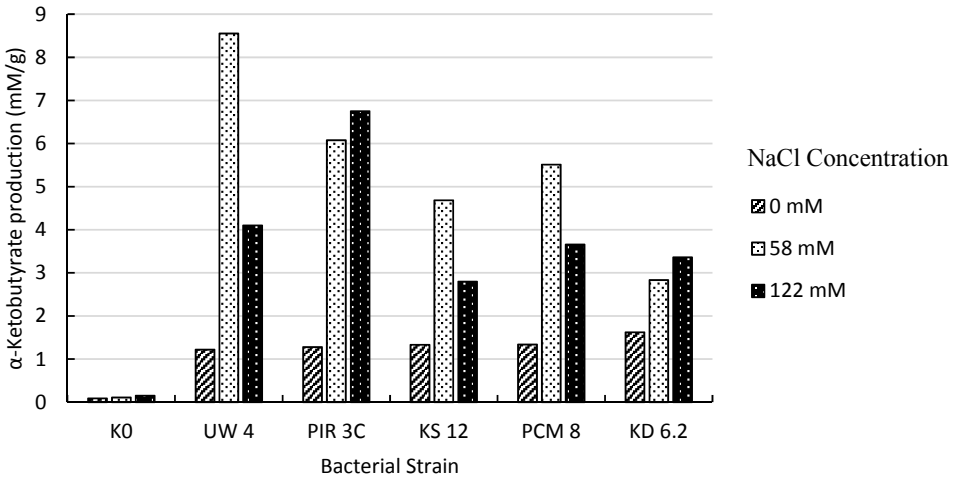

Figure 1. Effect of ACCD producing bacterial strains inoculation and two salinity stress condition (58 and 122 mM NaCl) on α-ketobutyrate production. (KO = un-inoculated bacteria, UW 4 = inoculated with *P. putida* UW4, PIR3C = *P. putida* PIR3C, PCM8 = *R. terrigena* PCM8, KD6.2 = *P. agglomerans* KD 6.2)

The positive control of *P. putida* UW4 produced high level of α-ketobutyrate at 58 mM NaCl concentration. The high α-ketobutyrate production at 122 mM NaCl concentration was produced by *P. putida* PIR 3C.

The α-ketobutyrate production of *P. putida* PIR3C and *P. aglomerans* KD6.2 increased with the increasing of NaCl concentration. *P. putida* UW4, *P. monteilii* KS 12, and *R. terrigena* PCM 8 were maximally produced α-ketobutyrate at 58 mM NaCl concentration and decreased at 122mM NaCl concentration.

Effect of ACCD Producing Bacterial Strains on Germination Percentage of Salinity Stressed Soybean Seedlings

Inoculating seeds with strain *R. terrigena* PCM 8 and *P. putida* PIR 3C showed the improvement of germination compared to the un-inoculated seeds. At low salinity levels, the effect of inoculation with the selected bacteria was statistically similar (Table 2). At 58 mM NaCl concentration, seeds inoculated with strain *R. terrigena* PCM 8 showed the maximum germination percentage, which was 5.4% higher than un-inoculated control, followed by seeds inoculated with strains *P. putida* PIR 3C and *P. putida* UW 4. At the highest salinity level (122 mM), seeds inoculated with *R terrigena* PCM 8 showed the maximum germination percentage (21% higher than the control), however the germination of these seeds was statistically similar to seeds that was inoculated with the other strains.

Table 2. Effect of selected bacteria inoculation on germination rate of soybean under different salinity levels

No	Bacterial Strain	NaCl Concentration (mM)		
		0	58	122
1	Control (KO)	96.7^{ab}	93.3^{abc}	71.7^{gs}
2	UW 4	98.3^{ab}	95^{ab}	81.7^{ef}
3	PIR 3C	100^{a}	96.7^{ab}	85^{de}
4	KS 12	96.7^{ab}	93.3^{abc}	80^{ef}
5	PCM 8	100^{a}	98.3^{ab}	86.7^{cde}
6	KD 6.2	95^{ab}	90^{bcd}	76.7^{fg}

Note: Values are the means of three replications. Values with the same superscripts within column indicate no significant difference with $P \geq 0.05$. (KO = un-inoculated bacteria, UW 4 = inoculated with *P. putida* UW4, PIR3C = *P. putida* PIR3C, PCM8 = *R. terrigena* PCM8, KD6.2 = *P. agglomerans* KD 6.2)

Classical Triple Response of Salinity Stressed Soybean Seedlings

This experiment was conducted to screen the most efficient endophytic bacteria producing ACCD for its growth promoting activity under salt-stressed conditions. The result showed that the shoot and root lengths

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decreased with increasing the levels of salinity, except the treatment with *P. putida* UW4 inoculation (Fig. 2 and Fig. 3). The treatment with 122mM NaCl concentration showed a maximum reduction in all observation variables but it varied in inoculation treatment.

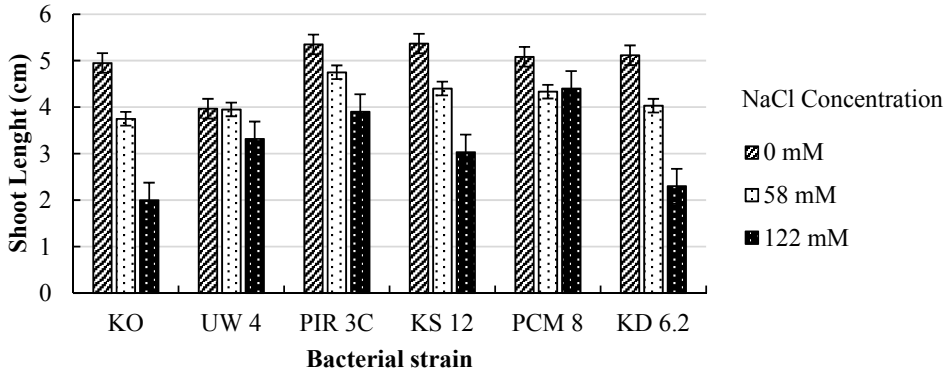

Figure 2. Classical triple response in soybean due to salinity; effect of selected bacteria inoculation and three salinity stress condition (0; 58; 122 mM NaCl) on shoot length of soybean sprouts. (KO = un-inoculated bacteria, UW 4 = inoculated with *P. putida* UW4, PIR3C = *P. putida* PIR3C, PCM8 = *R. terrigena* PCM8, KD6.2 = *P. agglomerans* KD 6.2).

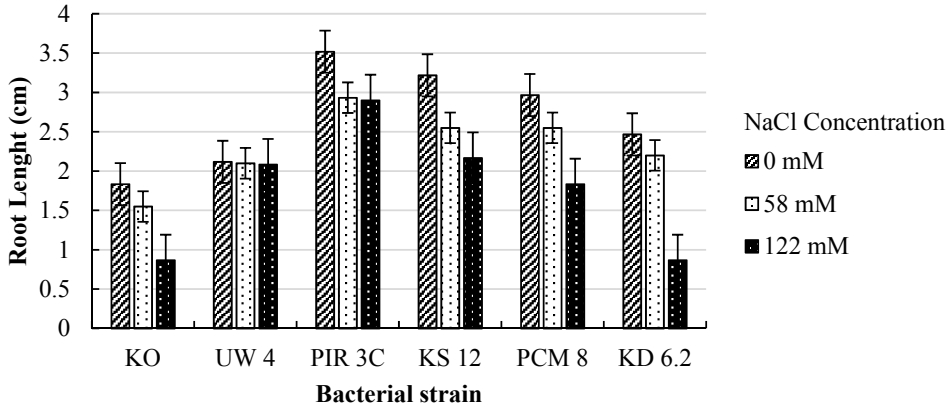

Figure 3. Classical triple response in soybean due to salinity; effect of selected bacteria inoculation and three salinity stress condition (0; 58; 122 mM NaCl) on root length of soybean sprouts. (KO = un-inoculated bacteria, UW 4 = inoculated with *P. putida* UW4, PIR3C = *P. putida* PIR3C, PCM8 = *R. terrigena* PCM8, KD6.2 = *P. agglomerans* KD 6.2).

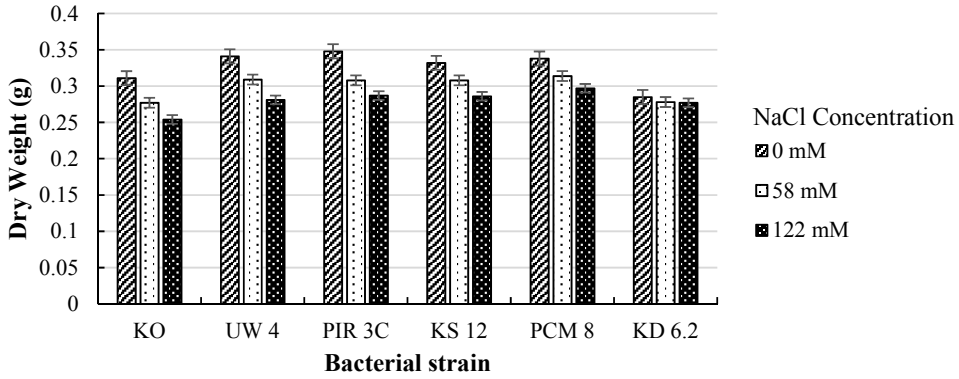

Figure 4. Classical triple response in soybean due to salinity; effect of selected bacteria inoculation and three salinity stress condition (0; 58; 122 mM NaCl) on dry weight of soybean sprouts. (KO = un-inoculated bacteria, UW 4 = inoculated with *P. putida* UW4, PIR3C = *P. putida* PIR3C, PCM8 = *R. terrigena* PCM8, KD6.2 = *P. agglomerans* KD 6.2).

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Inoculation with *P. putida* PIR 3C, *P. monteilii* KS 12 and *R. terrigena* PCM 8 resulted in the highest dry weight, shoot and root length (Fig 2, Fig. 3, and Fig. 4). These bacteria enhanced the shoot and the root length. They were also positively affected the ability of soybean plant growth under the stress conditions.

In Fig. 5, the seedlings show a response similar to the classical triple response. The seedlings are treated with bacterial strains *R. terrigena* PCM 8, *P. putida* PIR 3C, and *P. monteilii* KS 12 having a high reduction in ethylene production. High ethylene producing seedlings has thick hypocotyl and results in a reduction in seedling length, with swelling of the stem. The effect is more pronounced at a high NaCl concentration, 122mM. Inoculation with *R. terrigena* PCM 8 is more effective in decreasing stem diameter than the other strains.

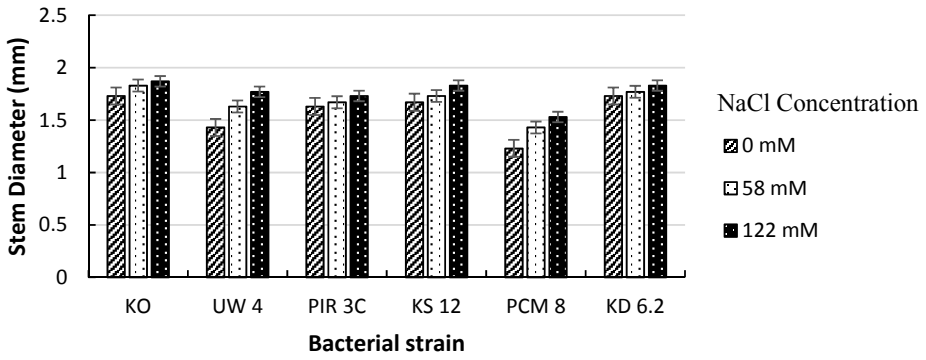

Figure 5. Classical triple response in soybean due to salinity; effect of selected bacteria inoculation and three salinity stress condition (0; 58; 122 mM NaCl) on stem diameter of soybean sprouts. (KO = un-inoculated bacteria, UW 4 = inoculated with *P. putida* UW4, PIR3C = *P. putida* PIR3C, PCM8 = *R. terrigena* PCM8, KD6.2 = *P. agglomerans* KD 6.2).

Table 3. The effect of bacterial inoculation and three levels salinity on the chlorophyll a, b, and total carotenoids (µg.ml^{-1}) content of soybean leaves

No.	Bacterial strains	NaCl concentrations (mM)	Concentration (µg/ml)		
			Chlorophyll a	Chlorophyll b	Carotenoids
1	KO	0	1.15^{cdefg}	1.05^{ab}	0.43^{efg}
		58	0.82^{efg}	0.57^{cd}	0.39^{fg}
		122	0.67^{g}	0.27^{d}	0.36^{g}
2	UW 4	0	1.26^{cdef}	0.52^{cd}	0.6^{abcde}
		58	1.26^{cdef}	0.5^{cd}	0.58^{bcdef}
		122	0.92^{defg}	0.44^{cd}	0.49^{cdefg}
3	PIR3C	0	1.88^{a}	0.88^{abc}	0.79^{a}
		58	1.36^{bcd}	0.66^{bcd}	0.71^{ab}
		122	1.15^{cdefg}	0.51^{cd}	0.58^{bcdef}
4	KS 12	0	1.68^{abc}	0.89^{abc}	0.66^{abc}
		58	1.2^{cdefg}	0.55^{cd}	0.62^{abcd}
		122	1.17^{cdefg}	0.5^{cd}	0.55^{bcdef}
5	PCM 8	0	1.82^{ab}	1.14^{a}	0.68^{abc}
		58	1.45^{abcd}	0.57^{cd}	0.56^{bcdef}
		122	1.18^{cdefg}	0.5^{cd}	0.5^{cdefg}
6	KD 6.2	0	1.25^{cdef}	0.76^{abc}	0.43^{defg}
		58	1.29^{cde}	0.52^{cd}	0.44^{defg}
		122	0.74^{fg}	0.25^{d}	0.33^{g}

Note: Values are the means of three replications. Values with the same superscripts within column indicate no significant difference with P ≥ 0.05. (KO = un-inoculated bacteria, UW 4 = inoculated with *P. putida* UW4, PIR3C = *P. putida* PIR3C, PCM8 = *R. terrigena* PCM8, KD6.2 = *P. agglomerans* KD 6.2)

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Effect of Salt Stress on Pigment Content of Leaf

The chlorophyll b content significantly decreased at high NaCl concentration-122 mM. Carotenoids content in soybean leaves did not significantly decrease by salinity level (Table 3). Treatment with bacterial inoculation showed a significantly different effect. Inoculation with *P. putida* PIR 3C and *R. terrigena* PCM 8 resulted in the highest chlorophyll a, b and carotenoids content. Inoculation of *P. putida* PIR 3C and *R. terrigena* PCM 8 bacteria were positively affected the ability of soybean plant growth under stress conditions.

Discussion

Effect of ACCD Producing Bacterial Strains on Plant Ethylene Production under Salinity Stress

The ethylene production increased with the increasing of NaCl concentration or salinity level (Table 1). This corresponds to the opinion of Govindasamy *et al*., 2009 who stated that stress in any form (including salinity) induced ethylene production in plants. Based on the results, *R. terrigena* PCM 8 showed the maximum reduction in ethylene production. This ACCD producing bacterial strains might have acted as a sink for the ACC and lowereds the level of stress ethylene synthesis in the treated seedlings. The high ACCD activity resulted in ACC will be more converted to α ketobutyrate than ethylene. *R. terrigena* PCM 8 has an ability in terms of the high of IAA producing. Both IAA production and ACCD activity might be acted synergistically in reducing the stress ethylene levels. Bacterial production of IAA is known to have an effect on plant ethylene production and root elongation. Bacterial IAA has been taken up by a plant and induces the activity of the enzyme ACC synthase, causing an increase in ACC. Consequently, ACC exudations are proportional to ACC production. It is suggested that increased levels of ACC exudations are facilitated by colonization of ACCD-producing bacteria and its utilization results in reduced ACC and ethylene concentrations (Glick *et al.*, 2007). *R. terrigena* PCM 8 with both IAA and ACCD producing activity will control the excess of ethylene production level and thereby lessen the ethylene feedback inhibition of IAA biosynthesis (Glick, 2014).

Strain *P. putida* PIR 3C, an ACCD activity producer, also gave maximum reduction in ethylene production. It was due to ACCD from these bacteria converted the ACC form plant, hence the ethylene production decreased. *P. agglomerans* KD 6.2 showed the minimum reduction in ethylene production because this strain has ACCD activity and IAA production lower than other selected bacteria. According to Govindasamy *et al.,* (2009), if bacteria with ACCD enzyme were presenting in the vicinity of the growing seedlings, the enzyme would cleave ACC and converted it into α-ketobutyrate and ammonium thereby preventing the oxidase conversion of ACC to ethylene. Hence, ethylene production will be much low in such plants.

The results of the current study clearly established the role of ACCD enzyme in limiting the amount of stress ethylene synthesis and were in agreement with the findings of earlier workers who demonstrated reduced ethylene production in plants treated with PGPR producing ACCD activity under heavy metal, flooding and salt stress conditions (Belimov *et al.*, 2001; Grichko and Glick, 2001; Nadeem *et al.*, 2010). The isolates with ACCD activity obtained in the present study may be used as bacterial inoculum for the improvement of the plant growth, particularly under unfavorable environmental conditions.

The Role of ACCD Producing Bacteria on The α-Ketobutyrate Level of Salinity Stressed Soybean Seedlings

The seedlings treated with *P. putida* PIR 3C and *R. terrigena* PCM 8 showed a high α-ketobutyrate production. Alpha ketobutyrate is one of the ACC deamination products. The α-ketobutyrate production increased with the increasing of ACCD activity value. The strains can significantly decrease the plant-ethylene production and it means that the more ACC is converted to *α-ketobutyrate* than ethylene formation, thereby producing a high level of α-ketobutyrate within the bacteria.

Strain *P. agglomerans* KD 6.2 showed a minimum α-ketobutyrate production. It might be due to the low availability of ACC

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substrate in the immediate vicinity of their roots and the activity of ACCD in bacteria which was lower than the other strains, thereby the level of α-ketobutyrate production within the bacteria was lower than the other strain.

The level of α-ketobutyrate produced by bacteria in soybean sprouts untreated with salinity stress showed that the α-ketobutyrate was a product of ACCD producing bacteria utilising indigenous ACC of sprouts as the source of N and ACC, converting it to α-ketobutyrate and ammonium. The α-ketobutyrate production is categorized as a low level because the amounts of ACC did not increase in soybean sprouts untreated with any salinity stress.

The α-ketobutyrate production usually increases with the increasing NaCl concentrations as higher NaCl concentrations can result in higher levels of ACC as a precursor ethylene. ACC will be converted to α-ketobutyrate by ACCD producing bacteria thereby increasing the level of α-ketobutyrate production (Glick, 2007). However, a different outcome was found with *P. putida* UW4, *P. monteilii* KS 12, and *R. terrigena* PCM 8 strains. Their α-ketobutyrate production peaked at 58 mM NaCl concentration and decreased at 122mM NaCl concentration. This may be due to the bacteria not fully utilizing the amount of ACC produced by the sprouts in a high NaCl concentration (122mM).

Effect of ACCD Producing Bacterial Strains on Germination Percentage of Salinity Stressed Soybean Seedlings

Higher salinity levels influence the ethylene production and affect the germination of both inoculated and un-inoculated seeds significantly. The increasing of salinity levels leaded to decreasing seed germination (Table 2). Several hypotheses have been proposed to explain the mechanisms of ethylene action in germinating seeds (Matto and Suttle, 1991; Matilla and Vazquez, 2008). Their results suggested that the ethylene produced in response to ROS regulates the length of the embryonic axis by increasing the size of root tip cells without increasing their number, and thereby regulated soybean seed germination.

The inoculation of soybean seeds with ACCD producing bacteria (*R. terrigena* PCM 8, *P. putida* PIR 3C and *P. monteilii* KS 12) did not only improve the growth of sprout soybean but also enhance seed germination under salt-affected conditions. The enhanced germination may be due to their ability in order to reduce the ACC produced during stress and, therefore, reduce the elevated ethylene level around the seeds (Nadeem *et al.*, 2013).

These results indicate that bacterial inoculation has improved the growth of soybean seedling (except strain *P. agglomerans* KD6.2) under salinity condition, as evidenced by the significant increase in the germination rate in the soybean seeds. Rueda-Puente *et al*., (2007) suggested that the possible role for growth enhancement under saline conditions is plant growth-promoting substances of bacteria.

Classical Triple Response of Salinity Stressed Soybean Seedlings

Inoculation with ACCD producing bacteria has increased the seedling length (except *P. putida* UW4), root elongation, and decreased stem (hypocotyl) diameter (except *P. agglomerans* KD 6.2) as compared with un-inoculated seed (KO). The results were in conformity with the findings that seed and/or root inoculation with certain bacteria decreases the endogenous ethylene levels. Furthermore, it also promotes the root growth through ACCD activity under gnotobiotic conditions (Belimov *et al*., 2002; Shaharoona *et al*., 2006a). The finding of this study showed that bacteria with more ACCD activity have more ability to decrease the intensity of ACC-induced classical “triple” response further confirms the premises that ACCD activity of bacteria which is responsible for decreasing endogenous ACC in inoculated plants.

The seedlings growth is normally limited by increasing concentration of NaCl. The concentration of NaCl as salinity stress has a significant effect on the height of soybean sprouts. The plants growing in salinity conditions will be dwarfed and stems tend to be thick by increasing salinity concentration. It is due to the cell propagation to the side which is more stimulated. The changes in cell shape are caused by the orientation of

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cellulose microfibrils that are deposited into the cell wall, more in the longitudinal direction, thus inhibiting the distribution that is parallel to the microfibril and only allows spreading to occur perpendicular to the microfibril (Salisbury and Ross, 1995).

The result showed that the shoot and the root lengths decreased with the increasing levels of salinity, except the treatment with *P. putida* UW4 inoculation (Fig. 2 and Fig. 3). It is due to the salinity stress affects the germination by preventing water and also introducing toxic ions into embryos or seedlings. The salinity stress also result in high ethylene production seedling. The research of Kasotia *et al.,* (2012) emphasized that the experimental watering of 50 ml 200 mM NaCl salt solution has shown a significantly reduced the emergence of soybean plants and in all observation variables such as root length, lateral root, shoot length, fresh and dry weight when salt was applied at the time of seeding. However, the percentage of emergence and all observation variables were significantly increased upon treatment with the ACCD producing bacteria.

Strain *P. putida* PIR 3C and *P. monteilii* KS 12 are more effective in increasing shoot and root length. It might be due to a high ACCD activity. The bacteria producing ACCD might be caused deamination of ACC by the enzyme leading to lowering of plant ethylene level and thereby facilitating the formation of longer roots (Glick, 2007).

Strain *R. terrigena* PCM 8 is more effective in decreasing stem diameter which might be due to variation in other traits of bacteria such as IAA production, nitrogen fixation, and root colonization that is additionally to ACCD activity (Shaharoona *et al*., 2006a).

The inoculation selected bacteria producing ACCD by *R. terrigena* PCM8, *P. putida* PIR3C and *P. monteilii* KS 12 on soybean seedlings effective to suppression endogenous production of ethylene and decreased the ethylene-imposed classical triple response in soybean seedlings, therefore, the plant seedling growth is improved.

Effect of Salt Stress and Bacterial Inoculation on Pigment Content

Data presented in Table 3 showed that salinity stress decreased the chlorophyll a, b and carotenoids content in the leaves of soybean seedling. Similar studies were also carried out by Kumari *et al.,* (2015) that the chlorophyll content (chlorophyll a, b and total) of soybean plants without PGPB inoculation was generally reduced under high salinity. This is due to NaCl concentration in the plant. The NaCl observed to be toxic to soybean and Na^+ and Cl^- are known to cause injuries to plant leaves (Kozlowski, 1997). Lower chlorophyll content would limit photosynthetic potential and lead to a decrease in biomass and production then inhibit the plant growth (Naumann *et al.,* 2008).

The high NaCl concentrations (122 mM) resulted in a significant decrease the chlorophyll b. These results indicate that NaCl stress has more effects on chlorophyll b than chlorophyll a (Houimli *et al.*, 2010).

Carotenoids are important pigments that can also act as antioxidants, protecting plasma membrane lipids from oxidative stress generated in plants exposed to salinity (Falk & Munné-Bosch, 2010). The data from the present study show that salinity does not significantly decrease carotenoids content.

The decrease in the pigment content recorded in the present study is related to the negative effect of salt stress on chloroplast and increased activity of chlorophyll degrading enzymes chlorophyllase through quenching of singlet oxygen. Stomatal limitation causes the reduction of CO_2 assimilation rate thus inhibits both the synthesis and activity of photosynthetic pigments (Debez *et al.,* 2008). The decrease in carotenoid due to salinity leads to degradation of β-carotene and formation of zeaxanthins, which are obviously involved in protection against photo inhibition (Sultana *et al.*, 1999).

The chlorophyll content (chlorophyll a, b and total) of soybean leaf was higher in plants inoculated with *P. putida* PIR 3C, *R. terrigena* PCM 8, and *P. monteilii* KS 12, respectively, compared to control plants under salinity. The above study has revealed that the Inoculation with ACCD producing bacteria has a positive effect on chloroplast development and photosynthetic system. According to Kumari *et al*. (2015), SJ-5 and AK-1 bacterial strains

had EPS and ACCD production activity suggesting their role in increased chlorophyll content in inoculated soybean plants.

It is concluded that three out of four ACCD producing endophytic bacteria used in this study increased soybean sprouts growth under salt-stressed condition.

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