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Salt tolerant endophytic bacteria from *carthamus tinctorius* and their role in plant salt tolerance improvement

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Abstract

Eight halotolerant endophytic bacterial species were isolated from root, stem, and leaf of *carthamus tinctorius* (safflower) plant. Eight isolates were selected according to further examination of their salt tolerance, and using 1-aminocyclopropane-1-carboxylic acid (ACC) as a sole nitrogen source. Two isolates from the total are able to utilize ACC as a sole nitrogen source and they were tested for (ACC) deaminase activity. The bacterial isolates were characterized and identified using 16S ribosomal DNA technique as *Bacillus cereus* and *Bacillus aerius*. Our study results showed that *Bacillus cereus* and *Bacillus aerius* were capable of consuming nearly ACC concentration in the DF-ACC medium after 24h incubation. This character of the capability of ACC utilization is related with plant growth promotion due to lowering stress ethylene level. This study suggested that the bacterial strains *B. cereus* (LB1) and *B. aerius* (SB1) are valuable biological plant growth promoters that could enhance salt tolerance in safflower plants under 100, 200, and 300 mmol l^{-1} NaCl levels resulting in an increase in plant growth and chemical composition, in comparison with the non-inoculated controls. Seedling irrigated with different concentrations of NaCl showed significant decreases in IAA and GB, while ethanol content was significantly increased compared to the control. In our studies the used bacterial strains have the ability to achieve systemic tolerance via production of ACC (1-amino cyclopropane-1-carboxylase) deaminase which has the ability to reduce the production of stress hormone ethylene. Our findings reported that the co-inoculation of

the two selected endophytic bacteria strains were successfully isolated from safflower seedlings significantly alleviated the harmful effects of salt stress, promoted plant growth and biomass yield.

Keywords: Endophytes, ACC deaminase activity, Salt stress, plant hormons

Introduction

Plant-associated bacteria that live inside plant tissues without causing any harm to plants are defined as endophytic bacteria. Endophytes seem to be ubiquitous in plant tissues, having been isolated from flowers, fruits, leaves, stems, roots and seeds of various plant species (Kobayashi and Palumbo 2000). High numbers of endophytes have been observed in the root interior of grasses, such as sugar cane (Olivares *et al*. 1996). It is capable of invading inner tissues including xylem vessels (Olivares *et al*. 1997). Most endophytes appear to originate from the rhizosphere through penetrating and colonizing root tissue from an access point to the xylem; however, some may be transmitted through the seed. Some microbes appear actively to penetrate plant tissues through invading openings or wounds, as well as actively using hydrolytic enzymes, such as cellulase and pectinase. Endophytic bactebria can promote plant growth and yield and can act as biocontrol agents, In addition to their beneficial effects on plant growth; they have considerable biotechnological potential to improve the applicability and efficiency of phytoremediation (Raaijmakers *et al*. 2008). Halotolerant bacteria are microorganisms that capable of growing in media of various NaCl levels (1-33%) or in the NaCl absence (Mayak *et al*. 2004). so, the hypothesis of the use of ACC deaminase-producing halotolerant bacteria to alleviate the effect of saline stress on plants by reducing ethylene levels was studied. ACC deaminase-producing salt-tolerant bacteria can survive well in a saline environment and that their beneficial properties help plants to ameliorate stress impacts.

Salinity is one of the most dangerous environmental limiting factors of the crop productivity, the crop plants are sensitive to salinity resulted from higher salt concentrations in the soil and these plants affected by increasing salinity day by day. For all important crops, average yields are about 20% and 50% of record yields; these losses are due to high soil salinity and drought (Shrivastava and Kumar, 2015). A wide range of adaptations are required to overcome salinity stress. However, such strategies being long drawn and cost intensive, so there is a need to develop simple and low cost biological methods for Salinity stress alleviation. Microorganisms could play an important role

in this respect by production of plant growth promoting hormones, synthesis of compatible solutes, and genetic diversity (Shrivastava and Kumar 2015). It has been previously reported that salinity cause an increased production of ethylene in plants, thereby accelerating leaf and petal abscission and organ senescence, leading to premature death. Reducing the stress-induced ethylene level can alleviate some of the effects of stress on plant. (Zahair *et al*., 2009).

Carthamus tinctorius L. its common name is Safflower. It belongs to Asteraceae family in the order of Asterales that contains about 22,750 genera and more than 1,620 species (Asgarpanah *et al*. 2013). *Carthamus* species probably originate from Southern Asia and is known to have been cultivated in China, India, Iran and Egypt almost from prehistoric times (Bae *et al*. 2002). Extracts from *C. tinctorius* especially oils are used in drug development and various pharmacological activities in the world. It is used as an analgesic, purgative and antidote to poisoning in traditional medicines (Zhou and Zhao 2009). Safflower is widely distributed in eastern and western Asia. The flower of safflower is used in folk medicine as an analgesic, antihypertensive crude drug, as well as a source of natural colorants, its seeds rich in c~-Iinoleic acid are commonly consumed as vegetable oil in Europe, and used clinically for the treatment of cataclasis, osteoporosis and rheumatoid arthritis in Korea (Kim 1992). Recent studies revealed that safflower seeds have been shown to improve lipid metabolism in high fat and cholesterol-fed (Cho *et al.* 2001). Several phenolic compounds, including serotonins, lignans and flavonoids with antioxidative and physiological actions have been isolated from safflower seeds (Zhang *et al*. 1997).

Materials and Methods

Isolation the salt tolerant entophytic bacteria from safflower plant parts

The roots, stems, and leaves were washed with tap water and then distilled water to remove the soil particles. They were excised and subjected to three steps of surface sterilization procedure; Step 1: Washed with 70% ethanol for 1 min and subsequently with distilled water. Step 2: Soaked in 0.1% mercuric chloride for 3 min and washed with distilled water for 2 times. Step 3: Soaked in 70% ethanol for 30 s and washed for 5 to 7 times with distilled water. The additional step was followed in this sterilization procedure, proposed by Gagne *et al*. (1987). The surface sterilized plant parts were aseptically sectioned into small pieces (0.1 cm thickness). The sections were made and placed on to the plates containing isolation medium (Nutrient agar) supplemented with of NaCl (0.1, 0.3,

0.6, 0.9 and 1.2 M) was determined in nutrient agar as the basal medium during incubation at 28°C for 3 day (janarthine *et al*. 2011) The bacterial growths associated with plant sections were purified by repeated plating on saline nutrient agar. Bacterial isolates were selected based on colony morphology and growth rate. To confirm that the plant surfaces were effectively decontaminated 1ml aliquots of the sterile distilled water that was used in the final rinse of surface sterilization procedures were plated onto nutrient agar medium N.A. Pure cultures of the halotolerant bacterial isolates were maintained in 30% glycerol at -80^{o}C.

Selection of bacterial isolates that contain ACC deaminase

Dilutions of this final salt tolerant culture are plated onto solid DF-ACC medium. The DF medium was prepared as described by Penrose and Glick (2003), the inoculated plates are incubated at the appropriate temperature no higher than 35°C because all of the known ACC deaminases are inhibited above this temperature for 3 days and the growth on the plates is checked daily. Even when apparently nitrogen-free agar is used, and no additional source of nitrogen is included in the medium, it is almost impossible to obtain plates with absolutely no bacterial growth but it is possible to get plates with very, very light growth. Solution of ACC was filter-sterilized through a 0.2µm membrane, and frozen at -20. Just prior to use, the frozen ACC, solution was thawed and added into autoclaved DF minimal medium to obtain the DF-ACC medium with a final ACC concentration of 3 mmol l^{-1}. Solid DF and DF-ACC media were supplemented with 1.5% (w / v) ultrapure agar (Sambrook and Russell 2001). Very poor growth was indicated as –ve ACC utilization and dense growth was +ve ACC utilization.

ACC deaminase activity

It is usually analysed according to Honma and Shimomura (1978), Penrose and Glick (2003) and Fu *et al*. (2009)) who measured the ACC deaminase activity using a colorimetric ninhydrin assay to detect the consumption of the ACC after 24 h growth in a minimal salt medium with initial concentration of 3 mmol l^{-1} from ACC. The 24 h consumption period is considered as enzyme induction period and the ACC concentration decreases during the 24 h consumption this assay is not appropriate for determining ACC deaminase activity. However, this ninhydrin assay of ACC (an amino acid with a cyclopropane ring) enables the detection of ACC consumption by bacteria, the present study was therefore conducted in an attempt to isolate and identify the diverse group of

halotolerant bacteria from safflower plant parts. Selected isolates were then checked for their ability to have ACC-deaminase activity and amelioration of salt stressed plants. In our study, a colorimetric 2, 4-dinitrophenylhydrazine assay of the product α-ketobutyrate and ninhydrin assay to determine consumption of the substrate ACC after 24 h growth in a minimal medium with an initial ACC concentration of 3 mmol l^{-1} were made.

Determination of the IAA production of bacterial halotolerants

Salkowski's colorimetric method was used to determine the IAA production ability by each isolate. The pure culture of each isolate was grown in a nutrient broth medium containing 0.1 mg/ml l-tryptophan and 5% NaCl and was incubated at 30°C for 2 days. After incubation, the broth was centrifuged, the supernatant was retained and 1 mL of supernatant was mixed with 2 mL of Salkowski's reagent (2% 0.5 $FeCl_3$in 35% $HClO_4$solution) and kept in the dark for minimum 30 min. Subsequently, the optical density (OD) was measured at 530 nm.

Identification of the bacterial isolates

For identification of the isolated bacteria, the genomic DNA was extracted using standard bacterial procedures (Sambrook *et al*., 1989). The primers used in the amplification of the 16S rDNA gene are forward primer (F1; AGA GTT TGA TCC TGG CTC AG) and reverse primer (R1; GGT TAC CTT GTT ACG ACT T**).** The PCR mixture was prepared as the following; 10 µL (10x) PCR buffer, 3 µL (50 mM) $MgCl_2$, 1 µL (20 pmole/µL) of each primer, 1 µL (10 mM) dNTPs mixture, 0.5 µL (2.5U) Taq DNA polymerase, 2 µL total DNA extract, and the volume is completed to 100 µL by SDH2O. PCR were carried out for 35 cycles under the following conditions: denaturation step at 94°C for 40 sec, annealing step at 55°C for 1 min, extension step at 72°C for 2 min and final extension at 72°C for 10 min. An aliquot of the PCR products (10 µL) was mixed with 2 µL of DNA loading buffer and analyzed by electrophoresis (15 V/cm, 60 min) on 0.7% horizontal agarose gel in TBE buffer containing 0.5 µg/mL ethidium bromide, then visualized on an UV transilluminator. Sequencing of the amplified fragments was performed at GATC Biotech, Constance, Germany. DNA Sequences were aligned at NCBI DataBase (www.ncbi.nlm.nlh.gov). Phylogenetic tree was then constructed by neighbour-joining method using TREEVIEW software (1.6.6) based on 16S rRNA gene sequences of some strains phylogenetically close to the isolated strain.

Seed treatment and gnotobiotic growth pot assays

Seed treatment was performed in accordance with Penrose and Glick (2003) with slight modifications. Briefly, halotolerant bacterial isolates were grown in TSA supplemented with 1 mol l^{-1} NaCl. The cells were then collected and resuspended in a Nitrogen free medium containing 1 mol l^{-1} NaCl and supplemented with 3 mmol l^{-1}ACC as a sole nitrogen source, and subsequently incubated for 24 h at 30^{o}C with shaking (120 xg) in order to induce ACC deaminase activity. After that, the cells were harvested, and washed by resuspension in sterile 0.03 mol l^{-1} MgSO4. Safflower seeds (Agriculture Research Center., Giza., Egypt) were surface sterilized by immersion in 70% ethanol for 1 min and 2% NaOCl for 30 s, followed by a thorough rinsing in sterilized distilled water (3-4 times). Then these surface-sterilized seeds were soaked in sterilized distilled water, or bacterial suspension (1×10^{8} CFU ml^{-1}) for 24 h. Following on from this, 250 ml of water, or water containing 100, 200, and 300 mmol l^{-1} NaCl, was added to previously sterilized seed germination pots (soil was previously autoclaved at 121^{o}C for 20 min). Surface sterilized seeds were transferred aseptically to growth pots. Eight seeds were placed in each pot for each treatment, and each treatment had five replicates. Seeds in growth pots treated only with water were used as the negative control, and seeds in growth pots treated only with salt solution were used as the positive control. In theory, the positive controls should be found to reduce growth in a manner very similar to the actual salt stress effects on plants in the field, whereas negative controls should provide the standard for any changes from the normal growth process of the plant. Data were collected regarding fresh weight, dry weight and other biochemical compounds.

Extraction and colorimetric assay of Indole-3-acetic acid (IAA)

Seedlings from both salt treated and control plants were collected for extraction of IAA after 30 days of treatment. Extraction of IAA was done according to protocol given by Horemans *et al.* (1984), then was tested and quantified using the method of Gordon and Weber (1951).

Gibberellic acid estimation

The estimation of gibberellic acid was performed by the spectrophotometric method following the protocol of Holbrook *et al*. (1961).

Determination of ACC in plant

Samples (1 g each) of leaves were cut into 4-mm pieces and extracted twice with 10 ml of 80% ethanol at 70°C for 30 min. The ethanol extracts were combined and evaporated in vacuo to dryness, resuspended in 0.5 ml chloroform and the ACC was extracted with 2 ml of water. The ACC content in the aqueous extract was determined by chemical conversion to ethylene according to the method of Lizada and Yang (1979).

Statistical analysis

The results are presented as the average means and standard error (SE) of replicates. The data were further analyzed for statistical significance using Duncan test analysis, and the difference between means was compared by a high-range. A *p*-value <0.05 indicated statistical significance. The data were discussed in terms of percentage variation, with respect to the control plants. Data plotting were carried out using Microsoft Excel 2010.

Results

Isolation the salt tolerant endophytic bacteria

Eight salt tolerant endophytic bacterial isolates from Safflower root, stem, and leaf were isolated after 24 hrs. incubation of previously surface sterilized Safflower plant parts. Bacterial colonies were found emerging from the ends of plant parts on minimum salt medium supplemented with gradient NaCl concentrations. Three bacterial isolates from leaf (designated as LB1, LB2 and LB3), three from stem (designated as SB1, SB2 and SB3), and two from root (designated as RB1 and RB2) were obtained but their salt tolerance were variable from one to another (Table 1). LB1, SB1 and RB1 tolerated till 1200mM but the other isolates began to be inhibited at 900mM and 1200mM (Table 1). The ability of all isolates for ACC utilization as a sole nitrogen source and IAA production were examined.

Table 1: Selection the most salt tolerant endophytic bacteria

Bacterial isolate	Source	tissue	0.1	0.3M NaCl	0.6M NaCl	0.9M NaCl	1.2M NaCl
LB1	Safflower	Leaf	+ve	+ve	+ve	+ve	+ve
LB2	Safflower	Leaf	+ve	+ve	-ve	-ve	-ve
LB3	Safflower	Leaf	+ve	+ve	+ve	+ve	-ve
SB1	Safflower	Stem	+ve	+ve	+ve	+ve	+ve
SB2	Safflower	Stem	+ve	+ve	+ve	+ve	-ve
SB3	Safflower	Stem	+ve	+ve	+ve	-ve	-ve
RB1	Safflower	Root	+ve	+ve	+ve	+ve	+ve
RB2	Safflower	Root	+ve	+ve	+ve	+ve	-ve

+ve sign referred to bacterial growth, -ve sign referred to no bacterial growth. The growth was detected on growth plates of solidified MSM plus NaCl gradient concentrations.

Selection the bacterial isolates of ACC utilization

Two bacterial isolates from the eight salt tolerant ones could use ACC as a sole nitrogen source; these two were related to Safflower stem and leaf. This selection was made using minimum salt medium and DF medium supplemented ACC as a sole nitrogen source. Based on growth degree of bacterial isolates on plates, comparison the growth of bacterial isolates with control (growth medium devoid of nitrogen source) that appeared very light growths on plates were made. Two bacterial isolates have grown densely on growth plates compared to other six ones that gave light growth as control. This test is considered as a preliminary test indicated that these two bacterial isolates could have ACC deaminase activity.

IAA production test

The all isolates tested for IAA production as an effective property of the bacteria to promote plant growth under normal and saline conditions. Six of them could produce IAA (Table 2) and the other two isolates could not produce IAA and gave negative results.

Table 2: Determination the ability of the eight endophytic bacterial isolates to produce IAA and utilize ACC as a sole nitrogen source.

Bacterial isolate	Tissue	IAA production	ACC utilization
LB1	Safflower leaf	+ve	+ve(dense growth)
LB2	Safflower leaf	-ve	-ve(light growth)
LB3	Safflower leaf	+ve	-ve(light growth)
SB1	Safflower stem	+ve	+ve(dense growth)
SB2	Safflower stem	-ve	-ve(light growth)
SB3	Safflower stem	+ve	-ve(light growth)
R1	Safflower root	+ve	-ve(light growth)
R2	Safflower root	+ve	-ve(light growth)

Identification of the most salt tolerant endophytic bacteria

LB1 and SB1 isolates was identified as *Bacillus cereus* and *Bacillus aerius* using 16S rDNA gene sequencing technique with maximum homology of 99%. *Bacillus cereus* and *Bacillus aerius* sequences were submitted in genbank and had accession numbers MG708176 and MG711593, respectively.

ACC determination by colorimetric ninhydrin assay

Ethylene glycol used as the solvent stabilized the ninhydrin reagent and its colour development depending on addition of ethylene glycol and ascorbic acid. Ethylene glycol used as the solvent stabilizer and ascorbic acid in the ninhydrin reagent prevented the ninhydrin from oxidation. The absorbance values of the most two salt tolerant safflower endophytic bacterial isolates were significantly lower than that of the diluted noninoculated DF-ACC medium (standard assay) (data not shown). *Bacillus cereus* and *Bacillus aer*ius consumed nearly all the 3 mmol l^{-1} of ACC in the DF-ACC medium after the24-h incubation (Fig 1), the color depth of the two bacterial cultures was visibly weaker compared with that of the non-inoculated DF-ACC medium (standard assay) (data not shown).

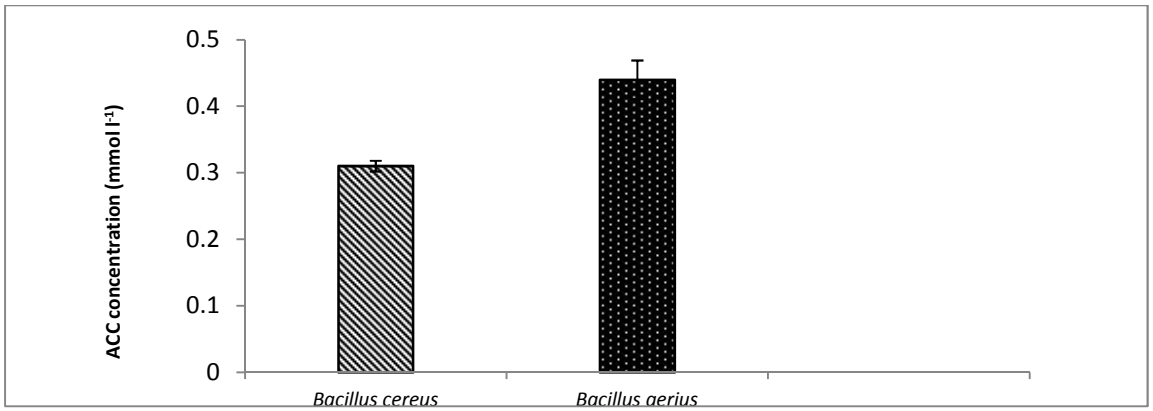

Fig 1: ACC concentrations remaining in the DF-ACC medium containing 3.0 mmol l^{-1} ACC after incubation of each ACC-utilizing bacterial isolate 24 h measured by the test tubes ninhydrin assay. Each data point represents the mean from triplicate determinations, and the error bar represents the standard error.

ACC deaminase activities of ACC-utilizing bacteria

ACC deaminase activity for the two ACC-utilizing bacterial isolates (Fig 2) used by the colorimetric 2, 4-dinitrophenylhydrazine assay represented that *Bacillus cereus* and *Bacillus aer*ius gave high µmol α-KB $h^{-1}mg^{-1}$ protein (2.4 and 1.8) (Fig 2). The colour depth of the two bacterial cultures was visibly darker compared with that of the noninoculated samples (standard assay) (data not shown).

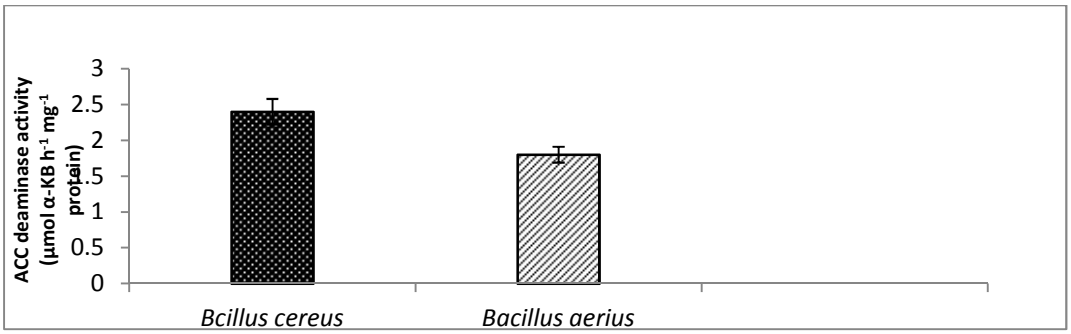

Fig 2: ACC deaminase activity of each ACC-utilizing bacterial isolate measured by the 2,4-dinitrophenylhydrazine assay for ketobutyrate mg $protein^{-1}$ h^{-1} after induction in the DF-ACC medium for 24 h. Each data point represents the mean from triplicate determinations and the error bar represents the standard error.

Interactive effect of irrigation with different concentrations of NaCl and inoculation with bacterial strains on growth and chemical composition of *Carthamus tinctorious* plant

A greenhouse pot experiment was conducted in Botany Department, Faculty of science, Fayoum University, during winter season (December, 2016) to investigate the effect of salinity alone or in

combination with inoculation with *Bacillus cereus*, *Bacillus aerius* or combination of them on growth and some metabolic activities of *Carthamus tinctorious*.

Growth criteria

Changes in growth criteria (fresh and dry matter) of safflower obtained from seeds inoculated with bacterial strains; *B. cereus*, *B.aerius* or *B. cereus* + *B.aerius* and followed by treatment with four concentrations (i.e 0,100,200 or 300mM) of NaCl are shown in Table (3). Fresh and dry matter of safflower seedlings were decreased gradually with increasing the NaCl level compared to the control (water). These parameters showed significant increases; 50.5 and 85.0%, and 101.5 and 119.7% by inoculation with bacterial strains *B. cereus* + *B.aerius* and *B. cereus*, respectively while, these parameters exhibited not significant differences with *B.aerius* when compared with the control. Under 100mM NaCl, *B. cereus* and *B. cereus* + *B.aerius* conferred significant increase in both growth criteria. These increases were 15.2 and 32.9%, and 43.4 and 115.6% for fresh and dry matter, respectively compared to the control. In contrast, SB failed to induce seedling growth of *C. tinctorious*, which reduced significantly compared to the control. Under 200mM NaCl, both *B. cereus* and *B. cereus* + *B.aerius* conferred the same growth criteria values, however with *B.aerius* growth criteria were further reduced compared to control. Under the stress conditions of 300mM NaCl, all bacterial strains failed to equal the growth parameters with the control.

Table 3: Effect of NaCl on fresh-dry weight of *carthamus tinctorius* Plant either inculated or not with *Bacillus cereus* (its symbol is *B. cereus*), *Bacillus aerius* (its symbol is *B.aerius*), and dual bacterial combination after 30 days old seedlings. Data are the mean of three replicates and error bars represent the standard errors of the means

Treatment	**Fresh weight (g)**	**Dry weight (g)**
0 mM NaCl	14.06 ± 0.12^{de}	1.73 ± 0.12^{fg}
100mM NaCl	11.46 ± 0.24^{fgh}	1.5 ± 0.06^{ghi}
200mM NaCl	10.8 ± 0.17^{ghi}	1.33 ± 0.08^{ij}
300mM NaCl	7.93 ± 0.48^{j}	1.06 ± 0.08^{j}
***(B. cereus* + *B.aerius)* + 0 mM NaCl**	28.33 ± 0.8^{a}	3.8 ± 0.12^{a}
***(B. cereus* + *B.aerius)* + 100 mM NaCl**	20.16 ± 0.6^{b}	3.73 ± 0.06^{a}
(*B. cereus* + *B.aerius*) + 200 mM NaCl	15.3 ± 0.85^{cd}	2.76 ± 0.18^{c}

***(B. cereus* + *B.aerius*) + 300 mM NaCl**	13.69±0.87^{de}	2.2±0.16^{de}
***B. cereus* +0 mM NaCl**	21.16±0.6^{b}	3.2±0.145^{b}
***B. cereus* +100 mM NaCl**	16.2±0.61^{c}	2.3±0.145^{d}
***B. cereus* +200 mM NaCl**	14.66±0.88^{cd}	2.2±0.115^{de}
***B. cereus* +300 mM NaCl**	13±0.83^{ef}	2.03±0.08^{e}
***B.aerius* +0 mM NaCl**	14.46±0.9^{cd}	1.93±0.12^{ef}
***B.aerius* +100 mM NaCl**	12.3±0.88^{efg}	1.66±0.08^{fgh}
***B.aerius* +200 mM NaCl**	10.33±0.88^{hi}	1.4±0.115^{hi}
***B.aerius* +300 mM NaCl**	6.16±0.78^{ij}	1.1±0.057^{j}

Each value represents mean ± SE of three replicated experiment. Each replicate was comprised of ten plants. Values in columns followed by different letters are significantly different at $P \leq 0.05$ followed by Duncan test.

Phytohormones content

Changes in idole-3acetic acid gibberellic acid and ethanol (express the activity of ACC deaminase) in seedling of *C. tinctorious* as a result of inoculation with bacterial strains LB, SB or LB+SB with our without salinity treatment are recorded in Table (4). Seedling irrigated with different concentrations of NaCl showed significant decreases in IAA and GB, while ethanol content was significantly increased compared to the control. Inoculation with LB, SB or LB+SB showed no significant difference when compared with control. In general irrigation of the inoculated seeds with different levels of NaCl significantly affects hormones contents when compared to corresponding controls. Under treatment with NaCl levels of 100,200or 300 mM, inoculation with LB+SB conferred significant increases in GA and IAA by 35.4& 244.1%, 60.4& 394.9 or 114.6 & 696.6%, respectively, however, inoculation with LB showed significant increase in IAA by 153.3, 242.8 or 515.3% respectively, while LB inoculants showed significant stimulation in GA content when treated with high level of NaCl (300mM) by 42.7% compared to control. Inoculation with SB exhibited no significant effect on GA & IAA contents with treatment of 100mM NaCl, while treatment of 200 or 300mM showed significant increases in both GA and IAA by 38.5 & 49.6% or 56.1 & 133.5%, respectively when compared to control. On the other hand, ethanol content which expresses ACC deaminase activity showed significant decrease although it's not lower than the control (water) but lower than corresponding treatments of different levels of NaCl 100,200 or 300mM without inoculation.

Table 4: Phytohormons gibberellic acid, indole-3-acetic acid and ethylene content in terms of ACC content in plant tissues inoculated with *Bacillus cereus*, *Bacillus aerius* and dual bacterial combination after 30 days old seedlings under non-salt (water) and salt (100, 200 or 300 mM of NaCl) conditions

Treatment	GB acid (mM/g F.W)	IAA (mM/g F.W)	Ethylene content in terms of ACC(µmol/g F.wt)
0 mM NaCl	16.00±0.67^{d}	2.36±0.05^{gh}	91.6±1.21^{g}
100mM NaCl	12.33±0.66^{e}	2.39±0.13^{fgh}	347.3±3.64^{b}
200mM NaCl	10.16±0.16^{f}	2.68±0.12^{fgh}	417.1±4.87^{b}
300mM NaCl	9.50±0.17^{f}	2.75±0.16^{fg}	651.9±11.62^{a}
***(B. cereus + B.aerius)* +0 mM NaCl**	15.00±0.33^{d}	5.98±0.49^{e}	89..7±0.56^{g}
***(B. cereus + B.aerius)* +100 mM NaCl**	21.66±0.66^{c}	8.12±0.43^{d}	107±4.12ef^{g}
(*B. cereus + B.aerius*) +200 mM NaCl	25.67±1.00^{b}	11.68±1.03^{c}	181.5±4.62^{cde}
***(B. cereus + B.aerius*)+300 mM NaCl**	34.33±4.33^{a}	18.8±0.85^{a}	184±2.05^{cde}
***B. cereus* +0 mM NaCl**	15.33±1.00^{d}	5.51±0.38^{e}	89.7±2.15^{g}
***B. cereus* +100 mM NaCl**	16.50±0.83^{d}	5.98±0.85^{e}	131.9±4.41^{defg}
***B. cereus* +200 mM NaCl**	17.16±0.16^{d}	8.09±0.41^{d}	204.2±3.96^{cd}
***B. cereus* +300 mM NaCl**	22.83±0.50^{c}	14.52±0.85^{b}	221.9±4.36^{c}
***B.aerius* +0 mM NaCl**	16.66±0.66^{d}	2.7±0.085^{h}	87.8±1.30^{g}
***B.aerius* +100 mM NaCl**	16.33±0.36^{d}	2.74±0.095^{fgh}	103.1±2.56^{fg}
***B.aerius* +200 mM NaCl**	22.16±1.16^{c}	3.53±0.13^{f}	175.2±2.24^{cdef}
SB +300 mM NaCl	24.98±1.68^{b}	5.51±0.51^{e}	101.9±0.88^{fg}

Each value represents mean ± SE of three replicated experiment. Each replicate was comprised of ten plants. Values in columns followed by different letters are significantly different at P≤0.05 followed by Duncan test.

Discussion

1-aminocyclopropane-1-carboxylic acid (ACC) activity is an effect marker for endophytic and other rhizospheric bacteria to enhance plant productivity and growth by lowering high ethylene levels produced under abiotic stresses as drought and salinity (Li *et al*. 2011). In our study, eight endophytic bacterial isolates were isolated and varied according to their salt tolerance degree and ACC utilization as a sole nitrogen source. The two of them were capable of ACC utilization as a sole nitrogen source, identified as *Bacillus cereus* and *Bacillus aerius* using16S rDNA sequence. This character of the capability of ACC utilization is related with plant growth promotion (Jacobson *et al*. 1994). More of gram-ve rods are PGPR such as *Enterobacter* sp and *Sporosarcina* sp that were isolated from pneumatophores; these isolates enhance growth of the entire plant by phosphate

solubilization and siderophore production as metabolites (Janarthine *et al*. 2011). Our study results showed that *Bacillus cereus* and *Bacillus aerius* were capable of consuming nearly ACC concentration in the DF-ACC medium after 24h incubation and (Fig 3), these results were supported by Li *et al*. (2011) study results which demonstrated that six ACC utilizing bacterial isolates obtained from *Sugarcane* rhizosphere and *Zea maize* stems belonging to the genus *Burkholderia, Pseudomonas*, and *Herbaspirillum* were capable of consuming nearly ACC concentration in the DF-ACC medium after 24h incubation.

Most environmental stresses are thought to result in overproduction of reactive oxygen species (ROS) in plant causing oxidative stress (Karuppananpandin *et al*., 2011; Sharma *et al*., 2012; Hemida *et al*., 2014; Rady and Hemida, 2016). Evidence suggest that salinity cause oxidative damage through excessive generation of oxygen radicals such as singlet oxygen ($^1O_2$), superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-) (Hemida *et al*., 2017). Or is associated with oxidative stress through altering antioxidant molecules levels in plants (Sharma *et al*., 2012; Rady and Hemida, 2016; Hemida *et al*., 2017). Excess of ROS triggers phytotoxic reactions such as lipid peroxidation, protein degradation and DNA mutation (Balakhnina *et al*., 2010a; Hemida *et al*., 2014). A wide range of adaptations strategies are required to overcome salinity stress. However, such strategies being long drawn and cost intensive, there is a need to develop simple and low cost methods for salinity stress alleviation management, which can be used on short term basis.

Microorganisms could play an important role in this respect; they can synthesize compatible solutes, produce plant growth promoting hormones, bio-control potential, and their interaction with crop plants (Shivastava and Kumer, 2015). Endophytic bacteria are of considerable interest due to their potential role in alleviating abiotic stress conditions (Brader *et al*., 2014). It has been confirmed in recent years that plant growth promoting properties (PGPEs) have the ability to colonize host plant's interior tissues and further build a beneficial symbiotic association with their host plants to improve host plant growth stress tolerance (Saravanakumar and Samiyappan, 2007).

In this study, we have isolated two strains of endophytic bacteria from safflower. These strains were *Bacillus cereus* and *Bacillus aerius*, which were selected and tested for plant growth promoting (PGP) mechanisms, ability of internal tissues colonizing, and their ability to tolerate salinity. Endophytic bacteria has also successfully colonized the host plants and cause stimulation in plant

growth criteria and synthesis of some biochemical compounds (Islam *et al*., 2016). The data obtained in this investigation is clearly demonstrated that NaCl, when applied at all levels of NaCl (100, 200 or 300 mM), significantly decreased plant growth parameters (fresh-dry matters). In this respect, extensive studies have been conducted concerning the inhibitory effects of salt stress on plant growth using different species and different methods to alleviate its effect (Basalah, 2010; Mousavi *et al*., 2013; Hemida *et al*., 2014; Rady *et al*., 2016; Hemida *et al*., 2017). The adverse effect of NaCl on plant growth has been attributed to changes in osmotic potential resulting from reduced water (Atek *et al*., 2006; Mousavi *et al*., 2013). Seeds of safflower inoculated with bacterial strains (e.g., *Bacillus cereus* (LB) and a combination of LB+SB) increased significantly growth parameters (fresh-dry) weight; Plant growth promoting endophytes (PGPEs) can promote plant growth directly via production of plant hormones, enhancing nutrient uptake (Santoyo *et al*., 2016; Li *et al*., 2016). A specific endophytic bacterium can promote plant development and growth at various stages during the life cycle of the plant using one or more of these mechanisms (Li *et al*., 2016). In this study, each of the endophytic bacteria tested for possessing at least one or more properties that were related to any plant growth promoting activities, including the ability of siderophere production, IAA production, nitrogen fixation, or ACC deaminase activity. So, the inoculants associated with different bacterial endophytes may possibly possess different plant growth promoting forces that offer them to enhance power of plant growth facilitation by utilization one or two of various mechanisms at different times during the life cycle of host plants. In recent years, endophytes have been used as one of practical measurements to alleviate salt stress and improve plant and yield under saline conditions (Ali *et al*., 2014; Li *et al*., 2016; Orhan, 2017). As soon as, in our studies the used bacterial strains have the ability to achieve systemic tolerance via production of ACC (1-amino cyclopropane-1-carboxylase) deaminase which have the ability to reduce the production of stress hormone ethylene as illustrated in Table (4).

Plants synthesize stress ethylene, which inhibits the plant growth as shown in Table (4), and this in accordance with Ali *et al*. (2014), Li *et al*. (2016) and Santoyo *et al*. (2016). Inoculation with bacterial strains *Bacillus cereus* (LB) and *Bacillus aerius* (SB) that contain the enzyme ACC deaminase help to reduce ethylene content as shown in Table (4) intern affected the plant growth promotion under salinity. Plant hormones are group of endogenous factors that help in transmitting signal between and within plant cells and also affect plant growth and development (Badu *et al*., 2012). Javid *et al*., (2011) have found that endogenous content of plant hormones such as ABA,

cytokinins, IAA and gibberellins changes in response to salt stress. This in accordance with our results in Table (4) as by increasing salt stress; IAA and GB content decreased significantly. Treatment of safflower with endophytic bacteria (*Bacillus cerus* and *Bacillus aerius*) enhanced the entire plant content of GA and IAA as shown in Table (4). These results are in agreement with Yedidia *et al*. (1999) who have stated that the colonization of endophytic bacteria enhanced the plant growth as a result of increasing the productivity and availability of plant hormones GA and IAA. Our results were also supported by Nia *et al*. (2012) who have studied the effect of inoculation of wheat plants by plant growth promoting bacteria (PGPB) isolated from saline soil on the content of hormones under sever water salinity, they have observed increase in hormonal content GA and IAA. In addition, Glick *et al*. (1998) have an envision of a complex cross talk between IAA and ethylene content in the plants inoculated by PGPB. Moreover, Asari *et al*. (2016), Abdeljalil *et al*. (2016) and Chen *et al*. (2016) have reported that the endophytic Bacillus species possess the potential of promotion of the production of phytohormones under stress conditions and this also support our results, this may attributed to that the PGPBs have the ability to produce plant growth promoting hormones which intern increase the hormonal reservoir in inoculated plants (Shahzad *et al*., 2016).

Conclusion

The utilization of beneficial endophytic bacteria provides an alternative approach to enhancing stress resistance in plants.The current study demonstrates the potential role of endophytic *B. cereus* and *B. aerius* in saline conditions. The experimental data revealed that the two isolates have the ability to grow in different saline conditions and tolerate to high salinity levels. The ability of two *Bacillus* species for IAA production, ACC deaminase activity and their ability for plant growth promotion under normal and stressful conditions were demonstrated. This study provides further encouragement for the utilization of phytohormone and ACC deaminase producing bacterial endophytes, such as *Bacillus cereus* and *Bacillus aerius* for the development of eco-friendly biofertilizers to enhance plant development and improve plant tolerance to salinity stress.

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