ACC Deaminase-Containing Rhizobacteria from Rhizosphere of *Zygophyllum coccineum* Alleviate Salt Stress Impact on Wheat (*Triticum aestivum* L.)

Ashraf Y. Z. Khalifa[1,2], Ashraf Metwally[1,3], Rebai Ben Ammar[1,4], Fatma Aly Farghaly[3]

(1) Biological Sciences Department, College of Science, King Faisal University, Saudi Arabia
(2) Botany and Microbiology Department, Faculty of Science, University of Beni-Suef, Egypt
(3) Botany and Microbiology Department, Faculty of Science, Assiut University, Assiut, Egypt
(4) Laboratory of Aromatic and Medicinal Plants, Center of Biotechnology of Borj-Cedria, Hammam-Lif, Tunisia.

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ABSTRACT

Plant growth-promoting bacteria are present in the rhizosphere, a soil zone around plant roots. An important mechanism of plant growth enhancement is the production of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase. Three ACC deaminase-producing bacterial strains were isolated from the rhizosphere of the halophytic plant *Zygophyllum coccineum* growing in Al-Uqair, Al-Ahsa, Saudi Arabia. The strains were identified as *Bacillus filamentosus*, *Janibacter indicus*, and *Brevibacterium casei* based on 16S rRNA sequence, and their taxonomic position was determined. Furthermore, the strains exhibited catalase and indoleacetic acid production and phosphate solubilization. The highest ACC deaminase activity was observed in *B. filamentosus* (~ 449 nmole α- ketobutyrate mg^{-1} h^{-1}), followed by *J. indicus* and *B. casei* (~ 66 nmole α- ketobutyrate mg^{-1} h^{-1}). Inoculation of wheat grains with each strain significantly enhanced growth. *Z. coccineum* rhizosphere harbors growth-promoting bacteria and ameliorated the negative effects of salt stress on wheat plants and may be used as an eco-friendly biofertilizer.

Key Words: Abiotic stress, Plant growth promoting bacteria, Salinity, Wheat, *Zygophyllum coccineum*

INTRODUCTION

The soil zone around plant roots, known as the rhizosphere, contains diverse microbial groups that significantly interact with roots. It is rich in nutrients secreted by plant roots that favor growth of different microbial groups. Plant growth-promoting rhizobacteria (PGPR) are found in the rhizosphere attached to plant roots and within plant tissues, and play pivotal roles in plant growth enhancement via direct and indirect mechanisms (Shameer and Prasad, 2018). Iron sequestration, plant hormone production, nitrogen fixation and phosphate solubilization are direct mechanisms for plant growth enhancement. Examples of the indirect mechanisms are antibiosis against microbial phytopathogens and improvement of abiotic stresses such as salt stress (Shameer and Prasad, 2018; Mishra, *et al.,*2018)

An important mechanism of PGPR-mediated plant growth enhancement is the production of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, which deaminases ACC, a precursor of ethylene in plants, and thereby reduces the ethylene content. Different bacterial groups inhabiting rhizospheric soil commonly consume ACC as the sole source of nitrogen. *Arthrobacter*, *Bacillus*, *Burkholderia phytofirmans*, *Pseudomonas fluorescens*, *Pseudomonas putida*, *Klebsiella*, *Serratia, Enterobacter* and *Microbacterium* are examples of ACC deaminase-containing bacterial species that have been reported to promote growth of different plants species (e.g., *Brassica napus* (canola), *Oryza sativa* (rice), and *Triticum aestivum* (wheat) (Sarkar *et al.,* 2018, Zhang *et al.,* 2018, Shameer and Prasad, 2018; Mishra, *et al.,* 2018).

Salinity is an important obstacle limiting agricultural production and expansion of cultivated areas, which affects most areas of Saudi Arabia and other arid regions. High salinity levels in irrigation waters, inefficient drainage, and elevated evaporation rates created by high temperatures are the main causes of soil salinity (Shrivastava and Kumar 2014). Plants under salt stress are

exposed to oxidative stress, ion toxicity, and osmotic stress, which adversely affect the morphology, physiology, and productivity of plants (Dumičić *et al.,* 2018). The use of rhizobacteria expressing ACC deaminase has become a promising approach owing to the dual beneficial roles of enhancing plant growth and ameliorating negative effects of salinity (Sarkar *et al.,* 2018). It has been recently reported that *Bacillus megaterium*, a nodule endophytic bacterium obtained from *Medicago polymorpha*, increases nodulation induced by the compatible microsymbiont, *Ensifer medicae*, and ameliorates salinity constraints on alfalfa (Chinnaswamy *et al.,* 2018).

Zygophyllum coccineum is a perennial herb with erect and branched stems and two-foliated leaves. Taxonomically, it belongs to the flowering plant family Zygophyllaceae, which is widely distributed in arid and semiarid regions. In Saudi Arabia, it grows across different regions such as Al Taif and Al-Ahsa (Alzahrani and Albokhari, 2017). *Z. coccineum* was reported to be abundant along the coastal strip of Al-Uqair (Youssef and Al-Fredan 2008). In addition, plant growth-promoting rhizobacteria (e.g., *Bacillus*, *Enterobacter*, and *Pseudomonas*) have been isolated from some members of the *Zygophyllaceae* family (including *Z. simplex*) growing in the Al Taif region, and exhibited antagonistic activities against certain phytophaogens such as *Fusarium oxysporum*, and *Sclerotinia sclerotiorum* (El-Sayed *et al.,*2014).

Although several reports highlighted the benefits of rhizobacteria (Bibi *et al.,* 2018, Khalifa and Almalki 2015, and Khalifa *et al.,* 2016), a few reports investigated rhizobacteria in association with halophytes, particularly in the Al-Ahsa District. For example, rhiozbacteria associated with roots and root-nodules of *Medicago sativa* growing in Al-Ahsa region, enhanced growth of some grain legumes such as *Pisum sativum* (Khalifa and Almalki 2015, and Khalifa *et al.,* 2016). A few reports of ACC deaminase-producing PGPRs isolated from Al-Ahsa District, or the ability of native PGPRs to promote plant growth or ameliorate salt stress effects. It has been reported that *Azotobacter* and *Streptomyces* obtained from rhizospheric soil of wheat roots, grown under elevated levels of salinity, west of Saudi Arabia, improved seed germination and growth of wheat plant under salt stress (Aly *et al*., 2012). Little is known about ACC deaminase-producing rhizobacteria in the rhizosphere of *Z. coccineum*. The goal of the current work was, therefore, to isolate, characterize, and identify ACC deaminase-producing rhizobacteria from the rhizosphere of *Z. coccineum*, a halophytic plant found in Al-Uqair, Al-Ahsa, and assess their roles in alleviating salt stress effects on wheat, an important economic cereal crop.

MATERIALS AND METHODS

Collection of *Z. coccineum* plants

Z. coccineum plants were uprooted and collected along with rhizospheric soils from Al-Uqair coast (25°39'35.8"N 50°11'25.6"E), Al-Ahsa, Saudi Arabia, in May, 2015. Soil characteristics and floristic composition of Al-Uqair were previously described (Youssef and Al-Fredan 2008). Breifly, the soil texture is sandy, the climate is tropical and arid, 29 different plant species including *Z. coccineum,* were reported (Youssef and Al-Fredan 2008). In the laboratory, 1 g rhizospheric soil of *Z. coccineum* was mixed with 50 mL of salt minimal medium DF salts with 3mM ACC as a sole nitrogen source (Dworkin and Foster 1958). Soil-inoculated 250-ml flasks were incubated in dark at 30 ºC, on a rotary shaker at 150 rpm, for 5 days. After the incubation period, aliquots (100 µL) from the obtained cultures were streaked onto salt minimal agar plates containing 0.5 % ACC and incubated at 30 ºC for 5 days. Pure colonies were re-streaked onto fresh agar plates. Obtained isolates were preserved by growing in soyatrptic broth for 24h then aliquots were transferred to sterilized 1.5 ml cryovial in 15 % glycerol as a final concentration, at -80 ºC. Aliquots of the cultures were stored in slant

screw-capped test tubes (13x100mm) at 4 ºC and maintained by further subculturing on fresh slants every 3-4 weeks for further use.

Morphological characteristics

Colony morphology of the bacterial isolates obtained was evaluated, and Gram staining was performed as previously described (Claus 1992).

Phenotypic characteristics

Bacterial strains were phenotypically characterized using the Biolog Gen III microtest system (Biolog, USA) following the manufacturer instructions.

Plant growth-promoting activities

Production of indole acetic acid (IAA). Bacterial strains were assayed for the production of IAA using the Salkowski reagent (Gordon and Weber 1951) as described earlier (Khalifa and Almalki 2015).

Solubilization of phosphate. The ability of bacterial strains to solubilize inorganic phosphate was tested on Pikovskaya agar as previously described (Pikovskaya 1948). The strains were grown on Pikovskaya agar containing tribasic calcium phosphate as the source of phosphate. The plates were incubated at 30°C for 72 h. The appearance of clear zones around bacterial colonies were considered a positive result.

Production of ACC deaminase. The ability of bacterial strains to produce ACC deaminase was tested following the method described by Penrose and Glick (2003). AAC activity of the bacterial strains was estimated from a standard curve using alfa ketobutyrate.

Production of catalase. Catalase production was assayed qualitatively by adding two drops of 5 % hydrogen peroxide to 50 µL of an actively growing bacterial culture of each strain. The appearance of gas bubbles was considered a positive result.

Antioxidant activity assays

Bacterial strains were grown in 250 mL flasks containing 50 mL of LB medium(per litre): 10 g peptone, 5 g yeast extract, 5 g sodium chloride (Bertani 2004) and incubated at 30 °C with shaking (150 rpm) for 24 h. After incubation, cell-free extracts were obtained by centrifugation of bacterial cultures at 4,000 x *g*, for 10 min at 4 °C. Antioxidant activities of the bacterial strains were estimated according to a previously described method (Heo *et al.,* 2005). Free radical scavenging activity was calculated from the equation [Absorbance (A) control – A sample/A control] × 100 and expressed as a percentage.

Identification of bacterial strains using 16S rRNA sequencing

Identification of the bacterial strains was carried out using the comparative sequence analysis of the 16S rDNA gene. Genomic DNA extraction, primers used, PCR conditions, and sequencing were performed as previously described (Khalifa and Bekhet 2018).

Phylogenetic analyses

The evolutionary history of the bacterial strains was obtained using the neighbor-joining method (Saitou and Nei 1987). Computation of the evolutionary distances was carried out using the maximum composite likelihood method (Tamura *et al.,* 2004) and MEGA v. 7 software (Kumar *et al.,* 2016).

Inoculation of wheat grains with PGPR under different salt concentrations

Pot experiment set-up. Healthy wheat grains (*Triticum aestivum* L.) were obtained from local markets and surface-sterilized. Bacterial inocula were prepared as described earlier (Gontia-Mishra *et al.,* 2017) with the following modifications. For single inoculation, 5 surface-sterilized wheat seeds were treated with 3×10^6 CFU mL^{-1} bacterial suspension of the desired strain for 24 h under aseptic conditions. For co-inoculation (1:1:1), 1 mL of each of the three bacterial strains was mixed and used for inoculation of wheat grains. Factorial experiments (two

different factors; concentrations of NaCl (25, 50 mM) and bacterial strain) in completely randomized design was performed using bacteria treated with three concentrations of NaCl (0, 25, and 50 mM). Inoculated grains were grown in plastic pots filled with 0.5 kg sterilized soil (1 clay: 1 sand ratio) in a growth chamber at 22 ºC, 50 % relative humidity, and 14 h photoperiod. Appropriate plant controls without added salt or bacteria were used. Experiments were performed in triplicate.

Physiological parameters. After 3 weeks, root and shoot dry weights were determined, and the dried tissues were used for biochemical analyses including estimation of free proline content.

Estimation of free proline content

Free proline accumulation was determined in roots and shoots according to the methods described earlier (Bates *et al.* 1973). Spectrophotometric absorbance was determined at 520 nm, with toluene serving as blank. The concentration of proline was calculated from a standard curve of L-proline and expressed as mole proline mg^{-1} DW.

Statistical design and analysis

Experiments were set up in a completely randomized design. Data were statistically analyzed using CoStat version 6.303 1998-2004 CoHort software (798 Lighthouse Ave PMP 320, Monterey, CA, 93940, USA). Analysis of variance (ANOVA) was performed to compare results. Least significance difference (LSD) test was used to compare the means at 5% significance level.

RESULTS AND DISCUSSION

Three different bacterial strains (ZCA4, ZCA8, and ZCA10) were obtained from the rhizosphere of the halophytic plant *Z. coccineum*, using a medium enriched with ACC. The strains were characterized based on phenotype, genotype and plant growth-promoting traits. The morphological and biochemical characteristics of the strains are shown in Table 1. All bacterial strains formed circular colonies with a continuous edge and were Gram-positive rods except ZCA8 which was ovoid-shaped. ZCA4 was an endospore-forming bacterium. These observations are in agreement with those of *Bacillus filamentosus* SGD-14^T (Sonalkar *et al.,* 2015), *Janibacter indicus* 0704P10-1^T (Zhang *et al.,*2014), and *Brevibacterium casei* NCDO 2048 (CMD1) (Collins *et al.,*1983).

Table (1): Morphological and biochemical characteristics of the bacterial strains isolated from rhizosphere *Z. coccineum*.

	B. filamentosus ZCA4	*J. indicus*, ZCA8	*Br. casei* ZCA10
Morphological characteristics			
Colony	Circular, slimy with entire edges	Circular, opaque, smooth, convex	Circular, opaque, smooth, convex
Pigmentation	Pale orange	Cream	Gray-white
Gram staining	Positive	Positive	Positive
Cell	Rod-shaped in filamentous chains	Ovoid-shaped	Rod-shaped
Endospore	Endospore-forming	Non-endospore-forming	Non-endospore-forming
Plant growth-promoting traits			
ACC deaminase (nmole α- ketobutyrate mg^{-1} h^{-1})	65.77±12.45	155.18±4.02	448.56±0.08
IAA production (μg ml^{-1})	1.77±0.42	2.19±0.39	5.49±0.39
Antioxidant activity using DPPH assay (%)	58.4±3.13	10.6±0.82	18.4±1.22

Table 1, cont.

	B. filamentosus ZCA4	*J. indicus*, ZCA8	*Br. casei* ZCA10
Phosphate solubilization	+	-	-
Catalase	+	+	+
Biochemical characteristics** using the Biolog Gen III microplate			
D-Turanose	/	+	+
N-Acetyl-D-galactosamine	/	+	+
L-Rhamnose	/	+	+
D-Serine	-	+	+
L-Serine	+	+	+
Bromo-succinic acid	-	+	+
Formic acid	+	+	+
Troleandomycin	-	+	-
Lincomycin	-	+	-
Vancomycin	-	+	+
Nalidixic acid	/	+	+
Rifamycin SV	-	+	-
Guanidine HCl	/	+	+
Tetrazolium violet	-	+	/
Lithium chloride	+	+	+
Sodium butyrate	+	+	+
pH 5	-	/	/
NaCl (8 %)	+	+	+
D-Serine	-	+	+
Minocycline	-	+	-
Niaproof 4	-	-	/
Potassium tellurite	+	/	+
Sodium bromate	-	+	/

+: Good growth, /: Weak growth and - : No growth. **: Only discriminatory characteristics are mentioned.

Phenotypic characteristics of the isolated strains

The biochemical and physiological characteristics of the isolated bacterial strains were obtained using the Biolog Gen III microtest system (Table 1). Strains ZCA4, ZCA8, and ZCA10 gave positive results in the majority of testers, 75 (79.79 %), 89 (94.68 %), and 84 (89.36 %), respectively, out of the 94 investigated traits (Table 1). Furthermore, weak growth was observed in up to five testers, and the strains were unable to grow in 3 – 14 testers. All bacterial strains displayed a good growth on a wide spectrum of carbon and nitrogen sources such as D-raffinose, D-maltose, pectin, L-aspartic acid, L-histidine and gelatin (Table 1). The strains grew in the presence of the maximum salt concentrations examined in the microtest station 8 % NaCl, potassium tellurite, lithium chloride, and at pH 6. In contrast, no strain was able to grow in the presence of tetrazolium blue (a dye) or of the antibiotic fusidic acid. Unlike the other two strains, ZCA8 exhibited resistance to the antibiotics troleandomycin, lincomycin, vancomycin, rifamycin SV, and minocycline, highlighting a potential intrinsic antibiotic resistance. Only ZCA4 was unable to metabolize bromo-succinic acid and D-serine. ZCA10 showed a weak growth on Niaproof 4, unlike the other two strains. These testers can be used to easily differentiate between the strains.

The observation that ZCA4 consumed an array of different carbon and nitrogen sources is in general agreement with that reported

for *B. filamentosus* SGD-14^T (Sonalkar *et al.,*2015), *J. indicus* 0704P10-1^T (Zhang *et al.,*2014), and *B. casei* NCDO 2048 (CMD1) (Collins *et al.,*1983). However, some differences do exist between our strains and the relevant type strains. For example, unlike SGD-14^T, strain ZCA4 consumed α-D-lactose and D-cellobiose and grew well in high concentrations of NaCl (12 %). Additionally, 0704P10-1^T tolerated lower NaCl concentrations (7 %) than ZCA8. These differences could be attributed to the presence or absence of specific genes that allow the strain to cope with diverse ecological niches. Consistent with this hypothesis, whole genome analysis showed that strains within the same bacterial species can have differences in functional genes (Konstantinidis *et al.,* 2006). All strains reacted positively to the following testers: D-raffinose, α-D-glucose, D-sorbitol, gelatin, pectin acid, D-trehalose, β -methyl-D-glucoside, D-galactose, myo-inositol, L-arginine, D-gluconic acid, L-lactic acid, β-hydroxy- D-,L- butyric acid, D-cellobiose, D-salicin, 3-methyl glucose, glycerol, L-aspartic acid, D-glucuronic acid, citric acid, α-keto-butyric acid, gentiobiose, N-acetyl-D-glucosamine, D-fucose, D-glucose- 6-PO4, L-glutamic acid, glucuronamide, α-keto-glutaric acid, acetoacetic acid, sucrose, N-acetyl-b- D-mannosamine, L-fucose, D-fructose- 6-PO4, L-histidine, mucic acid, D-malic acid, propionic acid D-aspartic acid, L-pyroglutamic acid, ouinic acid, L-malic acid, acetic acid, stachyose, N-acetyl neuraminic acid, D-saccharic acid, formic acid, 1% NaCl, 1% sodium lactate, aztreonam, pH 6, 4% NaCl, lithium chloride, sodium butyrate, 8% NaCl, p-hydroxy- phenylacetic acid, tween 40, dextrin, α- D-lactose, D -mannose, D-mannitol, glycyl-L-proline, L-galacturonic acid, methyl pyruvate, γ -amino-butryric acid, D-maltose, D-melibiose, D-fructose, D-arabitol, L-alanine, L-galactonic acid, lactone, D-lactic acid methyl ester and α-hydroxy- butyric.

Production of IAA by the isolated strains

IAA is known to enhance root elongation and production of lateral roots and root hair, enabling plants to appropriately access nutrients and water in the soil. IAA also increases the amount of exudate secretion by plant roots via loosening of plant cell walls. This favors the colonization of roots by other PGPRs (Forni *et al.,* 2017). Additionally, IAA enhances the activity of ACC synthetase, an enzyme responsible for ACC production, which can be used as a nitrogen source (Glick *et al.,* 1998). The isolated bacterial strains, ZCA4, ZCA8 and ZCA10, produced the plant growth-promoting hormone IAA at a concentration of 1.7, 2.2, and 5.5 µg mL^{-1}, respectively, when tryptophan (0.1 µg mL^{-1}) was added to the growth medium (Table 1). Results similar to those of ZCA4 have been reported for *Bacillus* strains (produced 1.5 µg mL^{-1}) obtained from rhizosphere of *Arachis hypogaea* (Haldar *et al.,* 2011). These results are much lower than those recorded by Passari *et al.,*(2015), who described that 14 actinomycetes strains obtained from seven medicinal plants produced IAA (10–32 µg mL^{-1}). However, in the same study, a few strains did not show any IAA production. Many *Bacillus* spp. have been reported to produce IAA at a concentration higher that that reported in our study. For example, *Bacillus megaterium* obtained from root-nodules of *Medicago sativa* growing in Al-Ahsa produced 40 µg mL^{-1} IAA (Khalifa and Almalki 2015). The ameliorative effect of IAA on salt-stressed plants could be achieved by reducing electrolyte leakage via maintaining cell membrane integrity (Kaya *et al.,* 2013).

Phosphate solubilization by the isolated strains

The three bacterial strains were assessed for their ability to solubilize phosphate using a previously described qualitative method (Pikovskaya 1948) and the results are shown in Table 1. Unlike, ZCA8 and ZCA10, ZCA4 gave a positive result. The appearance of a clear zone around each colony grown on Pikovskaya agar supplemented with calcium

triphosphate highlighted the bacterial capabilities for phosphate solubility. These observations are consistent with those of Khalifa and AlMalki (2015) and Sharma *et al.* (2013), who confirmed phosphate (P) solubility of rhizobacteria from *Bacillu*s species and actinobacteria, respectively. Despite its abundance in soil in both organic and inorganic forms, P is biologically unavailable for plant roots because it is mostly present as insoluble complexes (Sorty *et al.,* 2018). This limits plant growth and productivity. Nonetheless, many microbial spp. in soil do solubilize P complexes efficiently via production of acids and/or phytases (Sorty *et al.,*2018), and hence P can be easily absorbed by roots of plants to sustain growth and productivity.

ACC deaminase activity of the isolated strains

ACC deaminase activity of the bacterial strains are presented in Table 1. Strain ZCA8 displayed a higher ACC deaminase activity (155.2±4.02 nmol α-ketobutyrate mg^{-1} h^{-1}) than ZCA4 (65.77±12.45 nmol α-ketobutyrate mg^{-1} h^{-1}), but lower than ZCA10 (448.56±0.08 nmol α-ketobutyrate mg^{-1} h^{-1}). These activities are in general much lower than those reported for *B. epidermidis* RS448 (4.9 µmol α-ketobutyrate mg h^{-1}), *B. licheniformis* RS656 (3 µmol α-ketobutyrate mg h^{-1}) (Siddikee *et al.,* 2010), and *B. megaterium* (Chinnaswamy *et al.,*2018), highlighting the large difference in ACC deaminase activity between bacterial strains. It has been reported that a bacterium can promote plant growth with an ACC deaminase activity as low as 0.02 µmol α-ketobutyrate mg^{-1} h^{-1} (Penrose and Glick 2003).

Catalase activity of the isolated strains

Cultures of strains ZCA4, ZCA8, and ZCA10 showed gas bubbles when flooded with drops of hydrogen peroxide, indicating catalase production (Table 1). Catalase as an antioxidant enzyme plays an important role in protecting cells from damages caused by highly reactive oxygen species (ROS), such as hydrogen peroxide, hydroxyl and superoxide radicals that are released particularly under salinity stress (Gill and Tuteja 2010). Such ROS easily attack vital molecules such as proteins, DNA, and lipids (Gill and Tuteja 2010). Our strains could confer tolerance against salinity stress to plants via production of catalase. Our findings on catalase activities are consistent with previous reports, in which diverse halotolerant bacteria with catalase production enhanced plant growth under salinity stress conditions (Sharma *et al.,* 2016).

Antioxidant activity of the isolated strains

The DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay was used to estimate the antioxidant activity of the isolated bacterial strains, and the results obtained are presented in Table 1. The maximum scavenging potential of the corresponding cell-free extract was observed for the strain ZCA4 (58.35 %), followed by strains ZCA10 (18.4 %) and ZCA8 (10.6 %). The antioxidant activity of the positive control, ascorbic acid, was 85.5 %. Antioxidant activities of bacteria confer plant resistance to salt stress via eliminating ROS. These findings are consistent with those obtained by other researchers, who reported growth enhancement of rice seedling under salt stress when inoculated with a halotolerant bacterium, *Enterobacter* sp., via antioxidant activity (Sarkar *et al.,* 2018). It has been reported that abiotic stress such as salinity leads to production of excessive amounts of ROS in plant tissues (Zandalinas *et al.,* 2018).

Phylogenetic analysis of the isolated strains using 16S rRNA gene sequences

Comparative analysis of the 16S rRNA gene is a reliable tool not only for bacterial identification but also for revealing phylogenetic relationships. Strains ZCA4 (MG952579), ZCA8 (MG952580), and ZCA10 (MG952734) exhibited 16S rRNA gene sequences with 99.9 %, 99.7 %, and 99.4

% identities with those of *B. filamentosus* SGD-14^T (KF265351), *J. indicus* 0704P10-1^T (HM222655), and *B. casei* NCDO 2048^T (X76564), respectively. The 16S rRNA gene sequences of the bacterial strains have been deposited in NCBI GenBank. Accession numbers are given in Table 1. A neighbor-joining phylogenetic tree constructed using the 16S rRNA gene sequences of closed related species revealed the taxonomic position of our strains (Figure 1). ZCA4 clearly grouped with members of *Firmicutes* and *Bacillaceae*, whereas both ZCA8 and ZCA10 clustered with *Intrasporagiaceae* and *Brevibacteriaceae*, respectively. The latter two families are members of *Actinobacteria*. These findings show the diversity of rhizobacteria associated with *Z. coccineum.* Similarly, it has recently been reported that taxonomically-diverse plant growth-promoting rhizobacteria, including members of *Actinobacteria* and *Firmicutes*, are associated with roots of *Zygophyllum qatarense* and other halophytic plant species (Bibi *et al.,* 2018).

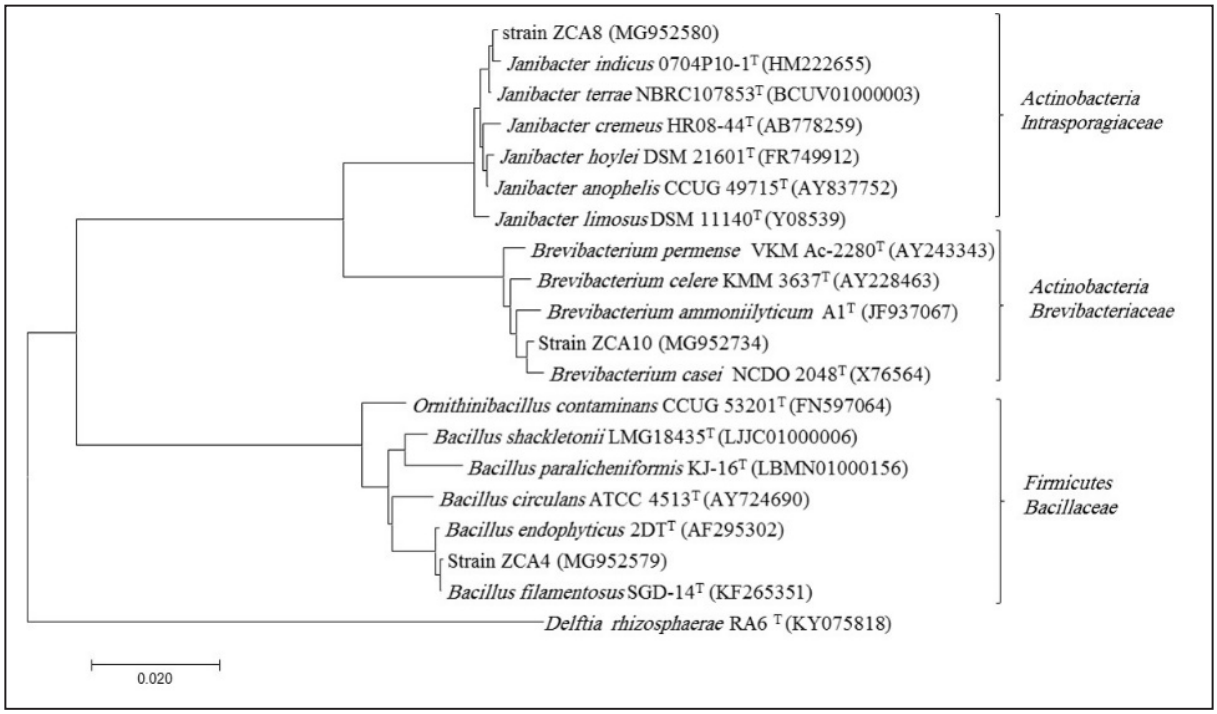

Fig. (1): Phylogenetic analysis of several bacterial strains based on 16S rRNA gene sequences.
The phylogenetic tree was constructed using the neighbor-joining method. The optimal tree with the sum of branch length = 0.29 is shown.

Inoculation of wheat grains with PGPR under different salt concentrations

In the present study, root dry weight of wheat plants reduced significantly in both treatments of NaCl. PGPR under salt stress significantly improved the root dry weights in all bacterial treatments, especially in the plants treated with mixture of the three bacterial strains at 50 mM NaCl, which increased to 169% compared to absolute control plants. In plants without NaCl and treated with the isolated PGPB, there was a significant reduction in root dry biomass, except in case of bacterial mixture treatment (Figure 2). The same negative effect was reported by Sezen et. al. (2016), who noticed that some isolates of PGPB inhibited some growth parameters of wheat plant when seeds subjected to these isolates individually. This negative effect on some growth parameters might be due to production of some kind of phytotoxins that affect the growth of inoculated plants negatively (Khalid *et al*, 2004). In shoots, only high dose of NaCl caused a significant inhibition in shoot dry weight of wheat plants untreated with

PGPB, but this inhibition was improved in plants treated with bacterial mixture (Figure 2). The results are in agreement with those of Bano and Fatima (2009), who showed that salt stress reduced root and shoot dry weights of *Zea mays* plants untreated with PGPB, while PGPB enhanced the growth. Egamberdieva and Hoflich (2003) observed that wheat plants inoculated with a mixture of *P. chlororaphis* and *P. extremorientalis* increases shoot dry weight under salt stress conditions. In addition, Stajković *et al*. (2011) reported that inoculation of bean plant with a mixture of *Rhizobium*, *Pseudomonas* spp., and *Bacillus* spp. improved shoot dry weight.

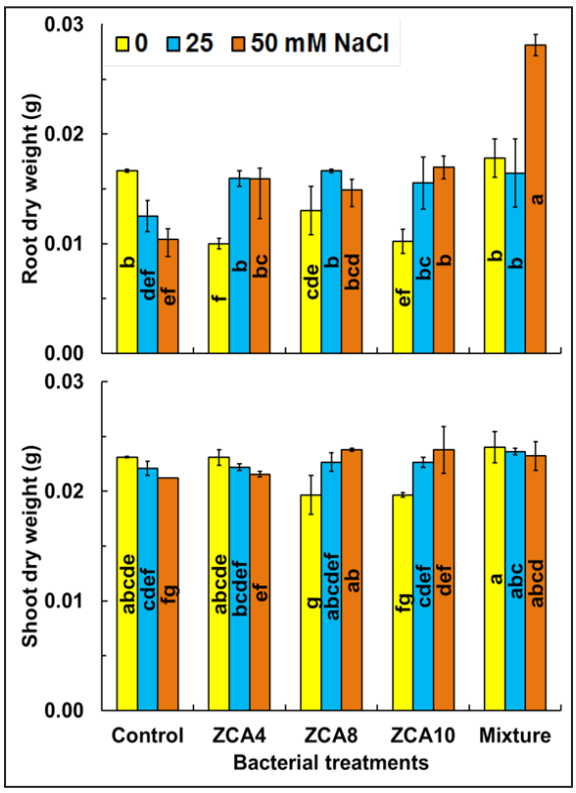

Fig. (2): Effect of NaCl and bacterial inoculation on root and shoot dry weights of 21 old wheat seedlings. Each value is the mean of three replicates and error bars represent ±standard deviation.

The inhibitory effect of salinity on plant growth is a result of reduced water absorption and nutrient uptake by the root system (Dolatabadian *et al*. 2011). Competition among Na^+ and Cl^- and essential nutrients, as N, Mg, and K, is another reason for the negative effects of NaCl stress on plants, as suggested by Heidari *et al*. (2011). In addition, Xiong and Zhu (2002) reported that salinity inhibits phytohormones synthesis and cell wall formation. Production of phytohormones, ACC deaminase, and antoxidant activity are three observed plant growth improvement mechanisms by PGPB. As shown in (Table 1), maximum ACC deaminase enzyme and IAA production was observed in isolate ZCA10, while the maximum antioxidant activity was reported in isolate ZCA4. Orhan (2016), reported that IAA secreted under salt stress by other halotolerant and halophytes species of PGPR, which improved root and shoot length and fresh biomass of the wheat plants. PGPR increased the antioxidants in plants compared to uninoculated plants. Also, in okra plant, Habib *et al*. (2016) reported that inoculation of plants with ACC deaminase-containing PGPR increased root and shoot biomass compared to uninoculated plants under salinity stress.

Under abiotic stresses, like salt stress and drought, plants protect themselves by inducing osmotically-active metabolites. Proline is one these osmolites, which accumulates in plant tissues in response to stress. In this experiment, 50 mM NaCl caused a significant increase in proline concentration in roots, while PGPB modulated this high concentration to a similar level of the absolute control plants. Interestingly, PGPB increased proline accumulation in shoots of unstressed plants. This is might be because proline concentration of tissues and plant organs are regulated by several interplaying factors as biosynthesis, degradation of proline rich proteins and intra/intercellular transport processes. In plants treated with 50 mM NaCl, proline concentration increased significantly in strain ZCA4, while decreased in plants treated with bacterial mixture (Figure 3). PGPB were reported to alleviate the negative effects of environmental stresses by different mechanisms, so multi-inoculants should have high efficiency than single inoculant, which means that PGPB working synergistically.

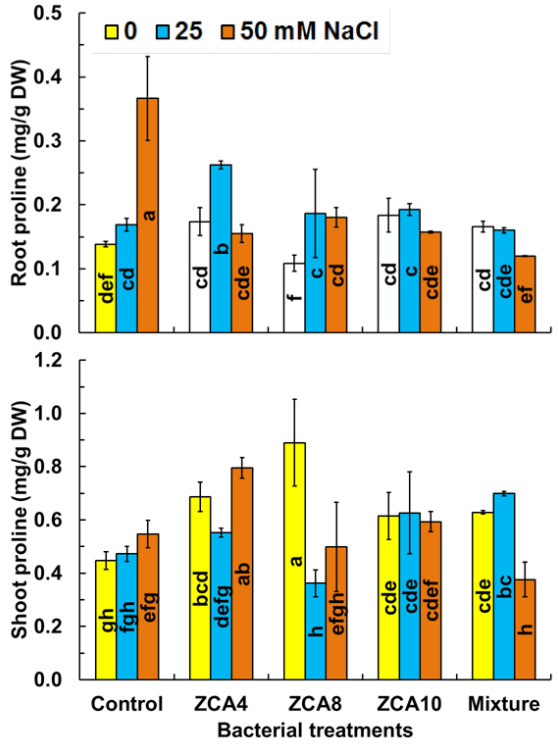

Fig. (3): Effect of NaCl and bacterial inoculation on root and shoot proline contents of 21 old wheat seedlings. Each value is the mean of three replicates and error bars represent ±standard deviation.

These results was in agreement with Nadeem *et al*. (2007), who showed that proline concentration increased due to saline stress, but decreased by PGPB treatment. The same results obtained by Kandowangko *et al*. (2009), who noticed that proline concentration increased by salt stress, but decreased due to PGPB inoculation. Also, Rojas-Tapias *et al*. (2012), found that proline content of *Zea mays* leaves increased by NaCl stress, while significantly decreased due to inoculating the plants with PGPB. Proline improves many enzymes activity, controls the cell pH and helps in detoxifying the effects of reactive oxygen species (Verbruggen and Hermans 2008).

CONCLUSIONS

This is the first report of ACC deaminase-producing rhizobacterial strains (*Bacillus filamentosus*, *Janibacter indicus*, and *Brevibacterium casei*) from the rhizosphere of the halophytic plant *Z. coccineum* growing in Al-Uqair, Al-Ahsa, Eastern region, Saudi Arabia. The results described in this study show that *Z. coccineum* roots harbor diverse bacteria that have the ability to ameliorate the negative effects of salt stress on wheat plants. However, the investigated strains should be tested with other crops to confirm the potential of these bacterial strains under salt stress. It could be concluded that multi-inoculants of PGPB have a critical role in ameliorating the negative effects of salinity stress. Thus, we recommended that seed pretreatment with PGPB may be useful as eco-friendly bio-fertilizer improving wheat growth under saline conditions.

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تخفيف تأثير الإجهاد الملحي في نبات القمح بواسطة البكتيريا المنتجة لإنزيم (ACC deaminase) والمعزولة من ريزوسفير نبات الرطريط

أشرف خليفة[2،1] و أشرف متولي[3،1] و الربعي بن عمار[4،1] و فاطمة علي فرغلي[3]

(1) قسم علوم الحياة، كلية العلوم، جامعة الملك فيصل، الأحساء، المملكة العربية السعودية
(2) قسم النبات والميكروبيولوجي، كلية العلوم، جامعة بني سويف، بني سويف، جمهورية مصر العربية
(3) قسم النبات والميكروبيولوجي، كلية العلوم، جامعة أسيوط، أسيوط، جمهورية مصر العربية
(4) مخبر النباتات الطبية والعطرية، مركز البيوتكنولوجيا بالقطب التكنولوجي ببرج السدرية، حمام الانف، الجمهورية التونسية

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الملخص

توجد البكتيريا المعززة لنمو النبات في منطقة الريزوسفير، وهي المنطقة الملاصقة مباشرة للمجموع الجذري للنبات. أحد الآليات المهمة التي تعزز نمو النبات بواسطة البكتيريا هو إنتاجها لإنزيم (ACC deaminase). في هذا البحث تم عزل ثلاث سلالات بكتيرية منتجة لهذا الإنزيم من ريزوسفير الرطريط المحب للملوحة، والنامي في منطقة العقير بمحافظة الأحساء. وقد تم تعريف تلك السلالات (*Bacillus filamentosus, Janibacter indicus,* and *Brevibacterium casei*) باستخدام تحديد التتابع النيوكليوتيدي للجين (16S rRNA) الريبوسومي، وتم تحديد أوضاعها التصنيفية. وقد أظهرت تلك السلالات قدرة على إنتاج إنزيم الكتاليز وإندول حمض الخليك وإذابة الفوسفات. لُوحظ أن أعلى نشاط لإنزيم (ACC deaminase) كان للسلالة (*B. filamentosus*)، تليها السلالة (*J. indicus*) ثم السلالة (*B. casei*). أظهر تلقيح حبوب نبات القمح بالسلالات المعزولة، منفردة أو مجتمعة، تحسنًا معنويًّا في النمو تحت ظروف الإجهاد الملحي. ومن هذه النتائج يمكن استنتاج أن البكتيريا المعزولة من ريزوسفير الرطريط تقلل من التأثير السلبي للإجهاد الملحي، ويمكن استخدامها كمخصب حيوي صديق للبيئة.

الكلمات المفتاحية: الإجهاد اللاحيوي، البكتيريا المعززة لنمو النبات، الرطريط، القمح، الملوحة.