J. Agr. Sci. Tech. (2016) Vol. 18: 855-864

Promotion of Wheat Growth under Salt Stress by Halotolerant Bacteria Containing ACC deaminase

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ABSTRACT

Salinity is a major abiotic stress that reduces crop productivity in arid and semiarid soils. About 25% of the country's arable land is affected by different levels of salt. A considerable part of this land is under wheat cultivation each year as the country's most important crop. ACC deaminase producing bacteria increase plant resistance to stress condition by reducing stress ethylene in a variety of environmental stresses such as salinity. In this study, 167 halotolerant bacterial strains were isolated from the saline habitats and screened for growth at different NaCl concentrations. These halotolerant bacterial strains were then tested for 1 AminoCyclopropane-1-Carboxylic acid (ACC) deaminase activity. Among six isolates of halotolerant bacteria containing ACC deaminase, the K78 strain produced the highest level of this enzyme. Phylogenetic analysis of the 16S rRNA gene sequence of this bacterium indicated that this strain belonged to *Bacillus mojavensis*. Inoculation of *Bacillus mojavensis* to salt stressed wheat plants produced an increase in root and shoot weight, chlorophyll content, and nutrient uptake in comparison with the un-inoculated soils. In summary, this study indicates that the use of ACC deaminase-producing halotolerant bacteria mitigates salinity stress effects on growth of wheat plants by reducing salt-stress-induced ethylene production.

Keywords: Abiotic stress, *Bacillus*, Ethylene production, Root growth , Salinity.

INTRODUCTION

Plant Growth Promoting Rhizobacteria (PGPR) comprise a group of beneficial bacteria that can be found in the rhizoplane and rhizosphere, the phyllosphere, or inside of plant tissues as endophytes (Glick, 1995; Fernandez-Aunión *et al*., 2010). PGPR enhance plant growth by direct and indirect mechanisms or a combination of both (Siddikee *et al*., 2010). Indirect mechanisms include the suppression of pathogens through the action of siderophores, and the production of antibiotics and extracellular hydrolytic enzymes (Swain *et al*., 2008). Direct mechanisms include altered nutrition through the fixation of atmospheric nitrogen; production of siderophores; producing phytohormones like auxins, cytokinins and gibberellins, solubilizing minerals like phosphorus and iron (Madhaiyan *et al*., 2006; Kang *et al*., 2009) or by the activity of 1-AminoCyclopropane-1-Carboxylic acid (ACC) deaminase, an enzyme that can cleave the plant ethylene precursor ACC that is typically increased by a wide variety of environmental stresses (Cheng *et al*., 2007; Indiragandhi *et al*., 2008; Zahir *et al*., 2009).

Salt stress is one of the major abiotic stress factors that affect almost every aspect of the physiology and biochemistry of a plant, resulting in a reduction in its yield. According to the FAO Land and Plant Nutrition Management Service (2008), over 6% of the world's land, which accounts for more than 800 million ha, is affected by different levels of salinity. Salt stress has previously been reported to cause an

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[DOR: 20.1001.1.16807073.2016.18.3.22.4]

increased production of ethylene in some plants, thereby accelerating leaf, petal and flowers abscission, increased yellowing of leaves, organ senescence, leading to premature death (Zahir *et al*., 2009). Much of the ACC, which is a precursor of ethylene produced under stress conditions, may be exuded from plant roots (Penrose and Glick, 2001) and ACC deaminase can hydrolyze ACC into into ammonia and α-ketobutyrate. Thus, PGPR that have ACC deaminase activity can be used to reduce the negative effects of salinity stress (Zahir *et al*., 2009; Siddikee *et al*., 2010). Halotolerant bacteria are a group of microorganisms able to grow in media containing a wide range of NaCl (1-33%) or in the absence of NaCl (Larsen, 1986). Hence, the present study was conducted in an attempt to isolate and characterize the diverse group of halotolerant bacteria from saline soils for their numerous PGP traits and to check the selected strains for their ability to ameliorate salt stress in wheat plants.

MATERIALS AND METHODS

Soil Sampling and Isolation of Halotolerant Bacteria

Soil samples from the rhizosphere of wheat plants were collected from non irrigated fields in different localities of Iran. A total of 30 soil samples were randomly collected from the rhizosphere of plants. The field-moist soil samples were sieved (< 2 mm), delivered in sealed plastic bags to the laboratory under refrigerated conditions, and stored at 4°C until the analysis. Ten-fold serial dilutions of the samples were made by mixing the soil with sterile saline water (0.9% NaCl), shaking for 20 minutes at 120 rpm, and then plating on agar medium (peptone، 15 g L^{-1}; tryptone, 5 g L^{-1}; dextrose, 2.5 g L^{-1}) modified with 5% (NaCl, and adjusted to a pH of 8.5 (Brisou *et al*., 1974). The plates were incubated at 30°C for 3-4 days. These were screened for salt tolerance and growth in nutrient broth amended with various concentrations of NaCl (0, 5, 10, 15, 20%). Table 1 indicates the growth of isolates at different salt levels. According to the classification of Kushner, the selected isolates were halotolerant bacteria. Pure cultures of the halotolerant bacterial strains were maintained in 30% glycerol at -80°C.

Table 1. The growth rate of isolates (K78 and K216) at different salt levels.

	OD[a] at 600 nm			
	NaCl (%)			
Isolate	0	2.5	5	10
K216	0.850	0.640	0.640	0.189
K78	0.869	0.659	0.670	0.228

[a] Optical density

Qualitative Assay of Utilization of ACC

The availability of 1-AminoCyclopropane-1-Carboxylic acid (ACC) as a nitrogen source is a consequence of the enzymatic activity of ACC deaminase (E.C. 4.1.99.4). ACC deaminase activity was checked according to the method of Glick (1995), with modifications. The bacteria were first cultured in a Tryptone Soya Agar (TSA) medium with 0.9 M NaCl. A solution of ACC (0.5 M) was filter sterilized (0.2 µm) and frozen at -20°C (Penrose and Glick, 2003). Halotolerant bacterial strains were streaked onto Nitrogen Fixing bacterium (NFb) medium supplemented with 3.0 mM ACC as a nitrogen source. Plates were incubated at 30°C for 12 days (Siddikee *et al*., 2010). The ability of a strain to utilize ACC was verified by maintaining the same strain in a control in the absence of any nitrogen source.

Quantification of ACC Deaminase Activity

ACC deaminase activity was assayed according to the method of Penrose and

[DOR: 20.1001.1.16807073.2016.18.3.22.4]

Glick (Penrose and Glick, 2003), which measures the amount of α-ketobutyrate produced when the enzyme ACC deaminase cleaves ACC. The µmole quantity of α-ketobutyrate produced by this reaction was determined by comparing the absorbance of a sample to a standard curve of α-ketobutyrate ranging between 0.1 and 1.0 nmol at 540 nm. A stock solution of α-ketobutyrate was prepared in 0.1M Tris-HCl (pH 8.5) and stored at 4°C. In order to measure the ACC deaminase activity, protein estimation was carried out according to Lowry *et al*. (1951).

Characterization of the Bacterial Isolates

The taxonomic identification of the bacterial isolates, including biochemical characterization and PCR amplification of the 16S rRNA, was performed at the Iranian Biological Resource Center (IBRC). The partial 16S rRNA gene sequences from isolate K78 was determined by the Macrogen Co. in South Korea (using ABI system 3730 XL) and was deposited in the NCBI database under Genbank Accession No: KP067954.

Evaluation of Halotolerant Bacteria Inoculation Effects

The halotolerant bacterial strains K78 (halotolerant and containing ACC deaminase), K216 (halotolerant and lacking ACC deaminase) were selected for the testing of growth promotion of wheat under salinity stress compared to the control (no inoculation). The whole plant including shoot and root system was weighed to determine the Fresh Weight per plant (FW/plant), and then oven dried at 60°C for 48 hours to determine the Dry Weight per plant (DW/plant) for each treatment (Al-Khaliel, 2010). Chlorophyll in plant samples was measured by chlorophyll meter (SPAD-502 meter). In recording the SPAD chlorophyll meter reading, care was taken to ensure that the SPAD meter sensor fully covered the leaf lamina and that interference from veins and midribs was avoided (Songsri *et al*., 2009). Leaves Relative Water Contents (LRWC) were used to evaluate water status and were measured at 37, 67 and 97 Days After Seeding (DAS) (Songsri *et al*., 2009). For the Membrane Stability Index (MSI) estimation ،two sets of plant material (0.1 g each) were each placed in 10 mL of double-distilled water (Sairam and Srivastava, 2001). One set was kept at 40°C for 30 minutes and its conductivity recorded using a conductivity bridge (EC40). The second set was kept in a boiling water bath (100°C) for 15 minutes and its conductivity was also recorded (EC100). The Membrane Stability Index (MSI) was calculated as:

$$MSI = 1 - \frac{EC40}{EC100}$$

Salt Tolerance Index (STI) of plants to different *EC* levels were determined according to Shetty *et al*. (1995) method. Phosphorus was determined by molybdovanadate method (Ryan *et al*., 2001); calcium and magnesium by complexometric; sodium and potassium were measured with flame photometry method (Page *et al*., 1982).

Statistical Analysis

Analysis Of Variance (ANOVA) was carried out on all the data. Significance at the 5% level was tested by Duncan's Multiple Range Test (DMRT) using the SAS package.

RESULTS

Isolation and Screening of Salt Tolerant Bacteria

A total of 167 halotolerant bacterial strains, isolated from 30 soil samples, were able to grow on Nutrient Agar (NA) (0.9% NaCl) plates. Colonies were selected based on color, shape، size, and abundance. These were screened for salt tolerance and growth

[DOR: 20.1001.1.16807073.2016.18.3.22.4]

in nutrient broth amended with various concentrations of NaCl. There was a gradual decrease in number of isolates growing at higher salt concentrations. Increase in concentrations of NaCl from 5 to 20% led to decrease in the bacterial isolates growth from 100 to 10.7%.

ACC Deaminase Activity

The 167 strains were tested for ACC deaminase activity, and 6 strains were found to be positive. Quantification of ACC deaminase activity showed wide variations (Table 2). The activity was highest in the strain Z53 (275 nmol α-ketobutyrate mg^{-1} protein h^{-1}) and strain K78 (253 nmol α-ketobutyrate mg^{-1} protein h^{-1}). Strain K78 was the most resistant to high concentrations of NaCl, and was therefore selected for greenhouse experiments. Strain K216 lacking ACC deaminase activity was selected for use as a negative control. The selected halotolerant bacterial strains were identified by 16S rRNA gene sequencing analysis to ascertain their taxonomic positions. The nucleotide sequences recovered from these bacterial strains were subjected to homology in the NCBI database. K78 and K216 isolates were most closely related to *Bacillus mojavensis* (100% sequence similarity) and (98% sequence similarity), respectively.

Halotolerant Bacterial Inoculation Effect on Wheat Growth

Analysis of fresh and dry weight showed root fresh and dry weight in K78 were significantly higher than the control. K78 increased root dry weight up to 28% as compared to the uninoculated control. Shoot fresh and dry weight decreased under salt stress conditions. This decrease was more pronounced as the exposure time to salinity increased. Shoot fresh and dry weight decreased by 64 and 54%, respectively. According to our results, the addition of K78 led to a significant increase in the shoot fresh and dry weight compared to the control (Figure 1). Relative chlorophyll content of leaves of inoculated and non inoculated plants was measured. Leaf chlorophyll contents of K78 and K216 plants were significantly ($P \leq 0.01$) reduced by increasing NaCl concentration. No significant difference between inoculated and non inoculated plants plants at 8 dS m^{-1} *EC* was observed. K78 plants had greater leaf chlorophyll content than K216 plants at 12, 14, and 16 dS m^{-1} treatments (Figure 2). The *LRWC* of salinity-stressed plants showed a gradual decrease with respect to the increasing salinization. The *LRWC* of salinity-stressed K78 and K216 plants was significantly greater than the control at 12 and 14 dS m^{-1} treatments. However, K78 was able to maintain a higher *LRWC* under salinity conditions, as compared to K216. Although the *MSI* was decreased at the high salinity levels, there was no statistically significant difference detected between the K216 plants and the control at different treatments. Inoculated plants with K78 strain, in the 14 dS m^{-1} level of salinity, significantly increased the *MSI* compared to the other treatments ($P \leq 0.05$). Results reported here indicate that increasing level of salinity led to a significant ($P \leq 0.01$) decrease in *STI* (Figure 2). Inoculated plants with K216 strain did not have any significant effect on *STI* in saline condition and the *STI* significantly decreased in high salinity treatments. But, K78 strain was able to significantly promote the *STI* in the

Table 2. ACC deaminase activity (nmol α-ketobutyrate mg^{-1} protein h^{-1}) of six halotolerant bacteria containing ACC deaminase in DF salt minimal medium supplemented with 0.9M NaCl and 0.5 mM ACC as a sole nitrogen source.

Strains	ACC deaminase activity
H41	47
H63	77
Z57	98
K216	-
Z53	275
K78	253
K15	48

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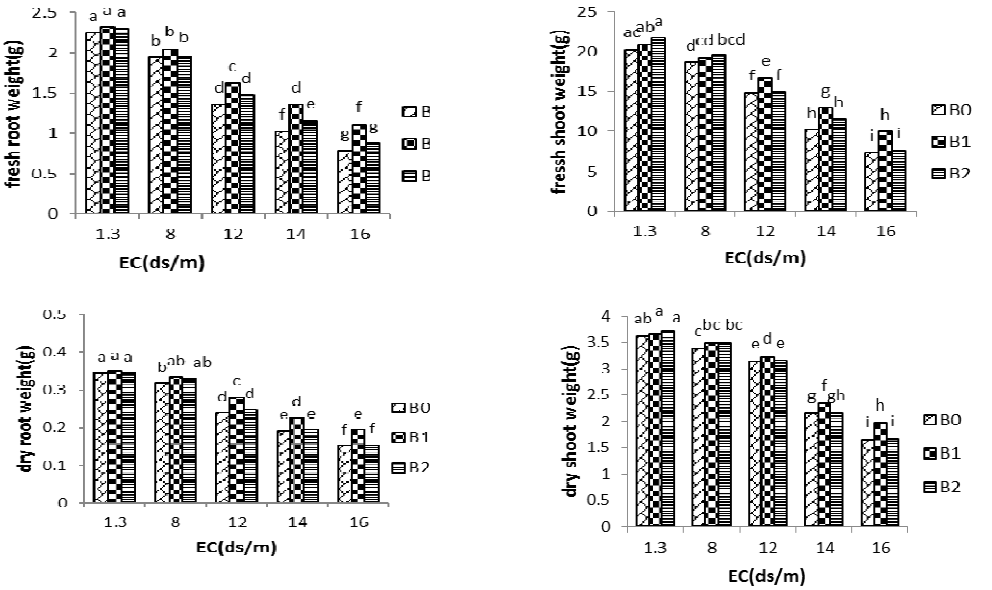

Figure 1. ACC deaminase-producing halotolerant bacteria inoculation effect on shoot and root weight of salt stressed wheat under greenhouse condition. (B0: Not treated with halotolerant bacteria; B1: Treated with K78, B2: Treated with K216).

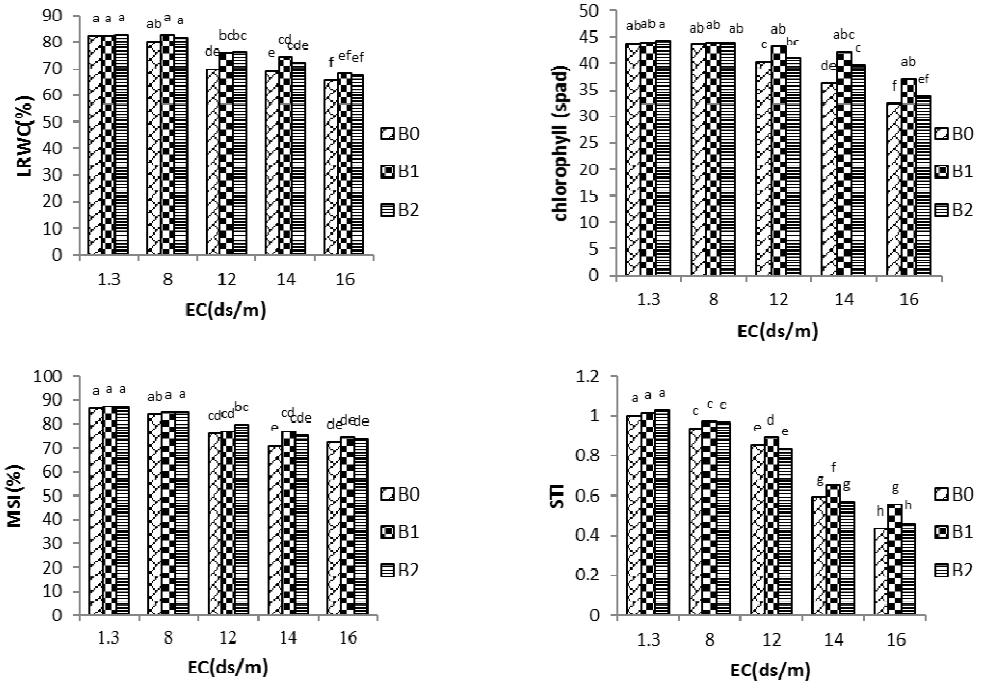

Figure 2. ACC deaminase-producing halotolerant bacteria inoculation effect on chlorophyll content, *LRWC, MSI* and *STI* of salt stressed wheat.

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presence of salt. The concentrations of phosphorus, potassium, calcium, magnesium, and sodium (ppm) ions in plants as a function of the *EC* of the nutrient solution are displayed in Table 3. These results indicate that increasing salt levesl led to a significant ($P \leq 0.01$) rise in the Na^{+}, Ca^{+2} and Mg^{+2} concentrations (ppm) of shoots, whereas there was a significant ($P \leq 0.01$) decrease in shoot P and K^{+} concentration with applied salt. Inoculation with K78 and K216 strains did not affect the P content under stressed conditions. Bacterial strains treatment significantly increased the plant K^{+} content as compared to the control ($P \leq 0.05$). In K78 and K216 treatments, a rise in the Ca and Mg concentrations with increasing *EC* was observed; however, the differences were not statistically significant.

DISCUSSION

PGPR are beneficial bacteria which have the ability to colonize the roots and promote plant growth through either direct or indirect mechanisms, which can be correlated with their ability to form biofilms, chemotaxis, and the production of exopolysaccharide, Indole Acetic Acids (IAA) and ACC deaminase. Salinity is one of the most important abiotic stresses that adversely affect plant growth and crop productivity. Plant species differ greatly in their salt tolerance mechanism. One approach to solve the salinity stress problem is the use of halotolerant bacteria. In the present study, a large number of halotolerant and halophilic bacteria were isolated, and screened for their tolerance levels of NaCl. The results showed that 71 and 11% of strains could grow well at 15 and 20% NaCl, respectively. Tolerance of bacterial strains to higher salinity levels was probably due to the synthesis of protective factors and adaptation of current environmental conditions. Ramadoss *et al*. (2013) found that only 25% (21 out of 84) isolates showed growth at 20% NaCl concentration. Siddikee *et al*. (2011) reported that different halotolerant bacteria were able to withstand high salt concentration (1.75M NaCl) and were able to facilitate plant growth promotion in the presence of growth inhibitory levels of salt. It has been reported that soil salinity plays a prominent role in the microbial selection process as environmental stress leads to reduced bacterial diversity (Damodaran *et al*., 2013). PGPR that contain the ACC deaminase activity could be helpful in sustaining plant growth and development under stress conditions by reducing the level of stress ethylene (Cheng *et al*., 2007; Zahir *et al*., 2009; Ali *et al*., 2014). In the present study, six out of the strains studied produced ACC deaminase. Strain K78, due to being the most resistant in high concentration of NaCl and having high level of the ACC deaminase activity, was selected for greenhouse experiments. Phylogenetic analysis of K78 halotolerant strain 16S rRNA gene sequence revealed it to belong to *Bacillus mojavensis*. ACC-deaminase activity has been widely reported in numerous genera of PGPR like *Azospirillum*, *Bacillus*, *Burkholderia*, *Pseudomonas* and *Rhizobium* (Shaharoona *et al*., 2006; Sharma *et al*., 2013; Barnawal *et al*., 2013; Nascimento *et al*., 2014) but has

Table 3. Halotolerant bacterial inoculation effects on nutrient accumulation (ppm) in the shoots of salt stressed wheat plants.

Source	*DF*	Sodium	Magnesium	Calcium	Potassium	Phosphorus
Salinity	4	2.08^{**}	0.31^{**}	3.08^{**}	5.71^{**}	0.039^{**}
Bacteria (K78)	2	0.003^{ns}	0.002^{ns}	0.088^{*}	0.24^{**}	0.0002^{ns}
Salinity×Bacteria (K78)	8	0.001^{ns}	0.0004^{ns}	0.014^{ns}	0.01^{*}	0.00001^{ns}
Error	30	0.006	0.001	0.017	0.05	0.0004

** $P \leq 0.01$; * $P \leq 0.05$, ^{ns} Not significance.

[DOR: 20.1001.1.16807073.2016.18.3.22.4]

not been reported previously in *Bacillus mojavensis.* In this study, halotolerant bacterial strains were tested for their growth promoting activity under salinity conditions at 1.3, 8, 12, 14, and 16 dS m^{-1}, by conducting experiments on wheat. The results showed that growth parameters were adversely affected by salinity. The reason may be due to the low water potential of soil, interference of the saline ions with the plant's nutrition or the toxicity of accumulated saline ions which caused the restriction of cell growth (Chookietwattana and Maneewan, 2012; Uddin and Juraimi, 2013). Several reports show that ACC deaminase-producing bacteria can affect on plant growth promotion under axenic conditions (Arif *et al*., 2010; Shahzad *et al*., 2010) and in drying soil (Belimov *et al*., 2008), flooded soil (Grichko and Glick, 2001), and normal soil (Ghosh *et al*., 2003; Dey *et al*., 2004; Shaharoona *et al*., 2006). An inoculation of the selected halotolerant bacterium containing ACC deaminase (K78 strain) has resulted in the increases in the fresh and dry weight, chlorophyll content; salt tolerance index, and K^+ concentrations of wheat especially at salinity levels of 12, 14, and 16 dS m^{-1} as compared to the uninoculated treatment and K216 strain that lacked the ability to produce the ACC deaminase. Decrease in fresh weight with higher salinity was due to accumulation of inorganic ions (Na^+ and K^+ ions), which resulted in decreased water level or accumulation of increased compatible solutes (Qurashi and Sabri, 2011). According to previous studies, water and salt stress had detrimental effects on fresh weight accumulation in young leaves of different tomato cultivars (Sturz *et al*., 2000). Increased fresh weight with bacterial inoculations reflects that bacteria might be involved in taking up Na^+ from the media making availability of water to plants or increasing the water status of plants (Afrasayab *et al*., 2010). Maximum reduction of 23% in chlorophyll was observed at 16 dS m^{-1} of *EC*. Bacterial inoculation significantly improved the photosynthetic pigments. Salinity diminishes the photosynthetic activities of plants under salt stress. In general, bacterial inoculations improved the chlorophyll pigments over respective non-inoculated treatments. Salt stress reduced the *LRWC* in both salt-stressed inoculated and non- inoculated wheat, but the *LRWC* was significantly higher in salt-stressed inoculated wheat than in salt-stressed non-inoculated plants at *EC* levels $\geq$ 12 and 14 dS m^{-1}. The index of *LRWC* indicates that *Bacillus mojavensis* improved water content of stressed wheat. *Bacillus mojavensis* can influence plant-water relations by altering the osmotic balance of the cells (Al-Khaliel, 2010). In the present investigation, the tolerance index of ACC deaminase-producing halotolerant bacteria inoculated plants (Average STI= 0.81) was significantly higher than non-inoculated stressed plants (STI= 0.68) providing additional evidence that the increased *STI* might be due to the beneficial effect of PGPR. Previously, ACC deaminase-producing plant growth promoting bacteria enhanced the salt tolerance of canola (Cheng *et al*., 2007) and tomato (Mayak *et al*., 2004). ACC deaminase producing halotolerant bacteria enhance root development and provide more surface area to explore more soil for water and nutrients, eventually leading to increased nutrient uptake (Siddikee *et al*., 2011). The concentrations of sodium, calcium and magnesium ions increased with salinity. Under salt stress, the osmotic pressure in the soil solution exceeds the osmotic pressure in plant cells due to the presence of high salt and, thus, reduces the ability of plants to take up water and minerals like K^+ .On the other hand, Na^+ ions can enter into the cells and have direct toxic effects on cell membranes, as well as on metabolic activities in the cytosol. These primary effects of salinity stress cause some secondary effects like reduced cell expansion, assimilate production, and membrane function, as well as decreased cytosolic metabolism and production of

[DOR: 20.1001.1.16807073.2016.18.3.22.4]

Reactive Oxygen Intermediates (ROSs). The results of this study indicate that the inoculation of ACC deaminase-producing halotolerant bacteria enhanced dry weight in roots, chlorophyll content, and nutrient uptake as well as salt tolerance of wheat plants by reducing the stress ethylene level.Thus, the *Bacillus mojavensis* could be an effective strain to promote the growth of wheat in saline soils.

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[DOR: 20.1001.1.16807073.2016.18.3.22.4]

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بهبود رشد گیاه گندم تحت تنش شوری با استفاده از باکتری متحمل به شوری مولدACC دآمیناز

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چکیده

شوری، یکی از مهمترین تنش‌های غیر زنده‌ای است که باعث کاهش قابلیت تولید محصول در خاک‌های مناطق خشک و نیمه خشک می‌شود. حدود ۲۵ درصد از اراضی زراعی کشور را خاک‌های شور تشکیل می‌دهند که هر ساله بخش قابل توجهی از این اراضی به کشت گندم به عنوان مهمترین گیاه زراعی کشور اختصاص داده می‌شود. باکتری‌های مولد ACC دآمیناز از طریق کاهش اتیلن تنشی در انواع تنش‌های محیطی از جمله شوری خاک باعث افزایش مقاومت گیاه در شرایط تنش می‌شوند.در این مطالعه ۱۶۷ جدایه باکتری تحمل کننده نمک از مناطق شور جداسازی شده و رشد آن‌ها در غلظت های مختلفNaCl بررسی شد. سپس این جدایه‌ها از نظر توان تولید ۱ – آمینو سیکلو پروپان ۱ -کربوکسیلیک اسید (ACC) دآمیناز در محیط حاویACC مورد آزمون قرار گرفت. از میان شش جدایه دارای توان تولید ACC دآمیناز، جدایه K78 دارای توان بالایی در تولید این آنزیم بود که نتایج حاصل از توالی خوانی ژن 16S rRNA نشان داد که این جدایه متعلق به سویه *Bacillus mojavensis* است. تلقیح با جدایه K78 به گیاه گندم تحت تنش شوری موجب اختلاف معنی‌دار در پارامترهای وزن ریشه و اندام هوایی، میزان کلروفیل و جذب عناصر غذایی در در مقایسه با تیمار شاهد (بدون تلقیح) شد. در نهایت این مطالعه نشان داد که استفاده از باکتری‌های متحمل به نمک مولد ACC دآمیناز می‌تواند از طریق کاهش میزان تولید اتیلن در شرایط تنش شوری رشد و نمو گیاه گندم را بهبود ببخشد.

[DOR: 20.1001.1.16807073.2016.18.3.22.4]