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Selection of efficient salt-tolerant bacteria containing ACC deaminase for promotion of tomato growth under salinity stress

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Abstract

For successful application of plant growth promoting bacteria (PGPB) in salt-affected soil, bioinoculant with salt-tolerant property is required in order to provide better survival and perform well in the field. The present study aimed to select the most efficient salt-tolerant bacterium containing 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase from eighty four bacterial strains and to investigate the effects of the selected bacterium on the germination and growth of tomato (Licopersicon esculentum Mill. cv. Seeda) under saline conditions. The Bacillus licheniformis B2r was selected for its ability to utilize ACC as a sole nitrogen source under salinity stress. It also showed a high ACC deaminase activity at 0.6 M NaCl salinity. Tomato plants inoculated with the selected bacterium under various saline conditions (0, 30, 60, 90 and 120 mM NaCl) revealed a significant increase in the germination percentage, germination index, root length, and seedling dry weight especially at salinity levels ranging from 30-90 mM NaCl. The work described in this report is an important step in developing an efficient salt-tolerant bioinoculant to facilitate plant growth in saline soil.

Key words: ACC deaminase, salt stress-induced ethylene, salt-tolerant bacteria

Introduction

Salt-affected soil can result in either the reduction of crop productivity or abandonment of many agricultural areas (Maas, 1990). Under high soil salinity, plant biosynthesizes ethylene by using 1-aminocyclopropane-1-carboxylic acid (ACC) as a precursor, and hence called salt stress-induced ethylene. Numerous reports have documented that ethylene could inhibit the elongation of plant root and shoot (Jusaitis, 1986; Penrose and Glick, 2003), suppress leaf expansion (Peterson *et al.*, 1991) and promote epinasty (Abeles *et al.*, 1992). Thus, reduction of salt stress-induced ethylene production in plant is one of the most crucial strategies to increase agricultural production in saline soil.

In the last decade, the concept of plant growth promoting bacteria (PGPB) containing ACC-deaminase for promotion of plant growth under environmental stress conditions has gained importance (Berg, 2009). These bacteria are capable of metabolizing ACC in the root of developing plants, thereby reducing the adverse effects of ethylene on plant growth (Ghosh *et al.*, 2003; Glick, 2005; Hontzeas *et al.*, 2006). However, the ability of inoculated bacteria to survive, outcompete with the native microflora, and colonize in the rhizosphere remains to be the challenge for successful application (Bashan, 1998) especially in saline soil since high salinity could influence the survival, growth and activity of microorganisms (Bremer and Krämer, 2000). The salt-tolerant ACC deaminase-containing bacteria could thus be advantageous over others to thrive in a new saline environment in the sufficient numbers to deliver beneficial effects on plants. Although the achievements of ACC deaminase-containing bacteria in promoting plant growth under various environmental stresses were reported immensely (Grichko and Glick, 2001; Dey *et al.*, 2004; Mayak *et al.*, 2004; Kausar and Shahzad, 2006; Shaharoona *et al.*, 2006; Belimov *et al.*, 2008; Zahir *et al.*, 2008), little information is available on the effectiveness of salt-tolerant bacteria containing ACC deaminase under high salinity (Nadeem *et al.*, 2006). Therefore, in this research study, attempts have been made to screen and select an efficient salt-tolerant ACC deaminase containing bacterium strain which could utilize ACC as a sole nitrogen source under salt- stressed conditions. Its effectiveness for growth promotion of tomato (*Licopersicon esculentum* Mill cv. Seeda) under *in vitro* and saline conditions has also been evaluated.

Materials and Methods

Microorganisms and culture conditions

Eighty-four bacterial strains, which were isolated from saline soil in Thailand, and identified on the basis of phenotypic characterization, and 16S rDNA sequence analysis from the previous work of Chookietwattana (2003), were used in the experiment. For inoculum preparation, bacterial strains were individually cultured at

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30°C for 24 h on halobacteria medium (Atlas, 1997) which contained the following ingredients per liter: $MgSO_4 \cdot 7H_2O$, 10.0 g; casein hydrolysate, 5.0 g; KCl, 5.0 g; disodium citrate, 3.0 g; KNO_3, 1.0 g; yeast extract, 1.0 g; $CaCl_2 \cdot 6H_2O$, 0.2 g; NaCl, 0.6 M; and agar, 15.0 g. Bacterial cells were scraped from the plates and diluted to obtain 10^8 CFU mL^{-1} by adjusting their optical density at 600 nm to approximately 1.0 using phosphate buffer (0.1 M, pH 7.0).

Screening of salt-tolerant bacterium containing ACC deaminase

The screening procedure comprised three consecutive screening steps. Each step was performed with three replicates. For a primary screening step, the ACC deaminase of eighty-four salt-tolerant bacterial strains were indirectly investigated for their abilities to utilize ACC as a sole nitrogen source by culturing them in 200 mL of DF salt minimal medium (Dworkin and Foster, 1958) supplemented with 0.6 M NaCl and 3.0 mM ACC as a sole nitrogen source. The cultures were incubated for 24 h at 30°C with shaking at 200 rpm and then measured the optical density (O.D.) at 600 nm by spectrophotometer. The first five bacterial strains with the highest O.D. were selected in this screening step. In secondary screening step, the influences of temperature and salinity on the growth of five selected bacterial strains were verified in order to indirectly determine the effects of temperature and salinity on their abilities to produce enzyme ACC deaminase. The effect of temperature was determined by growing the bacteria (1% inoculum) for 24 h in 200 mL of DF salt minimal medium supplemented with 0.6 M NaCl and 3.0 mM ACC as nitrogen source and incubating the cultures at 30, 35 and 45°C with shaking at 200 rpm. The effect of salinity was also evaluated by growing the bacteria in 200 mL of DF salt minimal medium supplemented with ACC (3.0 mM) as nitrogen source and NaCl over a range of 0.2, 0.4, 0.8, 1.2 and 1.6 M (1% inoculum) for 24 h at 30°C with shaking at 200 rpm. Bacterial growth was measured at 600 nm. Only the first three bacterial strains with the highest growth in a wide range of temperature and salinity were selected for further screening step. In tertiary screening step, the ACC deaminase activity of the three selected bacterial strains was determined according to the method of Penrose and Glick (2003). Bacterial strain which had the highest ACC deaminase activity was selected as an efficient salt-tolerant bacterium strain containing ACC deaminase.

Effect of the selected salt-tolerant bacterium containing ACC deaminase on plant growth

Tomato (*Licopersicon esculentum* Mill cv. Seeda) seeds were surface disinfected by immersion in 70% ethanol for 1 min, followed by 15 min in 0.9% (w w^{-1}) sodium hypochlorite. They were then washed three times with sterile distilled water. An inoculum of the efficient salt-tolerant bacterium containing ACC deaminase was prepared. The disinfected seeds were immersed in either the bacterial inoculum (10^8 CFU mL^{-1}) or phosphate buffer (0.1 M, pH 7.0) for one hour and were used as inoculated and uninoculated treatment, respectively. Germination assays were performed according to International Seed Testing Association (2005). Four replicates of 100 seeds were germinated in sterilized Petri dishes (9.0 cm diameter) containing two sheets of filter papers moistened initially with 4 mL of sterile distilled water supplemented with salinity at 0, 30, 60, 90, and 120 mM. The Petri dishes were then placed under artificial light which provided light intensity at 2,000 lux for 16 h daily and at a temperature of 28±2°C. Germination was observed daily. The germination percentage and germination index were calculated. Two weeks after germination, the root length of the seedlings was measured and then dry weight of the seedlings was determined by drying at 70°C for 3 d.

The survival and ability of the inoculated bacteria to colonize plant roots were assessed by the visualization of plant roots under the light and scanning electron microscopy (SEM). For SEM visualization, root pieces of interest were dissected about 1 cm from the whole roots and fixed in 2.5% glutaraldehyde prepared in 0.1 M phosphate buffer of pH 7.2 at 4°C for overnight. The fixed specimens were washed with phosphate buffer three times, each for 15 min. The specimens were further fixed in 1% osmic acid for 2 h at room temperature and rinsed with distilled water for three times, each for 15 min. The samples were frozen with liquid nitrogen for 2 min and then dehydrated using the freeze dryer. After that, the samples were gold coated and examined under a scanning electron microscope (JEOL, JSM-6460LV).

Statistical analysis

Means of bacterial growth were statistically compared using LSD values ($P < 0.05$). The experimental design for the study of germination and seedling growth assessment was two factors factorial arranged in a randomized completely block design; with four replications and 25 seeds per replicate. One way analysis of variance (ANOVA) was made to determine any significant differences between the groups at $P < 0.05$. All statistical analyses were performed by using the Statistix (NH Analytical Software, USA.).

Results and Discussion

Screening of salt-tolerant bacterium containing ACC deaminase

In the primary screening step, of the eighty-four bacterial strains, five bacterial strains namely: *Bacillus*

licheniformis B2r; *Halobacillus trueperi* A1W; *B. vietnamensis* Apa; *B. licheniformis* A8aa; and *B. vallismortis* A6aJ, were selected. This is not a surprising result though *Achromobacter*, *Burkholderia*, *Enterobacter*, and *Pseudomonas* have been recognized as well known ACC deaminase containing bacteria since several species of bacteria in the genus *Bacillus* are also reported as ACC deaminase producer (Ghosh *et al.*, 2003; Penrose and Glick, 2003). Unlike the well known Gram-negative ACC deaminase containing bacteria, *Bacillus* and *Halobacillus* are the Gram-positive endospore-forming bacteria. Their cell structure could assist them to survive well under stressful environmental conditions and thus provide more benefits to plants than the Gram-negative bacteria.

In the secondary screening step, the growth of five salt-tolerant bacterial strains in DF salt minimal medium supplemented with NaCl and ACC as a sole nitrogen source under various levels of temperature (30, 35 and 45°C) and salinity (0.2, 0.4, 0.8, 1.2 and 1.6 M) was observed (Table 1 and 2). In every level of temperature studies, the *B. licheniformis* B2r demonstrated the highest growth. Temperature variation between 30 to 45°C had no effect on the growth of *B. licheniformis* B2r and *Halobacillus trueperi* A1W. The average growth of *B. vietnamensis* Apa from 30 to 45°C were higher than *B. licheniformis* A8aa and *B. vallismortis* A6aJ (Table 1). Thus, *B. licheniformis* B2r, *H. trueperi* A1W and *B. vietnamensis* Apa were chosen as interesting strains in term of ability to utilize ACC at temperature stress condition.

In the study of salt influencing bacterial growth (Table 2), the growth of all bacterial strains was decreased as salinity increased. However, all bacterial strains were able to grow and utilize ACC as a sole nitrogen source at high salt concentration up to 1.6 M NaCl. *B. vietnamensis* Apa demonstrated the highest O.D. at the salinity ranging from 0.2-0.8 M. The growth of *H. trueperi* A1W was not affected by the addition of NaCl at 0.2-0.4 M to the DF salt minimal medium with ACC ($P \geq 0.05$). At 0.8 M NaCl salinity, its growth was dramatically reduced and much lowered than the growth of *B. licheniformis* B2r and *B. vietnamensis* Apa ($P \geq 0.05$). Nevertheless, there was no significant difference in the average growth among *B. licheniformis* B2r, *H. trueperi* A1W and *B. vietnamensis* Apa ($P \geq 0.05$). In addition, the salt concentration level of DF salt minimal medium at 0.4 M (20 g L^{-1}) in which these three strains could grow and utilize ACC as a sole nitrogen source was

Table 1: Growth of five ACC deaminase-containing bacteria in DF salt minimal medium supplemented with 0.6 M NaCl and 3.0 mM ACC as a sole nitrogen source under the various temperature levels. Values indicated were the optical density measured at 600 nm

Bacterial strain	Temperature			$\bar{X}$
	30°C	35°C	45°C	
B. licheniformis B2r	0.6879^{Aa}	0.8337^{Aa}	0.8380^{Aa}	0.7865^{a}
H. trueperi A1W	0.5746^{Aa}	0.5360^{Ab}	0.5746^{Ab}	0.5617^{ab}
B. vietnamensis Apa	0.1614^{Bab}	0.5347^{Ab}	0.5517^{Ab}	0.4159^{bc}
B. licheniformis A8aa	0.3950^{Aab}	0.4353^{Ab}	0.3994^{Ab}	0.4099^{bc}
B. vallismortis A6aJ	0.0390^{Bb}	0.5581^{Ab}	0.3621^{Ab}	0.3198^{c}

Each value represents mean of three replicates per treatment

^{ABC} Values with the same letter within rows indicate no significant difference with $P \geq 0.05$

^{abc} Values with the same letter within columns indicate no significant difference with $P \geq 0.05$

Table 2: Growth of five ACC deaminase-containing bacteria in DF salt minimal medium supplemented with ACC (3.0 mM) as a sole nitrogen source under the various salinity levels. Values indicated were the optical density measured at 600 nm

Bacterial isolate	NaCl (M)					$\bar{X}$
	0.2	0.4	0.8	1.2	1.6	
B. licheniformis B2r	0.5681^{Aab}	0.5157^{ABb}	0.1411^{BCa}	0.0251^{Cc}	0.0244^{Ca}	0.2549^{a}
H. trueperi A1W	0.6591^{Aa}	0.5284^{Aa}	0.0743^{Ba}	0.0263^{Bc}	0.0247^{Ba}	0.2547^{a}
B. vietnamensis Apa	0.6033^{Aab}	0.5610^{BCa}	0.1841^{BCa}	0.0270^{Cab}	0.0233^{Ca}	0.2797^{a}
B. licheniformis A8aa	0.5361^{Aab}	0.3887^{ABab}	0.0267^{Ba}	0.0283^{Ba}	0.0223^{Ba}	0.2004^{ab}
B. vallismortis A6aJ	0.3486^{Ab}	0.1889^{ABb}	0.0673^{Ba}	0.0284^{Ba}	0.0239^{Ba}	0.1314^{b}

Each value represents mean of three replicates per treatment

^{ABC} Values with the same letter within rows indicate no significant difference with $P \geq 0.05$

^{abc} Values with the same letter within columns indicate no significant difference with $P \geq 0.05$

much higher than the salt concentration level at only 5 g L^{-1} which was demonstrated by *Pseudomonas fluorescens* and *Pseudomonas putida* (Jalili *et al*., 2009). As compared to the study of Mayak *et al*. (2004), the growth of our three strains were also higher than the growth of *Achromobacter piechaudii* ARV8, an ACC deaminase containing bacterium, even though the *A. piechaudii* ARV8 was cultured in DF salt minimal medium with addition of 207 mM NaCl and without substitution of ACC to ammonium sulphate. It seemed, therefore, that the *B. licheniformis* B2r, *H. trueperi* A1W and *B. vietnamensis* Apa have the potential to utilize ACC as a sole nitrogen source under high salt stress. Their salt-tolerant properties could also confirm their abilities to survive and show the beneficial effects on promoting plant growth in salt-affected soils over the other ACC deaminase containing bacteria which lack salt-tolerant property. In order to elucidate an efficient salt- tolerant bacterium containing ACC deaminase, the tertiary screening step for comparing their ACC deaminase activities under salt stress was conducted. The results revealed that *B. licheniformis* B2r gave the highest ACC deaminase activity at 862 nmol h^{-1} (Figure 1). Its ACC deaminase activity was also found to be over the range of 170-280 nmol h^{-1} that was reported by Shaharoona *et al*. (2006).

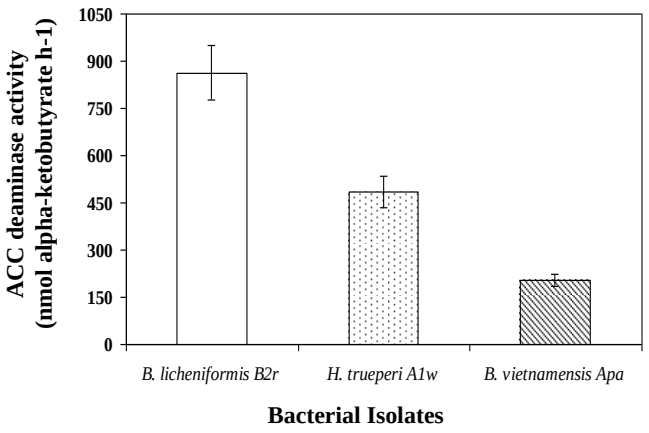

Figure 1: ACC deaminase activity of three ACC deaminase-containing bacteria in DF salt minimal medium supplemented with 0.6 M NaCl and 3.0 mM ACC as a sole nitrogen source. Values are the means of three replicates

From the results of a consecutive screening step, *B. licheniformis* B2r was selected as the efficient salt-tolerant bacterium containing ACC deaminase because it maintained the high growth in DF salt minimal medium having ACC as a sole nitrogen source under the wide ranges of temperature and salinity and also performed the highest ACC deaminase activity at 0.6 M NaCl.

Effect of the selected salt-tolerant bacterium containing ACC deaminase on plant growth

Salt stress is one of the major factors which limit the establishment of plants in different ways depending on plant species. The correlation between ethylene production and seed germination or seedling growth has been well documented (Smalle and Van der Straeten, 1997; Zapata *et al.*, 2004; Glick, 2005). The rationale of this study is based on the hypothesis that an inoculation of the selected salt-tolerant bacterium containing ACC deaminase, *B. licheniformis* B2r, could lower the biosynthesis of salt stress-induced ethylene within a plant which then eliminates the inhibitory effects of ethylene and subsequently enhance plant growth (Penrose and Glick, 2003; Glick, 2005; Hontzeas *et al.*, 2006). The results related to plant growth are summarized in Figure 2. It was noted that all plant growth parameters with respect to germination percentage, germination index, root length, and seedling dry weight decreased as the salinity increased. These results may be due to the low water potential of external medium, interference of the saline ions with the plant's nutrition or the toxicity of accumulated saline ions which caused the restriction of cell growth and lengthening the time needed for germination (Cuartero and Fernaández-Muñoz, 1999).

An inoculation of the selected salt-tolerant bacterium containing ACC deaminase has resulted in the increases in the germination percentage, germination index, root length, and seedling dry weight, especially at salinity 30, 60, and 90 mM as compared to the uninoculated treatments. These results are inline with the studies of Mayak *et al.* (2004); Kausar and Shahzad (2006) in which the effectiveness of rhizobacteria containing ACC deaminase for plant growth promotion under salinity stress was recorded. The results also coincided with the studies on effects of ACC deaminase in promoting plant growth cultivated 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). At 0 mM salinity, the effect of bacterial inoculation on germination percentage, root length, and seedling dry weight was less marked but the effect on germination index was still evidenced when comparing the inoculated treatments to their respective uninoculated treatments. These findings could be due to the fact that tomato is a moderate salt sensitive plant which can tolerate an EC_e (electrical conductivity of the saturated soil extracted) of about 2.5 dS m^{-1} (~ 25 mM NaCl) (Maas, 1990). In addition, a significant increase ($P < 0.05$) in the germination percentage and root length was observe at the salinity 30 mM while the positive

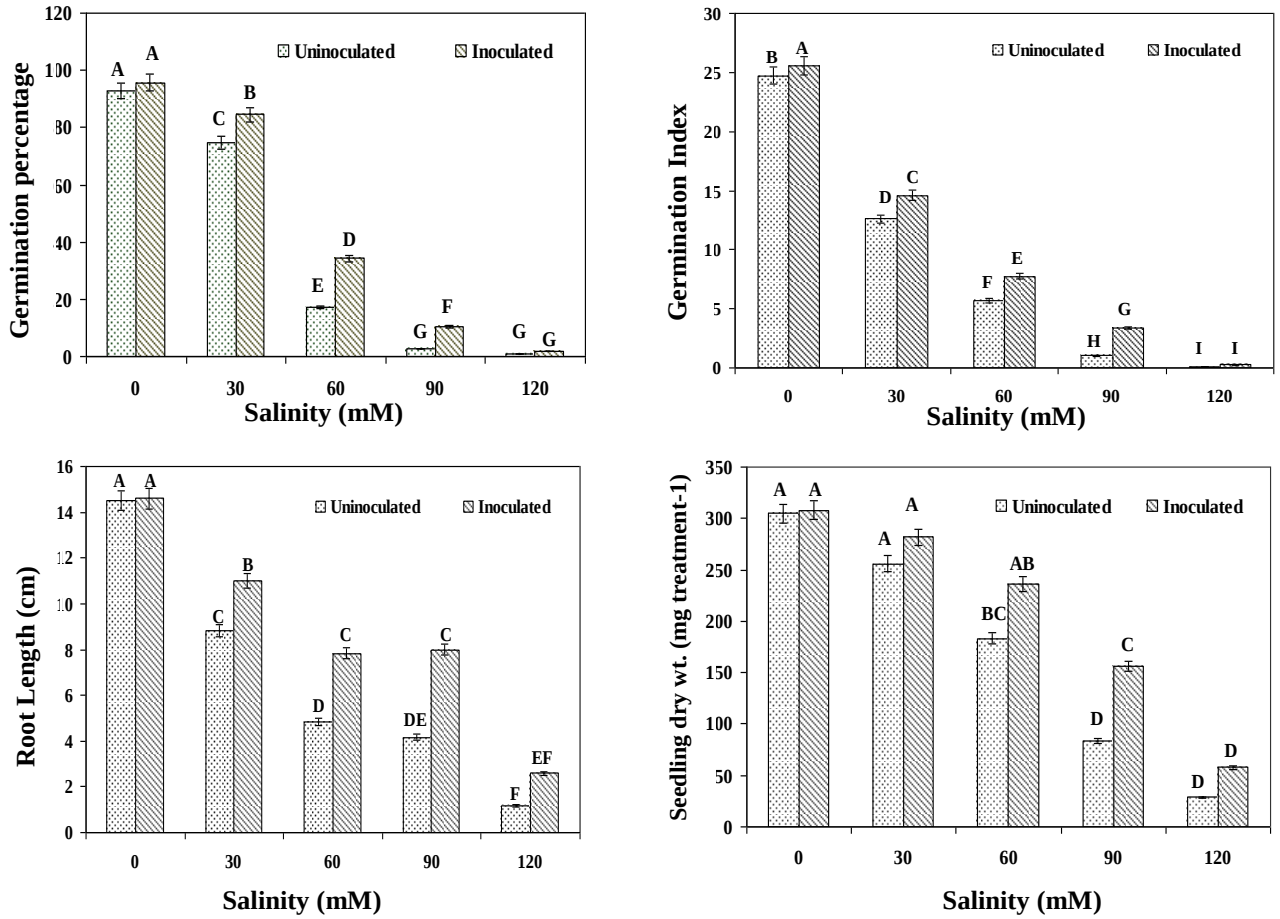

Figure 2: Effect of selected salt-tolerant bacterium containing ACC deaminase on tomato plants grown under diverse salinity. Each value represents mean of four replicates per treatment

[ABC] Values with the same letter above bar indicate no significant difference with $P \geq 0.05$.

effect on the seedling dry weight was observed at the salinity 60 mM. The reason why a significant increase of seedling dry weight was observed at higher salinity extent (60 mM NaCl) than seed germination and root length may be due to an intimate contact of seeds and roots with the salt solution while the shoots only experienced lower salt concentration taken up by the seedlings (Mayak *et al.*, 2004). These results are also in agreement with the findings of Nadeem *et al.* (2006) who found that the significant effects of inoculation with bacteria containing ACC deaminase on shoot/root fresh and dry weight could be observed at higher salinity levels (at electrical conductivity 8 and 12 dS m^{-1}). At 120 mM salinity, there were no significant differences ($P \geq 0.05$) in the germination percentage, germination index and seedling dry weight between the inoculated and uninoculated treatments. These results could be caused by the threshold for salt tolerance which vary among tomato strains. Nevertheless, a significant increase ($P < 0.05$) in the root length was still observed at 120 mM salinity which could infer to a high efficiency of the selected bacterium isolate in reducing the adverse effects of salt stress-induced ethylene at such a high salinity level (Li *et al.*, 2000). According to the results obtained, an effectiveness of bacterial inoculation on plant growth over uninoculated treatment might be related to a high root colonization ability of bacterial inoculants which was confirmed as in the Figure 3.

The results obtained in the present study clearly demonstrated that the selected salt-tolerant bacterium containing ACC deaminase, *B. licheniformis* B2r, can alleviate some of the detrimental effects of salt stress. A number of studies which related to the effects of *B. licheniformis* on promoting plant growth through mechanisms such as gibberellin production (Probanza *et al.*, 2002), phosphate solubilization (Rojas *et al.*, 2001) and

controlling bacterial blotch and seedlings diseases (Powell *et al*., 1990) have been reported. In addition, the results of pot trials also demonstrated an effectiveness of organic fertilizer inoculated with bacteria containing ACC-deaminase for increasing growth and yield of tomato over organic fertilizer without bacterial inoculation (Tahir *et al.*, 2006). Thus, the *B. licheniformis* B2r could be a promise strain for the production of effective ACC-deaminase bacteria-based biofertilizer for saline soil. However, an efficacy of either bioinoculant or biofertilizer (inoculated organic fertilizer) in field is still in need of further investigation.

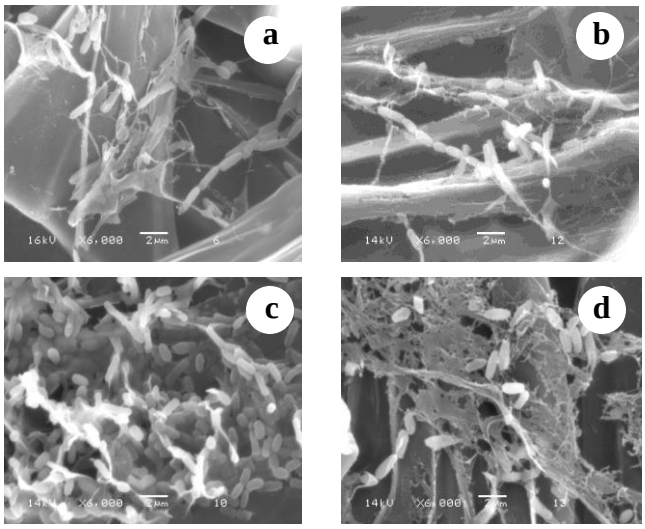

Figure 3: SEM micrographs of tomato roots in which their seeds were inoculated with the selected bacterial strain and grown under: (a) 0 mM NaCl for 7 d; (b) 30 mM NaCl for 7 d; (c) 30 mM NaCl for 14 d; and (d) 120 mM NaCl for 14 d

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