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NOTE

Halotolerant bacteria with ACC deaminase activity alleviate salt stress effect in canola seed germination

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Abstract Amelioration of salt stress effect on canola seed germination was investigated using 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase-producing plant growth promoting halotolerant bacteria. NaCl at 120 mM concentration reduced canola seed germination by 50 %. The inoculation of ACC deaminase-producing halotolerant *Brevibacterium epidermidis* RS15 and *Bacillus aryabhattai* RS341 at 120 mM NaCl significantly increased the seed germination with decreased seed ACC content. Notably, the hydrolytic enzymes activities like amylase, invertase, and protease also increased due to inoculation of RS15 and RS341 compared to uninoculated salt stress imposed germinating canola seeds. Ethylene emission of salt stress exposed eight-day-old canola seedlings was reduced by 35.4 and 41.1 % compared to uninoculated salt stressed control due to respective inoculation of RS341 and RS15. The amelioration of salt stress inhibitory effect on the canola seed germination was attributed to the inoculation of ACC deaminase-producing halotolerant bacteria modulating ethylene emission and inducing hydrolytic enzymes.

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Introduction

Seed germination is the most critical stage in plant ontogeny and is highly responsive to the environmental conditions (Kuriakose and Prasad 2007). The activation and/or synthesis of hydrolases (amylase, invertase, and protease), lipases, and phosphatases (Mayak et al. 2004) facilitates the availability of simpler substances and transported to the growing embryo to provide an energy source for seedling growth and development (Bernhardt et al. 1993). Salinity was reported to delay and decrease the seed germination in various crops like melon (Botia et al. 1998), tomato (Cuartero and Fernandez-Munoz 1999), wheat (Egamberdieva 2009), canola (Jalilia et al. 2009), and groundnut (Saravanakumar and Samiyappan 2007). The delay in germination caused by salinity was primarily attributed to osmotic effects by the excessive ion uptake and accumulation or reduced hydrolytic enzyme activities (Smith and Comb 1991; Dodd and Donovan 1999).

Like any other stress factors, salinity also increases ethylene biosynthesis via elevated levels of ACC (Mayak et al. 2004). Ethylene was known to be involved in seed germination under optimal conditions (Matilla and Matilla-Vazquez 2008), as well as in alleviating the inhibitory effect of the stressed environment on seed germination in many species (Kepczynski and Kepczynska 1997). Still the role of ethylene in germination remains controversial, as ethylene was also found to be inhibiting the germination of salt stressed alfalfa seeds (Zhenguo and Jundi 2001). Although several plant growth-promoting ACC deaminase bacteria reducing stress ethylene under various biotic and

abiotic stress conditions were well studied for their plant growth promotion effects (Glick et al. 1997), so far their role during seed germination under stress conditions is not known. The present study was conducted to test the effect of ACC deaminase-producing halotolerant bacteria on the modulation of ethylene level in germinating canola seeds under the influence of salt stress.

Materials and methods

Two halotolerant bacterial strains: *Brevibacterium epidermidis* RS15 (GU968456) and *Bacillus aryabhattai* RS341 (GU968473) with respective ACC deaminase activities of 2.37 and 1.11 μmol α-ketobutyrate mg^{-1} protein h^{-1} previously isolated from coastal saline soil of the Yellow Sea, Incheon, South Korea (Siddikee et al. 2010) were used in the present study.

Fifty surface-sterilized canola seeds (*Brassica campestris* L; Hungnong seeds, Seminis Korea Inc., Republic of Korea) per treatment in triplicate were used to check germination on sterilized filter paper (Whatman No. 2) soaked in a solution of 0, 75, 120, and 175 mM NaCl in Petri dishes. Germination test was carried out for 96 h at 28 ± 1 °C with a cycle of 12 h of dark followed by 12 h of light (18 μmol m^{-2} s^{-2}) in a plant growth chamber (DS 54 GLP, DASOL Scientific Co., Ltd., Republic of Korea). The number of seeds germinated was recorded at every 24 h. The rate of germination was estimated using a modified Timson index of germination velocity: $\sum G/t$, where G is the percentage of seed germination at 24 h intervals and t is the total germination time (Khan and Ungar 1985).

Halotolerant bacteria were grown in tryptic soy broth (TSB) supplemented with 5 % NaCl and cells were collected and re-suspended in 5 % NaCl containing liquid Jensen's nitrogen free (JNFb) medium supplemented with 3 mM ACC as sole nitrogen source and then incubated for 24 h at 30 °C with shaking (120 rpm) to induce ACC deaminase activity. After that, the cells were harvested, washed, and re-suspended in sterile 0.03 M $MgSO_4$ (10^8 cells ml^{-1}). The bacterial treatment to the seeds were given by immersing them in bacterial cells suspended $MgSO_4$ solution for 4–6 in shaker with 80 rpm. Seeds without any treatment served as negative control (NC) and seeds treated only with salt were used as positive control (PC). Fifty seeds per treatment were placed on sterilized filter paper (Whatman No. 2) in Petri dishes and incubated for 4 days. Every 12 h, the petri dish covers were removed at an open air circulation area to record the germination and to moisten the filter paper. The remaining seeds were used to assay the levels of ACC concentration and endogenous hydrolytic enzyme activities. From each treatment, 1 g of germinated seeds was stored at −80 °C to measure ACC concentration. ACC concentration was determined by following the protocol of Madhaiyan et al. (2006).

Another one gram of germinated canola seeds was used to assay the endogenous hydrolytic enzyme activity. The seeds under various treatments were ground in a mortar and made into paste in liquid nitrogen with cold 0.1 M phosphate buffer (5 ml) of respective pH (for amylase-pH 6.7, for invertase and protease-pH 7.0) and centrifuged at 8,000*g* for 15 min at 4 °C. The supernatants were collected and used for enzyme assay. Amylase and invertase activities were assayed as per the method described by Adewale and Oladejo (2009) and protease activity was assayed according to Reimerdes and Meyer (1976).

Ethylene emission from canola seedlings was measured following the protocol of Mayak et al. (2004) with slight modification. Canola seeds were surface sterilized, treated with halotolerant bacteria as described previously. Two pieces of filter paper were placed inside 120 ml narrow neck bottles, and 2 ml of sterilized de-ionized water was added to each bottle then autoclaved at 121 °C for 15 min. After cooling down to room temperature, 35 seeds were placed in each bottle then incubated at 28 °C. Three days after germination, inoculated seeds were irrigated with 2 ml of nitrogen free Hoagland's solution with the respective isolates (1×10^8 cfu ml^{-1}). Eight days after germination, the excess liquid was drained and 2 ml of 120 mM NaCl solution was added. Seeds and seedlings without any treatment served as NC and seedlings treated only with salt were used as salt PC. Additionally, salt stressed seedlings were treated with 2 ml of 10 μM $ZnCl_2$ (inhibitor of ACO and ethylene production) solution and used as chemical control (CC). Four hours after the addition of salt, the bottles were closed for 6 h with a rubber septum and the ethylene from the headspace was analyzed.

Results and discussion

Seed germination of several plants has been reported to decline with increasing salinity levels (Saravanakumar and Samiyappan 2007; Egamberdieva 2009; Jalilia et al. 2009). Cheng et al. (2007) reported that 150 mM NaCl reduced approximately 50 % growth of canola seedlings. In the current study, we evidenced canola seeds germinating at a higher rate of 96 % under normal condition in the absence of salt, was reduced to 50 % under 120 mM NaCl stress. Conversely, seed germination increased by 68.7 and 64.0 % due to respective inoculation of *B. epidermidis* RS15 and *B. aryabhattai* RS341 at 120 mM NaCl. Halotolerant bacterial inoculation showed more effectiveness at the lower salinity (75 mM NaCl) showing complete alleviation of the salt stress effect (Table 1). Several earlier studies have reported an increase in ethylene emission during seed germination

Table 1 Halotolerant bacterial inoculation effect on germination (%) of canola seed under salt stress

Treatments	Germination percentage[a]			
	0 mM NaCl	75 mM NaCl	120 mM NaCl	175 mM NaCl
Uninoculated control	96.6 ± 1.9	93.3 ± 3.8a	50.0 ± 3.8b	23.3 ± 5.1c
B. epidermidis RS15	ND	95.8 ± 2.4a	95.3 ± 2.4a	66.67 ± 4.8b
B. aryabhattai RS341	ND	95.8 ± 2.4a	83.4 ± 2.4a	62.5 ± 4.2b

ND not determined

[a] Values (% mean ± SE, $n = 3$) in column with same letters do not differ significantly at $P < 0.05$ (LSD)

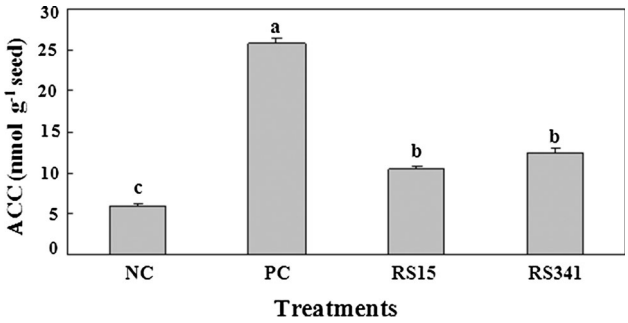

Fig. 1 Canola seeds germination at different levels of salt stress

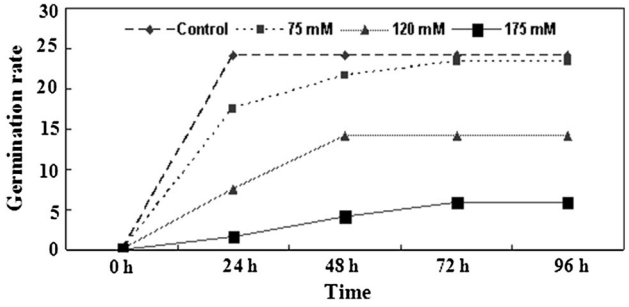

Fig. 2 Effect of ACC deaminase-producing halotolerant bacterial inoculation on ACC concentration in germinating canola seeds under salt stress. *NC* negative control, seeds not treated with halotolerant bacteria and salt, *PC* positive control, seeds not treated with halotolerant bacteria but exposed to 120 mM NaCl stress, *RS15, RS441* Inoculation, seeds treated with *B. epidermidis* RS15/*B. aryabhattai* RS341 and exposed to 120 mM NaCl stress. ACC concentration (nmol g^{-1} seed) with *same letters* do not differ significantly at $P < 0.05$ (LSD)

parallel to the salt concentration (Khan and Ungar 1985; Chang et al. 2010; Patricia et al. 2010). We also recorded a higher ACC content (25.9 nmol g^{-1} seed) in germinating canola seeds with 120 mM NaCl stress for 72 h. The inoculation of *B. epidermidis* RS15 and *B. aryabhattai* RS341 reduced the ACC content in the germinating canola seeds considerably (Fig. 1). Earlier studies have shown that inoculation of PGPR possessing ACC deaminase can break the ACC into ammonia and α-ketobutyrate, the immediate precursor of ethylene resulting in the reduction of ACC level (Mayer and Mayer 1989; Penrose and Glick 2001).

In angiosperms, the mobilization of polymers like seed proteins and carbohydrates to facilitate seed germination, seedling development, and successful plant establishment is crucial, as this phase of plant growth is thought to be the weakest in the life cycle of plants (Stebbins 1974). Canola seed germination reached its peak within 24 h, and thereafter no further increase in germination was recorded in control but salinity proportionately delayed the germination beyond 24 h with increasing salt concentration (Fig. 2). Several reports suggested that hyper-saline conditions could cause delayed germination (Prado et al. 1995) due to reduced hydrolytic enzyme activities and

Table 2 Effect of salt stress and bacterial inoculation on enzyme activities of germinating canola seeds

Treatment[a]	Enzyme activity[a]		
	Amylase[b]	Invertase[b]	Protease[b]
Negative control (NC)	695.8 ± 6.4a	325.9 ± 2.5a	913.7 ± 3.0a
Positive control (PC)	193.1 ± 2.8d	118.4 ± 0.7d	328.1 ± 1.7d
B. epidermidis RS15	453.6 ± 3.6b	262.1 ± 1.1b	807.4 ± 2.5b
B. aryabhattai RS341	415.3 ± 3.6c	244.4 ± 1.1c	748.8 ± 1.9c

All treatments were treated with 120 mM NaCl except Negative control

NC seeds not receiving any treatment, *PC* seeds receiving salt treatment alone

[a] Values (mean ± SE, $n = 3$) in column with same letters do not differ significantly at $P < 0.05$ (LSD)

[b] Unit; Amylase = μM maltose g FW^{-1} m^{-1}, Invertase = μM glucose g FW^{-1} m^{-1} , Protease = μM tyrosine g FW^{-1} m^{-1}

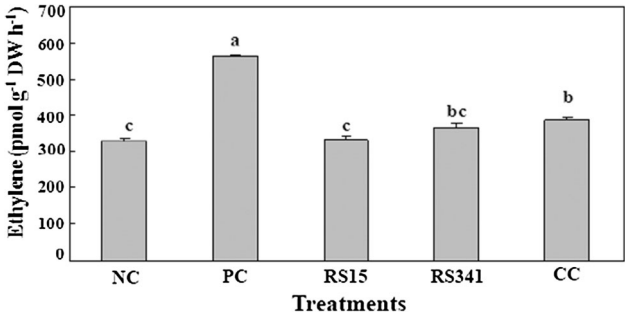

Fig. 3 Effect of ACC deaminase-producing halotolerant bacterial inoculation on ethylene production on canola seedlings under salt stress. *NC* negative control-seeds without any treatment, *PC* positive control-seeds exposed to 120 mM NaCl stress for 2 h, *RS15, RS441* Inoculation, seeds treated with *B. epidermidis* RS15/*B. aryabhattai* RS341 and exposed to 120 mM NaCl stress for 2 h, *CC* Chemical control-seedlings treated with 10 μM $ZnCl_2$ and exposed to 120 mM NaCl stress for 2 h.; Ethylene production (pmol g^{-1} DW h^{-1}) with *same letters* do not differ significantly at $P < 0.05$ (LSD)

mobilization of seed metabolites (Smith and Comb 1991). Abscisic acid (ABA) was reported as a potent inhibitor of storage protein mobilization (Garciarrubio et al. 1997) and galactomannan degradation in seeds of lettuce (*Lactuca sativa*), tomato (*Lycopersicon esculentum*) (Toorop et al. 1999). Contrastingly ethylene at low level, in an antagonistic way stimulated the activities of α-galactosidase and endo-β-mananase in *Arabidopsis* (Bialecka and Kepczynski 2007), lettuce (Nascimento et al. 2004) and tomato (Pirrello et al. 2006). The ethylene production during seed germination under optimal conditions (Matilla and Matilla-Vazquez 2008) as well as under stressed environments has been evidenced in many plant species (Kepczynski and Kepczynska 1997). Renata and Gniazdowska (2012) proposed a biphasic pattern of ethylene emission; a small peak with extremely low ethylene crucial for the activation of embryo axis during very early phase of germination and a larger peak during embryo expansion and radicle protrusion in a variety of seeds. An ideal balance in ethylene level during seed germination was emphasized for a successful physiological performance of the seed and plant establishment (Patricia et al. 2010). Therefore, we hypothesize that ethylene production during seed germination under salt stress is critically needed at optimum concentration and seed bacterization of halotolerant ACC deaminase bacteria can play a vital role in ethylene level modulation.

In our study, enhanced amylase, invertase, and protease activities evidenced in the ACC deaminase possessing halotolerant bacteria inoculated canola seeds than the uninoculated ones under salt stress (Table 2) may be attributed to the corresponding decrease of ACC content and simultaneous reduction in seed ethylene level (Fig. 3) to achieve an ideal balance between ethylene and ABA for effective stored food mobilization in seeds as earlier discussed by Patricia et al. (2010) in *S. virgata.* Therefore, in the current study, the amelioration of salt stress effect on seed germination could be due to the consequence of modulating ACC concentration by the ACC deaminase activity of halotolerant bacteria. Though we have found out a correlation of the decreased ethylene emission with increased seed enzyme activity in the salt stressed canola seed germination, the critical ethylene level needed to overcome salinity stress is yet to be understood.

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