Screening of Strong 1-Aminocyclopropane-1-Carboxylate Deaminase Producing Bacteria for Improving the Salinity Tolerance of Cowpea

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Abstract

Aim: To isolate rhizobacteria containing 1-aminocyclopropane-1-carboxylate (ACC) deaminase and evaluate the ability of selected bacteria for improving the growth of cowpea seedlings under salt stress conditions.

Methods: This study isolates salt-tolerant rhizobacteria which have strong ability to produce ACC deaminase and the phytohormone indol-3-acetic acid (IAA). Inoculation experiments with selected bacteria strains were used to verify the plant growth promoting activity of bacteria under salt stress conditions.

Result: Two isolates belong to Enterobacter cloacae and one isolate belongs to Pseudomonas sp. have been identified. Those rhizobacteria were found to be highly salt-tolerant at salinity level up to 10% NaCl. The selected bacterial strains were also capable to produce and secrete large amounts of ACC deaminase and the phytohormone IAA into the growth medium. Cowpea plants inoculated with ST3 strain revealed a significant increase in shoot length and shoot fresh weight over uninoculated control at the salinity level of 1.5% NaCl.

Conclusion: Three rhizobacterial strains belonging to the genera Enterobacter and Pseudomonas have been isolated. All three bacterial strains were identified as moderate halophiles and they can produce high levels of ACC deaminase and IAA. The strain Pseudomonas sp. ST3 showed the possible ability to promote the growth of cowpea under salt stress conditions.

Keywords: ACC deaminase; Cowpea, Enterobacter, Pseudomonas; Salinity tolerance

Introduction

Soil salinization from the agricultural standpoint is one of the most urgent problems in many areas worldwide, especially in agricultural countries like Vietnam. Salinization can be caused by saltwater invasion, long and severe drought, excessive use of chemical fertilizers. As a result, it can impact agricultural production, water quality, ecological health of streams and biodiversity. According to Qadir et al. [1], every day for the last two decades, about 2000 hectares of irrigated land in arid and semi-arid areas across 75 countries have been degraded by salt.

When exposed to salt stress conditions, plant tissues synthesize ethylene from its immediate precursor 1-aminocyclopropane-1-carboxylate (ACC). High levels of ethylene in plant tissues could inhibit the growth of root and shoot [2-4] as well as suppress leaf expansion [5]. Recently, researchers have found that several ACC deaminase producing bacteria can promote the growth of plants under salt stress conditions [6,7]. Such plant growth promoting bacteria (PGPR) belong to the genera such as Alcaligenes, Variovorax, Rhodococcus, Ochrobactrum and Bacillus [8,9]. Mechanism for this ability of PGPR is that enzyme ACC deaminase can hydrolyze ACC into ammonium and α-ketobutyrate, and as a result, the ACC level in plant tissues is decreased [10,11].

Beside the ability to produce ACC deaminase enzyme, some PGPR belonging to the genera Alcaligenes, Bacillus and Ochrobactrum sp. can also synthesize the phytohormone Indole-3-acetic acid (IAA) [8]. Previous studies have shown that inoculation with PGPR producing both ACC deaminase and IAA can enhance the salt tolerance and consequently improving the growth of plants under salt stress conditions. Such PGPR have shown the ability to improve the growth of soybean and maize under salt stress in the field conditions, however, these effects are still limited [12,13].

To date, little information is available on the isolation, identification and application of rhizobacteria for improving crops growing under salt stress conditions in Vietnam. Therefore, the present study aims to isolate the strong ACC deaminase and IAA producing rhizobacteria from salt-affected area of Danang, Vietnam. Furthermore, the abilities of selected rhizobacteria to promote the growth of cowpea under salt-stress conditions were also investigated.

Materials and Methods

Isolation of ACC deaminase producing rhizobacteria

Soil samples were collected from the rhizosphere of water spinach (Ipomoea aquatica L.) in the depth of 0-10 cm at Son Tra peninsula, Danang, Vietnam (16°10′ N, 108°22′ E). The rhizobacteria in soil samples were isolated by the method of Penrose and Glick [11]. The bacterial isolates observed by using this specific isolation method are
known to have the ability to use ACC as the sole nitrogen source. These selected isolates were stored at −80°C for further experiments.

Identification of bacterial strains

In brief, the purified genomic DNA from 3 isolates was used as the templates for the amplification of conserved region of the 16 s rRNA gene with the following universal primers: 27 F 5’-AGAGTTTGATCCTGGCTCAG-3’; 1492 R 5’-GTTACCTTGTGACGAC TT-3’. The PCR products were then checked on 0.8% agarose gel. The DNA band of the 16 s rRNA gene (approximately 1500 bp) was cut and purified by GeneJet Gel Extraction Kit (Thermo Scientific). The purified PCR products were then sent to Eurofins (Germany) for sequencing. The full-length of the 16 s rRNA gene sequence (1500 nucleotides) was determined by direct sequencing of PCR-amplified 16 s rDNA. The nucleotide sequences obtained were compared to the references of the 16 s rRNA gene sequences retrieved from GenBank database by using BLAST (NCBI, USA).

Analysis of the ACC deaminase activity

Selected bacterial strains were grown in tryptic soybean broth (TSB) medium at 30°C, 200 rpm for 24 h. Bacterial cells were then transferred to the Dworkin and Foster salt minimal medium (DF) containing 3 mM ACC as the sole nitrogen source and grown at 30°C, 200 rpm for 48 h to induce the production of ACC deaminase. Culture supernatant was collected at 12, 24, 36 and 48 h. The ACC deaminase activity was determined by measuring the production of α-ketobutyrate generated by the cleavage of ACC according to the protocol described by Penrose and Glick [11]. The amount of α-ketobutyrate produced by this reaction was measured by comparing the absorbance at 540 nm of the sample to a standard curve of α-ketobutyrate (Sigma-Aldrich Co, USA) ranging between 0.1 and 1.0 µM. The ACC deaminase activity was expressed as the amount of α-ketobutyrate produced per mg of protein per hour.

Analysis of the bacterial salt tolerance

Each bacterial strain was grown in 6 flasks containing 50 mL Nutrient Broth (NB) medium supplemented with the following concentrations of NaCl: 0%, 2%, 4%, 6%, 8% and 10% (w/v). The flasks were incubated at 30°C, 200 rpm for 48 h and the absorbance at 600 nm of each sample was measured afterwards. The standard deviation based on 3 independent cultivations.

Analysis of the IAA production

In order to analyze the IAA production, each bacterial strain was grown in DF salt minimal medium containing 50 mL distilled water and applied to the plant rhizosphere at day 7 based on 3 independent cultivations.

Effect of bacteria on plant growth under salt stress conditions

In this experiment, cowpea (Vigna unguiculata) seeds were surface-disinfected by immersion in 70% (v/v) ethanol plus 0.3% (v/v) Tween 80 for 5 minutes and later by solution containing 3% (w/v) HCl and 0.3% (v/v) Tween 80 for 20 minutes. Seeds were then washed three times with sterile distilled water. For germination, sterilized cowpea seeds were soaked in warm water (60°C) for 12 h and then covered with a clean cloth for 12 h. Soil for planting was sterilized at 121°C, 1.2 atm for 30 minutes in an autoclave and then transferred to planting pots (20 cm diameter x 15 cm height).

For each crop, four sets of pots with three replicates were prepared: control (applied water only), NaCl (applied 1.5% NaCl solution), T3 (applied ST3 cells solution), T3 + NaCl (applied ST3 cells solution and 1.5% NaCl solution). For each pot, 20 germinated cowpea seeds were transplanted at the same depth (approx. 2 cm below the surface). The plants were grown under natural light and temperature (14 h photoperiod, 25-35°C). Salinity was imparted to the plant by adding 1.5% (w/v) NaCl solution to the rhizosphere of the plants at day 7 after planting, three times a week. The bacterial strain ST3 was grown in DF salt minimal medium (10° CFU/mL) and centrifuged to collect the cell pellets. Bacterial cell pellets were then resuspended in 200 mL sterile distilled water and applied to the plant rhizosphere at day 7 after planting, once a week. Seedlings were harvested on day 30 for measuring the shoot length and shoot fresh weight.

Data regarding the effect of bacteria on shoot length and shoot fresh weight of cowpea were statistically analyzed by one-way analysis of variance (ANOVA), followed by Duncan’s multiple range test (DMRT) at p ≤ 0.05. Statistical analysis was performed using the R software for Windows (version 3.2.2).

Results

Screening of salt tolerant PGPB producing ACC deaminase

In the screening step, among twenty-five isolates selected from soil samples, only three isolates designated ST3, ST15 and ST23 were able to use ACC as the sole nitrogen source. The genomic DNA samples from those isolates were then isolated and used as templates for the amplification and subsequent sequencing of the 16 s rRNA genes. The 16 s rRNA gene sequence similarity values for isolates ST3, ST15, ST23, and related strains based on the partial sequence comparison were analyzed (data not shown). It was found that ST15 and ST23 belong to the Enterobacter genus, while ST3 showed highly close genetic relationship with Pseudomonas. In particular, ST15 and ST23 showed high 16S rRNA gene similarity of more than 99% with Enterobacter cloacae strain JNVU TW6 (accession number: 530252117). Therefore, the selected bacteria were named as Pseudomonas sp. ST3, E. cloacae ST15 and E. cloacae ST23, respectively. The obtained 16s rRNA gene sequences were then deposited into the GeneBank Nucleotide Sequence Database (NCBI) under accession numbers KU049661, KU049659 and KU049660 (not yet released) for strains ST3, ST15 and ST23, respectively. The phylogenetic tree between ST3, ST23, ST15 and related taxa (Figure 1).
Figure 1: Dendrogram showing the genetic relationships between ST3, ST15 and ST23 with related bacterial strains. ST3: Pseudomonas sp. strain ST3; ST15: E. cloacae strain ST15; ST23: E. cloacae strain ST23. Number indicates the mean genetic distance between different species.

Data of ACC deaminase assay revealed that all three selected strains showed the ACC deaminase activities ranging between 0.7 and 0.9 µM α-ketobutyrate/mg/h after 48 h of cultivation (Figure 2). Among three strains, ST3 showed the highest ACC deaminase activity of about 0.9 µM α-ketobutyrate/mg/h.

Figure 2: ACC deaminase activity of three bacterial strains (n=3, independent cultivations). ACC deaminase activity of selected bacterial strains grown in DF salt minimal medium supplemented with 3 mM ACC. White bars Pseudomonas sp. ST3; grey bars E. cloacae ST15; black bars E. cloacae ST23.

Screening of bacterial strains for high salinity tolerance showed that all three bacterial strains were able to grow well at the salinity level ranging between 0-6% (w/v) NaCl. The growth rates were highest for all bacterial strains in the medium supplemented with 2% (w/v) NaCl (Figure 3). Although the growth rate of the selected bacterial strains was decreased when concentrations of NaCl increased, those strains exhibited the ability to grow at high NaCl concentrations up to 10% (w/v).

Figure 3: Growth curves of three bacterial strains in the presence of six concentrations of NaCl. Growth of selected bacteria in NB medium supplemented with 0, 2, 4, 6, 8 and 10% NaCl. Lines indicate cell growth while symbols indicate supplemented NaCl concentrations. (A) Pseudomonas sp. ST3; (B) E. cloacae ST15; (C) E. cloacae ST23.

In this study, the plant growth promoting characteristic of bacterial strains based on the ability to produce phytohormone IAA was also investigated. When grown in DF salt minimal medium supplemented with 2 mg/mL tryptophan all three bacterial strains started to produce IAA after 12 h of cultivation and the amount of IAA reached the maximum values at 48 h (Figure 4). This result also demonstrated that the strain ST3 produced higher amount of IAA when compared to the two strains ST15 and ST23. Analysis showed that the highest positive correlation value (Pearson Correlation) between the IAA and ACC deaminase production was observed for strain ST3 (r=0.901, p ≤ 0.01).
Strains ST15 and ST23 had lower r values of 0.894 and 0.777 (p ≤ 0.01), respectively.

Figure 4: Concentration of the phytohormone (IAA) produced by three bacterial strains after 48 h of cultivation (n=3, independent cultivations). IAA production of bacterial strains grown in DF salt minimal medium supplemented with 2 mg/mL tryptophan. White bars *Pseudomonas* sp. ST3; grey bars *E. cloacae* ST15; black bars *E. cloacae* ST23.

**Figure 4:** Concentration of the phytohormone (IAA) produced by three bacterial strains after 48 h of cultivation (n=3, independent cultivations). IAA production of bacterial strains grown in DF salt minimal medium supplemented with 2 mg/mL tryptophan. White bars *Pseudomonas* sp. ST3; grey bars *E. cloacae* ST15; black bars *E. cloacae* ST23.

**Table 1:** Shoot length and shoot fresh weight of cowpea after 30 days of treatment.

| Treatment                      | Shoot length (cm) | Shoot fresh weight (g) |
|--------------------------------|-------------------|------------------------|
| Control (fresh water only)     | 14.1\(^{±}\) ± 0.17 | 18.7\(^{±}\) ± 0.08    |
| NaCl (with 1.5% NaCl solution) | 11.5\(^{±}\) ± 0.14 | 11.7\(^{±}\) ± 0.09    |
| T3 (with ST3 cells)            | 18.6\(^{±}\) ± 0.25 | 26.8\(^{±}\) ± 0.07    |
| T3+NaCl (1.5% NaCl with ST3 cells) | 14\(^{±}\) ± 0.1  | 22.7\(^{±}\) ± 0.05    |

Note: Means ± SEM (Standard error of means) of triplicates with 20 seedlings for each treatment (n=60). Mean values sharing different superscript letters in column are significantly different according to Duncan’s multiple range test (p ≤ 0.05).

**Effect of selected bacterial strains on growth of cowpea under salt stress conditions**

Among three selected bacterial strains, ST3 exhibited higher potential for improving growth of plant under salt stress conditions. Therefore, ST3 strain was selected for the inoculation experiment. In this experiment, strain ST3 was applied as an aqueous suspension to the rhizosphere of cowpea one week after planting. As shown in table 1, the application of ST3 cells to the pot T3 resulted in significantly increased of the shoot length and shoot fresh weight of cowpea seedlings (31.9% and 43.3% more than the uninoculated control, respectively). It was not surprised that the lowest values of shoot length and shoot fresh weight were observed for the pot NaCl due to the salt stress. However, the shoot length values increased up to 21.7% when strain ST3 was applied to the pot T3+NaCl as compared to the pot NaCl.

**Discussion**

This study indicates the effectiveness of ACC deaminase producing rhizobacteria for improving the salt tolerance of cowpea plants. In this study, soil bacteria have been isolated from rhizosphere of water spinach (*I. aquatica* L.). As reported by Yousif et al. [15], the growth of water spinach is markedly reduced under saline conditions. However, the existence of water spinach in salt-affected area suggesting that the rhizobacteria colonized its roots may contribute to the salt tolerance of this plant. According to the salt requirement, bacteria may be classified as: non-halophiles grow at NaCl concentration ranging between 0-2%; slight halophiles grow at 2-3% NaCl; moderate halophiles grow at 5-10% NaCl; and extremely halophiles grow at NaCl concentration greater than 10% [16]. Data of this study revealed that all three selected bacteria were able to proliferate at 10% NaCl and thus they can be classified as moderate halophiles. As reported by Bal et al. [8], the other ACC deaminase producing bacteria belonging to the genus *Bacillus* and *Ochrobactrum* can only grow in maximum NaCl concentration of about 6%. Bacterial strains belonging to the genus *Pseudomonas* and *Enterobacter* have been previously reported as salt tolerant organisms [17,18]. Paul and Nair reported that *Pseudomonas fluorescens* MSP-393 can produce osmolytes and salt-stress induced protein that overcome the negative effect of salt [19].
Data of this study demonstrated that all three selected strains were able to produce both ACC deaminase and IAA at significantly high concentrations. The ability to produce both components have been found in several bacterial species belonging to the genera such as *Bakholderia, Alcaligenes, Ochrobactrum* and *Bacillus* [20,21]. The ACC deaminase producing *P. fluorescens* spp. have been reported to produce IAA as well [6,22]. However, the capacity to produce IAA of those bacterial strains is significantly lower than our selected bacterial strains (Table 2).

| S. No | Bacterial strains | ACC deaminase activity (µM or Ketobutyrate/mg/h) | IAA (µg/mL) | References |
|-------|-------------------|---------------------------------------------|-------------|------------|
| 1     | *B. pumilus* SB1-ACC3 | 1.46                                        | 45.91       | [8]        |
| 2     | *B. licheniformis* B2r | 0.86                                        | N/A         | [23]       |
| 3     | *Enterobacter* sp. CS1 | 0.17                                        | N/A         | [7]        |
| 4     | *P. fluorescens* P4 | *                                        | 8.93        | [6]        |
| 5     | *P. fluorescens* ACC50 | 0.30                                        | 15.3        | [24]       |
| 6     | *Pseudomonas* sp. PDMZnCd2003 | N/A                                       | 11.15       | [25]       |
| 7     | *Pseudomonas* sp. Cre56 | *                                        | 18.5        | [22]       |
| 8     | *Pseudomonas* sp. ST3 | 0.90                                        | 62.7        | This study |

+: Positive; NA: Not Available

Table 2: The ACC deaminase activity and IAA production of different bacterial species.

Although the ability to produce ACC deaminase and IAA are particularly important for bacteria to improve the growth of plant under salt stress conditions, little information is available for the correlation between the ACC deaminase and IAA production ability among all selected strains. According to Glick [27], bacterial IAA together with endogenous plant-synthesized IAA can induce the transcription of ACC synthase and thus increase the level of ethylene in plant. However, as plant ethylene levels increase, ethylene will inhibit the IAA signal transduction and thereby limiting the extent that IAA can induce the ACC synthase transcription [28,29]. Furthermore, the large portion of synthesized ACC is degraded by the bacterial ACC deaminase. Therefore, the net result of the interaction between IAA and ACC deaminase is that the ethylene level in plant is reduced and IAA still stimulates the growth of plant.

The positive effects of the efficient salt-tolerant bacteria containing ACC deaminase for the growth, yield and disease suppression of different plants under salt stress conditions have been reported [18,26]. The role of *Pseudomonas* spp. for promoting salt resistance of rice and groundnut have also reported [24,30]. However, the effects of those bacterial strains for enhancing plant growth under salt stress conditions are still limited. Our data suggest that the *Pseudomonas* sp. strain ST3 is an effective rhizobacteria for enhancing the growth of cowpea under salt stress conditions. This could be due to the particularly high ACC deaminase and IAA production abilities of ST3 strain. *Pseudomonas* species have been reported to solubilize phosphate sources [31] and reduce the development of root-rot disease and enhance the yield of *Phaseolus vulgaris* L. [32]. Therefore, ST3 could be a promising candidate for potential biofertilizers in saline fields. Further work is needed to evaluate the effectiveness of ST3 strain for improving the plant growth under salt-affected field conditions.

**Conclusion**

Three rhizobacterial strains belonging to the genera *Enterobacter* and *Pseudomonas* have been isolated in this study. All three bacterial strains were salt resistance and they can produce high levels of ACC deaminase and IAA. The strain *Pseudomonas* sp. ST3 showed the possible ability to promote the growth of cowpea under salt stress conditions.

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