Indian Journal of Experimental Biology
Vol. 55, July 2017, pp. 463-468

# Exogenous expression of ACC deaminase gene in psychrotolerant bacteria alleviates chilling stress and promotes plant growth in millets under chilling conditions

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*Received 31 January 2017*; *revised 17 March 2017*

Endogeneous ethylene evolved during cold stress is a major limiting factor for plant growth which can be controlled by bacterial enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase (ACCD), by breaking down ACC, the precursor of ethylene. In the present study, we introduced ACCD expressing plasmid in ACCD negative psychrotolerant bacteria to study its effect on growth of finger and foxtail millet seedlings. ACCD negative *Sphingomonas* faeni ISO were selected and transformed with plasmid pRKACC containing the *acdS* gene. Inoculation of the millet seeds and studying physiological parameters when a cold stress of 4 and 10ºC was imposed showed that inoculation with ACCD expressing strains improved root and shoot length, biomass content of foxtail and finger millets seeds. Further, we also observed increased antioxidant activity in the plants by high levels of SOD, CAT, GPX, POD, APX and GR enzyme activity, and decreased proline content on inoculation with ACCD positive mutants. The enzyme ACC deaminase is thus be proved to be a potential strategy to alleviate cold stress in foxtail and finger millet by regulating endogenous ethylene evolved during stress conditions.

**Keywords:** ACC deaminase, *acdS* gene, Cold stress, Ethylene, Finger millet, Foxtail millet, *Sphingomonas faeni*

Low temperature or cold stress remains to be one of the major abiotic factors limiting growth, development and productivity of plants[1]. Several economically significant crops such as rice, millets, maize, soybean, banana, mango, papaya, and grapes are sensitive to low temperature[2]. Plants under low temperatures show poor early development owing to their detrimental effects especially during seedling stages. Plant hormones such as salicylic acid (SA), abscisic acid (ABA), ethylene, jasmonic acid (JA) have been found to induce cold acclimatization resulting in low temperature tolerance[3,4]. However, they affect the vegetative phase of growth and generally result in stunted growth with low productivity. The plant hormone ethylene also has a role in abiotic as well as biotic stresses, and has been observed to be harmful to plant growth[5]. Chilling temperatures can enhance the production of ethylene in plants by accumulation of its precursor molecule 1-aminocyclopropane-1-carboxylate (ACC) and conversion to ethylene in maize seedlings[6]. In short, stress in plants leads to accumulation of S-adenosylmethionine (SAM) which is converted into ACC by ACC synthase. Conversion of ACC to ethylene is catalyzed by ACC oxidase. Bacterial enzyme ACC deaminase (ACCD) can eliminate ACC accumulated in the plant tissues by converting ACC to α-ketobutyrate and ammonia, and thereby reducing ethylene evolved during stress[7].

Millets, small-seeded C4 panicoid crops (foxtail millet, pearl millet, finger millet, proso millet, etc.) are cultivated as food and fodder in dry areas of temperate, subtropical and tropical regions across the world[8,9]. These millets demand least inputs and possess remarkable nutritive value which emphasizes their importance as a food crop[8]. Despite several of these millets being able to tolerate a wide range of

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abiotic stress conditions, low temperatures are often detrimental for seedling growth, especially in chilling-sensitive species[10]. Cold delays both germination and plant growth, lengthens the crop cycle and increases production costs by demanding greater irrigation time[11]. Among millets, foxtail and to a lesser extent finger millet have gained focus of the research community with emphasis on studying the growth and molecular mechanism in response to abiotic stress[12-15]. Therefore, here, we studied the role of ACC deaminase in chilling stressed finger and foxtail millet plants by introducing an exogenously expressing ACC deaminase gene in bacteria and inoculation of the bacteria in stressed plants.

## Materials and Methods

#### Plasmids, strains and triparental mating

*Sphingomonas faeni* (ISO - psychrotolerant bacteria) was isolated in our lab and used in the present study[16]. The plasmid pRKACC was donated by Prof. BR Glick, University of Waterloo, Canada. Triparental mating using *E. coli* strains DH5α (pRKACC) - donor, HB101 (pRK2013) - helper and *S. faeni* ISO (acceptor) were used to introduce the *acdS* gene through transformation of plasmid pRKACC[17]. Transformed cells were selected based on their ability to grow in modified minimal medium containing sucrose as the sole carbon source amended with 10 µg/mL tetracycline[17].

#### Screening of *acdS* transformed mutants and ACC utilization

Transformation of *acdS* gene into wild type bacterial cells was confirmed by PCR analysis using the protocol of Kamala-Kannan *et al.*[18]. To study ACC utilization, transformed strains were initially grown in 5 mL of LB broth supplemented with 10 µL/mL of tetracycline. After growing them overnight on rotary shaker at 180 rpm, 2 mL aliquots of the cultures were centrifuged at 5000 rpm for 3 min at 4ºC. The pellets were then washed with 1 mL of DF media twice to remove excess antibiotic and were resuspended in DF-ACC media (DF media containing 3 mM) and incubated for 24 h on rotary shaker at 28-30ºC. At the end of 24 h, 2 mL the cultures were centrifuged at 5000 rpm for 3 min at 4ºC and the supernatants were collected. Aliquots of 60 µL of the supernatant were taken in separate PCR tubes and 120 µL of freshly prepared ninhydrin reagent was added. This mixture kept in boiling water bath for 30 min and absorbance was taken at 570 nm[19].

#### ACC deaminase activity of transformed mutants

Quantification of ACC deaminase activity in the transformed *S. faeni* ISO mutants were determined by growing them on nitrogen-free broth amended ACC (3mM) as sole nitrogen source[20]. The quantity of α-ketobutyrate produced as a result of enzymatic hydrolysis of ACC was estimated and protein content determined using Lowry method.

#### Cultivation of the seedlings

Foxtail and Finger millet seeds were procured from Sri Venkateshwara Agricultural University, Tirupathi, India and surface sterilized with 2% sodium hypochlorite solution containing 0.02% Tween20. At the end of surface sterilization, aliquots (100 mL) of water from the final wash were spread on nutrient agar to ensure efficiency of sterilization. Bacterial cultures *S. faeni* ISO and ACCD expressing mutants S37 and S42 were grown in LB until log phase followed by DF media and resuspended in 0.3M $MgSO_4$ after harvesting by centrifugation at 2600 rpm for 30 min. Surface sterilized seeds were then coated with wild-type, S37 and S42 strains with gentle shaking at 30 rpm for 4 h. Control seeds with incubated in sterile 0.3$MgSO_4$. The seeds were placed on solidified 0.5X Hoagland's solution (solidified using 2% plant agar, 5 seeds per plate).The seeds were germinated in dark for 3 days and divided into two sets. One set of both Foxtail and Finger millet seeds were transferred to 4ºC and the other set was transferred to 10ºC. The plates were maintained under chilling stress conditions for 7 days and then moved to 25ºC. Another set of seeds were continuously maintained at 25ºC. At the end of 10 days after inoculation all the seedlings were studied for growth and physiological biomarkers of stress[21].

#### Plant growth and stress biomarkers

Plant height, root length and fresh and dry biomass were measured[22]. Total chlorophyll, proline content and Lipid peroxidation in the seedlings was determined by Porra[23]; Bates *et al.*[24], Heath & Packer[25]. Superoxide dismutase (SOD), catalase (CAT), Peroxidase (POD) activity was assayed following methods described by Gao[26]. Ascorbate peroxidase (APX) was estimated by Nakano & Asada[27]. Glutathione peroxidase (GPX) was measured using the protocol of Hemeda and Klein[28]. Gluthathione reductase (GR) was analyzed by Foyer and Halliwell[29] method.

## Results and Discussion

Psychrotolerant bacterial strains (CSI, ISO, SR11, SR12 and IMDY) that were isolated from Himalayan soil sample were screened for ACC deaminase negative strain by growing them in DF media supplemented with and without ACC as the sole nitrogen source. Out of these five isolates, *Sphingomonas faeni* (ISO) was selected as the psychrotolerant strain negative for synthesis of ACC deaminase enzyme. The wild-type psychrotolerant *Sphingomonas faeni* ISO strain was successfully transformed with pRKACC plasmid and transformed mutants were selected based on resistance to tetracycline10µg/ml. Stability of the plasmid in the strain was studied by continuous culturing for 10 generations in the absence of tetracycline. PCR analysis identified the positive transformants which carried the ACC deaminase gene where 10 isolates (S14, S21, S22, S25, S29, S30, S35, S37, S39 and S42) showed positive results out of 42 (Fig. 1). Out of them, two mutants (S37 and S42) showing maximum ACC utilization (data not shown) with ACC deaminase activity (Table 1) was taken for further studies.

Physiologically evolved stress ethylene is generally reported to be detrimental to plant growth and can cause more damage in plants compared to the direct effects of the abiotic stress *per se*. Hence, it is necessary to study ethylene emission and its regulation by enzyme ACC deaminase[30-32]. In the present study, Fig. 2 shows the effect of ISO mutants (S37 and S42) on the growth of Finger and Foxtail millets under chilling stress of 4 and 10°C, respectively. At the end of 10 days, the mutant strains S37and S42 were able to improve robustness of plants observed through significant increase in plant dry weight [Fig. 3 C(i) and C(ii)]. Mutant strains S37 and S42 showed significantly high shoot length [Fig. 3 A(i) and A(ii)],

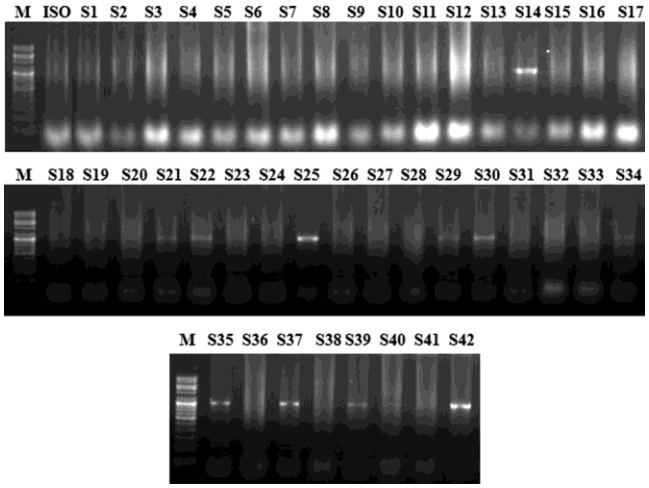


Fig. 1 — Confirmation of *acds* gene in the ISO mutants (S1 to S42) by PCR. [M, 100 DNA marker; ISO, wild-type *Sphingomonas faeni*]

Table 1 — ACC deaminase activity of ISO mutants (S37 and S42) and wild-type strain

| Bacterial Strains | ACC deaminase activity (nmol α-ketobutyrate $mg^{-1}$ protein $h^{-1}$) |
|---|---|
| ISO (*Sphingomonas faeni*) | 1.06±0.83 |
| S37 mutant | 73.23±2.01 |
| S42 mutant | 59.49±4.22 |

All values are expressed in Mean±SD (n=3)

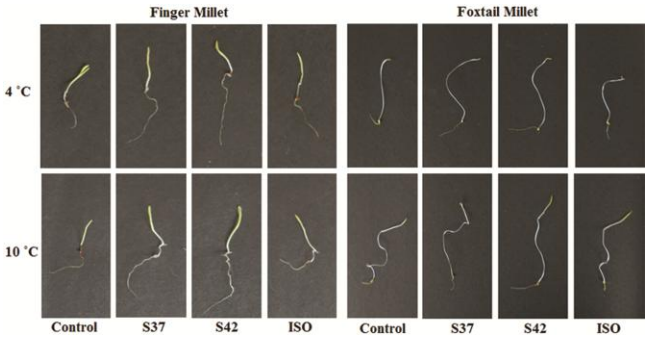


Fig. 2 — Effect of ISO mutants (S37 and S42) on the growth of finger and foxtail millets under chilling stress (4 ˚C and 10˚C).

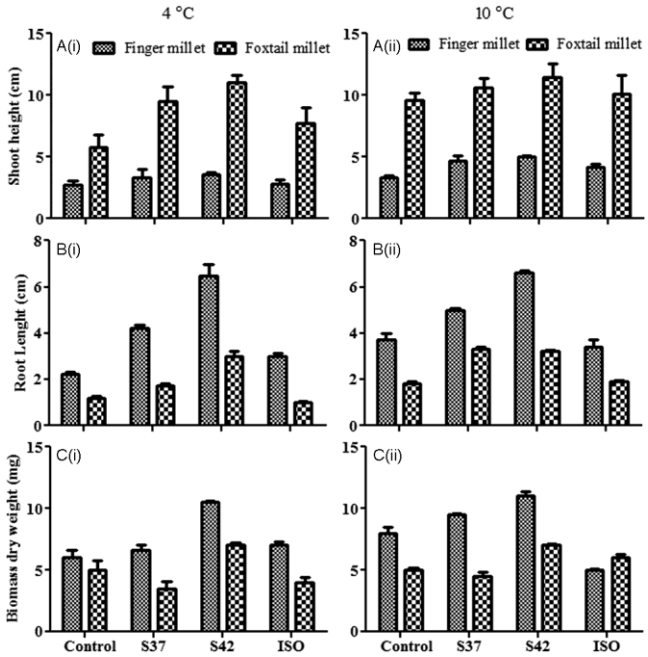


Fig. 3 — Effect of ISO mutants (S37 and S42) on A(i) and A(ii), shoot length ]; B(i) and B(ii), root length; and C(i) and C(ii) dry biomass of the finger and foxtail millets under chilling stress (4 and 10˚C). [All values are expressed in Mean±SD (n=3)]

root length [Fig. B(i) and B(ii)] compared to temperature control and wild type bacteria inoculated plants at 4 and 10°C (Fig. 2). These results were in accordance with the previously reported expression of exogenous ACC deaminase gene in the psychrotolerant bacterial strains *Flavobacterium* sp. OR306 and *Pseudomonas frederiksbergensis* OS211 resulted in an increased plant growth promoting potential[7]. The chlorophyll a and chlorophyll b content analysis showed that seeds treated with mutants S37 and S42 contained more chlorophyll compared to the wild-type treated seeds as well as control at both temperatures [Fig. 4 A(i) and A(ii)]. Such a phenomenon has also been observed as an effect of ACC deaminase enzyme[33]. Proline content was found to be reduced upon treatment with S37 and S42 compared to control and wild-type treatment (Fig. 4 B(i) and B(ii)]. Reduction of proline content has been observed as a response to stress alleviation in other plants including maize[34]. Lipid peroxidation measured in terms of MDA content showed that the seedlings treated with mutants S37 and S42 showed significant reduction in MDA levels against other treatments [Fig. 4 C(i) and C(ii)].

SOD, CAT, POD, APX, GPX and GR antioxidant enzyme levels were examined after experimental study. It was observed that the mutants increase the levels of these antioxidant enzymes to a significant level, and hence prevent the reduction of growth of these seedlings by bringing down the levels of ROS (Fig. 5). The current study shows a significant increase in the SOD specific activity in seedlings that were treated with mutants which kept under cold stress. This increase in SOD levels helps in lowering the risk of cellular damage and also increases the

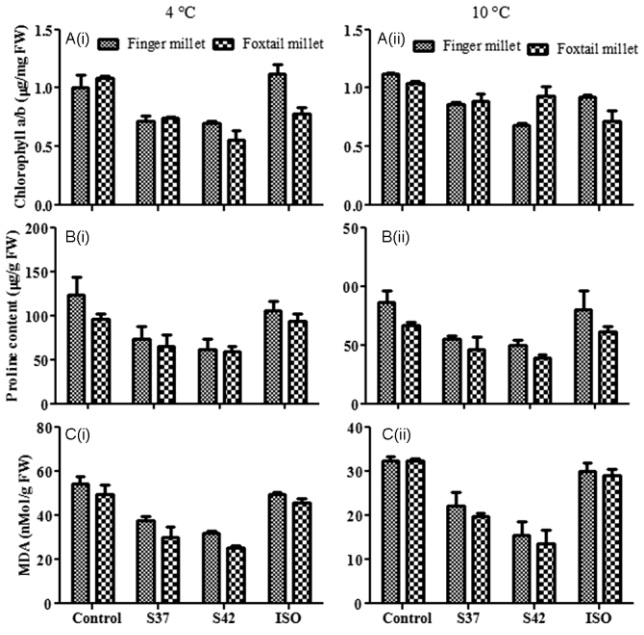


Fig. 4 — Effect of ISO mutants (S37 and S42) on A(i) and A(ii), Chlorophyll a/b ratio; B(i) and B(ii), Proline content; and C(i) and C(ii) Lipid peroxidation of the finger and foxtail millets under chilling stress (4 and 10˚C). [All values are expressed in Mean±SD (n=3)]

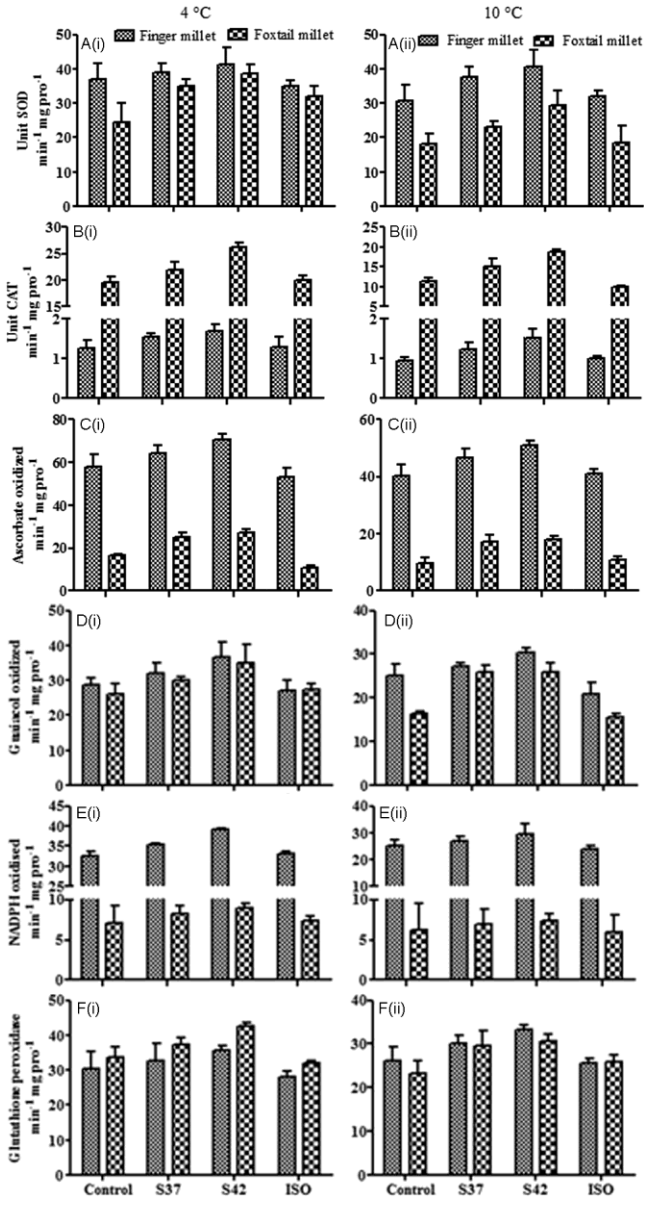


Fig. 5 — Antioxidant enzyme activity A(i) and A(ii), superoxide dismutase (SOD); B(i) and B(ii), catalase (CAT); C(i) and C(ii), ascorbate peroxidase (APX); D(i) and D(ii), guaiacol peroxidase (POD); E(i) and E(ii), glutathione reductase (GR); and F(i) and F(ii), glutathione peroxidase (GPX) of finger and foxtail millets treated with ISO mutant under chilling stress (4 and 10˚C). [All values are expressed in Mean±SD (n=3)]

tolerance capability of the seedling under stress [Fig. 5 A(i) and A(ii)]. There was also increase in stress induced CAT, APX, POD, GR and GPX specific activity observed in the treated seedlings when compared with the untreated controls [Fig. 5 B(i), B(ii), C(i), C(ii), D(i), D(ii), E(i), E(ii), F(i) and F(ii)]. Both APX and GPX contribute in plant growth by detoxification of $H_2O_2$. It was also observed that the total APX specific activity in both foxtail and finger millets was more than GPX specific activity. Hence, APX contributes more towards detoxifying $H_2O_2$ than GPX[35].

In the present study, the role of ACC deaminase in regulating the stress ethylene and the plant growth promoting activity was investigated in two varieties of small millets— Finger millets and Foxtail millets. We observed that the growth of the Foxtail and Finger millet seedlings reduced due to cold stress. Inoculation of the millet seeds with mutants S37 and S42 showed positive impacts on the growth parameters like root and shoot length and dry weight of the seedlings. Other reports studying stress ethylene also suggest that reduction of ethylene or its signaling factors in plants cause improvement in plant cold tolerance. Shi *et al*.[36] showed that, increase in ethylene levels led to reduced tolerance to freezing temperatures and blocking of ethylene biosynthesis as well as its signaling resulted in enhanced freezing tolerance. Therefore, it can be concluded that the exogenously expressed ACC deaminase gene in *Sphingomonas faeni* ISO strain alleviates chilling stress and promotes growth of Finger and Foxtail millets in a cold environment.

## Acknowledgment

We are thankful to Prof. BR Glick, University of Waterloo, Canada for providing the plasmid pRKACC. This research was supported by Basic Reseach Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2015R1A2A1A05001885), Republic of Korea.

## References

1 Miedema P, The effects of low temperature on *Zea mays* L. *Adv Agron*, 35 (1982) 93.

2 Larcher W, *Physiological Plant Ecology-Ecophysiology and Stress Physiology of Functional Groups*. (Springer, Berlin), 1995.

3 Lee TM, Lur HS, Lin YH & Chu C, Physiological and biochemical changes related to methyl jasmonate-induced chilling tolerance of rice (*Oryza sativa* L.) seedlings. *Plant Cell Environ*, 19 (1996) 65.

4 Janowiak F, Maas B & Dörffling K, Importance of abscisic acid for chilling tolerance of maize seedlings. *J Plant Physiol*, 159 (2002) 635.

5 Bleecker AB & Kende H, Ethylene: A gaseous signal molecule in plants. *Annu Rev Cell Dev Biol*, 16 (2000) 1.

6 Janowiak F & Dörffling K, Chilling-induced changes in the contents of 1-aminocyclopropane-1-Carboxylic Acid (ACC) and its N-malonyl Conjugate (MACC) in Seedlings of two Maize inbreds differing in chilling tolerance. *J Plant Pbysiol*, 147 (1995) 257.

7 Subramanian P, Krishnamoorthy R, Chanratana M, Kim K, Sa T, Expression of an exogenous 1-aminocyclopropane-1-carboxylate deaminase gene in psychrotolerant bacteria modulates ethylene metabolism and cold induced genes in tomato under chilling stress. *Plant Physiol Biochem*, 89 (2015) 18.

8 Otubo ST, Ramalho MAP, Abreu AFB & dos Santos JB, Genetic control of ~~low~~ Low Temperature Selection of Pearl Millet 149 temperature tolerance in germination of the common bean (*Phaseolus vulgaris* L.). *Euphytica*, 89 (1996) 313.

9 Richner A, Soldati WA & Stamp P, Shoot-to-root relations in field-grown maize seedlings. *Agron J*, 88 (1996) 56.

10 Dwivedi S, Upadhyaya H, Senthilvel S, Hash C, Fukunaga K, Diao X, Santra D, Baltensperger D & Prasad M, Millets: Genetic and Genomic Resources. In: *Plant Breeding Reviews*, Vol. 35, (Ed. Janick J;(), John Wiley & Sons, USA), 2012, 247.

11 Lata C, Gupta S & Prasad M, Foxtail millet: a model crop for genetic and genomic studies in bioenergy grasses. *Crit Rev Biotechnol*, 33 (2013) 328.

12 Khan Y, Yadav A, Bonthala VS, Muthamilarasan M, Yadav CB, & Prasad M, Comprehensive genome-wide identification and expression profiling of foxtail millet [*Setaria italica* (L.)] miRNAs in response to abiotic stress and development of miRNA database. *Plant Cell Tiss Org*, 118 (2014) 279.

13 Rahman H, Ramanathan V, Nallathambi J, Duraialagaraja S & Muthurajan R, Over-expression of a NAC 67 transcription factor from finger millet (*Eleusine coracana* L.) confers tolerance against salinity and drought stress in rice. *BMC Biotechnol*, 16 (2016) 35.

14 Rahman H, Jagadeeshselvam N, Valarmathi R, Sachin B, Sasikala R, Senthil N & Muthurajan R, (2014). Transcriptome analysis of salinity responsiveness in contrasting genotypes of finger millet (*Eleusine coracana* L.) through RNA-sequencing. *Plant Mol Biol*, 85 (2014) 485.

15 Lata C, Mishra AK, Muthamilarasan M, Bonthala VS, Khan Y & Prasad M, Genome-wide investigation and expression profiling of AP2/ERF transcription factor superfamily in foxtail millet (*Setaria italica* L.). *PLoS One*, 9 (2014) e113092.

16 Mageswari A, Subramanian P, Srinivasan R, Karthikeyan S & Gothandam KM, Astaxanthin from psychrotrophic *Sphingomonas faeni* exhibits antagonism against food-spoilage bacteria at low temperatures. *Microbiol Res*, 179 (2015) 38.

17 Nascimento F, Brígido C, Alho L, Glick BR & Oliveira S, Enhanced chickpea growth-promotion ability of a

*Mesorhizobium* strain expressing an exogenous ACC deaminase gene. *Plant Soil*, 353 (2012) 221.
18 Kamala-Kannan S, Lee KJ, Park SM, Chae JC, Yun BS, Lee YH, Park YJ & Oh BT, Characterization of ACC deaminase gene in *Pseudomonas entomophila* strain PS-PJH isolated from the rhizosphere soil. *J Basic Microbiol*, 50 (2010) 200.
19 Li Z, Chang S, Lin L, Li, Y, & An Q, A colorimetric assay of 1-aminocyclopropane-1-carboxylate (ACC) based on ninhydrin reaction for rapid screening of bacteria containing ACC deaminase. *Lett App Microbiol*, 53 (2011) 178.
20 Penrose DM & Glick BR, Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria. *Physiol Plant*, 118 (2003) 10.
21 Theocharis A, Bordiec S, Fernandez O, Paquis S, Dhondt-Cordelier S, Baillieul F, Clement C & Barka EA, *Burkholderia phytofirmans* PsJN primes *Vitis vinifera* L. and confers a better tolerance to low nonfreezing temperatures. *Mol Plant Microbe Interact*, 25 (2012) 241.
22 Barnawal D, Bharti N, Maji D, Chanotiya CS & Kaira A, 1-Aminocyclopropane-1- carboxylic acid (ACC) deaminase-containing rhizobacteria protect *Ocimum sanctum* plants during waterlogging stress via reduced ethylene generation. *Plant Physiol Biochem*, 58 (2012) 227.
23 Porra RJ, The chequered history of the development and use of simultaneous equations for the accurate determination of chlorophylls a and b. *Photosynth Res*, 73 (2002) 149.
24 Bates LS, Woldren RP & Teare ID, Rapid determination of free proline for water stress studies. *Plant Soil*, 39 (1973) 205.
25 Heath RL & Packer L, Photoperoxidation in isolated chloroplast. I. Kinetics and stoichiometry of fatty acid peroxidation. *Arch Biochem Biophys*, 125 (1968) 189.
26 Gao JF, Experimental techniques of plant physiology. Xi'an World Publishing Corporation (in Chinese), (2000).
27 Nakano Y & Asada K, Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. *Plant Cell Physiol*, 22 (1981) 867.
28 Hemeda HM & Klein BP, Effects of naturally occurring antioxidants on peroxidase activity of vegetable. *J Food Sci*, 55 (1990) 184.
29 Foyer CH & Halliwell B, The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. *Planta*, 133 (1976) 21.
30 Grichko VP & Glick BR, Amelioration of flooding stress by ACC deaminase containing plant growth-promoting bacteria. *Plant Physiol Biochem*, 39 (2001) 11.
31 Madhaiyan M, Poonguzhali S, Ryu J & Sa T, Characterization of 1-aminocyclopropane-1-carboxylate (ACC) deaminase containing *Methylobacterium oryzae* and interactions with auxins and ACC regulation of ethylene in canola (*Brassica campestris*). *Planta*, 226 (2007) 867.
32 Ali S, Charles TC & Glick BR, Amelioration of high salinity stress damage by plant growth-promoting bacterial endophytes that contain ACC deaminase. *Plant Physiol Biochem*, 80 (2014) 160.
33 Reed MLE & Glick BR, Growth of canola (*Brassica napus*) in the presence of plant growth-promoting bacteria and either copper or polycyclic aromatic hydrocarbons. *Canadian J Microbiol*, 51 (2005) 1061.
34 Krishnamoorthy R, Kim K, Subramanian P, Senthilkumar M, Anandham R & Sa T, Arbuscular mycorrhizal fungi and associated bacteria isolated from salt-affected soil enhances the tolerance of maize to salinity in coastal reclamation soil. *Agric Ecosys Environ*, 231 (2016) 233.
35 Subramanian P, Mageswari A, Kim K, Lee Y & Sa T, Psychrotolerant endophytic *Pseudomonas* sp. strains OB155 and OS261 induced chilling resistance in tomato plants (*Solanum lycopersicum* Mill.) by activation of their antioxidant capacity. *Mol Plant Microbe Interact*, 28 (2015) 1073.
36 Shi Y, Tian S, Hou L, Huang X, Zhang X, Guo H & Yang S, Ethylene signaling negatively regulates freezing tolerance by repressing expression of CBF and type-A ARR genes in Arabidopsis. *Plant Cell*, 24 (2012) 2578.