



Bacterization Effect of Culture Containing 1-aminocyclopropane-1-carboxylic Acid Deaminase Activity Implicated for Plant Development

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Authors' contributions

This work was carried out in collaboration between both authors. Author BSS designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript and managed literature searches. Author NS conducted the experiments related to 1-aminocyclopropane-1-carboxylic Acid Deaminase Activity. Both authors read and approved the final manuscript.

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ABSTRACT

ACC deaminase regulates the production of ethylene level of plants by metabolizing 1-aminocyclopropane-1-carboxylate (ACC) into α -ketobutyric acid and ammonia. It was observed that two important phyllospheric bacteria of Spinach (*Spinacia oleracea*) which are *Paenibacillus polymyxa* SNKp6 and *Bacillus thuringiensis* SNKr10 exhibit significant ACC deaminase activity which can help plant growth under stressful conditions like heavy metal contaminated sites. This growth activity was studied by *in vitro* seed germination of *Vigna radiata* plant. In the present study, we concluded that these two important bacterial strains can be helpful for plants to grow under stressful conditions and also help in their growth.

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1. INTRODUCTION

Phyllospheric bacteria are those bacteria lie on surface of plants leaves. These bacteria play important role for plants by forming mutual interaction with them. The phyllosphere is a very important biologically active interface between the aerial parts of the plant and the air. It precedes the fixation of carbon dioxide, release of molecular oxide and thus facilitates primary productivity. Microorganisms on leaf surfaces are said to be extremophiles as they are able to grow/tolerate high temperature from 40-55°C, Ultraviolet radiation and cool temperatures upto 5-10°C [1]. Many bacteria like *Methylobacterium* and *Pseudomonas* are found in phyllosphere and play useful role [2]. The microbial communities of leaves include different genera of algae, bacteria, filamentous fungi, yeasts and less frequently protozoa and nematodes. The plant growth promoting bacteria (PGPB) are those useful bacteria that helps the enhancement in plant's growth and yield [3], antimicrobial activity against phytopathogens (through HCN, siderophore antifungal activity), ammonia, biological nitrogen fixation and also by ACC deaminase activity. The plant growth promoting bacteria has been shown to be useful in many aspects of plant development and their response toward stress [4]. It plays important role as epinasty, leaf abscission, senescence and ripening of fruits [5]. Ethylene is produced mainly by two enzymes including ACC oxidase, ACC synthase. ACC is a precursor of ethylene in plant which is hydrolysed by ACC deaminase that help in growth and development of plants. 1-aminocyclopropane-1-carboxylate synthase (ACC synthase) is the enzyme that catalyzes ethylene synthesis by conversion of s-adenosyl methionine to 1-aminocyclopropane-1-carboxylate and ethylene synthesis involves the conversion of ACC, to ethylene with the help of ACC oxidase enzyme. The production of these enzymes by plants help the plants to grow under stress conditions during flooding, drought, presence of organic toxicants, metals, salt and flower wilting etc. [6]. Various plant growth promoting bacteria helps in resisting the plant during stressful conditions as *P. syringae* that is an epiphytic bacteria helping in reducing the plants ability to supercool conditions and avoid damage of plants from ice formation on their surface [7]. They binds to the seed coat, assimilate to its surface and then bacterium hydrolyzes ACC into α -ketobutyrate and ammonia by using ACC deaminase, thus the

level of ethylene lowered in developing plants [8]. These ACC deaminase genes thus regulate the ethylene level in plants and play important role in optimum growth even under stressed conditions.

Our resented investigation involves the study of important bacteria from medicinally important spinach plant's phyllosphere having properties to resist plants in stress environment with the help of ACC deaminase activity.

2. MATERIALS AND METHODS

2.1 Sample Collection

Spinach leaves were collected from different regions of Haryana (Chika, Karnal, Kurukshetra and Shahabad) region and from Punjab (Kapiyal and Sangrur) in India. Fresh leaves plucked and stored in sterile propylene bags and analysed for isolation process within 24 hours (h) [9].

2.2 Isolation of Phyllospheric Bacteria

The fresh leaves were washed properly using sterile distilled water and vortexed for 15-20 minutes (min.). Serial dilution of samples were prepared in range of 10^{-1} - 10^{-5} and 100 μ L of diluted samples were spreaded on respective media plates. Further, the plates were incubated at 35-37°C for 24-48 h. The isolates so obtained were preserved in 50% glycerol containing respective slants at 4°C [9]. The bacterial isolates were examined for their morphological features including colony size, elevation, shape, surface, colour and pigmentation etc. [10].

2.3 Screening of Bacterial Isolates for Plant Growth Promoting Attributes

The screening of isolated bacterial isolates for PGP attributes were done by examining their response towards phosphate solubilization, ammonia production, IAA production and *In vitro* biological nitrogen fixation. In phosphate solubilization, the bacterial isolates were observed on pikovskaya's agar media containing Bromothymol blue dye. The cultures were incubated at 35-37°C for 3-5 days [11]. The cultures were observed for zone of solubilization which indicated positive results. The ammonia production was confirmed by inoculating the respective fresh bacterial culture in 10 mL peptone water and incubated at 30-37°C for

48-72 h. Nessler's reagent (0.5 mL) was added and allowed to settle at room temperature (temp.). The appearance of brown colour indicated the positive test for ammonia production. The faint yellow was taken as weak ammonia producer and deep brown colour as strong ammonia producer [12].

Similarly, IAA production was confirmed by inoculating the respective bacterial isolate in Luria broth containing L-tryptophan (0.1%) and incubated at 35-37°C for 48-72 h. After incubation the culture broth was centrifuged at 10,000 rpm for 10 min. Two drops of o-phosphoric acid were added into 2 mL of supernatant along with 4 mL Salkowski reagent (50 mL, 35% of perchloric acid, 1 mL of 0.5 M FeCl₃ solution). It was observed for the development of pink colour within 2 h at room temperature [13]. *In vitro* BNF was observed by streaking the bacterial culture on nitrogen free Jensen's media along with addition of bromothymol blue (BTB) stain as an indicator dye and incubated at 35-37°C for 24-48 h. Yellow coloured zone around the colonies indicated the positive result of *in vitro* BNF [14].

2.4 Production Medium for ACC Deaminase Activity

2.4.1 Composition of DF minimal salt medium

4.0 g KH₂PO₄, 6.0g Na₂HPO₄, 0.2 g MgSO₄·7H₂O, 2.0 g glucose, 2.0 g gluconic acid, 2.0 g citric acid with trace elements: 1 mg FeSO₄·7H₂O, 10 mg H₃BO₃, 11.19 mg MnSO₄·H₂O, 124.6 mg ZnSO₄·7H₂O, 78.22 mg CuSO₄·5H₂O, 10 mg MoO₃, pH 7.2 with 0.3 g ACC as the sole source of nitrogen, 15 g agar, 1000 mL distilled H₂O.

2.4.2 ACC enzyme obtaining from bacterial cells

Bacterial cells prepared in minimal salt media were suspended in 1 mL of 0.1M Tris-HCl (pH 7.6), and transferred to 1.5 mL microcentrifuge tube. The contents of the 1.5 mL microcentrifuge tube were then centrifuged at 14,000 rpm for 5 min. and the supernatant was removed. The pellet was suspended in 600 µL of 0.1M Tris-HCl (pH 8.5). 300 µL of toluene was added to the cell suspension and vortexed at the highest setting for 30 sec. The toluenized cell suspension was immediately assayed for ACC-deaminase activity [15].

2.4.3 ACC-deaminase activity assay for bacterial samples

Two hundred microlitres of the toluenized cells were placed in a fresh 1.5 mL microcentrifuge tube; 20 µL of 0.5M ACC was added to the suspension, vortexed for short time, and then incubated at 30°C for 15 min. It was followed by addition of 1.0 mL of 0.56M HCl, the mixture was vortexed and centrifuged for 5 min. at 13,500 rpm at room temp. One mL of the supernatant was vortexed together with 800 µL of 0.56M HCl. After that, 300 µL of the 2,4-dinitrophenylhydrazine reagent (0.2% 2,4-dinitrophenylhydrazine in 2M HCl) was added to the glass tube, the contents were vortexed and then incubated at 30°C for 30 min. Following the addition and mixing of 2.0 mL of 2M NaOH, the absorbance of the mixture was measured at 540 nm [15,16]. ACC deaminase enzyme conc. was calculated by the formula shown as [16].

Units/mL =

$$\frac{\mu\text{mole of } \alpha \text{ ketobutyrate} \times \text{reaction volume}}{\text{Sample vol} \times \text{reaction time} \times \text{vol assayed}}$$

µmole of α ketobutyrate = µmole of α ketobutyrate equivalents released as per graph.

Reaction volume = total volume (in ml) of assay.

Sample vol = volume (in ml) of enzyme sample used.

Reaction time = time (in minutes) of reaction incubation.

Vol assayed = volume of reaction product (in ml) used in OD determination.

2.5 Stress Response Shown by Heavy Metal Resistance Activity

The selected bacterial isolates were tested for their resistance to heavy metals by agar dilution method [17]. Freshly prepared nutrient broth was amended with various heavy metal salts namely Hg (HgCl₂), Cu (CuSO₄), Pb (lead acetate; Pb (C₂H₃O₂)₂), Zn (ZnSO₄) and Cr (Potassium dichromate; K₂Cr₂O₇) at conc. of 25-400 µg/mL were inoculated with overnight grown cultures. Heavy metal tolerance was indicated by appearance of bacterial growth after incubating

the plates at 37 °C for 24-48 h that was checked by taking O.D. at 600 nm.

2.6 In vitro Seed Germination Assay

Seeds of useful plant like *Vigna radiata* (mung bean) seeds were collected and surface sterilized by soaking in sodium hypochlorite solution for 2-3 min. and washed with sterile distilled water for three times to remove the sodium hypochlorite solution [18]. Then seeds were allowed to inoculate with bacterial culture (10^8 cfu/mL) for 1-2 h, kept in 1% soft agar plates and then incubated for 3-5 days in the dark at the 30°C of seed germination. The seeds germination was evaluated and untreated seeds were taken as control. Germination efficiency along with seedling height, root/shoot length, wet/dry weight, % seed germination and vigor index was also analysed after incubation time and calculated by the formula shown as.

Germination (%) =

$$\frac{\text{No. of seeds germinated} \times 100}{\text{Total no. of seeds}}$$

Vigor index = Germination % × Seedling length (cm)

2.7 Statistical Analysis

Statistical analysis of all the data represented was done using SPSS version 16.0 Inc.

3. RESULTS AND DISCUSSION

The useful bacteria from spinach plants were isolated and studied for the important properties. These all results are discussed below:

3.1 Isolation

A total of 200 bacterial isolates were obtained using serial dilution technique on Nutrient Agar media. These bacterial isolates were collected on the basis of colony colour, elevation, size, shape and pigmentation, etc. The morphological study of 15 bacterial isolates having proficient plant growth promoting activities are discussed in Table 1. Our result outcomes of PGPB bacterial isolation from spinach phyllosphere was supported by findings of Verma et al. [19] who isolated total 89 bacteria from wheat phyllosphere and studied the bacterial diversity and their PGP attribute.

3.2 Screening for PGP Attributes

The 15 bacterial isolates out of 200 isolated colonies showed plant growth promoting activities like *Paenibacillus polymyxa* SNKp6 and *Bacillus thuringiensis* SNKr10 showed maximum phosphate solubilization, ammonia production, IAA production etc. after 48 h of incubation at 37 °C are as shown in Table 2.

The biological nitrogen fixation ability of our isolated phyllospheric bacterial isolates was favoured by the findings of Fürnkranz et al. [20]. They reported that γ -proteobacteria and cyanobacteria (*Nostoc* sp.) helps in improving the plant growth through biological nitrogen fixation in *Grias cauliflora*, *Carludovica drudei* and *Costus laevis* plants. Taurian et al. [21] also reported in favour of our findings by stating that form 37 bacterial isolates some phyllospheric bacteria help in promoting *Arachis hypogaea* L. plant growth by performing certain PGP activities like phosphate solubilization and IAA production etc. Similarly findings of Verma et al. [22] also favoured our result out comes by reporting that various methylotrophic bacteria isolated from wheat (*Triticum aestivum*) phyllosphere also help to promote the plants growth by IAA, phosphate solubilization and ammonia production.

We selected SNKp6 and SNKr10 bacterial isolates for the study of ACC deaminase as well as some other properties that are discussed below. The gram staining of SNKp6 and SNKr10 revealed that these as gram positive and rod shaped bacteria. The 16S rRNA sequence of these two selected bacterial isolates were also obtained and submitted to the NCBI. The Accession number received were KU569965 for *Paenibacillus polymyxa* SNKp6 and KU569966 for *Bacillus thuringiensis* SNKr10.

3.3 Calculation of ACC Deaminase Activity

In the present study, ACC deaminase activity was implemented by inoculating the selected bacterial isolates namely *Paenibacillus polymyxa* SNKp6 and *Bacillus thuringiensis* SNKr10 on modified DF broth medium and deaminase enzyme assay was done after an incubation period of 24 h at 30 °C using above described method by taking O.D. at 540 nm. We observed that SNKp6 produced significant amount of α -ketobutyrate and ACC deaminase enzyme activity in comparison to SNKr10 (Fig. 1).

Table 1. Morphological characteristics of some bacterial isolates

Isolate name	Shape	Elevation	Surface	Margin	Colour	Pigment
SNKp6	Round	Raised	Smooth	Entire	Creamish yellow	Light yellow
SNKr10	Irregular	Dispersed	Flat	Irregular	Cream	None
SNCh4	Round	Dispersed	Raised	Entire	Light yellow	Yellow
SNKr30	Round	Elevated	Flat	Smooth	Creamish	None
SNKr59	Round	Raised	Raised	Smooth	Creamish	None
SNKr60	Spherical	Raised	Raised	Smooth	Reddish	Red
SNKr62	Spherical	Dispersed	Raised	Smooth	Light pink	Pink
SNKr4	Spherical	Dispersed	Raised	Smooth	Light brown	Brownish
SNCh6	Irregular	Raised	Flat	Irregular	White	None
SNKr11	Irregular	Raised	Raised	Irregular	Reddish brown	None
SNSr36	Round	Dispersed	Raised	Smooth	Light pink	Pink
SNSr7	Spherical	Dispersed	Raised	Smooth	Creamish	None
SNKp8	Round	Raised	Smooth	Entire	Creamish	None
SNKp9	Round	Raised	Smooth shiny	Rough	Yellowish	Yellow
SNCh5	Round	Smooth	Flat	Entire	Deep brownish	Brown

Table 2. Plant growth promoting properties shown by various spinach phyllospheric bacterial isolates

Isolate name	Phosphate solubilization	NH ₃ production	IAA production (µg/mL)	Biological nitrogen fixation
<i>Paenibacillus polymyxa</i> SNKp6	+++	+++	39	+++
<i>Bacillus thuringiensis</i> SNKr10	+++	+++	34	+++
SNCh4	+++	+++	18	++
SNKr30	+++	+++	-	++
SNKr59	+++	+++	18	+++
SNKr60	+++	+++	19	+++
SNKr62	+++	+++	11	++
SNKr4	+++	+++	13	+
SNCh6	+++	+++	17	+
SNKr11	+++	+++	14	+++
SNSr36	+++	+++	17	+++
SNSr8	+++	+++	27	+++
SNKp8	+++	+++	24	+++
SNKp9	+++	+++	17	++
SNCh6	+++	+++	29	+++

*+++ = Very good producer; ++ = good producer

3.4 Heavy Metal Resistance Activity

Soil contamination with heavy metals is very serious problem as it causes various serious health problems by enter through food chains [23]. These two bacterial isolates *i.e.*

Paenibacillus polymyxa SNKp6 and *Bacillus thuringiensis* SNKr10 were used to check their growth at different conc. (25-400 µg mL⁻¹) of heavy metals like CuSO₄, HgCl₂, ZnSO₄, Pb as lead acetate and K₂Cr₂O₇. The isolate *Bacillus thuringiensis* SNKr10 showed maximum

capability to resist towards $25 \mu\text{g mL}^{-1}$ of Pb, $\text{K}_2\text{Cr}_2\text{O}_7$, CuSO_4 and HgCl_2 respectively and $50 \mu\text{g mL}^{-1}$ conc. of ZnSO_4 . *Paenibacillus polymyxa* strain SNKp6 also showed its maximum growth and resistance against $25 \mu\text{g mL}^{-1}$ of Pb, ZnSO_4 and HgCl_2 and upto $100 \mu\text{g mL}^{-1}$ of $\text{K}_2\text{Cr}_2\text{O}_7$ and CuSO_4 . Along with this, we also observed that these bacterial strains showed their resistance towards these heavy metals upto $400 \mu\text{g mL}^{-1}$ except for HgCl_2 . These results are discussed in Table 3; Fig. 2 by taking O.D. value at 600 nm. Our result outcomes are favoured by

findings of Pereira et al. [24] as they reported that *Rhodococcus erythropolis* EC 34, *Achromobacter* sp. 1AP2 and *Microbacterium* sp. 3ZP2 increased clover biomass maximally at 250 mg/kg of zinc contaminated soil therefore useful for phytoremediation of polluted soils. One of the other PGPB named *Micrococcus* sp. TISTR2221 also showed its stimulatory effects on the growth of *Zea mays* L. seedlings under toxic cadmium conditions [25] and therefore comparable to our findings.

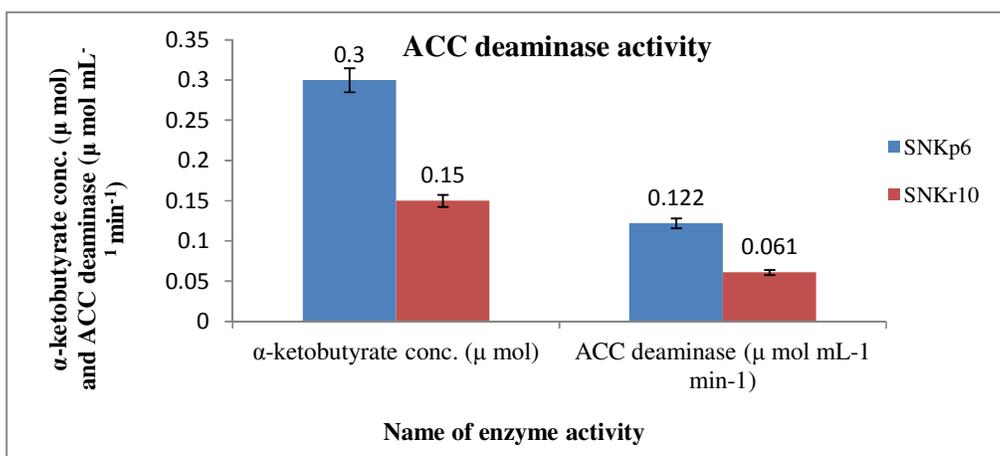


Fig. 1. α -ketobutyrate and ACC-deaminase activity shown by bacterial strains

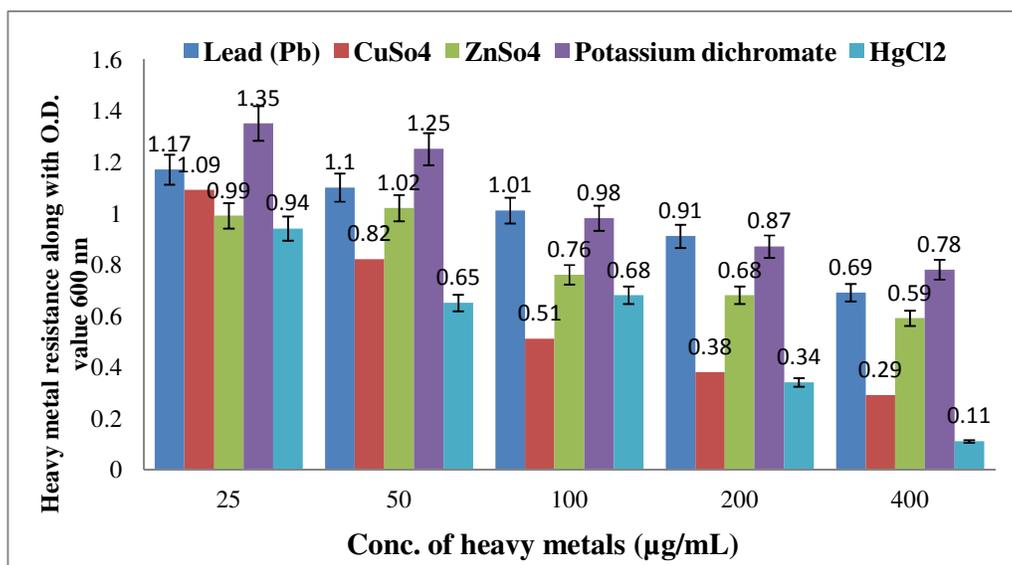


Fig. 2. Heavy metal resistance activity shown by SNKr10 with different concentration of heavy metals

3.5 *In vitro* Seed Germination Assay

In present study, we observed that isolates *Paenibacillus polymyxa* strain SNKp6 and *Bacillus thuringiensis* SNKr10 has shown significant plant growth activities when checked their effect on germination of *V. radiata* (Fig. 3). These bacterial treatment to seeds had shown a stimulatory effect on all seed germination parameters *i.e.* vigor index, germination rate, seedling height, wet/dry weight and root/shoot length as compared to the control. We observed that progressively 100% seed germination when seeds of *V. radiata* were treated with SNKr10 followed by SNKp6 having 98.33% seed germination while in control seed it was only 70.22%. Seedling height was also found to be significant in case of SNKp6 followed by SNKr10 in comparison to control (Fig. 4). The root and shoot length of *V. radiata* plants was also observed to be maximum in case of SNKp6 (11.97 cm; 12.9 cm) and less in SNKr10 treated seedlings in comparison to SNKp6 while little root/shoot length was observed in untreated

control (0.3 cm; 0.8 cm) (Fig. 5). The highest increase in wet and dry weight of *V. radiata* of seeds was in case of isolate SNKp6 less in seeds treated with SNKr10 (Fig. 6). The significant increase in seedling vigor index was also found in SNKp6 treated *V. radiata* seedlings followed by SNKr10 as compared to the control after 5 days of germination at 30°C (Fig. 7). Similar improvement in growth parameters by PGPB is also reported by Kefela et al. [26]. They reported that *Bradyrhizobium japonicum* IRAT FA3, *Paenibacillus polymyxa* and *Bacillus licheniformis* promote the seed germination rate up to 145% higher than the untreated control. Along with this, Zhao et al. [27] also reported that *Burkholderia phytofirmans* strain PsJN helps to increase the biomass and growth by improving the root and shoot growth of several plant species as in potatoes (*Solanum tuberosum* L.) by inducing various phytohormones and are also in favour of our result outcomes. Therefore, our bacterial effect on all these growth parameters of *V. radiata* was also observed to be significant as that of earlier reports of PGPB by researchers.

Table 3. Heavy metal resistance shown by SNKp6

Name of heavy metal	O.D. at 600 nm (SNKp6) (Mean ± S.D.)				
	25 ($\mu\text{g mL}^{-1}$)	50 ($\mu\text{g mL}^{-1}$)	100 ($\mu\text{g mL}^{-1}$)	200 ($\mu\text{g mL}^{-1}$)	400 ($\mu\text{g mL}^{-1}$)
Lead (Pb)	1.85±1.00	1.26±1.01	0.75±1.00	0.41±0.01	0.37±0.11
CuSO ₄	0.75±0.02	1.28±1.01	0.83±0.02	0.62±0.11	0.59±0.01
ZnSO ₄	1.16±0.001	1.07±0.19	0.88±0.01	0.54±0.01	0.74±0.10
Potassium dichromate	0.65±0.01	0.74±0.01	0.30±0.01	0.28±0.01	0.25±1.11
HgCl ₂	0.82±0.01	0.45±0.001	0.47±0.03	0.01±1.01	0.01±1.00
Lead (Pb)	1.85±0.01	1.26±1.01	0.75±0.12	0.41±1.00	0.37±1.02
CuSO ₄	0.75±1.00	1.28±1.00	0.83±1.12	0.62±1.00	0.59±0.01



Fig. 3. *In vitro* seed germination study of *Vigna radiata* seedling after treatment with bacterial isolates in comparison to untreated control

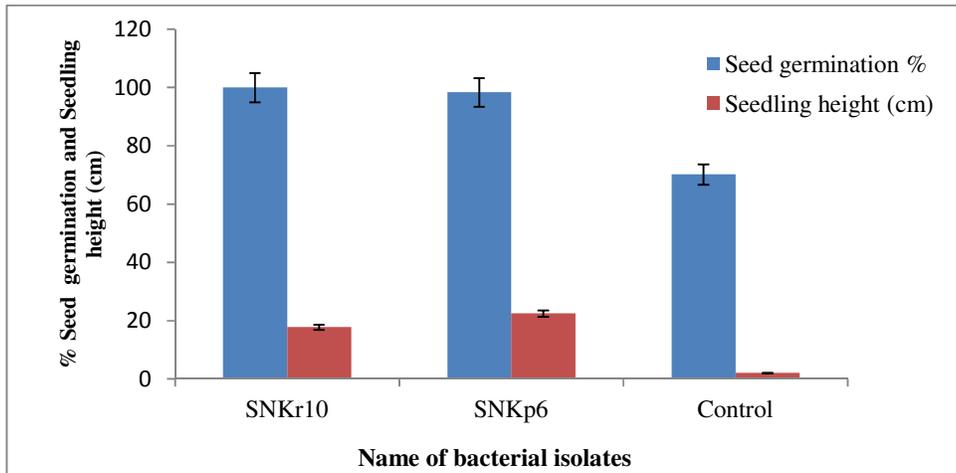


Fig. 4. Effect of bacterial strain on the % germination rate and seedling height of *Vigna radiata* seedlings

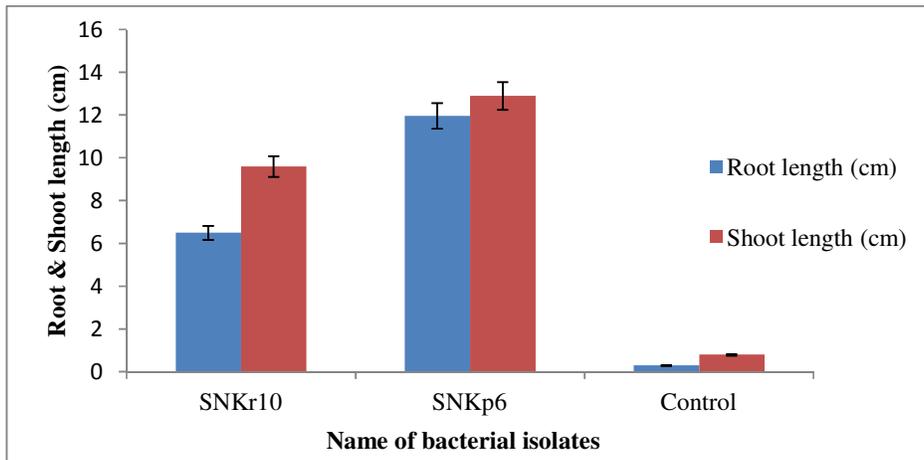


Fig. 5. Effect of bacterial strain on root and shoot length of *V. radiata* seedlings

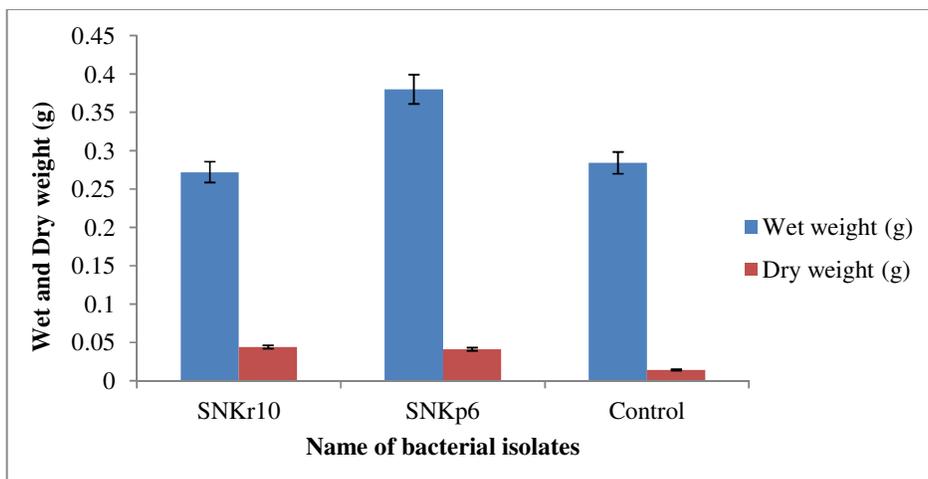


Fig. 6. Effect of bacterial strain on wet and dry weight of *V. radiata* seedlings

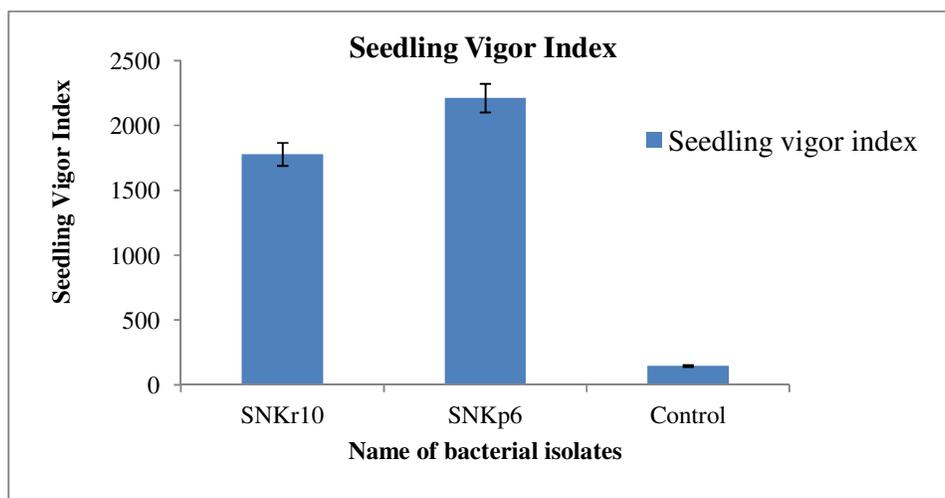


Fig. 7. Effect of bacterial strain on Vigor index of *V. radiata* seedlings

4. CONCLUSION

On the basis of our result outcomes, we can conclude that *Paenibacillus polymyxa* SNKp6 and *Bacillus thuringiensis* SNKr10 bacterial strain have the capability to grow under stress condition due to ACC deaminase activity. They have also shown the ability to grow in high conc. of heavy metals therefor; these can be useful for the sustainable agriculture by helping plants to resist and grow in such type of stressful conditions.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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