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# Plant growth promoting ability of ACC deaminase producing rhizobacteria native to Sunflower (*Helianthus annuus* L.)

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ABSTRACT

Rhizobacteria inhabiting the soil adhering to the root of the host plants are generally recognized to possess plant growth promoting properties. In the present study, a total of 120 rhizobacterial isolates from native soil samples of sunflower were isolated and screened for their ACC deaminase (ACCd) activity and plant growth promoting properties. A significant ACCd activity ranging between 322.55 and 2085.06 nmol of α-ketobutyrate $mg^{-1}$ of protein $h^{-1}$ was observed in 44 isolates with the maximum activity offered by *Bacillus subtilis* Rhizo SF 48. The rhizobacteria which produced more than 50% α-ketobutyrate were selected to evaluate their efficacy to promote the plant growth under laboratory and greenhouse conditions in sunflower. All the ten ACCd positive isolates were found to exhibit at least four different PGP traits and three were found antagonistic to *Fusarium oxysporum*. The seed treatment with ACCd producing PGPR also significantly enhanced both seed and vegetative growth parameters compared to control with a maximum enhancement offered by *B. subtilis* Rhizo SF 48. The findings of the study highlight the efficacy of ACCd producing PGPR in promoting growth parameters in sunflower plants upon seed treatment further warranting to test their ability in the suppression of biotic and abiotic stress.

## 1. Introduction

Plant growth promoting rhizobacteria (PGPR) are a wide range of beneficial microbes inhabiting the root surface of host plants that stimulate plant growth either by direct or indirect mechanisms (Hariprasad et al., 2014; Gowtham et al., 2018). The indirect mechanisms include the reduction in the level of disease, induction of systemic resistance and competition for nutrients (Lugtenberg and Kamilova, 2009; Babu et al., 2015; Tiwari et al., 2018), whereas direct mechanisms include the stimulation of root elongation, providing available nitrogen in simpler forms, production of phytohormones, rhizoremediation, iron chelation by siderophores, phosphate solubilization, etc. (Lucy et al., 2004; Chowdappa et al., 2013). The predominant PGPR residing in the rhizosphere include the isolates of the genera *Acinetobacter*, *Arthrobacter*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Pseudomonas*, etc. (Podile and Kishore, 2006; Babu et al., 2015; Gowtham et al., 2016).

Apart from the production of plant growth promoting properties, these beneficial PGPR are also known to perform some of the functions through specific enzyme activities, which provoke physiological changes in plants at the molecular level. Among these enzymes, bacterial 1-aminocyclopropane-1-carboxylate deaminase (ACCd, E.C.3.5.99.7) plays a role in the down regulation of ethylene production which in turn helps in the growth and development of plants (Saleem et al., 2007; Glick, 2014). The enzyme ACCd was first discovered by Honma and Shimomura in 1978. The enzyme is localized in the cytoplasm of the bacterium which degrades an immediate precursor of ethylene (ACC) in to α-ketobutyrate and ammonium (Glick, 1995; Farajzadeh et al., 2010).

The ACCd producing rhizobacteria are known to facilitate the growth of a variety of plants under stressed conditions such as flood, drought, salt and heavy metals by reducing the ethylene production (Glick, 1995; Saleem et al., 2007; Tiwari et al., 2018). Inoculation of plants with these bacteria leads to increased root growth and/or enhanced formation of lateral root hairs that can result in enhanced tolerance to abiotic stress. The bacteria of the genera *Agrobacterium, Bacillus, Burkholderia, Enterobacter, Methylobacterium, Pseudomonas* and *Rhizobium* have been reported as ACC deaminase producers with an ability to increase plant growth parameters in addition to induction of biotic and abiotic stress (Penrose and Glick, 2001; Madhaiyan et al.,

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2005; Saleem et al., 2007; Vaikuntapu et al., 2014; Tiwari et al., 2018).

Sunflower (*Helianthus annuus* L.) is one of the four major edible oilseed crops in the world and is mainly grown under rainfed conditions on a wide range of soils. In India, sunflower is grown in an area of about 4 lakh ha with an annual production of 2.11 lakh tons with a yield of 5275 hg ha$^{-1}$ (Food and Agriculture Organization of the United Nations, 2018). One of the significant obstacles to high yield and production in sunflower is the lack of synchronized crop cycle which directly depends on the adverse environmental conditions (Mwale et al., 2003). Recently, seed treatment with ACCd producing PGPR are progressively used to improve crop yields in red pepper, tomato, ground nut, *Panicum maximum*, etc. (Mayak et al., 2004; Saravanakumar and Samiyappan, 2007; Siddikee et al., 2011; Tiwari et al., 2018). Hence, in the present study rhizobacteria were isolated form sunflower native soil and were screened for their ability to produce ACCd enzyme along with their effect on plant growth parameters in sunflower.

## 2. Materials and method

### 2.1. Isolation of rhizobacteria from sunflower

A field survey was conducted to collect the rhizospheric soil samples across sunflower growing regions of Karnataka (India) during 2012–13. Rhizospheric soil adhering to the root surface of the plants were carefully collected and taken to the laboratory in polyethylene bags and stored at 4 °C. The collected soil samples were serially diluted up to $10^{-7}$, spread plated on nutrient agar (NA) medium and incubated at 37 °C for 24 h in an incubator. After incubation, each bacterial colony was picked from the edge with sterile inoculation needle, sub-cultured and used throughout the study.

### 2.2. Screening for ACCd producing rhizobacteria

#### 2.2.1. Primary screening

Each of the isolated rhizobacteria was inoculated on to the Petri plates containing ACC Dworkin and Foster minimal salt medium supplemented with 3 mM ACC as the sole nitrogen source (Dworkin and Foster, 1958). The inoculated plates were incubated at 37 °C for 48 h in an incubator and rhizobacteria growing on the medium was termed to be positive for the utilization of ACC and selected for further studies.

#### 2.2.2. Secondary screening

Quantitative measurement of ACCd activity of rhizobacteria was carried out according to the method of Honma and Shimomura (1978) with some modifications. The technique was employed to measure the amount of α-ketobutyrate produced when the enzyme ACCd cleaves ACC using a spectrophotometer. Each of the rhizobacteria which were positive for ACC utilization was inoculated onto a sterile DF salt minimal broth supplemented with ACC (3 mM) and incubated at 37 °C for 48 h in a rotary shaker at 150 rpm. After incubation, the sample was centrifuged at 10,000 rpm for 10 min. The collected supernatant was used to quantify the amount of α-ketobutyrate produced from the ACC positive rhizobacterial isolates by comparing the absorbance measured at 540 nm with the standard curve of α-ketobutyrate. The ACCd activity was expressed as nmol of α-ketobutyrate mg$^{-1}$ of protein h$^{-1}$.

### 2.3. Characterization of ACCd producing rhizobacteria for their plant growth promoting traits

All ACCd producing rhizobacteria were evaluated for their PGPR traits *in vitro*. Root colonization ability was carried out in test tubes containing water agar medium (0.6%) (Silva et al., 2003). Production of indole acetic acid was confirmed on Luria Bertani (LB) broth (1/10th strength) supplemented with L-tryptophan (500 μg mL$^{-1}$) following the method of Patten and Glick (2002). Siderophore production was determined by using King's B broth (1/10th strength) and blue indicator dye (Chrome Azurol S) as described by Schwyn and Neilands (1987). Hydrogen cyanide production was determined in the slants containing NA medium (1/10th strength) amended with glycine (4.4 g L$^{-1}$) and $FeCl_3.6H_2O$ (0.3 mM) by the method of Castric (1975). Halo zone formation on Pikovskaya's medium (Pikovskaya, 1948), protease medium amended with skimmed milk powder (1%) (Gupta et al., 2002), NA medium amended with colloidal chitin (8 g L$^{-1}$) (Renwick et al., 1991) and NA medium supplemented with carboxymethyl cellulose (CMC; 10 g L$^{-1}$) confirmed the solubilization ability of phosphate and production of protease, chitinase and cellulase, respectively.

### 2.4. Antagonistic nature of ACCd producing PGPR against Fusarium wilt of sunflower

All the ACCd producing PGPR isolates were subjected to *in vitro* antagonism against Fusarium wilt of sunflower caused by the pathogen *Fusarium oxysporum* (collected from the Culture Collection Center, Dept. of Studies in Biotechnology, University of Mysore) following the dual culture method (Idris et al., 2008). The rhizobacterial isolates were inoculated onto four corners of Petri plates containing PDA and incubated at 37 °C for 24 h. After incubation, the plates were inoculated with agar plug (5 mm diameter) comprising 7-day-old *F. oxysporum* at the center and incubated for 7 days at 28 ± 2 °C. After incubation, the radii of the fungal colony towards and away from the bacterial colony were measured and percent growth inhibition of *F. oxysporum* was calculated using the following formula:

$$\text{Growth Inhibition (\%)} = \frac{R - r}{R} \times 100$$

where, R is the maximum radius of the fungal colony grown in control plates and r is the radius of the fungal colony growing opposite to the bacterial colony.

### 2.5. Evaluation of pathogenicity of ACCd producing PGPR to sunflower

All the ACCd producing PGPR were evaluated for their pathogenicity to sunflower plants. In brief, three-week-old sunflower plants grown under greenhouse conditions were randomly wounded with $1 \times 10^8$ CFU mL$^{-1}$ of selected bacteria at the center of stem and leaf regions. The inoculated and un-inoculated plants were maintained under greenhouse conditions (25 ± 2 °C with 80% relative humidity) for seven days. The plants were monitored daily for the development of pathogenic symptoms like spots on leaves and stems, necrosis, wilting, etc. up to 7-days after wounding. The experiments were conducted in four replicates of ten plants for each treatment.

### 2.6. Morphological, biochemical and molecular characterization of ACCd producing PGPR

The ACCd producing PGPR were observed for their morphology on NA medium and also under a stereomicroscope for their morphological characters. Each of the bacterium was subjected to Gram's reaction and biochemical tests (Biochemical Characterization Kit, Hi-Media, Bangalore) and identified up to their genus level. The 16S rRNA gene was amplified in a thermal cycler (Eppendorf, Germany) using universal primers (2F, 5′-CCAGACTCCTACGGGAGGCCAGC-3′) and (2R, 5′-GCTGACGAGAGCCATGCAGCACC-3′). The PCR product was sequenced using ABI PRISM 3730XL DNA sequencer in Eurofins Genomics India Pvt. Ltd. (Bangalore, India). The nucleotide sequences obtained were analyzed for their homology with 16S rRNA gene sequences in the NCBI database using the BLAST search algorithm according to the method of Altschul et al. (1997). The sequences were deposited to the NCBI database and accession numbers were obtained. A phylogenetic tree was constructed by comparing each of the ACCd producing PGPR with the isolates showing highest similarities based on their 16S rRNA gene sequences available in the NCBI database. The

**Table 1**
**Screening of rhizobacteria for their ACCd activity.**

| Isolate | Pri. Scr. | Sec. Scr. | Isolate | Pri. Scr. | Sec. Scr. | Isolate | Pri. Scr. | Sec. Scr. | Isolate | Pri. Scr. | Sec. Scr. | Isolate | Pri. Scr. | Sec. Scr. | Isolate | Pri. Scr. | Sec. Scr. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Rhizo SF 1 | + | | Rhizo SF 21 | + | | Rhizo SF 41 | + | | Rhizo SF 61 | – | | Rhizo SF 81 | – | | Rhizo SF 101 | – | |
| Rhizo SF 2 | + | | Rhizo SF 22 | – | | Rhizo SF 42 | + | | Rhizo SF 62 | – | | Rhizo SF 82 | – | | Rhizo SF 102 | – | |
| Rhizo SF 3 | + | | Rhizo SF 23 | + | | Rhizo SF 43 | + | | Rhizo SF 63 | – | | Rhizo SF 83 | – | | Rhizo SF 103 | + | |
| Rhizo SF 4 | + | | Rhizo SF 24 | – | | Rhizo SF 44 | + | | Rhizo SF 64 | + | | Rhizo SF 84 | – | | Rhizo SF 104 | – | |
| Rhizo SF 5 | + | | Rhizo SF 25 | + | | Rhizo SF 45 | + | | Rhizo SF 65 | – | | Rhizo SF 85 | – | | Rhizo SF 105 | – | |
| Rhizo SF 6 | – | | Rhizo SF 26 | – | | Rhizo SF 46 | – | | Rhizo SF 66 | + | | Rhizo SF 86 | – | | Rhizo SF 106 | – | |
| Rhizo SF 7 | + | | Rhizo SF 27 | + | | Rhizo SF 47 | + | | Rhizo SF 67 | – | | Rhizo SF 87 | – | | Rhizo SF 107 | – | |
| Rhizo SF 8 | + | | Rhizo SF 28 | + | | Rhizo SF 48 | + | | Rhizo SF 68 | + | | Rhizo SF 88 | – | | Rhizo SF 108 | + | |
| Rhizo SF 9 | + | | Rhizo SF 29 | – | | Rhizo SF 49 | – | | Rhizo SF 69 | – | | Rhizo SF 89 | – | | Rhizo SF 109 | – | |
| Rhizo SF 10 | – | | Rhizo SF 30 | + | | Rhizo SF 50 | + | | Rhizo SF 70 | + | | Rhizo SF 90 | + | | Rhizo SF 110 | – | |
| Rhizo SF 11 | – | | Rhizo SF 31 | – | | Rhizo SF 51 | – | | Rhizo SF 71 | – | | Rhizo SF 91 | – | | Rhizo SF 111 | – | |
| Rhizo SF 12 | + | | Rhizo SF 32 | – | | Rhizo SF 52 | – | | Rhizo SF 72 | + | | Rhizo SF 92 | – | | Rhizo SF 112 | – | |
| Rhizo SF 13 | – | | Rhizo SF 33 | + | | Rhizo SF 53 | – | | Rhizo SF 73 | – | | Rhizo SF 93 | – | | Rhizo SF 113 | – | |
| Rhizo SF 14 | + | | Rhizo SF 34 | + | | Rhizo SF 54 | – | | Rhizo SF 74 | – | | Rhizo SF 94 | – | | Rhizo SF 114 | – | |
| Rhizo SF 15 | – | | Rhizo SF 35 | – | | Rhizo SF 55 | – | | Rhizo SF 75 | – | | Rhizo SF 95 | – | | Rhizo SF 115 | – | |
| Rhizo SF 16 | – | | Rhizo SF 36 | + | | Rhizo SF 56 | + | | Rhizo SF 76 | – | | Rhizo SF 96 | – | | Rhizo SF 116 | – | |
| Rhizo SF 17 | + | | Rhizo SF 37 | – | | Rhizo SF 57 | + | | Rhizo SF 77 | – | | Rhizo SF 97 | – | | Rhizo SF 117 | – | |
| Rhizo SF 18 | + | | Rhizo SF 38 | + | | Rhizo SF 58 | – | | Rhizo SF 78 | – | | Rhizo SF 98 | + | | Rhizo SF 118 | – | |
| Rhizo SF 19 | + | | Rhizo SF 39 | – | | Rhizo SF 59 | – | | Rhizo SF 79 | – | | Rhizo SF 99 | – | | Rhizo SF 119 | – | |
| Rhizo SF 20 | + | | Rhizo SF 40 | – | | Rhizo SF 60 | + | | Rhizo SF 80 | – | | Rhizo SF 100 | – | | Rhizo SF 120 | – | |


Note: Pri. Scr.- Primary screening and Sec. Scr.- Secondary screening of ACCd producing rhizobacteria; '+' indicates positive and '–' indicates negative for the experiment; 0%–100% indicates the per cent production of nmol of α-ketobutyrate $mg^{-1}$ of protein $h^{-1}$ compared to highest ACCd producing rhizobacteria (Rhizo SF 48 is considered as 100%).

phylogenetic tree was constructed using MEGA-X software by the neighbour-joining (NJ) method with the Kimura 2-parameter model.

### 2.7. Evaluation of plant growth promoting ability of ACCd producing PGPR

#### 2.7.1. Inoculum preparation

Each of the ACCd producing PGPR was grown in 100 mL of sterilized nutrient broth (NB) in 250 mL Erlenmeyer flask and incubated for 24 h at 37 °C in a rotary shaker at 150 rpm. After incubation, the bacterial culture was centrifuged at 8000 rpm for 10 min. The bacterial pellet was re-suspended in sterile distilled water and the bacterial cell count was adjusted to $1 \times 10^8$ CFU $mL^{-1}$.

#### 2.7.2. Seed bacterization

Sunflower (*Helianthus annuus* L.) seeds were surface sterilized in 4% NaOCl for 1 min and washed thrice with sterile distilled water. The surface sterilized seeds were bacterized overnight with ACCd producing PGPR suspension containing $1 \times 10^8$ CFU $mL^{-1}$ amended with CMC (0.2% w/v) and soaked for 12 h on a rotary shaker at 150 rpm (Silva et al., 2003). Seeds soaked in sterile distilled water with CMC served as control.

#### 2.7.3. Seed growth parameters

Each of the ACCd producing PGPR treated and untreated control seeds with four replicates of 100 seeds (10 seeds Petri $dish^{-1}$) were plated equidistantly on three layers of moistened blotter discs placed in Petri dish to evaluate percent seed germination (ISTA, 2005). Another set of treated seeds were subjected to between paper method with four replicates of 100 seeds (50 seeds paper $towel^{-1}$) (Abdul Baki and Anderson, 1973). Each set was incubated in an incubation chamber at 25 ± 2 °C for 7 days. After incubation, percent seed germination, root length and shoot length were recorded and vigor index was calculated

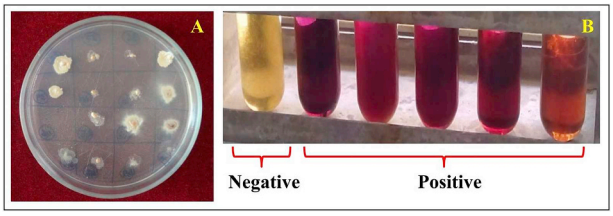



**Fig. 1.** ACC utilization by rhizobacteria on agar (A) and liquid media (B).

by using the following formula

$$\text{Vigor Index [VI]} = [(\text{Mean Root Length} + \text{Mean Shoot Length}) \times \% \text{ Germination}]$$

#### 2.7.4. Vegetative growth parameters

Sunflower seeds treated with ACCd producing PGPR and sterile distilled water were sown in poly cups (10 cm diameter) containing 2: 1: 1 red soil, sand and farmyard manure (FYM) which was previously autoclaved. The seedlings were watered daily and maintained at 25 ± 2 °C with 80% relative humidity (RH) for 30 days. The 30-day-old seedlings were uprooted carefully and used to record plant height, shoot fresh weight and shoot dry weight.

### 2.8. Statistical analysis

All the experiments were conducted in quadruplicates. The experimental data from laboratory and greenhouse were statistically analyzed separately and subjected to arcsine transformation and analysis of variance (ANOVA) using Statistical Package for the Social Sciences

**Table 2**
Plant Growth Promoting traits of ACCd producing rhizobacteria.

| Isolate | RC | IAA | Siderophore | HCN | PS | Protease | Chitinase | Cellulase |
|---|---|---|---|---|---|---|---|---|
| Rhizo SF 4 | + | + | – | – | – | – | + | – |
| Rhizo SF 7 | + | – | + | + | – | + | – | – |
| Rhizo SF 9 | + | + | – | – | + | – | – | – |
| Rhizo SF 23 | + | + | – | + | + | + | – | – |
| Rhizo SF 41 | + | – | + | – | + | + | – | – |
| Rhizo SF 44 | + | + | – | + | + | + | – | – |
| Rhizo SF 48 | + | + | – | + | + | – | + | + |
| Rhizo SF 64 | + | | – | – | – | – | – | – |
| Rhizo SF 90 | + | + | – | + | – | + | + | – |
| Rhizo SF 108 | + | + | + | – | – | + | – | + |

Note: RC – Root colonization, IAA – Indole Acetic Acid, HCN – Hydrogen cyanide, PS – Phosphate solubilization. '+' indicates positive and '–' indicates negative for the experiments.

(SPSS) IBM Statistics, Version 23 (SPSS Inc., Chicago, IL). The significant differences between the treatment mean values were determined by Highest Significant Difference (HSD) obtained by Tukey's test at $p \leq 0.05$ level.

## 3. Results

### 3.1. Isolation of rhizobacteria from sunflower

A total of 26 soil samples from seven sunflower growing districts of Karnataka were collected during the year 2012–2013. A total of 120 rhizobacterial isolates were isolated by serial dilution technique and each of the isolates were maintained on NA medium and glycerol stock solutions (Suppl. Table 1). All the isolated rhizobacteria were provided with a specific identity and stored at 4 °C until further use.

### 3.2. Screening for ACCd producing rhizobacteria

#### 3.2.1. Primary screening

The isolated rhizobacteria were grown on ACC supplemented DF media. The bacteria which were able to grow and utilize the nitrogen source (ACC) were selected as positive for ACCd production. Among the 120 rhizobacterial isolates, a total of 44 isolates were able to grow on media (Table 1 and Fig. 1A). The positive rhizobacteria were subjected to the quantification of α-ketobutyrate.

#### 3.2.2. Secondary screening

The rhizobacterial isolates (44 isolates) which were found positive in primary screening were inoculated on sterile DF salt minimal broth containing ACC (3 mM) to confirm the ability of the isolates to cleave ACC by quantifying the amount of α-ketobutyrate produced by the bacterial enzyme ACCd (Suppl. Table 2). It was noted that all the 44 rhizobacterial isolates were able to cleave ACC and produced α-ketobutyrate. A maximum of 2085.06 nmol of α-ketobutyrate mg of protein$^{-1}$ h$^{-1}$ was produced by Rhizo SF 48, while a minimum of 322.5 nmol α-ketobutyrate mg of protein$^{-1}$ h$^{-1}$ was observed with Rhizo SF 25 (Table 1 and Fig. 1B). The isolates which produced more than 50% of α-ketobutyrate compared to Rhizo SF 48 (maximum production) were selected for further studies.

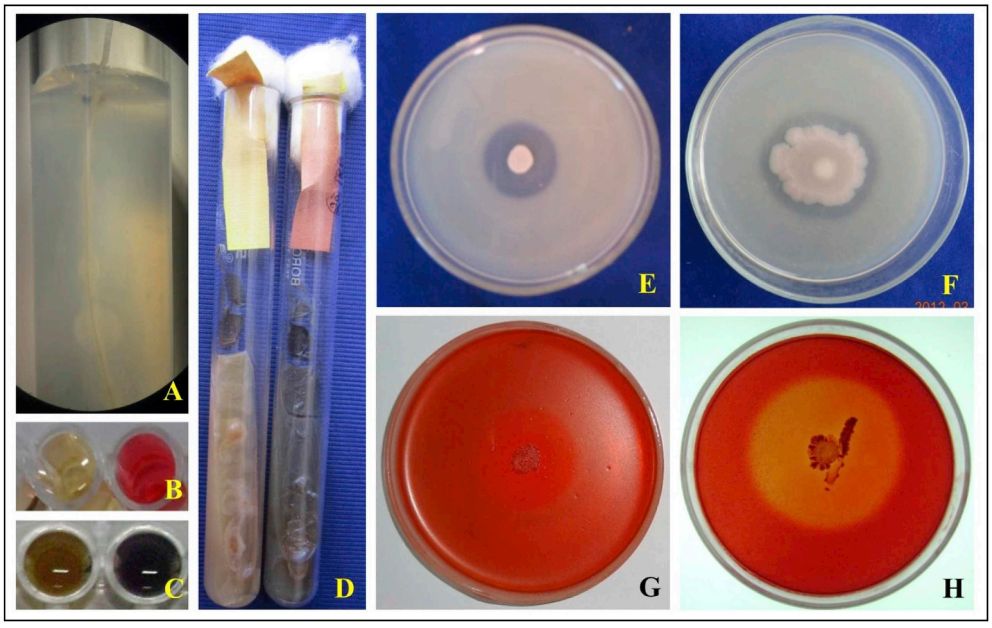



**Fig. 2.** Representative images for plant growth promoting traits of ACCd producing rhizobacteria. A: Root colonization; B: IAA production; C: Siderophore production; D: HCN production; E: Phosphate solubilization; F: Protease production; G: Chitinase production; H: Cellulase production.

**Table 3**
Antagonistic and Pathogenic nature of ACCd producing PGPR.

| Isolate | Antagonism* | Pathogenicity# |
|---|---|---|
| Rhizo SF 4 | – | – |
| Rhizo SF 7 | – | – |
| Rhizo SF 9 | – | – |
| Rhizo SF 23 | 43.54 ± 0.98[b] | – |
| Rhizo SF 41 | 35.70 ± 0.60[c] | – |
| Rhizo SF 44 | – | – |
| Rhizo SF 48 | 47.85 ± 1.23[a] | – |
| Rhizo SF 64 | – | – |
| Rhizo SF 90 | – | – |
| Rhizo SF 108 | – | – |

Values are means of four independent replicates. ± indicates standard errors. Means followed by the same letter(s) within the same column are not significantly ($p \leq 0.05$) different according to Tukey's HSD. *Antagonistic to *F. oxysporum*; #Pathogenic to sunflower plants and '–' indicates negative for the experiment.

### 3.3. Characterization of ACCd producing rhizobacteria for their plant growth promoting traits

All the ten ACCd producing rhizobacterial isolates were evaluated for their efficacy for plant growth promoting traits and the results are depicted in Table 2 and Fig. 2. All the ACCd producing PGPR were able to colonize the roots of sunflower (formation of turbid and milky zones along with slimy exudates surrounding the root segments), eight isolates were positive for IAA production (pink coloration on media after incubation), three isolates were positive for siderophore production (yellowish-orange coloration on media after incubation) and five isolates were found positive for HCN production (development of dark yellow to brown colour in picric acid strips). Similarly, the formation of halo zones around the inoculated rhizobacterial colonies on specific media confirmed the solubilization of phosphate (5 isolates) and production of protease (6 isolates) chitinase (3 isolates) and cellulase (2 isolates).

### 3.4. Antagonistic nature of ACCd producing PGPR against Fusarium wilt of sunflower

The ACCd producing PGPR isolates were screened for their antagonism against Fusarium wilt disease of sunflower caused by *F. oxysporum*. The results of antagonism study showed that, only Rhizo SF 23, Rhizo SF 41 and Rhizo SF 48 were found to inhibit the mycelial growth of *F. oxysporum* among the isolates tested (Table 3 and Suppl. Fig. 1). The ACCd producing PGPR, Rhizo SF 48 offered maximum percent of inhibition (47.85%), followed by Rhizo SF 23 which showed inhibition of 43.54%.

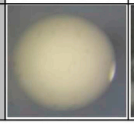
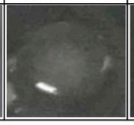
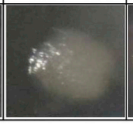
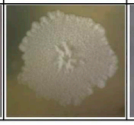
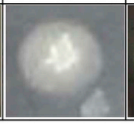
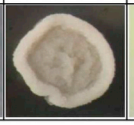
### 3.5. Evaluation of pathogenicity of ACCd producing PGPR to sunflower

The ACCd producing PGPR were evaluated for their pathogenicity to sunflower under greenhouse conditions. The pathogenicity results of the study revealed that, all the ACCd producing PGPR were non-pathogenic to sunflower plants as they did not produce any of the pathogenic symptoms in plants (Table 3). From the results it was confirmed that the selected bacterial isolates were non-pathogenic to sunflower.

### 3.6. Morphological, biochemical and molecular characterization of ACCd producing PGPR

The morphological (colony and microscopic characters) and biochemical characterization of each of the ACCd producing PGPR are given in Table 4 and Suppl. Table 3, respectively. The PCR amplification of the 16S rRNA gene generated bands in the region of 550–600 bp. The nucleotide sequences obtained were analyzed (compared with GenBank database) to identify the similarity using the NCBI BLAST at https://blast.ncbi.nlm.nih.gov/Blast.cgi. The partial 16S rRNA gene

**Table 4**
Morphological characteristics of ACCd producing PGPR.

| Morphology / Organism | Rhizo SF 4 | Rhizo SF 7 | Rhizo SF 9 | Rhizo SF 23 | Rhizo SF 41 | Rhizo SF 44 | Rhizo SF48 | Rhizo SF 64 | Rhizo SF 90 | Rhizo SF 108 |
|---|---|---|---|---|---|---|---|---|---|---|
| **COLONY CHARACTERS** | | | | | | | | | | |
| Shape | Round | Round | Round | Round | Wavy | Round | Round | Round | Round | Round |
| Colour | White/ opaque | Transparent | White /opaque | Milky white | Cream/opaque | Transparent | Milky white | White/ opaque | Milky white | Milky white |
| Pigmentation | No | Yellow to brown | No | No | No | Yellow to brown | No | No | No | No |
| Elevation | Doomed | Flat | Doomed | Flat | Flat | Flat | Slightly raised | Doomed | Slightly raised | Slightly raised |
| Surface | Smooth and shiny | Smooth and shiny | Shiny | Rough and non-shiny | Smooth and shiny | Smooth and non-shiny | Rough and non-shiny | Rough and non-shiny | Rough and non-shiny | Rough and non-shiny |
| Margin | Continuous | Continuous | Serrate | Serrate | Continuous | Continuous | Serrate | Serrate | Serrate | Serrate |
| Colony on NA |  |  |  |  |  |  |  |  |  |  |
| **MICROSCOPIC CHARACTERS** | | | | | | | | | | |
| Shape | Rods | Rods | Coccobacilli | Rods | Rods | Rods | Rods | Rods | Rods | Rods |
| Motility test | M | M | NM | M | M | M | M | M | M | M |
| Flagella | Yes | Yes | -- | Yes | Yes | Yes | Yes | Yes | Yes | Yes |

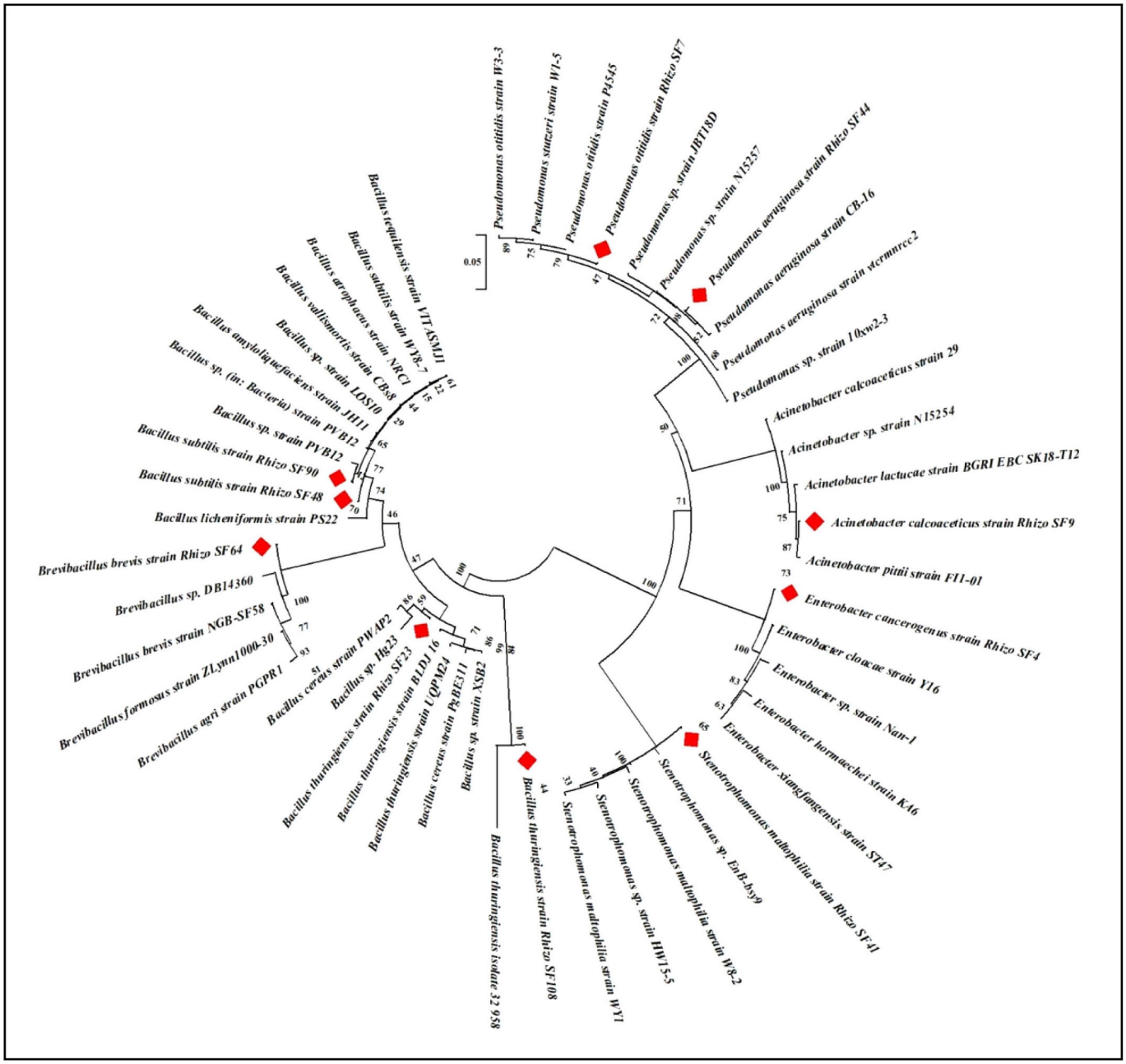



**Fig. 3.** Phylogenetic tree derived from Neighbour-Joining analysis showing the evolutionary relationship of ACCd producing rhizobacteria with their closest BLAST hits.

sequences of all the ACCd producing PGPR were deposited to GenBank of the National Center for Biotechnology Information (NCBI) and accession numbers were obtained (Suppl. Table 4). A phylogenetic tree was constructed to show the relationship between the ACCd producing PGPR isolates selected from the NCBI database (Fig. 3). The result showed a clear divergence of bacterial species by the formation of various clades and single membered clades with different ACCd producing PGPR across the dendrogram. The bootstrapping (1000) indicated that each branch corresponding to the particular clade of the species was well supported.

### 3.7. Evaluation of plant growth promoting ability of ACCd producing PGPR

#### 3.7.1. Seed growth parameters

Among the ten ACCd producing PGPR selected, only eight isolates offered significant ($p \leq 0.05$) enhancement in seed germination and seedling vigor compared to control. It was observed that Rhizo SF 48 isolate offered maximum improvement in seed germination (90%) and seedling vigor (1295.63) (Figs. 4, 5A and 5B), followed by Rhizo SF 23 isolate. The control offered a lower percent of seed germination (75.75%) and seedling vigor (601.14). Among the isolates evaluated, Rhizo SF 90 and Rhizo SF 108 treatment to seeds were not found significant compared to control.

#### 3.7.2. Vegetative growth parameters

Sunflower seeds treated with ACCd producing PGPR along with control were evaluated for their effect on vegetative growth parameters at 30-days after sowing. The results of the study revealed that, a significant ($p \leq 0.05$) increase in growth parameters were observed in ACCd producing PGPR treated plants when compared to control plants (Figs. 4, 5C and 5D). Among the ACCd producing PGPR evaluated, a maximum plant height (17.6 cm), shoot fresh weight (0.9 g) and shoot

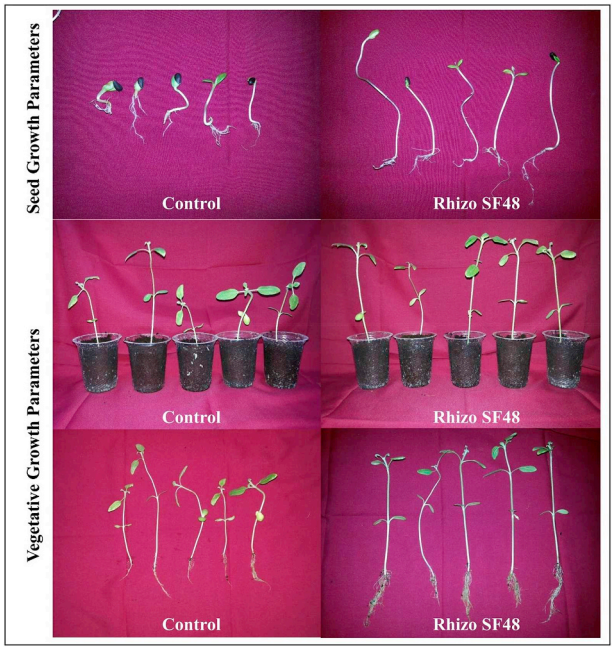



**Fig. 4.** Effect on plant growth promoting properties in sunflower upon seed treatment with ACCd producing Rhizo SF 48.

dry weight (0.42 g) were observed upon Rhizo SF 48 treatment, followed by Rhizo SF 23. The results showed that there was an increase of 0.25–0.8 fold in vegetative growth parameters in ACCd producing PGPR compared to control seedlings.

## 4. Discussion

The rhizosphere microbes which possess beneficial traits offer the first line of defense to the host plants to the invading pathogens. In the present study, seven major districts of sunflower growing regions of Karnataka were visited (during 2012–2013) to collect the rhizosphere soil (26 samples) and to isolate rhizobacteria. All the collected soil samples were serially diluted and a total of 120 bacterial isolates were isolated from the soil samples collected from different agro-climatic regions. In accordance with our studies, screening for plant growth promoting rhizobacteria was also carried out from sunflower for evaluation of their efficacy to improve plant growth from Brazil (Ambrosini et al., 2012). All the rhizobacterial isolates were screened for the utilization of ACC substrate in the DF minimal salt agar media. The screening results showed that, among the 120 isolates, 44 isolates were positive for ACCd production. Likewise, Barnawal et al. (2013) have screened the rhizobacterial isolates for ACCd production from the rhizospheric soil of lemongrass. The isolates which were found positive for ACCd production in agar media were further subjected for their ACC utilization rate in DF salt minimal broth containing ACC (3 mM) to confirm the quantification ability of the isolates to cleave ACC to produce α-ketobutyrate by ACCd enzyme as reported in the studies of Dworkin and Foster (1958). The quantification studies reported that all the isolates were able to produce α-ketobutyrate ranging between 322.5 and 2085.06 nmol mg of protein$^{-1}$ h$^{-1}$. It has been attributed that the quantification of the amount of α-ketobutyrate produced by the rhizobacteria is necessary to confirm the positive nature of the ACCd production. The method employed here is widely accepted for the identification of ACCd producing rhizobacteria (Penrose and Glick, 2001). Recently, Tiwari et al. (2018) have also screened rhizobacteria isolated from *Panicum maximum* soil for isolation of ACCd producing

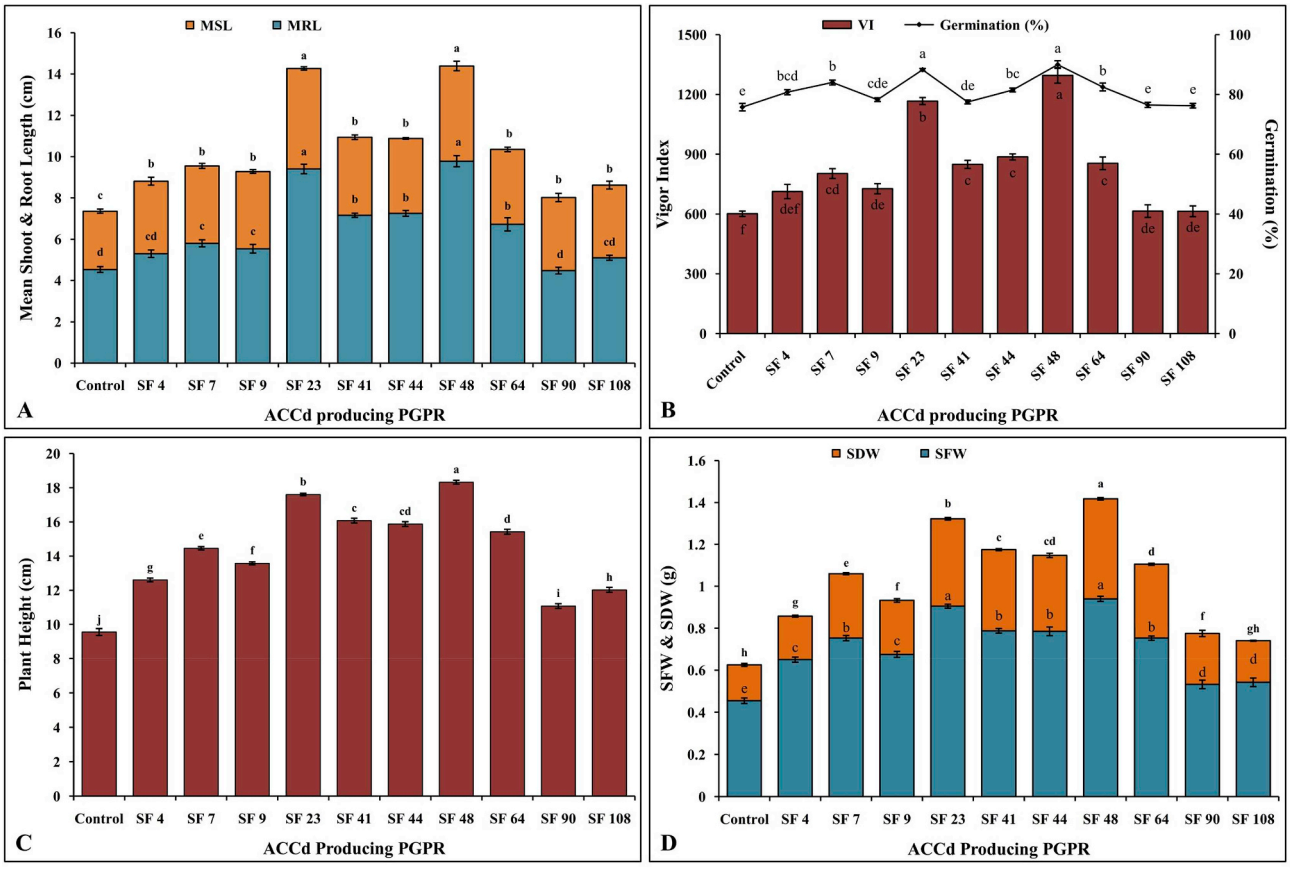



**Fig. 5.** Effect of ACCd producing PGPR on seed and vegetative growth parameters in sunflower. Each value is the mean for four replicates (n = 4) and bars sharing the same letters are not significantly ($p \leq 0.05$) different according to Tukey's HSD. The vertical bar indicates the standard error.

rhizobacteria through quantification of α-ketobutyrate.

The ACCd producing rhizobacteria were evaluated for their multiple plant growth promoting traits. All the ten selected ACCd producing rhizobacteria were efficient in offering at least four plant growth promoting traits evaluated. Raval and Desai (2012) have isolated bacteria from rhizosphere of sunflower which showed multiple-plant growth promoting traits like IAA production, phosphate solubilization and antifungal activity against plant pathogenic fungi. It has been documented that, rhizobacteria possessing beneficial traits stimulate plant growth by the production of hormones (IAA) and indirectly providing phosphorus and other mineral nutrients by nitrogen fixation (Vacheron et al., 2013). These multiple plant growth promoting abilities of rhizobacteria have been correlated to their non-pathogenic nature, disease suppressing and also abiotic stress induction (Gowtham et al., 2018; Tiwari et al., 2018). In accordance, Mayak et al. (2004) have also isolated rhizobacteria which possessed both plant growth promoting traits along with ACCd. Tiwari et al. (2018) have stated that rhizobacteria which are capable of showing multi-plant growth promoting traits along with ACCd have a better symbiotic association with the host plants irrespective of the agro-climatic conditions. In the study, morphological, biochemical and molecular characterization of all the positive isolates for ACCd were carried out to identify the isolates up to their species.

Further, ACCd positive isolates were evaluated for their antagonistic nature against stem wilt pathogen of sunflower caused by *F. oxysporum*. The results of the study revealed that among the 10 ACCd positive isolates, Rhizo SF 23, Rhizo SF 41 and Rhizo SF 48 showed inhibition towards the growth of *F. oxysporum*. Similarly, PGPR has been reported for their antagonistic nature against various pathogens including *F. oxysporum* (Hariprasad et al., 2014; Gowtham et al., 2016). This antagonistic nature of PGPR against plant pathogens is correlated with the production of secondary metabolites which obstruct the growth and progress of pathogens (Williams and Asher, 1996).

The sunflower seeds upon treatment with ACCd positive isolates offered enhanced seed and vegetative growth parameters. Among the isolates, Rhizo SF 48 offered maximum seed germination (90%) and vigor (1295). Similar observations were observed upon seed treatment with PGPR *Bacillus* spp. wherein a significant enhancement in the plant growth promoting properties was found in various crops (Mayak et al., 2004; Yuan et al., 2013; Tiwari et al., 2018). Likewise in the present study, maximum enhancement in vegetative growth parameters was also obtained upon treatment with *B. subtilis* Rhizo SF 48, followed by *B. thuringiensis* Rhizo SF 23. The ACCd producing PGPR isolates upon treatment to sunflower increased root and shoot elongation there by leading to enhanced plant growth. The results are in confirmation with previous studies, wherein seed treatment with ACCd producing PGPR enhanced the plant growth promotion to their host plants (tomato, pepper, lemon grass, *P. maximum*, etc.) (Mayak et al., 2004; Yuan et al., 2013; Tiwari et al., 2018). Interestingly, the most prominent beneficial effects of inoculation with native potential PGPR isolates have been reported in many host plants that lead to the improvement in their growth and health (Vaikuntapu et al., 2014; Tiwari et al., 2018). Further work on the evaluation of these potential ACCd producing PGPR isolates against biotic and abiotic stress suppression to the host plant is in progress.

## 5. Conclusions

The present study was conducted to screen the rhizobacteria native to sunflower for ACCd production with a potential for multiple plant growth promoting traits. About ten rhizobacteria isolates were found to be positive to ACCd production with beneficial properties (PGP traits) and they were also non-pathogenic to sunflower. The antagonistic studies revealed that, among the test bacterial isolates only Rhizo SF 23, Rhizo SF 41 and Rhizo SF 48 were able to inhibit the growth of Fusarium wilt pathogen. Further, from the results it was observed that, the isolated ACCd producing PGPR were able to significantly enhance the seed and vegetative growth parameters of sunflower there by warranting for their exploitation for suppression of biotic and abiotic stress.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101089.

## References

Abdul-Baki, A.A., Anderson, J.D., 1973. Vigor determination in soybean seed by multiple criteria 1. Crop Sci. 13 (6), 630–633.
Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25 (17), 3389–3402.
Ambrosini, A., Beneduzi, A., Stefanski, T., Pinheiro, F.G., Vargas, L.K., Passaglia, L.M., 2012. Screening of plant growth promoting rhizobacteria isolated from sunflower (*Helianthus annuus* L.). Plant Soil 356 (1–2), 245–264.
Babu, A.N., Jogaiah, S., Ito, S.I., Nagaraj, A.K., Tran, L.S.P., 2015. Improvement of growth, fruit weight and early blight disease protection of tomato plants by rhizosphere bacteria is correlated with their beneficial traits and induced biosynthesis of antioxidant peroxidase and polyphenol oxidase. Plant Sci. 231, 62–73.
Barnawal, D., Maji, D., Bharti, N., Chanotiya, C.S., Kalra, A., 2013. ACC deaminase-containing Bacillus subtilis reduces stress ethylene-induced damage and improves mycorrhizal colonization and rhizobial nodulation in *Trigonellafoenum-graecum* under drought stress. J. Plant Growth Regul. 32 (4), 809–822.
Castric, P.A., 1975. Hydrogen cyanide, a secondary metabolite of *Pseudomonas aeruginosa*. Can. J. Microbiol. 21 (5), 613–618.
Chowdappa, P., Kumar, S.M., Lakshmi, M.J., Upreti, K.K., 2013. Growth stimulation and induction of systemic resistance in tomato against early and late blight by *Bacillus subtilis* OTPB1 or *Trichoderma harzianum* OTPB3. Biol. Control 65 (1), 109–117.
Dworkin, M., Foster, J.W., 1958. Experiments with some microorganisms which utilize ethane and hydrogen. J. Bacteriol. 75 (5), 592.
Farajzadeh, D., Aliasgharzad, N., Bashir, N.S., Yakhchali, B., 2010. Cloning and characterization of a plasmid encoded ACC deaminase from an indigenous *Pseudomonas fluorescens* FY32. Curr. Microbiol. 61 (1), 37–43.
Food and Agriculture Organization of the United Nations, 2018. FAOSTAT Statistics Database. (Rome).
Glick, B.R., 1995. The enhancement of plant growth by free-living bacteria. Can. J. Microbiol. 41 (2), 109–117.
Glick, B.R., 2014. Bacteria with ACC deaminase can promote plant growth and help to feed the world. Microbiol. Res. 169 (1), 30–39.
Gowtham, H.G., Hariprasad, P., Nayak, S.C., Niranjana, S.R., 2016. Application of rhizobacteria antagonistic to *Fusarium oxysporum* f. sp. *lycopersici* for the management of Fusarium wilt in tomato. Rhizosphere 2, 72–74.
Gowtham, H.G., Murali, M., Singh, S.B., Lakshmeesha, T.R., Murthy, K.N., Amruthesh, K.N., Niranjana, S.R., 2018. Plant growth promoting rhizobacteria *Bacillus amyloliquefaciens* improves plant growth and induces resistance in chilli against anthracnose disease. Biol. Control 126, 209–217.
Gupta, A., Meyer, J.M., Goel, R., 2002. Development of heavy metal-resistant mutants of phosphate solubilizing *Pseudomonas* sp. NBRI 4014 and their characterization. Curr. Microbiol. 45 (5), 323–327.
Hariprasad, P., Chandrashekar, S., Singh, S.B., Niranjana, S.R., 2014. Mechanisms of plant growth promotion and disease suppression by *Pseudomonas aeruginosa* strain 2apa. J. Basic Microbiol. 54 (8), 792–801.
Honma, M., Shimomura, T., 1978. Metabolism of 1-aminocyclopropane-1-carboxylic acid. Agric. Biol. Chem. 42 (10), 1825–1831.
Idris, H.A., Labuschagne, N., Korsten, L., 2008. Suppression of *Pythium ultimum* root rot of sorghum by rhizobacterial isolates from Ethiopia and South Africa. Biol. Control 45 (1), 72–84.
ISTA, 2005. Proceedings of the international seed testing association. Internationalrules of seed testing. Seed Sci. Technol. 15, 1–9.
Lucy, M., Reed, E., Glick, B.R., 2004. Applications of free living plant growth-promoting rhizobacteria. Antonie Leeuwenhoek 86 (1), 1–25.
Lugtenberg, B., Kamilova, F., 2009. Plant growth-promoting rhizobacteria. Annu. Rev. Microbiol. 63, 541–556.
Madhaiyan, M., Poonguzhali, S., Lee, H.S., Hari, K., Sundaram, S.P., Sa, T.M., 2005. Pink-pigmented facultative methylotrophic bacteria accelerate germination, growth and yield of sugarcane clone Co86032 (*Saccharum officinarum* L.). Biol. Fertil. Soils 41 (5), 350–358.
Mayak, S., Tirosh, T., Glick, B.R., 2004. Plant growth-promoting bacteria that confer resistance to water stress in tomatoes and peppers. Plant Sci. 166 (2), 525–530.
Mwale, S.S., Hamusimbi, C., Mwansa, K., 2003. Germination, emergence and growth of

sunflower (*Helianthus annuus* L.) in response to osmotic seed priming. Seed Sci. Technol. 31 (1), 199–206.
Patten, C.L., Glick, B.R., 2002. Role of *Pseudomonas putida* indole acetic acid in development of the host plant root system. Appl. Environ. Microbiol. 68 (8), 3795–3801.
Penrose, D.M., Glick, B.R., 2001. Levels of 1-aminocyclopropane-1-carboxylic acid (ACC) in exudates and extracts of canola seeds treated with plant growth-promoting bacteria. Can. J. Microbiol. 47 (4), 368–372.
Pikovskaya, R.I., 1948. Mobilization of phosphorus in soil in connection with vital activity of some microbial species. Mikrobiologiya 17, 362–370.
Podile, A.R., Kishore, G.K., 2006. Plant-associated Bacteria. Plant Growth Promoting Rhizobacteria. Springer, Amsterdam, pp. 195–230.
Raval, A.A., Desai, P.B., 2012. Rhizobacteria from rhizosphere of sunflower (*Helianthus annuus* L.) and their effect on plant growth. Res. J. Recent Sci. 1 (6), 58–61.
Renwick, A., Campbell, R., Coe, S., 1991. Assessment of in vivo screening systems for potential biocontrol agents of *Gaeumannomyces graminis*. Plant Pathol. 40 (4), 524–532.
Saleem, M., Arshad, M., Hussain, S., Bhatti, A.S., 2007. Perspective of plant growth promoting rhizobacteria (PGPR) containing ACC deaminase in stress agriculture. J. Ind. Microbiol. Biotechnol. 34 (10), 635–648.
Saravanakumar, D., Samiyappan, R., 2007. ACC deaminase from *Pseudomonas fluorescens* mediated saline resistance in groundnut (*Arachis hypogaea*) plants. J. Appl. Microbiol. 102, 1283–1292.
Schwyn, B., Neilands, J.B., 1987. Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. 160 (1), 47–56.
Siddikee, M.A., Glick, B.R., Chauhan, P.S., jongYim, W., Sa, T., 2011. Enhancement of growth and salt tolerance of red pepper seedlings (*Capsicum annuum* L.) by regulating stress ethylene synthesis with halo tolerant bacteria containing 1-aminocyclopropane-1-carboxylic acid deaminase activity. Plant Physiol. Biochem. 49 (4), 427–434.
Silva, H.S.A., Romeiro, R.D.S., Mounteer, A., 2003. Development of a root colonization bioassay for rapid screening of rhizobacteria for potential biocontrol agents. J. Phytopathol. 151 (1), 42–46.
Tiwari, G., Duraivadivel, P., Sharma, S., Hariprasad, P., 2018. 1-Aminocyclopropane-1-carboxylic acid deaminase producing beneficial rhizobacteria ameliorate the biomass characters of *Panicum maximum* Jacq. by mitigating drought and salt stress. Sci. Rep. 8, 17513.
Vacheron, J., Desbrosses, G., Bouffaud, M.L., Touraine, B., Moënne-Loccoz, Y., Muller, D., Prigent-Combaret, C., 2013. Plant growth-promoting rhizobacteria and root system functioning. Front. Plant Sci. 4, 356.
Vaikuntapu, P.R., Dutta, S., Samudrala, R.B., Rao, V.R., Kalam, S., Podile, A.R., 2014. Preferential promotion of *Lycopersicon esculentum* (Tomato) growth by plant growth promoting bacteria associated with tomato. Indian J. Microbiol. 54 (4), 403–412.
Williams, G.E., Asher, M.J.C., 1996. Selection of rhizobacteria for the control of *Pythium ultimum* and *Aphanomyces cochlioides* on sugar-beet seedlings. Crop Protect. 15 (5), 479–486.
Yuan, J., Ruan, Y., Wang, B., Zhang, J., Waseem, R., Huang, Q., Shen, Q., 2013. Plant growth-promoting rhizobacteria strain *Bacillus amyloliquefaciens* NJN-6-enriched bio-organic fertilizer suppressed Fusarium wilt and promoted the growth of banana plants. J. Agric. Food Chem. 61 (16), 3774–3780.