

Effectiveness of Rhizobacteria Containing ACC Deaminase for Growth Promotion of Peas (*Pisum sativum*) Under Drought Conditions

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A series of experiments were conducted to assess the effectiveness of rhizobacteria containing 1-aminocyclopropane-1-carboxylate (ACC) deaminase for growth promotion of peas under drought conditions. Ten rhizobacteria isolated from the rhizosphere of different crops (peas, wheat, and maize) were screened for their growth promoting ability in peas under axenic condition. Three rhizobacterial isolates, *Pseudomonas fluorescens* biotype G (ACC-5), *P. fluorescens* (ACC-14), and *P. putida* biotype A (Q-7), were selected for pot trial on the basis of their source, ACC deaminase activity, root colonization, and growth promoting activity under axenic conditions. Inoculated and uninoculated (control) seeds of pea cultivar 2000 were sown in pots (4 seeds/pot) at different soil moisture levels (25, 50, 75, and 100% of field capacity). Results revealed that decreasing the soil moisture levels from 100 to 25% of field capacity significantly decreased the growth of peas. However, inoculation of peas with rhizobacteria containing ACC deaminase significantly decreased the “drought stress imposed effects” on growth of peas, although with variable efficacy at different moisture levels. At the lowest soil moisture level (25% field capacity), rhizobacterial isolate *Pseudomonas fluorescens* biotype G (ACC-5) was found to be more promising compared with the other isolates, as it caused maximum increases in fresh weight, dry weight, root length, shoot length, number of leaves per plant, and water use efficiency on fresh and dry weight basis (45, 150, 92, 45, 140, 46, and 147%, respectively) compared with respective uninoculated controls. It is highly likely that rhizobacteria containing ACC deaminase might have decreased the drought-stress induced ethylene in inoculated plants, which resulted in better growth of plants even at low moisture levels. Therefore, inoculation with rhizobacteria containing ACC deaminase could be helpful in eliminating the inhibitory effects of drought stress on the growth of peas.

Keywords: Rhizobacteria, ethylene, water stress, ACC deaminase, peas

Some rhizobacteria are beneficial to plants and affect plant growth positively through different mechanisms of action. All those bacteria inhabiting plant roots and influencing the plant growth positively by any mechanism are referred to as plant growth-promoting rhizobacteria (PGPR) [3, 10]. These PGPR could be employed as inocula after their isolation and identification. A diverse array of bacteria including species of *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Klebsiella*, *Enterobacter*, *Xanthomonas*, and *Serratia* has been shown to promote plant growth. During the last couple of decades, the use of PGPR for sustainable agriculture has increased tremendously in various parts of the world. Significant increases in growth and yield of agronomically important crops in response to inoculation with PGPR have been reported [3, 16–18].

Ethylene is a plant hormone that is involved in the regulation of many physiological responses [2]. Besides its physiological roles in different developmental stages, ethylene was originally regarded as a “stress hormone” because its synthesis in plants is increased by a number of biotic and abiotic stresses. Ethylene production has been often associated with reduced growth and premature senescence, and may therefore be an indicator of plant susceptibility to stresses such as drought and heat [14, 21].

One of the major mechanisms utilized by PGPR to facilitate plant growth and development is lowering of the ethylene levels by hydrolysis of 1-aminocyclopropane-1-carboxylic acid (ACC), the immediate precursor of ethylene in plants. The enzyme catalyzing this reaction (ACC deaminase) hydrolyzes ACC to α -ketobutyrate and ammonia [5]. It has been observed that plants that are inoculated with PGPR containing ACC deaminase are dramatically more resistant to the deleterious effects of stress ethylene that is synthesized as a consequence of stressful conditions

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such as flooding [6], heavy metals [4, 7], presence of phytopathogens [20], drought, and high salt contents [11, 12]. In most of these cases, it has been reported that the PGPR containing ACC deaminase significantly lowered the level of ACC in the stressed plants, thereby limiting the amount of stress ethylene synthesis and hence damage to the plant. Therefore, the use of plant growth promoting bacteria containing ACC deaminase may prove useful in developing strategies to facilitate plant growth in drought conditions. Without any genetic manipulation of the plant, it may be possible to productively cultivate a variety of crop plants under drought conditions, provided that the plants are grown in the presence of a suitable plant growth promoting rhizobacterium.

Keeping all this in view, a series of experiments were conducted under controlled conditions to study the effectiveness of rhizobacteria containing ACC deaminase for growth promotion of peas (*Pisum sativum*) under drought conditions.

Rhizobacteria containing ACC deaminase were isolated from the rhizosphere of wheat (ACC-1, ACC-2, ACC-4, ACC-5) and peas (ACC-6, ACC-9, ACC-10, ACC-13, ACC-14). For comparison, another previously isolated strain, *P. putida* biotype A (Q-7), that has shown the ability to improve growth and yield of maize under axenic and natural conditions [16, 17] was used. The ACC deaminase activity of the rhizobacterial isolates was assayed according to a modification of the method by Honma and Shimomura [9], which measures the amount of α -ketobutyrate produced when the enzyme ACC deaminase cleaves ACC. The ability of the different bacterial strains to colonize pea roots under axenic conditions was assessed using the method described by Shaharoon et al. [18]. The bacteria were identified using the Biolog identification system (Microlog System Release 4.2, U.S.A.).

Minimal salt medium containing ACC as substrate (N source) was used for preparation of inocula. Selected strains of bacteria were used for inoculation of the broth and incubated at $28 \pm 1^\circ\text{C}$ for 48 h under shaking (100 rpm) conditions. After incubation, the optical density was measured and a uniform population (10^8 – 10^9 CFU/ml) of all strains was maintained prior to seedling inoculation. Pea seeds were surface sterilized by exposure to 95% ethanol for five minutes and immersion in 0.2% HgCl_2 solution for 3 min. The seeds were then subjected to six washings with sterile distilled water. Thoroughly washed seeds were sown on sterilized filter paper sheets placed in Petri plates. Fifteen seeds were sown in each Petri plate with three replicates. Two ml of three-day-old broth of rhizobacteria ($\text{OD}_{550}=0.8$) was added to each Petri plate to moisten the filter paper sheets and the seeds were covered with another sterilized filter paper sheet. The plates were incubated in a growth chamber at $25 \pm 1^\circ\text{C}$. After 5 days, seed germination and root elongation (data not shown) were examined.

For the screening trial, glass jars were filled with sand and 40 ml of half-strength Hoagland solution [8] was added to the jars. The whole apparatus was autoclaved (25 min at 121°C) prior to the transplantation of seedlings. Surface-sterilized pea seeds were germinated on sterilized filter paper sheets in Petri plates. Uniformly germinated seeds were transplanted to the jars containing sand under aseptic conditions to eliminate the variation in growth, contributed by different endogenous germination rate/potential of the seeds. Five ml of 2-day-old inocula ($\text{OD}_{550}=0.8$) was applied to the seedlings growing in sand. For the uninoculated control, sterilized (autoclaved) inoculum was used. Twenty ml sterilized Hoagland solution was applied in the jars for providing nutrients. The jars were arranged according to a completely randomized design with three replicates, in the growth room maintained at 25°C day temperature and 18°C night temperature. After germination, 12 h of light was supplied daily. After 12 days, the root length, shoot length, and fresh weight of pea seedlings was recorded. The data were subjected to statistical analysis by using a completely randomized design and means were compared according to Dunnett's test using the Minitab statistical package (Minitab 15.1.0.0, © 2006 Minitab Inc.).

Two rhizobacterial isolates, *Pseudomonas fluorescens* biotype G (ACC-5) and *P. fluorescens* (ACC-14), that have maximum ACC deaminase activity and root colonization, respectively, and showed comparatively better growth promotion of peas under axenic conditions were selected for the pot trial. A previously isolated promising PGPR strain, *P. putida* biotype A (Q-7) [16, 17], was selected to test its efficacy for growth promotion under drought stress conditions.

The inocula for the pot trial were prepared by growing the selected PGPR strains. For the inoculation treatments, germinated seeds were dipped in respective broth ($\text{OD}_{550}=0.8$) containing selected ACC isolates, whereas for the uninoculated controls, the pea seeds were dipped in sterilized broth. Hoagland solution was used to provide nutrients to the growing seedlings. Four inoculated seeds were sown per pot that were filled with 700 g/pot sandy clay loam soil having pH 7.7, ECe 2 dS m^{-1} , and organic matter 0.61%. The nitrogen concentration in the soil was 0.13% and available P and extractable K were 10 and 370 mg/kg, respectively. Four soil moisture levels (25, 50, 75, and 100% of FC) were maintained by using autoclaved distilled water. The quantity of water lost was determined by weighing the pots after a two-day interval and the same quantity of water was added to the pot in order to maintain soil moisture levels. There were three replications for each treatment. The pots were arranged randomly according to CRD in a growth room under axenic conditions. After twenty four days of germination, the pea plants were harvested and data were recorded. For the measurement of water use efficiency, the transpiration rate was determined by

Table 1. Characterization of rhizobacteria containing ACC-deaminase and their growth promoting activity in peas under axenic conditions.

| Isolate | ACC deaminase activity (nmol α -ketobutyrate/g ⁻¹ biomass ^{1/2} h ⁻¹) | Root colonization (CFU/g ⁻¹ root) | Root length (cm) | Shoot length (cm) | Fresh weight (g) |
|----------------------|---|---|---------------------|----------------------|---------------------|
| ACC-1 | 8.8±2.1 | 6.0×10 ⁶ | 8.9 cd | 7.6 bc | 2.28 a |
| ACC-2 | 110.1±9.6 | 1.5×10 ⁷ | 13.3 a | 7.3 bcd | 1.59 cd |
| ACC-4 | 13.9±1.4 | 1.2×10 ⁷ | 11.0 b | 7.2 bcd | 1.57 cd |
| ACC-5 | 174.2±8.2 | 2.0×10 ⁷ | 12.9 a | 9.0 a | 2.15 a |
| ACC-6 | 13.6±0.5 | 4.0×10 ⁷ | 11.1 b | 7.5 bc | 1.74 bc |
| ACC-9 | 24.4±1.5 | 1.0×10 ⁷ | 10.0 bc | 7.7 bc | 1.69 bc |
| ACC-10 | 16.1±1.1 | 2.6×10 ⁷ | 10.1 bc | 8.2 ab | 1.54 cd |
| ACC-13 | 0.49±0.1 | 1.0×10 ⁷ | 10.6 bc | 8.1 b | 1.83 bc |
| ACC-14 | 25.7±3.4 | 8.0×10 ⁷ | 11.1 b | 8.0 b | 2.01 ab |
| Q-7 | 140.0±5.3 | 1.6×10 ⁷ | 9.7 bc | 6.9 cd | 1.78 bc |
| Uninoculated control | - | - | 7.4 d | 6.4 d | 1.32 d |
| <i>P</i> value | - | - | 0.0000 | 0.0001 | 0.0000 |

Means sharing the same letter in a column do not differ statistically according to Dunnett's test.

loss of weight of the pot after the two-day interval (original weight of the pot - weight of the pot after two days). The data were subjected to statistical analysis by using two factorial completely randomized designs using the Minitab statistical package (Minitab 15.1.0.0, © 2006 Minitab Inc.).

Results regarding the effect of inoculation with rhizobacteria on root length, shoot length, and fresh weight of peas under axenic conditions are been summarized in Table 1. It is evident from the data that inoculation with all the rhizobacteria containing ACC deaminase (except ACC-1) caused significant increases in the root length of peas that ranged from 20–80% over the uninoculated control. The shoot length of the pea seedlings was also significantly increased by inoculation with most of the rhizobacteria containing ACC deaminase that ranged from 7 to 41%, over the uninoculated control. Similarly, inoculation with rhizobacterial isolates containing ACC deaminase also caused significant increase in the fresh weight of pea seedlings (root+shoot) over the uninoculated control that ranged from 16 to 72%. Isolates *P. fluorescens* biotype G (ACC-5) and *P. fluorescens* (ACC-14) were found to be the most effective in increasing root length, shoot length, and fresh weight as compared with other isolates and these were selected for further experiments. This positive effect of inoculation could be due to a decrease in ethylene synthesis in inoculated roots. Many researchers have reported that under gnotobiotic conditions, seed and/or root inoculation with rhizobacteria promotes root growth through ACC deaminase activity [5, 11, 16–18]. We also found that the bacterial strain without ACC deaminase did not promote the growth of inoculated plants [19], confirming that the ACC deaminase trait of PGPR was primarily responsible for growth promotion.

The effect of inoculation with rhizobacteria containing ACC deaminase on the root and shoot lengths of peas at different moisture levels is clearly evident from Fig. 1. Results revealed that inoculation with all rhizobacterial isolates increased the root length of peas, but with variable

efficacy at different moisture levels. At the lowest soil moisture level (25% FC), *P. fluorescens* biotype G (ACC-5) caused maximum increase in root length that was

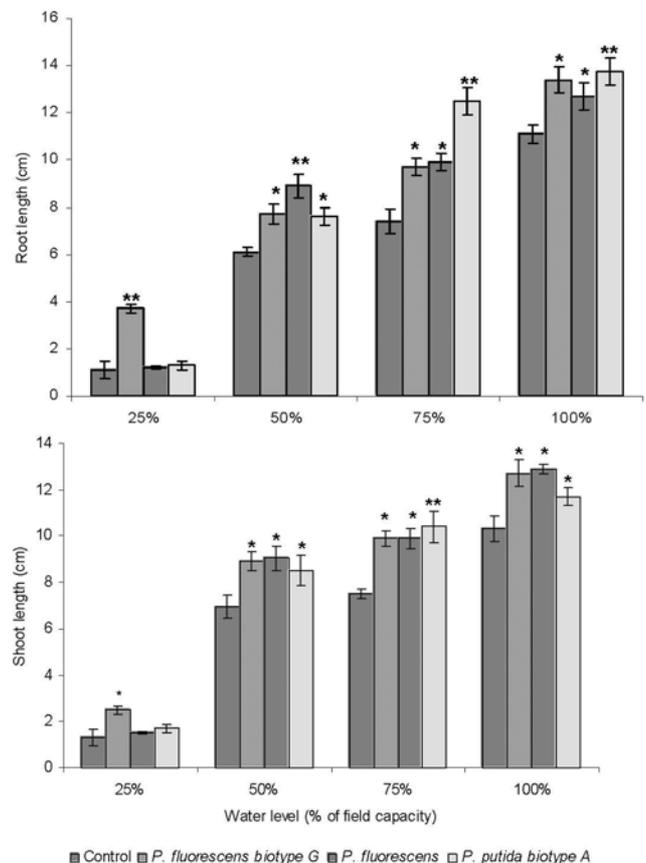


Fig. 1. Effect of inoculation with rhizobacteria containing ACC-deaminase on root and shoot length of pea seedlings at different soil moisture levels.

* Significantly different than respective uninoculated control at $P < 0.05$ according to Dunnett's test. ** Significantly different than respective uninoculated control $P < 0.01$ according to Dunnett's test.

2.3-fold higher as compared with the respective uninoculated control. Inoculation with *P. fluorescens* (ACC-14) showed promising increase of the root length at soil moisture of 50% FC that was 45% higher than the respective uninoculated control. At higher soil moisture levels (75 and 100% of FC), *P. putida* biotype A (Q-7) was found to be more effective as it caused 68% and 23% increases in root length over respective uninoculated controls, respectively. Similarly, the shoot length of peas was also increased in response to

inoculation at all moisture levels; nonetheless, the % increases in shoot length over respective uninoculated controls decreased with increasing moisture levels, as a 92% increase in shoot length over the respective uninoculated control occurred in the case of 25% of FC, whereas it was only 25% at 100% of FC.

The effect of inoculation of pea plants with rhizobacteria containing ACC deaminase on the fresh and dry weights (shoot+root) and number of leaves per plant of pea seedlings at different moisture levels is shown in Fig. 2. The data revealed that water stress applied at 25% of FC had the most negative effect on the fresh weight of pea seedlings. However, inoculation with rhizobacteria containing ACC deaminase reduced the effects of water stress applied owing to low soil moisture and, in most of the cases, significantly increased the fresh weight compared with their respective uninoculated control. Maximum increase in fresh weight was recorded in the case of pea plants inoculated with *Pseudomonas fluorescens* biotype G (ACC-5) under water stress applied at 25% FC and it was 45% higher than the respective uninoculated control. In the case of water stress applied at 50% FC, inoculation with *Pseudomonas fluorescens* (ACC-14) was found to be more promising as it caused maximum (53%) increase in the fresh weight of pea seedlings as compared with the respective uninoculated control. Like fresh weight, a maximum reduction in dry weight was recorded in plants exposed to soil moisture at 25% of FC. Overall, inoculation with rhizobacterial isolates resulted in significant increases in dry weight of pea seedlings exposed to water stress but with variable efficacy. At moisture level of 25% of FC, inoculation with PGPR isolate *P. fluorescens* biotype G (ACC-5) resulted in maximum (150%) increase in the dry weight as compared with the respective uninoculated control. At 50% of FC, inoculation with *P. putida* biotype A (Q-7) caused maximum increase in the dry weight of pea seedlings that was 108% higher than the respective uninoculated control. At moisture levels of 75% and 100% of FC, the performance of *P. fluorescens* biotype G (ACC-5) was better than the other isolates as it caused 150% and 189% increases in dry weight of pea seedlings as compared with the respective uninoculated control, respectively.

All the rhizobacterial isolates were also effective in significantly increasing the number of leaves per plant of peas at all moisture levels tested and resulted in up to 140, 29, 32, and 28% increases in number of leaves per plant as compared with their respective uninoculated controls (Fig. 2).

The effect of inoculation with rhizobacteria containing ACC deaminase on water use efficiency (per unit fresh or dry weight) is clearly visible from Fig. 3. It is evident from the results that the efficacy of rhizobacterial isolates in increasing water use efficiency varied with moisture in the soil. At low moisture level (25% of FC), *P. fluorescens*

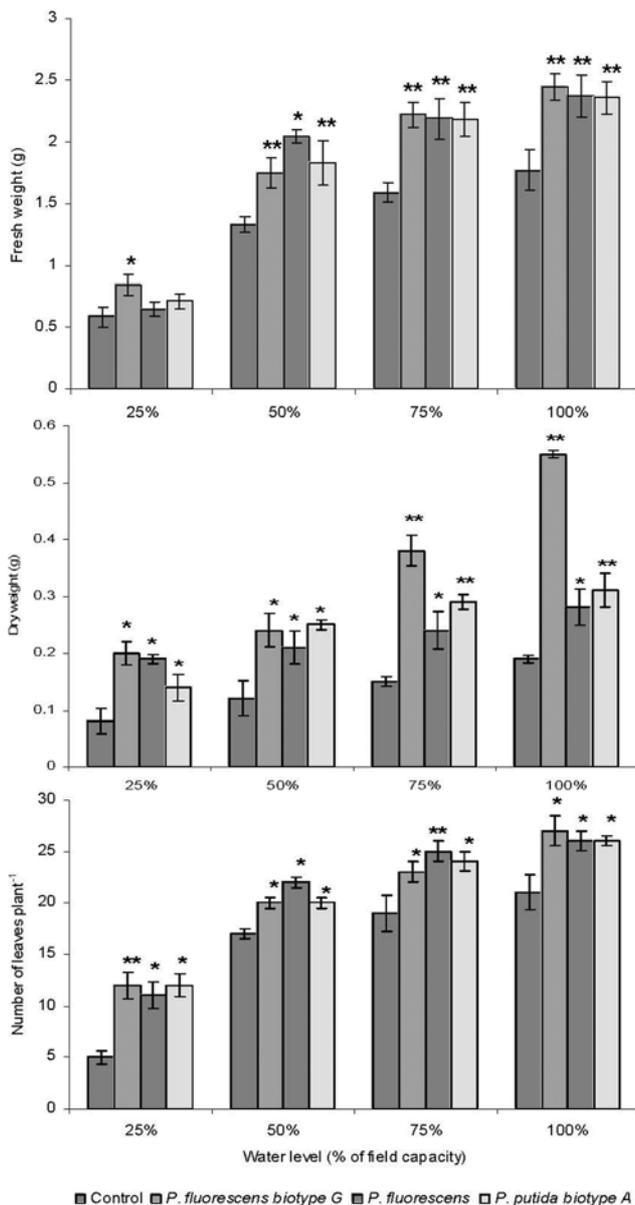


Fig. 2. Effect of inoculation with rhizobacteria containing ACC-deaminase on fresh and dry weight and number of leaves plant⁻¹ of pea seedlings at different soil moisture levels.

* Significantly different than respective uninoculated control at $P < 0.05$ according to Dunnett's test. ** Significantly different than respective uninoculated control $P < 0.01$ according to Dunnett's test.

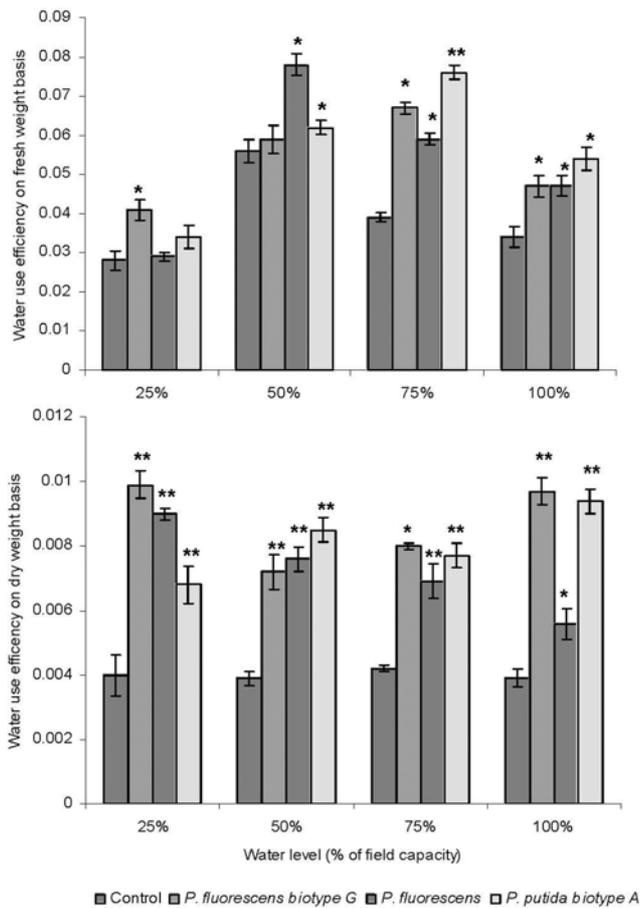


Fig. 3. Effect of inoculation with rhizobacteria containing ACC-deaminase on water use efficiency (calculated on fresh and dry weight basis) of pea seedlings at different soil moisture levels.

* Significantly different than respective uninoculated control at $P < 0.05$ according to Dunnett's test. ** Significantly different than respective uninoculated control $P < 0.01$ according to Dunnett's test.

biotype G (ACC-5) was found to be more promising as it caused maximum increases in WUE calculated on a fresh weight basis (46%) than respective uninoculated controls. At higher moisture levels (75 and 100% of FC), *P. putida* biotype A (Q-7) was found to be more effective in increasing the water use efficiency on a fresh weight basis, and these were 95 and 57% higher, respectively, than respective uninoculated controls. Similarly, inoculation was found to be effective in increasing the water use efficiency when calculated on a dry weight basis. In general, *P. fluorescens* biotype G (ACC-5) was found more effective than other isolates and caused 148, 85, 90, and 149% increases in water use efficiency (dry weight basis) at moisture levels of 25, 50, 75, and 100% of FC, respectively.

It is evident from the results that exposure of pea plants to decreasing soil moisture levels caused reduction in all growth parameters of peas. This effect could be due to the sensitivity of peas to drought stress that might have

affected different physiological and metabolic processes contributing to the growth and development processes of pea plants [15, 22]. Inoculation of pea plants with PGPR containing ACC deaminase partially or completely eliminated the "drought stress imposed effects" on root and shoot growth, fresh and dry weights, and number of leaves per plant of peas. This might be due to suppression of the stress-induced accelerated synthesis of ethylene by the ACC deaminase activity of these PGPR in the inoculated roots. Sharp increases in ACC levels and, consequently, ethylene synthesis in plants under drought stress conditions has been frequently reported [1, 12, 13]. Therefore, the inhibitory effects of ethylene induced by drought stress might have been eliminated through the ACC deaminase activity of the PGPR. This premise is supported from the observation that with increasing water stress, the % increases in growth parameters in response to inoculation increased. Many other researchers have also reported increased resistance to stresses like salt stress [11], flooding stress [6], heavy metals [4, 7], and pathogen stress [20] in response to inoculation with rhizobacteria containing ACC deaminase.

The increase in water use efficiency in response to inoculation may also be due to the production of more biomass, even at lower moisture levels. It is highly likely that water stress-induced ethylene might have been hydrolyzed in inoculated plants by their ACC deaminase activity, which resulted in production of more biomass per unit less quantity of water. Our results are in conformity with the finding of Mayak *et al.* [12], who reported increase in water use efficiency in response to inoculation with rhizobacteria containing ACC deaminase.

The efficacy of all the rhizobacterial isolates varied with the moisture level in the soil. It is highly likely that a decrease in water contents in soil might also have affected the colonization ability of the isolates differently. *Pseudomonas putida* biotype A (Q-7) could not perform well at lower moisture levels as compared with isolates that were obtained from the rhizosphere of wheat and peas (ACC-5 and ACC-14). It could be due to the fact that this isolate could not adapt to roots of peas, as in the case of rhizobacteria isolated from pea rhizosphere.

From these studies, it might be concluded that the rhizobacteria having ACC deaminase activity are effective in promoting plant growth and water use efficiency under drought conditions, by lowering the ethylene or ACC accumulation whose higher levels have inhibitory effects on root and shoot growth.

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