

## **PRELIMINARY SCREENING OF RHIZOBACTERIA CONTAINING ACC-DEAMINASE FOR PROMOTING GROWTH OF LENTIL SEEDLINGS UNDER AXENIC CONDITION**

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### **Abstract**

Nodulation and subsequent nitrogen fixation by lentil plants are inhibited by accelerated ethylene concentration in the root zone. Plant growth-promoting bacteria can help overcome these deleterious effects. Twenty seven isolates of rhizobacteria containing ACC-deaminase were isolated from the lentil rhizosphere by using dilution plate technique. A jar experiment was conducted under axenic condition for the screening of plant growth promoting rhizobacteria containing ACC-deaminase to promote growth of lentil seedlings. All the rhizobacterial isolates had the potential to modify the growth of lentil seedlings under axenic conditions. Results of jar study showed that inoculation with selected isolates increased the root length, shoot length, fresh root weight, fresh shoot weight, dry root weight and dry shoot weight of lentil seedlings up to 2.4, 2.3, 2.6, 2.7, 2.6 and 2.2 folds, respectively over uninoculated control. It is suggested that ACC-deaminase trait could be a useful approach for the screening of the effective PGPR to promote lentil growth under axenic conditions before testing their potential under natural conditions. The study also enabled us to select efficient PGPR isolates for co-inoculation with rhizobium to promote growth as well as nodulation in lentil grown in different regions of the country.

### **Introduction**

Various free-living soil bacteria that are capable of exerting beneficial effects on plants in culture or in a protected environment have potential for use in agriculture and can lead to increased yields of a wide variety of crops, are known as plant growth promoting rhizobacteria (PGPR) (Kloepper, 1994). The mechanisms by which PGPR promote plant growth are not fully understood, but are thought to include: (i) the ability to produce or change the concentration of the plant hormones indole-acetic acid (Mordukhova *et al.*, 1991), gibberellic acid (Mahmoud *et al.*, 1984), cytokinins (Tien *et al.*, 1979) and ethylene (Glick *et al.*, 1995; Arshad & Frankenberger, 2002); (ii) asymbiotic N<sub>2</sub> fixation (Boddey & Dobereiner, 1995; Kennedy *et al.*, 1997); (iii) antagonism against phytopathogenic microorganisms e.g. *Fusarium* spp. (Scher & Baker, 1982; Inam-ul-Haq *et al.*, 2007) by production of siderophores (Scher & Baker, 1982), chitinases (Renwick *et al.*, 1991), antibiotics (Shanahan *et al.*, 1992) and cyanide (Flaishman *et al.*, 1996); and (iv) solubilization of mineral phosphates and other nutrients (De Freitas *et al.*, 1997). In addition to the above described PGPR traits, some rhizobacteria can promote plant growth indirectly by affecting symbiotic N<sub>2</sub> fixation, nodulation or nodule occupancy (Cattelan *et al.*, 1999).

Many of the studies with PGPR show plant growth promotion, but only under gnotobiotic conditions (Shenbagarathai, 1993; Glick *et al.*, 1995; Shahroona *et al.*, 2006a) or in potted media (Fuhrmann & Wollum, 1989; Shahroona *et al.*, 2007) where these bacteria do not compete with normal array of soil microorganisms.

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Recently, it has been discovered that certain soil microorganisms contain an enzyme ACC-deaminase that hydrolyses ACC into ammonia and  $\alpha$ -ketobutyrate (Glick *et al.*, 1998; Shahroona *et al.*, 2006a) and decreases the amount of ACC, as well as ethylene, outside the germinating seeds, thereby acting as a sink for ACC. Decreased levels of ACC result in lower levels of endogenous ethylene, which eliminate the potential inhibitory effects of higher ethylene concentrations (Glick *et al.*, 1998; Yuhashi *et al.*, 2000). Ethylene is also known to affect several aspects of root development and nodule formation (Ligero *et al.*, 1991), including its action as an inhibitor of nodulation (Hirsch & Fang, 1994).

In addition, plants that are treated with PGPR containing ACC-deaminase are dramatically more resistant to the injurious effects of stress ethylene that is synthesized as a consequence of stressful conditions such as flooding (Grichko & Glick, 2001), the presence of phyto-pathogens (Wang *et al.*, 2000), drought (Zahir *et al.*, 2007) and high salt concentration (Mayak *et al.*, 2004; Nadeem *et al.*, 2007). It is highly likely that presence of PGPR containing ACC-deaminase on the roots of legumes could suppress accelerated endogenous synthesis of  $C_2H_4$  during the rhizobial infection and thus may facilitate nodulation. So, inoculation of legumes with competitive PGPR containing ACC-deaminase could be an effective and narrative approach for improving growth of lentil seedlings under axenic as well as under natural conditions.

Since the bacterial enzyme ACC-deaminase lowers the level of  $C_2H_4$  in roots, inoculation with PGPR containing ACC-deaminase could be effective for improving growth and nodulation in lentils. Keeping this in view, a study was planned to isolate and screen the indigenous PGPR strains from different locations of Punjab under axenic conditions.

### Materials and Methods

Rhizobacteria containing ACC deaminase isolated from the rhizosphere were screened for their growth promoting activity under gnotobiotic conditions.

**Isolation of rhizobacteria containing ACC-deaminase:** Several bacterial strains were isolated from the rhizosphere of lentil plants grown at different locations of Northern, Central and Eastern Punjab, Pakistan (Layyah, Faisalabad, Gujranwala and Sialkot). Plants were uprooted and brought to laboratory in polythene bags. The non-rhizosphere soil was removed from plant roots (40-55 days old) by gentle shaking. The rhizosphere soil adhered to roots was collected by dipping and gentle shaking in sterilized water under aseptic conditions. The soil suspension obtained was used to inoculate the DF minimal medium (Dworkin & Foster, 1958).

A total of twenty seven rhizobacterial colonies were isolated from the mixed culture (based on color, size, and shape by dilution plate technique using salt minimal medium containing ACC as sole nitrogen source (enrichment technique). The composition of salt minimal media containing ACC as sole nitrogen source in g L<sup>-1</sup> is as follows, KH<sub>2</sub>PO<sub>4</sub>, 1.36; Na<sub>2</sub>HPO<sub>4</sub>, 2.13; MgSO<sub>4</sub>.7H<sub>2</sub>O, 0.2; CaCl<sub>2</sub>.2H<sub>2</sub>O, 0.7; FeSO<sub>4</sub>.7H<sub>2</sub>O, 0.2; CuSO<sub>4</sub>.5H<sub>2</sub>O, 0.04; MnSO<sub>4</sub>.H<sub>2</sub>O, 0.02; ZnSO<sub>4</sub>.7H<sub>2</sub>O, 0.02; H<sub>3</sub>BO<sub>3</sub>, 0.003; CoCl<sub>2</sub>.6H<sub>2</sub>O, 0.007; Na<sub>2</sub>MoO<sub>4</sub>.2H<sub>2</sub>O, 0.004; Substrate ACC, 5 mM; Glucose, 1.0% dissolved in 1000 mL of distilled water.

**Table 1. Grouping of strains based on their ACC-metabolism assay.  
(Average of 4 replicates)**

PGPR isolate	Site	ACC utilization rate		
		High [OD <sub>550</sub> > 0.7]	Medium [OD <sub>550</sub> : 0.5-0.69]	Low [OD <sub>550</sub> < 0.5]
P1	Faisalabad	-	✓	-
P2	Faisalabad	-	-	✓
P3	Faisalabad	-	✓	-
P4	Faisalabad	-	-	✓
P5	Faisalabad	✓	-	-
P6	Faisalabad	-	-	✓
P7	Layyah	-	-	✓
P8	Layyah	-	-	✓
P9	Layyah	-	-	✓
P10	Layyah	✓	-	-
P11	Layyah	-	✓	-
P12	Layyah	-	✓	-
P13	Layyah	✓	-	-
P14	Layyah	-	✓	-
P15	Sialkot	-	✓	-
P16	Sialkot	-	✓	-
P17	Sialkot	-	-	✓
P18	Sialkot	-	✓	-
P19	Sialkot	-	✓	-
P20	Sialkot	-	-	✓
P21	Sialkot	-	-	✓
P22	Gujranwala	-	✓	-
P23	Gujranwala	-	-	✓
P24	Gujranwala	✓	-	-
P25	Gujranwala	✓	-	-
P26	Gujranwala	-	✓	-
P27	Gujranwala	-	✓	-

The minimal salt media was sterilized at 121°C for 20 minutes while substrate ACC was sterilized separately by filtering through 0.2 µm membrane filter. The collected rhizobacterial strains were purified by further streaking on fresh plates (2-3 times) and stored at -20 °C for subsequent use. The isolated plant growth promoting rhizobacterial strains containing ACC-deaminase were given codes as P1, P2, P3.....P27. The details are given in Table 1.

**ACC-metabolism assay:** ACC-metabolism assay (qualitative) was carried out to characterize the rhizobacterial strains for their ability to use ACC as a sole nitrogen source. The rhizobacterial strains were grown on two nitrogen sources (ACC and Ammonium Sulphate) and one mineral source (Magnesium Sulphate), to observe the growth rate of the strain, for ACC substrate in parallel to ammonium Sulphate. These strains were categorized into three groups, as strains with higher (OD<sub>550</sub> > 0.7), medium (OD<sub>550</sub>: 0.5-0.69) and lower (OD<sub>550</sub> < 0.5) ACC-metabolism rate depending upon their O.D value at 550 nm for ACC substrate as compared to Ammonium sulphate. ACC-metabolism assay was proceeded according to the method (modified) described by Jacobson *et al.*, (1994).

Isolates were inoculated in 5mL  $\frac{1}{2}$  TSB (tryptic soy broth). Cultures were incubated for 48 hours at room temperature along with shaking; these cultures were diluted ten times by 0.1 M of MgSO<sub>4</sub>. In this assay DF salts (Dworkin and Foster, 1958) were used. In 96-well micro-titer plate, 122  $\mu$ L DF salts were added in all wells. Fifteen  $\mu$ L 0.1 M MgSO<sub>4</sub> was added in lane 3, 6, 9, and 12, and 15 $\mu$ L 0.1 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was added in lane 2, 5, 8, and 11 while 15  $\mu$ L of thawed ACC was filled in the lane 1, 4, 7, and 10. For inoculation of each well 22  $\mu$ L bacterial culture was used. In uninoculated control wells, 22  $\mu$ L 0.1 M MgSO<sub>4</sub> was used in place of bacteria. Optical density (OD<sub>550</sub>) was measured after 0, 24, 48, 72, and 96 hours at 550 nm wave length. Value of ACC and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> well was compared along with MgSO<sub>4</sub> wells to determine the ability of bacteria to metabolize ACC.

**Screening rhizobacteria containing ACC-deaminase for growth promoting activity:** A growth room experiment was conducted on lentil under controlled conditions to screen rhizobacteria containing ACC-deaminase for their growth promoting activity under gnotobiotic conditions. Sterilized glass jars were used for the experiment. A broth was prepared by using minimal salt medium containing ACC as a source of nitrogen. Each test tube containing 60 mL sterilized broth was inoculated with selected strains of rhizobacteria. Rhizobacterial strains in the test tube were cultured in a shaking incubator (Firstek Scientific, Tokyo, Japan), at 30°C for 48 h (at 100 rpm). Culture broth was centrifuged for harvesting bacterial cells. Bacterial suspensions were concentrated by centrifugation at 45000 x g for 10 min and then washed three times with phosphate buffer. An optical density of 0.5 recorded at a wavelength of 535 nm, was achieved by dilution to maintain uniform cell density ( $10^8$ - $10^9$  CFU mL<sup>-1</sup>).

Lentil (*Lens culinaris* Medic) cv. Masoor-93 seeds were surface-sterilized by momentarily dipping them in 95% ethanol solution followed by dipping in 0.2% HgCl<sub>2</sub> solution for 3-5 minutes and 6-7 thorough washing with sterilized water. Surface-sterilized lentil seeds were inoculated by dipping for five minutes in the broth of respective rhizobacterial culture. Two sterilized filter paper sheets were soaked and saturated in suspension containing desired inoculum. The inoculated seeds were sandwiched in between soaked filter papers and were rolled and placed in sterilized glass jars. In the case of uninoculated control, seeds and filter papers were dipped in sterilized broth. Sterilized Hoagland nutrient solution (Hoagland & Arnon, 1950) of half strength was applied to bottom of the jars for seedling nutrition. Jars were arranged using completely randomized design with three replications for each treatment. Plants were incubated in a growth room at  $20 \pm 2$  °C with light supplied for 10 h and dark period for 14 h. After two weeks of incubation, plants were harvested and data regarding the growth parameters (root length, shoot length, fresh root weight, fresh shoot weight, dry root weight and dry shoot weight) were recorded. Standard error of means was calculated (Steel *et al.*, 1997).

## Results

Isolated rhizobacterial strains were first subjected to ACC-metabolism assay and later on these were tested for improving growth of lentil seedlings under axenic conditions. The results are described below:

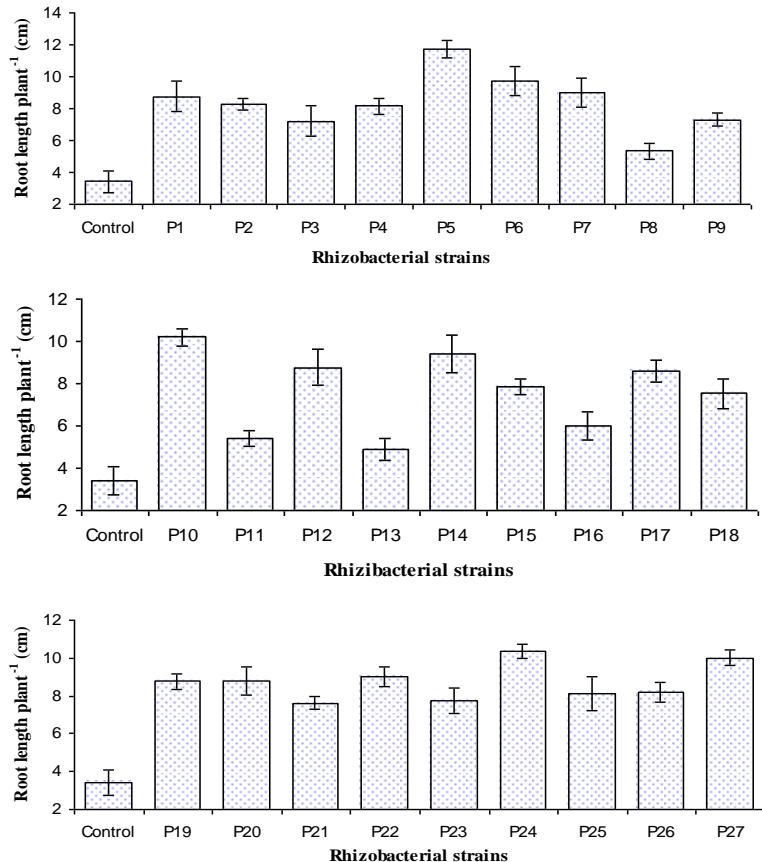


Fig. 1. Potential of different rhizobacterial isolates to promote root length of lentil seedlings under axenic conditions.

**ACC-metabolism assay:** The ability of rhizobacterial strains to utilize ACC as a sole source of N was determined. Results of ACC utilization assay revealed that all the 27 strains metabolized ACC (positive for possessing ACC-deaminase activity) but with different degrees of efficacy. On the basis of growth, measured in terms of  $OD_{550}$ , these 27 strains were divided into three groups (Table 1) i.e. High ( $OD_{550}>0.7$ ), Medium ( $OD_{550}: 0.5-0.69$ ), Low ( $OD_{550}<0.5$ ). These strains were tested for their growth promoting effect on lentil under axenic conditions.

**Effect of PGPR inoculation on seedling growth under axenic conditions:** Results of jar trial revealed that inoculation with rhizobacteria containing ACC-deaminase increased root elongation up to 2.4-folds over uninoculated control (Fig. 1). Isolates P5 and P24, out of twenty seven, were found to be the most effective, which showed significant improvement in the root elongation up to 2-folds over uninoculated control. The other effective isolates were P6, P10 and P27 which caused an increase of root elongation up to 1.8-fold higher over uninoculated control. Next remaining isolates also showed effectiveness in the range of 0.5-1.6 folds over uninoculated control but least effective isolate was P8 which showed 55.44% increase over uninoculated control.

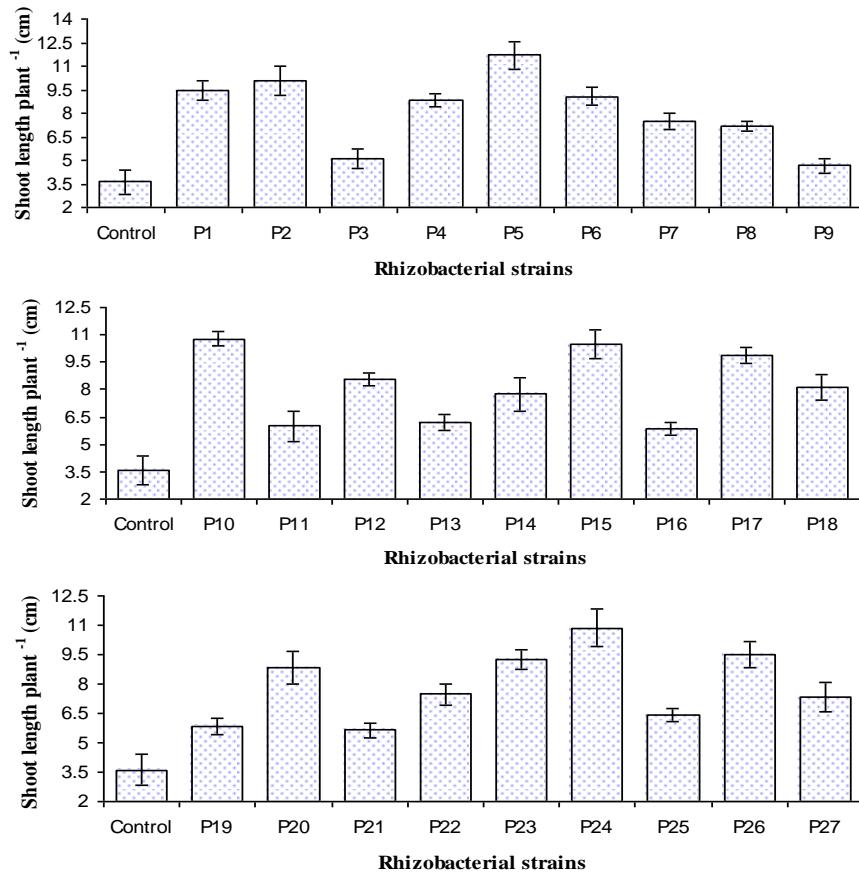


Fig. 2. Potential of different rhizobacterial isolates to promote shoot length of lentil seedlings under axenic conditions.

Increase in shoot length in response to PGPR inoculation was found more than 2.25-folds over uninoculated control (Fig. 2). The most promising increase was observed in case of inoculation with isolates P5, P10 and P24. Next effective isolates were P1, P2, P6, P15, P17, P23 and P26 which caused an increase of shoot length by more than 1.5-folds over uninoculated control. But the remaining isolates also showed effectiveness over uninoculated control but least effective isolate (P9) showed 30.0% increase over uninoculated control.

Results showed that inoculation with ACC-deaminase containing rhizobacteria increased root weight extensively that ranged from 2.0 to 2.61 folds as compared to uninoculated control (Fig. 3). Maximum increase was observed in case of P5, P10 and P24 isolates that increased root weight up to 2.0-folds over uninoculated control. The other effective isolates were P4, P7 and P19 that showed up to 1.8-folds higher root weight over uninoculated control. Next remaining isolates also showed effectiveness over uninoculated control.

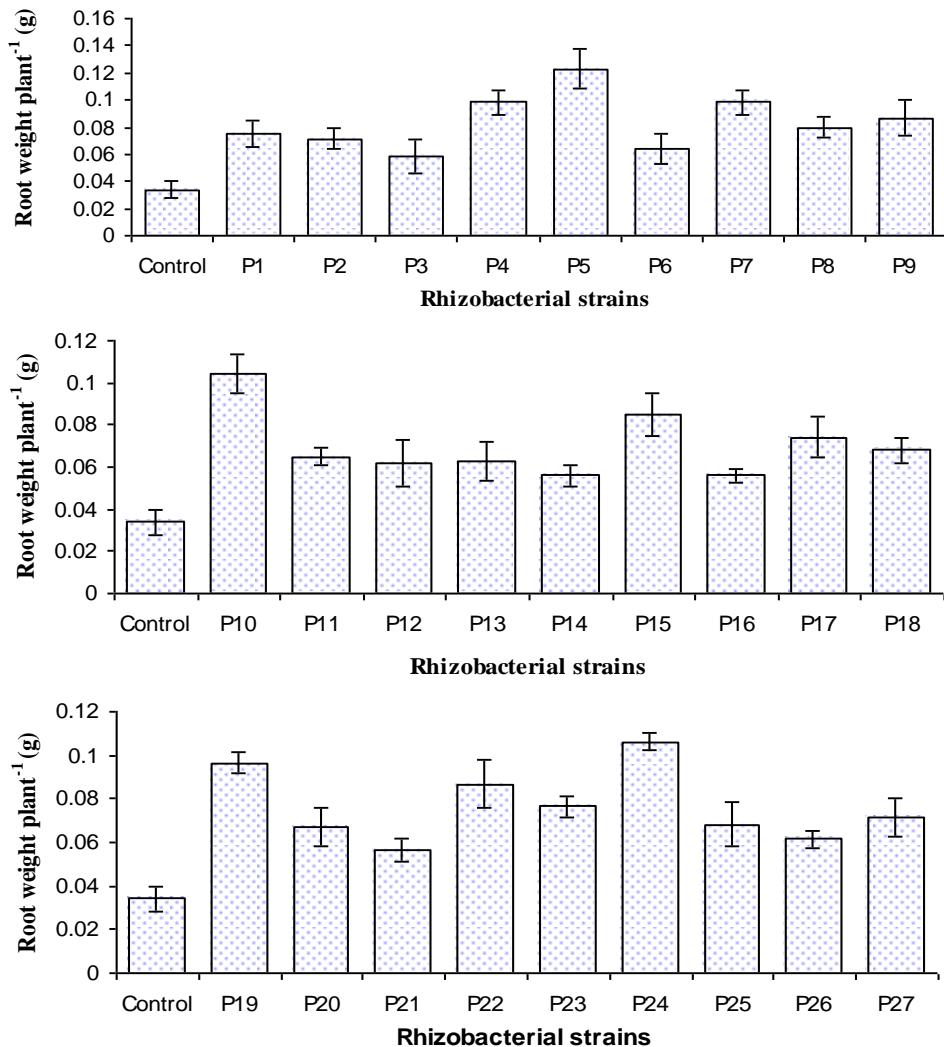


Fig. 3. Potential of different rhizobacterial isolates to promote root fresh weight of lentil seedlings under axenic conditions.

Data revealed that all the rhizobacterial isolates were effective in increasing shoot fresh weight significantly as compared to uninoculated control (Fig. 4). However, maximum shoot weight was observed in isolates P5, P6, P10, P14 and P24 that was up to 2.0-2.7 folds higher as compared to uninoculated control. Next effective isolates were P2, P4, P7, P9, P15, P20, P21, P22 and P26 which increased shoot fresh weight up to 1.5-1.9 folds over uninoculated control. Minimum increase in shoot weight was observed due to inoculation with isolate P27 that was up to 0.36 folds (36.0%) higher as compared to uninoculated control.

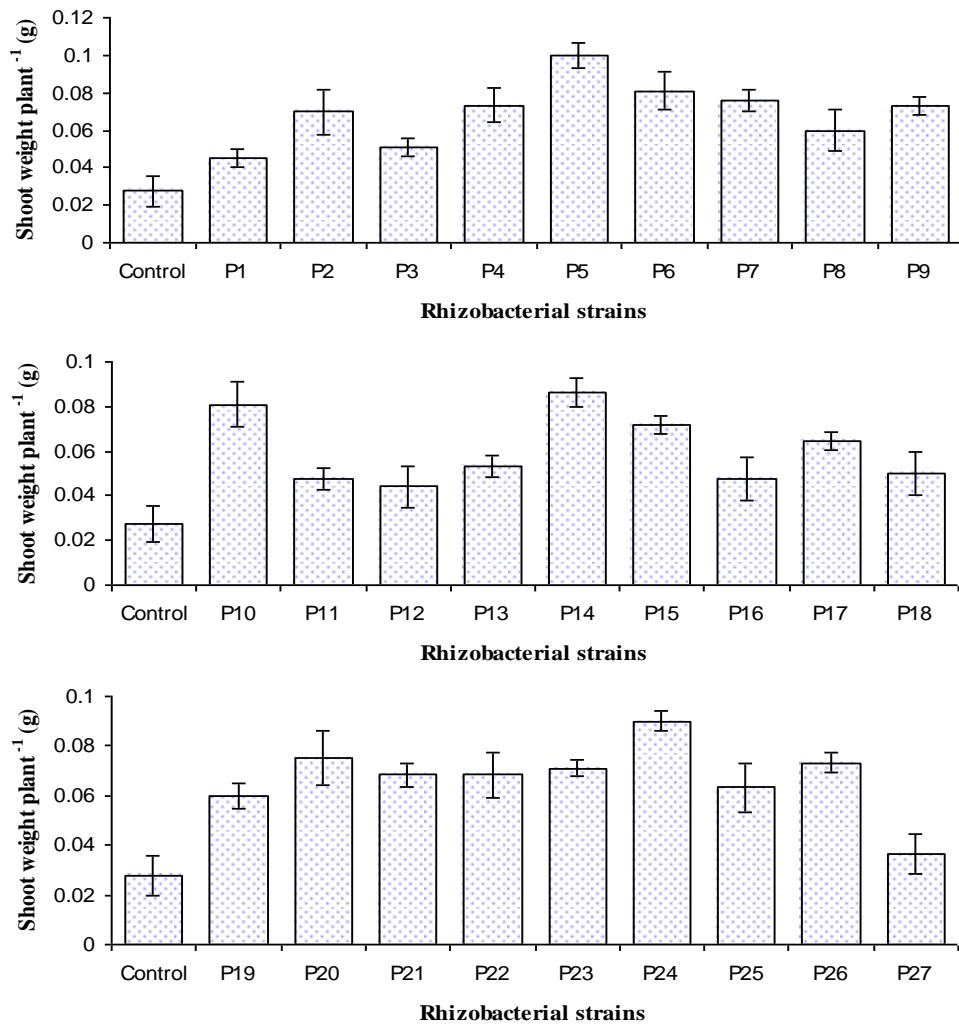


Fig. 4. Potential of different rhizobacterial isolates to promote shoot fresh weight of lentil seedlings under axenic conditions.

Increase in root dry weight in response to inoculation with PGPR containing ACC-deaminase was found up to 2.6-folds higher than uninoculated control (Fig. 5). Most promising increase was observed in case of inoculation with isolates P5, P10 and P24 that ranged from 2.0 to 2.6 folds as compared to uninoculated control. Next effective isolates were P1, P2, P4, P7, P8, P9, P15, P17, P18, P19, P20, P22, P23, P25 and P27 that gave from 1.0 to 1.87 folds increase in the root dry weight over uninoculated control while remaining isolates also showed effectiveness over uninoculated control that was up to 0.96 fold.

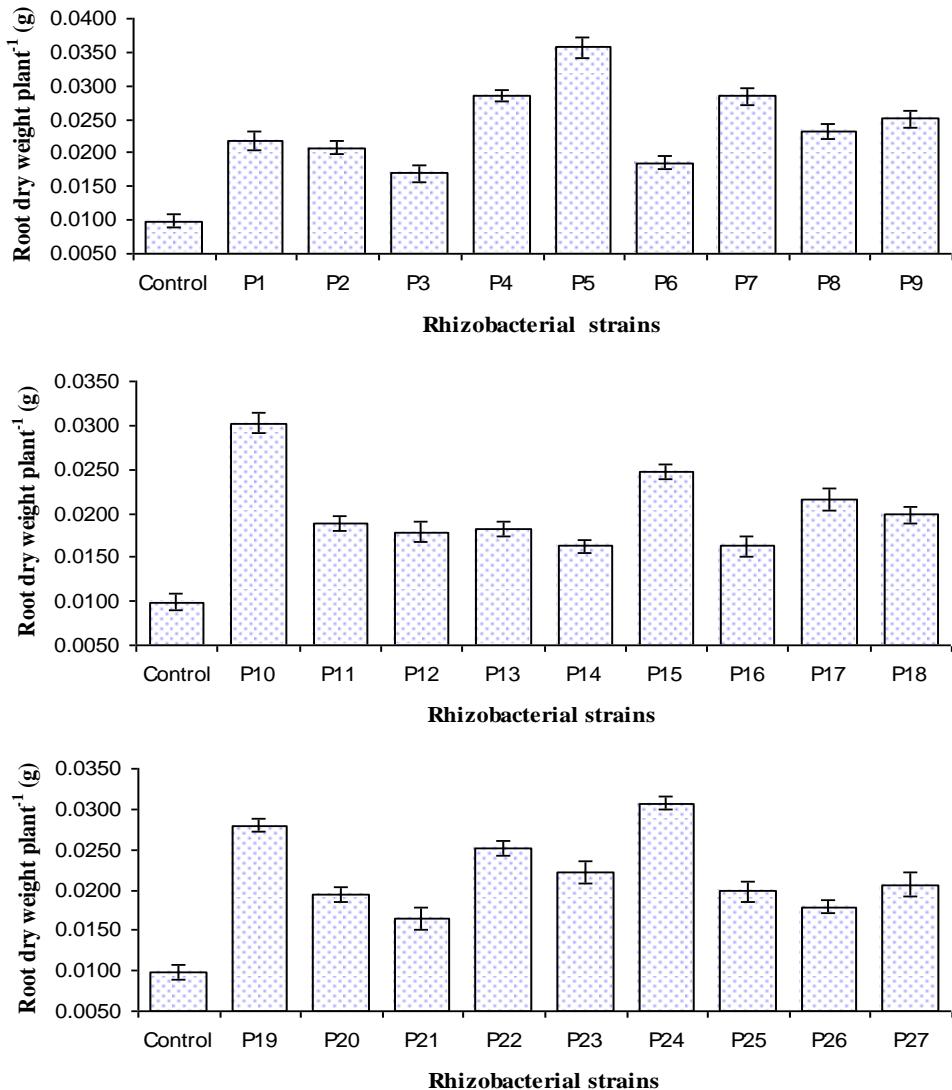


Fig. 5. Potential of different rhizobacterial isolates to promote root dry weight of lentil seedlings under axenic conditions.

Data showed that all the rhizobacterial isolates were effective in increasing shoot dry weight as compared to uninoculated control (Fig. 6). Maximum shoot dry weight was observed by inoculation with isolates P5, P10, P14 and P24 that was up to 2.22-folds higher as compared to uninoculated control. Next effective isolates were P2, P4, P6, P7, P9, P15, P16, P20, P21, P22, P23, P25 and P26 which gave 1.03 to 1.57 folds increase in shoot dry weight over uninoculated control. But other isolates also showed effectiveness in the range of 0.08- 0.93 folds over uninoculated control.

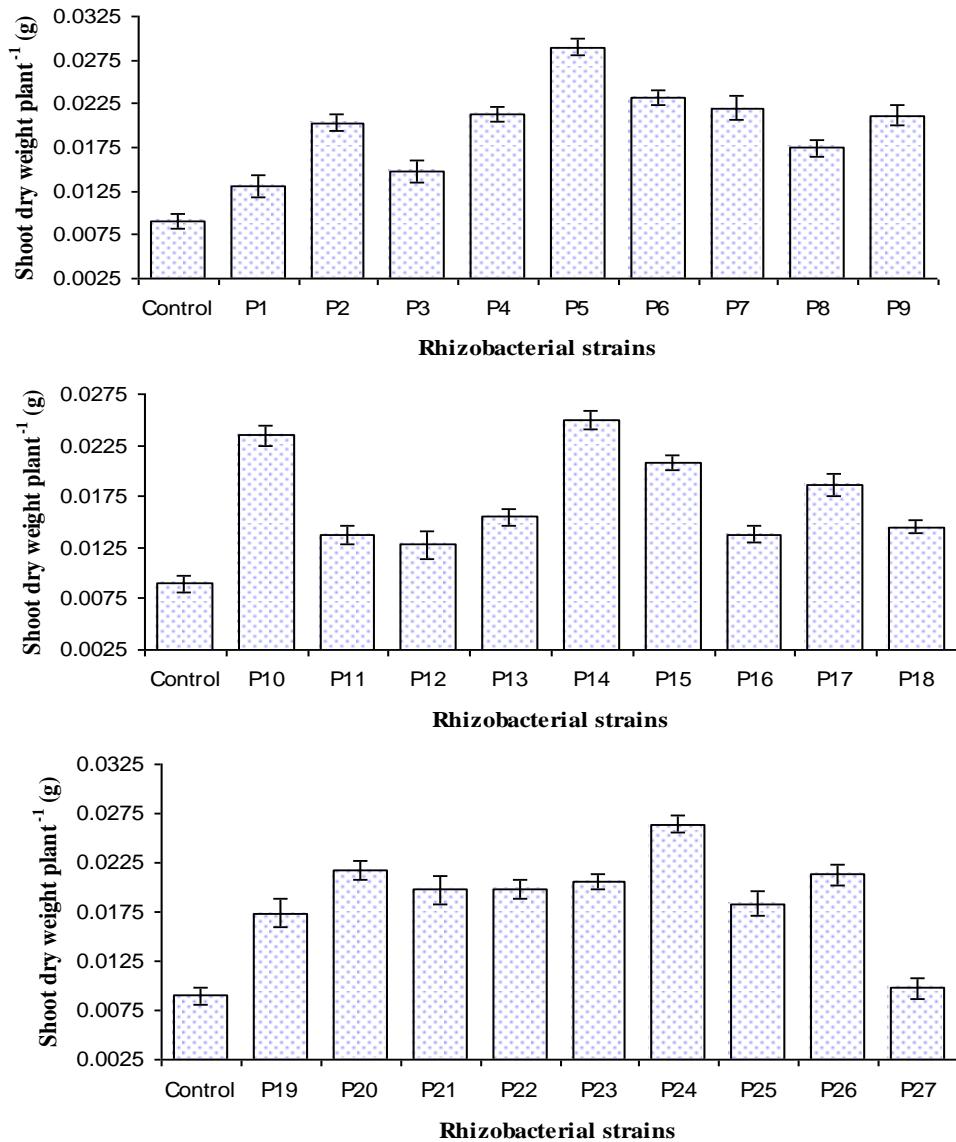


Fig. 6. Potential of different rhizobacterial isolates to promote shoot dry weight of lentil seedlings under axenic conditions.

Out of twenty seven rhizobacterial strains, isolates P5, P10 and P24 were the most effective PGPR in promoting lentil seedlings growth and could be further used with rhizobial culture for improving lentil growth as well as nodulation under natural conditions.

### Discussion

Some plant growth promoting rhizobacteria are capable of reducing higher levels of C<sub>2</sub>H<sub>4</sub> in plants through the activity of enzyme ACC-deaminase that hydrolyzes ACC into  $\alpha$ -ketobutyrate and ammonia, instead of ethylene (Glick *et al.*, 1998; Arshad *et al.*, 2007). Reduction in endogenous levels of ethylene in plants results in the formation of better root system. This study demonstrates the screening of rhizobacteria containing ACC deaminase to promote lentil growth under axenic conditions.

Isolated PGPR strains were tested for their potential to utilize ACC as a sole source of nitrogen (ACC-metabolism assay). Majority of the rhizobacteria isolated from various locations of Punjab were capable of utilizing ACC and showed their variable growth rate (O.D ranging from 0.40 to 0.80) on minimal salt medium containing ACC as a sole N source. The strains possessing relatively higher ACC utilization rate were more effective in promoting lentil seedling growth than the strains with lower ACC utilization rate. This reveals that lowering of C<sub>2</sub>H<sub>4</sub> (up to certain level) in plant is stimulatory because C<sub>2</sub>H<sub>4</sub> is involved in physiological processes of plants (Gomez *et al.*, 1998; Matilla, 2000; Arshad *et al.*, 2007).

Strains of PGPR containing ACC-deaminase were screened for their growth promoting activity in Jar experiment under gnotobiotic conditions. Results showed that most of the rhizobacterial strains were able to improve the root and shoot growth of inoculated lentil seedlings. It was observed that inoculation with strains possessing ACC deaminase significantly increased the root elongation up to 2.4-fold, shoot length up to 2.3-fold, dry/fresh root weight of lentil seedlings up to 2.6-fold and dry/fresh shoot weight of lentils seedlings up to 2.7-fold as compared to uninoculated control of lentil. It is very likely that these strains promoted root growth by lowering the endogenous inhibitory level of ethylene in roots because of their ACC deaminase activity which subsequently affected shoot growth positively. The results are in conformity with the findings of Shahroona *et al.*, (2006b) who reported that rhizobacteria with more ACC-deaminase activity had more ability to decrease the intensity of ACC-induced classical "triple" response which confirmed the premises that ACC-deaminase activity of rhizobacteria was responsible for decreasing endogenous as well as exogenous ACC in inoculated plant. Very recently, Shahroona *et al.*, (2006a) have reported a significantly positive correlation between ACC-deaminase activity and root elongation in maize due to inoculation with rhizobacteria under axenic conditions. Similar kind of findings have been reported by other scientists (Glick *et al.*, 1998; Belimov *et al.*, 2002; Dodd *et al.*, 2004; Mayak *et al.*, 2004; Nadeem *et al.*, 2007; Penrose *et al.*, 2001; Sergeeva *et al.*, 2005).

### Conclusions

These are preliminary studies for the selection of effective PGPR strains which will consequently be used for co-inoculation with rhizobium strains to improve nodulation in lentil. These studies have however, shown that the PGPR strains having high ACC-deaminase activity were better effective for improving the growth of lentil seedlings. Thus ACC-deaminase activity may be a useful criteria for the selection of effective plant growth promoting rhizobacteria.

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