



**Full Length Article**

## Rhizobacteria Capable of Producing ACC Deaminase Promote Growth of Velvet Bean (*Mucuna pruriens*) under Water Stress Condition

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### Abstract

Decreased water levels cause severe reduction in growth and yield of plants. Generally the plant growth is inhibited under water stress due to the high concentration of ethylene in the rhizosphere. Plant growth promoting rhizobacteria (PGPR) inhabiting the rhizosphere capable of producing 1-aminocyclopropane-1-carboxylate deaminase (ACCd) enzyme can also reduce the concentration of ethylene in plant by breaking down ACC, the immediate precursor of ethylene. Such PGPR were isolated from different parts of the province of Punjab and Khyber Pakhtunkhwa and screened for their ability to promote growth of velvet bean in axenic conditions. The results showed that some rhizobacterial strains significantly improved the growth of roots and shoots in normal and water stress condition. A significant increase was observed in root length (32%), shoot length (110%), root dry weight (50%) and shoot dry weight (60%) in response to inoculation compared to an uninoculated control. Four strains showed very encouraging results in promoting the growth of velvet bean. The ability of selected rhizobacteria to reduce the concentration of ethylene was confirmed by performing classical triple response bioassay in etiolated pea seedlings. The results of this study illustrate that ACCd active PGPR can be used for improving plant growth and yield of velvet bean under limited availability of water. © 2015 Friends Science Publishers

**Keywords:** Drought; Ethylene; Root growth; PGPR; Inoculation; Velvet bean

### Introduction

Water is an influential abiotic factor in determining plant growth, physiology and crop yield. Climatic changes caused uneven and erratic rainfall in various regions of the world, reducing fresh water availability to critical levels in some areas (Barnaba *et al.*, 2008; Chen *et al.*, 2013). Reduction and irregular rainfall decreases surface soil water available to plants and nutrient transport. Compared to other stresses, drought is a major factor limiting plant growth and yield (Belimov *et al.*, 2008; Lauteri *et al.*, 2014). Moreover, climate change scenarios predict increased intensity and duration of water stress in the future (IPCC, 2007). In Pakistan, the demand for water is expected to grow by a factor of 2.2 by 2050 (Bates *et al.*, 2008).

Increasing world population necessitates the improvement in agricultural practices for more food crop production under drought (Grierson *et al.*, 2011). The characteristics of the plants that confer water absorption are likely to be most vital to mitigate effects of drought (Ren *et al.*, 2007; Centritto *et al.*, 2009; Chaves *et al.*, 2009). Water absorption by the plant is linked to the root system (Bangash *et al.*, 2013). Therefore, recently attention is diverted to improve root system and functioning to enhance water use

efficiency, especially high yield with less water (Lynch, 2007; Ghanem *et al.*, 2011; Vacheron *et al.*, 2013).

Under stress condition, plant roots receive stress signals and transport chemical signals to upper part to limit the use of water for maintaining cellular water. Plant roots also release ACC which readily converts into ethylene (Davies and Zhang, 1991; Chen *et al.*, 2013). Ethylene is a phytohormone, which performs dual role in plant development. Ethylene helps plant growth and development in the early stages, while the high concentration of ethylene directly inhibits root growth (Wang *et al.*, 2002; Wilkinson and Davies, 2010). The impaired root system cannot get adequate water for plant growth and therefore reduce the yields (Bangash *et al.*, 2013; Glick, 2014).

In order to combat drought effects, plants use multiple strategies such as change in gas exchange and water relation (Liu *et al.*, 2005). Plant rhizosphere is usually known as a value added region for the beneficial microflora. The rhizosphere is typically rich in specific PGPR containing ACC deaminase enzyme (ACCd), which hydrolyzes ACC (ethylene precursor) and use it as the source of carbon and nitrogen (Honma and Shimomura, 1978; Belimov *et al.*, 2005; Glick, 2014). Bacteria adhere to the plant roots and continuously break ACC in root exudates into ammonium

and  $\alpha$ -ketobutyrate until a dynamic equilibrium between the roots and rhizosphere bacteria is maintained. Rhizobacteria reduce the concentration of ACC resulting in reduced production of ethylene (Nadeem *et al.*, 2013). The manipulation of bacterial community in the rhizosphere could possibly provide an economical and environment friendly way to improve crop yield by modulating the root system and functioning under low water conditions. For this purpose, rhizobacteria were isolated from the rhizosphere of velvet bean and screened for ACC deaminase activity. The selected strains of rhizobacteria were examined for their ability to promote growth of velvet bean in water stress conditions.

## Materials and Methods

### Soil Sampling and Isolation of Rhizobacteria with ACCd Activity

Rhizobacteria with ACCd activity were isolated from the rhizosphere of velvet bean. Velvet bean is not grown commonly in Pakistan, therefore soil samples were collected in polythene bags from different places (Haripur, Rawalpindi, Attock and Bahawalpur districts) to be used for growing velvet bean under control conditions. Velvet bean seeds were sown in small pots filled with different types of soil (200 g) separately. The pots were incubated at 25°C. After 60 d, plants were uprooted and non-rhizosphere soil was removed by gentle shaking. Roots with rhizosphere soil were stirred in sterile distilled water under aseptic conditions. This suspension was used for isolation of rhizobacteria through the dilution plate technique. About 250  $\mu$ L of the soil suspension from each dilution were placed on agar medium (Glick *et al.*, 1994). ACC (50 mg L<sup>-1</sup>) was used as the sole nitrogen source. Petri plates were incubated at 30°C for 48 h. Initially 253 rhizobacterial isolates were selected and examined again in growth medium spiked with the ACC. After 48 h of incubation, the optical density (OD) of the bacterial cultures was checked at 600 nm with a UV visible spectrophotometer. Thirty rhizobacteria showing the highest growth (OD) were selected to evaluate their potential to promote growth of velvet bean.

### Effect of Rhizobacteria on Growth of Velvet Bean

A petri dish experiment was performed to determine the effect of inoculation with ACCd active rhizobacteria on root growth of velvet bean. Velvet bean seeds were surface disinfected by soaking seeds in a solution of 95% ethanol for 20 sec, following a soak in 0.2% (w/v) HgCl<sub>2</sub> solution for 30 sec. Then, the seeds were rinsed with distilled sterilized water thoroughly to remove the seed disinfectant (Khalid *et al.*, 2004). Selected isolates were grown in liquid medium containing ACC for 48 h at 30°C. The sterilized seeds were then inoculated by soaking them in respective broth culture (OD 0.6 $\pm$ 0.02 at 600 nm) of 30 selected rhizobacteria for 10 min. In case of control treatment, the

seeds were dipped in the un-inoculated medium. The inoculated seeds were placed between two sterilized Whatman filter paper sheets. Five (5) mL of sterilized distilled water was also used to wet filter paper sheets. The plates were placed in the growth incubator at 25 $\pm$ 1°C for 10 d. The experiment was conducted in a completely randomized design with three replications. At the end of experiment, the data relating to root growth parameters was recorded.

Based on the results recorded for root growth, 10 effective isolates were selected to test their potential for promoting growth in glass jars. Velvet bean seeds were disinfected as described above. These seeds were placed on two filter paper sheets in a petri dish. Fifteen seeds were placed on each plate. The plates were incubated at 25 $\pm$ 1°C for germination. Pre-germinated velvet bean seeds were inoculated by immersing in the respective inocula (OD 0.6 $\pm$ 0.02 at 600 nm) and planted in glass jars containing 200 g of sterilized sand. For control, the seeds were immersed in sterilized medium. The jars with six replicates were placed in a growth chamber at 25 $\pm$ 1°C in a completely randomized design. In addition, five mL of half strength Hoagland solution (Hoagland and Arnon, 1950) was added to each jar. After 20 d, the plants were harvested and data on root length, dry root weight, shoot length and dry shoot weight were recorded.

### Effect of Selected Rhizobacteria on Growth of Velvet Bean under Water Stress

Based on the rhizobacterial performance to improve root growth in the jar experiment five isolates (G4, G9, H6, H38 and HS9) were selected to evaluate their ability to promote plant growth in water deficit condition. The seeds germinated with uniform size were planted in soil (jar experiment). The water level was maintained at 45% of the water holding capacity (WHC) by weighing the pots and the addition of water based on the weight loss. However, weight gain due to the growth of plants was ignored. Seedlings were inoculated after two days by injecting 10 mL of inoculum. For control, autoclaved liquid medium was used. The experiment was performed with three replications. After 30 days, root length, shoot length, root dry weight and shoot dry weight were recorded.

### Classical Triple Response Bioassay

To confirm the ACC-deaminase activity of selected rhizobacteria, classical triple response bioassay was performed with pea seeds (Shaharoon *et al.*, 2006). Two pea seeds were positioned in the folds of sterile filter papers and placed in jar covered with green foil to give the green light. ACC at 5 mM was used for seed treatment. All treatments were replicated three times. The jars were kept at 25 $\pm$ 1°C in the dark. After 10 days, the classical "triple" response was observed by measuring the length and diameter of the seedlings.

## Statistical Analysis

The collected data were analyzed statistically using Statistx version 9.0 and LSD post hoc test.

## Results

A total of 253 isolates were obtained from the rhizosphere of velvet bean. About 56% rhizobacteria had the ability to use ACC as the sole nitrogen source (Table 1). However, the bacterial biomass (OD) in a liquid medium varied from strain to strain. The maximum numbers of rhizobacteria with better growth in a medium of ACC were obtained from soil of Haripur district. Overall, 32% rhizobacteria (47 out of 147) showed higher growth ( $OD > 0.60$ ) in a liquid medium containing ACC as a nitrogen source, while other isolates exhibited slow growth rate.

The results of root growth (Plate experiment) showed that inoculation with rhizobacteria significantly improved root length of velvet bean compared to uninoculated control (Table 2). The highest mean root length was observed in the case of inoculation with the isolate HS9 (isolated from Haripur soil). Inoculation with different rhizobacteria had a positive effect on root dry weight. Isolate HS9 was also effective in improving the root dry weight of velvet bean plants and showed the highest average root dry weight among the tested isolates. Similarly, more lateral roots were recorded in the case of inoculation with HS9. Some other isolates (A45, BS10, G9, H6) also had a positive effect on the development of lateral roots compared to uninoculated seedlings. A negative effect of inoculation with F32 isolate on the lateral roots was observed.

The selected isolates also improved root length up to 52.4% compared to the uninoculated control (Fig. 1a). Maximum root length was observed in plants inoculated with HS9 isolate. The increase in shoot length in response to inoculation was 64.6% compared with uninoculated plants (Fig. 1b). Rhizobacterial isolate G9 was the more efficient to increase the shoot length compared with uninoculated plants. Three other isolates (BS10, H6 and H38) also improved the root and shoot length compared to uninoculated control. Maximum increase in root dry weight was observed in the case of inoculation with isolate G9, G28 and HS9 (Fig. 1c) and the increase was 85.7% over the control. Other tested isolates were found also effective in improving the root dry weight compared to the control. The results showed that shoot dry weight in the case of inoculation with rhizobacteria was 56.5% greater than uninoculated control (Fig. 1d). The most promising shoot dry weight (0.36 g pr plant) was recorded in case of H38 inoculation compared to control.

Five selected isolates (G4, G9, H6, H38 and HS9) were evaluated for their potential to promote growth of velvet bean under water stress conditions. Velvet bean plants were grown to 45% of the water holding capacity (WHC). Maximum root length was observed in case of

**Table 1:** Isolation of rhizobacteria producing ACC-deaminase from rhizosphere of velvet bean (*Mucuna pruriens* L.)

Sites	Soil sample code	Total isolates	ACC active isolates	Isolates having OD in ( $>0.6$ at 600 nm)
Bahawalpur	B	57	27	8
Faisalabad	F	45	16	7
Rawalpindi	G	72	45	13
Haripur	H	79	54	19

**Table 2:** Effect of rhizobacteria containing ACC-deaminase on the seed root growth of velvet bean

Isolate	Root length (cm)	Number of lateral root	Root weight (g per plant)
Control	2.10 ± 0.22	3 ± 0.20	0.006 ± 0.003
A18	3.69 ± 0.26	3 ± 0.17	0.017 ± 0.002
A16	1.94 ± 0.25	2 ± 0.20	0.007 ± 0.004
A45	3.33 ± 0.32	4 ± 0.21	0.022 ± 0.003
BS10	2.95 ± 0.28	4 ± 0.22	0.024 ± 0.001
B12	2.20 ± 0.14	3 ± 0.15	0.012 ± 0.001
B32	2.34 ± 0.16	3 ± 0.22	0.020 ± 0.002
F16	2.51 ± 0.22	4 ± 0.25	0.020 ± 0.002
F32	3.63 ± 0.32	2 ± 0.17	0.032 ± 0.005
G4	4.25 ± 0.33	3 ± 0.15	0.043 ± 0.005
G9	4.55 ± 0.44	4 ± 0.15	0.05 ± 0.001
G28	2.45 ± 0.27	3 ± 0.04	0.019 ± 0.00
H6	3.95 ± 0.25	4 ± 0.13	0.013 ± 0.002
H17	3.28 ± 0.22	3 ± 0.02	0.02 ± 0.003
H38	4.64 ± 0.38	3 ± 0.21	0.02 ± 0.004
HS9	4.85 ± 0.32	5 ± 0.16	0.069 ± 0.003

**Table 3:** Effect of rhizobacteria containing ACC-deaminase on the plant growth of velvet bean under water stress conditions

Isolate	Root length (cm)	Root weight (g per plant)	Shoot length (cm)	Shoot weight (g per plant)
Control	18.17 ± 1.7 <sup>a</sup>	0.20 ± 0.1 <sup>a</sup>	31.83 ± 4.4 <sup>a</sup>	0.50 ± 0.1 <sup>a</sup>
G4	21.83 ± 0.9 <sup>b</sup>	0.29 ± 0.1 <sup>bc</sup>	63.67 ± 4.4 <sup>b</sup>	0.67 ± 0.2 <sup>b</sup>
G9	23.20 ± 1.4 <sup>bc</sup>	0.30 ± 0.1 <sup>c</sup>	62.33 ± 3.4 <sup>b</sup>	0.77 ± 0.1 <sup>cd</sup>
H6	22.83 ± 1.1 <sup>b</sup>	0.28 ± 0.0 <sup>b</sup>	62.00 ± 2.9 <sup>b</sup>	0.73 ± 0.1 <sup>c</sup>
H38	24.00 ± 1.5 <sup>c</sup>	0.26 ± 0.1 <sup>b</sup>	62.33 ± 1.4 <sup>b</sup>	0.73 ± 0.1 <sup>c</sup>
HS9	23.50 ± 1.2 <sup>bc</sup>	0.30 ± 0.1 <sup>c</sup>	66.83 ± 2.5 <sup>c</sup>	0.80 ± 0.1 <sup>d</sup>

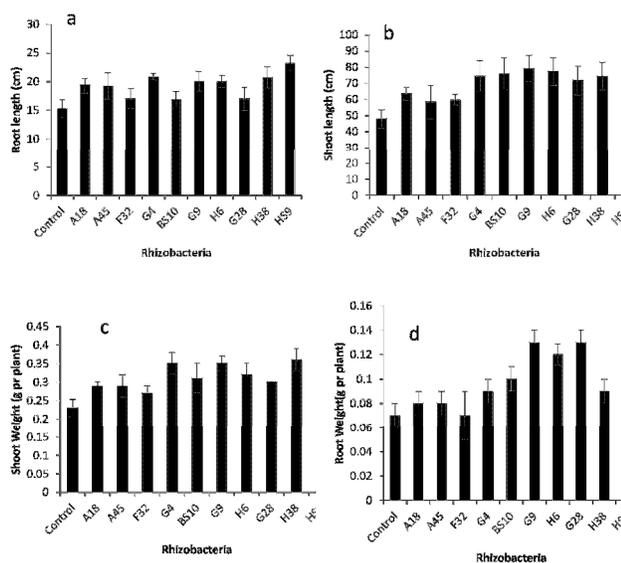
inoculation with isolate H38, which was 32.1% greater than uninoculated plants (Table 3). Likewise, HS9 and G9 showed a significantly higher root length than control. Rhizobacterial isolates HS9 and G9 had a positive effect on the root dry weight and up to 50% more root weight was observed compared to uninoculated control. Similarly, shoot length under water stress also improved in response to inoculation with selected rhizobacterial isolates. Shoot length was 110% higher for inoculation with isolate HS9 than uninoculated control. Other selected isolates also exhibited promising effect on shoot length of velvet bean plants. An increase of 60% in shoot dry weight was recorded upon inoculation with isolate HS9 compared to the control. In general, all isolates tested had a positive effect on the shoot weight compared to uninoculated control.

**Table 4:** Classical “triple” response of etiolated pea seedlings at different ACC concentrations (mM)

ACC concentrations	Shoot length (cm)	Shoot diameter (cm)	Root length (cm)
No ACC	5.11 a	0.53 d	5.33 a
2 mM	4.12 b	1.03 c	3.48 b
4 mM	3.14 c	1.74 b	3.88 b
6 mM	1.13 d	2.09 a	3.30 c

**Table 5:** Comparative effect of ACCd active rhizobacterial inoculation on etiolated pea seedlings in the presence of 5 mM ACC

5 mM ACC	Shoot length (cm)	Shoot diameter (cm)	Root length (cm)
ACC only	1.40 d	1.99 a	1.08 d
ACC + G4	1.60 c	0.60 c	1.92 b
ACC + G9	1.57 c	0.80 b	1.75 c
ACC + H6	1.68 bc	0.77 b	1.93 b
ACC + H38	1.77 ab	0.57 cd	2.0 ab
ACC + HS9	1.85 a	0.51 d	2.17 a



**Fig. 1:** Inoculation effect of ACC deaminase producing rhizobacteria on (a) root length, (b) shoot length, (c) dry root weight and (d) dry shoot weight of velvet bean seedlings in jar trial under control conditions

ACC-deaminase activity was confirmed by performing classical triple response bioassay (Table 4). The results revealed that the diameter of etiolated pea seedlings exposed to ACC increased considerably with increase in the concentration of ACC, while the length of the seedlings was reduced compared to the untreated control. Increased stem diameter and decrease in length of the seedlings was 74 and 77%, respectively over the untreated control. However, inoculation of seedlings exposed to ACC diluted the effect of ACC and caused 32% increase in shoot length and 280% decrease in shoot diameter of etiolated pea seedlings.

## Discussion

Rhizosphere soil was collected from different areas of Pakistan and used for isolation of ACCd active rhizobacteria. About 56% of the isolates were able to use ACC as a nitrogen source and grow in a medium enriched with ACC. However, rhizobacterial isolates showed substantial differences in growth potential in term of biomass. It has been reported that the bacteria isolated from rhizosphere soil of different crops have ACC deaminase enzyme that degrades available ACC (Glick *et al.*, 2007; Shaharoon *et al.*, 2008; Siddique *et al.*, 2011; Shahzad *et al.*, 2013). The bacteria isolated from different locations can also vary in their genetic composition and enzymatic activities involved in the cleavage of ACC (Bangash *et al.*, 2013). Probably for this reason rhizobacteria isolated from different locations had different effects on root growth of velvet bean plants. In general, the rhizobacteria with ACC deaminase activity showed a considerable improvement in the growth of roots compared with the uninoculated plants. ACCd active rhizobacteria possibly enhanced the root growth by reducing ethylene levels in the rhizosphere of velvet bean plant. It is widely known that PGPR hydrolyze ethylene precursor ACC, resulting in increased root growth (Belimov *et al.*, 2001; Mayak *et al.*, 2004; Shaharoon *et al.*, 2006; Chen *et al.*, 2013). The above findings are also supported by the results of the experiment under water stress condition. A significant improvement in the root and shoot growth was observed in plants inoculated with ACCd active bacteria compared to uninoculated plants. The rhizobacteria isolated from rainfed area (water stress) of Haripur district were more effective than other isolates. Ethylene is primarily known as a plant stress hormone (Arshad *et al.*, 2008). It is synthesized in higher concentrations in plants under stress conditions and inhibits the root growth. The rhizobacteria with ACCd enzyme lower ethylene synthesis and thus inhibitory effects on plants are reduced (Kang *et al.*, 2010; Shahzad *et al.*, 2010). The results show that rhizobacteria having ACCd could be effective to stimulate growth of velvet bean.

In addition, ACCd activity of selected rhizobacteria was confirmed by classical triple response of etiolated pea seedlings. Pea seeds are very sensitive to the high concentration of ethylene and exhibits typical responses (increased diameter and reduced length of seedlings) (Shahroon *et al.*, 2006). Inoculation with rhizobacteria containing ACC deaminase decreased the severity of ACC-induced classic response in etiolated pea seedlings. Furthermore, improvement in the length of the seedlings and root development implies that the negative effect of ACC can be reduced by inoculation with bacteria containing ACCd enzyme.

## Conclusion

Rhizobacteria with ACCd significantly improved the plant growth, especially the root development of velvet beans.

The improved root growth was possibly due to the ability of rhizobacteria to regulate ethylene levels in the rhizosphere. Best root system resulted in the promotion of shoot growth through increased supply of nutrients and water to the plant. The use of rhizobacteria containing ACCd is an environment friendly approach to increase agricultural productivity under stress condition.

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