

CHARACTERIZATION OF EFFECTIVE RHIZOBACTERIA ISOLATED FROM VELVET BEAN (*MUCUNA PRURIENS*) TO ENHANCE PLANT GROWTH

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Abstract

Rhizobacteria with plant growth promoting ability exist in association with plant roots and ameliorate over all plant development and yield. Numerous species of rhizobacteria have been identified with plant growth promoting ability, which can be attributed to multiple microbial characteristics. In the current study rhizobacterial isolates with best plant growth promotion traits were subjected to screening for plant growth promotion under axenic condition. The results of lab assays revealed that out of five rhizobacterial isolates three of bacterial isolate were Gram -ve and two of them were Gram +ve bacterial group. All isolates found positive for the auxin production and ACC-deaminase activity. The isolate HS9 showed highest ACC activity (331 α -ketobutyrate nmol mg⁻¹ biomass hr⁻¹) and auxin production (3.85 without L-TRP). PGPR increase plant growth by reducing the ethylene release and its inhibitory effects, the role of isolates to decrease ethylene effects was affirmed via classical triple response assay on velvet bean. Furthermore, isolate were assessed for resistance test, three efficient strains (G9, HS9 and H38) exhibited antibiotic resistance for streptomycin, kanamycin and rifampicin at 100 mg L⁻¹ in TSB medium. For the purpose of co-inoculation, all three isolates showed positive relation to grow together. The results concluded that rhizobacteria selected from rain fed areas were found effective to improve plant growth with their multiple growth enhancing traits. Therefore, PGPR with various characteristics could be a better option for inoculation and co-inoculation to improve plant growth in well watered and water stressed environment.

Key words: PGPR, ACC-deaminase, Ethylene, Antibiotic, Auxin.

Introduction

The rhizosphere is the root zone where soil, plant roots and soil biota interact with each other. Often these interactions are beneficial to the plants by improving the soil fertility and plant growth. On another hand, rhizode position from plant roots is a key energy supply for these microorganisms resulting in microbial abundance in the root vicinity. These microorganisms are known as plant growth promoting rhizobacteria (PGPR) (Glick, 2014; Masciarelli *et al.*, 2014; Dinesh *et al.*, 2015). The activity for growth promotion has been found in various genera of PGPR including *Enterobacter*, *Bacillus*, *Pseudomonas*, *Azospirillum*, *Klebsiella*, *Burkholderia*, *Arthrobacter* and *Serratia* (Zahir *et al.*, 2008; Kumar *et al.*, 2012; Ahemad & Kibret., 2014; Nadeem *et al.*, 2014).

Rhizobacteria improves plant growth through several mechanisms which include their ability to modulate phytohormones such as indoleacetic acid, gibberellic acid, cytokinins and ethylene (Saleem *et al.*, 2007; Singh, 2013), solubilize inorganic phosphate and ability to lower the concentration of stress ethylene. Ethylene is a phytohormone that is released in low concentrations under normal conditions; however, under stress condition the ethylene is released in high concentrations causing growth inhibition. Ethylene in high concentration is thus known as a stress hormone inhibiting root growth. Rhizobacteria producing ACC (1-aminocyclopropane-1-carboxylate) deaminase metabolizes ethylene precursor ACC (1-aminocyclopropane-1-carboxylate) in roots (Belimov *et al.*, 2007; Saleem *et al.*, 2007; Magnucka & Pietr, 2015). Rhizobacteria isolated from rain fed region have the ability

to mineralize ethylene precursor ACC into other compounds. The specific group of PGPR having ACC-deaminase enzyme utilize the root exuded ACC as carbon and nitrogen source by cleaving it into α -ketobutyrate and ammonia before its conversion into ethylene (Honma & Shimomura, 1978; Khalid *et al.*, 2006; Shahzad *et al.*, 2013; Bangash *et al.*, 2013; Saleem *et al.*, 2015; Subramanian *et al.*, 2015).

The reliable bioassay for plant ethylene synthesis is the classical triple response. Upon direct application of ethylene gas, the triple response was observed in pea seedlings. The triple response includes reduction in elongation, swelling of the hypocotyl and change in the direction of growth (horizontal) (Khalid *et al.*, 2006; Shaharoon *et al.*, 2006). PGPR from rainfed region shows promising effect to reduce ethylene release and to increase over all plant growth. Moreover the auxin is quantitatively the most abundant phytohormone involved in root initiation and plant development (Yasmin *et al.*, 2013; Etesami *et al.*, 2014).

Phosphorus (P) is one of the major essential macronutrients for plant growth and development (Kesaulya *et al.*, 2015). Usually soils have a high amount of total P; however, most of it is the insoluble form. Plants are unable to uptake the insoluble phosphorous (Masciarelli *et al.*, 2014). The conversion of the insoluble form of phosphorous to bioavailable form for plants is an important trait of PGPR. These rhizobacteria release organic acids such as citrate, lactate, succinate etc. which reduces the pH of the rhizosphere. The organic acids dissolve the mineral phosphates as a result of anion exchange or chelation of Fe and Al ions associated with phosphate. This leads to an

increase in the soluble P in the rhizosphere resulting in more uptake of P by the plants (Shahzad *et al.*, 2013; Kumar *et al.*, 2012).

Plant Growth Promoting Rhizobacteria enable the plant to survive under stress conditions and improve root development, plant growth and yield (Nadeem *et al.*, 2013; Dinesh *et al.*, 2015). Inoculation with PGPR strains shows promising enhanced effects on plant growth (Rajendran *et al.*, 2008). Augmentation of rhizosphere with single or combination of effective PGPR could be a possible and useful strategy to improve plant growth in well watered and in stressed conditions. The purpose of present study was to characterize the isolated rhizobacteria from rhizosphere of velvet bean for plant growth promoting traits and to evaluate for improving plant growth and development.

Materials and Methods

Sampling, isolation and screening of rhizobacteria: The soil samples were collected from rain fed and irrigated areas of Pakistan to grow velvet bean plants. About 50-60 days old plants of velvet bean were uprooted and the soil adhering to the roots were used for bacterial isolation. Dilution plate technique and ACC enriched mineral salt medium was used for the purpose of isolation and screening of ACC deaminase containing rhizobacteria.

Morphological characterization: Gram staining was performed by Ryu Non-staining KOH technique for rapid determining (Powers, 1995). A small loop of bacterial culture was mixed thoroughly with a drop of 3% KOH solution on a glass slide. If the reaction takes place and the solution becomes sticky then the isolate was characterized as gram negative and vice versa for the gram positive isolates.

Indole acetic acid (IAA) Production: Auxin production by selected isolates was determined in terms of IAA equivalents both with and without L-tryptophan (L-TRP) (Sarwar *et al.*, 1992). Culture filtrate (3.0 mL) was mixed with 2.0 mL of Salkowski reagent (98.0 mL of 35% HClO₄ + 2.0 mL of 0.5 M FeCl₃) and let it rest for 30 minutes. The development of color and intensity was measured on spectrophotometer at 535 nm and was compared with standard curve of IAA standard solutions.

Phosphate solubilization assay: To assess the ability of selected rhizobacterial isolates to solubilize inorganic phosphate, rock Phosphate was added to nutrient agar medium as an inorganic form of phosphate (Goldstein, 1986). A loop full of the respective bacterial culture was placed on the plates and incubated for one week at 28±1°C the bacterial cultures having a clearing zone were considered as positive for phosphate solubilization.

ACC-deaminase activity test: The ACC-deaminase activity of selected rhizobacterial strains was determined with modified protocol of Honma & Shimomura (1978) and Penrose & Glick (2003). The quantitative measurement of rhizobacterial enzymatic activity was determined by estimating the production of α -ketobutyrate through ACC break down by bacterial ACC deaminase enzyme. The concentration of α -ketobutyrate (mmol) formed in this reaction was observed by change in color of solution and

calculated by measuring the absorbance on spectrophotometer at 540 nm and sample absorbance was compared with standard curve of α -ketobutyrate.

Classical triple response bioassay: To affirm the presence of ACC-deaminase activity of selected rhizobacterial strain, conventional classical - triple response bioassay was executed on velvet bean seeds by following a protocol used by Shaharoon *et al.*, (2006). Two velvet bean seeds were placed in the folds of sterile filter papers and positioned in jar wrapped with green sheet. ACC at 5 mM concentration was applied with selected isolate. All of inoculated and control treatments were replicated three times. The jars were placed at 26±1°C in the dark. After ten days, "triple" response was observed regarding length and diameter of the bean seedlings.

Bacterial antagonism test: The effective strains were checked for their antagonism activity to grow together for co-inoculation purpose. The bacterial antagonism was evaluated by following the protocol of Stonier (1959). For the assay, test tubes and Petri plates were filled with MGY (mannitol-glutamate-yeast) broth. The test tubes were inoculated with each respective isolate and the inoculum was poured in agar plates and incubated at 29±1°C for 48h. Apart from this, 4mL of sterilized 1% agar phosphate buffer (cooled, 0.05 M, pH7) were filled in an Eppendorf. The liquid inoculum was mixed with liquid agar phosphate buffer after incubation at normal temperature and poured on an opposite strain plate and re-incubated for 48hr. The bacterial antagonism was observed with appearance of some clear zone around first strain spot, no clear zone was considered as the mutual growth production.

Antibiotic resistant bacterial mutant: Selected rhizobacterial isolates were assessed for their ability to show resistance against antibiotics. In current study, rifampicin and kanamycin were used for gram negative isolate while streptomycin for gram positive isolates. Selected isolates were allowed to grow on TSB (tryptic soy broth) medium with each respective antibiotic. Initially low antibiotics concentration (5 and 10 mg L⁻¹) were grown colonies were transferred to higher concentration of respective antibiotic (20 mg L⁻¹) and reached to 150 mg L⁻¹. The resistant isolates were further used for consortium preparation.

Application of selected strains on plant growth: To assess the plant growth promotion, the velvet bean seeds were surface sterilized by following the protocol of Khalid *et al.* (2004). The seeds were sown in jar filled with sterilized sand. In order to prepare rhizobacteria inocula, the selected isolates individually and in combination allowed to grow in liquid mineral salt medium for 24 hours at 80rpm in shaking incubator. After three days of germination the seedlings were inoculated by injecting 10mL of inoculum of respective isolate and consortium. After two week of experiment, plants were uprooted and data regarding root and shoot length and their dry weight were recorded. The experiment was performed with three replications.

Results and Discussion

The soil samples were collected from the agricultural land of irrigated and rainfed areas of Pakistan (Table 1). Rhizobacteria were isolated and screened on ACC enriched medium. About 56% (152) of all isolated strains showed the ACC-deaminase activity and overall 32% (47) of rhizobacterial isolates exhibited best proliferation in ACC liquid medium. Isolates were further screened for plant growth promotion activity (Saleem *et al.*, 2015). The maximum number of rhizobacteria that showed better growth on the ACC medium belonged to the soil of district Haripur (rainfed region) where agriculture depends solely on rainwater and crops often face water shortages during different stages of plant growth. The results imply that the bacteria isolated from the rainfed area and water

stress zone can utilize ACC more efficiently. The bacteria isolated from different places may vary in their genetic composition and enzymatic activities (Bangash *et al.*, 2013; Saleem *et al.*, 2015). Probably for this reason rhizobacteria isolated from different locations showed a varying potential to grow on ACC medium and could have variable effect on the growth of inoculated plants. Previously, a variety of bacteria isolated from different environments were found able to use ACC as a source nitrogen (Chinnadurai *et al.*, 2009; Siddikee *et al.*, 2011; Nadeem *et al.*, 2014; Magnucka & Pietr, 2015), however, rhizobacteria with ACC deaminase activity isolated from rainfed areas have not been explored much for their growth promoting activities. Recently, Bangash *et al.* (2013) reported that ACC deaminase producing bacteria isolated from rainfed areas of Pakistan had a great potential to promote wheat growth.

Table 1. Collection of soil sample from different agricultural lands.

Sampling site	Cropping system	Crop History	Texture	pH	ACC deaminase containing best grown and effective isolates
Rawalpindi	Rainfed	Wheat	Sandy Loam	7.5	6
Bhawalpur	Irrigated	Vegetable	Loam	7.6	8
Faisalabad	Irrigated	Wheat	Loamy Sand	7.7	7
Gujjar Khan	Rainfed	Wheat	Silt Clay Loam	7.4	7
Hari Pur	Rainfed	Wheat	Sandy Loam	8.5	9

Furthermore, the best effective screened isolates were examined for their morphological and biochemical characteristics that make the competent candidate for plant growth promotion. Based upon the results of Gram staining, the isolate G9, H6 and H38 belonged to gram negative while G4 and HS9 were Gram positive (Table 2). The plant growth promoting capability has been observed in wide range of both Gram positive and Gram negative (Babalola *et al.*, 2003; Ghosh *et al.*, 2003; Saleem *et al.*, 2007) also in rhizobia (Madhiyan *et al.*, 2006) and endophytes (Pandey *et al.*, 2005). The selected isolates produced IAA with and without L-TRP, the IAA concentration produced by PGPR ranged between 2.01-3.85 mgL⁻¹ and 3.5-7.5 mgL⁻¹ without and with L-TRP respectively (Table 2). L-TRP is considered as the immediate precursor for microbial IAA synthesis, therefore addition of L-TRP can increase the IAA production to several folds (Yasmin *et al.*, 2009; Kesaulya *et al.*, 2015). In some cases root exudates contain L-TRP as an organic compound which can be consumed by inhabiting PGPR. The isolate HS9 showed the best IAA production in both in presence as well as absence of L-TRP. Auxin (IAA) and ethylene both account are important phytohormone owing to their role in the regulation of plant development. IAA producing PGPR modulate the plant available auxin pool spatially and temporally that positively affect plant growth (Lopez *et al.*, 2007; Shi *et al.*, 2010; Shahzad *et al.*, 2013; Dinesh *et al.*, 2015; Etesami *et al.*, 2015). A significant correlation generally develops in auxin production and plant yield. Higher level of IAA production by *Pseudomonas* was recorded by other research workers (Asghar *et al.*, 2002).

Among the phytohormones, ethylene plays a dual role. At low concentration it acts as a growth regulator and promotes the development of adventitious roots,

whereas at high concentrations it inhibits the root growth (Zahir *et al.*, 2008; Nadeem *et al.*, 2013; Glick, 2014). Plant growth promoting rhizobacteria possessing ACC deaminase enzyme break down the ethylene precursor ACC that results in reduced ethylene synthesis. Because more ACC is exuded from plants under stress conditions, the bacteria present in the vicinity of the roots have greater potential to destroy ACC efficiently by using it as the source of nitrogen for their growth. In current study, result revealed that the selected rhizobacterial isolates possessed the ACC deaminase activity (Table 2). Isolates showed the best *in-vitro* ACC deaminase activity ranged from 299-33nmol mg⁻¹ biomass hr⁻¹ of α -ketobutyrate. Isolate HS9 was the best candidate to lower the inhibitory effects of ethylene through its ACC deaminase activity (Fig. 1). The ACC deaminase activity of PGPR was confirmed via conventional ethylene triple response test on velvet bean (Table 3 & 4). The results revealed an increase in stem diameter (83%) and (50%) reduction in the length of velvet bean seedling as compared to control, when exposed to various ACC concentrations. However, inoculation with ACC deaminase producing PGPR diluted the inhibitory effects of ethylene. The seedling length was 30% greater than the control whereas the diameter of the shoot was 71% lower than the control. The use of ACC deaminase producing rhizobacteria can be a useful tool for the selection of promising PGPR (Subramanian *et al.*, 2015). The selected PGPR strains (G9, H38 and HS9) were found positive, while the other (G4 and H6) were negative for phosphate solubilization activity. The phosphate activity of selected isolates also made them more competitive under water stress conditions. Rhizobacterial phosphate solubilization trait could be employed to dissolve inorganic phosphate and increase its availability for plants (Yasmin *et al.*, 2013; Kumar *et al.*, 2014; Kesaulya *et al.*, 2015).

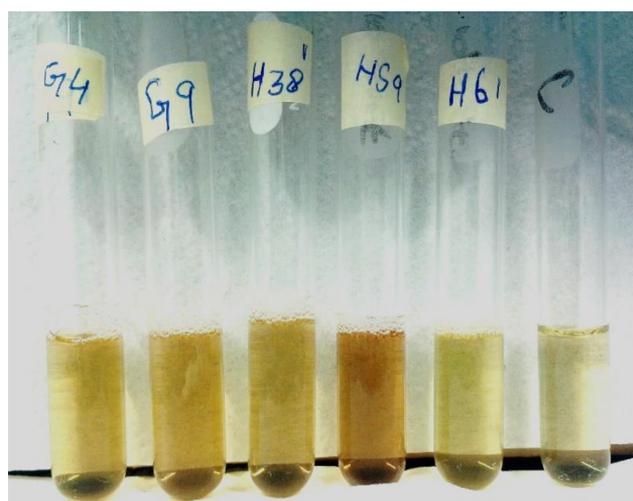


Fig. 1. *In vitro* ACC deaminase activity with five rhizobacterial strains.

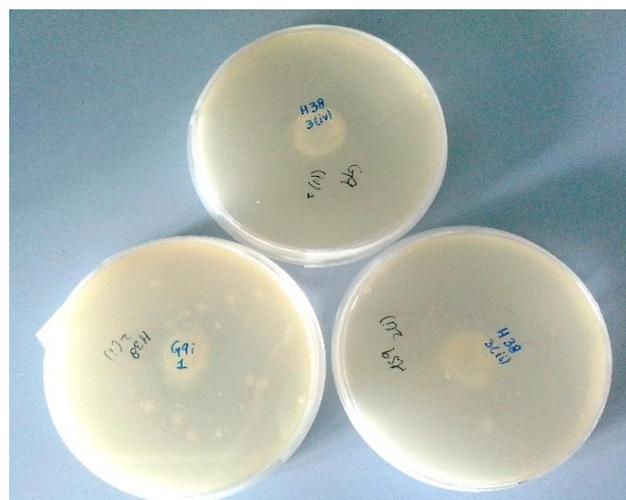


Fig. 2. All the three isolates (G9, HS9 and H38) grown together on MGY plates.

Table 2. Characterization of selected plant growth promoting rhizobacterial strains from velvet bean

Isolate	Gram Test	ACC-deaminase activity (α -ketobutyrate nmol mg ⁻¹ biomass hr ⁻¹)	Phosphate Solubilization Activity	IAA (mg L ⁻¹)		Quantitation Antagonism
				Without L-TRP	With L-TRP	
G9	G-ve	^a 299ab	+	3.05b	6.10a	^b H38 & HS9
G4	G+v	275cd	+	2.00c	2.75c	-
H6	G-ve	250d	-	2.03c	3.00bc	-
H38	G-ve	294bc	+	2.10c	3.50b	+ G9 & HS9
HS9	G+ve	331a	+	3.85a	6.75a	+ H38 & G9

^aMeans sharing the same letter in a column do not differ significantly according to Least Significant Difference test (P<0.05).

^bThese isolates can positively grow together

Table 3. Classical “triple” response of velvet bean seedlings at different ACC concentrations (mM)

ACC concentrations	Shoot Length (cm)	Shoot Diameter (cm)	Root Length (cm)
No ACC	4.11±0.28 ^a	0.43± 0.05	3.25±0.26
2 mM ACC	3.54±0.57	0.35±0.03	3.02± 0.33
4 mM ACC	2.63±0.34	1.42±0.27	2.67±0.25
5 mM ACC	2.03±0.29	1.69±0.32	2.50±0.46

^a Standard Error of the mean value

Table 4. Comparative effect of ACCd active rhizobacterial inoculation on velvet bean seedlings in the presence of 5 mM ACC

5 mM ACC	Shoot Length (cm)	Shoot Diameter (cm)	Root Length (cm)
ACC only	1.35d ^a	1.76a	0.80d
ACC + G4	1.60bc	0.60c	1.92a
ACC + G9	1.55c	0.65bc	1.65b
ACC + H6	1.68ab	0.77b	1.93a
ACC + H38	1.51c	0.54c	1.52c
ACC + HS9	1.75a	0.51c	1.91a

^aMeans sharing the same letter in a column do not differ significantly according to Least Significant Difference test (P<0.05).

In the current study three selected isolates (G9, H38 and HS9) were assessed for antibiotic resistance. The Gram +ve strain HS9 showed resistance to antibiotic streptomycin by exhibiting growth at TSB medium with 100 mgL⁻¹ of streptomycin. Similarly, the Gram-ve isolates were able to grow on kanamycin and rifampicin with same concentration, however isolate H38 did not show resistance at high concentration of antibiotic kanamycin. Jiang *et al.* (2008) reported that isolate J62 showed antibiotic resistance for various antibiotic concentrations kanamycin, streptomycin, ampicillin, tetracycline and rifampicin (100, 200, 500 and 50 µg ml⁻¹) respectively. Various bacterial genera such as *Azospirillum*, *Klebsiella*, *Enterobacter*, *Pseudomonas*, *Erwinia* and *Bacillus* appear to frequently colonize on roots (Yasmin *et al.*, 2009) and show resistance to several antibiotics. Plant growth promoting rhizobacteria resistant to antibiotics have advantage to survive better in the rhizosphere, that leads to dense root colonization and expression of beneficial effect on plant growth.

The best performing isolates can be used together as consortium (Laslo *et al.*, 2012) for better results. It is necessary to assess the isolates if they produce some antagonism by producing chemicals that would not allow the other strain to grow (Long & Azam, 2001). The results revealed that none of the isolate produced contrary antibiotic to the other isolates, thus all three isolates were able to grow together and any of the combination can be used for consortium (Fig. 2). The use of isolates as co-inoculation

could be a good strategy to obtain maximum beneficiary effects of competent PGPR (Barea *et al.*, 2005; Rajendran *et al.*, 2008; Chauhan *et al.*, 2015). Consortium of isolates can be used for the purpose of biofertilizers or rhizosphere augmentation.

Rhizobacteria possessing plant growth promoting traits were applied on velvet bean plant to evaluate their ability to promote growth of velvet bean (Table 5). The inoculated plants exhibited significant increase in root and shoot length compared to the un-inoculated control. The isolate HS9 showed the maximum increase of 47 and 86% in root and shoot length respectively, compared to the un-inoculated control. Similarly isolate HS9 showed maximum increase up to 28 and 30% in dry root weight and dry shoot weight respectively. The co-inoculation also found to give better results in comparison to the inoculation with a single isolate. The consortium of isolates G9 and HS9 gave maximum results in enhancing the weight of root and shoot by 60% and 93% respectively over the control.

The ACC deaminase activity along with other traits such as auxin production, phosphate solubilization, antibiotic production, root colonization might be helpful in improving the plant growth especially root growth. The improvement in root growth allows the plant to uptake more water and nutrients resulting in better growth and yield. Therefore PGPR containing various growth promoting traits along with ACC deaminase activity could be a better choice for inoculation of plants for their improved plant growth.

Table 5. Effect of selected isolate and their consortium on plant growth of velvet bean.

Isolate	Root length (cm)	Root weight (gm)	Shoot length (cm)	Shoot weight (gm)
Control	17.00±1.5 ^a	0.25±0.1	32.32±3.4	0.44±0.1
G9	20.06±1.2	0.28±0.1	50.32±3.6	0.58±0.1
H38	23.00±1.5	0.29±0.1	59.43±1.2	0.62±0.1
HS9	25.20±1.2	0.32±0.1	60.34±2.4	0.74±0.1
G9 + HS9	32.13±1.9	0.40±0.2	69.24±3.0	0.85±0.2
H38 + HS9	28.23±1.6	0.39±0.3	65.40±3.5	0.80±0.3

^a Standard Error of the mean value

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