BIOCHEMICAL AND FUNCTIONAL CHARACTERISATION OF ACC DEAMINASE PRODUCING FLUORESCENT *PSEUDOMONAS* INHABITING CHHATTISGARH SOILS

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ABSTRACT

Fluorescent *Pseudomonas* possesses a variety of promising properties which make it a better plant growth promoting bacteria. Their ability to promote plant growth through the production of Indole Acetic Acid (IAA), Phosphate solubilisation and HCN production are well known and studied. Besides these attributes they are also known to possess ACC deaminase enzyme which cleaves ACC, precursor of ethylene. During abiotic or biotic stress the production of ethylene is greatly enhanced, which is harmful for the normal growth of the plants. ACC deaminase containing fluorescent pseudomonads are able to alleviate the harmful effects of ethylene, thus these can be used as bioinoculants under biotic or abiotic stress conditions. In the present investigation eleven isolates of fluorescent *Pseudomonas* were isolated and characterised through various biochemical tests. They were tested for their ability to produce ACC deaminase enzyme, siderophore, IAA, Phosphate solubilisation and HCN production. All the isolates were able to utilise ACC as the sole nitrogen source but differed in their potential for ACC-deaminase activity *in vitro*. Highest ACC deaminase activity per hour was exhibited by P229 (43.3±0.60 µM a ketobutyrate/mg protein /h) while the lowest activity was observed in P5 (1.49±0.47 µM a ketobutyrate/mg protein /h). Isolates P141, P66 and P111 produced significant siderophore units while isolates P6, P166 and P229 were good IAA producer and phosphate solubiliser whereas only P166 had the ability to produce HCN. These isolates could be further tested under field conditions and based on the results the candidate isolates can be utilised as effective bioinoculants.

Key words: ACC deaminase, fluorescent *Pseudomonas,* IAA, HCN, Phosphate solubilisation, Siderophore

No: of tables- 6 No:of Figures :1 No : of References:42

Introduction

Plant growth promoting rhizobacteria (PGPR) are beneficial soil bacteria, which may facilitate plant growth and development both directly and indirectly (Glick, 1995). Direct stimulation may include providing plants with fixed nitrogen, phytohormones, iron that has been sequestered by bacterial siderophores, and soluble phosphate, while indirect stimulation of plant growth includes preventing phytopathogens (biocontrol) and thus, promote plant growth and development (Glick and Bashan, 1997). PGPR perform some of these functions through specific enzymes, which provoke physiological changes in plants at molecular level. These Plant Growth Promoting Rhizobacteria (PGPR) breaks down the immediate ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC) into ammonia and a ketobutyrate (a KB) by the production of ACC deaminase enzyme (Glick et al. 1994). Bacterial strains containing ACC deaminase can, in part, at least alleviate the stress induced ethylene-mediated negative impact on plants (Safronova et. al. 2006). The ACC deaminase metabolizes the root's ACC and checks the production of ethylene which otherwise inhibits plant growth through several mechanisms (Honma and Shimomura, 1978). The plants treated with bacteria containing ACC-deaminase may have relatively extensive root growth due to less ethylene (Burd et. al. 2000) and can better resist various stresses.Fluorescent *Pseudomonas* spp. are aerobic, gram-negative bacteria, ubiquitous in agricultural soils, and are well adapted to growing in the rhizosphere. Pseudomonads possess many traits that make them well suited as biocontrol and growth-promoting agents (Weller *et. al*. 1998). These include the ability to (i) grow rapidly *in vitro* and to be mass produced; (ii) rapidly utilize seed and root exudates; (iii) colonize and multiply in the rhizosphere and spermosphere environments and in the interior of the plant; (iv) produce a wide spectrum of bioactive metabolites (i.e., antibiotics, siderophores, volatiles, and growth-promoting substances); (v) compete aggressively with other microorganisms; and (vi) adapt to environmental stresses. In addition, pseudomonads are responsible for the natural suppressiveness of some soils to soilborne pathogens (Weller *et. al*. 2002).Therefore in the present investigation, an attempt was made to isolate and characterize ACC deaminase-producing fluorescent *Pseudomonas* with special reference to their functional characterisation.

Materials and Methods

Bacterial isolates and culture conditions

Strains of fluorescent *Pseudomonas* was isolated from the rhizospheric and non rhizospheric soils collected from different geographical locations of Chhattisgarh by the serial dilution agar plate method using King's B (proteose peptone, 20 g ; K_2HPO_4, 1.5 g; $MgSO_4$, 1.5 g; glycerol, 15 ml ; and Agar, 15 g; per liter) as selective medium (Table 1). Isolates were characterized on the basis of biochemical tests as per the procedures outlined in Bergey's Manual of Systematic Bacteriology (Sneath et al. 1986).To obtain pure cultures the colonies were further streaked on KM agar plates. Bacterial cultures were maintained at -80°C on King's B broth (Himedia) containing 50 % (w/v) glycerol and revived on King's B slants as per requirement.

Biochemical Characterisation

Fluorescent *Pseudomonas* isolates were characterised for fluorescent pigment production, gelatin liquefaction, casein hydrolysis, lipolytic activity and nitrate

reduction based on the method of Stanier et al.(1966) and Holt et al. (1994). Antibiotic sensitivity profile of the isolates was screened by testing resistance of the isolates to antibiotics Kanamycin (1 mg/ml) and Carbenicillin (0.1 mg/ml) by the streak plate method of Bauer *et al.* (1966). The ability of the bacterial strains to assimilate 35 carbohydrates was evaluated with HicarbohydrateTM kit (Himedia Laboratories,Mumbai, India). The tests were performed according to the recommendations of the manufacturer. Bacterial isolates were grown in King's B broth and incubated until the inoculum turbidity reached 0.5 O.D. at 600 nm. Each well of the kit was inoculated with 50 µl of these bacterial inoculums and incubated at 30° C for 24 h.

Qualitative estimation of ACC Deaminase activity

The utilization of 1-aminocyclopropane-1-carboxylic acid (ACC) as the only nitrogen source by the bacterial cultures was determined by growing the isolates in plates containing DF (Dworkin and Foster) salts minimal medium (Dworkin and Foster, 1958) supplemented with ACC. All the eleven *Pseudomonas* isolates were grown in 5 ml of Trypticase Soybean Broth (Himedia, India) and incubated at 28°C at 120 rpm for 24 h. The cells were harvested by centrifugation at 3000 *g* for 5 min. The pellet was washed twice with sterile 0.1 M Tris-HCl (pH 7.5), resuspended in 1 ml of 0.1 M Tris-HCl (pH 7.5) and spot inoculated on petri plates containing DF salts minimal medium (salts per litre: 4.0 g KH2PO4, 6.0 g Na2HPO4, 0.2 g MgSO4.7H2O, 2.0 g glucose, 2.0 g gluconic acid and 2.0 g citric acid with trace elements: 1 mg FeSO4.7H2O, 10 mg H3BO3, 11.19mg MnSO4.H2O, 124.6 mg ZnSO4.7H2O, 78.22 mg CuSO4.5H2O, 10 mg MoO3, pH 7.2) supplemented with 2 mM ACC (Sigma-Aldrich Co.,Mumbai, India.) as sole nitrogen source. Plates containing DF salts minimal medium without ACC served as negative control and with $(NH_4)_2SO_4$ (0.2% w/v) as positive control. The plates were incubated at 28°C for 72 h and growth of isolates was compared to negative and positive controls and was selected based on growth by utilizing ACC as nitrogen source.

Quantitative assay of ACC Deaminase

ACC deaminase activity (EC 4.1.99.4) was measured by the method of Honma and Shimomura (1978); Penrose and Glick (2003) with some modifications. This method measures the production of a-ketobutyrate and ammonia generated by the cleavage of ACC by the enzyme ACC deaminase. *Pseudomonas* isolates were grown in rich medium TSB (Trypticase soya broth) (5 ml) at 28°C until they reached stationary phase. The cells were collected by centrifugation, washed twice with 0.1 M Tris-HCl (pH 7.5), suspended in 2 ml of modified DF minimal medium supplemented with 2 mM of ACC. The culture was incubated at 28°C with shaking for another 36-72 h. The induced bacterial cells were harvested by centrifugation at 3,000 *g* for 5 min., washed twice with 0.1 M of Tris-HCl (pH 7.5), and resuspended in 200 µl of 0.1 M Tris-HCl (pH 8.5). The cells were labilized by adding 5% toluene (v/v) and then vortexed at the highest speed for 30 s. Fifty microlitres of labilized cell suspension was incubated with 5 µl of 0.3M ACC in an eppendorf tube at 28° C for 30 min. The negative control for this assay included 50 µl of labilized cell suspension without ACC, while the blank included 50 µl of 0.1 M Tris- HCl (pH 8.5) with 5 µl of 0.3 M ACC. The samples were then mixed thoroughly with 500 µl of 0.56 N HCl by vortexing and the cell debris was removed by centrifugation at 12, 000 *g*

for 5 min. A 500 µl aliquot of the supernatant was transferred to a glass test tube and mixed with 400 µl of 0.56N HCl and 150 µl of DNF solution (0.1 g 2,4-dinitrophenylhydrazine in 100 ml of 2N HCl); and the mixture was incubated at 28° C for 30 minutes. One ml of 2N NaOH was added to the sample prior to measuring the absorbance at 540 nm. The concentration of a -ketobutyrate in each sample was determined by comparison with a standard curve generated by preparing a-ketobutyrate solutions.

Characterisation for other plant growth promoting activity

Siderophore production

All the isolates were tested for other plant growth promoting activities. The siderophore production was determined quantitatively by performing the chrome azurol S (CAS) assay (Schwyn and Neiland 1987). Specific tests were carried out for identification of hydroxamate, and Catecholate types of siderophores following the standard methods (Arnow 1937). For selection of *Pseudomonas* isolates with high ability to produce siderophore, isolates were inoculated on King's B medium supplemented with a strong chelater 8- Hydroxyquinoline (50mg/L) (De Brito Alvarez and Gagne, 1995). The iron availability of this medium is too low and only the isolates with high ability of siderophore production can grow on this medium. Inoculated isolates were incubated at 28±2° C for 48-72hrs. Arnow's assay was used for quantification of catechole type of siderophore production. For qualitative estimation of siderophore quantification, actively growing cultures of *Pseudomonas* was inoculated to 20 ml King's B medium (Hi Media) in 50 ml Tarson tubes and incubated for 3 days at 28±2°C. The bacterial cells were removed by centrifugation at 3000 rpm for 5 min. Three ml of the culture supernatant was then mixed with 0.3 ml 5 N HCl solution, 1.5ml Arnow's reagent (10g $NaNO_2$, 10g $Na_2MoO_4.2H_2O$ dissolved in 50 ml distilled water) and 0.3ml 10 N NaOH. After 5-10 min the presence or absence of pink colour was observed and noted. Tetrazolium test is based on the capacity of hydroxamic acid to reduce tetrazolium salt by hydrolysis of hydroxymate groups using a strong alkali. The reduction and release of alkali shows red colour to a pinch of Tetrazolium salt when 1-2 drops of 2N NaOH and 0.1 ml of test sample is added. Instant appearance of a deep red colour indicated the presence of Hydroxamate siderophore. For $FeCl_3$ test one ml of the culture supernatant was mixed with freshly prepared 0.5 ml 2% aqueous $FeCl_3$ and observed for the presence and absence of deep red colour.For siderophore quantification, actively growing cultures of *Pseudomonas* was inoculated to 20 ml King's B broth (Hi Media) media in 100ml flasks and incubated for 3 days at 28±2°C. The bacterial cells were removed by centrifugation at 3000 rpm for 5 min. 0.5 ml of the culture supernatant was then mixed with 0.5 ml CAS solution and 10µl shuttling solution (sulfosalicyclic acid). After 20 mins of incubation, the colour obtained was determined using the spectrophotometer at 630 nm. Only King's B broth was used as blank while reference solution was prepared by adding CAS dye and shuttle solution to King's B and absorbance was recorded. Values of siderophore released in King's B was expressed in per cent siderophore units and calculated using the formula:

$$\left(\frac{Ar - As}{Ar}\right) \times 100$$

Where,

Ar = OD of reference solution,
As = OD of samples

Indole Acetic acid (IAA) production

For the production of indolic compounds, an active culture of *Pseudomonas* spp. was inoculated to 20 ml DF salts minimal media (Dworkin and Foster, 1958) in 100ml conical flasks and incubated for 3 days at 28±2°C. The medium was supplemented with L-Trp at a concentration of 1.02 g/L from a 5mM filter sterilized stock prepared in warm distilled water. After incubation for 72 hours, the grown bacterial cells were removed from the culture medium by centrifugation at 5,000 rpm for 5 min and the pH of the medium of all isolates was recorded. Then 1 ml of aliquot from the supernatant was mixed vigorously with 4 ml of Salkowski's reagent (Gordon and Webber, 1951) with blank as uninoculated DF salts minimal media. It was allowed to stand at RT for 20 min, before the absorbance at 535 nm was measured in colorimeter.

In vitro Phosphate Solubilization

Quantitative estimation of phosphate solubilisation was performed according to the method described by Murphy and Riley (1962). Isolates of *Pseudomonas* were inoculated to Pikovskaya broth medium (Himedia) in 100ml conical flasks and incubated for 5-7 days at 28±2°C. After incubation, the bacterial cells were removed from the culture medium by centrifugation at 5,000 rpm for 10 min and the pH of the medium of all isolates was recorded. Then 0.5 ml aliquot of the supernatant was mixed vigorously with 5 ml of chloromolybdic acid and 125 µl chlorostannous acid and allowed to stand at room temperature for 15-20 min. before the absorbance at 610 nm was measured in colorimeter. The standard was prepared from potassium dihydrogen phosphate (KH_2PO_4).

HCN production

The production of HCN compound was estimated by method of Wei. *et al.*, (1991). The cultures were grown on KM plates supplemented with 4.4 g/L glycine as a precursor molecule for hydrogen cyanide production and the filter paper strips soaked in saturated picric acid solution were exposed to the growing *Pseudomonas* isolates. The plates were incubated for 7 days at 28±2°C and observations were recorded as change in the colour of filter paper to brown as positive for HCN production.

Statistical analysis

On the basis of data derived from the carbon source utilization profiles, a matrix with binary code composing positive (1) and negative (0) values was made. Phenogram was constructed from the similarity matrices which were calculated using NTSYS (Numerical Taxonomy System Biostatistics) computer program on binary data of selected groups of primers detailed. Cluster analysis was done within the SAHN program by using UPGMA (unweighted pair-group method with arithmetic averages) method (Rohlf, 1998). The data were subjected to statistical analysis using WASP (Web Agri Stat Package) software (http://icargoa.res.in/wasp/ index.php). Critical difference at 0.01 & 0.05 level of significance was calculated for the observed values along with average and standard deviation. Mean values were compared by Duncan's multiple range test (Duncan,1955).

Result and Discussion

Biochemical Characterization

The isolates were characterised based on their utilization of various carbon sources, enzymes (proteases, gelatinases and lipases) production, nitrate reduction and sensitivity to antibiotics (Table 2). Isolates showed variability for traits such as gelatin

liquefaction, casein hydrolysis, lipolytic activity, nitrate reduction and antibiotic sensitivity test. Of the eleven fluorescent *Pseudomonas*, 3 isolates (27.27 %) showed proteolytic activity (casein hydrolysis) by inducing clear zones around the cells on skim milk agar medium, 9 isolates (81.81 %) showed lipolytic activity, 2 isolates (18.18%) were positive for gelatin liquefaction, 7 isolates (63.63 %) were negative for nitrate test and 4 isolates (36.36%) gave positive result for nitrate test (of which only P229 was positive before addition of zinc and 3 isolates P66, P111, and P153 showed positive response after addition of zinc).

In antibiotic sensitivity test all the isolates were resistant to antibiotic carbenicillin except P259 and P260. All isolates except P6, P141, P166, and P200 were found to be resistant to kanamycin. Isolate P229 was sensitive to both the antibiotics while P5, P66, P111 and P153 were resistant. Several strains within the family *Pseudomonadaceae* such as *P. putida* S12 show significant intrinsic resistance to multiple antibiotics (Kieboom and de Bont 2001)All the eleven isolates were citrate positive, whereas xylose, dextrose and mannose was utilised by all except P153 and P200. A varying degree of utilisation was observed for fructose, galactose, melibiose, L- arabinose, mannitol, xylitol, esculin hydrolysis, D-arabinose and malonate. None of the isolates were able to assimilate adonitol, arabitol, erythritol, a-methyl-D-gluconate, rhamnose, cellobiose, melezitose, a-methyl-D-mannoside, ONPG, D-arabinose, sorbose, maltose, sucrose, lactose, inulin, salicin, dulcitol, inositol, sodium gluconate, glycerol, raffinose, sorbitol, and trehalose.Numerical analysis of the biochemical data allowed the comparison among the isolates tested (Fig. 1). The similarity coefficient among the isolates ranged between 0.22 and 0.51. According to their level of similarity the isolates were distributed among three Phenons. Phenon 1, 2 and 3 consisted of 7 (P5, P6, P141, P166, P229, P259 and P260), 2 (P66 and P111) and 2 isolates (P153 and P 200) respectively. Grouping does not appear to be based on the quantity of ACC deaminase enzyme production by these isolates. Differential utilization of carbon sources by isolates of fluorescent *Pseudomonas* identified by Hi-carbohydrateTM kit test may play an important role in adapting to a variety of crop plants and soil types. Our results suggest that the ability of fluorescent pseudomonads to utilize specific organic substrates may be one important bacterial trait involved in the selection of soil borne fluorescent pseudomonads achieved by the plant.

Qualitative and quantitative assay of ACC Deaminase

Deamination of ACC into a-ketobutyrate and ammonia by the enzyme ACC deaminase was determined in qualitative and quantitative biochemical assay. All the eleven isolates were screened for ACC deaminase based on the enrichment method where ACC was used as the sole nitrogen source. All the isolates grew well on DF salt minimal medium supplemented with either ACC or ammonium sulphate serving as the sole nitrogen source which was compared with DF salt minimal medium without nitrogen source. Since all the isolates were able to utilise ACC in DF salts minimal media, and were assessed for their ACC-deaminase activity by quantifying the amount of a-ketobutyrate produced by the enzyme (Table 3). It was observed that isolates differed in their potential for ACC-deaminase activity. Highest ACC deaminase activity per hour was exhibited by P229 (43.3±0.60 μM a ketobutyrate/mg protein /h) followed by P200 (40.99±1.46 μM a ketobutyrate/mg protein /h) and P141 (40.88±0.18 μM a ketobutyrate/mg protein /h) while the lowest activity was observed in P5

(1.49±0.47 µM a ketobutyrate/mg protein /h). Ali et al. (2013) found that isolate SorgP4 utilized ACC as a sole source of nitrogen by the production of ACC deaminase enzyme and it showed the greater amount of ACC deaminase activity (3.71 ± 0.025 µM/mg protein/h of a-ketobutyrate) under non-stress and 1.42 ± 0.039 µM /mg protein/h of a-ketobutyrate under drought stress condition respectively. Similar results were obtained by Grichko & Glick (2000) where *Pseudomonas* ATCC17399/pRKACC strain produced significant amount of ACC deaminase enzyme (3.8 ±0.7 µM/mg protein/h of a-ketobutyrate). Ali *et. al*. 2013 found that isolate SorgP4 utilized ACC as a sole source of nitrogen by the production of ACC deaminase enzyme and it showed the greater amount of ACC deaminase activity (3.71 ± 0.025 µM/mg protein/h of a-ketobutyrate) under non-stress and 1.42 ± 0.039 µM /mg protein/h of a-ketobutyrate under drought stress condition respectively.

Characterisation for other plant growth promoting activity

Siderophore production

In soil, plant roots normally coexist with bacteria and fungi which may produce siderophores capable of sequestering the available soluble iron and hence interfere with plant growth and function. Siderophores are produced during extreme iron-depleted conditions for the solubilization of extracellular ferric iron by most bacteria and fungi. For selection of isolates with high ability to produce siderophore, isolates were inoculated on KMB supplemented with a strong chelater 8- Hydroxyquinoline. The isolates were categorized based on their growth on KMB medium supplemented with 8-Hydroxyquinoline as Luxuriant growing, medium growing and slow growing isolates. Luxuriant growth was exhibited by isolates P5, P66 and P111, while medium growing isolates were P6 and P260 and slow growing were P141, P153, P166, P229 and P259. Arnow's assay was performed to detect catechol type of siderophores. Colour change in the media was observed by only one isolate P153 indicating that only this isolate could produce catechol type of siderophore. All the isolates except P166 and P229 produced deep red colour on addition of Tetrazolium salt and NaOH which was used to test presence of hydroxamate type of siderophore, indicating that the isolates have capacity to reduce tetrazolium salt by hydrolysis of hydroxamate group in presence of strong alkali. $FeCl_3$ test was positive for all the isolates except P5, P6, P229 and P259. Only one isolate P153 gave positive response for Arnow's test, $FeCl_3$ test, tetrazolium test and HQ test (Table 4).
Carboxylate type of siderophore was determined by spectrophotometric test and the percentage of siderophore unit ranged from 22.37±1.31% to 80.15±0.15%. Among the eleven isolates, isolate P141 produced highest percent (80.15±0.15) of siderophore units followed by P66 (78.55±0.39%), P111 (77.63±0.26%) and P259 (75.79±2.63%). Minimum siderophore unit was observed for isolate P229 (22.37±1.31%) (Table 4). The role of siderophores in the control of diseases has been well documented by Baker et al. (1986).

Indole Acetic acid (IAA) production

The amount of IAA produced by different isolates of *Pseudomonas* in the media varied significantly which ranged from 1.5±0.18 to 16.55±0.04 µg/ml (Table 5). Efficacy of different isolates of fluorescent pseudomonads to produce IAA from L-tryptophan as precursors varied, the isolates P5 (13.86±0.28 µg/ml), P6 (9.14±0.28) µg/ml, P166 (1.78±0.47 µg/ml), P229 (16.55±0.04 µg/ml) and P260

(10.18±0.37 μg/ml) were identified as the high producer of IAA. The maximum production of IAA was observed for the isolate P229 (16.55±0.04 μg/ml) whereas P200 (1.5±0.18 μg/ml) was the lowest producer. Similar results were obtained by others (Narumiya *et al.*, 1979; Bano and Musarrat, 2003). Karnwal (2009) reported in his study that *Pseudomonas fluorescens* AK1 and *Pseudomonas aeruginosa* AK2 showed the best plant growth-promoting activity. These isolates were tested for

Fig. 1 Phenogram of fluorescent pseudomonads isolates based on their carbon source utilization profiles and various biochemical tests

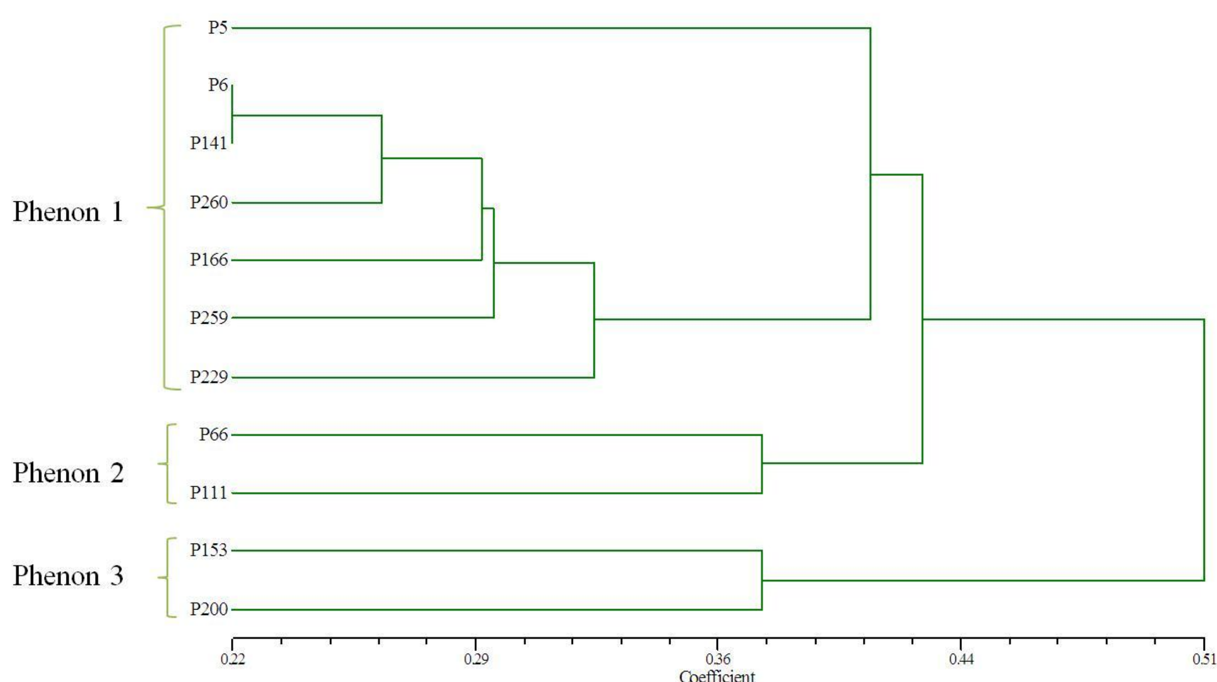

Table 1. Fluorescent *Pseudomonas* isolates used in the present study

S.No.	Isolates	Origin/Location
1	**P66**	Charama
2	**P141**	Kirda
3	**P200**	VIP road
4	**P229**	Virigpal, Jungle
5	**P260**	Brinjal, Kuteshwar
6	**P5**	Fenugreek, IGKV Horticulture, Raipur
7	**P6**	Cashew, IGKV Horticulture, Raipur
8	**P111**	Jaisekara
9	**P153**	Kurud (rice gram field)
10	**P259**	Brinjal, Kuteshwar
11	**P166**	Purur

Table 2. Distinct phenotypic characteristics revealed by fluorescent *Pseudomonas* isolates

Tests	5	6	66	111	141	153	166	200	229	259	260
1	-	-	-	-	-	-	-	-	-	-	-
2	+	+	+	+	+	-	+	-	+	+	+
3	-	-	-	-	-	-	-	-	-	-	-
4	+	-	-	-	-	-	-	-	-	-	-
5	+	+	+	+	+	-	+	-	+	+	+
6	-	+	+	-	+	-	+	-	+	+	+
7	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	-
9	-	+	+	+	+	-	+	-	+	+	+
10	-	-	-	-	-	-	-	-	-	-	-
11	-	-	+	+	+	-	+	-	+	-	+
12	+	+	+	+	+	-	+	-	+	+	+
13	-	-	-	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-	-	-	-	-
17	-	-	-	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-	-	-	-
19	-	-	-	-	-	-	-	-	-	-	-
20	+	-	-	-	-	-	-	-	-	-	-
21	-	-	-	-	-	-	-	-	-	-	-
22	-	-	-	-	-	-	-	-	-	-	-
23	-	-	-	-	-	-	-	-	-	-	-
24	-	-	-	-	-	-	-	-	-	-	-
25	-	-		-	-	-	-	-	-	-	-
26	-	-	-	-	-	-	-	-	-	-	-
27	-	-	-	-	-	-	-	-	-	-	-
28	-	-	-	-	-	-	-	-	-	-	-
29	+	+	+	-	+	-	+	-	+	+	+
30	-	-	-	-	-	-	-	-	-	-	-
31	-	-	+	+	-	+	+	-	-	-	-
32	+	+		+	+	-	-	-	+	-	+
33	+	+	+	+	+	+	+	+	+	+	+
34	+	+	+	-	-	-	+	-	-	+	-
35	-	-	-	-	-	-	-	-	-	-	-
36	R	R	R	R	R	R	R	R	S	S	S
37	R	S	R	R	S	R	S	S	S	R	R
38	-	-	NA	NA	-	NA	-	-	+	-	-
39	-	-	+	+	-	+	-	-	NA	-	-
40	-	-	+	+	-	+	-	-	-	-	-
41	-	-	+	-	-	+	-	-	-	-	-

42	+	+	+	-	+	-	+	+	+	+	+

Different tests: 1: Lactose; 2: Xylose; 3: Maltose; 4: Fructose; 5: Dextrose; 6:Galactose; 7: Raffinose; 8: Trehalose; 9: Melibiose; 10: Sucrose; 11: L-Arabinose; 12: Mannose; 13:Inulin; 14: Sodium gluconate; 15: Glycerol; 16: Salicin; 17: Dulcitol; 18: Inositol; 19: Sorbitol; 20: Mannitol; 21: Adonitol; 22: Arabitol; 23: Erythritol; 24: α-Methyl-D-mannoside; 25: Rhamnose; 26: Cellobiose; 27: Melezitose; 28: α-Methyl-D-mannoside; 29:Xylitol; 30: ONPG; 31: Esculin hydrolysis; 32: D-arabinose; 33: Citrate utilization; 34: Malonate utilization; 35: Sorbose; 36: Carbenicillin sensitivity; 37: Kanamycin sensitivity; 38: Nitrate test (Before adding Zn) 39: Nitrate test (After adding Zn) 40: Casein hydrolysis; 41: Gelatin hydrolysis ; 42: Lipase test; +: positive reaction; -: negative reaction; R: resistant; S: susceptible; NA: not applicable

Table 3. ACC deaminase activity of fluorescent *Pseudomonas* isolates

S.No.	Isolates	µM α ketobutyrate/mg protein /h
1	**P66**	39.69^{b}±0.77
2	**P141**	40.88^{b}±0.18
3	**P200**	40.99^{b}±1.46
4	**P229**	43.3^{a}±0.60
5	**P260**	35.63^{c}±0.17
6	**P5**	1.49^{e}±0.47
7	**P6**	$2.9d^{e}$±0.30
8	**P111**	3.99^{d}±0.09
9	**P153**	3.02^{de}±0.26
10	**P259**	3.89^{d}±0.06
11	**P166**	3.85^{d}±0.05
	Max	43.3^{a}±0.60
	Min	1.49^{e}±0.47
	CV	4.06
	CD0.01	2.48
	CD0.05	1.76
	Fcal	1171.37**

*Values are average of 2 replications; values after± represents standard error; CV=coefficient of variance; CD= critical difference; **Values are significant at 1% and 5% levels; As per Duncan's grouping means with the same letter are not significantly different*

Table 4. Production of various siderophores by fluorescent *Pseudomonas* isolates

S.No.	Isolates	siderophore units (%)	Arnow's test	$FeCl_3$ test	Tetrazolium test	HQ test
1	**66**	78.55^{ab}±0.39		Positive	Positive	+++
2	**141**	80.15^{a}±0.15		Positive	Positive	+
3	**200**	49.63^{d}±2.10		Positive	Positive	+

4	**229**	$22.37^{f}\pm1.31$				+
5	**260**	$71.05^{c}\pm2.63$		Positive	Positive	++
6	**5**	$53.55^{d}\pm0.65$			Positive	+++
7	**6**	$73.29^{bc}\pm4.86$			Positive	++
8	**111**	$77.63^{ab}\pm0.26$		Positive	Positive	+++
9	**153**	$74.34^{abc}\pm0.65$	Positive	Positive	Positive	+
10	**259**	$75.79^{abc}\pm2.63$			Positive	+
11	**166**	$60.3^{e}\pm0.20$		Positive		+
	Max	$80.15^{a}\pm0.15$				
	Min	$22.37^{f}\pm1.31$				
	CV	4.59				
	CD0.01	8.94				
	CD0.05	6.34				
	Fcal	96.01				

*Values are average of 2 replications; values after± represents standard error; CV=coefficient of variance; CD= critical difference; **Values are significant at 1% and 5% levels; As per Duncan's grouping means with the same letter are not significantly different*

Table 5. Quantification of Indole Acetic acid production by Fluorescent *Pseudomonas*

S.No.	Isolate no.	pH of the minimal medium 3DAI	IAA Production (µg/ml)
1	**66**	7.36	$5.55^{e}\pm0.471$
2	**141**	7.59	$5.85^{e}\pm0.75$
3	**200**	6.78	$1.78^{f}\pm0.47$
4	**229**	7.7	$16.55^{a}\pm0.04$
5	**260**	7	$10.18^{c}\pm0.37$
6	**5**	7.59	$13.86^{b}\pm0.28$
7	**6**	7.69	$9.14^{cd}\pm0.28$
8	**111**	7.34	$1.78^{f}\pm0.47$
9	**153**	7.23	$1.5^{f}\pm0.18$
10	**259**	7.59	$8.29^{d}\pm0.37$
11	**166**	7.62	$9.70^{c}\pm0.09$
	Max.		$16.55^{a}\pm0.04$
	Min.		$1.5^{f}\pm0.18$
	CV		7.32
	CD0.01		1.74
	CD0.05		1.23
	Fcal		156.33

*Values are average of 2 replications; values after± represents standard error; CV=coefficient of variance; CD= critical difference; **Values are significant at 1% and 5% levels; As per Duncan's grouping means with the same letter are not significantly different*

Table 6 ***In vitro*** **Phosphate solubilisation and HCN production by Fluorescent** ***Pseudomonas***

S.No.	Isolate no.	pH of the Pikovskaya's medium 7 DAI	Phosphate solubilized (µg/ml)	HCN production
1	P66	3.61	862.5[e]±6.25	
2	P141	3.72	562.5[j]±6.25	
3	P200	4.12	793.75[g]±12.5	
4	P229	3.48	1208.12[c]±1.87	
5	P260	3.67	825[f]±6.25	
6	P5	3.78	753.12[h]±3.12	
7	P6	3.6	2100[a]±6.25	
8	P111	3.71	645.62[i]±1.87	
9	P153	3.2	1368.75[b]±12.5	
10	P259	3.7	750[h]±6.25	
11	P166	3.52	1162.5[d]±6.25	Positive
	Max.	2100[a]±6.25		
	Min.	562.5j±6.25		
	CV	1		
	CD0.01	31.44		
	CD0.05	22.28		
	Fcal	3801.72		

*Values are average of 2 replications; values after± represents standard error; CV=coefficient of variance; CD= critical difference; **Values are significant at 1% and 5% levels; As per Duncan's grouping means with the same letter are not significantly different*

their ability to produce indole acetic acid in pure culture in the absence and presence of L-tryptophan at 50, 100, 200 and 500 µg/ml. Inoculation of canola seeds with *Pseudomonas putida* GR12-2, which produces low levels of IAA, resulted in 2 to 3 fold increase in the length of seedling roots (Glick *et al*., 1986; Caron *et al*., 1995). Ahamad et al. (2005) reportedmthat 11 isolates of pseudomonads from different cropmplants produced IAA without tryptophan in the range 5.34 to 22.4 mg/ml.

In vitro Phosphate Solubilization

Phosphorus frequently is the least accessible macronuntrient in many ecosystems and its low availability is often limiting to plant growth (Raghothama 1999). *In vitro* quantitative phosphate solubilization efficacy of different fluorescent pseudomonad isolates differed significantly both at 0.01 and 0.05 level amongst the isolates (Table 6). Quantitative estimation of soluble phosphate concentrations in Pikovskaya's broth was expressed as µg/ml and it ranged from 562.5±6.25 to 2100±6.25 µg/ml. The lowest value was observed for isolate P141 (562.5±6.25 µg/ml) and highest for isolate P6 (2100±6.25 µg/ml). Phosphate solubilization by isolates P153 (1368.75±12.5 µg/ml), P229 (1208.12±1.87 µg/ml), and P166 (1162.5±6.25 µg/ml) were significantly higher than all the other isolates. These candidate isolates can be used as microbial inoculants to improve soil fertility by releasing bound phosphorus

thereby increasing the crop yield potential. The production of organic acids and acid phosphatases play a major role in the mineralization of organic phosphorous in soil. Stimulation of different crops by plant growth promoting *Pseudomonas* isolate (s) with potential phosphate solubilization ability may help in exploiting large reserves of phosphorus present in most agricultural soils. Several *Pseudomonas* species have been reported among the most efficient phosphate-solublizing bacteria and as important bio-inoculants due to their multiple biofertilizing activities of improving soil nutrient status, secretion of plant growth regulators and suppression of soil-borne pathogens (Rodriguez and Fraga 1999; Gulati et al. 2008; Vyas et al. 2009).

HCN production

In the present investigation eleven isolates of different fluorescent pseudomonads were screened for its ability to produce hydrogen cyanide using glycine as its precursor molecule. The KMB plates were supplemented with 4.4 g/L glycine, incubated for 7 days at 28±2°C and observations were measured as colour of filter paper strips soaked in saturated picric acid solution turning to brown measured as positive for HCN production. Out of the 11 isolates only P166 exhibited positive response for its ability to produce HCN (Table 6). Microbial cyanogenesis has been demonstrated in many species of fungi (Hutchinson *et al*., 1973), but only in a few species of bacteria in the genera Chromobacterium and *Pseudomonas* (Patty, 1921). Glycine has usually been the indicated precursor of cyanide in fungi and bacteria (Brysk *et al*., 1969; Ward *et al*., 1971; Wissing *et al*., 1974). Microbial production of HCN has been reported as an important antifungal trait to control root infecting fungi (Ramette et al., 2003).

Conclusion

It is evident from the present study that the fluorescent pseudomonads under investigation are capable of producing plant growth promoting substances and ACC deaminase enzyme which is responsible to help the plants to resist abiotic and biotic stress conditions. These isolates can be tested under field conditions and the potential candidate can be used for the development of bioinoculants for crop plants under normal and stress conditions.

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