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CHARACTERIZATION OF FLUORESCENT *PSEUDOMONAS* STRAINS FOR THEIR ACC DEAMINASE AND KMBA PATHWAYS

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ABSTRACT

Present work deals with the characterization of isolated fluorescent pseudomonads based on their ability to utilize an unusual nitrogen source (e.g. 1-aminocyclopropane-1-carboxylate (ACC), L- methionine, α-keto γ-methylthiobutyric acid, KMBA) or other xenobiotic compound which provide a competitive advantage over other microorganisms in the rhizosphere. Most of isolated fluorescent pseudomonad showed the utilization of L- methionine as nitrogen source, while some rhizobacterial strain have utilized ACC as nitrogen source and only few strains e.g. G13, G29, G35, H4 and H9 showed the ability to utilize both ACC and L-MET as a nitrogen source and proved that both pathway is functional in these organisms. Most of isolated fluorescent pseudomonad showed the ability of ethylene production from deamination of L- methionine to produce α-keto γ-methylthiobutyric acid (KMBA). ACC deaminase activity was quantified in the strains and showed ACC deaminase activity at the range of 1-21 ug of α-ketobutyrate/mg of protein. Isolated fluorescent pseudomonad has shown high indole acetic acid (IAA) production at the range of 33-43µg/ml. Strain G29 has shown good ACC deaminase production as well as possess both L-methionine and ACC utilization pathway and thus it enhance the survival ability of these strains in the rhizosphere. So these strains could be considered as good PGPR strains for the application.

KEY WORDS: 1-aminocyclopropane-1-carboxylate, L- methionine, α-keto γ-methylthiobutyric acid, Indole acetic acid, Fluorescent pseudomonad

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INTRODUCTION

The soil contains a large number and variety of microorganisms and strains that are able to utilize an unusual carbon or nitrogen source such as an ACC or a xenobiotic compound should be able to proliferate and then persist longer than other microorganisms, especially in soils that contain these unusual compounds. For example, the ability of some bacteria to cleave ACC to ammonia and α-ketobutyrate may provide these strains with a competitive advantage over other microorganisms in the rhizosphere root and seed exudates contain a significant amount of ACC [1,2] because they can use ACC as a source of nitrogen [3]. The 1-aminocyclopropane-1-carboxylate (ACC) deaminase produced by some soil bacteria that catalyzes the degradation of ACC, a precursor of ethylene, as their source of nitrogen [4]. ACC degradation will ultimately reduce ethylene biosynthesis in the plant A number of plant growth-promoting bacteria contain the enzyme 1-aminocyclopropane- 1-carboxylate (ACC) deaminase, and this enzyme can cleave the plant ethylene precursor ACC and thereby lower the level of ethylene in a developing or stressed plant [5]. Plant growth promoting bacteria that contain the enzyme ACC deaminase, when bound to a plant root or to the seed coat of a developing seedling, may act as a mechanism for insuring that the ethylene level within the plant's tissues does not become elevated to the point where root (or shoot) growth is impaired. By facilitating the formation of longer roots and shoots, these bacteria may enhance the survival of some seedlings, especially during the first few days after the seeds are planted. Degradation of the ethylene precursor ACC by bacterial ACC deaminase releases plant stress and rescues normal plant growth under drought and salt stresses [6]. Production of IAA by PGPR increase root length, root surface area and the number of root tips, leading to enhanced uptake of nitrate and phosphorous

ACC deaminase in microorganisms

This enzyme was first isolated from *Pseudomonas* sp. strain ACP [7] and since then, it has been detected in a wide range of microbes including the fungus *Penicillium citrinum* [5], the yeast *Hansenula saturnus* [8] and a large number of bacteria including *Rhizobium leguminosarum* bv. *viciae*, *Rhizobium hedysari*, and *Mesorhizobium loti* [9]. ACC deaminase is a multimeric enzyme with a monomeric subunit molecular mass of approximately 35–42 kDa It is a sulfhydryl enzyme.While several D-amino acids, notably D-serine and D-cysteine can act as substrates for ACC deaminase (albeit less efficiently than ACC), L-serine and L-alanine are effective competitive inhibitors of the enzyme. ACC deaminase catalyzes a cleavage of ACC that includes cyclopropane ring fragmentation, and deamination of ACC to form a-ketobutyrate and ammonia. Despite the fact that its substrate ACC is plant produced, in those instances where it has been examined ACC deaminase is not a secreted enzyme. Rather, it is localized within the cytoplasm of the microorganism that produces it [10]. In this case, the substrate ACC is exuded by plant tissues [11, 12] and is then taken up by ACC deaminase containing microbe [12]. *Pseudomonas sp*. strain ACP and the yeast *Hansenula saturnus* are capable of utilizing the cyclopropanoid amino acid 1-aminocyclopropane-1-carboxylate (ACC) as a nitrogen source owing to induction of the enzyme ACC deaminase in these organisms [13].

Pathway of ethylene production via methionine

The first step in pathway of C_2H_4 biosynthesis in higher plants is the conversion of MET to S-adenosyl-methionine (SAM), catalyzed by methionine adenosyl transferase. Second step is conversion from SAM to ACC. From ACC it is converted to C2H4. The reaction is catalyzed by an enzyme known as ethylene forming enzyme [14, 15]. The ability of plant tissues to produce C_2H_4 depends on the presence of ACC [15], which may imply that the magnitude of C_2H_4 production by a plant tissues is regulated mainly by the availability of the substrate ACC. Methionine adenosyltransferase (ATP

methionine *S*-adenosyltransferase, catalyzes the conversion of methionine into *S*-adenosylmethionine (SAM), reaction 1; 1-Aminocyclopropane-1-carboxylate synthase (*S*-adenosyl- L-methionine methylthioadenosine lyase, catalyzes the synthesis of 1-aminocyclopropane-1-carboxylic acid (ACC) from SAM, reaction 2; ACC is converted to ethylene by ACC oxidase, reaction 3 [14,16]. The second pathway involves α- keto-γ-methylthiobutyric acid as an intermediate in production of ethylene.

Mechanism of plant root elongation by rhizobacteria that possess ACC deaminase

Ethylene is biosynthesized in higher plants from methionine via S-adenosylmethionine and ACC [11, 16]. The rate-limiting step in ethylene biosynthesis is the production of ACC by a ring-closing displacement of methylthioadenosine from S-adenosylmethionine in a reaction catalyzed by ACC synthase [11]. IAA produced in PGPR allows promotion of ACC synthase to produce ACC and is converted to ethylene by ACC oxidase. Ethylene produced in high amount would be deleterious for root elongation.

Mechanism of action of ACC Deaminase

The plant growth-promoting bacteria bind to the surface of either the seed or root of a developing plant; in response to tryptophan and other small molecules in the seed or root exudates, the plant growth-promoting bacteria synthesize and secrete the auxin indoleacetic acid (IAA), some of which is taken up by the plant. This IAA together with endogenous plant IAA, can stimulate plant cell proliferation and elongation, or it can induce the activity of ACC synthase to produce ACC. Some of the plant's ACC will be exuded along with other small molecules such as sugars, organic acids and amino acids. The exudates may be taken up by the bacteria and utilized as a food source of the rhizosphere bacteria. ACC may be exuded together with the other components of the root or seed exudates [2]. ACC may be cleaved by ACC deaminase and compounds produced that are readily further metabolized by the bacteria. The presence of the bacteria induces the plant to synthesize more ACC than it would otherwise need and also, stimulates the exudation of ACC from the plant (some of which may occur as a consequence of plant cell wall loosening caused by bacterial IAA). Thus, plant growth promoting bacteria are supplied with a unique source of nitrogen in the form of ACC that enables them to proliferate/survive under conditions in which other soil bacteria may not readily flourish. And, as a result of acting as a sink for ACC and lowering its level within the plant, the amount of ethylene that is produced by the plant is also reduced. Although it is entirely speculative at this point, the expression of ACC deaminase genes within nitrogen fixing nodules might decrease the rate of nodule senescence– as nitrogen fixation with its high energy demand could activate stress ethylene synthesis – and thereby effectively increase the amount of fixed nitrogen. The affinity of an enzyme for a particular substrate (i.e., the Km value) reflects more than the tightness of substrate binding, rather it has a profound effect on the kinetics of conversion of substrate into product [17]. When the Km values for the binding of ACC by ACC deaminase were determined for enzyme extracts of several different microorganisms at pH 8.5, the values ranged from 1.5 to 17.4 mM, indicating that the enzyme does not have a particularly high affinity for ACC[17]. There are two significant consequences of the low affinity of ACC deaminase for ACC. First, because the enzyme ACC oxidase (which catalyzes ethylene formation from ACC) has a much greater affinity for ACC than does ACC deaminase, the only way that ACC deaminase can effectively compete with ACC oxidase for ACC and thereby lower plant ethylene levels is for the amount of ACC deaminase to be much greater than the amount of ACC oxidase. This is likely to often be the case since ACC oxidase is an induced enzyme that is normally present in no senescent and non-stressed tissues in only very low levels. Second, since plant ACC levels are typically in the mM range and the Km is in the mM range, Michaelis–Menton kinetics indicate that every increase in ACC concentration will be accompanied by a parallel increase in the rate of ACC cleavage (i.e., when

Km > S then v ∞ S), independent of the level of enzyme present. Thus, the enzyme will immediately respond to a 2- to 3-fold increase in ACC levels (e.g., following environmental stress) by increasing, by 2-to 3-fold, the rate of conversion of ACC to ammonia and a-ketobutyrate.

Role of ACC deaminase on stress plants

Treatment of plant seeds or roots with ACC deaminase- containing bacteria typically reduces ACC and ethylene levels about 2- to 4-fold [2]. ACC and/or ethylene levels are generally reduced to a similar extent in transgenic plants that express a bacterial ACC deaminase under the control of either the 35S (constitutive) or rolD (root-specific) promoter [18], although ethylene levels have been reported to be decreased by more than 95% in some ripening transgenic tomato fruit [19]. Notwithstanding the often small reduction in ACC and ethylene levels, the protection afforded stressed plants through the action of ACC deaminase is often quite dramatic.

ACC Deaminase-Containing PGPB Decrease plant Stress Ethylene levels

Treatment of plant seeds or roots with ACC deaminase-containing bacteria typically reduces ACC and ethylene levels about 2- to 4-fold. Notwithstanding the often small reduction in ACC and ethylene levels, the protection afforded stressed plants through the action of ACC deaminase is often quite dramatic (and depending on the plant utilized and its age, ACC deaminase containing plant growth-promoting bacteria can reduce growth inhibition by 25-500%. For example, in laboratory experiments, treatment of a variety of plants (tomato, canola, lettuce, Indian mustard and tomato) with ACC deaminase-containing plant growth promoting bacteria protects the plants against damage from cold temperatures, drought [20], flooding [18], high salt [20], phytopathogens, polyaromatic hydrocarbons and several different metals including nickel [13], copper and lead [13]. Most of the experiments where a protective effect of ACC deaminase-containing plant growth-promoting bacteria on plant biomass and growth has been demonstrated have been conducted in either a greenhouse or growth chamber setting. Most of the experiments in which ACC deaminase-containing plant growth-promoting bacteria have been shown to reduce growth inhibition by various stressors were conducted with (ethylene sensitive) dicots including canola, tomato, tobacco, Indian mustard and mung bean. These bacteria are also effective (although to a lesser extent) with monocots such as wheat, rye and rice (all of which are somewhat less ethylene sensitive than the dicots). Present work dealt with the characterization of fluorescent pseudomonad strains for their IAA production and their ability to utilizing ACC as a N-source. Also the estimation of ACC deaminase activity and checking the presence of ACC/KMBA pathway in these strains.

MATERIALS AND METHODS

Identification bacterial cultures by biochemical methods

For the identification of fluorescent pseudomonad biochemical test like catalase test, oxidase Test, Hugh-Leifson´s Oxidation – Fermentation test, Gram staining and arginine dihydrolase test was performed using protocol as described in Bergey´s manual.

Identification bacterial cultures by molecular methods

Modified CTAB method was used for the extraction of genomic DNA. 1.5 ml of overnight grown cultures was centrifuged at 10,000 rpm for 5 minutes at 4° C. The supernatant was drained off and the pellet was resuspended in 200 µl of T. E. Pellet was vortexes vigorously to resuspend the pellet and then was kept at 60°C for 30 minute. 100µl of 3M NaCl was added to it followed by 80µl of 10% CTAB. It was mixed properly and then again kept at 60°C for 10 minutes. Equal volume of phenol-cholorform-isoamyl alcohol (25:24:1) was added and centrifuged at 10, 000 rpm for 12 minutes at 4°C. Aqueous phase was collected and 2-3 volume of chilled 100 % ethanol was added and kept for 1 hour. Further it was centrifuged at 10,000 for 10 minutes at 4°C and supernatant

was drained off and the pellet was again washed with 70% ethanol. The ethanol was allowed to evaporate and dried DNA was resuspended in sterile distilled water. Identification of fluorescent pseudomonad by molecular method involved the amplification of region including the 3' half of the 16S rDNA with the whole 16S-23S rRNA Internal Transcripted Spacer (ITS) sequence using specific primers (21]. Primer sequences ITS1F- 5'- AAGTCGTA ACA AG GTAG -3' and ITS2R- 5'- GACCATATATAACCCCAAG – 3' was used to get amplicon size of 560 bp.

IAA production by plant growth promoting rhizobacteria isolates

IAA estimation was done using Salkowski method[22].Minimal Media(g/100ml)-Na_2HPO_4 0.6g,KH_2PO_4 - 0.3g,NaCl - 50mg,NH_4Cl- 0.1g and D/W -100ml,Autoclaved at 10psi for 20 minutes.1M MgSO4-0.2ml,20% Glucose-2ml,0.1M $CaCl_2$ - 100µl, 1M MgSO4, 20% Glucose and 0.1 M CaCl2 were prepared separately and autoclaved at 10 psi for 20 minutes. And then were added according to need.IAA production was detected by the modified method as described by Brick et al., 1991 [23]. Quantitative analysis of IAA was performed using the method of Loper and Scroth [24] at the 50µg/ ml concentration of tryptophan. Cultures incubated for 48 hrs at 28°C on rotary shaker, centrifuged at 10,000g for 15minutes. The supernatent (2 ml) was mixed with two drops of orthophosphoric acid and 4 ml of Salkowski reagent (50ml, of 35% perchloric acid, 1ml of 0.5% FeCl3). Samples were incubated for 25 minutes at RT. Optical density was taken at 530nm with the help of spectrophotometer. IAA stock solution was prepared as 100 µg/ml in 50% ethanol. Concentration of IAA produced by cultures was measured with the help of standard graph of IAA (Hi-media) obtained in the range of 10-100µg/ ml.

Measurement of ACC deaminase activity

ACC deaminase activity was assayed according to the method of Honma and Shimomura[25] which measures the amount of *a*-ketobutyrate when the enzyme, ACC deaminase, cleaves ACC. The number of nmoles of *a*-ketobutyrate produced by this reaction is determined by comparing the absorbance at 540 nm of a sample to a standard curve of *a*- ketobutyrate ranging between 0.1 and 1.0 nmol. A stock solution of 100 mM *a*-ketobutyrate (Sigma-Aldrich Co.) was prepared in 0.1 M Tris-HCl pH 8.5 and stored at 4 °C. Just prior to use, the stock solution was diluted with the same buffer to make a 10-mM solution from which a standard concentration curve is generated. Each in a series of known *a*-ketobutyrate concentrations was prepared in a volume of 200 *u*l and transferred to a glass test tube (100x13 mm); each point in the series is assayed in duplicate. Three hundred *u*l of the 2,4-dinitrophenylhydrazine reagent (0.2 % 2,4-dinitrophenyl-hydrazine in 2 N HCl; Sigma-Aldrich Co.) was added to each glass tube and the contents were vortexed and incubated at 30 °C for 30 min during which time the *a*-ketobutyrate was derivatized as a phenylhydrazone. The color of the phenylhydrazone was developed by the addition of 2.0 ml of 2 N NaOH; after mixing, the absorbance of the mixture was measured at 540 nm. ACC deaminase activity was measured in bacterial extracts prepared in the following manner. Bacterial cell pellets, were each suspended in 1 ml of 0.1 M Tris-HCl, pH 7.6 and transferred to a 1.5-ml microcentrifuge tube. The contents of the 1.5-ml microcentrifuge tube were centrifuged at 16,000 x *g* for 5 min in a microcentrifuge and the supernatant was removed with a fine-tip transfer pipette. The pellet was suspended in 600µl of 0.1 M Tris-HCl, pH 8.5. 30µl of toluene was added to the cell suspension and vortexed at the highest setting for 30 s. 100µl aliquot of the "toluenized cells" was kept aside and stored at 4 °C for protein assay at a later time. The remaining toluenized cell suspension was immediately assayed for ACC deaminase activity.

Assay of ACC Deaminase activity

ACC deaminase assay was performed as per Glick et al, 2005[17]. All sample measurements was carried out in duplicate. Two hundred µl of

the toluenized cells was placed in a fresh 1.5-ml micro centrifuge tube; 20µl of 0.5M ACC was added to the suspension, briefly vortexed, and then incubated at 30 °C for 15 min. Following the addition of 1 ml of 0.56 N HCl, the mixture is vortexed and centrifuged for 5 min at 16,000 x *g* in a microcentrifuge at room temperature. One ml of the supernatant was vortexed together with 800µl of 0.56 N HCl in a clean glass tube (100x13 mm). Thereupon, 300 *µ*l of the 2,4-dinitrophenylhydrazine reagent (0.2 % 2,4-dinitrophenylhydrazine in 2 N HCl) was added to the glass tube, the contents vortexed and then incubated at 30 °C for 30 min. Following the addition and mixing of 2 ml of 2 N NaOH, the absorbance of the mixture was measured at 540 nm. The absorbance of the assay reagents including the substrate, ACC, and the bacterial extract were taken into account. After the indicated incubations, the absorbance at 540 nm of the assay reagents in the presence of ACC was used as a reference for the spectrophotometric readings; it is subtracted from the absorbance of the bacterial extract plus the assay reagents in the presence of ACC. The contribution of the extract, i.e., the absorbance at 540 nm of extract and the assay reagents without ACC, is determined and subtracted from the absorbance value calculated above. This value was used to calculate the amount of *a*-ketobutyrate generated by the activity of ACC deaminase.

Characterization of fluorescent pseudomonad strains for the presence of KMBA pathway

The presence of KMBA was determined by precipitation with 2,4-dinitrophenylhydrazine [26].The culture medium was separated from the mycelium by filtration, 1 ml of 0.1% 2,4-dinitrophenylhydrazine in 2 M HCl was added to 10 ml of culture filtrate and stirred at room temperature in total darkness. In the presence of KMBA a yellow precipitate will formed after 30 min.

Figure 1

KMBA pathway specific precipiatation by fluorescent pseudomonad strains

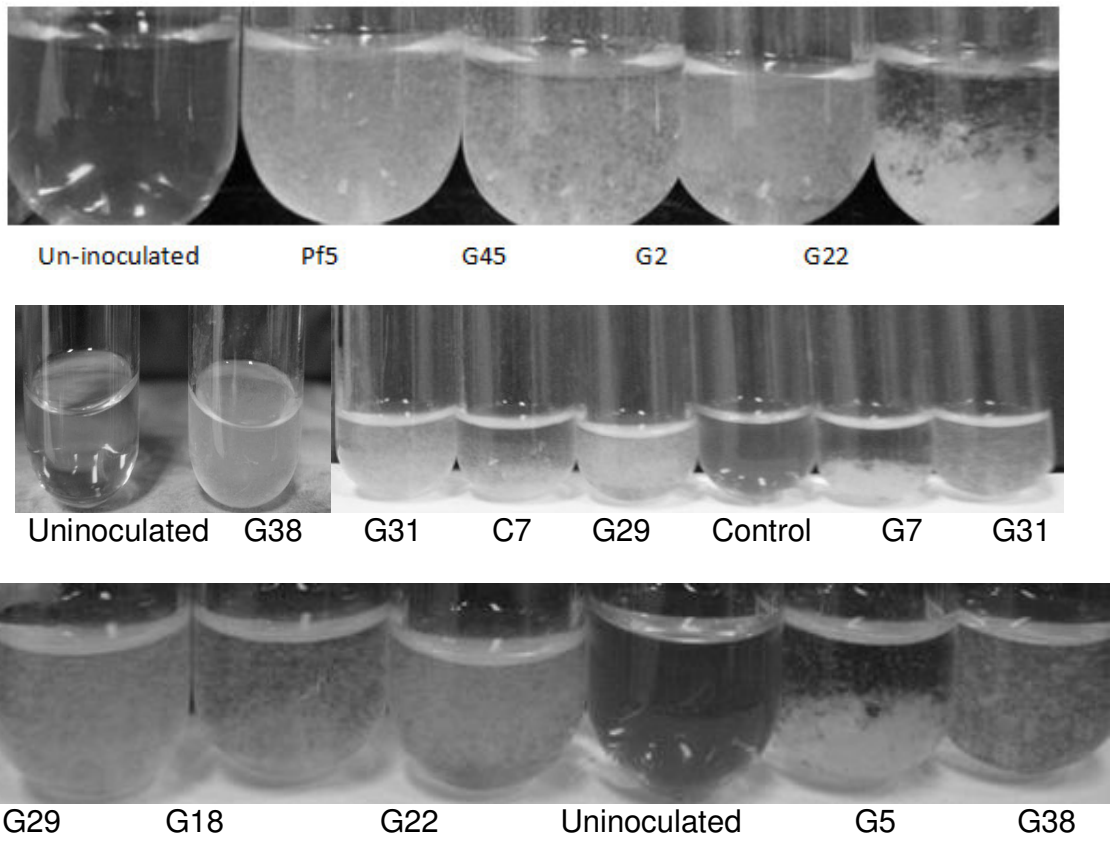

Table 1
IAA Production by rhizospheric fluorescent pseudomonads

Strains	IAA production without Tryptophan (ug/ml)	IAA production with tryptophan(ug/ml)
G45	30.64 ± 0.065	35.05 ± 0.032
G18	29.13 ±0.049	37.95 ±0.050
Pf-5	28.38 ± 0.045	33.76 ±0.011
G31	27.95 ±0.051	35.80 ±0.0175
G35	27.73 ±0.017	36.12 ±0.03
G25	27.63 ±0.047	43.01 ±0.0217
CHAO	27.52 ±0.032	34.73 ±0.011
G2	27.41 ±0.03	34.62 ±0.026
G20	27.09 ±0.02	36.77 ±0.081
G46	26.99 ±0.090	34.40 ±0.057
H4	26.23 ±0.020	34.40 ±0.057
G19	25.69 ±0.0040	36.93 ±0.0210

Table 2
Growth pattern of PGPR strains on the medium containing ACC as a sole nitrogen source.

Strong ACC utilizers			
STRAINS	ACC as a N source	NH4Cl as a N source	Without N source
G35	+++	+++	-
G19	+++	+++	-
P33	+++		
P36	+++	++	-
P35	+++	+++	-
Moderate ACC utilizers			
G29	++	+++	+
KH1	++	+	-
NR2	++	++	-
Weak ACC utilizers			
STRAINS	ACC as a N source	NH4Cl as a N source	Without N source
Pf-5	+	+	-
G14	+	+	-
G13	+	++	-
G46	+	+	-
H9	+	+	-

Table 3
Ability of PGPR strains to grow on medium containing ACC / L-MET as N-Source

Strains	ACC	L-Methionine
Pf-5	+	+
C7	-	+
G8	-	+
CHAO	-	+
C2	-	+
G45	-	+
H9	+	+
G29	++	+
G13	+	+
G16	-	+
G18	-	+
G38	-	+
G35	+	+
G2	-	+
G22	-	+
G5	-	+
H4	+	+
G31	-	+
G7	-	+

RESULTS AND DISCUSSION

Identification of strains by biochemical and molecular methods

Fluorescent pseudomonad was identified by fluorescent pseudomonad specific biochemical (catalase, oxidase, oxidative/fermentative and arginine dihydrolase test) and molecular identification methods (ITS amplification). All strains were gram negative and rod shaped and has showed amplification of 560 bp using fluorescent pseudomonad specific ITS primers.

Characterization of PGPR for Indole acetic acid (IAA) production

IAA is essential for intial growth of root and shoot apical meristems but the effects of IAA on plant seedlings are concentration dependent, i. e. low concentration may stimulate growth while high concentrations are inhibitory [27].Exogenous IAA is known to increase activity of ACC synthase [28] which catalyzes production of ACC in plants. From tryptophan, indole pyruvic acid is produced via tryptophan transaminase enzyme which is the first step in IAA biosynthetic pathway. Indolepyruvic acid is catalyzed by indolepyruvate decarboxylase for indole acetic acid production. IAA secreted by a bacterium may promote root growth directly by stimulating plant cell elongation or cell division or indirectly by influencing bacterial ACC deaminase activity. It is possible to say IAA and ACC deaminase work in concert to stimulate root elongation. But what concentrations ideal for a particular plant cannot be known as different plant seedlings respond differently to variable auxin concentrations and type of micro organisms [29]. Strain G25 exhibits highest IAA production when tryptophan is supplemented and G19 have low IAA production capacity in absence of precursor as shown in (Table.1). Concentration of IAA production ranges from 25-31µg/ml in strains which were not supplemented with tryptophan as a precursor. With tryptophan IAA production ranges between 33-43µg/ml. G25 and G18 strains exhibits high IAA production in presence of tryptophan compared to standard strain Pf5 from (Table.1). While other isolates are showing low production of IAA. The isolates showing good IAA production can be putative phytostimulants increasing the root, shoot length of the plants in which they are inoculated. Canola seeds with Pseudomonas putida GR12-2, which produces IAA, resulted in 2 - or - 3 fold increases in the length of seedling roots [30].L-tryptophan is the common substrate of pyrrolnitrin and indole-3-acetic acid biosynthetic pathways [24]. Weak production of the other isolates may reflect competition for the common substrate. The isolates having antifungal traits have the possibility of such competition for utilization of L tryptophan for antibiotic as well as IAA does exist this is affecting the production of IAA. And difference in production may be attributed to differences in the biosynthetic pathways preferred by different isolates.

Study of ACC deaminase activity in selected strains

ACC and NH4CL both act as a nitrogen source for PGPR strains. Below (Table.2) depicts strains utilizing either of the two nitrogen source. Strains G35, G19, exhibits the dense growth on plate containing ACC and G29, shows moderate growth and standard strain Pf-5 along with G14, G13, G46, H9 were weak ACC utilizers. When nitrogen source was not provided with plates containing minimal media then also some cultures such as G35, G19, G29 exhibited growth (Table.2). G29 strain has shown highest ACC deaminase activity i.e, 21 ug of α-ketobutyrate/mg of protein. Standard strain Pf-5 has exhibited low ACC deaminase activity, 1 ug of α-ketobutyrate/mg of protein. While other strains have not shown any detectable amount of ACC deaminase activity. Methionine is deaminated to produce α-keto γ-methylthiobutyric acid (KMBA), which is then oxidized to produce ethylene. The majority of soil microorganisms can derive ethylene from L-methionine (L- MET), while some rhizobacteria can hydrolyze ACC due to their ACC deaminase activity as reported earlier some strains having either ACC deaminase activity (e.g. *Pseudomonas Putida* biotype A, A7), or the ability to produce ethylene from L- MET (*Acinetobacter calcoaceticus*, M9) or both (*Pseudomonas fluorescens*, AM3) , using these

strains ethylene specific bioassay of a classical " triple" response in pea seadlings was used [12] for the effect of the inoculation with the rhizobacteria in the presence of 10mM ACC or L-MET. The exogenous application of ACC had a concentration-dependent effect on the etiolated pea seedlings in creating the classical "triple" response.The inoculation with *Pseudomonas putida* diluted the effect of ACC, which was most likely due to its ACC deaminase activity. In contrast, the inoculation of *P. fluorescens* in the presence of L-MET caused a stronger classical "triple" response in etiolated pea seedlings; most likely by producing ethylene from L-METand this is the first study on the comparative effect of rhiobacteria capable of utilizing ACC vs L-MET on etiolated pea seedlings. The proposed pathway reveals that L-MET is deaminated to KMBA, which is then degraded to C_2H_4, either by peroxidase or photochemically in the presence of a flavin [26].Culture Pf5, H9, G29, G13, G35, H4 were utilizing ACC pathway for ethylene production while others utilized KMBA pathway as shown in (Table.3).Strain H9, G29, G13,G35, H4 utilized both pathway simuntaneously (Table.3). Pf5 , G45, G2 , H4 were given yellow precipates when 2,4-Dinitrophenyl hydrazine(2,4- D) was added as depicted in Fig. 1 and un- inoculated did not give precipitate on addition of 2,4-D. Precipitates obtained in strains indicates that it undergoes KMBA pathway which is the intermediate product in ethylene biosynthesis via L- methionine. L- Met is converted to C2H4 by the transaminase pathway.

CONCLUSION

PGPR strain G18, G20 and G25 strain has shown high indole acetic acid (IAA) production. PGPR strain G19 and G35 have shown strong utilization potential for 1-aminocyclopropane-1-carboxylate (ACC) as a sole nitrogen source. Conversely G14, G13, G46, Pf-5, H9 have shown weak utilization of ACC as a nitrogen source. Pf5, H9, G29, G13, G35, H4 cultures were having ability to utilize both ACC and L-MET as a nitrogen source which signifies that both pathway is functional in these organisms. G29 have shown good ACC deaminase activity (8-21 ug of α-ketobutyrate/mg of protein).Strain G29 is good ACC deaminase producer as well as possess both L-methionine and ACC utilization pathway that will enhance the survival ability of these strains in the rhizosphere. So G29 could be considered as good PGPR strains for the application.

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