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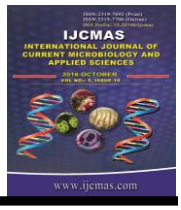

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Screening of 1-aminocyclopropane-1-carboxylic acid (ACC) Deaminase Producing Multifunctional Plant Growth Promoting Rhizobacteria from Onion (*Allium cepa*) Rhizosphere

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A B S T R A C T

The PGP (plant growth promoting) rhizobacteria are root colonizing bacteria which exist in symbiotic relationship with plants. The plant microbe relationship is used to increase crop yield in form of bioinculants applied during cultivation. A study resulted in isolating 61 rhizobacteria from *Allium cepa* (Onion) root rhizosphere. The screening was carried out to explore multifunctional plant growth promotion traits like Phosphate and Zinc solubilization, Ammonia production, IAA production, HCN production, Siderophore production, and ACC deaminase activity in laboratory condition. The ACC deaminase enzyme activity of bacteria is exploited to relive stress on plants aiming to enhance yield. The multifunctional isolate B10 and V15 revealed maximum 6 out of 7 characters tested. The studies on gnotobiotic seed germination and root elongation assay showed significant increase in treated root length and % germination compare to equivalent control. The multifunctional PGPR isolates B10 and V15 may show potential as a bioinoculant for increasing crop yields of Onion.

Introduction

By 2020 world's population is predicted to increase from ~7 billion to ~8 billion. To feed increasing population, it is necessary to increase agricultural productivity in a sustainable and environmentally friendly approach. Plants are continuously in contact with bacteria throughout their lifetime. Plant growth promoting rhizobacteria (PGPR) affect plant growth directly or indirectly by producing growth substances. It is well established that, in the rhizosphere, only 1-2% of bacteria promote plant growth (Antoun and Kloepper, 2001). The rhizosphere is a hot spot of microbial interactions due to the exudates released by plant roots, which constitute the main food source for microorganisms, leading to efficient geochemical cycling of nutrients. Therefore, screening and selection of effective PGPRs and their utilization in integrated practices is of great importance for enhancing the growth and yield of

agricultural crops along with maintaining the sustainability of agro-ecosystems (Bakthavatchalu *et al*., 2012). PGPR are broadly classified into three categories those that colonize the root surface and the close neighborhood (rhizobacteria), those that establish a symbiotic relationship with plants (symbiotic bacteria), and those that can enter into the root interior and colonize inside the plant (endophytic bacteria) (Bacon and Hinton, 2006). PGPR can modulate levels of the plant stress hormone 'ethylene' by producing 1-aminocyclopropane-1-carboxylate (ACC) deaminase enzyme by metabolizing ACC into a-ketobutyrate and ammonia (Glick *et al*., 2007).

While a considerable amount of both basic and applied work remains to be done before ACC deaminase producing plant growth promoting bacteria become a mainstay of plant agriculture, the evidence indicates that with the expected shift from chemicals to soil bacteria, the world is on the verge of a major paradigm shift in plant agriculture (Glick, 2014). Onion is an important vegetable crop more climate sensitive than others. Few studies are aimed at studying rhizobacteria in relation to onion plants (Reetha *et al*., 2014; Colo *et al*., 2014; Reddy *et al*., 1989). This research work aims at isolating multifunctional PGPR's of Onion rhizosphere showing growth promotion. The isolated PGPR's should exhibit ACC deaminase activity in addition to other traits.

Materials and methods

Chemicals and reagents

Aminocyclopropane-1-carboxylic acid, α-ketobutyrate**,** 2,4, Dinitro phenylhydrazine were purchased from Merk India Pvt.Ltd. Chrome azurol S reagent, microbiological media and media components were purchased from HiMedia India Pvt. Ltd. All the other chemicals purchased were reagent grade.

Sampling and Isolation of rhizobacteria

The Onion plant samples were collected (different growth stages) from diverse locations of Nashik District (Maharashtra, India) in sterile plastic bags. The samples were kept on ice bags during transport, and processed after refrigeration in laboratory. The roots of the plants were cut and washed gently with sterile distilled water to remove excessive adherent soil. The root samples were serially washed and vigorously vortex with sterile saline and aliquot of washed water was used to isolate bacteria using Streak plate and spread method on Nutrient agar and Soil extract Agar. The isolated colonies that developed on the plates were sub-cultured repeatedly to acquire pure single colony, which was preserved on agar slants for further characterization and identification .The pure cultures were preserved on nutrient agar slants. The Onion (*Allium cepa*) seeds were purchased from local market.

Screening of PGPR traits

The isolates were evaluated for following plant growth promoting traits.

Indole acetic acid production

The bacterial capacity to produce Indole acetic acid (IAA) was detected as described by Brick *et al.,* (1991). 1% tryptone water broth was inoculated with loop full of bacterial suspension and incubated for 48 hours at 30±2°C. The broth was centrifuged at 10000 rpm for 10 min and two drops of orthophosphoric acid were added to 2ml of cell free supernatant and the development of color was observed. After for 30 min in dark development of pink colour indicates IAA production.

Siderophore production test

The culture was tested for siderophore production by using Chrome-azurol S (CAS) medium which was described by Schwyn and Neilands (1987). The 24 h old cultures were spot inoculated on CAS medium and incubated at room temperature for 72 hours. Siderophore producing cultures were identified by formation of clear zone around the colonies.

Ammonia production test

The ammonia production was detected by Cappuccino and Sherman (1992) method using Nessler's reagent. Overnight grown bacterial cultures were inoculated in 3 ml 1% peptone broth and incubated at room temperature for 24 hrs on Shaker at 120 RPM. 0.5 ml of Nessler's reagent was added to the grown broth after incubation. The development of faint yellow to dark brown color indicated the production of ammonia.

Phosphate solubilization test

The phosphate solubilization test was performed on Pikovaskay's agar plates as described by Pikovaskay (1948). The 24 hour old cultures were spot inoculated on these plates and incubated for 72 hours at room temperature. Phosphate solubilization was detected by clear zone around the colonies.

Zinc solubilization

The isolates were spot inoculated on medium containing 0.1% Zinc oxide (Goteti *et al*., 2013). After the two days incubation Clear zone around the bacterial colony indicates zinc solubilizing ability of bacteria.

HCN production

The HCN production ability of isolates was determined using modified method of Castric (1975). The screw capped tubes Nutrient agar containing glycine (4.4g/L) slant was streaked with isolates. A Whatman Filter paper no. 1 strip soaked in 2% Sodium carbonate in 0.5% picric acid was placed on the top of the tube and was tightly capped. Tubes were incubated for 48 hours 30±2°C. Development of orange to red color on paper indicates HCN production.

ACC Deaminase production

ACC-deaminase activity of rhizobacteria was determined qualitatively by using yeast carbon base medium containing ACC as sole nitrogen source. Qualitative detection of ACC-deaminase activity of rhizobacteria was carried out according to modified methods of Honma and Shimomura (1978) and Penrose and Glick (2003). This method detects the amount of α- ketobutyrate produced when the enzyme ACC deaminase cleaves ACC. 0.3 M ACC stock was prepared by membrane (0.45 µ) filter sterilization and stored at -20^{O}C.

Characterization of isolates

The taxonomic attributes of selected isolates were determined using routine morphological (colonial, Gram staining and motility) and biochemical (catalase, oxidase, amylase and gelatinase) criteria.

Gnotobiotic root elonagation assay

The gnotobiotic root elongation assay is used as a method of assessing the effect of various bacterial strains on the growth of Onion seedlings. The three cultures (B10, V15 and were selected and assay was performed in triplicates.

Preparation of inoculum

The selected PGPR cultures were inoculated in sterile nutrient broth and incubated at

room temperature for 24 h under shaking (120 rpm) conditions. The cells were centrifuged to obtained pellet and pellet was re-suspended in saline. The washing of pellet was repeated two times. Finally, an Optical density of 0.5 at 600 nm was adjusted in saline and used for seed inoculation.

Seed surface sterilization

Uniform sized seeds were selected for assay. 30 seed were used per culture per plate. The onion seeds were surface sterilized by dipping in 95% ethanol solution for 2 min, 0.2% HgCl2 solution for 3 min and washed thoroughly with distilled water for 6 times.

Seed inoculation

Surface sterilized onion seeds were taken into another sterile Petri-plate and soaked in to inoculum for 30 mins. The seeds were shaken well so that fine coating will appear on the seeds. The equivalent distilled water treatment served as negative treatment. The seeds were placed equidistant on moist sterile filter paper sheets in petri plates. The plates were incubated at room temperature and left undisturbed. Plates were observed on daily basis and after 24 hrs 10 ml sterile distilled water was replenished.

Statistical analysis

The root and shoot lengths were measured to the nearest of millimeter and statistical analysis of data was done. The data were statistically tested by one way analysis of variance (ANOVA) and means were separated by Tukey Kramer HSD Post-hoc test using Minitab 14 software. Each treatment was analyzed with at least three replicates and a standard deviation (SD) was calculated and data are expressed in mean ± SD. The observations were considered significant when P was ≤ 0.05.

Results and Discussion

Isolation of rhizobacteria and Characterization of isolate

Ten diverse onion fields were selected for isolation of PGPR from rhizosphere. The outcome of isolation experiment was 61 bacterial isolate in pure culture. The isolates were qualitatively evaluated for 7 different Plant growth promotion traits. The selected six isolate also showed variation in morphological characters, biochemical characters and colony morphology. The goal of determining taxonomic character was to ensure that isolates of different types are studied (Table 2). Nonetheless, the identification of isolates up to species level will be followed depending on its potential in promoting growth and productivity. Reetha *et al.,* (2014) isolated indole acetic acid producing rhizobacteria *Pseudomonas fluorescence* and *Bacillus subtilis* from Onion rhizosphere enhancing its growth. Our approach of isolating and utilizing multifunctional PGP rhizobacteria for increasing crop yield is more lucrative for success of treatment. The dynamic soil and varied climatic conditions affect crop yield the most. To address the varied needs of plant growth isolate possessing multiple activities may prove more successful *In vivo*. According to Martínez-Viveros *et al.,* (2010) the PGPR often have more than one mechanism for enhancing plant growth and experimental evidence suggests that the plant growth stimulation is the net result of multiple mechanisms of action that may be activated simultaneously.

PGPR characters

The best six isolates exhibiting minimum 4 and maximum 6 traits out of 7 traits studied are shown in Table 1. The isolates with maximum number of PGPR traits were

selected for further studies 5 isolates were exhibiting IAA production character out of 6 selected. Loper and Schorth (1986) reported that 80% of bacteria isolated from rhizosphere exhibit IAA production character. Zinc (Zn) deficiency is a global nutritional problem in crops grown in calcareous soils that is important for the development and function of growth regulators (e.g. auxin) and chloroplast. Zinc deficient plants are stunted and have twisted, outward bending leaves affecting yield of crop (Rafique *et al*., 2008). All the isolate selected have zinc solubilization activity useful when zinc is not available in utilizable form. Figure 1 shows zone of clearance around isolate B10 and V15 colony revealing Zn solubilization. Phosphorus is important plant nutrient affecting overall plant growth and crop yield.

Table.1 Multiple PGPR traits of strains isolated from rhizosphere of *Allium cepa*

Isolate Code	ACC deaminase	IAA production	Siderophore production	Ammonia Production	HCN production	Solubilization	
						Zinc	Phosphate
NF5	-	+	-	+	-	+	+
NOR3	-	-	+	+	+	+	+
K3	-	+	+	+	-	+	+
B10	+	+	+	+	-	+	+
V15	+	+	-	+	+	+	+
T36	-	+	+	+	-	+	-

+ =detected, - = not detected

Table.2 Characterization of selected strains isolated from rhizosphere of *Allium cepa*.

Tests / Isolate codes	Gram character	Shape	Motility	Catalase	Oxidase	Amylase	Gelatinase
NF5	Positive	Cocci	-	+	-	-	-
NOR3	Negative	Rods	+	+	-	+	-
K3	Positive	Cocci	-	+	+	-	+
B10	Positive	Rods	+	+	-	-	-
V15	Positive	Rods	+	+	-	+	-
T36	Negative	Rods	-	+	+	-	+

+ =detected, - = not detected

Table.3 Seed growth promotion of *Allium cepa* seeds by Isolate B10, V15 and K3 compare to control

Treatment / Parameters	Control	B10	V15	K3
Germination rate (%)	73	86	83	80
Root length(cm)	2.49±0.67	3.87±1.59**	3.15±1.24*	3.19 ± 1.58^{NS}

Values are mean of three experiments ±SD, significantly different from the control at **P < 0.001, *P<0.05, ^{NS} = not significant by one way analysis of variance (ANOVA) with Tukey Kramer HSD Post-hoc test

Fig.1 Clear zone around isolate B10 and V15 colony showing Zn solubilization

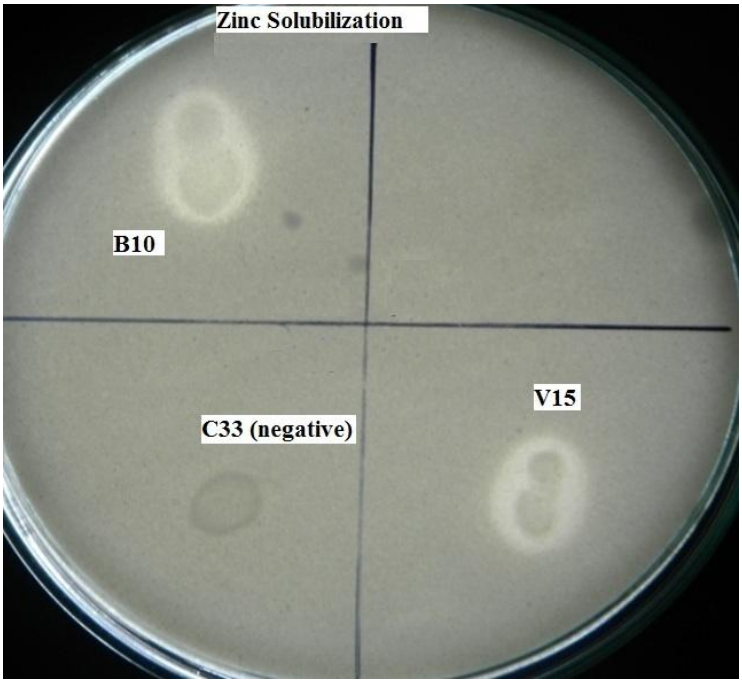


Fig.2 Clear zone around isolate B10 showing phosphate solubilization

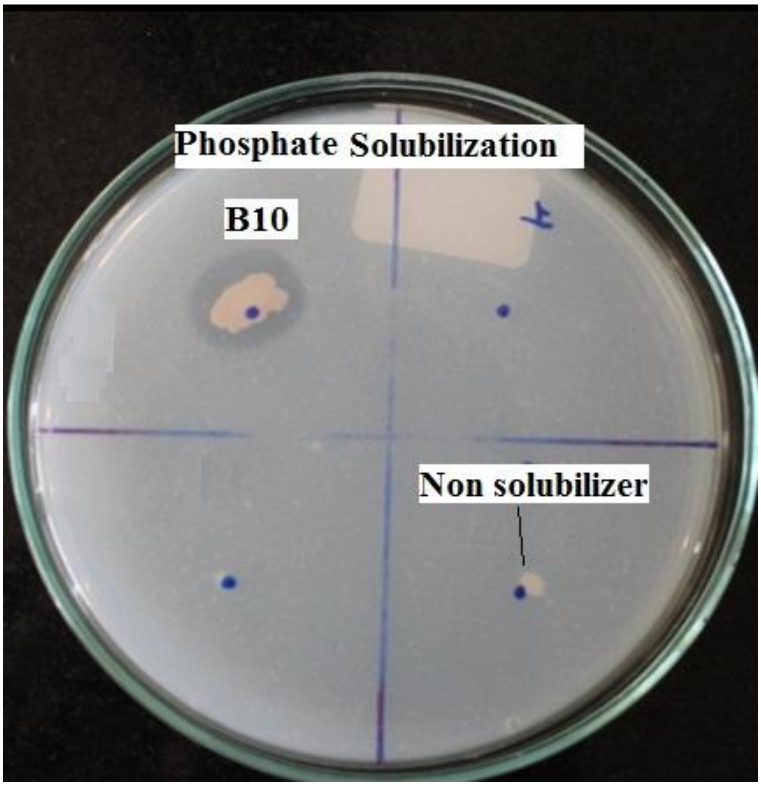


Fig.3 Production of HCN by Isolate V15

Fig.4 Production of ACC deaminase by Isolate V15

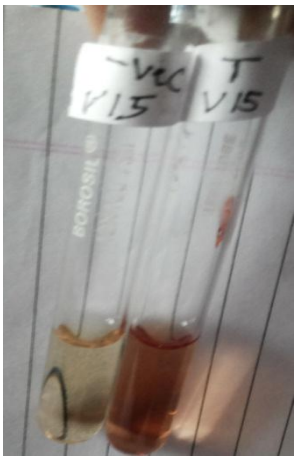
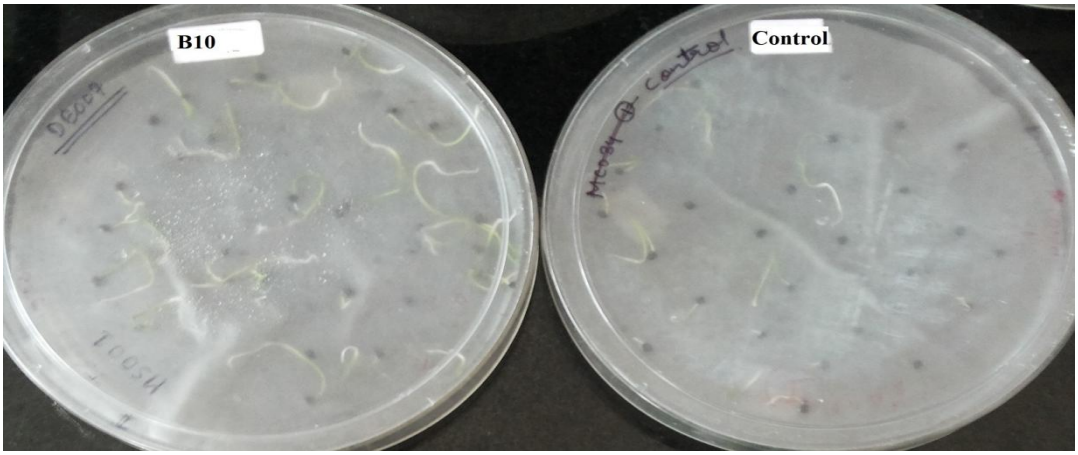


Fig.5 Allium cepa seed germination root elongation with respect to treated(B10) and control



In our study five isolates were exhibiting phosphate solubilization activity out of six selected. PGPR transform inaccessible compounds into forms accessible to plants by production of organic acid and phosphatase enzyme (Khan *et al*., 2014). Figure 2 shows zone of clearance around isolate B10 colony indicating phosphate solubilization. Siderophores are high affinity Fe^{3+} chelating compounds produced by microbes which take Fe^{3+} out of its compounds, bind it and form a Fe^{3+} siderophore complex, when accessible iron is lacking. Fe^{3+}-siderophore complex is transported to the surface of a bacterial cell or root cells, transported into the cell and reduced to Fe^{+2}. Plants are capable of binding the bacterial Fe^{3+} siderophore complex, thus PGPR help in providing the plant with iron (Colo *et al*., 2014). In our work, four siderophore producing strains were isolated and selected for further study.

The bacterial production of HCN has been reported as an important antifungal trait to control root infecting fungi and inducer of plant resistance. Figure 3 show representative production of HCN by Isolate B10.Ammonia production was detected in 95% of the bacteria isolated from rhizosphere of rice, mangroves and soils contaminated by effluent (Noumavo *et al*., 2015). All the selected isolates were NH_3 positive.

The ethylene is considered as a stress hormone, whose synthesis in plants is increased in harsh biotic and abiotic stresses. The higher levels of ethylene inhibit growth and development of plants (Glick *et al*., 2007). The enzyme ACC deaminase lowers level of ethylene by competing with enzyme ACC oxidase. The presence of ACC deaminase activity in rhizobacteria may relieve plant from stress in effect increasing crop yield. We found two isolate (B10 and V15) producing ACC deaminase enzyme. Figure 4 shows production of ACC deaminase by Isolate V15.

Gonotobiotic root elongation assay

The isolates B10, V15 and K3 were chosen to assess their effect on germination and root elongation in gnotobiotic condition. The B10, V15 and K3 treated seeds showed 86%, 83%, 80% germination respectively, significantly higher than control treatment 73% (Figure 5 and Table 3).The root lengths of isolate B10 and V15 were significantly higher than control, but significant difference was not observed between root length of K3 treatment and control. The shoot development was not observed in six days of incubation. ACC deaminase production may play significant role in germination and root elongation of onion seeds. Similar studies by Noumavo *et al.*, (2013) reported the promoter effect of rhizobacteria (*A. lipoferum*, *P. fluorescens*, *P. putida*) on germination and the plants growth of maize in plate assay. Li *et al.*, (2000) reported that mutant of the ACC deaminase gene from *Enterobacter cloacae* UW4 diminished the ability of the bacterium to promote the elongation of canola roots under gnotobiotic conditions.

In conclusion, ACC deaminase activity was detected in two isolates from onion rhizosphere coded as B10 and V15. The various multifunctional PGPR activities exhibited were IAA, ammonia, siderophore, HCN production and Zinc and phosphate solubilization by isolates. The isolate B10 and V15 significantly increased germination and root elongation compare to equivalent control in gnotobiotic assay. Therefore, it may be advantageous to use plant growth promoting rhizobacteria as a means to promote plant growth and increase crop yield by lowering ethylene levels rather than

relying on genetically modified plants or chemical fertilizer.

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