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RESEARCH ARTICLE

SCREENING OF PGPR CHARACTERS AMONG MICROBIAL POPULATION ISOLATED FROM RHIZOSPHERIC SOIL OF DHAINCHA *(SESBANIA BISPINOSA)*

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ABSTRACT

Plant growth promoting rhizobacteria (PGPR) are a group of beneficial microorganisms that colonize at plant rhizosphere, and induce plant growth by a wide variety of mechanisms. Over last couple of years, the use of PGPR is steadily increasing in modern agriculture as a possible alternative to replace chemical fertilizers, pesticides, and other supplements. In the present study, we have screened out a group of microorganism from the rhizospheric soil of Dhaincha (*Sesbania bispinosa*) on the basis of their PGPR characters. Twelve bacterial colonies with diverse growth features were isolated, and designated as DD1to DD12. They are characterized by their morphological, gram nature and biochemical properties. According to the efficiency finally three most efficient PGPR strains, *i.e.* - DD3, DD4 and DD6, were selected and further identified as *Escherichia coli*, *Pseudomonas fluorescens*, and *Burkholderia sp*. using 16 S rRNA sequencing technology. We believe using these PGPR strains individually or in combination as bio-fertilizer could be a viable step towards sustainable agriculture.

INTRODUCTION

The interaction between plant and microbes mainly plant growth promoting rhizobacteria (PGPR) are largely facilitated by the rhizopheric soil (Kloepper *et al*., 1980; Villacieros *et al*., 2003). PGPR are known to rapidly colonize the rhizosphere and suppress soil borne pathogens at the root surface (Rangajaran *et al*., 2003). These micro-organisms can also be beneficial to the plant by stimulating their growth and development through direct and indirect ways (Glick 1995; Gupta *et al*. 2000; Bloemberg and Lugtenberg, 2001; Moeinzadeh *et al*., 2010). According to published reports and hypothesis, the PGPRS mainly affect plant growth by - 1) enhancing Nitrogen supply to the host through Nitrogen fixation; 2) enhancing supply of some major plant nutrients (e.g. P, S, Fe); 3) suppressing soil borne pathogens (by the production of hydrogen cyanide, siderophores, antibiotics, enzymes like chitinase and/or competition for nutrients); 4) improving plant stress tolerance to drought, salinity, and metal toxicity; and / or 5) producing of phytohormones such as indole-3-acetic acid (IAA) (Gupta *et al*. 2000). Any bacterium possessing one or more of these above characters are known as PGPR. Now, Dhaincha (*Sesbania bispinosa*) is a crop generally cultivated for its nutritive value to soil. It is cultivated in monsoon season almost throughout India and grows well in loamy, clayey, black and sandy soils. It is an ideal green manure crop as it is fast growing, succulent, and easily decomposable with low moisture requirements. It forms both root and stem nodules in association with *Rhizobium sp.*, fixes more atmospheric N_2 and produces maximum amount of organic matter in the soil. It enriches concentration of Ca, P, S & micronutrients (Khan *et al*. 2010). It is a very important intercrop relayed with rice and other cereals. Nutrients derived from Dhaincha are very cheap as compared to those of chemical fertilizers. It increases physical and biological properties of soil, add organic matter to it and it gives long term residual effect to the following crops.

However, till date, no study has been found on PGPR association of Dhaincha plants. Moreover, a better knowledge of PGPR habiting rhizopheric soil and their implications on plant growth and yield might change traditional crop management practices. For a maximum exploitation of the plant-bacteria association, effective bacteria with PGPR

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characteristics must be isolated and characterised. The primary aim of this study was isolation and characterization of the plant growth promoting rhizobacteria (PGPR) inhabiting the rhizosphere of *S. bispinosa*.

MATERIAL AND METHODS

Collection of soil

Rhizosphere soil of *S. bispinosa* was collected from four different spots both from local farmer's field (around Kalyani area of Nadia district) and research plots of Horticultural faculty of Bidhan Chandra Krishi Viswavidyalay, State Agricultural University). Intact root system was plowed out and the rhizospheric soil samples at the vicinity of root were collected in sterilized polythene bags, brought to laboratory and kept in aseptic condition as far as possible for further use.

Isolation of bacteria

Bacterial strains from collected soil samples were isolated by soil dilution plate count technique. For the isolation, 1 gram of each soil samples collected from the various collection sites was dissolved in 10 ml sterile distilled water and mix well for 10 minutes. Serial dilution was made from each sample and appropriate dilution was spread on Nutrient agar plate and incubated at 28^0C for 42 hours. From the mixed population of microbes, microbial strains were isolated by single colony isolation from the nutrient agar medium. Streak plate method was used for obtaining pure culture, as and when required.

Preliminary characterization of bacteria

Morphological characteristics of the colony of each isolate were examined on Nutrient agar plates. All the isolates were streaked on Nutrient agar plates. After 3 days of incubation, different characteristics of colonies such as shape, size, elevation, surface, margin, colour, etc were recorded. Gram nature of each isolates was initially determined by using Crystal violet and Safranin staining according to standard microbiological protocol.

Screening for plant growth promoting activities of the isolates

Determination of Phosphate solubilization

Selected 12 isolates were placed on Pikovasky medium containing 5 g of tri-calcium phosphate [Ca_3 $(PO4)_2$] as sole phosphorus source for selectively screening of bacteria which have the ability to release inorganic phosphate from tri-calcium phosphate. After 3-days of incubation at 30˚C, phosphate solubilizing bacteria developed clear zones around colonies. All the isolates having phosphate solubilizing activity were preserved in the laboratory as a pure culture for their further investigation.

Detection of IAA production by the selected isolates

The bacterial isolates that show best P- solubilization ability, were selected for their phytohormone (Auxin) production ability.Test bacterial cultures inoculated in Nutrient Broth medium (Jensen's Media) containing L-tryptophan (5gm/ Lit), and incubated for 96 hour. Cultures were centrifuged at 10,000 rpm for 10 minutes. 2ml of supernatant was taken. 2 drops of Orthophosphoric acid and 4ml of Solawaski's reagent were added to it. (Solawaski's regent – 50ml of 35% Perchloric acid and 1ml of 0.5 (M) $FeCl_3$). Development of pink colour indicated production of IAA in the media. O.D. was taken at 540 nm. The produced IAA level was estimated by using a standard IAA graph.

The best IAA producing bacterial strain was screened for further study.

Detection of Siderophore production by selected isolates

Siderophore production was checked on solid CAS (Chromazurol S) universal blue agar plates (Schwyn and Neilands 1987). Actively growing cultures were spot inoculated on the CAS blue agar plate and incubated at 30° C for 48 h. Formation of yellow-orange halo around the colony indicated production and release of the siderophores on the agar plate.

Detection of Ammonia production by selected isolates

Each strain was tested for the production of ammonia in peptone water broth. (Peptone – 10 gm, NaCl 5 gm, Dist. water 1 lit pH- 7). Overnight broth cultures were inoculated in 10 ml peptone water and incubated at 30˚C for 4 days. After incubation period, Nessler's reagent (0.5 ml) was added to each tube. Development of **brown to yellow** colour was recorded as a positive test for ammonia production (Cappucino and Sherman 1992).

Detection of HCN production by selected isolates

Screening of bacterial isolates for hydrogen cyanide (HCN) production was determined as per methodology described by Castric. Bacterial cultures were streaked on nutrient agar medium containing 4.4 g per liter of glycine.

A Whatman filter paper No. 1 soaked in 0.5% picric acid solution (in 2% sodium carbonate) was placed inside the lid of a plate. Plates were sealed with paraffin and incubated at 30±0.1 °C for 4 days. Development of light brown to dark brown color indicated HCN production.

Selection of the best PGPR

The performances of all the selected bacterial isolates were critically considered in the light of Plant Growth Promoting activities and the best 3 Plant Growth Promoting Rhizobacterial strains were selected according to their multiple positive PGPR activities.

Biochemical characterization of the best 3 PGPR strains

These three best isolates which showed PGPR activity were further characterized by following standard biochemical tests as depicted bellow :-

Indole production

This test is done to determine if bacteria can breakdown the amino acid tryptophan into indole. In this, tryptone broth was inoculated and incubated at 37°C for 48 hrs. Development of red/pink layer on top of the media was checked after adding Kovac's reagent.

Methyl Red Test- Voges –Proskaur (MRVP)

This test is used to determine two things. The MR portion is used to determine conversion of glucose to acidic products like lactate, acetate and formate. The VP portion is used to determine conversion of glucose to acetone. Two sets of glucose peptone broth (peptone 5.0g; potassium dehydrogenate phosphate 5.0g; glucose 10.0g; distilled water 1 lit, pH 7.4) was inoculated with the isolates and incubated at 30°C for 72 hrs.

One set was used for methyl red test, in which development of colour was checked after adding 5 drops of methyl indicator(methyl red 5.0g; dissolved in 30ml of ethanol (95%) and diluted to 50 ml with distilled water). Second set was used for the Vogues Proskauer test, in which development of colour was checked after adding 0.6 ml of α-naphthol and 0.2 ml of KOH solution in 1 ml culture broth.

Citrate Utilization

This defines the ability of bacteria to convert citrate into oxaloacetate. In this media, citrate is the only carbon source available to the bacteria. If it cannot use citrate then it will not grow.

If it can use citrate, then the bacteria will grow and the media will turn a bright blue as a result of an increase in the pH of the media. Simmon's Citrate agar (ammonium dihydrogen phosphate 1.0g; dipotassium phosphate 1.0g; sodium chloride 5.0g; sodium citrate 2.0g; magnesium sulphate 0.2 g; bromothymol blue 0.8g; agar 15 g; distilled water 1 lit) slants was streaked with the isolates and incubated at 30 °C for 42 hours and color changes on slants were recorded.

Catalase Test

A drop of 3% hydrogenperoxide was added to 48 hrs. old bacterial colony on a clean glass slide and mixed using a sterile toothpick. The effervescence was checked for catalase activity.

Gelatin Hydrolysis test

This test was performed to determine the ability of the bacterial sample was inoculated into motility media using a needle and incubated at 30°C for 24 hours and checked for the bacterial migration.

H2S Production Test

SIM agar medium (peptone 30.0g; ferrous ammonium sulphate 0.2g; sodium thiosulphate 0.025g; agar 3.0g; distilled water 1 lit, pH 7.3) was inoculated with bacteria. Colour change was observed after 2 days incubation at 30°C

Urease Test

Christensen urea agar (urea 20.0g, NaCl5.0g; KH_2PO_4 2.0g; peptone 1.0g; glucose 1.0g phenol red 0.012g; agar 15.0g distilled water 1lit; pH 7) was inoculated with bacteria and incubated for 2-3 days at 30°C . Then the colour was observed.

Molecular Identification of the screened PGPR by16s rDNA sequencing method

Identification of the 3 PGPRs were done by 16s rRNA gene sequencing. The steps followed are-

1. Genomic DNA was isolated from the pure culture pellet.
2. The ~1.4kb rDNA fragment was amplified using high – fidelity PCR polymerase.
3. The PCR product was sequenced bi-directionally using the forward, reverse and internal primer.
4. The sequence data was aligned and analyzed to identify the bacterium and its closest neighbors.

The partial sequences were submitted to NCBI GenBank. The phylogenetic tree was made using Neighbor joining method.

RESULTS

Isolation of PGPR

Total 12 bacterial colonies with different growth characteristics were successfully isolated from the rhizosphere soil samples *of S. bispinosa* field from four different areas of Nadia district (three bacterial colonies from each of the soil sample) They were designated as DD1, DD2, DD3, DD4, DD5, DD6, DD7, DD8, DD9, DD10, DD11 AND DD12.

Morphological characteristics of PGPR isolates

The preliminary observation regarding the colony morphology of the isolated bacterial strains is presented in **Table 1.** The morphological characteristics of PGPR isolates widely varied. The isolates were found to be first growers. Some of the isolates produced circular and some irregular shaped colonies. They also differed in colour, margin, elevation but all are opaque and odorless. No pigmentation was observed in the colonies on Nutrient agar plates. Initially it was noted that out of 12 isolated colonies, 9 are whitish and 2 are yellow in colour, 7 are circular and 5 are irregular in form, 5 with lobate, 4 regular and 3 with entire margin.

Microscopic observation of PGPR isolates

Microscopic observation was performed to investigate shape and Gram reaction of the PGPR isolates. Mostly the bacterial cells were of rod shaped except the rest one, which was coccus. Both the type of cells were obtained either singly or in chain. Furthermore, it was noted that out of 12 isolates, 8 strains were Gram positive and rest 4 were Gram negative. The results clearly indicated that in Dhaincha rhizospheric soil, Gram positive bacteria were predominant than Gram negative bacteria.

Table 1 Morphological characteristics of 3-day-old colony of the bacterial isolates

Isolates	Colour	Shape	Margin	Elevation	Density	Odour	Pigmentation
DD1	white	circular	lobate	convex	opaque	Odorless	None
DD2	yellow	irregular	entire	flat	opaque	Odorless	None
DD3	white	circular	regular	convex	opaque	Odorless	None
DD4	white	circular	regular	flat	opaque	Odorless	None
DD5	white	circular	regular	flat	opaque	Odorless	None
DD6	yellow	circular	regular	convex	opaque	Odorless	None
DD7	white	irregular	lobate	flat	opaque	Odorless	None
DD8	yellow	circular	entire	flat	opaque	Odorless	None
DD9	white	irregular	lobate	raised	opaque	Odorless	None
DD10	white	circular	entire	convex	opaque	Odorless	None
DD11	white	irregular	lobate	convex	opaque	Odorless	None
DD12	white	irregular	lobate	flat	opaque	Odorless	None

Table 2 Shape and Gram nature of the bacterial isolates

Isolate	Shape of bacteria	Gram nature
DD1	Rod, cells are in chain	positive
DD2	Rod, cells are in chain	positive
DD3	Rod, single cell	negative
DD4	Rod, single cell	negative
DD5	Rod, single cell	positive
DD6	Rod, single cell	negative
DD7	Rod, cells are in chain	positive
DD8	Rod, single cell	positive
DD9	Rod, single cell	positive
DD10	Rod, cells are in chain	positive
DD11	Coccus, cells are in chain	negative
DD12	Rod, single chain	positive

PGPR activities of the Bacterial isolates

All the 12 bacterial isolates were then examined for their PGPR activities. First of all they were checked for phosphate solubilization activity. Among them 7 isolates (DD3, DD4, DD6, DD9, DD10, DD11 AND DD12) showed prominent phosphate solubilization zones**(Table 3).** Out of them 3 isolates showed sharp zones. Highest solubilization activity showed by the isolate DD6 followed by DD4 and DD3 and remaining four showed hazy zones.

Table 3 Bacterial isolates showing P-solubilization

Isolate	P-solubilizing ability
DD1	--ve
DD2	-ve
DD3	+ ve
DD4	+ ve
DD5	-ve
DD6	+ ve
DD7	+ ve
DD8	+ ve
DD9	+ ve
DD10	+ ve
DD11	+ ve
DD12	+ ve

Seven isolates which showed phosphate solubilization ability were further selected for their phytohormone (IAA) production ability. IAA productions by the seven isolates were estimated by their OD value taken in 540 nm, which ranges from 1260 ppm/lit to 180 ppm/lit in 8 days study. All the seven isolates were positive for IAA production. Among them isolate DD4 shows highest (1260 ppm) production of IAA followed by DD6 (1087 ppm) and DD3 (724 ppm) whereas the isolate DD11 shows lowest (180 ppm) production. All these seven IAA positive isolates were further screened for other PGPR activities like ammonia, siderophore and HCN producing ability.

Among them, five isolates (DD3, DD4, DD6, DD9, and DD11) showed positive results for the ammonia production, three (DD3, DD4, DD6) showed positive for HCN production and four (DD3, DD4, DD6, and DD12) shows positive results for Siderophore production activity **(Figure. 1)**. On the basis of this characterization these isolates were selected according to their multiple positive PGPR activities and as the isolates DD3, DD4 and DD6 showed highest results, were selected as the best.

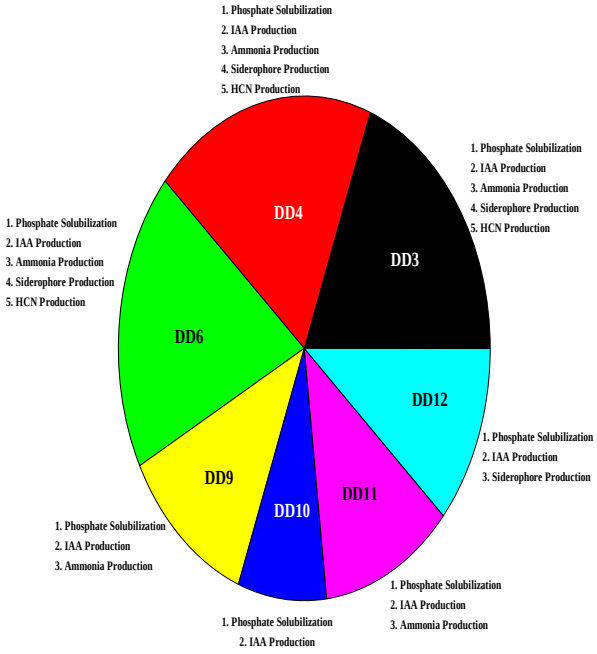


Figure 1 Multiple positive PGPR activities of the the isolates

Biochemical characterization of the best 3 PGPRs

The best three PGPRs were further characterized by a number of biochemical tests such as indole, MRVP, Citrate utilization, catalase, gelatin hydrolysis, H_2S production, urease and starch hydrolysis. The result is depicted in **Table 3**.

Molecular identification of the best 3 PGPRs

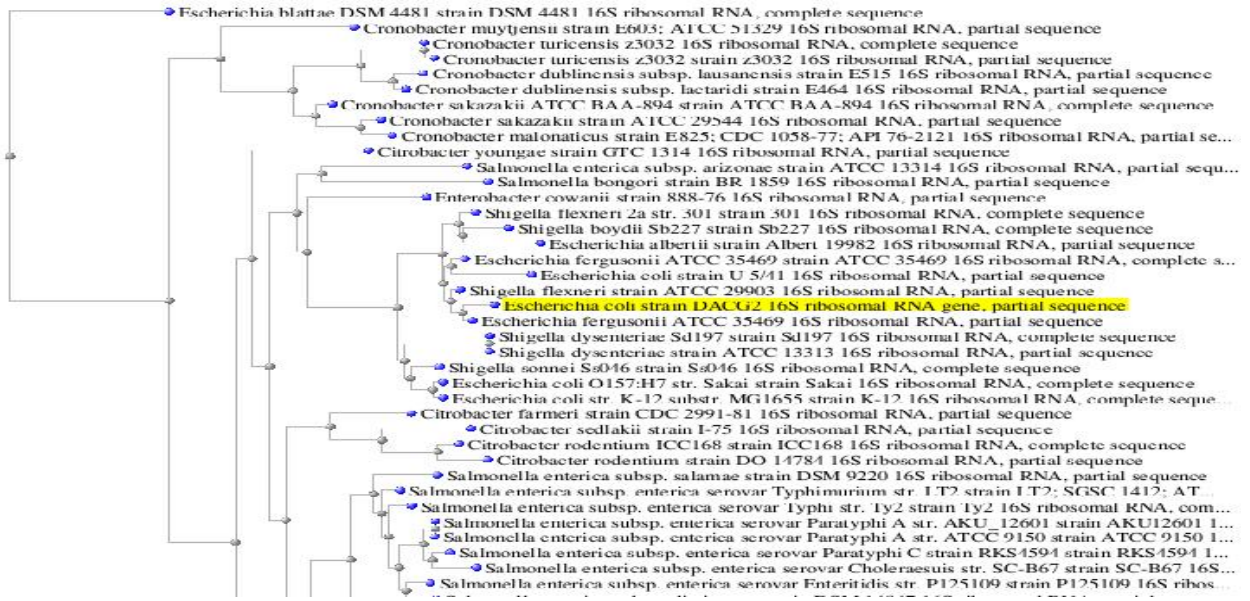
For the molecular identification, these two isolates were identified by 16 S rDNA sequencing. 16 S rDNA gene sequences compared with the available sequences in the databank with help of BLAST homology search. DD3 was identified as *Escherichia coli* DACG2 and deposited it in the GeneBank database with accession number JN858966 while DD4 was identified as *Pseudomonas fluorescens* strain

DACG3 (working GenBank flatfile Accession No. KP641168) and DD6 as *Burkholderia sp*. DACG1 (Gene Bank Accession No. JN639877).

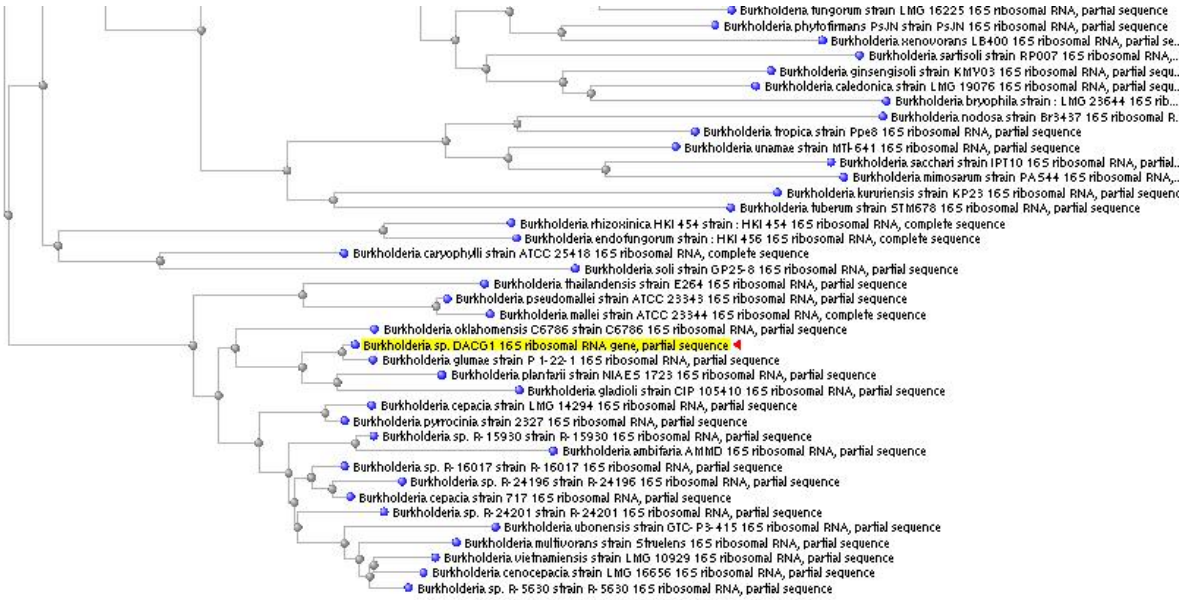
To be an effective PGPR, bacteria must be able to colonize roots because bacteria need to establish itself in the rhizosphere at population densities sufficient to produce the beneficial effects.

Table 3 Biochemical characterization of bacterial isolates

Isolates	Indole Test	Methyl-Red	V-P Test	Citrate test	Catalase Tests	Gelatin hydrolysis	H_2S production	Urease	Starch hydrolysis
DD3	+ve	+ve	-ve	-ve	+ve	-ve	+ ve	-ve	-ve
DD4	-ve	-ve	-ve	+ve	+ve	+ve	-ve	-ve	-ve
DD6	- ve	-ve	-ve	+ve	+ve	+ve	-ve	+ve	-ve



A



B

Figure 2 Phylogenetic position of - A: DD3 (*Escherichia coli* DACG2), and B: DD6 (*Burkholderia sp.* DACG1)

DISCUSSION

The exact mechanism by which PGPR stimulate plant growth is not clearly established, although several hypotheses such as production of phytohormones, suppression of deleterious organisms, activation of phosphate solubilization and promotion of the mineral nutrient uptake are usually believed to be involved (Kloepper *et al.*, 2004, Herman *et al.*, 2008).

There are many papers related to the advantages and screening of PGPR from crop plants particularly rice, maize and sugar cane but few on *S. bispinosa*. Little information about screening and using PGPR with Dhaincha is available. Though Dhaincha is an ideal green manure crop, which fixes more atmospheric N_2 and produces maximum amount of organic matter in the soil. In present study, beneficial bacteria were isolated from Dhaincha rhizosphere. Isolated bacteria were

screened for different plant growth promotion activities and characterized by biochemical tests. Phosphorus is one of the major nutrients, second only to nitrogen in requirement for plants. Most of phosphorus in soil is present in the form of insoluble phosphates and cannot be utilized by the plants (Sarwar and Frankenberger, 1994). The ability of bacteria to solubilize mineral phosphates has been of interest to agricultural microbiologists as it can enhance the availability of phosphorus and iron for plant growth. It has been reported that higher concentrations of phosphate-solubilizing bacteria are Commonly found in the rhizosphere soil as compared to nonrhizospheric soil (Whipps, 2001). In our experiments, out of twelve isolates seven were able to solubilize phosphate in the rhizosphere soil (Table 3). The isolate DD6 showed highest phosphate solubilization zone (20 mm) followed by DD4 and DD3.

IAA is one of the most important phytohormone which may function as important signal molecule in the regulation of plant development. It has been reported that IAA production by PGPR can vary among different species and strains, and it is also influenced by culture condition, growth stage and substrate availability (Spaepen *et al.*, 2007). Moreover, isolates from the rhizosphere are more efficient auxin producers than isolates from the bulk soil (Patten and Glick., 1996). All the seven phosphate solubilizing isolates are positive for IAA production (Table 5). Among them, three isolates DD3, DD4 and DD6 are found to be good producers of IAA (Table 5).

Another important trait of PGPR is the production of ammonia that indirectly influences the plant growth. All the isolates were able to produce ammonia.

Siderophore is one of the biocontrol mechanisms belonging to PGPR groups under iron limiting condition. PGPR produces a range of siderophore which have a very high affinity for iron. Therefore, the low availability of iron in the environment would suppress the growth of pathogenic organisms. This study has demonstrated that, out of seven, 4 PGPR isolates such asDD3 DD4, DD6, DD12 produced siderophores. Based on the result, it has been determined that, these 4 siderophore producing isolates were able to inhibit the phytopathogens.
HCN production by rhizobacteria has been postulated to play an important role in the biological control of pathogens (Voisard *et al.*, 1989). In the present work, three bacterial isolates were positive for HCN production, which acts as an inducer of plant resistance. Multiple PGP activities among PGPR have been reported by some other workers while such findings on indigenous isolates of India are less commonly explored (Abbas and Okon. 1993). Bacterial strains showing catalase activity must be highly resistant to environmental, mechanical and chemical stress. In the present study isolates DD3, DD4 and DD6 were also able to produce catalase.

It was clear from the present study, isolate DD3 *Escherichia coli* DACG2, DD4[*Pseudomonas fluorescens* strain DACG3(working GenBank flatfile Accession KP641168)] and DD6 *Burkholderia sp*. DACG1 were found to be the three most efficient PGPR which solubilized insoluble phosphorus, produced IAA, ammonia, HCN and catalase. Such type of study is necessary as it advocates that use of PGPR as inoculants or bio-fertilizer s is an efficient approach to replace chemical fertilizers.

CONCLUSION

It can be concluded from the above discussion that Plant growth promoting rhizobacteria are increasingly used for crop improvement and protection. In the same context, present study was focused for the isolation and characterization of PGPR from rhizosphere of the Dhaincha. Phosphate solubilization, Indole acetic acid production, Siderophore production, Hydrogen cyanide production and ammonia production was considered for the present study. Isolates with good plant growth promoting potentialities were characterized and the best three efficient isolate among them were identified. The morphological, cultural, biochemical and molecular characterization of these three efficient isolates revealed that the most efficient PGPR inhabiting in Dhaincha rhizosphere were Escherichia *coli* DACG2, *Pseudomonas fluorescens* strain DACG3 and *Burkholderia sp*. DACG1. The results are promising for design of potentially active plant growth promoting PGPR strain based formulation which would be beneficial for crop improvement and crop protection. The potential of this strain could be investigated in detail and field application shall be studied for its bio-control potential.

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