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PLANT GROWTH PROMOTING ACTIVITIES OF RHIZOBACTERIA ISOLATED FROM RHIZOSPHERIC SOILS OF RURAL BANGALORE, INDIA

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ABSTRACT

Soil is a dynamic ecosystem which provides support to plant life. Microorganisms inhabiting the rhizosphere region of soil play a key role in agriculture by promoting the exchange of plant nutrients and reducing the application of chemical fertilizers to a large extent. Engineering of rhizospheric region through exploitation of specific microorganisms leads to higher microbial diversity in the soil which in turn plays a significant role in maintaining the soil health. The present work envisages the isolation, screening and biochemical profiling of potent plant growth promoting rhizobacteria from various rhizospheric soils in and around Bangalore. Sixty isolates from rhizospheric region of fourteen different agricultural soils were screened for plant growth promoting traits such as phosphate solubilization, siderophore production, Ammonia, HCN & Phytohormone production. Twelve isolates that exhibited plant growth promotional traits were further subjected to screening for drought and salt tolerance. Among the twelve isolates, four potential isolates namely *Serratia marcescens*, *Pseudomonas aeruginosa* and *Acinetobacter pittii* were identified based on biochemical methods and 16SrRNA sequencing.

Keywords: Plant Growth Promoting Rhizobacteria; *Serratia marcescens*; *Pseudomonas aeruginosa*; *Acinetobacter pittii*; 16SrRNA sequencing

INTRODUCTION

Increasing population has exerted enormous pressure on farmers for higher crop yields. A notable enhancement in agricultural net output can be achieved by introducing various improved agricultural methods. Modern agricultural practices are heavily dependent on the use of chemical fertilizers, pesticides and various growth regulators. Although application of chemical fertilizers has resulted in improvement of agriculture production, this has led to the depletion of natural resource, environmental deterioration and loss of crop diversity.

Present day agriculture is not sustainable in the long run; hence, the concept of sustainable agriculture has emerged in recent years which emphasize more on the conservation of natural resources and environment. A need for sustainable agriculture can be achieved by engineering the rhizospheric microflora. The region of the soil surrounding the plant roots is termed as Rhizosphere. In this region, intense interactions between the plants and microbial partners (Bacteria and Fungi) take place. Beneficial bacteria inhabiting the rhizospheric region of the soil are known as plant growth promoting rhizobacteria (PGPR). PGPR comprises the heterogenous group of bacteria associated with plant roots; which can improve the quality of plant growth. PGPR engineers the rooting pattern, activates plant defense mechanisms and improves nutrient uptake in plants (Cruz *et al*., 2002; Barea *et al*., 2005).

Several plant growth promoting rhizobacteria such as *Rhizobium japonicum*, *Bradyrhizobium japonicum*, *Acetobacter diazotrophicus* L1, *Azotobacter chroococcum*, *Azotobacter beijerinckii*, *Azotobacter vinelandii*, *Bacillus cereus*, *Azospirillum* spp., *Pseudomonas fluorescens* etc. and fungi such as *Trichoderma*, *Piriformospora indica,* Arbuscular Mycorrhizal fungi *and Sebacin avermifera* have already been extensively studied and well documented for their positive effects on the plant growth promotion. These microorganisms not only help plant growth promotion but are also helpful in reducing the application of chemical fertilizers as much as possible. The exact mechanism involved in PGPR mediated plant growth promotion is not completely understood; however few mechanisms that are involved are as listed (i) solubilization of mineral phosphates and other nutrients present in the soil (ii) Nitrogen fixation (iii) phytohormones production such as Indole acetic acid, Gibberellic acid and cytokinins (iv) Antagonism against several plant pathogens via production of siderophores (v) Helps plants to take up available nutrients present in the soil.

Environmental factors such as climate, soil characteristics and activity of indigenous microbial flora of soil greatly influences the performance of plant growth promoting rhizobacteria. Hence, it is very much essential to develop efficient endogenous strains that can perform very well under varied environmental conditions (salinity, temperature, pH and heavy metals). The present study focuses on the screening of rhizobacteria exhibiting

exceptionally high growth promoting characters from fourteen different rhizospheric soils of rural Bangalore

MATERIALS AND METHODS

Collection and processing of soil samples

Soil samples were collected during the month of August 2018. Rhizospheric soil of crops like Corn, Mulberry, Brinjal, Coriander, Sugarcane, Tomato, Beetroot, Chickpea, Onion, Spinach, Groundnut, Capsicum, Ridge gourd and Pigeon Pea plant were collected using clean, dry zip lock pouch along with sterile spatula from different locations of Bangalore Rural areas.

Isolation and culture media

The samples were processed within 24 h from the time of collection. Soil samples were serially diluted using saline. Different dilutions such as 10^{-4}, 10^{-5} and 10^{-6} were plated on nutrient agar (NA) using standard microbiological techniques. The Petri-plates were incubated at 30±2°C for 24h. Isolated colonies were selected based on the morphological characteristics and pure cultures of the same were maintained on NA slants, stored at 4°C. Pure cultures were Gram stained following method of Vincent (1970).

All the isolates were screened for plant growth promoting traits. The isolates exhibiting highest growth promotional activities were identified by biochemical and molecular techniques.

Test for Plant Growth Promoting Traits (PGP)

All the isolates obtained were further screened for plant growth promotional traits such as phosphate solubilization, production of phytohormone, siderophore, HCN and ammonia. All the isolates obtained were further screened for plant growth promotional traits such as phosphate solubilization, production of phytohormone, siderophore, HCN and ammonia.

Phosphate Solubilization Assay

The ability of the isolates to solubilize phosphate was detected by spotting them on National Botanical Research Institute Phosphate- Bromophenol blue (NBRIP-BPB) medium comprising of Glucose-10g; Tricalcium phosphate - 5g; Magnesium Chloride-5g, Magnesium Sulphate- 0.25g, Potassium Chloride- 0.2g, Ammonium sulphate-0.1g, Bromophenol blue-0.025 g and Agar- 15g dissolved in 1000 ml sterile distilled water. The pH of the medium was adjusted to 5.8±0.2 and incubated at 37±2°C for 5days. Appearance of clear zone around the organism was considered as positive for phosphate solubilization. The plate containing uninoculated NBRIP-BPB media served as control(Nautiyal 1999).

Siderophore Production

Screening for the siderophore production was carried out by spot inoculating the isolates on chrome azurole S agar plate followed by incubation at 37±2°C for 4-5 days. Appearance of orange coloured colony was considered as positive for siderophore production (Schwyn and Neilands, 1987).

Ammonia Production

Isolates were inoculated into 5 ml sterile peptone water and incubated for 48 h at 37±2 °C. After the incubation period, 0.5 ml of Nessler's reagent was added. Development of Yellow to brown color was observed as positive test for ammonia production. Uninoculated medium was used as control (Cappuccino and Sherman, 1992).

Production of HCN

HCN production was carried out according to Bakker and Schipper (1987) with slight modifications. Isolates were inoculated into nutrient broth supplemented with 4% glycine. Whatman filter paper strips soaked in a solution of 2% Na_2CO_3 in 0.5% picric acid was placed towards the end of the test tube lid. Further, the test tubes were incubated at 32±2 °C for 48h. The change in the colour of filter paper from yellow to orange brown indicates positive for the HCN production.

Production of Plant Hormones

Indole Acetic Acid (IAA) Production

Screening of IAA production was carried out by inoculating the isolates in nutrient broth supplemented with 0.1% Tryptophan and incubated at 30±2°C for 48 h under continuous shaking conditions in an orbital shaking incubator (REMI cis24plus) at100 rpm/min. Following the incubation, culture was centrifuged at 5000 rpm. 1 ml of the supernatant was mixed with 2 ml of Salkowsky's reagent and kept at room temperature for 20 minutes (Gordon and Weber, 1951). Development of pink colour was considered as positive for IAA production.

Gibberellic Acid (GA) Production

Screening of Gibberellic acid production was performed by cultivating isolates in Nutrient broth supplemented with 1 % peptone and incubated under shaking conditions (120 rpm) at 30±2°C for 48 h. After the incubation period, cultures were centrifuged at 5000 rpm. pH of the supernatant was adjusted to 2.5 using HCl. Further, the supernatant was mixed with an equal amount of ethyl acetate. The amount of gibberellic acid present in the ethyl acetate phase was measured at 254 nm using UV Visible spectrophotometer (Bilkay *et al*., 2010).

Stress Tolerance

The isolates that exhibited plant growth promotional traits were further subjected to salt and drought stress in order to check their ability to grow and sustain in stressful environment.

Salt Tolerance

The isolates were cultivated in Nutrient Agar supplemented with varying concentrations of Sodium Chloride (NaCl) from 0mM to 240mM. Plates were incubated at 30±2°C for 48 h.

Drought Tolerance

To study the potential of the isolates to tolerate drought stress, an experiment was conducted using polyethylene glycol and mannitol. Isolates were inoculated on Nutrient Agar supplemented with different concentrations of PEG and mannitol ranging from 30 mM to 210 mM (Kumar *et al*., 2014). The media containing PEG and mannitol was autoclaved, inoculated with test organism and was incubated at 30±2°C for 48h. The isolates that were able to endure salt and drought stress were - subjected to characterization by biochemical and molecular methods (16S rRNA sequencing).

Biochemical Tests

Imvic and Citrate Utilization Test

Potential isolates were screened for indole, methyl red, Voges-Proskauer test as per standard protocols (Prescott and Harley, 2002). The Simmons citrate medium was prepared and the pH was set at 6.8. The plates were streaked with different bacterial isolates and incubated at 30±2°C for 48 hour and observed for the change in the colour of the medium.

Catalase Test

Bacterial isolates were streaked on clean glass slide and few drops of 3% H_2O_2 were added to the streaked culture. Appearance of bubbles confirmed the presence of catalase activity (Rorth and Jenson, 1967).

Oxidase Test

A loop full of test organism was streaked in a zigzag manner on the oxidase disk. A positive reaction was indicated by dark purple colour that developed in 10-15 seconds (Prescott and Harley, 2002).

Protease Production

Protease production of the strain was checked following the suitable protocol (Aboaba *et al*., 2006).Isolated microorganism were inoculated on skim milk agar plate and kept for incubation for 24 h. Appearance of clear zone around the colony was considered as positive for protease production

Amylase Assay

The amylase assay of test organism was carried out by starch hydrolysis test. 2h old grown culture of the test organism was streaked on the starch agar plate and incubated at 37±2°C for 24 h. The culture plate was flooded with Grams iodine to produce a blue coloured starch-iodine complex. Presence of clear halogen around the streaked organism was considered as positive for amylase production (Budi *et al*., 2000).

Carbohydrate Utilization Test

Carbohydrate utilization efficacy of isolates against various carbohydrates such as Glucose, Sucrose, Mannitol and Lactose was studied using triple sugar medium comprising of Tryptone-10g, carbohydrate source: glucose/ sucrose/ mannitol/ lactose-5gm, NaCl-15g, pH-7.5±0.2, distilled water-1000mL). Carbohydrate broth was prepared separately. 5mL of the broth was dispensed into the tubes and Durham's tubes were inserted in an inverted position into the tubes. The tubes were plugged, sterilized and inoculated separately and incubated at 37±2°C for 24-48h. A control was maintained without inoculation. The tubes were observed for the fermentation (Prescott and Harley, 2002).

Hydrogen Sulfide Production

The test cultures were stab inoculated onto hydrogen sulphide media (peptone-30g, beef extract-3gm, ferrous ammonium sulphate-0.2g, sodium thiosulphate-0.025g, pH-7.3±0.2, distilled water-1000mL). Tubes were incubated at 37±2°C for 24-48h. The formation of black precipitate in the medium indicates positive for the test (Holguin and Patten, 1999).

Genetic Identification and Phylogenetic Analysis

Effective isolates were further characterized molecularl

Identification of the isolated microorganism strains were done by 16S rRNA sequencing. Genomic DNA of the isolates were amplified with universal primer two7F1 (5 - AGAGTTTGATCMTGGCTCAG-3) and 1494Rc (5 - TACGGCTACCTTGTTAC GAC-3) in an exceedingly 25 µl reaction mixture containing 10X buffer (with 2.5mmol l-1 $MgCl_2$) 2.5 µl, 20 M^{-1} forward and reverse primer every 2 µl, dNTP mixture (2.5 mM) 3.0 µl, 0.5 µl of Taq DNA enzyme (2.5 U), Nuclease free water and fifty weight unit of DNA extract. DNA samples were amplified using DNA thermal cycler (T 100, BioRad, India. The PCR conditions were: initial denaturation for 3min at 94 °C, 30 cycles every consisting of denaturation for 1 min at 94 °C primer tempering for 1 min at 54 °C and extension at 72 °C for 5 min and a final elongation of 5 min at 72 °C. Amplified product was then refined using mistreatment Qiaquick PCR purification kit (Qiagen, USA). The refined amplified PCR product of 1.5 computer memory unit was send to Xcelris genetic science labs ltd (Xcelris Ahemdabad, India) for sequencing. Phyletic and molecular organic process analysis was done by mistreatment software package MEGA6.0 (Tamura *et al*., 2013).

RESULTS AND DISCUSSION

Isolation of Bacterial Cultures

A total of sixty bacterial isolates were isolated from the rhizospheric soil of different crop fields (Table 1). Out of sixty isolates, eighteen were found to be Gram positive rods, twenty-seven were Gram negative rods and fifteen were Gram positive cocci based on colony morphology and Gram staining method.

Plant Growth Promoting (PGP) Traits

All the sixty isolates were tested for the ability to exhibit growth promoting traits (Figure 2), among which twelve isolates showed positive for the all tested plant growth promotional traits such as phosphate solubilization, production of phytohormone, siderophore, HCN and ammonia (Table 2). The organisms' that exhibited plant growth promotional traits were subjected to further assays. The four isolates, Tomato21, Tomato-3, Groundnut-7, Groundnut-5 exhibited plant growth promotional traits among all the tested PGP traits.

Stress Tolerance

Effect of Sodium Chloride (NACL) on Growth of The Isolates

The four potential isolates- Tomato- 21, Tomato-3, Groundnut-7 and Groundnut-5 were able to tolerate NaCl concentration up to 210mM. They grew well on plates supplemented with 210mM NaCl concentration also (Figure 2).

Effect of PEG and Mannitol on growth of the isolates

Effect of drought stress on the growth of potential isolates was tested for their tolerance against different concentrations of PEG. Out of twelve isolates; Tomato- 21, Tomato-3, Groundnut-7, Groundnut-5 were able to tolerate PEG concentration of 2- 16 % (Fig. 3)

Identification of Bacterial Isolates

Among 12 isolates tested for stress tolerance studies, the four isolates- Tomato- 21, Tomato- 3, Groundnut-7 and Groundnut-5 which exhibited plant growth promoting traits as well as stress tolerance capability were further characterized using biochemical and molecular techniques.

Biochemical Characterization

Based on Gram staining, Tomato-21, Tomato-3, Groundnut-7 and Spinach-5 were found to be Gram (-ve) negative in nature. The isolates were observed positive for Catalase and Citrate utilization studies. Except Tomato-21, all three isolates exhibited starch hydrolysis and Triple sugar iron test (Table 3). All the four isolates were able to hydrolyze casein. Tomato -3 and Spinach -1 were found to be positive for utilizing carbohydrates like glucose, sucrose, mannitol and lactose.

Molecular Identification

Based on 16S rRNA sequencing, the four effective bacterial isolates were characterized. The blast analysis of the obtained sequence was performed and the sequences were deposited in the NCBI database (Table 4) for the phylogenetic tree construction using online tool-phylogeny.fr (Figure 4).

DISCUSSION

Soil microflora mostly consists of free living micro-organisms such as bacteria, fungi, actinomycetes and AM fungi. Microorganisms inhabiting this region of soil play an imperative role in agriculture by promoting the exchange of plant nutrients and thereby reducing the dependency on chemical fertilizers to a large extent. Plant growth promoting rhizobacteria are one such promising microorganism that falls under beneficial section. PGPR have the ability to colonize the surface or inner part of roots, as a result play a prime role in influencing the plant growth and development directly or indirectly (Gerhardt *et al*., 2009; Gordan and Weber, 1951). PGPR strains must possess the capability to colonize root surface as they need to establish themselves in the rhizosphere at population densities sufficient to exhibit the wide beneficial effects.

In the present study, 60 isolates were isolated from the rhizospheric soils of Corn, Mulberry, Brinjal, Coriander, Sugarcane, Tomato, Beetroot, Chickpea, Onion, Spinach, Groundnut, Capsicum, Ridge gourd and Pigeon Pea plant. Out of 60 isolates, 12 potential isolates that possessed plant growth promoting traits were selected. Selected 12 isolates were further subjected to salt and drought stress on plates supplemented with varied concentrations of NaCl and Polyethylene Glycol. Among 12 isolates 4 best isolates that exhibited tolerance to NaCl and Polyethylene Glycol were characterized using biochemical and molecular techniques. Four potential isolates were found to be gram negative and tested positive for the ability to utilize citrate as a carbon source. Utilization of citrate is thought to play a significant role in competitive root colonization and maintenance of bacteria in roots (Turnball *et al*., 2001; Weisskopf *et al*., 2011). These strains also showed positive for catalase activity and casein hydrolysis.

Four best/ potential isolates that exhibited tolerance to NaCl and Polyethylene Glycol isolated from the rhizospheric soils of tomato, groundnut and spinach were identified as *Pseudomonas aeruginosa* (MK691419), *Serratia marcescens* (MK691417), *Acinetobacter pittii* (MK691420) and *Serratia marcescens* (MK691418) based on 16SrRNA sequencing.

Several studies on the application of *Pseudomonas aeruginosa*, *Serratia marcescens* and *Acinetobacter pittii* for plant growth promotional activities are available in the literature. *Acinetobacter* sp. PUCM1022 isolated from rhizosphere of *Pennisetum glaucum* significantly enhanced the shoot height, root length, and root dry weights of pearl millet seedlings in pot experiments when compared with the untreated controls (Zamin *et al*., 2011). Likewise, Verma and Shahi (2015) have also reported the phytohormone production, phosphate solubilization, siderophore producing ability and antifungal activity of Pseudomonas spp., Bacillus spp. and Acinetobacter spp. isolated from the rhizosphere of wheat. Similarly, Daur *et al*. (2018) have investigated the positive effects of *Acinetobacter pittii* on the physiology, nutrient uptake, improvement in the relative water content, photosynthetic pigments, growth and yield of alfalfa under arid conditions. Dual inoculation of *Acinetobacter junii* and AM fungi significantly increased the growth and yield of tomato and bell pepper plants (Tallapragada *et al*., 2015). Acinetobacter strains were found effective in the enhancement of the growth of *Vigna radiate*, *Vigna unguiculata*, *Abelmoschus esculentus* and *Dolichos lablab* (Patel *et al*., 2017). *Serratia marcescens* TRS-1 has been reported as a plant growth promoter with having the ability to reduce root rot disease of tea (Chakraborty *et al.,* 2011).In a similar study, *Serratia marcescens* AL2-16 is known to fix atmospheric nitrogen, solubilize inorganic phosphate and produce good quantity of siderophore and phytohormones and thus can be employed as a potential biofertilizer for the growth enhancement of *Achyranthes aspera* L (Devi *et al*., 2011). Two isolates of *Pseudomonas aeruginosa* depicted a significant level of growth enhancement of chili plants under greenhouse condition (Linu *et al*., 2019).Soil treated with the native Plant growth promoting bacterium *Pseudomonas aeruginosa* RRALC3 in combination with inorganic fertilizer enhanced the growth, nutrient and carbon content of the native legume *Pongamia pinnata* (Radhapriya *et al*., 2015).

CONCULSION

Deeper understanding of the performance of these isolates on the growth of plant could unravel the mechanism and potential of these PGPR exhibiting multiple Plant growth promoting traits. Isolation and screening of bacteria possessing plant growth promoting rhizobacteria for a number of growth promoting attributes can be a promising approach/strategy for the improvement of plant growth and suppression of various diseases.

Thus the prevalence of *Pseudomonas aeruginosa*, *Serratia marcescens* and *Acinetobacter pittii* (with multiple plant growth-promoting traits) in the rhizospheric region can be employed to improve the growth and health of plants thereby helping farmers to increase crop productivity in a better way.Thus the prevalence of *Pseudomonas aeruginosa*, *Serratia marcescens* and *Acinetobacter pittii* (with multiple plant growth-promoting traits) in the rhizospheric region can be employed to improve the growth and health of plants thereby helping farmers and agriculture industry.

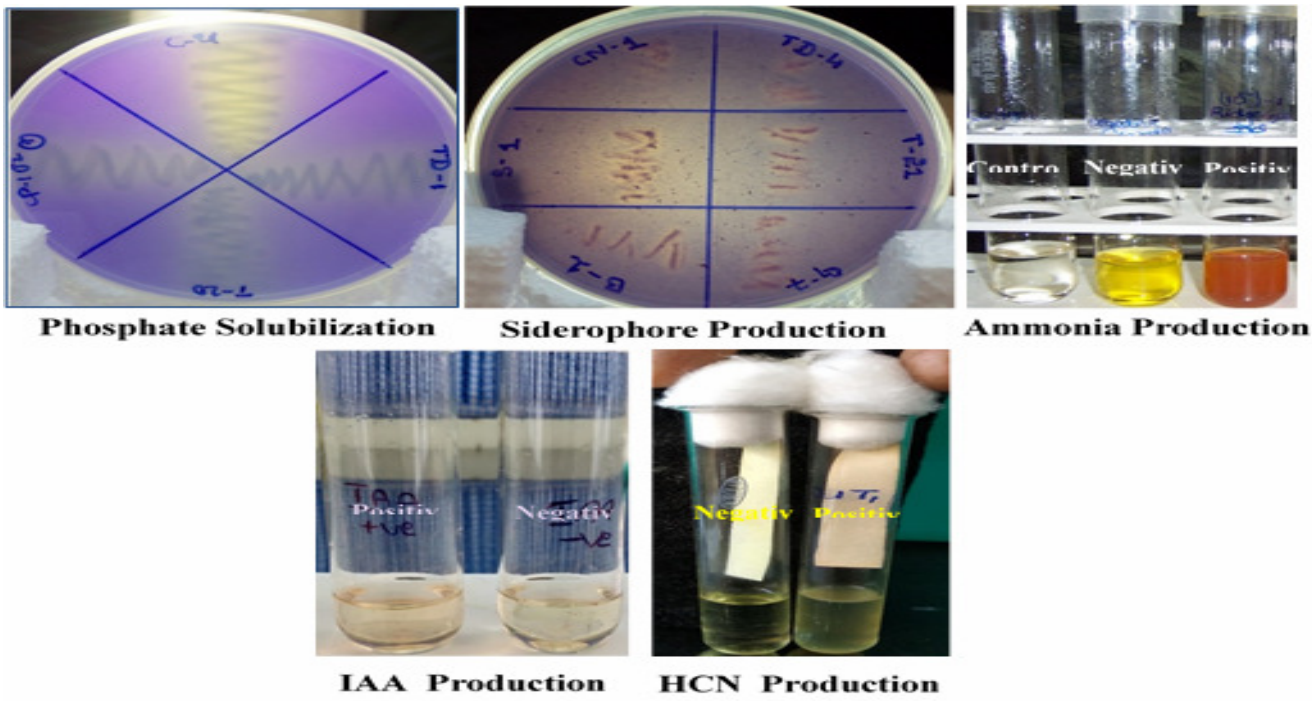

Fig. 1 : Plant Growth Promoting Traits of Isolated Rhizobacteria

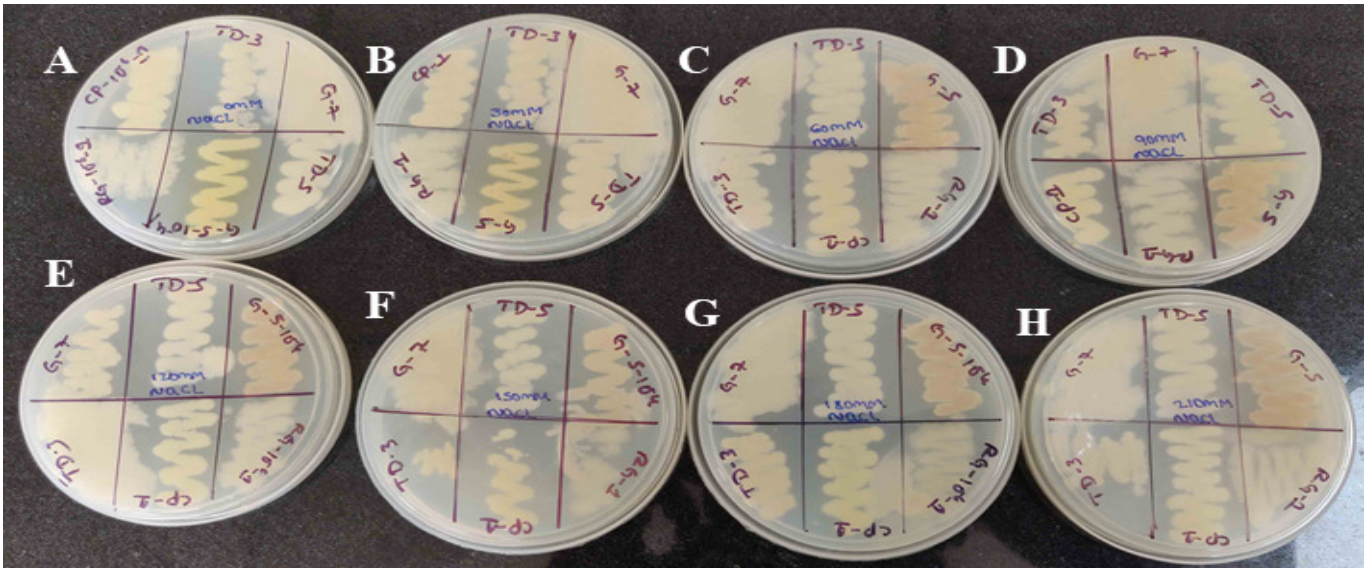

Fig. 2 : Growth of bacterial isolates on Nutrient Agar Supplemented with varied concentrations of NaCl

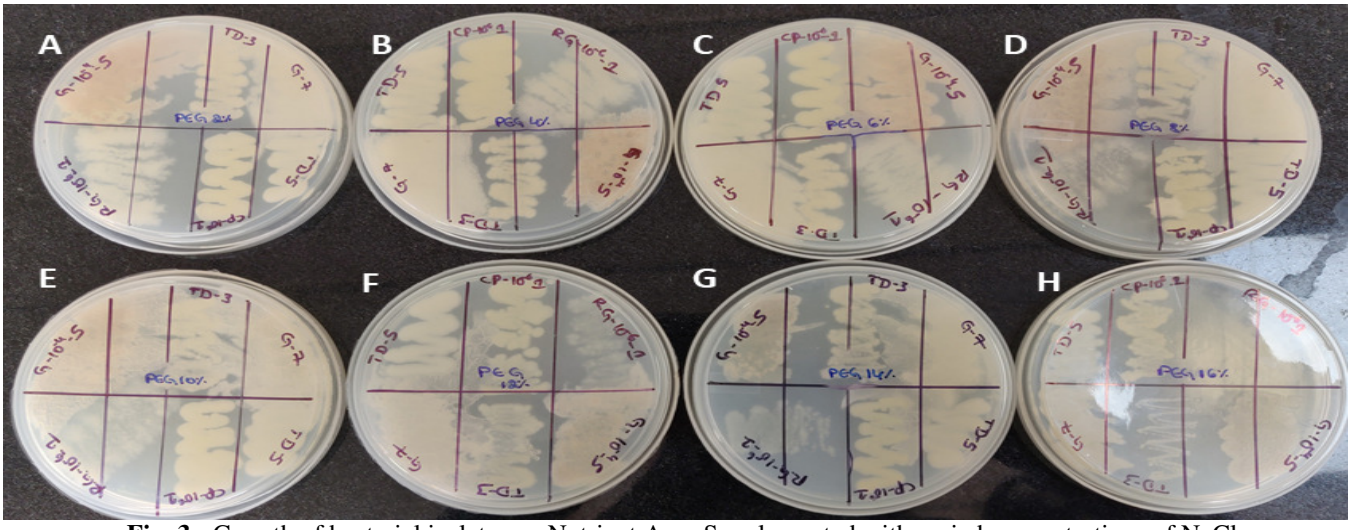

Fig. 3 : Growth of bacterial isolates on Nutrient Agar Supplemented with varied concentrations of NaCl

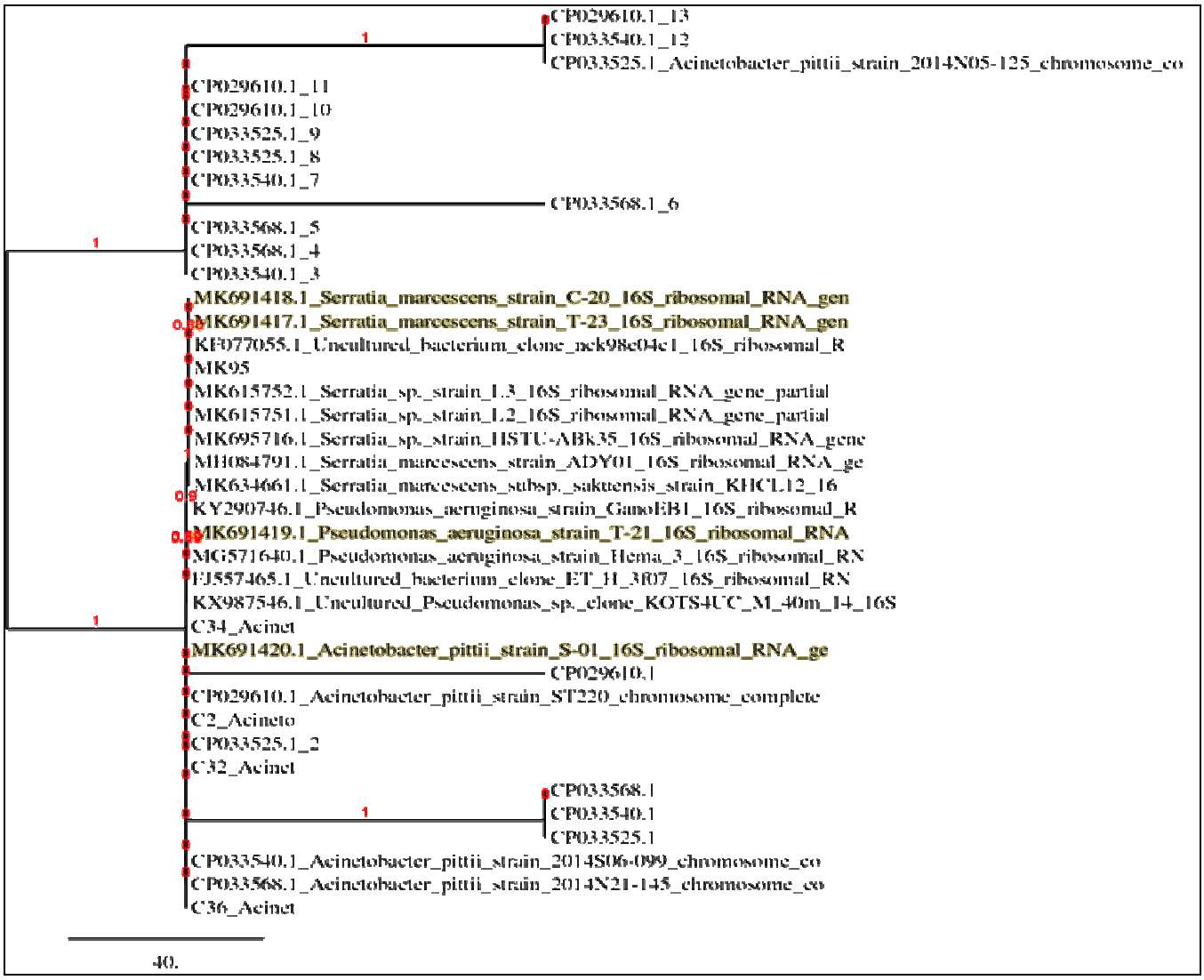

Fig. 4 : Phylogenetic tree of potential isolates was constructed using phylogeny.fr

Table 1: Isolation of rhizobacteria from various crop fields

Sl. No.	Crop	Location	Number of isolates
1.	Brinjal	Tathaguni	06
2.	Beetroot	Dibburahally	04
3.	Chick pea	Bommanahally	03
4.	Coriander	Beerappanahally	05
5.	Corn	Chikkadibburahally	07
6.	Capsicum	Tathaguni	02
7.	Groundnut	Beerappanahally	05
8.	Mulberry	Talakayalabetta	06
9.	Onion	Dimbarlahally	05
10.	Pigeon pea	Bommanahally	01
11.	Ridge gourd	Yalagalahally	06
12.	Spinach	Dibburahally	03
13.	Sugarcane	Bhashetihally	03
14.	Tomato	Thimmasandra	04
	Total isolates		60

Table 2: Production of growth promoting abilities of rhizobacteria

Sl. No.	Isolates	Phosphate	Siderophore	Ammonia	HCN	Indole acetic acid	Gibberellic acid
1	Tomato-3	+++	+++	+++	+++	+++	+++
2	Tomato-21	++++	++++	++++	++++	++++	++++
3	Groundnut-7	+++	+++	+++	+++	+++	+++
4	Groundnut-5	++++	++++	++++	++++	++++	++++

5	Spinach-1	++++	++++	++++	++++	++++	++++
6	Toor dal-3	+++	+++	+++	+++	+++	+++
7	Toor dal-5	++++	++++	++++	++++	++++	++++
8	Coriander-20	+++	+++	+++	+++	+++	+++
9	Onion-8	++++	++++	++++	++++	++++	++++
10	Brinjal-2	++++	++++	++++	++++	++++	++++
11	Beetroot-9	+++	+++	+++	+++	+++	+++
12	Chickpea-1	++++	++++	++++	++++	++++	++++

Note: '++++' indicates more production, '+++' indicates less production

Table 3: Biochemical characteristics of selected bacterial isolates

Sl. No.	Biochemical test	Tomato-21	Tomato-3	Groundnut-7	Spinach-1
1	Indole Test	+	-	+	-
2	Methyl Red Test	-	-	-	-
3	Voges-Proskauer Test	_	+	-	+
4	Citrate Utilization Test	+	+	+	+
5	Catalase Test	+	+	+	+
6	Oxidase Test	+	-	-	-
7	Hydrogen sulfide Test	+	-	-	-
	Carbohydrate Test				
	Glucose	-	+	-	+
8	Sucrose	-	+	-	+
	Mannitol	-	+	-	+
	Lactose	-	+	-	+
9	Casein hydrolysis	+	+	+	+
10	Starch hydrolysis	-	+	+	+
11	Triple sugar iron Test	-	+	+	+

Table 4 : Binomial name and accession numbers of potential bacterial isolates isolated in the present study

Sl. No.	Bacterial Isolate	Source	Binomial name	NCBI accession no.
1.	Tomato-21	Tomato	*Pseudomonas aeruginosa*	MK691419.1
2.	Tomato-3	Tomato	*Serratia marcescens*	MK691418.1
3.	Groundnut-7	Groundnut	*Acinetobacter pittii*	MK691420.1
4.	Spinach-1	Spinach	*Serratia marcescens*	MK691417.1

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Conflict of interest: The authors declare that there is no conflict of interest

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