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MOLECULAR CHARACTERIZATION OF PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR) ISOLATED FROM PADDY RHIZOSPHERE ZONE OF PADDY (*ORYZA SATIVA* L.)

Biotechnology

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ABSTRACT

BACKGROUND: Rhizosphere is the zone of soil influenced by the secretions of root and is considered to be a "hot spot" of microbial colonization. In the rhizosphere region the plant-microbe interaction may be beneficial, neutral, variable, or deleterious for plant growth. The rhizobacteria that exhibit beneficial effect on plant growth and development are termed Plant growth Promoting Rhizobacteria. **AIM:** To study the characterization of genomic DNA from different strains of rhizobacteria of *Oryza sativa* using *Pseudomonas* specific 16SrRNA gene PCR primers. **METHODOLOGY:** The serial dilution agar plate method or viable plate count method was used for isolation of rhizosphere bacteria. Molecular characterization of rhizobacterial isolates was performed on the basis of 16S rRNA sequence analysis. The rhizobacterial isolates were cultured for 48 hours and the genomic DNA of the isolates was extracted. 16s rRNA gene was amplified by PCR using 100 ng of genomic DNA employing universal primers 27F (5'- GTTTGATCCTGGCTCAG-3') and 1494R (5'-ACGGCTACCTTGTTACGACTT-3') and also by universal primers 27F (5'-AGAGTTTGATCTTGGCTCAG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3'). **RESULTS:** Thirty-one rhizobacteria were isolated from Paddy growing fields of Madhubani. The genomic DNA of each of the isolates was cleaved with restriction endonucleases, and the genomic DNA fragments of different size were separated by Agarose gel electrophoresis and probed with cloned DNA fragment. The rhizobacterial isolates were identified as *Bacillus subtilis, Bacillus megaterium, Bacillus pumilus, Bacillus polymorpha, Lactobacillus acidophillus, Lactobacillus brevis* and *Lactobacillus plantarum*, B. *subtilis, B. megaterium, B. pumilus* and *B. polymorpha* etc. **CONCLUSIONS:** 31 rhizobacterial isolates were characterized at molecular level. All these isolates have multifunctional activities viz., IAA production, Phosphate solubilization, Siderophore production, nitrification etc. On the basis of present results, it can be concluded that these rhizobacterial strains could be useful for bio-fertilizer individually or as a consortium in order to be used for an alternative approach to chemical fertilizers.

KEYWORDS

Rhizosphere, Cicer arietinum, Molecular characterization, 16S rRNA

INTRODUCTION

Rhizosphere is regarded as the narrow zone of the soil that is directly influenced by the root secretions. It is considered as a "hot spot" of microbial colonization and activity[1] and the largest ecosystem on earth with huge energy flux[2] (Barriuso *et al*., 2008). Soil which is not a part of the rhizosphere is considered as bulk soil[3]. The rhizosphere zone of soil contains many bacteria, actinomycetes and fungi that feed on sloughed-off plant cells, proteins and sugars secreted by roots. These organic substances are termed rhizodeposition. Except for some pathogenic microorganisms, many rhizosphere microbes improve the growth and health of plants by various mechanisms viz. nitrogen fixation and production of plant growth hormones[4, 5]. The different species of plants regulate the biodiversity and biogeography of the rhizobacteria[6-8].

In the rhizosphere region the plant-microbe interaction may be beneficial, neutral, variable, or deleterious for plant growth. The rhizobacteria that exhibit beneficial effect on plant growth and development are termed Plant growth Promoting Rhizobacteria (PGPR)[9, 10]. The different strains of PGPR-bacteria are responsible for growth promotion activities of plants[11]. This includes the ability to produce or change the concentration of plant growth hormones viz. Indole acetic acid (IAA), Gibberellic acid (GA), Cytokinin, and Ethylene; fix atmospheric dinitrogen; suppress or inhibit the growth of deleterious microorganisms by production of siderophore, β-1,3-glucanase, chitinases, antibiotics and cyanide; and dissolve phosphates and other nutrients. *Azotobacter* and *Azospirillum* are known to plant growth due to their ability to fix atmospheric dinitrogen, and plant growth stimulating hormone Indole acetic acid (IAA) is also involved in this process[12]. The use of phosphate solubilizing bacteria is also known to increase plant growth[13].

More than 90% of soil bacteria are not culturable. However, using advances techniques of biochemistry and molecular genetics viz. phospholipid fatty acid analysis (PLFA), nucleic acid isolation and hybridization. Polymerase chain reaction (PCR)-based methods, rRNA sequencing, percentage G+C content and DNA re-association between bacteria, restriction fragment length polymorphism (RFLP), amplified ribosomal DNA restriction analysis (ARDRA), cloning and sequencing techniques and microarrays rhizosphere bacteria have been successfully identified and characterized[14-16].

The most common bacteria of rhizosphere include the species of *Pseudomonas, Bacillus, Rhizobium, Agrobacterium, Alcaligenes, Azotobacter, Beijerinckia, Mycobacterium, Flavobacter, Cellulomonas, Micrococcus* and *Streptomyces* that cover up to 15% of the total surface of root[17]. The predominant bacterial strains in the rhizosphere include Gram-negative, rod-shaped, non-sporulating bacteria which belong to the groups Proteobacteria and Actinobacteria[18, 16]. Species of *Pseudomonas* are the most abundant rhizobacteria. Therefore, Gram-negative bacteria have greater capacity to utilize root exudates and are hence stimulated by rhizodeposition, while the Gram-positive bacteria are rather inhibited[19]. The aerobic bacteria are relatively lesser in the rhizosphere due to the reduced oxygen levels[20]. Gram-positive, rods or cocci and spore forming bacteria like *Bacillus* and *Clostridium* are relatively lesser. However, certain strains of *Bacillus* constitute the chief Gram-positive bacteria that inhabit the rhizosphere, followed by *Arthrobacter* and *Franckia*[2]. In crops like strawberry, oilseed, potato, wheat and rice etc. strains of Gram-positive *Bacillus* are more numerous than the gram-negative bacteria[14, 24-]. This may be due to the ability of Bacillus to form endospores and produce antimicrobial substances that inhibit the growth of other microbial competitors. Thus, it can be stated that the rhizosphere is richest ecological zone in relation to the microbial diversity[27,28].

Rice (*Oriza sativa* L.) is a staple food cultivated in in India, China, Japan, Pakistan, SriLanka and countries of South eastern Asia. The rhizosphere of rice holds a high diversity of rhizobacteria. *Pseudomonas fluorescence* and *Burkholderia pyrrocinia* are two main rhizobacteria of rice[29]. Therefore, it is essential to study the diversity of rhizobacteria in paddy growing fields at the molecular level. The present investigation is aimed to study the isolation of genomic DNA and characterization of different strains of rhizobacteria isolated from Paddy at the molecular level using *Pseudomonas* specific 16SrRNA gene PCR primers.

MATERIALS AND METHODS

Soil samples from the rhizosphere zone of Paddy growing in Madhubani were collected. The serial dilution agar plate method or viable plate count method was used for isolation of rhizosphere bacteria. About 10 gram of rhizosphere soil was added in 100 ml sterile water blank and shaked for 15 minutes on a magnetic shaker. The soil sample was then serially diluted to 10^{-2} to 10^{-6}. For this purpose, 10 ml of the soil suspension in agitated form was added to 90 ml of water to make the total volume to 100 ml. Serial dilutions of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, …10^{-7} were prepared by pipetting measured volumes (1 ml or 10 ml) into additional dilution banks (having 99 ml or 90 ml sterile water).

Finally, 1 ml aliquot of various dilutions were added to sterile Petri dish to which 15 ml of the sterilized, cooled and molten (at 45^{0}C) nutrient agar media (for bacteria) or Czapek Dox agar media (for actinomycetes). The dilutions of 10^{-3} to 10^{-6} were selected for enumeration of actinomycetes and 10^{-4} to 10^{-7} for bacteria relative to their proportion in the soil. Upon solidification, the Petri plates were incubated in an inverted position in a B.O.D. incubator for 7 days at 25±2^{0}C. The number of colonies appeared on the dilution plates were counted with the help of colony counter, and the average number was multiplied by the dilution factor to determine the number of microbes per gram or milliliter of the soil sample or Colony Forming Unit (CFU) per gram. The serial dilution agar-plate experiments were carried out in replicates of five.

Molecular characterization of rhizobacterial isolates was performed on the basis of 16S rRNA sequence analysis. The rhizobacterial isolates were cultured for 48 hours and the genomic DNA of the isolates was extracted by the method of Sambrook *et al*[30]. The rhizobacterial isolates were grown in minimal medium-1 (MM-1) and minimal medium-2 (MM-2) broths at 30^{0}C with shaking at 160g for 48 hours. MM-1 consisted of following ingredients:

KH_2PO_4:0.2 g/L: NH_4Cl0.25 g/L: KCl0.5 g/L: $CaCl_2.2H_2O$0.15 g/L: NaCl

1.0 g/L: $MgCl_2.6H_2O$ 0.62 g/L: Na_2SO_4 2.84 g/L: HEPES10 mM: pH6.8

MM-2 consisted of following ingredients:

$MgSO_4$0.5 g/L: $K_2HPO_4$1.3 g/L: Ca $(NO_3)_2$.06 g/L: Glucose0.06 g/L: Casamino acids 0.001 g/L

pH7.5: HEPES = [4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid.

The genomic DNA was also isolated according to Sharma and Singh[31]. For this purpose, pellets from 20 mL of stationary-phase cells were suspended in 50 mM Tris HCl, pH 8.0 containing 20 % sucrose, treated with 1 mg/mL of lysozyme after addition of EDTA to a final concentration of 25 mM, and lysed in 0.5 % SDS (Sodium do decyl sulphate). This was then digested with 40 mg/mL of RNase and 20 mg/mL of proteinase K, and DNA was banded in CsCl2 gradients in the presence of ethidium bromide, precipitated with isopropanol, washed with 70 % ethanol, and lyophilized. Pellets were suspended in distilled water and the DNA concentrations were determined by absorbance at 260 nm.

16s rRNA gene was amplified by PCR using 100 ng of genomic DNA employing universal primers 27F (5'- GTTTGATCCTGGCTCAG-3') and 1494R (5'-ACGGCTACCTTGTTACGACTT-3') (Sigma-Aldrich, USA) as suggested by Kalam *et al*[32] and by universal primers 27F (5'-AGAGTTTGATCTTGGCTCAG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3'). according to Turner *et al*[33]. Reaction mixture (25 µL) was prepared for full-length 16s rRNA gene amplification. It was denatured at 94^{0}C for 2 minutes, followed by 30 cycles consisting of denaturation at 94^{0}C for 1 minute; primer annealing at 52^{0}C for 1:30 minutes, primer extension at 72^{0}C for 2 minutes and finally extension at 72^{0}C for 10 minutes in a thermal cycler. The amplified products of 16s ribosomal gene were separated on 1 % agarose gel in 0.5 X TE (Tris-EDTA) buffer containing 2 µL ethidium bromide (20 mg/mL). λ Hind-III was used as a size marker. The gel was viewed under UV light. The amplified PCR products for full length 16s rRNA gene were sent to the sequencing company. After receiving the sequences for individual isolates, these 16s rDNA sequences were conducted by using the BLASTIN program for NCBI web site (http://www.ncbi.nlm.nih.gov)[34].

RESULTS

Thirty-one rhizobacteria were isolated from Paddy growing fields of Madhubani. These included *Bacillus subtilis, B. megaterium, B. pumilus, B. polymyxa, Lactobacillus acidophyllus, L. brevis, L. plantarum, Micrococcus luteus, Azotobacter beijerinckii, Azotobacter chroococcum, Pseudomonas putida, Pseudomonas fluorescens, Pseudomonas aeruginosa, Pseudomonas plecoglossicida, Arthrobacter chroococcum, Citrobacter sp. Streptococcus salivaris, Staphylococcus saprophyticus, Erwinia amylovora, Enterobacter hormechei, Agrobacterium fabrum, Flavobacterium anhuiense, Acinetobacter soli, Burkholderia* sp., *Rhizobium* sp., *Serratia* sp., *Nitrosomonas* sp., *Nitrosococcus* sp., *Nitrobacter* sp., *Nitrospira* sp. and *Nitrosovibrio* sp. Their total genomic DNA was isolated and characterized using 16S rRNA sequencing method. The molecular identification of each of the rhizobacterial isolates was determined on the basis of 16S rDNA sequence analysis. The rhizobacterial strains were also identified by RFLP (Restriction Fragment Length Polymorphism) analysis. The genomic DNA of each of the isolates was cleaved with restriction endonucleases, and the genomic DNA fragments of different size were separated by Agarose gel electrophoresis and probed with cloned DNA fragment. The results obtained have been presented in Table-1; Figure-1 and 2.

Table-1: Rhizosphere Isolates Of *Oryza Sativa* Growing In Paddy Fields Of Madhubani And Their Identity On The Basis Of 16S rRNA Gene Sequence (BLAST analysis)

Rhizobacterial isolates ID	Source of Isolation, Rhizosphere of	16S rDNA fragment length	Genus/Species identified	Similarity percent	Gene Bank Accession number
PR1	*Oryza sativa*	935 bp	*Bacillus subtilis*	100 %	MTR7511
PR2	*Oryza sativa*	993 bp	*Bacillus megaterium*	99 %	MTR7512
PR3	*Oryza sativa*	992 bp	*Bacillus pumilus*	99 %	MTR7513
PR4	*Oryza sativa*	994 bp	*Bacillus polymorpha*	99 %	MTR7514
PR5	*Oryza sativa*	1012 bp	*Lactobacillus acidophillus*	96 %	MTR7515
PR6	*Oryza sativa*	1015 bp	*Lactobacillus brevis*	96 %	MTR7516
PR7	*Oryza sativa*	1008 bp	*Lactobacillus plantarum*	96 %	MTR7517
PR8	*Oryza sativa*	1105 bp	*Micrococcus luteus*	99 %	MTR7518
PR9	*Oryza sativa*	1325 bp	*Azotobacter beijerinckii*	99 %	MTR7519
PR10	*Oryza sativa*	895 bp	*Azotobacter chroococcum*	99 %	MTR7520
PR11	*Oryza sativa*	1038 bp	*Pseudomonas putida*	99 %	MTR7521
PR12	*Oryza sativa*	1035 bp	*Pseudomonas fluorescens*	99 %	MTR7522
PR13	*Oryza sativa*	1006 bp	*Pseudomonas aeruginosa*	99 %	MTR7523
PR14	*Oryza sativa*	1036 bp	*Pseudomonas plecoglossicida*	99 %	MTR7524
PR15	*Oryza sativa*	1011 bp	*Arthrobacter chroococcum*	96 %	MTR7525
PR16	*Oryza sativa*	1015 bp	*Citrobacter sp.*	97 %	MTR7526
PR17	*Oryza sativa*	1350 bp	*Streptococcus salivaris*	96 %	MTR7527
PR18	*Oryza sativa*	1345 bp	*Staphylococcus saprophyticus*	97 %	MTR7528
PR19	*Oryza sativa*	1065 bp	*Erwinia amylovora*	95 %	MTR7529
PR20	*Oryza sativa*	1378 bp	*Enterobacter hormeichi*	98 %	MTR7530
PR21	*Oryza sativa*	1017 bp	*Agrobacterium fabrum*	100 %	MTR7531
PR22	*Oryza sativa*	973 bp	*Flavobacterium anhuiense*	97 %	MTR7532
PR23	*Oryza sativa*	1162 bp	*Acinetobacter soli*	99 %	MTR7533
PR24	*Oryza sativa*	1103 bp	*Burkholderia sp.*	99 %	MTR7534
PR25	*Oryza sativa*	1205 bp	*Rhizobium sp.*	94 %	MTR7535
PR26	*Oryza sativa*	935 bp	*Serratia sp.*	95 %	MTR7536

Pr27	*Oryza sativa*	1015 bp	*Nitrosomonas sp.*	93 %	MTR7537
PR28	*Oryza sativa*	1060 bp	*Nitrosococcus sp.*	93 %	MTR7538
PR29	*Oryza sativa*	1065 bp	*Nitrobacter sp.*	94 %	MTR7539
PR30	*Oryza sativa*	1107 bp	*Nitrospira sp.*	95 %	MTR7540
PR31	*Oryza sativa*	936 bp	*Nitrosovibrio sp.*	95 %	MTR7541

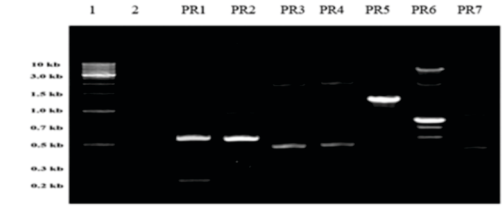

Figure-1: PCR pattern of seven rhizobacterial isolates. Lane 1 DNA molecular mass standard (Generular 2-Log DNA ladder; Size indicated in the left-hand margin); Lane-2, no DNA control (sterile water); Lanes PR1-PR7, Rhizobacterial isolates of Paddy.

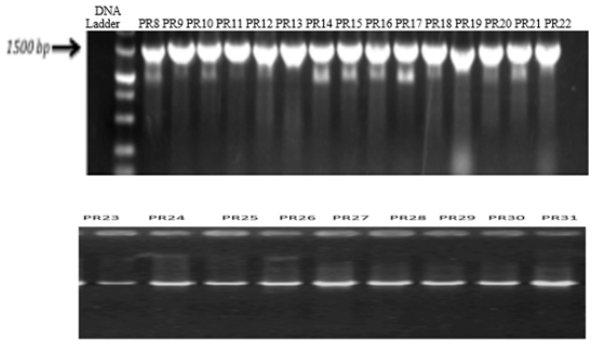

Figure-2: Gel electrophoresis of 16S rRNA gene sequence fragments of Rhozobacterial isolates PR8-PR31; PR8: *Micrococcus luteus*, PR9: *Azotobacter beijerinckii*, PR10: *Azotobacter chroococcum*, PR11: *Pseudomonas putida*, PR12: *Pseudomonas fluorescens*, PR13: *Pseudomonas aeruginosa*, PR14: *Pseudomonas plecoglossicida*, PR15: *Arthrobacter chroococcum*, PR16: *Citrobacter* sp., PR17: *Streptococcus salivaris*, PR18: *Staphylococcus saprophyticus*, PR19*: Erwinia amylovora*, PR20: *Enterobacter hormeichi*, PR21: *Agrobacterium fabrum*, PR22: *Flavobacterium anhuiense*, PR23: *Acinetobacter soli*, PR24: *Burkholderia* sp., PR25: *Rhizobium* sp., PR26: *Serratia* sp., PR27: *Nitrosomonas* sp., PR28: *Nitrosococcus* sp., PR29: *Nitrobacter* sp., PR30: *Nitrospira* sp. PR31: *Nitrosovibrio* sp.

DISCUSSION

Rhizosphere is a narrow zone of the soil which is directly influenced by the root secretions. It is regarded as a "hot spot" of microbial colonization and the largest ecosystem on the earth with huge energy flux.

Thirty-one PGPR isolates (PR1 to PR31) were screened and characterized at the molecular level (Table-1). Amplicons of approximately 1500 bp were obtained after of the 16S rDNA. The analysis of the 16S rRNA gene sequences were performed by NCBI-BLAST, database. The direct sequencing of amplified PCR products was scored the maximum identities of 93 % to 100 % homology in molecular identification of the Plant growth promoting rhizobacterial isolates. The rhizobacterial isolates PR1, PR2, PR3, PR4, PR5, PR6 and PR7 were identified as *Bacillus subtilis, Bacillus megaterium, Bacillus pumilus, Bacillus polymorpha, Lactobacillus acidophillus, Lactobacillus brevis* and *Lactobacillus plantarum* respectively. B. *subtilis, B. megaterium, B. pumilus and B. polymorpha* showed 99 % to 100 % homology, whereas *L. acidophillus, L. brevis* and *L. plantarum* showed 96 % homology in their molecular identification (Table-1; Figure-1).

The rhizobacterial strains PR8, PR9, PR10, PR11, PR12, PR13, PR14, PR23 and PR24 showed 99 % homology and were identified as *Micrococcus luteus, Azotobacter beijerinckii, Azotobacter chroococcum, Pseudomonas putida, Pseudomonas fluorescens, Pseudomonas aeruginosa, Pseudomonas plecoglossicida, Acinetobacter soli* and *Burkholderia* sp. respectively (Table-1; Figure-2). Isolates PR20 and PR21 showed 98 % and 100 % homology and identified as *Enterobacter hormeichi* and *Agrobacterium fabrum* respectively. The isolates PR15 and PR17 showed 96 % homology and identified as *Arthrobacter chroococcum* and *Streptococcus salivaris* respectively. The strains PR15 and PR18 were identified as *Citrobacter* sp. and *Staphylococcus* saprophyticus respectively with 97 % homology. The strains PR19, PR22, PR25 and PR26 showed 95 %, 94 %, 94 % and 95 % homology, and were identified as *Erwinia amylovora, Flavobacterium anhuiense, Rhizobium* sp. and *Serratia* sp. respectively. Similarly, the strains of nitrifying bacteria viz., PR27, PR28, PR29, PR30 and PR31 showed 93 %, 93 %, 94 %, 95 % and 95 % and were identified as *Nitrosomonas* sp., *Nitrosococcus* sp., *Nitrobacter* sp., *Nitrospira* sp. and *Nitrosovibrio* sp. respectively (Table-1; Figure-2). The present findings gain support from the work of Kay Thi Oo *et al*[35] and Sadaf Kalam *et al*[36] who also identified the rhizobacterial strains as PGPR for sustainable agriculture. Although rhizobacteria are largely represented by the species of Pseudomonas and Bacillus, many authors have also reported rhizobacteria belonging to Enterobacteriaceae such as *Serratia, Pantoea* and *Enterobacter* strains and some other families[37].

CONCLUSIONS

In the present investigation, 31 rhizobacterial isolates were characterized at molecular level. All these isolates have multifunctional activities viz., IAA production, Phosphate solubilization, Siderophore production, nitrification etc. On the basis of present results, it can be concluded that these rhizobacterial strains could be useful for bio-fertilizer individually or as a consortium in order to be used for an alternative approach to chemical fertilizers. Formulation of rhizobacteria based biofertilizers on large scale for sustainable agriculture development require further investigation at scientific level.

Conflict Of Interest: Authors declare no conflict of interest directly or indirectly.

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