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Full Length Research Paper

Rhizosphere of rice plants harbor bacteria with multiple plant growth promoting features

Shailesh K. Shahi[1], Ashutosh K. Rai[1], Madhu B. Tyagi[2], Rajeshwar P. Sinha[3] and Ashok Kumar[1]*

[1]School of Biotechnology, Banaras Hindu University, Varanasi – 221005, India.
[2]Department of Botany, MMV, Banaras Hindu University, Varanasi – 221005, India.
[3]Centre of Advanced Study in Botany, Banaras Hindu University, Varanasi – 221005, India.

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114 diazotrophic bacteria from the rice rhizosphere of five districts of Eastern Uttar Pradesh (India) were isolated and screened for plant growth promoting (PGP) activities employing standard microbiological and biochemical techniques. All these isolates showed nitrogenase activity in the range of 0.23 to 1.72 µmol C_2H_4 mg^{-1}protein h^{-1}. Further analysis showed that 84 (73.68%) isolates were Indole-3-acetic acid (IAA) producer; the value of IAA production ranged from 10.1 to 353.0 µg IAA mg^{-1} protein. IAA production occurred solely in the medium supplemented with tryptophan. P solubilization activity was observed in 28 (24.56%) isolates, the activity being in the range of 38.50 to 321.0 P released µg mg^{-1} protein. 45 (39.46%) isolates were capable of producing siderophore, the range of production being 4.50 to 223.26 µg mg^{-1} protein. Analysis of molecular diversity was made by amplified ribosomal DNA restriction analysis (ARDRA) and denaturating gradient gel electrophoresis (DGGE), which exhibited distinct differences among all the isolates. Of the 114 isolates, twenty one (21) isolates showed multiple plant growth promoting traits and were potent in terms of PGP activities. These isolates were identified on the basis of 16S rDNA sequencing and belonged to the genera *Pantoea, Bacillus, Microbacterium*, *Pseudomonas, Sphingomonas, Ancylobacter, Enterobacter, Advenella*, *γ-proteobacterium* strain VA3S1*, Rhizobium* and *Agrobacterium.* Findings of this study suggest that certain isolates may be exploited for developing a potential source of biofertilizer.

Key words: Plant growth promoting rhizobacteria, N_2 fixation, amplified ribosomal DNA restriction analysis, indole-3-acetic acid, siderophore, denaturing gradient gel electrophoresis, temperature gradient gel electrophoresis

INTRODUCTION

The microbe-plant interaction in the rhizosphere can be beneficial, neutral, variable, or deleterious for plant growth and development. During the last few decades, a large array of bacteria including species of *Pseudomonas, Azospirillum, Azotobacter, Klebsiella, Enterobacter, Alcaligens, Arthobacter, Burkholderia, Bacillus* and *Serratia* have been isolated from rhizosphere of various crop plants (Kloepper et al., 1989; Glick, 1995; Ladha and Reddy, 2000; Franche et al., 2009; Venieraki, 2011). Majority of these bacteria harbor a number of plant growth promoting (PGP) features such as fixation of atmospheric dinitrogen (N_2), mineral P solubilization, and production of phytohormones (IAA and gibberellic acid), siderophores and certain antagonistic compounds which are effective in controlling plant diseases (Miyamoto et al., 2004; Richardson et al., 2009). Interest in the beneficial plant growth promoting rhizobacteria (PGPR) has increased tremendously in recent years due to their potential as biofertilizers

*Corresponding author. E-mail: kasokbt@rediffmail.com. Tel: 091-542-2368331/6701593. Fax: 091-2368693/2368174.

Abbreviations: PGP, Plant growth promoting; **IAA,** indole-3-acetic acid; **ARDRA,** amplified ribosomal DNA restriction analysis; **DGGE,** denaturating gradient gel electrophresis; **FISH,** fluorescence *in situ* hybridization; **SSCP,** single strand conformation polymorphism.

(Vessey, 2003; Richardson et al., 2009; Compant et al., 2010).

Importance of IAA production by PGPR has been widely acknowledged (Kennedy et al., 2004; Roesch et al., 2007; Ashrafuzzaman et al., 2009). IAA secreted by the bacteria promotes root growth directly by stimulating plant cell elongation or cell division or indirectly by influencing bacterial 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity (Pedraza et al., 2004). Similarly, role of P solubilizing bacteria in plant growth and development has been well documented (De Freitas et al., 1997; Richardson et al., 2009). Using radio-labelled insoluble P, it has been demonstrated that microbes indeed transfer solubilized P to the plants. Equally important is the production of siderophores by PGPR especially under iron-limited conditions (Ladha and Reddy, 2000; Ahmad et al., 2008). Many bacteria and fungi are capable of producing more than one type of siderophore and benefit plants in one or other way (Dimkpa et al., 2008).

With the development of newer molecular techniques and approaches such as rRNA sequencing, random amplified polymorphic DNA (RAPD), FISH (fluorescence *in situ* hybridization), DGGE (denaturating gradient gel electrophoresis), SSCP (single strand conformation polymorphism) and TGGE (temperature gradient gel electrophoresis), it has now been possible to characterize both culturable and non-culturable bacteria (Schmalenberger et al., 2001; Sørensen et al., 2009; Venieraki et al., 2011). Nevertheless, the complex network of interactions which comprises life in the rhizosphere of crop plants is still poorly understood and its disclosure is expected to reveal various unhidden facts (Sørensen et al., 2009).

Till date, majority of the workers have considered one or two plant growth promoting characters of a bacterium in their study; reports dealing with the screening of multiple PGP characters in an individual species are sporadic. In this study, an attempt was made to screen the presence of multiple PGP characters and construct a road map of diazotrophic bacteria associated with rice rhizosphere of five districts of Eastern Utter Pradesh (India).

MATERIALS AND METHODS

Sample collection and isolation of bacteria

10 distantly located rice fields from each of the five districts namely, Ajamgarh, Chandauli, Ghazipur, Jaunpur and Varanasi of Eastern Uttar Pradesh (UP), India, were selected for this study. In general, rice variety Mansoori and Malviya dhan-36 were cultivated in these fields. Sampling was done at flowering stage in the month of November, 2007. The rhizospheric soil samples were collected from each field by uprooting the rice plants (four plants from each corner and one from the middle of each field) followed by transferring the soil adhered to roots in sterile polythene bags. After proper mixing, 10 g soil of each field was transferred to a 250 ml Erlenmeyer flask containing 90 ml sterile distilled water and shaken (120 rpm) for 30 min. Serial dilutions (up to 10^{-5}) were made and 0.1 ml aliquots were spread on to $JNFb^-$ solid agar plates (Dobereiner, 1995) which contained (in g/L); malic acid (5.0), K_2HPO_4 (0.6), KH_2PO_4 (1.8), $MgSO_4.7H_2O$ (0.2), NaCl (0.1), $Na_2MO_4.2H_2O$ (0.002), Fe-EDTA (4 ml 1.4%) and KOH (4.5), pH was adjusted to 5.8. All the plates were incubated at 37°C in an incubator. After 4 to 5 days of incubation, distinct bacterial colonies appearing on the plates were selected and maintained for several generations in the aforementioned medium to confirm the diazotrophic nature of various isolates. Distinct morphotypes of bacteria were screened on the basis of colony color, pigment formation, elevation, shape and size. For testing the formation of a pellicle, various isolates were grown in semi-solid medium (0.15% agar-agar) without shaking.

Estimation of nitrogenase activity

Nitrogenase activity was measured by acetylene reduction assay as per the method of Stewart et al. (1967). Equal amount of inoculum from each isolate was added in 3-ml semi-solid (0.15% agar-agar w/v) $JNFb^-$ medium in a 7-ml vacutainer tube (Becton-Dickinson, Rutherford, NJ, USA) and grown for 96 h at 30°C. Acetylene was injected in each tube by a hypodermic syringe to attain 10% final concentration and the tubes were incubated at 30°C without shaking. At the desired time interval, 0.2-ml gas phase was withdrawn and the ethylene formed was analyzed in a 5700 Nucon Gas Chromatograph (Nucon Engineers Ltd., New Delhi) fitted with Porapak R column and flame ionization detector. N_2 was used as the carrier gas.

Indole-3-acetic acid (IAA) production

The production of indole acetic acid (IAA) was determined as per the method of Gordon and Weber (1951) using pure IAA as standard. Briefly, 1.5 ml of culture grown in $JNFb^-$ medium supplemented with tryptophan (100 µg/ml) for 72 h was centrifuged at 12,000 rpm for 5 min and in the resulting supernatant (1 ml), 2 ml $FeCl_3$-$HClO_4$ reagent was added. Absorbance of pink color was measured at 530 nm after 25 min in a Milton Roy 1201 UV-Vis Spectrophotometer (Milton Roy Company, USA).

Phosphate solubilisation

Test for phosphate solubilization was made following the method of Goldstein (1986). The appearance of clearing zone around bacterial colonies after 96 h of growth at 30°C was used as indicator for positive P solubilization. Plates inoculated with heat-killed cells served as control. Quantitative estimation of P was made as per the method described by Jha and Kumar (2007).

Siderophore assay

Test for the production of siderophore was made according to the method of Neilands (1981). Accordingly, chrome azurol S (CAS) agar plates containing: 1 mM chrome azurol S, $FeCl_3.6H_2O$ (1 mM final concentration) made in 10 mM HCl, and N, N-cetyl trimethyl ammonium bromide (2 mM) (CTAB) was prepared. Spot inoculation of test culture was made on CAS-agar plates and incubated at 30°C for 72 h. Yellow to orange halo zone appearing around the colonies was recorded as positive test for siderophore production.

16S rDNA amplification and sequencing

Genomic DNA was extracted employing DNEasy extraction kit

(Qiagen, Germany) according to the instructions of manufacturer. 16S rDNA gene (1.5 kb) was amplified using universal primer in a final volume of 50 µl. The PCR reaction mix included; 1.5 U of *Taq* DNA polymerase (Bangalore Genei, India), 1 X PCR assay buffer with 1.5 mM $MgCl_2$, 50 pmol of each forward and reverse primers (Integrated DNA Technologies, USA), 125 µM of each dNTPs (Bangalore Genei, India) and 50 ng template DNA. The sequence of primer used was 8f 5'-AGA GTT TGA TYM TGG CTC AG-3' and 1495r 5'-CTA CGG CTA CCT TGT TAC GA-3' where Y = C or T and M = A or C. Thermal cycle was set as: initial denaturation at 94°C for 3 min, 35 cycles of 1 min at 94°C, 1 min at 58°C and 2 min at 72°C followed by final extension at 72°C for 5 min and storage at 4°C. Partial 16S rRNA fragment of V2-V3 region (400 bp) was amplified by the primer set; Fd 5'-ACT GGC GGA CGG GTC AGT AA-3' and rev. 5'-CGT ATT ACC GCG GCT GCT GG-3'. Thermal cycle was set as: an initial denaturation at 95°C for 3 min followed by 30 cycles of 95°C for 1 min, 50°C for 1 min, 72°C for 1 min 10 s, and final extension at 72°C for 5 min.

Sequencing of 16S rDNA was done with forward primer from both the ends by the di-deoxy chain termination method in an automated DNA sequencer (ABI Prism; Model 3100, Applied Biosystems, USA). 16S rDNA sequence was compared to the GenBank database using the algorithm BLASTN program to identify the most similar 16S rDNA (Altschul et al., 1997). The most similar sequences were further aligned by CLUSTAL (version 2.0.10). Phylogenetic tree was inferred by the neighbor-joining method using MEGA version 4.0 at 1000 bootstrap (Tamura et al., 2007).

Amplified ribosomal DNA restriction analysis (ARDRA)

16S rDNA (1.5 kb) amplified by PCR was purified and subjected to restriction digestion by *Alu*I and *Rsa*I according to the instructions of manufacturer (New England Biolabs, UK). Restriction digestion was done in a final volume of 25 µl containing 1 X restriction enzyme buffer, 0.30 µl (3.0 U) restriction enzyme and 15 µl PCR products. After mixing, samples were incubated for 6 h in a water-bath preset at 37°C. Reaction was terminated by heat inactivation of restriction enzymes at 70°C for 20 min .The samples were analyzed by agarose gel (3%) electrophoresis and monitored on gel documentation unit (BioRad Laboratories, USA).

Denaturing gradient gel electrophoresis (DGGE)

16S rDNA (200 bp) was amplified with the forward primer containing GC clamp at 5' end (f352T: 5'- CGC_CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG TGG C- 3' and 519r: 5'-ACC GCG GCT GCT GGC AC- 3') in 25 µl of reaction mixture containing; 1XPCR buffer, 2.5 mM $MgCl_2$ (Bangalore Genei, India), 100 ng of the template DNA, 25.0 pmol each of the forward and reverse primers, 250 µM each of dNTPs, and 1 U of *Taq* DNA polymerase (Bangalore Genei, India). The touchdown PCR program was performed with thermal cycle set as; initial denaturation at 95°C for 5 min, followed by 6 cycles of 95°C for 1 min, 65°C for 1 min, and 72°C for 1 min, in which the annealing temperature was reduced by 0.5°C/cycle from the preceding cycle, and then 24 cycles of 95°C. Perpendicular DGGE was performed with "The Decode Universal Mutation Detection System" (BioRad Laboratories, USA). A uniform gradient gel of 0 to 100% denaturant was prepared which was changed several times so as to optimize suitable concentration (30 to 70% denaturant showed the best result).

RESULTS

With a view to isolate N_2-fixing bacteria, selective medium (medium devoid of inorganic combined nitrogen sources) was used which resulted in the isolation of 321 diazotrophic isolates from 50 rhizospheric soil samples of five districts of Eastern Uttar Pradesh (Table 1). However, routine examination revealed that several isolates were common in each district, such isolates were excluded and finally 114 isolates exhibiting distinct morphotypes in terms of colony morphology including size, colour, elevation, mucilage formation, and harbouring certain plant growth promoting activities were selected. With a view to find out most probable number (MPN), serial dilution technique was employed which showed population density in the range of 3.34×10^6 to 1.33×10^7 CFU g^{-1} of rhizospheric soil of all the five districts.

Growth in $JNFb^-$ semi-solid medium (0.15% agar-agar) showed the formation of pellicle by majority of the isolates. The colour of pellicles varied from whitish to creamy to yellowish and marked differences in the location of pellicles were observed (1 to 2 mm below the surface of the medium in some isolates and 4 to 12 mm in others) in different isolates. Preliminary screening of plant growth promoting (PGP) characters namely, IAA and siderophore production, and P solubilization suggested the presence of one or two or all the three aforementioned characters in different isolates (Table 1). Assay of nitrogenase activity revealed significant differences in acetylene reduction activity by all the isolates. Out of the 114 isolates, the highest rate of nitrogenase activity was noted in V1S7 (1.72 µmol C_2H_4 mg^{-1} protein h^{-1}) followed by G1S3 (1.65 µmol C_2H_4 mg^{-1}protein h^{-1}), the lowest rate (0.23 µmol C_2H_4 mg^{-1} protein h^{-1}) was detected in JP5S6. Test of IAA production by cultures grown in $JNFb^-$ medium supplemented with 100 µg ml^{-1} tryptophan showed positive result in 84 (73.68%) isolates (Table 1). Quantitative estimation of IAA showed the highest production by the isolate J2S5 (353.0 µg IAA mg^{-1} protein), the lowest content was noted in G4S10 (10.10 µg IAA mg^{-1} protein). However, none of the isolates were capable to produce IAA in the absence of tryptophan in the medium. Test of P solubilization activity showed positive result in 28 (24.56%) isolates (Table 1). Further analysis revealed that the isolate AJ 2-3 had the highest level of P solubilization activity (321.0 µg mg^{-1} protein) followed by AJ 1-4 (280.50 µg mg^{-1} protein), the lowest value (38.50 µg mg^{-1} protein) was noted in JP2S6. Similarly, test of siderophore production exhibited positive result for 45 (39.46%) isolates. The value of siderophore (hydroxamate type) production by different isolates was in the range of 4.50 to 223.26 µg mg^{-1} protein. Out of all the isolates, V1S7 showed the highest value (223.26 µg mg^{-1} protein) and the lowest value (4.50 µg mg^{-1} protein) was noted in VB5S7. Detailed screening of PGP characters in all the 114 diazotrophic isolates revealed that 21 isolates (18.42%) were endowed with three PGP characters (IAA and siderophore production and phosphate solubilization) and 15 isolates (13.15%) lacked the aforementioned characters. Further screening of isolates for double

Table 1. Isolation of diazotrophic plant growth promoting bacteria from rice rhizosphere of five districts of Eastern UP[a].

District	Distinct isolate	IAA producer[b]	P-solubilizer[b]	Siderophore producer[b]
Azamgarh	22	16 (72.72)[c]	8 (36.36)	10 (45.45)
Chandauli	11	9 (81.81)	2 (18.18)	3 (27.27)
Ghazipur	23	16 (69.56)	5 (21.73)	10 (43.47)
Jaunpur	25	22 (88.00)	5 (20.00)	6 (24.00)
Varanasi	33	21 (63.63)	8 (24.24)	16 (48.48)
Total	114	84 (73.68)	28 (24.56)	45 (39.46)

[a] All isolates were grown under identical conditions for testing PGP characters. [b] The range of nitrogenase activity, IAA production, P solubilization and siderophore production among all the 114 isolates was; 0.23 to 1.72 µmol C_2H_4 mg^{-1}protein h^{-1}, 10.1 to 353.0 µg IAA mg^{-1} protein, 38.50 to 321.0 P released µg mg^{-1} protein, and 4.50 to 223.26 µg mg^{-1} protein, respectively. Background value obtained in set without culture was adjusted to zero (as blank) so as to get actual value in test samples. Results are based on three experiments conducted separately but under identical conditions. [c] Value in parenthesis represents %.

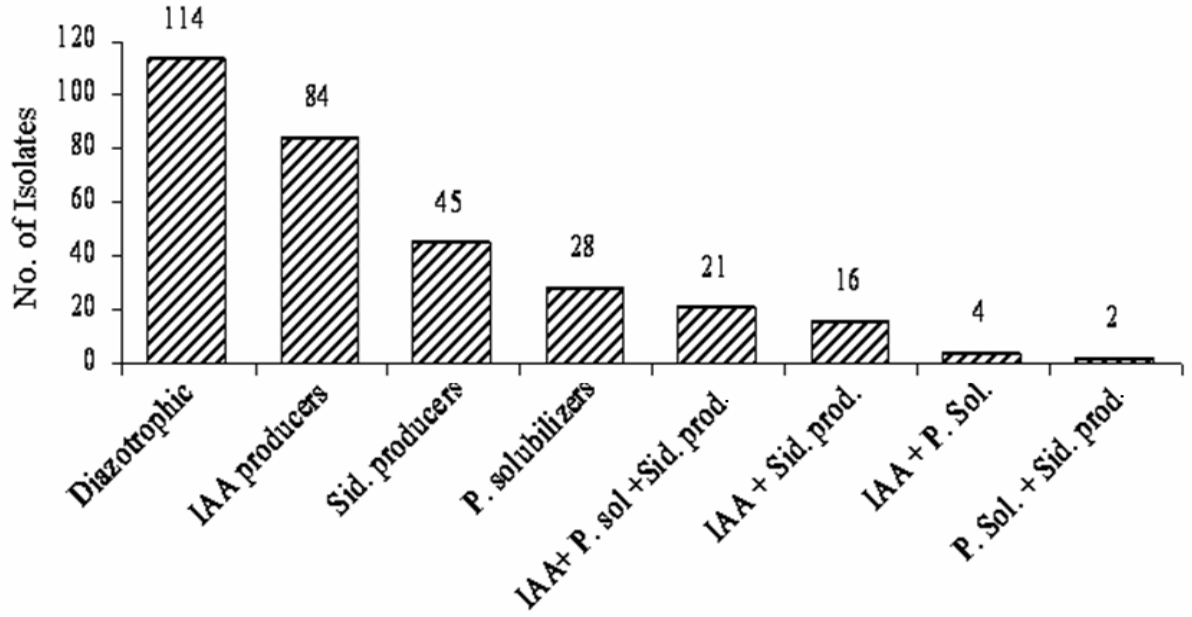

Figure 1. Occurrence of various plant growth promoting features among different isolates. 114 isolates were screened for individual PGP characters wherein 84 were IAA producers, 45 siderophore producers and 28 were P solubilizers. Among these three groups, screening for the presence of combination of characters was made. Tests made for PGP characters among the three groups resulted in screening of isolates with more than one character. This included 21 isolates with three characters (IAA^++ $siderophore^+$ + P^+) and 22 isolates with two characters which included IAA^+ + $siderophore^+$ (16), IAA^+ + P^+ (4) and P^++S^+ (2). The number of isolates with two characters is excluding 21 isolates which had three characters.

characters showed that 16 isolates were IAA + siderophore producers, 4 contained IAA+ P solubilizing characters and 2 had P solubilizing + siderophore producing characters. It is pertinent to mention that the number of isolates with double characters shall increase if the number of isolate showing triple characters (21) is included in aforementioned groups since these 21 isolates also share double characters (Figure 1). The number of isolates with separate combination of characters is shown in Figure 1.

Once it became evident that all the 114 isolates indeed show significant differences in morphological and PGP characters, it was desirable to analyze molecular diversity employing PCR assay. Typical representation of ARDRA pattern of 23 isolates from rhizospheric soil of Ghazipur district is shown in Figure 2. It is evident that in majority of the isolates, 7 to 8 bands were noted if 16S rDNA was digested with *Alu*1 or *Rsa*1. It is also evident from the restriction pattern that the *Alu*I showed better distinguishing pattern than *Rsa*1 (Figure 2A and B). In order to search homology among these isolate, combined ARDRA pattern based on *Alu*1 and *Rsa*1 digestion was used for cluster analysis. It is evident from the data of Figure 3 that all the isolates could be grouped in three

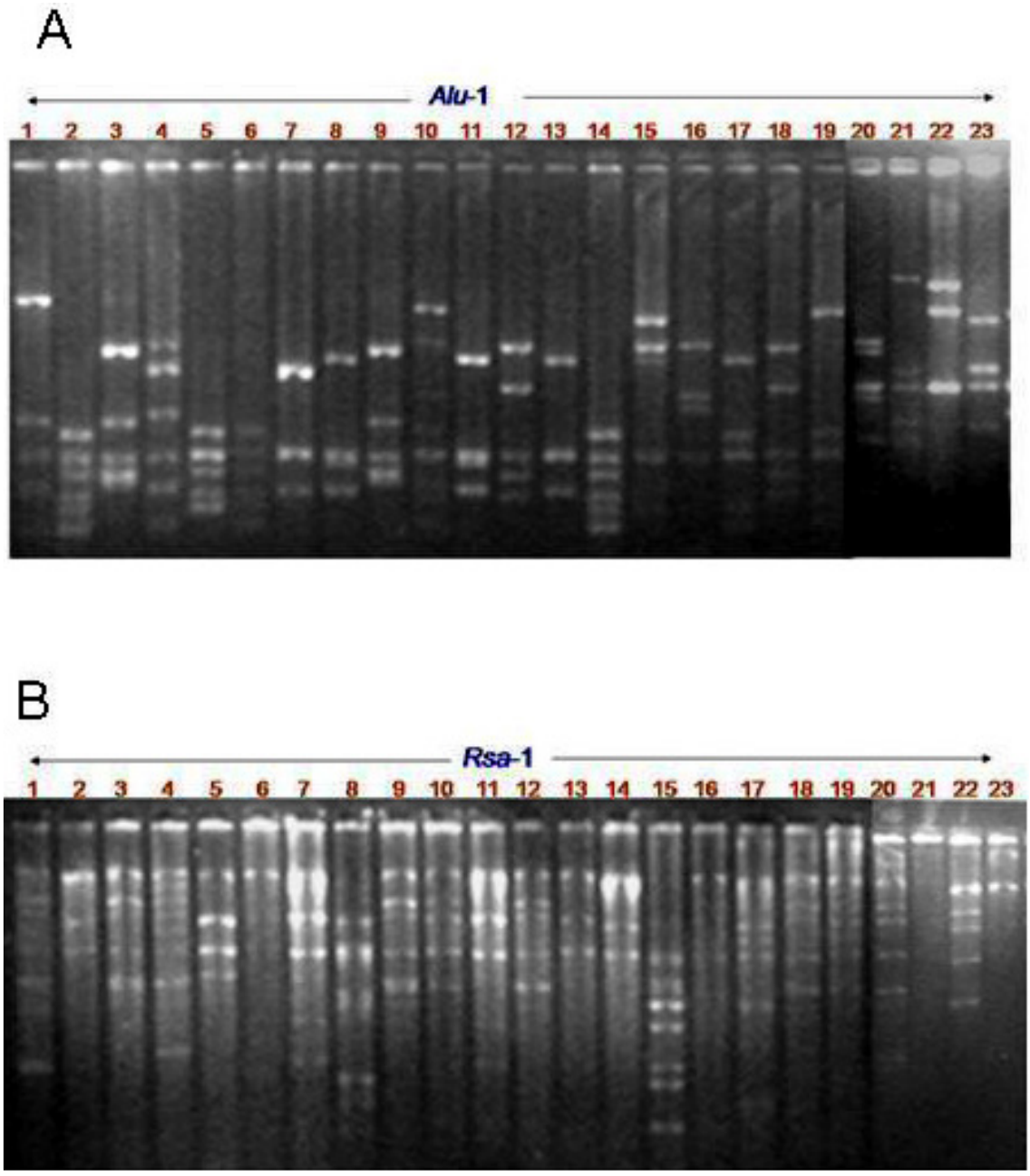

Figure 2. Typical representation of ARDRA pattern of various isolates of Ghazipur district. 16S rDNA (1.5 kb) was digested with *Alu1* (A) and *Rsa1*(B). Lanes 1 to 23; 1- G1S3, 2- G3S10, 3- G3S8A 4- G3S12, 5- G4S10, 6- G6S10, 7- GS1S1, 8- GS1S2, 9- GS1S9, 10- GS3S3, 11- GS4S4, 12-GS7S1, 13-GS8S2, 14-GS8S5, 15-G4S11, 16-G5E, 17-G5S9, 18- GS2S6, 19- GS7S4, 20-G6S6, 21-GS3S1, 22-GS3S5, and 23-GS4S2. G-Ghazipur, G1, 3, 4, 5 and 6- Ghazipur collection site, and S- isolate number from individual site.

clusters. Clusters A, B and C included 2, 19 and 2 isolates respectively and none of the isolates showed 100% homology to any group. Cluster A showed 68% similarity with cluster B and C whereas cluster B and C showed 70.60% similarity to each other (Figure 3). Similarly, isolates of Varanasi (33) were found in four clusters including, A, B, C and D consisting 22, 3, 7 and 1 isolates, respectively. Cluster A showed 76.2% similarity with cluster B, cluster C showed 73.9% similarity with cluster A and B while cluster D showed 73.25% similarity with cluster A, B and C. Isolates of Jaunpur, Azamgarh and Chandauli districts were also found in three clusters and none of the isolates showed 100% homology to each other. That all the isolates of five districts are indeed different also became evident from DGGE analysis. Typical representation of DGGE pattern of 19 isolates of

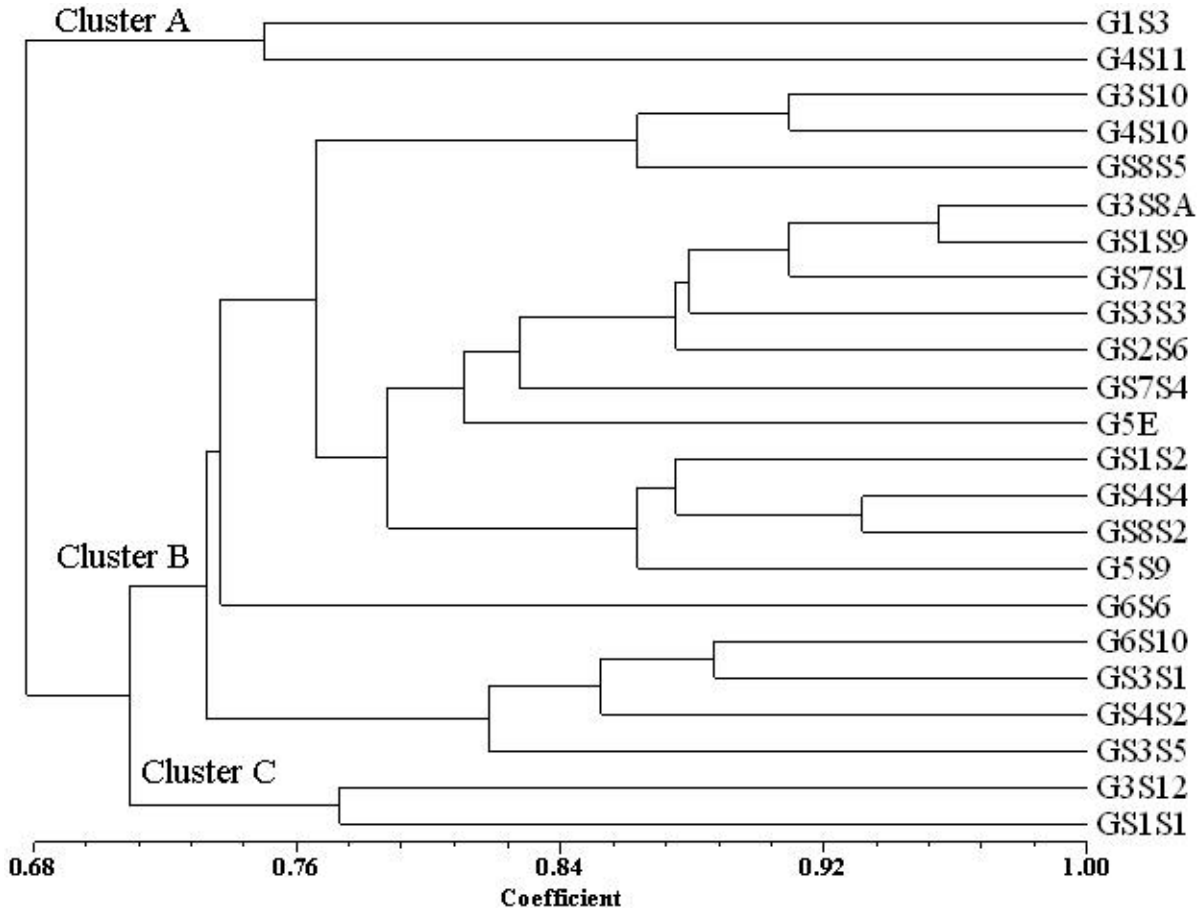

Figure 3. Dendrogram based on restriction digestion of 16S rDNA (1.5 kb) with *Alu1* and *Rsa1*. Phylogenetic tree was constructed using NTSYS PC 2.1 software.

Varanasi district is shown in Figure 4. It is evident that the location of desired band of 16S rDNA (200 bp) of all the isolates is quite distinct. Almost identical DGGE pattern was noted by the isolates of remaining four districts.

Comparative analysis of PGP features of all the 114 isolates revealed that 21 isolates namely AJ 1-4, AJ 2-3, AJ 3-2, AJ 3-3, AJ 4-5, AJ 5-2, AJ 6-1, AJ 9-2, AJ 10-4, C2S1, C3S5, GS1S1, G1S3, J2S10, J1S9, J2S5, V1S7, VA2S3, VA3S1, VB4S6, and VA4S7 seem potential as these isolates have relatively higher level of nitrogenase activity, IAA and siderophore production, and P solubilisation. Accordingly, response of rice variety Saryu-52 to V1S7 (*Pantoea agglomerans*) was tested in pot experiments. It was observed that inoculation with the isolate V1S7 caused pronounced effects on growth of the rice variety Saryu-52 in terms of increased root and shoot length, fresh weight and chlorophyll *a* content. Though, significant increase was noted in all the parameters studied, there was maximum Ca. 54.35 % increase in root length of test plant inoculated with V1S7 (data not included). This aroused our interest to identify all the 21 isolates anticipating their potential role as bioinoculant in future study. To achieve this, full length 16S rDNA (1.5 kb) of C3S5 and partial 16S rDNA (400 bp) of remaining 20 isolates were amplified and sequenced. On the basis of sequence homology available in GenBank, all these isolates were identified and the accession number was obtained (Table 2). After confirming the tentative taxonomic assignment, it was desirable to reveal homology of these isolates with other genus/species available in GenBank sequence data base. Accordingly, identical sequences of known 20 bacterial strains were retrieved from sequence database and homology search was made with 21 isolates of this study. It is evident from the analysis of data that isolates reported in this study shared close homology with respective species/genus available in the database (Figure 5).

DISCUSSION

The plant rhizosphere is a versatile and dynamic ecological environment of intense microbes- plant interactions for harnessing essential micro- and macro-nutrients from a limited nutrient pool (Jeffries et al., 2003; Compant et al., 2010). The previous notion is reflected

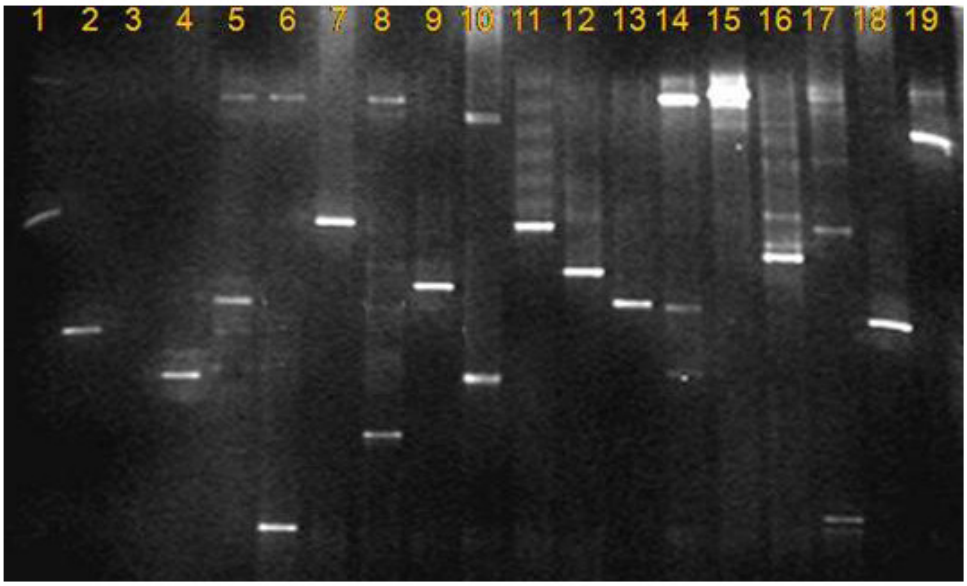

Figure 4. DGGE of 16S rDNA amplified product (200 bp) from 19 isolates of Varanasi district: Lanes 1 to 19: 1-VB4S7A, 2- VB7S4, 3-VA7S7, 4-VB5S10, 5- VB4S6, 6-VB1S2, 7- V1S6, 8-VB3S1, 9-VA8S1, 10-VA1S6A, 11-VB5S5, 12-VB7S5, 13-VA2S3, 14- VA7S1, 15- VA3S1, 16-VA1S2, 17- VB1S5, 18- VB5S7, and 19-VA4S7. V-Varanasi; 1, A and B-represent collection site, and S isolate number from individual site.

from our findings since we could successfully isolate 114 distinct isolates from rice rhizosphere of five districts of Eastern UP. Differences in bacterial species in rice rhizosphere of individual district could be due to the differences in soil types and/or agro-climatic conditions. Our results are in agreement with earlier reports where vast differences in bacterial assemblages were noted in rhizosphere of a particular plant species growing at different locations (De Weger et al., 1995; Ladha and Reddy, 2000; De Los Santos et al., 2001; Roesch et al., 2007; Venieraki et al., 2011). In general, rhizosphere of plants has been reported to be the preferred niche for the growth of diazotrophic bacteria (Ladha and Reddy, 2000). Preference of bacteria for rhizospheric association is thought to be mainly due to low tension of oxygen in root zone especially for diazotrophs. Additionally, metabolites of varying nature are secreted by the roots which favor growth of diverse types of bacteria in the root zone (Franche et al., 2009). Exudates of roots contain a variety of organic C and N compounds which are utilized by a vast assemblage of bacteria (Compant et al., 2010). Most probably, this may be one of the possible reasons for the presence of different types of bacteria in rhizosphere of a particular plant species.

Existence of multiple PGP characters among rhizobacteria has been reported by certain workers (Ahmad et al., 2008), however such reports in indigenous isolates of India are less common. Findings of this study clearly demonstrated the presence of one or more than one types of PGP characters in majority of the isolates. However, among all the PGP traits of the bacteria, the frequency of IAA-producers was found much higher than siderophore producers and P solubilizers. Our findings are consistent with earlier reports where similar trend was reported in different bacteria (De Weger et al., 1995; Ahmad et al., 2008). Such a difference in distribution of PGP characters among various bacteria seems to be governed by the genetic set up of an individual isolate; nevertheless it would be worthwhile to characterize the gene (s) involved in the expression of a particular trait. It is possible that gene(s) related to each PGP character is present in an individual isolate but during the course of time it lost its function.

Tryptophan acts as a precursor of IAA biosynthesis, production of IAA by bacteria occurs rarely in its absence (Glick, 1995; Pedraza et al., 2004; Jha and Kumar, 2007; Roesch et al., 2007). This is also evident from our results where all the isolates showed IAA production solely in the medium supplemented with tryptophan. Since availability of tryptophan in soil is limited, soil rich in organic matter especially due to litter decomposition shall favor the growth of IAA-producers since the chances of tryptophan availability are expected to increase. *In situ* detection of IAA in various soil types with or without inoculation of bacteria may resolve the aforementioned issue.

Reports showing production of metabolites beneficial to plants, such as, phytohormones, siderophores, certain antibiotics and P solubilization by various bacteria have evoked confusion about the specific role of a particular PGP trait (Bottini et al., 2004; Kennedy et al., 2004;

Table 2. Identification based on 16S rRNA gene sequencing and affiliation of the isolates in the GenBank database.

Isolate code	Bacterial strain	Accession number	Highest match (%)	Matching strain of GenBank with accession number
AJ 1-4	*Pseudomonas* sp. AJ 1-4	GU056817	97	*Pseudomonas putida* strain SP3 HQ005307
AJ 2-3	*Enterobacter* sp. AJ 2-3	GU056818	99	*Enterobacter asburiae* strain T90 HQ407265
AJ 3-3	*Pantoea* sp. AJ 3-3	GU056819	98	*Pantoea* sp. isolate R8 AJ002811
AJ 3-2	*Ancylobacter* sp. AJ 3-2	GU056816	99	*Ancylobacter abiegnus* Z-0056 GU247895
AJ 4-5	*Sphingomonas* sp. AJ 4-5	GU056820	98	*Sphingomonas* sp. B331 GQ169809
AJ 5-2	*Sphingomonas* sp. AJ 5-2	GU056821	90	*Sphingomonas* sp. B331 GQ169809
AJ 6-1	*Rhizobium* sp. AJ 6-1	HM031382	100	*Rhizobium* sp. 8 (2011) HQ880671
AJ 9-2	*Enterobacter* sp. AJ 9-2	GU056824	99	*Enterobacter cloacae* strain PYPB08 JF346895
AJ 10-4	*Pseudomonas putida* AJ 10-4	HM031383	98	*Pseudomonas putida* strain SP3 HQ005307
C2S1	*Agrobacterium* sp. C2S1	HM031385	99	*Agrobacterium* sp. JY08 HM134254
C3S5	*Sphingomonas* sp. C3S5	FJ012066*	99	*Sphingomonas* sp. PVS9 HM484368
GS1S1	*Pseudomonas* sp. GS1S1	GU056822	99	*Pseudomonas* sp. NP15b EU580707
G1S3	*Bacillus megaterium* G1S3	EU040256	100	*Bacillus megaterium* strain E5 JF416939
J1S9	*Bacillus megaterium* J1S9	EU040252	100	*Bacillus megaterium* strain J9B-47 HQ238799
J2S10	*Microbacterium* sp. J2S10	EU040255	99	*Microbacterium* sp. e23 (2011) HQ652552
J2S5	*Sphingomonas* sp. J2S5	GU056827	100	*Sphingomonas* sp. SO1a AB453305
V1S7	*Pantoea agglomerans* V1S7	EU040249	99	*Enterobacter* sp. BR3359 HQ677831
VA2S3	*Advenella incenata* VA2S3	GU056831	96	*Advenella incenata* isolate 4 AM944735
VA3S1	*Gamma proteobacterium* VA3S1	GU056832	100	Uncul.*Gamma proteobacterium* _clone NL5BD-03-H07 FM252496
VA4S7	*Pseudomonas* sp.VA4S7	GU056833	100	*Pseudomonas* sp. MACL14 EF198250
VB4S6	*Rhizobium* sp VB4S6	GU056834	79 (Max. Identity, 97%)	*Rhizobium* sp. KNUC364 EU239147

AJ- Azamgarh, C- Chandauli, G- Ghazipur, J-Jaunpur and V- Varanasi. Others as in Figure 2.

Roesch et al., 2007). However, there are a number of reports which have clearly demonstrated that bacteria having any one of the PGP characters may show plant growth enhancement (Ladha and Reddy, 2000; Vessey, 2003). For example, several isolates improve the supply of P to plants as a consequence of their ability to solubilize insoluble P, certain isolates cause root elongation by IAA production and a few which produces siderophores are responsible for increased availability of iron. Henceforth, a strain having multiple PGP characteristics is expected to show better response than those possessing single PGP trait. We have successfully isolated 21 isolates which were found to have multiple plant growth-promoting traits and thereby seem to be potential isolate. Their role in plant growth stimulation was evident from the result of pot experiment where growth of the rice variety Saryu-52 in terms of root and shoot length, fresh weight and chlorophyll *a* content increased significantly following inoculation with V1S7 (*E. agglomerans*). However, it would be desirable to examine whether all the traits of PGP are expressed simultaneously or at different phases of growth of the bacteria.

DNA-based techniques are well documented to provide a comprehensive measure of the diversity and composition of rhizospheric bacteria (Schmalenberger et al., 2001; Miyamoto et al., 2004). We used ARDRA and DGGE techniques to investigate diversity among 114 isolates of rhizospheric soil of five districts of Eastern UP.

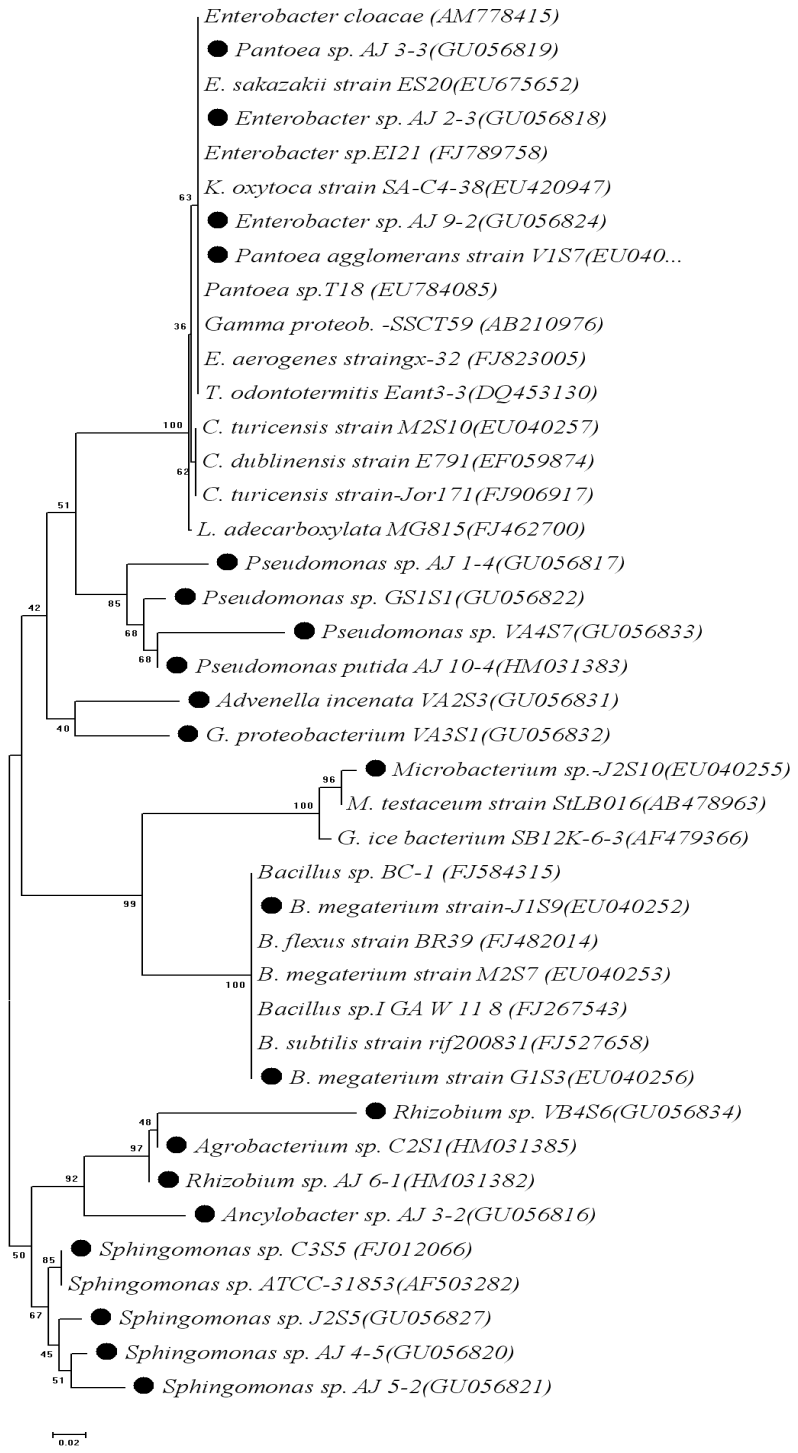

Figure 5. Phylogenetic relatedness based on 16S r-DNA sequences of 41 identified bacterial isolates. Homology search of 21 isolates of the present study was made with the sequences of 20 isolates of NCBI GenBank. Scale bar 0.02 substitution per nucleotide position.

ARDRA profile indeed showed distinct banding pattern after restriction digestion with *Alu*1 and *Rsa*1.DGGE profile, where a single reproducible band with higher intensity was considered, also showed diversity among different isolates. That these isolates indeed differ among each other became evident from the cluster analysis which showed grouping of all the isolates under different groups. From the data of DNA finger printing, it may be

concluded that there does exist significant molecular diversity among 114 isolates, a fact which is also supported by morphological characteristics and PGP characters of the isolates reported herein. Additionally, identification of 21 potential PGP isolates based on 16S rDNA sequencing also supports the prevalence of diversity among 114 rhizospheric isolates recovered from rice fields of five districts of Eastern UP. These all 21 isolates belonged to different species/strains of a genus, the more common being the species of *Pseudomonas* and *Sphingomonas* followed by *Enterobacter*. Species of *Ancylobacter, Pantoea* and *Advenella incenata* has not been reported from rice field soil of India. To our knowledge, this is the first comprehensive study made on PGPR of rice rhizosphere from Eastern Uttar Pradesh, India.

In conclusion, this study resulted in constructing a road map of important rhizospheric bacteria of rice rhizosphere and demonstrated the presence of multiple PGP characters in vast majority of the isolates. Results obtained suggest that certain isolates may be exploited for the development of efficient bioinoculant for enriching the soil fertility and maintaining growth and development of plants. However, large scale field trials with selected strains are needed to screen the novel strain.

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