Isolation and Characterization of Rhizobacteria from *Brassica juncea L.* plant

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ABSTRACT

Rhizospheric area of plant is populated with diversified array of numerous bacteria. The current investigation was undertaken to isolate, characterize and identify plant growth promoting rhizobacteria (PGPR) from rhizosphere of *Brassica Juncea L. (*a variety of mustard). Identification was made with the combined effort of morphological and biochemical characterization. I6s rRNA sequencing technique was also used for some selected strains. On the basis of systematic and scientific investigations a comparative study of each isolate was made. The identified bacterial strains were belonged to genus proteus, Escherichia, Staphylococcus, Achromobacter, Lysinibacillus, Pseudomonas, Bacillus, Streptococcus and enterococcus. It was concluded that a diverse group of bacterial population exist in the rhizosphere of *Brassica Juncea* L.

Keywords: PGPR, rhizosphere, 16S rRNA, phylogenetic analysis, *Brassica Juncea* L.

1. INTRODUCTION

Sustainability in agriculture and security of food depends on a healthy and fertile soil. Much progress had been done in improving both but they exert a mounting pressure on natural resources. Chemical fertilizers and pesticides are applied mainly to provide essential nutrients and to combat pathogenetic diseases to soil-plant system respectively. Plant Growth Promoting Rhizobacteria (PGPR) can be exploited in agriculture as an environmentally friendlier alternative to chemical fertilizers and pesticides, which pollute the environment (Sarathchandra et al, 2001). PGPR are a heterogeneous group of bacteria that can be found in the rhizosphere, at root surfaces and in association with roots, which can improve the quality of plant growth directly or indirectly. In last few decades a large array of bacteria has reported to enhance plant growth (Ahmad et al, 2008). PGPR are free-living soil bacteria, isolated from the rhizosphere, when applied as seed or crop inoculation, it promote plant growth and yield by several mechanism e.g. phytohormones production, provide available nitrogen by biological nitrogen fixation, available phosphorus by phosphate solubilization and suppression of phytopathogen (Vessey, 2003; Akhtar *et al*., 2012). They are involved in various biotic activities of the soil ecosystem to make it dynamic for nutrient turn over and sustainable for crop production (Ahemad et al, 2009). There are so many evidences are present which proves that microorganisms affect plant fitness by direct or indirect effects on plant functional traits such as nutrition provision, changes to photosynthesis, increase in plant growth and development and stress tolerance(Friesen et al. 2011;Vander heijden et al. 2008). Knowledge of the native microbial population, their characterization and identification is required for understanding the distribution and diversity of microbes in the rhizosphere of specific crops (Keating et al.,1995;Chahboune et al.,2011). With increasing awareness about the chemical fertilizers based agricultural practices, it is important to find region specific bacterial strains which can be used as a growth promoting inoculum to achieve more crop production.(Deepa et al.,2010). Keeping in mind the study was planned to isolate the native strains from rhizosphere of Brassica juncea. These bacterial strains were characterized and screened in vitro for PGP potentials and identified.

2. RESEARCH METHODOLOGY

2.1Collection of the samples

Rhizospheric soil samples had been collected from the fields of *Brassica juncea L.* grown and cultivaed in Meyar village of Nalanda district in Bihar, India. Samples of rhizospheric soil tightly adhering to roots had been collected during vegetative and flowering stages showing good and healthy growth. Samples were stored in sterilized sample collection bags and packed for transport to laboratory for further study.

2.2Isolation of bacteria

By using serial dilution method the rhizospheric soil samples had been processed within 24 hours for isolaing most predominant PGPR.For isolation of PGPR strains fresh roots were washed under running tap water and then surface sterlized with 5% NaOcl. After surface sterlization the root samples were again washed three times with sterile distilled water. The root samples were crushed with mortar and pestle. Serial dilutions were prepared from grounded roots.

Soil tightly adhering to roots were serially diluted for isolating microorganism from rhizosphere. 0.1ml of the alliquotes were transferred to different culture media which included Nutrient agar, Luria-Bertini (LB) agar, Macconky agar, Blood agar and spreaded over the entire media surface.

Plates were then incubated at 37^{oC} till the appearance of different colonies. The plates having growing colonies were treated as master plates and used for further study. Morphologically different colonies were picked and streaked on fresh nutrient agar medium in order to obtain pure culture.

2.3 Morphological and Biochemical characterization

All the bacterial isolates were coded as AN1, AN2, AN3, AN4, AN5, AN6, AN7, AN8, AN9, AN10, and AN11 and subjected to microscopic examination. Colony morphology (size, shape, colour, margin, elevation and texture) were observed with the oil immersion objective of the bright field microscope. The Grams reaction was performed as per standard protocol (Vincent et al 1970). Biochemical characterizations of all the isolates such as indole, Methyl red, Voges-Proskauer test, citrate, oxidase and catalase were examined according to standard methods (Cappuccino et al., 1992). The microbial isolates were identified accordance with the Bergey's manual of determinative bacteriology(Holt et al.,1994).

2.4 Molecular characterization

Morphological and biochemical characterization are sometimes not much helpful for identification of all isolates. Strains which were not identified by above were subjected to identification based on 16S rRNA sequencing. DNA was isolated from the culture by the CTAB method and quality was evaluated on 1.2% Agarose Gel, a single band of high-molecular weight DNA has been observed. Isolated DNA was amplified with *16S rRNA* Specific Primer (**8F**and **1492R**) using Veriti® 99 well Thermal Cycler (Model No. 9902). A single discrete PCR amplicon band was observed (**Figure 1**). The PCR amplicon was enzymatically purified and further subjected to Sanger Sequencing. Bi-directional DNA sequencing reaction of PCR amplicon was carried out with **8F** and **1492R** primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. The sequence of all the strains were submitted to National centre for biotechnology information(NCBI) Data Bank for accession numbers .Consensus sequence of *16S rDNA* was generated from forward and reverse

sequence data using aligner software. The *16S rDNA* sequence was used to carry out BLAST alignment search tool of NCBI Genbank database. Based on maximum identity score first Fifteen sequences were selected and aligned using multiple alignment software program ClustalW. Distance matrix was generated using RDP database.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou et al.,1987). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. The analysis involved 16 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).

3. RESULTS AND DISCUSSIONS

The study had been undertaken to isolate different rhizobacterial strains from Brassica juncea L. Total eleven rhizobacterial isolates were isolated and differentiated by their morphological and biochemical characters. The result shows different colony morphologies. Colony size ranges from pinhead, small, medium and large. Whereas shape includes round, circular, oval and irregular. Colour of isolates are tan, cream, golden yellow, yellow, green and white. Margin is entire and wavy. Elevation of isolates includes convex, raised, flat and umbonate. Texture is generally found mucoid some smooth and dry. All morphological characters are tabulated in Table No.1. biochemical characters of isolates also varies. Out of eleven, six strains shows Gram positive whle five shows Gram negative reaction. Three strains are indole positive and eight strains are indole negative. Methyl red test revealed that seven isolates were positive and four were negative. Three isolates were VP positive and eight were negative. Simmon's citrate shows that seven isolates were citrate positive and four were negative. Five strains were oxidase positive whereas six were negative. Nine strains were catalase positive and two strains were catalase negative. The biochemical characteristics of rhizobacterial strains is summarized in Table 2.

Table.1 Morphological Characters of the isolates

S. No.	Isolate code	SIZE	SHAPE	COLOUR	MARGIN	ELEVATION	TEXTURE
1	AN1	Small	Round	Tan	Entire	Convex	Mucoid
2	AN2	Small	Round	Cream	Entire	Raised	Mucoid
3	AN3	Pinhead	Circular	Golden-yellow	Entire	Convex	Smooth
4	AN4	Large	Circular	Yellow	Entire	Raised	Mucoid
5	AN 5	Small	Circular	Yellow	Entire	Flat	Mucoid
6	AN6	Medium	Oval	Green	Wavy	Umbonate	Mucoid

7	AN7	Large	Irregular	White	Wavy	Umbonate	Dry
8	AN8	Small	Round	Tan	Entire	Convex	Mucoid
9	AN9	Medium	Oval	yellow	Wavy	Umbonate	Mucoid
10	AN10	Pinpoint	Circular	White	Entire	Low convex	Mucoid
11	AN11	Pinpoint	Circular	Cream	Entire	Convex	Dry

Table.2 Biochemical Characters of isolates

S.No.	Isolate code	Gram staining	Indole	MR	VP	Citrate	Oxidase	Catalase
1	AN1	-	+	+	-	-	-	+
2	AN2	-	+	+	-	-	-	+
3	AN3	+	-	+	+	+	-	+
4	AN4	-	+	-	-	+	+	+
5	AN5	+	-	+	-	-	+	+
6	AN6	-	-	-	-	+	+	+
7	AN7	+	-	-	+	+	+	+
8	AN8	-	-	+	-	+	-	+
9	AN9	-	-	+	-	+	+	+
10	AN10	+	-	+	-	+	-	-
11	AN11	+	-	-	+	-	-	-

The identification of the isolates was done using the morphological and biochemical properties with the help of Bergey's Manual of Determinative Bacteriology. AN1 was identified as Proteus vulgaris, AN2 as Escheria coli, AN3 as Staphylococcus aureus, AN6 as pseudomonas aureginosa, AN7 as Bacillus subtilis, AN8 as Proteus mirabilis, AN10 as Streptoccus pyogens, AN11 as Enterococcus faecalis. Rhizobacterial strains AN4, AN5 and AN9 were identified by 16S rRNA gene sequence. Isolated DNA was amplified and enzymatically purified and quality checked. A single discrete band of 1500 bp was found as shown in figure 1. Purified PCR amplicon were further processed or Sanger sequencing and consenus sequence of 1299 bp in case of AN4,1488 bp in case of AN5 and 1301 bp 16S rDNA was generated from forward and reverse sequence data using aligner software. Based on the sequences of strains AN4, AN5 and AN9, BLAST search result found that AN4 shows similarity with Achromobacter xylosoxidans strain R8-558 (Accession Number:JQ659958.1). AN5 showed similarity with Lysinibacillus macroides partial, isolate AVS1 (Accession Number: HG326429.1). AN9 showed similarity with Achromobacter sp. FHS22 (Accession Number: KT154933.1). Accession no for nucleotide sequence of AN4 is MG461692, AN5 is MG493189 and AN9 is MG461693.

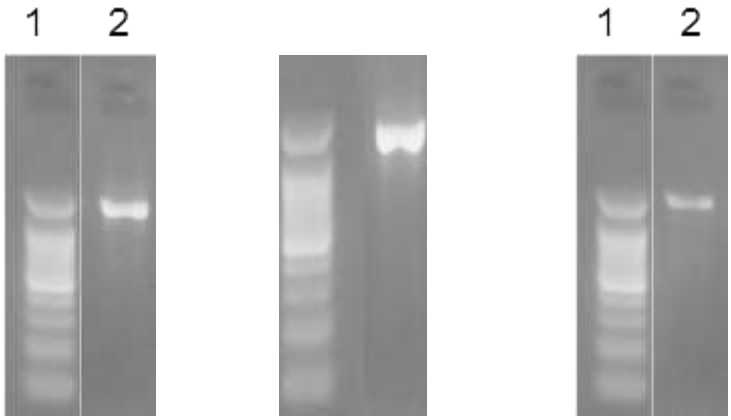

Figure 1. 1.2% Agarose gel showing single 1500 bp of *16S rDNA* amplicon. Lane 1: 100bp DNA ladder; Lane 2: *16S rDNA* amplicon of AN4, AN5 and AN9 respectively.

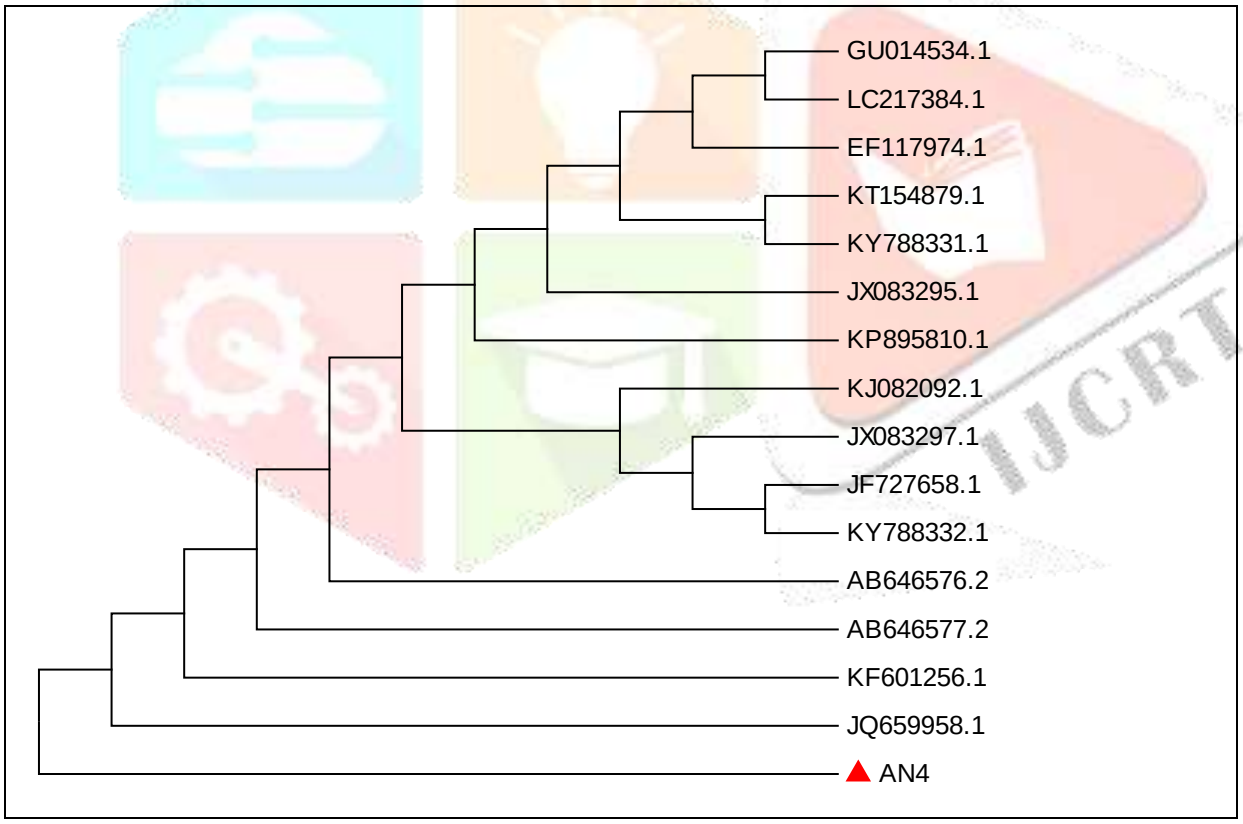

Figure 2. Phylogenetic tree of AN4

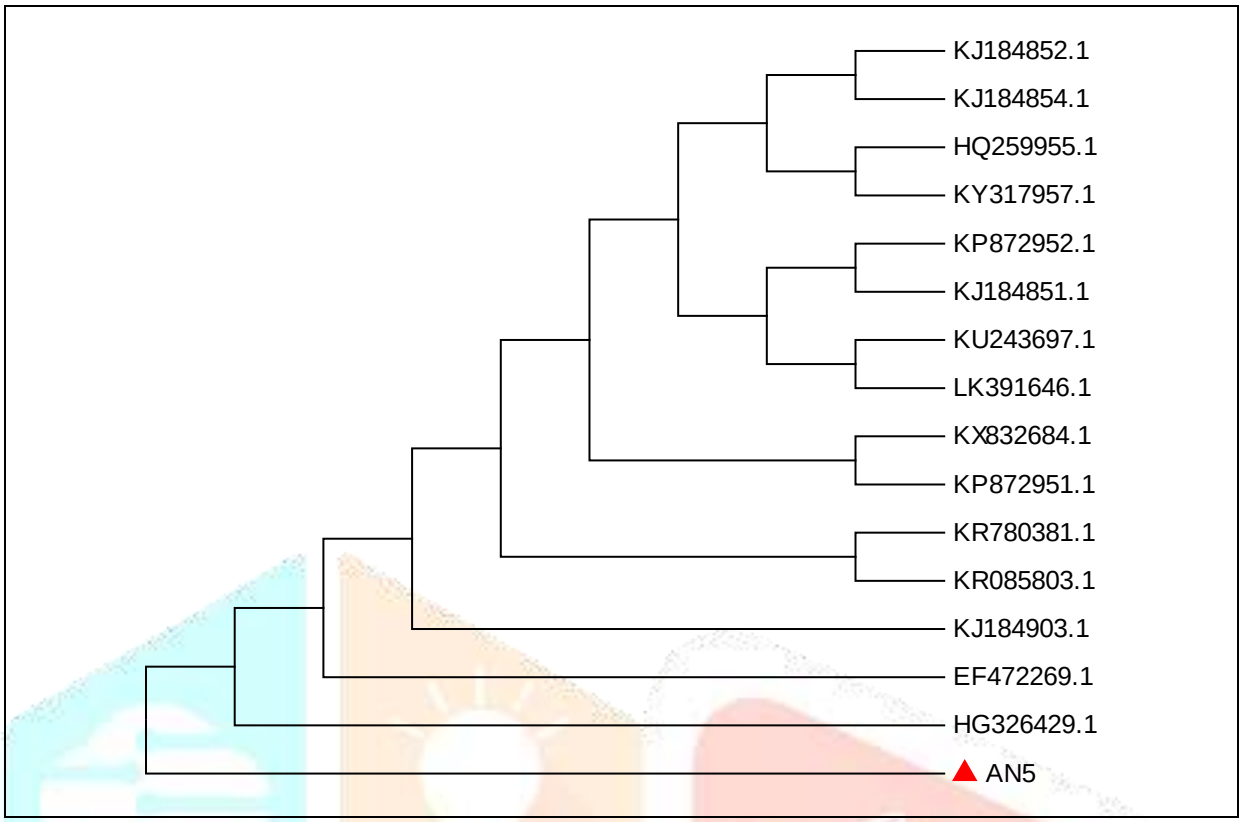

Figure 3. Phylogenetic tree of AN5

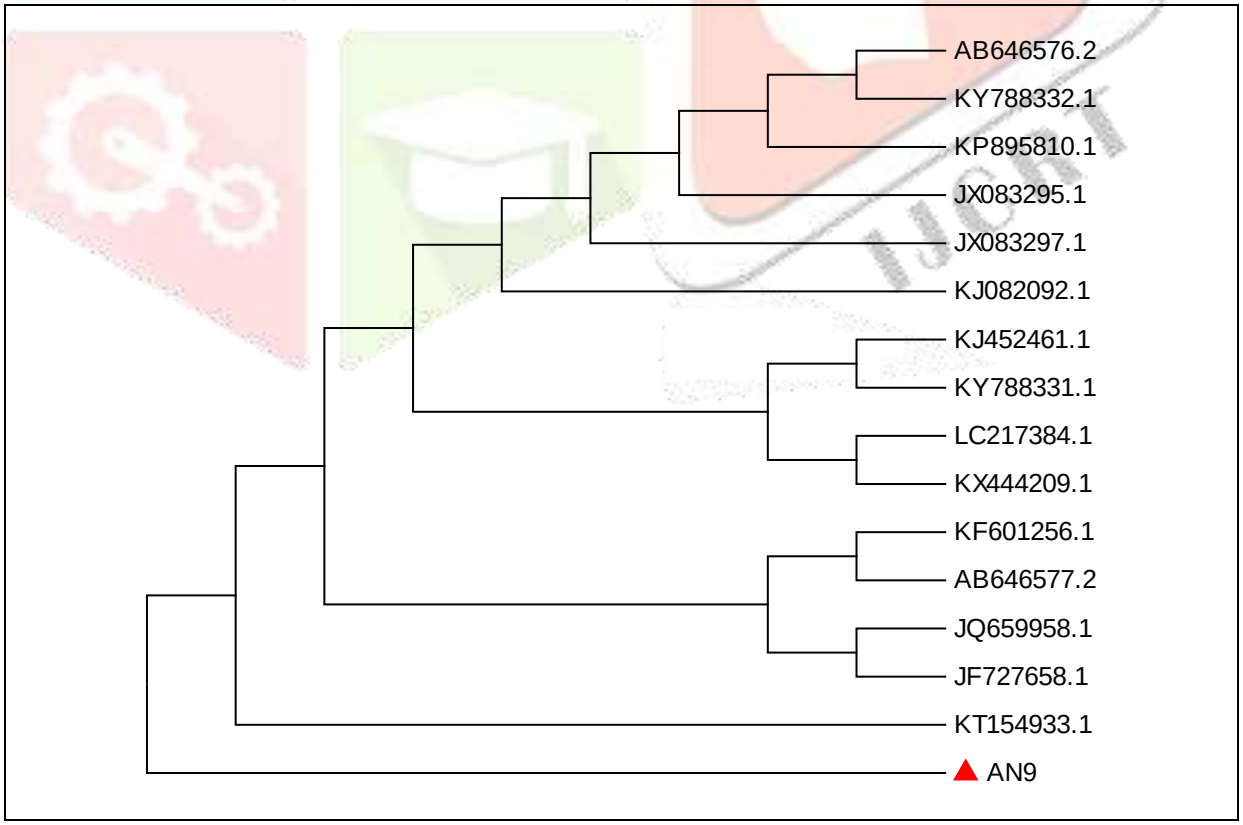

Figure 4. Phylogenetic tree of AN9

Catalase positive strains are found to be highly resistant to environmental, mechanical and chemical stress enhances the growth, seed emergence, crop yield and contribute to the protection of plant against certain pathogen and pests (Dey et al 2004; Herman et al 2008; kleopper et al 2004). Nine out of eleven strains that is 81.8% of strains shows positive for catalase.

Molecular phylogeny extends our knowledge about microorganisms present in rhizosphere. Comparative sequence analysis is currently the most widely used approach for microbial identification.

CONCLUSION

To conclude eleven bacterial isolates were identified from rhizosphere of Brassica juncea. Isolated strains revealed diverse morphological, physiological and biochemical behaviour. The microbial diversity can prove to be a valuable future resource in agricultural progress. Further more research work is required to explore much more about the role of these isolates in the metabolism of plant.

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