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ESTIMATION OF GENETIC DIVERSITY OF PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR) STRAINS FOUND IN DIFFERENT AREAS OF PAKISTAN USING RAPD AND 16S rRNA ANALYSIS

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ABSTRACT

The present study was an approach to provide the information about the diversity of rhizobacteria involved directly or indirectly in the plant growth and development. Among molecular markers, RAPD proved to be the most promising tool to delineate rhizobacterial strains. Seventeen bacterial strains of rhizobacteria were isolated from different parts of Pakistan (Sargodha, Mianwali, Karak and Faisalabad). RAPD analysis of these strains has shown a high degree of polymorphism. Range of genetic similarity was observed 0.543-0.927. Cluster analysis resulted into three clusters (I, II and III). Cluster I comprised of bacterial strains collected from Faisalabad, cluster II comprised of Strains collected from Sargodha while cluster III included strains from Mianwali. Strains isolated from the Karak region were not making any cluster. These results have shown that soil conditions and local environment has a significant impact on genetic make-up of bacterial strains. Further 16S rRNA analysis of the strain was performed to validate the results. Results of 16S rRNA analysis have revealed that most dominating species were Bacillus sp. and two strains were also showing homology with *Brevibacterium halotolerans*. The findings of this study were very promising for the application of PGPRs for plant growth improvement.

Key words: Rhizobacteria, RAPD, Genetic diversity, Soil, 16S rRNA, Plant growth, Sequencing.

INTRODUCTION

Soil microorganisms subsidize plant growth by enhancing nutrient availability by recycling (Lynch *et al.* 1985), enhance root health by providing a defense against pathogens (Weller, 1988) or enhancing nutrient uptake (Kennedy and Smith, 1995). Among the microbes the rhizobacteria are the most important group which perform these jobs basically by increasing the soil productivity, and indirectly producing some chemicals which increase the soil fertility. The utility of rhizobacteria for enhancing productivity has been implemented for the better soil fertility (Aragno, 2005). Especially, the biocontrol plant growth-promoting bacteria (biocontrol-PGPB) and plant growth-promoting bacteria (PGPB) are auspicious for their possible use in maintaining ecological balance for agriculture (Davison, 1988; Park and Kloepper, 2000; Hallmann *et al.* 1997). Plant growth promoting rhizobacteria (PGPR) are helpful for plant growth (Bashan *et al.* 1995), working as biocontrol (Bolwerk *et al.* 2003; Ashrafuzzaman *et al.* 2009), producing plant hormone-like chemicals and dropping of plant ethylene level (Glick, 1995) and initiation of resistance against pathogens (Van Loon *et al.* 1998). PGPR have adopted some defensive mechanisms against a variety of toxic metals (Park and Kloepper, 2000; Burd *et al.* 2000) and provide protection to plants against pathogens and pests (Park and Kloepper, 2000; Ashrafuzzaman *et al.* 2009).

The microbes inhabiting rhizosphere are influenced by certain characteristics of plant (Grayston *et al.* 1998; Marilley and Aragno, 1999; Germida and Siciliano, 2001; Marschner *et al.* 2004) and soil (Latour *et al.* 1996; Marschner *et al.* 2001), agronomic applications (Kennedy and Smith, 1995; Alvey *et al.* 2003) and also infections caused by mycorrhizae (Marschner *et al.* 2001). Ecological theories suggest that microbial communities with greater microbial diversity would be less susceptible to invasion by pathogens (Matos *et al.* 2005). Evaluation of environmental risk factor is necessary before utilization of indigenous or non- indigenous microbes for agricultural improvement (Aragno, 2005; Joseph *et al.* 2012). A comprehensive evaluation of genetic diversity and the environmental impact can be useful for the introduction of a new microorganism into the environment (Van Veen *et al.* 1997).

Various studies have been focused on evaluation of genetic diversity amongst soil bacteria (Yap *et al.,* 1996: Head *et al*., 1998; Upadhyay *et al*., 2009; Hayat *et al.* 2013). Study of genetic diversity of soil bacteria has been found promising in enhancing soil fertility (Saharan and Nehra, 2011). Previous studies using 16S rRNA

showed the association of PGPR strains found in Pakistan with genus Bacillus (Hayat *et al.* 2013).

As the prior sequence information is not pre-requisite in RAPD (Yasmin *et al.* 2008) it is very promising technique in microbes and has been used extensively for the evaluation of genetic diversity in microbes (Bereswill *et al.* 1995; Birch *et al.* 1997; Saharan and Nehra, 2011). The 16S rRNA genes being an associated component of DNA of all organisms and highly conserved can be utilized as biological markers to identify organisms at species, genus and family level. Phylogenetic analysis of soil bacteria can be done with 16S rRNA sequence analysis and their classification into new genera is done (Hayat *et al.* 2013). Pakistani soil houses millions of microbes including certain unique ones, only found in Pakistan. There are very few reports on genetic diversity of rhizobacteria in Pakistan. Therefore, conservation and characterization of such agriculturally important microbes are suggested for its maximum use by the next generations. The aim of the present study was the estimation of genetic diversity and to study phylogenetic relationships among rhizobacteria strains habituating different areas in Pakistan.

MATERIAL AND METHODS

The experimental work was accomplished in the Department of Bioinformatics and Biotechnology (GCUF) and Plant Genomic & Molecular Breeding Lab (PGMB), Agricultural Biotechnology Division, National Institute for Biotechnology and Genetic Engineering (NIBGE) to study phylogenetic relationships among different rhizobacterial strains. Isolation of bacterial strain from soil samples collected from different areas was done in GCUF while RAPD and 16S rRNA analysis was conducted in NIBGE.

Collection and isolation of Rhizobacteria strains: The soil samples used in the study for the collection and isolation of rhizobacteria strains were obtained from different cotton crop growing fields of Faisalabad, Sargodha, Mianwali and Karak. Cotton plants up rooted along with adequate amount of non rhizosphere soil in polythene bags (2 kg size: 23 to 32 cm) were brought instantly to the laboratory and were air-dried. The samples were gently shaken to remove the non rhizosphere soil, leaving only the rhizosphere soil, which is strongly stuck to the roots. Under aseptic conditions for the collection of the rhizosphere soil from the roots, the samples were dipped and gently shaken in sterilized water. The glucose peptone agar medium (GPAM) was inoculated with soil suspension and by streaking three to four times in the fresh medium pure cultures were obtained (Mahler and Wollum, 1982). Bacterial culture was grown in LB broth. Prolific growth was observed in 17 bacterial isolates (Figure 1). These isolates were selected and stored for further studies due to their unique morphological appearance on agar medium (Table 1).

RAPD analysis: A total of 21 decamer primers were selected on the basis of their performance in PCR reactions including OPE-01 through OPE-06, OPE-09, OPE-10, OPE-13 through OPE-16, OPC-17, OPK-08, OPX-08, OPN-02, OPN-05, OPN-06, OPW-07, OPW-02 and OPK-09. Extraction of Genomic DNA was done for each of bacterial strains by the method of Doyle (1987), spectrophotometrically quantified and checked on 0.8% agarose gels. Total 50 ml reaction volume was made for PCR containing 10 mm Tris–HCl (pH 8.3), 50 mm KCl, 3 mm $MgCl_2$, 100 mm, each of dATP, dCTP, dGTP, dTTP, 30 mg of primer, 0.001% (w/v) gelatin, 20 ng of genomic DNA and 2 units of Taq polymerase. PCR reagents Taq polymerase, along with buffer, $MgCl_2$ dNTPs, and gelatin was purchased from MBI Fermentas, USA. 1.2% gel (w/v) was used to visualize the amplification of products. Staining of the agarose gels was done for 30 min with ethidium bromide after electrophoresis. The electrophoresis documentation and analysis system 120 (Kodak Digital Science, Japan) was used to take photographs (Figure 2).

Statistical analysis: Scoring of all visible and unambiguous fragments amplified by the primers was made by visual observation. Amplifications (band in each position) were scored as present or absent (Alvey *et al.* 2003). Euclidean distances were estimated with the help of this data. These distances were further utilized to make a dendrogram using the unweighted pair group method of arithmetic means (UPGMA).

16S rRNA analysis: For amplification of the 16S rRNA genes by PCR, forward primer f (5'-AACACATGCAAGTCGAAC-3') (Lane, 1991) and reverse primer 1387r (5'-ACGGGCGGTGTGTACAAG-3') (Marchesi *et al.* 1998) were used which are designed to amplify the conserved region of bacterial 16S rRNA genes and produce an approximately 1300bp fragment. The total volume of 50 µL of PCR mixture comprised of 25 µL *Taq* polymerase buffer, 0.5 µL *Taq* polymerase, 5 µL DNA template, 8 µL dNTPs, 1 µL each primer (10 pmol) and 9.5 µL d_3H_2O. The first step was the initial denaturation at 95 °C for 2 min, it was followed by 35 cycles of denaturation at 94 °C for 30 Sec, annealing at 55 °C for 30 Sec, and elongation at 72 °C for 1 min, last step was the final extension for 5 min at 72 °C. The presence of specific PCR products was checked on 1% agarose (w/v) gel electrophoresed for 45 min at 70V in TAE 1X buffer. Amplicons were further purified by Gel/PCR DNA fragments extraction kit (Fermentas, USA).

Sequence Analysis of 16S rRNA Gene: DNA fragments extraction kit (Fermentas, USA) was used for the purification of the PCR products of 16S rDNA. Five of

the strains were selected for further sequencing and Bioinformatics analysis. The characterization of each sequence compared to the GenBank database was done by The BLASTN program (www.ncbi.nlm.nih.gov). The sequences were aligned with the ClustalW program (www.ebi.ac.uk). ClustalX2 (Larkin *et al.* 2007) was used to construct the Neighbor-joining tree and bootstrap analysis of samples.

RESULTS AND DISCUSSION

Plant growth promoting Rhizobacteria (PGPRs) colonize plant roots and employ advantageous effects on the development and growth of plants by a variety of processes. The actual procedure behind this is still unknown: however, several theories like phytohormones production, fight against deleterious organisms, initiation of phosphate solubilization (Verma *et al.* 2007), and enhanced mineral nutrient uptake is most likely involved (Glick, 1995). PGPR have been proven to contribute to the enhancement of the growth, emergence of seeds and augmented crop yield, and also protect plants against certain pathogens and pests (Kokalis-Burelle *et al.* 2006; Ashrafuzzaman *et al.* 2009). Randomly amplified polymorphic DNA (RAPD) profiles have proven to be a promising tool for investigation of genetic diversity (Yasmin *et al.* 2008). This method was used by different researchers (Gajbhiye *et al.* 2007) for genetic analysis of Bacillus isolates, (Paffetti *et al.* 1996) for the study of genetic diversity of an Italian Rhizobium meliloto population (Bevivino *et al.* 1998) and for characterization of *Burkholderia cepacia* population. 16S rRNA sequence analysis has also been used to classify and reclassify soil bacteria phylogenetically into new genera (Hayat *et al.* 2013).

In this study, the genetic diversity of the plants growth promoting rhizobacteria was determined in order to select the better strains. We screened seventeen samples with the RAPDs primers and 16S rRNA, these samples were collected from cotton growing fields of different regions of Pakistan (Faisalabad, Sargodha, Mianwali, Karak). We selected cotton crop because cotton has a significant economic importance (Shaheen *et al*., 2014). Faisalabad and Sargodha are cotton growing area while in Mianwali and Karak region cotton is grown occasionally. We selected these diverse areas to study genetic diversity of PGPRs. The analysis of genetic diversity of the rhizobacteria isolates was done to validate the diversity amongst the rhizospheric soil. In the present study a total of 21 RAPD primers were utilized to find out the genetic diversity among 17 rhizobacteria strains. Total number of amplicons produced by these 21 decamer primers is 557. OPE-03 produced a minimum number of amplicons while OPW-02 produced the maximum number of amplicons. All the bacterial strains elucidated a high degree of polymorphism with decamer primers. RAPD profile based genetic similarity matrix for the seventeen bacterial strains was attained using Nei and Lei's coefficient of similarity (Table 2). The genetic similarity of seventeen bacterial strains was high, ranging from 0.543-0.927%. The genotypes BACT -13 and BACT -17 had the greatest genetic similarity (0.927). The second highest genetic similarity was estimated between BACT -8 and BACT - 10 (0.905).

A dendrogram of 17 genotypes of PGPR bacteria was constructed based on the RAPD primer scoring profile (Figure 3) using Unweighted Pair Group Method of Arithmetic means (UPGMA). RAPD analysis has shown a high degree of genetic similarities among seventeen cultivars. UPGMA analysis resulted into dendrogram with three major clusters I, II & III. The grouping of genotypes into three distinct clusters actually indicates the difference in their lineage and the grouping. The genotypes included in the cluster I are BACT-1, BACT-4, BACT-8, BACT-10, BACT-14. These all strains were collected from Faisalabad region. Whereas, genotypes included in cluster 'II' are BACT-2, BACT-9, BACT-13, BACT-17, BACT-12, BACT-16, BACT-11, and BACT-15. These all strains were collected from Sargodha region. The genotypes BACT-3, BACT-7 were collected from Mianwali region and they form a different cluster III. The genotype BACT-5 & BACT-6 collected from Karak are not making any cluster. The genetic divergence among these strains was high. The cluster analysis and similarity matrix also indicate that bacterial strains collected from the same region are showing a high degree of similarity with each other. Other studies were also showing the similar results, bacterial strains collected from the same field were showing close resemblance then other field samples (Roesti *et al.* 2006).

The biodiversity studies have been conducted in microbial communities to assess the effects of environmental agitations mostly at the genus and species level. Truly, the study of factors which influence the biodiversity at the intraspecific level is vital for assessing the effect of exogenous microorganisms on closely related local bacteria. Furthermore, plant and cultivar type can also affect the genetic makeup of rhizobacteria (Paffetti *et al.* 1996). The strains which are in cluster I are showing similarity and showing genetic divergence from cluster II & III.

Table 1. Rhizobacterial strains with their native regions.

Region	Name of Bacterial Strains
Faisalabad	BACT-1, BACT-4, BACT-8, BACT-10 and BACT-14
Sargodha	BACT-2, BACT-9, BACT-13, BACT-17, BACT-12, BACT-16, BACT-11, BACT-15
Mianwali	BACT-3, BACT-7
Karak	BACT-5, BACT-6

Table 2. Similarity Matrix for Nei and Lei's Coefficient of 17 Plants Growth promoting rhizobacteria strain. Numbers Both in First Column and Row Represent the plants growth promoting rhizobacteria strain of the table.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
1	1																
2	0.754	1															
3	0.746	0.750	1														
4	0.832	0.707	0.698	1													
5	0.651	0.716	0.672	0.644	1												
6	0.616	0.644	0.612	0.621	**0.543**	1											
7	0.720	0.793	0.871	0.690	0.672	0.621	1										
8	0.806	0.767	0.698	0.836	0.681	0.638	0.733	1									
9	0.767	0.841	0.772	0.737	0.694	0.711	0.806	0.780	1								
10	0.815	0.759	0.698	0.853	0.655	0.655	0.733	0.905	0.806	1							
11	0.754	0.784	0.776	0.698	0.690	0.629	0.776	0.724	0.806	0.724	1						
12	0.720	0.802	0.750	0.690	0.681	0.621	0.793	0.741	0.823	0.741	0.767	1					
13	0.793	0.832	0.806	0.780	0.731	0.659	0.841	0.797	0.862	0.815	0.841	0.832	1				
14	0.823	0.810	0.741	0.819	0.698	0.655	0.776	0.853	0.858	0.905	0.767	0.793	0.858	1			
15	0.759	0.806	0.772	0.711	0.677	0.608	0.789	0.728	0.819	0.780	0.901	0.772	0.871	0.789	1		
16	0.746	0.802	0.776	0.707	0.681	0.621	0.784	0.733	0.823	0.759	0.793	0.845	0.823	0.819	0.797	1	
17	0.815	0.905	0.836	0.784	0.759	0.698	0.871	0.810	0.918	0.828	0.862	0.888	**0.927**	0.897	0.875	0.879	1

16S rRNA gene analysis for each isolate resulted in amplification of a single band of approximately 1300 base pairs (Figure 4). Five strains from each cluster resulted from the RAPD analysis were used for sequencing and bioinformatics analysis to classify the rhizobacteria. The Bioinformatics analysis of the sequences using the BLASTN program (Table 3) has shown a high degree of similarity, the highest value for each isolate was more than 85% by E-value 0. The similarities of the isolates were found with genetically diverse species, mostly of *Bacillus* sp., like *Bacillus mojavensis, Bacillus safensis, Bacillus pumilus, Lactobacillus murinus, Bacillus amyloliquefaciens, Bacillus subtilis, Bacillus vallismortis, Brevibacterium halotolerans, Bacillus tequilensis, Lysinibacillus fusiformis, Bacillus subtilis, Bacillus axarguiensis*. The evolutionary relationships were elucidated by constructing the phylogenetic tree using 16S rRNA gene partial sequences (Figure 5). The phylogenetic tree has shown that the 5 isolates were showing a high degree of relatedness with each other. Three of the isolated strains were found to belong to bacillus sp. While two of the strains were showing highest similarity with *Brevibacterium halotoleras* which was in accordance with the previous studies (Hayat *et al.* 2013).

Table 3. Selected strains of rhizobacteria for 16S rRNA and their highest similarities.

Bacterial isolate(Region)	Sequence length	Highest Blast Score	Bacterial strain
01 (Faisalabad)	1310bp	99%	*Bacillus mojavensis*
16 (Sargodha)	1322bp	92%	*Bacillus safensis, Bacillus pumilus, Lactobacillus murinus*
06 (Karak)	1324bp	98%	*Bacillus amyloliquefaciens, Bacillus subtilis, Bacillus vallismortis*
05 (Karak)	1315bp	98%	*Brevibacterium halotolerans, Bacillus tequilensis, Lysinibacillus fusiformis, Bacillus subtilis, Bacillus axarguiensis*
07 (Mianwali)	1315bp	98%	*Brevibacterium halotolerans, Bacillus mojavensis, Bacillus tequilensis, Lysinibacillus fusiformis, Bacillus subtilis*

Figure 1. Growth of bacterial colonies on agar plates.

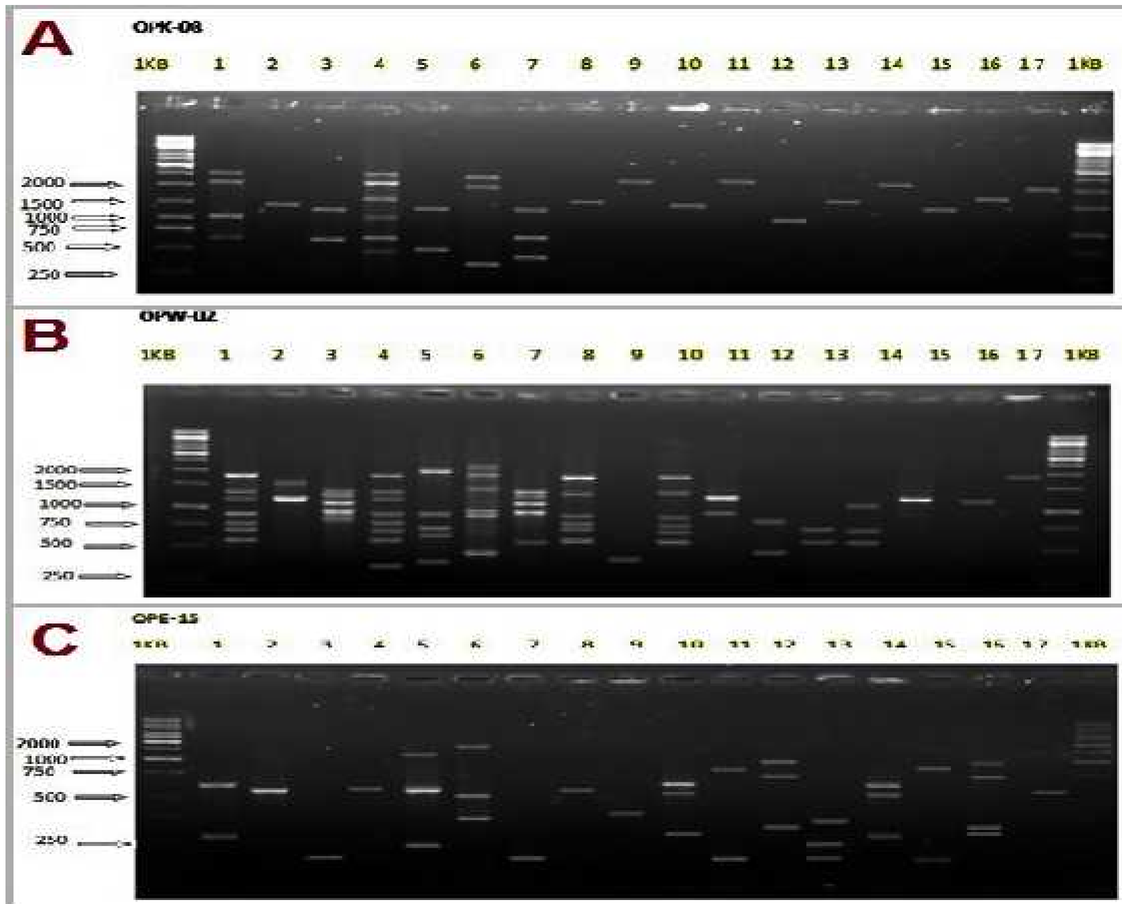

Fig 2. A) Agarose gel picture of PCR amplification with OPK-08, B) Agarose gel picture of PCR amplification with OPW-02, C) Agarose gel picture of PCR amplification with OPE-15; Lane name with respective isolates; (1Kb ladder), 1(BACT-1), 2(BACT-2), 3(BACT-3), 4(BACT-4), 5(BACT-5), 6(BACT-6), 7(BACT-7), 8(BACT-8), 9(BACT-9), 10(BACT-10), 11(BACT11), 12(BACT-12), 13(BACT-13), 14(BACT-14), 15(BACT-15), 16 (BACT-16), 17(BACT-17), (1Kb ladder).

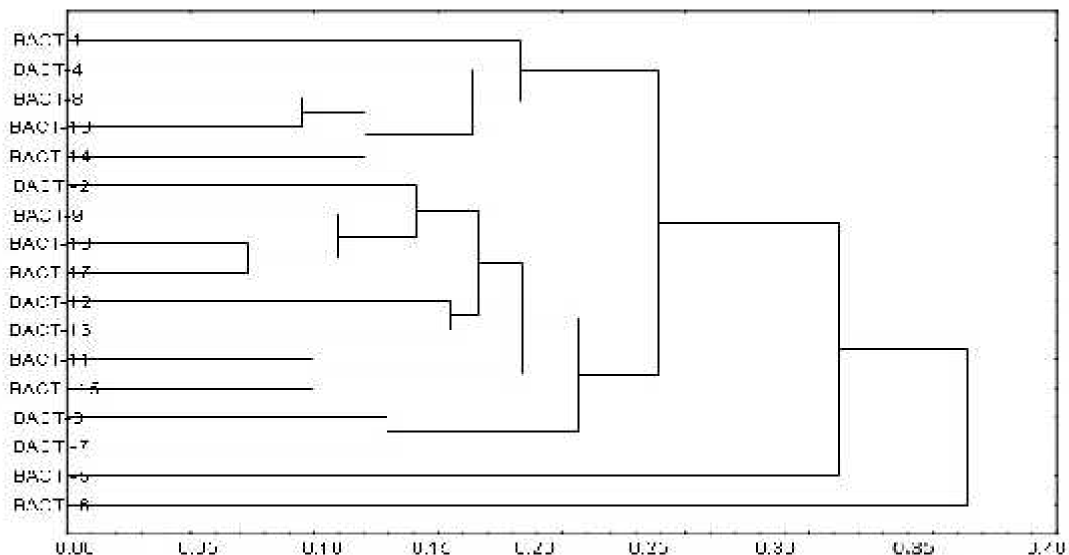

Fig 3. Dendrogram of 17 strain plants growth promoting rhizobacteria genotypes.
Method of Arithmetic means (UPGMA). The scale is based on Nei and Lei's coefficient of similarity.

Fig. 4. Agarose gel electrophoresis of PCR amplified 16SrRNA; Lane name with respective isolates; (1Kbladder), 1(BACT-1), 2(BACT-2), 3(BACT-3), 4(BACT-4), 5(BACT-5), 6(BACT-6), 7(BACT-7), 8(BACT-8), 9(BACT-9), 10(BACT-10), 11(BACT-11), 12(BACT-12), 13(BACT-13), 14(BACT-14), 15(BACT-15), 16(BACT-16), 17(BACT-17), (1Kb ladder).

Fig. 5. Dendrogram based on 16S rRNA gene partial sequences showed the evolutionary relationship among the isolates.

Conclusions: A high degree of similarity was observed in the isolates; hence it was indispensable to study the strains at the DNA level for providing a perfect fingerprint for its identification. RAPD being a simple technique was used to delineate the bacterial strains and 16S rRNA was utilized further to authenticate the results.

Most of the strains were found to belong to Bacillus sp. which is found to be dominating PGPR species in Pakistan. This study will prove to be a crucial part of studies being conducted to improve soil health in Pakistan.

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REFERENCES

Alvey, S., C. H. Yang, A. Buerkert, and D. Crowley (2003). Cereal/legume rotation effects on rhizosphere bacterial community structure in West African soils. Biol. Fertility. Soils 37(2): 73-82.

Aragno, M. (2005). The rhizosphere a hot spot of bacterial diversity? In. Satyanarayana, T. and B. N. Johri (eds.) Microbial Diversity Current Perspectives and Potential Applications. I.K. International Pvt. Ltd.,New Dehli: 261-284.

Ashrafuzzaman, M., F. A. Hossen, M. R. Ismail, A. Hoque, M. Z. Islam, S. Shahidullah and S. Meon (2009). Efficiency of plant growth-promoting rhizobacteria (PGPR) for the enhancement of rice growth. Afr. J. Biotechnol 8(7): 1247-1252.

Bashan, Y., M. E. Puente, M. N. Rodriguez-Mendoza, G. Toledo, G. Holguin, R. Ferrera-Cerrato and S. Pedrin (1995). Survival of Azospirillum brasilense in the Bulk Soil and Rhizosphere of 23 Soil Types. Appl. Environ. Microbiol 61(5): 1938-1945.

Bereswill, S., P. Bugert, I. Bruchmuller and K. Geider (1995). Identification of the fire blight pathogen, Erwinia amylovora, by PCR assays with chromosomal DNA. Appl. Environ. Microbiol 61(7): 2636-2642.

Bevivino, A., S. Sarrocco, C. Dalmastri, S. Tabacchioni, C. Cantale and L. Chiarini (1998). Characterization of a free living maize rhizosphere population of Burkholderia cepacia: effect of seed treatment on disease suppression and growth promotion of maize. FEMS. Microbiol. Ecol. 27(3): 225-237.

Birch, P. R., L. J. Hyman, R. Taylor, A. F. Opio, C. Bragard and I. K. Toth (1997). RAPD PCR-based differentiation of Xanthomonas campestris pv. phaseoli and Xanthomonas campestris pv. phaseoli var. fuscans. Eur. J. Plant. Pathol 103(9): 809-814.

Bolwerk, A., A. L. Lagopodi, A. H. Wijfjes, G. E. Lamers, T. F. Chin-A-Woeng, B. J. Lugtenberg and G. V. Bloemberg (2003). Interactions in the tomato rhizosphere of two Pseudomonas biocontrol strains with the phytopathogenic fungus Fusarium oxysporum f. sp. radicis-lycopersici. Mol. Plant Microbe Interact 16(11): 983-993.

Burd, G. I., D. G. Dixon and B. R. Glick (2000). Plant growth-promoting bacteria that decrease heavy metal toxicity in plants. Can. J. Microbiol. 46(3): 237-245.

Davison, .J (1988). Plant beneficial bacteria. Nat. Biotechnol. 6(3): 282-286.

Doyle, J. J. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. 19: 11-15.

Gajbhiye, A., M. Bhandarkar, A. Dongre and S. Meshram (2007). Genetic Diversity Evaluation of Bacillus isolates using Randomly Amplified Polymorphic DNA molecular marker. Rom. Biotech. Lett. 12(3): 3277.

Germida, J. and S. Siciliano (2001). Taxonomic diversity of bacteria associated with the roots of modern, recent and ancient wheat cultivars. Bio. Fertility Soils 33(5): 410-415.

Glick, B. R. (1995). The enhancement of plant growth by free-living bacteria. Can. J. Microbiol 41(2): 109-117.

Grayston, S. J., S. Wang, C. D. Campbell and A. C. Edwards (1998). Selective influence of plant species on microbial diversity in the rhizosphere. Soil. Biol. Biochem 30(3): 369-378.

Hallmann, J., Quadt-Hallmann, A., W. Mahaffee and J. Kloepper (1997). Bacterial endophytes in agricultural crops. Can. J. Microbiol 43(10): 895-914.

Hayat, R., R. Khalid, M. Ehsan, I. Ahmed, A. Yokota and S. Ali (2013). Molecular characterization of soil bacteria for improving crop yield in pakistan. Pak. J. Bot 45(3): 1045-1055.

Head, I.M., J.R. Saunders and R.Q.W. Pickup (1998). Microbial evolution, diversity, and ecology: a decade of ribosomal RNA analysis of uncultivated microorganisms. Microb. Ecol 35: 1-21.

Joseph, B., R. Ranjan Patra and R. Lawrence (2012). Characterization of plant growth promoting rhizobacteria associated with chickpea (*Cicer arietinum* L.). Int. J. Plant Prod 1(2): 141-152.

Kennedy, A. and K. Smith (1995). Soil microbial diversity and the sustainability of agricultural soils. Plant and soil 170(1): 75-86.

Kokalis-Burelle, N., J. Kloepper and M. Reddy (2006). Plant growth-promoting rhizobacteria as

transplant amendments and their effects on indigenous rhizosphere microorganisms. Appl. Soil. Ecol 31(1): 91-100.

Lane, D. (1991). 16S/23S rRNA sequencing. Nucleic acid techniques in bacterial systematics: 125-175.

Larkin, M. A., G. Blackshields, N. Brown, R. Chenna, P. A. McGettigan, H. McWilliam, F. Valentin, I. M. Wallace, A. Wilm and R. Lopez (2007). Clustal W and Clustal X version 2.0. Bioinformatics 23(21): 2947-2948.

Latour, X., T. Corberand, G. Laguerre, F. Allard and P. Lemanceau (1996). The composition of fluorescent pseudomonad populations associated with roots is influenced by plant and soil type. Appl. Environ. Microbiol 62(7): 2449-2456.

Lynch, J. and E. Bragg (1985). Microorganisms and soil aggregate stability Adv. Soil. Sci (pp. 133-171): Springer.

Mahler, R., I. Ag Wollum (1982). Seasonal fluctuation of Rhizobium japonicum under a variety of field conditions in North Carolina. Soil. Sci 134(5): 317-324.

Marchesi, J. R., T. Sato, A. J. Weightman, T. A. Martin, J. C. Fry, S. J. Hiom and W. G. Wade (1998). Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Appl. Environ. Microbiol 64(2): 795-799.

Marilley, L. and M. Aragno (1999). Phylogenetic diversity of bacterial communities differing in degree of proximity of Lolium perenne and Trifolium repens roots. Appl. Soil. Ecol. 13(2): 127-136.

Marschner, P., D. Crowley and C.H. Yang (2004). Development of specific rhizosphere bacterial communities in relation to plant species, nutrition and soil type. Plant. Soil 261(1-2): 199-208.

Marschner, P., C-H. Yang, R. Lieberei, D. E. Crowley (2001). Soil and plant specific effects on bacterial community composition in the rhizosphere. Soil Biol. Biochem. 33(11): 1437-1445.

Matos, A., L. Kerkhof and J. Garland (2005). Effects of microbial community diversity on the survival of Pseudomonas aeruginosa in the wheat rhizosphere. Microb. Ecol 49(2): 257-264.

Paffetti, D., C. Scotti, S. Gnocchi, S. Fancelli and M. Bazzicalupo (1996). Genetic diversity of an Italian Rhizobium meliloti population from different Medicago sativa varieties. Appl. Environ. Microbiol 62(7): 2279-2285.

Park, K. S. and J. W. Kloepper (2000). Activation of PR-1a Promoter by Rhizobacteria That Induce Systemic Resistance in Tobacco against Pseudomonas syringae pv. tabaci. Biol. Control 18(1): 2-9.

Roesti, D., R. Gaur, B. Johri, G. Imfeld, S. Sharma, K. Kawaljeet and M. Aragno (2006). Plant growth stage, fertiliser management and bio-inoculation of arbuscular mycorrhizal fungi and plant growth promoting rhizobacteria affect the rhizobacterial community structure in rain-fed wheat fields. Soil. Biol. Biochem 38(5): 1111-1120.

Saharan, B.S. and V. Nehra (2011). Plant growth promoting rhizobacteria: a critical review. Life Sci. Med. Res 21: 1-30.

Shaheen, T., Y. Zafar and M. Rahman (2014). Single nucleotide polymorphism analysis of ubiquitin extension protein genes (UBQ) of *gossypium arboreum* and *gossypium herbaceum* in comparison with *arabidopsis thaliana.* Pak. J. Bot 46(2): 605-608.

Upadhyay. S.K., D. P. Singh and R. Saikia (2009). Genetic Diversity of Plant Growth Promoting Rhizobacteria Isolated from Rhizospheric Soil of Wheat Under Saline Condition. Curr Microbiol DOI 10.1007/s00284-009-9464-1

Van Loon, L., P. Bakker and C. Pieterse (1998). Systemic resistance induced by rhizosphere bacteria. Ann. Rev. phytopathol 36(1): 453-483.

Van Veen, J. A., L. S. van Overbeek and J. D. van Elsas (1997). Fate and activity of microorganisms introduced into soil. Microbiol. Mol. Biol. Rev 61(2): 121-135.

Verma, R., A. S. Naosekpam, S. Kumar, R. Prasad and V. Shanmugam (2007). Influence of soil reaction on diversity and antifungal activity of fluorescent pseudomonads in crop rhizospheres. Bioresour. Technol. 98(7): 1346-1352.

Weller, D. M. (1988). Biological control of soilborne plant pathogens in the rhizosphere with bacteria. Annu. Rev. Phytopathol 26(1): 379-407.

Yap, W.H., X. Li, W.L. Soong and J.E. Davies (1996). Genetic diversity of soil microorganisms assessed by analysis of HSP 70 (DNAK) sequences. Ind. J. Microbiol 17: 179-184.

Yasmin, T., N. Tabbasam, I. Ullah, M. Asif, Y. Zafar (2008). Studying the extent of genetic diversity among *Gossypium arboreum* L. genotypes/cultivars using DNA fingerprinting. Genet. Resour. Crop. Ev. 55(3): 331-339.