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MOLECULAR CHARACTERIZATION OF RHIZOSPHERE BACTERIAL COMMUNITIES ASSOCIATED WITH WHEAT (*TRITICUM AESTIVUM* L.) CULTIVARS AT FLOWERING STAGE

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ABSTRACT

There is limited knowledge available on rhizosphere bacterial communities associated with different cultivars. To contribute in this perspective the present research was conducted, based on 16S rRNA bacterial diversity in the rhizosphere soil of two wheat cultivars i-e Chakwal-50 and Fareed-06 at flowering stage to investigate the hypothesis: cultivar difference affects the composition of rhizosphere bacterial communities. The 16S rRNA gene was amplified by 27 forward and 1492 reverse primers and cloned using TA cloning vector. The plasmid DNA of transformants was subjected to RFLP analysis by *Hind III, X BaI* restriction enzymes and sequenced by M13 Forward and Reverse primers. The results revealed heterogeneity in the bacterial communities of both rhizosphers that may correlates to variation in N-NO_3, Mn, Fe and Co contents, soil moisture contents, total organic carbon and organic matter of rhizosphere soil**.** The two 16S-rRNA rhizosphere libraries covered 8 phyla, 14 classes, 14 orders, 23 families, 33 genera along with un-classified and un-characterized lineages that may represents novel lineages. At phylum, class, order, family and genera level 13, 50, 36, 78, 91 % divergence was observed. The sequence results confirmed the presence of beneficial bacteria in rhizosphere soil that are potentially effective for plants and soil health.

Keywords: Rhizosphere soil, Wheat cultivars, 16S rRNA gene library, TA cloning vector, RFLP.

INTRODUCTION

Among crops, wheat is the most important cereal crop in world and counted as major staple food crop of Pakistan (Mahmood *et al.,* 2013). There exists a plethora of bacterial communities in the rhizosphere of wheat and other plants that stimulate plant growth and improves agriculture in several ways like N_2 fixation, production of plant growth promoting hormones, metabolites, helps in uptake of nutrients by the plants, stimulates root growth and morphology and suppresses disease. They not only improve soil health status but also have pivotal role in forest regeneration and phytoremediation of soil (Gray and Smith, 2005; Lucy *et al*., 2004; Zehnder *et al*., 2001; Hameeda *et al*., 2008). These functions can be scrutinized well by studying the plants microbe interaction, unluckily 95-99% microbial diversity in soil is unable to culture by standard techniques (Nichols, 2007). It is impossible to study bacterial diversity in a short time using traditional culture techniques, these techniques fails to provide suitable ecological niches that usually present in natural environment required to support complete spectrum of microbial diversity.

The field of metagenomics gives comprehensive information about total genomic analysis of uncultured and cultured microbial communities in a very short time that's impossible by just 16S rRNA analysis involving culture dependent approach. The Phylogeny and ecology of microorganisms are assessable only by culture-independent technique (Handelsman, 2004). These advances also make easy the assignment and detection of new metabolic capabilities to taxonomic groups (Bejaet *et al*., 2000; Venter *et al*., 2004) and provides fundamental steps to identify unclassified bacteria up to order/family/genera and species level. No doubt metagenomics have great role in understanding plant microbes' interaction, in this way novel (both functional and conserved) genes, their products and metabolic pathways can be exploited by this biological wealth of genetic pool (Cowan, 2000). The identified valuable genes may also be transferred into culturable bacteria and there is need to explore and to improve methods of culturability (van Elsas *et al*., 2008) to get maximum benefits from metagenomics approach. The field of metagenomics has role in expanding the sequence data deposited in Genbank.

The major factors that effects the microbial communities in rhizosphere (van de Putten *et al*., 2007; Berg and Smalla, 2009: Frank *et al*., 2006) includes root exudates, root morphology, soil type, plant species and cultivar type, but conclusive information extensively unavailable explaining to what extent cultivar difference affects the diversification of associated bacterial communities. To investigate it we designed hypothesis: different cultivars of same plant species possesses

variation in bacterial communities harboring in their rhizosphere. To test hypothesis, the rhizospheric soil samples were collected at flowering stage of wheat varieties growing in NARC, Islamabad Pakistan. The soil physico-chemical properties were determined and culture Independent technique was adopted along with Restriction fragment length polymorphism (RFLP) followed by 16S-rRNA as phylogenetic marker to get insight to rhizosphere bacterial communities.

MATERIALS AND METHODS

Rhizospheric Soil Sampling: Wheat plants of cultivars i-e Freed-06 (Hussain *et al*., 2010) and Chakwal-50 (Mahmood *et al*., 2013) were collected in replicates from 8 different plots at NARC, Islamabad (latitude: 33°40'N and longitude: 73°10'E) during flowering stage. The roots adhering rhizospheric soil were kept in polythene bags and shifted to laboratory in coolers containing ice to avoid any sort of degradation after 1 hour of sampling. In the laboratory the rhizospheric soil was removed by brushing, homogenized and composited samples were made and finally sieved through 2mm seiver and stored at -80ºC for DNA extraction whereas for physico-chemical properties, the soil was kept at 4ºC and air dried before use.

Soil Physico-chemical Properties: The soil texture was measured by hydrometer method followed by Gee and Bauder (1986). The organic matter was determined by titration method devised by Walkley and Black (1934). Whereas soil organic carbon was determined using the method of Walkley (1947). The available macro and micronutrients in rhizosphere soil i-e Na, K, Mg, Ca, Fe, Mn, Zn and Cu were measured on atomic absorption spectrometer using the microwave-assisted acid digestion method developed by Kingston (1994) whereas P and NO_3-N contents were determined following the Ammonium Bicarbonate-DTPA method of Soltanpour and Schwab (1977).

Extraction of DNA from soil and PCR amplification of 16S rRNA gene: DNA was extracted directly from rhizosphere soil using Fast DNA™ SPIN Kit using FastPrep®-24 instrument (MP Biomedicals, USA). The presence of DNA was confirmed by agarose gel electrophoresis and the concentration was measured by Nanodrop spectrometer (NanoDrop 2000c, Thermo Fisher Scientific).

The amplification of 16S rRNA gene from rhizospheric soil was done in a thermo cycler (Biometra, Germany) using universal primers 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (TACGGTTACCTTGTTACGACTT) (Lane *et al*., 1991; Turner *et al*., 1999). The reaction mixture of 25 µL contained 1µL of template DNA (> 50 ng/µl), 1 µL 10 mM dNTPs, 5 µL of 5X buffer, 0.5 µL of 10 mM dNTPs, 1 µL of 50 mM $MgCl_2$, 1 µL of forward and reverse primers (10 µM), 0.25 µL of BSA (10 mg/ml), 0.25 µL of *Taq* DNA polymerase [(5U/µL); Fermentas] and rest of the volume adjusted by autoclaved filter sterilized H_2O. After initial denaturation at 95°C for 3 min, samples were cycled for 35 cycles through the following temperature profile: denaturation at 95°C for 20 sec, annealing at 60°C for 20 sec, extension at 72°C for 45 seconds plus one additional cycle for chain elongation at 72°C for 3 min. Amplified PCR products were separated by gel electrophoresis on 1.5 % (w/v) agarose and purified using Gene JET™ Gel Extraction Kit (Fermentas).

Cloning of 16S rRNA Gene: InsTAclone™ PCR Cloning Kit (Fermentas) was used for ligation of PCR product. The 5:1 vector: insert ratio of purified PCR product was obtained using the formula:
ng of vector × kb size of insert/ kb size of vector × insert:vector molar ratio = ng of insert

The ligation reaction contained 30 µL of ligation reaction contained 2 µL pTZ57R/T vector, 10 µL purified PCR product, 6 µL ligation buffer (10X), 1.0 µL T4 DNA ligase and 11 µL sterile de-ionized water (nuclease free). Ligation was performed overnight at 16ºC and transformed in to competent cells of *E. coli* Top-10 (Chen *et al*., 1972) using the heat shock method (Sambrook *et al*., 1989). The transformants were recovered on LB agar plates containing 25 µL of 100 mg/ mL ampicillin, 30 µL IPTG (24 mg mL^{-1}) and 40 µL X-GAL (20 mg mL^{-1}) at 37 ºC for overnight and the presence of insert was confirmed my PCR using M13 F (tgtaaaacgacggccagt) and M13 R (ggaaacagctatgaccatg) primers**.** Plasmid DNA from individual clones was purified using the Gene JET™ Plasmid Mini-prep Kit (Fermentas) and stored at -20 ºC. Plasmid DNA was subjected to RFLP analysis by two restriction enzymes: *HindIII* and *XbaI*. Sequencing of 16S rRNA gene was done using M13 F and M13 R primers by Macrogen, South Korea.

Sequence alignment and construction of Phylogenetic Tree: Sequence were compared using the BLAST algorithm (www. ncbi.nlm.nih.gov/BLAST) and taxonomic hierarchy of sequences was determined with the "Classifier" algorithm (Wang *et al*., 2007) provided at Ribosomal Database Project (http://rdp.cme.msu.edu). Sequence alignments and phylogenetic tree construction were done with CLC DNA work bench version 6.6.1 or with Geneious software version 6.1. Phylogenetic trees were constructed using the Neighbour-joining method and 100 bootstrap replicates were used (Hillis and Bull, 1993). The partial 16S rRNA gene sequences obtained have been deposited in EMBL-Genbank under the accession numbers: HE818630 to HE818695.

Statistical analysis of data: was done by factorial analysis of variance (ANOVA) and completely

randomized design test (CRD) with Tuckey HSD using Statistix software, version 8.1.

RESULTS

In the present study two PCR based 16S-rRNA gene libraries were constructed from rhizosphere soil of Freed-06 and Chakwal-50 by cloning using *E. coli* top 10 strain and bacterial diversity was compared along with comparison of physico-chemical properties of rhizosphere soil of two cultivars. A total 101 clones with PCR insert were obtained and the plasmid DNA from transformants was grouped in to 66 categories based on digestion pattern of 16S-rRNA gene by two restriction enzymes: *Hind III* and *XbaI*. The sequences results covered 1300-1500bp and variations were confirmed in sequences on the basis of Blast using NCBI site. The sequences were aligned and phylogenetic tree was constructed using Neighbour-joining method (Figure 1 and Figure 2).

On the basis of comparison with reference species submitted in Genbank, the 16S rRNA sequences of clones showed affiliation with 8 phyla: Proteobacteria, Firmicutes, Acidobacteria, Actinobacteria, Gemmatimonadetes, Bacteroidetes, Planctomycetes and Chloroflexi. However, the phylum Bacteroidetes was not found in 16S-RNA gene library created from rhizosphere of Chakwal-50 (Figure 1 and Figure 2).

The Phylum Proteobacteria was the dominant phylum (27%) in the rhizosphere library of Fareed-06 whereas in the rhizosphere library of Chakwal-50 the proteobacteria was the second dominant phyla comprised of 23% of clones respectively. The diversity of Phylum Proteobacteria was greater in the rhizosphere of Fareed-06 as compared to Chakwal-50. The rhizosphere of Fareed-06 encompassed four classes of Proteobacteria: α proteobacteria, β proteobacteria, γ proteobacteria and δ proteobacteria (Table 2) with both classified and unclassified genera, the nine classified genera included *Azoarcus*, *Methylobacterium*, *Microvirga*, *Skermanella*, *Bradyrhizobium*, *Rubellimicrobium*, *Balneimonas*, *Mesorhizobium* and *Lysobacter* conversely the rhizosphere library of Chakwal-50 shared only two classes: α proteobacteria and δ proteobacteria with following classified genera: *Rhodoplanes* and *Bradyrhizobium* (Table 3).

The phylum Actinobacteria was the second superseding phylum in rhizosphere library of Fareed-06 consisted of 20% of total clones obtained from rhizosphere library but the %age allocation of members of this phylum was highest in the rhizosphere soil of Chakwal-50 then other phyla, shared 27% of total clones. All the 16S rRNA sequences from both rhizosphere soils fell in to one class: Actinobacteria but a 25% difference in bacterial composition was found at order level as the rhizosphere library of Fareed-06 enfolded one additional order Rubrobacteridae that was not found in the rhizosphere soil of Chakwal-50. The following characterized genera Solirubrobacter, *Pseudosporangium*, *Nonomuraea* were found in the rhizosphere of Fareed-06 along with unclassified genera. The rhizosphere of Chakwal-50 possessed genera includes: *Arthrobacter*, *Modestobacter*, *Aciditerrimonas*, *Streptomyces*, *Conexibacter*, *Nocardioides*, *Micromonospora*, *Ilumatobacter* and *Dactylosporangium.*

The Chloroflexi was the third largest phylum in the rhizosphere library of Fareed-06 (Table 2) accounted for 17% of total clones but least dominant in the rhizosphere of chakwal-50 (Table 3) with sharing of 4% of total clones, primarily represented by one common class Anaerolinae in both rhizospheric soils. The phylum Bacteroidetes was the 4th largest phylum in the rhizosphere soil of Fareed-06 accounted for 15% of total clones with three characterized genera: *Ohtaekwangia*, *Flavisolibacter* and *Ferruginibacter* and some unclassified representatives. The 16S-rRNA sequence of the clones from both rhizosphere libraries indicated resemblance with phylum Firmicutes, however the sequences analysis of clones indicated more diversity in rhizosphere of Fareed-06, harbored two families: Bacillaceae and Paenibacillaceae (Table 2).

The 16S-rRNA sequences from both rhizospheres revealed comparability with un-identified representatives of phylum Gemmatimonadetes, *Gemmatimonas* was the only one identified genera in both rhizosphere. The diversity of phylum Acidobacteria was more prevalent in the rhizosphere of Chakwal-50 (Figure 2; Table 3) and it was the third largest phylum in that rhizosphere soil harbored uncultured representatives of class Acidobacteria_GP6 and Acidobacteria_GP4 and different from Fareed-06 that contained representatives having similarities with Acidobacteria_GP10. On the other hand the diversity of phylum Planctomycetes was greater in the rhizosphere of Fareed-06 harboring both classified and unclassified genera. The genus Planctomyces affiliated members were only found in rhizosphere of Freed-06 and *Zavarzinella* members were common in rhizosphere of Chakwal-50.

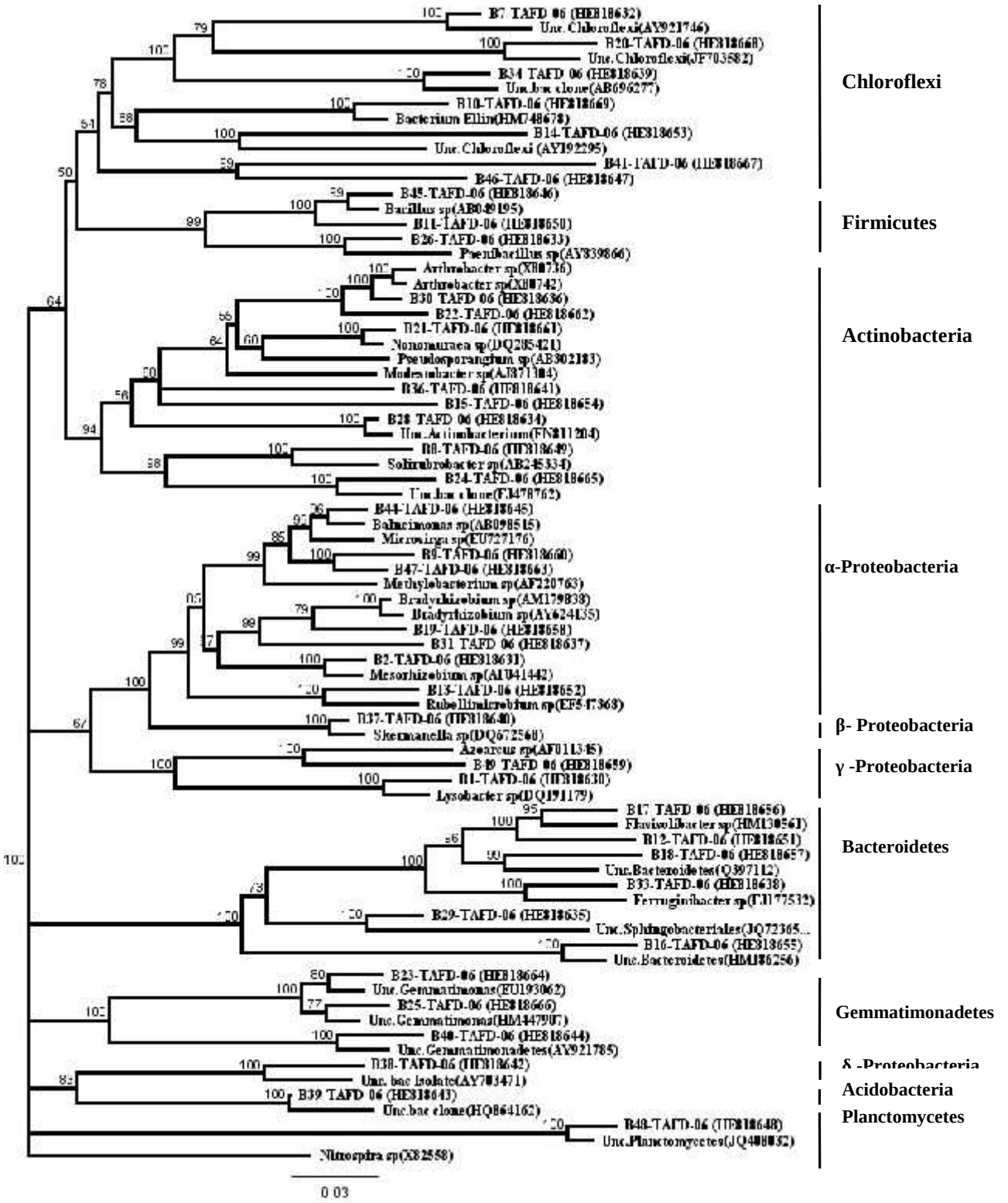

Figure 1. Neighbour Joining tree constructed from 16S-rRNA sequences obtained from rhizosphere of cultivar Fareed-06, growing at NARC, Islamabad, Pakistan. The number at the nodes of the phylogenetic tree are percentages indicating the levels of bootstrap support based on neighbour joining analysis of 1,00 bootstrap replicates. The scale bar representing the substitution per site. Bootstrap values supporting the grouping of cultured or un-cultured bacterial sequence to their respective lineages, mentioned next to the nodes.

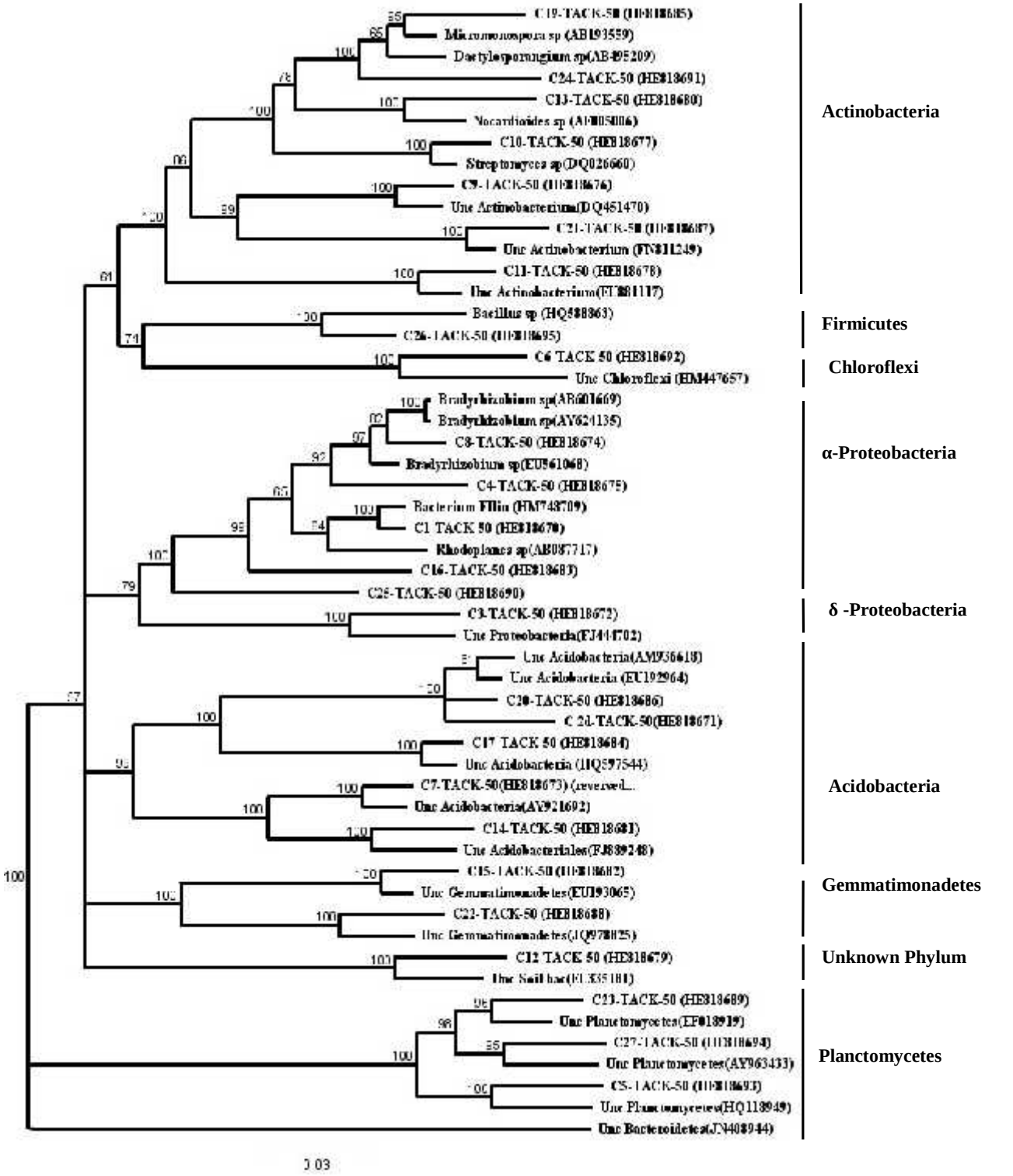

Figure 2. Neighbour Joining tree constructed from 16S-rRNA sequences obtained from rhizosphere of cultivar Chakwal-50, growing at NARC, Islamabad, Pakistan. The number at the nodes of the phylogenetic tree are percentages indicating the levels of bootstrap support based on neighbour joining analysis of 1,00 bootstrap replicates. The scale bar representing the substitution per site. Bootstrap values supporting the grouping of cultured or un-cultured bacterial sequence to their respective lineages, mentioned next to the nodes.

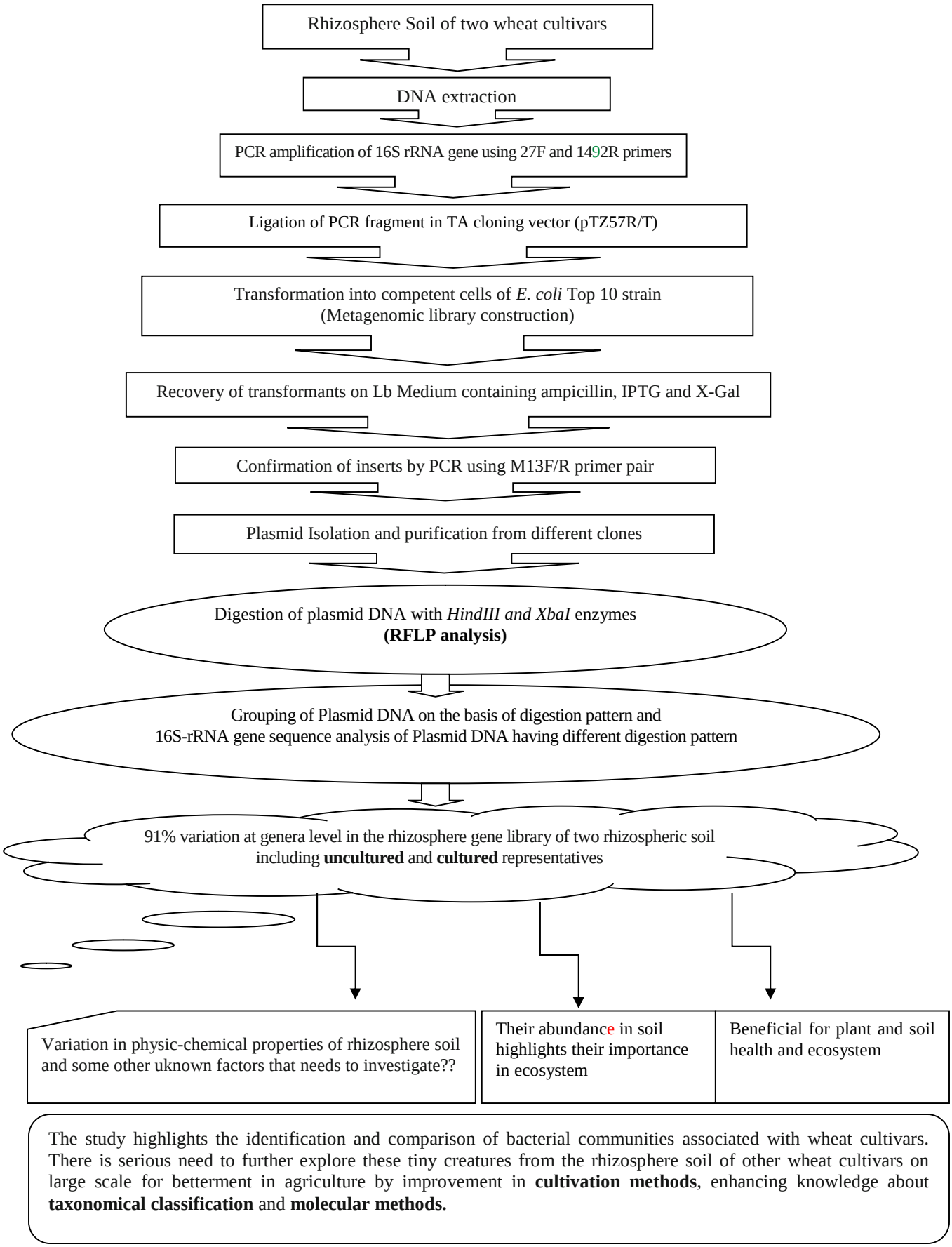

Figure 3. Schematic representation of work done.

Table 1. Physico-chemical properties of rhizosphere soil of wheat cultivars.

Wheat cultivars	pH	EC (dS/m)	Organic matter (%)	Total organic Carbon	Soil Texture	Soil moisture contents (%)	Nutrient concentration (mg/kg)										
							N-N O_3	P	Na	K	Ca	Mg	Mn	Zn	Fe	Co	Cr
Fareed-06	7.3	0.36	1[a]	0.68[a]	Sandy loam	50[a]	16.9[a]	15.3[a]	4.7[a]	5.8[a]	9.3[a]	6.2[a]	2.2[b]	2.7[a]	291[a]	39[b]	10.6[a]
Chakwal-50	7.3	0.34	0.79[b]	0.47[b]	Sandy loam	39.4[b]	13.4[b]	14.9[a]	4.1[a]	5.5[a]	9.0[a]	5.9[a]	4.2[a]	2.5[a]	175[b]	56.1[a]	11.1[a]

The soil texture, moisture contents, nutrients concentration including P, Na, K, Ca, Mg, Zn and Cr were same in both rhizospheric soil whereas the organic matter %, total organic carbon, N-NO_3, Mn, Fe and Co contents found differ. The means that share same letters are insignificantly different at $p<0.05$

Table 2. Class-level, Order-level and Family level distribution of different phyla encountered in rhizosphere soil samples of Wheat variety: Freed-06 based on 16S rRNA clone library construction in *E. coli* Top 10 strain using TA cloning vector.

No. of Clones	Phyla	Rhizosphere soil of Freed-06		
		Class-level distribution	Order-level distribution	Family-level distribution
11	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae
				Methylobacteriaceae
				Bradyrhizobiaceae
			Rhodospirillales	Rhodospirillaceae
		Betaproteobacteria	Rhodocyclales	Rhodocyclaceae
		Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae
		Deltaproteobacteria	Novel	Novel
1	Acidobacteria	Acidobacteria_Gp10	Novel	Novel
		Novel	Novel	Novel
3	Firmicutes	Bacilli	Bacillales	Bacillaceae
				Paenibacillaceae
8	Actinobacteria	Actinobacteria	Solirubrobacterales	Solirubrobacteraceae
			Actinomycetales	Micromonosporaceae
			Rubrobacteridae	Streptosporangiaceae
			Acidimicrobiales	Micrococcaceae
				Geodermatophilaceae
				Novel
6	Bacterioidetes	Sphingobacteria	Sphingobacteriales	Chitinophagaceae
		Bacteroidetes_incertae_sedis		Novel
				Novel
3	Gemmatimonadates	Gemmatimonadates	Gemmatimonadales	Gemmatimonadaceae
			Novel	Novel
7	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolineaceae
		Novel	Novel	Novel
1	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae

Table 3. Class-level, Order-level and Family level distribution of different phyla encountered in rhizosphere soil samples of Wheat variety: Chakwal-50 based on 16S rRNA clone library construction in *E. coli* Top 10 strain using TA cloning vector.

No. of Clones	Phyla	Rhizosphere of Chakwal-50		
		Class-level distribution	Order-level distribution	Family-level distribution
6	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae
				Bradyrhizobiaceae
		Delt proteobacteria	Myxococcales	Novel
5	Acidobacteria	Acidobacteria_Gp4	Acidobacteriales	Acidobacteriaceae
		Acidobacteria_Gp6	Novel	Novel
		Novel	Novel	Novel
1	Firmicutes	Bacilli	Bacillales	Bacillaceae
7	Actinobacteria	Actinobacteria	Acidimicrobiales	Acidimicrobineae"_incertae_sediss
			Actinomycetales	Acidimicrobiaceae
			Solirubrobacterales	Streptomycetaceae
				Nocardioidaceae
				Micromonosporaceae
				Conexibacteraceae
2	Gemmatimonadates	Gemmatimonadates	Gemmatimonadales	Gemmatimonadaceae
1	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolineaceae
3	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae

DISCUSSION

The result findings of current study provides evidence of the existence of numerous well studied lineages together with poorly described and un-cultured bacterial candidates with marked variations (91 % at genera level) in community composition of two rhizosphere libraries that supported the hypothesis. The two 16S-rRNA rhizosphere libraries covered 8 phyla, 14 classes, 14 orders, 23 families, 33 genera along with un-classified and un-characterized lineages that may represents novel lineages (Table 2 and Table 3). The 3 out of 33 genera: *Bacillus*, *Gemmatimonas* and *Bradyrhizobium* were found common in both rhizospheres, deviation in diversity was also seen at class (50 %), order (36 %) and family (78 %) level. The variation may relate to difference in physico-chemical properties: total organic carbon, organic matter%, soil moisture contents, $N\text{-}NO_3$, Fe, Mn and Co contents of two rhizosphere soil (Table 1). Complexity and heterogeneity in bacterial community related to several factors, the important one physical and chemical properties of soil (Preem *et al*., 2012; Brockett *et al*., 2012).

Diversification of phylum Proteobacteria in rhizosphere soil (Anne *et al*., 2009) had been confirmed by molecular methods (Teixeira *et al*., 2010; Yergeau *et al*., 2009). The 16S-rRNA sequences in both rhizospheres showed the dominancy of class alpha-proteobacteria (Figure 1 and Figure 2; Table 2 and Table 3) with well characterized members than rest of three classes, however, variation at class, order, family and genera was observed in both rhizospheres. The members of phylum Proteobacteria are well known for their role in agriculture, possesses several plant growth promoting mechanisms including: phytohormones, polyamine, siderophore production, phosphate solubilization (Perrig *et al*., 2007; Antoun *et al*., 1998), and nitrogen fixation etc (Islam *et al*., 2013), these cheapest and environmental friendly tiny creatures are the best alternative of chemical fertilizers/pesticides. There is serious need to further explore them.

Both the rhizosphere libraries showed 80% divergence on the basis of family level distribution pattern of phylum Actinobacteria (Table 2 and Table 3). The difference was best resolved at genera level and 100% deviation was found. However the generic level diversity was well characterized and diverse in the rhizosphere of Chakwal-50. Actinobacteria is counted as one among the largest bacterial phyla whose members have active contribution in plants microbe interaction (Coombs and Franco, 2003), possessed variable physiological and metabolic properties, versatile lifestyles; wide genomic heterogeneity reflects their wide distribution (Ventura *et al*., 2007).

The members of Acidobacteria are physiologically diverse and ubiquitous and some being acidophilic, the ecology and metabolism of these bacteria is not yet known properly, they have role in ecosystems maintenance, as they are abundantly present in soils (Barns *et al*., 2007; Quaiser *et al*., 2003; Rappe and Giovannoni, 2003; Kuske *et al*., 1997; Eichorst *et al*., 2007). The phylum consisted of eight groups (Barns *et al*., 1999; Hugenholtz *et al*., 1998) and three of eight groups containing cultured members. In current study

100% dissimilarity was found in both rhizospheres regarding distribution of members belongs to phylum Acidobacteria. In both rhizospheres the 16S-rRNA sequences were comparable with only uncultured representatives. Advances had been made to culture the uncultured members of Acidobacteria present in soil by the extension of incubation period, use of dilute nutrient broth and by the use of suitable additive for cultivation as: Gellan gum and $CaCl_2$ (Janssen *et al*., 2002; Davis *et al*., 2011).

Bacterioidetes are anaerobic bacteria that are commonly present in soil, in sediments and sea water (George and Garrity, 2010). The rhizosphere of Fareed-06 enfolded representatives comprised of two classes: Sphingobacteria and Bacteroidetes_incertae_sedis having both culturable and uncharacterized members with diverse origin. None of the representative from rhizosphere soil of Chakwal-50 fell under this phylum (Figure 2; Table 3). The sphingobacteriaceae affiliated members of phylum Bacteroidetes have been reported previously as psychrotolerant bacteria and as plant growth promoting bacteria (Pankratov *et al*., 2007; Mehnaz *et al*., 2010).

The family Bacillaceae of Phylum Firmicutes was commonly found in the rhizosphere of both cultivars whereas Paenibacillaceae was restricted to rhizosphere soil of Fareed-06 only (Table 2). The species of genus *Bacillus* and *Paenibacillus* have been isolated from various plants, act as PGPRs, have role as biocontrol agents (Ortiz-Castro *et al*., 2008; Timmusk *et al*., 2005). The genera *Paenibacillus* possessed nif H gene due to which they have role in nitrogen fixation (Weid *et al*., 2002). The un-identified lineages of phylum Gemmatimonadetes were found dominant in the rhizosphere of both cultivars. The members of this phylum are aerobes, widely distributed in nature and usually found from sludge, have un-identified lineages with few cultivatable (Zhang *et al*., 2003) members. Production of metabolically active compound as homospermidine a kind of polyamines had been revealed within this phylum (Hosoya *et al*., 2006), also capable of accumulating polyphosphate.

Planctomycetes and Chloroflexi (green non-sulphur bacteria) are widely distributed (Castenholz and Pierson, 1995; Morris *et al*., 2006) in nature, counted as not-yet culturable bacteria (Janssen, 2006) with few cultured representatives. The chloroflexi are capable of chlorophototrophy (light-energy conversion based upon chlorophyll). Whereas Planctomycetes have association with diatoms (Morris *et al*., 2006), have genes for transport and assimilation of NO^{-3} to diatoms. They also have ability to drive energy through cleavage of sulfated macromolecules produced by algae (Glockner *et al*., 2003). The identified sequences from both rhizosphers showed affiliation with Chloroflexi and Planctomycetes phyla were 100% uncultured. The family Anaerolineaceae of phylum Chloroflexi was common family in both of the rhizosphers. However unclassified representatives were more diverse with high no of clones in Freed-06 rhizosphere. Planctomycetaceae was the common family of phylum Planctomycetes in both rhizospheres with uncharacterized candidates (Table 2 and Table 3), 100% variation was observed at generic level.

The present study contributed to some extent to enhance the knowledge about diversity and comparison of rhizosphere bacterial communities of two wheat cultivars. Serious attempts are required to work on bacterial systematics to characterize taxa/ groups properly; the submission of sequences with properly described taxonomy and designing of primers that covers broad spectrum of phyla is required. The exact picture of species composition of bacterial diversity and their role in ecosystem may possible by going inside of their functions by enhancing the understanding about knowledge of molecular analysis: metagenomics and meta transcriptomics. Conversely upgrading the methods of cultivation by screening suitable additives are required besides metagenomics approach. Working with culture dependent and independent methods encourages understanding well the phenomenon of plants microbe interaction and maximum benefits can be obtained from the beneficial micrflora existing in the rhizosphere. The exact composition of root exudates, rizodeposits and soil physico-chemical properties also help to understand this phenomenon.

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