A comparative study of bacterial diversity based on culturable and culture-independent techniques in the rhizosphere of maize (Zea mays L.)

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Objective: Maize is an important crop for fodder, food and feed industry. The present study explores the plant-microbe interactions as alternative eco-friendly sustainable strategies to enhance the crop yield.

Methodology: Bacterial diversity was studied in the rhizosphere of maize by culture-dependent and culture-independent techniques by soil sampling, extraction of DNA, amplification of gene of interest, cloning of desired fragment and library construction.

Results: Culturable bacteria were identified as Achromobacter, Agrobacterium, Azospirillum, Bacillus, Brevibacillus, Bosea, Enterobacter, Microbacterium, Pseudomonas, Rhodococcus, Xanthomonas genera. For culture-independent approach, clone library of 16S ribosomal RNA gene was assembled and 100 randomly selected clones were sequenced. Majority of the sequences were related to Firmicutes (17%), Acidobacteria (16%), Actinobacteria (17%), Alpha-Proteobacteria (7%), Delta-proteobacteria (4.2%) and Gemmatimonadetes (4.2%) However, some of the sequences (30%) were novel that showed no homologies to phyla of cultured bacteria in the database. Diversity of diazotrophic bacteria in the rhizosphere investigated by analysis of PCR-amplified nifH gene sequence that revealed abundance of sequences belonging to genera Azoarcus (25%), Aeromonas (10%), Pseudomonas (10%). The diazotrophic genera Azotobacter, Agrobacterium and Zoogloea related nifH sequences were also detected but no sequence related to Azospirillum was found showing biasness of the growth medium rather than relative abundance of diazotrophs in the rhizosphere.

Conclusion: The study provides a foundation for future research on focussed isolation of the Azoarcus and other diazotrophs found in higher abundance in the rhizosphere.

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community in Pakistan. Securing sustainable yields requires a detailed knowledge of genetic and environmental factors that influence crop. Developed nations have used extensive breeding and management strategies for maximizing yields with higher inputs of fertilizers and insecticides. As the environmental and economic concerns of using these chemical increased. Alternative strategies are being employed to enhance the cropping system sustainability using eco-friendly approaches while retaining the competitive crop yields. The interaction between rhizosphere microbiota colonizing the plant root plays an important role in crop yield. The plant-microbe interactions taking place between “plant growth promoting rhizobacteria (PGPR) and plant root” mediate plant’s nutrient acquisition and disease tolerance. These rhizobacteria are a diverse group of microbes like Azospirillum, Azotobacter, Bacillus, Burkholderia, Herbaspirillum and Pseudomonas (Bhattacharyya and Jha, 2012; Qaisrani et al., 2014; Ayyaz et al., 2016) and involved in plant stimulation by atmospheric N₂-fixation, phytoremediation production, antagonism against pathogens, phosphate solubilization, siderophore production and biofilm formation. The beneficial effects of PGPR inoculation have been studied on various crops including maize (Bhattacharyya and Jha, 2012; Sheng et al., 2012; Zaheer et al., 2016).

Traditionally, analysis of bacterial communities and diversity has been dependent upon the cultivation of the microbes from the environment. However, culture-based studies provide limited information of community structure because majority of bacteria cannot be cultured in laboratory due to lack of information on specific growth requirements. As a result a large proportion of microbial population remained un-explored. Advancement in culture-independent techniques like sequence analysis of amplicons of 16S rRNA and nifH genes from soil DNA, has facilitated microbial diversity studies by comparing composition, richness, and structure of the prokaryotic communities in soil and other environments (Mirza et al., 2014; Hakim et al., 2018). These studies have even facilitated soil microbiologists to make more focused attempts to isolate useful microbes.

Previously, analysis of bacterial taxa associated with maize was based on culturable fraction or culture-independent fraction (Sanguin et al., 2006) separately. No data is available on the comparative analysis or the nifH based analysis of culture-dependent and culture-independent fractions of rhizosphere communities from the rhizosphere of maize. In present study maize rhizosphere soil samples were collected and investigated the culturable fraction of bacterial community from maize by isolations on growth media, followed by 16S rRNA based identification of isolates. Bacterial diversity studies were extended to non-culturable fraction by extracting DNA directly from that soil for PCR amplification of 16S rRNA and nifH genes, followed by sequence and phylogenetic analysis.

2. Methodology

2.1. Analysis of bacterial diversity through culture-dependent technique

Rhizospheric soil and roots samples of maize plants (variety FSH 810) were collected from experimental fields of NIBGE (National Institute for Biotechnology and Genetic Engineering). The field soil was a sandy loam and bacteria were isolated on LB (Luria-Bertani) agar and NFM (Nitrogen-Free Medium) medium (Okon et al., 1977) using serial dilution plating technique. Colonies with different shape, size and color purified separately through subculturing on the same medium. Nitrogen fixers were obtained by enrichment technique, root pieces (5–10 mm length) were inoculated along with rhizospheric soil to NFM medium and after 5–6 enrichments, single colonies were purified on LB plates. Colony morphology was studied after 24 h of incubation at 28 ± 2 °C.

2.2. PCR amplification and cloning

CTAB (Cetyl Trimethyl Ammonium Bromide) method was used to extract total genomic DNA from pure bacterial strains. 16S ribosomal RNA gene was amplified by primers PH: 5′-AAGGAGGTGATCCACGCGCCA-3′ and PA: 5′-AGAGTTGTAACTTGGCTCAG-3′ using conditions reported by Qaisrani et al. (Qaisrani et al., 2014). The PCR products were cloned in pTZ57/R vector (Fermentas, Germany), confirmed by restriction analysis and sequenced commercially from Macrogen, Korea. Molecular phylogenetic analysis of strains were done as per Zaheer et al. (2016) study.

2.3. Analysis of bacterial diversity by culture-independent technique

2.3.1. Soil sampling and DNA extraction

Rhizospheric soil samples were collected from three maize plants and pooled to prepare a composite sample. From this composite sample two sub-samples (0.5 g each) were used for extraction of soil DNA. Soil DNA was extracted using Fast DNA Spin Kit (MP Biomedicals Inc, France).

2.3.2. PCR amplification, cloning and library construction

To amplify 16S rRNA gene from soil DNA, primers and reaction conditions were same as reported earlier by Qaisrani et al. (2014). For amplification of nifH; PolF and PolR primers was used. PCR conditions were the same as Qaisrani et al. (2014); except annealing temperature of 48 °C. The PCR products were cloned in pTZ57/R vector (Fermentas, Germany), confirmed by restriction analysis. 100 clones were randomly selected and sequenced commercially from Macrogen, Korea. For nifH, PCR products of nifH from six independent reactions were combined and cloned. Forty clones were selected randomly and sequenced.

3. Results

3.1. Bacterial diversity using culture-dependent method

Ten isolates were identified as Bacillus, four as Azospirillum brasilense, two Pseudomonas stutzeri, three Stenotrophomonas spp., two Enterobacter spp. and one each of Brevibacillus, Agrobacterium, Bosea and Microbacterium sp., based on 16S rRNA gene analysis (Table 1). Bacillus genera came as dominant genera in culturable population followed by Azospirillum sp. and Stenotrophomonas sp. (Fig. 1).

3.2. Bacterial diversity revealed by 16S rRNA gene sequence analysis

Out of 100 clones sequenced randomly from 16S rRNA clone library, 70 clones provided the good read-length and sequence information (Table 2). Most of the clones (30%) were related to the uncultured bacterial sequences, which did not show any similarity with the known phyla or taxa (Fig. 2). Firmicutes, acidobacteria, actinobacteria, alpha-proteobacteria, delta-proteobacteria, and gemmatimonadetes were the major phyla found.

3.3. Diversity of diazotrophs revealed by nifH sequence analysis

Twenty clones provided the sequence information out of 40 clones sequenced from nifH gene clone library. Among the nifH sequences obtained in the present study, 85% sequences showed similarity with those of cultivable diazotrophs and the remaining 35% showed sequence similarity with non-culturable bacteria.
The nifH sequences similar to culturable diazotrophs belonged to genera Aeromonas, Agrobacterium, Azoarcus, Azotobacter, Bacillus, Pseudomonas and Zoogloea, which showed culturing with their respective member in phylogenetic analysis (Fig. 3).

4. Discussion

Investigation of bacterial communities and diversity in the plant’s rhizosphere is very important as these microbes exert direct beneficial or pathogenic effect on plants. Despite the abundance of bacterial species in the rhizosphere, more than 99% of these species cannot be cultured that include 31 bacterial phyla. Metagenomic analysis provided detailed information of microbial diversity, composition, richness, structure and function (Mirza et al., 2014). Comparison of culturable and non-culturable community will help to determine the structurally abundant, functionally viable and potentially valuable bacteria that can ultimately be used as inoculum to influence the plant health in a positive manner.

Limited studies are available regarding the bacterial diversity in maize rhizosphere. In the present study, bacterial diversity was compared by using culture-dependent technique and culture-independent technique. Among the culturable population obtained dominant (37%) were members from Bacillus spp. which have been widely stated in the rhizosphere of different plants (Hakim et al., 2018). Bacterial isolates showing plump rods with vibroid motility in N-free semi solid NFM medium, showed high sequence similarity with Azospirillum brasilense strains. Azospirilla have been isolated from many crops including cereals, legumes and grasses (Qaisrani et al., 2014; Ayyaz et al., 2016).

Among others, following genera Pseudomonas stutzeri, Stenotrophomonas maltophilia Enterobacter, Agrobacterium, Microbacterium, Bosea, and Brevibacillus spp. were obtained. From maize rhizosphere, the isolation of Pseudomonas, Enterobacter, Microbacterium...
neimengense and Agrobacterium spp. have been described. Isolation of *Stenotrophomonas* from the rhizospheric soil of *Astragalus bisulcatus* and similarly from the rhizospheric soil of sugarcane *Brevibacillus* has been reported. The genus *Bosea* has been isolated from the agriculture soil (Qaisrani et al., 2014; Hakim et al., 2018). The low sequence homologies (<90%) obtained for *Agrobac-

| Clone ID | Accession # of the clones | Description | Maximum similarity (%) in the databank and accession number |
|----------|---------------------------|-------------|-------------------------------------------------------------|
| MRS1     | HE585109                  | Uncultured (U) bacterium | EU160410 (98)                                                 |
| MRS2     | HE585110                  | Bacillus sp. | AJ315064 (95)                                                 |
| MRS3     | HE585111                  | U. Myxococcales bacterium | EU445232 (95)                                                 |
| MRS4     | HE585112                  | U. bacterium | EU676444 (94)                                                 |
| MRS5     | HE585113                  | U. Actinobacter sp. | JX505257 (98)                                                 |
| MRS6     | HE585114                  | Actinomadura sp. | AF131317 (95)                                                 |
| MRS7     | HE585115                  | U. bacterium | FJ935277 (84)                                                 |
| MRS9     | HE585116                  | Pseuobacillus validus | GU191921 (95)                                                 |
| MRS11    | HE585117                  | U. Acidobacterium | DQ151404 (99)                                                 |
| MRS12    | HE585118                  | U. Acidimicrobium | FJ514175 (94)                                                 |
| S1       | HE995450                  | U. Bacillus sp. | HE646746 (99)                                                 |
| S2       | HE995441                  | U. Gemmatimonas sp. | HM447783 (96)                                                 |
| S3       | HE995452                  | U. Bacillus sp. | JQ793577 (97)                                                 |
| S4       | HE995443                  | U. bacterium | JN177890 (98)                                                 |
| S5       | HE995444                  | U. acetobacteraceae | EU193084 (98)                                                 |
| S6       | HE995454                  | U. Gemmatimonadetes | AV921764 (100)                                                |
| S7       | HE995446                  | U. Acidobacteria | HM447891 (98)                                                 |
| S8       | HE995447                  | U. Nocardioides | HE662540 (98)                                                 |
| S9       | HE995448                  | U. Acidobacterium | HE646770 (76)                                                 |
| S10      | HE995449                  | U. Acidobacteriaceae | EU792108 (93)                                                 |
| S11      | HE995450                  | U. Acidobacter | EU792108 (93)                                                 |
| S12      | HE995451                  | Syntrophobacter fimarumodians | EU266858 (92)                                                |
| S13      | HE995452                  | U. acidobacterium | JN409041 (93)                                                 |
| S14      | HE995453                  | U. Acidobacteriaceae | HM438249 (99)                                                 |
| S15      | HE995454                  | U. Conexibacter sp. | FJ551841 (97)                                                 |
| S16      | HE995455                  | U. Acidimicrobium | FJ551841 (97)                                                 |
| S17      | HE995456                  | U. Acidobacteriaceae | HM438240 (99)                                                 |
| S18      | HE995457                  | U. bacterium | JN030403 (98)                                                 |
| S19      | HE995458                  | U. Sphingomonadales | FJ89322 (98)                                                   |
| S20      | HE995459                  | Bacillus sp. | AB082678 (85)                                                 |
| S21      | HE646745                  | Streptomyces prasinopsis | JX182604 (99)                                                |
| S22      | HE646746                  | Bacillus subterreus | NR104749 (99)                                                 |
| S23      | HE646747                  | U. bacterium | JF913025 (95)                                                 |
| S24      | HE646748                  | Terrabacteri sp. | EU436595 (98)                                                 |
| S25      | HE646749                  | U. bacterium | GQ306031 (93)                                                 |
| S26      | HE646750                  | U. bacterium | JN417563 (99)                                                 |
| S27      | HE646751                  | U. Bacillus sp. | JN082282 (99)                                                 |
| S28      | HE646752                  | U. bacterium | HM437987 (98)                                                 |
| S29      | HE646753                  | Catenulis poraacidiphila | CP001700 (97)                                                |
| S30      | HE646754                  | U. bacterium | HM37969 (99)                                                   |
| S31      | HE646755                  | Agrobacterium tumefaciens | JF513176 (97)                                                |
| S32      | HE646756                  | U. Rubrobacteriaceae | FJ552011 (98)                                                 |
| S33      | HE646757                  | U. Bacillus sp. | AY082367 (99)                                                 |
| S34      | HE646758                  | U. Actinobacterium | JN37890 (97)                                                   |
| S35      | HE646759                  | U. Chloroflexi | HQ397103 (96)                                                  |
| S36      | HE646760                  | U. Bacillaceae bacterium | JQ793415 (99)                                                |
| S37      | HE646761                  | U. delta-Proteobacterium | KF247583 (95)                                                |
| S38      | HE646762                  | U. bacterium | JQ248756 (96)                                                 |
| S39      | HE646763                  | U. bacterium | JN038819 (93)                                                 |
| S40      | HE646764                  | Nonomuraea sp. | KC417349 (99)                                                  |
| S41      | HE646765                  | U. Rhizobiales | HM447746 (94)                                                  |
| S42      | HE646766                  | Acidobacteriaceae | HM438242 (90)                                                 |
| S43      | HE646767                  | U. Bacteroidetes bacterium | KC49976 (98)                                                  |
| S44      | HE646768                  | U. Gemmatimonas sp. | HM438475 (96)                                                 |
| S45      | HE646769                  | U. bacterium | JN178474 (98)                                                 |
| S46      | HE646770                  | U. Acidobacteria bacterium | JQ957800 (97)                                                |
| 58       | HE798162                  | U. bacterium | FJ52787 (98)                                                   |
| 59       | HE798163                  | U. bacterium | JQ69654 (98)                                                   |
| 60       | HE798164                  | U. bacterium | JN037819 (98)                                                  |
| 61       | HE798165                  | U. bacterium | JN037990 (84)                                                  |
| 62       | HE798166                  | U. bacterium | JN869020 (94)                                                  |
| 63       | HE798167                  | U. Sphingomonas sp. | JN628042 (99)                                                 |
| 64       | HE798168                  | U. bacterium | K554081 (97)                                                   |
| 65       | HE798169                  | U. alpha-Proteobacterium | KF437571 (97)                                                |
| 66       | HE798170                  | U. Actinobacterium | HQ13825 (98)                                                   |
| 67       | HE798171                  | Bacillus sp. | AB062678 (99)                                                  |
| 69       | HE798173                  | Alpha-Proteobacterium bacterium | KF43757 (96)                                                |
| 70       | HE798174                  | Nocardioidea sp. | NR_044185 (99)                                                 |
| 71       | HE798175                  | U. bacterium | FJ555754 (97)                                                  |
| 72       | HE798176                  | Janibacter sp. | JN644568 (90)                                                  |
terium, Bosea and Stenotrophomonas spp. might be due to the partial 16S ribosomal RNA gene sequences gained in the present study for sequence comparison.

The cultivation-independent analysis demonstrated that majority of the sequences (78.6%) obtained from the soil DNA derived 16S rRNA clone library were related to the uncultured bacteria. About 30% of the total cloned sequences showed no similarity with the known phyla or taxa and were considered as novel sequences. Other genera detected were firmicutes (17%), acidobacteria (16%), actinobacteria (17%), alpha-proteobacteria (7%), delta-proteobacteria (4.2%) and gemmatamonedales (4.2%). Abundance of proteobacteria was earlier reported in canola (Kaiser et al., 2001) and two pasture soils followed by actinomycetes (McCaig et al., 1999). Moreover, abundance of α-proteobacteria was reported in rice clone libraries along with acidobacteria, firmicutes, bacteriodetes groups (Arjun and Harikrishnan, 2011).

Similarities of clone sequences showing relatedness to culturable bacteria were further computed to find the PGPRs among...
them. In the 16S rRNA clone library, sequences related to Bacillus sp., Syntrophaceae, Kaistobacter sp, Sphingomonadales, Streptomyces sp, Janibacter sp, Nocardioides sp, Azospirillum sp, Sphingomonas sp, Rubrobacteraceae and Nonomuraea were detected. Members of these genera have been earlier known as PGPR, exhibiting one or more plant-beneficial traits including enzyme production like ACC-deaminase, bio-control potential against Fusarium and improve the phytoremediation ability of Brassica juncea grown-up in contaminated soil with glyphosate (Qaisrani et al., 2014; Ermakova et al., 2010). Bacillus strains produce antifungal compounds, siderophores and HCN that help plant for optimum growth and exhibit bioremediation potential for Chromium (Cr).
contaminated soils (Kathiravan et al., 2011). The Burkholderia strains have been described as to produce ACC-deaminase, siderophores and anti-fungal compounds for maize growth promotion (Byrt et al., 2011). Herbicide resistant characters in Kaistobacter and Nocardoides strains have also been described. Sphingomonas and Streptomyces have been reported as biocontrol agents and produce siderophores and enzymes. Bioremediation potential of Streptomyces for Cr has been reported (Sheng et al., 2012). The sequences associated to Terribacillus sp. Acidobacteria; Gemmata sp., Gemmimonas sp. Chloroflexi and Actinobacterium were also detected during this study but no PGPR activity has been reported for the members of these groups so far.

When the culture-dependent data was compared with the culture-independent data, Azospirillum, Achromobacter, Rhodococcus and Bacillus genera were detected in the clone library but sequences related to Pseudomonas were not detected using culture-independent technique. Although, the number of clones sequenced were not in large quantity and were randomly selected but Pseudomonas were lacking among the 70 clones. From the rhizosphere of maize, using ITS the presence of acidobacteria, actinobacteria, bacteroidetes, chloroflexi, firmicutes, gemmatimonadetes and proteobacteria have been reported (Chauhan et al., 2011). Moreover, Enterobacter, Erwinia, Klebsiella, Pseudomonas, Stenotrophomonas and Bacillus were reported as predominant while Achromobacter, Lysinibacillus and Paeucbacillus as rare genera in maize rhizosphere (Paola et al., 2011). Comparing the data of present study with those of published on this subject it is clear that proteobacteria, actinobacteria, bacteroidetes acidobacteria, firmicutes, chloroflexi, planctomycetes, gemmatimonadetes are the most dominant bacteria in the rhizosphere of maize.

Regarding the diversity of functional gene nifH, the sequences related to nifH of Azospirillum sp. (25%), Pseudomonas stutzeri (10%), Aeromonas (10%), Azobacter (5%), Agrobacterium (5%), Zoogloea oryzae (5%) and Bacillus (5%) were detected. Moreover 35% sequences showed no similarity with the nifH of cultured bacteria. These results suggest that maize rhizosphere favors the growth and presence of diverse diazotrophs that can have the potential to enhance the crop productivity. The nifH sequences in soil DNA related to those of Pseudomonas were detected but nifH gene could not be amplified from the pure cultures of Pseudomonas retrieved from rhizosphere of maize. The incidence of nitrogen-fixation in Pseudomonas genus has been long discussed. P. stutzeri strain are rare nitrogen-fixer (Mirza et al., 2006) and in most cases positive identification of these strains based on DNA-techniques were not engaged at the time of their isolation. nifH sequences related to Zoogloea genus from maize rhizosphere were found. The presence of nitrogen fixing Zoogloea has been reported from the soils of Pakistan and was used as PGPR for sugarcane (Mirza et al., 2001). Among the clones of nifH gene obtained in the present study, 25% were related to Azoarcus. Isolation of Azoarcus strains from kallar grass of Pakistani saline soils was initially reported by Reinhold-Hurek et al. (1993) and was extensive studied there within host plant. Since then, no Azoarcus could be isolated from rhizosphere although extensive work was carried out on the isolation of diazotrophs and other PGPR. As a result, Azoarcus was considered as of rare occurrence and uncommon among the diazotrophic population in Pakistani soils. However presence of nifH sequences related to Azoarcus in maize rhizosphere necessitate intensification of isolation attempts to obtain pure cultures of this important bacterium for inoculum production for maize or other crops grown in the country. Contrary to the Azoarcus, four Azospirillum strains were identified from the rhizosphere of maize using culture-dependent technique but no 16S rRNA or nifH sequence related to this diazotrophic genus was detected among both the libraries (nifH, 16S rRNA) constructed from soil DNA. This reflects the biasness of the growth medium used in this study rather than the relative abundance of Azospirillum in the rhizosphere. Future studies based on next-generation sequencing technology may enable detection of these and other important PGPR in the maize rhizosphere of maize.

5. Conclusion

This study has provided a basis for the future research on “non-culturable” PGPRs and the diazotrophic population present in the rhizosphere of maize especially a rarely cultivated but frequent colonizer Azoarcus. More focused approach should be used for targeted cultivation of this diazotroph and exploit its potential to enhance nitrogen acquisition of plant. The information will help to identify potential PGPR for maize inoculation as many of the strains identified might have direct or indirect part in plant stimulation. Moreover, several other bacteria were detected that might have potential for bio-remediation of contaminated-soils or the production of useful enzymes for industrial purposes.

Declaration conflicts of interest

None.

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