

Original Research Article

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Microbiome of Groundnut (*Arachis hypogaea* L.) Rhizosphere Infected with *Macrophomina phaseolina* Root Rot

M. V. Parakhia* and B. A. Golakia

Department of Biotechnology, Junagadh Agricultural University, Junagadh, Gujarat, India

*Corresponding author

ABSTRACT

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This study reports the analyses of the rhizospheric microbiome of the groundnut (*Arachis hypogaea* L.) from Gujarat, India. Samples were collected using standard protocols and 16S rRNA gene V3-V4 region amplicon sequencing was performed to identify the microbial communities prevalent in the rhizosphere. A total of 9527329 quality checked reads with the average read length 232 bp were analysed using QIIME to reveal the abundance of Acidobacteria, Proteobacteria, and Planctomycetes. The diversity analysis for the community highest 116 species were observed in infected sample.

Introduction

In the world where the population keeps increasing at an alarming rate while food is inadequate, it is compulsory to new alternatives to feed the growing population need to be identified. The answer lies in harnessing and exploiting the beneficial interactions between plants and their rhizospheric microbiome to increase plant health and productivity. An understanding of the mechanisms that govern such interactions is essential to increase plant health and productivity.

To amplify the food production over use of conventional chemical pesticides from the

years also has resulted in adverse effects on the environment and non-target organisms. The Groundnut & other crops in India often suffer from various type of root rot & wilt diseases caused by fungi. Among these the dry root rot caused by *Macrophomina phaseolina* (Tassi) Goid has been noticed to cause 33.33 per cent seed rotting and 23.80 per cent post emergence mortality (Gupta and Kolte, 1981).

To study the dry root rot disease conventional microbiological methods were used. in these method only cultivable microbes and its interaction can be studied. it is identified that 98% microbes are not cultivable means cannot grown in laboratory media. it is

require to study the role of uncultivable microbe's on infection to the plant. The rhizosphere zone around the root is the largest microorganisms ecology in the world. The rhizomicrobiome has the capability to help fight numerous biotic and abiotic stresses as well as enhance plant health and productivity. rhizomicrobiome study of microorganisms cultivable as well as uncultivable to understand the interaction effect of microorganisms on plant productivity and health.

Microbiome is defined as the totality (Cultivable and Uncultivable) of microorganisms and their collective genetic material present in or on the plant or in another environment.

Metagenomics combines the power of genomics, bioinformatics, and systems biology. Operationally, it is novel in that it involves study of the genomes of many organisms simultaneously. It provides new access to the microbial world; the vast majority of microbes cannot be grown in the laboratory and therefore cannot be studied with the classical methods of microbiology. Although community ecology is not new to microbiology, the ability to bring to bear the power of genomics in the study of communities initiates an unparallel opportunity.

Normally the plant associated microbes are studied by culture based methods. Unfortunately this culture based methods could not represent the actual microbial composition of the environment. Culture based methods are, most often, known to have bias towards few bacterial groups. Microbes that cannot grow on artificial media can study using direct nucleic acid extracted from samples. In the recent times high-throughput technologies are developed to expose the interactions between complex microbial

communities and their hosts. For prokaryotes, sequencing of 16S rRNA gene is the most common method used for the identification of different species and strains. By analyzing on 16S rRNA gene region, it is possible to taxonomically group different bacteria.

Along with increasing plant productivity, the soil microbiome also provides an important role in disease-suppressive soils. The ability of a soil to suppress disease is of key importance in measuring soil productivity (Janvier *et al.*, 2007). There are many PGPRs that aid in disease suppression via the release of antimicrobial or antifungal compounds that deter plant pathogens (Garbeva *et al.*, 2004; Weller *et al.*, 2002).

Elevated levels in species richness and diversity produce high functional redundancy within the soil microbiome, allowing it to quickly recover during stress (Nannipieri *et al.*, 2003; Yin *et al.*, 2000). The high functional redundancy in soil microbial diversity also confers protection against soil-borne diseases (Brussaard *et al.*, 2007; Garbeva *et al.*, 2004; Mendes *et al.*, 2011; Nannipieri *et al.*, 2003). Consequences of interaction between rhizosphere microbiome, especially the dominant microbial community and dry root rot fungus, *Macrophomina* have not been systematically analyzed. Unraveling the composition and function of the groundnut rhizosphere microbiome is not only important for our basic understanding of host-microbe interactions but also of great agronomic and ecological importance as well. Analysis of groundnut rhizosphere microbiome is of great commercial importance for groundnut farming as the crop is susceptible to several pathogens. Therefore, there is a need to decipher the microbial composition of groundnut rhizosphere and their influence on economically important diseases like dry root rot.

With these background a thesis project on "Utilizing groundnut (*Arachis hypogaea* L.) rhizosphere microbiome for biological control of dry root rot" is envisaged with the following objectives.

Materials and Methods

Sample collections

Rhizosphere soil of groundnut variety GG-20 was collected. Samples were collected from selected sites having different type of soils (table no.3.1) with grown GG-20 groundnut variety having healthy and/or dry root rot infected plant for microbiome study.

DNA Extractions and sequencing

DNA was extracted from all 10 samples using The Power Soil® DNA Isolation Kit with minor modification and accordance to manufacture's instruction by MoBio laboratories Inc. Sample library preparation and sequencing were carry out as per ion torrent S4 sequencing kit guideline.

Data analysis

Reads obtained from sequencer were converted to bam file and Fastq format in ion torrent server

Quality control for reads

Primary quality control by the machine in-build software to reduce ambiguous reads and final fastq file used for the secondary quality checking using the CLC genomics workbench 20.0. In that CLC processed for the adapter, barcodes, ambiguity and primer, duplicate reads are screened and removed.

Binning process

Reads are used for mapping with using databases RDP, Greengen, and SILVA for

16S region. Binning process were carry out in MG-RAST (<https://www.mg-rast.org/>) and QIIME (<http://qiime.org/>). QIIME software was used for analysis of 16S rDNA sequencing data. QIIME is comprehensive software comprising of tools and algorithms such as Fast Tree for heuristic based maximum-likelihood phylogeny inference (Price *et al.*, 2010).

Ion Reporter (<https://ionreporter.thermofisher.com/ir/>) tool were used to identify the genus or species level of microbes present in complex polybacterial research samples, using both Greengenes and premium MicroSEQ ID 16S rRNA reference databases. Ion Reporter metagenomics workflow: Unaligned binary data files (Binary Alignment Map, BAM) generated by the Ion Torrent Ion S5 were uploaded to Ion Reporter and analysed using default settings. Calls were made by assessing the most specific unique taxonomic level identified using the mapped sequences.

The Ion Reporter metagenomics workflow was developed for primer information, classification information, percent ID, and mapping information. Also identified population diversity for whole metagenome at taxonomic level with the interactive display

Data process and visualisation

Data of QIIME and MG RAST were processed using MEGAN (<https://bio.tools/megan>) and STAMP (<https://beikolab.cs.dal.ca/software/STAMP>) tools and data was further process and selection for OTU were narrow down manually. Selected OTU were shorted on the base of abundance and uniqueness.

Selected Data/OTU were compared on the basis of sample site variation and status of groundnut (Healthy/Infected). Data also

analyzed manually for selected genus and species richness in the samples/sites.

Diversity analysis

Using QIIME (Observed-species, Shannon, Simpson and Good-coverage) alpha diversity through 8 indices was analyzed. Beta diversity was also calculated by QIIME (Version 1.9.1) on unweighted Jackknifed. Cluster analysis was preceded by principal component analysis (PCA). Beta-diversity provides a compute of similarity, or dissimilarity, of one microbial composition to another. Beta-diversity is typically calculated on the OTU/ASV/species composition tables directly (after normalization), but can be calculated using abundances at higher taxonomic levels.

While alpha diversity is a measure of the diversity (or complexity) within samples, beta diversity refers to the diversity between samples. This is essentially a measure of how similar or dissimilar the samples are, and is usually represented by a distance matrix which is then used to do Principal Coordinates Analysis (PCOA). The result of this is an ordination plot of multiple dimensions, where each sample is a point and the distance between the points represents the similarity of those samples (closer together = more similar). I used these plots to explore the data and see which variables explain the similarity or dissimilarity of groups of samples (Aitchison and Greenacre, 2002).

Results and Discussion

To study microbial diversity and population associated with healthy and root rot infected groundnut GG20 plant rhizosphere. Rhizospheric soil samples were collected from the 5 different sites. From each site collected 10 healthy and 10 root rot infected plants. out of them 20 sample make two samples, one

was healthy sample and one was root rot infected sample. Samples were collected from five different type of soil location. So total 10 samples in which 5 healthy plant rhizosphere and 5 rppt rot infected plant rhizosphere soil. Total DNA was extracted direct from all 10 rhizosphere soil using The Power Soil[®] DNA Isolation Kit. to check the DNA quality performed the gel electrophoresis and got the intact DNA as shown in Figure 1.

Microbial population was studied using the metagenomics approach. after the DNA was extracted, prepare the amplicon using the primer set (16S primer set V2-4-8 16S primer set V3-6,7-9) provided in the 16S[™] Metagenomics Kit from thermo fisher. using these primer set prepared the sequencing libraries. sequencing was performed using the Next generation sequencer ION S5 (Ion-torrent) of the prepared libraries of all 10 samples. during the sequencing samples were separated by using the IonExpress barcode_001 to Ion Express barcode_010. observations were recorded from the NGS as Figure 2.

Sequencing data was extracted from the the Sequencer. Total 2.5 GB data in fastQ format. this data included unprocess total reads 1,07,68,140 with the mean length was 233bp. debarcoding of the sample data gave the following data reads (Table 1).

All sequenced reads were analyzed using the MG-RAST (Metagenomics RAST: metagenomics.anl.gov) server version 3.5, which is an open source service made up of a high-throughput pipeline built for high-performance computing of metagenomes. Screening against a large group of protein and nucleotide databases, MG-RAST allows for the automated assignment of metagenomic sequences to their respective phylogenetic and functional groups for ease of data interpretation, in addition to providing tools

for comparative metagenomics. The pipeline, implemented in Perl, utilizes a combination of open source components which include the SEED framework (Anderson *et al.*, 2005), Sun Grid Engine, SQLite, and NCBI Blast (Gopalakrishnan *et al.*, 2011).

It is important to check the quality and coverage of the sequencing data using the CLC Genomics Workbench 20.0. analysed the data obtained from the sequencer.

Table.1 Untrimmed raw data representing No. of reads and Mean length present in samples

S.N.	Sample	Bases in MB	Reads	Mean Read length
1	Healthy-1	241.32	1022740	235
2	Infected-1	507.05	1245313	236
3	Healthy-2	149.48	635376	235
4	Infected-2	155.23	658089	235
5	Healthy-3	275.30	1125961	244
6	Infected-3	242.10	1073193	225
7	Healthy-4	264.10	1154472	228
8	Infected-4	186.61	827429	225
9	Healthy-5	198.11	863458	229
10	Infected-5	211.36	921298	229

Table.2 Family, Genus and Species no. of (operational taxonomic unit) OTU presented in all 10 metagenomes

Sr. No.	Sample	Family OTU	Genus OTU	Species OTU
1	H1	336361	113208	38262
2	I1	817965	271234	90693
3	H2	201247	60035	19225
4	I2	215198	81489	26290
5	H3	478039	225603	99773
6	I3	420035	163045	53588
7	H4	481866	207818	78772
8	I4	325684	136267	46115
9	H5	366968	156751	55638
10	I5	325480	129548	41767

Fig.1 Gel of DNA extracted from all 10(H1-H5 and I1-I5) samples

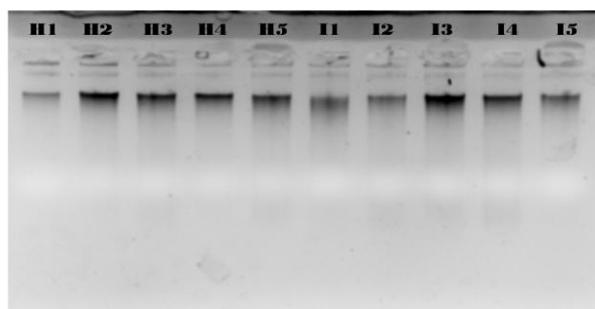


Fig.2 Sequencing run summary representing chip image (Ion 530) and read length

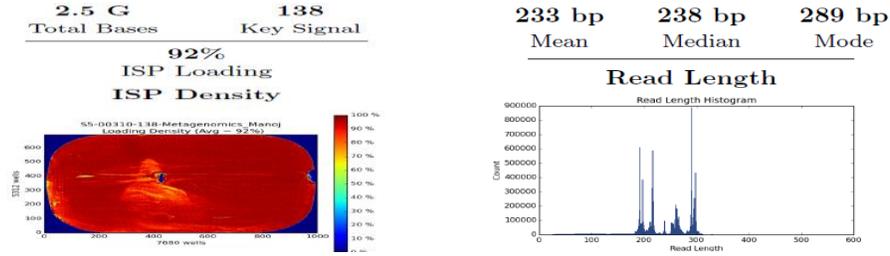


Fig.3 The α -diversity of samples representing the species numbers and standard deviation

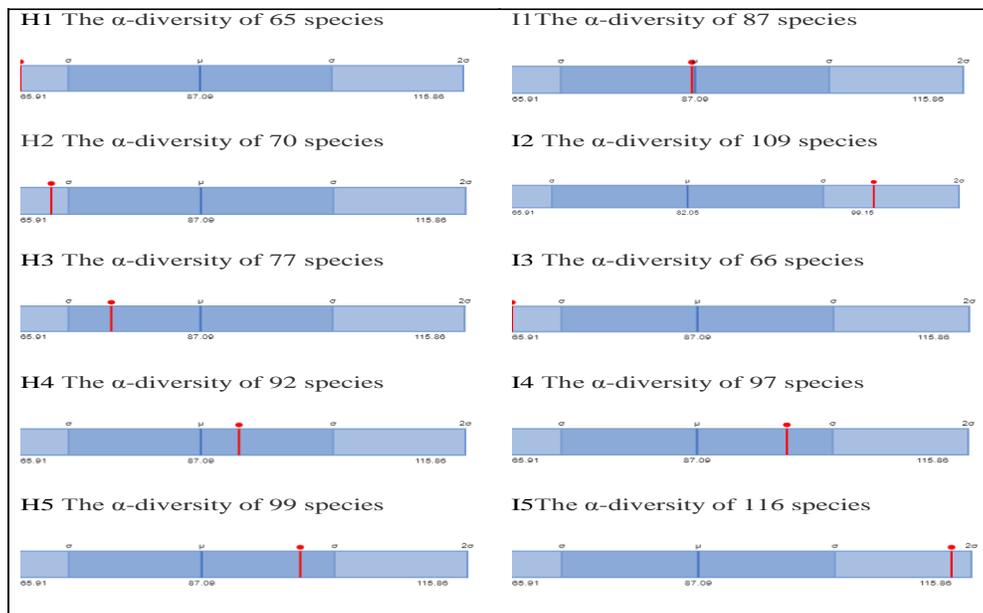


Fig.4 OTU numbers representing Family, Genus and Species of groundnut variety GG-20 Rhizospheric metagenomes of 5 different location of Gujarat at healthy and infection stage. (X-axis represent sample and Y-axis represent OTU)

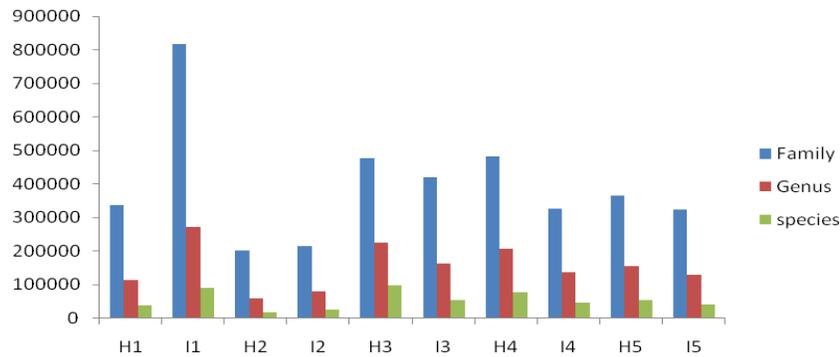
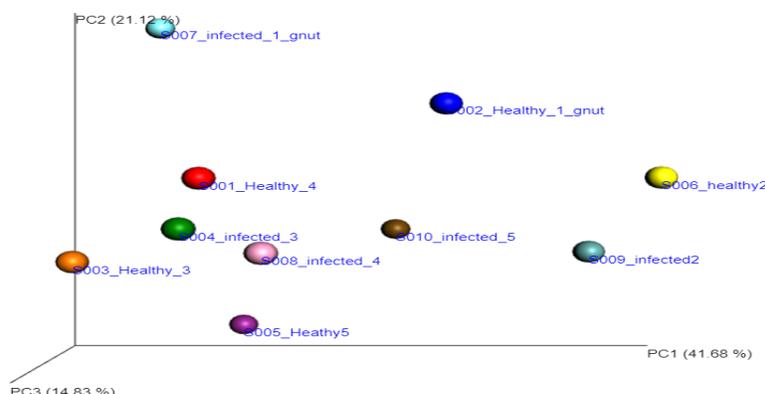


Fig.5 Principle component analysis based on unweighted unifracs 2d reveals sequence based grouping of Family, Genus and species level in A) Healthy H1, H2, H3, H4 and H5. B) Infected I1, I2, I3, I4 and I5 C) Healthy and Infected samples



GC Content of sequencing data

GC content of any sequencing data is specific for strains and the GC content of a gene region can impact its coverage, with regions having 50–60% GC content receiving the highest coverage while regions with high (70–80%) or low (30–40%) GC content having significantly decreased coverage. Our result based on CLC analysis all the samples sequencing data had GC content 50-65% so we can say that our sequencing data have sufficient coverage. The PHERD score of all the samples were between 17-32 and most of the sequences score was 30. So it was indicated that the quality of data was good and have 99.99% accuracy.

Bacterial diversity analysis

Alpha-diversity

The alpha-diversity of the microbial metagenomes in each site was calculated to assess both species richness and evenness. The below image shows the range of α -diversity values in the study 'Groundnut metagenome'. The min, max, and mean values are shown, with the standard deviation ranges (σ and 2σ) in different shades. The α -diversity

of this metagenome is shown in red. Alpha diversity summarizes the diversity of organisms in a sample with a single number. The α -diversity of annotated samples can be estimated from the distribution of the species-level annotations. According to the result the infected soil sample shows more diversity than the healthy soil sample (Figure 3).

Analysis using QIIME pipeline

Sequence data further processed using the Quantitative Insights Into Microbial Ecology (QIIME). It is an open-source bioinformatics pipeline for performing microbiome analysis from raw DNA sequencing data (McPherson *et al.*, 2018) (Caporaso *et al.*, 2010). From all the 10 metagenomes, no. of valid reads were uploaded in QIIME. The low quality reads were removed before the uploading. Second the raw sequences data also uploaded in MGRAST open source pipeline to analyse metagenome (<http://api.metagenomics.anl.gov/api.html>). In this software reads were blast against the ribosomal database, genebank data base and converted to OTU (Operational Taxonomic Unit). In MGRAST the OTU analysed up to genus while in QIIME analysed up to the species. From the QIIME pipeline we got no. of OTU for

family, genus and species as Table 2. We observed that no. of OTU for family, genus and species were different from location to location mens different in H1, H2, H3, H4 and H5 samples. while the no. of family OTU high in H4 samples and low in H2 samples. no. of genus OTU was found high in H3 samples and low in H2 sample. same trend was observed for no. of species OTU (Fugure4).

Beta diversity analysis

To compute the differences in microbial community composition between samples, in QIIME Bray-Curtis dissimilarity was calculated and visualized in a Principle component analysis (PCA) plot. The pattern of separation between samples indicated distinct microbial community composition between the healthy and infected rhizosphere soil as well as between soil types (Figure 5).

At family, Genus and species level community composition two axes explained more than 60% of microbial community variance between samples, with samples clearly separated by soil type on the first axis (41.68% explained variance). The community structure and composition of the plant associated microbiome depends on several factors, such as the soil properties, plant nutritional status, climate, plant genotype, and even the developmental stage of the host plant (Bulgarelli *et al.*, 2015; Pii *et al.*, 2016; Trognitz *et al.*, 2016; Turner, *et al.*, 2013). The microbial composition of healthy and infected samples were distinct.

Present study is that the microbial population was divers from location to location and the status of plant health. the antagonistic microorganisms such as bacteria and fungi are an alternative source for controlling the plant pathogens. some bacterial species form healthy samples have plant growth promoting

activities. while some bacterial species have characteristics to suppress the growth of plant pathogen or to protect the plant from the pathogen. microbiome of rhizosphere manipulation will be useful to control the plant disease. microbiome manipulated by adding of the some beneficial microbes into the rhizosphere.

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