Chapter 13
Metagenomics of Plant Rhizosphere and Endophytic Association: Concepts and Applications

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Abstract Microbes in the rhizosphere influence plant growth, productivity, susceptibility, and resistance to biotic and abiotic stresses. Various studies have been reported to show diversity and activity of microbes are as high in plants as in endophytes and rhizosphere. The roots harbor more diverse microbes than any other part of the plant. The soil type and its management also influence the microbial diversity. The microbial communities can enhance and facilitate pathogen defense and their role in environmental remediation through different mechanisms. Metagenomics is a growing field that helps understand the genomes in the microbial communities. The high resolution of uncultured microbes and the correlation of the function with the environment can be achieved using functional metagenomics. New emerging subdisciplines of metagenomics are Metatranscriptomics and Metaproteomics, which provide further functional analysis of microbial communities. Integrative metagen"omics" approach results in comprehensive information for the community from genes to RNA to proteins and metabolites. In this chapter, we discuss the plant rhizosphere; types of metagenomics analysis such as 16S (for bacteria), whole metagenomics, and 18S/ITS (for fungus); and application of metagenome associated with rhizosphere and endophytes.

Keywords Metagenome · 16S · ITS · Rhizosphere · Endophytes

13.1 Introduction

Rhizosphere plays an important role in microbial-mediated processes like plant growth promotion, plant protection, and pathogenesis. Rhizosphere is the soil neighboring the roots which is most exposed to the influence of plant's root exudates

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(Soni et al. 2017). The rhizosphere microbiology has received significant amount of attention, as it influences the plant both directly and indirectly improving their fitness and health (Sapkota et al. 2015). Rhizosphere microbiota interaction helps plants to deal with abiotic stress and diseases and improves the exchange of substances such as nitrogen fixation, phosphate solubilization, and production of plant growth hormones or by acting as a biocontrol agent to help against pathogens and tolerance to various stresses (Tsurumaru et al. 2015; Elias et al. 2016; Majeed et al. 2015; Massart et al. 2015; Reinhold-Hurek et al. 2015; Vega-Avila et al. 2015; Gallart et al. 2018). It differs from the normal soil because of the biological and physicochemical processes happening due to the plant and microbial association such as root growth, water and nutrient uptake, respiration, and rhizodeposition (López et al. 2012).

Approximately, 10^{10}–10^{11} bacterial cells are present in 1 gram soil (Claire Horner-Devine et al. 2003) belonging to 10^{3}–10^{4} species (Curtis et al. 2002), but approximately 1 gram of plant tissue estimates 10^{9} bacterial cells (Chi et al. 2005) which shows the vast diversity of microbes in the rhizosphere. The microbiome includes various functional gene pool from prokaryotic to eukaryotic associated with various habitats of a plant-like rhizosphere and rhizoplane and plays a crucial role in plant protection (Abd-Elsalam et al. 2010; Mendes et al. 2011; Lakshmanan et al. 2014). The structure of microbial communities in the rhizosphere is largely influenced by ambient condition, soil properties, plant genotype, cultivars, and developmental stages of the plant (Broeckling et al. 2008; Qiao et al. 2017). Different plant species host specific microbial communities when grown in the same soil, i.e., plants are able to shape their rhizosphere microbiome (Aira et al. 2010; Berendsen et al. 2012; Bazghaleh et al. 2015; Berlanas et al. 2019).

Different approaches of metagenomics help to provide insight on many of the important aspects such as taxonomic diversity, which organisms are present, and functional metagenomics, what are their roles (Vieites et al. 2009) which in turn allows to characterize microbes in the given environmental sample. It detects the species and also helps understand the metabolic activities and functional roles of the microbes in a given sample (Langille et al. 2013). As some of the microorganisms are culturable under laboratory practices and some are not, still they all are life forms based on DNA as a genetic information can be studied by Metagenomics; this makes this approach very important and extensive.

13.2 Study of Microbial Community in Plant Rhizosphere and Endophytic Association

13.2.1 Sampling, DNA Extraction, and Sequencing

The rhizosphere of a plant is collected along with its adhering soils, refined, and made free from root hairs before processing for metagenomic DNA extraction. To study the microbial community associated with the various crop cycle, the soil can

be collected at specific growth stages. For example, the sampling of the rhizosphere soil can be prior to the onset of blooming stage in order to analyze the microbial community structure and function before the most critical stage of the crop cycle. The sample after collection should be stored in −80 °C until the metagenomic DNA extraction is performed (Prabha et al. 2019). The collected rhizosphere is subjected to isolation of the DNA using 2–5 gm of the rhizosphere soil sample by any specialized DNA isolation kit or manual isolation method.

To study the endophytic microbial community roots and leaves, samples are collected, and surface sterilization is performed by repeated immersion in 70% (v/v) ethanol for couple of mins and then 2.5% (v/v) sodium hypochlorite (NaOCl) for 5 min (Barra et al. 2016). Sterile distilled water is used to rinse the roots. The roots and leaves are cut in small pieces, frozen in liquid nitrogen, macerated and homogenized with a mortar and pestle, and followed by storage in −80 °C until DNA extraction. DNA isolation can be performed using kit or manual isolation method (Zhang et al. 2019).

The quality of the extracted DNA is determined using NanoDrop and Qubit. The extracted DNA should be subjected to agarose-gel electrophoresis for quality check. There are two main methods for studying microbial community, namely, amplicon-based and shotgun metagenomics. For amplicon-based sequencing, gene-specified (16S/ITS/18S) primer is designed with Illumina adapters. PCR amplification is performed using the forward and reverse universal primers. The PCR products are purified, and the purified products are used for sequencing on Illumina Sequencer. For shotgun metagenome sequencing, the isolated high-quality DNA is used for metagenomic library preparation with respect to the selected metagenomics approach. This library is used for high-throughput sequencing through NGS platforms (Prabha et al. 2019).

13.2.2 Methods of Metagenomics Analysis

There are two main methods for microbiome analysis using high-throughput omic techniques amplicon-based and shotgun metagenomics as shown in Fig. 13.1. In amplicon-based method, primers are designed to amplify a specific gene such as 16S rRNA for bacteria/archaea, 18S for Eukaryotes, and ITS for fungi, from the genomes present in a given sample. The sequences are then clustered into operational taxonomic units (OTUs), and further taxonomic abundance and diversities are compared across samples. Shotgun metagenomics refer to the study of entire genomic material in the microbiome of a sample. It can shed light on the structure and organization of genomes, gene function, and their evolutionary relationships (Roumpeka et al. 2017).

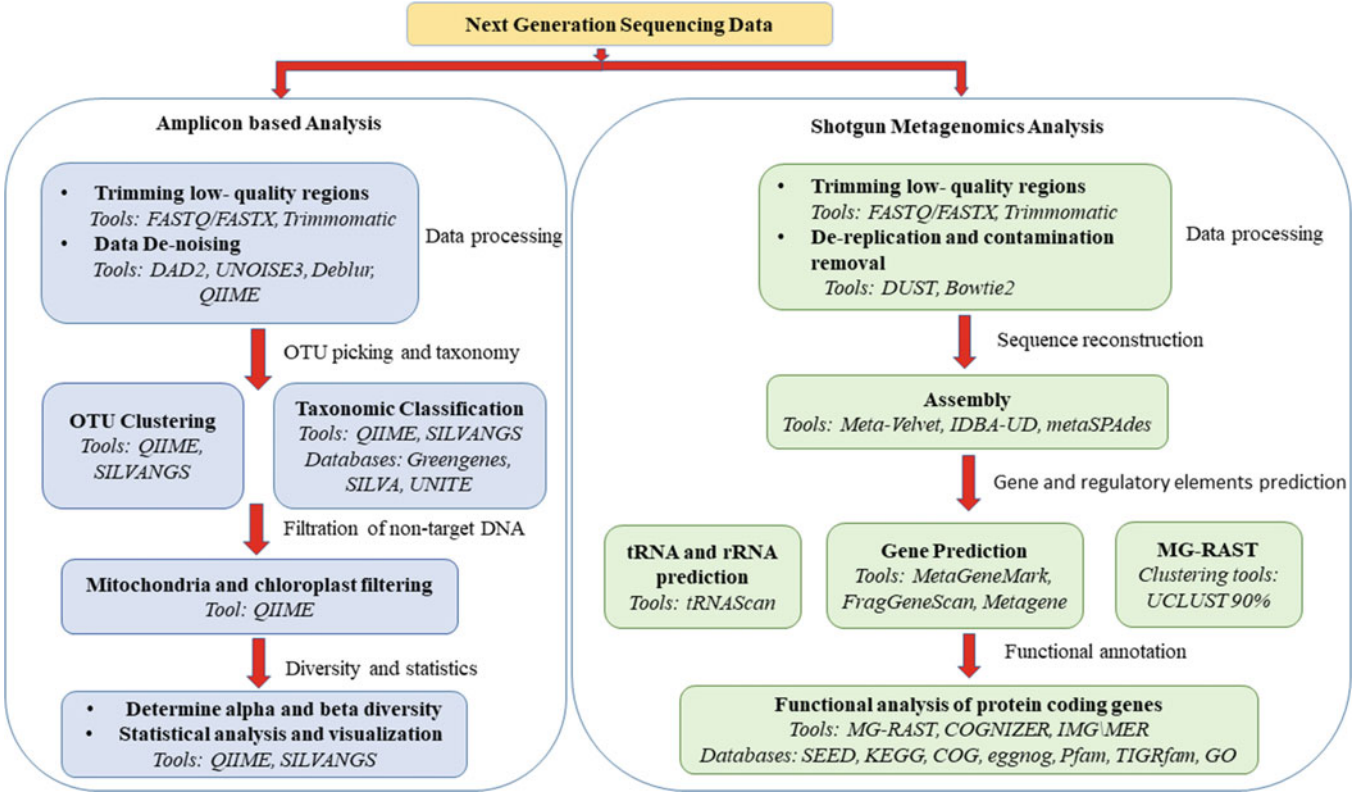

Fig. 13.1 Application of NGS for analyzing rhizospheric and endophytic microbial community

13.2.2.1 Amplicon-Based Metagenomics (16S/18S/ITS)

The prokaryotic 16S ribosomal RNA gene (16S rRNA) is approximately 1500 bp long and contains 9 hypervariable regions (V1–V9) flanked by conserved regions (Chakravorty et al. 2007). These variable regions of 16S rRNA are frequently used in taxonomic classifications in diverse microbial communities. Internal transcribed spacer (ITS) is a highly variable sequence that lies between the 16S and 23S rRNA genes and is of great importance in distinguishing fungal species (Bromberg et al. 2015). The length of ITS regions may vary from 50 bases to several kbs. ITS1 and ITS2 genes were observed to be the most appropriate marker for fungal phylogenetic analysis because of their variable regions, conserved primers, and multicopy nature of the genome (Cuadros-Orellana et al. 2013). The fungal taxonomical studies are based on the nuclear ribosomal gene cluster, which includes 18S or small subunit (SSU), 5.8S subunit, and 28S or large subunit (LSU) genes.

There are six most popular pipelines which are widely used for amplicon-based analysis (Table 13.1): three *OTU based*, QIIME (Kuczynski et al. 2012), MOTHUR (Schloss et al. 2009), and USEARCH-UPARSE (Edgar 2010, 2013), and three *ASV level based*, DADA2 (Callahan et al. 2016), QIIME2-Deblur (Amir et al. 2017), and USEARCH-UNOISE3. The OTU-based three pipelines cluster sequences at 97% identity into operational taxonomic units (OTUs). The latter three pipelines attempt to reconstruct exact biological sequences called amplicon sequence variants (ASVs) present in the sample (Marizzoni et al. 2020; Prodan et al. 2020).

Preprocessing of the sequenced reads: The raw reads are subjected to demultiplexing and quality assessment followed by removal of poor-quality reads prior to analysis (Plummer et al. 2015). Most commonly used tool is Trimmomatic (Bolger et al. 2014). The high-quality PE reads are merged into a unique sequence prior to data analysis. FLASH (Fast Length Adjustment of Short Reads) (Magoč and Salzberg 2011) is used to stitch overlapping paired end reads into single end long reads in 16S analysis. In QIIME-uclust and QIIME 2-Deblur, reads can be filtered and merged externally using USEARCH. DADA2 utilizes a model-based approach for correcting amplicon errors, and reads are merged after denoising of data. After quality filtration and merging of reads, chimeric reads are removed and remaining sequences are clustered into OTUs.

OTU Picking: Clustering of high-throughput 16S sequences into biologically meaningful operational taxonomic units (OTUs) is a challenging task. In OTU picking, 16S sequences are clustered at a certain level of sequence similarity (default 97%). There are three different approaches for OTU picking: de novo, closed-reference, and open-reference. (1) de novo OTU picking method: input sequences are aligned against one another and sequences that align with greater than a user-specified percent identity belongs to the same OTU, without any external reference sequence collection. (2) Closed-reference OTU picking method: sequences are first aligned to a reference sequence collection and any sequences which does not match reference sequence at a user-defined percent identity threshold is excluded from downstream analyses. (3) Open-reference OTU picking method: reads are first

Table 13.1 List of tools and databases

Category	Tools	References
Shotgun	CLARK	Ounit et al. (2015)
	Centrifuge	Kim et al. (2016)
	IDBA-UD	Peng et al. (2012)
	KRAKEN	Wood and Salzberg (2014)
	MetaVelvet	Namiki et al. (2012)
	MetaVelvet-SL	Sato and Sakakibara (2014)
	Ray Meta	Boisvert et al. (2012)
	SOAPdenovo2	Luo et al. (2012)
	metaSPAdes	Nurk et al. (2017)
	MetAMOS	Treangen et al. (2013)
	KAIJU	Menzel et al. (2016)
	Prodigal	Hyatt et al. (2010)
	FragGeneScan	Rho et al. (2010)
	MetaGeneAnnotator	Noguchi et al. (2008)
	MetaGeneMark	Zhu et al. (2010)
	Glimmer-MG	Kelley et al. (2011)
	Kraken2	Wood et al. (2019)
	MetaMaps	Dilthey et al. (2019)
	Megan	Huson and Weber (2013)
	MetaPhlAn	Segata et al. (2012)
	MG-RAST	Wilke et al. (2016)
16S/18S/ITS	QIIME/QIIME2	Caporaso et al. (2010)
	Mothur	Schloss et al. (2009)
	USEARCH	Edgar (2010)
	UPARSE	Edgar (2013)
	UNOISE	Edgar (2016)
	DADA2	Callahan et al. (2016)
	Deblur	Amir et al. (2017)
	PipeCraft	Anslan et al. (2017)
	LotuS	Hildebrand et al. (2014)
	AMPtk	Palmer et al. (2018)
	PIPITS	Gweon et al. (2015)
Functional 16S analysis	PICRUSt	Langille et al. (2013)
Databases	SILVA	Quast et al. (2012)
	Greengenes	DeSantis et al. (2006)
	Ribosomal database (RDP)	Cole et al. (2007)
	KEGG	Ogata et al. (1999)
	GhostKOALA	Kanehisa et al. (2016)
	SEED	Overbeek et al. (2005)
	eggnog	Powell et al. (2014)
	COG/KOG	Tatusov et al. (2000)
	PFAM	Bateman et al. (2004)

(continued)

Table 13.1 (continued)

Category	Tools	References
	TIGRFAM	Haft et al. (2003)
	Reactome	Fabregat et al. (2016)
	MetaCyc	Caspi et al. (2016)
	UNITE	Kõljalg et al. (2013)

aligned to a reference sequence database, and any reads which fail to align are clustered de novo (Rideout et al. 2014). OTU picking method comprises of taxonomic assignment, sequence alignment, and tree-building steps.

Taxonomic Assignment: A crucial step in microbiome amplicon analysis is taxonomic assignment. Taxonomic classification of 16S/18S sequences is accomplished using one of these databases: Greengenes, SILVA, RDP, or NCBI 16S/18S microbial database. The Greengenes database (McDonald et al. 2012) contains Bacteria and Archaea taxonomic information. The SILVA database (Quast et al. 2013; Yilmaz et al. 2014) is designed for Bacteria, Archaea, and Eukarya taxonomic details and is primarily based on phylogenies for small subunit rRNAs (16S for prokaryotes and 18S for Eukarya). The RDP database (Cole et al. 2007, 2014) contains 16S rRNA sequences from Bacteria, Archaea, and 28S rRNA sequences for fungi (Eukarya) available from the International Nucleotide Sequence Database Collaboration (INSDC) (Cochrane et al. 2016) databases (Balvočiūtė and Huson 2017). The most popularly used ITS database for taxonomic assignment is UNITE (Nilsson et al. 2019). In the case of rhizosphere and endophytes, we have an influence of the plant parts. To avoid the non-microbiota such as chloroplast and mitochondria from the data, which are expected due to the presence of the plant part, are removed to obtain only the microbiota using QIIME (Zhang et al. 2019).

Diversity Analysis: Whittaker in 1960 and 1972 described three different types of measures of biodiversity: alpha, beta, and gamma diversity. Alpha diversity is defined as diversity of organisms within a sample or ecosystem and is usually expressed by the number of species (i.e., species richness) in ecosystem. Beta diversity measures difference in diversities across the sample or ecosystem. Gamma diversity measures the diversity of a larger unit such as a region or landscape (Navas-Molina et al. 2013). Alpha diversity measures richness, dominance, and evenness using various diversity metrics such as richness, Chao1, Shannon index, and inverse Simpson index. Beta diversity metrics are namely phylogenetic and non-phylogenetic metrics such as Bray-Curtis distance, Euclidean distance, and unifrac weighted and unweighted that can be calculated using QIIME package and phyloseq R package.

Functional Analysis: The functional composition of 16S microbial communities can be performed using PICRUSt. Ancestral-state reconstruction algorithm is used to predict the gene families and then combines gene families to estimate the composite metagenome. It provides the insight about the metabolic activities and functional roles of the microbes in the sample. The result of the annotation for predicted gene family counts is orthologous groups of the gene families or KOs, COGs, or Pfams (Langille et al. 2013).

13.2.2.2 Shotgun Metagenomics

Metagenomics, also referred to as WGS- or shotgun-metagenomics, allows researchers to comprehensively sequence and study the entire genomic material present in the microbiome sample. Sequencing the genomes of all organisms present in metagenomic sample can furnish detailed information of the structure and organization of genomes, function of predicted genes, evolutionary relationships, and identification of novel genes (Roumpeka et al. 2017). The extensive advantage of metagenomic approach is that it provides high taxonomic and functional resolution. Insight into gene functions and characterization of specific strains of these microbial communities from rhizosphere/endophytes can reveal plant growth promotion predicted coding genes (Romero et al. 2019).

A wide range of bioinformatic tools are available to execute the shotgun metagenomic analysis as shown in Table 13.1. The bioinformatics analysis generally includes the following steps: (a) the assembly of sequenced metagenomic fragments to construct contiguous sequences, (b) gene prediction from assembled sequences, and (c) identification of domains, their functions, and metabolic pathways for the putative proteins (Roumpeka et al. 2017).

Preprocessing of Sequenced Reads: Based on quality assessment of sequenced data, reads are trimmed to retain high-quality pair-end data. Most commonly used trimming tools are Trimmomatic (Bolger et al. 2014) and Cutadapt (Martin 2011) that remove low-quality bases from both terminals of each sequence. Removal of bad quality reads greatly improved the accuracy and contig lengths of resulting assembly.

Metagenomic Assembly: To assemble all of the genomes present within a metagenomic sample, we have many tools based on de novo metagenomic assemblers which uses de Bruijn graph approach for assembly (Pevzner et al. 2001). One of the widely used metagenomic de novo assembler is MetaVelvet (Afiahayati et al. 2015; Namiki et al. 2012). For a given set of metagenomic reads, it first constructs a large de Bruijn graph, and then mixed de Bruijn graph is decomposed into subgraphs which can be used to construct longer contiguous genome sequences. It is reported that MetaVelvet tool surpasses other commonly used assemblers like IDBA-UD (Peng et al. 2011, 2012) and Ray Meta (Boisvert et al. 2012). Another method which metagenomics assembler commonly uses is K-mer-based method: KRAKEN (Wood and Salzberg 2014), CLARK (Ounit et al. 2015), KAIJU (Menzel et al. 2016), and Centrifuge (Kim et al. 2016) are the popular tools which used this method. K-mer based methods extract kmers from each read pair, and heuristic searches were performed against the user-specified database. They are ultrafast, and sensitivity depends on the choice of the database. Another framework which combines available bioinformatics tools into a metagenomic analysis pipeline is MetAMOS (Treangen et al. 2013). This pipeline first assembles the metagenome reads, and scaffolds are created. Finally, in post-assembling stage, assembled scaffolds are annotated and taxonomically classified.

Gene Prediction: Annotating the assembled data and predicting genes and regulatory elements are important steps in a metagenomic analysis pipeline. A metagenomic gene-finding algorithm, MetaGeneAnnotator (Noguchi et al. 2008), can predict genes from uncharacterized metagenomic communities. Glimmer-MG (Kelley et al. 2012), an extension of Glimmer which is a popular bacterial gene prediction tool, clusters metagenomic data which likely belong to the same organism and also considers insertions and deletions during the gene prediction. FragGeneScan (Rho et al. 2010) is another tool based on hidden Markov models (HMMs), specifically designed to predict fragmented genes directly without the need of assembly; however, the software can also run on assembled sequences. MetaGeneMark (Zhu et al. 2010) is an ab-initio gene prediction tool specifically designed for metagenome sample to identify protein coding regions.

Taxonomic classification: Many software has recently been deployed to classify metagenomics data taxonomically and estimate their taxonomic abundance profiles. Certain bioinformatics tools like CosmosID, Inc. (CosmosID, Inc., Rockville, MD, USA), Kraken2 (Wood et al. 2019), MetaMaps (Dilthey et al. 2019), and MetaPhlAn (Segata et al. 2012) are designed to identify taxonomic level till species, subspecies, and strain level using assembled/unassembled metagenomic data. MG-RAST (Glass et al. 2010; Wilke et al. 2016) is a widely used metagenomics analysis web-server which can identify taxonomic information below the genus level.

Functional Annotation: To infer functional annotation from metagenomics data, many reference databases like KEGG (Kanehisa et al. 2012), COG/KOG (Tatusov et al. 1997), eggNOG (Powell et al. 2012), PFAM (Punta et al. 2012), and TIGRFAM (Selengut et al. 2007) are available. MetaCyc (Caspi et al. 2016) is considered as largest comprehensive database of curated metabolic pathways and enzymes from all domains of life. Reactome (Fabregat et al. 2016) is another open-source and curated database of biological pathways. The metabolic pathway analysis can also be done using GhostKOALA (Kanehisa et al. 2016). It correlates taxonomy with their functional annotation, and user can visualize metabolic pathways from different taxa in the same map.

13.2.2.3 Metatranscriptomics and Metaproteomics

Metatranscriptomics and metaproteomics are reasonably recent subtypes of metagenomics, which enables us to look into functional analysis of microbial communities (Ghosh et al. 2019). The study of microbial communities based on RNA sequencing in a complex ecosystem is known as metatranscriptomics (Zhang et al. 2017). The co-expressed gene clusters of the ecologically relevant trends are identified followed by the transcripts abundance, and functional annotation is studied in the environmental samples (Oyserman et al. 2016). To get high-quality RNA from the environment samples is the biggest challenge associated with this method. However, it is an efficient approach to elucidate gene expression and has the capability to discover novel gene in the microbial community (Frias-Lopez et al. 2008; Tartar et al. 2009).

The study of proteome expressed in the microbial community at a particular time is known as metaproteomics. This method allows to discover the microbial activities based on the metabolic pathways in the microbial ecosystem (Zampieri et al. 2016). Metaproteomics is an emerging field along with metagenomics, which allows to characterize the proteins from a microbiota such as human gut (Petriz and Franco 2017). The study of metagenomics, metatranscriptomics, and metaproteomics provides information of the functional dynamics, activities, and production capabilities of microbial community (Simon and Daniel 2011).

13.3 Future Perspective and Applications

Recent studies have highlighted the plant-plant and plant-microbe interactions along with their complexities as an interlinked ecosystem. It is inhabited by diverse microbial communities that are structurally and functionally affected by plant and soil type (Yurgel et al. 2019). Genomics has given rise to metagenomics, an approach that will enable us to explore the as-yet-uncultured microbes which represents the vast majority of organisms in most environments on earth. The high-throughput and "omics" techniques could shed light on the composition and structure of beneficial rhizobiome communities and what role the host may play in the enrollment and control of its microbiome.

Crop production is reliant on pesticides to manage diseases and pests and on chemical fertilizers to provide sufficient nutrients to enhance crop yields. However, the wide use of pesticides and chemically synthesized fertilizers may lead to pesticide resistance pathogens, environmental pollution, contamination of surface along with the groundwater, and detrimental effects on humans, beneficial soil microbes, and other organisms (Liu et al. 2018). One way to address these issues is to utilize rhizosphere engineering which may lessen our dependency on agrochemicals by substituting their functions with beneficial microbes and biodegradable biostimulants and can manipulate plant/microorganism interactions accordingly (Ryan et al. 2009).

There are increasing evidences to suggest that the rhizobiome can enhance plant growth directly, improve drought tolerance, and play important role in environmental remediation through different mechanisms (Jones et al. 2019). The microbe-mediated nutrient uptake, disease resistance, and stress tolerance are some examples of microbial functions crucial to agricultural production systems. Moreover, they are engaged in the secretion of a diverse range of chemicals that can be classified as signaling compounds, and may serve as nutrient solubilizers (Verma et al. 2018). Microbes in the rhizosphere could serve as candidate taxa for biofertilizers and growth supplements and may act as proficient innovative tools for the sustainability of agro-ecosystems. Understanding the hidden mechanisms of the host-based selection of microbiome could further guide insight into microbiome-based breeding programs (Poudel et al. 2019).

Recent metagenomic approaches can help in deciphering these interactions in a comprehensive manner and can enable us to have a reasonable agriculture yield with improved crop management. Besides this, the researcher also suggests that the rhizosphere microflora can benefit plants by increasing tolerance to abiotic stresses like temperature, salinity, and heavy metal stress. It also increases plant-defensive measures by protecting against deadly pathogens through microbial antagonism (Jones et al. 2019).

Rhizosphere has been witnessed as one of the most crucial interfaces for life on earth. The microbial root colonization activates multiple types of physical and chemical interconnections between microbes and plants. Rhizodeposition of discrete exudates acts as an important substrate for the soil microbial community, and there is complex coaction between this community and type of compounds released (Ramakrishnan et al. 2009). The culture-independent rhizosphere and endosphere microbe's analysis will provide insight on plant-microbe interaction, by understanding the variability of beneficial microbes in a various different environment which will, in turn, help crop management practices. Using the metagenomics information from a different niche, we can modulate the composition of root microbiomes to improve crop growth and health (Rascovan et al. 2016).

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