Molecular Tools to Explore Rhizosphere Microbiome

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Abstract

Rhizosphere microbial diversity plays an important role in plant health and agricultural sustainability. Several scientific groups have developed a wide range of methodologies for analyzing the structure, diversity, and functions of microbial populations to better understand rhizosphere biology and rhizosphere–microbe interactions. In this chapter we will discuss some of the advanced molecular tools available to explore microbial diversity of rhizosphere.

Keywords

Rhizosphere · Microbiome · Omics technology · Bacteria

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2.1 Introduction

Rhizosphere microbiomes play an important role in plant health and sustainability. Several scientific groups have developed a wide range of methodologies for analyzing the structure, diversity, and functions of microbial populations to better understand rhizosphere biology and rhizosphere–microbe interactions. It has been suggested that microbial inoculants are promising components for integrated solutions to agro-climatic issues because inoculants possess the capacity to influence the plant growth (Compant et al. 2010; Lugtenberg and Kamilova 2009), enhance nutrient availability, and uptake and improve plant health (Adesemoye et al. 2009; Yang e al. 2009; Berendsen et al. 2012; Packialakshmi et al. 2020). Further, plants have evolved to adjust with biotic and abiotic stresses in association with rhizosphere microbiome (Lemanceau et al. 2017). Some recent findings have shown that soil microbiome can directly and indirectly interact with the plants, improving their fitness and health (Sapkota et al. 2015).

2.2 Rhizosphere Microbiome

Soil is the mother and media for all the biological processes on the earth. Soil nurtures numerous flora and fauna in it. It also provides basic habitat for crop plants and the rhizosphere soil which is the most active part of soil provides a balanced atmosphere for many biological processes which directly or indirectly influences plant growth and development. Soil also contains billions of microorganisms which influences various biological processes (McNear Jr 2013). Microorganisms like fungi, bacteria, nematodes, actinomycetyes, archaea present in soil at different proportions. The number and activities are more in rhizosphere soil when compared to outside the rhizosphere zone. These microbiomes are involved in the various biological processes which can regulate plant growth and development positively and negatively. As plant growth promoters they help in better crop growth and development. Species of *Trichoderma*, mychorhizal fungi helps in performing these functions. On the other hand, they also cause numerous diseases like wilts, root rots, damping off, etc. which serious hamper crop growth and development. The native microbial communities play an important role in biogeochemical cycles of essential elements such as nitrogen, carbon, phosphorous. Apart from this, they also help in organic matter decomposition and remineralization of the elements (Pierre-Alain et al. 2007). A better understanding of these biological processes is critical for maintaining plant health thereby feeding ever-growing population of the planet earth (Morrisseey et al. 2004). Before this it is important to understand the diversity of different microbiomes in soil which gives an idea of exploiting their role for beneficial functions.

2.2.1 Bacterial Diversity

The bacterial community found in the rhizosphere is known for its colonization around the roots due to availability of nutrients, and composition, and it affects the plant growth directly or indirectly (Alawiye and Babalola 2019). The plant is able to specifically select microorganisms for rhizosphere colonization from the large pool of microbes living in the surrounding soil (Rosier et al. 2016). It was reported that rhizosphere habitats large number of bacterial population and the population densities in the rhizoplane range from 10^5–10^7 CFU g/1 of fresh weight (Bais et al. 2006). The rhizosphere microbiome has a strong effect on plant health by facilitating nutrient acquisition and helping plants to tolerate abiotic stresses (Pérez-Jaramillo et al. 2015). Several beneficial microorganisms (bacterial and fungal) have plant growth promotion activities or strengthen the defenses of the plant against pathogens and insects (Mendes et al. 2011; Pieterse et al. 2014; Goel et al. 2017; Kumar et al. 2019a, b).

To characterize the bacterial diversity and composition, molecular techniques have successful been applied. These methods facilitate characterization of representative microorganisms on the basis of biomolecules which includes Nucleic acid based either DNA or RNA based Fingerprinting techniques, restriction fragment length polymorphism (RFLP), denaturing/temperature gradient gel electrophoresis (DGGE/TGGE)ARDRA, RISA, DNA Microarray, Real Time PCR (Q-PCR), fluorescent in situ hybridization, Dot blot, Clone library sequencing), Protein based (Protein microarray), fatty acid/lipid based characterization includes (Microbial lipid analysis). Generally, 16S rRNA is used as a phylogenetic marker gene for microbial diversity analysis because this gene is remarkably well conserved through billions of years of evolution (Hugenholtz and Tyson, 2008; Soni and Goel 2010). This conservation allows amplification and analysis from bacteria and archaea, revealing the taxonomic distribution and evolutionary relationships among microorganisms.

In the advancement of genomic technologies, high-throughput sequencing techniques have allowed to characterize the genome without culturing them known as culture independent methods. In addition, community level analysis of microbial diversity is also performed using advanced genomics tools using DNA, RNA or protein as initial sample. These techniques allow the identification of entire bacterial diversity of any sample, tissue includes metagenomics, metaproteomics, proteogenomics, metatranscriptomics, metabolomics, whole genome sequencing, G+ C fractionation.

Rhizospheric bacterial diversity has been characterized in several crops species including bacterial communities associated with arabidopsis (Lundberg et al. 2012; Bulgarelli et al. 2012), barley (Bulgarelli et al. 2015), wheat and maize (Mazzola et al. 1995; Peiffer et al. 2013). Bacterial diversity in maize rhizoplane showed the abundance of genera *Bacillus, Arthrobacter, Listeria,* and *Sporolactobacillus* followed by *Azotobacter*, *Micrococcus,* and *Pseudomonas* genera (Cavaglieri et al. 2009). PCR-RFLP techniques used to explore the seasonal variation of the microbial community and the microbial succession of rice rhizoplane and identified the microbial diversity (Ikenaga et al. 2002). Knief et al. (2012) applied

metaproteogenomic analysis of microbial communities in the rhizosphere of rice. The diversity of bacterial endophytes from rice roots were analyzed using 16S RNA amplicon sequencing and identified microbes having plant growth promoting and antagonistic activities against bacterial and fungal pathogens (Kumar et al. 2020).

Culture dependent and independent bacterial diversity in Duckweed (*Spirodela polyrhiza*) an aquatic plant and identified the number of bacterial lineages includes *Alphaproteobacteria, Betaproteobacteria, Bacteroidetes,* and *Verrucomicrobia* (Matsuzawa et al. 2010). The rhizoplane-associated bacterial diversity was also analyzed using the high-throughput 16S rRNA amplicon sequencing strategy (Knief 2014). Furthermore, the application of next-generation sequencing (NGS) techniques may be more powerful tool that possibly helpful in the detection and identification of microbial communities in plants. NGS enables rapid analysis of the composition and diversity of microbial communities using culture independent amplicon or shotgun based sequencing in several habitats including rhizosphere (Trujillo et al. 2015; Soni et al. 2017; Goel et al. 2018). The 16S ribosomal RNA (rRNA) gene multiplex amplicon sequencing by PacBio sequencer targeting target the V1–V9 regions was performed. The community-based culture collection (CBC) recovered 399 unique bacteria representing 15.9% of the rhizosphere core microbiome and 61.6–65.3% of the endophytic core microbiomes of sugarcane stalks (Armanhi et al. 2018). Rhizospheric microbiome of *Lathurus sativa* was analyzed using illumina based NGS approach (Kumar et al. 2018a, b). By using paired-end sequencing on an Illumina sequencer identified a number of OTUs (n = 637) in rhizosphere samples of apple trees with the higher abundance of proteobacterial class of bacteria (Singh et al. 2019), diversity and composition of bacterial communities in rhizosphere soils of *Panax ginseng,* bacterial genera, namely *Asticcacaulis, Actinomadura, Knoellia, Rhodomicrobium, and Nakamurella* were detected from the soil of rusty root-affected (Wei et al. 2020). Rhizospheric bacterial communities of *Adenium obesum, Aloe dhufarensis* and *Cleome austroarabica* were explored using next-generation sequencing approaches (Khan et al. 2020).

2.2.2 Fungal Diversity

The soil has many species of fungi, and so far 80,000 or more species have been taxonomically named and described based on their distinguishing characters. These fungi function both active and inactive roles. Our current knowledge on soil fungal biodiversity is largely based on their morphological features like fruiting bodies in the environment, or, characters of mycelia on artificial/selective isolation media under laboratory conditions. Both these methods have certain limitations which are the obstacles for their detection, and diversity analysis. Fungi are the successful soil inhabitants due to their high capacity to withstand fluctuating environmental conditions (Sun et al. 2005). Fungi can be found in almost all the environmental conditions (Frac et al. 2018). The fungal species, their diversity and numbers are controlled by numerous biotic (presence of plants and other microbes) and abiotic

(soil texture, structure, temperature, soil pH, moisture, salinity, and alkalinity) conditions (Lopez-Bucio et al. 2015; Rouphael et al. 2015). Fungi perform both beneficial and harmful functions in plants. As beneficial microbes, they got the capability to produce a number of extracellular enzymes helps in different functions like break down of organic matter, decomposing soil components, and provide various nutrients for metabolic functions of plants (Zifcakova et al. 2016).

Several researchers have carried out experiments to analyze the fungal population in different cropping systems and their effect on growth and development of crop plants. Qin et al. (2017) determined the impact of various mulching techniques (furrow-ridge) rhizosphere fungal diversity of potato under continuous cropping, and found that, furrow planting with half mulch have highest population of fungi (89%). They also found that the rhizosphere soil was dominated by Zygomycota, Chytridiomycota, Ascomycota, Basidiomycota, and unidentified fungal communities. Similarly Tan et al. (2017) analyzed rhizosphere soil and root endogenous fungal diversity and composition in response to continuous cropping of *Panax notoginseng*, Chinese ginseng. They found that, continuous cropping becomes vulnerable to fungal pathogen attack. Ascomycota, Zygomycota, Basidiomycota, and Chytridiomycota were the dominant phyla observed during continuous cropping of *Panax notoginseng*. Fungal diversity was less in diseased plant's rhizosphere than healthy plants. This study clearly indicated that, diseased rhizosphere soil will have less biodiversity of fungal species than healthy. The present study also found that, soil organic matter and pH play greatest impact on microbial community composition in different cropping systems. Twenty soil samples collected from crop fields of Nanjangud Taluk, Karnataka were analyzed for fungal diversity; it was found that, ten species of fungi belonging to seven genera were prominent. The predominant genus was *Aspergillus, Penicillium,* and *Mucor* species (Chandrashekar et al. 2014). The study suggested that, the microclimate and soil properties greatly influence fungal communities and biodiversity. The abondance, composition, activity, and diversity in rhizosphere also influenced by alteration in soil micro environment (Cycon and Seget 2009). Many investigations also concluded that, soil moisture and temperature play significant role in soil microbial communities (De Curtis et al. 2012). Soil moisture plays a significant role in increasing the activities of soil fungi (Zou et al. 2010). Molecular techniques were followed to analyze the fungal diversity in the wheat rhizosphere by Smit et al. (2010). They followed sequencing of cloned PCR-amplified genes encoding 18 s rRNA and temperature gradient gel electrophoresis (TGGE), and found that, Ascomycota, Basidiomycota, Zygomycota, and chytridiomycota were the predominant species in wheat rhizosphere. Study conducted in China to estimate fungal biodiversity in a forest soil ecosystem using ITS sequence reads indicated that, Basidiomycota (47.8%), Ascomycota (32.4%), and zygomycota (13.4%) were the major fungal communities observed, but, basidiomycetes fungi found to be dominant among the three phyla (Hanif et al. 2019). Studies have also indicated that, the soil fungal diversity also influenced by root released compounds in different cropping systems (Garbeva et al. 2004). Rhizopshere soils of potato, eggplant, and peanut shown decreased level of bacteria and actinomycetes as the population of fungi increased over continuous cropping

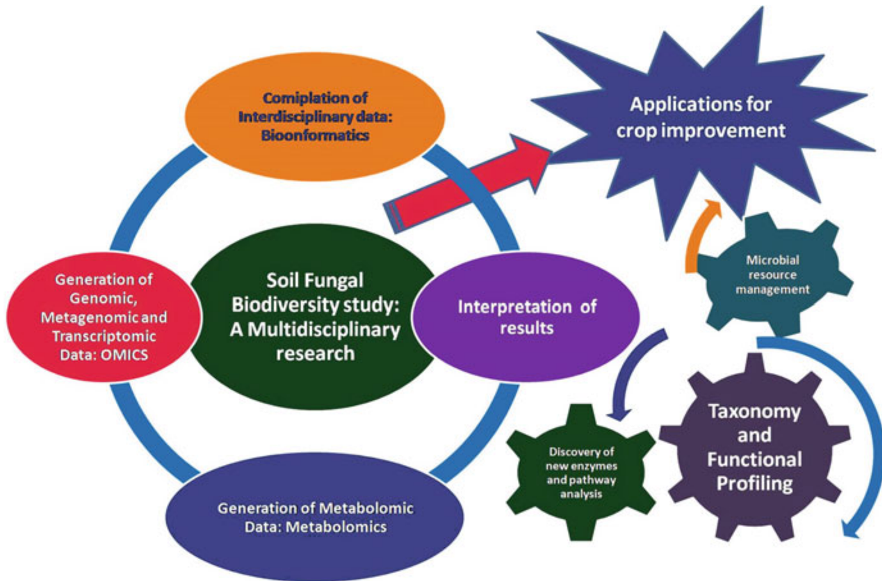

Fig. 2.1 Application of multidisciplinary research for exploring soil fungal diversity for crop improvement

system (Li and Guo 2014). Finally, it can be concluded that, the fungal diversity in rhizosphere soil greatly influenced by type of crop plants grown, cropping system, soil ecology, soil physical and chemical properties. Crop rotation, tillage will produce a number of changes on fungal diversity with a particular ecological importance (Lupwayi et al. 2010). Exploring soil fungal diversity is a multidisciplinary subject and, Fig. 2.1, gives a complete picture of utilization of soil fungal diversity for crop production and other areas.

2.3 Omics Technology

2.3.1 Genomics

Genomics is the branch of genetics that deals with the analysis of the genomes. The genome represents the haploid set of genes or chromosomes within an organism. Its mapping, sequencing, or any other analysis is known as genomics that comes under the branch of genetics. It may be classified as structural and functional genomics (Wang et al. 2020). Structural genomics involves the location, sequence, and physical characterization of the genes within a genome (Fig. 2.2). While, functional genomics refers the analysis of gene functions and regulation (Forouhar et al. 2007). Nowadays, genomics becomes very popular to sequence and analyze the whole genome of an organism. It has been expanded to the functional aspect of the entire

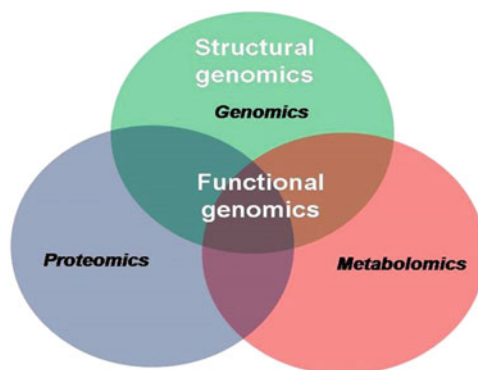

Fig. 2.2 Mutual relationship among the omics approaches

genome, i.e. transcriptomics (the study of RNA), proteomics (the study of proteins), and metabolomics (the study of metabolites) (Soni et al. 2015; Suyal et al. 2017, 2018, 2019b). Moreover, the combinations of various "meta-" and "-omics" technologies have made it beneficial to humankind, especially in medical, industrial, and agricultural fields (Rawat et al. 2019; Suyal et al. 2014b, 2019c). Although several genomics tools and techniques are emerging day by day, here, the basic technologies are being discussed briefly. These methods and techniques are the basis of genomics and lie in the heart of advanced technologies.

2.3.1.1 Polymerase Chain Reaction (PCR)

This technique was originally isolated by Kary Banks Mullis in 1983, for which he got Nobel Prize in 1993. This technique has revolutionized the whole molecular biology field and is relevant till today. It allows the amplification of target DNA fragments extracted from any source. Moreover, in combination with gel electrophoresis techniques, viz. agarose gel electrophoresis, denaturing gradient gel electrophoresis (DGGE); temperature gradient gel electrophoresis, etc. it offers several benefits to the researchers and has increased our understanding in microbial community analysis (Kumar et al. 2018a, b; Rajwar et al. 2018; Joshi et al. 2019).

2.3.1.2 Restriction Fragment Length Polymorphism

Restriction enzymes are endonucleases that can cleave DNA at specific sites. They are also called molecular scissors. These enzymes are widely used to map the genomes (O'Donnell et al. 2020). Restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), plasmid fingerprinting, etc. are some techniques that explore the principle of restriction endonucleases.

2.3.1.3 DNA Sequencing

DNA sequencing is the most significant advancement in genomics (Kumar et al. 2014; Suyal et al. 2014a; Shukla et al. 2015). It allows the identification of the nucleotide sequences in a given genome. Nowadays, high-throughput, automated, efficient, and reliable next-generation sequencing technologies are available which made it easier to sequence and analyze the whole genome. In recent years,

Microfluidics and Fluorescent Activated Cell Sorting (FACS) becomes popular to sequence single cells. This technique involves tagging, isolation, and sequencing of fluorescent cells (O'Donnell et al. 2020).

2.3.1.4 DNA Cloning

This technique involves the transfer of a DNA segment from one cell to another to make its identical copies in vivo (O'Donnell et al. 2020). In recent years, several vector systems have been developed that can accommodate various types and sizes of DNA fragments, viz. plasmids, hybrid vectors (cosmid, phagemid), phages, artificial chromosomes (Yeast artificial chromosome, bacterial artificial chromosome).

2.3.1.5 Hybridization Techniques

This technique measures the level of genetic similarity between two different nucleic acid molecules by allowing their complementary sequence to combine and separate. DNA dissociation/re-association kinetic analysis and fluorescent in situ hybridization (FISH) are basic methods that employ this principle. Furthermore, DNA microarray analysis is an advanced technique that is based on it. It involves hybridization between a probe and DNA fragment on a chip known as DNA chip. In most cases, DNA chips involve a single genome; however, multiple genomes can also be analyzed. A technique "representational difference analysis (RDA)" analyzes the variations among the strains of a species concerning previously sequenced representative. This method involves the combination of PCR, DNA sequencing, and DNA–DNA re-association kinetics. It is a very popular method to analyze the prokaryotic genomes because they can vary significantly in their genome size (Barcellos et al. 2009).

The combination of genomics with other omics technologies is frequently being used in the study of rhizospheric microorganisms (Giri et al. 2015; Suyal et al. 2015a; Goel et al. 2017). Moreover, blending the bioinformatics tools with these technologies has opened newer insights into microbial ecology research and development.

2.3.2 Metagenomics

Genomic methods limit analysis of those microorganisms that can be cultured. It is widely accepted that only 0.1–1% (depending upon the environmental sample) of microorganisms can be grown on synthetic growth media. This leaves more than 99% of the microbial diversity unexploited (Suyal et al. 2015b, c, 2019a). Moreover, various environmental stresses force bacteria to enter under viable but nonculturable state that again reduces their accessibility through genomics approach. Thus, cultivation dependent microbial identification can underestimate the microbial diversity. To overcome the difficulties and limitations associated with cultivation technologies, metagenomics has emerged as a potential tool (Soni and Goel 2011; Soni et al. 2016; Soni et al. 2017; Joshi et al. 2017; Kumar et al. 2019a, b). It involves the direct

extraction of nucleic acids from the environment. However, when isolating metagenomic DNA from the environment samples, three major issues are important that need to be taken into consideration. The first one is the DNA should be extracted from such source that has a broad a range of microorganisms. Secondly, during the DNA extraction steps, DNA shearing must be avoided. Thirdly, the DNA must be free from contaminating substances which interfere with downstream DNA processing such as restriction and ligation because the bacterial community composition is significantly influenced by the efficiency of DNA extraction method (Lai et al. 2006).

In general the analysis of DNA provides information on structural diversity of environmental sample and does not allow conclusion on metabolic activity or gene expression of members of that community. This information can be attained by isolating mRNA from the environmental samples followed by cDNA synthesis through Reverse Transcriptase PCR and targeting this cDNA for the downstream processes. However, there are several technological challenges regarding quality and stability of the RNA because of short half-life of mRNA and presence of RNases (Jensen et al. 2017).

2.3.3 Transcriptomics

Recent advances in DNA sequencing technologies have revolutionized the rhizosphere biology by elucidating the microbial composition with deeper coverage through metagenomics. However, metagenomics could not provide the functional insight, thus rendering the functional role of active rhizospheric microbiome elusive. Transcriptomics and metatranscriptomics are therefore sought as they can elucidate both structure and function of the active rhizospheric microbiome thus complementing metagenomics data. Transcriptomics is the study of total RNA complement expressed under certain environmental condition. However, metatranscriptomics refer to the high-throughput sequencing of total RNA isolated from the environmental sample. The two most popular metatranscriptomics tools to study rhizosphere are RNA sequencing and gene expression microarray.

2.3.3.1 RNA Sequencing

RNA sequencing (RNA-seq) is a technique to sequence and quantify RNA molecules in the sample with NGS technology. RNA-seq reveals the complete transcriptome with qualitative and quantitative insight of mRNA, rRNA, and tRNA and currently considered gold standard for gene expression analysis. The first step in this technique involves isolation of high quality RNA from the rhizospheric soil followed by conversion into cDNA fragments (a cDNA library) which are subsequently sequenced by NGS. Urich et al. (2008) first time used “Double-RNA approach” to characterize function and structure of the soil microbial communities by sequencing both rRNA and mRNA in a single metatranscriptome. Previous studies highlighted the active rhizospheres microbiome of wheat (*Triticum*

aestivum), oat (*Avena strigosa*), pea (*Pisum sativum*), and grapevine (*Vitis vinifera*) through metatranscriptomics (Turner et al. 2013; Berlanas et al. 2019).

2.3.3.2 Gene Expression Microarray

Microarray is a collection of microscope probes attached on solid surface used for high-throughput expression analysis and comparative genomic hybridization studies (Martínez et al. 2015). It has also been used to monitor gene expression and bacterial identification in different environment samples. Mendes et al. (2011) used a microarray based approach to characterize the rhizosphere microbiome and detected 33,000 bacterial and archaeal species. Previously, metabolic capabilities of maize, pea, and alfalfa rhizosphere have been documented with functional gene microarray (Li et al. 2014). Effect of *Rhizobium leguminosarum* biovar *viciae* inoculation on gene expression of pea, alfalfa, and sugar beet rhizosphere was studied in the past using microarray which revealed the presence of conserved factors for plant colonization (Ramachandran et al. 2011) (Fig. 2.3).

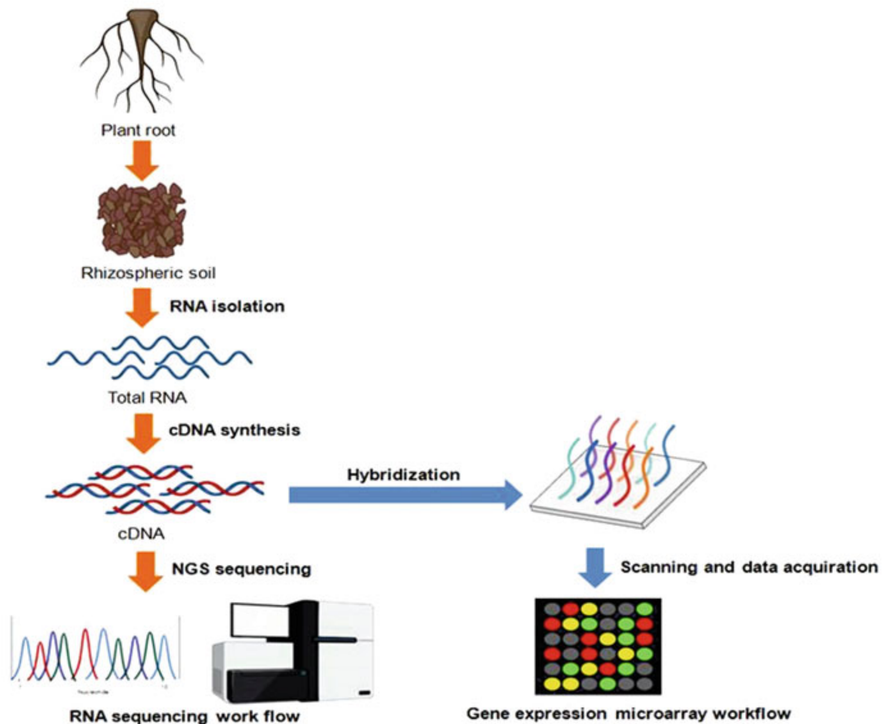

Fig. 2.3 Workflow for the rhizospheric metatranscriptomics

2.3.4 Proteomic Approaches

Recent advances in microbial ecology research taking researchers for studying microorganisms in their ecologies without cultivating in laboratory media. This is helping us to get access to large number of uncultivable microbes which may have tremendous potential in solving many problems of basic science. In this view, proteomics has provided new opportunities in assessing soil microbial diversity and functions. It is the most appropriate and alternate approach to metagenomics where useful information on key biological players which carryout frontline metabolic activities to solve mystery of adoption capabilities of soil microbes in a given ecology (Ploetze et al. 2015).

Further, proteomics is a system biology approach and considered as a logical choice for investigating the plant–microbe interactions. Here the investigations were based upon two-dimensional gel electrophoresis (2-D) which is a good choice for rapid identification of major proteome differences in microbes and their interactive ecologies (Rampitsch and Bykova 2012). This is a functional genomics or system biology approach allowing the study of protein expression of an organism or to obtain protein map of all the proteins expressed. Proteomics is complementary to genome sequencing, gives information on the non-model microorganisms and their activities in a given environment (Muller et al. 2007; Weiss et al. 2009). While the expression profiling of gene provides information at the level of transcript accumulation, proteomics provides information on all the expressed proteins. Information such as location and time in which each functional protein accumulates its level of accumulation and posttranslational modifications to the proteins can be obtained through proteomic approach. Moreover, proteomics also provide more precise information on gene expression since the functional product of most of the genes are not the RNA but the protein. In this approach, most separations for proteins analysis were done with 2-dimentional gel electrophoresis (2-D). The identification of the separated proteins has been aided by sequence library available in the database. Initially the protein spot is from agarose gel is eluted and subjected for electrolytic cleavage where peptide fragments are formed. Matrix-assisted laser desorption/ ionization time of light (MALDI-TOF) mass spectrometric analysis (MS) will be performed to analyze the cleaved peptide fragments. MALDI-TOF analysis generates a list of peptide masses for the cleaved fragments. The size of the fragment is the specific characteristic of the protein which can be predicted from gene sequence. The sequence results for a protein can be compared with database of a calculated/submitted peptide masses for each open reading frame (ORF) in the genome. If there is no match found in sequence database, the proteins can be analyzed by peptide sequencing.

Furthermore, proteomics has a wide range of applications. One such application is detection and diagnosis of plant diseases, their management. Diversity analysis of microbiomes associated with soil, water, and other ecologies. In addition, study of plant diseases, resistance or immunity is benefiting tremendously from proteomics. A fruitful approach in plant pathology has been to perform proteomic experiments on pathogens grown in vitro, or, where their artificial culture is not possible (rusts,

mildews) on a partially purified fungal structure (Bindschedler et al. 2009; Song et al. 2011). The utilization of proteomics to explore biological control agents and their mechanisms is gaining much more attention. The interactions between a potential biological control agent, a phytopathogen, and a plant (tripartite interaction) bring significant changes to the plants proteome and metabolome (Chinnasamy 2005). Microarray technology will be adopted for use in proteomics. Here array of antibodies for a large number of proteins on a chip to analyze for the changes at protein level in an analogous way to how mRNA changes are currently measured. Proteomics has wider application in characterization of intercellular proteins which gives insights in to microbial functions in rhizosphere soil. Biological control of rice brown spot disease, caused by a deadly pathogen *Helminthosporium oryzae* was studied. Tripartite interaction between pathogen-biocontrol agent (Bacillus)—rice was investigated using proteomic approach. Nine proteins including ribulose 1,5 bisphosphate carboxylase, ATP synthase, serine/threonine protein kinases, 2-cys-peroxiredoxin, trehalase-phosphatase, and 50S ribosomal proteins were detected using 2-D PAGE analysis followed by differential expression using MALDI-TOF mass spectrometry (Prabhukarthikeyan et al. 2019). These proteins may help in plant metabolism and defense response against brown spot pathogen. A complex process governing the interactions between host plants with symbiotic microorganisms and vice versa in case of mycorrhizae has been studied through proteomic approach (Bona et al. 2011). Recently, Wang et al. (2011) have applied proteomic approach to study the expression of proteins in rhizosphere soil during the interactions between crop and soil microbes. 2-D polyacrylamide gel electrophoresis, 2D-differential gel electrophoresis, and mass sprectrometry were employed to study expression of gene involved in interaction between plant pathogen, nitrogen fixing bacteria in legumes through bacterial proteomic analysis (Cheng et al. 2010). Thus, proteomics is an appropriate and most useful approach in solving the complex problems of plant–microbe interactions.

2.3.5 Metaproteomic Approaches

Metaproteomics is the most recent and new approach within the “Omics” umbrella is gaining significant importance. This approach investigates the expression pattern of proteins from a complex biological system and gives a direct evidence of physiological and metabolic activities of microbiome. Metaproteome characterization from a biological system will enhance the knowledge of understanding of microbial world and liking microbial communities to ecological functions (Wang et al. 2014). Metaproteomics otherwise is a technology of harnessing the power of high performance mass spectrometry to identify the suite of proteins that control metabolic activities in microbial communities (Hettich et al. 2013). In recent years, the availability of extensive metagenomic sequences from various microbial communities has extended post genomic era to a new exiting area of research.

Metaproteomics even though in earlier stage has shown its potential with regard to functional gene expression within microbial habitats in relation to ecologies. The

interaction of these microbial communities with surrounding environment is also assessed through metaproteomic analysis (Wang et al. 2011). This approach is one of the best approaches in soil microbial community analysis. Metaproteomics can be performed in four major steps, 1. Rhizosphere soil Sample collection, 2. Protein extraction, 3. Purification and fractionation; MS analysis, and finally 4. Protein interpretation and bioinformatics analysis (Wang et al. 2014). Two major work flows for metaproteomic analysis have been developed: (1) Sodium dodecyl sulfate polyacrylamide gel electrophoreses (SDS-PAGE) coupled either with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF-TOF) mass spectrometry (MS) analysis or with electrospray ionization source tandem MS (ESI-MS/MS) analysis. (2) Liquid chromatography coupled with electrospray ionization source tandem MS (LC-ESI-MS/MS).

Metaproteomic analysis of rhizosphere soil is very useful and powerful scientific to solve the mystery of interactions between plants and microorganisms in the soil ecosystem. Role of these microbial communities will help us in utilizing this information in enhancement of yield. Wang et al. (2011) standardized method for extraction of protein from different soil samples and identified 1000 separate spots with high reproducibility stained in 2-DE gels. 189 spots represented 122 proteins on a 2-DE gel of rice samples identified by MALDI-TOF/TOF-MS successfully. These identified proteins mainly originated from rice and microorganisms which were involved in various metabolic activities like protein, energy, nucleotide, and secondary metabolism as well as signal transduction and stress resistance. Similarly in Sugarcane, metaproteomic analysis combined with community level physiological profiles analysis (CLPP) of rhizosphere soil was carried out to understand the reason for sugarcane yield decline. Significant results were found that, sugarcane rationing induced significant changes in soil enzyme activities, the catabolic microbial community, and, the expression level of soil proteins. They influences biochemical processes in the rhizosphere ecosystem and mediated sugarcane and microbial interactions (Lin et al. 2013). Comparative metaproteomic analysis identified that, 38 proteins were differentially expressed in ratoon sugarcane soil which were responsible for yield decline. Knief et al. (2012) carried out the metaproteomic analysis of microbial communities (bacteria and archaea) in the phyllopsphere and rhizosphere soil of rice. Total of 4600 identified proteins obtained from metaproteomic database, they indicated one carbon conversion process in the rhizosphere and phyllopshere. Rhizosphere was dominated by proteins involved in methanogenesis and methanotrophy and phyllosphere by Methylobacterium. These proteins were mainly involved in transport process and stress responses in phyllosphere. Dinitrogenase, reductase were exclusively found in rhizosphere despite the presence of *nifH* genes (Knief et al. 2012).

2.3.6 Metabolomics

Metabolomics is the qualitative and quantitative study of low molecular weight metabolites (<1KDa). Metabolomics serves as a powerful tool for detection,

quantification, and elucidation of molecular interactions in the rhizosphere. In rhizospheric niche, majority of plant-to-microbe and microbe-to-microbe communication is mediated by small metabolites. Exploring these metabolites in rhizosphere explicate different molecular interactions operating at the plant microbe interface which further reveals several critical signaling pathways involved in plant growth promotion, plant disease, defense priming and induces systemic resistance. Thus metabolomics enhances our understanding of molecular and cellular pathways operating in rhizosphere.

Typical mass spectrometry (MS) based metabolomics has three major steps. First step is sample preparation which involves extraction of metabolites through organic solvents or solid phase extraction. Second step is the separation and detection, where metabolites are separated through different chromatographic methods based on the nature of metabolites and then detected by the mass analyzers. Gas chromatography-mass spectrometry (GC-MS) is preferred for volatile and thermally stable compounds which separate metabolite through gas chromatography and detect through quadrupole, qTOF (Quadrupole Time-of-Flight) or QqQ (triple-quadrupole) mass analyzers (van Dam and Bouwmeester 2016). On the other hand, liquid chromatography-mass spectrometry (LC-MS) mostly use normal phase (NP) or reverse phase (RP) chromatography to separate metabolites based on their polarity. With LC-MS, soft ionization like electron spray ionization (ESI) is the most preferred ionization method as it provides accurate mass determination. Capillary electrophoreses (CE) coupled with TOF mass analyzer is used for intermediate primary metabolic pathways (glycolysis, tricarboxylic acid cycle, and pentose phosphate pathway) (Mhlongo et al. 2018). Finally data is analyzed with freely available software like MarVis1, Mzine, MAVEN, Metaboanalyst, and MetAlign or commercial software like Markerlynx, Profiling solutions, and Mass profiler pro.

Several primary and secondary metabolites (non-volatile and volatile) have been documented as major messengers between plant roots and PGPR establishing mutualistic relationship (van Dam and Bouwmeester 2016). Metabolomics of rhizosphere was previously used to study various plant growth modulatory compounds like ACC deaminase, auxins, abscisic acid, cytokinins, gibberellins, jasmonic acid, salicylic acid, and siderophores produced by microorganisms (Mhlongo et al. 2018). Similarly, metabolomics has been the ideal tool to study signaling molecules of root nodule symbiosis like flavonoids (bacterial nod gene induces) and lipochitooligosaccharides (product of nod genes). Rothballer et al. 2018 studied the role of AHLs (acyl homoserine lactones) and its degradation products in quorum sensing of rhizospheric bacteria. Metabolomics has also demonstrated the change is microbial community in grass (*Avena barbata*) rhizosphere with bacterial succession dynamics with respect to substrate preference in changing root exudates over the course of development (Zhalnina et al. 2018). However, the cost of equipment, limited public reference database, and lack of proper expertise make metabolomics more challenging than the DNA sequencing based approaches.

2.3.7 Phenomics

Phenomics is defined as the systematic study of phenotypes on a genome-wide scale. In other words, phenomics is set of multidimensional approaches to study how genome of an organism translates into the full set of phenotypic traits. However, prediction of the phenotype from the genotype is not state forward because large number of genes interact with themselves and the environment to produce the phenotype. Metagenomics has provided an access to complete genotype of rhizospheric microorganisms to genus, species, and subspecies level. However, large fractions of the genes in metagenomics have no assigned function and even the ascribed functions for most of the genes are based on DNA sequence homology.

Apart from the traditional techniques of phenotypic characterization, transcriptomics, proteomics and metabolomics are the widely used tools which provide enormous phenomic data, thus elucidating the phenomics of rhizospheric microorganisms (Houle et al. 2010). Phenotypic features (phenome) of the rhizobia have been studied for their classification and placing them into different cross inoculation groups. Phenomics have also been very useful to study the plant pathogen interactions and host-pathogen co-evolution at molecular level.

Complexity of biological processes at cellular and developmental level needs to be addressed with high quality digital phenotypic data. Recently, global *Escherichia coli* promoter activity was accessed using PFIboxes to obtain high quality phenome data of gene expression under the 15 different antibiotics stress (French et al. 2018). Growth measurement is the key phenotype for accessing microbial fitness in any ecosystem. Automated microbial phenomics framework was developed to records and analyzes over 100,000 growth curves in parallel (Zackrisson et al. 2016). However, limited high-throughput tools are available to study the phenomics of rhizospheric bacteria. Therefore, development of high-throughput and high-resolution phenotyping tools are required to address the detailed phenomics of rhizospheric microorganisms.

2.4 Conclusion

The rhizospheric microbial diversity from Himalayan agro-ecosystems has been revealed by author group extensively. It has been observed that the microbial diversity from *Phaseolus vulgaris* rhizosphere was varied significantly in Kumaun and Grahwal Himalayan regions of Uttarakhand. Contrary to the Kumaun where, genus *Pseudomonas* was predominant; Garhwal Himalayan *P. vulgaris* rhizosphere was inhabited primarily by *Sphingomonas* (Suyal et al. 2019c). However, among the diazotrophs, genus *Rhizobium* was predominant throughout the Uttarakhand Himalayas (Suyal et al. 2015b). Here we can conclude our discussion in a short statement that is the use of more advanced molecular tools may help us to reveal several unexplored microbiomes which ultimately benefitted to sustainable agriculture.

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