23 Molecular Microbial Biodiversity Assessment in the Mycorrhizosphere

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1 Introduction

The microbial flora within the soil plays a crucial role in ensuring the plant's well-being and the richness of the soil. These organisms have niche activities that contribute either through nutrient uptake, nutrient cycles, suppression of disease, growth enhancement and many more processes (Jacobi et al. 2017; Muller et al. 2016). While studying the soil microbial structure, it has been noted that mycorrhizae also play a role in the root ecosystem. This therefore has resulted to the widening of the rhizosphere terminology to mycorrhizosphere, which includes the fungal component of this community (Sehgal and Sagar 2017). As mycorrhizae and the soil microorganisms contribute to the overall well-being and productivity of plants, the understanding of the interactions involved between the plant-microbe-soil is absolutely crucial. The understanding derived from these interactions is imperative in improving soil health and crop production.

A major group of fungi in the root system is the arbuscular mycorrhizal (AM) which is known to form symbiosis with the host root systems. Currently at least 160 taxa have been identified and a brief analysis via molecular techniques has indicated that these numbers are conservative. Research conducted on soil microbiology has shown that bacterial communities also interact with the AM fungi in the root. They affect the root-fungi interaction directly through (i) provision of energy, (ii) exudates that improve AM function such as germination, growth, receptivity and recognition, (iii) alteration of soil pH, and (iv) exudates that inhibit the detrimental organisms in the soil. Indirectly, these bacteria can affect the growth, yield, soil structure and root exudates in a mycorrhizae based interaction. The direct impact of the soil bacteria interaction with the root and the mycorrhizae has mostly been

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positive in greenhouse trials (Ross 1980; Tommerup 1985; Wilson et al. 1988). Frequent reports have cited that AM improves plants nutrient uptake and improves disease resistance in their host. Other organisms such as N fixers and P solubilizer are known to work with AM in jointly improving plants growth and development (Puppi et al. 1994).

Now that we have accomplished the definition of the microbial composition within this area, we arrive now at a problem that is constantly faced by soil microbiologists which is the appropriate tools to study the community, diversity and structure. The initial techniques that were utilized by microbiologists such as general serial dilution, plating and the biochemical assays have all met with their limitations especially when addressing soil microorganisms that are tedious or difficult to culture. As group of non-culturable and difficult organisms make up a large portion of soil microbes, it is essential that these organisms are identified so that their role and function within the ecosystem is understood (Amann et al. 1995). The endosymbionts remain largely unexplored and require elucidation for better understanding of the microbial diversity in the ecosystems (Bianciotto et al. 1996, 2000). Therefore, to study the mycorrhizae population and the immense unculturable and culturable organisms within the soil, technologies that are high throughput and able to screen large quantities of material quickly and accurately is required. Through the advent of molecular biology, several molecular biology and omics platforms have been established which enable us to address the need to analyze large microbial samples, including unculturable organisms, at high accuracy, at improved costing and reduced time (Hugenholtz et al. 1998, 2001; Quince et al. 2009). The molecular assessment techniques have provided means to study various soil ecosystems (Elshahed et al. 2008; Finlay and Medzhitov 2007; Liu et al. 2007). This chapter endeavors to provide an overview of molecular assessment tools that are available and their applications and limitations in studying the mycorrhizosphere community with the overall aim of using this information to enhance plant well-being and positively contributing to sustainable agriculture.

2 Molecular Detection of Microorganisms in the Mychorrhizosphere

Compared to the morphological and biochemical methods that have been employed to date, the molecular approaches promise better opportunities to analyze the full diversity of the microbial community. The continuous advancement in technologies and platforms related to molecular studies allows for rapid profiling of communities to identify microbial groups present and thus making the information readily available for mutual benefit of scientists from various different fields (Fakruddin and Mannan 2013).

As mentioned above, all methods utilized for the analysis of the mycorrhizosphere has to be inclusive of mycorrhizae and other fungal and bacterial species found within the sphere. Therefore there is a continuous quest for methods that would provide precise coverage of microbial diversity at ideal cost and at a time

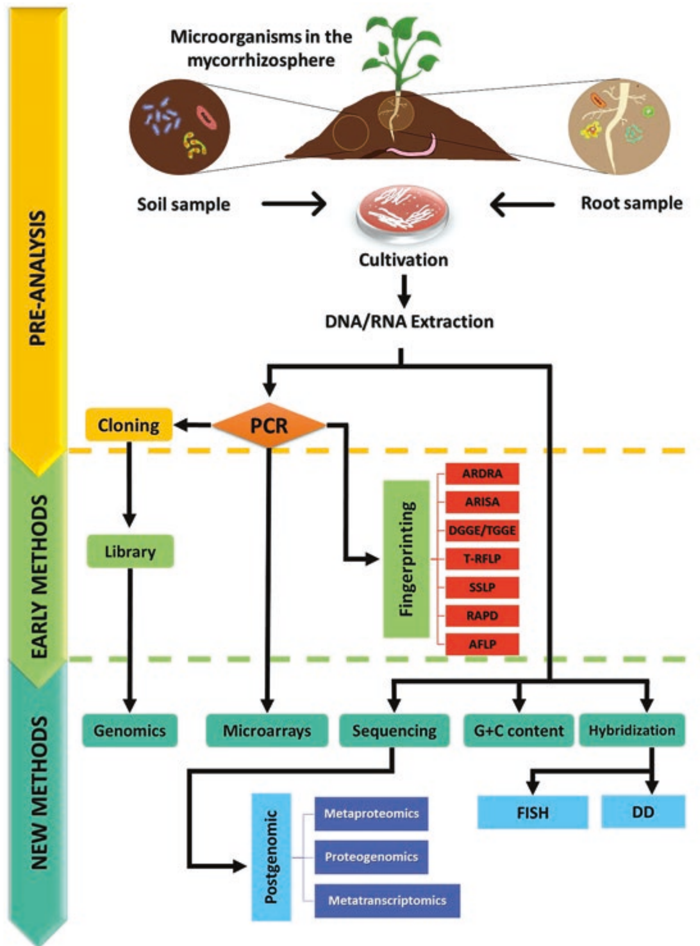

Fig. 23.1 Links most of the methods available to analyze soil sample from the mycorrhizosphere. This figure provides processes that encompass all the analysis starting from the early methods up to the current cutting-edge platforms available to analyze soil samples

effective manner. Figure 23.1 shows the diagrammatic representation of various methods that may be utilized to conduct microbial analysis on the mycorrhizosphere and the components within this zone.

2.1 Nucleic Acid Isolation

As in most molecular techniques especially those that require PCR, nucleic acid is a routine requirement that needs to be fulfilled. Appropriate soil and root samples are needed for successful isolation of nucleic acid. Samples are collected aseptically from the roots and the soil aggregates around the root within the mycorrhizosphere.

This would provide the DNA representation of organisms including symbionts that are found within the root and the area surrounding them.

2.2 PCR Amplification

Most molecular identification techniques have been divided into PCR and non-PCR based. A large number of microbial community and structure analytical tools have been developed utilizing the PCR technique. The PCR technique is developed on the basis of PCR amplification involving specific target genes that are either prokaryotic or eukaryotic based and in certain cases genes that are genus, species or function specific (González and Saiz-Jiménez 2005). Some of the commonly used marker genes are the 16S (prokaryote) and the 18S (eukaryote) small subunit ribosomal RNA (rRNA) (Dakal and Arora 2012; González and Saiz-Jiménez 2005). These genes have been used over the years and have been consistent in their results that they have been regarded as a gold standard for identification of microbes from environments. The reason for their stability in performance is largely attributed to the ubiquitous nature of these genes in prokaryotic and eukaryotic organisms, as they are structurally and functionally conserved. Henceforth, the variability in the conserved region can be used for identification (Rastogi and Sani, 2011) and for the estimation of the divergent point between species. These gold standards have been utilized by researchers and have since classified all living forms into Eukarya, Bacteria and Archaea (Neelakanta and Sultana 2013; Rastogi and Sani 2011).

Previous studies have shown that the above-mentioned 16S and 18S genes have been used efficiently in the detection and identification of bacteria and fungi present in the mycorrhizosphere (Nadarajah 2017). However, we need to note that the 16S gene may be present in multiple copies in a genome and thus it may be useful to have alternative markers. Some researchers have used genes such as *rpoS, gyrB* and *recA* in their studies of the microbial communities (Case et al. 2007; Tacão et al. 2005; Waleron et al. 2008). Although these genes show promise in reflecting the evolutionary history and diversity within a community (van Elsas et al. 2006), the limited availability of sequence databases for these genes in contrast to 16S and 18S hampers the extensive use of these candidates. However, it is hoped that the continuous submission of data on these alternative candidates to databases will eventually result in these genes being used routinely in soil microbial analysis. One definite indication for a need of new alternative marker genes comes from the difficulty in resolving pseudomonads through the utilization of 16S rRNA. This is due to the fact that pseudomonads have slightly distinct roles and these functions are supported by different sets of accessory genes. Costa et al. (2007) reported that the global regulator *gacA* gene was able to resolve the pseudomonads at a higher resolution compared to the universal 16S rRNA gene. Amplification of genes from DNA/RNA of microbial communities such as a*moA*, *nifH*, *nirK*, *nirS*, and *dsrA* facilitated studies on microbial processes such as nitrogen fixation, denitrification and sulfate reduction. Microbial catabolic diversity can be elucidated through advanced studies on enzymes-coding genes that are involved in carbon utilization.

In addition to the 18S rRNA, another commonly used molecular marker in the identification of fungi is the internal transcribed spacer region located between 18S and 28S rRNA which consists of internal non-coding regions ITS1, ITS2 and 5.8S rRNA gene. These regions are highly conserved and may be beneficial in studies that aim to show the similarities between evolutionarily distant organisms and sequences with high genetic variability (ITS regions) which will especially be useful in determining genera and species. Apart from that, the ITS regions are of particular importance in molecular diagnostics of molds, because they are present in all fungi in a large number of copies, which increases the sensitivity and specificity of the PCR reaction (Atkins and Clark 2004; Ciardo et al. 2007, 2010). Further, other than the ITS primers mentioned above (especially IT1 and ITS4 which is widely used), certain studies have also utilized universal eukaryotic primers such as NS31 (Simon et al. 1992) in combination with AM2 and AM3 (Santos-González et al. 2007) which produces the amplified 5.8S rRNA gene. The PCR products amplified from environmental DNA can be analyzed by (i) genetic fingerprinting, (ii) clone libraries, or (iii) by combination of these techniques or (iv) new next generation technologies.

Besides the standard PCR process, quantitative PCR or qPCR is being applied in analysis of DNA extracted from soil. The extracted DNA is subjected to qPCR to quantify the number of target genes of 16S or any other functional genes (*amoA, rpo,* or *nifH*). Though it has been successfully utilized in soil studies (Kolb et al. 2003), this method provides a bias picture of the number of targets and does not detect similar genes with slightly varied sequence or similar function. Nonetheless, this method is still quite efficient at portraying the effects of the environment on the gene and gene expression and thus is efficient in mapping the diversity of the microbial communities in various environmental conditions within the soil or the mycosphere.

2.3 Preparation of Library

Following PCR amplification is the construction of libraries that carry the amplified PCR product. The establishment of the cloned libraries provide a means to analyze PCR products obtained from 16S and 18S rRNA genes. A metagenomic analysis of any given microbial community involves the construction of libraries which involves the isolation of metagenome DNA, the fragmentation, cloning and transformation, followed by screening and bioinformatics analysis of the clones (Mocali and Benedetti 2010).

There are plasmids such as cosmids, fosmids and BACs that may be utilized in the construction of these libraries depending on the size of inserts involved. In most studies conducted to date the preferred host for cloning and expression studies of the metagenome is *Escherichia coli*. However, over the years there are new host that have been included into the repertoire such as *Pseudomonas aeruginosa*, *Rhizobium leguminosarum* and *Streptomyces lividans*. These host have been chosen for some specific application such as analysis and detection of bioactive compounds (Mocali and Benedetti 2010; Streit and Schmitz 2004).

These metagenomic libraries are then subjected to analysis based on the objective(s) of the study, which could be anything from determining the presence of a gene to the identification of clones with a desired function. Some of these activities may not require the sequencing of the libraries as it may be involved in identification of a specific gene, enzymes or metabolites. For instance, a study on the mycorrhizosphere indicates that the genes of interest may be directly involved in process of nitrogen fixation, nutrient acquisition, quorum sensing and others. However, in projects that require determination of community diversity and structure, there is a need for sequencing which would incur a higher cost into the projects. Hence it is quite common for projects like this to include a prescreening strategy such as fingerprinting to ensure smaller number of clones are subjected to the process of sequencing followed by analysis that adopts bioinformatics tools (Coutinho et al. 2013; Deja-Sikora et al. 2007; Gonzalez et al. 2003; Mocali and Benedetti 2010; McNamara et al., 2006). The following section will address the importance and application of fingerprinting techniques.

2.4 Fingerprinting Techniques

The genetic fingerprinting technique prompts to electrophoretically analyzing PCR based products that have been amplified from metagenomic DNA. There are several types of fingerprinting tools that have been developed over the years that may be utilized in the microbial fingerprinting of the mycorrhizosphere. These techniques include: ARDRA (amplified rDNA restriction analysis), ARISA (automated ribosomal intergenic spacer analysis), SSCP (single strand conformation polymorphism), T-RFLP (terminal restriction fragment length polymorphism) and DGGE/TGGE (denaturing/temperature gradient gel electrophoresis).

Fingerprinting techniques have been used in the detection of microbial cells and in visualizing the quantitative profiles of the composition within a given ecosystem. Conducting genetic fingerprinting has permitted the researchers to explore the diversity within a community especially for communities that involve non-culturable and difficult organisms. Although a composition of the community is provided, this method by no means provides a direct taxonomic identification of microorganisms (Dakal and Arora 2012; González and Saiz-Jiménez 2005). The basic procedure of this protocol is the isolation of a given sample DNA, which is followed by amplification of any specific genes mentioned above and visualization of the product on an electrophoretic gel. The banding profiles generated from these amplified products represents the data which will be analyzed (Muyzer 1999; Rastogi and Sani 2011).

2.4.1 Amplified Ribosomal RNA Restriction Analysis (ARDRA)

ARDRA is utilized in monitoring the communities within changing environments. In this particular technique, the rDNA is amplified via PCR and digested using restriction enzyme before visualization of the restricted fragments via gel electrophoresis. This technique allows the capture of microbial community structure information but unfortunately it does not give a picture on diversity and phylogeny (Cetecioglu et al. 2012; Rastogi and Sani 2011). ARDRA-ITS allows the inquiry of

microorganisms without any information on the genome organization. The conserved domain within the amplified rDNA is interrupted by the non-coding variable of ITS1 and ITS 2, which allows for differentiation. This is useful to exhibit the differences at the species and subspecies levels. However one of the major limitation of the ARDRA technique is that it does not provide any details about the microbial population present in the sample (Gich et al. 2000).

2.4.2 Automated Ribosomal Intergenic Spacer Analysis (ARISA)

ARISA has been efficiently used to shed light on the richness and diversity of microbial communities. This culture independent method was developed towards the end of the twentieth century to differentiate between the size and nucleotide variation within the intergenic spacer region that exists between the 16S and 23S ribosomal subunits (Cardinale et al. 2004; Fisher and Triplett 1999; Popa et al. 2009). The variation within the intergenic spacer region is analyzed within an automated capillary laser detection system. This method of analysis utilizes universal primers that cause multiple peaks and limits the ability of the system. In addition, it is very difficult to interpret results for fingerprints obtained for uncultured microorganisms (Popa et al. 2009).

2.4.3 Denaturing or Temperature Gradient Gel Electrophoresis (DGGE/TGGE)

Denaturing or temperature gradient gel electrophoresis are molecular techniques based on PCR-amplified molecular markers (16S rRNA or 18S rRNA genes) separated by gradient polyacrylamide gels electrophoresis based on either chemical gradient (denaturing) or temperature gradient (Rastogi and Sani 2011). Both these techniques involve partial denaturation of DNA within domains that is largely dependent on the DNA sequences of these domains. Differences in nucleotide sequences will cause difference in temperature of melting for this particular domain and therefore result in variable migration rates through the polyacrylamide gel (Muyzer 1999; Muyzer et al. 1993; Muyzer and Smalla 1998; Więckowicz 2009).

These techniques allow for the detection of approximately 50% of differences in sequence of fragments which can go up to 500 bp. Besides providing the ability to determine the differences between these fragments, this technique also has the added advantage of excision of respective bands from the gel for amplification followed by sequencing. The sequence data obtained from these fragments may be utilized to generate phylogenetic correlations of the microbial diversity in a given sample. However, one limitation of this technique lies in the short fragments generated i.e. up to 500 bp. These short fragments make it a bit hard to separate the fragments effectively to make concrete interpretation of the results. However from literature review of past studies of microbial diversity and communities, the DGGE/TGGE techniques have been successfully used to interpret the microbial communities of bacteria (Gaylarde et al. 2012; Piñar et al., 2009, 2013), cyanobacteria (Gaylarde et al. 2012), archaea (Piñar et al. 2001a, b) and fungi (Giacomucci et al. 2011). As the mycorrhizosphere has all these groups of organisms, this technique remains a method of choice for microbial diversity studies.

2.4.4 Terminal Restriction Fragment Length Polymorphism (T-RFLP)

The T-RFLP method is a marriage between multiple techniques, which includes RFLP, PCR, nucleic acid electrophoresis, and comparative genomics. This fingerprinting technique is used as a supplement to the ARDRA method. The only difference between the ARDRA technique and T-RFLP is that one of the two primers used in this technique is fluorescent labeled (Liu et al. 1997; Więckowicz 2009). The amplified fragment is then restricted with enzymes and fractionated through polyacrylamide gel electrophoresis. As a consequence of the digestion, only the labeled fluorescent end is detected in the gel profiles and these detected bands greatly simplify the analysis of any microbial population in soil (Cetecioglu et al. 2012; Rastogi and Sani 2011). The variation in the number, size and peak height obtained from the analysis of these restriction fragments will provide the data on the biodiversity of the population. However for complete quantitative analysis of the polymorphisms of the restricted bands, the resulting banding profiles may be compared against configured databases to provide valuable comparative community analysis (Rastogi and Sani 2011). This method is applied in identifying the strains, comparative analysis on microbial communities and the estimation of phylogenetic divergence within the community. Community dissection at a higher level may be obtained by inclusion of primers that are specific to phylogenetic groups in the T-RFLP protocol.

2.4.5 Single Strand Conformation Polymorphism (SSCP)

This technique differentiates samples based on the migration mobility in polyacrylamide gel resulting from the variation in the protein structure. This variation is caused by differences in the secondary structure of folded DNA which is a result of sequence differences of single-stranded DNA (ssDNA). Therefore, any given population of fragments of the same size may separate with different mobilities in a non-denaturing PAGE due to the variable conformational change. All fragment lengths analyzed are of uniform size i.e. approximate range of 150–400 bp. Unlike the other gel techniques, this method does not require GC clamped primers nor does it require gel gradients (Cetecioglu et al. 2012; Rastogi and Sani 2011). The SSCP-PCR is ideal to detect polymorphisms that results from mutation in the DNA which contributes in conformational change (Orita et al. 1989). In some circumstances, this technique has been used as an alternative to the DGGE/TGGE. The disadvantages of this systems is that the fragments are between 150–400 bp and that these single stranded DNA fragments are able to form multiple conformations that may be represented as multiple bands (Cetecioglu et al. 2012; Rastogi and Sani 2011).

2.4.6 Random Amplification of Polymorphic DNA (RAPD)

RAPD is based on PCR of randomly chosen single primers that anneal to complimentary sequences in the DNA (Agrawal and Shrivastava 2013). Once these primers are annealed in inverted orientation to the template, several bands are amplified. The products are then fractioned through a gel and the presence or absence of the polymorphic bands in the profile allows for the polymorphism assay. RAPDs are

able to distinguish isolates to their taxonomic level based on the primers used. However, while the RAPD method is quick and convenient, this technique has its glitches in reproducibility therefore requiring optimization in every fingerprinting exercise to ensure robustness of data. This technique has been used to elucidate the genetic difference and species diversity in many environments studied (Singh et al. 2005).

2.4.7 Amplification Fragment Length Polymorphism (AFLP)

AFLP is a more robust and stringent method with reproducibility and ability to provide quantifiable data. This method produces a more complex fingerprint compared to RAPD. To provide this quality of data, the technique requires good quality and quantity of DNA in addition to requiring reasonably good experimental skill set (Karp et al. 1996). While AFLP is suitable for determination of genetic distance, mapping and fingerprinting analysis, this method is not amenable for use in comparative genomics involving fast evolving microbes. AFLP is not suitable for use in homologous genomes analysis too (Karp et al. 1996).

2.4.8 Restriction Fragment Length Polymorphism (RFLP)

RFLP is a technique used where restriction endonucleases are used to digest DNA of organisms. Different organisms with different genome content are likely to be digested at different locations within the genome by the same endonuclease. The fragments generated will be different not just in size but in numbers too. The DNA fragments are generally digested with different endonucleases and the profiles are visualized via gel electrophoresis. Therefore, the restriction profiles visualized are able to distinguish the differences between species and also up to strain levels (Avise 1994). Compared to RAPD, the RFLP techniques provides several advantages as follows: (i) any DNA source may be utilized for the analysis; (ii) their codominance is independent of the environment, and (iii) markers mapped to a population are not stressed but rather the effect of phenotypic mutations.

2.5 DNA Sequencing

The Sanger's sequencing method has been used for more than a decade. This method has since been improved on for better efficacy, cheaper cost and rapid data generation (Mecler and Nawrot 2007; Rastogi and Sani 2011). Over the last few years, several new next generation sequencing techniques have been developed using primarily platforms such as 454-based/pyrosequencing and Illumina/Solexa's Genome Analyzer (Margulies et al. 2005). These high-throughput technologies have since become a method of choice for metagenomes and metatranscriptome sequencing projects. The pyrosequencing technique enables the sequencing of DNA or RNA samples from the soil (Lauber et al. 2009; Roesch et al. 2007; Urich et al. 2008). This technique leaves out library generation, template preparation and capillary sequencing (Rothberg and Leamon 2008). The multi-parallelism of the 453 system allows the generation of 450-bp reads of thousands to millions run at once. The

Solexa platform offers higher throughput compared to the 454 but at smaller read lengths. While sequencing is generally looked upon as an unbiased technique, it still is dependent on the quality and quantity of the DNA or RNA. The 454 platform can be used together with the Illumina/Solexa platform where the 454 can generate a longer read and the Illumina/Solexa can fill in the gaps in the data through its high throughput (Quince et al. 2009). Through sequencing it is possible to obtain the information on the most abundant of species to the most rare organisms in the biosphere giving novel insight into the soil microbial communities (Elshahed et al. 2008; Liu et al. 2007; Roesch et al. 2007). Some of the limitations of these methods are in the financial and analysis of large datasets generated through bioinformatics. This method still remains as the most detailed tool for study of microbial diversity, community structure and gene expression (metatranscriptomics) across diverse soils (Lauber et al. 2008, 2009; Urich et al. 2008). We assume that with time, this technology will improve in sensitivity and therefore supersede any other techniques such as the microarray (Lauber et al. 2008, 2009; Roesch et al. 2007; Urich et al. 2008). Programs such as MEtaGenome Analyzer are used to align and assemble the sequence obtained into a finished sequence. These sequences are made available in databases such as National Center for Biotechnology Information (NCBI) and Genomes Online Database (GOLD) for common use by the research community.

2.6 Bioinformatic Tools and Databases Used in Metagenomics

In order for us to make sense of the large amount of data that is generated from soil microbiology studies, the bioinformatics tools and databases are a crucial medium to support the analysis and information generation from these studies. Determination of a sequence homology between an investigated product and thousands of sequences collected in public (National Center for Biotechnology Information NCBI, GenBank), or commercial databases is possible by using suitable computer programs, such as BLAST which is among the most widely used ones (Mecler and Nawrot 2007). The BLAST algorithm is a heuristic program which performs “local” alignments, based on shortcuts, and its task is to conduct a quick search (Tatusova and Madden 1999). An advantage of the molecular tools such as metagenome analysis is the ability for this method to also elucidate the non-culturable and problematic organisms whether from soil or any environment.

2.7 Determination of the DNA Base Ratio (Mole Percent G+C)

A classical genotyping method used in determination of bacterial taxa is the mole percentage of cytosine plus guanosine where the G+C percentage has been reported to be between the range of 20–80% in the bacterial world (Vandamme et al. 1996). The G+C percentage can be determined through thermal denaturation method, HPLC and the buoyant density method (De Ley 1970; Mandel and Marmur 1968; Mesbah et al. 1989). It has been reported that microorganisms differ in the G+C

content and related groups differ slightly in their G+C percentage (i.e. 3–5%) (Nüsslein and Tiedje 1999; Tiedje et al. 1999). Through density gradient centrifugation based on G + C content, the fractionation of the total community DNA is determined. The fractionated profile will then provide the information on the relative abundance of any genus or taxa. These profiles can be analyzed further using techniques such as DGGE/ARDRA to provide greater detail on the community diversity.

2.8 Fluorescence In Situ Hybridization (FISH)

The fluorescent hybridization probe technique is employed to detect the presence of rRNA at cellular level with the aid of an epifluorescence microscope. This technique enables correlations to be made with regards to cell metabolic state through the intensity of fluorescent signals in cell. Over the years this technique has advanced in the type of fluorescent dyes developed which have better sensitivity, and multiple fluorochromes. The signals have also been amplified through reporter enzymes, where the catalyzed reporter deposition FISH, with tyramide-labeled fluorochromes, allows enhanced signal emissions (Rogers et al. 2007). Further FISH has been used in combination with secondary-ion mass spectrometry (SIMS) where 16S rRNA probes are used to identify microbes by *in situ* NanoSIMS imaging (Li et al. 2008). This technique is suitable for the detection of microbial density and metabolic state in any given soil sample (Caracciolo et al. 2010).

2.9 DNA: DNA Hybridization (DDH)

This technique allows for the entire genome comparison between strains based on nucleotide level similarities/dissimilarities. In this technique, all the steps that comprise extraction, denaturation and incubation of the sample DNA are conducted in conditions that allows for hybridization and re-association. As comparisons are down to the nucleotide level, the DDH technique is able to differentiate to the species level the organisms within the soil sample. In conducting the DDH analysis a 70% standard was stipulated while a 97% delineation was recommended for the 16S rRNA gene sequence homology (Goris et al. 2007) for species level differentiation. However, this method is not suitable for differentiation at the genus level (Krieg and Holt 1984). In addition, there has also been some inquiry into the suitability of utilizing data obtained from short oligonucleotides and mispairing to extrapolate to whole genomes. Currently, the conversion of DNA-DNA hybridization to whole genome sequence similarities is rather unachievable (Vandamme et al. 1996). There are three forms of hybridization available: The Southern blotting which enables the identification of DNA molecules through DNA/RNA probes, Northern blotting involves RNA molecules analyzed with RNA/DNA probes and finally, Western blot whereby proteins are probes with specialized antibody probes.

2.10 Microarray

For the microarray method the soil DNA that is obtained in fluorescent labeled and brought in contact with the microarray. The array contains thousands upon thousands of oligo-probes that are either 16S based (Phylochip) or functional gene related (Geochip) which hybridizes to the soil DNA at homologous positions. Following hybridization, the signal output from the chips is digitally analyzed. Through the phylogeny relationship analysis (Phylochip) and the functional analysis of the population (Geochip), a high throughput picture is obtained of the heterogeneity of the microbial samples. In a highly diverse sample such as soil, distinguishing complexity may prove to be problematic. When highly abundant 16S rRNA genes fragments are available, cross hybridization becomes an issue due to shared sequence similarities to non-target probes which results in weak signals that are false positive. The currently available phyloarrays can be paired with various techniques, which include 16S cloning, and sequencing or the utilization of fingerprinting techniques such as PCR DGGE. Other than the phyloarray, the Geochip has been utilized successfully to studies the nutrient recycling processes in the soil sample from the Antarctic (Yergeau et al. 2007) where the association between the abundance of these functional genes corresponded to their respective abiotic factors. Functional gene array accompanied with quantitative PCR and enzyme assays has greatly facilitated in validating the microarray hybridization results and thus provides a reliable method on deriving information on the functional element of the microbe (He et al. 2007; Neelakanta and Sultana 2013; Yergeau et al. 2007). However, the lack of robustness and the inability to produce data on novel sequence types is a constraint to the application of the functional gene array. Hence, the information can only be accessed based on the existing breadth of known functions/genes (DeSantis et al. 2007; Yergeau et al. 2007). Despite such challenges, the microarray provides a quick glimpse at the functionality of soil and mycorrhizospheric microbial population (Van Elsas and Boersma 2011).

2.11 Reverse Sample Genome Probing (RSGP)

RSGP is a technique that has been employed to analyze microbiota and to determine dominancy within these species. In this method, the genomic DNA will be isolated from pure cultures and hybridized to determine fragment that underwent cross-hybridization less than 70% which is then followed by the preparation of genomic arrays and finally random labeling of total communities and internal standards. This method is useful only when low diversity is observed in the mixture of total community DNA and internal standard (Greene and Voordouw 2003).

2.12 Postgenomic Approaches

The *in situ* gene expression of microbe can't be deduced from DNA-based molecular approaches (Rastogi and Sani 2011). Therefore postgenomic approaches such as metatranscriptomics and metaproteomics are applied with the available comprehensive metagenomic databases to connect the genetic potential to the functionality in microbial communities (Rastogi and Sani 2011).

2.12.1 Metaproteomic

Metaproteomic is a study on proteins retrieved from environmental microorganisms at a certain point in a microbe's life cycle. (Keller and Hettich 2009; Wilmes and Bond 2006). It functions mainly by providing valuable insights into the interactions between proteins and data on the quantity of proteins. In doing so, there is an opportunity for the elucidation of physiological roles of microbial communities (Keller and Hettich 2009). For example from a soil sample, a few important proteins, enzymes, and chaperones associated in the biodegradation of chlorophenoxy acid were identified through proteomic analysis (Benndorf et al. 2007; Rastogi and Sani 2011). Metaproteomic study encompasses the extraction of proteome from a sample from environment followed by separation of the proteome through one and two-dimensional electrophoresis to produce a proteofingerprint of community and finally the digestion of protein spots that will be then identified through several analyzes (Rastogi and Sani 2011). The advancement in techniques such as chromatography and mass spectroscopy (MS-based proteomics) has enabled microbiologists to perform the profiling of the proteome of microbiota which are high-throughput (Rastogi and Sani 2011). Besides, services provided in the Web like ExPASy (Expert Protein Analysis System; http://www.expasy.org/) provides various tools to identify and characterize the protein mass fingerprinting data (Rastogi and Sani 2011).

2.12.2 Proteogenomics

Most of the protein sequences obtained through proteomic analysis could not be identified with certainty as proteins are poorly related to the available database sequences. As a consequence, protein sequences remain unidentified in terms of their functionality and phylogenetic characteristics (Rastogi and Sani 2011). To overcome this limitation, a new technique known as proteogenomics which integrates metaproteomic and metagenomic approaches has effectively increased the identification of the sequences of protein where the sample of which the proteins were extracted and subjected to metagenomic analysis (Banfield et al. 2005). This method was adopted in a study conducted on phyllosphere bacterial communities which results in an increased number of identified protein, suggesting that most of the microbial communities in phyllosphere were different genetically as compared to those readily available in databases (Delmotte et al. 2009).

2.12.3 Metatranscriptomics

Metatranscriptomics encompass random sequencing of mRNA transcripts obtained from microbiota at a given location and period (Moran 2009). While metagenomics provides information on the genes, this technique further examines the global transcription of genes to comprehend the activity and expression of microbial genes in their natural environments. This technique also surveys the differential expression of genes and their regulation in accordance to the changing environment (Rastogi and Sani 2011). Transcriptomic study can be done by isolating the RNAs in the microbe and selecting the mRNA by synthesizing the cDNA through the portrayal of poly-A tail. However, due to the lack of the poly-A tail in prokaryotic species, rRNA will have to be coextracted together with mRNA and this may lead to massive background sequences (Bashiardes et al. 2016; Rastogi and Sani 2011). Over the years, some improvements have been made to overcome this limitation whereby mRNAs are selectively enriched through subtractive hybridization of rRNA for gene transcript analysis. Besides, double-RNA method is also used in a study to analyze the community based on the total RNA pool which provides a means to study the structure and biochemical properties of microbes all in one go (Urich et al. 2008; Rastogi and Sani 2011). This study produced rRNA tags and mRNA-tags that facilitated understanding of the phylogenetic composition of soil microbial communities from sandy soil samples (Rastogi and Sani 2011). Another study successfully discovered transcripts associated to various biogeochemical processes. (Poretsky et al. 2005).

2.13 Concluding Remarks

In this chapter, we addressed the issue of assessing the microbial community within the mycorrhizosphere. As mentioned above, this zone has not been extensively studied mainly due to the inability at times to separate the organisms (endosymbionts) from the host and to also culture some of the bacteria and fungi in the lab. These have posed an obstacle on obtaining a clear picture of the soil ecosystem. Throughout the chapter we have provided a background on the various techniques that are now available for those who seek to decipher the mycorrhizosphere community. We begin with the basic DNA and RNA extraction to the library construction and the application of PCR techniques in fingerprinting of the samples. The more recent techniques however such as microarray and sequencing provide larger amount of information on the microbiota that is evident within the soil community.

Through the availability of the multiple techniques that have been outlined and the continuous advancements made in each technology we posit that with time we will be able to gather core information on the microbial structure and community within the mycorrhizosphere. However more importantly, we need clearly defined objectives and scope of research and use the techniques or combination of techniques to get a better overview of the ecosystem. In addition, while information of microbial diversity is useful, we need to focus on the functionality of these organisms. The molecular based post-genomic techniques such as metagenomics,

proteogenomic and metatranscriptomics has provided a new level of understanding into the different and fascinating processes that occurs within the microbial communities. Through the utilization of the above tools, the interactions within the microcosm might be directly assessed.

However, given the overall nature of these methods, it is strongly recommended that to obtain a better overview of the ecosystem, studies should:

1. Directly analyse microorganisms based on molecular methods
2. Detect microbial activities through methods that enable *in situ* analysis
3. Isolate and question the contribution of these organisms in their given eco-physiological behavior and thence use this to predict their *in situ* behavior.

Through the information derived from the molecular studies conducted on the soil sample, we believe that various questions with regards to relationships, diversity, products and application may be answered. However as with any knowledge, the more we unravel, the more questions will arise. Figure 23.2 provides a diagrammatic representation of the outcome of molecular soil analysis.

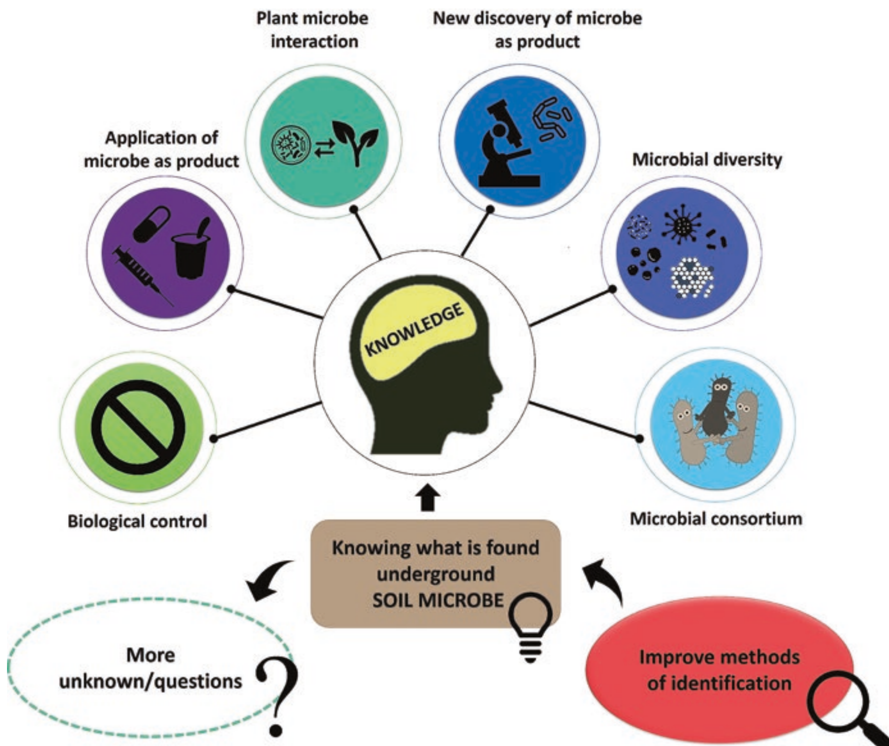

Fig. 23.2 Shows how the information derived from the mycorrhizosphere may be used to answer several questions with regards to soil health and the advancement of knowledge and techniques

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