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Review

Genomics as a potential tool to unravel the rhizosphere microbiome interactions on plant health

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ABSTRACT

Intense agricultural practices to meet rising food demands have caused ecosystem perturbations. For sustainable crop production, biological agents are gaining attention, but exploring their functional potential on a multi-layered complex ecosystem like the rhizosphere is challenging. This review explains the significance of genomics as a culture-independent molecular tool to understand the diversity and functional significance of the rhizosphere microbiome for sustainable agriculture. It discusses the recent significant studies in the rhizosphere environment carried out using evolving techniques like metagenomics, metatranscriptomics, and metaproteomics, their challenges, constraints infield application, and prospective solutions. The recent advances in techniques such as nanotechnology for the development of bioformulations and visualization techniques contemplating environmental safety were also discussed. The need for development of metagenomic data sets of regionally important crops, their plant microbial interactions and agricultural practices for narrowing down significant data from huge databases have been suggested. The role of taxonomical and functional diversity of soil microbiota in understanding soil suppression and part played by the microbial metabolites in the process have been analyzed and discussed in the context of 'omics' approach. 'Omics' studies have revealed important information about microbial diversity, their responses to various biotic and abiotic stimuli, and the physiology of disease suppression. This can be translated to crop sustainability and combinational approaches with advancing visualization and analysis methodologies fix the existing knowledge gap to a huge extend. With improved data processing and standardization of the methods, details of plant-microbe interactions can be successfully decoded to develop sustainable agricultural practices.

1. Introduction

Spontaneous expansion of the world population has increased the demand for larger quantities and improved quality in food production, which has placed tremendous pressure on the existing agricultural system. The primary threat to agricultural productivity is the abiotic and biotic stresses posed on the crops growing in the field due to environmental perturbations. Multiple physical, chemical, and biological interactions create a dynamic environment that decides the functional and structural differentiation of the root rhizosphere. Plant-specific polysaccharides such as root mucilage determine the macro- assemblage of soil and subsoil particles and affect penetration resistance (Haas et al., 2009) and hydraulic properties of the soil (Benard et al., 2019). Soil microorganisms form a vital component of the ecosystem by maintaining soil fertility by decomposing organic matter and nutrient recycling (Harris, 2009; Srivastava et al., 2017). The rhizosphere is a complex assemblage, where plants, microorganisms, and soil form a domain for legions of biotic and abiotic activities (Mueller et al., 2019). Microorganisms coexist in complex arrays in which interactions among members are essential for community assembly and ecosystem function (Hallam and McCutcheon, 2015).

Bacteria, the most predominant domain on earth, inhabit various layers of soil and different tissues of plants and play vital roles by interacting with the surroundings. Several culture methods were used for identifying these organisms to understand the diversity of rhizosphere (Rovira, 1991), though later it was found that only 1% of the total population is culturable (Rappé and Giovannoni, 2003). This constraint was overcome to an extent by the introduction of molecular methods

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and automation of Sanger sequencing (Sanger et al., 1977; Garrido-Cardenas et al., 2017). Even with the development of the sequencing method, the multidimensional interactions inside the rhizosphere between plant and soil, plant and microorganisms, and microorganism to microorganism remained an inaccessible area for research. Metagenomics evolved as a genomic tool by combining a set of techniques, where the microbial genome is used as the template for identifying the diversity and functional genes along with the aid of bioinformatics to derive information from the obtained data. With the success of the metagenomics approach in understanding the potential of the rhizospheric microbiome, metatranscriptomics and metaproteomics studies are initiated as a combination of molecular techniques and bioinformatics tools to reveal the complex microbial interactions in the active rhizospheric zone.

In this review, the complexity of the rhizosphere as an ecosystem and the significance of rhizospheric microorganisms, particularly the role of plant growth-promoting bacteria in sustainable agriculture, is discussed. The role of biological agents for plant growth promotion, their mechanisms, and constraints in field application in various soils are reviewed in detail. The evolution of molecular techniques from Polymerase chain reaction to 'omics' studies and the concept of engineering the rhizospheric microbiome for improving crop productivity are also discussed along with their challenges and limitations. The review covers the insights derived from the research on the topic and discusses the possibilities that the technique holds in the future for improved crop productivity. A diagrammatic representation of the research perspectives covered in this review is given in Fig. 1.

2. Rhizosphere as a complex microbial ecosystem

The rhizosphere is a dynamic region driven by complex interactions between plants and the organisms associated with the root (de Luna et al., 2005). Cardon and Whitbeck (2011) stated that the organic carbon influx, mineral nutrients, and water determines the path of energy flow and community structure of the rhizospheric area and thereby influences microbial activity, ecosystem processes, and even patterns of soil development. Another factor that determines the rhizospheric nature is the chemical transformations provided by root exudates and secondary metabolites generated from microbial degradation of organic compounds (Koo and Cho, 2006). These chemical molecules attract microbes to proliferate in the carbon-enriched zone and contribute to improved fertility of the root ecosystem (Lugtenberg and Kamilova, 2009). The plant-soil interaction determines the spatial and chemical composition of the soil (Angers and Caron, 1998; Philippot et al., 2013). Physical factors such as pH, organic matter, and nutrient availability determine the microbial community structure (Grayston et al., 2004). Kuzyakov and Domanski (2000) reviewed that around 20% to 50% of the photosynthate is being transported to the below-ground plant structures, and approximately 17% is released to the soil environment, which serves as the growth substrates for the rhizospheric organisms. In return, they benefit plants by providing nutrients, phytohormones and also by giving resilience to abiotic stress such as heat, high salt, or drought, and phytopathogens (Paterson et al., 2007). They also help in the degradation of organic matter, biogeochemical cycling, soil formation, soil fertility, and atmospheric trace gas formation (Hinsinger et al., 2009). The soil particles adhering to the root structures are affected by the root exudates, which determine the microbial composition of the area. The rhizospheric ecosystem is populated with beneficial organisms like nitrogen-fixing bacteria, phosphate solubilizing bacteria, arbuscular mycorrhizal fungi, macroinvertebrates, protozoa, and soil-borne pathogens competing for organic matter available in the soil (Mendes et al., 2015), which contributes directly to the plant growth and productivity.

Fig. 1. Workflow of 'omics' research in Rhizosphere Microbiome.

Symbionts such as mycorrhizal fungi play an essential role in the translocation of minerals (Johnson and Graham, 2013), suppression of soil-borne pathogens (Whipps, 2001), biological nitrogen fixation (Guimarães et al., 2012), and the formation of stable soil aggregates (Miller and Jastrow, 2000). They also help in the uptake of trace elements like iron by secreting siderophores (Koppisch et al., 2005), and in the absence of such organisms, plants themselves will release phytosiderophores for improving the solubility of iron in the rhizosphere (Walker and Connolly, 2008). They also provide support to plants under biotic stress through antibiosis (Haas and Défago, 2005), competition (Dawson et al., 2001), parasitism (Mela et al., 2012), and inhibiting quorum sensing of the pathogen, which affects their virulence (Lin et al., 2007). They can also modulate the systemic resistance of the plant's immune system by activating pathways like the jasmonic acid/ethylene pathway or various defense genes like MPK3 and MPK6. (Hartmann and Schikora, 2012; Zamioudis and Pieterse, 2012). Reports have shown that rhizobacteria support plants during stress conditions such as drought, flood, and high salinity (Jorquera et al., 2012; Mayak et al., 2004; Grichko and Glick, 2001). In their studies, Dodd and Pérez-Alfocea (2012) and Berg et al. (2014) compiled the mechanism of action of microbes on stress conditions by modifying water homeostasis, plant energetics, regulation of root uptake of toxic ions, and root shoot signalling, which helps in saline tolerance.

2.1. Biological agents for plant growth stimulation

The conventional agricultural practices have been significantly supported by the agrochemicals for improving soil fertility and pest control, favoring increased crop productivity. Prolonged unscientific usage of chemical fertilizers showed its deleterious effects demanding a more soil-friendly approach. With the knowledge about the influence of the microbial community on the rhizospheric soil, individual bacteria as biological agents came into field application (Zarb et al., 2005). Bioinoculant or microbial inoculants are the cost-effective and eco-friendly formulation of beneficial microorganisms which act on nutrient acquisition and disease resistance of plants and thereby improves soil fertility. They are used in the field by either introducing them in seeds or soils or as activators to the beneficial microflora in the soil (Bhattacharyya and Jha, 2012). Rather than following the 'one microbe' approach, synergistic features of an assemblage of microorganisms called 'concerted rhizosphere communities' are being used in the formulations of bioinoculants (Bakker et al., 2012; Mendes et al., 2013). These organisms are encapsulated or adhered in a supporting material called the carrier, which will deliver the organism in good condition when applied to the field (Arora et al., 2008). Carriers can be made of soil, lignocellulosic biomass, or inert materials like polyacrylamide gels or alginate (Bashan and Holguin, 1998). Microorganisms such as *Azospirillum*, *Azotobacter*, *Pseudomonas* sp., *Bacillus* sp. can be cultured in vitro and packed in materials like clay, charcoal, and compost to provide better shelf life. The major challenge in developing an effective bioinoculant is its successful interaction in the field with the native microbiome and should withstand the physical stresses like soil, water, and pH along with the negative effect from soil-borne pathogens (van Veen et al., 1997; Tyc et al., 2014). Most of the strains showing improved activity *in vitro* have failed to work on the field due to interferences from various stresses. Several rhizoremediation studies have reported reductions in the population of the introduced organisms (van Veen et al., 1997; Matos et al., 2005; Kröber et al., 2014).

Endophytes colonize in the tissues and hold intimate interaction with the host plant, which makes them a better candidate as biocontrol agents compared to rhizospheric microorganisms (Thomas and Reddy, 2013; Thomas and Sekhar, 2014). Apart from using for plant growth improvement, the rhizobacteria can also be applied for cleaning up polluted soils. The process is coined as rhizoremediation by combining phytoremediation and bioaugmentation (Kuiper et al., 2004) and was proved as an efficient method compared to physical processes like excavation and incineration. The plant root exudates attract microorganisms which can decompose the pollutant and help in bioaugmentation. Following the concept of probiotics in the human gut microflora, Fgaier and Eberl (2011) theoretically suggested the usage of siderophore-producing organisms for eradicating soil-borne pathogens. Kawasaki et al. (2012) showed that *Trifolium* and other legumes were able to grow in polycyclic aromatic hydrocarbons polluted soil with predominating microorganisms like *Verrucomicrobia* and Actinobacteria, which suggests that they have a major role in phytoremediation.

2.1.1. Plant growth-promoting rhizobacteria (PGPR) as a biological agent

Beneficial natural soil microflora colonized in the rhizospheric soil and root surfaces are termed as PGPR, and they enhance the plant growth through various mechanisms (Bhattacharyya and Jha, 2012). Widely used commercial bioinoculants are PGPR, and they are applied as whole organisms, microbial metabolites, or by seed inoculation. Various mechanism of action by these organisms improves soil fertility and limits the usage of chemical fertilizer and pesticides (Beneduzi et al., 2012). The two types of soil bacteria that show the capacity to act as PGPR are rhizospheric bacteria that are found around the root of plants and endophytic bacteria that are present inside the plant root tissues. Enhanced fertility and crop production are achieved through various mechanisms like nitrogen fixation, phosphate solubilization, and by the production of indole-3-acetic acid, siderophore, and hydrogen cyanide. They also bring about biocontrol activity by producing various secondary metabolites like antibiotics against various soil or plant pathogenic agents like bacteria, fungi, and nematodes (Bhattacharyya and Jha, 2012). The mechanism of action of PGPRs is widely reviewed in many studies and is diagrammatically represented in Fig. 2. Though several groups of organisms like *Acetobacter*, *Azotobacter*, *Flavobacterium,* and *Burkholderia* are involved in this process, the largest studied genera are *Pseudomonas*, *Enterobacter*, *Bacillus,* and *Erwinia.*

2.1.1.1. PGPR supporting plant nutrition. Plants require 16 essential elements for their growth, of which carbon, hydrogen, and oxygen can be assimilated through the atmosphere, soil, and water, whereas the remaining elements like nitrogen, phosphorus, iron, and potassium need to be supplemented through fertilizers. Though the atmospheric air consists majorly of nitrogen, bioavailable nitrogen forms in the soil are presumably low, and it is the major factor determining arid soil fertility. Biological nitrogen fixation reduces nitrogen to ammonia and is carried out by diazotrophic bacteria and archaea (Dixon and Kahn, 2004) with the help of a highly conserved enzyme called nitrogenase which catalyzes the process. *Frankia* and *Rhizobia* are the two most studied groups among the nitrogen-fixing bacteria (Saharan and Nehra, 2011). Studies state that only 20% of the nitrogen is fixed by free-living nitrogen-fixing bacteria like *Acetobacter diazotrophicus, Azoarcus*, and Cyanobacteria (Das et al., 2013). Different marker genes like *nir-K* (nitrite reductase), *nif-H* (nitrogenase reductase), *nar-G* (nitrate reductase), *nos-Z* (nitrous oxide reductase), and *amo-A* (ammonia-oxidizing gene) have been used for the genomic survey of various nitrogen cycling gene families as well as microbial families involved in the nitrogen fixation (Tu et al., 2016; Anand et al., 2017). *Klebsiella oxytica* M5I, which is the first organism to be fully genetically characterized for nitrogen fixation, was found to have eight different operons for *nif* genes coding for the enzymes supporting nitrogen fixation.

In organic phosphate solubilization, major enzymatic activity occurs from non-specific phosphatases and phytases. Phosphatases dephosphorylates phosphoester or phosphoanhydride bonds in organic matter and the phytases release phosphorus from phytic acid produced by phosphate-solubilizing bacteria (PSB) (Rodríguez et al., 2006). For solubilizing inorganic mineral phosphates, most of the PSB produce organic acid like gluconic acid (Rodríguez and Fraga, 1999), while some organisms like *Synechococcus* sp. produces phosphoenolpyruvate carboxylase for solubilization (Rodríguez et al., 2006). Inoculation of

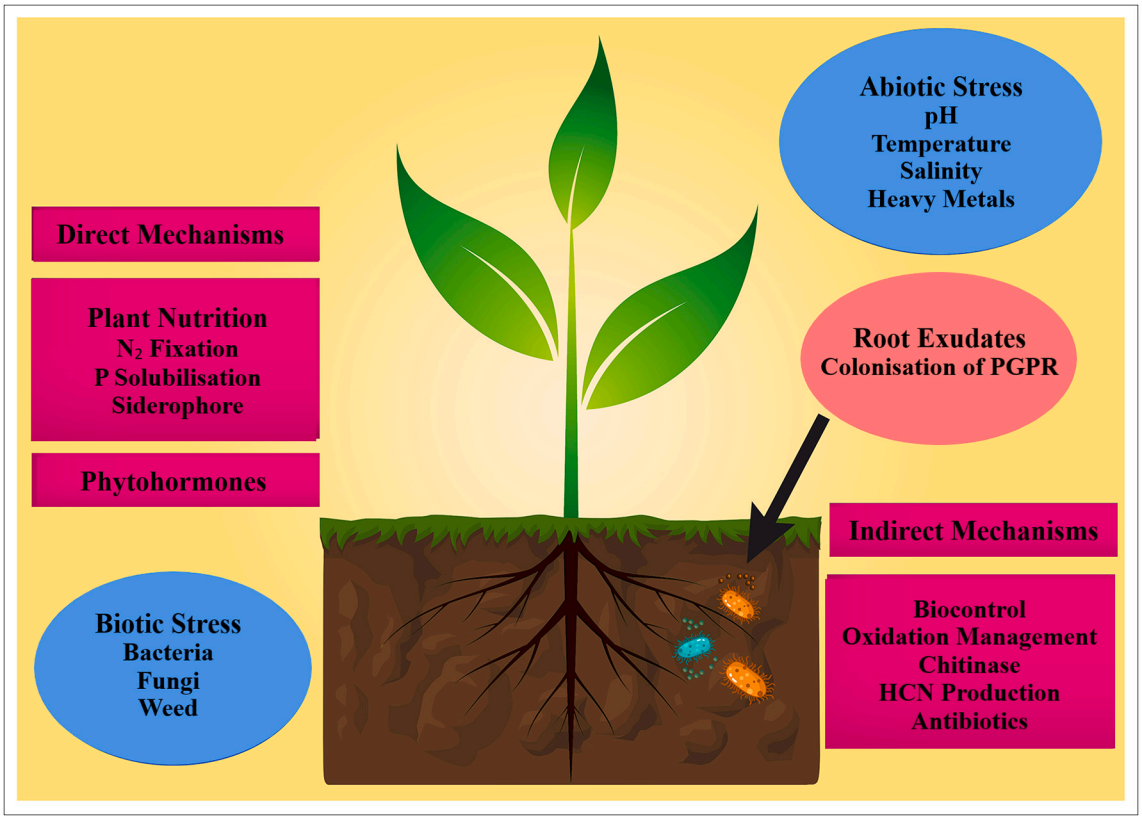

Fig. 2. Role of PGPR on plant health.

chickpeas with *Rhizobium* sp. and *Bacillus* sp. showed a 2-fold increase in yield and a 4-fold increase in phosphorus content in grains (Rudresh et al., 2005). The root rhizosphere provides an excellent source for the isolation of phosphate solubilizing organisms with improved activity. Vazquez et al. (2000) found that metabolic activities of phosphate solubilizing microbes from rhizospheric regions are higher compared to those isolated from other sources. Whereas some other studies have reported salt, temperature, and pH tolerant bacterial isolates with better phosphate solubilizing properties from rhizoplane than the rhizosphere or root-free soil. These stress-tolerant strains can, therefore, be used as model organisms to study the physiological and biochemical mechanisms involved in solubilization under stressed conditions (Johri et al., 1999; Khan et al., 2007).

Iron serves as a major cofactor for enzymes which plays essential roles in growth and metabolism. PGPR can release siderophores and can attract iron towards the rhizosphere (Payne, 1994). For example, *Pseudomonas fluorescens* and *P. aeruginosa* are reported to be producing siderophores pyochelin and pyoverdine (Haas and Défago, 2005). In soil, siderophores released from PGPRs bind with iron and are received by specific receptors which can identify bacterial ferric-siderophore complexes (Beneduzi et al., 2012). The affinity of siderophores towards iron varies, and the high-affinity siderophores were reported from *Pseudomonas* sp. Pyoverdine from *P. aeruginosa* with higher iron uptake capacity can inhibit organisms that produce less potent siderophores in iron-depleted media (Kloepper et al., 1980). Therefore, siderophores can provide an antagonistic effect on the plants by competing with pathogenic bacteria for iron uptake (Beneduzi et al., 2012).

2.1.1.2. PGPR producing phytohormones. Phytohormones like auxins, gibberellins, ethylene, cytokinins, and abscisic acid regulate soil fertility by either promoting or inhibiting plant growth under various stresses (Patten and Glick, 1996; Arshad and Frankenberger Jr, 2012). Indole-3-acetic acid (IAA) regulates organogenesis, tropic responses, and cellular responses, particularly in cell division, elongation, and differentiation (Asgher et al., 2015). Various bacteria use diverse pathways for IAA production, which elicits rapid and long-term responses varying from pathogenesis to phytostimulation (Goswami et al., 2016). *Azospirrilum brasilense* is the most studied organism that can produce 90% of IAA by L-tryptophan independent pathways, whereas the remaining 10% is produced using L-tryptophan, which are secreted as precursor molecules in the roots (Jha and Saraf, 2015). Cytokinins enhance root development and root hair formation, retarded root elongation, and improved shoot initiation (Goswami et al., 2016). They also have a huge role in leaf expansion, branching, chlorophyll production, and delayed senescence (Wong et al., 2015). *Pseudomonas*, *Azospirillum,* and *Bacillus* are known to produce cytokinins from the root rhizosphere of barley, canola, bean, and *Arabidopsis* (Alexandre et al., 1996; Persello-Cartieaux et al., 2001). Zeatin and isopentenyl adenine (IPA) are also cytokinins that have been found in culture filtrates of *Rhizobium* sp., *Bradyrhizobium,* and *Pseudomonas fluorescens* (Goswami et al., 2016). Gibberellins aids in various physiological developments in higher plants which include seed germination, flowering, fruiting, and elongation of stems (Hedden and Phillips, 2000; Iqbal et al. (2011). They can also improve seed germination and growth under stress conditions (Manjili et al., 2012). Though bacterial genera which can produce gibberellins are rare, the species reported are *Bacillus pumilus, B. licheniforms, Acetobacter diazotrophicus,* and *Herbaspirillum seropedicae* (Bastián et al., 1998; Gutiérrez-Mañero et al., 2001). Among the plant hormones, abscisic acid (ABA) is the fast-acting hormone in terms of drought stress, where ABA signaling and ABA-responsive genes cause stomatal closure, regulates the growth of root and shoot, and water content of plants for adaptation (Cutler et al., 2010; Wilkinson and Davies, 2010). They can also reverse the damages caused by salinity stress (Gómez-Cadenas et al., 2002), drought (Bano et al., 2012), and cold stress (Li et al., 2014). Studies have reported salicylic acid (SA), another phytohormone produced by PGPR, can modulate stress tolerance by activating stress-activated signal pathways

like the production of antioxidant enzymes (Ahmad et al., 2011; Zhou et al., 2014; Silva et al., 2017). et al

As the phytohormones play a significant role as a plant growth regulator, exogenous supplementation of phytohormones can improve plant growth during stress conditions. Exogenous application of SA has been found to be effective in reversing salinity-induced inhibition (Azooz et al., 2011; Fahad and Bano, 2012). Mora-Herrera and Lopez-Delgado (2007) found that the application of ABA has reduced stress by inhibiting the production of free radicals by regulating the peroxidase enzyme. Rice seedlings exposed to drought stress when treated with ABA showed improved photosynthetic capacity, stomatal regulation, and deeper root systems to assist in nutrient and water acquisition (Spollen et al., 2000; Vysotskaya et al., 2009). Therefore, as a survival mechanism for improved crop production during various stress conditions, exogenous application of various phytohormones can be considered as a good agricultural practice (Iqbal et al., 2011).

2.1.1.3. PGPR as antagonists and biocontrol agents. Along with supporting the physiological activities of plants, PGPR can indirectly support plant growth by acting as antagonists through the production of bacteriocins and antibiotics. Bacteriocins are antibacterial molecules that can kill the organism in its proximity. Colicins, the bacteriocin produced by *E. coli*, is the most represented molecules from Gram-negative organisms in this context (Beneduzi et al., 2012). Compared to other strains, bacteriocins from *Bacillus* sp. are of agricultural importance due to their broad spectrum of activity. For example, the bacteriocin Bac IH7 promotes the growth of tomato and musk melon. Thuricin 17 (Th17) is another bacteriocin studied extensively for its legume and non-legume growth promotion and seed germination during salt stress (Subramanian and Smith, 2015). Besides bacteriocins, PGPR produces antibiotics that protect from deleterious activities of plant pathogens by inhibiting the synthesis of their cell membranes and affecting their cellular structures. Pyrrolnitrin is an example of an antibiotic produced by *P. fluorescens* BL915 strain against *Rhizoctonia solani* in cotton plants (Kumudini et al., 2017). 2,4-diacetylphloroglucinol (DAPG) and phenazine are antibiotics produced by *Pseudomonas* sp., against *Pythium* sp., and *F. oxosporum*, respectively (Chin-A-Woeng et al., 2003). Antibiotics from *Bacillus* sp., such as polymyxin, circulin, and colistin are active against both Gram-positive and Gram-negative organisms and fungi (Ageitos et al., 2017). Along with antibiotic production, the property of sporulation makes *Bacillus* a right candidate with high stability for formulations in agricultural field application.

Exploring the role and functional importance of beneficial organisms to plants is important for agriculture as they can enhance the growth and development of crops by producing known secondary metabolites and also by inducing the biosynthesis of structurally unknown metabolites (van de Mortel et al., 2012). A general prediction is not possible due to the complex nature of the soil environment (Young and Crawford, 2004) and the vast diversity of soil microbial communities (Torsvik et al., 1990). Though plant species play a significant determining factor for the microbial community population (Edwards et al., 2015), some other studies stated soil geochemistry as the primary determinant (Breidenbach et al., 2016) along with the growth stage of the plants (Sun et al., 2014) as influencing factors.

Despite their beneficial action on the rhizosphere, there are constraints such as their highly specific and targeted mode of action. Meanwhile, reports states that the application of bioinoculants has caused adverse effects such as the minimized acquisition of zinc and copper, which are important for plant health (Weber et al., 2018). Though a huge data set is available, the implementation of microbial inoculants in the soil is not entirely successful due to the multiple constraints, including varying plant species and their varying environments. Although the bioformulations are found to deliver a promising future for improved crop production in laboratory conditions, the field implementation, as well as economic advantage in terms of agricultural economics, are still argumentative. PGPR dynamics with the native microflora as well as the stabilization measures to be implemented for the targeted action on plant roots are areas that still need proper research works. Irrespective of the laboratory condition, in the field, PGPR usually takes longer duration for their beneficial action as the reproduction and colonization are harder to establish considering the competition and nutrient availability. To overcome these constraints to a larger extend, region-specific microbial strains are preferred for crop improvement (Basu et al., 2021). Considering these factors, it would be appropriate to observe a 'pre-application' period prior to the application of PGPR for the analysis of soil biochemical parameters. Seasonal variations in temperature, humidity, and rainfall of the region should also be considered for the selection of bioinoculants in a specific region. Depending upon the environmental pressure of the region and crops cultivated, rotation of various complementing stress-tolerant bioinoculants over the seasons can also be attempted for better performance on productivity. There are reports of long-term fumigation of soil affecting the soil community and subsequently the action of PGPR in soil (Dangi et al., 2017). Therefore, during the pre-application period, chemical and physical treatments of soil that affects the microbial community structure should be avoided. Paterson et al. (2007) stated that the major challenge in this field is to identify the drivers and real-time shifts in the soil microbial community structure and its practical significance in response to perturbations and environmental pressures. The most important process in the PGPR application is the colonization period, during which the bacteria prepare their own niche within the host plant before initiating its effects on them (Kumar et al., 2016). Zhang et al. (2019) reported that the pre inoculation of pepper seedlings with *Bacillus* sp. improved rhizosphere richness as well as the diversity of the soil and subsequently crop productivity. This explains that the prolonged colonization period can improve the effect of PGPR on the rhizosphere. Therefore, it is highly recommended that the agricultural practices can be modified for a prolonged colonization period with minimal stresses in terms of nutrient availability, and abiotic factors can effectively improve the potential benefits of the bioinoculants.

The minimal shelf life of the PGPR bioformulations and failure of carriers in maintaining the desirable environment on the field after application affects their effectiveness in agriculture applications. To provide the optimal colony count on the field, the carrier should provide oxygen and moisture for maintaining microbial growth and as well be cheap and accessible (Rebah et al., 2002). Therefore, multiple novel formulations for successful field delivery are being suggested in various studies. Application of nanomaterials as carriers in the form of nano-fibers, nano-fertilizers, and nano-pesticides are suggested by Nayana et al. (2020) for improved plant uptake of PGPR. However, the negative impacts of the nano-materials such as silver, titanium, zinc oxide, silica, and gold engineered metal nanoparticles on the soil ecosystem should be further explored prior to application.

3. Molecular tools for rhizospheric community analysis

Traditional cultural approaches have revealed the undoubted role of the rhizospheric microbiome in plant productivity, but techniques for understanding unculturable organisms and their ecology demands a combination of multilayer technologies (Mendes et al., 2013). Advances in molecular techniques and 'omics' studies have helped ecologists to correlate the functional roles of microbiota in their ecosystem (Singh et al., 2005). Broad-scale community analyses like BIOLOG, phospholipid fatty acid analysis (PLFA), Denaturing gradient gel electrophoresis (DGGE), DNA hybridization assays have always gained attention due to the vast diversity data they generate. Combinations of these identification and characterization techniques were also tried in understanding the complex ecosphere. Microbial identification methods like BIOLOG and PLFA were coupled for studying the community composition of rhizospheric soils (Söderberg et al., 2004). Their lower resolution of the community analysis was compromised to an extent by rRNA gene

analysis techniques (Singh et al., 2004). Amplification of ribosomal genes along with fingerprinting techniques like Restriction Fragment Length Polymorphism (RFLP) analysis, Amplified rDNA Restriction Analysis (ARDRA), cloning, and sequencing provided genetic diversity of the whole rhizospheric community (Torsvik and Øvreås, 2002). Various molecular methods are developed for studying single cells to the whole community using the combination of microscopy and specific tagging techniques like reporter gene technology. Direct visualization microscopic techniques like scanning electron microscopy were used for studying bacterial colonization. For example, Fakhouri et al. (2001) noticed antagonistic interaction between biocontrol agents *Pseudomonas* sp. and *Fusarium oxysporum* on the tomato roots. But conventional SEM method involves dehydration of the sample; therefore, a modified technique called environmental scanning electron microscopy (ESEM) was developed in which the specimen chamber can be operated slightly above the saturation vapor pressure of water (Sørensen et al., 2009). In this technique, sample preparation does not require desiccation or coating with gold or palladium because the sample environment can be dynamically altered within the system for hydration and dehydration. This helps in studying materials in their natural state, though standardization of the technique is challenging as the wetness of each material varies (Muscariello et al., 2005). Other than SEM, application of epifluorescent microscopy with general or specific dyes was attempted for studying the rhizospheric community. Unspecific DNA staining with Acridine (DeLeo et al., 1997) or DAPI or green fluorescent SYBR Green II (Weinbauer et al., 1998) was used for studying specific root exudates and observation of fungal zoospores in the plant roots. This technique provided advantages like the visualization of single proteins as well as differential staining for multiple proteins on thin sections of soil samples. For studying colonization of bioinoculants, specific cell stains like strain-specific fluorescent antibodies were used with the help of confocal laser scanning microscopy (Hansen et al., 1997; Kirchhof et al., 1997). Taxonomic rRNA targeting oligonucleotide probes and strain-specific monoclonal antibodies for detecting specific cellular activities were developed, which introduced advanced technique like Fluorescent in-situ Hybridization (FISH) for studying active bacterial population, qualitative/quantitative colonization studies, and interactions between roots, plants, and microbes (Assmus et al., 1997; Kutter et al., 2006; Kreuzer et al., 2006; Watt et al., 2006). FISH is a widely used technique in rhizospheric studies because it is relatively inexpensive, and probes are commercially available (Silverman and Kool, 2007).

Monitoring of single bacteria during their early stages of growth, colonization, and interaction with the bacterial community can be achieved by creating mutants of the same with a bioluminescent or green fluorescent protein (Gage et al., 1996; Ramos et al., 2000). Another widely used approach is the application of reporter genes containing bacteria called reporter bacteria, which can be analyzed using fluorescent microscopy. They are otherwise called whole-cell biosensors or bacterial bioreporters or monitor strains. They have reporter genes that encode a product related to a specific metabolic activity or gene expression as a response to the bioavailable fraction of compounds in their surroundings. This compound should be specific to the strain under study, and the cellular response can be analyzed using fluorescent microscopy (Sørensen et al., 2009). Specific and non-specific reporter bacteria were extensively used for studying colonization, metabolic activity, stress response, and the response of specific bioinoculants to elements or compounds (Jaeger III et al., 1999; Unge et al., 1999; Sorensen et al., 2008).

Apart from visualization methods, cultivation-independent molecular methods were adopted for microbial community studies in the 1990s with the availability of PCR and other DNA-based techniques. Phylogenetic information based on the molecular marker small subunit (SSU), which is the 16S rRNA gene of prokaryotes and 18S rRNA gene of eukaryotes, revolutionized the community studies. This led to the development of different databases like GenBank, based on nucleic acids and proteins (Sorensen et al., 2008), and helped in visualization studies of mRNA using FISH probes and antibodies. For example, it was successfully used for identifying nitrate-reducing bacteria from a mixed microbial community in a biological wastewater treatment system (Mota et al., 2012). They used fluorophore coupled nucleotides in mRNA and used fluorescence assisted cell sorting (SmRFF) to detect, sort, and identify the specific bacteria without having any previous knowledge about the community.

3.1. Metagenomic DNA libraries

Staley and Konopka (1985) described 'the great plate count anomaly', where the magnitude difference between the number of cells under the microscope to the cells that form colonies on agar plate was considerably low. This explained the difficulty in representing a niche with culturable microorganisms. Carl Woese (1987) stated the 16S rRNA molecule as the evolutionary chronometer, and it revolutionized an era of 16S rRNA sequencing studies unraveling microbial diversity and ecology. Though the term 'metagenomics' was coined by Handelsman et al., 1998, it emerged in the late 1980s as a method of extraction of total DNA from a selected environment (Olsen et al., 1986; Pace et al., 1986). The polymerase chain reaction was used to amplify the 16S rRNA gene selectively, and clone libraries were created to study the diversity of the ecosystem. The sequences representing the amplified gene would be aligned and were used to identify the phylogenetic relationships between the culturable and unculturable species (Ntushelo, 2013).

In metagenomics analysis, the total genome extracted from the rhizosphere is digested, ligated, and transformed into a suitable host. The clone libraries are then screened either for functional or phylogenetic analyses. This has helped in identifying the functional attributes of soil microbiota, identification of unculturable organisms as well as for identifying and characterizing novel enzymes and metabolites from the microorganisms such as lipases, proteases, amylase, membrane proteins, antibiotics, and antibiotic resistance enzymes having industrial importance (Henne et al., 2000; Santosa, 2001; Gupta et al., 2002; Lorenz et al., 2002; Richardson et al., 2002; Yun et al., 2004; Daniel, 2005; Lee, 2005). However, the efficiency of the techniques depends on multiple factors like the representation of the microbial community present in the sample, DNA isolation method, host-vector systems, and the screening methods (Daniel, 2005). The most common contaminant of soil and rhizospheric DNA is humic acid, and during the purification process, DNA may get sheared, which will affect the library construction. Therefore, DNA extraction method with cation exchange resins followed by density gradient or centrifugation are found to be having less sheared DNA but with lower concentration (Jacobsen and Rasmussen, 1992; Lindahl et al., 1995; Courtois et al., 2001; Gabor et al., 2003). According to Daniel (2005), a small insert library which is having a high copy number would help in detecting weakly expressed unexplored genes but may have to screen large numbers to get a positive clone. Though large insert libraries are efficient in identifying the enzyme activities, pathways and for the partial genomic characterization of microorganisms, technical difficulties like low copy number and limited gene expression make it challenging.

Once the DNA library is created, there are mainly two methods for screening the library. One is using pre-existing DNA-based information, where a library that carries inserts with the known gene of interest can be identified, and the second type is based on the gene expression, which shows a phenotypic variation on screening. Oligonucleotides with known loci or gene sequences are used for constructing primers and are used for screening. This technique is mostly used for identifying community diversity in the soil. Methods based on a phenotypic variation on the screening medium are used for identifying enzymes and metabolites. When it is coupled with a promoter-less fluorescent tag, induction of genes can be visualized in the presence of the inducing substrate (substrate-induced gene-expression screening, SIGEX) (Uchiyama et al., 2005). Once the positive clones are identified, sequencing of the inserts is done using Sanger's method, shotgun sequencing, or pyrosequencing

(Leveau, 2007). Automation of Sanger sequencing, which can sequence up to 100 kilobases per run was a breakthrough in metagenomics, and it continuously evolves with the establishment of massive sequencing techniques (Garrido-Cardenas et al., 2017). Next-generation sequencing techniques like AB SOLiD, Illumina, 454 Roche, Ion Torrent, and even third-generation sequencing platform like Oxford Nanopore have been used in the various analysis of soil-based library (Qin et al., 2010; Tun et al., 2012; Mitra et al., 2016; Leggett et al., 2016) and is discussed in detail in the latter part of the review.

To improve the chance of identifying a gene of interest, enrichment of a subpopulation is also attempted. For example, the community will be separated in terms of pore size for identifying bacteriophage community from deep-sea sediments (Edwards and Rohwer, 2005; Hallam et al., 2004) or by affinity purification or differential lysis (Tringe and Rubin, 2005). Enrichment in terms of DNA level is also followed by using metagenomic DNA as a PCR template for finding the gene of interest (Nesbø et al., 2005). Stable isotope probing is another technique for identifying an organism that uses a specific substrate by adding C^{13} or N^{15} as isotopes in the subpopulation. The unlabeled DNA will be separated from labeled DNA with the help of density gradient centrifugation and will be used as the template for cloning (Friedrich, 2006).

Lorenz and Eck (2005) have reviewed that metagenomics "provides industry with an unprecedented chance to bring biomolecules into industrial application". For example, it helped in identifying multiple novel enzymes like esterases from the soil and drinking water (Elend et al., 2007), halotolerant thermostable tannase from cotton field soil (Yao et al., 2011), an alkaline serine protease from goat surface skin (Pushpam et al., 2011), xylanase from China Holstein cow rumen (Cheng et al., 2012), novel bioactive molecules and pathways such as biotin from horse excrement (Entcheva et al., 2001), a novel protease inhibitor gene from uncultured marine microorganisms (Jiang et al., 2011), and multiple acid resistance genes from planktonic and rhizospheric communities from Tinto River (Guazzaroni et al., 2013). Metagenomic libraries generated from forest soil were screened for novel enzyme activities by Arjun et al. (2018). They found a novel L asparaginase enzyme which showed enhanced antitumor activity on human leukemia cell lines with better substrate affinity than the commercially available enzyme of bacterial origin. Tyson et al. (2004) identified the members of an acid mine drainage using metagenomics, among which the majority were unculturable. Similarly, Whitaker and Banfield, 2006found single nucleotide polymorphisms in the biofilm community, which shed light on the population dynamics and evolution of microbial communities in extreme conditions. Therefore, though the technique has revealed only the 'tip of the iceberg' of the bacterial community dynamics from different environments, including the rhizosphere, it has gained access to the information that helps to understand the biology and evolution of microorganisms irrespective of the culturable status (Leveau, 2007).

3.1.1. *Metagenomic analysis of plant growth-promoting bacteria*

Metagenomics elucidates huge information about unculturable bacteria, particularly organisms with plant growth promotion traits. It contributes to improving crop productivity by discovering novel genes and metabolites or by identifying novel unculturable microorganisms with plant growth-promoting potential (Leveau, 2007). Mendes et al. (2014) investigated the taxonomic and functional diversities of microorganisms in the bulk soil and rhizosphere of soybean plants to study the rhizosphere community assembly processes. They found that the assembly of the microbial community in the rhizosphere is based on niche-based processes where the major influencing factors are the plant and environmental factors affecting the soil. Direct amplification of bacterial DNA from different plant tissue samples and application of modern bioinformatics tools helped in analyzing the bacterial community composition and its phylogenetic structure (Manter et al., 2010). Metagenomic analysis of rice roots revealed that the major bacterial community belongs to the members of γ-proteobacteria, specifically enterobacter-related endophytes, and rarely reported endophyte species of δ- and ε-proteobacteria (Sun et al., 2008). Similarly, Tsurumaru et al. (2015) proved that the dominant bacterial community associated with the taproot of sugar beet (*Beta vulgari* L.) is Alphaproteobacteria, followed by the Actinobacteria and the Betaproteobacteria. Another metagenomic study of the sorghum root and stem microbiome revealed that though the two tissues differ in bacterial composition, both were dominated by agriculturally important genera such as *Microbacterium*, *Agrobacterium*, *Sphingobacterium*, *Herbaspirillum*, *Erwinia*, *Pseudomonas,* and *Stenotrophomonas* (Maropola et al., 2015).

Functional screening of the rhizospheric community under biotic and abiotic stresses has proven beneficial in identifying novel bacterial metabolites and pathways (Sasse et al., 2018). Tsurumaru et al. (2015) analyzed the microbial community associated with the taproot of sugar beet (*Beta vulgaris*) for identifying genes showing plant growth-promoting traits like nitrogen fixation, phosphate solubilization, siderophore production, and 1-aminocyclopropane-1-carboxylic acid (ACC) production. The study revealed that the frequently detected gene was for Beta 1,3-glucanase production for plant disease suppression along with genes for phosphate solubilization and ACC deaminase production. The genes or IAA and nitrogen fixation were found to be mostly absent, which shows that they do not contribute to sugar beet growth. Such functional analysis of the rhizospheric microbiome will aid in providing knowledge about plant growth promoters which will help in crop management practices. Apart from rhizospheric bacteria, endophytic bacteria are also known for their production of antibacterial and antifungal compounds, and their abundance and possibility of metabolic signaling suggests chances of the multidimensional network of interaction and symbiosis in the plant endosphere. This was confirmed by the metagenomic analysis of the endophytic rice microbiome, which revealed three quorum-sensing systems – the autoinducer-2 system, the diffusible signal factor system, and the N-acyl homoserine lactone (AHL) system (Sessitsch et al., 2012; Miliute et al., 2015).

β- glucosidase plays a prominent role in the process of fertilization by contributing to the carbon cycle and organic matter depletion. Biver et al. (2014) identified new β- glucosidase with a broad spectrum of activity and thermostability by screening metagenomic libraries constructed with DNA isolated from the topsoil of winter wheat (*Triticum aestivum*) field. Similarly, a function-based screening approach was attempted by Tan et al. (2014) for identifying phytases from the soil cultivated with winter wheat and identified two novel phytases, among which one is an unusual histidine acid phosphatase family phytase. In contrast, the second phytase belongs to a new type, which is encoded by multiple open reading frames (ORFs) and is different from all phytases known to date.

Several techniques were employed to study the antibiotic function of rhizosphere microbial communities against plant pathogens. Studies were conducted using pathogens (indicator organisms) like *Erwinia* sp., *Xanthomonas* sp., *Fusarium* sp., and *Rhizoctonia* sp. to understand the antibiotic activity of the rhizosphere microbial communities through metagenomics library-based functional screening. (Chin-A-Woeng et al., 1998; Rangarajan et al., 2003; Emmert et al., 2004; Kim et al., 2006). While in some PGPR studies, HPLC and colorimetric assays were used for screening IAA and nitrogen-fixing enzymes clones from the library, and immunoaffinity chromatography for screening cytokines and their metabolites (Timmusk et al., 1999; Radwan et al., 2002; Leveau and Lindow, 2005; Tejera et al., 2005). Several chitinases producing clones for biocontrol of plant pathogens were identified by observing clear zones on solid media (Leveau and Preston, 2008). Another approach for plant pathogen suppression is the usage of biosurfactants. Sachdev and Cameotra (2013) have suggested the possibility of identification of biosurfactant-producing microorganisms from heavy metal contaminated areas of soil through metagenomics functional screening. Some of the products/genes derived/identified through the functional screening of metagenomic libraries generated from rhizospheric and suppressive disease soils are listed in Table 1.

Table 1
List of products/ genes derived through functional metagenomics.

Target gene/ product	Source	Host/Vector	Size of insert	Assay	Uses	Reference
Esterase, Est D2	Rhizospheric soil sample from South Korea	*E. coli*/Fosmid	1630 bp	Growth assay	Food industry, Detergent, Pharmaceuticals	Lee et al., 2010
Nickel resistance	Rhizospheric soil from acid mine drainage	*E. coli*/ Plasmid	2.5 kb	Growth assay	Genetic engineering	Mirete et al., 2007
Salt resistance	Modern salinity rhizosphere from Spain	*E. coli* / Plasmid	420–2341 bp	Growth assay	Genetic engineering	Mirete et al., 2015
Fungal antagonism	Forest rhizospheric soil	*E. coli* / Fosmid	35 kb	Growth inhibition assay	Biocontrol	Chung et al., 2008
ACC deaminase	Rhizospheric soil samples from Maize	*E. coli* / Plasmid		Competition assay	Bioprospecting	Jin et al., 2015
CelRH5, a novel endo-β-1,4-glucanase	Rhizospheric sample from non-fertilised grassland	*E. coli* / Fosmid	1080 bp	Substrate assay	Food, paper pulp and biofuel industry	Wierzbicka-Wos et al., 2019
Chitinase	Phytopathogen suppressive soil	*E. coli* /Fosmid	240 bp	Growth assay	Biocontrol	Hjort et al., 2009
Na^+ (Li^+/H^+ antiporter)	Sugar beet soil	*E. coli* / Plasmid	1185 bp	Heterologous complementation		Majernik et al., 2001
Utahmycins A and B	Soil sample from Utah	*Streptomyces albus*/ Cosmid			Antibacterial, antimalarial, anticancer	Bauer et al., 2010
Protease	Banana Rhizosphere	*E. coli* / Fosmid	2010 bp-2881 bp	Toxicity assay	Biocontrol	Chen et al., 2018

Pesticide abatement is a public concern when it comes to environmental safety. Pyrethroids and pyrethrins are typical insecticides used in the agricultural field and removing them from the field demands enzyme pre-treatment by pyrethroid-hydrolyzing esterase. A novel pyrethroid-hydrolyzing esterase gene was successfully cloned from the soil, which showed broader substrate specificities and higher activity (Li et al., 2008). This finding proved metagenomics as a tool for identifying ideal candidates for rhizoremediation. Though the rhizosphere is stated as the most active site, seeds also form essential habitat for microbes (Nelson, 2004). Studies conducted by Truyens et al. (2015) revealed that microbes in the seed spermosphere and endosphere could promote seed germination and enhance plant growth during both abiotic and biotic stress. In tropical regions, epiphytic fungi like *Penicillium* sp. and *Fusarium* sp. were found to improve seed germination (Tamura et al., 2008). Apart from identifying novel catalysts and active molecules, metagenomic analysis is done for identifying profiles of antibiotic resistance genes (ARGs) and their co-occurrence patterns in 10 typical environmental samples by Li et al. (2015). They have found resistance genes of aminoglycoside, bacitracin, β-lactam, chloramphenicol, macrolide-lincosamide-streptogramin, quinolone, sulphonamide, and tetracycline, which are widely used for treatment in the medical and veterinary field.

Plant-microbe interactions in biodiverse and extreme environments have been proved beneficial in identifying their adaptive strategies against abiotic stress factors (Verma et al., 2017). Ecosystems like the Amazon rain forest, which are best known as a biodiversity hotspot, harbors unique microbial diversity and, therefore, unique plant-microbe interaction. Fonseca et al. (2018) characterize the bulk and rhizospheric soil samples from the region using the shotgun sequencing method for studying its functional and taxonomic characterization. They found diverse fungi and bacterial in the rhizosphere, whereas the archaeal population was found to be enriched in bulk soil samples. The rhizospheric samples were over-represented by *Nitrobacter hamburgensis* and *Rhodopseudomonas palustris*, which helps in nitrogen cycling, and also by *Oligotropha carboxidovorans* for carbon cycling, which shows the functional importance of these two pathways in maintaining a healthy soil ecosystem. Hu et al. (2020) studied the effects of geological factors on the diversity and composition of soil bacterial communities in the Tibetan plateau. They found that inclusion of the geological factors has explained 67.9% and 35.9% variations in plant and bacterial community variations, respectively, and thereby helped in improved understanding up to 27.6% in variations in the functioning of the soil ecosystem. Other than the environmental stresses, the productivity of crops reduces due to replanting disease, where productivity drop occurs in fields when the same or closely related fruit or nut crops are replanted. Though it happens worldwide, the reason behind the process is not apparent with the existing studies. For the first time, using shotgun metagenomics, Radl et al. (2019) studied the taxonomic and functional variations happening in the soil rhizosphere due to apple replanting. When compared with the control sample, they found that Actinobacteria, which plays a significant role in the decomposition of soil organic matters, were significantly reduced in the rhizosphere affected by replant disease. They also found that in affected soil, genes associated with stress sensing are up-regulated, whereas genes linked to nutrient uptake and sensing were up-regulated in control soil. Genes responsible for the degradation of aromatic compounds were found to be less abundant in the affected soil, and that explained the accumulation of phenolic compounds in the affected rhizospheric soil.

3.2. Metaproteomics

Metagenomics laid the foundations for a series of approaches investigating RNA (metatranscriptomics), proteins (metaproteomics), and metabolites (meta-metabolomics), all providing novel insights into the metabolic capacity of a given microbiota (Schlaeppi and Bulgarelli, 2015). Metaproteomics, otherwise called community proteomics, is the study of all the protein samples recovered from various environmental sources. It uses a wide variety of biochemical techniques like 2D polyacrylamide gel electrophoresis (PAGE), 2D–difference gel electrophoresis, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS), liquid chromatography, and stable isotope probing to identify and characterize proteins from environmental sources (Bestel-Corre et al., 2004; VerBerkmoes et al., 2009; Cheng et al., 2010). The experimental strategy of the technique can be broadly outlined as a protein profile generation from the total protein extracted from the environment using 2D gel electrophoresis for either linking it with the genetic structure of the community or for functional community analysis by protein digestion and mass spectrometry (Maron et al., 2007). There are two approaches followed in metaproteomic studies, which are the top-down approach and the bottom-up approach. The bottom-up approach demands protein digestion followed by liquid chromatography–mass spectrometry (LC-MS) analysis for protein identification, whereas top-down approach can detect intact separate protein using LC-MS without protein digestion. Most of the soil rhizosphere studies follow the bottom-up approach due to its sensitivity, technical feasibility, and the possibility of broader applications. However, it has a

disadvantage that the digested peptides or several peptides may not annotate to a single individual protein and leads to interrupted information (Abiraami et al., 2020). In the top-down approach, since there is no digestion step, increased coverage of protein in primary structure can be procured, which may give additional information of alternative splicing and post-translational modification (Kelleher, 2004). This method is used for identifying PGPR for improved yield and disease-resistant phenotypes (Vorholt et al., 2017). However, when used with MS, the peptides are easily fragmented, fractionated, and ionized, which leads to reduced data quality (Santos et al., 2016). Moreover, if an abundant protein species is present, it will get refragmented multiple times and reduces the chances of identifying novel proteins (Durbin et al., 2016).

Metaproteomic studies are used for studying soil fertility, plant-microbe interaction, nutrient cycling, and bioremediation. The first metaproteogenomic study of plant-associated microorganisms was to analyze the bacterial communities in the phyllosphere of *Arabidopsis*, soybean and clover plants. It showed remarkable consistency with the dominant bacterial taxa and the proteins identified in the different plants (Delmotte et al., 2009). Wang et al. (2011) characterised the metaproteome of rhizospheric soil of crops like rice, sugarcane, tobacco, *Pseudostellariae heterophylla* and *Rehmanniae* sp. using 2D gel electrophoresis and identified 122 proteins using MALDI-TOF/TOF-MS. They found that almost one-third of the total protein, which could play a significant role in the ecosystem, could not identify using the technique. Knief et al. (2012) compared the bacterial composition of phyllosphere and rhizosphere of rice cultivar using metaproteomic study and found that microbial community based on their functionality differs in the two zones. They observed that protein for methanotrophy and methanogenesis were found in the rhizosphere, whereas methanol-based methylotrophy related genus *Methylobacterium* dominated within the protein repertoire of the phyllosphere microbiota. Moreover, proteins related to stress response and transport process were more prominent in phyllosphere than rhizosphere. Bona et al. (2019) characterized the bacterial community of *Vitis vinifera* using metaproteomics approach to elucidate the taxonomical and functional aspects of bacterial diversity in an Italian vineyard. The total protein was analyzed using MS-MS and identified using Mascot (Matrix Science Inc., Boston, MA, USA) and BLAST2GO. They found that the genera *Streptomyces*, *Bradyrhizobium* and *Pseudomonas* are the most actively participating community in protein expression and were majorly involved in protein and nitrogen metabolism.

Shifts in microbial community and composition are strong indicators of biological activity, quality, and crop productivity of terrestrial agroecosystems (Edmeades, 2003). The mode of action of various stimulants like biological and chemical fertilizers and their impact on microbial shift were elucidated in detail using metaproteomic studies. Mattarozzi et al. (2020) combined mass spectrometry-based soil metaproteomics with multiple enzymatic assays to study the effect of two biostimulants compositions, B1 and B2, on maize plants. They hypothesised that biostimulants act by activating specific metabolic activities in plant growth-promoting bacteria without changing the taxonomic profile of the plant roots. Using liquid chromatography-high resolution mass spectrometry for protein identification, they found that the rhizosphere of B1 treated samples were found to have enhanced enzymatic phosphorus and glucose activities, whereas B2 showed carbohydrate, organic substance, and phosphorus metabolism. The biostimulants enhanced the action of bacteria *Bradyrhizobium japonicum*, *Hyalangium minutum*, *Stenotrophomonas rhizophila*, *Variovorax paradoxus* and *Paenibacillus macerans* without affecting the taxonomic profile of the maize rhizosphere.

Similarly, Chen et al., 2019used a metaproteomic approach to study the impact of different ratio of nitrogen fertilisers at various stage of rice plant development without affecting the constant total nitrogen supply, soil protein expression and microbial community structure during the plant growth. The study followed two nitrogen application regimes. The first one was a traditional nitrogen application with three sessions including 60, 30 and 10% during pre-transplanting, tillering and panicle initiation stages, respectively. The second one was the efficient nitrogen application consisted of four sessions, including 30, 30, 30, and 10%, and the fourth session was extended to the anthesis stage. The study adopted a combined approach of soil metaproteomics and Terminal Restriction Fragment Length Polymorphism (T-RFLP) approach to understand the biological processes in the rhizosphere. They found that soil enzymes and nitrogen utilization efficiency, improved crop yield during tradition nitrogen application. T-RFLP and qPCR showed a significant reduction in denitrifying bacteria during efficient nitrogen application but improved the nitrifying bacteria. The study showed the influence of microbial-derived proteins linked to nitrogen fixation, carbohydrate metabolism, energy and defence were upregulated on the usage of efficient nitrogen application, suggesting the change of pattern in fertilizer application for improved crop productivity. The metaproteomic study can be used to evaluate agricultural practices like ratooning of sugarcane. Lin et al. (2013) did a comparative proteomic analysis of ratoon cane and plant cane and suggested that though theoretically, the yield of ratoon cane is low, plant proteins related to carbohydrate and amino acid metabolism and stress response were upregulated in ratoon cane soil compared to plant cane and hence improves soil quality. They reported that 24.77% of soil proteins are of bacterial origin, and the upregulated microbial proteins play a significant role in membrane transport and signal transduction. Bao et al. (2014) combined metaproteomics and spatial resolution of catalyzed reporter deposition-FISH (CARD-FISH) to link nitrogen fixation and methane oxidation to family *Methylocystaceae*, which inhabit in vascular bundles and epidermal cells of rice root. Metaproteomic studies revealed the presence of nitrogenase complex-containing nitrogenase reductase (*nif*H) and the alpha subunit (*nif*D) and a beta subunit (*nif*K) of dinitrogenase along with methane monooxygenase proteins and CARD-FISH showed localization of Methylocystaceae members in the vascular bundles and epidermal cells of rice roots.

The metaproteomic study can help in identify the stress responses induced by the bacterial community by gene expression and corresponding proteins under various abiotic stresses (Fatima and Arora, 2019). For example, serpentine soil is known for its high concentration of heavy metals like cobalt, copper and nickel and is considered an extreme condition (Baker et al., 2010). The plants growing in such conditions have evolved into either metallophytes or hyperaccumulators to achieve metal tolerance (Baker et al., 2000). Hyperaccumulators are used for phytoremediation or phytomining for commercial production of metal product or catalyst (Chaney et al., 2007). Mattarozzi et al. (2017) used a metaproteomic approach applying liquid chromatography-high resolution mass spectrometry (LC-HRMS) analysis to study the dominant bacterial metabolic processes in the metal tolerant plant *Biscutella laevigata* and metal hyperaccumulator plant *Noccaea caerulescens* inhabiting serpentine soils. They generated a bacterial protein database using 16S rRNA profiling and used for protein identification using LC-MS data. The bacterial species represented in the rhizosphere of *B. laevigata* and *N. caerulescens* were *Phyllobacterium*, *Microbacterium oxidans*, *Pseudomonas oryzihabitans*, *Stenotrophomonas rhizophila* and *Bacillus methylotrophicus*. The major proteins from the rhizosphere were found to be involved in response to a stimulus like histidine kinase and a response regulator histidine kinase TcrY along with membrane transport and signal transduction genes. Nutrient stress is another abiotic stress for soil microorganisms, and there are various survival mechanisms followed by bacterial cells under nutrient limitation. In contrast, Gram-positive organisms form spores as a surviving mechanism, whereas Gram-negative bacteria achieve a viable but non-culturable state (VBNC) for overcoming limited nutrient or water availability (Atlas, 1998). However, the mechanism of transition of these organisms to a culturable state is least studied. Hence, Giagnoni et al., 2018studied the effects of gluconate or water in transforming soil-borne *Cupriavidus metallidurans* strain CH34 from the VBNC state to culturable by inoculating into artificial soils followed by bacterial

metaproteomic analysis. They observed that when cells changed from VBNC state to culturable, the proteins linked to cell shape and protein synthesis were rapidly downregulated, whereas stress-related proteins were upregulated. When turned back to culturable organisms by providing gluconate, multiple proteomic profiles like protein and energy metabolic pathways were upregulated. When water was used for the transition to culturability, only six proteins were upregulated, in which one was to degrade the sigma factors involved in bacterial resistance during nutrient starvation. This study can serve as a model for studying metabolic changes during the transition of bacteria from culturability to the VBNC state by releasing organic compounds and can use to "engineer" the bacterial community for improved fertility (Fierer et al., 2010a, 2010b).

3.3. Metatranscriptomics

The term defines the technique to identify functional genes that are transcriptionally active as mRNAs during the sampling, which will help to understand the metabolic process of the microbial community (Simon and Daniel, 2011; de Menezes et al., 2012; White III et al., 2017a, 2017b). The advantage of this technique is that profiling of microbial community based on relative abundance on multiple samples across kingdoms can be achieved without the biases of PCR (Turner et al., 2013a, 2013b). Though metagenomic studies help identify the potential pathways present in the ecosystem, metatranscriptomic studies are a better tool in understanding 'instantaneous' regulatory responses in 'live' microbial systems (Moran, 2009). Though studies prove that root exudates play a major role in assembling microbial community in the rhizosphere, a general picture of communication between plant development, root exudation and microbiome assemblage is still unclear. Therefore Chaparro et al., 2014studied the dynamics of rhizospheric microflora during different stages of development from seedling to plant using metatranscriptomics. They found that plant selects different microbes at different stages of development, specifically for their functions. They were able to identify around 81 unique transcripts during different stages of growth, including streptomycin for disease suppression. This technique has also helped identify the active microbial population in rhizosphere soil by correlating metabolic functions to the bacterial community Kim et al. (2014a, 2014b). To study the mechanism and factors affecting the modulation of root exudation by rhizosphere microbiome, Korenblum et al. (2020) studied the tomato rhizosphere microbiome using a split root hydroponic system where each root side is treated with a different microbiome. They found a mechanism called systemically induced root exudation of metabolites (SIREM), where the rhizospheric microbiome determines the chemical composition of root exudate using a systemic root to root signalling mechanism. For example, secretion of acyl sugars as secondary metabolites is triggered by the local colonization of genus *Bacillus* sp. Similarly, when there is a change in root rhizospheric community, there were transcriptome and metabolomic changes observed in leaf and root microbiome, which states that the rhizospheric microbiome drives the chemical and molecular changes through SIREM and regulates soil conditioning.

Understanding microbial gene expression under different environmental conditions will help in optimizing the plant-microbe interaction and improve crop productivity in plants, particularly cereals like maize (*Zea mays* L.). They require the application of a massive amount of nitrogenous fertilizers resulting in an increase in expense for agriculture. The microbial expression profile of two maize line differing in their nitrogen use efficiency (NUE) was compared through mRNA sequencing and found that microbial processes on plants with high and low NUE were varying particularly in nitrogen cycling (Rosenblueth et al., 2018; Pathan et al., 2018). The rhizosphere of high NUE favoured ammonification and nitrification processes, whereas in low NUE maize favoured genes encoding for the denitrifying process for preferring longer residence time for nitrogen in the rhizosphere. To improve the nitrogen utilization efficiency of the plants, different approaches like using single bacteria or a mixture of bacteria as PGPR were applied in the soil. The effect of a single and a mixed inoculum of PGPRs like *Rhizobium phaseoli*, *SinorhizobiumamericanumAzospirillum brasilense*, *Bacillus amyloliquefaciens* and *Methylobacterium extorquens* on nitrogen fixation in one moth old maize plants was studied (Gómez-Godínez et al., 2019). The focus of their study was the metatranscriptomic profiling of *R. phaseoli* as a single species and as in mixed inoculum. They found that plants inoculated with mixed inoculum have more biomass than plants inoculated with single species or non-inoculated plants. The RNA transcripts analysis proved that in the mixed inoculum, there was up-regulation of multiple genes in *R. phaseoli* coded for ATP binding protein, sugar ABC transporter permease, and MFS transporter. These genes might be playing a significant role in coordinating functional interactions between the microorganisms. They also found variations in gene regulation in *Rhizobium* sp., when grown on liquid media and surfaces. When in the liquid medium, transcriptome analysis of the organism showed a downregulation of genes coding for motility encoding flagellar synthesis because chemotaxis and mobility are required only when attached to a surface (Balsanelli et al., 2016). The remarkable observation derived from the study was the presence of many hypothetical genes which are found to be upregulated during the microbial interactions, showing the lack of knowledge in genes involved in microbial cross-talking during metabolic processes.

Singh et al. (2018) have used the metatranscriptomics approach for studying rhizoremediation capacity by the rhizomicrobiome of crop plants to remove aromatic compounds and xenobiotics from the soil. They found that the bacterial community of the soil contaminated with aromatic amine compounds, carbazoles, naphthalene, phenols, biphenyls, and xenobiotics is dominated by proteobacteria, firmicutes, actinobacteria, and cyanobacteria and their transcripts required for the metabolism of these contaminants. Transcriptomic studies have proved the involvement of multiple genes in the biodegradation of hydrocarbon, suggesting that interspecies interactions facilitate the process (Kotoky et al., 2018). The transcriptome of phytoremediating crop *Salix* sp. grown in petroleum hydrocarbon contaminated and non-contaminated soil was analyzed. They analyzed the resultant contigs after assembly and were found to be majorly represented by fungal gene expression from Ascomycota and Basidiomycota along with bacterial gene expression for biofilm and stress reduction, suggesting a tripartite mutualistic interaction between plant, fungi, and bacteria during the process (Gonzalez et al., 2018). Modes of action of biocontrol agents like *Pseudomonas* sp., on molecular level after colonization on roots, were analyzed using a transcriptomic approach. Kandaswamy et al., 2019performed root transcriptome analysis using microarray to understand the molecular changes on rice plant roots followed by PGPR colonization. They found 61 transcripts coding for cell wall modification, secondary metabolite production and defence response along with the salicylic acid (SA) responsive pathogenesis-related protein. On confirmation by HPLC and real-time PCR for chemical profiling and gene expression, they found that the beneficial bacteria provides immunity for the rice plants by stimulating the plant defence responses ('priming') like modifying SA production and protects itself from these responses by altering the rhizospheric chemical composition.

3.4. Challenges and Limitation of the 'omics' approach

Creating a representative sample from a complex ecosystem like rhizosphere or soil is the first and foremost challenge faced in microbial community analysis. Most of the studies trying to review 'complete' microbial community often focus on explaining the entire functions with the prominent group. The abundant microbial population are the most exploited group and, to understand the least abundant species, enrichment protocols are practised such as, size selection which will create biases in the study. Most of the microbial community experiments consider multiple perspectives like metagenome, metatranscriptome, metaproteome, metabolome and multiple measurable environmental

parameters for gaining a complete analysis of the ecosystem. However, the disadvantage of this technique is that correlating patterns of transcriptional variation with a single parameter is not possible in this case, as multiple parameters are involved in the study. It is desirable to consider a smaller number of parameters while looking for transcriptional differences so that experimental parameters can be monitored and hypothesized by the observation (Carvalhais et al., 2013). In the metagenomic analysis, clone library generation and functional screening of the clone using the surrogate host are the major steps. However, according to Gonzalez et al. (2018), gene expression on the surrogate host poses some challenges as the functional screening of host bacteria with highly diverse physiology could cause unclear data generation. Other major limiting factors for conducting metagenomic analysis are its intense labour and economics. Quality DNA extraction to a single run of the procured sequence demands commercial kits, and dense instrumentation are limitations to its application.

The bottleneck of the metaproteomic technique lies in extraction method, sample preparation, instrumentation, and data analysis among which protein extraction and data analysis influence the results the most (Starke et al., 2019). Though it has the advantage of identifying gene functions, metabolic activities, protein abundance and protein-protein interaction over DNA/RNA based techniques like metagenomics and metatranscriptomics, they have limitation in identifying intracellular proteins and may have interruption from proteins of other than the bacterial origin (Keller and Hettich, 2009). The identification of extracellular enzymes in the soil, such as phosphatases, cellulases is limited using the technique as they are firmly adhered to soil particles and humic acids and are challenging. Therefore, the profile generated from the sample may not guarantee complete coverage of the metabolites (Starke et al., 2019). Metaproteomic studies identify the functional dimensions of the microbial community identified through metagenomic analysis. However, it requires information about the evolution of the microbial community, which demands new technologies. Technologies for protein extraction and separation are evolving compared to nucleic acid research, which affects sample quality and integrity. The technique has improved tremendously over the years and has the potential to deliver better information by integrating solutions for pitfalls like extraction technique optimization, improved protein databases for analysis and understanding of lesser abundant proteins in soil rhizosphere, which will help in describing soil ecology and it is functioning more effectively.

Transcriptomic analysis requires quality mRNA, which should be stored in lower temperature like -80 °C or an RNA protection solution due to its shorter half-life. It is difficult to differentiate it from other RNA types like tRNA, rRNA and miRNA present in the soil (Simon and Daniel, 2011). In bacteria and archaea, transcription and translation take place simultaneously which causes a lower abundance of transcripts. This can be overcome by an enrichment method or by amplification of the RNA by commercially available kits. As RNA is easily degradable, reverse transcription is carried out for analysis. During the construction of cDNA from the mRNA, there are chances of errors as there are templates with high levels of homology (Cocquet et al., 2006). In most of the gene expression studies of rhizospheric soil, microarrays and real-time quantitative reverse transcription PCR is used. These methods demand previous information of the sequences to design specific primers. Even with the usage of a specific primer, complete coverage of the gene cannot be guaranteed. Though there are lots of genome databases available, they contain only a fraction of the full diversity of genes present in natural environments, which may limit the study (Cocquet et al., 2006). Similarly, when comparing data between metagenomic studies and metatranscriptome studies, it was observed that the genes involved in RNA, protein metabolism and carbohydrate metabolism are over-represented in transcriptomics (Urich et al., 2008). It could be because of the prevalence of transcripts of essential cell functions compared to other processes. Moreover, differences in transcription kinetics of the same gene in different population and the poorer correlation between RNA levels and proportional protein synthesis have generated significant setbacks for the studies (Maron et al., 2007).

Genomic and proteomic level interactions between plant species and beneficial bacteria are well exploited in laboratory conditions, whereas the 'omics' analysis of PGPR, post-application in soil, needs more clarity. Soil site suitability of winter crops was studied by Mandal et al. (2020) using geostatistical and visualization methods. Soil characteristics such as texture, pH, Nitrogen, Phosphorus, Potassium, electrical conductivity, etc., were studied based on grids and analyzed statistically using principal component analysis, inverse distance weighting (IDW) interpolation and reclassification methods to predict the appropriate irrigation and crop management practices. The study suggested the development of combinative methodology by bringing together the tools for PGPR activity visualization and the geostatistical formulations for the soil classification.

Mechanisms of stabilization and survival at the plant level over the climatic period should be exploited to implement better agricultural practices. An instrument-based method for visualization such as Portable X-ray fluorescence (PXRF) spectrometry for enhanced soil profile visualization was suggested by Sun et al. (2020), but they are expensive and possess practical difficulties in field applications. Menger et al. (2020) reported a fluorescent dye-based method to evaluate the coverage of pesticide application in the soil. For the visual identification, a fluorescent dye that can be visualized with a lightbox, is sprayed on samplers attached to leaves and is analysed using algorithms to understand the coverage of pesticides. Similarly, fluorescent dye-based methods can be utilized for analysing the PGPR colonization in situ and their sustainability over climatic stress. For example, *Pseudomonas* sp. is a widely used plant growth promoting bacteria and is known for their production of fluorescent pigments such as pyoverdine. Indicator strips for the detection of such signatory molecules can be utilized for visualizing their establishment and sustainability on field. Nanomaterial based visualization techniques can be developed based on the formulations used and software for analysis can be developed for the real time stratification of the soil based on PGPR presence. The advantage of using nanotechnology-based indicator strips is that they are eco-friendly and can be added along with nanomaterial-based formulations. Apart from identifying PGPR activity directly, there are small set of genes expressed on the plant roots in response to beneficial rhizobacterial activity. Valette et al., 2020identified genes involved in diterpenoid phytoalexin synthesis and plant defence, commonly expressed in rice cultivars during plant-bacterial interaction. Microarray strips or indicator strips of such molecules can also be considered for detecting the efficacy and sustainability of PGPR in situ. Characterizing various steps of root colonization and sustainability along with the protein profiling of PGPR using 'omics' approaches at various stresses can shed more light to the molecular mechanisms involved in the process.

4. Characterization of rhizospheric microbiome by next-generation sequencing (NGS)

Next-generation sequencing is the most cost-efficient and time-saving tool used for the sequencing of single genomes or metagenomes. Before the invention of NGS, the studies were mainly aimed at analysing gene segments that are of standard size, which have repetitive sequences and genes that are of medical importance. NGS platforms perform high throughput sequencing, during which millions of fragments of DNA from a single sample are sequenced in unison and are characterised by two parameters which are mean read length and throughput (White III et al., 2017a, 2017b). NGS technology can undertake sequencing of both massive and single molecule by delivering long and short reads. Short read sequences are considered highly accurate with a base pair read lengths of 100–300 bp and are later assembled to draft genomes. Though accurate, when assembled, there are difficulties in arranging repetitive regions or genome arrangements like insertions or deletions. On the other hand, long read sequences range from

10 to 50 kb read lengths with higher error rates than short reads. For example, for phylogenetic studies, long reads are preferred, whereas a single nucleotide polymorphism study demands a lesser error rate and hence limiting the samples per run, in turn, will increase the cost of analysis. There are two approaches followed in sequencing, of which metabarcoding relies on amplification based on marker genes and in shotgun metagenomics, where samples are randomly sequenced and assembled (Knief, 2014). Amplicon sequencing focus on the amplification of marker genes like 16 rRNA for bacteria and internal transcribed spacer 1 (ITS1) for fungus. Once amplified, the generated data are processed through various bioinformatic tools to convert into meaningful information. Amplicon sequencing is used for assessing the taxonomic profile of the sample and thereby predicting its functional diversity. The sequences generated from amplicon and shotgun sequencing are compared with known sequences of microorganisms in public databases like NCBI, and even specified databases for properties like disease suppression are available (DeSantis et al., 2006).

Pyrosequencing was the first alternative available for Sanger sequencing and is a DNA sequencing technology based on the sequencing-by-synthesis principle. Though it has the advantages of accuracy and flexibility, high reagent cost and error rate are stated as disadvantages when it comes to extensive data processing (Fakruddin et al., 2013). Following this, NGS platforms like Illumina, Ion Torrent, PacBio and Nanopore have gained their significance in sequencing technology. They are widely used depending upon the query to be resolved (Slatko et al., 2018). Illumina offers a longer read technology which can deliver up to 8–15 kbp, which was proved to be beneficial in genomic assembly and binning of microbial communities (White III et al., 2016). PacBio and Oxford Nanopore platforms offer low to medium throughput when compared to Illumina. Oxford Nanopore has demonstrated the longest read length (up to 90 kbp) but is highly error prone. In contrast, Illumina offers overlapping insert libraries with medium throughput, which is useful in low abundance phenotype studies (White III et al., 2017a, 2017b).

NGS is used for studying microbial community abundance, community structure, their metabolic potential, plant-microbe interaction, and community expression under various abiotic stresses. Some of the studies on rhizosphere utilising different NGS platforms during the past five years are summarised in Table 2.

Though next-generation sequencing has revolutionised the omics field, there are limitations to the technique which limits its usage. Extraction bias, presence of free/high molecular weight nucleic acids from dead organisms are some of the obstacles that restricts the use of this platform. Since complete coverage of genome or metagenome cannot be guaranteed by a single extraction method, different extraction procedures are needed to ensure complete coverage of the data (White III et al., 2017a, 2017b). They cannot differentiate between active microbial population on specific metabolic processes like C cycling, which demands a combination of techniques for better understanding of community. For example, to study the active fungal population in carbon assimilation, Hannula et al. (2020) combined DNA-Stable Isotope Probing (SIP) with NGS by providing $^{13}CO_2$ to grassland plants. On pyrosequencing, they found that members of Ascomycota, Mucuromycota and Basidiomycota, which forms 58% of the fungal population, were using fresh rhizodeposits as their carbon source, whereas the rest of the community, including mycelial Basidiomycota were either received carbon source a week later or not utilising recently fixed carbon at all.

5. Role of bioinformatics in metagenomic data analysis

The evolution of metagenome research is linearly correlated with the evolution of sequencing methods. The newer sequence technologies include 454 lifesciences, Solexa/Illumina and Applied Biosystems SOLiD sequencing, offer more accuracy and longer read lengths. The broad set of data generated in terms of short read and long read sequences are linked biologically with the help of bioinformatics tools. All these metagenomic outputs are shared globally through public domains in a standard format of data submission by Genomic Standards Consortium (GSC) to define 'minimum information about a genome sequence' (MIGS) and the 'minimum information about metagenome sequence' (MIMS) for better data capture and exchange (Field et al., 2008). The bioinformatic tools have helped in arranging microbes according to their taxonomy, metabolism, habitat prediction, genome reconstruction, and structural variation (Hiraoka et al., 2016) and are proven efficient in various fields like pathology, agriculture, and forensics (Fierer et al., 2010a, 2010b; Li et al., 2011; Qin et al., 2012; Coughlan et al., 2015). Several tools are available for phylogenetic and functional analysis of bacterial metagenomic data, generated such as MG-RAST, EBI metagenomics, and SILVA. Bioinformatic servers like PHACCS are also available for analyzing viral metagenomic data. However, the metagenomic studies of viral and fungal genome need progression (Mocali and Benedetti, 2010).

Taxonomic assignment of the procured sequences demands the precision of metagenomic or amplicon sequences. The analysis can be carried out either using reference method where obtained data is directly given into the reference database as in RefSeq (Tatusova et al., 2014) or SILVA (Quast et al., 2012) or reference-free method, where clustering of sequences are done for marker genes like 16S rRNA for phylogenetic identification and sulfate thioesterase (*soxB*) or methylcoenzyme M reductase (*mcrA*) genes for functional gene identification

Table 2
Representation of rhizospheric research carried out using different NGS platforms.

Platform	Study	Total number of sequences/reads	Mean read length	References
Pyrosequencing	Analysis of soil/ rhizospheric bacterial diversity or endophytic bacteria	9295–5,199,102	35–456 bp	Nam et al., 2015; Akinsanya et al., 2015; Osman et al., 2017; Xiong et al., 2015
	To evaluate the performance of primer pairs in metabarcoding studies	799,429	14,204 (± 12,013)	Beckers et al., 2016
	Biological control on plant rhizosphere microbiome	7087–94,677	2482 ± 349	Xue et al., 2015; Saravanakumar et al., 2017; Liu et al., 2018
	Disease suppression	471,419		Hamid et al., 2017
	Diversity studies of rhizosphere microbiome of hyperaccumulator plants	46,260		Lopez et al., 2017
Illumin	Rhizosphere community analysis	4238–75,578		Shang et al., 2016; Huaidong et al., 2017; Chen et al., 2020; Shi et al., 2015; Yong-hong, 2018
	Secondary metabolite production	797,716 -819,368		Baraniya et al., 2016
	Impact of virus on bulk and rhizosphere soil	9.5 to 25 million		Bi et al., 2020
	Disease suppression	234,396		Han et al., 2017
Ion Torrent	Diversity of rhizospheric Microbiomes	3,558,577-8,191,610	98 bp-228 bp	Kavamura et al., 2018; Urbina et al., 2018; Fonseca et al., 2018
	Effect of nutrient on soil bacterial community	468,657 -457,615		Cardinale et al., 2019
	Disease suppression	2524–868,608	181	Ros et al., 2017; De Corato et al., 2019

as operational taxonomic units (OTUs) (Chen et al., 2009). CD–HIT, UCLUST AND UPARSE are examples of reference-free methods used for gene identification (Edgar, 2010; Fu et al., 2012; Edgar, 2013). The major challenge in clustering amplicon sequences in the presence of chimeric sequence generated during PCR which may alter the result, and this can be surpassed through software packages like AmpliconNoise and ChimeraSlayer (Haas et al., 2011; Quince et al., 2011).

Reconstruction of the single genomic sequences is done by metagenomic binning and assembly. Long range contiguity is another approach for single-cell genome construction where long sequences from different regions of the single genome are ligated for genome reconstruction (Dostie and Dekker, 2007). Bioinformatic tools like CONCOCT and MetaBAT can perform bin sequencing according to their species using species-specific tetranucleotide contigs (Alneberg et al., 2014; Kang et al., 2015). Other than the identification of community, functional genes and their transcripts, microbial proteins are also extracted and compared for understanding the microbial activity. OpenMS (Sturm et al., 2008) and Trans-proteomic Pipeline (Keller and Shteynberg, 2011) are the most common proteomic tool libraries available. Most of the digested protein samples from the proteome are identified using mass spectrometry analysis, and the data is obtained from standard proteomic software packages. At the same time, there are limitations for the analysis of metaproteomic data on these software packages. For example, as metaproteomes consist of huge species variations, it demands more memory, algorithms, and workforce for data evaluation. Another factor is that if the taxonomic detail of a proteome extracted is unavailable, the chances of identifying them from a protein database are limited and there are identical peptides in homologous proteins which may cause misinterpreted results (Schlüter et al., 2008; Herbst et al., 2016; Locey and Lennon, 2016). Considering these limitations, new metaproteomic databases like Galaxy-P (Jagtap et al., 2015), MetaPro-IQ (Zhang et al., 2016) and MetaProteomeAnalyser (Muth et al., 2015) were introduced, but with most of the available software packages, single peptide identification is the preferable study (Segal-Salto et al., 2016). The Unique Peptide Finder of the UniPept server developed to facilitate the selection of unique peptides for a certain taxon, and this can be used for taxonomic identification of protein extracted (Mesuere et al., 2016).

Massive investment for human resource and infrastructure is required to standardize samples, data collection protocols, high-throughput analytical methods and computational tools for analyzing large sets of complex data. To standardize the data collection, processing and interpretation of the data generated, under the direction of U.S. National Institute of Standards and Technology, various agencies such as The Genomic Standards Consortium (Field et al., 2011), American Academy of Microbiology (Reid and Greene, 2013), Unified Microbiome Initiative (Alivisatos et al., 2015), Report on the Fast-Track Action Committee on Mapping the Microbiome (Stulberg et al., 2016), Earth Microbiome Project (Gilbert et al., 2014), and National Microbiome Initiative (Handelsman, 2016) are collaborating (Sharma et al., 2020). The collective categorization and analysis of the functional aspects of phytobiome as well as microbiome by these unification of research agencies can 'fix' the knowledge gap to a large extend. Considering these developments, it would be a better idea to conceptualize and evolve region wise microbiome databases specific to crop improvement, following the example of human genome diversity project. Database development by a regional committee can identify and emphasize the data generation based on region specific PGPRs, relevant crops, agricultural practices followed, and the stress factors to be considered according to the climatic variations. This can integrate information of regional importance for field application and also can eliminate irrelevant information which is time effective during the screening of data. With the astronomical number of gene sequences submitted which creates impracticality in screening them, it is recommended that the clustering of genes based on the core genome can be considered as it will help in reducing the magnitude several times along with improved accuracy.

6. Scope of metagenomics to decipher suppressive soil

Disease suppressive soils provide long-lasting and stable protection against soil-borne pathogens. They also benefit the crop production cost by eliminating the need for pesticide usage. Though the molecular mechanisms of disease resistance are not yet understood, the beneficial organism like *Streptomyces* sp., have been identified and exploited for the elucidation of the phenomenon (Heinsch et al., 2017). Based on the nature of the activity, soil suppression is broadly classified into specific and general suppression. General suppression is described as the property of soil to prevent the soil-borne pathogens, which effectively improves the disease resistance of the soil microbiome (Weller et al., 2002). The root pathogens reside in the soil as dormant structures where their growth is fuelled by the root or seed exudates and results in root infection. General suppression degrades the root exudates and limits their availability for the growth stimulation of infectious organisms (Cook et al., 2014). However, this general suppressiveness was not transferable and can be manipulated by adding large quantities of pathogen inoculum (Baker and Cook, 1974). Specific suppression is more effective and often results from a specific individual or group of microorganisms. Generally, it is superimposed with general suppression and is transferrable. When specific suppression is operating, microbial populations contributing to the property will be numerous, and it may take years to identify the common and recurring components of microbial communities that contribute to pathogen suppression (Weller et al., 2002).

Mendes et al. (2011) extracted metagenomic DNA from various types of soil that exhibited different levels of disease suppressiveness and detected a total of 33,346 bacterial and archaeal OTUs. The predominance of bacterial phyla ranged from 1% for the Chloroflexi and Cyanobacteria to 20% and 39% for the Firmicutes and Proteobacteria. The conclusion derived from the study points out that the diversity of the bacterial population is the determining factor of antagonistic activity than the abundance of a particular species. Mostly the antagonistic action may occur from the uncultured fraction of the soil microbiota (Steinberg et al., 2007) and hence unlocking this antagonistic potential by metagenomics techniques is possible. One such attempt was carried out by METACONTROL project by van Elsas et al., 2008 by compiling seven European laboratories with an objective for exploring anti-phytopathogen loci, such as polyketide class in antibiotics and chitinase biosynthesis. The observations from the study stated that global scale gene mapping would be an important approach for the future works such as mining soil for genes and pathways, identifying the function of hitherto uncultured microorganisms and characterizing soil based on its function and diversity. It also provides an insight into industrially significant secondary metabolites like chitinase with antifungal property (Berini et al., 2017).

Amplicon-based metagenomics was attempted to profile soil microbiota to study the yield decline of strawberry due to a fungal pathogen *Verticillium dahliae* (Xu et al., 2015). The reasons elucidated from the study stated that other than the physical factor like wet soil, the loss in suppressiveness could be due to the absence of dynamic groups like *Bacillus* and *Pseudomonas* populations or nematophagous fungus like *Paecilomyces*. Similarly, Penton et al. (2015) revealed that differences associated with disease suppressiveness of soils to *Rhizoctonia solani* on wheat were correlated to a loss in less than 40 fungal genera, including several endophytic species and mycoparasites.

Gómez Expósito et al. (2017) suggested a combination of techniques for effectively identifying the complex interactions and responses in different phyla of suppressive and conductive soils. The study stated that documentation of differences in relative abundance between bacterial and fungal communities in suppressive and conducive soils by network analyses could be instrumental in zooming in on specific microbial consortia. On the analysis of a soil sample suppressive to Fusarium wilt

of strawberry, NGS revealed an increase in *Streptomyces* population. On genome mining, it was found that the production of a thiopeptide compound called conprimycin by *Streptomyces*, plays a significant role in the process and a chemogenomic approach suggested the activity of the metabolite by interfering with fungal cell wall synthesis (Cha et al., 2016). Chapelle et al., 2016experimented on a combination of metagenomic and metatranscriptomic analyses in the rhizobacterial community of sugar beet plants in a *Rhizoctonia*-suppressive soil and made a significant finding that upon infection, oxidative stress activates the survival-related genes of the rhizobacterial communities resulted in biofilm formation, mobility and production of diverse secondary metabolites. Other than affecting sugar beet plants, *R. solani* AG-8 affects cereals like wheat cultivation by causing stunted plants with reduced tillers and grain production (Paulitz et al., 2010). Hayden et al. (2018) conducted a comparative metatranscriptomics of wheat rhizosphere in *R. solani* AG-8 suppressive and non-suppressive soil in South Australia. On differential expression based on mRNA annotation, they found that the dominant taxa present in non-suppressive soil are *Arthrobacter* sp. and *Pseudomonas* sp., *Stenotrophomonas* sp. and *Buttiauxella* sp. for the suppressive samples. There was a greater expression of polyketide cyclase, antimicrobial biosynthesis gene called terpenoid (*dxs*) and cold shock proteins (*csp*). Non-suppressive soil samples were found to be dominated by the expression of antibiotic genes like non-heme chloroperoxidase (*cpo*) and phenazine biosynthesis protein F (*phzF*). Genes coding reactive oxygen species (ROS) and superoxide radicals (*sod, cat, ahp, bcp, gpx1, trx*) were found in large numbers in non-suppressive soil samples which could be due to the root infection by *R. solani* AG-8. De Corato (2020) has suggested some promising agricultural practices that provides pathogen protection for crops. Practices such as intercropping peanut with medicinal herbs, long term application of organic wastes, bio fumigation, crop rotations, etc. can deliver suppression against pathogens by manipulating the microbial composition of the soil and gathering chemical compounds for antagonism. The author has also reviewed that 2,4-diacetylphloroglucinol (DAPG) an antibiotic produced mostly by *Pseudomonas* sp., is often associated with disease suppression.There are chemical methods, PCR based and probe hybridization approaches to detect the level of DAPG, as an indicator molecule for the suppression in an ecosystem and to detect its producer. Hansen et al., 2021developed a whole cell sensor for the detection of DAPG as well as the DAPG producing organism by combining repressor *phl*F from DAPG biosynthesis pathway module and *lac*Z as the output indicator. From the studies conducted, it is clear that during disease conduction and suppression in soil, molecular interplay occurs between plants and microbial community and involves intricate interactions between various plant and microbial metabolites. Therefore, a combinational approach of different techniques starting from gene (metagenomics) to metabolite production (metabolomics) should ensure unbiased interventions for understanding the complex processes involvement in antagonistic action of soil and should develop molecular assessment tools that aid the implementation of eco-friendly and cost-effective methods of agricultural practices.

7. Engineering rhizospheric microbiome for improving crop productivity

'Microbiome' is defined as a multi-species community of microorganisms in a specific environment and 'microbiome research' emphasises community-level analyses with data derived from genome-enabled technologies (Stulberg et al., 2016). Out of the different ecosystem explored like gut, skin, forest, ocean, deep subsurface, and acid mine drainage, soil microbiome is a well-attempted ecosystem in terms of community analysis and biomolecule extractions. Though plants are covered with microbes below and above the soil surfaces, the intricate intervention of plant-microbe association happens in root and seeds. When a plant undergoes biotic or abiotic stress, the rhizospheric microbiome is altered by recruiting beneficial microbes to the system (Bakker et al., 2013). For example, microbiome research on contaminated soils has identified potential growth-promoting organisms which can help in crop improvement in stressed soils. Another example is *Helianthus tuberosus*, a high biomass crop used for bio-ethanol production was inoculated with such bacteria and showed improved yield and less cellular stress in heavy metal contaminated soils (Montalbán et al., 2017). Drought-sensitive pepper grown in desert farming showed root microbiome with bacteria which increases photosynthesis and plant biomass production under drought stress. De Beer and Stoodley (2004) stated that the biocontrol bacteria should act synergistically with the commensals; otherwise, such non-antagonistic strains can affect their growth. These dynamic changes in the beneficial microbiome by the plant can be profiled and studied for crop quality and productivity (Berendsen et al., 2012).

In-situ microbiome engineering is an approach which alters the microbiome in its native environment without the need for individual domestication in the laboratory. It can be either applied with low specificity for large scale community changes (e.g., microbiota transplants) or specifically designed for an individual microbial member with lower impact on the entire community (e.g., engineered probiotics). The application of this approach can increase crop yield by improved nutrient absorption and contribute significantly to rhizoremediation (Sheth et al., 2016). Hussain et al. (2019) created a microbial consortium with *Bacillus subtilis* and *Serratia marcescens* which was fabricated into a composite with poly (vinyl alcohol)/poly (vinyl pyrrolidone) and plasticised with glycerol to form a seed coat for canola (*Brassica napus L.*) seed using electrospinning. They claim that biocomposite coated seeds have improved soil fertility by pH maintenance and increased nutrient uptake. Kim and Anderson (2018) found that root colonising pseudomonads can boost plant performance by producing metabolites and enzymes and can enhance the disease resistance through biofilm formation on root rhizosphere. They suggested that beneficial multifunctional effects of such root colonisers make them potential candidates as probiotics and can contribute to sustainable yield and quality in agricultural production. Williams and Marco (2014) showed that the phyllosphere microbiota of laboratory-grown plants and field-grown plants are different and stated that microbiota transplantation of the field microbiota to lab-grown plants is possible. They observed using pyrosequencing that the transplanted *Escherichia coli* O157:H7 survived in the lab-grown plants and altered the dominant taxa in the lab-grown lettuce plants. A successful application of the microbiota transplantation was made by Kwak et al. (2018) by transplanting rhizosphere microbiota from resistant plants to suppress disease symptoms in susceptible plants. From 16S rDNA sequencing and taxonomic binning method, they found that the resistance to *R. solanacearum*-disease is caused by a flavobacterium, named TRM1, and found that it could suppress the infection in pot experiments. These mesocosm experiments using 'omics' approach hold potential to plant-specific tailor-made probiotics to mitigate plant diseases.

Fortified bacterial consortium is a novel concept developed against nematode infestation in cucumber by combining 3 PGPR bacteria and 3 plant extracts (Panpatte et al., 2021). Apart from imparting disease resistance, a better understanding of plant microbe interactions can identify indicator molecules involved in the phytomicrobiome signalling pathways under different stages of interactions and abiotic stresses using 'omic' approaches. Developing biosensors from these indicator molecules or usage of immobilized bacteria as bioreporters can help in identifying the changes in abiotic factors such as nutrient and pollutant contamination as well as dynamics in the microbial community.

8. Future perspectives and conclusion

Rhizosphere microbial diversity and their beneficial/detrimental effect on plant growth is a complex area as they are entangled with multiple biological and chemical assemblages. It plays a crucial role in plant growth, homeostasis, and survival through its complex chemical

interactions with the plants and the information connecting these interactions between microbial community and plant species and various factors affecting them hold immense potential for sustainable agriculture. Detailing of the adaptive dynamics of the ecosystem to various physical and chemical factors are limiting with the basic culture-dependent system. Using the technical capabilities of the culture-independent 'omics' studies, the functional and taxonomic diversity of this interplays are deciphered to improve soil fertility. Combining traditional approaches with advanced next-generation sequencing techniques has proved a practical methodology for understanding the phylogenetic and functional correlation of the bacterial community, but there are limitations to the state. There are the least explored areas, such as studying the effect of monoculture of crops on indigenous rhizospheric bacterial community, the role of protozoans, bacteriophages, and virus on soil fertility or plant pathogenesis. As the microbial community act on the soil in mutualistic symbiosis, identifying fungal and eukaryotic members and their mechanism of action is equally important as that of bacteria. As bacteria is the dominant domain in the soil ecosystem, other domains like protozoa or eukaryotic domain like insects are lesser represented for their contribution to maintaining soil fertility. Apart from the diversity studies, the formulations proven efficient in laboratory conditions to fail to deliver in situ. The intricate interactions between indigenous microflora, biotic and abiotic factors to PGPR inoculants are overly complex and are often not addressed in the controlled environment. It can be comprehended from the existing data that stress tolerant PGPR strains that complement the indigenous microflora can deliver improved beneficial plant interactions. Successful field delivery of processed bacterial formulations can be explored with advancing techniques like nanotechnology with proper monitoring ensuring environmental safety. This demands real time analysis and visualization techniques post application that are cost effective and technically simple to be operated on the field. Tagging biomolecules or microorganisms with fluorescently labelled dyes or nanomaterials can be used for their detection after application on field or for stratification of the soil based on their nutrient and mineral composition, for the addition of soil amendments. Pre application and post application period with minimal external inhibitions such as fumigation or application of chemical fertilizers should be considered for ensuring prolonged colonization and activity of the applied formulations. Similar to crop rotation, rotation of microbial inoculants can also be tried for ensuring complementary action between microbial communities. During plant growth promotion and soil suppression, root exudates and microbial metabolites form an integral part of molecular interplay and more studies are needed to elucidate the role of these signature molecules as they can be used as biosensors or indicators for understanding the soil activities on field.

De-contamination of the polluted soil is an area of immediate attention due to the decrease in cultivable soil and a rising need for crop productivity. The rhizoremediation mechanisms of individual microorganisms, microbial population shifts in the contaminated soil, genes/protein/metabolites for bioremediation and development of a soil decontaminating microbial consortium are the future area of interests developed in the rhizoremediation approach. Fortifying the rhizospheric community by biostimulation and bioaugmentation can be considered as a management strategy for engineering rhizospheric microbiota. Apart from the microbial abundance, there are multiple rate-limiting constraints such as pH, moisture, micronutrients, etc., that can be explored further for understanding the remediation process. In short, the flexibility of the analysis techniques can be manipulated according to the objectives to be addressed by combining basic chemical or biological techniques with mathematic models and integrated network analysis. Untangling the complex networks in soil with the large scale of multiple variables is possible with evolving ultra-fast next-generation sequencing technologies.

'Omics' studies rely on biochemical, molecular biology and bioinformatics tools for data generated from the sources, among which bioinformatics tools decide the quality of data generated. The choice of bioinformatics tool to process a large amount of sequenced data generated from NGS with minimal errors is still a challenge. Binning and assembly of the massive data obtained from ever-evolving ultra-fast sequencing methods require pipelines with huge data storage, fewer error rates and high processing speed for accurate results. Metadata generation from sources require replicates for confirmation of results, but the cost of techniques causes a lesser number of replication and results in incomplete or error-prone reference databases. Unreviewed data based on predictions and assumptions can affect the quality of the reference databases which calls for improved stringency in data addition particularly on protein and genome databases. The requirement of developing biological reference databases with high processivity for handling massive data with a user-friendly interface demands combined efforts of bioinformaticians, microbiologist and ecologists. Databases of regional importance with information regarding crops of regional importance, their rhizospheric microflora, crop management practices, etc. can reveal vast information about indigenous microbial and plant population. However, there are attempts to overcome the lack of shared metadata, which would help in addressing data storage issues and standardization of shared data, including a reduction in partial sequences for accurate interpretation.

Even with the shortcomings, undoubtedly, it can be stated that the 'omics' approach endowed significant contributions in translating rhizospheric research for sustainable agriculture. Evolution of cutting-edge technologies in molecular biology and bioinformatics will deliver an improved image of plant-microbe interactions and the multi-variant networks of the rhizosphere.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author contribution

First author and corresponding author conceptualized the idea. First author prepared the manuscript. Second author edited the manuscript and provided additional inputs for improving the manuscript. Corresponding author edited and processed the manuscript for final submission.

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