ISSN: 0975-833X

Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 17, Issue, 04, pp.32387-32392, April, 2025
DOI: https://doi.org/10.24941/ijcr.48784.04.2025

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

RESEARCH ARTICLE

METAGENOMIC STUDY OF RHIZOSPHERIC SOIL FOR EXPOUNDING PGPR DIVERSITY

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ARTICLE INFO

Article History:
Received 20th January, 2025
Received in revised form
19th February, 2025
Accepted 26th March, 2025
Published online 26h April, 2025

Key words:

Metagenomics, Rhizosphere, PGPR and Microbial Community.

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ABSTRACT

Metagenomic has proven to be a powerful tool for investigating microbial diversity and functional potential within complex environments. Its application has steadily expanded, particularly in exploring the rhizospheric soil microbiome. The rhizosphere—the narrow region of soil influenced by root exudates— notably contribute in plant growth-promoting rhizobacteria (PGPR), which play essential roles in enhancing plant growth. These beneficial bacteria contribute through various mechanisms such as nitrogen fixation, phosphate solubilization, and phytohormone production. Material and methods: Two soil sample from different rhizosphere were collected from Jeevkar Nagar (garden area) and Ishwar Farm (sorghum field) from two different location of Surat, India. Further Bacterial DNA was isolated, purified and quantified. After library preparation samples was sequenced using Sequencing with ion torrent s5 plus system and analysed using alpha diversity analysis method. Results: In our sample analysis three major group of bacterial community dominate the diversity of particular soil microflora respectively, Proteobacteria (76.89 %), Actinobacteria (12.8) and Firmicutes (2.36 %) with minor presence of other bacterial community. Microbial community taxonomic analysis shows 20, 21 phyla; 40,39 class; 55,52 order; 53,50 family; and 55,51 genus and species in sample 1 and sample2 respectively. Conclusions: This study highlights the effectiveness of metagenomics in uncovering the hidden diversity and functional capabilities of PGPRs in rhizospheric soils. The insights gained pave the way for the development of advanced microbial strategies to promote sustainable agriculture and enhance soil health.

Citation: Jitendra K Pal, Jinal H Naik, Roshni Shukla and Harshvadan Patel, 2025. *"Metagenomic study of rhizospheric soil for expounding pgpr diversity". International Journal of Current Research, 17, (04), 32387-32392.*

INTRODUCTION

The rhizosphere is a narrow zone of soil directly influenced by plant roots and is home to a diverse array of microbial communities—including bacteria, fungi, protozoa, and archaea—that are vital to plant health. Often regarded as a plant's secondary genome, this microbiome plays a crucial role in plant development. Among these microorganisms, plant growth-promoting rhizobacteria (PGPR) are particularly important due to their ability to enhance nutrient availability, suppress pathogens, and support overall plant growth (Mohanty *et al*., 2021 and Ghosh, *et al*., 2024). Root exudates—comprising carbohydrates, sugars, organic acids, vitamins, flavonoids, nucleotides, enzymes, hormones, inorganic ions, and volatile compounds—act as chemical signals that facilitate interactions between plant roots and soil microorganisms (Hayat, *et al*., 2012). These exudates shape the rhizosphere into one of the most dynamic and biologically active ecosystems, attracting a wide range of beneficial microbes(Gouda *et al*., 2018). PGPR are among the most influential members of this community, contributing to plant health through nitrogen fixation, phosphate solubilization, and the production of phytohormones. Microbial populations are significantly higher in the rhizosphere compared to bulk soil (Giri *et al*., 2005), emphasizing its importance in sustainable agriculture. A deeper understanding of the biology, classification, and functional capacities of PGPR is essential to reduce reliance on chemical fertilizers and pesticides. Metagenomics offers a culture-independent approach to studying microbial communities, enabling comprehensive characterization of soil microbiota (Nam et.al.,2023). By directly extracting and sequencing 16S rRNA genes (Culligan *et al*., 2013). Metagenomic methods provide valuable insights into both the taxonomic structure and functional potential of the rhizosphere microbiome (N. Sabale *et al*, 2020 andCallahan *et al*., 2019). This study aims to employ metagenomics to characterize the taxonomic diversity of PGPR within rhizospheric soil associated with plant growth promotion and also focuses on studies that have expanded our understanding of PGPR diversity in rhizospheric soils, highlighting how metagenomic sequencing technologies have been utilized to unravel both the taxonomic composition and functional potential of microbial communities associated with

plant roots (AbdElgawad, *et al*., 2020 andMasenya *et al*., 2024).

MATERIAL AND METHODS

Sample collection: Two soil sample from different rhizosphere were collected from Jeevkar Nagar (garden area) and Ishwar Farm (sorghum field) from two different location of Surat, India(latitude 25_19018.062400 N, longitude 82_59014.240400 E) at 22$^{\overline{O}}$C on 24th February 2024. Rhizosphere soil Samples were accomplished by digging soil at 10 cm depth and 50 gram each sample were collected in sterile bag with appropriate label mark on them and stored at 4º C in the laboratory until further analysis (Sharma and Kaur2021).

DNA Extraction: Extraction of microbial DNA was performed using modified CTAB method (Saghai-Maroof *et al*. 1984; Shrestha *et al*., 2018). For that, 200mg of each rhizosphere soil sample was taken and homogenised with 1.5 ml of extraction buffer. Cell lysis of prepared mixture was performed by adding 20 µL lysozyme, and mixture was incubated at 37 °C for 30 min. Mixture was treated for protein solubilization and digestion with 200 µL SDS (10%) and 10 µL Proteinase K (1 mg/mL) respectively and incubated for 1 h at 65ºC in water bath and centrifuged at 2750×gfor 5 min. Supernatant was collected and extraction of residual pellets were performed by adding 1.5 ml of extraction buffer and homogenized twice as described above. Collected supernatant was mixed with an equal volume of chloroform: Isoamyl alcohol (24:1 v/v) followed by vigorous shaking. The aqueous phase was retained by centrifugation, and then 0.6 volume of isopropyl alcohol was used to precipitate dissolved DNA molecules. For proper sedimentation the DNA was passed through the silica column and centrifuge at 8000 rpm for 1 min. The obtained DNA was washed by 500µL AW1 and AW2 (washing buffers) by centrifuge at 8,000 rpm and 12,000 rpm for 1 min respectively. The dry spin was performed to remove excess of wash buffer. Followed by addition of 25 µL 1×TE buffer to the column and DNA elution was carried out by centrifugation at 10,000 rpm for 1 min (Natarajan *et al*., 2016).

Quality control: Quality of DNA was checked by using a Qubit 3.0 (Thermo Fisher Scientific) with Hs Qubit Reagents. 1 µL of elute DNA was added to 199 µL of qubit buffer and 1 µL qubit dye. The concentration of extracted DNA was measured and samples having concentration of 1.5–2.0 ng/µL were selected for further processing.

Library preparation: Extracted DNA was further analysed for 16S rRNA analysis usingIon 16S™ Metagenomics kit. 2 µL of DNA sample was mixed with 15 µL of environmental master mix. 3 µL of 16S primer set. Samples were processed in thermocycler with the following thermal conditions: 95°C for 10 min; then 25 cycles of 95°C for 30 s, 58°C for 30s, 72°C for 30 s; and finally, 72°C for 7 min. Amplification products were purified using magnetic beads (Jones *et al*., 2022)

Purify the amplification products: Amplified Product was mix with 54 µL of vortex magnetic bead reagent. Mixed properly and incubated for 5 min at room temperature. The plates were placed in a magnetic rack until solution get cleared. After that, Supernatant was discarded carefully and pellets were washed with 700 µL of 70% Ethanol for 30 sec. Step was repeated twice. Residual ethanol was removed and beads were allowed to air dry for 4 min. Plates were removed from magnetic rack and treated with 15 µL nuclease free water. Plates were placed in magnetic rack for 1 min. Supernatant was collected into new vial (Dias *et al*., 2014).

DNA End repair and Library Construction: 20 µL of end repaired buffer and 1 µL of end repair enzyme was added to 100 µL of sample consist of 75 µL of amplicons (10-100 ng) each sample. Sample was vortexed and incubated at room temperature for 20 min. Amplicons were purified using previously described purification method. DNA was further processed for ligation and nick repair mechanism with 100µL of reaction mixture which consist of 10 µL 10 X Ligase Buffer, 2 µL of Ion P1 adaptor, 2 µL of Ion Xpress^{TM} Barcodes, 2µL NTP mix, 2 µL of DNA, 8 µL of Nick replair polymerase and 49 µL of Nuclease-free water. Reaction tubes were placed in thermal cycle with thermal conditions: 25°C for 15 min, 72°C for 5 min. Adapter-ligated and nick-repaired DNA was then purified using magnetic beads and eluted in Low TE buffer (Ribarska *et al*., 2022).

Library Amplification: Library was amplified using 100 µL of Platinum TM PCR Super Mix High Fidelity, 5 µL of Library amplification Primer Mix and 25 µL of unamplified library with thermal conditions: 95°C for 5 min; then 7 cycles of 95°C for 15 s, 58°C for 15 s, 70°C for 1 min; and then finally 70°C for 1 min. Amplified library was purified with 195 µL of impure beads. Purified DNA was eluted and stored in 20 µL of low TE (Gupta, *et al*., 2019).

Sequencing with ion torrent s5 plus: Libraries were prepared for sequencing using oil amplification to template the libraries onto beads and loaded onto chips using the Ion Chef Instrument. Transfer the diluted libraries to PCR tubes and cap them properly. Insert prepared reagent trays and cartridges into the instrument. Load Ion 550 chips into the chip loading station on the Ion Chef. At the end of the run, Ion Chef will inform you. Carefully remove the loaded chips and continue the sequence. Prepare and load the Ion s5 sequencing reagents. Insert the loaded Ion 550 chip into the sequencer's chip and secure it. The duration of the sequence depends on the type of chip and the length of the reading, which usually takes several hours. The sequencer notifies you when it is finished and saves the sequence data (Lee, J. Y. 2023). Carefully remove the used chip. Transfer sequence data to a bioinformatics workstation. Use torrent suite for quality control. Trimming, and analysis (Reuter, *et al*., 2015 and Ashraf *et al*., 2022 and)

Statistical analysis: Alpha diversity analysis was utilized to determine the abundance and diversity of various species. The richness of bacterial diversity was estimated using observed species, the Chao1 index, Simpson, and Shannon. (Navgire *et al*., 2022 and Andermann, T. *et al*., 2022)

RESULTS

Quality checking of extracted DNA: DNA samples was evaluated by using 0.8% Agarose gel electrophoresis. The concentration of the sample was found respectively 383.42 and 99.16 ng/µL.

Raw data statistics: The Sequencing data for each sample was subjected to initial quality control to assess the quantity and quality of the reads. The raw data statistics are summarized in

Table 1. Summary of Raw Data Statistics

S/n	Samples	Base pair	>=Q20 base	Reads
1	Sample 1	1,840,199,985	1,568,192,589	10,827,206
2	Sample 2	8,764,4001	70,545,996	5,39,303

Table. This discrepancy indicates a higher sequencing depth and data yield for Sample 1. The richness of bacterial diversity was estimated using observed species, the Chao1 index, Simpson, and Shannon. In sample 1, the species richness is found higher.

Chao1 index: The Chao1 index is used to estimate the species richness, i.e., the total number of species, within a given community. Theimage illustrates a Chao1 rarefaction plot used to estimate species plot utilized in ecological and biological research for approximating species richness. The x-axis spans from 0 to 80,000 sequences per sample, while the y-axis spans from 0 to 300 in Chao1 measure for sample 2. the Chao1 measure increases, as the number of sequences per sample increases, indicating a higher estimated species richness. Chao 1 index plot for samples 1 & 2 is mentioned in Fig 1 (A and B). In sample 1 species richness is higher compared to sample 2.

Shannon index: The Shannon Diversity Index, is used to measure of species diversity within a community that reflect species richness and evenness. The result shows the correlation between sequence per sample and refraction measure of Shannon index, which reflects the total number of distinct species present. The x-axis spans from 0 to 7,000,000 sequences per sample, while the y-axis spans from 0 to 7 in Simpson index for sample 1. The x-axis spans from 0 to 80,0000 sequences per sample, while the y-axis spans from 0 to 7 in Simpson index for sample 2. Simpson index also increases, as the number of sequences per sample increases, indicating a higher estimated species richness. Simpson 1 index plot for samples 1 & 2 is mentioned in Fig 1(C & D). In sample 1 species richness is higher compared to sample 2.

Simpson index: The Simpson Diversity Index, used measure of species diversity within a community. This index illustrates number of species present and their proportional representation (evenness), it is symbolized as D. The graph depicts the correlation between sequence per sample and refraction measure of Simpson index, The x-axis spans from 0 to 7,000,000 sequences per sample, while the y-axis spans from 0 to 1 in Simpson index for sample 1. The x-axis spans from 0 to 80,0000 sequences per sample, while the y-axis spans from 0 to 1 in Simpson index for sample 2. Simpson index also increases, as the number of sequences per sample increases, indicating a higher estimated species richness. Simpson 1 index plot for samples 1 &2 is mentioned in Fig 1(E & F). In sample 1 species richness is higher compared to sample 2.

Krona chart: The Krona chart was used to displays the taxonomic identification and relative abundance of bacteria isolated from agricultural soil. Each concentric circle represents different taxonomic levels: phylum, class, order, family, genus, and species. Analysis of sample 1, shows the highest diversity of Proteobacteria (57.5%), Actinobacteria (31.73%) and Firmicutes (5.7 %) with other phylum of bacteria while sample 2, shows Proteobacteria (76.89 %), Actinobacteria (12.8) and Firmicutes (2.36 %) with presence of other bacterial communities in moderation A 16S Metagenomic analysis revealed Proteobacteria as the dominant phylum in both soil samples (57.58% in Sample 1 and 76.89% in Sample 2). Proteobacteria play important role in soil fertility, plant growth and development. Actinobacteria is second highest phylum find in soil (31.73% in sample 1 and 12.8 % in sample 2) (Ashraf, *et al*., 2022).Actinobacteriaplay multiple roles in soil such as decomposes organic matter, soil quality, involves in nutrient cycle, produce antibiotics that suppress plant pathogen, crop yield. Firmicutes are assists with nutrient cycling by degrading organic matter and produce some beneficial enzymes. Some Firmicutes can also be plant pathogen Fig 3 (A & B).

CLASS: The most abundant bacteria at class level were Alphaproteobacteria, betaproteobacteria, Gammaproteobacteria, and Actinobacteria were represented across the two samples. 16S Metagenomics analysis revealed *Alphaproteobacteria* (41.53%) as the dominant class in soil sample1 and Gammaproteobacteria (32.11%) as the dominant in soil sample 2. This class encompasses beneficial plant growth-promoting rhizobacteria (PGPR) that contribute to plant growth through various mechanisms such as Nitrogen fixation, Phosphate solubilization, Siderophore production, Phytohormone synthesis, Pathogen suppression, Enzymes (e.g., protease, cellulose), Antibiotics (e.g., streptomycin, oxytetracycline) and the breakdown of inorganic substances (Mhete *et al*., 2020). Actinobacteria was found in analyzed soil samples **(**Sample 1- 31.37% and Sample 2- 12.71%**)** which are important for the cycling of organic matter because they generate a large number of extracellular enzymes that aid in crop development and prevent the growth of several plant diseases in the rhizosphere, also they contribute to the improvement of soil health (Bhatti *et al*., 2017) Fig. 3 (C & D).

ORDER: The most abundant bacteria at order level of Actinomycetales (22.25%) have the dominant in soil sample 1 and Xanthomonadales (15.44%) as the dominant in soil sample 2. The order, Rhizobiales, Sphingomonadales are present in the analyzed both soil samples. Actinomycetales induced the nitrogen metabolizing enzyme such as glutamine synthetase, and nitrate reductase. It is also increased nitrogen-contain amino acids. This is enhanced seed quality (AbdElgawad *et al*., 2020).The order Sphingomonadales is class of the Alphaproteobacteria, and phylum of the Proteobacteria (Kalnenieks *et al*., 2020). The benefits of Sphingomonadales are promoting plant growth and strongly associated ability to produce Phytohormone and extracellular enzymes, which enhance nutrition of water and minerals. These rhizobacteria are known to promote plant growth and have been well-reported from the phylloplane, rhizoplane, and rhizosphere. By producing plant hormones such salicylic acid (SA), indole-3-acetic acid (IAA), and zeatin (is a type of cytokinin). Sphingomonas species facilitate plant germination and growth (Asaf *et al*., 2020).While enterobacterial contribute to process like decomposition and nutrient cycling also interact plant roots and various ways Fig. 3 (E & F).

FAMILY: The *Sphingomonadaceae (*11.72%) occupied the highest richness and dominant in soil sample 1 and *Xanthomonadaceae (*14.13%) in samples 2.*Sphingomonadaceae, yphomicrobiaceae Acidimicrobiaceae, Xanthomonadaceae,* and *Enterobacteriaceae,* are known for their plant growth promoting attributes in the soil. (Kalnenieks *et al*., 2020). The benefits of Sphingomonadales are promoting plant growth and strongly associated ability to produce Phytohormone and extracellular enzymes, which enhance

nutrition of water and minerals (Asaf *et al*., 2020).The abundance of *Sphingomonadaceae* (11.72%)was found in analyzed soil sample 1 Fig.3 (G & H).

Genus and Species: The highest abundance of *Rhodoplanes sp.*in analyzed soil sample 1(1.52%) is found. The dominant *Nevskiaramosa* in was found in analyzed soil sample 2 (1.12%). Fig 4 (A and B) respectively. *Rhodoplanes* species are phototrophic bacteria present in rhizospheric soil(Weon*et al*., 2008).Various bacterial family such as *Rhodoplanes sp., Enhydrobacteraerosaccus, Skermanellaaerolata,* and *Nevskiaramosaetc* are known for their plant growth promoting attributes in the soil. (Premalatha *et al*., 2015). They are contributed in soil health and soil fertility (Singh *et al*., 2022). Fig 4 A and B.

DISCUSSION

The present metagenomic study on rhizospheric soil ecosystem provides valuable insights into the diversity and composition of plant growth-promoting rhizobacteria (PGPR). Through the application of high-throughput sequencing technologies, the study revealed a highly complex and diverse microbial consortium (Lee, J. Y. 2023).These microbial groups are renowned for their multifaceted plant-beneficial functions, including nitrogen fixation, phosphate solubilization, siderophore production, and the biosynthesis of phytohormones. Chao1 rarefaction plot used to estimate species richness. The number of sequences per sample and the Chao1 measure, reflects the total number of distinct species present. The Chao1 measure increases, as the number of sequences per sample increases, indicating a higher estimated species richness. Chao 1 index in sample 1 The Shannon Diversity Index, provides a quantitative assessment of the distribution of species abundance in a given ecosystem species richness is higher in sample 1compared to sample 2.

The Simpson Diversity Index, is used to measure of species diversity within a community for number of species present (species richness) and their proportional representation (evenness), in present study sample 1 species richness is higher compared to sample 2. Krona, visualization tool was used to find out to exploration of relative abundances and confidences within the complex hierarchies of metagenomic classifications. A 16S Metagenomics analysis revealed Proteobacteria as the dominant phylum in both soil samples (57.58% in Sample 1 and 76.89% in Sample 2). This phylum encompasses beneficial plant growth-promoting rhizobacteria (PGPR) that contribute to plant growth through various mechanisms: like Nitrogen fixation: Increases nitrogen availability for plants Phosphate solubilization Siderophore production Phytohormone synthesis Pathogen suppression Enzymes: Produce various important enzymes (e.g., protease, cellulose, and hydrogenase Antibiotics. Proteobacteria play important role in soil fertility, plant growth and development (Khan, *et al*., 2023). Some Proteobacteria can also be harmful, causing plant diseases. Actinobacteria is second highest phylum find in soil (31.73% in sample 1 and 12.8 % in sample 2). It is play multiple roles in soil such as decomposes organic matter, soil quality, involves in nutrient cycle, produce antibiotics that suppress plant pathogen, crop yield while the Firmicutes (sample 1 5.7% and 2.31%) are assists with nutrient cycling by degrading organic matter and produce some beneficial enzymes. Some Firmicutes can also be plant pathogen. Krona and 16S Metagenomics analysis revealed *Alphaproteobacteria* (41.53%) as the dominant class in soil sample1 and Gammaproteobacteria (32.11%) as the dominant in soil sample 2. This class encompasses beneficial plant growth-promoting rhizobacteria (PGPR) that contribute to plant growth through various mechanisms such as Nitrogen fixation, Phosphate solubilization, Siderophore production, Phytohormone synthesis, Pathogen suppression, Enzymes (e.g., protease, cellulose), Antibiotics (e.g., streptomycin, oxytetracycline). While the abundance of Actinobacteria in analyzed soil samples (Sample 1- 31.37% and Sample 2- 12.71%) which are important for the cycling of organic matter because they generate a large number of extracellular enzymes that aid in crop development and prevent the growth of several plant diseases in the rhizosphere, they contribute to the improvement of soil health Other bacterial communities inhabit in soil sample 1 and 2 were Deltaproteobacteria (5.6%) and (6.05), Betaproteobacteria (4.33% and 12.63%), Bacilli (4.04%) and 1.17%) respectively with some other minor bacterial communities which have very diverse impact on ecology and environment. (Bhatti *et al*., 2017). In this study the most abundant bacteria at order level of Actinomycetales (22.25%) have the dominant in soil sample 1 and Xanthomonadales (15.44%) as the dominant in soil sample 2. The order, Rhizobiales (16.7%) while Sphingomonadales (13.17%) are present in both soil with some other minor bacterial communities. Actinomycetales is use for improved soil fertility and plant production. It is increased nitrogen availability in soils. Which induced the nitrogen metabolizing enzyme such as glutamine synthetase, and nitrate reductase (Oteino *et al*., 2015). It is also increased nitrogen-contain amino acids, sugars, organic acids, fatty acids, antioxidant phenolics, mineral and vitamins level in treated legume seeds by actinomycetes. This is enhanced seed quality also (AbdElgawad *et al*., 2020). While order Sphingomonadales The promote plant growth and strongly associated ability to produce Phytohormone and extracellular enzymes, which enhance nutrition of water and minerals (Kalnenieks *et al*., 2020). These rhizobacteria are known to promote plant growth by producing plant hormones such salicylic acid (SA), indole-3-acetic acid (IAA), and zeatin.Sphingomonas species also facilitate better seed germination and growth also (Asaf *et al*., 2020).

The sample analysis for 16s metagenomic found family Sphingomonadaceae (11.72%) occupied the highest richness and dominant in soil sample 1 and Xanthomonadaceae (14.13%) occupied the highest richness and dominant in soil samples 2. Bacteria from various family such as Sphingomonadaceae (11.72% and 12.93%), Hyphomicrobiaceae (7.1% and 1.42%) Acidimicrobiaceae,(5.84 % and 2.41%) Xanthomonadaceae (1.15% and 14.13%), and Enterobacteriaceae (1.5% and 14.05%) respectively in soil sample 1 and 2, are known for their plant growth promoting attributes in the soil. This family encompasses beneficial plant growth that contribute to plant growth through various mechanisms such as Nitrogen fixation, Phosphate solubilization, and Pathogen suppression (Masenya *et al*., 2024). The benefits of Sphingomonadales are promoting plant growth and strongly associated ability to produce Phytohormone and extracellular enzymes, which enhance nutrition of water and minerals (Kalnenieks *et al*., 2020). In case of genus and species, the highest abundance of *Rhodoplanes sp.* is found *in* analyzed soil sample 1 and 2 (1.52% and 0.69) respectively. *Rhodoplanes species*are

phototrophic bacteria present in rhizospheric soil. They are contributed in soil health and soil fertility (Singh *et al*., 2022). *Nevskiaramosa* in was found dominant in analyzed soil sample 2 (1.12%), (Weon *et al*., 2008). *Enhydrobacter*, a rare genus with its single species *Enhydrobacteraerosaccuss*. recognized as a member of the family *Rhodospirillaceae* within the class *Alphaproteobacteria* (Premalatha *et al*., 2015. Our study finding shows that both soil sample collected from garden area and sorghum field soil shows significant diversity and differentiation of different bacterial community with their own beneficial activities for plant growth and development.

CONCLUSION

Taxonomic analysis of the microbial community revealed 20 and 21 phyla; 40 and 39 classes; 55 and 52 orders; 53 and 50 families; and 55 and 51 genera and species in Sample 1 and Sample 2, respectively. The present study shows the large microbial diversity in both soil samples which establishes the power of metagenomics in opening the hidden diversity and functional potential of PGPRs in rhizospheric soils. Metagenomics has significantly advanced the field of microbial ecology also. It has not only led to the discovery of numerous novel genes and metabolic pathways but also improved our understanding of how these genes contribute to the function and structure of microbial communities across different ecosystems.

Key words: Metagenomics, Rhizosphere, PGPR and microbial community.

ACKNOWLEDGEMENTS

We acknowledge the fund received from Vanita Vishram women's university and Gene Xplore – Diagnostics & Research Centre Pvt. Ltd. for their collaborationand technical support.

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