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ORIGINAL PAPER

Targeted metagenome sequencing reveals the abundance of *Planctomycetes and Bacteroidetes* in the rhizosphere of pomegranate

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Abstract
Agricultural productivity of pomegranate can be enhanced by identifying the crop-associated microbial diversity in the rhizosphere region with respect to plant growth promoters and other beneficial organisms. Traditional culture methods have limitations in microbial screening as only 1–2% of these organisms can be cultured. In the present study, 16S rRNA amplicon-based metagenomics approach using MinION Oxford Nanopore platform was employed to explore the microbial diversity in the rhizosphere of pomegranate Bhagwa variety, across variable soil depths from 0 to 5 cms (R2), 5–10 cms (R4) and 10–15 cms (R6), using bulk soil as the control. Across all the three layers, significant variations in pH, nitrogen content and total fungal count were observed. 16S rRNA analysis showed the abundance of planctomycetes, *Pirellula staleyi,* followed by bacteroidetes, *Flavisolibacter LC59* and *Niastella koreensis* across the various soil depths in the rhizospheric soil samples. Pathway prediction analysis indicated arginine and proline metabolism (gamma-glutamyl putrescine oxidase) and hydrogen sulfide biosynthesis as the most abundant pathway hits. Comparative abundance analysis across layers showed the R6 layer with the maximum microbial diversity in terms of highest dimension of variation (79.2%) followed by R4 and R2 layers ($p < 0.01$). Our analysis shows the significant influence of root zone in shaping microbial diversity. This study has reported the presence of *Planctomycetes*, *Pirellula staleyi* for the first time in the pomegranate field.

Keywords Bulk soil · DNA sequencing · *Punica granatum* · Microbiomics · Rhizosphere · Soil metagenomics

Introduction

Pomegranate (*Punica granatum*) is a very important fruit crop belonging to the Punicaceae family that is widely farmed around the world and is prominent in India's dry regions (Melgarejo-Sánchez et al. 2021). Karnataka is the second largest producer of pomegranate after Maharashtra (Saxena et al. 2018). In Karnataka, India, major varieties grown are Bhagwa, Ganesh, Mridula (Vasanth Kumar 2009). Apart from its agricultural value, the health advantages of pomegranate have been investigated in relation to illness states such as diabetes, high blood pressure, and inflammatory activities (Asgary et al. 2014). The antioxidant activity, nutritional status, and total phenol content of pomegranate peel extracts in water and methanol were investigated previously by Middha et al. (Middha et al. 2013). Various metabolites, such as steroids, were discovered, as well as other components like flavonoids, anthraquinone, proanthocyanidins, and phenolic compounds like gallic acid. Usha et al (2020) critically reviewed anticancerous property of pomegranate (Usha et al. 2020). Bhagwa is a highly commercially viable variety of pomegranate, found to be the most suitable and promising variety of pomegranate for dry regions of Karnataka. Compared to other elite varieties like Ganesh, fruit quality is outstanding fetching an overall acceptance score of 90.8/100 compared to Ganesh with the score of 57.8/100 (Prasad et al. 2013). The variety is commonly used as it is also suitable for high-density planting in pomegranate

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orchards (Saroj and Sharma 2017). Our lab also annotated the whole genome of pomegranate (Bhagwa *var*.) and provided new insights into the pharmacological properties of the secondary metabolites (Usha et al. 2022). All these factors gave a justification for the inclusion of Bhagwa variety of pomegranate in our experimental study.

Microorganisms serve a critical part in a variety of sectors, including medical, engineering, industry, and agriculture (Sloan et al. 2006). As a result, using biotechnological tools to investigate the genetic and biological variety of these bacteria will aid in the discovery of novel genes, enzymes, and chemicals of interest. Many microorganisms cannot be cultivated using traditional microbiological methods. To address these issues, DNA-based molecular approaches that can characterize microorganisms present in any material without the need for culturing have been developed (McSweeney et al. 2006). As described by Streit and Schmitz (2004), methodologies such as 16S rRNA gene analysis have the limitation of only disclosing the taxonomic features of bacteria and not their functional role (Streit and Schmitz 2004). With the advancement of modern technology, such as 'metagenomics,' taxonomic diversity as well as functional characterization of microbes in environments like soil, water, ancient animal remains, or the digestive system of animals and humans (Ghazanfar and Azim 2009) can be investigated without the need for microbes to be cultured in lab conditions. Metagenomics aids in the isolation of species like Verrucomicrobia, Acidobacteria, Armatimonadetes, and methanogenic Archaea that are not cultivable using traditional culture methods (Cardenas and Tiedje 2008). 16S rRNA sequencing using NGS platforms have helped to unravel the microbial diversity of any ecosystem which is otherwise a challenge to explore through wet lab studies. The advantage of nanopore over other NGS platforms is the generation of long reads facilitating the sequencing of entire 16S rRNA region from V1-V9 which in turn facilitates higher resolution for bacterial identification unlike Illumina platform where short reads limit the sequencing to V1-V2 or V3-V4 (Santos et al. 2020).

The interplay between microbes and host–microbe interaction results in rhizosphere microbial community differentiation that can be explored using 16S rRNA-targeted sequencing and shotgun metagenome as demonstrated in experiment with barley having abundance of Comamonadaceae, Flavobacteriaceae, and Rhizobiaceae in the rhizosphere (Bulgarelli et al. 2015). Citlali et al. (2018) discovered that, in addition to soil type, host plant and species had a significant impact on microbial diversity reporting the dominance of Proteobacteria, Firmicutes, Actinobacteria, Acidobacteria, and Bacteroidetes associated with Crassulacean Acid Metabolism plants (Citlali et al. 2018). Pathogenesis, secretion, phage interactions, and nutrition mobilization were all linked to the functional annotation of features associated with microbial diversity.

The metagenomics approaches like pyrosequencing have also facilitated the discovery and utilization of new industrially important enzymes from shift in the microbial community abundance to bacteria involved in chitin breakdown, including phyla such as actinobacteria, proteobacteria, and proteobacteria (Jacquiod et al. 2013). The impact of physico-chemical properties of soil on the microbial community, especially the soil depth, has been investigated (Yan et al. 2019) wherein 16S rRNA targeted sequencing using Illumina MiSeq platform revealed a decrease in alpha diversity across a depth of 0–300cms in legume crop cultivation (*Robinia pseudoacacia* and *Trifolium repens*) with an abundance of *Nitrospira* in top soil. Phosphorus concentration decreased across the depth. Similar results were reported by Kuramae et al. employing real-time PCR and microarray methodologies based on phylogeny targeting 16S rRNA (Kuramae et al. 2012). The C:N ratio and soil phosphorus level were more important factors in defining microbial diversity. Firmicutes were determined to be the most prevalent phylum in deciduous forest soils of Netherlands. *Bacillus* and *Clostridium* OTUs were shown to be associated with high phosphate and pH, implying that the bacterial population can be used as a proxy for soil properties. There are reports of availability of soil resources having profound influence on functional diversity of microbes in spruce plantations with the organic horizon revealing an abundance of sequences for glycoside hydrolases in comparison with enriched glycoside transferases in mineral horizon (Uroz et al. 2013). Although there are few publications on traditional screening of microbial flora using normal microbiological techniques, there have been no reports using metagenomics strategy to examine microbial diversity in the rhizosphere of pomegranate (Shahaby et al. 2016). Our lab previously reported the adaptations of *Fusarium* in the rhizosphere from wilt-infected pomegranate field (Das et al. 2021). Therefore, the current study on microbial diversity in the rhizosphere of *Punica granatum* using amplicon targeted sequencing on a nanopore sequencing platform was an attempt to correlate soil characteristics based on depth with the selective microbial community associated in the rhizosphere compared to bulk sample, as well as to identify the prominent and abundant bacterial species associated with the rhizosphere.

Materials and methods

Site description and sampling

Collection of soil sample

Soil samples were collected from Doddamarali, Chikkaballapur Taluk of Karnataka (Chikkaballapur, Doddamarali, 13.3669°N, 77.7373°E), where the pomegranate variety, Bhagwa is cultivated (Fig. 1a, b). The farming practice followed an integrated approach of conventional and organic farming. The irrigation practice followed in the field was drip irrigation. The total numbers of pomegranate plants in the field were 429 in an area of 1 acre. The plants were in 13 columns and 33 rows. The average yield recorded was 9 t/acre. The average temperature recorded was 28–30 °C, humidity of 43%, wind speed 13 kmp, wind direction NE. Monocropping was practiced with no tillage. Samples were collected from all the corners and centre of the plot randomly from the one-acre land of pomegranate crop. The soil samples were collected from the rhizosphere of the plant and the bulk soil away from the root zone (two feet away from root). For each type of sample, the depths covered for sample collection were 0–5 cms, 5–10 cms, 10–15 cms.

Physiochemical characterization and total microbial count estimation

Since the soil characteristics play a very important role in shaping the microbial diversity, the study of soil characteristics with respect to the physico-chemical parameters was very crucial in this study. These parameters should be well defined and recorded before exploring the microbial diversity. Thorough analysis of physico-chemical properties of soil including pH, electrical conductivity, NPK, organic carbon, micronutrients such as Zn, B, Mn, Fe, Cl, Cu, bacterial and fungal count were carried out for samples drawn from bulk and rhizosphere soil in the pomegranate field. The analysis was done for soil samples taken from various depths, 0–5 cm, 5–10, and 10–15 cm (Fig. 2).

The pH and electrical conductivity were measured using electrometric method. Total nitrogen was estimated using Kjeldahl method, phosphorus using spectrophotometer and potassium using flamephotometer. Titration method was followed for organic carbon and chlorine. Atomic absorption spectrometry (AAS) was used for Fe, Cu, Mn, B, Zn. Total bacterial count was enumerated using microbiology of food

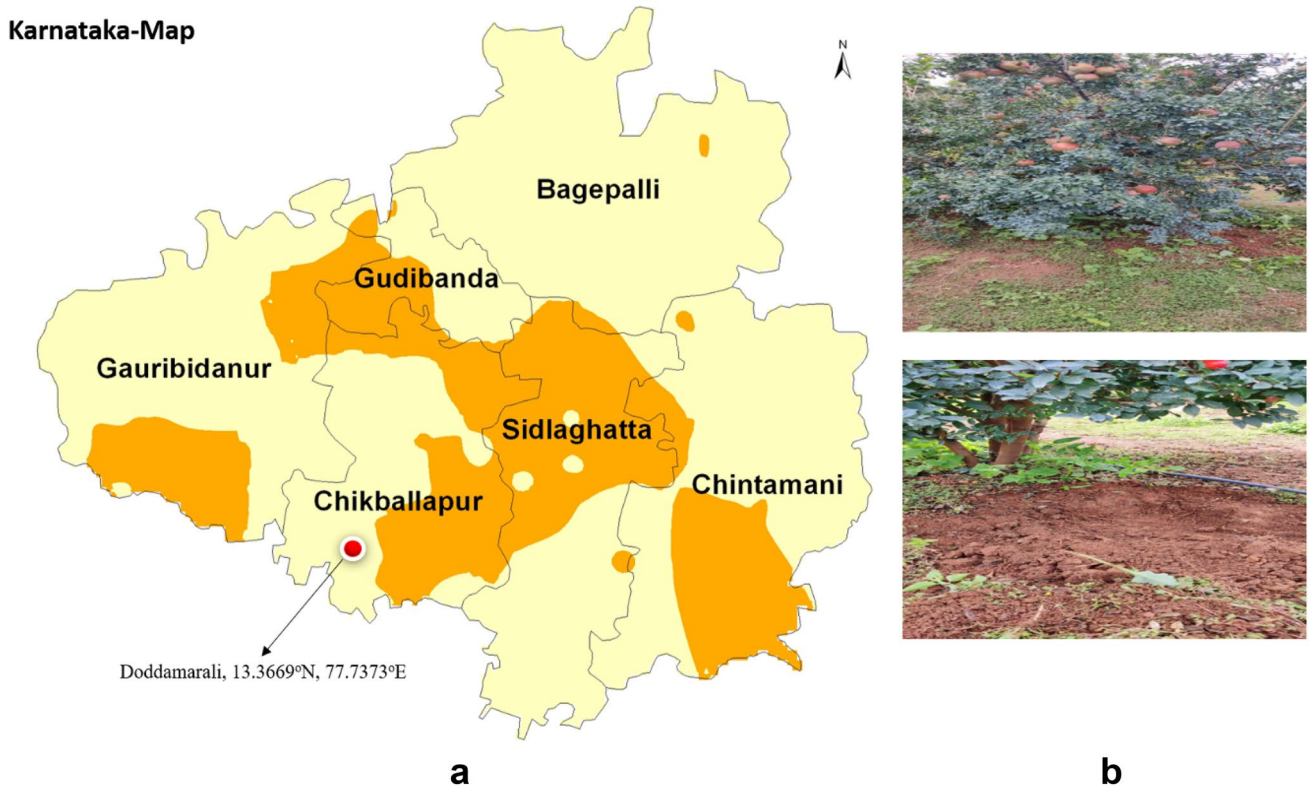


Fig. 1 **a** Study area map showing the site of soil sample collection in Chikkaballapur district of Karnataka. **b** Pomegranate field in Doddamaralli, Chikkaballapur where Bhagwa variety is grown

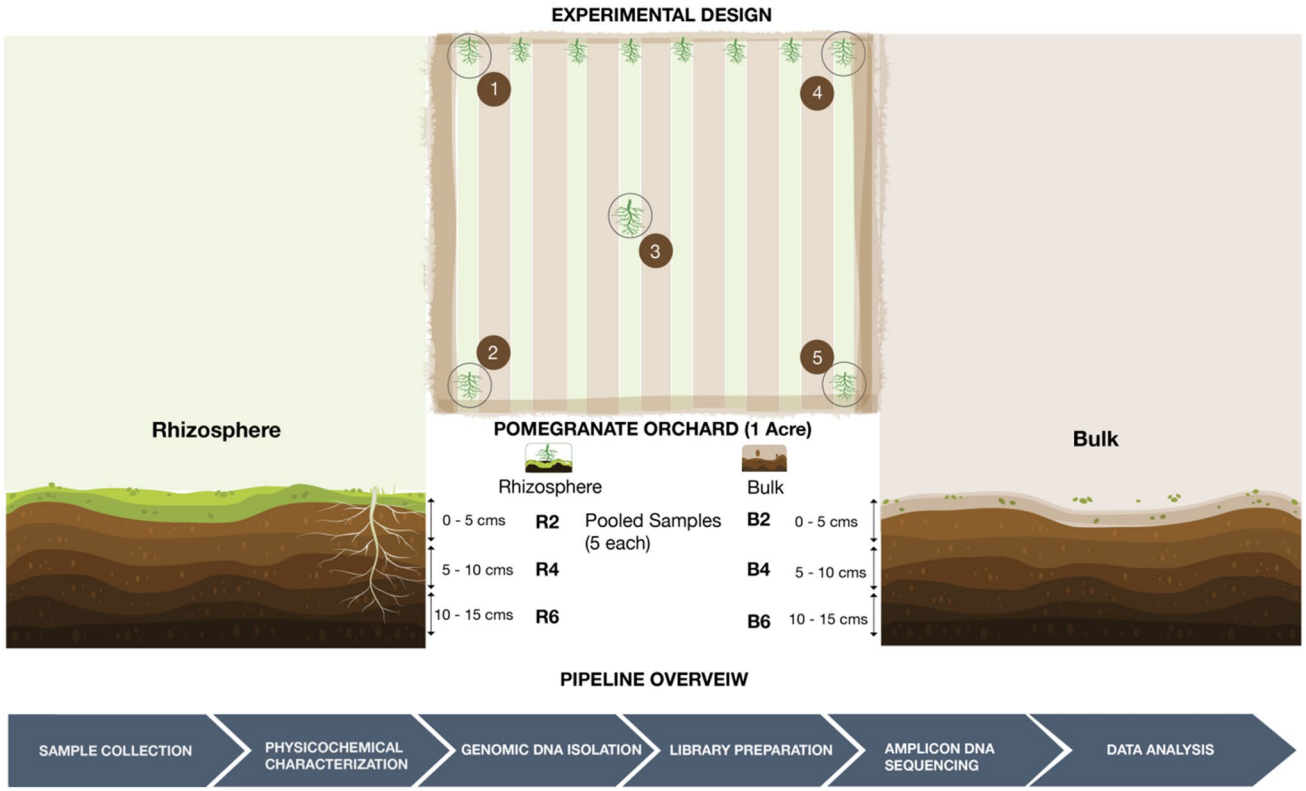


Fig. 2 Experimental design

and animal feeding stuffs, horizontal method for the enumeration of micro-organisms colony-count technique at 30 °C; Bureau of Indian Standards (IS 5401:2002) and total fungal count using method for yeast and mold count of foodstuffs & animal feeds; Bureau of Indian standards (IS 5403:1999).

Sample preparation, microbial community, DNA extraction and sequencing

The 16S targeted metagenomics sequencing was carried out to explore the microbial diversity of the soil samples from rhizosphere and bulk regions at various depths of 0–5 cms, 5–10 cms, 10–15 cms.

DNA extraction, QC and amplification of 16S rRNA gene

DNA extraction was done for all the six samples through commercially available kits such as QIAGEN, ZYMO RESEARCH DNA extraction as per the manufacturer's recommendation. Extracted DNA from all the samples were subjected to NanoDrop 1000 spectrophotometer (Thermoscientific) and GEL Check before being taken for PCR amplification: The NanoDrop readings of 260/280 at an approximate value of 1.8–2 was used to determine the quality of DNA.

40 ng of extracted DNA was used for amplification along with 10 pM of each primer. Universal primers 16F: 5′ AGA GTTTGATCMTGGCTCAG 3′ and 16R: 5′ TACGGYTAC CTTGTTACGACTT 3′ were used. 25 cycles of PCR were carried out maintaining the following conditions: Denaturation at 95 °C for 15 s, annealing at 60 °C for 15 s, elongation at 72 °C for 2 min, final extension at 72 °C for 10 min and hold at 4 °C. The amplified 16S PCR product was purified and subjected to GEL check and NanoDrop QC. The NanoDrop readings of 260/280 at an approximate value 1.8–2 is used to determine the DNA's quality. Sequencing was performed taking 1 µg of DNA template using Oxford Nanopore MinION platform.

Bioinformatics workflow

EPI2ME 16S analysis workflow followed by Quantitative Insights into Microbial Ecology (QIIME) was used to perform genus-level identification from single reads; with access to basecalled files for detailed investigations at the species and sub-species level. The phylogeny analysis of query sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment. The workflow was designed to BLAST basecalled sequence against the NCBI 16S bacterial database, which contains 16S sequences from different organisms. Each read

was classified based on percentage coverage and identity. The quality check for the sequences obtained was also subjected to QIIME workflow. KEGG pathway prediction was adopted to find the major pathway hits in rhizosphere soil.

Data analysis

Microbiome analyst, the web-based tool was used for data processing and statistical analysis. A total of six samples (B2, B4, B6, R2, R4, R6) and 1270 features or taxa were present in the current study. The sample data contained a total of six samples and one sample variables. The OTUs were annotated as SILVA label. Data Integrity Check was carried out before data analysis and Library Size was determined for the inspection of each sample. Data filtering was carried out after integrity check. A total of 579 low abundance features were removed based on prevalence and 25 low variance features were removed based on interquartile range. The number of features that remained after the data filtering step was 217.

For data normalization, the data were stored as a table with one sample per column and one variable (taxa or OTU) per row. Normalization was done using data rare faction and total sum normalization. Following that, no data transformation was performed.

Visualization of taxonomic composition in bulk and rhizosphere samples were done using Stacked bar/area plot and pie chart. Rarefaction curve analysis, used to present relationship between number of OTUs and number of sequences was performed using the modified function ggrare originated from ranacapa package2. Heat tree method was adopted to compare abundance of different taxonomic levels present in various depths of bulk and rhizosphere. Heat tree analysis was performed using R package metacoder package3. The prominent taxa in each sample was analyzed using this method.

Community profiling

Alpha diversity, Beta Diversity and Core microbiome analysis were carried out for the samples for community profiling.

Alpha diversity analysis This method was used to measure the diversity present within rhizosphere and bulk soil samples for each depth individually as well as for bulk and rhizosphere in general. Alpha diversity analysis was performed using the phyloseq package4. The results were plotted across bulk and rhizosphere samples and reviewed as box plots for each group or experimental factor. Alpha diversity measures across all the samples of bulk and rhizosphere for given diversity index as well as the diversity distribution using box plot for the given group/ experimental factor was calculated.

Beta diversity analysis This method was adopted to compare the microbial diversity between bulk and rhizosphere soil samples to arrive at the variance and similarities between samples. Each sample in bulk and rhizosphere soil were compared to every other five samples. Similarity or distance between samples was measured using Bray–Curtis distance method. Ordination-based Principle Coordinate Analysis (PCoA) plot was prepared. Beta diversity analysis was performed using the phyloseq package5. Ordination plot was represented in 2-D, Statistical significance was estimated using PERMANOVA.

Hierarchical clustering The rhizosphere and bulk soil data were subjected to hierarchical cluster analysis. Hierarchical clustering was performed with the hclust function in package stat and depicted as a heatmap.

Core microbiome analysis To identify the core taxa that remain unchanged in their composition across the rhizosphere and bulk samples, Core Microbiome Analysis was carried out. Sample prevalence and relative abundance (fractions) of taxa was identified using R package microbiome. The result was represented in the form of heatmap of core taxa or features where *Y*-axis represents the prevalence level of core features across the detection threshold (Relative abundance) range on *X*-axis.

Wet laboratory validation

Wet laboratory analysis was carried out to confirm the organisms identified through 16S rRNA targeted sequencing. Soil samples from R2 and B2 layers were serially diluted and plated on nutrient agar media for estimating the CFU (Colony Forming Units). Selective media were prepared for *E.coli, S. typhi* and *Pseudomonas aeruginosa*. Mac Conkey agar was used to culture *E.coli* and *S. typhi* whereas Centrimide Agar was used for culturing *Pseudomonas aeruginosa*.

Isolation of organisms from soil using selective media

Media preparation Ready to use Mac Conkey agar media (SRL, 76875) was prepared for isolation of *E.coli* and *S. typhi*, ready to use Cetrimide agar media (Himedia, MH024) was prepared for culturing *Pseudomonas aeruginosa* from the soil sample. Media was autoclaved at 15 psi pressure at 121 °C for 15 min.

Procedure of plating Test samples (1 ml) were tenfold diluted in 9 ml of water (10^{-1}). This was further serially diluted to (10^{-2}) and (10^{-3}). All three dilutions were plated on selective media by spread plate technique. The plates were incubated in both aerobic chamber at 37 °C for

24–48 h. After the incubation period colonies were observed and colony characterization was carried out.

Results

Physicochemical characterization of the rhizosphere and bulk soil

The pH value showed significant variation between bulk and rhizosphere ($p < 0.05$). The rhizosphere soil was found to be more alkaline (7.61–7.73) than bulk soil (6.85–7.1). Within each sample, considerable variation was not present among the layers. The soil was found to be neutral to alkaline with appreciable amount of macronutrients and micronutrients. There were differences in the nutrient status between bulk and rhizosphere soil, rhizosphere soil having better nutrient status than bulk. Electrical conductivity did not show significant variation between rhizosphere and bulk soil ($p > 0.05$) (Table 1).

With respect to total nitrogen content, the rhizosphere layers showed significant difference with the top layer, R2 having highest (0.2%) followed by R6 the bottom layer (0.18%) in the study ($p < 0.05$), whereas there was no significant difference with respect to bulk sample layers. Rhizosphere sample had significantly higher total nitrogen content than bulk ($p < 0.05$). The total phosphorous and potassium content did not show any variation between the samples and within the layers in both the cases. Organic carbon content did not show any significant difference between bulk and rhizosphere samples, nor among the layers. Fe content and Cu content did not show significant variation between rhizosphere sample and bulk sample ($p > 0.05$) as well as among the layers. Mn and Zn content also did not differ between rhizosphere and bulk samples. In case of Zn, within bulk sample the mean value showed significant difference ($p < 0.05$), between B4 and B6, B4 with maximum Zn content (25.2 ppm) whereas there was no significant variation within the rhizosphere layers (Table 1).

Total bacterial count did not show any variation between bulk and rhizosphere sample as well as within the layers. Total fungal count showed variation between bulk and rhizosphere with rhizosphere sample having increased CFU (176) compared to bulk (122.67), $p < 0.05$ (one tail t test). Within the sample, layer wise variation was not significant (Table 1).

To conclude, the rhizosphere sample showed significant variation in physio-chemical parameters across soil depth for pH, N, and total fungal content compared to bulk sample. No significant variation was reported in case of Mn, Cu, Cl, Zn, P & K levels. The P and K content remained the same across the soil depth in both the samples (0.01%). With respect to the soil physicochemical properties in the rhizosphere

Table 1 Physiochemical characterization of soil and total microbial count estimation

	pH	EC (µs/cm)	N (%)	P (%)	K (%)	C (%)	Cl (ppm)	Fe (%)	Cu (ppm)	Mn (ppm)	Zn (ppm)	B (ppm)	Total Bacterial Count/g (CFU)	Total Fungal Count/g (CFU)
B2	6.96	112	0.17	0.01	0.01	0.8	10	0.85	22	8.1	24	2.8	2600	110
B4	6.85	91	0.14	0.01	0.01	0.72	8	0.81	21.2	7.7	25.2	2.9	2420	136
B6	7.11	76	0.13	0.01	0.01	0.65	7	0.84	22.5	7.9	23.6	3	2542	122
B_A	6.97	93	0.15	0.01	0.01	0.72	8.33	0.83	21.9	7.9	24.27	2.9	2520.67	122.67
SD	0.13	18.08	0.02	0	0	0.08	1.53	0.02	0.66	0.2	0.83	0.1	91.88	13.01
R2	7.73	135	0.20	0.01	0.01	0.92	15	0.98	26.9	9.5	24.8	3.4	1968	154
R4	7.64	111	0.17	0.01	0.01	0.78	12	0.89	24.5	8.9	26.1	3.3	2140	176
R6	7.61	124	0.18	0.01	0.01	0.86	14	0.92	27.6	8.5	29.2	3.7	2640	198
R_A	7.66	123.33	0.18	0.01	0.01	0.85	13.67	0.93	26.33	8.97	26.7	3.47	2249.33	176
SD	0.06	12.01	0.01	0	0	0.07	1.53	0.05	1.63	0.5	2.26	0.21	349.09	22

B_A bulk soil average, R_A rhizosphere soil average, EC refers to electrical conductivity of the soil, the total microbial count was enumerated in terms of colony forming units. $p < 0.05$, one tailed t test

sample, the highest pH and nitrogen content were reported in R2, and fungal CFU in R6 layer (Table 1).

Soil depth and microbial diversity

The rhizosphere and bulk soil samples from various depths of 0–15 cms of pomegranate field (Bhagwa variety) collected and processed for 16S rRNA gene high-throughput sequencing using nanopore platform gave maximum read count for R6 (6805) and minimum read count for B6 (5408) with a total read count of 34,252 (Table S1). The DNA extracted from all the six layers of both rhizosphere and bulk samples were subjected to quality check using NanoDrop (Table S2). The alpha diversity did not show significant difference among rhizosphere soil layers based on the Chao 1 index ($p > 0.05$). Within bulk sample, there was significant variation between B2 and B4, with B2 showing the highest alpha diversity Cha0 1 index in terms of species richness (207.06 and 190.93), $p < 0.05$ (Table 2).

Alpha-diversity measure was estimated using Chao1index at OTU level represented in the form of boxplots. The maximum species richness in terms of OTU was associated with bulk compared to rhizosphere (Fig. 3b).

The rarefaction curves for both the bulk and rhizosphere sample revealed that the sequencing depth was adequate to explore the maximum number of species in terms of OTU (approximately 101 species) (Fig. 3a). Principal coordinates analysis (PCoA) was used to visualize the beta diversity between the rhizosphere and bulk sample with respect to the various layers in each sample. Bray Curtis distance represented the distance between the samples that reflected the dissimilarities between the samples and layers in terms of microbial diversity (Fig. 3c). Rhizosphere samples were more scattered showing higher microbial diversity than the bulk samples.

Hierarchical clustering was performed with the hclust function in package stat and was represented in the form of Heat Map depicting the clustering of organisms in both bulk and rhizosphere. The most abundant phylum in both the samples were proteobacteria (15%), followed by Firmicutes (10%), Planctomycetota (10%), Chloroflexi (9%), Acidobacteriota (7%) and Actinobacteriota (5%) comprising more than 50% of the total (Fig. 4, Figure S1).

Similarly, core microbiome analysis to identify the taxa that remain unchanged in the bulk and rhizosphere samples at OTU level was also analyzed and represented in the form of heat map where the Proteobacteria, Firmicutes, Planctomycetota, Chloroflexi, Acidobacteriota and Actinobacteriota represented the core microbiome (Figure S2).

Correlation analysis to establish the relationship among the organisms and pattern search to identify the Phylum correlated with Firmicutes to know the trend of association were depicted in the form of heat maps. Fermicutes showed strong negative correlation with Acidobacteriota, Planctomycetota, Verrucomicrobiota, Abditibacteriota and Gemmatimonadota whereas strong positive correlation was shown with Chloroflexi, Patescibacteria and Cyanobacteria ($p < 0.05$) (Figure S3).

Univariate analysis, RNA seq method, metagenomeSeq were compared for differential abundance analysis. Linear discriminant analysis (LDA) effect size was also calculated and error estimation was done through Random Forest method. The STAMP software was used for statistical analysis to prepare the Bar plots as well as the scatter plots for layer-wise comparison between the top, mid and bottom layers of rhizosphere and bulk as well as between rhizosphere sample and bulk sample in general. The analysis gave a detailed account of the most abundant and prominent organisms in each layer of rhizosphere compared to bulk. The most abundant organism in R2 was found to be *Pirellula staleyi* (50%) compared to B2 (15%). The abundant organism in B2 was *Candidatus solibacter usitatus* (80%) as clearly reflected in the profile bar compared to R2 (38%). Similar results were reported for B4 Vs R4 and B6 Vs R6. The same trend reflected for rhizosphere and bulk sample layers in general (Figs. 5a, b, 6a, b, Table 3).

Table 2 Alpha diversity analysis

	Sample type	Samples	Variable	Value
1	bulk	b2	Chao1	207.06 ± 3.78
2	bulk	b4	Chao1	190.923 ± 3.34
3	bulk	b6	Chao1	201.33 ± 3.40
4	rhizosphere	r2	Chao1	201.56 ± 4.63
5	rhizosphere	r4	Chao1	200.06 ± 2.70
6	rhizosphere	r6	Chao1	209.50 ± 5.55

Pathway predictions

KEGG Pathway prediction gave the first hit as arginine and proline metabolism (gamma-glutamyl putrescine oxidase) followed by purine metabolism, monobactam biosynthesis, sulfur metabolism, secondary metabolite synthesis and microbial metabolism in diverse environments in case of rhizosphere soil ($p < 0.05$) (Table 4, Fig. 7).

KEGG Pathway prediction of rhizosphere soil samples revealed the predominance of gamma-glutamyl putrescine oxidase activity responsible for argine and proline metabolism followed by cysNC; bifunctional enzyme involved in sulphur metabolism.

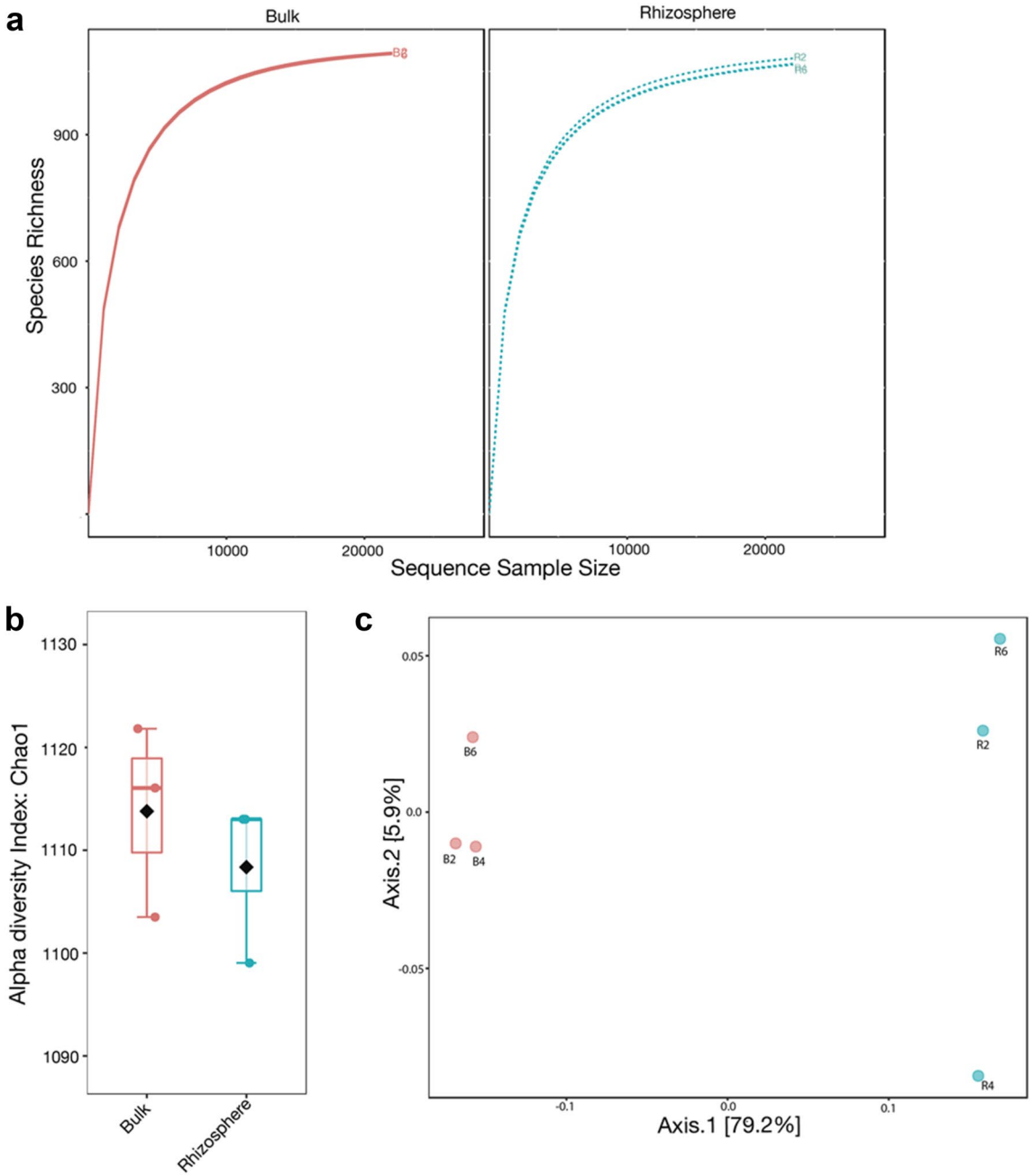


Fig. 3 **a** Rarefaction curves **b** Alpha diversity index/ box plot **c** Beta diversity analysis using Bray Curtis distance method. Rare faction curve denoting the species richness and the plateau denoting optimal sequencing depth. Alpha diversity measure using Chao1 at OTU level represented as boxplot. Each boxplot represents the diversity distribution of a group present within Sample Type class [Statistical significance: *p*-value: 0.52514; [*t*-test] statistic: 0.70804]. Beta diversity analysis was performed using the phyloseq package5. Statistical significance was calculated using [PERMANOVA] *F*-value: 14.505; *R*-squared: 0.78384; *p*-value < 0.01

Wet laboratory validation: cultural characteristics of isolated organisms in selective media

In the wet lab studies, Rhizosphere soil sample reported more bacterial microbial load in terms of CFU in Nutrient Agar media ($723 \times 10^2 \pm 4.35$) compared to the Bulk Sample $554 \times 10^2 \pm 2$ (Table S3 and Figure S4). The bacterial species that could be cultured in selective media were *E coli, Salmonella, Pseudomonas aeruginosa* and *Enterobacter aerogenes*. Colony characterization of *E coli* in Mac Conkey agar revealed Pink colonies with entire margin and microscopic observation revealed rod shaped organisms, whereas *Salmonella* colonies appeared colourless, smooth and transparent. The CFU was more for

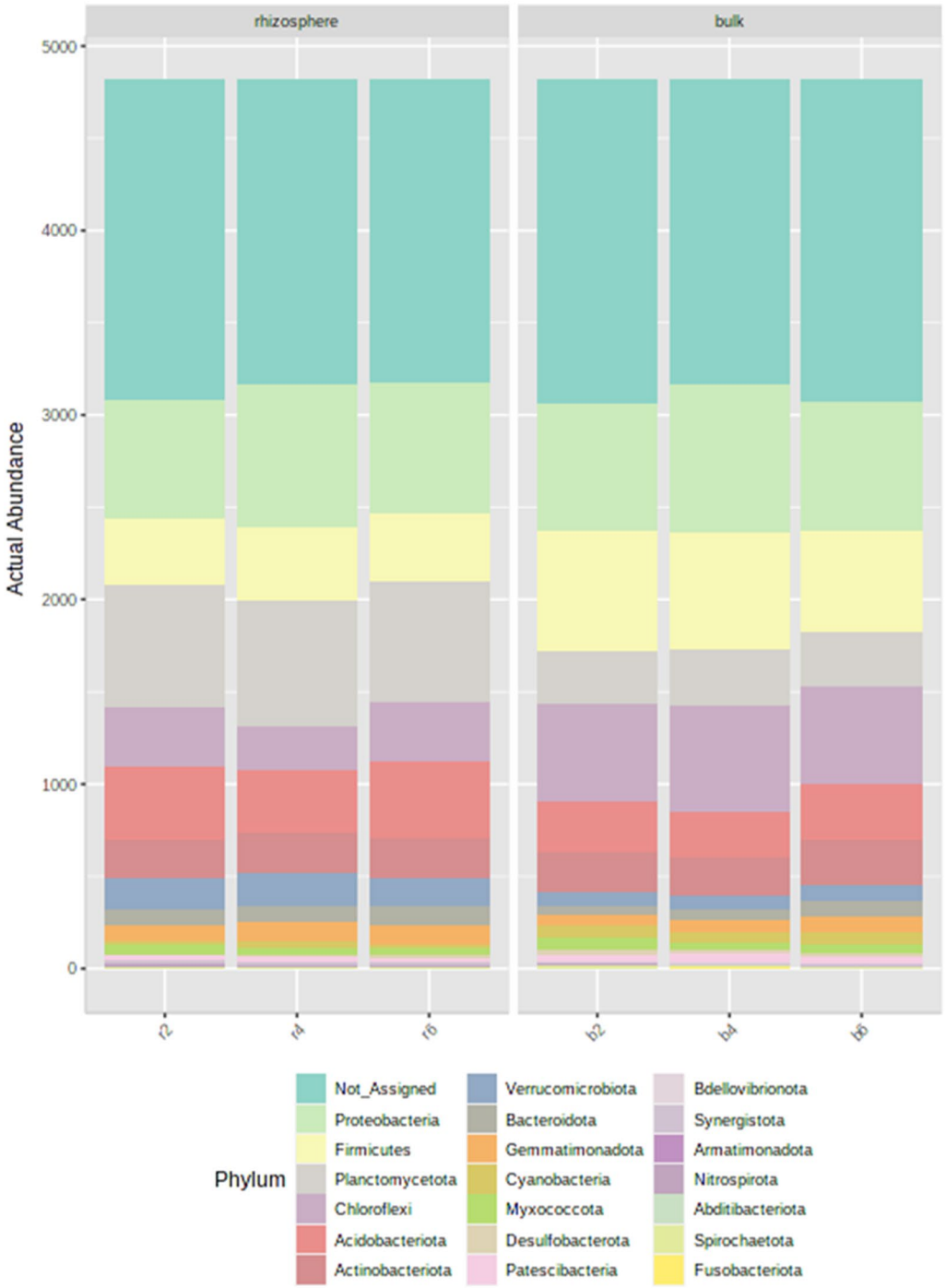
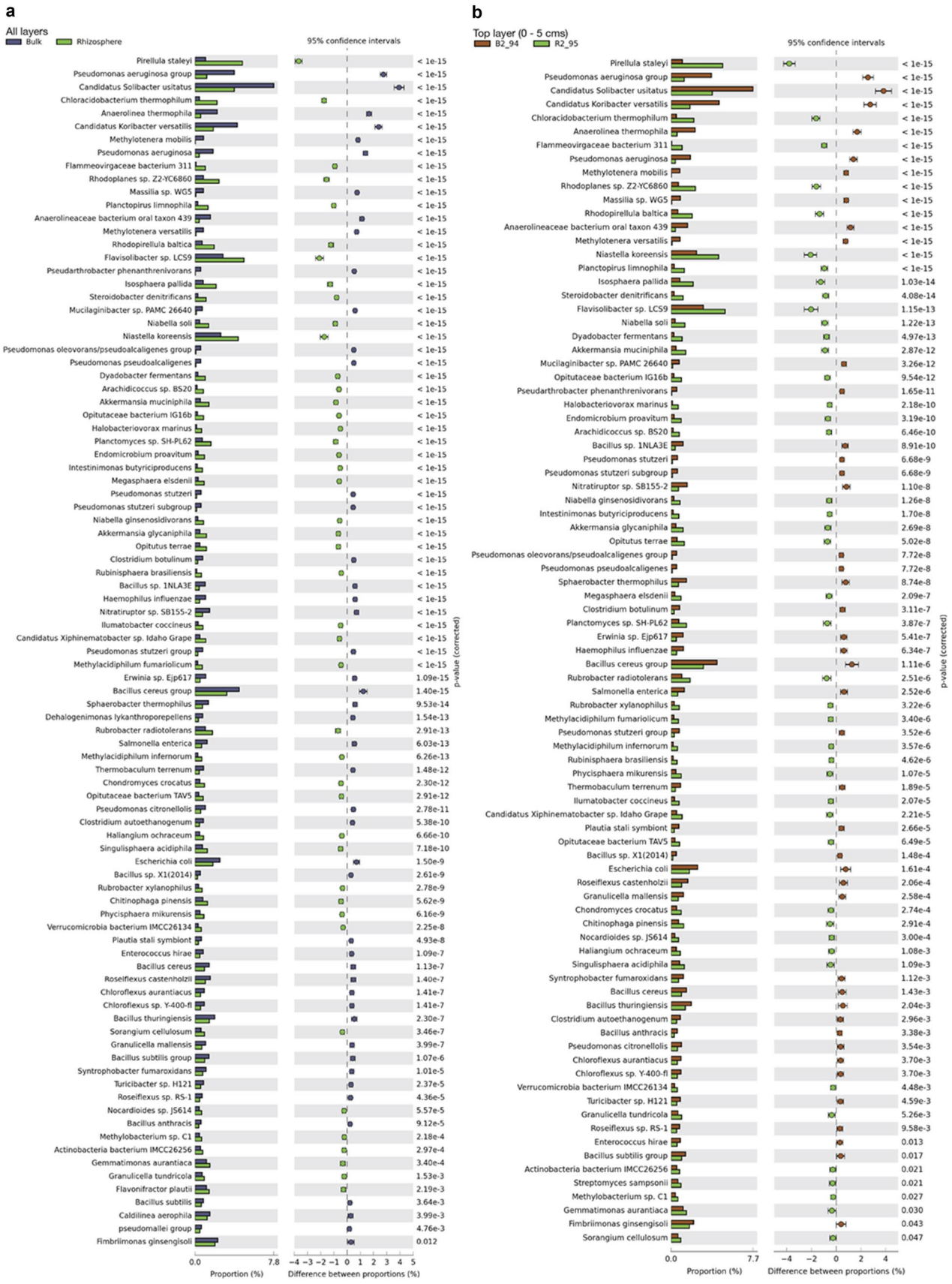


Fig. 4 Heat map representing the abundance at phylum level with proteobacteria showing maximum abundance

Salmonella compared to *E coli* with Bulk sample reporting more CFU (Table S4, Figure S5).

Colony characterization of *Pseudomonas aeruginosa* in Cetrimide agar revealed yellowish green colonies with curled margin and microscopic observation revealed rod shaped organisms, whereas *Enterobacter aerogenes* colonies appeared blue, smooth and mucoid. The CFU was more for *Enterobacter aerogenes* compared to *Pseudomonas aeruginosa* with bulk sample reporting more CFU (Table S5, Figure S6).


a
All layers
Bulk
Rhizosphere
95% confidence intervals
Pirellula staleyi
Pseudomonas aeruginosa group
Candidatus Solibacter usitatus
Chloracidobacterium thermophilum
Anaerolinea thermophila
Candidatus Koribacter versatilis
Methylotenera mobilis
Pseudomonas aeruginosa
Flammeovirgaceae bacterium 311
Rhodoplanes sp. Z2-YC6860
Massilia sp. WG5
Planctopirus limnophila
Anaerolineaceae bacterium oral taxon 439
Methylotenera versatilis
Rhodopirellula baltica
Flavisolibacter sp. LCS9
Pseudarthrobacter phenanthrenivorans
Isosphaera pallida
Steroidobacter denitrificans
Mucilaginibacter sp. PAMC 26640
Niabella soli
Niastella koreensis
Pseudomonas oleovorans/pseudoalcaligenes group
Pseudomonas pseudoalcaligenes
Dyadobacter fermentans
Arachidicoccus sp. BS20
Akkermansia muciniphila
Opitutaceae bacterium IG16b
Halobacteriovorax marinus
Planctomyces sp. SH-PL62
Endomicrobium proavitum
Intestinimonas butyriciproducens
Megasphaera elsdenii
Pseudomonas stutzeri
Pseudomonas stutzeri subgroup
Niabella ginsenosidivorans
Akkermansia glycaniphila
Opitutus terrae
Clostridium botulinum
Rubinisphaera brasiliensis
Bacillus sp. 1NLA3E
Haemophilus influenzae
Nitratiruptor sp. SB155-2
Ilumatobacter coccineus
Candidatus Xiphinematobacter sp. Idaho Grape
Pseudomonas stutzeri group
Methylacidiphilum fumariolicum
Erwinia sp. Ejp617
Bacillus cereus group
Sphaerobacter thermophilus
Dehalogenimonas lykanthroporepellens
Rubrobacter radiotolerans
Salmonella enterica
Methylacidiphilum infernorum
Thermobaculum terrenum
Chondromyces crocatus
Opitutaceae bacterium TAV5
Pseudomonas citronellolis
Clostridium autoethanogenum
Hallangium ochraceum
Singulisphaera acidiphila
Escherichia coli
Bacillus sp. X1(2014)
Rubrobacter xylanophilus
Chitinophaga pinensis
Phycisphaera mikurensis
Verrucomicrobia bacterium IMCC26134
Plautia stali symbiont
Enterococcus hirae
Bacillus cereus
Roseiflexus castenholzii
Chloroflexus aurantiacus
Chloroflexus sp. Y-400-fl
Bacillus thuringiensis
Sorangium cellulosum
Granulicella mallensis
Bacillus subtilis group
Syntrophobacter fumaroxidans
Turicibacter sp. H121
Roseiflexus sp. RS-1
Nocardioides sp. JS614
Bacillus anthracis
Methylobacterium sp. C1
Actinobacteria bacterium IMCC26256
Gemmatimonas aurantiaca
Granulicella tundricola
Flavonifractor plautii
Bacillus subtilis
Caldilinea aerophila
pseudomallei group
Fimbriimonas ginsengisoli
< 1e-15
1.09e-15
1.40e-15
9.53e-14
1.54e-13
2.91e-13
6.03e-13
6.26e-13
1.48e-12
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6.66e-10
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2.61e-9
2.78e-9
5.62e-9
6.16e-9
2.25e-8
4.93e-8
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1.13e-7
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2.97e-4
3.40e-4
1.53e-3
2.19e-3
3.64e-3
3.99e-3
4.76e-3
0.012
p-value (corrected)
0.0
7.8
Proportion (%)
−4 −3 −2 −1 0 1 2 3 4 5
Difference between proportions (%)
b
Top layer (0 - 5 cms)
B2_94
R2_95
95% confidence intervals
Pirellula staleyi
Pseudomonas aeruginosa group
Candidatus Solibacter usitatus
Candidatus Koribacter versatilis
Chloracidobacterium thermophilum
Anaerolinea thermophila
Flammeovirgaceae bacterium 311
Pseudomonas aeruginosa
Methylotenera mobilis
Rhodoplanes sp. Z2-YC6860
Massilia sp. WG5
Rhodopirellula baltica
Anaerolineaceae bacterium oral taxon 439
Methylotenera versatilis
Niastella koreensis
Planctopirus limnophila
Isosphaera pallida
Steroidobacter denitrificans
Flavisolibacter sp. LCS9
Niabella soli
Dyadobacter fermentans
Akkermansia muciniphila
Mucilaginibacter sp. PAMC 26640
Opitutaceae bacterium IG16b
Pseudarthrobacter phenanthrenivorans
Halobacteriovorax marinus
Endomicrobium proavitum
Arachidicoccus sp. BS20
Bacillus sp. 1NLA3E
Pseudomonas stutzeri
Pseudomonas stutzeri subgroup
Nitratiruptor sp. SB155-2
Niabella ginsenosidivorans
Intestinimonas butyriciproducens
Akkermansia glycaniphila
Opitutus terrae
Pseudomonas oleovorans/pseudoalcaligenes group
Pseudomonas pseudoalcaligenes
Sphaerobacter thermophilus
Megasphaera elsdenii
Clostridium botulinum
Planctomyces sp. SH-PL62
Erwinia sp. Ejp617
Haemophilus influenzae
Bacillus cereus group
Rubrobacter radiotolerans
Salmonella enterica
Rubrobacter xylanophilus
Methylacidiphilum fumariolicum
Pseudomonas stutzeri group
Methylacidiphilum infernorum
Rubinisphaera brasiliensis
Phycisphaera mikurensis
Thermobaculum terrenum
Ilumatobacter coccineus
Candidatus Xiphinematobacter sp. Idaho Grape
Plautia stali symbiont
Opitutaceae bacterium TAV5
Bacillus sp. X1(2014)
Escherichia coli
Roseiflexus castenholzii
Granulicella mallensis
Chondromyces crocatus
Chitinophaga pinensis
Nocardioides sp. JS614
Haliangium ochraceum
Singulisphaera acidiphila
Syntrophobacter fumaroxidans
Bacillus cereus
Bacillus thuringiensis
Clostridium autoethanogenum
Bacillus anthracis
Pseudomonas citronellolis
Chloroflexus aurantiacus
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Verrucomicrobia bacterium IMCC26134
Turicibacter sp. H121
Granulicella tundricola
Roseiflexus sp. RS-1
Enterococcus hirae
Bacillus subtilis group
Actinobacteria bacterium IMCC26256
Streptomyces sampsonii
Methylobacterium sp. C1
Gemmatimonas aurantiaca
Fimbriimonas ginsengisoli
Sorangium cellulosum
< 1e-15
1.03e-14
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2.18e-10
3.19e-10
6.46e-10
8.91e-10
6.68e-9
6.68e-9
1.10e-8
1.26e-8
1.70e-8
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7.72e-8
7.72e-8
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3.11e-7
3.87e-7
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3.57e-6
4.62e-6
1.07e-5
1.89e-5
2.07e-5
2.21e-5
2.66e-5
6.49e-5
1.48e-4
1.61e-4
2.06e-4
2.58e-4
2.74e-4
2.91e-4
3.00e-4
1.08e-3
1.09e-3
1.12e-3
1.43e-3
2.04e-3
2.96e-3
3.38e-3
3.54e-3
3.70e-3
3.70e-3
4.48e-3
4.59e-3
5.26e-3
9.58e-3
0.013
0.017
0.021
0.021
0.027
0.030
0.043
0.047
p-value (corrected)
0.0
7.7
Proportion (%)
−4 −2 0 2 4
Difference between proportions (%)

◂**Fig. 5** **a**, **b** Differential abundance Analysis of bacterial species. The figure depicts extended error bar plots denoting the samples (**a**) mid-layer (**b**) bottom layer of the corresponding bulk B (in brown) and rhizosphere sample R (in green). The axes denote the corrected p-values against the proportion of reads and difference between the proportions with 95% confidence intervals (colour figure online)

Discussion

Physicochemical properties of soil and microbial diversity

There was significant variation in certain parameters of rhizosphere soil compared to Bulk soil such as alkalinity of rhizosphere soil compared to bulk though the significant difference in pH was not detected among the layers in rhizosphere and bulk soil. In general, soil of pomegranate filed was found to be neutral to alkaline in nature. The soil pH has profound influence in shaping the microbial diversity as already reported (Tan et al. 2020) especially with respect to alpha diversity. Tan and his coworkers reported that in the pH range of 5.23–8.86, there was a positive correlation with the OTU in maize cropping system. This also supports the rhizosphere specific microbial diversity with the prevalence of *Pirellula staleyi* and *Flavisolibacter* obtained in our study compared to bulk soil. Similar findings establishing the correlation of soil pH and microbial community were reported in the permafrost region of North Eastern China (Ren et al. 2018).

Electrical conductivity did not show significant variation between rhizosphere and bulk soil ($p > 0.05$). The significant increase in nitrogen content in the rhizosphere sample compared to bulk could be attributed to the plant secretions and other exudates along with the presence of litter in the rhizosphere region. The finding that the abundance of Planctomycetes member *Pirellula staleyi* which can degrade putrescine exudate from plant roots could also be the reason for augmentation of nitrogen content in the rhizosphere (Lu et al. 2022). The higher nitrogen content in rhizosphere would have contributed to higher number of organisms/biomass in rhizosphere reflected in terms of higher read counts obtained in sequencing than in bulk. The absence of the root effect was reflected in bulk sample with relatively lower nitrogen content. Similar results of relatively higher nitrogen content in the top soil (Zhou et al. 2008; Tang et al. 2018).

The total P, K, Fe, Cu and Mn and organic carbon content did not vary between the samples and within the layers in rhizosphere and bulk soil. The increased fungal CFU in rhizosphere could also be attributed to the plant root effect compared to bulk soil. Another reason could be the zero-tillage practice adopted in the farming system of pomegranate (Bhattarai 2015).

Community profiling

The phyloseq package employed to calculate the alpha diversity revealed only significant difference between B2 and B4, with B2 showing the highest alpha diversity Cha0 1 index in terms of species richness (207.06 and 190.93) which could be also correlated with the highest read count reported in B2 sample (5810). Similar decrease in alpha diversity across soil depth was also reported in legume fields of China (Yan et al. 2019). *Candidatus solibacter usitatus* dominated in all the bulk layers.

The bacterial community among the rhizosphere soil layers almost remained the same with *Pirelulla staleyi* and *Flavisolibacter sp* LCS9 being the most abundant in all the three layers. The absence of change in microbial diversity could be due to the negligible variations in physicochemical properties of rhizosphere soil across the depth.

Beta diversity

Beta diversity analyzed through Phyloseq package 5 and visualized through PCoA adopting Bray- Curtis distance method showed higher diversity for rhizosphere samples, all the three layers well separated, R6 showing the maximum diversity in terms of highest dimension of variation (79.2%) and second highest dimension of variation for B6 (5.9%). B2 and B4 samples were clustered together showing similarity in the microbial diversity. The higher F value and R^2 value (14.505 and 0.78384) gives a clear indication that the root zone effect can play a major role in shaping the microbial diversity supporting the higher beta diversity in rhizosphere soil. Similar findings were reported in the rhizosphere and bulk samples of *Ranunculus glacialis* along a high alpine altitudinal gradient (Praeg et al. 2019).

The hierarchical clustering and core microbiome analysis through R package microbiome depicted in the form of heat map revealed higher prevalence of Proteobacteria, Firmicutes, Planctomycetota, Chloroflexi, Gammaproteobacteria and Actinobacteriota. Similar findings of abundance of these phyla were reported in biofuel cropping system (Zhang et al. 2017), fallow field (Ko et al. 2017) and unfertilized aerable soil (Wang et al. 2018). These findings lead to the conclusion that the common phyla remain unaltered irrespective of farming practices.

Fermicutes showed strong negative correlation with Acidobacteriota, Planctomycetota, Verrucomicrobiota, Abditibacteriota and Gemmatimonadota whereas strong positive correlation was shown with Chloroflexi, Patescibacteria and Cyanobacteria though Fermicutes shared dominance with Planctomycetota which is an interesting finding.

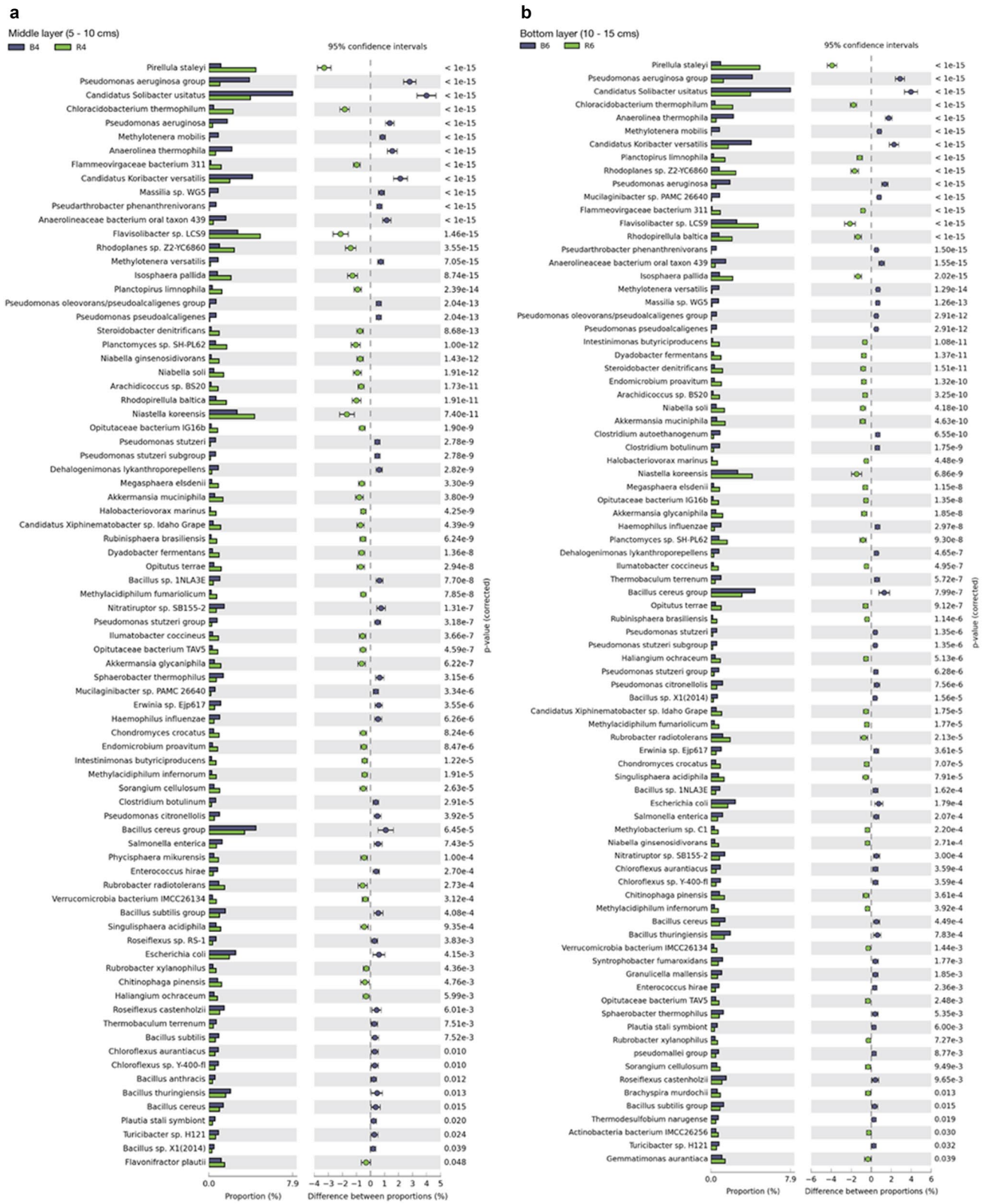

Fig. 6 **a**, **b** Differential abundance Analysis of bacterial species. The figure depicts extended error bar plots denoting the samples (**a**) mid-layer (**b**) bottom layer of the corresponding bulk B (in brown) and rhizosphere sample R (in green). The axes denote the corrected *p*-values against the proportion of reads and difference between the proportions with 95% confidence intervals (colour figure online)

Table 3 List of organisms at species level in the order of abundance (OTU) present in various layers

Name	Max	B2_	R2_	B4_	R4_	B6_	R6_
Flavisolibacter sp. LCS9	513	322	513	276	488	266	484
Pirellula staleyi	502	112	491	114	445	100	502
Niastella koreensis	452	253	452	269	435	275	423
Candidatus Solibacter usitatus	820	818	392	805	394	820	405
Bacillus cereus group	458	458	308	453	338	453	316
Rhodoplanes sp. Z2-YC6860	255	72	231	100	241	85	255
Conexibacter woesei	270	270	225	238	209	235	256
Chloracidobacterium thermophilum	229	53	216	44	229	40	222
Isosphaera pallida	222	86	210	81	209	86	222
Rhodopirellula baltica	217	67	201	66	165	81	217
Rubrobacter radiotolerans	196	108	181	93	148	122	196
Candidatus Koribacter versatilis	478	478	178	418	199	416	178
Escherichia coli	264	264	175	256	193	250	172
Fimbriimonas ginsengisoli	243	224	174	243	211	238	216
Planctomyces sp. SH-PL62	168	77	147	63	168	83	166
Gemmatimonas aurantiaca	157	118	147	126	157	113	146
Akkermansia muciniphila	145	50	140	54	133	58	145
Bacillus thuringiensis	207	200	136	207	158	200	137
Niabella soli	141	40	131	47	141	54	139
Flavonifractor plautii	153	109	128	117	148	126	153
Planctopirus limnophila	144	31	127	31	122	26	144
Singulisphaera acidiphila	135	84	127	68	112	78	135
Opitutus terrae	127	58	127	46	115	45	104
Pseudomonas aeruginosa group	426	403	124	390	104	426	127
Chitinophaga pinensis	139	74	120	82	121	86	139
Caldilinea aerophila	154	154	116	149	126	154	124
Steroidobacter denitrificans	119	29	114	22	97	37	119
Akkermansia glycaniphila	121	47	113	51	114	49	121
Flammeovirgaceae bacterium 311	115	6	105	17	115	11	96
Candidatus Xiphinematobacter sp. Idaho Grape	112	55	105	41	112	54	108
Dyadobacter fermentans	105	27	105	33	96	28	103
Granulicella tundricola	101	68	101	76	86	70	92
Roseiflexus castenholzii	167	167	100	147	102	155	112
Bacillus subtilis group	157	143	100	157	99	130	93
Bacillus cereus	153	153	97	138	99	143	89
Opitutaceae bacterium IG16b	97	26	97	23	83	22	77
Brachyspira murdochii	97	91	97	84	82	64	95
Phycisphaera mikurensis	96	47	96	46	91	60	80
Endomicrobium proavitum	107	29	95	34	81	33	107
Chondromyces crocatus	96	54	95	44	96	48	95
Streptomyces sampsonii	96	67	93	72	96	96	79
Megasphaera elsdenii	93	37	93	27	89	28	88
Sorangium cellulosum	101	69	91	50	101	61	93
Haliangium ochraceum	96	51	86	49	80	43	96
Niabella ginsenosidivorans	96	28	85	22	96	39	78
Actinobacteria bacterium IMCC26256	81	57	81	59	79	50	74
Acidobacterium capsulatum	94	84	80	94	89	88	77
Opitutaceae bacterium TAV5	84	39	80	31	84	47	81
Arachidicoccus sp. BS20	87	20	78	20	87	22	85
Rubrobacter xylanophilus	78	32	78	38	67	41	69
Ilumatobacter coccineus	91	34	76	35	91	25	74

Table 3 (continued)

Name	Max	B2_	R2_	B4_	R4_	B6_	R6_
Bacillus subtilis	94	90	73	94	60	71	56
Intestinimonas butyriciproducens	84	21	73	25	66	18	84
Terriglobus saanensis	73	68	73	72	62	63	66
Syntrophobacter fumaroxidans	122	122	72	103	85	118	74
Nocardioides sp. JS614	71	36	71	41	59	50	66
Sphaerobacter thermophilus	154	154	70	137	69	125	84
Methylacidiphilum fumariolicum	79	27	70	22	73	34	79
Nitratiruptor sp. SB155-2	160	160	69	146	68	141	86
Granulicella mallensis	123	123	68	95	70	109	67
Halobacteriovorax marinus	68	14	68	15	66	15	66

Table 4 Pathway prediction

Pathway Id	Bpool	Std Dev	Rpool	Std Dev	Significance *P*-value	
K09471	299.67	9.07	433	33.15	0.04	ko:K09471 puuB, ordL; gamma-glutamylputrescine oxidase [EC:1.4.3.-]
K00955	283.67	4.73	393	17.58	0.04	ko:K00955 cysNC; bifunctional enzyme CysN/CysC [EC:2.7.7.4 2.7.1.25]
K02419	287	2	324.67	4.73	0.04	ko:K02419 fliP; flagellar biosynthesis protein FliP
K00480	228	13.45	324.33	23.25	0.04	ko:K00480 E1.14.13.1; salicylate hydroxylase [EC:1.14.13.1]
K02279	249.33	2.89	323	24.25	0.05	ko:K02279 cpaB, rcpC; pilus assembly protein CpaB
K00612	258.33	8.02	322.67	27.23	0.05	ko:K00612 nodU; carbamoyltransferase [EC:2.1.3.-]
K16092	316	3.46	320	22.27	0.05	ko:K16092 btuB; vitamin B12 transporter
K14161	229.33	3.06	318	9.17	0.05	ko:K14161 imuB; protein ImuB
K09812	272	1	318	7.21	0.05	ko:K09812 ftsE; cell division transport system ATP-binding protein
K10979	228.67	2.52	318	15.72	0.05	ko:K10979 ku; DNA end-binding protein Ku
K02073	280.67	15.18	317.67	9.45	0.05	ko:K02073 metQ; D-methionine transport system substrate-binding protein
K03293	257.67	16.5	317.67	24.09	0.05	ko:K03293 TC.AAT; amino acid transporter, AAT family
K06162	241.33	9.07	317	18.25	0.05	ko:K06162 phnM; alpha-D-ribose 1-methylphosphonate 5-triphosphate diphosphatase [EC:3.6

Microbial abundance and soil depth

Phylum level, Proteobacteria dominated followed by Firmicutes, Planctomycetota, Chloroflexi, Gammaproteobacteria and Actinobacteriota. *Pirellula staleyi,* belonging to Phylum Planctomycetes was the most abundant species discovered in the study in the rhizosphere region compared to Bulk soil. Similar group of phyla were reported by Li et al 2021 (Li et al. 2021). It is very difficult to get the pure cultures of this phylum, only metagenomics approaches have been successful in unveiling the various species of Plantomycetes (Elshahed et al. 2007). Following *Pirellula, Flavisolibacter LC59, Niastella koreensis* dominated the Rhizosphere layers from R2 to R6 compared to Bulk soil. Another species of *Flavisolibacter, Flavisolibacter nicotianae* was also reported in *Nicotiana tabacum* rhizosphere (Li et al. 2019). *Candidatus solibacter usitatus* followed by *Pseudomonas aueruginosa* were more abundant across the bulk soil layers of Pomegranate Variety Bhagwa. The rhizosphere layers had few organisms which were unique to only pomegranate root zone such as Flammeovirgaceae bacterium 311 (R2, R4, R6) and *Halobacteriovorax marinus* (R2, R4, R6) (Table 3).

The role of *Halobacteriovorax* in predating the soil pathogens reported could be linked to our study with the possible role of the organism in helping the plant growth by controlling the soil pathogens in the rhizosphere zone (Oyedara et al. 2016). The presence of *Flammeovirgaceae bacterium* was also reported in the roots of *Salicornia europaea* in Central Poland (Furtado et al. 2019).

Bulk soil had more number of organisms which were very unique to Pomegranate such as *Methylotenera versatilis, Methylotenera mobilis, Massilia sp* WG5, *Pseudobacter phenanthrenivorans, Pseudomonas pseudoalcaligenes* (B2, B4, B6) and *Mucilaginibacter sp* PAMC 26640 (Figs. 5a, b, 6a, b, Table 3, Table S6). This leads to the finding that the rhizosphere of pomegranate field had a subset of microorganism found in Bulk soil. This finding is also in line with the reports earlier where the microbial community in the

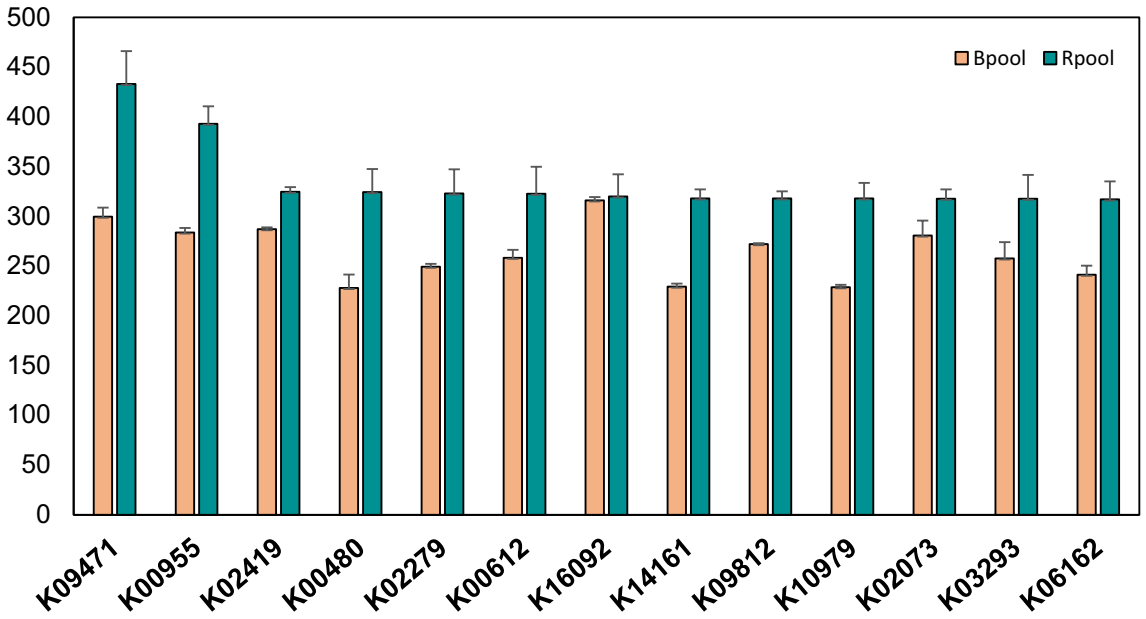

Fig. 7 Pathway Prediction. The figure depicts the various significant ($p < 0.05$) pathway hits and their respective KEGG Ids from the pooled samples Bpool representing the Bulk layer and the Rpool representing the Rhizosphere. The sequenced data from various soil depths of rhizosphere and bulk samples were deposited in SRA repository (Table S7)

rhizosphere was analyzed through Shot Gun Metagenomics (Mendes et al. 2014).

Pathway prediction

The first hit which showed significant increase in the rhizosphere sample is arginine and proline metabolism pathway involving gamma-glutamylputrescine oxidase for putrescene catabolism. This pathway is involved in stress response as well as regulation of putrescene (Schneider et al. 2013). The second hit was related to bifunctional enzyme CysN/CysC involved in sulfur metabolism, hydrogen sulfide biosynthesis. This protein is involved in step 1 and 2 of the subpathway that synthesizes sulfite from sulfate. Proteins known to be involved in the 3 steps of the subpathway are Sulfate adenylyltransferase subunit 2 (cysD), Bifunctional enzyme CysN/CysC (cysNC) Bifunctional enzyme CysN/CysC (cysNC) and phosphoadenosine phosphosulfate reductase (cysH). This sub pathway is part of the hydrogen sulfide biosynthesis pathway, which is itself is an integral part of Sulfur metabolism. This finding is also in line with the study done by Elshahed (Elshahed et al. 2007) where the sulfur reduction mechanism of the organism is described as a strategy of survival under anaerobic conditions, i.e., the metabolic functions assigned to planctomycetes like *Pirellula* which showed highest abundance in rhizosphere.

H_2S has great positive impact on plant root development and ABA-mediated abiotic stress response through stomatal closure (Li et al. 2022). Since *Pirellula,* belonging to planctomycetes has the ability to reduce sulfur and convert to H2S, it can positively influence the growth and development of pomegranate variety Bhagwa. Another major pathway hit was with respect to the one associated with xenobiotic compound degradation involving salicylate hydroxylase which could be the reason for the bioremediation of xenobiotic compounds used in the pomegranate field thereby protecting the plant and the soil ecosystem from toxicity.

The fact that the rhizosphere community selection of microbes is influenced by the functional attributes of the microorganism was also reported in a similar study where in comparative analysis was carried out in terms of the taxonomical composition and functional pathways of the rhizosphere and bulk soil using 16S rRNA amplicon based sequencing (Yan et al. 2019).

Wet lab studies

The major drawback in exploring the microbial diversity in various ecosystems is the fact that only 1–2% of these microorganisms can be cultured in the lab. Metagenomics approaches can efficiently unveil the diversity of bacterial community (Cardenas and Tiedje 2008). The validation of 16S rRNA targeted amplicon sequencing was done through wet lab studies which could provide the data regarding presence of only four species out of 101 species obtained through metagenomics approach. *E coli, Salmonella, Pseudomonas aeruginosa* and *Enterobacter aerogenes* were the species that could be cultured. This further emphasize the

importance of culture independent techniques in exploring the microbial diversity.

The rhizosphere soil reported higher CFU/ml than the bulk soil. The number of reads obtained through metagenomics approach was also higher in rhizosphere soil highlighting the root effect in enhancing the bacterial population compared to bulk soil. The existing studies in pomegranate have only employed conventional culture techniques to isolate the microbes from rhizosphere where only few organisms could be cultured (Shahaby et al. 2016). Only 13 bacterial species could be isolated out of which *Pseudomonas aeruginosa, Bacillus subtilis., Streptomyces species* were common to both the studies. The role of *Pseudomonas aeruginosa* could be conferring systemic resistance to pathogens in plants like beans through salicylic acid production (de Meyer et al. 1999; Audenaert et al. 2002).

Conclusion

This study involving metagenomics approach is the pioneer attempt in exploring microbial diversity of pomegranate rhizosphere**.** Our study also reports the presence of *Pirellula staleyi* for the first time in the pomegranate field**.** Being a planctomycetes member, it can enhance the plant growth by the production of H_2S influencing root growth and ABA mediated abiotic stress response positively. The ability to degrade putrescine can also facilitate the nitrogen content enhancement in the root zone and better association of beneficial bacteria like *Pseudomonas fluorescens* (Kuiper et al. 2001). Present study is a systematic approach in understanding the microbial diversity of rhizosphere and the influence of the physicochemical properties of pomegranate soil on microbial diversity of pomegranate. The study has revealed the existence of specific set of microorganisms unique to rhizosphere and has significantly contributed to the enhancement of database of microorganisms in the rhizosphere of pomegranate variety Bhagwa.

The findings from the study leads to the fact that the insignificant variations within the topsoil of rhizosphere contributed to consistent microbial diversity across the soil depth. The study has confirmed the role played by the host in defining the distinct microbial diversity through the root activity which is a subset of bulk microbiome. The presence of beneficial organisms in the rhizosphere such as *Pirellula staleyi*, *Flavisolibacter LC59, Niastella koreensis* and the unique organisms such as flammeovirgaceae bacterium 311 and *Halobacteriovorax marinus* already proven to have functional role in plant protection which could not be explored through conventional culture techniques makes our study very relevant and interesting. The plant growth promoting activity of *Flavisolibacter* has already been explored (Xiao et al. 2017). *Niastella koreensis* was earlier isolated from the Ginseng soil in Korea and proven to have alkaline phosphatase activity that makes it a good candidate for biofertilizer having phosphate solubilization trait (Weon et al. 2006). These organisms can be used as effective plant growth promoting rhizobacteria and biocontrol agents in pomegranate fields.

Presently shotgun metagenomics are ongoing, and the data generated through whole genome sequencing would go one step ahead in adding pertinent data with respect to the functional genes of the microbes revealed through 16S rRNA sequencing involved in enhancement of plant productivity and plant protection as biocontrol agents.

The pot studies would be used for the validation of Insilco data generated. The outcome would be the development of biofertilizers, and biocontrol agents identified through metagenomics approach. The thorough understanding of the functions of the gene with respect to the metabolic pathways prevalent in the farming system and the impact on plant growth and productivity would in turn help the farmer to adopt ecofriendly and sustainable farming practices.

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Declarations

Competing interests The authors declare no competing interests.

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