







Surveillance of Root-associated Microbiome of Oxalogenic *Colocasia esculenta* (Linn) Plant Reveals Distinct Bacterial Species Diversity

Mangesh Vasant Suryavanshi^{1,2*} , Nidhi Bharti³ , Shrikant Subhash Bhute^{1,4} , Vipin Chembili² , Pooja Chavan⁵  and Yogesh Shripad Shouche¹ 

¹National Centre for Microbial Resource, National Centre for Cell Science, Pashan, Pune - 411021, India. ²Yenepoya Research Centre, Yenepoya University, Derlakatte, Mangalore - 575018, India. ³Department of Botany, Savitribai Phule Pune University, Pune - 411007, India. ⁴School of Life Sciences, University of Nevada, Las Vegas, Las Vegas, NV, 89154, USA. ⁵Department of Mechanical Engineering, Zeal College of Engineering and Research, Narhe, Pune - 411 041, India.

Abstract

Oxalic acids are widely distributed in tissues of various plants that can exacerbate the effect on other plant-grazing animals including humans. Bacterial communities had been demonstrated to specify with the rhizosphere of host type and which can differ with oxalogenic plant. The present study has been conducted with the primary objective of understanding the root-associated microbial communities in *Colocasia esculenta*, an oxalogenic plant and to recognize possible bacterial species that present the potential of having their capability to metabolize oxalates. Of the 852 sequences obtained, 311 corresponded to rhizosphere (S), 250 to rhizoplane (P) and 291 were from non-rhizospheric (NS) soil. *Flavobacteriaceae*, *Enterobacteriaceae*, *Moraxellaceae* and *Pseudomonadaceae* were the major contributors in the rhizoplane microbial community assemblage. *Paenibacillaceae* was the major contributor to the rhizospheric microbial community. The findings of the study showed that the rhizoplane, owing to the characteristic root exudates, has a distinctive composition of microbial partners as compared to the rhizosphere and bulk soil communities.

Keywords: *Colocasia esculenta*, rhizosphere soil, microbial community

*Correspondence: mangeshnccs@gmail.com, Tel.: +91-824-2204668 Ext: 2035

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INTRODUCTION

Oxalate (ethane-1,2-dioic acid) is found in diverse environments such as soil and gastrointestinal tracts; oxalate metabolizing bacteria commonly known as oxalotrophic bacteria that can metabolize oxalate for carbon and energy source are often enriched in such environments. In fact, only bacteria have the ability to degrade oxalate and remove it from the environments. Rhizosphere of the the oxalogenic plants is one such niche for such oxalotrophic bacteria.^{1,2} Recent literature has demonstrated that oxalotrophy phenomenon is involved in root colonization by plant-associated bacterial species like *Burkholderia*³ that may have a positive role in plant growth.

Oxalates, especially calcium oxalate (CaOx) crystals have been recorded in many plant species⁴ that can accumulate in plant-eating insects and animals including humans through a complex food network. Most of the times, some plant produces these oxalate crystals as a defense against the herbivory. However, the ingestion of oxalates can be toxic to mammals and may lead to hyperoxaluria and urolithiasis⁵. In fact, plants release oxalate in surrounding soil via root exudates⁶ and also contribute to the CaOx pool in the soil during the decay process⁷. The global oxalotrophic bacterial diversity has not been fully explored, diverse habitats can be explored with the aim of isolating and cultivating oxalotrophic bacteria. These oxalotrophic bacteria could then be utilized in the health and agriculture sectors to cope up with the increasing oxalate concentrations. *Colocasia esculenta* commonly known as arum has been documented to have oxalogenic properties⁸ and the present study has been conducted with the primary objective of understanding the rhizospheric microbial communities in an oxalogenic plant with the prospects of recognizing possible bacterial species for their capability to metabolize oxalates.

MATERIALS AND METHODS

Plant and sampling

Naturally growing *Colocasia esculenta* (Local name: Arum) plants were collected in sterile polypropylene containers from a botanical garden near National Centre for Cell Science, situated in Savitribai Phule Pune University campus, Pune,

Maharashtra, India (Latitude: 18°34' N, Longitude: 73°58' E). The plant roots and surrounded area were selected for sampling the non-rhizospheric (NS), rhizospheric (S) and rhizoplane (P) soil fractions. Soil from roots was fractionated into the three compartments following the previously described method⁹. Samples were selected after the randomization where the soil samples from six to eight arum plants were pooled, resulting in three independent biological replicates. The rhizosphere sample was collected by the gentle scraping of the portion with soil particles closely adhering to the root surface by using a sterile spatula and, root system without soil particles was considered as the rhizoplane sample. DNA from all the three fractions was isolated using PowerSoil® DNA isolation kit (MO BIO Laboratories, Inc. Carlsbad, CA) as per the manufacturer's protocol provided.

Denaturing Gradient Gel Electrophoresis and qPCR for 16S rRNA gene

The fingerprint pattern of the all tested sample was generated by 16S rRNA gene PCR-DGGE and qPCR assay described elsewhere^{10,11}. Briefly, PCR-DGGE was carried out on each community DNA with modified linker primers: 341F-GC (5'-CGCCCGCCGCGCGGGCGGGCGGG GCGGGGCACGGGGGGC CTACGGGAGGCA GCAG-3') and 518R (5'-ATTACCGCGTCTGCTGG-3') for total diversity^{10,12}.

Quantitative estimation of the total bacterial population is achieved through 16S rRNA gene target and which was done in a triplicate using qPCR assays as described earlier¹¹. Average values of the triplicate of the single sample were used for enumerations of 16S rRNA gene copy numbers. Absolute count was derived for each sample using standard curves after the manual curation. All the quantitative values were scaled-up and represented in per gram of soil sample.

16S rRNA gene amplification and sequence data processing

16S rRNA gene was amplified in triplicate for each sample by polymerase chain reaction using bacteria-specific primers (8F: 5'-AGAGTTTGATCCTGGCTCAG-3' and 907R: 5'-CCGTCAATTCCTTTRAGTTT-3'). Purified PCR products were then ligated to TOPO 2.1 TA vector using TOPO 2.1 TA cloning kit (Invitrogen, USA) and the vector containing 16S copies were then

transformed into electrocompetent *E. coli* cells. Nearly 400 transformants were selected and sequenced using ABI Prism 3730 XL DNA Analyser (Applied BioSystems Inc., USA) as described earlier¹³. Sequences were assembled and quality checked using ChromasPro v1.34 (<http://www.technelysium.com.au/ChromasPro.html>), checked for orientation using orientation checker¹⁴ and aligned in Clustal X2¹⁵. Chimeric sequences were removed using Mallard v1.02¹⁰ and anomalous sequences removed using Pintail¹⁶. A total of 852 good quality sequences were analyzed using QIIME-Quantitative Insight into Microbial Ecology v1.9¹⁷. Operational Taxonomic Units (OTUs) were picked using an open reference method with a *uclust_ref* tool¹⁸ using default 97% similarity value, followed by picking the most abundant sequence as the OTU representative sequence against the SILVA123 database (12.10-release)¹⁹.

Taxonomic assignments to each generated sequence

We utilized two independent approaches to assign the taxonomy to each sequence after the OTU generation. In the first approach, each representative OTU was utilized for taxonomical classification using a SILVA123 database with 97% identity as a reference. In an alternative approach, the same representative OTU file were utilized for taxonomical classification through BLAST using curated type strain 16S rRNA gene database on the EzTaxon-e server (<http://www.ezbiocloud.net>)²⁰. For every OTU, the first hit was selected with 97% similarity cut-off and taxonomically identified at the species level. These identified OTUs were used for assessing the differences in the bacterial population between the three samples.

PICRUSt analysis

The functional metagenome imputation was done using the bioinformatic method as described earlier [21]. Briefly, OTUs from a closed reference-based OTU picking approach were utilized to bin the quality sequences. We used the Greengenes database v.13.5 for at 97% sequence similarity level cut-off to taxonomic profiling. The biom table utilized for PICRUSt analysis with available parameters at <http://huttenhower.sph.harvard.edu/galaxy/>. As steps mentioned, the OTU table was normalized for the 16S rRNA gene copy number. Next to that ascertains to for predicting metagenome with the help of the KEGG

database at three different KEGG levels (L1 to L3). Additionally, prediction of oxalate bioconversion pathways present in imputed metagenome was made using KEGG Orthology (KO) database²² as mentioned elsewhere¹⁰.

Statistical analysis

Statistical analysis was done as mentioned elsewhere¹⁰. Briefly, statistical inferences were done in the STAMP-Software package for Taxonomic and Metabolic Profiling v2.08²³. Taxonomic profiling including genus-level taxonomy (L6) biom file containing all the relative abundance values, generated using QIIME command *summarize_taxa.py* analyzed for qualitative ways using GraphPad Prism tool²⁴. To compare 16S rRNA gene sequence libraries for the dispersion obtained from the three root compartments, here we treating each gene sequences as separate points in space with hundreds of dimensions, *K*-shuff, a statistical tool based on PHYLIP was used²⁵. The diversity, species richness (OTUs) in the non-rhizosphere, rhizosphere and rhizoplane regions were analyzed by Simpson and Shannon indices along with goods coverage calculated by *alpha diversity.py* in QIIME environment and in DOTUR algorithm.

Nucleotide sequence accessions

Good quality 16S rRNA gene partial sequences of an average 850 base pair generated in this study which were deposited in Genbank-NCBI database with the following accession numbers: KF784899 - KF785750.

RESULTS

Microbial diversity

The fingerprinting pattern of the three root zones as analyzed through DGGE indicate a marked difference in the patterns. The actual bacterial count as obtained through qPCR indicates higher bacterial count in rhizosphere and lowest in rhizoplane as indicated by the following values: NS= 8.9×10^9 , S= 6.1×10^{10} and P= 9.2×10^4 counts/gm of the soil sample. A total of 852 sequences were successfully retrieved from the three root compartments. Of these 852, 311 corresponded to rhizosphere (S), 250 to rhizoplane (P) and 291 were from non-rhizospheric (NS) soil. The 852 sequences obtained were clustered into representative 504 OTUs based on the open reference OTU clustering with 97% cutoff. This representative OTU file was used for BLAST analysis over EzTaxon-Server

(Validly Culturable bacterial database), further resulting in 272 OTUs (97% cut off). Bacterial OTUs (271) were identified with species-level whereas 01 OTU corresponded to eukaryote. 148 sequences were identified and 160 unidentified at the species level in the rhizospheric soil. 208 sequences identified and 41 were unidentified at the species level in the rhizoplane soil. Similarly, in the non-rhizospheric soil, 273 sequences were identified and 17 rendered unidentified

(Supplementary File S1).

The taxonomic assignment of each sequence to define OTUs was based on a similarity threshold of 97% to the SILVA database. Alpha diversity through non-parametric indicators: Simpson and Shannon were computed to evaluate community diversity characteristics of overall bacteria associated with the *Colocasia esculenta* roots (Fig. 1).

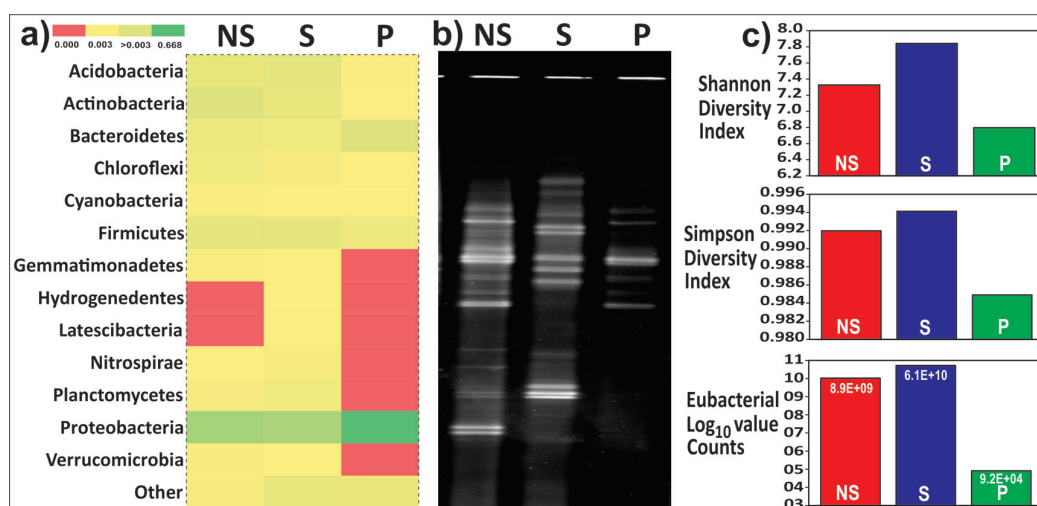


Fig. 1. Comparison of bacterial diversity in root-associated soil (Database used SILVA123) a) Heatmap for phylum-level diversity b) Fingerprint of root-associated microbiome by PCR-DGGE c) Graphical representation of alpha diversity indices and actual eubacteria count by quantitative PCR.

Table 1. Eubacterial taxonomic features of root-associated diversity

Diversity Features	Clone library for microbiome study		
	NS	S	P
Total Sequences (N)	291	311	250
dOTUs ^a	172	228	60
Shannon (H) ^b	4.93049	5.1482	2.94576
Simpson (D) ^c	0.00578268	0.00780002	0.112161
IKF ^d	0.3049	0.2935	0.2554
sOTUs ^e	195	262	152
Species level assignments ^f	273	148	208
Exclusion ^g	1	3	1
Newer to species level assignments ^h	17	160	41

^a Number of OTUs formed at 97 % sequence similarity using DOTUR.

^b Shannon diversity index, $H = -\sum[(n/N)\ln(n/N)]$; at Hmax, n= N.

^c Simpson's index, $D = \sum n(n-1)/N(N-1)$

^d IKF index derived from K Shuff algorithm.

^e Number of OTUs formed at 97 % sequence similarity using the Silva123 database by open-reference OTU generation in OIIME environment.

^f Number of library sequences at 97 % sequence similarity using EzTaxon database to the sOTUs.

^g Number of library sequences having Eukaryotic taxonomy assignments.

^h Number of library sequences fails to taxonomic assignments at 97 % sequence similarity using EzTaxon database to the sOTUs.

Bacterial community composition and structure

Taxonomic analysis based on relative abundance revealed bacterial members of phylum *Proteobacteria* were recorded relatively higher abundance across all samples S (48%), P (78%), and NS (40%). *Firmicutes* in rhizosphere (S) soil (28%), *Actinobacteria* (16%) in the non-rhizospheric soil whereas, *Bacteroidetes* (12%) in the rhizoplane soil were found to the second most abundant groups. The P and S showed a complete absence of members from the phyla *Cyanobacteria*, *Gemmatimonadetes* and *Planctomycetes* in contrast to the NS soil. Members from the phylum *NC10* (0.68%), were exclusive to the rhizosphere soil. *Acidobacteria*, *Chloroflexi*, *Nitrospirae*, and *Verrucomicrobia* were completely absent in the

rhizoplane (P) soil samples (Fig. 1). K-shuff analysis showed a greater difference in microbial diversity of rhizoplane from bulk soil and rhizosphere as suggested by higher *AreaK* values generated (Table 1).

At the family level, the OTUs were characterized by 101 different families. Microbial communities from non-rhizospheric soil (NS) corresponded to 86 families. In contrast, the rhizoplane (P) microbial communities corroborated to only 17 families. *Flavobacteriaceae*, *Enterobacteriaceae*, *Moraxellaceae*, and *Pseudomonadaceae* were the major contributors in the rhizoplane microbial community assemblage. *Paenibacillaceae* was the major contributor to the rhizospheric microbial community (Fig. 2).

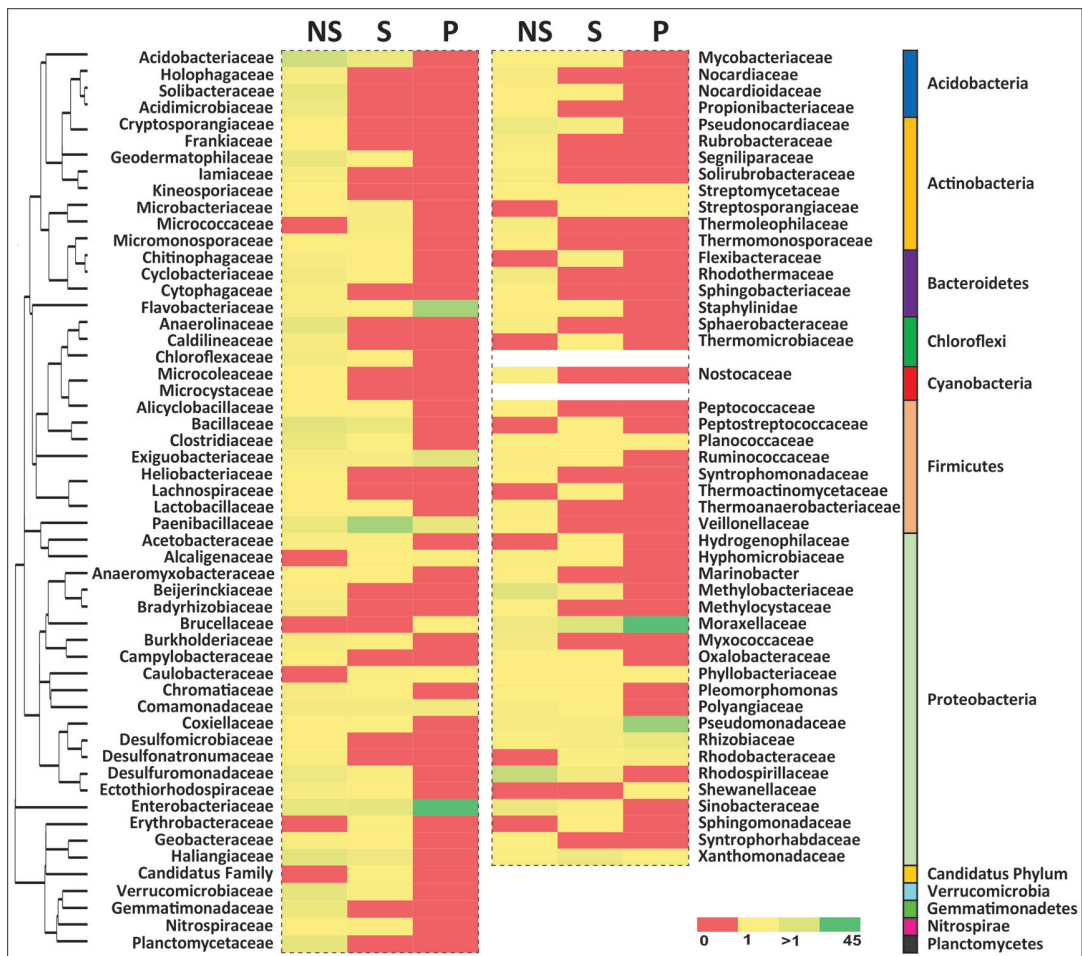


Fig. 2. Heatmap for the family level abundance of root-associated soil microbiome (Database used SILVA123). Abundance in each library shown in the actual number and the clustering of each family-level taxa with dendrogram is derived with the help of farthest neighbour (constrained) method.

The bacterial species, particularly those belonging to the phylum *Firmicutes*, present among these communities are demonstrated through a Venn diagram to gauge the relationships between these communities (Fig. 3). The results illustrate that only 3 species were shared among all the three root zones. There were no species belonging to *Firmicutes* that were shared by non-rhizospheric (NS) and rhizoplane (P) soil samples. However, 6 bacterial species were shared by non-rhizospheric and rhizospheric soil samples. 6 bacterial species were exclusively present in the rhizoplane compartment which constituted of species belonging to genus *Exiguobacterium*, *Paenibacillus*, and *Solibacillus*.

Functional imputed metagenome

We compared the functional potential of the microbial communities in soil fractions (NS, P, and S), used bioinformatics tool- PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States). Overall predicted genes that are more abundant in the rhizosphere were mainly associated with viz. Cellular Processes and Signalling, Cell Growth and Death, Carbohydrate Metabolism, Glycan Biosynthesis and Metabolism and Transcription (Fig. 4). Apart from other functional capabilities, other microbial metabolism enclosure was dominant (Supplementary File S2). PICRUSt was also employed to study the oxalate metabolism genes in the rhizosphere (Table 2) and bacterial families harboring oxalate metabolizing capacity genes are mentioned in Supplementary File S3.

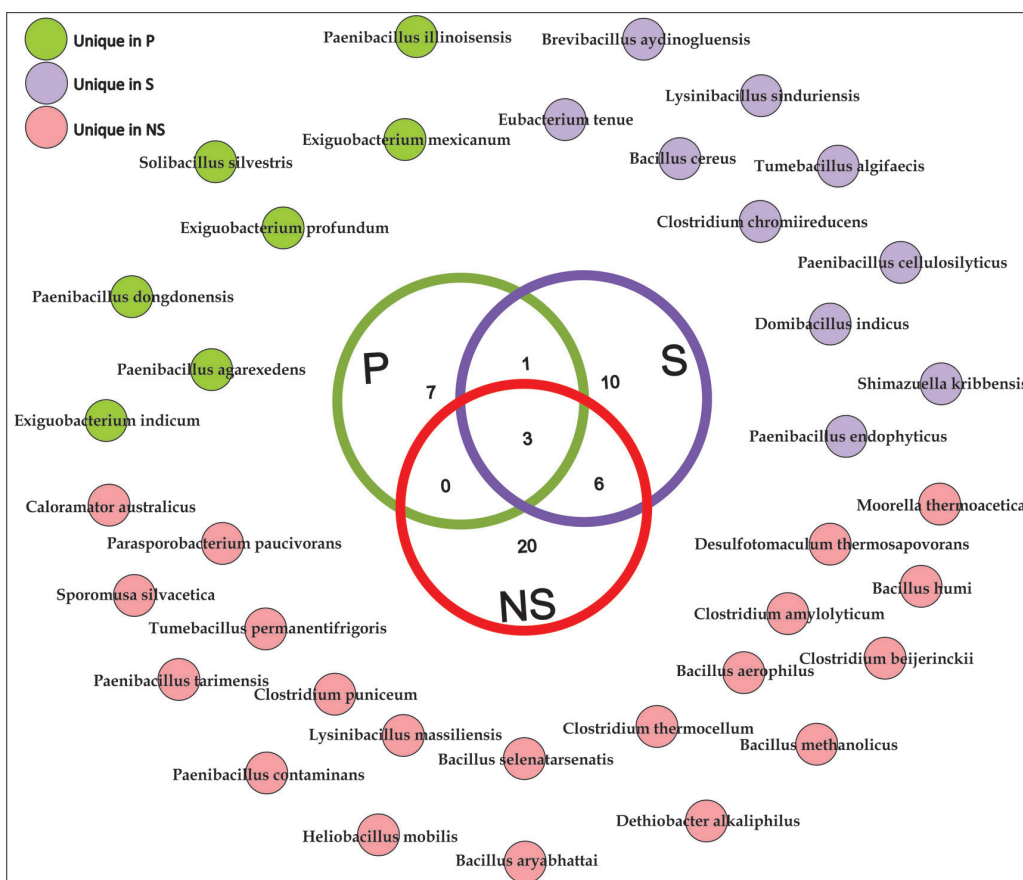


Fig. 3. Chart showing the sharing of all observed bacterial species of Firmicutes phylum for root-associated soil microbiome. These species-level taxonomic assignments were derived at 97 % sequence similarity using EzTaxon-e server.

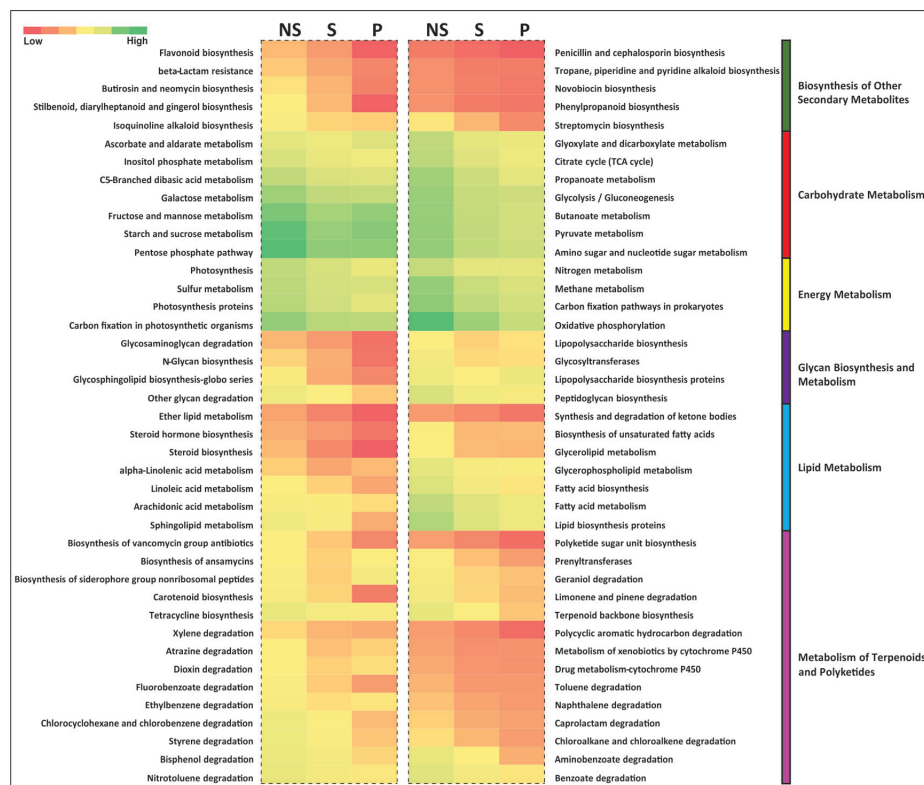


Fig. 4. Functional capacities regarding with metabolism of the root associated soil microbiome. This heatmap showed relative abundance of representative functions which are derived from the PICRUSt tool and greengene 13.8 database.

DISCUSSION

This culture-independent molecular investigation offers new visions into the structure and composition of native microbial communities *Colocasia esculenta* associated root zones. Importantly, the species level identification was revealed with the dual approach.

Methods to calculate approximately the microbial diversity yet have advanced to comprehend the population and diversity of microorganisms in varied ecosystems²⁶. The 16S rRNA sequences based characterization of microbial communities has been accepted and adopted as the standard method in microbial ecology and a vast number of up-to-date open-source sequence analysis tools such as “mothur”²⁷, “QIIME”²⁸, or “RDP”²⁹ assist the analysis of the large number of sequences generated by modern, advanced and colossal parallel sequencing methods under the umbrella of Next Generation Sequencing (NGS). However, one of the shortcomings of NGS methodologies is the short

read length and that sequencing the complete 16S rRNA gene of all-inclusive communities is still expensive and operationally convoluted. The present study follows the traditional approach of generating clone banks followed by the Sanger sequencing method for studying the microbial ecology of the root compartments of *Colocasia esculenta*. The method to utilize PCR products generated by a single primer pair rather than using separate primer pairs for every variable 16S rRNA region reduces the impact of different primer pairs on the constitution of the PCR products, which can impinge on species composition and species richness (SR) estimates. The method of analysis involves the formation of OTUs based on the reference database SILVA followed by taxonomic assignments using the EzTaxon-e server. Since the clone libraries generated were sequences of length more than 850 bp, it was preferred to complete the taxonomical classification through BLAST analysis using the EzTaxon-e server. The higher cut-off percentage (97%) and e-value were used

Table 2. Bacterial enzymes describing the oxalate metabolizing potentials in the microbiome derived from the imputed metagenomic approach. Selected oxalate metabolizing ability governing genes in microbiome as mentioned earlier by Suryavanshi et. al., 2016¹³

Enzymes	KEGG Orthology ID	ID Abundance of genes in microbiome study			KEGG_ Description
		NS	S	P	
oxalyl-CoA decarboxylase	K01577	0.0022	0.0016	0	oxalyl-CoA decarboxylase [EC:4.1.1.8]
formyl-CoA transferase	K07749	0.0239	0.0219	0.0069	formyl-CoA transferase [EC:2.8.3.16]
oxalate/formate antiporter	K08177	0.0107	0.0099	0.0122	MFS transporter, OFA family, oxalate/formate antiporter
formyl-CoA hydrolase	K01067	0.0059	0.0059	0.0081	acetyl-CoA hydrolase [EC:3.1.2.1]
NAD-dependent formate dehydrogenase	K08349	0.0003	0.0012	0.0106	formate dehydrogenase-N, beta subunit [EC:1.2.1.2]
oxalate decarboxylase	K01569	0.0076	0.0040	0.0016	oxalate decarboxylase [EC:4.1.1.2]

to minimize the misidentification of the taxa.

The diversity of microbes inhabiting the numerous niches of the root and rhizosphere differs¹². Study of the *Arabidopsis* rhizosphere and bulk soil using 16S rRNA sequencing did not indicate any major difference in the microbial communities, whereas, only a minor decrease was observed in the OTU richness of the rhizosphere microbial population³⁰. Meta-transcriptomic analysis of the rhizosphere and bulk soil indicated that the soil compartments do not show considerable variation in microbial diversity when only the numbers of microbial taxa were considered. However, in our study, a clear distinction between rhizosphere and bulk soil diversity was observed at the genus level and not the phylum level.

Roots offer distinct microhabitats at the soil-root interface: rhizosphere soil, rhizoplane, and endo-rhizosphere¹². However, the effectiveness of major nutrients and other micronutrients can be confined to the bacterial population to the *Colocasia* plant³¹. Otherwise, rhizosphere effects on the microbial community could be the possible reason to differentiate metabolic potential in the soil to dissolve organic carbon pool³². In the present study, the results indicate a clear distinction in the microbial community diversity at the phylum level as well. The complete absence of members of the phyla *Cyanobacteria*, *Gemmatomonadetes* and *Planctomycetes* from the rhizosphere and rhizoplane microbial population supports the role of root exudates in significantly

influencing and designing the microbiome. Recent studies involving the exploration of the rhizoplane population have suggested a greater difference in their bacterial population in comparison to the bulk soil. Experimental studies corroborate the idea that root exudates play a major role in shaping a specialized community which may also have prominent host-specific microbiome signatures. We detected increased relative abundances of *Proteobacteria* in the rhizosphere and rhizoplane, compared with the bulk soil. The phyla *Bacteroidetes* and *Proteobacteria* are by and large deemed as copiotrophic microorganisms (or r-strategists), which grow within environments of high nutrient accessibility³³. The copiotrophs can proliferate rapidly in rhizospheric substrate 'hotspots' with elevated concentrations of root exudates. *Proteobacteria* phylum has been showed to have the highest richness (OTUs for *frc* and *oxc*), followed by *Actinobacteria* and *Firmicutes*³⁴. Since *Colocasia esculenta* is an oxalogenic plant and has been known to release oxalates in the root exudates, the preponderance of *Proteobacteria* in the rhizosphere and rhizoplane microbial communities indicates the oxalotrophic activity which might be a major functional trait of the communities associated. Nunes da Rocha et al. demonstrated that groups associated with *Verrucomicrobia* might be essential to the rhizosphere of several plants, such as grasses, leek, and potatoes³⁵. On the other hand, a non-*Streptomyces* actinomycete, isolated from the rhizosphere of an elephant ear plant

(*Colocasia esculenta*) in Bangkok, Thailand which was identified as a novel *Saccharomonospora colocasiae* sp. strain S265^T 36.

The K-shuff analysis indicates the spatial and structural variability between the communities represented by higher *AreaK* values. Larger the value of *AreaK*, more distant are the compared communities and that share fewer members between them. The results also predict the compositional difference between the communities. The K-shuff analysis of the three root compartment libraries supported the previous observations statistically. The results maintained the fact that there is a substantial structural difference in the rhizoplane microbial communities in comparison to the other two libraries (rhizosphere (S) and non-rhizosphere (NS)). Detection of some of the core species which are present throughout the samples from like NS, S, and P suggests that there may be negligence of rhizospheric effect governed by host exudates around the roots and could be the example of invaders demonstration. Since oxalogenic plants could act as good source oxalate exudates and of which enrichment of oxalotrophic bacteria as one of the rhizospheric populations. Parallel objectivity was to identify the microbes from the natural environment which have the putative function of oxalotrophy and can possibly be developed into probiotics that can be utilized in the treatment of kidney stones in near future. Studies have indicated a positive role of the probiotics in the management of kidney stones. *In lieu* of the objective mentioned, microbes exclusive to the three root compartments were identified. This analysis focused only on the members of the *Firmicutes*, and can be linked to other studies on human subjects showed that the gut microbiome of humans is rich in *Firmicutes* and *Bacteroidetes*³⁷. The acclimatization to obtain nutrients and mechanisms of carbohydrate and energy metabolism from the neighboring environment is of major significance to bacteria for their existence in the corresponding ecological niche. Imputed metagenomic analysis performed in this study suggested considerable insight into such mechanisms.

In congruence with the present study, *Arabidopsis thaliana* has been found to host

distinct microbial communities in the root compartments (i.e., rhizosphere, rhizoplane, and endosphere)^{30,38,39}, *Oryza sativa*⁴⁰, or *Populus deltoides*⁴¹. Previous studies have corroborated the fact that plants profile their individual microbiome from bulk soil, which is host-specific⁴². Clone library approach has its an intrinsic limitation for bulk sequences generation, we anticipate the next-generation sequencing platforms for longer reads but, we revealed the eubacterial soil population for its species-level diversity found around the *Colocasia*.

CONCLUSION

The present study attempts to explore the root-associated microbial players in the *Colocasia esculenta*. It is observed that the rhizoplane, owing to the characteristic root exudates, has a distinctive composition of microbial partners as compared to the rhizosphere and bulk soil communities. Field experiments and an intensive microbial community analysis are required to enhance our knowledge of the *Colocasia* root-associated bacterial flora.

SUPPLEMENTARY INFORMATION

Supplementary information accompanies this article at <https://doi.org/10.22207/JPAM.14.1.57>

Additional file: Additional Figs. S1, S2 and S3.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

MVS and SSB contributed to the data generation and drafted the manuscript. NB, VC, YSS interpretation supervised and reviewed the manuscript. NB, VC, and PC helped for the preparation of figures, interpretation and draft improvisation. All the authors read and approved the manuscript.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript. All the 16S rRNA gene sequences generated during this study were deposited in the Genbank-NCBI database under the accession numbers: KF784899 - KF785750.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals.

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