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Metagenomic analysis reveals the predominance of *Candidatus Patescibacteria* in the rhizosphere of arecanut palms in yellow leaf disesase (YLD) endemic areas of India

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Arecanut (*Areca catechu* L.) is an important plantation and industrial crop cultivated predominantly in South and South-East Asia, especially in India, China and Malaysia. Arecanut production has been hampered by environmental and disease pressures, especially the increased incidence of yellow leaf disease (YLD) in countries like India and China (Nampoothiri *et al*., 2000; Wang *et al*., 2020). The rhizosphere microbiome has been linked with beneficial aspects such as plant health by enhancing plant growth, meeting the nutrient requirements of plants, and imparting abiotic and biotic stress tolerance (Olanrewaju *et al*., 2019). Modern molecular techniques have been employed to unravel the rhizosphere microbiome composition, relative abundance, and their correlation to many factors such as the host plants, edaphic factors, soil physico-chemical properties, the initial microbial load and climatic factors. The rhizosphere microbiome has been investigated recently in a few perennial crops like citrus (Xu *et al*., 2018) and grapevine (Berlanas *et al.,* 2019). Few studies have focused on microbial interactions within the rhizosphere, especially in arecanut palms. Mohan *et al*. (2019) investigated the microbiome of arecanut palms and a recent study by Li *et al.* (2021) reveals the association between the arecanut microbiome and root rot. However, there are no reports delineating the arecanut rhizosphere microbiome under YLD endemic conditions. Studies on the composition and diversity of rhizosphere microbiomes are critical in finding ways and measures of improving plant health and productivity under YLD endemic field conditions.

This study compared rhizosphere microbiomes between healthy and YLD affected arecanut palms by amplicon sequencing. The arecanut rhizosphere soil samples were collected during the South West monsoon season during the year 2019, coinciding with the peak symptomatic period, from YLD endemic regions of Sullia taluk, Dakshina Kannada district, Karnataka State, India. The soils were collected from the apparently healthy (YLD-AHR) palms, intensely diseased (YLD-DIR) palms and the non-rhizosphere (YLD-NR) regions. Samples were collected from four different geographic locations (two separate fields each from Aranthodu and Allette, Sullia taluk, Dakshina Kannada district, Karnataka, India) and from around three palms per site. Soil samples were collected from the active root zones, 30-45 cm away from the palm trunk core and at 5-45 cm depth, where the root system was denser from the surface. The three samples collected from each of the palms were pooled together, stored in sterile bags on dry ice immediately, and brought to the laboratory for further processing within 24 hrs of sampling.

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The sampled roots with rhizosphere soil particles attached were placed in sterile tubes containing physiological solution (9 g L^{-1} NaCl); the tubes were vortexed for 5 min to detach the soil particles and then centrifuged at 4000 rpm for 5 min. The supernatant was discarded and the remaining soil fraction was used for DNA extraction.

The metagenomic DNA of the rhizosphere samples (YLD-AHR, YLD-DIR and YLD-NR) were extracted using the QIAamp® DNA Microbiome Kit (Qiagen, Germany). The V3-V4 regions of the *16S rRNA* gene were amplified using (KAPA) HiFi HotStart Ready Mix (Roche, Switzerland) and the 341F and 785R primers (Klindworth *et al*., 2013). Paired-end sequencing (2 × 300 bp) of the templates was performed on an Illumina Miseq platform (Illumina, USA). The amplicons were purified, and adapters were added to sequence the libraries. Library preparation was done and quantified using the fluorometric method (Rengarajan *et al*., 2002)

The data quality of the raw reads was checked by FastQC (Andrews, 2010) and MultiQC (Ewels *et al*., 2016). The reads were trimmed (20 bp) from the 5' end to remove the degenerate primers. The trimmed reads were processed to remove adapter sequences and low-quality bases using Trimgalore (Krueger, 2019.). The QC passed reads were imported into Mothur (Schloss *et al*., 2009), and the pairs were aligned to form contigs. The contigs were initially screened for errors. High-quality contigs were then checked for identical sequences, and the duplicates were merged. After this process, the gaps and the overhang at the ends of the contigs were removed and processed for chimera removal, which might have formed due to errors in PCR conditions. UCHIME algorithm (Edgar *et al*., 2011) was used to flag contigs with chimeric regions. The filtered contigs were processed, classified, and clustered into Operational Taxonomic Units (OTUs). Using an agglomerative clustering algorithm, sequences were placed to respective OTUs at 97 per cent sequence similarity with USEARCH (Edgar, 2010). A representative sequence of each OTU was further used to estimate the bacterial diversity using the Metagenomics Rapid Annotation pipeline (Silva v.132 database) (Quast *et al*., 2013) to obtain the taxonomical diversity of bacteria and archaea. The raw sequence pertaining to this study was deposited in the NCBI-SRA database Repository under Bio-project-PRJNA721704.

Candidatus Patescibacteria was frequently identified as a novel bacterial phylum in the rhizosphere/soil of arecanut palms. This phylum comprised 57 OTUs and was about 65 per cent more abundant in the rhizosphere than in non-rhizosphere regions. The relative occurrence of Phylum *Candidatus Poatescibacteria* in YLD-AHR, YLD-DIR and YLD-NR soil samples were 60.86, 29.57 and 9.58 per cent, respectively (Fig.1). Similarly, under Bacteria (Kingdom), its pooled abundances were about 4.81 per cent, 2.05 per cent and 1.49 per cent, respectively, for YLD-AHR, YLD-DIR and YLD-NR samples (Table 1). The predominant classes documented under Phylum *Candidatus Patescibacteria* were *Parcubacteria* (39.92%), *Saccharimonadia* (32.14%), ABY1 (14.20 %), *Microgenomatia* (5.28 %), *Gracilibacteria* (4.47 %), and other *Patescibacteria* (3.99%). The predominant genera based on relative abundance were *Parcubacteria-Candidatus Moranbacteria, Candidatus Kaiserbacteria, Candidatus Yanofskybacteria, Candidatus Nomurabacteria, Candidatus Adlerbacteria, Candidatus Zambryskibacteria, ABY1-Candidatus Magasanikbacteria, Candidatus Kerfeldbacteria, Candidatus Komeilibacteria, Candidatus Kuenenbacteria, Microgenomatia-Candidatus Levybacteria, Candidatus Woesebacteria,* and *Saccharimonadia-Candidatus Saccharimonas.*

Candidatus Moranbacteria recorded a relative abundance of 8.13 per cent and 1.06 per cent in YLD-AHR and YLD-DIR, respectively, and even not recorded in YLD-NR. It reveals that the arecanut palm selectively enriches this *Candidatus Moranbacteria* in its rhizosphere, but its synergism and biological significance have not yet been revealed in rhizosphere of any crops. Genes involved in the downstream transformation of pyruvate to acetyl-CoA and acetate detected in *Candidatus Moranbacteria* and *Candidatus Yanofskybacteria*, suggest that these lineages might ferment carbon compounds to acetate (Vigneron *et al*., 2019), the elite carbon source for the growth and metabolism of the culturable microflora.

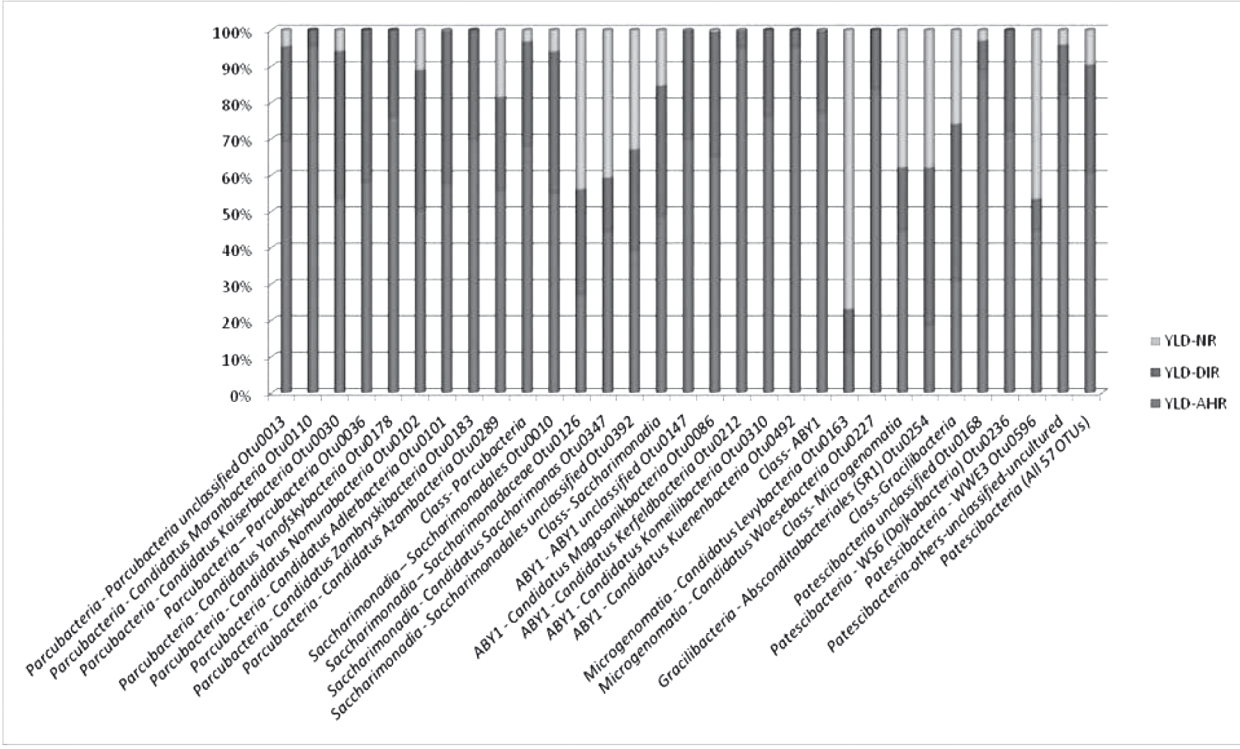

Fig. 1. Relative occurrence (%) of *Candidatus Patescibacteria* OTUs in arecanut rhizosphere of YLD endemic region

Further, the synergistic interaction of *Candidatus Patesic bacteria* with many acetate-utilizing microorganisms was also suggested (Vigneron *et al.,* 2019). The chitin degradation ability *of Candidatus Moranbacteria* (GH18) (Vigneron *et al*., 2019) infer that these organisms could help degrade fungi remains in the rhizosphere, thereby improving the root health.

On the other hand, YLD-DIR showed a relative abundance of *Candidatus Kaiserbacteria* (6.82%), supporting its abundance in the oxic rhizosphere (Herrmann *et al*., 2019). Further, *Candidatus Kaiserbacteraceae* shows hydrochemical preferences for ammonia-oxidizing bacteria (Herrmann *et al*., 2019), supported by a very high relative abundance of Betaproteobacteriales-Nitrosomonadaceae OTU (34.11%) in the YLD endemic arecanut rhizosphere (Paulraj *et al*., 2021). This interaction of *Candidatus Kaiserbacteria* and *Nitrosomonadaceae* and other nitrogen transformation potential taxa, *viz*., *Rhizobiales*, *Myxococcales* in the rhizosphere could have impaired rhizosphere nitrogen transformation cycle (RNTC) (Paulraj *et al*., 2021) in the arecanut YLD rhizosphere environment.

A very high abundance of *Parcubacteria* in the arecanut rhizosphere (44.55% in YLD-AHR and 38.95% in YLD-DIR) was recorded.

The presence of an average of 29±12 carbohydrate-active enzyme (CAZy) genes per *Patescibacteria* genome suggests the potential of these microbes for the degradation of complex carbon substrates. Also, the genome has the potential to encode enzymes to counter oxidative stress (enzymatic resistance to oxygen) (Vigneron *et al., 2019*). Further, the lack of known respiratory pathways suggests syntrophic/symbiotic and fermentative lifestyles of these bacteria (Wrighton *et al*., 2012; Castelle *et al*., 2018).

The class *Saccharimonadia* showed a relative abundance of 25.83 per cent in YLD-AHR, 38.84 per cent in YLD-DIR and 51.55 per cent in YLD-NR in correlation with the predominance of fermentable carbon in the rhizosphere and accumulation of debris in YLD-NR (Ferrari *et al*., 2014). *Candidatus Magasanikbacteria* showed relatively equal abundance in both the healthy and diseased rhizosphere (4.04% in YLD-AHR and 4.12% in YLD-DIR). Interestingly, previous reports reveal that

Table 1. Relative abundance of *Candidatus Patescibacteria* in YLD endemic arecanut rhizosphere

Class - Genus - OTU	*Patescibacteria* contig (Nos) in YLD rhizosphere soil			*Patescibacteria* OTUs relative abundance (%) in bacteria			*Patescibacteria* OTUs relative abundance (%) in phylum patescibacteria		
	YLD-AHR	YLD-DIR	YLD-NR	YLD-AHR	YLD-DIR	YLD-NR	YLD-AHR	YLD-DIR	YLD-NR
Parcubacteria - Parcubacteria unclassified Otu0013	1909 ± 86.15	723± 42.47	124± 4.88	1.17±0.27	0.43±0.12	0.12±0.01	10.88± 1.65	6.79± 1.40	6.01± 0.79
Parcubacteria - Candidatus Moranbacteria Otu0110	1754± 85.24	79±3.14	0.00	0.99±0.03	0.05±0.00	0.0	8.13± 1.49	1.06± 0.20	0.00
Parcubacteria - Candidatus Kaiserbacteria Otu0030	829± 41.85	625±29.89	90± 2.89	0.51±0.14	0.39±0.08	0.08±0.01	4.71± 0.91	6.82± 0.92	4.04± 0.57
Parcubacteria – Parcubacteria Otu0036	810± 33.88	584±26.85	0.00	0.49±0.12	0.36±0.09	0.00	5.05± 0.97	6.23± 0.99	0.00
Parcubacteria - Candidatus Yanofskybacteria Otu0178	507± 31.20	158±9.76	0.00	0.32±0.08	0.10±0.02	0.00	2.42± 0.50	1.58± 0.28	0.00
Parcubacteria - Candidatus Nomurabacteria Otu0102	329± 19.86	252±17.67	72± 4.55	0.21±0.06	0.15±0.04	0.05±0.01	1.75± 0.36	2.19± 0.55	2.81± 0.79
Parcubacteria - Candidatus Adlerbacteria Otu0101	328± 15.94	239±12.20	0.00	0.20±0.05	0.15±0.04	0.00	2.01± 0.48	2.47± 0.53	0.03± 0.01
Parcubacteria - Candidatus Zambryskibacteria Otu0183	177±9.10	77±4.12	0.00	0.11±0.03	0.05±0.01	0.00	1.14±0.27	0.76± 0.15	0.00
Parcubacteria - Candidatus Azambacteria Otu0289	97± 6.34	44±2.26	32± 2.83	0.06±0.02	0.03±0.01	0.02±0.01	0.49± 0.11	0.47± 0.10	1.37± 0.48
Class- *Parcubacteria*	7147± 290.88	3035±153.72	339± 7.11	4.30±0.86	1.85±0.46	0.30±0.01	38.95± 5.24	30.88± 4.95	14.93± 1.13
Saccharimonadia – Saccharimonadales Otu0010	3472± 81.77	2450±55.57	375± 5.63	1.96±0.21	1.6±0.05	0.32±0.03	28.79± 4.00	35.31± 3.48	15.60± 0.51
Saccharimonadia – Saccharimonadaceae Otu0126	456± 23.92	482±21.84	735±5.10	0.27±0.07	0.37±0.09	0.63±0.03	4.95±1.39	9.56± 2.05	33.73± 2.49
Saccharimonadia - Candidatus Saccharimonas Otu0347	149± 10.63	49±3.63	136± 5.29	0.09±0.02	0.03±0.01	0.11±0.01	1.88± 0.61	0.72± 0.22	5.21± 0.91
Saccharimonadia - Saccharimonadales unclassified Otu0392	66± 4.89	46±1.92	55± 2.37	0.04±0.01	0.03±0.01	0.05±0.01	0.31± 0.08	0.89± 0.19	2.02± 0.30

Class- *Saccharimonadia*	4143± 100.89	3027±62.32	1301± 16.18	2.36±0.30	2.03±0.16	1.12±0.07	35.94± 5.87	46.48± 4.67	56.56± 2.81
ABY1 - ABY1 unclassified Otu0147	823±4 1.75	354±15.48	0.00	0.44±0.03	0.22±0.03	0.0	4.25± 0.75	3.85± 0.43	0.09± 0.03
ABY1 - *Candidatus Magasanikbacteria* Otu0086	654± 31.5	344±19.71	0.00	0.40±0.09	0.21±0.05	0.0	3.37± 0.54	3.33± 0.58	0.21± 0.08
ABY1 - *Candidatus Kerfeldbacteria* Otu0212	745± 37.31	37±3.15	0.00	0.42±0.01	0.02±0.01	0.0	3.39± 0.67	0.30± 0.10	0.04± 0.02
ABY1 - *Candidatus Komeilibacteria* Otu0310	211± 13.14	65±5.28	0.00	0.13±0.03	0.04±0.01	0.0	1.04± 0.22	0.53± 0.16	0.00
ABY1 - *Candidatus Kuenenbacteria* Otu0492	261± 19.23	13±0.76	0.00	0.13±0.03	0.01±0.00	0.0	1.35± 0.36	0.25± 0.07	0.00
Class- ABY1	2896± 116.12	839±44.19	7± 0.51	1.63±0.09	0.51±0.10	0.00	14.36± 1.90	8.50± 1.17	0.35± 0.09
Microgenomatia - Candidatus Levybacteria Otu0163	76± 1.97	81±0.81	76± 1.97	0.04±0.00	0.05±0.00	0.43±0.12	0.62± 0.08	1.13± 0.05	14.57± 4.07
Microgenomatia - Candidatus Woesebacteria Otu0227	367± 19.00	71±2.74	0.0	0.20±0.01	0.05±0.00	0.00	1.83± 0.34	1.04± 0.18	0.00
Class- *Microgenomatia*	624± 27.42	241±6.17	528± 38.55	0.34±0.02	0.16±0.00	0.43±0.12	3.34±0.44	3.35± 0.34	14.68± 4.11
Gracilibacteria - Absconditabacteriales (SR1) Otu0254	126± 6.13	289±13.96	126±6.13	0.07±	0.21±0.06	0.22±0.02	1.15± 0.27	5.51± 1.11	10.33± 0.45
Class-*Gracilibacteria*	369± 11.97	504±15.52	306± 7.10	0.20±0.03	0.36±0.07	0.27±0.04	2.84± 0.44	8.78± 1.55	11.90± 0.56
Patescibacteria unclassified Otu0168	472± 32.01	43±2.17	0.00	0.24±0.04	0.03±0.0	0.0	2.36± 0.59	0.46± 0.07	0.67± 0.22
Patescibacteria - WS6 (*Dojkabacteria*) Otu0236	230± 8.99	89±1.50	0.00	0.13±0.00	0.06±0.0	0.0	1.28± 0.14	1.40±0.19	0.00
Patescibacteria - WWE3 Otu0596	26± 1.33	5±0.27	27± 2.39	0.02±0.0	0.00	0.03±0.01	0.12± 0.02	0.05± 0.01	0.91± 0.32
Patescibacteria-others-unclassified-uncultured	862± 46.91	147±3.29	43±2.27	0.46±0.05	0.10±0.00	0.04±0.01	4.56± 0.82	2.01± 0.15	1.58± 0.32
Patescibacteria (All 57 OTUs)	16041±347.03	7793±160.57	2524± 49.50	4.81±0.01	2.05±0.04	1.49±0.03	100.00	100.00	100.00

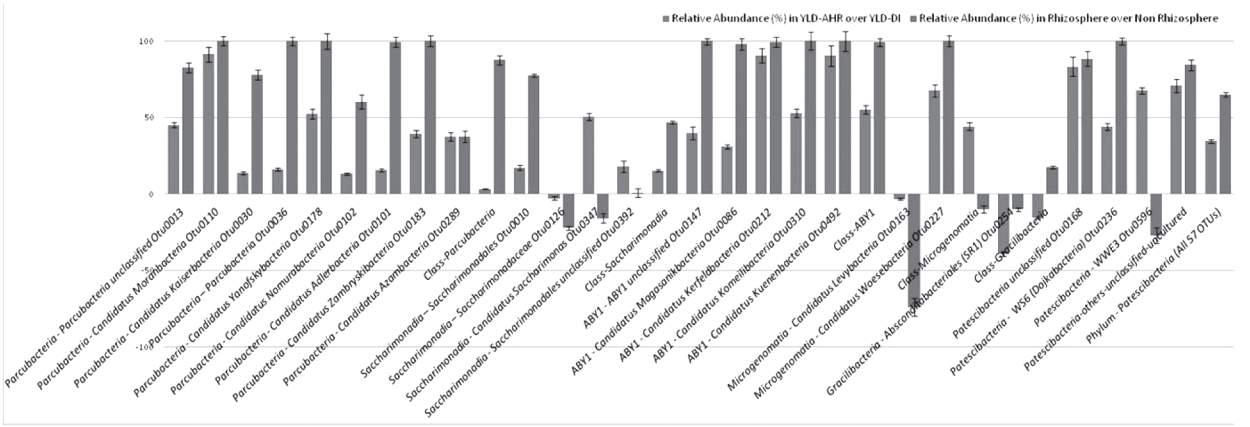

Fig. 2. Comparative relative abundance of *Candidatus Patescibacteria* OTUs in yellow leaf disease intensive, apparently healthy and non-rhizosphere soils

Cand. *Magasanikibacterales* show a correlation with the iron-oxidizing *Gallionella* and thiosulfate-oxidizing *Sulfuricella,* indicating the co-localization of *Candidatus Patescibacteria* and other key autotrophs (Brown *et al*., 2015). Some of these abundant autotrophic taxa are known to be involved in nitrogen, sulfur and iron cycling. For instance, *Candidatus* ABY1 recorded a relative abundance of 18.05 per cent in YLD-AHR and 10.77 per cent in YLD-DIR compared to 0.28 per cent in the non-rhizosphere (YLD-NR) region. Thus the classes ABY1 and *Candidatus Gracilibacteria* are more enriched and show co-occurrence with autotrophic organisms, involved in nitrogen, sulfur, and iron cycling, and predominant chemolithoautotrophs. Further, earlier reports of genome analysis of some *Parcubacteria* species indicate a potential involvement in nitrogen cycling (Castelle *et al*., 2017; León-Zayas *et al*., 2017), corroborated by the fact that nitrite reductase encoding genes in the genomes of *Candidatus Patescibacteria* has been ascribed to nitrite detoxification mechanisms rather than anaerobic respiration or denitrification (Castelle *et al*., 2018).

The candidate phyla radiation (CPR) is a diverse group of uncultured bacterial lineages with inadequately understood metabolic functions. CPR bacteria were initially recovered from groundwaters and aquifers, where they have been found to account for up to 20 per cent of the microbial community (Kantor *et al*., 2013). Phylogenetic and taxonomic analyses suggested the reclassification of the CPR into a single phylum, *Candidatus Patescibacteria,* with 14 classes known so far (Hug *et al*., 2016). YLD endemic region receives more than 3000 mm rainfall during the southwest monsoon season, which could have favoured the runoff and subsurface leaching of excess rainwater, facilitating the preferential mobilization of *Candidatus Patescibacteria* from hilltop soils into the subsurface and rhizosphere. The fact that surface charge is negative for soil mineral particles and the negative charge of *Candidatus Patescibacteria* cells (Koyama *et al*., 2013) could contribute to generally favourable conditions for cell dispersal in soil. The abundance and community structure of *Candidatus Patescibacteria* might depend not only on the ambient hydrochemical conditions but also on the availability of other partners. *Candidatus Patescibacteria* encodes large cell surface proteins, most likely involved in attaching to other microorganisms (Castelle *et al*., 2018). Members of *Candidatus Patescibacteria* are especially abundant in the ultra-small fraction of cells, optimized for the uptake of the sparse nutrients. Further, the lack of functional genes essential for amino acid or nucleotide biosynthesis in these taxa would have inevitably led to dependencies on other organisms. Hence, a host-dependent lifestyle co-localized organism's association (Brown *et al*., 2015; Castelle *et al*., 2018) might have optimized their abundance in the rhizosphere during the monsoon.

The ability to metabolize sugar compounds under oxic and anoxic conditions (Albertsen *et al*., 2013) and the high abundance of transporter and glycoside hydrolase genes (Brown *et al*., 2015; Castelle *et al*., 2017), together with the high surface-to-volume ratio of ultra-small cells, are considered as favourable traits for the uptake of such compounds which are generally available in low concentrations in the oligotrophic conditions during the monsoon seasons in the rhizosphere. Fermentative metabolism, independent of inorganic electron acceptors, prevails in the *Candidatus Patescibacteria* (Brown *et al*., 2015; Nelson and Stegen, 2015) explain their predominance throughout the rhizosphere niche system. Hence, it is proposed that various factors such as import from the soil and community differentiation driven by rhizosphere niche conditions, including the availability of organic resources and potential hosts, symbiotic or mutualistic interaction with other rhizosphere bacterial communities, could have greatly determined the abundance of *Candidatus Patescibacteria* in arecanut rhizosphere. Similarly, Zhang *et al*. (2018) observed a high abundance of *Candidatus Patescibacteria* in seepage collected beneath maize-planted agricultural soils.

The role of *Candidatus Patescibacteria* in biosphere community dynamics and their ecological significance are largely unknown. However, it is apparent that this group could contribute to community stability and a significant microbial role in rhizosphere environmental conditions. Our findings report *Candidatus Patescibacteria* OTUs, under various classes, families and genera and their predominance in the arecanut rhizosphere in YLD regions. The factors that could have driven the relative abundance of *Candidatus Patescibacteria* in the rhizosphere are presented. To conclude, the synergistic interaction of some of the members of *Candidatus Patescibacteria* and other major phyla, *viz*., *Betaproteobacteria*, *Planctomycetes*, *Actinobacteria*, and *Chloroflexi, etc*. and their nutritional requirements, variable responses of the plant's innate immune system, the influence of microbe-microbe interactions, or possible interactions with the plant host and significance for the sustainability of crop husbandry are future prospects that warrant research.

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