Research Article

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Microbial insight into rhizosphere of arecanut palms of Wayanad using metagenomics

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Abstract

The rhizosphere bacterial diversity of a plant is considered to play an essential role in mediating plant as well as soil health. An attempt to explore the bacterial diversity in the rhizosphere of arecanut palms in Wayanad was done to obtain an understanding of dominant bacterial phylotypes and the status of nutrient concentrations in rhizosphere soil and plants. Since arecanut production in Wayanad is facing a decline, a study to understand the rhizosphere conditions of healthy palms essentially provided insight into what strategies needed to be adopted for improvement of arecanut cultivation. The nutrient imbalance involving increased iron in soil and deficiencies of calcium, magnesium, zinc, and boron in the Arecanut rhizosphere was found to be an evident reason for the decline in production. Apart from that, the biological activities in the rhizosphere by the diversity of microorganisms were studied to understand the dominant bacterial phyla and genera present in the Arecanut rhizosphere. The presence of various important bacterial phyla like Proteobacteria, Actinobacteria, Acidobacteria, Firmicutes, and Bacteroidetes revealed the presence of various beneficial soil microorganisms and emphasized the need to enhance or augment the population of native microflora for efficient nutrient cycling by increasing the organic content of the soil. Since organic carbon is an essential requirement to support bacterial diversity, proper management practice that encompasses organic carbon amendment along with proper nutritional management could enhance bacterial diversity as well as health of the arecanut palms. The study indicated that the dominant bacterial phyla contained various beneficial microorganisms that can be exploited for improving nutrient recycling in the arecanut rhizosphere.

Keywords: Arecanut, metagenomics, microflora, rhizosphere, soil

Introduction

Arecanut (*Areca catechu* L.) is an important plantation crop cultivated predominantly in the southern states of India. The crop has always been of profound significance due to its integral role in culture and commerce in the Indian context. According to DASD (https://dasd.gov.in/images/kerala/arecanut area-production and productivity in india.pdf), the area under this crop as per 2015-16 values is 472 thousand hectares with production close to 774 thousand tonnes and productivity of 1638 kg ha^{-1}. The scenario in Kerala in the year 2015-16 for arecanut showed a total area of 99.12 thousand hectares with a production of 132.45 thousand tonnes and productivity of 1336 kg ha^{-1}, in which the Wayanad district majorly contributes to the production.

Wayanad district belongs to Central and South Sahyadris, hot, moist, sub-humid to humid eco-subregion (ICAR) (19.2), with sandy clay loam soils being the major soil type. Arecanut, being one of the major plantation crops cultivated in Wayanad district, covered a total area of 12.7 thousand hectares in the year 2011. The crop is being challenged by various biotic and abiotic stresses thereby resulting in decreased productivity, especially in Wayanad district. The increased incidence of yellowing disease, as well as decreased productivity, has led to a decline in the total arecanut production in Wayanad. Adopting cultivation practices that put emphasis on improved soil physico-chemical and biological conditions by organic matter recycling in the rhizosphere increases soil health in relation to soil microflora and enzyme activities (Bhat *et al*., 2008).

Improved rhizosphere microflora has been observed to be linked with plant health and hence, rhizosphere microflora is reported to impart

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benefits like increased plant growth, abiotic stress tolerance as well as suppression of plant pathogens (Berendsen *et al*., 2012; Mendes *et al*., 2013). With the help of culture-independent technologies like metagenomics, more information about the role of these microorganisms are expected to be found out (Compant *et al*., 2010). The current study was undertaken to unravel the prokaryotic diversity in the rhizosphere of arecanut palms, which give an insight into various beneficial microorganisms associated with arecanut. These beneficial microorganisms could be exploited for rhizosphere engineering and thereby improve arecanut growth and soil health in Wayanad region of Kerala.

Material and methods

Collection of rhizosphere soil samples

Soil samples were collected from various arecanut plantations across Wayanad district of Kerala, India. The rhizosphere soil samples from the active root zones within 15-30 cm from the roots and 0-15 cm deep from the surface were collected, processed and stored in refrigerated conditions for further analysis.

Samples were collected from 7 locations near Ambalavayal region of Wayanad, where arecanut plantations were observed to be in healthy conditions compared to other low lying fields. The farmers' fields were maintained by the annual application of cow dung and organic manure with no regular application of fertilizers. Arecanut palms of about 10-15 years age, belonging to Mangala variety were selected for the study since they were predominantly grown in that area. The index leaf, which is the middle portion of the fourth leaf from the apex, (Bhat and Sujatha, 2013) was also collected from the palms for nutrient analysis. Three samples from each palm were collected and pooled to obtain representative results.

Analysis of soil physico-chemical characters and leaf nutrient contents

The soil samples were subjected to the analysis of physico-chemical parameters. The soil pH and EC were estimated using methods illustrated by Jackson (1958). The soil organic carbon was estimated using the Walkley and Black method (Walkley and Black, 1934) and total nitrogen using the Micro-Kjeldahl method (Jackson, 1973). The available Ca and Mg was estimated using spectrophotometry (Hesse, 1971), available S by extraction using 0.15 per cent $CaCl_2$ followed by turbidimetry (Massoumi and Cornfield, 1963) and available P using ascorbic acid reduced molybdophosphoric blue method (Watanabe and Olsen, 1965). The concentration of available Fe, Mn, Zn, and Cu was analyzed using 0.1N HCl extract (Sims and Johnson, 1991) and available B using azomethine-H extract method (Berger and Truog, 1939) using atomic spectrophotometer.

The index leaf samples were dried, powdered and subjected to the analysis of plant nutrient concentration. The concentrations of N, P, K, Ca and Mg were analyzed as per the methods illustrated by Jackson (1973). The concentration of S was estimated using turbidimetry (Hart, 1961) and the concentrations of Fe, Mn, Zn and Cu using diacid method for atomic spectrophotometry (Lindsay and Norwell, 1978) and B using colorimetry of diacid digest using azomethine-H (Wolf, 1974). The values obtained are a mean of three replications.

Biological characterization of soil samples

The biological properties analyzed consisted of microbial biomass carbon using the fumigation-extraction method (Vance *et al*., 1987) and culturable microflora using dilution plate method (Clark, 1965). A mean of three replications was taken at multiple dilutions to obtain the most representative result. The population of bacteria was enumerated using nutrient agar, fungi using Martin's Rose Bengal agar, actinobacteria on Kenknight and Munaier's agar, diazotrophs on Jensen's agar, P-solubilizers using Pikovskaya's agar, fluorescent pseudomonads using King's B agar, *Trichoderma* on *Trichoderma* specific agar and *Bacillus* using heat treatment followed by plating on nutrient agar (Weaver, 1994).

Soil DNA extraction, sequencing and diversity analysis

The metagenomic DNA was extracted using the MN nucleospin soil DNA extraction kit (Macherey Nagel, Germany) to obtain good quality metagenomic DNA. DNA extraction was done in three replications and pooled to obtain a representative result. Two samples namely, CH-5 and CH-7 were selected for metagenomic analysis, since the plantations from which the samples were collected were observed to be properly maintained

compared to other fields. Since arecanut is a perennial crop, the rhizosphere microflora has been designed as a growth of the plant for few years and hence more number of replications were also not required.

The V3-V4 regions of the 16S rRNA gene were amplified using the specific primers (Klindworth *et al*., 2013). The Illumina adapter overhang sequences were then added and target regions were amplified. Library preparation was done and quantified using fluorometric method. The denatured library was then subjected to MiSeq sequencing.

The Fastq sequences were subjected to the bioinformatics downstream processing using the QIIME workflow. The Fastq sequences with a sequence length cut off 250 bp were subjected to quality checking *viz.,* base quality, base composition, and GC content. The sequences were then trimmed off to remove spacer and conserved regions and then subjected to FLASH program to obtain a consensus V3-V4 region. The chimeric sequences were removed using the UCHIME program implemented by USEARCH tool. The pre-processed reads thus obtained were clustered to obtain the operational taxonomic units (OTUs) using Uclust program implemented by QIIME.

The number of OTUs picked up after removing the singletons, were further used for the estimation of bacterial diversity using various annotation tools. The Fastq sequences were analyzed using the Metagenomics Rapid Annotation pipeline (MG-RAST) 4.0.3 to obtain the taxonomical diversity of bacteria and archaea (Meyer *et al*., 2008). The taxonomic profiles were created using the MG-RAST pipeline to compare the sequences against RDP database at 97 per cent identity and an E-value cut off of 5.

Results and discussion

Physico-chemical properties of arecanut rhizosphere and leaf nutrient status

Arecanut palms with no symptoms of biotic and abiotic stresses were studied from seven locations. Plantations adopted proper cultivation practices compared to plantations in other regions were identified, to obtain the representative physico-chemical and biological parameters of the rhizosphere. The plantations in Ambalavayal were observed to be in healthy condition compared to the arecanut plantations in lower lands with poor drainage affected. Studies have shown that continuous manure application has been inevitable to obtain desirable yield in arecanut (Bhat and Sujatha, 2012). The study indicated that inadequacies in manuring and proper management of plantations have resulted in reduced yield and productivity of arecanut.

The soil samples were laterite with loamy texture and ranged from slightly acidic to neutral pH. The nutrient content analysis revealed a deficiency of calcium, magnesium, zinc and boron with a mean value of 395.8 mg kg^{-1}, 90.5 mg kg^{-1}, 2.11 mg kg^{-1} and 0.38 mg kg^{-1} respectively, while other nutrients were mostly present in adequate concentration when compared to the optimum soil nutrient concentrations (Bhat *et al.,* 2012) (Table 1).

The leaf nutrient analysis revealed an increased concentration of iron, copper, and potassium with average values of the seven samples were 640.143 mg kg^{-1}, 58.617 mg kg^{-1} and 0.659 per cent respectively (Table 2). Deficiency of nutrients such as zinc and boron were also observed when compared to the standardized leaf nutrient concentration norms (Bhat and Sujatha, 2013). Since, an increased concentration of iron was observed in the soil as well as leaf tissues, followed by low zinc and boron indicated the result of antagonistic interaction of iron with the minerals, especially zinc resulting in its deficiency (Fargeria, 2001). The imbalance in the nutrient concentrations in the rhizosphere indicates the need for the adoption of improved nutrient management strategies for improving the cultivation aspects.

Culturable microbial diversity and major functional groups

The culturable diversity of bacteria, fungi, actinobacteria, N-fixers, P-solubilizers, fluorescent pseudomonads, *Trichoderma* and *Bacillus* provided the microbial insight on different functional groups of microorganisms prevalent in the rhizosphere of arecanut palms. Bacterial, fungal and actinobacterial populations were enumerated and the mean values were observed as 4.0×10^6, 2.9×10^3 and 1.1×10^3 cfu g^{-1} fresh soil samples respectively. The increased population of nitrogen fixers was observed in samples CH-1 and CH-2,

Table 1. Physico-chemical parameters of arecanut rhizosphere soil samples

Sample	pH	EC (dS m^{-1})	Bulk density (mg m^{-3})	OC (%)	N (%)	P (kg ha^{-1})	K (kg ha^{-1})	Ca (mg ha^{-1})	Mg (mg kg^{-1})	S (mg kg^{-1})	Fe (mg kg^{-1})	Mn (mg kg^{-1})	Zn (mg kg^{-1})	Cu (mg kg^{-1})	B (mg kg^{-1})
CH-1	5.5	0.05	1.33	0.56	0.168	15.51	81.76	116.50	54.50	3.14	65.62	9.74	2.87	1.01	0.28
CH-2	5.6	0.03	1.33	0.43	0.056	13.21	26.88	116.30	21.40	2.88	88.00	3.00	0.34	0.57	0.32
CH-3	5.4	0.12	1.25	1.51	0.112	18.38	285.60	573.00	160.40	6.28	22.72	45.14	2.73	0.66	0.44
CH-4	5.4	0.10	1.17	1.02	0.392	17.23	291.20	459.10	95.90	6.54	37.54	19.43	1.16	0.51	0.46
CH-5	6.1	0.14	1.33	1.00	0.224	29.29	212.80	435.00	75.40	5.23	28.00	20.53	3.03	1.03	0.44
CH-6	5.4	0.50	1.11	1.76	0.448	18.95	892.64	346.50	148.70	10.98	118.80	85.97	2.91	5.21	0.48
CH-7	7.3	0.13	1.33	1.00	0.056	22.97	257.60	723.90	77.70	10.98	66.01	75.18	1.74	1.70	0.24

Table 2. Nutrient status of arecanut index leaf samples

Samples	N (%)	P (%)	K (%)	Ca (%)	Mg (%)	S (%)	Fe (mg kg^{-1})	Mn (mg kg^{-1})	Zn (mg kg^{-1})	Cu (mg kg^{-1})	B (mg kg^{-1})
CH 1	1.89	0.175	0.573	0.342	0.108	0.506	713	67	31.1	39.2	14.285
CH 2	2.14	0.229	0.639	0.489	0.149	0.798	726	90.7	26.3	57.7	15.238
CH 3	2.11	0.118	0.516	0.322	0.084	0.541	669	48.1	21.8	20.5	3.809
CH 4	2.48	0.09	0.63	0.513	0.130	0.850	305	67.5	28.4	9.8	19.999
CH 5	2.38	0.304	0.714	0.488	0.157	1.154	656	113	29.6	78.4	17.143
CH 6	2.25	0.386	0.861	0.545	0.220	1.622	709	121.6	37.8	93.4	5.714
CH 7	2.6	0.234	0.681	0.469	0.148	1.043	703	90	31.9	62.5	7.619

while that of fluorescent pseudomonads were found in CH-6 and CH-7. Among the various groups of microorganisms, population of *Bacillus* was found to be high in the arecanut rhizosphere. Phosphate solubilizers were also observed in the arecanut rhizosphere except the samples CH-4 and CH-5 (Table 3). The population of culturable microorganisms may be considered insufficient as the total microbial biomass carbon was found to be less in the arecanut rhizosphere (Table 3). Microbial biomass carbon is a measure of the weight of microorganisms in soil and it acts as an indicator of soil quality. Since, the microbial biomass carbon is found to be less in acidic soils, as the low pH only supports the growth of acid-tolerant microorganisms, the microbial biomass carbon is a factor that is highly dependent on soil management practices (Schnürer *et al*., 1985). Even though the presence of culturable microorganisms was observed, an enhancement of the active microflora is required as indicated by the microbial biomass carbon in the soil.

A comparatively high population of bacteria and fungus indicated the functioning of active rhizosphere for nutrient cycling and various other fitness benefits including the production of plant growth hormones, disease suppression, and improved soil structure. The population of *Bacillus*, *Trichoderma,* and total bacteria and fungus were observed to be comparatively predominant. An increase in the microbial activity can be achieved by increasing the organic carbon in the soil. The typical bacterial microflora in the arecanut rhizosphere using culture-dependent studies indicated the presence of *Bacillus, Arthrobacter, Micrococcus,* and *Pseudomonas* corresponding to the results obtained in the current study (Bopaiah and Bhat, 1981).

Table 3. Culturable microbial diversity observed in the rhizosphere of arecanut palms in Wayanad

Samples	Microbial biomass carbon (µg g^{-1})	Microbial population in cfu/g^{-1} fresh soil							
		Bacteria $x10^6$	Fungi $x\ 10^3$	Actinobacteria $x\ 10^3$	P-Solubilizers $x\ 10^3$	N-fixers $x\ 10^3$	*Trichoderma* $x\ 10^2$	Fluorescent pseudomonads $x\ 10^3$	*Bacillus* $x\ 10^4$
CH-1	734.10	16.2	19.3	Absent	15	10.8	Absent	20	19.4
CH-2	825.80	19.6	9.5	Absent	15	8.8	Absent	1.25	23.6
CH-3	912.10	92.5	24.2	5.3	2.3	2.8	3.2	4.5	12.5
CH-4	554.67	48.2	39.2	13.6	Absent	Absent	4.8	0.1	6.60
CH-5	190.68	19.8	31.2	8.8	Absent	Absent	Absent	2.4	1.85
CH-6	286.69	41.8	38.6	12.4	6.3	Absent	10.5	0.1	4.15
CH-7	200.62	42.0	42.2	41.3	6.4	1.3	16.5	2.4	6.85

Bacterial and archaeal diversity in arecanut rhizosphere

The bacterial diversity from the arecanut rhizosphere indicated the presence of various phylotypes that are considered to be directly influenced by plant health and soil factors. The usage of metagenomic tools has enabled to study the total bacterial diversity avoiding the plate count anomaly that prohibits the possibility to study unculturable microorganisms. The study of the arecanut rhizosphere with low pH and the above

mentioned nutritional parameters have provided some insight into the bacterial diversity. Even though low soil pH is considered to harbor less number of bacterial communities (Fierer and Jackson, 2006), the arecanut rhizosphere was found to be an active zone for bacterial diversity with 5.68,677 and 3,12,307 OTUs in CH5 and CH7 respectively probably due to the presence of active roots. Since root exudates are highly essential or shaping the rhizosphere microflora (Dennis *et al*., 2010), an abundant bacterial diversity in the arecanut rhizosphere could be correlated to the secretion of root exudates. The major phyla that constituted the bacterial diversity included Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes, Acidobacteria and Verrucomicrobia in various proportions (Fig. 1). Among these dominant phyla, Proteobacteria was found to be the most dominant one with 23 per cent of the total bacterial population in CH-5 and almost 21 per cent of the total bacterial population in CH-7 followed by Actinobacteria, with 11.75 per cent of total bacterial population in CH-5 and 17.6 per cent in CH-7. The dominance of the phyla as indicated by the metagenomic study could also indicate the abundance of phenolics apart from compounds like sugars, amino acids and other organic substances (Chaparro *et al*., 2014), especially in arecanut rhizosphere.

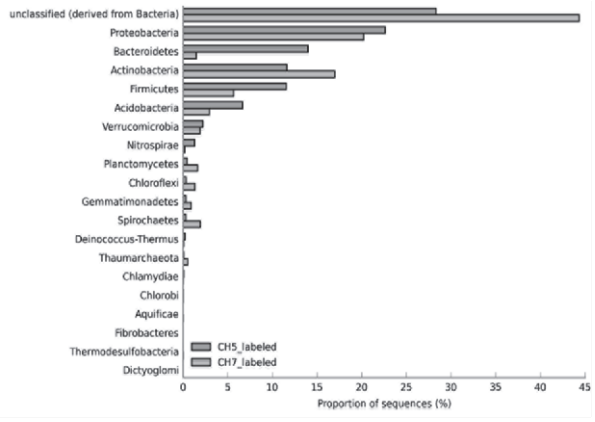

Fig 1. A percentage comparison of various phylum at 99 per cent confidence level was obtained. The comparison of bacterial populations in samples CH-5 and CH-7 were done to observe Proteobacteria, Bacteroidetes, Actinobacteria, Firmicutes and Acidobacteria to be active members of the arecanut rhizosphere microflora

Another aspect of these bacterial phyla on the probable fitness benefits obtained by the arecanut palms by their presence could also be considered. Members of the phylum Proteobacteria and Actinobacteria were recorded to be active members of disease suppressive soils and observed to possess plant beneficial characters including plant growth hormone production, ACC deaminase activity as well as antagonistic activities against plant pathogens (Robleto *et al*., 1988; Palaniyandi *et al*., 2013). The phylum Proteobacteria has been considered to be the most abundant one in the soil ecosystem due to the physiological and nutritional diversity within the phylum (Janssen, 2006). The presence of Proteobacteria in arecanut rhizosphere in large population could indirectly indicate a higher degree of nutrient cycling mediated by microorganisms happening in the rhizosphere.

The phylum Acidobacteria, found to be a significant part of the bacterial population in the arecanut rhizosphere, is also speculated to be an inevitable part of the microbiome involved in maintaining plant health and soil quality (Kalam *et al*., 2017). Although unculturable, the members of this bacterial phylum were found to be present in large numbers indicating the metabolic diversity present in the arecanut rhizosphere ecosystem. A previous study has indicated the presence of Acidobacteria to be high in metabolically active soil with high degree of copiotrophic condition (Lee *et al*., 2008).

The predominant bacterial genera in the arecanut rhizosphere were observed to be *Arthrobacter, Burkholderia, Bacillus, Pedobacter, Acidobacter* and various others. Even though the correlation between a lot of the bacterial taxa are not correlated with the rhizosphere soil conditions due to complexities present in the ecosystem, many of the abundant bacterial genera observed in the arecanut rhizosphere were observed to be capable of plant growth promotion through various activities as indicated by various studies (Tilak *et al*., 2005). Studies using metagenomics have revealed the importance of *Pseudomonas, Burkholderia, Bacillus* and many other bacterial genera to be active members of plant growth-promoting rhizobacteria (Leveau, 2007).

Among the prokaryotic diversity, archaebacteria were observed to constitute only 0.09 and 0.6 per cent of the total prokaryotic

population in samples CH-5 and CH-7 respectively, with the dominant phylum being Thaumarchaeota. The phylum was observed to constitute 93 per cent of the total archaebacterial population in CH-5 and 99 per cent in CH-7. The insight into the archaebacterial diversity in the arecanut palm rhizosphere showed the abundance of phylum Thaumarchaeota in both CH-5 and CH-7 (Fig. 2). Previous studies have revealed the presence of ammonia monooxygenase for oxidizing ammonia into hydroxylamine. The archaea performing ammonia oxidation could also influence the growth of both plants and microbes associated and also act as the main performer in nutrient cycling (Stieglmeier, 2014).

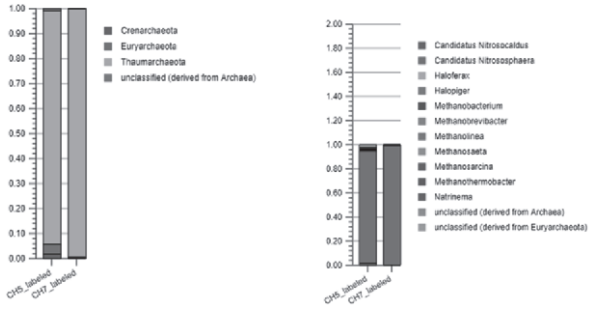

Fig. 2. A comparative analysis of population of archaeabacteria indicated a selectively high population of Thaumarchaeota at phylum level and that of Candidatus Nitrososphaera at genus level

A study on the arecanut based cropping system revealed that organic matter recycling and proper nutrient management to be essential for maintaining the active rhizosphere microflora as well as enzyme activities in the soil (Bhat *et al*., 2008). The analysis of microbial diversity also indicated high microbial population in rhizosphere soil samples with high organic compounds compared to those with low organic carbon, as indicated by the culturable bacterial and fungal population in samples CH-3 and CH-6. Even though it is established that the plant iron nutrition significantly affects the rhizosphere bacterial community (Yang and Crowley, 1999), the current study could not establish the impact of nutritional factors on rhizosphere bacterial community, even though influence by organic carbon was hinted. This study could provide details for further investigations to understand the intricate relationship between the plant health, rhizosphere microorganisms and soil factors that need to be understood for evolving improved cultivation practices for arecanut in Wayanad where the crop is being faced by a plethora of biotic and abiotic stresses.

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