A Comparative Study on the Microbial Communities of *Rhynchophorus ferrugineus* (Red Palm Weevil)-Infected and Healthy Palm Trees

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Abstract

Several studies have investigated palm trees' microbiota infected with red palm weevil (RPW) (*Rhynchophorus ferrugineus*), the major pest of palm trees. This study compared the microbial communities of infected and uninfected palm trees in the Hail region, Northern Saudi Arabia, determined by high-throughput 16S rRNA gene sequencing by Illumina MiSeq. The results indicated that taxonomic diversity variation was higher for infected tree trunk than the healthy tree trunk. Soil samples from the vicinity of healthy and infected trees did not have a significant variation in bacterial diversity. *Myxococcota*, *Acidobacteriota*, and *Firmicutes* were the dominant phyla in RPW-infected tree trunk, and *Pseudomonadaceae* was the most prominent family. This study is the first report on the characterization of RPW-infected and healthy palm trees' microbiome.

Keywords *Rhynchophorus ferrugineus* · Red palm weevil · Microbial community · Palm tree · Illumina MiSeq · Hail region · Northern Saudi Arabia

1 Introduction

Date palm tree (*Phoenix dactylifera* L.) holds a significant cultural and economic status in many Middle East regions since ancient times. The Kingdom of Saudi Arabia is among the top three date-producing countries globally, with date production amounting to 1.3 million tonnes, accounting for nearly 17% of the global date production. Consequently, it is also the world leader in the cultivation of palm trees, and currently, there are around 28.5 million palm trees in the country. However, infections by several insect pests, fungal and bacterial pathogens and other pathogenic agents, nematode diseases, birds, weeds, and succession by other species are the critical issues in palm tree cultivation, which directly affects date production.

The red palm weevil (RPW) *Rhynchophorus ferrugineus* Olivier (Coleoptera: Curculionidae), an invasive wood-boring insect, is the most dangerous and destructive pest to date palm trees and ornamental palm trees in this region [1]. This insect has been designated as a Category 1 date palm pest in the Middle East by the United Nations Food and Agriculture Organization [2] and is often referred to as the 'cancer' of palm trees. RPW is a native of South East Asia, but foreign trade of contaminated plant parts over the last two decades has facilitated its spread and infestation in

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the Middle East [3]. *Rhynchophorus ferrugineus* infects the palm tree by injuring the apical regions, armpit palms, or digging gallery. Infection is established by the presence of necrosis, oozing of a brown viscous liquid, or the presence of perforation on the surface of the trunk. When infection is more advanced, there is a collapse and a sudden drying of palms and abnormal umbrella-shaped canopy is noticed. Heavily infected trees show loss of all their palms and rotting of the trunk, and this is often followed by death of the palm tree. Due to the impact of RPW, hundreds of thousands of infested palm trees die each year [4], resulting in substantial ecological, environmental, and economic losses.

Considering the economic and social effects of RPW infestations, the focus on control and eradication of this pest has significantly increased in the last decades. Evaluating the efficacy of various chemical and bio-control methods has been the theme of most research work in this field [5, 6]. Compared to unrestricted pesticide use, biological control involving the biological system administration is more effective and environmental friendly [7, 8]. In this aspect, the microbial profile of RPW has not been adequately understood, although an intracellular endosymbiont, '*Candidatus nardonella*' has been identified in RPW [9, 10]. Several studies have been carried out to characterize gut bacterial population in RPW [11, 12]. Other studies aimed at identifying possible bacterial pathogens in RPW to develop bio-control strategies against this insect [13, 14]. Studies by Pu et al. have shown that *Bacillus thuringiensis* (Bt) can be used as a potential bio-pesticide for RPW control and can effectively minimize damage to palm trees [15]. In another study from our own institutes, we found that the genera *Aspergillus* and *Fusarium* spp were highly associated with RPW [16]. Another study found that different types of bacilli were associated with RPW [17].

Another strategy for eradicating pathogenic infections in plants is by controlling phytoplasmas, which are pathogenic bacteria inhabiting plants and transmitted by insect vectors such as Hemiptera [18]. Although removal of infected plant that serves as source inoculum helps mitigate disease to some extent [19, 20], efficient control has not been found to date. Understanding an infected plant's microbial community profile compared with an uninfected and healthy plant's microbial profile will help develop an effective bio-control strategy or find out new bacteria. There have been no reports on microbial communities of the date palm tree, diseased or healthy. This study was carried out to examine the contrast of microbial communities among RPW-infected palm trees and healthy trees as a primary data source for forthcoming comprehensive studies. To date, there are no reports on microbiome analysis of palm trees and the effect of RPW infestation on the host-microbial community.

2 Materials and Methods

2.1 Study Site

The study site is located in the Hail region of Northern Saudi Arabia (27°59'05.7" N and 41°45'46.3" E). Climate is hot in summers and cold in winters, with the temperature ranging from 34 to 44 °C in the summer and 2–16 °C in winters. Average annual precipitation ranges from 10 to 20 cm, and the site is vulnerable to extreme conditions like storms and droughts. Several species of palm trees dominate the flora of this region as shown in Fig. 1.

Fig. 1 **a** Red palm weevil documented on palm tree trunk, **b** infected tree trunk, **c** healthy tree trunk

2.2 Sample Collection

Sampling was carried out at the study site during September 2020, when RPW disease infestations were observed in the palm trees. Samples were collected from infected tree which contain numerous egg and larvae. Presences of more than 50 adult pest in and around the tree were observed. All infected palm tree had multiple perforations which could count for more than five. Trunks destroyed from the middle could reasonably be assumed to be created by RPW. Uninfected or healthy trees were selected by observing plants with no trunk perforations and absence of egg and larvae of the insect. Both infected and healthy tree samples were taken from the trunk tissue. The trees were photographed in the field, followed by the removal of the trunk from the base. Trunk samples (measuring 1 cm in diameter and 5 cm in length) were cut by a sterile knife, further sized down to 0.5 cm in thickness and 0.8 cm in diameter, placed in an Eppendorf tube and stored in liquid nitrogen for transport. Additionally, samples of soil close to the trees' base were collected using a knife or a small hatchet after removing debris and litter from the surface. Samples were then placed in a plastic collecting box labeled with a unique number and returned to the laboratory. Totally 12 samples were collected. Three samples were obtained from the trunk and soil each of a palm tree that had never been infected by RPW, whereas the other three samples were collected from the trunk and soil of RPW-infected palm.

2.3 DNA Extraction, PCR, and 16S rRNA Sequencing

DNA was isolated from one or more representative trunk for each different palm tree, tentatively identified based on RPW infections, using an EZNA HP Plant DNA Kit (Omega). Genomic DNA was amplified using 1541R 5' AAGGGG TGATCCAGCCAGCCCA-3' and 8F 5' AGAGTTTGATCC TGGCTCAG-3' primers [18, 21]. The 16S ribosomal RNA gene was amplified using polymerase chain reaction (PCR) (at 95 °C for 3 min, followed by 35 cycles at 98 °C for 30 s, then for 30 s at 55 °C, 72 °C for 1 min, and at the end of the extension for 5 min at 72 °C). PCRs were carried out in a 25- μ l mixture containing 1 μ l of each universal primer (8F and 1541R); 3 μ l of DNA template; 7.5 μ l of nuclease-free water and 12.5 μ l of GoTaq® Green Master Mix (Promega™ Corporation, WI). Agarose gel electrophoresis was carried out using 1 μ l of SYBR Safe DNA Gel Stain (Thermo Fisher Scientific) fluorescent dye; assess the amplification of the 16S rRNA region.

2.4 Preparation of 16S rRNA Amplicon Libraries and Illumina MiSeq Sequencing

By targeting the V4 region of the bacterial 16S rRNA gene, Index PCR was carried out by the dual-index strategy for

primer design as described in an earlier study [19, 20, 22]. In short, dual-index PCR reaction was carried out in 25- μ l reaction mixture consisting of 3 μ l of DNA template, 2.5 μ l of AccuPrime™ PCR buffer II, 0.5 μ l of individually index primers, 0.1 μ l of AccuPrime™ Taq DNA Polymerase (Invitrogen, Carlsbad, CA), and 18.4 μ l of nuclease-free water according to the manufacture's protocol. PCR amplifications were performed with an initial denaturation for 3 min at 95 °C. Temperature cycling at 95 °C for 30 s and 55 °C for 30 s primer annealing, and extension at 72 °C for one minute for 35 cycles. Using SequelPrep kits (Invitrogen), the exact size amplicons were conducted according to the manufacturer's procedure to purify and normalize subsequent positive magnification. After pooling the library, the concentrations of amplicons were calculated using an Agilent bioanalyzer.

The amplicon library was counted through qPCR using the same primers and PerfeCta NGS library quantification kits (Quanta Biosciences Inc., Beverly, MA, United States) for actual exact concentration, according to the manufacturer's directions. During counting, the library was denatured, diluted, and sequenced on an Illumina MiSeq to suitable concentrations. Sequencing reads were downloaded in Fastq file format from the Illumina Basespace server. At read 1, the sequences were demultiplexed, and read 2 was obtained with a length of approximately 250 bp. Practical taxonomic parts were defined and classified by 95% matching with a reference in the SILVA database. Further analysis was carried out using Mothur v1.41.1 Pipeline [22]. Nonmetric multidimensional scaling (NMDS) plots were acquired based on the Bray–Curtis index using PAST 3.18 software [23]. Statistical analysis was carried out using standard methods and LEfSE based on Linear Discriminant Analysis (LDA).

2.5 Statistics

Data analysis was done using R software environment for statistical computing and graphics version 4.0.3 (R Core Team, 2021). Categorical data were represented by frequency and percentage, and non-normal continuous data were represented using median [1st Quartile, 3rd Quartile]. A comparison between the groups was made using the Kruskal–Wallis test and Pairwise Wilcoxon Rank Sum Test (PWRST). $p < 0.05$ was considered significant.

3 Results

3.1 Diversity of Microbial Population between Diseased and Healthy Palm Trees

In the study area, five plants were infected among 300 plants. The infections were very typical of RPW with plant foliar damage as well as the trunk contain numerous egg

and larva. These resulted in observable tissue damage and fermentation of trunk area of the plant. Red palm weevil is a hidden pest most of its life cycle developing inside the palm tree except the adult which is coming out of the tree rarely.

A total of 1620 bacterial amplicon sequence variants (ASV) were obtained (median value) from soil and trunk of healthy and infected palm trees. Using the Kruskal–Wallis test, it was found statistical significance on the diversity between the family, genus and phylum of microbial community of soil samples and trunk samples of healthy and diseased palm trees as evident from comparison of median values ($p < 0.05$ for all pairs) (Table 1). However, no significant differences were observed between the family, genus or phylum of healthy and diseased samples of soil or tree trunk as analyzed by unpaired Wilcoxon test (Table 2). Tree trunk samples had more diversity and richness of microbial community as compared to soil samples. Besides, no significant differences in diversity in the microbial community in the soil samples collected from around healthy trees and the soil from infected trees were observed. In contrast, the highest community richness and diversity were observed in diseased tree trunk samples compared to the healthy tree trunk (Fig. 2).

3.2 Bacterial Community Structure at Taxonomic Levels between Diseased and Healthy Palm Trees

Illumina MiSeq was used to characterize bacterial strains into at various taxonomic level viz. phylum, families and genus. A comparison between microbial community of healthy and RPW-infected palm tree trunk was then drawn to elucidate the community structure at various taxonomic levels.

Table 3 shows the different phylum found in the healthy and infected palm tree trunk. It was observed that trunk samples collected from infected palm tree had maximum bacterial diversity represented by 27 different phyla. Of the 27 different phyla, 10 phyla were dominant with more than 5000 ASVs. On the other hand, uninfected palm tree trunk presented 20 different phylum levels with only seven dominant phyla repeated more than 5,000 times. Also, *Proteobacteria* and *Bacteroidota* were the dominant phyla with the highest representation in both healthy and infected trunk samples (Fig. 3). Additionally, phyla *Myxococcota*, *Acidobacteriota*, and *Firmicutes* were found exclusively in the sample from the infected trunk and were absent in healthy tree samples.

At the family level, 480 different families were identified in both healthy and infected trunk samples. In samples from RPW-infected trees, diversity at the family level

Table 1 Diversity of microbial population between diseased and healthy palm trees was compared by Kruskal–Wallis test

Factor		Taxonomic level			
		Family	Genus	Phylum	<i>p</i> -Value
Soil	<i>N</i>	318	519	32	< 0.0001
	Median [Q1, Q3]	36.83 [8.67, 224.92]	25.33 [6.67, 124.83]	177.5 [31.92, 2088.83]	
Infected soil	<i>N</i>	380	716	32	< 0.0001
	Median [Q1, Q3]	42.17 [8.58, 228.42]	25.17 [6.33, 101.42]	145 [21.83, 2014.67]	
Trunk	<i>n</i>	218	366	21	0.0006
	Median [Q1, Q3]	55.5 [8, 227.5]	31.17 [6.75, 183.33]	809.33 [31.33, 2443]	
Infected trunk	<i>n</i>	328	553	28	< 0.0001
	Median [Q1, Q3]	41 [11.25, 191.33]	20.67 [6, 100]	210.67 [14.67, 3467.83]	

n Number of sequences; $p < 0.05$ is considered significant

Table 2 Distribution of sequences at various taxonomic levels was compared by Wilcoxon test

Taxonomic level		Soil	Infected soil	<i>p</i> -Value	Trunk	Infected trunk	<i>p</i> -Value
Family	<i>n</i>	318	380	0.94	218	328	0.84
	Median [Q1, Q3]	36.83 [8.67, 224.92]	42.17 [8.58, 228.42]		55.5 [8, 227.5]	41 [11.25, 191.33]	
Genus	<i>n</i>	519	716	0.55	366	553	0.02
	Median [Q1, Q3]	25.33 [6.67, 124.83]	25.17 [6.33, 101.42]		31.17 [6.75, 183.33]	20.67 [6, 100]	
Phylum	<i>n</i>	32	32	0.75	21	28	0.56
	Median [Q1, Q3]	177.5 [31.92, 2088.83]	145 [21.83, 2014.67]		809.33 [31.33, 2443]	210.67 [14.67, 3467.83]	

$p < 0.05$ is considered significant

Fig. 2 Diversity of microbial population between diseased and healthy palm trees

Table 3 Distribution of dominate bacterial phylum in relation to the infection trunk substrate and no infection palm tree trunk

ASVID	Healthy trunk (CT)		Infected trunk (IT)	
	No. of ASVs	% Distribution	No. of ASVs	% Distribution
<i>Myxococcota</i>	0	0	5489	2
<i>Verrucomicrobiota</i>	14,582	5.6	6816	2
<i>Acidobacteriota</i>	0	0	9593	3
<i>Planctomycetota</i>	7330	2.8	12,835	5
<i>Firmicutes</i>	0	0	13,288	5
<i>Chloroflexi</i>	5677	2.19	18,026	7
<i>Patescibacteria</i>	22,424	8.6	20,352	7
<i>Actinobacteriota</i>	33,571	13	30,958	12
<i>Bacteroidota</i>	62,492	24	31,235	12
<i>Proteobacteria</i>	112,643	43.5	116,780	45
Total	258,719	100	256,372	100

IT presented infected palm tree trunk, CT presented uninfected palm tree trunk

was represented by 328 different families. On the contrary, 217 different families were characterized in samples from healthy tree trunk samples. A total of 26 different families were recorded for their richness due to greater number (> 5000 times) (Fig. 4). Of the 26 diversity-rich families, 15 families were from the infected tree and 11 from healthy tree samples. The *Pseudomonadaceae* family found in infected tree trunk has the highest distribution (20%) among the various recorded families in both infected and healthy tree samples (Table 4).

At the genus level, up to 554 different genera were identified in the microbiome of infected trunk and 367 genera in the microbiome of healthy trunk. The microbial community of infected trunk was dominated by *Pseudomonadaceae* (20%) and *Chitinophagaceae* (7%). On the other hand, *Caedibacteraceae* (10%) and *Intrasporangiaceae*

(9%) were the dominant families in uninfected tree trunk samples (Table 5; Fig. 5).

Overall observations in trunk and soil are shown in Figs. 6 and 7.

4 Discussion

Many researchers have established, directly or indirectly gut microflora of the RPW is responsible for the degradation of palm tree. Some researchers focused on the bacterial study and some are on fungi. From our department in a previous study fungi related with the RPW were studied; *Aspergillus niger* contributed to the highest number of fungal isolates among all species, followed by *Aspergillus*

Fig. 3 Distribution and domains phyla in two different substrates (IT and CT)

Fig. 4 Distribution and dominant families in two different substrates (IT and CT)

Table 4 Distribution of the dominant families presented in healthy trees (CT) and infected trees (IT)

ASVID	IT	% Distribution	CT	% Distribution
<i>Enterococcaceae</i>	5332	4	0	0
<i>Enterobacterales</i>	5394	4	0	0
<i>Microscillaceae</i>	5637	4	0	0
<i>Xanthobacteraceae</i>	5639	4	0	0
<i>Xanthomonadaceae</i>	5668	4	0	0
<i>Erwiniaceae</i>	5954	4	0	0
<i>Enterobacteriaceae</i>	6349	4	0	0
<i>Saccharimonadales</i> LWQ8	6635	5	0	0
<i>Anaerolineae</i> SBR1031 A4b	7084	5	0	0
<i>Flavobacteriaceae</i>	7114	5	0	0
<i>Pirellulaceae</i>	7698	5	0	0
<i>Solirubrobacterales</i> 67–14	7797	5	0	0
<i>Chitinophagaceae</i>	10,553	7	0	0
<i>Moraxellaceae</i>	10,726	7	0	0
<i>Pseudomonadaceae</i>	28,781	20	0	0
<i>Thioalkalspiraceae</i>	0	0	5022	3
<i>Actinobacteria</i> PeM15 PeM15	0	0	5092	3
<i>Burkholderiales</i> B1-7BS	0	0	5679	4
<i>Cryomorphaceae</i>	0	0	8522	6
<i>Pleomorphomonadaceae</i>	0	0	10,552	7
<i>Saccharimonadales</i> S32	6635	7	12,910	8
<i>Intrasporangiaceae</i>	0	0	14,100	9
<i>Caedibacteraceae</i>	0	0	15,307	10
<i>Chitinophagales</i> 37–13	10,553	7	20,673	14
<i>Sphingobacteriales</i> S15–21	0	0	27,072	18
<i>Rickettsiales</i> SM2D12	0	0	27,338	18
Total	143,549	100	152,267	100

Table 5 Distribution of the dominant genera presented in both genera (IT and CT)

ASVID	(IT) ASV	%	(CT) ASV	%
<i>Enterobacterales</i>	0	0	5022	5
<i>Rhizobiaceae</i>	5394	6.2	5835	6
<i>Segetibacter</i>	0	0	6387	7
<i>Gryllotalpicola</i>	0	0	6509	7
<i>Sphingomonas</i>	0	0	6862	7
<i>Flavobacterium</i>	6410	7	8521	9
<i>Asticcacaulis</i>	0	0	8935	9
<i>Sphingomonadaceae</i>	0	0	11,841	12.3
<i>Saccharimonadaceae</i> TM7a	6635	8	12,910	13.4
<i>Mucilagibacter</i>	0	0	22,924	23
<i>Enterococcus</i>	5332	6	0	0
<i>Chloroflexi</i> <i>Anaerolineae</i>	7084	8	0	0
<i>Chitinophagales</i>	7562	9	0	0
<i>Solirubrobacterales</i>	7797	9	0	0
<i>Acinetobacter</i>	10,720	12.5	0	0
<i>Pseudomonas</i>	28,740	33.5	0	0
Total	85,674	100	95,746	100

flavus and *Fusarium solanum* [16]. However, present study deals with bacterial isolates.

In that context, they could isolate many fermenting bacteria from the gut. In a study with germ free RPW larvae, it was proved that there is cross-talk between RPW and its gut microbiota [23]. RPW gut microbiota exhibited a highly stable microbial community with low variance in abundance across different life stages and host plants. In a study from Italy, they found many facultative and obligate anaerobic bacteria with a fermentative metabolism, mainly *Dysgonomonas*, *Lactococcus*, *Salmonella*, *Enterobacter*, *Budvicia*, *Entomoplasma*, *Bacteroides* and *Comamonas* in RPW gut. These bacteria may be directly or indirectly responsible for the RPW larvae thrive [24, 25]. In the present study, efforts were made to isolate microorganism from the infected site and correlate between healthy plants.

In the present study, only two plants were included, the number of sample was low because while sampling requires to cut open the tree which causes huge economic loss to farmer as well as the study was aimed to have a pilot study by using next-generation sequencing.

Fig. 5 Distribution and dominant genera in two different substrates (IT and CT)

A significant number of microbes are present in animals and plants' bodies, carrying out multiple interactions that can modulate the roles and behaviors within the complex. Like every other biological species, the microbial community is subject to succession. This study investigated the bacterial community in soil and tree trunk from healthy and diseased palm trees infected by *Rhynchophorus ferrugineus* (Red Palm Weevil) under field conditions. Till date there have been no reports on the microbial community structure RPW-infected palm trees. In our study, we found significant differences in the microbiological diversity of RPW-infected palm trees and healthy trees. Previous studies have shown that plant infection by pathogens has a profound effect on the microbial community of the plant host [26, 27]. The findings of this study, therefore, align with previously reported literature.

The microbial diversity was more prominently marked in tree trunks as compared to soil samples. In this analysis, soil samples were taken from the surface, partially explaining the lack of significant variations in diversity between soil from areas surrounding healthy and diseased trees. On the other hand, the difference in bacterial community diversity was significant in the trunk samples of diseased and healthy palm trees. Proença et al. [28], in their studies on pine wilt disease, found that infection can increase the diversity of endophytic wood-colonizing bacteria in the host *P. pinaster* trees. They also noticed that the endophytic bacterial community differed as the disease progressed, suggesting the importance of the host plant microbiome's disease progression development.

The time difference between the appearance of first symptoms after RPW infestation and the tree's death is crucial for the evolution of the bacterial community in the diseased

tree. Considering the pathology of disease progression from initial latent infection followed by necrosis and finally death, it is imperative that different bacterial communities are involved in the various stages of the disease. However, in this study, samples were collected and analyzed from only one stage of the disease. Therefore, it is necessary to investigate the bacterial community at different stages of RPW infection and in the soil at different depths after disease occurrence.

Plants host individual and diverse microbial communities that are important for their survival, according to new evidence for their close relationship with their microbiota [29]. Understanding their relatedness using genome-based and high-throughput techniques remains highly pursued in microbiome research. In this study, we identified microbial communities at various taxonomic levels using the Illumina Seq technique. The soil microbiota is essential for suppressing soil-borne plant pathogens and improving the natural suppressiveness of soil. Microbiome disruption causes specific perturbation, which changes and shapes the network of soil microbial populations, increasing resistance to phytopathogens and diseases. Similar results were obtained in our study where bacterial population of the palm trees were same as that of the soil samples collected from the vicinity of the trees. Considering this, the ASVs were analyzed for the samples collected from the trunk since the difference in diversity in soil samples was not much marked in this study.

The number of phyla, families and genus was significantly high in infected tree trunk as compared to healthy tree trunk. In the case of phyla, *Myxococcota*, *Acidobacteriota*, and *Firmicutes* were exclusively detected in the diseased tree trunk. Members of these phyla have been recorded to be pathogenic in some cases. Among the families detected in

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Fig. 7 Different types of culture yielded from the infected and healthy trunk

plant development or the growing season. By understanding the variation and composition of the bacterial community of citrus, we can conduct RPW control treatments better and harness the plant's natural microbial population. This study will help to establish better management and treatment strategies.

5 Conclusions

Significant differences for the bacterial community structure and function between healthy and diseased trees were observed in trunk and soil samples of palm trees, suggesting that RPW can only affect the host bacteria community structure and function in certain anatomic regions of the host tree. Our study proved that taxonomic diversity variation was higher for infected tree trunk than the healthy tree trunk. Soil samples from the vicinity of healthy and infected trees did not have a significant variation in bacterial diversity, proving that changes in the microbial community are mostly present in the trunk regions. The bacterial community in the healthy trunk was dominated by *Acidobacteria* (phylum) and *Mucilaginibacter* (genus), whereas infected trunk, *Proteobacteria* (phylum), *Firmicutes* (phylum), and *Pseudomonas* (genus) were dominant. Further investigation of the host-microbial community and function in different stages of RPW and the rhizosphere is needed to elucidate the effect of RPW on the microbiome of palm trees. Our data suggest that not only gut microbes of RPW, other microbes commonly present on rhizosphere might contribute or augment the death of palm tree when it gets infected with the RPW. However further *in vitro* study is essential to prove the same.

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