Detection and Identification of Phytoplasma of *Cleome rutidosperma* in Areca Palm Yellow Leaf Disease Field

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Abstract [**Objectives**] The paper was to detect and identify the phytoplasma of *Cleome rutidosperma* in areca palm yellow leaf disease (YLD) field in Wenchang City, Hainan Province, China. [**Methods**] The nested PCR technique was employed to amplify the phytoplasma 16S rDNA of *C. rutidosperma* samples, followed by sequence analysis. Concurrently, this study examined *C. rutidosperma* in YLD field, collecting symptomatic leaves for phytoplasma detection. [**Results**] The 16S rDNA sequence of the *C. rutidosperma* witches'-broom phytoplasma was found to be identical to that of the HNWC5 strain associated with areca palm yellows phytoplasma, leading to the identification of this phytoplasma as belonging to the 16SrII-A subgroup. Field investigations revealed a higher incidence of *C. rutidosperma* in areca palm fields, with symptoms of leaf yellows observed in six of these fields. Quantitative PCR (qPCR) analysis confirmed the presence of phytoplasma infection in these instances. [**Conclusions**] Through the analysis of geographical distribution, sequence alignment, and field occurrence data, a significant correlation has been identified between witches' broom disease and YLD. It is proposed that the former may act as an intermediate host for the areca palm yellows phytoplasma.

Key words Areca palm yellow leaf disease; Phytoplasma; *Cleome rutidosperma*; Identification; Detection

1 Introduction

Areca palm yellow leaf disease (YLD) is a severe condition resulting from phytoplasma infection. In the initial stages of infection, the lower leaf tips of the areca palm plant exhibit yellowing. As the disease progresses to the intermediate stage, the crown of the tree begins to shrink. Ultimately, in the late stage, the condition leads to a characteristic bunchy top appearance and culminates in the death of the plant. In 2008, it was reported that the disease impacted several cities and counties, including Qionghai, Wanning, Lingshui, Qiongzhong, Sanya, Ledong, and Baoting, with the affected area exceeding 2 000 hm^2. By early 2021, the disease had disseminated to 18 cities and counties in Hainan Province, with the total affected area surpassing 32 102.38 hm^2. This figure represents 27.89% of the total cultivation area of areca palm in the province, with a notably high incidence concentrated primarily in the central and eastern regions of Hainan[1-2]. Currently, the incidence of YLD in areca palm field ranges from 10% to 30%. In severely affected areas, this incidence can reach as high as 90%, leading to a reduction in production of 70% - 80%, and in some cases, complete cessation of production[1]. According to preliminary statistics, the annual economic loss attributed to YLD exceeds 2 billion yuan, significantly impacting farmers' efforts in poverty alleviation and the overall development of the rural economy[3].

Phytoplasma is a type of obligate pathogenic bacterium that parasitizes the phloem cells of plants. It is primarily transmitted by phloem-feeding insects, including leafhoppers, planthoppers, and psyllids[4-5]. Currently, it has been reported that the vector insect responsible for YLD in India is *Proutista moesta* Westwood, whereas the vector insect associated with YLD in China is *Icerya seychellarum*[1,6]. Phytoplasma exhibits a broad host range, with phytoplasma-related diseases being particularly prevalent in temperate and tropical regions. These pathogens have been documented to infect over 1 000 plant species[7-8]. Phytoplasma-infected plants encompass a variety of categories, including food crops, fruit trees, vegetables, forage crops, and weeds, which collectively result in substantial economic losses[9-13].

Cleome rutidosperma DC. is an annual herb that belongs to the genus *Cleome* within the family Cleomaceae. This species is primarily distributed across Singapore, Malaysia, Indonesia, and other regions of Southeast Asia, as well as in the Chinese provinces of Guangdong, Guangxi, Fujian, Yunnan, and Hainan. It has been introduced to China as an ornamental plant and is classified as a low-risk invasive species[14]. *C. rutidosperma* possesses significant potential value in both medicinal applications and ecological conservation[15-17]. Furthermore, it is frequently encountered as a common weed in areca palm field. In October 2022, the research team identified a significant area of *C. rutidosperma* leaves exhibiting symptoms including witches'-broom, yellowing, and little leaf within the YLD field located in Wenchang City, Hainan Province, China. It is hypothesized that these symptoms may be attributed to a phytoplasma infection, suggesting a potential correlation between YLD and the observed symptoms in *C. rutidosperma*. Consequently, a molecular technique was employed to clone the 16S rDNA of phytoplasma from a sample of *C. rutidosperma*. This cloned sequence was subsequently compared with the phytoplasma associated with YLD. Furthermore, we examined the prevalence of *C. rutidosperma* in areca palm field in Wenchang, Qionghai, and Wan-

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ning, Hainan, and collected symptomatic leaves for phytoplasma detection. This study offers a theoretical foundation for research on the disease cycle and the prevention and control of YLD.

2 Materials and methods

2.1 Materials

2.1.1 Test materials. The *C. rutidosperma* witches'-broom samples were collected from the YLD field in Wenchang, Hainan, while the healthy samples were obtained from the field in the same region. A total of three samples from each category were collected and subsequently stored at −20 ℃ for future analysis.

2.1.2 Main agents. The 2 × *Taq* PCR Mix, plant genome DNA extraction kit, and DNA purification recycling kit were procured from Tiangen Company. The pMD18-T vector was obtained from TaKaRa, while the DH5α competent cells were acquired from Shanghai Weidi Biotechnology Co., Ltd. The primary instruments utilized in this study included the WB100-2 constant temperature water bath, the 5810R centrifuge, the KZ-5F-3D high-speed and low-temperature tissue grinding instrument, and the Biometra TOne 96G PCR instrument.

2.2 Methods

2.2.1 Extraction of total DNA from leaves of *C. rutidosperma*. According to the instructions provided with the plant genomic DNA extraction kit, sample processing and DNA extraction were conducted in accordance with the specified protocols. The extracted DNA was subsequently stored at −20 ℃ for future use.

2.2.2 PCR amplification of phytoplasma 16S rDNA. The nested PCR method was utilized for amplification, employing DNA from *C. rutidosperma* witches'-broom as the template. DNA extracted from healthy plants served as a negative control, while ddH_2O was used as a blank control. The initial step of nested PCR amplification of the phytoplasma 16S rDNA gene involved the use of primers P1/P7, as designed by Schneider *et al.*[18]. The subsequent step employed primers R16F2n/R2, which were developed by Gundersen *et al.*[19], for the amplification of the same gene. The sequence of primers utilized in this study is presented in Table 1 and was synthesized by Sangon Biotech (Shanghai) Co., Ltd. The amplification reaction system consisted of 12.5 μL of 2 × *Taq* PCR Mix, 8.5 μL of ddH_2O, 1.0 μL of upstream and downstream primers (10 μmol/L), and 2.0 μL of DNA template. The procedure for the amplification reaction was conducted as follows: predenaturation at 95 ℃ for 5 min, denaturation at 95 ℃ for 45 sec, annealing at the specific temperatures for each primer as indicated in Table 1 for 45 sec, and extension at 72 ℃, with the duration calculated at a rate of 1 kb/min of amplification length. The amplified products were visualized using UV imaging following 1% agarose gel electrophoresis.

2.2.3 Recovery and purification, target gene cloning and sequencing of PCR products. DNA purification and recovery kit was utilized to recover and purify the target band in accordance with the provided instructions. Subsequently, the target fragment was ligated to the pMD18-T vector following the manufacturer's guidelines. The recombinant plasmid was then introduced into DH5α competent cells following the specified protocol, and the cells were cultured at 37 ℃ on LB medium supplemented with ampicillin (Amp). Individual colonies were isolated and confirmed through colony PCR, and the positive bacterial cultures were subsequently sent to Shenzhen BGI Genomics Co., Ltd. for sequencing.

Table 1 Information of primers used in this study

Primer name	Sequence(5'-3')	Fragment size//bp	Annealing temperature//℃
P1	AAGAGTTTGATCCTGGCTCAGGATT	1 800	48
P7	CGTCCTTCATCGGCTCTT		
R16F2n	GAAACGACTGCTAAGACT	1 200	55
R16R2	TGACGGGCGGTGTGTACAAACCCCG		

2.2.4 Sequence analysis of 16S rDNA and construction of phylogenetic tree. The target fragment underwent homologous retrieval of phytoplasma utilizing the BLASTn online tool available on the NCBI website (https://www.ncbi.nlm.nih.gov/) to determine its association with phytoplasma. The 16S rDNA gene sequences corresponding to each group or subgroup of phytoplasma were retrieved from the GenBank database. These sequences were subsequently compared with the target fragment using DNAMAN software to conduct a preliminary assessment of the classification status of the phytoplasma. Additionally, the target fragment was uploaded to the GenBank database. Using MEGA 7.0 software and the maximum likelihood method (ML), a bootstrap value of 1 000 was established to construct a phylogenetic tree based on the 16S rDNA gene sequences. Additionally, an analysis was conducted to examine the correlation between the *C. rutidosperma* witches'-broom phytoplasma and the areca palm yellows phytoplasma.

2.2.5 Occurrence of *C. rutidosperma* in areca palm field and detection of its phytoplasma. The survey was conducted in October 2023, focusing on three cities and counties in the eastern part of Hainan: Wenchang, Qionghai, and Wanning, which are primarily engaged in the cultivation of areca palm. In Wenchang, the investigation encompassed four sites located in Zhongxing, Tanniu, Penglai, and Wencheng towns. In Qionghai, four sites were examined in Wanquan, Tayang, Jiaji, and Changpo towns. Similarly, in Wanning, the survey covered four locations in Xinglong, Longgun, Shangen, and Wancheng towns. A random inspection was conducted to investigate five areca palm fields exhibiting yellowing symptoms across each township. The longitude and latitude of each location were recorded, along with the yellowing rate of YLD. Additionally, statistical analyses were performed to assess the occurrence and incidence of *C. rutidosperma* in the areca palm fields. Samples displaying symptoms were collected for DNA extraction, and these samples were subsequently analyzed using a general quantitative polymerase chain reaction (qPCR) detection method for phytoplasma[20]. Occurrence ratio of *C. rutidosperma* = Frequency of occurrence of *C. rutidosperma* /Total number of areca palm fields under investigation. Incidence ratio of *C. rutidosperma* = Number of areca palm fields exhibiting diseased *C. rutidosperma* /Total number of areca palm fields under investigation. Detection rate of phytoplasma = Positive rate of phytoplasma detection/Total number of detections × 100%.

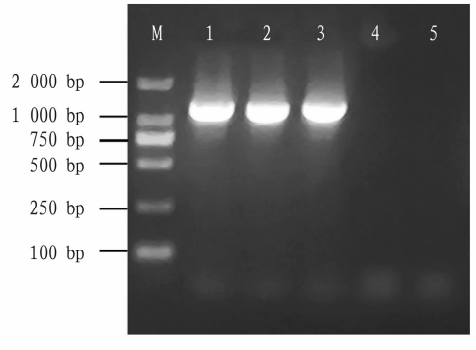
3 Results and analysis

3.1 Symptoms of infected *C. rutidosperma* The infected *C. rutidosperma* plants were identified in a YLD park in Wencheng Town, Wenchang City, Hainan Province (19°33′9″ N, 110°48′5″ E). The affected area encompassed approximately 30 m^2 and exhibited a pattern of consecutive lesions. In comparison to healthy specimens, the infected plants displayed characteristic witches'-broom symptoms, including shortened stem segments and dense clumping, along with leaf yellowing and reduction in leaf size, resulting in a more slender growth form (Fig. 1).

NOTE A – B. Symptoms of diseased plants; C. Healthy plants in the field.

Fig. 1 Symptoms of diseased plants of *Cleome rutidosperma*

3.2 PCR amplification The products of PCR amplification were subjected to analysis via agarose gel electrophoresis. As illustrated in Fig. 2, the amplification band corresponding to the 16S rDNA gene measured approximately 1 200 bp. Furthermore, no amplification bands were observed in the negative control and blank control, indicating that the PCR amplification system was free from contamination and that the size of the 16S rDNA gene fragment met the expected criteria. Preliminary assessments suggest that the pathogenic symptoms observed in *C. rutidosperma* are attributable to phytoplasma infection, and the strain has been provisionally designated as *C. rutidosperma* witches'-broom phytoplasma Hainan strain.



NOTE M. DL2000 DNA Marker; 1 – 3. *Cleome rutidosperma* witches'-broom samples; 4. Negative control; 5. Blank control.

Fig. 2 The results of 16S rDNA amplification products by gel electrophoresis

3.3 Sequence analysis of 16S rDNA gene The target fragment was successfully cloned and sequenced, yielding 1 248 bp of 16S rDNA gene fragment (GenBank No.: PP780014). The 16S rDNA sequences obtained from the three samples exhibited consistency. BLASTn analysis conducted to retrieve the target fragment sequence from the NCBI website indicated that the 16S rDNA of *C. rutidosperma* witches'-broom phytoplasma exhibited a similarity of over 99.68% to the 16S rDNA of the 16SrII group phytoplasma across 100 sequences. The 16S rDNA gene sequences of the *C. rutidosperma* witches'-broom phytoplasma were analyzed and compared with those of various phytoplasma groups and subgroups. The findings indicated that the 16S rDNA of the *C. rutidosperma* witches'-broom phytoplasma exhibited 100% similarity to that of the areca palm yellows phytoplasma HNWC5 strain, which belongs to the 16SrII-A subgroup (GenBank No.: OQ586072). Furthermore, the similarity with the cotton phyllody phytoplasma (GenBank No.: EF186827) and the candidatus phytoplasma aurantifolia (GenBank No.: U15442), both classified within the 16SrII group, was found to be greater than 97.92%. In contrast, the similarity with other phytoplasma groups was comparatively low, remaining below 92.04% (Table 2). The phylogenetic tree of the 16S rDNA gene of *C. rutidosperma* witches'-broom phytoplasma was constructed using the maximum likelihood method implemented in MEGA 7.0 software. As illustrated in Fig. 3, the evolutionary tree of the 16S rDNA gene demonstrates that the *C. rutidosperma* witches'-broom phytoplasma and members of the 16SrII group occupy a distinct branch. The bootstrap support value of 100 indicates a strong phylogenetic relationship between the *C. rutidosperma* witches'-broom phytoplasma and the members of the 16SrII group. *C. rutidosperma* phytoplasma was found to cluster and branch independently from the areca palm yellows phytoplasma, with a support value of 100. This finding indicated that *C. rutidosperma* phytoplasma was the closest relative to the areca palm yellows phytoplasma (Fig. 3). The known areca palm yellows phytoplasma HNWC5 strain is classified within the 16SrII-A subgroup of phytoplasmas[21]. Thus, based on gene sequence comparison analysis, the *C. rutidosperma* witches'-broom phytoplasma is also categorized within the 16SrII-A subgroup of phytoplasmas.

3.4 Investigation on occurrence of *C. rutidosperma* in areca palm field and detection of its phytoplasma A comprehensive investigation was conducted on a total of 60 areca palm fields across three cities and counties, which represent the primary locations for areca palm cultivation in Hainan Province. As illustrated in Table 3, the occurrence rate of *C. rutidosperma* was recorded at 34 out of 60 in the areca palm fields of each township surveyed. This finding indicates a higher probability of occurrence for *C. rutidosperma* within the areca palm fields. In the study involving 60 areca palm fields, only 6 exhibited symptoms of yellowing, characterized by the yellowing of leaf tissue while the veins remained green. The severity of these symptoms observed during the field investigation was less pronounced than that associated with witches'-broom yellowing, as identified in the study (Fig. 4). The presence of phytoplasma was confirmed through qPCR analysis. All samples exhibiting symptoms tested positive for phytoplasma, thereby indicating an infection. However, the concentration of phytoplasma detected by qPCR was found to be low (Fig. 5).

Table 2 Comparison of 16S rDNA gene similarity between *Cleome rutidosperma* witches'-broom phytoplasma and members of phytoplasma groups

Phytoplasma name	Strain	Host	Geographical origin	Classification	GenBank accession No.	Similarity//%
Areca palm yellows phytoplasma	HNWC5	*Areca catechu*	China	16SrII-A	OQ586072	100.00
Cotton phyllody phytoplasma	CoP	*Gossypium* spp.	Burkina Faso	16SrII-C	EF186827	98.16
Candidatus phytoplasma aurantifolia	WBDL	*Citrus* × *limon*	*	16SrII-B	U15442	97.92
Peach X-disease phytoplasma	BF	*Catharanthus roseus*	USA	16SrIII-A	HQ589203	92.04
Almond witches'-broom phytoplasma	AlmWB-P1	*Prunus persica*	Lebanon	16SrIX-D	AF455040	90.93
Aster yellows phytoplasma	AY-WB	*Callistephus chinensis*	*	16SrI-A	AY389828	90.42
Elm yellows phytoplasma	EY1	*Ulmus pumila*	*	16SrV-A	AY197655	90.28
Brinjal little leaf phytoplasma	BLL16	*Solanum melongena*	India	16SrVI-A	MW273754	90.91
Periwinkle virescence phytoplasma	MaPV	*Catharanthus roseus*	Malaysian	6SrXXXII	EU371934	91.12

NOTE " * " signifies the absence of corresponding information.

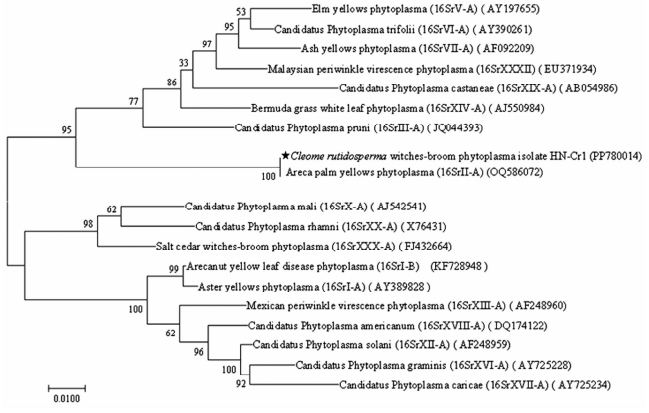
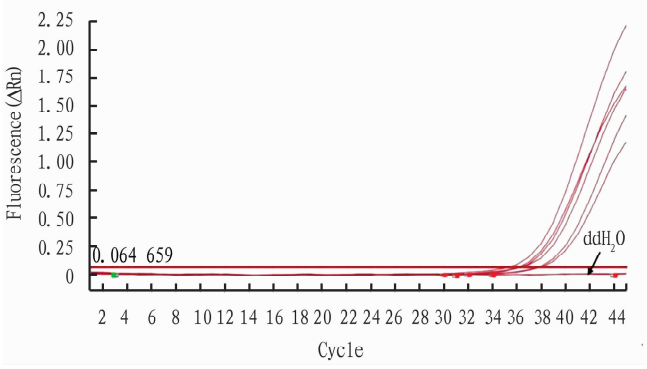


Fig. 3 Phylogenetic tree based on 16S rRNA gene

Table 3 Investigation and phytoplasma detection results of *Cleome rutidosperma*

City/County	Township/town	Proportion of occurrence	Incidence rate	Detection rate of phytoplasma in diseased samples//%
Wenchang	Zhongxing	1/5	0	
	Tanniu	1/5	0	
	Penglai	4/5	0	
	Wencheng	4/5	2/5	100
Qionghai	Wanquan	4/5	2/5	100
	Tayang	4/5	0	
	Jiaji	2/5	0	
	Changpo	3/5	1/5	100
Wanning	Xinglong	3/5	1/5	100
	Longgun	4/5	0	
	Shangen	3/5	0	
	Wancheng	1/5	0	
Total	34/60	6/60	100	

Fig. 4 Field symptoms of *Cleome rutidosperma* in the survey



NOTE The amplification curve suggests that the sample is positive for the detection of phytoplasma.

Fig. 5 Phytoplasma detection of diseased samples of *Cleome rutidosperma*

4 Discussion

Phytoplasma infections can significantly affect cash crops. A notable example is the coconut lethal yellowing disease, which is attributed to phytoplasma infections from the 16SrI, 16SrIV, 16SrXI, 16SrXIV, 16SrXXII, and 16SrXXXII groups. This disease poses a severe threat to coconut cultivation. In Côte d'Ivoire alone, it is estimated that 12 000 t of coconuts are lost each year due to this disease, with an additional 7 000 hm^2 of coconut plantations at risk[22]. Sugarcane white leaf disease, which is attributed to the infection by members of the phytoplasma 16SrXI group, represents a significant quarantine concern. This disease poses a serious threat to sugarcane cultivation in various Asian countries and results in economic losses exceeding 20 million US dollars annually in Thailand alone[23]. Phytoplasma diseases not only undermine biodiversity but also represent a significant threat to the agricultural economy; consequently, the implications for human activities should not be overlooked. The identification of phytoplasma is crucial for the diagnosis of phytoplasma-related diseases. Currently, the classification and identification of tentative species of phytoplasma primarily rely on the characteristics of the 16S rDNA gene sequence, as well as variations in transmission vectors, host plants, and molecular levels[24]. This study, utilizing 16S rDNA sequence comparison analysis, identified the *C. rutidosperma* witches'-broom phytoplasma as a member of the 16SrII-A subgroup. This represents the first identification of a phytoplasma as-

sociated with witches'-broom, yellows, and little leaf symptoms in the current context. In 2020, *C. rutidosperma* witches'-broom disease was first reported in Taiwan, China, and was identified as an infection by a member of the 16SrII-V subgroup of phytoplasmas[25]. This study aimed to compare the 16S rDNA sequences of *C. rutidosperma* witches'-broom phytoplasma with those from Taiwan, China (GenBank No.: MN213634). The analysis revealed a similarity of 99.68%, with the only variation occurring at the upstream primer sequence position. In this study, the universal primer R16F2n was utilized as the upstream primer. The 16S rDNA sequence of the *C. rutidosperma* witches'-broom phytoplasma from Taiwan, China, was analyzed using virtual RFLP via the iPhyClassifier online tool[26]. The analysis revealed that the virtual RFLP maps generated for 17 restriction enzymes were identical to the reference maps for members of the 16SrII-A subgroup (GenBank No.: L33765), resulting in a similarity coefficient of 1.00. In 2024, phyllody in *C. rutidosperma* was reported in Hainan, China, and identified as an infection caused by a member of the phytoplasma 16SrII-A subgroup[27]. The 16Sr DNA sequence obtained in this study was found to be completely consistent with the 16Sr DNA sequence of the *C. rutidosperma* witches'-broom phytoplasma. This finding indicates that the symptoms of witches'-broom, yellowing, little leaf, and phyllody observed in *C. rutidosperma* are attributable to phytoplasma infection.

Furthermore, the location of occurrence for the *C. rutidosperma* witches'-broom phytoplasma identified in this study coincided with that of the areca palm yellows phytoplasma belonging to the 16SrII group, as reported by the same research group in the same areca field[21]. In this study, it was determined that the 16S rDNA sequence of the *C. rutidosperma* witches'-broom phytoplasma exhibited 100% similarity to that of the areca palm yellows phytoplasma. Among the 60 yellows disease cases investigated, six areca palm fields displayed yellowing symptoms of *C. rutidosperma*, which were confirmed to be caused by phytoplasma infection. However, due to the low concentration of the phytoplasma, molecular identification through nested PCR amplification was not feasible. Consequently, based on the geographical location, sequence analysis, and the incidence of the disease observed during field investigations, it is hypothesized that the *C. rutidosperma* witches'-broom disease may be attributed to the infection by areca palm yellows phytoplasma, which could serve as the intermediate host for this pathogen. Currently, the pathogen inoculation technology for YLD has not been developed. Consequently, this study was unable to identify intermediate hosts through inoculation, which will be a significant focus in future research.

The diverse array of natural hosts for phytoplasma may facilitate its adaptation to various ecological niches and enhance the transmission of associated diseases, thereby complicating efforts for disease prevention and control[28]. It has been reported that *Paulownia fortunei* (Seem.) Hemsl. infected with *Paulownia* witches' broom (PaWB) harbors a phytoplasma that has been detected in seven species of plants. The phytoplasma associated with infected *P. fortunei* is classified within the 16SrI-D subgroup[29]. This finding suggests that these seven plant species may serve as intermediate hosts for the phytoplasma responsible for the witches' broom disease in *P. fortunei*. When Tang Yan[30] investigated the host range of phytoplasma associated with jujube witches' broom, the 16S rDNA sequences amplified from various plant species, including *Prunus* spp., *Ailanthus altissima* (Mill.) Swingle, *Diospyros kaki* Thunb., *Vitex negundo* var. *heterophylla* (Franch.) Rehd., *Erigeron canadensis* L., and *Ulmus pumila* L., exhibited 100% similarity to those amplified from jujube. The homology with the phytoplasma 16SrV-B subgroup was notably high, leading to the conclusion that these six plant species may serve as potential field hosts for the phytoplasma associated with jujube witches' broom. Brown *et al.*[31] identified 46 weed species within the park that exhibited a high incidence of coconut lethal yellowing disease. The findings indicated that the phytoplasma responsible for lethal yellowing in coconuts was detected in *Cleome rutidosperma*, *Macroptilium lathyroides*, and *Stachytarpheta jamaicensis*. It can be observed that in areas with a high incidence of phytoplasma disease, the same phytoplasma strain is frequently identified in adjacent plants. Consequently, interrupting the disease cycle of phytoplasma is of paramount importance for its prevention and management. In addressing the prevention and control of YLD, it is essential to enhance the detection and verification of phytoplasma in the intermediate hosts within the areca palm yellow disease-affected areas. Furthermore, measures should be implemented to eliminate the transmission pathways of the intermediate hosts associated with YLD in order to mitigate the progression of this affliction.

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