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DETECTION OF PHYTOPLASMAS ASSOCIATED WITH KALIMANTAN WILT DISEASE OF COCONUT BY THE POLYMERASE CHAIN REACTION

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ABSTRACT

Coconut is the second Indonesia's most important social commodity after rice. There are more than 3.6 million hectares of coconut plantations in Indonesia equivalent to one third of the total world coconut area. However, the production and productivity of the coconut are very low and unstable for various reasons, including pests and diseases. Kalimantan wilt (KW) disease causes extensive damage to coconut plantation. In previous investigations, bacteria, fungi, viruses, viroids and soil-borne pathogens such as nematodes were tested, but none of them were consistently associated with the disease. The objective of this research was to detect and diagnose the phytoplasma associating with KW. Two DNA extraction methods, namely a modification of CTAB method involving grinding coconut trunk tissue in pre-warmed CTAB instead of liquid nitrogen, and a small scale DNA extraction method, were used to prepare DNA from coconut trunk tissues. Research results showed that both methods were found equally suitable for preparing DNA from coconut trunk tissues for PCR analysis. The phytoplasmas aetiology of KW has been proved by the nested PCR approach using P1/P7 and R16F2n/R16R2 primer combinations. The study has further demonstrated that the nested PCR approach can be employed to effectively detect the presence of phytoplasma both in infected and in symptomless coconut trunk tissues. Phytoplasma DNA was amplified from 95 out of 116 samples (81.9%). Based on source of samples, phytoplasma DNA was amplified from KW infected and symptomless samples, 95.1% and 67.3% respectively. This study confirmed that KW is caused by phytoplasma.

Key words : Coconut, *Cocos nucifera* L., plant disease, Kalimantan wilt disease, phytoplasma, polymerase chain reaction, Central Kalimantan

ABSTRAK

Deteksi phytoplasma yang berasosiasi dengan penyakit layu Kalimantan pada kelapa dengan reaksi rantai polymerase

Kelapa merupakan komoditi sosial kedua setelah padi di Indonesia dengan luasan areal lebih dari 3.6 juta ha pertanaman, ekuivalen dengan sepertiga luas kelapa dunia, hal ini menjadikan Indonesia sebagai negara produsen kelapa terluas di dunia. Sekarang ini produksi dan produktivitas kelapa sangat rendah dan tidak stabil yang disebabkan oleh berbagai alasan termasuk serangan hama dan penyakit. Penyakit layu Kalimantan telah mengakibatkan kerugian yang besar pada pertanaman kelapa. Penelitian sebelumnya untuk mengetahui penyebab penyakit dilakukan dengan menguji bakteri, cendawan, virus, viroid dan patogen tanah seperti nematoda tetapi tidak ada yang secara konsisten berasosiasi dengan penyakit layu Kalimantan. Penelitian ini bertujuan untuk mendeteksi dan mendiagnosa phytoplasma sebagai penyebab penyakit yang berasosiasi dengan layu Kalimantan. Penelitian ini menggunakan dua metode untuk mengekstraksi DNA yaitu metode CTAB yang biasanya menggunakan nitrogen cair dimodifikasi dengan menghancurkan sampel tanaman pada CTAB yang dipanaskan, dan metode skala kecil. Hasil penelitian menunjukkan bahwa kedua metode yang digunakan menghasilkan DNA yang sama baiknya untuk analisis PCR. Teknik nested PCR menggunakan kombinasi primer P1/P7 dan R16F2n/R16R2 dapat membuktikan bahwa penyebab penyakit layu Kalimantan adalah phytoplasma. Teknik ini juga secara efektif dapat mendeteksi phytoplasma dalam jaringan tanaman kelapa yang sudah terinfeksi maupun yang belum menunjukkan gejala penyakit. DNA phytoplasma dapat dideteksi pada 95 sampel dari 116 sampel (81.9%) yang dianalisis. Berdasarkan jenis sample yang diperiksa ternyata phytoplasma dapat dideteksi pada sample yang terinfeksi maupun yang belum menunjukkan gejala penyakit masing-masing 95.1% dan 67.3%. Hasil penelitian ini mengkonfirmasi bahwa penyakit layu Kalimantan disebabkan oleh phytoplasma.

Kata kunci: Kelapa, *Cocos nucifera* L., penyakit tanaman, penyakit layu Kalimantan, phytoplasma, reaksi rantai polymerase, Kalimantan Tengah

INTRODUCTION

Coconut palm is an economically important perennial crop that supports the livelihood of many Indonesian families. At least three million families and hundreds of thousands of workers are engaged in coconut growing, copra processing, coconut oil milling and trading. Coconut is the Indonesia's second most important social commodity after rice. In 2000, there were more than 3.6 million hectares of coconut plantations in Indonesia equivalent to one third of the total world coconut area (DJUNAEDI, 2003). This makes Indonesia the largest coconut producing country in terms of area. The crop is predominantly (95%) cultivated by smallholders with acreage of farmers' own about 1-2 ha, therefore it is vital importance to the rural economy.

Coconut production and productivity has been very low and unstable for various reasons, such as old age, slow rate of replanting, poor management practices, pest and disease damages. Among other diseases, Kalimantan wilt has caused extensive damage to coconut plantations in Central Kalimantan. Kalimantan wilt was first seen by farmers in 1978, and an outbreak of the disease was reported in 1988 in East Kotawaringin and Kapuas districts, Central Kalimantan (SITEPU *et al*., 1988). The disease has caused serious losses, because many infected palms were destroyed resulting serious economic losses to thousands of farm families, whose income depends directly or indirectly upon coconut. In previous investigations (WAROKKA, 1999), bacteria, fungi, viruses, viroids and soil-borne pathogens such as nematodes have been tested for as causes of KW,

but none have been consistently associated with the disease.

Symptomatology has been one of the major criteria for preliminary diagnosis of putative phytoplasmal diseases (LEE and DAVIS, 1992). Symptom remission following tetracycline treatment has been considered essential in proving that these organisms cause the plant disease. This approach has been used to confirm the phytoplasmal aetiology of coconut diseases such as Lethal Disease (LD) (KAIZA, 1987), Kaincope disease (STEINER, 1976) and Lethal Yellowing (LY) (McCOY, 1972). The same result also found in KW, coconut palm infected with KW and treated with oxytetracycline antibiotic resulting in remission of symptoms compare to palms treated with penicillin without remission of symptom (unpublished data). This result indicated that KW is infected by phytoplasma.

During the last decade, amplification of phytoplasma DNA by Polymerase Chain Reaction (PCR) assays has provided a reliable and specific means for detecting these plant pathogens in both plant and insect hosts (LEE *et al*., 2000), and these assays have significantly advanced diagnostics for phytoplasmal diseases (LEE and DAVIS, 1992). PCR assays provide a much more sensitive means than serological tests or DNA-DNA hybridization assays for detection of phytoplasmas. The specificity of PCR amplification, and hence pathogen detection is based on two synthetic oligonucleotide primers which flank the target DNA sequences to be amplified and hybridize to opposite strands (SAIKI *et al*., 1988). The primers base pair to and define each end of the target sequence on opposite strands, so that DNA synthesis by the enzyme proceeds across the region between them, ensuring simultaneous copying of both strands of the selected fragment. The extension product of one primer then serves as template for the second primer.

Phytoplasmas are phloem-inhibiting organisms. According to ZIMMERMANN (1979), if phytoplasmas can only be transported via the sieve tubes, their direction of movement in plants is expected to be from mature leaves to phloem sinks such as roots, young leaves, inflorescence, fruits and the apical meristem. Therefore, phytoplasma concentrations are probably quite low in most mature leaves. Partial evidence supporting such a distribution of the LY phytoplasma in coconut palms has been obtained previously by electron microscopy (THOMAS, 1979).

To overcome the problems associated with detection of low titers of phytoplasma in plant tissue, a nested-PCR assay has been designed to increase sensitivity and specificity for the amplification of phytoplasmas. This assay is performed by preliminary amplification using a universal primer pair followed by use of a second primer pair on the products from the first PCR (GUNDERSEN and LEE, 1996; LEE *et al*., 1994).

A major limitation to acquiring important etiological and epidemiological information concerning KW is lack of sensitive and specific method for detection and diagnosis of phytoplasma. The objective of this research was to detect and diagnose of the phytoplasma associated with KW in Central Kalimantan, Indonesia.

MATERIAL AND METHODS

Plant Sample Collecting

In order to detect phytoplasmas associated with coconut palm, extensive collections of coconut trunk samples were performed at the 12 plots in Samuda, Central Kalimantan from January 2001 to June 2003. All coconut palm samples were taken by using a hand drill to bore a hole in to the trunk about 10cm deep, 0.75 - 1 m above the ground. About 5g of coconut drilling wood materials was taken using aluminium foil, transferred to falcon tubes with desiccants. The drilling holes were plugged with a piece of leaf frond wood and painted with insecticide to avoid penetration of pests. After each drilling, the drill bit was sterilised with a 3% sodium hypochlorite solution then rinsed with water to avoid cross contamination from plant to plant.

Total samples collected were 116 consisting of 61 typical KW symptom, and 55 symptomless KW.

Total DNA Extractions from Plant Tissue

Two different procedures were used for extraction of DNA from small quantities of plant material for detecting the phytoplasma. The first procedure was using cetyl trimethyl ammonium bromide (CTAB) method of DOYLE and DOYLE (1990). The second procedure was the small scale DNA extraction method of ZHANG *et al*. (1998). Both procedures were modified and adopted for routine DNA extraction. For the first method, the modification was the omission of freezing the tissue and the incubation temperature was 65^{o}C for 30 minutes. Thus, fresh tissue was directly ground into a paste with a mortar and pestle, and added to pre-heated DNA extraction buffer. For the second method liquid nitrogen was used, and the incubation temperature was 60^{o}C for 20 minutes.

The CTAB Method

Five grams of coconut trunk drilling samples were poured into clean and sterile Falcon tube containing 15 ml of pre-heated (65^{o}C) CTAB buffer. The sample was incubated at 65^{o}C for 30 minutes with caps loosely on the tubes, and occasional swirling. After incubation, the lysate was cooled to room temperature, and extracted with an

equal volume of chloroform-isoamyl alcohol (24:1, v/v), and mixed gently. The mixture was centrifuged at 3,000 x g for 10 minutes to separate the phases, and the aqueous phase was carefully removed using a pasteur pipette to a clean falcon tube. Precipitation of nucleic acids from the aqueous phase was by addition of 0.6 volume of cold isopropanol or 2.5 volumes of cold absolute ethanol and one tenth volume of 3 M sodium acetate. The mixture was left overnight at – 20°C, then centrifuged at 10,000 x g for 30 minutes to pellet the nucleic acids. After centrifugation, the supernatant was removed and the pellet dried in a vacuum desiccator. Each pellet was dissolved in 0.5 ml of 1 x TE buffer, pH 8.0, then transferred to a microfuge tube, and stored at 4°C. If the DNA extracted was contaminated with tannins and other undesirables, the following clean-up steps were carried out. An equal volume of phenol-chloroform-isoamyl alcohol (25:24:1, v/v) was added to the tube containing DNA, mixed gently then spun at 3,000 x g for 10 minutes. The aqueous phase was transferred to a clean tube and an equal volume of chloroform-isoamyl alcohol (24:1, v/v) added, mixed gently and spun at 3,000 x g for 10 minutes. The aqueous phases were again transferred to a clean tube, 2.5 volumes of absolute ice cold ethanol was added and 1/10th volume of 3M NaAc. The mixture was left at – 20°C for 20 minutes or overnight, and centrifuged at 10,000 x g for 20 minutes to pellet the DNA. The tubes were dried upside down on a piece of paper towel for 5 minutes, 1 ml of cold 70% (v/v) ethanol was added to the pellets, which were washed gently by inverting the tube several times, and spun at 10,000 x g for 10 minutes. The supernatant was decanted and the pellet dried and dissolved in a minimal volume of 1 x TE buffer and stored at 4°C for short term use, or at – 20°C for long term use.

Small Scale DNA Extraction Method

About 300 mgs of coconut trunk samples were frozen in plastic beakers with liquid nitrogen then scraped into a chilled mortar and ground to powder. The powder was transferred into a 1.5 ml Eppendorf tube containing 800 µl of pre-heated (60°C) CTAB buffer, and thoroughly mixed by vortexing. The entire mixture was held at 60°C for 20 minutes. During incubation, the mixture was briefly vortexed several times. After the incubation, the lysate was cooled on ice, then chloroform-isoamyl alcohol (24:1, v/v) was added to fill the tube and the mixture was vortexed vigorously, then centrifuged at 3,000 x g for 10 minutes in an Eppendorf Centrifuge model 5414. The aqueous phase was removed and transferred to a clean tube, and re-extracted with chloroform-isoamyl alcohol. Precipitation of nucleic acids from the aqueous phase was by addition of 600 µl ice cold isopropanol, and invertion to mix gently. Mixtures were then centrifuged at 10,000 x g for 8 minutes to pellet the DNA. Pellets were washed in 70% (v/v) cold ethanol and dried under vacuum or allowed to air dry. Each pellet was dissolved in 100 µl of 1 x TE buffer and stored at – 20°C.

PCR Analysis

Amplification of 16S ribosomal DNA was performed using the nested PCR approach. The phyto-plasma universal primer pairs P1 (5'-AAG AGT TTG ATC CTG GCT CAG GAT T-3') (DENG and HIRUKI, 1991) / P7 (5'-CGT CCT TCA TCG GCT CTT-3') (SMART *et al*., 1996) and R16F2n (5'-GAA ACG ACT GCT AAG ACT GG-3') / R16R2 (5'-TGA CGG GCG GTG TGT ACA AAC CCC G-3') (GUNDERSEN and LEE, 1996) were used to prime the amplification of fragments of ribosomal DNA. Primer pair P1/P7 mediates the amplification of a 1.8-kilobase (kb) fragment containing nearly the entire 16S rRNA gene, the interspacer region, and a portion of the 5' end of the 23S rRNA gene. Primer pair R16F2n/R16R2 mediates amplification of a 1.25-kb fragment of the 16S rRNA gene internal to the P1/P7 priming sites.

Amplifications were performed in 25 µl reactions using "Ready To Go PCR beads™" (Pharmacia Biotech) containing 0.5 µl each of the appropriate forward and reverse primer (from 10 pMol μl^{-1} stock solutions) and sterile double distilled water was added to this solution to a final volume of 24 µl. DNA concentrations were not adjusted after extraction, but used as template. One µl of DNA was used per reaction tube. Reactions were overlaid with 50 µl of mineral oil and were performed in a Hybaid Omnigene thermocycler (Hybaid Ltd, U.K). Reaction conditions for first round PCR using P1/P7 primers were 95°C for 3 minutes followed by 30 cycles of denaturation 94°C for 30 seconds, annealing 53°C for 1 minute and 30 seconds, 72°C for 1 minute and 30 seconds; and followed by a final extension step of 72°C for 10 minutes. One µl of the P1/P7 reaction product was used as the template in the second round PCR using primer pair R16F2n/R16R2. For the second round, reaction conditions were 95°C for 3 minutes followed by 35 cycles of denaturation 94°C for 30 seconds, annealing 56°C for 1 minute and 30 seconds, 72°C for 1 minute and 30 seconds; and followed by a final extension step of 72°C for 10 minutes. Aliquots of 6 µl of the PCR products obtained from each amplification were analyzed by 1% (w/v) agarose gel electrophoresis in 1 x TBE buffer (90mM Tris-borate; 2mM EDTA, pH 8.0) containing ethidium bromide with final concentration of 0.5 $\mu g\ ml^{-1}$. The sample loading into the gel slots was mixing with 2 µl of gel loading buffer (0.25% (w/v) bromophenol blue + 40% (w/v) sucrose in water). Electrophoresis was carried out at 60 V for 1 hour, and the stained gel was then

visualised under ultra violet light examination and recording was done on the UV transilluminator (Eagle Eye).

RESULTS

Total samples collected was 116 consisting of 61 typical KW symptom and 55 symptomless KW, numbers of samples collected in each plot is shown in Table 1. DNA was extracted using both the CTAB method and small scale extraction method from 116 coconut trunk samples from palms showing typical KW symptoms and symptomless. Both method give good quality of DNA extracted. All DNA samples were screened by nested PCR for the presence of phytoplasmas using P1/P7 and R16F2n/R16R2 primer combinations. Results of the first round amplification of the samples extracted by CTAB method showed that after 35 cycles of amplification, the universal primer pairs P1/P7 amplified the DNA of phytoplasma from the Ghanaian samples only, which served as a positive control for a DNA band visible on a gel. The amplification product was the expected size of about 1.8 kb. No amplification product was visible after processing DNA from KW infected samples, except the phytoplasma from Ghana which serve as positive control (Figure 1). An aliquot of 1 µl of DNA product of first round PCR was then subjected to nested PCR. The resultant amplification product was 1.25 kb which is the right size for phytoplasma amplify with R16F2n/R16R2 primer combinations (Figure 2). Results of this study showed that 95 out of 116 (81.9%) coconut trunk samples collected from 12 plots positively detected phytoplasma. Base on the samples collected, total of 58 out of 61 (95.1%) and 37 out of 55 (67.3%) respectively from KW infected and symptomless positively detected phytoplasma (Table 1).

Figure 1. Amplification of coconut KW infected samples extracted by CTAB method, with P1/P7 primers. Lane 1, 1 kb ladder; 2-13, coconut trunk samples; 14, water; 15, coconut phytoplasma from Ghana

Gambar 1. Amplifikasi sampel terinfeksi KW yang diekstraksi dengan metode CTAB menggunakan primer P1/P7. Lajur 1, 1 kb ladder; 2-13, sample kelapa; 14, air sebagai kontrol negatif; 15, phytoplasma kelapa dari Ghana

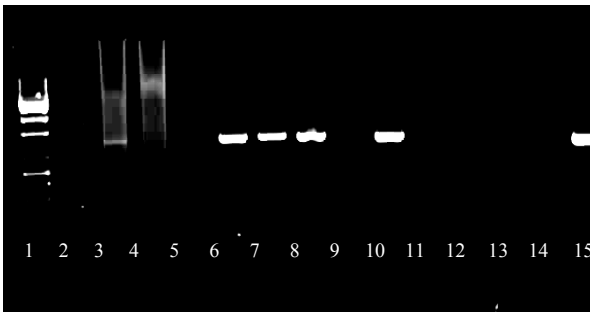

Figure 2. Amplification of coconut KW infected samples extracted by CTAB method, with R16F2n/R16R2 primers. Lane 1, 1 kb ladder; 2-13, coconut trunk samples; 14, water; 15, coconut phytoplasma from Ghana

Gambar 2. Amplifikasi sampel terinfeksi KW yang diekstraksi dengan metode CTAB menggunakan primer R16F2n/R16R2. Lajur 1, 1 kb ladder; 2-13, sample kelapa; 14, air sebagai kontrol negatif; 15, phytoplasma kelapa dari Ghana

Table 1. Number of coconut KW samples tested and samples positive for phytoplasmas
Tabel 1. Jumlah sampel yang diuji dan sampel positif terdeteksi phytoplasma

Locality /plot *Lokasi/plot*	KW infected samples *Sampel terinfeksi*		Symptomless samples *Sampel belum bergejala*		Total *Total*	
	Number of samples *Jumlah sampel*	Positive phytoplasma *Positif phytoplasma*	Number of samples *Jumlah sampel*	Positive phytoplasma *Positif phytoplasma*	Number of samples *Jumlah sampel*	Positive phytoplasma *Positif phytoplasma*
Plot # 1	7	6	4	4	11	10
Plot # 2	3	3	5	4	8	7
Plot # 3	5	5	4	3	9	8
Plot # 4	5	5	3	3	8	8
Plot # 5	6	6	5	3	11	9
Plot # 6	5	5	4	1	9	6
Plot # 7	6	5	5	4	11	9
Plot # 8	5	5	5	3	10	8
Plot # 9	5	5	5	3	10	8
Plot # 10	6	6	5	4	11	10
Plot # 11	4	4	5	2	9	6
Plot # 12	4	3	5	3	9	6
	61	58 (95.1%)	55	37 (67.3%)	116	95 (81.9%)

Amplification of KW samples extracted by the small scale extraction method gave similar results with the previous method described above. The first round amplification using P1/P7 primers amplified visible DNA products for the Ghanaian samples only (data not shown). An aliquot of 1 µl of the first round product served as template for the nested PCR using R16F2n/R16R2 gave amplification products of the expected size of 1.25 kb (Figure 3).

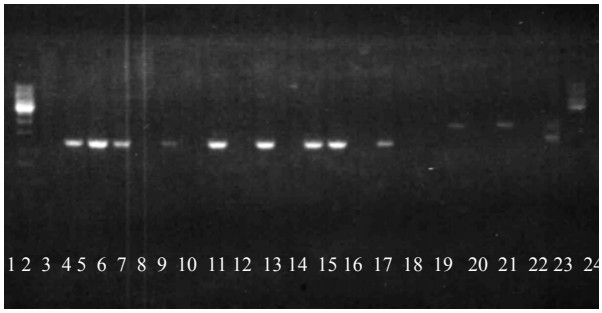

Figure 3. Amplification of coconut KW infected samples extracted by the small scale extraction method, with R16F2n/R16R2 primers. Lane 1 and 24, 1 kb ladder; 2-21, coconut trunk samples; 22, water as negative control; 23, coconut phytoplasma from Ghana as positive control

Gambar 3. Amplifikasi sampel terinfeksi KW yang diekstraksi dengan metode skala kecil menggunakan primer R16F2n/R16R2. Lajur 1 dan 24, 1 kb ladder; 2-21, sampel kelapa; 22, air sebagai kontrol negatif; 23, phytoplasma kelapa dari Ghana sebagai kontrol positif

DISCUSSION

The PCR is the most recent and most sensitive approach for the detection of phytoplasmas. A number of assays based on this have been developed to detect a wide variety of phytoplasmas. The comparative ease to use, speed and sensitivity makes it a very versatile tool and permits many applications to the study of phytoplasmas. For sensitive detection of phytoplasmas, primers based on Mollicutes 16S rRNA genes have been used to selectively amplify phytoplasma DNA from mixtures with host DNA (AHRENS and SEEMULLER, 1992; DENG and HIRUKI, 1991; LEE *et al*., 1993; NAMBA *et al*., 1993). The sensitivity of detection obtained using pathogen-specific primers based on the cloned phytoplasma sequences have been reported to substantially exceed the lower limits of detection by hybridization with the respective DNA probes (SCHAFF *et al*., 1992; HARRISON *et al*., 1994).

Because of the phytoplasmas associated with plant diseases have not yet been obtained in pure culture *in-vitro*, infected hosts serve as sources of phytoplasma DNA for molecular detection by PCR. In their hosts, phytoplasmas are present in relatively low titers and represent a minute amount of total components. Thus, in order to obtain optimal results of phytoplasma detection in coconut trunk tissues, a nested PCR approach with P1/P7 and R16F2n/R16R2 primer combinations has been routinely used to screen for the presence of the phytoplasmas in KW infected palms. The used of P1/P7 primer combinations in the first round PCR amplifies the entire sequence of the 16S gene, but no bands from KW infected palms were visible on gels, except the LDG phytoplasma which was used as a positive control. In the second round, R16F2n/R16R2 primers amplified a phytoplasma DNA band from 58 (95.1%) out of 61 KW infected samples from various plots. This result is different from those for Lethal Yellowing (LY) and Lethal Disease (LD). HARRISON (1996) reported 100% detection in the DNA from spear leaves of LY infected palms, and MPUNAMI (1997) reported 71% only for LD infected palms.

PCRs employing primer pairs derived from ribosomal or non-ribosomal DNA sequences have been used to detect the LY phytoplasma in leaves or inflorescence and in trunk tissues or roots of coconut palm (HARRISON *et al*., 1999). Such studies have also shown that the LY phytoplasmas are more readily detected in immature than mature tissues (CORDOVA, 2000; ESCAMILLA *et al*., 1995; HARRISON *et al*., 1995). MPUNAMI (1997) reported that phytoplasma DNA was detected in all meristematic tissues sampled, including the petioles of very young leaves, the area below the growing point, root tips, inflorescences and the spear leaf. The highest concentrations were found in the petioles of young unopened leaves, the root tips, and the area below the growing point. The proportion of phytoplasma DNA recovered from total DNA was significantly influenced by the plant part used for DNA extraction (KOLLAR *et al*. 1990). HARRISON *et al*. (1991) reported that pigeon pea witches'-broom phytoplasma DNA formed 0.67% of total DNA extracted from periwinkle stems.

Studies of other DNA samples from various diseases proved positive for phytoplasma with the P1/P7 and R16F2n/R16R2 primers. Based on the results for the P1/P7 primer pair, it is clear that the phytoplasmas detected in these samples were not identical to the KW phytoplasma.

DNA extraction methods of both the CTAB and the small scale provided a good template for amplification of phytoplasma DNA. A total of 95 out of 116 (81.9%) samples, 57 of KW infected and 38 of symptomless palms, respectively, were positive for phytoplasmas. The modified CTAB method with the omission of the freezing samples in liquid nitrogen will be very useful for preparing DNA from freshly collected coconut trunk tissue. Under ICOPRI laboratory conditions, where regular supplies of liquid nitrogen are sometimes difficult to obtain, the usefulness of the modified CTAB extraction method without liquid nitrogen cannot be over emphasized. On the other hand, the use of the small scale method is particularly convenient for

extracting small amounts of tissue and for assays of insect vectors. Thus, this method reduced the amount of the chemicals and reagents, and is relatively inexpensive compared to commercially available nucleic acid extraction kits (ZHANG *et al*., 1998).

By use of the nested PCR approach, this study has confirmed the phytoplasmal aetiology of KW. The study has further demonstrated that nested PCR can be employed to effectively detect the presence of phytoplasmas both in infected and in symptomless coconut trunk tissues.

CONCLUSIONS

Coconut trunk drilling samples are suitable to be used for DNA extraction of KW phytoplasma.

The modification of the CTAB DNA extraction method of both DOYLE and DOYLE (1990) and ZHANG *et al*., (1998) could be routinely used for detection of phytoplasma associated with KW. The study showed that 95 out of 116 (81.9%) samples detected phytoplasma, 57 (95.1%) of KW infected and 38 (67.3%) of symptomless palms respectively.

The use of nested PCR assay has proved and confirmed the phytoplasma aetiology of KW disease of coconut in Central Kalimantan.

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