Developing a polymerase chain reaction based detection method for coconut root (wilt) phytoplasma

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Abstract

Coconut (*Cocos nucifera* L.) is an economically important perennial oil-yielding crop cultivated in Kerala and other states of India. Root (wilt), which afflicts coconut cultivation in Kerala and bordering districts of Tamil Nadu, is a debilitating disease due to which an estimated 968 million nuts are lost every year. Consistent presence of phytoplasma only in tissues of diseased palms was detected by electron microscopy and is considered as the etiology of the disease. DNA from various tender tissues of healthy and root (wilt) diseased coconut palms was isolated by two methods for using as template in polymerase chain reaction (PCR) to detect phytoplasma. The total genomic DNA isolation method using liquid nitrogen yielded higher amount of DNA than phytoplasma-enrichment method, which yielded lower amount of DNA but free from host plant contaminants. Among various tissues, tender petiole followed by midribs yielded highest concentration of DNA by both the methods. DNA was amplified by PCR using phytoplasma-specific oligomers sets viz., Ahrens and Seemuller forward and reverse, P1/P7, P1/P6, P4/P7, fU5/rU3, Rohde forward/reverse primers, R16F2/R2 and R16mF2/R1. The P4/P7 pair amplified two DNA fragments of 680 and 450 bp from DNA of diseased tissues but not from healthy palm tissues. None of the other primer set amplified DNA fragments from diseased and healthy coconut tissues. Amplification by nested PCR using R16F2n/R2n primer too did not produce positive results from DNA products of first reaction. PCR detection of DNA fragments by P4/P7 set only in root (wilt) diseased coconut tissues is an indication of the presence of phytoplasma.

Key words: Universal phytoplasma primers, DNA, 16S and 23S rDNA, coconut tissues, PCR amplification

Introduction

Coconut (*Cocos nucifera* L.) is an economically important perennial oil-yielding crop grown in Kerala and other states of the country. Root (wilt) disease (RWD), which afflicts coconut cultivation in Kerala and bordering districts of Tamil Nadu, is characterized by abnormal inward bending of leaflets accompanied by yellowing and marginal necrosis of leaves and necrotic blackening in inflorescences in certain cases (Radha and Lal, 1972). These symptoms constitute the visual detection method of the disease. The affliction results in debilitation and gradual decline in yield of the palm, due to which an estimated 968 million nuts are lost every year. Consistent presence of phytoplasma in tissues of roots, tender rachilla, petioles and developing leaf bases of diseased palms as detected by electron microscopy and its total absence in healthy palms was considered as proof of the etiology of the disease (Solomon *et al.*, 1983a). The evidences for phytoplasma as the causal agent of RWD are also based on therapeutic effect of oxytetracycline-HCl antibiotic in inducing remission of symptoms in a significant number of palm trials, (Pillai *et al.*, 1991) and transmission of the disease from diseased palms to healthy coconut seedlings through insect vector *Stephanitis typica* (Dist.) (Mathen *et al.*, 1987). The major impediment limiting research on phytoplasma disease is the inability of the researchers to cultivate the pathogen *in vitro* (McCoy *et al.*, 1983).

Besides electron microscopy, various techniques like light microscopy with Giemsa and Diene's stains, fluorescent microscopy using aniline blue and Hoechst 33258 and DAPI stain were used for detecting the pathogen (Anon. 1985; Solomon *et al.*, 1987). However, these phytoplasma-detection techniques are indirect and

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non-specific and cannot distinguish one phytoplasma from another. Immunotechniques using polyclonal antiserum or monoclonal antibodies are highly specific methods used for diagnosis of phytoplasma and diseases caused by them (Fos *et al.*, 1992; Saeed *et al.*, 1993; Chang *et al.*, 1995 and Seddas, *et al.*, 1996). Earlier, a disease-specific polyclonal antiserum was raised and used for the detection of RWD (Solomon *et al.*, 1983b) Recently, production of a polyclonal antiserum against the RWD phytoplasma for detection of phytoplasma in infected tissues was reported (Sasikala *et al.*, 2002). Notwithstanding the sensitivity of the serological techniques, production of polyclonal antiserum requires high purity antigen. Many of the purification processes comprise long-drawn out steps during which most of the antigen is lost and thus it is very difficult to obtain high-titre antiserum.

Of late, polymerase chain reaction (PCR) method using primers derived from 16S rDNA sequences has been found useful and sensitive in the detection of phytoplasmas in host plants. A number of primer sets based on sequences of the conserved 16S rDNA region have been developed and used for the detection of various phytoplasmas (Deng and Hiruki, 1991; Ahrens and Seemuller, 1992; Lee *et al.*, 1993; Rohde *et al.*, 1993; Smart *et al.*, 1996). Analysis of DNA homology in the highly conserved genes encoding ribosomal RNA and protein have shown that phytoplasma comprise a coherent set of pathogen distinct from other prokaryotes (Gundersen *et al.*, 1994; Seemuller *et al.*, 1994). In this paper, we record the results of the PCR study carried out for detection of phytoplasma in root (wilt) diseased coconut using several phytoplasma-specific primers.

Materials and Methods

Plant materials

Coconut palms between 10 and 12 years of age displaying typical RWD symptoms were identified from plantation of CPCRI, Regional Station, Kayangulam, Kerala. All the diseased coconut palms showed necrotic symptoms in inflorescences, foliar yellowing and necrosis in leaves. For comparison, symptomless coconut palms, presumably healthy, were identified from the same plantation. The sample leaf tissues from these palms were subjected to serological testing with RWD-specific antiserum for confirmation of the disease status (Solomon *et al.*, 1983b). Whole palm crowns were collected in the field and brought to the laboratory, where they were sampled for various tissues by destructive sampling. Tissues of rachilla of unopened inflorescences, petioles, midrib and lamina of tender leaves, heart and submeristematic portions were collected from 28 root (wilt) diseased coconut palms and 12 healthy palms. A total of 140 tissue samples from diseased palms and 60 tissue samples from healthy palms were collected and used for DNA isolation.

DNA isolation

DNA from coconut tissues was isolated by two methods and compared for yield of DNA from various types of tissues and their ability to act as templates for PCR amplifications primed by several oligomers that recognize specific conserved DNA sequences of the 16S and 23S rDNA of phytoplasmas.

Method 1 - This method was according to Doyle and Doyle (1990). Fresh tissue (3 g) was chopped into small pieces and ground to fine powder using liquid nitrogen and suspended in CTAB buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 10 mM Tris-HCl, 0.2% β-mercaptoethanol, pH 8.0) in 9 ml of buffer per gram of tissue. The suspension was incubated at 65 °C for 60 min and centrifuged at 4 °C at 11,000 g for 10 min. The supernatant was extracted with an equal volume of chloroform: isoamyl alcohol (24: 1). The extraction with chloroform: isoamyl alcohol mixture was repeated till no visible precipitate was present at the interface. The final aqueous layer was mixed gently with 0.7 volume of ice-cold isopropanaol, which was left overnight at 4 °C and centrifuged at 15,000 g for 20 min to precipitate DNA. The DNA pellet was washed in 95% ethanol and was air-dried. Then it was suspended in 500 µl double distilled sterile water.

Method 2 - This method was according to Kirkpatrick *et al.* (1987) and Ahrens and Seemuller (1992). Fresh tissue (5 g) was chopped into small pieces and ground in 30 ml of ice-cold grinding buffer (125 mM potassium phosphate, 10% sucrose, 0.15% bovine serum albumin-fraction V (BSA), 2% polyvinylpyrrolidone-10 (PV-10) and 0.53% ascorbic acid, pH 7.6). The tissue grinding was done with the help of acid-purified river sand to disrupt the cells and the homogenate was centrifuged at 4 °C for 4 min at 800 g. The supernatant was collected and centrifuged at 11,000 g for 30 min and the resulting pellet was suspended in DNA extraction CTAB buffer (Doyle and Doyle, 1990) and further steps of DNA extraction were carried out following the protocol described in the Method 1.

All the DNA preparations were quantified by spectrophotometry and purity was checked by 1% agarose gel electrophoresis. The DNA samples isolated by Doyle and Doyle method were treated with RNase at 10 µg/ml and incubated at 37 °C for 30 min. The DNA was reprecipitated with chloroform: isoamyl alcohol (24:1) in the presence of 3 M sodium acetate and two volumes of ethanol. The pellet was washed in 70% ethanol twice, air-dried and resuspended in double distilled sterile water.

Primers and PCR conditions

PCR was performed to standardize different parameters for amplification of 16S rDNA sequence of phytoplasma. Phytoplasma specific primer pairs, Ahrens and Seemuller forward and reverse, P1/P7, P1/P6, P4/P7, fU5/rU3, Rohde forward/reverse primers, R16F2/R2 and R16mF2/R1 were used in the study (Table 1). PCR was carried out in 40 µl volume containing 1x PCR buffer (Genei, Bangalore), 1.5 to 3 mM $MgCl_2$, 100 - 250 µM of four dNTPs, 0.4 to 1.0 µM each primer, 0.5 to 2 unit *Taq* DNA polymerase (Genei, Bangalore) and 25 to 500 ng of template DNA, the details of which are given in Table 2. The reaction mixture was subjected to 30 to 45 amplification cycles using the following parameters: initial heating at 94 & 95 °C for 2 to 5 min denaturation at 94 and 95 °C for 30 sec to 1 min annealing at 50 to 60 °C for 45 sec to 2 min, extension at 72 °C for 1 to 2 min and the final extension step was at 72 °C for 5 to 10 min. The DNA isolated from healthy coconut palms and water served as negative controls. The DNA of aster yellows phytoplasma was used as positive control for amplification.

Table 1. Phytoplasma-specific universal primers used for amplification of coconut root (wilt) phytoplasma DNA in this study

Primer	Sequence (5' – 3')	Reference
A&Sf	acg aaa gcg tgg gga gca aa	Ahrens & Seemuller (1992)
A&Sr	gaa gtc gag ttg cag act tc	Ahrens & Seemuller (1992)
P1	aag agt ttg atc ctg gct cag gat t	Deng & Hiruki (1991)
P4	gaa gtc tgc aac tcg act tc	Kirkpatrick *et al.*, (1994)
P6	tgg tag gga tac ctt gtt acg act ta	Deng & Hiruki (1991)
P7	cgt cct tca tcg gct ctt	Smart *et al.*, (1996)
fU5	cgg caa tgg agg aaa ct	Lorenz *et al.*, (1995)
rU3	ttc agc tac tct ttg taa ca	Lorenz *et al.*, (1995)
Rohde-f	gag tac taa gtg tcg ggg caa	Rohde *et al.*, (1993)
Rohde-r	aaa aac tcg cgt ttc agc tac	Rohde *et al.*, (1993)
R16F2	acg act gct gct aag act gg	Lee *et al.*, (1993)
R16R2	tga cgg gcg gtg tgt aca aac ccc g	Lee *et al.*, (1993)
R16mF2	cat gca agt cga acg ga	Gundersen & Lee (1996)
R16mR1	ctt aac ccc aat cat cga c	Gundersen & Lee (1996)
R16F2n	gaa acg act gct aag act gg	Gundersen & Lee (1996)
R16R2n	tga cgg gcg gtg tgt aca aac ccc g	Gundersen & Lee (1996)

Table 2. Summary of PCR ingredients concentration and thermocycling conditions tested with various primer combinations

Ingredients concentration	First PCR	Nested PCR
$MgCl_2$	1.5, 2.0, 2.5 & 3.0 mM	2.5 mM
dNTPs	100, 150, 200 & 250 µM	200 µM
Primers	0.4, 0.8 & 1.0 µM	1.0 µM
Polymerase	0.5, 1.0, 1.5 & 2.0 Unit	1.0 Unit
Template	25, 50, 100, 150, 200, 250, 400 & 500 ng	1 µl from1:40 dilution of first PCR product
Thermocycling conditions		
Initial heating temp.	94 & 95 °C	94 °C
Initial heating time	2, 3, & 5 min	3 min
Denaturation temp.	94 & 95 °C	94 °C
Denaturation time	30, 45 sec & 1 min	30 sec
Annealing temp.	50 to 60 °C	55 °C
Annealing time	45 sec, 1 & 2 min	45 sec
Extension temp.	72 °C	72 °C
Extension time	1 & 2 min	30 sec
Final step extension temp.	72 °C	72 °C
Final step extension time	10 min	5 min
No. of reaction cycle	30, 35, 40 & 45	35

Nested PCR

The product from initial PCR was used for nested PCR reaction with primer pair R16F2n/R2n. One µl from 1: 40 dilution of first PCR product was used as template for nested PCR amplification for 35 cycles. Nested PCR was carried out with concentrations of ingredients as given in the Table 2 and with initial heating at 94 °C for 3 min, denaturation at 94 °C for 30 sec, annealing at 55 °C for 45 sec, extension at 72 °C for 30 sec and final extension at 72 °C for 5 min (Gundersen and Lee, 1996).

Analysis of PCR products

About 10 µl of each PCR product was electrophoresed on a 1.0% agarose gel in 0.5x TBE buffer (1x is 45 mM Tris borate and 1 mM EDTA, pH 8). The gel was stained with ethidium bromide and the DNA was visualized and documented with an UV transilluminator.

Results and Discussion

Work was initially carried out to select suitable method and appropriate tissue from coconut for DNA isolation. In this investigation, youngest and fresh tender tissues from coconut palms were used for DNA isolation since greatest concentration of phytoplasmal bodies are found in actively growing young tissues, where the concentration of active phloem cells and functional sieve elements are highest resulting in maximizing isolation of phytoplasma DNA with host plant DNA. Mature tissues, however, have fewer numbers that too of degenerated forms and the concentration of phytoplasmas decreases progressively in older leaf bases (Solomon *et al.*, 1987). In general both the methods used for extraction of DNA from coconut tissues were found to be effective and yielded DNA that was of adequate quantity for PCR. However, the quantity and quality of DNA obtained from coconut tissues varied between the extraction methods adopted and type of tissues used for isolation. Of the two procedures used for DNA isolation, the method of Doyle and Doyle (1990) in which tissues were ground in liquid nitrogen prior to DNA isolation yielded higher quantity of DNA generally along with RNA as detected in electrophoresis. The higher yield of DNA in Doyle and Doyle method is due to extraction of total genomic nucleic acids of the tissue. The procedure of Kirkpatrick *et al.*, (1987) modified by Ahrens and Seemuller (1992) yielded lower quantity but of good quality with least amount of co-precipitating contaminants. Since Ahrens and Seemuller method of DNA isolation yielded better quality DNA, the DNA prepared by this method would be better suited for use as template in PCR detection of phytoplasma DNA from diseased coconut tissues. This phytoplasma enrichment procedure was initially developed as a means to increase the percentage of phytoplasmal DNA in nucleic acid extracts from diseased plants and hence relative amount of DNA from phytoplasmas would be more in this method of DNA preparation. Borth *et al.*, (1999) reported this method of phytoplasma enrichment followed by DNA isolation resulted in the co-isolation of contaminating substances contributing to the viscosity of DNA that are inhibitory in PCR assay. In this investigation, it was found that DNA isolated by phytoplasma-enrichment method produced quality DNA free from co-contaminants. Among the various coconut tissues used for DNA isolation, petiole followed by midrib tissues yielded maximum amount of DNA (Table 3) and the ratio of A_{260}/A_{280} of the DNA samples isolated from various coconut tissues are also given in the same Table.

Table 3. Quality and quantity of DNA extracted from various tissues of coconut by two methods

Tissue	Method 1 A_{260}/A_{280}	Method 1 Yield (μg/ mg tissue)	Method 2 A_{260}/A_{280}	Method 2 Yield (μg/ mg tissue)
Petiole	1.83	1.40	1.96	0.26
Midrib	1.73	1.00	2.0	0.22
Heart tissue	1.69	0.90	1.92	0.18
Rachilla	1.82	0.80	1.86	0.16
Lamina	1.80	0.67	1.90	0.17

Of the eight phytoplasma-specific oligomers set tested, only the universal primer set P4/P7 amplified two DNA fragments. The amplification fragments were 680 and 450 bp after 35 cycles of amplification at the following thermocycling conditions: initial denaturation at 95 °C for 2 min denaturation at 94 °C for 1 min annealing at 55 °C for 1 min and chain extension at 72 °C for 1.5 min with the concentration of PCR ingredients of 2.5 mM $MgCl_2$, 200 M of dNTPs, 0.4 μM of each primer, one unit of DNA polymerase and 100 ng template DNA prepared using the total genomic DNA isolation method of Doyle and Doyle (Figure 1 and 3) and 25 ng of DNA prepared using the phytoplasma DNA isolation method of Ahrens and Seemuller (Figure 2 and 3). None of the other phytoplasma-specific universal primer pairs tested in this investigation primed the DNA sequences from root (wilt) diseased coconut tissues. The P4/P7 primer set showed positive amplification from all DNA preparations made from 140 tissue samples collected from 28 root (wilt) diseased coconut palms. All the primer pairs, including P4/P7 primer set, tested did not amplify DNA fragments from nucleic acids isolated from healthy palms and water control. The P4/P7 pair amplified a single fragment of 500 bp from positive control DNA of aster yellows phytoplasma. The more

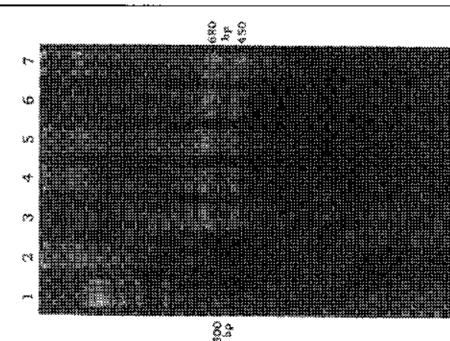

Fig. 1. Polymerase chain reaction amplified phytoplasma DNA from coconut using P4/P7 primer pair. DNA was isolated by Doyle & Doyle method from root (wilt) diseased and healthy coconut tissues. Lane 1: 500 bp DNA ladder, lane 2: DNA from healthy coconut tissue (petiole) and lane 3-7: DNA from rachilla, petiole, midrib, lamina and heart tissue of root (wilt) diseased palms showing two DNA fragments of 450 and 680 bp.

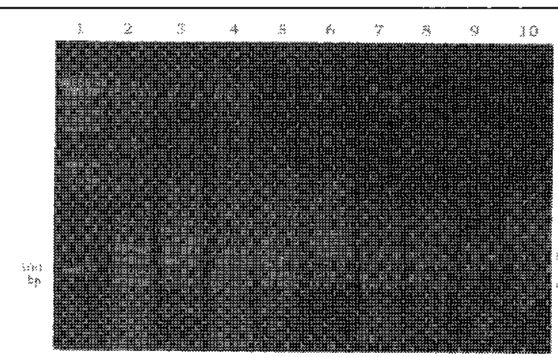

Fig. 2. Polymerase chain reaction amplified phytoplasma DNA from coconut using P4/P7 primer pair. DNA was isolated by Ahrens & Seemuller method from root (wilt) diseased and healthy coconut tissues. Lane 1: 500 bp DNA ladder; lanes 2 & 7: DNA from rachilla; lanes 3 & 8: DNA from petiole; lane 4: DNA from midribs; lane 5: DNA from lamina; lane 6: DNA from heart tissues of root (wilt) diseased palms showing two DNA fragments of 450 and 680 bp; lane 9: DNA from healthy coconut tissue (petiole) and lane 10: water control.

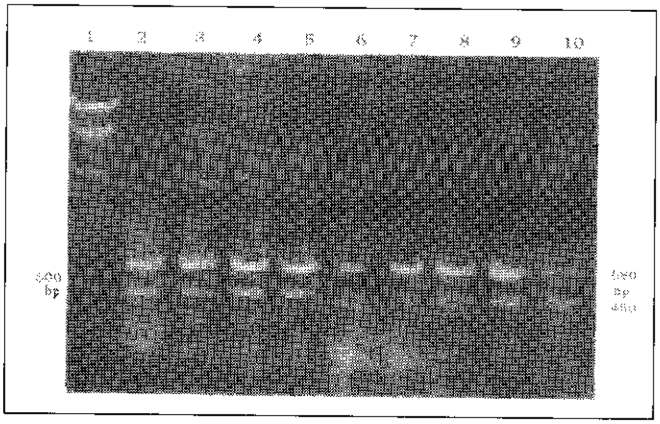

Fig. 3. Polymerase chain reaction amplified phytoplasma DNA from coconut using P4/P7 primer pair. Lanes 2-6: DNA isolated from root (wilt) coconut tissues by Doyle & Doyle method and lanes 7-10: DNA isolated from root (wilt) diseased coconut tissues by Ahrens & Seemuller method. Lane 1: 500 bp DNA ladder; lanes 2 & 7 : DNA from rachilla; lanes 3 & 8: DNA from petiole; lanes 4&9: DNA from midribs; lanes 5 & 7: DNA from heart portions and lane 6: DNA from lamina tissues of root (wilt) diseased coconut palms showing two DNA fragments (450 and 680 bp)

sensitive nested primer set too produced no DNA fragment from both diseased and healthy coconut palms. Amplification with as low as 25 ng of template DNA prepared from coconut tissues using Ahrens and Seemuller method proves that this method of DNA isolation contained more amount of phytoplasma DNA in the preparations.

The P4/P7 primer set amplifies a DNA fragment of 0.5 kb from 1275 bp of 16S to 59 bp of 23S rDNA including interspacer region of phytoplasma (Tymon *et al.*, 1998). Although in this investigation, the amplicons obtained from DNA of phytoplasma infected coconut tissues were not identical to the earlier reported results, the detection of DNA fragments of 450 and 680 bp only from root (wilt) diseased palms indicates the presence of phytoplasma DNA in the diseased tissues. Hence, it is hypothesized that the coconut RWD phytoplasma may be different from other phytoplasmas and this phytoplasma DNA may have two priming sites for the P4/P7 primer pair. Detection of a single DNA fragment of 650 bp from root (wilt) diseased coconut tissues using the same primer pair has recently been reported (Sharmila *et al.*, 2004). Contrary to this, in this investigation, it was not found amplifying a single fragment of 650 bp from diseased coconut palms.

PCR is a sensitive tool to detect DNA sequences and provides a convenient means for obtaining amplification of DNA of phytoplasmas from total genomic DNAs of diseased host plants with specific primers under controlled thermocycling conditions. Correct DNA product of phytoplasma DNA is obtained when thermocycling conditions are precisely standardized. PCR reactions have been used to detect phytoplasmas with high sensitivity and specificity from a wide variety of host plants using appropriate primer combinations (Harrison *et al.*, 1994; Gundersen and Lee 1996; Schneider and Gibb, 1997). In this investigation, several primer combinations were used for detecting DNA of phytoplasma from infected coconut tissues and these primer combinations are universal in their ability to prime PCR amplification from many distinct phytoplasmas (Saeed and Cousin, 1995; Lorenz *et al.*, 1995). The fact that the P4/P7 primer set amplified DNA fragments consistently only from root (wilt) diseased tissues but not from healthy tissues implies the presence of phytoplasma DNA in the diseased palms thus confirming the phytoplasmal etiology of root (wilt) disease of coconut. Further studies using newly designed phytoplasma-specific universal primers and standardization of optimal concentration of PCR ingredients and thermocycling conditions will yield correct phytoplasma DNA fragment from root (wilt) diseased coconut tissues. This will help in designing a set of primers specific to coconut root (wilt) phytoplasma for sensitive detection, which can ultimately be used to identify diseased palms and seedlings where a requirement of phytoplasma-free plant materials is essential.

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