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Comparison of different DNA isolation methods for PCR detection of coconut root (wilt) phytoplasma

Rintu Rebecca Jacob and M. Mayilvaganan*

Central Plantation Crops Research Institute, Regional Station, Krishnapuram 690 533, Kayangulam

Abstract

Six DNA isolation methods comprising three total genomic DNA isolation procedures *viz.*, the Doyle & Doyle, the Kobayashi and the Qiagen DNeasy Plant Maxi kit and three phytoplasma-enrichment DNA isolation procedures *viz.*, the Ahrens & Seemuller, the Lee and the Tymon, were used for extraction of DNA from four types of young tissues (inflorescence, petiole, midrib and heart tissue) of root (wilt) diseased and healthy coconut palms. Two phytoplasma-specific primer pairs, Pc399/P1694 and PA2F/R, with one nested primer pair, NPA2F/R, were tested for amplification of phytoplasma DNA from root (wilt) diseased coconut tissues. The total genomic DNA isolation methods generally yielded higher amount of DNA than the phytoplasma-enrichment DNA isolation methods. However, the phytoplasma-enrichment DNA isolation procedures yielded higher quality DNA in terms of absorption ratio ($A_{260/280}$) than the total genomic DNA isolation methods. Among the total genomic DNA isolation methods, the Doyle & Doyle method yielded highest quality and quantity of DNA and amongst phytoplasma-enrichment DNA isolation methods, the Ahrens & Seemuller yielded higher amount of DNA and the Lee method yielded higher quality and quantity of DNA respectively. In tissues, petiole was found to produce highest quality and quantity of DNA. The Pc399/P1694 primer pair did not prime the amplification of phytoplasma DNA sequences from root (wilt) diseased coconut tissue DNA extracts. Direct priming with PA2F/R primer pair followed by nested priming with NPA2F/R amplified a 485 bp fragment from DNA extracts prepared by phytoplasma-enrichment DNA isolation methods such as the Lee and the Ahrens & Seemuller from petiole and heart tissues of only root (wilt) diseased coconut palms. Detectable amplified product was, however, obtained with higher amount of template DNA.

Key words : Doyle & Doyle method, Lee method, Ahrens & Seemuller method, coconut tissues, phytoplasma-specific primers, 16S and 23S rDNA, amplification

Introduction

Coconut is an economically important perennial oil-yielding crop of southern India. Root (wilt) disease (RWD), a debilitating malady widely prevalent in the southern area of Kerala and also in a few adjoining areas of neighboring Tamil Nadu, is caused by phytoplasma (Solomon *et al.*, 1983a). Phytoplasmas are wall-less, pleomorphic, non-helical and non-culturable prokaryotic and systemic pathogen that belong to the class *Mollicutes*, which inhabit the sieve elements of phloem cells in the plant and are known to cause more than 700 diseases (Weintraub and Beanland, 2006). Phytoplasmas are commonly detected by electron microscopy (Marcone and Ragozzino, 1995; Hwang *et al.*, 1997), staining and light microscopy (Parente *et al.*, 1994; Chang *et al.*, 1996), serological techniques (Gomez *et al.*, 1996; Seddas *et al.*, 1996) and DNA-based molecular techniques (Lee *et al.*, 1992,1997). However, polymerase chain reaction (PCR) assays using primers derived from sequences of 16S and 23S rRNA gene including 16S-23S interspacer region is a useful sensitive method in the detection of phytoplasma in infected plant and insect hosts. In the case of root (wilt) disease, previously, DNA was isolated from coconut tissues by two procedures and eight universal phytoplasma-specific primer pairs were tested for PCR amplification of phytoplasma DNA, but only one primer pair amplified two nonspecific amplification products from root (wilt) diseased coconut tissues (Mayilvaganan *et al.*,

*Author for correspondence

2006). A close scrutiny of literature revealed the availability of several other methods for isolation of DNA from plant tissues for the PCR detection of phytoplasma. Three more phytoplasma-specific primer pairs including a nested primer pair have been designed from 16S-23S rDNA sequences for sensitive detection of phytoplasma (Heinrich *et al.*, 2001; Skrzeczkowski *et al.*, 2001). In the present work, different DNA isolation methods were used to extract DNA from various tissues of coconut in order to find out the suitable procedure(s) to meet the above-mentioned requirements for the PCR detection of coconut root (wilt) phytoplasma. The comparison of the efficiency of the different extraction procedures is based on the quality as absorption ratio ($A_{260/280}$) and yield of DNA and evaluation of DNA for suitability to act as template for PCR amplification of phytoplasma DNA using new primer pairs.

Materials and Methods

Plant material

Coconut palms between 10 and 12 years of age displaying typical RWD symptoms were identified from the 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 test 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.

DNA extraction

DNA was isolated from four types of tissues *viz.* immature inflorescences, midribs, petiole and heart tissue by six procedures and compared for quality and yield of DNA and their ability to act as templates for PCR amplification primed by oligomers that recognize specific conserved sequence of the 16S and 16S-23S interspacer region of rRNA gene of phytoplasma. Among the six methods employed for DNA extraction, the first three *viz.*, Doyle & Doyle (1990), Kobayashi (Kobayashi *et al.*, 1998) and Qiagen kit were total genomic DNA isolation procedures and other three *viz.*, Ahrens and Seemuller (1992), Lee (Lee *et al.*, 1993) and Tymon (Tymon *et.al.*,) 1998were methods of DNA isolation from phytoplasma-enriched pellets. Each procedure of DNA extraction was performed on six number of each type of tissue.

All the DNA pellets were dissolved in sterile water, quantified and purity was checked by spectrophotometry by measuring absorption at 260 and 280 nm.

PCR amplification and analysis

For amplification of phytoplasma DNA from nucleic acids extracted from various types of tissues of RWD coconut palms with six different procedures, phytoplasma-specific primers Pc399/P1694 (Skrzeczkowski *et al.*, 2001), PA2F/R with nested primer pair NPA2F/R (Heinrich *et al.*, 2001) were tested in the study. With Pc399/P1694 primer pair, PCR was performed in 40 µl volume containing 1.5 mM $MgCl_2$, 150 µM of each dNTP, 0.5 µM each primer, 1 unit *Taq* DNA polymerase (Genei, Bangalore) and 100 to 500 ng of template DNA. Amplification consisted of an enzyme activation step at 95 °C for 5 min and then 33 cycles of denaturation at 94 °C for 30 sec, annealing at 60 °C for 1 min, extension at 72 °C for 2 min and the final extension step was at 72 °C for 10 min. With PA2F/R, PCR was performed in 40 µl volume containing 1.75 mM $MgCl_2$, 250 µM of each dNTP, 20 picomoles of each primer, 1 unit of *Taq* DNA polymerase (Genei, Bangalore). Template DNA of 100 to 500 ng was used for direct PCR with PA2F/R and the PA2F/R product was diluted 1:5, 1:10, 1:20 and 1:40 and 1, 2, 5 µl was used for nested PCR with NPA2F/R. The following thermocycling programmes were used for PA2F/R PCR: 35 cycles of denaturation at 94 °C for 30 sec, annealing at 60 °C for 1 min 30 sec, extension at 72 °C for 45 sec with a final extension step at 72 °C for 10 min. For NPA2F/R, PCR was performed with 35 cycles of denaturation at 94 °C for 30 sec, annealing at 60 °C for 30 sec, extension at 72 °C 45 sec and a final extension step at 72 °C for 10 min. The DNA isolated from healthy coconut palms and water served as negative controls and the DNA of Australian grapevine yellow phytoplasma was used as positive control for amplification. PCR product was electrophoresed on 1.2% 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

The quality and quantity of DNA obtained from various tissues of coconut varied widely depending upon the extraction method and the particular tissue used for isolation. The quality as absorption ratio ($A_{260/280}$) and yield of DNA by the total genomic DNA extraction methods *viz.*, Doyle & Doyle, Kobayashi and Qiagen kit are presented in Table 1. The data show that among the three methods of total genomic DNA extractions, the quality and yield of DNA by the Doyle & Doyle method

Table 1. Quality and yield of total genomic DNA extracted from tissues of coconut

Tissue	Doyle & Doyle Method		Kobayashi Method		Qiagen DNA kit	
	Quality (A_{260}/A_{280})	Yield (µg/ mg tissue)	Quality (A_{260}/A_{280})	Yield (µg/ mg tissue)	Quality (A_{260}/A_{280})	Yield (µg/ mg tissue)
Inflorescences	1.82	1.29	1.80	1.31	1.74	0.79
Midribs	1.78	1.24	1.74	1.13	1.72	0.72
Petioles	1.87	1.72	1.84	1.44	1.78	0.89
Heart tissues	1.83	1.47	1.78	1.26	1.74	0.84

Values are the mean of six extractions from each type of tissue

Table 2. Quality and yield of DNA extracted by phytoplasma enrichment methods from tissues of coconut

Tissue	Ahrens & Seemuller Method		Lee method		Tymon method	
	Quality (A_{260}/A_{280})	Yield (µg/ mg tissue)	Quality (A_{260}/A_{280})	Yield (µg/ mg tissue)	Quality (A_{260}/A_{280})	Yield (µg/ mg tissue)
Inflorescences	1.83	0.24	1.85	0.22	1.80	0.06
Midribs	1.76	0.27	1.83	0.17	1.73	0.11
Petioles	1.94	0.30	2.00	0.26	1.86	0.09
Heart tissues	1.88	0.26	1.88	0.23	1.81	0.08

Values are the mean of six extractions from each type of tissue

is higher in a range of 1.78 - 1.87 and 1.24 - 1.72 µg/mg fresh tissue. The quality and yield of DNA of the other two methods are lower than that of Doyle & Doyle method. Among the four types of tissues tested for DNA isolation, petiole yielded highest quality and quantity of DNA from all three methods of total genomic DNA isolation.

The quality and yield of DNA by three phytoplasma-enrichment DNA isolation methods *viz.*, Ahrens & Seemuller, Lee and Tymon are summarized in Table 2. Among these three methods, DNA obtained from the Lee method was of higher quality than the other two methods and the absorption ratio ($A_{260/280}$) of tissues are in the range of 1.83 – 2. However, yield of DNA by the Ahrens & Seemuller method was higher in a range of 0.24 – 0.30 µg/mg fresh tissue than the Lee and the Tymon methods. As in the case of total genomic DNA isolation methods, the petiole tissue produced higher quality and quantity DNA by all the three phytoplasma-enrichment DNA extraction methods.

In general, the total genomic DNA isolation methods yielded higher quantity of DNA than the phytoplasma-enrichment DNA isolation methods. Nevertheless, the phytoplasma-enrichment DNA isolation methods yielded higher quality DNA than the former. When the six protocols were compared for mean quality of DNA, the Lee method followed by Ahrens & Seemuller method produced highest quality DNA (Fig. 1). When the six protocols were compared for mean yield of DNA, the Doyle & Doyle method yielded highest quantity of DNA and the Tymon method yielded lowest amount of DNA (Table 2). As mentioned earlier, the petiole tissue yielded highest quality and quantity of DNA by all but one procedure. The immature midribs yielded highest amount of DNA by the Tymon method.

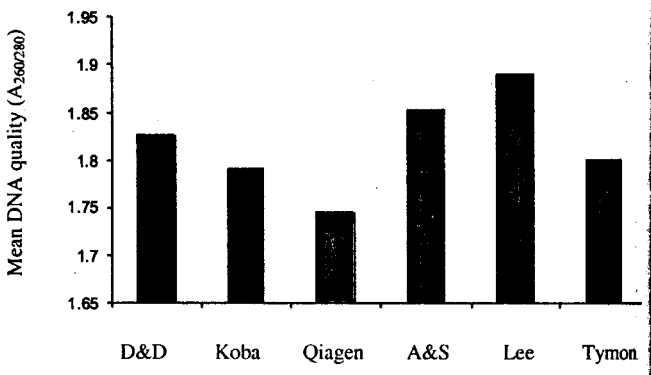

Fig. 1. Comparison of mean quality as absorption ratio ($A_{260/280}$) of DNA of six different isolation methods. D&D: Doyle & Doyle method; Koba: Kobayashi method; Qiagen: Qiagen DNeasy Maxi Plant kit method; A&S: Ahrens & Seemuller method; Lee: Lee method and Tymon: Tymon method.

It has been reported that young petiols contain greatest concentration of active phloem and phytoplasma bodies (Escamilla *et al.*, 1995; Harrison *et al.*, 1995). Mature tissues of hardy crops like coconut contain fewer phytoplasma bodies in degenerated form (Thomos, 1979; Solomon *et al.*, 1987). Harrison *et al.* (1994) too found immature leaf bases obtained from lethal yellowing affected palms, including coconut palm, is suitable for isolating DNA and can be used for detection of phytoplasma by PCR.

In a previous study (Mayilvaganan *et al.*, 2006), the total genomic DNA isolation method of Doyle & Doyle and phytoplasma-enrichment DNA isolation method of Ahrens & Seemuller were used for extraction of DNA from root (wilt) coconut tissues. These methods of DNA isolation were also included in this piece of investigation for comparison with other methods for their relative performance. The results obtained in this study on quality and quantity of DNA are very similar to the previous work that total genomic DNA isolation method of Doyle & Doyle yielded higher amount of DNA where as the phytoplasma-enrichment DNA isolation method of Ahrens & Seemuller yielded higher quality DNA. It is obvious that phytoplasma-enrichment DNA isolation method yields less amount of DNA as it involves preparation of crude phytoplasma pellet and extraction of DNA from the phytoplasma-enriched pellet. The phytoplasma enrichment DNA isolation procedures were developed as a means to increase the percentages of phytoplasma DNA in nucleic acid extracts from diseased host tissues and hence the relative amount of DNA from phytoplasma would be more and the co-precipitating host plant contaminants would be less in these methods of DNA extraction. Contrary to these results, Borth *et al.* (1999) reported that the Lee method yielded higher amount of DNA than Ahrens & Seemuller method in papaya and macadamia plants for the detection of phytoplasma. However, in terms of quality of DNA, the Lee method gave highest quality DNA with low amount of co-precipitating contaminants as evident from the absorption values, which are in total agreement with the results of Borth *et al.* (1999).

The Pc399/P1694 oligomers did not prime the amplification of phytoplasma DNA sequences in DNA extracts from both root (wilt) diseased and healthy coconut tissues that were tested under the standard PCR component concentrations and thermocycling conditions according to Skrzeczkowski *et al.* (2001). The first PCR amplification with primer pair PA2F/R followed by nested amplification with NPA2F/R produced 485 bp amplicon, which is of appropriately-sized product expected of the primer pairs, from DNA isolated from petiole and heart tissues by the Lee and the Ahrens & Seemuller methods using the PCR components concentration and thermocycling conditions given by Heinrich *et al.* (2001) (Fig. 2). However, detectable amplified product was obtained only when more than 500 ng of template DNA used in the direct PCR with PA2F/R primer pair and when the direct PCR product was diluted in 1:5 and 5 µl of diluted DNA was used as template in the nested PCR amplification. The primer pairs PA2F/R and NPA2F/R did not amplify DNA fragments from DNA isolated from healthy coconut palms and water control but amplified 485 bp amplicon from positive control of Australian grapevine yellow phytoplasma DNA.

Previous attempts to amplify phytoplasma DNA from root (wilt) diseased coconut tissues with eight universal phytoplasma-specific primer pairs *viz.*, Ahrens and Seemuller forward /reverse, P1/P7, P1/P6, fU5/rU3, Rhode forward/ reverse, R16F2/R2 and R16mF2/R1 and with nested primer pair R16F2n/R2n, resulted in unspecific amplification of two DNA fragments of 680 and 450 bp by only P4/P7 primer pair (Mayilvaganan *et al.*, 2006). In this study, the PA2F/R and NPA2F/R primer pairs amplified specific DNA amplicon from root (wilt) diseased

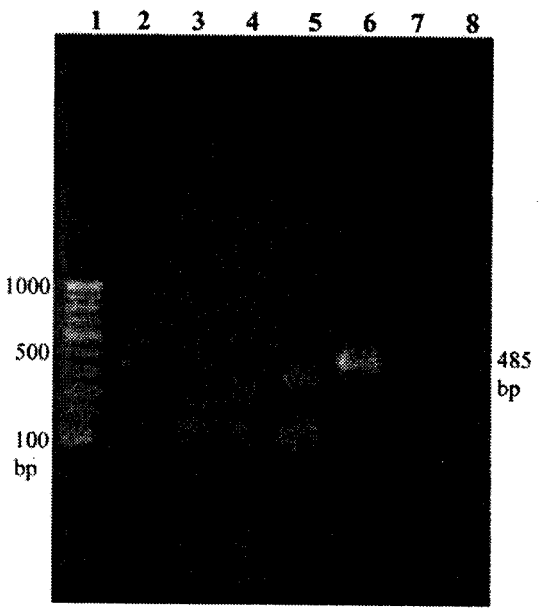

Fig. 2: Nested polymerase chain reaction amplification of the phytoplasma DNA from root (wilt) diseased coconut tissues using PA2F/R and NPA2F/R primer pairs. Lane 1: 100 bp DNA ladder; Lane 2: DNA from petiole tissue by the Lee method; Lane 3: DNA from heart tissue by the Lee method; Lane 4: DNA from petiole tissue by the Ahrens & Seemuller method; Lane 5: DNA from heart tissue by Ahrens & Seemuller method; Lane 2 – 5: DNA from root (wilt) diseased coconut tissues; Lane 6: DNA of Australian grapevine yellows phytoplasma (positive control); Lane 7: DNA from petiole tissue of healthy coconut palm (negative control) and Lane 8: water control.

coconut tissues. These universal primers were designed after extensive analysis of the phytoplasma and higher plant DNA sequences currently available in the databases for sensitive detection of phytoplasmas in woody and tree crops (Skrzeczkowski *et al.*, 2001; Heinrich *et al.*, 2001).

Concentration of phytoplasma cells in host plants is sparse and shows significant variations depending on the season (Seemuller *et al.*, 1998; Heinrich *et al.*, 2001) and the phytoplasma titer is very low in woody plant hosts (Kartte and Seemuller, 1991; Lederer and Seemuller, 1991). The sensitivity and suitability of molecular methods in detection of phytoplasma from hardy tree crops, particularly PCR methods largely depends on the amount of phytoplasma cells or its DNA in the preparation and obviously obtaining pure DNA concentrated enough for precise detection and analysis is very important.

When the PA2F/R PCR products was analysed after 35 cycles of amplification, visible amplified product was not obtained and only after nested amplification with NPA2F/R, detectable amount of DNA was obtained from the DNA isolated by phytoplasma-enrichment DNA isolation procedures such as the Lee and Ahrens & Seemuller. As mentioned earlier, the phytoplasma-enrichment DNA isolation methods ensures enhanced amount of phytoplasma DNA in the preparation. Coconut, which is a hardy tree crop, is reported to contain fewer and evenly distributed phytoplasma cells in root (wilt) diseased (Solomon *et al.*, 1987) and in lethal yellowing affected coconut palms (Parthasarathy, 1974; Thomos, 1979). From such plants, direct PCR, a single amplification with a series of 35 cycles with a pair of oligomers failed to detect phytoplasmas. In such cases, nested PCR assays using another primer pair are used to reamplify the DNA from the first PCR product to greatly increase the sensitivity of the detection. The main purpose of the study was to identify appropriate DNA extraction method(s) and to select suitable primer pairs for sensitive amplification of phytoplasma DNA from root (wilt) diseased coconut tissues. From the results of the present study, it is concluded that phytoplasma-enrichment isolation methods such as the Lee and the Ahrens & Seemuller would be appropriate methods for DNA isolation from root (wilt) diseased coconut tissues and direct and nested amplification with PA2F/R and NPA2F/R primer pairs would be useful for further studies on coconut root (wilt) phytoplasma.

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