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Real-time PCR technique for detection of arecanut yellow leaf disease phytoplasma

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Abstract The Yellow Leaf Disease, with phytoplasmal etiology, is a serious disease affecting arecanut palms and causing substantial yield loss. Phytoplasmas are cell wall less, unculterable, phloem limited plant pathogens generally detected using microscopy, serology and molecular techniques. Here we report a SYBR green based real time PCR approach for detection of arecanut yellow leaf disease phytoplasma. We designed efficient primers for SYBR green based real time PCR to overcome the problems in conventional PCR. Primers QPF2/QPR2, designed from highly conserved phytoplasma 16S rRNA gene was used to amplify DNA preparation from spindle leaf tissues of symptomatic palms using real time PCR. A unique melting peak at 82.3±0.5 °C was observed for symptomatic arecanut samples. The PCR products were further purified, sequenced and analysed using BLASTn. The sequences showed 99 % nucleotide identity with Indian arecanut yellow leaf disease phytoplasma sequences in the database. Two representative sequences were also deposited in the Genbank database. The present study thus devised a platform for rapid and sensitive detection of phytoplasma associated with the arecanut yellow leaf disease.

Keywords Arecanut yellow leaf · Phytoplasma · Real-time PCR · SYBR green

Phytoplasmas are plant pathogenic Mollicutes causing a large number of diseases in crop plants accounting to huge losses and hence have immense economic importance in the agricultural scenario. Phytoplasmas are phloem bound, cell wall less, plant pathogens transmitted by sap sucking insect vectors. They cannot be cultured in vitro in cell free media and hence their detection in hosts relies up on microscopy, serological assay or molecular techniques. The molecular detection of phytoplasma relies on nested PCR amplification of 16S rDNA. With the advent of real time PCR, accurate, specific and quick detection of plant pathogens including phytoplasma is materialized. Real time PCR primers and assays have been established for phytoplasma diagnostics in many important crop species. DNA intercalating dyes like SYBR Green and Eva Green enable easy detection in real time in a simple assay (Manimekalai et al. 2011and Martini et al. 2007). However, more specific detection is possible with probe based real time PCR assays (Nikoli et al. 2010).

The arecanut palm (*Areca catechu* L.) is widely cultivated in the tropics and has diverse use ranging from the mastigatory purpose to the use as an ornamental palm. The palm is affected by a number of pests and diseases throughout its life. One of the major diseases of the arecanut palm in south India is the YLD. We had reported that phytoplasma associated with the disease is phylogenetically related to Rice Yellow Dwarf phytoplasma and belongs to 16SrXI group, subgroup B (Manimekalai et al. 2010, 2013). Being a member of the 16SrXI-B group, routine detection of the YLD phytoplasma is done by nested PCR amplification 16S rDNA with primers specific to 16SrXI-B group. Detection of YLD phytoplasma often becomes a tricky task due to low titer of phytoplasma in the infected palm, presence of PCR inhibitors and high percent of false positives due to cross contamination with nested PCR assay. In this backdrop we initiated an effort to develop a specific and efficient real time PCR based detection system for rapid and consistent detection of Arecanut YLD phytoplasma.

Four primer pairs were picked from related sequences of arecanut YLD phytoplasma. Two set of primers, QPF1/QPR1 and QPF2/QPR2 were designed based on the multiple sequence alignment of arecanut yellow leaf phytoplasma

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Table 1 Details of primers used for realtime PCR assay

Primer	Forward primer	Reverse primer	Product size (bp)
QPF1- QPR1	CGCAACCCTTGTCATTAGTT	CCAATTTTTCATTGGCAGTC	70
QPF2-QPR2	ACGCTTAACGTTGTCCTGCT	TGCCTCAGCGTCAGTAAAGA	146
QPF3-QPR3	GGGCCTATAGCTCAGTTGGT	AATGGACTTGAACCATCGAC	65
F1-R1	TCTAAGGTAGGGTCGATGA	GGACTTGAACCACCGACCTC	218

sequences isolated with XIF-XINR primers (JN967905.1, JN967906.1, JN967904.1) and other one set of primer, QPF3/QPR3, was picked from 16S to 23S ribosomal RNA intergenic spacer region from coconut root wilt phytoplasma amplified with P1-P7 primer set (FJ794816.1) (Table 1). Primer specificity was analyzed through Primer-BLAST and BlastN (Altschul et al. 1990) tools in order to avoid cross-specificity with other bacterial sequences. The primers were checked for qualities such as GC percent, annealing temperature, PCR efficiency, primer complexity and dimer formation using FASTPCR 6.1 (Kalendar et al. 2009). Designed primers were synthesized from Integrated DNA Technologies (IDT), USA. Another primer pair F1/R1, used in our earlier work for detection of coconut root wilt phytoplasma, was included in the present study (Manimekalai et al. 2011). Then we examined the suitability and efficiency of the 4 different primer sets for phytoplasma detection in arecanut through real time PCR. We tried to identify the primer pair most suited for diagnostics of arecanut phytoplasma keeping in view the low concentration of phytoplasma in the host and the presence of PCR inhibitors in samples. The plant polyphenolic and polysaccharide molecules present in the DNA preparation from diseased samples often cause inhibition of the PCR amplification (Marzachi 2004).

Spindle leaves were collected from ten YLD symptomatic arecanut palms (a1 to a10) from Sullia, Karnataka. Grassy shoot diseased sugarcane sample collected from Sugarcane Breeding Institute, Coimbatore, was used as the positive control. Healthy arecanut samples were collected from a disease free location, Kidu in Karnataka. The DNA was extracted from 3 g spindle leaf tissue sample using a modified phytoplasma enrichment protocol (Ahrens and Seemuller 1992) and dissolved in TE buffer (pH 8). The DNA concentration was measured in a spectrophotometer and the DNA preparations were diluted to 25 ng/μl for further analysis. The samples were tested in real time PCR in a Stratagene MX3005P machine (Agilent Technologies). The assay also included a positive control sugarcane sample and 2 NTCs (No template controls) and healthy arecanut sample. Brilliant II Fast SYBR Green QPCR Master mix (Stratagene) was used for the reaction mixture. Each reaction mix of 15 μl included 50 ng of template DNA, 160 nM each of forward and reverse primers, 7.5 μl of 2X Brilliant II Fast SYBR Green QPCR Master mix and nuclease free water to make up the volume. The real time PCR cycle included a pre-incubation at 95 °C for 10 min (segment 1) followed by 40 cycles of amplification at 95 °C for 10 s and 55 °C for 30 s (segment 2) and a melting curve analysis at 95 °C for 10 s, 55 °C for 30 s and 95 °C for 30 s (segment 3). Ct value was calculated automatically by plotting fluorescence intensity against the number of cycles. Data analysis was performed using MxPro software. The real time PCR products were run on 1 % agarose gel and amplicons were eluted and sequenced. Sequence analysis using Blastn was performed to verify the fidelity of the amplification products.

In the real time PCR assay with 4 different primer sets, primers F1/R1 could not amplify the arecanut YLD phytoplasma while the primers QPF1/QPR1 and QPF3/QPR3 gave inconsistent results. The presence of PCR inhibitors in the DNA preparations could be the reason. The primers QPF2/QPR2 gave consistent amplification with high number of positives. All the 10 symptomatic arecanut samples studied and the sugarcane sample gave positive amplification signals with this primer pair. However no amplification was obtained for the NTC. The cycle threshold values (Ct values) for the diseased arecanut samples ranged between 30.05 and 34.44 (Table 2). SYBR Green binds to any double stranded DNA molecule and hence can give signals for non specific products

Table 2 The mean cycle threshold (Ct) values of the samples and the corresponding melting temperature (Tm)

Sample name	Ct (dR)	Tm product	Amplification
NTC	No Ct	–	–
a1	31.7	82.34	+
a2	34.17	81.86	+
a3	31.46	81.87	+
a4	30.05	81.87	+
a5	31.27	81.87	+
a6	34.44	81.88	+
a7	30.76	81.88	+
a8	31.61	81.88	+
a9	34.02	81.88	+
a10	32.73	81.39	+
SCGS	22.41	82.37	+
Healthy	38.6	80	–

Tm obtained during melt curve analysis of the amplified product in real time PCR with primers QPF2/QPR2; SYBR Green is the reporter molecule; +/- indicates positive/negative for amplification

and primer dimmers also. So the dissociation curve analysis was performed to evaluate the specificity of the amplification product. A unique melting peak at 82.3±0.5ºC was obtained for all the symptomatic arecanut samples and the sugarcane sample. The NTC showed no peak. The unique melting peak indicated a single amplification product. Hence specific detection is possible with this method on account of melting peak characteristic of the phytoplasma gene fragment. For the healthy arecanut samples, a lower melting peak, corresponding to primer dimer was obtained. In the agarose gel electrophoresis of real time PCR products, amplicons of expected size, 146 bp, were obtained for all samples with primers QPF2/QPR2. No amplification was seen for the NTC. Blastn analysis of the sequences confirmed that the sequences corresponded to arecanut YLD phytoplasma 16S rDNA. Two representative sequences were deposited in the GenBank database (Accession Numbers - KF768254, KF768255).

In this study, a rapid and efficient real time PCR protocol has been established for the detection of phytoplasma associated with Indian YLD of arecanut. The real time PCR technique saves much time taken for the molecular detection as there is no need for the second PCR and agarose gel electrophoresis for amplicon detection. Moreover there are fewer chances for sample cross contamination as the sample handling steps are less. The real time PCR method has been applied for detection of phytoplasma in many plant hosts. In our earlier studies we established the real time PCR based detection system for coconut root wilt phytoplasma (Manimekalai et al. 2011). Martini et al. (2007) employed the SYBR Green based real time PCR for detecting *Ca*. P. prunorum from infected apricot trees and its insect vector. Quantification of phytoplasma titer in host and in different tissues of the host is also possible with this tool. Torres et al. (2005) quantified the apple proliferation group phtyoplasmas using real time PCR conjugated with fluorescent SYBR Green.

The current management strategy for the arecanut YLD is the removal of diseased palms and planting of disease free materials. This needs quick and early detection which is likely only through molecular techniques. The SYBR Green based real time PCR assay developed in the present study is certainly an improvement over the conventional nested PCR based molecular detection. This will enable rapid and sensitive detection of arecanut YLD phytoplasma and hence will be of use in devising the disease management strategies.

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