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ORIGINAL ARTICLE

Nested and TaqMan® probe based quantitative PCR for the diagnosis of Ca. Phytoplasma in coconut palms

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Abstract

The root (wilt) disease caused by phytoplasma (Ca. Phytoplasma) is one of the major and destructive occurs in coconut gardens of Southern India. As this organism could not be cultured in vitro, the early detection in the palm is very much challenging. Hence, proper early diagnosis and inoculum assessment relay mostly on the molecular techniques namely nested and quantitative PCR (qPCR). So, the present study qPCR assay conjugated with TaqMan® probe was developed which is a rapid, sensitive method to detect the phytoplasma. For the study, samples from different parts of infected coconut palms *viz*., spindle leaflets, roots and the insect vector—leaf hopper (*Proutista moesta*) were collected and assessed by targeting 16S rRNA gene. Further, nested PCR has been carried out using p1/p7 and fU5/rU3 primers and resulted in the amplification product size of 890 bp. From this amplified product, specifically a target of 69 bp from the 16S rRNA gene region has been detected through primers conjugated with Taqman probe in a step one instrument. The results indicated that the concentration of phytoplasma was more in spindle leaflets (8.9×10^5 g of tissue) followed by roots (7.4×10^5 g of tissue). Thus, a qPCR approach for detection and quantification of coconut phytoplasma was more advantageous than other PCR methods in terms of sensitivity and also reduced risk of cross contamination in the samples. Early diagnosis and quantification will pave way for the healthy coconut saplings selection and management under field conditions.

Keywords Coconut · Phytoplasma · Root (wilt) disease · 16S rRNA gene · Quantitative PCR · TaqMan® probe

Introduction

India is the third largest producer of coconut in the world (21,665 million nuts) and contributes major production of nearly 70% by southern states of India. The maximum production of 7429 million nuts and productivity of 13,732 was recorded in Kerala followed by Tamil Nadu which accounts 6171 million nuts and 13,423 nuts ha^{-1}, respectively in India (Coconut development board 2015–2016) [1]. Coconut palm is affected by more than 50 diseases globally and among them phytoplasma causing disease is the major destructive one and cause huge yield losses namely root (wilt) disease in India [39, 46], Kalimantan wilt disease in Central Kalimantan [45], lethal yellowing in Mexico, Ghana, Tanzania

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[43], Florida [6] and Lethal decline in Kenya, West and East Africa [32]. Root wilt disease in coconut was first observed in Kerala state, India during 1882 [7] and presently causes 35% yield loss may extend up to 80% in severe cases with the annual losses estimated around 968 million nuts [40]. The wilt disease causing phytoplasma is cell wall-less mollicutes [1] is an obligate parasite transmitted by phloem-feeding insects [lace bug (*Stephanitis typica*) and plant hopper (*Proutista moesta*)] resides endocellularly in insects and phloem tissues of coconut palms. The detection of phytoplasma in salivary glands and brain tissue of lace bugs, *S. typica* offered acquisition access and incubation period on root (wilt) diseased palm [31] and experimental transmission of phytoplasma from diseased palm to healthy 2-year old coconut seedlings under insect proof conditions confirmed its vectoral role. Further, the presence of 650 bp product in the genomic DNA of *Proutista moesta* shows the insect being a vector of KWD phytoplasma [16]. After the well establishment of phytoplasma, the root (wilt) major symptoms like flaccidity, yellowing and marginal necrosis will be identified. The early diagnosis in the palm is very much challenging.

As they have still not been cultured in vitro, their diagnosis relies mainly on molecular techniques [22]. Dot hybridization assays using cloned phytoplasma DNA or their complementary RNA probes were used for detection and differentiation of phytoplasma in past [26]. However, the complete diagnostic procedure is laborious and requires several post amplification steps [21]. Alternatively, the PCR assays using phytoplasma-probing primers are most useful for diagnosis of phytoplasma diseases. Several universal- and species-specific primers have been designed for routine detection of phytoplasma from various host plants [28]. At this juncture, the nested PCR assay is developed to increase the sensitivity and specificity of phytoplasma detection [20]. This can be employed effectively to detect the presence of phytoplasma both in infected and symptomless coconut palms [50] and also can detect even at lower concentration in the plant tissue [20]. The nested PCR requires two rounds of PCR and further needs standardization of dilution of first PCR and followed by a qualitative assay [30]. Alternatively, SYBR Green and TaqMan® probes are used most commonly for the diagnosis of phytoplasma through real time PCR (quantitative PCR, qPCR) [49]. SYBR Green-based qPCR was used successfully for the detection of Candidatus *Phytoplasma* prunorum [29] and apple proliferation [48]. When compared to classical PCR procedure, the nested PCR and quantitative PCR are considered to be more accurate and sensitive for early detection and quantification of pathogens, for which the sampling strategies, DNA extraction and PCR conditions are to be standardized for each of the pathogens. No attempt was made so far to standardize the procedures for phytoplasma infecting coconut crop. Hence, in the present study, it was hypothesized that combining Nested PCR and qPCR assays may increase the sensitivity of detection of coconut root (wilt) causing phytoplasma with less time consumption. So, the aim of the study is to evaluate nested PCR for the early detection and quantitative PCR for the quantification of phytoplasma (in palm sample and insect vector) causing root (wilt) disease in coconut plantations.

Materials and methods

Survey and sampling of coconut

Roving survey was conducted in root (wilt) affected areas of Tamil Nadu, India namely Coimbatore, Dindugul, Tirunelveli, Tirupur and Theni districts. Totally 75 palms were selected at each 5 acre garden. The disease incidence was recorded in the palms based on the expression of major foliar symptoms like of flaccidity, yellowing and necrosis along with GPS coordinates. The per cent disease incidence was calculated by using the formula [51].

$$\text{Disease incidence} = (\text{No. of palms infected}/\text{Total no. of palms observed}) \times 100$$

In the infected coconut gardens, the spindle leaflet (unopened leaves) samples were collected from three locations at adjoining the areas of Kerala border like Coimbatore, Theni and Tirunelveli districts. Totally five root (wilt) affected palm spindle leaflets were randomly collected from each location and used for detection purpose under laboratory assay.

Twenty-four coconut palms showing phytoplasma infection were selected to study the inoculum density. The pooled samples of 8 palms were collected from spindle leafles, 4th petiole leaflets from spindle and roots of diseased palms separately. The same sets of samples were also collected from 24 healthy coconut palms (Each sample have pooled of 8 palms), which served as control. The insect vector, *Proutista moesta* (50 insects per palm) were collected from root (wilt) infected coconut palms using aspirator and used for the extraction of DNA. Each 8 palms pooled samples were treated as one replication, totally three replications were maintained for this experiment. Before isolation of DNA, all the collected samples were stored at 4 °C under laboratory conditions for further use.

DNA extraction

Modified CTAB method [50] was used for the extraction of total DNA from leaflet and root samples of coconut for the

detection of phytoplasma. Described by Jones [25]; DNA from insects by Doyle and Doyle [15] and the DNA pellet was air dried and suspended in 35 µl of TE buffer.

All the extracted DNA was quantified photometrically (ND 1000, Thermo Scientific, USA), diluted to 20 ng/µl working stock and stored at − 20 °C.

Nucleotide sequence analysis of 16S rDNA gene revealed that coconut root (wilt) phytoplasma is very closely related to the date palm leaflet symptomless phytoplasma under the members of 16S rDNA II group. Coconut root (wilt) phytoplasma (GQ861513) and date palm leaflet symptomless phytoplasma (EU119395) shared a 16S rDNA sequence similarity of 88%. Hence, this has been targeted.

Nested PCR

Nested PCR targeting 16S rRNA gene of phytoplasma causing coconut root (wilt) disease was performed using the primer sets p1/p7 followed by fU5/rU3 (Fig. 1) as described [12]. The first-round PCR was performed with universal phytoplasma primers p1 [13] and p7 [42] and 10 times diluted first round PCR products were used as template for second-round PCR using the group specific forward primer fU5 [28] and reverse primer rU3 [35] and the band of expected size was gel-purified using spin columns (Bangalore genei, India) according to the manufacturer's instructions and cloned using pTZ57R/T vector supplied with TA cloning kit (Fermentas, USA) prior to sequencing. Sequencing reactions were performed using ABI prism terminator cycle sequencing ready reaction kit and electrophoresis of the products were carried out on an Applied Biosystems (Model 3100) automated sequencer. The identity of 16S rDNA sequence was established by performing a similarity search against the GenBank database (http://www.ncbi.nih.gov/BLAST) and the sequence obtained in this study have been submitted to GenBank database under Accession Number JQ697495.

TaqMan® probe based qPCR assay

The quantitative PCR assay was performed for assessing the inoculum of phytoplasma in different locations and parts of root (wilt) infected coconut palms. Primers 16SCF and 16SCR and TaqMan probe CP[a]16S probe (Table 1) were designed to be specific for the novel coconut root (wilt) phytoplasma and amplify a 69 bp segment from bases 66 to 135 of JQ697495.

The transformed *E. coli* (harbouring pTZ57R/T) vector inserted with 16S rRNA gene of phytoplasma served as standard for qPCR. The colony PCR was performed with M13 forward and reverse primers (Fermentas, USA) were used to re-amplify the 16Sr RNA gene of phytoplasma fragment along with vector region at both the ends, purified by QIAquick PCR purification kit (Qiagen, Germany)

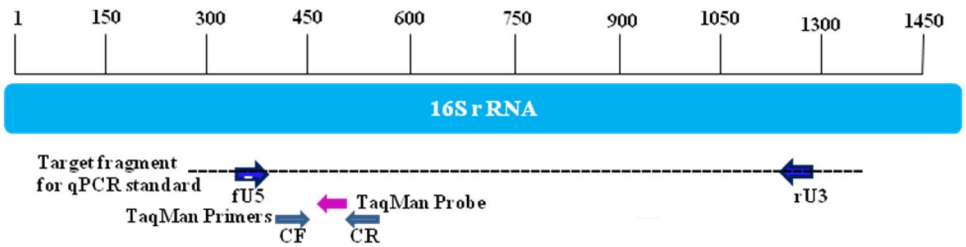

Fig. 1 Positions of primers used in this study. p1 and p7 are used for the first round nested qPCR; fU5 and rU3 for second round nested PCR; CF and CR for TaqMan® qPCR and P is the probe sequence used for TaqMan qPCR. The details of these primers were presented in Table 1

Table 1 Primer sequences and their positions in ribosomal genes of phytoplasma used in the study

Primer	Sequence	Positions in ribosomal genes	Annealing temperature
p1	5′AGAGTTTGATCCTGGCTCAGGATT3′	6–30 in 16S rRNA	48 °C
p7	5′GTCCTTCATCGGCTCTT3′	68–51 in 23S rRNA	
fU5	5′CGGCAATGGAAACT3′	369–386 in 16S rRNA	57 °C
rU3	5′TTCAGCTACTTTGTAACA3′	1251–1231 in 16S rRNA	
CF	5′ACTATCTTGACGATATTCAATGA3′	66–88 in 16S rRNA	60 °C
CR	5′CCAGCAGCCGCGGTAATACATAGG3′	110–134 in 16S rRNA	
P[a]	5′6FAM -CCGGCAAACTATGTG -NFQ3′	95–110 in 16S rRNA	

[a]TaqMan® probe labelled at the 5′ end with 6-carboxy-fluorescein (6FAM), and at the 3′ end with Non fluorescent quencher (NFQ)

and quantified thrice photometrically (ND 1000, Thermo Scientific, USA).

Standard curve for 16Sr RNA gene of phytoplasma was created using tenfold series of the cloned and re-amplified 16Sr RNA gene PCR product of coconut root (wilt) phytoplasma. For qPCR, new set of primers and Taqman probe labeled with FAM (6-carboxyfluorescein) at 5′ end and (NFQ) non-fluorescent quencher at 3′ end were designed (Table 1) and their position in 16S rRNA gene of phytoplasma was presented in Fig. 1. Three independent dilution series were carried out resulting in DNA concentrations between 7.82 ng/µl and 7.82 fg/µl. DNA concentrations could be converted to target molecule numbers per µl [24]. The three dilution series have been run along with different parts of coconut root (wilt) infected palm samples.

qPCR was performed in a total volume of 10 µl using PCR tubes supplied with the quantitative PCR thermocycler (ABI stepone RT-PCR, USA). The DNA template (2 µl each of phytoplasma DNA and 10-fold diluted standards) was added with 8 µl of master mix consisting of 5 µl of 2× TaqMan mix, 0.5 µl of 20× assay mixture (primers and probe), 2.4 µl of nuclease free water and 0.1 µl bovine serum albumin (20 mg/ml). qPCR was started at 60 °C for 30 s (Pre-PCR holding stage), 95 °C for 10 min (initial activation of Thermostart DNA polymerase) followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min and 60 °C for 30 s and data acquisition at each stage during an additional temperature step at 80 °C for 15 s. The fluorescent signals detection at each cycle was visualized through stepone software version2 (Applied Biosystems, USA). The standard curve for all the 10-fold diluted template DNA were plotted against the threshold cycle (CT) and the validity of standard curve was evaluated using R^2 value (> 0.99). In order to reconfirm the standard curve calibration and to reduce the handling errors, two sets of standard curves were made and validated. Similarly, the post run PCR mixture was run in agarose gel electrophoresis with 2 µl of loading buffer and single bands were visualized under UV transilluminator and photographed in gel documentation system (Applied Innotech, USA). The quantification of 16S rRNA gene target present in the samples has been done by the software provided by ABI systems, UK and calculated the target number per g of sample [3].

Statistical analysis

International Rice Research Institute (IRRI) Biometrics unit, the Philippines was developed the software of IRRISTAT version 92 has been used for the analysis of this experiment [19]. Before analyzing the experimental data (the percentage values of the disease indices) were converted into arc sine transformed values.

Results

Survey

Roving survey on the occurrence of root (wilt) disease was conducted in different villages of Coimbatore, Dindigul, Tirupur, Tirunelveli and Theni districts of Tamil Nadu, India. Based on the GPS coordinates, the distribution of coconut root (wilt) map was constructed and presented in the Fig. 2. Among the villages surveyed, maximum incidence of root (wilt) disease recorded in Melagudalur village (68.08%) followed by Mani Nagaram village (61.59%) of Cumbum block in Theni district and Ambarampalayam village (58.08%) of Pollachi South block in Coimbatore district. The average incidence of root (wilt) disease was maximum in Theni district (21.11%) followed by Tirunelveli district (9.41%) and there was no incidence in Dindigul and Tirupur districts.

Nested PCR

The Nested PCR primers p1/p7 followed by fU5/rU3 targeting 16S rRNA gene of coconut root (wilt) causing phytoplasma resulted highly reproducible amplicon with a size of 890 bp without any non-specific amplicon backgrounds, while the healthy coconut samples did not shown any amplification (Fig. 3). The BLAST search of sequence obtained from the nest PCR showed 98% similarity with coconut root (wilt) phytoplasma (Accession Number KP739433), sugarcane white leaf phytoplasma (KR020692) and candidatus phytoplasma Oryza (MG760454).

Detection and quantification by qPCR

All the 10-fold diluted standard DNA were run with qPCR by TaqMan® probe method targeting about 69 bp conserved region inside the 890 bp template in triplicate. The fluorescence signal obtained from each dilution and from each cycle was correlated in a normalized plot. The graph clearly identified the minimum threshold cycle as well as the regression between the threshold cycle and the template concentrations. The slope between CT and the template copies/µl had a positive regression ($R^2 = 0.99$) and from that slope it is possible to calculate the target copy number from standards and different location samples (Fig. 4). With the help of standard copy number/µl, the occurrence of phytoplasma per µl of DNA extracted from leaf sample was calculated and transformed into copies/g leaf tissues. The threshold cycle detecting phytoplasma by qPCR, copies of phytoplasma per µl of DNA extracted and per gram of leaf tissue were compared between three locations (Table 2). The data clearly indicated that all the three replication leaf samples

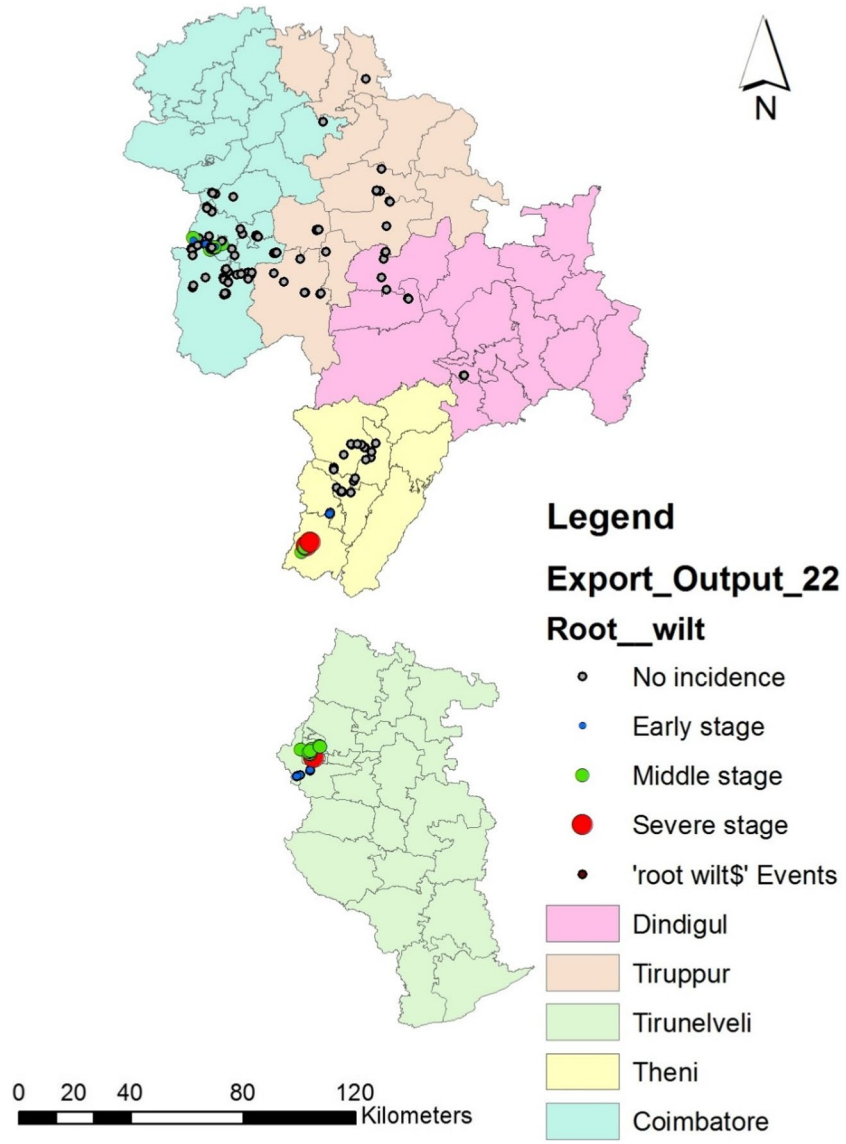

Fig. 2 Distribution of coconut root (wilt) disease in Tamil Nadu, India. Nil represents no incidence, early stage—0–10% incidence; middle stage—10–25% incidence; advanced stage—more than 25% incidence

collected from three different locations of Tamil Nadu had a concentration level ranging from 5.4×10^5 to 10.6×10^5 phytoplasma per gram tissue. Among the three locations, Cumbum leaf samples harboured the maximum phytoplasma concentration (10.6×10^5) and minimum in Pollachi area leaf samples (5.4×10^5). The post-run agarose gel electrophoresis after qPCR clearly revealed that there is no non-specific amplification and also reconfirmed the amplification of 69 bp target (Fig. 5).

The different parts of root (wilt) affected coconut palm samples were collected from severely infected root (wilt) palm samples in Tamil Nadu were assayed for phytoplasma concentration by TaqMan® probe along with eight 10-fold diluted standards. Normalized plot and the standard curve against CT clearly correlated the standards and unknown samples (Fig. 6; $R^2 = 0.95$). With the help of standard copy number/µl, the occurrence of phytoplasma per µl of DNA extracted from different parts of root wilt infected palm samples were calculated and transformed into copies/g leaflet tissues. The threshold cycle detecting phytoplasma by qPCR, copies of phytoplasma per µl of DNA extracted and per gram of leaf tissue were compared between different parts of root (wilt) infected coconut palm samples (Table 3). The results showed that all the samples collected from different parts of root (wilt) infected coconut palm had a concentration level ranging from 5.48×10^4 to 8.9×10^5 copies of phytoplasma per gram tissue. Among the different parts, spindle leaves have maximum concentration of phytoplasma (8.9×10^5 per g) followed by roots (7.4×10^5 per g) and minimum in 4th spindle leaf lets (5.4×10^4 per g). Post-run agarose gel electrophoresis after qPCR confirmed that there was no non-specific amplification apart from the targets (Fig. 7).

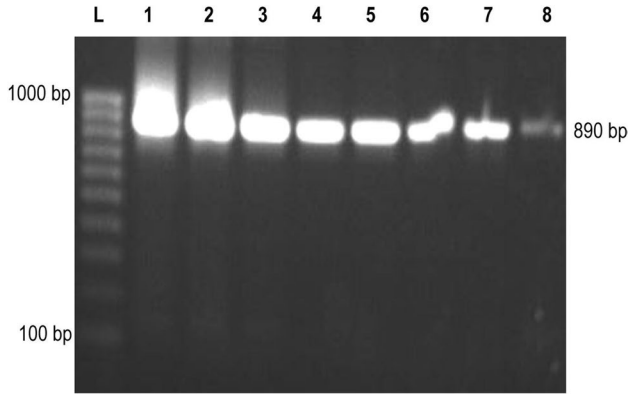

Fig. 3 Amplification of 16S rRNA gene from Coconut root (wilt) disease infected tissues by Nested PCR. Lane M—1 Kb ladder; 1—root (wilt) palm sample from Cumbum; 2—root (wilt) palm sample from Thenkasi; 3—root (wilt) palm sample from Pollachi; 4—Spindle leaf let; 5—4th petiole sample; 6—roots; 7—insect vector; 8—Positive Control

Discussion

Phytoplasmas have been identified as one of the major pathogens in grapevine, sugarcane and coconut causing severe epidemics and yield losses [14]. As this phytoplasmas are still not having been cultured in vitro, their diagnosis in the affected coconut palms mainly depends on the molecular techniques like PCR targeting ribosomal DNA. Due to the failure of conventional PCR to detect the phytoplasma quantitatively and it is laborious and less reproducible [41]. Hence, to avoid this flaw, quantitative PCR can be utilized for the detection as well as quantification (phytoplasma concentration) in the different plant tissues. The main aim of this protocol is to know the quantum of inoculum to cause and damage the crop plants [18]. Further, the main use of nested PCR is to detect phytoplasma or mysterious phytoplasma in its lower concentration in the plants and also in the mixed infection of related pathogens. In case of trees having infected with phytoplasma, detection can be done only by nested PCR before the symptom expression. Primer pairs were selected from 16S rRNA contained ribosomal or non-ribosomal regions for nested PCR

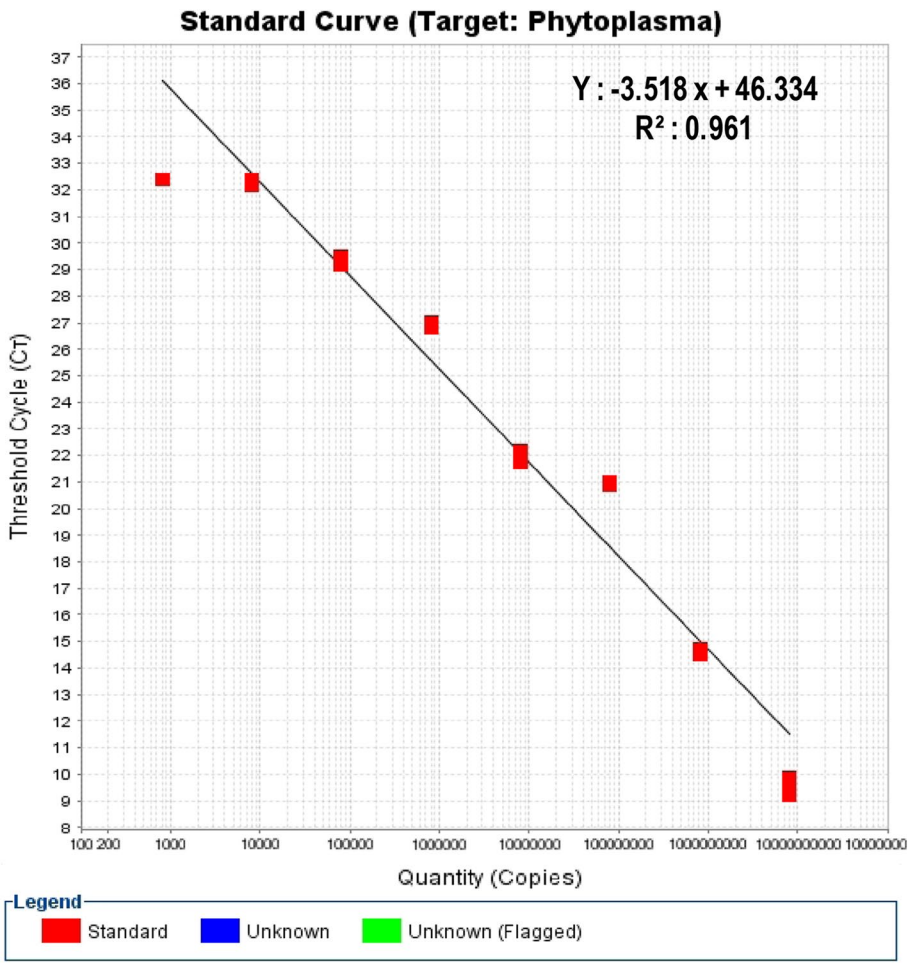

Fig. 4 Standard curves generated during qPCR using TaqMan® probe for copy number against threshold cycle (CT). The standard refers the 10-fold diluted known concentration of 16S rRNA gene of phytoplasma. Samples refer the amplified product from different locations based on the disease incidence

Table 2 Concentration of phytoplasma quantified by TaqMan® probe in root (wilt) affected coconut palms collected from different locations of Tamil Nadu, India

Locations	CT[a]	Phytoplasma 16S rRNA gene	
		Copies/µl	Copies ($\times 10^5$ per g of tissue)
Coimbatore	35.16 (±0.28)	625.38 (±102.14)[c]	5.47 (±0.89)[c]
Theni	34.33 (±0.39)	1220.02 (±526.57)[a]	10.68 (±0.46)[a]
Tirunelveli	34.99 (±0.40)	1003.7 (±64.46)[b]	8.78 (±0.57)[b]

Values are mean (±SE) (n = 5) obtained from two runs of qPCR; values in each column followed by same letter are not significant ($p < 0.05$) according to Duncan's Multiple Range Test

[a]Average threshold cycle (±SE) (n = 3) obtained during TaqMan® qPCR

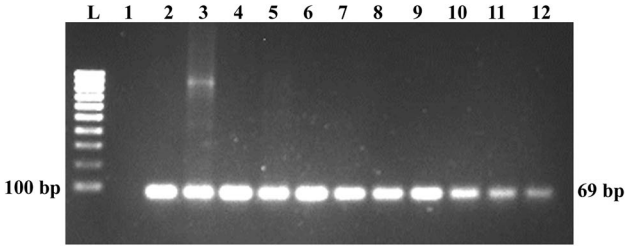

Fig. 5 Post-qPCR agarose gel electrophoresis of 16S rRNA gene amplicons from 10-fold diluted standards and samples from different locations of coconut root (wilt) infected leaf samples. Lane L—100 bp ladder; lane 1—negative control; 2 to 9—10-fold diluted standards (7.82 ng/µl–78.2 fg/µl); 10—Theni sample, 11—Tirunelveli sample; 12—Coimbatore sample

have been detected in coconut leaves, inflorescence, trunk tissues and roots of lethal yellowing disease affected coconut palms [25]. In the present study, Nested PCR resulted in highly reproducible amplicon with a size of 890 bp without any non-specific amplicon backgrounds, while the healthy coconut samples did not shown any amplification. Further, the BLAST search of sequence obtained from the nest PCR showed 98% similarity with coconut root (wilt) phytoplasma (Accession Number KP739433), sugarcane white leaf phytoplasma (KR020692) and candidatus phytoplasma Oryza (MG760454). This nested PCR approach was highly precise for the detection of phytoplasma in immature tissue than matured one [9]. Hence, nested PCR is highly specific than conventional PCR for the detection of phytoplasma [4]. In our study, the highest concentration of phytoplasma was identified in unopened leaves (petiole), root tip than growing point of young tissues. Similarly, Warokka et al. [50] detected and quantified the phytoplasma in plant parts like meristematic tissue including the below growing point, petiole of young leaves, root tips, inflorescence and spear leaves. In addition, quantitative PCR assay has already been successfully used to detect phytoplasma in grapevine [2], chrysanthemum [11], potato [10], apricot prunus [29] and apple [48]. In the present study, the PCR standardization and quantification of phytoplasma causing root (wilt) of coconut was performed in quantitative PCR using TaqMan® probe. The PCR product of 16S rRNA gene of phytoplasma from the diseased coconut palms served as standard template and the quantification procedure was developed. The results of the investigation clearly revealed that TaqMan® probe is the ideal tool for both detection as well as quantification of phytoplasma from coconut palm. The results are in agreement with the earlier findings that TaqMan® probe coupled quantitative PCR targeting any gene of phytoplasma can be carried out successfully without any non-specificity [18].

For assessing the efficiency of quantitative PCR, critical temperature (CT) is the important criteria for the quantification and its direct influence on the target concentration of the phytoplasma. Increased CT values are resulted in lower the concentration of target and vice versa. To evaluate the efficiency of qPCR assay, R^2 (regression coefficient) will be used for the negative correlation. In the present study, the regression coefficient of 0.99 and 0.95 derived from 10-fold diluted known concentration of 16S rRNA gene of phytoplasma as from standard curve, which reveals that low concentrations of target was detected by the efficiency of quantitative PCR using TaqMan® probe. The CT values ranged from 9 to 32 recorded in the present experiment revealed the range of detection limits from 10^2 to 10^9 phytoplasma per µl reaction. It is also suggested that this standard could be developed as a curve to quantify the unknown samples. The high regression coefficient obtained in this experiment was supported by several other workers in different plant species [23]. The standard curve development is very critical in quantifying phytoplasma by qPCR as a small error may result and exaggerated values to predict the phytoplasma concentrations [49]. The accuracy of TaqMan® probe is to have very sharp standard curve due to the probe used for reporting the fluorescence. As and when the amplicon of target hybridize the probe, the reporter emits fluorescence detected as signal, which is absent in other qPCR chemistries like SYBR Green assay [17]. The SYBR Green assay bound the amplicon without any specificity and gave the fluorescence as signal, which may be for non-specific minor amplicons too [17]. This causes the poor resolution of SYBR Green over TaqMan® probe in qPCR assay [33].

In the present investigation, different parts of the infected plants were assessed by qPCR method. As reported by Li et al. [27], it is very important to find out the plant part which was colonized by the pathogen to detect, identify and quantify the phytoplasma by quantitative PCR. Similar investigations in apple and related crops associated with phytoplasma was carried out by Torres et al. [48] and concluded that leaf ribs in case of pear and bud in case of apricot and Japanese plum contained phytoplasma. Maximum

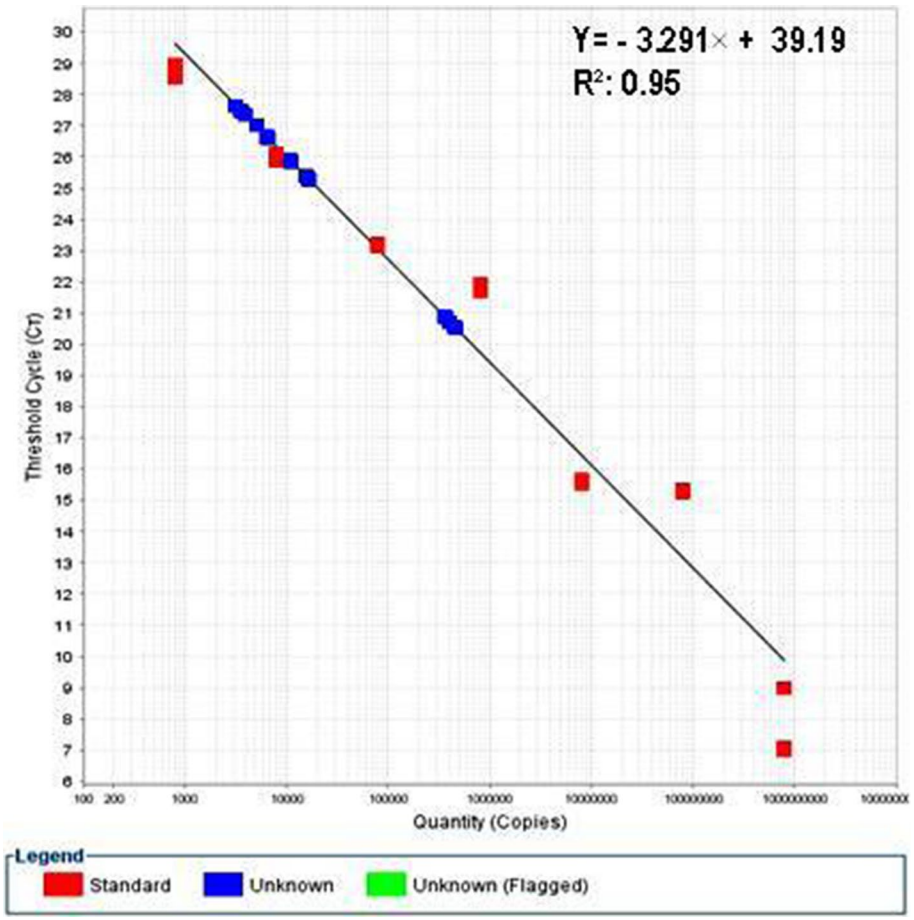

Fig. 6 Standard curve generated during qPCR using TaqMan® probe for copy number against threshold cycle (CT). The standards refer the 10-fold diluted known concentration of 16S rRNA gene of phytoplasma. Samples refer the amplified product from different parts of infected coconut palms

Table 3 Concentration of phytoplasma in different parts of root (wilt) affected palms quantified by TaqMan® probe qPCR

Different parts of coconut palm	CT[a]	Phytoplasma 16S rRNA gene	
		Copy nos./µl	Copies ($\times 10^5$/g of tissue)
Spindle leaflets	34.49 (±0.54)	1025.54 (±150.87)[a]	8.97 (±1.32)[a]
4th petiole leaflets	34.92 (±0.36)	632.83 (±134.34)[e]	5.54 (±1.18)[e]
Roots	34.66 (±0.42)	846.26 (±131.86)[b]	7.40 (±1.15)[b]
Insect vector	34.76 (±0.52)	652.72 (±64.46)[d]	5.71 (±0.57)[d]

Values are mean (±SE) (n = 5) obtained from two runs of qPCR; values in each column followed by same letter are not significant ($p < 0.05$) according to Duncan's Multiple Range Test

Values are mean (±SE) (n = 5) obtained from two runs of qPCR; each run have 3 replications. Values in each column followed by same letter are not significant ($p < 0.05$) according to Duncan's Multiple Range Test

[a]Average threshold cycle (±SE) (n = 3) obtained during TaqMan® qPCR

concentration of phytoplasma per g of tissue with an average CT value of 27.9 (pear), 31.89 (apricot) and 26.64 (Japanes plum) was reported in those plant parts. As the quantitative PCR diagnosis and quantification was used for the first time in phytoplasma causing root (wilt) disease of coconut palm, it is essential to identify the portion of the affected palm colonized in different concentrations by phytoplasma. Further this pathogen is transmitted in nature by insect vectors *viz.*, lace bug, *S. typica* and plant hopper, *P. moesta* as evidenced by experimental transmission studies under controlled conditions [37], different parts of palm including spiral leaflets, 4th petiole leaflets from spindle and roots, hence the insect samples were included for phytoplasma quantification by quantitative PCR. Among the portions investigated, the unopened spindle leaves harboured highest phytoplasma concentration followed by roots and 4th petiole leaflets.

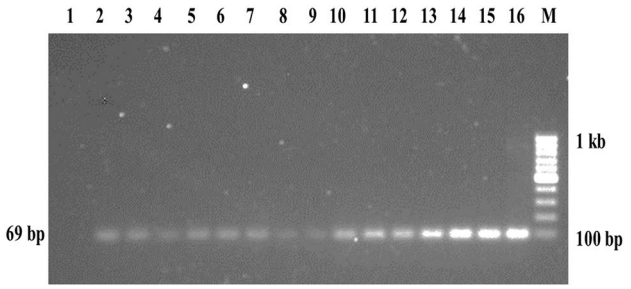

Fig. 7 Post-qPCR agarose gel electrophoresis of 16S rRNA gene amplicons from 10-fold diluted standards and samples from different parts of infected coconut palm. Lane L—100 bp ladder; 1—negative control; 2,3—spindle leaflets; 3, 4—4th petiole leaflets; 5, 6—roots; 7, 8—insect vector; 9 to 16—10-fold diluted standards (7.82 fg/µl–78.2 ng/µl)

This result clearly demonstrated that the phytoplasma colonized the spindle leaves of coconut at first then roots spread through trunk to root and caused the wilt disease. As the disease spread mediated by insects, the quantitative analysis of phytoplasma concentration in plant hopper was also substantially comparable with plant samples which was reported in grapevine yellows [2], coconut yellow decline [36] and other plants [5]. Experimental transmission studies conducted in the past proved that the transmission of the disease through the lace bug, *Stephanitis typica* (Distant) [Heteroptera: Tingidae] in the field [34] and in the insect proof house [44] and *Proutista moesta* by Edwin and Mohankumar [16], Rajan [37], Rajan et al. [38], Solomon [47]. Result of the present study revealed that the sampling, spindle leaves of coconut would be the best target for detecting and assessing the phytoplasma concentrations in diseased palms. To our knowledge this is the first report for the quantification of phytoplasma concentration in coconut palms affected by root (wilt) disease by qPCR conjugated TaqMan® probe method.

Conclusion

Nested and qPCR are the convenient and sensitive method to diagnose the unculturable phytoplasma in infected coconut gardens along with quantification to assess the disease severity. In future, this molecular method could be a quick diagnostic tool to identify the pathogens inoculum load to predict the epidemiology. Further, the present results showed that the Taqman® probe based qPCR could able to identify as well as to quantify the concentration of phytoplasma in different parts of palm. This improved analytical method could be used as a reliable diagnostic procedure to evolve the management of coconut root (wilt) in future.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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