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Evidence of 16SrXI group phytoplasma DNA in embryos of root wilt diseased coconut palms

Ramaswamy Manimekalai · Smita Nair · V. P. Soumya

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Abstract The root wilt disease (RWD) is a major phytoplasma disease of coconut palms in South India. Phytoplasma belonging to the 16SrXI group has been associated with the disease. In our earlier studies we detected phytoplasma from leaves, inflorescence and roots of symptomatic palms in the nested PCR. In this work we examined the embryo from diseased palms for the presence of phytoplasma through semi-nested PCR assay with primers 1F7/7R3 - 1F7/7R2 specific for phytoplasma 16S rRNA gene. Out of 270 embryos collected in four rounds of sampling from two different locations, positive amplification was obtained for 45 (16.67 %) embryos. Sequencing and blastn analysis confirmed the presence of coconut root wilt phytoplasma. To check the potential seed transmission of phytoplasma, mature nuts from diseased palms were germinated in poly bags under disease free conditions and seedlings were sampled for DNA isolation and nested PCR analysis. But phytoplasma DNA could not be detected in any of the seedlings raised in poly bags.

Keywords Nested PCR · Seedlings

Introduction

Coconut palm (*Cocos nucifera* L.), a vital plantation crop in the tropical countries of the world, is rightly known as 'Kalpavriksha' in Sanskrit meaning 'tree which gives all that is necessary for living'. Pests and diseases cause economic loss mainly to farmers in developing countries. The root wilt disease (RWD) of coconut is a non-lethal, debilitating malady gradually reducing the vigor of the affected palms (Solomon et al. 1999). It is a major production constraint in southern India causing an estimated yield loss of 968 million nuts annually. The most consistent and diagnostic symptom of RWD in India is the characteristic bending of the leaflets termed flaccidity, leaf yellowing, necrosis, impaired stomatal regulation and damaged root system. The symptoms are obvious only in palms that are more than 30 months old (Butler 1908). The molecular detection and phylogeny of phytoplasma proved the association of 16SrXI group phytoplasma with coconut RWD (Manimekalai et al. 2010).

Coconut palms across the world are affected by numerous phytoplasmal diseases like lethal yellowing in Jamaica (Myrie et al. 2007); Florida (Harrison et al. 1994); Mexico (Harrison et al. 2002) and Nigeria (Ekpo and Ojomo 1990); yellow decline in Malaysia (Nejat et al. 2009) and Kalimantan wilt in Indonesia (Warokka et al. 2006). Phytoplasmas are cell wall less, unculturable plant pathogens representing a distinct, monophyletic clade within the class mollicutes and classified in to '*Candidatus* Phytoplasma' genus (IRPCM 2004). Phytoplasmas have been detected in most organs of infected plants, where they colonize the sieve tubes of the phloem (Christensen et al. 2005). Oropeza et al. (2011) studied the *in planta* distribution of lethal yellowing phytoplasma in coconut palms and reported that phytoplasma move from photosynthetic source tissue to sink tissue via the phloem mass flow process. They observed higher levels of phytoplasma in the stem, young leaves, inflorescence, stem apex and root apex. They could also observe phytoplasma DNA in 28 % of the 394 embryos studied through nested PCR assay. In earlier works, Nipah et al. (2007a) and Cordova et al. (2003) reported the detection of lethal yellowing phytoplasma in coconut embryo DNA samples. No phytoplasma could be detected in seedlings from diseased palms in any of the above studies. However the presence of phytoplasma in embryos raises concern over possible seed transmission. Calari et al. (2011) reported the molecular detection of phytoplasma in

R. Manimekalai (✉) · S. Nair · V. P. Soumya
Molecular Biology Laboratory, Central Plantation Crops Research Institute, Kasaragod 671 124, Kerala, India
e-mail: rmanimekalaiicar@gmail.com

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seedlings of winter oilseed rape, tomato and corn obtained from seeds of diseased mother plants. In our earlier studies on coconut root wilt phytoplasma in India, the molecular detection using 16S rDNA specific primers revealed the presence of phytoplasma in leaves, inflorescence and roots of diseased palms. The RWD is a non lethal debilitating disease and hence the diseased palms retain the nut production capacity. So with the occurrence of phytoplasma in inflorescence samples there are chances of phytoplasma detection from embryos of diseased palms. However, no work has been conducted so far to study the embryos or seedlings of diseased palms. In this backdrop, the present study was undertaken to examine the embryos from diseased coconut palms for the presence of RWD phytoplasma through molecular detection techniques. The seedlings raised from nuts of diseased palms, grown under disease free conditions, were also studied to get an insight in to the possible vertical transmission of RWD phytoplasma in the host plant.

Materials and methods

Collection of nuts and embryo excision

Mature nuts were harvested from root wilt symptomatic palms in two locations of south India viz., Kayankulam and Theni. Initially the study was carried out with 78 nuts from disease endemic region, Kayankulam. The samples included three coconut cultivars viz., CGD (Chowghat Green Dwarf), WCT (West Coast Tall) and COD (Chowghat Orange Dwarf) varieties. Second round of sampling was done from Kayankulam and 24 CGD, 23 WCT and 24 COD nuts were collected. Samples also included nuts from the same bunch in a single palm. Again, a third round of sampling was done to collect 71 mature nuts from the same palms in Kayamkulam. Additionally, 50 mature nuts were sampled from root wilt symptomatic palms in Theni district of Tamil Nadu.

The nuts were split open and the embryos were scooped out along with the surrounding endosperm portion using a cork borer. The embryos were then removed from the endosperm portion using a scalpel blade. Embryos were surface sterilized with 1 % sodium hypochlorite for 20 min and were then washed 5 times with sterile distilled water prior to DNA extraction.

DNA extraction, PCR, amplicon sequencing and sequence analysis

Total DNA was extracted using Qiagen DNeasy Plant Mini Kit according to the manufacturer's instructions. Phytoplasma 16S rDNA specific semi nested primers 1F7/7R3 - 1F7/7R2 (Manimekalai et al. 2010) were used to amplify the DNA samples through nested PCR assay. Grassy shoot diseased sugarcane sample was used as positive control. PCR assays were performed in 15 μl volume containing 50 ng of DNA template, 0.2 μM of each primer, 150 μM of each dNTPs, 0.5U of Taq DNA polymerase (Bangalore Genei) and 1X PCR buffer with 1.5 mM $MgCl_2$. The first round amplification with primers 1F7/7R3 was performed to 35 cycles in a Veriti flex (Applied Biosystems) thermocycler under the following conditions: initial denaturation at 95 °C for 2 min followed by 35 cycles of 1 min denaturation at 94 °C, 1 min annealing at 60 °C and 1 min and 30 s primer extension at 72 °C followed by a final extension at 72 °C for 10 min. The products of first PCR were diluted 1:4 with sterile water and 2 μl of each dilution then used as template during 35 cycles of PCR with nested primer pair 1F7/7R2. Each run included a no template control (NTC). The PCR products were checked on 1 % agarose gel stained with ethidium bromide and visualized in a gel documentation system (Syngene Bioimaging System). The amplicons of expected size were eluted with Gel Elute Kit (Sigma) and sequenced. The sequences were subjected to similarity search using Blastn.

Field germination of nuts

For field germination studies, mature nuts from healthy and diseased palms were collected and sown in poly bags inside the net house under disease free conditions. Eight nuts from healthy coconut palms were collected from Kasaragod and 29 nuts from diseased palms were collected from Kayankulam. Seed germination was observed. Leaves from germinated seedlings were sampled 4 months after sowing for DNA extraction and PCR analysis.

Results

Phytoplasma detection in embryos

Intact DNA was obtained from all the embryo samples. Out of the 78 embryo DNAs sampled first, nested PCR products of expected size were obtained for 4 samples from Kayankulam. Similar sized amplification product was seen for the positive control while the NTC remained clear. The amplified products were sequenced and sequence analysis using blastn revealed that sequences showed homology with phytoplasma 16S rRNA gene and had 99 % nucleotide identity with root wilt disease phytoplasma 16S rRNA gene.

In the second round sampling, 5 COD, 5 WCT and 1 CGD samples tested positive with primers 1F7/7R3 - 1F7/7R2. In the third round samples, 5 COD, 5 WCT and 1 CGD embryos gave positive amplification (Fig. 1). Samples from palms G_3BP11, G_3BP8, $G_6$32 and $B_3$126, gave consistent positive result in repeated sampling studies. For the 50 embryo samples collected from Theni, 19 tested positive for phytoplasma in the nested PCR assay. Overall, 45 out of the 270 embryos

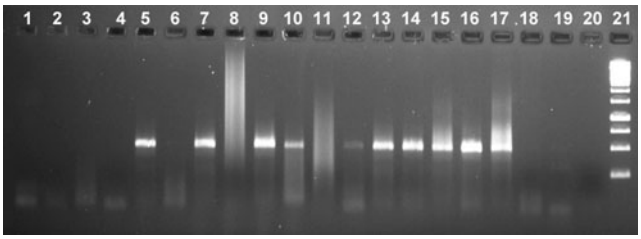

Fig. 1 Nested PCR amplification of embryo DNA samples with primers 1F7/7R3 - 1F7/7R2 (amplicon size - 490 bp). Lanes 1–19 – Embryo samples from diseased palms. Lane 20 – No template control. Lane 21 – 1Kb DNA ladder (band size from bottom – 250 bp, 500 bp, 750 bp, 1, 000 bp etc.)

studied here showed positive for phytoplasma DNA, thus giving 16.67 % positive amplification.

Seed germination and PCR studies on seedling samples

All the 8 healthy nuts in the poly bags germinated 3 months after sowing. Of the 29 nuts from diseased palms sown, only 18 germinated, the germination percentage being 62.1 %. In the PCR amplification studies, none of the seedling samples gave positive amplification with primers 1F7/7R3 - 1F7/7R2.

Discussion

The role of seed transmission in the spread of phytoplasma is a topic of debate as of now. The known mode of phytoplasmal spread is through sap sucking insect vectors and through vegetative propagules. Broadly defined as phloem limited plant pathogens, their movement to embryos is considered unlikely due to lack of vascular connection between developing embryo and plant. But reports are available on the detection of lethal yellowing phytoplasma from embryos of diseased coconut palms (Cordova et al. 2003; Nipah et al. 2007a, b; Oropeza et al. 2011). So far, there are no reports on positive detection of lethal yellowing phytoplasma in seedlings from diseased palm nuts. In our study, we tested 270 embryos from root wilt diseased coconut palms and obtained positive amplification for 45 embryos (16.67 %) in the nested PCR. In a study on lethal yellowing of coconut, Oropeza et al. (2011) reported that 28 % of the 394 embryos from infected palms tested positive for phytoplasma.

In the current study, 11 palms of WCT, 11 palms of COD and 13 palms of CGD varieties of coconut were sampled. Of these, consistent positive results were obtained for embryo samples collected from palms G_3BP11, G_3BP8 (COD), $G_6$32 (CGD) and $B_3$126 (WCT). Moreover, the samples also included nuts from the same bunch of a palm. Of the 8 embryos of G_3BP14 sampled from the same bunch, 3 gave positive amplification. This shows that not all the embryos in a bunch give identical results.

With the presence of phytoplasma DNA in embryos of mature nuts from diseased palms, it is significant to see whether the disease affects seed germination. Eight nuts from healthy and 29 nuts from diseased palms sown in poly bags under disease free conditions were observed. All the 8 nuts from healthy palms germinated in 3 months while only 18 out of the 29 nuts from diseased palms sown in poly bags germinated. Oropeza et al. (2011) reported reduced germination in seeds from lethal yellowing positive symptomatic palms compared to those from negative symptomless ones. In contrast, Nipah et al. (2007a) observed a higher germination for fruits from infected palms compared to fruits from healthy palms in case of Cape St Paul wilt disease. The witches broom disease of lime was found to affect seed germination and the seeds from diseased plants showed reduced germination percentage (Faghihi et al. 2011).

Since the diseased nuts retain the germination capacity, the possible seed transmission of coconut RWD needs to be addressed. We tested the seedlings germinated in poly bags for the presence of phytoplasma using nested PCR assay. But the seedlings showed negative for phytoplasma. However, the number of seedlings raised from disease affected palms was less due to poor germination percentage. In earlier studies on lethal yellowing phytoplasma in coconut, seedlings from nuts of diseased palms tested negative for phytoplasma (Nipah et al. 2007a, b; Oropeza et al. 2011). Faghihi et al. (2011) reported the presence of phytoplasma in the seed coats of lime affected by witches' broom disease, but they could not detect phytoplasmal DNA in the excised embryos, seedlings or plantlets derived from seed of infected trees thus ruling out the possibility of seed transmission. On the contrary, seedlings of winter oilseed rape, tomato and corn obtained from seeds of diseased mother plants showed positive amplification for phytoplasma in the nested PCR. In each case the seedlings carried phytoplasmas belonging to the ribosomal groups retrieved in the infected mother plants (Calari et al. 2011). The probable vertical transmission of phytoplasma in the insect vectors has been reported (Tedeschi et al. 2006 and Kawakita et al. 2000). But studies on the seed transmission of phytoplasma in the host plant are in the preliminary stage. Our report gives a preliminary insight into the occurrence of phytoplasma in the embryos from root wilt diseased palms. The potential seed transmission of coconut root wilt phytoplasma needs to be examined further.

Phytoplasmas are phloem-limited plant pathogens seen in the sieve tubes of diseased plants (Gasparich 2010). Nevertheless, evidence of phytoplasmas in phloem parenchyma and cell types other than phloem sieve elements has been reported previously (Siller et al. 1987). The PCR technique employed in the present work detects only the existence of phytoplasma DNA in the embryos. However it cannot selectively discriminate viable from nonviable phytoplasma (Cordova et al. 2003). There can be a possibility that the phytoplasma DNA

enters the embryos and is somehow protected against nuclease digestion (Nipah et al. 2007a).

Conclusion

The present work undoubtedly proves that the 16SrXI Group phytoplasmal DNA could be detected from embryos of root wilt diseased coconut palms.

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