Molecular detection of phytoplasmas associated with root wilt disease in coconut seedlings

R. Manimekalai*, Soumya V.P., Sathish Kumar R. and G.V. Thomas
Central Plantation Crops Research Institute, Kasaragod 671 124, Kerala, India
*DIT-Agri Bioinformatics Promotion Centre, Kasaragod 671 124, Kerala
E-mail: rmanimekalai@rediffmail.com

Root (wilt) disease is a major production constraint in coconut, causing an estimated loss of 968 million nuts. The most prominent symptom of the disease includes a characteristic bending of leaflets (flaccidity), foliar yellowing and marginal necrosis. (Solomon *et al*, 1999). The symptoms are only obvious in palms that are more than 30 months old (Butler, 1908). The microscopic and serological evidence reveals phytoplasma as a pathogen associated with root (wilt) disease (Sasikala *et al,* 1998).

Phytoplasmas, formerly known as mycoplasma like organisms, are a group of plant pathogenic organism, without a cell wall, and are unculturable prokaryotes. They are associated with diseases in more than 300 plant species worldwide. The inability to culture them has greatly hampered research efforts to understand various aspects of phytoplasmal biology such as pathogenicity, vector relationship, and pathogen-host interactions (Guo *et al*, 2000).

PCR is the most suitable diagnostic tool for the detection of phytoplasma (Namba *et al*, 1993). But, the molecular detection is often hindered by their irregular distribution and low concentration in host plants, especially in woody perennials. Specific primers based on 16S rRNA gene are used for detection of phytoplasma in root (wilt) diseased coconut palms (Manimekalai *et al*, 2010).

The disease is non-lethal but causes severe yield loss. Besides, there is no control measure for preventing the disease. The only way is to use healthy explants for production of new plants. Therefore, the objective of this research was to detect presence of phytoplasma associated with root (wilt) disease in coconut seedlings.

Plant sample collection

Inflorescence, spindle leaf, mature leaf and root tissue samples of 15 symptomatic palms were collected from CPCRI plot from Kayamkulam. The spindle leaf-let samples were collected from twenty seedlings in seed production plot at CPCRI (RS), Kidu.

* Corresponding author

DNA extraction

DNA was extracted from the stalk of tender inflorescence, mid-rib of young leaf, mature leaf and meristem of root tips using phytoplasma enrichment protocol. Three grams of these tissue samples were frozen in liquid nitrogen and ground to a fine powder in a mortar and pestle. To this 0.25 g of polyvinyl polypyrrolidone (PVPP), of molecular weight 40000 was added, mixed well and transferred to a centrifuge tube containing 10 ml phytoplasma grinding buffer (Tris-HCl pH8, 100mM, sucrose 10%, bovine serum albumin.15%, ascorbic acid250mM).The mixture was kept in ice for 15 minutes. Then, the mixture was centrifuged at 5000 rpm for 5 min. at 4°C. The supernatant was transferred to a fresh centrifuge tube and again centrifuged at 13000 rpm for 25 min. at 4°C. The pellet was taken and dissolved in 1 ml SDS buffer (SDS - 2%, Tris-HCl pH8100mM, NaCl1.4 M, EDTA PH8, 20mM). This was kept at 60°C for 1 hr. Equal volume of chloroform isoamyl alcohol (24:1) was added to it and centrifuged at 13000 rpm for 15 min. at 4°C. The aqueous phase was transferred to fresh tubes. Equal volume of isopropanol was added to the tubes containing the aqueous phase. The tubes were incubated for 1 hr at -20°C, then centrifuged at 13000 rpm for 15 min. at 4°C. The supernatant was discarded; the pellet was dissolved completely in 400 microliter of TE buffer (Tris HCl pH-8-10mM, EDTA PH-8-1mM). To that, 1/10 volume of 3M sodium acetate and 2 volumes of ice-cold absolute alcohol was added. The tubes were incubated at -20°C for 2-20 hrs. Then, the tubes were centrifuged at 13000 rpm for 15 min at 4°C. The supernatant was discarded and the pellet was washed in 80% ethanol. The Pellet was then dissolved in 100 microliter of TE buffer, stored at low temperature. The total DNA was estimated at a 260nm (1 OD=50μg/μl). DNA from twenty healthy seedlings collected from Kidu was extracted by using DNAeasy plant minikit (QIAGEN).

Primer designing

The specific primer pair was designed for coconut root wilt phytoplasma based on multiple sequence alignment of different phytoplasma using clustalX. The oligonucleotide sequence corresponding to 16S and 23S region was used for primer designing. Oligomer sequence with minimum degeneracy was selected. The primers were checked for quality (GC percent, annealing temperature, presence of dimers) using the software FASTPCR programme. Many primers were designed and tested for amplification of phytoplasmal DNA. The primer pairs 1F7/7R3, 3fwd and 3rev, were designed for the first PCR and 1F7/7R2, 3fwd and 5rev primers were designed for sensitive and efficient nested PCR. The specific primer amplified coconut phytoplasma and it also amplified sugarcane phytoplasma.

PCR amplification

DNA from different tissues of 15 diseased palms was amplified using direct and nested PCR. The phytoplasma-specific primer pairs 1F7/7R3 and 3fwd/3rev were derived

from 16S rRNA gene sequences. PCR assays were performed in 15 volumes containing 50ng of DNA template, 0.2 μM of each primer, 150μM of each dNTP, 0.5U of Taq DNA polymerase (Bangalore Genei), 1X PCR buffer with 1.5mM $MgCl^2$. First round of amplification with 1F7 /7R3 and 3fwd and 3 rev primers were performed for 35 cycles in a Mycycler (BIO-RAD) thermocycler under the following conditions: 1 min. denaturation at 94°C (initial 2 min. at 95°C for the first cycle), 1min. annealing at 60°C and 72°C for 1 min. and 30 seconds, and followed by a final extension step of 72°C for 10 min. The products of 3fwd/3rev and 1F7/7R3 primed PCR were diluted 1:4 with sterile water and 2μl of each dilution was then used as template during 35 cycles of PCR with nested primer pair 1F7/7R2 and 3fwd/5rev; for the second-round reaction conditions were 95°C for 2 min. followed by 35 cycles of denaturation at 94°C for 1 min. Annealing 60°C for 1 min. 72°C for 1 min. 30 seconds, and followed by a final extension step of 72°C for 10 min.

Positive controls contained DNA from diseased sugarcane and negative controls contained water substituted for test DNA. The final PCR products were analyzed in 1.2 % agarose in 1X TBE buffer (90mM Tris borate, 2mM EDTA pH. 8) containing ethidium bromide. The gels were documented using an image analyzer.

Sequencing

The PCR products of diseased samples were gel-eluted using QIAGEN gel extraction kit. The eluted products were sent for direct sequencing.

The DNA was extracted from different tissues (inflorescence, spindle leaf, root) samples of 15 root (wilt) coconut palms collected from CPCRI, Kayamkulam plot. Polymerase chain reaction of DNA samples was done using the primer pair 1F7/7R3 nested with 1F7/7R2. Out of 15 symptomatic palms, 8 palms showed up as positive for PCR, inflorescence samples from 6 palms were positive for PCR and only 3 root samples showed positive, revealing uneven distribution of phytoplasma (Fig.1).

Initial PCR and semi-nested PCR of DNA from spear leaflets of 20 healthy seedlings [collected from seed production plot of CPCRI (RS), Kidu] using primer pairs 3fwrd/3rev and 3fwd/5rev did not show any amplification (Fig .2). The positive sugarcane sample sample showed amplification at 1250bp. No amplification was seen in water Control.

Amplification of phytoplasma gene sequence from symptomatic palms containing low titers of pathogen is often fraught with difficulty, especially in coconut. Nested PCR assay is performed to overcome problems related to sensitivity of phytoplasma detection.

The presence of phytoplasmas in seedlings has major implications in coconut breeding and distribution of coconut germplasm. The seedlings produced in diseased areas cannot

be planted in disease [free regions a that would cause introduction of phytoplasmas into these clean areas.

Phytoplasmas couldn't be detected in the seedlings collected from seed-raising plot of CPCRI (RS), Kidn. Therefore PCR be used for detection of phytoplasma in planting material of coconuts mainly to avoid use of infected planting material. In future, a molecular kit comprising PCR and QPCR will be routinely used for testing the mother palms used for root (wilt) resistant planting material production.

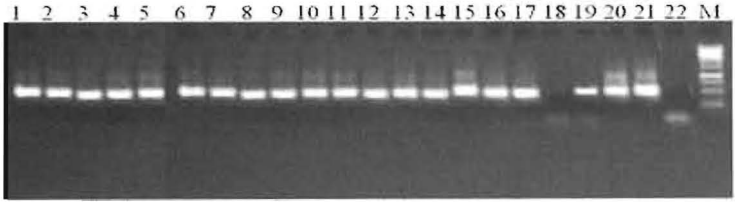

Fig 1. Amplification of root wilt disease phytoplasma using 1F7/7R3, followed by nested PCR 1F7/7R2

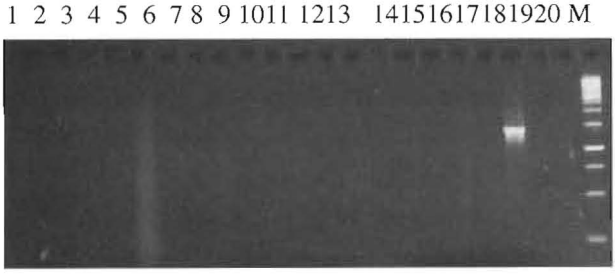

Fig 2. No amplification in seedling samples using 3 Fwd/3 Rev, followed by semi-nested 3 Fwd/5 Rev

Lane 1 to 17

Lane 18 – sugarcane positive samples

Lane 19 – water control

M – 1 Kb ladder

REFERENCES

Guo,Y.H., Cheng, Z. M. and Walla, J.A. 2000. Amplification of the 23S rRNA gene and its application in differtiation and detection of phytoplasmas. *Can .J. pl. Pathol.*,**22**:380-386.

Manimekalai, R., Soumya, V.P., Sathish Kumar, R., Selvarajan, R., Krishna Reddy, M., Sasikala, George V. Thomas, Rajeev, M. and Baranwal, V.K. 2010. Molecular detection of 16Sr XI group phytoplasma associated with root (wilt) disease of coconut (*Cocos nucifera* L.) in India. *Pl. Dis.*, **94**:636.

Namba, S., Kato, S., Iwanami, S., Oyaizu, H., Shiozawa, H. and Tsuchizaki, T. 1993. Detection and differentiation of plant pathogenic mycoplasma like organisms using polymerase chain reaction. *Phytopathol,* **83:768**-791

Sasikala M., Mathen K., Govindankutty M.P., Solomon J.J. Geetha, L 1988. Transmission of a mycoplasma-like organism from *Cocos nucifera* with root (wilt) disease to *Catharanthus roseus* by *Cassytha filiformis. Netherlands J. Pl. Patholo.* **94**:191-194.

Solomon, J,J., Nair, C.P.R., Srinivasan, N., Gunasekaran, M., and Sasikala, M. 1999. Coconut root wilt –The malady and Remedy. *J. Plantation Crops*, **27**:71-92.