Bulletin of Insectology ***60*** *(2): 161-162, 2007*
ISSN 1721-8861

Are phytoplasmas the etiological agent of yellow leaf disease of *Areca catechu* in India?

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Abstract

Phytoplasmas are fastidious, pleomorphic wall less bacteria known to cause diseases of several hundreds of crop plants all over the worldwide and are transmitted by sap-feeding insects. It was reported by conventional disease detection methods, that the etiological agent of yellow leaf disease of areca in India was a phytoplasma. However, these studies did not confirm the presence of this agent conclusively. Hence, the study was initiated to verify the possible presence of phytoplasma in yellow leaf affected areca samples using nested PCR assay. The results suggest no phytoplasma association with this disease of areca palms in examined samples from India.

Key words: *Areca catechu*, nested PCR, yellow leaf disease, phytoplasma, detection.

Introduction

The areca nut palm (*Areca catechu* L.) is one of the most important commercial crops in India. The economic product is the fruit, called areca-nut, which is the most popular chewing substance in south east Asia. It is also used in socio-religious practices, in ayurvedic medicines against leucoderma, leprosy, cough, fits, worms anaemia and obesity. Areca tannins from areca nut are found to have inhibitory activities on reverse transcriptase enzyme (Hattori *et al*., 1993).

Yellow leaf disease (YLD) was first observed as early as in 1914, however, the ambiguity regarding the etiology still prevails. About 25,000 acres of areca garden have been affected by YLD. As the name of the disease indicates, initial symptoms are the yellowing of leaves in the inner whorl, gradually spreads to the outer whorl of the crown. Stem of the affected palms becomes spongy and friable, the conducting strands get destroyed. In advanced stages, the stem breaks off at the top. Rotting of the roots is also observed. Nuts are reduced in size and kernel turns into black (Rawther, 1976).

Phytoplasma involvement as etiological agent of YLD was reported by various workers. Electron microscopic studies revealed the presence of pleomorphic, mycoplasma-like organisms in sieve cells, sieve tube members and companion cells of phloem of YLD affected palms (Nayar and Selsikar, 1978; Selsikar and Wilson, 1981), and was called as ANYLP (Wilson, 2005). In the present study we made an effort to identify the etiological agent by polymerase chain reaction assays.

Materials and methods

The YLD affected leaf samples were collected from YLD affected areca gardens in Karnataka (figure 1A). The healthy samples were also collected from unaffected areca gardens in a different locality. The samples were transported to the laboratory at 4 °C.

Total nucleic acid (DNA) from samples was prepared by following two protocols. In one method, procedures of Zhang *et al*. (1998) with slight modifications were followed. Leaf samples of about 1 g were crushed into fine powder with liquid nitrogen and homogenized with 5 mL of pre-heated extraction buffer and incubated at 65 °C for 20 minutes. During incubation, mixture was vortexed several times. Nucleic acids were extracted with the equal volume of phenol: chloroform. DNA was re-extracted with an equal volume of ice-cold 99.5% isopropanol, and incubated at -20 °C for 1 hour followed by centrifugation at 3,500 rpm for 30 minutes. After rinse the pellet with 80% ethanol, air dry, the nucleic acid was resuspend in 100 µl of nucleic acid free water. In the second method, 2 g of total leaflet was subjected to nucleic acid preparation as described by Prince *et al*. (1993). Total DNA was resuspended with 100 µl of TE buffer.

For the direct PCR, total DNA isolated from samples were used as templates without dilutions and also with dilutions of 1: 10 and 1: 20. Phytoplasma specific universal primer P1/P7 were used. One µL of direct PCR product was diluted to 1: 25 before using as template for nested PCR. In the first set of nested PCR, R16F2n/R2 primers were used as described by Gundersen and Lee (1996). For the further confirmation a second set of nested PCR was carried out using R16mF2/R16mR1 primers as per Khan *et al*. (2006) with the products of P1/P7 PCR. DNA isolated from periwinkle infected with aster yellows phytoplasma (AY) and elm yellows phytoplasma (EY) were used as positive controls and DNA extracted from healthy periwinkle was used as negative control in polymerase chain reactions. Results were analysed in 1% agarose gel in 1 X TAE stained with ethidium bromide.

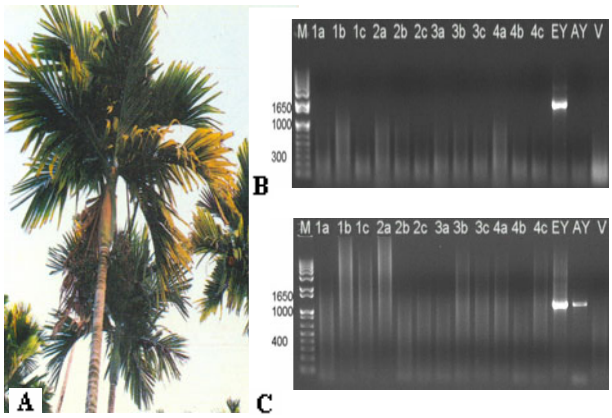

Figure 1. (A): Diseased areca palm showing symptoms (B): 1 % agarose gel showing the direct PCR results. Sample number 1 and 2 were the DNA extracted from diseased palms and sample 3 and 4 from healthy palms. Lane M-DNA marker; in lane 1a, 2a, 3a and 4a 1 µl of total DNA was used as template in PCR without dilution; in lane 1b, 2b, 3b, 4b, total DNA was diluted 1: 10 before using as template in PCR; in lane 1c, 2c, 3c, 4c, total DNA was diluted 1: 20 before using as template in PCR. Lane EY, elm yellows phytoplasma; lane AY, aster yellows phytoplasma, and lane V, DNA from healthy periwinkle. (C): 1 % agarose gel showing the nested PCR result obtained with R16F2n/R16R2 primers. Lanes were marked as in B. (In colour at www.bulletinofinsectology.org).

Results

No amplification was detected in first PCR except for the lane corresponding to the elm yellow phytoplasma infected periwinkle (figure 1B and 1C). In nested PCR with R16F2n/R16R2, all the areca samples and negative control did not show any amplification, where as, both the positive controls, i.e., EY and AY showed amplification. Amplification of AY in second PCR indicates the efficacy of nested PCR. In the second set of nested PCR with R16mF2/ R16mR1 primers, no amplification was visualised except for positive controls (data not shown).

Discussion

Phytoplasmal etiology of YLD of areca nut in India was proved by electron microscopic experiments and also by simple staining techniques (Nampoothiri, 2000). Furthermore a vector of the disease was identified as *Proutista moesta* (Ponnamma *et al*., 1991). This further supports the phytoplasmal hypothesis. But, treatment of palms with tetracycline did not confirm the phytoplasmal etiology. Hence, the present research was initiated to verify the possible association of phytoplasma by PCR with phytoplasma specific universal primers for all the known phytoplasma. Further, we followed two different methods for the isolation of DNA, in order to eliminate the possibilities of inefficiency of DNA preparation with areca samples.

It was reported, yellow leaf disease of areca-nut in Hainan was caused by phytoplasma, and it was detected by PCR using universal primer R16F2/R2 primers. In our experiments with 60 samples of areca (40 infected and 20 healthy), no amplification was observed by nested PCR. At the end, we also tried with universal bacterial primers to confirm the possibility of association of endoparasitic bacteria, but no amplification was detected. This results suggests that the causative agent of YLD in Indian subcontinent may be different from YLD of areca-nut in Hainan.

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