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Reliable improved molecular detection of coconut lethal yellowing phytoplasma and reduction of associated disease through field management strategies

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Abstract

The relentless spread of the fatal disease lethal yellowing throughout the coconut growing areas is having a serious economic impact on many vulnerable communities, and therefore effective management is required. Phytoplasmas from the 16SrIV group are the associated agents. Improved detection methods for this phytoplasma DNA were developed including: a multiplex direct-PCR system, PCR on 16S rRNA and hemolysin genes; a SybrGreen system based on the *GroEL* gene; and real-time PCR using TaqMan probes based on the 16S rDNA and *GroEL* gene. With these methods increased sensitivity and specificity for the detection of this phytoplasma together with quantization capability. These improved methods are part of a package for the integrated management of lethal yellowing spread that is currently tested in coconut farms in Jamaica and show that diseased incidence can be reduced.

Key words: Lethal yellowing, phytoplasmas, management, disease incidence.

Introduction

The relentless spread (Myrie *et al*., 2006) of the fatal disease lethal yellowing (LY) throughout the coconut growing areas is having a serious economic impact on many vulnerable communities. Approximately one million two hundred thousand coconut trees were destroyed in 15 years and therefore effective management of LY spread is required. These improved methods (Black approach) are part of a package for the integrated management of LY spread that include the following strategies: strict weekly surveillance; immediate removal and destruction of LY infected trees; replanting with coconut plants selected for high yielding and LY resistance, proper weed control; and a good fertilization regime. This package is currently being tested in coconut farms in Jamaica, and the results from these tests have shown LY incidence can be reduced.

Phytoplasma from the 16SrIV group is the associated agent (Harrison *et al*., 2002) systemically colonizing phloem tissues and leading ultimate death of coconut palm. It is therefore important to improve detection methods for LY phytoplasma DNA. Real Time and conventional PCRs with primers designed from ramdomly cloned phytoplasma DNA have allowed for phytoplasma detection. It was reported by Dollet *et al.* (2006) that the primer pair P1/P7 was capable of amplifying DNA region in *Bacillus sp* and other bacteria. Therefore new systems of detection are required.

Materials and methods

Seventy (70) tissue samples were collected from coconut palms with and without symptoms of LY disease. In addition, seven samples were collected from coconut trees with different stages of the disease. Total nucleic acids were extracted employing Doyle and Doyle (1990) method. Ten insect [*Haplaxius* (*Myndus*) *crudus*] samples were also collected from coconut fields. Universal ribosomal primer pair P1/P7 (Deng and Hiruki, 1991; Smart *et al*., 1996), LY phytoplasma group specific primer pair LY16Sr/LY16Sf (Harrison *et al*., 2002a) HemR1(5'T'CACGGAGACGACGAAGGATTAG'3) /F1(5'TTCCACCATATTCATCAACAACAA'3), *gro*ELR1(5'CTTTAGGACCAAAAGGTACT'3)/F1 (5'GAAGAACAACAACCACTATC'3) and *gro*ELR2 (5'CGATAATGCTGGAGATGGGACTACT'3)/F2 (5'GAACTACAGCGGCTCCTGTTGTAAT'3) were used in conventional PCRs. Real time PCR systems were used for direct detection of phytoplasma associated lethal yellowing of coconut palms. TaqMan primers (503LY16S-ANYF/503LY16S-ANYR) and probes (503LY16S-ANYM) were designed base on the 16S ribosomal RNA sequences. A SybrGreen system based on the *GroEL* gene were also developed. Comparison was made between the two PCR systems. A multiplex direct-PCR system, simultaneously amplifying two targets of interest (16S rRNA and hemolysin genes) were developed. Four farms (Nutt River Farm, Belvedere, Ecovillage and Spring Garden) were selected for employing the improved method (Black's approach). Three farms (Chiquita, Needham Pen and Fair Prospect) did not used the Black's approach.

Results

Coconut lethal yellowing phytoplasma was detected by conventional PCR using the lethal yellowing group

Table 1. Primers and TaqMan sequences for lethal yellowing phytoplasma used in Real Time PCR.

Name	Orientation	Sequence (5'- 3')	Amplicon length	Location
503LY16S-ANYF	Forward	GCTAAGTCCCCACCATAACGT	180 bp	16S rRNA
503LY16S-ANYR	Reverse	CGTGTCGTGAGATGTTAGGTTAAGT	180 bp	16S rRNA
503LY16S-ANYM	Probe	FAMCCCCTGTCGTTAATTG-NFQ	180 bp	16S rRNA

Table 2. Lethal yellowing deaths at Nutts River Farm.

Years	Months												Total
	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	
2007	19	9	7	9	16	8	13	9	15	4	9	4	122
2008	26	12	18	15	29	26	26	11	11	7	9	6	196
2009	4	4	5	4	5	4	5	4	3	1	5	9	53
2010	2	0	2	1	0	2	0	1	0	0	0	2	10
Total	51	25	32	29	50	40	44	25	29	12	23	21	381

specific primers, Hem primers, *gro*EL primers and in real time PCRs systems using TaqMan probes and primers (table 1). In conventional PCR fewer samples tested positive. Analysis of samples from coconut palms that lost their canopies for 4 months tested positive in real time PCR for LY phytoplasma. Insect samples tested positive for LY phytoplasma in real time PCR using TaqMan probes. SybrGreen system based on the *GroEL* gene have accurately detected LY phytoplasma. Multiplex direct-PCR system, have simultaneously amplified two targets of interest; 16S rRNA and hemolysin genes (figure 1). The four farms that employed the Black's approach saw a significant decline in lethal yellowing disease. The most notable reduction was on the Nutts River Farm in St. Thomas, Jamaica (figure 1). A significant decrease was noted in 2009 and 2010. The month of October had the lowest deaths of coconut trees and the month of January had the highest deaths (table 2). Farms, which had not used the Black's approach experienced a upsurged in the lethal yellowing disease (10,000 coconut palms lost in 6 years).

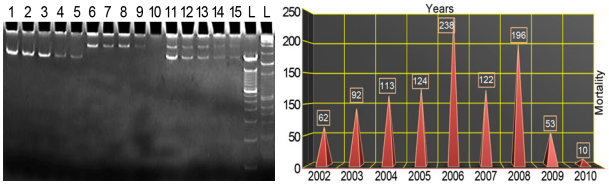

Figure 1. Left: 1-5 diseased samples amplified with P1/P7, 6-10 diseased samples amplified with Hem primers; 11-15 samples simultaneously amplified with P1/P7 and Hem primers in a single PCR. Right: lethal yellowing deaths at Nutts River Farm.

Discussion

Early detection of lethal yellowing diseased trees is an important plank of the Black's approach. Real time PCRs used in this study have shown that it has the capability to detect lethal yellowing disease tree at the onset of the first symptom. Conventional PCR is not as sensitive as real time PCR as samples that exhibit no band or faint band in the conventional PCR had given a strong positive signal when amplified in real time PCR. The amplification of two or more genes simultaneously in the multiplex direct PCR system has contributed to the accurate identification of disease trees. It also reduces the amount of PCRs required for disease confirmation. This has also increase the efficiency the Black's approach. It is clear from the data collected that Black's approach had significantly reduced lethal yellowing disease. Farms that were not employing the Black's approach were devastated by the disease. The insect tested positive for the lethal yellowing phytoplasma does not make them a vector of the disease.

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