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Improving molecular diagnostics for the detection of lethal disease phytoplasma of coconut in Ghana

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Abstract

Accurate and timely detection is important for the control of lethal disease of coconut in Ghana. To improve on the detection of the phytoplasmas involved, multiplex PCR with an in-built internal control and a real-time loop mediated isothermal amplification (LAMP) were used to eliminate false negative results and minimise cross-over contamination. Real-time LAMP provided a fast and reliable means of diagnosis.

Key words: Coconut, LAMP assay, multiplex PCR, Ghana, phytoplasma.

Introduction

Lethal disease (LD) of coconut locally called Cape Saint Paul Wilt disease (CSPWD) is the most important disease of coconut in Ghana and is associated with a phytoplasma. Early and accurate detection of the disease is an important trigger for the initiation of containment measures which includes felling of infected palms. Routine detection of the disease is carried out using PCR with ribosomal and non ribosomal primers such as P1 and P7 (Deng and Hiruki, 1991; Smart *et al*., 1996) and primers based on the *secA* gene (Hodgetts *et al*., 2008).

The loop mediated isothermal amplification (LAMP) technique is fast becoming a popular diagnostic tool for plant pathogens and assays for detecting the CSPWD phytoplasma have been developed (Tomlinson *et al*., 2010). The technique uses *Bst* polymerase which has a strand displacement activity in conjunction with 4-6 specially designed primers that recognise 6-8 regions of the target DNA respectively, thereby making it very specific. Many methods have been developed for detecting LAMP reaction products including real-time methods (Bekele *et al*., 2011). A multiplex PCR involving primers for amplifying both pathogen and plant DNA was used to ascertain the presence of inhibitors in the plant materials and to concurrently test the efficiency of extracting DNA from woody coconut tissues. A real-time LAMP assay that reduces the risk of cross-over contamination was used to amplify and identify the CSPWD phytoplasma in infected samples.

Materials and methods

Coconut trunk borings collected from symptomless and CSPWD infected West African Tall (WAT) ecotype and from symptomless hybrids of the Malayan Yellow Dwarf (MYD) and Vanuatu Tall varieties (VTT) (i.e MYD x VTT) located in diseased fields in the Western region of Ghana were the sources of coconut and phytoplasma DNA used in the study. DNA was extracted with a modified protocol of Daire *et al.* (1997) using CTAB buffer.

PCR was carried out by multiplexing primers for amplifying the *sec A* gene from phytoplasma DNA and primers for amplifying a microsatellite marker CncirF3 from coconut DNA (Lebrun *et al*., 2001). For the *SecA* gene the primers CSPWDSecAFor2 (CGAGATGCA GATCGTTTTG) and CSPWDSecARev2 (CCATCACC AAATTGACGTCC) were used. Since the proportion of pathogen DNA was expected to be significantly lower than that of the plant DNA, the volumes of the pathogen primers used were twice as much as those of the plant primers.

The LAMP primers used are described in Tomlinson *et al.* (2010) and amplicons were detected in real-time following the protocol of Bekele *et al.* (2011). The LAMP products were analysed in terms of the time taken to provide positive result and the melting temperatures of the amplicons (Tm) used to validate the results.

Results

A 380 kb fragment (approximately) from the coconut DNA and a 290 kb amplicon (approximately) from the DNA of CSPWD infected samples were amplified with the plant and pathogen primers, respectively, in the multiplex PCR. Positive results from infected plant samples appeared either as double bands on the gel representing the plant and pathogen fragments or as single bands with sizes corresponding to the expected pathogen fragment size (figure 1). Single bands amplified with the plant primers indicated that the palms were likely to be uninfected. Absence of bands for both plant and pathogen DNA indicated either a lack of DNA or PCR inhibition. LAMP amplicons were observed to begin to form as early as in 12 minutes and results of the LAMP assay were also comparable to those from the PCR analysis (table 1).

Table1. Comparison of PCR and LAMP assays results.

Variety	Sample number	No. Infected*	Positives samples (PCR)	(LAMP)
WAT	98	34	29**	29**
MYD x VTT	10	0	1	1

* Infected palms from field sampling including diseased palms and palms which had died from infection.
** 28 of the samples were the same assays. A sample each from the dead palms was tested by two assays.

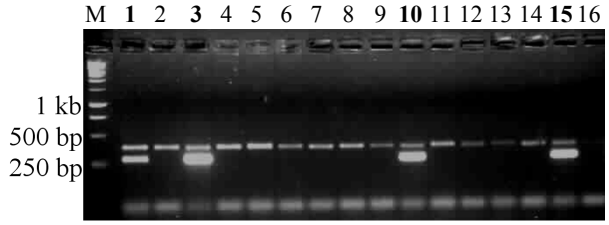

Figure 1. PCR amplification of plant and pathogen DNA. Lanes 1, 3, 10 and 15: Amplification of plant and pathogen DNA. Lanes 2, 4-9, 11-14: Amplification of plant DNA. Lane 16: No template control.

Discussion

Several PCR primers have been developed for amplifying the CSPWD phytoplasma, however, these assays do not have an internal control to guard against false negatives resulting from PCR inhibition or a lack of phytoplasma DNA. Multiplexing primers for amplifying both plant and phytoplasma DNA ensured that samples which gave negative reactions, particularly those from infected palms, were re-extracted to ascertain the effectiveness of the extraction protocol. The results showed that it is difficult to obtain DNA from tissues of palms that had died from the disease as repeated extractions and analyses for most of such samples continually produced negative results.

Real-time LAMP, which, is performed in a closed system and does not require post- amplification manipulations ensures that false positive results arising from cross over contamination are reduced if not eliminated. The technique apart from producing similar results as PCR, has the advantage of detecting amplicons in a relatively short time as compared to PCR. The LAMP technique is currently being trialled for in-field use.

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References

BEKELE B., HODGETTS J., TOMLINSON J., BOONHAM N., NIKOLIC P., SWARBRICK P., DICKINSON M., 2011.- Use of a real-time isothermal assay for detecting 16SrII and XII phytoplasmas in fruit and weeds of the Ethiopian Rift Valley.- *Plant Pathology*, 60: 345-355.

DAIRE X., CLAIRE D., REINERT W., BOUDON E., 1997.- Detection and differentiation of grapevine yellows phytoplasmas belonging to the elm yellows group and to the stolbur subgroup by PCR amplification of non-ribosomal DNA.- *European Journal of Plant Pathology*, 103: 507-514.

DENG S., HIRUKI C., 1991.- Amplification of 16S rRNA genes from culturable and non-culturable mollicutes.- *Journal of Microbiological Methods*, 14: 53-61.

HODGETTS J., BOONHAM N., MUMFORD R., HARRISON N. A., DICKINSON M., 2008.- Phytoplasma phylogenetics based on analysis of *SecA* and 23S rRNA gene sequences for improved resolution of candidate species of '*Candidatus* Phytoplasma'.- *International Journal of Systematic and Evolutionary Microbiology*, 58: 1826-1837.

LEBRUN P., BAUDOUIN L., BOURDEIX R., KONAN J. L., BAKER J. H. A., ALDAM C., HERRAN A., RITTER E., 2001.- Construction of a linkage map of the Rennel Island Tall coconut type (*Cocos nucifera* L.) and QTL analysis for yield characters.- *Genome*, 44: 962-970.

SMART C., SCHNEIDER B., BLOMQUIST C. L., GUERRA L. J., HARRISON N. A., AHRENS U., LORENZ K. H., SEEMÜLLER E., KIRKPATRICK B. C., 1996.- Phytoplasma–specific PCR primers based on sequences of the 16 – 23S rRNA spacer region.- *Applied and Environmental Microbiology,* 62: 2988-2993.

TOMLINSON J. A., BOONHAM N., DICKINSON M., 2010.- Development and evaluation of a one-hour DNA extraction and loop-mediated isothermal amplification assay for rapid detection of phytoplasmas.- *Plant Pathology*, 59: 465-471.

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