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Research Paper

Developing molecular tools capable of identifying, characterizing and distinguishing phytoplasma strains responsible for the emerging lethal yellowing disease (LYD) of coconut in Nigeria

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Abstract. Lethal yellowing disease (LYD) of coconut palms is caused by a wall-less prokaryote in the genus *Ca. Phytoplasma*. Phytoplasmas contained different groups and subgroups. Molecular techniques together with symptoms in host plants, host plants species, host plant variety and geographical location are required for identification and classifying phytoplasmas into various group and subgroups. On this basis, the phytoplasma associated with the LYD of coconut belong to the subgroup in the Coconut Lethal Yellows Group. Phytoplasma-specific PCR assays generally utilize primers specific to the conserved regions of the 16SrRNA gene and to the variable 16S-23S intergenic region. Universal primers and group-specific primers have been developed and are used routinely to detect, characterize and distinguish phytoplasma infections in host plants and insect vectors. However, the unreliable results for LYD detection in samples using previously available strain specific primers has necessitated the need to develop new primers that are efficient and reproducible in detecting LYD, including possible new emerging LYD phytoplasma variants. A database of more than 60 phytoplasma spacer region (SR) sequences now exists, which should facilitate the identification of other phytoplasma clade-specific PCR primers. Hence, the need to explore molecular methods and procedures that will enhance the detection and characterization of the phytoplasma strains responsible for the LYD of coconut in Nigeria.

Keywords: LYD, PCR, phytoplasma, primers, spacer region.

INTRODUCTION

The coconut palm's (*Cocos nucifera* L.) domestication are affected by array of diseases, which include; anthracnose, black scorch, bole rot, bud rot, dry basal rot, Ganoderma butt rot, leaf blight, leaf spot, premature nut fall, powdery mild dew, root rot, thread blight, and lethal yellowing disease (LYD) among others. LYD, the deadliest disease affecting the coconut palm, also affects at least 40 species in other Arecaceous genera throughout the world (Danyo, 2011). Coconut palm has been the main species investigated due to its economic importance in some countries (Ekhorutomwen *et al*., 2016). Various forms of LYD are caused by phytoplasmas-plant parasitic, wall-less, phloem-limited prokaryotes. Their wall-less nature makes it difficult to culture them, limiting the in-vitro studies that can be performed (Eziashi and Omamor, 2010). Studies on

Table 1. Current distribution and range of lethal yellowing-type diseases of coconut palms in Africa.

Location	Disease name	16Sr Subgroup	Host	References
Ghana, Côte d'Ivoire, Nigeria, Togo, Cameroon, Benin	Cape St. Paul wilt, CSPW Keta disease, Kaincopé, Kribi disease or Côte d'Ivoire lethal yellowing disease	XXII-B	*C. nucifera*	Dabek *et al*., 1976; Tymon *et al*., 1998; Makarova *et al*., 2012; Harrison *et al*., 2014; Osagie *et al*., 2015; Arocha-Rosete *et al*., 2015
Mozambique	Coconut lethal yellowing disease (CLYD)	IV-B IV-C XXII-A	*C. nucifera*	Córdova *et al*., 2014; Harrison *et al*., 2014; Bila *et al*., 2015a
Nigeria	Awka wilt disease	XXII-A	*C. nucifera*	Ekpo and Ojomo, 1990; Tymon *et al*., 1998; Wei *et al*., 2007; Osagie *et al*., 2015
Tanzania, Kenya	CLD	IV-C	*P. dactylifera*, *C. nucifera*	Tymon *et al*., 1998; Córdova *et al*., 2014

Source: Adapted from Gurr *et al.* (2016).

Until the end of the 1990s, phytoplasmas associated with "maladiede Kain copé" in Togo, "Awka wilt" in Nigeria and CSPWD in Ghana were thought to fall within the 16SrIV group (Tymon *et al*., 1998). Recently, they were included in a new group, 16SrXXII (Wei *et al*., 2007), incidentally cited as "Ca. P. cocosnigeriae," where particularly the phytoplasma associated with "Awka wilt" was classified as a new subgroup designated to 16SrXXII-A (Tymon *et al*., 1998; IRPCM, 2004; Arocha-Rosete *et al*., 2014, 2015).

phytoplasmas have so far been in-situ, taking advantage of their presence in various hosts to perform various assays including bio-imaging, immunological and molecular procedure. Coconut LYD diagnosis was based uniquely on symptomatology inspection, which is somewhat a complex task because the symptoms differ across varieties of coconut palms, and other biotic (Fusarium wilt) and abiotic (potassium deficiency) stressing factors induce similar symptoms, hindering an accurate and conclusive diagnosis (Monjana *et al*., 2010). Nowadays, molecular based identification has been widely used to quickly detect pathogens, and that of phytoplasmas is not an exception. The wide distribution of the phytoplasma responsible for LYD of coconut and the gross similarities in common symptoms, though, the sequence and detail of symptom progression can vary based on the phytoplasma group, geographical location, host plant species, and variety (Dollet and Fabre, 2006; Harrison *et al*., 2014). It is not surprising then, that the high genetic variability of phytoplasma's, often reflected in their geographic distribution, may hinder the efficacy of a universal diagnostic method based on the amplification of the variable conserved regions, such as a 16S rDNA-based PCR. The unreliable results for LYD detection in samples using previously available strain specific primers for PCR (Mazivele *et al*., 2018) have necessitated the need to develop precise molecular tools capable of identifying, characterizing and distinguishing strains of phytoplasma responsible for the LYD of coconut in Nigeria. (Table 1)

MOLECULAR MARKERS

Molecular markers are often referred to as genetic markers. They are simply defined as simply landmarks on chromosomes that serve as reference points to the location of other genes of interest when a genetic map is constructed (Acquaah, 2007). Molecular markers are often used in molecular biology and biotechnology to identify a particular sequence of DNA in a pool of unknown DNA. The rationale of markers is that an easy-to-observe trait (marker) is tightly linked to a more difficult-to-observe and desirable trait. When a marker is observed or detected, it signals that the trait of interest is present (by association). Genetic markers can be detected at both the morphological level and the molecular or cellular level (Acquaah, 2007).

Classification of molecular markers

Generally molecular markers are classified in various ways, including a genetic basis and an operational basis. There are enzyme-based markers (isozymes) and DNA-based markers (require hybridization between a probe and homologous DNA segment(s) within the genome). PCR-based molecular markers have the advantage of requiring small amounts of DNA and being relatively quick

to assay (Acquaah, 2007). On the basis of genetic characteristics, molecular markers may be grouped into two general categories:

1. Single-locus, multiallelic, codominant markers. Examples are RFLPs (restriction fragment length polymorphisms) and microsatellites (SSRs - simple sequence repeats). Microsatellites are capable of detecting higher levels of polymorphisms than RFLPs (Acquaah, 2007).
2. Multilocus, single-allelic, dominant markers. Examples are AFLPs (amplified fragment length polymorphism) and RAPD (random amplified polymorphic DNA) (Acquaah, 2007).

Furthermore, Semagn *et al.* (2006), listed several molecular markers in alphabetical order as follows; allele specific associated primers (ASAP), allele specific oligo (ASO), allele specific polymerase chain reaction (AS-PCR), amplified fragment length polymorphism (AFLP), anchored microsatellite primed PCR (AMP-PCR), anchored simple sequence repeats (ASSR), arbitrarily primed polymerase chain reaction (AP-PCR), cleaved amplified polymorphic sequence (CAPS), degenerate oligonucleotide primed PCR (DOP-PCR), diversity arrays technology (DArT), DNA amplification fingerprinting (DAF), expressed sequence tags (EST), inter-simple sequence repeat (ISSR), inverse PCR (IPCR), inverse sequence-tagged repeats (ISTR), microsatellite primed PCR (MP-PCR), multiplexed allele-specific diagnostic assay (MASDA), random amplified microsatellite polymorphisms (RAMP), random amplified microsatellites (RAM), random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), selective amplification of microsatellite polymorphic loci (SAMPL), sequence characterized amplified regions (SCAR), sequence specific amplification polymorphisms (S-SAP), sequence tagged microsatelite site (STMS), sequence tagged site (STS), short tandem repeats (STR), simple sequence length polymorphism (SSLP), simple sequence repeats (SSR), single nucleotide polymorphism (SNP), single primer amplification reactions (SPAR), single stranded conformational polymorphism (SSCP), site selected insertion PCR (SSI), strand displacement amplification (SDA), and minisatellite also called variable number of tandem repeats (VNTR). Additionally, there is also a DNA barcoding markers (DBM). Although some of these marker types are very similar (e.g., ASAP, ASO and AS-PCR), some synonymous (e.g., ISSR, RAMP, RAM, SPAR, AMP-PCR, MP-PCR, and ASSR; Reddy *et al.*, 2002), and some identical (e.g., SSLP, STMS, STR and SSR), there are still a wide range of techniques for researchers to choose upon (Semagn *et al.*, 2006). One of the main challenges is, therefore, to associate the purpose(s) of a specific project with the various molecular marker types.
It is important to note that various systems are used to assay molecular markers (Semagn *et al.*, 2006).

DEVELOPMENT OF PHYTOPLASMA STRAIN SPECIFIC PRIMERS

The development of molecular techniques such as PCR and DNA sequencing has increased the specificity, sensitivity and the detection time of phytoplasma from symptomatic and asymptomatic plants and insect vectors (Kirkpatrick *et al.*, 1987; Lee *et al.*, 1993; Webb *et al.*, 1999; Christensen *et al.*, 2004). Phytoplasma-specific PCR assays generally utilize primers specific to conserved regions of the 16SrRNA gene and to the variable 16S-23S intergenic region (Rhode *et al.*, 1993; Davis and Lee, 1993; Lee *et al.*, 1994; Zhang *et al.*, 1998). Furthermore, all phytoplasmas examined to date contain two rRNA operons, which appear to be identical (Schneider and Seemu¨ller, 1994). Since there are fewer evolutionary constraints on this portion of the rRNA operon, there is generally greater variation in the spacer region (SR) sequence than in that of the 16S gene (Barry *et al.*, 1991). Several sequences of the phytoplasma 16S-23S rRNA SRs reveals that the conserved region is highly variable, hence, its use for the development of group-specific phytoplasma PCR primers (Smart *et al.*, 1996). A database of more than 60 phytoplasma SR sequences now exists (Kirkpatrick *et al.*, 1994), which should facilitate the identification of other phytoplasma clade-specific PCR primers.

Universal primers (P1/P6, P1/P7 and F2n/R2; Lee *et al.*, 1991, 1992; Smart *et al.*, 1996) have been developed and are used routinely to detect phytoplasma infections in host plants and insect vectors, while group-specific primers (G813/AKSR primer for Cape Saint Paul Wilt (CSPW) (Tymon *et al.*, 1997); RhodeF/R for coconut lethal disease (CLD) (Rohde *et al.*, 1993); Phyto3F/R, Phyto14F/R primers coconut lethal yellows disease (CLYD) (Mazivele *et al.*, 2018); BF/BR, S1/S2, 15F/15R, 21F/21R for aster yellows phytoplasma (Zhang *et al.* 2004)) have been used to distinguish and characterize phytoplasma strains. For example, Zhang *et al.*, 2004, developed aster yellows strain-specific primers capable of distinguishing strains of phytoplasma (aster yellows witches"-Broom (AY-WB) (16SrI-A), aster yellows severe (AY-S) (16SrI-B), aster yellows bolt white (AY-BW) (16SrI-B), aster yellows bolt distortion no. 2 (AY-BD2) (16SrI-B), and aster yellows bolt distortion no. 3 (AY-BD3) (16SrI-B)) responsible for the aster yellows in lettuce and aster plant; i.e. AY-WB can be distinguished from the other aster yellows phytoplasma strains with the use of primer pair BF/BR, while AY-WB and AY-S can be distinguished from the other strains with the use of primer pair S1/S2. 15F/15R, 21F/21R primers pair are used to distinguish AY-BD2 and AY-BD3 from other strains of aster phytoplasma.

In order to design and develop strain specific primer for the phytoplasma responsible for LYD in Nigeria, one can adopt the procedure of Zhang *et al.* (2004), Dollet Fabre (2006) and Mazivele *et al.* (2018); where P1/P7 universal primers can be used to amplify the ribosomal sequence.

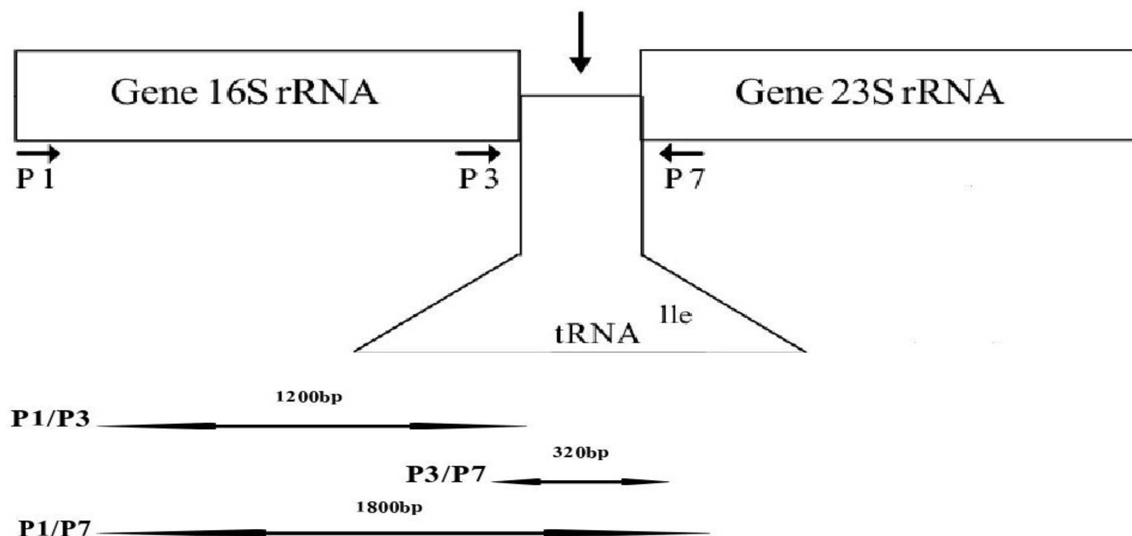

Figure 1. Diagrammatic representation of a phytoplasma rRNA operon including the 16S, the 23S rRNA genes and the intergenic spacer region (SR). The positions of oligonucleotide primers used in PCR analysis are represented as arrows (Modified from Smart *et al*., 1996).

The amplified DNA specific to LYD can then be cut from the gel, purified, cloned and sequenced using appropriate protocol (Figure 1). The design can be based on insertion sequence, genomic DNA or PCR product. Furthermore, one can then align the sequences specific to each strain, and then, specific primers for PCR is constructed to amplify phytoplasma strains responsible for LYD of coconut.

One can also develop multiple primers for multiplex PCR assay by designing phytoplasma species specific primer pairs based on various conserved genes including 16S rRNA, ribosomal protein gene operon (rp), elongation factor TU (tuf), putative preprotein translocase Y (secY), immunodominant protein (idp) and chaperonin protein (groEL) genes to simultaneously detect groups, subgroups and strains of phytoplasmas (Kim *et al*., 2011). To design phytoplasma species-specific primers, all available published DNA sequences data of 16S rRNA genes, groEL genes, idp genes, tuf genes, rp genes and secY genes of the same phytoplasma species can be retrieved from the National Center for Biotechnology Information (NCBI) database, and aligned with available sequences for each phytoplasma species (Table 2). On the basis of all alignment, each gene sequence, the conserved regions of the sequences for specific phytoplasma species can be selected and searched against the NCBI database. Then, the most stable gene for each phytoplasma species can thereafter be selected to design species-specific primers and used for PCR. Each designed primer pair can be tested by PCR analysis with the target DNA template of 'Ca. Phytoplasma' species and the resulted PCR fragment can be sequenced. The designed primers can then be primed with the sequences from groEL, idp, tuf, rp, secY and 16S rRNA genes (Kim *et al*., 2011).

PCR METHODS FOR PHYTOPLASMA DETECTION

The following 3 PCR methods are commonly used for phytoplasma detection and assays;

- Direct PCR
- Nested PCR
- Multiplex PCR

Direct PCR: the direct PCR amplification is a method in which a sample is added directly to an amplification reaction without being subjected to prior DNA extraction, purification, or quantification (Cavanaugh and Bathrick, 2018).

Nested PCR: Nested PCR is a modification of PCR that was designed to improve sensitivity and specificity. Nested PCR involves the use of two primer sets and two successive PCR reactions. The first set of primers is designed to anneal to sequences upstream from the second set of primers and are used in an initial PCR reaction. Amplicons resulting from the first PCR reaction are used as template for a second set of primers and a second amplification step. Sensitivity and specificity of DNA amplification may be significantly enhanced with this technique. However, the potential for carryover contamination of the reaction is typically also increased due to additional manipulation of amplicon products. To minimize carryover, different parts of the process should be physically separated from one another, preferably in entirely separate rooms (Carr *et al*., 2010). Amplicons from nested PCR assays are detected in the same manner as in direct PCR above.

Multiplex PCR: In a multiplex assay, more than one

Table 2. Primers description.

Phytoplasma Primer name	Primer type specificity	Primer sequence (5′- > 3′)	Amplicon size (pb)
Phyto3F/R	Specific for CLYD	GCACGAAAGCGTGGGGAGCA CCCCACCTTCCGGTAGGGAT	763
Phyto14F/R	Specific for CLYD	GATTAACGCTGGCGGCGTGC CGTGGTTTGACGGGCGGTGT	1371
G813/AKSR	Specific for CSPW	CTA AGTGTC GGG GGT TTC C TTG AATAAG AGG AAT GTG G	892
Rhode F/R	Specific for CLY	GAG TACTAA GTG TCG GGG CAA AAA AAC TCG CGT TTC AGC TAC	560
BF/BR	Specific for AY-WB	AGGATGGAACCCTTCAATGTC GGAAGTCGCCTACAAAAATCC	900
S1/S2	Specific for AY-WB, AY-S	CGCTAACAAATGTAAAGGCAAG CTTTAATAGGACTATGAGGG	490
15F/15R	Specific for AY-BD2, AY-BD3	CCCTCAAACCCACGAAGTT TACTGTGTTCCCTTACTCC	390
21F/21R	Specific for AY-BD2, AY-BD3	CCAATCATTTAGATAAAATTGATACC TGTAGTTGAGTTCTATGTAGC	700
F2n/R2	Universal for all phytoplasmas	GAAACGACTGCTAAGACTGG TGACGGGCGGTGTGTACAAACCCCG	1250
P1/P7	Universal for all phytoplasmas	AAG AGTTTG ATC CTG GCT CAGGAT T CGTCCTTCATCGGCTCTT	1800

Note: When primer(s) are developed to amplify specific regions in the genome, and when such primer is not reproducible during PCR procedures, such primer(s) are not considered as marker(s).

target nucleic acid sequence can be amplified by using multiple primer pairs in a reaction mixture. As an extension to the practical use of PCR, this technique has the potential to produce considerable savings in time and effort within the laboratory without compromising on the utility of the experiment. Multiplex PCR is a widespread molecular biology technique for amplification of multiple target sequence in a single PCR experiment. The multiplex PCR is of two types; Single Template PCR and Multiple Template PCR. The Single Template PCR uses a single template which can be genomic DNA along with several pairs of forward and reverse primers to amplify specific regions within a template, while the Multiple Template PCR uses multiple templates and several primer sets in the same reaction tube. Presence of multiple primers may lead to cross hybridization with each other and the possibility of mis-priming with other templates (http://www.premierbiosoft.com/tech_notes/index.html).

Other methods such as; restriction fragment length polymorphism (RFLP) analysis, primed PCR, heteroduplex mobility assay (HMA), loop mediated isothermal amplification (LAMP), DNA sequencing are also used for phytoplasma detection, differentiation and classification.

RFLP analysis uses restriction endonucleases, enzymes that recognize and cleave certain segment of DNA. A limitation of the method is that the presence of a mutation cannot be detected unless that mutation happens to fall within the recognition sequence of the restriction enzyme being used for digestion of the PCR

products (Arens, 1999).

The commonly used primed PCR include; arbitrarily primed polymerase chain reaction (AP-PCR) and Chimeric or triplet repeat primed polymerase chain reaction (RP-PCR). The arbitrarily primed polymerase chain reaction (AP-PCR) is a PCR-based DNA fingerprinting technique using primers whose nucleotide sequence is arbitrarily chosen (Welsh and McClelland, 1990; Williams *et al*., 1990). This method has also been called random amplified polymorphic DNA (RAPD). Chimeric or triplet repeat primed PCR is defined as a PCR method that generates different sized amplicons due to multiple annealing sites on the template.

HMA is a fast and inexpensive method for determining relatedness between DNA sequences (Delwart *et al*., 1993). Heteroduplexes are formed when two non-identical but closely related single-stranded DNA fragments anneal. Such molecules will have structural distortions at mismatched base pairs and at unpaired bases where an insertion or a deletion in the nucleotide sequence has occurred (Upchurch *et al*., 2000). Heteroduplex mobility assay (HMA) is based on the principle that DNA heteroduplexes formed between related sequences have a reduced mobility in polyacrylamide gels which is proportional to their degree of divergence (Wang and Hiruki, 2001). Heteroduplexes migrate more slowly than a homoduplex in polyacrylamide gel electrophoresis. The extent of this retardation has been shown to be proportional to the degree of divergence between the two DNA sequences. The presence of an unpaired base is known to influence the mobility of a heteroduplex more than a mismatched nucleotide (Wang and Griffith, 1991; Upchurch *et al*., 2000). The HMA method has also been used to characterize the variability of plant virus and phytoplasma diseases (Marinho *et al*., 2008).

LAMP technique is becoming increasingly important as a rapid diagnostic tool for phytoplasmas, which is capable of being used to process large numbers of samples cheaply and is reportedly suitable for field use (Fukuta *et al*., 2003; Tomlinson *et al*., 2010; Hodgetts *et al*., 2011; Yankey *et al*., 2011; Keremane *et al*., 2015; Kogovšek *et al*., 2015). One main advantage of LAMP over conventional PCR (and the often nested-PCR protocols) is that the LAMP protocol can be completed in less than an hour on a simple (heatblock) or using more sophisticated equipment (GenieII, Optigene and quantitative PCR platforms) giving a digital display of data (Gurr *et al*., 2016).

DNA sequencing is the process of determining the order of nucleotides (adenine, cytosine, guanine and thymine) in DNA. Several methods have been developed for DNA sequencing; the basic methods (Maxam-Gilbert sequencing, Chain-termination methods), advanced methods (shortgun sequencing, Bridge PCR), and high-throughput methods (Single-molecule real-time sequencing (Pacific Biosciences), Ion semiconductor (Ion Torrent sequencing), Pyrosequencing, Sequencing by synthesis (Illumina), Combinatorial probe anchor systhesis (cPAS-BGI/MGI), Sequencing by ligation (SOLiD sequencing), Nanopore DNA sequencing, Chain termination (Sanger sequencing), Massive parallel signature sequencing (MPSS), Polony sequencing, DNA nanoball sequencing, Heliscope single molecule sequencing, Next generation sequencing (NGS)). There are also several methods currently in development; these includes; Tunnelling currents DNA sequencing, Sequencing by hybridization, Sequencing with mass spectrometry, Microfluidic Sanger sequencing, Microscopy-based techniques, RNAP sequencing, *In vitro* virus high-throughput sequencing (Gilbert, 1980; Pettersson *et al*., 2009). The use of automated DNA sequencers has enhanced DNA sequencing in the laboratory.

PCR PROCEDURES FOR PHYTOPLASMA DETECTION

As phytoplasmas occur in relatively low concentrations in infected palms (Thomas and Norris, 1980), sensitivity of detection is an important feature of any diagnostic assay. Amplification of phytoplasma DNA sequences using the polymerase chain reaction (PCR), which requires minute amounts of target DNA (Saiki *et al*., 1988) is one of the most sensitive techniques for the detection of phytoplasmas in their plant and insect hosts, and for monitoring their distribution and ecology (Dollet and Fabre, 2006). Rapid detection of phytoplasma in plants and vectors has resulted in many research advances, including screening of putative vectors and is now a vital part in the research of phytoplasma-associated diseases (Duduk and Bertaccini, 2011; Eziashi *et al*., 2014, Marcone, 2014). When sampling for phytoplasmas, Harrison *et al*. (1999) found that testing the immature leaves from around the apical meristem, which is rich in phloem, is the most reliable source of phytoplasma detection in palms. However, once palms are symptomatic, PCR testing of the phloem from the palm trunk (drilling a hole of about 10 to 15 cm into the trunk) is a non-destructive method of successful phytoplasma detection (Harrison *et al*., 2002).

TOTAL GENOMIC DNA EXTRACTION FROM PALMS AND INSECT VECTORS

Palms tissues is frozen in liquid nitrogen, or diced and directly ground to a paste with a laboratory mortar and pestle. DNA can thereafter be extracted from a known milligram of the grounded palm tissues using DNeasy plant mini kit or a conventional extraction method in the presence of CTAB buffer. When sampling for phytoplasma in insect vectors, DNA can be extracted from whole insects or insect heads using appropriate DNA conventional extraction protocol or a DNA extraction kit. DNA from insect and plant tissue is diluted in Tris-EDTA (TE; pH 7.4, 10 mM) to 50 ng/µl and used as template for PCR (Elateek, 2010). Note that when using DNA extraction kits, the manufacturer's instructions are strictly followed.

OPTIMIZING SAMPLE SIZE REQUIRED FOR INITIAL SCREENING OF INSECT AND PLANT SAMPLES

According to Elateek (2010), DNA extracts from individual phytoplasma-infected leafhoppers is pooled in groups of eight and used as template for PCR. DNA from eight individual phytoplasma-infected (PI)-leafhoppers and eight individual non-infected leafhoppers reared in the laboratory are extracted. 1µl of DNA from each PI-leafhopper extract is pooled to be used as positive control (8AY) for the experiment (a total of 8 µl) and 1µl of DNA from each non-infected leafhoppers is pooled to be used as negative control (8N) for the experiment (a total of 8 µl). Also, 1µl of DNA from 7 phytoplasma-infected leafhoppers are pooled (7PI) with 1µl of DNA from a non-infected leafhopper (1N). Different ratios of DNA from phytoplasma-infected leafhoppers and non-infected leafhoppers is pooled together (8PI:0N; 7PI:1N; 6PI:2N; 5PI:3N; 4PI:4N; 3PI:5N; 2PI:6N; 1PI:7N; 0PI:8N) to be tested for the optimum sample size to accommodate the design of the DNA extraction kit when an extraction kit is to be used. 1µl aliquot of DNA extract from each pooled sample will be used in PCR for each combination to be tested.

For plant samples, DNA from a known milligram palm tissues from eight individual phytoplasma-infected (PI)-plants and eight individual non-infected plants are extracted. 1µl of DNA from each PI-infected plant is pooled to be used as positive control (8AY) for the experiment (a total of 8µl) and 1µl of DNA from each non-infected plants is pooled to be used as negative control (8N) for the experiment (a total of 8µl). Also, 1µl of DNA from 7 PI-infected plants are pooled together (7AY) with 1µl of DNA from non-infected plants (1N). Different ratios of DNA from PI-infected plants and non-infected aster plants are pooled together (8PI:0N; 7PI:1N; 6PI:2N; 5PI:3N; 4PI:4N; 3PI:5N; 2PI:6N; 1PI:7N; 0PI:8N) to be tested for the optimum sample size. 1µl aliquot of DNA extract from each pooled sample is used in PCR for each combination tested. Therefore, pools of eight DNA extracts from individual leafhoppers or plants can be used to identify positive and negative samples.

PCR REACTION CONDITIONS FOR PHYTOPLASMA DETECTION IN LEAFHOPPER AND PLANT SAMPLES

The following PCR reaction conditions as described by Mazivele *et al*. (2018), can be adopted for phytoplasma detection in leafhopper and plant samples. 2ng of the test sample are added to 15 µl of Qiagen PCR Core kit (Qiagen, Germantown, USA) containing 1X buffer, 0.5X of Qsolution, 0.3mM of dNTP's, 0.3µM of primers, 0.2 units/ml of Taq Polimerase. The PCR reactions are performed in the Eppendorf thermocycler. Optimal parameters for test primer pairs are set: 95°C for 3 min, followed by 94°C for 30 s, 59.3°C for 30 s, 72°C for 55 s, for 40 cycles and 72°C for 10 min. PCR conditions for an optimal annealing temperature can be established for test primer(s) using a temperature gradient-PCR protocol. PCR products (10 µl) are separated by horizontal gel electrophoresis in 1.5% agarose in 1X TTE buffer (Tris-Triton-EDTA; 1M Tris-HCl (pH 8.0), 0.25 M EDTA, Triton X-100) for 20 min at 230 V 50 Hz. Gels are stained in dilute GelRed™ (6X in water), and DNA visualized under UV light and photographed using the Kodak Electrophoresis Documentation and Analysis System (EDAS) 290 (Eastman Kodak Company, New Haven, CT). PCR assays can also be carried out in a PTC-200 thermocycler with a gradient alpha block (MJ Research Inc., Waltham, MA).

CONCLUSION

LYD is a serious disease threatening the coconut palm existence. Phytoplasma strains distribution within and between location of the coconut plantations needs molecular techniques for their easy and rapid detection. Till date except for universal primers, no strain-specific primers have been developed for the detection, characterization and distinguishing strains of phytoplasma responsible for LYD of coconut in Nigeria. However, the unreliable results for LYD detection in samples using previously available strain specific primers has necessitated the need to develop new primers that are efficient and reproducible in detecting LYD, including possible new emerging LYD phytoplasma variants in Nigeria. Hence, the need to develop strain-specific primers for detecting, characterizing, and distinguishing strains of phytoplasma responsible for LYD of coconut in Nigeria. Furthermore, molecular techniques are used as a diagnostic tool to identify phytoplasmas in a group-specific manner, which is necessary for epidemiological studies of the LYD, analysis of vector hosts, and formulation of disease control strategies. The ability to detect specific groups of phytoplasmas would also benefit plant importation and quarantine agencies.

REFERENCES

Acquaah G (2007). 'Molecular markers'. In: Principles of plant genetics and breeding. Blackwell Publishing Limited, USA. pp. 247-249.

Arens M (1999). Methods for subtyping and molecular comparison of human viral genomes. Clin. Microbiol. Rev. 12:612-626.

Arocha-Rosete Y, Konan-Konan JL, Diallo AH, Allou K, Scott J (2015). Analyses based on the *16S rRNA* and *secA* genes identify a new phytoplasma subgroup associated with a lethal yellowing-type disease of coconut in Côte d'Ivoire. Phytopathol. Mol. 5:S57.

Arocha-Rosete, Y, Konan Konan JL, Diallo AH, Allou K, Scott JA (2014). Identification and molecular characterization of the phytoplasma associated with a lethal yellowing-type disease of coconut in Côted'Ivoire. Can. J. Plant Pathol. 36:141-150.

Barry T, Colleran G, Glennon M, Dunican LK, Gannon F (1991). The 16s/23s ribosomal spacer region as a target for DNA probes to identify eubacteria. PCR Methods Appl. 1:51-56.

Bila J, Mondjana A, Samils B, Högberg N (2015). High diversity, expanding populations and purifying selection in phytoplasmas causing coconut lethal yellowing in Mozambique. Plant Pathol. 64:597-604.

Carr J, Williams GD, Hayden TR (2010). Molecular diagnostics. In: Techniques and application for the clinical laboratory. pp. 289-300.

Cavanaugh SE, Bathrick AS (2018). Direct PCR amplification of forensic touch and other challenging DNA samples: A review. Forensic Sci. Genet. 32:40-49.

Córdova I, Oropeza C, Puch-Hau C, Harrison N, Collí-Rodríguez A, Narvaez M (2014). A real-time PCR assay for detection of coconut lethal yellowing phytoplasmas of group 16SrIV subgroups A, D and E found in the Americas. J. Plant Pathol. 96:343-352.

Christensen NM, Nicolaisen M, Hansen M, Schulz A (2004). Distribution of phytoplasmas in infected plants as revealed by real-time PCR and bio imaging. Mol. Plant-Microbe Int. 17:1175-84.

Dabek AJ, Johnson CG, Harries HC (1976). Mycoplasma-like organisms associated with Kaincopé and Cape St. Paul Wilt Diseases of coconut palms in West Africa. *PANS*, 22:354-358.

Danyo G (2011). Review of scientific research into Cape St. Paul Wilt Disease (CSPWD) of coconut in Ghana. Afr. J. Agric. Res. 6(45):67-4578.

Davis RE, Lee IM (1993). Cluster-specific polymerase chain reaction amplification of 16SrDNA sequences for detection and identification of mycoplasmalike organisms. Phytopath. 83:1008-11.

Delwart EL, Shpaer EG, Mccutchan FE, Louwagie J, Grez M, Rübsamen-Waigmann H, Mullins JI (1993). Genetic relationships determined by a DNA heteroduplex mobility assay: analysis of HIV-1 *env* genes. Science 262:1257-1261.

Dollet M, Fabre S (2006). Variability and diagnosis of Coconut Lethal Yellowing Syndromes in Africa. 16th International Congress of the International Organization for Mycoplasmology (IOM), Cambridge, United Kingdom, 9-14.

Duduk B, Bertaccini A (2011). Phytoplasma classification: taxonomy based on 16Sribosomal gene, is it enough? Phytopathog. Mol. 1:3-13.

Ekhorutomwen OE, Eziashi EI, Adeh SA, Omoregie KO, Okere CI (2016). Insight on the possibility to control lethal yellowing disease (LYD) in coconut palms using CRISPR/Cas9 system and bio-control (entomopathogens) among other measures. Int. J. Agric. Earth Sci. 2(2):2489-0081.

Ekpo E, Ojomo E (1990). The spread of lethal coconut diseases in West Africa: incidence of Awka disease (or bronze leaf wilt) in the Ishan area of Bendel state of Nigeria. Principles, 34:143-146.

Elateek S (2010). Molecular and biochemical genetic studies on some leafhopper transmitted plant pathogens. PhD **Dissertation. p. 128.**

Eziashi EI, Omamor IB (2010). Lethal yellowing disease of the coconut palms (*cocos nucifera* l.): An overview of the crises. Afr. J. Biotechnol. 9(54):9122-9127.

Eziashi EI, Omamor IB, Aisueni NO, Aisagbonhi CI, Airede CE, Ikuenobe CE, Oruade-Dimaro EA, Odewale JO, Osagie IJ (2014). Screening Potentials Weed Species as Alternate Hosts of Insect Vectors of the Lethal Yellowing Disease (LYD) of Coconut Palms (*Cocos nucifera* L.) in Nigeria. Brit. J. Appl. Sci. Technol. 3(1):123-130.

Fukuta S, Kato S, Yoshida K, Mizukami Y, Ishida A, Ueda J (2003). Detection of tomato yellow leaf curl virus by loop-mediated isothermal amplification reaction. J. Virol. Methods. 112:35-40.

Gilbert W (1980). DNA sequencing and gene structure. Nobel lecture.

Gurr GM, Johnson AC, Ash GJ, Wilson, BAL, Ero MM, Pilotti CA, Dewhurst CF, You MS (2016). Coconut Lethal Yellowing Diseases: A Phytoplasma threat to palms of global economic and social significance. Front. Plant Sci. 7:1521.

Harrison NA, Cordova I, Richardson P, Dibonito R (1999). “Detection and diagnosis of lethal yellowing,” eds C. Oropeza, J. L., Verdeil, G. R., Ashburner, R. Cardeña, F. and J. M. Santamaría (Dordrecht:Springer), in Current Adv. Coconut Biotechnol. 35:183-196.

Harrison NA, Womack M, Carpio ML (2002). Detection and characterization of a lethal yellowing (16SrIV) group phytoplasma in Canary Island date palms affected by lethal decline in Texas. Plant Dis. 86:676-681.

Harrison NA, Davis RE, Oropeza C, Helmick EE, Narváez M, Eden-Green S (2014). ‘*Candidatus* Phytoplasma palmicola’, associated with a lethal yellowing-type disease of coconut (*Cocos nucifera* L.) in Mozambique. Int. J. Syst. Evol. Microbiol. 64:1890-1899.

Hodgetts J, Tomlinson J, Boonham N, González-Martín I, Nikoli´c, P, Swarbrick P (2011). Development of rapid in-field loop-mediated isothermal amplification (LAMP) assays for phytoplasmas. Bull. Insectol., 64:S41-S42.

IRPCM (2004). ‘*Candidatus* Phytoplasma’, a taxon for the wall-less, non-helical prokaryotes that colonize plant phloem and insects. Int. J. Syst. Evol. Microbiol. 54:1243-1255.

Keremane ML, Ramadugu C, Rodriguez E, Kubota R, Shibata S, Hall DG (2015). A rapid field detection system for citrus huanglongbing associated *‘Candidatus* Liberibacter asiaticus’ from the psyllid vector, *Diaphorina citri* Kuwayama and its implications in disease management. Crop Protect. 68:41-48.

Kim YH, Win NK, Back CG, Yea MC, Yim KO, Jung HY (2011). Multiplex PCR assay for simultaneous detection of Korean quarantine phytoplasmas. Plant Pathol. J. 27(4):367-371.

Kirkpatrick BC, Stenger DC, Morris TJ, Purcell AH (1987). Cloning and detection of DNA from a non-culturable plant pathogenic mycoplasma-like organism. Science, 238:197-200.

Kirkpatrick BC, Smart CD, Gardner SL, Gao JL, Ahrens U, Ma¨urer R, Schneider B, Lorenz KH, Seemu¨ller E, Harrison NA, Namba S. Daire X (1994). Phylogenetic relationships of plant pathogenic MLOs established by 16/23S rDNA spacer sequences. IOM Lett. 3:228-229.

Kogovšek P, Hodgetts J, Hall J, Prezelj N, Nikoli´c P, Mehle N. (2015). LAMP assay and rapid sample preparation method for on-site detection of flavescence dorée phytoplasma in grapevine. Plant Pathol. 64:286-296.

Lee IM, Davis RE, Hiruki C (1991). Genetic relatedness among clover proliferation mycoplasma like organisms (MLOs) and other MLOs investigated by nucleic acid hybridization and restriction fragment length polymorphism analyses. Appl. Environ. Micro. 57:3565-69.

Lee M, Gundersen DE, Davis RE, Chiykowski LN (1992). Identification and Analysis of a Genomic Strain Cluster of Mycoplasmalike Organisms Associated with Canadian Peach (Eastern) X Disease, Western X Disease, and Clover Yellow Edge. J. Bacteriol. 174:6694-6698.

Lee IM, Gundersen DE, Hammond RW, Davis RE (1994). Use of mycoplasmalike organisms (MLO) group-specific oligonucleotide primers for nested PCR assays to detect mixed- MLO infections in a single host plant. Phytopath. 84:559-566.

Lee IM, Hammond RW, Davis RE, Gundersen DE (1993). Universal amplification and analysis of pathogen 16S rDNA for classification and identification of mycoplasmlike organisms. Phytopathology 83:834-842.

Makarova O, Contaldo N, Paltrinieri S, Kawube G, Bertaccini A, Nicolaisen M (2012). DNA barcoding for identification of *‘Candidatus* Phytoplasmas’ using a fragment of the elongation factor Tu gene. *PLoS ONE*, 7:e52092.

Marcone C (2014). Molecular biology and pathogenicity of phytoplasmas. Ann. Appl. Biol. 165:199-221.

Marinho VLA, Fabre S, Dollet M (2008). Genetic variability among isolates of *Coconut lethal yellowing phytoplasmas* determined by Heteroduplex Mobility Assay (HMA). Trop. Plant Pathol. 33(5):377-380.

Mazivele MOM, Nuaila V, Colombo MM, Taviani E (2018). Promising primers for detection of phytoplasma causing coconut lethal yellowing disease in Mozambique. Phytoparasitica, 46:301-308

Monjana A, Santos L, Vaz A, Caniço MS, Nuaila V, Massingue F, Massinga J (2010). Manual Prático para Identificação e Maneio do Amarelecimento letal do coqueiro. MTF/MOZ/098/STF (STDF 230)-FAO.

Osagie IJ, Ojomo EE, Pilet F (2015). Occurrence of Awka wilt disease of coconut in Nigeria for one century. Phytopathog. Mol. 5:S61.

Pettersson E, Lundeberg J, Ahmadian A (2009). “Generations of sequencing technologies”. Genomics, 93(3):105-11.

Reddy MP, Sarla N, Siddiq EA (2002). Inter simple sequence repeat (ISSR) polymorphism and its application in plant breeding. *Euphytica*, 128:9-17.

Rhode W, Kullaya A, Mpunami A, Becker D (1993). Rapid and sensitive diagnosis of mycoplasmalike organisms associated with lethal disease of coconut palm by a specifically primed polymerase chain reaction for the amplification of 16SrDNA. *Oleagineux*, 48:319-322.

Saiki RK, Gelfand DH, Stoffell S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Elrich HA (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science, 239:487391.
Semagn K, Bjørnstad Å, Ndjiondjop MN (2006). An overview of molecular marker methods for plants. Afr. J. Biotechnol. 5(25):2540-2568.
Schneider B, Seemu¨ller E (1994). Presence of two sets of ribosomal genes in phytopathogenic mollicutes. Appl. Environ. Microbiol. 60:3409-3412.
Smart CD, Schneider B, Blomquist L, Guerra J, Harrison NA, Ahrens U, Lorenz KH, Seemüller E, Kirkpatrick BC (1996). Phytoplasma-specific PCR primers based on sequences of the I6S-23S rRNA spacer region. App. Environ. Microbiol. 62: 2988-2993.
Tymon AM, Jones P, Harrison NA (1998). Phylogenetic relationships of coconut phytoplasmas and the development of specific oligonucleotide PCR primers. Ann. Appl. Biol. 132:437-452.
Tymon, AM, Jones P, Harrison NA (1997). Detection and differentiation of African coconut phytoplasmas: RFLP analysis of PCR-amplified 16S rDNA and DNA hybridization. Ann. Appl. Biol. 131: 91-102.
Thomas DL, Norris RC (1980). The use of electron microscopy for lethal yellowing diagnosis. Proceedings of the Floridu State Horticultural *SocieQ*. 93:196-199.
Tomlinson JA, 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 Pathol. 59:465-471.
Upchurch DA, Shankarappa R, Mullins JI (2000). Position and degree of mismatches and the mobility of DNA heteroduplexes. Nucleic Acids Res. 28(12):E69-e69.
Wang YH, Griffith J (1991). Effects of bulge composition and flanking sequence on the kinking of DNA by bulged bases. Biochemistry, 30:1358-1363.
Wang K, Hiruki, C (2001). Use of heteroduplex mobility assay for identification and differentiation of phytoplasmas in the aster yellows group and the clover proliferation group. Phytopathology, 91:546-552.
Webb DR, Bonfiglioli RG, Carraro L, Osler R, Symons RH (1999). Oligonucleotides as hybridization probes to localize phytoplasmas in host plants and insect vectors. Phytopath. 89:894-901.
Wei W, Davis RE, Lee IM, Zhao Y (2007). Computer-simulated RFLP analysis of 16SrRNA genes: identification of ten new phytoplasma groups. Int. J. Syst. Evol. Microbiol. 57:1855-1867.
Welsh J, McClelland M (1990). Fingerprinting genome using PCR with arbitrary primers. Nucleic Acid Res. 18:7213-7218.
Williams JG, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acid Res. 18(22):6531-5.
Yankey EN, Swarbrick D, Sickinson M, Tomlinson J, Boonham N, Nipah JO (2011). Improving molecular diagnostics for the detection of lethal disease phytoplasma of coconut in Ghana. Bull. Insectol. 64:S47-S48.
Zhang Y, Uyemoto JK, Kirkpatrick BC (1998). A small-scale procedure for extracting nucleic acids from woody plants infected with various phytopathogens for PCR assay. J. Virol. Method, 71:45-50.
Zhang J, Hogenhout SA, Nault LR, Hoy CW, Miller SA (2004). Molecular and symptom analyses of phytoplasma strains from lettuce reveal a diverse population. Phytopathol. 94:842-49.

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