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1 **DETECTION OF *FUSARIUM OXYSPORUM* F. SP. ELAEIDIS CAUSING FUSARIUM WILT OF OIL**
2 **PALM USING LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP)**

3 *Kwasi Adusei-Fosu*
4 *[1]Scion, Forest Protection, 49 Sala Street.*
5 *3010 Rotorua New Zealand.*
6 *+6473435559*
7 *Kwasi.adusei-fosu@scionresearch.com*
8
9 *[2]Matthew Dickinson*
10 *University of Nottingham-United Kingdom.*
11 *School of Biosciences.*
12 *Plant Science Division.*
13 *LE12 5RD.*

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29 **Abstract**

30 *Fusarium oxysporum* f. sp. *elaeidis* (FOE) a pathogen that causes fusarium wilt disease in oil palm can be detected
31 using polymerase chain reaction (PCR) but very time consuming. Loop-Mediated Isothermal Amplification (LAMP)
32 was used to rapidly detect *Fusarium oxysporum* f. sp. *elaeidis* (FOE) in oil palm seedlings. Eight additional *Fusarium*
33 *oxysporum* isolates collected from symptomatic oil palm trees (i.e. presumed-FOE as their pathogenicity was not
34 confirmed) and five other non-FOE isolates were sampled from symptomatic mature oil palm trees and tomato
35 respectively to broaden the scope of the research. The identities of FOE, presumed-FOE and non-FOE were
36 established via sequencing. LAMP primers designed for detecting FOE or presumed-FOE were based on partial
37 sequences of *Secreted In Xylem* (*SIX8*) and *P-450* cytochrome. The earliest detection time for *SIX8* and *P-450*
38 cytochrome primers were 4:00 mins and 6:45 mins respectively with both recording late time for detection at 26:30
39 mins. Annealing derivative curves were used for assessing the level of specificity for both *SIX8* and *P-450* cytochrome,
40 but none of the LAMP primers could distinguish between FOE, presumed-FOE and non-FOE.

41 **KEY words: *Fusarium oxysporum* f. sp. *elaeidis, LAMP, Secreted In Xylem gene, P450 Cytochrome-oxidase,***
42 ***Diagnostics***

43 **Introduction**

44 The use of the LAMP could aid on-site detection of diseased plants [1] which drastically reduces the quantity of
45 samples to be transported to the laboratory. This marked the beginning of several attempts made to develop real-time
46 PCR equipment for use in the field [12, 22, 26].

47

48 LAMP is a rapid amplification method employing a strand displacing *Bst* DNA polymerase and 4 – 6 primers, two of
49 which are 'fold back' primers [14, 19] which form stem-loop motifs with self-priming capability (Fig. 1.0). The
50 primers used are two sets, the internal primers and external primers. Subsequent studies have found the use of
51 additional 'loop primers', which bind to the loop structures and greatly reduce the reaction times [14], resulting in a
52 total of 6 primers. The 60 – 65 ^{o}C reaction temperature combined with a minimum of four primers makes LAMP a
53 highly specific reaction. The high level of specificity results from the requirement for primers to bind up to eight
54 regions of the target sequence. This results in an amplification scheme where the priming sequence is copied with
55 each round of replication and remains tethered to the previous amplicon resulting in a concatenated product of
56 alternating sense / anti-sense repeats of varied length. This results in large amounts of amplicons which can be used
57 for further studies in detection [5].

58

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59 LAMP is one of the most well established methods for isothermal amplification of nucleic acids to date. The technique
60 has been used as a molecular tool for the detection of several plant pathogens over recent years [7, 16, 26] including
61 fungi [11, 18, 24]. There are several reports on LAMP for detecting *Fusarium* spp. [4, 6, 8, 17, 18]. LAMP assay
62 could detect and differentiate *F. oxysporum* f. sp. *lycopersici* (*Fol*) race 1 isolates based on the *SIX4* and *SIX5* genes
63 using three primer sets [4]. The usefulness of the analysis of fungal cultures by direct analysis of surface scrapings
64 from agar plate cultures, direct testing of single infected barley grains, and detection of *Fg* in total genomic DNA
65 isolated from bulk samples of ground wheat grains has been demonstrated [18]. LAMP has been used to successfully
66 quantify genomic DNA of *F. oxysporum* f. sp. *cubense* (*Foc*-TR4) in soil samples [29]. The sensitivity of the LAMP
67 has also been reported [3]. Even though PCR and LAMP assays would successfully detect positive infected samples
68 of tomato with *Fol*, considering the time, safety, cost and simplicity, the latter technique was overall superior [4]. In
69 addition, the real-time application with the Optigene-system (Optigene, UK) has several advantages such as easy
70 mobility or portability of the detection device. This makes it portable for field work. Unfortunately no report has been
71 published on LAMP tool/assay to detect isolates of *Fusarium oxysporum* f. sp. *elaeidis* (FOE). Hence, there was the
72 need to develop LAMP primers to detect FOE in inoculated oil palm seedlings and *Fusarium oxysporum* isolates
73 collected from symptomatic mature oil palm trees (i.e. presumed-FOE as their pathogenicity had not been tested
74 although they were collected from symptomatic oil palm in the field) to enable faster screening of oil palm seedlings
75 prior to transplanting them to field to ensure disease free oil palm seedlings were planted.
76
77 ***Materials and methods***
78 *DNA extraction, PCR and DNA sequencing*
79 A total of 40 strains were used including eight *Fusarium oxysporum* isolates (presumed-FOE) collected from
80 symptomatic oil palm mature trees (*Elaeis guineensis*), four FOE (BOP-B5, NORP-N5, OPRI-5 and 16F) isolates
81 confirmed to be pathogenic against oil palm seedlings and the remaining non-FOE isolates collected from tomato.
82 The identities of all the isolates were confirmed via sequencing. Mycelia (50 - 100 mg) from cultured FOE, presumed-
83 FOE and non-FOE were isolated from PDA plates using a sterile surgical blade. Tissue disruption was carried out
84 using glass beads and homogenizer (FastPrep®) at a speed of 6.5 ms^{-1} for a total time of 45 s in the presence of liquid
85 nitrogen. DNA extractions of the cultures were then carried out using DNeasy Plant Mini Kit (Qiagen) according to
86 the manufacturer's protocol. Polymerase chain reaction (PCR) of various regions of the template DNA was performed
87 using primer pairs of interest (Table. S1). PCR was carried out in 30 µl volumes consisting of 15 µl of master mix
88 (MangoTaq™ DNA Polymerase), 1 µl (of 10 pmol / ul) each of all primer pairs mentioned in separate reaction
89 mixtures, 12 µl sterile distilled water and 1 µl of template DNA of the isolates of interest. The reaction was performed

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90 in a BIO-RAD S1000 Thermal Cycler with the amplification conditions of 95 °C for 2 min for initial denaturation,
91 followed by 35 cycles of denaturation at 95 °C for 2 min, annealing at temperatures suitable for amplification for each
92 primer pair of interest and extension/elongation at 72 °C for 1 min 30 sec. The final extension was set at 72 °C for 5
93 min. PCR products were cleaned using the QIAquick PCR Cleanup kit (Qiagen) following manufacturer's instruction
94 followed gel electrophoresis using 1 kb ladder (Promega). Sequencing reactions were performed by Fisher Scientific
95 or MyGATC. BLAST searches were performed using the GenBank sequence database to confirm the identity of the
96 fungal isolates sequences based on the *Secreted In Xylem* (*SIX*) gene and *P450 cytochrome oxidase* used for amplifying
97 the rDNA. The output from BLAST algorithms was used to query any unknown sequences against the database of all
98 the fungal gene regions. These sequences were subsequently used to design LAMP primers.
99
100
101 ***Loop-Mediated Isothermal Amplification* (*LAMP*) *primer design***
102 *Loop Mediated Isothermal Amplification* (*LAMP*) *assay*
103 The LAMP primers (Table S1) were self-designed from partial sequences based *Secreted In Xylem* (*SIX8*, *SIX10* and
104 *SIX13*) gene and *P450 cytochrome oxidase. Fusarium oxysporum* f. sp. *elaeidis* (FOE) detection assay was done by
105 preparing the LAMP primer mix which consisted of 152 µl sterile distilled water with primer concentration of 10 µM
106 each of the F1, B1, F2, B2 primers and 2 µM each of FIP, BIP primers. Master mix for eight reactions was prepared
107 which consisted of 23 µl of LAMP primer mix, 46 µl sterile distilled water and 115 µl Optigene master mix (dNTPs,
108 *Bst* DNA polymerase and $MgCl_2$). A final volume of 21 µl in each of the eight LAMP tubes consisted of 20 µl of the
109 reaction mix dispensed into each of the LAMP tubes and 1 µl genomic DNA. The detection time was set to 30 min
110 for all reactions for each primer set.
111
112 ***Results for Loop-Mediated Isothermal Amplification* (*LAMP*)**
113 LAMP assays were developed for two sets of genes, those encoding *Secreted In Xylem* (*SIX*) and the cytochrome
114 *P450*. Results to confirm the presence or absence of FOE as well as detection time in genomic DNA of all isolates
115 used for the study are shown in Tables 2 & 3. The amplification and derivative curves generated were also observed
116 to confirm the specificity of the products amplified (example is shown in Fig. 2). Generally, amplification was
117 observed at 65 ^{0}C. *SIX8* gene could amplify all FOE, some presumed-FOE and non-FOE isolates (Table 2). *SIX10* and
118 *SIX13* could not detect FOE and presumed-FOE isolates. Generally, the detection times varied among all the genes
119 (*SIX8* or *P450*) that were used (Tables 2 & 3). The time of detection for all the *SIX* genes was between 4:00 min to
120 29:15 min. *P450 cytochrome* detected isolates at 6:45 min (Table 3). Both *SIX8* and P450 *cytochrome* LAMP primers

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121 designed recorded a detection time below 30 min in FOE and presumed-FOE isolates. The LAMP primers were
122 randomly tested on the sampled roots of oil palm seedlings that showed symptoms of FOE infection to confirm the
123 sensitivity of the assay for on-site detection, and these assays were positive with the *SIX8* gene primer set but not
124 consistent, hence results not shown.
125
126 ***Discussions***
127 The research showed that LAMP primers could detect both FOE within inoculated oil palm seedlings and presumed-
128 FOE (symptomatic oil palm in fields). The time for detection FOE or presumed-FOE using *SIX* gene and P450
129 *cytochrome* primers differed but all could detect either FOE or presumed-FOE within 30 min compared to PCR which
130 is time consuming. Although there are reports on Loop-Mediated Isothermal Amplification (LAMP) assays for
131 detecting several *formae speciales* (f.spp.) for *Fusarium* [1, 4, 8] and other plant pathogens [27] this is the first time
132 an attempt has been made to use LAMP for detection of *Fusarium oxysporum* f. sp. *elaeidis* (FOE).
133
134 *Secreted In Xylem (SIX)* gene, are small, secreted and well known to be cysteine-rich, first identified in the xylem sap
135 of tomato infected with *Fol* [9, 23]. Loop Mediated Isothermal Amplification (LAMP) was successfully designed
136 based on *SIX* genes to distinguish *Foc* from other plant pathogenic fungi [4, 27]. The detection of *Fol* with LAMP
137 was achieved based on the 28S rRNA regions [3, 27] but unable to distinguish between pathogenic races of *Fol*
138 isolates. The LAMP assay using the *SIX8* was positive for FOE, presumed-FOE and non-FOE. The presence or
139 absence of some of the *SIX* genes in FOE, presumed-FOE and non-FOE used in this study is congruent with a study
140 that showed that as of now, only fourteen *SIX* (1-14) genes have been identified and most share similarities with each
141 other or with other fungi [28]. The LAMP assay for *SIX8* was faster for detection but detection time varied from one
142 isolate to the other and differed as well among FOE, presumed-FOE and non-FOE. This could be because of the
143 differences in the genomic DNA concentrations used in the study and the presence of some inhibitors as well which
144 influenced the time of amplification. LAMP *SIX8* primer in this study, could not directly detect FOE (OPRI-5, BOPP-
145 5, NORP-5 and 16F) isolates that were artificially inoculated into soil. On the contrary, other research could directly
146 detect *Fol* race 1 in soil artificially inoculated based on primers designed for *SIX4* and *SIX5* [4]. Similarly, LAMP as
147 an effective tool for detecting *Foc* race 4 isolate in soils has been reported [20].
148
149 It is reported that the *P450 cytochrome* are distributed widely in many organisms [15]. *P450 cytochrome* has been
150 associated with pathogenicity in some fusarium such as the *F. oxysporum* f. sp. *cubense* (*Foc*) [25]. In this study, *P450*
151 *cytochrome* was detected in FOE, presumed-FOE and non-FOE isolates. The level of *P450 cytochrome* differences

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152 such as the copy numbers or gene families, significantly varies biologically across kingdoms, phyla and species [15].
153 Furthermore, *P450 cytochrome* share conserved overall protein architecture and have many conserved sequences,
154 despite the higher level of diversity in the *P450 cytochrome* [15]. These characteristics of the *P450 cytochrome* may
155 have contributed to the presence in FOE, presumed-FOE and non-FOE as well as the varying time of detection. The
156 variation in time of detection using *P450 cytochrome* LAMP primers could be because the differences in the genomic
157 DNA concentrations isolated from FOE, presumed-FOE and non-FOE.
158
159 Generally, LAMP primers developed in this study, either the *P450 cytochrome* or *SIX8* genes represents an extremely
160 rapid [10] diagnostic tool for FOE. However, as at now, the assays lack the specificity required to discriminate between
161 FOE, presumed-FOE and non-FOE isolates. However, the primers provided could potentially be used to detect or
162 screen FOE as it is host specific to oil palm seedlings especially in nurseries to prevent the spread and introduction of
163 the pathogen in various oil palm plantation sites.
164

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LIST OF FIGURES

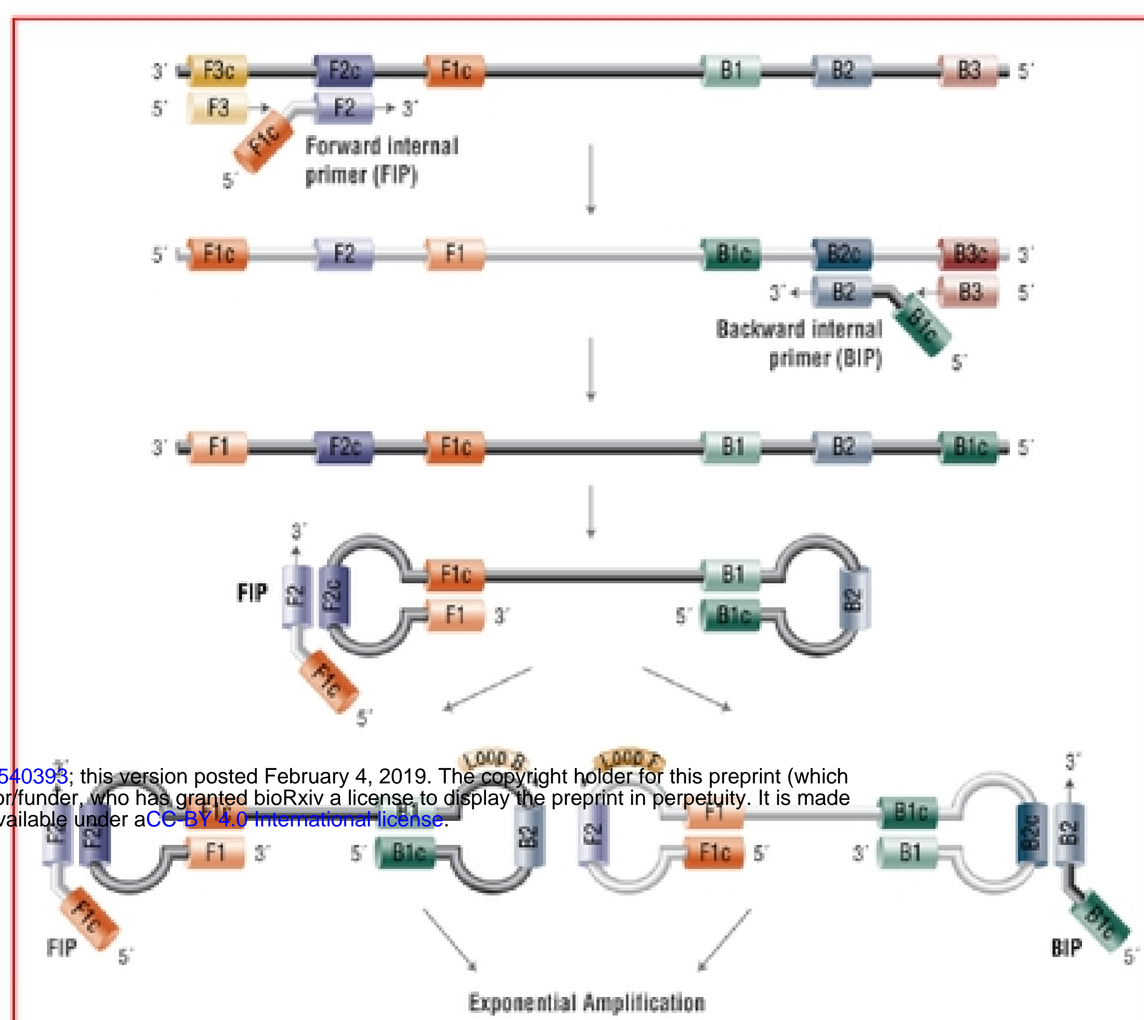

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Figure. 1. Loop-mediated Isothermal Amplification (LAMP) uses 4 - 6 primers recognizing 6 - 8 distinct regions of target DNA. A strand-displacing DNA polymerase initiates synthesis and 2 of the primers form loop structures to facilitate subsequent rounds of amplification (Source:https://www.neb.com/applications/dna-amplification-and-pcr/isothermal-amplification).

Figure

Table.1. List of selected *Fusarium oxysporum* isolates collected from symptomatic oil palm and other fusarium isolates (non-FOE)

Fusarium species	Isolate code	Location
F.oxysporum* f. sp. *elaeidis	IVORY COAST-16F	Ivory Coast
F.oxysporum* f. sp. *elaeidis	DR CONGO-F3	DR Congo
F.oxysporum f. sp. *elaeidis*	DR CONGO-Z/CBS217.49	DR Congo
F.oxysporum f. sp. *elaeidis*	SURINAME-S/CBS783.83	Suriname
F.oxysporum f. sp. *elaeidis*	GHANA NORPALM-N2	Ghana
F.oxysporum f. sp. *elaeidis*	GHANA NORPALM-N3	Ghana
F.oxysporum* f. sp. *elaeidis	GHANA NORPALM-N5	Ghana
F.oxysporum f. sp. *elaeidis*	GHANA BOPP-B4	Ghana
F.oxysporum f. sp. *elaeidis*	GHANA BOPP-B5	Ghana
F.oxysporum* f. sp. *elaeidis	GHANA OPRI-5	Ghana
F.oxysporum f. sp. *elaeidis*	GHANA OPRI-11	Ghana
F.oxysporum f. sp. *elaeidis*	GHANA OPRI-18	Ghana
*F.*oxysporum sp.	Fus126B-3	ADAS
*F.*oxysporum sp.	Foxy150-5	ADAS
F.oxysporum sp.	Foxy sp-8	ADAS
*F.*oxysporum sp.	Foxy2571-9	ADAS
F.oxysporum sp.	Foxy sp-10	ADAS
Fusarium sp.	Fus-11	ADAS
*F.*oxysporum sp.	FoxyBX14/153b-A	ADAS
*F.*oxysporum sp.	FoxyBX14/153a-B	ADAS
*F.*oxysporum f. sp. *lycopersici*	Foxy.Forla-C	UoN
*F.*oxysporum f. sp. *lycopersici*	Foxy.Forlb-D	UoN
Fusarium solani 1	F1	UoN
Fusarium solani 2	F2	UoN
Fusarium oxysporum f.sp. *radicis-lycopersici*	F3/ FORL A	UoN
Fusarium oxysporum f.sp. *radicis-lycopersici*	F4/ FORL B	UoN
*F.*oxysporum BX15/4a	F5	UoN
*F.*oxysporum BX15/4b	F6	UoN
*F.*oxysporum BX15/4c	F7	UoN
*F.*oxysporum f. sp. *cubense* (FOC2A)	F8	UoN
*F.*oxysporum f. sp. *cubense* (FOC2B)	F9	UoN
*F.*oxysporum BX14/153a	F10	UoN
*F.*oxysporum BX14/153b	F11	UoN
*F.*oxysporum BX14/153c	F12	UoN
*F.*oxysporum BX14/152a	F13	UoN
*F.*oxysporum BX14/128a	F14	UoN
*F.*oxysporum BX14/168d	F17	UoN
*F.*oxysporum f. sp. *lycopersici* race 1	F18	Alison Jackson (UK)
*F.*oxysporum f. sp. *lycopersici* race 2	F19	Alison Jackson (UK)
*F.*oxysporum f. sp. *radices lycopersici*	F20	Alison Jackson (UK)

F.oxysporum* f. sp. *elaeidis = FOE (i.e. Pathogenic to oil palm)

F.oxysporum f. sp. *elaeidis* = presumed-FOE (i.e. Pathogenicity not confirmed in oil palm though sampled from symptomatic oil palm trees)

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All Result Tables and list of primers