

Development of a loop-mediated isothermal amplification assay for rapid and sensitive detection of *Fusarium oxysporum* f. sp. *cubense* race 4

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Abstract *Fusarium* wilt (Panama disease), caused by the fungus *Fusarium oxysporum* f. sp. *cubense* race 4 (Foc race 4), is one of the most destructive diseases affecting banana (*Musa*). Early and accurate detection of Foc race 4 is essential to protect the banana industry. We developed a novel and highly specific loop-mediated isothermal amplification (LAMP) assay for the detection of Foc race 4 based on a SCAR marker sequence. The detection limit for this assay was 10 fg per 25 µl reaction in pure culture and DNA amplification was completed within 60 min. The assays detected 69 different isolates of Foc race 4 from geographically distinct counties in China, and no cross-reaction was observed with other fungal pathogens. When 26 infected and eight healthy looking but infested banana samples naturally from different fields were examined, the detection rate of LAMP was 100 %. The LAMP assay developed in this study was simple, fast, sensitive, and specific, and can be used in the field to detect Foc race 4 in infected banana plant tissue in resource-poor settings.

Keywords *Fusarium oxysporum* f. sp. *cubense* race 4 · Loop-mediated isothermal amplification (LAMP) · PCR · Sequence-characterised amplified region (SCAR)

Introduction

Fusarium wilt (Panama disease), caused by the fungus *Fusarium oxysporum* f. sp. *cubense* (Foc), is one of the most destructive diseases affecting banana (*Musa*) (Ploetz 2006; Stover 1962), and results in serious damage in Southeast Asia (Molina et al. 2008). Foc race 4 affects cultivars that produce more than 80 % of the world's bananas, including the Cavendish and plantain subgroups, and it poses a serious threat to a multi-billion dollar industry and the stability of millions of farmers. To control the spread of Foc within China, a suitable detection method, which is rapid, cost-effective and efficient is required in banana growing areas.

Fusarium species can be identified morphologically, but differences between species are difficult to establish. *Fusarium oxysporum* formae speciales (f. sp.) and races within a species can be determined only by time-consuming procedures such as pathogenicity tests in host plants and vegetative compatibility group (VCG) tests (Bentley et al. 1998; Nauta and Hoekstra 1994). The problem is further complicated by the occurrence of saprophytic strains of *F. oxysporum* that are morphologically identical to *F. oxysporum* f. sp.

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cubense but are nonpathogenic opportunistic invaders. Methods based on polymerase chain reaction (PCR) were developed to distinguish between Foc race 4 and other non-Foc race 4 isolates (Dita et al. 2010; Lin et al. 2009). Highly specific diagnostic assays can be developed by developing proper amplification primers, but the method requires expensive laboratory instrumentation and reagents.

Recently, a novel technique called loop-mediated isothermal amplification (LAMP) has been developed (Nagamine et al. 2001; Notomi et al. 2000). LAMP is based on the principle of autocycling strand displacement DNA synthesis performed by *Bacillus stearothermophilus* (*Bst*) DNA polymerase to detect a specific DNA sequence (Notomi et al. 2000). This technique uses four to six primers that recognise six to eight regions of the target DNA, and provides very high specificity (Nagamine et al. 2002; Notomi et al. 2000). The technique can be performed under isothermal conditions ranging between 60 °C and 65 °C, and it produces large amounts of DNA (Notomi et al. 2000). The reaction shows high tolerance to biological contaminants (Kaneko et al. 2007), which can help avoid false-negative results due to inactivation of the enzyme (a common problem in PCR). Although LAMP amplification products can also be detected by gel electrophoresis, this procedure reduces the suitability for field application. However, SYBR Green I can be used to detect amplification by a simple visual inspection. One advantage of this technique is that expensive equipment is not required since the temperature is not cycled during the amplification process. LAMP technology has been successfully applied to detect for example *Meloidogyne enterolobii* (Niu et al. 2012), *Erwinia amylovora* (Moradi et al. 2012), *Botrytis cinerea* (Tomlinson et al. 2010), and citrus bacterial canker (Rigano et al. 2010). However, this technology has not yet been applied to detect Foc race 4 from banana plant tissue.

Numerous techniques have been developed to study the population biology and diversity of pathogens, including restriction fragment length polymorphism (RFLPs), random amplified polymorphic DNAs (RAPDs), amplified fragment length polymorphisms (AFLPs), microsatellites, single nucleotide polymorphisms (SNPs), and mitochondrial haplotype analyses (Martin et al. 2012). RAPDs generate dominant markers that can provide a large number of polymorphic bands with no requirement on sequence

information. Based on RAPD markers, SCAR (Sequence Characterized Amplified Regions) primers can be developed to offer a powerful tool to monitor the performance of biological control programmes (Karam et al. 2008). In this study, we developed a sensitive and rapid diagnostic LAMP assay to detect Foc race 4 by targeting a SCAR of genomic DNA (GenBank : EF155535) submitted by our team previously. This is the first report on the detection of Foc race 4 using the LAMP method.

Materials and methods

Fungal isolates and DNA extraction

The origin, host affiliation, and number of isolates used in this study are listed in Table S1. We tested 79 *F. oxysporum* f. sp. *cubense* isolates of two described races (69 of race 4 and 10 of race 1) sampled from a wide geographic distribution (Qi et al. 2006), as well as 17 isolates from 11 different *F. oxysporum* formae speciales, 32 isolates of 19 different *Fusarium* spp., and 33 other fungi and bacteria isolated from banana and other hosts. Isolates were stored as monoclinal cultures grown on sterile moist filter paper, dried, and stored at 4 °C.

Total DNA was extracted from mycelium of each isolate with the CTAB procedure as described previously (Zhang et al. 2006). DNA samples from infected tissues (stem and leaves) were extracted according to the method of Tooley et al. (1997). A sample (10 mg) of diseased tissue (stem or leaf) was cut from each plant, placed into 10 µl of freshly prepared 0.5 M NaOH and macerated with a plastic pestle. After the tubes were centrifuged at 12,000 ×g for 5 min, 5 µl of supernatant was removed and immediately diluted with 195 µl 100 mM Tris (pH=8.0). Soil DNA samples collected from infected fields were extracted by using a FastDNA® SPIN Kit for Soil from BIO 101® Systems (Q-Biogene Ltd, Morgan Irvine, CA, USA). Purified DNA was quantified using a spectrophotometer, and aliquots were diluted to 100 ng µl⁻¹ in sterile distilled water as stocks at -20 °C.

Detection of Foc race 4 by PCR

Two hundred twenty-eight arbitrary oligonucleotides (10-mer primers) were used as primers for

PCR with a set of Foc race 4 and non-Foc race 4 isolates. One oligonucleotide fragment of approximately 400 bp from Foc race 4 isolates (but not from non-Foc race 4 isolate) was amplified. After testing a large collection of isolates, S22 (5'-TGCCGAGCTG-3') was used for further investigation. In all race 4 isolates, a DNA fragment of approximately 400 bp was amplified. This PCR product was cloned and sequenced. This sequence was submitted to the GenBank nucleotide sequence database by our group in 2006 (GenBank accession number: EF155535). Based on the sequence, a specific SCAR primer pair Foc-F/Foc-R was designed (Table 1), and the sensitivity of the primers Foc-NF/Foc-NR was also evaluated. PCR was performed according to standard PCR protocols using a PTC-200 PCR apparatus (MJ Research) (35 cycles of 1 min at 95 °C, 1 min at 60 °C, 1 min at 72 °C, followed by 1 cycle of 10 min at 72 °C). PCR sensitivity was evaluated using 100 ng, 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, and 1 fg per 1 µl DNA concentration in a 25 µl reaction. Amplification products were separated by gel electrophoresis on 2.0 % agarose gels, stained with ethidium bromide, and visualised under UV light.

LAMP primer design

Based on the *F. oxysporum* f. sp. *ubense* race 4 sequence (GenBank accession number: EF155535), a set of LAMP primers, comprising two outer (F3 and B3) and two inner (FIP and BIP) primers, were designed using LAMP primer software PrimerExplorer V4 (<http://primerexplorer.jp/elamp4.0.0/index.html>; Eiken Chemical Co., Japan) (Fig. 1). Loop primers (LF and LB) were designed manually. The designed primer sequences for *F. oxysporum* f. sp. *ubense* race 4 are shown in Table 1.

LAMP reaction and detection

The LAMP reaction was performed using a Loopamp DNA amplification kit (Eiken Chemicals Co. Ltd, Tokyo, Japan) in a 25 µl volume. The reaction mixture contained 40 pmol each of FIP and BIP, 5 pmol each of F3 and B3 primers, and 20 µmol l⁻¹ each of loop primers F-Loop and B-Loop, 2 µl template DNA (~10 ng), 1 µl *Bst* DNA polymerase (8 units), and 12.5 µl 2×reaction mix prepared in the kit. Negative controls containing nuclease-free water instead of DNA were included in each assay. To identify the optimal temperature and time for the visual detection

Table 1 Primers used in this study

Primer type	Primer	Primer sequences (5'to 3')
RAPD	S22	5'-TGCCGAGCTG-3'
SCAR	FOC-F	5'-ATATGAATGACTCGTGGCACG-3'
	FOC-R	5'-GCTGGG AATGCGACGGTAT-3'
	FOC-NF	5'-GATGAGATTGAAGGACCTCTTCG-3'
	FOC-NR	5'-TCTAGTTCCTTGGAGTAGTTTCGG-3'
ITS	ITS1	5'-TCCGTAGGTGAACCTGCGG-3'
	ITS4	5'-TCCTCCGCTTATGATATGC-3'
PCR	FOC-F	5'-ATATGAATGACTCGTGGCACG-3'
	FOC-N	5'-GCTGGGAATGCGACGGTAT-3'
	FOC-NF	5'-GATGAGATTGAAGGACCTCTTCG-3'
	FOC-NR	5'-TCTAGTTCCTTGGAGTAGTTTCGG-3'
LAMP	F3	5'-AGGACCTCTCGAATGGCA-3'
	B3	5'-GACGCTGCAGCTATGACAA-3'
	FIP	5'-GGTGGCTCAATAGCCCAGTGAA-CCGATACCTGTGAAGTCGC-3'
	BIP	5'-CGACATCATCAGCATCTCCGCT-AGCTTTGGCTCTTGTGACAG-3'
	F-loop	5'-GCCTAATTGAACATTCAGTATAAAC-3'
	B-Loop	5'-ACTCCAAGGAACTAGACGACG-3'

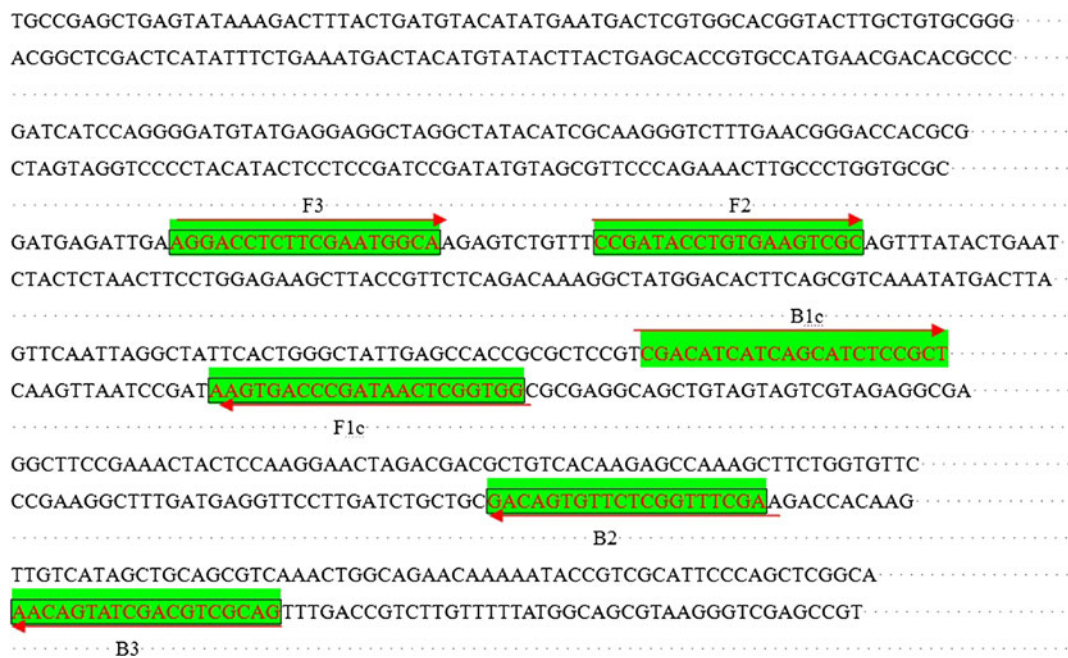


Fig. 1 Location and sequence of LAMP primer sets targeting the Foc race 4 isolate sequence (GenBank accession number: EF155535). Partial sequence of Foc race 4 and the location of

four LAMP primers (F3, B3, FIP [F1c-F2], and BIP [B1c-B2]). Arrows indicate the direction of extension

of LAMP amplification, the reactions were performed in 60 °C, 62 °C, 63 °C, and 65 °C water baths for 15, 30, 45, 60, and 75 min. Finally, the reaction was terminated by heating the reaction mixture at 80 °C for 10 min. Two methods were used to analyze DNA amplification, including electrophoresis in 2 % agarose gels stained with ethidium bromide and direct visual inspection of the LAMP product with SYBR Green I by the naked eye or under UV light.

Specificity and sensitivity of LAMP

To determine the LAMP specificity for the target Foc race 4, 69 Foc race 4, 10 Foc race 1, 11 different *F. oxysporum* formae speciales, 19 different *Fusarium* spp., and 33 other fungi and bacteria isolated from banana and other hosts (Table 1) were subjected to LAMP assays. The specificity of the reaction was confirmed through *Hind*III (Takara) restriction enzyme digestion analysis and sequencing. The LAMP products were purified and digested by *Hind*III (Takara) restriction enzyme at 37 °C for 3 h. Digested products were analyzed by 2.0 % agarose gel electrophoresis, and the individual DNA bands were purified and cloned into an *Hind*III-restricted

pBI121 vector and transformed into *E. coli* cells, and selected clones were subjected to sequencing. To determine the detection limit of Foc race 4 by LAMP, assays were performed using serial 10-fold dilutions (from 100 ng to 0.1 fg) of pure Foc race 4 genomic DNA. Amplification was monitored as described above. Sensitivity tests were repeated in triplicate.

LAMP testing of diseased banana

To develop field application of LAMP as a diagnostic tool for Foc race 4 surveys and management, more than 15 banana *Fusarium* wilt samples (stem and leaves) induced by Foc race 4 and 10 soil samples collected from a greenhouse and inoculated with the pathogen were surveyed using the LAMP method. Infected plants were grown in a growth chamber, as described previously (Saravanan et al. 2003). To prepare pathogen inoculums, sand and maize powders were mixed at a ratio of 19:1 and autoclaved for 2 h. The sterilised medium was inoculated by transferring a disc of pathogenic culture grown on PDA slants and incubating at room temperature for 3 weeks. Pathogen inoculations were performed as previously described (Borges et al. 2004). Non-infested banana plants were

used as controls. We also collected 26 infected and eight healthy looking but pathogen infested banana samples from different fields in the Fujian province for LAMP detection. DNA extraction was performed and the LAMP reactions were performed at 63 °C for 60 min. Amplification was monitored with gel electrophoresis and SYBR Green I.

Results

Detection of Foc race 4 using PCR

The specificity of the PCR primers was assessed using a collection of 69 Foc race 4 isolates and 92 isolates of other non-Foc race 4 fungi (Table S1). The primer pair Foc-F/Foc-R amplified a unique DNA fragment of approximately 364 bp (Fig. 2) in all Foc race 4 isolates from different provinces in China; however, this primer pair did not yield amplification products when using isolates of any other non-Foc race 4 fungi. Internal transcribed spacer (ITS) universal primers ITS1/ITS4,

designed to amplify a ribosomal DNA fragment of approximately 544 bp in Foc race 4, yielded positive PCR reactions in all fungal isolates tested (data not shown). Furthermore, no amplification of banana genomic DNA was achieved using the primer pair Foc-F/Foc-R. The sensitivity of the primer sets Foc-F/Foc-R in a 25 µl reaction was 100 pg of pure template of Foc race 4 total genomic DNA.

Optimization of LAMP assay

LAMP was performed using Foc race 4 DNA as template to determine the optimal temperature and reaction time. Optimization of LAMP reaction conditions (temperature and time) revealed that the ideal settings for the primer set were 63 °C for 60 min. Thus, the LAMP assays were performed under these conditions. As expected, the typical ladder-like pattern on 2.0 % agarose gel electrophoresis was observed in all positive samples, but not in the negative controls. Under visual fluorescence detection with SYBR Green I, positive or negative results were easily determined;

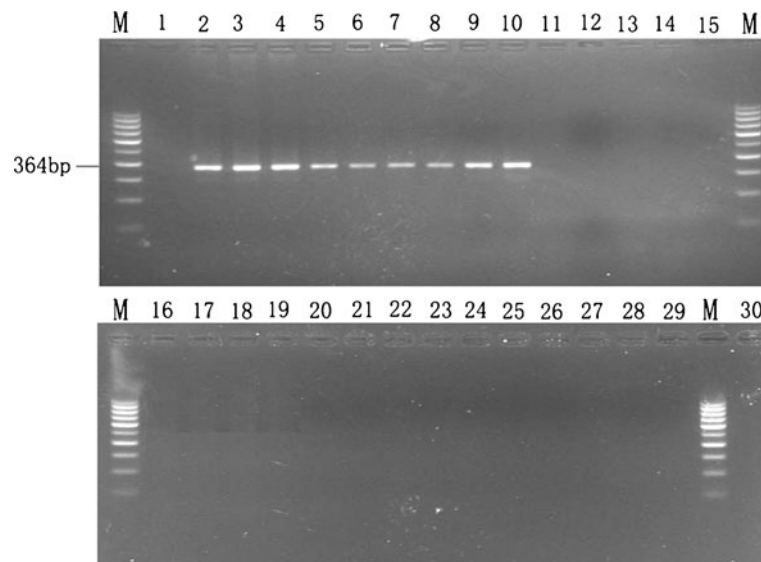


Fig. 2 Agarose gel electrophoresis of PCR-amplified products using the specific primers Foc-F/Foc-R. Lanes M are the molecular weights of the 100 bp ladder marker (Takara Biotechnology Co., Ltd., Otsu, Japan); lane 1, negative control (sterile distilled water); lanes 2–4, *F. oxysporum* f. sp. *cubense* race 4 isolates from Fujian; lanes 5–7, *F. oxysporum* f. sp. *cubense* race 4 isolates from Guangdong; lanes 8–10, *F. oxysporum* f. sp. *cubense* race 4 isolates from Hainan; lane 11, *Fusarium oxysporum* f. sp. *cubense* race 1; lane 12, *F. oxysporum* f. sp. *vasinfectum*; lane 13, *F. oxysporum* f. sp. *cucumerinum*; lane

14, *F. oxysporum* f. sp. *lycopersici*; lane 15, *F. oxysporum* f. sp. *melonis*; lane 16, *F. oxysporum* f. sp. *batatas*; lane 17, *F. oxysporum* f. sp. *pisii*; lane 18, *F. oxysporum* f. sp. *phaseoli*; lane 19, *F. oxysporum* f. sp. *niveum*; lane 20, *F. oxysporum*; lane 21, *F. eguisei*; lane 22, *F. verticillioides*; lane 23, *F. nivale*; lane 24, *F. culmorum*; lane 25, *F. graminearum*; lane 26, *F. solani*; lane 27, *Mycosphaerella fijiensis*; lane 28, *Colletotrichum musae*; lane 29, *Alternaria solani*; lane 30, *Ralstonia solanacearum*. The same results were obtained in all four replicates

positive reactions appeared green, while the negative control remained orange. Fluorescence detection results were consistent with the results from agarose gel electrophoresis (data not shown).

LAMP assay specificity

LAMP specificity was examined using DNA templates extracted from a large collection of 69 *Foc* race 4 isolates and 92 isolates of other non-*Foc* race 4 fungi (Table S1). After incubation at 63 °C for 60 min, *Foc* race 4 strains gave a positive reaction, whereas no amplification was observed for the other non-*Foc* race 4 fungi species (Fig. S1). In addition, the identities of the amplified products were confirmed through direct sequencing, in which the sequences obtained were perfectly matched with the expected DNA sequences (data not shown). These results indicated that the LAMP technique developed in this study is highly specific for *Foc* race 4.

Detection limit of LAMP assay

To determine the detection limit, LAMP reactions were performed using 10-fold serial dilutions of pure *Foc* race 4 DNA, and the amplicons were detected both by agarose gel electrophoresis and by visual inspection. As shown in Fig. 3, the minimum detection concentration required for the LAMP assay was 10 fg genomic DNA. These observations also showed that, based on visual inspection, the results of the LAMP assay were correlated with the gel electrophoresis (Fig. 3a and b).

Evaluation of the LAMP assay using diseased plant material

To demonstrate the applicability of the LAMP method in field samples, this method was evaluated using plant materials infested with *Foc* race 4. Banana plants that exhibited symptoms of yellowing of the leaf margins and vascular discolouration (typically observed in plants infected by *Foc* race 4) were collected in a greenhouse. The LAMP assay was used to detect the presence or absence of *Foc* race 4 in plant tissue at optimised temperatures and times. A total of four *Foc* race 4-infected and three healthy banana plant tissues were analysed. Purified DNA was used as a positive control while sterile distilled water was used as a

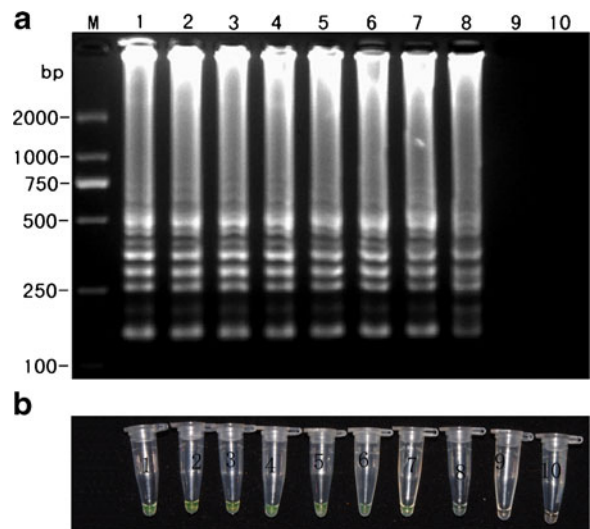


Fig. 3 Sensitivity of LAMP assay to detect *Foc* race 4 using serial DNA dilutions. **a** LAMP assay detected by agarose gel electrophoresis. **b** LAMP assay and visual inspection by adding SYBR Green I dye observed under visible light. The same results were obtained in all three replicates. Lanes M are the DNA molecular marker; lanes 1–10, amplified products using DNA at concentrations of 100 ng, 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg, and 0.1 fg in a 25 μ l LAMP reaction

negative control. All four infected samples were positive for *Foc* race 4 by LAMP while none of the healthy banana corms showed any bands (Fig. 4a). These observations also showed that (based on visual inspection) the results of the LAMP assay were correlated with the results seen after gel electrophoresis (Fig. 4a and b). These results were indicative of the high detection capability of the LAMP assay. Furthermore, the LAMP assay detected *Foc* race 4 in 34 of 34 (26 infected and eight healthy looking but pathogen infested banana) collected from different fields. The detection rate of LAMP was 34/34 (100 %) for the collected samples in this study. All reactions were repeated at least twice with consistent results. Two randomly selected samples with LAMP-amplified products had 100 % identical DNA sequences to all previously sequenced *Foc* race 4 isolates. Thus, the DNA isolated from plants was from *Foc* race 4 isolates.

Discussion

Fusarium wilt (Panama disease) is a major threat to the global banana industry. Rapid and accurate detection

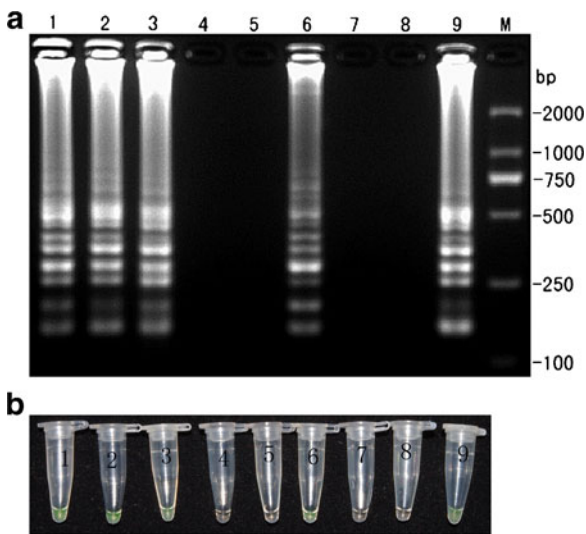


Fig. 4 LAMP detection of Foc race 4 from infected (lanes 1, 2, 3, 6) and uninfected (lanes 4, 5, 7) banana using LAMP. **a** LAMP assay detected by agarose gel electrophoresis. **b** LAMP assay and visual inspection by adding SYBR Green I dye observed under visible light. Three independent replicates gave the same results. Purified Foc race 4 DNA was used as a positive control (lane 9) and DNA from healthy banana plants was used as a negative control (lane 8). Lane M represents the DNA molecular markers

of Foc race 4 is vital to prevent the introduction and spread of Foc race 4 into non-endemic areas, and to minimise and control damage to the banana industry. Recently, PCR-based methods have been developed to detect Foc race 4 (Dita et al. 2010; Lin et al. 2009). These methods are fast, specific, and sensitive, but cannot be carried out in the field because they require equipment and facilities that are not easily portable. We developed a novel, equipment-free method to detect Foc race 4 using the LAMP technique. The amplification efficiency is extremely high because there is no time loss due to thermal cycling, and inhibition reactions at later stages are less likely to occur (unlike in standard PCR). In addition, LAMP amplifies DNA to higher concentrations than PCR, making it convenient for visualization of the products without gel electrophoresis. The LAMP reactions could be completed within 1 h by incubating at 63 °C. The results were visually inspected using SYBR Green I and amplicons were confirmed through gel electrophoresis. In this study, the assay specifically amplified only Foc race 4 and no cross-reactivity was observed for other *Fusarium* or fungal species. These results

demonstrate that LAMP is highly specific for the amplification of Foc race 4, detecting it with high efficiency. Furthermore, the LAMP assay can be successfully applied to examine crude DNA isolated from infected plant tissue samples and the use of LAMP assays to test crude extracts has been demonstrated by previous studies (Li et al. 2011; Niu et al. 2012). Therefore, compared to conventional methods including PCR, the LAMP assay requires less time, costs less, and is easier to operate, significantly increasing the efficiency of Foc race 4 diagnosis and management. Although our LAMP assay is sensitive, specific, and rapid, the CTAB DNA extraction method is not convenient in rural areas and more convenient DNA extraction method should be developed.

For the LAMP assay, the buffer, primers, and DNA polymerase are mixed and incubated at 63 °C in a regular laboratory water bath or heat block that provides a constant temperature. The LAMP primers were selected from regions of the Foc race 4 SCAR marker sequence that are highly specific to Foc race 4. The six primers (F3, B3, FIP, BIP, F-loop, and B-loop) targeted eight regions of Foc race 4 (Fig. 1), providing additional levels of specificity compared to PCR primers (targeting two regions). Of the 69 Foc race 4 and 92 non-Foc race 4 strains tested, the LAMP assay had 100 % inclusivity and 100 % exclusivity.

Several studies have reported the use of the LAMP method to detect various pathogens using an expensive real-time turbidimeter for reaction confirmation (Mori and Notomi 2009; Nakao et al. 2010; Yamazaki et al. 2010). The use of expensive equipment decreases the versatility of LAMP and limits the use of this procedure, especially in developing countries. Thus, rapid and unambiguous visual inspection of LAMP results is essential for diagnostics and SYBR Green I has been developed to allow for visual discrimination of positive samples. Generally, the addition of SYBR Green I is made after incubation and colour change in positive amplifications is from orange to green, which could be judged under natural or UV light, and agreed with the gel electrophoresis results. However, SYBR Green I results in weak fluorescence under UV light in the negative reaction (Niessen and Vogel 2010; Niu et al. 2012). Considering the disadvantage in visual observation on SYBR Green I, the use of SYBR Green I should be analysed carefully.

The LAMP protocol described in this study represents a very sensitive, specific, and rapid diagnostic protocol for Foc race 4 detection. To the best of our knowledge, this is the first study to use the LAMP technique to detect Foc race 4. With the development of LAMP, a diagnostic kit makes Foc race 4 detection become a simple routine testing assay in the field. This protocol is very useful for detecting low levels of Foc race 4 in banana plant tissues and can be used to confirm early stages of Foc race 4 infection at a relatively low level. We recommend that this technique be applied routinely in diagnostics and in corm surveillance so that fungi-carrying banana can be identified during the early stages of infection and management can be devised before the infection becomes epidemic.

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