

Loop-mediated isothermal amplification (LAMP) based detection of *Colletotrichum falcatum* causing red rot in sugarcane

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Received: 16 December 2014 / Accepted: 31 March 2015 / Published online: 11 April 2015
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Abstract Red rot, caused by *Colletotrichum falcatum*, is a destructive disease prevalent in most sugarcane-producing countries. Disease-free sugarcane planting materials (setts) are essential as the pathogen spreads primarily through infected setts. The present study was undertaken to develop a loop-mediated isothermal amplification (LAMP) assay for the detection of *C. falcatum*. *C. falcatum* genomic DNA was isolated from pure mycelium culture and infected tissues. Four sets of primers corresponding to a unique DNA sequence specific to *C. falcatum* were designed. Specificity of the LAMP test was checked with DNA of another fungal pathogen of sugarcane, *Puccinia melanocephala*, as well as two closely-related species, *Colletotrichum fructivorum* and *Colletotrichum acutatum*. No reaction was found with the three pathogens. When *C. falcatum* DNA from pure culture was used in a detection limit analysis, sensitivity of the LAMP method was observed to be ten times higher than that of conventional PCR; however, sensitivity was only 5 times higher when DNA from *C. falcatum*-infected tissues was used. Using the LAMP assay, *C. falcatum* DNA is amplified with high specificity, efficiency, and rapidity under isothermal conditions. Moreover, visual judgment of color change in <1 h without further post-amplification processing makes the

LAMP method convenient, economical, and useful in diagnostic laboratories and the field.

Keywords *Colletotrichum falcatum* · LAMP · Red rot · Sugarcane

Introduction

Red rot, a major disease of sugarcane (*Saccharum* spp. hybrids) caused by the fungus *Colletotrichum falcatum* Went [teleomorph, *Glomerella tucumanensis* (Speg.) Arx and E. Muller], is reported to occur worldwide and may cause severe losses in sucrose yield [1–4]. The disease is spread primarily through infected setts [5, 6]. In several parts of India, red rot epidemics cause significant yield losses, including reports of 100 % loss under conditions favorable for disease development [7]. Some highly productive Indian commercial sugarcane varieties have succumbed to this disease [8]. In Louisiana, red rot causes poor establishment of cane stands as disease develops in planted stalks, rather than standing cane [1, 3, 9]. The characteristic symptoms of sugarcane red rot are reddening of the internal tissues, with interrupted red and white patches perpendicular to the axis of the stalk developing at later stages. Hence, early detection of disease is required. Additionally, latent infection occurs frequently, making visual diagnosis impossible. If such canes are planted, the spread of disease would increase many fold. To a large extent, red rot can be prevented if disease-free setts are used as planting material [7]; therefore, a sensitive and reliable technique is needed to detect the pathogen in the propagative material.

Disease diagnosis through conventional methods involving pathogen isolation and characterization is labor-

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intensive and time-consuming. Although immunological and molecular diagnostic tools have received attention as an alternative to conventional methods, these have several limitations. Enzyme-linked immunosorbent assay (ELISA) is one technique routinely used in several laboratories due to its sensitivity and ability to simultaneously analyze many samples in a single microplate. Hiremath and Naik [10] reported rapid diagnosis of sugarcane red rot infection in the planting materials by using a dot immunobinding assay (DIBA). Polyclonal antisera have been developed using a set of *C. falcatum* proteins to detect *C. falcatum* in sugarcane through ELISA, DIBA and Western blot methods [11]. However, these techniques are restricted due to false positive results caused by cross-reaction of antibodies with plant debris or unrelated organisms [12]. Both end point and real-time PCR molecular techniques have been used in detection of fungal pathogens in plant tissues [13–19], owing to increased specificity and sensitivity compared to more traditional experiments. But these techniques require costly instruments like thermocyclers and electrophoresis equipment and laboratory sophistication to handle the assays. Recently, a sequence characterized amplified region (SCAR) marker has been developed based on the ~560 bp DNA fragment sequence amplified with RAPD primer OPE-01 to detect *C. falcatum* [7].

Loop-mediated isothermal amplification (LAMP) is a novel DNA amplification method wherein four specific primers are used to amplify target DNA in about 1 h with strand displacement DNA polymerase under isothermal conditions [20]. These four specific primers are referred to as outer (F3 and B3) and inner (FIP and BIP) primers and are designed based on the six regions of the target DNA. Of the two inner primers, one initiates the LAMP reaction while the other, in conjunction with one outer primer, hybridizes to the opposite end of the target DNA. The second outer primer facilitates DNA synthesis following strand displacement and induces stem-loop structure formation. An accumulation of 10^9 copies of target DNA are observed in <1 h [20]. For efficient amplification, concentrations of inner primers are usually kept 4–10 times higher than those of outer primers [20–22]. The other critical component in a LAMP reaction is the Mg^{2+} concentration, where higher concentrations lead to the stabilization of incorrect primer template binding, thus resulting in decreased specificity of LAMP [21]. Currently, LAMP is mainly applied in the fields of medicine, virus detection, and food safety testing, with fewer applications for the detection of fungi, bacteria, nematodes in plants, and insects [22–26]. The first LAMP technology developed for an economically important sugarcane pathogen was for the detection of *Leifsonia xyli* subsp. *xyli*, the bacterium that causes ratoon stunt [21]. In addition, we have recently reported an RT-LAMP assay to detect Sugarcane mosaic virus and Sorghum mosaic virus

in sugarcane [27]. In the present investigation, a LAMP protocol was developed for the detection of *C. falcatum* in sugarcane. This research is important globally in not only advancing our ability to acquire disease-free material from a vegetatively propagated sugarcane crop, but also in proper disease control, survey analysis, and quarantine management.

Materials and methods

Materials and designing of primers

Two isolates of *C. falcatum* (strains cf1010-2 and cf11/05) were grown on PDA medium for 1 week. Mycelium was harvested to isolate the genomic DNA using SDS buffer (100 mM Tris-HCl pH 8.0, 50 mM EDTA, 100 mM NaCl, and 2 % SDS). DNA was also extracted from 200 mg red rot-infected sugarcane tissues using hexadecyltrimethylammonium bromide (CTAB) method as described earlier [28]. Quality and concentration of DNA were tested using a NanoDrop ND-1000 Spectrophotometer (Thermo Fischer Scientific, Wilmington, DE, USA) followed by equilibration through agarose gel electrophoresis using standard concentrations of λ DNA. Nucleic acids were stored at -20 °C.

A 442 bp DNA fragment specific to *C. falcatum* [7, ACC no JN545852 (*Glomerella tucumanensis* Cf86032)] was used to design the LAMP primers. This DNA fragment was obtained from the *C. falcatum* genome while performing PCR with a SCAR marker (SCAR-F 5'-CCTACCCAACCGAGTATCG-3' and SCAR-R 5'-GCGCAGCTTGCTCTCAAGAGC-3'), which was designed using a 566 bp DNA fragment amplified by RAPD primer OPE-01 from *C. falcatum* isolate Cf 86032 [7]. In a homology search of NCBI, this sequence was found only in *C. falcatum* and is highly conserved. OptiGene LAMP Designer software (OptiGene, Horsham, West Sussex, UK) was used to design two outer primers (F3 and B3) and two inner primers (FIP and BIP), which recognize a total of six distinct regions of the 442 bps specific sequence of *C. falcatum* (Table 1). In total, six sets of LAMP primers (RRSC1, RRSC2, RRSC3, RRSC4, RRSC5, and RRSC6) were generated wherein some of the primers were common (Table 1). These primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA). FIP and BIP primers were HPLC purified.

LAMP assay mixture

The six sets of LAMP primers were screened to identify the most efficient primer sets following the procedure of Wang et al. [29]. The reaction was carried out in a 25 μ l mixture

Table 1 Oligonucleotide primers used for loop-mediated isothermal amplification (LAMP) of *C. felcatum*

Primer name	Primer set name	Type	Position in clone ^a	Sequence (5'–3')
rrF3-1	RRSC1	Forward outer	49–66	GTGGCTCAAGAGGACATG
rrB3-1	RRSC1	Reverse outer	329–312	GTTGATGATCCGTGCTGG
rrFIP-1	RRSC1	Forward inner	149–132 and 80–99	ACCTTCACCGCCCTCTGACATTCTGATTGGCTTGGAGA
rrBIP-1	RRSC1	Reverse inner	184–204 and 251–233	GCCACACTCGTCACTCTAACCATGTC1TAGTTGCAGTGCC
rrF3-2	RRSC2	Forward outer	49–66	GTGGCTCAAGAGGACATG
rrB3-2	RRSC2	Reverse outer	329–312	GTTGATGATCCGTGCTGG
rrFIP-2	RRSC2	Forward inner	139–119 and 80–99	CCCTTGACAACGTC AACCCATTCTGATTGGCTTGGAGA
rrBIP-2	RRSC2	Reverse inner	184–204 and 251–233	GCCACACTCGTCACTCTAACCATGTCTTAGTTGCAGTGCC
rrF3-3	RRSC3	Forward outer	49–66	GTGGCTCAAGAGGACATG
rrB3-3	RRSC3	Reverse outer	329–312	GTTGATGATCCGTGCTGG
rrFIP-3	RRSC3	Forward inner	149–131 and 76–95	ACCTTCACCGCCCTCTGAGGAGCATTCTGATTGGCTT
rrBIP-3	RRSC3	Reverse inner	184–204 and 251–233	GCCACACTCGTCACTCTAACCATGTCTTAGTTGCAGTGCC
rrF3-4	RRSC4	Forward outer	49–66	GTGGCTCAAGAGGACATG
rrB3-4	RRSC4	Reverse outer	270–252	AGGGTCCTCCTTCTTTCC
rrFIP-4	RRSC4	Forward inner	149–132 and 80–99	ACCTTCACCGCCCTCTGACATTCTGATTGGCTTGGAGA
rrBIP-4	RRSC4	Reverse inner	184–204 and 251–233	GCCACACTCGTCACTCTAACCATGTCTTAGTTGCAGTGCC
rrF3-5	RRSC5	Forward outer	74–92	CGGGAGCATTCTGATTGG
rrB3-5	RRSC5	Reverse outer	329–312	GTTGATGATCCGTGCTGG
rrFIP-5	RRSC5	Forward inner	160–141 and 92–110	TGTGCTGCCGTACCTTCACCTTGGAGAGGGAGAGCAT
rrBIP-5	RRSC5	Reverse inner	184–204 and 251–233	GCCACACTCGTCACTCTAACCATGTCTTAGTTGCAGTGCC
rrF3-6	RRSC6	Forward outer	75–93	GGGAGCATTCTGATTGGC
rrB3-6	RRSC6	Reverse outer	329–312	GTTGATGATCCGTGCTGG
rrFIP-6	RRSC6	Forward inner	160–141 and 93–111	TGTGCTGCCGTACCTTCACCTTGGAGAGGGAGAGCATG
rrBIP-6	RRSC6	Reverse inner	184–204 and 251–233	GCCACACTCGTCACTCTAACCATGTCTTAGTTGCAGTGCC

^a Nucleotide position refers to the nucleotide sequence of *Glomerella tucumanensis* (accession number JN545852)

containing a final concentration of 0.2 μM F3 and B3, 1.6 μM FIP and BIP, 1× ThermoPol buffer [50 mM KCl, 20 mM Tris–HCl, pH 8.8, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1 % Tween 20), 0.8 M betaine, 6 mM MgSO₄, 8 U *Bst* DNA polymerase] (New England Biolabs, Ipswich, MA, USA), 1.4 mM dNTPs, and 1 ng of double-stranded target DNA. Ten microliters of SYBR Green I dye (1:100 dilution, Life Technologies, Grand Island, NY, USA) were added to tube caps before reaction initiation and kept separate until assay completion to avoid potential aerosol contamination. All reaction mixtures were incubated at 63 °C for 1 h, followed by 80 °C for 5 min in a water bath to terminate the reaction. Tubes were briefly centrifuged to allow the dye and reaction to mix. Products were stored at 4 °C. Each assay was done in duplicate and repeated a minimum of three times for verification.

Analysis of LAMP products

The LAMP products were detected visually by mixing 10 μL of SYBR Green I dye (1:100 dilution) with reactions

following termination [24] as described above. Samples that turned yellowish green were considered to be positive, while those samples that remained orange were assumed to be negative [30]. In addition, a 3.5 μL aliquot of all LAMP products were electrophoresed on a 2 % agarose gel containing SYBR Safe DNA gel stain (Life Technologies, Grand Island, NY, USA) and visualized under UV light. The presence of a ladder-like DNA amplification product was considered a positive reaction, while lanes containing no visible bands were considered negative reactions [20].

Optimization of Mg²⁺ concentration

To optimize the Mg²⁺ concentration in the reaction mixture, a total of nine different Mg²⁺ concentrations (2.00, 2.50, 3.00, 3.25, 3.50, 3.75, 4.00, 4.25 and 4.50 mM) were tested. These values represent the final Mg²⁺ concentrations in the 25 μl reaction mixture. The concentration of other components remained constant. Reactions were performed using 1.0 ng of *C. falcatum* genomic DNA. Sterilized water was used as the negative control.

Specificity of LAMP

DNA from another fungal pathogen of sugarcane (*Puccinia melanocephala*) as well as two other species of *Colletotrichum*, namely *Colletotrichum fructivorum* and *Colletotrichum acutatum*, was used to test the specificity of the LAMP protocol developed for the detection of *C. falcatum* DNA. Reactions were performed at the optimum Mg^{2+} concentration (4.0 mM) using sterilized water as the negative control.

Sensitivity of detection amongst LAMP and conventional PCR assays

To compare the detection sensitivities between LAMP and conventional PCR, different concentrations of target DNA, isolated either from pure mycelium culture of *C. falcatum* or pathogen-infected sugarcane tissue, were used to perform the test. Detection limits represent the lowest DNA concentrations at which positive results were observed. Reactions were performed at the optimum Mg^{2+} concentration (4.0 mM) using pathogen-free tissue and sterilized water as negative controls. The conventional PCR using SCAR primers (SCAR-F 5'-CCTACCCAACCGAGTATCG-3' and SCAR-R 5'-GCGCAGCTTGCTCTCAAGAGC-3') was carried out in a 20 μ l reaction mixture containing 67 mM Tris-HCl (pH 8.0), 16.6 mM ammonium sulphate, 0.45 % w/v BSA, 3.5 mM $MgCl_2$, 150 μ M each of dATP, dCTP, dTTP and dGTP, 7.5 pmol (15 ng) each of both reverse and forward primers and 0.5 units *Taq* DNA polymerase. The PCR was performed under the following conditions: 1 cycle at 94 °C for 3 min, followed by 40 cycles, each consisting of a denaturation step at 94 °C for 1 min; an annealing step at 66 °C for 45 s; and an extension step at 72 °C for 1 min. Amplification products were electrophoresed on a 2 % agarose gel containing SYBR Safe DNA gel stain (Life Technologies, Grand Island, NY, USA) and visualized under UV light.

Results

Optimization of LAMP

Of the six sets of primers, use of three primer sets, RRSC1, RRSC4 and RRSC5, resulted in a positive reaction when the concentration of the inner and outer primer pairs was kept at an 8:1 ratio and the concentration of Mg^{2+} at 8.0 mM. The mixtures which showed a positive reaction turned yellowish green, while the mixtures showing no reaction remained orange (Fig. 1a). Agarose gel electrophoresis of the reaction products yielded similar results where positive reactions produced intense ladder-like bands (Fig. 1b). Of the three

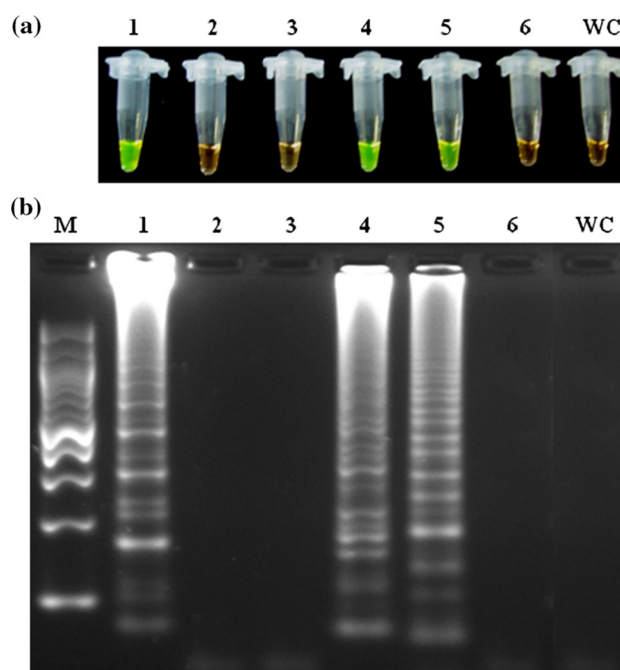


Fig. 1 Screening of six loop-mediated isothermal amplification (LAMP) primer sets (1–6) using *Colletotrichum falcatum* genomic DNA extracted from pure mycelium culture. Lane M 100 bp DNA ladder; lanes 1–6, six different LAMP primer sets (1–6). WC indicates water control. **a** LAMP reaction causing change in color. **b** LAMP products electrophoresed on 2 % agarose gel

primer sets, RRSC1 showed the most intense green color and was used in subsequent reactions for the optimization of Mg^{2+} concentration. At 2.00, 2.50, 3.00, 3.25, 3.50 and 3.75 mM Mg^{2+} , no color change or DNA amplification pattern were observed in either control (sterilized water) or target DNA (*C. falcatum*) samples. However at 4.0 mM Mg^{2+} , only the tube bearing target DNA showed a positive reaction (Fig. 2a). When the concentration of Mg^{2+} was increased, all tested samples turned yellowish green, including the blank control (sterile distilled water). Similar results were also observed in the detection by agarose gel electrophoresis (Fig. 2b).

Optimized LAMP method for the detection of *C. falcatum* and specificity test

The LAMP assay for detecting *C. falcatum* in sugarcane was established based on the optimized reaction conditions described above. The LAMP protocol showed a positive reaction with target DNA isolated from two different strains of *C. falcatum* (Fig. 3a, b). As expected, target DNA of the sugarcane brown rust pathogen, *P. melanocephala*, did not show any reaction. Additionally, when target DNA isolated from conidia of *C. fructivorum* and *C. acutatum* were used along with DNA isolated from

Fig. 2 Effect of $MgSO_4$ concentration on LAMP. Lane M 100 bp DNA ladder; lanes 1, 3, 5, 7, 9, 11, 13, 15, 17 are without *Colletotrichum falcatum* DNA; lanes 2, 4, 6, 8, 10, 12, 14, 16, 18 are with *C. falcatum* DNA (1 ng extracted from pure mycelium culture). WC indicates water control. **a** LAMP reaction causing change in color. **b** LAMP products electrophoresed on 2 % agarose gel

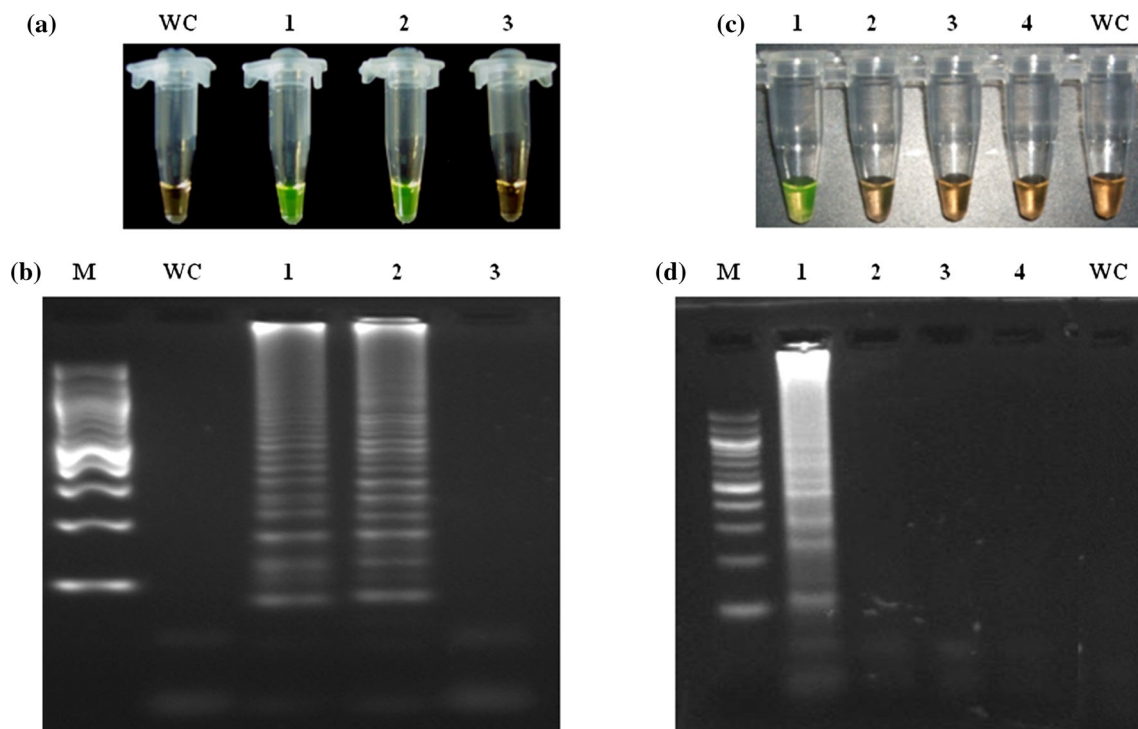
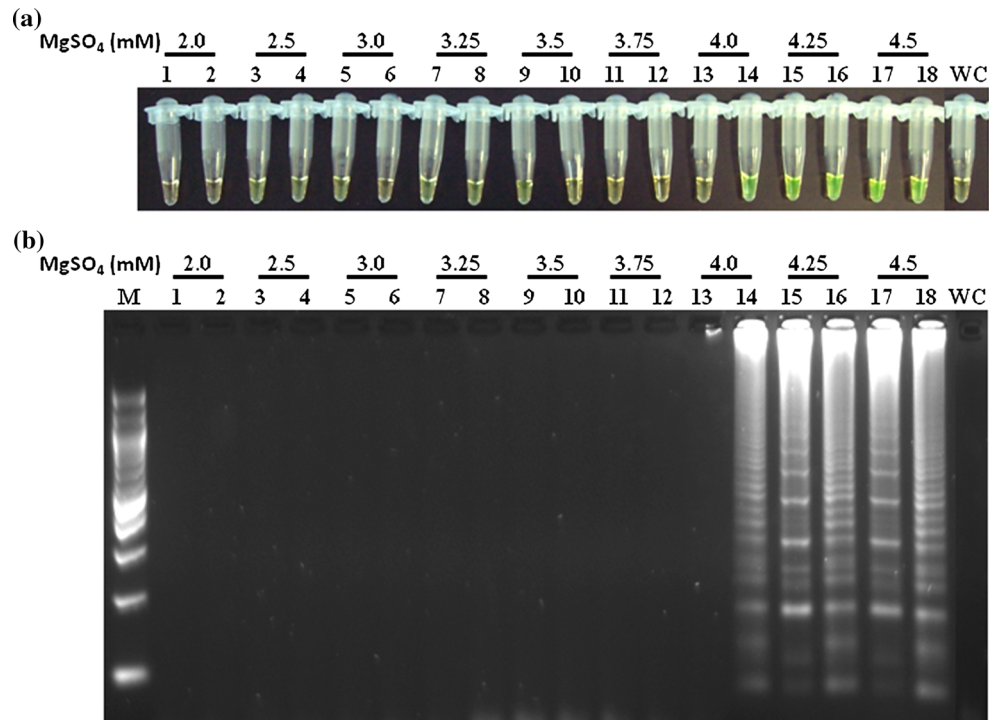


Fig. 3 Specificity test of LAMP primers. **a** LAMP reaction causing change in color. Lane M 100 bp DNA ladder; lane 1, *Colletotrichum falcatum* DNA (cf1010-2) of pure mycelium culture; lane 2, *C. falcatum* DNA (cf11/05) of pure mycelium culture; lane 3, *Puccinia melanocephala* DNA isolated from spores. WC indicates water control. **b** LAMP products electrophoresed on 2 % agarose gel.

c LAMP reaction causing change in color. Lane 1, *C. falcatum* DNA isolated from infected tissues; lane 2, DNA isolated from *Colletotrichum fructivorum* conidium; lane 3, DNA isolated from *Colletotrichum acutatum* conidium; lane 4, DNA isolated from uninfected sugarcane tissues. WC indicates water control. **d** LAMP products electrophoresed on 2 % agarose gel

C. falcatum-infected tissue, a positive reaction was obtained only with *C. falcatum* (Fig. 3c, d).

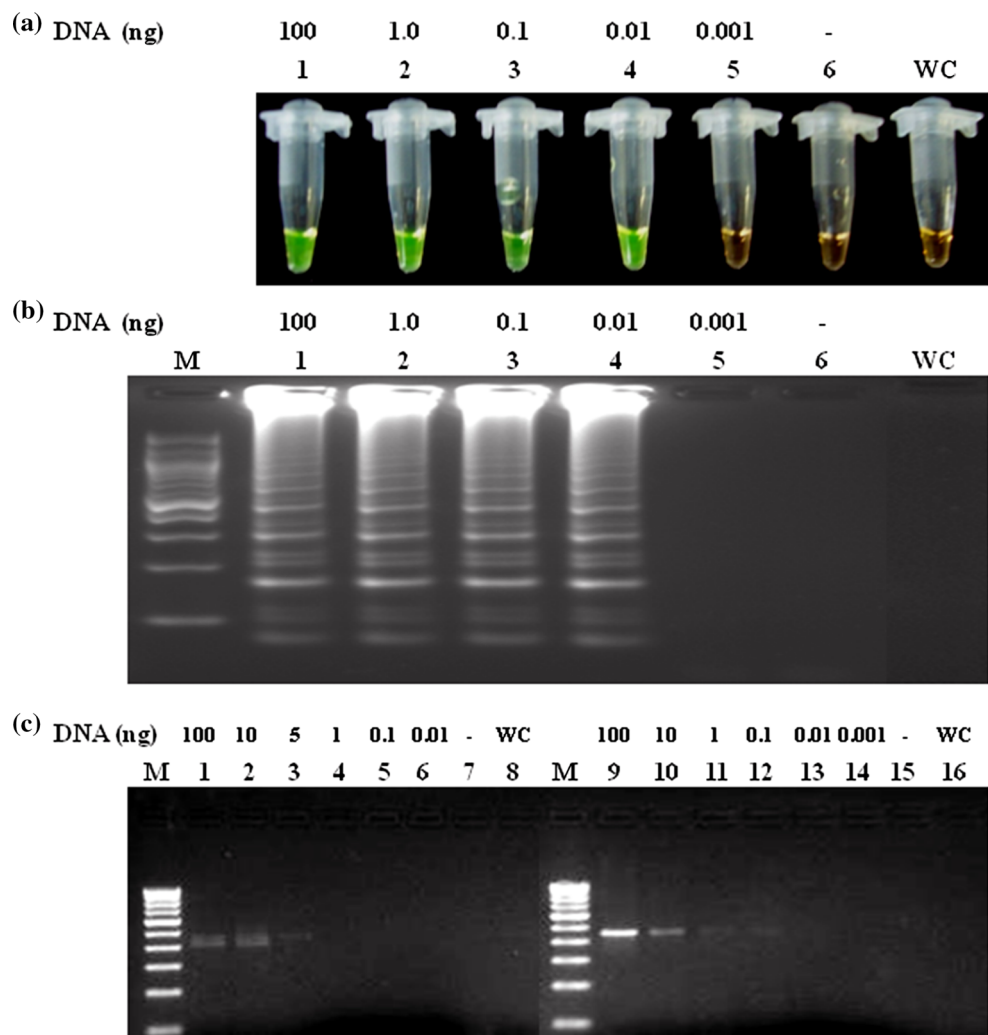
LAMP sensitivity comparison

Serial dilutions of the target DNA were made at the following concentrations: 100, 1.0, 0.1, 0.01 and 0.001 ng. Positive reactions were observed in tubes containing 0.01 ng DNA and greater when the optimized conditions were used (Fig. 4a, b). At 0.001 ng, no reaction was observed in either color change or DNA amplification. To compare the sensitivity of the LAMP protocol with conventional PCR, a SCAR marker was used to amplify the target DNA isolated both from *C. falcatum*-infected tissue and pure *C. falcatum* culture. Sensitivity limits were 0.1 ng with target DNA isolated from pure culture and 5 ng with target DNA isolated from red rot-infected tissues (Fig. 4c). Neither control (negative or sterilized water) gave a positive reaction.

Discussion

In light of the critical requirement for disease-free sugarcane setts, a rapid method of disease detection is highly desirable. The LAMP assay is a relatively new technique where the reaction assay is performed in a single tube incubated at one temperature for approximately 1 h. A visual color change of the reaction mixture is used to indicate the presence of the target DNA. The LAMP assay has exhibited good potential for diagnosis of various plant pathogens [21–23]. For the first time, we developed a LAMP assay for the detection of *C. falcatum* that causes red rot in sugarcane. As reported earlier, the LAMP assay is useful for rapid detection and diagnosis because it can be efficiently performed with limited resources and has the potential to be performed under field conditions [20–22, 25, 31]. The isothermal conditions required by LAMP can be met easily using a water bath or simple thermostat-linked heating block. The amplification efficiency of the LAMP method is

Fig. 4 Detection limit comparison between different amplification assays. **a** LAMP detection of *Colletotrichum falcatum* by reaction color change using 100, 1, 0.1, 0.01 and 0.001 ng of input target DNA isolated from *C. falcatum*. **b** Gel electrophoresis of LAMP products for *C. falcatum* at the indicated concentrations. **c** Conventional PCR using SCAR marker to detect *C. falcatum* using DNA isolated from infected tissues (lanes 1–6) and *C. falcatum* pure mycelium culture (lanes 9–14) at the indicated concentrations. – depicts tubes and wells used as negative controls. WC indicates water control. Lane M 100 bp DNA ladder



extremely high because all reactions are conducted at a constant, optimal temperature suitable for the enzyme. Involvement of four primers targeting six regions of the target genome makes the diagnostic tool very sensitive, specific, and fast because the sequences of these regions and corresponding primers assure the specificity of the reaction, and the structures of the primers ensure loop initiation and elongation of the targeted DNA [20]. Hence, it is important that these primers be developed from conserved regions of the target DNA. One reason for the false positive reactions, as reported earlier [21], may be aerosol deposition of the positive samples into controls. This is easily avoided by using SYBR green in the lid of the reaction tube before reaction initiation, which can be mixed after the reaction is completed to induce color change. Gel electrophoresis and visualization of positive samples involving hazardous chemicals like ethidium bromide is avoided because disease detection through LAMP is possible through color change.

In order for the LAMP primers to work effectively, it is necessary to calibrate the four required primers (F3, B3, FIP and BIP) with Mg^{2+} concentration. Guan et al. [24] also reported that optimization of different primer concentrations and the ratios between inner and outer primers (F3 and B3) was essential for the development of a LAMP assay to detect genetically modified soybean events [24]. In the present investigation, 4.0 mM Mg^{2+} concentration was the most effective compared to 5.75 mM in the case of ratoon stunt in sugarcane [21]. Although the critical value of the Mg^{2+} concentration differed, the Mg^{2+} concentration is considered to be the most critical component in optimizing LAMP assays [22–25]. At concentrations higher than the optimum, positive results were observed even in negative samples, such as in the reaction observed at 4.25 mM and above in the present study (Fig. 2). Since *Taq* polymerase is a Mg^{2+} dependent enzyme, the optimal concentration of Mg^{2+} is critical to the success of the PCR [32]. In presence of high Mg^{2+} concentrations, primers usually bind to an incorrect template and stabilize double stranded DNA which prevents complete denaturation, resulting in decreased specificity and reduced product yield [32]. On the other hand, variable $MgCl_2$ resulted in the formation of concentration gradients within the $MgCl_2$ solution supplied with the DNA polymerase and thus resulted in unreliable data [32]. Nie [22] also made a similar observation on Mg^{2+} concentration while detecting Potato virus Y through reverse-transcription loop mediated isothermal amplification assay.

Under the optimum level of Mg^{2+} (4.0 mM), the detection limit of our LAMP assay was 0.01 ng with target DNA isolated from *C. falcatum*, which was 10 times more sensitive when compared to conventional PCR where a minimum of 0.1 ng DNA was required for detection of

DNA isolated from pure *C. falcatum* culture. In addition, the LAMP sensitivity was five hundred times greater than conventional PCR using target DNA from *C. falcatum*-infected tissues. Liu et al. [21] also reported LAMP to be 10 times more sensitive than conventional PCR for the sugarcane ratoon pathogen when DNA was isolated from stem juice. However, the detection level was almost the same when target DNA was isolated from tissues.

The LAMP assay developed in the present study is specific for *C. falcatum* and because of its low detection limit (0.01 ng DNA), is useful for early detection of the disease. We also demonstrated the specificity of LAMP as it did not react with DNA of another common fungal pathogen of sugarcane (*P. melanocephala*), or other species of *Colletotrichum* (*C. fructivorum* and *C. acutatum*). Furthermore, the LAMP primers used in this study were designed to target the same 442 bp fragment amplified using a SCAR marker demonstrated by Nithya et al. [7]. When this marker was employed, the target sequence was successfully amplified from all 31 isolates/races obtained in India without amplifying sequences in other closely related *Colletotrichum* species. Similar results were obtained by our group after using the SCAR sequence primers for the two strains isolated in Louisiana (data not shown). Hence, we have confidence that our assay can detect a wide variety of *C. falcatum* strains regardless of geographic region. Further research using more geographically diverse isolates/strains in our LAMP assay will be conducted to determine this.

This work indicates the successful development of a visual, rapid detection method for *C. falcatum* causing red rot in sugarcane. This is a simple time-saving diagnostic tool where the reaction can be completed in < 1 h in a single tube under isothermal conditions. Thus, it offers a sensitive and efficient method for diagnosis of *C. falcatum* and has the potential to be applied to the detection of other pathogens in sugarcane in research and diagnostic laboratories, as well as, under field conditions. The manifestation of diseases can be minimized through appropriate control measures if they are accurately detected using a better diagnostic tool like LAMP.

Acknowledgments Amaresh Chandra is grateful to the Department of Biotechnology, Government of India, New Delhi, India for awarding the DBT-CREST Fellowship (BT/IN/DBT-CREST Awards/05/AC/2012-12). Amber Keizerweerd and Michael Grisham are thankful for financial support provided by the United States Department of Agriculture, Agriculture Research Service (CRIS Project: 6410-22000-013-00D). Drs. J. W. Hoy and Vinson Doyle are greatly appreciated for their help in providing the *C. falcatum*, *C. fructivorum*, and *C. acutatum* isolates. We also thank Dr. Duncan Clark of OptiGene Limited for his assistance in primer design.

Conflict of interest The authors declare that they have no conflict of interest.

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