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RAPID AND VISUAL DETECTION OF *COLLETOTRICHUM GLOEOSPORIOIDES* ON *ANOECTOCHILUS* USING LOOP-MEDIATED ISOTHERMAL AMPLIFICATION ASSAY

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A B S T R A C T

Anthracnose, caused by *Colletotrichum gloeosporioides*, is among the most destructive diseases to the plant genus *Anoectochilus* in China. *C. gloeosporioides* can enter and spread through a commercial field via asymptomatic infected plants. Early and accurate detection of *C. gloeosporioides* is essential to controlling this disease in the field. To enable rapid detection of the pathogen on symptomless *Anoectochilus* plants, we developed a convenient, cost-effective, and highly sensitive loop-mediated isothermal amplification (LAMP) method. Four LAMP primers were designed based on the internal transcribed spacer (ITS) sequence of *C. gloeosporioides*. All 25 *C. gloeosporioides* isolates collected from geographically distinct counties in China yielded positive results in the LAMP assay. No cross-reaction was observed with other fungal pathogens. A sensitivity assay showed that the PCR and LAMP assays had detection limits of 10 pg and 10 fg of genomic DNA per 25-µL reaction, respectively. Furthermore, we used the PCR and LAMP assays for the detection of *C. gloeosporioides* DNA from naturally infected *Anoectochilus* plants. The LAMP assay developed in this study is simple, fast, sensitive, and specific, and can be used in the field to detect *C. gloeosporioides* in infected plant tissue.

Keywords: *Colletotrichum gloeosporioides*, detection, LAMP, sensitivity.

INTRODUCTION

Anoectochilus (*Anoectochilus roxburghii*) is listed in the earliest known Chinese Materia Medica and has been used as a traditional Chinese medicine (Cai *et al*. 2003). Anthracnose, caused by *Colletotrichum gloeosporioides*, is considered to be one of the most destructive plant diseases and leads to serious losses in yield and quality of *Anoectochilus* (Chen *et al*. 2015). This disease can severely damage *Anoectochilus* plants in warm and humid areas. Anthracnose lesions can form on seedlings, and the disease affects all aboveground parts of the *Anoectochilus* plant. Given the current situation, the disease poses a real threat to the future of the *Anoectochilus* industry in China (Wang *et al*. 2013; Chen *et al*. 2015).

The availability of a rapid, reliable, and sensitive method for early detection of the pathogen could facilitate epidemiological studies and the implementation of appropriate control measures. However, *C. gloeosporioides* are difficult to identify because *C. gloeosporioides* species complex is defined genetically on the basis of multi-gene phylogenies, and twenty two species plus one subspecies were accepted within the *C. gloeosporioides* complex (Weir *et al*. 2012; Liu *et al*. 2013). Moreover, traditional diagnostic methods for the identification of plant pathogens are time consuming. These methods also require considerable expertise, as they require differentiation between species based on morphological characteristics (Senaratna *et al*. 1991; Martinez-Culebras *et al*. 2000).

Common methods currently used for the detection of *C. gloeosporioides* are based on the conventional polymerase chain reaction (PCR) amplification (Mills *et al*. 1992; Chen *et al*. 2006; Tapia-Tussell *et al*. 2008). This is a valuable tool for investigating latent infection and the early stages of disease. However, PCR amplification based detection has several intrinsic disadvantages including the requirement for rapid thermal cycling, insufficient specificity, and rather low amplification efficiency (Parida *et al*. 2008; Chen *et al*.

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2013). Taking such disadvantages into account, several methods have been developed that do not need temperature cycling or rapid heating and cooling including nucleic acid sequence-based amplification (NASBA), loop-mediated isothermal amplification (LAMP), helicase-dependent amplification (HDA), rolling circle amplification (RCA), recombinant polymerase amplification (RPA), and multiple displacement amplification (MDA) (Gabrielle *et al*. 1993; Notomi *et al*. 2000; Gill *et al*. 2008). In contrast, the LAMP method was adapted for the detection of plant pathogens under isothermal conditions using a simple incubator, such as a water bath or heating block, and the strand displacement-DNA synthesis activity of *Bst* DNA polymerase (Notomi *et al*. 2000; Nakao *et al*. 2010).

The LAMP method was developed to amplify nucleic acids with high specificity, sensitivity, and rapidity under isothermal conditions (Nakao *et al*. 2010). The method is easy to perform, and is based on the reaction of a DNA polymerase with strand displacement activity and a set of two specially designed inner primers (FIP and BIP) and two outer primers (F3 and B3). The end LAMP products can be visualized with the naked eye by adding DNA-intercalating dyes or by measuring the increase in turbidity derived from magnesium pyrophosphate formation (Mori and Notomi 2009; Mori *et al*. 2013). The simplicity of the LAMP method, which does not require a thermal cycler, makes it suitable for field testing.

In this study, we developed a LAMP assay based on internal transcribed spacer (ITS) regions for the sensitive and rapid detection of *C. gloeosporioides* using calcein-$MnCl_2$ as an indicator. This is the first study on the detection of *C. gloeosporioides* that targets the ITS region in genomic DNA using a LAMP method. The LAMP protocol described in this study represents a specific, sensitive, and rapid diagnostic protocol for *C. gloeosporioides* detection.

MATERIALS AND METHODS

Isolates and DNA extraction: The origin, host, and numbers of isolates used in this study are listed in Table 1. We tested 25 *C. gloeosporioides* isolates sampled from a wide geographic distribution in China, as well as 28 isolates of other non-*C. gloeosporioides* fungi. Fungi were cultured for approximately 5 d in potato-dextrose agar (PDA) (Kuramae-Izioka 1997). Fungal mycelia were grown in potato-dextrose broth for approximately 5 d. The mycelia were harvested by filtration and freeze-dried for 36 h. DNA was extracted using either the cetyltrimethyl ammonium bromide (CTAB) procedure (Chen *et al*. 2013) or a Fast Quant RT Kit with gDNase (Qiagen, Hilden, Germany) according to the manufacturer's instructions as previously described (Chen *et al*. 2013). Purified DNA was quantified using a spectrophotometer, and aliquots were diluted to 100 ng μL^{-1} in distilled water and stored at –20°C. This DNA was used as template in a loop-mediated isothermal amplification (LAMP) test.

Table 1. Fungal isolates used in the LAMP assay.

Specie[a]	Host	No. of isolates	Source	LAMP[b]	PCR[b]
Colletotrichum gloeosporioides	*Anoectochilus roxburghii*	9	Yongtai, Fujian, China	+	+
Colletotrichum gloeosporioides	*Anoectochilus roxburghii*	8	Minqing, Fujian, China	+	+
Colletotrichum gloeosporioides	*Anoectochilus roxburghii*	5	Nanping, Fujian, China	+	+
Colletotrichum gloeosporioides	*Capsicum annuum*	1	Fujian, China	+	+
Colletotrichum gloeosporioides	*Mangifera indica*	1	Fujian, China	+	+
Colletotrichum gloeosporioides	*Citrus reticulata*	1	Fujian, China	+	+
Colletotrichum acutatum	*Eriobotrya japonica*	1	Fujian, China	_	_
Colletotrichum circinans	*Allium fistulosum*	1	Fujian, China	_	_
Colletotrichum coccodes	*Solanum tuberosum*	1	Fujian, China	_	_
Colletotrichum corchori	*Corchorus capsularis*	1	Fujian, China	_	_
Colletotrichum falcatum	*Saccharum officinarum*	1	Fujian, China	_	_
Colletotrichum fragariae	*Fragaria ananassa*	1	Fujian, China	_	_
Colletotrichum glycines	*Glycine max*	1	Fujian, China	_	_
Colletotrichum graminicolum	*Zea mays*	1	Fujian, China	_	_
Colletotrichum gossypii	*Gossypium spp*	1	Fujian, China	_	_
Colletotrichum higginsianum	*Brassica pekinensis*	1	Fujian, China	_	_
Colletotrichum lini	*Linum usitatissimum*	1	Fujian, China	_	_
Colletotrichum lycopersici	*Lycopersicon esculentum*	1	Fujian, China	_	_
Colletotrichum orbiculare	*Cucumis sativus*	1	Fujian, China	_	_
Magnaporthe oryzae	*Oryza sativa*	1	Fujian, China	_	_

Fusarium oxysporum	*Anoectochilus roxburghii*	1	Fujian, China	_	_
Pythium ultimum	*Anoectochilus roxburghii*	1	Fujian, China	_	_
Rhizoctonia solani	*Anoectochilus roxburghii*	1	Fujian, China	_	_
Fusarium moniliforme	*Gossypium hirsutum*	1	Fujian, China	_	_
Fusarium graminearum	*Triticum aestivum*	1	Fujian, China	_	_
Verticillium dahliae	*Solanum melongena*	1	Fujian, China	_	_
Gloeosprium piperatum	*Capsicum frutescent*	1	Fujian, China	_	_
Alternaria solani	*Solanum melongena*	1	Fujian, China	_	_
Alternaria alternata	*Solanum lycopersicum*	1	Fujian, China	_	_
Botryosphaeria rhodina	*Psidium guajava*	1	Fujian, China	_	_
Magnaporche oryzae	*Oryza sative*	1	Fujian, China	_	_
Sclerotinia sclerotiorum	*Brassica oleracea*	1	Fujian, China	_	_
Botrytis cinerea	*Solanum lycopersicum*	1	Fujian, China	_	_
Rizoctonia solani	*Brassica campestris*	1	Fujian, China	_	_

[a]All isolates were maintained in the collection of Fujian Academy of Agricultural Sciences.

[b]Note that presence (+) or absence (-) are based on the presence of a LAMP reaction or PCR product of expected size.

LAMP primers design: Alignment of the internal transcribed spacer (ITS) nucleotide sequences of *C. gloeosporioides* (GenBank accession number KM357453 and referenced to Chen *et al*., 2015) was performed using the GenBank/National Centre for Biotechnology Information (NCBI) website. Sets of four primers for LAMP were designed to target the ITS region of *C. gloeosporioides*. The LAMP primer software Primer Explorer (ver. 4) (http://primerexplorer.jp/elamp4.0.0 /index.html; Eiken Chemical Co., Tokyo, Japan) was used to design the primers. A set of four primers including a forward inner primer, (FIP:5'-GCCACTACCTTTGAGGGCCTAC-TTTCAACCCTCAA GCTCTGC-3'), a backward inner primer, (BIP:5'-CGGAGCCTCCTTTGCGTAGTAA-GGGTTTTACGGC AAGAGTCC-3'), and two outer primers (F3:5'-ATGCCTGTTCGAGCGTCA-3' and, B3: 5'-TCCGAGGTCAACCTTTGGAA-3') was chosen from the candidate primer sets (Figure 1).

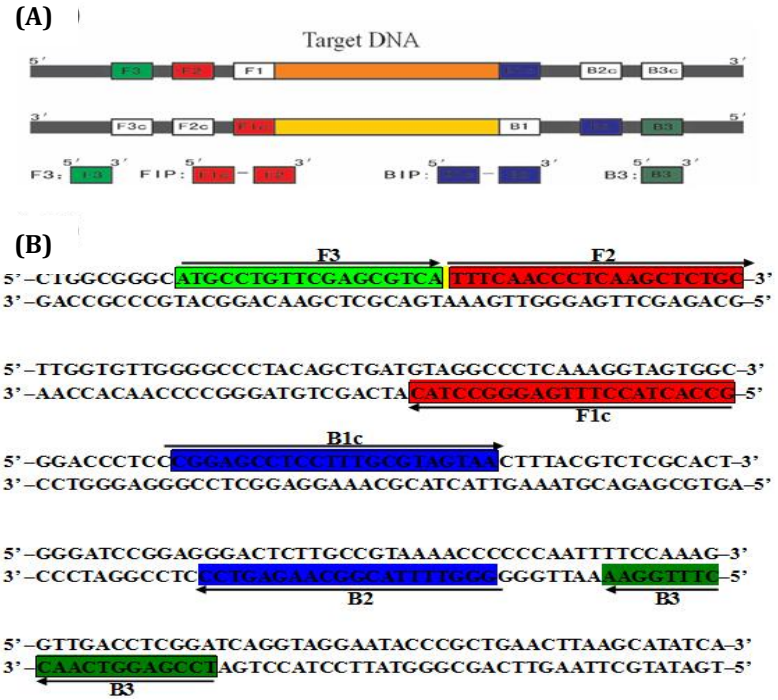

Figure 1. Location and partial sequence of LAMP primer sets targeting the specific ITS sequence. (A) Schematic diagram of LAMP primers. (B) Nucleotide sequences of target DNA used for the LAMP primers [F3, B3, FIP (F1c-F2), and BIP (B1c-B2)] for the ITS. FIP is a hybrid primer consisting of the F1c and F2 sequences; BIP is a hybrid primer consisting of the B1c and B2 sequences. Arrows indicate the extension direction.

Optimization of the LAMP assay: LAMP reactions were performed in volumes of 25 µL. The reaction mixture contained 40 pmol each of FIP and BIP primers, 5 pmol each of F3 and B3 primers, 20 ng of template DNA, 8 units of *Bst* DNA polymerase, and 12.5 µL of a 2× reaction mix, which contained 10× Thermopol buffer, 25 mM dNPTs, 5 M betaine (Sigma, St. Louis, MO, USA), 50 mM $MgSO_4$, 2% Tween 20, and 1 µL of 50 µM calcein-500 µM $MnCl_2$. Negative controls containing nuclease-free water instead of DNA were included in each assay. To identify the optimal temperature and time for visual LAMP amplification, the reactions were performed in water baths at 61, 62, 63, 64, and 65°C for 30, 45, 60, and 75 min. The reactions were terminated by heating at 85°C for 10 min. The LAMP product was detected by naked-eye inspection or agarose gel electrophoresis to determine the optimal reaction conditions (Chen *et al*. 2013). The results correlated well with gel electrophoresis. The optimal reactions under isothermal conditions were 65°C for 60 min followed by 85°C for 10 min. All of the experiments were repeated at least three times.

LAMP assay specificity: To determine the specificity of LAMP for the target *C. gloeosporioides*, 25 *C. gloeosporioides* isolates and isolates of 28 other fungi (Table 1) were subjected to LAMP assays. The LAMP amplification products were detected as described above. All of the experiments were repeated at least three times.

LAMP assay sensitivity: To determine the sensitivity of LAMP, 10-fold dilutions ranging from 100 ng to 10 fg of purified *C. gloeosporioides* DNA were made and used to determine the analytical sensitivity of the LAMP test. Based on the ITS sequence, we designed a specific PCR primer pair, CG-F/CG-R and the specificity and sensitivity of the CG-F/CG-R primers was evaluated to compare the LAMP assays. 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 65°C, 1 min at 72°C, followed by 1 cycle of 10 min at 72°C). The LAMP amplification products were detected as described above. PCR amplification products were separated by gel electrophoresis on 2.0% agarose gels, stained with ethidium bromide, and visualized under UV light. All of the experiments were repeated at least three times.

LAMP detection of diseased samples: To assess the field application of LAMP for *C. gloeosporioides* investigation and management, 32 *Anoectochilus* samples infected with *C. gloeosporioides* from various natural fields in the Fujian province were assayed using LAMP assays. Non-infected *Anoectochilus* were used as controls. DNA extraction from the infected plants was performed as previously described (Chen *et al*. 2013). The LAMP reactions were performed at 65°C for 60 min. Amplification was monitored with gel electrophoresis. *C. gloeosporioides* infection was confirmed using the conventional culture method for the identification of plant pathogens based on morphological characteristics (Weir *et al*. 2012). All of the experiments were repeated at least three times.

RESULTS AND DISCUSSION

Optimization of the LAMP assay: LAMP was performed using *C. gloeosporioides* DNA as template to determine the optimal temperature and reaction time based on the amount of product. The best results were achieved when the reaction temperature and time were 65°C and 60 min, respectively. Following the LAMP reaction, the positive reactions turned green, while the negative ones remained orange. Furthermore, the positive LAMP reactions produced a characteristic ladder of multiple bands on a 2% agarose gel, whereas no amplification was detected in the negative control (Figure 2).

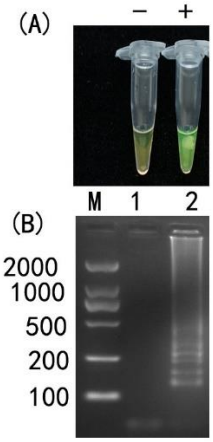

Figure 2. Detection of *C. gloeosporioides* using the optimized LAMP method. (A) LAMP assay and visual inspection using calcein observed under visible light. (B) The LAMP assay was evaluated using 2.0% agarose gel electrophoresis. Positive reactions turned green after the addition of calcein. Lane 1, negative control; Lane 2, *C. gloeosporioides*; and Lane M, DL2000 DNA markers. Tube (+) represents the positive *C. gloeosporioides*; Tube (–) represents the negative control.

Specificity of the LAMP assay: LAMP specificity was examined using DNA templates extracted from 25 *C. gloeosporioides* isolates and 28 isolates of non-*C. gloeosporioides* fungal species (Table 1). After incubation at 65°C for 60 min, the *C. gloeosporioides* samples showed a positive reaction, whereas no amplification was observed for the non-*C. gloeosporioides* samples (Figure 3). In addition, the identities of the amplified products were confirmed through direct sequencing. The lowest band from the amplicons was purified and cloned into the pMD18-T vector (Takara Biotechnology Co., Ltd., Otsu, Japan), and the insert was sequenced (Takara); the sequences we obtained were perfect matches with the expected DNA sequences (data not shown). These results indicated that the LAMP assay we developed was highly specific to *C. gloeosporioides*.

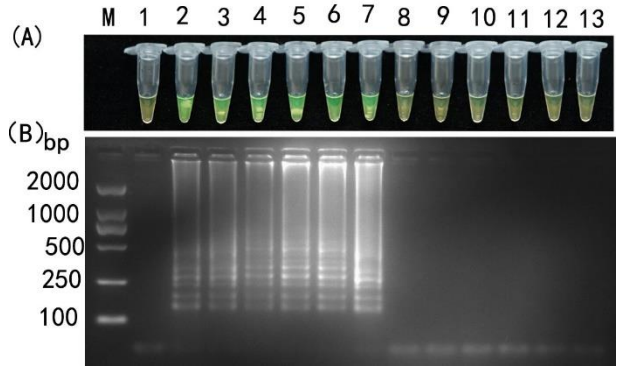

Figure 3. Specificity of the LAMP assay for detection of *C. gloeosporioides*. (A) LAMP assay and visual inspection by pre-addition of calcein for fluorescence observed under visible light. (B) LAMP assay detected by agarose gel electrophoresis. Lane M, DL2000 DNA marker; Lane 1, negative control (sterile distilled water); Lanes 2–7, *C. gloeosporioides*; Lane 8, *C. glycines*; Lane 9, *C. higginsianum;* Lane 10, *C. lycopersici;* Lane 11, *Fusarium oxysporum*; Lane 12, *Alternaria solani*; and Lane 13, *Sclerotinia sclerotiorum*. The same results were obtained in all four replicates.

Sensitivity of the LAMP assay: To determine the detection limit, the sensitivities of the PCR and the LAMP assay were assessed using 10-fold serial dilutions of the total DNA extracted from *C. gloeosporioides*. In conventional PCR, the CG-F/(5'-CGGAGGATAACCAAACTCTG-3') /CG-R: (5'-CGAGACGTAAAGTTACTACGC-3') primer pair detected up to 10 pg of purified DNA per 25 µL of reaction volume (Figure 4). In contrast, the minimum detection limit for the LAMP assay was 10 fg of genomic DNA (Figure 4). That is, the sensitivity of the LAMP assay was 1,000-fold higher than that of conventional PCR with a detection limit of 10 fg for a 60-min reaction. Our results suggest that the LAMP assay is more sensitive than PCR based assay. These observations also showed that, based on visual inspection, the results of the LAMP assay were consistent with gel electrophoresis.

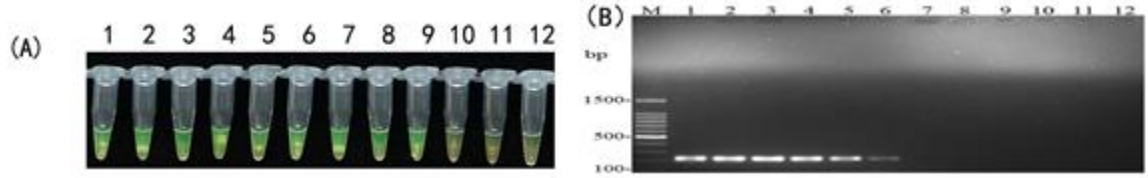

Figure 4. Sensitivity of the PCR and LAMP assays for detection of *C. gloeosporioides* using serial DNA dilutions. (A) LAMP assay and visual inspection by pre-addition of calcein for fluorescence observed under visible light. (B) PCR assay assessed using 2.0% agarose gel electrophoresis. Lane M, DL 1,500 DNA ladder markers; Lanes 1–12, amplified products using DNA at 1000 ng, 100 ng, 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg, 100 ag, and 10 ag in a 25-µL reaction. The same results were obtained in all four replicates.

LAMP assays of infected *Anoectochilus*: To demonstrate the applicability of the LAMP method in field samples, 32 *Anoectochilus* samples gathered from natural fields in Fujian, China, were tested using the LAMP and PCR assays. Among the 32 *Anoectochilus* samples, 28 samples were positive by the LAMP assay and 22 were positive by PCR, and the pathogen was detected in all 28 samples using the conventional culture method. This was not the case for four *Anoectochilus* samples in which *C. gloeosporioides* was not detected by the conventional culture method. These results indicate the high detection capability of the LAMP assay.

Anthracnose, caused by *C. gloeosporioides*, is a major threat to *Anoectochilus* production (Chen *et al*. 2015). Rapid and accurate detection of *C. gloeosporioides* is vital to prevent the introduction and spread of *C. gloeosporioides* into non-endemic areas, and to control and minimize damage to *Anoectochilus* production industries. Although rapid detection of *C. gloeosporioides* by PCR has been reported (Mills *et al*. 1992; Chen *et al*. 2006), we failed to detect *C. gloeosporioides* in field samples using this method. In addition, the PCR reaction products are not visible to the naked eye, but must be visualized by staining with ethidium bromide following gel electrophoresis. Taking such disadvantages into account, we have developed the first visual detection method for *C. gloeosporioides* based on ITS sequences using the LAMP method. The diagnosis of anthracnose in agricultural efforts using morphological microscopic observation and PCR-based molecular methods has also been developed (Senaratna *et al*. 1991; Martinez-Culebras *et al*. 2000; Tapia-Tussell *et al*. 2008). Compared with morphological microscopic observation and conventional PCR, the LAMP assay presented here is more advantageous owing to its simpler operation, more rapid reaction, and easier detection. In this study, the optimal temperature and time of LAMP for the detection of *C. gloeosporioides* were determined to be 65°C and 60 min, respectively. Because LAMP is conducted at one temperature, no time is wasted by changes in temperature, as is the case for thermal cycling with PCR. Moreover, LAMP requires only a standard laboratory water bath or heat block that can provide a constant temperature of 65°C. Another very important advantage of LAMP is that the amplified products can be detected visually by simply adding the dye calcein, i.e., electrophoresis is not required. Because the LAMP assay is simple and relatively easy to perform, it should be useful even for those laboratories and research institutes that are unfamiliar with PCR or other methods of molecular analysis.

In this study, the LAMP method used for *C. gloeosporioides* detection was found to be highly sensitive; it detected 10 fg of genomic DNA, while, the detection limit for *C. gloeosporioides* by PCR was 10 pg (Figure 4). This indicates that LAMP is 1000 times more sensitive than standard PCR. This increased sensitivity makes LAMP a better choice than PCR for the diagnosis of lower concentrations of *C. gloeosporioides*. Furthermore, this LAMP assay can be applied successfully to the diagnosis of relatively crude DNA isolated from plant samples. Thus, when compared with conventional methods, LAMP has the advantages of saving time, being low in cost, and being easy to conduct, which significantly increase the efficacy of *C. gloeosporioides* diagnosis and management. However, as in the case of gel electrophoresis analysis, a colorimetric assay using intercalating dye is associated with an increased risk of contamination for other subsequent LAMP reaction solutions (because the assay requires opening the tubes). To avoid such contamination, separate rooms should be used for LAMP setup and analysis. Fortunately, calcein has been developed to allow visual discrimination of positive LAMP samples (Tomita *et al*. 2008; Zhang *et al*. 2012) and we used the pre-addition of calcein-$MnCl_2$ before incubation; the color change from orange to green in positive LAMP amplifications can be judged under natural light and UV light, and was consistent with gel electrophoresis results. Compared with other methods used to visually detect endpoints, such as those based on the visualization of turbidity (Mori *et al*. 2001; Dai *et al*. 2012), the addition of DNA intercalating dyes (Hill *et al*. 2008; Niu *et al*. 2012; Duan *et al*. 2014), and the use of calcein, this method is simpler (Tomita *et al*. 2008; Chen *et al*. 2013). Furthermore, calcein can be added before incubation so that the reaction tubes need not be opened, which reduces the risk of cross contamination.

The ITS region is highly conserved among *Colletotrichum* spp. It contains ITS1, the 5.8S RNA, and ITS2 between the 18S RNA and 28S RNA gene fragments (Martinez-Culebras *et al*. 2000; Peres *et al*. 2002). We developed four primer pairs for LAMP based on the ITS region of *C. gloeosporioides*; our results suggest that the assay and primers are specific and sensitive enough to differentiate *C. gloeosporioides* from other *Colletotrichum* and fungal species. Previous reports have also indicated that the ITS region is sufficiently variable to allow specific discrimination of all *Colletotrichum* species, and this locus can be used for PCR-based detection of *Colletotrichum* species (Martinez-Culebras *et al*. 2000; Peres *et al*. 2002; Chen *et al*. 2006). In this study, the ITS-based LAMP assay was also found to be suitably sensitive and specific for testing artificially and naturally infected *Anoectochilus* plants.

To our knowledge, this is the first study to use the ITS-LAMP technique to detect *C. gloeosporioides*. This method is useful for detecting low levels of *C.*

gloeosporioides in plant tissues. Thus, samples infected with *C. gloeosporioides* can be identified in the early stages of infection, and management measures can be designed before the infection becomes epidemic.

In summary, we have established a LAMP assay and demonstrated that it is more sensitive, specific, and practical for the detection of *C. gloeosporioides* than previous methods. Therefore, this new LAMP assay has the potential to be useful for monitoring and controlling the occurrence of *C. gloeosporioides* in *Anoectochilus* production industries.

CONCLUSION

In this study, we developed a loop-mediated isothermal amplification (LAMP) technology to rapidly detect *C. gloeosporioides*. The high sensitivity and specificity and its ease of use make this assay ideal for use in resource-limited settings such as in developing countries.

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REFERENCE

Cai, W., Xiao, H., and Fan, X. 2003. A review of research on *Anoectochilus roxburghii*. *Subtropical Plant Sci* 32, 68-72.

Chen, L., Chu, C., Liu, C.D., Chen, R.S., and Tsay, J.G. 2006. PCR-based detection and differentiation of anthracnose pathogens, *Colletotrichum gloeosporioides* and *C.truncatum*, from Vegetable Soybean in Taiwan. *J. Phytopathol* 154, 654-662.

Chen, Q., Li, B., Liu, P., and Weng, Q. 2015. First report of anthracnose caused by *Colletotrichum gloeosporioides* on *Anoectochilus roxburghii* in China. *Plant Dis* 100, 531.

Chen, Q., Li, B., Liu, P., Lan, C., Zhan, Z., and Weng, Q. 2013. Development and evaluation of specific PCR and LAMP assays for the rapid detection of *Phytophthora melonis*. *Eur J Plant Pathol* 137, 597-607.

Dai T, Lu C, Lu J, Dong S, Ye W, Wang Y, and Zheng X. 2012. Development of a loop-mediated isothermal amplification assay for detection of *Phytophthora sojae*. *FEMS Microbiol Lett* 334, 27-34.

Duan, Y., Ge, C., Zhang X., Wang J. and Zhou M. 2014. Development and evaluation of a novel and rapid detection assay for *Botrytis cinerea* based on loop-mediated isothermal amplification. *PLoS ONE* 9(10): e111094.

Gabrielle, M.E., van der, V., Schukkink, R.A.F., Gemen, B., and Klatser, S. 1993. Nucleic acid sequence-based amplification (NASBA) for the identification of mycobacteria. *Microbiology.* 139, 2423-2429.

Gill, P., and Ghaemi, A. 2008. Nucleic acid isothermal amplification technologies—A Review. *Nucleos Nucleot Nucl* 27,:224–243.

Hill, J., Beriwal, S., Chandra, I., Paul, V.K., and Kapil, A. 2008. Loop-mediated isothermal amplification assay for rapid detection of common strains of *Escherichia coli*. *J Clin Microbiol* 46, 2800–2804.

Kuramae-Izioka, E. E. 1997. A rapid, easy and high yield protocol for total genomic DNA isolation of *Colletotrichum gloeosporioides* and *Fusarium oxysporum*. *Revista Unimar*. 19, 683-689.

Liu, F., Damm, U., Cai, L., and Crous, P. W. 2013. Species of the *Colletotrichum gloeosporioides* complex associated with anthracnose diseases of *Proteaceae*. *Fungal Divers.* 61, 89-105.

Martinez-Culebras, P.V., Barrio, E., Garcia, M.D., and Querol, A. 2000. Identification of *Colletotrichum gloeosporioides* responsible for anthracnose of strawberry based on the internal transcribed spacers of the ribosomal regions. *FEMS Microbiol Lett* 189, 97-101.

Mills, P. R., Sreenivasaprasad, S., Brown, A. E. 1992. Detection and differentiation of *Colletotrichum gloeosporioides* isolates using PCR. *FEMS Microbiol Lett*. 98,137-143.

Mori, Y., Kanda, H., and Notomi T. 2013. Loop-mediated isothermal amplification (LAMP): recent progress in research and development. *J infect chemother* 19, 404-411.

Mori, Y., and Notomi T. 2009. Loop-mediated isothermal amplification (LAMP): a rapid, accurate, and cost-effective diagnostic method for infectious diseases. *J infect chemother*. 15, 62-69.

Mori, Y., Nagamine, K., Tomita, N., and Notomi, T. 2001. Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. *Biochem Bioph Res Co*. 289, 150-154.

Nakao, R., Stromdahl, E., Magona, J., Faburay, B., Namangala, B., Malele, I., Inoue, N., Geysen, D., Kajino, K., Jongejan, F., and Sugimoto, C. 2010. Development of loop-mediated isothermal amplification (LAMP) assays for rapid detection of *Ehrlichia ruminantium*. *BMC Microbiol*. 10:296.

Niu, J., Jian, H., Guo, Q., Chen, C., and Wang, X. 2012. Evaluation of loop-mediated isothermal amplification (LAMP) assays based on 5S rDNA-IGS2 regions for detecting *Meloidogyne enterolobii*. *Plant Pathol* 61, 809–819.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., and Hase, T. 2000. Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res*. 28(12): E63.

Parida, M., Sannarangaiah, S., Dash, P. K., Rao, P. V. L. and Morita, K. 2008. Loop mediated isothermal amplification (LAMP): a new generation of innovative gene amplification technique; perspectives in clinical diagnosis of infectious diseases. *Rev Med Virol* 18, 407-421.

Peres, N. A. R., Kuramae, E. E., Dias, M. S. C., and de Sousa, N. L. 2002. Identification and characterization of *Colletotrichum* spp affecting fruit after harvest in Brazil. *J. Phytopathol*, 150, 128–134.

Senaratna, L. K., Wijesundera, L. C., and Liyanage, A. S. 1991. Morphological and physiological characters of two isolates of *Colletotrichum gloeosporioides* from rubber (Hevea brasiliensis) *Mycol Res*, 95, 1085-1089.

Tapia-Tussell, R., Quijano-Ramayo, A., Cortes-Velazquez, A., Lappe, P., and Larque-Saavedra, A.2008. PCR-based detection and characterization of the fungal pathogens *Colletotrichum gloeosporioides* and *Colletotrichum capsici* causing anthracnose in papaya (*Carica papaya* L.) in the Yucatan Peninsula. *Mol Biotechonol*. 40, 293-298.

Tomita, N., Mori, Y., Kanda, H., and Notomi, T. 2008. Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. *Nat Protoc*. 3, 877-882.

Wang, W., Su, M., Li, H., Chang, Q., He, E., and Liu, M. 2013. Effect of germicides on the diseases and growth of *Anoectochilus roxburghii*. *Subtropical Plant Sci* 42, 249-251.

Weir B. S. *et al*. The *Colletotrichum gloeosporioides* species complex. 2012. *Stud. Mycol* 73,115-180.

Zhang, X., Li, M., Cui, Y., Zhao, J., Cui, Z., and Li, Q. 2012. Electrochemical behavior of calcein and the interaction between calcein and DNA. *Electroanal*. 24, 1878-1886.