Research

Rapid Detection of *Alternaria* Species Involved in Pear Black Spot Using Loop-Mediated Isothermal AmplificationXue Yang,^{1,2,3} Yong-Jie Qi,^{4,5} Mohamed N. Al-Attala,^{1,6} Zheng-Hui Gao,^{4,5} Xing-Kai Yi,^{4,5} Ai-Fang Zhang,^{1,2,3} Hao-Yu Zang,^{1,2,3} Chun-Yan Gu,^{1,2,3} Tong-Chun Gao,^{1,2,3} and Yu Chen^{1,2,3,†}¹ Institute of Plant Protection and Agro-products Safety, Anhui Academy of Agricultural Sciences, Hefei 230031, China² Scientific Observing and Experimental Station of Crop Pests in Hefei, Ministry of Agriculture, China³ Laboratory of Quality & Safety Risk Assessment for Agro-Products, Hefei, Ministry of Agriculture, China⁴ Institute of Horticulture, Anhui Academy of Agricultural Sciences, Hefei 230031, China⁵ Key Laboratory of Genetic Improvement and Ecophysiology of Horticultural Crop, Anhui Province, China⁶ Plant Pathology Unit, Plant Protection Department, Desert Research Center, Cairo 11753, Egypt

Abstract

Alternaria species are the most important fungal pathogens that attack various crops as well as fruit trees such as pear and cause black spot disease. Here, a loop-mediated isothermal amplification (LAMP) assay is developed for the detection of *Alternaria* species. *A. alternata* cytochrome b (*cyt-b*) gene was used to design two pairs of primers and amplified a 229-bp segment of *Aacyt-b* gene. The results showed that LAMP assay is faster and simpler than polymerase chain reaction (PCR). LAMP assay is highly sensitive method for the detection of about 1 pg of genomic DNA of *A. alternata* by using optimized concentration of MgCl₂ (4 mM) in final LAMP reaction. In contrast, the limit of detection was 1 ng of target DNA via conventional PCR. Among the genomic

DNA of 46 fungal species, only the tubes containing DNA of *Alternaria* spp. except *A. porri*, *A. solani*, and *A. infectoria* changed color from orange to yellowish green with SYBR Green I including the main pathogens of pear black spot. The yellowish green color was indicative of DNA amplification. Moreover, LAMP assay was used for testing infected tissues among 22 healthy and diseased pear tissues; the orange color changed to yellowish green for infected tissues only. Altogether, we conclude that *cyt-b* gene can be used for the detection of *Alternaria* spp. via LAMP assay, which is involved in pear black spot disease.

Keywords: fungi, fruit, tree fruits, pathogen detection

The genus *Alternaria* is ubiquitous and includes both plant pathogenic and saprophytic species (Dang et al. 2015). *Alternaria* spp. may damage crops in the field or cause postharvest decay (Logrieco et al. 2003; Peever et al. 2004). This genus includes more than 280 species and causes at least 20% of crop damage (Gannibal 2015). *Alternaria* spp. are able to produce various secondary metabolites. Over 260 different metabolites have been reported to be produced by this genus. Several *Alternaria* metabolites known as potent mycotoxins (e.g., alternariol, alternariol methyl ether, altenuene, and tenuazonic acid) have an important role in pathogenesis on plants (Lou et al. 2013; Meena et al. 2017). Moreover, *Alternaria* spp. have

a wide host range, e.g., apple, blueberry, date palm, lemon, pear, potato, strawberry, tomato, and wheat (Al-Nadabi et al. 2018; He et al. 2018; Ito et al. 2004). Pear (*Pyrus* spp.) is one of the most common fruits and has a long history as an economic crop in commercial cultivation in more than 50 countries (Bao et al. 2008; Bassil and Postman 2010; Terakami et al. 2009; Wolko et al. 2010). More than 35 different disease causing agents (bacterial, fungal, viral, and nematodes) affect different parts of pear trees and represent a constraint on the pear industry worldwide (Zhang et al. 2011).

Pear black spot disease (PBS) can cause necrosis on pear leaves, twigs, and fruits. The symptoms first appear as small purple dots on the leaves of young shoots near to the ground. Then, dots grow to circular spots and become purplish black or brown. The same symptoms appear on the pear fruits but are black and slightly sunken (Tanahashi et al. 2016). Appropriate conditions such as high temperature and humidity make PBS disease more serious and severe (Reis et al. 2007). PBS is caused by several species of *Alternaria* such as *A. alternata*, *A. gaisen*, *A. tenuissima*, *A. ventricosa*, and *A. yaliinficiens*. Now, *A. ventricosa* and *A. yaliinficiens* have been isolated and identified from the export Ya Li Chinese pear in the United States (Roberts 2005, 2007). Until now, none of these species has been isolated from pear in China.

The routine technique for the detection and identification of *Alternaria* spp. often requires culture isolation, and morphological and physiological characterization (Simmons 2007). This process is tedious and time-consuming, whereby it takes several days to weeks to obtain a diagnostic result. Finding easier, highly sensitive and specific method is an important task for the detection and identification of plant pathogens in the primary stages or before their outbreak on plants. Currently, many different methods are available for this purpose, such as polymerase chain reaction (PCR), nested PCR, real-time PCR, helicase-dependent amplification (HDA), and recombinase polymerase amplification (RPA) (Lau and Botella 2017;

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Miles et al. 2015). In 2000, Notomi developed a new technique for the amplification of DNA called loop-mediated isothermal amplification (LAMP). LAMP is a highly sensitive and specific method for the detection of different organisms as compared with other molecular methods. Four or six primers are needed for completing amplification at the optimum temperature. The LAMP method can use deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) as template for the amplification. LAMP assay has been reported in many previous studies for the detection and identification of many different plant pathogens (Dai et al. 2012; Duan et al. 2014; Niessen and Vogel 2010; Niu et al. 2012; Tomlinson et al. 2007, 2010; Yang et al. 2018; Zhang et al. 2018).

Cytochrome b (*cyt-b*) gene has been characterized for different plant pathogens, such as *Puccinia recondita* f. sp. *tritici*, *P. graminis* f. sp. *tritici*, *A. solani*, etc. (Grasso et al. 2006). The *cyt-b* gene contains slowly and rapidly evolving codon positions (Farias et al. 2001). Many previous studies have used the mitochondrial *cyt-b* gene

for the identification, exploration of evolution, classification, and phylogenetic analysis of many organisms (Aquadro and Greenberg 1983; Brown et al. 1979) such as *Aspergillus* spp. and *Fusarium solani* (He et al. 2011; Wang et al. 1998, 2000). The *cyt-b* gene can be used as phylogenetic marker for analyzing the relationships among cichlid fish (Farias et al. 2001).

The objective of this work was to develop a LAMP assay, based on the *cyt-b* gene, for the detection and identification of *Alternaria* spp. This method gives us a more effective and reliable detection technique as compared with other in vitro traditional assessment methods. Moreover, the LAMP assay is useful for laboratories and field identification of pathogens in developing countries, as it is easier and cheaper than other methods.

Materials and Methods

Fungal strains and plant materials. The fungal strains used in this study (Table 1) were obtained from the cultures stored at Anhui

Table 1. List of fungal species used for testing the specificity in the LAMP assay

Species	Isolates ID	Host	Location	Source ^a	LAMP
<i>Alternaria alternata</i> (strain 1)	DSL-1	Dangshan Suli	Dangshan, Suzhou, Anhui, China	AAAS	+
<i>A. alternata</i> (strain 2)	XXPGL	Pingguoli	Xiaoxian, Suzhou, Anhui, China	AAAS	+
<i>A. alternata</i> (strain 3)	XXHJL	Whangkeumbae	Xiaoxian, Suzhou, Anhui, China	AAAS	+
<i>A. alternata</i> (strain 4)	XXSJL	Shuijingli	Xiaoxian, Suzhou, Anhui, China	AAAS	+
<i>A. alternata</i> (strain 5)	XXZSL	Zaosu	Xiaoxian, Suzhou, Anhui, China	AAAS	+
<i>A. alternata</i> (strain 6)	XXFSL	Housui	Xiaoxian, Suzhou, Anhui, China	AAAS	+
<i>A. alternata</i> (strain 7)	XXGL	Gongli	Xiaoxian, Suzhou, Anhui, China	AAAS	+
<i>A. alternata</i> (strain 8)	XXXL	Xuehuali	Xiaoxian, Suzhou, Anhui, China	AAAS	+
<i>A. alternata</i> (strain 9)	XJXL	Korla pear	Kuerle, Xinjiang, China	AAAS	+
<i>A. alternata</i>	CFCM-1	Strawberry	Changfeng, Hefei, Anhui, China	AHIQ	+
<i>A. alternata</i>	DSPG-1	Apple	Dangshan, Suzhou, Anhui, China	AAAS	+
<i>A. alternata</i>	J-Aa	Orange	—	AHIQ	+
<i>A. alternata</i>	NM-Aa	Lemon	—	AHIQ	+
<i>A. alternata</i>	YC-Aa	Tobacco	—	AHIQ	+
<i>A. alternata</i>	FQ-Aa	Tomato	—	AHIQ	+
<i>A. tenuissima</i>	DSL-2	Pear	Dangshan, Suzhou, Anhui, China	AAAS	+
<i>A. gaisen</i>	3.7807	—	—	CGMCC	+
<i>A. infectoria</i>	3.7808	—	—	CGMCC	-
<i>A. arborescens</i>	3.15534	—	—	CGMCC	+
<i>A. citri</i>	3.10272	—	—	CGMCC	+
<i>A. mali</i>	3.7809	—	—	CGMCC	+
<i>A. porri</i>	3.7810	—	—	CGMCC	-
<i>A. solani</i>	FQZY	—	Nanjing Agriculture University	CGMCC	-
<i>A. longipes</i>	3.17855	—	—	CGMCC	+
<i>Ascochyta erobotryae</i>	SXPP-1	Loquat	Shexian, Huangshan, Anhui, China	AAAS	-
<i>Aspergillus niger</i>	FDPT-1	Grape	Feidong, Hefei, Anhui, China	AAAS	-
<i>Aspergillus flavus</i>	SXGJ-1	Orange	Shexian, Huangshan, Anhui, China	AAAS	-
<i>Botryosphaeria dothidea</i>	DSL-4	Pear	Dangshan, Suzhou, Anhui, China	AAAS	-
<i>Botrytis cinerea</i>	FCT-1	Peach	Fanchang, Wuhu, Anhui, China	AAAS	-
<i>Colletotrichum gloeosporioides</i>	DSL-3	Pear	Dangshan, Suzhou, Anhui, China	AAAS	-
<i>Coniella granati</i>	HYSL-1	Pomegranate	Huaiyuan, Bengbu, Anhui, China	AAAS	-
<i>Coniothyrium diplodiella</i>	FDPT-2	Grape	Feidong, Hefei, Anhui, China	AAAS	-
<i>Fusarium oxysporum</i>	CFXG-1	Watermelon	Changfeng, Hefei, Anhui, China	AAAS	-
<i>Glomerella acutata</i>	WHYT-1	Nectarine	Wuhu, Anhui, China	AAAS	-
<i>Glomerella cingulata</i>	LSSL-1	Pomegranate	Lieshan, Huainan, Anhui, China	AAAS	-
<i>Monilinia fructicola</i>	HSYT-1	Cherry	Huangshan, Anhui, China	AAAS	-
<i>Nigrospora sphaerica</i>	HSMHT-1	Kiwifruit	Huangshan, Anhui, China	AAAS	-
<i>Penicillium purpurogenum</i>	DSL-5	Pear	Dangshan, Suzhou, Anhui, China	AAAS	-
<i>Pestalotiopsis punicea</i>	LSSL-2	Pomegranate	Lieshan, Huainan, Anhui, China	AAAS	-
<i>Pestalotiopsis theae</i>	SXPP-2	Loquat	Shexian, Huangshan, Anhui, China	AAAS	-
<i>Pestalotiopsis clavispora</i>	TXLM-1	Blueberry	Tunxi, Huangshan, Anhui, China	AAAS	-
<i>Phomopsis fukushii</i>	DSL-6	Pear	Dangshan, Suzhou, Anhui, China	AAAS	-
<i>Phomopsis punicea</i>	LSSL-3	Pomegranate	Lieshan, Huainan, Anhui, China	AAAS	-
<i>Phomopsis amygdalina</i>	WHT-2	Peach	Wuhu, Anhui, China	AAAS	-
<i>Plasmopara viticola</i>	FDPT-2	Grape	Feidong, Hefei, Anhui, China	AAAS	-
<i>Sclerotinia sclerotiorum</i>	FDYT-1	Cherry	Feidong, Hefei, Anhui, China	AAAS	-

^a AAAS: Anhui Academy of Agricultural Sciences; AHIQ: Anhui Entry-Exit Inspection and Quarantine Bureau; CGMCC: China General Microbiological Culture Collection Center. + and - indicate the presence and absence of the expected 229-bp DNA fragment amplified by LAMP primers. — means no information available from the source.

Academy of Agricultural Sciences (AAAS), Anhui Entry-Exit Inspection and Quarantine Bureau (AHIQ), and China General Microbiological Culture Collection Center (CGMCC). The fungal strains preserved at AAAS were collected from different orchards and markets. These strains are monosporic isolates and are identified according to their morphological features and ITS gene sequence BLAST results. Fungal infected pears showing black spot disease were collected from the field situated in Anhui Province of China. The infected tissue samples were taken from under the husk of the infected pear fruit and placed on potato dextrose agar (PDA) dishes. The isolates were characterized by the length of colonies on PDA at 25°C in the dark for 1 week. Then, the cultures were stored at 4°C (Ramjagathesh and Ebenzar 2012; Simmons 1967, 1981).

DNA extraction. The biomass of each fungal strain was frozen in liquid nitrogen for breaking the cells and immediately physically ground using a mortar and pestle. Genomic DNA (gDNA) of all the fungal pathogens was extracted using the Fungal DNA Mini Kit (Omega Bio-tek, China), according to the manufacturer's protocol. The quantity of gDNA for each sample was determined by using the Nanodrop (NanoVue Plus, GE Healthcare Life Sciences), and was found between 50 and 100 ng/μl.

Primer design. A specific region of the *A. alternata* *cyt-b* gene (JQ437357) was selected based on basic local alignment search tool (BLAST) results from NCBI sequence database. The specific site, showing no similarity to genes from any other fungal genera (as per data available in May 2017), was located from 2312 to 2541 bp (a 229-bp region) in the *cyt-b* gene (Fig. 1A and B). Two pairs of LAMP primers for amplifying these 229 bp from the *cyt-b* gene were designed based on the specific region using the Primer explorer V4 software program (<http://primerexplorer.jp/e/>) (Eiken Chemical Co., Ltd., Tokyo, Japan). The software recommended three sets of primers; we chose the best set of four primers according to the results of Primer explorer V4 software program. The primers were

synthesized by Invitrogen Trading Shanghai Co., Ltd. (Table 2). The sequence of LAMP primers used in this study is shown in Figure 1. F1c and F2 together formed the forward inner primers (FIP), while B1c and B2 together formed the backward inner primers (BIP) (Notomi et al. 2000). The outer primers F3 and B3 were designed to start the LAMP reaction.

LAMP reaction. To optimize the reagents, the LAMP reaction was prepared by mixing 2 μl of 10× isothermal amplification buffer (New England Biolabs), different concentrations of MgCl₂ (25 mM, Sangon, China) (2 mM, 3 mM, 4 mM, 5 mM, and 6 mM), 2 μl of dNTP Mix (10 mM, Sangon, China), 0.6 μl of FIP and BIP primers (40 μmol), 0.6 μl of F3 and B3 primers (10 μmol), 1 μl of DNA (50 ng), 1.2 μl of betaine (5 M) (Sigma, U.S.A.), and 0.8 μl of *Bst* 2.0 WarmStart DNA polymerase (8,000 U/ml) (New England Biolabs). Additionally, ddH₂O was added to make the final volume 20 μl. SYBR Green I (Sangon Biotech, Shanghai) was not added to the reaction mixture, but 0.25 μl of SYBR Green I was added to each tube after amplification. Reaction tubes were placed in a thermal cycler (BIO-RAD, Singapore). After amplification, LAMP reactions were examined based on the change in color of SYBR Green I, and gel electrophoresis (2% agarose gel for 50 min at 80 V). Each

Table 2. Sequence information of LAMP primers used for amplification of the target sequence in *cyt-b* gene

Primer name	Type	Sequence 5'→3'
F3	Forward outer	GGCTCCTTTGAATGGTAAC
B3	Backward outer	TGTCTTCAACCAAGCATCA
FIP (F1c-F2)	Forward inner primer	CGTTCGAAGTAATCTATAAATCGG ATTCAATAGGCAAATGGGGA
BIP (B1c-B2)	Backward inner primer	AATGGATCAAGAACGTTCAACG AATTGGTTGTGGGGTACTC

Fig. 1. Design of LAMP primers for the detection of a specific region in the *cyt-b* gene (JQ437357) in the *Alternaria alternata* genome. **A**, Schematic illustration of the LAMP primers used in this study. F1c and B1c are complementary to F1 and B1, respectively. **B**, Arrows show the locations of the LAMP primers.

treatment was replicated three times and the experiment was repeated twice.

Optimization of LAMP reaction. Different concentrations of $MgCl_2$ (25 mM), i.e., 2 mM, 3 mM, 4 mM, 5 mM, and 6 mM, were used to determine the optimal LAMP reaction. Further, the mixture tubes were incubated at 65°C for 30 min, 45 min, 60 min, 75 min, and 90 min to determine the optimal reaction time. Each treatment was replicated three times, and the experiment was repeated twice.

Specificity test of LAMP reaction. To study the specificity of LAMP, 46 different fungi were used in the study including 9 *A. alternata* strains isolated from different pear cultivars, 6 strains of *A. alternata* from different plant hosts, 9 strains of other *Alternaria* spp., and 22 other different fungal species belonging to other genera (Table 1). LAMP reaction conditions were based on optimization of experiments as mentioned above. The assay was evaluated based on the change in the color of SYBR Green I in amplification tubes. The experiment was repeated twice.

Sensitivity test of LAMP and PCR assays. To compare the sensitivity of PCR and LAMP, different concentrations of gDNA of *A. alternata* ranging from 10 µg to 100 fg/µl were used. For PCR, F3 and B3 primers were used to amplify the same specific region (229 bp) of *cyt-b* gene. PCR conditions included initial denaturation at 95°C for 3 min; 35 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 10 min. The total volume of the PCR was 25 µl, containing 9.5 µl of ddH₂O, 1 µl of genomic DNA of *A. alternata*, 1 µl of each primer (F3 and B3) (10 mM), and 12.5 µl of 2× Taq Master Mix (Novoprotein). After the amplification, PCR products were examined by running on a 1% agarose gel for 30 min at 120 V. The DNA ladder DL2000 was used for size references. For LAMP, the reaction conditions were based on the optimization experiments. The PCR and LAMP experiments were repeated twice.

Detection of black spot pathogen in pear samples. Twelve samples of healthy and eight samples of infected pear fruits were collected from the different fields of Anhui Province. About 10 g of plant tissue from each sample was used for the DNA extraction. The DNA was extracted using the Plant DNA Mini Kit (Omega Bio-tek, China) according to the manufacturer's protocol and used as a template for LAMP. The DNA of *A. alternata* and the distilled water were used as positive and negative control, respectively. The amplification was analyzed using SYBR Green I. The experiment was repeated twice.

Results

Designing of LAMP primers and assay. The Primer explorer V4 software program was used for designing two pairs of LAMP primers using *cyt-b* gene sequence. In the conventional PCR, the F3 and B3 primers (forward and backward LAMP primers) were used for better DNA detection on 1.0% agarose gel electrophoresis (Fig. 1). In LAMP tubes, yellowish green color with SYBR Green I was indicative of a positive reaction in comparison with the negative control (Figs. 2, 3, and 4). The results confirmed that LAMP could be used for detection and identification of *Alternaria* spp.

Optimized LAMP reaction. LAMP reaction was prepared using *A. alternata* gDNA as a template to determine the optimal reaction conditions for the amplification. Results showed that the best detection time of well-formed bands was 60 min, where the color of LAMP reaction changed to yellowish green. It was indicative of a positive reaction (Fig. 2A and B). In addition, the best concentration of $MgCl_2$ (25 mM) was 3.2 µl (4 mM) in the final LAMP reaction (Fig. 2C).

Sensitivity of LAMP and PCR assays. To investigate the sensitivity of LAMP assay, different concentrations of gDNA of *A. alternata* ranging from 10 µg to 100 fg/µl in a ninefold serial dilution of DNA, were used in the study. The DNA amplification was observed in all concentrations from 10 µg to 1 pg but not in lower concentrations. The negative control showed no amplification under the same conditions (Fig. 3A). In the PCR assay, positive amplification reactions were observed at concentrations from 10 to 1 µg/µl (Fig. 3B). The above results showed that the LAMP assay was more sensitive than PCR assay.

Specificity of LAMP assay. Using 46 genomic DNAs of different fungal species, the color of SYBR Green I in the tubes containing gDNA of *A. alternata*, all six pathotypes and nine strains of *Alternaria* spp. changed to yellowish green except in *A. porri*, *A. solani*, and *A. infectoria* tubes after 1 h of reaction time at 65°C. These results are indicative of positive reactions. The other 22 fungal genera and the negative control showed an orange color, suggesting no DNA amplification (Figs. 4 and 5). All results are shown in Table 1.

Detection of black spot pathogens in pear using LAMP. The LAMP assay was used for detecting *Alternaria* pathogenic fungi in pear tissues. The results showed that the LAMP assay succeeded for the detection of *Alternaria* spp. in the infected pear tissues among healthy ones. These results indicate that this specific region of the *A. alternata cyt-b* gene (JQ437357) can be used for the detection and identification of black spot pathogens on pear (Fig. 6).

Discussion

Alternaria species are the most commonly found fungi and can be detected in the soil and organic matter around the world. At least 268

Fig. 2. Optimization of LAMP reaction for the detection of *Alternaria alternata*. Tubes and Lanes (A, B) are 30 min, 45 min, 60 min, 75 min, and 90 min, respectively. N is negative control (no DNA). A, 2% agarose gel showing products from the LAMP reaction. B, Visualization by SYBR Green I with different timings. C, Visualization by SYBR Green I with different concentrations of $MgCl_2$. N is negative control (no DNA). Tubes 2–6 contain different $MgCl_2$ concentrations including 2 mM, 3 mM, 4 mM, 5 mM, and 6 mM, respectively. The yellowish green color indicates a positive reaction, which can be easily distinguished from the reddish orange color indicating no reaction in the negative control under normal light.

metabolites are produced by *Alternaria* species. Some metabolites from *Alternaria* fungi have a toxic effect on other organisms (Lou et al. 2013). *A. alternata* causes symptoms in leaves and fruits by producing phytotoxins (AK toxins I and II) (Kodama et al. 1990; Maekawa et al. 1984; Tsuge et al. 2013). The fungal infection decreases the quality and quantity of pear fruit and has been continuously reported from countries such as China, Japan, and the United States (Roberts 2005, 2007; Sanada et al. 1988). Diagnosis of plant disease in the early fungal infection stage is one of the most challenging jobs for scientists and farmers. Morphological identification is now an ineffective method because microbiologic culture-based identification needs more time and space and is usually unreliable. Therefore, an effective, rapid, and reliable method for detecting different plant pathogens is needed. The LAMP assay achieves all these requirements and can successfully be used for the detection of many different plant pathogens (Almasi et al. 2013; Dai et al. 2012; Denschlag et al. 2012; Parida et al. 2005; Tomlinson et al. 2007, 2010; Vaagt et al. 2013), as well as animal pathogens (Iwamoto et al. 2003; Saei et al. 2011).

In this study, two pairs of primers (F3/B3 and FIP/BIP) were used to target a specific region of *cyt-b* gene (JQ437357) of *A. alternata* fungus for the detection and identification of *Alternaria* spp. (Table 2). The specific region in the *cyt-b* gene ranged from 2312 to 2541 bp (229-bp region) (Fig. 1) showed no homology with any other genera in BLAST result of NCBI database.

The optimal temperature, time duration, DNA quality, and Mg^{2+} ion concentrations affect the successful amplification reaction in LAMP method (Goto et al. 2009). We investigated the effect of $MgCl_2$ concentration in the LAMP reaction. The results showed that

the best concentration was 4 mM of $MgCl_2$ in LAMP reaction. In addition, we found that the optimized reaction conditions for successful amplification of DNA were 65°C and 60 min. Many different dyes have been used previously to detect successful amplification of

Fig. 4. The PCR and LAMP assay for *Alternaria alternata* detection in different infected pear samples. **A**, Tubes and **B**, Lanes 1–9 are from different cultivars: Pingguoli, Whangkeumbae, Shuijingli, Zaosu, Housui, Dangshan Suli, Xuehuali, Korla pear, Gongli, respectively. Tube and Lane N is the negative control (no DNA).

Fig. 3. Sensitivity of LAMP and PCR test for the detection of *Alternaria alternata* using different quantities of gDNA. **A**: Sensitivity of the LAMP test. Tubes 1–9, template DNA concentrations (10 µg, 1 µg, 100 ng, 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, and 100 fg, respectively); N, negative control (no DNA). **B**: Sensitivity of the PCR test. M indicates the DL 2,000-bp DNA size marker; lanes 1–9 are template DNA concentrations (10 µg, 1 µg, 100 ng, 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, and 100 fg, respectively); and lane N is negative control (no DNA).

DNA, such as hydroxynaphthol blue (HNB), SYBR Green I, magnesium pyrophosphate, ethidium bromide, and Genefinder. In this study, LAMP reaction tube was examined via SYBR Green I, which allowed the detection of differences between positive and negative reactions using the naked eye (Figs. 2, 3, 4, and 5). This is a simple method that visually differentiates between positive and negative reactions without demand for any special equipment.

Previous studies reported that the sensitivity of the LAMP reaction is generally higher than that of conventional PCR (Goto et al. 2009; Mori et al. 2004; Niessen and Vogel 2010; Zhao et al. 2015). This study reports the sensitivity limit of LAMP primers for the detection of *Alternaria* spp. using gDNA of *A. alternata*. The amplification limit of the LAMP assay was approximately 1 pg of *A. alternata* target DNA, compared with 1 ng of *A. alternata* target DNA through conventional PCR. The results demonstrate the high sensitivity of LAMP primers.

Fig. 5. Specificity of the LAMP assay visualized by SYBR Green I color change. **A:** *Alternaria alternata* from different host and other *Alternaria* species. Tubes 1–10 are *A. alternata*, *A. tenuissima*, *A. gaisen*, *A. arborescens*, *A. infectoria*, *A. solani*, *A. mali*, *A. longipes*, *A. porri*, and *A. citri*; Tubes 11–17 are *A. alternata* pathotypes (pear pathotype, apple pathotype, orange pathotype, strawberry pathotype, lemon pathotype, tomato pathotype, and tobacco pathotype, respectively). **B:** Lane 1 is *A. alternata*, showing amplification; Lanes 2–23 are the other tested fungi, showing no amplifications. Lane N is the negative control (no DNA).

Fig. 6. Evaluation of the LAMP assays using infected and healthy pear samples with visualization of color change by SYBR Green I in amplification tubes. Tube 1 is the positive control (DNA). Tube 2 is the negative control (no DNA). Tubes 3, 5, 6, 9, 10, 12, 14, 15, 17, 18, 20, 22 are infected samples. Tubes 4, 7, 8, 11, 13, 16, 19, 21 are healthy samples.

In addition, we studied the specificity of LAMP primers for the identification of the black spot pathogen among other different fungal species. The results showed that LAMP primers could detect *A. alternata* species-group and *Alternaria* spp. among different genera, but LAMP primers can't detect *A. porri*, *A. solani*, and *A. infectoria* (Table 1; Fig. 5). The results also show that there is no cross-reactivity with other fungi from different genera or negative control. Our test is easier and faster as compared with the conventional PCR method for the detection and identification of *Alternaria* spp. This result also suggests that *cyt-b* gene might be different with several *Alternaria* species, and the results are compatible with other reports in which *cyt-b* gene was used to identify many other fungi as *Aspergillus* spp. and *F. solani* (Aquadro and Greenberg 1983; Brown et al. 1979; He et al. 2011; Wang et al. 1998, 2000).

Moreover, the LAMP assay proved to be a rapid and easy method for the detection of black spot pathogens in infected tissues of pear (Fig. 6), as all the three pathogens causing pear black spot reported from China belong to the *Alternaria* genus. This indicates that the LAMP method is efficient for the detection of pathogen from infected tissues, which confirms some previous studies conducted on the detection of different plant pathogens (Almasi et al. 2013; Dai et al. 2012; Denschlag et al. 2012; Parida et al. 2005; Tomlinson et al. 2007, 2010; Vaagt et al. 2013; Yang et al. 2018).

Finally, there are many advantages of the LAMP assay which make it the most preferred method for the detection of plant pathogens; it is simple, fast, cost effective, can be directly judged using naked eye, and doesn't need any special processing or electrophoresis device. Moreover, either DNA or RNA can be used as a template for amplification in LAMP assay. However, LAMP assay has some limitations and defects, as it is easily contaminated, needs a source of light, and requires some skill for assessing the results (Chowdry et al. 2014; Mitsunaga et al. 2013; Mori et al. 2001).

Conclusively, the LAMP assay can be useful for the rapid detection of *Alternaria* spp. including *A. alternata* on infected pears. The LAMP technique excludes the need for sophisticated equipment such as PCR machines, gel electrophoresis, and gel-imaging systems for the detection of *Alternaria* spp. in the laboratory. The LAMP method is quite sensitive for *A. alternata* pathotypes detection and could be a useful tool for pathologists in designing integrated control programs for black spot fungal disease and many other diseases caused by *Alternaria* species.

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