q-LAMP Assays for the Detection of *Botryosphaeria dothidea* Causing Chinese Hickory Canker in Trunk, Water, and Air Samples

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Abstract

Trunk canker disease caused by *Botryosphaeria dothidea* with a prolonged latent infection phase poses a serious threat to Chinese hickory production. To further understand the epidemiological characteristics and develop reasonable management techniques, a quantitative loop-mediated isothermal amplification (q-LAMP) assay was developed to quantitatively monitor *B. dothidea* in hickory plants, water, and air samples. Specific primers were designed based on the different sites of the β -tubulin sequence between *B. dothidea* and other fungi commonly found on Chinese hickory. At the optimum reaction temperature of 65.9°C, this loop-mediated isothermal amplification (LAMP) assay can

specifically distinguish *B. dothidea* from other tested fungi. The limit of detection of LAMP assays for *B. dothidea* was 0.001 ng/ μ l of pure genomic DNA and 10 spores per 1 ml of water. The q-LAMP assay enables rapid detection of *B. dothidea* within 60 min in hickory trunk, water in hickory forests, and spores captured on tapes. These results provide a powerful and convenient tool for monitoring *B. dothidea*, which could be applied widely in epidemiology, forecast, and management of tree canker disease.

Keywords: pathogen detection, tree nuts

Chinese hickory (*Carya cathayensis* Sarg), a deciduous tree of Juglandaceae, is a well-known fruit and woody oil tree species native to China. This species has a distinctive fragrance and high nutritional value, and it is mainly distributed in the Tianmu Mountain area and Changbei District at the junction of Zhejiang and Anhui provinces in China (Ma et al. 2009; Wang 2004; Zhu et al. 2008). At present, there are about 1,300,000 ha of Chinese hickory cultivated in China, with an average annual output value of \$260 million and a processing output value of \$509 million (Zhang et al. 2018). The widespread outbreak of canker disease caused by *Botryosphaeria dothidea* seriously threatens the sustainable development of the Chinese hickory industry (Yunwei et al. 2015; Zhang and Xu 2011; Zhang et al. 2015). *B. dothidea*, one of the most common fungal pathogens damaging trees worldwide, infects the hosts through wounds, and it breaches the outer bark and colonizes the phloem, vascular cambium, and xylem. This blockage causes a weakening and decay of the wood at the infection sites (Slippers et al. 2007). This pathogenic fungus can easily be missed during the management time point or simply pass undetected by quarantine systems because of its prolonged latent infection or endophytic phase (Marsberg et al. 2017). Canker symptoms generally occur on trunks of hickory below ~200 cm, and few lesions are observed on other parts, such as twigs, branches, and nuts. The occurrence period of trunk canker is from late March to late November annually, and the pathogen overwinters around the diseased tissues with obvious latent infection characteristics (Dai et al. 2017; Yang et al. 2009). The *B. dothidea* fungus exists in hickory trees all year round, and the obvious canker symptoms will not be observed until April when is too late to start treatment of the disease. At present, this disease is observed by the naked eye, and thus, a big gap exists in the preliminary prediction of this disease.

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Previous studies conducted in California pistachio orchards have confirmed *B. dothidea* to be a water splash-dispersed pathogen with relatively short distances of spore dispersal (Ahimera et al. 2004; Michailides 1993). Under moist conditions, pycnidia of *Botryosphaeria* spp. produce pycnidiospores that are generally exuded in a gelatinous matrix forming cirrhi, a ribbonlike mass of spores (Ahimera et al. 2004; Michailides 1993; Phillips 2002; Úrbez-Torres et al. 2006). In addition, there is still a lack of research on the role of wind in the dissemination of *B. dothidea* conidia. Our previous research indicated that the time of the first fungicide spray, which should be carried out before the initial spore dispersal, greatly impacts efficacy. Additionally, we speculated that *B. dothidea* overwintered on cankers on trunks with obvious latent infection (Dai et al. 2017). However, little is known about the epidemiology and inoculum dispersal of *B. dothidea* infecting Chinese hickory, and the transmission routes of *B. dothidea* among hickory forests and the characteristics of infection in the trunk are still vague. Furthermore, the hickory forest is a complex and open ecological environment. To explore the transmission characteristics of *B. dothidea*, it is very important to quantitatively detect trunk canker fungi on trunks and detect a small number of conidia in water and air between hickory forests. The use of traditional microscopic counting after capturing spores in the forest requires professional classification by technicians, and a long time is required to observe and identify the spores. In view of the complexity of environmental samples and human subjectivity, microscopic counting does not always provide reliable data. A real-time PCR technique was developed and adapted to help quantify canker control by measuring the abundance of the pathogen (Zhu et al. 2016). However, traditional PCR technology is more frequently used for qualitative detection of pathogens, and it is susceptible to interference from PCR inhibitors, dust, and other pathogens in the environment. It is difficult to quantitatively detect low concentrations of captured spores. A new time-saving, low-cost, highly specific, and sensitive nucleic acid amplification method, loop-mediated isothermal amplification (LAMP), has been gradually used (Chotiwan et al. 2017). The results of LAMP can be judged by either turbidity or end products that can be visible to the naked eye with fluorescent reagents, such as SYBR Green I (Tomito et al. 2008), within 60 min under the condition of a constant temperature. The purpose of this study was to establish a quantitative loop-mediated isothermal amplification (q-LAMP) detection technique for the detection of trunk canker fungus in hickory; quantitatively determine the abundance of this fungus in trees, air, and water accurately and quickly; and monitor the change in the content of the fungus so as to provide guidance for the prediction and management of trunk canker fungus in hickory.

Materials and Methods

Fungal isolates. Isolates of *B. dothidea* and commonly isolated endophytic fungi of hickory plants sampled from *C. cathayensis* in Lin'an District, Hangzhou, China are listed in Table 1. Isolates were maintained on potato dextrose agar (PDA) slants at 4°C.

DNA extraction from mycelium, trunk, and spores. Mycelial DNA was extracted using a Rapid Fungi Genomic DNA Isolation Kit (Sangon Biotech) according to the manufacturer's protocol. DNA from the trunk and conidia was extracted by a Plant Genomic DNA Kit (TIANGEN BIOTECH) according to the manufacturer's protocol. For DNA isolation from diseased trunk tissues, 3 g of phloem lesions were collected and put into an FW100 high-rotation speed disintegrator (Taisite). After 2 min of crushing, 400 mg of tissues were added with liquid nitrogen to grind the sample with a pestle. Although a small amount of liquid nitrogen remained, the samples were rapidly ground, and this procedure was repeated three to four times. For DNA extraction from conidia, *B. dothidea* were cultured on PDA medium at 25°C for 3 days in darkness and then, under a 12-h light/dark condition; a certain number of pycnidia were selected, placed in a centrifuge tube with 1.5 ml of sterile water, and filtered using a 125-µm sieve. Suspensions were centrifuged for 5 min at 6,800 rpm using a Sorvall Legend MicroCL 17R Centrifuge (Thermo Scientific). Then, 80% of the volume was drained, and the pellet was resuspended in the remaining 20% sample volume by vortexing (Gehesquière et al. 2013). The samples were transferred to a fresh tube and thoroughly mixed with 200 ml of 10% Chelex-100 solution (Jiliang et al. 2011), 200 ml of 10% sodium dodecylsulfate (SDS) solution, and 0.4 g of acid-washed glass beads (Aladdin). This mixture was treated at speed 6.0 for 40 s by the FastPrep-24 Instrument (MP Biomedicals), and it was then heated in a water bath at 100°C for 5 min; the oscillation heating steps were repeated three times followed by refrigeration at -20°C for 2 min. Each sample was centrifuged for 5 min at 10,000 rpm, and DNA was extracted from the supernatants using a Plant Genomic DNA Kit following the manufacturer's instructions. DNA concentrations were determined spectrophotometrically, and the extracted DNA was stored at -20°C for use in PCR and q-LAMP analyses.

Amplification and analysis of the β-tubulin sequence of *B. dothidea* from Chinese hickory. The β-tubulin gene of *B. dothidea* was PCR amplified using the primers T1-5'-AACATGCGTGAGA TTGTAAGT-3' and Bt2b-5'-ACCCTCAGTGTAGTGACCTTGGC-3' (Lisboa et al. 2016). The reactions contained the following reagents: 12.5 µl of 2× Taq Master Mix, 1 µl of primers of T1 and Bt2b-5', 1 µl of DNA sample, and double-distilled water (ddH₂O) to adjust the volume to 25 µl. The thermal cycling of the PCR program was 94°C for 5 min and 35 reaction cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 40 s, with an extension at 72°C for 5 min. The PCR products were separated by 1% agarose gel electrophoresis, and genomic DNA was purified using a SanPrep Column DNA Gel Extraction Kit (Sangon). The purified PCR products were sequenced by GenScript. The sequence analysis and homology blasts were conducted.

Sequences of β-tubulin for *Botryosphaeria corticis* (GenBank accession no. EU673107), *Botryosphaeria eucalyptorum* (GenBank accession no. AY236920), *Botryosphaeria fabicerciana* (GenBank accession no. KF779068), *Botryosphaeria lutea* (GenBank accession no. AY236923), *Botryosphaeria parva* (GenBank accession no. AY236912), *Botryosphaeria rosaceae* (GenBank accession no. KX197101), *Pestalotiopsis microspora* (GenBank accession no. KU377338.1), *Trichoderma viride* (GenBank accession no. Z15055.1), *Fusarium proliferatum* (GenBank accession no. KJ544178.1), *Nigrospora chinensis* (GenBank accession no. KY019579.1), and *Daldinia* sp. (GenBank accession no. KU684130.1) were downloaded from GenBank to compare the differences in the amplified β-tubulin sequences between *B. dothidea* from Chinese hickory and other fungi.

LAMP primer design and screening. According to the difference in the β-tubulin area, four sets of LAMP primers were designed using Primer explorer V5 software (<http://primerexplorer.jp/e/>). The LAMP assay was performed in 25-µl reaction mixtures, including a

final concentration of 4.8 U Bst DNA polymerase (New England Biolabs), 2.5 µl of 10× TernoPol buffer, 1 mM dNTPs, 5 mM Mg²⁺, 1.6 µM FIP and BIP, 0.2 µM F3 and B3, 0.6 M betaine, 150 µM hydroxynaphthol blue (HNB; metal ion indicator), and 1 µl of DNA sample (100 ng/µl). The volume was adjusted to 25 µl with nucleic acid-free water. The LAMP reactions were incubated at 65.9°C for 60 min, and each process was repeated at least three times. Thereafter, the best set of primers screened that showed the most intensive sky blue color was used for the q-LAMP assay performed later.

q-LAMP reaction mixtures. The q-LAMP assay was performed in a 20-µl reaction mixture, including a final concentration of 8 U Bst DNA polymerase (New England Biolabs), 2 µl of 10× TernoPol buffer, 1 mM dNTPs, 6.25 mM Mg²⁺, 1.6 M FIP and BIP, 0.125 µM F3 and B3, 0.6 M betaine, 1.2 µl of 10×SYBR Green I (TaKaRa), and 1 µl of DNA sample (-0.001 to 100 ng/µl). The volume was adjusted to 20 µl with nucleic acid-free ddH₂O. The LAMP reactions were incubated at 65.9°C for 60 min and performed using a CFX96 thermocycler (BioRad).

Determination of optimum reaction temperature for the q-LAMP assay. To better facilitate the reaction, a series of constant temperatures was set up (e.g., 67.0, 66.6, 65.9, 64.5, 62.8, 61.4, 60.5, and 60.0°C). The screening results can be determined based on fluorescence intensity and reaction time.

Verification of the specificity of the q-LAMP assay. Each q-LAMP series consisted of three replicate reactions containing DNA (at a concentration of 0.1 ng/µl) of the *B. dothidea* and non-*B. dothidea* strains listed in Table 1. The reaction was treated at the selected optimum temperature.

Establishment of a DNA standard curve for mycelium and spore samples. Mycelial DNA samples were 10-fold diluted in water and used as templates for the q-LAMP assay. The final concentrations of DNA were 100, 10, 1, 0.1, 0.01, and 0.001 ng/µl. DNA of mycelia and six 10-fold dilution series of conidia (1.0×10^5 /ml to 1.0×10^0 /ml) were placed into the instrument and allowed to react. No-template control reactions were included in which the DNA was substituted with nucleic acid-free water. For quantification, two standard curves for pure DNA from mycelia and conidia were constructed separately by analyzing the association of logarithmic values of the initial template concentration and cycle threshold (Ct) values.

Detection of *B. dothidea* in the trunk by q-LAMP assays. In the woods of Taiyang Town, Lin'an District, Hangzhou City, the phloem tissues of hickory trees were collected at intervals of 0, 5, 10, and 20 cm from a targeted canker lesion. During 2018, tissues from a total of 100 targeted canker lesions of different trees were collected from Taiyang Town. The absolute quantitative method was used to react the sample with a standard sample simultaneously using a CFX96 thermocycler. Extraction of DNA from samples was done using the methods described earlier. The cycle threshold values of the sample and the standard sample were recorded. The standard curve obtained by diluting the standard sample was used to determine the amount of DNA of *B. dothidea* in the sample.

Table 1. Isolates of *Botryosphaeria dothidea* and commonly isolated endophytic fungi isolates of hickory plants used in this study

Species	Isolate no.	Source	Year
<i>B. dothidea</i>	BD16-1	Lin'an District, Hangzhou, China	2016
<i>B. dothidea</i>	BD17-1	Lin'an District, Hangzhou, China	2017
<i>B. dothidea</i>	BD18-1	Lin'an District, Hangzhou, China	2018
<i>Pestalotiopsis microspora</i>	PM18-1	Lin'an District, Hangzhou, China	2018
<i>Trichoderma viride</i>	TV18-1	Lin'an District, Hangzhou, China	2018
<i>Fusarium proliferatum</i>	FP18-1	Lin'an District, Hangzhou, China	2018
<i>Nigrospora chinensis</i>	NC18-1	Lin'an District, Hangzhou, China	2018
<i>Alternaria oregonensis</i>	AO18-1	Lin'an District, Hangzhou, China	2018
<i>Daldinia</i> sp.	DS18-1	Lin'an District, Hangzhou, China	2018

Monitoring *B. dothidea* conidia from artificial and natural water samples by q-LAMP assays. Three independent dilution series of 10-ml samples were established in 50-ml conical flasks using rainwater and 100 mg of field soil collected in the hickory forests and containing 1,000, 100, 10, or 0 conidia. These samples were concentrated to 1-ml volumes to extract DNA using the methods mentioned above. The DNA samples were submitted to q-LAMP analysis using the optimized thermocycling conditions.

In the forests infected with *B. dothidea*, five open metal containers (30 × 20 × 10 cm) were randomly placed at a distance of 50 cm from a symptomatic trunk (Gehesquière et al. 2013). The containers were placed before a predicted period of rainfall, and rainwater in each container was collected the next day. Using the centrifugal scheme mentioned above, 100-ml water samples collected in each container were concentrated to 1 ml, and then, the same method was used to extract the DNA of the conidia. The DNA samples were submitted to q-LAMP analysis.

Monitoring *B. dothidea* conidia from artificial and natural air samples by q-LAMP. The spores of *B. dothidea* were added to Melinex tapes (2 × 2 cm) on a clean bench, and the numbers of spores were 1,000, 100, and 10 per tape. The tapes with spores were cut and placed in a 2-ml centrifuge tube, and the conidial DNA was extracted according to the methods mentioned above. The DNA samples were submitted to q-LAMP analysis, and the amplification time was recorded. According to the established standard curve for conidia, the number of spores was calculated and compared with the number of spores added artificially to evaluate the accuracy of the q-LAMP detection results.

An airborne spore catcher (Jiaduo) containing Melinex tapes was set up in a *C. cathayensis* forest in Taiyang Town, Lin'an District, Hangzhou City. Beginning on 13 May 2018, samples were collected every 7 days five consecutive times. The DNA of conidia was extracted and submitted to q-LAMP analysis. The number of spores on the tape was calculated based on the results of the q-LAMP reaction.

Results

Primer design and validation. Four sets of primers (Table 2) were designed for LAMP detection of *B. dothidea* based on the different sites of the β -tubulin sequence between the *B. dothidea* isolates and other fungi (Fig. 1). The experimental results show that primer group A has excellent sensitivity and high efficiency (not shown in detail).

Optimum reaction temperature for the q-LAMP assay. The fluorescence quantitative results showed that the strongest fluorescence intensity and the shortest reaction time were obtained when the reaction

temperature was 65.9°C (Supplementary Fig. S1). Thus, 65.9°C was adopted as the reaction temperature to carry out the q-LAMP assays.

Specificity verification of the q-LAMP assay. At the optimum reaction temperature of 65.9°C, the reference strains were validated by LAMP with the screened primer group A. The color of the positive amplifications in *B. dothidea* LAMP tubes changed from violet to blue when detected with HNB reagents, whereas the color remained violet in the other fungi and the blank tube with 1-h reaction periods (Fig. 2A). The LAMP products were separated by 1% agarose gel electrophoresis; ladder-like patterns were observed in the positive reactions but not in negative controls (Fig. 2B). In addition, the specificity of primer group A also was verified by q-LAMP assay. Fluorescence signals were detected in DNA samples containing *B. dothidea*, but no fluorescence signals were detected in the other six fungal DNA and control samples (Fig. 2C).

Establishment of a DNA standard curve for mycelium DNA and spores. Serial dilutions (100, 10, 1, 0.1, 0.01, and 0.001 ng/ μ l) of the mycelial genomic DNA of *B. dothidea* were used as templates to determine the analytical sensitivity of the LAMP assays. The amplification times of each concentration were 16.32, 18.68, 22.43, 26.63, 30.84, and 33.26 min, respectively. The limit of detection (LOD) of LAMP assays for independently detecting *B. dothidea* DNA from mycelia was 0.001 ng/ μ l (Fig. 3A). Defining $\log(\text{DNA concentration value}/0.0001)$ as the x axis and the corresponding CT value as the y axis, a standard curve was constructed; that is:

$$y = -3.5823x + 37.233$$

(Fig. 3B), the DNA concentration calculation formula is:

$$10^{\left(\frac{37.233-y}{-3.5823}\right)-4}$$

and the correlation coefficient $R^2 = 0.9904$, which showed a good linear relationship.

The serial dilutions (10^5 , 10^4 , 10^3 , 10^2 , and 10^1 spores per milliliter) of the genomic DNA of spores were used as templates to determine the analytical sensitivity of LAMP assays. The amplification times of each concentration were 16.67, 21.51, 28.69, 36.26, and 43.61 min, respectively. The LOD for detecting *B. dothidea* spores was 10 spores per milliliter (Fig. 3C). The standard curve was established based on the association of logarithmic values (x) of the number of spores per milliliter and cycle threshold values (y):

Table 2. The sequences of the loop-mediated isothermal amplification primers used in this study

Primer set name and primer name	Type	Sequence (5' to 3')
A		
F3-1	Forward outer	TGTCCATAGGTTACCTCCA
B3-1	Reverse outer	AGATGGTCTGCCTGTGGT
FIP-1	Forward inner	CAAAGTCAGCGGATGCAGCGACCGCCAATGCGTAAG
BIP-1	Reverse inner	AGGGTAACCAAATCGGTGCTGCGTCGATTTCGCGTTAGCGG
B		
F3-2	Forward outer	CCTCCCGACCATCTTTTTCG
B3-2	Reverse outer	TGTTCTCCACGCTGAGA
FIP-2	Forward inner	CGGGTGTTTTGGCAACAAACCATGCATCGCGCTGACTTTG
BIP-2	Reverse inner	CCGCTAACGCGAATCGACACCGACTTACACGCCAGAGCC
C		
F3-3	Forward outer	CGGCAATGCGTAAGTCTC
B3-3	Reverse outer	GACTTACACGCCAGAGCC
FIP-3	Forward inner	GCAGCACCGATTGTGGTTACCCTCTCCCGACCATCTTTTTCGC
BIP-3	Reverse inner	TGGTTTGTGTGCCAAAACACCCGCGGAGATGGTCTGCCTGT
D		
F3-4	Forward outer	TCCATAGGTTACCTCCAGA
B3-4	Reverse outer	GACTTACACGCCAGAGCC
FIP-4	Forward inner	GCAGCACCGATTGTGGTTACCCTGCGTAAGTCTCCTCCCGA
BIP-4	Reverse inner	TGGTTTGTGTGCCAAAACACCCGCGGAGATGGTCTGCCTGT

$$Y = -6.863x + 49.937$$

(Fig. 3D); the calculation used to determine the number of spores is:

$$10^{\left(\frac{49.937 - y}{6.863}\right)}$$

and the correlation coefficient $R^2 = 0.9922$, which showed a good linear relationship.

Application of q-LAMP assays detecting *B. dothidea* from trunks. For the samples collected from the infected trunks in Taiyang Town (TY-0, TY-5, TY-10, and TY-20) (Fig. 4A and B), the q-LAMP assay results showed that the amplification times of trunk samples were 23.99, 26.41, 31.54, and 33.82 min (Fig. 4C). According to the DNA concentration calculation formula;

$$10^{\left(\frac{37.233 - y}{3.5823} - 4\right)}$$

the DNA concentration values corresponding to each sample were 0.498, 0.105, 0.004, and 0.001 ng/μl, respectively. Therefore, there

is a phenomenon in which DNA of *B. dothidea* was detected from asymptomatic trunk tissues, and the amount of DNA of *B. dothidea* decreases as the distance from the lesion increases.

Application of q-LAMP assays detecting *B. dothidea* in water. Using the established q-LAMP detection system, it was found that the amplification times of spore samples added quantitatively to artificially prepared water samples were 29.31, 36.40, and 43.54 min. According to the calculation formula:

$$10^{\left(\frac{49.937 - y}{6.863}\right)}$$

the numbers of spores corresponding to each sample were 1,012.83, 93.86, and 8.55, respectively. The q-LAMP detection results of spore number were consistent with those of artificial additions (Fig. 5).

Moreover, it was found that the amplification times of spore samples in forest water samples were 30.93, 33.22, 34.00, 37.45, and 39.09 min (Supplementary Fig. S2). According to the calculation formula:

Fig. 2. Specificity of the quantitative loop-mediated isothermal amplification detection system for *Botryosphaeria dothidea*, **A**, on the basis of HNB color change; **B**, on the basis of gel electrophoresis, lane 1: *Trichoderma viride*; 2: *Fusarium proliferatum*; 3: *Pestalotiopsis microspora*; 4: *Nigrospora chinensis*; 5: *Alternaria oregonensis*; 6: *Daldinia* sp. 7: CK; 8: BD16-1; 9: BD17-1; 10: BD18-1; M: DNA marker. **C**, Fluorescence signals detected in DNA samples containing *B. dothidea*. RFU: relative fluorescence units.

Fig. 3. Standard curve of mycelium DNA and spore for *Botryosphaeria dothidea*. **A**, A representative amplification plot for 10-fold serial dilution containing 100 ng to 0.001 ng of DNA. **B**, Standard curve derived from absolute quantification of 10-fold serially diluted DNA from mycelium of *B. dothidea*. **C**, A representative amplification plot for 10-fold serial dilution containing 10^5 to 1 spores/ml. **D**, Standard curve derived from absolute quantification of 10-fold serially diluted DNA from spores of *B. dothidea*. RFU: relative fluorescence units.

$$10^{\left(\frac{49.937-y}{6.863}\right)}$$

the numbers of containers A to E spores corresponding to each sample were 588.17, 272.77, 209.99, 65.99, and 38.107, respectively.

Application of q-LAMP assays detecting *B. dothidea* in air samples. Using the established q-LAMP detection system, the amplification times of spore samples quantitatively added to the Melinex tape were 29.42, 35.94, and 43.48 min. According to the calculation formula:

$$10^{\left(\frac{49.937-y}{6.863}\right)}$$

the numbers of spores corresponding to each sample were 976.11, 109.49, and 8.73, respectively. The q-LAMP detection results of spore number were consistent with those of artificial additions (Fig. 6).

Spore samples from Melinex tapes in the spore catcher located in forests in Taiyang Town, Lin'an District, Hangzhou City were quantified. It was found that the amplification times of samples 1 to 4 were 38.01, 39.07, 39.85, and 40.42 min. According to the calculation formula

$$10^{\left(\frac{49.937-y}{6.863}\right)}$$

the numbers of spores corresponding to each sample were 54.70, 38.28, 29.51, and 24.38, respectively, whereas sample 5 did not show any amplification reaction (Supplementary Fig. S3).

Discussion

This study focuses on the development, validation, and application of the q-LAMP detection system. It is very important to explore a stable and sensitive detection system for the detection and quantification of *B. dothidea* in trunk, water, and air samples. As a laboratory analysis and routine method, PCR is the most widely used. However, it not only requires a high level of instrumentation in the amplification stage, but also, it has a long reaction time. At the same time, the Taqman-based real-time PCR assay also has this shortcoming. Therefore, rapid, specific, and economic q-LAMP assays are absolutely a good choice for the detection of *B. dothidea*. LAMP is an innovative technique for gene amplification and a simple diagnostic tool for the early detection (Tomito et al. 2008).

Fig. 4. The samples collected from the infected trunk for quantitative loop-mediated isothermal amplification assays. **A**, Location of sample collection points in Hangzhou City, China. **B**, Schematic diagram of sample collection on the trunk. **C**, A representative amplification plot of the sample from Taiyang Town. RFU: relative fluorescence units.

To extract DNA from lesions more efficiently and quickly, three different methods were tried in this study: the improved CTAB method (Gao and Qiao-Ming 2008; Japelaghi et al. 2011), the commonly used SDS-CTAB binding method (Hua-Hui et al. 2010; Niu et al. 2010; Wei-Long et al. 2013), and the TIANGEN Plant Genomic DNA Kit (not shown in this article). Bark is thick and hard, and it is rich in polysaccharides, polyphenols, tannins, pigments, and other secondary metabolites. It has been found that the DNA separated by the simple CTAB method is brown because of polyphenol oxidation. Polysaccharides, tannins, and other substances combine with DNA to form a viscous gelatinous substance. The modified CTAB method and the commonly used SDS-CTAB binding method cannot prevent coprecipitation of the polysaccharide and the nucleic acid. The extracted genomic DNA has low purity and cannot be used to perform stable LAMP. However, using an FW100 high-rotation speed disintegrator to crush bark and then extract DNA according to the requirements of the TIANGEN Plant Genomic DNA Kit not only extracts high-quality samples but also, is time consuming. For the extraction of spore DNA from trunk canker fungus, to increase the sensitivity and sample size of the *B. dothidea* spores detection assay in water, a centrifugation-based method was used to concentrate the conidia in a smaller sample volume (Gehesquière et al. 2013). In addition, the combination of the Fsat-prep instrument and the TIANGEN Plant Genomic DNA Kit was used for the first time in this study to obtain

Fig. 5. Quantitative loop-mediated isothermal amplification assays detecting artificially added conidia in water samples. Ct: cycle threshold.

Fig. 6. Quantitative loop-mediated isothermal amplification assays detecting artificially added conidia in air samples. Ct: cycle threshold.

high-purity spore DNA, which was of great help for subsequent LAMP detection.

Chinese hickory lives in a complex natural environment. There are not only *B. dothidea* but also, other endophytic fungi in the diseased trunk. Therefore, how to specifically detect *B. dothidea* in such an environment is very important for the occurrence and development of the disease. In this study, several endophytic fungi on Chinese hickory were isolated, and primers were successfully designed to specifically detect trunk canker fungus based on the difference in the area of β -tubulin.

Using the q-LAMP detection technology established in this study, samples with no symptoms on the trunk surface can be detected. In addition, the q-LAMP assay results of trunk canker fungus showed that, with the increase of the distance between the collection site and the visible lesions, the DNA quantity of the detected trunk canker fungus decreased continuously, but when the collection distance exceeded 20 cm, the detection success rate was greatly reduced; of course, there was also the situation that the collection site was not infected by the trunk canker fungus. At present, the surgical removal of canker lesions is an important technique to control trunk canker disease on Chinese hickory (Yang et al. 2009). Our results indicated that *B. dothidea* existed not only in the canker lesions but also, in the tissues around them. Therefore, treatment of the trunk not limited to the lesions will be expected to have a better outcome. Trunk canker fungus has the characteristics of latent infection; in the absence of visible lesions, we cannot determine whether Chinese hickory trees have been infected by trunk canker fungus. In future research, it is still necessary to optimize and improve this technology to detect trunk canker fungus successfully and accurately before the appearance of visible lesions. However, this technology can monitor changes of spores in water and air, which is very important for the prediction and prevention of Chinese hickory trunk canker. Previous studies suggested that *B. dothidea* in a California pistachio orchard was a water splash-dispersed pathogen with relatively short distances of spore dispersal (Ahimera et al. 2004; Michailides 1993). In additional studies, we will adopt this technique to monitor the changes of spores in water and air for successive years to analyze their correlations with the outbreak of trunk canker disease. This can give more information about the epidemiology and inoculum dispersal of *B. dothidea* infecting Chinese hickory.

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