Original article

Rapid detection by a loop-mediated isothermal amplification assay based on *EF-1α* gene for stem rot on *Cymbidium ensifolium*

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Summary

Based on the Elongation factor 1α (*EF-1α*) sequence of *Fusarium oxysporum*, a set of four LAMP (Loop-mediated isothermal amplification) primers were designed, and a rapid, simple and sensitive method for the visualization detection of *Fusarium oxysporum* of stem rot on *Cymbidium ensifolium* established. The template DNA from infected stems were used for a one-step LAMP which was carried out under different temperature and time conditions. The optimal temperature and duration were found to be 64°C for 60 min by SYBR Green I as a fluorescence indicator and electrophoresis in agarose gels for detection that with LAMP products in this study. LAMP assay was used for specific evaluation of all strains, only *F. oxysporum* of *Cymbidium ensifolium* turned yellow-green, and the other strains were all orange, including the other diseases on *Cymbidium*. The detection limit of *F. oxysporum* by LAMP was 1 pg genomic DNA per 25 μL reaction, while the limit of detection of routine polymerase chain reaction (PCR) was 10 pg. The positive ratios of LAMP, PCR, and traditional tissue isolation for detecting *F. oxysporum* of stem rot samples with typical symptoms in *Cymbidium ensifolium* were 35/35 (100%), 31/35 (88.6%) and 29/35 (82.8%), respectively. In conclusion, the establishment of LAMP assay provides a new technical platform for the real-time monitoring and detection for stem rot on *Cymbidium ensifolium*. It meets the need of rapid detection, diagnosis, and real-time monitoring of the pathogen in production.

Keywords

Cymbidium ensifolium, *Fusarium oxysporum*, Polymerase Chain Reaction, loop-mediated isothermal amplification, pathogen detection

Significance of this study

What is already known on this subject?

- Stem rot caused by *Fusarium oxysporum* is one of the main diseases of *Cymbidium ensifolium*, which has severely hit the orchid industry. Therefore, a rapid and accurate detection method for stem rot of *C. ensifolium* is needed in production. In recent years, LAMP technology has been widely used in pathogen detection, and the detection time is shorter than the conventional PCR method. SYBR Green I can also be added to realize the visualization of detection results. Therefore, this paper designed a set of specific primers and established a LAMP visual detection method for stem rot of *C. ensifolium*.

What are the new findings?

- In this paper, *EF-1α* (Elongation factor-1α) gene sequence of stem rot pathogen *F. oxysporum* of *Cymbidium ensifolium* was used as the target sequence, and a set of specific primers was designed. The optimal reaction temperature and time were determined to be 65°C and 60 min, respectively. Specific verification showed that the reaction product of *F. oxysporum*, a stem rot pathogen of *C. ensifolium*, was yellow-green (positive). Sensitivity test results show that the minimum concentration of LAMP detection is 1 pg μL⁻¹, which is 10 times higher than conventional PCR. The detection rate of 35 field samples was 100% by the LAMP method.

What is the expected impact on horticulture?

- The LAMP system for rapid detection of *F. oxysporum* on stem rot of Jianlan established in this study has the advantages of strong specificity, high sensitivity, visible detection results, low false positive, low cost and high speed, which can improve the accuracy and success rate of prevention and control of stem rot of horticultural crops.

Introduction

Stem rot caused by *Fusarium oxysporum* is one of the main diseases affecting *Cymbidium ensifolium* (Yao et al., 2018a). It restricts the production, reduces the quality of flowers, and causes serious economic losses in most parts of the world (Huang et al., 2018). *Fusarium oxysporum* on *C. ensifolium* occurs in the young through the reproductive growth stages (Yao et al., 2018a). Pathogens infect the plants through stems, especially through wounds, invade the vascu-

lar systems, and secrete mycotoxins during infection. Consequently, symptoms of infected plants include necrosis at the base of stem, wilting of leaves, yellowish-brown of leaves, and eventually withering away to death (Yao et al., 2018a). It is difficult to identify the stem rot of *C. ensifolium* until it begins to exhibit typical yellow and wilting symptoms. Effective management approaches to control the stem rot of plant adopt resistant cultivars, cultivation method, and fungicides

(Lan et al., 2018). However, despite these measures, a single control measure is not totally effective, so it is still difficult to control stem rot. *Fusarium oxysporum* or *Fusarium* spp. can survive for years in soil under various conditions (Franco et al., 2018; Armitage et al., 2018), which made crop rotation measures ineffective. On the other hand, there are no resistant cultivars of *C. ensifolium*. At present, the most effective method to control the disease is to use large amounts of fungicides. However, it will bring about pesticide residues in crops and is bad for the environment (Sinyangwe et al., 2016; Chen et al., 2018). Early accurate and reliable diagnosis of plant disease is the basis of integrated disease management (Yao et al., 2013). Thus, it is of great significance to develop a simple, convenient, economical, and sensitive assay for the early monitoring and detection of *F. oxysporum* before the onset of symptoms.

So far, many methods have been developed to detect *F. oxysporum*, such as traditional biological methods, PCR, nested PCR, and real-time PCR (Souza et al., 2017; Li et al., 2012; Ling et al., 2016). The diversity and complexity of symptoms and pathogens gives difficulties in rapidly and accurately diagnosing the disease with traditional biological methods (Yao et al., 2018b). Meanwhile, PCR approach has been extensively used to detect plant pathogens (Kim et al., 2018; Crutcher et al., 2016; Sasaki et al., 2015), but it has intrinsic drawback, including a relatively long operation time and a complicated process, which limit its application in poorly equipped departments and districts. Recently, LAMP method was established for nucleic acid amplification (Herrera et al., 2018; Almasi, 2019). Compared with PCR, LAMP method is

much faster and can be completed to reaction within 1 h by water bath under isothermal conditions (Tian et al., 2017). In addition, the specificity of PCR was lower than that of LAMP, because four–six LAMP primers that recognize regions can be properly hybridized to the target site before amplification. Furthermore, DNA dyes can be directly measured with the LAMP product and simply observing the color changes with the naked eye (Herrera et al., 2018; Du et al., 2019). Because its features of fast detection, simple and convenient operation, and direct visual judgment, LAMP has been successfully used in pathogen detection, including *F. oxysporum* f. sp. (Ayukawa et al., 2016; Shan et al., 2019), *Colletotrichum* spp. (Chandra et al., 2015), *Phytophthora* spp. (Li et al., 2019), *Salmonella* spp. (Yang et al., 2016), and virus (Oloniniyi et al., 2017). Currently, no attempt to design LAMP primers for detecting *F. oxysporum* on *C. ensifolium* has been reported. Because of its specialized forms and obvious individual polymorphism of *F. oxysporum* (Booth, 1971; Ibrahim et al., 2017), it is easy to be affected and mutated by the environment (Zhang et al., 2013). It is possible that LAMP of *F. oxysporum* assays have differences in sensitivity and specificity. So the selection of target genes and the properties of primers are crucial for the specificity and sensitivity of the LAMP assay. It was found that *EF-1α* gene sequences were conserved to a certain extent in *Fusarium* species and among *Fusarium* strains with different specialization types, and there were abundant changes among species, making it a better molecular detection target than rDNA-ITS, RAPD, and *β-tubulin* sequences (Wei et al., 2013). Thus, we used the target sequence of the *EF-1α* gene to design specific primers to detect *F. oxysporum*.

TABLE 1. Isolates tested in the LAMP assay.

Species ^a	Host	Number of isolates	Locality	LAMP ^b
<i>Fusarium oxysporum</i>	<i>Cymbidium ensifolium</i>	6	Fujian, China	+
<i>Fusarium oxysporum</i>	<i>Cymbidium ensifolium</i>	4	Guangdong, China	+
<i>Fusarium oxysporum</i>	<i>Cymbidium ensifolium</i>	5	Zhejiang, China	+
<i>Fusarium oxysporum</i>	<i>Musa nana</i>	2	Fujian, China	+
<i>Fusarium oxysporum</i>	<i>Cucumis sativus</i>	3	Fujian, China	+
<i>Fusarium sporotrichioides</i>	<i>Pisum sativum</i>	3	Fujian, China	–
<i>Fusarium solani</i>	<i>Solanum lycopersicum</i>	2	Fujian, China	–
<i>Fusarium graminearum</i>	<i>Triticum aestivum</i>	1	Jiangsu, China	–
<i>Fusarium nivale</i>	<i>Triticum aestivum</i>	1	Jiangsu, China	–
<i>Fusarium moniliforme</i>	<i>Oryza sativa</i>	1	Jiangsu, China	–
<i>Fusarium sambucinum</i>	<i>Solanum tuberosum</i>	1	Heilongjiang, China	–
<i>Fusarium acuminatum</i>	<i>Medicago sativa</i>	1	Gansu, China	–
<i>Fusarium avenaceum</i>	<i>Avena sativa</i>	1	Gansu, China	–
<i>Exserohilum turcicum</i>	<i>Zea mays</i>	3	Fujian, China	–
<i>Bipolaria maydis</i>	<i>Zea mays</i>	3	Fujian, China	–
<i>Phomopsis asparagi</i>	<i>Asparagus officinalis</i>	1	Fujian, China	–
<i>Botryosphaeria rhodina</i>	<i>Psidium guajava</i>	1	Fujian, China	–
<i>Verticillium dahliae</i>	<i>Solanum melongena</i>	1	Fujian, China	–
<i>Colletotrichum gloeosporioides</i>	<i>Cymbidium ensifolium</i>	1	Fujian, China	–
<i>Alternaria kikuchiana</i>	<i>Pyrus pyrifolia</i>	1	Fujian, China	–
<i>Alternaria alternata</i>	<i>Cymbidium ensifolium</i>	2	Fujian, China	–
<i>Phytophthora vignae</i> Pruss	<i>Vigna</i> sp.	1	Fujian, China	–
<i>Magnaporthe oryzae</i>	<i>Oryza sativa</i>	1	Fujian, China	–
<i>Botrytis cinerea</i>	<i>Solanum lycopersicum</i>	1	Fujian, China	–
<i>Ralstonia solanacearum</i>	<i>Solanum tuberosum</i>	1	Fujian, China	–

^aAll isolates of *Fusarium oxysporum* and other species and fungi were maintained in the collection of the Fujian Academy of Agricultural Science.

^bNote that presence (+) or absence (–) are based on the presence of a LAMP product of the expected size.

porum on *C. ensifolium* in diseased tissue. LAMP is a detection technology with intuitive results, and this method was considered to be cost-effective for researchers with limited budgets and to be used in the field.

Materials and methods

Strains and culture conditions

The strains contain 20 strains of *F. oxysporum*, 11 strains of other *Fusarium* spp., and 17 strains of other fungi species (Table 1). The strains were cultured in 100 mL of potato-dextrose broth by shake cultivation (180 rpm) at 28°C for 4–5 days. After that, the mycelia was filtered from the tested isolate, dried by vacuum freezing, and stored at -80°C.

DNA extraction

Genomic DNA was extracted by plant genomic DNA kit (Tiangen, Beijing, China). The concentration of DNA was measured by NanoDrop 2000c spectrophotometer (Thermo Fischer Scientific, Waltham MA, USA). The template DNA detected by LAMP was diluted in ddH₂O as required. All samples were stored at -20°C.

LAMP primer design

The primers were designed by software (<http://lamp-designer.software.informer.com/>) based on the conserved region of *EF-1α* gene of *F. oxysporum*. The primers were designed by the software Primer Explorer v. 4 (<http://primer-explorer.jp/elamp4.0.0/index.html>; Eiken Chemical Co., Tokyo, Japan). A set of primers with the highest sensitivity and specificity, which is the forward inner primer (FIP) and reverse inner primer (BIP), the two outer primers (F3 and B3). Primer sequences are shown in Table 2 and synthesized by Sangon (Shanghai Sangon Biotech Co., Ltd., Shanghai, China).

Conditions for LAMP assay

LAMP reaction was performed in 25 µL volume consisting of 0.2 µM each of outer primers F3 and B3, 1.6 µM each of inner primers FIP and BIP, 1.0 µL target DNA sample (50 ng), 8 U *Bst* DNA polymerase, 20 mM Tris-HCl, 10 mM (NH₄)₂SO₄, 6.0 mM MgSO₄, 50 mM KCl, 0.8 mM Betaine, 1.4 mM dNTPs, and added ddH₂O to the total volume of 25 µL. 1.0 µL SYBR green I was added to the tube lid and the tube was sealed.

The positive control was DNA of *F. oxysporum*, and the negative control was ddH₂O.

The optimal reaction temperature and time of the LAMP were further screened according to the method of Li et al. (2018).

Specificity and sensitivity of LAMP assay

The specificity of LAMP primers was tested by DNA of 20 *F. oxysporum* strains, 11 other *Fusarium* spp. strains, and 17 other fungi species strains (Table 1), and the positive and negative control, respectively, are *F. oxysporum* and ddH₂O. To verify the sensitivity of LAMP, the genome DNA of *F. oxysporum* was diluted with ddH₂O from 1 ng µL⁻¹ to 100 ag µL⁻¹, and the same samples were used for PCR reaction. The LAMP tests were conducted under the conditions as described above, and detected by the SYBR Green I and gel electrophoresis. Three replicates were analyzed to each sample.

Detection of diseased plants

To verify the practicability of the LAMP method, 35 samples of *C. ensifolium* stem tissue with symptoms of stem rot collected from Fujian, Guangdong, and Zhejiang provinces in China (Table 3) were tested by LAMP. Healthy samples of *C. ensifolium* and ddH₂O were used for the negative controls, and the positive control was *F. oxysporum* of *C. ensifolium*. According to the method of Yao et al. (2018a), DNA was extracted from the collected samples for LAMP test. LAMP tests were executed with the conditions above, which were detected by the SYBR Green I and gel electrophoresis. Meanwhile, samples collected above were detected by PCR and identified morphology. Three replicates were analyzed to each sample.

Results

Conditions for LAMP reactions

The DNA of *F. oxysporum* was used for the LAMP test to confirm the best temperature and time of reaction. The trapezoidal bands of the test products appeared at 65°C and 66°C, with a temperature of 65°C having the strongest intensity (Figure 1a). Then optimize the reaction time at 65°C. The positive results were obtained in 60 to 90 min by gel electrophoresis (Figure 1b), while the trapezoidal bands was the strongest at 60 min (Figure 1b). Therefore, the best reaction conditions of the LAMP assay for detection of *F. oxysporum* are at 65°C for 60 min (Figure 2).

TABLE 2. Nucleotide sequences of LAMP primers used in this study.

Primer	Sequence (5'–3')
F3	GCGTTTGCCCTCTTAACCAT
B3	GCATGAGCGACAACATACCA
FIP	CGAGCTCAGCGGCTTCCTATT-CACAACCTCAATGAGTGCGT
BIP	TGACAAGCTCAAGGCCGAGCAGGAGTCTCGAACTTCCAGA

TABLE 3. Diseased plant stem and root samples used in this study and numbers of positive detections of *Fusarium oxysporum*.

Source locations	No. of samples	Positives in LAMP	Positives in PCR	Positives in isolation
Fujian, China	15	15	14	12
Guangdong, China	10	10	9	9
Zhejiang, China	10	10	8	8
Total	35	35	31	29

FIGURE 1. Screening of the optimal temperature and optimal time for LAMP. (a) The temperature of LAMP for *Fusarium oxysporum* was detected by 2.0% agarose gel electrophoresis. (b) The optimal time of LAMP for *F. oxysporum* was detected by 2.0% agarose gel electrophoresis. Lane M: DL 2000 DNA marker.

Specificity of LAMP assay

F. oxysporum and other fungi pathogenic were applied to test the LAMP assay specificity. LAMP reaction products with *F. oxysporum* appeared the characteristic trapezoidal bands of on the agarose gel, and the color turned to yellow-green (Figure 3, not all data shown). Nevertheless, containing other *Fusarium* spp. or other fungi species strains were negative (Figure 3). The visual results were consistent with gel electrophoresis results. In addition, the specificity of LAMP on the detection for *F. oxysporum* we showed in the assay.

Sensitivity of LAMP assay

The verification sensitivity of LAMP was detected by 10 times dilution ($10 \text{ ng } \mu\text{L}^{-1}$, $1 \text{ ng } \mu\text{L}^{-1}$, $100 \text{ pg } \mu\text{L}^{-1}$, $10 \text{ pg } \mu\text{L}^{-1}$, $1 \text{ pg } \mu\text{L}^{-1}$, $100 \text{ fg } \mu\text{L}^{-1}$, $10 \text{ fg } \mu\text{L}^{-1}$, $1 \text{ fg } \mu\text{L}^{-1}$) of DNA of *F. oxysporum*. From Figure 4a–b, the minimum concentration was 1 pg in 25 μL while the limit of PCR detection is 10 pg (Figure 4c). The LAMP assay was 10 times more sensitive than PCR.

FIGURE 2. Detection of *Fusarium oxysporum* by the optimized LAMP system. (a) The specificity of LAMP for *F. oxysporum* was detected by 2.0% agarose gel electrophoresis. Lane 1: *F. oxysporum*; lane 2: negative control; lane M: DL 2000 DNA marker. (b) The specificity of LAMP for *F. oxysporum* was detected by SYBR Green I. Tube 1: *F. oxysporum*; tube 2: negative control.

FIGURE 3. Specificity of LAMP detection for *Fusarium oxysporum* and other different strains (not all data shown). (a) The specificity of LAMP for *F. oxysporum* strains and other pathogens was detected by 2.0% agarose gel electrophoresis, and the positive reaction had a ladder-like pattern. Lane M: marker DL 2000; lane 1: *F. oxysporum*, lane 2: *Colletotrichum gloeosporioides*; lane 3: *Fusarium moniliforme*; lane 4: *Botrytis cinerea*; lane 5: *Alternaria alternata*; lane 6: *Phytophthora vignae* Pruss; lane 7: *Fusarium solani*; lane 8: negative control. (b) The specificity of LAMP for *F. oxysporum* was detected by the naked eye. Positive samples produce a green color (tube 1 containing 100 ng of *F. oxysporum* DNA), whereas the negative control (tube 8) and other different pathogens remained orange (tubes 2–7).

FIGURE 4. Sensitivity of the LAMP assay to detect serially diluted genomic DNA (from 10 ng to 1 fg) with *Fusarium oxysporum*. (a) The LAMP assay detected by 2.0% agarose gel electrophoresis. Lane M: DL 2000 DNA marker; lanes 1–8: amplified products using DNA at concentrations of 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, and 1 fg in a 25-μL LAMP reaction. (b) Visual appearance of LAMP reactions from the diluted genomic DNA. (c) Agarose gel electrophoresis analysis of the PCR products.

FIGURE 5. Application of LAMP for detecting *Fusarium oxysporum* in naturally infected plant tissue (not all data shown). (a) Agarose gel electrophoresis analysis of the LAMP products. Lane M: DL 2000 DNA marker; lane 1: root disease tissue; lane 2: stem disease tissue; lane 3: root disease tissue; lane 4: stem disease tissue; lane 5: stem disease tissue; lane 6: healthy tissue. (b) Visual inspection of the LAMP products. Tube 1: root disease tissue; tube 2: stem disease tissue; tube 3: root disease tissue; tube 4: stem disease tissue; tube 5: stem disease tissue; tube 6: healthy tissue.

Detection of diseased plants

The LAMP assay was used for detecting samples, which displaying stem rot of *C. ensifolium* from the field in Fujian, Guangdong and Zhejiang provinces. The results showed that stems displaying obvious symptoms were positive (Figure 5, not all data shown). The detection rates of *F. oxysporum* of *C. ensifolium* samples for LAMP, PCR, and identified morphology detected *F. oxysporum* of stem samples with obvious symptoms were 35/35 (100%), 31/35 (88.6%) and 29/35 (82.8%). As expected, the LAMP assay was more sensitive than PCR or identified morphology method (Table 3). The results indicated the feasibility of the LAMP assay to directly detect stem rot-suspect collected from the field.

Discussion

LAMP method is a simple, economical and rapid nucleic acid isothermal amplification method without complex equipment, and the results can be judged visually using SYBR Green I (Pang et al., 2019; Feng et al., 2019). LAMP assay uses a set of 4–6 primers, by hybridizing 6 to 8 binding sites with different regions of the target gene, expressing great specificity (Lan et al., 2018). In recent years, LAMP technology has been extensively applied in plant disease early warning with remarkable results (Xu et al., 2017). In this study, the forward inner primer (FIP) and reverse inner primer (BIP), the two outer primers (F3 and B3) were designed with *EF-1α* sequence of *C. ensifolium F. oxysporum*, and optimized the reaction conditions. Finally, visualisation of the LAMP assay for *F. oxysporum* of *C. ensifolium* was established. All *F. oxysporum* isolates in the study showed color changes, but other isolates, including *Fusarium* spp. such as *F. moniliforme*, *F. sambucinum*, *F. solani*, and other strains were negative. The results of agarose gel electrophoresis and LAMP visual affirmed that the target of LAMP amplification was

correct. Therefore, the LAMP primers have high specificity for *F. oxysporum* in this study, while it further confirmed high specificity of the LAMP assay. Okiro et al. (2019) and Meng et al. (2016) reported that the sensitivity of LAMP assay was 10–1,000 times higher than that of PCR. LAMP reactions are faster, more sensitive, more intuitive, and less complex than PCR. In this study, the limit of LAMP sensitivity was 10 times higher than that of PCR. Lu et al. (2015) reported the development of LAMP assay targeting the CYP51C element for visual detection of *F. oxysporum*; Ayukawa et al. (2016) introduced detection of *Fusarium oxysporum* f. sp. *lycopersici* (Fol) race 1 by the primer targeting for the *SIX4* and *SIX5* genes. However, their sensitivity was lower than the LAMP assay targeting the *EF-1a* gene in this study. Ghosh et al. (2016) reported the reaction using minimum 10 fg of DNA (LAMP) for LAMP assay targeting the *EF-1a* gene. So, *EF-1a* gene conserved sequence is suitable for designing LAMP primer, although it has been reported that the detection sensitivity of some *F. oxysporum* is relatively high.

LAMP primers of that may be unsuitable for the other specific types of *F. oxysporum*. Therefore, there have many reports the use of LAMP to detect *F. oxysporum* or *F. oxysporum* f. sp. of different crops in recent years (Almasi, 2019; Chen et al., 2018; Franco, 2018; Ayukawa et al., 2016; Peng et al., 2014; Lu et al., 2014). Compared with conventional PCR and identified morphology, the LAMP assay was faster and easier and can be directly observed by naked eye, which is convenient for field real-time monitoring. The approach described here provides an efficient strategy for early monitoring of plants infected by *F. oxysporum* with LAMP assay, which is an effective method to control stem rot of *C. ensifolium*.

Conclusion

A simple, economical, specific, and sensitive LAMP method was found for detection of *F. oxysporum* of *C. ensifolium*. The LAMP assay was faster and easier, and can be directly observed by visualizing, and on the other hand the early monitoring of stem rot of *C. ensifolium* in the field. It will guide researchers or farmers to adopt effective measures to reduce economic losses in the *C. ensifolium* industry.

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