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Establishment and Optimization of a Dual Loop-Mediated Isothermal Amplification (LAMP) Rapid Detection System for *Sclerotinia sclerotiorum* and *Alternaria alternata*

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Abstract

In order to effectively address the problem of the decline in quality and yield of *C. morifolium* cv. Chuju caused by the simultaneous infection of two pathogenic fungi, the existing LAMP technology (a detection method that can be achieved without expensive equipment) provides a window period for disease prevention. Developing a LAMP detection method that can simultaneously detect different pathogens and has high sensitivity has become the key to solving this problem. In this study, we first designed specific primers based on the conserved sequences of the two common pathogens of *C. morifolium* cv. Chuju, namely *Sclerotinia sclerotiorum* and *Alternaria alternata*, for LAMP amplification. Subsequently, we gradually optimized each aspect of the LAMP method to enhance the detection efficiency and sensitivity. The LAMP assay showed high specificity for both pathogens and a detection limit of 1.43×10^{-6} ng/μL, which is about 100-fold higher than that of conventional PCR. These improvements aid in the effective monitoring and early diagnosis of disease during the cultivation of *C. morifolium* cv. Chuju, which will contribute to improved disease management and reduced economic losses.

Keywords

LAMP, *Chrysanthemum morifolium* (Ramat) Tzvel. cv. Chuju, *Sclerotinia sclerotiorum*, *Alternaria alternata*

1. Introduction

Chrysanthemum morifolium (Ramat.) Tzvel. cv. Chuju, abbreviated as *C. morifolium* cv. Chuju, is a nationally recognized geographical-indication product and

is ranked first among the "Four Famous Medicinal Jujubes" in China. It is a unique germplasm resource of Chuzhou that combines medicinal and edible value as well as interesting regional and cultural value [1]-[3]. Despite its importance, production is threatened by two major diseases: sclerotiniose caused by *Sclerotinia sclerotiorum* and black spot disease caused by the necrotrophic fungus *Alternaria alternata*, both of which cause substantial annual yield losses worldwide [4]. Following infection by *A. alternata*, leaves usually form small, almost circular brown lesions that gradually increase in size and number, eventually leading to leaf wilt and possible plant death [5]-[7]. Similarly, *S. sclerotiorum* infection can cause stem and tissue decay, often resulting in whole plant collapse, while black spot disease can severely damage foliage, disrupt flowering and may also cause complete plant mortality [8] [9]. These threats underscore the urgent need for rapid, economical and efficient diagnostic methods. Field-deployable detection tools would be a useful aid for timely management and supply new reference data for the control of *S. sclerotiorum* [10] and *A. alternata* [11].

Conventional polymerase chain reaction (PCR) is the most widely used method for the rapid detection of plant pathogens [12]-[14]. While PCR is a well-established tool in molecular diagnostics, it is not without inherent limitations, including the requirement for precise thermal cycling, potential problems with specificity, and relatively modest amplification efficiency [15]-[17]. Considering these constraints, we developed a field-applicable nucleic acid amplification technique that can detect *S. sclerotiorum* and *A. alternata* without requiring a thermal cycler.

Loop-mediated isothermal amplification (LAMP), first proposed by Notomi *et al.* in 2000 [18], has proven to be a powerful nucleic acid amplification strategy and has obvious advantages in the detection of plant pathogens [19]-[21]. The method has been adapted extensively in microbiological diagnostics and has led to the rapid identification of bacteria [22], viruses [23], and fungi [24]. Despite these advances, a LAMP assay that can detect *S. sclerotiorum* and *A. alternata* simultaneously has not been reported.

In the present study, we developed a LAMP assay based on the ribosomal DNA intergenic spacer region [25] [26]. Our results confirm that the assay is highly specific and efficient. This newly developed LAMP method provides useful reference information for the surveillance and management of *S. sclerotiorum* and *A. alternata*, which cause sclerotiorum and black spot diseases, respectively.

2. Materials and Methods

2.1. Fungal Strains, Culture Conditions, and DNA Extraction

S. sclerotiorum and *A. alternata* isolates were collected from naturally infected *C. morifolium* cv. Chuju plants in the greenhouse at the School of Biological Science and Food Engineering, Chuzhou University, China. Additional fungal pathogens used in this study were obtained from the laboratory's preserved culture collection. *S. sclerotiorum* reference strain KY798875.1 and *A. alternata* reference strain MH560609.1 were further utilized for optimization of the LAMP assay, to deter-

mine the detection limit, and as positive controls for both the LAMP and PCR reactions.

The fungal pathogen strains used in this study were cultured in PDA medium at 25˚C for 72 h [27]. When the mycelium had grown to about two-thirds of the plate surface [28], the mycelial mass was carefully picked up using a sterile inoculation loop and placed in a 1.5-mL centrifuge tube.

2.2. DNA Extraction

Genomic DNAs were extracted using the protocol provided with the Omega Fungal Genomic DNA Extraction Kit (Omega). DNA concentrations and purity were measured using a Nanodrop spectrophotometer, and the purified samples were then stored at −20˚C.

2.3. Primer Design and Specificity Checks

The LAMP primers were designed using the online Primer Explorer V5 software (http://primerexplorer.jp/lampv5e/index.html) with the default settings according to the ribosomal DNA intergenic spacer (IGS) regions of *S. sclerotiorum* and *A. alternata* from the National Center for Biotechnology Information database (https://www.ncbi.nlm.nih.gov/). For *S. sclerotiorum*, primers targeted the IGS1 subregion (287 - 842 bp); for *A. alternata*, primers targeted the IGS2 subregion (312 - 905 bp). The selected loci contain species-specific motifs unique to each pathogen, ensuring amplicon uniqueness: the core LAMP amplicons are ladder-like fragments (a typical LAMP characteristic) of 216 bp (*S. sclerotiorum*) and 248 bp (*A. alternata*). Inner (FIP/BIP) and loop (LF/LB) primers annealed to 6 and 4 conserved sites of the target IGS loci, respectively, to enhance species-specific amplification. Comprehensive in silico verification was performed following standard fungal diagnostic protocols [29], ensuring the primers' discrimination ability.

2.4. Establishment and Optimization of the LAMP Reaction System

The LAMP assays for *S. sclerotiorum* and *A. alternata* were carried out using *Bst* DNA polymerase as the key enzyme with ddH_2O as a negative control instead of the DNA template. Reactions were performed in a total volume of 25 μL in 1.5 mL microcentrifuge tubes that were incubated in a water bath at 65˚C for 60 min [30]. After amplification, SYBR Green I was added to the reaction mixture for visual detection: a fluorescent green color indicated a positive reaction, and an orange color indicated a negative result [31]. To further verify the results, the reaction products were examined by 1% agarose gel electrophoresis. The presence of a ladder-like band pattern was interpreted as a positive outcome, while the absence of a band pattern signified a negative outcome [32].

To optimize the LAMP reaction system, a series of single-factor experiments were performed to assess the effects of important reaction components. The parameters tested were magnesium ion concentration (2 - 12 $mmol \cdot L^{-1}$), dNTPs (0.8

- 1.6 mmol·L^{-1}), the ratio of inner to outer primers (16:1 - 2:1), and betaine concentration (1.0 - 1.8 mol·L^{-1}). Each experiment was conducted following the previously described LAMP protocol, and the results were analyzed to determine the best conditions for efficient amplification. Each group of experiments was repeated three times. The optimal parameters were determined based on the color development situation and in accordance with the principle of economy.

2.5. Optimization of LAMP Reaction Conditions

A two-step approach was used to optimize the LAMP reaction conditions, focusing on reaction time and temperature. LAMP mixtures with and without *Alternaria* DNA as the template were prepared and incubated for 60 min at five different temperatures: 61˚C, 62˚C, 63˚C, 64˚C, and 65˚C.

After the optimal temperature was determined, the reaction time was optimized at this temperature with six reaction time intervals: 45, 50, 55, 60, 65, and 70 minutes. All reaction time experiments were carried out using the same procedures as those for temperature optimization.

2.6. Specificity and Sensitivity of the LAMP Reaction System

The specificity of the LAMP assay was tested with DNA from *S. sclerotiorum*, *A. alternata* and five other phytopathogenic fungi (*Saccharomyces cerevisiae*, *Mortierella rostafinskii*, *Fusarium chlamydosporum*, *Rhizopus arrhizus* and *Talaromyces annesophieae*). The diluted DNA sample was used to assess the sensitivity of the LAMP assay, which was initially quantified at 143.4 ng/μL with a nucleic acid-protein analyzer and was diluted 10-fold in serial dilutions, resulting in DNA sample concentrations of 10^{-1} to 10^{-9} of the original sample. The results were assessed by both fluorescence detection and PCR, according to the procedures mentioned in the above sections.

2.7. DNA Template Range Detection

To test the application of the LAMP assay for various sample types, four tissues of infected *C. morifolium* cv. Chuju plants were chosen: rhizosphere soil, roots, stems, and leaves. DNA was isolated from each tissue and used as a template in the LAMP reaction. The performance of the assay was determined in terms of fluorescence-based chromogenic changes and agarose gel electrophoresis patterns, and the detection range and sensitivity of the assay were determined for different plant tissues.

- Plant DNA extraction

Genomic DNA from *C. morifolium* cv. Chuju was isolated according to a modified CTAB procedure [33] [34]. Pre-warmed CTAB buffer (65˚C) was added to the powder, followed by incubation at 65˚C for 45 min with shaking. After centrifugation at 12,000 rpm for 20 min at room temperature, the supernatant was collected. An equal volume of chloroform:isoamyl alcohol (24:1, v/v) was added, and the mixture was centrifuged again at 12,000 rpm for 20 min. The supernatant

was then transferred to a fresh tube, and two-thirds volume of pre-cooled isopropanol (−20˚C) was added, gently mixed, and incubated for 5 minutes. DNA was pelleted by centrifugation at 8000 rpm for 10 minutes, and the supernatant was discarded. The pellet was washed 2 - 3 times with 75% ethanol (8000 rpm, 10 minutes per wash), resuspended in pre-cooled 95% ethanol, mixed, and centrifuged at 12,000 rpm for 20 minutes. After removal of the ethanol, the pellet was air-dried and dissolved in 200 μL TE buffer. To eliminate residual RNA, 1 μL RNase (10 mg·mL^{-1}) was added, and the solution was incubated at 37˚C for 1 hour. The purified DNA was finally stored at −20˚C.

- Soil DNA extraction

For DNA extraction from soil, 0.25 g of rhizosphere soil was carefully weighed from diseased chrysanthemum plants [35] and transferred into a sterile 1.5-mL centrifuge tube. DNA extraction was carried out with a commercial soil genomic DNA extraction kit, with separate extractions for rhizosphere soils collected from plants with sclerotinia disease and black spot disease. Following extraction, the DNA samples were kept at −20˚C to preserve their integrity for later molecular analyses [36].

3. Results

3.1. Establishment of the LAMP Reaction System

The LAMP primers were listed in Table 1, including two inner primers (FIP and BIP), two outer primers (F3 and B3), and loop primers (LF and LB). The LAMP reaction system consists of DNA template, *Bst* DNA polymerase, magnesium ions (Mg^{2+}), dNTPs, and betaine.

Table 1. Sequence-specific LAMP primers for *Sclerotinia sclerotiorum* and *Alternaria alternata*.

Primer name	Sequence (5' - 3')	bp
S-F3	TGCCTGTTCGAGCGTCAT	18
S-B3	AGTTCAGCGGGTATCCCTA	19
S-FIP	GCCGCCACTGATTTTAGAGCCTTTTCAACCCTCAAGCTCAGC	41
S-BIP	TCGTTACAGGTTCTCGGTGTGCCCTGATCCGAGGTCAACCAT	42
S-LF	GCCATTACTGACATGGACTCAA	22
A-F3	GGATGCTAGACCTTTGCTGA	20
A-B3	ACATTGCGCCCTTTGGTAT	19
A-FIP	TAGCTTTGCTGGAGACTCGCCTTAGAGAGTGCGACTTGTGCT	42
A-BIP	GAGACAAGACGCCCAACACCAAAAGGGCATGCCTGTTCG	39
A-LF	GCCTACTGGTTTCGGAGCGC	20
A-LB	AGCTTGAGGGTACAAATGACGCT	23

3.2. Optimization of the LAMP Reaction System

In the optimization of the reaction system, SYBR Green I staining and gel electrophoresis results indicated that all the tested Mg^{2+} concentrations resulted in green fluorescence and ladder-like bands (**Figure 1**). Considering reagent efficiency, 2.0 mM was chosen as the optimal Mg^{2+} concentration. For dNTPs, the strongest fluorescence and best electrophoresis bands were obtained in the range of 1.2 - 1.6 mM (**Figure 2**); 1.2 mM was selected to minimize the use of reagents. Betaine, which minimizes non-specific amplification [37], resulted in the greatest fluorescence and brightest bands at 1.6 M (**Figure 3**), which was determined to be the optimal concentration. Primer optimization using fluorescence and gel electrophoresis showed that the optimal inner-to-outer primer ratio was 2:1, with final concentrations of 18 μmol·L^{-1} (inner primers), 9 μmol·L^{-1} (outer primers), and 4 μmol·L^{-1} (loop primers).

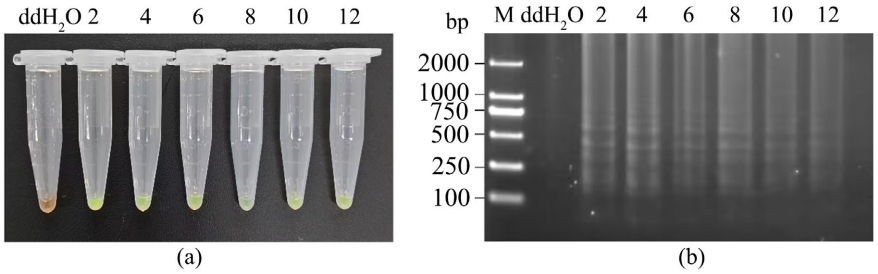

Figure 1. Optimization results of Mg^{2+} concentration. Numbers indicate different Mg^{2+} concentrations (mmol/L) ((a) LAMP result; (b) PCR result; M, marker; the same as follows.).

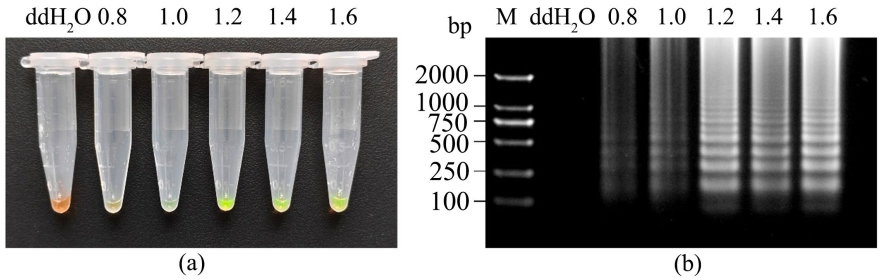

Figure 2. Optimization results of dNTP concentration. Numbers indicate different dNTP concentrations (mmol/L).

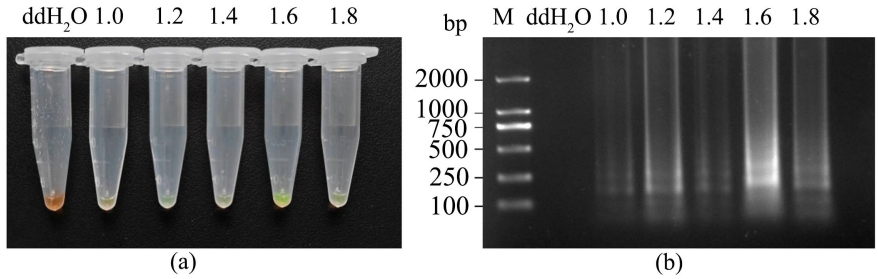

Figure 3. Optimization results of betaine concentration. Numbers indicate different betaine concentrations (mol/L).

3.3. Optimization of LAMP Reaction Conditions

SYBR Green I staining and gel electrophoresis analysis revealed that the LAMP reaction produced the maximum amplification at 60˚C, as indicated by the strong green fluorescence and clear ladder-like bands (Figure 4). Evaluation of reaction times from 45 to 70 min showed that all durations yielded ladder-like bands; however, the highest fluorescence and brightest electrophoresis bands were observed at 45 min (Figure 5). Taking into consideration the amplification efficiency and time efficiency, 45 minutes was determined to be the optimal reaction duration at a constant temperature.

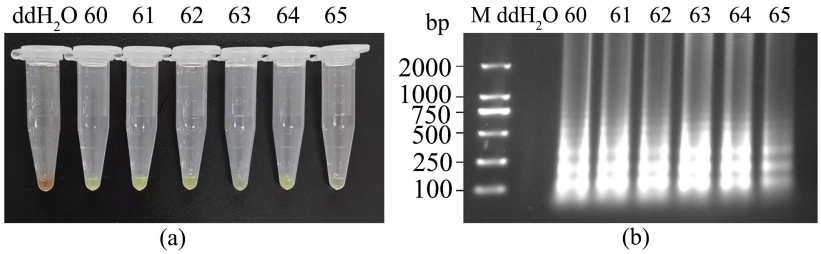

Figure 4. Optimization results of reaction temperature. Numbers indicate different reaction temperatures (˚C).

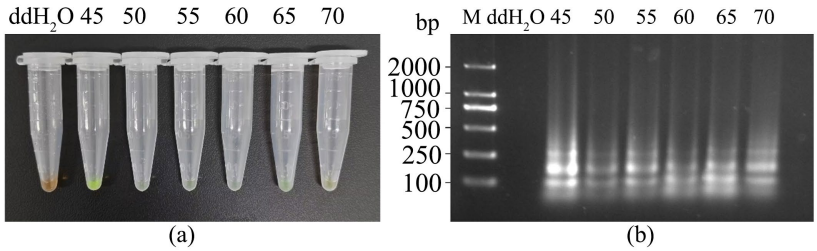

Figure 5. Results of reaction time optimization. Numbers indicate different reaction times (min).

3.4. Specificity of the LAMP Assay

Seven pathogenic fungal DNA samples were tested to determine the specificity of the LAMP assay. Reactions with *S. sclerotiorum* and *A. alternata* templates resulted in clear ladder-like bands on agarose gel and intense green fluorescence after SYBR Green I staining (Figure 6). In contrast, LAMP reactions with DNA from the other fungal pathogens had no detectable bands and were orange-yellow fluorescent, indicating negative results.

3.5. Sensitivity of the LAMP Assay

Serial 10-fold dilutions of extracted DNA were used as templates for both LAMP and PCR amplification. SYBR Green I staining revealed that LAMP reactions gave rise to green fluorescence between 10^{-1} to 10^{-8} ng·μL^{-1} of DNA, and the 10^{-9} ng·μL^{-1} dilution showed orange fluorescence, indicating the absence of amplification (Figure 7). The detection limit of the LAMP assay was 1.43×10^{-6} ng·μL^{-1}, which was compared with 1.43×10^{-4} ng·μL^{-1} for PCR.

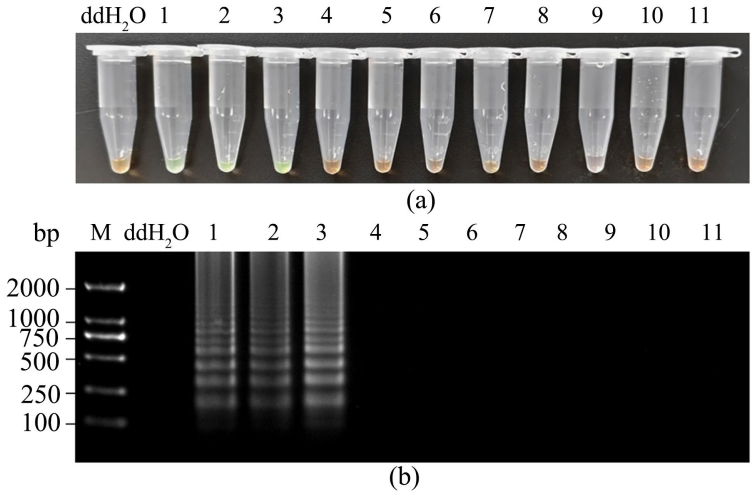

Figure 6. Specific detection of the pathogens of chrysanthemum sclerotinia and black spot diseases using the LAMP reaction system.

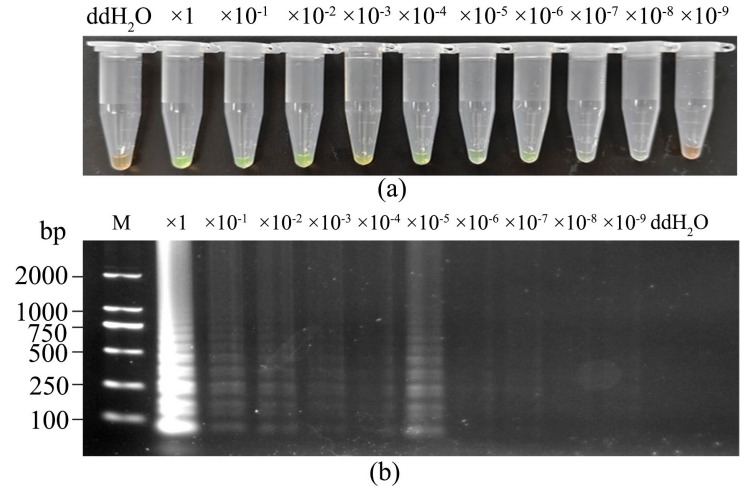

Figure 7. Sensitivity of LAMP for detection of *Alternaria alternata* genomic DNA. 1, original solution of *A. alternata* DNA (143.4 ng/μL); 2-10, 10× gradient dilution solutions in sequence.

3.6. DNA Template Range Detection

The LAMP assay was tested with DNA templates from various tissues of *C. morifolium* cv. Chuju. For sclerotinia detection, DNA from root and stem tissues showed greater SYBR Green I fluorescence and more intense ladder-like bands on gels than DNA from rhizosphere soil and leaves (**Figure 8(a)**), indicating greater detection efficiency in root and stem tissues.

By comparison, the LAMP amplification for black spot disease produced consistent positive results in each of the four tissue types (root, stem, leaf, and rhizosphere soil). All the templates showed strong green fluorescence and well-defined ladder-like bands, whereas no amplification was detected in the negative control (**Figure 8(b)**), thus proving the reliable and stable performance of the assay in different tissues.

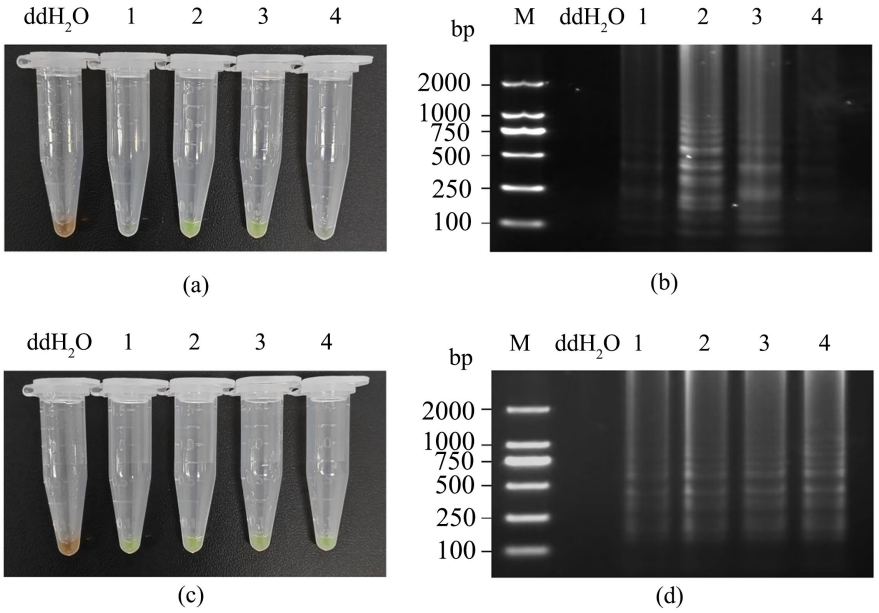

Figure 8. Results of DNA template range detection. (a) and (b), LAMP results; (c) and (d), PCR results. DNA templates from 1 (soil), 2 (root), 3 (stem), 4 (leaf).

To eliminate the interference of potential amplification inhibitors on detection performance, an internal amplification control (IAC) (a non-target exogenous DNA fragment with specific LAMP primers) was co-amplified in all plant/soil extract reactions, and a spike-in recovery check was performed by adding a known concentration of *S. sclerotiorum*/*A. alternata* genomic DNA (1.43×10^{-2} ng/μL) to each tissue/soil extract sample. Results confirm that there was no significant amplification inhibition in the plant/soil extracts used in this study, and the observed differential detection performance across tissues (e.g., higher efficiency in root/stem for *S. sclerotiorum*) reflects the real pathogen distribution in *C. morifolium* cv. Chuju.

4. Discussion

LAMP technology has demonstrated significant potential for the detection of microbial pathogens [38] because of its high specificity, sensitivity, ease of operation, and ability to reveal direct visual results [39]. In comparison to conventional detection methods and immunological assays, which are often labor-intensive and time-consuming, LAMP overcomes these limitations and is especially suitable for the early detection of soil-borne diseases under field conditions [20]. In the current work, the dual LAMP system developed for chrysanthemum sclerotinia and black spot disease showed the practicality of the LAMP system in plant disease diagnostics. By using species-specific primers, the assay provides a method to accurately identify *S. sclerotiorum* and *A. alternata* without the use of expensive equipment, offering a fast and accessible detection method for grassroots agricultural applications.

Optimization of the LAMP reaction system is critical for achieving efficient am-

plification. In this study, single-factor experiments were applied to determine the optimal concentrations of components, and synergies in reaction conditions under different gradients and concentrations were revealed. This observation reflects the multifactorial nature of LAMP reactions. Future studies could use orthogonal experimental designs to minimize factor interactions and further increase system stability. In terms of detection performance, the optimized LAMP assay showed a minimum detection sensitivity of 1.43×10^{-6} ng·μL^{-1}, which was about 100-fold higher than conventional PCR. These results are consistent with previous reports showing the superior sensitivity of LAMP in comparison to PCR [40]-[42], which highlights the benefits of this method for detecting low-abundance pathogens.

Applicability tests showed that root and stem tissues gave better detection for chrysanthemum sclerotia, and consistent detection was seen for all types of samples for black spot disease. These results provide practical information for sampling in the field for diagnosis. Unlike traditional methods that require isolation and culturing of pathogens, which can be time-consuming, the LAMP system provides the ability to directly detect pathogens from DNA extracted from soil or diseased plant tissues. This approach eliminates the need for pathogen culture, greatly reduces the time of detection, and aids in early-stage disease prevention. Moreover, the assay enables rapid diagnostics at grass-root agricultural units without pathogen purification [43], reducing technical barriers for field applications.

At present, the detection resolution of this LAMP system is limited to the genus level, and it can accurately detect *S. sclerotiorum* and *A. alternata*, but it cannot distinguish specific species or strains within the genus. Furthermore, the evaluation of specificity was based on control samples that have low lineage coverage, excluding closely related species (e.g., other *Sclerotinia* spp.) and symbiotic microorganisms associated with *C. morifolium* cv. Chuju, such as rhizosphere probiotics and endophytic fungi. As a result, the anti-interference capability and specificity of the assay in complex natural microbial communities require further validation.

Overall, the LAMP system developed in this study proved to have good performance in specificity, sensitivity, and practical applicability. However, the limitations discussed indicate areas for improvement in the future. Beyond optimizing the reaction system by orthogonal experimental design, we would recommend the following strategies: 1) design species-specific primers to improve taxonomic resolution and allow differentiation at the strain level; 2) expand the diversity of control samples to include closely related pathogens (e.g., *Sclerotinia* spp.) and *C. morifolium*-associated symbiotic microorganisms (rhizosphere probiotics and endophytic fungi) to rigorously validate assay specificity; and 3) include field sample testing to assess anti-interference performance under complex environmental conditions. Implementing these refinements will provide a more robust technical framework for accurate disease prevention and control in *C. morifolium* cv. Chuju.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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