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Rapid and specific detection of *Peronospora belbahrii* in basil (*Ocimum basilicum* L.) using a LAMP assay

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Abstract

Basil downy mildew, caused by the oomycete *Peronospora belbahrii*, can decrease whole basil crops in days, making it a top concern for basil growers. In this study, a Loop-Mediated Isothermal Amplification (LAMP)-based biosensor was developed for the rapid detection of *P. belbahrii* in *Ocimum basilicum* L. A fragment of a gene with unknown function was used as the target sequence to design primers for LAMP reactions. The optimal incubation temperature and time for the most efficient primer set were determined. Under standardized conditions, the LAMP assay exhibited at least 1000-fold higher sensitivity than conventional PCR, detecting attogram levels (1 copy) of synthetic DNA. Detection of the pathogen from genomic DNA (gDNA) of *P. belbahrii* spores confirmed the sensitivity of the assay. In terms of specificity, the LAMP assay showed negative results for gDNA from pathogenic fungi affecting basil and positive results for gDNA from diseased basil leaves. Finally, the LAMP assay could facilitate the rapid detection and diagnosis of basil downy mildew in infected tissues, providing information on basil health and, consequently, informing decisions regarding control management in *O. basilicum* fields.

Keywords: basil, molecular diagnostics, downy mildew, oomycete, loop-mediated isothermal amplification.

Introduction

Peronospora belbahrii is an obligate biotrophic oomycete that causes downy mildew in basil (*Ocimum basilicum* L.), one of the most destructive diseases threatening basil production worldwide [1]. Disease incidence in commercial crops can reach 20-30%, leading to severe yield and quality losses and significant economic impacts estimated in the tens of millions of dollars annually [2, 3]. Characteristic symptoms include interveinal chlorotic lesions on the upper leaf surface and dense gray-purple sporulation on the abaxial surface, which accelerate senescence, necrosis, and eventually plant death [4, 5].

Diagnosis of *P. belbahrii* remains difficult because the pathogen has not been cultured *in vitro* [6]. Consequently, diagnosis is commonly based on symptom observation and microscopic detection of sporangia, which is unreliable at early infection stages when symptoms are not yet visible [7, 8]. Moreover, morphological similarities among oomycetes can complicate microscopic diagnosis, particularly during early infection stages or when sporulation is limited. Accurate identification often requires specialized expertise, increasing the risk of misdiagnosis when relying solely on morphological traits [9, 10]. Since the disease spreads rapidly and can devastate fields within days, early and accurate detection is necessary to mitigate outbreaks and implement timely control strategies [11]. In this context, the development of rapid, sensitive, and specific molecular diagnostic tools has become imperative to improve early detection and enhance disease management [12].

Molecular diagnostic techniques have traditionally focused on identifying plant pathogens through species-specific molecular markers. However, achieving species-level specificity within the Oomycetes remains challenging because many taxa are obligate biotrophs, and the available genomic resources for *P. belbahrii* reamin limited [13, 14].

Most available molecular techniques for oomycete detection are based on conventional polymerase chain reaction (PCR). However, PCR is time-consuming, requires expensive instrumentation, and demands a considerable amount of DNA, which may be insufficient during early or latent infection. These limitations highlight the need to develop portable, rapid, and field-deployable molecular methods that maintain high analytical sensitivity and specificity [15].

Loop-Mediated Isothermal Amplification (LAMP) has emerged as a valuable alternative for molecular diagnosis of plant pathogens. This technique enables DNA amplification under isothermal conditions, providing high sensitivity and specificity with minimal equipment requirements [16, 17, 18, 19]. LAMP reactions are typically completed within 60 minutes, and results can be assessed visually through colorimetric changes or agarose gel electrophoresis [20, 21]. These advantages make LAMP particularly suitable for early detection of obligate biotrophic pathogens in resource-limited or field settings [22]. LAMP assay has already been reported for diverse phytopathogens (viruses, bacteria, fungi, nematodes, oomycetes, etc.) in different plant species, for example: Bean wilt virus 2 (BBWV-2) in patchouli (*Pogostemom cablin*) [23], *Pseudomas syringae* in pea (*Pisum sativum*) [24], *Fusarium oxisporum* in chickpea (*Cicer arietinum*) [25], and *Globodera rostochiensis* on potato (*Solanum tuberosum*) [26].

Within oomycetes, LAMP assays have been successfully applied to *Phytophthora ramorum*, *P. kernoviae*, and *P. nicotianae* [27], *Pythium terrestres*, *P. spinosum*, and *P. huanghuaiense* in soybeans [28, 29], as well as *Bremia lactucae* [30] and *Peronospora destructor* [31]. Despite ongoing progress in molecular diagnostics, the lack of rapid and deployable detection tools for obligated biotrophic pathogens continues to limit early disease surveillance. This gap is particularly critical for *P. belbahrii*, whose rapid epidemic development necessitates diagnostic strategies that support timely phytosanitary interventions in basil crops.

Therefore, this study aimed to develop and validate a LAMP assay for the rapid, specific, and sensitive detection of *P. belbahrii* in *O. basilicum*.

Results

LAMP primer design

For orthologous sequence identification, BLAST analysis was performed using the NCBI database on genomes from the *Peronospora* genus, specifically *P. belbahrii* (GeneBank: GCA_002864105.1, GCA_920618645.1, GCA_921012115.1, and GCA_902712285.1), *P. effusa* (GeneBank: GCA_021491655.1 and GCA_002245715.1), and *P. destructor* (GeneBank: GCA_011800735.1 and GCA_947650345.1). No matching orthologous sequences were detected in *P. farinosa* or *P. tabacina*. (Figure 1).

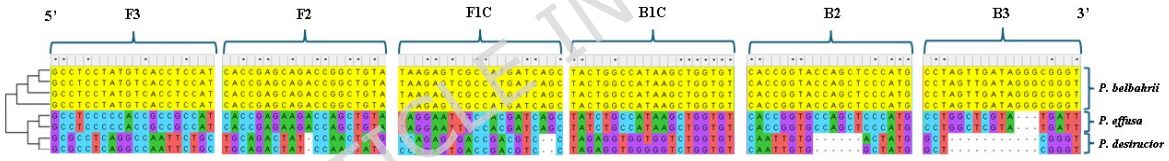

Figure 1. Phylogenetic tree and alignment sequences of *Peronospora species*. A phylogenetic analysis was performed using the Neighbor-Joining method [32], and the percentage of replicate trees in which associated taxa clustered in the bootstrap test (1,000 replicates), using the MEGA11 software [33]. The highlighted region in yellow indicates a consensus sequence and full complementarity among different sequences from *P. belbahrii*

The results of the sequence search are reported in Supplementary Material 1, as well as the site recognized by the *P. belbahrii*-specific primers, showing no relationship with the sequences of the other species. Three sets of primers named PbLAMP-1, PbLAMP-2, and PbLAMP-3 were obtained (Table 1). The sequences used for primer design (MRBU01006465.1 and CAKLCB010000276.1) present 100% identity and little or similarity with sequences from other species of the *Peronospora* genus (Figure 2).

Table 1. Sequences of specific primers designed for *P. belbahrii*.

Panel	Primers	Sequence (5'-3')
1	F3	ACCGGAATTGGGCTGTCT
	B3	GCAAGGTATCGAACTACGCC

	FIP	TGCTATGCTGCTGTGGTTGCTTGGTCTTGACGGTCTCTCA
	BIP	CGAGAAGAGACCACAGCCACAGAATTGTGGCTATGTCGGCA
2	F3	GCCTCCTATGTCACCTCCAT
	B3	ACCCGCCCTATCAACTAGG
	FIP	TACAGCCGGTCTGCTCGGTGTAAGAGTCGCCATGATCAGC
	BIP	CACCGGTACCAGCTCCCATGACACCAGCTTATGGCCAGTA
3	F3	GGATCGGACTTGCCACAG
	B3	GTGGCTATGTCGGCATGG
	FIP	TGGTTGCTGCTTCATGGCGGGGAATTGGGCTGTCTTGGT
	BIP	GCATAGCAACAGCGCTAGCGAGGACGGTATCCTGCAGGTAA
	LF	GAACTGCCAGAGAGTGAGCG
	LB	CCCGAGAAGAGACCACAGC

Figure 2. Schematic representation of the positions and sequences of the *P. belbahrii*-specific primers of the PbLAMP-2 used in LAMP analysis; F1c/F2 and B2c/B1, which constitute the FIP and BIP primers, respectively.

LAMP Optimization and sensitivity for P. belbahrii identification

To determine the sensitivity of the LAMP panel, DNA extracted from sporangia of *P. belbahrii* was used. The detection limit of *P. belbahrii* DNA was up to 5 $\times 10^{-1}$ attograms (1 copy) based on visual detection by colorimetry, while in agarose gel electrophoresis it was 1,000 copies. PCR amplification products with 200 bp bands were observed from 5.94 $\times 10^{-1}$ femtograms (1,000 copies) to 5.94 $\times 10^{-2}$ femtograms (1,000,000 copies) (Figure 3). Sequencing showed 98.1% identity for spore DNA and 98.79% identity for synthetic DNA (NCBI, GenBank database ID: PX516868, and PX516869 respectively).

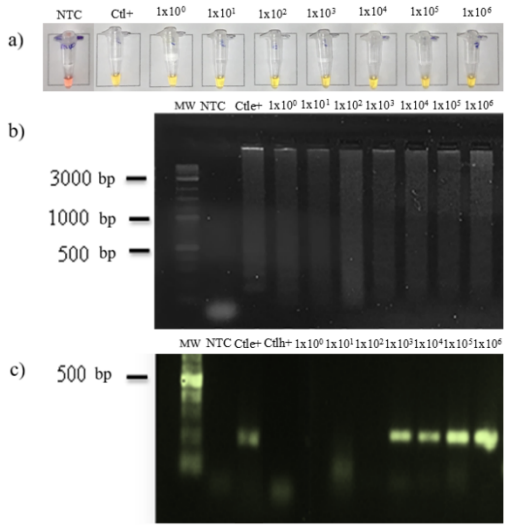

Figure 3. Analytical sensitivity of the LAMP assay for *P. belbahrii*. a) Visual by colorimetric LAMP. b) LAMP visualization by 2% agarose gel electrophoresis. MM) Molecular marker (1 Kb); NTC) Negative control; Ctl+) Spore DNA; 1×10^0) 1 copy of synthetic DNA (0.59 atg); 1×10^1) 10 copies of synthetic DNA (5.9 atg); 1×10^2) 100 copies of synthetic DNA (59.4 atg); 1×10^3) 1,000 copies of synthetic DNA (0.594 ftg); 1×10^4) 10,000 copies of synthetic DNA (5.94 ftg); 1×10^5) 100,000 copies of synthetic DNA (59.4 ftg); 1×10^6) 1,000,000 copies of synthetic DNA (594 ftg) and c) Visualization by PCR. MM). Molecular marker (N3231S); NTC). Negative control; Ctle+). Spore DNA; Ctlh+) DNA from symptomatic leaves; 1×10^0) 1 copy of synthetic DNA; 1×10^1) 10 copies of synthetic DNA; 1×10^2) 100 copies of synthetic DNA; 1×10^3) 1,000 copies of synthetic DNA; 1×10^4) 10,000 copies of synthetic DNA; 1×10^5) 100,000 copies of synthetic DNA; 1×10^6) 1,000,000 copies of synthetic DNA (Supplementary Material 2)

Temperature and time criteria

The thermal conditions of the LAMP reaction were tested using various temperatures and amplification times. Optimal amplification was observed at 65°C (Figure 4), requiring 60 minutes for both colorimetric detection and visualization on agarose gel (Figure 5).

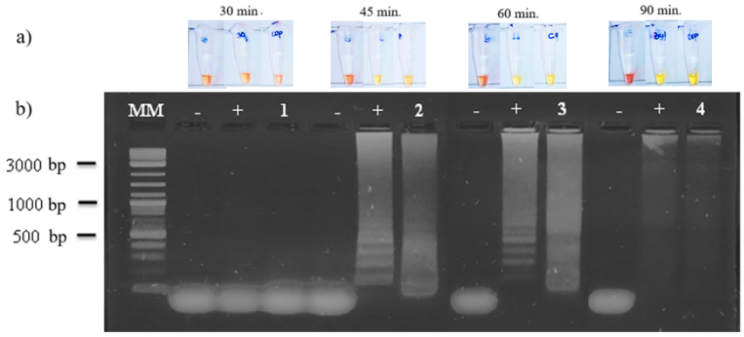

Figure 4. Optimal LAMP assay time for *P. belbahrii*. a) Visualized by colorimetric LAMP and b) Visualized by 2% agarose gel electrophoresis. MM: molecular marker (1 Kb); 30 minutes: -) : negative, +) : spore DNA, 1) synthetic DNA; 45 minutes: -) negative, +) spore DNA, 2) synthetic DNA; 60 minutes: -) negative, +) spore DNA, 3)

synthetic DNA; 90 minutes: -) negative, +) spore DNA, 4) synthetic DNA (Supplementary Material 4)

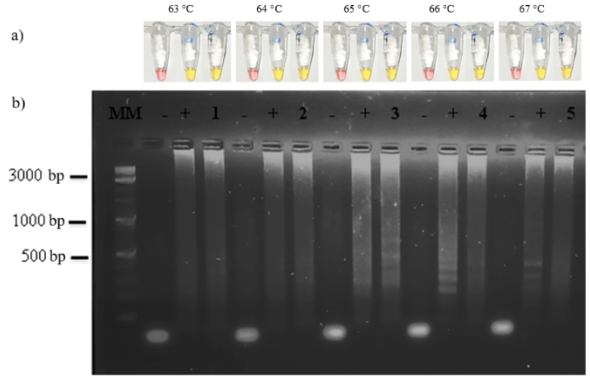

Figure 5. Optimal temperature for the LAMP assay for *P. belbahrii.* a) Visualized by colorimetric LAMP and b) Visualized by 2% agarose gel electrophoresis. MM: molecular marker (1 Kb); 63°C: -) negative, +) spore DNA, 1) synthetic DNA; 64°C: -) negative, +) spore DNA, 2) synthetic DNA; 65°C: -) negative, +) spore DNA, 3) synthetic DNA; 66°C: -) negative, +) spore DNA, 4) synthetic DNA; 67°C: -) negative, +) spore DNA, 5) synthetic DNA (Supplementary Material 3)

LAMP specificity

The specificity of the LAMP assay was tested using DNA from different species of plant pathogenic fungi of the genus *Fusarium* spp. and *Macrophomina phaseolina*, which can infect basil. For the specificity test, two *P. belbahrii* DNA samples were positive controls, along with four basil tissue samples, one showing chlorosis in its leaf blade. The LAMP products were positive in both the positive samples and the chlorotic plant tissue sample. Conversely, samples of *Fusarium* spp. and *M. phaseolina* tested negative in the LAMP assay (Figure 6). To allow direct comparison of amplification patterns under identical experimental conditions, plant material and non-target species were analyzed within the same electrophoretic run. The LAMP assays matched visually with the electrophoresis results. The LAMP method's sensitivity was 10,000-fold greater than PCR's when detecting *P. belbahrii* spores and synthetic DNA.

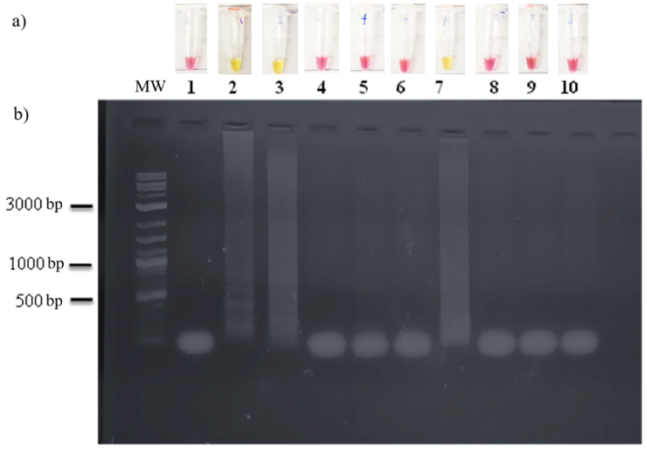

Figure 6. Specificity of the LAMP assay for the detection of *P. belbahrii*. a) Colorimetric LAMP assay, and b) visualization by 2% agarose gel electrophoresis. MM: Molecular marker; 1: negative control; 2: spore DNA; 3: synthetic DNA; 4: DNA

from healthy basil leaves 1; 5: DNA from healthy basil leaves 2; 6: DNA from healthy nursery basil leaves; 7: Nursery leaves with chlorosis; 8: *Fusarium* spp. 1; 9: *Fusarium* spp. 2; 10: *M. phaseolina* (Supplementary Material 5)

Detection of P. belbahrii in biological samples

The two plants from the nursery were confirmed to have *P. belbahrii* in their leaves. Following exposure to a humid, dark environment, the plants showed brown spores and necrosis after three days, confirming the presence of *P. belbahrii* and the LAMP test result (Figure 7).

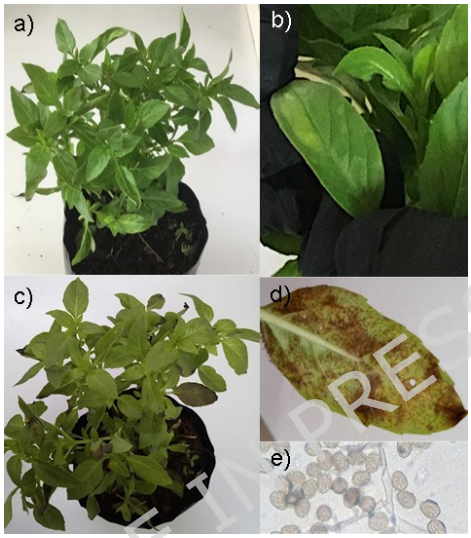

Figure 7. Presence of downy mildew on basil. a) Plants with signs on leaves, b) Leaf with signs, c) Plants with downy mildew, d) Leaf with a brown spore cluster on the underside of the leaf, e) Morphology of *P. belbahrii*

Discussion

Basil downy mildew, caused by *P. belbahrii*, remains a serious global concern for *O. basilicum* L. production due to its rapid spread, high virulence, and obligate biotrophic nature [34]. Infection by *P. belbahrii* requires high humidity and at least six hours for successful colonization, beginning with sporangial germination on the leaf surface [35]. Under favorable environmental conditions, the disease can cause complete crop loss within 10 days, affecting both yield and quality [2]. Therefore, timely diagnosis of *P. belbahrii* in *O. basilicum* plants at early stages enables effective control of its spread.

The LAMP assay developed in this study represents a significant advance in the rapid and specific detection of *P. belbahrii*. By targeting a gene fragment of unknown function that is conserved across *P. belbahrii* isolates but divergent from other *Peronospora* species, the designed PbLAMP-2 primer set provided high diagnostic accuracy and specificity. The use of four primers that recognize six distinct regions of the target gene aligns with previous designs that report detection of 1 to 50 fg for the identification of *Pseudomonas syringae* in tomato, Sugarcane mosaic virus (SCMV), Sorghum mosaic virus (SrMV) in *Saccharum officinarum*, and *Candidatus*

Phytoplasma asteris in *Cucumis sativus* [36, 37, 38], ensuring selective amplification and minimizing the risk of cross-reaction.

The results of this work confirmed that the PbLAMP-2 panel consistently amplified *P. belbahrii* DNA, with optimal efficiency at 65°C for 60 min, and that both colorimetric and electrophoretic methods could clearly visualize amplification products. Thus, the convenience of point-of-care panels, such as biosensors that detect color changes, enables fast, reliable diagnosis without specialized equipment [39, 40].

The detection sensitivity analysis of *P. belbahrii*, which evaluated PCR and LAMP techniques, showed that LAMP is more sensitive, detecting up to 1 theoretical copy, unlike PCR's limit of 1,000. The results align with other reports that compare the techniques. LAMP being more sensitive for the diagnosis, for example, of Impatiens Necrotic Spot Virus (INSV) in ornamental plants, being 60 times higher [41]; 10 times higher for Tomato Chlorotic Spot Orthotospovirus (TCSV), a pathogen of *Solanum lycopersicum* [42]; 100 times higher for *Plasmopara viticola* in *Vitis vinifera* [43], and up to one thousandfold for *Rhizoctonia solani* and *Macrophomina phaceolina* in *Glicine max* [44]. This high sensitivity is attributed to the LAMP mechanism, which enables continuous and exponential amplification of DNA under isothermal conditions using strand-displacing polymerases. In this sense, the PbLAMP-2 assay developed here provides a valuable diagnostic alternative for detecting trace amounts of *P. belbahrii* DNA.

Importantly, the novelty of this study lies in the development of the first LAMP assay specifically designed for the detection of *P. belbahrii*, for which rapid diagnostic tools remain extremely limited. Compared with conventional PCR, the LAMP assay offers substantial operational advantages, including shorter reaction times, minimal equipment requirements, and the possibility of visual detection without advanced laboratory infrastructure. Although reagent costs may be comparable or slightly higher, the technique significantly reduces technical complexity and processing time, making it particularly suitable for routine surveillance and early phytosanitary decision-making in basil production systems [25].

Assay specificity was also verified through experiments using DNA from *M. phaseolina* and *Fusarium* spp., pathogens frequently associated with basil crops [45, 46, 10, 47]. No amplification was observed in these non-target species, confirming that the primers selectively target *P. belbahrii* sequences. This result highlights the importance of performing specificity tests using DNA from regional pathogens. Similarly, [41] used LAMP for detecting grape downy mildew, emphasizing the need for regional disease forecasting based on pathogen abundance and climate. The present findings reinforce the diagnostic reliability of the PbLAMP assay, making it suitable for regional surveillance programs in areas where mixed infection and pathogens co-occurrence are common.

The PbLAMP-2 assay successfully detected *P. belbahrii* DNA from synthetic fragments, biological samples containing sporangia, and infected basil leaves without downy mildew symptoms. Similar results [48], demonstrating that the LAMP assay was sensitive, detecting 0.001 ng of target DNA extracted from a pure culture of *Phytophthora infestans* and 0.05 ng of crude DNA extracted from infected leaves. Notably, the PbLAMP-2 assay detected *P. belbahrii* DNA in basil leaves up to three

days before sporulation, demonstrating the potential as an early diagnostic tool. The above represents an advantage, as pre-symptomatic detection enables preventive management strategies, such as fungicide application, environmental control, or removal of infected plants, to be implemented before large-scale outbreaks occur. The ability of LAMP to detect early infections has been documented in other systems, including Impatiens Necrotic Spot Virus (INSV) in ornamental plants [40] and Tomato Chlorotic Spot Orthotospovirus in tomato [41], highlighting its relevance for integrated pest management.

The validation strategy employed in this study aligns with previously published LAMP-based diagnostic assays, where analytical sensitivity, specificity testing, and validation using biological samples are considered adequate for methodological studies. Nevertheless, future work should evaluate the assay under broader field conditions to further support its applicability.

The methodological approach employed here, combining bioinformatic identification of conserved sequences, LAMP primer design, and molecular validation, illustrates a model framework applicable to other obligate biotrophic pathogens lacking *in vitro* cultures. This strategy expands the possibilities for phytopathogens such as *Pseudoperonospora cubensis*, *Bremia lactucae*, and *Hyaloperonospora arabidopsis*, for which limited genomic information has hindered the development of specific detection tools [49, 50, 51]. Adopting LAMP technology for these pathogens can support early warning systems and integrated pest management programs, thereby improving crop resilience in high-value horticultural systems.

Early identification of *P. belbahrii* can substantially reduce epidemic risk by enabling preventive management strategies before symptom onset.In this context, the proposed PbLAMP-2 assay represents not only a diagnostic advance but also a practical tool for strengthening plant health surveillance systems in high-value crops such as basil.

Future studies should focus on validating this assay under variable field conditions, integrating it with portable detection platforms. Additionally, scaling up this approach to detect multiple downy mildew pathogens could lead to the development of a multiplexed LAMP system for routine surveillance of oomycete diseases in horticultural crops.

Conclusion

This study reports the first LAMP-based biosensor for the specific detection of *P. belbahrii* in *O. basilicum*. The assay's high sensitivity, strong specificity, and straightforward visualization make it a practical and accessible molecular tool for early pathogen detection. Its implementation can improve disease surveillance, reduce losses associated with basil downy mildew, and serve as a reference for developing a diagnostic assay for other obligate biotrophic plant pathogens.

Materials and methods

Spore sampling, isolation, and DNA extraction

A sample of 100 basil leaves showing signs of downy mildew was randomly collected from an organic production system in San José del Cabo, Baja California Sur, Mexico (23°10’15.528’’N, 109°40’31.218’’W). The basil leaves were collected from private agricultural land, and permission for sample collection was obtained from the landowner. Samples were stored at 4 °C and transported to the Food Science and Technology Laboratory of the Universidad Autónoma de Baja California Sur for immediate processing. Sporangia and spores from the underside of basil leaves were obtained by superficially washing spores with sterile distilled water. DNA extraction was performed using a 3% CTAB (Hexadecyltrimethylammonium bromide) extraction buffer (3% CTAB; 1.4 M NaCl; 20 mM EDTA; 1 mM Tris-HCl; pH 8, 0.2% β-mercaptoethanol). The extraction buffer was added to 300 milligrams of the collected biomass as described by Stewart & Via [52]. DNA concentrations were estimated using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, U.S.A.), and DNA integrity was analyzed by 2% agarose gel electrophoresis.

Endpoint PCR for species-specific amplification of P. belbahrii sequence

To detect *P. belbahrii*, a species-specific conserved gene (NCBI Genome: PBM13950) with unknown function was used. Next, Endpoint PCR amplification was performed using the primers: (Fw) 5´ACAACTCGCAGTCTGGATG 3´ and (Rv) 5´TGCAGGCGTAGTTCGATACC 3´ [53] (Standish et al., 2022). PCR reactions were performed in a Techne thermocycler (TC-412, CMB UK) using the New England BioLabs Taq DNA Polymerase with Standard Taq Buffer kit (NEB #M0273, Massachusetts, U.S.A.) in a final volume of 12.5 µL, containing 1X Buffer, 200 µM dNTPs, 0.2 µM Fw, 0.2 µM Rv, 0.33 U Taq polymerase, 100 ng/µL DNA template, and 4.68 µL nucleic acid- and nuclease-free H_2O. The amplification conditions consisted of an initial denaturation of 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 sec, annealing at 50 °C for 30 sec, extension at 72 °C for 30 sec, and a final extension at 72 °C for 5 min.

LAMP primer design

From the nucleotide sequences of a gene with an unknown function (MRBU01006465.1) of *P. belbahrii* [53], was chosen as the target region for LAMP primers. Its full length is 1060 bp and its overall GC content is 57.69%. The 200 bp fragment (positions 15102 to 15266) from *P. belbahrii* was selected as the target sequence for LAMP primer design. In this study, the gene sequences of *P. belbahrii* (MRBU01006465.1, CAKKTJ010000165.1, CAKLCB010000276.1, and CACTHD011001378.1) and the related species cluster two *P. effusa* sequences (CP090806.1 and NPIT01100608.1) and two *P. destructor* sequences (WBRY01000018.1 and CANTFM01000057.1) from the database obtained at NCBI were aligned to identify highly conserved regions of a gene with unknown function using MUSCLE

The resulting sequences were then aligned with Clustal Omega online tool, and a phylogenetic tree was constructed with the Mega X software. Based on this alignment, LAMP-specific primers were designed using the Primer Explorer V.4 software (https://primerexplorer.jp/e/) following the criteria of Notomi et al. [16]. Three sets of primers were obtained in the LAMP assay for *P. belbahrii*, named PbLAMP-1, PbLAMP-2, and PbLAMP-3. The primers were synthesized by T4OLIGO (Irapuato, Mexico). A positive control, consisting of the target DNA sequence synthesized by

INTEGRATED DNA TECHNOLOGIES (Iowa, U.S.A.), was used in the analytical standardization of the primer panels.

LAMP Optimization

An initial LAMP amplification assay was performed using Bst 2.0 DNA polymerase (New England BioLabs, Massachusetts, U.S.A.), following the standard LAMP protocol recommended by the manufacturer, to evaluate the suitability of the three primer panels. Based on the evaluation , using 10^6 copies of synthetic DNA at 65 °C for 45 min, and then in agarose gel electrophoresis, the PbLAMP-2 primer set was selected because it consistently produced positive amplification with both synthetic DNA and *P. belbahrii* DNA, while no amplification was observed in the negative control (NTC) of the three primer panels. Once the primer set was chosen, LAMP evaluation was carried out by colorimetry in a total volume of 25 µL containing 1.6 µM of the internal primers PbLAMP-2FIP and PbLAMP-2BIP, 0.2 µM of the external primers PbLAMP-2F3 and PbLAMP-2B3, WarmStart Colorimetric LAMP 1X master Mix with UDG, brought to 24 µL with molecular biology grade water, and 1 µL of synthetic DNA. To determine the optimal reaction temperature, samples were incubated at 63-67 °C for 30, 45, 60, and 90 minutes. Each reaction was carried out in triplicate and included a positive control (CTL+) with 0.38 ng of DNA from biological material of *P. belbaharii* sporangia and spores, as well as a negative control (without template) containing molecular biology grade water (NTC). LAMP assays were performed in a Techne thermal cycler (TC-412, Cambridge, UK). LAMP amplification products were analyzed visually by color change after incubation and electrophoresis in 2% agarose gel, stained with GelRed (Biotium, CA, U.S.A.) as an intercalator, and visualized in a Bio-Rad UV transilluminator (UV transilluminator 2000, CA, U.S.A.).

Sensitivity

The sensitivity of the PbLAMP-2 panel assay was evaluated using serial dilutions of synthetic DNA: 1,000,000; 100,000; 10,000; 1000; 100; 10 and 0 copies (594, 59.4, 5.94, 0.594, 0.059.4, 0.0059 and 0.00059 fg respectively). The copy number was estimated using the following formula: $(C)(M)(1x10^{-15}mol/fmol)\ (6.022x10^{23})\ =$ copy number/µL. where "C" is the original concentration of synthetic DNA in ng/µL and "M" is the molecular weight in fmol/ng. Five replicates were performed for each dilution, including one NTC reaction per amplification batch to rule out false positives. All dilutions were subjected to the LAMP assay at 65 °C for 60 minutes, which were the optimal reaction conditions. The amplification products were inspected visually and analyzed by electrophoresis on a 2% agarose gel to determine the minimum number of copies required for detection. To compare the sensitivity regarding the endpoint PCR, amplifications were performed according to the specifications described in the endpoint PCR section, with five replicates for each serial dilution using the primers PbLAMP-2 F3 and PbLAMP-2 B3. PCR products that amplified with F3 (5´GCCTCCTATGTCACCTCCAT 3´) from both spore and synthetic DNA of *P. belbahrii* were sequenced (Macrogen Genome Center, Republic of Korea).

LAMP specificity and validation

DNA from representative plant pathogenic fungal species was used to test the LAMP assay's specificity; these species included *Macrophomina phaseolina* and two *Fusarium* spp., isolated from diseased plants that can also infect basil. Three samples

were from healthy leaves (CTL-) and one sample was from a leaf with signs of the disease (CTL+). Specificity was assessed by amplification at 65 °C for 60 minutes to determine the primer specificity. The LAMP assay was performed as described above using primers from the PbLAMP-2 panel and optimal reaction conditions.

Detection of P. belbahrii in O. basilicum samples

To detect the presence of *P. belbahrii* in *O. basilicum* plants, 300 mg of leaves were taken from two apparently healthy plants and another group of two plants: one with signs of yellowing at the apical part of the leaf, and the other, apparently healthy. After sampling, the nursery plants were placed in a humid, dark environment for three days. The aim was to promote the development of *P. belbahrii*, identify whether the assay can detect the pathogen before the appearance of signs, and validate it in biological samples. The biological material was processed to obtain nucleic acids as previously described, followed by LAMP amplification using the PbLAMP-2 panel with a 65 °C incubation for 60 minutes.

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Authors' contributions

EAS, MRC, and MRA performed the laboratory work. EAS, MRB, APE, and MRA analyzed data results. MRB, BM, CGT, and MRA conceptualized research. All authors read, discussed, and approved the manuscript.

Conflict of Interest

The authors declare no conflicts of interest.

Data Availability

The datasets generated and/or analyzed during the current study are available in NCBI, GenBank database ID: PX516868, and PX516869

Figures captions

Fig. 1 Phylogenetic tree anatomy of *Peronospora*. A phylogenetic analysis was performed using the Neighbor-Joining method (Saitou & Nei, 1987), and the percentage of replicate trees in which associated taxa clustered in the bootstrap test (1000 replicates), using the MEGA11 software (Tamura et al., 2021)

Fig. 2 Schematic representation of the positions and sequences of the *P. belbahrii*-specific primers used in LAMP analysis

Fig. 3 Analytical sensitivity of the LAMP assay for *P. belbahrii*. A) Visual by colorimetric LAMP. B) LAMP visualization by 2% agarose gel electrophoresis. MM) Molecular marker (1 Kb); NTC) Negative control; Ctl+) Spore DNA; 1×10^0) 1 copy of synthetic DNA (0.59 atg); 1×10^1) 10 copies of synthetic DNA (5.9 atg); 1×10^2) 100 copies of synthetic DNA (59.4 atg); 1×10^3) 1,000 copies of synthetic DNA (0.594 ftg); 1×10^4) 10,000 copies of synthetic DNA (5.94 ftg); 1×10^5) 100,000 copies of synthetic DNA (59.4 ftg); 1×10^6) 1,000,000 copies of synthetic DNA (594 ftg) and C) Visualization by PCR. MM). Molecular marker (N3231S); NTC). Negative control; Ctle+). Spore DNA; Ctlh+) DNA from symptomatic leaves; 1×10^0) 1 copy of synthetic DNA; 1×10^1) 10 copies of synthetic DNA; 1×10^2) 100 copies of synthetic DNA; 1×10^3) 1,000 copies of synthetic DNA; 1×10^4) 10,000 copies of synthetic DNA; 1×10^5) 100,000 copies of synthetic DNA; 1×10^6) 1,000.000 copies of synthetic DNA

Fig. 4 Optimal temperature for the LAMP assay for *P. belbahrii.* A) Visualized by colorimetric LAMP and B) Visualized by 2% agarose gel electrophoresis. MM: molecular marker (1 Kb); 63°C: -) negative, +) spore DNA, 1) synthetic DNA; 64°C: -) negative, +) spore DNA, 2) synthetic DNA; 65°C: -) negative, +) spore DNA, 3) synthetic DNA; 66°C: -) negative, +) spore DNA, 4) synthetic DNA; 67°C: -) negative, +) spore DNA, 5) synthetic DNA

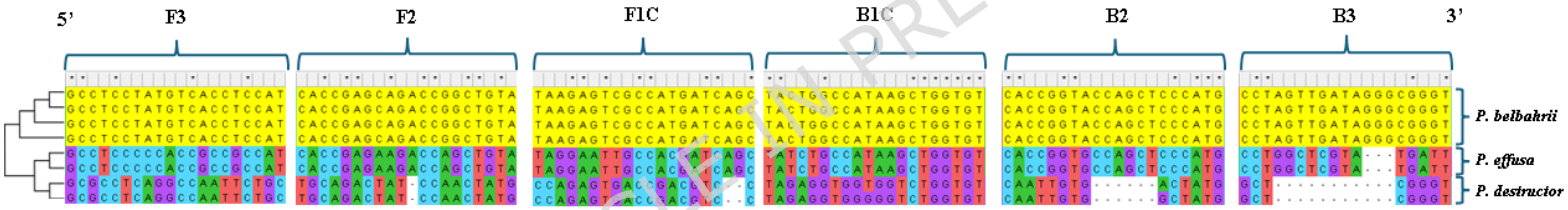
Fig. 5 Optimal LAMP assay time for *P. belbahrii*. A) Visualized by colorimetric LAMP and B) Visualized by 2% agarose gel electrophoresis. MM: molecular marker (1 Kb); 30 minutes: -) : negative, +) : spore DNA, 1) synthetic DNA; 45 minutes: -) negative, +) spore DNA, 2) synthetic DNA; 60 minutes: -) negative, +) spore DNA, 3) synthetic DNA; 90 minutes: -) negative, +) spore DNA, 4) synthetic DNA

Fig. 6 Specificity LAMP assay for identification of *P. belbahrii*. A) Colorimetric LAMP assay, and B) *P. belbahrii* detected by 2% agarose gel electrophoresis. MM: Molecular marker; 1: negative control; 2: spore DNA; 3: synthetic DNA; 4: DNA from healthy basil leaves 1; 5: DNA from healthy basil leaves 2; 6: DNA from healthy nursery basil leaves; 7: Nursery leaves with chlorosis; 8: *Fusarium* spp. 1; 9: *Fusarium* spp. 2; 10: *M. phaseolina*

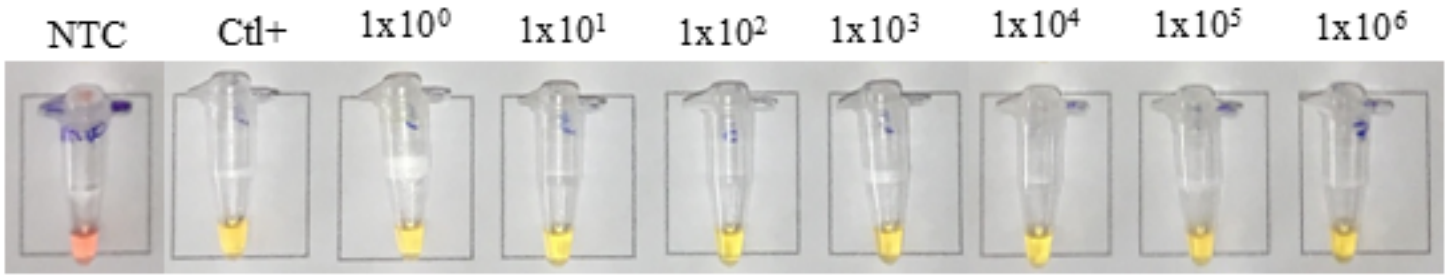
Fig. 7 Presence of downy mildew on basil. A) Plants with signs on leaves, B) Leaf with signs, C) Plants with downy mildew, D) Leaf with a brown spore cluster on the underside of the leaf, E) Morphology of *P. belbahrii*

Table caption

Table 1. Sequences of specific primers designed for *P. belbahrii*.



a)



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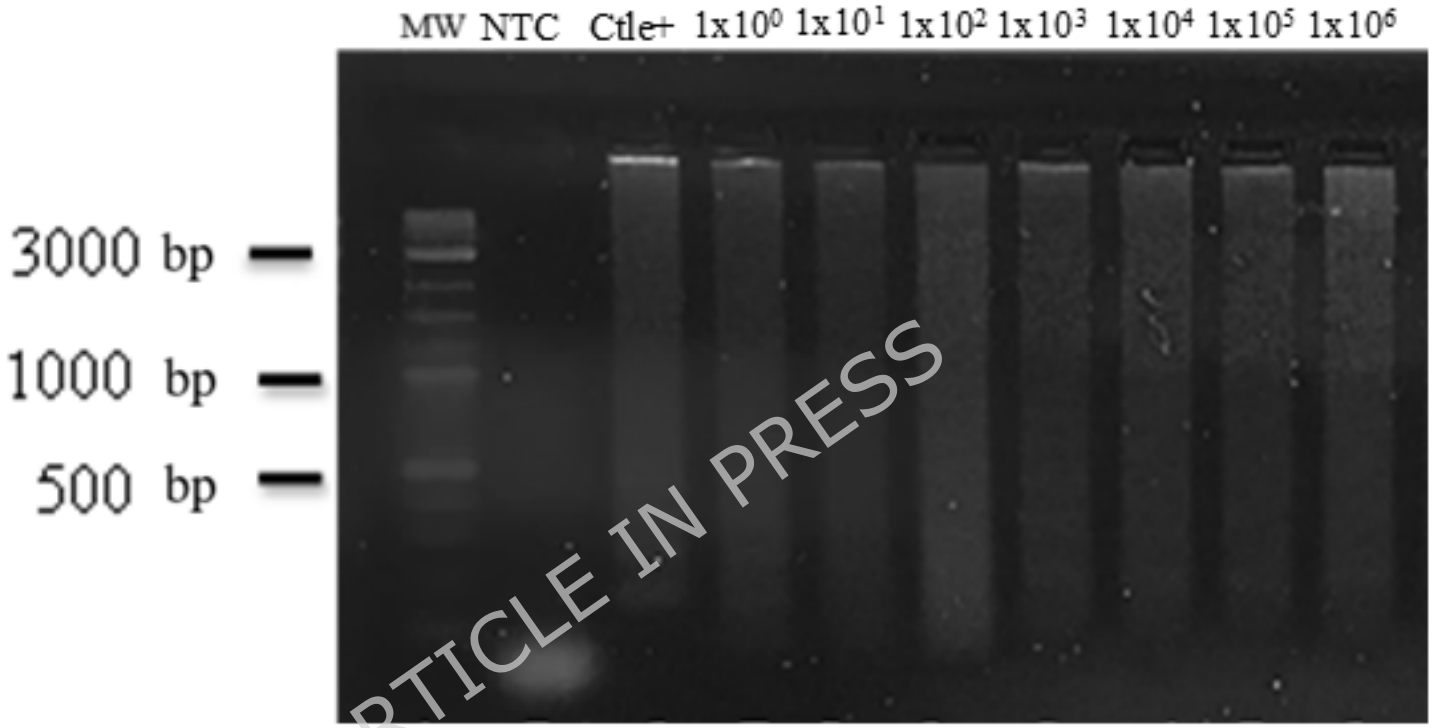

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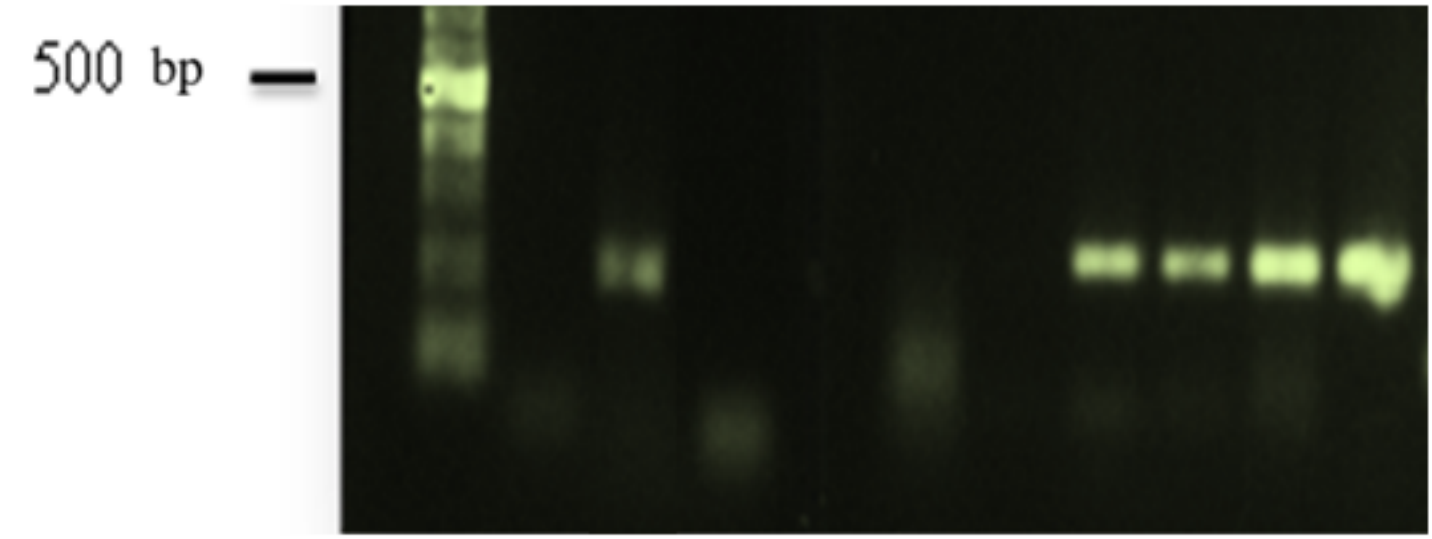

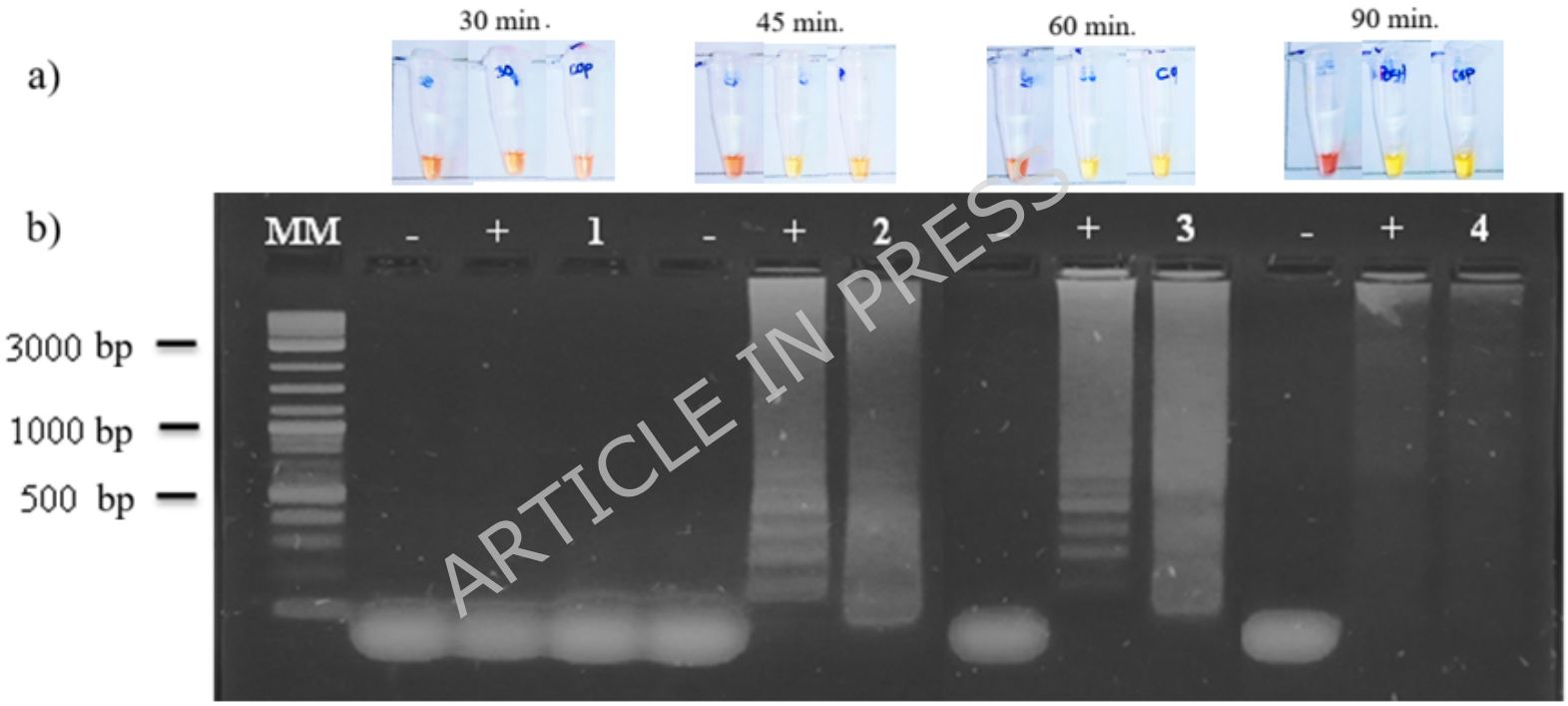

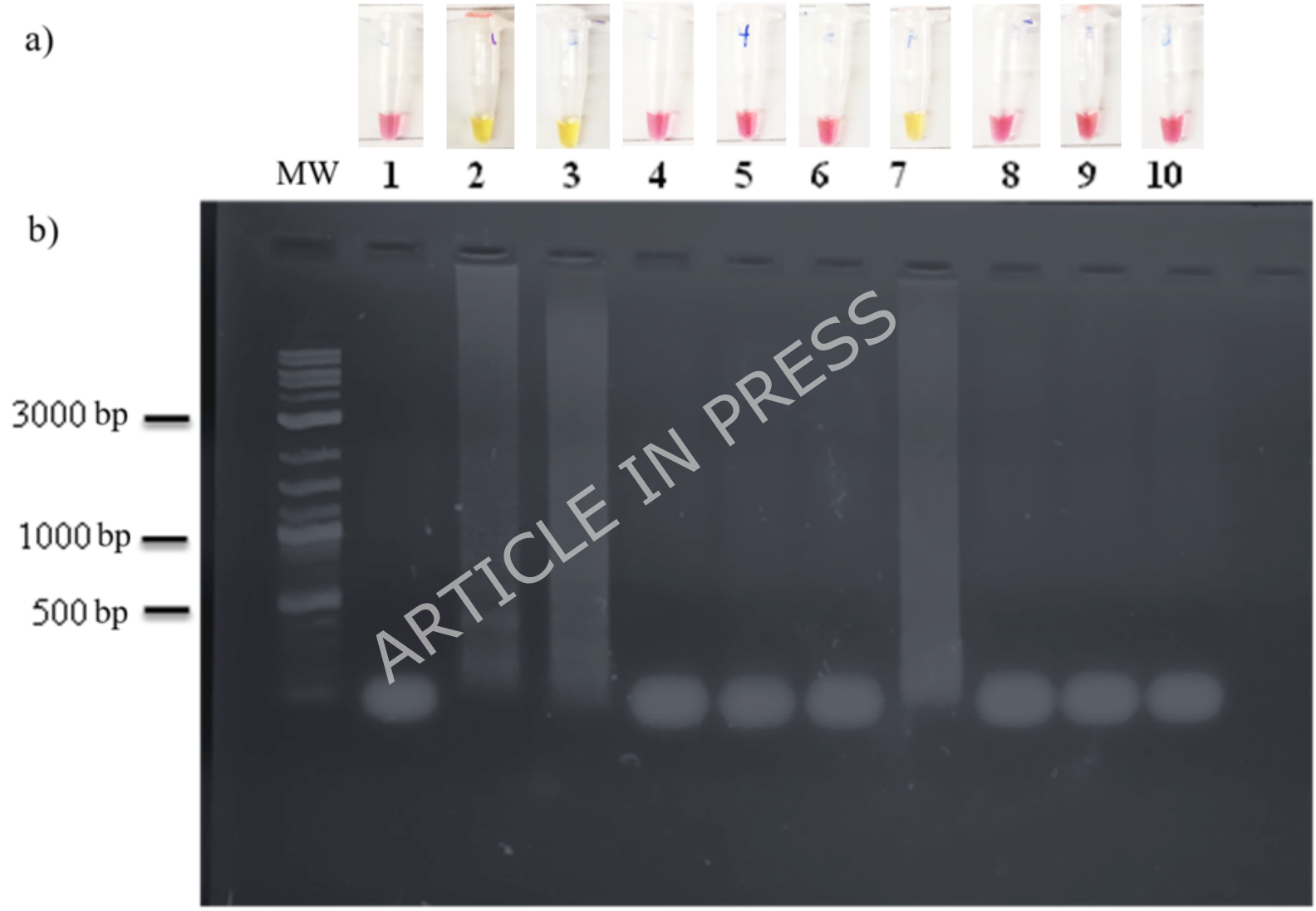

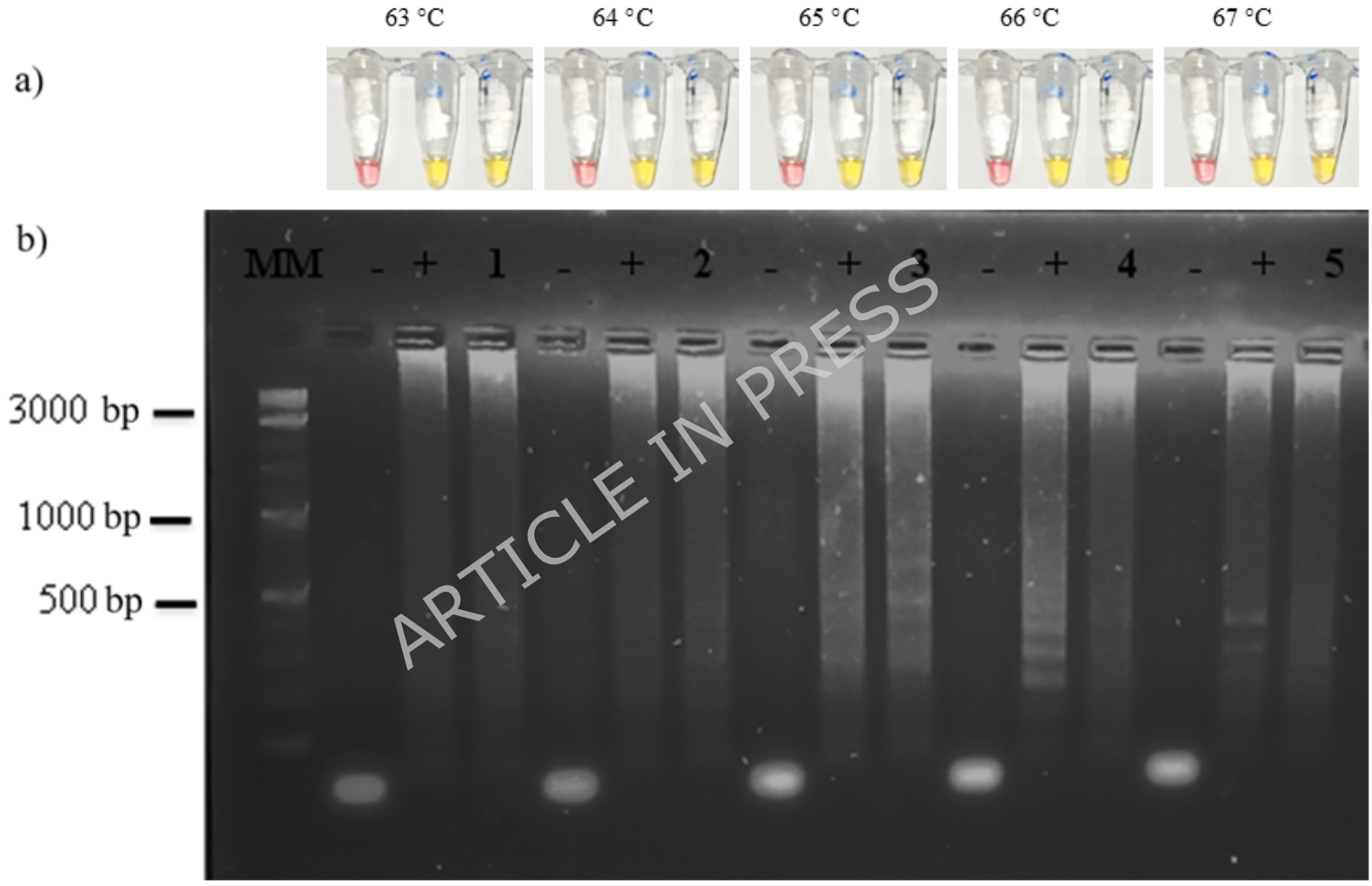


a)
b)
c)
d)
e)