Rapid Loop-Mediated Isothermal Amplification Assay for the Detection of Root Rot in Soybean Caused by Phytophthora sojae

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LAMP Primer design

Table S1 Nucleic acid sequences of four LAMP primer sets tested for specificity, with "Ps-ITS-LAMP 3" selected for use

Primer sets		**Primer sequence Primer sequence (5' to 3')**	GenBank
Ps-ITS-LAMP 1	F3	GCGTGACGTTGTTGGTTGT	MT367710.1
	B3	AAGCCAAGCCTCACACAG	
	FIP	CGCGAATCGAACACTCCTCCATCTGCCTGTATGGCCAGTC	
	BIP	TATGGTTGGCTTCGGCTGAACATACGGTTCACCAGCCCAT	
	LF	GCAGCAGACAAACCGGTCG	
	LB	ATGCGCTTATTGGATGCTTTTCC	
Ps-ITS-LAMP 2	F3	CCACGTGAACCGTATCAACA	KU211332.1
	B3	CATCCACTGCTGAAAGTTGC	
	FIP	ATAGAGCCCGCCACACAGCATGCTCTGTGTGGCTGTCT	
	BIP	CCTCCTCGTGGGGAACTGGATAGACTTTCGTCCCCACAGTA	
	LF	CCGCCGACTTTGACATCGA	
	LB	GAGCCCACTTTTTAAACCCATTCTT	
Ps-ITS-LAMP 3	F3	**CCACGTGAACCGTATCAACA**	**AY590266.1**
	B3	**CATCCACTGCTGAAAGTTGC**	
	FIP	**ATAGAGCCCGCCACACAGCATGCTCTGTGTGGCTGTCT**	
	BIP	**CCTCCTCGTGGGGAACTGGATAGACTTTCGTCCCCACAGTA**	
	LF	**GCCGCCGACTTTGACGT**	
	LB	**GAGCCCACTTTTTAAACCCATTCTT**	
Ps-ITS-LAMP assay 4	F3	GGCGTTTAATGGAGGAGTGT	AY590266.1
	B3	CGCAGAGACAACACAGAGTT	
	FIP	CTACGGTTCACCAGCCCATACCGTTGGCTTCGGCTGAACAA	
	BIP	TGAGGCTTGGCTTTTGAACCGGCCAAATGGATCGACCCTCG	
	LF	CAGCAGGAAAAGCATCCAATAAGC	
	LB	GTTGCGAAGTAGGGTGGCG	

Table S2 Sensitivity, specificity, and accuracy of nested PCR and conventional PCR assays for diagnosing Phytophthora root rot in soybean seedlings using a 5 μL DNA sample

	Nested PCR	PCR
True positives	32/32	6/32
False positives	0	0
True negatives	34/34	34/34
False negatives	0	26
Assay sensitivity	100%	18.75%
Assay specificity	100%	100%
Assay accuracy	100%	60.6%

Protocol S1: DNA Extraction Protocol

** This protocol applies to uninfected and infected soybean plant samples. Additionally, to prevent cross-contamination, we ensured that uninfected and infected samples were handled separately throughout the protocol.*

Before beginning the protocol, clean and disinfect the fume hood with a 10% bleach solution, leaving it on for 10 minutes, then rinse with deionized water before use. Clean the mortar and pestle with soap, deionized water, and a 10% bleach solution, rinsing thoroughly between uses.

I. Sample Preparation

1. The required volume of cetyltrimethylammonium bromide (CTAB) buffer was preheated and incubated at 60°C for 15 minutes.
2. Infected parts of the soybean plant were cut and isolated from the roots and stem.
3. The samples were placed in a mortar, liquid nitrogen was added, and they were ground with a pestle until a fine powder was formed.
4. A total of 80 mg from each sample was measured and placed into labeled microcentrifuge tubes.
5. 700 µL of CTAB buffer was added to the samples, which were briefly vortexed and centrifuged for 15–30 seconds.
6. The samples were then incubated at 60°C for 30 minutes.

II. DNA Extraction

1. New 1.5 mL microcentrifuge tubes were prepared and labeled according to the corresponding samples.
2. After incubation, the samples were centrifuged at 12,000 × *g* for 1 minute, and 200 µL of the supernatant was transferred to the respective tubes.
3. 500 µL of 3 M NaCl was added to each sample tube, followed by vortexing and centrifugation at 12,000 × *g* for 1 minute.
4. 150 µL of the resulting supernatant was transferred to a new 1.5 mL tube already containing 200 µL of the previously extracted supernatant (from Step 2).
5. 350 µL of 100% ethanol was added to each sample tube, followed by brief vortexing.
6. 700 µL of supernatant from each sample was transferred to spin columns and centrifuged at 12,000 × *g* for 1 minute.
7. The collection tubes were discarded, and the mini-columns were transferred to new 2 mL collection tubes.

III. Purification of Extracted DNA

1. 500 µL of Wash 1 buffer was pipetted into each spin column and centrifuged at 12,000 × *g* for 1 minute.
2. The mini-columns were transferred to new 2 mL collection tubes.
3. 500 µL of Wash 2 buffer was pipetted into each spin column and centrifuged at 12,000 × *g* for 1 minute.
4. The mini-columns were transferred again to new 2 mL collection tubes.
5. The columns were centrifuged at 12,000 × *g* for 1 minute to remove excess buffer.

IV. Final Elution

1. The mini-columns were transferred to new 1.5 mL microcentrifuge tubes.
2. 100 µL of nuclease-free water was added to each mini-column, followed by centrifugation at 12,000 × *g* for 1 minute.
3. The mini-columns were discarded, and the 1.5 mL tubes were closed, labeled, and stored at -20°C until further use.

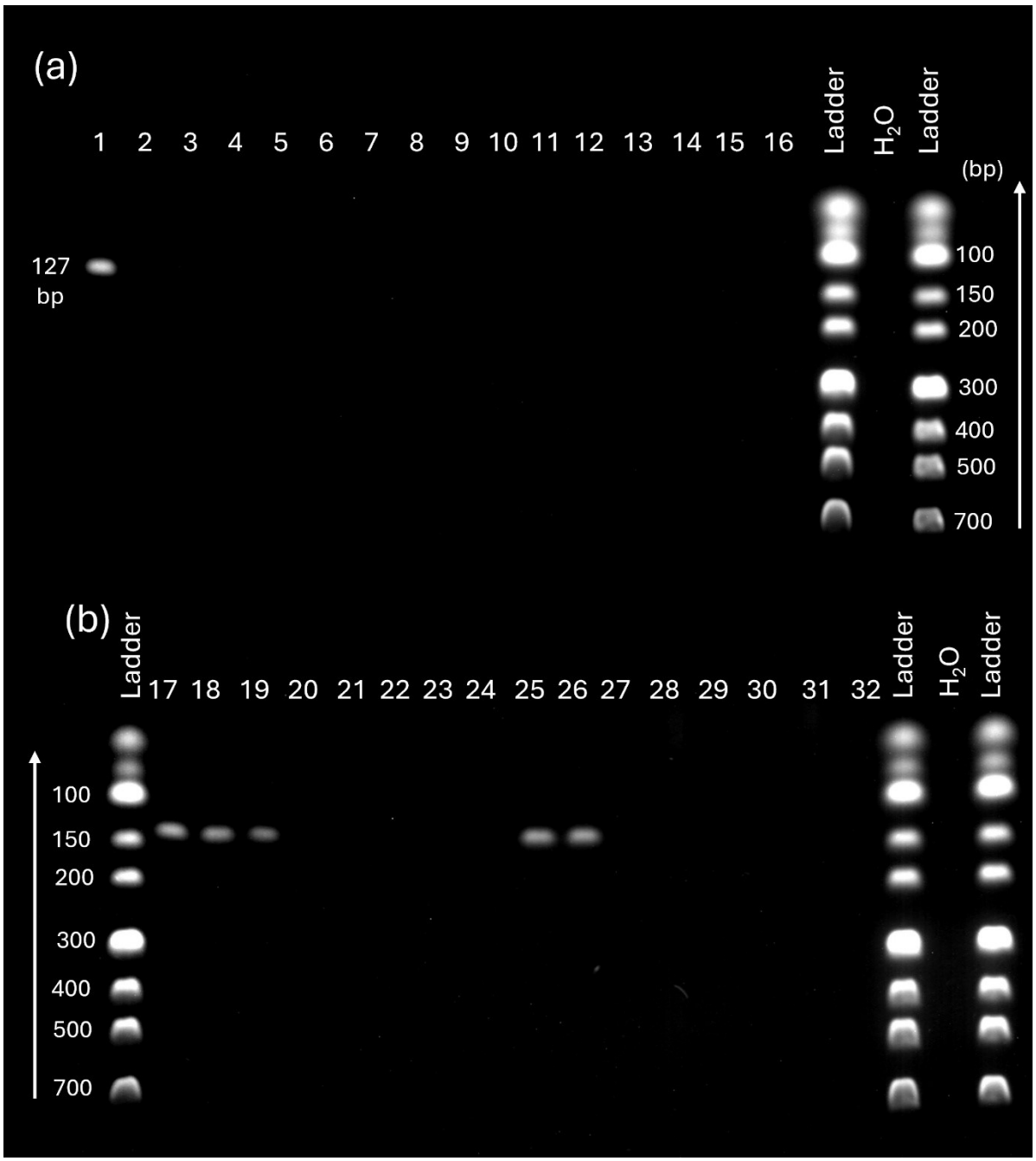


Figure S1. Conventional PCR results for the detection of *P. sojae* in DNA extracted from 32 inoculated soybean seedlings, each tested in duplicate. Samples were run on two 2% agarose gels (Low Range DNA Ladder used as a size marker): (a) seedlings 1–16 and (b) seedlings 17–32. Amplicons were visualized under UV light. Only 6 out of 32 samples showed bands at the expected size (127 bp), highlighting the limited sensitivity of the conventional PCR assay. NTC: no-template control (H_2O). Gels ran from bottom to top.

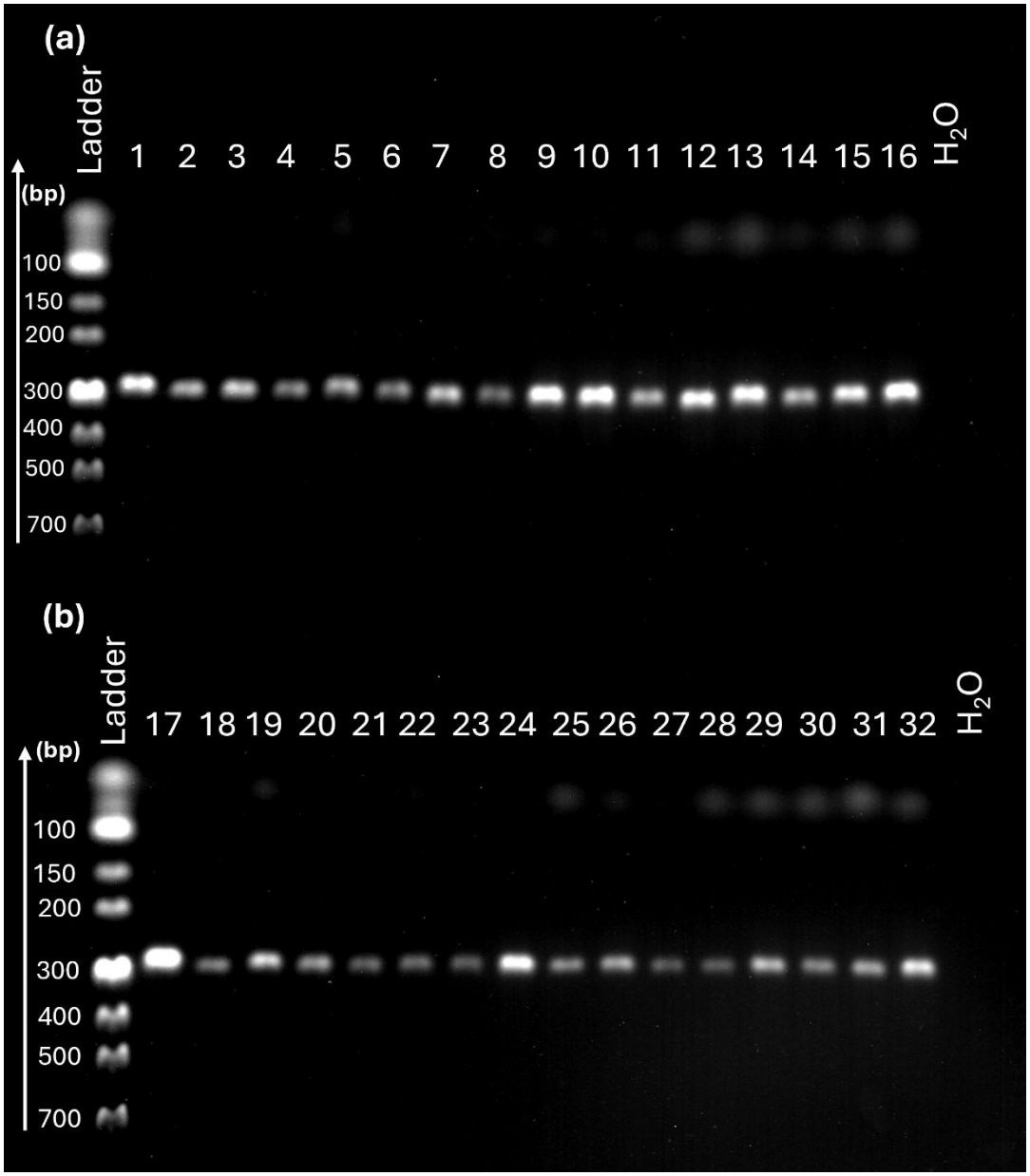


Figure S2. Nested PCR of DNA from *P. sojae*-infected soybean seedlings on 3% agarose gels (Low Range DNA Ladder used as a size marker). (a) samples 1–16, (b) samples 17–32; all infected samples show bands at the expected 267 bp, confirming detection; negative control: no-template (H_2O). Gels ran from bottom to top.

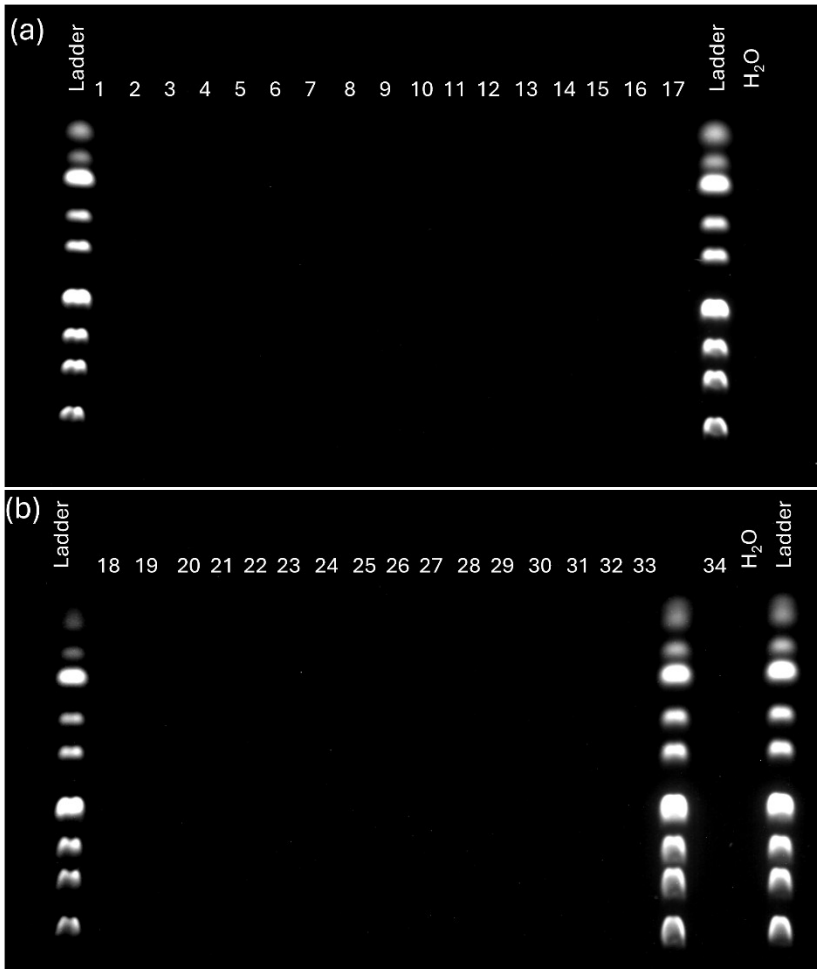


Figure S3. Gel electrophoresis analysis of nested PCR products from 34 control (uninfected) soybean seedlings. DNA was extracted and tested from seedlings labeled (a) 1–17 and (b) 18–34. No amplification was observed on the 2% agarose gel, confirming the absence of *P. sojae* infection. H_2O was used as a no-template control (NTC). Low Range DNA Ladder used as a size marker. Gels ran from bottom to top. Each sample was tested in duplicate.

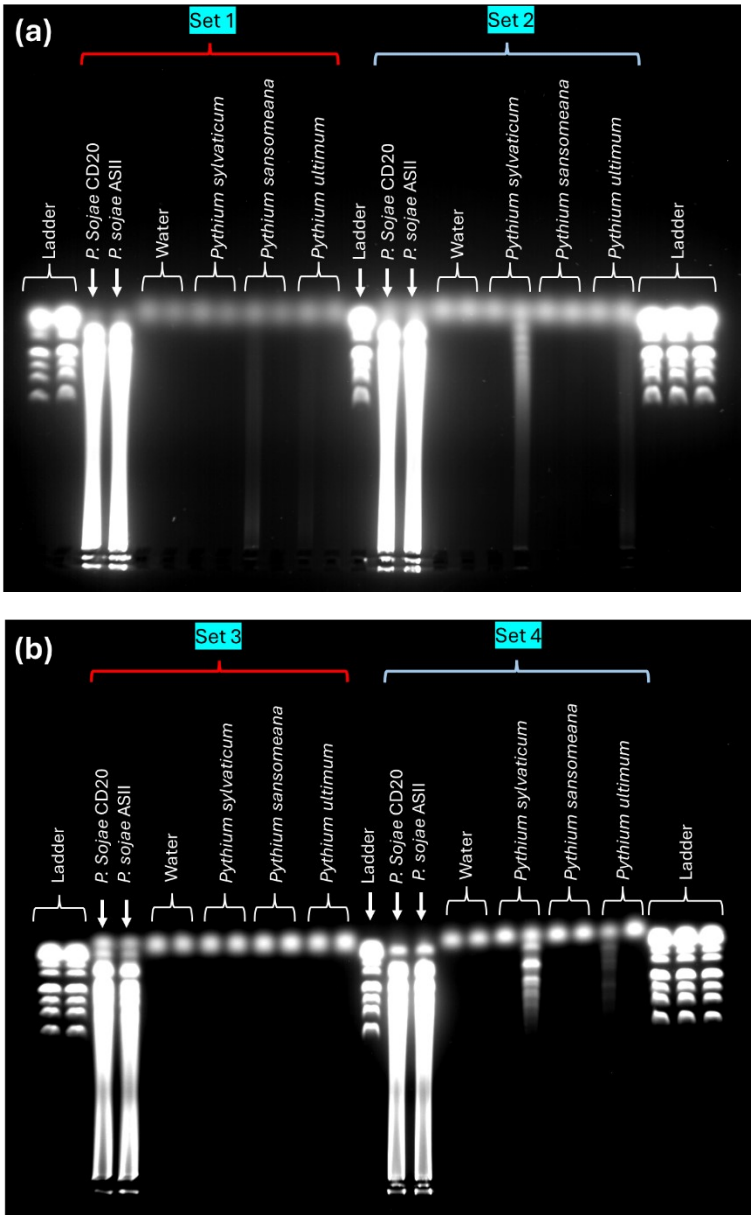
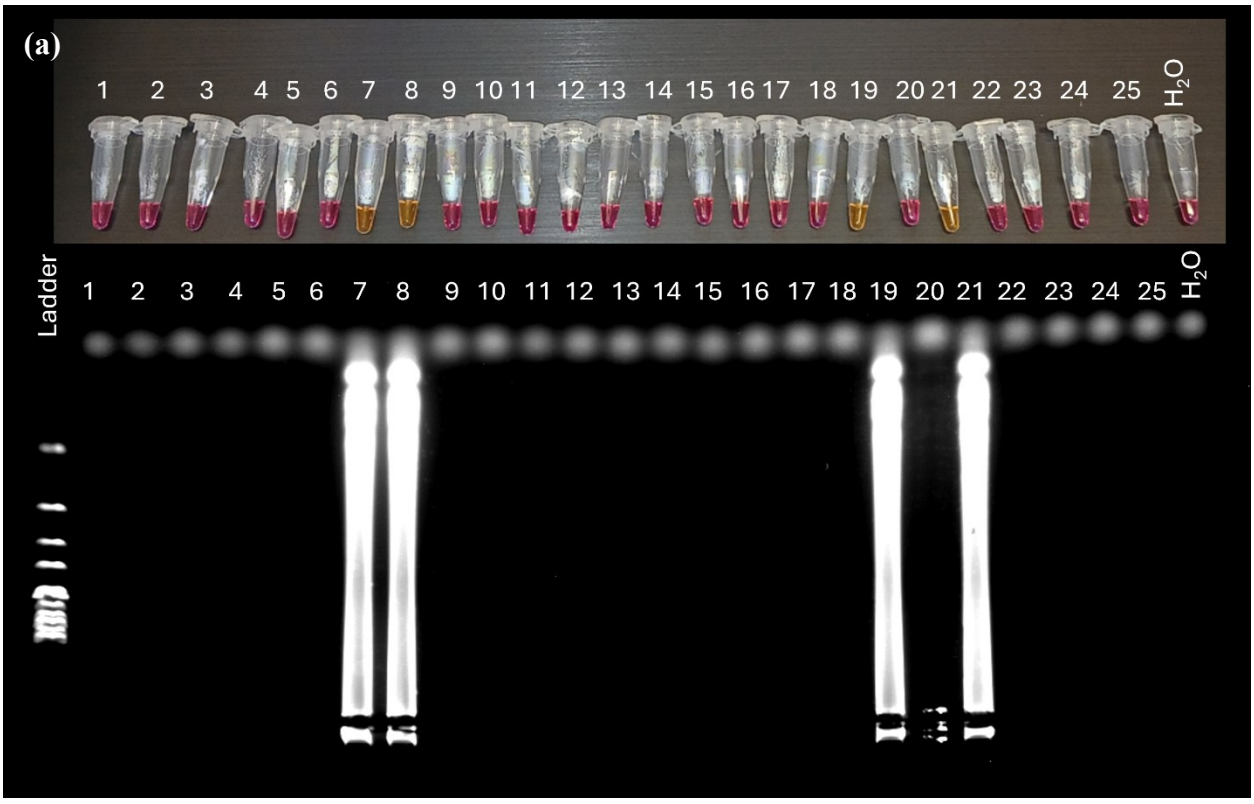


Figure S4 Agarose gel electrophoresis (1%) of loop-mediated isothermal amplification (LAMP) products from four primer sets designed for *Phytophthora sojae* detection, tested against *Phytophthora sojae* (strains CD20 and AS11) and three Pythium species (*Pythium sylvaticum*, *Pythium sansomeana*, and *Pythium ultimum*). The reaction mixtures were incubated at 65°C for 30 minutes. (a) Results for Ps-ITS-LAMP 1and Ps-ITS-LAMP 2. (b) Results for Ps-ITS-LAMP 3 and Ps-ITS-LAMP 4. Ps-ITS-LAMP 3 was selected as the most effective, demonstrating the highest specificity and clarity. 1 kb DNA ladder used as a size marker. Gels ran from bottom to top. The experiment was run twice and in duplicate for each primer set.


(a)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 H2O
Ladder
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 H2O

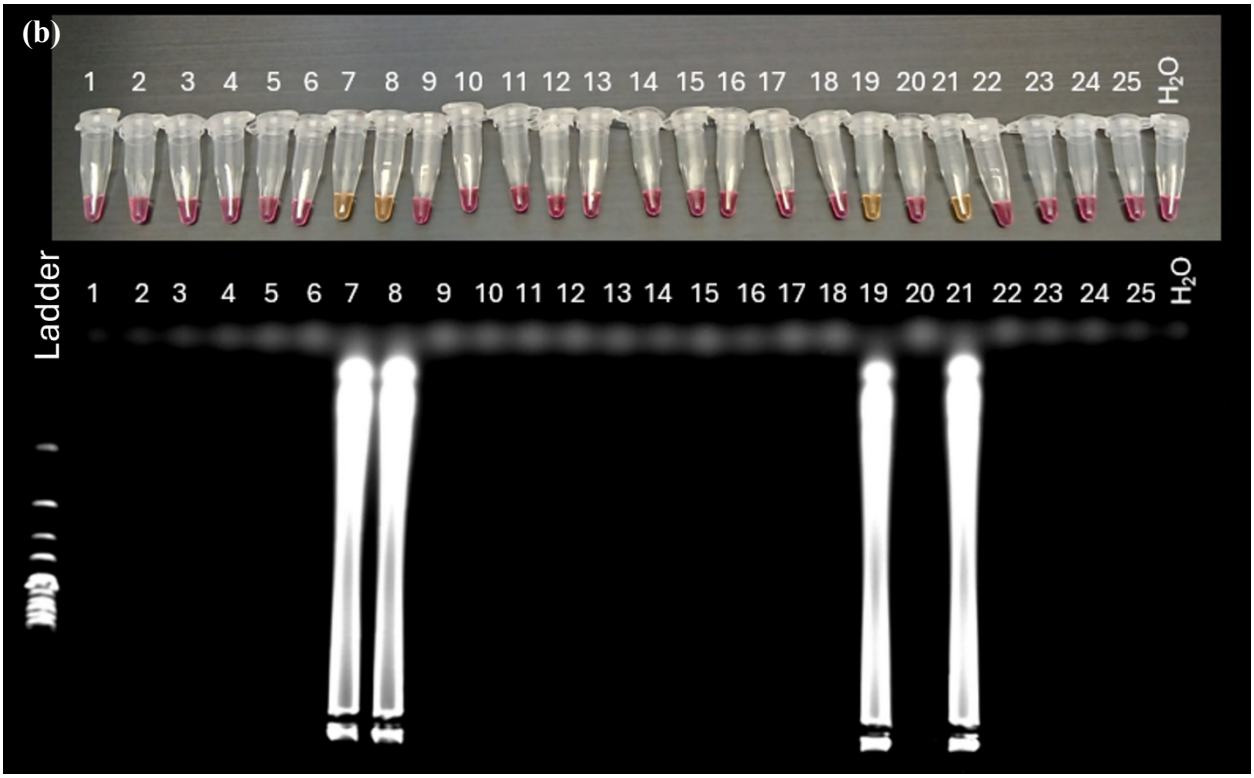

(b)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 H2O
Ladder
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 H2O

Figure S5 Specificity evaluation of the LAMP assay for detecting *Phytophthora sojae* isolates. A panel of 25 species was blind-tested and labeled 1–25. (a, b) Colorimetric LAMP detection results, where a positive reaction is indicated by a color change from pink to yellow. Gel electrophoresis analysis was performed to confirm amplification, with smearing patterns denoting successful LAMP reactions and the absence of smears indicating no amplification. 1 kb DNA ladder used as a size marker. Gels ran from bottom to top. Species corresponding to each number are as follows:

1 – *Pythium sylvaticum*, 2 – *Pythium ultimum*, 3 – *Phytophthora sansomeana*, 4 – *Fusarium oxysporum*, 5 – *Fusarium solani*, 6 – *Pythium torulosum*, 7 – *Phytophthora sojae* As11, 8 – *Phytophthora sojae* Cd20, 9 – *Pythium irregulare*, 10 – *Pythium heterothallicum*, 11 – *Rhizoctonia solani*, 12 – *Clonostachys rosea*, 13 – *Diaporthe longicolla*, 14 – *Diaporthe caulivora*, 15 – *Sclerotinia sclerotiorum*, 16 – *Cadophora gregata* genotype A, 17 – *Pythium attrantheridium*, 18 – *Pythium oopapillum*, 19 – *Phytophthora sojae* As11, 20 – *Phytophthora cactorum*, 21 – *Phytophthora sojae* Cd20, 22 – *Phytophthora cinnamomi*, 23 – *Pythium intermedium*, 24 – *Phytophthora nicotianae*, and 25 – *Pythium recalcitrans*. Repeated entries of *P. sojae* isolates (As11 and Cd20) represent distinct biological replicates**.**

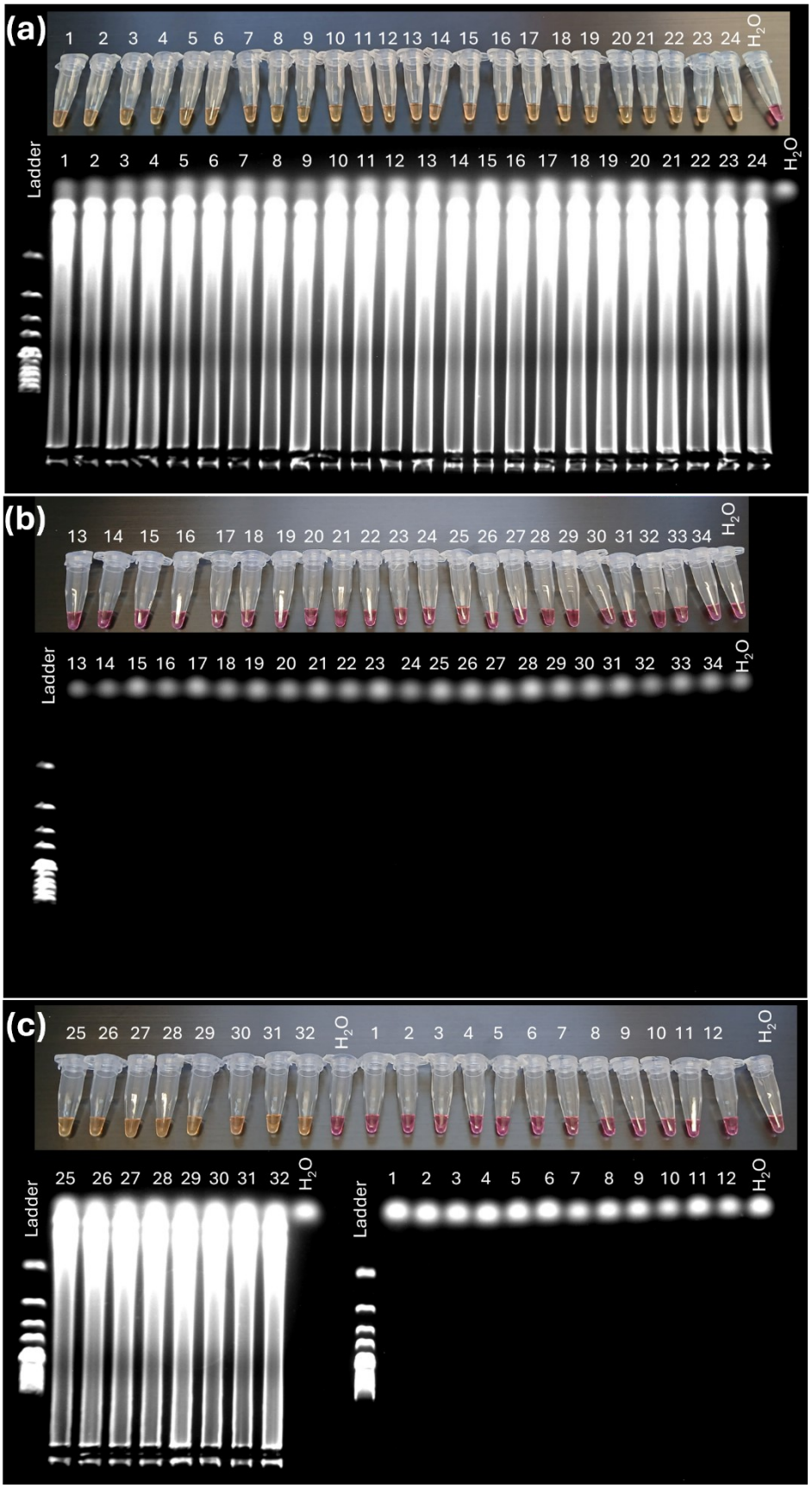

(a)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 H2O
Ladder
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 H2O
(b)
13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 H2O
Ladder
13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 H2O
(c)
25 26 27 28 29 30 31 32 H2O 1 2 3 4 5 6 7 8 9 10 11 12 H2O
Ladder
25 26 27 28 29 30 31 32 H2O
Ladder
1 2 3 4 5 6 7 8 9 10 11 12 H2O

Fig. S6 Replicate 2 of naked-eye detection of *Phytophthora sojae* using 1% agarose gel electrophoresis of loop-mediated isothermal amplification (LAMP) products from 34 control (uninfected) and 32 *P. sojae*-infected seedlings. (1 kb DNA ladder used as a size marker). Gels ran from bottom to top.

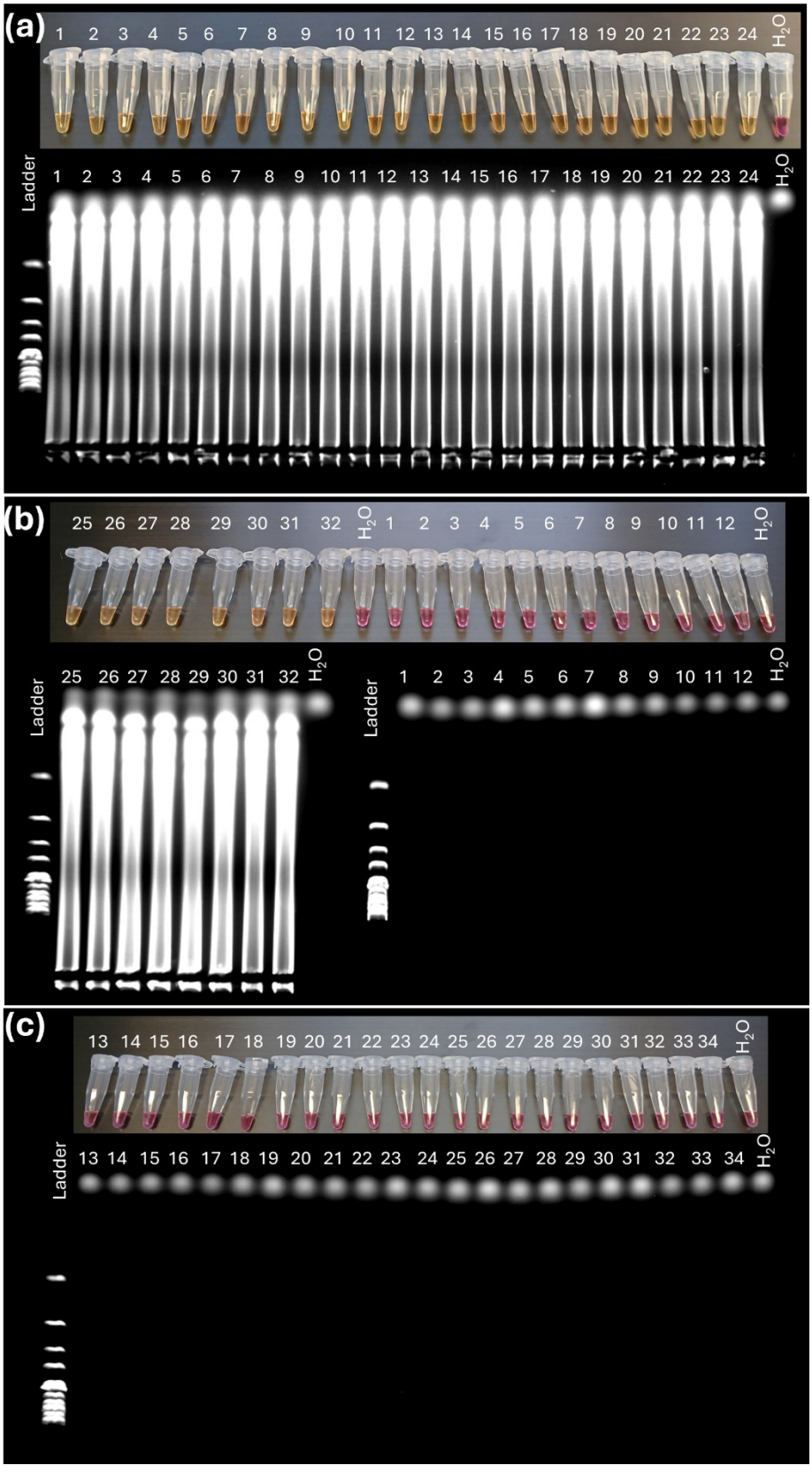

(a)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 H2O
Ladder
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 H2O
(b)
25 26 27 28 29 30 31 32 H2O 1 2 3 4 5 6 7 8 9 10 11 12 H2O
Ladder
25 26 27 28 29 30 31 32 H2O
Ladder
1 2 3 4 5 6 7 8 9 10 11 12 H2O
(c)
13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 H2O
Ladder
13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 H2O

Fig. S7 Replicate 3 of naked-eye detection of *Phytophthora sojae* using 1% agarose gel electrophoresis of loop-mediated isothermal amplification (LAMP) products from 34 control (uninfected) and 32 *P. sojae*-infected seedlings. (1 kb DNA ladder used as a size marker). Gels ran from bottom to top.

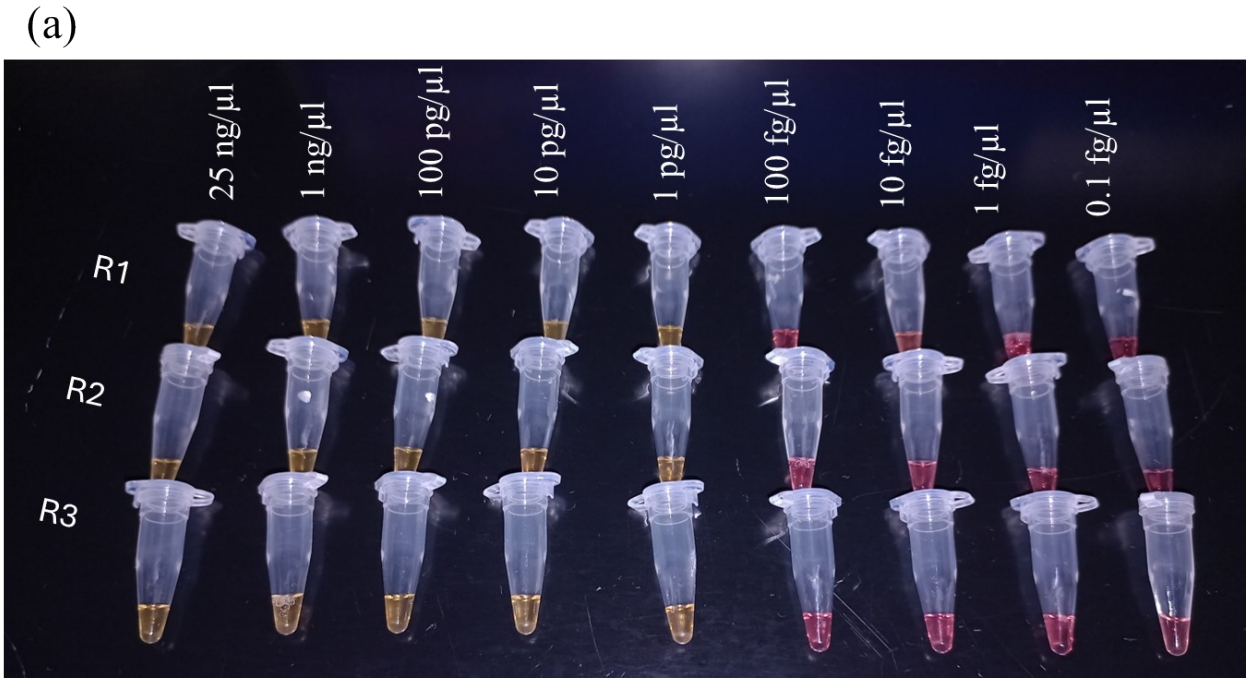


Fig. S8 Analytical Sensitivity of the LAMP Assay. The limit of detection (LOD) of the LAMP assay was determined using serial dilutions of genomic DNA from P. sojae isolate CD20. For each Lamp reaction, 1 µL of DNA template was used, with concentrations ranging from 15 ng/µL to 10 fg/µL.. (a) Colorimetric detection demonstrates positive

reactions with a color change from pink to yellow. (b) Results were confirmed using a 1% agarose gel, showing characteristic DNA smears for positive samples, (1 kb DNA ladder used as a size marker). Gels ran from bottom to top. Each assay was tested in three experimental replicates (R).