Rapid and sensitive detection of *Pseudocercospora eumusae* pathogen causing eumusae leaf spot disease of banana by loop-mediated isothermal amplification (LAMP) method

R. Thangavelu¹ · P. Ganga Devi¹

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Abstract

A rapid and sensitive loop-mediated isothermal amplification (LAMP) method was developed for the specific detection of *Pseudocercospora eumusae*. The LAMP primers designed based on the specific SCAR (Sequence Characterized Amplified Region) marker sequence of *P. eumusae* proved highly specific to *P. eumusae* and there was no cross reactivity with closely related *Pseudocercospora* spp. (*P. fijiensis* and *P. musicola*) and 17 other leaf spot causing fungal pathogens of banana. The developed LAMP method exhibited greater sensitivity as the minimum detectable concentration of *P. eumusae* genomic DNA was 10 pg/μl which was 100 times lower than that of conventional PCR (1 ng/μl). This method also detected the target pathogen from crude DNA of the mycelium and single leaf spot tissues which eliminates laborious purification steps in DNA isolation and requires less operational time. To the best of our knowledge, this is the first report on the development of this LAMP method to specifically detect and diagnose *P. eumusae* pathogen from pure cultures and leaf spot tissues.

Keywords Crude DNA · Loop-Mediated Isothermal Amplification method · *Pseudocercospora eumusae* · Optimization

Introduction

Eumusae leaf spot disease caused by *Pseudocercospora eumusae* is a serious threat to banana production in several Asian countries [India, Sri Lanka, Thailand, Malaysia, Vietnam, and some Indian Ocean Islands (Mauritius)] and Nigeria and the estimated yield losses in India are between 20 and 50% (Arzanlou et al. 2008; Churchill 2010; Gangadevi and Thangavelu 2014). Generally, there are three main leaf spot diseases that affect banana viz., black Sigatoka caused by *P. fijiensis*, yellow Sigatoka caused by *P. musicola* and eumusae leaf spot caused by *P. eumusae*. The morphological identification of these fungal species is difficult since they produce similar disease symptoms (Jones 2000). In addition, these fungi can co-exist in the same lesion and, therefore, these *Pseudocercospora* species can be misidentified on the

basis of morphological similarities alone (Maxwell et al. 2005; Arzanlou et al. 2008). Moreover, minor differences exist between the ascospores (size, shape and germination pattern) and conidial morphology, making the differentiation of *P. fijiensis*, *P. musicola* and *P. eumusae* very difficult. Furthermore, the ascospores and conidial structures are not easily produced in culture. Therefore, there is a need for the development of molecular markers to specifically detect *P. eumusae* which causes severe yield losses in banana.

The loop-mediated isothermal amplification (LAMP) method described by Notomi et al. (2000) is currently being applied in the fields of medicine, food safety testing and the detection of bacteria, fungi, viruses and nematode in plants and insects (Fukuta et al. 2004; Nie 2005; Guan et al. 2010; Nemoto et al. 2009). LAMP offers a rapid, accurate, and cost-effective diagnosis of infectious diseases. This method is able to amplify a gene from a single copy to 10⁹ copies in 1 h at constant temperature, typically in the range of 60–70 °C (Notomi et al. 2000) and hence it requires just simple equipment such as a water bath or temperature block (Notomi et al. 2000). The *Bst* DNA polymerase employed in this method with strand-displacement activity (Hafner et al. 2001) along with two forward and backward inner primers (FIP, BIP) and outer primers (F3, B3) which recognize six

R. Thangavelu and P. Ganga Devi contributed equally to this article.

✉ R. Thangavelu
rtbanana@gmail.com

¹ ICAR, National Research Centre for Banana,
Tiruchirappalli 620 102, Tamil Nadu, India

separate regions within a target DNA (Tomita et al. 2008). The LAMP amplified products can simply be detected by visual assessment through the naked eye thereby eliminating the need for gel electrophoresis.

LAMP has already been used for the detection of viral and fungal diseases in banana. Jun Peng et al. (2012) developed a LAMP method for the rapid detection of banana bunchy top virus (BBTV) and banana streak virus (BSV) infections. A Real-Time Fluorescence LAMP method was developed for the rapid and quantitative detection of *Fusarium oxysporum* f. sp. *cubense* (Tropical Race 4) in the soil by Zhang et al. (2013). However, so far, no attempt has been made to use the LAMP method for the accurate diagnosis and detection of *P. eumusae* in banana. Therefore, in the present study, we developed a LAMP method for a rapid detection of *P. eumusae* from pure cultures as well as from single leaf spot tissues.

Materials and methods

Fungal strains used in the study

Ninety-nine fungal isolates of *P. eumusae* collected from different states of India and 16 other leaf spot fungal pathogens isolated from banana were acquired from the Pathology laboratory, ICAR- NRC for Banana, Tiruchirappalli, Tamil Nadu, India. All the isolates used in this study were tested for their pathogenicity under controlled conditions in glass house and are listed with their respective GenBank accession numbers in Table 1.

Genomic DNA preparation from fungal cultures

Genomic DNA from 15 day old cultures of *P. eumusae* isolates and 7 days old cultures from the other fungal pathogens as mentioned in Table 1 was extracted and purified as per the method of Raeder and Broda (1985). Briefly, 100 mg of fungal mycelium was frozen using liquid nitrogen, powdered thoroughly and suspended in 800 µL of extraction buffer (200 mM Tris-HCl pH 8.5, 25 mM NaCl, 25 mM EDTA, 0.5% SDS). The tubes were incubated for 2 h at 37 °C and centrifuged at 16,000×g for 10 min. The supernatant was transferred to a new tube and extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1 v/v/v) and chloroform-isoamyl alcohol (24:1 v/v). The DNA was precipitated with isopropanol (1:1 v/v) and 3 M sodium acetate. The DNA pellet was washed with 70% ethanol and re-suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The purification of the DNA was performed by adding 5 µL RNase A (10 mg/ml), incubated at 37 °C for 1 h and then the suspension was re-extracted with chloroform-isoamyl alcohol. Finally, the DNA was precipitated

with 100% isopropanol (1:1 v/v), washed with 70% ethanol, resuspended in TE buffer and finally stored at –20 °C until use.

Genomic DNA preparation from single leaf spot tissues

The genomic DNA of single leaf spot samples of the 99 samples was extracted as per the method described by Johanson et al. (1994). Briefly, a single leaf spot (stage 5 and 6) was excised and placed in a sterile mortar containing 2 ml of cetyltrimethyl ammonium bromide extraction buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0). After 3 min of incubation, it was ground and 800 µL of the leaf extract was transferred to a 2 ml sterile microfuge tube. To this, 50 µL of 20% Poly vinyl pyrrolidone (PVP) was added, incubated at 65 °C for 30 min and centrifuged at 16,000×g for 5 min at 4 °C. Then, the supernatant was transferred to a new tube and extracted twice with 600 µL of chloroform-isoamyl alcohol (24:1). Finally, the DNA was precipitated by adding 0.5 volume of 5M sodium chloride and 0.7 volume of 100% isopropanol and incubated at –20 °C for 30 min. The DNA pellet was rinsed with 70% ethanol, re-suspended thoroughly in TE buffer and stored at –20 °C for further use.

Genomic DNA used in the study

The genomic DNA of *P. eumusae* (target pathogen) isolates of different states of India, genomic DNA of other leaf spot fungal pathogens of banana, genomic DNA of *P. eumusae*, *P. musicola*, *P. fijiensis* and *P. musae* of different banana growing countries obtained from CIRAD, France by MTA between ICAR-NRC for Banana, Tiruchirappalli, India and CIRAD, France (Table 1) and the genomic DNA of leaf spot tissues obtained from 69 different banana germplasm accessions/cultivars grown in eight different states of India (Table 2) were used for the validation of the LAMP primers.

RAPD analysis

RAPD analysis was performed in a total volume of 25 µL reactions containing 10× PCR buffer, 2 mM of MgCl₂, 10 picomoles of primers, 0.25 mM of dNTPs and 1.5 units of Taq DNA polymerase (Genei, Bangalore, India). Amplification conditions were programmed as follows: Initial denaturation at 94 °C for 20 s followed by 40 cycles of 94 °C for 20 s, 36 °C for 1 min, 72 °C for 1 min and a final extension step at 72 °C for 8 min. Totally, 40 RAPD primers (OPA1-20 and OPB 1–20 series) with 60 to 70% GC content (Operon Technologies Inc. Alameda, CA) were used.

The RAPD-PCR using the primer OPB 10 generated a unique amplicon at 1300 bp specific to *P. eumusae*. This

Table 1 Details of genomic DNA samples used in the study and their reaction to LAMP method

S. no.	Code	Organism	Origin	No. of DNA samples	GenBank accession no.	Reaction to LAMP method
1	Pe1–18 21–37 39–81	<i>Pseudocercospora eumusae</i>	Tamil Nadu, India	66	GU168024–GU168040 KC966842–KC966858 KC966860–KC966902	+
2	Pe 19, 20, 91–94	<i>P. eumusae</i>	Maharashtra, India	6	KC966840, KC966841 KC966912–KC966915	+
3	Pe 38	<i>P. eumusae</i>	Tripura, India	1	KC966859	+
4	Pe 82, 97, 98	<i>P. eumusae</i>	Kerala, India	3	KC966903 KC966918 KC966919	+
5	Pe 83, 90	<i>P. eumusae</i>	Assam, India	2	KC966904 KC966911	+
6	Pe 84–86	<i>P. eumusae</i>	Andhra Pradesh, India	3	KC966905–KC966907	+
7	Pe87–89	<i>P. eumusae</i>	Karnataka, India	3	KC966908–KC966910	+
8	Pe 95–96	<i>P. eumusae</i>	Uttar Pradesh, India	2	KC966916, KC966917	+
9	Pe 99	<i>P. eumusae</i>	Calcutta, India	1	–	+
10	Pe 100, 101	<i>P. eumusae</i> ^a	Thailand	2	–	+
11	Pe 102	<i>P. eumusae</i> ^a	Sri Lanka	1	–	+
12	Pe 103	<i>P. eumusae</i> ^a	Vietnam	1	–	+
13	Pe 104	<i>P. eumusae</i> ^a	Nigeria	1	–	+
14	Pe 105	<i>P. eumusae</i> ^a	Malaysia	1	–	+
15	Pm1,2	<i>P. musicola</i> ^a	Australia	2	–	–
16	Pm3	<i>P. musicola</i> ^a	Guinea, Africa	1	–	–
17	Pm 4, 5	<i>P. musicola</i> ^a	Cameroon	2	–	–
18	Pm 6, 7	<i>P. musicolav</i>	Colombia	2	–	–
19	Pm 8	<i>P. musicola</i> ^a	Brazil	1	–	–
20	Pm 9	<i>P. musicola</i> ^a	Guadeloupe, France	1	–	–
21	Pm 10	<i>P. musicola</i> ^a	Martinique, France	1	–	–
22	Pf 1, 2	<i>P. fijiensis</i> ^a	Australia	2	–	–
23	Pf 3	<i>P. fijiensis</i> ^a	Samoa	1	–	–
24	Pf 4	<i>P. fijiensis</i> ^a	Papua New Guinea	1	–	–
25	Pf 5	<i>P. fijiensis</i> ^a	New Caledonia, France	1	–	–
26	Pf 6	<i>P. fijiensis</i> ^a	Philippines	1	–	–
27	Pf 7	<i>P. fijiensis</i> ^a	Colombia	1	–	–
28	Pf 8	<i>P. fijiensis</i> ^a	Venezuela	1	–	–
29	Pf 9	<i>P. fijiensis</i> ^a	Panama	1	–	–
30	Pf 10	<i>P. fijiensis</i> ^a	Gabon, Africa	1	–	–
31	Pmu 1	<i>P. musae</i> ^a	Australia	1	–	–
32	OLS 7	<i>P. marksii</i>	India	1	MF380502	–
33	OLS 23	<i>P. musae</i>	India	1	MF380516	–
34	OLS 8	<i>Acremonium</i> sp.	India	1	MF380503	–
35	OLS 10	<i>Stagonospora</i> sp.	India	1	MF380505	–
36	OLS 12	<i>Nigrospora sphaerica</i>	India	1	MF380507	–
37	OLS 1	<i>Corynespora</i> sp.	India	1	KU898065	–
38	OLS 2	<i>Phoma</i> sp.	India	1	KU898066	–
39	OLS 3	<i>Alternaria</i> sp.	India	1	KU898067	–
40	OLS 11	<i>Alternaria longissima</i>	India	1	MF380506	–
41	OLS 21	<i>Alternaria alternata</i>	India	1	MF380515	–
42	OLS 4	<i>Exserohilum</i> sp.	India	1	KU898068	–
43	OLS 5	<i>Curvularia</i> sp.	India	1	KU898069	–
44	OLS 6	<i>Cladosporium</i> sp.	India	1	MF380501	–
45	OLS 9	<i>Cladosporium</i> sp.	India	1	MF380504	–
46	OLS 18	<i>Microdochium</i> sp.	India	1	MF380512	–
47	OLS 19	<i>Phomopsis</i> sp.	India	1	MF380513	–

Table 1 (continued)^aDNA obtained from CIRAD, France

+ Indicates positive LAMP reaction, – indicates negative LAMP reaction

unique band was excised from the gel and the DNA was purified using Genelute gel purification kit (Sigma, USA). The purified DNA was then cloned and transformed into pGEM[®]-T easy vector system (Promega, USA). The plasmid DNA was isolated from transformed colonies as described by Birnboim and Doly (1979). The plasmid DNA was checked for the presence of inserts by digesting with *EcoRI* and sequenced at M/S. Xcelris lab, Ahmedabad, Gujarat India.

Primer design and LAMP reaction

The LAMP primers specific to *P. eumusae* were designed based on the specific SCAR marker sequence of *P. eumusae* derived from RAPD analysis. The four LAMP primers: FIP-forward inner primer (consisted of F1c and F2), BIP-backward inner primer (consisted of B1c and B2) and outer primers (F3 and B3) that are capable of recognizing six distinct regions on the target DNA of *P. eumusae* pathogen were designed using Primer explorer V3 software program (<http://primerexplorer.jp/e/>) and the same is depicted in Fig. 1.

The optimization of LAMP reagents was performed using a range of concentrations of *Bst* DNA polymerase (1–8 U/ml), dNTPs (0.2–2 mM), MgSO₄ (2–8 mM), primers (0.2–2 mM) and betaine (0.5–0.8 M) (Notomi et al. 2000). To determine the optimal reaction temperature and time, the LAMP reaction mixtures were incubated at 56–65 °C for 15, 30, 45, 60, and 90 min. A total volume of 25 µl for the LAMP reaction contained 0.48 µM FIP and BIP, 0.12 µM concentrations of F3 and B3, 2 mM dNTPs mix, 2.0 U of *Bst* DNA polymerase large fragment (New England Biolabs, Inc., Beverly, MA), 1× ThermoPol buffer (20 mM Tris–HCl, 10 mM (NH₄)₂SO₄, 10 mM KCl, 2 mM MgSO₄, 0.1% Triton X-100, pH 8.8) (New England Biolabs), 4 mM MgSO₄ and 1 µl of template DNA (25 ng). The reaction was incubated in a thermal cycler at 65 °C for 60 min. The visual detection of LAMP products was performed by the addition of 2 µl of 1000× SYBR[®] Green I nucleic acid gel stain (Invitrogen, Carlsbad, California). The LAMP products (7 µl) were also analyzed by gel electrophoresis for the confirmation of amplification by the LAMP primers.

Specific detection of *P. eumusae* from fungal mycelium and single leaf spot tissue

The specificity of the LAMP method was tested using genomic DNA of 105 isolates of *P. eumusae*, closely related *Pseudocercospora* spp. (10 isolates each of *P. fijiensis* and *P.*

musicola) and 17 other leaf spot causing fungal pathogens of banana. The genomic DNA (99 nos.) from single leaf spot tissue of banana were also used for the detection of *P. eumusae* by LAMP method. The optimized LAMP reaction was performed on all the samples used in this study and amplified products were by gel electrophoresis as stated above. The LAMP primers were also validated using mixtures of DNA samples of *P. eumusae*, *P. fijiensis* and *P. musicola* for assessing their specificity for *P. eumusae*.

Sensitivity of the LAMP method

The sensitivity of the LAMP method was assessed by comparing it with conventional PCR using genomic DNA of the target pathogen at concentrations ranging from 0.01 to 10,000 pg. For conventional PCR, the outer primers F3 and B3 were used for amplification and the reaction consisted of 0.2 mM dNTPs, 0.4 µM each of forward and reverse primers, 3 U of Taq DNA polymerase, 1× Taq polymerase buffer and 1 µl of DNA template. No template control was also set using nuclease-free water. The reaction was carried out with an initial denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, extension at 72 °C at 45 s and a final extension at 72 °C for 10 min. The PCR products were analysed using 1.5% agarose gel electrophoresis, stained with ethidium bromide and examined under UV light (302 nm).

Detection of *P. eumusae* from crude DNA of fungal mycelium and eumusae leaf spot tissues

Tissues of eumusae leaf spot were collected from different banana cultivars belong to different genomic groups [Rasthali (Silk-AAB), Grand Naine (AAA) and Monthan (cooking banana-ABB)]. The crude DNA from a pure culture of *P. eumusae* and eumusae leaf spot tissue was extracted using 800 µl of extraction buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris–HCl, pH 8.0), incubated for 5 min and centrifuged at 16,000×g for 5 min at 4 °C. To the supernatant, 50 µl of 20% freshly prepared PVP was added, incubated at 65 °C for 15 min and centrifuged at 16,000×g for 5 min at 4 °C. The crude DNA was then precipitated by adding 0.5 volume of 7.5 M potassium acetate and 0.7 volume of 100% isopropanol and incubated at –20 °C for 30 min. Then, the crude DNA pellet thus obtained was washed with 70% ethanol and re-suspended in sterile distilled water.

Table 2 Reaction of genomic DNA samples from leaf spot tissues of banana cultivars grown in different states of India to LAMP method

S. no.	Code	Cultivar	Genomic status	Origin	Reaction to LAMP method
1.	1LT	Kachkel	ABB	ICAR-NRCB, Trichy—Tamil Nadu (TN)	+
2.	2LT	Krishna Sagar	AAB	ICAR-NRCB, Trichy—TN	+
3.	3LT	Myndoli	AAB	ICAR-NRCB, Trichy—TN	+
4.	4LT	Sabri	AAB	ICAR-NRCB, Trichy—TN	+
5.	5LT	Sirumalai	AAB	ICAR-NRCB, Trichy—TN	+
6.	6LT	Balbisia	BB	ICAR-NRCB, Trichy—TN	+
7.	7LT	Vannan	AAB	ICAR-NRCB, Trichy—TN	+
8.	8LT	Ladan	AAB	ICAR-NRCB, Trichy—TN	+
9.	9LT	Red Banana	AAA	ICAR-NRCB, Trichy—TN	+
10.	10LT	Singalaji	ABB	ICAR-NRCB, Trichy—TN	+
11.	11LT	Nendran	AAB	ICAR-NRCB, Trichy—TN	+
12.	12LT	Poovan	AAB	ICAR-NRCB, Trichy—TN	+
13.	13LT	Grand Naine	AAA	ICAR-NRCB, Trichy—TN	+
14.	14LT	Hill Banana	AAB	ICAR-NRCB, Trichy—TN	+
15.	15LT	Monthan	ABB	ICAR-NRCB, Trichy—TN	+
16.	16LT	Channasakkar	AAB	ICAR-NRCB, Trichy—TN	+
17.	17LT	Laalukali	AAB	ICAR-NRCB, Trichy—TN	+
18.	18LT	Kluteperod	BBB	ICAR-NRCB, Trichy—TN	+
19.	1LM	Grand Naine	AAA	Jalgoan, Maharashtra	+
20.	2LM	Grand Naine	AAA	Jalgoan, Maharashtra	+
21.	3LM	Grand Naine	AAA	Jalgoan, Maharashtra	+
22.	4LM	Grand Naine	AAA	Jalgoan, Maharashtra	+
23.	5LM	Grand Naine	AAA	Jalgoan, Maharashtra	+
24.	19LT	NeyPoovan	AB	Kadavur, Karur—TN	+
25.	20LT	NeyPoovan	AB	Aravakurichi, Karur—TN	+
26.	21LT	Honda	AAB	ICAR-NRCB, Trichy—TN	+
27.	22LT	Honda	AAB	ICAR-NRCB, Trichy—TN	+
28.	23LT	Bungan	AAB	ICAR-NRCB, Trichy—TN	+
29.	24LT	Monthan	ABB	ICAR-NRCB, Trichy—TN	+
30.	25LT	Rasthali	AAB	Kulithalai, Karur—TN	+
31.	26LT	Rasthali	AAB	Kulithalai, Karur—TN	+
32.	27LT	Madhukar	AAA	ICAR-NRCB, Trichy—TN	+
33.	28LT	Soneri	AAB	ICAR-NRCB, Trichy—TN	+
34.	29LT	Ambeli	AAB	ICAR-NRCB, Trichy—TN	+
35.	30LT	Mysorebale	AAB	ICAR-NRCB, Trichy—TN	+
36.	31LT	Madhukar	AAA	ICAR-NRCB, Trichy—TN	+
37.	32LT	Mysorebale	AAB	ICAR-NRCB, Trichy—TN	+
38.	33LT	Mysorebale	AAB	ICAR-NRCB, Trichy—TN	+
39.	34LT	Saapakal	AAB	ICAR-NRCB, Trichy—TN	+
40.	35LT	Soneri	AAB	ICAR-NRCB, Trichy—TN	+
41.	1LTr	Grand Naine	AAA	Tripura	+
42.	2LTr	Grand Naine	AAA	Tripura	+
43.	3LTr	Grand Naine	AAA	Tripura	+
44.	36LT	Soneri	AAB	ICAR-NRCB, Trichy—TN	+
45.	37LT	Mannan	AAB	ICAR-NRCB, Trichy—TN	+
46.	38LT	Williams	AAB	ICAR-NRCB, Trichy—TN	+
47.	39LT	Singapur	AAB	ICAR-NRCB, Trichy—TN	+
48.	40LT	Singapur	AAB	ICAR-NRCB, Trichy—TN	+

Table 2 (continued)

S. no.	Code	Cultivar	Genomic status	Origin	Reaction to LAMP method
49.	41LT	Poovan	AAB	ICAR-NRCB, Trichy—TN	+
50.	42LT	Poovan	AAB	ICAR-NRCB, Trichy—TN	+
51.	43LT	Pachanadan	AAB	ICAR-NRCB, Trichy—TN	+
52.	44LT	Pachanadan	AAB	ICAR-NRCB, Trichy—TN	+
53.	45LT	Saba	ABB	ICAR-NRCB, Trichy—TN	+
54.	46LT	Mysorebale	AAB	ICAR-NRCB, Trichy—TN	+
55.	47LT	Tongat	AA	ICAR-NRCB, Trichy—TN	+
56.	48LT	Kanaibansi	AA	ICAR-NRCB, Trichy—TN	+
57.	49LT	Kullan	AAB	ICAR-NRCB, Trichy—TN	+
58.	50LT	Rasthali	AAB	Sittanatham, Manaparai—TN	+
59.	51LT	Robusta	AAA	ICAR-NRCB, Trichy—TN	+
60.	52LT	Ladies finger	AAB	ICAR-NRCB, Trichy—TN	+
61.	53LT	Numaran	AAB	ICAR-NRCB, Trichy—TN	+
62.	54LT	Laalipoovan	AB	Vayampatti, Manaparai—TN	+
63.	55LT	Tongat	AA	ICAR-NRCB, Trichy—TN	+
64.	56LT	Ladan small	AAB	ICAR-NRCB, Trichy—TN	+
65.	57LT	Soniyal	AAB	ICAR-NRCB, Trichy—TN	+
66.	58LT	Matti	AA	ICAR-NRCB, Trichy—TN	+
67.	59LT	Sirumalai	AAB	ICAR-NRCB, Trichy—TN	+
68.	60LT	Karpuravalli	ABB	ICAR-NRCB, Trichy—TN	+
69.	61LT	Rasthali	AAB	Pudhukadai, Cuddalore—TN	+
70.	62LT	Poovan	AAB	Bathalakundu, Dindigul—TN	+
71.	63LT	Matti	AA	ICAR-NRCB, Trichy—TN	+
72.	64LT	Red Banana	AAA	ICAR-NRCB, Trichy—TN	+
73.	65LT	Nendran	AAB	Kalaiyur, Cuddalore—TN	+
74.	66LT	Sarapoovan	AAB	Kondapalli, Muttadi—TN	+
75.	67LT	Hoobale	ABB	ICAR-NRCB, Trichy—TN	+
76.	68LT	Jamupalem	AB	ICAR-NRCB, Trichy—TN	+
77.	69LT	Jahaji	AAA	ICAR-NRCB, Trichy—TN	+
78.	70LT	Madhuranga	AAB	ICAR-NRCB, Trichy—TN	+
79.	71LT	Somai	AB	ICAR-NRCB, Trichy—TN	+
80.	72LT	Thozhuvan	AAB	ICAR-NRCB, Trichy—TN	+
81.	1LK	Kadali	AB	Ernakulam, Kerala	+
82.	2LK	Kadali	AB	Ernakulam, Kerala	+
83.	1LAs	Malbhog	AAB	Jorghat, Assam	+
84.	1LAp	Mortamon	AAB	Kovvur, Andhra Pradesh	+
85.	2LAp	Monthan	ABB	Kovvur, Andhra Pradesh	+
86.	3LAp	Karpuravalli	ABB	Kovvur, Andhra Pradesh	+
87.	1LKa	Monthan	ABB	Nadia, Karnataka	+
88.	2LKa	Cultivar Rose	AA	Mohanpur, Karnataka	+
89.	3LKa	Karibale	AAB	Sirumuli, Karnataka	+
90.	2LAs	Malbhog	AAB	Jorghat, Assam	+
91.	6LM	Grand Naine	AAA	Jalgoan, Maharashtra	+
92.	7LM	Grand Naine	AAA	Jalgoan, Maharashtra	+
93.	8LM	Grand Naine	AAA	Jalgoan, Maharashtra	+
94.	9LM	Grand Naine	AAA	Jalgoan, Maharashtra	+
95.	1LUp	Basarai dwarf	AAA	Kushinagar, Uttar Pradesh	+
96.	2LUp	Basarai dwarf	AAA	Kushinagar, Uttar Pradesh	+

Table 2 (continued)

S. no.	Code	Cultivar	Genomic status	Origin	Reaction to LAMP method
97.	3LUp	Basarai dwarf	AAA	Faizabad, Uttar Pradesh	+
98.	3LK	Nendran	AAB	Kannur, Kerala	+
99.	4LK	Rasthali	AAB	Wayanad, Kerala	+
100.	Healthy leaf-HLD1	Rasthali	AAB	ICAR-NRCB, Trichy—TN	—
101.	Healthy leaf-HLD2	Grand Naine	AAA	ICAR-NRCB, Trichy—TN	—
102.	Healthy leaf-HLD3	Karpuravalli	ABB	ICAR-NRCB, Trichy—TN	-

+ shows positive reaction, – shows negative reaction

Fig. 1 Design of LAMP primer for specific detection of *P. eumusae* pathogen. FIP composed of complementary sequence F1c plus the sequence F2; internal primer BIP is composed of sequence B2 plus the complementary sequence to B1c

Results

Primer design and optimization of the LAMP reaction

Of the 40 different RAPD primers only the primer OPB 10 generated a unique amplicon of 1300 bp size specific to *P. eumusae*. Out of nine sets of primers that were screened, only one set of forward and backward inner primers viz., FIP1L-GTCTGGAAGCACGCCAATCTGT-TACTTG

AAGGCATGCACCAC and BIP1L-TTCGCAACCTCATCGACGTTGT-TCCAGTACTCATTGGCCTCC along with outer primers F3-GCTCGAGCGAAGATGAAAGT and B3-AGCTGCACCAGAATGTTCTTC specifically detected the target DNA (Table 3). This was observed by the colour change of LAMP products from orange to yellowish green upon addition of 1000× SYBR green I. The LAMP products also produced a ladder-like pattern which confirmed the amplification of target pathogen *P. eumusae* by the LAMP primers (Fig. 2).

Table 3 Primers used in LAMP for the detection of *P. eumusae*

Primer name	Sequence
Outer primers	
F3	GCTCGAGCGAAGATGAAAGT
B3	AGCTGCACCAGAATGTTCTTC
Inner primers	
FIP1L	
F2	TACTTGAAGGCATGCACCAC
F1c	GTCTGGAAGCACGCCAATCTGT
BIP1L	
B1c	TCCAGTACTCATTTGGCCTCC
B2	TTCGCAACCTCATCGACGTTGT

Optimization of LAMP reaction

During LAMP optimization process, the effect of Mg^{2+} concentration, amount of *Bst* DNA polymerase, effect of addition of betaine and the reaction temperature and time

were tested. The best LAMP reaction was observed at a temperature of 65 °C, incubation time of 60 min, 2.5 U of *Bst* DNA polymerase, 0.48 μ M of FIP and BIP, 0.12 μ M of F3 and B3 primer, 6 mM $MgSO_4$ concentration and 0.5 M betaine. It was also noted that $MgSO_4$ at a concentration of 6 mM is a critical step in the LAMP method. Other concentrations did not amplify the DNA of the target pathogen.

Detection of *P. eumusae* from fungal mycelium and single leaf spot tissues

The LAMP primers (F3, B3, FIP1L and BIP1L) used in this study amplified all the isolates of *P. eumusae* used in this study. The LAMP amplified products showed a color change from orange to yellowish green and a visible ladder-like DNA pattern for all the *P. eumusae* isolates (Table 1).

Fig. 2 **a** Detection of *P. eumusae* from the fungal mycelium and single leaf spot tissue obtained from different states of India by LAMP assay. 1–10—Genomic DNA from fungal mycelium (1–2—Tamil Nadu, 3—Maharashtra, 4—Tripura, 5—Kerala, 6—Assam, 7—Andhra Pradesh, 8—Karnataka, 9—Uttar Pradesh, 10—Malaysia), 11–18—Genomic DNA from single leaf spot tissue (11—Tamil Nadu, 12—Maharashtra, 13—Tripura, 14—Kerala, 15—Assam, 16—Andhra Pradesh, 17—Karnataka, 18—Uttar Pradesh). **b** Testing the specificity of the LAMP assay using DNA samples from other non-target fungi (19–20—*P. eumusae* from Tamil Nadu; 21–22—*P. fijiensis*; 23–24—*P. musicola*; 25—*Corynespora* sp. 26—*Phoma* sp., 27—*Alternaria* sp., 28—*Exserohilum* sp., 29—*Curvularia* sp.), 30—Non-template control

Detection of *P. eumusae* from single leaf spot tissues

The primers used in the LAMP method amplified the genomic DNA of all the *P. eumusae*. The LAMP primers showed positive reaction only for the DNA of *P. eumusae* extracted from leaf spot of varying sizes (stages 4–6) and showed negative reaction for DNA extracted from healthy leaf spot tissues (Table 2). These results indicated that the LAMP primers designed in this study were able to detect and diagnose the *P. eumusae* present in the leaf spot tissues.

Specificity of LAMP method

The specificity test of the LAMP primers showed that the DNA of *P. eumusae* was amplified. The non-target organisms and non-template control retained the original orange color and showed any ladder-like DNA pattern (Table 1). Besides, the specificity of the LAMP method checked using different combinations of mixtures of DNA (*P. eumusae* + *P. musicola*, *P. fijiensis* + *P. musicola*, *P. eumusae* + *P. fijiensis* and *P. eumusae* + *P. fijiensis* + *P. musicola*) revealed that the LAMP primers were highly specific to *P. eumusae* by showing the positive reaction of LAMP only in the tubes containing DNA of the target pathogen *P. eumusae* (Fig. 3).

Sensitivity of LAMP method

The LAMP method amplified the genomic DNA of *P. eumusae* at a concentration of the 1 $\mu\text{g}/\mu\text{l}$ (Fig. 4a). The conventional PCR was able to amplify the genomic DNA of *P. eumusae* only when the concentration was about 1000 $\mu\text{g}/\mu\text{l}$ and produced an amplicon of 200 bp (Fig. 4b). Thus, it can be inferred that the sensitivity of LAMP method was 100 fold higher than that of the conventional PCR.

Fig. 3 Specificity of LAMP assay with mixtures of DNA of closely related *Pseudocercospora* spp of banana 1—*P. eumusae* + *P. fijiensis* + *P. musicola*; 2—*P. eumusae* + *P. fijiensis*; 3—*P. eumusae* + *P. musicola*; 4—*P. fijiensis* + *P. musicola*; 5—*P. fijiensis*; 6—*P. musicola*; 7—non template control

Fig. 4 Sensitivity of LAMP assay (a) vs. Conventional PCR (b). 1–7—Genomic DNA of *P. eumusae* of varying concentrations. 1—10 ng, 2—1 ng, 3—0.1 ng, 4—10 μg , 5—1 μg , 6—0.1 μg , 7—0.01 μg , 8—non template control

Validation of LAMP method using crude DNA of fungal mycelium and leaf spot tissues

The LAMP method validated using the crude DNA showed specific amplification of target pathogen by showing positive reaction only in the tubes containing crude DNA extracted from the mycelium of *P. eumusae* and leaf spot infected tissue samples. The tubes with no template control crude DNA from other leaf spot pathogens and healthy leaf tissue resulted in the negative reaction as it retained the original orange color.

Discussion

Early and specific detection of plant pathogens is important for appropriate and accurate control measures. *Pseudocercospora eumusae* is a serious disease of bananas in several parts of the world. Nucleic acid-based diagnostics by conventional and real time PCR for the detection of *P. eumusae* from pure cultures and dry leaves were reported by Arzanlou et al. (2007). However, PCR-based methods are greatly limited due to its reduced efficiency, being labour intensive, time consuming and needing extensive sample preparation (Fakruddin 2011). Furthermore, the PCR-based diagnostics need expensive thermal cyclers, gel electrophoresis, gel imaging systems and the use of hazardous ethidium bromide (Tomita et al. 2008). To overcome all these limitations, this study developed a LAMP method which can amplify the DNA with high specificity,

efficiency and rapidity under isothermal conditions for the rapid detection of *P. eumusae* from pure cultures as well as leaf spot tissue.

Several authors reported the rapid amplification of many fungal pathogens by the LAMP method. For example, Tomlinson et al. (2010) designed LAMP primers based on the intergenic spacer (IGS) of nuclear ribosomal DNA (rDNA) to detect *Botrytis cinerea*. Zhang et al. (2013) designed LAMP primers from the sequences of IGS region of the nuclear ribosomal operon of *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (*Foc*-TR4) for the detection of *Foc*-TR4 in soil samples. Similarly, Mohammed Amin Almasi et al. (2013) designed LAMP primers based on the sequences of 28 s ribosomal RNA gene and reported successful detection of *Fusarium oxysporum* f. sp. *lycopersici*. In this study, LAMP primers were designed based on the specific SCAR marker sequence of *P. eumusae* derived from RAPD analysis. The designed LAMP primers (F3, B3, FIP 1L and BIP 1L) were specific for the target pathogen *P. eumusae*. The primers did not amplify the DNA of closely related *Pseudocercospora* species.

Compared to conventional PCR, the LAMP method is a simpler, more feasible and rapid method. It requires only 1 h of reaction time and a thermal block or water bath for the detection of pathogen. Conversely, the conventional PCR requires 3 h of reaction time and also certain costly equipment including thermal cycler, gel system and gel scanner. Furthermore, the sensitivity of LAMP method developed in this study was 100 times higher than the conventional PCR. It has been reported that different *Pseudocercospora* species can co-exist in a single lesion (Maxwell et al. 2005; Arzanlou et al. 2008) and hence the identification of the disease based on the symptoms alone is very difficult. This study has shown that the LAMP primers can specifically detect *P. eumusae* DNA from a sample containing a mixture of DNA of *P. eumusae* and *P. fijiensis* and *P. musicola*. Therefore, the problem of misidentification of *P. eumusae* based on morphological similarities of the disease symptoms would be resolved and the detection and diagnosis of the eumusae leaf spot disease made easier and simple. The LAMP method developed in the present study has detected the target pathogen (*P. eumusae*) directly from the DNA isolated from single leaf spot tissue and may not require culturing of the pathogen for its detection. This implies that eumusae leaf spot disease can be identified within 4 h. In contrast, in the normal method of identification which includes culturing of the pathogen, DNA extraction and purification etc. needs a minimum of 17 days for the determination of the target pathogen. Besides, the LAMP method was able to identify *P. eumusae* from crude DNA samples from the mycelium and leaf spot tissues of the disease.

Conclusion

In the present study, the LAMP method was successfully developed, standardized and validated for the rapid detection and diagnosis of *P. eumusae*. Therefore, this study would be useful for the early and accurate diagnosis of the pathogen which in turn paves the way for the implementation of management practices at an early stage of disease development. In addition, this method helps to reduce the number and amount of crop protection chemicals to be used and thereby resulting in less environmental pollution. To the best of our knowledge, this is the first report on the development of LAMP method to specifically detect *P. eumusae*.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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