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DETECTION OF *PHYTOPHTHORA NICOTIANAE* INDUCED CITRUS GUMMOSIS BY THE LOOP-MEDIATED ISOTHERMAL AMPLIFICATION

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Abstract

Citrus fruits are indeed one of the prime fruits of Pakistan. The decline of citrus orchards due to gummosis became a potential threat to successful production of fruit during the last few years and is now continuously hammering farmer's as well as the economy of Pakistan. In field, control of gummosis is significantly depending on quick and exact detection of *Phytophthora nicotianae.* The current study used a highly sensitive loop-mediated isothermal amplification (LAMP) test with hydroxynaphthol blue (HNB) as a fluorescent indicator to detect *P. nicotianae.* On the basis of translation elongation factor 1-alpha (EF1α) sequence of *P. nicotianae*, four different specific LAMP primers were designed. The LAMP assay showed positive results on different 40 isolates of *P. nicotianae* that were collected from different districts of Punjab, Pakistan. While none of the cross reaction was seen with other different pathogens. From 25 µL of reaction mixture, the detection frontier of *P. nicotianae* was 10 pg genomic DNA. It is concluded that the detection of *P. nicotianae* can be performed from infected tissues by the LAMP assay, which is simple, fast, sensitive and specific.

Key words: LAMP, Pathogen, Oomycetes, Fruits, Orchards, Pakistan

Introduction

Phytophthora gummosis of citrus is an economically important disease that causes enormous citrus production losses each year worldwide (Graham & Menge, 1999; Matheron & Porchas, 2002). *Phytophthora* has emerged as a major threat to citrus industry throughout the world. Citrus growers have been in trouble due to this disease for the last few years in Punjab Pakistan. Citrus crop is attacking by *Phytophthora* species at all stages from seedling to post harvest and storage conditions. It attacks at seedling stage and cause damping off disease, destruction of the fibrous root system, gummosis, foot and crown rots, brown rot and post-harvest decay of fruits (Graham & Menge, 1999). To date, twelve distinct *Phytophthora* species have been identified in various citrus-growing regions worldwide. These species attack citrus crops and are responsible for significant crop losses (Naqvi, 2003). Recently two more species *P. insolita* and *P. humicola* have been isolated from the rhizosphere of citrus plants. Among them, the most widespread and important *Phytophthora* species are *P. citrophthora* and *P. nicotianae* which cause different citrus diseases (Ippolito *et al*., 2002a; Naqvi, 2006). Phytophthora gummosis develops rapidly under wet, cool conditions and causes 46 % losses worldwide (Teng & James, 2002).

Now a days, the citrus industry depends on postharvest or pre-planting, visual disease diagnosis to reduce economic losses. A number of management approaches including the application of chemicals and soil fumigants as well as cultural practices have been used to minimize the losses caused by *Phytophthora*. However, the use of chemicals is a potential threat to non-target organism's i-e. plants, plant products and consumers, so it is necessary to use alternative measures that are eco-friendly, effective and less expensive. Suitable diagnostic techniques using a quick, less expensive and more efficient assay are needed for the management of citrus diseases. Rapid procedures that can be deployed in the field to reduce the time between sample collection and detection could help to limit the spread of citrus diseases. Therefore, in the application of DNA based technology, the PCR method offers great potential to detect plant pathogens more efficiently because of its speed, flexibility, specificity and sensitivity (Ersek *et al*., 1994).

Previously, detection as well as diagnosis techniques based on conventional isolation on V8 medium, soil baiting methods, immuno-detection assays, and traditional PCR (polymerase chain reaction) have been formulated in order to identify and diagnose the *Phytophthora nicotianae* (Lacourt & Duncan, 1997; Khaliq *et al*., 2018). Diagnosis of *P. nicotianae* as well as *P. citrophthora* from the soil and roots through conventional detection method based upon selective media (Ippolito *et al*., 2002b), it was confirmed to diagnose the pathogen at the target species. Based on enzyme immuno-assays ELISA technique is very sharp to confirm the occurrence of *Phytophthora* species at inferior densities (Timmer *et al*., 1993). A species-specific oligonucleotide hybridization investigation has been established to identify two species of *P. citrophthora* and *P. parasitica* (Goodwin *et al*., 1990a, b). While PCR-based detection approaches were widely used for rapid and simple detection of various pathogens (Ippolito *et al*., 2002a; Bonants *et al*., 1997; Schena *et al*., 2004). The internal transcribed spacer (ITS) section of ribosomal DNA is a fast and feasible detection in order to quickly recognize notorious species of *Phytophthora* as well as unidentified isolates (Lee *et al*., 1993; Ristaino *et al*., 1998; Crawford *et al*., 1996; Cooke & Duncan, 1997). The specific identification of *P. nicotianae* and *P. citrophthora* from plant roots and soils was assessed using an ITS-targeting polymerase assay that was designed (Ippolito *et al*., 2002a). Meng and Wang demonstrated precise primers for the validation of *P. nicotianae* against different fungal species (Meng & Wang, 2010). These primers have been used in the detection of oomycetes pathogen from infected tobacco and citrus tissues, soil samples and water

suspensions. The use of PCR based methods provide an accurate and rapid finding of *P. nicotianae* in the water trials at a suitable level which allows the citrus growers to make timely management strategies. Although polymerase based recognition means providing quicker, more consistent and more subtle tools for *P. nicotianae* identification when associated with old morphological techniques. Consequently, the advancement of simple rapid, and profitable recognition approaches is still required for the specific finding of *P. nicotianae*.

The LAMP (Loop-mediated isothermal amplification) assay is regarded as one of the most popular methods for nucleic acid amplification since it is simple, quick, sensitive, and specific (Tomita *et al*., 2008). Using the LAMP assay and a set of four to six specificity designed primers, six to eight target gene sequences can be determined (Notomi *et al*., 2000). Before the reaction, HNB (hydroxynaphthol blue) is used for the identification and detection of LAMP products (Goto *et al*., 2009). Positive and negative reactions are differentiated by color changes i.e., sky-blue color is the indication of a positive reaction while purple color is the indication of a negative reaction.

Successfully LAMP has been developed to detect several *Phytophthora* species including *P. sojae* (Dai *et al*., 2012), *P. melonis* (Chen *et al*., 2013), *P. capsici* (Dong *et al*., 2015), *P. infestans, P. cinnamomi* (Hansen *et al*., 2016) and *P. agathidicida (*Winkworth *et al*., 2020). Previously, assays for the detection of *P. nicotianae* were developed based on nested PCR and LAMP, as well as the Ras-related protein gene Ypt1 (Li *et al*., 2015). Isolates of *P. nicotianae* were obtained from plant tissues and soils infected with tobacco black shank disease. Another study developed the LAMP-based detection approach for the swift diagnosis of *P. nicotianae*, using three distinct DNA extraction methods (Hieno *et al*., 2019). The objective of the current study was to develop a LAMP test for detecting the gummosis pathogen *P. nicotianae*, using specific EF1α region primers. Citrus gummosis and fungal DNA samples were utilized to evaluate the diagnostic specificity and sensitivity of this LAMP test. The newly developed LAMP assay, notable for its speed, specificity, and high sensitivity, successfully detected *P. nicotianae*.

Material and Methods

Isolation, culture collection and DNA extraction of Pathogens: Isolates of *P. nicotianae* were originally obtained from diseased samples of gummosis and root rot of citrus collected from various districts of Punjab, Pakistan. The isolates of *P. capsici* and *P. infestans* were obtained from the Laboratory of Phytophthora Diseases at the Agricultural Environment and Resources Institute (AERI) of the Yunnan Academy of Agricultural Sciences (YAAS) in Kunming, China. Further, in this study, oomycetes and fungal isolates were maintained in our laboratory (Table 1). Different Oomycete pathogen isolates were grown for 3-4 days at 25°C in V8 broth medium (Rajput *et al*., 2015). Fungal isolates were cultivated for 4-5 days at 28-30°C in PDB (potato dextrose broth) medium (Arslan, 2015). The mycelium of each isolates were collected on Whatman No. 1 filter paper by vacuum filtration and stored at -70°C for 48 h. Genomic DNA extractions were performed by the CTAB cetyl-trimethylammonium bromide method (Rajput *et al*., 2021). For LAMP assay, a template of each purified DNA from all isolates were used in this study.

Primer designing for LAMP: We employed an alignment of the EF1α (translation elongation factor 1-alpha) sequence found in the (National Center for Biotechnology Information NCBI) databases to find conserved areas in the Oomycete pathogens including *Phytophthora* species, which is closely related to *P. nicotianae*. Using the web software Primer Explorer V4 (http://primerexplorer.jp/elamp4.0.0/index.html; Eiken Chemical Co., Ltd., Tokyo, Japan), different target gene primer sets were created for the specificity and sensitivity assay. Accordingly, specific LAMP primers and their pairs were chosen, as well as their placements relative to the target DNA employed in this work (Fig. 1 & Table 2) shows a set of four primers for *P. nicotianae* based on the EF1α gene: forward and backward outer primers (F3 and B3), forward and backward inner primers (FIP: F1c-F2 and BIP: B1c-B2, respectively).

Reactions involving LAMP: Using a Veriti Thermal Cycler, LAMP reactions were kept at 62°C for 60 minutes (Thermo Fisher Scientific). 2 µl DNA template was included in each 25 µl reaction mixture (0.8-1.6 M) 0.8 mol l^{-1} betaine (100-120 mM) 120 µmol l^{-1} hydroxynaphthol blue (Santa Cruz Biotechnology, Inc.) (0.2-2 mM) dNTPs (Thermo Fisher Scientific), 1.4 mmol l^{-1} (2-8 mM) $MgSO_4$ 6 mmol l^{-1} (Sigma-Alrich), 2.5 µl 10X isothermal DNA buffer (0.32-0.64 U μL^{-1}) (1.4-2 M) 8 U Bst DNA polymerase (New England Biolabs) 2 µmol l^{-1} FIP and BIP primers (0.2-0.8 M) 0.2 µmol l^{-1} F3 and B3 primers, 0.8 µmol l^{-1} loop primer (Dai *et al*., 2012). LAMP experiments employed a *P. nicotianae* DNA template as a positive control and a double distilled H_2O template as a negative control. All of the experiments were carried out twice.

Assays for LAMP sensitivity and specificity: The LAMP sensitivity assay was carried out using 10-fold serial dilutions of pure *P. nicotianae* DNA isolated from various isolates (from 100 ng to 10 fg) (Table 1). The dilution series was made with H2O that had been doubly distilled. Following DNA amplification, the outcomes of a mix reaction of hydroxynaphthol blue and $MgSO_4$ in a colour change from violet to blue (Dai *et al*., 2012). For LAMP specificity assay, forty isolates of *P. nicotianae*, eight isolates from different oomycetes pathogens and eleven isolates from other fungal pathogens were used (Table 1). All isolates were identified prior to LAMP testing through PCR reaction using the ITS primers (White *et al*., 1990). All isolates' genomic DNA was measured and diluted to 1 ng μl^{-1} using a NanoDrop ND-1000 spectrophotometer. Each DNA sample was tested twice with each primer set, with a positive control of *P. nicotianae* DNA template and a negative control of double distilled H_2O template included in this experiment. The response mix shifted from violet to blue in both LAMP sensitivity and specificity assays after 60 minutes of incubation, and these samples were judged positive.

Table 1. List of isolates used to determine sensitivity and specificity for LAMP.

Species	Host	Sources	Number of isolates	LAMP
Colletotrichum gloeosporioides	Citrus	Faisalabad, Pakistan	1	-
Alternaria alternata	Tomato	Faisalabad, Pakistan	1	-
Alternaria alternata	Potato	Faisalabad, Pakistan	1	-
Fusarium equiseti	Grapes	Nari, Afghanistan	2	-
Fusarium solani	Shisham	Faisalabad, Pakistan	1	-
Fusarium oxysporum	Chilli	Faisalabad, Pakistan	1	-
Curvularia verruculosa	Grapes	Kunar, Afghanistan	2	-
Curvularia hawaiiensis	Grapes	Quetta, Pakistan	1	-
Pythium aphanidermatum	Chilli	Hyderabad, Pakistan	1	-
Pythium aphanidermatum	Sesame	Faisalabad, Pakistan	2	-
Phytophthora capsici	Chilli	Hyderabad, Pakistan	1	-
Phytophthora capsici	Chilli	Faisalabad, Pakistan	1	-
Phytophthora capsici	Snapbean	KunMing, China	1	-
Phytophthora infestans	Tomato	Faisalabad, Pakistan	1	-
Phytophthora infestans	Potato	Faisalabad, Pakistan	1	-
Phytophthora infestans	Potato	KunMing, China	1	-
Phytophthora nicotianae	Citrus	Sargodha, Pakistan	8	+
Phytophthora nicoatinae	Citrus	Mandi Baha ud din, Pakistan	2	+
Phytophthora nicoatinae	Citrus	Nankana Sahib, Pakistan	2	+
Phytophthora nicoatinae	Citrus	Faisalabad, Pakistan	4	+
Phytophthora nicoatinae	Citrus	Toba Tek Singh, Pakistan	6	+
Phytophthora nicoatinae	Citrus	Jhang, Pakistan	1	+
Phytophthora nicoatinae	Citrus	Sahiwal, Pakistan	4	+
Phytophthora nicoatinae	Citrus	Lodhran, Pakistan	3	+
Phytophthora nicoatinae	Citrus	Okara, Pakistan	1	+
Phytophthora nicoatinae	Citrus	Vehari, Pakistan	2	+
Phytophthora nicoatinae	Citrus	Khanewal, Pakistan	3	+
Phytophthora nicoatinae	Citrus	Bahawalpur, Pakistan	2	+
Phytophthora nicoatinae	Citrus	Bhakkar, Pakistan	1	+
Phytophthora nicoatinae	Citrus	Layyah, Pakistan	1	+

Table 2. Loop-mediated isothermal amplification primer sequences are listed below.

Primer name	Sequence (5'-3')	Length
Forward outer primer (F3)	TCGACTGAAGTCAAGTCCGT	20 nt
Backward outer primer (B3)	AAGGGCACCCAGGACTTC	18 nt
Forward inner primer (FIP) (F1c + F2)	GACAACGTCGGCTTCAACGTCA-CGAGATGCACCACGAGTCT	41 nt
Backward inner primer (BIP) (B1c+B2)	GAACGTGTCGGTGAAGGAGCT-ACTCCAAGAACGACCCGG	39 nt

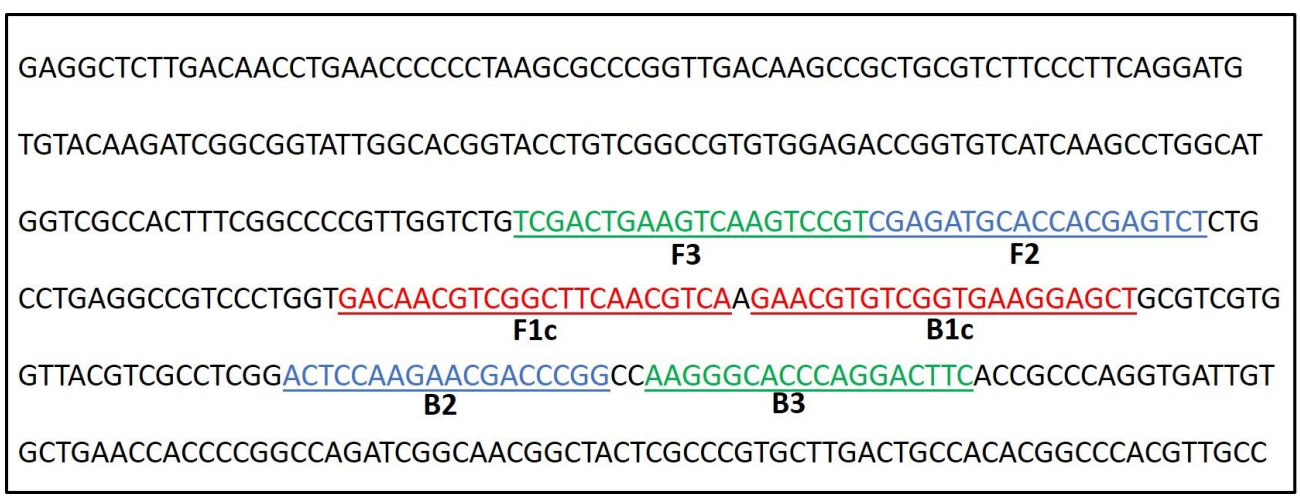

Fig. 1. Position and sequence sets of LAMP primers from the *P. nicotianae* elongation factor 1-alpha (EF1) gene. (F3 and B3) are two of the four LAMP primer sites (FIP: F1c-F2 and BIP: B1c-B2).

Fig. 2. For the detection of *P. nicotianae*, a modified LAMP technique was used. Under visible light, hydroxynaphthol blue was employed for visual assessment of the LAMP test. In the presence of hydroxynaphthol blue, positive reactions turned blue. The positive *P. nicotianae* is represented by tube (+), whereas the negative control is represented by tube (-).

Assessment of pathogens from citrus tissues and soil: To assess the field application of LAMP detection for *P. nicotianae*. 258 citrus plant tissues and contaminated soil samples from several orchards in Punjab Province were collected for LAMP and PCR testing. DNA was extracted from naturally infected citrus tissues (stem or root) and uninfected citrus tissues were used as control (Sagar & Das, 2017). For the analysis of soil samples at a depth of 06-18 cm and stored at 4°C, a soil sampler was employed. Before extraction, one gramme of soil was crushed and sieved, and DNA was extracted using a DNeasy PowerSoil DNA Isolation Kit (QIAGEN) according to the manufacturer's instructions. The LAMP reactions were carried out for 60 minutes at 62°C. Using the traditional culture method, the infection of *P. nicotianae* was confirmed based on morphological characteristics (Erwin & Ribeiro, 1996).

Results

LAMP assay for Optimization: To control the reaction time and temperature, an optimized experiment was done using 100 ng *P. nicotianae* DNA as the template. At 62°C for 60 minutes, positive LAMP reaction temperature and duration were recorded (Fig. 2). Under these conditions, the LAMP assay was completed satisfactorily. Under visible light, hydroxynaphthol blue can be used to successfully detect visual fluorescence. Positive reaction LAMP findings appeared blue, while the negative control remained violet (Fig. 2).

LAMP sensitivity assay: For serial dilution the LAMP test was developed through a 10-fold series (100 ng - 10 fg) of pure genomic DNA templates of *P. nicotianae* were detected through visual examination of the LAMP product with HNB. To determine the limit detection amount of the LAMP sensitivity assay, it was 10 pg. Decreasing concentrations of *P. nicotianae* DNA are shown from left to right, whereas the change of colour from violet to blue. The result of LAMP assay by minimum detection concentration was 10 pg. The sensitivity of the LAMP reaction was evaluated in at least three replicates of each dilution (Fig. 3).

LAMP specificity tests: The successful specificity of the LAMP assay was developed through the detection of genomic DNA from 40 isolates of *P. nicotianae*, 08 different Oomycetes pathogens and 11 fungal isolates (Table 1). Therefore, after 60 min of incubation at 62°C the *P. nicotianae* isolated from infected citrus gummosis in the different areas of Punjab province, as well as the standard *P. nicotianae* strain, all exhibited positive reactions (Fig. 4 and Table 1), while no results were detected from other fungal and oomycetes pathogens with no color change showed a specificity of this LAMP evidence for distinguishing *P. nicoatinae* from the other pathogens (Fig. 4).

Citrus tissues and soil assessment by using LAMP assay: In field, we used the PCR and LAMP tests to detect *P. nicotianae* from infected tissues and soil obtained from several districts in Punjab province. Both methods for detecting the presence of *P. nicotianae* were evaluated using the extracted genomic DNA (Figs. 5 and 6). The LAMP assay calculated 108/258 (41.8%) positive reactions for *P. nicotianae* isolate sample ratio while conventional PCR calculated 134/258 (51.9%) positive reactions for *P. nicotianae* isolate sample ratio (Table 3). These findings suggested that *P. nicotianae* is a major cause of Phytophthora gummosis and citrus root rot in these areas. Furthermore, the findings revealed that the LAMP assay may be utilized to detect *P. nicotianae* directly in infected field samples and diagnose Phytophthora gummosis and citrus root rot.

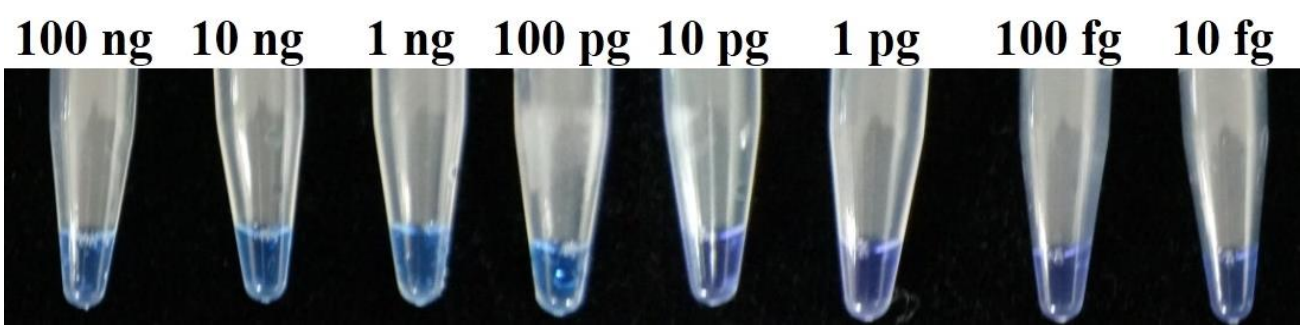

Fig. 3. The sensitivity test was performed using the LAMP method using a 10-fold *P. nicotianae* DNA dilution series through HNB. Positive colour change from violet to blue occurs as a result of LAMP reactions. The detection limit for the LAMP test was 10 pg, and the DNA amounts of *P. nicotianae* were measured from left to right.

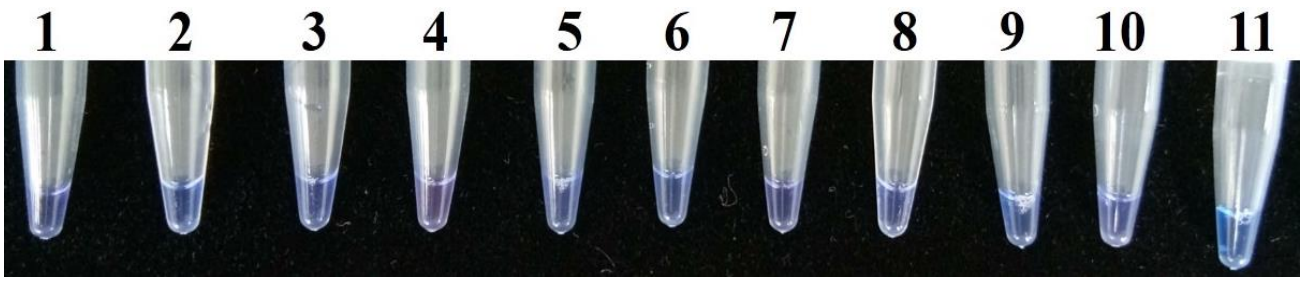

Fig. 4. Specificity of LAMP detection for *Phytophthora nicotianae* and other pathogens. Lane 1, *Colletotrichum gloeosporioides*; lane 2, *Alternaria alternate*; lane 3, *Fusarium equiseti*; lane 4, *Fusarium solani*; lane 5, *Fusarium oxysporum*; lane 6, *Curvularia verruculosa*; lane 7, *Curvularia hawaiiensis*; lane 8, *Pythium aphanidermatum*; lane 9, *Phytophthora capsici*; lane 10, *Phytophthora infestans*; and lane 11, *Phytophthora nicotianae* (standard isolate strain). The specificity of LAMP for *P. nicotianae* detection by HNB. In above figure, 1-10 tubes were observed violet colour as a negative deduction, whereas the standard *P. nicotianae* isolate was showed sky blue colour as a positive deduction after the reaction.

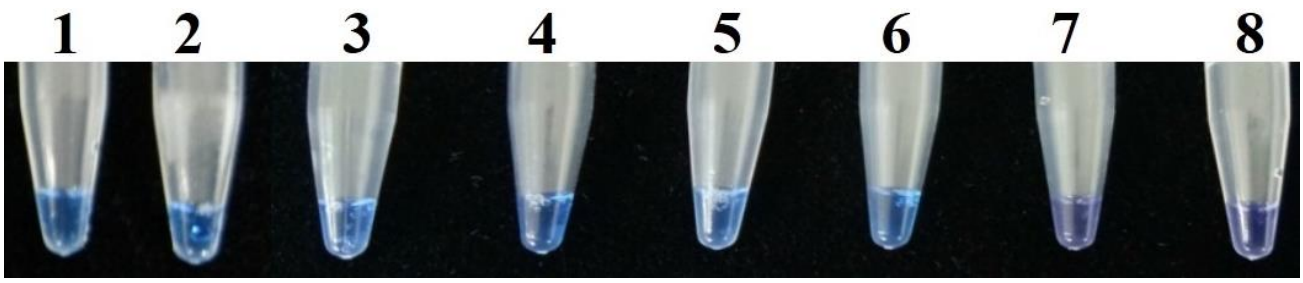

Fig. 5. The LAMP test's effectiveness in detecting the presence of *P. nicotianae* zoospores in soil. From left to right, the quantity of Zoospores decreased (1000, 500, 200, 100 and 50). Lane 1: Positive control (standard *P. nicotianae* strain); lanes 2–6: soil with varying amounts of *P. nicotianae* zoospores; lane 7: *P. nicotianae*-free soil as a control; lane 8: negative control.

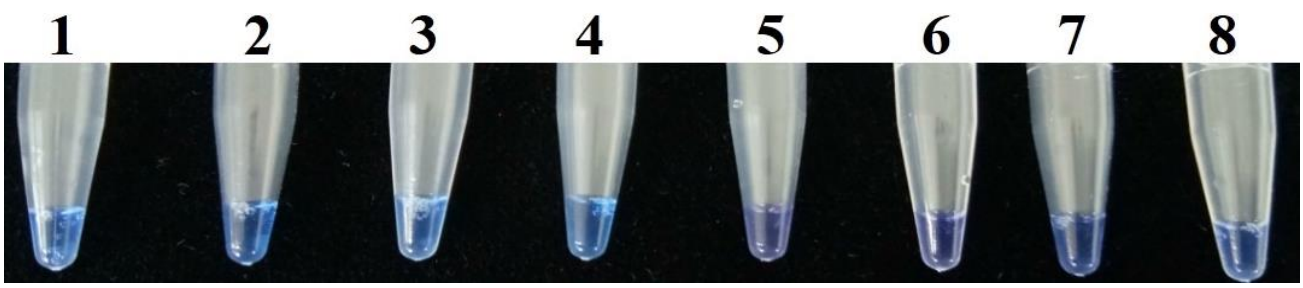

Fig. 6. LAMP test detection of *P. nicotianae* in inoculated citrus stem and roots. Positive control (standard *P. nicotianae*) line 1; DNA from inoculated tissues line 2-4; DNA from healthy tissues line 5-7; negative control line 8.

Discussion

Citrus gummosis disease is caused by *P. nicotianae*, which is a severe danger to the citrus sector globally (Rajput *et al*., 2020). *P. nicotianae* must be identified quickly and precisely in order to diagnose the condition and treat it effectively. However, there are now two detection methods available, both of which have some drawbacks in common practical usage. Traditional morphological methods are inefficient and require specialist knowledge, whereas PCR-based detection approaches are expensive, requiring costly instruments and equipment (e.g., thermal cycling). When compared to conventional PCR, LAMP is one of the most efficient methods for detecting *P. nicotianae* because it is easier to use, more rapid and sensitive, and provides concise detection. In this investigation, we used DNA isolated directly from citrus gummosis samples to construct a simple, quick, and highly sensitive LAMP test for the detection of *P. nicotianae*. LAMP primers targeting the *P. nicotianae* translation elongation factor 1-alpha (EF1α) gene. It was discovered that 62°C for 60 minutes was the best temperature for identifying *P. nicotianae* DNA (Fig. 2). This shows that detecting *P. nicotianae* may be done in a short length of time (1 hour), and that the procedure's speed and simplicity make it more desirable than other conventional and molecular detection approaches. Another significant advantage of LAMP is that amplified products can be visually detected simply by adding the hydroxynaphthol blue (HNB) dye, eliminating the need for electrophoresis. Because of its simpler technique, the LAMP test could be beneficial even for laboratories and research institutes that are unfamiliar with PCR and other molecular diagnostics. The simplest technique to detect LAMP product with the naked eye is to look for white turbidity (a reaction by-product) caused by the concentration of magnesium phosphate (Mori *et al*., 2001).

In terms of sensitivity, up to 10 pg of template DNA in a 25 µl reaction mixture produced a measurable amplification result using DNA taken from pure cultures. The decreasing trends of *P. nicotianae* DNA concentrations are clearly displayed from left to right, whereas the colour change from violet to blue and the LAMP assay's least detection concentration was 10 pg (Fig. 3). LAMP primers have been developed for the detection of various Phytophthora species, including, *P. sojae*, *P. melonis*, *P. capsici*, *P. infestans*, *P. cinnamomi*, *P. agathidicida* (Dai *et al*., 2012; Chen *et al*., 2013; Dong *et al*., 2015; Hansen *et al*., 2016; Dai *et al*., 2019; Winkworth *et.al.,* 2020).

Previously, based on the LAMP assays and Ras-related protein gene Ypt1-based, for the identification of *P. nicotianae* were described (Li *et al*., 2015) and *P. nicotianae* isolates were isolated from infected plant tissues and soils of tobacco black shank disease. Although the nuclear rDNA ITS, rDNA LSU, -tubulin, EF-1, and Ypt1 sequences and the mitochondrial cox1, cox2, and cox spacer sequences and 160 species of Phytophthora were used for the LAMP detection and based on multiple alignments, the ITS and Ypt1 sequences were selected and 11 sets of candidate LAMP primers were designed for rapid diagnosis of this pathogen (Hieno *et al*., 2019). With the use of primers targeting the EF1a gene, Siegieda's team was able to detect two target pathogens, Phytophthora spp. and *Phytophthora cactorum*, using a LAMP test. Regardless of the tested DNA isolation method, the team determined that the LAMP detection limit for the optimized reaction for Phytophthora spp. was 3 pg/L and 300 fg/L for *P. cactorum* was 3 pg/L (Siegieda *et al*., 2021). The 40 *P. nicotianae* and non-*P. nicotianae* isolates had 100 percent inclusiveness and exclusivity in the LAMP specificity assay. Furthermore, the LAMP assay was used to detect *P. nicotianae* from infected tissues and soil samples obtained from several citrus fields in Punjab, and to compute the sample ratio of the isolates (Table 3). Because of its high performance and inexpensive cost, the LAMP approach is better suited for detecting *P. nicotianae* in the field.

Table 3. List of different citrus areas to collect isolates for sensitivity and specificity results.

Collection source	Samples from citrus	LAMP positive	PCR positive
Sargodha	29	14	11
Mandi Baha ud din	13	8	6
Nankana Sahib	11	6	5
Faisalabad	14	7	4
Toba Tek Singh	21	12	9
Jhang	12	5	3
Sahiwal	23	14	12
Lodhran	18	10	8
Okara	15	7	5
Vehari	22	11	10
Khanewal	27	14	13
Bahawalpur	17	9	7
Bhakkar	12	7	6
Layyah	24	10	9

Phytophthora species have been detected utilizing LAMP detection methods in a variety of investigations, including real-time PCR, real-time turbidimeter, colorimetric, fluorescent, and lateral flow device for reaction confirmation (Dai *et al*., 2012; Tomlinson *et al*., 2010; Aglietti *et al*., 2019; Ristaino *et al*., 2020; Hudson *et al*., 2020). Based on the Ypt1 gene for reaction confirmation, Chen and colleagues developed nested PCR and LAMP techniques to detect *P. melonis*. Both approaches for detecting 10 fg genomic DNA yielded the same sensitivity result (Chen *et al*., 2013). Another study used the LAMP test to detect two oomycete pathogens; *P. ramorum* and *P. kernoviae* using a lateral-flow device (LFD) integrated into the amplification products (Tomlinson *et al*., 2010). Additionally, *P. sojae* was found using the LAMP method by Dai and colleagues, and the reaction was then verified using an expensive real-time turbidimeter. Although the LAMP approach's flexibility was constrained by the use of expensive equipment, which discouraged its implementation, particularly in underdeveloped countries (Dai *et al*., 2012).

The LAMP approach used in this work is a quick, specific, and highly sensitive diagnostic protocol for *P. nicotianae* detection. To the best of our knowledge, this is the first report of the EF1a application for the detection of *P. nicotianae*. This approach is more practical for detecting low levels of *P. nicotianae* in plant tissues, as well as checking the early stages of *P. nicotianae* infection at a low level. As a result, *P. nicotianae*-carrying citrus can be easily discovered in the early stages of infection, and a care strategy can be devised before the infection spreads.

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Competing Interests: Authors have declared that no competing interests exist.

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