

## Application of loop-mediated isothermal amplification (LAMP) assay for rapid and sensitive detection of fungal pathogen, *Colletotrichum capsici* in *Capsicum annuum*

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### Abstract

*Colletotrichum capsici* is an economically important pathogen that severely affects the fruits of chilli (*Capsicum annuum*) plants. Loop-mediated isothermal amplification (LAMP) assay was used for accurate and sensitive detection of *C. capsici*. Sequences of  $\beta$ -tubulin gene of *C. capsici* were used as target template to design LAMP primers. The primers were very specific to *C. capsici* and did not react against any other seed-borne fungi of chillies. LAMP assay was sensitive and could detect as little as 10 fg  $\mu\text{L}^{-1}$  of *C. capsici* pathogen in comparison with 1 ng  $\mu\text{L}^{-1}$  of *C. capsici* detection using polymerase chain reaction. Further test detection of infected chilli seeds collected from different locations in India showed that the LAMP assay was more sensitive than the PCR-based detection of *C. capsici*. This LAMP assay is simple, fast and sensitive in detection of *C. capsici* infected field samples, tissue culture materials and for schedule screening of plant quarantine materials under exchange. In the present study, LAMP assay has been used for fast and sensitive detection of *C. capsici* pathogen.

### Key words

*Colletotrichum capsici*, *Capsicum annuum*, Fungal pathogens, LAMP assay

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### Introduction

Chilli (*Capsicum annuum* L.) is an important vegetable crop grown throughout the world. It is affected by many diseases, including fruit rot. The pathogen *Colletotrichum capsici* (Syd.) (Butler & Bisby) is the causal agent of the anthracnose disease in chillies found both in subtropical and tropical areas of several countries (Than *et al.*, 2008). *C. capsici* is an important pathogen which is naturally abundant, haploid, and anamorphic state of the genus *Glomerella* observed in chillies (Shenoy *et al.*, 2007). Suitable detection methods using a quick, cheap and competent assay is urgently required for the effective control of this pathogen. Rapid methodology can be performed in both field and

greenhouse and help in reducing the time for sampling and further diagnosis could effectively reduce the spread of this pathogen. Currently, the most common methodology for quick detection of *C. capsici* is based on polymerase chain reaction (Nayaka *et al.*, 2009). However, PCR has few basic disadvantages like requirement of high-tech thermal cycler, time requirement, low specificity, and very less amplification products (Freeman *et al.*, 2002). Thus, the less time-consuming loop-mediated isothermal amplification (LAMP) method is used to avoid PCR technique, thus, allowing master reaction mixture to be incubated in a water bath (Gill and Ghaemi, 2008). This novel LAMP methodology has been successfully used for the detection of plant pathogenic bacteria (Rigano *et al.*, 2010), fungi (Duan *et al.*, 2014;

Tomlinson *et al.*, 2010), plant viruses and viroids (Boubourakas *et al.*, 2009; Peng *et al.*, 2012).

The principle of LAMP assay is very simple and the reaction carried out under isothermal conditions, employing a *Bst* DNA polymerase with a strand-displacing activity that involves a set of primers. These primers can recognise target sequences on the template DNA to be amplified. The amplified products of the template DNA contain single-stranded loops, which allow selected primers to bind without the need for repeated cycles of thermal denaturation of target region of test pathogen DNA (Notomi *et al.*, 2000). LAMP products can be visualised with unaided eye *via* different methods such as by addition of DNA intercalating dyes such as SYBR Green I, or by addition of hydroxynaphthol blue (HNB) (Goto *et al.*, 2009), or by estimating the turbidity enhanced level derived from magnesium pyrophosphate (Mori *et al.*, 2001; Kikuchi *et al.*, 2009). LAMP products can also be visualised as a DNA marker like banding pattern on 2% gel electrophoresis (Ma *et al.*, 2010). The LAMP assay can also carried out in real time which results in quantitative detection of test pathogens (Bekele *et al.*, 2011). Moreover, without the need of any special equipment, LAMP assay can amplify DNA with high specificity and efficiency compared to PCR. Thus, this assay is more suitable for the early detection and identification of plant pathogens in agricultural field conditions.

In view of the above, in the present study LAMP assay was carried out for sensitive and specific detection of latent infection of *C. capsici* in chilli seeds, and to evaluate the comparative performance of LAMP and PCR-based detection of naturally infected chilli seed samples collected from different states of India.

## Materials and Methods

**Isolation and maintenance of fungal cultures :** Ten isolates of *C. capsici* were isolated from infected chilli seeds that were collected from various chilli growing states of India and other two non-host species of *Colletotrichum*, mainly *Colletotrichum gloeosporioides* and *Colletotrichum lindemuthianum*. For the specificity test, seedborne pathogens such as *Alternaria alternata*, *Drechslera rostrata*, *Fusarium solani*, *Macrophomina phaseolina*, *Aspergillus flavus* and *Trichoderma viride* were also isolated. All the fungal cultures were purified by modified single spore isolation techniques (Akhtar *et al.*, 2014), and these cultures were further maintained on potato dextrose agar medium (HiMedia, India) with regular sub culturing and stored at 4 °C.

### Extraction of genomic DNA

**DNA extraction from fungal cultures :** For fungal genomic DNA extraction, all the test fungi were cultivated in potato dextrose broth (20 g glucose and 4 g potato extract in 1 l H<sub>2</sub>O)

medium on a shaker for 7 days at 24 °C and later collected on sterile filter paper and stored at -70°C until further use. Approximately, 100 mg of frozen-dried fungal mycelium were ground in liquid nitrogen using sterile pestle and mortar, and the fungal genomic DNA was isolated using HiPurA™ fungal DNA purification kit (HiMedia, India) as per manufacturer's instructions. The purified DNA was quantified by Nanodrop spectrophotometer (Thermo Fisher Scientific, Mumbai, India), and the final concentration of the template DNA was adjusted to 25 ng µl<sup>-1</sup> for PCR and LAMP analysis.

**LAMP primer design :** LAMP primers (Fig. 1a) were designed using the *C. capsici* β-tubulin gene sequence (accession number JX856129.1) using PRIMER EXPLORER V4 software (Eiken Chemical Co. Ltd, Tokyo, Japan). A forward inner primer FIP (5'- CAACTCCGACGTGGCCAGAG-CATGGCCTCGACAGCAAC-3') consisted of F1c (the complementary sequence of F1, 5'-CAACTCCGACGTGGCCAGAG-3', nt 183–164) and F2 (5'-CATGGCCTCGACAGCAAC-3', nt 123–140), and a backward inner primer BIP (5'-CAATGGAACCTCGGAGCTCCAG-TAAAACGCGTGGGACTGTAG-3') of B1c (the complementary sequence of B1, 5'-CAATGGAACCTCGGAGCTCCAG-3', nt 208–229) and B2 (5'- TAAAACGCGTGGGACTGTAG-3', nt 289–270). The outer primers F3 (5'- TCGAACAGGCAGAACATCTC-3', nt 96–115) and B3 (5'- CGAAGGAGCGAAGGTCAAGT-3', nt 328–310) were used for LAMP reaction (Fig. 1). Specificity of primers was carefully checked by basic local alignment search tool (BLAST), NCBI against human and other fungi DNA sequences in public GenBank databases.

**LAMP and PCR reactions :** The LAMP assay for *C. capsici* was performed in a 25 µl mixture containing 25 ng of template DNA, 5 pmol of each F3 and B3 primers, 40 pmol of each FIP and BIP primers, 2.5 µl 10x Buffer (200 mM Tris-HCl, 100 mM KCl, 100 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 20 mM MgSO<sub>4</sub>, 0.1% Triton X-100, pH 8.8 at 25 °C), 4 mM MgCl<sub>2</sub>, 0.8 mM dNTPs, 0.8 M betaine and 1.0 µl of *Bst* DNA polymerase (8 U µl<sup>-1</sup>). The mixture was incubated at 94 °C for 5 min, and 65 °C for 45 min and then the reaction was terminated by incubation at 82 °C for 10 min. The LAMP products were visually detected by adding 2 µl 10x SYBR Green I solution. The LAMP reaction products were further analysed by electrophoresis on 2.0% agarose gels, and 100 bp DNA plus ladder (Fermentas, Thermo Fisher Scientific, India) was used as molecular size standard for the agarose gels.

PCR amplifications were carried out in a reaction volume of 25 µl containing 25 ng of template DNA. PCR detection assay for *C. capsici* was conducted using species specific primers: CC1F1-5'ACCTAACTGTTGCTTCGGC G3' and CC2R2-5' AAATTTGGGGGTTTACGGC 3' with

an expected amplicon size of 447 bp. The reaction mixture consisted 10x PCR buffer, 10 mM dNTPs, 1 U Taq DNA polymerase, and 0.2  $\mu$ M of forward and reverse primer each. All the PCR reaction components were obtained from Fermentas, Thermo Fisher Scientific, India. The amplifications were performed using a Thermal Cycler (GenePro PCR, Bioer, Portsmouth, NH, USA). The amplification programme used is as follows: initial denaturation at 92 °C for 3 min, 35 cycles of denaturation at 92 °C for 1 min, annealing at 56 °C for 1 min, and extension at 72 °C for 1 min, with final extension at 72 °C for 10 min. All the amplified PCR products were resolved by electrophoresis on 1.2 % agarose, stained with ethidium bromide and photographed using the Gel Documentation System (AlphaImager® Corporation, Santa Clara, CA, USA).

**Specificity test :** LAMP specificity for *C. capsici* was analysed using pure culture template DNA of *C. capsici*, six other seedborne plant-pathogenic fungi and two non-host species of *Colletotrichum*. LAMP assay was performed as described earlier at 65 °C for 45 min and the reaction was terminated by incubating at 82 °C for 10 min. The assays were analyzed based on colour change of SYBR Green I and was further confirmed by gel electrophoresis. Each test fungal sample was represented by three replications, and the experiment was performed at least three times.

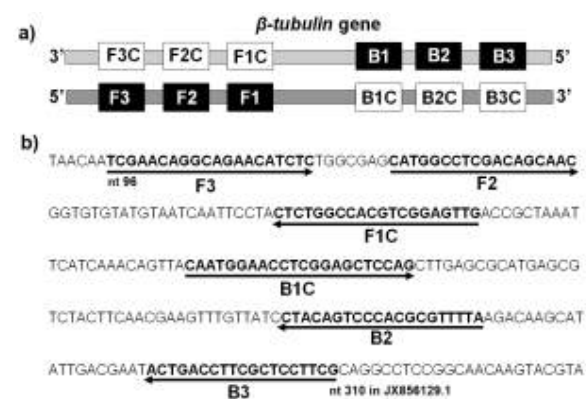
**Sensitivity test :** To analyse the sensitivity of *C. capsici* LAMP assay, the genomic DNA extracted from *C. capsici* was adjusted to 25 ng  $\mu$ l<sup>-1</sup> as a constant for LAMP and PCR assays. It was further diluted to 10 ng  $\mu$ l<sup>-1</sup> in a 10-fold series (10 ng  $\mu$ l<sup>-1</sup> – 10 fg  $\mu$ l<sup>-1</sup> dilutions) to analyze the detection efficiency limit of *C. capsici* LAMP assay in comparison with the standard PCR. After the LAMP reactions were complete, the LAMP products were visualised by adding of SYBR Green I, and the PCR samples were analyzed by gel electrophoresis. Each test fungal sample was represented by three replications, and the experiment was performed at least three times.

**Performance evaluation :** To evaluate the performance of LAMP method for diagnosis of *C. capsici*, 31 chilli seed samples collected from different states of India were examined for the LAMP assay and standard PCR. For each sample, the seeds were grinded, and its total DNA was extracted as mentioned above. Then they were further processed for the LAMP and PCR assay. When the LAMP reactions were over, the samples were analyzed based on their colour change and further confirmed by gel electrophoresis as described in previous section.

## Results and Discussion

Ten isolates of *C. capsici*, two non-host *Colletotrichum* species-*C. gloeosporioides* and *C.*

*lindemuthianum* and other seed-borne fungi of chilli viz. *D. rostrata*, *A. alternata*, *F. solani*, *M. phaseolina*, *A. flavus* and *T. viride* were used to test the specificity of LAMP assay. Positive reactions were observed in all *C. capsici* infected samples, whereas other *Colletotrichum* spp. or isolates of seed-borne fungi of chilli did not show any reactions (Table 1) (Fig. 2). Ladder-like pattern on 2% gel electrophoresis was observed in all the isolates of *C. capsici* but not in other seed-borne fungi or negative controls (Fig. 2). Based on the clear visual detection with addition of SYBR Green I, both positive and negative controls were easily detected after the LAMP reaction. All the infected samples of *C. capsici* appeared to have a fluorescent green colour, whereas negative control or other seed-borne fungi remained orange in colour. The LAMP assay perfectly distinguished between *C. capsici* and other fungal pathogens, mainly the LAMP primers designed in this research work were only specific for *C. capsici*. The LAMP assay produces pyrophosphate ions, which bind to Mg<sup>2+</sup> ions and form a white precipitate of magnesium pyrophosphate which can be viewed by naked eye. To ensure high specificity, no positive products were amplified from the DNA of non-host *Colletotrichum* species, i.e., *C. gloeosporioides* and *C. lindemuthianum*, and other seed-borne fungal pathogens of chilli seeds. In the present study, it was observed that the reaction time and efficiency improved with F3, B3, FIP and BIP primers only in the specific detection of *C. capsici*, i.e., at 45 min, this seed-borne pathogen was detected. Similar results have also been reported by Peng *et al.* (2012) where banana streak virus was

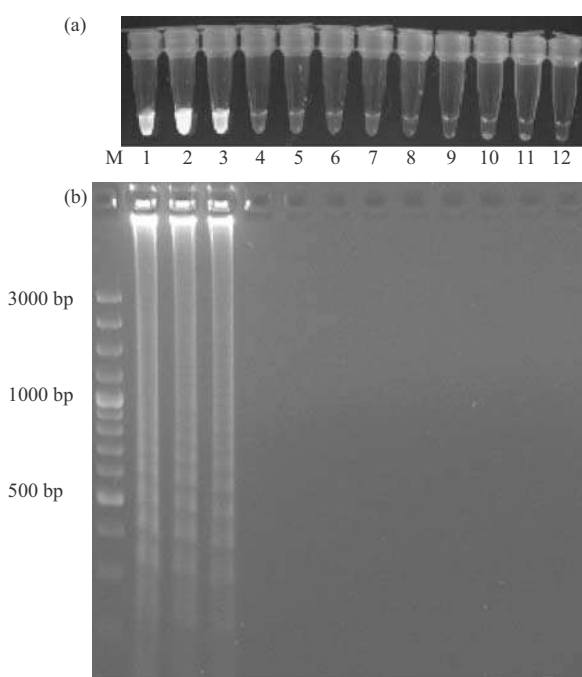


**Fig. 1 :** Design of LAMP primers specific for *Colletotrichum capsici* based on  $\beta$ -tubulin sequences. (a) Schematic representation of LAMP-amplified regions. (b) Design of inner and outer primers from  $\beta$ -tubulin sequences (JX856129.1) from *C. capsici* genome and their relative positions inside the sequence. The F1c is complementary to the F1c region of the template sequence, as are B2 and B3. The B1c is identical to the B1c region of the template sequence, as are F3 and F2. The forward primer sequence is indicated above the template sequence, and the reverse primer sequence is indicated below the template sequence

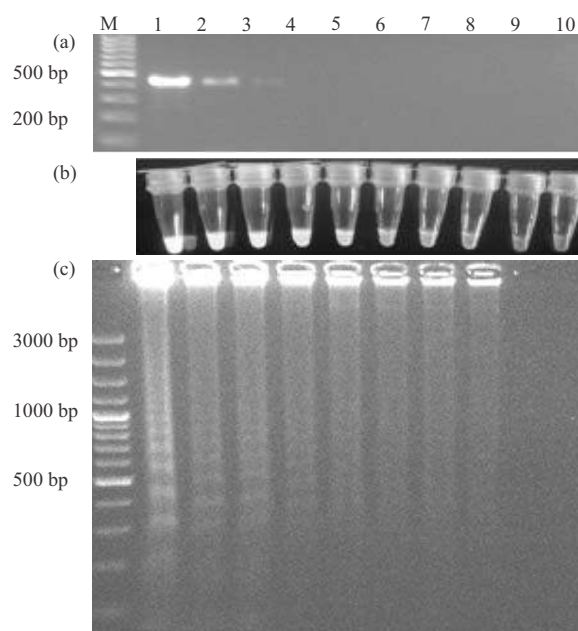
**Table 1** : Specificity of LAMP assay for detection of pathogen, *Colletotrichum capsici* in chilli (*Capsicum annuum*)

Isolates	Host	Origin	LAMP	PCR
<i>Colletotrichum capsici</i>	<i>Capsicum annuum</i>	Karnataka, India	+	+
<i>C. capsici</i>	<i>C. annuum</i>	Karnataka, India	+	+
<i>C. capsici</i>	<i>C. annuum</i>	Karnataka, India	+	+
<i>C. capsici</i>	<i>C. annuum</i>	Karnataka, India	+	+
<i>C. capsici</i>	<i>C. annuum</i>	Uttar Pradesh, India	+	+
<i>C. capsici</i>	<i>C. annuum</i>	Uttar Pradesh, India	+	+
<i>C. capsici</i>	<i>C. annuum</i>	Maharashtra, India	+	+
<i>C. capsici</i>	<i>C. annuum</i>	Maharashtra, India	+	+
<i>C. capsici</i>	<i>C. annuum</i>	Maharashtra, India	+	+
<i>C. gloeosporioides</i>	<i>Mangifera indica</i>	Tamil Nadu, India	-	-
<i>C. lindemuthianum</i>	<i>Phaseolus vulgaris</i>	New Delhi, India	-	-
<i>Drechslera rostrata</i>	<i>C. annuum</i>	Karnataka, India	-	-
<i>Alternaria alternata</i>	<i>C. annuum</i>	Karnataka, India	-	-
<i>Fusarium solani</i>	<i>C. annuum</i>	Uttar Pradesh, India	-	-
<i>Macrophomina phaseolina</i>	<i>C. annuum</i>	Uttar Pradesh, India	-	-
<i>Aspergillus flavus</i>	<i>C. annuum</i>	Maharashtra, India	-	-
<i>Trichoderma viride</i>	<i>C. annuum</i>	Maharashtra, India	-	-

+, positive reaction; -, negative reaction.



**Fig. 2** : Specificity test of LAMP assay for the detection of *Colletotrichum capsici*. (a) Colour changes of LAMP amplification product after adding 2 µl of 10x SYBR Green I; (b) Agarose gel electrophoresis of LAMP products. Lane M, 100 bp DNA plus molecular weight marker; Lanes 1-3: *C. capsici* DNA, lane 4: *C. gloeosporioides*, lane 5: *C. lindemuthianum*, lane 6: *A. alternata*, lane 7: *D. rostrata*, lane 8: *F. solani*, lane 9: *M. phaseolina*, lane 10: *A. flavus*, lane 11: *T. viride* and lane 12: negative control

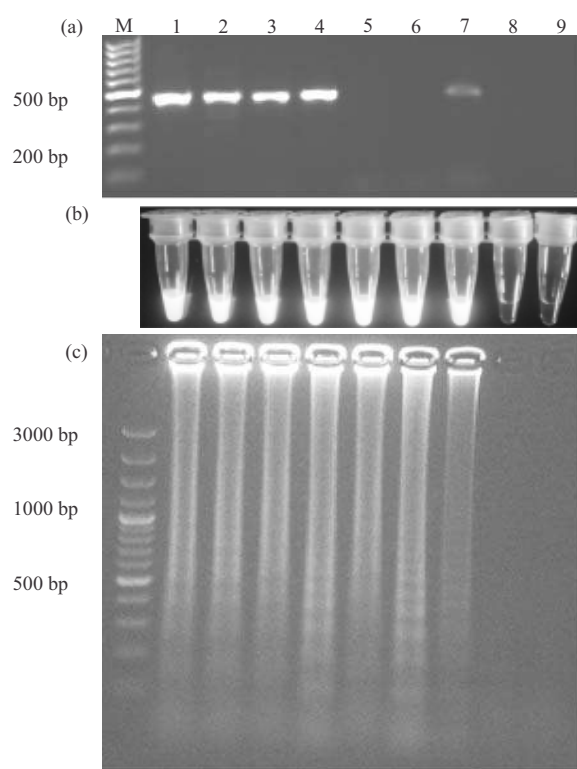


**Fig. 3** : Results of the sensitivity test for LAMP assay for *Colletotrichum capsici* detection in comparison with PCR assay. (a) PCR-based specific detection of *C. capsici* (447 bp) indicating lane M: 100 bp DNA ladder; lane 1: 25 ng µl<sup>-1</sup> of *C. capsici* DNA; lane 2: 10 ng µl<sup>-1</sup>; lane 3: 1 ng µl<sup>-1</sup>; lane 4: 100 pg µl<sup>-1</sup>; lane 5: 10 pg µl<sup>-1</sup>; lane 6: 1 pg µl<sup>-1</sup>; lane 7: 100 fg µl<sup>-1</sup>; lane 8: 10 fg µl<sup>-1</sup>; lane 9: healthy control and lane 10: negative control respectively. (b) Colour changes of LAMP amplification product after adding 2 µl of 10x SYBR Green I; tubes 1-10 as mentioned above for PCR. (c) Agarose gel electrophoresis of LAMP products indicating lane M: 100 bp DNA plus ladder; lane 1-10, as mentioned above for PCR



**Table 2 :** Performance evaluation of LAMP assay for detection of pathogen, *Colletotrichum capsici* in chilli (*Capsicum annuum*)

Sources	Number of samples	LAMP	PCR
Karnataka	11	11	9
Orissa	2	2	2
Maharashtra	5	5	3
Uttar Pradesh	13	12	11

**Fig. 4 :** Performance evaluation of LAMP assay for *Colletotrichum capsici* detection from field collected samples in comparison with PCR assay. (a) PCR based specific detection of *C. capsici* (447 bp) indicating lane M: 100 bp DNA ladder; lane 1: positive control-*C. capsici* template DNA; lane 2-7: *C. capsici* infected field samples; lane 8: healthy control and lane 9: negative control respectively. (b) Colour changes of LAMP amplification product after adding 2  $\mu$ l of 10x SYBR Green I; tubes 1-9 as mentioned above for PCR. (c) Agarose gel electrophoresis of LAMP products indicating lane M: 100 bp DNA plus ladder; lane 1-9, as mentioned above for PCR. (lane 5 and 6 showed positive reaction in LAMP, whereas not amplified in PCR reaction)

detected by LAMP assay. The LAMP primer set in the present study was designed (Fig. 1) using the partial region of  $\beta$ -tubulin gene, which has species-specific sequences. Earlier studies have shown that the  $\beta$ -tubulin region is useful in early detection of wheat stripe rust fungal species (Huang *et al.*, 2011).

To analyze the sensitivity of LAMP reaction, assays were performed using serially diluted ( $10 \text{ ng } \mu\text{l}^{-1}$  to  $10 \text{ fg } \mu\text{l}^{-1}$ ) pure *C. capsici* DNA. The sensitive assay, when compared with standard PCR (447 bp), showed the detection limit up to  $1 \text{ ng } \mu\text{l}^{-1}$  of pure *C. capsici* DNA. In the case of LAMP assay, different concentrations of test fungal DNA showed that minimum detection concentration required for LAMP assay was  $10 \text{ fg } \mu\text{l}^{-1}$  and it was noted by direct visual inspection of LAMP product with SYBR Green I indicated by a colour change from orange to fluorescent green. (Fig. 3). The detection limit was 10,000 times higher than that of the standard PCR. Previous reports have suggested the LAMP assay to be highly sensitivity than PCR method (Xu *et al.*, 2010; Peng *et al.*, 2012; Fukuta *et al.*, 2013; Takahashi *et al.*, 2014).

It was found that the positive sample ratio was 30/31 (96.7%) for LAMP assay and 25/31 (80.6%) for standard PCR method (Table 2) (Fig. 4). The LAMP assay might be useful for the detection of test pathogen under natural conditions as it is much simpler and faster as compared to the standard PCR. These special characteristics of LAMP increase its versatility and perfect suitability for usual testing in quarantine laboratories.

It should be noted that LAMP assay does not require expensive reagent and equipments. Compared to the standard PCR method, LAMP assay is related with simple and fast detection (< 70 - 80 min). A regular laboratory water bath that can provide a stable temperature ( $65^\circ\text{C}$ ) can be used in this LAMP assay. The LAMP assay developed in the present study used four primers: F3, B3, FIP, and BIP. To determine the optimal time (30 min, 45 min and 60 min) and temperature for the detection of *C. capsici*, the LAMP reaction was performed at  $61^\circ\text{C}$ ,  $63^\circ\text{C}$ ,  $65^\circ\text{C}$  and  $67^\circ\text{C}$  using the LAMP primers (as mentioned above) with appropriate negative control. It was noted that at  $65^\circ\text{C}$  and 45 min, LAMP products changed from orange to green colour after addition of SYBR Green I in *C. capsici* infected samples (data not shown). Therefore, temperature of  $65^\circ\text{C}$  and time of 45 min was selected for the detection of *C. capsici* and for further evaluation of infected seed samples.

In summary, the results of the study revealed that the LAMP assay is useful for the early detection and identification of plant pathogenic *C. capsici*. This LAMP assay could be used as a detection tool for simple and quick diagnosis of chilli plants infected with *C. capsici* in agricultural fields and in quarantine laboratories.

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