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RESEARCH ARTICLE

Rapid detection of *Fusarium oxysporum* f. sp. *ciceris* from disease infested chickpea fields by loop-mediated isothermal amplification

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ABSTRACT:*Fusarium* wilt (FW) in chickpea, caused by major fungal pathogen, *Fusarium oxysporum* f. sp. *ciceris* (Foc) results in10-40% economic losses worldwide. In field condition, several soil borne pathogens produce similar FW like symptoms in chickpea. Therefore, by visual symptoms, distinguish and diagnosis of FW disease is very difficult in field. Hence, there is real need of an accurate, rapid and inexpensive diagnosis method for FW. In this study, we developed hydroxynaphthol blue dye based a loop-mediated isothermal amplification (LAMP) assay targeting *ef1á* gene sequence for rapid diagnosis of FW in chickpea. The reaction was optimized using minimum 10 fg of Focg DNA at 63°C for 60 min. The characteristic sky blue colour and ladder like band in agarose gel of the final LAMP products indicated the positive reaction. This LAMP based tool will be very helpful to rapid diagnosis and monitoring the chickpea FW in the field.

Keywords: *Fusarium oxysporum* f. sp. *ciceris*, LAMP, Hydroxynaphthol blue, Detection

Chickpea (*Cicer arietinum* L.) is the second largest cultivated legume crop grown in 54 countries, including the parts of subtropical South Asia, Africa, Australia and temperate and Mediterranean regions. India is the largest producer of chickpea and accounts 68.47% of total global production. In India, chickpea represents 50.34% of total pulse production with 35.16% of total pulse cultivating area.

Within the various biotic stresses,*Fusarium* wilt (FW), caused by seed and soil borne fungus *Fusarium oxysporum* (Foc) is the major fungal pathogen, results in economic losses ranging from 10-40% worldwide. It is estimated to cause 10-15% yield loss annually in India (Sharma *et al.,* 2016), but can result in 100% losses under favourable conditions. As the general symptoms of FW and dry root rot (DRR) disease are similar, in field these are often visually undistinguishable. In both cases affected plants show foliar chlorosis and causes mortality of the plants usually in patches in field. Therefore, this presents a real need of rapid, reliable and easy detection method of Foc for better management of FW disease in chickpea. In recent years, PCR-based methods have been developed to detect different microorganisms, including fungus (Sharma *et al.,* 2015), bacteria, virus (Tarafdar *et al.,* 2013, 2017) etc. However, those methods are expensive and time consuming, and required trained persons. Loop-mediated isothermal amplification (LAMP) is an alternative amplification technology, is highly sensitive, less time-consuming than conventional PCR-based methods, and does not require any thermal cycler. Recently, LAMP assay is being used for the detection of pathogenic fungi (Ghosh *et al.,* 2017, 2015) bacteria (Pan *et al.,* 2011) and viruses (Jeong *et al.,* 2004).

The purpose of the present study, the LAMP method has been applied first time for the detection of Foc from purified fungal DNA samples and infected chickpea field samples as well. The developed LAMP assay effectively detected Foc with high specificityand sensitivity.

MATERIALS AND METHODS

Fungal culture

A total of five Foc isolates representing five races isolated from different agro-ecological zones of India (Foc 1 from South zone for race 1, Foc 14 from Central zone for race 2,Foc 11 from North East plain zone for race 4, Foc 9 from North West plain zone for race 6 and Foc 58 from North Hill zone for new identified race) (Sharma *et al.,* 2014) and two *Rhizoctonia bataticola* (RB) isolates were used in the present study to validate the LAMP assay (Table 1). All the isolates are maintained in International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Telangana State, India for further use.

DNA extraction

All the five isolates of Foc and two of RB were grown in PDB and incubated in a rotary shaker at 120 rpm at 25 ± 1°C for five days. Mycelia were harvested through filter papers, and washed 2-3 times with sterile distilled water to remove excess of salts. The DNA from the fungal isolates was extracted by modified cetyl trimethyl

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ammonium bromide (CTAB) method. 200 mg mycelium was grinded in liquid nitrogen and transferred into 1.5 ml pre-warmed (65°C) DNA extraction buffer [1 M Tris–HCl (pH 8.0), 0.5 M EDTA (pH 8.0), 5 M NaCl and 2% CTAB], mixed well by gentle shaking and incubated at 65°C in a water bath for 10 min and followed the method given by Sharma et al (2014). Similarly, genomic DNA from the roots of infected chickpea plants from fields was extracted using Pure Link plant total DNA purification kit (Invitrogen, USA) followed by manufacturer's instructions. Quantity and quality and of extracted DNA was evaluated on 0.8% agarose gel as well as by spectrophotometric analysis and stored at -20°C for further use.

Primer design

A total of six LAMP primers used in this study were taken from published literature of our lab (Ghosh *et al.,* 2014). The primers were designed in LAMP designer software program (http://lamp-designer.software.informer.com/) based on the conserved region of Foc elongation factor 1 alpha (*Ef1á*) gene. *In-silico* evaluation of those primers was done in nBLAS Ttools using NCBI sequence database.

Standardisation of LAMP reaction

The LAMP assay was standardised with Foc DNA as the template. The best results were obtained when a 25 µL volume of mixture was containing 2.0-µl primer mixture (2.5 µM each of F3 and B3 primers and 20 µM each of FIP, BIP, Loop F, and Loop B primers), 1 mM dNTPs, 1.28 M betaine, 20 mM Tris-HCl (pH 8.8), 4 mM $MgCl_2$, 10 mM KCl, 2 mM $MgSO_4$, 10 mM $(NH4)_2SO_4$, 0.1% Triton X-100, 8 U of Bst DNA polymerase, 150 µM HNB, and 1 µL of target DNA. The mixture was prepared in 0.2 mL microcentrifuge and kept in water bath with prefixed temperature. A positive LAMP reaction was indicated by a sky blue colour. The LAMP products were subjected to agarose gel electrophoresis. The ladder like characteristic bands in the gel was evident of the positive reaction.

Optimization of LAMP reaction conditions and sensitivity

The LAMP reaction mixtures were incubated for 60 min from 57°C to 63°C to determine the optimal temperature for the reactions. Then, the LAMP assay was performed to determine the optimal reaction time. The assay was

Table 1. The details of fungal and plant samples used in this study

Sample	Collected from	Visual detection	LAMP detection
Fungal isolates			
Foc 1	Patancheru, Telengana State	NA	+
Foc 9	Hisar, Haryana	NA	+
Foc 11	Dholi, Bihar	NA	+
Foc 14	Junagadh, Gujarat	NA	+
Foc 58	Dhaulakaun, Himachal Pradesh	NA	+
RB 6	BP 10, ICRISAT	NA	-
RB 26	BIL 2, ICRISAT	NA	-
Field samples			
Sample 1	BIL 06C, ICRISAT	FW	+
Sample 2	BL 02, ICRISAT	FW	+
Sample 3	BIL 06D, ICRISAT	FW	+
Sample 4	BUS 1, ICRISAT	FW	-
Sample 5	BM 08B, ICRISAT	FW	+
Sample 6	BIL 07D, ICRISAT	FW	-
Sample 7	BIL 06B, ICRISAT	FW	+
Sample 8	BP 02, ICRISAT	FW	+
Sample 9	BP 13, ICRISAT	DRR	-
Sample 10	BM 08B, ICRISAT	DRR	+
Sample 11	BP 02, ICRISAT	DRR	-
Sample 12	BIL 06B, ICRISAT	DRR	-
Sample 13	BS 07A, ICRISAT	DRR	+

NA: not applicable; FW: Fusarium wilt; DRR: dry root rot; '-': negative reaction; '+': positive reaction

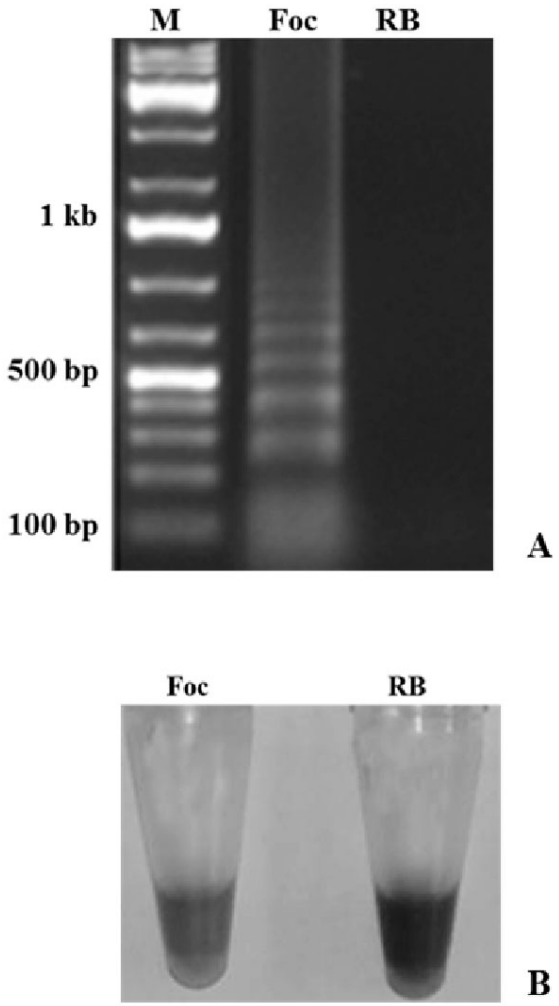

Fig. 1. Detection of *Fusarium oxysporum* f. sp. *ciceris* by LAMP assay. (A) The ladder like pattern in agarose gel electrophoresis is indicating the positive reaction with Foc DNA. (B) HNB visual detection of Foc. The positive reaction becomes sky blue and the negative ones remains violet.

conducted for 15, 30, 45, 60 and 90 min at the optimal reaction temperature. The reactions were terminated by heat inactivation at 80°C for 10 min. LAMP sensitivity was assayed using 10 folds serially diluted FocDNA ranged from 10 ng to 10 fg. The purified DNA was dissolved in double-distilled water, and 1 µl of the solution was used as the template for LAMP. The LAMP reaction was assessed based on HNB-visualized colour change and then on gel electrophoresis.

Evaluation of the LAMP assay

A total of five Foc isolates collected from diverse geographical locations represents predominant races in India and two RB isolates were subjected to LAMP assays. To confirm the applicability of LAMP in field diagnosis, the method was evaluated with the field sample infested by Foc and RB. The chickpea plants showed typical symptoms of FW and DRR were collected from different experimental fields of ICRISAT.

RESULTS

Optimization of LAMP reaction

The optimization of the LAMP reaction reagents using Foc DNA as a template and the reagents was discussed in material and methods section. The optimal time and temperature of LAMP reaction for Focwere determined. The LAMP reactions were assayed in a range of time points from 15 to 90 min at 60°C. The amplification was observed for the all-time points, but the best amplification was obtained at 60 min. Conversely, LAMP was conducted with a range of test temperatures from 57°C to 63°C for identifying the optimize temperature. The positive signals were found for allthe temperatures, but strongest sky blue signals by HNB visualization and most intense band in gel electrophoresis was found when LAMP assay was conducted at 63°C for 60 min (results not shown).

Sensitivity of the LAMP assay

The sensitivity of the LAMP assay for Foc detection was measured using fungal DNA as a template under optimized condition. Serially diluted fungal DNA from 10 µg to 1 fg were used in this assay. But it was found that only up to 10 fg the LAMP products consisted of very faint ladder like DNA fragments in gel electrophoresis. However, up to 100 fg DNA produced a detectable visually positive signal by HNB. The results specifiedthat the detection limit for Foc in LAMP assay were 10 fg DNAin gel electrophoresis and 1 pg DNA by HNB visualization (data not shown).

Evaluation of LAMP assay with diseased plant samples

The DNA of all the five Foc isolates collected from diverse geographical locations in India were tested by LAMP showed the positive reaction with a change to sky-blue colour by HNB visualization. But none of the DNA from RB isolates was showed positive signals either in gel electrophoresis or by HNB visualization.The result of LAMP assay using DNA from each fungal pathogen, Foc and RB is shown in Fig. 1. For the field evaluation of developed LAMP assay, a total of 13 plant samples were collected from different experimental chickpea fields of ICRISAT, Patancheru. Of them eight were suspected as apparently infested by Focand five were infested by RB,only in consideration of typical visual symptoms. To detect the Foc in field samples, the LAMP assay was carried out using the root DNA at optimised temperatures and times. The visually accurate detection rate of Foc was only 75%, as out of eight samples two were failed to give positive signals in LAMP assay. On the other hand, out of five field samples suspected as RB infested, two were given positive signals in Foc LAMP assay (Table 1). The results indicated that the visual diagnosis on the basis of field symptoms was always could not be accurate and insignificant.

DISCUSSION

In this study, we are first reporting LAMP mediated an easy and rapid detection method for detection of Foc from field samples. The optimal condition of LAMP assay was determined to be 63°C for 60 min. As LAMP assay conducts in isothermal condition, there is no chance to mislay in temperature with the time like normal thermal cycling PCR. The advantage of LAMP is that it requires only a heat block or regular laboratory bath which can provide a constant temperature e.g. for Foc required 63°C. In the present LAMP assay, for detection of Foc we used six Foc-*Ef1á* gene specific primers, F3, B3, FIP, BIP, LF and LB. So, there is less probabilities of false and unspecific amplification from DNA of other microbes and environmental samples. To confirm the specificity of developed LAMP assay, we used DNA extracted from RB and DRR diseased chickpea plants in the reactions. No changes in colour of LAMP reaction proved its reliability and specificity.

Another importance of LAMP is that electrophoresis is not essential for visualizing amplified products which can be easily analysed by adding hydroxynaphthol blue (HNB)dye.The change of assay colour to sky blue indicates the positive LAMP reaction which is visible in naked eye. Conversely, the LAMP assay is simple and it would be possible for those research institutes or laboratories that are unaware with PCR and other molecular methods. The easiest way of detecting positive LAMP reactions in naked eye is to examine the white turbidity within the products resulted by magnesium pyrophosphate accumulation as a by-product (Ghosh et al 2015).

The high sensitivity of LAMP reaction significantly improved the detection efficiency of Foc in present study. It was found that only 100 fgFoc DNA is needed to be present in the DNA sample for efficient diagnosis of FW in chickpea. Therefore, this LAMP assay can be used for early detection of Foc in chickpea fields for better management of FW disease. In this study, we observed that our developed LAMP assay effectively diagnosed *Fusarium* wilt in chickpea which is misinterpreted with DRR disease by considering of visual symptoms in field condition.

CONCLUSION

This is the first report of detection of *Fusarium* wilt in chickpea fields using LAMP assay. Our developed method will be very applicable for quick and extensive disease diagnosis in chickpea fields for monitoring and better management of disease complex.

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