



## Rapid detection of Tobacco streak virus (TSV) in cotton (*Gossypium hirsutum*) based on Reverse Transcription Loop Mediated Isothermal Amplification (RT-LAMP)



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### ABSTRACT

Tobacco Streak Virus (TSV) belongs to the genus *Ilarvirus* of the family *Bromoviridae* an emerging pathogen posing threat to the crop species worldwide. Identification of symptoms due to TSV infection by visual observation of plants often results in misdiagnosis as symptoms produced by this virus can match with those reflecting physiological and nutritional disorders affecting cotton. Development of diagnostic tools with rapidity will have immense role to play in detection and management of the emerging virus. The protocol for rapid diagnosis of TSV infected samples by using Reverse Transcription-Loop Mediated Isothermal Amplification (RT-LAMP) was optimised and this is the first report of its use for diagnosis of TSV on cotton and Soybean. The colorimetric detection for diagnostic simplicity of amplified RT-LAMP product by using different dyes lead to enhanced applicability of this technique. The RT-LAMP diagnostic tool can be utilized not only for laboratory research but also for quarantine and field diagnosis of this important emerging pathogen affecting cotton.

The productivity of cotton crops is mainly affected by various biotic and abiotic factors during diverse growth stages of the crop. Among a range of biotic factors, incidence of viral diseases causes serious threat to cotton crop. *Tobacco streak virus* (TSV) is an emerging and potent pathogen causing severe crop loss in cotton in recent years (Arun Kumar et al., 2008). TSV is a member of genus *Ilarvirus* (family: *Bromoviridae*) and known to be transmitted by thrips in the cotton growing zones of South and Central India (Sdoodee and Teakle, 1987; Prasada Rao et al., 2003). Incidence of TSV in India was first diagnosed in Groundnut and Sunflower affected with Peanut Stem Necrosis Disease (PSND) and Sunflower Necrosis Disease (SND) respectively (Prasada Rao et al., 2000; Ramaiah et al., 2001 and Reddy et al., 2002). TSV has a broad host range comprising of more than 200 plant species belonging to 30 different families (Fulton, 1985; Brunt et al., 1996; EPPO, 2005). In cotton, tobacco streak virus has been reported to cause a maximum of 62.7% yield loss (Rageshwari et al., 2017). Whereas the association of TSV with bud blight of soybean was earlier reported from USA (Fagbenle and Ford, 1970; Rebedeaux et al., 2004) and Brazil (Costa and Cravalho, 1961; Almeida et al., 2005) where it contributed to yield

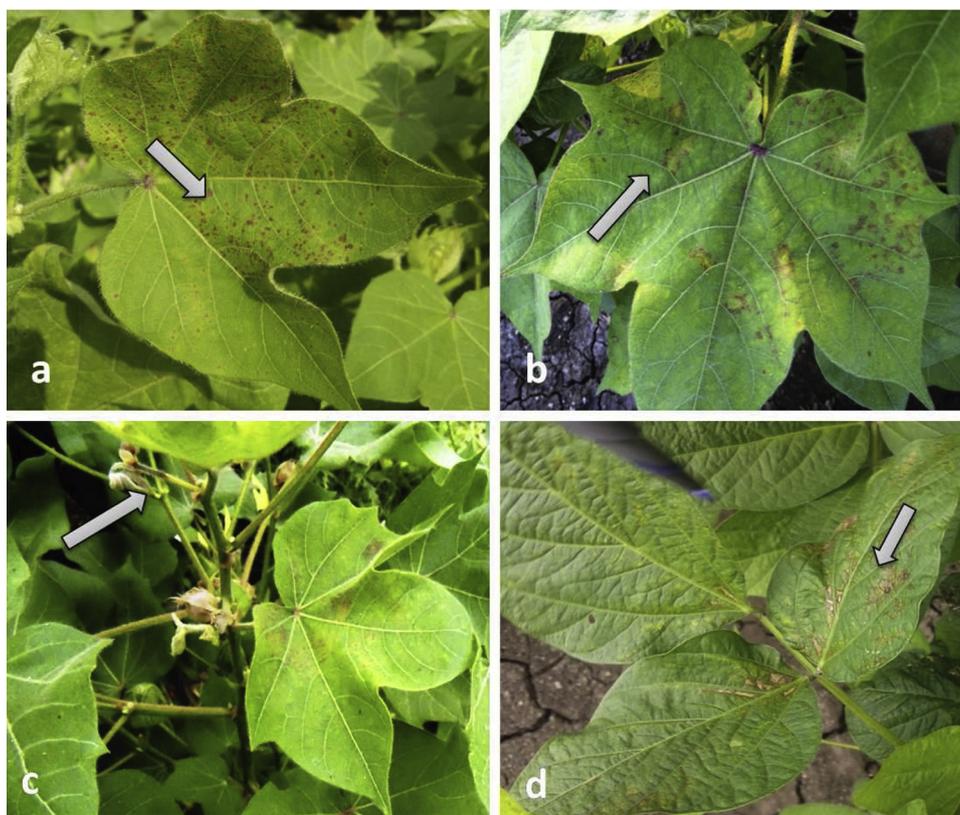
losses of 25 and 100%, respectively. Since, diagnosis of specific virus infection in plant is a challenging task, management of viral disease is becoming difficult in agriculture. Recently, advancement in biochemistry and biotechnology propelled invention of simpler and cost effective diagnostic tools for detection and management of many pest and diseases affecting crops.

The symptoms of TSV on cotton consisting of dark purple necrotic spreading lesions diffuse ring spots with numerous necrotic lesions, necrosis of bracts and square on upper surface (Fig. 1a–c), veinal necrosis, and reduction in leaf size of the infected plants which resemble the symptoms developed by physiological and nutritional disorders. Whereas the characteristic symptoms caused by the TSV in soybean are stunting of plant and necrotic patches on the leaves of growing tip of plants (Fig. 1d). Since, the diagnosis of TSV is difficult to distinguish from the physiological and nutritional maladies; there is vital need to develop complementary tool for diagnosis of this menacing TSV. Presently, the TSV prevalence is being diagnosed through serological and nucleic acid based techniques such as ELISA and RT-PCR in Cotton (Bhat et al., 2002; Arun Kumar et al., 2008, Vinod kumar et al., 2017

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**Fig. 1.** Symptoms of TSV infection on Cotton (a–c) and Soybean (d): a) Dark purple necrotic spreading lesions b) diffuse ring spots with numerous necrotic lesions c) Burning of bracts and square on upper surface d) TSV infected soybean plant showing necrotic patches on the leaves.

**Table 1**

List of Primers used in RT-LAMP and RT-PCR assay.

Oligo name	5' < —Sequence— > 3'	Number of nucleotides	Length of Product	Acce. no./Reference
<b>LAMP-Primer</b>				
TSV F3	GCGAAATGCCGCTAGAGC	18 mers	243bp	KX394690
TSVB3	CGGTA AAACTCGTCTCCGA	20 mers		
TSV FIP	TTCGCTTGAGGGCGGAAAC-AACGCGAATGCTCGAATGAC	40 mers		
TSV BIP	TCGCAAAGCGAGTGAATGGTCT-CCCCTCGACTATGGTCTTGA	42 mers		
<b>PCR Primer</b>				
TSV-CP/F-1	ATGAATACITTTGATCCAAGG	20 mers	602bp	Arun Kumar et al. (2008)
TSV-CP/R-1	TCAGTCTTGATTACCAG	18 mers		

and Rageshwari et al., 2017) and other crop plants including soybean (EPPO, 2005; Abtahi and Kuhi, 2008; Sujitha et al., 2016; Bhaskara Reddy et al., 2014; Vemana et al., 2014; Rajamanickam et al., 2016). However, in recent past, Loop-mediated isothermal amplification (LAMP) assays emerged as useful and robust technique in the field of diagnostics because of its inexpensive and rapidity (Nagamine et al., 2001; Notomi et al., 2000). LAMP and reverse transcription LAMP (RT-LAMP) have been used as a method of choice to detect wide range of plant viruses (Banerjee et al., 2016; Bhat et al., 2013; Li et al., 2013; Jeong et al., 2015). However, the RT-LAMP based detection of TSV infection in crop species is not yet reported. The aim of the present study was to develop robust RT LAMP assay for rapid diagnosis of TSV infection in cotton.

To optimize the RT-LAMP protocol, infected and healthy leaf samples of Cotton (*Gossypium hirsutum*) crop at 90 DAS and soybean were collected from experimental fields of ICAR-CICR, Nagpur during Kharif 2015–16. To validate the standardised RT-LAMP protocol, leaf samples of cotton showing symptoms of TSV infection were collected from different locations of six districts of Vidarbha and Khandesh region of

Maharashtra State during Kharif 2016-17 (Supplementary Table 1), quickly frozen in liquid nitrogen and stored at -80 °C till further use. Total RNA was purified using Spectrum Plant RNA isolation Kit (Sigma Aldrich) as per the manufacturer's instructions. One µg of total RNA was reverse transcribed (RT) to cDNA using Super Script RIII First Strand Synthesis Kit (Life technologies). To design LAMP primers, nucleotide sequences coding for TSV coat protein reported on different host species such as cotton, soybean and other plant species were retrieved from NCBI Gene bank, subjected for multiple alignment and homology search to identify the conserved region (Supplementary Table 2). LAMP primers were designed for identified conserved region using Primer ExplorerV4 software (<http://primerexplorer.jp/e/>) and custom oligos were synthesised from Integrated DNA technologies (Coralville, Iowa, USA). The primer sequence details and schematic representations of position of primers on target sequences are depicted in Table 1 and Fig. 2. The temperature and time essential for RT-LAMP assay was optimized at 65 °C isothermal conditions for 60 min. The test samples of cotton and soybean were subjected for the assay with final volume of 25 µl reaction comprising 2.5 µl 10x Thermopol buffer (1 µl

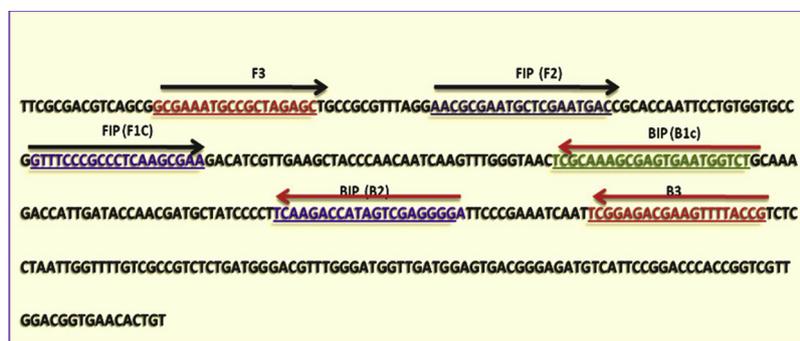


Fig. 2. Graphical representation of position of target sequences (Primers) on conserved region of Tobacco streak virus selected by multiple sequence alignment.

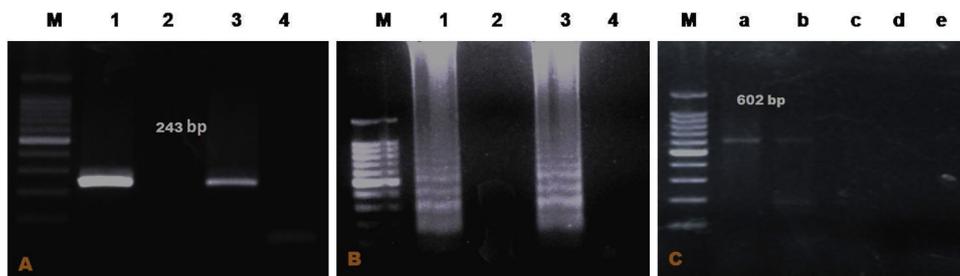
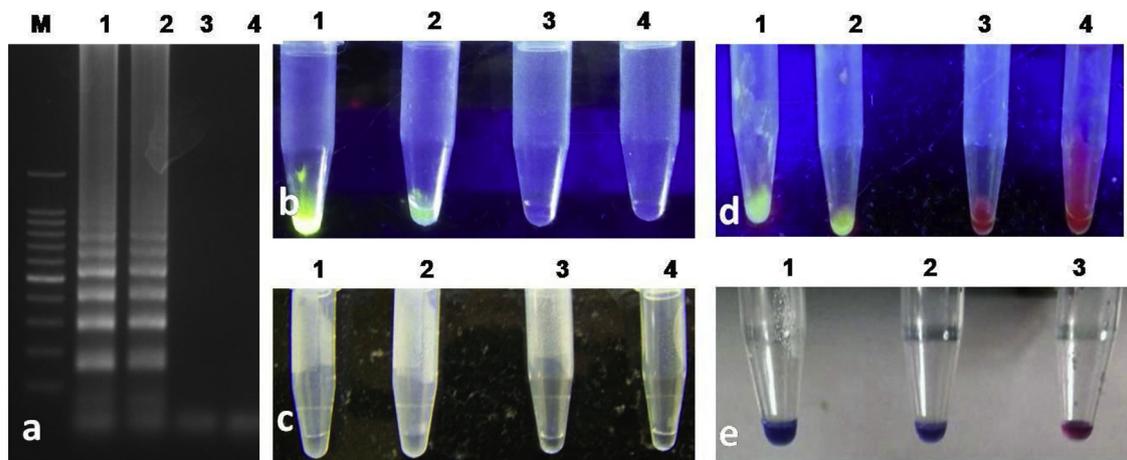


Fig. 3. A) TSV specific amplification using F3 and B3 of LAMP primers (243 bp amplicon size) B) Detection of TSV infection by using RT-LAMP. C) TSV specific amplification using Coat protein gene specific primers (602 bp amplicon size) Lane M 100 bp DNA Ladder 1 = TSV infected cotton sample, 2 = Uninfected healthy cotton sample, 3 = TSV infected soybean sample, 4 = Uninfected healthy Soybean sample, a = TSV infected cotton sample, b = TSV infected soybean sample, c = Uninfected healthy cotton sample, d = Uninfected healthy soybean sample, e = No template control.

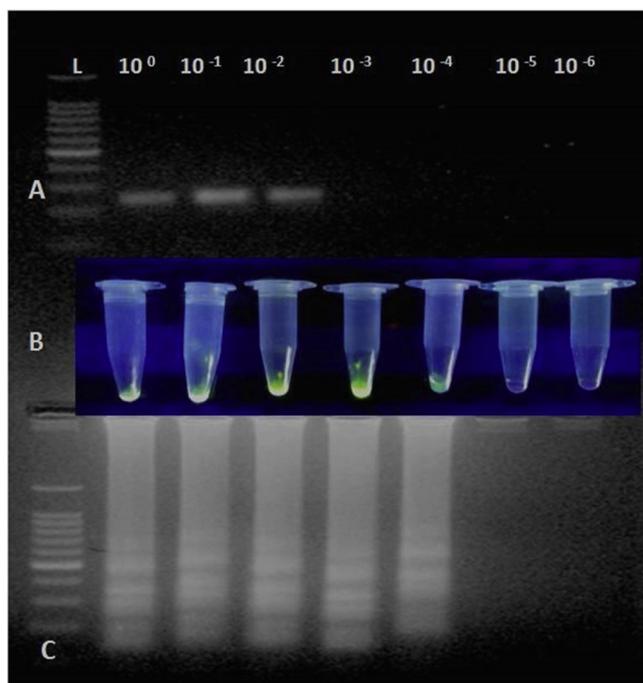
of *Bst* DNA polymerase large fragments 8 U/ $\mu$ l (8000 U/ml, New England Biolab), 0.4  $\mu$ l of dNTPs (10 mM), 2  $\mu$ l each of 0.8 mM TSV-FIP and TSV-BIP primers (HPLC purified), 0.5  $\mu$ l each of TSV-F3 and TSV-B3 primers (0.8 mM), 3  $\mu$ l (1 mM) of 5 M Betaine (Sigma Aldrich) 1.5  $\mu$ l of 8 mM MgSO<sub>4</sub>, 1  $\mu$ l of cDNA template and 10.6  $\mu$ l RNase/DNase free ddH<sub>2</sub>O supplement. The reaction tubes were incubated in water bath at optimised temperature (65 °C) for 60 min followed by heat inactivation at 80 °C for 5 min for termination of assay. The amplified RT-LAMP product was separated and visualised on 1.5% agarose gel (Fig. 3B). Colorimetric detection of the amplified product was executed by addition of 1  $\mu$ l DNA binding dyes such as SYBR® Safe DNA gel stain (1000x) and ethidium bromide (1 mg/ml) per each reaction and visualised under UV transilluminator. Hydroxyl Naphthol Blue (HNB) based detection with 1  $\mu$ l of HNB (120 mM) added prior to incubation followed by visual observation was also done. To validate and confirm the results of RT-LAMP assay, conventional RT-PCR reaction (Initial denaturation-94 °C for 2 min followed by 30 cycle of 94 °C for 30 s, 48 °C for 1 min and 72 °C for 1 min with final extension of 72 °C for 5 min) was carried out by using specific primers for coat protein gene sequences of TSV from published literature (Arun kumar et al., 2008) and F3 and B3 primers (Table 1) designed for present study. To compare the difference of sensitivity between RT-LAMP and RT-PCR, cDNA which was synthesized from total RNA extracted from TSV infected cotton leaves were serially diluted 10-fold from 10<sup>0</sup> to 10<sup>-6</sup> ng and used as template in LAMP and PCR reactions. The reaction volume was 25  $\mu$ l and they were carried out at 65 °C for 60 min; heating at 80 °C for 5 min to terminate the reaction. 10  $\mu$ l of the reaction products from LAMP and PCR were electrophoresed on 1.5% agarose gel in TBE stained with ethidium bromide.

Amplified product obtained by performing RT-LAMP assay at optimised reaction conditions produced typical ladder like appearance or banding pattern on agarose gel confirms the presence of TSV in infected samples. Specificity of the RT-LAMP reaction is revealed by no amplification in the healthy sample, no template and blank controls (Fig. 3B). Appearance of turbidity in the reaction tube of positive samples during amplification process (approximately after 30 min) implied the preliminary affirmation about possible results. However, changes in the

colour of reaction to sky blue in positive and violet in negative samples by addition of HNB dye confirms the presence of virus. Whereas, change from orange to green fluorescence and yellowish fluorescence after addition of SYBR Safe DNA gel stain and ethidium bromide respectively under transilluminator warrant the TSV presence. Though all the colorimetric methods provided the efficient detection, HNB and SYBR Safe® DNA gel stain dye (Fig. 4) may be preferred as method of choice as compared to ethidium bromide based on the safety, cost and sensitivity features of the former dyes (Goto et al., 2010 and Almasi et al., 2013). Our experimental results clearly showed the standardised RT-LAMP protocol coupled with different colorimetric assay system (Fig. 4). The results obtained with optimised RT-LAMP assay were further confirmed by conventional RT-PCR. The resultant RT-PCR reaction produced a desired amplicon size of 602 bp and 243 bp using TSV-CP-F1/R1 and F3 and B3 respectively, corroborate the results of standardised RT-LAMP protocol (Fig. 3A & C). To validate, total 31 leaf samples of cotton were tested, out of which 27 showed the presence of TSV titre RT-LAMP assay (Supplementary Table 1 and Supplementary Fig. 1). In the present investigation, we have also compared the RT-PCR with RT-LAMP for assessment of rapidity and sensitivity. Although the sensitivity of the LAMP assay and the RT-PCR assay was comparable at high virus titre, whereas the LAMP assay was by a 10-fold dilution factor found more sensitive than the PCR assay for the detection of TSV infection (Fig. 5). RT-LAMP was found to be more rapid, sensitive and less time consuming than the RT-PCR in congruence with the results reported elsewhere (Bhat et al., 2013; Duan et al., 2014; Helen and Gerhard, 2013; Siljo and Bhat, 2014; Li et al., 2015; Sasi et al., 2015). In fact, this is the first report of use of RT-LAMP tool for rapid diagnosis of TSV on cotton and Soybean. Further, the colorimetric detection for diagnostic simplicity of amplified RT-LAMP product by using different dyes lead to enhanced applicability of this technique in field of diagnostics. Colorimetric assay support the application of RT-LAMP as capable diagnostic tool for epidemiological studies of TSV especially in laboratories having less amenities including the unavailability of sophisticated equipments. Salient features of RT LAMP in terms of requirement of minor equipments, less time, more options for visualisation offer more economical advantage over RT-PCR. These limitations



**Fig. 4.** Colorimetric detection of TSV RT-LAMP reaction of Cotton and Soybean: (a) 1.5% agarose gel electrophoresis (b) SYBR Safe DNA gel stain under UV transilluminator. (c) Turbidity of the reaction mixture. (d) Ethidium bromide (0.5 ug) visualization under UV transilluminator. (e) Hydroxy naphthol blue dye 150Um- 1ul. M = GeneRuler™ 100 bp Plus DNA Ladder, 1 = cDNA template from TSV infected soybean leaf sample. 2 = cDNA template from TSV infected Cotton leaf sample. 3 = cDNA template from uninfected soybean leaf sample. 4 = cDNA template from uninfected Cotton leaf sample. Waterbath temperature 65 °C for 60 min. Terminated the reaction 80 °C for 10 min.



**Fig. 5.** Visual assessment of sensitivity of LAMP assay :A) Result of conventional PCR using F3 and B3 of LAMP primers (243bp amplicon). B) Colorimetric detection by using SYBR Safe DNA gel stain. (C) LAMP assay on the basis of 1.5% agarose gel electrophoresis L = GeneRuler™ 100 bp Plus DNA Ladder; lane  $10^0$ – $10^{-6}$  indicated the 10 fold serially diluted cDNA concentrations in LAMP reaction. Waterbath temperature 65 °C for 60 min followed by terminate the reaction 80 °C for 10 min.

in current PCR-based techniques have spurred the development of RT-LAMP protocol based on isothermal amplification of target nucleic acids. This advance diagnostic protocol can be effectively utilized in laboratory research of quarantine matters and field diagnosis of TSV, an important emerging pathogen of cotton, soybean and other crop species. Also the rapid diagnosis of TSV infection even at low titre of viruses would mean reliable diagnosis of diseases and timely conveyance of pest management inputs, which show the way to reduced plant protection costs and misuse of pesticides.

#### Conflict of interest

The author of this study has no conflict of interest.

#### Ethical approval

This article does not contain any studies with human participants or animals.

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#### Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.jviromet.2019.04.018>.

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