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Fast detection of *Southern tomato virus* by one-step transcription loop-mediated isothermal amplification (RT-LAMP)

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Abstract

Southern tomato virus (STV) is a double stranded RNA (dsRNA) virus belonging to genus *Amalgavirus* (family Amalgamaviridae*)* which has been detected in tomato plants showing stunting, fruit discoloration and size reduction. A one step reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay was developed for detection of. STV in total RNA or sap extracts (obtained just by grinding in buffer) from STV-infected tomato plants by using a set of three pair of primers designed in basis of the STV gene encoding the putative coat protein. Amplification products were visualized by gel electrophoresis or direct staining of DNA. The sensitivity of RT-LAMP was identical to that of the conventional RT-PCR and less affected to presence of polymerase inhibitors. STV was detected by RT-LAMP in different tomato tissues, i.e. leaves, roots, fruits and seeds. Also the virus was successfully detected by RT-LAMP from sap extracts obtained from field tomato plants whereas conventional RT-PCR did not. Results of this work show that RT-LAMP plants is a specific, rapid and cheap procedure to detect STV and it could be implemented on field surveys and sanitation programs.

Southern tomato virus (STV) of genus *Amalgavirus* (family Amalgaviridae) is a double stranded RNA virus (dsRNA) of about 3.5 kb containing two open reading frames (ORFs) which are partially overlapped. The 5'-proximal ORF1 codes for the putative coat protein (CP) whereas the ORF2 contains typical motifs of an RNA-dependant RNA-polymerase (RdRp) (Sabanadzovic et al., 2009). STV was first detected in USA (Missisipi and Imperial Valley of California) and in South-Western Mexico in tomato (*Solanum lycopersicum*) plants of different varieties showing symptoms of stunting, fruit discoloration, and reduced fruit size (Sabanadzovic et al., 2009). Afterwards, STV was detected in tomato plants showing similar symptoms in Spain, Italy, France, China and Bangladesh (Candresse et al., 2015, Iacono et al., 2015, Padmanabhan et al., 2015b, Padmanabhan et al., 2015a, Verbeek et al., 2015). STV is transmitted by seed at rates higher than 70% but "horizontal" transmission by vectors is unknown (Sabanadzovic et al., 2009). In addition, the role played by STV on development of described tomato symptoms remains unclear because the virus is frequently detected on mixed infections with other viruses such as *Pepino mosaic virus* (PepMoV) and *Tomato mosaic virus* (ToMV) and sometimes in symptomless tomato plants. A specific and sensitive method for STV detection is essential to increase the knowledge on these important features and to implement sanitation programs.

In this work, we developed a fast and sensitive method for STV detection based in a one step reverse transcription loop-mediated amplification under isothermal conditions (RT-LAMP) (Mori and Notomi, 2009). This method has been reported to be at least as sensitive as the conventional RT-PCR and has the advantage to avoid the use of expensive thermal cycling instruments since the retrotranscription and nucleic acid amplification is performed under isothermal conditions (Soliman and El-Matbouli, 2006). In addition, RT-LAMP has been reported to be less sensitive to inhibition of nucleic acid polymerases than conventional

RT-PCR and therefore less prone to false negatives (Boonham et al., 2004, Francois et al., 2011). RT-LAMP is gaining popularity in human medicine (Parida et al., 2008) and more recently on detection of different plant pathogens (Tomlinson et al., 2010, Bühlmann et al., 2013) due to its speed, robustness and simplicity.

Fresh tissue of leaf, root and fruit (200 mg) or seeds (pooling five seeds per sample) from virus-infected or non- infected tomato plants was grinded in a power homogenizer TissueLyser (Qiagen, Hilden, Germany) with liquid nitrogen. Sap extracts were prepared by resuspension in 500 µl of STE (100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA pH 7.5) and homogenization with a vortex mixer for 2 min. Total RNAs were extracted by using a standard protocol based on phenol:chloroform:isoamyl alcohol extraction followed by ethanol precipitation (Ferriol et al., 2011). STV specific primers were designed in basis of complete nucleotides sequences of STV isolates BD-13 from Bangladesh (KT634055), NC12-03-08 from California (USA) (KT852573), Mexico-1 from Mexico (EF442780) and MS-7 from Mississippi (USA) (EU413670) which were retrieved from GenBank and aligned by using the Clustal W program (Larkin et al., 2007) to identify conserved STV genomic regions. Nucleotide identities between these STV isolates were very high, ranging from 99.65 to 99.88 %. The CP region was chosen as the amplification target. A set of three STV-specific primer pairs was designed by using the LAMP Designer 1.12 sofware (Premier Biosoft,, Palo Alto, CA): the forward outer F3-CP (5′-GAGACTCAGAGGAATGACATG-3′), reverse outer B3-CP (5′-CCTCCTTGACTTGCTTCC-3′), forward inner FIP-CP (5′-TTGGTTGCGATCAAGTCCTCCGAGGAGGAGATCGCTGAT-3′), reverse inner BIP-CP (5′-GAGCCTGTTAGCCGGTATGTGGGGTTGAACTGCTGGTAAA-3′), loop forward LoopF-CP (5-CGCTTGACAATCTTACGCTG′-3), and loop reverse LoopR-CP (5′-TGAGTGACTACGAGTTGAACTG -3′). The RT-LAMP reaction was performed by adding

1,5 µl of sample previously denatured at 95°C for 5 min in a eppendorf tube to a final reaction volume of 25 µl containing 1x Isotermal Amplification Buffer (20 mM Tris-HCl, 10 mM (NH_4) SO_4, 50 mM KCl, 2 mM $MgSO_4$, 0.1% Tween 20), 1.4 mM dNTPS, 5 U WarmStar RT (New England Biolabs, Ipswich, MA), 8 U Bst DNA polymerase (New England Biolabs) and the three STV specific primer pairs (0.2 µM F3-CP and B3-CP, 1.6 µM FIP-CP and BIP-CP, 0.4 µM LoopF-CP and LoopB-CP). The mixture was incubated at 68ºC for 1 h in a water bath and heated at 80ºC for 10 min to stop the reaction. The amplification products obtained were analyzed by electrophoresis in 1.5% agarose gels and visualized by using Gel Red (Biotium Inc., Fremont) under UV light. To simplify the procedure, the amplification products were also visualized directly in the eppendorf tubes by adding 5µl of Gel Red in the reaction mix and observation of fluorescence under UV.

The reliability and specificity of RT-LAMP was assessed by using total RNA extracts obtained from leaf tissues of STV-, PepMV-, ToMV- infected and non-infected tomato plants. Amplification products were only observed by agarose electrophoresis analysis in samples corresponding to STV-infected tomato plants but not in those of PepMV-, ToMV- infected and non-infected plants (Fig. 1A). Also, only the eppendorf tubes of samples corresponding to STV-infected tomato plant showed fluorescence under UV light after adding the Gel Red in the reaction mix (Fig 1B).

To compare the sensitivities of RT-LAMP and conventional RT-PCR, 10-fold serial dilutions from a total RNA extract obtained from leaf tissue of a STV-infected tomato plant and adjusted to the concentration of 100 ng/µl were prepared and amplified by both methods. The conventional RT-PCR was carried out as previously described (Sabanadzovic et al., 2009). The amplification products obtained with both RT-LAMP and conventional RT-PCR

were analysed and visualized by electrophoresis in agarose gels. Both detection techniques showed identical sensitivities allowing the detection of STV in the dilution 10^{-2} of total RNA extracts, equivalent to the concentration of 1 ng/µl (Fig 2). The conventional RT-PCR failed to detect STV from non-diluted total RNA extracts (100 ng/µl) whereas RT-LAMP gave a clear positive signal which supports that RT-LAMP is less sensitive to polymerase inhibitors than conventional RT-PCR (Francois et al., 2011). The sensitivity for STV detection by RT-LAMP was similar to those reported for other plant viruses such as *Potato virus Y*, *Prunus necrotic ringspot virus* and *Tomato torrado virus* (Przewodowska et al., 2015, Zong et al., 2014, Budziszewska et al., 2016).

The distribution of STV within the infected plants was analyzed by RT-LAMP of total RNA extracts obtained from STV-infected tomato leaf, root and fruit or seeds. Equivalent extracts from non-infected tomato plants were used as negative controls. Amplification products were observed by electrophoresis or fluorescence in eppendorf tubes under UV light in all STV-infected plant tissues and seeds, but not in the equivalent extracts of non-infected plants (Fig. 3). This result shows that STV is distributed in all tissues including seeds in and that RT-LAMP is sensitive enough to detect it.

To determine if RNA extraction can be avoided, sap extracts and 10-fold serial dilutions of leaf tissue of STV-infected and non- infected tomato plants were analyzed. Amplification products were observed by both electrophoresis in 1.5% agarose gels or fluorescence of reaction mix in eppendorf tubes under UV light from samples corresponding to dilutions 10^{-1} and 10^{-2} but not in those corresponding to non-diluted or dilution 10^{-3} of sap extracts from the STV-infected plant sap extract. In addition, no amplification products were observed on none of samples corresponding to non-infected tomato plants. The equivalent

131 assay was performed by conventional RT-PCR, but no amplification products were observed
132 from none of samples corresponding to non-diluted or diluted sap extracts. As we mentioned
133 above, the presence of inhibitors of nucleic acid polymerases in the low processed sap
134 extracts might be the cause of the lack of STV amplification by the conventional RT-PCR or
135 by RT-LAMP from non-diluted sap extract (Boonham et al., 2004, Francois et al., 2011).

136 The robustness of the STV detection by RT-LAMP from sap extracts (dilutions 10^{-1}
137 and 10^{-2}) was assayed by testing 50 tomato plants of the local variety ´Valenciano` collected
138 from five plots in Valencian Community and results were compared with those obtained by
139 conventional RT-PCR from both sap (dilutions 10^{-1} and 10^{-2}) and total RNA extracts of the
140 same tomato plants (data not shown). STV was detected by RT-LAMP in nine tomato plants
141 collected in two different plots and amplification products were observed by both
142 electrophoresis in agarose gels and observation of fluorescence of the reaction mix in
143 eppendorf tubes under UV light. Conventional RT-PCR from total RNA extracts showed an
144 amplification product of expected size in the same nine tomato plants, but failed to amplify
145 the virus in all sap extracts. These results show that RT-LAMP by using the low processed
146 sap extracts is a useful method to detect STV from field tomato samples, avoiding the costly
147 and time consuming procedures of viral RNA extraction.

148 In conclusion, the RT-LAMP assay developed in this study is a specific, sensitive,
149 rapid and cost-effective method for STV detection and could be implemented for
150 epidemiological studies on field surveys, as well as sanitation programs of governmental
151 institutions, seed companies and plant suppliers.

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Figures

Figure 1. Aanalysis of amplification products by electrophoresis in a 1.5% agarose gel (Panel A) or by fluorescence visualization under UV light after adding Gel Red in reaction mix tubes (Panel B) obtained by RT-LAMP from total RNA extracts of leaf tissues of STV-infected tomato plants (lanes 1-3), PepMV-, ToMV- and non-infected tomato plants (lanes 4 to 6, respectively). Lane M corresponds to 1Kb Plus DNA molecular weight marker (Thermo Fisher Scientific, Waltham, MA). The order of the samples is the same in both panels.

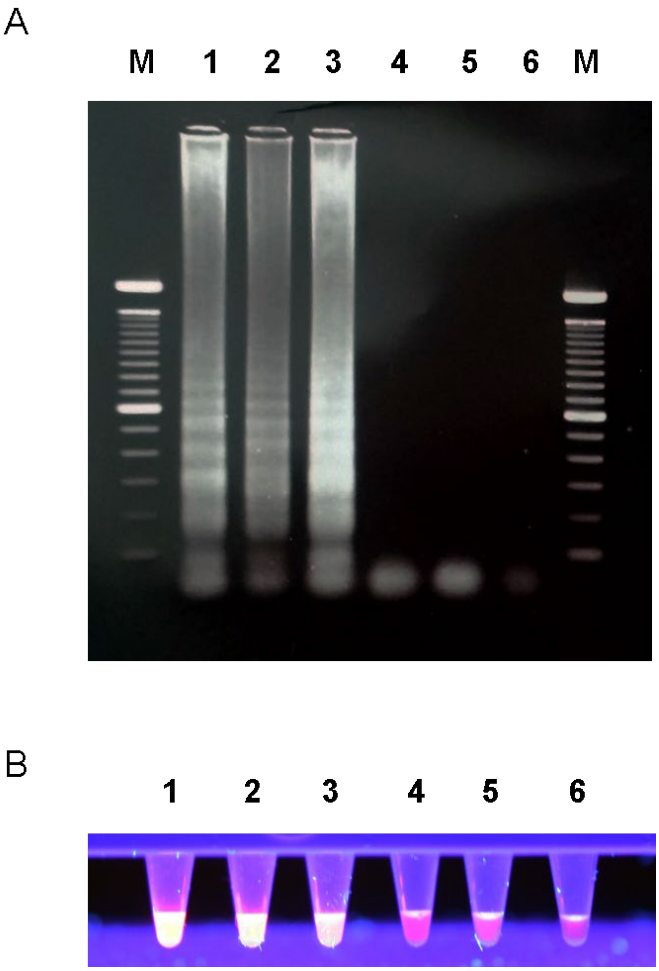
Figure 2. Electrophoresis in 1.5% agarose gels of amplification products obtained by RT-AMP (Panel A) and conventional RT-PCR (Panel B) from a RNA total extract (100 ng/ µl) (lane 1) and 10-fol serial dilutions (lanes 2-6) of leaf tissue of a STV-infected tomato plant. Lane M corresponds to 1Kb Plus DNA molecular weight marker.

Figure 3. Analysis of amplification products by electrophoresis in 1.5% agarose gel (Panel A) or by fluorescence visualization under UV light after adding Gel Red in reaction mix tubes (Panel B) obtained by RT-LAMP from total RNA extracts of seed, leaf, fruit and root tissues of a STV-infected tomato plant (lanes 1 to 4, respectively) and the equivalent extracts of a non-infected tomato plant (lanes 5 to 8, respectively). Lane M corresponds to 1Kb Plus DNA molecular weight marker.

Figure 4 Electrophoresis in 1.5% agarose gels of amplification products obtained by RT-LAMP (Panel A) and conventional RT-PCR (Panel B) from sap extracts and 10-fol serial dilutions of a STV-infected tomato plant (lanes 1, 3, 5 and 7, respectively) and of a non-

infected tomato plant (lanes 2, 4, 6 and 8, respectively). Lane M corresponds to 1Kb Plus DNA molecular weight marker. At the bottom of panel A we show the fluorescence visualization under UV light after adding Gel Red in reaction mix tubes of samples corresponding to RT-LAMP amplification.

Fig. 1



278 **Fig. 2**

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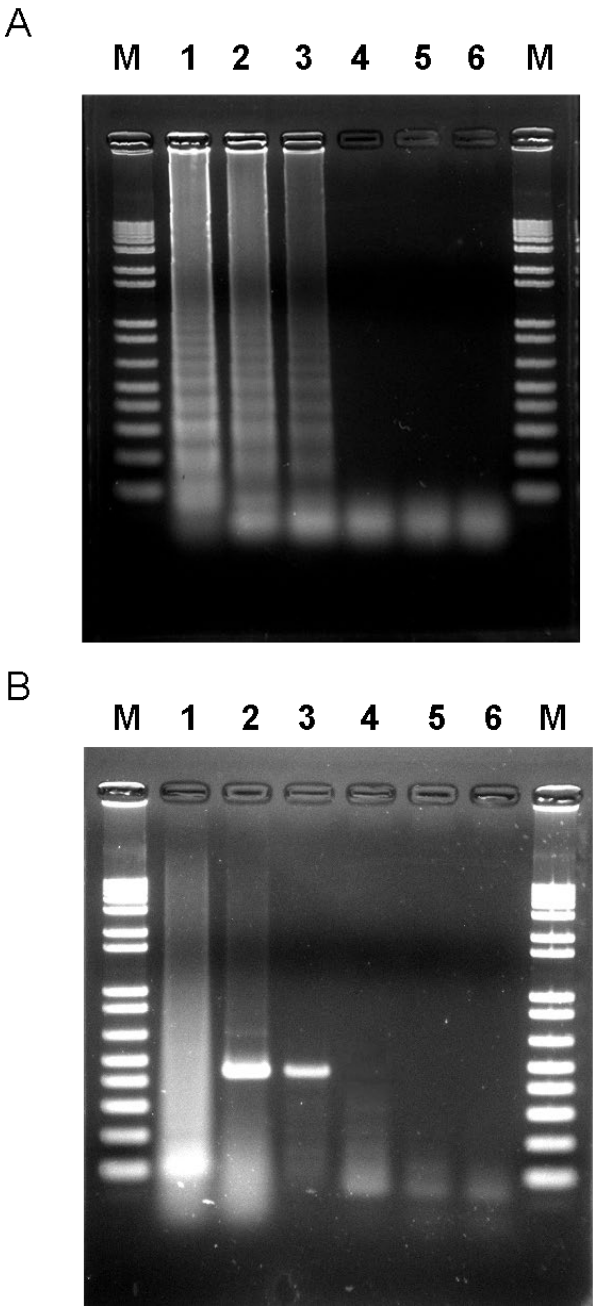

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285 **Fig. 3**

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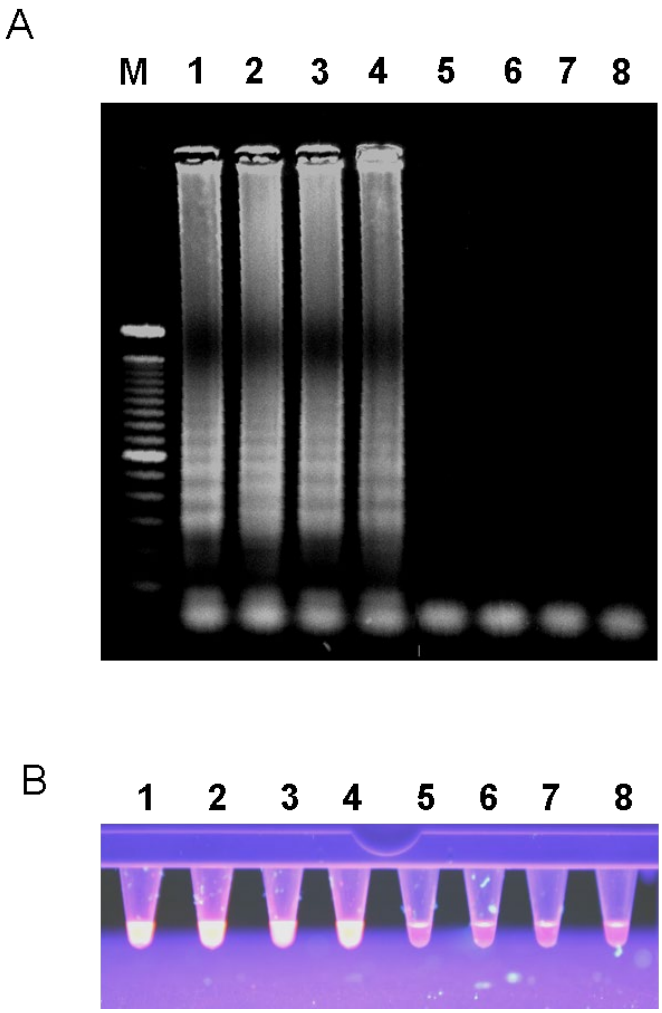

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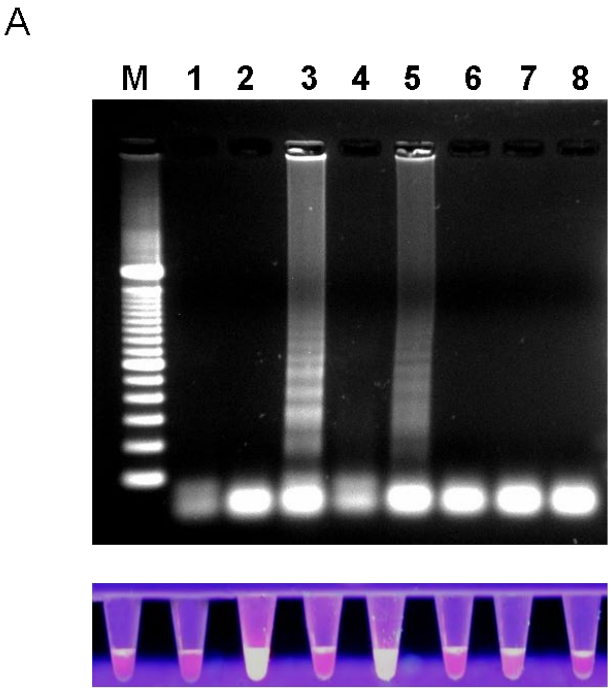
Fig. 4



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