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Efficient serological and molecular methods for the detection of tomato spotted wilt virus

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Abstract

Background: Tomato spotted wilt virus (TSWV) is a globally distributed orthotospovirus that threatens tomato production, causing serious yield and quality losses. Reliable diagnostics are crucial for timely detection, resistance breeding, and phytosanitary control.
Aims: In this study, we evaluated several serological and molecular methods for detecting TSWV in tomato plants and seeds.
Methods: First, we propagated and isolated TSWV from *N. glutinosa* plants for use as a positive control in subsequent experiments. Next, we performed the following assays: tissue blot immunoassay (TBIA), dot blot (DB), conventional PCR, one-enzyme RTX-PCR, immuno-capture RT-LAMP (IC-RT-LAMP), and recombinase polymerase amplification (RPA).
Results: The TSWV yield was approximately 2 mg per 100 g infected tissue, and maintained strong reactivity with monoclonal antibodies even after 100-fold dilution. Among serological assays, TBIA and DB were reliable for routine screening, with TBIA particularly suited to large-scale applications. PCR and RTX-PCR confirmed infections with high specificity, although infection rates in inoculated tomato plants remained relatively low (~20%). IC-RT-LAMP demonstrated high sensitivity and rapid visual detection, while RPA enabled positive identification within 25 min at a constant temperature, making it highly attractive for field use.
Conclusions: The integration of rapid immunoassays with sensitive molecular methods provides an efficient, cost-effective workflow for TSWV surveillance, supporting resistance breeding and management of this major pathogen.

Keywords: dot blot, immune capture-reverse transcription PCR, one-enzyme reverse transcription-PCR, recombinase polymerase amplification, tissue blot immunoassay, tomato spotted wilt virus.

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Abbreviations: DB - dot blot; dpi - day post inoculation; IC - immune capture; LAMP - loop-mediated isothermal amplification; LFA - lateral flow assay; one-enzyme RTX-PCR - PCR using RTX enzyme for one enzyme RT-PCR; RPA - recombinase polymerase amplification; RT - reverse transcription; TBIA - tissue blot immunoassay; TSWV - tomato spotted wilt virus.
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Introduction

Tomato spotted wilt virus (TSWV; species *Orthotospovirus tomatomaculae*, family Tospoviridae) (Kuhn et al., 2023), is a negative strand RNA-virus containing envelope structures, which makes it unique among plant viruses (de Haan et al., 1991). TSWV ranks among the most destructive plant viruses worldwide. First described in Australia in 1919, TSWV has since attained a global distribution, infecting over 1 000 plant species across more than 85 families, including key agricultural crops such as tomato (*Solanum lycopersicum*), pepper (*Capsicum annuum*), groundnut (*Arachis hypogaea*), and various ornamentals (Parrella et al., 2003; Pappu et al., 2009). Infected plants typically exhibit chlorotic or necrotic spots, wilting, stunted growth, and in severe cases, complete crop failure, resulting in considerable economic losses, particularly in *Solanaceous* and *Asteraceous* crops (Roselló et al., 1996; Latham and Jones, 1998).

TSWV is transmitted in a circulative-propagative manner by several thrip species, primarily *Frankliniella occidentalis*, *Thrips tabaci*, and *Scirtothrips dorsalis*. The virus is acquired during the larval stages when thrips feed on infected plant tissues and is transmitted persistently following viral replication within the insect (Nagata et al., 2002; Ullman et al., 2005; Montero-Astúa et al., 2016). Its extensive host range, persistent vector transmission, and ability to overcome resistance through genetic reassortment and mutation intensify its impact in both open-field and protected cultivation systems (Boiteux and de Ávila, 1994; Adkins, 2000).

Accurate and timely diagnosis of TSWV is critical for outbreak management, limiting disease spread, and selecting resistant genotypes in breeding programs. Current diagnostic approaches include both serological and molecular methods. Moreover, targeting for the early stage plant disease detection applications, a new hyperspectral non-destructive method has been developed as well as the use of a hand-held Raman spectrometer for detection of early stages of the viral infection (Wang et al., 2019; Juárez et al., 2024; Orecchio et al., 2025). Serological assays, especially the double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA), are widely used for high-throughput screening due to their affordability, simplicity, and rapid turnaround (Clark and Adams, 1977). However, DAS-ELISA can be less sensitive during early infection stages or in samples with low viral titres and may yield false negatives depending on tissue type and sampling time (Dijkstra and de Jager, 1998). Nevertheless, two other methods based on the immunodetection can be easily used – Tissue blot immunoassay (TBIA), which transfers the plant sap onto nitrocellulose membrane by simply pressing the plant tissue (Filardo et al., 2022), and dot blot (DB) successfully used for liquid samples which are pipetted simply onto nitrocellulose membrane. The subsequent immunodetection is performed using specific antibodies, secondary antibodies conjugated with the alkaline phosphatase and the final visualisation using the substrate of alkaline phosphatase (Hsu, 2009).

To address the limitations of serological methods, molecular diagnostics such as reverse transcription polymerase chain reaction (RT-PCR) and reverse transcription quantitative PCR (RT-qPCR) have become the gold standard owing to their superior sensitivity and specificity (Mumford et al., 1994; Roberts et al., 2000). RT-PCR assays typically target conserved regions of the nucleocapsid or movement protein genes, allowing for the detection of latent or asymptomatic infections. TaqMan-based RT-qPCR further facilitates absolute quantification of viral load in both plant tissues and insect vectors (Debreczeni et al., 2011). The immuno-capture RT-PCR (IC-RT-PCR) combines the specificity of the antigen-antibody bond with the sensitivity of an amplification method (Mulholland, 2009).

Emerging isothermal amplification methods, such as loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA) or RT-RPA, offer promising alternatives for rapid, field-based detection. LAMP assays targeting TSWV have demonstrated high sensitivity with results obtainable within 30 min, without the need for complex equipment (Notomi et al., 2000; Piepenburg et al., 2006; Lee et al., 2021; Iturralde Martinez and Rosa, 2023). Similarly, CRISPR/Cas-based diagnostic platforms are gaining attention for their ultra-sensitive and specific detection capabilities (Mahas et al., 2021; Zhang et al., 2021; Jaybhaye et al., 2024).

Despite the high accuracy of molecular techniques, they often require specialised laboratory infrastructure and trained personnel, limiting their use in large-scale or in-field settings. Therefore, an integrated diagnostic strategy is increasingly recommended, combining initial high-throughput screening using immunodetection with confirmatory molecular assays for critical or ambiguous samples. This dual approach enhances diagnostic reliability, supports resistance breeding programs, and strengthens phytosanitary surveillance systems.

Given the significant global economic burden imposed by TSWV and the need for robust early detection, this study presents a comprehensive evaluation of serological (TBIA, DB) and molecular (PCR, RTX-PCR, IC-RT-PCR, RPA) diagnostic methods for the detection of TSWV in tomato. We assess and compare their performance, sensitivity, specificity, and practical applicability to inform the development of standardised diagnostic protocols for both research and crop protection initiatives.

Materials and methods

Virus source: The commercially available TSWV isolate was purchased from the German Collection of Microorganisms and Cell Cultures (*DSMZ, Leibniz Institute*, Science Campus Braunschweig-Süd, Germany), as a lyophilised sample of *Nicotiana tabacum* leaves (catalog number: PV – 1265; *GenBank*: ON924228 - ON924230).

Plant material: For primary virus propagation, *Nicotiana glutinosa* L. plants were used. To prepare host material

for virus isolation, commercially available tomato plants (*Solanum lycopersicum* L.) cv. Start were employed. For the evaluation of molecular methods, commercially available tomato plants cv. Gallant were used. All experiments were repeated at least three times, results from one of these experiments are presented here.

In order to develop a cost-effective method for diagnosing TSWV in commercially grown tomatoes on a large scale, we collaborated with Hanácká zemědělská společnost Jevíčko a.s. (HZSJ), an agricultural company based in Jevíčko, Moravia, Czech Republic. The collaboration focused on two tomato cultivars, Plumola and Lucioso, which are commercially grown in hydroponic greenhouses in Chornice. Moreover, for the survey of TSWV incidence, we analysed 148 tomato plant samples collected between years 2023 and 2025. These samples represented a wide range of cultivars (Balkonové, Bejbíno, Blumko, Dafne, Delikano, Denár, Gallant, Hroznové, Jergus, Mandat, Merrygold, Nagina, Odat, Orbit, Pálava, Pedro, Perun, Rose Crush, Rose Lady, Rubinka, Start, Tipo, Tornádo, Toro, Valdo), obtained from private gardens and field plots across different locations in the Czech Republic.

For the purposes of early virus management, we focused on testing seeds harvested from infected, PCR-positive, and symptomatic tomato plants. Seeds were extracted from ripened tomato fruits and immersed in water for 12 h at room temperature with continuous stirring. Residual fruit pulp was then removed by gently rubbing the seeds against a metal kitchen mesh. The seeds were subsequently placed on filter paper and allowed to dry thoroughly at room temperature for approximately one week. One batch of seeds was processed by DB analysis (*see* below), while the other batch was sown in soil and maintained in a cultivation room under controlled conditions, as described above, for subsequent testing of the seedlings.

TSWV propagation: *N. glutinosa* plants were cultivated in a growth chamber under controlled conditions (20°C, 16-h day/8-h night photoperiod) and mechanically inoculated at the four-leaf stage. Two hours prior to inoculation, the plants were placed in the dark. Lyophilised, commercially purchased leaves containing TSWV were soaked for 1 h in 10 mM potassium phosphate buffer (pH 7.0) at a 1:200 (mass:volume) ratio, and subsequently homogenised using a mortar and pestle. The resulting sap was inoculated onto 2 leaves per *N. glutinosa* plant by rubbing with carborundum powder (400 - 500 mesh). Following inoculation, the plants were kept in the dark until the next day, after which they were returned to the growth chamber under the controlled conditions described above. Symptoms were monitored regularly until they progressed to necrosis. At this stage, all symptomatic leaves were tested for the presence of TSWV, collected, and stored at –20°C for subsequent use.

Tomato plants at the four-leaf stage (cvs. Start and Gallant) were inoculated in the same manner as *N. glutinosa*. The inoculum was prepared from symptomatic *N. glutinosa* leaves that had tested positive by one-enzyme RTX-PCR. These leaves were stored at –20°C and then homogenised in 10 mM potassium phosphate buffer (pH 7.0) at a 1:10 (mass:volume) ratio.

TSWV isolation: TSWV isolation was performed from systemic leaves of tomato cv. Start, collected on the 21st day post-inoculation (dpi), following Kikkert et al. (1997), with major modifications. Briefly, the leaves were harvested and crushed in a blender with extraction buffer (0.1 M phosphate buffer, pH 7.0; 0.01 M Na_2SO_3) at a 1:5 (mass:volume) ratio, and the homogenate was filtered through cheesecloth. The resulting juice was centrifuged at 10 000 × g for 15 min at 4°C. The pellet was resuspended in 0.01 M Na_2SO_3 to a volume equal to one-fifth of the original extraction buffer and stirred for 30 min. Following centrifugation (8 000 × *g* for 15 min at 4°C), the supernatant was ultracentrifuged in a *50.2 Ti* rotor (*Beckman Coulter*, Brea, CA, USA) at 27 000 rpm for 1 h at 4°C. The resulting pellet was then dissolved by stirring overnight in 0.01 M Na_2SO_3, at a volume corresponding to one-tenth of the original extraction buffer.

The homogenate was subjected to a second ultra-centrifugation in a *50.2 Ti* rotor at 27 000 rpm for 2.5 h at 4°C, after which the resulting pellet was resuspended in the extraction buffer to a volume equal to 1/200 of the original extraction buffer and stirred for 1 h. Finally, the homogenate was centrifuged at 10 000 × *g* for 10 min at 4°C, the supernatant collected, and its spectrum measured using a *NanoDrop* spectrophotometer (*Thermo Scientific*, Wilmington, DE, USA). The entire procedure was performed at low temperature, either on ice or at 4°C.

The concentration of the isolated TSWV was estimated by comparing the intensity of bands in TSWV serial dilutions with those of known concentrations of isolated potato virus X (property of the Laboratory of Virology, IEB) on SDS-PAGE gels stained with *Coomassie Brilliant Blue R-250* (*CBB R-250*; *see* below). Despite the structural and molecular differences between TSWV and PVX, the effects of sample preparation, especially the presence of the denaturing agent SDS and heat treatment, allow for such a comparison. The immunoreactivity of the isolated TSWV and its tenfold serial dilutions was tested using DB analysis followed by the immunodetection with a specific monoclonal antibody (*see* below).

RNA isolation and cDNA preparation: Total RNA was isolated from approximately 100 µg of plant material using *RiboEx™ LS* (*GeneAll® Biotechnology*, Seoul, Korea), according to the manufacturer's instructions. Briefly, around 100 mg of ground plant tissue was mixed with 0.75 mL of *RiboEx™ LS* and vortexed for 5 min at room temperature. After adding 0.5 mL of chloroform, the mixture was vortexed for 2 min at room temperature. Following centrifugation at 12 000 × *g* for 15 min at 4°C, the upper aqueous phase was transferred to a fresh tube. An equal volume of isopropanol was then added, gently mixed, and left to precipitate at room temperature for 10 min.

After centrifugation at 12 000 × *g* for 10 min at 4°C, the supernatant was discarded and the pellet washed with 0.5 mL of 75% ethanol. Centrifugation was repeated at

7 500 × *g* for 10 min at 4°C, and the pellet was washed a second time as described above. After removing any residual ethanol, the pellet was left to air-dry at room temperature for 5 min. Finally, the RNA was resuspended in 40 µL of *DEPC*-treated water, and its concentration, spectrum, and A_{260}/A_{280} ratio were measured using a *NanoDrop* spectrophotometer (*Thermo Scientific*, Wilmington, DE, USA).

Prior to RT, the RNA concentration was adjusted to 500 - 1 000 ng/µL. cDNA synthesis was performed using the *RevertAid First Strand cDNA Synthesis Kit* with random hexamers (*Thermo Scientific*™, Wilmington, DE, USA), following the manufacturer's instructions. Primer annealing was carried out for 5 min at 65°C, and RT proceeded at 25°C for 5 min followed by 42°C for 1 h.

Primer design: To ensure that the designed primers were suitable for detecting a broad range of TSWV variants, we performed an *in-silico* analysis using the *NCBI BLAST* tool. All primers were compared with numerous TSWV isolates originating from different plant hosts and collected from various regions around the world, covering distinct phylogenetic clades. The *BLAST* analysis showed that the primers share high sequence similarity with these global isolates, suggesting strong detection versatility. Moreover, alignment of the primer binding regions revealed complete nucleotide conservation across all analysed sequences, supporting their reliable use for TSWV detection in diverse field samples.

PCR: For PCR detection, colourless *DreamTaq*™ *Hot Start PCR Master Mix* (*Thermo Fisher Scientific*, Waltham, MA, USA) was used together with TSWV_F3 and TSWV_B3 primers (Table 1). The template consisted of 5× diluted cDNA, and the positive control was cDNA generated from RT using isolated TSWV as a template at a starting concentration of 40 ng/µL. The negative control comprised 5× diluted cDNA obtained from RT using isolated tomato brown rugose fruit virus (property of the Czech Agrifood Research Center) as a template at a starting concentration of 40 ng/µL, as well as a reaction in which H_2O was added instead of template.

The cycling conditions were as follows: 5 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 56°C, and 30 s at 72°C; followed by a final extension of 10 min at 72°C. PCR products were separated on a 2% agarose-TBE gel containing *MidoriGreen* stain (*NIPPON Genetics EUROPE GmbH*, Düren, Germany) and visualised using the *ChemiDoc™ MP Imaging System* (*Bio-Rad*, Hercules, CA, USA).

One-enzyme RTX-PCR: Detection of TSWV in *N. glutinosa* and tomato plants was carried out directly from plant tissue, following Hoffmeisterová et al. (2022), using a forward primer (TSWV_q1F) and a reverse primer (TSWV_q1R) at a final concentration of 0.4 µM each (Table 1) (Paul et al., 2021). The reactions were performed under the following conditions: 10 min at 68°C (corresponding to RT), immediately followed by 33 cycles of PCR (98°C for 10 s, 60°C for 20 s, and 72°C for

Table 1. Used primers. The 5' end of TSWV-rpa-F primer was labeled with a FAM fluorophore, whereas the 5' end of the TSWV-rpa-R primer was labeled with biotin.

Primer name	Product size (bp)	Primer sequence (5' to 3')	Final concentration in reaction premix (µM)			Design
			End-point PCR One-step RTX-PCR	LAMP	RPA	
TSWV_F3	330	TCAAGCCTATGGATTACCTCT		0.25		Paul et al., 2021
TSWV_B3		TCTCACTGTAATGTTCCATAGC		0.25		
TSWV_FIP	168	GGTCGATCCCGAGATCCTTGTAGCTTCAGTTGATAGCTTTGAG		0.4	0.3	Paul et al., 2021
TSWV_BIP		ACACCAGGGAAGCCTTAGGAACCTTCTTCACCTGATCTTCATT		0.4	0.3	
TSWV_loopF	not used separately	AGCCAAGACAACACTGATCAT		0.1		Paul et al., 2021
TSWV_loopB		AAGTTTGCACTGTGCTGAAA		0.1		
TSWV_q1F	179	AATCCGCATGTAGCACCCTC	0.4			this study
TSWV_q1R		GACAGCTCCCATCCAAAGCA	0.4			
TSWV-rpa-F	303	ATGTCTGACATTCCTGAAGAATCGTC			0.1	this study
TSWV-rpa-R		ATAAGAGGTAAGCTACCTCCCAGCAT			0.1	

20 s), with a final elongation step at 72°C for 5 min. PCR products were separated on the agarose gel as described above.

IC-RT-LAMP: The IC of TSWV was performed with minor optimisations, following Mulholland (2009), using a specific monoclonal anti-TSWV antibody (*DSMZ*, *Leibniz Institute*, Science Campus Braunschweig-Süd, Germany). Briefly, 20 µL of anti-TSWV IgG (diluted 1:100 in 0.1 M Na_2CO_3, pH 9.5) was pipetted into 0.2 mL tubes and incubated overnight at 37°C. The tubes were then washed three times with 200 µL of PBS containing *Tween 20*. Tomato leaves were ground in PBS + *Tween 20* (1:5, mass:volume) using a mortar and pestle, and the homogenate was centrifuged at 10 000 × *g* for 10 min at room temperature. The supernatant was loaded into the pre-prepared anti-TSWV-coated tubes and incubated for 3 h at 37°C. After washing three times with 200 µL of PBS + *Tween 20*, 20 µL of *DEPC*-treated water was added to each tube. The tubes were then incubated for 5 min at 80°C, after which the water was removed. Positive and negative controls were prepared by adding 20 µL of either isolated TSWV (0.6 µg/µL) or H_2O instead of the supernatant of the plant crude extract during the IC step.

RT was performed directly in the pre-prepared tubes using the *RevertAid First Strand cDNA Synthesis Kit* with random hexamers (*Thermo Scientific*™, Wilmington, DE, USA), following the manufacturer's instructions. No additional template was added; therefore, the template volume was replaced with water.

The LAMP reaction was performed according to Paul et al. (2021), using a *Bst 3.0 DNA Polymerase* (*New England Biolabs*, Ipswich, MA, USA). Briefly, the 20 µL reaction mixture contained 1.5× *Isothermal Amplification Buffer II* (*New England Biolabs*, Ipswich, MA, USA), 6 mM $MgSO_4$, 1 M betaine, 0.75 mM of each dNTP, 0.25 µM TSWV_F3 primer, 0.25 µM TSWV_B3 primer, 0.4 µM TSWV_FIP primer, 0.4 µM TSWV_BIP primer, 0.2 µM TSWV_loopF primer, 0.2 µM TSWV_loopB primer (Table 1), 8 U of *Bst 3.0 DNA Polymerase*, and 1 µL of template. The undiluted reaction mixture after RT was used as the template. The reaction conditions were as follows: incubation for 60 min at 65°C, followed by enzyme deactivation for 5 min at 80°C. Products were separated on the agarose gel as described above.

RPA: The *Reverse Transcriptase Recombinase Polymerase Amplification* (*RPA*) *Kit* was purchased from *Amplfuture Biotechnology* (Changzhou, China). The 50 µL reaction system consisted of 30 µL buffer A, 2.5 µL buffer B, 0.1 µM each of forward and reverse primers (TSWV-rpa-F, TSWV-rpa-R), 1 µL RNA template, and 15.5 µL H_2O. After thorough mixing, the reaction mixture was incubated at 42°C for 30 min. The RPA products were mixed with 50 µL of Tris-saturated phenol:chloroform:isoamyl alcohol (25:24:1, v:v:v) DNA extraction solution, followed by centrifugation at 12 000 × *g* for 5 min. The supernatant was collected for further analysis by agarose gel electrophoresis or lateral flow assay (LFA). For agarose gel electrophoresis, a total of 7.5 µL of purified RPA products was subjected to electrophoresis on a 1.5% agarose-TBE gel. The amplification results were subsequently visualised. The LFA of amplification products was performed using the lateral flow test strips containing a gold pad coated with FAM-labeled antibodies, a test line immobilised with biotin-specific antibodies, and a control line coated with goat anti-mouse secondary antibodies. Ten microliters of RPA products were diluted 10-fold, and the entire volume was applied to the sample well of the test strip.

For application of RPA to test used set of tomatoes, RPA was performed using a *Lyo-ready RPA Kit* (*Lyo-ready Enzymes and Reagents for Recombinase Polymerase Amplification*, *Invitrogen*™, *Fisher Scientific*, Waltham, MA, USA), following the manufacturer's instructions. Briefly, the 20 µL reaction mixture contained 1× RPA reaction buffer, 0.2 mM of each dNTP, 0.3 µM forward primer and 0.3 µM reverse primer (*see* below) (Table 1), 0.6 µg *Lyo-ready T4 UvsX Protein*, 0.6 µg *Lyo-ready T4 UvsY Protein*, 8 µg *Lyo-ready T4 Gene 32 Protein*, 3 U *Lyo-ready Bst DNA Polymerase*, 0.5 µL template (undiluted cDNA), and 14 mM $MgCl_2$. As recommended by the manufacturer, primers of at least 30 nucleotides were used; therefore, TSWV_FIP and TSWV_BIP primers (Table 1) with the length more than 40 nt were employed. The template consisted of 20× diluted cDNA from tomato samples. The positive control was cDNA obtained from RT using isolated TSWV as a template, starting at a concentration of 40 ng/µL. The negative control was diluted cDNA obtained from RT using isolated tomato brown rugose fruit virus (property of the Czech Agrifood Research Centre) as a template, also at 40 ng/µL, and a reaction with H_2O instead of template was included. The entire preparation was kept on ice. All premix components, except $MgCl_2$, were thoroughly mixed, pipetted into microtubes, and the templates added. Finally, $MgCl_2$ was added separately to each tube. Reactions were incubated in a thermoblock at 40°C for 25 min, then immediately kept on ice. RPA products were separated on the agarose gel as described above.

SDS-PAGE/*CBB R-250* staining: The isolated and specifically diluted TSWV was further diluted 1:1 in standard SDS-PAGE sample buffer containing 6 M urea, incubated at 65°C for 20 min, and loaded onto a 12% polyacrylamide gel. Proteins were separated according to Laemmli (1970).

After electrophoresis, the gel was washed three times for 1 min each in H_2O, stained overnight with *CBB R-250* solution, and destained following the protocol of Brunelle and Green (2014).

TBIA: TBIA was performed following Lin (1990). The entire procedure was carried out at room temperature. Briefly, systemic leaves of infected tomato plants were cut with a razor blade and gently pressed onto a nitrocellulose membrane (*Amersham*™ *Protran*™ *Premium* 0.45 µm, *Cytiva*, Wilmington, DE, USA) for approximately 2 s. Leaves from uninfected tomato plants were used as

negative controls and were printed in the same manner. For the positive control, 1 µL of isolated TSWV (0.6 µg/µL) was pipetted (dot-blotted) onto the membrane. After printing or pipetting, the membrane was allowed to air-dry for at least 20 min. The membrane was then processed using the immunodetection procedure (*see* below).

The main experiment was conducted as follows: 20 tomato plants were inoculated, while 5 were left uninoculated as negative controls. Tissue printing was performed at 7th, 14th, 21st, and 28th dpi, at which point the experiment concluded. Two parallel layouts for tissue printing were used: in the first, all plants, including controls, were printed onto a single membrane on days 7, 14, 21, and 28. In the second layout, each plant, along with one of the five negative controls, was printed separately onto an individual membrane at 7th, 14th, 21st, and 28th dpi, providing detailed insight into the progression of infection over time.

For tissue printing of tomatoes from the commercial greenhouse of HZSJ in Chornice, greenhouse staff printed leaves from approximately seven-month-old plants onto a membrane, which was then delivered to the IEB for immunoprocessing. Leaves from around five-month-old tomato plants grown in private gardens and greenhouses were collected, kept cool, and delivered to the IEB within one day, where they were printed and immunoprocessed.

The presence of virus in plants grown from seeds harvested from TSWV-infected tomato plants was detected immunochemically after printing the stems of approximately three-week-old seedlings onto a nitrocellulose membrane (*Amersham™ Protran™ Premium* 0.45 µm, *Cytiva*, Wilmington, DE, USA).

DB: DB analysis followed by immunochemical detection of TSWV in tomato seeds was performed according to Marlow and Handa (1987). Briefly, 34 seeds harvested from infected plants were crushed separately in 50 µL of PBS, and the homogenate was centrifuged at 10 000 × *g* for 10 min at room temperature. Subsequently, 1 µL of the supernatant was pipetted (dot-blotted) onto a nitrocellulose membrane (*Amersham™ Protran™ Premium* 0.45 µm, *Cytiva*, Wilmington, DE, USA). After air-drying for at least 20 min, the membrane was blocked, and the presence of TSWV was detected immunochemically (*see* below). As a positive control, 1 µL of isolated TSWV (0.6 µg/µL) was dot-blotted alongside the tissue-printed samples.

Immunochemical detection using monoclonal anti-TSWV antibody: The tissue-printed or dot-blotted nitrocellulose membrane was incubated with blocking buffer (1% bovine serum albumin in 5% semi-skimmed dry milk in PBS + 0.05% *Tween 20*) for 1 h. After washing three times for 5 min each in PBS + 0.05% *Tween 20*, the membrane was incubated overnight with the primary antibody (monoclonal anti-TSWV antibody, 1:1 000 in PBS + 0.05% *Tween 20*; *DSMZ*, *Leibniz Institute*, Science Campus Braunschweig-Süd, Germany).

Following three washes of 5 min each in PBS + 0.05% *Tween 20*, the membrane was incubated for 3 h with secondary antibodies conjugated to alkaline phosphatase (polyclonal *Goat Anti-Rabbit IgG Antibody*, *Alkaline Phosphatase Conjugate*, 1:20 000 in PBS + *Tween 20*; *Sigma-Aldrich*, *Merck*, Darmstadt, Germany). After washing three times for 5 min in PBS + *Tween 20*, the membrane was incubated with alkaline phosphatase substrate solution (*SigmaFAST™ BCIP®/NBT* tablet, *Sigma-Aldrich*, *Merck*, Darmstadt, Germany) until the colour reaction was fully developed.

Results

TSWV propagation in *N. glutinosa*: Following the mechanical inoculation of 12 *N. glutinosa* plants with lyophilised, commercially sourced *N. tabacum* leaves containing TSWV, only seven plants developed symptoms by the 18th dpi. These plants were maintained until the 21st dpi, when the first necrotic lesions appeared on the systemically infected leaves (Fig. 1*A*,*B*). The presence of TSWV in symptomatic plants was verified by one-enzyme RT-PCR (data not shown).

TSWV propagation in tomato plants cv. Start: Twenty tomato plants cv. Start were mechanically inoculated, and the onset of symptoms was subsequently monitored. The first symptoms appeared at approximately 20th dpi, and the plants continued to grow for a further week without developing necrosis. By around 28th dpi, 14 plants displayed clear symptoms (Fig. 1*D*). The presence of TSWV was confirmed by PCR in symptomatic plants. Thereafter, leaves exhibiting symptoms were collected and stored at –20°C.

TSWV isolation: Following TSWV isolation, the quality and purity of the viral preparation were assessed by SDS-PAGE and visualised on a CBB R250-stained gel

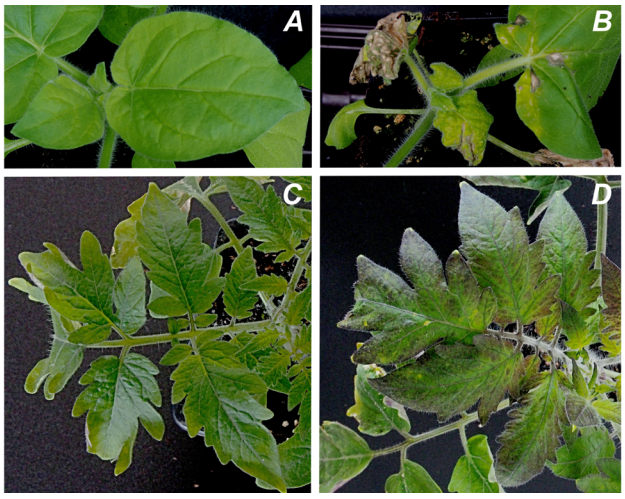

Fig. 1. Symptoms of TSWV on two different experimental plant species – *N. glutinosa* (*A*,*B*) and tomato cv. Start (*C*,*D*). Uninfected *N. glutinosa* (*A*) and infected *N. glutinosa* (*B*), both at 21st dpi; uninfected tomato cv. Start (*C*) and infected tomato cv. Start (*D*), both at 28th dpi.

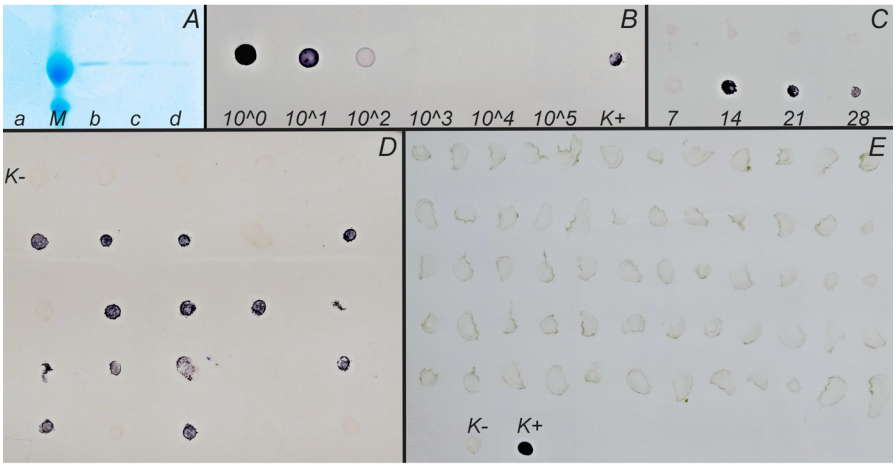

Fig. 2. TSWV detection using SDS-PAGE/*Comassie Brilliant Blue R-250* staining and DB and TBIA followed by immunodetection. The isolated TSWV was separated on a 12% polyacrylamide gel, stained with *Comassie Brilliant Blue R-250* and destained in a destaining solution (*A*). M: *BLUelf* prestained protein marker (*FroggaBio*, Buffalo, NY, USA); 18 and 24kDa; a: uninfected plant; b: isolated TSWV (undiluted, *i.e.*, 0.6 µg/µL); c: isolated TSWV (2× diluted); d: isolated TSWV (4× diluted). TBIA of isolated TSWV (10-fold serial dilution; *B*). The numbers represent the dilution of the isolated TSWV (the undiluted concentration 10^0 = 0.6 µg/µL). K+ represents an infected plant. TBIA of the tomato plant nr. 23 – infection over time (*C*). Numbers represent days post inoculation (dpi). *Upper row*: uninfected plant; *lower row*: infected plant. TBIA of 25 tomato plants (*D*). K- marks a row of five uninfected plants, followed by four rows of inoculated plants. TBIA of 60 tomato plants grown in a commercial greenhouse (HZSJ, a.s., Chornice, Czech Republic; *E*). K-: healthy plant; K+: 0.6 µg of isolated TSWV.

(Fig. 2*A*). The concentration of TSWV was estimated using the same method (data not shown), with a yield of approximately 2 mg of virus per 100 g of infected tomato leaves. The reactivity of the isolated TSWV was evaluated using a specific monoclonal antibody in a 10-fold serial dilution assay by DB. The virus preparation showed strong reactivity in the undiluted and 10-fold diluted samples, and detectable but reduced reactivity at the 100-fold dilution (Fig. 2*B*).

PCR and RTX-PCR: The PCR was run using cDNA from tomato, cv. Gallant as template and appropriate primers according to the Table 1. There were shown bands of 330 bp in two positive samples of nine inoculated tomato plants, the rest was uninfected as well as three uninoculated control plants (Fig. 3*A*).

One-enzyme RTX-PCR: One-enzyme RXT-PCR was performed using the supernatant of the ground leaf samples as the template, with the appropriate primers according to Table 1. Bands of the expected size (179 bp) were observed in two positive samples from nine inoculated tomato plants; the remaining plants were uninfected, as well as three uninoculated control plants (Fig. 3*B*).

Using a one-enzyme RTX-PCR assay, we surveyed the incidence of TSWV in 148 tomato plant samples collected from field plots and private gardens. Only a single sample, originating from a private garden in Rašov, tested positive for the virus. In contrast, none of the greenhouse-grown tomatoes of cultivars Plumola and Lucioso (HZSJ, Chornice) were found to be infected over three consecutive years of testing. These findings indicate that the incidence of TSWV in tomato crops in the Czech Republic is currently very low.

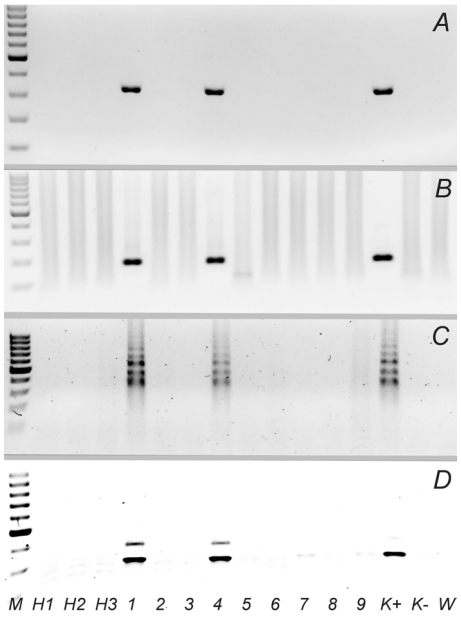

Fig. 3. Different molecular methods of detection of TSWV in tomato, cv. Gallant plants. PCR (*A*), one-enzyme RTX-PCR (*B*), IC-RT-LAMP (*C*), RPA (*D*). One-enzyme RTX-PCR and IC-RT-PCR were performed using directly the supernatant of leaf-homogenate in suitable buffer, the other methods were performed after RNA isolation and subsequent reverse transcription using cDNA as template. M: *GeneRuler™ 100 bp DNA Ladder* (*Life Technologies Czech Republic s.r.o.*, Prague, Czech Republic), adjusted fragment: 500 bp. H1, H2, H3: healthy/uninfected plants; 1 - 9: experimental plants nr. 1 - 9; K+: cDNA obtained from RT of purified TSWV (0.3 µg TSWV into 10 µL of RT-mixture); K-: cDNA obtained from RT of purified tomato brown rugose fruit virus (ToBRFV) (property of Czech Agrifood Research Centre; 0.5 µg ToBRFV into 10 µL of RT-mixture); W: water instead of template.

IC-RT-LAMP: The IC-RT-PCR template was originally ground tomato cv. Gallant leaves from which the TSWV was captured using the specific antibody on the walls of the tubes. In the second step, RT was performed directly in the tubes. Finally, in the LAMP reaction, the cDNA was used as the template. The agarose gel showed the typical positive-LAMP-reaction band patterns in two positive samples of nine inoculated tomato plants; the rest were uninfected, as well as three uninoculated plants (Fig. 3*C*).

RPA: RPA amplification was performed using the primers TSWV-rpa-F and TSWV-rpa-R (Table 1) with nucleic acid templates from TSWV-infected and healthy samples. The purified RPA products were analyzed by agarose gel electrophoresis, which showed that the target band was detected only in the infected samples, whereas no band was observed in the healthy controls (mock) (Fig. 4*A*). Subsequently, the diluted RPA products were tested using lateral flow assay strips, which had a gold pad coated with FAM-labeled antibodies, a test line immobilised with biotin-specific antibodies, and a control line coated with goat anti-mouse secondary antibodies. The results showed that both the test and control lines were visible for the TSWV-infected samples, while only the control line appeared for healthy plant samples (mock) (Fig. 4*B*).

For the applications purposes, the cDNA from tomato plants cv. Gallant was used as a template for RPA. On the gel, bands were visible in two positive samples of nine inoculated tomato plants while rest of samples including three uninoculated control plants as well as the negative controls (Fig. 3*D*) showed any product.

TBIA and DB: At 21st dpi, one leaf per tomato cv. Start was tissue-printed onto a nitrocellulose membrane, and the presence of TSWV was detected immunochemically using the specific monoclonal anti-TSWV antibody. Fourteen of the twenty inoculated plants were tested positive for TSWV, while the remaining six inoculated plants and five uninoculated plants were negative/uninfected (Fig. 2*D*).

The progression of the infection over time was subsequently estimated using plants nr. 13, 14, 21, and 23, in which the infection was observed at 21st dpi, as described above. The intensity of the tissue-print colour correlates with the virus titre. Thus, the trend of TSWV infection development was the same for all four plants: no virus was detected at 7th dpi, the infection was fully developed by 14th and 21st dpi and the virus titre decreased at 28th dpi. For the purposes of this paper, only the results for plant no. 23 are shown (Fig. 2*C*).

The leaves of tomatoes produced in the HZSJ company's greenhouses were tissue-printed by the greenhouse staff on site. After delivery to the IEB/Czech Agrifood Research Centre, the membrane was immunochemically developed. No positive plants were detected (Fig. 2*E*), nor were any detected in tomatoes collected from private gardens and greenhouses in the Moravian region (data not shown).

Furthermore, to meet the needs of crop companies for early virus detection, extracts from seeds harvested from TSWV-infected plants were tested *via* DB and subsequent immunoprocessing. No positive samples were detected among the 34 seeds tested (data not shown). Additionally, tomato plants grown from seeds harvested from TSWV-infected plants were tissue-printed and immunoprocessed. No positive samples were detected among the 19 tested tomato seedlings (data not shown).

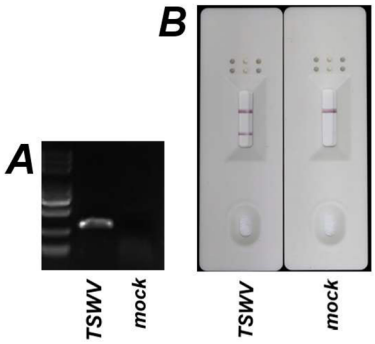

Fig. 4. Different methods of RPA detection of TSWV. RPA products were visualised by electrophoresis (*A*) or by lateral flow assay using strips (*B*). TSWV: TSWV infected plant; mock: healthy plant.

Discussion

TSWV is one of the most destructive plant viruses worldwide owing to its extensive distribution and capacity to infect more than 1 000 plant species, including economically important crops such as tomato, pepper and groundnut (Adkins, 2000). In addition to characteristic symptoms such as chlorotic or necrotic lesions, wilting and stunted growth, infection in plants of the *Solanaceae* family can result in complete crop failure and substantial economic losses. Consequently, outbreak management, limitation of disease spread and the incorporation of resistant genotypes into breeding programs are of critical importance (Pappu et al., 2009; Turina et al., 2012; Oliver and Whitfield, 2016).

Current diagnostic approaches include both serological and molecular techniques. In this study, we compared several of these methods, namely PCR, one-enzyme RTX-PCR, IC-RT-LAMP, RPA, DB, and TBIA, using tomatoes of different cultivars grown under experimental conditions, in commercial greenhouses, and in private gardens and greenhouses. Alongside conventional PCR, particular emphasis was placed on methods that can be performed without costly laboratory equipment or which enable the processing of samples by less experienced personnel directly under field conditions. The comparison focused on accuracy, sensitivity, specificity, and practicality, with the ultimate aim of supporting the development of standardised diagnostic protocols suitable both for research and for agricultural disease management (Boonham et al., 2014; Martinelli et al., 2015; Fox and Mumford, 2017; Massart et al., 2017).

Classical molecular methods such as PCR and qPCR are highly sensitive but require skilled operators, RNA extraction and reverse transcription kits, as well as costly

laboratory equipment such as thermocyclers and light cyclers. Following amplification, products either undergo additional analysis (*e.g.*, DNA electrophoresis) or require careful interpretation (*e.g.*, qPCR data analysis). This highlights the demand for novel molecular approaches that avoid some of the critical steps of conventional assays while maintaining high sensitivity and specificity (Notomi et al., 2000; Fu et al., 2024).

One such approach is one-enzyme RTX-PCR, which employs a homemade enzyme with reverse transcriptase/DNA polymerase activity (developed in the Laboratory of Virology, IEB; Hoffmeisterová et al., 2022) without compromising sensitivity or specificity. Plant material is homogenised in the buffer, centrifuged and the supernatant directly used as the reaction template, enabling rapid processing of large numbers of samples. Reverse transcription and amplification occur in a single tube, thereby minimizing the risk of contamination. Domestic production of the enzyme further enhances cost-effectiveness, making one-enzyme RTX-PCR a promising alternative to conventional RT-PCR or RT-qPCR (Wu et al., 2024).

The IC-RT-LAMP method bypasses RNA extraction by immobilizing virus particles on the tube surface with specific antibodies. Reverse transcription then takes place in the same tube, reducing contamination risk. Amplification relies on *Bst* polymerase and three primer pairs under isothermal conditions, allowing the assay to be run in a simple heating block rather than a thermocycler. However, for TSWV, only monoclonal antibodies proved effective (data not shown). Because these become unstable at working concentrations, higher antibody consumption is required over time, making the assay less cost-effective. Similar RT-LAMP methods using cDNA as template have recently been optimised for TSWV detection, showing higher sensitivity than conventional RT-PCR and potential for in-field deployment with simplified extraction procedures (Caruso et al., 2024).

RPA employs a protein complex (including single-stranded DNA-binding proteins and recombinase) that enables rapid isothermal amplification within minutes in a heating block. However, since the template must be RNA or cDNA, RNA isolation followed by reverse transcription is still required. As the newest method assessed in this study, RPA is currently relatively expensive, reflecting its limited commercialization (Piepenburg et al., 2006; Kersting et al., 2014; Lobato and O'Sullivan, 2018). However, against manufacturer's instructions we tested successfully not only primers about 30 nt long (in our case more than 40 nt), but also shorter ones with conventional length around 25 nt.

Despite their comparatively lower sensitivity, serological methods remain widely used in plant virology. The most common is DAS-ELISA, though this requires laborious sample preparation and carries a risk of handling errors. By contrast, TBIA involves pressing plant tissues onto nitrocellulose membranes, enabling rapid and simple sampling that can be performed by non-specialists in the field, as we demonstrated in our collaboration with HZSJ, a Czech large-scale hydroponic tomato producer. Following drying, membranes are processed *via* blocking and antibody-based immunodetection. In the case of TSWV, only the monoclonal antibody was reliable, though its instability at working concentrations necessitates its use in larger batches of samples processed within a short timeframe (approximately one week) (Clark and Adams, 1977; Rubio et al., 2020).

Overall, molecular methods are more sensitive than serological assays. Nevertheless, TBIA is valuable for large-scale preliminary testing in the field (Filardo et al., 2022), with suspected samples subsequently confirmed by molecular assays. Imaging-based detection approaches have also been recently explored for TSWV, with promising results in *Capsicum* germplasm (Mensah et al., 2024). Such tools may serve as an initial screening prior to molecular confirmation. In comparative inoculation experiments, three uninoculated and nine mechanically inoculated tomato cv. Gallant plants were tested, only two inoculated were tested positive (Fig. 3). As TSWV is naturally transmitted in a circulative-propagative manner by thrips, mechanical inoculation appears inefficient for this virus. Even in *Nicotiana* species, regarded as highly susceptible to diverse viruses (Llamas-Llamas et al., 1998), infection rates did not reach 100%. TBIA results (Fig. 2*D*) indicated more positives than molecular assays, possibly reflecting reduced viral activity during serial passages. Of 12 *N. glutinosa* plants inoculated with lyophilised *N. tabacum* leaves, only seven were positive. Subsequent inoculation into tomato cv. Start yielded 14 positives out of 20 plants. However, when tomato cv. Gallant was inoculated using cv. Start leaves stored at –20°C, only two of nine plants tested positive. These results suggest that TSWV infectivity decreases markedly during passage and prolonged storage at –20°C, confirming live plant passage as the most reliable maintenance method (Ruark-Seward et al., 2020). Moreover, differences in cultivar susceptibility/resistance may play a role, which could explain the low incidence of TSWV infection among the tested cultivars grown in the Czech Republic, a factor that warrants further investigation. Seed transmission tests, involving seeds and seedlings from infected plants, revealed no evidence of TSWV transmission. However, recent studies show that in *Capsicum annuum* the virus can be transmitted through seeds, being localised to the endosperm and even detected in second-generation seedlings (Wang et al., 2022). More broadly, virus transmission during seed development depends on host defense responses and the action of viral suppressors, as highlighted in a recent review (Escalante et al., 2024). Our results therefore underline the need for more detailed studies of seed tissues, plant developmental stage and host genotype to establish the true potential for TSWV seed transmission.

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