Accepted manuscript

Bados, J.P., J. Gómez, A. Pérez, J. Salazar, and M. Marín. 2025. Molecular detection of three RNA viruses in tomato (*Solanum lycopersicum* L.) leaf tissue and seeds in Antioquia (Colombia). Revista Colombiana de Ciencias Hortícolas 19(1), e18368. Doi: https://doi.org/10.17584/rcch.2025v19i1.18368

Molecular detection of three RNA viruses in tomato (*Solanum lycopersicum* L.) leaf tissue and seeds in Antioquia (Colombia)

Detección molecular de tres virus de RNA en tejido foliar y semillas de tomate (*Solanum lycopersicum* L.) en Antioquia (Colombia)

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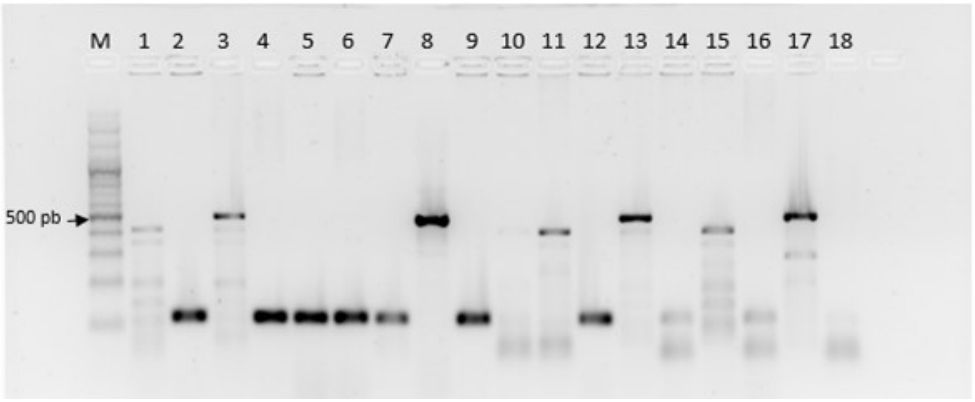

RT-PCR detection of PYVV using primers PYVV_F_CP/ PYVV_R_CP. Photo: J.P. Bados

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Short title: MOLECULAR DETECTION OF THREE RNA VIRUSES IN TOMATO

Doi: https://doi.org/10.17584/rcch.2025v19i1.18368

Received: 16-10-2024 Accepted: 03-02-2025 Published: 14-03-2025

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ABSTRACT

Viruses are a significant threat to tomato crops (*Solanum lycopersicum*) worldwide. In Colombia, 20 species of viruses from 10 genera have been reported in tomato, with 9 of them being RNA viruses that cause varying levels of damage. This study in Antioquia evaluated the presence of three RNA viruses: potato virus Y (PVY), potato yellow vein virus (PYVV) and Southern tomato virus (STV) in different tomato seeds and plants using RT-qPCR, conventional RT-PCR and Sanger sequencing. The study included 15 leaf tissue samples of the Chonto variety and 15 seed samples of various tomato varieties (Cherry, Chonto, Yellow, Kidney, San Marzano, and Mexican husk tomato). The results showed high levels of PVY in leaf tissue (93.3%) and seeds (46.6%), while PYVV was detected in 60% of leaf samples and 26.6% of seed samples. STV was found in 93.3% of seeds but was not detected in any leaf samples. Sanger sequencing confirmed the presence of these viruses, and phylogenetic analyses placed the isolates in clades previously reported for these viruses in Colombia. These findings underscore the need for a tomato seed certification program in Colombia to ensure viral-free seeds.

Additional key words: diagnosis; plant virology; RT-qPCR; sequencing; Solanaceae.

RESUMEN

Los virus son una amenaza importante para el cultivo de tomate (*Solanum lycopersicum*) en el mundo. En Colombia, se han reportado en tomate cerca de 20 especies de virus de 10 géneros; 9 de los cuales son de RNA y presentan diferentes niveles de patogenicidad. En este trabajo se evaluó mediante RT-qPCR, RT-PCR convencional y secuenciación Sanger, la presencia de tres virus de RNA: potato virus Y (PVY), potato yellow vein virus (PYVV) y Southern tomato virus (STV), en semillas y cultivos de tomate en Antioquia. Las evaluaciones incluyeron 15 muestras de tejido foliar de la variedad Chonto y 15 de semillas de diferentes variedades (Cherry, Chonto, Amarillo, Riñón, San Marzano y Tomatillo verde mexicano). Los resultados indicaron altos niveles de incidencia para el virus PVY tanto en tejido foliar (93.3%) como en semillas (46.6%), mientras que el PYVV se detectó en el 60 y 26.6% de las muestras, respectivamente. El STV se encontró en el 93.3% de las semillas, pero no se detectó en ninguna muestra foliar. La ocurrencia de estos virus se confirmó mediante secuenciación Sanger, y los análisis filogenéticos indicaron la ubicación de los aislamientos en clados previamente reportados para dichos virus en Colombia.

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Estos resultados llaman la atención sobre la necesidad de establecer en el país un programa de certificación de semilla de tomate por su sanidad viral.

Palabras clave adicionales: diagnóstico; virología vegetal; RT-qPCR; secuenciación; Solanaceae.

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) stands as a cornerstone of global agriculture, with a staggering market value of 93.9 billion dollars per year and a production surpassing 180 million tons (FAO, 2022). In Colombia, this crop sprawls over 1,796 ha, contributing to an annual production of 822,818 t. Antioquia, a leading grower, holds a substantial share of nearly 25% of the national production (MinAgricultura, 2021).

Viruses are among the most numerous and limiting groups of phytopathogens in tomato cultivation worldwide, with a report of at least 312 species from 22 families and 39 genera (Rívarez *et al.*, 2021). DNA viruses are the most numerous (220 species), especially those of the *Begomovirus* genus. In comparison, 84 species of viruses with RNA genomes belonging to 18 families have been reported, and mainly associated with the genera: *Tobamovirus*, *Orthotospovirus*, *Potyvirus*, *Cucumovirus*, *Ilarvirus*, *Alfamovirus*, *Potexvirus*, *Crinivirus*, *Cytorhabdovirus*, *Alphanucleorhabdovirus*, *Torradovirus*, *Nepovirus* and *Amalgavirus* (Rívarez *et al.*, 2021; 2023). In Colombia, about 20 virus species have been reported infecting this crop, belonging to a genus with a ssDNA genome (*Begomovirus*) (Vaca-Vaca *et al.*, 2012) and nine RNA genera (*Amalgavirus*, *Alphaendornavirus*, *Tobamovirus*, *Cucumovirus*, *Potyvirus*, *Tospovirus*, *Nepovirus*, *Torradovirus* and *Crinivirus*) (Morales *et al.*, 2009; Tamayo and Jaramillo, 2013; Muñoz *et al.*, 2016, 2017; Gallo *et al.*, 2020a, b). Of these viruses, three species stand out for their apparent differences in pathogenicity on this crop: potato virus Y (PVY; *Potyvirus*), potato yellow vein virus (PYVV; *Crinivirus*), and Southern tomato virus (STV; *Amalgavirus*) (Gil *et al.*, 2013; Gallo *et al.*, 2020b).

PVY is a virus recognized worldwide for its aggressiveness on different solanaceous plants, including potatoes, peppers, and tomatoes (Petrov, 2014; Prigigallo *et al.*, 2019; Rodríguez *et al*., 2016). In tomato, it induces mild mottling and different necrotic patterns, including veinal and spot necroses on leaves and sometimes streaks on petioles and stems (Tsedaley, 2015). PVY has rapid nucleotide mutation and genome recombination rates that contribute to the emergence of

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novel strains, changing the virulence of the virus, usually towards increased aggressiveness to worse symptomatology of the plants (Prigigallo *et al.*, 2019). PVY is mainly transmitted by at least 50 species of aphids (Hemiptera, Aphididae) such as: *Myzus persicae*, *Aphis nasturtii*, *Macrosiphum euphorbiae*, and *Aulacorthum solani*, by mechanical means or by asexual seed (e.g. potato tubers) (Ogawa *et al.*, 2008; Tsedaley, 2015).

PYVV is a virus that has a significant impact on potato crops in South America, causing losses of up to 50% in the *Solanum tuberosum* L. group Andigena cv. Diacol Capiro and more than 25% in the *S. tuberosum* group Phureja cv. Criolla Colombia (Guzmán *et al.*, 2012; Hernández-Guzmán and Guzmán-Barney, 2014). In tomato, the virus was initially thought to cause sporadic vein-associated yellowing, but it has been found to induce chlorosis, necrosis, leaf wrinkling, and fruit deformation (Salazar *et al.*, 2000; Gallo *et al.*, 2020a). PYVV is transmitted by the whitefly species *Trialeurodes vaporariorum* (Hemiptera, Aleyrodidae) and can also be easily dispersed by potato tubers (Salazar *et al.*, 2000). The efficiency of transmission of PVY and PYVV by the sexual seed of tomato is currently unknown, although their detection in tomato seeds has been previously carried out in Colombia (Gallo *et al.*, 2020b).

Unlike the two previous viruses, STV is considered a persistent virus, and apparently, its infection alone did not induce symptoms or cellular ultrastructure changes (Elvira-González *et al.*, 2020). However, when found in co-infections with other viruses, it does induce symptoms such as stunting of the growing tips, fruit discoloration, and reduced fruit size (Sabanadzovic *et al.*, 2009; Alcalá *et al.*, 2017). STV virions are not yet observed (Hao *et al.*, 2023), and its epidemiology remains unclear, as it is only known to be vertically transmitted through seeds at high rates (up to 90%) (Sabanadzovic *et al.*, 2009; Rívarez *et al.*, 2021).

PVY, PYVV, and STV are viruses with RNA genomes. The first consists of about 700 nm long and 11 nm wide flexuous particles, with a ssRNA+ genome of approximately 9,700 nt. It has a genome-linked protein (VPg) at the 5' terminus and a Poly-A region at the 3' end (Karasev and Gray, 2013). The expression of its genome occurs from a polyprotein of 3,061-3,063 amino acids, which is subsequently cleaved by viral proteases to yield nine functional proteins, including those involved in RNA replication and other non-structural and structural proteins (Tsedaley, 2015). PYVV has three genomic segments (RNA1: 8,035 nt; RNA2: 5,399 nt; RNA3: 3,892 nt) individually encapsidated in flexible particles between 650 and 900 nm (Livieratos *et al.*, 2004; Chaves *et al.*, 2014). RNA 1 encodes for a small protein with a transmembrane domain

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(p7) and the proteins associated with virus replication (L-Pro, MTR, HEL, and RdRp). RNA2 encodes for five proteins (HSP70h, p60, CP, p7, and p10), and RNA3 encodes for the minor capsid (CPm) and two proteins of unknown function (p4 and p26) (Livieratos *et al.*, 2004; Chaves *et al.*, 2014). STV has a double-stranded RNA (dsRNA) genome of 3.5 kb in length, which contains two overlapping open reading frames (ORFs): ORF 1 encodes for the 42 kDa putative coat protein (CP or p42), and ORF 2 encodes for the RNA-dependent RNA-polymerase (RdRp) by +1 ribosomal frameshifting (Sabanadzovic *et al.* 2009; Elvira-González *et al.*, 2020).

Given the RNA nature of these viruses, they can be effectively detected by real-time RT-PCR (RT-qPCR) tests. In previous evaluations using the RT-qPCR with SYBR Green, Gallo *et al.* (2020a, b) evaluated the presence of these and other RNA viruses (e.g. PVX, PVS) in tomato crops and seeds collected in Antioquia during 2016-2017, finding high levels of incidence of PVY, PYVV and STV (Gallo *et al.*, 2020a, b). For this reason, the primary goal of this study was to monitor the present status of these viruses in tomato leaf tissue and seed samples collected in Antioquia. Additionally, we aimed to assess their genomic characteristics by conducting Sanger sequencing of the coding region for the viral capsid.

MATERIALS AND METHODS

Plant material

Fifteen asymptomatic leaf samples (5x) of the Chonto tomato type were randomly obtained from four fields in the municipality of Marinilla, Antioquia. Two crops were from El Socorro area (6° 10' 43" N; 75° 16' 17" W; 2100 m.a.s.l.) with four and three samples, and two crops were from Alto del Mercado area (6° 10' 43" N; 75° 18' 19" W; 2200 m a.s.l.) with four samples each. Leaf samples were stored at -80°C until further use. Additionally, 15 samples (~0.5 kg/sample) of tomato fruits of different varieties were acquired from supermarkets in the city of Medellin, Antioquia. These included eight Cherry tomatoes (*S. lycopersicum* var. Cerasiforme), three Chonto tomatoes (*S. lycopersicum* var. Santa Cruz), one yellow tomato (*S. lycopersicum* var. Amarillo), one kidney tomato (*S. lycopersicum* var. Milano; SLT13), one San Marzano tomato (*S. lycopersicum* var. Marzano dell'Agro Sarnese-Nocerino), and one Mexican husk tomato (*Physalis ixocarpa*). From each fruit sample, the seeds were carefully extracted, left to ferment for 3 d, washed with distilled water, and then dried at room temperature.

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RNA extraction and cDNA synthesis

Total RNA was isolated from leaf samples using the GeneJET Plant RNA Purification kit (Thermo-Fisher Scientific) with 50 mg of leaf tissue, while the RNeasy Plant kit (Qiagen) was employed with 100 mg of seeds, following the manufacturer's instructions. RNA concentration and purity were evaluated by spectrophotometry at 260 and 280 nm using a Nanodrop 2000C (Thermo-Fisher Scientific). Subsequently, cDNA synthesis was carried out using primers: Oligo-dT, PYVV_R_CP, and STV_R for PVY, PYVV, and STV, respectively (Tab. 1). The cDNA reactions, comprising 20 µL, entailed 200 U of ExcelRT reverse transcriptase enzyme (SMOBIO), 1X RT buffer, 0.5 mM dNTPs, 20 pmol of each primer, 20 U of RNase inhibitor (RNAok, SMOBIO), and 50 ng μL^{-1} of total RNA. Before the addition of RT, the reactions were incubated in a T3 thermal cycler (Biometra) at 70°C for 5 min, followed by 1 min on ice, and subsequently incubated at 25°C for 10 min, 45°C for 50 min, and finally 85°C for 5 min to deactivate the enzyme.

Table 1. Primers used in this work.

Virus	Primers	Sequences	Amplicon size (bp)	Reference
STV	STV_F3	GAGACTCAGAGGAATGACATG	233	Elvira-González *et al*., 2017
	STV_B3	CCTCCTTGACTTGCTTCC		
	STV_F	GCTGCCAACGTTCCTCTTACG	78	Gallo *et al*., 2020b
	STV_qR	GAAAGTAGCGCTTGTCGATACC		
PVY	PVYF	ACGTCCAAAATGAGAATGCC	479	Nie and Singh, 2001
	PVYR	TGGTGTTCGTGATGTGACCT		
	PVY-1_FP	CCAATCGTTGAGAATGCAAAAC	77	Singh *et al*., 2012
	PVY-1_RP	ATATACGCTTCTGCAACATCTGAGA		
	PVY_F_CP_To	TACTCGGGCAACTCAATCAC	109	This work
	PVY_R_CP_To	CCAAACCATAAGCCCATT		

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		CATC		
PYVV	PYVV_F_CP	TCAGGTTAGAGCAGACA GAGG	495	Álvarez-Yepes *et al*., 2017
	PYVV_R_CP	AGTTGCTGCATTCTTGAA CACG		
	PYVV_F_CP	TCAGGTTAGAGCAGACA GAGG	115	Álvarez-Yepes *et al*., 2017
	qPYVV_R_CP	AGGTCTCAGGATCTGGAT CAACT		

Virus detection

The RT-qPCR technique with SYBR Green was employed to detect PVY, PYVV, and STV. The reaction consisted of 2 µL of previously generated cDNA and the 2X Fast Q-PCR Master Mix (SYBR, ROX) kit (SMOBIO) in a total volume of 10 µL. This comprised 5 µL of the mix, 0.4 µL of the primers PVY1FP/PVY1RP, PYVV_F_CP/qPYVV_R_CP, and STVF/STVqR (Tab. 1), and 2.2 µL of sterile ultrapure water. Due to difficulties in obtaining amplifications for PVY, a new primer´s pair (PVY_F_CP_To/PVY_R_CP_To) was designed with the sequences available in GenBank for isolates reported in tomato using the Primer-Blast software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). For PVY confirmation, three amplicons were separated via 2% agarose electrophoresis, purified from the gel using the GeneJET Gel Extraction kit (Thermo-Fisher Scientific), and subjected to Sanger sequencing in both directions using an ABI Prism 3730xl equipment from Macrogen (South Korea).

The qPCR amplification program comprised an initial step at 95°C for 5 min, followed by 35 cycles of 95°C for 15 s and 52°C for 60 s, utilizing a Rotor-Gene Q-5plex Platform (Qiagen). Fluorescence acquisition occurred after each amplification cycle. Positive samples were defined as those with threshold cycle (Ct) values below cycle 35 and amplicons with a melting temperature (Tm) of ±1°C respecting those previously reported in literature (Muñoz *et al.*, 2017; Gallo *et al.*, 2020a, b). All reactions included both positive (*S. lycopersicum* virus infected tissue) and no template controls (NTC).

RT-PCR and Sanger Sequencing

To confirm the presence of the three viruses, a segment of their capsid coding region was amplified in a minimum of five samples using the One-Step RT-PCR method with the ExcelRT

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[RP1100] kit (SMOBIO). Independent reactions were conducted with primers PVY_F/PVY_R, PYVV_F_CP/PYVV_R_CP, and STV_B3/STV_F3, which amplify fragments of 475 bp, 495 bp, and 233 bp, respectively (refer to Tab. 1 for specific details). One-step RT-PCR reactions were followed according to the manufacturer's instructions (SMOBIO). The amplification program included a reverse transcription step at 42°C for 30 min, followed by an initial denaturation at 94°C for 3 min, 40 cycles of 94°C for 50 s, 52°C for 50 s, 72°C for 1 min, and a final extension at 72°C for 5 min, performed in a T3000 thermal cycler (Biometra).

After amplification, the resulting amplicons underwent separation via 1.5% agarose electrophoresis. At least three bands were purified from the gel using the GeneJET Gel Extraction kit (Thermo-Fisher Scientific) to enable Sanger sequencing in both directions on an ABI Prism 3730xl instrument from Macrogen (South Korea). Upon receipt of the sequences, consensus sequences were generated, and their viral nature was authenticated using BLASTn (https://blast.ncbi.nlm.nih.gov/). Subsequently, the sequences were aligned utilizing the MUSCLE program (Edgar *et al*., 2004) with sequences available in GenBank for each virus originating from diverse countries and hosts. These alignments were then utilized to produce identity matrices at the nucleotide level and to construct phylogenetic trees using the Maximum Likelihood method and Tamura-Nei model with 1,000 bootstrap replicates using the Mega 11.0 software (Tamura *et al.*, 2021). Finally, the sequences were deposited in GenBank and the assigned accession numbers were PQ469630 to PQ469635.

RESULTS

RNA extraction and cDNA synthesis

Total RNA extractions performed on leaf tissues resulted in 94 to 982.8 ng μL^{-1} and 260/280 nm absorbance ratios of 1.78 to 2.1, indicating adequate quantities and purity of the obtained RNA. In the case of seeds, the RNA concentration was lower (24.3 to 206.4 ng μL^{-1}), reflecting the higher number of structural tissues (e.g. seed coat, endosperm) and the low expression levels of this organ. However, the purity obtained was still appropriate for the virus evaluation, with 260/280 nm absorbance ratios of 1.76 to 2.07.

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Virus detection

The results of RT-qPCR tests showed that the Chonto variety leaf tissue samples had 93.3% incidence of PVY virus and 60% of PYVV. STV was not found in any of the leaf samples despite three independent replicates. Among the samples, eight showed co-infection of PVY and PYVV, particularly from the two fields in the Alto del Mercado area. Six samples only had PVY, while one had PYVV (Tab. 2).

Table 2. Detection results of three RNA viruses in leaf tissues of tomato variety Chonto.

Leaf tissue		PVY		PYVV		STV	
Sample	**Field**	**Ct**	**Tm**	**Ct**	**Tm**	**Ct**	**Tm**
SL1	Alto del Mercado 1	>35	(-)	20	79.54	>35	(-)
SL2	Alto del Mercado 1	28.24	79.0	>35	(-)	>35	(-)
SL3	Alto del Mercado 1	28.83	78.8	15	76.76	>35	(-)
SL4	Alto del Mercado 1	28.38	79.0	12	77.01	>35	(-)
SL5	Alto del Mercado 2	25.80	79.0	10	76.68	>35	(-)
SL6	Alto del Mercado 2	27.44	79.0	11	76.59	>35	(-)
SL7	Alto del Mercado 2	27.06	79.3	20	79.28	>35	(-)
SL8	Alto del Mercado 2	24.79	79.3	15	77.1	>35	(-)
SL9	El Socorro 1	26.16	79.3	>35	(-)	>35	(-)
SL10	El Socorro 1	24.75	79.2	>35	(-)	>35	(-)
SL11	El Socorro 1	24.80	79.3	>35	(-)	>35	(-)
SL12	El Socorro 1	25.62	79.5	20	76.5	>35	(-)
SL13	El Socorro 2	24.65	79.5	25	81.5	>35	(-)
SL14	El Socorro 2	26.75	79.5	>35	(-)	>35	(-)
SL15	El Socorro 2	26.51	80.0	>35	(-)	>35	(-)
Incidence percentage		**93.3%**		**60%**		**0%**	
Positive control		24.79	79.8	10	77.58	24	82.4
Negative control		>35	(-)	>35	(-)	>35	(-)

In addition, this research revealed important findings about the prevalence of these viruses in seeds. The STV had the highest incidence at 93.3%, followed by PVY at 46.6% and PYVV at 26.6%. Notably, one sample (SLT2) was virus-free, while two samples had a triple viral

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infection, five had a double infection of PVY and STV, and two had PYVV and STV. In five samples, only STV was detected (Tab. 3).

Table 3. Detection results of three RNA viruses in seeds of different tomato varieties.

Seeds		PVY		PYVV		STV	
Sample	**Variety**	**Ct**	**Tm**	**Ct**	**Tm**	**Ct**	**Tm**
SLT1	Cherry tomato	30.92	79.2	>35	(-)	25	81.18
SLT2	Cherry tomato	>35	(-)	>35	(-)	>35	(-)
SLT3	Cherry tomato	>35	(-)	>35	(-)	23	81.5
SLT4	Cherry tomato	31.02	78.5	>35	(-)	25	81.43
SLT5	Cherry tomato	>35	(-)	>35	(-)	25	81.54
SLT6	Cherry tomato	32.06	79.5	>35	(-)	19	81.33
SLT7	Cherry tomato	>35	(-)	>35	(-)	21	81.61
SLT8	San Marzano tomato	30.44	79.0	>35	(-)	20	81.71
SLT9	Yellow tomato	30.51	79.0	>35	(-)	22	81.36
SLT10	Mexican husk tomato	29.87	79.5	33.7	76	24	81.33
SLT11	Cherry tomato	30.08	79.5	32.5	78.1	19	81.5
SLT12	Chonto tomate	>35	(-)	>35	(-)	21	81.54
SLT13	Kidney tomato	>35	(-)	>35	(-)	19	81.26
SLT14	Chonto tomate	>35	(-)	31.6	78.4	10	81.28
SLT15	Chonto tomate	>35	(-)	31.2	78.05	23	81.42
Incidence percentage		**46.6%**		**26.6%**		**93.3%**	
Positive control		10	78.87	18.5	78.1	25	82.2
Negative control		>35	(-)	>35	(-)	>35	(-)

In the analysis of amplification and denaturation curves (Tm) for each virus in tomato leaf tissues, it was observed that PYVV had the lowest Ct values for positive samples (average Ct=16.4, SD=5.07), with some as low as Ct=10, indicating high levels of viral titer in these tissues. In contrast, PVY had an average Ct=26.41 and a narrower range of variation (SD=1.43). The Tm values for PYVV were consistent with previous reports by Álvarez-Yepes *et al.* (2017), Muñoz *et al.* (2017), and Gallo *et al.* (2020a, b), with an average value of 77.88°C and a high standard deviation (SD=1.78) due to the presence of two variants of the virus in tomato,

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characterized by Tm values around 77.02°C and 79.84°C (Muñoz *et al.*, 2017). In the case of PVY, new primers (PVY_F_CP_To/ PVY_R_CP_To) were designed to account for sequences of virus isolates from tomato. Upon confirmation of the identity of the three sequenced amplicons, average Tm values of 79.26°C and a low level of variation between the mentioned values (SD=0.3) were obtained (Fig. 1).

For seed samples, PVY was found in the positive samples with an average Ct value of 30.7 (SD= 0.72) and Tm values similar to those found in leaf tissues (Tm=79.17°C, SD=0.37). On the other hand, PYVV was detected at much higher Ct values (Ct=32.25, SD=1.1), a significant difference compared to the levels found in leaf tissues, and with an average Tm of 77.63°C (SD=1.1). STV had the highest viral titer in the seed tissue, with Ct values as low as 10 and an average of 21.14 (SD=3.91). The Tm values were in a very narrow range (81.18 to 81.71°C) with an average of 81.42°C and a standard deviation of only 0.14, indicating low variation for the amplified region among the isolates of this virus (Fig. 1).

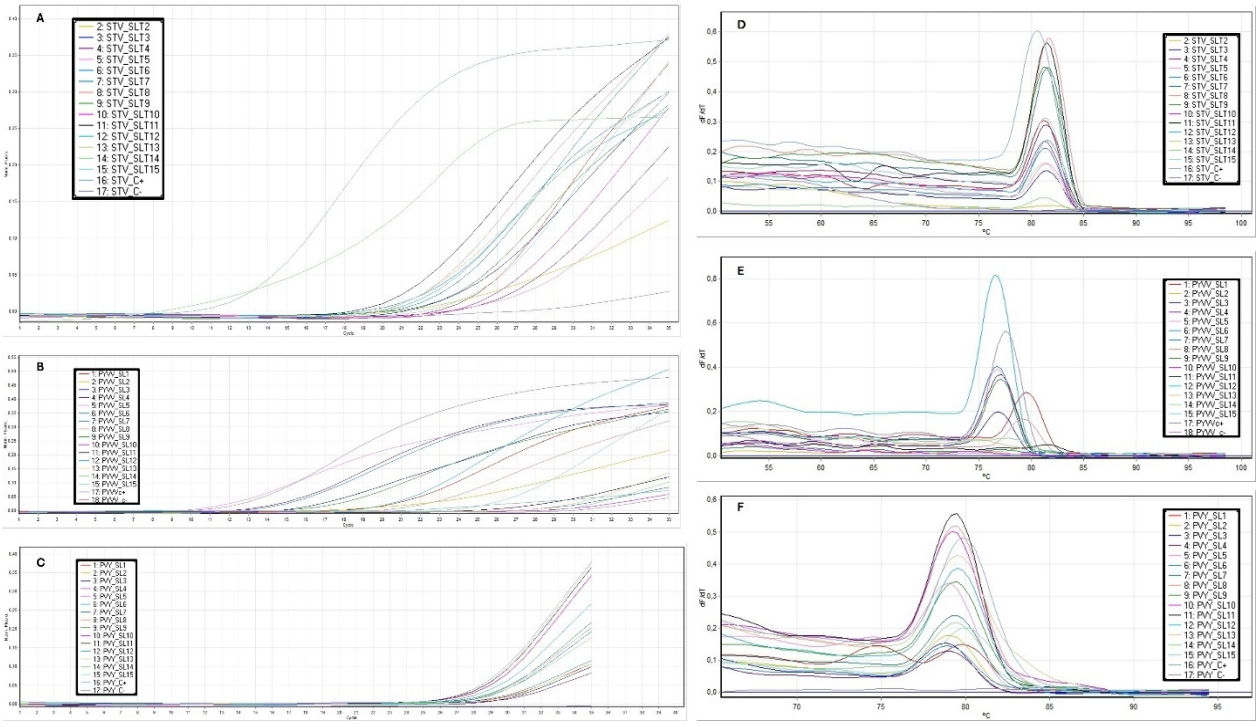

Figure 1. Detection by RT-qPCR with SYBR Green of three RNA viruses in tomato leaf tissue and seeds. A and D: amplification and denaturation curves for STV in seeds. B and E: amplification and denaturation curves for PYVV in leaf tissue. C and F: amplification and denaturation curves for PVY in leaf tissue.

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RT-PCR and Sanger sequencing

In most cases, the one-step RT-PCR reactions produced the expected amplicon sizes (PVY: 475 bp, PYVV: 495 bp, and STV: 233 bp). Three amplicons for each virus were extracted from agarose gels (Fig. 2) and then sequenced using the Sanger method. The consensus sequences confirmed their viral nature, showing high levels of identity after performing Blastn. Specifically, the PVY amplicons exhibited identity levels >97.85%, with a coverage of 100% and E value= 0.0 to 2^{-179}, when compared to accessions of different isolates of this virus from Colombia and Ecuador originating from tamarillo (*S. betaceum*), tomato, potato (*S. tuberosum* and *S. phureja*), and cape gooseberry (*Physalis peruviana*).

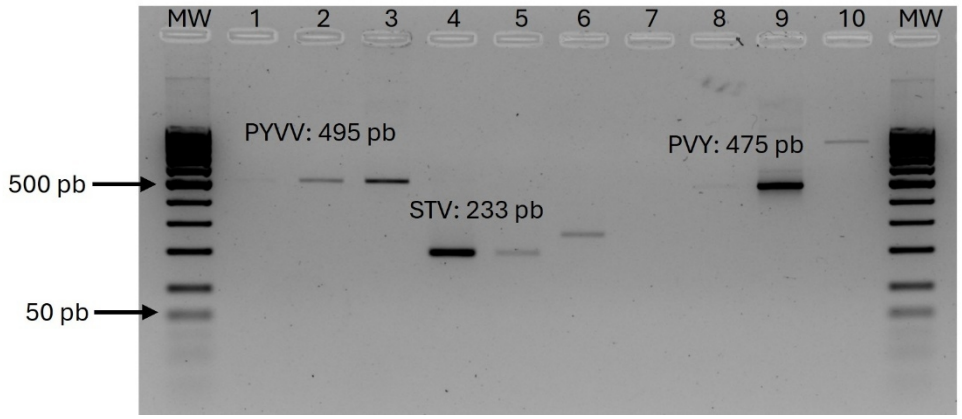

Figure 2. Purified amplicons obtained by One-Step RT-PCR for PYVV (1-3), STV (4-6), and PVY (8-10). Sizes of the expected products are shown on the gel. For STV (6) and PVY (10), the amplicons of unexpected size were found to correspond to a region of the host genome. Molecular weight marker (MW): PCR Ranger 100 bp DNA Ladder (Norgen).

The phylogenetic analysis separated the PVY isolates into two different clades, with a genetic distance D=0.072 between both groups (expressed as the number of base substitutions per site) (Fig. 3A). The first clade corresponds to isolates previously associated with the strain PVY^{N}, showing a shallow level of variation (D=0.002). The second clade, in addition to the tomato isolate SL11 (PQ469639), included two isolates from tamarillo from Ecuador, showing a higher level of internal genetic variation (D=0.03-0.05). The other two clades generated in the analysis correspond to the ordinary (PVY^{O}) and the PVY^{NTN} strain, associated with Potato tuber necrotic ringspot disease (PTNRD), which usually includes recombinant isolates of this virus (Karasev and Gray, 2013).

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In the case of PYVV, the two sequences showed high levels of identity and coverage (>99%) compared to two different groups of accessions derived from Colombian isolates of this virus. The SL8 isolate (PQ469633) had 99.75% identity with respect to MK563997, HQ620551, HQ620552, MN119295, and JF718296 (100% coverage; E value=0.0), which were mainly obtained from potato and tomato in Colombia. In contrast, the SL3 isolate (PQ469632) shared >99.75% identity with the KX589279 and KX589280 accessions and 96.81% with the KX573902 accession, obtained in 2015 and 2016 from tomato crops in Antioquia. The phylogenetic tree showed this situation by dividing the two isolates into a basal group only occupied by tomato isolates and a general clade that includes isolates not only from tomatoes but also from potatoes, showing a genetic distance greater than 0.05 between both clades (Fig. 3C).

On the other hand, the two STV sequences (PQ469634 and PQ469635) were highly identical (>99.31%; 100% coverage, E value=1^{-67} to 5^{-66}) to those reported in GenBank for different accessions including OP548653, MK610257, OP484999, MN095716, and OR596870 from virus isolates on tomato from different countries around the world (e.g. South Korea, China, Canada, Colombia). Phylogenetic analysis placed the two STV sequences from this work in a global clade that showed very low levels of internal variation (D<0.0069) and separated from a second clade consisting of three tomato STV isolates from Korea, Switzerland, and Germany, although with a very small genetic distance (D<0.021) (Fig. 3B).

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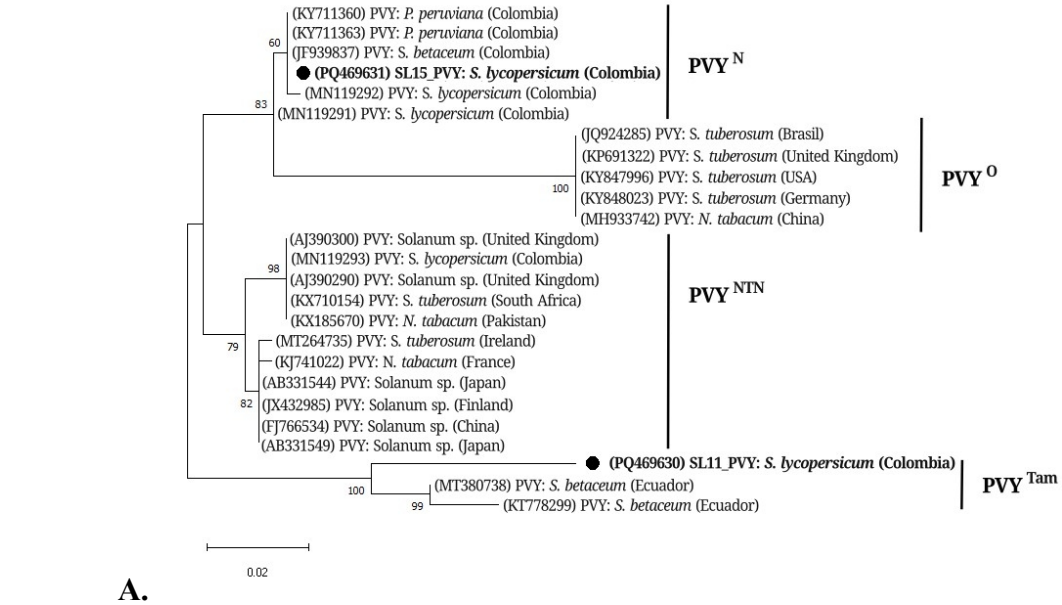
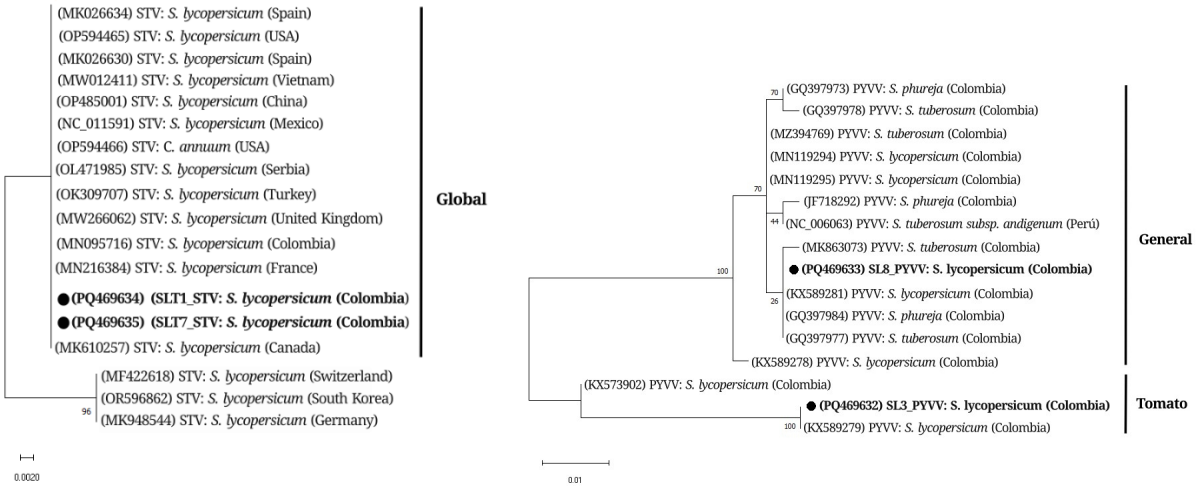


A.



B. **C.**

Figure 3. Phylogenetic trees from partial sequences of the capsid region for A) PVY, B) STV, and C) PYVV, obtained in this study on tomato in Antioquia and complemented with sequences of these viruses from different countries and hosts. The trees were generated using the Maximum Likelihood method and Tamura-Nei model, with 1,000 bootstrap replicates. The branch lengths are measured in number of substitutions per site. Accessions in bold correspond to the isolates sequenced in this work.

DISCUSSION

In this study, we evaluated the presence of three RNA viruses (PVY, PYVV, and STV) in tomato leaf tissues and seeds from Antioquia (Colombia) using RT-qPCR with SYBR Green. The results showed very high levels of incidence of PVY (93.3%) and PYVV (60%) in leaf tissues, and STV (93.3%) in seeds. Additionally, PVY was found in 46.6% of the seed samples and

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PYVV in 26.6%. These findings align with previous research in Antioquia, where high levels of viral infection in tomatoes have been reported. For example, Gallo *et al.* (2020b) found incidence levels of 100% for PYVV, 94.4% for PVX, 77.8% for PVY, 72.2% for STV, and 5.6% for PVS in tomato plants from the eastern region of Antioquia; while Muñoz *et al.* (2016; 2017) reported the presence of PVY and PYVV in tomato leaf tissues at levels of 88.8 and 72.7%, respectively.

The high incidence of STV in tomato seeds is consistent with previous reports of its vertical transmission in tomato at levels up to 90% (Alcalá *et al.*, 2017; Elvira-González *et al.*, 2018). In Colombia, Gallo *et al.* (2020b) found that STV was present in 80% of the tomato seed samples evaluated. It is noteworthy that STV was not detected in any of the 15 leaf samples of the Chonto tomato variety used in this study, despite being present in the positive controls and even after three independent repetitions of the entire experiment.

In a recent study, Hao *et al.* (2023) utilized in situ Hybridization with digoxin-labeled sense and antisense RNA probe assays to demonstrate the presence of STV in infected tomato leaves, young stems, and various seed tissues, including seed-coat, endosperm, and embryo. Their findings indicate that STV is a systemic infectious virus. Given the high incidence of this virus in seeds, together with the availability of various molecular tests for its detection (RT-qPCR SYBR Green, RT-qPCR Taqman, RT-LAMP) (Elvira-González *et al.*, 2017; 2018; Gallo *et al.*, 2020b), as well as its robust localization in seeds, the detection of STV could potentially serve as a biomarker for assessing the viral health of tomato planting material. This is particularly relevant for seeds and explants used in early-stage *in vitro* propagation to obtain certified seeds of this vegetable. Consequently, the quantitative evaluation of STV presence in seeds or mother plants subjected to chemotherapy, cryotherapy, or thermotherapy could be instrumental for a tomato seed certification program. In this sense, Pagán (2022) highlighted that while vertical transmission of plant viruses through seeds has been recognized for a century, it was often regarded as less significant. Recent advances in detection techniques, however, have led to a sixfold increase in the identification of seed-transmitted viruses, with some seeds showing 100% infection rates. Furthermore, persistent viruses transmitted vertically such as STV, are now known to be common in both wild plants and crops.

Regarding the detection of PVY, this virus has been extensively documented to affect tomato plants in various countries worldwide (Petrov, 2014; Rívarez *et al.*, 2021), including Colombia (Muñoz *et al.*, 2016; Gallo *et al.*, 2020a, b). Despite the unknown impact of sexual seed

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transmission of this virus in tomatoes, our study found it in 46.6% of seed samples and in 93.3% of the leaf tissue samples. This suggests exceptionally efficient transmission through various routes such as aphids, plant-to-plant contact, and mechanical transmission (e.g. pruning and harvesting) in the tomato production agroecosystems of Antioquia. Furthermore, this study identified two variants of the virus in tomato. One variant was linked to the necrotic strain (PVY^{N}), while the other was associated with a group of recombinant variants (PVY^{Tam}) not commonly reported in potato or tobacco and mainly found in Colombia and Ecuador (Henao-Díaz *et al.*, 2013; Green *et al.*, 2020). This discovery enhances our understanding of the substantial diversity exhibited by this viral species and may be associated with the fact that the primers regularly used for the detection of PVY in Colombia (PVY-1_FP/PVY-1_RP, Singh *et al.*, 2012; PVY_Col_RV2/PVY_Col_qFV2, García *et al.*, 2023) failed to yield appropriate results, necessitating the design of new primers (PVY_F_CP_To/PVY_R_CP_To) based on the sequences of these recombinant variants and the conventional strains.

Finally, the study highlights the importance of PYVV in the tomato virome in Colombia, as previously reported by Muñoz *et al.* (2017), Villamil-Garzón *et al.* (2014) and Gallo *et al.* (2020a). Urgent research is needed to assess the impact of PYVV infection on tomato yield and fruit quality since studies have shown that in certain plant-virus systems, such as *S. tuberosum*/PYVV and *S. phureja*/PYVV, losses up to 50% can occur (Guzmán *et al.*, 2012; Hernández-Guzmán and Guzmán-Barney, 2014). Furthermore, the detection of PYVV in tomato seeds requires more investigation to determine if the virus is effectively transmitted through sexual seeds or if its presence is due to external contamination, as observed with other plant viruses (Marín and Gutiérrez, 2016). Phylogenetic analysis confirmed the presence of a second lineage of PYVV in Colombia, consisting only of isolates found in tomato, as previously reported by Muñoz *et al.* (2017). This result raises the need to update the primers and probes used for the detection of PYVV in conventional and real-time RT-PCR reactions, as the existing ones were designed using sequences from the general clade and primarily derived from potatoes (Offei *et al.*, 2004; López *et al.*, 2006; Álvarez-Yepes *et al.*, 2017).

This study highlights the urgent need to improve the tomato seed certification program in Colombia. The high incidence of the three viruses examined in this study, as well as others commonly found in this crop (e.g. begomovirus, tobamovirus), underscores the crucial importance of robust seed certification processes and epidemiological assessments. These

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measures are essential for reducing the economic impact of these viruses and for implementing effective preventive measures. Fortunately, highly sensitive detection methods, such as those used in this study, are available to support these efforts.

CONCLUSION

In this study, the molecular technique of RT-qPCR using SYBR Green with specific primers was applied to identify viruses in leaf tissues of the Chonto variety of tomato obtained from different fields in Antioquia. The study revealed a high incidence of PVY (93.3%) and PYVV (60%) viruses. Additionally, STV was found in 93.3% of tomato seed samples from six varieties marketed in the department of Antioquia (Colombia).

The phylogenetic analysis using Sanger sequences from a partial region of the capsid confirmed that three viruses were present in tomato crops/seeds in Antioquia. It also identified two distinct genetic lineages for PVY (PVY^{N} and PVY^{Tam}) and PYVV (General clade and Tomato clade). Additionally, the two sequenced STV isolates were located in a Global clade with samples from various countries worldwide.

Conflict of interests: All authors edited and reviewed the manuscript, and they declare no conflict of interest regarding the subject under study.

Author's contributions: J.P. Bados, J. Gómez, A. Pérez, J. Salazar: Experimental work on virus detection, data analysis, manuscript review, and editing. M. Marín: funding acquisition, sample collection, data analysis, manuscript writing.

Acknowledgment: This study was part of project #60939: "Molecular detection of RNA viruses in tomato leaf tissue and seeds in Antioquia using RT-qPCR and LAMP," founded by the Faculty of Sciences at the Universidad Nacional de Colombia Sede Medellín. The samples were collected under a Framework Permit for Collection for Non-Commercial Scientific Research Purposes, granted by ANLA to the Universidad Nacional de Colombia according to Resolution 000697 of 2024.

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