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Comparative analysis of mechanical inoculation methods for Tomato spotted wilt orthotospovirus (TSWV) in tomato

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ABSTRACT

Tomato spotted wilt orthotospovirus (TSWV) is a pathogen that causes significant yield and quality losses in 1000 plant species in 84 families, including tomato, pepper, lettuce, tobacco, and various weeds. TSWV, a member of the Orthotospovirus genus within the *Bunyaviridae* family, is characterized by an isometrically enveloped particle. The virus is transmitted both mechanically and via vectors. This study evaluated the effects of two different mechanical inoculation methods, each using a different buffer solution, for in vitro TSWV inoculation. Tomato plants germinated from seeds were infected with TSWV using these two distinct mechanical inoculation methods, and infection was subsequently confirmed via PCR. The presence of TSWV in the infected plants was further validated by Sanger sequencing. Disease incidence and quantitative characteristics of TSWV-infected tomato plants were measured and statistically analyzed to compare the efficiency of each inoculation method and buffer combination. Differences in disease incidence rates and quantitative properties were observed between the two inoculation methods. One of the mechanical inoculation methods, in combination with its respective buffer, demonstrated a 30% higher transmission efficiency for TSWV, emphasizing the impact of both inoculation technique and buffer composition on successful virus transmission.

1. Introduction

Among the world's fresh vegetable production of approximately 1.14 billion tons, tomato (*Solanum lycopersicum*), a member of the *Solanaceae* family, has a share of 16% with 186 million tons. China ranks first in world tomato production with a production of 64 million tons, followed by India with a production of 20 million tons (Food and Agriculture Organization 2022).

Tomato spotted wilt orthotospovirus (TSWV), which belongs to the *Orthotospovirus* group of the Bunyaviridae family, is a plant virus with an isometrically enveloped particle. TSWV has a single-stranded linear RNA and a tripartite genome structure consisting of 8.9 kb L-RNA, 5.4 kb M-RNA, and 2.9 kb S-RNA (de Haan et al. 1990). One of the three RNAs is negative, while the other two have ambisense sense polarity (Usta et al. 2023). TSWV causes serious yield and quality losses in tomato production areas in the world. TSWV has been reported to cause disease in nearly 1000 plant species in 84 families, including tomato, pepper, lettuce, tobacco, peanut, artichoke, eggplant, chicory, ornamental plants, and weeds, all of which are members of the Solanaceae family (Gordillo et al. 2008).

Transmission of TSWV occurs mechanically and via vector thrips. Nine thrips genera belonging to the *Thrips sp*., *Scirtothrips sp*., and *Frankliniella sp.* can carry the disease in a circulative and propagative manner (Ogada and Poehling 2015). Among these species, *Frankliniella occidentalis* is an effective species in the transmission of TSWV (Coutts and Jones 2005). The most striking symptoms of TSWV in plants are bronzing on the leaves, black spots, black spots on the stem, wilting and deformation in the fruits (Güldür 1995). The fruits of plants showing these symptoms cannot be consumed fresh and become unusable, and also lose their economic value as industrial tomatoes (Turhan and Korkmaz 2006).

In the protection against TSWV, efforts are being made to prevent the transmission of TSWV by growing virus-free plants, sanitation, eradication, disinfection, and using pesticides against vectors. However, applying all these methods alone or together is not sufficient for an effective fight against TSWV. For this reason, resistant commercial tomato varieties were obtained by using breeding methods with the Sw-5 resistance gene, which is known to be resistant against TSWV (Dianese et al. 2011). However, as a result of a point mutation in the C118Y and T120N regions of the gene encoding the NSm protein in the TSWV genome, TSWV gained the ability to break the Sw-5 resistance gene, thus inactivating the hypersensitive reaction response (Fidan and Sari 2019). To date, several studies have focused on the effect of different mechanical inoculation methods on plant varieties (Hull 2009; Zhao et al. 2025). The effect of TSWV infection on different commercial tomato varieties was determined, and it was found that there were significant decreases in the TSWV-infected plant biomass (Ramkat et al. 2006). The changes in TSWV-infected tomato varieties were examined by comparing healthy plants in the early, middle, and late stages. Significant decreases in inflorescences, number of flowers,

number of fruits, fruit length, fruit circumference, chlorophyll content, and fruit yield were observed when plants were compared with healthy plants in the early stage of the infection compared to the middle and late stages (Farooq and Akanda 2007). It was determined that the yield of TSWV-infected tomato plants decreased by 42.1% and the market value decreased by 95.5%. Also, a 26.61% decrease in the weight of TSWV-infected plants, a 20.18% reduction in the number of leaves, a 10.94% decrease in leaf area, and an 11.93% reduction in fruit size were observed (Şevik 2007).

The objective of this study was to determine the effects of TSWV infection on host growth and physiological parameters with two different mechanical inoculation methods with different phosphate buffers and to observe the reactions under TSWV disease conditions in tomato variety.

2. Materials and Methods

2.1. Growing conditions and plant material

This experiment was carried out under greenhouse conditions at Aydın Adnan Menderes University Faculty of Agriculture in the autumn season of 2023. The greenhouse tomato variety 'Uyum RZ F1' (Ertürk Fide Tarım, Antalya) was used as plant material (susceptible to TSWV). Tomato seedlings (*Solanum lycopersicum*) were transferred into a mixture of peat and perlite (1:1) in sterilized plastic containers (200 x 180 mm). The tomato variety was grown in the growth chamber maintained under light conditions of 22±5°C, 60% relative humidity, 8/16 photoperiod, and all plants were watered with tap water every 3 days.

2.2. Experimental design

Two different mechanical inoculation methods containing different phosphate buffers were used for TSWV transmission. A total of 20 plants were used for each mechanical inoculation method. The inoculation process was carried out with pestle, sponge, and brush contamination tools. These 20 plants were divided into 5 plants for each inoculation tool and 5 plants used for negative controls.

2.3. Mechanic inoculation of TSWV

The TSWV used in this study is a common strain (TSWVAntRB), which causes black spots containing light and dark-green areas in tomatoes. The tomato leaves infected by TSWV (provided by the Plant Protection Department, Akdeniz University, Türkiye) were used as inoculum for 2 different mechanic inoculation experiments (Table 1).

Table 1. Chemical contents used in two different mechanical inoculation methods.

	Experiment I (0.01 M Phosphate Buffer)	**Experiment II (0.05 M Phosphate Buffer)**
Material and Gradients	0.340 g - KH_2PO_4 0.445 g - Na_2HPO_4-$2H_2O$ 1 g - Carborundum powder	0.5253 g - KH_2PO_4 10.93 g - Na_2HPO_4-$2H_2O$ 1 g - Na_2-NO_3 0.1 ml - Merkaptoetanol

According to the suggestion of Hull (2009), in experiment I, inoculum was prepared by grinding TSWV infected tissues at the rate of 1:6 (wt vol^{-1}) tissue to buffer ratio in freshly prepared ice-cold 0.01 M potassium phosphate buffer, pH 7.0, containing 0.1% carborundum powder with a chilled mortar and pestle. Debris was removed by squeezing the extract through a cotton bud. The inoculum was kept on ice until the inoculation was completed and was applied by rubbing both surfaces of the leaf with a mortar, sponge and brush on tomato plants at four leaf stages (8 to 10 days after planting). After inoculation, the plants were sprayed with distilled water and kept in two growth chambers having the same environmental conditions.

According to the suggestion of Oğuz et al. (2009), in experiment II, inoculum was prepared by grinding infected tissues at a rate of 1:6 (wt vol^{-1}) tissue to buffer ratio in freshly prepared ice cold 0.05 M potassium phosphate buffer, pH 7.0, containing 0.1% mercaptoethanol with a chilled mortar and pestle. Debris was removed by squeezing the extract through a cotton bud. The inoculum was kept on ice until the inoculation was completed and was applied by rubbing both surfaces of the leaf with a mortar, sponge, and brush on tomato plants at four leaf stages (8 to 10 days after planting). Tomato leaves were abraded with carborundum powder, and inoculum was applied by rubbing both surfaces of the leaf with a mortar, sponge, and brush. After inoculation, the plants were sprayed with distilled water and kept in two growth chambers having the same environmental conditions. Distilled water was inoculated into the 5 plant groups in each experiment with the same contamination tools used in mechanical inoculation and was used as a negative control.

2.4. Disease incidence

Plants were scored for the severity of TSWV symptoms on days 7, 14, and 21 after inoculation, using a scale of 1 to 5 modified by Bora and Karaca (1970). Accordingly, they were scored as (0) no symptoms, (1) mild chlorotic spots on the leaf, (2) severe chlorotic spots on healthy leaves, (3) chlorotic spots on the leaf and shortening in height, (4) deformities and color changes in the fruit, and (5) the plant is dead.

2.5. Physiological parameters

All leaves on the plants were counted, and average values were given as 1 per plant. Plant height was determined by measuring the stem length from the soil surface to the stem tip. Stem diameter (mm $plant^{-1}$) was measured with a digital caliper (OEM KMP300, Germany) on three parts of the stem and calculated by the arithmetic mean. Chlorophyll measurements were made in 3 leaves using the SPAD (Konica, Minolta) device, and their averages were taken. Following these measurements, the harvested plants were divided into plant parts (root, leaf, stem). Root systems were carefully washed with tap water to remove soil debris. Root length (cm plant-1) was measured as the distance from the soil surface to the tip of the longest root. Then, the samples were dried in a ventilated oven at 65°C for 72 hours, and their dry weights were recorded (Aksu et al. 2017; Şevik and Cansız 2021; Shokoohi 2023).

2.6. Detection of TSWV by RT-PCR

Total nucleic acid (TNA) samples were extracted from the samples of TSWV infected tomato plants to determine the presence of the TSWV (Svanella-Dumas et al. 2000). The quality of the isolated TNA was measured on the nanodrop, and a range was determined between 1.7 and 2.0. RT-PCR analysis was performed using 1-Step Hot Start Master Mix® (Thermo Fisher, Massachusetts, USA). 12.5 µl Hot-Start Master Mix, 0.5 µl Verso enzyme, and 1.25 µl RT-Enhancer were used for amplification in the PCR stage. An inoculum plant infected with TSWV was used as a positive control. The following PCR-specific primers of

TSWV (Mumford et al. 1994). (F: 5'-AATTGCCTTGCAACCAATTC-3' and R: 5'-ATCAGTCGAAATGGTCGGCA-3') was used. The amplification conditions for the PCR reaction was as follows 50°C 15 min, 95°C 35 min for 1 cycles 94°C for 1 min, 55°C for 1 min, and 72°C 1 min for 35 cycles; 5 min for 72°C and for each 25 µl sample mixture (Fidan and Sari 2019). The data was analyzed using the electrophoresis method.

2.7. *Statistic analysis, sequencing and phylogenetic relationships*

ANOVA was conducted to identify statistically significant variances induced by infections across the measured parameters. An inoculum plant infected with TSWV was used as a positive control. The study used 2 different experiment designs, a fully randomized plot design with 5 replicates, and Duncan's Multiple Range Test (SPSS Statistic Tool) which was used to detect statistically significant variations in means (P<0.05).

In this study, two PCR-positive products from tomato were selected for further analysis.

The two selected PCR products were sent to Macrogen (Istanbul, Turkey) for sanger sequencing. The nucleotide sequences from each chosen sample were obtained using BioEdit software, both in forward and reverse nucleotides. Subsequently, these sequences underwent analysis via BLAST (Basic Local Alignment Search Tool) at NCBI (The National Center for Biotechnology Information) to confirm their identity and compare to other isolates around the world for their phylogenetic relationships (Fidan and Sari 2019).

3. Results

3.1. *Disease incidence and symptom visualization*

Experiment I showed more yellowing symptoms while Experiment II showed necrotic spots. Visual assessment of tomatoes revealed brown necrotic spots on the leaves and stem, drying, and bruising on the lower leaf surface (Figure 1).

From the scale values obtained according to the 0-5 scale, the % disease severity was calculated using the Townsend-Heuberger formul (Bora and Karaca 1970). The obtained symptom and disease scale data were analyzed using the Duncan test in the SPSS program. In the mechanical inoculation method used in Experiment I, pestle and brush contamination tools gave the highest disease scale, while in Experiment II, the highest values were obtained in pestle infection tools (Table 2).

Table 2. Disease scale in two different mechanical inoculation methods

Design/Tools	Total plant	Disease Scale	Duncan[a]
Experiment I - Pestle	5	23%	0.930
Experiment I - Brush	5	24%	0.932
Experiment II - Pestle	5	26%	0.982
Negative control	5	0%	0.000

When TSWV was transferred to tomato plants using two different mechanical inoculation methods, necrotic spots and yellowing on the leaves and black spots on the stem were observed, which are the characteristic symptoms of TSWV. A similar study detected TSWV typical symptoms such as black spots and yellowing on both the fruit and leaves in infected pepper plants (Salamon and Szabó 2016).

3.2. *Physiological parameters*

In Experiment I (Pestle), a 12.22% decrease in the number of leaves was observed with the mechanical inoculation method. In other experiments, a decrease was also observed compared to the negative control plants, but it was not found to be significant. TSWV infection did not cause a significant change in the leaf number in tomato plants where both mechanical inoculation methods were applied. The number of leaves in negative control plants was almost equal. TSWV infection caused a significant change in the chlorophyll content in tomato plants where both mechanical inoculation methods were applied. A significant decrease (P<0.05) was observed compared to the negative control plants. The chylopril content in the negative control

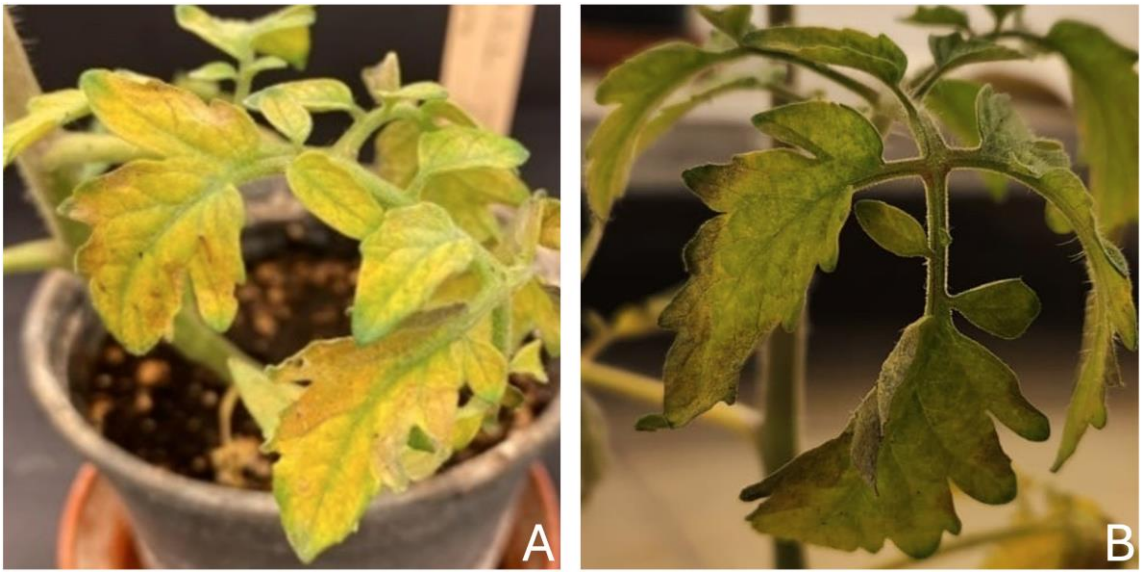

Figure 1. Symptoms triggered on leaves by TSWV infection in tomatoes. Experiment I showed more yellowing symptoms, while Experiment II showed necrotic spots. Experiment I (A); Experiment II (B).

plants showed a value of approximately 40–50 SPAD units. The highest chlorophyll decrease was observed in the mechanical inoculation method in Experiment II (Pestle) with a rate of 69.18% (Table 3).

Among the mechanical inoculation methods, Experiment II (Brush), Experiment II (Pestle), and Experiment I (Pestle) methods caused a significant decrease (P<0.05) in plant height. Experiment II (Sponge) showed the highest plant height compared to other mechanic inoculation methods.

TSWV infection caused a change in the stem diameter in tomato plants where both mechanical inoculation methods were applied. The highest stem diameter change was observed in Experiment I (Pestle), with a rate of 11.22% (Table 4).

In the experiments, a decrease was observed compared to the negative control plants. The most significant decrease was found to be in Experiment II (Pestle). A 29.06% decrease in the root length was observed with the mechanic inoculation method. In virus disease conditions, reductions of plant weight were statistically significant (P<0.05). However, the highest reduction in dry weight in experiments (33.33%) was observed in 'Experiment II (Pestle),' followed by 'Experiment II (Brush)' (28.88%). The results show that TSWV caused a reduction in dry weight (P<0.05) (Table 5).

Tomato spotted wilt orthotospovirus (TSWV) infection induces discernible physiological impacts on economically significant plant species. This investigation scrutinized the effects of a TSWV infection on plant morphology and physiology, particularly focusing on the varietal differences and the efficacy of different mechanical inoculation methods. The results indicated that TSWV-infected plants exhibited diminished plant heights compared to their healthy counterparts, with statistically significant variations among mechanic inoculation methods. Notably, the Experiment II-Brush method caused the most substantial reduction in plant height. Similarly, prior research demonstrated a 3% reduction in plant height due to an infection of Cucumber mosaic virus (CMV) (Salaudeen 2015). Root length was also adversely affected by TSWV infection, corroborated by findings from previous studies on tomato plants (Hossain et al. 2016). Moreover, TSWV exerted physiological repercussions on root area and length (McKinney and Tillman 2017).

Mechanically inoculated TSWV-infected plants exhibited reduced stem diameter, with the experiment I pestle method yielding the most pronounced effects. Consistent with earlier observations (Hossain et al. 2016), reductions in stem diameter were evident in TSWV-infected tomato plants. Furthermore, a decline in stem diameter was noted in TSWV-infected tobacco plants (Trojak-Goluch et al. 2016). Dry weight analysis revealed a significant reduction in TSWV-inoculated tomato plants, with the Experiment II-Pestle method yielding the most notable decrease. TSWV diminished dry weight in infected eggplant plants (Alam and Elçi 2021), while a similar study observed comparable effects in infected pepper plants (Moskova et al. 2021). Chlorophyll content in TSWV-infected plants decreased following mechanical inoculation, consistent with findings by

Table 3. Number of leaves and chlorophyll content in TSWV-infected tomato plants

Experiments/Parameters	Leaf Number (piece plants^{-1})**			Chlorophyll Content**		
	Control	Infected	Change (%)	Control	Infected	Change(%)
Experiment I (Pestle)	44.20	38.80	(-) 12.22	42.50	23.78	(-) 44.00
Experiment I (Brush)	45.50	41.40	(-) 9.01	44.30	26.04	(-)41.12
Experiment I (Sponge)	46.40	46.00	(-) 0.86	39.56	24.04	(-)39.22
Experiment II (Pestle)	42.10	39.00	(-) 7.35	40.12	12.36	(-)69.18
Experiment II (Brush)	40.00	39.80	(-) 0.5	42.57	19.40	(-)54.45
Experiment II (Sponge)	43.50	40.40	(-) 11.21	41.34	21.56	(-)47.80

**(P<0.05) (-) shows decrease.

Table 4. Plant height and stem diameter in different TSWV-infected tomato plants

Experiments/Parameters	Plant height (cm plants^{-1}) **			Stem diameter (mm plant^{-1})**		
	Control	Infected	Change (%)	Control	Infected	Change(%)
Experiment I (Pestle)	54.60	42.60	(-) 21.98	4.545	4.035	(-)11.22
Experiment I (Brush)	57.85	46.40	(-) 19.79	4.560	4.244	(-)6.93
Experiment I (Sponge)	56.70	49.20	(-) 13.23	4.530	4.228	(-)6.67
Experiment II (Pestle)	58.00	44.40	(-) 23.45	4.550	4.636	(+)1.89
Experiment II (Brush)	60.20	42.80	(-) 28.87	4.600	4.256	(-)7.48
Experiment II (Sponge)	59.10	46.40	(-) 21.52	4.400	4.40	(-)3.64

**(P<0.05) (-) shows decrease, (+) shown increase.

Table 5. Root length and dry weights in different TSWV-infected tomato plants

Experiments/Parameters	Root length (cm plants^{-1}) **			Dry weights (g plant^{-1})**		
	Control	Infected	Change (%)	Control	Infected	Change(%)
Experiment I (Pestle)	24.60	19.50	(-) 20.73	2.75	2.35	(-)14.55
Experiment I (Brush)	25.85	21.40	(-) 17.22	2.60	2.10	(-)19.23
Experiment I (Sponge)	24.70	18.90	(-) 23.50	2.53	2.50	(-)1.18
Experiment II (Pestle)	26.50	18.80	(-) 29.06	2.85	1.90	(-)33.33
Experiment II (Brush)	26.20	18.90	(-) 27.86	2.70	1.92	(-)28.88
Experiment II (Sponge)	25.10	18.60	(-) 25.89	2.50	1.88	(-)24.80

**(P<0.05) (-) shows decrease.

Pazarlar et al. (2013) regarding Tobacco mosaic virus (TMV) infection in pepper plants. TSWV-infected plants exhibited a decrease in the number of leaves, but there was no significant decrease compared to healthy ones. A similar study revealed that TMV infection reduced the number of leaves in different cultivars (Pazarlar et al. 2013).

3.3. Molecular analysis and sequencing

The total nucleic acid was isolated from leaf samples taken from TSWV-inoculated plants, and the products were amplified by PCR using TSWV-specific primers. The presence of TSWV was detected in all samples amplified with TSWV-specific primers (Fig. 2).

TSWV demonstrated a sequence identity of 98.65% when compared to the TSWV isolate identified in Zimbabwe (MG602671.1). It also exhibited a 97.30% identity with isolates from Korea (KC261968.1). Similarly, it shared a 97.30% identity with the TSWV isolate found in pepper plants in Italy (MH763621). Furthermore, analysis revealed a 97.30% similarity with the Capsicum annum isolate originating from South Korea (OM022891.1). In addition, the tomato TSWV isolate displayed a 97.30% identity with a tomato isolate from France (MK792774.1) (Fig. 3).

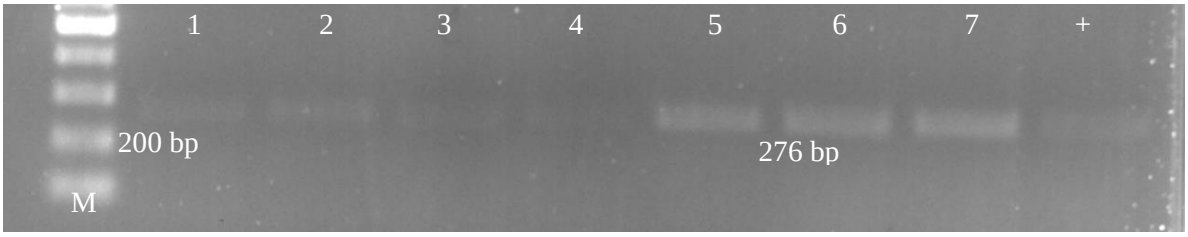

Figure 2. PCR analysis of infected tomatoes with TSWV-specific primers. Marker (100 bp); M, 1,2,3,4,5,6,7; TSWV positive samples, positive control; +.

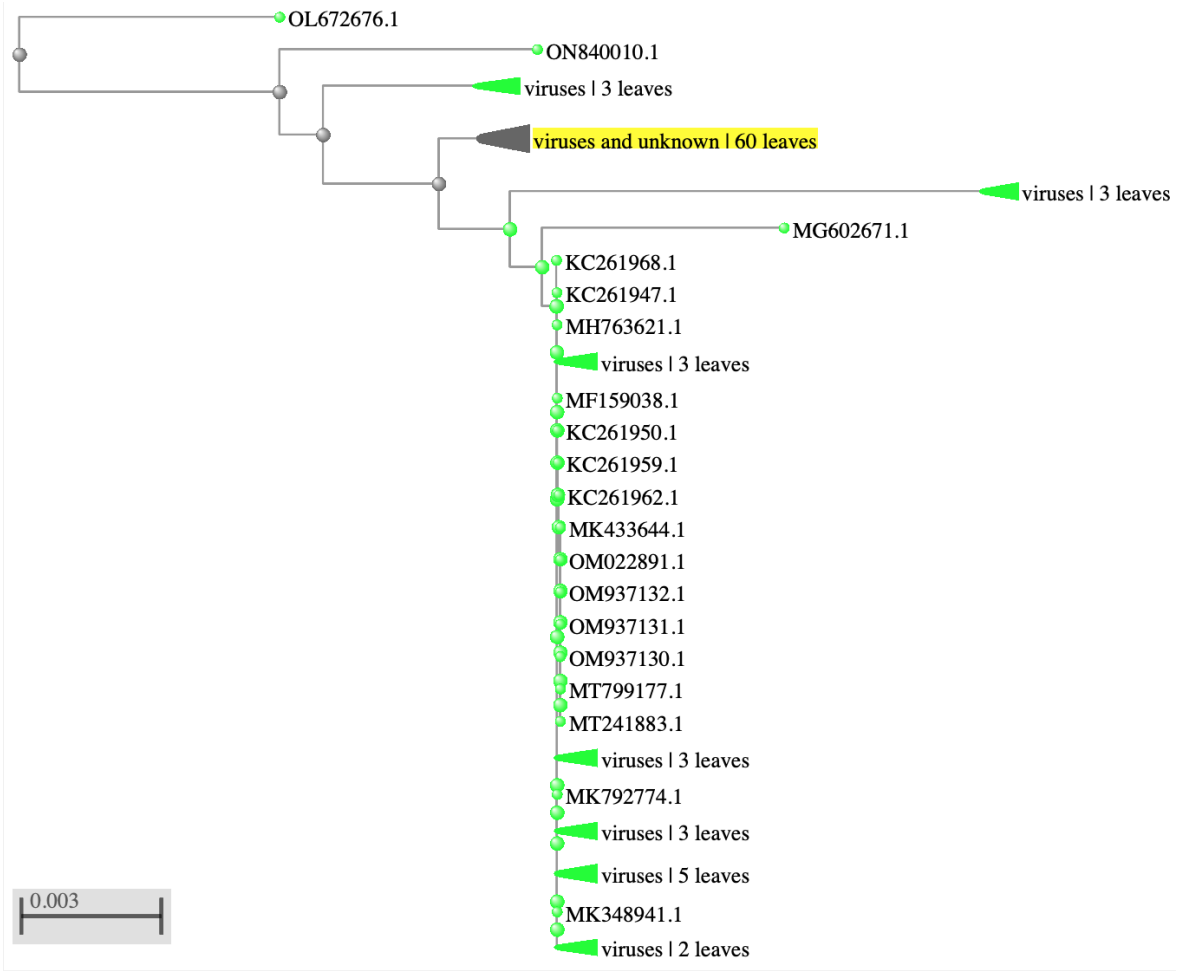

Figure 3. Phylogenetic tree showing the similarity rate of the TSWVAntRB isolates used in the study with other isolates using Sanger sequences. Values greater than 95% are in the tree. Green: Viral sequence groups with the indicated number of leaves. Yellow: mixed cluster of viral and unidentified sequences.

The total nucleic acids were isolated from TSWV-infected plants. PCR was performed with specific primers using TNAs, and the expected band length (276 bp) was observed as a result of gel electrophoresis. These primers for the detection of TSWV were used in similar studies, and the expected band sizes were determined (Erilmez and Oz 2023; Caruso et al. 2024). The TSWV isolates were divided into two origins, namely Asia and Europe (Lian et al. 2013). The sequence comparison of TSWVAntRB isolates with that of isolates in NCBI returned a similarity ratio of 93-98% (López et al. 2011). A similar study shows the similarities emerging with the phylogenetic tree are consistent with Fidan and Sarı (2019), which confirms the accuracy of the results obtained.

4. Conclusions

The efficiency of the two different mechanic inoculation methods for TSWV on tomato plants was examined in the current study. From this study, it was determined that Experiment II has shown a significant negative effect on physical parameters (plant height, stem diameter, root length, dry mass, chlorophyll contents) of tomato plants. In Experiment II, the mechanical inoculation tool pestle achieved the most effective virus transmission. Morever, Experiment I showed a negative effect on the number of tomato leaf reductions of tomato plants, but this effect was not significant.

Specific primers used to detect TSWV in tomato plants showed positive results, while sequence and phylogenetic analyses confirmed the TSWV isolate. Statistical analysis indicated that Experiment II provides more effective virus inoculation by negatively affecting physical parameters in virus-infected plants. Moreover, notable distinctions were observed between two different mechanic inoculation methods.

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