

ENHANCING RNA ISOLATION EFFICIENCY
FOR TOMATO BROWN RUGOSE FRUIT VIRUS
(ToBRFV) DETECTION IN NON-PRIMARY HOST
WEEDS AND DETERMINATION OF HOST POTENTIAL

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Abstract

Tomato brown rugose fruit virus (ToBRFV) is considered an emerging disease and a viral epidemic affecting tomato consumers. The aim of this research is to optimize the isolation of ToBRFV from the weeds *Solanum nigrum* and *Chenopodi album* and to determine its potential for use as a test plant. The detection and study of ToBRFV in these non-primary hosts requires optimized RNA isolation protocols due to challenges such as low viral load, plant inhibitors, and variations in tissue properties. This paper explores innovative approaches to improve the efficiency of RNA isolation from *S. nigrum* and *C. album*, offering a comparative analysis with standard isolation methods and proposing novel solutions to increase RNA yield and purity. By addressing the unique biochemical challenges posed by these plants, our work provides an essential foundation for accurate ToBRFV diagnosis and further virological research. The leaves taken from the samples showing symptoms were applied using SDS-LiCl method with and without phenol, CTAB-LiCl methods and the RNA isolation kit was used. Among the RNA isolation techniques, used in this study, the most suitable one was found as modifying SDS-LiCl from which good quality RNA was able to be isolated. When the two plants were evaluated in terms of their potential as indicator plants, *S. nigrum* plant was

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found to be superior to *C. album* plant in terms of longevity and resistance to different climatic conditions, not only showing local symptoms but also having the potential to carry the virus systemically.

Key words: tomato brown rugose fruit virus, RNA isolation, *Solanum nigrum*, *Chenopodium album*, weeds

Introduction. Tomato is produced both for domestic consumption and for international trade, for this reason, it can be found throughout the year [1]. Global food security faces ongoing risks from emerging plant viruses, which lead to outbreaks that diminish both the yield and quality of the crops essential to food supply chains [2]. Tomato brown rugose fruit virus (ToBRFV) is an emerging RNA virus that primarily infects tomatoes, causing significant crop losses and posing a global threat to tomato production. First identified in greenhouse tomato plants in Jordan during the spring of 2015, the virus was traced back to an outbreak in Israel in 2014 [3, 5, 6, 8]. ToBRFV is currently present in 47 countries and five continents [4, 9].

In general, weeds can adversely affect crops, or they often become the main hosts for plant viruses and serve as insect vectors at crop harvests [10, 11, 14]. Weeds play a significant role in plant viral epidemiology, especially when it comes to virus overwintering and dissemination. ToBRFV may infect agricultural weed species that may allow the persistence of the virus in periods with no tomato production. However, the role of weeds in the disease epidemiology of ToBRFV is unknown. In this context, weeds can serve as reservoirs for the virus, posing a threat to cultivated crops. This is a critical concern, as these plants are commonly found in agro-ecosystems and can act as potential sources of viral inoculum. The risk is even greater if the weeds are asymptomatic, making the presence of the virus harder to detect [12, 15, 16]. Further confirmatory in vivo assays are still required to demonstrate that ToBRFV-infecting weeds can potentially also infect tomato and therefore be relevant to ToBRFV outbreaks [7]. Since its first identification, ToBRFV has spread rapidly, making early detection and molecular characterization essential for controlling its spread. In addition to infecting tomato and pepper, ToBRFV can also be experimentally transmitted to alternative hosts, such as *S. nigrum* and *C. album* [3]. These hosts can serve as model plants for studying virus-host interactions in future. However, the isolation of high-quality RNA from these plants, especially for viral studies, poses several challenges. Suitable integrated ToBRFV management necessarily requires monitoring of potential secondary hosts, by the removal of infected plants, and continuous monitoring of cultural practices [13]. Understanding the role of these alternative hosts is crucial for developing effective disease prevention measures and reducing the risk of ToBRFV transmission in susceptible crops.

Plant gene expression analysis, diagnosis of plant virus diseases cloning, transcriptomic and other research often need to isolate and purify RNA from tissues

and high purity, high concentration and good integrity RNA is always a must [17]. The physical and chemical properties of polysaccharides closely resemble those of RNA, making co-precipitation with polyphenols more likely, which in turn compromises the overall quality of the RNA [15,16,18]. Various commercial kits are also available, although these kits are expensive to use for routine plant disease diagnosis and especially for a large number of samples [19].

This study aims to evaluate and optimize existing RNA extraction protocols to improve the yield and quality of viral RNA from *S. nigrum* and *C. album*, enabling better detection and characterization of ToBRFV. Also the other aim of this study was to discover new weed-virus interactions.

Materials and methods. Plant material and virus inoculation. *S. nigrum* and *C. album* plants were grown under controlled greenhouse conditions at the Faculty of Agriculture, Erciyes University. ToBRFV-infected leaf samples were obtained by mechanical inoculation using sap extracted from infected tomato plants. The plant tissue of symptomatic leaves was first homogenized into 0.01 M sodium phosphate buffer pH 7.0 (1 g tissue to 1 mL buffer). The resulting extract was rubbed on carborundum-dusted leaves of *S. nigrum* and *C. album* plants at the four-leaf stage [8, 11]. Non-infected plants were used as negative controls. In mechanical inoculation, a ToBRFV isolate was used (NCBI accession number PQ314730). Leaf samples of *C. album* and *S. nigrum* plants from above and below the inoculated leaf were collected for nucleic acid extractions 4 weeks after inoculations. The symptoms were also recorded.

Molecular characterization of ToBRF isolates. PCR amplicons were sequenced directly and analyzed using the basic local alignment search tool (BLAST). Multiple alignments of the nucleotide sequences of these isolates and reference isolates for each ToBRFV strain obtained from GenBank were performed using BioEdit. Cluster analysis was done with the Mega X program using the neighbour-joining method with nucleotide identity distances. Bootstrap analyses with 1000 replicates were performed to estimate the support for inferred phylogenies.

RNA extraction protocols. Four different isolation protocols were tested to determine the most effective method for isolating ToBRFV RNA from experimental hosts. The following methods were evaluated: SDS-LiCl: This method follows the SDS-Phenol based extraction and RNA precipitation with sodium acetate and LiCl [20]; CTAB-LiCl (Cetyltrimethylammonium Bromide) Method: Commonly used for plants with high levels of polysaccharides and phenolics [20]. Modified SDS-LiCl Precipitation Method: Specifically optimized for viral RNA isolation and GeneJET Plant RNA Purification Mini Kit (Thermo Fisher Scientific, USA) for the isolation of RNA. These applications have been performed differently from the SDS-LiCl method. The tissue (0.12 g) was ground (0.12 g) into a fine powder in mortar and pestle using liquid nitrogen. Afterwards, 600 μ L RNA extraction buffer was added to the ground tissue and the crushing process was continued, ensuring that the tissue was completely disintegrated. If tissue

does not dissolve well, the extraction buffer volume can be increased up to 1 mL and phenol was not used. RNA quality was assessed using spectrophotometry (A260/A280 and A260/A230 ratios) and agarose gel electrophoresis. RNA yield was quantified using a NanoDrop spectrophotometer (Shimadzu spectrophotometer, Japan). The presence and integrity of ToBRFV RNA were confirmed via RT-PCR using ToBRFV-specific primers.

Results and discussion. *Phylogenetic analyses of ToBRF isolates.*

When the sequences of PCR amplicons obtained from ToBRFV isolates were analyzed, 99% identity with the PQ282223.1 (Eskisehir province of Turkey), OR792460.1 (China) and OM515235.1 (Netherlands) isolates was detected. The phylogenetic analyses showed a similarity within the cluster contained in isolate Acc. No. OR792460.1 from China, obtained from tomato and isolate Acc. No. OM515235.1 from Netherlands, obtained from tomato (Fig. 1).

Symptomological observations on tomato brown rugose fruit virus inoculated *Solanum nigrum* and *Chenopodi album* plants. Inoculated *S. nigrum* plants with ToBRFV were systemically infected in the newly emerged two top leaves 10–15 days post-inoculation. Initial symptoms included mild chlorosis, followed by more pronounced mosaic patterns on the upper leaves. As the infection progressed, leaves exhibited mild curling (Fig. 2).

C. album exhibited symptoms more rapidly than *S. nigrum*, with visible signs appearing 5–7 days post-inoculation. The symptoms were chlorotic spots and chlorotic rings on the inoculated leaves. These symptoms expanded to non-inoculated leaves, nonindicating systemic infection. Symptomatic leaves turned completely yellow and fell off in a short time (Fig. 2).

In this study, it was observed that ToBRFV caused different symptoms in *S. nigrum* and *C. album* plants. Both plant species are susceptible to the virus and show signs of infection. However, the differences in symptoms between *S. nigrum* and *C. album* plants suggest that these plants show different susceptibilities to ToBRFV. There was no transmission by seed in ToBRFV inoculated *C. album* plants. These observed differences in symptom development suggest that *S. nigrum* may support systemic ToBRFV infection, while *C. album* is more susceptible to localized infection and may lack the capability to facilitate virus movement to non-inoculated tissue. This distinction implies that *S. nigrum* could serve as a more effective host in long-term studies of ToBRFV's systemic behaviour, while *C. album* might be a better model for studying localized resistance mechanisms. Furthermore, the resilience of *S. nigrum* displaying intense symptoms yet surviving over 240 days and tolerating extreme environmental conditions indicates its utility as a stable inoculum source for studying ToBRFV transmission and virulence under variable conditions. Weeds may act as an alternative host of phytopathogens, serving as sources of inoculum and playing a significant role in disease epidemiology [11]. In a study conducted by SALEM et al. [8] *S. nigrum* was similarly identified as the host of ToBRFV. *S. nigrum* is known to naturally host

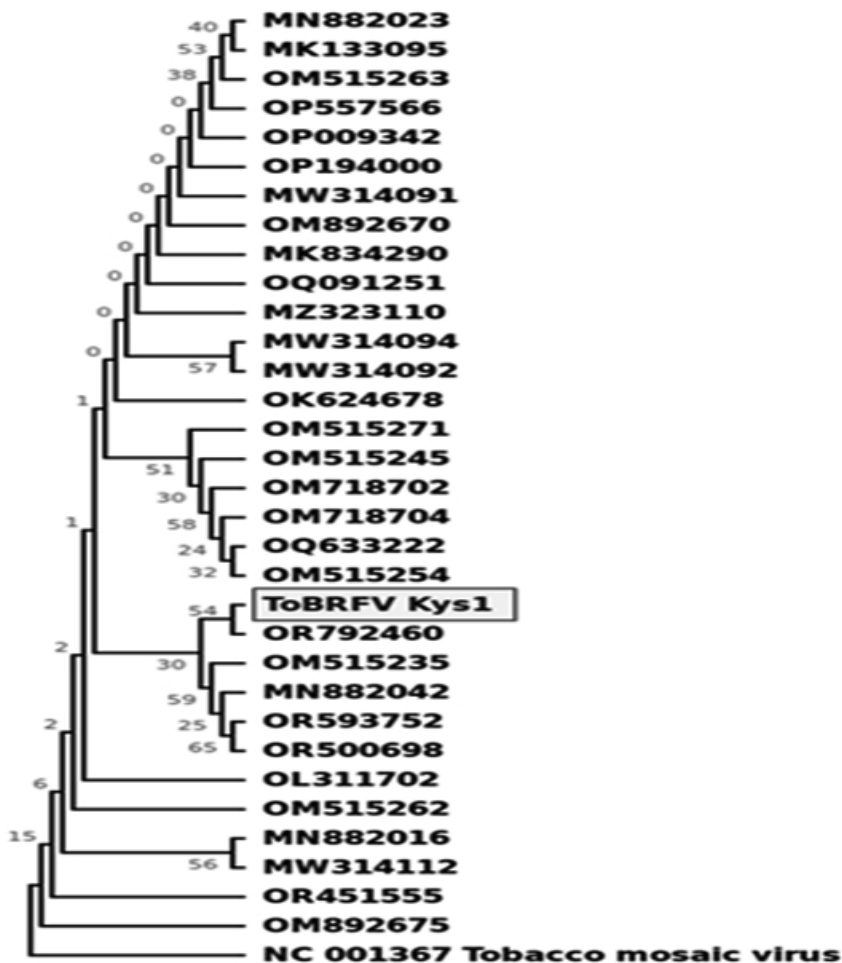


Fig. 1. Phylogenetic analysis of ToBRFV isolates using neighbour-joining method with 1000 bootstrap value. Samples studied in the current experiment were shown as blue square. The rest of the sequences were downloaded from NCBI database

many plant viruses [8]. However, the suitability of *S. nigrum* as an experimental host for ToBRFV has not been evaluated.

ToBRFV inoculated *S. nigrum* plant showed intense symptoms and survived for more than 240 days. Also, the resistance of the solanum plant to extreme conditions (e.g., drought, high temperature) was better than of the tobacco plant. Using the *S. nigrum* plant as an inoculum source, we applied the inoculum obtained from this plant to the *Nicotiana tabacum* Cv. Samsun indicator plant by mechanical inoculation. Intense chlorotic spots were observed on *Nicotiana tabacum* Cv. Samsun plant. The use of *S. nigrum* as a source of inoculum may allow for the study of its effects on various biological processes and potential applications in plant pathology research.

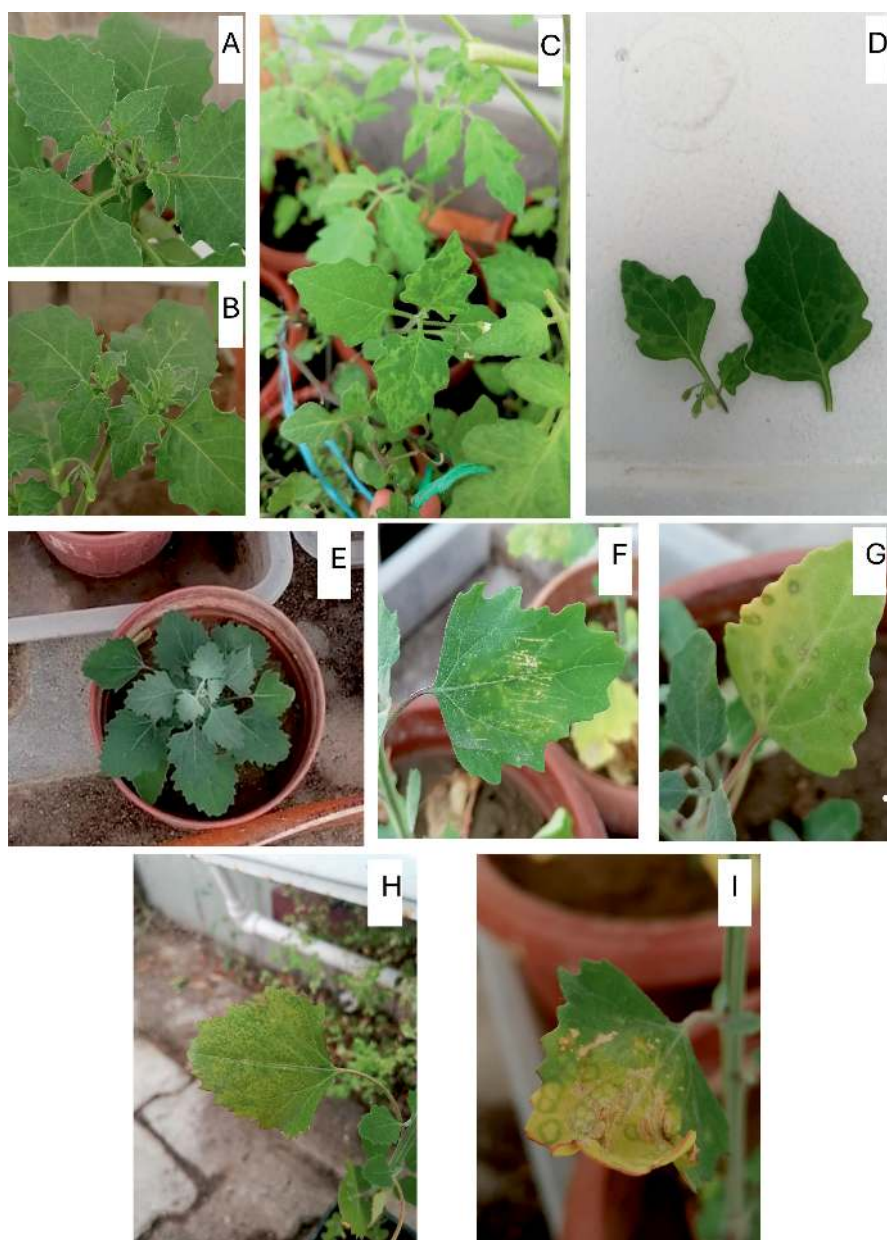


Fig. 2. ToBRFV on *S. nigrum* and *C. album* plant (A: Virus uninoculated *S. nigrum* plant; B, D: dark green blistering; C: mosaic-like symptoms, E: *C. album* plant without virus inoculation; F: chlorotic spots; G, I: chlorotic ring spots; H: dark green mottling)

RNA yield and quality. Different methods for extracting RNA from different plants have varying degrees of effectiveness [17]. The physical and chemical properties of polysaccharides closely resemble those of RNA, making co-precipitation with polyphenols more likely, which in turn compromises the

overall quality of the RNA. Studies have shown that *C. album* and *S. nigrum* contain many polysaccharides, phenols, proteins and other secondary metabolites [15,16,18].

Using conventional RNA extraction methods it is difficult to obtain high quality RNA from *S. nigrum* and especially *C. album*. By trying SDS-LiCl [20] and CTAB-LiCl to extract *C. album* and *S. nigrum* RNA, the results were not ideal. Using GeneJET Plant RNA Purification Mini Kit to extract these plants RNA, the results were suitable, but the price for purchasing commercial kits was relatively high. Thus, our goal was to develop an easy, affordable, and effective RNA extraction process so that plants strong in polysaccharides and polyphenols could yield high-quality RNA.

In this study, extracted total RNA was verified by quantitative nucleic acid detection and RT-PCR analysis. The concentration of total RNA was detected. OD value as one of the ways of detecting the purity of RNA has been widely used [17].

RNA isolated from the T1 sample with the commercial kit was with low concentration, indicating very little nucleic acid, compared to other samples. OD260/280 was extremely high, suggesting significant contamination, possibly from residual solvents or proteins. A low OD260/230 ratio indicated that the sample was heavily contaminated with substances such as phenol, salts, or organic solvents. The T2 sample showed a moderate nucleic acid concentration, but its quality was compromised due to significant contamination. The low OD260/280 ratio suggested the presence of protein impurities, while the very low OD260/230 ratio indicated substantial contamination with salts, phenols, or other organic solvents. The T3 and T4 samples had a high nucleic acid concentration, which was suitable for many applications. However, the OD260/280 ratio is slightly elevated, suggesting possible minor impurities in RNA. T3 and T4 samples were of decent quality with good nucleic acid concentrations. In contrast, T1 and T2 showed significant contamination, especially with proteins and organic compounds (Table 1).

The T1, T2, T3, and T4 samples show high nucleic acid concentrations and acceptable OD260/280 ratios, suggesting minimal contamination using the Modified SDS-LiCl method. The OD260/230 ratio is also favourable, indicating low levels of contamination (Table 1). This method also demonstrated better removal of contaminants, which is crucial for sensitive molecular techniques. RNA from *C. album* was more difficult to isolate cleanly than from *S. nigrum*, likely due to higher levels of secondary metabolites, but the optimized protocols showed considerable improvement over standard methods.

RT-PCR analyses of RNAs isolated using different isolation methods. RNA isolated using the modified SDS-LiCl method showed superior performance in downstream applications, with clearer RT-PCR bands for ToBRFV-specific amplification (Fig. 3).

T a b l e 1

Concentration and purity of total RNA isolated from *C. album* and *S. nigrum* leaves using the GeneJET Plant RNA Purification Mini Kit, Modified SDS-LiCl method, SDS-LiCl method, CTAB-LiCl method

	No.	Plant organ	OD260/280	OD260/230	Concentration (ng/μL)
GeneJET Plant RNA Purification Mini Kit	T1	<i>C. album</i> leaf	3.20	0.24	65.56
	T2	<i>C. album</i> leaf	1.61	0.67	129.58
	T3	<i>S. nigrum</i> leaf	2.13	1.23	160.29
	T4	<i>S. nigrum</i> leaf	2.14	1.77	177.22
	P	<i>S. lycopersicum</i> leaf	2.22	1.66	141.31
Modified SDS-LiCl method	T1	<i>C. album</i> leaf	2.45	1.36	110.78
	T2	<i>C. album</i> leaf	2.17	2.59	108.44
	T3	<i>S. nigrum</i> leaf	2.13	1.63	149.91
	T4	<i>S. nigrum</i> leaf	2.38	1.68	167.45
	P	<i>S. lycopersicum</i> leaf	2.14	2.45	124.13
SDS-LiCl method	T1	<i>C. album</i> leaf	4.76	0.48	5.24
	T2	<i>C. album</i> leaf	3.82	0.53	22.57
	T3	<i>S. nigrum</i> leaf	2.34	1.47	14.12
	T4	<i>S. nigrum</i> leaf	3.62	0.64	9.52
	P	<i>S. lycopersicum</i> leaf	2.19	1.70	36.45
CTAB-LiCl method	T1	<i>C. album</i> leaf	0.80	0.14	0.94
	T2	<i>C. album</i> leaf	4.89	0.35	2.10
	T3	<i>S. nigrum</i> leaf	1.78	0.51	20.47
	T4	<i>S. nigrum</i> leaf	2.07	0.69	13.13
	P	<i>S. lycopersicum</i> leaf	2.71	0.75	61.18

When the RNA isolation steps with the SDS-LiCl method were applied without using Phenol, higher quality RNA isolation was achieved in both *C. album* and *S. nigrum*. The results of RT-PCR analyses performed on cDNA libraries constructed using these RNAs as templates confirm this fact. RT-PCR amplification of ToBRFV was more reliable and sensitive with RNA isolated using the optimized protocol, indicating its suitability for both diagnostic and research applications. Modified SDS-LiCl method described here provided a robust method to obtain high-yield and high-quality RNA from *C. album* leaves rich in bioactive compounds, such as polysaccharides and polyphenols.

Since plant species are diverse, no universal protocol has been described to isolate nucleic acid from a wide range of species. Nucleic acid can be extracted from plant tissues using a variety of techniques, most of which are expensive, time-consuming, and not generally practical. In general, RNA extraction protocols and commercial kits are limited to specific species and/or tissues, and commercial diagnostic kits have been predominantly used in studies related to the detection of ToBRFV [7, 20].

When comparing RNA isolation methods, the Modified SDS-LiCl method demonstrated distinct advantages over the SDS-LiCl and CTAB-LiCl particularly

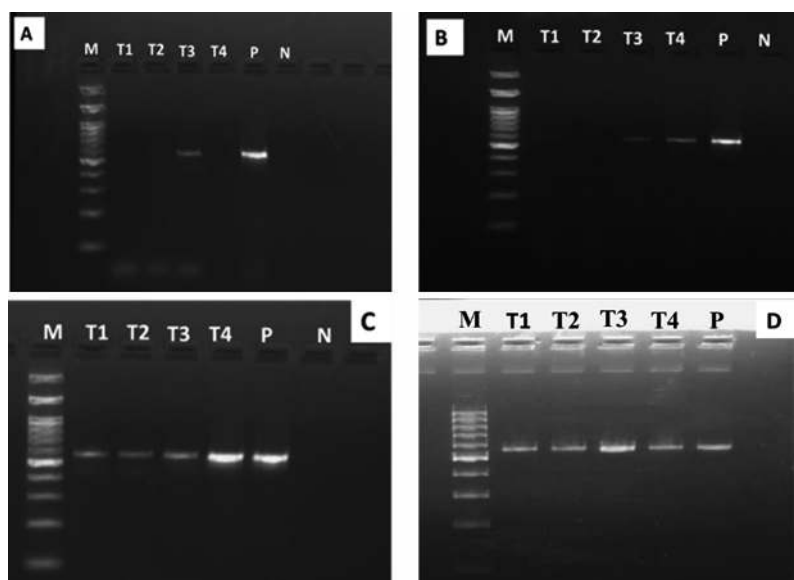


Fig. 3. RT-PCR analysis performed using ToBRFV-F/R primers (M: DNA ladder, T1, T2: *C. album*, T3, T4: *S. nigrum*, P: positive control +, N: negative control -) (A: SDS-LiCl method; B: CTAB-LiCl; C: Modified SDS-LiCl method; D: GeneJET Plant RNA Purification Mini Kit)

in terms of purity and yield. The modified SDS-LiCl method gave similar results to the commercial kit. Various commercial kits are also available, although these kits are expensive to use for routine plant disease diagnosis and especially for a large number of samples [19]. The comparison of the two RNA isolation methods demonstrates clear differences in RNA yield and purity. The GeneJET Plant RNA Purification Mini Kit yielded RNA with acceptable concentrations for some samples (T3, T4, and P) but showed significant contamination in T1 and T2, as indicated by low OD260/230 ratios and deviations in OD260/280 ratios. In contrast, the Modified SDS-LiCl method produced RNA with higher purity across all samples, as reflected in more balanced OD260/280 and OD260/230 ratios, particularly for T1 and T2, which showed marked improvement compared to the commercial kit.

Conclusion. The findings of this study highlight the importance of optimizing RNA extraction methods for the detection of ToBRFV in non-primary hosts. The Modified SDS-LiCl method demonstrated significant improvements in RNA yield and purity compared to conventional extraction techniques. Among the tested plants, *S. nigrum* exhibited systemic infection with ToBRFV, making it a more suitable indicator plant for virological studies, while *C. album* displayed localized symptoms and rapid leaf deterioration. The challenges in extracting RNA from *C. album* were likely due to its higher levels of secondary metabolites, yet the optimized method successfully enhanced RNA purity and reduced contamination. Additionally, RT-PCR analysis confirmed that the RNA isolated

using the Modified SDS-LiCl method provided clearer and more reliable results, emphasizing its suitability for molecular diagnostics. Given that weeds can serve as viral reservoirs, the study underscores the need for continued monitoring of alternative hosts to prevent ToBRFV transmission in agricultural systems. The proposed RNA isolation method offers a cost-effective and efficient alternative to commercial kits, making it a valuable tool for future plant virology research.

Overall, this study underlines the necessity to reconsider control measures in tomato growing practices before planting the following growth cycle since weeds might carry a primary inoculum of ToBRFV. The study presents an optimized RNA isolation protocol that significantly improves the yield and purity of RNA from *S. nigrum* and *C. album*, enabling more efficient detection and characterization of ToBRFV. This protocol can serve as a foundation for future research into ToBRFV and other plant viruses, ultimately contributing to better disease management in agriculture.

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