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Tomato brown rugose fruit virus spreads through pruning, contaminated soil and water

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Abstract

Tomato brown rugose fruit virus (ToBRFV; *Tobamovirus fructirugosum*), a newly identified member of the genus *Tobamovirus*, has rapidly spread across all continents since its first report in 2015, causing severe damage to global tomato production. However, the transmission characteristics of this virus remain insufficiently studied, and field research on ToBRFV transmission pathways at a large scale is often challenging due to numerous uncontrollable factors under natural conditions. In this study, we constructed ToBRFV infectious clones and systematically simulated the effects of pruning practices, plant debris retention, soil reuse, and irrigation with contaminated water on viral transmission under controlled greenhouse conditions. Disease incidence was quantitatively assessed at multiple time points following infection through different transmission routes. Our results demonstrate that mechanical contact, soil, and contaminated water irrigation can all mediate ToBRFV transmission with varying efficiencies. By establishing a small-scale greenhouse simulation platform, this study provides evidence that multiple factors collectively contribute to ToBRFV dissemination. These findings significantly advance our understanding of the viral transmission mechanisms in agricultural settings and offer critical insights for developing effective control strategies.

Keywords – greenhouse – infectious clone – transmission characteristics

Introduction

Viruses classified in the genus *Tobamovirus* (family *Virgaviridae*) include several economically significant pathogens, such as tobacco mosaic virus (TMV; *Tobamovirus tabaci*), tomato mosaic virus (ToMV; *T. tomatotessellati*), cucumber green mottle mosaic virus (CGMMV; *T. cucumeris*) and tomato brown rugose fruit virus (ToBRFV; *T. fructirugosum*) (Dombrovsky & Smith 2017, Zhang et al. 2022). These viruses are very stable and can remain intact in plant debris, soil, agricultural tools, building surfaces, and worker facilities, surviving for extended periods even under extreme environmental conditions (Broadbent & Fletcher 1963, Broadbent et al. 1965). Tobamoviruses may be transmitted by many routes, including seeds, mechanical contact, bees, infected soil residues, and contaminated water or aerosols (Broadbent 1965a, Broadbent & Fletcher 1963, Broadbent 1965b, Broadbent et al. 1965, Castello et al. 1995, Fillhart et al. 1997, Okada et al. 2000, Dombrovsky & Smith 2017, Darzi et al. 2020).

Seedborne infection by tobamoviruses is prevalent, with infectivity persisting in seeds for several years. Although most tobamoviruses have relatively low seed transmission rates, even minimal transmission can facilitate disease spread or pose critical threats to newly cultivated areas (Dombrovsky & Smith 2017). Viral seed transmission primarily occurs through infected embryos but tobamoviruses mainly infect the seed coat (testa) and endosperm (Broadbent 1965a, Reingold et al. 2014). Seed transmission of these viruses is largely mechanically mediated, for example, through root damage during seedling transplantation, which renders roots susceptible to infection by contaminated seed coats (Dombrovsky & Smith 2017).

Under natural field conditions, tobamoviruses can be directly transmitted from infected to healthy plants when leaves rub against each other due to wind action. Additionally, indirect transmission may occur through virus-contaminated hands, pruning tools, clothing, equipment, or even animals that have contacted infected plant sap (Frane & Bantarri 2001).

Tobamoviruses can also persist long-term in infected plant debris (leaves, roots, fruits, etc.) or soil organic matter. For instance, tobacco mosaic virus survived nearly two years in root debris within soil, and for even longer in dry soil (Fletcher 1969, Broadbent 1976). Similarly, cucumber green mottle mosaic virus, which infects cucurbit crops, remained viable in soil for 17 months after removal of infected watermelon plants (Lovelock et al. 2022). Soil contamination by tobamoviruses primarily results from the incorporation of infected plant residues during successive crop cycles (Broadbent et al. 1965). When healthy plant roots grow and contact virus-infected debris, the pathogens enter through micro-wounds or root hairs. Once root infection occurs, tobamoviruses can systemically spread to the aboveground parts of infected plants (Broadbent 1965b, Allen 2009).

Several studies have detected plant viruses, particularly highly stable tobamoviruses, in environmental water samples. Tobacco mosaic virus has been isolated from several rivers in Germany and Italy, as well as from two forest ditches and a stream in Croatia (Koenig & Lesemann 1985, Piazzolla et al. 1986, Pleše et al. 1996). Infectious tomato mosaic virus was obtained from various water sources in Croatia, Germany, and New York, USA (Büttner & Nienhaus 1989, Jacobi & Castello 1991, Pleše et al. 1996). Remarkably, tomato mosaic virus RNA was detected in 500- to approximately 140,000-year-old glacial ice cores drilled in Greenland (Castello et al. 1999), and infectious tomato mosaic virus particles were identified even in clouds and fog (Castello et al. 1995). Bačnik et al. (2020) identified multiple tobamoviruses, including tobacco mosaic virus, tomato mosaic virus, pepper mild mottle virus (PMMoV; *Tobamovirus capsici*), and tobacco mild green mosaic virus (TMGMV), in influent and effluent water from a wastewater treatment plant in central Slovenia, with some viruses confirmed to be infectious (Bačnik et al. 2020). Additionally, pepper mild mottle virus has been found in seawater and river water (Rosario et al. 2009, Hamza et al. 2011). In hydroponic systems, tomato mosaic virus released from infected plant roots into nutrient solutions can infect other plants through root uptake without requiring aerial contact or mechanical transmission, subsequently inducing disease symptoms (Pares et al. 1992).

Certain insects can mechanically transmit tobamoviruses. For instance, Okada et al. detected tobacco mosaic virus in bumblebees (*Bombus terrestris*), pollen loads, and hive materials, demonstrating that bumblebees can transmit tobacco mosaic virus among tomato plants, facilitating virus spread in greenhouse environments (Okada et al. 2000). Darzi et al. found that the important pollinator *Apis mellifera* (Italian honeybee) can transmit cucumber green mottle mosaic virus (Darzi et al. 2018). Under laboratory conditions, Qi et al. confirmed that *Myzus persicae* (green peach aphid) can transmit cucumber green mottle mosaic virus between *Nicotiana benthamiana* plants through physical contact (Qi et al. 2021).

Since its initial detection in 2014 in commercial tomato greenhouses in Israel, tomato brown rugose fruit virus has spread to over 35 countries worldwide, with severe impacts on tomato production (Salem et al. 2016, Luria et al. 2017, Zhang et al. 2022, Salem et al. 2023). For decades, the *Tm-2²* resistance allele had provided commercial tomato varieties with broad-spectrum resistance against tobamoviruses (Lanfermeijer et al. 2004) but tomato brown rugose fruit virus is able to overcome this resistance (Luria et al. 2017). Tomato brown rugose fruit virus is known to infect over 40 plant species across four families (*Amaranthaceae*, *Apocynaceae*, *Asteraceae*, and

Solanaceae), although tomato and pepper are the only known naturally infected agricultural crops (Zhang et al. 2022, Salem et al. 2023). Comprehensive and precise analysis of the transmission characteristics of tomato brown rugose fruit virus is a prerequisite for developing effective containment strategies. Existing research indicates that viral particles are primarily present on the seed surface (seed coat) rather than inside the embryo, resulting in an extremely low actual seed-to-seedling transmission rate (0.08%–2.8%) (Davino et al. 2020, Salem et al. 2022). However, the 100% contamination rate on the surface of seeds from ToBRFV-infected fruits leads to further dissemination and spread of the disease in new growing areas (Salem et al. 2022). The virus can persist for extended periods on various greenhouse surfaces and is difficult to completely remove through routine handwashing (Skelton et al. 2023). Significant progress has been made in studying transmission via environmental media, confirming that contaminated soil (Klein et al. 2023, Molad et al. 2024) and irrigation water (Mehle et al. 2023) are effective sources of transmission. As an emerging pathogen, the transmission characteristics and epidemiological patterns of tomato brown rugose fruit virus remain insufficiently characterized and lack systematic investigation. Under natural conditions, conducting large-scale field studies on the transmission routes of tomato brown rugose fruit virus is often challenging due to numerous uncontrollable factors. In this study, we utilized an infectious tomato brown rugose fruit virus clone to establish a small-scale greenhouse simulation platform. Under highly controlled conditions, we systematically simulated the effects of agricultural practices (pruning), retention of diseased plant residues, soil reuse, and irrigation with contaminated water on tomato brown rugose fruit virus transmission. The results confirmed that, like other tobamoviruses, tomato brown rugose fruit virus can be transmitted through pruning activities, contaminated soil and water. We further quantified disease incidence rates in test plants at various time points following different transmission routes. These findings provide robust experimental data and theoretical foundations for understanding tomato brown rugose fruit virus transmission dynamics and developing effective disease management strategies.

Materials & Methods

Plant growth conditions

Tomato (cultivar: 'Fenguan') and *N. benthamiana* plants were cultivated in a greenhouse under 16-hour light/8-hour dark cycles at 24°C.

Construction of tomato brown rugose fruit virus infectious clone

Three pairs of primers targeting conserved regions of the tomato brown rugose fruit virus genome were designed. Three genomic fragments of tomato brown rugose fruit virus were amplified from pepper disease samples collected in Yuanmou County, Yunnan Province. A near-full-length tomato brown rugose fruit virus genome sequence was obtained by overlap PCR. Subsequently, this full-length tomato brown rugose fruit virus genome was ligated into the pCB301 vector via homologous recombination to generate the tomato brown rugose fruit virus infectious clone.

Preparation of tomato brown rugose fruit virus inoculum

The tomato brown rugose fruit virus infectious clone was infiltrated into leaves of 4-week-old *N. benthamiana* plants. Approximately 7 days post-inoculation, newly emerged symptomatic leaves from the top of plants were collected and used as the virus source for tomato brown rugose fruit virus transmission experiments.

Tomato brown rugose fruit virus transmission experiment

Mechanical transmission experiment of tomato brown rugose fruit virus

To realistically simulate pruning operations in agricultural production, this experiment was designed using sterilized surgical scissors to cut the apical leaves of tomato plants and detect

whether tomato brown rugose fruit virus could be transmitted. Tomato seedlings grown in potting soil were used when they developed 4 true leaves. For the control group, the apical leaves of healthy tomato plants were first cut, and then the same scissors were used to continuously cut the apical leaves of 30 healthy seedlings (one pot). For the experimental group, scissors were first used to cut diseased plants infected with tomato brown rugose fruit virus, followed by the same operation, simulating pruning under normal conditions and tomato brown rugose fruit virus-contaminated conditions, respectively. The experiment included two cutting frequencies: the first involved making two initial cuts on the apical leaves of healthy or infected tomato plants, followed by making two consecutive cuts on each subsequent test plant; the second increased the cutting frequency to four times, making two initial cuts on the apical leaves of healthy or infected plants, followed by making four consecutive cuts on each test plant. Sampling was performed at 3, 6, 9, 12, 15, and 30 days after the cutting operation.

Soil-borne transmission experiment of tomato brown rugose fruit virus

This experiment simulated the residue of diseased plant material in soil by thoroughly grinding infected *N. benthamiana* leaves and mixing them with potting substrate at a ratio of 20 g leaves per 2 kg substrate. The control group consisted of potting substrate mixed with the same quantity of ground healthy *N. benthamiana* leaves. Healthy seedlings were transplanted into the prepared substrates, with special care taken to minimize root damage during transplantation and to prevent any contact between seedling leaves and the soil. Sampling was conducted at 5, 10, 15, 20, and 30 days after the first true leaves had fully expanded.

Tomato brown rugose fruit virus-contaminated soil replanting experiment

For both control and experimental groups, 30 healthy tomato seedlings (with 4 true leaves) of similar growth status were agroinfiltrated with either pCB301-GUS (control) or pCB301-ToBRFV (experimental group). After 23 days (when tomato brown rugose fruit virus had systemically infected the experimental plants), all tomato plants from both groups were uprooted, and residual roots in the soil were carefully removed using a mesh filter.

The processed soils from both control and experimental groups were then repacked into small pots. Healthy tomato seedlings were transplanted into these pots, with special care taken to minimize root damage during transplantation and to prevent any contact between seedling leaves and the potting mix. Sampling was performed at 5, 10, 15, 20, and 30 days after the first pair of true leaves had fully expanded.

Tomato brown rugose fruit virus transmission through contaminated water experiment

Newly germinated tomato seedlings were transplanted into potting mix in the greenhouse, with special care taken to minimize root wounding during the process. For the experimental group, 20g of tomato brown rugose fruit virus-infected *N. benthamiana* leaves were thoroughly ground and mixed into 1L of water, which was then applied through bottom irrigation to cultivate the tomatoes. The control group received the same treatment using healthy *N. benthamiana* leaf extract. Watering was performed at 5-day intervals. Sampling time points were set at 5, 10, 15, 20, and 30 days after the first pair of true leaves had fully expanded.

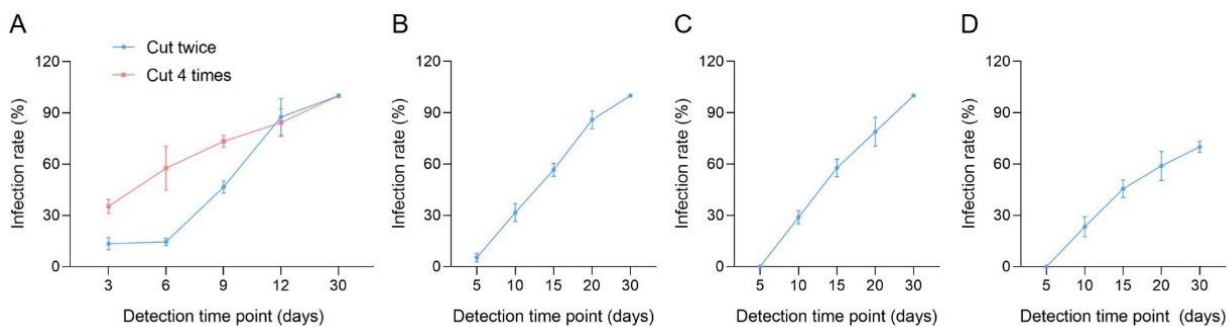
In all the aforementioned transmission experiments, both the control and experimental groups consisted of 30 tomato plants each, with three biological replicates established. During sampling, one newly emerged apical leaf was collected from each tomato plant. Prior to each sampling, surgical scissors and forceps were sterilized with 75% ethanol. Total RNA was then extracted from the tomato samples using the TRIzol method, followed by reverse transcription into cDNA. PCR amplification was performed using tomato brown rugose fruit virus CP-specific primers (Forward: 5'-ATGTCTTACACAATCGCAACTC-3'; Reverse: 5'-CCATTGTAAACCGGATGCAC-3'). The amplification products were separated by 1% agarose gel electrophoresis and visualized under a UV imaging system. Samples displaying bands were recorded as positive, while those without

bands were recorded as negative, after which the corresponding disease incidence rates were calculated.

Results

To study the transmission pattern of tomato brown rugose fruit virus under controlled greenhouse conditions, we first constructed an infectious clone of tomato brown rugose fruit virus, with the specific method referred to in the "Materials & Methods" section. Experiments involving inoculating *N. benthamiana* and tomato plants with this infectious clone demonstrated that the clone possesses infectivity. Subsequently, we utilized this infectious clone to conduct experiments analyzing the transmission characteristics of tomato brown rugose fruit virus.

To simulate pruning operations in agricultural production, this study designed experiments using both sterile and tomato brown rugose fruit virus-contaminated surgical scissors to perform apical leaf excision of healthy tomato plants. Subsequent monitoring confirmed viral transmission through this mechanical contact. When healthy tomato seedlings were pruned twice with contaminated scissors, disease incidence reached 10% by day 3 post-inoculation (dpi), progressively increasing to 100% by day 30 (Figure 1A). Quadruple pruning with infected scissors demonstrated similarly progressive infection patterns, with significantly higher incidence rates at 3, 6, and 9 dpi compared to doubly-pruned plants (Figure 1A). Control plants pruned with sterile scissors had no tomato brown rugose fruit virus infection, as expected. These results conclusively demonstrate that tomato brown rugose fruit virus can be efficiently transmitted through contaminated pruning tools.



Figs 1 – Tomato brown rugose fruit virus can be transmitted through contaminated tools, soil, and water. (A) Infection rates at different time points in the pruning experiment. (B) Infection rates at different time points in the soil transmission experiment. (C) Infection rates at different time points in the replanting experiment. (D) Infection rates at different time points in the water transmission experiment. Detailed experimental methods and settings are provided in the materials and methods. The x-axis represents the selected time points for calculating the incidence rate, while the y-axis indicates the tomato brown rugose fruit virus infection rate detected by RT-PCR. Each experiment was performed with three biological replicates.

To investigate whether tomato brown rugose fruit virus can be transmitted through contaminated soil, infected *N. benthamiana* leaves were thoroughly ground and mixed into potting substrate to simulate the residual presence of diseased plant material in soil. The control group consisted of potting substrate mixed with an equivalent amount of healthy *N. benthamiana* leaf homogenate. Healthy tomato seedlings were transplanted into these substrates containing different *N. benthamiana* leaf mixtures and cultivated. Samples were collected at 5, 10, 15, 20, and 30 days after the emergence of the first true leaves. As shown in Figure 1B, the infection rates in the experimental group at 5, 10, 15, 20, and 30 days were 5%, 31%, 68%, 85%, and 100% respectively, while the control group showed no infection, indicating that tomato brown rugose fruit virus can be transmitted through contaminated soil.

We further examined whether healthy seedlings could become infected when replanted in the same soil after removal of tomato brown rugose fruit virus-infected plants. For the control and experimental groups, 30 healthy tomato seedlings at a similar growth stage were agroinfiltrated with pCB301-GUS (control) or pCB301-ToBRFV. After 23 days (by which time tomato brown rugose fruit virus had systemically infected the experimental group plants), all tomato plants were uprooted, and new healthy seedlings were planted in the original soil. Infection rates were detected by sampling at 5, 10, 15, 20, and 30 days after the emergence of the first true leaves. As shown in Figure 1C, no tomato brown rugose fruit virus was detected in any experimental group plants at day 5, while infection rates at days 10, 15, 20, and 30 were 28.9%, 57.8%, 78.9%, and 100% respectively. No virus was detected in control plants at any timepoint. These results demonstrate that tomato brown rugose fruit virus particles released into soil by living infected plants are sufficient to cause disease in healthy plants.

We then conducted an experiment using tomato brown rugose fruit virus-contaminated water versus normal tap water to irrigate tomato seedlings transplanted into virus-free potting mix in the greenhouse. Infection rates were assessed by sampling at 5, 10, 15, 20, and 30 days after the emergence of the first true leaves. As shown in Figure 1D, no tomato brown rugose fruit virus was detected in any experimental plants at day 5, while infection rates at days 10, 15, 20, and 30 were 23.3%, 45.6%, 58.9%, and 70.0% respectively. The control group irrigated with tap water remained uninfected. These results demonstrate that tomato brown rugose fruit virus can be transmitted through contaminated water sources.

Discussion

Tomato brown rugose fruit virus has been listed by Chinese plant virologists as one of the top ten viruses impacting agricultural economies due to its severe damage to tomato and pepper quality, rapid transmission, and significant effects on agricultural production safety, product quality, and farmers' income in affected regions (Wu et al. 2024). It has also been classified as a quarantine pathogen by multiple countries (Salem et al. 2023). Understanding tomato brown rugose fruit virus's transmission characteristics is crucial for developing effective control measures and minimizing losses.

This study utilized an infectious tomato brown rugose fruit virus clone to systematically analyze its multi-route transmission characteristics in a controlled environment. By incorporating the virus into the growing substrate and irrigation water, the viral inoculation dose was increased, resulting in more consistent disease development in plants. This method established rigorous and stable experimental conditions for investigating the transmission characteristics of tomato brown rugose fruit virus.

Our results corroborate and complement the growing body of literature, collectively advancing a more comprehensive and quantitative understanding of tomato brown rugose fruit virus epidemiology. Our data indicate that pruning is a relatively direct and efficient driver of transmission during agricultural practices. This finding provides a key mechanistic explanation for the highly aggregated spatial distribution of the virus in greenhouses observed by González-Concha et al. (2021), suggesting that frequent farming operations such as pruning and sucker removal facilitate the spread of the virus from initially infected plants to surrounding healthy ones.

Our positive results regarding soil and irrigation water transmission form a coherent body of evidence with the studies by Klein et al. (2023), Molad et al. (2024) and Mehle et al. (2023). Together, these studies confirm that soil and water bodies not only serve as reservoirs for the virus but also function as effective transmission media. This implies that in greenhouses, the virus may spread through direct pathways such as "infected plant → pruning → tools → healthy plant," or through indirect pathways like "infected plant → fallen leaves/root systems → soil/water source → healthy plant." These routes collectively constitute a multidimensional and complex transmission network. Due to varying initial viral concentrations, definitive conclusions cannot be drawn regarding the relative efficiencies of soil versus waterborne transmission.

No single measure will be sufficient to eradicate this highly pathogenic virus and therefore early detection and integrated control measures are critical. These include planting resistant tomato varieties to reduce disease severity, implementing highly sensitive seed and seedling quarantine to avoid infected planting materials, and applying seed treatments like hot water immersion or chemical disinfection before sowing. During farming operations (transplanting, pruning, tying, spraying, harvesting), disinfecting tools and clothing can minimize the risk of contact transmission. Optimal planting density ensures proper ventilation and light penetration, while balanced fertilization enhances plant resistance. Maintaining field sanitation through prompt removal of infected plants and regular monitoring of irrigation water enables early outbreak detection and timely intervention. Recent findings that tomato brown rugose fruit virus infects various weeds like *Portulaca oleracea* and *Conyza canadensis* suggest these may serve as intermediate hosts during winter or fallow periods (Salem et al. 2022a), necessitating weed control around production areas. Additionally, studies confirm that insects such as bumblebee *Bombus terrestris* and *Tuta absoluta* can mechanically transmit tomato brown rugose fruit virus (Levitzky et al. 2019, Caruso et al. 2024), significantly increasing field transmission risks and highlighting the importance of insect control. Public awareness campaigns about tomato brown rugose fruit virus, improved agricultural quarantine knowledge, and dissemination of scientific control techniques are also vital for effectively containing this virus.

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