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Epidemiological Insights Into Water and Soil-Mediated Transmission of Tomato Mottle Mosaic Virus

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ABSTRACT

Tomato mottle mosaic virus (ToMMV) poses a growing threat to tomato and pepper crops, yet its epidemiology remains largely unexplored. This study aimed to investigate water- and soil-mediated transmission of ToMMV under controlled conditions. The experiment involved mechanical inoculation of tomato plants, which demonstrated that infectious ToMMV particles remained viable in water up to 27 weeks at a 10^{-2} dilution. In hydroponic systems, ToMMV present in the nutrient solution caused systemic infection in bait plants within 5–17 weeks, depending on the severity of root injury, while infection was not observed in plants with intact roots even after 38 weeks of exposure. The transmission of the pathogen via irrigation was also confirmed in an experiment where seedlings and seed-derived plants grown in substrate (roots were not deliberately injured) were irrigated with ToMMV-contaminated water, resulting in infection 6 and 10 weeks after the start of exposure, respectively. The soil-mediated transmission process was further verified by planting healthy seedlings and seeds into previously contaminated substrate, resulting in infection within a period of 3–18 weeks. These findings emphasise the need for upgrading integrated management strategies, including water quality monitoring and reliable, rapid diagnostics, to mitigate the spread of ToMMV in greenhouse production systems. To support the development of effective monitoring strategies, the study also assessed the performance of two isothermal amplification assays for potential on-site detection of ToMMV in water. A recombinase-polymerase amplification (RPA) assay exhibited sensitivity comparable to that of reverse transcription-quantitative PCR and reliably detected ToMMV in crude water samples without RNA extraction.

1 | Introduction

Emerging plant diseases and pest outbreaks pose a significant threat to food security, public health and biodiversity and can lead to substantial economic consequences (Anderson et al. 2004). In recent decades, emerging plant viruses have increasingly impacted the global economy. A notable example is the tomato brown rugose fruit virus (ToBRFV; virus species *Tobamovirus fructirugosum*, genus *Tobamovirus*, family *Virgaviridae*), which currently poses the greatest threat to tomato (*Solanum lycopersicum*) production and, to a lesser extent, pepper (*Capsicum* spp.) cultivation (Kon et al. 2024). Another relatively recently discovered tobamovirus, tomato mottle mosaic virus (ToMMV; *Tobamovirus maculatessellati*), also infects these crops. First reported in 2013 in tomato samples from Mexico (Li et al. 2013), ToMMV has since been detected in plants across various countries and continents (EPPO Global Database 2024). ToMMV has also been detected in tomato and pepper seeds intercepted in several countries (Fowkes et al. 2022; Kon et al. 2024; Lovelock et al. 2020). As an example, analyses of official seed samples of tomatoes and peppers conducted in Slovenia showed the presence of ToMMV in five tomato seed samples, all of which originated

from China (Tiberini et al. 2022). Separately, the analysis of a historical seed collection including seed originating in France, the Netherlands and Spain, carried out by Schoen et al. (2023), indicated that ToMMV was probably already present in those countries before it was first described.

The symptoms caused by tobamoviruses can be severe and vary depending on factors such as plant species, cultivar, environmental conditions, physiological conditions and co-infection with other viruses or other pathogens (Klap et al. 2020; Luria et al. 2018; Mazumder et al. 2024; Yilmaz and Batuman 2023). Specifically, for ToMMV, symptoms in tomato include leaf mottling and mosaic (mild to severe), leaf deformations such as crinkling, curling and shoestring, fruit deformations and stunted growth (Ambrós et al. 2017; Mazumder et al. 2024; Nagai et al. 2018). In peppers, symptoms of ToMMV include leaf chlorosis, mottling, mosaic, shrinking and necrosis (Li et al. 2014, 2017). As a member of the *Tobamovirus* genus, ToMMV possesses biological properties that enable rapid spread, resulting in high epidemic potential and a potentially significant impact on agriculture. Viruses of the *Tobamovirus* genus are seed-borne and they are not vector transmissible; they have a thermal inactivation point of 90°C allowing them to survive in plant sap for many years (Adams et al. 2017; Dombrovsky and Smith 2017; Mazumder et al. 2024). This thermal stability and general virion stability enables their abundant presence in soil, as well as in irrigation and drainage water, where they remain infectious over long periods of time (Caruso et al. 2022; Li et al. 2016).

Since its discovery, research on ToMMV has primarily focused on the development of reliable diagnostic methods, molecular characterisation, host identification and various aspects of *Tm-2*^{2}-mediated resistance (Fowkes et al. 2022; Kimura et al. 2023; Kon et al. 2024; Li et al. 2020; Mazumder et al. 2024; Tettey et al. 2022; Tiberini et al. 2022; Zhang et al. 2022). However, the epidemiology of ToMMV is still largely unknown despite its increasing detection in various regions and its potential threat to tomato and pepper production. Although insights can be gained from the extensive research on well-studied tobamoviruses, especially ToBRFV, which has been extensively studied in recent years (Caruso et al. 2022; Mehle et al. 2023; Oladokun et al. 2019; Panno et al. 2020; Salem et al. 2022; Zhou et al. 2024), there is little direct evidence on the transmission and spread of ToMMV, particularly with regard to its potential for transmission via water and soil.

Water-borne transmission of ToBRFV has been confirmed in both major cropping systems: hydroponics and soil-based. Recent studies have demonstrated that ToBRFV can persist in recirculating nutrient solutions and spread efficiently in hydroponic systems, leading to rapid disease outbreaks (Mehle et al. 2023; Zhou et al. 2024). Given the similarity of ToMMV to ToBRFV, it is crucial to investigate whether ToMMV follows a similar transmission route and whether water sources, including irrigation systems, can serve as pathways for the virus. Water-borne transmission has been demonstrated to accelerate the emergence of diseases, affecting crops such as tomatoes and peppers (Mehle et al. 2018).

The use and management of irrigation water is a key factor in crop production, as it can influence both plant health and the potential for pathogen transmission. Hydroponic systems, which use significantly less water than soil-based cultivation, are becoming increasingly popular for specific crops and have the potential to help combat global water scarcity (Sambo et al. 2019). The adoption of soilless cultures, whether open or closed hydroponic systems, is increasing on a global scale. These systems present an alternative for growers dealing with soil-related issues such as nematodes, pathogens and nutrient imbalances (Schnitzler 2004; Stanghellini and Rasmussen 1994). However, recirculating nutrient solutions in hydroponics can facilitate the spread of water-borne plant pathogens, whether from the water source or introduced during distribution. This increases the risk of disease outbreaks if the system is not carefully managed (Stewart-Wade 2011). It is therefore essential to assess the risk of plant virus spread and implement the necessary management practices before recirculating used water to prevent crop losses (Bandte et al. 2009). The findings on the water-linked epidemiology of other tobamoviruses suggest that water-mediated transmission may play a role in the epidemiology of ToMMV, warranting further investigation. If water-mediated transmission is confirmed for ToMMV, it would require the development and validation of a highly sensitive test for ToMMV in water matrices that allow for reliable and accurate detection. Of utmost importance would be tests that can potentially be used for on-site detection, facilitating rapid response measures to mitigate the spread of the virus within greenhouse environments. The implementation of effective water management strategies, founded upon reliable methodologies and meticulous crop monitoring, is imperative to mitigate the risk of widespread outbreaks.

Another important epidemiological pathway for tobamoviruses is soil-borne transmission, which has not yet been investigated for ToMMV. Globally, the number of soil-transmitted plant viruses is relatively low; however, once the soil is contaminated, eradication is very costly and difficult (Roberts 2014). This is particularly evident in the context of continuous (monoculture) tomato and pepper production in greenhouses, where relocation of production sites is a particularly challenging endeavour. Despite the fact that soil-borne viruses are transmitted at lower rates than vector-borne viruses, this can nevertheless have significant consequences for agriculture. Once an infection is established via the roots, it has the potential to become systemic in the upper parts of the plant, and other transmission methods can readily spread the virus (Broadbent 1965; Klein et al. 2023). The hypothesis that the tobamovirus tobacco mosaic virus (TMV) is soil-borne was first proposed by Beijerinck (cited by Hiruki and Teakle 1987), and subsequent research has confirmed this as a recognised mode of transmission (Broadbent 1976; Broadbent et al. 1965; Fletcher 1969). In view of the recent observation of soil-mediated transmission of ToBRFV (Dombrovsky et al. 2022; Klein et al. 2023) in which plant roots acquire the virus from contaminated soil and spread it systemically, it is crucial to undertake the investigation into the potential for soil transmission of ToMMV.

The main objective of this study was to enhance the global understanding of the epidemiology of tobamoviruses by providing data on water- and soil-mediated transmission of ToMMV. We designed and conducted experiments to assess the potential role of contaminated water in both hydroponic and conventional production systems, as well as the role of contaminated growing

substrate in the epidemiology of ToMMV. Specifically, our study aimed to answer four key questions: (1) Can ToMMV be released from the roots of infected plants into irrigation water? (2) How long does ToMMV remain infectious in water? (3) Can ToMMV infect plants when irrigated with contaminated water? (4) Can contaminated growing substrate serve as a transmission medium for ToMMV? Additionally, we evaluated two available diagnostic tests for their suitability for on-site detection of ToMMV in water samples. This could support proactive water monitoring in commercial greenhouses and facilitate rapid containment measures in the event of an outbreak.

2 | Materials and Methods

All experiments described in this study were conducted in a quarantine greenhouse. The temperature was maintained at 22°C ± 2°C during the 16-h light period and 19°C ± 2°C during the 8-h dark period. All tomato seedlings used in these experiments were produced from virus-free seeds (cv. Roma VF or Moneymaker). They were planted in 9.5 cm pots filled with growth substrate (Fruhstorfer Erde Aussaat und Stecklingserde) and maintained in growth chambers under the temperature and light conditions described above. Two weeks after sowing, seedlings were transplanted into new pots (9.5 cm in diameter) filled with fresh substrate and kept in the growth chamber until use, at a stage characterised by two or three fully developed lower leaves (for the mechanical inoculations).

2.1 | Experimental Material and Handling of Test Plants and Nutrient Solution Samples

The ToMMV isolate PV-1267 (obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH—henceforth referred to as DSMZ) was propagated on tomato (*S. lycopersicum*) cv. Roma VF and cv. Moneymaker. In brief, two or three fully developed lower leaves of tomato plants were dusted with carborundum powder (400 mesh, VWR Chemicals) prior to inoculation. The inoculum was prepared by extracting infected leaf tissue in a 0.02 M phosphate buffer (pH 7.4) supplemented with 2% polyvinylpyrrolidone 10,000. Test plants were mechanically inoculated with a few drops (approximately 300–500 μL) of the inoculum. Five to 10 min after inoculation, the plants were rinsed with tap water to remove any remaining abrasive material and kept in a quarantine greenhouse.

Test plants for the experiments described below were mechanically inoculated using either a plant extract (prepared at a 1:10 ratio of leaf tissue in phosphate buffer), spiked water samples, or nutrient solution samples, with approximately 300–500 μL of each inoculum applied per plant. For mechanical inoculation, two to three fully developed leaves on intact test tomato plants were first dusted with carborundum powder and then inoculated with the 300–500 μL of inoculum. The test plants were then rinsed with tap water to remove the excess of the used abrasive, 5–10 min following inoculation. The inoculated test plants were watered according to their water requirements and monitored on a weekly basis for symptom development for at least 4 weeks after inoculation. ToMMV infection was confirmed by testing the extracted RNA from newly developed leaves using the reverse transcription-quantitative real-time PCR (RT-qPCR) developed by Tiberini et al. (2022). All RT-qPCR assays in this study were performed in three technical replicates, unless otherwise stated, and no significant variation was observed (the variation among technical replicates was within ±0.5 of the mean C_q value for C_q values below 30).

The RNeasy Plant Mini Kit (Qiagen) was used to extract total RNA from leaf material (approximately 200 mg) according to the manufacturer's instructions, with minor modifications: no 2-mercaptoethanol was added to the RLT buffer, and in the final step of the protocol two consecutive washes with 50 μL (total of 100 μL) of RNase-free water prewarmed to 65°C were used to elute the RNA from the column. The success of RNA extraction in all samples was tested by RT-qPCR with *nad5*-specific primers and a probe (Botermans et al. 2013). RNA extraction was considered successful for all samples with a C_q value for *nad5* of less than 33. RNA from nutrient solution samples was extracted using the QIAamp Viral RNA Mini Kit (Qiagen). The success of the RNA extraction from nutrient solution samples was assessed based on the results of luciferase control RNA (Promega), which was spiked into each sample at a known concentration (2 ng per sample) immediately prior to RNA extraction and tested by RT-qPCR using luciferase RNA-specific primers and probe (Toplak et al. 2004). Analysis of the C_q values obtained for the luciferase control RNA showed that the extractions were successful and that there was no inhibition. Negative controls were included in all RNA extractions and RT-qPCR runs to monitor possible contamination during the procedures.

RT-qPCR testing was performed on a regular basis on control plants cultivated in the same chamber of the quarantine greenhouse as the experimental plants. The purpose of this testing was to monitor for any unintended (adventitious) spread of ToMMV during handling in the greenhouse.

2.2 | Identifying the Maximum Dilution of ToMMV-Infected Plant Material Capable of Infecting Test Plants

Tomato leaves cv. Moneymaker (1 g) approximately 4-week-old infected with the ToMMV were homogenised with a hand-held homogeniser (Bioreba) in 10 mL of tap water, using extraction bags with a synthetic intermediate layer for filtration (Universal Extraction bags 12 × 15 cm, Bioreba). Serial tenfold dilutions were prepared using tap water as diluent. RNA was extracted from these dilutions using the QIAamp Viral RNA mini kit and tested by RT-qPCR.

For each dilution, 300–500 μL was applied to four tomato cv. Moneymaker plants (Figure 1A), which were about 2 weeks old and had their lower leaves dusted with carborundum powder before inoculation. As a negative control for the mechanical inoculation procedure, two plants were treated with non-contaminated tap water, which was confirmed to be free of ToMMV by RT-qPCR. The development of symptoms on the test plants was monitored for up to 4 weeks. RT-qPCR analysis was performed on RNA extracted from a pooled sample of all four test plants, either when symptoms became visible or, if

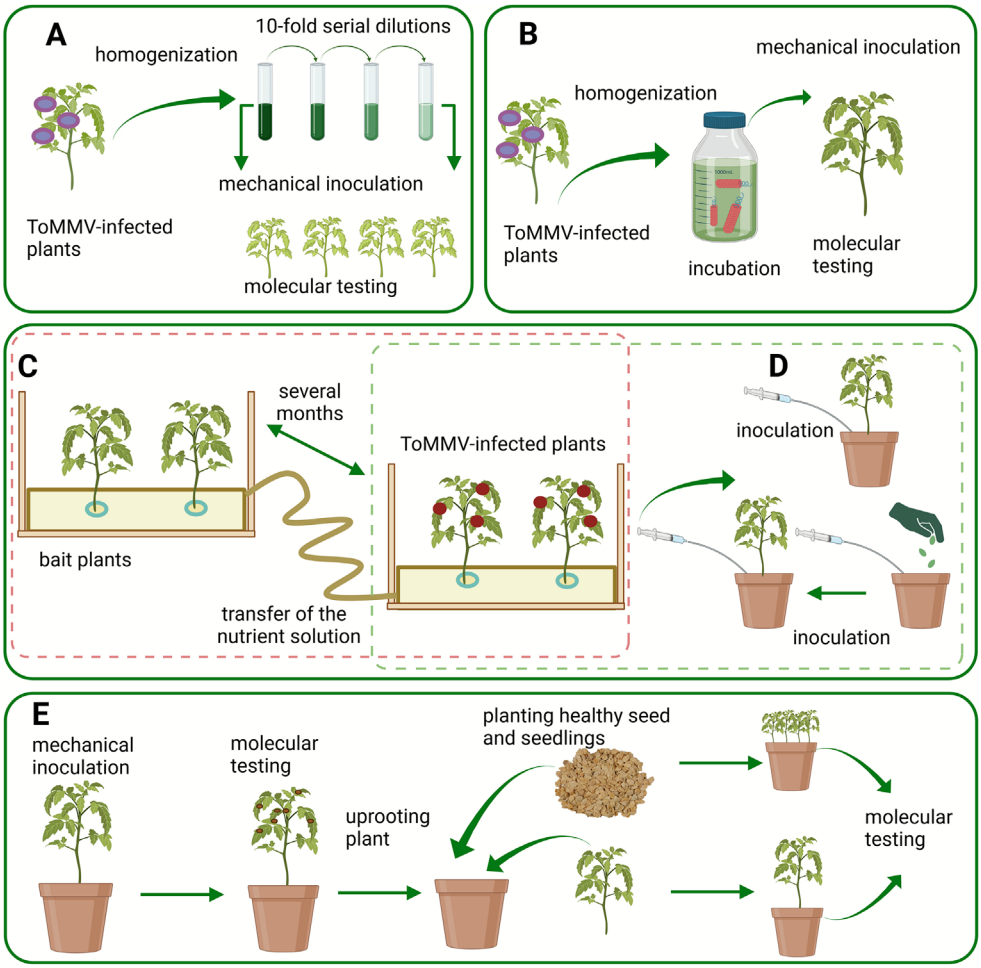

FIGURE 1 | Diagram of the experiments conducted: (A) Research on identifying the highest tomato mottle mosaic virus (ToMMV) dilution that can infect tomatoes. (B) Setup of the study on ToMMV survival in water. (C) Examination of water-mediated transmission in a hydroponic system. (D) Investigations into ToMMV transmission from substrate contaminated by irrigation with contaminated water to both planted tomato seedlings and seedlings grown from planted seeds. (E) Examination of ToMMV transmission with contaminated growing substrate. Created in https://BioRender.com.

no symptoms appeared, 4 weeks after mechanical inoculation. Only newly developed leaves that had emerged after inoculation were included in the pooled samples.

2.3 | Persistence of ToMMV in Water

To determine the survival time of ToMMV in a water environment, a spike source was prepared by homogenising 1 g of ToMMV-infected tomato leaves (cv. Roma, approximately 4 weeks old) in 10 mL of tap water using a hand-held homogeniser (Bioreba) and Universal Extraction bags and used to spike 1 L of tap water. Three dilutions of ToMMV-infected plant material in water (10^{-2}, 10^{-4} and 10^{-6}) were prepared. The contaminated water samples were stored in 1 L beakers covered with a double layer of gauze and perforated aluminium foil to allow limited air exchange (to mimic natural conditions) while minimising external contamination. The beakers were kept in a quarantine greenhouse under the conditions described above and sampled and tested weekly. Testing was conducted as follows: RNA was extracted using the QIAamp Viral RNA Mini Kit and assayed with RT-qPCR. Infectivity of ToMMV was also evaluated weekly by mechanical inoculation of ToMMV dilutions on four test plants of tomato cv. Roma VF as described in Section 2.1 (Figure 1B). Four weeks post-inoculation, RNA was extracted from a pooled sample of newly grown leaves from all test plants and tested by RT-qPCR. Each week, two control plants inoculated with non-contaminated (tested for ToMMV) tap water were also included in the study.

2.4 | Possibility of Water-Mediated Transmission of ToMMV in Experimental Hydroponic Systems

Four separate experiments using a test hydroponic system were designed to study the possibility of water-mediated transmission of ToMMV. Six ToMMV-infected tomato plants (cv. Roma VF, approximately 4 weeks old) whose roots were thoroughly rinsed to remove the growth substrate were transplanted into plastic pots with a diameter of 9.5 cm filled with rockwool substrate (Grotop Master Dry) and further grown on in a glass tank (0.6 × 0.4 × 0.4 m) filled with nutrient solution (Johnson et al. 1994). Each week, a freshly prepared nutrient solution was

added to replenish the amount consumed by the plants. After 1–2 weeks, healthy 2-week-old tomato seedlings (cv. Roma VF, 5–10 cm tall) grown in substrate (Fruhstorfer Erde Aussaat und Stecklingserde) were introduced as bait plants in other separate tanks. For Experiment 4, the tank with bait plants was established after the end of Experiment 3. Each experimental tank contained six bait plants whose roots were thoroughly rinsed to remove the growth substrate before being transplanted into plastic pots with a diameter of 9.5 cm filled with rockwool substrate.

The bait plants were irrigated with a nutrient solution from the tank containing the infected plants as follows. To prevent contact between the infected plants and the bait plants, these tanks were placed in separate greenhouse chambers. To prevent contact between the nutrient solution and the upper parts of the bait plants, a styrofoam sheet (3 cm thick) was placed in the tanks about 5 cm above the bottom. A pump (Statuary fountain pump PondoCompact 300; Pontec) and plastic tubing system were used to drain the nutrient solution from the root zone of the tank with infected plants into a glass bottle, which was then transferred to the separate greenhouse chamber containing the tanks with the bait plants (Figure 1C). This was repeated each week (in total for 38 weeks) during the experiment. The contaminated nutrient solution was directed to the root zone of the bait plants using a plastic funnel. Occasionally, the roots of the infected plants were stirred by the water flow from a pump. The roots of bait plants in Experiment 1 were not wounded, in Experiment 2 they were gently wounded (stirred by water flow from a pump), and in Experiments 3 and 4 they were severely wounded (stirred by hand). This root injury was done to simulate real hydroponic conditions where root damage is expected due to the presence of macrobiota and root growth through rockwool or other similar substrates. To prevent algal growth in the experimental tanks, the bottoms of all tanks were covered with aluminium foil.

Samples of the nutrient solution and leaf tissue were collected at regular intervals from both infected and bait plants and analysed as described above. Each week, leaf samples were pooled from six bait plants within each of the four experimental tanks. Additionally, six plants from the infected tank were occasionally tested as pooled samples. In addition, the infectivity of ToMMV in the nutrient solution (from the tank with infected plants) was tested by mechanical inoculation of tomato plants as previously described. Several control plants grown in the same greenhouse chamber with bait plants and fresh nutrient solutions were also tested to monitor possible contamination.

2.5 | ToMMV Transmission by Irrigation of Growing Substrate With Contaminated Water

To test the possibility of ToMMV transmission with irrigation of growing substrate with contaminated water we performed two experiments (Figure 1D). In the first experiment four 10-day-old tomato seedlings (cv. Roma VF), approximately 5–10 cm tall, were planted in 18 cm diameter plastic pots filled with growth substrate (Fruhstorfer Erde Aussaat und Stecklingserde). In the second experiment five tomato seeds (cv. Roma VF) were sown in each of the four pots with the growth substrate. During the first 4 weeks, 50 mL of contaminated nutrient solution (obtained from the tank with the infected tomato plants) was added to the substrate in each pot once a week using a syringe. Subsequently, 100 mL of the contaminated nutrient solution was added to each pot twice a week to provide sufficient water for the growing plants. Care was taken to ensure that the green parts of the bait plants did not encounter the syringe or the contaminated nutrient solution.

To monitor contamination, two control plants were used, which were watered with the same amount of non-contaminated nutrient solution and kept in the same greenhouse chamber.

Each week, RNA was extracted from a pool of newly grown leaves of bait and control plants and analysed by RT-qPCR.

2.6 | Transmission of ToMMV Through Contaminated Growing Substrate

To assess the potential for ToMMV transmission through contaminated growing substrate, tomato seedlings and seeds (cv. Roma VF and Moneymaker) were planted in contaminated growing substrate. This contaminated growing substrate was prepared by growing inoculated tomato plants in plastic pots filled with growing substrate (see Section 2.5). Once the plants were confirmed to be infected (by RT-qPCR), they were allowed to grow for an additional 3–4 weeks before being uprooted, ensuring as much of the root system as possible was removed. New seeds (eight pots with five seeds each) and seedlings (12 pots with one seedling each) were then planted in the same growing substrate (Figure 1E). The plants were then watered according to their water requirements with non-contaminated tap water. To monitor for contamination, two control plants were grown in the same greenhouse chamber in non-contaminated substrate. The young leaves of both the test and control plants were analysed weekly by RT-qPCR.

2.7 | Comparison of ToMMV Specific Tests Potentially Applicable for On-Site Testing of Water Samples

To evaluate the potential use of two different tests for the on-site testing of water samples, the recombinase-polymerase amplification (RPA) test and the loop-mediated amplification (LAMP) test were examined. The RPA test used the AmplifyRP XRT kit for ToMMV (XSC 22800; Agdia). This test is known for its rapid detection capabilities and ease of use in field conditions. The RPA test was performed according to the manufacturer's instructions using the AmpliFire isothermal fluorometer device (Agdia).

The LAMP test developed by Kimura et al. (2023) was adapted for use with the Isothermal Master Mix from Optigene. This adaptation was necessary because the chemistry used in the original article was not available on the Slovenian market. The test was considered positive if the amplification curve was exponential. The expected T_m (melting temperature) on the Genie II (Optigene) device was between 83.5°C and 84.5°C. The time of positivity (T_p) in minutes was noted. The test was negative if no exponential amplification curve was observed.

Both tests were tested for sensitivity and specificity on extracted RNA from different samples (see Section 3.6). RNA from leaf material was extracted as described in Section 2.1. RNA extraction from seed samples was performed with GH+ buffer as described in Appendix 1 of PM7/146(2) (EPPO 2022). The results of RT-qPCR were used for comparison.

RPA was further evaluated on crude homogenate from infected ToMMV leaves (PV-1267), diluted in concentrated non-contaminated irrigation water. This concentration was achieved using an 8-mL CIM QA monolith column (BIA Separations) on an AKTA Purifier 100 FPLC system (GE Healthcare), according to the method described by Bačnik et al. (2020).

3 | Results

3.1 | Identifying the Maximum Dilution of ToMMV-Infected Plant Material Capable of Infecting Test Plants

The tests of dilution of ToMMV-infected plant material in water confirmed that a higher viral load is required to detect the virus following mechanical inoculation of test plants based on symptoms and when using RT-qPCR testing of test plants compared to using RT-qPCR to test contaminated water (Table 1). With test plants assays, the presence of infectious ToMMV was confirmed by the presence of symptoms and with RT-qPCR up to a dilution of 10^{-5}. In contaminated water, ToMMV RNA was reliably detected by RT-qPCR up to a dilution of 10^{-9}. To optimise resource utilisation, dilutions of 10^{-1} and 10^{-2} were omitted from testing because they were anticipated to produce positive outcomes, thereby minimising greenhouse occupancy, labour and chemical consumption.

3.2 | Persistence of ToMMV in Water

At a dilution of 10^{-2} ToMMV can remain infectious in water for up to 27 weeks, whereas at a dilution of 10^{-4}, we could only confirm its infectivity immediately after preparation of the dilution. In the experiment with the dilution of 10^{-6}, we could not even confirm the infectivity at the time of preparation of the dilution. On the other hand, we were able to detect the RNA of ToMMV in prepared dilutions stored at room temperature for much longer, at least 30 weeks in all three dilutions. Throughout the entire experimental period, we observed fluctuations in C_q values in the three dilutions tested. In the 10^{-2} and 10^{-4} dilutions, the C_q values remained relatively stable. In the 10^{-6} dilution, C_q values remained relatively low during the initial weeks, then increased between weeks 8 and 9, after which they stabilised (Figure 2).

3.3 | Possibility of Water-Mediated Transmission of ToMMV in Experimental Hydroponic Systems

We demonstrated that infectious particles of ToMMV are released from the roots of infected plants into the nutrient solution. This was verified at several time points by mechanical inoculation of test plants with the nutrient solution, which resulted in infection in weeks 5, 11, 14 and 19 of the experiment (data not shown). ToMMV RNA was detected in the nutrient solution of the tank with infected plants in the first weeks after the infected plants were introduced (with $C_q \leq 30$, Figure 3). We confirmed that contaminated water can serve as a source for new infections of plants in hydroponics via their roots. We observed ToMMV replication in the leaves of previously healthy tomato plants after 17 weeks when we occasionally injured the roots only gently, and after 5 or 14 weeks when we occasionally severely wounded the roots of the test plants (Figure 3B–D, respectively). In the experiment in which the roots were not injured (Figure 3A), there was no infection of the tomato leaves of

TABLE 1 | Results of the tomato mottle mosaic virus (ToMMV) sap dilution series infectivity assays.

	ToMMV-contaminated water	Test plants[a]	
Dilution	RT-qPCR C_q[b]	Symptoms[c]	RT-qPCR C_q[d]
10^{-1}	11	NT	NT
10^{-2}	15	NT	NT
10^{-3}	18	+	9
10^{-4}	22	+	8
10^{-5}	25	+	8
10^{-6}	28	–	undet
10^{-7}	31	–	undet
10^{-8}	30	–	undet
10^{-9}	33	–	undet
10^{-10}	undet	–	undet
10^{-11}	undet	–	undet
10^{-12}	37	–	undet
10^{-13}	undet	–	undet
10^{-14}	undet	–	undet
10^{-15}	undet	NT	NT
10^{-16}	37	NT	NT
10^{-17}	undet	NT	NT
10^{-18}	undet	NT	NT
NC	undet	–	undet

Abbreviations: –, symptoms were not visible; +, symptoms visible; NC, negative control; NT, not tested; undet, no signal obtained with reverse transcription-quantitative PCR (RT-qPCR).
[a]Each dilution was inoculated on four tomato plants, except NC, which was inoculated on two tomato plants.
[b]The presence of ToMMV RNA in water samples investigated by RT-qPCR. The average C_q values of three replicates are given.
[c]Symptoms observed were leaf curling, shoestring, bubbling and mosaic.
[d]The plants were tested when symptoms appeared (12 days post-inoculation [dpi] for the 10^{-3} dilution, and 17 dpi for the 10^{-4} and 10^{-5} dilutions); if no symptoms were observed test plants were tested 4 weeks after mechanical inoculation.

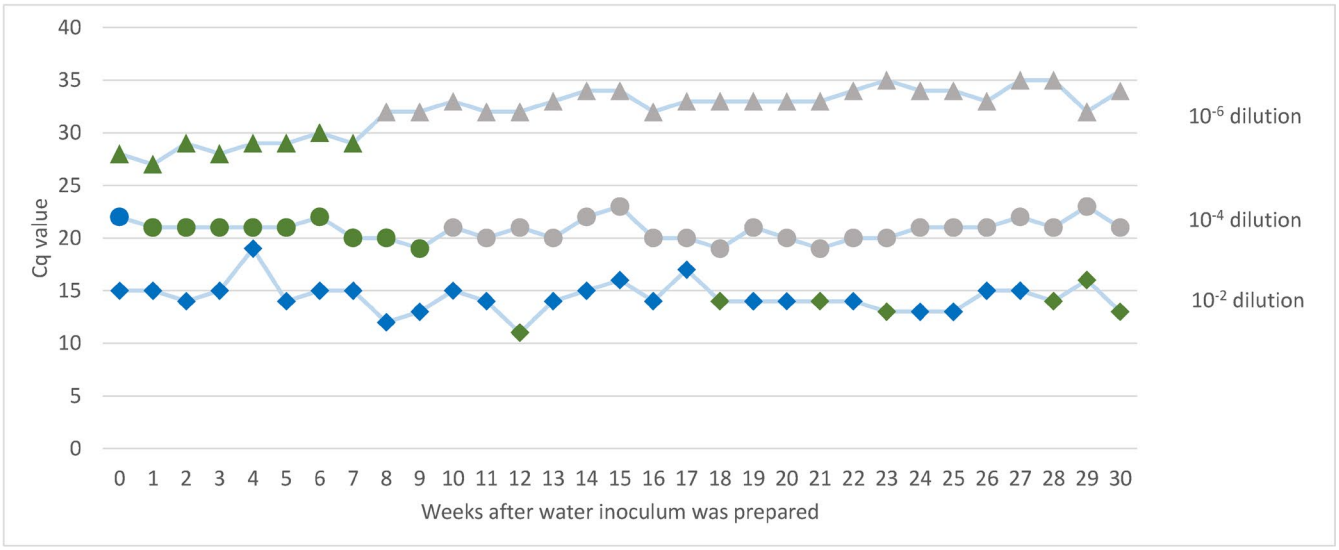

FIGURE 2 | Detection of tomato mottle mosaic virus (ToMMV) RNA by reverse transcription-quantitative PCR in artificially contaminated water stored in the quarantine greenhouse. Points where ToMMV infectivity was confirmed by inoculating test plants are marked in blue on the graph. Points where infectivity was tested but not confirmed are shown in green. Points shown in grey where infectivity in water was not tested, although ToMMV RNA was still detected.

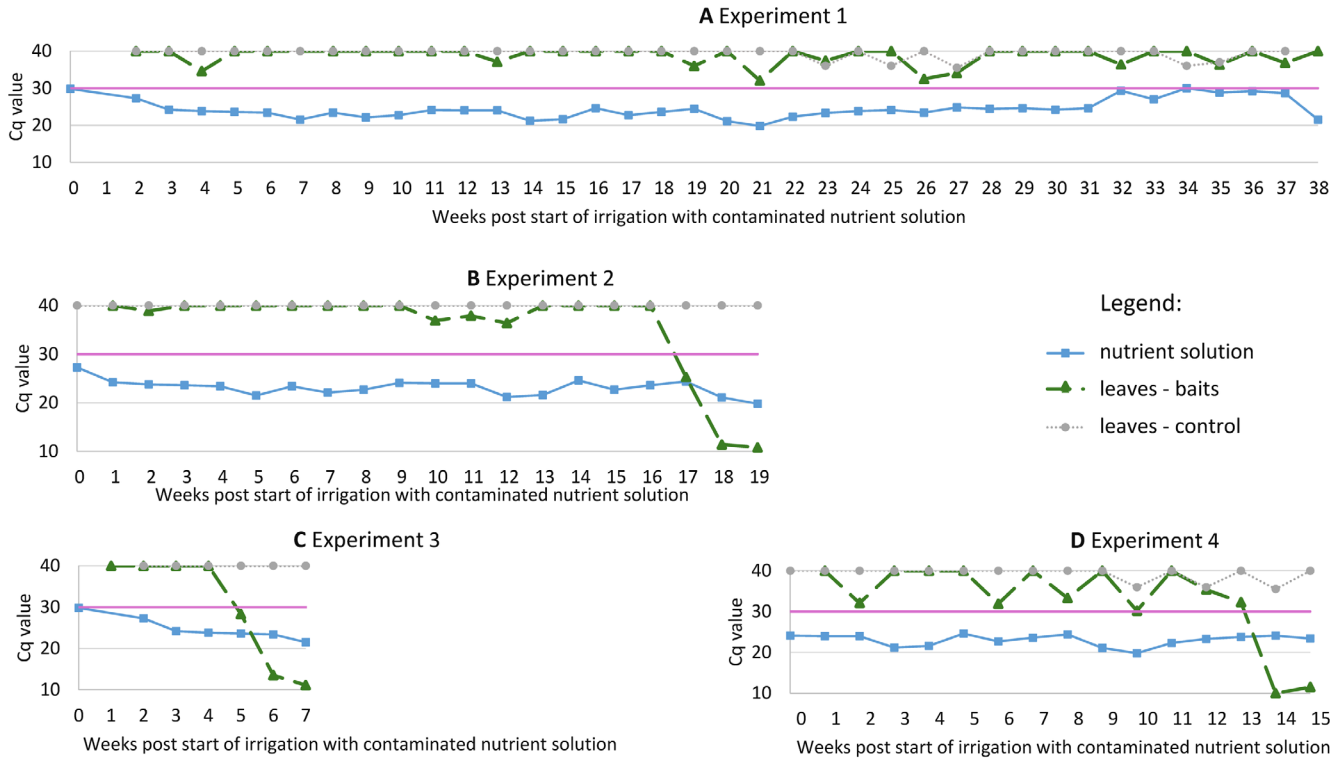

FIGURE 3 | Evaluation of tomato mottle mosaic virus (ToMMV) infection in tomato plants grown in an experimental hydroponic system with ToMMV-contaminated nutrient solution in four separate experiments: (A) Experiment 1: Roots were not wounded; (B) Experiment 2: Roots were gently wounded; (C) and (D) Experiments 3 and 4: Roots were severely wounded. *y*-axes show C_q values from reverse transcription-quantitative PCR. The plotted C_q value represents the average of three replicates. The *x*-axes show the weeks after the start of irrigation with contaminated nutrient solution. The pink line shows the C_q value of 30 below which the result was considered positive.

the bait plants even 38 weeks after the plants were placed in the experimental tank. The roots of the bait plants were not tested in this study, as it was not possible to distinguish between a potential root infection and a superficial infection caused by the nutrient solution.

3.4 | ToMMV Transmission by Irrigation of Growing Substrate With Contaminated Water

Using the same ToMMV-contaminated nutrient solution from the previous experiment to irrigate growing substrate containing

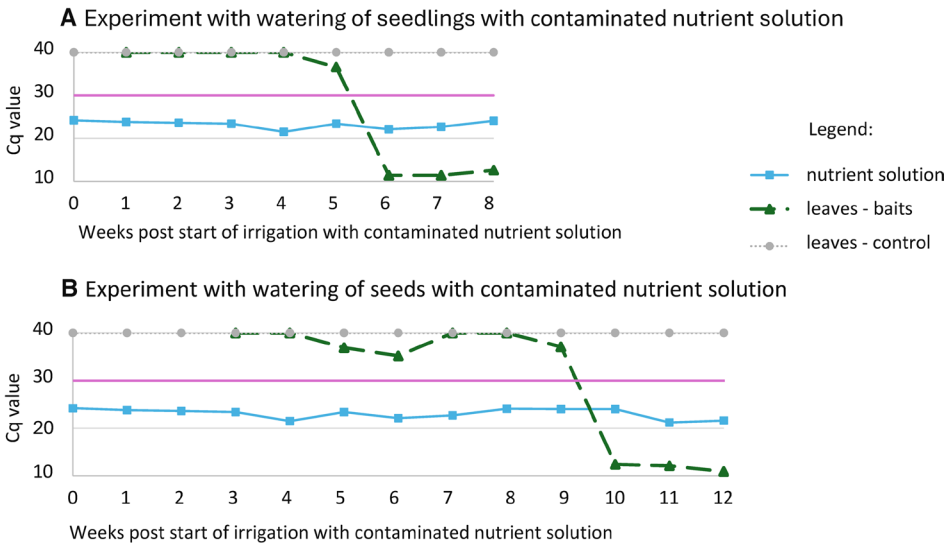

FIGURE 4 | Evaluation of tomato mottle mosaic virus (ToMMV) infection in tomato plants grown in substrate, where tomato (A) seedling and (B) seeds were watered with ToMMV-contaminated nutrient solution. *y*-axes show C_q values from reverse transcription-quantitative PCR. The plotted C_q value represents the average of three replicates. The *x*-axes show the weeks after the start of irrigation with contaminated nutrient solution. The pink line shows the C_q value of 30 below which the result was considered positive.

TABLE 2 | Detection of tomato mottle mosaic virus (ToMMV) by reverse transcription-quantitative PCR (RT-qPCR) in test plants grown in contaminated growing substrate.

Testing material	Repetition of the experiment	Time elapsed (weeks)[a]
Seedlings planted in contaminated soil	1	5
	2	3
	3	14
Seeds sown in contaminated soil	1	9
	2	18

[a]Weeks after tomato seeds or seedlings planted in contaminated growing substrate were confirmed to be ToMMV positive by RT-qPCR (C_q values obtained from these plants at that time ranged from 9 to 17).

initially healthy tomato seeds and seedlings, we confirmed that contaminated water can indeed infect plants in a soil-based system. When contaminated water was injected into the growth substrate containing healthy tomato seedlings, we observed infection on the leaves after 6 weeks of irrigation (Figure 4A). Similarly, when contaminated water was applied to the growth substrate in which uninfected tomato seeds had been sown, infection was detected on the leaves of the plants that developed from these seeds 10 weeks after sowing (Figure 4B).

3.5 | Transmission of ToMMV With Contaminated Growing Substrate

We confirmed the transmission of ToMMV via contaminated growing substrate. Healthy tomato seedlings planted in contaminated growing substrate (after ToMMV-infected plants were uprooted) were confirmed as infected by RT-qPCR 3–14 weeks after planting (Table 2). Similarly, when healthy tomato seeds were sown in the contaminated growing substrate, the resulting plants become infected 9–18 weeks after sowing. In all experiments, plants were irrigated with non-contaminated tap water.

3.6 | Evaluation of ToMMV-Specific RPA and LAMP Test

We evaluated the specificity and sensitivity of two tests with potential for on-site detection of ToMMV in water samples. Both tests successfully detected the two tested ToMMV isolates (Tables 3 and 4). A comparative analysis of the two isothermal tests, conducted on a panel of non-target tobamoviruses with all samples tested in duplicate and RT-qPCR used as the reference method, showed that the RPA test produced a false positive result for paprika mild mottle virus (which also yielded a high C_q value in RT-qPCR), whereas the LAMP assay correctly yielded a negative result (Table 3). However, in terms of sensitivity, the RPA assay performed better than the LAMP assay (Table 4), showing slightly higher sensitivity that was comparable to that of RT-qPCR. Based on this advantage, the RPA assay was subsequently employed to test water samples directly, without prior RNA extraction, to simulate real-world on-site testing conditions. Our results indicate that ToMMV can be reliably detected in water samples using the RPA assay without RNA extraction, even at relatively low viral concentrations, up to at least 1:1000 dilution.

4 | Discussion

Plant viruses of agricultural importance—including members of the *Tombusviridae*, *Virgaviridae*, *Alphaflexiviridae* and *Bromoviridae* families—are widely present in the environment and pose a significant risk to crop production (Betancourt 2023). Their ability to persist in terrestrial and aquatic environments increases the likelihood of disease outbreaks, leading to substantial crop losses. A recent and particularly concerning example is

TABLE 3 | Results of testing different isolates of tobamoviruses and healthy samples with the tomato mottle mosaic virus (ToMMV)-specific recombinase-polymerase amplification (RPA) and loop-mediated amplification (LAMP) assays.

Virus	Virus name	Matrix[a]	ID	Collection	Dilution factor	*nad5* (C_q)[b]	ToMMV (C_q)[b]	Agdia RPA	LAMP[c]
BPeMV	Bell pepper mottle virus	L	PV-0170	DSMZ (Germany)	1×	24	undet	neg	neg
CGMMV	Cucumber green mottle mosaic virus	L	JKI32360	JKI (Germany)	25×	31	undet	neg	neg
ObPV	Obuda pepper virus	L	PV-1176	DSMZ (Germany)	25×	23	undet	neg	neg
ORSV	Odontoglossum ringspot virus	L	PV-1048	DSMZ (Germany)	25×	28	undet	neg	neg
PaMMV	Paprika mild mottle virus	L	PV-0606	DSMZ (Germany)	25×	26	35	pos	neg
PMMoV	Pepper mild mottle virus	L	PAS 487	GEVES (France)	25×	26	undet	neg	neg
PMMoV	Pepper mild mottle virus	L	PAS 486	GEVES (France)	1×	25	undet	neg	neg
RMV	Ribgrass mosaic virus	L	PV-0145	DSMZ (Germany)	1×	22	undet	neg	neg
SFBV	Streptocarpus flower break virus	L	PV-1058	DSMZ (Germany)	1×	23	undet	neg	neg
SHMV	Sunn-hemp mosaic virus	L	PV-0156	DSMZ (Germany)	1×	25	undet	neg	neg
TMGMV	Tobacco mild green mosaic virus	L	JKI32436	JKI (Germany)	25×	24	undet	neg	neg
TMV	Tobacco mosaic virus	L	JKI32434	JKI (Germany)	25×	32	undet	neg	neg
TMV	Tobacco mosaic virus	L	PAS 601	GEVES (France)	25×	26	undet	neg	neg
ToBRFV	Tomato brown rugose fruit virus	L	PV-1236	DSMZ (Germany)	25×	24	undet	neg	neg
ToMMV	Tomato mottle mosaic virus	L	PV-1267	DSMZ (Germany)	25×	26	15	pos	pos (21)
ToMMV	Tomato mottle mosaic virus	S	NIB V 414	Bejo Zaden BV (Netherlands)	2×	22	29	pos	pos (41)
ToMV	Tomato mosaic virus	L	JKI23645	JKI (Germany)	25×	24	undet	neg	neg
ToMV	Tomato mosaic virus	L	PAS 603	GEVES (France)	25×	23	undet	neg	neg
ToMV	Tomato mosaic virus	L	PAS 598	GEVES (France)	1×	25	undet	neg	neg
ToMV	Tomato mosaic virus	L	PAS 599	GEVES (France)	1×	23	undet	neg	neg
YMoV	Youcai mosaic virus	L	PV-0527	DSMZ (Germany)	1×	24	undet	neg	neg

(Continues)

TABLE 3 | (Continued)

Virus	Virus name	Matrix[a]	ID	Collection	Dilution factor	*nad5* (C_q)[b]	ToMMV (C_q)[b]	Agdia RPA	LAMP[c]
Healthy samples									
Healthy tomato seed		S	D1977/23 + D93/23	NIB (Slovenia)	25×	30	undet	neg	neg
Healthy tomato seed		S	D1977/23	NIB (Slovenia)	25×	29	undet	neg	neg
Healthy tomato leaves		L	/	NIB (Slovenia)	1×	30	undet	neg	neg

Abbreviations: /, no data available; neg, sample was negative; pos, sample was positive; undet, no signal obtained with reverse transcription-quantitative PCR (RT-qPCR).
[a]L, leaves; S; seeds.
[b]The results of RT-qPCR analysis for *nad5*/ToMMV. The average C_q value of two technical replicates is given.
[c]In parentheses, Tp in minutes is given.

ToBRFV, which has been shown to spread through hydroponic water systems in both commercial greenhouses and in laboratory settings (Mehle et al. 2023; Zhou et al. 2024). These findings underscore the urgency of addressing water-borne virus spread in agriculture, even though this mode of transmission may occur only occasionally. Notably, even a single infected plant can result in an outbreak at a production site, as tobamoviruses are known to spread rapidly from plant to plant through contact and by handling during cultivation.

In our study, we provide the first insights into the water-mediated epidemiology of ToMMV—focusing on hydroponic and soil-based growing systems—for which such information was previously unavailable.

We initiated our experiments with the aim of determining the highest dilution of ToMMV in water that could still result in successful infection of tomato test plants via mechanical inoculation. The outcomes of these initial trials served as the foundation for designing subsequent experiments. Mechanical transmission—used as an indicator of viral infectivity—was successful up to a dilution of 10^{-5}, leading us to conclude that the likelihood of ToMMV transmission through mechanical means remains high when RT-qPCR detects viral RNA with C_q values of ≤ 25 in water samples. However, ToMMV nucleic acids were detectable by RT-qPCR down to a dilution of 10^{-9}.

As an example, Mehle et al. (2023) reported that ToBRFV RNA was detectable by RT-qPCR down to a dilution of 10^{-12}, with successful mechanical transmission observed up to a dilution of 10^{-8}. However, it is important to note that in both our study and that of Mehle et al. (2023), the absolute concentration of virus used to prepare the dilution series was not determined. This limitation prevents direct conclusions about the relative virulence or infectivity of the two viruses. Interpreting results from serial dilutions requires considering the initial viral concentration, as it affects detection sensitivity and inoculation success. Without this, comparisons across viruses or conditions may be misleading. Future studies should quantify initial viral load (e.g., via RT-qPCR standard curves or digital droplet RT-PCR) for more accurate assessments. However, this limitation did not affect our study's goal of evaluating infection potential rather than precise infectivity thresholds.

Tobamoviruses are characterised by their extreme virion stability, allowing them to survive in water and soil, as well as on working surfaces for extended periods (Adams et al. 2017; Broadbent et al. 1965; Giesbers et al. 2024; Li et al. 2016; Mehle et al. 2014; Skelton et al. 2023). Therefore, the next aim in our study was to determine for how long ToMMV can persist in water. Our results demonstrated that ToMMV remains infectious in contaminated water for approximately 6 months at high concentrations. At intermediate concentrations, we confirmed infectivity only immediately after the preparation of the contaminated water. The lowest concentration tested was below the limit of detection with test plants. However, viral RNA was still detectable in contaminated water samples even after 7 months. Comparative studies on other plant viruses indicate varying survival times in water and nutrient solution under greenhouse conditions. For instance, ToBRFV remains infectious for up to 4 weeks, tomato mosaic virus (ToMV) for

TABLE 4 | Results of testing tomato mottle mosaic virus (ToMMV) dilutions with the recombinase-polymerase amplification (RPA) and loop-mediated amplification (LAMP) assays.

Virus	ID	Dilution factor	ToMMV C_q [a]	Agdia RPA	LAMP[b]
ToMMV (RNA)	PV-1267[d]	2.5×10^{1}	15	+	+ (21)
		2.5×10^{2}	18	+	+ (24)
		2.5×10^{3}	21	+	+ (27)
		2.5×10^{4}	24	+	+ (31)
		2.5×10^{5}	28	+	+ (41)
		2.5×10^{6}	31	+	+ (59)
		2.5×10^{7}	34	+	−
		2.5×10^{8}	undet/39[c]	−	−
RNA from healthy tomato leaves used as a diluent			−	−	−
ToMMV (RNA)	NIB V 414[e]	2×10^{0}	29	+	+ (41)
		2×10^{1}	32	+	+ (49)
Water used as a diluent			undet	−	−
ToMMV (crude homogenate)	PV-1267[f]	1×10^{1}	NT	+	NT
		1×10^{2}	NT	+	NT
		1×10^{3}	NT	+	NT
Concentrated uncontaminated water sample used as diluent			undet	NT	NT

Abbreviations: −, sample was negative; +, sample was positive; NT, not tested; undet, no signal obtained with reverse transcription-quantitative PCR (RT-qPCR).
[a]The results of RT-qPCR analysis for ToMMV. The average C_q values of two technical replicates are given.
[b]Tp in minutes is given in parentheses.
[c]Different results for the same sample tested in two parallels (note that each sample was tested in two parallels and only in case of different results the results of both parallels are given).
[d]RNA solution from leaves of healthy tomato was used as diluent for RNA of ToMMV isolate PV-1267.
[e]RNase-free water was used as a diluent of RNA of ToMMV isolate NIB V 414.
[f]Crude extract of ToMMV isolate PV-1267 was diluted in concentrated uncontaminated water sample.

6 months, pepino mosaic virus (PepMV) for 3 weeks and potato virus Y (PVY) for 1 week (Mehle et al. 2014, 2023; Pares et al. 1992). Notably, Mehle et al. (2023) showed that ToBRFV at high and medium concentrations remains infectious for up to 4 weeks, while at lower concentrations, infectivity is limited to 1 week. These findings, along with our own, underscore the critical role of ascertaining the initial virus concentration in determining survival duration. Although we did not perform absolute quantification, our use of serial dilutions demonstrates how different starting concentrations influence virus persistence. Environmental factors also significantly influence virus persistence. For example, PVY survives for up to 10 weeks at 4°C—compared to 1 week at 20°C—suggesting that lower temperatures enhance stability (Mehle et al. 2014). In addition to virion stability, temperature and concentration, other factors such as pH, the presence of organic matter and microbial communities can affect virus degradation in water (Molad et al. 2024). Sample handling and experimental design further contribute to variability in virus detectability. Freshly prepared dilutions are more likely to contain infectious particles than those stored for extended periods, due to the natural decline in infectivity over time, and this effect that becomes particularly evident when the amount of virus in the solution is limited. Moreover, differences in the number and susceptibility of test plants, as well as inoculation methods, can influence the outcome of infectivity assays. Taken together, these findings highlight the complexity of virus survival in aqueous environments. When interpreting results of virus persistence in water, it is essential to consider all these factors.

In addition to the previously addressed aspects, we also investigated whether ToMMV can be released from the roots of infected plants into the nutrient solution and subsequently infect healthy plants through their roots. Our experiments confirmed that ToMMV can indeed be transmitted in a hydroponic-like experimental system. Infected plants released infectious virus particles into the nutrient solution, as demonstrated by successful mechanical inoculation of test plants with this solution at weeks 5, 11, 14 and 19 of the experiment. Furthermore, C_q values of the nutrient solution used for irrigating bait plants were generally equal to or below 25, which—based on our dilution series experiment—indicates a high likelihood of mechanical transmission. The only exceptions were at the beginning of Experiments 1–3, when the virus likely required time to accumulate, and during weeks 32–37 of Experiment 1, when higher C_q values were observed. This latter increase was probably due to the addition of larger volumes of fresh nutrient solution, which temporarily diluted the virus concentration in the water. The increased water demand was probably driven by intensive plant growth, elevated external temperatures and

enhanced transpiration. Finally, we confirmed that virus particles released from roots of infected plants into the nutrient solution were capable of infecting healthy plants in the hydroponic system via root uptake. This mode of transmission is consistent with previous findings on other environmentally stable pathogens, including ToBRFV, PepMV, PVY and potato spindle tuber viroid (PSTVd) (Mehle et al. 2014, 2023; Schwarz et al. 2010). The dynamics of ToMMV infection in our study varied depending on the extent of root injury. In plants with severely wounded roots, infection occurred within 5–14 weeks. In those with mild root damage, infection was delayed, occurring around 17 weeks. Notably, no infection was observed in plants with intact roots, even after 38 weeks of exposure. A similar infection pattern was reported by Mehle et al. (2023) in experiments where the roots of bait plants were wounded. However, a key difference was observed: ToBRFV was able to infect plants with intact roots after 22 weeks of exposure (Mehle et al. 2023). This discrepancy may be attributed to several factors, including the potentially higher virulence of the ToBRFV isolate used in their study or differences in the susceptibility to the used virus isolates of the tomato cultivars employed (cv. Roma VF in our experiments vs. cv. Moneymaker in Mehle et al. 2023) or the difference in the initial virus concentration. Although ToMMV was not able to infect bait plants in hydroponic system when their roots remained intact, such a scenario is highly unlikely in real agricultural settings, where root damage commonly occurs due to natural root growth, interactions with soil macrobiota and routine handling practices. These conditions may facilitate root-mediated virus transmission, underscoring the importance of considering this pathway in disease management strategies for hydroponic and soil-based cultivation systems.

Another important question we addressed regarding the water-linked epidemiology of ToMMV was whether virus-contaminated water could infect plants when used for irrigation of plants in growing substrate. Tomato seedlings and plants grown from healthy seeds in a growing substrate became infected 6 and 10 weeks after the start of exposure, respectively. This difference in the infection dynamics suggests that the developmental stage of the plant plays an important role in the efficiency and timing of infection. Seedlings, which already possess a developed root system, provide a larger surface area for interaction with the contaminated substrate. In addition, micro-injuries caused by transplanting may facilitate viral entry. In contrast, plants grown directly from seed have limited initial contact with the substrate, and their root systems are less developed in the early stages, potentially delaying virus uptake. However, it is also possible that infection in these plants occurred during germination, as has been reported for some other viruses (Teakle and Morris 1981).

Beyond water-mediated transmission, we were interested in the role of soil-mediated transmission of ToMMV, especially considering that soil-based cultivation remains predominant across Europe (Eurostat 2022). Given the lack of experimental data, we aimed to provide initial insights into the potential role of soil in the epidemiology of ToMMV. Soil transmission has been documented for various tobamoviruses (Allen 1981; Antignus et al. 2005; Broadbent 1965; Fletcher 1969; Li et al. 2016), with tomato mosaic virus shown to persist in root debris for nearly 2 years (Broadbent 1976; Fletcher 1969). Soil-mediated transmission has also been confirmed for ToBRFV under both experimental and natural conditions (Dombrovsky et al. 2022; Klein et al. 2023; Luria et al. 2017). Additionally, research conducted by Molad et al. (2024) showed that the infectivity of soil naturally contaminated with ToBRFV was profoundly reduced by 184 days after the soil pile was collected and cleared of roots and plant debris. Our study further confirms the soil-borne transmission of ToMMV, as healthy tomato seedlings and seedlings grown from healthy seeds became infected within 3–18 weeks of being planted in previously contaminated growing substrate. These findings highlight the role of growth substrate as a reservoir for ToMMV and underscore the need for further research on the persistence of ToMMV under real-world production conditions.

Our results provide new insights into the potential for ToMMV transmission via water and contaminated growing substrates, highlighting important considerations for sustainable agricultural practices. In intensive production systems, where recycled or shared water sources are commonly used to conserve water, such practices may inadvertently facilitate virus spread. As water scarcity continues to challenge agriculture (Yadav et al. 2022), these findings emphasise the necessity to integrate plant health safeguards into water management strategies. It is therefore imperative that early detection is prioritised, given that even minimal quantities of virus present in irrigation water have the potential to initiate an infection and contribute to its subsequent dissemination (Mehle et al. 2018). The need for highly sensitive and reliable diagnostic tools is further emphasised by the findings of Mehle et al. (2023), who detected ToBRFV in drain water from a commercial tomato greenhouse 1 week before any visible virus-like symptoms appeared in the crop. As part of the Euphresco project 'Validation of molecular diagnostic methods for the detection and identification of tomato mottle mosaic virus' (2022-A-394), we conducted a comprehensive review of available molecular methods for detecting ToMMV (Mehle et al. 2024) and we used knowledge gained in that project to kick-off the part of the research in this study related to the detection of ToMMV. Our results showed that the RPA assay demonstrated sensitivity comparable to RT-qPCR, making it a promising tool for fast and reliable virus detection in the field. To further improve on-site diagnostics, future research should focus on validating the RPA assay using real agricultural water samples and exploring its integration with virus concentration methods, such as Centricon 70-Plus filtration units, which have already shown promising performance (Maksimovic Carvalho Ferreira et al. 2022).

In conclusion, our study provides valuable insights into the epidemiology of ToMMV, showing that contaminated water and growing substrate can serve as important sources of infection under favourable conditions. Because our results related to growing substrate are based on preliminary experiments, further research is needed to determine how long ToMMV can remain infectious in soil or substrate, and which practices could help prevent its transmission. These findings highlight the importance of good water and soil management, as well as the need for reliable diagnostic tools to detect the virus early. To develop effective control strategies, close collaboration between researchers, growers and policymakers will be essential.

Author Contributions

Ana Vučurović: investigation, writing – original draft preparation, writing – review and editing; **Jakob Brodarič:** investigation, writing – review and editing; **Irena Bajde:** investigation, writing – review and editing; **Miha Kitek:** investigation, writing – review and editing; **Nataša Mehle:** conceptualisation, supervision, writing – original draft preparation, writing – review and editing.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding author.

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