

Evidence of the internalization of animal caliciviruses via the root of growing strawberry plants and dissemination to the fruit

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ABSTRACT

Human norovirus (NoV) is the leading cause of foodborne disease in the U.S. and epidemiological studies have shown that fresh produce is one of the major vehicles for the transmission of human NoV. However, the mechanisms of norovirus contamination and persistence in fresh produce are poorly understood. The objective of this study is to determine whether human NoV surrogates, murine norovirus (MNV-1) and Tulane virus (TV), can attach and become internalized and disseminated in strawberries grown in soil. MNV-1 and TV were inoculated to the soil of growing strawberry plants at a level of 10^8 PFU/plant. Leaves and berries were harvested over a 14 day period and the viral titer was determined by plaque assay. Over the course of the study, 31.6% of the strawberries contained internalized MNV-1 with an average titer of $0.81 \pm 0.33 \log_{10}$ PFU/g. In comparison, 37.5% of strawberries were positive for infectious TV with an average titer

of $1.83 \pm 0.22 \log_{10}$ PFU/g. A higher percentage (78.7%) of strawberries were positive for TV RNA with an average titer $3.15 \pm 0.51 \log_{10}$ RNA copies/g as determined RT-qPCR. In contrast, no or little virus internalization and dissemination was detected when TV was inoculated into bell peppers grown in soil. Collectively, this data demonstrates; (i) virally contaminated soils can lead to the internalization of virus via plant roots and subsequent dissemination to the leaf and fruit portions of growing strawberry plants; and (ii) the magnitude of internalization is dependent on the type of virus and plant.

INTRODUCTION

Human norovirus (NoV) is the major cause of non-bacterial gastroenteritis, contributing to over 95% of the non-bacterial acute gastroenteritis and an estimated 58% of all foodborne illnesses reported annually in the U.S. (1-4). In recent years, fresh produce has been identified as a leading cause of foodborne illness in the U.S. (3, 5, 6). Human NoV alone accounts for over 40% of the fresh produce related illnesses reported each year in the U.S. (1, 2, 4-8). Human NoV has been attributed to outbreaks in many diverse types of produce including: fresh cut fruit, lettuce, tomatoes, melons, salads, green onions, strawberries, blueberries, raspberries, salsa, as well as many others (5-7, 9-15). Human NoV is resistant to common disinfectants, has a low infectious dose, and is highly stable in the environment which contributes to the high prevalence of foodborne outbreaks associated with the virus and its presence and persistence in food commodities (3, 16, 17).

Foods can become contaminated with human NoV at any point from farm to fork, including production, processing, and preparation. Infected food handler contamination and exposure to fecally contaminated water are thought to be the most frequent modes of human NoV contamination of foods (2, 5, 10, 18). The use of irrigation water that is contaminated with fecal matter is considered a significant cause of viral contamination in soft berries (19-22). A multi-country study conducted in Europe investigating human NoV prevalence in soft red fruit obtained directly from processing companies or retail markets found viral RNA in 7-34% of samples (2, 22). These findings implicate that human NoV entered the farm to fork continuum during production. In a survey of the foodborne human outbreaks in the U.S. from 2001-2008, production or processing was determined to the point of contamination in 3 of 109 outbreaks associated with leafy greens and/or fruit or nuts (2). However, in approximately 50% of the single commodity human NoV outbreaks the point of contamination could not be determined and viral contamination prior to preparation could have occurred in these instances (2). In September/October 2012 a large outbreak of human NoV associated with the consumption of frozen strawberries sickened nearly 11,000 individuals in Germany (19). Genotyping revealed GI.9, GII.6 and GII.13/GII.16 norovirus strains in the implicated berries and in patient stool samples (19). The multiple strains detected in this outbreak is consistent with the source being water contaminated with human feces, which would result in multiple viral strains being present (19). Human NoV contamination of berries via irrigation water has the potential to lead to human NoV outbreaks, however, the modes of contamination and persistence of the virus in growing berries is poorly understood.

The major challenge in human NoV research is that it cannot be grown in cell culture so much of the understanding of human NoV molecular biology, pathogenesis, and environmental stability has come from the study of other caliciviruses, including murine norovirus (MNV-1) and Tulane virus (TV). An ideal surrogate virus should be cultivable in the laboratory and resemble human NoV closely in regards to size, genetic make-up, receptor binding, cell tropism, and disease manifestation. MNV-1 has been used extensively as a surrogate for the study of human NoV and is currently the only cultivatable member of the genus *Norovirus*. MNV-1 has been shown to be more stable at low pHs when compared to other surrogates such as feline calicivirus (FCV), which indicates it may have similar environmental stability as human NoV (23). Tulane virus (TV) is a newly recognized surrogate for human NoV and is member of the genus *Recovirus* within *Caliciviridae* (24). TV was shown to be have similar pH stability as MNV-1 at ranges from pH 3 to pH 8 (25). TV causes enteric infection in primates and also recognizes histo-blood group antigens as a cellular attachment receptor, similar to human NoV(24). Thus, TV is thought to be a better surrogate for human NoV.

Recently, we and others have demonstrated that human NoV and surrogates (MNV and TV) were efficiently internalized via root of leafy greens and disseminated to the shoot and leaf portions in hydroponically grown leafy greens (26). In addition, it was shown that the rate of internalization was reduced when leafy greens were grown in soil (27-30). However, whether viruses can be internalized and disseminated to fruits is not known. The high number of human NoV soft berry associated outbreaks warrants investigation into the modes by which the virus contaminates and persists in these crops. The goal of this study is to evaluate whether human NoV surrogates can become

internalized through the roots of growing strawberry plants and be disseminated to the fruits. The determination of whether viruses can become internalized within the fruits of growing plants is imperative to develop prevention and control measures to ensure the safety of berries.

MATERIALS AND METHODS

Viruses and cell culture. Murine norovirus (MNV-1) was generously provided by Dr. Herbert W. Virgin IV, Washington University School of Medicine and Tulane virus (TV) was a generous gift from Dr. Xi Jiang at Cincinnati Children's Hospital Medical Center. MNV-1 and TV were propagated in confluent monolayers of the murine macrophage cell line RAW 264.7 and the monkey kidney cell line MK2-LLC (ATCC, Manassas, VA), respectively. RAW 264.7 cells were cultured in high-glucose Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), at 37°C under a 5% CO₂ atmosphere. For growing MNV-1 stock, confluent RAW 264.7 cells in T-150 flasks were infected with MNV-1 at a multiplicity of infection (MOI) of 0.1. After 1 h incubation at 37°C, 15 ml of DMEM with 2% FBS was added. The virus was harvested 2 days post inoculation by three freeze-thaw cycles and low speed centrifugation at 1000 × *g* for 30 min. MK2-LLC cells were cultured in low serum Eagle's minimum essential medium (Opti-MEM, Invitrogen), supplemented with 2% FBS, at 37°C under a 5% CO₂ atmosphere. For growing TV stock, MK2-LLC cells in T-150 flasks were washed with Hank's balanced salt solution (HBSS) and subsequently infected with TV at an MOI of 0.1. After 1 h incubation at 37°C, 15 ml of

Opti-MEM with 2% FBS was added. The virus was harvested 2 days post inoculation and subjected to three freeze-thaw cycles, followed by centrifugation at $1000 \times g$ for 30 min. Viral titer was determined by plaque assay.

Tulane virus stability in strawberry and green pepper puree. Strawberry puree was prepared by blending 25g of fresh strawberries with 25ml of ddH₂O. The pH of the strawberry puree was 4.02. To determine the stability of TV in strawberry puree, 3 replicates were prepared by diluting TV 1:10 in strawberry puree to an approximate titer of $10^{6.0}$ pfu/ml. TV inoculated strawberry puree was stored at room temperature for 14 days, and the survival of TV was determined at day 0 (before virus inoculation), 1, 3, 7, and 14 post inoculation by standard plaque assay.

Green pepper puree was prepared by blending 25g of fresh green peppers with 25ml of ddH₂O. The pH of the green pepper puree was 5.47. To determine the stability of TV in green pepper puree, 3 replicates were prepared by diluting TV 1:10 in green pepper puree to an approximate titer of $10^{6.0}$ pfu/ml. TV inoculated green pepper puree was stored at room temperature for 14 days, and the survival of TV was determined at day 0 (before virus inoculation), 1, 3, 7, and 14 post inoculation by standard plaque assay.

Plant growth, virus inoculation, sample collection, and virus internalization. A schematic diagram of experimental design was shown in Fig.1A. Strawberry plants (*Fragaria ananassa*) and bell pepper plants (*Capsicum annuum*) were purchased from a local greenhouse center (Strader's, Columbus, OH) and grown under greenhouse conditions until the emergence of fruit. After the observation of at least one strawberry

or pepper per plant, the plants were moved to the lab and grown under the conditions of 12 h light and 12 h dark, at 20°C and 50-65% relative humidity (RH). Strawberry plants were inoculated at the base of the plant with 20ml of 1×10^7 pfu/ml virus (MNV-1 or TV), in two separate 10 ml treatments 2 h apart on the same day. Following inoculation, the surface of the soil was covered with parafilm to limit viral contamination of the aerial portions of the plant. At days 0 (before viral inoculation), 1, 3, 7, and 14 a samples leaves (10g) and all berries or peppers from each plant were harvested. All tissues were submerged in 2000ppm chlorine following harvest to remove any virus present on the surface of the plant. Previously, both MNV-1 and TV have been shown to be completely inactivated by 2000ppm chlorine treatment (25). Following chlorine treatment, leaves and berries were rinsed for 5 min in ddH₂O and next residual chlorine was inactivated by submersion of tissues in 0.25M sodium thiosulfate. Prior to homogenization all leaves and berries were weighed and colorimetric measurements were collected on all harvested berries. The leaf samples were homogenized using liquid nitrogen and mortars and pestles and homogenized tissue was resuspended in 5mL phosphate buffered saline (PBS). Individual strawberries were placed in sterile stomacher bags and 10ml PBS was added. The strawberries were stomached for 120sec and the homogenate was collected. All homogenates were centrifuged at $1000 \times g$ for 30 min to remove cellular debris. The virus containing supernatant was transferred to a new collection tube for viral enumeration by plaque assay and RT-qPCR and for the measurement of pH.

Pepper plants were inoculated with TV only, and were harvested and processed as described above for strawberries. However, no color measurement or pH measurements

were evaluated for the peppers. Viral enumeration was conducted using plaque assay and RT-qPCR.

Virus enumeration by plaque assay. MNV-1 and TV were quantified by plaque assay in RAW 264.7 and LLC-MK2 cells, respectively. Briefly, cells were seeded into six-well plates (Corning Life Sciences, Wilkes-Barre, PA) at a density of 2×10^6 cells per well. After 24 h incubation, RAW 264.7 and MK2-LLC cell monolayers were infected with 400 μ l of a 10-fold dilution series of MNV-1 or TV, respectively, and the plates were incubated for 1 h at 37°C with gentle agitation every 10 min. The cells were overlaid with 3 ml of Eagle minimum essential medium (MEM) containing 1% agarose, 2% FBS, 1% sodium bicarbonate, 0.1 mg of kanamycin/ml, 0.05 mg of gentamicin/ml, 15 mM HEPES (pH 7.7), and 2 mM L-glutamine. After incubation at 37 °C and 5% CO₂ for 2 days, the plates were fixed in 10% formaldehyde. The plaques were visualized by staining with 0.05% (w/v) crystal violet. Viral titer was expressed as mean log₁₀ plaque forming unit (PFU)/ml \pm standard deviation.

Enumeration of TV genomic RNA by real time reverse transcriptase PCR (RT-qPCR). In strawberry and pepper plants inoculated with TV, RT-qPCR was used to detect genomic RNA. Briefly, total RNA was extracted from samples using an RNeasy Kit (Qiagen), followed by reverse transcription and real-time PCR. First strand cDNA was synthesized by SuperScriptase III (Invitrogen) using the primer TVRT (5'-AATTCCACCTTCAACCCAAGTG -3'), which targets the VP1 gene of TV. The VP1 gene was then quantified by real-time PCR using custom Taqman primers and probes

(Forward primer: 5'-TTGCAGGAGGGTTTCAAGATG-3') (Reverse primer: 5'-CACGGTTTCATTGTCCCCATA-3') (Probe: 5'-FAM-TGATGCACACATGTGGGA-NFQ-3') on a StepOne real-time PCR machine (Applied Biosystems). PCR reaction and cycling parameters followed the manufacturer's protocol (Invitrogen). Briefly, TaqMan Fast Universal Master Mix was used for all reactions. For cycling parameters, a holding stage at 95°C was maintained for 20 sec prior to cycling, followed by 50 cycles of 95°C for 1 sec for annealing and 60°C for 20 sec for extension. Standard curves and StepOne Software v2.1 were used to quantify genomic RNA copies. Viral RNA was expressed as mean log₁₀ genomic RNA copies/ml ± standard deviation.

Statistical analysis. All experiments were performed in triplicate. Statistical analysis was performed by one-way multiple ANOVA using Tukey's family comparisons or basic linear regression analysis using Minitab 16 statistical analysis software (Minitab Inc., State College, PA). A *P* value of <0.05 was considered statistically significant.

RESULTS

MNV-1 dissemination to berries via root internalization in soil grown strawberry plants. Strawberry plants were purchased from a local greenhouse center and grown under greenhouse conditions until the emergence of fruit. Strawberry plants were inoculated at the base of the plant with 2×10^8 pfu of MNV-1. At days 0 (before viral inoculation), 1, 3, 7, and 14, leaves and all berries from each plant were harvested and the viral titer in each tissue was determined by plaque assay. At day 1 post inoculation, 1 of

5 leaves (20%) tested positive for infectious MNV-1 and only 1 of 20 (5%) harvested strawberries tested positive for MNV-1 (Fig.1B). The level of infectious MNV-1 recovered from strawberry tissues increased on day 3 post inoculation. 4 of 5 (80%) of leaf samples tested positive for infectious MNV-1, with an average titer in the positive leaves of $1.64 \pm 0.28 \log_{10}$ PFU/g (Fig.1B). In harvested berries, 8 of 13 (61.5%) tested positive for infectious MNV-1 and the average titer in the positive strawberries was found to be $1.11 \pm 0.65 \log_{10}$ PFU/g (Fig.1B). At day 7 post inoculation, 3 of 5 (60%) of the leaves and 3 of 5 (60%) of berries tested positive for infectious MNV-1, with average titers in the positive samples 1.90 ± 0.32 and $0.84 \pm 0.35 \log_{10}$ PFU/g in leaves and berries, respectively (Fig.1B). By day 14, no berries could be recovered from the plants, and only 1 of 5 of the strawberry plant leaves were positive for infectious virus at this time-point (Fig.1B). Of the 20 leaf samples harvested over the 14 day study period, 9 (45%) were positive for infectious MNV-1 with an average titer of $1.64 \pm 0.44 \log_{10}$ PFU/g (Fig.2B). Overall, 38 strawberries were harvested during the 14 day study period and 12 tested positive for infectious MNV-1, which correlates to 31.6% of the strawberries containing internalized MNV-1. The average titer of the 12 positive berries was $0.81 \pm 0.334 \log_{10}$ PFU/g (Fig.2B).

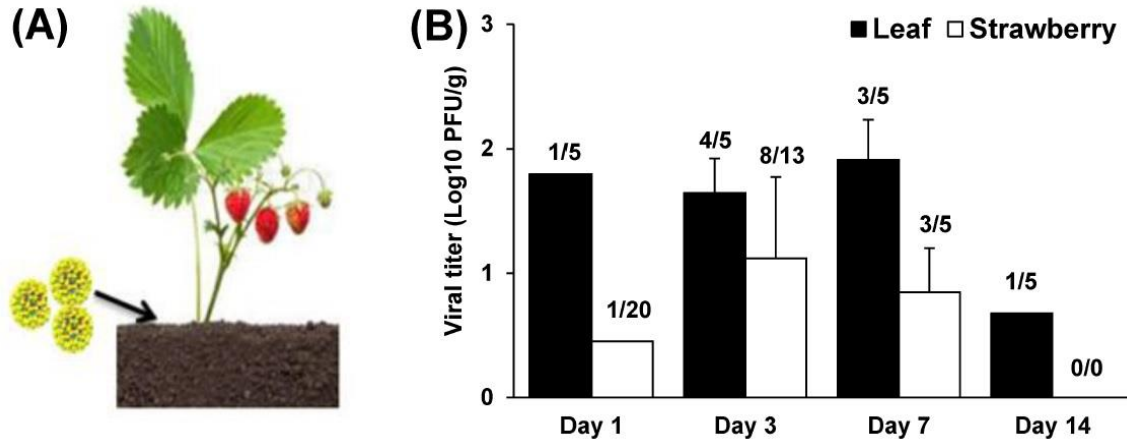


FIG 1. MNV-1 internalization and dissemination in strawberry plants grown in soil.

(A) Schematic diagram of virus inoculation in strawberry growing in soil. Strawberry plants were inoculated at the base of the plant with 2×10^8 PFU of MNV-1. At days 0 (before viral inoculation), 1, 3, 7, and 14 leaves and all berries from each plant were harvested. **(B) Viral titer in leaves and strawberries.** Viral titer in each tissue was determined by plaque assay. Bars represent the average PFU/g of positive strawberry plant tissues ± 1 standard deviation. ## indicate the number of samples positive for infectious virus out of the total number of samples tested.

TV dissemination to berries via root internalization in soil grown strawberry plants.

Strawberry plants were inoculated at the base of the plant with 2×10^8 pfu of TV. At days 0, 1, 3, 7, and 14, leaves and all berries from each plant were harvested and viral titer in each tissue was determined by plaque assay. At day 1 post inoculation, none of the harvested leaves (0 of 5) or berries (0 of 13) tested positive for infectious TV. The level of infectious TV recovered from strawberries tissues increased on days 3, 7, and 14 post inoculation. At day 3 post inoculation, 3 of 5 (60%) of leaves tested positive for infectious virus with an average titer in the positive samples of $2.59 \pm 0.69 \log_{10}$ PFU/g

(Fig. 2A). 4 of 12 (33.3%) of berries tested positive for infectious TV at day 3 post inoculation with an average titer in the positive berries of $1.86 \pm 0.58 \log_{10}$ PFU/g recovered (Fig. 2A). Day 7 post inoculation yielded positive detection of infectious TV in 3 of 5 (60%) of leaves and 12 of 18 (66.7%) of berries, with titers of 2.18 ± 0.72 and $2.03 \pm 0.44 \log_{10}$ PFU/g, respectively (Fig. 2A). On day 14 post inoculation, 3 of 5 (60%) leaves tested positive for infectious TV with an average titer in the positive samples of $1.45 \pm 0.23 \log_{10}$ PFU/g (Fig. 2A). Only 4 berries were recovered on day 14 post inoculation, and 2 tested positive for infectious TV with an average titer in the positive samples of $1.60 \log_{10}$ PFU/g. Of the 20 leaf samples collected over the study period, 9 (45%) were positive for infectious TV titer, which was the same as MNV-1 in leaves (Fig.2B). In addition, the average titer of TV in leaves was $2.07 \pm 0.52 \log_{10}$ PFU/g (Fig. 2B), which was similar to MNV-1 dissemination in leaves ($1.64 \pm 0.44 \log_{10}$ PFU/g) ($P>0.05$). Overall, 48 berries were harvested of which 18 (37.5%) tested positive for infectious TV, with an average titer in the TV positive berries over the course of the study being $1.83 \pm 0.22 \log_{10}$ PFU/g (Fig. 2B), which was significantly higher than that of MNV-1 ($0.81 \pm 0.334 \log_{10}$ PFU/g) ($P<0.05$). In addition, TV-positive rate (37.5%) in strawberry was relatively higher than MNV-1-positive rate in strawberry (31.6%).

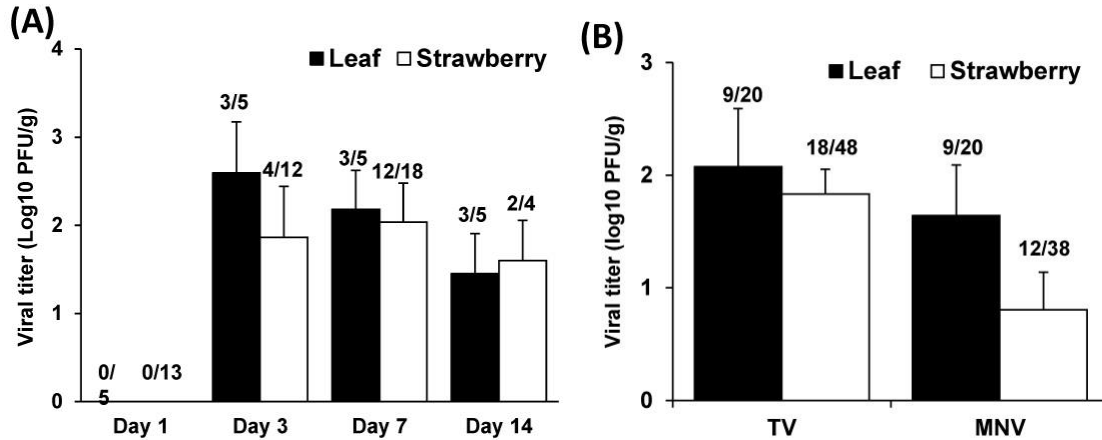


FIG 2. TV internalization and dissemination in strawberry plants grown in soil.

(A) TV titer in leaves and strawberries. Strawberry plants were inoculated at the base of the plant with 2×10^8 PFU of TV. At days 0 (before viral inoculation), 1, 3, 7, and 14 leaves and all berries from each plant were harvested and viral titer in each tissue was determined by plaque assay. Bars represent the average PFU/g of positive strawberry plant tissues ± 1 standard deviation. #/# indicate the number of samples positive for infectious virus out of the total number of samples tested. **(B) Total number of MNV-1 and TV positive leaves and strawberries.** Bars represent the average titer of the positive leaf and strawberry samples over the 14 day study period ± 1 standard deviation for both TV and MNV-1. #/# indicate the number of samples positive for infectious virus out of the total number of samples tested.

Detection of TV dissemination to berries via root internalization in soil grown strawberry plants using RT-qPCR. The level of TV RNA in inoculated strawberries was also determined by RT-qPCR. At day 1 post inoculation, 2 of 5 (40%) leaves and 6 of 13 (46.2%) berries were positive for TV RNA with average titers in the positive samples of 3.84 ± 0.20 and 2.86 ± 0.19 log₁₀ RNA copy/g, respectively (Fig. 3). 4 of 5

(80%) leaves and 11 of 12 (91.7%) berries were positive for TV RNA on day 3 post inoculation with average titers in the positive samples of $3.95 \pm 0.21 \log_{10}$ RNA copy/g in leaves and $3.33 \pm 0.37 \log_{10}$ RNA copy/g in berries (Fig. 3). On day 7 post inoculation, 4 of 5 (80%) leaves were positive for TV RNA with an average titer in the positive leaves of $3.39 \pm 0.26 \log_{10}$ RNA copy/g (Fig.3). All berries tested positive for TV RNA on day 7 post inoculation (18 of 18) with an average titer of $2.96 \pm 0.51 \log_{10}$ RNA copy/g (Fig.3). On day 14 post inoculation, all leaf samples were positive for TV RNA (5 of 5) with an average titer of $3.05 \pm 0.11 \log_{10}$ RNA copy/g detected (Fig. 3). 2 of 4 (50%) berries harvested on day 14 post inoculation were positive for TV RNA with an average titer in the positive samples of $2.35 \log_{10}$ RNA copy/g (Fig. 3). Overall, 78.7% of the harvested strawberries were positive for TV RNA with an average level of $3.15 \pm 0.51 \log_{10}$ RNA copy/g.

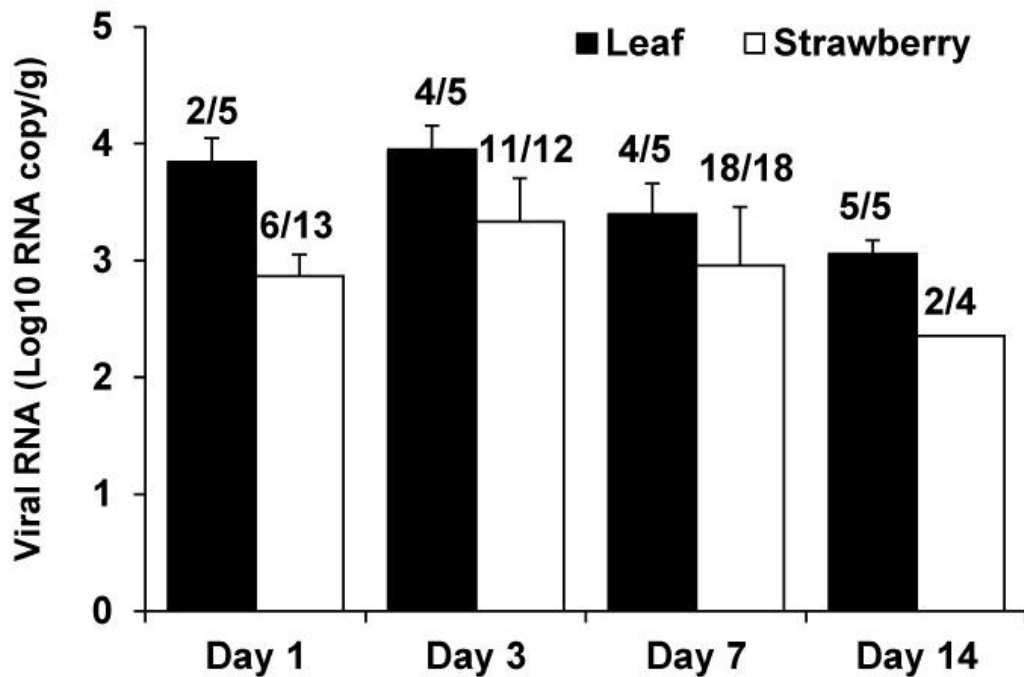


FIG 3. Detection of TV internalization and dissemination in strawberry plants grown in soil using RT-qPCR. Strawberry plants were inoculated at the base of the plant with 2×10^8 pfu of TV. At days 0 (before viral inoculation), 1, 3, 7, and 14 leaves and all berries from each plant were harvested and viral titer in each tissue was determined by RT-qPCR. Bars represent the average RNA copy/g of positive strawberry plant tissues ± 1 standard deviation. #/# indicates the number of samples positive for TV RNA out of the total number of samples tested.

Stability of TV in strawberry puree. To determine the stability of TV in strawberry puree, 3 replicates of TV were diluted 1:10 in strawberry puree to an average titer of $5.79 \pm 0.52 \log_{10}$ PFU/ml (Fig. 4). TV inoculated strawberry puree was stored at room temperature for 14 days, and the survival of TV was determined at day 0, day 1, day 3, day 7, and day 14 post inoculation by standard plaque assay. At day 1 post inoculation

the titer of TV was $5.26 \pm 0.07 \log_{10}$ PFU/ml, which was not significantly different from the initial titer ($P>0.05$) (Fig. 4). At day 3 post inoculation the TV titer was $4.74 \pm 0.14 \log_{10}$ PFU/ml. By day 7 post inoculation the TV titer had dropped by approximately 2 logs to $3.57 \pm 0.09 \log_{10}$ PFU/ml (Fig. 4). On the final time point tested, day 14 post inoculation, the TV titer was $2.66 \pm 0.24 \log_{10}$ PFU/ml (Fig. 4).

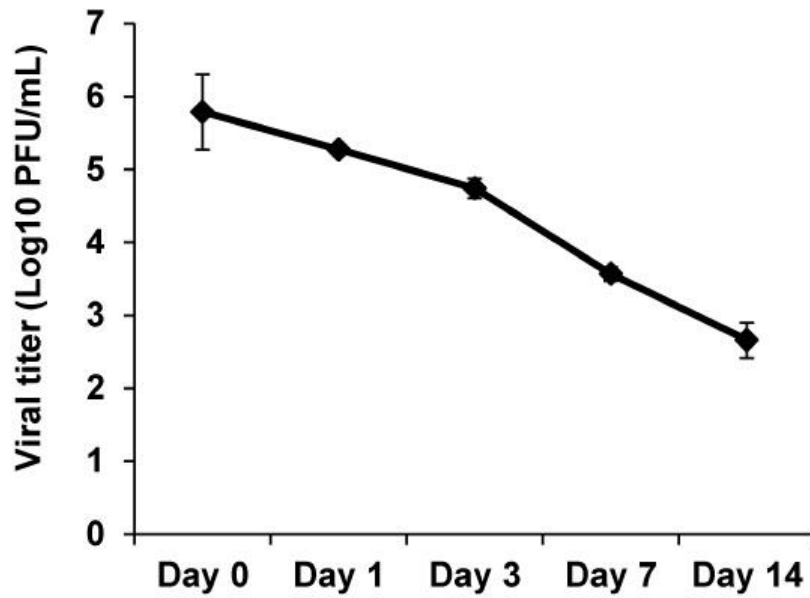


FIG 4. Determination of TV stability in strawberry puree. TV was diluted 1:10 in strawberry puree to an approximate titer of $10^{6.0}$ pfu/ml. TV inoculated strawberry puree was stored at room temperature for 14 days, and the survival of TV was determined at day 0, 1, 3, 7, and 14 post inoculation by standard plaque assay. Each data point represents the average of three replicates \pm standard deviation.

TV internalization via the root of pepper plants and dissemination to the fruits.

We next determined whether TV can become internalized in other fruits. Bell peppers were chosen as they are true fruiting plants in which the fruit develops from the ovary with seeds inside. This is in contrast to strawberries which are aggregate accessory fruits, the flesh of the fruit is formed from a receptacle that supports the ovary and the “seeds” on the outside of the fruit are actually ovaries that contain the true seeds. Pepper plants were purchased from a local greenhouse center and grown under greenhouse conditions until the emergence of fruit. Pepper plants were inoculated at the base of the plant with 2×10^8 pfu of TV. At days 0, 1, 3, 7, and 14 leaves and all peppers from each plant were harvested and viral titer in each tissue was determined by plaque assay and RT-qPCR. No infectious TV was recovered from peppers at any time-point throughout the study. There was minimal detection of infectious TV in the leaf tissues, 2 samples were positive on day 1 and day 3 post inoculation with average titers of 1.87 and 1.13 \log_{10} PFU/g detected respectively, and only 1 sample was positive on day 7 with an average titer of 0.69 \log_{10} PFU/g. Similarly, no TV RNA was found to be present in the peppers at any day post inoculation and only a few of the leaf tissues tested positive for viral RNA. On day 1 and day 3 post inoculation 3 leaf samples were positive for TV RNA at levels of 2.91 ± 0.65 and $1.98 \pm 0.48 \log_{10}$ RNA copy/g, respectively. On day 7 post inoculation only 1 leaf sample was positive for TV RNA with a titer of 1.87 \log_{10} RNA copy/g.

Stability of TV in green pepper puree. The stability of TV in green pepper puree was determined. Three replicates of TV were diluted 1:10 in green pepper puree to an average titer of $6.07 \pm 0.07 \log_{10}$ PFU/ml (Fig. 5). TV inoculated green pepper puree was

stored at room temperature for 14 days, and the survival of TV was determined at day 0, day 1, day 3, day 7, and day 14 post inoculation by standard plaque assay. At day 1 post inoculation the titer of TV was $5.36 \pm 0.12 \log_{10}$ PFU/ml (Fig. 5). At day 3 post inoculation the TV titer was $5.28 \pm 0.13 \log_{10}$ PFU/ml. By day 7 post inoculation the TV titer had dropped to $4.37 \pm 0.14 \log_{10}$ PFU/ml (Fig.5). On the final time point tested, day 14 post inoculation, no infectious TV was recovered from the green pepper puree (Fig.5).

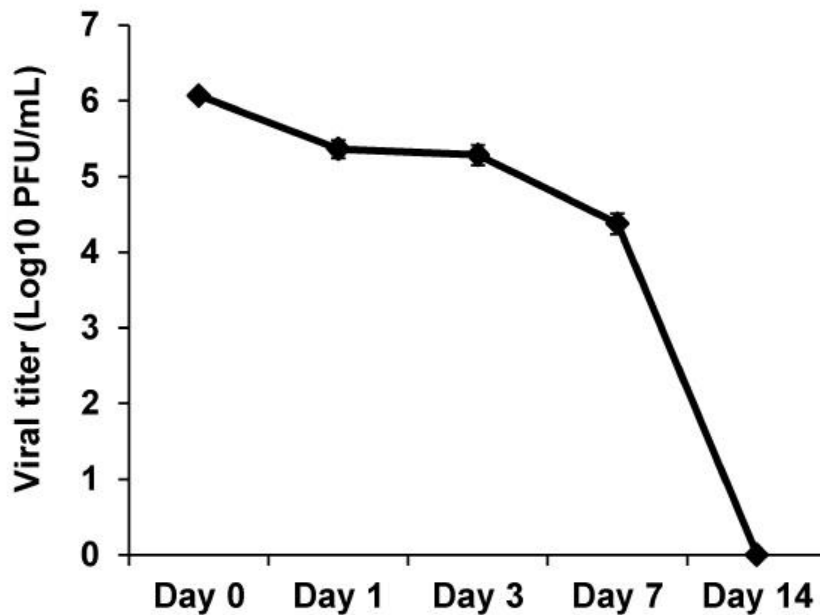


FIG 5. Determination of TV stability in green pepper puree. TV was diluted 1:10 in green pepper puree to an approximate titer of $10^{6.0}$ pfu/ml. TV inoculated green pepper puree was stored at room temperature for 14 days, and the survival of TV was determined at day 0, 1, 3, 7, and 14 post inoculation by standard plaque assay. Each data point represents the average of 3 replicates \pm standard deviation.

DISCUSSION

Strawberries are known to be a high risk food for human NoV contamination; however the interaction of the virus with growing plant systems and the mechanisms of viral persistence in plants are poorly understood. Several human NoV outbreaks have been associated with the consumption of soft berries, many of which could be linked to the use of irrigation water sources contaminated with sewage (10, 11, 13, 14). The fresh produce market is global and in many outbreaks the product is imported from countries that have their own production practices and food safety management systems which may not regulate irrigation water sources (31). The use of virally contaminated irrigation water may distribute the virus on the plant tissues, but there is little data available on whether viral contaminants in the water supply can become internalized via the root of growing produce and disseminated to other plant tissues. In this study, we found for the first time that human NoV surrogates (MNV-1 and TV) can be internalized via the roots of growing strawberry plants and be disseminated to the berry during soil growth. Overall, there was no significant difference for the positive rate and virus titer in leaves for MNV-1 and TV. However, the positive rate and titer of TV in strawberry were higher than those of MNV-1 in strawberry. This suggests that the type of virus may affect the internalization efficiency in strawberries. In contrast, no or minimal internalization was detected in bell peppers under the same conditions, suggesting that the type of plant significantly affects the rate of virus internalization in plant tissues.

To date, no study has evaluated whether enteric viruses can be internalized within the fruit of growing strawberry plants. Recently, a study was conducted to evaluate the presence of pathogenic human and zoonotic viruses in field grown strawberries after

irrigation with surface water (32). Berries and irrigation water were tested for the presence of human rotavirus (RV), human NoV GI, human NoV GII, and hepatitis E virus (HEV). Of the total 60 strawberries tested, 16 were positive for GI human NoV, 2 for human RV, and 1 for swine HEV (32). Although no virus was detected in the irrigation water samples, possibly due to low concentration of virus in the water source, a significant amount of the strawberry plots (7 of 16) were found to be positive for virus 1 hour following irrigation (32), suggesting that the irrigation water contained viruses. Though this study did not specifically evaluate the presence of internalized viruses in strawberries, the drip irrigation system would minimize exposure of the berry surface to irrigation water which could indicate that viral internalization was occurring.

The kinetics of virus internalization and dissemination was determined in strawberries using two human NoV surrogates (TV and MNV). Overall, 38 strawberries were harvested during the 14 day study period and 12 tested positive for infectious MNV-1, which correlates to 31.6% of the strawberries containing internalized MNV-1. 48 berries were harvested of which 37.5% tested positive for infectious TV. Overall, TV-positive rate in strawberry was higher than MNV-1 positive rate in strawberry although TV and MNV-1 had the same positive rate (45%) in leaves (Fig.2B). In addition, the titer of infectious TV ($1.83 \log_{10}$ pfu/g) recovered from berries was significantly higher than MNV-1 ($0.81 \log_{10}$ pfu/g) ($P < 0.05$). Interestingly, it was previously found that internalization and dissemination rate of TV in hydroponically grown Romaine lettuce was also higher compared to MNV-1 (26). Similarly, Hepatitis A virus was found to be internalized more efficiently than MNV-1 in hydroponically grown spinach and green onion (28). This evidence indicates that the type of pathogen influences the rate of

internalization, and it appears that MNV-1 is less efficiently disseminated into fruit portion of the strawberry plants compared to TV.

Though infectious virus was recovered from strawberry tissues, it was relatively low compared to the level of inoculum. Similar results were obtained in a study that evaluated the dissemination of bacteriophage f2 when applied to the roots of hydroponically growing beans and found that less than $2 \log_{10}$ PFU/g of virus could be recovered from the beans when the plants were exposed to 10^{10} PFU/mL of phage (33). It is possible that dissemination to seeds or fruits is less efficient compared to other plant tissues such as stems and leaves. The complexity of the growth matrix, such as soil versus hydroponic growth, has been established as one of the major variables impacting the rate of pathogen internalization in crops (15, 34-38). A study evaluating the internalization of MNV-1 in both soil and hydroponically grown green onions and spinach, failed to detect any infectious virus from the plants grown in soil (28). However, using a hydroponic growth system up to $4.66 \log_{10}$ PFU/g of MNV-1 was recovered from green onions and viral RNA was detected in both the green onions and spinach (28). Several studies have detected positive viral internalization using hydroponic growth systems, but few have had success with soil grown plants (27-30, 39). Given the fact that during symptomatic human NoV infection the virus can shed at levels of 10^{10} virus particles/g feces, it is possible that high levels of virus similar to our experimental conditions could be present in irrigation water leading to viral internalization by field grown strawberries.

There was an increase in the recovery of TV RNA from strawberries compared to infectious virus. At day 1 post inoculation, 40% leaves and 46.2% berries were positive

for TV RNA with average titers in the positive samples 2.8-3.8 log₁₀ RNA copy/g. 80% leaves and 91.7% berries were positive for TV RNA on day 3 post inoculation with average titers in the positive samples of 3-4 log₁₀ RNA copy/g. On day 7 post inoculation, 80% leaves and 100% of berries were positive for TV RNA with an average titer in the positive samples of approximately 3 log₁₀ RNA copy/g. Overall, 78.7% of the harvested berries were positive for TV RNA with an average titer of 3.15 ± 0.51 log₁₀ RNA copy/g. Several studies have found that the level of internalized viral RNA was increased compared to the level of recovery of infectious virus (26, 28-30). This could be due to the lower detection limit of RT-qPCR based assays compared to infectivity assays or due to the fact that RT-qPCR assays do not discriminate between RNA from infectious and non-infectious viral particles. Another possible reason for the low level of virus recovery from strawberry tissues could be inactivation of the virus by low pH or by compounds naturally present in the plant. The average pH of the strawberries harvested in this study ranged from pH 3-5, with lower pH (pH 3-4) values measured for ripe berries. Although TV was shown to be stable in pH ranging from 2-8 for 30 min (25), it is possible that some viruses can be inactivated upon exposure to low pH for a long time period. In addition, other compounds, such as polyphenols (anthocyanins, flavanols, flavonols) or phenolic acids (hydroxybenzoic acid, hydroxycinnamic acid) present in the strawberries could potentially have anti-viral capabilities that lead to reduced viral recovery from strawberry tissues. Total strawberry extracts have been shown to inhibit the replication of coxsackievirus B1 and influenza A, and this inhibition was enhanced using the anthocyanin fraction of the extract, so it possible that these compounds may also affect calicivirus stability (40). We evaluated the stability of TV in strawberry puree

and found that after 14 days the titer of TV was reduced by approximately 3 logs. This indicates that other components present in the strawberry puree, and perhaps 14 day exposure to low pH, did reduce TV infectivity.

In contrast to MNV and TV internalization in strawberry, no infectious TV or viral RNA was recovered from pepper tissues when they were inoculated with same amount of TV. To rule out the possibility that TV can directly be inactivated upon internalization to pepper, we determined the survival of TV in pepper puree at room temperature for 14 days. We found that the stability of TV in pepper puree was comparable to TV in strawberry puree during 7 days of incubation time (compare Figs. 4 and 5). This suggests that the type of plant significantly affects the internalization rate in plant tissue. However after 14 days of storage at room temperature no infectious virus was detected (Fig. 5). This indicates that green peppers contain substances that can inhibit viral infectivity after longer term exposure. Bell peppers do not contain capsaicin, the lipophilic chemical associated with the burning sensation upon consumption of peppers, but contain other chemicals such as para-coumaric acid and 3-isobutyl-2-methoxypyrazine distinct from strawberries which may have impacted viral infectivity and recovery. Differences in pathogen internalization have been observed between different plant types in other studies (15, 28, 34, 36, 37, 41-43). For example, it was found that internalization of *Salmonella enterica* serovar Typhimurium via the leaf was variable for seven different kinds of produce, and was highest for arugula and iceberg lettuce compared to basil, red lettuce, Romaine lettuce, parsley, and tomato (43). Similarly, another study showed *S. Typhimurim* internalization was found to be higher in green onions compared to iceberg lettuce. Variability in the colonization of spinach by

Escherichia coli O157:H7 has even been observed between different spinach cultivars (37). In terms of viruses, MNV-1 was found to be internalized more efficiently in green onions compared to spinach in a floating hydroponic system (28).

The use of fecally contaminated irrigation water poses a major risk to the safety of soft berries and other types of fresh produce. In light of the recent large scale outbreak of human NoV associated with the consumption of strawberries in which a contaminated water source has been implicated as the source of virus, it is imperative to determine the ecology and persistence of human NoV in growing crops. In this study it was shown that virally contaminated soils can lead to the internalization of virus via plant roots and subsequent dissemination to the leaf and fruit portions of growing strawberry plants. In addition it was found that the magnitude of internalization was dependent on the type of virus and plant. To our knowledge, this is the first demonstration of human NoV surrogates internalizing via plant roots and disseminating to fruits in a soil growth system. Internalized viruses would be protected from all surface decontamination practices, and hence pose a significant risk to public health. The identification of the mechanisms of persistence of enteric viruses in soft berries can lead to the development of novel intervention strategies targeted at internalized viruses within fruit tissues.

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