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## Internalization and dissemination of human norovirus and Tulane virus in fresh produce is plant dependent

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### Abstract

Human norovirus (NoV) is a leading cause of fresh produce associated outbreaks. Previous research indicates that the roots of growing leafy greens and berries internalize human NoV. However the effect of plant type and inoculum level on internalization rates has not been directly compared. In this study we compared the internalization and dissemination rates of human NoV and its surrogate, Tulane virus (TV) in green onion, radishes, and Romaine lettuce. We also evaluated the effect inoculum level and plant growth matrix on the rate of viral internalization. In the hydroponic growth system, we detected internalization and dissemination of human NoV RNA in green onions. In hydroponically growing green onions inoculated with high titer TV, we found higher rates of internalization and dissemination compared to green onions inoculated with low titer TV. In soil growth systems, no infectious TV was detected in either green onion or radishes. However, in Romaine lettuce plants grown in soil approximately  $4 \log_{10}$  PFU/g was recovered from all tissues on day 14 p.i. Overall, we found that the type of plant, growth matrix, and the inoculum level influences the internalization and dissemination of human NoV and TV.

### Keywords

Human norovirus; Tulane virus; Internalization; Fresh produce; Green onion; Radish

## 1. Introduction

Human norovirus (NoV) is one of the major causes of acute gastroenteritis worldwide (Allwood et al., 2004; Atmar and Estes, 2006; DiCaprio et al., 2013; Glass et al., 2009;

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Robilotti et al., 2015). The virus is a leading cause of foodborne illness in the United States, causing nearly 58% of foodborne disease (Dicaprio et al., 2013; Scallan et al., 2011). Fresh produce has been identified as a leading cause of foodborne illness and is a major high risk food associated with human NoV outbreaks in the US (Hall et al., 2013; Li et al., 2012; Lynch et al., 2009). Vegetable row crops (such as leafy greens) and fruits were responsible for 30% and 21%, respectively, of human NoV foodborne outbreaks in the US (2009–2012) (Hall et al., 2012). Vegetables and crops can be contaminated with human NoV at any point from farm to fork. Evidence has shown that the source of pre-harvest contamination mainly comes from soil, fertilizer, or irrigation water. Though human NoV is a major contributor to fresh produce associated outbreaks, the modes of contamination and persistence of the virus in vegetables remains poorly understood.

Human NoV is a non-enveloped single stranded positive sense RNA virus of the family *Caliciviridae* (Robilotti et al., 2015). The major challenge in human NoV research is that currently there is no robust cell culture system for the virus. Recently, two cell culture systems were developed for human NoV, but have not yet been optimized to produce high viral titers (Ettayebi et al., 2016; Jones et al., 2015). Therefore, much of the understanding of human NoV molecular biology, pathogenesis, and environmental stability has come from the study of surrogate viruses (Li et al., 2012; Richards, 2012). Many viruses within the family *Caliciviridae* have been utilized as human NoV surrogates, including murine norovirus (MNV), feline calicivirus (FCV), and Tulane virus (TV) (Cannon et al., 2006; Cromeans et al., 2014; Farkas, 2015; Hirneisen and Kniel, 2013b; Kniel, 2014; Li et al., 2012). Tulane virus (TV) is a newly recognized surrogate for human NoV and is member of the genus *Recovirus* within *Caliciviridae* (Farkas et al., 2008). TV was shown to have similar pH stability to human NoV and other surrogates at ranges from pH 3 to pH 8 (Hirneisen and Kniel, 2013b). TV causes enteric infection in primates and also recognizes histo-blood group antigens as a cellular attachment receptor, similar to human NoV (Farkas et al., 2010).

Internalization of pathogens in growing produce is considered one of the potential routes for contamination of fresh produce (Erickson, 2012; Heaton and Jones, 2008; Warriner et al., 2003; Warriner and Namvar, 2010). Bacterial pathogens, such as *Salmonella enterica* serovar Typhimurium and *Escherichia coli* O157:H7, have been shown to be internalized in vegetables including lettuce, radishes, alfalfa and green onions (Erickson et al., 2010a,b; Erickson et al., 2010a,b; Franz et al., 2007; Ge et al., 2012, 2014; Golberg et al., 2011; Hora et al., 2005; Jablasone et al., 2005; Kroupitski et al., 2009; Mitra et al., 2009; Pu et al., 2009; Sharma et al., 2009; Warriner et al., 2003). Human NoV has also been shown to be internalized in growing produce such as lettuce, spinach, and green onion (DiCaprio et al., 2015a,b; Dicaprio et al., 2012; DiCaprio et al., 2015a,b; Esseili et al., 2012; Hirneisen and Kniel, 2013a; Wei et al., 2010, 2011). However, few studies exist where multiple types of produce have been evaluated for viral internalization in parallel. In addition, the influence of initial inoculum levels on viral internalization in different types of produce has not been evaluated extensively. In this study, green onion, radishes, and Romaine lettuce were selected to evaluate the effect of growth matrix, inoculum level, and vegetable type on the internalization of human NoV and TV in fresh produce.

## 2. Materials and methods

### 2.1. Viruses and cell culture

Human NoV GII.4 strain 707 (GenBank accession number JQ798158) was originally isolated from an outbreak of acute gastroenteritis in Ohio. The virus genomic RNA was quantified by reverse transcriptase quantitative PCR (RT-qPCR) and then stored at  $-80^{\circ}\text{C}$ . TV was generously provided by Xi Jiang at Cincinnati Children's Hospital. TV was propagated in confluent monolayers of the monkey kidney cell line MK2-LLC (ATCC, Manassas, VA). MK2-LLC cells were cultured at  $37^{\circ}\text{C}$  in a 5%  $\text{CO}_2$  atmosphere in low-serum Eagle's minimum essential medium (Opti-MEM; Invitrogen, Carlsbad, CA) supplemented with 2% FBS. Before virus infection, MK2-LLC cells were washed with Hanks' balanced salt solution (HBSS) and subsequently infected with TV at an MOI of 1. After 1 h of incubation at  $37^{\circ}\text{C}$ , 18 ml of Opti-MEM with 2% FBS was added. The virus was harvested 2 days post infection by three freeze thaw cycles followed by centrifugation at  $3000 \times g$  for 10 min to remove cell debris. After centrifugation, the supernatant was collected and virus stocks were stored at  $-80^{\circ}\text{C}$ . TV titer was determined by plaque assay.

### 2.2. Virus enumeration by plaque assay

TV was quantified by plaque assay in LLC-MK2 cells by seeding the cells into 6-well plates (Corning Life Sciences, Wilkes-Barre, PA) at a density of approximately  $3 \times 10^6$  cells per well. After one day incubation, cell monolayers were infected with 400  $\mu\text{l}$  of a 10-fold dilution series of samples, respectively, and the plates were incubated for 1 h at  $37^{\circ}\text{C}$  with gentle agitation every 10 min. The cells were then overlaid with 2.5 ml of overlay medium (Eagle's minimum essential medium with 1% agarose, 2% FBS, 1% sodium bicarbonate, 15 mM HEPES, and 100 units/ml penicillin, kanamycin, and streptomycin) (Invitrogen). After incubation at  $37^{\circ}\text{C}$  for 2 days, the cells were fixed with 10% formaldehyde (Fisher Scientific, Waltman, MA) overnight. The plaques were visualized by staining with 0.05% (wt/vol) crystal violet (Sigma-Aldrich, St. Louis, MO).

### 2.3. Quantification of human NoV by RT-qPCR

Human NoV RT-qPCR was carried out as previously described (22). Total RNA was extracted from 100  $\mu\text{l}$  of sample using an RNeasy kit (Qiagen, Hilden, Germany), followed by RT-qPCR. Firststrand cDNA was synthesized with SuperScript III reverse transcriptase (Invitrogen) using the primer VP1-P1 (5'-TTATAATACACGTCTGCGC CC-3'), which targets the VP1 gene of human NoV. The VP1 gene was then quantified by real-time PCR using custom primers and TaqMan probe (Forward primer, 5'-CACCGCCGGGAAAATCA-3', reverse primer, 5'-GCCTTCAGTTGGGAAATTTGG-3', and reporter, 5'-FAM- ATTTGCAGCAGTCCC NFQ-3') on a StepOne real-time PCR machine (Applied Biosystems, Foster City, CA). TV RNA was also quantified by RT-qPCR. First strand cDNA was synthesized by SuperScriptase III (Invitrogen) using the primer TVRT (5'-AATTCCACCTTCAACCCAAGTG -3'), which targets the VP1 gene of Tulane virus. The VP1 gene was then quantified by real-time PCR using custom primers and TaqMan probe (Forward primer: 5'-TTGCAGGAGGGTTTCAAGATG-3') (Reverse primer: 5'-CACGGTTTCATTGTCCCCATA-3') (Probe:5'-FAM-TGATGCACACATGTGGGA-NFQ-3'). PCR 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 s prior to cycling, followed by 50 cycles of 95 °C for 1 s for annealing and 60 °C for 20 s for extension. A standard plasmid for human NoV and TV was constructed by inserting the sequence of entire ORF2 (VP1 gene) into pGEM T-easy vector (Promega, Madison, WI). The purified plasmids for human NoV and TV with known concentration was then ten-fold serial diluted to generate a standard curve for RT-qPCR. Standard curves and StepOne software version 2.1 were used to quantify genomic RNA copies. Viral RNA was expressed as the mean log<sub>10</sub> genomic RNA copies/ml ± standard deviation.

#### 2.4. Plant cultivation

Seeds of green onion (*Allium fistulosum* L.) and radish (*Raphanus sativus*) were purchased from Livingston Seed (Columbus, Ohio) and stored at 4 °C. Before sowing, the seeds were soaked for 1 day at room temperature (20 °C) in a 500 ml beaker of tap water. Subsequently, the water was drained and the seeds were covered with 2 layers of wet Kimwipes (Fisher Scientific, USA) for 2 days to accelerate seed germination. After germination, the seeds were planted in 4 inch plastic pots and grown in plant growth chamber under long day conditions (22 °C with 16/8 light/dark photoperiod) in Metro-Mix® 300 soil (Sun Gro Horticulture, USA).

Two month old green onions and Romaine lettuce and one month old radishes were used for experiments. For growth in the hydroponic system, plants were removed from soil and roots were washed thoroughly with tap water to remove soil and then placed in racks above feed water. Feed water was aerated using a pump and antibiotics (100 units/ml penicillin, kanamycin, and streptomycin) were added to limit microbial growth. The feed water was not replaced during the study period. After viral inoculation, only the roots contacted the feed water and a plastic barrier separated the aerial plant tissues and feed water to limit cross contamination.

#### 2.5. Viral inoculation and sample collection for hydroponically grown green onion and radish

For green onion grown hydroponically, TV was diluted in feed water to result in a high titer ( $1.0 \times 10^6$  PFU/ml) and low titer ( $1.0 \times 10^4$  PFU/ml) virus concentration in the feed water. Human NoV was diluted in the feed water of hydroponically growing green onions to achieve  $1.0 \times 10^5$  RNA copy/ml. For hydroponically grown radishes, only the high concentration ( $1.0 \times 10^6$  PFU/ml) of TV was tested. For each treatment group, a 10 ml sample feed water was collected before plants were inserted and was used a control to determine the stability of TV in feed water without plants. In addition, uninoculated control groups were set up for each treatment group. The total volume of the hydroponic feed water following virus dilution was 400 ml and the initial virus titer was determined using plaque assay (TV) or RT-qPCR (human NoV). Negative control plants were kept in separate tanks and no virus was added.

At days 0 (before viral inoculation), 1, 3, 7, and 14, four plants were harvested for each condition and a sample of the feed water was collected. To minimize cross contamination,

the upper most leaves were harvested first, followed by shoots, and roots were harvested last (Fig. 1A, Fig. 1B). Harvested tissues were placed in individual sterile plastic bags and each tissue was weighed. Next, the surface of each tissue was decontaminated by submersion in 1000 ppm chlorine followed by rinsing with water and inactivation of residual chlorine with 0.25 M sodium thiosulfate. Samples were then homogenized by grinding with mortars and pestles with 5 ml PBS (pH 7.0). Sample homogenates were transferred to 15 ml tubes and centrifuged at  $3000 \times g$  for 10 min to remove cellular debris and the virus-containing supernatant was then transferred to a new collection tube. No virus concentration step was performed prior to detection. Detection of viruses in plant tissue samples and feed water samples was determined by RT-qPCR (human NoV) and plaque assay (TV). The detection limit for RT-qPCR was determined to be 5 RNA copy/ml using dilution of plasmid standard in each sample matrix. The detection limit for plaque assay was determined to be 10 PFU/ml by diluting TV stock in each sample matrix. Three technical replicates were executed during RT-qPCR analysis and two technical replicates were used for plaque assay for each sample.

## **2.6. Viral inoculation and sample collection for soil grown green onion, radish, and Romaine lettuce**

20 ml of  $1.0 \times 10^6$  PFU/ml TV was applied to the root zone of either green onion, radish, or Romaine lettuce grown in soil. The virus solution was inoculated slowly into the soil 0.5 cm from the soil surface to limit the contamination of shoot and leaves. The surface of the soil was covered with plastic film to limit viral cross contamination. The sample collection procedure was the same as described above, with the exception of the harvesting of the plant root. After harvesting leaves and shoots, roots were washed with tap water to remove soil debris. All harvested tissues were surface decontaminated with 1000 ppm chlorine and the remainder of the tissue processing was the same as described above.

## **2.7. Plant sap collection and virus inoculation**

Growing green onions and radishes were divided into three portions, leaves, shoots, and roots, as previously described. Each portion was subjected to tissue sap (fluid) extraction by a hydraulic plant sap press (Spectrum, Plainfield, IL USA) following the manufacturer's procedures. From each tissue, 5 ml tissue fluids were collected separately in a 15 ml tube and TV was added to the sap from each tissue to achieve a viral titer of  $1 \times 10^6$  PFU/ml. At days 1, 3, 7, and 14, the virus titer in each sap was determined by plaque assay. As a positive control, TV suspended in PBS was also tested in the same manner.

## **2.8. Statistical analysis**

Data is presented as mean  $\pm$  1 standard deviation (SD). Statistical analysis by One-way ANOVA was performed by SPSS 19 (IBM, USA). Least significant difference (LSD) was used to compare the difference of treatments. *P*-values less than 0.05 were considered statistically significant.

### 3. Results

#### 3.1. Internalization and dissemination of human NoV in hydroponically grown green onions

Human NoV was inoculated to the feed water of hydroponically growing green onions at a concentration of  $1.0 \times 10^5$  RNA copies/ml and plants were harvested at days 1, 3, 7, and 14 post inoculation. Human NoV RNA was detected in all green onion tissues on all study days. The level of human NoV RNA in the root was the highest on day 1 post inoculation ( $6.0 \times 10^4$  RNA copies/g) and decreased on each following treatment day. On day 14, the virus RNA in the roots was reduced significantly compared to days 1, 3, and 7 to  $4.2 \times 10^3$  RNA copies/g ( $P < 0.05$ ) (Fig. 2). Similar to the results of the roots, the highest level of human NoV RNA in the shoots was detected on day 1 post inoculation and decreased on days 3, 7, and 14. The level of human NoV RNA on day 1 post inoculation was  $5.9 \times 10^4$  RNA copy/g (Fig. 2). The level of human NoV RNA was reduced significantly at days 3, 7 and 14 post inoculation compared to day 1— $1.8 \times 10^3$ ,  $3.2 \times 10^2$ ,  $1.8 \times 10^3$  RNA copy/g, respectively ( $P < 0.05$ ) (Fig. 2). The highest level of human NoV RNA detected in the leaves was also on day 1 post inoculation at a level of  $4.6 \times 10^4$  RNA copy/g (Fig. 2). Compared to the other tissues, the level of human NoV RNA in the leaves was more stable from days 1–14 post inoculation. On days 7 and 14 post inoculation the level of human NoV RNA in the leaves was  $1.0 \times 10^4$  and  $1.4 \times 10^4$ , respectively (Fig. 2).

The level of human NoV RNA in the feed water used to grow the green onions hydroponically had a significant reduction from  $1 \times 10^5$  RNA copy/ml to  $2.9 \times 10^4$  RNA copy/ml on day 1 post inoculation ( $P < 0.05$ ). The level of human NoV RNA in the feed water continued to decrease over the study period and on day 14 post inoculation the RNA level was  $1.3 \times 10^3$  RNA copy/ml.

#### 3.2. Internalization and dissemination of TV in hydroponically grown green onions and radishes

Two different starting titers of TV in the feed water for hydroponically growing green onions were tested being high,  $1.0 \times 10^6$ , and low,  $1.0 \times 10^4$  PFU/ml. For the plants in the low titer TV treatment group, the highest TV titer detected was in the roots on day 1 post inoculation at a level of  $1.6 \times 10^1$  PFU/g (Fig. 3A). The titer in the roots decreased on day 3 post inoculation to  $1.1 \times 10^1$  PFU/g and on days 7, 14 post inoculation the level of virus decreased significantly and was below the detection limit (BDL) ( $P < 0.05$ ). There was no detection of infectious TV in any of the leaf or shoot tissues harvested at any time-point. There was a slight decrease ( $<1$  log) in the level of TV detected in the feed water on day 1 post inoculation and the infectious titer remained stable at this level up to day 7. There was an approximate  $2.5 \log_{10}$  PFU/ml reduction in the feed water TV titer on day 14 post inoculation. In the control feed water samples (without plants), the titer was slightly higher ( $0.5$ – $1 \log_{10}$  PFU/ml) compared to the feed water used to grow the green onions at all time-points.

In the hydroponically growing green onions which received the high titer TV treatment, the highest level of internalized virus detection was in the roots on day 1 post inoculation at  $1.9$

$\times 10^5$  PFU/g (Fig. 3B). The level of TV in the roots decreased significantly at each subsequent time point ( $P < 0.05$ ). On days 3, 7, and 14 the titer of TV detected in the roots was  $6.9 \times 10^4$ ,  $2.1 \times 10^3$ ,  $2.3 \times 10^1$  PFU/g, respectively (Fig. 4). In the shoots of green onions in the high level TV treatment group, the virus was detected at day 1 at a level of  $1.7 \times 10^2$  PFU/g. On day 3 post inoculation the titer in the shoots increased significantly ( $P < 0.05$ ) to  $1.3 \times 10^3$  PFU/g (Fig. 3B). The level of TV in the shoots was BDL on days 7 and 14 post inoculation. No infectious TV was detected in the green onion leaves at any time point. In the feed water of high titer TV treatments, there was an approximate 3.6 log<sub>10</sub> PFU/ml reduction in the feed water on day 14 post inoculation compared to day 0. In the control feed water samples (without plants), there was no significant reduction in TV titer over the 14 day study period ( $P > 0.05$ ).

RT-qPCR was used to determine the level of TV RNA in harvested green onion samples. The green onions inoculated with a low dose of TV had detectable RNA in the roots on day 1 post inoculation at level of  $5.8 \times 10^4$  RNA copy/g (Fig. 4A). This high level of RNA remained detectable in the roots for the duration of the study (Fig. 4A). There was no TV RNA detected in either the shoots or the leaves of green onions inoculated with a low dose of TV. When green onions were inoculated with the high dose of TV there was also a high level of TV RNA detected in the roots on day 1 post inoculation  $9.0 \times 10^5$  RNA copies/g and remained at this level until day 14 post inoculation (Fig. 4B). There was also TV RNA detected in the shoots of green onions inoculated with the high dose of TV on days 1 and 3 post inoculation at levels of  $3.2 \times 10^4$  and  $4.2 \times 10^4$  RNA copies/g, respectively. However, there was no RNA detected in the shoots at days 7 or 14 post inoculation. No TV RNA was recovered from the leaves of green onions inoculated with a high dose of TV.

Hydroponically growing radish plants also had the feed water contaminated with a high titer of TV. In contrast to green onion, there was no infectious TV recovered from any radish tissue at any time point in the study. In the feed water used to grow the radish plants, there was no reduction in infectious TV titers between day 0 and day 14 post inoculation.

### 3.3. TV internalization and dissemination in soil grown green onions and radishes

The soil of growing green onion plants was inoculated with  $1.0 \times 10^6$  PFU of TV and tissues were harvested on days 1, 3, 7, and 14 post inoculation. TV was not detected in any green onion tissue on any day post inoculation. Soil growing radish plants were similarly inoculated with TV with the same lack of recovery of infectious TV from radish tissues.

### 3.4. TV internalization in Romaine lettuce plants grown in soil

The soil of growing Romaine lettuce plants was inoculated with  $1.0 \times 10^6$  PFU of TV and tissues were harvested on days 1, 3, and 14 post inoculation. At day 1 post inoculation, TV was detected in the roots of lettuce plants at a level of  $5.24 \times 10^3$  PFU/g. No infectious TV was detected in the shoots or leaves at day 1 post inoculation (Fig. 5). On day 3 post inoculation TV was recovered in the shoots ( $7.2 \times 10^1$  PFU/g) and leaves ( $1.0 \times 10^2$  PFU/g) of Romaine lettuce, as well as the roots (Fig. 5). All harvested tissues were positive for TV on day 14 post inoculation with  $6.1 \times 10^4$  PFU/g in roots,  $4.4 \times 10^4$  PFU/g in shoots, and  $8.7 \times 10^4$  PFU/g in leaves (Fig. 5).

### 3.5. Stability of TV in green onion and radish saps

Tissue sap from the leaves, shoots, and roots of both green onions and radishes were prepared and the stability of TV in these saps was tested over 14 days. TV was stable in all green onion saps tested with no significant decreases in infectious TV titers over the 14-day study period (Fig. 6). TV was also stable in radish saps obtained from leaves and shoots (Fig. 7). However, in the root sap obtained from radishes the titer of TV was significantly decreased compared to controls on days 3, 7, and 14 ( $P < 0.05$ ) (Fig. 7).

## 4. Discussion

The consumption of fresh fruits and vegetables continues to increase in many countries and fresh produce is an important vehicle for foodborne disease transmission. The outbreaks associated with fresh produce have resulted in considerable public health and economic burdens (Moore et al., 2015). Among the pathogens associated with fresh produce outbreaks, an increased incidence of infections and outbreaks are attributed to foodborne viruses, most notably human NoV (Baert et al., 2011). It has been demonstrated that various types of plants are susceptible to internalization of human viral pathogens during growth (DiCaprio et al., 2015a,b; DiCaprio et al., 2012; DiCaprio et al., 2015a,b; Hirneisen and Kniel, 2013a). Various factors have the potential to affect the ability of human pathogens to internalize in growing plants including the growth substrate (soil vs. hydroponic solution), plant developmental stage, pathogen type, inoculum level, plant species and cultivar, abiotic and biotic stresses (DiCaprio et al., 2015a,b; DiCaprio et al., 2012; DiCaprio et al., 2015; Hirneisen and Kniel, 2013a). In this study, different plants types, inoculum levels, and growth matrices were tested to determine their effect on the internalization and dissemination of human NoV.

Previous research has shown that the level of pathogen inoculum used to contaminate plants can greatly influence the rate of bacterial internalization and dissemination (Pu et al., 2009). For example, in *Arabidopsis thaliana*, low inoculum levels ( $10^4$  CFU/ml) of *Salmonella* Typhimurium were able to colonize the root surface but did not invade the lateral root junctions. When the inoculum level was increased to  $10^6$  CFU/mL, the bacteria were able to internalize into the root (Cooley et al., 2003). In this study, two different levels of TV inoculum, high ( $1 \times 10^6$  PFU/ml) and low ( $1 \times 10^4$  PFU/ml), were used to contaminate the feed water of hydroponically growing green onions. TV was detected in roots of green onions in both the high and low inoculum groups. The high inoculum group had infectious virus detected in the aerial portions of the plant while no virus was detected in these tissues when a low virus inoculum was applied.

Unlike bacteria that have the ability to replicate in the phyllosphere, viruses are unable to replicate outside of their host cell. However, in general it would be expected that higher inoculum levels would result in higher levels of internalization (Hirneisen and Kniel, 2013a). This was the conclusion made in another study in which higher inoculums of *E. coli* O157:H7 in spinach showed higher contamination incidences (Pu et al., 2009). In another study, the internalization of *Salmonella* Typhimurium was highly dependent on the concentration of the pathogen present in irrigation water. Irrigation water containing 5 Log CFU/mL *Salmonella* Typhimurium resulted in limited incidence of internalization in lettuce,

while 8 Log CFU/mL *Salmonella* Typhimurium in the irrigation water, significantly increased the internalization frequency. Similar to these studies, we found that increased TV contamination levels in green onions lead to increased internalization of the virus in hydroponically grown green onion.

In addition, we evaluated the internalization of human NoV in hydroponically growing green onion. We found that high levels of human NoV RNA were detected in all harvested green onion tissues starting on day 1 post inoculation. The levels of RNA decreased over the study period; however RNA was still detectable in all tissues on day 14 post inoculation. The data indicates that human NoV internalized via the root of the green onions and transports to the leaves.

We also used RT-qPCR to evaluate the level of internalized TV RNA in this study. We found that high levels of TV RNA were detected starting on day 1 post inoculation and that the level of RNA remained at this level at all subsequent time points. However, this differs from the internalization results obtained for TV using plaque assay as the method of detection, in which lower levels of internalization were detected and there were reductions in the level of virus internalized at later time points. This could be due to the fact that RT-qPCR is more sensitive than plaque assay and that RNA detection alone may not accurately reflect the recovery of infectious viruses in these samples. In previous studies in hydroponically growing Romaine lettuce, it was shown that the levels of internalized TV RNA was higher than the levels of infectious virus that could be recovered from the same samples (Dicaprio et al., 2012). In addition it was found that employing an RNase pre-treatment to degrade viral RNA that was not found in an intact viral capsid reduced the level of TV RNA detection (Dicaprio et al., 2012). Similarly, in studies investigating murine norovirus (MNV-1) and hepatitis A virus (HAV) internalization in green onions and spinach more samples were found to be positive for viral RNA compared to infectious virus (Hirneisen and Kniel, 2013a).

Two types of growth substrates, soil and hydroponic solution are commonly used in pathogen internalization studies conducted with plants. Pathogen internalization has been detected more often in hydroponically grown plants compared to plants grown in soil (Dicaprio et al., 2012). In this study, both hydroponic and soil growing green onions were contaminated with TV at a level of  $1 \times 10^6$  PFU/ml. Similar to previous studies, we found that internalization of TV via the root of hydroponically growing green onion occurred while no TV internalization occurred in soil grown green onions.

The same phenomena was also observed with HAV and MNV, which were not detected in green onion plants grown in soil for up to 20 days (Hirneisen and Kniel, 2013a). Compared to soil systems, a drastic increase in virus internalization was observed in hydroponic systems with both HAV and MNV internalized up to 4 log RT-qPCR units (Hirneisen and Kniel, 2013a). Most investigators have suggested that the motility in hydroponic solution may provide more opportunity for uptake and internalization into leafy green plants compared to soil systems (DiCaprio et al., 2015a,b). The virus capsid has an ionic charge and it may remain bound to charged particles in the soil matrix reducing viral uptake by the roots (DiCaprio et al., 2015a,b). It is also possible that root damage induced by transplanting

of seedlings into the hydroponic system also increased internalization in this study and others (Dicaprio et al., 2012; Franz et al., 2007).

The type of plant has also been shown to influence the rate of bacterial pathogen internalization (Erickson, 2012; Ge et al., 2012; Golberg et al., 2011; Hirneisen and Kniel, 2013a). In this study, no virus internalization occurred in radishes while there was viral internalization detected in green onions and Romaine lettuce. Internalization of *Salmonella* Typhimurium was also observed in lettuce and radish but not cress or spinach seedlings (Jablasone et al., 2005). In the same study, radish seedlings were susceptible to *Salmonella* internalization while mature radishes were resistant, indicating the importance of plant age on pathogen internalization and dissemination (Jablasone et al., 2005). The radishes used in this study were mature (30 days old). In our study we found no significant decrease in the level of virus detected in the feed water of hydroponically growing radishes. This indicates that no viral uptake occurred via radish root and it is possible that these mature radishes were not permissive to pathogen internalization.

Previously it was shown TV was internalized and disseminated in growing strawberry plants while the virus was not detected in peppers of growing green pepper plants (DiCaprio et al., 2015a,b). It was found that the green peppers had a significant anti-viral effect on TV and that this may play a role in the lack of internalization in bell pepper plants (DiCaprio et al., 2015a,b). In this study, we found that the levels of TV detected in Romaine lettuce increased over the study period, which is indicative of internalization and dissemination with no inhibition. However, the internalization and dissemination kinetics was different in green onions with high levels of internalized virus detected on day 1 post inoculation and decreasing over the study period. This may indicate that an inhibitory compound in the green onion may be inactivating the virus. Green onions contain a wide array of phenolic compounds which have the potential to act as antimicrobials (Siddiq et al., 2013). We found that sap from the green onion root lead to a 1-log reduction in infectious TV only after 14 days of exposure.

Previously, it was found that a component of radishes, *trans*-4-methylthio-3-butenyl isothiocyanate (TMBI), possessed antimicrobial activity (Park et al., 2016). In addition, the pigment found in the skin of radishes, anthocyanins, have also been shown to have antiviral activity. Different cyanidin glycosides present in radishes have antiviral activity against influenza A and B viruses and herpes-1 virus (Knox et al., 2003; Suzutani et al., 2003). Our data indicates that radish roots are not permissive to virus penetration and internalization; therefore, we investigated whether radishes had antiviral properties. A maximum 1-log reduction in infectious TV titer was achieved after 14 days of exposure of TV to radish sap, indicating only minimal antiviral effects.

In conclusion, we have shown that i) human NoV and TV can be internalized in hydroponically grown green onions but not in soil grown green onions, ii) the magnitude of TV internalization is influenced by the level of virus present, and iii) different types of plants have different susceptibility for viral internalization.

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## Abbreviations

<b>NoV</b>	norovirus
<b>TV</b>	Tulane virus
<b>MNV</b>	murine norovirus
<b>FCV</b>	feline calicivirus
<b>CPE</b>	cytopathic effect
<b>HBGAs</b>	histo-blood group antigens
<b>DMEM</b>	Dulbecco's modified Eagle medium
<b>FBS</b>	fetal bovine serum
<b>MOI</b>	multiplicity of infection
<b>Opti-MEM</b>	low serum Eagle's minimum essential medium
<b>HBSS</b>	Hank's balanced salt solution
<b>RT-PCR</b>	reverse transcriptase polymerase chain reaction
<b>RT-qPCR</b>	real time reverse transcriptase polymerase chain reaction

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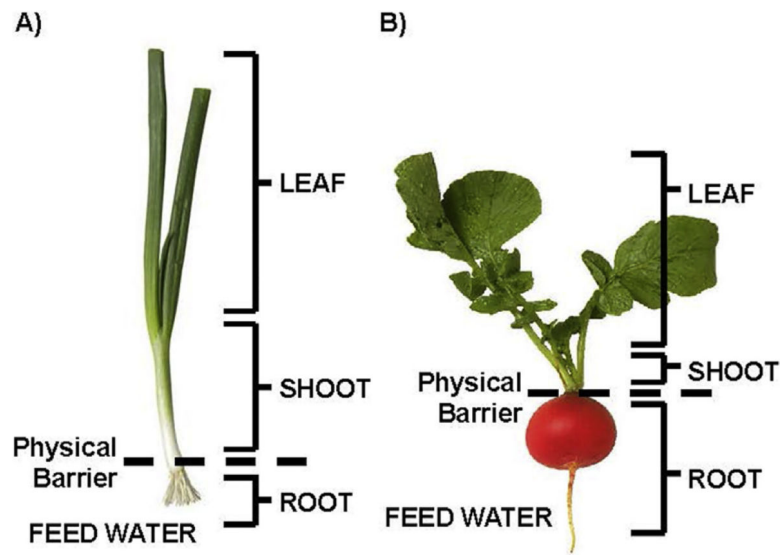
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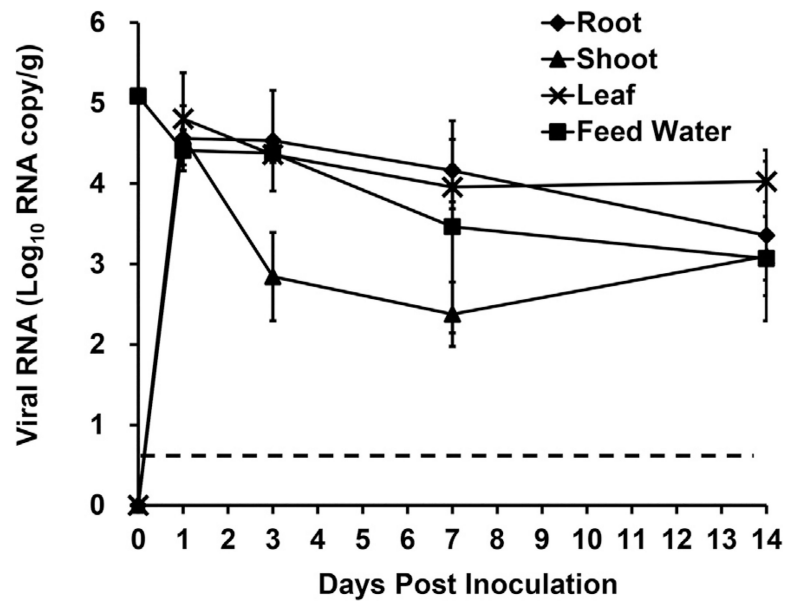
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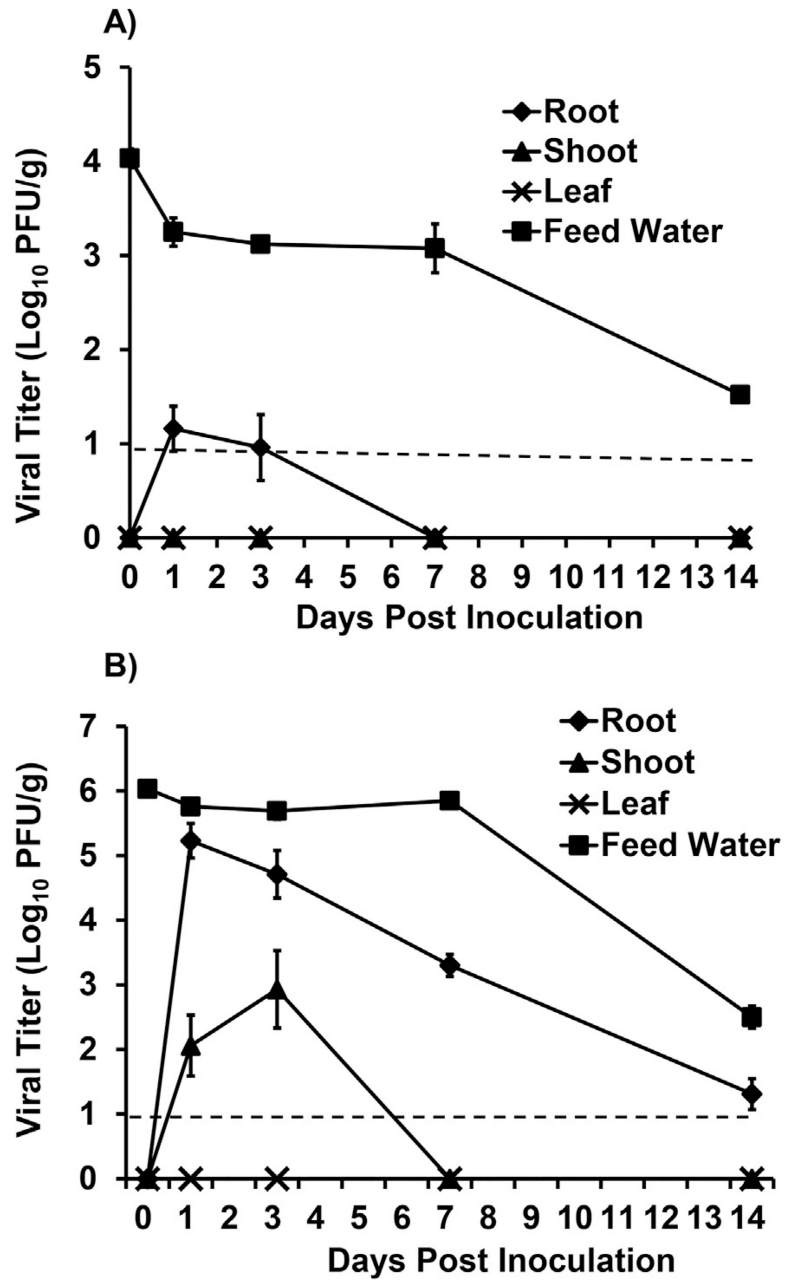
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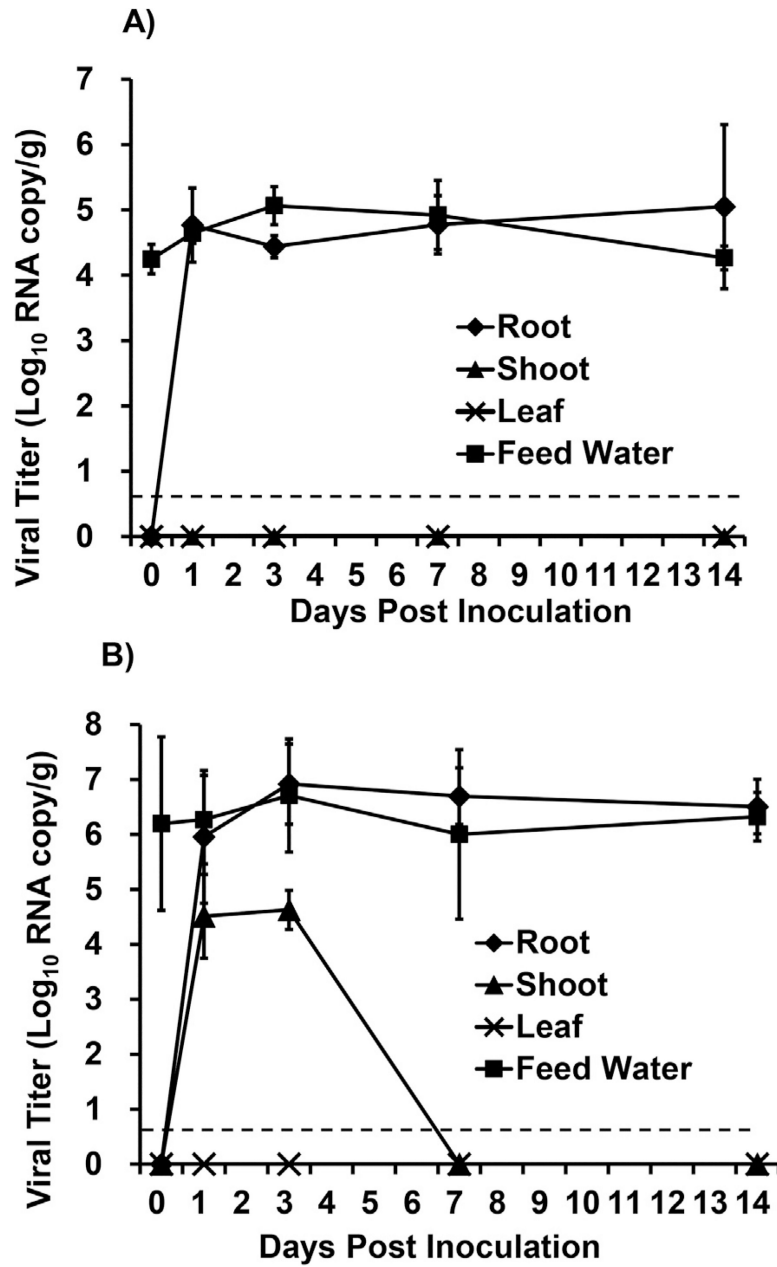
**Fig. 1. Schematic representation of harvested tissues of green onion (A) and radish (B).** Leaf tissue represents the aerial tissues of the starting 2 inches above the root juncture and was harvested first. Shoot tissue represents the 2 inch portion of the aerial tissue connected to the root juncture and was not in contact with the feed water, which was harvested second. Root tissue consists of the bulb and all roots and was in direct contact with the feed water and was harvested third. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 2. Human NoV internalization in hydroponically grown green onion.** Internalization kinetics plot was determined by RT-qPCR and results are reported as log<sub>10</sub> RNA copies/g. Data points were the averages of six replicates. Error bars represent  $\pm 1$  standard deviation. Dotted line represents the LOD.

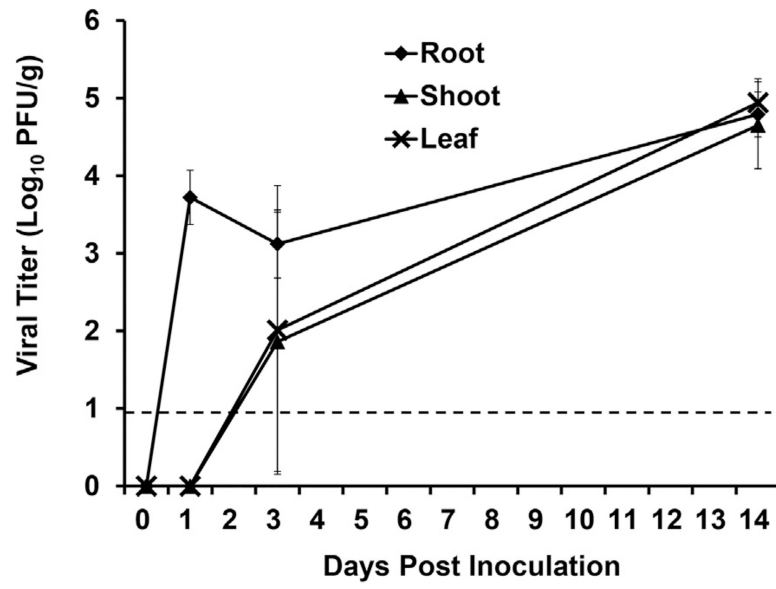


**Fig. 3. TV internalization in hydroponically grown green onions given low (A) and high (B) inoculum.** Viral titer is reported as log<sub>10</sub> PFU/g. Data points were the averages of six replicates. Error bars represent ±1 standard deviation. Dotted line represents the LOD.

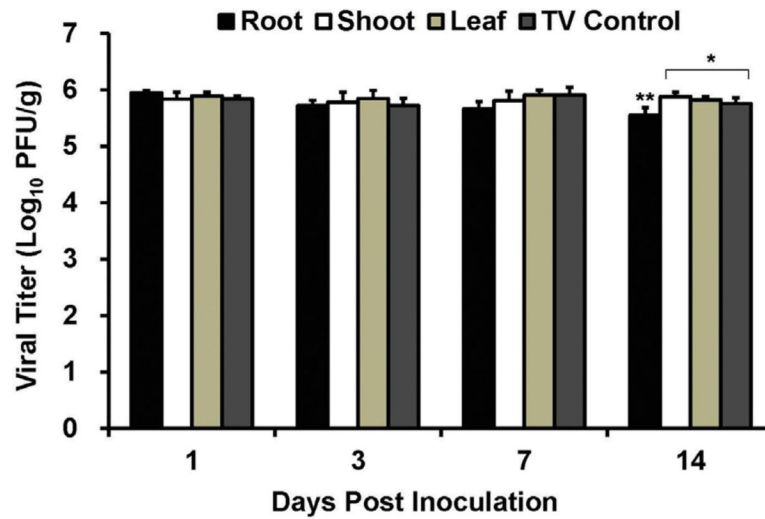


**Fig. 4. TV RNA detection in hydroponically grown green onion given low (A) and high (B) inoculum.**

Internalization kinetics plot was determined by RT-qPCR and results are reported as log<sub>10</sub> RNA copies/g. Data points were the averages of six replicates. Error bars represent ±1 standard deviation. Dotted line represents the LOD.

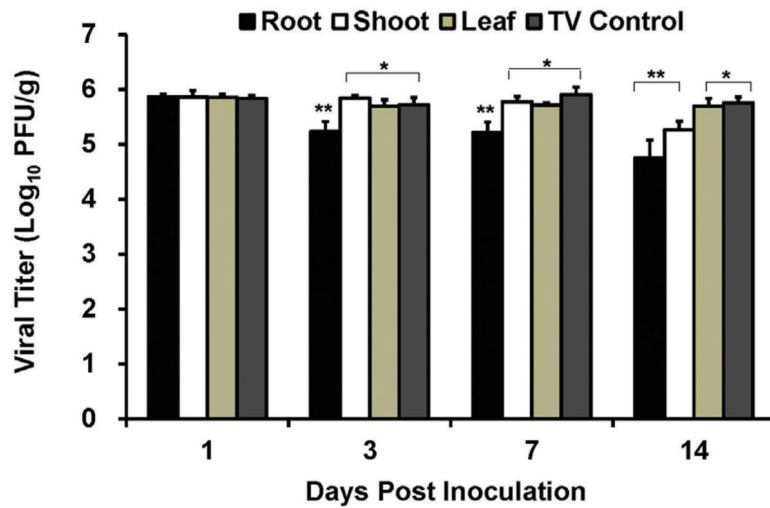


**Fig. 5. TV internalization in soil grown Romaine lettuce.**  
Viral titer is reported as log<sub>10</sub> PFU/g. Data points were the averages of six replicates. Error bars represent  $\pm 1$  standard deviation. Dotted line represents the LOD.



**Fig. 6. Stability of TV in green onion sap.**

TV was diluted 1:10 in green onion sap from root, shoot, and leaf to an approximate titer of  $1 \times 10^6$  PFU/ml. TV inoculated green onion sap 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 six replicates  $\pm$  standard deviation. \*\* denotes that values are significantly different ( $P < 0.01$ ) compared to TV control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 7. Stability of TV in radish sap.**

TV was diluted 1:10 in radish sap from root, shoot, and leaf to an approximate titer of  $1 \times 10^6$  PFU/ml. TV inoculated green onion sap 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. \*\* denotes that values are significantly different ( $P < 0.01$ ) compared to TV control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)