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Survival and Transfer of Murine Norovirus within a Hydroponic System during Kale and Mustard Microgreen Harvesting

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Hydroponically grown microgreens are gaining in popularity, but there is a lack of information pertaining to their microbiological safety. The potential risks associated with virus contamination of crops within a hydroponic system have not been studied to date. Here a human norovirus (huNoV) surrogate (murine norovirus [MNV]) was evaluated for its ability to become internalized from roots to edible tissues of microgreens. Subsequently, virus survival in recirculated water without adequate disinfection was assessed. Kale and mustard seeds were grown on hydroponic pads (for 7 days with harvest at days 8 to 12), edible tissues (10 g) were cut 1 cm above the pads, and corresponding pieces (4 cm by 4 cm) of pads containing only roots were collected separately. Samples were collected from a newly contaminated system (recirculated water inoculated with ~3 log PFU/ml MNV on day 8) and from a previously contaminated system. (A contaminated system without adequate disinfection or further inoculation was used for production of another set of microgreens.) Viral titers and RNA copies were quantified by plaque assay and real-time reverse transcription (RT)-PCR. The behaviors of MNV in kale and mustard microgreens were similar ($P > 0.05$). MNV was detected in edible tissues and roots after 2 h postinoculation, and the levels were generally stable during the first 12 h. Relatively low levels (~2.5 to ~1.5 log PFU/sample of both edible tissues and roots) of infectious viruses were found with a decreasing trend over time from harvest days 8 to 12. However, the levels of viral RNA present were higher and consistently stable (~4.0 to ~5.5 log copies/sample). Recirculated water maintained relatively high levels of infectious MNV over the period of harvest, from 3.54 to 2.73 log PFU/ml. Importantly, cross-contamination occurred easily; MNV remained infectious in previously contaminated hydroponic systems for up to 12 days (2.26 to 1.00 PFU/ml), and MNV was detected in both edible tissues and roots. Here we see that viruses can be recirculated in water, even after an initial contamination event is removed, taken up through the roots of microgreens, and transferred to edible tissues. The ease of product contamination shown here reinforces the need for proper sanitation.

Hydroponics is gaining in popularity, with consistent growth across the United States (1). Specifically growing microgreens hydroponically is a new trend in the food industry (1). Microgreens have been defined as salad crop shoots harvested for consumption within 10 to 20 days of seedling emergence (2). Microgreens are considered a gourmet food to add taste, color, and texture to dishes; they mainly appear in fine and upscale restaurants and have been gaining attention and popularity during the past few years due to the fresh appearance and health benefits (3, 4). Both microgreens and sprouts are usually grown within indoor facilities under controlled environmental conditions to minimize potential contamination of foodborne pathogens. However, microgreens are different from sprouts. Generally, microgreens have two fully developed cotyledon leaves, with the first pair of true leaves emerged or partially expanded, and during harvest they are cut above the soil line, whereas sprouts are mainly soaked in the water and younger, with the cotyledon not opened or just opened.

With the increasing consumption of microgreens, concern over a situation similar to the sprout boom is occurring. As previously reported, sprouts have been involved in at least 55 foodborne outbreaks across the world, with illnesses ranging from as few as one person to as large as thousands of people (5; CDC Foodborne Outbreak Online Database [http://wwwn.cdc.gov/foodborneoutbreaks]). While no outbreak has been documented associated with microgreens so far, as outlined in this study, they are at risk for potential contamination. The U.S. Food and Drug Administration (FDA) has yet to define commodity-specific guidelines regarding microgreens.

Human norovirus (huNoV) causes over 5 million illnesses each year in the United States and is the most common viral etiology of foodborne illnesses (6). It is likely that an individual may experience an average of 5 episodes of norovirus gastroenteritis within a lifetime (7). Produce safety is of great concern as fresh produce serves as the major vehicle for huNoV transmission (8, 9). Produce that is consumed raw or with little or no processing may become contaminated with huNoV during postharvest handling (e.g., irrigation water and amendments) and processing (e.g., washing and packing) and also through contact with infected individuals who may handle the produce or seeds (10–12). Previously, huNoV has been detected in surface water and groundwater with various frequencies (13, 14). It is likely that viruses can be spread by water, and internalization of huNoV through root uptake of produce via polluted irrigation water is one of the potential

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FIG 1 Hydroponic systems used in this study, including the control, kale, and mustard systems.

routes for plant contamination (12, 14). However, without an appropriate cell culture model, the behavior of huNoV is still not well understood.

In order to predict the characteristics of huNoV, murine norovirus (MNV) with close genetic and antigenic relatedness has been widely used (15, 16). MNV was the first norovirus to be propagated in cell culture and is clustered in norovirus genogroup V (GV) (16). For these reasons, along with the fact that MNV is nonpathogenic, MNV was chosen as the surrogate for huNoV in this study.

Epidemiology suggests that a great number of produce-associated illnesses are caused by viruses (9, 17), resulting in the study of internalization of viruses in plants. It has been observed that plants that were grown in artificially contaminated hydroponic systems can take up viral pathogens (18–24). The driving force of water absorption facilitates internalization, and humidity in the plant-growing environment significantly affects transpiration (22). In addition, factors such as root integrity (20), virus type (19), and inoculation level (22) can affect the levels of virus internalization. However, it is still poorly understood whether virus internalization occurs in produce grown in contaminated hydroponic systems and if virus particles can accumulate in edible plant tissues (24).

In this study, kale and mustard microgreens were selected as a model to better understand the virus uptake, persistence, distribution, and transmission in microgreens grown in an artificially contaminated hydroponic system. MNV, an huNoV surrogate, was used to investigate the behavior of human norovirus.

MATERIALS AND METHODS

Hydroponic system. Microgreens were grown at the Fisher Greenhouse at the College of Agriculture and Natural Resources, Newark, DE. The nutrient film technique (NFT) hydroponic system was prepared by tilting three platforms at a 30° angle in order to allow water to flow through the system (Fig. 1). Each set had 4 trays and its own water vessel containing 4,000 ml of tap water supplemented with 30 ml of nutrient solution A, which contained 120.0 g/liter $Ca(NO_3)_2$ (YaraLiva, Tampa, FL), and 30 ml of solution B, which contained 120.0 g/liter 5-11-26 Hydro-Sol (Peters Professional, Dublin, OH), 1.17 g/liter $MgSO_4$ (Giles Chemical, Waynesville, NC), and 0.58 g/liter Sprint 330 (Becker Underwood, Ames, IA). A pump was placed in the water vessel and pumped water to the top of the system through a tube at a constant rate (~10 ml/s). The pump was set to run continuously over time, cycling on for 5 min and then off for 10 min. The water flowed down the platforms due to gravity and then back into the water vessel and recirculated through the NFT system.

Disinfection of the hydroponic system. After each trial of experiments was completed, the hydroponic system was disinfected. Recirculated water and microgreen plants, including hydroponic pads, were removed. The system was then sprayed with 5% (vol/vol) bleach in water (Clorox, Oakland, CA), and the water vessel was filled with 8,000 ml of tap water containing 5% bleach. The circulation system was maintained with running water throughout for 24 h. After complete disinfection, the system was rinsed thoroughly with tap water first, and then 10,000 ml of tap water was recirculated for another 24 h to remove the chlorine residues. All samples (including microgreens and water) tested negative before inoculation.

Plant cultivation. Seeds of microgreens, including kale (*Brassica napus*) and mustard (*Brassica juncea*) (Johnny's, Winslow, ME) were planted and grown on Micro-Mats hydroponic grow pads (Handy Pantry, West Springville, UT), which were soaked in circulating water. Three individual sets of 12 Micro-Mats hydroponic grow pads (33.00 by 6.35 cm) were placed in each hydroponic system (3 pads/tray) including kale, mustard, and a positive control with no plants and circulating virus alone. On day 0, seeds were distributed evenly on pads; and each pad had 6.75 g kale and 3.75 g mustard seeds, respectively. Water was supplemented with a nutrient solution and recirculated by pumping, as described above in the section "Hydroponic system." The microgreens were germinated and ready to harvest beginning on day 8 to day 12. The temperature of the greenhouse was 22.3°C, with an average humidity of 51%. Seeds were germinated in 12 h of daylight at an average radiation of 1,057.2 J/cm^2 and 12 h of dark at 0.4 J/cm^2 daily. (Greenhouse parameters were provided by the Priva greenhouse monitoring system.)

Inoculation of circulating water. Each of three water vessels held a total volume of 4,000 ml fresh feed water that was inoculated with 200 ml of MNV on day 8 at a starting titer of ~3.5 log PFU/ml. The microgreens were maintained in virus-inoculated feed water from days 8 to 12. An inoculated positive control was included in circulating water without seeds on the pads. A negative control was also included in a smaller setting due to space limitations. Due to evaporation, freshwater was added to maintain the initial water level daily, but no additional virus inoculum was added after the initial virus inoculation.

Sample collection. The virus titer in water was monitored throughout the experiment. Water samples (10 ml) were collected directly from each water vessel at each sampling time, including controls taken prior to virus inoculation without a further concentration step. Starting on day 9, microgreens (edible portion) and root pads were sampled, respectively. The microgreens (edible portion) were cut 1 cm above the pad with pruners (Fiskars, Sauk City, WI): 10 g of microgreen samples (kale or mustard) and correspondingly two pieces of 4-cm by 4-cm pads containing roots without the edible portion were collected separately in two homogenizer bags (Fisher Scientific, Pittsburgh, PA) and treated as one microgreen edible tissue sample and one root sample. Microgreen edible tissue and root samples were mixed with 10 ml and 5 ml phosphate-buffered saline (PBS [pH 7.2]), respectively. Samples were then smashed with a 16-ounce rubber mallet hammer (Craftsman, Hoffman Estates, IL), followed by stomaching for 2 min. The homogenates were collected and transferred to new collection tubes. Samples were then frozen at −20°C for less than 2 weeks before being processed by plaque assay and real-time reverse transcription (RT)-PCR. Chloroform extraction was conducted prior to analysis at a ratio of 1:1 (vol/vol) to avoid the interference of bacteria and

TABLE 1 Internalization of MNV in kale and mustard microgreens grown hydroponically within the first 12 h

Microgreen	Virus titer, log PFU/sample (ratio of positive counts/samples tested)[a]			
	2 h	4 h	8 h	12 h
Edible tissues				
Kale	A 3.47 ± 0.24 A (7/7)	A 3.62 ± 0.12 A (9/9)	A 3.59 ± 0.14 A (9/9)	A 3.50 ± 0.15 A (8/8)
Mustard	A 3.47 ± 0.27 A (9/9)	A 3.69 ± 0.20 A (9/9)	A 3.53 ± 0.27 A (9/9)	A 3.58 ± 0.32 A (9/9)
Roots				
Kale	A 1.98 ± 1.14 A (9/9)	A 2.63 ± 0.24 B (9/9)	A 2.66 ± 0.28 B (9/9)	A 2.68 ± 0.34 B (9/9)
Mustard	A 2.59 ± 0.51 A (9/9)	A 2.75 ± 0.44 A (9/9)	A 2.79 ± 0.50 A (9/9)	A 2.77 ± 0.33 A (9/9)

[a] Values are means ± SD from three trials with three samples each. Values in columns with the same preceding letter indicate no significant difference in virus titers when comparing the edible tissue/root between kale and mustard at each sampling time. Values in rows with the same following letter indicate no significant difference in virus titers within the portions of kale or mustard over time. Virus titers that accumulated in edible tissues were consistently significantly greater than those in roots within the first 12 h.

tissue residue. After phase separation by centrifugation (6,000 × *g*, 10 min, at 4°C), the aqueous phase was retained for analysis without further concentration steps. Inhibitor controls were included by addition of MNV directly into environmental samples (smashed microgreen edible and root tissues, as well as water samples) followed by chloroform extraction. It was determined that plant and water matrices have little effect on virus titers in both assays.

Virus survival and uptake in the newly contaminated hydroponic system. The newly contaminated hydroponic system was obtained by MNV inoculation in recirculated water on day 8. The survival and transfer of virus were monitored in two separate studies, including short periods (12 h) and long periods (harvesting time from day 8 to day 12). Virus uptake in the first 12 h was investigated directly following inoculation. Water and microgreen (edible tissue and roots) samples were collected at 2, 4, 8, and 12 h to determine the rate of detectable virus taken up by the microgreens. In addition, virus survival and transfer during harvesting from days 8 to 12 were measured. The amount of virus detected from external surface of microgreen edible tissues by rinsing the surface instead of smashing the tissues was consistently under the detection limit, indicating no external contamination occurred.

Cross-contamination in a previously contaminated hydroponic system. Three sets of hydroponic systems, including kale, mustard, and an unplanted control, were first used for growing microgreens, and MNV was inoculated on day 8 as described previously, to obtain virus-contaminated hydroponic systems. After being harvested on day 12, microgreens, pads, and water were removed without washing or disinfection, and the hydroponic system was considered previously contaminated. Immediately, a new set of pads and microgreen seeds were applied for germination without inoculation, and the extent of virus transfer to these microgreens was determined from days 8 to 12 via the previously contaminated system. The titer of virus present in the freshwater was also monitored from day 0 to day 12.

Detection of background flora in the hydroponic system. Sampling for bacterial growth was performed in triplicate from a newly contaminated system (on days 8, 9, and 12) and from a previously contaminated system (on days 1, 8, and 12) simultaneously. Samples were collected from each system, including water and microgreen edible tissues and roots. The samples were serially diluted in sterile PBS (pH 7.2) and enumerated on tryptic soy agar (TSA) (Remel, Lenexa, KS) to monitor the background flora. In addition, the water samples were analyzed using Colilert according to the manufacturer's instructions with Quanti-Tray/2000 (IDEXX, Westbrook, ME) to detect coliforms and *Escherichia coli*.

Virus propagation and infectivity quantification. Murine norovirus (MNV-1) (a gift from Herbert Virgin, Washington University School of Medicine, St. Louis, MO) was cultured in RAW 264.7 cells (ATCC TIB-71) in Dulbecco's modified Eagles medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Mediatech, Manassas, VA), 100 U/ml penicillin G–streptomycin–0.25 μg/ml amphotericin B (HyClone, Logan, UT), 2 mM L-alanine/L-glutamine (Gibco, Carlsbad, CA), and 1 mM sodium bicarbonate (Cellgro, Manassas, VA). Cells were infected with MNV at a multiplicity of infection (MOI) of 1. After 48 h of infection of 80 to 90% confluent monolayers, complete cytopathic effect (CPE) was observed. Viruses were obtained by three cycles of freeze-thawing of infected cells, followed by centrifugation at 2,000 × *g* for 15 min. The supernatant was filtered through a 0.2-μm-pore membrane filter (Thermo, Rochester, NY) before being stored at −80°C.

Quantification of infectious virus. An MNV plaque assay was performed similarly to previous studies (16, 25). Briefly, after RAW 264.7 cells reached 80 to 90% confluence in 6-well plates (Costar, Corning, NY), 100 μl of 10-fold serial dilutions of MNV sample prepared in Hanks' balanced salt solution (HBSS) (Cellgro, Manassas, VA) was dispensed over monolayers in duplicate. The plates were incubated at 37°C with 5% CO_2 for 1 h with gentle agitation every 15 min followed by addition of 2-ml overlays. MNV-1 overlays consisted of 1.5% agarose (Lonza Sea-Plaque, Rockland, ME) with complete Eagle's minimal essential medium (MEM) (HyClone, Logan, UT) supplemented with 2% FBS, 100 U/ml penicillin G–streptomycin–0.25 μg/ml amphotericin B, 2 mM L-alanine/L-glutamine, and 1 mM sodium pyruvate. After the incubation period (typically 48 h for MNV), 1 ml of 0.2g/liter neutral red (Fisher Scientific, Fair Lawn, NJ) was added to each well followed by a 2- to 5-h incubation. Titers of virus were determined and expressed as PFU with a limit of detection of 10 virus particles/sample.

Virus genome quantification by real-time RT-PCR. MNV RNA was extracted and reverse transcribed into cDNA using the QIAamp viral RNA minikit (Qiagen, Valencia, CA) and Omniscript RT kit (Qiagen) as reference protocols, respectively. The primers used for MNV were the forward primer 5′-TCTTCGCAAGACACGCCAATTTCAG-3′ and reverse primer 5′-GCATCACAATGTCAGGGTCAACTC-3′ (26). Real-time PCRs were performed in a total reaction volume of 20 μl containing 10 μl SYBR green PCR master mix (Qiagen), 2 μl cDNA, and primers (described above) with the protocol from QuantiTect SYBR green PCR kit (Qiagen). Reactions were run on a Rotor-Gene Q thermocycler (Qiagen) under the following conditions: 95°C for 10 min followed by 40 cycles of 94°C for 15 s, annealing temperature 59°C for 30 s, followed by dissociation step which is 60°C for 15 s, and finally 90°C for 15 s. SYBR green signals were read in every cycle, and the logarithm of the increment in fluorescence was plotted versus the cycle number with a fixed threshold level for all runs. The detection limits for MNV were determined to be ~100 genomic copies/ml of sample solution. Virus quantity was determined by comparison to a standard curve and expressed as genomic copies. The positive controls tested were MNV stocks. Negative controls were also collected during the harvesting period, which consisted of the environmental samples (microgreen edible tissues, roots, or water) without virus inoculation.

Statistical analysis. Experiments were conducted in triplicate. In each trial, samples were collected in triplicate, except for water samples (one replicate), and then each of those samples was analyzed in duplicate. Results are reported as means ± standard deviations (SD). The kinetics of

TABLE 2 Survival of MNV in recirculated water during harvest of kale and mustard microgreens within the first 12 h

Water sample	Virus titer, log PFU/ml[a]				
	0 h	2 h	4 h	8 h	12 h
Control	2.26 ± 0.15	2.87 ± 1.11	2.82 ± 1.04	2.69 ± 1.05	2.68 ± 0.99
Kale	2.27 ± 0.24	2.89 ± 0.91	2.82 ± 0.84	2.75 ± 1.49	3.14 ± 1.01
Mustard	2.35 ± 0.39	2.28 ± 0.58	1.91 ± 0.19	2.15 ± 0.15	1.91 ± 0.13

[a] Values are means ± SD from three trials with three samples each. No significant difference in virus titers was observed when comparing the water samples from the control, kale, and mustard systems on each sampling day. No significant difference in virus titers was observed in water samples from the control, kale, or mustard system over time.

MNV survival in the recirculated water were characterized by fitting the plaque assay data from the both newly and previously contaminated systems to linear, exponential, and Weibull models, respectively. The statistical criterion applied to distinguish among the survival models was the *P* value. Data were analyzed by analysis of variance (ANOVA) on JMP software version 11.2 (SAS Institute, Inc., Cary, NC), and significant differences were indicated if *P* was <0.05.

RESULTS

MNV was efficiently taken up via roots and transferred into edible tissues during the first 12 h from virus inoculated in water (short-term study). At full maturation on day 8, MNV was inoculated into the circulating water at a starting titer of 2.63 ± 0.66 log PFU/ml. The amount (log PFU per sample) and ratio (number of positive samples over number of samples tested) of MNV disseminated in kale and mustard microgreens 2, 4, 8, and 12 h postinoculation are shown in Table 1. The rates of occurrence of MNV were similar in kale and mustard microgreens ($P > 0.05$), and MNV was present in all edible tissues and roots tested. High levels of MNV were detected in both kale and mustard edible tissues as soon as 2 h postinoculation, with an average of 3.47 log PFU/sample, and the levels were stable during the first 12 h without significant change ($P > 0.05$) (Table 1). A similar trend of MNV genomic materials (~4 log copies/sample) was observed in edible tissues (see Fig. S1A in the supplemental material). As expected, MNV was found in roots since hydroponic pads that contained roots were soaked in the virus-contaminated water. The levels of MNV detected in the roots 2 h postinoculation were 1.98 and 2.59 log PFU/sample for kale and mustard, respectively (Table 1). The viral levels in kale roots significantly increased to 2.63 log PFU/sample 4 h postinoculation ($P < 0.05$) and were maintained up to 12 h with slight increase, whereas, the titers detected in mustard roots were stable at ~2.7 log PFU/sample over that time (Table 1). The number of MNV genomic copies in kale and mustard roots was ~5 to 6 log copies/sample, and this amount was stable within the first 12 h (see Fig. S1B). Importantly, the titer of infectious MNV in roots was significantly lower than that in edible tissues ($P < 0.05$). These results suggest that MNV was efficiently taken up via roots, internalized, and transferred into microgreen edible tissues.

Furthermore, the viral titer in recirculated water was also monitored during the 12-h period. Compared with the original inoculation levels, MNV in water was maintained at ~2 log PFU/ml without significant reduction in all three systems (control, kale, and mustard) over that time (Table 2). This trend was also confirmed by detection of MNV genomic materials present in water (see Fig. S2 in the supplemental material).

MNV remained infectious and gradually decreased in the roots and edible tissues of kale and mustard microgreens during the harvesting period (long-term study). In order to observe virus behavior for a longer period of time, the survival and transfer of MNV from days 9 to 12 were also determined. The starting titer of MNV in recirculated water (day 8) was 3.42 ± 0.49 log PFU/ml, and Table 3 shows MNV uptake and transfer at days 8 to 12. The rates of occurrence of MNV remained similar in kale and mustard, with contamination observed in more than half of the samples (Table 3). The number of positive samples decreased over time. MNV was detected in both kale and mustard edible tissues 1 day postinoculation, with average titers of 2.30 ± 1.02 and 2.49 ± 0.39 log PFU/sample, respectively (Table 3). The levels of infectious MNV in both kale and mustard edible tissues gradually decreased through day 12. At day 12, the titers dropped to 1.55 ± 1.17 and 1.61 ± 0.93 log PFU/sample, respectively (Table 3). The level of MNV detected in mustard edible tissues was significantly higher at days 9 and 10 than that at day 12 ($P < 0.05$), but no significant difference was observed in kale (Table 3). MNV genomic materials were persistent in edible tissues at ~4 log copies/sample (see Fig. S3A in the supplemental material). Similarly, infectious MNV was also detected in the root samples of kale and mustard on all days tested, with a decreasing trend (Table 3). The viral titers in the kale and mustard roots on day 9 were 2.53 ± 0.28 and 2.23 ± 0.37 log PFU/sample, respectively, and dramatically decreased to <1.50 log PFU/sample on day 12 ($P < 0.05$) (Table 3). It is interesting that the viral titers in roots were close to that found in edible tissues during days 9 to 12 (Table 3). An increased number of MNV genomic copies (~4 to 5 log copies/sample) was observed in roots, but the number remained stable over time (see Fig. S3B).

TABLE 3 Internalization of MNV in kale and mustard microgreens grown hydroponically from days 9 to 12

Microgreen	Virus titer, log PFU/sample (ratio of positive counts/samples tested)[a]			
	Day 9	Day 10	Day 11	Day 12
Edible tissues				
Kale	A 2.30 ± 1.02 A (8/9)	A 2.23 ± 0.92 A (8/9)	A 1.96 ± 1.16 A (7/9)	A 1.55 ± 1.17 A (6/9)
Mustard	A 2.49 ± 0.39 A (9/9)	A 2.37 ± 0.37 A (9/9)	A 2.12 ± 0.36 AB (9/9)	A 1.61 ± 0.93 B (7/9)
Roots				
Kale	A 2.53 ± 0.28 A (9/9)	A 2.14 ± 0.34 A (9/9)	A 2.01 ± 0.79 AB (8/9)	A 1.47 ± 0.85 B (7/9)
Mustard	A 2.23 ± 0.37 A (9/9)	A 1.71 ± 0.98 AB (7/9)	A 1.51 ± 0.88 AB (7/9)	A 1.42 ± 0.82 B (7/9)

[a] Values are means ± SD from three trials with three samples each. Values in columns with the same preceding letter indicate no significant difference in virus titers when comparing the edible tissue/root between kale and mustard on each sampling day. Values in rows with the same following letter indicate no significant difference in virus titers within the portions of kale or mustard over time.

TABLE 4 Survival of MNV in recirculated water during the harvest of kale and mustard microgreens from days 8 to 12

Water sample	Virus titer, log PFU/ml[a]				
	Day 8	Day 9	Day 10	Day 11	Day 12
Control	A 3.54 ± 0.49 A	A 3.36 ± 0.66 A	A 3.21 ± 0.67 A	A 3.09 ± 0.63 A	A 2.73 ± 0.65 A
Kale	A 3.26 ± 0.40 A	A 3.11 ± 0.20 AB	A 2.85 ± 0.29 AB	A 2.68 ± 0.25 B	A 2.75 ± 0.16 B
Mustard	A 3.46 ± 0.72 A	A 3.21 ± 0.85 A	A 3.20 ± 0.37 A	A 3.10 ± 0.39 A	A 2.77 ± 0.52 A

[a] Values are means ± SD from three trials with three samples each. Values in columns with the same preceding letter indicate no significant difference in virus titers when comparing the water samples from the control, kale, and mustard systems on each sampling day. Values in rows with the same following letter indicate no significant difference in virus titers in water samples from the control, kale, or mustard system over time.

However, the numbers of genomic copies in roots were slightly lower than that within the first 12 h (see Fig. S1B and S3B).

In addition, the viral titers in recirculated water were measured daily from day of inoculation (day 8) until the microgreens were completely harvested (day 12). The starting titer of the recirculated water used for this long-term study (days 9 to 12) was approximately 1 log PFU/ml higher than that used for the short-term study (0 to 12 h). As virus uptake occurred via the root to microgreen edible tissues over the period of harvest, the level of viruses present in water gradually decreased (Table 4). The titer in water after inoculation (day 8) was 3.26 ± 0.40 log PFU/ml for kale, which was significantly higher than that on days 11 and 12, with values of 2.68 ± 0.25 and 2.75 ± 0.16, respectively ($P < 0.05$). Virus titers also decreased in control and mustard water; however, no significant difference was observed over this period. The number of MNV genomic copies remained at >3 log copies/ml in all water samples tested (control, kale, and mustard), with no significant reduction from day 8 to day 12 ($P > 0.05$) (see Fig. S4 in the supplemental material). These results suggested that MNV was stable and persistent in recirculated water.

Cross-contamination of MNV in a previously contaminated hydroponic system easily occurred. Immediately following the completion of the original study, an identical experimental design was used and procedures were performed without disinfection, and the potential risks associated with the previous contaminated system were investigated. Contamination occurred in almost all of the samples examined (Table 5). Generally, lower titers of infectious MNV (~1.5 to 2.5 log PFU/sample) were found in both edible tissues and roots than in the newly contaminated system (Table 5). The virus titers in the edible tissues of kale and mustard at day 12 were 2.61 log PFU/sample, significantly higher than titers on day 8, which were 1.99 ± 0.82 and 2.26 ± 0.28, for kale and mustard, respectively ($P < 0.05$) (Table 5). The number of MNV genomic copies present in edible tissues persisted at ~4 log copies/sample (see Fig. S5A in the supplemental material). This indicates that viruses can accumulate in microgreen edible tissues over longer exposure times (days 0 to 12). MNV was detected in roots as well. Viral titers in kale roots were consistent at ~2 log PFU/sample, whereas viral titers increased in mustard roots from ~1.5 to 2.2 log PFU/sample (Table 5). The number of genomic copies present in roots dropped to ~3 log copies/sample (see Fig. S5B), which was significantly lower than the number found in newly contaminated system (see Fig. S3B in the supplemental material). This provides evidence that the integrity of MNV genomic materials decreases gradually in roots.

Greater than 2 log PFU/ml infectious MNV was detected in freshwater at day 0 after the inoculated water was discarded; however, these MNV levels were significantly lower than those in the original newly contaminated hydroponic system (Table 6). Viral titers decreased over time from ~2 log PFU/ml at day 0 to ~1 log PFU/ml at day 12 in all systems tested ($P < 0.05$) (Table 6). MNV genomic materials also gradually decreased through day 12 without significant difference ($P > 0.05$) (see Fig. S6 in the supplemental material). These results demonstrate that MNV can persist in the environment and is able to remain infectious over a long period of time. Without proper cleaning and sanitation, viruses were transferred and contaminated the whole hydroponic system via recirculated water. Even with fairly low titers, MNV was efficiently internalized and disseminated in microgreens grown hydroponically.

Bacterial background in the hydroponic system. The bacterial background in the hydroponic system was examined in this study. In the newly contaminated hydroponic system, the bacteria flora present in water increased from day 8 to 9 with an average of 2.22 ± 0.25 log CFU/ml to >5.35 log CFU/ml (see Table S1 in the supplemental material). The level of bacteria at day 9 was similar to that at day 12, indicating bacterial levels were maintained over the harvest period. The highest bacterial levels of ~8 log PFU were

TABLE 5 Internalization of MNV in kale and mustard microgreens grown in a previously contaminated hydroponic system

Microgreen	Virus titer, log PFU/sample (ratio of positive counts/samples tested)		
	Day 8	Day 9	Day 12
Edible tissues			
Kale	A 1.99 ± 0.82 A (8/9)	A 2.43 ± 0.15 AB (9/9)	A 2.61 ± 0.14 B (9/9)
Mustard	A 2.26 ± 0.28 A (9/9)	A 2.49 ± 0.12 B (9/9)	A 2.61 ± 0.14 B (9/9)
Roots			
Kale	A 2.03 ± 0.20 A (9/9)	A 2.09 ± 0.17 A (9/9)	A 2.17 ± 0.18 A (9/9)
Mustard	A 1.69 ± 0.66 AB (8/9)	A 1.52 ± 0.89 A (7/9)	A 2.21 ± 0.11 B (9/9)

[a] Values are means ± SD from three trials with three samples each. Values in columns with the same preceding letter indicate no significant difference in virus titers when comparing the edible tissue/root between kale and mustard on each sampling day. Values in rows with the same following letter indicate no significant difference in virus titers within the portions of kale or mustard over time.

TABLE 6 Survival of MNV in recirculated water in a previously contaminated hydroponic system

Water sample	Survival by plaque assay, log PFU/ml[a]								
	Day 0	Day 1	Day 2	Day 3	Day 5	Day 8	Day 9	Day 10	Day 12
Control	A 2.26 ± 0.02 A	A 2.00 ± 0.03 B	A 1.94 ± 0.10 B	A 1.81 ± 0.07 BC	A 1.65 ± 0.14 CD	A 1.68 ± 0.07 CD	A 1.47 ± 0.12 E	A 1.61 ± 0.13 DE	A 1.44 ± 0.13 E
Kale	A 2.24 ± 0.23 A	A 2.15 ± 0.12 AB	A 2.01 ± 0.12 AB	AB 1.99 ± 0.21 AB	B 1.89 ± 0.13 BC	A 1.67 ± 0.19 CD	A 1.59 ± 0.14 DE	A 1.50 ± 0.08 DE	AB 1.32 ± 0.15 E
Mustard	A 2.11 ± 0.12 A	A 2.08 ± 0.14 A	A 1.87 ± 0.04 A	AB 1.93 ± 0.07 B	A 1.52 ± 0.07 B	A 1.51 ± 0.22 B	A 1.32 ± 0.15 B	A 1.45 ± 0.05 B	B 1.00 ± 0.30 C

[a] Values are means ± SD from three trials with three samples each. Values in columns with the same preceding letter indicate no significant difference in virus titers when comparing the water samples from the control, kale, and mustard systems on each sampling day. Values in rows with the same following letter indicate no significant difference in virus titers in water samples from the control, kale, or mustard system over time.

detected in kale and mustard samples, with no significant change over the time of harvesting ($P > 0.05$) (see Table S1). Interesting, the starting levels of bacteria detected in water from the previously contaminated hydroponic system were much higher, with an average of >4 log CFU/ml, and remained stable over the 12 days (see Table S2 in the supplemental material). Similar levels of bacteria were also present in kale and mustard edible tissues and roots from the previously contaminated hydroponic system. In addition, the Colilert method showed *E. coli* was absent in the recirculated water (data not shown). The number of coliforms was much lower, but the growth trend of coliforms was similar to that determined by TSA plate count (see Tables S3 and S4 in the supplemental material).

Kinetics of MNV survival in recirculated water. Three different models were compared to evaluate the kinetics of virus survival in recirculated water. Compared with the exponential and Weibull models, the linear model was the best fit for water samples obtained from a newly contaminated hydroponic system (see Table S5 in the supplemental material), whereas, both the linear and Weibull models are appropriate to estimate the trend of MNV in the recirculated water from a previously contaminated hydroponic system (see Table S5).

DISCUSSION

Use of hydroponic systems for growing produce crops along with aquaculture or aquaponics-raised fish is increasing, but there is limited information pertaining to the microbiological safety of microgreens. Epidemiological data revealed that huNoV is a leading cause of produce-associated outbreaks (9). Recent surveillance of produce-associated outbreaks in the United States and the European Union from 2004 to 2012 indicated that >50% of outbreaks were caused by huNoV and recommended the produce industry follow the Good Agricultural Practices guides and avoid contamination by food handlers who are infected by huNoV (8). Microgreens can be contaminated by huNoV at any point from farm to table. Even though microgreens share some similarities with sprouts, there are currently no standards or practices for microgreen production. HuNoV can contaminate irrigation water via cross-contamination. It is possible that food handlers in a greenhouse may introduce viruses to water when they are infected asymptomatically. In this study, a nutrient film technique hydroponic system was chosen as a model to mimic the large-scale production of microgreens and investigated for the potential risks of virus transfer and survival after a contamination event. Our results show that MNV can be efficiently taken up and internalized into microgreen edible tissues via roots through contaminated recirculated water as soon as 2 h post-water inoculation. Importantly, we found that MNV remained in the system once water and plants were removed and could survive for a long period of time in microgreens as well as in the hydroponic system. Without appropriate cleaning and disinfection procedures, virus cross-contamination could easily occur where viruses were infectious in the new set of plants grown in the previously contaminated hydroponic system. The levels of MNV used here were similar to the levels of huNoV detected in environmental samples (e.g., water samples), ranging from ~10 to ~5×10^4 genomic copies/ml (27–29). However, those levels were much lower than those of huNoV shed in vomit and feces (up to 3×10^7 viral particles/episode of vomiting or up to 10^{11} genomic copies/g of stool sample) (30).

Infectious MNV (~2 to 3 log PFU/sample) was detected in

both kale and mustard microgreen edible tissues as soon as 2 h following inoculation. During the first 12 h following inoculation and recirculation of contaminated water, the titers were relatively stable. It is possible that virus uptake occurred immediately after inoculation, and quickly saturated plant edible tissues. The saturation can also be confirmed by the stable levels of genomic materials detected in microgreen edible tissues and roots (see Fig. S1 in the supplemental material). Similarly, Hirneisen et al. determined that the levels of MNV internalized in green onions within a floating hydroponic system were also consistent with an average of ~4 log PFU/sample from day 1 to day 5 after inoculation, suggesting that saturation was reached within 24 h (31). Ward and Mahler reported that the uptake and transfer of bacteriophage f2 occurred rapidly in bean plants within 16 h after exposure, and virus reached the maximal levels in stems and upwards in leaves via cut roots (20), whereas DiCaprio et al. used Romaine lettuce in a hydroponic growth system with one-time-inoculated water with aeration, and they found that MNV internalized in Romaine lettuce increased and reached the peak titer on day 3 post-water inoculation (18). Chancellor et al. used fluorescent microspheres to investigate hepatitis A virus uptake in green onions and determined that florescence accumulated and nearly doubled between days 1 and 2 and reached a plateau at day 7 (32). In this study, higher titers of MNV reached peaks within a shorter time in both microgreen edible tissues and roots. The variations in the length of time required to reach the peak virus concentration may be attributed to the experimental protocol, such as the virus types and inoculation levels, procedures, types of plants, growth stage, integrity of roots, and hydroponic system (18–22, 31, 32). The growth stage of plants in this study was much younger (days 8 to 12), whereas the plants used in previous studies mentioned above were at least 3 weeks old following germination (20, 22). It is possible that root growth can increase contact surface with recirculated water; allowing the microgreens to concentrate more viruses in their tissues. Low humidity may facilitate virus uptake. Wei et al. observed a 10-fold-higher internalization of MNV at 70% humidity compared to that at 99% humidity, as humidity significantly affects transpiration (22). The environmental humidity of microgreen growth conditions was much lower (~51%), which may increase the rate of transpiration, resulting in higher levels of viruses being internalized. In addition, the water was continuously recirculated in the system, which may facilitate virus uptake. The roots being bound to hydroponic pads can increase the direct contact with and time of exposure to the surrounding recirculating water. Moreover, the stage of plant development may present different composition of carbohydrates (e.g., monosaccharide and raffinose) (33), which can potentially affect the virus binding affinity. Esseili et al. found that norovirus virus-like particles were likely to bind older and younger leaves differently by cell wall materials (23).

From days 9 to 12, the concentration of infectious MNV present on microgreens remained persistent, with only a slight decrease (~1 log PFU/sample), whereas Ward and Mahler observed a 2- to 3-log reduction in the level of bacteriophage f2 in bean plant tissues (roots, stem, and leaves) within a week (20). It is likely that the hydroponic pads absorbed viruses from the recirculating water. Interestingly, microgreen edible tissues contained slightly more infectious viruses than the number of viruses present in roots, regardless of the type, but there was no significant difference ($P > 0.05$). This is similar to what was observed in previous studies. Chancellor et al. used fluorescent microspheres as a biomarker to determine how hepatitis A virus contaminates green onions. Chancellor's group observed that significantly more fluorescence was detected at the bottom than at the middle or top of green onion 1 day postinoculation; however, the levels in those three sections (top, middle, and bottom) became similar as the time increased (32). Also, the levels of MNV, Tulane virus, and huNoV GII.4 RNA distributed in Romaine lettuce sections from a hydroponic system, including roots, shoots, and leaves, were also similar (18); occasionally the levels of viruses (MNV, Tulane virus, or hepatitis A virus) detected in leaf and shoot/stem sections of plants were higher than those in roots (18, 31, 32). On the contrary, Ward and Mahler found distinctly lower levels of virus in leaves than in roots of 3-week-old bean plants, and they considered that the interiors of the plants act as molecular sieves and permit only a portion of bacteriophage f2 to be moved from one barrier to the next (20). These observed differences may be explained by the maturity of the plants and the differences between the sections, such as components and structures, as well as the persistence of viruses under different conditions.

The MNV concentrations present in the recirculating water for the long-term study gradually decreased during the experimental period, as virus was taken up by microgreens. However, in most cases, no significant difference was observed between kale/mustard and control water for both short- and long-term studies. It is possible that the decrease in virus titers in water was not detectable. The hydroponic study was conducted on a large scale, and 4,000 ml of virus-inoculated water was circulated. The viruses transferred into the microgreens might be negligible. By comparing different models, it was determined that the linear model was the best fit for all of the water samples ($P < 0.05$), which may be useful to predict the behavior of MNV in a similar environment (see Table S5 in the supplemental material).

We also found that MNV survived in the previously contaminated hydroponic system (up to 16 days) and could be continuously circulated. Studies have shown that MNV can survive in water for a month with ~1-log PFU/ml reductions (34). Here in the previously contaminated hydroponic system, even after the inoculated circulated water was removed, without proper cleaning or disinfection, the system on its own still reserved a large amount of viruses on the surfaces of water vessel and hydroponic platforms. Cross-contamination could easily occur within a hydroponic system, and MNV were easily transferred to microgreen seeds. It is very likely that all portions of microgreens would be contaminated by virus during germination (35), and the surface of microgreens may provide sites for virus accumulation. During the harvesting period (days 8 to 12), viruses were detected in the microgreen edible tissues and roots. The viruses detected in edible tissues may also include those present on the external surface. The information obtained here enforces the need for proper sanitation and provides useful information required for development of preventative strategies. MNV was measured by both plaque assay and real-time RT-PCR, providing different perspectives on virus behavior. The genomic copies in microgreen edible tissues were consistent over the time (see Fig. S1A, S3A, and S5A in the supplemental material). With relatively large amounts of viruses available in the water used in this study, microgreen edible tissues may become saturated with MNV, and the capsids of MNV were more likely to be damaged rather than the genomic materials. Interestingly, the genomic materials degraded faster when the vi-

rus was located in microgreen roots (see Fig. S1B, S3B, and S5B). The possible toxic effects of the plants on viruses (viral capsids or genomic materials), especially different sections of the plant, should be further explored (36, 37).

Generally, the behaviors of MNV in both kale and mustard were similar, and no significant difference was observed. This may be explained by the fact that they both belong to *Brassica* species. In the future, it will be interesting to investigate virus uptake among different genera of plants.

In conclusion, virus inoculated into water was taken up into the edible tissues of the microgreens via the roots. The internalization of viruses into produce poses a potential risk, as it will become more difficult to remove or inactivate them (32). Besides, if the system is not properly disinfected or cleaned, cross-contamination can occur. This study on survival and transfer of MNV in a hydroponic system demonstrates that it is important to identify the routes of virus contamination and provides useful information to develop efficient preventative strategies and to better conduct risk assessment regarding viral contamination in hydroponics.

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