Internalization of Murine Norovirus 1 by *Lactuca sativa* during Irrigation[▽]

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Romaine lettuce (*Lactuca sativa*) was grown hydroponically or in soil and challenged with murine norovirus 1 (MNV) under two conditions: one mimicking a severe one-time contamination event and another mimicking a lower level of contamination occurring over time. In each condition, lettuce was challenged with MNV delivered at the roots. In the first case, contamination occurred on day one with 5×10^8 reverse transcriptase quantitative PCR (RT-qPCR) U/ml MNV in nutrient buffer, and irrigation water was replaced with virus-free buffer every day for another 4 days. In the second case, contamination with 5×10^5 RT-qPCR U/ml MNV (freshly prepared) occurred every day for 5 days. Virus had a tendency to adsorb to soil particles, with a small portion suspended in nutrient buffer; e.g., ~ 8 log RT-qPCR U/g MNV was detected in soil during 5 days of challenge with virus inoculums of 5×10^8 RT-qPCR U/ml at day one, but < 6 log was found in nutrient buffer on days 3 and 5. For hydroponically grown lettuce, ~ 3.4 log RT-qPCR U of viral RNA/50 mg of plant tissue was detected in some lettuce leaf samples after 5 days at high MNV inoculums, significantly higher than the internalized virus concentration (~ 2.6 log) at low inoculums ($P < 0.05$). For lettuce grown in soil, approximately 2 log RT-qPCR U of viral RNA/50 mg of plant tissue was detected in lettuce with both high and low inoculums, showing no significant difference. For viral infectivity, infectious MNV was found in lettuce samples challenged with high virus inoculums grown hydroponically and in soil but not in lettuce grown with low virus inoculums. Lettuce grown hydroponically was further incubated in 99% and 70% relative humidities (RH) to evaluate plant transpiration relative to virus uptake. More lettuce samples were found positive for MNV at a significantly higher transpiration rate at 70% RH, indicating that transpiration might play an important role in virus internalization into *L. sativa*.

Human norovirus (HuNoV) is the leading food-borne pathogen in the United States, accounting for approximately 60% of food-borne disease annually (16). Recently, there have been increasing outbreaks of HuNoVs associated with vegetables and fruits (6, 8, 15). The facts that (i) fresh produce is consumed raw or with minimum preparation, (ii) postharvest biocidal sanitizing is ineffective for removal and inactivation of virus on fruits and vegetables (2), and (iii) the infectious dose of HuNoV is as low as a few particles (3) make the consumption of contaminated produce a risk to consumer health. Consumption of fresh produce, such as leafy greens, contaminated with HuNoVs is now recognized as a common cause of gastroenteritis (7). Leafy greens may be contaminated preharvest through the use of irrigation water containing fecal material (14). River, canal, pond, or well water used for irrigation may be exposed to human enteric viruses due to leakage of sewage water or from animal production zones close to produce fields. It has been shown that virus can attach to the leaf surface and internalize through stoma and cuts on the leaf during direct contact with virally contaminated water (23). However, as viral particles are small, at approximately < 100 nm in diameter, another possible route of contamination through irrigation water is the internalization of human enteric viruses through

roots into the edible tissues of leafy greens during plant water absorption. To date, few studies have been conducted on the uptake of enteric viruses by plants via contaminated irrigation water, and the literature is inconsistent on the likelihood and quantity of internalized virus when roots are intact or damaged (2, 21, 22).

The objective of this study was to evaluate the likelihood and concentration of murine norovirus 1 (MNV) (a widely used surrogate of HuNoV) that may be taken up by lettuce during irrigation. Two scenarios were considered which mimic (i) a severe one-time contamination (e.g., a flooding occurrence or vast amounts of fecal material brought into surface water) and (ii) irrigation water containing a lesser concentration of viruses delivered over several days (e.g., water constantly being exposed to septic tank leakage or sewage water contaminating irrigation water).

MATERIALS AND METHODS

Viruses. Murine norovirus 1 (MNV) was propagated with RAW 264.7 cells cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco-Invitrogen Co.) as described by Wobus et al. (25). Viruses were purified from infected cells through three freeze-thaw cycles. The supernatant was recovered by centrifugation at $2,500 \times g$ for 15 min and was stored at -80°C until use. MNV concentration was determined by endpoint dilution, with the highest dilution providing a positive signal on reverse transcriptase quantitative PCR (RT-qPCR), defining one RT-qPCR unit (RT-qPCR U). The titer of the initial stock of MNV was $\sim 1 \times 10^9$ RT-qPCR U/ml. The MNV plaque assay was conducted with confluent RAW 264.7 cells grown in 12-well plates for 24 to 48 h as previously described (25). The infectivity concentration of the MNV stock was $\sim 1 \times 10^8$ PFU/ml.

Lettuce (*Lactuca sativa*) plant and virus challenging. Lettuce seeds were purchased from a local store (Lancaster, PA) and surface sanitized with 70% ethanol

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for 10 min. The seeds were then washed with sterile distilled water, air dried overnight in a biosafety hood, and stored in the dark before use. Nutrient solution used to culture lettuce was prepared as described by Korkmaz et al. (10) with hydrosol (product no. 5N-4.7P-22K; Grace-Sierra, Milpitas, CA), magnesium sulfate, iron chelate (Sprint330; Ciba-Geigy, Greensboro, NC), and calcium nitrate. The nutrient solution contained 210 mg/liter K, 200 mg/liter N, 129 mg/liter C, 66 mg/liter S, 48 mg/liter P, 20 mg/liter Mg, 4 mg/liter Fe, 0.5 mg/liter Mn, 0.5 mg/liter B, 4 mg/liter Fe, 0.15 mg/liter Cu, 0.15 mg/liter Zn, and 0.10 mg/liter Mo.

For virus challenge studies, two concentrations of virus were evaluated, each representing a specific environmental scenario. To assess the situation of severe one-time contamination (e.g., flooding), 5×10^8 RT-qPCR U/ml MNV was applied to lettuce plants for 24 h, followed by removal of the virus solution, which was then replaced with virus-free nutrient solution every day for up to 5 days. To evaluate the situation of constant contamination with a relatively low quantity of virus, lettuce was grown with 5×10^5 RT-qPCR U/ml MNV and replaced with fresh nutrient solution containing the same concentration of virus every day for 5 days.

Lettuce grown hydroponically or in soil was used to evaluate the internalization of virus during irrigation. All lettuce was grown in a green house at 22 to 24°C. Lettuce seeds were sprouted and grown, and after 20 days plants were used for the virus study. For hydroponically grown lettuce, lettuce seeds were sprouted in 22-mm-square by 37-mm-deep cubes of oasis (Griffin, MA), as previously described (10), and placed in a container, and nutrient solution (50 ml; alone or with the addition of MNV) was added to the container without contact with the lettuce plant. For lettuce grown in soil, Peat-Lite (Sun Gro, Vancouver, Canada) was placed in a plant pot (Griffin, MA) and placed in a container. Nutrient solution (50 ml) was added to the container and delivered through the soil by capillary force and therefore had no direct contact with the leaf surface. MNV was diluted in nutrient solution to 5×10^8 or 5×10^5 RT-qPCR U/ml, and 50 ml of solution was applied to lettuce as described above.

To evaluate the effect of transpiration on virus uptake, lettuce growing hydroponically was challenged with 1×10^8 RT-qPCR U/ml MNV and grown at 99% relative humidity (RH) in a dew chamber (Percival, IA) or at 70% RH in a growth chamber (Conviron, Manitoba, Canada) at ~22°C for 24 h, with 12 h of fluorescent light and 12 h of darkness. Lettuce leaf samples were collected and analyzed as described below.

Sample analysis. For both high and low inoculums, nutrient solution and soil samples were collected and analyzed on days 1, 3, and 5. The viral RNA was extracted from the nutrient solution or soil samples as described by Wei et al. (24), using the QIAamp viral RNA minikit (Qiagen, CA). Leaf samples from lettuce plants were challenged with virus as described above and collected on days 1, 3, and 5. Leaf samples (50 mg) were frozen with liquid nitrogen and ground with a beadbeater (Biospec, OK) for 10 s. Then 500 μ l RLT lysis buffer (RNeasy plant minikit, Qiagen, CA) was added to a homogenized lettuce sample and further bead beaten for 30 s. The RNA was extracted from lettuce samples (50 mg of leaves collected from three lettuce plants in each replicate) by using an RNeasy plant minikit in accordance with the manufacturer's instructions. Total RNA was eluted with 60 μ l RNase-free water and stored at -80°C until use.

Two-step RT-qPCR was used to quantify virus concentration. The RT step was performed in 20- μ l volumes, including 2.0 μ l $10\times$ buffer, 2.0 μ l deoxynucleoside triphosphate (dNTP) (5 mM), 1.0 μ l each primer (5 μ M), 0.1 μ l RNase inhibitor, 1 μ l reverse transcriptase, 10.9 μ l RNase-free H₂O, and 2 μ l RNA, by use of the Sensiscript RT kit (Qiagen, CA) and amplified at 37°C for 60 min. The qPCR was performed in 20- μ l volumes containing 10 μ l $2\times$ SYBR green mix, 1.2 μ l of each primer (5 μ M), 2 μ l cDNA, and 5.6 μ l H₂O by using a QuantiTect SYBR green PCR kit (Qiagen, CA). The amplification cycle was 95°C for 15 min, 40 cycles of 94°C for 15 s, 64°C for 30 s, and 72°C for 30 s, followed by a dissociation step of 95°C for 15 s, 60°C for 15 s, and 95°C for 15 s. The primers for MNV were developed by Hsu et al. (9). To generate a standard curve, virus was serially diluted and $\sim 10^7$ to $\sim 10^2$ RT-qPCR U of virus was added to 50-mg lettuce samples, followed by RNA extraction, and applied to RT-qPCR as described above. A standard curve was generated from three independent trials of virus inoculation, RNA extraction, and RT-qPCR. The quantification of viral RNA in nutrient solution and soil samples was conducted as described by Wei et al. (24).

To evaluate the infectivity of internalized MNV, 50-mg lettuce leaf samples were homogenized in 5 ml Hanks' balanced salt solution (HBSS) by using a Tissuemiser homogenizer (Fisher Scientific, PA). The lysates were then applied to a QiaShredder (Qiagen, CA) and centrifuged at $10,000 \times g$ for 2 min. The supernatant was then serially diluted with HBSS buffer and assessed in a plaque assay as described above.

Transpiration rate measurement. To measure the transpiration rate at two humidities, lettuce grown hydroponically in the oasis cube was placed at the

FIG. 1. MNV left in the soil or nutrient solution for 5 days of virus internalization under two conditions: one-time severe contamination (5×10^8 RT-qPCR U/ml MNV added into nutrient solution at day 1 and replaced with virus-free solution afterward) (hydroponic solution [◆], soil [▲], and nutrient solution for soil [■]) and low levels of constant contamination (5×10^5 RT-qPCR U/ml MNV added into nutrient solution every day) (hydroponic solution [X], soil [●], and nutrient solution for soil [○]).

opening of a flask. Air was applied to the water in the flask through a pipe connected with an air pump to avoid anaerobic conditions. The whole flask was covered with plastic film to prevent evaporation. The transpiration rate was measured by weight loss of the whole flask plus the plant after 1 h of transpiration (12), and the experiments were conducted in four replicates.

Statistical analysis. Statistical analysis was conducted with Tukey's test using JMP8 software (SAS, Cary, NC). For hydroponically grown lettuce or that grown in soil, experiments were replicated at least four times, with three lettuce plants in each replicate. For samples in the RH study, experiments were replicated eight times, and leaf samples were collected from one lettuce plant in each replicate. Statistical differences were considered when P values were <0.05 .

RESULTS

Concentration of MNV in nutrient solution or soil after 5 days of virus challenge. Fresh nutrient solution (50 ml with or without added virus) was added to a container holding the hydroponic oasis cubes or the soil pots every day, and approximately 10 to 30 ml remained after 24 h, depending on evaporation rates, relative humidity, and environmental conditions within the green house. The remaining volume was collected for analysis of virus concentration and replaced with fresh nutrient solution, to the original volume of 50 ml. Soil samples were also collected to evaluate the virus associated with soil particles.

For the one-time severe contamination situation of hydroponically grown lettuce, ~ 7.4 and 6.4 log RT-qPCR U/ml MNV were detected in nutrient solution after 3 and 5 days, respectively. This was true even after the removal of the original solution containing 5×10^8 RT-qPCR U/ml MNV after 1 day, which was replaced with virus-free solution every day until day 5 (Fig. 1). In this situation, some of the original virus solution may have been held in the oasis cube and mixed with virus-free buffer over the 5 days, and thus the virus could not be completely removed but steadily reduced during 5 days. For soil challenged with the solution of 5×10^8 RT-qPCR U/ml MNV, ~ 8.3 log RT-qPCR U/g MNV was detected in soil after 1 day and had no significant reduction after 5 days (7.8 log) ($P < 0.05$). However, only ~ 6 log RT-qPCR U/ml MNV was found in nutrient buffer for the plants grown in soil after

TABLE 1. MNV viral genome detected in lettuce leaves grown hydroponically^a

Day	Control	Log RT-qPCR U MNV ($\pm$ SD)/50 mg lettuce sample (no. of positive samples/no. of total replicates) for:	
		5×10^8 MNV/ml	5×10^5 MNV/ml
1	ND	3.9 ($\pm$ 2.0) (2/5)	2.3 ($\pm$ 0.05) (2/6)
3	ND	3.8 ($\pm$ 1.4) (6/6)	2.3 ($\pm$ 0.5) (4/6)
5	ND	3.4 ($\pm$ 0.5) (4/6)	2.6 ($\pm$ 0.2) (2/6)

^a ND, not detected. Detection limit is ~ 1.5 log RT-qPCR U MNV/50 mg lettuce tissue. Each replicate contained three lettuce plants.

both 3 and 5 days, which is significantly lower than the virus concentration in soil ($P < 0.05$). Thus, compared to plants grown hydroponically in oasis cubes, virus was likely to be adsorbed to soil particles, with a small portion of particles dissociated and suspended in nutrient buffer.

In the situation of low levels of constant contamination (soil or nutrient solution with fresh samples of 5×10^5 RT-qPCR U/ml virus every day), MNV concentration remained constant over the 5-day period and ~ 5 to 6 log RT-qPCR U/ml or U/g virus was detected in both nutrient solution and soil after 5 days (Fig. 1).

MNV internalization into lettuce grown hydroponically or in soil. For lettuce grown hydroponically, virus was detected in some replicates of leaf samples for both high and low MNV inoculums at days 1, 3, and 5 (Table 1). At days 3 and 5, lettuce challenged with 5×10^8 RT-qPCR U/ml MNV had significantly higher virus internalization than lettuce grown with 5×10^5 RT-qPCR U/ml MNV every day ($P < 0.05$). This may have resulted from (i) a higher MNV concentration in nutrient solution during the 5-day period (Fig. 1) and (ii) virus sustained in lettuce after uptake. Cell culture assays indicated that MNV internalized into lettuce leaves was still infectious when high virus inoculums were used (Table 2); however, the concentration of infectious MNV was significantly lower than that determined using qRT-PCR detection of the viral genome.

For lettuce grown in soil, approximately 1.7 to 2.4 log RT-qPCR U/50 mg MNV was detected in some leaf samples of lettuce grown at both high and low inoculums at days 1, 3, and 5, except for samples with 5×10^5 RT-qPCR U/ml at day 1 (Table 3); there was no significant difference detected concerning the concentrations of internalized viruses between lettuce

TABLE 3. MNV viral genome detected in lettuce leaves of lettuce grown in soil^a

Day	Control	Log RT-qPCR U MNV ($\pm$ SD)/50 mg lettuce sample (no. of positive samples/no. of total replicates) for:	
		5×10^8 MNV/ml	5×10^5 MNV/ml
1	ND	2.0 ($\pm$ 0.1) (2/4)	ND (0/4)
3	ND	2.3 ^b (1/4)	1.7 ^b (1/4)
5	ND	2.0 ($\pm$ 0.05) (3/4)	2.4 ($\pm$ 0.5) (2/4)

^a ND, not detected. Detection limit is ~ 1.5 log RT-qPCR U/50 mg lettuce tissue. Each replicate contained three lettuce plants.

^b No standard deviation was obtained because one sample showed positive results.

plants grown at high and low inoculums at days 3 and 5. As mentioned above, virus particles may have been adsorbed to soil particles and therefore may be less accessible to uptake into lettuce leaf tissues than to the virus suspended in the nutrient buffer. Although approximately 8.7 RT-qPCR U/ml MNV was inoculated in the simulation of a one-time severe contamination event, ca. 6 log/ml was detected in the nutrient solution at days 3 and 5. This amount was similar to the concentration of virus in buffer in the situation of low levels of constant contamination, which may explain the similar internalizations at both inoculums. In cell culture infectivity assays, infectious MNV was detected in three replicates of lettuce samples grown in soil at high inoculums after 5 days, indicating a potential risk to food safety.

Effect of RH on MNV internalization into lettuce grown hydroponically. To evaluate the effect of transpiration on the uptake of human viruses, lettuce grown hydroponically was challenged with 1×10^8 RT-qPCR U/ml MNV under conditions of very high RH (99%) (to minimize transpiration) and low RH (70%) (to favor transpiration). The transpiration rate at 70% RH was approximately 10-fold that corresponding to 99% RH (Table 4), and only one out of eight lettuce samples showed positive internalization of MNV at 99% RH, with ~ 2.7 log RT-qPCR U/50 mg (Table 4). However, for plants grown at similar temperatures but at 70% RH, MNV was detected in seven out of eight lettuce samples, with ~ 2.6 log RT-qPCR U/50 mg observed. This indicated that transpiration may play an important role for virus uptake through the roots.

TABLE 2. Infectivity of MNV internalized into lettuce grown hydroponically or in soil

Day	PFU ($\pm$ SD)/50 mg lettuce tissue of lettuce grown ^a :			
	Hydroponically		In soil	
	5×10^8 MNV/ml	5×10^5 MNV/ml	5×10^8 MNV/ml	5×10^5 MNV/ml
1	10 ($\pm$ 14)	0 ($\pm$ 0)	0 ($\pm$ 0)	ND
3	323 ($\pm$ 589)	0 ($\pm$ 0)	0 ^b	0 ^b
5	11 ($\pm$ 13)	0 ($\pm$ 0)	3 ($\pm$ 6)	0 ($\pm$ 0)

^a The means presented are from the leaf samples in which virus was detected. ND, no infectivity data because no viral RNA was detected in lettuce leaves.

^b No standard deviation was obtained because there was one replicate of sample.

TABLE 4. MNV internalization into hydroponically grown lettuce at two humidity levels (RH) for 24 h^a

% RH	Control	Log RT-qPCR U MNV ($\pm$ SD)/50 mg lettuce sample (no. of positive samples/no. of total replicates) for 1×10^8 MNV/ml	Transpiration rate ($\pm$ SD) (g/cm ² /h)
99	ND	2.7 (1/8)	0.0031 ($\pm$ 0.002)
70	ND	2.6 ($\pm$ 0.09) (7/8)	0.032 ($\pm$ 0.009)

^a ND, not detected. Detection limit is ~ 1.5 log RT-qPCR U/50 mg lettuce tissue. Each replicate contained one lettuce plant.

DISCUSSION

Internalization of virus by lettuce was observed in two irrigation water contamination situations. It was demonstrated that in a one-time severe contamination situation, virus could not be removed from the plants by the replacement of fresh buffer mimicking fresh water; instead, a large quantity of MNV particles were associated with the soil, and some virus particles remained suspended in the buffer. Thus, it is likely that even after a flood, viruses remaining in the soil may contaminate clean irrigation water and subsequently contaminate plants. As stated by the LGMA (California Leafy Green Products Handler Marketing Agreement) accepted food safety practices (13), fields in a flooded area should be left for 60 days before planting, or this may be shortened to 30 days with appropriate soil testing. However, further research and risk assessment are needed regarding the survival of virus in soil after a flooding incident, due to the likelihood of viral contamination of leafy greens through root internalization or plant surface contact and the potential for viral persistence inside/on plant tissue and eventually transmission to consumers. In a situation of low levels of constant contamination, virus internalization occurred >1 day later than that in the one-time severe contamination; however, this still suggests that continuous use of irrigation water with a low quantity of viruses could pose a risk of contamination. Thus, regular testing of irrigation water or groundwater for virus may help to reduce the risk of contamination; however, representative samples can be hard to obtain due to the inconsistent presence of virus in water (1).

Previous results are inconsistent among the studies that have been conducted assessing the impact of internalization of human enteric viruses or bacteriophage into produce during hydroponic or traditional growth conditions (4, 17, 21, 22). It was reported that <2 log PFU of bacteriophage f2/g plant tissue was detected in the shoots of hydroponically growing bean (*Phaseolus vulgaris* L.) challenged with $\sim 10^{10}$ PFU/ml f2 at roots (22). Calicivirus was occasionally detected in the edible parts of romaine lettuce grown hydroponically or in soil with total virus inoculums of 10^6 to 10^9 RT-qPCR U; in similar experiments using human norovirus, no virus was found in any plants, indicating that the frequency of contamination via roots was rare even when plants were exposed to high concentrations of virus (21). However, Chancellor et al. (4) showed 100% positive detection of hepatitis A virus RNA inside green onions grown in soil as well as hydroponically. Poliovirus was recovered from the leaves of tomatoes grown in soil injected with only 10^3 to 10^4 PFU/ml virus once every week (17).

The inconsistent results may be a result of (i) variant plant properties for virus penetration and uptake and (ii) different experimental parameters of produce growth, irrigation, and soil conditions, etc. Zhu et al. (26) evaluated the uptake and translocation of nanoparticles in plants and found that with a size of 0.02 to 2 μm (identical to or larger than the size of enteric viruses and bacteriophage) and with a slightly negative charge, the nanoparticles were detected and distributed in pumpkin stems and leaves. However, no uptake was observed with lima bean plants (*Phaseolus limensis*), indicating the various responses of plants to nanoparticles.

Water content and water movement in soil are important for viral contamination, as indicated by the increased number of

viruses recovered from produce surfaces with an increase in soil moisture content (18). Low water content could increase virus particle attachment to soil-water interfaces, favor virus adsorption, and result in retention of virus movement in soil with a lesser access to plants (20). To favor the virus uptake, in this study saturated soil conditions were maintained with nutrient buffer during the entirety of the experiments. Nutrient solution was added to a container, and liquid moved up toward the soil through capillary force. This may explain in part why more virus internalization, especially with low inoculums, was observed in our study than in some of the previous research discussed above (17, 21). However, further experiments are needed to monitor soil conditions, soil water content, and virus movement to evaluate their effects on virus uptake by plants.

Transpiration is the driving force for water absorption, and the majority (96%) of water is taken up by the plant through transpiration (11). Humidity is a major factor controlling plant transpiration, and high humidity will reduce the diffusion of water out of the leaf and lower the transpiration rate, and transpiration will cease if humidity reaches 100% (5, 19). Transpiration was assessed in this study to identify a potential role in the movement of viruses into the plant tissue. In this study, lettuce grown under conditions of 99% humidity in order to minimize transpiration showed a significantly lower frequency of virus internalization than lettuce grown under conditions of a 70% RH chamber which had a 10-fold-higher transpiration rate. Thus, transpiration is likely to be a major force for virus uptake through roots.

In conclusion, MNV was taken up by romaine lettuce through the roots via contaminated irrigation water and reached edible leaf tissue. The internalization of human enteric viruses into produce during irrigation is possible under favorable conditions, and the fact that some internalized virus remained infectious poses a threat to food safety. Furthermore, the virus may be taken up in a passive manner by transpiration. The exact method of virus internalization under different environmental conditions, such as soil water content and environmental relative humidity, is still unclear.

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