Journal of Food Protection, Vol. 78, No. 8, 2015, Pages 1472–1480
doi:10.4315/0362-028X.JFP-14-518
Copyright ©, International Association for Food Protection

Postharvest Survival of Porcine Sapovirus, a Human Norovirus Surrogate, on Phytopathogen-Infected Leafy Greens

MALAK A. ESSEILI,[1]† ASHLINA CHIN,[2]† LINDA SAIF,[1] SALLY A. MILLER,[2] FENG QU,[2] MELANIE L. LEWIS IVEY,[2,3]* AND QIUHONG WANG[1]*

[1]*Food Animal Health Research Program, Department of Veterinary Preventive Medicine, and* [2]*The Department of Plant Pathology, Ohio Agricultural Research and Development Center, The Ohio State University, Wooster, Ohio 44691; and* [3]*The Department of Plant Pathology and Crop Physiology, Louisiana State University AgCenter, Baton Rouge, Louisiana 70803, USA*

MS 14-518: Received 30 October 2014/Accepted 9 April 2015

ABSTRACT

Leafy greens are increasingly being recognized as an important vehicle for human noroviruses (HuNoV), which cause recurring gastroenteritis outbreaks. Leafy greens often become infected by phytopathogens in the field, which may cause symptoms on the edible parts. Whether plant pathogen infections enhance the survival of HuNoV on leafy greens is unknown. Lettuce and spinach plants were infected with a bacterium, *Xanthomonas campestris* pv. *vitians* strain 701a, and with *Cucumber mosaic virus* strain Fny, respectively. The survival rate of porcine sapovirus (SaV), a HuNoV surrogate, on infected and noninfected postharvest leaves was then assessed. In addition, acibenzolar-*S*-methyl, a commercial chemical elicitor of plant systemic defense, was used to assess whether stimulating the plant host defense affects the postharvest survival of SaV. Leaves harvested from control and treated plants were inoculated with SaV and incubated for 7 days at 4°C. The infectivity (tissue culture infectious dose affecting 50% of the culture [$TCID_{50}$]/ml) and RNA (genomic equivalent/ml) titers of SaV were assayed using immunohistochemistry staining and SaV-specific TaqMan real-time reverse transcription PCR. Our results showed that cucumber mosaic virus Fny induced mild, nonnecrotic symptoms on spinach leaves and had no effect on SaV survival. In contrast, *X. campestris* pv. *vitians* 701a induced small localized necrotic lesions and significantly enhanced SaV survival on lettuce leaves. Treatment with acibenzolar-*S*-methyl was effective in reducing *X. campestris* pv. *vitians* 701a–induced lesions on infected lettuce plants but had no direct effect on SaV survival when used on healthy lettuce plants. These findings indicate that phytopathogen-induced necrotic lesions may enhance the postharvest survival of HuNoV on lettuce leaves. Therefore, preventive measures aiming to maintain healthy plants and minimize preharvest biological damage are expected to improve the safety of leafy greens.

The Centers for Disease Control and Prevention estimates that, each year, 48 million Americans contract a foodborne illness, leading to 128,000 hospitalizations and 3,000 deaths *(10)*. The highly contagious human noroviruses (HuNoVs) cause 58% of all foodborne illnesses in the United States *(39)* and are also the leading cause of foodborne illnesses worldwide *(28)*. NoV gastroenteritis symptoms include diarrhea, vomiting, and nausea; however, infections can also be asymptomatic. Although the disease is usually self-limiting, significant morbidity and mortality can occur among children, the elderly, and the immune compromised. The virus has a low infectious dose (~18 to 1,000 viral particles), is shed in both stools and vomitus (~10 to 11 log genomic equivalents [GE]/g), and can persist for a prolonged period of time in the environment *(20, 45)*. These characteristics allow the virus to spread easily through droplets, contaminated food, water, fomites, and person-to-person contact. Transmission through water, food, and food preparation areas is now recognized as a major source of foodborne outbreaks.

Leafy greens can be readily exposed to enteric pathogens at the farm level through exposure to contaminated irrigation water, sewage run-off, and soil manure amendments *(6)*. During the last decade, HuNoV foodborne disease outbreaks associated with leafy vegetables have increased substantially. Between 2009 and 2012, a total of 4,318 NoV outbreaks were reported, resulting in 2,512 hospitalizations and 304 deaths *(21)*. Leafy greens were the primary vehicle of HuNoV foodborne outbreaks, and 90% of the outbreaks involved food preparation settings, such as restaurants and catering or banquet facilities. Therefore, in view of the importance of leafy greens in HuNoV outbreaks, knowledge of the factors affecting HuNoV survival on leafy greens is essential to develop proper preventive measures.

* Authors for correspondence. Tel: 330-263-3960; Fax: 330-263-3677; E-mail: wang.655@osu.edu (Q.W.). Present address: Department of Plant Pathology and Crop Physiology, Louisiana State University, 302 Life Sciences Building, Baton Rouge, LA 70803, USA. Tel: 225-578-8537; Fax: 225-578-1415; E-mail: MIvey@agcenter.lsu.edu (M.L.L.I.).
† These authors contributed equally to this study.

Downloaded from http://meridian.allenpress.com/doi/pdf/10.4315/0362-028X.JFP-14-518 by guest on 26 November 2022

Leafy greens are exposed to various biotic and abiotic stress factors in the field. Infection with phytopathogens poses an important stress on the health of the plants, altering the plant's physiological functions and host defenses in ways that could be lethal to susceptible plants *(4, 16, 29)*. However, plant diseases can be asymptomatic or mildly symptomatic, which may not prevent the produce from being marketed *(49)*. One such phytopathogen, *Cucumber mosaic virus* (CMV), is ubiquitous and infects a wide range of plants, particularly species of the cucurbits, tomato, pepper, celery, and spinach *(8, 34)*. Additionally, the bacterial phytopathogen *Xanthomonas campestris* pv. *vitians* exerts a significant effect on leafy salad vegetables *(8)*. However, diversity in both CMV and *X. campestris* pv. *vitians* phytopathogens has been reported, and both may cause varying symptoms *(31, 37)*. For example, *X. campestris* pv. *vitians* strain 701a causes small localized necrotic spots on lettuce, tomato and pepper, whereas other strains of the bacterium may cause more severe systemic diseases *(37)*. Phytopathogen infection weakens the defenses of susceptible plants and may cause further damage and wounds *(23)*. These wounds, as well as cuts and bruises caused by harvesting and processing *(5, 9)*, may provide easy access to human enteric pathogens which attach preferentially to cut edges and injuries *(44)*. For example, a number of studies reported that enteric bacterial pathogens, such as *Escherichia coli* O157:H7 *(1, 13, 26)* and *Salmonella enterica (3, 49)*, were more prevalent on mechanically damaged and phytopathogen-infected produce, respectively, than on healthy plants. Therefore, it is essential to determine whether phytopathogen infections showing mild symptoms on leafy greens pose an increased risk to consumers after contamination with HuNoVs, which have also been shown to attach to cut edges and injured tissues *(14, 18)*.

Controlling phytopathogen infections is a major challenge to food production worldwide. Plants have evolved a range of mechanisms to protect themselves against pathogens by inducing local or systemic acquired resistance (SAR) *(41)*. For successful colonization, plant pathogens must counteract the host defense responses *(41)*. The SAR response is an induced immune mechanism in plants that provides broad-spectrum resistance against phytopathogens and is mediated by the production of several plant signals, including salicylic acid, for which acibenzolar-*S*-methyl (ASM) is a commercially available synthetic analogue (e.g., the so-called plant activator Actigard) *(43)*. ASM is used as a prophylactic agent in the field to protect plants from phytopathogens, including *X. campestris* pv. *vitians* and CMV *(22, 30, 43)*. In view of the reported correlation between plant defense response to phytopathogens and the reduction of human bacterial pathogens *(25, 40, 42)*, it is important to assess whether induced host defense plays a role in the survival of HuNoV. Therefore, the objectives of this study were to evaluate the postharvest survival of porcine sapovirus (SaV), a HuNoV surrogate, on leafy greens infected with phytopathogens and to assess whether enhancing plant host resistance with ASM will directly affect SaV survival on healthy plants. Toward these objectives, we used two kinds of commonly consumed leafy greens, romaine lettuce and spinach, and two phytopathogens, *X. campestris* pv. *vitians* strain 701a for lettuce and CMV strain Fny for spinach. Since HuNoV is still refractory to growth in routine cell culture, we used SaV, an established suitable enteropathogenic calicivirus surrogate of HuNoV *(15, 47)*.

MATERIALS AND METHODS

Plant growth conditions. Seeds of romaine lettuce cultivar Little Caesar and four spinach varieties, Baby's Leaf Hybrid, Giant 157, Salad Fresh, and Teton (Burpee, Warminster, PA) were grown in 200-cell trays containing Fafard Super Fine germinating mix (Conrad Fafard, Aguwam, MA) under greenhouse conditions as described previously *(15)*. At 2 weeks of age, the seedlings were transferred to 15-cm-diameter pots containing pasteurized Wooster sandy loam soil. The plants were fertilized biweekly using Osmocote slow release fertilizer and placed inside plastic trays in a completely randomized design. Water was added to the trays daily without directly contacting the leaves.

Inoculation with phytopathogens. The four spinach varieties were tested at age 5 weeks for susceptibility to 4 viruses: tomato bushy stunt virus, tomato spotted wilt virus, turnip mosaic virus, and CMV (strain Fny). The viruses were propagated in *Nicotiana benthamiana* plants to obtain fresh inoculum according to standard procedures *(24)*. Symptomatic *N. benthamiana* leaves were homogenized in inoculation buffer (50 mM NaPO4, pH 7.0, and 1% Celite 545), after which 1 ml of the homogenate was rubbed onto the surface of two older spinach leaves, directly across from each other, using a sterile gloved finger. Control spinach plants were rub inoculated with 1 ml of sterile water. Of the four spinach varieties, Baby's Leaf Hybrid grew the fastest and consistently exhibited viral symptoms for all four viruses tested. CMV was chosen for subsequent experiments because it did not cause death of the Baby's Leaf Hybrid spinach plants after two weeks but exhibited mild symptoms, such as leaf turning, rugosity, and mosaic on the young leaves (Fig. 1B). CMV infections were confirmed in spinach tissues by performing enzyme-linked immunosorbent assay with a commercial kit (Agdia, Elkhart, IN) according to the manufacturer's instructions. Briefly, spinach leaves were ground at a 1:10 ratio (tissue weight in grams per buffer volume in milliliters) in extraction buffer (0.01 M sodium sulfite, 8 M polyvinylpyrrolidone, 0.03 M sodium azide, 2% chicken albumin, and 2% Tween 20 in 0.01 M phosphate-buffered saline [PBS]) before being incubated on CMV antibody-coated 96-well plates. Infected and noninfected tobacco plants were processed similarly and used as positive and negative controls, respectively. Thereafter, the plates were incubated under dark conditions with a freshly prepared enzyme conjugate consisting of the detection antibody and alkaline phosphatase conjugate diluted in 0.01 M PBS supplemented with 0.2% bovine serum albumin and 8 M polyvinylpyrrolidone. Then, the substrate *p*-nitro-phenyl phosphate was diluted in a buffer consisting of 0.5 mM magnesium chloride hexahydrate, 10% ethanolamine, and 0.03 M sodium azide (pH 9.8) before being added to the plates. Unless otherwise specified, all buffers were adjusted to pH 7.4 and all incubation steps were performed at 25°C for 2 h. Following each incubation step, plates were washed seven times with 0.01 M PBS–Tween 20 (0.05%).

Downloaded from http://meridian.allenpress.com/doi/pdf/10.4315/0362-028X.JFP-14-518 by guest on 26 November 2022

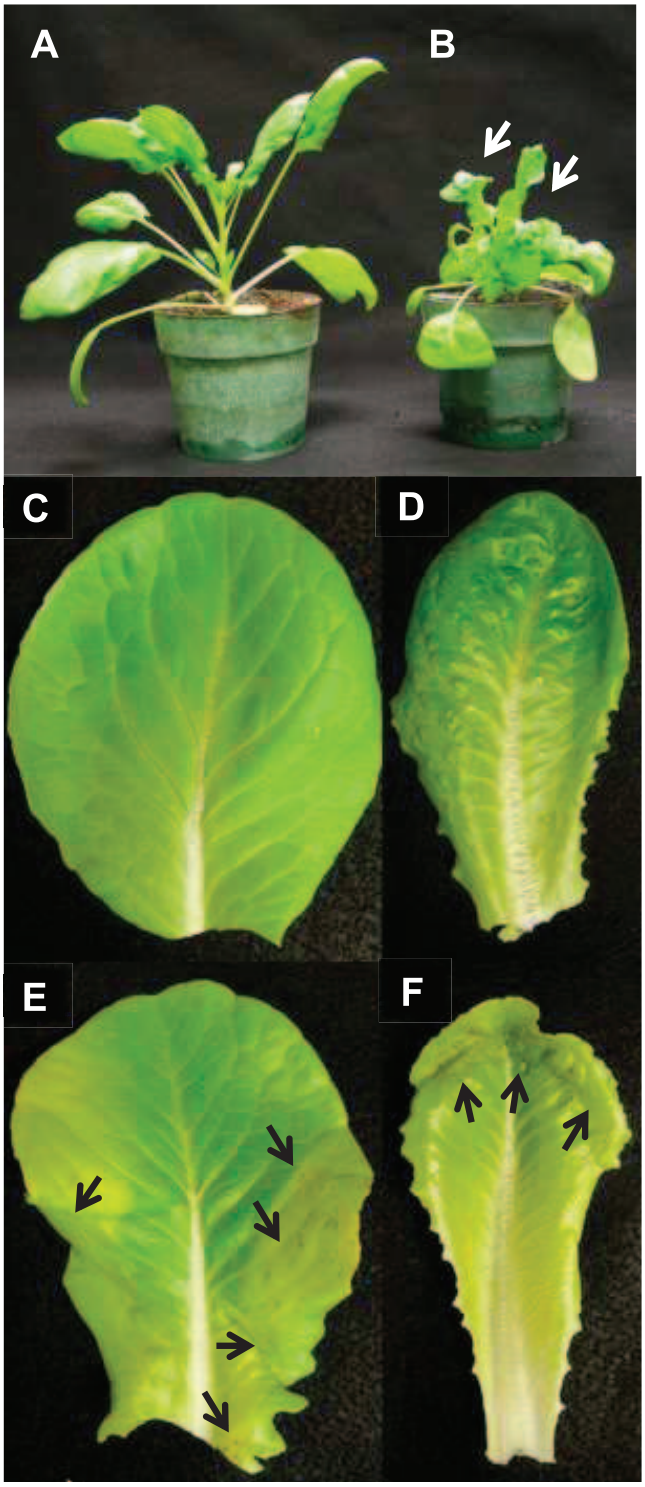

FIGURE 1. *Phytopathogen-infected plants. Control (A) and CMV Fny–infected (B) spinach plants showing leaf turning, rugosity, and mosaic on the younger infected leaves (indicated by arrows) at 2 weeks postinfection, and control older (C) and younger (D) and* X. campestris *pv.* vitians *701a–infected older (E) and younger (F) lettuce leaves showing chlorosis, leaf curling, and small, circular necrotic lesions (arrows) on infected leaves at 2 weeks postinfection.*

The absorbance at 405 nm was measured using a microplate reader, and the CMV antigen levels were quantified using the multimode analysis software (DTX 880 system, Beckman Coulter, Woburn, MA).

Lettuce plants at 4 weeks of age were infected with *X. campestris* pv. *vitians* strain 701a, previously shown to cause localized necrotic spot symptoms in romaine lettuce *(1)*. The *X. campestris* pv. *vitians* 701a inoculum was prepared by washing a pure culture grown on yeast dextrose carbonate agar medium for 48 h at 37°C with sterile deionized water. The concentration of bacteria was adjusted to an optical density at 600 nm of ~0.15 (~10^8 CFU/ml). Prior to inoculation, lettuce plants were misted for 3 h in a mist chamber (24 s every 10 min). Four older leaves on the lettuce plants were then inoculated by spreading 500 μl of the bacterial suspension onto the leaf surface with gloved fingers. Control plants were rub inoculated with 500 μl of sterile deionized water. The inoculated plants were returned to the mist chamber for continued misting (with 12 h of daylight) until necrotic lesions developed (approximately 2 weeks later). Symptomatic leaves showed chlorosis and leaf curling and developed small, circular necrotic lesions (Fig. 1E and 1F). To confirm the infection, leaves from infected and control plants were weighed and surface sterilized in 70% ethanol for 10 s. Thereafter, the samples were shaken in 0.01 M PBS for 30 min at 15 × *g*, and 1 ml of the supernatant was serially diluted 10-fold in 0.01 M PBS. Subsequently, 100-μl aliquots from each of the dilutions were spread on yeast dextrose carbonate agar plates in duplicate. The plates were incubated at 37°C for 72 h, after which emerging CFU were enumerated from dilutions showing 20 to 200 colonies. Dark-yellow mucoid colonies on yeast dextrose carbonate agar plates inoculated with leaf extracts, as well as the expected bacterial concentrations in the leaves, indicated successful infection of lettuce with *X. campestris* pv. *vitians* 701a.

Optimizing the rate of ASM application for lettuce plants. Since SaV only showed enhanced survival on lettuce infected with *X. campestris* pv. *vitians* 701a, the commercial formulation of ASM, Actigard 50WG, was used to induce resistance in lettuce. However, in order to reduce the chances of phytotoxicity and stunted growth, different rates of ASM application were tested (0.25, 5, 0.75, and 1 mg of product per plant dissolved in 100 ml of water). ASM was applied to 4-week-old lettuce plants after the soil was allowed to dry for 48 h and then again 1 week later. Each pot was drenched with 100 ml of the ASM solution, while negative-control plants received 100 ml of water. In preliminary studies, 100-ml amounts of liquid were absorbed into the 10-cm pots without runoff. One week after ASM application, the weight and phytotoxicity symptoms of treated and nontreated plants were compared. None of the treated plants exhibited any apparent stunting or burning due to ASM application, nor did they exhibit any symptoms different from the negative control. Therefore, the highest application rate, 1 mg per plant, was chosen. In addition, to confirm that the selected ASM application rate activated plant host defense, *X. campestris* pv. *vitians* 701a was used to induce lesions in lettuce pretreated with ASM. Specifically, lettuce plants grown as described above were divided into two groups: one group was treated with ASM and challenged with *X. campestris* pv. *vitians* 701a 3 days after the second treatment of ASM (as described above), while the second was only treated with 701a (positive control). Ten younger leaves and 10 older leaves were harvested from both groups and assessed for lesion occurrence. Also, the diameters of 10 randomly selected lesions per leaf age per treatment were measured under a compound microscope.

Propagation of SaV. The SaV Cowden strain was propagated using LLC-PK cells (ATCC CL-101) as previously described *(15, 47)*. Briefly, LLC-PK cells were cultured at a density of 3 × 10^6 cells per flask (175 cm^2) and incubated for 3 days at 37°C.

Downloaded from http://meridian.allenpress.com/doi/pdf/10.4315/0362-028X.JFP-14-518 by guest on 26 November 2022

The cells were washed, and then SaV was inoculated at a multiplicity of infection of 0.1 and incubated for one hour at 37°C. The culture medium containing the virus was minimal essential medium supplemented with 1% nonessential amino acids, 1% antibiotic–antimycotic cocktail (Invitrogen, Carlsbad, CA), and 50 μM glycochenodeoxycholic acid (Sigma-Aldrich, St. Louis, MO). The cells were incubated for an additional 3 days at 37°C. SaVs were released by applying three cycles of freezing-thawing. The virus was separated from cell debris by centrifugation at 2,500 × *g* for 20 min at 4°C. The supernatants containing SaVs were aliquoted, stored at −80°C and used in all subsequent experiments. Virus titers were assayed using reverse transcription quantitative real-time PCR (RT-qPCR) and infectivity assays as described below. The infectious titer of the SaV stock was $\sim 10^6$ tissue culture infectious dose affecting 50% of the culture ($TCID_{50}$)/ml, corresponding to an RT-qPCR titer of $\sim 10^{10}$ genomic equivalent (GE)/ml.

Contamination of plant leaves with SaV, a HuNoV surrogate. Spinach and lettuce plants (at 5 and 4 weeks of age, respectively) were challenged with phytopathogens as described above. Two weeks postinfection, leaves were removed and inoculated with SaV to mimic postharvest contamination. Specifically, leaves (younger and older) from control and CMV Fny–infected spinach, control and *X. campestris* pv. *vitians* 701a–infected lettuce, and control and ASM-treated lettuce plants were each spot inoculated with 1 ml of SaV at 5.35 ± 0.24 log $TCID_{50}$/ml or with minimal essential medium (Life Technologies, Carlsbad, CA) as a negative control. The SaV droplets were allowed to dry in a biosafety level 2 hood for 2 h. Inoculated leaves were individually bagged using Whirl-Pak bags (Nasco, Salida, CA) and incubated at 4°C for 7 days. At 2 h postinoculation (p.i.) (defined as day zero) and day 7 p.i., leaves were processed to determine SaV infectivity and RNA titers as described previously *(15)*. Briefly, viruses were eluted from the samples using minimal essential medium supplemented with 1% antibiotic–antimycotic cocktail (Invitrogen) and 2% heat-inactivated (60°C for 1 h) fetal bovine serum (HyClone, Thermo Scientific, Logan, UT). Samples were shaken vigorously (vortexing for 1 min, followed by shaking at 250 rpm for 10 min at 4°C), and the resulting solutions were transferred to sterile 50-ml Falcon tubes and centrifuged at 3,724 × *g* for 10 min to remove bacterial cells and plant debris. The supernatants were ultracentrifuged at 112,700 × *g* for 1.5 h to concentrate the viruses. The resulting pellets were suspended back to the original volume of 1 ml in sterile 1× PBS (pH 7.4). The latter was used for infectivity assays in LLC-PK cells following the immunohistochemical staining procedure and for assessing the RNA titers using SaV-specific RT-qPCR as described below.

RNA extraction. Viral RNA was extracted from 500 μl of the processed leaf samples using the RNeasy Minikit (Qiagen, Valencia, CA). A sterile water sample was extracted with every run to serve as an RNA extraction control, in addition to the experimental plant leaf control samples. Prior to RNA extraction, samples were treated with RNase A (0.5 μg/μl) (Invitrogen) for 1 h at 37°C. The RNA was eluted in 50 μl of nuclease-free water and stored at −20°C.

Real-time RT-PCR for the detection of SaV. One-step TaqMan SaV-specific RT-qPCR was used to estimate the virus concentration in plant samples, as described previously *(47)*. Briefly, 2 μl of each sample was mixed with 18 μl of master mix prepared using the Qiagen Onestep RT-PCR kit. The master mix contained 1× PCR buffer, 400 μM deoxynucleoside triphosphates, 200 nM each primer,100 nM TaqMan probe, 0.8 μl of enzyme mix, and 0.4 U/μl of RNAsin (Promega, Madison, WI). The amplification cycle consisted of the RT step (50°C for 30 min) and one cycle at 95°C for 15 min, followed by 45 cycles of 95°C for 15 s and 57.5°C for 1 min. RT-qPCR was performed with a Mastercylcer realplex (Eppendorf, Hauppauge, NY). Negative-control (nuclease-free water) and positive-control (SaV of known threshold cycle value) samples were included with each run. Each sample was tested in duplicate. An internal RNA control was spiked into randomly selected negative samples to check for RT-PCR inhibitors as described previously *(47)*. None of the lettuce RNA samples showed any PCR inhibitors. The final SaV RNA titer was calculated using the equation $y = -0.2914x + 12.851$ ($R^2 = 0.99$), where y is the SaV log qRT-PCR titer (GE per reaction mixture volume) and x is the threshold cycle value, as described previously *(15, 47)*.

SaV infectivity assay. The infectivity titers of SaV eluted from plant leaves were estimated in LLC-PK cells using immunohistochemical staining as described previously *(47)*. In short, LLC-PK cell monolayers in 96-well plates were infected in quadruplicate with serially diluted (1:10) samples and incubated for 72 h at 37°C. The cells were fixed for 30 min at room temperature using 3.7% formalin in PBS and were thereafter permeabilized for 5 min using 1% Triton X-100 in PBS. The primary antibody used was generated in our laboratory following hyperimmunization of guinea pigs with viruslike particles of porcine SaV *(15, 47)*. The secondary antibody used was horseradish peroxidase-conjugated goat anti-guinea pig IgG (H+L) (KPL, Gaithersburg, MD). Both antibodies were diluted in PBS supplemented with 2% nonfat dry milk and incubated with the cells for 1 h at 37°C. Following each incubation step, the cells were washed with PBS–Tween 20 (0.05%) three times. Finally, SaV-infected cells were visualized after treatment with horseradish peroxidase substrate (aminoethylcarbazole [AEC]) using the AEC kit (Sigma-Aldrich) for 2 h at room temperature. SaV-infected cells appeared with red cytoplasm and clear nuclei under light microscopy. The wells with infected cells were scored as positive, and the viral titers were estimated following the Reed-Muench equation for the calculation of $TCID_{50}$/ml.

The weights of the younger and older leaves did not differ significantly for lettuce or spinach. However, the weights of the leaves differed significantly between spinach and lettuce for both younger (0.36 ± 0.02 versus 1.55 ± 0.13 g, respectively) and older (0.46 ± 0.02 versus 2.41 ± 0.22 g, respectively) leaves. To avoid biases in SaV titer calculation due to differences in the weights between lettuce and spinach leaves, the SaV inoculum used was fixed to 1 ml per replicate sample and the droplets were spread to approximately similar surface areas (a 6-cm-diameter circle, ~ 30 cm^2) on all leaves. Since our processing protocol involved a concentration step and suspension back to the original inoculum volume, the SaV infectivities and RNA titers were reported in $TCID_{50}$ and GE per milliliter of recovered viruses instead of per gram of leaf tissues.

Statistical analyses. GraphPad Prism version 5 (GraphPad Software, La Jolla, CA) was used for statistical analyses. The entire data set was log transformed, and the titers on day 7 p.i. were subtracted from the average titers on day zero to calculate the 7-day log reduction in titer for each replicate sample. Two-way analysis of variance followed by the Bonferroni posttest was used to determine significant differences between the log reductions in SaV titers of control and treated leaves and younger and older leaves. Pearson product-moment correlation analysis was used to determine the correlation between SaV infectivity titers and RNA

Downloaded from http://meridian.allenpress.com/doi/pdf/10.4315/0362-028X.JFP-14-518 by guest on 26 November 2022

titers derived from both spinach and lettuce experiments. Each experiment was repeated twice using newly grown plants with triplicate samples per time point per leaf age per treatment. Differences in means were considered significant when the P value was <0.05 and are denoted in the figures by asterisks as follows: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$. Data are expressed as mean results ± standard errors.

RESULTS

Infection with CMV Fny did not affect SaV survival on spinach leaves. Systemic spread of CMV Fny occurred, as both younger and older spinach leaves were infected. The CMV antigen levels (quantified by the absorbance at 405 nm) did not differ significantly between younger and older leaves (1.81 ± 0.14 versus 1.26 ± 0.26). However, mild symptoms were more apparent on younger than on older leaves (Fig. 1A and 1B). On day zero, the SaV infectivity titers on control younger and older leaves were similar to those on CMV Fny–infected spinach leaves (3.32 ± 0.19 and 3.5 ± 0.18 versus 3.29 ± 0.16 and 3.69 ± 0.14 log $TCID_{50}$/ml, respectively). In addition, the SaV RNA titers on control younger and older leaves were similar to those on CMV Fny–infected spinach leaves (7.38 ± 0.21 and 7.35 ± 0.15 versus 7.25 ± 0.29 and 7.50 ± 0.15 log GE/ml, respectively). On day 7 p.i., the log reductions in SaV infectivity titers did not differ significantly between control and CMV-infected plants for both younger and older leaves (Fig. 2A). Similarly, the log reductions in SaV RNA titers did not differ significantly between control and CMV-infected plants for both younger and older leaves (Fig. 2B).

Infection with *X. campestris* pv. *vitians* 701a significantly enhanced SaV survival on lettuce leaves. Both younger and older lettuce leaves were infected; however, the bacterial titers in older leaves were significantly higher than in younger leaves (6.65 ± 0.17 versus 5.94 ± 0.2 log CFU/g, respectively). In addition, younger leaves exhibited significantly fewer lesions (4.3 ± 0.3 versus 7.4 ± 0.5 lesions per leaf) and smaller lesion diameters (2.4 ± 0.2 versus 4.9 ± 0.5 mm per lesion) than older leaves. On day zero, the SaV infectivity titers on control younger and older leaves were similar to those on infected lettuce leaves (3.39 ± 0.11 and 2.96 ± 0.13 versus 2.98 ± 0.13 and 2.88 ± 0.12 log $TCID_{50}$/ml, respectively). In addition, the SaV RNA titers on control younger and older leaves were similar to those on infected lettuce leaves (6.25 ± 0.1 and 5.84 ± 0.17 versus 6.44 ± 0.13 and 5.8 ± 0.13 log GE/ml, respectively). On day 7 p.i., the log reductions in SaV infectivity titers differed significantly between control and *X. campestris* pv. *vitians* 701a–infected plants for younger and older leaves (Fig. 3A). However, the log reductions in SaV RNA titers did not differ significantly between control and *X. campestris* pv. *vitians* 701a–infected plants for both younger and older leaves (Fig. 3B).

SaV survived similarly on healthy spinach and lettuce leaves but differentially on CMV Fny– versus *X. campestris* pv. *vitians* 701a–infected leaves. On day zero, the SaV infectivity titers on control younger and older spinach leaves were similar to those on control lettuce leaves (3.32 ± 0.19 and 3.54 ± 0.13 versus 3.39 ± 0.11 and 2.96 ± 0.13 log $TCID_{50}$/ml, respectively). On day 7 p.i., the log reductions in SaV infectivity titers on control spinach younger and older leaves were similar to those on control lettuce leaves (2.24 ± 0.45 and 1.87 ± 0.09 versus 1.98 ± 0.21 and 1.96 ± 0.25 log $TCID_{50}$/ml, respectively). However, CMV Fny–infected spinach leaves showed significantly higher log reductions in SaV infectivity titers than did *X. campestris* pv. *vitians* 701a–infected lettuce leaves for both younger and older leaves (Fig. 2A and 3A). However, the SaV log reductions in RNA titers did not differ significantly among controls or infected plants for either younger or older leaves (Fig. 2B and 3B).

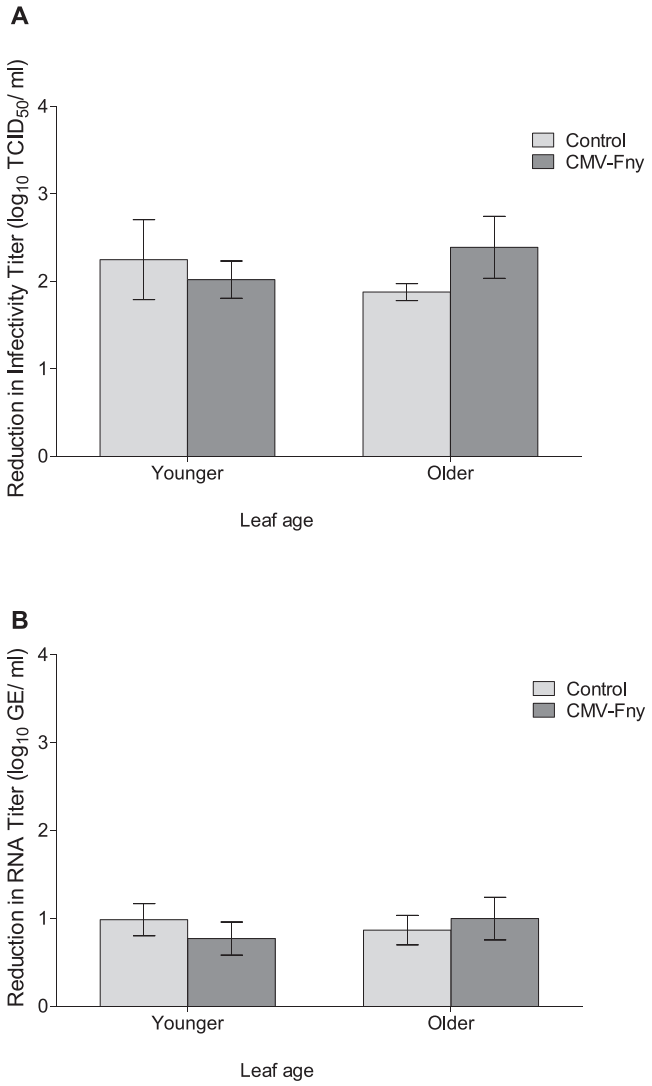

FIGURE 2. *Log reductions at 4°C in SaV infectivity ($TCID_{50}$/ml) (A) and RNA (GE/ml) (B) titers 7 days after inoculation of control and CMV Fny–infected spinach younger and older leaves with SaV.*

ASM pretreatment significantly reduced *X. campestris* pv. *vitians* 701a–induced symptoms on lettuce leaves. To ensure that the rate of application of ASM (1 mg per plant) activated lettuce plant host defenses, ASM-treated lettuce plants were challenged with *X. campestris* pv. *vitians*

Downloaded from http://meridian.allenpress.com/doi/pdf/10.4315/0362-028X.JFP-14-518 by guest on 26 November 2022

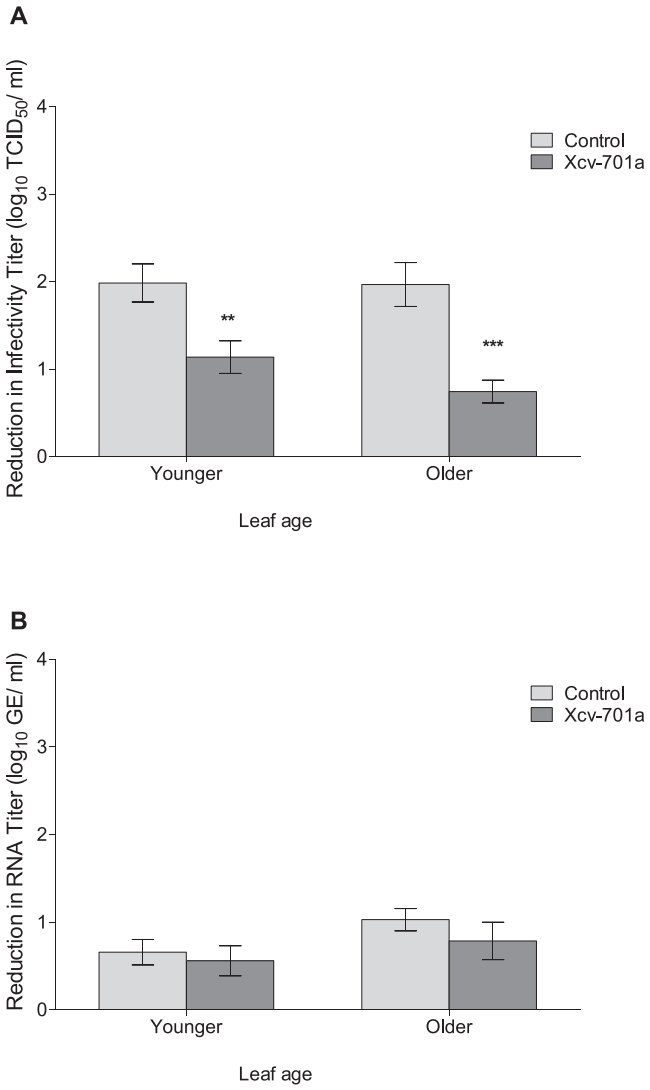

FIGURE 3. *Log reductions at 4°C in SaV infectivity ($TCID_{50}$/ml) (A) and RNA (GE/ml) (B) titers 7 days after inoculation of control and* X. campestris *pv.* vitians *701a–infected lettuce younger and older leaves with SaV. Asterisks denote significant differences in SaV mean titers in comparison to the results for the control (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).*

701a and then compared with positive controls (non–ASM-treated plants with *X. campestris* pv. *vitians* 701a infection). ASM treatment reduced the number and diameter of *X. campestris* pv. *vitians* 701a–induced lesions on both younger and older lettuce leaves. Specifically, the ASM-treated group exhibited, on average, a significantly lower number of lesions than the positive control group on both younger (0.7 ± 0.2 versus 4.3 ± 0.3 lesions per leaf) and older (2.8 ± 0.2 versus 7.4 ± 0.5 lesions per leaf) leaves. In addition, the ASM-treated group showed significantly smaller lesions (in diameter) than the positive control group on both younger (0.6 ± 0.1 versus 2.4 ± 0.2 mm per lesion) and older (1.6 ± 0.2 versus 4.9 ± 0.5 mm per lesion) leaves.

ASM pretreatment had no direct effect on SaV survival on healthy lettuce leaves. SaV survival on healthy leaves of lettuce plants that were pretreated with ASM and on those that received no treatment was compared. On day zero, the SaV infectivity titers on control younger and older leaves were similar to those on ASM-treated leaves (2.58 ± 0.21 and 2.49 ± 0.14 versus 2.51 ± 0.19 and 2.26 ± 0.16 log $TCID_{50}$/ml, respectively). In addition, the SaV RNA titers on control younger and older leaves were similar to those on ASM-treated leaves (6.37 ± 0.1 and 6.42 ± 0.13 versus 6.48 ± 0.16 and 6.28 ± 0.14 log GE/ml, respectively). On day 7 p.i., the log reductions in SaV infectivity titers did not differ significantly between control and ASM-treated plants for younger or older leaves (Fig. 4A). In addition, the log reductions in SaV RNA titers did not differ significantly between control and ASM-infected plants for either younger or older leaves (Fig. 4B).

Correlation between SaV infectivity and RNA titer. On day zero, the SaV infectivity titers correlated significantly ($P < 0.0001$, $R^2 = 0.34$) with the RNA titers derived from both spinach and lettuce experiments. However, on day 7 p.i., the correlation was not significant, with an R^2 of

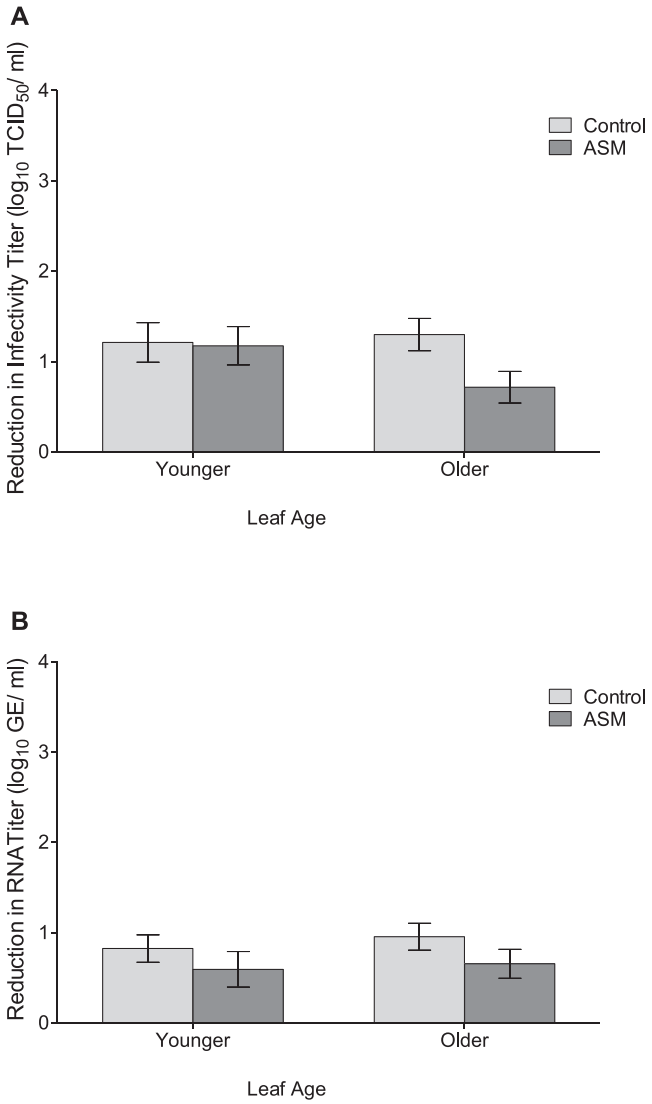

FIGURE 4. *Log reductions at 4°C in SaV infectivity ($TCID_{50}$/ml) (A) and RNA (GE/ml) (B) titers 7 days after inoculation of control and ASM-pretreated lettuce younger and older leaves with SaV.*

Downloaded from http://meridian.allenpress.com/doi/pdf/10.4315/0362-028X.JFP-14-518 by guest on 26 November 2022

0.01. In addition, the day 7 log reductions in infectivity and RNA titers were not significantly correlated.

DISCUSSION

Although the epidermis of fruits and vegetables is covered with a cuticle that provides a primary barrier against biological and physical damage, tissue damage often occurs during production, harvest, and postharvest handling. Mechanical damage to fresh produce caused by harvesting and processing (e.g. bruises, wounds, broken trichomes, and cracks in the leaf cuticle) has been shown to enhance the attachment, growth, and survival of human bacterial pathogens, such as *E. coli* O157:H7 *(9, 32)*. Damage to produce tissues can also occur due to infection with plant pathogens *(9),* creating suitable niches for enteric pathogens to exploit *(1, 19)*. Infections with phytopathogens can be asymptomatic or display mild symptoms, so the infected produce can still be marketed. To mimic this scenario, we used a strain of the virus CMV (Fny strain) which infects spinach, resulting in mild (on younger leaves) or no symptoms (on older leaves) *(12)*. Also, we used strain 701a of the bacterium *X. campestris* pv. *vitians,* which was isolated from lettuce in Ohio *(37)* and is known to cause small localized lesions *(1)*. Therefore, these infected leafy greens would be suitable for marketing and so could serve as a model for two different scenarios (presence of nonnecrotic and necrotic symptoms) in which phytopathogens may influence the survival of HuNoV after harvest and during food preparation and storage prior to consumption.

Infections with the bacterial phytopathogen *X. campestris* pv. *vitians* have been more prevalent over the past decade *(3, 37)*. The bacterium *X. campestris* pv. *vitians* strain 701a infects the leaves of lettuce plants, penetrating through natural openings, such as stomata, causing the progressive development of brown localized necrotic lesions *(37)*. Our results showed that the survival of the HuNoV surrogate SaV was significantly enhanced on *X. campestris* pv. *vitians* 701a–infected leaves exhibiting localized necrotic lesions. Both younger and older leaves showed significantly smaller reductions in SaV infectivity titers, albeit with different levels of significance. Similarly, previous studies reported higher survival of enteric bacteria pathogens, such as *E. coli* O157:H7, on lettuce infected with phytopathogens *(1, 9)*. In those studies, the formation of necrotic lesions was suggested to promote the growth of bacterial enteric pathogens. In our study, significant differences in lesion numbers and sizes (diameters) and bacterial densities were detected between younger and older leaves. The latter may be the result of several factors, including (i) inoculation of *X. campestris* pv. *vitians* 701a onto older leaves, allowing more time for the development of symptoms before the bacteria spread to newer younger leaves, (ii) greater nutrient leaching from the surface of older than of younger leaves, supporting more growth of bacterial plant pathogens *(1, 2),* (iii) increased leaching of nutrients from leaves with larger numbers of necrotic lesions *(46),* (iv) differences in age-dependent leaf susceptibility to phytopathogens *(24),* and (v) induced resistance in non-invaded tissues as a result of infection of other tissues on the same plant *(30)*. In addition, a previous study showed that younger, noninoculated lettuce leaves supported fewer lesions and lower *X. campestris* pv. *vitians* densities than older, inoculated leaves *(38)*. On the other hand, in our study, CMV Fny inoculated onto older leaves infected spinach plants systemically but showed nonnecrotic lesions and no effect on the survival of SaV. Similar to our results, previous studies showed no effect on the preharvest survival of enteric bacterial pathogens *E. coli* O157:H7 and *S. enterica* serovar Typhimurium in spinach and lettuce infected with phytopathogens and exhibiting nonnecrotic lesions, such as yellow spots *(19, 23)*. Taking these results together, the significant enhancement of SaV survival on *X. campestris* pv. *vitians* 701a–infected lettuce leaves and the absence of measurable effect in CMV Fny–infected spinach leaves is likely due to the presence of necrotic lesions induced by *X. campestris* pv. *vitians* 701a and their absence in CMV-infected plants. Furthermore, previous studies showed that *X. campestris* pv. *vitians* strains isolated from lettuce (including strain 701a) possess pectinolytic activity *(37, 38)*. Pectinases cause the degradation of plant cell wall pectins, thus enhancing the pathogen's ability to invade the plants' interior. Damaged cell walls expose cell wall carbohydrates, which have been shown to specifically bind to HuNoV VLPs *(14)*. In addition, a previous study reported a significant increase in the abundance of sugars on *X. campestris*–infected tomato leaves manifesting necrotic lesions compared with the sugar abundance on healthy leaves *(2)*. Therefore, damaged cell walls in necrotic lesions are expected to enhance the persistence and survival of HuNoV on lettuce postharvest. This is of particular importance because attached viruses are more difficult to wash off or inactivate, thus posing an increased risk to consumers.

Healthy leaves of both spinach and lettuce retained infectious SaV by day 7 at 4°C. Similar results were previously reported for hepatitis A virus, where infectious virus was still detectable on healthy lettuce leaves stored at 4°C for 9 days *(11)*. In addition, our study showed enhanced survival of the HuNoV surrogate SaV on *X. campestris* pv. *vitians* 701a–infected versus noninfected lettuce leaves. Therefore, preventive measures should not only address minimizing the initial contact of leafy greens with HuNoV sources but should also address controlling phytopathogen infections. Controlling bacterial phytopathogens on food has been shown to reduce the growth of enteric bacterial pathogens *(17, 27),* but to our knowledge, no similar studies have been performed for enteric viruses, such as HuNoV. Furthermore, in our study, SaV showed similar survival patterns on younger and older leaves of both healthy and CMV-infected spinach leaves. Although CMV weakens the host defenses by inhibition of both the salicylic and jasmonic acid defense pathways *(31),* the virus did not cause any noticeable wounds or necrotic lesions on the surface of the leaves where SaV was applied. Collectively, this suggests that CMV-induced suppression of host defenses had no effect on SaV survival after harvesting. In contrast, SaV survival differed significantly in *X. campestris* pv. *vitians* 701a–infected and noninfected lettuce leaves,

Downloaded from http://meridian.allenpress.com/doi/pdf/10.4315/0362-028X.JFP-14-518 by guest on 26 November 2022

suggesting that in addition to necrotic lesions (discussed above), host defense may be a factor indirectly affecting SaV survival in lettuce. Our results confirmed previous studies showing that ASM was able to induce the necessary host defenses to suppress the severity of *X. campestris* pv. *vitians* 701a–induced symptoms on lettuce *(50)*. Following the confirmation that the ASM application rate used in our study was effective in inducing host plant defenses, we applied ASM as intended to be used in the field, i.e., as a crop protectant prior to any pathogen attack. Our results showed that enhancing host defenses in healthy lettuce plants had no direct effect on postharvest survival of SaV. However, given that (i) the plant SAR response is induced by pathogens that cause necrotic lesions, such as *X. campestris* pv. *vitians* 701a *(41),* (ii) ASM is a stimulator of SAR *(43, 50)* and was shown in our study to reduce *X. campestris* pv. *vitians* 701a–induced surface lesions, and (iii) the enhancement of SaV survival on *X. campestris* pv. *vitians* 701a–infected lettuce, the use of ASM may be expected to protect lettuce against *X. campestris* pv. *vitians* 701a and, thus, indirectly reduce the survival of SaV on infected lettuce plants.

The infectivity titers of SaV correlated significantly with the RNA titers only after inoculation (i.e., at day zero). However, since there was no significant correlation on day 7 p.i., the SaV RNA titers could not be used to predict its infectivity. This may be due to various inactivating factors present on the leaf surface or released during processing *(7, 15, 36)* that might cause damage to viral particles. Damaged viral particles can lose their infectivity while retaining RNA, thus altering the correlation between infectivity titers and RNA titers. Since HuNoV continue to be refractory to growth in routine cell culture and the RNA titer cannot be used to predict its infectivity, it is important to use suitable culturable enteropathogenic calicivirus surrogates, such as porcine SaV, to mimic HuNoV survival. Furthermore, SaVs are directly relevant to human health, since SaVs are transmitted via the fecal-oral route and recent studies have suggested that human SaV-associated gastroenteritis is becoming more prevalent worldwide *(35)*. Certain porcine SaVs and NoVs are genetically and/or antigenically closely related to the human strains, suggesting the potential for zoonotic infections *(33, 48)*.

In conclusion, we showed that (i) SaV contamination of leafy greens persists under postharvest storage conditions, (ii) *X. campestris* pv. *vitians* 701a infection of lettuce promotes SaV survival, likely due to the induction of necrotic lesions, and (iii) the use of ASM reduced the symptoms of *X. campestris* pv. *vitians* 701a on lettuce and, thus, may indirectly reduce the survival of SaV on infected lettuce plants. Therefore, necrotic lesion–inducing phytopathogens may increase the risk of foodborne illness caused by HuNoV for consumers of fresh leafy green vegetables. While plant outer leaves with large necrotic lesions may be removed from the food chain prior to or during preparation, smaller necrotic lesions on inner leaves may not be noticed and serve to improve the survival of human pathogens. While there is no evidence of a direct effect of ASM on the survival of SaV, the plant disease resistance inducer may indirectly reduce the risk of foodborne illness to consumers by reducing the incidence and severity of necrotic symptoms. Therefore, efforts to minimize plant disease in the field prior to harvest should help to improve the safety of fresh produce.

ACKNOWLEDGMENTS

This project was supported by Agriculture and Food Research Initiative competitive grant 2011-67017-30067 from the U.S. Department of Agriculture's National Institute of Food and Agriculture. Salaries and research support were provided by state and federal funds provided to the Ohio Agricultural Research and Development Center (OARDC), The Ohio State University. We thank Drs. Issmat Kassem and Ken Shenge for their critical review of the manuscript.

REFERENCES

1. Aruscavage, D., S. A. Miller, M. L. Ivey, K. Lee, and J. T. LeJeune. 2008. Survival and dissemination of *Escherichia coli* O157:H7 on physically and biologically damaged lettuce plants. *J. Food Prot.* 71: 2384–2388.
2. Aruscavage, D., P. L. Phelan, K. Lee, and J. T. LeJeune. 2010. Impact of changes in sugar exudate created by biological damage to tomato plants on the persistence of *Escherichia coli* O157:H7. *Food Microbiol. Saf.* 75:187–192.
3. Barak, J. D., and A. S. Liang. 2008. Role of soil, crop debris, and a plant pathogen in *Salmonella enterica* contamination of tomato plants. *PLoS One* 3:e1657.
4. Bebber, D. P., T. Holmes, D. Smith, and S. J. Gurr. 2014. Economic and physical determinants of the global distributions of crop pests and pathogens. *New Phytol.* 202:901–910.
5. Berger, C. N., D. J. Brown, R. K. Shaw, F. Minuzzi, B. Feys, and G. Frankel. 2011. *Salmonella enterica* strains belonging to O serogroup 1,3,19 induce chlorosis and wilting of Arabidopsis thaliana leaves. *Environ Microbiol.* 13:1299–1308.
6. Beuchat, L. R., and J. H. Ryu. 1997. Produce handling and processing practices. *Emerg. Infect. Dis.* 3:459–465.
7. Bidawid, S., J. M. Farber, and S. A. Sattar. 2001. Survival of hepatitis A virus on modified atmosphere-packaged (MAP) lettuce. *Food Microbiol.* 18:95–102.
8. Blancard, D., H. Lot, and B. Maisonneuve. 2006. A color atlas of diseases of lettuce and related salad crops: observation, biology and control. Academic Press, Burlington, MA.
9. Brandl, M. T. 2008. Plant lesions promote the rapid multiplication of *Escherichia coli* O157:H7 on postharvest lettuce. *Appl. Environ. Microbiol.* 74:5285–5289.
10. Centers for Disease Control and Prevention. 2011. Estimates of foodborne illness in the United States. Available at: http://www.cdc.gov/foodborneburden/. Accessed 8 January 2014.
11. Croci, L., D. De Medici, C. Scalfaro, A. Fiore, and L. Toti. 2002. The survival of hepatitis A virus in fresh produce. *Int. J. Food Microbiol.* 73:29–34.
12. Ding, S. W., B. J. Anderson, H. R. Haase, and R. H. Symons. 1994. New overlapping gene encoded by the cucumber mosaic virus genome. *Virology* 198:593–601.
13. Dingman, D. W. 2000. Growth of *Escherichia coli* O157:H7 in bruised apple (*Malus domestica*) tissue as influenced by cultivar, date of harvest, and source. *Appl. Environ. Microbiol.* 66:1077–1083.
14. Esseili, M. A., Q. Wang, and L. J. Saif. 2012. Binding of human GII.4 norovirus virus-like particles to carbohydrates of romaine lettuce leaf cell wall materials. *Appl. Environ. Microbiol.* 78:786–794.
15. Esseili, M. A., Q. Wang, Z. Zhang, and L. J. Saif. 2012. Internalization of sapovirus, a surrogate for norovirus, in romaine lettuce and the effect of lettuce latex on virus infectivity. *Appl. Environ. Microbiol.* 78:6271–6279.
16. Flood, J. 2010. The importance of plant health to food security. *Food Secur.* 2:215–231.

Downloaded from http://meridian.allenpress.com/doi/pdf/10.4315/0362-028X.JFP-14-518 by guest on 26 November 2022

17. Frampton, R. A., A. R. Pitman, and P. C. Fineran. 2012. Advances in bacteriophage-mediated control of plant pathogens. *Int. J. Microbiol.* 2012:326452.
18. Gandhi, K. M., R. E. Mandrell, and P. Tian. 2010. Binding of virus-like particles of Norwalk virus to romaine lettuce veins. *Appl. Environ. Microbiol.* 76:7997–8003.
19. Ge, C., C. Lee, E. Nangle, J. Li, D. Gardner, M. Kleinhenz, and J. Lee. 2014. Impact of phytopathogen infection and extreme weather stress on internalization of Salmonella Typhimurium in lettuce. *Int. J. Food Microbiol.* 168-169:24–31.
20. Glass, R. I., U. D. Parashar, and M. K. Estes. 2009. Norovirus gastroenteritis. *N. Engl. J. Med.* 361:1776–1785.
21. Hall, A. J., M. E. Wikswo, K. Pringle, L. H. Gould, U. D. Parashar, and Division of Viral Diseases, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention. 2014. Vital signs: foodborne norovirus outbreaks—United States, 2009-2012. *Morb. Mortal. Wkly. Rep.* 63:491–495.
22. Herman, M. A. B., S. Restrepo, and C. D. Smart. 2007. Defense gene expression patterns of three SAR-induced tomato cultivars in the field. *Physiol. Mol. Plant Pathol.* 71:192–200.
23. Hora, R., K. Warriner, B. J. Shelp, and M. W. Griffiths. 2005. Internalization of *Escherichia coli* O157:H7 following biological and mechanical disruption of growing spinach plants. *J. Food Prot.* 68: 2506–2509.
24. Hull, R. 2014. Plant virology, 5th ed. Elsevier Academic Press, Amsterdam.
25. Iniguez, A. L., Y. Dong, H. D. Carter, B. M. Ahmer, J. M. Stone, and E. W. Triplett. 2005. Regulation of enteric endophytic bacterial colonization by plant defenses. *Mol. Plant Microbe Interact.* 18:169–178.
26. Janisiewicz, W. J., W. S. Conway, M. W. Brown, G. M. Sapers, P. Fratamico, and R. L. Buchanan. 1999. Fate of *Escherichia coli* O157:H7 on fresh-cut apple tissue and its potential for transmission by fruit flies. *Appl. Environ. Microbiol.* 65:1–5.
27. Janisiewicz, W. J., W. S. Conway, and B. Leverentz. 1999. Biological control of postharvest decays of apple can prevent growth of *Escherichia coli* O157:H7 in apple wounds. *J. Food Prot.* 62: 1372-5.
28. Koo, H. L., N. Ajami, R. L. Atmar, and H. L. DuPo. 2010. Noroviruses: the principal cause of foodborne disease worldwide. *Discov. Med.* 10:61–70.
29. Kunkel, B. N., and D. M. Brooks. 2002. Cross talk between signaling pathways in pathogen defense. *Curr. Opin. Plant Biol.* 5:325–331.
30. Loebenstein, G. 2009. Local lesions and induced resistance. *Adv. Virus Res.* 75:73–117.
31. Mochizuki, T., and S. T. Ohki. 2012. *Cucumber mosaic virus*: viral genes as virulence determinants. *Mol. Plant Pathol.* 13:217–225.
32. Mukherjee, A., D. Speh, A. T. Jones, K. M. Buesing, and F. Diez-Gonzalez. 2006. Longitudinal microbiological survey of fresh produce grown by farmers in the upper midwest. *J. Food Prot.* 69: 1928–1936.
33. Nakamura, K., Y. Saga, M. Iwai, M. Obara, E. Horimoto, S. Hasegawa, T. Kurata, H. Okumura, M. Nagoshi, and T. Takizawa. 2010. Frequent detection of noroviruses and sapoviruses in swine and high genetic diversity of porcine sapovirus in Japan during fiscal year 2008. *J. Clin. Microbiol.* 48:1215–1222.
34. Palukaitis, P., M. J. Roossinck, R. G. Dietzgen, and R. I. Francki. 1992. *Cucumber mosaic virus*. *Adv. Virus Res.* 41:281–348.
35. Pang, X. L., B. E. Lee, G. J. Tyrrell, and J. K. Preiksaitis. 2009. Epidemiology and genotype analysis of sapovirus associated with gastroenteritis outbreaks in Alberta, Canada: 2004-2007. *J. Infect. Dis.* 199:547–551.
36. Reinders, R. D., S. Biesterveld, and P. G. Bijker. 2001. Survival of *Escherichia coli* O157:H7 ATCC 43895 in a model apple juice medium with different concentrations of proline and caffeic acid. *Appl. Environ. Microbiol.* 67:2863–2866.
37. Sahin, F., P. A. Abbasi, M. L. L. Ivey, J. Zhang, and S. A. Miller. 2003. Diversity among strains of *Xanthomonas campestris* pv. *vitians* from lettuce. *Phytopathology* 93:64–70.
38. Sahin, F., and S. A. Miller. 1997. Identification of the bacterial leaf spot pathogen of lettuce, *Xanthomonas campestris* pv. *vitians*, in Ohio, and assessment of cultivar resistance and seed treatment. *Plant Dis.* 81:1443–1446.
39. Scallan, E., R. M. Hoekstra, F. J. Angulo, R. V. Tauxe, M. A. Widdowson, S. L. Roy, J. L. Jones, and P. M. Griffin. 2011. Foodborne illness acquired in the United States—major pathogens. *Emerg. Infect. Dis.* 17:7–15.
40. Schikora, A., I. Virlogeux-Payant, E. Bueso, A. V. Garcia, T. Nilau, A. Charrier, S. Pelletier, P. Menanteau, M. Baccarini, P. Velge, and H. Hirt. 2011. Conservation of Salmonella infection mechanisms in plants and animals. *PLoS One* 6:e24112.
41. Seo, S., and K. R. Matthews. 2012. Influence of the plant defense response to *Escherichia coli* O157:H7 cell surface structures on survival of that enteric pathogen on plant surfaces. *Appl. Environ. Microbiol.* 78:5882–5889.
42. Shirron, N., and S. Yaron. 2011. Active suppression of early immune response in tobacco by the human pathogen Salmonella Typhimurium. *PLoS One* 6:e18855.
43. Smith-Becker, J., N. T. Keen, and J. O. Becker. 2003. Acibenzolar-S-methyl induces resistance to *Colletotrichum lagenarium* and *cucumber mosaic virus* in cantaloupe. *Crop Prot.* 22:769–774.
44. Takeuchi, K., C. M. Matute, A. N. Hassan, and J. F. Frank. 2000. Comparison of the attachment of *Escherichia coli* O157:H7, *Listeria monocytogenes, Salmonella typhimurium,* and *Pseudomonas fluorescens* to lettuce leaves. *J. Food Prot.* 63:1433–1437.
45. Teunis, P. F., C. L. Moe, P. Liu, S. E. Miller, L. Lindesmith, R. S. Baric, J. Le Pendu, and R. L. Calderon. 2008. Norwalk virus: how infectious is it? *J Med Virol.* 80:1468–1476.
46. Tukey, J. H. B., and J. V. Morgan. 1963. Injury to foliage and its effect upon tbe leaching of nutrients from above-ground plant parts. *Physiol. Plant.* 16:557–564.
47. Wang, Q., Z. Zhang, and L. J. Saif. 2012. Stability of and attachment to lettuce by a culturable porcine sapovirus surrogate for human caliciviruses. *Appl. Environ. Microbiol.* 78:3932–3940.
48. Wang, Q. H., V. Costantini, and L. J. Saif. 2007. Porcine enteric caliciviruses: genetic and antigenic relatedness to human caliciviruses, diagnosis and epidemiology. *Vaccine* 25:5453–5466.
49. Wells, J. M., and J. E. Butterfield. 1997. Salmonella contamination associated with bacterial soft rot of fresh fruits and vegetables in the marketplace. *Plant Dis.* 81:867–872.
50. Yigit, F. 2011. Acibenzolar-*S*-methyl induces lettuce resistance against *Xanthomonas campestris* pv. *vitians*. *Afr. J. Biotechnol.* 10: 9606–9612.

Downloaded from http://meridian.allenpress.com/doi/pdf/10.4315/0362-028X.JFP-14-518 by guest on 26 November 2022