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Inactivation of internalized and surface contaminated enteric viruses in green onions

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ABSTRACT

With increasing outbreaks of gastroenteritis associated with produce, it is important to assess interventions to reduce the risk of illness. UV, ozone and high pressure are non-thermal processing technologies that have potential to inactivate human pathogens on produce and allow the retention of fresh-like organoleptic properties. The objective of this study was to determine if UV, ozone, and high pressure are effective technologies compared to traditional chlorine spray on green onions to reduce enteric viral pathogens and to determine the effect of location of the virus (surface or internalized) on the efficacy of these processes. Mature green onion plants were inoculated with murine norovirus (MNV), hepatitis A virus (HAV) and human adenovirus type 41 (Ad41) either on the surface through spot inoculation or through inoculating contaminated hydroponic solution allowing for uptake of the virus into the internal tissues. Inoculated green onions were treated with UV (240 mJ s/cm^2), ozone (6.25 ppm for 10 min), pressure (500 MPa, for 5 min at 20 °C), or sprayed with calcium hypochlorite (150 ppm, 4 °C). Viral inactivation was determined by comparing treated and untreated inoculated plants using cell culture infectivity assays. Processing treatments were observed to greatly affect viral inactivation. Viral inactivation for all three viruses was greatest after pressure treatment and the lowest inactivation was observed after chlorine and UV treatment. Both surface inoculated viruses and viruses internalized in green onions were inactivated to some extent by these post-harvest processing treatments. These results suggest that ozone and high pressure processes aimed to reduce the level of microbial contamination of produce have the ability to inactivate viruses if they become localized in the interior portions of produce.

1. Introduction

Viral contamination of pre-harvest produce is a food safety concern and should be considered in the design and implementation of produce production practices and post-harvest intervention strategies. Data on viral outbreaks associated with produce is limited; however, a recent survey testing packaged leafy greens in Canada, Belgium and France for human noroviruses confirmed that the presence of human NoV RNA was between 28 and 50% of samples (Baert et al., 2011). While these sequences were not associated with documented outbreaks, the results suggest that packaged produce is a source for enteric viruses (Baert et al., 2011). Large produce outbreaks including the 2003 HAV green onion outbreak that resulted in over 1000 illnesses and 4 deaths highlighted the risk of enteric virus contamination of produce (Wheeler et al., 2005).

Hepatitis A virus (HAV), human norovirus, and human adenovirus type 41 (Ad41) are considered important enteric viruses from a public health and economic perspective. Due to the inability to assess human norovirus infectivity in cell culture, murine norovirus (MNV), a common human norovirus surrogate, is used in this study. Ad41 was chosen as a comparison to MNV and HAV for internalization and processing treatments as well as for the fact that it is a common environmental contaminant. The morphological structure and genome of Ad41 are completely different from MNV and HAV. Ad41 is a double stranded DNA genome packed inside of a large (90–100 nm in diameter) icosahedral capsid characterized by penton fibers that project from each apex which are important for viral attachment to host cells (Favier et al., 2002). MNV and HAV are small single stranded RNA icosahedral viruses approximately 30–40 nm in diameter. Due to the differences in size, structure, and genetic makeup, these viruses have the potential to behave differently on produce surfaces and also under varying processing parameters.

Enteric viruses can contaminate agricultural environments through the application of manure, biosolids, pesticides and compost, through foodhandlers in the field, and through water contact, either by irrigation or flooding. Recent research has highlighted the possibility for enteric viruses to internalize into plant tissues though both cut edge and stomata on the edible portions (Wei et al., 2010a) and through root uptake (Hirneisen et al., 2012b). Both MNV and HAV have been shown to be internalized in plants through root uptake, particularly when grown in contaminated hydroponic systems; however, internalization observed in soil grown crops was rare (Hirneisen et al., 2012b). Internalization,

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as used in this study, is defined as the uptake of human enteric pathogens through the roots into the intercellular spaces between plant cells and in the plant vasculature tissues, xylem and phloem (Hirneisen et al., 2012b). One concern with the potential for the internalization of human pathogens into the vascular tissues of produce crops is that these pathogens could be protected from inactivation by post-harvest processing intervention strategies. Once viruses are internalized, it is assumed that their elimination may be a greater challenge since traditional sanitation measure usually target the pathogens on the surface of produce (Doyle and Erickson, 2008; Li et al., 2012; Wei et al., 2010a). Due to the food safety risks associated with consumption of fresh produce, preventative processing strategies are a promising means to reduce viral loads. Non-thermal processing technologies including ultraviolet light (UV), ozone, and high pressure processing have shown to be effective for a wide range of enteric viruses (Hirneisen et al., 2010); however, the effectiveness of these technologies on internalized viruses as compared to contamination on produce surfaces remains unknown.

The objective of this study is to determine if nonthermal processing technologies including UV, ozone and pressure are able to inactivate HAV, Ad41 and MNV present in the internal vascular tissues of green onions. The efficacy of these processes was compared to viral inactivation achieved by a traditional post-harvest chlorine spray on green onions. Additionally, comparing the inactivation of surface inoculated viruses to internalized viruses provides insight into the efficacy of intervention strategies aimed at reducing viral titers.

2. Materials and methods

2.1. Cell culture and viral propagation

MNV-1 (generously provided by H. Virgin, Washington University, St. Louis, MO) was propagated in the RAW 264.7 cell line cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco-Invitrogen, CA) supplemented with 1% penicillin/streptomycin, 1% sodium bicarbonate, and 1% glutamate. Ad41 (ATCC # VR-930TM) was cultured on human embryonic kidney (HEK) 293 cells (ATCC # CRL-1573TM) in Eagle's Minimal Medium (MEM) (Mediatech, VA) supplemented with 1% penicillin/streptomycin, 1% sodium bicarbonate, 1% sodium pyruvate and 1% MEM non-essential amino acids. HAV (ATCC VR-1402) was propagated in fetal rhesus monkey kidney cells (FRhK-4) (ATCC CRL 1688) using Dulbecco's Modified Eagle's Medium (DMEM) (Mediatech, Manassas, VA) supplemented with 1% penicillin/streptomycin and 1% sodium bicarbonate. All media were supplemented with 2% fetal bovine serum (FBS) (Mediatech) for maintenance or 10% FBS for cell growth.

Virus was propagated in cell culture by inoculating confluent flasks with virus at a multiplicity of infection (MOI) of 0.5. Flasks were incubated at 37 °C for 48 h for MNV and Ad41 or for 15 d for HAV. Viruses were purified from infected cells through three freeze-thaw cycles to lyse cells and the supernatant containing the virus was recovered by centrifugation at 2500 ×*g* for 15 min and stored at −80 °C.

2.1.1. Green onion production

Green onion, guardsman bunching, (*Allium fistulosum x cepa*) seeds were purchased from Johnny's Selected Seeds (Winslow, Maine). Green onion seeds were germinated in ReadyEarth soilless medium in the misting room of the Fisher Greenhouse (College of Agricultural and Natural Resources, University of Delaware). After germination (10 d), green onion seedlings were transferred to ProMix soilless medium and grown at 18 °C and 65% humidity in a biocontrol growth chamber in the Fisher Greenhouse.

2.1.2. Inoculation of green onions

After 45 days of growth, green onions were removed from soil substrate, washed in tap water to remove excess soil substrate and placed in Hoagland's hydroponic solution (Sigma) inoculated with Ad41, MNV or HAV at a final concentration of 10^4 log Most Probable Number (MPN) units/mL or log PFU/mL. After 5 d in virus inoculated hydroponic solution, green onions were removed from the solution. To ensure that viruses were not present externally, green onions were cut at the bottom of the bulb above where the hydroponic solution touched before processing treatments (approximately 5 cm). To inoculate the surface of green onions, plants were removed from soil substrate and washed in tap water to remove excess soil substrate. To be consistent with the green onions inoculated via internalization, green onions were cut about 5 cm above the roots before surface inoculation and allowed to dry for no longer than 30 min. Green onions were spot inoculated by distributing many small droplets of virus over the surface. The final concentration of Ad41, MNV and HAV on the green onion surface was 10^5 log MPN units, 10^6 log PFU and 10^6 log MPN units, respectively. Due to the size limitations of these small scale processing methods, green onions were cut into 2.5 cm pieces before treatments and each piece was inoculated and treated individually. Green onions were then treated by chlorine, UV, ozone and pressure. Treatments on virus inoculated Hanks' Balanced Salt Solution (HBSS) served as positive controls to assess the efficacy of these processes on the viruses. Uninoculated green onions served as negative controls.

2.1.3. Chlorine spray

Green onions were sprayed with 150 ppm calcium hypochlorite kept at 4 °C and pH 7.5 to mimic production practices (Suslow, 2000). Green onions were sprayed with calcium hypochlorite for 20 s on each side (total 40 s of spraying) and allowed to sit in the sprayed chlorine solution on the plant for 2 min before chlorine was quenched by stomaching green onions in 8 mL of PBS with 2 mL of 5% sodium thiosulfate for 5 min.

2.1.4. UV treatment

UV treatment of green onions was performed as previously described by Fino and Kniel (2008). UV radiation was generated using a low pressure G36T6 model 4136 germicidal light unit, which emits UV light at 253.7 nm (Fuller Ultraviolet, Frankfort, Ill.) contained inside an enclosed chamber approximately 1 m in length. The interior was fully covered with a highly reflective material (Solar Bright, Fuller Ultraviolet) that increased the UV light intensity and minimized the shadowing effect of irregularly shaped samples. The intensity of the light was measured by a UV meter calibrated to read specifically at 253.7 nm (Spectronics, Westbury, N.Y.), placed in a defined spot and at the same distance that samples would be exposed. The light was suspended in the chamber directly over the sample, and the intensity was changed by modifying the distance between the light and the sample. During treatment the chamber was fully closed. Inoculated green onions were placed in the center of a sterile Pyrex glass petri dish. Green onions were treated by UV at 240 mW s/cm^2. Inoculated green onions placed in petri dishes without UV treatment (0 mW s/cm^2) served as the controls.

2.1.5. Ozone treatment

Ozone treatment of the green onions was performed as described by Hirneisen et al. (2011). Green onions in sterile water were treated with bubbling gaseous ozone from an ozone generator (Golden Buffalo, Orange, CA) designed to produce 0.9 g of ozone/h at a flow rate of 2.4 L/min (6.25 ppm). The ozone gas was delivered through nonreactive plastic tubing into a flask containing green onions in 45 mL ddH_2O on a magnetic stirrer. Ozone amount and residual were monitored (HACH Company, Loveland, CO). Samples were ozonated for 10 min during which the contents of the beaker were stirred to ensure dispersal of the ozone. Ozone was produced and delivered throughout the duration of the treatment times. At the end of each treatment time, the residual ozone was quenched with 2 mL of 5% sodium thiosulfate. Inoculated green onions placed in 45 mL of sterile water without applied ozone (0 min) served as the controls.