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Human Enteric Pathogen Internalization by Root Uptake into Food Crops

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Abstract

With an increasing number of outbreaks and illnesses associated with produce contaminated before harvest, understanding the potential and mechanisms of produce contamination by enteric pathogens can aid in the development of preventative and post-harvest processing measures to reduce microbial populations. Enteric pathogens localized at subsurface sites on leafy green plant tissue prevent their removal during washing and inactivation by sanitizers. Root uptake of enteric pathogens and subsequent internalization has been a large area of research with results varying due to differences in experimental design, systems tested, and pathogens and crops used. The potential for uptake of foodborne pathogen, both bacterial and viral, through roots into food crops is reviewed. Various factors shown to affect the ability of human pathogens to internalize include growth substrate (soil vs. hydroponic solution), plant developmental stage, pathogen genus and/or strain, inoculum level, and plant species and cultivar. Several mechanisms of internalization ("active" vs. "passive") of bacteria to plant roots have also been hypothesized.

Introduction

Outbreaks of foodborne illnesses have been increasingly linked to the consumption of fruits and vegetables. For a number of these produce-related outbreaks, the origin of contamination has been traced back to the farm; however, in many outbreaks, definitive identification of the mode of produce contamination remains unknown. Produce can become contaminated with viral or bacterial pathogens in the field through soil, feces, or water used for irrigation, through application of manure, biosolids, pesticides, and fertilizers, and through dust, insects, and animals, or during postharvest (harvesting equipment, transport containers, animals, insects, and dust) and by food handlers in food service establishments (Beuchat, 2002). Data from the Center for Science in the Public Interest database revealed that produce outbreaks accounted for 13% of foodborne outbreaks during 1990–2005 (deWaal and Bhuiya, 2007). The 2006 outbreak linked to *Escherichia coli* O157:H7–contaminated spinach that resulted in 205 confirmed cases and three deaths served as a catalyst for research efforts to ensure the safety of leafy greens (CDC, 2006). The response to these incidents has led to the adoption of new recommendations intended to improve produce safety (Commodity Specific Food Safety Guidelines for the Production and Harvest of Lettuce and Leafy Greens, 2010).

From these events emerged research efforts aimed to answer the question of pathogen internalization through root uptake of plants. Internalization as used in this review 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. Understanding pathogen interactions with different produce commodities in the growing environment is useful to minimize risk of contamination, develop processes for pathogen reduction in pre- and post-harvest environments, and to prioritize research initiatives to investigate these issues that most greatly impact produce safety. The ability of plants to internalize foodborne pathogens, including both bacteria and viruses, through intact roots during growth is a topic that has received much debate over the last few years (Solomon and Sharma, 2009) and therefore will be a focus of this review.

Internalization into Crops by Pathogenic Bacteria

Many studies have assessed the ability of crops to internalize human bacterial pathogens through root uptake. These studies have employed different experimental approaches that may account for the large variation in results (Tables 1 and 2).

Effect of plant growth substrate

In internalization studies conducted with bacterial pathogens and produce crops, two types of growth substrate, soil and hydroponic solution, were used. Bacterial internalization

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Table 1. Enteric Bacteria Internalization into Leafy Greens

Plant	*Pathogen*	*Growth medium*	*Inoculationm method*	*Plant age*	*Results: internalization*	*Reference*
Leafy greens (spinach, lettuce, parsley)	*Escherichia coli* O517:H7 (*stx* negative)	Field-grown	Drip irrigation or compost (2, 4, or 6 log CFU/mL)	Transplanted to field 8 weeks, inoculated 1, 8, and 10 weeks after transplantation	No internalization was detected in any leaf tissues; detection in root occurred at one sampling time.	Erikson *et al.*, 2010
Lettuce (romaine and iceberg)	*E. coli* O157:H7	Soil	10^4 and 10^6 CFU/g	Seedlings with 4–5 leaves were transplanted and inoculated 30 days after transplanting.	Heat and drought stress applied individually or in combination did not promote internalization.	Zhang *et al.*, 2009a
Lettuce (iceberg, romaine, leaf)	*E. coli* O157:H7 (5 strains)	Soil	Inoculated 3 and 6 log CFU/mL by manure, soil, and water	Inoculated when 3–4 leaves present, analyzed on days 26 and 60 post-inoculation	All samples were negative for bacterial at all inoculums, routes of inoculation, and times post-inoculation.	Zhang *et al.*, 2009b
Lettuce	*Salmonella* Newport	Soil	10^6 and 10^8 CFU/g	Inoculated 20 days, analysis on days 2 and 7 post-inoculation	No *Salmonella* was present in the aerial parts of the plant tissues.	Bernstein *et al.*, 2007a
Lettuce	*E. coli* O157:H7, *Salmonella* Typhimurium	Hydroponic Hoagland's Solution (10%)	7 log CFU/mL	8-day seedlings inoculated, harvested after 18 days	No *E. coli* O157:H7 in surface sterilized roots; *Salmonella* Typhimurium detected in surface sterilized root but not shoots	Franz *et al.*, 2007
		Soil	9 log CFU/mL	Inoculated 14 and 18 days, plants harvested on 35 days	*E. coli* O157:H7 showed the highest internalization prevalence; plants with internal contamination weighed less than plants without internalized pathogens.	
Lettuce (crisphead)	*E. coli* O517:H7	Soil	Contaminated manure (10^4 CFU/g) added to soil	Inoculated 3-week-old seedlings, analyzed	No internalization	Johannessen *et al.*, 2005
Lettuce	Fluorescent microspheres, *E. coli* O157:H7	Soil	10^9 particles or CFU	Mature lettuce plants were inoculated and harvested 1, 3, and 5 days post-inoculation.	Fluorescent microspheres and *E. coli* O157:H7 were detected on all days sampled in internal tissues.	Solomon and Matthews, 2005
Lettuce (green ice)	*E. coli* O157:H7	Manure amended soil	Inoculated manure with 8, 6, and 4 log CFU/g and added to lettuce flats	Days 3, 6, and 9 post-planting seedlings were cut 1 cm above the soil surface.	*E. coli* O157:H7 was visualized at depths of up to 45 μm below the tissue surface; edible portions can become contaminated through transport by the root system.	Solomon and Matthews, 2002
Spinach	*E. coli* O157:H7	Soil (drench)	6 log CFU/mL	Inoculated at 4-leaf stage, analysis on days 0, 7, and 14	No internalization into leaf observed by direct plating and qPCR; bacterial presence on roots observed by confocal laser microscopy on day 7 and 14	Mitra *et al.*, 2009
Spinach	*E. coli* O157:H7	Hydroponic solution	Low (5 logs) or high (8.5 logs)	Plants (12-day) inoculated, allowed to grow for 21 days	*E. coli* O157:H7 was recovered from shoot tissue from 3 replicates on days 14 and 21.	Sharma *et al.*, 2009
		Pasteurized soil		Plants (7-day) transferred to inoculated pasteurized soil (125 g)	Microscopic analysis showed *E. coli* cells in root hairs after 7 days in soils with high population; no cells observed on day 21 or 28	
Spinach	*E. coli* P36	Hydroponic solution	10^7 CFU/mL, 10^2–10^3 CFU/mL	Inoculated seeds and cultivated for 35 days; inoculated 13 days in hydroponic system	Seed inoculum: *E. coli* presence on root exterior (7 log) and interior (4 log) Hydroponic inoculum: *E. coli* presence on roots	Warriner *et al.*, 2003a
Spinach	*E. coli O157:H7* Ph1 (bioluminescent)	Soil microcosms	10^6 CFU/mL	Spinach roots were damaged either by mechanical (knife) or biological (*P. syringae* DC3000) disruption.	Undamaged plants: E. coli internalized into roots, but not leaves No internalization into root or leaves after biological or mechanical disruption of spinach roots	Hora *et al.*, 2005
Cabbage	*E. coli*	Sewage spill into the water source used for irrigation	1.7 million gallons of unchlorinated filtered sewage was released into a creek used for irrigation.	A field containing 15 acres of cabbage was irrigated on the day the contaminated water flowed past the field; cabbage samples collected 7 days post- irrigation	$^3/_4$ plots had *E. coli* roots positive Unable to isolate *E. coli* from leaves corresponding to the positive root samples or stagnant water samples Six different serotypes of nonpathogenic *E. coli* associated with the cabbage roots	Wachtel *et al.*, 2002b
Cress, radish, spinach, lettuce, and mustard	*E. coli* O157:H7, *Salmonella* Typhimurium, *Listeria monocytogenes*	Hydroponic solution	Seeds were soaked in bacterial cell suspension (2 log CFU/mL)	Plants surface sterilized, seedlings analyzed on day 9	*E. coli* O157:H7 was recovered from external and internal tissues of all plants. *Salmonella* internalized only into radish and lettuce seedlings. *Listeria* was not recovered from surface-sterilized samples	Jablasone *et al.*, 2005
Green ice leaf lettuce	*E. coli* O157:H7	Soil	Manure-amended soil or irrigation water (1, 2, 3, 4 log CFU/g)	Young plants (12-day) and old plants (30-day) were analyzed at 1, 10, 20, and 30 days post-exposure.	7% of all 12-day-old surface-sterilized plants were positive; 11% of 30-day-old surface-sterilized plants were positive at 15 days post-inoculation.	Mootian *et al.*, 2009

CFU, colony-forming units.

Table 2. Enteric Bacterial Internalization into Non-leafy Green Produce

Plant	*Pathogen*	*Growth medium*	*Inoculation method*	*Plant age*	*Results: internalization*	*Reference*
Tomato	*Salmonella* Montevideo	Pine bark	Irrigation water (350 mL of 10^7 CFU/mL) every 14 days and seed stock (10^7 CFU/mL)	Analyzed on day 70	No *Salmonella* was detected in stem scar, fruit pulp, leaves, or stems; a few root samples were positive.	Miles *et al.*, 2009
Tomato	*Salmonella* (Montevideo, Michigan, Poona, Hardford, Enteridis)	Hydroponic (Hoagland's solution)	Hydroponic solution inoculated with 4.5 log CFU/mL	Inoculated on day 7, analyzed on day 16	Detected in hypocotyls and cotyledons; *Salmonella* Montevideo and *Salmonella* Michigan were dominant serovars	Guo *et al.*, 2002
Maize	*Escherichia coli*	Hydroponic solution	7 log CFU/mL	Inoculated on day 15	Positive internalization, *more E. coli* present in plants with decapitated roots, no internalization into soil grown maize	Bernstein *et al.*, 2007b
Barley	*Salmonella* Typhimurium, *Listeria (L. monocytogenes, L. ivanoii,* and *L. innocua)*	Hydroponic (Hoagland's) solution	8 log CFU	Grown for 2 weeks, roots and shoots harvested and analyzed by enumeration, FISH, and PCR	*Salmonella* was internalized in roots (5–6 log CFU/g) and in shoots after 4 weeks; no endophytic colonization could be detected for *Listeria* species	Kutter *et al.*, 2006
Alfalfa seeds, *Medicago truncatula*	*Salmonella* Cubana, Infantis, Typhimurium *E. coli* K12 and *E. coli* O157:H7 *Klebsiella pneumonia*	Hydroponic solution	7, 6, 5, 4, 3, 2, 1, 0 CFU/ seedlings	Plants harvested 5 and 7 days after inoculation	*Salmonella* Cubana had similar colonization behavior as *Klebsiella*, but only at the 4 highest inoculations *E. coli* O157:H7, at high inoculum levels, colonized the interior regions at higher levels than K12. 4 lowest levels of inoculation, *Klebsiella* colonized the interior of alfalfa in the highest number	Dong *et al.*, 2003
Green ice leaf lettuce	*E. coli* O157:H7	Soil	Manure-amended soil or irrigation water (1, 2, 3, 4 log CFU/g)	Young plants (12-day) and old plants (30-day) analyzed at 1, 10, 20, and 30 days post-exposure	7% of all 12-day-old surface-sterilized plants were positive; 11% of 30-day-old surface-sterilized plants were positive at 15 days post-inoculation.	Mootian *et al.*, 2009
Arabidopsis thaliana	*E. coli* O157:H7 *Salmonella* Newport	Hydroponic solution, soil, and autoclaved soil	Pipetted bacterial suspension directly on roots or suspended seeds in bacterial suspension	6–7 days post-germination and seeds	GFP-labeled bacteria were observed within the root, but not in the vasculature.	Cooley *et al.*, 2003
Peanut	*Salmonella* Typhimurium, GFP	Hydroponic solution	Seed soaked in 10^7 CFU/ mL	Seedlings were analyzed, fixed for immunohistochemistry	*Salmonella* was detected in every major tissues (cortical, vascular, epidemermal, and pith).	Deering *et al.*, 2012

CFU, colony-forming units; GFP, green fluorescent protein; FISH, fluorescent *in situ* hybridization; PCR, polymerase chain reaction.

into plant tissues through the roots was generally observed in studies where plants were grown in inoculated hydroponic solution (Bernstein *et al.*, 2007b; Dong *et al.*, 2003; Franz *et al.*, 2007; Guo *et al.*, 2002; Jablasone *et al.*, 2005; Kutter *et al.*, 2006; Sharma *et al.*, 2009; Warriner *et al.*, 2003a) as opposed to inoculated soil, in which little or no internalization was observed (Bernstein *et al.*, 2007a,b; Erikson *et al.*, 2010; Johannessen *et al.*, 2005; Miles *et al.*, 2009; Mitra *et al.*, 2009; Sharma *et al.*, 2009; Zhang *et al.*, 2009a,b). Some investigators have suggested that the motility of *E. coli* O157:H7 in hydroponic solution may provide more opportunity for uptake and internalization into leafy green plants than in soil (Sharma *et al.*, 2009). However, exceptions to this trend have been noted (Franz *et al.*, 2007; Mootian *et al.*, 2009; Solomon *et al.*, 2002). For example, Solomon and Matthews (2005) microscopically observed *E. coli* O157:H7 below the tissue surface in edible portions of the green ice lettuce when grown in manure-amended soil inoculated with *E. coli* O157:H7. A portion (7%) of green ice leaf lettuce plants was positive for *E. coli* O157:H7 when grown in contaminated soil (Mootian *et al.*, 2009). Franz *et al.* (2007) proposed that the difference in internalized bacterial populations from plants grown in contaminated soil versus hydroponic solution was due to root damage sustained from growth in soil which allowed for greater internalization of *E. coli* O157:H7 into the roots.

Sterilized or pasteurized soil has also been used to assess the potential of bacterial internalization into crops. Cooley *et al.* (2003) compared autoclaved and non-autoclaved soil on *Salmonella* Newport and *E. coli* O157:H7 survival and internalization in *Arabidopsis thaliana*. These authors believed that the endogenous bacteria and *Enterobacter absuriae* present in soil colonized root tissue surfaces more efficiently than *Salmonella* Newport or *E. coli* O157:H7, resulting in the suppression of growth of these pathogens. The effect of the soil matrix on bacterial internalization and survival was also investigated by Sharma *et al.* (2009). *E. coli* O157:H7 was unable to translocate into root or shoot vascular tissue of spinach plants when inoculated into pasteurized soils (Sharma *et al.*, 2009). The reduction of Gram-negative microorganisms present in soil through pasteurization did not enhance the ability of *E. coli* O157:H7 or non-pathogenic *E. coli* to internalize to spinach tissues. The authors also observed that soil placed stress *on E. coli* populations, as low initial populations of approximately 3.2–4 log CFU/mL in the hydroponic solution grew by 1–2 log CFU/mL between days 0 and 7; however, in pasteurized soils, low populations (3.9–4.4 log CFU/g) *of E. coli* declined to less than 1 log CFU/g by day 21. High populations of *E. coli* (7.6–8.0 log CFU/g) declined to 2.6–4.5 log CFU/g soil by day 28, indicating the physiological stress placed on *E. coli* cells in soil. It was hypothesized that the stress placed on *E. coli* O157:H7 cells in soils, along with their limited motility in soil, may have prevented the ability of these cells to internalize into spinach root tissues. Total carbon levels have also been shown to affect the survival of *E. coli* O157:H7 in soils as well, imparting various levels of physiological stress and potentially affecting the organism's ability to internalize to root tissues (Vidovic *et al.*, 2007).

Effect of environmental stress

Several internalization studies focused on the effects of extreme environmental conditions and plant damage on root uptake of pathogenic bacteria in food crops. Zhang *et al.* (2009a) tested if high temperatures and drought conditions during growth of romaine and iceberg lettuce affected the root uptake of *E. coli* O157:H7. Romaine and iceberg lettuce plants grown in inoculated soil were heat stressed under two conditions: either 2 days of 36°C during the day (12 h) and 15°C at night (12 h), or 3 days of 32°C during the day (12 h) and 15°C at night (12 h). Two watering conditions were also tested: a moist treatment in which lettuce was watered daily during the heat treatment and a drought treatment whereby lettuce was not watered during the heat treatments. Analysis of plant and soil samples suggested that heat and drought stress did not impact the susceptibility of plants to be contaminated through uptake by *E. coli* O157:H7. *E. coli* O157:H7 survived for longer durations in moist (unstressed) soils compared to in heat-stressed soils.

The wounding of the root tips from soil may also place stress on plants and affect bacterial uptake through roots. Wounding has the potential to expose vascular tissue to pathogenic bacteria. After root decapitation, internalization of *Salmonella* Newport in lettuce leaves occurred more in plants with damaged roots (Bernstein *et al.*, 2007a). When root decapitation was performed on maize grown in *E. coli* O157:H7-contaminated hydroponic solution, internalization of the bacteria occurred (Bernstein *et al.*, 2007b). Other investigators have postulated that root damage to lettuce plants was one potential factor where larger numbers of *E. coli* O157:H7 cells were internalized in leaves when plants were grown in soil (more root damage) than in hydroponic media (less root damage) (Franz *et al.*, 2007). Although root damage can potentially increase the likelihood of internalization of *E. coli* in maize and lettuce, several studies examining spinach plants minimize the role that mechanical damage of roots can have on the frequency of internalized cells. In spinach plants, mechanical damage of root tissues did not increase the likelihood of internalization through uptake (Hora *et al.*, 2005). In spinach grown in hydroponic media, Sharma *et al.* (2009) observed higher levels of internalization of *E. coli* O157:H7 in roots of spinach plants grown hydroponically than in those grown in soil. In these experiments, no internalized cells were recovered from leaf tissues. Root tissue is more likely to be less damaged when plants are grown in hydroponic growth substrate than in soil; the authors hypothesize that increased motility of *E. coli* O157:H7 cells may have led to a more intimate attachment to root tissue, permitting more frequent internalization of *E. coli* O157:H7 in spinach plants grown hydroponically compared to those grown in soil. The results of these studies indicate that root damage does not universally promote the increased frequency of uptake and internalization of pathogenic bacterial cells; uptake and internalization of enteric bacterial pathogens cannot be generalized, and there are likely specific pathogen–plant interactions.

Effect of pathogen strain and serovar

Similar to the effect of the growth substrate, pathogen strain or serovar was shown to play a large role in internalization of the pathogen. Internalization was shown to be dependent on *Salmonella* serovar in one study. Guo *et al.* (2002) inoculated hydroponic growth substrate with a five-serovar *Salmonella* cocktail (Motevideo, Michigan, Poona, Hartford,

and Enteriditis); *Salmonella* was detected in the hypocotyls, cotyledons, stems, and leaves of tomato plants. Polymerase chain reaction (PCR) analysis of recovered *Salmonella* from tomato plant tissues revealed that serotypes Montevideo and Michigan were most prevalent and that Enteritidis, Hartford, and Poona were not detected in any tomato tissue samples (Guo *et al.*, 2002). Similarly, *Salmonella* serovars Cubana, Infantis, and Typhimurium showed different abilities to internalize and colonize alfalfa sprouts when seeds were inoculated under the same environmental conditions (Dong *et al.*, 2003). In this study, only *Salmonella* Cubana was able to match the level of colonization of the plant pathogen *Klebsiella pneumoniae* to the interior of alfalfa roots and hypocotyls (Dong *et al.*, 2003).

Listeria spp. were not able to internalize plant tissues (Jablasone *et al.*, 2005; Kutter *et al.*, 2006). Jablasone *et al.* (2005) compared the internalization of *L. monocytogenes* to *Salmonella* Typhimurium on inoculated seeds of cress, radish, spinach, lettuce, mustard, carrots, and tomatoes. Under the same experimental conditions, *Salmonella* Typhimurium internalized into the roots, whereas *L. monocytogenes* did not. In another study, *L. monocytogenes, L. ivanovii,* and *L. innocua* were not found to be associated with the internal portions of barley sprouts by fluorescent *in situ* hybridization, whereas *Salmonella* Typhimurium was (Kutter *et al.*, 2006). Both *Salmonella* and *Listeria* were detected on the external root surfaces (main roots, side roots, and root hairs).

When species were compared, *E. coli* O157:H7 showed the highest probability of internalization and contamination compared to *Salmonella* Typhimurium for both surface sterilized and non-surface sterilized shoots in soil grown lettuce plants (Franz *et al.*, 2007). Conversely, Dong *et al.* (2003) found that when comparing *Salmonella* serovars, *E. coli* O157:H7 and *E. coli* K12 to the plant pathogen *Klebsiella pneumoniae, S.* Cubana had the highest internalized populations of the human pathogens. In this same study by Dong *et al.* (2003), when comparing *E. coli* O157:H7 to *E. coli* K12, *E. coli* O157:H7 colonized the interior of sprouts in higher numbers compared to *E. coli* K12.

Effect of inoculum level

Internalization studies varied greatly with regard to the population of bacteria inoculated into the growing substrate. It would be expected that higher inoculum levels would result in higher levels of internalization; however, experimental results do not fully support this hypothesis. For example, inoculation of tomatoes with irrigation water contaminated with *S.* Montevideo at a population of 7 log CFU/mL every 14 d for 70 days resulted in no detected internalization in the stem scar, fruit pulp, or leaves or stems of the plant. In this study five of 24 roots samples were positive for *S.* Montevideo (Miles *et al.*, 2009). A possible explanation for the lack of internalization into the edible portions of the tomato plant despite extremely high levels of contamination could be due to the use of non-hydroponicpine bark growth substrate, potentially decreasing the potential for pathogen root uptake. The use of pine bark may not have provided an opportunity for bacterial cells to form intimate attachments to root tissue (as hydroponic growth substrates do), minimizing opportunities for internalization through root uptake. The use of pine bark as a growth substrate may have physiologically stressed bacterial cells (not providing available nutrients for bacterial growth), again negatively affecting their ability to internalize to root tissue. Similar results were also observed by Sharma *et al.* (2009) whereby limited internalization (only detectable by enrichment) in spinach plants was observed despite continuous exposure to high populations (approximately 7 log CFU/mL) of *E. coli* O157:H7 in inoculated pasteurized soil. The physiological stress of soil on the inoculated *E. coli* and conditions limiting bacterial motility may have affected the internalization potential. In hydroponic solution, internalized populations (up to 4 log CFU) were detected after 14 days without enrichment. However, it was also observed that high levels of inoculation (8-9 log CFU) resulted in bacteria internalized into plant tissues (Solomon and Matthews, 2005; Solomon *et al.*, 2002).

Several investigators reported that higher bacterial inocula lead to more uptake and internalization, and lower inocula results in little to no internalization. Mootian *et al.* (2009) inoculated *E. coli* O157:H7 at low levels (1, 2, 3 and 4 log CFU/g) in manure amended soil and irrigation water to determine whether exposure of lettuce plants to these low populations would result in detectable levels of the pathogen. Detection of internalized *E. coli* O157:H7 only occurred after sample enrichment, indicating that the level of contamination of lettuce tissues was extremely low; lettuce plants exposed to 10^1 CFU/g of *E. coli* O157:H7 were detected as positive (Mootian *et al.*, 2009). In a study evaluating the effect of *Salmonella* inoculum level and motility on the potential for uptake and internalization in *Arabidoposis thaliana* plants, low inoculum levels (10^4 CFU/mL) of *Salmonella* Typhimurium strains defective in flagellin synthesis or motility functions were able to colonize the root surface but did not invade the lateral root junctions (Cooley *et al.*, 2003). When the inoculum level was increased to 10^6 CFU/mL, these flagellin-deficient *Salmonella* strains were able to internalize into the root. Cooley *et al.* (2003) suggested that as the inoculum level of the bacteria increases, the probability increases that the bacteria are translocated to more sites on root tissue and more likely to be internalized.

Effect of plant type, age, and exposure time

In bacterial internalization studies, the many inherent variables and those under study make it difficult to assess specifically the role of plant type. Jablasone *et al.* (2005) inoculated seeds of cress, radish, spinach, lettuce, mustard, carrots and tomatoes with *E. coli* O157:H7 and *Salmonella* Typhimurium. *E. coli* O157:H7 was found to internalize into all plant types, whereas *Salmonella* Typhimurium was internalized into radish and lettuce seedlings. The authors suggested that the antimicrobial constituents of root vegetables may be responsible for limited bacterial growth and internalization of human pathogenic bacteria.

Differences in the developmental stages of plant roots systems as they mature may influence the ability of enteric microbes to interact with, enter plant roots and travel to the leaf tissue (Mootian *et al.*, 2009). Both plant age at the inoculation/contamination event and extent of time of exposure play a role in possible internalization of the bacterial pathogen. Leaf age has been shown to influence the growth and survival of both *Salmonella* and *E. coli* O157:H7 communities with young lettuce leaves being associated with a greater risk of contamination (Brandl and Amundson, 2008). Jablasone

et al. (2005) soaked seeds in a bacterial suspension of *E. coli* O157:H7 and *Salmonella* Typhimurium and recovered bacteria from internal tissues nine days after growth. When seeds were inoculated with *E. coli* P36 and young spinach plants were harvested for analysis 35 days later, approximately 4 log of *E. coli* was detected in the interior of roots (Warriner *et al.*, 2003a). However, in this study when 13-day-old seedlings were transferred to a hydroponic system inoculated with *E. coli* P36, colonization of spinach was limited to the exterior of the roots. Warriner *et al.* (2003a) reasoned that bacterial growth on the seeds was likely sustained by the exudates released during germination, enabling *E. coli* to colonize both the exterior and the interior of the seedling. Another study indicated that internalized *E. coli* O157:H7 bacterial cells were observed by epifluorescent microscopy in surface-sterilized root tissue of younger spinach plants, seven days after inoculation, compared to older spinach plants 14 days after inoculation (Sharma *et al.*, 2009). Contrary to these studies, other investigators have shown older plants to uptake human enteric bacteria more than younger plants. Bernstein *et al.* (2007a) compared the ability of *Salmonella* Newport to contaminate the above-ground tissues of 17- and 33-day-old lettuce plants. In this study, both 17- and 33-day-old lettuce plants were inoculated with *Salmonella* and analyzed for internalization two and seven days post-inoculation. No contamination was found in the 17-day old plants, but *Salmonella* was recovered from internal tissues of the 33-day-old plants, two days after inoculation. *Salmonella* Newport was also not able to internalize into 20-day old plants, and Bernstein *et al.*, (2007a) suggested that plant age affected bacterial entry through plant roots. Similarly, Mootain *et al.* (2009) observed a greater percentage of 30-day-old green ice leaf lettuce plants (11%) containing internalized *E. coli* O157:H7 compared to 7% of 12-day-old plants with internalized *E. coli* O157:H7.

Another factor affecting the detection of internalized pathogenic bacteria in crops is the ability for bacterial cell survival in the soil or in aerial tissues of the plant over a period of time. Mootian *et al.* (2009) concluded this was likely the case where at the end of the cultivation period (42 days), only two of the 42 non-surface sterilized maize samples tested positive by enrichment for *E. coli* O157:H7 and no bacteria were recovered from surface sterilized leaf tissue. In a study where soil was inoculated by water contaminated with *E. coli* O157:H7, bacteria were visible by confocal microscopy on the rhizosphere of one spinach plant on day seven; by day 14, *E. coli* O157:H7 was visible on the root surface of 4 out of 9 plants (Mitra *et al.*, 2009). While, there is a greater association of the bacteria with the spinach roots in the older plants, these results do not show uptake of the bacteria into the plant tissue.

Localization of bacteria within plant tissues

The location of internalized bacterial pathogens is of interest to determine if the bacteria are able to internalize not only into the root tissues, but also into the edible portions of the plant. The presence of bacterial human pathogens in the aerial parts of plants is dependent upon several processes including root internalization, short and long distance transport in the plant and survival or multiplication within the plant tissue (Bernstein *et al.*, 2007b). In some studies, bacteria were found to be present in surface-sterilized roots but not detected in the leaves, indicating that bacteria were able to penetrate the root tissue of plants grown in soil or hydroponic systems, but could not translocate to edible portions of the plants (Bernstein *et al.*, 2007a; Franz *et al.*, 2007; Mitra *et al.*, 2009; Sharma *et al.*, 2009; Wachtel *et al.*, 2002a; Warriner *et al.*, 2003a). Other studies have observed the presence of bacterial pathogens internalized into edible leaf portions (Solomon and Matthews, 2005; Solomon et al, 2002). Cooley *et al.* (2003) inoculated a hydroponic solution with green fluorescent protein (GFP)–labeled *Salmonella* Newport and *E. coli* O157:H7 and observed the interaction of these pathogens with *Arabidopsis thaliana* sprouts microscopically. Inoculated bacteria on the roots were concentrated initially at the root tips and the branch point, possibly due to the availability of nutrients at those locations. Inoculated bacteria were rarely able to penetrate into the vasculature, and bacteria were not observed to be moving systemically within the plant (Cooley *et al.*, 2003). Contradictory to the Cooley *et al.* (2003) study, Kutter *et al.* (2006) observed the colonization of barley roots by *Salmonella* Typhimurium by fluorescent *in situ* hybridization (FISH). *Salmonella* was detected in the intracellular spaces of the root cortex, and its dissemination through the entire plant was detected by PCR. Kutter *et al.* (2006) suggested that this systemic spread could have occurred through the water transport in the vascular system once the bacteria were able to colonize these tissues. Similarly, *Salmonella* Typhimurium was observed to be associated with every major tissue (cortical, vascular, epidermal, and pith) of peanut plants when seeds were inoculated with 10^7 CFU/mL (Deering *et al.*, 2012).

Mechanism of internalization

Controversy exists as to whether bacterial internalization via root uptake is a passive or active event by either the plant or pathogen. Several studies have shown pathogen specific interactions with crops. Additionally, cell surface moieties affect internalization, as *Salmonella* Typhimurium strains defective in flagellin synthesis or motility functions were able to grow on the surface of the root, but were unable to invade the root (Cooley *et al.*, 2003). The authors predicted that the bacteria used their flagella to position themselves near the developing lateral root, increasing their potential for internalization. This suggests that internalization into plant routes is an active process dependent upon the plant and pathogen. On the other hand, Solomon and Matthews (2005) determined that bacterial factors (surface moieties, appendages, and adaptive responses) were not necessary for root uptake, as both microspheres and bacterial cells were able to internalize into the root and leaves of lettuce. Lettuce plants were inoculated with fluorescent microspheres and *E. coli* O157:H7 for comparison, because they are similar in size (1 μm in diameter). Solomon and Matthews (2005) concluded that *E. coli* O157:H7 entry into plant tissues was mediated by the plant rather than by specific bacterial factors and supports the passive mechanism of internalization.

Inoculation of *Arabidopsis thaliana* with GFP-labeled *S. enterica* and *E. coli* O157:H7 showed invasion of the roots at the lateral root junctions (Cooley *et al.*, 2003). These GFP-labeled bacteria were found within the primary root but not in the vasculature. Oppositely, Itoh *et al.* (1998) observed *E. coli* O157:H7 in and on the xylem elements of radish sprouts when seeds were contaminated. Scanning confocal laser microscopy images of alfalfa roots showed extensive colonization of

enteric bacteria on cracks in the lateral roots (Dong *et al.*, 2003), suggesting that bacteria may enter the plant through these cracks. The results of these studies indicate that bacterial surface moieties can influence the ability of the pathogen to interact with plant roots, but specific bacterial mechanisms that may promote internalization remain ill-defined.

Internalization of Viral Pathogens into Crops

While most research efforts have focused on the possibility of internalization of human pathogenic bacteria into crops, human enteric viruses also pose a threat to produce safety. From 1973 to 2006, 60% of U.S. foodborne outbreaks associated with the consumption of leafy greens were caused by noroviruses, while *Salmonella* and *E. coli* each only accounted for 10% of outbreaks (Herman *et al.*, 2008). While many outbreaks caused by foodborne viruses involve foods contaminated by food handlers, several outbreaks have been associated with environmental contamination of fresh produce (CDC, 1971; Dentinger *et al.*, 2001; Rosenblum *et al.*, 1990; Wheeler *et al.*, 2005). One of the largest outbreaks of hepatitis A virus (HAV) in the United States was linked to consumption of green onions contaminated with HAV imported from Mexico in 2003, leading to over 1000 illnesses and four deaths (Wheeler *et al.*, 2005). The exact means of contaminated green onions implicated in the outbreak is unknown, but the green onions were believed to have been grown in conditions in which the crops were contaminated through farm workers or water used for irrigation, rinsing, processing, coolin, and icing (Wheeler *et al.*, 2005).

Intrinsic differences exist between enteric viruses and bacteria, including size and surface structure. Viral persistence and motility in soil and hydroponic solution will affect their ability to internalize to root systems, and may vary from the results observed with bacteria. Limited research has been conducted on the ability of enteric viral pathogens to internalize into plant tissue via root uptake. Methods and results of these studies are summarized in Table 3. Similar to studies with human pathogenic bacteria, growth substrate played a large role in the ability of viruses to internalize through root uptake. Studies using hydroponic solution as the growth substrate showed greater viral uptake than plants grown in soil (Chancellor *et al.*, 2006; Hirneisen *et al.*, 2009; Hirneisen and Kniel, 2010; Oron *et al.*, 1995; Urbanucci *et al.*, 2009; Ward and Mahler, 1982; Wei *et al.*, 2011). These studies also assessed other factors that have the potential to influence root uptake of enteric viruses into plant tissues, including severed tips, water quality, and the common use of non-pathogenic surrogates because human noroviruses are noncultivable.

In a study by Ward and Mahler (1982), severing the roots at the midpoint of corn and bean plants grown in hydroponic systems resulted in a significantly greater internalization of bacteriophage f2 (10^6 PFU/g of plant tissue) as compared to intact roots (10^2 PFU/g of plant tissue). A gradient effect in the distribution of phage within the bean plant was observed, with more phage particles present in the stem than in the leaves, suggesting the interior tissues of the plant act as molecular sieves and permit only a portion of the phage to continue to the next barrier (Ward and Mahler, 1982). Water quality was observed to affect poliovirus internalization into tomato plants irrigated with subsurface irrigation systems. Poliovirus was not detected in either the leaves or tomato fruit when plants were inoculated with poliovirus contaminated

Table 3. Enteric Virus Internalization into Food Crops

Virus	*Crop*	*Growth conditions*	*Results*	*Reference*
Bacteriophage f2	Bean and corn	Hydroponic solution inoculated with 10^{10} PFU/mL phage f2	Maximal phage concentrations of 106 PFU/g in plants with cut roots	Ward and Mahler, 1982
Poliovirus	Tomato	Inoculated via subsurface irrigation in soil	Virus not detected in either leaves or tomatoes, wastewater; positive internalization with tap water	Oron *et al.*, 1995
HAV/ fluorescent microspheres	Green onion	Soil Hydroponic solution	No internalization HAV vaccine and microspheres internalized	Chancellor *et al.*, 2005
CaCV, human NoV	Lettuce	Soil inoculated by contaminated water Hydroponic	CaCV detected in lettuce in a few samples, NoV not internalized CaCV internalized, NoV not internalized	Urbanucci *et al.*, 2009
Murine norovirus	Romaine lettuce	Hydroponic solution; high dose (10^8 RT-qPCRU/mL) one time and low dose (10^5 RT-qPCRU/mL) constant exposure; 70% and 99% humidity conditions	MNV levels in lettuce challenged with one high dose higher than with constant low dose exposure; 10-fold higher internalization at 70% humidity	Wei and Kniel, 2011
HAV/MNV	Green onion and spinach	Soil	HAV and MNV were not internalized with the exception of 1 spinach plant out of 10 sampled positive for internalized HAV.	Hirneisen *et al.*, 2009
		Hydroponic	Both HAV and MNV were detected in leaves, roots and stems of spinach, and green onions at titers up to 4 log RT-qPCR units/plant.	Hirneisen *et al.*, 2010

PFU, plaque-forming unit; CaCV, canine calcivirus; NoV, norovirus; HAV, hepatitis A virus; MNV, murine norovirus.

wastewater; however, poliovirus was detected in the leaves of plants irrigated with poliovirus-contaminated tap water (Oron *et al.*, 1995). The authors suggested that poliovirus was closely bound to organic matter in the wastewater and therefore unable to internalize into the plant.

The importance of virus type and use of surrogates in hydroponic internalization studies was also studied (Chancellor *et al.*, 2006; Urbanucci *et al.*, 2009). When both the HAV vaccine and fluorescent microspheres were internalized into green onions grown in hydroponic solution were assessed, the HAV vaccine was not detected in green onion, but fluorescent microspheres were (Chancellor *et al.*, 2005). One critique of this study was that fluorescent microspheres are not an appropriate surrogate for enteric viruses in internalization studies because of the size differential between the viruses (10—30 nm) and the microspheres (1—10 μm) (Rawsthorne *et al.*, 2009). The potential for internalization of human norovirus and canine calicivirus (CaCV) into lettuce plants grown in contaminated hydroponic cultures was assessed (Urbanucci *et al.*, 2009). CaCV was detected in lettuce leaves for all samples grown hydroponically and in four of 28 leaves when lettuce was grown in soil, whereas noroviruses (NoVs) were not detected in any lettuce tissue grown in either hydroponic or soil systems. Differences in the capacity of the two viruses to internalize were concluded to be due to the differences in surface properties (Urbanucci *et al.*, 2009). The difference in these results examining viral internalization illustrates the importance of using human pathogenic viruses in experiments.

Hirneisen and Kniel (2010) observed internalization of murine norovirus (MNV) and HAV into spinach and green onion plants when grown hydroponically but not in soil systems (Hirneisen *et al.*, 2009). MNV and HAV were detected by quantitative polymerase chain reaction (qPCR) in all portions of both green onion and spinach plants including edible portions at concentrations up to 10^4 real-time (RT)–qPCR/plant. The internalization and uptake of MNV, a surrogate for human NoV, into romaine lettuce was assessed in two irrigation water contamination systems: a one-time severe contamination event and a low constant contamination (Wei *et al.*, 2011). Lettuce grown in hydroponic systems challenged once at a high dose (10^8 RT-qPCRU/mL) had significantly higher levels of internalized virions compared to lettuce irrigated daily with 10^5 RT-qPCRU/mL MNV. Cell culture assays indicated that MNV internalized into lettuce leaves were still infectious. Transpiration is the driving force of water absorption by the plant from the growing substrate (Kramer *et al.*, 1983), and humidity is a major factor controlling plant transpiration, as high humidity will reduce the diffusion of water out of the leaf and slow the transpiration rate (Conger and Portier, 2001; Tanner and Beevers, 2001). Wei *et al.* (2011) assessed MNV internalization in romaine lettuce plants when grown under conditions of 70% and 99% humidity. Lettuce grown in 70% humidity resulted in a 10-fold higher transpiration rate and significantly greater internalization of MNV as compared to plants grown in 99% humidity. These results suggest that viruses may be taken up in a passive manner by transpiration (Wei *et al.*, 2011).

Conclusion

Increasing numbers of outbreaks involving environmental contamination of produce have resulted in questions about the attachment and interactions of pathogens on and in plants. This review primarily addresses pathogen uptake through roots because the potential internalization of human enteric pathogens into the vascular tissue from the root could protect the pathogen from any post-harvest processing treatments. From the current studies reviewed here, it is difficult to determine which conditions can promote the internalization of human foodborne pathogens by root uptake into crops because these studies vary extensively in experimental design, results, and produce production systems. This variability in design and results likely better represents the realities and risks of internalization after a contamination event during produce production. Taking into account these variations, several conclusions can be reached: (1) uptake through internalization is a plant–pathogen specific interaction; (2) the plant growth substrate used plays a large role in the uptake of both bacterial and viral pathogens in plants; (3) intact, healthy, noninjured roots seem to discourage the uptake of bacteria cells and viruses into plants; and (4) generally, the presence of internalized pathogens in roots of plants does not directly correlate with internalized pathogens in the edible or foliar tissues of crops. In addition, contaminated soil, for the most part, resulted in little to no observed internalization as compared to contaminated hydroponic solution. For those studies where internalization was observed in soil-grown crops, internalization was sporadic and at low levels. Generally, environmentally stressful plant growth conditions did not promote internalization. While these results vary, the risk of root uptake of pathogens into produce through the roots from contaminated soil is relatively low. Future internalization studies to be conducted with enteric pathogens should include realistic plant growth conditions, along with realistic pathogen contamination levels encountered in production systems.

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