Internalization of Fresh Produce by Foodborne Pathogens

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Abstract

Recent studies addressing the internalization of fresh produce by foodborne pathogens arose in response to the growing number of recent and high profile outbreaks involving fresh produce. Because chemical sanitizing agents used during harvest and minimal processing are unlikely to reach enteric pathogens residing within plant tissue, it is imperative that paths for pathogen entry be recognized and minimized. Using both microscopy and microbial enumeration tools, enteric pathogens have been shown to enter plant tissues through both natural apertures (stomata, lateral junctions of roots, flowers) and damaged (wounds, cut surfaces) tissue. In studies revealing preharvest internalization via plant roots or leaf stomata, experimental conditions have primarily involved exposure of plants to high pathogen concentrations ($\geq$6 log g^{-1} soil or 6 log ml^{-1} water), but those pathogens internalized appear to have short-term persistence. Postharvest internalization of pathogens via cut surfaces may be minimized by maintaining effective levels of sanitizing agents in waters during harvesting and minimal processing.

INTRODUCTION

During the past decade, many outbreaks of enteric pathogen infection have been associated with fresh produce. Contamination of fresh produce is an important public health concern because no pathogen "kill" step is applied during harvest or minimal processing. In addition, with produce contamination there is the potential for internalization of pathogens, and as such the bacteria would likely not be exposed to disinfectant washes. This review examines studies done on pathogen internalization of produce.

DEFINITION OF PATHOGEN INTERNALIZATION

Pathogen internalization has different definitions depending on the arena in which it is used. In biological sciences, it refers to the process by which one item traverses past the surface of a mass and is incorporated within that mass. In actuality, classification that an item is internalized is dependent on the methodology used to discriminate between surface and internalized locations. Consequently, there exists a wide assortment of locations in which internalized human enteric pathogens may reside within fresh produce, including the vasculature, intercellular tissues, and stomata or cracks of the cuticle, as well as entrapped within crevices.

METHODS TO DETECT PATHOGEN INTERNALIZATION

Several approaches have been used to determine that enteric pathogens have been internalized into plant tissue. One of the simplest approaches is to physically remove the surface layer of a plant and enumerate within the inner tissues the pathogens, as was applied to studies of apple tissue and green onions (Janes et al. 2005, Chancellor et al. 2006). Another common approach is to expose the plant to pathogens at one location, such as soil and roots, and assay for pathogens at a different part of the plant, such as leaves, making the assumption that their presence at that site would have occurred only through systemic transfer (Guo et al. 2002; Solomon & Matthews 2005; Bernstein et al. 2007a,b; Urbanucci et al. 2009; Habtesellassie et al. 2010). To ensure that inadvertent transfer of pathogens from the contamination source to the nonexposed plant parts does not occur in this latter approach, both plastic barriers and uninoculated soil have been used to prevent direct contact or aerial splash between soil and the leaves (Girardin et al. 2005, Solomon & Matthews 2005, Bernstein et al. 2007a, Erickson et al. 2010a). Despite this precautionary measure, there still exists the possibility that pathogens had moved from the soil to the aerial portion of the plant through capillary action on the exterior surfaces of the plant, with subsequent internalization occurring at those locations. Moreover, in the absence of a physical barrier, aerosolization of pathogen-contaminated soil particles to the phyllosphere (aboveground) plant tissues is another possible mode of contamination in such studies. Although this contamination mode has been discounted because of the absence of contamination of control (uncontaminated) pots within the same chamber (Pu et al. 2009, Habtesellassie et al. 2010), it does not rule out the possibility that microcurrents within the chamber could transfer the pathogen over the short distances that exist between the soil surface and phyllosphere tissue in the same pot. Such a possibility could explain the occasional observation of contamination of phyllosphere tissue in spinach (Pu et al. 2009) or lettuce (Habtesellassie et al. 2010) plants exposed to contaminated soil.

Microscopic analysis of plant tissues is a more definitive approach for validating the internalization of pathogens. In one of the first studies exploring internalization of plant tissue, tissues were exposed to rabbit anti–*Escherichia coli* O157:H7 polyclonal fluorescent antibody, and fluorescence microscopy was used to visualize the presence of *E. coli* O157:H7 in and on the walls of vessels

just beneath the epidermis of the hypocotyls of contaminated radish sprouts (Itoh et al. 1998). In a subsequent study, surface-sterilized sprouts germinated from mung bean seeds exposed to either a suspension of *E. coli* or *Salmonella* Montevideo were found to contain viable green fluorescing bacteria when exposed to the LIVE/DEAD BacLight stain (Warriner et al. 2003b).

Scanning electron microscopy (SEM), which is often used to examine surface topography, has also been a useful tool to determine internalization of pathogens in leafy greens. Several investigators have published SEM micrographs of bacterial infiltration into stomata or damaged tissue (Ells & Hansen 2006, Keskinen et al. 2009, López-Gálvez et al. 2010), and SEM images of *E. coli* K-12 cells were observed inside vasculature and intercellular spaces of tissue near the fracture faces of spinach leaves (Wang et al. 2010). However, a disadvantage to SEM is that because it is subject to high pressures, fixation and critical point drying of the tissue are needed to preserve the structure and shape of the bacteria, but this process can cause distortion of morphology (Little et al. 1991) and obviates the ability to determine the pathogen's viability. Therefore, the most common microscopy tool employed to visualize internalization of pathogens has been confocal microscopy (**Table 1**). Using this tool, in-focus images at a number of microscopic depths can be obtained, and three-dimensional reconstructions of topologically complex objects can be resolved. Recognition of pathogen cells with this technology is enabled by inserting a fluorescently labeled protein plasmid into the pathogen or applying fluorescent antibodies specific for that pathogen to the pathogen-infected tissue samples. Some common observations from studies employing confocal microscopy are the presence of pathogen cells at various depths beneath the surface of plant tissues and the absence of pathogen cells on the surface of chlorine-disinfected tissues. Another common feature among studies employing confocal microscopy is that the microbial challenge conditions usually involve exposing the plant tissues to very high pathogen cell numbers (10^6 to 10^9 CFU ml^{-1}), which reflects the low sensitivity of this type of microscopy to detect pathogens.

The most common method used to determine the internalization of pathogens in plant tissues is to apply a disinfectant treatment and then either enumerate or grow by enrichment culture the pathogens from the surface-disinfected tissue. Several investigators have used 70% ethanol to disinfect lettuce and tomato fruit, roots, stems, and leaves (Klerks et al. 2007b, Hintz et al. 2010); however, when used by itself, the surface disinfection efficiency against *Salmonella enterica* serovar Dublin was determined to be only 99.8% (Klerks et al. 2007b). Immerson for 1 to 20 min of plant tissue in high concentrations of chlorine (500 to 20,000 ppm) has also been commonly applied for surface disinfection (Buchanan et al. 1999; Dong et al. 2003a,b; Warriner et al. 2003a; Duffy et al. 2005; Jablasone et al. 2005; Pu et al. 2009; Zhang et al. 2009a). Dong et al. (2003a) confirmed that disinfection of tissues (inoculation dose of enteric bacteria not specified) using 500 ppm chlorine in combination with 0.1% sodium dodecyl sulfate and 0.2% Tween 20 was based on either (*a*) the absence of fluorescently labeled bacteria in tissues examined under confocal microscopy, (*b*) the absence of any bacterial growth on an agar-containing growth medium to which the surface-disinfected tissues were applied (tissue prints), or (*c*) the rare presence or lack of bacterial growth in the last set of washes used to rinse the tissues. However, a more recent study using a chlorine disinfectant treatment revealed recoveries of 3.7–4.4 log CFU *E. coli* O157:H7 per lettuce leaf or root from disinfected tissue compared with 8 log CFU *E. coli* O157:H7 per leaf or root from nondisinfected tissue (Zhang et al. 2009a). Moreover, *E. coli* O157:H7 could be recovered from lettuce leaves and roots by direct plating (limit of detection of <0.6 log CFU per leaf piece or root) even when the samples had been treated with 2,500 or 5,000 ppm chlorine (10 or 20 min exposure time) or with 10,000 ppm chlorine (10 min exposure time). *E. coli* O157:H7 was not detected by direct plating when tissues were treated with 10,000 ppm chlorine for 20 min, but was observed in 16 of 18 tissue prints following this chlorine treatment. In comparison, a surface disinfection

Table 1 Studies employing confocal microscopy for detection of internalized pathogens in produce

Produce exposure conditions to pathogen	Observations	Reference
Lettuce pieces were submerged overnight at 7°C in an *E. coli* O157:H7 suspension (10^7 CFU ml^{-1}), washed with sterile deionized water twice, treated with 20 ppm chlorine for 5 min, rinsed twice in sterile deionized water, then stained with fluorescein isothiocyanate–conjugated antibody against *E. coli* O157:H7 and propidium iodide (results in live cells staining green to yellow and dead cells staining yellow to red)	■ Many live cells were entrapped 20 to 100 μm below the surface in stomata and cut edges ■ *E. coli* O157:H7 attached to the whole cut surface and penetrated to the interior of the cut edges, where they remained viable even after chlorine treatment	Seo & Frank 1999
Lettuce pieces were submerged at 22°C for 20 min in an *E. coli* O157:H7 suspension (10^7, 10^8, or 10^9 CFU ml^{-1}), held at 4°C for 24 h, treated with 200 ppm chlorine for 5 min at 22°C, submerged in sterile deionized water, then stained with fluorescein isothiocyanate–conjugated antibody against *E. coli* O157:H7 and propidium iodide (results in live cells staining green to yellow and dead cells staining yellow to red)	■ Most *E. coli* O157:H7 cells on the surface of leaves were removed or inactivated following chlorine treatment ■ Viable cells were observed in stomata and near cut edges of tissues treated with chlorine; however, no viable cells were observed at cut surfaces ■ Penetrating cells were mostly found at the junction of lettuce cells	Takeuchi & Frank 2000
Alfalfa seeds were immersed in a suspension of *Salmonella* Stanley (10^7 CFU ml^{-1}) for 5 min at 22°C. Seeds were germinated and grown at 22°C for 7 d before analysis of tissues	■ *Salmonella* was present at a depth of 12 μm within intact sprout tissue	Gandhi et al. 2001
Lettuce plants were exposed to 200 ml of an *E. coli* O157:H7 suspension (10^7 CFU ml^{-1}), then the edible portion of the plant was harvested 1, 3, or 5 d later and surface disinfected with 80% ethanol and 0.1% $HgCl_2$ for 5 or 10 min	■ The target pathogen was visualized at depths of up to 45 μm below the tissue surface	Solomon et al. 2002
Carrot slices were immersed in suspensions of *E. coli* O157:H7 (10^7 CFU ml^{-1}) for 30 min followed by rinsing with sterile distilled water	■ *E. coli* O157:H7 cells were found surrounding carrot cells and in intracellular spaces ■ Cellular structure of the carrot and pathogen was differentiated with the use of Nile blue stain ■ Fluorescein isothiocyanate–labeled antibodies specific for *E. coli* O157:H7 bound to cells at 60 to 80 μm below the carrot tissue surface	Auty et al. 2005

Italian **parsley** (10 g bunch) was immersed in suspension of *Salmonella* (8 l of 10^6 CFU ml^{-1} in 0.1% peptone water) equilibrated to 5, 25, or 35°C for 3 or 15 min, then the parsley was drained and allowed to dry for 1 h at 25°C	■ *Salmonella* was internalized regardless of the dip temperature ■ *Salmonella* cells were found near the stomata and within cracks in the cuticle ■ Immersion for longer times resulted in higher numbers of internalized cells	Duffy et al. 2005
Lettuce plants were surface irrigated with 30 ml of FluoSpheres (10^8 fluorescent microspheres per ml) and sectioned plant samples were examined 1, 3, or 5 days postchallenge	■ The FluoSpheres were present in internal portions of stem and leaf tissues, suggesting transport of the spheres from the root upward into the edible tissue	Solomon & Matthews 2005
Lettuce leaves with leaf tip burn lesions were immersed in a suspension of *E. coli* O157:H7 (10^5 CFU ml^{-1}) followed by incubating the leaves for 24 h at 28°C	■ *E. coli* O157:H7 cells were found in high densities not only on the surface of the plant cells but also inside the dead tissue and stomata	Brandl 2008
Lettuce pieces were preconditioned for 20 min under dark, laboratory neon light, or high-intensity bulb conditions prior to submerging in *Salmonella enterica* inoculum (8 log CFU ml^{-1}) for 2 h under light or dark conditions and subsequent analysis	■ Numerous *Salmonella* cells were observed beneath stomata and in the intercellular space in the underlying parenchyma cells	Kroupitski et al. 2009
Leaves of **lettuce** (iceberg, romaine, and ruby red), **arugula**, **parsley**, **basil,** and **tomato** were first submerged in 30 ml of sterile distilled water while being exposed to high light intensity (100 μE $m^{2(-1)}$ s^{-1}) for 20 min, then the leaves were exposed to 30 ml of a *Salmonella* suspension (10^8 CFU ml^{-1}) for 2 h followed by washing the leaves twice in sterile distilled water	■ Incidence of internalized *Salmonella* was determined by calculating the percentage of microscopic fields containing GFP-labeled *Salmonella* of the 30 microscopic fields examined ■ The prevalence of *Salmonella* was highest in iceberg lettuce (81%) and arugula (88%), with lower prevalences in basil (46%), red lettuce (20%), romaine lettuce (16%), parsley (2%), and tomato (0.6%) leaves ■ A higher incidence of internalization occurred in iceberg lettuce harvested in summer compared with other seasons	Golberg et al. 2011
Lettuce pieces were submerged in 10 ml of green fluorescent protein–labeled *Salmonella* (10^8 CFU ml^{-1}) for 2 h at 25°C before rinsing for 1 min with 50 ml sterile distilled water	■ Fluorescent bacteria were found within stomata and at various depths below the leaf surface in the intercellular space ■ Three-dimensional reconstruction model of the fluorescent images supported the finding regarding the internal localization of *Salmonella*	Kroupitski et al. 2011

treatment that involved dipping the tissue in 80% ethanol for 10 s followed by immersion in 0.1% $HgCl_2$ for 10 min yielded only 3 of 18 *E. coli* O157:H7–positive leaf prints and was superior to chlorine treatment for inactivation of *E. coli* O157:H7 on lettuce leaves. A disadvantage to using mercury as a disinfectant for this purpose is the exorbitant costs associated with waste disposal and the relatively long treatment times. As an alternative, Erickson et al. (2010a) determined that dipping tissues in 1% $AgNO_3$ for 10 s was as effective as the $HgCl_2$ treatment. Similarly, Franz et al. (2007) determined that $AgNO_3$ was 99.9993% effective in leaf surface sterilization compared with 99.994% for a 5-s treatment with 10,000 ppm chlorine followed by a 5-s treatment with 70% ethanol. Effectiveness of a 12,000 ppm chlorine treatment could be increased to 99.999997% by exposing the treated tissues to UV light (254 nm) for 10 min per side (Li et al. 2008).

Once the tissue has been surface disinfected, it is often processed for enumeration of internalized pathogens by either pummeling (stomaching) the tissue in the presence of an extractant (Warriner et al. 2003a,b; Duffy et al. 2005; Nthenge et al. 2007; Wang et al. 2010) or grinding the tissue in the presence of an extractant (Franz et al. 2007; Zhang et al. 2009a,b,c; Erickson et al. 2010a,b,c; Ongeng et al. 2011). On the basis of this author's knowledge, no study comparing these processes has been published, although theoretically, there are limitations to either process that could affect the detection of internalized pathogens. In grinding the tissue, internalized pathogens may be exposed to plant tissue antimicrobials during the extraction process, whereas in pummeling, pathogens may be dislodged from stomata and cut tissue sites but not from other less exposed internal sites.

PATHOGEN INTERNALIZATION DURING HARVESTING OF LETTUCE

Harvesting iceberg lettuce for bagged salads involves not only cutting the head from the plant but also coring the head in the field. In the industry, this practice is referred to as field coring or cut and core. The practice is accomplished manually by field workers using a handheld device consisting of a stainless steel blade, shaft, and cylindrical coring ring. Because of the close proximity between the soil and cutting the heads from the plants, cross-contamination of this tool may occur. Hence, the potential and degree to which enteric pathogens could be transferred from the soil to cored lettuce during field coring has been investigated in two recent studies. In a study by McEvoy et al. (2009), a single contaminated coring knife artificially contaminated with 5 log CFU of *E. coli* O157:H7 successively contaminated at least nineteen lettuce heads. Similarly, field coring devices inoculated by contacting soil containing *E. coli* O157:H7 at 2.7 or 1.7 log CFU g^{-1} transferred the pathogen to 10 and 5 consecutively processed heads, respectively (Taormina et al. 2009). Moreover, lettuce cores remained positive for the pathogen after spraying with 100 ppm chlorine for 2 min. Given that no viable *E. coli* O157:H7 were observed at cut surfaces of lettuce pieces subjected to a 200 ppm chlorine treatment (Takeuchi & Frank 2000), it is likely that the *E. coli* O157:H7 detected in these chlorine-treated lettuce cores infiltrated the cut tissues.

PATHOGEN INTERNALIZATION DURING POSTHARVEST MINIMAL PROCESSING

Fresh-cut produce has been defined by the International Fresh-Cut Produce Association as any fresh fruit or vegetable that has been physically altered from its original form (by peeling, trimming, washing, and cutting) to obtain a 100% usable product that is subsequently bagged or prepackaged (Schneider et al. 2004). On the basis of research studies that are described below, these operations

together with the process of vacuum cooling create conditions that promote internalization of pathogens when they are in close proximity to plant tissues.

Temperature is the single most important factor in maintaining postharvest quality. In the case of crops such as lettuce, spinach, and celery, which have a high surface-to-volume ratio, vacuum cooling is used to quickly remove heat from the tissue. Temperature reduction in these products occurs as a result of energy consumed during water evaporation at low pressure; however, water from a recirculating reservoir is also sprayed on the produce to avoid moisture loss (Suslow et al. 2003). In a study designed to address the food safety risks associated with this method of cooling, levels of infiltration of surface-inoculated *E. coli* O157:H7 into lettuce tissue were significantly greater (1 log CFU g^{-1}) with vacuum-cooled product than in product not cooled by vacuum (Li et al. 2008). The tissue of vacuum-cooled product was imaged with laser scanning microscopy, and enlarged lettuce stomata were observed, suggesting a possible mechanism for the increased penetration of pathogen cells.

Hydrocooling (showering produce with chilled water or immersing produce in cold water baths) or washing with water colder than the product temperature also enhances internalization of pathogens into many types of produce. Under these conditions, a negative pressure differential is produced as a result of air spaces within the produce item being contracted. Types of produce in which internalization of pathogenic bacteria occurs through the stem scar as a result of the negative pressure differential include tomatoes (Zhuang et al. 1995), apples (Buchanan et al. 1999, Burnett et al. 2000), mangoes (Bordini et al. 2007), and oranges (Eblen et al. 2004). Infiltration of pathogens into produce exposed to an aqueous medium does not necessarily require a negative temperature differential, as pathogen internalization from exposure to water has been demonstrated in almonds (Danyluk et al. 2008), pecans (Beuchat & Mann 2010), lettuce (Takeuchi & Frank 2000), parsley (Duffy et al. 2005), and tomatoes (Callejas et al. 2011). Moreover, the numbers of internalized cells increased when product was immersed in water for longer periods of time (Duffy et al. 2005) or the product was recently damaged or had exposed cut surfaces (Takeuchi & Frank 2000, Eblen et al. 2004, Janes et al. 2005, Fatemi et al. 2006). Given the ease in which pathogens may be internalized into produce tissue during processing, it is imperative that pathogens are not present in the wash or cooling waters by maintaining effective levels of sanitizing agents. Although sanitizing agents such as chlorine may reduce surface contamination, they have little or no effect on pathogens already lodged within cuticle cracks, stomata, and other harborage sites of produce (Seo & Frank 1999, Duffy et al. 2005, López-Gálvez et al. 2010).

PREHARVEST PATHOGEN INTERNALIZATION OF PLANT LEAVES OR STEMS

In addition to pathogen internalization occurring in postharvest plant tissue, many studies have revealed internalization of several different types of human enteric pathogens into phyllosphere (aboveground) plant tissue in a preharvest setting (**Table 2**). Similar to postharvest tissue, damaged preharvest plant tissue may serve as one site of entry. For example, leaf tip burn lesions, a common physiological disorder of lettuce, led to dense populations of *E. coli* O157:H7 cells accumulating both internally and externally (Brandl 2008). Even a gentle rubbing of contaminated lettuce leaves with a sterile glass rod increases internalization of *E. coli* O157:H7 compared with leaves that are left untouched after spraying leaves with the pathogen (Erickson et al. 2010c). Disruption to the plant cuticle is one of the most common manifestations of tissue damage, and these areas are not only sites where increased access to nutrients by epiphytic (surface) bacteria occur, thereby increasing their chance for survival and growth (Harapas et al. 2010), but also are sites where the bacteria can infiltrate plant tissue. A recent study examined the potential for *E. coli* O157:H7

Table 2 Internalization of human enteric pathogens into leaves or stems

Plant	Treatment conditions and sampling times	Surface disinfection	Results	Reference
Green onions	Nine and eleven weeks after seeding, 50 U of **hepatitis A** vaccine were added to green onion leaves, then the above-ground portion of plants were sampled three weeks and one week later, respectively	Outer layers removed	■ Hepatitis RNA detected inside green onions sampled (4 of 4) but not on the outside	Chancellor et al. 2006
Arabidopsis thaliana	Leaves were dipped for 5 min in 3 ml of inoculum (8.8 log ***Listeria monocytogenes*** per ml) containing 0.02% Silwet surfactant (without the surfactant, the leaves did not submerge in the aqueous suspension), then were rinsed in distilled water to remove unattached cells before analyzing leaves 24 h later	None	■ Confocal microscopy allowed visualization of *L. monocytogenes* inside the stomatal openings on the leaf surface and also observed in intercellular spaces, deeper in the leaf tissue ■ No evidence of intracellular *L. monocytogenes*	Milillo et al. 2008
Lettuce, iceberg	Pieces were preconditioned for 20 min under dark, laboratory neon light, or high-intensity bulb conditions prior to submerging in ***Salmonella enterica*** inoculum (8 log CFU ml^{-1}) for 2 h under light or dark conditions and subsequent analysis	None	■ Using confocal laser scanning microscopy, confined to the surface primarily with almost no *Salmonella* penetration observed following incubation in the dark ■ Incubation under light resulted in aggregation of bacteria near open stomata and invasion into the inner leaf tissue ■ Mutants defective in motility and chemotaxis had significantly reduced internalization	Kroupitski et al. 2009
Lettuce	Leaves were spot inoculated (20 μl 6–7 log CFU ***E. coli* O157:H7** ml^{-1}) onto plants on days 3, 30, and 60 after transplanting seedlings and analyzed one to three times after inoculation until harvest (57 to 67 days after transplanting)	80% ethanol followed by 0.1% mercury chloride	■ All 212 surface-sanitized leaf samples from plants with inoculated leaves were negative for internalized *E. coli* O157:H7, regardless of plant age at time of inoculation or the location of the leaf (abaxial versus adaxial) receiving the inocula	Zhang et al. 2009b
Spinach	Irrigation water containing ***E. coli* O157:H7** at 10^2, 10^4, or 10^6 CFU ml^{-1} was applied to spinach 48 and 69 days after transplantation of seedlings into fields, then leaves were sampled immediately after spraying and at weekly intervals up to harvest (76 days posttransplantation)	80% ethanol followed by 0.1% mercury chloride	■ Internalized *E. coli* O157:H7 was only detected on the day of exposure in leaves (5 of 20 samples) dosed at 10^6 CFU ml^{-1} but was not detected 7, 14, or 28 days later.	Erickson et al. 2010b

Lettuce	Irrigation water containing ***E. coli* O157:H7** at 10^8 CFU ml^{-1} was sprayed onto either the abaxial (lower) or adaxial (upper) side of leaves of field-grown lettuce under sunny or shaded conditions, then samples were collected for analysis of internalized populations 0, 1, 2, 5, 7, and 14 days later	1% silver nitrate	■ *E. coli* O157:H7 was internalized into lettuce leaves initially at populations of 2.3-3.2 log CFU g^{-1} ■ Pathogen still detected by enrichment culture in abaxial-sprayed leaves 14 days later but not detected on days 5 and 2 in leaves sprayed on the adaxial side under sunny and shaded conditions, respectively	Erickson et al. 2010b
Lettuce and spinach	***E. coli* O157:H7** (10^6 or 10^8 CFU ml^{-1}) was sprayed onto the abaxial and adaxial sides of leaves in one set of plants, and the pathogen was applied as fine drops to the abaxial side of leaves of another set of plants prior to gently spreading the inoculum over the surface using a glass rod with rounded end and analyzing the leaves of both sets after 48 h	80% ethanol followed by 0.1% mercury chloride	■ No internalized *E. coli* O157:H7 was detected in leaves 48 h after being spray inoculated with the 10^6 CFU ml^{-1} inoculum ■ When leaves were sprayed with a 10^8 CFU ml^{-1} inoculum, internalization as determined by enrichment culture occurred in 70%–90% of the abaxial-sprayed leaves and 30%–40% of the adaxial-sprayed leaves ■ Rubbing the leaf after droplet inoculation led to internalized *E. coli* O157:H7 counts of 2.7 and 2.9 log CFU g^{-1} in spinach and lettuce leaves, respectively	Erickson et al. 2010c
Spinach	***Cryptosporidium parvum*** (1,000 oocysts per ml) was sprayed on spinach leaves two weeks after seedlings had emerged then leaves were sampled two, three, and five days after exposure	None	■ Using confocal microscopy, oocysts were observed to infiltrate through the stomatal openings in spinach leaves and to persist at the mesophyll level	Macarisin et al. 2010
Lettuce, romaine	Intact whole lettuce leaves were submerged in **murine norovirus** solutions (5 log PFU ml^{-1}) with and without biosolids or swine manure	Wiped with 1% Virkon	■ Approximately 3 log PFU was internalized by both intact and cut lettuce leaves for murine norovirus in biosolids, implying that viruses were internalized through entry other than open cuts ■ Murine norovirus in biosolids had significantly higher levels of internalization than pure murine norovirus or murine norovirus in manure	Wei et al. 2010
Basil, parsley, tomato, arugula, lettuce (iceberg, romaine, red)	Pieces were preconditioned for 20 min under high intensity light prior to suspending in ***Salmonella* Typhimurium** (8 log CFU ml^{-1}) for 2 h before rinsing and analyzing	None	■ Using confocal laser scanning microscopy, the following incidences of internalized cells were observed: iceberg lettuce (81%); arugula (88%); romaine lettuce (16%); red lettuce (20%); basil (46%); parsley (1.9%); and tomato (0.6%)	Golberg et al. 2011

to be internalized into baby spinach or lettuce that had been cut and then regrown for a second crop. Whether cut five days prior or immediately prior to spraying with contaminated irrigation water (10^7 *E. coli* O157:H7 CFU ml^{-1}), the leafy green stems initially contained internalized *E. coli* O157:H7 at levels of 3–4 log CFU g^{-1} (M.C. Erickson, C.C. Webb, J.C. Diez-Perez, and M.P. Doyle, unpublished data). However, this initial population was not persistent, as internalized *E. coli* O157:H7 were not detected in samples collected two weeks later, which was likely to be in response to the activities of plant defenses (to be discussed below).

A number of surface structures of uninjured plant tissue serve as potential sites of bacterial infiltration, including stomata, hydathodes, and trichomes (Huang 1986). Stomata are minute pores in the epidermis of plant leaves or stems that are involved in gas exchange and water transpiration. Responding to environmental conditions, a pair of guard cells controls the opening and closing of these pores. Hydathodes have structures similar to stomata except the guard cells associated with hydathodes do not function to regulate aperture opening. Occurring on the marginal teeth or serrations of leaves or at the leaf tips of *Brassica* plants, hydathodes permit water in liquid form to be excreted in the morning hours when the soil is wet and transpiration from closed stomata is absent. These water droplets are in continuous contact with the water in the vascular system and together with resident pathogens may be drawn back into the leaf when stomata open and transpiration occurs. Trichomes are hairlike or bristlelike epidermal projections of diverse form, structure, and function but are typically fragile and readily collapse under slight pressure to expose the underlying tissue. The density of all three of these surface structures varies from species to species, age of the plant, and region of the leaf (Huang 1986). Hence, it is not unexpected to find accompanying differences in internalization of enteric pathogens. For example, Golberg et al. (2011) determined that the prevalence (percentage of microscopic fields with $\geq$1 fluorescently labeled bacterium) of *Salmonella* Typhimurium beneath the epidermis of leaves was greatest in arugula (88%), followed by iceberg lettuce (81%), basil (46%), red lettuce (20%), romaine lettuce (16%), parsley (1.9%), and tomato (0.6%), when leaves had been submerged in a suspension of 10^8 CFU ml^{-1}. Differences have been observed in the location of pathogens within leaves, with Erickson et al. (2010b) determining that pathogen internalization in lettuce leaves sprayed with irrigation water containing *E. coli* O157:H7 at 10^8 CFU ml^{-1} occurred with greater persistence in leaves sprayed on the lower or abaxial side (up to 14 days) than in leaves sprayed on the upper or adaxial side (2 days).

To date, most studies of enteric bacterial pathogen internalization of plant tissue exposed the tissue to very high concentrations of the pathogen ($\geq$8 log CFU ml^{-1}). When lettuce leaves were spot inoculated with a lower concentration (7 log CFU ml^{-1}), internalized *E. coli* O157:H7 was absent from all 424 surface-sanitized leaf samples regardless of plant age at the time of inoculation or the location at which the pathogen was applied to the leaf (abaxial versus adaxial) (Zhang et al. 2009b). Similarly, pathogen internalization did not occur in spinach leaves when plants were sprayed in the field with irrigation water containing *E. coli* O157:H7 at concentrations as high as 4 log CFU ml^{-1} and only occurred in 25% of samples when sprayed with irrigation water containing *E. coli* O157:H7 at 6 log CFU ml^{-1} (Erickson et al. 2010b). On the basis of these results and the method of pathogen introduction, it is likely that immigrant bacterial cells remained at the site of introduction and internalized only when the bacterial cell landed directly on stomata. Monier & Lindow (2005) determined that different modes of administering bacteria to plant surfaces could affect the fate of immigrant bacterial populations. If administered in small aerosol particles, the bacteria most likely remain at the site of initial impaction during inoculation. However, if immigrant cells are applied in large droplets, typical of rain splash, cells most likely move across the leaf surface and collect at those sites where internalization can occur. The importance of a moisture film residing on plant surfaces for short intervals is illustrated by a recent study in which

leafy green plants were misted repeatedly over a short interval with *E. coli* O157:H7–contaminated water (10^3 CFU ml^{-1}) to simulate spray irrigation in a field setting. In that study, internalization in 56 of 180 parsley samples and 82 of 240 spinach samples were *E. coli* O157:H7 positive by enrichment culture when plants were exposed to repeated spray over a 20-min period (M.C. Erickson, C.C. Webb, and M.P. Doyle, unpublished data). Maintaining a moist environment for an extended period of time on spinach and parsley therefore appeared to facilitate pathogen movement and entry into plant stomata.

Conditions other than moisture that facilitate internalization of pathogens into plant leaves or stems have also been explored. For example, the type of suspending media affected the internalization of murine norovirus into romaine lettuce leaves with greater internalization occurring when the virus suspension contained human biosolids compared with a virus suspension containing animal manures or no wastes (Wei et al. 2010). Several surface structures of enteric bacterial pathogens (flagella and the type 3 secretion system) have been determined to play a role in bacterial adherence to leafy green produce (Xicohtencatl-Cortes et al. 2009) and are necessary for bacterial cell translocation and internalization into plant tissue apertures. Kroupitski et al. (2009) revealed that mutations eliminating *Salmonella* motility and chemotaxis inhibited bacterial internalization into iceberg lettuce and that applying light and dark conditions and treatment with fusicoccin, which forced stomatal opening, revealed that motile salmonellae are attracted to nutrients produced de novo by photosynthetically active cells.

PREHARVEST PATHOGEN INTERNALIZATION OF FRUITS

Leaves and stems are not the only aerial plant tissues subject to internalization of enteric human pathogens. Plant fruits can also be internalized by enteric pathogens. For example, 43% and 40% of tomatoes from tomato plants receiving stem inoculation with salmonellae before and after flower set, respectively, were positive for *Salmonella*, whereas inoculating flowers with salmonellae resulted in 25% of tomatoes internalized by *Salmonella* (Guo et al. 2001). *Salmonella* invasiveness of tomatoes; however, is serovar dependent. Inoculating tomato plant flowers with *S.* Montevideo resulted in internal populations of 10^2 CFU g^{-1} tomato, whereas when flowers were exposed to *Salmonella* serovars Javiana, Newport, Hadar, Enteritidis, Typhimurium, Dublin, Senftenberg, or Infantis, the pathogen was detected within tomatoes by enrichment culture only (Shi et al. 2007). Moreover, internalization of *Salmonella* into fruit can be affected by the presence or absence of plant pathogens or bacteria antagonistic to *Salmonella*. For example, the presence of either *Enterobacter* or *Bacillus* spp. reduced the occurrence of *Salmonella* in tomatoes (Shi et al. 2009) and reduced internalization also occurred when a plant's bacterial diversity was high (Gu et al. 2011). In contrast, the presence of the plant pathogen *Erwinia tracheiphila* facilitated the internalization of *Salmonella* in developing cantaloupe fruit (Gautam et al. 2011). This latter response implies that *Salmonella* colonizes plant tissues differently than plant pathogens. This concept is supported by results from a high throughput screening study for *Salmonella* promoters differentially regulated inside tomato fruits in which only one *Salmonella* gene, *argG*, was common in the infection of plants by *Salmonella* and by plant pathogens (Teplitski et al. 2009).

PREHARVEST PATHOGEN INTERNALIZATION OF ROOTS OR GERMINATING SEEDS

Other avenues by which human enteric pathogens may infiltrate plant tissues are through germinating seeds and roots (**Table 3**); however, there are differences in the degree of invasiveness by different bacterial pathogens at these sites. For example, alfalfa seedling roots exposed directly

Table 3 Internalization of human enteric pathogens via roots or seeds of plants

Plant	Treatment conditions	Surface disinfection	Results	Reference
Radish sprouts	Seeds were soaked in water containing ***Escherichia coli* O157:H7** (3 log CFU ml^{-1}) and then sprouts were grown at 18–25°C for 7 days before analysis	80% ethanol followed by 0.1% mercury chloride	■ *E. coli* O157:H7 was detected by culture in 7 of 20 surface-disinfected hypocotyls ■ Using microscopy, *E. coli* O157:H7 was visualized in the inner tissues and stomata of cotyledons of radish sprouts	Itoh et al. 1998
Alfalfa sprouts	Seeds were inoculated by immersion in a suspension of ***Salmonella* Stanley** (7 log CFU ml^{-1}), germinated, and were grown for 7 days before analysis	None	■ Laser scanning confocal microscopy revealed bacteria were present at a depth of 12 μm within intact sprout tissue	Gandhi et al. 2001
Tomatoes	Plant roots were exposed to ***Salmonella*-contaminated hydroponic solution (4.6 log CFU ml^{-1}) for 1–9 days, then hypocotyls, cotyledons, stems, and leaves were analyzed	None	■ Following 1 day of exposure, *Salmonella* was detected in hypocotyls/cotyledons, stems, and leaves at populations of 3.0 and 3.4 log, and 0.00 CFU g^{-1}, respectively, whereas after 9 days of exposure, populations were 4.0, 3.7, and 3.6 log CFU g^{-1}, respectively	Guo et al. 2002
Lettuce	Treatment 1: Seeds were placed in soil inoculated with 4, 6, or 8 log CFU ***E. coli* O157:H7** g^{-1}. Seedlings were sampled 3, 6, and 9 days postplanting. Treatment 2: 200 ml of 7 log CFU ***E. coli* O157:H7** ml^{-1} was added to soil of mature plants (avoided splash) then plants were harvested on days 1, 3, and 5 postinoculation	Treatment 1: Seedlings disinfected with 80% ethanol followed by 0.1% mercury chloride Treatment 2: Mature plants were not surface disinfected	■ Treatment 1: Using confocal microscopy, the target pathogen was visualized at depths up to 45 μm below the tissue surface ■ Cells were restricted to the intercellular space ■ Viable cells were recovered from 0 of 22, 4 of 22, and 6 of 22 seedlings that had been exposed to soil containing 4, 6, and 8 log CFU g^{-1}, respectively ■ Treatment 2: 40% to 80% of mature plants were positive for *E. coli* O157:H7	Solomon et al. 2002
Tomatoes	Five-week-old plants were grown in methyl bromide–fumigated soil contaminated with ***Salmonella*** (5.8 log CFU) and/or nematodes (*Meloidogne incognita*) at 4.0–4.3 log eggs, then plant samples were analyzed 2 and 4 weeks after inoculation	None	■ Analysis of roots, galls, stems, and leaves failed to reveal the presence of *Salmonella* even upon wounding the roots with nematodes	Beuchat et al. 2003

Arabidopsis thaliana (thale cress)	Treatment 1: Plants were grown in hydroponic medium, and the roots (5–7 day postgermination) were exposed to ***Salmonella enterica* serovar Newport** or ***E. coli* O157:H7** at 4 or 6 log CFU ml^{-1}, then plants were sampled over the next 4 days Treatment 2: Seedlings were transplanted into unautoclaved and autoclaved soil contaminated with ***S. enterica* serovar Newport** or ***E. coli* O157:H7** at 8 log CFU g^{-1}, then plants were sampled over the next 30 days	None	■ Treatment 1: Root inoculation led to contamination of the entire plant, indicating that the pathogens were capable of moving on or within the plant in the absence of competition ■ Treatment 2: Survival of pathogens on leaves and flowers of soil-grown plants decreased as the plants matured, but both pathogens were detectable for at least 21 days	Cooley et al. 2003
Alfalfa	Seedlings were exposed to inoculum (***Klebsiella pneumoniae* 342, *E. coli* O157:H7, *E. coli* K-12, *S. enterica* serovars Cubana, Infantis, Typhimurium** and ***S. enterica* strain 8137**) in an agar growth medium (2–5 log CFU per plant), and roots and hypocotyls were analyzed 5 days later	1% bleach /0.1% SDS/0.2% Tween 20	■ All *Salmonella* isolates colonized the interiors of the seedlings in large numbers at the lowest exposure concentration, although the extent of colonization varied with the isolate ■ Among the bacterial strains tested, *K. pneumoniae* 342 and *E. coli* isolates were the strongest and weakest endophytic colonizers of alfalfa, respectively	Dong et al. 2003a
Alfalfa, *Arabidopsis*, *Medicago truncatula*, wheat, rice	Seedlings were exposed in an agar-based nutrient system to ***K. pneumoniae* 342** or ATCC13883 (10^0 to 10^7 CFU ml^{-1}), then roots and hypocotyls were analyzed 6 days later	0.6% sodium hypochlorite/0.1% SDS/0.2% Tween 20	■ *K. pneumoniae* ATCC13883 colonized the interior of the host plant, but the highest colonization levels were generally 100-fold less than that obtained from *K. pneumoniae* 342, and those levels required at least 1,000 cells in the inocula ■ Monocots were colonized endophytically in higher numbers than the dicots	Dong et al. 2003b

(*Continued*)

Table 3 (*Continued*)

Plant	Treatment conditions	Surface disinfection	Results	Reference
Spinach	Treatment 1: Seeds were submerged in 7 log ***E. coli*** ml^{-1} for 20 min prior to sowing and growing in soil systems and plants were analyzed after 35-day growing period Treatment 2: 13-day-old seedlings were exposed to ***E. coli***–contaminated hydroponic solution (2 or 3 log CFU ml^{-1}) for 16 days prior to analysis Treatment 3: 20-day-old seedlings were transplanted into ***E. coli***–contaminated soil (2 log CFU g^{-1}), then plants were harvested periodically up to day 42	4,000 ppm chlorine	■ Treatment 1: Internalized *E. coli* could not be recovered from leaves but was recovered from roots (1.9 log CFU g^{-1}) of plants derived from inoculated seeds ■ Treatment 2: *E. coli* was recovered from surface-disinfected root tissue (1.4–1.6 log CFU g^{-1}) but not from within leaves ■ Treatment 3: On day 42, *E. coli* was recovered from surface-disinfected root tissues (3.8 log CFU g^{-1}) but not from within leaves	Warriner et al. 2003a
Mung bean sprouts	Seeds were exposed to a suspension of ***E. coli*** or ***Salmonella* Montevideo** (7 log CFU ml^{-1}) for 20 min, then germinated samples (in agar-based systems) were assayed at 24-h intervals	20,000 ppm chlorine	■ Four days after exposure, *E. coli* was detected in apoplastic fluid and extracts of surface-disinfected hypocotyls at 5.3 log CFU g^{-1}, whereas *Salmonella* was detected at 5.5 and 5.1 log CFU g^{-1}, respectively	Warriner et al. 2003b
Spinach	Five-week-old plants in commercial grade soil were irrigated with 20 ml of 6 log CFU ***E. coli* O157:H7** ml^{-1}, then plants were analyzed 7 days later	2,000 ppm sodium hypochlorite	■ *E. coli* O157:H7 was recovered from all surface-disinfected roots (33 of 33 samples) but not surface-disinfected leaves (0 of 33 samples)	Hora et al. 2005
Lettuce, spinach, radish, cress	Seeds were soaked in a pathogen (***E. coli* O157:H7, *Salmonella***, or ***Listeria monocytogenes***) cell suspension (2 log CFU ml^{-1}), germinated at 15°C on dampened sterile filter paper, and grown in a solidified hydroponic system before analyzing plant samples on days 9 and 49	10% sodium hypochlorite	■ *E. coli* O157:H7 was not recovered from within the tissue of mature plants but was internalized in seedlings of all plant types ■ Internalization of *Salmonella* was observed in lettuce and radish but not spinach seedlings ■ *L. monocytogenes* did not internalize within seedlings	Jablasone et al. 2005
Lettuce	***E. coli* O157:H7** or **FluoSpheres** (30 ml of 8 log CFU or particles per ml) were applied to soil containing 45-day-old plants then plants were analyzed 1, 3, and 5 days after exposure	None	■ Laser scanning confocal microscopy revealed that FluoSpheres were present within root tissue and leaf stem tissue ■ Numbers of FluoSpheres and *E. coli* O157:H7 cells in plant tissue were similar	Solomon & Matthews 2005

Green onions	Nine and eleven weeks after seeding, 50 U of **hepatitis A** vaccine were added to soil at the base of plants, then the aboveground portion of plants were sampled 3 and 1 week later, respectively	Outer layers removed	■ Hepatitis RNA was detected inside green onions sampled (4 of 4) but not on external surfaces	Chancellor et al. 2006
Barley	Seedlings were exposed to ***S. enterica*** or ***Listeria* spp.** (1 ml of 8 log CFU ml^{-1}) 1–2 days after transplantation in an axenic system and then harvested 1–4 weeks later	1% chloramine T solution	■ Endophytic colonization of *Listeria* spp. was not observed ■ Systemic spreading of *S. enterica* to the plant shoot (stems and leaves) was demonstrated ■ *S. enterica* was found as endophytic colonizer of barley roots and was at 6 log CFU g^{-1} after surface disinfection	Kutter et al. 2006
Lettuce (romaine)	Seventeen-, twenty-, and thirty-three-day old plants were exposed to contaminated potting medium (6 log CFU ***S. enterica* serovar Newport** g^{-1}), then aboveground plant parts were analyzed 2 and 7 days after inoculation	None	■ Leaves of lettuce plants with intact and damaged roots harbored *Salmonella* at 500 and 5,130 CFU g^{-1} of leaf, respectively, at 2 days postinoculation but not 5 days later	Bernstein et al. 2007a
Maize	Plant roots (undamaged roots, decapitated root tips, or entire root system removed) were exposed to contaminated hydroponic medium (7 log CFU ***E. coli*** ml^{-1}), then shoots of plants were analyzed 48 h after inoculation	Mone	■ Penetration and transport of *E. coli* into the aboveground parts of plants occurred with all inoculation treatments ■ *E. coli* cell numbers in shoots of plants with damaged roots or removed root systems were 27.8 and 23.9 times higher, respectively, than that in plants with intact roots	Bernstein et al. 2007b
Lettuce, cv Tamburo	Three ml of 9 log CFU ***E. coli* O157:H7**, ***S.* Typhimurium 110**, or ***S. typhimurium*** 119 ml^{-1} was added to soil 14 and 18 days after seeding, then plants were analyzed after 35 days	1% silver nitrate	■ *E. coli* O157:H7, *S.* Typhimurium 110, and *S.* Typhimurium 119 were found in disinfected leaf samples at prevalences of 29%, 23%, and 15% and cell numbers of 4.0, 2.6, and 2.4 log CFU g^{-1}, respectively	Franz et al. 2007

(*Continued*)

Table 3 (*Continued*)

Plant	Treatment conditions	Surface disinfection	Results	Reference
Lettuce	Treatment 1: Seeds were planted in ***S. enterica***–contaminated, manure-amended soil (7 log CFU g^{-1}), then the aboveground portions of plants were analyzed after 6 weeks Treatment 2: Plants were grown in Hoagland's agar and then ***S. enterica*** (10 µl of 7 log CFU ml^{-1}) was pipetted into the agar near the roots before analyzing shoots 7 days later	70% ethanol	■ Treatment 1: 3 out of 28 plants were positive for endophytic colonization of *S.* Dublin after surface disinfection ■ No endophytic colonization was observed for surface-disinfected plants grown on soil contaminated with *S.* Enteritidis or *S.* Typhimurium ■ Treatment 2: prevalence of lettuce plants endophytically colonized were 59%, 85%, 93%, 85%, and 89% for *Salmonella* serovars Dublin, Enteritidis, Montevideo, Newport, and Typhimurium, respectively ■ Prevalence was not affected by the lettuce cultivar (Tamburo, Cancan, Nelly)	Klerks et al. 2007a
Lettuce	Seeds were germinated in soil inoculated with ***S. enterica* serovar Dublin** at 7 log CFU g^{-1} and harvested 6 weeks later	70% ethanol	■ Three of fifty-six surface-disinfected plants were positive for *Salmonella*	Klerks et al. 2007b
Lettuce	Lettuce seedlings were exposed for 3, 7, or 14 days to ***E. coli*** O157:H7 (5 or 7 log CFU ml^{-1}) in a hydroponic system before leaves were collected and analyzed	80% ethanol followed by 0.1% mercury chloride	■ Five of twenty-four and five of twenty-three leaves were positive for internalized *E. coli* O157:H7 when plants were exposed to a pathogen suspension at 5 or 7 log CFU ml^{-1}, respectively	Nthenge et al. 2007
Arabidopsis thaliana	Fourteen-day-old plant roots were exposed to hydroponic solutions containing ***S. typhimurium*** at 8 log CFU ml^{-1} for 2 days, then plants were analyzed two weeks after exposure	1% bleach, 0.1% SDS, 0.2% Tween 20	■ Salmonellae grew on and in *Arabidopsis* roots two weeks after infection but were not detectable in leaf homogenates from these plants	Schikora et al. 2008
Parsley	Three times at three-day intervals, 500 ml of 7.6 log CFU ***S. enterica* serovar Typhimurium ml^{-1}** was drip irrigated on soil containing parsley plants (20–40 cm high), then samples were taken 1 day after the first and third irrigation	70% ethanol	■ For samples collected after the first irrigation, *Salmonella* was detected in leaves at 2.0 log CFU g^{-1} ■ For samples collected after the third irrigation, *Salmonella* was detected in leaves at 4.1 log CFU g^{-1}	Lapidot & Yaron 2009

Tomatoes	Treatment 1: Seeds were soaked in 8 log CFU ***S. Montevideo*** ml^{-1} prior to growing plants, then plants were sampled when tomatoes were judged to be at red-ripe stage Treatment 2: Tomato plants were irrigated 1 to 6 times with 350 ml of 7 log CFU ***S. Montevideo*** ml^{-1} with exposures spaced at 14-day intervals, then plants were sampled when tomatoes were judged to be at red-ripe stage	Roots dipped in 1% chlorine solution followed by 70% ethanol; Tomatoes surface disinfected with 70% ethanol	■ Treatment 1: Plants grown from contaminated seeds did not yield any fruit or plant tissues positive for *S.* Montevideo ■ Treatment 2: All 24 stem and leaf sections tested negative for internalized *Salmonella* ■ Only 5 of 24 root samples tested positive for internalized *Salmonella* ■ No tomatoes were positive for *Salmonella* in either the stem scar or the fruit pulp	Miles et al. 2009
Spinach	Ten ml of 6 log CFU ***E. coli* O157:H7** ml^{-1} was injected into the root zone of spinach plants, then primary root and plant leaves were analyzed 0, 7, and 14 days after inoculation	70% ethanol followed by 10% sodium hypochlorite	■ Internal colonization was observed in 10 of 60 samples when plants were exposed to *E. coli* O157:H7 in the root zone	Mitra et al. 2009
Lettuce	Young and mature lettuce plants (12 and 30 days of age, respectively) were grown in soil, manure-amended soil, or irrigated with water containing 1, 2, 3, or 4 log CFU ***E. coli* O157:H7** g^{-1} or ml^{-1}, then plants were harvested at 1, 10, 20, and 30 days postexposure and aboveground tissue analyzed	80% ethanol followed by 0.1% mercury chloride	■ Following enrichment culture, 9% (26 of 288) of surface-disinfected plants exposed at young age were positive for *E. coli* O157:H7 ■ 7.5% (9 of 120) of surface-disinfected plants exposed at mature age were positive for *E. coli* O157:H7	Mootian et al. 2009
Spinach	The soil of spinach plants was inoculated once (1 ml of 3 or 7 log CFU ***E. coli* O157:H7** ml^{-1}) at one of five successive weeks during the growing cycle, then leaves were sampled on the third day and weekly after inoculation until final harvest (day 44)	2% calcium hypochlorite	■ Among 120 spinach plant samples examined for internal leaf contamination, only one was *E. coli* O157:H7 positive	Pu et al. 2009

(*Continued*)

Table 3 (*Continued*)

Plant	Treatment conditions	Surface disinfection	Results	Reference
Spinach	Treatment 1: Seedlings were planted in pasteurized soils containing 3.8–4.4 or 7.6–8.1 log CFU ***E. coli* O157:H7** g^{-1} then plants were sampled at weekly intervals up to 4 weeks Treatment 2: Seedlings were exposed to ***E. coli* O157:H7** hydroponic solution at either a low (4.6–5.2 log CFU ml^{-1}) or high (8.4–8.9 log CFU ml^{-1}) population for 21 days, then plants were sampled at weekly intervals up to 4 weeks	0.1% mercury chloride followed by 80% ethanol	■ Treatment 1: *E. coli* O157:H7 was not detected in surface-disinfected tissues of spinach plants ■ Fluorescent *E. coli* cells were microscopically observed in root tissues in 23 (21%) of 108 spinach plants grown in inoculated soils ■ Treatment 2: *E. coli* O157:H7 was internalized sporadically in tissues of spinach plants grown in hydroponic medium inoculated with low bacterial populations ■ Internalized *E. coli* O157:H7 was recovered from shoot tissues of spinach plants exposed to high populations for 14 days (3.7 log CFU per shoot) and 21 days (4.4 log CFU per shoot)	Sharma et al. 2009
Lettuce	Treatment 1: In hydroponic and soil systems, roots of lettuce plants were exposed to **canine calicivirus** solution containing 8 or 10 log PCR-U, then leaves were analyzed for up to 67 h after inoculation Treatment 2: Seedlings were grown in turf briquettes watered daily for up to 11 days with 5 log PCR-U of **human norovirus G2**, then the whole plant, without roots, was analyzed	None	■ Treatment 1: 5 of 12 leaf samples were positive for canine calicivirus when plants were exposed in a hydroponic system, whereas 1 of 12 leaf samples was positive for virus when plants were exposed in soil system ■ Treatment 2: 12 of 12 samples were negative for human norovirus G2	Urbanucci et al. 2009
Lettuce (iceberg, romaine, and leaf)	Seedlings were transplanted and then ***E. coli* O157:H7** suspension was added to soil to give 3 or 6 log CFU g^{-1}, with root and leaf samples taken for up to 60 days after seedlings were transplanted	80% ethanol followed by 0.1% mercury chloride	■ All 270 surface-disinfected leaf samples were negative for internalized *E. coli* O157:H7 ■ All surface-disinfected root samples from 3 log CFU g^{-1} exposure and 148 of 150 surface-disinfected root samples from 6 log CFU g^{-1} exposure were negative for *E. coli* O157:H7	Zhang et al. 2009b

Lettuce (romaine and iceberg)	30 days after transplanting seedlings, ***E. coli* O157:H7** suspension was added to soil to give 4 or 6 log CFU g^{-1}, then plants were exposed during the day (12 h) to either 23°C (3 days), 32°C (3 days), or 36°C (2 days), followed by analysis of root and leaf tissue	80% ethanol followed by 0.1% mercury chloride	■ All 144 surface-disinfected leaf samples were negative for internalized *E. coli* O157:H7 ■ 143 of 144 surface-disinfected root samples were negative for *E. coli* O157:H7	Zhang et al. 2009c
Lettuce, spinach, and parsley	***E. coli* O157:H7**–contaminated compost (10^3 or 10^5 CFU g^{-1}) was applied to field plots prior to transplantation of spinach, lettuce, or parsley plants, then plants were collected and analyzed 2, 7, 13, and 49 days later	1% silver nitrate	■ Internalized *E. coli* O157:H7 was not detected (limit of detection was 1 log CFU ml^{-1}) in leaves or roots of plants collected at any of the sampling times	Erickson et al. 2010a
Spinach	Irrigation water containing ***E. coli* O157:H7** at 10^2, 10^4, or 10^6 CFU ml^{-1} was applied to the base of field-grown plants at 0, 55, and 69 days after transplantation, then plants were collected and analyzed initially and at weekly intervals (up to 3 weeks) after application	80% ethanol followed by 0.1% mercury chloride	■ During the early or late portion of the growing season, internalized *E. coli* O157:H7 was not detected in leaves or roots exposed to any of the inoculum concentrations ■ In mid-season exposed plants, internalized *E. coli* O157:H7 was detected by enrichment culture in surface-disinfected roots 7 days after exposure (5 of 30 samples), but was not detected in roots 14 or 22 days after exposure	Erickson et al. 2010a
Lettuce	Treatment 1: ***E. coli*** was added via manure to soil to achieve 7 log CFU g^{-1} soil prior to seeding, then lettuce phyllosphere was sampled on days 32 and 50 Treatment 2: On days 17 and 32, ***E. coli***–contaminated irrigation water (20 ml, 6 log CFU ml^{-1}) was added to uncontaminated soil in pots growing lettuce plants (avoided splash), then lettuce phyllosphere was sampled on days 27 and 41	None	■ Average *E.* coli count for both modes of introduction was 2.5 log CFU g^{-1} for lettuce phyllosphere samples collected from the middle growth stage ■ *E. coli* was not detected in late growth stage phyllosphere samples	Habteselassie et al. 2010

(*Continued*)

Table 3 (*Continued*)

Plant	Treatment conditions	Surface disinfection	Results	Reference
Tomato	Plants in steam-treated soil were irrigated with 250–350 ml of 7 log CFU ***S. enterica* Newport** ml^{-1} every 7 days and then sampled at 5 stages of growth	70% ethanol	■ Salmonellae were detected in 13 of 20 root samples, 8 of 20 stem samples, 2 of 20 leaf samples, and 2 of 32 fruit samples	Hintz et al. 2010
Cabbage	***E. coli* O157:H7–** or ***S.* Typhimurium**–contaminated manure was incorporated into soil at the time of transplantation, 56 days posttransplantation, or 105 days posttransplantation to obtain 4 or 7 log CFU g^{-1} of soil, then cabbage leaves were analyzed at harvest (120 days posttransplantation)	1% silver nitrate	■ No internalization in cabbage leaf tissues occurred when soil was contaminated at 4 log CFU g^{-1} initially ■ Internalized *E. coli* O157:H7 and *S.* Typhimurium were observed in leaves at harvest (18 of 18) when soil was contaminated at the time of transplantation with 7 log CFU g^{-1}	Ongeng et al. 2011

to a suspension of *Klebsiella pneumoniae* 342 had greater endophytic colonization than if exposed to several strains of *Salmonella* and were the least colonized by *E. coli* K-12 (Dong et al. 2003a). Franz et al. (2007) determined that more lettuce plants were contaminated endophytically with *E. coli* O157:H7 than *S.* Typhimurium when seedlings were exposed to the pathogens through soil. However, when introduced on the seeds of carrots, cress, lettuce, radish, spinach, or tomatoes, both *E. coli* O157:H7 and *Salmonella* were present within the tissue of seedlings, but *Listeria monocytogenes* was not (Jablasone et al. 2005). A similar response was observed in barley roots where *Salmonella* invaded the roots but *Listeria* spp. did not (Kutter et al. 2006). Differences in the extent of internalization have even been observed when different strains of *Salmonella* were exposed to alfalfa seedlings (Dong et al. 2003a). With lettuce plants, endophytic colonization was 59%, 85%, 93%, and 89% for *Salmonella* serovars Dublin, Enteritidis, Montevideo, and Typhimurium, respectively (Klerks et al. 2007a). These differences in responses suggest that endophytic colonization of pathogens is an active process and may not simply involve passive diffusion into the intercellular spaces of plants.

The vehicle of pathogen introduction can influence the cell exposure concentration of the pathogen at which internalization of plant roots or germinating tissues occurs. For example, when enteric pathogens in an agar-based medium (Dong et al. 2003a) or suspended in a hydroponic medium (Warriner et al. 2003a) were introduced directly onto plant roots, pathogen internalization into plant tissue occurred when exposed to bacterial cell concentrations as low as 2 log CFU ml^{-1}. In contrast, *E. coli* O157:H7 was not internalized into roots of lettuce plants grown in a growth chamber when the pathogen was applied through a soil matrix at cell populations as high as 6 log CFU g^{-1} (Zhang et al. 2009b,c). Similarly, in a field study in which *E. coli* O157:H7 was applied to soil through contaminated irrigation water, with *E. coli* O157:H7 populations in soil ranging from 2 to 5 log CFU g^{-1}, internalization of the pathogen into spinach roots was only observed in 16% of samples at one of the three application times (Erickson et al. 2010a). In a second trial, when *E. coli* O157:H7-contaminated compost was added to soil with *E. coli* O157:H7 populations as high as 4 log CFU g^{-1} soil, pathogen internalization was not observed at any sampling time up to harvest (Erickson et al. 2010a). Pathogen internalization was also not observed in lettuce or spinach seedlings when seeds were sown in field plots containing soil initially contaminated with *E. coli* O157:H7 at levels ranging from 2.5–5.4 log CFU g^{-1} (M.C. Erickson, C.C. Webb, J.C. Diez-Perez, and M.P. Doyle, unpublished data). These results suggest that some biological, chemical, or physical characteristics of soil impede pathogens from being internalized into plant roots. This conclusion is also supported by studies in which prevalence for internalization of *Salmonella* or *E. coli* O157:H7 was greater in hydroponic-grown leafy greens than in soil-cultivated leafy greens (Franz et al. 2007, Sharma et al. 2009). It was, therefore, no surprise that in a study conducted to determine the lowest *E. coli* O157:H7 cell numbers in soil that could provide consistent and significant pathogen internalization of leafy green roots from contaminated soil matrices, populations of 7 log CFU g^{-1} or higher were required (M.C. Erickson, C.C. Webb, and M.P. Doyle, unpublished data). However, sporadic internalization of *E. coli* O157:H7 in spinach roots from pathogen-contaminated soil has been observed at lower *E. coli* O157:H7 populations in soil, but in that case the soil had been pasteurized to reduce indigenous soil bacteria levels (Sharma et al. 2009). Hence, it is surmised that indigenous soil bacteria are one of the contributing factors in soil that impedes plant tissues from internalizing enteric pathogens into roots.

An observation made in a study addressing pathogen internalization via roots was that *E. coli* O157:H7 was recovered from the tissues of spinach seedlings but not from mature plants (Jablasone et al. 2005). It was suggested that the age of the plants could be an important factor in the promotion of processes that facilitate internalization, with younger plants being more

susceptible to pathogen internalization than older plants (Warriner & Namvar 2010). This premise no longer holds merit, as Mootian et al. (2009) observed internalization of *E. coli* O157:H7 in 10.4% of 30-day-old lettuce plants compared with 6.4% internalization in 12-day-old lettuce plants. It is possible the potential for internalization decreases over time following the pathogen's introduction into the soil, as exemplified by the observation of internalization of *E. coli* O157:H7 and *S.* Typhimurium in cabbage plants cultivated on soil amended with contaminated manure only at the time of transplantation (Ongeng et al. 2011). This limited time period for internalization may occur in response to the limited time period in which pathogen cells are free to move within interstitial spaces of the soil matrix. Lapidot & Yaron (2009) have determined that bacteria in close contact with soil particles adsorb to the soil particles and can move only short distances, even under saturated conditions. Similarly, transfer of furrow-inoculated *Salmonella* was not detectable across beds of cantaloupe and honeydew melon (Lopez-Velasco et al. 2011). The introduction of pathogenic cells via a water vehicle and the continued presence of water in soil were therefore suggested as key factors for bacterial cells to move to the root zone and be available for internalization. In support of this hypothesis, pathogen internalization was observed in lettuce plants when *E. coli* O157:H7–contaminated irrigation water was added to the soil matrix but not when the plants were transplanted into soil containing comparable levels of the pathogen (Mootian et al. 2009). Moreover, support for a role of soil moisture facilitating internalization was found in a recent study in which lettuce plants were initially irrigated with water containing high cell numbers of *E. coli* O157 (8 log CFU ml^{-1}) and maximum pathogen internalization occurred in lettuce, spinach, and parsley roots (2.9–3.3 log CFU g^{-1}) when soil was saturated with water compared with roots (0.4–1.7 log CFU g^{-1}) that were in soil that was only moist (M.C. Erickson, C.C. Webb, and M.P. Doyle, unpublished data).

Studies addressing internalization of enteric pathogens in plant tissue roots revealed entry mostly occurs at lateral root junctions (Cooley et al. 2003; Dong et al. 2003a,b) where root exudates are concentrated. The potential for internalization at damaged sites on roots has been addressed in several studies, but the results are conflicting. Removal of seminal root and root hairs from spinach roots (Hora et al. 2005), exposure of spinach or tomato roots to nematodes (Beuchat et al. 2003, Hora et al. 2005), and cutting off segments of lettuce roots (Urbanucci et al. 2009) do not affect the level of internalization of pathogens into these plants. In contrast, lettuce leaves harbor internally nearly 10 times the level of *Salmonella* in plants with decapitated roots compared with leaves of plants with undamaged roots (Bernstein et al. 2007a). Similarly, *E. coli* cell numbers in shoots of maize plants with decapitated roots were 27.8 times higher than those found in shoots from plants with intact roots (Bernstein et al. 2007b).

DISPOSITION OF INTERNALIZED ENTERIC PATHOGENS IN PLANT TISSUES

Once enteric pathogens enter plant tissues, the issue as to whether they continue to pose a food safety risk is dependent on the fate of those cells. Both pathogen survival and potential for systemic transfer of internalized pathogen cells within plant tissues have been addressed in multiple studies.

Under conditions in which internalization of pathogens occurred at root sites, subsequent transfer and detection in leaves or fruit has been dependent on the initial pathogen cell numbers and matrix surrounding the roots. When lettuce plants were grown in a hydroponic system containing 10^5 or 10^7 CFU ml^{-1}, surface-sterilized lettuce leaves were found to contain *E. coli* O157:H7 in 5 of 24 and 5 of 23 samples, respectively (Nthenge et al. 2007). Similarly, when lettuce seedlings were cultivated in potting soil and watered twice with a 10^9 CFU ml^{-1} inoculum, *E. coli* O157:H7, *S.* Typhimurium 110, and *S.* Typhimurium 119 were found in 29%, 23%, and 15% of

surface-sterilized leaf samples, respectively (Franz et al. 2007). Mobilization of *E. coli* O157:H7 to lettuce, spinach, and parsley aerial tissue was less frequent (7%) when roots were exposed to soil irrigated with water containing 10^8 CFU ml^{-1} (M.C. Erickson, C.C. Webb, and M.P. Doyle, unpublished data) and did not occur with lettuce or spinach plants in a field study when soil populations contained 2–5 log CFU g^{-1} (Erickson et al. 2010a). The critical role of microbial numbers in the mobilization of pathogens from root tissue was corroborated by the finding of infectious murine norovirus in lettuce leaves collected from hydroponic- or soil-cultivated plants exposed to a high norovirus inoculum and their absence in leaves of plants exposed to a low norovirus inoculum (Wei et al. 2011). In much the same manner as occurs with leafy greens, transfer of *Salmonella* from roots to leaves or fruits of tomato plants follows a similar pattern as the surrounding matrix and pathogen cell numbers vary. For example, salmonellae are associated with the stems of tomato plants grown hydroponically in an inoculated nutrient solution (Guo et al. 2002), but only sporadically from stems (8 of 20 samples), leaves (2 of 20 samples), and fruit (2 of 32 samples) of tomato plants cultivated in soil that was irrigated with 10^7 CFU ml^{-1} at seven-day intervals (Hintz et al. 2010). *Salmonella* was not detected in leaves, stems, or fruit samples taken from soil-cultivated tomato plants irrigated every other day with water containing *S.* Enteritidis at 10^5 CFU ml^{-1} (Jablasone et al. 2004) or in fruit taken from soil-cultivated plants irrigated every two weeks with 10^7 CFU ml^{-1} (Miles et al. 2009).

The disposition of pathogens internalized at leaf sites has also been investigated. Mobilization does not appear to be an issue based upon studies employing syringe infiltration of *Arabidopsis* (thale cress) and spinach leaves with *Salmonella* and *E. coli* O157:H7, respectively, and the absence of detection of these pathogens in neighboring leaves (Schikora et al. 2008, Mitra et al. 2009). However, persistence of internalized pathogen cells varies and appears to be dependent on their initial pathogen cell numbers. When *E. coli* O157:H7 was detected initially in 5 of 20 surface-sanitized leaves of spinach plants sprayed in the field with an inoculum of 10^6 CFU ml^{-1}, no internalized pathogen was detected in leaves seven days later (Erickson et al. 2010b). Similarly, *E. coli* O157:H7 internalized within leaves (25 of 40 samples) immediately following five consecutive days of a 20-min spray exposure of spinach plants to a low dose inoculum (10^3 CFU ml^{-1}) but was not detected two days later (M.C. Erickson, C.C. Webb, and M.P. Doyle, unpublished data). In contrast, internalized *E. coli* O157:H7 persisted for up to two weeks in leaves of lettuce plants spray inoculated in the field with an inoculum of 10^8 CFU ml^{-1}; however, greater survival occurred in leaves sprayed on the abaxial side (up to 14 days) compared with leaves sprayed on the adaxial side (two days) (Erickson et al. 2010b).

PLANT DEFENSES AND THEIR ROLE IN THE FATE OF INTERNALIZED ENTERIC PATHOGENS

Plant defenses participate directly in the regulation of endophytic plant pathogens (Jones & Dangl 2006, Pieterse & Dicke 2007), and it is plausible that many of these same pathways are used by plants to target internalized human enteric bacteria. Results of several studies support this concept. Iniguez et al. (2005) determined that ethylene, a signal molecule for induced system resistance in plants, decreased endophytic colonization of *S. enterica* in alfalfa roots, and determined that a *Salmonella* mutant defective in flagella and the type III secretion system colonized the interior of plants to a greater extent than the wild-type strain. Furthermore, increased *Salmonella* colonization was observed in an *Arabidopsis* mutant with defective systemic acquired resistance defenses compared with plants with active defenses (Schikora et al. 2008). Moreover, using cDNA amplified fragment length polymorphism transcriptome analysis, distinct gene expression profiles were observed for lettuce plants not colonized and those colonized by *S. enterica* serovar Dublin

(Klerks et al. 2007b). Given that a variety of stressors may activate plant defenses (Pieterse & Dicke 2007), survival of internalized enteric pathogens within plant tissue could be affected by those conditions. For example, there were less internalized *E. coli* O157:H7 in contaminated lettuce leaves exposed to cabbage loopers or thrips or that had been damaged mechanically than in leaves that were not mechanically damaged or not exposed to insects (Erickson et al. 2010c). On the basis of these limited studies, further study into the nature of human pathogen–plant interactions is warranted to understand the role of baseline or inducible plant defenses on the fate of internalized enteric pathogens.

SUMMARY POINTS

1. Internalization of plants by foodborne pathogens can occur at a number of different infiltration sites and in a number of different produce commodities, both preharvest and postharvest.
2. At exposure concentrations that would likely be encountered in the field, the data support the conclusion that pathogen infiltration through roots or seeds is not likely to occur when plants are cultivated in soil systems.
3. The presence of a moisture film appears to be a critical factor contributing to a pathogen's ability to reside on leaf surfaces and to migrate and infiltrate into stomata.
4. Studies exploring the fate of foodborne pathogens internalized into produce preharvest via either roots or leaf stomata suggest that such pathogens are transient endophytic residents when the original source of contamination is removed from the plant vicinity.
5. Enteric foodborne pathogens can lodge in stomata or be entrapped in crevices of produce exposed to contaminated water postharvest.
6. Cut produce surfaces introduced during harvesting and minimal processing furnish sites that are especially amenable to penetration by enteric pathogens.
7. Effective levels of sanitizing agents should be maintained in the wash or cooling waters during harvesting and minimal processing to avoid cross-contamination and internalization of pathogens into produce, especially at cut surfaces.
8. Further research is needed to better understand the role of plant defenses in minimizing the internalization and persistence of internalized foodborne pathogens in plant tissues.

DISCLOSURE STATEMENT

The author is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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