6
Internalization of Human Pathogens within Growing Salad Vegetables

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Introduction

It is recognized that prevention of degenerative diseases can be aided by increasing the proportion of salad vegetables in the daily diet. This has led to a rapid increase in consumer demand for salad and fruit produce, with the current global market being estimated as over US$ 130b (FDA, 2001). However, the incidence of foodborne illness associated with consumption of salad vegetables and fruits has also increased (*Figure 6.1*) (O' Mahony *et al.*, 1990; Lin *et al.*, 1996; Little *et al.*, 1997; Tauxe *et al.*, 1997; Beuchat, 1998; Kaneko *et al.*, 1999; Szabo *et al.*, 2000). The rise in foodborne illness associated with salad vegetables can be attributed partly to higher consumption rates, but also to the increase in ready-to-eat vegetable types. Here, the increased handling of produce can augment cross-contamination, and cutting/shredding operations release nutrients that could sustain bacterial growth (FDA, 1998; Cornell, 2000). The introduction of organic produce, where manure is typically applied instead of chemical fertilizers, has also been implicated. However, there is no evidence to suggest that organic produce carries a higher risk of being contaminated with human pathogens than does other produce (McGrath, 2000; Sagoo *et al.*, 2001).

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Abbreviations: EHEC, enterohaemorrhagic *Escherichia coli*; gfp, green fluorescent protein; SEM, scanning electron microscopy; UV, ultraviolet.

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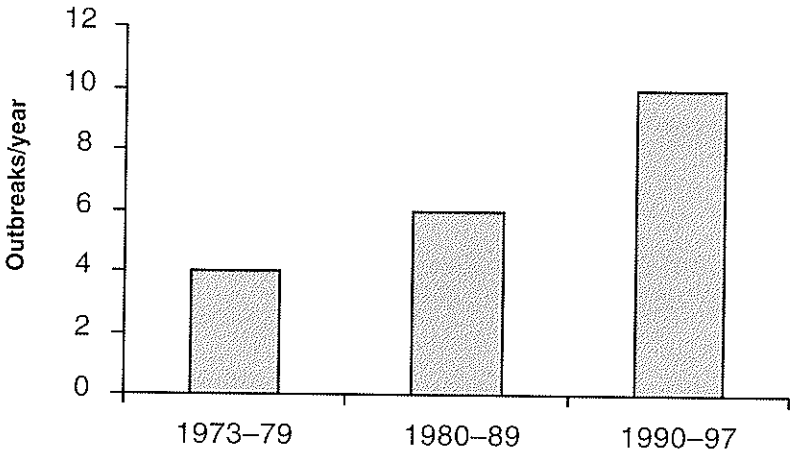

Figure 6.1. Outbreaks of foodborne illness associated with salad vegetables (source: Centre for Disease Control and Prevention, USA).

The human pathogens implicated in foodborne illness associated with salad vegetables are *Salmonella*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Yersinia enterolitica*, and *Aeromonas hydrophila* (FDA, 2001). A survey performed in the UK found that *L. monocytogenes* was present in 7% of salads (Sizmur and Walker, 1988). This figure rose to 19% in a 1990 survey (Velani and Roberts, 1991) and to 25% in 1993 (Harvey and Gilmour, 1993). Across the EU, the isolation of pathogens in salad vegetables is high, with an estimated 30% being positive for *L. monocytogenes* (Harvey and Gilmour, 1993; Garcia-Gimeno *et al.*, 1996) and 7% for *Salmonella* (Joce *et al.*, 1990). One of the largest outbreaks in the UK (and across the EU) was linked to imported lettuce contaminated with *Shigella sonnei* (Kapperud *et al.*, 1995). In global terms, the largest outbreak was recorded in Japan 1996, where over 9000 people were taken ill as a result of consuming radish sprouts contaminated with *E. coli* O157:H7 (National Institute of Infectious Diseases, 1997).

The increase in foodborne illness associated with salad vegetables and fruit has led to several reviews covering the major foodborne illness outbreaks, human pathogens of most concern, and post-harvest processing (Lund, 1992; Beuchat, 1998; Carmichael *et al.*, 1999; Heard, 2000; FDA, 2001). In the following review, a hitherto understudied aspect of salad vegetable contamination, that of the internalization of human pathogens within pre-harvest growing salad vegetables, will be described.

Post-harvest biocidal washing of salad vegetables

The post-harvest washing of salad vegetables is the primary intervention step to remove field-acquired contamination (Brackett, 1992; Beuchat and Ryu, 1997; Beuchat, 1999; Carmichael *et al.*, 1999; NACMCF, 1999a; Taormina and Beuchat, 1999). The wash process is composed of an initial treatment in potable water to remove soil, and is followed by a biocidal wash typically containing sodium hypochlorite as the antimicrobial agent. This can be performed in a batch or continuous

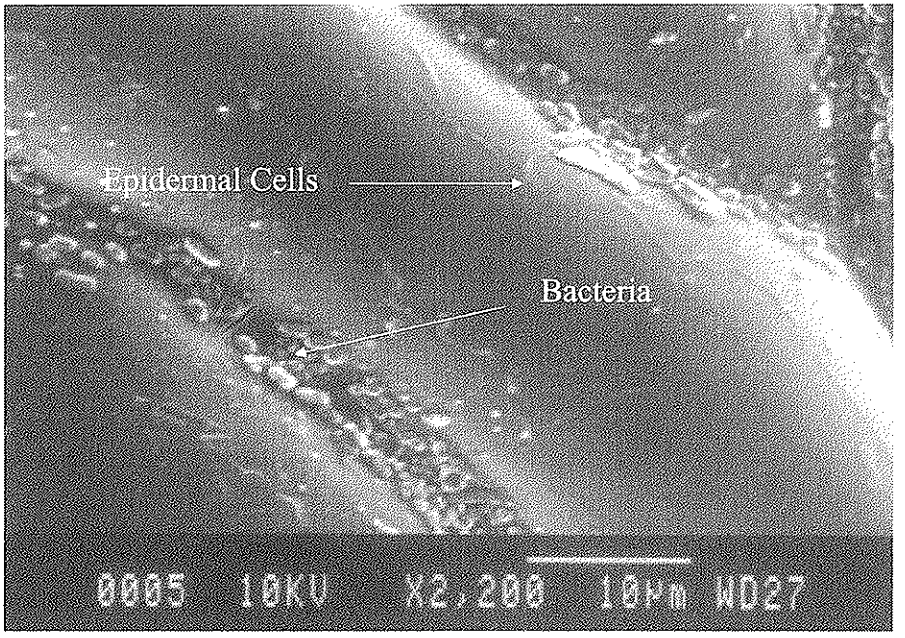

Figure 6.2. Cryogenic scanning electron micrograph of bacteria on the surface of an unwashed salad spinach leaf (courtesy of Keith Warriner, Rachel Jones and William M. Waites, University of Nottingham, unpublished data).

process, spray or dip, depending on the nature of the product (Beuchat *et al.*, 1998). However, regardless of the type of washing applied, a reduction of <2 log cfu/g of bacterial numbers is achieved, leaving a significant residual level of survivors. The limitation of currently used hypochlorite-based biocidal washing has been proposed to be through the rapid sequestering of free chlorine via organic matter (White, 1972), the low concentration applied (100–200 ppm) and inappropriate water pH (FDA, 1998). This has led to the application of alternative antimicrobial agents, such as trisodium phosphate, chlorine dioxide, ozone, hydrogen peroxide, and peroxyacetic acid (Sapers and Simmons, 1998; Singh *et al.*, 2002). The potential of organic acids in decontaminating vegetables/fruits (as part of an organic process) has also received attention (Ohsone *et al.*, 1999). Such chlorine substitutes are effective at decreasing bacterial cell numbers in free suspension, or when bacteria are artificially inoculated on the surface of vegetables/fruits. However, the efficacy of decontaminating naturally contaminated produce is, at best, only marginally improved over chlorine (Zhuang and Beuchat, 1996). This would suggest that the limitation of post-harvest biocidal washing is not solely attributed to the stability of antimicrobial agents, but also the interaction of bacteria with growing plants in the field. It is well documented that once pathogens become associated with salad vegetables, they can remain viable, even in the presence of a competitive microflora and/or in modified air packaging (Francis and O'Beirne, 1998). In this respect, given the low efficacy of post-harvest biocidal washing to remove contamination from vegetables, the microbiological quality of the raw product is of great importance. There are three basic modes of bacterial interaction

with growing plants. Specifically, bacteria on the surface of plants (epiphytes), those that gain access through damaged tissue, and endophytic bacteria that can become internalized via root colonization.

Epiphytic bacteria

Epiphyte (surface) bacterial populations are typically Gram negative, with pseudomonads forming the dominant type recovered (Lund, 1992). The growth of bacteria on leaf surfaces is supported by the sugars and amino acids deposited from guttation fluid during transpiration (Mercier and Lindow, 2000). Bacteria, including human pathogens (Brandl and Mandrell, 2000), tend to aggregate between the grooves of epidermal cells, but less so across the waxy epidermal layer (*Figure 6.2*). Although the leaf surface can provide a habitat for human pathogens, desiccation and exposure to solar UV radiation can be detrimental to cell viability (Suslow, 2001; Brandl and Mandrell, 2002). However, a degree of protection of bacterial cells can be achieved through incorporation into biofilms. It has been estimated that 10–40% of the total bacteria on the surface of parsley and broad-leaf endive are associated with biofilms (Morris *et al.*, 1998). The resistance of biofilms to chemical disinfectants is well established, and is thought to be a limiting factor of post-harvest biocidal wash treatment (Carmichael *et al.*, 1999; Fett, 2000; Takeuchi and Frank, 2000). However, when washed leaves are viewed under SEM, very few bacterial cells are visible (*Figure 6.3*), suggesting that surface-located bacteria are relatively easily removed.

Internalization of bacteria through natural openings and damaged plant tissue

It has been demonstrated that human pathogens can also be protected from biocidal washing by being located in the sub-surface structures of plants (Ryall and Pentzer, 1982; Seo and Frank, 1999; Han *et al.*, 2000; Takeuchi and Frank, 2000, 2001; Burnett and Beuchat, 2001). The leaf surface has numerous stomata that can provide entry into the substomatal cavity (*Figure 6.4*), and bacteria can reside within the inner leaf spaces, thereby being protected from biocidal wash treatment. *E. coli* O157:H7 inoculated onto lettuce leaves has been shown to survive biocidal washes by being located in stomata, or to a greater extent, when they are able to find entry into the inner part of the leaf through cut edges (Beuchat, 1999; Seo and Frank, 1999). A similar finding has been reported for apples inoculated with *E. coli* O157:H7 (Buchanan *et al.*, 1999), and tomatoes with *Salmonella* (Zhuang *et al.*, 1995). Damage caused by spoilage bacteria/fungi can also enable human pathogens to enter the inner plant tissue and thereby become protected (Wells and Butterfield, 1997, 1999).

Endophytic bacteria

The presence of microorganisms within healthy tissue of vegetables was described in the 1960s (Samish and Etinger-Tulczynsha, 1962), but only very recently has it been accepted that bacteria can reside in the internal structures of undamaged plants (Sturz and Nowak, 2000). The endophytic bacterial population of plants is known to be diverse, comprising of both Gram-positive and Gram-negative cells (Bell *et al.*, 1995;

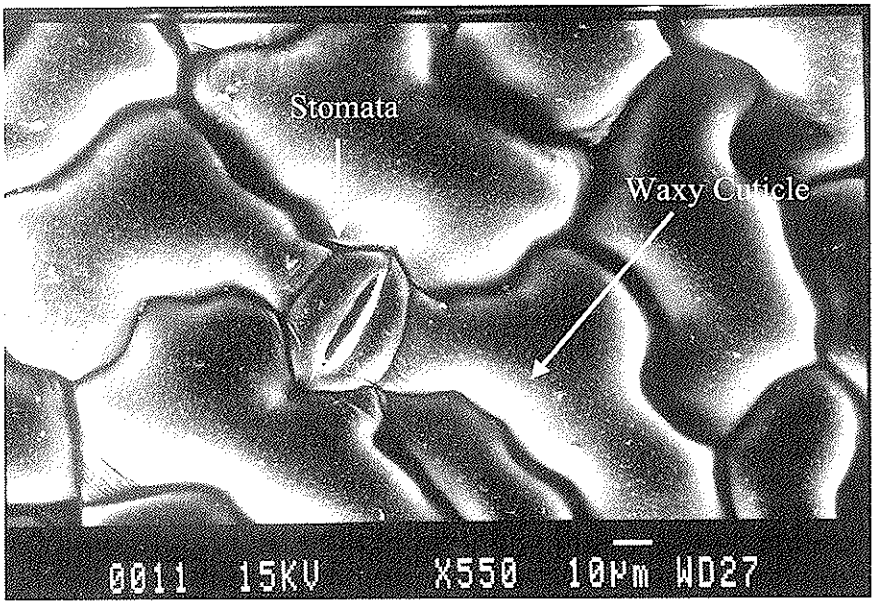

Figure 6.3. Cryogenic scanning electron micrograph of a washed salad spinach leaf (courtesy of Keith Warriner, Rachel Jones and William M. Waites, University of Nottingham, unpublished data).

Quadt-Hallman *et al.*, 1997a,b; Kobayashi and Palumbo, 2000). To date, the majority of work has focused on plant pathogens and those that promote growth, or suppress pathogens (Weller, 1988; Stoltzfus *et al.*, 1997; Chanway, 1998; James and Olivares, 1998; Reinhold-Hurek and Hurek, 1998; Azevedo *et al.*, 2000; Sturz and Nowak, 2000; Elbeltagy *et al.*, 2001). However, up to 50% of bacterial types recovered have no adverse or positive effect on plant health (Sturz *et al.*, 1998), suggesting that specialized invasion mechanisms may not be entirely necessary for internalization (Bell *et al.*, 1995; Wilson, 1995).

ESTABLISHMENT OF ENDOPHYTIC POPULATIONS

In recent years, a substantial research effort has been devoted to attempts to modify the endophytic populations of plants in order to increase the numbers of beneficial bacteria present. However, such studies have met with limited success, primarily due to the large knowledge gaps that exist regarding the factors affecting the composition of the bacterial endophytic microflora (Hallmann *et al.*, 1997; Sturz and Nowak, 2000). It is accepted that adapted rhizobacteria can compete against artificially introduced bacteria, but it is likely that additional factors are involved (Hozore and Alexander, 1991).

The introduction of bacteria into the endophytic microflora of plants can occur from populations present in seeds. For example, plant pathogenic pseudomonads have been known to infiltrate seeds and become established in the mature plant (Baker, 1972). Bacteria can also find access to the inner parts of plants during seed

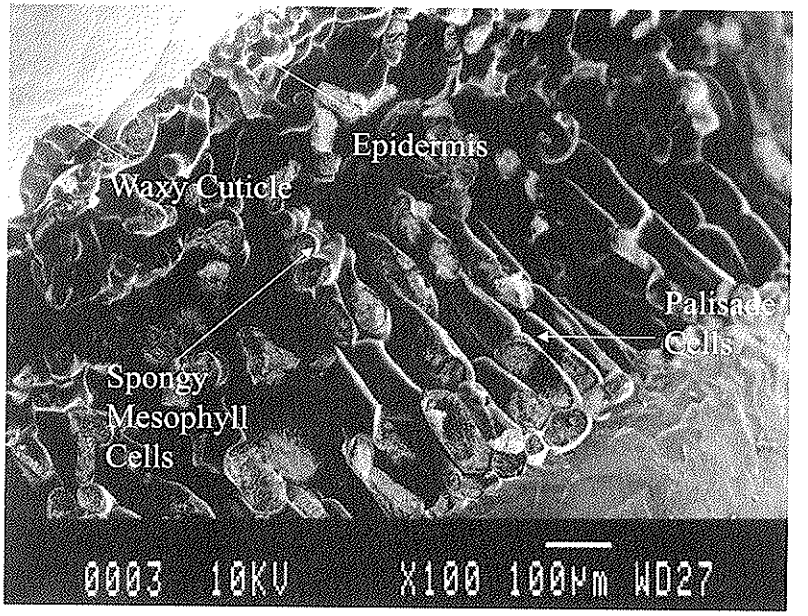

Figure 6.4. Cryogenic scanning electron micrograph of a spinach leaf in cross section. Shown is the epidermis covered by the waxy cuticle layer. Palisade cells are filled with chloroplasts and carry out photosynthesis in the leaf. The spongy mesophyll cells act as a temporary nutrient store and are surrounded by air gaps that could potentially provide protective sites for bacteria (courtesy of Keith Warriner, Rachel Jones and William M. Waites, University of Nottingham, unpublished data).

germination. In this period, the seed releases a mixture of carbohydrates and peptides that can attract surrounding bacteria in the rhizosphere (Andrews *et al.*, 1982; Joce *et al.*, 1990; Hara-Kudo *et al.*, 1997; Troxler *et al.*, 1997). Although exudates support bacterial growth within the rhizosphere, access into the inner apoplastic space is restricted by protective border cells on the root surface (Hawes *et al.*, 2000). However, bacteria can gain entry via germinating radicals (Gagne *et al.*, 1987; Ndoye *et al.*, 1994; Barraquio *et al.*, 1997) or secondary roots (Agarwal and Shende, 1987), where they can persist in localized sites (Hallman *et al.*, 1997). Bacteria localized in the apoplastic fluid surrounding the root cells (symplast) are restricted in entering the xylem via the Casparian strip, which is a thickened cell wall impregnated with a water-insoluble substance, suberin (*Figure 6.5*). In immature plants, the protective structures are not fully formed, and enable entry of bacteria into the xylem, which develops into a continuous tube transporting water and minerals up to the leaves (Peterson *et al.*, 1981; Kloepper *et al.*, 1992; Lamb *et al.*, 1996; Troxler *et al.*, 1997). Although internalization of bacteria is most prominent in young plants, it has been reported that the rhizobacterium *Pseudomonas aureofaciens* can become internalized into developed corn plants (Lamb *et al.*, 1996). There is also evidence to suggest partial localized degradation of protective structures by plant virus infection (Brugidou *et al.*, 1998), which could potentially enable access to the xylem by bacteria.

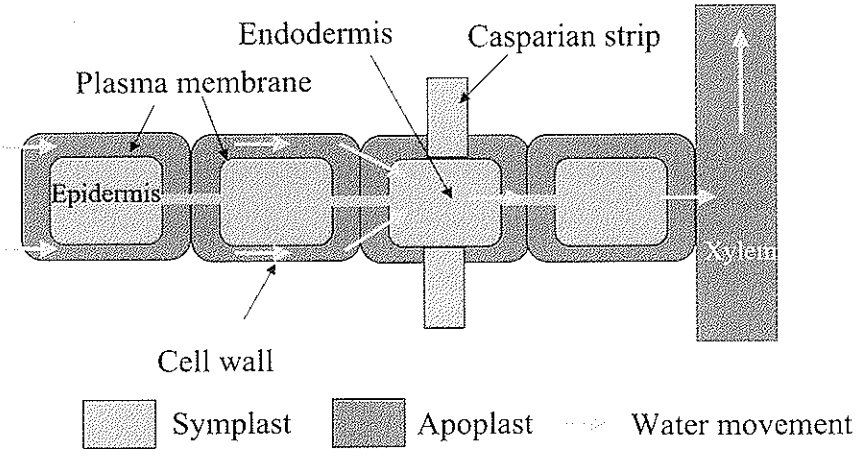

Figure 6.5. Schematic representation of structures found within plant roots. The apoplast fills the space between the cell wall and plant cells. To enter into the xylem, water must pass through the symplast of the endodermal cells. The movement of bacteria is restricted by the plant cell plasma membranes and the Casparian strip within the root.

Human pathogens as endophytes

Compared to human pathogens, such as *L. monocytogenes* (Dowe *et al.*, 1997) and *Aeromonas* spp. (Isonhood and Drake, 2002), that naturally inhabit the soil/water environment, the survival of enteric human pathogens (for example, *E. coli* and *Salmonella)* would be considered to be low. However, both *E. coli* O157:H7 (Barker *et al.*, 1999, Bolton *et al.*, 1999) and *Salmonella* (Marsh *et al.*, 1998) have been demonstrated to survive in soil over prolonged periods of time (>80 days). The persistence of enteric pathogens has been linked to the climatic conditions. For example, *Salmonella enterica* serovar Typhimurium and *E. coli* inoculated into manure-fertilized soil persisted to a greater extent in mid–late summer compared to in spring (Natvig *et al.*, 2002). It has also been reported that *E. coli* O157:H7 persists to a greater extent in soil with rooted grass (only a 1–2 log cfu/g reduction after 130 days at 18°C), compared to when present in river water or cattle manure (Maule, 2000). In a similar study, the survival of *E. coli* O157:H7 in soil was enhanced by colonizing the roots of rye grass or alfalfa plants (Gagliardi and Karns, 2002). It was also noted that clover or hairy vetch did not enhance the persistence of *E. coli* O157:H7, suggesting that the effect is plant specific. The same effect has been reported for *L. monocytogenes*, where association with radish or parsley, but not carrots, enhanced the survival of the bacterium, compared to when inoculated into soil alone (Al-Ghazali and Al-Azawi, 1990; van Renterghem *et al.*, 1991).

The key to successful colonization of plants is the ability of the bacterium to utilize the carbon and energy sources released by roots (Roberts *et al.*, 2000). Interestingly, Ji and Wilson (2002) were able to predict the success of biocontrol bacteria to suppress the plant pathogen *Ps. syringae* by determining the nutritional similarity index (NOI). Here, 52 carbon sources known to be present in tomato plants were

tested against a range of bacteria. By using catabolic mutants of *Ps. syringae* that had a range of NOI of between 0.07 and 0.90, a direct correlation between root colonization and nutritional similarity index was found. *E. coli* (used as a control) had an NOI of 0.83 relative to *Ps. syringae*, and was comparable to successful root colonizers. Although the authors had not determined the interaction of *E. coli* with tomato plants, the NOI model would suggest that the bacterium may compete successfully in the rhizosphere.

INTERNALIZATION OF HUMAN PATHOGENS IN DIFFERENT PLANT TYPES

Given that human pathogens can colonize certain plant roots, there is a potential for such bacteria to become internalized into the endophytic microflora, but this has only been studied in a narrow range of vegetables and fruits. Such studies have been aided by the advent of cell labelling techniques exploiting green fluorescent protein (gfp) in combination with laser confocal microscopy (Dumas *et al.*, 1999; Brandl and Mandrell, 2000). Green fluorescent protein is a fluorescent protein originally isolated from the jellyfish *Aequorea victoria*. The key benefit of gfp is the ability to fluoresce under UV light in the absence of an energy source or other cellular co-factors, thereby enabling *in situ* visualization with minimum disruption to cell physiology. The gene encoding for gfp can be readily inserted and expressed in bacterial cells using plasmid vectors. However, for the plasmid to be retained and replicated within the host cell, selective pressure (typically using an antibiotic) needs to be applied. Therefore, when studying plant–microbial interactions over extended periods where selective agents cannot be used, the gfp phenotype can be readily lost.

Sprouted seeds

The internalization of human pathogens into growing plants has been identified in sprouted seeds (radish and alfalfa). The specific interest in sprouted seeds has arisen due to the large number of foodborne illness cases associated with such products, and the failure of post-harvest washing to remove contamination (NACMCF, 1999b). The main reason for this is that the high humidity (RH >90%) and temperature (20–25°C) used for sprout cultivation is also suitable for the growth of human pathogens (de Roever, 1999). Studies have shown that radish (Hara-Kudo *et al.*, 1997; Itoh *et al.*, 1998) and alfalfa (Joce *et al.*, 1990; Ponka *et al.*, 1995; Mahon *et al.*, 1997; Taormina and Beuchat, 1999) sprouts cultivated from seeds inoculated with either *E. coli* O157:H7 or *Salmonella* typically internalize the bacteria within plant structures so that they survive surface sterilization treatment, even by potent antimicrobial agents such as $HgCl_2$. Gandhi *et al.* (2001) inoculated alfalfa seeds with gfp-tagged *Salmonella* Stanley. The bacterium was found in the sub-surface areas of the root, hypocotyls, and cotyledons of the formed sprouts. A similar distribution of *E. coli* O157:H7 on alfalfa sprouts derived from inoculated seeds has also been reported (Taormina and Beuchat, 1999). However, Charkowski *et al.* (2002) reported differences in the growth and distribution of gfp-tagged *Salmonella enterica* and *E. coli* O157:H7 on alfalfa sprouts. *S. enterica* reached log 3.2 cfu/g, compared with *E. coli* (log 2.3 cfu/g) after two days of sprouting. *E. coli* O157:H7 preferentially colonized the roots, whilst *Salmonella* colonized the seed coat and roots (Charkowski *et al.*, 2002). Itoh *et al.*

(1998) inoculated radish seeds with *E. coli* O157:H7 and, by using immunofluorescent microscopy, the bacteria could be visualized on the inner tissue of stoma, and beneath the epidermis of the hypocotyls of the sprouts.

Lettuce

Lettuce has been used as a model vegetable to demonstrate the internalization of human pathogens primarily because of its commercial importance. Watchel *et al.* (2002a) determined the interaction of gfp-labelled non-pathogenic and EHEC *E. coli* with lettuce seedlings in an adherence assay. Here, 48 h germinated lettuce seeds were introduced into suspensions of *E. coli* (*ca.* 10^6 cfu/ml) and incubated overnight at 20°C. Interestingly, the pathogenic *E. coli* cells adhered to the roots to a greater extent than those of the non-pathogenic strains tested. The authors suggested that this could be due to the attachment of the bundle-forming pili of pathogenic *E. coli*, which is implicated in cell wall attachment in animals (Giron *et al.*, 1991). However, when additional non-pathogenic strains were tested, the attachment was found to be comparable with the O157:H7 strains studied. When lettuce seedlings were viewed under a confocal microscope, the bacteria were observed within the deep grooves and tips of seed coats, root hairs, and the emerging radical. The same authors also cultivated lettuce within soil microcosms irrigated with water containing different inoculation levels (10^8, 10^6, 10^4 and 10^2 cfu/ml) of *E. coli* O157:H7. The planted lettuce seeds were grown in the inoculated soil for up to 10 days, and *E. coli* counts associated with the roots, hypocotyl, and cotyledon subsequently determined (Watchel *et al.*, 2002a). At the highest dose level, but not at lower cell densities, *E. coli* was associated with the roots of plants by day 3. *E. coli* numbers at the lower doses progressively increased over the cultivation period to reach levels of 10^3–10^4 on roots. Although the majority of *E. coli* was recovered from the roots, lower numbers (*ca.* 2 log cfu/g) were associated with hypocotyls and cotyledons of 10-day-old lettuce plants. Evidence that the *E. coli* had become internalized into the inner plant tissue was obtained using confocal laser microscopy. Here, *E. coli* was observed within the vascular system of hypocotyls (Watchel *et al.*, 2002a).

Soloman *et al.* (2002) propagated lettuce seedlings in soil inoculated with gfp-labelled *E. coli* O157:H7 (at cell densities of 10^8, 10^6 or 10^4 cfu/g). At days 3, 6 and 9, samples were taken with the roots separated from the leaves. In this instance, the plant samples were washed to remove surface-located bacteria and subsequently surface sterilized using a combination of 80% v/v ethanol and 0.1% $HgCl_2$. The authors reported that *E. coli* O157:H7 could be recovered from the internal tissue of seedlings inoculated with the highest cell density (i.e. 10^8 cfu/g), but not at the lower inoculation levels applied. Fluorescent microscopy of seedlings showed that *E. coli* was present at depths of up to 45 μm below the outer leaf surface. Subsequent experiments used contaminated irrigation water, containing 10^7 cfu/ml *E. coli* O157:H7, to water 50-day-old plants. Care was taken to prevent direct contact of the leaves with soil. *E. coli* O157:H7 was recovered from the leaves, but as no surface sterilization treatment was performed, it is unclear whether these were internalized (Soloman *et al.*, 2002).

Cabbage

Cabbage crops accidentally irrigated with creek water contaminated with *E. coli*

resulted in the organism being recovered from the roots of plants, but not the edible leaves (Watchel *et al.*, 2002b). No studies were performed to determine if the *E. coli* had been internalized. From ribotyping studies, six different *E. coli* types (all non-pathogenic) were recovered from cabbage roots. When the different *E. coli* strains were introduced to lettuce seedlings and incubated overnight, a range of adherence strengths were observed, ranging from very high to low (Watchel *et al.*, 2002b). This reinforced the view that the interaction of *E. coli* (and presumably other human pathogens) is strain dependent.

Tomatoes

Salmonella inoculated on to flowers and stems of tomatoes survived for 49 days, and were recovered internally from the ripened fruit (Guo *et al.*, 2001). Contact of tomatoes with soil inoculated with *Salmonella* also enabled the bacterium to infiltrate into the inner tissue (Guo *et al.*, 2002a). The same authors also observed that *Salmonella* can be internalized into the inner tissue of tomato plants in hydroponic nutrient solution inoculated with log 4.46–4.65 cfu/ml of the pathogen. Here, five different *Salmonella* were introduced into hydroponic nutrient solution of 7-day-old tomato seedlings. One set of plants had roots removed, whereas the other set remained intact (Guo *et al.*, 2002b). However, in this study, the authors did not rely on surface sterilization to demonstrate internalization of *Salmonella*. Instead, conclusions were based on a comparison of the number of *Salmonella* recovered from hypocotyls and cotyledons derived from intact plants (where the roots would form a barrier to internalization), or those that had been detached from the roots. Within 1 day of exposure to the *Salmonella* cocktail, the pathogen had been recovered from the stems (3.01 log cfu/g) and hypocotyls (3.40 log cfu/g) of plants, regardless of whether the roots were intact or detached (Guo *et al.*, 2002b). From analysis of the cells recovered from tomato plants, *Salmonella* serotype Montevideo was most prevalent. This may have been expected, considering that *S.* Montevideo has previously been implicated in several foodborne illness outbreaks associated with tomatoes (Lin and Win, 1997).

Fruit

It has been reported that *Bacillus subtilis* L-forms (i.e. those with modified or no cell wall) introduced into the stolons of strawberry plants not only remained viable but also moved freely within the plant tissues and into daughter plantlets (Ferguson *et al.*, 2000). Intact fruit on trees have very low bacterial counts, and no evidence has been obtained to suggest that human pathogenic bacteria can internalize into produce during the ripening period (Riordan *et al.*, 2001). However, apples soaked in suspensions of *E. coli* O157:H7 readily take up the bacterium into the inner core tissues (Buchanan *et al.*, 1999) and these bacteria survive biocidal washing (Burnett and Beuchat, 2002). Internalization of bacteria into apples can also occur if fruit is dropped on to contaminated soil, or possibly by contamination from insects or birds (Wallace *et al.*, 1997).

E. coli O157:H7 (10^7 cfu/ml) inoculated on to stem scars of oranges were recovered from the internal tissue of the fruit after a 3 h storage period at 4°C (FDA, 1999). A further storage time of 5 days resulted in the decline of *E. coli* numbers to *ca.* 0.6 log cfu/g. However, numbers on oranges stored at 21°C increased by 2 log cfu/g. The

survival of *E. coli* O157:H7 was not associated with the high acid resistance of the bacterium, but its protection by being located in segregated vesicles within the orange (FDA, 1999).

INTERNALIZATION OF ENTERIC VIRUSES INTO GROWING PLANTS

Seymour and Appleton (2001) have provided a review of the association of enteric viruses with salad vegetables and fruit. Numerous foodborne illness outbreaks associated with lettuce (Rosenblum *et al.*, 1990; Nygard *et al.*, 2001), strawberries (Niu *et al.*, 1992), diced tomatoes (Williams *et al.*, 1995), and raspberries (Noah, 1981) contaminated with enteric viruses have been reported. The persistence of enteric viruses has been found to be plant dependent. For example, hepatitis A persists for longer on lettuce, compared to carrot or fennel (Croci *et al.*, 2002). The authors also noted that current post-harvest biocidal washing was not totally effective at removing the virus from artificially inoculated salad vegetables (Croci *et al.*, 2002). Poor sanitation and handling has been proposed to be a key source of viruses recovered from fruit and vegetables (Seymour and Appleton, 2001). The possibility that enteric viruses could interact with growing plants in the field has not been considered to any great extent, due to the inability of viruses to multiply in the environment and also their low resistance to UV solar radiation. However, by gaining access to protected sites, such as roots or closed foliage, the survival time can be up to 60 days (Smith, 1982). The internalization of enteric viruses into the inner tissue of plants has not been shown, but the potential has been realized for several years (Tierney *et al.*, 1977; Smith, 1982). Studies have been performed with tomato plants grown in soil irrigated with water containing poliovirus (10^3–10^4 pfu/ml). Here, the poliovirus was occasionally recovered from leaves of plants, but none were detected in the tomato fruit, even when the roots were artificially damaged (Oron *et al.*, 1995). The poliovirus application was far higher than typically encountered in the natural environment (0.1–10 pfu/ml). Therefore, the risk of internalization of enteric viruses was considered to be low (Oron *et al.*, 1995).

The internalization of feline calicivirus and bacteriophage MS2 into growing cress cultivated in inoculated soil has been reported (Kirkham *et al.*, 2002). When the cress was harvested, each virus type could be recovered from the edible portion of plants. The authors did not rely on surface sterilization to prove internalization. Instead, the inoculated soil was overlaid with agar to prevent virus transfer to the upper part of the growing plant (Kirkham *et al.*, 2002).

The uptake of viruses may be viewed as a significant risk, although this may provide an opportunity in controlling human pathogenic bacteria using bacteriophages. A study using apples and melons inoculated with *Salmonella* and treated with lytic phage decreased pathogen numbers by >3 log cfu/g (Leverentz *et al.*, 2001). However, the acidic pH of the fruit was thought to be detrimental to phage survival, thereby limiting its efficacy to completely inactivate the inoculated *Salmonella*.

Conclusions

It is established that the presence of endophytic bacteria in salad vegetables and fruit can limit the efficacy of post-harvest biocidal wash treatments. The evidence obtained

to date would suggest that internalization of pathogens in seedlings (alfalfa, radish, lettuce, and tomato) is relatively common. However, very little work has been directed at establishing the persistence of human pathogens (bacteria and enteric viruses) up to the point of harvest. It is also noteworthy that many studies use artificially high inoculum levels (up to 10^8 cfu/g) that may not be encountered in the natural environment. In addition, the interaction of human pathogenic bacteria is dependent on both the bacterial strain and plant type. This would make risk assessment difficult in light of the wide diversity of crops, cultivated under a wide range of climatic conditions, and exposed to an abundance of different human pathogen strains. The potential of plant pathogens and other rhizobacteria to facilitate the internalization of human pathogens in the field also has to be considered.

An added problem is the means by which low numbers of internalized human pathogens can be detected, considering that only 10 cells of *E. coli* O157:H7 can cause infection. By using gfp-labelled bacteria in conjunction with confocal laser microscopy, the numbers of bacteria have to be high and close to the surface of the plant tissue to be visualized. In contrast, relying on simple plate counts of macerated samples, acidic products or antibacterial/antiviral compounds released could kill or inhibit bacteria/viruses (Konowalchuk and Spires, 1976; Burnett and Beuchat, 2001). It is likely that any human pathogens internalized within plant tissue would be localized in compartments away from such antimicrobial agents. Regardless of this factor, the potential of human pathogens to become internalized within growing plants enforces the conclusion that post-harvest biocidal washing cannot be relied upon to remove contamination. As a result, it is clear that down-stream intervention steps are required. However, considering that in the field it is not possible to control all sources of contamination, this may prove problematic.

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