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ORIGINAL ARTICLE

Variable agronomic practices, cultivar, strain source and initial contamination dose differentially affect survival of *Escherichia coli* on spinach

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Keywords
Escherichia coli O157:H7, phyllosphere, rhizosphere, spinach, strain source, surrogate.

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Abstract

Aims: Greenhouse and field trials were conducted under different agronomic practices and inoculum doses of environmental *Escherichia coli* and attenuated *E. coli* O157:H7, to comparatively determine whether these factors influence their survival on leaves and within the rhizosphere.

Methods and Results: *Hydroponic conditions: E. coli* spray-inoculated at log 4 CFU ml^{-1} was recovered from leaf surfaces at a mean population of 1·6 log CFU g^{-1} at 15 days. *E. coli* O157:H7 sprayed at log 2 or 4 CFU ml^{-1} levelled off on spinach leaf surfaces at a mean average population of 1·4 log CFU g^{-1} after 14 days, regardless of initial dose. Quantitative recovery was inconsistent across leaf developmental age. *Field conditions:* Average populations of *E. coli* O157:H7 spray-inoculated at log 1·45 or 3·4 CFU m^{-2} levelled off at log 1·2 CFU g^{-1} over a 14-day period. Pathogen recovery from leaves was inconsistent when compared to regularly positive detection on basal shoot tissue. Pathogen recovery from soil was inconsistent among sampling locations. Moisture content varied up to 40% DW and was associated with 50% ($P < 0·05$) decrease in positive locations for *E. coli* O157:H7 but not for *E. coli*.

Conclusions: Overall, similar populations of environmental *E. coli* and *E. coli* O157:H7 were recovered from plants despite differences in inoculum dose and agronomic conditions. Strain source had a significant impact on the quantitative level and duration of survival on leaves and in soil. Water availability appeared to be the determinant factor in survival of *E. coli* and *E. coli* O157:H7; however, *E. coli* showed greater environmental fitness.

Significance and Impact of the Study: Persistence of surrogate, indicator *E. coli* and *E. coli* O157:H7, irrespective of variable growing conditions in spinach is predominantly limited by water availability, strain source and localization within the plant. These findings are anticipated to ultimately be adopted into routine and investigative pathogen testing protocols and mechanical harvest practices of spinach.

Introduction

The ability of *Escherichia coli* to survive and multiply in various environments outside an animal host, such as soil, water and on plants, is dependent on the availability of resources, energy status, inducible stress adaptations and the abiotic conditions present in those environments (Winfield and Groisman 2003). Resource accessibility and availability are related with local conditions intrinsic of the habitat, but in general as a chemoheterotroph; pH, salt, water and carbon source availability limit its survival (Wilson and Lindow 1994; Beattie and Lindow 1999;

Wilson *et al.* 1999; Vital *et al.* 2008). Significant uncertainty surrounds the specific temporal aspects of postcontamination survival sequences and outcomes during leafy greens production despite recent efforts to describe host: pathogen interactions under growth chamber (Patel *et al.* 2009), greenhouse (Pu *et al.* 2009) and field conditions (Erickson *et al.* 2010a; Wood *et al.* 2010; Moyne *et al.* 2011). The degree to which these reports accurately reflect the fate and persistence of *E. coli* O157:H7 under variable conditions, including agronomic practices, cultivar, associated microbiota, and microclimates within the major U. S. production centre, the Central Coast of California, is left largely to speculation.

The occurrence of a contamination event by strains of *E. coli* O157:H7 is of great concern because of its reported low infectious dose, associated low dose–response thresholds among susceptible populations, and the potential to remain viable in the environment as non-culturable cells (Kolling and Matthews 2001; Oliver 2005; Liu *et al.* 2010; Van Elsas *et al.* 2011). To better design strategic and risk-based sampling regimes to detect this pathogen, it will be important to more fully characterize the plant host conditions that affect survival of a broader group of *E. coli* in these habitats. It has been shown that induction of adaptive mechanisms to starvation leads to its persistence within the harsh phyllosphere environment (Brandl 2006). All pathogenic strains of *E. coli*, some commensal *E. coli,* and a broader group of environmental *E. coli* contain genomic regions that encode genes associated with traits for adherence, invasion, colonization and transport functions that are involved in their survival in soil and water (Touchon *et al.* 2009). Moreover, RpoS proteins of *E. coli* have a global role in adaptation and tolerance to diverse stress conditions including high osmolarity (Muffler *et al.* 1996) and low pH (Bearson *et al.* 1996), while for *E. coli* O157:H7, survival and growth were reported in sterile freshwater with low carbon concentrations (Vital *et al.* 2008). The objective of this study was to compare the survival of environmental (generic) *E. coli* and attenuated (nontoxigenic) *E. coli* O157:H7 populations on spinach grown under hydroponic, as a model system, and field conditions in a major commercial production region. The potential for survival in soil as well as spatial distribution on the plant by the applied attenuated *E. coli* O157:H7 was also evaluated.

Materials and methods

General growing conditions

Soilless media: Spinach seeds (*Spinacia oleracea* L. cv Whale, Shasta) were grown in hydroponic systems as described by Gutiérrez-Rodríguez (2009), and similarly described by Sharma *et al.* (2009). A total of four experiments, two in hydroponic conditions and two in coir–vermiculite horticultural mix, were conducted. Plants were transplanted into the hydroponic system when they developed 4–5 fully expanded leaves.

The nutrient solution was composed of 150 ppm total *N* at a pH of 5·8, Ec (electrical conductivity) of 1·8 dS m^{-1}, and the solution was replaced weekly. For analysis, plants were divided into different organs: leaf 6 (older), leaf 12 (younger), the basal region of shoot emergence, small leaves (unexpanded leaves), other leaves besides leaf 6 and 12 known as 'leaf material', roots and the potting mix. The standard growing cycle was 21 days. Harvested plant material was immediately processed after collection.

Field evaluations

Spinach seeds (*Spinacia oleracea* L. cv Barbados and Avenger) were sown following normal commercial practices on 152-cm-wide raised seedbeds during the months of June through November of 2009 in the Salinas Valley of California. Air and soil temperature during spinach cultivation is described in Fig. 1 and highlights standard industry growing conditions during this time of the year in the Salinas Valley. Fields were managed under standard fertilizer and pest management practices by cooperating growers. Total applied *N* fertilization was 168 kg ha^{-1} in the form of ammonium nitrate and urea (UN32) as the main *N* source. Plants were cultivated in a sandy loam soil series composed of 72% sand, 17% silt and 11% clay.

Inoculation studies

Plants cultivated in the greenhouse were inoculated with individual generic strains of *E. coli* or a cocktail of three

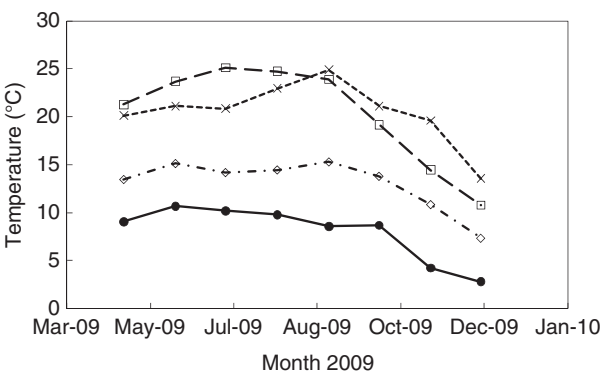

Figure 1 Average air and soil temperature in the Salinas Valley during May through December 2009 as reported by CIMIS weather station no. 89. Symbols represent (x) average maximum temperature; (•) average minimum temperature; (◇) average air temperature and (□) average soil temperature.

strains (TVS 353, 354 and 355, originally isolated from irrigation water, lettuce and a sandy loam soil, respectively, in the Salinas region). *E. coli* O157:H7 strains used in this study are non-toxigenic isolates (lacking *stx*1 and *stx*2) ATCC 700728 (*a*PTVS 154) and ATCC 43888 (*a*PTVS 155) and will be known through the text as *E. coli* O157:H7 cocktail. An antibiotic-resistant derivative strain for tolerance to rifampicin (80–100 mg l^{-1}) was isolated for all isolates used in this study via spontaneous mutation and used to minimize interference with other bacteria and to facilitate the detection and recovery (Beuchat *et al.* 2001).

Both generic *E. coli* and *E. coli* O157:H7 strains were separately grown in 9 ml of tryptic soy broth (BD Diagnostics, Sparks, MD, USA) supplemented with 80 mg l^{-1} of rifampicin at 37°C for 18 h. After incubation, cultures were centrifuged at 1500 ***g*** for 10 min. The pellet was resuspended and washed twice with Butterfield's phosphate buffer (BPB) (Whatman Inc., Piscataway, NJ, USA), and 200 μl was plated on TSA + PPMR [Tryptic soy agar (TSA; BD Diagnostics, Sparks, MD, USA)] amended with MUG (4-methylumbelliferyl-β-D-glucuronide; Fischer Scientific Inc., Waltham, MA) (0·1 mg ml^{-1}), pentachloronitrobenzene (PCNB; Amvac Chemical Corp., Newport Beach, CA) (5 mg ml^{-1}) and rifampicin (Fischer Scientific, 100 mg l^{-1}) and incubated at 37°C overnight. PCNB was used in the plating media to inhibit growth of naturally occurring rifampicin-resistant fungi that could compete for available nutrients in the plate with our inoculated strains. After incubation, the bacteria was resuspended and decanted from the plate using BPB into a beaker containing 300 ml of the buffer. The OD of the solution was adjusted to 0·725 at 600 nm and used to achieve a nominal inoculum concentration of log 2·0, 4·0 and 5·0 CFU ml^{-1}. Inoculum for field trials was prepared as mentioned earlier and adjusted to deliver a nominal, uniform inoculum concentration of log 1·45 or 3·4 CFU m^{-2} at time zero. Targeted concentrations were confirmed by plating in TSA+PPMR.

Plants were spray-inoculated late afternoon just before dusk in greenhouse and field trial locations. Spray inoculation was achieved in the greenhouse using a spray bottle with the nozzle adjusted to deliver a large droplet size and a plastic bag covering the pot to prevent external contamination. Under field conditions, a hand-held pump-pressurized sprayer was used to apply the inoculum. This system was also set to deliver a large droplet size. Additionally, under greenhouse conditions, each potting plug was inoculated with a 10-ml aliquot of each *E. coli* strain. Plant tissue and potting mix from the greenhouse were evaluated at 4, 7, 9, 14 and 21 days, while for field plants, evaluation was conducted at 1, 7, 14 and 21 days. Soil evaluations were performed at 7, 14 and 21 days postinoculation. The test bacteria were also introduced to soil in the field environment by inoculation at eight focal points within an 80-m-length bed. The location selected for our field trials was repeatedly tested for the presence of background *E. coli* with resistance to doses of rifampicin used in recovery of applied strains, and all tests were negative (data not shown). A thermally sealed infusion sachet filled with sterile sand saturated with 2% milk powder was infused with the bacterial isolates to achieve approximately log 6 CFU per sachet.

Bacterial recovery and enumeration

Greenhouse and field soil

Hydroponic solution with and without the presence of coir–vermiculite mix was tested to assess the survival of *E. coli* O157:H7 without the presence of root exudates and to determine the level of transfer of the inoculated bacteria in the coir–vermiculite mix into the solution. Evaluations were made at 2, 4, 6, 12 and 18 days postinoculation, collecting a 10-ml aliquot, which was centrifuged for 15 min at 1500 ***g***. The pellet was then resuspended with 200 μl of BPB and plated on TSA+PPMR.

For coir–vermiculite mix, a 50-g plug was used for recovery of generic *E. coli* or attenuated strains of *E. coli* O157:H7. During the establishment and later cultivation of spinach in the field, irrigation was not equally distributed across the selected area. Consequently, it was divided into two sections (A and B) based on water content within each section. For field soil inoculation trials, soil samples consisted of a 10 × 15 cm soil core taken underneath the sachet from eight locations along the planted rows. Both types of sample sources were mixed with 0·1 mol l^{-1} sodium phosphate +0·05% Tween 20 at a 2 : 1 ratio (buffer/soil), and tenfold serial dilutions were made using BPB to enumerate bacterial populations. When populations were below the standard limit of detection by direct plating, a 100-ml aliquot was extracted from the aqueous phase of the soil suspension, mixed with 100 ml of 2 × mEHEC™; Biocontrol, Bellevue, WA, USA) for *E. coli* O157:H7 or buffer peptone water (Difco Laboratories, Detroit, MI) for *E. coli* both amended with rifampicin (100 mg l^{-1}) and incubated for 18 h at 37°C to determine presence/absence survival of all applied isolates.

Standardization of bacterial enumeration

To standardize the reporting of recovered isolates from soil, the water content of field soil was estimated by drying between 70 and 90 g of collected soil for 5 days at 70°C. After drying, the moisture content was determined by weighing in the pre-tared vessel and expressed on a percentage basis from the original weight of the sample.

Plants

Hydroponic cultivation: At each evaluation point, plant material was harvested and organized into sample type (older, younger, the basal region of shoot emergence, unexpanded leaves, 'leaf material', roots and potting mix); 6–8 plants were analysed at each time point. Each plant section was placed inside a 0·5-l stomacher bag (NASCO Whirl-Pak®, Salida, CA), pulsified (Pulsifier; Microgen Bio Products, Camberley, Surrey, UK) for 30 s and ten-fold dilutions were made with BPB. TSA-PPMR or CHROMagarO157 (Becton Dickinson BBl, Franklin Lakes, NJ) amended with rifampicin (CHROM-O157-Rif) were used for bacterial recovery. When necessary, qualitative detection of *E. coli* O157:H7 below the level of plating recovery was carried out by probe-based PCR (Biocontrol; Assurance GDS-O157, Bellevue, WA, USA) following protocols provided by the supplier.

Field conditions: Field grown spinach was harvested at dawn by cutting the leaves by hand with sterile scissors. Leaves were separated from the petioles using aseptic techniques, and each portion was placed inside individual plastic bags and stored inside a pre-cooled ice chest for transport back to the University of California Davis where the plants were held for 24 h at 0°C before processing. Leaves and petioles were transferred to a 1-l stomacher bag (NASCO Whirl-Pak®), and mEHEC was added at a 2 : 1 ratio. Leaves and petioles were pulsified as described earlier, after which the supernatant was streaked on TSA-PPMR and incubated at 37°C for 24 h. The supernatant was also enriched at 42°C for 18 h; after which the enriched liquid was streaked onto CHROM-O157-Rif for colony confirmation and when necessary molecular confirmation was assessed by probe-based PCR specific for *E. coli* O157:H7 (Campbell *et al.* 2001).

Statistical analysis

A randomized complete block design was used in all greenhouse and field studies. Plant distribution within this randomization was followed for all evaluations. One-way ANOVA/'LSD test' with $\alpha = 0{\cdot}05$ was used to determine whether there were significant differences between treatments.

Results

Persistence of *Escherichia coli* and *Escherichia coli* O157:H7 on hydroponic solution and potting mix

Lower survival capacity of attenuated *E. coli* O157:H7 inoculated at log 6 CFU ml^{-1} into hydroponic solution (total *N* concentration of 150 ppm) was observed without the presence of potting mix and spinach seedlings. Populations were reduced by five log CFU g^{-1} within in 16 days of inoculation (Table 1). Similar results were observed for hydroponic solutions with 75 and 250 ppm total *N* (data not shown). During 14 days of hydroponic cultivation, there was constant transfer of inoculated *E. coli* from the potting mix (plug) to the solution. After 7 days of inoculation, spinach seedlings had germinated and the solution was replaced with new non-contaminated solution. Re-contamination of this solution either came from the previously inoculated potting mix (plug) or from the contaminated walls of the hydroponic container. At 18 days, the transfer of *E. coli* from the potting mix into the hydroponic solution was not detected by direct plating or after enrichment on selective media (Fig. 2).

Survival in potting mix of individual strains of *E. coli* was strain dependent (Fig. 3). During the first 15 days

Table 1 Survival of attenuated *Escherichia coli* O157:H7 (two isolate mixture) in hydroponic solution (150 ppm Total *N*) in the absence of plant or potting mix over 16 days post-inoculation

Days after inoculation	Bacteria recovered* (log CFU g^{-1})
1	5·1 ± 0·2 a
4	3·5 ± 0·3 b
8	2·1 ± 0·5 c
12	1·6 ± 0·5 c
16	0·62 ± 0·08 d

*Initial inoculum dose was log 6 CFU ml^{-1}. Values represent averages of three replicates per evaluation point plus standard error. Means with dissimilar letters are significantly different between time points, LSD.05.

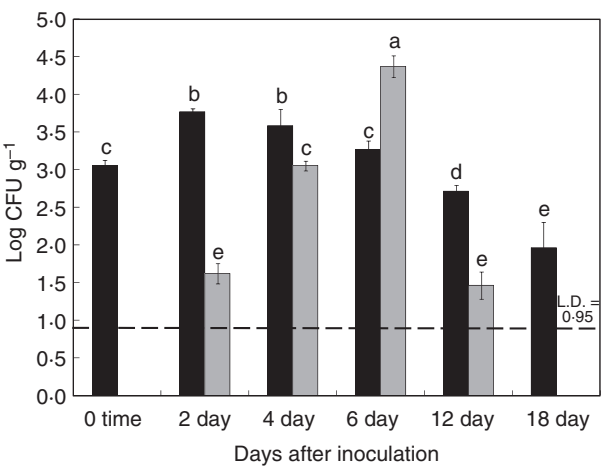

Figure 2 Survival of a generic *Escherichia coli* cocktail on potting mix (■) and hydroponic solution (▒) up to 18 days postinoculation at log 5 CFU ml^{-1}, (limit of detection was 0·95 CFU g^{-1}). Means with dissimilar letters are significantly different between treatments, LSD.05.

after inoculation, strain TVS355 appeared to have greater environmental fitness than strains TVS353 and 354 based on residual cell population densities. As with plants, described in more detail below, the survival of a cocktail of these strains in potting mix inoculations seems to better mimic the survival of an environmentally fit strain (355) when considering inoculation under identical environmental conditions. Recovered populations at 7 and 15 days for strain TVS 355 were quantitatively equivalent to the recovered populations from the cocktail after 21 days postinoculation.

Persistence of *Escherichia coli* and *Escherichia coli* O157:H7 in soil under field conditions

Under field settings, soil water content appeared to be a major factor influencing survival of *E. coli* and *E. coli* O157:H7 cocktails. Soil water content lower than 9% (section B) for avirulent *E. coli* O157:H7 and 5% for generic *E. coli* disproportionately reduced populations over 21 days of evaluation relative to areas identically inoculated but receiving more uniform irrigation (Table 2). Under our experimental conditions, nonpathogenic *E. coli* had greater environmental fitness than the applied attenuated *E. coli* O157:H7. Viable populations were detected after 14 and 21 days of inoculation for *E. coli*, while none were recoverable by enrichment for *E. coli* O157:H7 (Table 2).

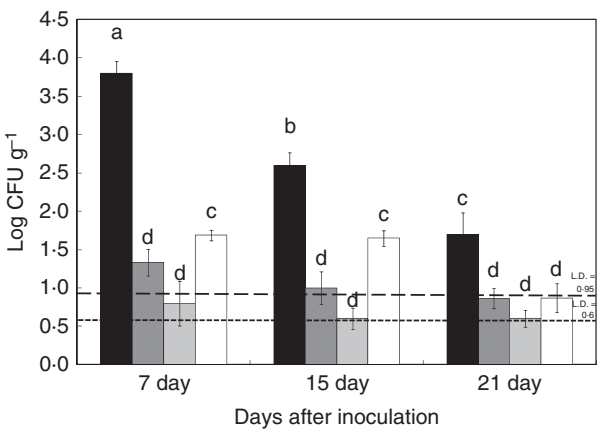

Figure 3 Survival of generic *Escherichia coli* strains in potting mix up to 21 days postinoculation under greenhouse conditions. Inoculum doses: mixed inoculum (■) 4·6 CFU ml^{-1} (limit of detection was 0·95 CFU g^{-1}). For individual strains, 2·3 CFU ml^{-1} TSV 353 (▩), 354 (▩) and 355 (□) (limit of detection was 0·6 CFU g^{-1}). Total number of soil plugs evaluated was 15 per time point and inoculum. Means with dissimilar letters are significantly different between treatments, LSD.05.

Inoculum dose, Strain source and persistence of *Escherichia coli* and *Escherichia coli* O157:H7 on spinach

Inoculum dose under the tested conditions does not appear to have an impact on the overall equilibrium survival of bacteria on the surface of spinach, irrespective of growing conditions (Tables 3 and 4). Greenhouse- and field-inoculated plants with the combined *E. coli* (TVS 353, 354 and 355) and *E. coli* O157:H7 (*a*PTVS 154 and 155) cocktail had similar residual populations after 15 days of inoculation. Average survival population for all cultivars was log 1·4 CFU g^{-1} and remained constant through 21 days postinoculation under greenhouse conditions (Tables 3 and 4; Fig. 4).

Table 3 Temporal survival of attenuated *Escherichia coli* O157:H7 on greenhouse-inoculated spinach plants

	Recovery of *E. coli* O157:H7 (log CFU g^{-1})		
Spinach cultivar	Day 1	Day 7	Day 12–14
Whale*	4·4 ± 0·14 a	2·1 ± 0·21 c	1·5 ± 0·30 d
Shasta†	3·6 ± 0·04 b	2·1 ± 0·37 c	1·3 ± 0·23 d

Means with dissimilar letters are significantly different between and within treatments, LSD.05.
*Inoculum dose was log 5 CFU ml^{-1}. Values represent averages of 20 plants ± standard error per time point.
†Inoculum dose was 4·2 CFU ml^{-1}. Values represent averages of six plants ± standard error per time point.

Table 2 Survival of a generic *Escherichia coli* and attenuated *E. coli* O157:H7 in soil under field conditions according to soil moisture content

	Per cent (%) water content*						Number of positive samples†					
	Field section A‡			Field section B‡			Field section A‡			Field section B‡		
Treatments	7D	14D	21D	7D	14D	21D	7D	14D	21D	7D	14D	21D
E. coli O157:H7	11·7	6·6	7·7	9·3	5·2	5·8	2/4	0/4	0/4	2/4	0/4	0/4
E. coli	11·7	6·8	6·7	8·7	6·2	5·4	2/4	3/4	2/4	1/4	1/4	1/4

*Data represent average % of water content within four distinct locations.
†Data represent number of positive locations for the respective bacterium among locations. All samples were positive after 1 day of inoculation.
‡Each field section was 37·5 m long (total 75 m) and consisted of four distinct locations that received similar irrigation patterns. Positive results were annotated after enrichment for days (D) 14 and 21 and without enrichment for day 7.

Table 4 Temporal survival of attenuated *Escherichia coli* O157:H7 on field-inoculated spinach plants

	Recovery of *E. coli* O157:H7 (log CFU g^{-1})		
Spinach cultivar	Day 1	Day 7	Day 14
Barbosa*	3·4 ± 0·16 a	1·6 ± 0·25 b	1·4 ± 0·40 b
Barbosa†	1·5 ± 0·18 b	1·3 ± 0·20 b	1·3 ± 0·32 b
Avenger*	3·1 ± 0·07 a	1·4 ± 0·26 b	1·0 ± 0·06 c

Means with dissimilar letters are significantly different between treatments, LSD.05.
*Inoculum dose was log 3·4 CFU m^{-2}.
†Inoculum dose was log 1·45 CFU m^{-2}. Values for all cultivars represent averages of eight repetitions each of 150 g.

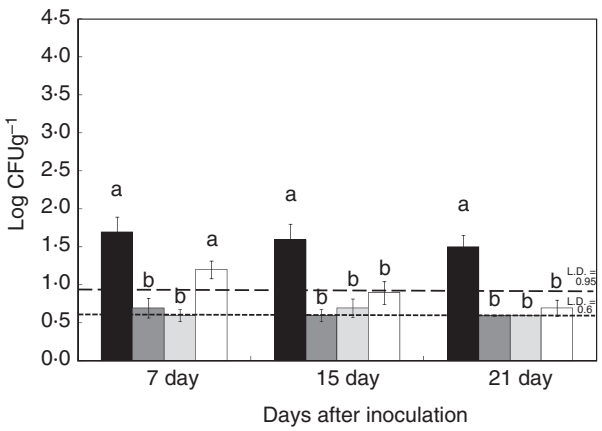

Figure 4 Survival of generic *Escherichia coli* strains on spinach plantlets cultivated in the greenhouse after 21 days of initial soil inoculation. Total number of plantlets per time point and inoculum was 5. Inoculum: mixed inoculum (■) 4·5 CFU ml^{-1} (limit of detection was 0·95 CFU g^{-1}). For individual strains, 2 CFU ml^{-1} TSV 353 (■), 354 (■) and 355 (□) (limit of detection was 0·6 CFU g^{-1}). Means with dissimilar letters are significantly different between treatments, LSD.05.

In addition, *E. coli* strains isolated from the same approximate geospatial location, but different spinach-associated environment, appeared to decline in population at different rates over time despite having identical inoculum doses (Figs 3 and 4). An *E. coli* strain isolated from a lettuce production soil (355) in the Salinas Valley and inoculated on spinach plantlets or potting mix appeared to have greater environmental fitness after 7 and 15 days, respectively, than strains isolated from irrigation water (353) or Romaine lettuce surfaces (354) (Figs 3 and 4).

Additionally, certain tissues of the spinach plants were observed to be more supportive to populations of *E. coli* than others (Fig. 5) despite being ostensibly inoculated with the same dose. It was not determined, within these experiments, whether this is a reduced rate of bacterial cell death or a population-wide balance narrowly favouring plant microsite growth over death.

Recovery of *E. coli* across leaf stages was generally inconsistent. Recovery from basal shoot tissue and unexpanded leaves, however, was generally consistent across the 21-day postinoculation interval. Interestingly, a cocktail of these same strains appears to enhance environmental fitness above that determined for the individual component strains within exposure to the same environment.

Under field conditions, survival of the avirulent *E. coli* O157:H7 dual isolate mixture on spinach leaves was inconsistent among sampling locations and evaluations dates (Tables 4 and 5). Air maximum and soil average temperatures varied between 25 and 15°C during May through December 2009 (Fig. 1) and are considered normal for the Salinas Valley.

Over the 14-day evaluation period following inoculation, there was a significant ($P < 0·05$) reduction in bacterial

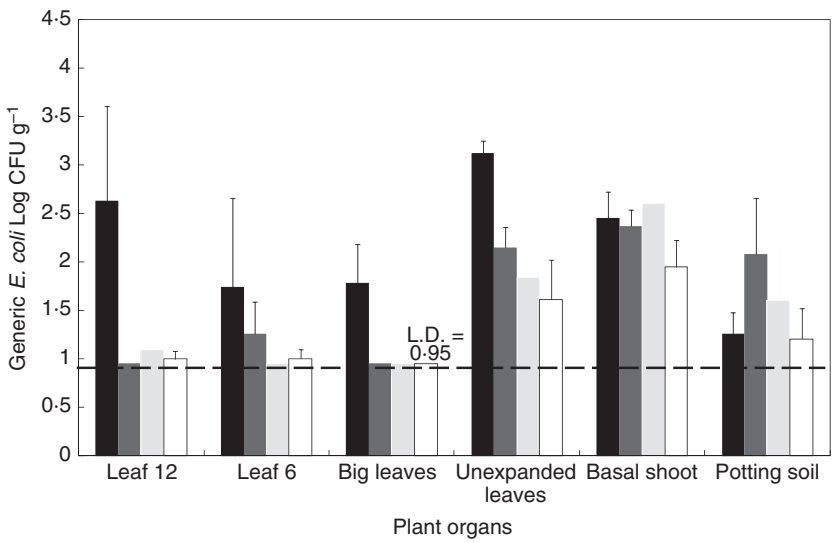

Figure 5 Recovery of a cocktail of three generic strains (353, 354 and 355) of *Escherichia coli* from different spinach plant organs inoculated at log 4 CFU ml^{-1} after 21 days of inoculation in the greenhouse. Days or recovery: 0 time (■), 9 day (■), 14 day (■) and 21 day (□) postinoculation. The limit of detection was 0·95 CFU g^{-1}. Values represent averages of 20 plants with standard deviations as error bars to indicate variability.

Table 5 Survival of attenuated *Escherichia coli* O157:H7 cocktail on spinach plants grown under field conditions according to leaf tissue locale

Days after inoculation	Number of positive samples*			
	Leaves		Petioles	
	Direct plating	After enrichment	Direct plating	After enrichment
1	9/9	9/9	6/6	6/6
7	3/9	6/9	4/6	6/6
14	1/9	3/9	2/6	4/6

*Data represent number of positive leaves and petioles from 9 to 6 field replications, respectively, each of 150 g. Inoculum dose was log 3·4 CFU m^{-2}.

populations on leaves and petioles; however, this reduction was less pronounced on spinach petioles (Tables 4 and 5). At 14 days, only 11% of the 150 g composite samples was still positive for *a*PTVS 154 or 155 by direct plating, whereas 33% of the paired, associated petioles from those leaves were positive (Table 5). After enrichment, the number of positive samples detected increased on both plant tissues. In this comparison as well, a twofold increase in the number of positive samples for petioles when compared to leaves was observed.

Discussion

Environmental and uniformity of crop management factors during cultivation not only impact the quality of vegetables (Gruda 2005) but also the survival capacity of generic *E. coli* and *E. coli* O157:H7 in the rhizosphere and phyllosphere (Ibekwe *et al.* 2009). In contrast to other studies, such as Wilson *et al.* (1999), inoculum dose under the tested conditions does not appear to have an impact on the overall equilibrium survival of bacteria on the surface of spinach, irrespective of growing conditions. These results suggest that other factors independent of inoculum dose may have greater impact on survival, at least for experimentally applied inoculum. Several reports have highlighted the potential of *E. coli* O157:H7 to remain viable in the environment as non-culturable cells and could be one of the factors influencing this overall equilibrium survival and is reflected in the recovery capacity of applied isolates in this study (Kolling and Matthews 2001; Oliver 2005; Liu *et al.* 2010; Van Elsas *et al.* 2011).

Wood *et al.* (2010) evaluated the influence of UV radiation on the persistence of *E. coli* in the phyllosphere of spinach, and although a strong relationship between UV exposure and bacterial density in the phyllosphere was not established, it is indicative of the nature and impact of environmental stress to which enteric pathogens are exposed.

Desiccation is another stress factor that influences enteric pathogen survival in the phyllosphere and abiotic surfaces (Delaquis *et al.* 2007; Moretro *et al.* 2010). Erickson *et al.* (2010a) indicated that survival of *E. coli* O157:H7 on spinach leaves was greater on the abaxial side of the leaves. Such experimentally derived observations highlight the importance that UV exposure and desiccation have on bacterial survival in the phyllosphere. In evaluating rates of inoculum death, it is worth noting that both inoculation methods utilized by Erickson *et al.* (2010a), Wood *et al.* (2010) and more recently by Moyne *et al.* (2011) were reported to have been conducted during daylight conditions.

Our inoculations were done just prior to twilight in an effort to reduce the stress associated with rapid desiccation and solar UV irradiation associated with early morning inoculations. The rate of desiccation is lessened by this timing factor as Salinas Valley twilight and night conditions are heavily influenced by marine conditions which extended the duration of leaf wetness not associated with irrigation (Fig. 1). Thus, the extended survival of the test strains relative to other reports could be associated in part with these marked differences in stress factors and inoculation method.

Therefore, our results underscore the importance of inoculation method and environmental conditions on our ability to assess enteric pathogen fitness in the environment and develop predictive standards and microbiological limits for inclusion in industry guidance documents or regulatory policy (Mitra *et al.* 2009).

Strain fitness as determined in this study and by other authors is another important factor that influences *E. coli* and *E. coli* O157:H7 survival under different environmental conditions (Winfield and Groisman 2003) and that is associated with enhanced metabolic diversity, antibiotic resistance and carbon utilization preferences (Durso *et al.* 2004). *Escherichia coli* strains used in this study were isolated from the Salinas Valley from plant, water or soil surfaces. These isolate source characteristics concomitant with the location of our experimental plots provided our study with a reference point that could provide a more detail understanding of how indicator *E. coli* behaves relative to pathogenic forms and the validity of the selected surrogates to conduct risk assessment studies. Similar observations for other surrogates isolated from a cabbage field were determined by Patel *et al.* (2009) in their growth chamber experiments with spinach, reinforcing the importance of strain selection for environmental studies and fitness among nonpathogenic *E. coli*.

In our study, our soil isolated strain also had the greatest persistence under greenhouse or field conditions either

in the phyllosphere or in the rhizosphere of spinach. Normal commercial agronomic practices in spinach and lettuce cultivation in the Salinas Valley involve frequent soil cultivation, the potential for multiple crop cycles per season, and intensive pest management practices to achieve higher yields (Koike *et al.* 2011). Such harsh conditions may select for levels of stress tolerance among environmental *E. coli*. Further comparative studies may determine whether isolates recovered from soil, in general, have elevated fitness when compared to isolates randomly obtained from irrigation water and lettuce surfaces.

It has been suggested that plant colonization is an important part of the enteric pathogen life cycle and that during this stage enteric bacteria can exist as epi- or endophytes (Brandl 2006; Teplitski *et al.* 2010). Therefore, upon arrival in the surface of plants, location of deposition and localized re-distribution within the plant has a significant impact on enteric survival. Under our greenhouse and field trials, recovery of *E. coli* and *E. coli* O157:H7 was greatly enhanced at the base of the shoot, in developing tissue and at the petioles of the leaves. These plant tissues are the areas closest to persistent free moisture at petiole junctions, nutrient availability, rapid cell division and protection from UV irradiation and desiccation, which provide optimal conditions for bacterial survival. Brandl and Amundson (2008) indicated the potential role of young tissue in the proliferation of *E. coli* O157:H7 and *Salmonella enterica* on lettuce. Our results suggest that these same tissues in spinach tissues may be ideal reservoirs for enteric pathogen survival in an open environment. *Escherichia coli* has also been suggested to have biphasic lifestyles consisting of host-independent and host-associated phases (Van Elsas *et al.* 2011) in relation to variable environmental conditions. Observed results suggest that the basal shoot and unexpanded leaves could be conducive environments for such parallel biphasic styles favouring survival.

A number of abiotic and biotic factors including moisture, pH and competitive microbiota influence the survival of *E. coli* in soil (Lang *et al.* 2007; Ibekwe *et al.* 2009; Van Elsas *et al.* 2011). *Escherichia coli* and *Escherichia coli* O157:H7 survival in hydroponic solution was brief and independent of nitrogen concentration in the solution. This was not expected as the pH was kept at a constant and survival-permissive 5·8; iron and other inorganic nutrients were readily available and conductivity values were equal to or below 2 dSm^{-1} and recognizing the capacity of *E. coli* O157:H7 to assimilate nitrate (Brandl and Amundson 2008).

Commercial hydroponic conditions of leafy greens include protecting the solution from sunlight (UV light), adjusting pH and solution recirculation (Gruda 2005). Our data suggest that if a contamination event were to occur in a hydroponic system, *E. coli* would be able to contaminate the cultivation system in the absence of a disinfection treatment. It also highlights the importance of organic nutrient availability coming either from root exudates or from the potting mix. A large proportion of the organic carbon released into the rhizosphere of plants is derived from photosynthesis and influenced by the plant physiological status (Neumann and Römheld 2000). Durso *et al.* (2004) measured carbon source utilization by *E. coli* and *E. coli* O157:H7 and found many organic carbon sources that can be utilized for growth. Sharma *et al.* (2009) reported similar interactions in hydroponic culture comparing generic *E. coli* and an isolate of *E. coli* O157:H7. Several of these compounds, including sucrose, sorbitol and organic acids, are present in root exudates and could explain in part the enhanced survival of *E. coli* and *E. coli* O157:H7 observed when roots and potting mix were present in the hydroponic solution.

Survival of *E. coli* and *E. coli* O157:H7 in soil under our experimental conditions was mainly influenced by strain source and water availability despite the presence of powder milk, added as a protective osmoticum, in our inoculation sachets. Significant variability in the recovery of our test strains was also observed despite the use of such a nutrient containing inoculation matrix. Our results are not in full agreement with those reported by Patel *et al.* (2009) but follow similar survival patterns as those described by Erickson *et al.* (2010b) for similar inoculum doses. Although higher inoculation doses were used by Patel *et al.* than those used in our experimental plots, the extended survival observed in their experiments could be attributed to constant environmental conditions provided by the growth chamber and reduced water loss. This was not the case for our field plots and those from Erickson *et al.* (2010b).

Overall, generic *E. coli* and *E. coli* O157:H7 populations recovered from hydroponic or field grown spinach spray-inoculated at log 4 CFU ml^{-1} resulted in highly similar equilibrium populations. It is hypothesized that this observation is because of either persistent multicellular aggregates or a balance between limited growth, emigration and death as suggested by Brandl (2006). It seems plausible to anticipate that even a uniform contamination event will not lead to uniform distribution within the spinach tissue or in the soil. These field observations strongly suggest the need for further evaluation of sampling design for pathogen detection systems on leafy greens. Overall, protected and open-environment cultivation had similar negative effects on the survival of *E. coli*, but a greater negative impact on survival of attenuated *E. coli* O157:H7 was experienced. The selection and validation of appropriate and predictive surrogates for

diverse pathogen tolerances remain an important area to pursue.

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References

Bearson, S.M., Benjamin, W.H., Swords, W.E. and Foster, J.W. (1996) Acid shock induction of RpoS is mediated by the mouse virulence gene mviA of Salmonella typhimurium. *J Bacteriol* **178**, 2572–2579.

Beattie, G.A. and Lindow, S.E. (1999) Bacterial colonization of leaves: a spectrum of strategies. *Am Phytopathol Soc* **89**, 353–359.

Beuchat, L.R., Ward, T.E. and Pettigrew, C.A. (2001) Comparison of chlorine and a prototype produce wash product for effectiveness in killing *Salmonella* and *Escherichia coli* O157: H7 on alfalfa seeds. *J Food Prot* **64**, 152–158.

Brandl, M.T. (2006) Fitness of human enteric pathogens on plants and implications for food safety. *Annu Rev Phytopathol* **44**, 367–392.

Brandl, M.T. and Amundson, R. (2008) Leaf age as a risk factor in contamination of lettuce with *Escherichia coli* O157:H7 and *Salmonella enterica*. *Appl Environ Microbiol* **74**, 2298–2306.

Campbell, G.R., Prosser, J., Glover, A. and Killham, K. (2001) Detection of *Escherichia coli* O157:H7 in soil and water using multiplex PCR. *J Appl Microbiol* **91**, 1004–1010.

Delaquis, P., Bach, S. and Dinu, L.D. (2007) Behavior of *Escherichia coli* O157:H7 in leafy vegetables. *J Food Prot* **70**, 1966–1974.

Durso, L.M., Smith, D. and Hutkins, R.W. (2004) Measurements of fitness and competition in commensal *Escherichia coli* and *E. coli* O157:H7 strains. *Appl Environ Microbiol* **70**, 1435–1443.

Erickson, M.C., Webb, C.C., Diaz-Perez, J.C., Phatak, S.C., Silvoy, J.J., Davey, L., Payton, A.S., Liao, J. *et al.* (2010a) Surface and internalized *Escherichia coli* O157:H7 on field grown spinach and lettuce treated with spray-contaminated irrigation water. *J Food Prot* **73**, 1023–1029.

Erickson, M.C., Webb, C.C., Diaz-Perez, J.C., Phatak, S.C., Silvoy, J.J., Davey, L., Payton, A.S., Liao, J. *et al.* (2010b) Infrequent internalization of *Escherichia coli* O157:H7 into field grown leafy greens. *J Food Prot* **73**, 500–506.

Gruda, N. (2005) Impact of environmental factors on product quality of greenhouse vegetables for fresh consumption. *Crit Rev Plant Sci* **24**, 227–247.

Gutierrez, E. (2009) Influence of nitrogen on spinach leaf fragility, composition, and postharvest quality. MSc thesis at http://gradworks.uni.com/14/72/1472612.html. (accessed 10 November 2011).

Ibekwe, M.A., Grieve, C.M., Papiernik, S.K. and Yang, C.H. (2009) Persistence of *Escherichia coli* O157:H7 on the rhizosphere and phyllosphere of lettuce. *Appl Microbiol* **49**, 784–790.

Koike, S., Cahn, M., Cantwell, M., Fennimore, S., LeStrange, M., Natwick, E., Smith, R.F. and Takele, E. (2011) *Spinach Production in California*. Oakland: University of California. ANR publication 7212.

Kolling, G.L. and Matthews, K.R. (2001) Examination of recovery in vitro and in vivo of nonculturable *Escherichia coli* O157:H7. *Appl Environ Microbiol* **67**, 3928–3933.

Lang, N.L., Bellett-Travers, M.D. and Smith, S.R. (2007) Field investigations on the survival of *Escherichia coli* and presence of other enteric micro-organisms in biosolids-amended agricultural soil. *J Appl Microbiol* **103**, 1868–1882.

Liu, Y., Wang, C., Tyrell, G. and Li, X.F. (2010) Production of Shiga-like toxins in viable but nonculturable *Escherichia coli* O157:H7. *Water Res* **44**, 711–718.

Mitra, R., Cuesta-Alonso, E., Wayadande, A., Talley, J., Gilliand, S. and Fletcher, J. (2009) Effect of route of introduction and host cultivar on the colonization, internalization, and movement of the human pathogen *Escherichia coli* O157:H7 in spinach. *J Food Prot* **72**, 1521–1530.

Moretro, T., Heir, E., Mo, K.R., Habimana, O., Abdelgani, A. and Langsrud, S. (2010) Factors affecting survival of shiga-toxin-producing *Escherichia coli* on abiotic surfaces. *Intern J Food Microbiol* **138**, 71–77.

Moyne, A.L., Sudarshana, M.R., Blessington, T., Koike, S.T., Cahn, M.D. and Harris, L. (2011) Fate of *Escherichia coli* O157:H7 in field-inoculated lettuce. *Food Microbiol* **28**, 1417–1425.

Muffler, A., Traulsen, D.D., Lange, R. and Hengge-Aronis, R. (1996) Post-transcriptional regulation of the sigma S subunit of RNA polymerase in *Escherichia coli*. *J Bacteriol* **178**, 1607–1613.

Neumann, G. and Römheld, V. (2000) The release of root exudates as affected by the plant physiological status. In *The Rhizosphere: Biochemistry and Organic Substances at the Soil-Plant Interface* ed. Pinton, R., Varanini, Z. and Nannipieri, Z. pp. 41–93. New York, NY: CRC Press.

Oliver, J.D. (2005) The viable but non-culturable state in bacteria. *J Microbiol* **43**, 93–100.

Patel, J., Millner, P., Nou, X. and Sharma, M. (2009) Persistence of enterohaemorrhagic and nonpathogenic *E. coli* on spinach leaves and in the rhizosphere soil. *J Appl Microbiol* **108**, 1789–1796.

Pu, S., Beaulieu, J.C., Prinyawiwatkul, W. and Ge, B. (2009) Effects of plant maturity and growth media bacterial

inoculum level on the surface contamination and internalization of *Escherichia coli* O157:H7 in growing spinach leaves. *J Food Prot* **72**, 2313–2320.

Sharma, M., Ingram, D.T., Patel, J.T., Millner, P.D., Wang, Z., Hull, A.E. and Donneberg, M.S. (2009) A novel approach to investigate the uptake and internalization of *Escherichia coli* O157:H7 in spinach cultivated in Soil and hydroponic medium. *J Food Prot* **72**, 1513–1520.

Teplitski, M., Barak, J.D. and Schneider, K.R. (2010) Human enteric pathogen in produce: un-answered ecological questions with direct implications for food safety. *Curr Opin Biotechnol* **20**, 166–171.

Touchon, M., Hoede, C., Tenaillon, O., Barbe, V., Baeriswyl, S. and Bidet, P. (2009) Organized genome dynamics in the *Escherichia coli* species results in highly diverse adaptive paths. *PLoS Genet* **5**, e1000344.

Van Elsas, J.D., Semenov, A.V., Costa, R. and Trevors, J.T. (2011) Survival of *Escherichia coli* in the environment: fundamental and public health aspects. *ISME: Multidiscip J Microbiol Ecol* **5**, 173–183.

Vital, M., Hammes, F. and Egli, T. (2008) *Escherichia coli* O157 can grow in natural freshwater at low carbon concentrations. *Environ Microbiol* **10**, 2387–2396.

Wilson, M. and Lindow, S.E. (1994) Inoculum density-dependent mortality and colonization of the phyllosphere *by Pseudomonas syringae*. *Appl Environ Microbiol* **60**, 2232–2237.

Wilson, M., Hirano, S. and Lindow, S.E. (1999) Location and survival of leaf-associated bacteria in relation to pathogenicity and potential for growth within the leaf. *Appl Environ Microbiol* **65**, 1435–1443.

Winfield, M.D. and Groisman, E.A. (2003) Role of nonhost environments in the life style of *Salmonella* and *Escherichia coli*. *Appl Environ Microbiol* **69**, 3687–3694.

Wood, J.D., Bezanson, G.S., Gordon, R.J. and Jamieson, R. (2010) Population dynamics of *Escherichia coli* inoculated by irrigation into the phyllosphere of spinach grown under commercial production conditions. *Inter J Food Microbiol* **143**, 198–2004.