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Fate of *Escherichia coli* O157:H7 in field-inoculated lettuce

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ABSTRACT

Impact of drip and overhead sprinkler irrigation on the persistence of attenuated *Escherichia coli* O157:H7 in the lettuce phyllosphere was investigated using a split-plot design in four field trials conducted in the Salinas Valley, California, between summer 2007 and fall 2009. Rifampicin-resistant attenuated *E. coli* O157:H7 ATCC 700728 (BLS1) was inoculated onto the soil beds after seeding with a backpack sprayer or onto 2- or 4-week-old lettuce plant foliage with a spray bottle at a level of 7 log CFU ml^{-1}. When *E. coli* O157:H7 was inoculated onto 2-week-old plants, the organism was recovered by enrichment in 1 of 120 or 0 of 240 plants at 21 or 28 days post-inoculation, respectively. For the four trials where inoculum was applied to 4-week-old plants, the population size of *E. coli* O157:H7 declined rapidly and by day 7, counts were near or below the limit of detection (10 cells per plant) for 82% or more of the samples. However, in 3 out 4 field trials *E. coli* O157:H7 was still detected in lettuce plants by enrichment 4-weeks post-inoculation. Neither drip nor overhead sprinkler irrigation consistently influenced the survival of *E. coli* O157:H7 on lettuce.

1. Introduction

California produces over 70% of the annual U.S. leafy green vegetable crop (Cooley et al., 2007; FDA, 2009). Numerous outbreaks of *Escherichia coli* O157:H7 have been associated with the consumption of leafy green vegetables in the U.S. since 1996 (FDA, 2009). Where traceback investigations were possible, lettuce and leafy greens grown in Kern County (CDPH, 2008), San Benito County (CDC, 2006) and Salinas Valley in Monterey County, have been implicated as sources of contamination (Cooley et al., 2007). Although, contaminated irrigation water, wildlife, and animal-based soil amendments have been suspected in some outbreaks, epidemiological investigations typically occur long after produce has been harvested, and thus the exact source of the outbreak strain(s) and the specific conditions that lead to contamination are usually unknown (Suslow et al., 2003). The leafy greens industry and government regulatory agencies have focused on water, wildlife, soil amendments, and worker hygiene in developing programs aimed at reducing the risk of contamination of leafy greens in the field. Since 2007, essentially all commercially-grown lettuce in California is subject to the California Leafy Greens Marketing Agreement (www.caleafygreens.ca.gov/members/resources.asp). Integral to the marketing agreement are a number of metrics including limits for generic *E. coli* in irrigation water.

Numerous studies have attempted to elucidate pre- and post-harvest factors that influence the interaction of *E. coli* O157:H7 and leafy greens (Delaquis et al., 2007; Theofel and Harris, 2009). Under laboratory and field conditions, *E. coli* O157:H7 has been shown to survive for extended periods of time in water (Wang et al., 2002), manure, or manure-amended soil (Islam et al., 2004, 2005). To date, most of the preharvest studies on the survival of *E. coli* O157:H7 on lettuce plants have been conducted in the laboratory under controlled environmental conditions, in part because of the challenges associated with conducting field trials (Cooley et al., 2006; Franz et al., 2005; Jablasone et al., 2005; Solomon et al., 2002). *E. coli* O157:H7 could be detected for up to 20 days from greenhouse lettuce plants that were inoculated with a single spray or soil surface application of the bacteria. Under greenhouse or growth chamber conditions of high humidity and warm temperatures, *E. coli* O157:H7 populations were shown to increase when inoculated onto lettuce seeds (Jablasone et al., 2005) or plants (Brandl and Amundson, 2008). Higher populations were consistently observed on younger Romaine leaves, suggesting that leaf age may impact colonization (Brandl and Amundson, 2008).

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Laboratory-based studies, although important, cannot completely mimic the range of environmental conditions and stresses likely encountered in the field, particularly temperature and humidity fluctuations, UV exposure, and wind. To date, there are limited published data on the behavior of *E. coli* O157:H7 on growing lettuce plants under field conditions (Ercolani, 1979; Erickson et al., 2010a,b; Islam et al., 2004), and none for lettuce grown in the Salinas Valley.

In studies carried out in a southern U.S. state (Georgia) in late October, Islam et al. (2004) applied an attenuated strain of *E. coli* O157:H7 to lettuce plants via soil amended with inoculated manure composts or via spraying inoculated water one time directly onto seedlings. The inoculated organism could be detected on the lettuce plants from all treatments for 77 days after seedlings were planted (sampling terminated) and in the soil through day 126 (inoculated water treatment) and day 154 (inoculated manure compost treatments) after planting.

In contrast, a recent similar study did not find *E. coli* O157:H7 contamination of leafy green lettuce after applying contaminated compost to the soil (Erickson et al., 2010a). The authors attributed their dissimilar findings to the application of uncontaminated soil over the contaminated soil, preventing the transfer from soil to plant. Additional research found that when lettuce leaf surfaces were spray inoculated, *E. coli* O157:H7 could be detected 7 days after inoculation only if the level of inoculum applied was higher than 6 log CFU/ml (Erickson et al., 2010b). Survival of *E. coli* O157:H7 was longer on the abaxial surface (underside) of the leaves than on the adaxial surface (Erickson et al., 2010b).

Lettuce is seeded in the Salinas Valley from late January through late September, and harvested mid-March through late November and early December. Fields are irrigated by drip, furrow, overhead sprinkler, or a combination of methods. With drip and furrow irrigation the lettuce foliage remains dry, whereas with overhead sprinkler irrigation the water falls onto edible portions of the plants and the soil surface.

The objective of this study was to determine the impact of drip and overhead sprinkler irrigation, two of the most commonly used irrigation methods on lettuce ranches, on the fate of *E. coli* O157:H7 in the phyllosphere of Romaine lettuce grown in the Salinas Valley. Four field trials were conducted in two different locations in the Salinas Valley over 3 years. Non-pathogenic BSL1 *E. coli* O157:H7 (ATCC 700728) was applied to the soil soon after seeding, but before lettuce seed germination, or on lettuce plants at different times during plant growth. Here we report on the bacterial population dynamics on the plants we monitored for up to 5 weeks after inoculation.

2. Materials and methods

2.1. Bacterial strain, culture conditions, and inoculum preparation

Because of the inappropriateness of using toxigenic *E. coli* O157:H7 in field studies, we selected *E. coli* O157:H7 ATCC 700728, a nontoxigenic strain classified as BSL1, for the lettuce inoculation. A spontaneous rifampicin-resistant mutant of *E. coli* O157:H7 ATCC 700728 was isolated by step-wise exposure to increasing concentrations of rifampicin and selecting colonies that were resistant to rifampicin at 120 μg/ml. Growth rates of the parent and mutant strains in broth were shown to be identical (data not shown). With 50 μg/ml of rifampicin in agar media we were able to easily differentiate *E. coli* O157:H7 colonies from the indigenous microorganisms present in the lettuce phyllosphere. PCR was used to confirm that the strain does not have *stx1* and *stx2* genes but has the single base mismatch at +93 in the *uidA* gene, characteristic of *E. coli* O157:H7. Other virulence genes such as the enterohemolysin *hlyA* and the intimin *eaeA* genes were detected by PCR with the primers described by Wang et al. (2002).

A stock culture of rifampicin-resistant *E. coli* O157:H7 ATCC 700728 was streaked onto tryptic soy agar (TSA) supplemented with 50 μg/ml of rifampicin and incubated overnight at 37 °C. A single isolated colony was streaked onto TSA supplemented with 50 μg/ml of rifampicin, and plates were incubated overnight at 37 °C. Cells were resuspended directly from the plate in 0.1% peptone water and centrifuged at 10,000× *g* for 2 min. Washing was repeated three times and cells were resuspended in 0.1% peptone water to achieve an optical density of 1 at 600 nm, corresponding to approximately 1×10^9 CFU/ml. Cells were enumerated on TSA plates supplemented with 50 μg/ml rifampicin, and the inoculum was held overnight at 4 °C.

2.2. Field studies

Four field trials were conducted in the Salinas Valley during summer 2007 (7/20/07 to 9/14/07), spring 2008 (4/16/08 to 6/24/08), spring 2009 (5/6/09 to 7/6/09), and fall 2009 (9/8/09 to 11/16/09). A split-plot design was used to evaluate the two main treatment effects: drip irrigation and overhead sprinkler irrigation. Three blocks were established for each irrigation treatment. Each block was 44 m long with nine 1-m wide beds. For the drip irrigation treatment, drip tape was installed 7.5–10 cm below the soil surface in the center of the beds prior to seeding. All beds were direct seeded according to standard commercial practice with two rows of Romaine lettuce cv. Green Towers (*Lactuca sativa*) per bed, spaced 30 cm apart (equidistant from the drip tape in the drip treatment beds). After seeding, but prior to the first irrigation, a pre-emergent herbicide, pronamide (Kerb 50 W, Dow Agrosciences), was applied to all beds at the rate of 2.24 kg/ha.

Plants were initially thinned at the four to six true-leaf stage so that remaining plants were 25 cm apart. Ten unfarmed beds were retained between the drip and overhead irrigation blocks to reduce drift from the overhead sprinkler irrigation. Sub-treatments, such as inoculum level and time of inoculation, were applied randomly in each block on one bed. In each block, two untreated lettuce beds were used as a buffer between the sub-treatment beds to prevent cross contamination.

Similar to commercial operations the first two irrigations of the field trials were with overhead sprinklers to water the beds thoroughly and promote uniform germination of the lettuce seeds. Sprinklers were spaced in a square 9.1 m by 9.1 m pattern. After the first two sprinkler irrigations, the drip treatment beds were irrigated with drip tape until the completion of the trial. After emergence, the crop was irrigated two times per week. Drip-irrigated plots were watered 2–4 h per irrigation, and sprinkler-irrigated plots were watered 1.5–2.5 h per irrigation. The total amount of water applied to the crops varied from 28 to 46 cm, depending on the weather conditions.

The crop was thinned to a final population of 65,000 plants/ha, approximately 30 days after planting. After thinning, the sprinkler-irrigated plots were fertilized once at 80 kg nitrogen/ha; for the drip-irrigated plots, nitrogen fertilizer was applied through the drip tape in three applications totaling approximately 80 kg/ha.

2.3. Environmental factors

Temperature and humidity were recorded every 15 min with a HOBO weather station data logger (Onset, Bourne, MA) located within the field during the spring and fall 2009 trials. Precipitation data were retrieved from the Salinas South weather station (California Irrigation Management Information System, CIMIS #89)

through the University of California Integrated Pest Management website (www.ipm.ucdavis.edu/index.html).

2.4. Field inoculation

Lettuce or soil was inoculated between 9 and 11 a.m. for all field trials. Two types of inoculation were used for delivering *E. coli* O157:H7 ATCC 700728 onto the lettuce plants or on the soil surface. Spray bottles were used to inoculate individual 4-week-old lettuce plants just after thinning in summer 2007, spring 2008, spring 2009, and fall 2009. The bottles were calibrated to deliver a dose adjusted to 10^7 CFU per spray (for all trials) and 10^5 CFU per spray (for summer 2007); approximately 1 ml was delivered in a single spray. A backpack sprayer containing inoculum adjusted to 10^7 CFU/ml was used to spray the soil surface of the lettuce bed just before lettuce seed germination (5 days after planting) during spring 2009, and to spray 2-week-old lettuce plants during fall 2009. The inoculum concentration was verified by enumeration on TSA supplemented with 50 μg/ml rifampicin.

2.5. Plant sampling

To collect samples, entire lettuce plants were removed by cutting at the base with a sterile scalpel, approximately 3 cm above the ground. Small plants were pooled in 532-ml Whirl-Pak bags (Nasco, Ft. Atkinson, WI) larger plants were collected separately in polypropylene bags (30.5 cm by 30.5 cm, Bitran).

When soil was inoculated pre-emergence, 10 plants per block were collected and pooled in one Whirl-Pack bag 7 days after soil inoculation. At day 15, 50 plants per block were collected and pooled in five separate Whirl-Pak bags (10 plants per bag). All samples were brought to the laboratory from the field in a cooler with ice-packs, held at 4 °C, and analyzed within 48 h.

After inoculating 2-week-old plants, lettuce samples were collected randomly starting at day 0 and weekly thereafter until 28 days post-inoculation (dpi). The size of the bacterial population delivered effectively per plant was evaluated by sampling the plants immediately after inoculation at 0 h post-inoculation (hpi) and 2 hpi. Ten plants per block were collected and pooled in one Whirl-Pak bag. At 7 dpi, 12 plants per block were collected and pooled in three separate Whirl-Pak bags (4 plants per bag). At days 14 and 21, 20 plants per block were collected separately and bagged individually. At day 28, 40 plants per block were collected and bagged individually for a total of 240 plants at day 28 (40 plants × 3 blocks × 2 treatments).

After inoculating 4-week-old plants, samples were collected randomly among the inoculated lettuce beds from day 0 (0 and 2 h) until 3–5 weeks after inoculation. Each lettuce head was bagged individually upon collection. The total number of samples collected at each time is provided in Table 1. Non-inoculated plants (control) were collected concurrently at the time of inoculation (day 0) in all trials and throughout the trial only during summer 2007.

For all experiments conducted on 2- or 4-week-old inoculated plants, lettuce samples collected through the first 7 days were brought to the laboratory from the field in a cooler with ice-packs, held at 4 °C, and analyzed for *E. coli* O157:H7 within 48 h. Samples collected at 14 days and later were transported, without cooling, to the laboratory for detection of *E. coli* O157:H7 through enrichment.

2.6. Inoculum recovery and quantification

For bacterial enumeration or enrichment, the entire lettuce head was homogenized. When the lettuce head weighed less than 50 g, the entire sample was placed into a sterile Whirl-Pak bag with 100 ml of 0.1% peptone water and homogenized in a Stomacher 400 laboratory blender (Seward, Westbury, NY) for 2 min at medium speed. Lettuce weight increased with sampling time (Table 1); therefore, heads weighing more than 50 g were divided into outer and inner leaves and placed in as many bags as needed (up to 50 g per bag) and processed as described previously. For spring and fall 2009 field trials, only the outer leaves were processed with enrichment, at the last sampling point. *E. coli* O157:H7 ATCC 700728 was enumerated by plating cell suspensions on TSA (supplemented with 50 μg/ml rifampicin) with an automated spiral plater (Autoplate 4000, Spiral Biotech Inc., Boston, MA), and incubating overnight at 37 °C. When necessary to improve the limit of detection, samples were filtered onto disposable analytical filter units (0.45 μm; Nalgene, Rochester, NY). Filter membranes were removed and placed on plates of CHROMagar O157 (BD, Franklin Lakes, NJ) (Bettelheim, 1998) supplemented with 50 μg/ml rifampicin, and incubated overnight at 37 °C.

Indigenous bacteria on the uninoculated control plants were retrieved by sample preparation as described above. Cell suspensions were spiral plated on TSA plates, incubated at 37 °C for 24 h, and colonies were counted. Cell suspensions obtained after stomaching were also plated on TSA supplemented with 50 μg/ml rifampicin to evaluate the number of rifampicin-resistant bacteria, and on CHROMagar O157 to evaluate if *E. coli* O157:H7 bacteria were naturally present on uninoculated lettuce.

2.7. Enrichment

Samples were enriched at a 1:2 (wt:vol) ratio of lettuce to tryptic soy broth (TSB) supplemented with 50 μg/ml rifampicin, or

Table 1
Sampling after inoculation of 4-week-old Romaine lettuce plants: number of lettuce heads (*n*) and mean weights of heads at collection times.

Days post-inoculation	Number of samples and lettuce head weight							
	Summer 2007[a]		Spring 2008		Spring 2009		Fall 2009	
	n	wt (g)[b]	n	wt (g)	n	wt (g)	n	wt (g)
0[c]	60	16 ± 4	120	19 ± 9	120	6 ± 3	120	2 ± 1
2	60	30 ± 6	120	28 ± 14	120	9 ± 4	120	4 ± 2
7	60	63 ± 15	120	63 ± 24	120	27 ± 13	120	7 ± 4
14	60	202 ± 55	120	193 ± 53	120	107 ± 41	120	21 ± 10
21	90	335 ± 92	120	434 ± 110	150	253 ± 93	120	65 ± 22
28	ND[d]	ND	120	767 ± 193	150	489 ± 191	156	129 ± 42
35	ND	ND	ND	ND	ND	ND	360	216 ± 43

[a] The number of samples collected during summer 2007 includes all samples collected for both treatments with a low and high level inoculum.
[b] Results are expressed as the mean weight ± SD.
[c] Day 0: $n = 24$ at 0 h and $n = 36$ at 1 h post-inoculation for summer 2007; $n = 60$ at 0 h and $n = 60$ at 2 h post-inoculation in all other trials.
[d] ND, not determined.

at 20 g soil to 200 ml TSB supplemented with 50 μg/ml rifampicin, and incubated for 18 h at 42 °C. After enrichment, broth was spiral plated onto CHROMagar O157 supplemented with 50 μg/ml rifampicin to confirm the presence of *E. coli* O157:H7. Plates were incubated at 37 °C overnight.

2.8. Soil sampling and analysis

Prior to lettuce inoculation, soils were sampled and analyzed for the presence of wild-type *E. coli* O157:H7. On day 0 of all trials, five soil samples per bed were collected randomly from the surface of all 18 beds (3 beds per block); samples were removed from the top 15 cm of soil with an auger (diameter 2.5 cm) and bulked into a plastic bag. After thoroughly mixing the bulked samples, 20-g subsamples were added to 90 ml of Butterfield's phosphate buffer (Hardy Scientific, Santa Clara, CA) and vortexed for 1 min. Samples were spiral plated onto CHROMagar O157, and incubated at 37 °C overnight.

Soil samples were also collected as described above and evaluated for the presence of *E. coli* O157:H7 ATCC 700728. In the spring 2009, some of the soil was inoculated with *E. coli* O157:H7 pre-emergence. This soil was sampled at 0 and 2 hpi and 7 and 15 dpi. When lettuce plants were inoculated at 4 weeks after seeding, soil was collected from the area surrounding those plants on day 21 of the summer 2007 trial and on the last sampling day (28 or 35) of the other trials. These samples were processed for bacterial enumeration as described above and spiral plated on CHROMagar O157 supplemented with 50 μg/ml rifampicin or enriched for the presence of *E. coli* O157:H7 ATCC 700728 as described in section 2.7.

2.9. DNA template preparation

Colonies presumptively identified as *E. coli* O157:H7 were confirmed by real-time PCR. For each plant determined to be positive by enrichment at 7 and 14 dpi during summer 2007, we selected 12 rifampicin-resistant mauve colonies on CHROMagar O157. For spring 2008 and 2009, we similarly selected one isolate for each plant that was positive by enrichment at 7, 14, 21, and 28 dpi. The DNA template was isolated from a 1-ml overnight culture incubated at 37 °C in Luria-Bertani broth (LB; BD, Franklin Lakes, NJ). The cell culture was washed twice with water by centrifugation at 10,000× *g* for 2 min, resuspended in 1 ml of water, and boiled for 10 min. After centrifugation of the boiled extract (10,000× *g* for 2 min), 1 μl of the supernatant was used for the real-time PCR reaction.

2.10. Real-time PCR

Amplification of *stx*1, *stx*2 and *uid*A genes was performed on the ICycler real-time detection system (Bio-Rad, Hercules, CA) with Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). Primers to amplify *uid*A, *stx*1 and *stx*2 genes were designed as described by Yoshitomi et al. (2006). The different components were added to the real-time PCR mixture at the following concentrations: 0.25 μM for reverse and forward primers, 1X Power SYBR Green PCR Master Mix and, immediately prior to PCR, 1 μl of prepared template. For each plate, *E. coli* O157:H7 strain H1730 (isolate from lettuce outbreak containing both *stx*1 and *stx*2 genes) and *E. coli* strain K12 were used as positive and negative controls, respectively. Cycling conditions were performed in a two-step PCR, with an initial polymerase activation of 94 °C for 10 min, followed by 40 cycles of denaturation at 94 °C for 20 s, and an annealing/extension step at 63 °C for 25 s. After completion of 40 PCR cycles, melt curve data were generated by increasing the temperature from 60 to 95 °C at 0.2°C/10 s and recording the fluorescence. Identification of an isolate as positive for the gene of interest was determined by a positive Ct value and the corresponding melting temperature.

2.11. Data analysis

The detection limit by direct plating was 200 CFU/plant (2.3 log CFU/plant) at day 0 and 400 CFU/plant (2.6 log CFU/plant) at days 2 and 7. By using a combination of plating and filtration, our detection limit was further reduced to 10 CFU/plant. When *E. coli* O157:H7 was not detected by plating, filtration, or enrichment, sample counts were treated statistically as zero. When cells were detected only by enrichment, a value of 9 CFU/plant was assigned for calculation of the mean. Microbial data (CFU/plant) were log transformed before being statistically analyzed with JMP software (SAS Institute Inc., Cary, NC). Analysis of variance (ANOVA) was performed to compare *E. coli* O157:H7 populations among plants treated with drip or overhead sprinkler irrigation at different growing times. Pearson's chi-square test and two-tailed Fisher's exact test were performed to examine the distribution of plants that tested positive by enrichment in drip- or overhead sprinkler-irrigated beds. Results with P values of <0.05 were considered significant.

3. Results

3.1. Phyllosphere bacteria

Higher levels of indigenous bacteria were present in the phyllosphere of non-inoculated plants during spring and fall 2009 compared to summer 2007 and spring 2008 (Table 2) at day 0. Plants irrigated by overhead sprinkler had significantly higher levels of indigenous bacteria than plants irrigated by drip during both spring trials (Table 2). In contrast, the irrigation method did not significantly influence the level of indigenous bacteria during the summer 2007 or fall 2009 trials.

3.2. Detection of E. coli O157:H7 in soil

Wild-type *E. coli* O157:H7 was not detected in any of the soil samples collected from the lettuce plots before inoculation for all four field trials. Similarly, *E. coli* O157:H7 ATCC 700728 was not recovered in the soil samples collected from areas around the *E. coli*-inoculated plants at the end of each field trial.

3.3. Survival of E. coli O157:H7 in summer 2007 trial

A preliminary trial was conducted in summer 2007 to evaluate the inoculum level and sample size needed to quantify *E. coli* O157:H7 on lettuce plants from the time of inoculation through

Table 2
Impact of irrigation method on indigenous bacterial populations in the lettuce phyllosphere.

Irrigation method	Indigenous bacteria (log CFU/g)[a]			
	Summer 2007	Spring 2008	Spring 2009	Fall 2009
Sprinkler	3.7 ± 0.2 A[b]	3.8 ± 0.3 A	4.7 ± 0.1 A	5.4 ± 0.2 A
Drip	3.4 ± 0.4 A	3.4 ± 0.4 B	4.0 ± 0.1 B	5.2 ± 0.2 A
(Both treatments)	3.4 ± 0.3 (3)[c]	3.6 ± 0.4 (3)	4.4 ± 0.7 (2)	5.3 ± 0.7 (1)

[a] Values represent the mean population size (log CFU/g) ± SD measured at day 0 on non-inoculated plants; $n = 15$.
[b] Within columns, means followed by different letters are significantly different at $P < 0.05$.
[c] Within the row, means followed by different numbers are significantly different at $P < 0.05$.

development of lettuce heads to approximate commercial harvest size. In addition, lettuce plants sampled from uninoculated beds (control) were subjected to the same microbiological analysis as for *E. coli* O157:H7-inoculated plants. Bacteria resistant to rifampicin were never recovered from the control plants in the absence of a filtration or enrichment step. When rifampicin-resistant indigenous bacteria were recovered from control plants, they were easily differentiated from *E. coli* O157:H7 by plating the filtrate or the enrichment broth on CHROMagar O157. *E. coli* O157:H7 was not retrieved from the uninoculated plants at any sampling time during the preliminary trial.

When plants were sampled immediately after inoculation (0 h), average *E. coli* O157:H7 populations of 4 or 3 log CFU/plant were achieved with the high (10^7 CFU/ml) or low (10^5 CFU/ml) level inoculum concentrations, respectively (Table 3). *E. coli* O157:H7 populations declined rapidly after inoculation by 1–2 log during the first hour and most plants had less than 10 CFU/plant by 2 days post-inoculation (Table 3). The population size was highly variable from one plant to another: at day 2, eight plants out of 30 inoculated with the high level inoculum had *E. coli* O157:H7 counts ranging from 1 to 179 CFU, and two plants out of 30 inoculated with the low level inoculum had bacterial counts of 6 and 117 (Table 3). An enrichment method rather than plating was employed at days 7, 14, and 21. *E. coli* O157:H7 was detected by enrichment through 14 days for both inoculum concentrations (Table 3). However, at 21 dpi, *E. coli* O157:H7 was neither detected on the plants nor in the soil sampled from around the plants (section 3.2) at either inoculum concentration. A high inoculum level resulted in a higher frequency of positive plants through day 14 (Table 3). Thus, in subsequent field trials, plants were inoculated only with a high inoculum level (10^7 CFU/ml) and the number of samples processed at each collection time was increased.

3.4. Survival of *E. coli* O157:H7 in inoculated soil and emerging lettuce plants

In spring 2009, the field was irrigated just prior to application of *E. coli* O157:H7 onto the soil surface of the seeded beds. The amount of *E. coli* O157:H7 effectively delivered was calculated to be 4.7 ± 0.4 log CFU/g of soil at 0 h; the population decreased to 4.2 ± 0.4 log CFU/g in the first 2 h after inoculation. By day 7, *E. coli* O157:H7 was detected in soil samples by enrichment from two out of six inoculated beds but from none of the 60 lettuce plants tested. Fifteen days after inoculation, *E. coli* O157:H7 could not be recovered by enrichment, either from the soil or from the 300 sampled lettuce plants that were growing in the inoculated soil.

Table 3
Survival of *E. coli* O157:H7 ATCC 700728 on lettuce plants (summer 2007 trial).

Time post-inoculation	*E. coli* O157:H7 (log CFU/plant) or number of lettuce plants with detectable *E. coli* O157:H7[a]		
	Control	Low initial inoculum (5 log CFU/ml)	High initial inoculum (7 log CFU/ml)
0 h	0	3.6 ± 0.4	4.2 ± 0.8
1 h	0	2.3 ± 1.3	3.4 ± 1.1
2 days	0/30	2/30 (6, 117)[b]	8/30 (1, 2, 4, 19, 20, 92, 127, 179)[b]
7 days	0/30	1/30	11/30
14 days	0/30	1/30	7/30
21 days	ND[c]	0/30	0/60

[a] Values represent the mean population size (log CFU/plant) ± SD at 0 h ($n = 12$) and 1 h ($n = 18$), or as the ratio of the number of plants in which *E. coli* O157:H7 was detected by plating (day 2) or by enrichment (days 7 and 14) over the total number of plants sampled.
[b] Number of CFU obtained per plant are indicated for day 2 samples.
[c] ND, not determined.

3.5. Survival of *E. coli* O157:H7 after inoculation of 2-week-old lettuce plants

A rapid decrease in *E. coli* O157:H7 population from 5.0 ± 0.5 log CFU/g at 0 h to 2.5 ± 0.4 log CFU/g at 2 h was observed after inoculation of 2-week-old lettuce plants (combined drip and overhead irrigation samples) (fall 2009). At days 7 and 14, *E. coli* O157:H7 was detected only by enrichment in 50% and 47% of the plants tested, respectively. At 21 dpi, one plant out of 120 was positive for *E. coli* O157:H7, and at 28 dpi or 2 weeks before the end of the field trial, *E. coli* O157:H7 was not detected on any of the 240 lettuce plants tested by enrichment.

3.6. *E. coli* O157:H7 population dynamics after inoculation of 4-week-old lettuce plants (2008 and 2009 trials)

Within each of the three trials in 2008 and 2009, *E. coli* O157:H7 population size was not significantly different between inoculated plants irrigated by drip or overhead sprinkler at each post-inoculation time through day 7 (Table 4). Therefore, counts from both irrigation treatments for each trial were averaged in order to compare the data (Fig. 1). In these trials, the size of the bacterial population effectively delivered to each plant was influenced by plant weight (provided in Table 1), which reflected leaf surface area. Higher levels of *E. coli* O157:H7 were recovered from spring 2008 plants (19 g average weight) compared to spring and fall 2009 plants (6 and 2 g average weight, respectively) at 0 h (Table 4).

The decrease in *E. coli* O157:H7 population was similar in all three field trials (Table 4, Fig. 1). A 2- to 3-log decrease in *E. coli* O157:H7 population size was observed during the first 2 h post-inoculation (Table 4) and the population size declined to an average of <1 log CFU/plant at day 7 (Fig. 1). Temperature and humidity were similar in spring and fall 2009 trials during the approximate 2-h inoculation period. Temperatures were between 16 and 18 °C for both trials; relative humidity decreased from 76 to 65% and from 74 to 72% for the spring and fall trials, respectively.

Enrichment techniques were used to detect *E. coli* O157:H7 as early as 2 days after inoculation because 42%, 85%, and 74% of the plants tested for spring 2008, spring 2009, and fall 2009 trials, respectively, did not yield colonies with filtered samples. By day 7, for all field trials, at least 82% of the lettuce plants had *E. coli* O157:H7 levels of less than 10 cells; therefore, plants sampled at day 14 and later were processed only by enrichment. *E. coli* O157:H7 could be recovered by enrichment through the end of the trial; 28 days in spring 2008 and 2009 and 35 days in fall 2009 (Fig. 2). The percentage of *E. coli* O157:H7-positive plants remained consistently higher during the spring 2008 trial compared to the spring and fall 2009 trials (Fig. 2).

Table 4
Effect of irrigation method on *E. coli* O157:H7 populations after spray inoculation of 4-week-old lettuce plants.

Time post-inoculation	Irrigation method	*E. coli* O157:H7 (log CFU/plant)[a]		
		Spring 2008	Spring 2009	Fall 2009
0 h	Sprinkler	6.4 ± 0.2	5.8 ± 0.5	5.4 ± 0.6
	Drip	6.1 ± 0.3	5.6 ± 0.3	5.1 ± 0.9
2 h	Sprinkler	4.6 ± 1.1	2.9 ± 0.9	2.4 ± 0.9
	Drip	4.1 ± 0.6	2.5 ± 1.1	2.6 ± 1.0
2 or 3 days[b]	Sprinkler	1.7 ± 0.9	0.8 ± 0.6	1.2 ± 1.2
	Drip	1.3 ± 0.5	0.8 ± 0.5	1.2 ± 1.2
7 days	Sprinkler	0.9 ± 0.5	0.8 ± 0.6	0.6 ± 0.5
	Drip	0.7 ± 0.5	0.6 ± 0.5	0.8 ± 0.6

[a] Results are expressed as the mean population size (log CFU/plant) ± SD; $n = 30$ at 0 and 2 h, $n = 60$ at 2, 3, and 7 days.
[b] Sampling for trials was done at day 2 in spring 2008 and 2009, and at day 3 in fall 2009.

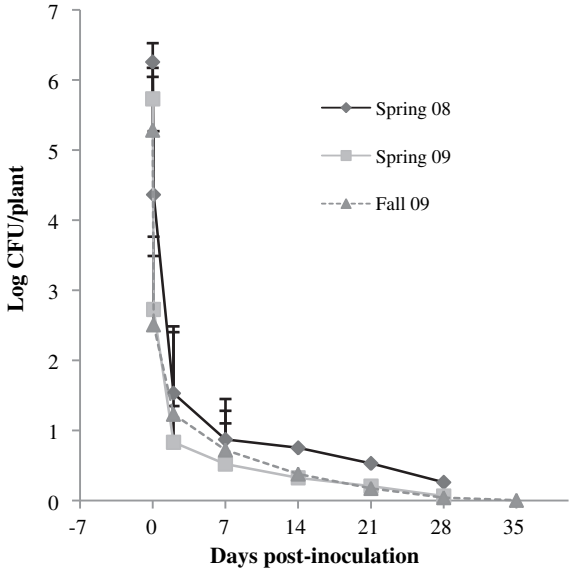

Fig. 1. *E. coli* O157:H7 population dynamics on lettuce leaf surfaces during spring 2008, spring 2009, and fall 2009 trials. Romaine lettuce was inoculated 4 weeks after planting. Each data point represents the mean population size of *E. coli* O157:H7 ± SD; SD is shown only for sampling times (days 0, 2 or 3, and 7) when a plate count was possible. From 14 to 28 dpi, *E. coli* O157:H7 was detected only by enrichment. For all field trials, $n = 60$ at 0 and 2 h post-inoculation, and $n = 120$ at 2 or 3, 7, and 14 dpi. For the spring 2008 trial, $n = 120$ at 21 and 28 dpi. For the spring 2009 trial, $n = 150$ at 21 and 28 dpi. For the fall 2009 trial, $n = 120$ at 21 dpi and $n = 156$ at 28 dpi.

Although irrigation method did not impact population size through day 7, on later sampling days the irrigation method did influence the percentage of plants positive for *E. coli* O157:H7. The number of positive samples was significantly greater among plants irrigated by overhead sprinkler than plants irrigated by drip at day 28 during spring 2008 and at days 7 and 14 during spring 2009 (Fig. 2). In contrast, at day 7 during fall 2009, *E. coli* O157:H7 was detected on a greater number of plants irrigated by drip than on plants irrigated by overhead sprinkler (Fig. 2). During the spring 2008 and 2009 trials, no rainfall was recorded between the time of inoculation and the end of the trials; however, during the fall 2009 trial, 35 mm of rain fell during the first 7 days following inoculation. Field temperature and humidity readings, which were collected only for the 2009 trials, indicated that humidity was higher during the first 2 weeks following inoculation in the fall than in the spring (Fig. 3).

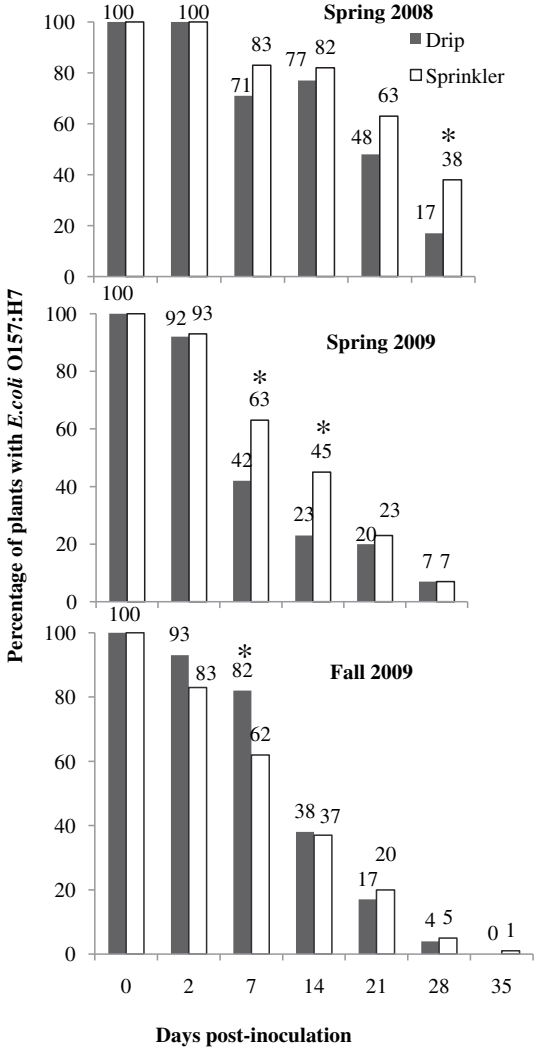

Fig. 2. Effect of drip and sprinkler irrigation on *E. coli* O157:H7 survival. From 14 to 28 days post-inoculation, *E. coli* O157:H7 was detected only by enrichment. $n = 60$ at 0, 2, 7 and 14 days post-inoculation for all trials. $n = 60$ at day 21 and 28 post-inoculation for the spring 2008 trial. $n = 75$ at day 21 and 28 post-inoculation for the spring 2009 trial. $n = 120$ at day 21 and 156 at day 28 post-inoculation for the fall 2009 trial. * indicates statistical difference as determined by Pearson's chi-square test.

3.7. *E. coli O157:H7 localization*

After inoculation of the 4-week-old plants, each lettuce head collected before day 14 during spring 2008 and 2009 and before day 28 during fall 2009 was bagged and processed as one sampling unit. At later sampling times, when the average head weight was greater than 100 g, each head was separated into outer and inner leaves before analysis. For these field trials, *E. coli* O157:H7 was detected primarily on the outer leaves only, and on both outer and inner leaves in less than 30% of the plants (Table 5). *E. coli* O157:H7 was detected on the inner leaves in less than 1% of the plants that tested positive by enrichment (Table 5) at 21 dpi (spring 2008), 14 dpi (spring 2009), and 28 dpi (fall 2009). Therefore, only the outer leaves were tested by enrichment at 28 dpi in spring 2008, at 21 and 28 dpi in spring 2009, and at 35 dpi in fall 2009.

3.8. *Confirmation of E. coli O157:H7 ATCC 700728*

A total of 668 isolates were analyzed by real-time PCR for detection of the Shiga toxin-producing genes *stx*1 and *stx*2 and the *uid*A genes. Presence of the target genes was indicated through analysis of both primary fluorescent curves and melt profiles. *E. coli* O157:H7 strain ATCC 700728 does not have *stx1* and *stx2* genes that encode the Shiga toxin, but has the single base mismatch at +93 in the *uidA* gene, characteristic of the *E. coli* O157:H7 strains we detected by real-time PCR. All the recovered bacteria tested negative for *stx*1 and *stx*2 genes and positive for *uid*A, which confirms their identity as *E. coli* O157:H7 ATCC 700728.

4. Discussion

The field trials described in this paper were conducted in the Salinas Valley where a majority of the commercial lettuce is produced. Few field trials have used *E. coli* O157:H7 because of the obvious barriers to releasing large quantities of this organism into the environment. Islam et al. (2004) used attenuated *E. coli* ATCC 43888 (missing *stx1* and *stx2* genes) labeled with a plasmid containing genes encoding a green fluorescent protein (GFP) and resistance to ampicillin (B6914) and more recently, Erickson et al. (2010a,b) used a cocktail of similarly marked and attenuated

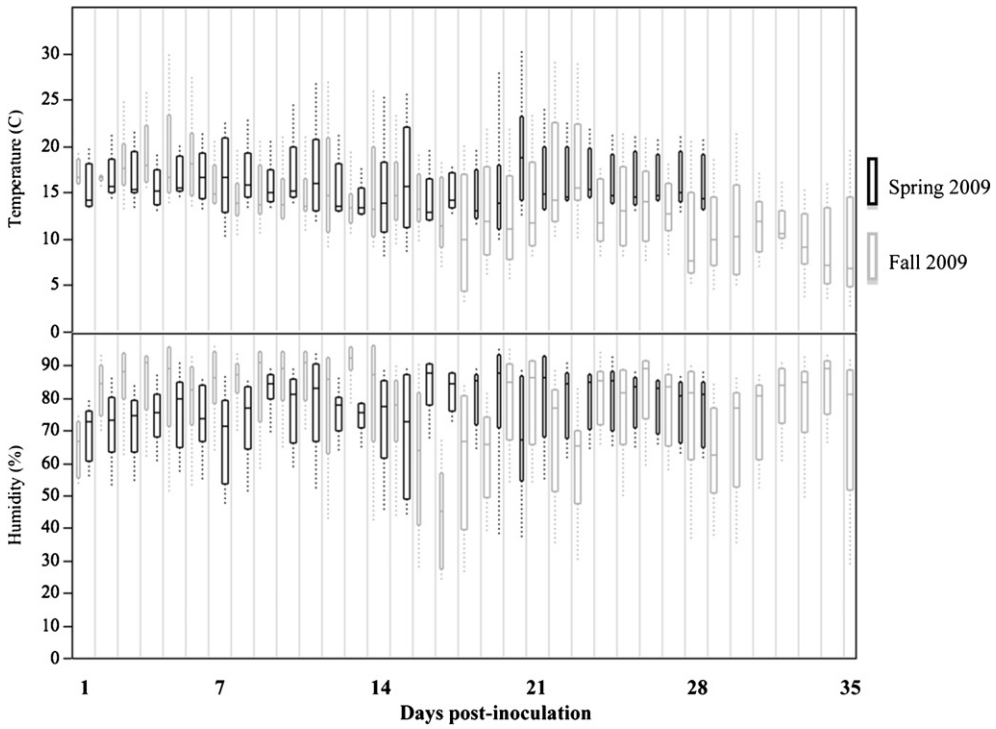

Fig. 3. Temperature and humidity during spring and fall 2009. Data are presented in a boxplot graph where the bottom and top of the box are the 25th and 75th percentile and the end of the whiskers are the minimum and maximum of all the data.

E. coli O157:H7. Permission was given to use a single attenuated strain of *E. coli* O157:H7 and two, ATCC 700728 and ATCC 43888, were considered. Both are missing *stx1* and *stx2* genes but ATCC classifies them as BSLI and BSLII, respectively. We conducted preliminary experiments in the laboratory comparing the survival of ATCC 700728 and ATCC 43888 on lettuce plants grown under low humidity. No difference in survival on the lettuce was observed between the two strains (data not shown). A plasmid-borne label such as GFP was not acceptable to the committee granting the permit for these experiments and, therefore, a rifampicin-resistant variant of ATCC 700728 was selected for the field trials.

We evaluated the survival of an attenuated strain of *E. coli* O157:H7 applied to the soil after lettuce seeding but prior to germination, or to the growing lettuce plants 2 or 4 weeks after seeding. The inoculum level used in our study was likely orders of magnitude greater than would normally be expected in irrigation water, which in the Salinas Valley is largely from wells. However, contamination of well water is possible prior to foliar contact as it is not always pumped directly into irrigation lines. The method of inoculum application used in the study was designed to provide a single uniform aqueous contamination of the field that might occur from contaminated irrigation water or from contaminated foliar spray. The total volume of inoculum applied to each plant was much less than would be expected during typical overhead irrigation, which can last from 2 to 4 h (Jackson et al., 1996). It is unknown how lower contamination levels and higher application volumes would translate to levels of *E. coli* per plant. The preliminary field trial we conducted in summer 2007 demonstrated the difficulty in detecting *E. coli* O157:H7 by plate count and the large number of samples required to retrieve the attenuated strain of *E. coli* O157:H7 even by enrichment of whole plants. The rapid population decrease after inoculation necessitated the application of the high inoculum concentrations that were used to facilitate comparison between irrigation treatments in the following field trials.

Table 5
Localization of *E. coli* O157:H7 on outer and/or inner leaves of lettuce plants.

E. coli O157:H7 location	Percentage of *E. coli* O157:H7-positive plants[a]			
	Spring 2008		Spring 2009	Fall 2009
	14 dpi[b]	21 dpi	14 dpi	28 dpi
Outer and inner leaves	24 (23)[c]	30 (20)	8 (2)	0 (0)
Outer leaves only	66 (63)	69 (46)	81 (21)	100 (7)
Inner leaves only	10 (9)	1 (1)	1 (3)	0 (0)
Total positive/total analyzed	79 (95/120)[d]	56 (67/120)	39 (26/66)	0.6 (7/156)

[a] Inner and outer leaves were analyzed separately only when plants weighed more than 100 g.
[b] dpi, days post-inoculation.
[c] Value in parentheses indicates number of plants that tested positive for *E. coli* O157:H7 by enrichment.
[d] Ratio in parentheses indicates number of plants that tested positive for *E. coli* O157:H7 by enrichment per total number of plants analyzed.

The incidence of *E. coli* O157:H7 in water sampled in the Salinas Valley is extremely low (Cooley et al., 2007). Recovery of *E. coli* O157:H7 from a small number of samples is only possible by enrichment and concentration; therefore, quantification of the organism is usually not done (Cooley et al., 2007). Current leafy greens metrics (www.caleafygreens.ca.gov/members/resources.asp) set thresholds for generic *E. coli* levels for preharvest water at 126 MPN/100 ml (geometric mean) and 235 MPN/100 ml (max) for foliar application, or at 126 MPN/100 ml (geometric mean) and 576 MPN/100 ml (max) for non-foliar application. The underlying assumption is that there is a relationship between microbial levels in irrigation water and the possibility that leafy greens will become contaminated. However, Cooley et al. (2007) demonstrated that in the Salinas watershed generic *E. coli* was a poor indicator of the presence of *E. coli* O157:H7. The correlation between levels of generic *E. coli* and incidence of *E. coli* O157:H7 was insignificant at most individual sampling sites (Cooley et al., 2007).

In all four field trials, a very rapid population decline of *E. coli* O157:H7 was observed, which was independent of the inoculum level, time of planting, or irrigation method. It is possible that part of the observed drop in population was due to attachment of the bacteria to the leaf surface and our failure to remove these attached cells with the stomaching method employed here. However, other studies in our laboratory with inoculated plants grown in the greenhouse or growth chamber suggest that the methods used here recover the majority of the cells applied (data not shown).

The ability to retrieve *E. coli* O157:H7 from lettuce plants was influenced by the time of inoculation. *E. coli* O157:H7 could not be retrieved from lettuce leaves at any time point when applied to the soil before seed germination. When 2-week-old plants were inoculated, *E. coli* O157:H7 could not be recovered from lettuce plants at 28 dpi (2 weeks before the end of the field trial). As early as 2 days after inoculation of 4-week-old plants, *E. coli* O157:H7 was detected only by enrichment for some samples. The population size of *E. coli* O157:H7 was highly variable among plants during the first 2 days. By 7 dpi, 82% or more of the lettuce plants had counts below the limit of detection. However, when *E. coli* O157:H7 was inoculated onto 4-week-old plants, the organism could be detected by enrichment throughout the experiment through harvest for three of the four trials. Our inability to recover *E. coli* O157:H7 by enrichment after 14 dpi during the summer 2007 trial may have been due to the lower number of samples analyzed. However, the number of plants analyzed and the enrichment of the entire head far exceeds the testing standards routinely used in commercial or regulatory practice.

Our results confirm findings from previous studies on the fate of *E. coli* O157:H7 in lettuce fields that demonstrated persistence of the bacteria throughout the growing season to harvest (Ercolani, 1979; Erickson et al., 2010b; Islam et al., 2004). Islam et al. (2004) similarly applied an attenuated *E. coli* O157:H7 strain B6914 onto leaf lettuce grown in Georgia, USA. Irrigation water was inoculated at log 5 CFU/ml and applied by spraying lettuce plots one time with a hand sprayer 3 weeks after seedlings were transplanted (in late October). Unlike the current study, *E. coli* O157:H7 could be detected in the soil and on lettuce plants for 15 and 8 weeks after inoculation, respectively. However, the *E. coli* strain and lettuce variety differed from the current study, the water application rate (2 L per 8 m^2) was much higher by volume than the rate used for the current study, and the weather (temperature and rainfall) conditions were not the same.

Environmental conditions as well as microorganisms in the phyllosphere are thought to contribute to the fate of pathogens in the field (Brandl, 2006, 2008; Heaton and Jones, 2008). The leaf surface provides limited nutrients, and rapid fluctuations in temperature and humidity are typical (Lindow and Brandl, 2003). Direct-seeded lettuce fields are typically watered with overhead irrigation for the first 2 weeks after seeding. Thereafter, drip-irrigated seedlings received all water at or just below the soil surface. In the Salinas Valley, rainfall is rare from May through October, and total precipitation averages ~6 mm during this time. However, due to the coastal location of the Salinas Valley, leaf wetness can periodically occur from the early, pre-dawn hours through mid-morning (10:00, Pacific Standard Time) for much of the growing season (Scherm et al., 1995). Thus, for most of the day, periods of significantly different free moisture levels would be expected on leaves of plants grown using the two irrigation methods employed for this study. O'Brien and Lindow (1989) compared the ability of *E. coli* and *Pseudomonas syringae*, a plant-associated bacteria, to grow on plants under wet and dry conditions. The ability of *E. coli* to multiply on plants was similar to *P. syringae* under wet conditions, whereas under dry conditions, population sizes of *E. coli* were significantly lower than for *P. syringae*. Under laboratory conditions, Brandl and Amundson (2008) demonstrated that a warm incubation temperature and the presence of free water on Romaine leaves favored growth of *E. coli* O157:H7. The relative field humidity in the current study fluctuated between a minimum of 40% to a maximum of 100% and the overhead sprinkler provided water on the leaves, and thus creating conditions conducive for bacterial growth. Although overhead irrigation significantly increased the percentage of *E. coli* O157:H7-positive plants detected at the end of the spring 2008 trial, the irrigation method did not significantly impact the percentage of lettuce plants that were positive for *E. coli* O157:H7 at the end of either 2009 trials. It is possible that *E. coli* O157:H7 did multiply in discrete locations on some plants where conditions were favorable. We cannot exclude the possibility that some of the observed persistence of *E. coli* O157:H7 was due to localized multiplication which would not be detected with our sampling methodology and would be difficult to demonstrate with any field trial. At the field level, high populations of *E. coli* O157:H7 were not sustained.

A number of studies have explored the potential of *E. coli* O157:H7 to grow on lettuce leaves in growth chamber or greenhouse settings (Brandl and Amundson, 2008; Franz et al., 2007; Ibekwe et al., 2007; Jablasone et al., 2005; O'Brien and Lindow, 1989; Solomon et al., 2002). However, competition between the natural flora and enteric pathogens has been demonstrated on lettuce plants. In a study with two prevalent epiphytic bacteria isolated from pathogen-contaminated plants, Cooley et al. (2006) found that *Wausteria paucula* promoted *E. coli* O157:H7 survival on leaf lettuce but *Enterobacter asburiae* repressed the growth of the pathogen (Cooley et al., 2006). Such studies highlight the impact that natural flora may have on field survival of enteric pathogens that contaminate lettuce and other crops grown in outdoor environments.

The potential for internalization into the vascular system of plants has been shown to be greater in seedlings than in mature plants (Jablasone et al., 2005). In the field, internalization of *E. coli* O157:H7 in leafy green lettuce was reported when *E. coli* O157:H7 was applied at 8 log CFU/ml by spraying the abaxial side of the leaves, and could be detected up to 14 days post-inoculation (Erickson et al., 2010b). In contrast, *E. coli* O157:H7 was not detected internally in the leaves when applied in the soil via compost (Erickson et al., 2010a). We did not investigate the potential of *E. coli* O157:H7 to be internalized but we evaluated the potential for *E. coli* O157:H7 migration on the plants.

Lettuce plants were inoculated when they were at the eight- to twelve-leaf stage. As lettuce plants grow, new leaves are formed in the center of the plant. With the overhead sprinkler treatment, we expected to observe a random distribution of *E. coli* O157:H7 between inner and outer leaves, but this was not the case. At sampling days closer to harvesting time, *E. coli* O157:H7 was recovered mainly on the outer leaves of the lettuce plant. During plant development, *E. coli* O157:H7 did not move readily from the outer leaves where the inoculation occurred, to the inner leaves, but appeared to largely remain located on the outer leaves. At harvest, it is typical for Romaine lettuce to be hand cut and for many outer leaves to be removed from the head and discarded in the field. Thus, lettuce leaves contaminated early in the production cycle are less likely to be included in retail packages.

Under the field conditions examined in the present study, neither drip nor overhead irrigation had a consistent impact on the survival of *E. coli* O157:H7. For both irrigation methods, increasing the interval from the time of contamination to the point of harvest significantly decreased the likelihood that the *E. coli* O157:H7 would be present in the harvested product. With the high inoculum levels used in this study, the organism could be detected by enrichment on a small but measurable fraction of inoculated heads

of lettuce through to harvest. The infectious dose for *E. coli* O157:H7 is generally reported to be as few as 10 cells (FDA, 2010). Under favorable conditions, even small numbers of *E. coli* O157:H7, could potentially become a hazard during postharvest handling. Plant tissue damage caused by bruising, cutting, or plant pathogens promotes the growth of *E. coli* O157:H7 (Aruscavage et al., 2008; Brandl, 2008). Packaged chopped or shredded lettuce can support the growth of *E. coli* O157:H7 when the storage temperature is not maintained below 8 °C (Abdul-Raouf et al., 1993; Lang et al., 2004; Li et al., 2001; Theofel and Harris, 2009). However, those studies were carried out with laboratory-cultured *E. coli* O157:H7 inoculated onto harvested lettuce and may not be representative of field-inoculated product. The contribution to foodborne illness of a small percentage of plants carrying low levels of environmentally-stressed *E. coli* O157:H7 is unknown.

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