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Survival of attenuated *Escherichia coli* O157:H7 or surrogate in field-inoculated lettuce

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Summary

A better understanding of the movement and fate of *Escherichia coli* O157:H7 in lettuce field is needed to provide information on contamination risks and strategies to avoid future outbreaks of foodborne illness. To date, research has primarily focused on the behavior of *E. coli* on cut leafy greens and very little information is available on the association of *E. coli* O157:H7 on growing lettuce plants. In August 2007 we conducted an initial lettuce field trial in the Salinas valley to get a better understanding of *E. coli* O157:H7 survival on plants in the field. Preliminary experiment allowed us to develop rifampicin mutants for the three strains under evaluation: two attenuated (non-pathogenic) strains of *E. coli* O157:H7: ATCC 43888 and ATCC 700728 and one surrogate, *Citrobacter youngae*. A series of experiment were conducted in the laboratory to select the organism to be used for the field trial. Survival of attenuated *E. coli* O157:H7 or surrogate *C. youngae* was identical for the three strains tested either on lettuce pieces under high humidity storage or on whole plants in a dry environment. Liquid and agar culture preparation methods did not influence survival although sample to sample variability was greater with liquid culture. On lettuce pieces under high humidity, initial and final counts were similar after 24 h of incubation at room temperature. In contrast, on whole plants in a dry environment, counts decreased by 100-fold within 24 h. Because of its classification as a BSL1 strain, rifampicin-resistant attenuated *E. coli* O157:H7 Strain ATCC 700728 was selected for inoculation onto 4-week old field lettuce. A split plot design was used for the field trial to evaluate the two main treatment effects: drip and overhead irrigation and the three subtreatments: uninoculated control and initial inoculation with 10^7 and 10^5 CFU/plant. One hour after inoculation, a high variability in counts was observed among plant samples and a decrease between 1 to 2 log CFU/plant was recorded for both levels of inoculation. After 2 days, two and eight of 30 plants yielded counts ranging from 1 to 179 cells per plant for the low and high inoculum levels, respectively. At day 7, 14, and 21 plants were scored as positive or negative based on enrichment of the sample (individual heads of lettuce). Positive samples were detected at day 14 (one and seven of 30 for low and high inoculum levels, respectively), but none of 90 samples were positive at day 21. Differences were not detected between irrigation methods although there were unexplained differences in survival among the beds. *E. coli* O157:H7 Strain ATCC 700728 was not detected in soil 30 days after inoculation.

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Objectives

Phase I

1. To isolate and characterize antibiotic-resistant strains of an appropriate non-pathogenic *E. coli* or surrogate organism for inoculation of lettuce in the field.
2. To evaluate inoculation preparation, and inoculation and recovery methods for the selected organism and to convert these methods into standard microbiological operating procedures for use in the field.

Phase II

1. Evaluate the persistence of attenuated (non-pathogenic) *E. coli* O157:H7 or non-pathogenic surrogates inoculated onto lettuce grown under field conditions (drip and overhead irrigation) in the Salinas valley.

Procedures

Bacterial suspension preparation. Stock cultures of rifampicin-resistant or nalidixic acid-resistant *E. coli* O157:H7 ATCC 700728 and ATCC 43888 were streaked on tryptic soy agar (TSA) with 50 µg/ml of rifampicin or 120 µg/ml of nalidixic acid. These strains were tested in our laboratory and both were confirmed as negative by PCR for *stx1* and *stx2* genes. Both strains have a single base mismatch at +93 in *uidA* gene, characteristic of *E. coli* O157:H7, that could be detected by PCR. For the liquid culture preparation, a single colony was inoculated in 2 ml TSB supplemented with the appropriate antibiotic and incubated overnight at 37°C and 150 rpm. Bacteria were harvested by centrifugation (10,000 g for 2 min) and resuspended in 0.1% peptone buffer. Washing was repeated three times and cells resuspended in 0.1% peptone buffer. For the

plate culture, one colony was streaked on TSA with 50 μg/ml of rifampicin and incubated overnight at 37°C. Cells were suspended directly from the plate in 0.1% peptone and centrifuged (10,000 g for 2 min). Washing was repeated three times and cells were resuspended in 0.1% peptone buffer.

Plant growth conditions: Romaine lettuce (*Lactuca sativa* cv. Parris Island) seeds were grown in an environmental chamber with a light intensity of 230 μm m^{-2} s^{-2}, 25% humidity, 12 h photoperiod, 18°C during the night and 22°C during the day. After inoculation, plants were grown at 22°C with a 12 h photoperiod and 25% humidity.

Inoculum recovery and quantification. Lettuce samples were homogenized in a stomacher for 2 min at medium speed in 0.1% peptone. The recovered bacterial suspension was plated with a Spiralplater on TSA with 50 μg/ml rifampicin. When necessary to improve the limit of detection, samples were filtered onto disposable analytical filter units (0.45 μm, Nalgene). Filter membranes were removed and placed on CROMAgar™ 0157 (BD, Franklin Lakes, NJ) (1) supplemented with 50 μg/ml rifampicin.

Field studies: A split plot design was used for the field trial to evaluate the two main treatment effects: drip and overhead irrigation. Three subtreatments (uninoculated control and inoculation with 10^7 and 10^5 CFU/plant of rifampicin-resistant *E. coli* O157:H7 ATCC 700728) were applied randomly and replicated three times per irrigation type. Each replicate or sampling block (total 18) was 40 inches (wide) by 36 inches (deep) and a total length of 145 bed feet. In between the drip and overhead irrigation, 10 unfarmed beds prevented drift from the overhead irrigation. Romaine lettuce was inoculated after thinning (4 weeks after planting) by spraying the prepared cell suspension per plant. Sampling was conducted at 0 and 1 hour, 2, 7, 14, and 21 days. Unless otherwise noted, 90 plants were sampled at each sampling date, 30 plants per subtreatment, five plants per sampling block.

Soil sampling and analysis: Soil samples were collected from each of 18 blocks throughout the field. Five random samples per block were taken from the top layer (15 cm) with an auger and bulked. After being thoroughly mixed in a clean plastic bag, 10 g subsamples were homogenized with 90 ml 0.1% peptone buffer in a stomacher. Detection of *E. coli* O157:H7 was performed by plating serial dilution on CROMAgar™ 0157 (BD, Franklin Lakes, NJ) (1) for the sampling at day 0. Detection of *E. coli* O157:H7 ATCC 700728 was performed by plating serial dilution on CROMAgar™ O157 suplemented with 50 μg/ml rifampicin for the sampling at day 28. Colonies presumptively identified as *E. coli* O157:H7 were confirmed by real-time PCR.

DNA template preparation: DNA template was isolated from 1 ml overnight culture grown in Luria Broth (LB) at 37°C. Cell culture was washed twice with water by centrifugation at 12,000 g for 2 min, resuspended in 1 ml water, and boiled for 10 min. After centrifugation, 0.5 μl was added to the real-time PCR reaction.

Real-time PCR: Amplification of *stx*1, *stx*2 and *uid*A genes was performed on the ICycler real time detection system (Bio-Rad) with Power SYBR Green PCR Master Mix (Applied Biosystems). Primers to amplify *uid*A, *stx*1 and *stx*2 genes were designed as described by Yoshitomi *et al*. (2). The different components were added to the real-time PCR mixture in the

following concentrations: 0.25 μM for reverse and forward primer, 1X Power SYBR Green PCR Master Mix and immediately prior to PCR, 0.5 μl of prepared template. For each plate *E. coli* strain K12 was used as a negative control and *E. coli* O157:H7 strain H1730 (isolate from lettuce outbreak containing both *stx*1 and *stx*2 genes) was used as a positive control. Cycling conditions were performed in a two-step PCR, with an initial polymerase activation of 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 20 s, and an annealing/extension step at 63°C for 25 s. After completion of 40 PCR cycles, melt curve data was generated by increasing the temperature from 60 to 95°C at 0.2°C/10 s and recording fluorescence. Identification of an isolate as positive for the gene of interest was determined by positive Ct value and corresponding melting temperature.

Results:

Phase I: The survival of antibiotic-resistant attenuated *E. coli* O157:H7 or *C. youngae* (selected as a surrogate) was evaluated using two different inoculation preparation methods. Romaine lettuce purchased at a local retail market was cut into squares (10 X 10 cm) and inoculated with 20 μl of inoculum applied to the leaf surface in several droplets. After holding the lettuce for 2 hours in a biosafety hood to allow the inoculum to dry, samples were placed in a plastic bag with a moist towel and held at ambient temperature for an additional 22 h. In addition, Romaine lettuce plants were grown in an environmental growth chamber. The inoculum was either sprayed or spotted onto the plants and the plants were held for 24 h in the laboratory at ambient temperature prior to sampling.

1) Comparison between mutants resistant to nalidixic acid and rifampicin.

To facilitate differentiation of lettuce-colonizing bacteria from the inoculum, nalidixic acid and rifampicin-resistant *E. coli* O157:H7 ATCC 43888, ATCC 700728 and *C. youngae* mutants were isolated using standard procedures. Romaine lettuce purchased from a retail store had background populations (aerobic plate counts) that ranged from 3 to 5 log CFU/g. After 2 h in the biosafety hood, a reduction in the background population of approximately 1 log CFU/g was observed. However, after 22 h of storage at ambient temperature, an increase from initial levels of 1 to 3 log CFU/g was noted. No colonies were observed when controls were plated onto agar plates containing 50 μg/ml of rifampicin. In contrast, when controls were plated onto agar containing 120 μg/ml of nalidixic acid many colonies were observed especially when the background population was high. Therefore, rifampicin-resistant isolates were used in all further studies.

2) Survival of attenuated *E. coli* O157:H7 or *C. youngae* inoculum prepared from solid or liquid culture and inoculated at high or moderate density onto lettuce pieces.

Romaine lettuce pieces (10 X 10 cm) were inoculated with *E. coli* O157:H7 ATCC 43888 or ATCC 70728 or *C. youngae* at high (2 X 10^6 cells/lettuce piece) or moderate (2,000 cells/lettuce piece) concentrations. Similar behavior among the two *E coli* O157:H7 and *C. youngae* was observed at the high inoculum level (Fig. 1). The estimated and recovered levels of inoculated bacteria were approximately the same immediately after inoculation when the inoculum was still wet suggesting efficient recovery from the lettuce. Greater variability was observed in counts among samples 2 and 24 h after inoculation, particularly for those samples inoculated with liquid culture. For this reason, inoculum was prepared from cells collected from agar plates for all further studies. Similar data were obtained in a second study with *E. coli* O157:H7 ATCC 43888

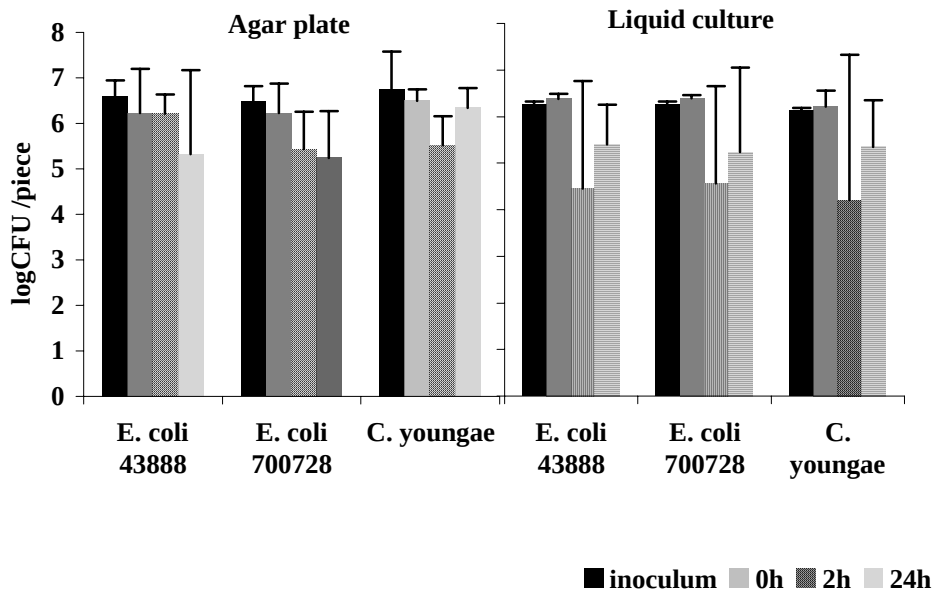

Figure 1. Bacterial survival after inoculation of lettuce pieces at high concentration (2 X 10^6 CFU/piece). Bacteria were harvested from agar plates or broth.. Each bar represent the mean of four replicate samples from each of three trials (n=12). The estimated delivered inoculum was calculated by determining populations in the inoculum preparation. Other data represent counts recovered from lettuce samples.

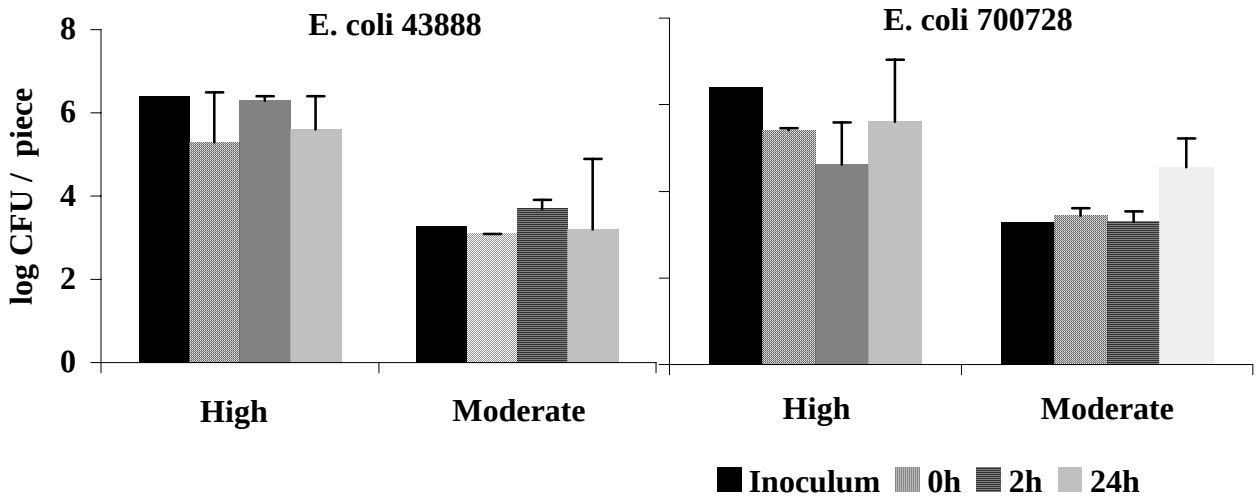

Figure 2: Survival of *E. coli* 43888 and 700728 inoculated onto lettuce pieces at high (2 X 10^6 CFU/piece) and moderate (2,000 CFU/piece) concentration. Each bar represent the mean of four replicate samples from a single trial (n=4). The estimated delivered inoculum was calculated by determining populations in the inoculum preparation. Other data represent counts recovered from lettuce samples.

and ATCC 700728 inoculated at high and moderate concentrations (Fig. 2). However, increases in the population of both strains by as much as 2 log CFU/piece was observed after 24 h in some samples that were inoculated at moderate concentration.

3) **Development of methods for recovering bacteria inoculated at a low density.**
When lettuce pieces were inoculated at 200 CFU/lettuce piece the cells could not be detected using standard methods. To improve the limit of detection, lettuce pieces were stomached in 50 ml of buffer and then the liquid was passed through a sterile filter. The filter was placed directly onto agar medium and colonies appearing on the filter were enumerated. This step improved the limit of detection from 200 to 1 CFU/lettuce piece (Fig. 3). Although initial 1-log reductions were observed within the first 2 h after inoculation, the population of *E. coli* ATCC 43888 increased by 2 log CFU/piece after 24 hours.

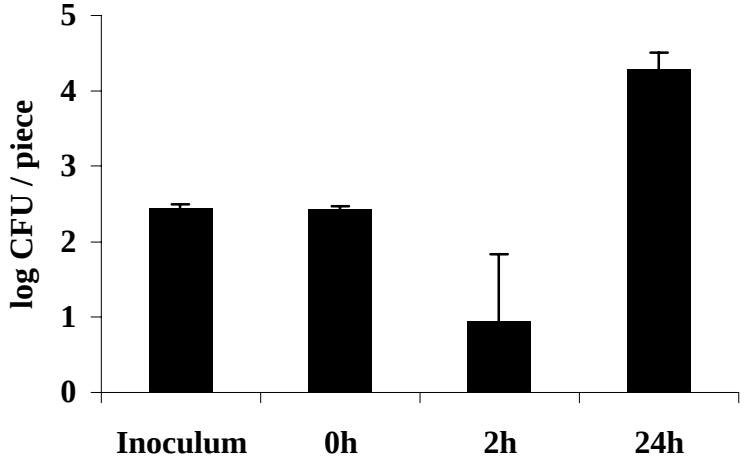

Figure 3. Survival of *E. coli* O157 43888 (harvested from agar plates) after inoculating lettuce pieces (10 X 10 cm) at low concentration (approximately 200 cells/leaf) and recovery using sample filtration. Each bar represent the mean of four replicate samples from a single trial (n=4). The estimated delivered inoculum was calculated by determining populations in the inoculum preparation. Other data represent counts recovered from lettuce samples.

4) Comparison of methods for inoculating lettuce plants
For field use, inoculation by spraying is more practical than spotting individual plants. Spray and spot inoculation delivery methods were compared on lettuce plants that had been grown for 4 weeks in an environmental growth chamber. The number of bacteria recovered immediately after spraying was lower than the number of bacteria inoculated by the spot method (Fig. 4). However, not all of the inoculum delivered by spraying hit the lettuce leaves which could explain the lower than estimated populations. Populations decreased by 2 and 4 log CFU/plant with spot and spray inoculation, respectively 24 h after inoculation. Sample to sample variability in counts was greater for plants inoculated by the spot method.

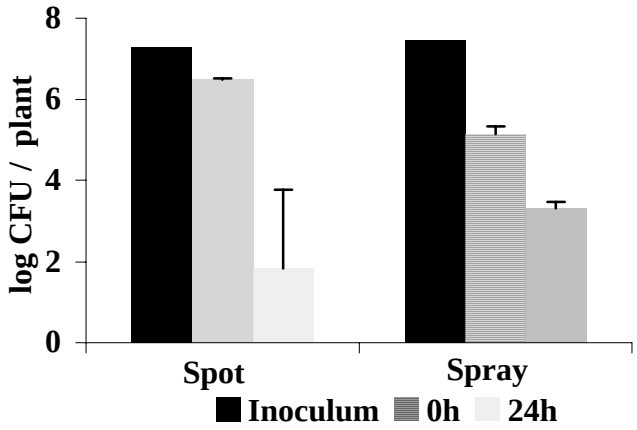

Figure 4. Comparison of *E. coli* O157 43888 survival on plants inoculated by the spray or spot method. For each treatment 10 plants were inoculated with an estimated 2 X 10^7 cells. Each bar represents the mean of five replicate plants from a single trial (n=5). The estimated delivered inoculum was calculated by determining populations in the inoculum preparation. Other data represent counts recovered from lettuce samples.

Phase II: We compared the survival of attenuated rifampicin-resistant *E. coli* O157:H7 ATCC 700728 inoculated at two levels onto lettuce irrigated by drip or overhead sprays.

1) Survival of attenuated *E. coli* O157:H7 in field inoculated lettuce.
Lettuce plants were inoculated by spraying with an estimated 10^5 or 10^7 CFU/plant (Fig. 5). Spray bottles were calibrated to distribute uniformly 1.2 ml of cell suspension but depending on the surface area of the plants, the number of bacteria effectively distributed on each plants was much lower (Fig.5). Control plants were inoculated with 1.2 ml of 0.1% peptone buffer.

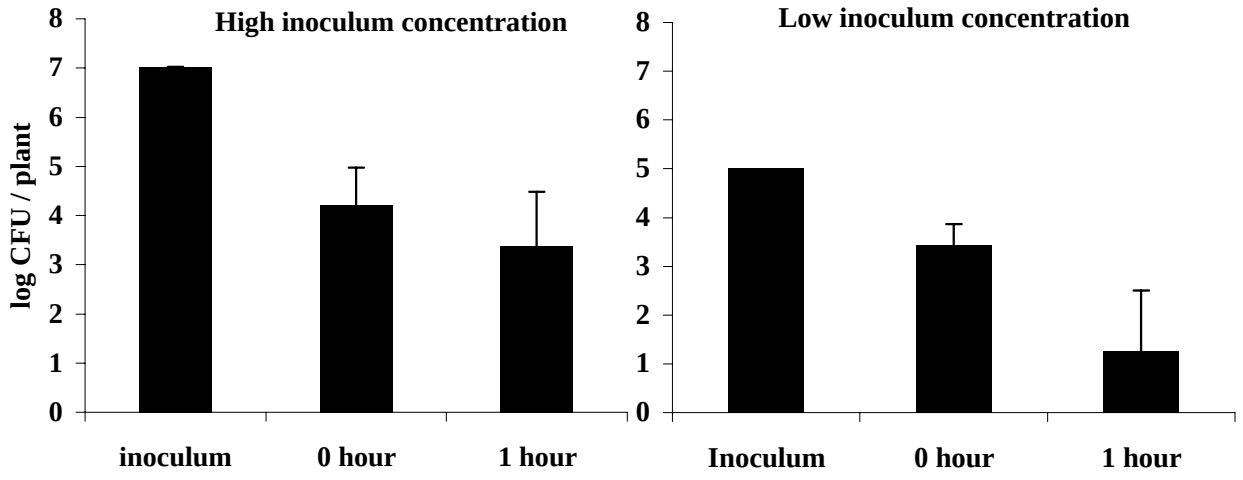

Figure 5. Bacterial survival after spraying lettuce plants with. an estimated 10^5 or 10^7 CFU/plant. Each bar represent the mean of 12 replicate samples at 0 hour (n=12) and 18 replicate samples at 1 hour (n=18) from a single trial. The estimated delivered inoculum was calculated by determining populations in the inoculum preparation. Other data represent counts recovered from lettuce samples.

The background population growing aerobically on TSA was between 3 to 4 log CFU/g of lettuce. At each sampling time we performed the same number and type of analyses for the control plants and *E. coli* O157:H7 inoculated plants. Bacteria resistant to rifampicin were never recovered from the control plants in the absence of an enrichment step. Rifampicin resistant bacteria were recovered from control plants incubated in enrichment broth but none of them gave the reaction typical of *E. coli* O157:H7 (mauve colonies) on CHROMagar.

To enumerate bacteria on the plants, 12 plants were selected randomly for each inoculum concentration immediately after inoculation. Average populations of 3 or 4 log CFU/plant were achieved for the low and high inoculum concentrations, respectively. After 1 hour, no moisture was visible on the plants. For both inoculum levels, a reduction of 1 to 2 log CFU per plant was observed at this time (Fig. 5). Higher variability in counts was observed among plant samples after 1 h. Samples were filtered in order to detect *E. coli* in the lettuce inoculated at the low inoculum concentration.

Two days after the field inoculation, *E.coli* O157:H7 ATCC 700728 was detected in 2 and 8 plants out of 30 at low and high inoculum, respectively (Table 1). Levels detected ranged from 1 to 179 CFU per plant. Because of the low number of plants that had detectable *E.coli* O157:H7 ATCC 700728 an enrichment rather than plating method was employed at 7, 14 and 21 days.

Table 1. Survival of *E.coli* O157:H7 ATCC 700728 on randomly-selected lettuce plants. Results are expressed as the number of plants where *E. coli* O157:H7 ATCC 700728 was detected by plating (day 2) or by enrichment (days 7, 14 and 21) over the total number of plants sampled.

	Number of lettuce plants with detectable *E. coli*/total plants tested (number of cells per plant recovered by filtration)	
Sampling day	High initial inoculum	Low initial inoculum
2	8/30 (1 ,2, 4, 19, 20, 92, 127,179)	2/30 (6, 117)
7	11/30[1]	1/30[1]
14	7/30[1]	1/30[1]
21	0/60	0/30

Survival of *E.coli* O157:H7 ATCC 700728 was measured by evaluating the number of plants where *E.coli* O157:H7 ATCC 700728 was detected after enrichment. *E.coli* O157:H7 ATCC 700728 was detected over 14 days for both concentrations of inoculum (Table 1). The method of irrigation did not make an apparent difference in *E.coli* O157:H7 ATCC 700728 survival (Table 2). Plant location in the field appeared to influence on *E.coli* O157:H7 ATCC 700728 survival although there was no obvious explanation for this observation. Two blocks H3 and H4 consistently had a higher number of plants with *E.coli* O157:H7 ATCC 700728 (Fig. 6).

After 21 days *E.coli* O157:H7 ATCC 700728 was neither detected on the plants nor in the soil at either inoculum concentration.

Table 2. Survival of *E.coli* O157:H7 ATCC 700728 on lettuce plants irrigated by overhead sprinkler or drip. Results are expressed as the number of plants where *E. coli* O157:H7 ATCC 700728 was detected by plating (day 2) or by enrichment (days 7 and 14) over the total number of plants sampled.

	Irrigation Method (number of samples with detectable *E. coli*/total samples tested)	
Sampling days	Sprinkler	Drip
2 day	4/15	4/15
7 day	5/15	6/15
14 day	2/15	5/15

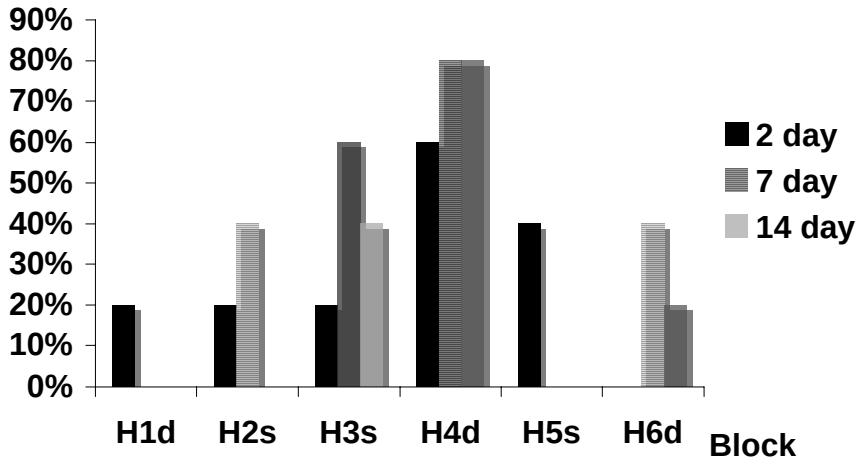

Figure 6. Percentage of plants where *E.coli* O157:H7 ATCC 700728 was detected by plating (day 2) or by enrichment (days 7 and 14). Each block (H1 to H6) represents one replicate. H1, H4 and H6 blocks were irrigated with drip. H2, H3 and H5 blocks were irrigated with sprinkler.

2) Detection of *E. coli* O157:H7 in soil

Before we inoculated the lettuce field with *E. coli* O157:H7 ATCC 700728, we sampled the soil to determine the presence of wild-type *E. coli* O157:H7. Soil samples were collected from each of 18 blocks throughout the field. Five random samples per block were taken from each block and bulked. Although we isolated a total of six colonies on CHROMagar that were the mauve color characteristic of *E. coli* O157:H7, further analysis with real-time PCR proved that these bacteria were false positive. Four weeks after we inoculated the field with *E. coli* O157:H7 ATCC 700728, we repeated the same sampling procedure, this time using CHROMagar with 50 µg/ml of rifampicin and were not able to retrieve any viable *E. coli* O157:H7 ATCC 700728.

3) Identification of bacteria recovered from field trial as *E. coli* O157:H7 ATCC 700728

For each plant determined to be positive by enrichment at 7 and 14 days, we recovered 12 isolates that were both rifampicin resistant and had mauve colonies after plating on CHROMagar (Table 3). A total of 240 bacteria was further submitted to real-time PCR analyses for detection of the Shiga toxin producing genes *stx*1 and *stx*2 and *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 have the single base mismatch at +93 in *uidA* gene, characteristic of *E. coli* O157:H7 strains as we detected by real-time PCR. All the recovered bacteria tested negative for amplification of *stx*1 and *stx*2 genes and positive for amplification of *uid*A confirming their identity as *E. coli* O157:H7 ATCC 700728 (Table 3).

Table 3. Detection of *stx*1, *stx*2 and *uid*A genes in bacteria recovered from lettuce inoculated with *E. coli* O157:H7 strain ATCC 700728.

Sampling day	Number of plants with *E.coli*/ Total plants tested	Number of bacteria tested	*Stx*1 detection	*Stx* 2 detection	*uid*A detection
7	12/60	144	144 negative	144 negative	144 positive
14	8/60	96	96 negative	96 negative	96 positive

References

1. **Bettelheim, K. A.** 1998. Reliability of CHROMagar O157 for the detection of enterohaemorrhagic *Escherichia coli* (EHEC) O157 but not EHEC belonging to other serogroups. J. Appl. Microbiol. **85:**425-428.
2. **Yoshitomi, K. J., K. C. Jinneman, and S. D. Weagant.** 2006. Detection of Shiga toxin genes stx1, stx2, and the +93 uidA mutation of *E. coli* O157:H7/H-using SYBR Green I in a real-time multiplex PCR. Mol Cell Probes **20:**31-41.