California Leafy Greens Research Board
Final Report April 1, 2008 to March 31, 2009

I. Abstract

Project Title: Survival of attenuated *Escherichia coli* O157:H7 ATCC 700728 in field-inoculated lettuce.

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Summary: A better understanding of the behavior of *E. coli* O157:H7 in the lettuce production environment is important for the development of effective mitigation strategies. For these reasons, in August 2007, an initial field trial was established in the Salinas valley to monitor and quantify the survival of a non-pathogenic strain of *E. coli* O157:H7 (strain ATCC 700728) on leaf-inoculated Romaine lettuce plants. The data obtained in 2007 were used to refine sampling and analysis protocols for field trials in June and August 2008 (described in this report) and to develop laboratory conditions that could be used to mimic field conditions. Under laboratory conditions, factors that could affect survival of *E. coli* O157:H7 on lettuce leaves such as plant age, inoculum preparation and strain of *E. coli* O157:H7 were evaluated. None of the studied factors had a significant impact on *E. coli* O157:H7 survival on lettuce plants. For the 2008 field trials, a split plot design was used to evaluate the two main treatment effects: drip and overhead irrigation. Plants were inoculated using a liquid suspension at a level of 10^7 CFU/lettuce plant with non-pathogenic *E. coli* O157:H7 strain ATCC 700728 4 weeks after planting in the June 2008. Unfortunately, the August 2008 lettuce was significantly impacted by extensive bird damage and inoculation was delayed to 6 weeks after planting. For both trials the population of *E. coli* O157:H7 declined rapidly during the first hours after inoculation. By day 7 of the June 2008 trial, 82% of the lettuce plants had less than 10 cells of *E. coli* O157:H7; however, 93% of the plants were positive by plating or enrichment. At the predicted time of harvest (8 weeks after planting) or 28 days post-inoculation, 33% of the plants were positive *E. coli* O157:H7 by enrichment. During the June 2008 trial, irrigation had an impact on the survival of *E. coli* O157:H7: the number of positive plants was significantly higher in blocks that were irrigated by sprinkler when compared to drip. However we could not confirm this result in August 2008 as the bird damage modified the size of lettuce between the blocks and lettuce heads irrigated by drip were larger than lettuce heads irrigated by sprinkler. Differences between the size of drip and sprinkler irrigated plants resulted in higher *E. coli* O157:H7 populations on plants irrigated by drip compared to plants irrigated by sprinkler at every sampling time. At 8 weeks after planting or day 21 after inoculation 90% of the lettuce plants were positive for *E. coli* O157:H7 ATCC 700728 by enrichment. Populations of *E. coli* O157:H7 applied in a liquid suspension rapidly declined to very low levels in the first few hours after inoculation. Although populations are low the number of plants that were positive for *E. coli* O157:H7 by enrichment remained high. Future work should focus on the characteristics of these persistent populations and to better characterize the impact of irrigation method on survival of this organism in the production environment.

II. Main Body of Report

Project Title: Survival of attenuated *Escherichia coli* O157:H7 ATCC 700728 in field-inoculated lettuce.

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Objectives:

Phase I

1. To compare the survival of *E. coli* O157:H7 ATCC 700728 and ATCC 43888 under laboratory settings. Demonstration of similar behavior of the two strains will strengthen the justification for use of ATCC 700728 in field trials.
2. To evaluate the impact on survival of inoculum level, age of plant, and inoculum preparation method including acid adaptation and starvation. These studies will help to identify environmental factors that have the greatest impact on survival of E. coli O157:H7 on lettuce plants.
3. To evaluate the survival of isolates of *E. coli* O157:H7 ATCC 700728 recovered from field trial 1 on day 14 with laboratory strain *E. coli* O157:H7 ATCC 700728 to determine if isolates that survive the longest in the field are more robust than the original culture.

Phase II

To evaluate the persistence of attenuated (non-pathogenic) *E. coli* O157:H7 ATCC 700728 inoculated onto lettuce grown under field conditions (drip and overhead irrigation) in the Salinas Valley.

Procedures

Bacterial suspension preparation. Stock cultures of rifampicin-resistant *E. coli* O157:H7 ATCC 700728 and ATCC 43888 were streaked on tryptic soy agar (TSA) with 50 μg/ml of rifampicin. These strains were confirmed 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 strains that was detected by PCR. For liquid culture preparation, a single colony was inoculated in 2 ml of TSB supplemented with the appropriate antibiotic and incubated overnight at 37°C and with shaking at 200 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.

Acid-adapted cells were prepared by culturing them for 18 h at 37°C in TSB supplemented with 10 g/l of glucose (Buncic and Avery 1998). Starved cells were prepared by pelleting an 18-h culture grown in TSB, suspending in saline solution (0.85% NaCl, pH 6.6), and incubating for 48 h at 37°C (Ryu and Beuchat 1998).

Inoculation of lettuce plants. Romaine lettuce seeds were grown in an environmental chamber with a light intensity of 230 μm m^{-2} s^{-2}, 12 h photoperiod, 18°C during the night and 22°C during the day. All plants were grown in Sunshine mix 1 (Sun Gro Horticulture Distribution, Inc, Bellevue, WA). After 4 weeks plants were inoculated by spraying with a bacterial suspension (1 ml per plant at different concentrations). After inoculation, plants were held in the laboratory at 22°C with a 12 h-photoperiod and at approximately 25 to 30% relative humidity.

Inoculum recovery and quantification. Inoculated lettuce plants were homogenized with 0.1% peptone in a stomacher for 2 min at medium speed. The recovered bacterial suspension was plated with a Spiralplater on TSA supplemented 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). The filter membrane was removed and placed on CROMAgar™ O157 (BD, Franklin Lakes, NJ) (Bettelheim, 1998) supplemented with 50 μg/ml rifampicin.

Field inoculation. A split plot design was used for field trials to evaluate the two main treatment effects: drip and overhead irrigation. Three replicates (or blocks) were established for each treatment. One block included nine beds seeded with Romaine lettuce that measured 40 inch wide x 145 feet long. In between the drip and overhead irrigation, 10 unfarmed beds prevented drift from the overhead irrigation. Two trials were conducted in June and August 2008. Plants were inoculated with a spray bottle calibrated to deliver 10^7 CFU/plant of rifampicin-resistant *E. coli* O157:H7 ATCC 700728. Romaine lettuce was inoculated 4 weeks after planting in June 2008 and 6 weeks after planting in August 2008.

Field sampling, inoculum recovery and quantification. Sampling was conducted at 0 and 2 hours, 2 days, 7 days and once per week thereafter up to the time that the plants were considered ready to harvest. At each sampling day, 20 plants were selected per block for a total of 120 plants. During the first hours after inoculation (day 0), 10 plants were sampled at both 0 and 2 hours. All samples, collected up to 7 days after inoculation, were brought to the laboratory from the field in a cooler with ice-packs, held at 4°C, and analyzed within 48 h. Samples collected at later sampling dates were brought to the laboratory from the field without cooling (samples were

for enrichment only). These samples were held at 4°C upon arrival in the laboratory and until they were processed.

For bacterial enumeration or enrichment, the entire lettuce head was homogenized in a Stomacher (Seward) for 2 min at medium speed in 0.1% peptone. When the lettuce head weight was between 0 to 25 g, 50 ml peptone buffer was added to the stomaching bag. When the lettuce head weight was between 25 to 50 g, 100 ml peptone buffer was added to the stomaching bag. Lettuce heads weighing more than 50 g were cut into smaller pieces and distributed over multiple bags. Bacterial suspension was enumerated with a spiral plate count method on TSA with 50 μg/ml rifampicin. When necessary to improve the limit of detection, 5-ml samples were filtered onto disposable analytical filter units (0.45 μm, Nalgene). Filter membranes were removed and placed on CROMAgar^{TM} O157 (BD, Franklin Lakes, NJ) (1) supplemented with 50 μg/ml rifampicin.

Enrichment. When the levels were below that achieved with direct plating, 100 g of lettuce or 20 g of soil was added to 200 ml tryptic soy broth (TSB) with 50 μg/ml rifampicin and incubated for 18 h at 42°C. The entire head of lettuce was enriched using this procedure. If the lettuce weighed more than 100 g it was split into smaller portions (separating inner and outer leaves). The enrichment broth was Spiralplated on CROMAgar^{TM} O157 with 50 μg/ml rifampicin to confirm the presence of *E. coli* O157:H7. Heads of lettuce were scored either positive or negative for *E. coli* O157:H7.

Soil sampling and analysis. Soil samples were collected from each of 18 blocks throughout the field at day 0 before inoculation. 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, 20 g subsamples were vortexed with 90 ml 0.1% peptone buffer. Detection of *E. coli* O157:H7 was performed by plating serial dilution on CROMAgar^{TM} 0157 (BD, Franklin Lakes, NJ) (1) for the sampling.

At harvesting time, soil samples were collected from the top layer of soil surrounding the *E. coli* O157:H7-inoculated plants. Five random samples per block were collected and processed as described above. Detection of *E. coli* O157:H7 ATCC 700728 was performed by plating serial dilutions on CROMAgar^{TM} O157 supplemented with 50 μg/ml rifampicin and by enrichment.

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 10,000 g for 2 min, resuspended in 1 ml water, and boiled for 10 min. After centrifugation, 1 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*. (Yoshitomi et al., 2006). 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. *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 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 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.

Statistical analysis: By using a combination of plating and filtration, our lower detection limit was 10 CFU/plant. When cells were not detected by direct plating and filtration but only by enrichment, a value of 9 CFU/plant was assigned for calculation of the mean. *E. coli* O157:H7 samples not detected by plating, filtration or enrichment were treated statistically as zero. Microbial data (CFU/plants) were log transformed and statistical analyses were carried on with Jump (SAS Institute Inc.). Data were analyzed by 1 way analysis of variance (ANOVA) to determine statistical differences between treatment means.

Results:

Phase I:

We previously reported that the behavior of *E. coli* O157:H7 on field-inoculated lettuce was closer to the behavior of *E. coli* O157:H7 on lettuce plants held at low relative humidity than on cut lettuce leaves held at high humidity. A large number of factors potentially impact the ability of *E. coli* O157:H7 to survive on lettuce plants. Inoculated lettuce plants grown under laboratory conditions were used to evaluate factors that may have significant impact on bacterial survival in the field.

1) Comparison of the survival of *E. coli* O157:H7 ATCC 700728 and ATCC 43888 and *Citrobacter youngae*. When inoculated on cut lettuce pieces (see previous report), we did not observed differences in the survival of several *E. coli* O157:H7 isolates and *Citrobacter youngae*. For this reason, we rifampicin resistant *E. coli* O157:H7 ATCC 700728 was selected for field trials. However, survival in the field bore no similarity to survival on cut lettuce pieces. In preliminary experiments we demonstrated that survival on lettuce plants held at low relative humidity in the laboratory more closely mimicked that in the field. The survival of *E. coli* O157:H7 ATCC 700728 and ATCC 43888 and *C. youngae* was compared on plants growing in the laboratory. As observed in the field, a relatively large reduction (2 log CFU/ plant) of population size occurred within the first 2 days after inoculation for all tested strains (Fig.1). The population size dynamic during the course of the experiment remained statistically identical for all bacteria further justifying the use of ATCC 700728 in field trials.

2) Effect on *E. coli* O157:H7 survival of inoculum level, genotype and age of plant, and stresses. A large number of variables potentially impact the ability of *E. coli* O157:H7 and other organisms to survive in the environment. These include conditions under which the cells originally grew and subsequent exposure to various stresses (e.g., desiccation, humidity, UV, low or high pH, antimicrobials, heat) (O'Brien and Lindow 1989; Buncic and Avery 1998; Ryu and Beuchat 1998; Uesugi, Danyluk et al. 2006). Other factors include, strain, levels of inoculum, inoculum carrier, timing of inoculation, and method of inoculation (Beuchat, Farber et al. 2001; Lang, Harris et al. 2004).

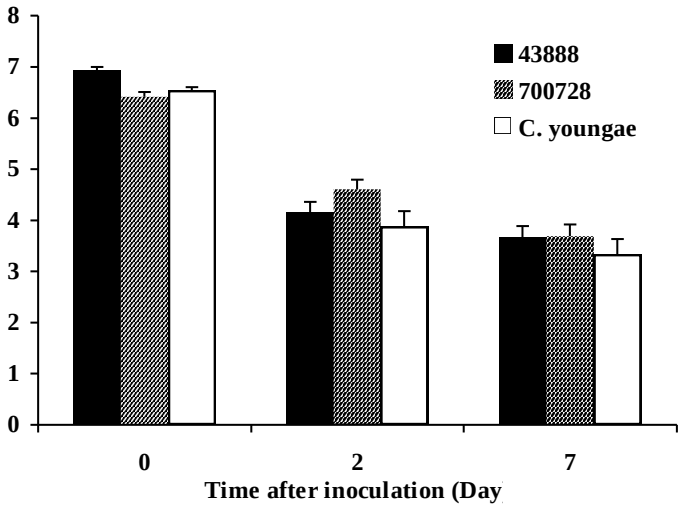

Figure 1: Comparison of bacterial survival on lettuce plants among *E. coli* O157:H7 strain ATCC 700728, ATCC 43888 and *C. youngae*. ATTC 43888 and 700728 are non-pathogenic strains of *E. coli* O157:H7 and *C. youngae* was considered a potential surrogate of *E. coli* O157:H7. Each bar represent the mean of three experiments with five plant samples per experiment (n=15); error bars indicate the standard error of the mean.

The decline in bacterial population was similar for two inoculum levels (10^7 or 10^5 CFU/ plant) evaluated over 7 days (Fig. 2). A reduction of 2 log CFU was observed during the first 2 days and then a reduction of 1 log CFU in the following 7 days for both inoculum levels.

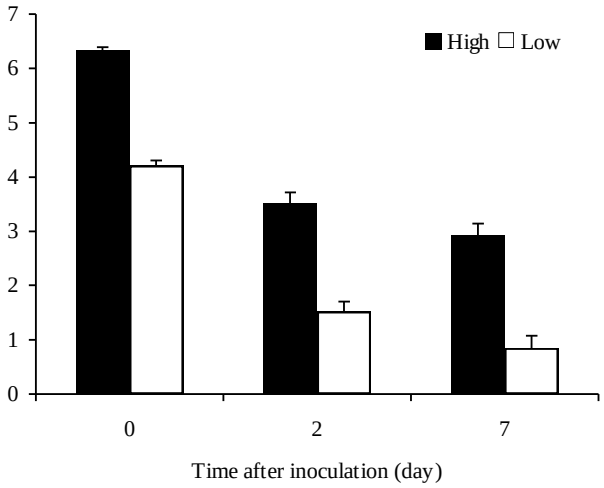

Figure 2: Effect of inoculum level on *E. coli* O157:H7 ATCC 700728 survival on lettuce plants. Romaine lettuce were inoculated with two level inoculum 10^7 (High) and 10^5 (Low) CFU/plant. Each bar represent the mean of two experiments with five plant samples per experiment (n=10); error bars indicate the standard error of the mean.

Survival of *E. coli* O157:H7 ATCC 700728 was identical when inoculated onto 4 and 6 week-old Romaine lettuce (fig. 3).

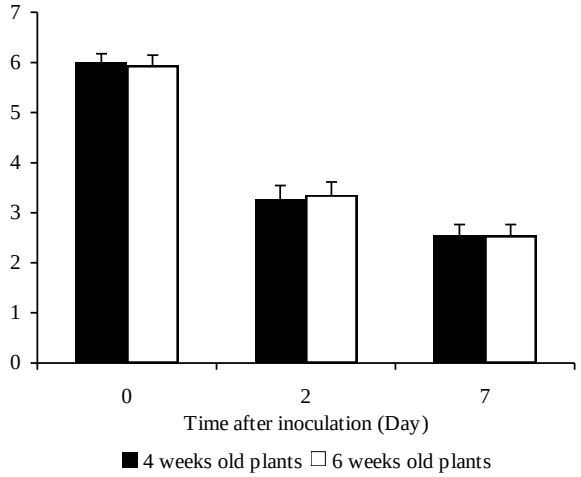

Figure 3: Effect of plant age on *E. coli* O157:H7 ATCC 700728 survival. Romaine lettuce was inoculated with 10^7 CFU/ plant. Each bar represent the mean of three experiments with five plant samples per experiment (n=15); error bars indicate the standard error of the mean.

Acid-adapted cells of *E. coli* O157:H7 have an increase resistance to heat making them potentially more resistant to environmental stress (Singh et al., 2006). On lettuce plants, acid-adapted *E. coli* O157:H7 ATCC 700728 did not confer a better environmental fitness to the bacteria: survival rate was statistically identical to the control (Fig. 4). In contrast, starvation pretreatment decreased the survival of *E. coli* O157:H7 (Fig. 4).

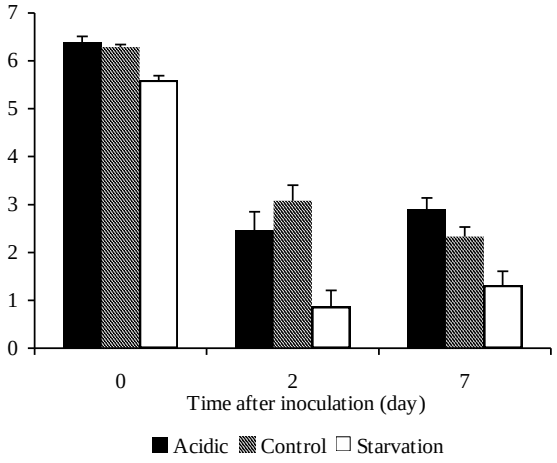

Figure 4: Effect of acidic and starvation culture treatment on *E. coli* O157:H7 ATCC 700728 survival. Romaine lettuce was inoculated (10^7 CFU/ plant) with a bacterial cell suspension grown under acidic or starvation conditions. Each bar represent the mean of three experiments with five plant samples per experiment (n=15); error bars indicate the standard error of the mean.

3) Evaluation of the survival rate of *E. coli* O157:H7 ATCC 700728 recovered from the field. Bacteria that are found on leaves surfaces have presumably adapted to their environment. We retrieved isolates of *E. coli* O157:H7 from our first field trial 14 days after inoculation in order to evaluate their epiphytic fitness. A survival comparison was conducted in the laboratory between the retrieved field strain (H3A1) and the original strain of *E. coli* O157:H7 ATCC 700728 (Fig. 5). Survival between the original strain and the field isolate were not significantly different.

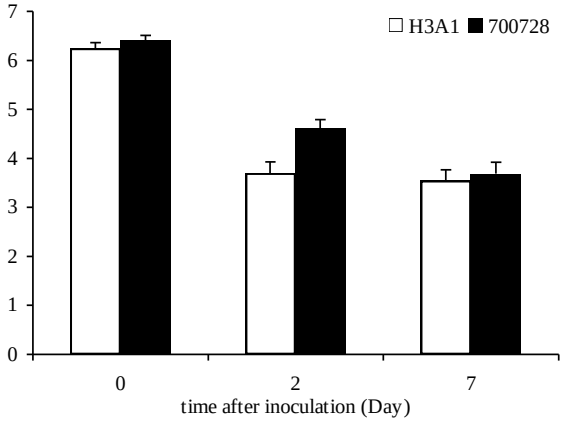

Figure 5: Survival comparison between isolate H3a1 recovered from field trial on day 14 and isolate 700728 used for field inoculation. Each bar represent the mean of three experiments with five plant samples per experiment (n=15); error bars indicate the standard error of the mean.

Phase II

Survival of *E. coli* O157:H7 during June 2008 trial.

Based on the information from the August 2007 field trial (see previous report) we inoculated Romaine lettuce 4 weeks after planting and just after thinning with a nontoxigenic strain of *E. coli* O157:H7 ATCC 700728 at an inoculum level of 10^7 CFU/ml. The size of bacterial population effectively delivered per plant, was evaluated by sampling plants just after inoculation (0 hour). As observed in the previous trial, *E. coli* O157:H7 population declined rapidly during the first hours from 6.3 log CFU/plant to reach an average of 1.5 log CFU/plant at day 2 (Fig. 6).

Enrichment techniques were used to detect *E. coli* O157:H7 as earlier as 2 days after inoculation where 42% of the plants tested were less than 10 CFU per plant. By 7 days, 82% of the lettuce plants had less than 10 cells of *E. coli* O157:H7. Therefore plants, sampled at day 14 and after, were processed only by enrichment. However, even at very low level, *E. coli* O157:H7 persisted until harvesting time or 28 days after inoculation. The percentage of plant hosting *E. coli* O157:H7 decreased from 100% at day 2 to 33% at day 28 (Fig. 7).

In order to assess *E. coli* O157:H7 distribution, lettuce heads were separated in outer and inner leaves at 7 and 21 days. *E. coli* O157:H7 was detected mainly on the outer leaves of the lettuce plant (Table 1). Thus, at 28 days post-inoculation, only the outer leaves were tested by enrichment.

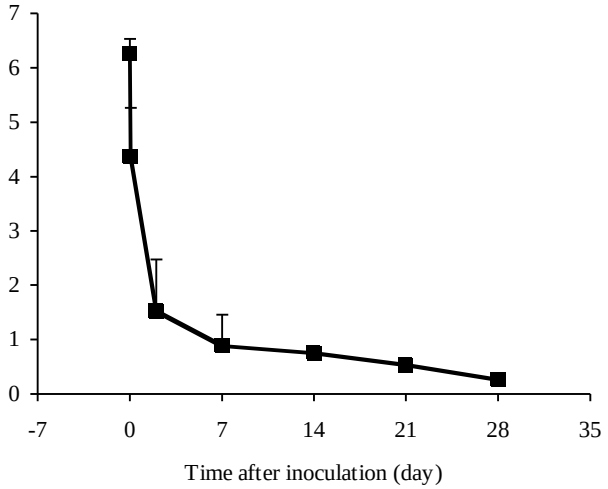

Figure 6: *E. coli* O157:H7 survival on lettuce plant during June 2008 trial. Romaine lettuce was inoculated 4 weeks after planting and was harvestable at day 28. Each point represents the mean population size of *E. coli* O157:H7 ± SD (n=60 at 0 and 1 hour, n=120 at day 2, 7, 14, 21 and 28). SD is shown only for sampling time (day 0, 2 and 7) when a plate count was possible. From 14 to 28 days after inoculation *E. coli* O157:H7 was detected only by enrichment.

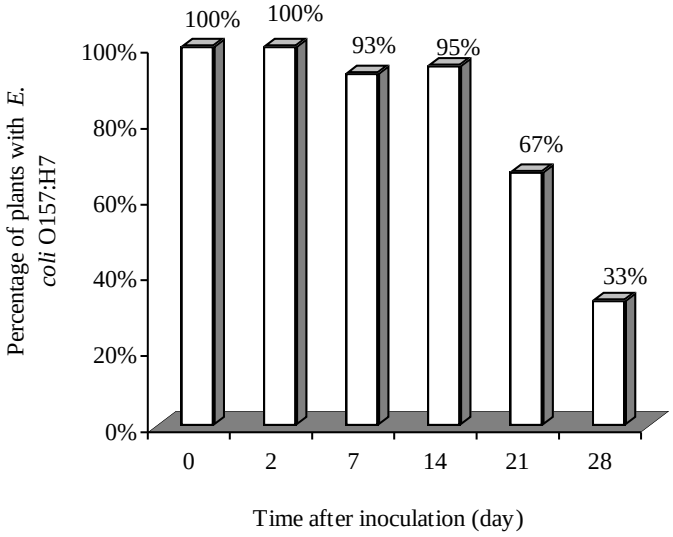

Figure 7: Percentage of plants positive for *E. coli* O157:H7 by enrichment during June 2008 trial. Plants were harvested at day 28. n=120

Table 1: Localization of *E. coli* O157:H7 ATCC 700728 on lettuce inner (I) or outer leaves (O). + O detected by enrichment on outer leaves, +I detected by enrichment on inner leaves.

Sampling day	(+O, +I)	(+O,-I)	(-O, +I)	(-O,-I)
14	19%	53%	8%	20%
21	17%	38%	1%	44%

E. coli O157:H7 was detected in a higher number of plants irrigated by overhead sprinkler than plants irrigated by drip (Fig. 8).

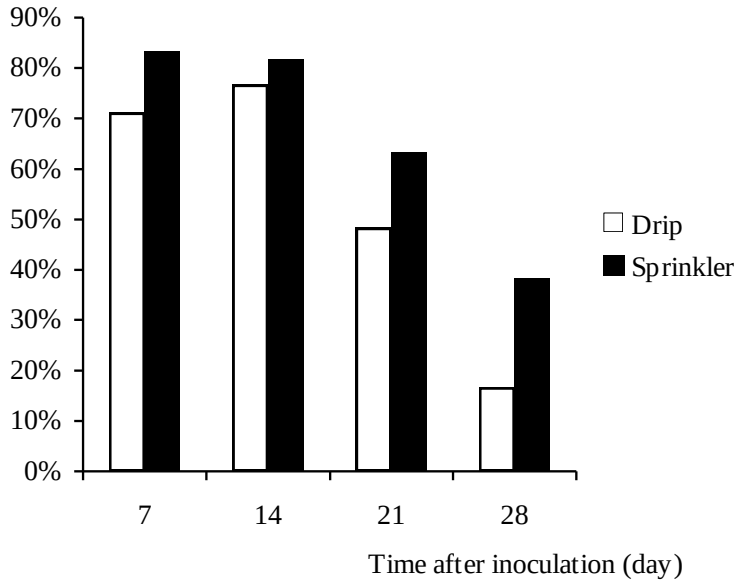

Figure 8: Effect of the irrigation method on *E. coli* O157:H7 survival. Percentage of plants having *E. coli* O157:H7 was determined by enrichment (n=60).

Survival of *E. coli* O157:H7 during September 2008 trial.

The second field trial was established at the same location in August 2008. Four weeks after planting, it was noted that birds were feeding on the lettuce and the damage was more extensive on lettuce irrigated by sprinkler than by drip. Bacterial plant inoculation was delayed until six weeks after planting in order for the lettuce plants to recover. Fertilizer was applied in the lettuce beds irrigated by sprinkler to stimulate growth. However lettuce heads irrigated by drip remained larger than lettuce heads irrigated by sprinkler (Fig. 9) for the remainder of the trial.

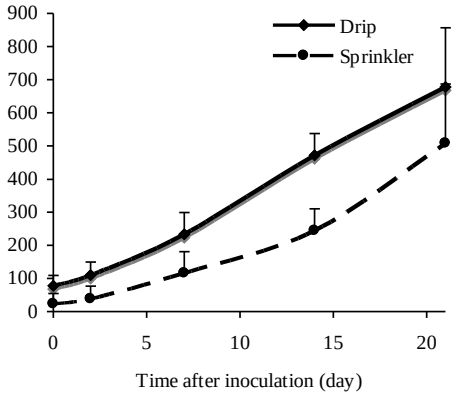

Figure 9: Lettuce growth curve during the August 2008 trial.

E. coli O157:H7 ATCC 700728 was applied at a level of 10^7 CFU/plant. The size of bacterial population effectively delivered per plant, was evaluated by sampling plants just after inoculation (0 hour). The *E. coli* O157:H7 population size remained higher on plants irrigated by drip compared to plants irrigated by sprinkler at every sampling time (Fig. 10). These differences were mainly due to the difference in plant size caused by the bird damage. *E. coli* O157:H7 population decreased from 7.4 log CFU/plant to 5 log CFU per plant irrigated by drip and from 7.1 log CFU/plant to 3 log CFU per plant irrigated by sprinkler at day 2 (Fig. 10). The percentage of plants hosting *E. coli* O157:H7 remained high during all the trial period. At time of harvest (21 days after inoculation) 90% of the lettuce plants were positive for *E. coli* O157:H7 (Fig. 11A). No significant differences in *E. coli* O157:H7 persistence were observed between the plants irrigated by drip or by sprinkler (Fig. 11B).

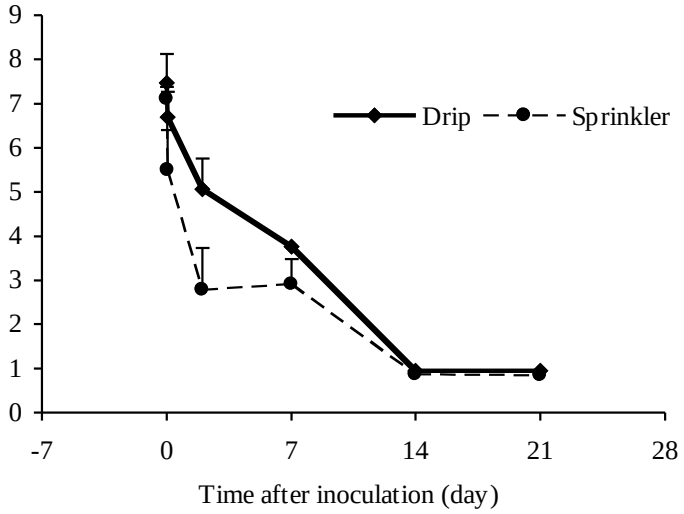

Figure 10: *E. coli* O157:H7 survival on lettuce plant during September 2008 trial. Romaine lettuce was inoculated 6 weeks after planting and was harvestable at day 21. Each point represents the mean population size of *E. coli* O157:H7 ± SD (n=30 at 0 and 1 hour, n=60 at day 2, 7, 14, and 21). SD is shown only for sampling time (day 0, 2 and 7) when a plate count was possible. From 14 to 21 days after inoculation *E. coli* O157:H7 was detected only by enrichment.

Detection of *E. coli* O157:H7 in soil. Before inoculation of the lettuce field soil was sampled to determine the presence of wild-type *E. coli* O157:H7. For both trials, soil samples were collected from each of 18 blocks throughout the field. Five random samples per block were taken from each block and bulked. Prior to field-inoculation, *E. coli* O157:H7 was never retrieved in either trials.

At harvesting time, we sampled again the soil in the row where lettuce plants were inoculated with *E. coli* O157:H7 ATCC 700728. Detection of *E. coli* O157:H7 ATCC 700728 was performed by plating serial dilution and by enrichment. *E. coli* O157:H7 ATCC 700728 was not retrieved.

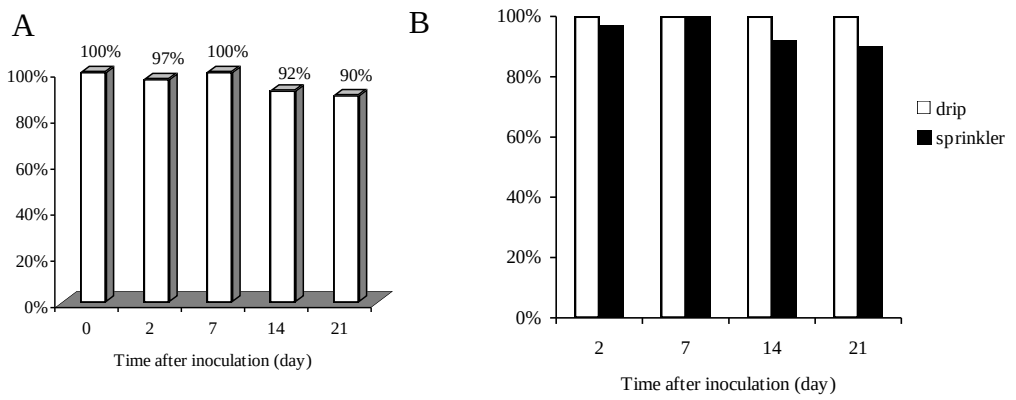

Figure 11: A) Percentage of plants hosting *E. coli* O157:H7 during the August 2008 trial (n=120). B) Effect of the irrigation method on *E. coli* O157:H7 survival (n=60).

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

For each plant determined to be positive by enrichment we recovered one isolate that were both rifampicin resistant and had mauve colonies after plating on CHROMagar (Table 2). A total of 317 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 2). The non-toxigenic strain did not acquire the Shiga toxin by horizontal gene transfer indicating that the potential for a non-toxigenic strain to become toxigenic after field inoculation remained extremely low.

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

Trial	Sampling day	Number of positive plants/ Total plants tested	Number of bacteria tested	*Stx*1 detection	*Stx*2 detection	*uid*A detection
June 2008	7	92/120	92	92 negative	92 negative	92 positive
	14	95/120	95	95 negative	95 negative	95 positive
	21	67/120	67	67 negative	67 negative	67 positive
	28	33/120	33	33 negative	33 negative	33 positive
August 2008	21	104/120	30	30 negative	30 negative	30 positive

Conclusion:
Using laboratory control conditions that mimic environmental field conditions, we evaluated potential factors that can affect *E. coli* O157:H7 survival on lettuce leaves. plant age, inoculum preparation and *E. coli* O157:H7 strains were compared. Under low humidity, the only factor that had a significant impact on survival of *E. coli* O157:H7 was preparing the cells under starvation conditions.

The fate of *E. coli* O157:H7 ATCC 700728 was similar in the 2007 and 2008 field trials. The population size declined very rapidly during the first two days. In 2007 E. coli O157:H7 ATCC 700728 was not detected at day 21 but in 2008 this organism could be detected on plants (less than 10 CFU/plant) by enrichment up to day 28 (approximate harvest). Because of the very rapid decline of the pathogen population and the very low pathogen level on the plant (less than 10CFU/plant), we increased the sampling number from 30 in 2007 to 120 in 2008 per sampling day. This could be one of the reason we were able to detect the *E. coli* O157:H7 persistence until harvesting time in 2008. However we could not rule out that differences in field location could explain the differences observed between the result in August 2007 (Watsonville) and 2008 (Salinas).

In a controlled environment, the availability of water on the plant surface is an important factor for *E. coli* O157:H7 survival and potentially growth. During the June 2008 trial, irrigation had a significant impact on the survival of *E. coli* O157:H7: the number of *E. coli* O157:H7 contaminated lettuce was higher in the blocks irrigated by sprinkler than were irrigated by drip. However we could not confirmed this result during August 2008 trial, the damage caused to the lettuce completely modified the lettuce growth rate between the blocks irrigated by sprinkler and by drip. Results obtained from the three trials we conducted, were difficult to compare due to differences in sampling and setting. Thus we were not able to determine the seasonal effect on *E. coli* O157:H7 survival.

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