Project Title

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

CPS - CALIFORNIA LEAFY GREENS RESEARCH PROGRAM

FINAL PROJECT REPORT, DUE APRIL 30, 2010

Project Title

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

Project Period

April 1, 2009 through March 31, 2010

Principal Investigator

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Objective

Objective 1: Evaluate the persistence of attenuated (non-pathogenic) E. coli O157:H7 ATCC 700728 inoculated onto Salinas valley field-grown lettuce: 1) irrigated by drip or overhead sprinklers; and 2) planted in early (May) and late (July) summer.

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Final Report

What were the original objectives of the project? Simply restate your objectives from the grant proposal.

Objective 1: Evaluate the persistence of attenuated (non-pathogenic) *E. coli* O157:H7 ATCC 700728 inoculated onto Salinas valley field-grown lettuce: 1) irrigated by drip or overhead sprinklers; and 2) planted in early (May) and late (July) summer.

What were the actual outcomes and accomplishments of the project? Describe how they were achieved and how they compare/relate to the original objectives and outcomes in your grant proposal.

Abstract:

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, we conducted two field trials in spring (May seeding) and fall 2009 (September seeding) 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. A split plot design was used to evaluate the two main treatment effects: drip and overhead irrigation. Approximately 4 weeks after seeding plants were inoculated (1 ml/plant) with a liquid suspension (log 7 CFU/ml) of *E. coli* O157:H7 ATCC 700728. For both trials, a rapid population size decline of 3 log was recorded during the first 2 hours. The population size was 0.8 ± 0.5 log CFU/plant at day 2 for the spring trial and 1.2 ± 1.2 log CFU/plant at day 3 for the fall trial. Enrichment techniques were used to detect *E. coli* O157:H7 as earlier as 2 days after inoculation where 85%, during spring, and 74%, during fall, of the plants tested were below the limit of detection (10 CFU/plant). By 7 days, more than 90% of the lettuce plants for both trials were below the limit of detection for *E. coli* O157:H7. However, *E. coli* O157:H7 could be recovered from lettuce plants by enrichment through 28 (spring) or 35 (fall) days postinoculation. The percentage of *E. coli* O157:H7-positive plants decreased from 93% at day 2 to 7% at day 28 (spring) and from 88% at day 2 to 0.6% at day 35 (fall). For both trials there were no significant differences is counts of *E. coli* O157:H7 determine on plants from day 0 to day 7. For enriched samples, significant differences in the number of positive plants identified were identified on only a few of the sample days. Greater numbers of positive plants were identified for overhead sprinkler irrigation for the first 2 weeks postinoculation in spring while the opposite was true for the first week of the fall trial. However, during the first week after inoculation of the fall trial, 35 mm of rain fell which likely impacted the results.

Summary of Findings:

The present study was a continuation of work that began in 2007. The results from the present study were consistent with our previous work. The time from the "contamination" or inoculation event to the point of harvest significantly influences the probability of isolating *E. coli* O157:H7 from lettuce plants. When the soil was inoculated prior to emergence, *E. coli* was isolated from the soil for up to 15 days post inoculation but could not be recovered from the plants. When plants were inoculated, the levels of *E. coli* O157:H7 rapidly declined and were only isolated

after 7 days by enrichment; the percent positive plants steadily declined post inoculation. A significantly higher number of positive plants was observed with overhead irrigation on some but not all sampling points; no significant difference was observed at the end of the trials.

Results:

<u>Survival of *E. coli* O157:H7 after soil inoculation and on 2-week old plants:</u>

Just before plant germination or 5 days after seeding, we uniformly applied *E. coli* O157:H7 ATCC700728 on the surface of one bed in each block. The inoculum concentration was adjusted log 7 CFU/ml and the amount effectively delivered was calculated to be 4.7 ± 0.4 log CFU/g of the surface layer (approximately 1-cm) soil. Because the field was irrigated shortly before inoculation, the *E. coli* O157:H7 population size decreased slowly during the first 2 h to 4.2 ± 0.4 log CFU/g. However, after 7 days *E. coli* O157:H7 was detected only in 2 out of 6 blocks and only by enrichment. Fifteen days after inoculation, *E. coli* O157:H7 ATCC700728 could not be retrieved from the soil. Pathogen transfer from the soil to the plant was not observed: *E. coli* O157:H7 was never retrieved from plants (10 to 50 per block) sampled at 7 or 15 days post-inoculation. For the 2-week old plants inoculated in fall 2009, a rapid decrease in *E. coli* O157:H7 population from 5 ± 0.5 log CFU/g at 0 h to 2.5 ± 0.4 log CFU/g at 2 h was observed. At days 7 and 14, *E. coli* O157:H7 was detected only by enrichment in 50% and 47% of the plants tested, respectively. At day 21 after inoculation, one plant out of 120 was positive for *E. coli* O157:H7, and at day 28 *E. coli* O157:H7 was not detected on any of the 240 lettuce plants enriched.

<u>Survival of *E. coli* O157:H7 after 4-week old plant inoculation:</u>

In 2009 we conducted two field trials, one in spring and one in fall. As in our previous trials, Romaine lettuce was inoculated 4 weeks after planting and just after thinning with a *E. coli* O157:H7 ATCC 700728 at an inoculum level of 10^7 CFU/ml. The size of bacterial population effectively delivered per plant (time 0) was 5.7 ± 0.4 log CFU/plant in the spring trial and 5.3 ± 0.8 log CFU/plant in the fall trial (Fig 1A). A rapid population size decline of 3 log was recorded during the first 2 h to 2 days. Populations were 0.8 ± 0.5 log CFU/plant at day 2 in the spring trial and 1.2 ± 1.2 log CFU/plant at day 3 during the fall trial (Fig. 1A). The *E. coli* O157:H7 population size was not significantly different for plants irrigated by drip or overhead sprinkler. Enrichment techniques were needed to detect *E. coli* O157:H7 as early as 2 days after inoculation where 85%, during spring, and 74%, during fall, of the plants tested had levels less than 10 CFU/plant. By 7 days, more than 90% of the lettuce plants for both trials were below the limit of detection by plating (10 cells/plant). Therefore, plants sampled at day 14 and after were processed only by enrichment. *E. coli* O157:H7 was detected by enrichment through 28 days post-inoculation during spring and 35 days post-inoculation during fall. The percentage of positive plants decreased from 93% at day 2 to 7% at day 28 during spring and from 88% at day 2 to 0.6% during fall (Fig. 1B). The number of positive plants was similar in the spring and fall trials except at day 7 where a higher number of positive plants was recorded in the fall. During the first week of the fall trial significant rainfall was recorded and may have influenced the results.

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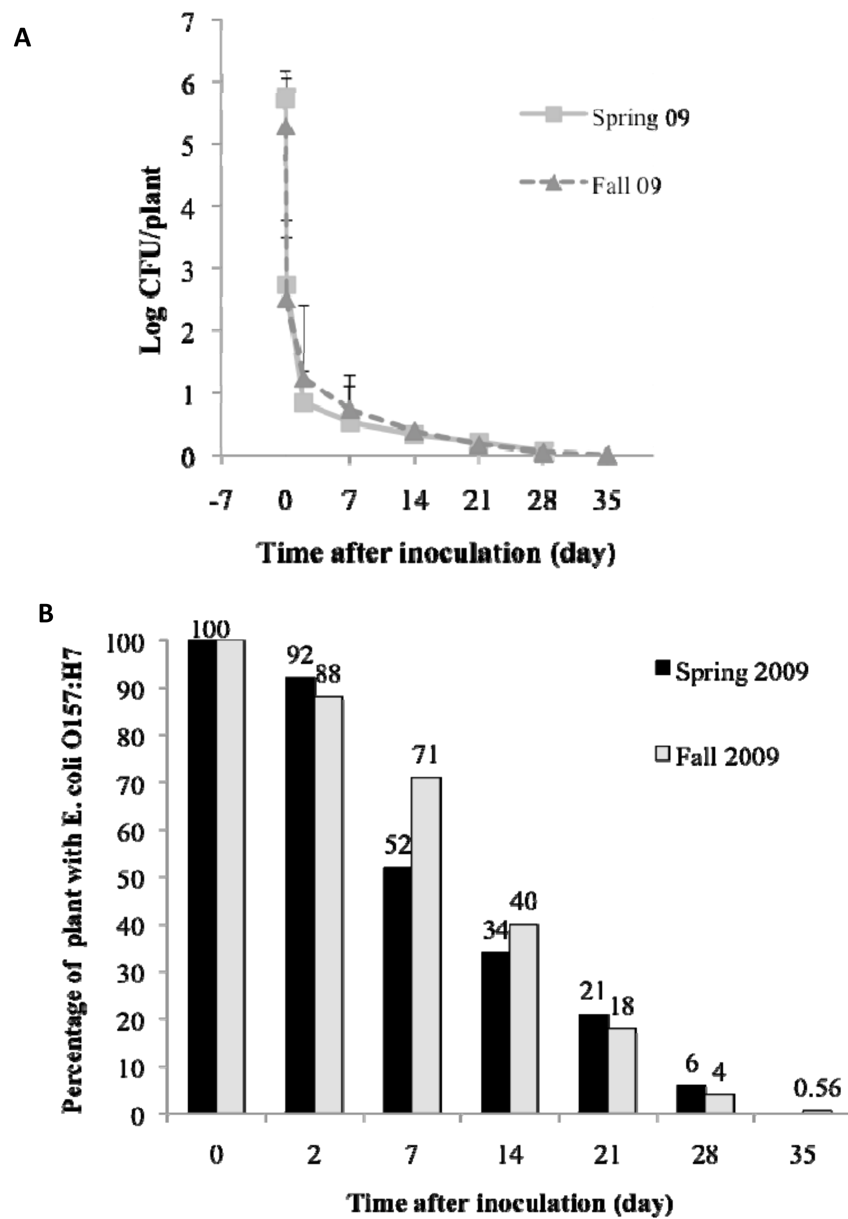

Figure 1: *E. coli* O157:H7 survival on lettuce plant. A) Population dynamic. B) Percentage of plants positive for *E. coli* O157:H7 after enrichment.

A) Romaine lettuce was inoculated 4 weeks after planting and was harvestable at day 28 during spring and at day 35 during fall. Each point represents the mean population size of *E. coli* O157:H7 ± SD. 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. For both graph, data were combined for drip and overhead sprinkler irrigation treatment. n=120 at day 2, 7 and 14 post-inoculation for both trials. n=150 at day 21 and 28 post-inoculation for spring trial. n=120 at day 21, 156 at day 28 and 360 at day 35 post-inoculation during fall.

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Spring and fall seasons gave opposite results regarding the influence of irrigation on *E. coli* O157:H7 survival. Overhead sprinkler significantly increased the frequency of plants tested positive by enrichment during at day 7 and 14 (Fig. 2A) postinoculation in the spring trial. However, the percent positive plants was higher in plant irrigated by drip at day 7 in the fall trial. During the fall trial, 35 mm of rain fell in the first 7 days following inoculation; rainfall was not recorded during the spring trial. However all differences disappeared toward the end of the field trial and at harvesting time 7%, in spring, and 1%, in fall, of the plants were contaminated with *E. coli* O157:H7 independently of the irrigation system used.

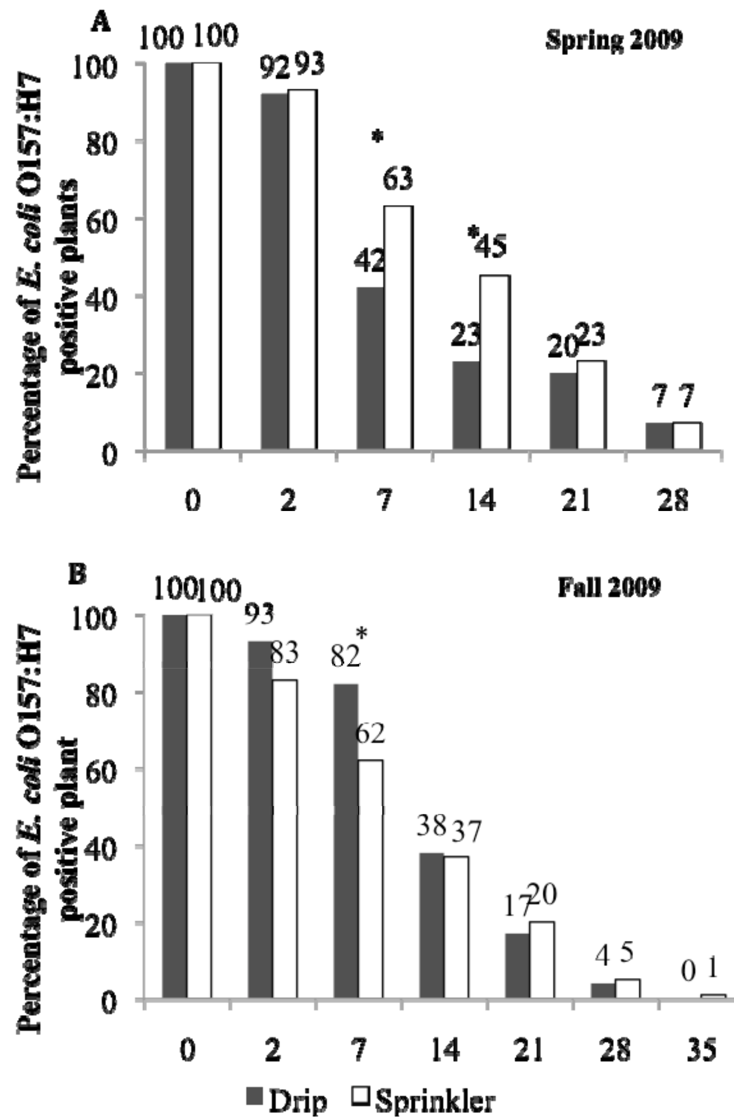

Figure 2: Effect of the irrigation method on *E. coli* O157:H7 survival.

Percentage of plants positive for *E. coli* O157:H7 was determined by enrichment. n=60 at day 0, 2, 7 and 14 post-inoculation for both trials. n=75 at day 21 and 28 post-inoculation for spring trial. n=60 at day 21, 78 at day 28 and 180 at day 35 post-inoculation during fall. * indicates statistical difference as determined by Pearson's chi-square.

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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. 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 not retrieved. 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.

Identification of bacteria recovered during spring field trial as *E. coli* O157:H7 ATCC 700728. For each plant determined to be positive by enrichment we selected one isolate that was both rifampicin resistant and had mauve colonies after plating on CHROMagar containing rifampicin. A total of 140 isolates (distributed over the entire trial) 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 but has 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 tested bacteria were 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.

What methods or resources were used to gather data?

Bacterial suspension preparation. Stock cultures of rifampicin-resistant *E. coli* O157:H7 ATCC 700728 were streaked on tryptic soy agar (TSA) with 50 µg/ml of rifampicin. A single colony was inoculated in 2 ml of TSB supplemented with 50 µg/ml of rifampicin and incubated overnight at 37°C with shaking at 200 rpm. Cell culture was spiral plated to produce a uniform lawn on TSA plates with 50 µg/ml of rifampicin. *E. coli* O157:H7 cells were suspended directly from the plate in 0.1% peptone and enumerated by dilution and plating on TSA plate with 50 µg/ml of rifampicin. The cell suspension stock was kept at 4°C for 12 hours until final dilution to prepare the inoculum.

Field inoculation. The spring trial was conducted from 5/6/09 to 7/6/09 and the fall trial from 9/8/09 to 11/16/09. A split plot design was used to evaluate the two main treatment effects: drip and overhead sprinkler irrigation. Three replicates (or blocks) were established for each irrigation treatment. Each block, measuring 1 m wide by 44 m long, included nine beds seeded with Romaine lettuce. Ten unfarmed beds were retained between the drip and overhead irrigation blocks to reduce drift from the overhead sprinkler irrigation. Sub-treatments, such as level of inoculation or 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.

Two different types of inoculation were used for delivering *E. coli* O157:H7 ATCC 700728 on the soil surface or onto the lettuce plants. 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 or to spray 2-week old lettuce plants during fall 2009. Spray bottles were used to inoculate individual 4-week old lettuce plants just after thinning in spring and fall. The bottles were calibrated to deliver an inoculum adjusted to

10^7 CFU per spray. The inoculum concentration was verified by enumeration on TSA supplemented with 50 µg/ml rifampicin.

Lettuce sampling following soil inoculation (5 days after planting). Sampling was conducted at 7 and 15 days after soil inoculation. At day 7, 10 plants were collected per block and pooled for further processing. At day 15, 50 plants per block were collected and pooled in five separate 7-oz stomaching bags. 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. Samples were analyzed by enumeration, filtration and enrichment.

Lettuce sampling following inoculation of 2-week old plants. Ten plants per block were collected at 0 h and 2 h and pooled in one Whirl-Pak bag for further processing. Twelve plants per block were collected at day 7 and pooled in three separate Whirl-Pack bags. Twenty plants per block were collected separately for a total of 120 plants at days 14 and 21. Forty plants per block were collected separately for a total number of 240 plants at day 28.

Lettuce sampling following inoculation of 4-week old plants. 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 or 28 days for the spring trial or 35 days for the fall trial. During the first hours after inoculation (day 0), 10 plants were sampled at both 0 and 2 hours. At 7 and 14 days post-inoculation, 20 plants were selected per block for a total of 120 plants. At 21 and 28 days post-inoculation, 25 plants were selected per block for a total of 150 plants for the spring trial. At 21, 28 and 35 days 120, 156 and 360 plants were selected for the fall trial. 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 processed by enrichment only). These samples were held at 4°C upon arrival in the laboratory and until they were processed.

Inoculum recovery and quantification
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. For both trials only the outer leaves were processed with enrichment at the last sampling point. 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™ O157 (BD, Franklin Lakes, NJ) (Bettelheim 1998) supplemented with 50 µg/ml rifampicin.
Enrichment. When the levels were below detection limit 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 spiral plated on CROMAgar™ 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.

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Soil sampling and analysis.

Soil sampled before inoculation was 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. 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 with a sterile spoon and bulked. After soil inoculation, 5 random samples were collected from each of the 6 inoculated surface bed and bulked.

After being thoroughly mixed in a clean plastic bag, 20 g subsamples were vortexed for 1 min 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) (Bettelheim 1998). Detection of *E. coli* O157:H7 ATCC 700728 was performed by plating TSA 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 JMP (SAS Institute Inc.). 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 and two-tailed Fisher analysis were performed to examine the distribution of plants tested positives by enrichment in drip or overhead sprinkler irrigated beds. Results with *P*-values of < 0.05 were considered significant.

What unexpected outcomes (positive and negative) resulted from the project, and how did you manage them?

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The major unexpected outcome occurred with the late summer (August) planting. The lettuce seed failed to germinate. After consulting with Mary Zischke we decided to plant a second crop in September, representing an early fall rather than late summer planting. These data turned out to be useful and complementary to the spring trial but did not allow us to achieve our second objective.

We added two inoculation scenarios: inoculation of the soil surface pre-emergence and inoculation of 2-week old plants. Both of these additional inoculation points provided complementary and useful data.

Describe any collaborative efforts involved in planning and/or implementing this project.
Dr. Anne-Laure Moyne, Staff Research Associate, Western Center for Food Safety, UC Davis was responsible for execution of the field trial, data collection and analysis. Michael D. Cahn, Farm Advisor, Irrigation and Water Resources, and Steve T. Koike Farm Advisor, Plant Pathology, Cooperative Extension Monterey County were responsible for coordination of the irrigation, planting and maintenance of the lettuce. Sharon Benson, USDA-ARS, Salinas manages the field trials that take place on USDA property in Salinas.

Tyann Blessington, graduate student, Mohammed Azam, undergraduate student, Neha Dhawan, undergraduate student, Susan Geiger, undergraduate student, Marie Peden, intern, Timothy Ryner, graduate student, Luxin Wang, postdoc and Irene Zhao, technician all contributed to sample collection and processing on a full or part-time basis.

Mary Zischke, Director, California Leafy Greens Research Program also assisted with troubleshooting during the project.

Did you have the necessary funds to fully implement this project?
As described in the original proposal, the funds provided by CPS were sufficient to cover approximately 50% of the costs associated with this project. The remaining costs were covered by a grant through the Western Center for Produce Safety.

Describe any changes that occurred to the original budget.
None.

Give a brief narrative breakdown of how the grant funds were spent.
Salary and Benefits
Funding was used to support the salary and benefits for staff who assisted with inoculation, harvest and processing of lettuce samples for each of the two trials. Salary for Dr. Moyne was covered by the Western Center for Food Safety (see below).
Materials and Supplies
Funding for supplies includes standard microbiological disposables, nonselective and selective microbiological media, and other disposable and expendable laboratory supplies, including DNA extraction and PCR amplification kits. These funds also include charges for waste disposal. Funds from the CPS grant were used for one trial and supplies for the second trial were covered by the Western Center for Food Safety.
Travel

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Funds of were used to travel to the field to collect samples (2 trials) at least weekly trips were made for each field trial (gas, car rental) (10 to 11 trips per trial). . Some of these trips required an overnight stay in Salinas in order to harvest, transport, and process the samples in one day. Travel was also covered, in part, by the Western Center for Food Safety.

USDA ARS Salinas Subcontract

Costs for farming were $10,596 for 2 1-acre field trials.

Collaborator scope of work

Preparation of land for planting with lettuce, disking field after trial. Irrigation of lettuce and other cultural practices in cooperation with UC Cooperative Extension and PI.

Cahn Subcontract – UC Cooperative Extension Salinas (2 – 1 acre field trials)

Funds of $14,700 were used for Salinas-based collaborator expenses.

Collaborator scope of work

Establish and manage lettuce field trial, including installing drip and sprinkler irrigation systems, planting lettuce, overseeing cultural practices such as the irrigations, cultivation, thinning, fertilizer side dress, and removing irrigation system and equipment at end of trial.

List publications and presentations resulting from this grant. Provide a digital copy with the report. State if there have been no publications or publications.

Moyne, A-l, M.R. Sudarshana, T. Blessington, S.T. Koike, M.D. Cahn, and L.J. Harris. 2010 Fate of *Escherichia coli* O157:H7 in field-inoculated lettuce. Submitted to Applied and Environmental Microbiology, March 2010.

This manuscript includes data from the CPS grant as well as two previous years of funding from the California Leafy Greens Research Program and the Western Center for Produce Safety. A copy of the manuscript will be provided after it has gone through scientific review and has been accepted for publication.

Updates of the research were provided to the California Leafy Greens Research Program meetings in October 2009 and March 2010 as well as to a PMA meeting in Anaheim, CA in September 2009.

Appendices may include raw data, calculations, graphs, and other quantitative materials that were part of the research, but would be distracting in the report itself.

None.

Give any suggestions which might be helpful to CPS in making future grants similar to yours.

No suggestions.