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CPS - CALIFORNIA LEAFY GREENS RESEARCH PROGRAM

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Project Title

Comparison of surrogate *E. coli* survival and epidemiology in the phyllosphere of diverse leafy green crops

Project Period

April 1, 2009 through April 30, 2010

Principal Investigator

Trevor Suslow
University of California, Davis
tvsuslow@ucdavis.edu
530.754.8313

Co-Principal Investigators

Steven Koike
UC Cooperative Extension – Monterey County
stkoike@ucdavis.edu
831.759.7356

Richard Smith
UC Cooperative Extension – Monterey County
rifsmith@ucdavis.edu
831.759.7357

Michael Cahn
UC Cooperative Extension – Monterey County
mdcahn@ucdavis.edu
831.759.7377

Objectives

Objective 1: Compare the foliar survival and growth of specific isolates of nonpathogenic *E. coli* to non-toxigenic (attenuated) *E. coli* O157:H7 on diverse types and cultivars of lettuce and leafy greens during late spring and late summer conditions.

Objective 2: Rapid Response Field Survey: Conduct microbiological grid-analysis of grower/handler fields, identified as positive for EHEC, to localize natural contamination events in relation to the presence of presumptive indicators.

Objective 3: Monitoring survival of attenuated *E. coli* O157:H7 on field grown lettuce produced under different fertilizer levels.

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Materials and Methods:

Preharvest Survival

Bacterial suspension preparation and field inoculation

Three nonpathogenic *E. coli* strains isolated from water (PTV 353), lettuce (PTV 354) and soil (PTV 355) with spontaneous, natural resistance to the antibiotic rifampicim (100µg/L) were selected for this study. Also, each strain had a distinct DNA-fingerprint. A mixture of two strains of attenuated *E. coli* O157:H7 (PTVS 154 and PTVS 155), rifampicim resistant (100µg/L) and both lack the shigatoxin genes *stx*1 and *stx*2, were also selected for a parallel experiment. All strains were grown at 37ºC for 18 hours on TSA+rif+Pyruvate. Several characteristic colonies were transferred to 5 mL of Butterfield's Phosphate Buffered Saline (PBS) to create a suspension of approximately log 9 CFU/ml and 100 µL spread onto selective agar and incubated for 18 hours to create a uniform lawn. Cells were harvested by gently scrapping from the agar surface with a sterile rubber spatula, washed in PBS, pelleted, resuspended in PBS, and then serially diluted in PBS to the desired target dose.

The different beds containing the different crops were divided in three areas: Control (uninoculated), nonpathogenic (cocktail generic *E. coli*) and pathogenic (cocktail attenuated *E. coli* O157:H7). There were three replications. Inoculum was applied with a CO_2 powered, handheld, backpack sprayer (I do not know the technical specifications of the sprayer) with a concentration of 4.3 log CFU/ml and it was sprayed 2 L/100ft. Each cocktail was sprayed separately. Leaves were harvested by hand into PE polybags at different time points, placed in a pre-chilled insulated cooler with gel-ice. A triple layer of 'butcher-paper" was placed over the still partially frozen gel-ice to prevent freeze-injury to plant samples. Samples were transported to the Mann Lab and held at 2.5°C (36.5°F). Recovery by direct detection and enrichment culture were conducted using lab-standard procedures (See Figure 1 for schematic)

Postharvest Evaluations

Plant material

Mizuna (*Brassica rapa* cv. Japonica), Tatsoi (*Brassica rapa* var. rosularis), Red Chard (*Beta vulgaris* var. cycla) and Lettuce (*Latuca sativa*) 'Pinecrest' from the 2009 Spring Mix trial described above were collected at grower-defined peak maturity. Leaves were placed in PE polybags and placed in a pre-chilled insulated cooler with gel-ice. A triple layer of 'butcher-paper" was placed over the still partially frozen gel-ice to prevent freeze-injury to plant samples. Samples were transported to the Mann Lab and held at 2.5°C (36.5°F)

Sample preparation and treatments

The leafy greens were harvested separately at 24 hours and 7 days after inoculation. The plant material was processed in a clean room, separate from areas designated for

pathogen inoculum preparation and handling and with practical protection against lab cross-contamination for a field grown crop. Leaves with defects such as yellowing, decay, cuts and bruising were discarded. A spring mix salad was prepared for each inoculated group with the following proportions (based on weight): 15% Mizuna, 15% Tatsoi, 15% Red Chard and 55% lettuce 'Pinecrest'. The mixture was prewashed for 1 min with tap water to remove traces of soil and organic matter. The performance of liquid chlorine dioxide (ClO_2), applied as a concentration of 3 mg/L (3 ppm), was tested on disinfection of leaves and in preventing process water-based cross-contamination. This treatment was compared to a standard disinfection with sodium hypochlorite (NaClO) set at 20 ppm pH 6.5 and to a treatment based on Cl+T128 additive. A ratio of 300g of spring mix salad in 5 L disinfectant solution was used for each replicated washed sample unit. A small, model wash tank was fitted with a pressurized air-line terminating in an air diffuser/bubbler to generate a vigorous 'Jacuzzi-type' agitation system for leaves. A sterilized perforated plastic board was used to hold leaves submerged just below the water bath surface to ensure full water contact during disinfection contact time. All process-wash comparisons were performed for 2 min followed by a shower-rinse step with tap water for 1 minute. A brief hand de-watering step was applied for easily drained and removed wash water. The mixture of leaves was further placed in a foodservice-scale manually spin dryer to remove more tightly adhering water.

To evaluate treatment efficacy, 50 mL of process water was collected for each washing step (prewashing, tank-washing, finishing rinse, and centrifuge de-watering) and mixed with and equal volume of sterile Neutralizing Buffer (NB).

Recovery and Detection – Water samples

Twenty ml (10 ml process water + 10 ml NB) of the process water samples were filtered through an Isogrid™ hydrophobic bacteriological membrane (Neogen Corp) and placed onto selective/differential agar media for the applied strains of *E. coli* (TSA+rif100+MUG+Pyruvic) and incubated for 4 hours at 22°C and moved to 42°C for an additional 20-22h. The results are expressed as CFU/10 ml.

Recovery and Detection – Plant samples

For brevity, a schematic for our recovery and detection plan is provided in Figure 1.

Confirmation and Identification of surrogate E. coli by Pulse-Field Gel Electrophoresis

To further confirm the identity of applied generic *E. coli* recovered from field samples, 10% of total characteristic rifampicin-resistant colonies recovered from ChromECC agar, from plant and water samples, were purified and processed for DNA fingerprinting by a standardized PFGE method. This method was shown, in prior field research, to differentiate the three applied strains and, though originally isolated from Salinas region farms, tested to be unlikely in a given environmental location among the high diversity of generic *E. coli* encountered.

Results:

The survival of all applied *E.coli* was very low in this first trial attempt. The field trial location made available was not ideal and limited results were obtained in 2009 efforts.

Detectable survival was essentially zero following 18 and 48h of the first foliar application and a second identical inoculation was made on Day 5 after the first. Survival was highly variable and sporadic among the different Spring Mix varieties (Table 1). The low initial survival following both sprays was, in large part, influenced by warm windy weather immediately after application of the low doses used. The second application post-spray survival was influenced by four-days of intermittent rainfall and observations of an increase in recoverable populations was suggested by the outcomes of postharvest evaluations (See Figure 2). Although differences were observed in log CFU decline from initial inoculum delivered per gram, it is premature to ascribe any significance to the reliability of these data due to problems encountered in irrigation uniformity, weed density, and other trial management concerns. We hope to repeat this trial in the near future. We feel the usefulness of this data resides in the observation that even under these stress-inducing conditions, survival of the applied *attE.coli* O157:H7 was confirmed by enrichment-based detection (Table 1 and Fig. 3) at 8 and 14 days post-inoculation.

The postharvest wash processes compared in this preliminary study, as anticipated, reduced but did not eliminate the field-applied inoculum (Fig 2 and 4). Process water treatments appear to provide a high degree of risk reduction in regards to cross-contamination potential as suggested by the low recovery in wash and rinse volumes tested by filtration (Fig. 3). However, forceful removal of tightly adhering water during the centrifuge de-watering step makes it apparent that detachment of residual viable cells from the leaf surfaces is possible. This contaminated water could represent an operational process unit step where cross-contamination may occur in the absence of adequate preventive measures. This observation would need additional verification under experimental and commercial conditions. Due to the limitations of available plant material and survival of inoculum, as described above, this aspect of our planned research effort was severely truncated.

Objective 2 – *Analysis of sampling and pathogen detection in controlled inoculation and natural contamination events.*
Specific experiments were conducted to preliminarily determine the validity of standard compliance-monitoring schemes for pathogen detection of spinach, as an example of the dynamics of survival on mini-greens.

Materials and Methods:
Field growing conditions
Spinach seeds (*Spinacia oleracea* L. cv Barbados) were sowed following normal commercial practices during September 2009 in the Salinas Valley. Experimental plots were managed under standard fertilizer and pest management practices by cooperative growers. Total N fertilization was 168 Kg/ha with ammonium nitrate and urea (UN32) as the main N source. Plants were cultivated in sandy loam soil composed of 72% sand, 17% silt and 11% clay. Plants were harvested at dawn in parallel with commercial harvest practices. A total of 3 experiments were done. Plants were harvested by hand

to prevent damage that could induce excessive loss of turgor. Samples were transported in a pre-cooled insulated cooler with gel-ice to the University of California Davis. Samples were stored for 24h at 0C before evaluations began.

Bacterial Strains

Controlled contamination events used two attenuated strains of *E. coli* O157:H7 at an inoculum concentration of log 0.3 and 0.56 CFU/m^2 per planted bed were used.

Sampling Design

Field samples were collected at 24hr, 7, 14 and 28 days post-inoculation. Individual leaves and composites up to 150g were used in the recovery of *E. coli* O157:H7 with and without disinfection with 1% $AgNO_3$.

Detection and Recovery

Leaf samples were taking as individual leaves and placed in a small stomacher bag (NASCO Whirl-Pak®) or processed as larger composites in appropriate size stomacher bags and proper dilutions were made with Butterfield's phosphate buffer (BPB). Tryptic soy broth amended with rifampicin (100mg/L) (TSB-rif), ECC amended with rifampicin (100mg/L) (ECC-Rif) or TSA-Rif were used for bacterial recovery. When necessary qualitative detection of att*E. coli*O157:H7 below the level of plating recovery was carried out by probe-based PCR.

Results

To assess detection of post-inoculation survival over time, 150g of leaves were used in the analysis and this represented approximately 165 and 220 leaves. The observed differences in the number of leaves per 150g of spinach were associated with variation in leaf area.

The number of positive groups of spinach by direct plating for att*E. coli* O157:H7 was higher for the inoculum dose of log 0.56 CFU/m^2 when compared to the lower inoculum at any point of evaluation (Tables 2-5). After 7 days post inoculation (dpi), 50% or less of the samples analyzed were positive for the pathogen by direct plating for any inoculum dose. Additionally, samples collected after 7 dpi were also stored at 0C for 20 days. After this interval, 150g samples were used in the recovery of att*E. coli* O157:H7. After direct plating, no viable cells were recovered; however after enrichment, 50% of the log 0.56 CFU/m^2 was still viable by enrichment while none were detected for the log 0.3 CFU/m^2.

Differences in pathogen recovery between trials were also observed. Trial 1 was cultivated during the months of June-July while Trial 2 was grown during August-September 2009. Overall, after 18 days post-inoculation (dpi), viable populations were detected by direct recovery for both inoculum doses while at 28 days viable populations without enrichment were detected only for the higher inoculum dose.

Twenty eight days post- inoculation, for Trial 1, only the high inoculum plots had detectable recovery after enrichment in 30% of the replicated samples collected. For Trial 2 at 18 dpi, a regional weather system provided intermittent rains for 4 consecutive days. After sampling at day 28 (6 days after cool, wet weather) direct plating recovery of att*E. coli*O157:H7 as well as detection by enrichment was higher than Trial 1 at the same

evaluation point for both inoculum doses. This represented an apparent increase above 18 dpi evaluations of enriched samples from the same plot areas.

Silver nitrate, as a bulk disinfectant treatment, was able to reduce significantly, but not eliminate, the population of bacteria present in groups of 150g of spinach. In both trials and inoculum doses the number of positive groups after disinfection and enrichment was low. However, silver nitrate was not able to reduced populations to a zero-detection level. As expected the number of positive groups of spinach for att*E. coli* O157:H7 was lower for the low inoculum after disinfection. Viable cells were still detected at 18 dpi for the high inoculum dose after surface disinfection.

Individual leaves from the inoculated plants in the field were also used in the evaluations with the idea of determining the variability in the population of att*E. coli* O157:H7 within a known initial contamination (Table 5). After 7 dpi more than 50% of the non-disinfected leaves analyzed from the high inoculum dose were positive for the pathogen in both trials while less than 50% were positive for the low inoculum dose. At 14 dpi in Trial 1 33 and 17% of the leaves were positive for the pathogen without disinfection for the high and low does, respectively. At 18 dpi, 20 and 13% of the leaves without disinfection were positive for the pathogen in Trial 2.

These data provide further evidence that uniform "contamination" does not result in uniform survival on leafy greens, with the current example of spinach. Random field sampling and processing of relatively small masses of tissue (25g standard per process certified methods) may provide insufficient sensitivity to achieve the desired detection goals in preharvest and finished product testing schemes. Our data provides a foundation and support for future studies to more systematically standardize larger plant mass sample design. In addition, we feel our results begin to define critical thresholds for survival of *E.coli* O157:H7 arriving in contaminated irrigation water under field conditions. Inoculum levels of log 2 CFU/ml applied to the full bed area of emerged spinach had very low levels of survival under the seasonal conditions. We are unaware of any irrigation water source in CA or AZ being characterized as approaching within 50-fold or less of this level of contamination. Naturally, this does not exclude or preclude irrigation water from strong consideration as a source of contamination. We have yet to understand the role of isolated aggregates or a bolus of contamination carried in irrigation water from triggering sporadic illness or outbreaks.

Objective 3: *Monitoring survival of attenuated E. coli O157:H7 on field grown lettuce produced under different fertilizer levels*

For brevity, due to the page-restrictions within the CPS reporting guidelines, the details of Methods and a full inclusion of outcomes of this Objective will not be included but will become available in the Final Report to the CA Leafy Greens Research Program and in peer-reviewed journal manuscripts.

In brief, Nitrogen dose had no measureable effect on survival of *attE.coli* O157:H7 applied as a foliar spray at three different developmental stages (rosette, mid-growth and two-week preharvest) with log 4.2 CFU/ml on each date (Fig. 5). Survival by enrichment was detected in plants treated close to harvest for only a few replications. Additional time in the field further reduced detectable survival to one of sixteen 150g sample groups processed.

Tables and Figures

Table 1. Summary of outcomes of 1st and 2nd controlled contamination of Spring Mix Trial in Salinas Valley, CA (Oct-Nov, 2009) with generic E.coli and attEcO157:H7

	Approx. Log Decline Day 1*		**Day 14 Presence/Absence****
Plant Type	Generic E. coli Mix	attEcO157:H7 Mix	
Mizuna	3.1 ± 0.5	4.3 ± 0.1	Positive (2/2)
Arugula 1	3.2 ± 0.3	4.3 ± 0.1	Negative (0/2)
Arugula 2	3.2 ± 0.4	4.2 ± 0.2	Negative (0/2)
Tatsoi	2.3 ± 0.2	4.2 ± 0.2	Positive (2/2)
Green Chard	2.3 ± 0.2	3.8 ± 0.3	Not done
Red Chard	3.5 ± 0.2	4.5 ± 0.1	Positive (2/2)
Green Romaine	3.5 ± 0.2	4.4 ± 0.1	Not done
Red Romaine	3.7 ± 0.3	4.4 ± 0.1	Not done
Green Oak Leaf	3.6 ± 0.3	4.4 ± 0.1	Positive (3/3)
Red Oak Leaf	4.4 ± 0.2	4.3 ± 0.2	Not done
Green leaf	4.1± 0.2	4.4 ± 0.1	Not done
Red Leaf	4.3 ± 0.2	4.3 ± 0.2	Not done
Tango	4.4 ± 0.1	3.7 ± 0.2	Positive (3/3)
Lollo Rosa	2.5 ± 0.4	4.1 ± 0.2	Not done
Spinach 1	3.9 ± 0.1	3.9 ± 0.1	Not done
Spinach 2	3.3 ± 0.4	4.4 ± 0.1	Negative (3/3)

* Populations at Time 0+ 2h determined to be approximately log 4.5 CFU/g for each bacterial mixture. Log Decline is T0+2h recovery – T24 recovery.

** Day 14 detection of attEcO157:H7 after 2nd spray inoculation following selective enrichment. Not all types of spring mix are represented due to problems in those plot areas. Numbers represent positive replications of total replications harvestable.

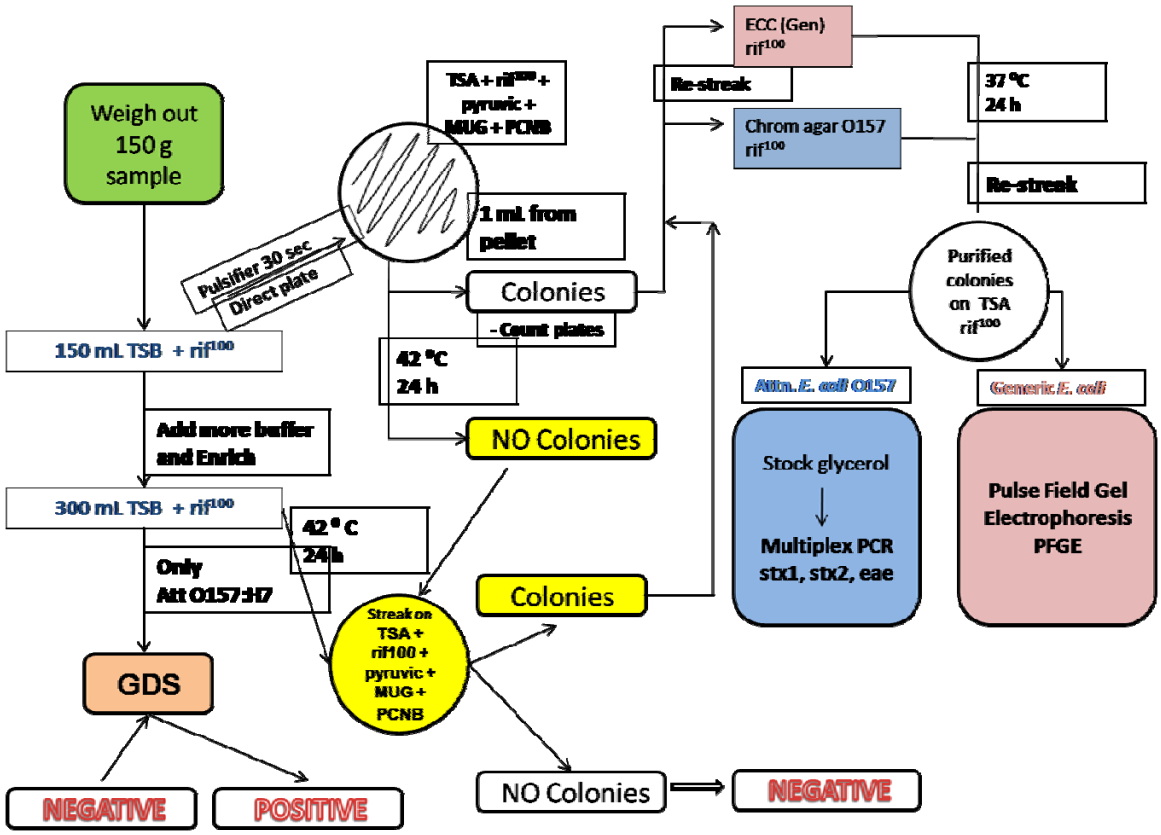

Figure 1: Schematic plant sample process plan for applied generic *E. coli* surrogate isolates and attenuated E. coli O157:H7 applied in controlled field trials of diverse "Spring Mix" leaf greens.

Key Terms in Chart–

TSB or TSA+rif ^{100}(Tryptic Soy Broth or Agar with 100 ppm rifampicin to selectively recover applied isolates that are resistant to this antibiotic).

Pyruvic – Pyruvic acid is added to direct detection media to aid in resuscitation of environmentally stressed cells.

MUG – a substrate modified by both generic E. coli and attenuated E. coli O157:H7 that causes the colonies to be fluorescent under UV light.

PCNB – A fungicide added to our recovery media that we determined to be essential for inhibition of soil fungi that interfere with recovery and enumeration of agar media.

GDS – Biocontrol GDSO157; a PCR-based kit as one method for verification of the presence of the attenuated *E. coli* O157:H7 in initial broth enrichments.

stx 1, stx2, eae – Colonies that appear positive on differential agar media for EcO157:H7 are tested by multiplex PCR to confirm they lack genes shigatoxin (stx 1 and 2) but have another semi-diagnostic virulence marker, eae. The presence of other markers are tested for in subsequent evaluations for positive colonies, as needed.

PFGE – The three generic E. coli each have a unique DNA fingerprint and this technique is used as a conformation step and to identify the distribution of survival of each strain in the mixture over time and on individual leafy-type.

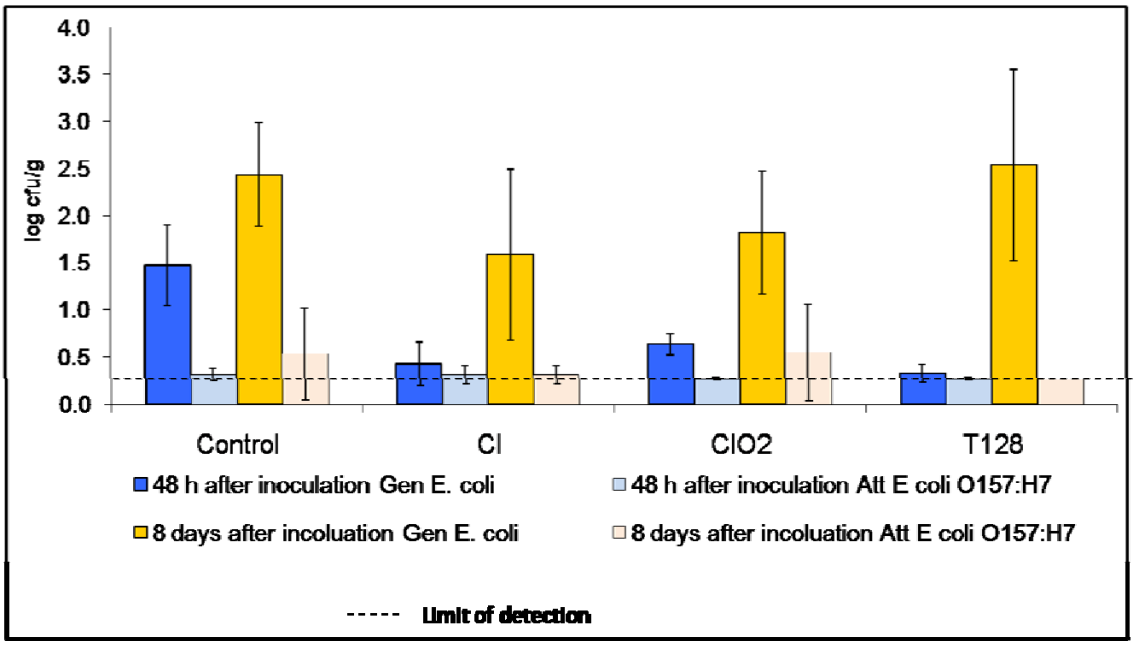

Figure 2: Survival comparison between generic *E. coli* and attenuated *E. coli* O157.H7

Recovery of field applied generic *E. coli* from process water

	DISINFECTANT	STEP	SCORE MEMBRANE	MPN (cfu/10mL)
8 DAYS AFTER INOCULATION	Cl	PREWASHING	7	7
		WASHING	0	0
		RINSED	0	0
		CENTRIFUGED	188	200
	ClO_2	PREWASHING	37	37
		WASHING	0	0
		RINSED	3	0
		CENTRIFUGED	32	32
	T128	PREWASHING	412	476
		WASHING	0	0
		RINSED	0	0
		CENTRIFUGED	744	1001

Figure 3: Preliminary assessment of postharvest wash process water cross-contamination potential and performance on a composite field inoculated "Spring Mix salad" at four unit operational steps.

	DISINFECTANT	REP	GDS (*E. coli* O157)		DISINFECTANT	REP	GDS (*E. coli* O157)
48 HOURS AFTER INOCULATION	Cl	1	POSITIVE	8 DAYS AFTER INOCULATION	Cl	1	POSITIVE
		2	POSITIVE			2	POSITIVE
		3	POSITIVE			3	POSITIVE
	ClO_2	1	POSITIVE		ClO_2	1	POSITIVE
		2	POSITIVE			2	POSITIVE
		3	POSITIVE			3	POSITIVE
	T128	1	POSITIVE		T128	1	POSITIVE
		2	POSITIVE			2	POSITIVE
		3	POSITIVE			3	POSITIVE

Figure 4: Detection of *attE. coli* O157:H7 from postharvest-washed composite field inoculated "Spring Mix salad" with different process water treatments. Detection was conducted using GDSO157 PCR kits and culture confirmations were performed on selective/differential media. Selected colonies were confirmed to be rifampicin resistant and shigatoxin-negative.

Table 2. Direct detection recovery of att*E. coli* O157:H7 from field spinach (150g) with and without surface disinfection relative to time (days) post-inoculation.

	Initial Foliar Spray Inoculum Dose per Area of Planted Bed							
Days after inoculation*	Log 0.56 CFU/m^2				Log 0.3 CFU/m^2			
	Not Disinfected		Disinfected		Not Disinfected		Disinfected	
	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
7 day	3/ 6	4/8	2/6	2/8	2/6	2/8	2/6	0/8
14 day	1/6	ND	1/6	ND	0/6	ND	0/6	ND
18 day	ND	3/ 8	NA	0/8	ND	2/8	NA	0/8
25-28day	1/6	2/8	NA	NA	0/6	1/8	NA	NA

*At 18hrs post- inoculation all leaves from both inoculation doses without disinfection were positive for *E. coli* O157:H7. Values represent number of positive groups of total replications by direct plating. ND refers to *not done* at this time point and NA refers to *none available* for plant material at this time of evaluation.

Table 3. Enrichment enhanced detection of att*E. coli* O157:H7 from non-disinfected spinach (150g) relative to time (days) post-inoculation.

	Initial Foliar Spray Inoculum Dose per Area of Planted Bed			
Days after inoculation	Log 0.56 CFU/m^2		Log 0.3 CFU/m^2	
	Trial 1	Trial 2	Trial 1	Trial 2
7 day	6/6	4/8	3/6	3/8
14 day	3/6	ND	2/6	ND
18 day	ND	3/8	ND	2/8
25-28 day	2/6	5/8	0/6	3/8

Values represent number of positives groups of total replications after enrichment and detection by GDSO157 and selective media recovery. ND refers to *not done* at this time point.

Table 4. Enrichment enhanced detection of att*E. coli* O157:H7 from spinach (150g) disinfected with 1% silver nitrate, relative to time (days) post-inoculation.

	Initial Foliar Spray Inoculum Dose per Area of Planted Bed			
Days after inoculation	Log 0.56 CFU/m^2		Log 0.3 CFU/m^2	
	Trial 1	Trial 2	Trial 1	Trial 2
6 day	2/6	2/8	2/6	1/8
14 day	2/6	ND	1/6	ND
18 day	NA	1/8	NA	0/8

Values represent number of positives groups of total replications after enrichment and detection by GDSO157 and selective media recovery. ND refers to *not done* at this time point and NA refers to *none available* for plant material at this time point.

Table 5. Direct detection recovery of att*E. coli* O157:H7 from individually processed leaves of field spinach with and without surface disinfection relative to time (days) post-inoculation.

	Initial Foliar Spray Inoculum Dose per Area of Planted Bed							
Days after inoculation*	Log 0.56 CFU/m^2				Log 0.3 CFU/m^2			
	Not Disinfected		Disinfected		Not Disinfected		Disinfected	
	T1	T2	T1	T2	T1	T2	T1	T2
7 day	16/24	23/30	3/24	5/30	11/24	16/30	1/24	2/30
14 day	8/24	ND	0/24	ND	4/24	ND	0/24	ND
18 day	NA	6/30	NA	3/30	NA	4/30	NA	0/30

*At 18hrs of inoculation all 24 or 30 leaves from both inoculation doses without disinfection were positive for *attE. coli* O157:H7. Values represent number of positives individual leaves of total leaves by direct plating for non-disinfected leaves and for disinfected leaves after enrichment. ND refers to *not done* for this time point and NA refers to *none available* for plant material at this time point.

N-Dose		Week 1				Week 2				Week 3				Week 4				Week 5			
		Rep 1	Rep 2	Rep 3	Rep 4	Rep 1	Rep 2	Rep 3	Rep 4	Rep 1	Rep 2	Rep 3	Rep 4	Rep 1	Rep 2	Rep 3	Rep 4	Rep 1	Rep 2	Rep 3	Rep 4
0	A	Y	Y	Y	Y	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	B					N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	C									N	Y	Y	Y	Y	Y	N	N	Y	N	N	N
75	A	Y	Y	Y	Y	N	N	Y	Y	N	N	N	N	N	N	N	N	N	N	N	N
	B					Y	N	Y	N	N	N	N	N	N	N	N	N	N	N	N	N
	C									Y	N	Y	Y	Y	N	N	N	N	N	N	N
150	A	Y	Y	Y	Y	N	Y	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	B					N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	C									Y	N	Y	Y	N	N	N	N	N	N	N	N
225	A	Y	Y	Y	Y	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	B					N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	C									Y	Y	Y	Y	Y	N	N	N	N	N	N	N
300	A	Y	Y	Y	Y	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	B					N	N	Y	N	N	N	N	N	N	N	N	N	N	N	N	N
	C									Y	Y	N	N	N	Y	N	N	N	N	N	N

Figure 5: Survival of a mixture of two attenuated *E.coli* O157:H7 rifR strains (log 4.2 CFU/ml) sprayed onto Romaine lettuce treated with 0 (field-residual N levels only) or supplemented with 75, 150, 225, or 300 lbs total N applied as AN 20. Applications were made at the developmental stages rosette (A), mid-growth (B), and two-weeks pre-harvest (C. Composite leaf samples of 150/g per plot replication were analyzed weekly and rated as Positive (Y-red fill) or Negative (N-green fill) based on either direct plating or enrichment-enhanced detection. Plants in Group C were also sampled and analyzed one week past harvest typical maturity.