Project Title

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

PI: Trevor Suslow, Department of Plant Sciences, UC Davis

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

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.

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 rifampicin (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), rifampicin 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 were spread onto selective agar and incubated for 18 hours to create a uniform lawn. Cells were harvested by gently scraping the agar surface with a sterile rubber spatula, then 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 using a two nozzle spray boom and Teejet 8005 spray tips (selected to reduce spray drift); the sprayer was set at approx. 25-27 psi 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 polyethylene bags at the different time intervals after inoculation, 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).

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 originally isolated from Salinas region farms. Due to the broad level of natural diversity among non-pathogenic *E. coli,* the specific genetic fingerprints of each strain would be highly unlikely to be found randomly at the trial location unless part of the inoculation mixture.

Results:

The survival of all applied *E. coli* was very low in this first trial attempt within a commercial operation location, rather than a designated experimental field. The isolated field trial location provided was not ideal for crop management, especially weed management, and limited results were obtained in our 2009 efforts. Detectable survival was essentially zero 18 and 48h after the first foliar application. A second identical inoculation was made on Day 5. 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. 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) at 8 and 14 days post-inoculation. Pulsed Field Gel Electrophoresis analysis of applied surrogate isolates from a Spring Mix field trial show patterns representing individual colony fingerprints with restriction enzyme Xba1 verifying isolates TVS 353 (Water) TVS 354 (Plant) TVS 355 (Soil) from approximately 10% of randomly collected isolates taken from direct plating on TSA rif 100 mg/l or CHROMECC with 80 mg/L of rifampicin (Appendix Figure 1). From the composite analysis, TVS 354 was the dominant type recovered across sample dates at 66%, followed by TVS 353 (21%) and TVS 355 (13%).

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.

Opportunities for involvement in Rapid Response event analyses were less frequent than anticipated but detailed assessments of initial presumptive pathogen detections on product or environmental samples necessitated a greater investment of time and resources in a highly dynamic and interactive process with growers, handlers, and processors. An overview of the conditions and sequence of events is presented.

RR7 – Limited Feral Pig Intrusion of Romaine Lettuce Immediately Preceding Harvest

Initial contact events

- Preharvest scouting observed pig intrusion on edge of two lettuce blocks
- UCD notified of animal intrusion event
- Irrigation terminated; 30-ft buffer zone delimited as no-harvest zone
- UCCE conducts site evaluation same day; initial samples taken
- UCCE conducts indicator *E.coli* enumeration on lettuce damaged or in immediate proximity to pig tracks.
- UCD team arrives next day for additional lettuce samples
- Fecal scat, soil, lettuce collected and processed at UCD

Results

- No EHEC detected from fecal droppings
- No EHEC or Salmonella detected on lettuce within and beyond LGMA 30-ft no-harvest zone
- Elevated indicator E. coli present on lettuce closest to immediate area of fecal droppings (Figure 1 and 2)
- Indicator E. coli levels at field baseline level on plants along path of intrusion and within 30-ft buffer zone (Fig 1 and 2)
- Pulsed Field Gel Electrophoresis patterns suggested several *E. coli* present on lettuce in impacted zone (Appendix 1 Fig. 2)

Key Learning's

- Preharvest scouting was effective
- Detectable transfer from animals and fecal scat appears highly limited
- Lack of irrigation event post-intrusion may have significantly limited transfer
- LGMA no-harvest zone appeared more than adequate in this case

RR8 – Two Risk Factor Concerns: Deer Intrusion and Irrigation Source

Initial contact events

- UCD notified of animal intrusion and concern for surface irrigation source having elevated indicator *E. coli*
- Company field personnel collect deer pellets on product in delimited zone and transfer to UCD along with water samples collected on site

Results

  - High populations of indicator *E. coli* present in deer pellets but no EHEC detected, More than one sub-type of *E.coli fou*nd in individual deer fecal pellet samples (Appendix 1 Figure 3)
- Indicator E. coli levels in water dropped below LGMA use-termination level on consecutive days of collection (< 235 MPN/100ml)
- Filtration concentration and enrichment of water reveals presumptive E. coli O157:H7 by GDSO157 PCR
  - Subsequent colony confirmation of *E. coli* O157:H7 from plates
- Repeat sampling of irrigation source 72h after initial reveals same result
  - Subsequent colony confirmation of *E. coli* O157:H7 from plates
- Crop destroyed and reservoir put out of use at ranch
- No further tests allowed. Residual samples destroyed.

RR9 - Presumptive E. coli O157:H7 Positive on Bagged Spinach

Initial contact events

- Commercial lab detects positive for *E.coli* O157:H7 on finished product
- Shipper contacts UCD and offers opportunity for analysis

- Fortuitously, UCD and UCCE already in area for trial work; site evaluation was made on same day as notification
    - No obvious site risk factors observed
    - Crop was approaching harvest maturity during period of intense rainfall followed by unseasonably warm weather and minor rain events (Oct 13-25 2009; Figure 3)
- Samples taken from pallets in commercial cooler in lot on same day and returned to UCD for analysis
    - 13 pallets X 2 bags per pallet

Results

- Widespread PCR-positives detected by UCD (150gm units) {Figure 4}
    - Standard and novel E. coli O157:H7 and EHEC analysis
        - ❖ GDSO157, uidA, TPEC, Shigatoxin
        - ❖ 17/26 2lb bags were positive for O157 and EHEC
    - 80 colonies from sample bags 2, 11, and 20 were re-streaked
    - Sample 11: 5/80 Positive for uidA (Himathongkham et al. 2007) diagnostic for *E. coli* O157:H7
    - Sample 20: 5/80 Positive for uidA
- Original commercial lab sample provided to UCD and analyzed
    - Distribution analysis yields low frequency (2/5) in standard sample units (25g)
        - ❖ Routine tests utilize a single 25g sample, therefore adding the original sample as a positive one may consider the lab had a 3 in 6 (50:50) chance of detection to disqualify this product from marketability.
    - Culture from commercial lab not consistent with typical E. coli O157:H7 by determinative tests
- Analysis of 190 individual leaves (150 gm) results in 16% positive for *E. coli* O157:H7
- Distribution analysis yields low frequency in standard sample units (25g)
    - Unusual results noted for real-time PCR against common virulence markers
    - Unusual colony phenotype results noted in efforts to conduct culture confirmation

Key Learning's

- Weather events preceding harvest are possible contributors

- No preharvest factors associated with adjacent land use or with irrigation source, no animal intrusion apparent during risk assessments, to explain contamination event.
- Atypical *E. coli* triggered GDS-O157 positive reactions
- Standard product sample mass (25g) could easily have missed contamination event
- If pathogen testing is to be done, preharvest and harvested product analysis would likely be improved if sample size (g/lot) was increased on a risk-based approach. For example, harvest after a significant rain event would trigger an increased sample collection and analysis.

RR10 – Presumptive Salmonella and EHEC Positive in Preharvest and Harvested Spinach

Initial contact events;

- Widespread presumptive positives for Salmonella and EHEC detected in preharvest samples and harvested product (but not released to market) in adjacent blocks
- UCD contacted to assist in analysis
- Site evaluation conducted next day; samples taken from field and cooler

Results

- EHEC detection confirmed in multiple harvested and retained product samples but not from n=60 samples taken from unharvested adjacent block
- Salmonella detected in soil samples in unharvested block but not RR10 product samples (Appendix 1 Figure 4)
- RR10 Salmonella isolates did not match original product sample Salmonella (Appendix 1 Figure 4)
- Retained harvested lots destroyed and unharvested lot disced into soil
- Key risk factor appeared to be irrigation water source. Completion of analysis is ongoing

- **Key Learning's**
  - Multiple potential pathogens may be present in environmental or crop input sources but persistence may be highly ephemeral under prevailing conditions.

- Environmental Salmonella is non-H2S type (no black colonies) and could be missed in typical analysis
- Detecting pathogens in irrigation sources may not be readily correlated with detectable contamination of crop or product
- The actual source of contamination could not be determined. Field observations by grower and food safety managers cited a period of high bird intrusion during crop growth as a possible factor.

RR11 – Confirmed Salmonella Positive on Arugula

Initial contact events;

- Commercial lab detects culture confirmed Salmonella
    - UCD contacted, other obligations delay site evaluation for 10 days
    - Irrigation terminated in the interval

Results

- No Salmonella detected on n=30 samples of 75 gm from production block
- Culture confirmed Salmonella detected in environmental samples from irrigation source (Appendix 1 Figure 4 and Table 1)

Key Observation

- Survival of Salmonella on arugula under field conditions following initial contamination event may have been limited and could not be confirmed after 10 days under ambient conditions.

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

The lab-based study that prompted grower interest in this objective (Brandl and Amundson, 2008) suggested that survival and growth of pathogenic *E. coli* O157:H7 may be influenced by nitrogen content in lettuce leaves. Leaves containing higher levels of nitrogen and associated leaf exudates of higher nitrogen apparently favored survival and growth of this pathogen in model research conducted in a growth chamber that was maintained at near 100% relative humidity. To preliminarily test the relationship of this potential risk factor in a practical field setting, a romaine lettuce fertilizer trial was superimposed with an inoculation experiment using a mixture of two attenuated *E. coli* O157:H7 strains. Isolates PTVS 154 and PTVS 155 were the same as described in Objective 1.

The lettuce crop was grown at the Hartnell Experimental Research Facility under the supervision of R. Smith and M. Cahn according to standard commercial practices with the exception of variable nitrogen applications. The nitrogen levels established were 1) unfertilized control; 2) low fertility treatment (75 lbs N/ac); 2) moderate fertility treatment (150 lbs N/ac); 3) commercial rate (225 lbs N/ac); and high fertility treatment (300 lbs N/ac). Treatments within each dose plot were accomplished by applying soluble nitrogen (Ammonium nitrate - AN20) into a drip irrigation system in four weekly applications beginning at the thinning stage (30 days after planting). Plots were four beds wide (40 inches OC) by 150 feet long and replicated four times in a randomized complete block design.

The number of N treatments in the trial were increased by two, relative to the proposed plan and, due to a very low level of survival following inoculation at the relative low dose of bacteria, a decision was made to focus on detectable recovery following enrichment culture only.

As reported in Brandl and Amundson, the inoculum dose was adjusted to log 4.3 CFU/ml, with approximately 2-10 ml applied per plant as a spray, depending on plant age. Spray application of PTVS 154 and 155 was conducted with a CO2-pressurized back-pack sprayer 1-3 times during the growth cycle: 1) rosette; 2) mid-growth; and 3) at two-weeks preharvest. Three treatment intervals corresponded to the first three sampling dates; one spray, two sprays, and three sprays; the final simulated contamination 14 days before harvest maturity. Inoculum preparation, dose adjustment, inoculation, and enumeration were as described in Objective 1.

Seven days following the first inoculation, 5-20 lettuce plants (depending on plant age +size) were collected across the length of each replicate plot and assayed for viable populations at 7, 14, 21, and 28 days post-inoculation. Each head was topped and tailed and further chopped into approximately 4 in^2 pieces to include older and younger leaves. All pieces from one plot were blended and a randomly selected composite of 150g was processed by direct plating following sample massaging and hand-maceration in 300 ml of TSB and parallel enrichment in the bulk volume to recover rifampicin-marked strains, as described in Obj. 1.

Results

From an initial inoculum of approximately log 4.3 CFU/ml applied to Romaine plants from one to three times, no attE.coliO157:H7 rif colonies were observed by direct plating at any date for any treatment level. Evidence for viable residual contamination of applied strains was detected on plants within 14 days of the initial contamination date (Fig. 5) but rarely after that date and not associated with levels of N-fertilization. As presented in Table 2, nitrogen dose had no demonstrable or practical effect on detectable survival of *att E.coli* O157:H7 applied as a foliar spray (Fig. 5). Survival detection by enrichment revealed positive plants in populations of 360 individuals at 14 days but never beyond.

Survival by enrichment was detected in plants treated close to harvest for only one of 360 plants among the various treatment replications receiving all three applications. The one positive plant composite was associated with the plots not receiving additional N-applications above field residuals. As only one sample was positive following enrichment and due to utilization of resources for Objective 2, we abandoned the effort to develop an assessment of residual viable populations on an expanded set of plants or as internalized populations.

Literature Cited

Brandl, M. T., and Amundson, R. 2008. Leaf age as a risk factor in contamination of lettuce with Escherichia coli O157:H7 and Salmonella enterica. Appl. Environ. Microbiol. 74:2298-2306

Himathongkham, S., M. L. Dodd, J. K. Yee, D. K. Lau, R. G. Bryant, A. S. Badoiu, H. K. Lau, L. S. Guthertz, L. Crawford-Miksza, and M. A. Soliman. 2007. Recirculating Immunomagnetic Separation and Optimal Enrichment Conditions for Enhanced Detection and Recovery of Low Levels of Escherichia coli O157:H7 from Fresh Leafy Produce and Surface Water. Journal of Food Protection® **70:**2717-2724.

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 plant growth or crop management in those plot areas. Numbers represent positive replications of total replications harvestable.

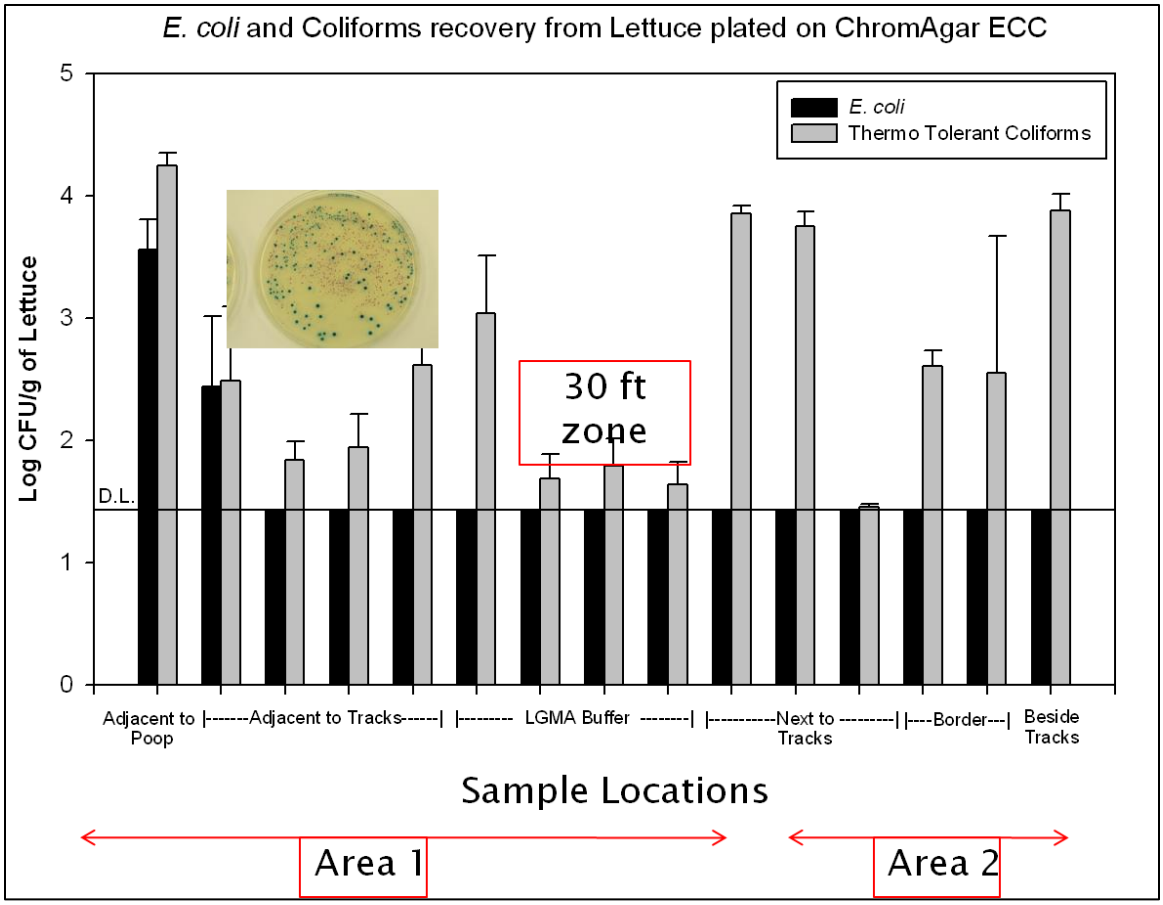

Figure 1- Recovery of fecal coliforms and generic E.coli from Romaine lettuce in relation to spatial proximity to areas of feral pig intrusion fecal deposits and tracks through rows.

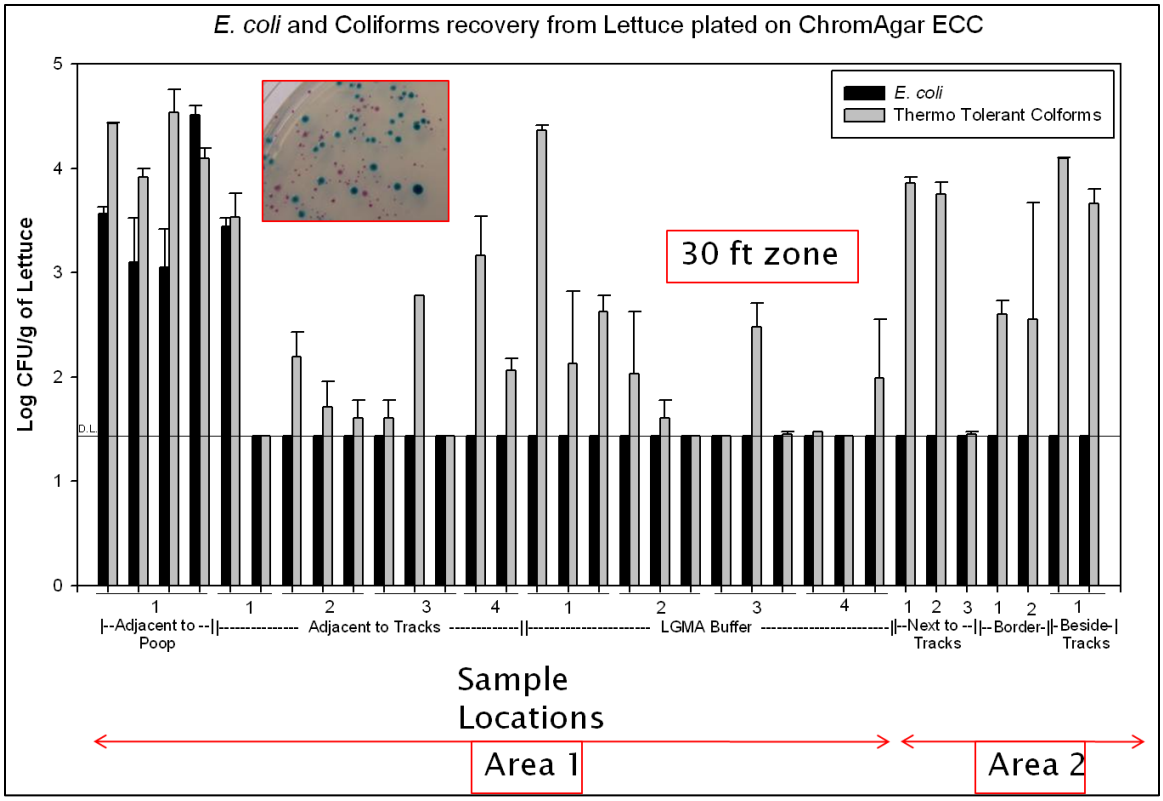

Figure 2- Details of recovery of fecal coliforms and generic E.coli from Romaine lettuce in relation to spatial proximity to areas of feral pig intrusion fecal deposits and tracks through rows.

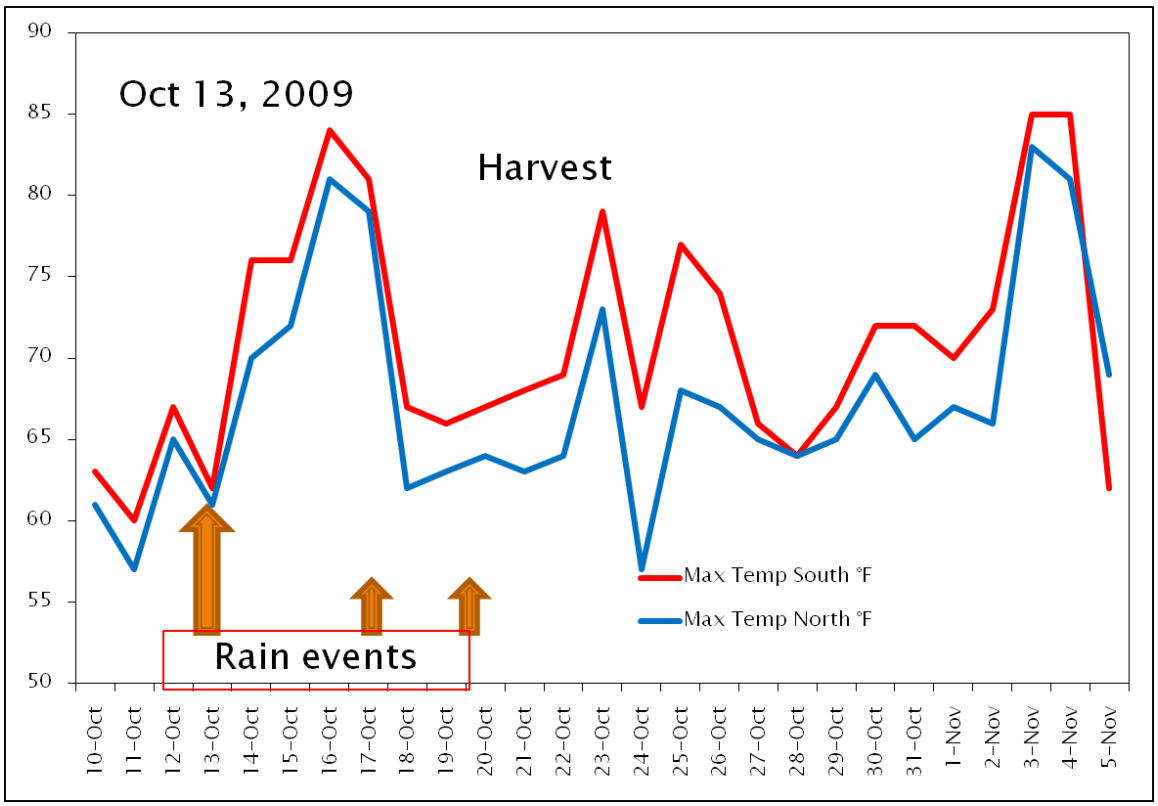

Figure 3 – Maximum air temperature profile for period surrounding Rapid Response event #9. Data obtained from CIMIS database for Salinas South and Salinas North. Greatest single day rainfall accumulation exceeding 3 inches occurred on October 13, 2009.

Pallet –Tag Sequence and Sample Randomization Ranking

Pallet #	Sample #	GDSO157	Ranking	multiplex PCR			
				stx1	stx2	eae	hylA
439	24	+	2	+	+	+	+
	14	+	11	-	+	+	+
440	4	R		-	-	+	-
	8	+	20	-	-	+	-
441	18	-		+	+	-	+
	26	-		-	+	+	+
442	15	-		-	+	-	+
	19	R		+	-	+	-
496	1	R		-	-	+	-
	3	-		+	-	-	-
497	20	+	14	-	-	+	+
	21	+	10	-	-	+	-
498	6	+	28	-	+	+	+
	23	+	18	+	+	-	+
499	2	+	8	-	+	+	+
	5	+	3	-	+	+	+
500	17	-		-	+	+	+
	22	+	9	-	+	+	+
530	11	+	12	-	-	+	+
	13	+	16	+	-	+	-
531	7	+	1	+	+	+	+
	12	+	23	+	+	-	+
532	9	+	6	-	-	+	-
	25	R		-	-	+	-
533	10	+	15	+	+	+	+
	16	+	27	-	+	+	+

Figure 4 – Overview of results from analysis of finished product bags of spinach from 13 pallets of a single contaminated lot. Sample bags were randomized prior to analysis by GDSO157 and multiplex PCR. Red cells (+) designate positive determination by GDS while white cells in the column (-) were negative. Cells highlighted in yellow (R) were close to crossing the threshold for a positive reaction. Due to positive detection by GDS and presence of all four virulence markers (stx 1, stx2, eae, and hylA) in the complex enrichment culture, a more detailed analysis of pallet 531 Bag 7 and 533 Bag 10 was conducted.

Table 2: Effect of nitrogen fertilizer applications to Romaine lettuce on foliar survival of *attE.coli* O157:H7 in relation to timing of a water-borne contamination event

Applied Nitrogen (lbs/ac)	Lettuce Crop Stage during spray contamination (# positive/4 reps at harvest)		
	Rosette (1 spray	Mid-growth (2-sprays	2-weeks preharvest (3-sprays)
0	0/4	0/4	1/4
75	0/4	0/4	0/4
150	0/4	0/4	0/4
225	0/4	0/4	0/4
300	0/4	0/4	0/4

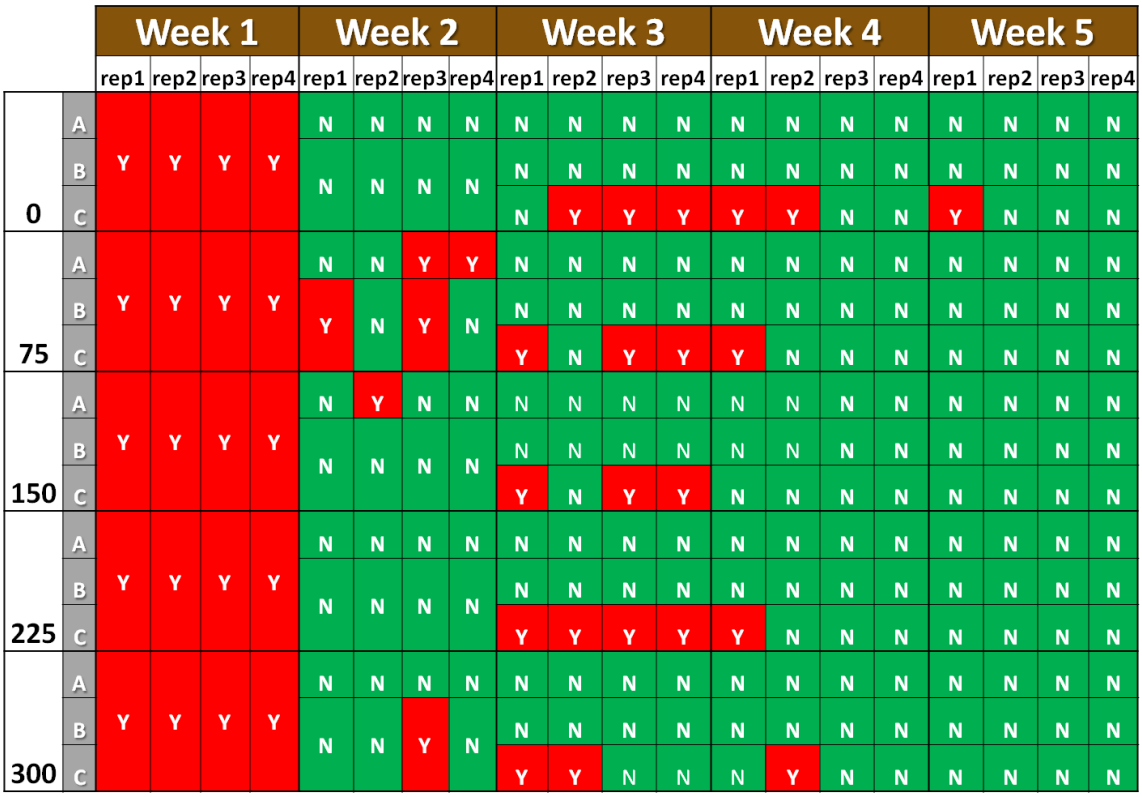

Figure 5. Representative outcome of spray-inoculation with an approximate log 4.0 CFU/ml of a two-stain mixture of attE.coli O157:H7 onto Romaine lettuce treated with o, 75, 150, 225, or 300m lbs/ac of total nitrogen. Red rectangles (Y) in red were positive by enrichment of 150 g of sample tissue with O157-specific PCR and colony confirmations. Green rectangles (N) were negative for the applied rifampicin resistant isolates. Treatment A was spray-inoculated once, B- twice, and C-three times during growth.