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CENTER FOR
PRODUCE SAFETY

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Project Title
Examination of the survival and internalization of *E. coli* on spinach under field production environments

Project Period
January 1, 2009 through December 31, 2009

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

Co-Principal Investigator
Mike Cahn
UC Cooperative Extension – Monterey County
mdcahn@ucdavis.edu
831.759.7377

Collaborating Investigators
Richard Smith
UC Cooperative Extension – Monterey County
rfsmith@ucdavis.edu
831.759.7357

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

Objectives:
Objective 1: Monitor survival of generic and attenuated O157:H7 *E. coli* strains on foliage of field grown spinach.

Objective 2: Determine whether field grown spinach can absorb and transport *E. coli* from roots to foliage (internalization).

Research Report
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Principal Investigator: Steven Koike
University of California Cooperative Extension
Monterey County

Collaborating Investigators: Michael Cahn, Richard Smith, Grace McClellan
University of California Cooperative Extension
Monterey County

Trevor Suslow, Adrian Sbodio
Department of Plant Sciences
University of California at Davis

Introduction:

Due to recent food contamination outbreaks involving the extensive leafy green vegetable acreage in California, it is imperative to obtain information on the biology, ecology, and epidemiology of both generic and pathogenic *E. coli* under coastal California agricultural field conditions. Because few studies have been conducted under these conditions, our practical understanding of how *E. coli* operates in the field is presently incomplete. Applied field-oriented research is also needed so that industry and regulators can make informed decisions on growing practices, programmatic risk reduction and audit compliance metrics, and emerging regulatory food safety policies for the field. This project attempted to develop information on how nonpathogenic generic and attenuated O157:H7 *E. coli* strains persist under Salinas Valley farming conditions with a specific focus on spinach.

Objectives:

1. Monitor survival of generic and attenuated O157:H7 *E. coli* strains in soil and on foliage in a field spinach production environment.

2. Determine whether *E. coli* can be internalized by roots of field grown spinach and be systemically transported to foliage (internalization).

General methods and procedures:

The intent of these experiments was to simulate *E. coli* contamination situations in a commercial farm environment and evaluate subsequent survival of the inoculated bacteria. All experiments were conducted on an isolated commercial farm lot (identified as SVR-57) in the Salinas Valley. Prior to planting, the soil was prepared and formed into 80-inch wide seedbeds according to standard commercial practices. Fields were planted in commercial fashion with spinach according to standard specifications: 39 lines of seed on 80-inch wide beds, seed density of 3.5 million live seed/acre. Following planting, experiment plots were marked out onto these beds. Experimental design was a randomized complete block with four replications.

Inoculations made to soil or plants used mixtures of either three rifampicin-resistant generic *E. coli* strains (TVS strains 353, 354, and 355) originally isolated from the Salinas Valley or two attenuated O157:H7 *E. coli* strains (PTVS strains 90 and 93). Inocula were delivered either in a liquid spray suspension or in a sand and organic carrier mixture in a mesh bag. Liquid spray inoculum simulated a contaminated water source and was applied with a CO_2 powered, handheld, backpack sprayer. Mesh bag inoculum simulated a contaminated point source and was placed on top of the planted beds. Experiments always included a non-inoculated control.

After inoculation, the seeded field was turned over to the grower cooperator who produced the spinach according to standard practices. The field was irrigated with overhead sprinklers for the duration of the growing period. Ditches were cut across the field at the front of each section to allow run-off water to drain away from the plots and prevent cross contamination. Standard fertilization was applied to the crop (400 pounds of 13-0-16 applied pre-plant at listing; 200 pounds of urea 46-0-0 as top-dress on the spinach). Aphids, other insect pests, and downy mildew disease were managed by commercial applications of insecticides and fungicides.

During the course of the experiments, soil, plant, and water samples were collected and tested for the inoculated generic or attenuated strains. Soil and plants were processed in the lab and the resulting liquid extracts dilution plated onto Tryptic Soy Agar plates amended with 100 µg/liter rifampicin (rif), 100 µg/liter 4-methylumbelliferyl-beta-D-galactopyranoside (MUG), 100 µg/liter of the pentachloronitrobenzene fungicide (PCNB), and 1 g/liter pyruvic acid.

To determine presence/absence of *E. coli*, an enrichment step was also completed in which 25 ml of supernatant was mixed with 75 ml of Tryptic Soy Broth amended with 100 µg/liter rifampicin (TSB+rif). This mixture was incubated for 18 hours at 42º C before plating and incubating on amended TSA as described above.

Water samples were tested in two ways. For each sample, 15 ml were withdrawn, centrifuged, and the resulting supernatant removed. The remaining pellet was re-suspended and plated onto either TSA+rif+MUG+PCNB+PA (for plots inoculated with generic *E. coli*) or CHROMAgar™ O157+rif (for plots inoculated with attenuated O157:H7 *E. coli*). Secondly, water samples were also tested using the QuantiTray 2000 Colilert System®. 100 ml of collected water were mixed with Colilert reagents, dispensed into the assay trays, sealed, and incubated at 37 °C for 48 hours according to manufacturer recommendations.

Outcomes and accomplishments:

Summary for objective 1. Various *E. coli* strains (mixtures of either rifampicin-resistant generic *E. coli* or rifampicin-resistant attenuated O157:H7 *E. coli*) applied as water-based sprays

or mixed with sand and placed in mesh bags to simulate point sources of contamination did not survive in soil for long periods of time under commercial growing conditions in the Salinas Valley. Spray or bag inoculum was not recovered, via direct plating, from the spinach plants growing through inoculated soil or next to bag inoculum. However, when mature spinach plants were spray inoculated and immediately disked into the soil, inoculated bacteria were recovered from field plots for over 85 days.

Summary for objective 2. When various *E. coli* strains were inoculated onto spinach roots by using a subsurface drip irrigation system, the above ground foliage did not test positive for the *E. coli* strains when using direct plating methods. Surface sterilizing plants with mercuric chloride followed by enrichment culture resulted in only one of 80 whole plants being positive for the rifampicin-resistant generic *E. coli*.

Objective 1.

Persistence in soil: sprayed inoculum. Both the generic and attenuated O157:H7 *E. coli*^{rif} strains, spray inoculated to soil at high rates (10^8 CFU/ml), post-seeding but pre-emergence, were recovered from soil for relatively short periods of time (Appendix, Fig. 1). In general, a 100-fold (2 log) and 100,000-fold (5 log) reduction from 0 days-post-inoculation (dpi) to 8 dpi and 15 dpi, respectively, was observed for generic *E. coli*. A 5 log reduction from 0 dpi was encountered by 8 dpi for the attenuated *E. coli* O157:H7 strains. By one day after inoculation, all inoculated soil samples contained bacterial populations that were significantly lower than the original inoculum concentrations delivered to the soil surface. By 15 days after inoculation, recovery was below the detection limit by standard direct plating for both strains but the generic *E. coli* were still detectable following a centrifugation concentration enrichment. The attenuated O157:H7 strain declined at a faster rate compared to the generic strain (Fig. 2). We did not recover either generic or attenuated O157:H7 *E. coli*^{rif} strains in uninoculated plots, indicating that inter-plot contamination did not occur to a detectable level.

Persistence in soil: point source mesh bag inoculum. Both the generic and attenuated O157:H7 *E. coli*^{rif} strains, inoculated to the soil by placing mesh bag inoculum on the tops of the beds post-seeding but pre-emergence, were recovered from soil adjacent to the bags (0 cm distance) for relatively short periods of time (Fig. 3). By three days after inoculation, all inoculated soil samples contained bacterial populations that were significantly lower than the 1 dpi recovered concentrations. By 15 days after inoculation, recovery at 0 cm distance was below the standard direct plating detection limit for both strains but the generic *E. coli* were still detectable following a centrifugation concentration enrichment. The generic and attenuated O157:H7 strains declined at comparable rates (Fig. 2). We did not recover either generic or attenuated O157:H7 *E. coli*^{rif} strains in uninoculated plots, indicating that inter-plot contamination did not occur to a detectable level.

For soil samples taken further away from the point source inoculum mesh bags, low populations of the generic strain were found on day 1 only at both 25 and 50 cm distances (Fig. 3). After day 1, no generic strains were recovered at any time until the experiment was ended after 15 days. From the 25 and 50 cm distances, no attenuated strains recovered at any time until the experiment was ended after 15 days (Fig. 3).

Persistence on inoculated spinach plants: For all plant samples collected and tested by direct plating onto TSA amended medium, no generic or attenuated O157:H7 *E. coli*^{rif} strains were recovered for any inoculum concentration or at any sample date. We also did not recover either generic or attenuated O157:H7 *E. coli*^{rif} strains in uninoculated plots, indicating that inter-plot contamination did not occur to a detectable level.

Inoculum transported in irrigation runoff. Runoff irrigation water was collected from furrows on 3, 17, and 29 days-post-inoculation (dpi). Using the Colilert/QuantiTray 2000 system for analysis of *E. coli* in these water samples, only those plots treated with sprayed generic *E. coli* on the soil surface had substantially higher populations of total *E. coli* on Day 3 as compared to background *E. coli* positives in non-sprayed plots. Analysis of positive QuantiTray wells revealed that *E. coli* in runoff water from the generic sprayed and mesh bag plots were rifampicin-resistance while those recovered from non-treated plots were rifampicin-sensitive and therefore not the applied strains.

Inoculating plants at different development stages. In this field experiment, conducted twice, we applyed controlled dose contamination to emerged and developing spinach leaves at First True Leaf (FTL), FTL + 7 days, and FTL + 14 days. For plants treated at FTL stage, recovery from collected leaves was possible only from 1 of 3 and 2 of 3 composite samples taken from the 576 and 57,600 MPN/100 ml doses in Trial 1 (September 2009) and none recovered in Trial 2 (October 2009). Within 2 weeks all applied bacteria were not detectable. For plants treated at FTL +7, only 1 of 3 samples yielded detectable populations from the 57,600 MPN/100 ml dose of generic *E. coli* in Trail 1 and 2. Lastly, for plants treated at FTL + 14 days, *E. coli* O157:H7^{rif} strains were detected in 1 of 3 or 2 of 3 samples at the 235 and 576 or 57,600 MPN/100 ml dose, respectively in Trial 1 but were not detectable in Trial 2. At FTL+14, generic *E. coli* were not detectable in Trial 1 but recoverable in 3 of 3 and 1 of 3 samples from 5,760 and 57,600 MPN/100 ml doses, respectively.

Post-harvest survival in soil. When generic and attenuated O157:H7 *E. coli*^{rif} strains were inoculated onto the mature spinach crop and the plants incorporated into the soil, both strains were recoverable from field soil for an extended period of time (Fig. 4). From the day of crop incorporation (day 0) through 85 days-post-inoculation (dpi), our periodic sampling continued to recover both generic and attenuated strains (Fig. 4). Because of these recoveries, we had the grower irrigate all plots on 88 dpi and re-disk the plots on 101 dpi. For the 106 dpi soil sample, neither generic nor attenuated O157:H7 strains were recovered by direct plating (Fig. 4).

Objective 2.

Inoculating roots and testing for internalization of *E. coli*: Bacterial inoculum was delivered to the spinach roots via a subsurface drip irrigation system. Inoculated water that was withdrawn directly from the subsurface drip tape tested positive for the respective strains. Irrigation water inoculated with generic *E. coli* resulted in a log 4.22/ ml contamination level and the attenuated O157:H7 inoculated water had a log 3.82/ ml level. When spinach foliage was tested by direct plating for either of the inoculated strains, no positive results were obtained. When plant tissues were processed in an enrichment step, only one sample tested positive for either strain (sample taken at 21 days from one of the generic *E. coli* replications).

Soil collected adjacent to the subsurface drip tapes tested positive, by direct plating, for both generic and attenuated O157:H7 *E. coli*^{rif} strains, confirming that viable inoculum was delivered to the subsurface soil area (Fig. 5). Populations of both generic and attenuated O157:H7 *E. coli*^{rif} strains declined rapidly over time. We did not recover either generic or attenuated O157:H7 *E. coli*^{rif} strains from water control plots (Fig. 5).

Gathering data:

Information on field survival of *E. coli* was dependent on extensive sampling of soil, plant, and water samples from replicated field plots. Samples were processed and bacteria cultured in both the UC Cooperative Extension lab in Salinas and the Suslow research lab at the UC Davis campus.

Unexpected outcomes:

One of the most significant and satisfying findings during this project was documenting the extended survival of attenuated *E. coli* O157:H7 strains on crop residue following incorporation into the soil. Though not wholly unexpected, due to the predicted increased survival in association with nutrient-rich spinach, the extent of persistence was in dramatic contrast to all our prior studies on soil survival with both lettuce and spinach. Applied as a simulation of contaminated irrigation water, persistence rarely extended beyond one week. This survival for over 85 days on plant residues is more consistent with studies conducted by researchers in Georgia with the same strains inoculated into soil with manure and compost. This outcome supports the general belief that these pathogen surrogates reasonably reflect the survival behavior under varying levels of environmental stress and warrants further examination.

Collaborations:

Collaborations were an essential and critical component of this study. We obtained outstanding cooperation from the grower and field manager who allowed us to use a commercial field for this study. The several spinach crops were grown exactly like a commercial planting, so we had excellent cooperation from the seed company that provided the seed, planting contractor, fertilizer supplier, and pest control company.

Funding:

The overall project cost more to complete than originally budgeted. In particular, travel and personnel expenses were higher than expected for our UC Davis team. There were no changes to the original budget. Budget funds were used for the technician, SRA, and student assistant salaries. Funds were used to purchase the many supplies needed for collecting samples, processing samples, growing bacterial strains, preparing inoculum, and identifying recovered bacteria. Funds were used for traveling to the experimental site (UC Davis team) and for renting a field vehicle (UCCE Monterey team).

Publications and presentations:

Steven Koike presented current progress and updates on this project at the following meetings:

Update on food safety and leafy green vegetables. California Department of Food and Agriculture, Plant Pest Detection Branch seminar. Sacramento. July 16, 2009.

Survival and internalization of *E. coli* on spinach under field production conditions. Food Safety Innovations Workshop. Produce Marketing Association Fresh Summit Convention. Anaheim. October 3, 2009.

Field sanitation and microbiology. Hartnell College seminar: Food safety for harvest operations. Salinas. October 9, 2009.

Field persistence of foodborne pathogens: Salinas Valley studies with *E. coli*. 2009 Vegetable Crops Continuing Conference. UC Davis. December 10, 2009.

Trevor Suslow presented current progress and updates on this project at the following meetings:

Decoding the Microbiology of Fresh Produce. National Restaurant Association Quality Assurance Executive Study Group. Sacramento. April 1, 2009.

On-Farm Issues and Methods to Minimize the Risk of STECs and other Enteric Pathogens. Third Governor's Conference on Ensuring Food Safety. Lincoln, Nebraska. May 5, 2009.

Central Coast Leafy Greens Grower Food Safety Research Update. Salinas. May 19, 2009.

Overview of Produce and Pathogens. AOAC International 125th Annual Meeting. Washington, D.C. June 26, 2009.

Central Coast Leafy Greens Grower Food Safety Research Update. Santa Maria. August 3, 2009.

E. coli survival and epidemiology. California Leafy Greens Research Board. Mid-term research reports. Seaside. October 13, 2009.

E. coli survival and epidemiology. California Leafy Greens Research Board. Annual research reports. Coalinga. March 16, 2010.

Publication: An abstract was prepared for the Produce Marketing Association Fresh Summit Convention (October 3, 2009) and the upcoming Center for Produce Safety First Annual Research Symposium (to be held June 23, 2010).

Acknowledgments:

We acknowledge the support of the Center for Produce Safety and the leafy greens industry in California. We thank Bonnie Fernandez (Center for Produce Safety) and June Rasmussen (UC Cooperative Extension) for help with project administration. This project would not be possible without our industry cooperators: Steve Adams, David Costa, Gilbert Hernandez. Special thanks to Grace McClellan and Adrian Sbodio for overseeing field and lab operations, and to Joe Sproul and Kevin Vaughn (Wilbur Ellis) for assistance with field pest control. We thank NewStar for donating spinach seed for the field trials. Thanks to the following for their help with this project: Patty Ayala, Christopher Bettiga, Jianlong Bi, Nick Lumbreras, Sharid Kamal, Kat Kammeijer, Ian Kile, Eric Lauritzen, Kim Vu.

Appendix

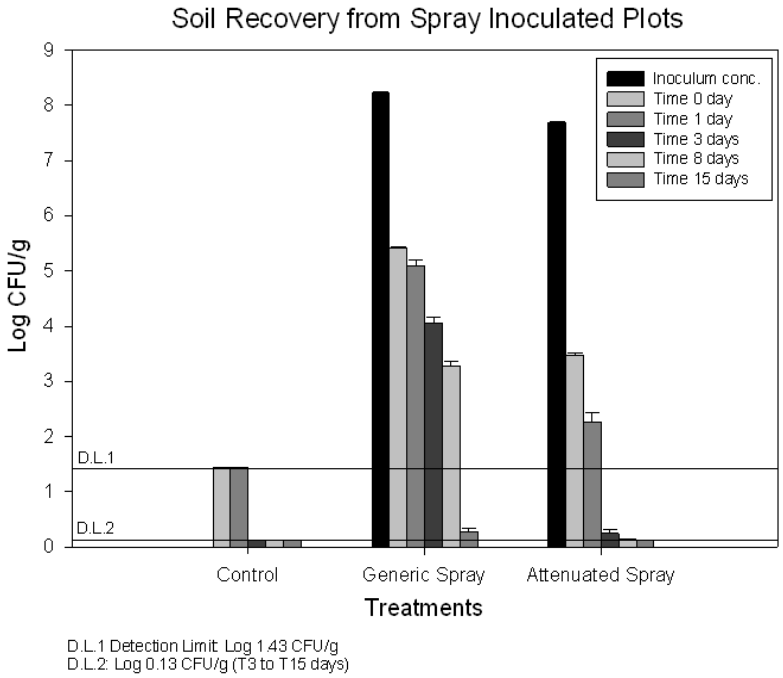

Fig. 1. Recovery of generic and attenuated O157:H7 *E. coli*[rif] strains that were spray inoculated to soil after spinach was seeded but prior to seedling emergence. D. L. = detection limit.

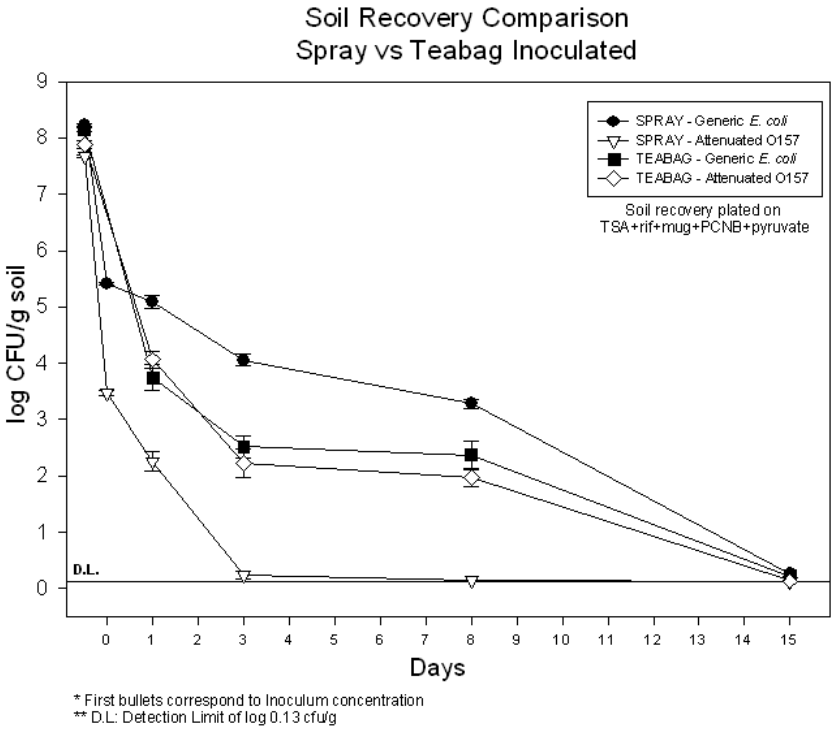

Fig. 2. Recovery over time of generic and attenuated O157:H7 *E. coli*[rif] strains that were inoculated to soil as spray inoculum or inoculum in mesh bags. D. L. = detection limit.

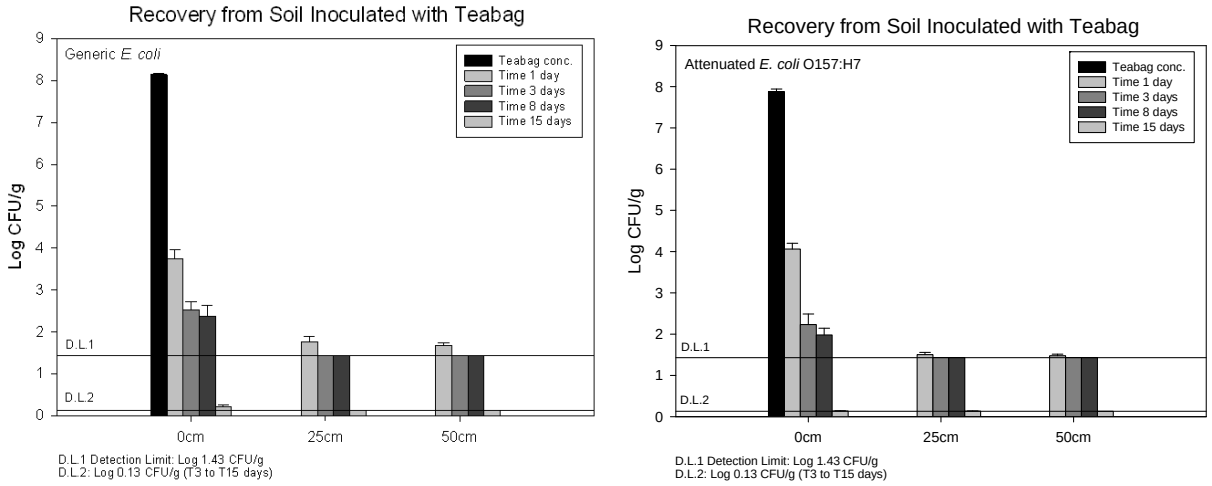

Fig. 3. Recovery of generic and attenuated O157:H7 *E. coli*^{rif} strains that were inoculated to soil by placing mesh bag inoculum onto bed tops after spinach was seeded but prior to emergence. Soil was taken adjacent to the bags (0 cm) and at 25 and 50 cm away. D. L. = detection limit.

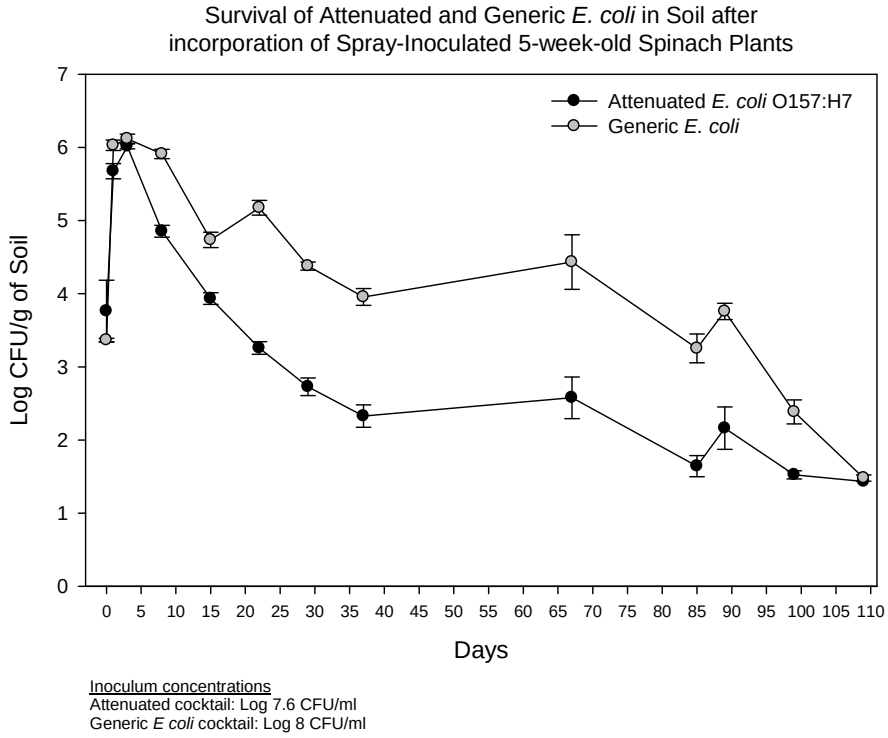

Fig. 4. Recovery over time of generic and attenuated O157:H7 *E. coli*^{rif} strains inoculated to spinach plants at crop maturity. Spinach was then disked and incorporated into the soil.

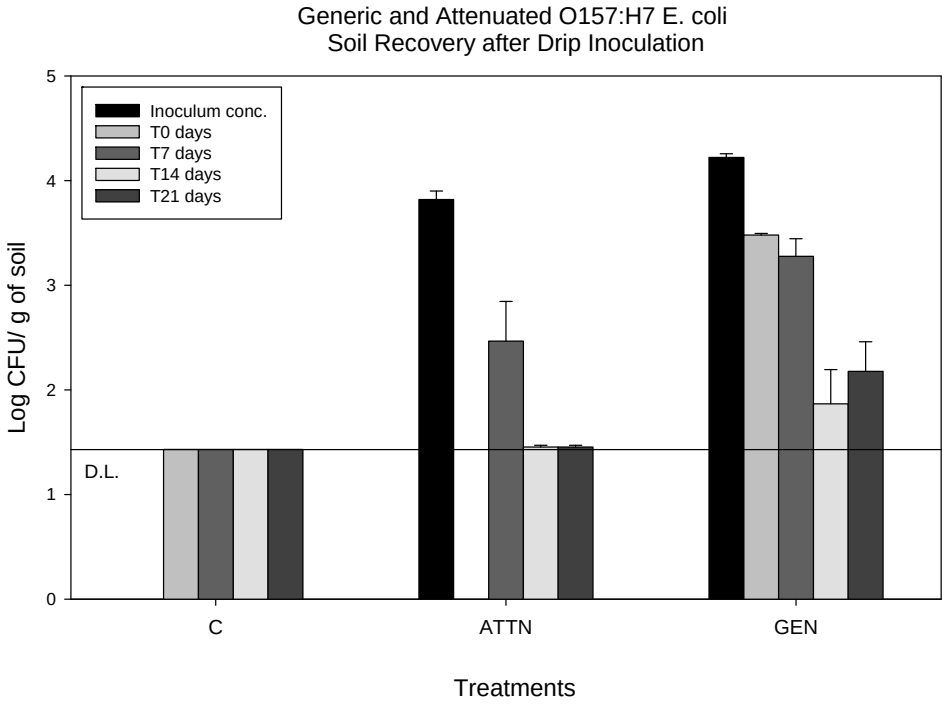

Fig. 5. Recovery of generic and attenuated O157:H7 *E. coli*^{rif} strains inoculated to subsurface soil and spinach roots by injecting inoculum into subsurface drip tape. D. L. = detection limit.