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Survival and Dissemination of *Escherichia coli* O157:H7 on Physically and Biologically Damaged Lettuce Plants

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ABSTRACT

The ecology of the vegetable leaf surface is important to the survival of enteric pathogens. Understanding changes in ecological parameters during the preharvest stages of production can lead to development of approaches to minimize the hazard of contamination of fresh fruits and vegetables with foodborne pathogens. In this study, survival levels of *Escherichia coli* O157 over a 10-day period were compared among traumatically injured, phytopathogen-damaged, and healthy lettuce plants. Leaves from lettuce plants cracked along the central vein, plants infected with *Xanthomonas campestris* pv. *vitians,* and healthy plants were inoculated with *E. coli* O157:H7. The presence of *E. coli* O157:H7 populations on inoculated leaves and noninoculated leaves of these same plants was determined for 10 days. The density of *E. coli* O157:H7 decreased over time on the inoculated leaves regardless of the treatment. The population of *E. coli* O157:H7 remained higher on traumatically injured leaves than on healthy plants ($P < 0.001$). *E. coli* O157:H7 was detected on leaves other than the direct inoculation site of the enteric pathogen in all three treatments groups. Preharvest damage, especially that caused by traumatic injury, impacted the survivability of *E. coli* O157:H7. Maintaining healthy plants and minimizing physical damage around the time of harvest might improve the safety of fresh produce.

Although the phyllosphere of plants presents a harsh environment for enteric pathogens to survive, leafy green vegetables have been implicated in several outbreaks of foodborne illnesses *(19).* This location is subject to rapid and large fluctuations in temperature, humidity, and osmotic pressure that restrict bacterial proliferation on leaf surfaces *(24).* In addition, competition for limited water and nutrients makes it difficult for transient microorganisms, such as enteric pathogens, to survive on leaf surfaces *(13).* However, damage to leafy vegetables may alter the phyllosphere microenvironment, aiding in both the attachment and survival of enteric pathogens, such as *Escherichia coli* O157:H7. For example, mechanically damaged wounds of apples supported 1- to 3-log increase in growth of *E. coli* O157 compared to nondamaged apple tissues *(10).* Likewise, Seo and Frank *(17)* showed that *E. coli* O157:H7 was more likely to attach at cut sites on lettuce leaves than at intact sites. Infection of plants, and subsequent lesion formation, can also alter the microenvironment of leaf surfaces and aid in the attachment and survival of foodborne pathogens *(2).* Some fungi enhance the proliferation of foodborne pathogens on fruits and vegetables *(15, 22). Salmonella* was more common on fresh produce that had a bacterial soft rot infection than on healthy fresh produce *(23).* Despite these advances in knowledge, the extent to which biotropic phytopathogenic bacteria enhance the survivability of *E. coli* O157:H7 on leafy vegetables has not previously been reported.

Foodborne pathogens that can disseminate from a contamination site may be able to find nutrient pools to aid in their survival. Dissemination of *E. coli* O157:H7 from the root of plants to aerial parts of the plants has been shown from direct inoculation of the leaf and from the soil. Cooley and colleagues *(4)* showed that on sterile *Arabidopsis thaliana* plants, *E. coli* O157 moved from an inoculation site on the root to the shoot of the plant. *E. coli* O157:H7 can also translocate from contaminated soil to the edible portions of lettuce plants by dissemination from the root *(18).* The potential for foodborne pathogens contaminating the aerial parts of plants to disseminate systemically and proliferate in areas with protection, e.g., biofilms, and nutrient-rich pools has not been fully explored.

Damaged plant tissues exude carbohydrates and proteins that can be used as nutrient sources by microorganisms living on leaf surfaces. In contrast, injured plant cells can release antimicrobial agents that can inhibit microbial populations *(1).* Thus, the balance between factors favoring microbial growth and suppression are in delicate balance. Nutrients are not homogeneously distributed on leaf surfaces, and thus, motility of the microorganism is important for survival *(11).* Nutrient pools can also differ on individual leaves on the same plant. For example, more leached nutrients are available on the surface of older leaves than young leaves *(21).* The objective of this study was to determine the extent to which phytopathogen-induced and

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traumatic tissue damage of lettuce leaves influenced the survival and dissemination of *E. coli* O157:H7 in edible portions of the plant.

MATERIALS AND METHODS

Bacterial strains. *Xanthomonas campestris* pv. *vitians* strain 701a, which is resistant to ampicillin, was isolated from lettuce plants *(16),* and a green fluorescent protein–expressing, ampicillin-resistant strain of *E. coli* O157:H7 strain B6914 *(5)* that does not contain Shiga toxin I or II genes was maintained on Luria agar (Acumedia, Lansing, Mich.). A single colony of *X. campestris* pv. *vitians* was used to inoculate 7 ml of Luria broth, and the culture was incubated at 28°C for 48 h. *E. coli* O157:H7 was inoculated in the same way but incubated at 37°C for 24 h. One milliliter of *X. campestris* pv. *vitians* culture was transferred to 300 ml of Luria broth and incubated for an additional 48 h at 30°C and was used to inoculate lettuce plants.

Lettuce treatments. Little Caesar lettuce seeds (W. Atlee Burpee and Co., Warminster, Pa.) were hot-water treated using the procedure described by Miller and Lewis Ivey *(14),* to remove potential bacterial and fungal contaminants. The seeds were direct-planted into 288-cell trays containing Fafard Super-Germinating Mix (Conrad Fafard, Aguwam, Mass.) and grown under greenhouse conditions (22 to 25°C) without fertilizer application until the four-leaflet stage (~3 weeks). Seedlings were transferred to 15-cm-diameter pots containing sterile Wooster sandy loam soil and placed into three separate greenhouses with automated misting capabilities. In three greenhouses, all plants were maintained at 100% relative humidity in the greenhouses by misting with deionized water for 36 s, five times per hour. The plants had a weekly fertilizer application until they were 7 weeks old with Peter's 20-20-20 applied at a rate of 120 g/liter. Treatments were applied after the plants were 7 weeks old. A total of 36 plants were used during the first experimental replicate. The entire experimental protocol was repeated once with an additional 27 plants.

In the first greenhouse, 12 plants were spray inoculated with *X. campestris* pv. *vitians* strain 701a (approximately 10^8 CFU/ml) until runoff. Misting in all greenhouses was discontinued once necrotic lesions were observed (10 to 14 days after inoculation) on the *X. campestris*–infected plants. The lesions were confirmed to be caused by *X. campestris* by identifying bacterial streaming from the lesion. At that time, the outer leaves of 12 plants (approximately six leaves/plant) in a second greenhouse were mechanically damaged by bending the leaves in half widthwise to crack the central vein and sprayed with distilled water (to simulate the conditions used for pathogen infection). In a third greenhouse, 12 plants were only sprayed with sterile deionized water until runoff. Twenty-four hours after mechanical injury, all plants were moved to one of three biosafety level 2 growth chambers. Immediately after being placed in the growth chambers, sterile Whirl-Pak (Nasco, Modesto, Calif.) bags cut at the bottom and inverted were used to cover the five to seven interior leaves on each lettuce plant and separate them from the *E. coli* O157 inoculation that was to follow. The bags were cinched at the base of the crown, and the cut end was open to the air (Fig. 1). All growth chambers were maintained on a 12-h photo period with 25°C daytime temperatures and 20°C nighttime temperatures. Relative humidity was maintained at 55%.

***E. coli* O157:H7 inoculation.** All outer, uncovered leaves on each lettuce plant (about five to seven leaves per plant) were evenly swabbed using a cotton applicator dipped in an overnight culture of *E. coli* O157:H7 strain B6914 (approximately 10^7 CFU/ml). A new applicator was used for each plant. The leaves were air dried in the growth chamber for 1 h before data were collected. In addition to the *E. coli* O157:H7–inoculated plants, each chamber contained two additional plants that were not inoculated with *E. coli* O157:H7.

FIGURE 1. *Isolation of internal leaves from outer inoculated leaves. The closed end of the Whirl-Pak bag was cut with a sterile scalpel and is at the top in this picture. The yellow tie end (not visible) was placed over the isolated leaves and secured at the base of the plant.*

***E. coli* O157:H7 enumeration.** To determine the initial inoculation dose, one outer leaf was removed from each of 10 plants per treatment, and the individual leaf was placed in a sterile Whirl-Pak bag and weighed. Sterile buffered peptone water (Acumedia) was added to each bag at a 1:10 (wt/vol) dilution and stomached (five strokes per s; IUL Instruments, Barcelona, Spain) for 90 s. The homogenate was further diluted (10-fold serial dilutions) and plated onto Luria agar plates amended with cycloheximide (100 mg/ml; Sigma-Aldrich, St. Louis, Mo.) and ampicillin trihydrate (100 mg/ml; Fisher Biotech, Subiaco, Wash.); this medium is hereafter referred to as LAcyc-amp. The plates were incubated at 30°C for 48 h. Colonies fluorescing green were counted. This procedure was repeated on days 3, 6, and 10 after inoculation.

Enumeration of *E. coli* O157:H7 on noninoculated lettuce leaves. Interior leaves that were covered with a Whirl-Pak bag, 75 mm above the soil surface of three plants, in each treatment group were aseptically removed on days 3, 6, and 10 with a sterile razor blade. Sterile buffered peptone water was added to the leaves in a 1:1 dilution (wt/vol) and ground with a tissue grinder. The ground leaves were further macerated in a laboratory stomacher (five strokes/s) for 60 s. Serial dilutions were plated on LAcyc-amp medium and incubated for 48 h at 30°C. Fluorescent green colonies were counted.

Each sample of the tissue homogenate (inoculated and noninoculated leaves) was also enriched for detection of low populations of *E. coli* O157 by adding 40 ml of sterile buffered peptone water to the remaining solution in each Whirl-Pak bag and incubating them at 37°C for 24 h. Seventy-five microliters of the overnight enrichment was streaked onto one LAcyc-amp medium and incubated at 37°C for an additional 24 h. The presence of fluo-

TABLE 1. *Average* E. coli *O157 log counts at each time interval for each treatment (10 plants per treatment per time interval)*

Time (days)	*E. coli* O157 log counts per treatment on lettuce plants that were:		
	Undamaged	Physically damaged	*X. campestris* infected
0	6.48	5.53	5.48
3	6.16	5.47	4.94
6	5.14	4.90	4.75
10	3.29	5.33	4.19

rescent green colonies from the streaked plate indicated that the sample was positive for *E. coli* O157.

Enumeration of *X. campestris* pv. *vitians* on infected leaves. Colonies typical of *X. campestris* (yellow colonies similar to those from the inoculum plate) were counted on the LAcyc-amp plates from the *Xanthomonas*-infected plants after 72 h at 30°C.

Statistical analyses. A repeated-measures analysis of variance was used to determine the effects of treatment and time on *E. coli* O157 counts (SAS version 9.1 software, SAS Institute Inc., Cary, N.C.). Subsequently, significant differences in the bacterial counts among the three treatments (within day) and among sample dates (within treatments) were identified by Tukey's test. The relationship between populations of *X. campestris* and *E. coli* O157:H7 counts on *Xanthomonas*-infected leaves was determined by Pearson's correlation using SAS (version 9.1) software. Differences between treatments for the number of plants with noninoculated leaves that were positive for *E. coli* O157 at each time internal were determined by the chi-square test using SAS (version 9.1).

RESULTS

While *E. coli* O157:H7 was added to each leaf in a similar manner, the initial counts were higher on the control plants than on both the *Xanthomonas*-infected and the physically damaged lettuce plants ($P < 0.002$). In the statistical model, significant interaction effects were noted between treatment and day; thus, the effect of treatment was analyzed independently within day, as well as between-day within treatment. No significant differences in *E. coli* O157:H7 counts between the three treatments were identified on days 3 and 6 (Table 1). However, the physically damaged plants had significantly higher *E. coli* O157 counts on day 10 than on the control plants ($P < 0.001$). The *E. coli* O157:H7 counts on *Xanthomonas*-infected plants were not significantly different from the damaged or control plants on day 10 (Table 1). The average *X. campestris* population on the infected leaves was 7.25 log CFU/g leaf and did not significantly change over the 10 days of the study. When the log counts of *X. campestris* and *E. coli* O157:H7 were compared (Table 1), no correlation was found ($P > 0.05$) between the populations.

A decrease in absolute numbers of *E. coli* O157:H7 was observed on the control plants after 6 days ($P = 0.01$), and the numbers continued to decrease significantly over the 10-day period. By day 10, *E. coli* O157 counts had decreased about 3 log CFU/g on the control plants ($P < 0.001$). The *E. coli* O157 counts on the *Xanthomonas*-infected plants also had decreased significantly by day 10 ($P = 0.008$). The physically damaged plants had no significant change in *E. coli* O157 counts over the 10 days ($P > 0.5$). Given the differences in counts immediately after inoculation, the change in population numbers were also compared among the three treatments over the 10 days. The magnitude of decrease from day 1 to day 10 was greater in the control plants than in the *Xanthomonas*-infected and mechanically damaged lettuce plants ($P < 0.001$) (Fig. 2).

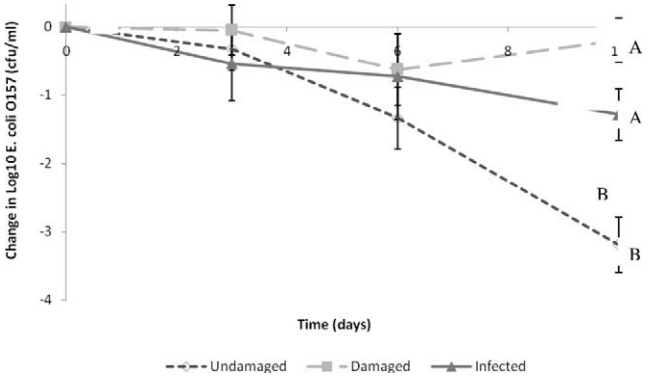

FIGURE 2. *Change in log-transformed* E. coli *O157 counts between time 0 and days 3, 6, and 10. The values were determined by subtracting the log counts for each plant at each time period from the count at time 0 and finding the average for each treatment given equation log* N/N_0*, where* N *is the count at time* t *and* N_0 *is the initial count.*

None of the direct-plated samples from noninoculated leaves yielded bacterial colonies above the threshold of detection (approximately 10 CFU/g). Enrichment cultures were positive for *E. coli* O157:H7 for all three treatments by day 3. On day 3, each set of noninoculated leaves for each treatment was positive for the presence of *E. coli* O157:H7 (Fig. 3). On days 6 and 10 no significant differences between healthy plants and damaged plants in the

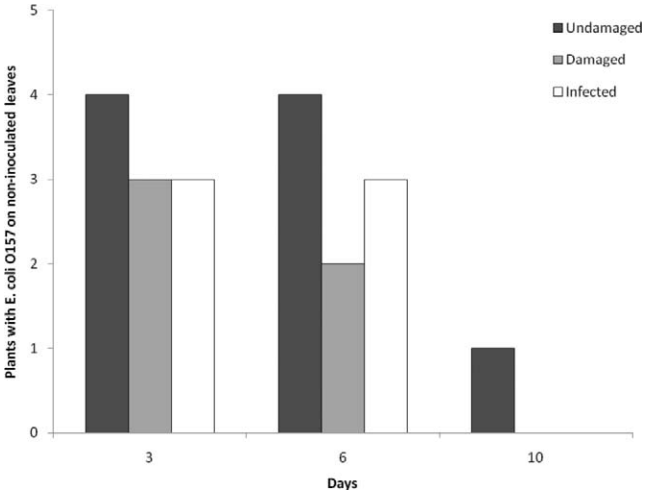

FIGURE 3. *Number of plants in each treatment with* E. coli *O157 present on noninoculated leaves at three different time periods. There were no significant differences between the treatments at each time period* ($P > 0.05$)*. Six plants were used per treatment per time interval.*

number of plants positive for *E. coli* O157:H7 on noninoculated leaves were identified ($P > 0.05$). By day 10, only 1 plant (undamaged) of all 18 plants tested was culture positive for *E. coli* O157:H7 at a noninoculated location.

DISCUSSION

Over the 10-day experiment reported herein, *E. coli* O157 populations declined quickly and greatly (3 log) on healthy lettuce plants, but survival was enhanced on plants damaged by *X. campestris* and mechanical damage. Clearly, damaged leaves release more nutrients onto the leaf surface *(21).* Jablasone and colleagues *(9)* demonstrated that *E. coli* O157 populations decreased on the surface of alfalfa seedlings as a function of nutrient availability. Likewise, the larger amount of nutrients expected on damaged leaves in this study may have contributed to the observed increased survival of *E. coli* O157:H7 in the phyllosphere.

Plant tissues can be disrupted by even the slightest contact. For example, Tukey and Morgan *(21)* showed that simply brushing squash with a cloth can cause nonpermanent microscopic damage that increases leaking of nutrients. The method used in this project to inoculate *E. coli* O157:H7 likely caused some damage to the epidermal hairs on leaves. All treatments, even the control, likely had some increase in the leaching of nutrients, possibly enhancing the survival of the organism over what might occur on an undisturbed plant tissue. However, when plants are contaminated in the field with foodborne pathogens they are likely disrupted by the source of the contamination, be it a deposit of manure, insect predation, the hoof or foot of an animal, or the hands of a worker *(1).*

The role of mechanical and biotrophic tissue damage on the survival of *E. coli* O157 in apples has been evaluated. In one study, mechanical damage was shown to increase proliferation on apples *(10).* In contrast, infection with the biotrophic phytopathogen *Glomerella cingulata* did not increase the numbers of *E. coli* O157:H7 in apples *(15).* No net increases in bacterial numbers were observed in these experiments, only prolonged survival in damaged tissues compared to healthy tissues.

In this study, plants were maintained at 55% relative humidity and between 20 and 25°C. These conditions are not ideal for enteric pathogen proliferation. *Salmonella* had higher populations on cilantro leaves when grown at 37°C than at 22°C and decreased in population size when incubated at 60% relative humidity *(2). Salmonella* Montevideo on tomatoes stored at 60% relative humidity and 22°C did not increase over a 10-day period. As the relative humidity increased incrementally to 97%, *Salmonella* proliferated on the tomato surface *(7).* However, these persistent high levels of humidity are not typical of environmental conditions under which vegetables are produced in the United States. For the most part, the microenvironment on plants is unfavorable for microorganism survival because of several environmental factors, including fluctuating relative humidity and temperature *(12).* Lesions on *Xanthomonas*-infected plants and wounds on the mechanically damaged plants improved survival of *E. coli* O157:H7 in this study. Brandl and Mandrell *(2)* demonstrated that leaf lesions could provide protection from environmental stress. *E. coli* O157:H7 attaches preferentially to cut or injured surfaces and could penetrate into the cuts, protecting the pathogen from the environment *(17, 24).*

Some epiphytic microorganisms have been shown to be beneficial and others antagonistic to the survival and proliferation of *E. coli* O157:H7 and other pathogens *(3, 11).* In a previous study, the presence of *Pseudomonas syringae* did impact the persistence of *E. coli* O157:H7 on lettuce when the two microorganisms were simultaneously coinoculated on plants *(6).* The present study showed that *X. campestris* was not antagonistic to *E. coli* O157:H7, but it is uncertain if its presence had a beneficial effect. Although *E. coli* O157:H7 counts did not increase in plants infected with *X. campestris,* neither did *E. coli* O157:H7 decrease as much as on healthy plants. It was not determined if the increased survival was due directly to a factor secreted by *X. campestris* or if it was only indirectly attributable to the change in microenvironment of the necrotic lesions. We hypothesize that the necrotic lesions formed by *Xanthomonas* infections are essential for the improved survival of *E. coli* O157:H7 and the differences observed were not simply due to the presence of the phytopathogen. The injured surfaces where cuticle damage has occurred and water is available are important factors in the binding of *E. coli* O157:H7 to leaf surfaces *(24).* Furthermore, Seo and Frank *(17)* showed that cut edges had more bacterial attachment.

Since the plants in this experiment were not sterile, the flora could contain other microorganisms that were antagonistic to *E. coli* O157:H7 and nutrient availability could have been limited on the noninoculated leaves, since these were the younger leaves on the plants. The inability to compete with the resident species for nutrients or the inability to find nutrients were possible causes of the decrease in the presence of *E. coli* O157:H7 on the noninoculated leaves over time. The presence of *Enterobacter asburiae* can decrease *E. coli* O157:H7 populations on lettuce leaves *(3).* In addition, young growing leaves leach fewer nutrients than older leaves *(20).* Since nutrient pools are sporadic across the leaf surface, bacteria reside at loci where leached nutrients are plentiful *(12).* The health and growing conditions of plants impact the survival *E. coli* O157 on lettuce plants. *E. coli* O157:H7 is not reported to penetrate through the cuticle on plant surfaces *(24),* so it is possible that damage caused by either physical or biological means provides a contact site for *E. coli* O157:H7 to attach and survive.

The presence of *E. coli* O157:H7 on noninoculated leaves 3 days after inoculation of other leaves on the same plant showed that dissemination occurred for all three treatments without detectable differences between the treatments. Solomon et al. *(18)* showed that *E. coli* O157:H7 disseminated to aerial parts of lettuce plants from the root system. It was not determined whether the dissemination of *E. coli* O157:H7 in these plants occurred through migration on the surface or via internalization. Regardless of the route of contamination and the specific factors governing the survival at these distal sites, it is important to note that even with large inoculation doses, *E. coli* O157:H7 was detect-

able only at sites other than the inoculation site by enrichment culture procedures only and only for short periods of time on most plants. As such, the contamination of sites that have not had direct contact with the microorganism poses a much smaller food safety risk than those areas that have had direct microbial contamination. Removal of outer leaves, those visibly contaminated with soil, or those that have been traumatically injured prior to consumption will reduce the likelihood of foodborne pathogen exposure. Further understanding of the factors that contribute to persistence, survival, and dissemination could help determine when produce is most susceptible to foodborne pathogen contamination and help provide practical approaches to prevent foodborne disease.

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