Research Paper

Effect of Route of Introduction and Host Cultivar on the Colonization, Internalization, and Movement of the Human Pathogen *Escherichia coli* O157:H7 in Spinach

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ABSTRACT

Human pathogens can contaminate leafy produce in the field by various routes. We hypothesized that interactions between *Escherichia coli* O157:H7 and spinach are influenced by the route of introduction and the leaf microenvironment. *E. coli* O157:H7 labeled with green fluorescent protein was dropped onto spinach leaf surfaces, simulating bacteria-laden raindrops or sprinkler irrigation, and survived on the phylloplane for at least 14 days, with increasing titers and areas of colonization over time. The same strains placed into the rhizosphere by soil infiltration remained detectable on very few plants and in low numbers (10^2 to 10^6 CFU/g fresh tissue) that decreased over time. Stem puncture inoculations, simulating natural wounding, rarely resulted in colonization or multiplication. Bacteria forced into the leaf interior survived for at least 14 days in intercellular spaces but did not translocate or multiply. Three spinach cultivars with different leaf surface morphologies were compared for colonization by *E. coli* O157:H7 introduced by leaf drop or soil drench. After 2 weeks, cv. Bordeaux hosted very few bacteria. More bacteria were seen on cv. Space and were dispersed over an area of up to 0.3 mm^2. The highest bacterial numbers were observed on cv. Tyee but were dispersed only up to 0.15 mm^2, suggesting that cv. Tyee may provide protected niches or more nutrients or may promote stronger bacterial adherence. These findings suggest that the spinach phylloplane is a supportive niche for *E. coli* O157:H7, but no conclusive evidence was found for natural entry into the plant interior. The results are relevant for interventions aimed at minimizing produce contamination by human pathogens.

To address the rise in the incidence of foodborne illness in the United States, it is critical to better understand the routes of human pathogen contamination of field-grown produce and the nature of the interactions between human pathogens and plants (10). Numerous routes of crop contamination exist (4), and plant-microbe relationships may be influenced by the plant species and cultivar, the pathogen, the means of pathogen introduction, and environmental features, including the nature of the phylloplane niche. Although some researchers have predicted that the plant environment would be hostile to enteric pathogens (4), other research groups (13, 14, 16, 21, 23, 24, 26, 27) have reported that both *Escherichia coli* O157:H7 and *Salmonella enterica* can invade plant interiors and move systemically. Both the surfaces and interior spaces of plants are suitable and supportive habitats for many plant pathogens, including enterobacteria in the genera *Erwinia*, *Pantoaea*, *Enterobacter*, and *Serratia*. Recent work (17) has indicated that *Serratia marcescens*, the causal agent of yellow vein disease of cucurbits (7), colonizes different plant tissues and translocates systemically within different transport tissues (xylem or phloem), depending upon its route of infection. Such evidence raises the question of whether some pathogens adapt to multiple plant niches, possibly utilizing different colonization or virulence mechanisms depending upon the plant microenvironment. The objective of this study was to test the hypothesis that the relationship(s) formed in the field by *E. coli* O157:H7 with spinach are similarly influenced by the route of introduction and by the nature of the plant surface microenvironment. The hypothesis was tested by monitoring bacterial location, density, and movement after introduction via four different routes, each designed to mimic a different natural pathway of contamination, on three different spinach cultivars with different leaf surface morphologies. The results of this work provide insights useful in the design of strategies to minimize plant contamination by human pathogens.

MATERIALS AND METHODS

Plant growth. *Spinacea oleracea* cv. Tyee, a savoy-leaf spinach variety, was used for all experiments. For the cultivar comparison study, the hybrid smooth-leaf cv. Space and the delicate cordate-leaf cv. Bordeaux, which is a common constituent in popular bagged “spring mixes,” were added. Seeds were sown in Sungro potting mixture (Park Seed Wholesale, Inc., Greenwood, SC), and plants were fertilized with Miracle Gro fertilizer twice weekly and maintained in growth chambers (12-h light:12-h dark photoperiod at 24°C). Plants were inoculated at the four-leaf stage except where indicated.

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**Bacterial strains and inoculum preparation.** A mixture of five strains of green fluorescent protein (GFP)–expressing *E. coli* O157:H7 (provided by M. Doyle, Center for Food Safety, University of Georgia, Griffin) was used in all experiments. The strains were ATCC 43888 (human feces), EO122 (cattle), K3995 (spinach), K4492 (clinical isolate from lettuce), and F4546 (alfalfa sprouts) (29). Before use, the bacteria were subcultured three times (1% inoculum, incubated at 37°C for 18 h) in tryptic soy broth supplemented with 100 μg/ml ampicillin. Culture volumes containing equal amounts of each strain were mixed and diluted in phosphate-buffered saline (PBS), pH 7.4, to a final concentration of 10⁶ CFU/ml.

**Comparison of inoculation methods.** Spinach plants with four true leaves (approximately 4 weeks old) grown in commercial potting mixture in 4-in. (10-cm) pots in the growth chamber were inoculated as described by Luo (17). The four inoculation methods described below were designed to simulate different natural pathways by which *E. coli* O157:H7 might encounter spinach seedlings in the field and to explore possible resulting host-microbe interactions. Inoculated plants were placed in a clear Plexiglas box (24 by 24 by 48 in. [61 by 61 by 122 cm]) with top or side panel ventilation under fluorescent tube lights (40 watts; Plant and Aquarium, General Electric Co., Fairfield, CT) until used. For each inoculation treatment (see below for descriptions), a total of 87 spinach plants (27 for microscopy and 60 for enumeration) were inoculated with *E. coli* O157:H7. Fifty-four spinach plants (18 for microscopy and 36 for enumeration and BAX PCR assay) were inoculated with PBS (control). The presence, numbers, and locations of bacteria were assessed beginning on day 0 by plate counts and enrichment culture and at 1 day postinoculation (dpi) by confocal laser scanning microscopy (CLSM). Additional samples were assessed by CLSM, plate counts, enrichment culture, and BAX PCR assay at 7 and 14 dpi. The time between inoculation and day 0 sampling (for plate counts and enrichment culture) was 0 to 3 h. Each treatment was replicated three times in separate experiments. In each treatment and each replication, 15 plants inoculated with *E. coli* O157:H7 were examined using plate counts and BAX PCR assay (45 plants total), and 9 plants inoculated with *E. coli* O157:H7 were examined using CLSM (27 plants total). For each replication, 15 control plants were inoculated with PBS (45 plants total), of which 9 were examined using plate counts and BAX PCR assay and 6 were examined using CLSM. A fourth replication was included for plate counts and BAX PCR assay only.

**Surface sterilization.** Spinach plants or plant parts used for culturing and BAX PCR assay were weighed and then surface sterilized in 70% ethanol (30 to 60 s), 10% sodium hypochlorite (1 min), and three washes (1 min each) of sterilized distilled water. The roots of plants inoculated by soil drench were rinsed in sterilized water to remove soil before surface sterilization.

**Leaf drop inoculation.** To simulate bacterial introduction through contaminated sprinkler irrigation water, rain splash from contaminated soil, insect excreta or salivation, or other droplet-based sources, the bacterial suspension was dropped onto the surface of spinach leaves. Four leaves per plant were marked with indelible ink (for microscopy) or by pressure from a pipette tip (for enumeration and BAX PCR assay) at six spots, three on each side of the midrib. The abaxial leaf surface usually has significantly more stomata and is generally a more hospitable environment for phylloplane residents than is the adaxial surface. Plants were divided into two groups. For plants in one group, a 10-μl drop of inoculum (mixed *E. coli* O157:H7 strains, 10⁶ cells per ml) was spotted at each mark and allowed to dry. Each dried spot occupied an area of approximately 2 mm², and the total inoculum per leaf was 1.2 × 10⁶ bacterial cells. Plants of the other group received PBS drops as controls. The leaves were processed for bacterial enumeration immediately (within 3 h) and at 7 and 14 dpi and were processed for CLSM at 1, 7, and 14 dpi.

**Soil drench inoculation.** The soil drench treatment was designed to determine whether *E. coli* O157:H7, which could be present in contaminated soil near spinach roots, would be able to colonize root surfaces or enter into root tissue. A 25-ga, 1.5-in. (3.8-cm) needle fitted onto a syringe was inserted 1 to 2 in. (2.5 to 5 cm) below the soil surface, and finger pressure was used to deliver 10 ml of bacterial suspension (10⁶ cells per ml) or PBS (control) into the root zone. Both the primary root and the leaves of the plant were analyzed. Bacterial presence and numbers were monitored in all replications through colony counts and BAX PCR assay. Enumeration was done immediately and at 7 and 14 dpi. In the first replication, plant roots and stems were sectioned and examined by CLSM at 1, 7, and 14 dpi. In the second and third replications, bacteria were monitored at the same time points by confocal microscopy of whole plant mounts and examination of surrounding soil samples.

**Stab inoculation.** A 10-μl droplet of bacterial suspension (10⁶ cells per ml, mixed strains of *E. coli* O157:H7) or PBS (control) was placed on the plant surface at the crown (the root-stem junction), and a small multiprong inoculating fork (8) was stabbed 10 times through the droplet into the interior plant tissue. At 1, 7, and 14 dpi, a series of 15 to 20 thin, hand-cut cross sections were taken at the point of inoculation and on either side of the point of inoculation extending as far as 1 cm above and 1 cm below the point of inoculation. These sections were examined by CLSM. The plants were monitored for the presence and growth of bacteria by plate counts (immediately and 7 and 14 dpi) using the remainder of the plant from which the three samples had been excised.

**Pressure inoculation.** To determine whether bacteria present within the spinach leaf interior, regardless of the route of entry, can survive and/or move within the plant, bacterial inoculum (10⁶ cells per ml, mixed strains of *E. coli* O157:H7) was forced through stomatal openings into the spinach leaf intercellular spaces by pressing the orifice of a needleless syringe against the abaxial leaf surface and applying slow hand pressure on the plunger until the appearance of a spreading, water-soaked area below the epidermis confirmed inoculum infiltration into the intercellular spaces. One milliliter of inoculum (0.25 ml in each of four places) was introduced per leaf, and two leaves of each plant were inoculated. Bacterial survival and growth within the internal tissues were monitored using plate counts and CLSM at 1, 7, and 14 dpi.

**Colonization of *E. coli* O157:H7 on different spinach cultivars.** The susceptibility of different spinach cultivars and leaf surface morphologies to colonization by *E. coli* O157:H7 was assessed using savoy-leaf cv. Tyee along with the semi-smooth-leaf cv. Space and the cordate-leaf cv. Bordeaux. A total of 72 plants (18 per replication) of each cultivar were inoculated with *E. coli* O157:H7 by the leaf drop method, and 45 plants (15 per replication) received PBS (control). A total of 45 plants (15 per replication) of each cultivar treated with *E. coli* O157:H7 and 27 plants (9 per replication) treated with PBS were examined by plate counts and BAX PCR assay (immediately and at 7 and 14 dpi). A total of 9 (3 per replication) *E. coli* O157:H7–treated plants and 18 (6 per replication) PBS-treated plants of each cultivar were examined by scanning electron microscopy (SEM) and CLSM at...
1 and 14 dpi. A fourth replication was included for bacterial enumeration only.

The same experiment was repeated using the soil drench inoculation method with the same numbers of plants, treatments, and replications.

**Enumeration of E. coli O157:H7 on or in inoculated spinach plants.** The methods used for E. coli O157:H7 detection and enumeration were as previously described (9) with slight modifications. To enumerate bacteria inside the plant, whole plant samples taken at 0, 7, and 14 dpi were surface sterilized as previously described and then macerated with sterile mortars and pestles. Appropriate dilutions were plated onto violet red bile agar (VRBA) supplemented with 100 μg/ml ampicillin and incubated at 37°C for 18 h. To maximize the chances of detecting bacteria present below the detection limit for enumeration, macerated samples also were by inoculation into GN Hajna (Difco, Becton Dickinson, Sparks, MD) broth supplemented with 50 ng/ml cefoxitin, 10 mg/ml cefsulodin, and 8 mg/ml vancomycin for 18 h at 37°C. The resulting enrichment cultures were streaked onto sorbitol MacConkey agar supplemented with 100 μg/ml ampicillin and onto CHROMagar O157 supplemented with 100 μg/ml ampicillin. All antibiotics were from Sigma-Aldrich (St. Louis, MO). Data were analyzed by analysis of variance (ANOVA) with the statistical analysis SAS 9.1 software (20). Means were separated by Tukey's test.

**Detection of E. coli O157:H7 using the BAX PCR assay.** The presence and identity of bacteria in the enriched samples was confirmed by PCR assay using a Quilicon BAX (R) System Q7 instrument (DuPont Corp., Wilmington, DE) programmed to optimize specific detection of E. coli O157:H7, following the manufacturer’s instructions.

**Tissue preparation for microscopy.** Leaf pieces were fixed as described previously (17) in 4% paraformaldehyde and then rinsed and stored in PBS at 4°C until used. Before examination, pieces were incubated in 0.2 M phosphate buffer at 4°C for 15 min.

SEM was used to examine plants inoculated by the leaf drop method in one experiment. For SEM, samples were moved to 1% osmium tetroxide at 4°C for 1 h, washed (15 min at 4°C) in 30% ethanol, and dehydrated by 15-min incubations in a graded series of chilled ethanol solutions: 50, 70, 95, 100, 100, and 100% (3). The tissues were dried (20 min) in a CDPD30 critical point dryer (BAL-TEC), coated with gold (Balzers Union MED 010 Au/Pt coater, BAL-TEC), and examined using a Quanta 600 field-emission gun environmental SEM (FEI Company, Hillsboro, OR). For each experimental replication and for each spinach cultivar, three E. coli O157:H7–inoculated plants (nine total) and two PBS-inoculated plants (six total) were sampled at 1 and 14 dpi. From each sampled plant, 12 square leaf pieces (0.5 cm², 6 per leaf) within the area of inoculation were excised and pooled in 4% paraformaldehyde and then macerated in 4% paraformaldehyde in PBS, and a leaf piece was removed from one of the nine resulting tubes for examination by SEM.

A Leica TCS SP2 system attached to a Leica DMRE confocal laser scanning microscope (Leica Microsystems, Bannockburn, IL) was used for CSLM. A 488-nm excitation wavelength, produced by krypton-argon lasers, was used to monitor GFP expression. For each inoculation treatment in each replication and for each of the three spinach cultivars, 2 E. coli O157:H7–inoculated leaf pieces (6 total) and 2 PBS-inoculated leaf pieces (6 total) were sampled at 1 dpi and 6 E. coli O157:H7–inoculated leaf pieces (18 total) and 2 PBS-inoculated leaf pieces (6 total) were sampled at 14 dpi. Pieces were obtained from the same sample pool prepared for SEM. Optical sections were taken at 0.3- to 2-μm intervals, and images were processed using Photoshop CS version 7.0 software (Adobe Systems Inc., San Jose, CA).

For both SEM and CSLM, 10 to 15 microscopic fields (0.06 mm² each for CSLM, and of various sizes for SEM) per leaf piece were examined.

**Detection of internalization after soil drench inoculation using real-time PCR assay.** Total genomic DNA from the upper leaves of spinach plants inoculated via soil drench with bacteria or buffer were extracted at 1, 7, and 14 dpi using the DNasey Plant Maxi Kit (Qiagen, Valencia, CA), and the bacterial genomic DNA was extracted using the DNasey Blood and Tissue Kit (Qiagen). In each replication, three plants each of cvs. Tyee, Space, and Bordeaux received E. coli O157:H7 and two received PBS. Primers GTAAGTTACACTATAAAAGCACCCTGC-F and TCTGTGGGATGGTAATAAATTITTTT-G (mean Ct, 17.8) for the E. coli gene eae (22) were used to detect extracted bacterial genomic DNA as one positive control, and primers CGTCCGATCCAGATTATCCA-F and CAACATCGGATATATATAAGCGCAAACT-TG-R (mean Ct, 18.1) for the plant cytomegalo virus gene cox (28) served as the plant genomic DNA internal control. Real-time PCR was performed using the Quantitect SYBR Green PCR Kit (Qiagen) and an iQ5 thermal cycler (Bio-Rad, Inc., Hercules, CA), following the manufacturer’s recommendations. PCR annealing conditions were 55°C for 30 s in all cases. Data were analyzed using an ANOVA, and means were compared by Tukey's means separation test (P = 0.05) of the PROC GLM (20). A dilution series of E. coli genomic DNA (100, 50, 25, 10, and 1 ng) was used to standardize the real-time PCR detection threshold. SYBER green fluorescence was detected at all dilutions, close to cycle 15, and all results fit a single melt curve. Subsequent PCRs were performed using 1 ng of bacterial genomic DNA.

**RESULTS**

Colonization of E. coli O157:H7 on or in spinach plants resulting from different inoculation methods. Estimates of E. coli O157:H7 titers within inoculated spinach plants that were surface sterilized before evaluation (titers determined by plate counts on antibiotic-supplemented VRBA) are shown in Table 1. All control samples were negative. As expected, plants inoculated with E. coli O157:H7 by pressure infiltration, which forces bacteria into the intercellular spaces of the leaf interior, yielded very high bacterial densities, similar to their inoculation levels, at all time points. The other treatments yielded very few positive results. E. coli O157:H7 was detected at 0 dpi by plate counts on VRBA for all inoculation methods except leaf drop, for which bacteria were not detected until 7 dpi. For all treatments except pressure infiltration, bacterial titers were highest immediately after inoculation and lowest at the later time points. For example, when the plants were inoculated by soil drench, E. coli O157:H7 was observed in 7 of 20 whole-plant samples immediately after inoculation, but numbers dropped to only 2 and 1 of 20 samples at 7 and 14 dpi, respectively (Table 1). Except for the pressure infiltration, the highest bacterial density and highest proportion of positive plants were in the soil drenching group, and the lowest proportion was in the stab inoculation group.
group; in which only 3 of 60 samples yielded bacterial counts higher than 1 log CFU/g.

**Detection of E. coli O157:H7 in spinach plants after enrichment.** To maximize our ability to detect *E. coli* O157:H7 within spinach plants even when bacterial titers were below the detection limit of the direct count method (<1 log unit), half of each sample collected for direct plate counts was inoculated into enrichment medium (GN broth with antibiotics) and incubated for 18 h at 37°C. Enriched samples were plated onto differential media (sorbitol MacConkey agar and CHROMagar), and BAX PCR assay was performed using the manufacturer's *E. coli* O157:H7-specific primers. The number of plants positive for *E. coli* O157:H7 was higher for the enriched samples evaluated with the BAX PCR assay than for the nonenriched samples evaluated by direct plate counts (Table 2). However, 100% of pressure inoculated plants were positive, and of the other treatment groups the soil drench and stab inoculation methods retained the highest and lowest percentages of positive results, respectively.

**Potential internalization of E. coli O157:H7 and susceptibility of different spinach cultivars.** Spinach cvs. Tyee, Space, and Bordeaux were inoculated with the five-strain mixture of *E. coli* O157:H7 by the leaf drop and soil drench methods. Samples were collected and analyzed, except that in this experiment the roots of plants inoculated by soil drench were discarded and only leaves were analyzed to allow detection of possible bacterial translocation. No *E. coli* O157:H7 was detected by direct plating in any of the leaf samples inoculated by either leaf drop or soil drench; however, enrichment cultures yielded a few positive plants in each case (Table 3).

**Colonization of spinach cultivars by E. coli O157:H7 as assessed by CLSM: leaf drop inoculation.** CLSM did not reveal any bacteria on the surfaces of PBS-inoculated control plants. On the abaxial (inoculated) phylloplane of plants inoculated by leaf drop, a mean of 6, 4, or 2 cells per microscope field (0.06 mm²) was found for cvs. Tyee, Space, and Bordeaux, respectively, at 1 dpi (Fig. 1A). At 7 dpi, bacterial density had increased in cvs. Tyee and Space in the same 0.06 mm² areas, and bacteria were concentrated in furrows between epidermal cells (Fig. 1B); however, bacterial numbers on cv. Bordeaux were similar to those at 1 dpi. At 14 dpi, the numbers had increased to more than could be counted in cvs. Tyee and Space, but the mean was still only 3 cells per field on cv. Bordeaux. Movement beyond the inoculation site was evident in cvs. Tyee and Space but not in cv. Bordeaux, as indicated by

### Table 1. Detection of Escherichia coli O157:H7 by culturing on violet red bile agar

<table>
<thead>
<tr>
<th>Inoculation method</th>
<th>Treatment group</th>
<th>0 dpi</th>
<th>7 dpi</th>
<th>14 dpi</th>
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<tr>
<td></td>
<td>No. of positive plants/total examined</td>
<td>E. coli (log CFU/g)</td>
<td>No. of positive plants/total examined</td>
<td>E. coli (log CFU/g)</td>
</tr>
<tr>
<td>Leaf drop</td>
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<td>&lt;1</td>
<td>0/12</td>
</tr>
<tr>
<td></td>
<td>Inoculated</td>
<td>0/20</td>
<td>&lt;1</td>
<td>4/20</td>
</tr>
<tr>
<td>Stab</td>
<td>Control</td>
<td>0/12</td>
<td>&lt;1</td>
<td>0/12</td>
</tr>
<tr>
<td></td>
<td>Inoculated</td>
<td>2/20</td>
<td>2.5 ± 0.3 c</td>
<td>1/20</td>
</tr>
<tr>
<td>Soil drench</td>
<td>Control</td>
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<td>&lt;1</td>
<td>0/12</td>
</tr>
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<td></td>
<td>Inoculated</td>
<td>7/20</td>
<td>2.3 ± 0.5 b</td>
<td>2/20</td>
</tr>
<tr>
<td>Pressure</td>
<td>Control</td>
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<td>&lt;1</td>
<td>0/12</td>
</tr>
<tr>
<td></td>
<td>Inoculated</td>
<td>20/20</td>
<td>5.6 ± 0.4 A</td>
<td>20/20</td>
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* Because plants were surface sterilized before the assay, bacteria detected are presumably in the leaf interior.

### Table 2. Presence of E. coli O157:H7 detected by BAX PCR assay in spinach plants after sample enrichment in GN broth containing antibiotics

<table>
<thead>
<tr>
<th>Inoculation method</th>
<th>Treatment</th>
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<th>7 dpi</th>
<th>14 dpi</th>
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<td>0/12</td>
<td>0/12</td>
<td>0/36</td>
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<td>1/20</td>
<td>19/60</td>
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<td>0/12</td>
<td>0/12</td>
<td>0/12</td>
<td>0/36</td>
</tr>
<tr>
<td>E. coli O157:H7</td>
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<td>2/20</td>
<td>3/20</td>
<td>10/60</td>
<td></td>
</tr>
<tr>
<td>Soil drench</td>
<td>Control</td>
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<td>0/12</td>
<td>0/36</td>
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<td>Control</td>
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<td>20/20</td>
<td>20/20</td>
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</table>

* Because plants were surface sterilized before the assay, bacteria detected are presumably in the leaf interior.
### TABLE 3. Presence of E. coli O157:H7 detected by BAX PCR assay after enrichment in three varieties of spinach inoculated by leaf drop and soil drench

<table>
<thead>
<tr>
<th>Inoculation method</th>
<th>Cultivar</th>
<th>Treatment</th>
<th>No. of positive plants/total examined</th>
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</thead>
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<td>Tyee</td>
<td>Control</td>
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<tr>
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<td></td>
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<tr>
<td></td>
<td>Space</td>
<td>Control</td>
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<td></td>
<td>E. coli O157:H7</td>
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</tr>
<tr>
<td></td>
<td>Bordeaux</td>
<td>Control</td>
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</tr>
<tr>
<td></td>
<td></td>
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</tr>
<tr>
<td>Soil drench</td>
<td>Tyee</td>
<td>Control</td>
<td>0/12 0/12 0/12 0/36</td>
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<tr>
<td></td>
<td></td>
<td>E. coli O157:H7</td>
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</tr>
<tr>
<td></td>
<td>Space</td>
<td>Control</td>
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<tr>
<td></td>
<td></td>
<td>E. coli O157:H7</td>
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<tr>
<td></td>
<td>Bordeaux</td>
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<tr>
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<td></td>
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</table>

Colonization of spinach cultivars by E. coli O157:H7 as assessed by CLSM: pressure infiltration. After pressure inoculation into the intercellular spaces of the leaf mesophyll, no bacteria were detected with CSLM in PBS-inoculated control plants. In plants receiving the E. coli mixture, bacteria were visible in the spaces between mesophyll cells near the point of inoculation (Fig. 2A), but their numbers decreased over time. At 1 dpi, each of the ten 0.06-mm² microscope fields examined by CLSM contained an average of 33 bacterial cells. At 7 dpi, bacteria were still found near the original inoculated area, but for eight 0.06-mm² fields there was a mean of only eight cells per field (Fig. 2B and 2C). Bacterial cells were not seen at any other site in the leaf interior. At 14 dpi, only one bacterial cell was found among nine 0.06-mm² fields examined. Hand-cut sections of the leaf lamina and petioles at various sites and distances from the point of inoculation, including midribs, mesophyll, petioles, and regions of the leaf blade, also were examined at each time point. No bacteria were observed in the leaf interior of these sections or at any other site. For inoculated test plants, bacteria were detected on eight (88%) of nine plants at 1 dpi, four (44%) of nine plants at 7 dpi, and one (11%) of nine plants at 14 dpi.

Colonization of spinach cultivars by E. coli O157:H7 as assessed by CLSM: stab inoculation. After stab inoculation of spinach plant stems, all PBS-inoculated plants and almost all E. coli–inoculated plants were negative by CSLM (Table 4). A few bacteria were seen in the intercellular spaces in a leaf section from only 1 of the 27 inoculated plants examined in a section cut from the point of inoculation at 14 dpi. Also at 14 dpi, a few bacteria were visible on the surfaces of lateral roots, root hairs, and soil particles. Bacteria were never seen in any of the stem sections excised at any distance from the point of inoculation, up to 1 cm above and 1 cm below the inoculation point, from any of the 45 inoculated plants.

Colonization of spinach cultivars by E. coli O157:H7 as assessed by CLSM: soil drench. No bacteria were detected by enrichment culturing of samples from plants whose roots were drenched with PBS (Table 4). No bacteria were observed by CSLM on or within the roots of drench-inoculated plants until 7 dpi, when a single bacterial cell was present on the rhizoplane of only one of nine plants.

### FIGURE 1. Confocal laser scanning micrographs of spinach (cv. Tyee) phylloplane showing fluorescent E. coli O157:H7 on the abaxial spinach leaf surface after inoculation by leaf drop. (A) At 1 dpi, very few bacteria are visible. (B) At 7 dpi, bacteria are visible in the furrows between adjacent epidermal cells; numbers are higher than at 0 dpi. (C) At 14 dpi, bacteria persist, often in aggregates, on the leaf surface.
examined. At 14 dpi, bacteria were visible on the root surfaces of four of the nine plants.

Comparison of the leaf surface colonization of *E. coli* O157:H7 on three different spinach cultivars. Nine *E. coli* O157:H7–inoculated plants and six PBS-inoculated plants of each spinach cultivar (the savoy-type cv. Tyee, the semismooth cv. Space, and the cordate-leaf cv. Bordeaux) were examined by SEM at 1 and 14 dpi and by CLSM at 1, 7, and 14 dpi. No bacteria were present on control plants inoculated with PBS. Confocal laser scanning micrographs revealing the spatial distribution of *E. coli* O157:H7 that was introduced by the leaf drop method are shown in Figures 3 and 4.

At 1 dpi, bacterial distribution on the leaf surface (estimated from six 0.06-mm² microscope fields) appeared similar on all cultivars. Most bacteria were located on or closely adjacent to stomata (Fig. 3).

At 14 dpi, in cv. Tyee large aggregates of bacteria were distributed over areas ranging from 0.06 mm² (one microscope field) to 0.15 mm² and often were clustered in furrows between epidermal cells and near stomata. In cv. Space, bacteria were more sparsely distributed over areas ranging from 0.06 to 0.3 mm²; on this cultivar furrow aggregation was not as noticeable as it was on cv. Tyee, but stomatal proximity was common. Because very few bacterial cells were present on cv. Bordeaux, no distribution pattern was apparent. In all cultivars and at all time points, the bacteria were more prevalent in the proximity of stomata (Fig. 3), which were almost always open.

In leaf sections cut from the points of inoculation and viewed by CLSM, no bacteria were found inside the leaf tissue. No evidence of bacterial internalization was found by CLSM at any time point.

Results of SEM were similar to those of CLSM, except that at 1 dpi no bacterial cells were visible by SEM in any of the cultivars (data not shown). SEM also revealed a possible stronger attachment (based on bacterial numbers remaining after chemical sterilization) in cv. Tyee than in cv. Space and cv. Bordeaux at 14 dpi (Fig. 4A). On cv. Tyee, bacteria clustered in the furrows between epidermal cells, whereas on cvs. Space (Fig. 4B) and Bordeaux (Fig. 4C), the relatively few bacteria present on the leaf surface were not concentrated in the furrows. These results were consistent among samples and experimental replications. In all instances, plants treated with only PBS remained negative (data not shown).

Evidence of internalization using the soil drench method of inoculation and detection via plate counts and real-time PCR assay. A real-time PCR assay was used to complement plate counts and the BAX PCR assay to evaluate the internalization potential of *E. coli* O157:H7 when introduced into the rhizosphere of spinach cultivars by soil drench inoculation. A dilution series of extracted *E. coli* O157:H7 DNA was first subjected to PCR using the *eae* primers to create a standard graph, and Ct values (the point at which the increase in fluorescence becomes linear) were obtained (data not shown). No evidence of internalization was found by real-time PCR assay in the foliage of

<table>
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<th>Inoculation method</th>
<th>1 dpi</th>
<th>7 dpi</th>
<th>14 dpi</th>
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<td><em>E. coli</em> O157:H7</td>
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<td><em>E. coli</em> O157:H7</td>
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<td>0/6 (0)</td>
<td>1/9 (11)</td>
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<td>Pressure</td>
<td>8/9 (88)</td>
<td>0/6 (0)</td>
<td>4/9 (44)</td>
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plants whose roots had been drenched with *E. coli* O157: H7. We probed extracted plant genomic DNA with both the plant-specific *cox* primers and the *E. coli-*specific *eae* primers in separate reactions. Both the product generated by the *cox* primers from plant genomic DNA and the product generated by the *E. coli* primers from bacterial genomic DNA expressed fluorescence in the 15th cycle. A first peak represented fluorescence from the bacterial genomic DNA (produced by the *E. coli eae* primers), and a second peak represented the plant genomic DNA (produced by the *cox* primers). If *E. coli* were present in the bacteria-treated leaves, PCR with the *E. coli eae* primers should result in a peak similar to that of the bacterial DNA control. However, minor below-threshold fluorescence at the 35th cycle (far later than that in the controls) was not accompanied by a corresponding third peak, indicating that the fluorescence was more likely due to random amplification than to a true positive result. The Ct values were similar regardless of the date of sampling (1, 7, or 14 dpi) or the spinach cultivar (Tyee, Space, or Bordeaux).

**DISCUSSION**

The overall goal of our research was to characterize the potential for *E. coli* O157:H7 to colonize, internalize, and translocate within spinach plants. To examine possible routes of entry and contamination of leafy greens by *E. coli* O157:H7, we designed several inoculation methods to mimic different pathways of potential field contamination. Leaf drop mimics contamination by bacteria-laden raindrops or sprinkler irrigation, stab inoculation simulates natural wounding by blowing sand or insect feeding, and soil drench is akin to the contamination of soil in the rhizosphere. Our results suggest that under some of our treatment conditions *E. coli* O157:H7 can and does colonize spinach leaf or root surfaces. As expected, the bacterial presence or absence and titer on or within spinach plants differed depending on the mode and location of introduction and the initial inoculum titer. Bacterial titers within the inoculated plant samples, as determined by plate counts, were very low in most of the treatments except for pressure infiltration. With this inoculation method, which did not duplicate a natural contamination event, bacteria are forced into the plant interior through open stomata. When introduced directly into leaf interiors in this manner, the pathogen survived for at least 2 weeks.

Even after pressure inoculation of bacteria into the plant interior, there was no evidence of titer increase and the only a suggestion of within-plant translocation was a small cluster of fluorescent cells seen in the intercellular spaces of midrib-associated cortical cells in a single thin section of a cv. Tyee plant at 1 dpi (data not shown). The bacteria may have been prevented from traversing symplastic cellular barriers during the pressure inoculation process. In contrast, after leaf drop, stab, and soil drench in-
oculation we occasionally detected E. coli O157:H7 by BAX PCR assay and direct plate counts in plant organs distal to the inoculation site. Our detection of E. coli O157:H7 by BAX PCR assay in a few samples of surface sterilized leaves is consistent with an interpretation of infrequent internalization and translocation. That finding also is consistent with similar reports of others. Franz et al. (13), studying internalization of E. coli O157:H7 in lettuce, detected 3.95 log CFU/g inside the leaves after inoculating the soil, and Solomon et al. (23) found transmission of E. coli O157:H7 from contaminated manure and irrigation water into lettuce plant tissue. The seeming discrepancy in results, even within our study, is likely due to differences in tissue preparation techniques used for each assessment. Bacteria were detected by BAX PCR assay and enumerated by direct plate counts from whole plant samples that had been surface sterilized, but we cannot rule out the possibility that a few bacteria could have escaped the surface sterilization process, thereby remaining on the leaf surfaces. Confirmation of these findings will be an important goal of future research.

Despite remaining questions, it is clear that E. coli O157:H7 colonized leaf surfaces and, regardless of the inoculation method used, was present and viable for up to 2 weeks after inoculation. The bacteria often clustered near stomata, which were open in most instances and could have served as a direct route for pathogen entry. E. coli O157:H7 strain mixtures used in the leaf drop experiments survived for up to 14 days, and the titers and the area of phylloplane colonization increased during this period. These strains of E. coli O157:H7 seem well suited for epiphytic leaf survival. In contrast, the same mixtures introduced into the rhizosphere remained detectable on only a few plants and were present on these plants in very low numbers, which decreased with time. The rhizosphere may present a less supportive environment for this human pathogen. Of the inoculation methods tested in this study, stem puncture inoculations were the least likely to result in bacterial colonization or multiplication.

Microscopy results of some treatments were not completely consistent among the replicates of our experiments and among the test methods. The inability to see bacteria in plant tissues at some time points at which bacteria were recovered by culture may have been due to quenching of the fluorescence from the GFP-tagged E. coli O157:H7 strains, but microscopic monitoring of both lab cultures and plant samples revealed that the signal remained strong. Thus, the failure to see fluorescing bacteria in some such treatments could indicate loss of the GFP-tagged plasmid.

The recent outbreaks of E. coli O157:H7 infection associated with leafy greens (1) has led the scientific community to rethink the possible epiphytic survival of enteric pathogens. Until recently, research has focused on the survival of human pathogens within human and animal hosts. The recent produce outbreaks necessitate further investigation of the survival of the E. coli O157:H7 inside and on the surface of plants. Very low numbers of bacterial cells are required to trigger foodborne illness and related deaths.

To address specifically the question of whether E. coli O157:H7 within leaf mesophyll intercellular spaces could persist, multiply, spread, or translocate within the plant, we inoculated a mixture of GFP-tagged E. coli O157:H7 strains into leaf interiors using the pressure of a needleless syringe and then monitored the fluorescing bacteria by CLSM, plate counts, and PCR assay. Our results revealed that the plant environment is conducive to bacterial survival for at least 2 weeks, but we saw no evidence of multiplication, colonization of internal tissues, or translocation within the plant, suggesting that such movement within plants may be rare.

To our knowledge, this study is the first to compare susceptibility of several spinach cultivars differing in leaf topography to E. coli O157:H7 colonization and to investigate the colonization patterns. We compared the rough-surface cv. Tyee, the semismooth-leaf cv. Space, and the cordate-leaf cv. Bordeaux. After the introduction of bacteria by leaf drop, bacterial clusters could be seen by CLSM in the furrows between the epidermal cells, often near stomata. Higher bacterial numbers on cv. Tyee than on cvs. Space and Bordeaux could reflect the more prominent ridges and valleys on cv. Tyee, which likely provide protected niches favorable to bacterial survival and replication. Alternatively, the high bacterial titers on cv. Tyee could reflect a specific plant-bacterial interaction that resulted in stronger bacterial adherence to the leaf surface or protection from the harsh chemical treatments and critical point drying associated with leaf preparation for microscopy. Reports by others that E. coli O157:H7 can form biofilms on surfaces (19, 21, 25) would be consistent with this finding. Although we saw no evidence of biofilms, the question remains an important topic for future research. A third possibility would be that nutrients on the phylloplane of cv. Tyee are more suitable for growth of E. coli O157:H7. Our finding that Bordeaux, a red-veined, arrow-leaved, mildew-resistant cultivar bred especially for baby leaf production (11), hosted extremely low levels of bacteria compared with the other cultivars suggests that further research on cultivar differences in spinach and other leafy greens should be a high priority.

The fact that bacterial titers were generally lower in our cultivar comparison experiment than in the previous experiment was a minor concern. The difference could have resulted from differences in plant age at the time of inoculation. Plants were at the 4-leaf stage in the first experiment but at the 8- to 12-leaf stage for the comparison experiment. These differences could also explain the low titers of E. coli O157:H7 (<1 log) in the positive plants of the cultivar comparison experiments. Age of the plant has been reported to influence E. coli O157:H7 and Salmonella enterica internalization; Brandl and Amundson (5) found that at warm incubation temperatures and in the presence of free water, populations of these two bacteria were consistently higher on young lettuce leaves than on more mature leaves, both pre- and postharvest.

Others have investigated the ability of E. coli O157:H7 to enter plant interiors and to translocate within plant vascular tissue. Although Bernstein et al. (2) could find no evidence of E. coli O157:H7 internalization in maize plants...
grown in bacteria-drenched soil, they concluded that both internalization and translocation occurred in maize seedlings that were grown in an E. coli O157:H7–spiked (9.3 × 10⁶ CFU/ml) hydroponic system after finding the bacterium in the plant leaves. Solomon et al. (23) also reported evidence of E. coli O157:H7 internalization in lettuce plants, but only at very high inoculum levels, and Eblein et al. (12) found that both Salmonella and E. coli internalized in orange fruits. In contrast, Hora et al. (15) found no evidence of E. coli internalization after severing the roots of growing spinach plants, dipping the cut surface in inoculum, and then repotting the plants. Even the addition of nematodes to the bacterial inoculum yielded no evidence of internalization.

Our studies confirm and extend current understanding of the ability of E. coli O157:H7 to survive as an epiphyte on the spinach phylloplane. We have negligible evidence of natural entry into the plant interior. A small number of E. coli O157:H7 cells were detected by enrichment and/or PCR assay of surface-sterilized spinach plants, and although these cells may not have entered the plant, but we cannot rule out the possibility that a few bacteria escaped the surface sterilization because the method we used was recently shown to be not completely reliable (29).

Bacterial survival in the plant’s internal environment might require special capabilities to combat plant defense mechanisms and to survive in an environment that is very different from that on the phylloplane. Plant pathogenic bacteria have evolved responsive defense and gene regulatory mechanisms that facilitate their entry into and colonization of plant interiors and allow these bacteria to circumvent the plant’s surveillance mechanism. There is no evidence that such plant adaptation mechanisms operate in human enteric pathogens, but such adaptions could arise in the future. For example, certain strains of the cosmopolitan enterobacterium Serratia marcescens cause cucurbit yellow vine disease (CYVD) (18). Non-CYVD strains of this bacterial species occupy diverse niches as soil or water resident saprophytes, plant endophytes, insect pathogens, and even opportunistic human pathogens. However, none of these other strains tested is able to cause CYVD (6).

Repetitive sequence PCR fingerprinting and DNA-DNA hybridization revealed significant differentiation between CYVD-causing S. marcescens strains and strains from other niches (30). The genomes of CYVD strain Z01-A and an endophytic strain of rice (R02-A) differed significantly when assessed by suppressive subtractive hybridization. A pool of DNA sequences were specific to CYVD pathogenic strains of S. marcescens, and gene sequencing revealed that a phage gene cluster and a genome island containing a type 1 fimbrial (pilus) gene cluster present in phytopathogenic strains were absent in other strains (30). It will be interesting to examine such characteristics in plant-colonizing strains of enteric human pathogens.

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