Preharvest Internalization of *Escherichia coli* O157:H7 into Lettuce Leaves, as Affected by Insect and Physical Damage

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**ABSTRACT**

Environmental pests may serve as reservoirs and vectors of zoonotic pathogens to leafy greens; however, it is unknown whether insect pests feeding on plant tissues could redistribute these pathogens present on the surface of leaves to internal sites. This study sought to differentiate the degree of tissue internalization of *Escherichia coli* O157:H7 when applied at different populations on the surface of lettuce and spinach leaves, and to ascertain whether lettuce-infecting insects or physical injury could influence the fate of either surface or internalized populations of this enteric pathogen. No internalization of *E. coli* O157:H7 occurred when lettuce leaves were inoculated with 4.4 log CFU per leaf, but it did occur when inoculated with 6.4 log CFU per leaf. Internalization was statistically greater when spinach leaves were inoculated on the abaxial (underside) than when inoculated on the adaxial (topside) side, and when the enteric pathogen was spread after surface inoculation. Brief exposure (~18 h) of lettuce leaves to insects (5 cabbage loopers, 10 thrips, or 10 aphids) prior to inoculation with *E. coli* O157:H7 resulted in significantly reduced internalized populations of the pathogen within these leaves after approximately 2 weeks, as compared with leaves not exposed to insects. Surface-contaminated leaves physically injured through file abrasions also had significantly reduced populations of both total and internalized *E. coli* O157:H7 as compared with nonabraded leaves 2 weeks after pathogen exposure.

Fresh fruit and vegetable consumption in the United States has increased dramatically during the past two decades. This trend has been accompanied by an increased number of outbreaks of infections associated with consumption of fresh produce that may have been contaminated at the farm level. Many potential preharvest sources of contamination of produce have been identified through experimental studies in the laboratory and field. Possible sources include manure, manure compost, sewage sludge, irrigation water, runoff water from livestock operations, and exposure of produce to feces from wild and domestic animals (2). In addition, several soil inhabitants can serve as reservoirs and vectors of zoonotic pathogens. For example, dispersal of *Escherichia coli* O157:H7 in soil and vermicomposts was aided by the presence of earthworms (35), and *E. coli* O157:H7 was found in slugs on a sheep farm (30). Free-living bacterivorous nematodes are capable of ingesting *E. coli* O157 and *Salmonella* (20) and might then serve as vectors for contaminating preharvest fruits and vegetables coming in contact with soil (21). Laboratory experiments have determined that the darkling beetle can acquire and harbor *Campylobacter* from an environmental source (31), and cockroaches can serve as vectors for foodborne pathogens (23). Airborne insect pests, such as the fruit fly and housefly, can harbor *E. coli* on their bodies or in their guts and may contaminate plant and food surfaces by their excreta and by regurgitation (3, 16, 27, 29, 34). Hence, it is conceivable that other insect pests (e.g., aphids, leafhoppers), which can transmit plant pathogens to lettuce (39), could also serve as primary vectors of human pathogens.

In addition to their role as primary vectors, insects may also indirectly affect the behavior of plant and foodborne pathogens through their feeding activities and damage inflicted to the plant surface (18). Several outcomes of insect feeding activities could lead to increased survival or growth of enteric pathogens. Insertion of the insect’s stylet could physically introduce a surface pathogen into the phloem, or pathogens may be introduced into damaged cells during mastication, where they would then have access to nutrients needed for survival and growth. Second, honeydew (sugary excretions by feeding insects) could serve as a nutrient source for foodborne pathogens on the plant surface and increase their survival. Last, foodborne pathogens could...
penetrate the gelatinous saliva plug that remains once the insect finishes feeding and moves away from the site. With regard to this latter pathway, the type of damage inflicted may influence the extent of pathogen internalization. Huckaba and Coble (15) found that penetration of acifluorfen, a herbicide, was not significant when a piercing-sucking insect had fed on soybean leaves, whereas penetration was significant when a rasping-sucking wound was produced.

In addition to dissemination of enteric pathogens on plants, insect feeding activities could also have an adverse effect on pathogen survival. Plants have evolved a wide range of defense mechanisms to cope with biotic and abiotic stresses that are mediated by signal transduction involving phytohormones such as jasmonate, salicylate, and abscisic acid. Of these pathways, the jasmonate pathway plays a dominant role in insect-induced, mite-induced, and wound-induced plant responses (6), whereas the salicylate pathway plays a dominant role in plant pathogen–induced plant responses (4). These lines of distinction are not absolute and there is a high degree of interaction among these phytohormones (28). Hence, plant defense pathways produced in response to one type of stress may be effective against other subsequent types of stress. As an example of cross-protective mechanisms, growth of mites has been reported reduced on cotton plants that had either been abraded or previously exposed to mite feeding, compared with undamaged controls (19). Similarly, it is feasible that plant defenses activated in response to insect feeding or other physical damage could also be detrimental to enteric pathogen survival. The objective of this study was to investigate the fate of *E. coli* O157:H7 applied to the surface of lettuce leaves that had either been exposed to insects that commonly infest leafy greens or that had been injured through physical abrasion.

MATERIALS AND METHODS

**Bacterial strains and culture conditions.** Five strains of green fluorescent protein (GFP)–labeled *E. coli* O157:H7 (E0143 [meat isolate], C7927 [human isolate], K262 [human isolate], C0083 [cattle feces isolate], and E0139 [beef jerky isolate]) were obtained from the University of Georgia, Center for Food Safety culture collection, for use in this study. The protocol used to transform these strains had been described by Jiang et al. (17). An ampicillin-resistant marker in the GFP plasmid aided in the selective growth of *E. coli* O157:H7 when ampicillin was present in growth media. Hence, strains were grown at 37°C for 24 h either on tryptic soy agar (TSA-Amp; Acumedia, Lansing, MI) or in TSA-Amp (25 l, ca. 10^9 CFU/ml of suspension). Equal volumes of each strain of GFP-labeled *E. coli* O157:H7 were combined to obtain a five-strain mixture. Serial dilutions of the five-strain *E. coli* O157:H7 mixture were made in sterilized deionized water to give 10^9 or 10^8 CFU/ml. The cells were acclimatized for 24 h to the low-nutrient medium, and then used as an inoculum for lettuce or spinach leaves.

**Source of leafy greens.** Green leaf lettuce (*Lactuca sativa* L., cv. Two Star) and smooth spinach (*Spinacia oleracea* L., cv. 7-Green F1) were seeded in trays containingMiracle-Gro Moisture Control Potting Mix (Miracle Grow Co., Marysville, OH) and placed in an environmental growth chamber (2.8-m width, 2.8-m length, and 2.0-m height) under 70% humidity, which was set for a 12-h light cycle at 20°C and a 12-h dark cycle at 15°C. Once seedlings had sprouted three to four leaves, they were transplanted into 10.2-cm-diameter pots containing ca. 250 g of top soil (Sims Bark Co., Tuscumbia, AL), and these pots were then exposed to the same environmental growth chamber conditions as used for seedlings. Water (50 to 75 ml) was applied to soil of these plants at 2-day intervals. Smooth spinach plants of a harvestable size were also removed from a field plot by digging around the plant to avoid damaging the roots. After removal of loose soil from around the roots, the plants were transplanted into 10.2-cm-diameter pots containing top soil and acclimatized to the environmental growth chamber conditions described above for 3 to 5 days prior to use in experimental studies.

**Experimental treatments.** To evaluate the method of application of inoculum, two replicate trials of a preliminary experiment were conducted on successive weeks by using growth chamber–cultivated lettuce and field-transplanted spinach plants. In each replicate trial, the GFP-labeled *E. coli* O157:H7 inoculum (25 μl, ca. 10^6 or 10^8 CFU/ml [ca. 4.4 log or 6.4 log CFU, respectively]) was applied in a hood (11.4 cm/m^2/s light reading) as a fine mist to the abaxial (underside) surface of single leaves of lettuce and spinach of each of 10 plants of harvestable size and the adaxial (upper side) surface of lettuce and spinach leaves of a second set of 10 plants. To another 10 plants of both spinach and lettuce, the inoculum was applied in fine drops to the abaxial surface of a leaf and spread gently over the surface by using a glass rod with a rounded end. After inoculation, all plants were placed for 48 h in the environmental growth chamber under the conditions described above. On removal from the growth chamber, the 10 plant leaves from plants in each treatment were divided into two groups. Five inoculated leaves in one group were analyzed for populations of GFP-labeled *E. coli* O157:H7, whereas the second 5 inoculated leaves received a surface disinfection treatment with an 80% ethanol–0.1% mercury chloride wash (38) prior to analysis for number and presence (by enrichment) of internalized pathogen populations.

For a study involving insects, two experimental approaches were undertaken. In the first experimental approach, the adaxial surfaces of two leaves on each of 50 plants were inoculated with GFP-labeled *E. coli* O157:H7 (25 μl of 10^8 CFU/ml) on day 0 by using the method of droplet and gentle rubbing application,
described above. After holding plants in the environmental growth chamber overnight (ca. 18 h) under conditions described previously, the two inoculated leaves from each of the 50 plants were covered with a small organza bag (Petal Garden, Inc., Binghamton, NY) before dividing into five groups of 10 plants each and introducing to a treatment group, one of four types of insects: 5 cabbage loopers, two to three instar (Trichoplusia ni (Hübner)), 10 aphids (Myzus persicae (Sulzer)), 10 thrips (Frankliniella fuscus (Hinds)), or 15 whiteflies (Bemisia tabaci (Gennadius)). No insects were introduced to the remaining 10 plants and served as the positive control. All plants were then held in the environmental growth chamber until the following day, at which time the insects were removed and the treated leaves of 5 plants from each treatment group were analyzed for total and internalized GFP-labeled E. coli O157:H7. Treated leaves from another 5 plants of each treatment group were analyzed for total and internalized GFP-labeled E. coli O157:H7 approximately 2 weeks later.

Plants used in the second experimental approach of the insect study were also inoculated with E. coli O157:H7 and exposed to insects but in a sequence different from the plants in the first approach. Two leaves from each of 50 plants were individually encased in an organza bag, divided into five treatment groups, and insects (5 cabbage loopers, 10 aphids, 10 thrips, or 15 whiteflies) inserted into the bags of four treatment groups. Insects and bags were removed from leaves the following day, at which time the E. coli O157:H7 inoculum was applied to the leaves in the same manner as described above. Plants were replaced in the environmental growth chamber under conditions described previously and held for ca. 18 h until the following day, at which time leaves from 5 plants were analyzed from each treatment group for total and internalized GFP-labeled E. coli O157:H7. Another 5 plants from each treatment group were analyzed for total and internalized GFP-labeled E. coli O157:H7 approximately 2 weeks later.

In both experimental approaches involving insects, two replicate trials were conducted. The first replicate trial was conducted with lettuce plants that were 30 days posttransplantation, whereas the second replicate trial was conducted with lettuce plants that were 45 days posttransplantation. In each trial, one leaf from each plant was analyzed for total number of GFP-labeled E. coli O157:H7 on the leaf surface and in internal tissues. The second treated leaf on a plant was surface sanitized with ethanol and HgCl₂ prior to analysis for internalized GFP-labeled E. coli O157:H7.

A third experimental study was conducted to determine the fate of GFP-labeled E. coli O157:H7 on spinach leaves that were subsequently injured through physical abrasion. In each of the two replicate trials, both spray and drop-spread inoculation methods described previously were utilized for application of GFP-labeled E. coli O157:H7 inoculum (45 to 60 µl of 8.9 to 9.2 CFU/ml) to the center portion of the abaxial surface of each of 50 leaves (ca. 8 cm²) from 60-day-old spinach plants. Ten inoculated plant leaves from each inoculation method and 10 uninoculated leaves were immediately harvested for enumeration of total populations of E. coli O157:H7. The remaining plants containing inoculated leaves were placed back in the growth chamber and held under conditions described previously. After 24 h, a metal file was used to make three 0.5-cm abrasions along the outer abaxial leaf edge of half of the inoculated leaves from each inoculation method. Plants were then held in the growth chamber for an additional 24 h before sampling 20 leaves each of both abraded and unabraded inoculated leaves from each inoculation method. Ten of the leaves were subjected to enumeration of total populations of E. coli O157:H7, while the other 10 leaves were subjected to surface sanitation by using 1% silver nitrate (11), followed by enumeration or enrichment analysis. In addition, 10 uninoculated leaves from plants housed in the growth chamber were sampled for total E. coli O157:H7 populations and another 10 uninoculated leaves for internalized E. coli O157:H7 populations. Twelve days later, a similar sampling protocol for inoculated and uninoculated leaves was followed.

Enumeration and enrichment of GFP-labeled E. coli O157:H7. Surface-sanitized lettuce and spinach leaves along with leaves designated for total E. coli O157:H7 enumeration were added to 0.1% peptone water (1:9, leaves:peptone [wt/vol]) and macerated with a mortar and pestle. Samples were removed from this macerated mixture and either directly surface plated on TSA-Amp or serially diluted with 0.1% peptone prior to surface plating, incubating at 37°C for 24 h, and counting green fluorescent colonies. For enrichment, 1.0 ml of macerated tissue suspension was added to 9 ml of TSB-Amp, incubated for 24 h at 37°C, and streaked onto TSA-Amp, and plates were incubated for 24 h at 37°C before examining for green fluorescent colony formation. The limit of detection for plate count enumeration was 2 log CFU/g, whereas the limit of detection for enrichment culture was 1 log CFU/g. In the preliminary study where enrichment culture was used, the number of positive samples out of the total number of samples analyzed by this method was recorded. In the insect as well as in the physical damage studies, those samples that did not yield any colonies by direct plate count but were positive and negative by enrichment culture were assigned populations of 1.0 log and 0.0 log CFU/g, respectively, for purposes of calculating mean log populations for treatments.

Statistical analysis. E. coli O157:H7 populations obtained from plate count enumeration assays were converted to log CFU per gram before statistical analysis. Enumeration data were then analyzed by analysis of variance (ANOVA) by using the StatGraphics Centurion XV software package (StatPoint, Inc., Herndon, VA). When statistical differences were observed (P < 0.05) with ANOVA, differences among sample means were determined with Fisher’s least-significant difference test. Incidence data obtained from enrichment culture assays in the preliminary study were subjected to the chi-square test.

RESULTS

No internalized E. coli O157:H7 was detected in individual lettuce leaves 48 h after being spray inoculated with 4.4 log CFU of the pathogen per leaf (data not shown). In contrast, pathogen internalization was observed 48 h after leaves were spray inoculated with a higher number of E. coli O157:H7 (6.4 log CFU per leaf) (Table 1). Total (internalized plus surface) populations of E. coli O157:H7 on these leaves ranged from 6.7 to 7.4 log CFU/g. When the spray was applied as a mist, internalized E. coli O157:H7 could only be detected by enrichment; however, statistically higher incidences were observed in contaminated abaxial (under side) as compared with adaxial (upper side) side of spinach leaves. In an attempt to cover the entire leaf surface with the inoculum, droplets were applied to the abaxial side of leaves and gently spread over the surface with a rounded glass rod. This procedure led to internalized E. coli O157:H7 counts of 2.7 ± 0.8 and 2.9 ± 1.1 log CFU/g in spinach and lettuce leaves, respectively.

Two approaches were used to evaluate the effect of insect pests on populations of surface and internalized E.
coli O157:H7 in lettuce plants. Treatments differed in terms of the sequence of exposure of leaves to E. coli O157:H7 and test insects. In both cases, the E. coli O157:H7 was applied by drops and rubbing in order to have the entire abaxial leaf surface covered and to ensure that insect activity and pathogen deposition would be at the same locations. Due to the different times between exposure of leaves to the pathogen and analysis (2 or 14 days and 1 or 13 days for experimental approaches 1 and 2, respectively), total (surface and internalized) populations of E. coli O157:H7 were slightly lower when pathogen had been applied to the leaves before exposure to the insects (Table 2) as compared with leaves inoculated with E. coli O157:H7 after they had been exposed to the insects (Table 3). In the first experimental approach, when E. coli O157:H7 was applied to the leaves before exposure to the insects, total populations of E. coli O157:H7 were not significantly (P > 0.05) affected by the presence of the insects on day 2 (Table 2). In contrast, on day 14, 12 days after removal of the insects, the total population of E. coli O157:H7 on leaves that had been exposed to cabbage loopers was significantly less (P ≤ 0.05) than the population on control leaves that had not been exposed to insects or to the population on leaves exposed to whiteflies. In the second experimental approach in which E. coli O157:H7 was applied to leaves on which insects had been feeding, the total E. coli O157:H7 population on leaves immediately after exposure to cabbage loopers (day 1) was significantly higher than was the population on leaves exposed to aphids; however, by day 13, leaves that had been exposed to thrips had significantly lower populations as compared with the control leaves (Table 3).

After exposure of inoculated lettuce leaves to insects, the populations of internalized E. coli O157:H7 in leaves exposed to insects in experimental approach 1 (Table 4) were not significantly different. In contrast, exposure of leaves to insects prior to application of the pathogen (experimental approach 2) resulted on day 13 in lower populations of internalized E. coli O157:H7 in leaves exposed to cabbage loopers, aphids, and thrips, as compared with the control (Table 5).

In a study designed to control the level of physical damage applied to plants, inoculated leaves that were surface abraded on day 1 had on day 14 significantly lower total populations of E. coli O157:H7 as compared with leaves that were not injured, regardless of the method used to inoculate the leaves (Table 6). Lower internalized populations were also observed on day 14 in abraded leaves as compared with non-abraded leaves when the drop-spread method of inoculation was used (Table 7).

**DISCUSSION**

Internalization of E. coli O157:H7 into leafy green leaves was dependent on the population sprayed onto the surface. Internalization was not observed when individual lettuce leaves were sprayed with 4.4 log CFU of E. coli O157:H7; however, internalization was observed when sprayed with 6.4 log CFU per leaf (Table 1). These data are in agreement with the results of a field study where

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**TABLE 1. Internalized Escherichia coli O157:H7 48 h after mist application of inoculum of 6.4 log CFU per leaf to the leaf surface**

<table>
<thead>
<tr>
<th>Plant</th>
<th>% incidence</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lettuce</td>
<td>70</td>
<td>0.0736</td>
</tr>
<tr>
<td>Spinach</td>
<td>90</td>
<td>0.0115</td>
</tr>
</tbody>
</table>

* Total (internalized plus surface) cell numbers of E. coli O157:H7 in and on leaves 48 h after application of inoculum ranged from 6.7 to 7.4 log CFU/g for all treatments.

* Percent positive samples by enrichment out of total number of samples analyzed.

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**TABLE 2. Total (surface plus internalized) population of Escherichia coli O157:H7 on lettuce leaves inoculated with E. coli O157:H7, followed by brief exposure to common insect pest before holding plants in growth chamber (experimental approach 1)**

<table>
<thead>
<tr>
<th>Insect</th>
<th>Log CFU/g (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 2</td>
</tr>
<tr>
<td>Cabbage loopers (5)</td>
<td>5.3 ± 0.3 c</td>
</tr>
<tr>
<td>Aphids (10)</td>
<td>4.8 ± 0.8 c</td>
</tr>
<tr>
<td>Whiteflies (15)</td>
<td>4.8 ± 0.7 c</td>
</tr>
<tr>
<td>Thrips (10)</td>
<td>5.5 ± 0.2 c</td>
</tr>
<tr>
<td>Control (no insects)</td>
<td>5.2 ± 0.5 c</td>
</tr>
</tbody>
</table>

* Leaf inoculated with E. coli O157:H7 and held for ~1 day and then exposed to insects for ~1 day.

* The detection limit (1.0 log CFU/g) was used to calculate the mean log value when a sample did not yield any colonies by direct plate count but was positive by enrichment culture. When E. coli O157:H7 was not detected by plate count enumeration or by enrichment culture, the samples were assigned a value of 0.0. Values not followed by the same letter are significantly different (P ≤ 0.05).

* Days after exposure to E. coli O157:H7 inoculum.

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**TABLE 3. Total (surface plus internalized) population of Escherichia coli O157:H7 on lettuce leaves briefly exposed to common insect pests, followed by inoculation with E. coli O157:H7 before holding plants in growth chamber (experimental approach 2)**

<table>
<thead>
<tr>
<th>Insect</th>
<th>Log CFU/g (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
</tr>
<tr>
<td>Cabbage loopers (5)</td>
<td>6.3 ± 0.4 d</td>
</tr>
<tr>
<td>Aphids (10)</td>
<td>5.0 ± 0.5 c</td>
</tr>
<tr>
<td>Whiteflies (15)</td>
<td>5.4 ± 0.7 cD</td>
</tr>
<tr>
<td>Thrips (10)</td>
<td>5.8 ± 1.1 d</td>
</tr>
<tr>
<td>Control (no insects)</td>
<td>5.8 ± 0.4 d</td>
</tr>
</tbody>
</table>

* Leaf exposed to insects for ~1 day and then inoculated with E. coli O157:H7.

* The detection limit (1.0 log CFU/g) was used to calculate the mean log value when a sample did not yield any colonies by direct plate count but was positive by enrichment culture. When E. coli O157:H7 was not detected by plate count enumeration or by enrichment culture, the samples were assigned a value of 0.0. Values not followed by the same letter are significantly different (P ≤ 0.05).

* Days after exposure to E. coli O157:H7 inoculum.
internalization of attenuated strains of *E. coli* O157:H7 occurred in spinach plants exposed to $10^6$ CFU/ml of spray-contaminated irrigation water but not $10^4$ CFU/ml of irrigation water (10). Together, these studies suggest that a minimum threshold population may be required for internalization to be detected. This threshold population is likely affected by the conditions used for cultivation of the plants and would thus account for the observation that *E. coli* O157:H7 was internalized in 3 to 32% of spinach leaves drop inoculated with 4.8 log CFU per leaf (22).

Stomata, serving as pores for gas exchange, are the most likely portal by which *E. coli* O157:H7 is internalized into leaves. In support of this route of entry, several plant-pathogenic bacteria have been documented to enter through plant stomata (14). *E. coli* O157:H7 cells have also been demonstrated to penetrate stomata of lettuce and spinach leaves exposed to an inoculum postharvest (32, 33, 36). Given that stomata are generally found in higher densities on the abaxial side of leaves compared with the adaxial side (7, 24), this distribution could explain the higher incidences of *E. coli* O157:H7 internalization observed in abaxial-inoculated spinach leaves as compared with adaxial-inoculated spinach leaves (Table 1).

Opening of stomata is controlled by a number of environmental factors including light, carbon dioxide concentration, and humidity (26). For safety reasons in this study application of the misted *E. coli* O157:H7 inoculum to the leaf surfaces was conducted in a hood in which light radiation was significantly reduced compared with that of the growth chamber in which the plants were transferred and held. Under the reduced-light conditions encountered during mist spraying, some stomata may have been closed or partially open and would have restricted internal access to the pathogen at that time. Internalization of *E. coli* O157:H7 is likely to have occurred in the growth chamber but would be dependent on either migration of the pathogen to the stomatal openings or internal transfer of the pathogen at insect feeding sites.

### TABLE 4. Internalized population of *Escherichia coli* O157:H7 on lettuce leaves inoculated with *E. coli* O157:H7, followed by brief exposure to common insect pest before holding plants in growth chamber (experimental approach 1)

<table>
<thead>
<tr>
<th>Insect (no./leaf)</th>
<th>Leaf inoculated with 4.8 log CFU per leaf</th>
<th>Leaf exposed to insects for 1 day and then inoculated with <em>E. coli</em> O157:H7</th>
<th>Leaf exposed to insects for ~1 day and then inoculated with <em>E. coli</em> O157:H7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cabbage loopers (5)</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Aphids (10)</td>
<td>1.1 ± 1.0</td>
<td>0.2 ± 0.4</td>
<td>0.2 ± 0.4</td>
</tr>
<tr>
<td>Whiteflies (15)</td>
<td>1.2 ± 1.4</td>
<td>0.5 ± 0.7</td>
<td>0.5 ± 0.7</td>
</tr>
<tr>
<td>Thrips (10)</td>
<td>0.8 ± 1.3</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Control (no insects)</td>
<td>1.1 ± 1.1</td>
<td>0.6 ± 1.2</td>
<td>0.6 ± 1.2</td>
</tr>
</tbody>
</table>

*Leaf inoculated with *E. coli* O157:H7 and held for ~1 day and then exposed to insects for ~1 day.*

*The detection limit (1.0 log CFU/g) was used to calculate the mean log value when a sample did not yield any colonies by direct plate count but was positive by enrichment culture. When *E. coli* O157:H7 was not detected by plate count enumeration or by enrichment culture, the samples were assigned a value of 0.0.

*Days after exposure to *E. coli* O157:H7 inoculum.*

### TABLE 5. Internalized population of *Escherichia coli* O157:H7 on lettuce leaves briefly exposed to common insect pests, followed by inoculation with *E. coli* O157:H7 before holding plants in growth chamber (experimental approach 2)

<table>
<thead>
<tr>
<th>Insect (no./leaf)</th>
<th>Log CFU/g (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cabbage loopers (5)</td>
<td>0.2 ± 0.4 AB</td>
</tr>
<tr>
<td>Aphids (10)</td>
<td>0.6 ± 1.0 AB</td>
</tr>
<tr>
<td>Whiteflies (15)</td>
<td>0.9 ± 0.8 BCD</td>
</tr>
<tr>
<td>Thrips (10)</td>
<td>1.6 ± 1.3 DE</td>
</tr>
<tr>
<td>Control (no insects)</td>
<td>1.8 ± 1.2 E</td>
</tr>
</tbody>
</table>

*Leaf exposed to insects for ~1 day and then inoculated with *E. coli* O157:H7.*

*The detection limit (1.0 log CFU/g) was used to calculate the mean log value when a sample did not yield any colonies by direct plate count but was positive by enrichment culture. When *E. coli* O157:H7 was not detected by plate count enumeration or by enrichment culture, the samples were assigned a value of 0.0.

*Days after exposure to *E. coli* O157:H7 inoculum.*

To facilitate complete coverage of the leaf surface with *E. coli* O157:H7, thus ensuring that both insect activity and pathogen deposition would be at the same locations, the inoculum was applied as small droplets to the abaxial surface of leaves and gently spread with a glass rod. Internalization of *E. coli* O157:H7 into leaves was greater by using this method of application compared with leaves sprayed with a mist (Table 5). It is our contention that spreading the pathogen would have increased the probability for *E. coli* O157:H7 to be located on or near stomata; however, we also acknowledge the possibility that damage to trichomes and epidermal cells or the development of cracks in the waxy cuticle layer occurred by the spreading action, thereby facilitating internalization of *E. coli* O157:H7 at these damaged sites.

The number of insects applied to an individual leaf was based on the expectation that minimal damage to the leaf would occur and that their activity would be sufficient to exert an effect, if it existed, on internalization or the fate of *E. coli* O157:H7. Selection of insects for this study was based on their relative prevalence in lettuce fields as well as differences in feeding behaviors (1). Cabbage looper larvae produce ragged holes where they have chewed on the tissue. Whiteflies and aphids, however, feed through a stylet that punctures the tissue and sucks out the plant juices; hence, only small holes are produced from their activity. Thrips feed by rasping on the plant surface while sucking out the juices. *E. coli* O157:H7 populations on the leaf surface and internally appeared to have been influenced by the different feeding behaviors of the test insects and whether damage occurred prior to or after exposure to the pathogen. For example, the highest level of total *E. coli* O157:H7 contamination occurred on day 1 when the pathogen was applied to leaves on which cabbage loopers had fed (Table 3). Exposed tissue within the cabbage looper tracks could be a source of nutrients for *E. coli* O157:H7, thereby
facilitating its survival and possibly growth. A similar explanation was offered for a study wherein *E. coli* applied within minutes of infliction of minor shoot injury to glasshouse celery, Cos lettuce, and chive plants persisted at higher concentrations than when applied to uninjured plants (12). Stimulation of plant defenses, on the other hand, could account for the observation that decreased populations of *E. coli* O157:H7 occurred at 13 days postinoculation in several insect-exposed leaves as compared with control leaves. For example, total *E. coli* O157:H7 populations in leaves exposed to thrips (Table 3) and internalized populations in leaves exposed to cabbage loopers, thrips, and aphids (Table 5) were significantly less than were populations in control leaves when the pathogen was applied to leaves after insect exposure. The absence of significant differences between internalized populations for insect-infested and control leaves when *E. coli* O157:H7 was applied prior to insect exposure and activity may be indicative that insects may facilitate pathogen internalization through their feeding activity, and these increases counter any plant defenses raised in response to the feeding injuries. As support for this explanation, leaves contaminated by the drop-spread method and then physically damaged through file abrasion contained significantly reduced internalized populations of *E. coli* O157:H7 as compared with non-abraded leaves 2 weeks later (Table 7). A similar trend was noted in leaves contaminated by the mist spray method, but the differences were not statistically significant, possibly due to the low numbers of cells initially internalized via this method.

In plants, defenses against insects, plant pathogens, and physical injury are typically regulated by a network of interconnected and antagonistic signal transduction pathways involving salicylic acid and jasmonic acid–ethylene. For example, resistance of the plant *Arabidopsis* to bacterial plant pathogens is predominantly dependent on salicylic acid (4). Components of the jasmonate- and ethylene-signaling pathways that induce resistance to insects (13), however, have also been demonstrated to contribute to resistance against several plant pathogens (8). Hence, caterpillars of the herbivore *Pieris rapae* that have stimulated the production of jasmonic acid and ethylene in *Arabidopsis* led to the reduction of disease caused by *Pseudomonas syringae*, presumably due to ethylene’s ability to sensitize tissue to respond faster to salicylic acid (5). In other cases, it has been demonstrated that herbivores appear to capitalize on the antagonistic interaction between salicylic acid and jasmonic acid signaling by suppressing induced defenses or modulating the defense-signaling network (25). For instance, herbivorous nymphs of the silver leaf whitely activated the salicylic acid signaling pathway, whose activity was antagonistic to induction of the jasmonic acid defenses (37). Based on these tripartite interactions between plants, insects, and plant pathogens, it is tempting to conjecture that plant defenses induced in response to feeding insects in this study may also have had a supplemental function by killing internalized pathogens such as *E. coli* O157:H7.

### TABLE 6. Total (surface plus internalized) population of *Escherichia coli* O157:H7 on spinach leaves inoculated with *E. coli* O157:H7, followed by abrasion damage to leaf surface and holding plants in growth chamber

<table>
<thead>
<tr>
<th>Inoculation method</th>
<th>Condition</th>
<th>Day 0†</th>
<th>Day 2</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mist spray</td>
<td>Not abraded</td>
<td>7.0 ± 0.4 E</td>
<td>4.1 ± 1.5 C</td>
<td>2.7 ± 1.2 a</td>
</tr>
<tr>
<td></td>
<td>Abraded</td>
<td>4.2 ± 1.8 C</td>
<td>3.0 ± 1.9 b</td>
<td>1.5 ± 0.9 a</td>
</tr>
<tr>
<td>Drop-spread</td>
<td>Not abraded</td>
<td>7.3 ± 0.7 E</td>
<td>5.7 ± 1.3 d</td>
<td>3.0 ± 1.9 b</td>
</tr>
<tr>
<td></td>
<td>Abraded</td>
<td>5.3 ± 1.2 d</td>
<td>1.1 ± 1.1 a</td>
<td>1.1 ± 1.1 a</td>
</tr>
</tbody>
</table>

† Leaf inoculated with *E. coli* O157:H7 on abaxial surface and held for ~1 day before making 3 abrasions (0.5 cm each) around the edge of the abaxial surface. All noninoculated leaf samples held in the growth chamber for similar periods were negative by enrichment for *E. coli* O157:H7.

### TABLE 7. Internalized *Escherichia coli* O157:H7 on spinach leaves inoculated with *E. coli* O157:H7, followed by abrasion damage to leaf surface and holding plants in growth chamber

<table>
<thead>
<tr>
<th>Inoculation method</th>
<th>Condition</th>
<th>Log CFU/g (mean ± SD)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mist spray</td>
<td>Not abraded</td>
<td>1.6 ± 1.6 CD</td>
</tr>
<tr>
<td></td>
<td>Abraded</td>
<td>1.1 ± 1.2 BC</td>
</tr>
<tr>
<td>Drop-spread</td>
<td>Not abraded</td>
<td>3.1 ± 1.1 E</td>
</tr>
<tr>
<td></td>
<td>Abraded</td>
<td>1.9 ± 1.1 D</td>
</tr>
</tbody>
</table>

† Leaf inoculated with *E. coli* O157:H7 on abaxial surface and held for ~1 day before making 3 abrasions (0.5 cm each) around the edge of the abaxial surface. All noninoculated leaf samples held in the growth chamber for similar periods were negative by enrichment for *E. coli* O157:H7.

The detection limit (1.0 log CFU/g) was used to calculate the mean log value when a sample did not yield any colonies by direct plate count but was positive by enrichment culture. When *E. coli* O157:H7 was not detected by plate count enumeration or by enrichment culture, the samples were assigned a value of 0.0. Values not followed by the same letter are significantly different (P ≤ 0.05).

† Days after exposure to *E. coli* O157:H7 inoculum.
In summary, *E. coli* O157:H7 may be internalized into lettuce and spinach leaf tissue, but the population of *E. coli* O157:H7 required for this event to occur in lettuce was higher than 4.4 log CFU per leaf. Contamination in the field with this number of *E. coli* O157:H7 is unlikely, thereby minimizing the risk of internalization of lettuce leaves. On the rare occasion that such an event would occur, internalization of *E. coli* O157:H7 may be minimized by plant defenses that are induced in response to intrusive insect activities or physical injury.

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REFERENCES


