Identification of the Cellular Location of Internalized *Escherichia coli* O157:H7 in Mung Bean, *Vigna radiata*, by Immunocytochemical Techniques

AMANDA J. DEERING,1* ROBERT E. PRUITT,2 LISA J. MAUER,1 AND BRADLEY L. REUHS1

1Department of Food Science and 2Department of Botany and Plant Pathology, Purdue University, West Lafayette, Indiana 47907, USA

MS 11-015: Received 10 January 2011/Accepted 24 March 2011

ABSTRACT

*Escherichia coli* O157:H7 has been associated with numerous outbreaks involving fresh produce. Previous studies have shown that bacteria can be internalized within plant tissue and that this can be a source of protection from antimicrobial chemicals and environmental conditions. However, the types of tissue and cellular locations the bacteria occupy in the plant following internalization have not been addressed. In this study, immunocytochemical techniques were used to localize internalized *E. coli* O157:H7 expressing green fluorescent protein in germinated mung bean (*Vigna radiata*) hypocotyl tissue following contamination of intact seeds. An average of 13 bacteria per mm3 were localized within the sampled tissue. The bacteria were found to be associated with every major tissue and corresponding cell type (cortex, phloem, xylem, epidermis, and pith). The cortical cells located on the outside of the vascular bundles contained the majority of the internalized bacteria (61%). In addition, the bacteria were localized primarily to the spaces between the cells (apoplast) and not within the cells. Growth experiments were also performed and demonstrated that mung bean plants could support the replication of bacteria to high levels (107 CFU per plant) following seed contamination and that these levels could be sustained over a 12-day period. Therefore, *E. coli* O157:H7 can be internalized in many different plant tissue types after a brief seed contamination event, and the bacteria are able to grow and persist within the plant.

There have been increased efforts to reduce the potential for human bacterial pathogen contamination of fresh produce throughout the growing, harvesting, and postharvest processes due to the increase in the number of outbreaks that have been associated with fresh fruits and vegetables in recent years (22). Sprouts are often examined because of their frequent association with outbreaks and their susceptibility to contamination during the growing process. The seeds are soaked in water in large batches and then exposed to high temperature (25 to 35°C) and humidity for germination (10). These conditions are not only highly favorable to the growth of bacterial pathogens but also favorable for cross-contamination events. Sprouts are also problematic because the entire intact plant is consumed, often raw or lightly cooked, making the internalization of bacteria in any part of the plant tissue a potential health risk to the consumer (24).

Many different surface sanitizers have been examined for their potential to reduce the likelihood of contamination in fresh produce (2, 3, 9, 13); however, their efficacy is greatly dependent on the sanitizing agent being in direct contact with the target pathogen. It has been shown that bacteria can enter the plant through natural openings, such as stomata, broken trichomes, and lenticels (18). These areas can provide protection to the bacteria from the effects of the sanitizers, and this has been shown to occur in apple (4) and tomato (26) fruits.

Various methods and strategies have been conducted to examine the possibility of internalization of human pathogenic bacteria in plants. Sampling and plating collected exudate from plant tissue that had been contaminated and surface sterilized following contamination has been a common approach in determining if bacteria were internalized within the plant (25). This method, however, does not account for bacteria that could potentially be in the natural openings of the plant, which can provide protection and allow the bacteria to survive the sterilization process.

Internalization of bacteria in plants has also been examined using both conventional epifluorescence microscopy and laser scanning confocal microscopy by taking advantage of *Salmonella* spp. and *Escherichia coli* O157:H7 strains that have been constructed to express green fluorescent protein (GFP) (5, 23). While useful data have been obtained by using these techniques, the microscopic analysis can only be carried out with fresh tissue due to the fact that GFP is no longer fluorescent following conventional fixation techniques. Precise localization of bacteria in unfixed tissue that has been sectioned is difficult due to the movement of bacteria during the sectioning process as well

* Author for correspondence. Tel: 765-496-3861; Fax: 765-494-7953; E-mail: adeering@purdue.edu.
as the high level of background autofluorescence present in most fresh plant tissue. Sectioning of the fresh tissue is not required for laser scanning confocal microscopy, but fluorescent signals from bacteria containing GFP can sometimes bleed from one optical section to the next, leading to equally imprecise bacterial localizations.

In this study, the internalization of *E. coli* O157:H7 in mung bean was examined. The growth and persistence of *E. coli* O157:H7 associated with mung bean plants were determined by contaminating seeds, allowing them to germinate under normal growth conditions, and then analyzing the plant tissue. Immunocytochemical techniques were then used to localize the internalized bacteria within the sampled tissue using fluorescence microscopy. Using these techniques, the exact cell types the bacteria occupy within the plant, as well as an estimate of the total number of the target bacteria within the tissue, were determined.

**MATERIALS AND METHODS**

**Bacteria.** *E. coli* O157:H7 B6-914 GFP-91 (hereafter referred to as *E. coli* O157:H7–GFP) was used in this study (7). The strain was constructed by transforming *E. coli* O157:H7 B6-914 with a GFP plasmid, pGFP (cDNA vector, Clontech, Mountain View, CA) containing the ampicillin resistance gene for selection. The strain does not produce Shiga-like toxins (Stx1 and Stx2) and was chosen for safety advantages. Differences in growth kinetics between the transformed strain and the parental strain have not been observed (7). In addition, the lack of the toxin genes does not influence the growth characteristics of the bacteria (11). Bacteria were stored at −80°C as 7% dimethyl sulfoxide freezer stocks and cultured in Luria-Bertani (LB) broth supplemented with ampicillin (100 µg/ml; LB + AMP) at 37°C.

**Contaminated water uptake by mung bean seeds.** Overnight cultures of *E. coli* O157:H7–GFP were grown in LB + AMP at 37°C with shaking. The culture was washed three times to remove the growth media; cells were centrifuged for 6 min at 1,400 × g, the supernatant was removed, and the resulting pellet was resuspended in 0.1 M phosphate buffer (pH 7.0) with a mortar and pestle. The number of bacteria stored at −80°C as 7% dimethyl sulfoxide freezer stocks and cultured in Luria-Bertani (LB) broth supplemented with ampicillin (100 µg/ml; LB + AMP) at 37°C. Bacteria were then grown in LB + AMP at 37°C with shaking. The culture was washed three times to remove the growth media; cells were centrifuged for 6 min at 1,400 × g, the supernatant was removed, and the resulting pellet was resuspended in 0.1 M phosphate buffer (pH 7.0) with a mortar and pestle. The number of bacteria then determined by using Excel (Microsoft 2008, Version 12.2.8).

**Enumeration of *E. coli* O157:H7–GFP from mung bean plants.** A culture of *E. coli* O157:H7–GFP was prepared as described above. Mung bean seeds were soaked for 30 min in a 10^7-CFU/ml culture of *E. coli* O157:H7–GFP in distilled water. The seeds were then ground using a mortar and pestle in 10 ml of 0.1 M phosphate buffer (pH 7.0), plated on LB + AMP plates, and incubated overnight at 37°C. The remaining 30 seeds were each placed into an individual sterile test tube (25 by 150 mm) containing 25 ml of sterile 0.8% soft-top agar. The tubes were sealed with Parafilm and incubated in a growth chamber at 25°C with 50% relative humidity and a 16/8 light cycle. At 3, 6, 9, and 12 days after contamination, five plants were removed and ground in 10 ml of 0.1 M phosphate buffer (pH 7.0) with a mortar and pestle. The number of bacteria from each plant was determined by plating on LB + AMP plates and incubating overnight at 37°C. The average CFU per plant was then determined for each sample time.

**SDS-PAGE and Western blot analysis.** Total protein samples were extracted from 3- and 6-day-old mung bean seedlings by grinding leaf, root, and hypocotyl sections in 1× sodium dodecyl sulfate (SDS) sample buffer (50 mM Tris-HCl [pH 6.8], 2% SDS, 0.1% bromophenol blue, 10% glycerol, 100 mM dithiothreitol). The protein samples were then boiled for 10 min and centrifuged for 2 min at 14,000 × g. *E. coli* O157:H7–GFP bacteria were grown overnight in LB + AMP, and a crude extraction of the GFP was performed by using an abbreviated protocol from a Wizard Plasmid Purification system (Promega, Madison, WI); 1.5 ml of culture was centrifuged for 3 min at 16,000 × g, and the resulting supernatant was removed and resuspended in 250 µl of the resuspension solution provided in the kit. An equal volume of provided cell lysis solution was added, and the tube was inverted three times and incubated for 4 min at room temperature. The sample was then centrifuged at 8,200 × g for 10 min, the supernatant was transferred to a fresh tube, and an equal volume of 1× sample buffer was added and vortexed. Then, 10 µl of each sample was loaded and run on an SDS-polyacrylamide gel (12% separating gel and 4% stacking gel) at 120 V in 1× SDS–polyacrylamide gel electrophoresis (PAGE) Running Buffer (25 mM Tris, 250 mM glycine, and 0.1% SDS).

For the Western blot analysis, the proteins from the gel were transferred to a polyvinylidene fluoride membrane overnight at 55 V in continuously circulating 1× transfer buffer (47 mM Tris-base, 38 mM glycine, and 0.04% SDS) at 4°C. The blots were then washed and incubated in blocking buffer (4% nonfat dry milk in Tris-buffered saline with Tween 20 [TBST: 150 mM NaCl, 20 mM Tris-base, and 0.05% Tween 20, pH 7.5]) for 2 h at room temperature with rotation. The membrane was then incubated in a 1:50 dilution of anti-GFP primary antibody (Living Colors A.v. peptide antibody, affinity purified rabbit immunoglobulin G fraction, Clontech Laboratories, Inc., Mountain View, CA) in 0.5× blocking buffer solution for 2 h at room temperature with rotation. The membrane was washed three times in TBST for 10 min each time and then incubated in a 1:2,500 secondary alkaline phosphatase conjugated antibody (anti-rabbit immunoglobulin, Sigma, St. Louis, MO) in 0.5× blocking buffer solution for 2 h at room temperature with rotation. The membrane was removed and washed three times for 10 min each with TBST. Substrate (Western Blue, Promega) was then added to the membrane and allowed to develop at room temperature to visualize the detection of GFP.

**Fixation and paraffin embedding of plant tissue.** Hypocotyl sections from seedlings grown from contaminated seeds as described above were harvested at 3 days after germination (DAG) into fixative (4% formaldehyde and 0.5% glutaraldehyde in 100 mM phosphate buffer [pH 7.0]) and fixed overnight at 4°C with rotation. The samples were washed with buffer, dehydrated through a graded ethanol series (30, 50, 75, 95, and 100%), and infiltrated with liquid paraffin at 58°C using *tert*-butyl alcohol as the intermediate solvent. The samples were embedded in paraffin, and the blocks were serially sectioned at 10 µm with a rotary microtome (Microm HM-330, Walldorf, Germany). The sections were floated by using water on glass microscope slides coated with 0.01% poly-L-lysine and dried overnight at 38°C (20).

**Immunocytochemistry for *E. coli* O157:H7–GFP detection.** Slides containing paraffin sections were dewaxed twice in
FIGURE 1. Western blot from crude GFP extracted from E. coli O157:H7 cells. Lane 1, E. coli–GFP and total protein from mung bean plants 3 and 6 DAG (days after germination). A positive signal is observed only in the E. coli–GFP sample (arrow indicates ~26-kDa GFP) and not in any other lane. Lane 2, 3-DAG root; lane 3, 3-DAG hypocotyl; lane 4, 3-DAG leaf; lane 5, 6-DAG root; lane 6, 6-DAG hypocotyl; lane 7, 6-DAG leaf.

Microscopy and experimental control. The slides from a total of three seed-contaminated plants were examined using a Nikon (Melville, NY) Microphot-FXA fluorescence microscope under the appropriate filter set. Paired fluorescence and differential interference contrast digital images were collected with a MicroFire digital camera (Optronics, Goleta, CA). The cellular location and tissue type were recorded for every bacterium localized in the sections. A positive signal was recorded only if the following criteria were fulfilled: (i) fluorescence was observed only under the appropriate filter set and not with other filter sets, (ii) the size of the positive signal was approximately 1 μm (the approximate size of an E. coli bacterium), and (iii) the shape was either rod-like or circular (cross section of the typical rod-shaped E. coli bacterium). In addition, the following experimental controls were performed: (i) tissue collected from plants that were incubated in buffer only (no bacteria present) were treated as previously described and examined by immunocytochemical techniques to ensure signal was not present; and (ii) slides containing blocking buffer only, primary anti-GFP antibody only, and secondary Alexa Fluor 568 antibody only were examined to ensure that signal was not present. The collected images were cropped and reduced in resolution by using Adobe Photoshop CS3, and scale bars were added by using ImageJ (19). The concentration of bacteria in the plant tissue (number of bacteria per mm$^2$) was calculated by measuring the length and width of the hypocotyl and treating the total volume as a cylinder.

RESULTS AND DISCUSSION

Contamination and persistence of E. coli O157:H7–GFP in mung bean. The process of soaking mung bean seeds prior to germination is commonly used by growers in industry (15). Seeds used for sprout production are particularly vulnerable to pathogen contamination during this process due to the capacity of a dry seed to imbibe large volumes of the germination liquid and any bacteria that could be present. In this study, mung bean seeds that were soaked for 12 h in water contaminated with $10^7$ CFU of E. coli O157:H7–GFP per ml experienced an average weight change of 116% (standard deviation, 25.7%). Thus, these dry seeds were able to imbibe large amounts of liquid during germination. An average of $10^8$ CFU per seed was enumerated from the mung bean seeds that were soaked for only 30 min in the contaminated water, thereby demonstrating that high levels of bacteria that were present in the germination liquid can be taken up by the seeds. After 3 days of growth, an average of $10^7$ CFU per plant could be enumerated from the plants, and this level of bacterial growth was sustained over the 12-day sampling period. Although the initial concentration of the target bacteria used for contamination was higher than what might be normally found in the environment, these results are important because they demonstrate that dry seeds can retain high levels of bacteria following a short exposure to the contaminated germination liquid and that the bacteria are subsequently able to grow and persist within the plant.

Western blot and experimental control analysis. A Western blot was performed to test the specificity and cross-reactivity of the anti-GFP antibody. A positive GFP band (~26 kDa) was observed only in the lane with the crude GFP extraction from the E. coli O157:H7–GFP cells (Fig. 1). This showed that the primary anti-GFP antibody
FIGURE 2. Fluorescence and bright-field paired images of E. coli O157:H7–GFP bacteria localized in seed-contaminated mung bean tissue by using immunocytochemical techniques. The arrow indicates the location of the bacterium within the tissue: A, epidermal; B, cortical; C, vascular; D, pith. Larger objects that fluoresce more faintly are chloroplasts that are also visible in the bright-field images.
used for the immunocytochemistry experiments was specific to the GFP associated with the E. coli O157:H7–GFP cells and that there was no cross-reactivity with the total proteins extracted from the leaf, hypocotyl, or root of 3- and 6-DAG mung bean plants. All immunocytochemical experimental control samples (see “Materials and Methods”) were examined by fluorescence microscopy, and a positive signal was not observed in any of the samples.

**Immunocytochemical detection of bacteria internalized in mung bean sprouts.** Sections of hypocotyls from several contaminated mung bean seedlings were collected and chemically fixed to preserve the morphology of the tissue and immobilize any bacteria that were present. These samples were processed, embedded in paraffin, and sliced into 10-μm serial sections. Following immunocytochemical detection, every section from each hypocotyl segment (approximately 200 sections for each segment) was examined for the presence of fluorescent signals consistent with the expected size and shape of the bacteria used for contamination. This method enabled the examination of the entire interior of each hypocotyl segment for the presence and location of internalized bacteria.

An average of 13 bacteria per mm\(^3\) (a total of 822 bacteria) were localized within the three sampled mung bean hypocotyl tissue sections. The bacteria were found to be associated with every major tissue and corresponding cell type: epidermal (Fig. 2A), cortex (Fig. 2B), vascular (Fig. 2C), and pith (Fig. 2D). The distribution of internalized E. coli O157:H7 cells within the mung bean tissue is shown in Table 1. The cortical cells located on the outside of the vascular bundles contained the majority of bacteria. Bacteria were also localized to the vascular tissue, parenchyma cells comprising the pith, and epidermal cells. Much of the vascular tissue was undifferentiated due to the young age (3 days after germination) of the tissue when it was sampled. If a specific cell type (i.e., sieve element or tracheary element) could not be distinguished in the vascular tissue, the bacteria localized to these areas were said to be found in the vascular tissue but were not assigned a specific cell type. When a definitive tissue type anywhere else in the sampled tissue could not be assigned to a localized bacterium, the cell type was designated as unknown. The ability of E. coli O157:H7 to be internalized in a variety of tissue types of mung bean plants was demonstrated. Furthermore, the presence of bacteria internalized to the vascular tissue indicates the possibility that the bacteria can move and spread throughout the plant. It was found that seeds that are contaminated could possibly pose a food safety risk because the resulting germinated plants are likely harboring internalized bacteria that can persist for long periods and may potentially be vehicles for the transmission of the E. coli O157:H7 to humans.

**Apoplastic localization pattern of E. coli O157:H7 in mung bean.** The bacteria internalized in the mung bean tissue were primarily localized to the spaces between the cells (apoplast) and not within the cells (symplast). When a positive signal was recorded for a bacterium, the location within the cell (symplastic versus apoplastic) was also documented. The approximate 2:1 ratio of apoplastic to symplastic cellular location (actual ratio, 1.81:1) observed is what would be expected if the bacteria were present in the areas between the horizontal and vertical surfaces of the stacked plant cells. The apparent localization of the bacteria to the symplast of the cell was likely the result of the sectioning process: The bacterium may have actually been located in the apoplast of the cell either above or below the observed symplast. The probability that nearly all bacteria were localized to the apoplast was further supported by micrographs collected from fresh mung bean hypocotyl that had been seed contaminated (as described) and free-hand sectioned. Figure 3 is representative of the micrographs collected, in which it can be seen that bacteria localized primarily to the apoplastic spaces in the plant. The apoplastic localization of the bacteria is therefore not likely a result of the treatments performed upon the tissue for the immunolocalization experiments but reflects where most of the bacteria are found in the tissue.

The pathogenesis of E. coli O157:H7 in humans involves the bacteria adhering to the extracellular regions of the host cell. This complex process (12), not described in detail here, ultimately causes extensive changes to the ultrastructure of the cell resulting in inflammation and the inability of the cell to absorb nutrients. This is unlike the intracellular mode of pathogenesis for other bacteria, such as Salmonella spp., whereby the bacterium is able to induce phagocytosis by the host cell and once internalized, causes damage and destroys the host cell (17). Based on the mode of E. coli O157:H7 pathogenesis in humans, it is intriguing that E. coli O157:H7 would also localize to the apoplastic spaces in the plant tissue as was observed from both the fixed and fresh mung bean tissue examined. While this could be due to the plant cell wall acting as a barrier, this is not the case for all human enteric bacteria since Salmonella Typhimurium was found to have a symplastic localization pattern in peanut tissue (6). These results indicate that the bacterial locations of cellular colonization in plants may be similar to what is found in humans.

The data presented here give insight into the possible endophytic lifestyle of the human enteric pathogen, E. coli O157:H7, in plants. There have been several other reports showing that endophytic bacteria, such as Acetobacter diazotrophicus (21) and Bacillus megaterium (14), can be localized to the intercellular spaces of plant tissues.
The apoplast of the plant is rich in pectin, a family of polysaccharides that is composed primarily of galacturonic acid; hemicelluloses that contain arabinose, xylose, and glucose; and other polysaccharides. Various proteins, such as glycoproteins, are also associated with the apoplast. The apoplastic spaces, therefore, may be a preferred location within the plant due to the many available compounds that the bacteria could utilize as a carbon source for growth.

Potential food safety concerns and conclusions. The infective dose for *E. coli* O157:H7 has been reported to range from 1 to 100 CFU (8, 16). Low numbers of bacteria are sufficient to cause illness, especially in the young, the elderly, and immunocompromised individuals (17). The number of bacteria localized (822) from the sampled mung bean tissue would likely be sufficient to cause disease in humans if consumed. The number of bacteria actually present in each mung bean plant, however, could potentially be 10 times higher than what was observed, because the bacterial cells need to be disrupted to allow the primary antibody to bind to the GFP. Therefore, the cells that were cut during the sectioning process would likely be the only bacteria that were detected by the described methods. Since the bacterial cells are ~1 μm in length and could be stacked end to end in the 10-μm sections of plant material, only 1 cell in 10 is likely to be cut during the sectioning process. Consequently, the number of bacteria internalized in the tissue could be as high as 130 bacteria per mm³ (a total of 8,220 bacteria localized), potentially creating a food safety risk.

This is the first report to date that systematically identifies and quantifies the tissue and cellular locations (i.e., in the areas between the cell walls or inside the cell) of internalized *E. coli* O157:H7 cells within the plant. These results are important for understanding not only which tissues the bacteria target for internalization but also how the organisms are able to grow and persist within the plant. From the data presented here, seed contamination events appear to be of particular importance due to the likelihood that the resulting plants are harboring internalized bacteria, although further research is needed to determine how long the internalized bacteria persist in older plants. Determining ways to prevent contamination events from occurring and developing ways to treat and handle fresh produce that have internalized bacteria are among the areas of future research that will help to reduce the number of outbreaks that occur due to the consumption of contaminated fresh produce.

ACKNOWLEDGMENTS

This project was funded in part by the Agricultural Research Service of the U.S. Department of Agriculture (USDA-ARS) project no. 1935-42000-049-00D. The authors thank Drs. Ron Turco and Mussie Habetselassie for providing the *E. coli* O157:H7-GFP strain used in this study.

REFERENCES

1230  DEERING ET AL.


