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Research Paper

Listeria monocytogenes Internalizes in Romaine Lettuce Grown in Greenhouse Conditions

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ABSTRACT

Listeria monocytogenes has been implicated in a number of outbreaks involving fresh produce, including an outbreak in 2016 resulting from contaminated packaged salads. The persistence and internalization potential of *L. monocytogenes* in romaine lettuce was evaluated, and the persistence of two *L. monocytogenes* strains was assessed on three romaine lettuce cultivars. Seeds were germinated, and plants grown in three soil types (i.e., standard potting mix, autoclaved potting mix, and top soil) and sterile soft-top agar for up to 21 days. Average CFU per gram of *L. monocytogenes* on seeds and plants was calculated from five replicates per harvest day. Up to 8.2 log CFU/g *L. monocytogenes* persisted on romaine lettuce plants (Braveheart cultivar) grown in soft-top agar, while those grown in commercial potting mix (initial soil aerobic plate count of 4.0×10^4 CFU/g) had a final concentration of 5.4 log CFU/g, and autoclaved commercial potting mix had a final concentration of 3.8 ± 0.2 log CFU/g after a 21-day period. Pathogen levels dropped below the limit of detection (2 log CFU/g) by day 18 in 75% topsoil (initial soil aerobic plate count of 4.0×10^1 CFU/g); this did not occur in sterile media. Although *L. monocytogenes* strain differences and presence of a clay coating on seeds did not affect persistence, differences were observed in *L. monocytogenes* growth and survival among cultivars. To assess internalization, seeds were inoculated with *L. monocytogenes* expressing green fluorescent protein. Three plants were fixed, paraffin embedded, and sectioned; localization was studied by using standard immunohistochemistry techniques. A total of 539 internalized *L. monocytogenes* cells were visualized among three 20-day seedlings. *L. monocytogenes* cells were located in all major tissue types (pith followed by cortex, xylem, phloem, and epidermis). The presence of *L. monocytogenes* in the plant vasculature suggests potential for transport throughout the plant into edible tissue.

Key words: Immunohistochemistry; Internalization; *Listeria monocytogenes*; Microscopy; Persistence; Romaine lettuce

Listeria monocytogenes remains a major food safety concern, particularly in ready-to-eat foods. On an annual basis, *L. monocytogenes* accounts for nearly 1,600 cases of listeriosis in the United States, with 99% of the cases resulting from foodborne transmission *(22)*. Produce has historically been considered a low-risk food in likelihood of causing listeriosis *(27)*. An outbreak occurred in the United States and Canada in 2016, resulting from contaminated packaged salads *(8)*. Since 2010, listeriosis outbreaks have been caused by contaminated celery, cantaloupe, sprouts, and apples *(7, 14)*. The 2011 listeriosis outbreak from cantaloupe was the second-most deadly bacterial foodborne outbreak in U.S. history *(6)*. Fresh produce is especially concerning as a transmission vehicle because it is often minimally processed and consumed raw.

There are numerous sources of potential contamination in the preharvest period, including soil, irrigation water, improperly composted manure, and roaming animals. A recent study has shown that up to 17.5% of produce fields are contaminated with *L. monocytogenes (24)*. Attachment is necessary for a bacterium to colonize and potentially internalize into the edible portions of a plant, but the mechanisms for attachment to produce have not been well elucidated for *L. monocytogenes*. For example, Gorski et al. *(15)* found that flagella are necessary for some, but not all strains, of *L. monocytogenes* to colonize plants and that the fitness of colonization was improved by functional motility of the flagella. Additionally, *L. monocytogenes* can incorporate into a heterogeneous biofilm formed by epiphytic bacteria or form its own biofilm on a plant surface *(2, 12)*. Once attached, persistence on the plant is partially dependent on the bacteria's ability to survive environmental stressors (e.g., UV exposure and temperature fluctuations), as well as its ability to utilize plant leachates as a nutrient source *(4)*. Biofilms and colonization of plant microsites (e.g., along veins or in spaces between epidermal cells) provide a protective environment that may support microbial proliferation *(2, 19)*. Internalization is yet another way foodborne pathogens can gain access to a protected environment. For example, damaged tissue, such as a tear in the waxy plant cuticle, could provide a site of entry. Additional entry sites include cracks in the seed coat or minute tears in the root tissue during germination. Bacteria that are present near germinating radicles are exposed to nutritional exudates from the growing plant, which can

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encourage microbial growth and increase access to the plant *(13)*.

Romaine lettuce is one of the fastest growing crops in the United States, in terms of production, export, and consumption *(25, 26)*. There have been multiple recalls in recent years due to *L. monocytogenes* contamination *(28),* and a listeriosis outbreak was linked to bagged lettuce in 2016 *(1)*. Sanitizers that are used in the fresh produce industry to treat produce only contact the external portions of the fruit or vegetable. Therefore, if bacteria are inside the plant, they are not exposed to the sanitizer and therefore not killed. Understanding the ability of foodborne pathogens to grow and remain on or in plant tissue can inform preharvest control strategies to prevent contamination, as well as can indicate the degree to which postharvest sanitization methods must be successful to prevent cross-contamination during washing and disease from consumption of contaminated produce.

In this study, we investigated the growth and persistence of *L. monocytogenes* on romaine lettuce postcontamination of seeds. Immunohistochemical techniques and fluorescence microscopy were used to examine the internalization potential and localization patterns of *L. monocytogenes* in the romaine hypocotyl.

MATERIALS AND METHODS

Bacterial strains and romaine cultivars. *Listeria monocytogenes* strains 10403S (laboratory-type strain, serotype 1/2a, lineage II) and FSL J1-194 (human clinical isolate, serotype 1/2b, lineage I) were used in this study *(5, 21)*. These isoaltes were selected to represent two major *L. monocytogenes* lineages, and 10403S is a widely used laboratory strain for which a green fluorescent protein (GFP)–expressing mutant was available. DH-L1039 (*L. monocytogenes* 10403S with pH-*hly gfp*-PL3 integrated into the $tRNA^{Arg}$ locus) was used to express GFP *(23)*. All bacterial strains were stored at −80°C in brain heart infusion (BD, Sparks, MD) broth supplemented with 25% glycerol. Strains were cultured by using brain heart infusion broth and incubated overnight (16 to 18 h) at 30°C with shaking at 200 rpm. Romaine cultivars Braveheart, Sun Valley, and Sunbelt were used in this study; seeds were obtained from a large commercial grower and are among the most common commercial cultivars currently in use. All cultivars had a germination rate of 95% or higher. All seeds came with a clay coating as used in industry. Braveheart cultivar seeds without a clay coating were used to test the effect of seed coating on persistence.

Seed inoculation, romaine lettuce growth conditions, and *L. monocytogenes* enumeration. Each strain of *L. monocytogenes* was cultured overnight as detailed previously. Each culture was centrifuged and suspended in 0.1 M phosphate buffer (pH 7.0) to approximately 10^8 CFU/ml. A high inculum was used to investigate proof of concept and to increase the likelihood of detecting persistent contamination; we acknowledge that the probability of natural contamination at this level is unlikely. Eighty seeds of each cultivar were soaked in 10^8 CFU/mL *L. monocytogenes* for 30 min at room temperature with rotation at 8 rpm (Labquake, Labindustries, Inc., Berkeley, CA). Seeds were air dried in a biosafety cabinet for 1 h. To enumerate initial seed contamination levels, five seeds were weighed and individually ground in 10 mL of 0.1 M phosphate buffer with a mortar and pestle; mortar and pestle were sterilzed between samples. Dilutions of the homogenate were plated on modified Oxford agar (Difco, BD); plates were incubated for 2 days at 30°C. Inoculated seeds were individually and aseptically placed into sterile test tubes (25 by 200 mm) containing 20 mL of 0.8% soft-top agar (Bacto, Difco, BD) immediately after drying; tubes were sealed with parafilm. Seeds in soft-top agar were grown in a biosafety level 2 greenhouse maintained at an average temperature of 28°C with a 16:8 h light-dark cycle for the duration of the experiment for up to 60 days. Seeds soaked in 0.1 M phosphate buffer (pH 7.0) for 30 min with rotation served as negative controls for each trial. Clay-coated seeds were used in this study to reflect industry practices. Seeds without clay coating were treated similarly to test the effect of seed coating on *L. monocytogenes* persistence. Growth trials were also performed in commercial potting mix (Metro-Mix 510, Sungro, Agawam, MA), autoclaved commercial potting mix, and 75% top soil. Soils were prepared by mixing 9.46 L of either Professional grower's potting mix (Sun Gro Sunshine Redi-Earth, Bellevue, WA) or 75% top soil (mixed with 25% Sun Gro Metro-mix 510; Bellevue, WA) with about 12 g of Marathon 1% Granular Insecticide (OHP, Inc., Mainland, PA). Soils were distributed into planter trays, and seeds were planted 2.54 cm below the soil surface. Planter trays were placed into larger holding trays with water and slow-release fertilizer (Osmocote, Scotts Miracle-Gro, Marysville, OH). All persistence trials were conducted in a biosafety level 2 greenhouse maintained at an average temperature of 28°C with a 16:8 h light-dark cycle for the duration of the experiment. Seedlings were harvested every 3 days from seed inoculation (day = 0) to day 21.

Five seedlings were enumerated, as described for the initial seed contamination; average CFU per gram of plant was determined. A single untreated negative control seedling was harvested at each time point to confirm absence of *L. monocytogenes*. Commercial potting mix and 75% topsoil were tested for *L. monocytogenes* by using a modified U.S. Food and Drug Administration *Bacteriological Analytical Manual* (BAM) enrichment protocol. Briefly, 25 g of soil was stomached (Stomacher 400 Circulator, Seward, Davie, FL) with 225 mL of Buffered Listeria Enrichment Broth (Difco, BD) for 1 min at 280 rpm. The homogenate was incubated for 4 h at 30°C, followed by addition of 900 μL of *Listeria* selective enrichment supplement (3.6 mg/mL sodium hydroxide, 9 mg/mL nalidixic acid, 2.25 mg/mL acriflavine, and 11.25 mg/mL cycloheximide in 10% methanol aqueous solution; Sigma-Aldrich, St. Louis, MO). Samples were incubated at 30°C for an additional 44 h; 100 μL of the enrichment was spread plated onto modified Oxford agar plates and incubated at 30°C for 48 h.

Statistical analyses. Persistence data were analyzed via two-factor analysis of variance ($P < 0.0001$) by using JMP 11 statistical software (SAS Institute Inc., Cary, NC), and individual differences were tested by using Tukey's honestly significant difference test ($\alpha = 0.05$). Treatment, day, and treatment × day interactions were used as predictive variables, with log CFU per gram as the response. Comparisons were made among trails after day 0, as colonies enumerated from the seeds directly after the brief contamination event greatly varied between the different trials. Additionally, data collected beyond day 21 were for observational purposes and not included in statistical analysis, as data were collected on varying days.

Romaine tissue fixation, embedding, and immunohistochemistry. Braveheart cultivar seeds inoculated with *L. monocytogenes*–GFP as described previously were planted in commercial potting mix and placed in the greenhouse as described previously.

Hypocotyl sections were cut from three 20-day-old plants and treated as described in Deering et al. *(10)*. Briefly, hypocotyl sections were placed in fixative, dehydrated through a graded ethanol series, and permeated with paraffin oil. The samples were paraffin embedded and serially sectioned into 10-μm sections. The 910 resulting sections were floated onto microscope slides coated with 0.01% poly-L-lysine and dried overnight at 38°C. Immunohistochemistry procedures for *L. monocytogenes*–GFP detection were conducted as described in Deering et al. *(10)*. Briefly, slides were incubated with blocking buffer, a 1:100 dilution of the primary anti-GFP antibody (Living Colors A.v. Peptide Antibody, affinity-purified rabbit immunoglobulin G (Clontech Laboratories, Inc., Mountain View, CA), washed, and incubated with a 1:2,500 dilution of fluorophore-conjugated secondary antibody (Alexa Fluor 568 Goat Anti-Rabbit Immunoglobin G, Invitrogen, Carlsbad, CA). Total protein was extracted from root, stem, and leaf sections of noninoculated 30-day-old plants grown in commercial potting mix in the greenhouse and from *L. monocytogenes* 10403S-GFP to evaluate cross-reactivity and reactivity, respectively, by standard Western blot, as previously described *(10)*. Crude protein was extracted 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, and 100 mM dithiothreitol). Proteins were separated by SDS–polyacrylamide gel electrophoresis and transferred overnight in a mini transblot cell to a polyvinylidene difluoride membrane at 55 V, 4°C in 1× transfer buffer (47 mM Tris base, 38 mM glycine, 0.04% SDS, and 20% methanol). The membrane was incubated with a 1:1,000 dilution of the primary anti-GFP antibody in 0.5× blocking buffer for 2 h at room temperature with rotation, washed 3× for 10 min with Tris-buffered saline with Tween (TBST) pH 7.5, and then incubated with a 1:2,500 dilution of secondary alkaline phosphatase–conjugated antibody (goat anti-rabbit immunoglobulin G, Sigma), for 2 h at room temperature with rotation. The membrane was washed 3× for 10 min with TBST pH 7.5. The bands were developed with 10 mL of alkaline phosphatase substrate (Western Blue, Promega, Madison, WI) for 5 min.

Determining location of *L. monocytogenes* in romaine tissue by fluorescent microscopy. Hypocotyl sections from three Braveheart plants previously fixed in paraffin were examined by using a Nikon Microphot-FXA fluorescence microscope (Nikon, Melville, NY). Both fluorescence, as well as differential interface contrast images, were taken by using a MicroFire digital camera (Optronics, Goleta, CA). The slide and section number, as well as tissue type and apoplastic or symplastic localization, were recorded for each *L. monocytogenes* cell. The following criteria were used to identify *L. monocytogenes* cells: (i) signals observed only under the green excitation filter set (to accommodate Alexa Fluor 568: excitation 578 nm and emission 603 nm) and not under other filter sets, (ii) signals restricted to 1 to 1.5 μm in length (typical length of *L. monocytogenes*), and (iii) morphology of the signal was either rod or circular (cross section of *L. monocytogenes* cell). Control slides treated independently with blocking buffer, primary antibody, or secondary antibody were used to evaluate background signal. Plants that had not been inoculated with *L. monocytogenes* were also fixed, sectioned, and analyzed to ensure absence of signal. Images were cropped by using Adobe Photoshop CC (Adobe Systems, San Jose, CA), and scale bars were added by using ImageJ (National Institutes of Health, Bethesda, MD). *L. monocytogenes* concentration in plant tissue (bacteria per cubic millimeter) was assessed by considering each hypocotyl as a cylinder and calculating the volume from the measured values of height and width.

Internalized bacteria localization patterns were analyzed by using SAS 9.1 statistical software (SAS Institute Inc.). A mixed model with a Poisson distribution and plant random effects were used for analysis. Tissue type and distance from the center of the hypocotyl were modeled as fixed effects. Differences in least-squares means were used to assess significant pairwise comparisons of tissue type by using a multivariate Tukey-Kramer multiple comparison procedure; significance was defined as $P < 0.05$.

RESULTS AND DISCUSSION

In this study, we investigated the growth and persistence of *L. monocytogenes* on romaine lettuce and used immunohistochemistry to examine the internalization localization patterns of *L. monocytogenes* in the romaine hypocotyl. We found that (i) commercial clay coating does not impact *L. monocytogenes* persistence, (ii) significant differences in *L. monocytogenes* persistence exist among cultivars and soil and media types, and (iii) *L. monocytogenes* internalized in all major tissue types of the hypocotyl with the majority localizing in the pith in seeds grown in greenhouse conditions. Although this study is limited to defined laboratory conditions that may not directly represent field conditions, it demonstrates proof of concept.

Industry clay coating of romaine seeds minimally impacts *L. monocytogenes* persistence over time. All romaine seeds were provided by a commercial grower, using clay-coated seeds to facilitate mechanized planting. We tested whether the presence of this clay coating significantly impacted persistence of *L. monocytogenes*; there were no statistically significant differences from days 3 and 21 (Fig. 1). The presence of a clay coating affected day 0 *L. monocytogenes* levels, as varying levels of clay coating remained after inoculation; wetting from the inoculum partially dissolved the coating. *L. monocytogenes* levels were consistent through ≥60 days, which is equivalent to time to harvest for the cultivars used in this study (Fig. 1). In all trials, *L. monocytogenes* levels, irrespective of cultivar or strain, recovered to ≥7.0 log CFU/g on day 3 (Figs. 1 through 3). Day 0 *L. monocytogenes* concentrations for all trials in the study were not included in the statistical analysis due to highly variable initial inoculum counts because of degradation of the clay seed coat during inoculation. Including day 0 in the statistical analyses would artificially inflate growth measurements that could be attributed to coating degradation of the seed sampled. *L. monocytogenes* concentrations were below the detection limit (reported as 2.0 log CFU/g) on Sunbelt and Braveheart seeds on day 0. During inoculation of the Sunbelt seeds, the coating disintegrated. To determine if *L. monocytogenes* attached directly to the seed in the absence of clay coating, uncoated Braveheart and Sun Valley seeds were subjected to the same inoculation and enumeration methods. This also resulted in *L. monocytogenes* concentrations below the limit of detection (data not shown). However, efforts will be made in future studies to minimize the effects of inoculum inconsistencies. This study has shown that even a brief

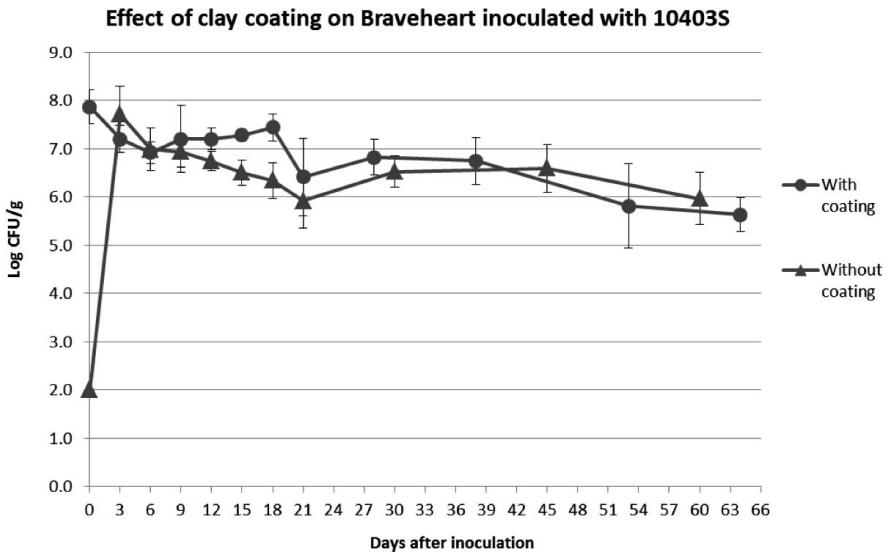

FIGURE 1. *Effect of presence of seed clay coating on* L. monocytogenes *persistence throughout a 60-day period. Data points for each day reported are based on the average CFU per gram values of five replicates grown in soft-top agar in greenhouse conditions. Error bars denote standard deviations.*

contamination period of 30 min can result in *L. monocytogenes* attachment to seeds and that it can grow and persist for times potentially extending through to the harvest period in defined laboratory conditions tested, but to a much more limited extent in soil. Further efforts to understand variables (e.g., soil type, clay coating, and cultivar) that allow or prevent pathogenic bacteria to grow and remain on or in plant tissue can inform preharvest control strategies, as well as can indicate the degree to which postharvest sanitation methods must be successful to prevent outbreaks from consumption of contaminated produce.

***L. monocytogenes* persists on romaine lettuce for up to 60 days in soft-top agar.** In this study, we evaluated the effect of cultivar, strain, growth media, and seed coating on *L. monocytogenes* persistence on romaine lettuce. To test cultivar differences, Braveheart, Sun Valley, and Sunbelt romaine cultivar seeds were inoculated with either *L. monocytogenes* 10403S or FSL J1-194 (Fig. 2). *L. monocytogenes* concentration on day 0 was highly variable between cultivars; the effect of seed coating on recovered inoculum is discussed in the following. On day 3, *L. monocytogenes* 10403S concentrations were similar among cultivars (7.2 ± 0.3 log CFU/g on Braveheart, 7.3 ± 0.2 log CFU/g on Sun Valley, and 7.6 ± 0.6 log CFU/g on Sunbelt); there were no significant differences among cultivars through day 15. There were significant differences of *L. monocytogenes* 10403S levels among on all three cultivars on day 21. Specifically, the highest concentration of 10403S was recovered from Sunbelt (8.25 log CFU/g) (Fig. 2A). We observed more variation in *L. monocytogenes* FSL J1-194 levels among cultivars during the 21-day period (Fig. 2B). There was a significant difference in FSL J1-194 levels on day 18 between Braveheart and Sunbelt cultivars. Final concentrations of FSL J1-194 were 7.22 log CFU/g on Braveheart, 6.05 log CFU/g on Sun Valley, and 6.59 log CFU/g on Sunbelt after a 21-day period (Fig. 2B). Given the limitations of our study design, cultivar differences may manifest after extended periods (e.g., after 18 days) in romaine lettuce due to growth or persistence or both over time. Nevertheless, *L. monocytogenes* can persist at high levels for periods equivalent to commercial time to harvest (approximately 60 days).

***L. monocytogenes* persists longer on plants grown in synthetic medium compared with soil-based media.** To test potential impact of growth medium on persistence, Braveheart cultivar seeds were inoculated with *L. monocytogenes* 10403S and planted in four substrates (soft-top agar, commercial potting mix, autoclaved commercial potting mix, and 75% top soil; Fig. 3). *L. monocytogenes* was not detected in any of the soil-based media prior to use in the experiments. *L. monocytogenes* persistence at higher levels in seeds grown in sterile soft-top agar after a 21-day period (6.4 ± 0.8 CFU/g), followed by commercial potting mix (5.4 ± 0.3 log CFU/g), autoclaved commercial potting mix (3.8 ± 0.2 log CFU/g), and 75% top soil (2.0 ± 0.0 log CFU/g; below detection limit) (Fig. 3A). Differences in *L. monocytogenes* concentration levels were more pronounced once extended to at least 60 days (Fig. 3B). *L. monocytogenes* concentrations were below the detection limit by day 18 in 75% top soil, by day 30 in autoclaved commercial potting mix, and by day 45 in commercial potting mix. Only contaminated seeds grown in sterile soft-top agar were able to persist until the typical harvest period for romaine lettuce. *L. monocytogenes* on contaminated seeds grown in soil or potting mixes types did not persist until a typical harvest period (approximately 60 days) (Fig. 3B) and below the detection limit by day 30. Aerobic plate counts were measured for 75% top soil mixture (4.0×10^4 CFU/g) and commercial potting mix (4.0×10^1 CFU/g). Native microflora may have contributed to limiting *L. monocytogenes* persistence as observed in other studies *(9, 17, 30),* but it was not tested directly in this study. Additionally, there were apparent differences in relative humidity between soft-top agar tubes and soil trials, as condensate developed in soft-top agar tubes. Although measurements of relative humidity were not recorded, the greenhouse environment fluctuates due to to ventilation capabilities. In addition to

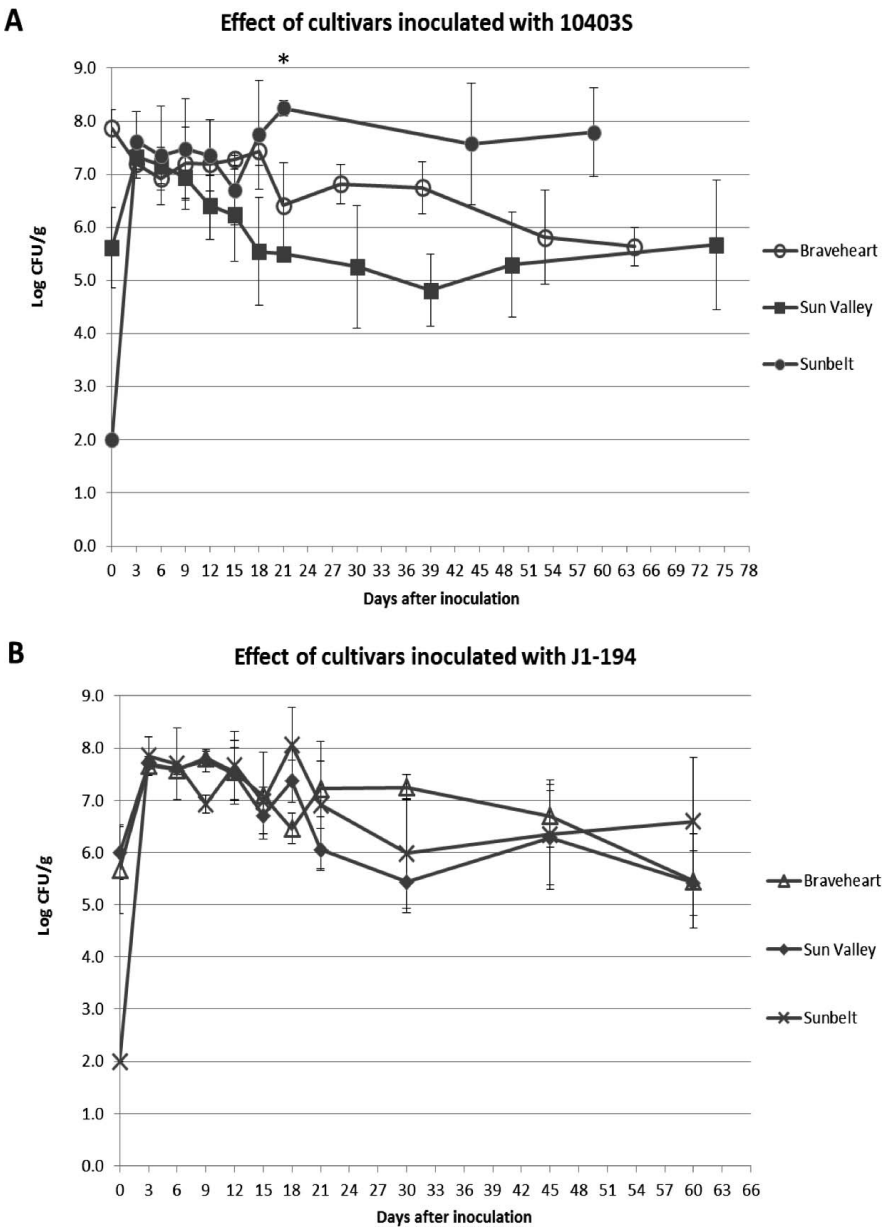

FIGURE 2. *Comparison of* L. monocytogenes *persistence among three different lettuce cultivars inoculated with either* L. monocytogenes *10403S (A) or* L. monocytogenes *FSL J1-194 (B) grown in soft-top agar in greenhouse conditions. Data points for each day reported are based on the average CFU per gram values of five replicates. Error bars denote standard deviations. An asterisk indicates statistically significant differences among cultivars.*

limited microflora, high relative humidity has been shown to enhance microbial growth *(3, 18)*.

***L. monocytogenes* is able to localize in all major tissue types of romaine lettuce grown in commercial potting mix in greenhouse conditions.** Immunohistochemistry was used to identify internalization patterns of *L. monocytogenes* among romaine lettuce tissue types in three 20-day-old plants grown in commercial potting mix in a biosafety level 2 greenhouse. Immunohistochemistry allowed us to identify internalization patterns of *L. monocytogenes* among romaine lettuce tissue types. We used a fluorophore-conjugated antibody to circumvent the need for direct visualization of GFP, as fixation results in loss of GFP fluorescence. Additionally, epifluorescence microscopy was used instead of laser scanning confocal microscopy, as fluorescent signals in the latter can bleed from one optical section to the next. Similar immunohistochemistry internalization analysis methods have been used in other studies *(11, 16)*. Total protein extracts from stem, leaf, and root sections of two separate, noninoculated plants were analyzed via Western blot to evaluate (i) antibody specificity and (ii) absence of cross-reaction with romaine lettuce (data not shown).

We calculated the density of *L. monocytogenes* to be 3.9 cells per mm^3 of plant tissue. As *L. monocytogenes* is typically 1 to 1.5 μm *(20)*, multiple cells could fit within a single 10-μm section of tissue; therefore, the concentration of *L. monocytogenes* cells is likely underestimated in this study. Additionally, only disrupted bacterial cells are detected, which is necessary to detect GFP. A total of 539 *L. monocytogenes* cells were localized among three hypocotyl sections of 20-day-old romaine lettuce plants germinated from inoculated seeds. *L. monocytogenes* was found to be associated with every major tissue type, including the epidermis (Fig. 4A), cortex (Fig. 4B), pith (Fig. 4C), and vascular tissue (Fig. 4D). Location of *L. monocytogenes* by tissue types is detailed in Table 1. *L.*

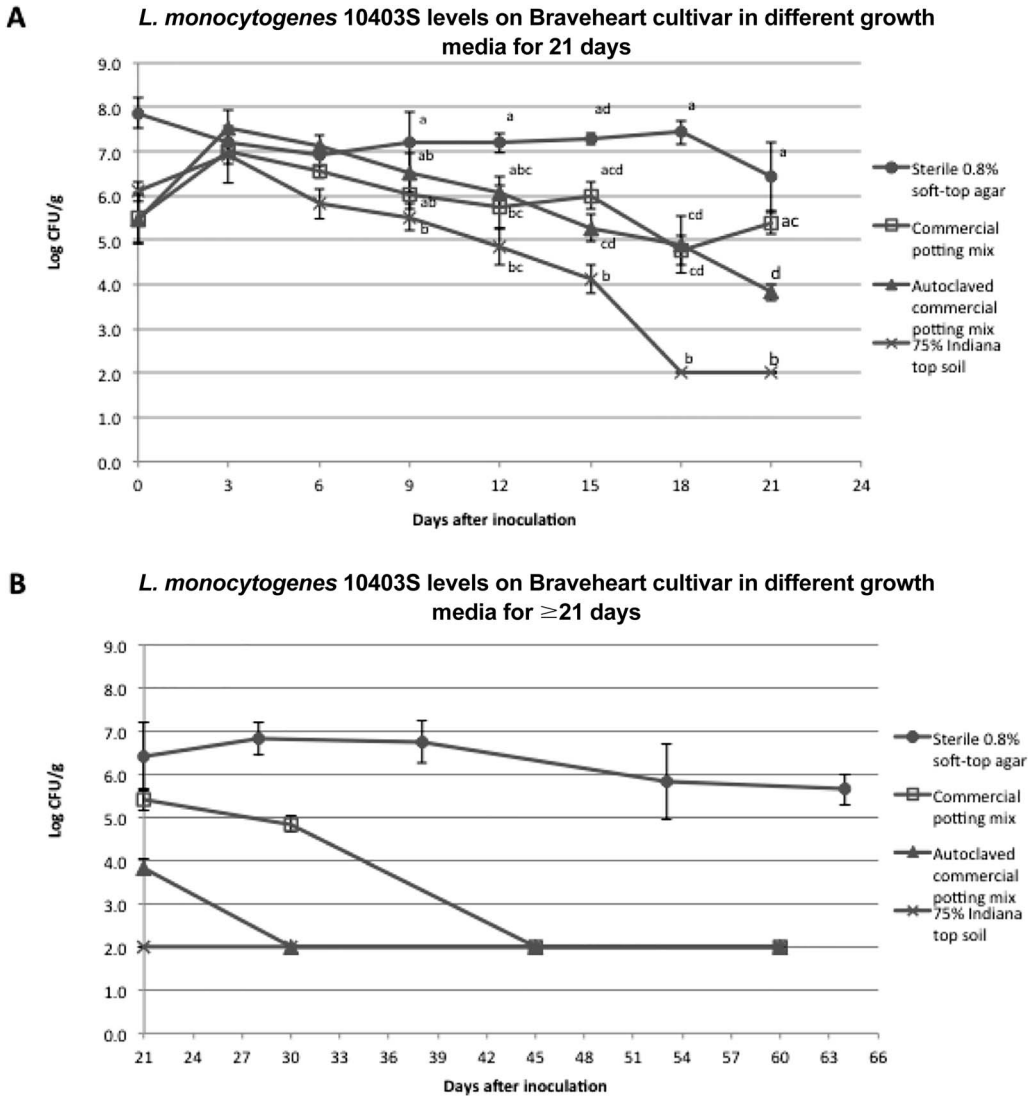

FIGURE 3. *Effect of growing medium (soft-top agar, commercial potting mix, autoclaved commercial potting mix, and 75% topsoil) on Braveheart cultivar inoculated with* L. monocytogenes *10403S for a 21-day period (A) versus a 60-day period (B) grown in greenhouse conditions. Data points for each day reported are based on the average CFU per gram values of five replicates. Error bars denote standard deviations. Distinct letters in (A) represent statistically significant differences between data points for a specific day (Tukey's honestly significant difference;* $P < 0.05$*). Values reported at 2.0 log CFU are below the detection limit.*

monocytogenes cells found in any unidentifiable or inadequately differentiated tissue type were classified as unknown, which often occurs in sections at the beginning or end of the hypocotyls. There was no significant difference among plants. Pairwise comparisons showed all combinations of known tissue types were significantly different ($P < 0.05$), except for the epidermis compared with phloem and cortex compared with xylem. No fluorescence was detected from controls. The majority of *L. monocytogenes* cells (34.1%) were localized in the pith, which is the innermost part of the stem. Any *L. monocytogenes* located in this region would be especially difficult to mitigate using current lettuce-sanitizing processes. Internalized bacteria were also found in the xylem whose purpose allows for a continuous route of transport of water and minerals from the roots of the plant through to the leaves, allowing *L. monocytogenes* transmission throughout the plant, where it then has the potential to migrate into other tissue types. Other internalization studies have also corroborated pathogen internalization and movement through plant vasculature *(16, 29)*. Future studies combining lower inoculums and fieldlike growth conditions (e.g., topsoil) are needed to further investigate *L. monocytogenes* transmission and persistence in romaine lettuce.

***L. monocytogenes* localized similarly in apoplastic and symplastic romaine lettuce tissue.** Using immunohistochemistry and microscopy, we differentiated apoplastic (extracellular) or symplastic (intracellular) localization for *L. monocytogenes* cells (Table 1). We hypothesized that there would be a higher proportion of *L. monocytogenes* localized in symplastic compared with apoplastic plant tissue; we anticipated that the observed ratio would be 2:1, based on previous studies *(10, 11)*. However, a ratio of 1:1.07 symplastic to apoplastic localization was found, which indicates that the mechanism

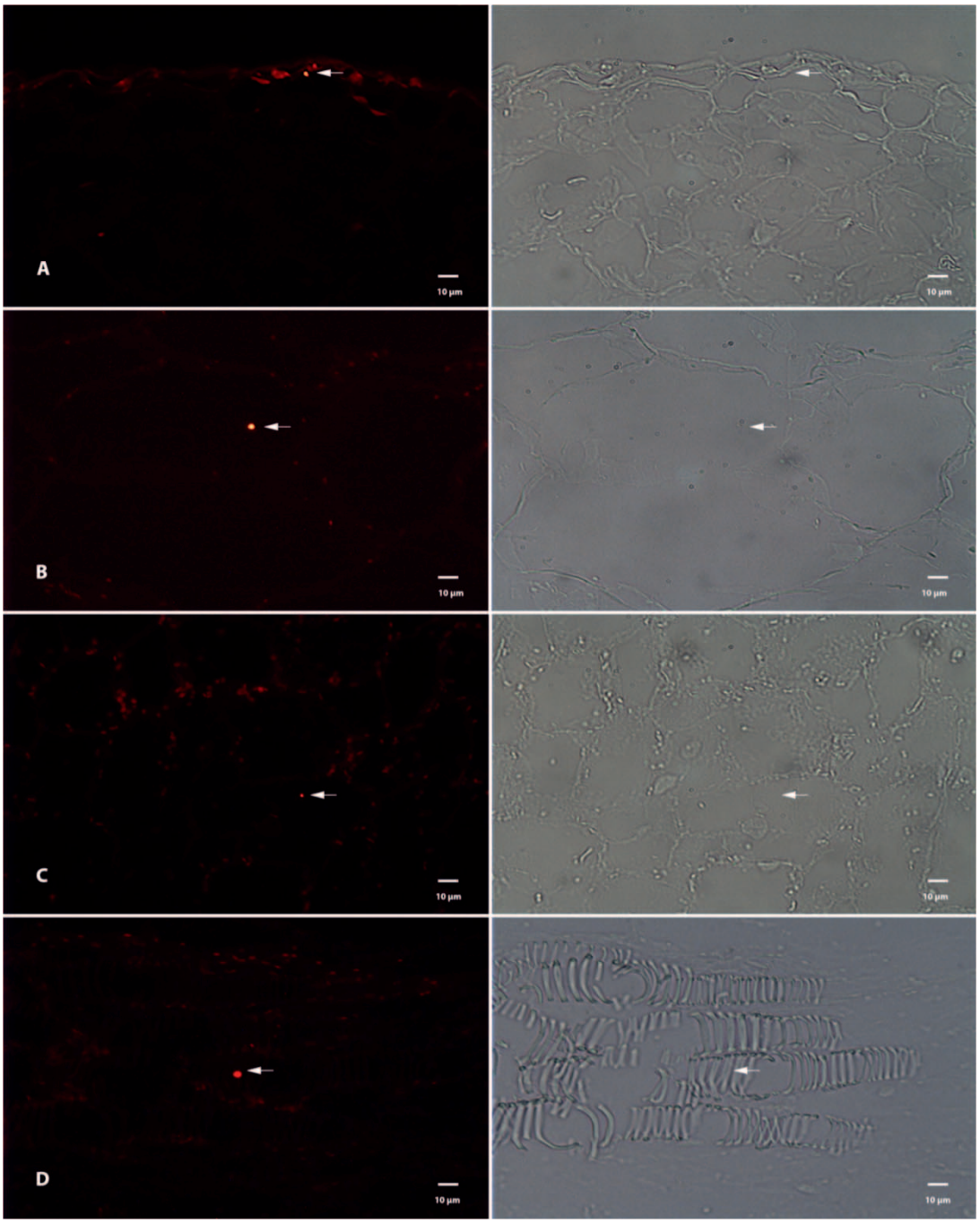

FIGURE 4. *Fluorescence and brightfield paired images of* L. monocytogenes *internalized in romaine lettuce tissue grown in potting soil in greenhouse conditions.* L. monocytogenes *was able to internalize into every major tissue and associated cell type of the hypocotyl section following contamination (initial starting concentration of approximately* 10^8 *CFU/ml) for 30 min. The arrow points to the location of the bacterium in each set of paired micrographs. The letters represent the following tissue types: (A) epidermis; (B) cortex; (C) pith; and (D) vascular tissue. Scale bar = 10 µm.*

of internalization in romaine lettuce differs from mammalian cells, though the mechanism remains largely unknown. Young seedlings lack developed defense mechanism or essential protective barriers, such as the Casparian strip, to prevent entry of bacteria. Without the Casparian strip, the passive flow of water and solutes is not blocked, and bacteria can be pulled into the plant, gain entry to the xylem, and potentially be transported through the rest of the developing plant *(31)*. Alternatively, *L. monocytogenes* could have entered through cracks in the seed coat or at sites of emerging lateral roots before or during germination and could have spread to the various tissue types simply by being present at tissue-differentiating sites.

This study demonstrates that *L. monocytogenes* is able to persist and internalize in romaine lettuce tissue in laboratory conditions. A relatively short exposure time of 30 min can result in contaminated seeds, which then grow into contaminated plants and can harbor internalized bacteria. This study and others suggest that reliance on surface sanitization may be an insufficient intervention

TABLE 1. L. monocytogenes *hypocotyl localization by tissue type*

Tissue type[a]	*L. monocytogenes* count by tissue type, no. (%)[b]	No. (%) by location in the plant cell:	
		Apoplastic[c]	Symplastic[d]
Epidermis	24 (4.5)	16 (66.7)	8 (33.3)
Cortex	113 (20.9)	59 (52.2)	54 (47.8)
Pith	184 (34.1)	102 (55.4)	82 (44.6)
Xylem	93 (17.3)	35 (37.6)	58 (62.4)
Phloem	48 (8.9)	27 (56.3)	21 (43.7)
Unknown[e]	77 (14.3)	Not determined	Not determined
Total	539 (100)	239 (44.3)	223 (41.4)

[a] Romaine lettuce tissue type.
[b] *L. monocytogenes* cells in a specific tissue type expressed as a total of all *L. monocytogenes* cells found, regardless of tissue type.
[c] Apoplastic *L. monocytogenes* cells in or touching the plant cell wall within a specific tissue type.
[d] Symplastic *L. monocytogenes* cells clearly within the boundaries of the plant cell wall.
[e] *L. monocytogenes* cells that could not be clearly classified into a tissue type or location within plant cell due to regions of undefined or undifferentiated tissue.

strategy, which warrants consideration of new alternatives. Minimizing exposure of seeds to human foodborne pathogens, such as *L. monocytogenes,* is a feasible preventive measure. Other recognized prevention methods, such as those outlined by Good Agricultural Practices, should continue to be implemented. Investigating other routes of contamination (e.g., through stomata) are critical to understanding the mechanisms that lead to foodborne pathogens internalized within plant tissues. Future field studies are needed to further understand the true risk associated with contaminated seeds.

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