The Endophytic Lifestyle of *Escherichia coli* O157:H7: Quantification and Internal Localization in Roots

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ABSTRACT

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The foodborne pathogen *Escherichia coli* O157:H7 is increasingly associated with fresh produce (fruit and vegetables). Bacterial colonization of fresh produce plants can occur to high levels on the external tissue but bacteria have also been detected within plant tissue. However, questions remain about the extent of internalization, its molecular basis, and internal location of the bacteria. We have determined the extent of internalization of *E. coli* O157:H7 in live spinach and lettuce plants and used high-resolution microscopy to examine colony formation in roots and pathways to internalization. *E. coli* O157:H7 was found within internal tissue of both produce species. Colonization occurred within the apoplast between plant cells. Furthermore, colonies were detected inside the cell wall of epidermal and cortical cells of spinach and *Nicotiana benthamiana* roots. Internal colonization of epidermal cells resembled that of the phytopathogen *Pectobacterium atrosepticum* on potato. In contrast, only sporadic cells of the laboratory strain of *E. coli* K-12 were found on spinach, with no internal bacteria evident. The data extend previous findings that internal colonization of plants appears to be limited to a specific group of plant-interacting bacteria, including *E. coli* O157:H7, and demonstrates its ability to invade the cells of living plants.

Additional keywords: endophyte, rhizosphere.

Fresh produce (minimally processed fruit and vegetables) is now recognized as an important source of foodborne pathogens; in particular, verotoxigenic *Escherichia coli* (VTEC) and the O157:H7 serogroup (47). Contaminated fruit and vegetables account for ≈20% of all reported outbreaks of VTEC (27,47) and notable outbreaks have occurred from spinach (11), lettuce (12,26), and sprouted seed (8). Contamination of produce can occur at any point in the food chain, although contaminated irrigation water and contamination from farm or wild animals are thought to be important sources preharvest on growing crops (14,45). Preharvest contamination is likely to result in the presence of bacteria in the soil, where they can exploit the relatively favorable environment of the rhizosphere (1,4). Recent evidence has shown that human-pathogenic enterobacteria can colonize plants as alternative hosts (4,6,31) and can enter plant tissue, where they may be protected from postharvest sanitation processes (18,21), posing a potential health risk. From the protected niche of the rhizosphere, colonization of the roots and, potentially, aerial plant tissue can occur, as reported for VTEC (7) and other endophytic bacteria (25,28,29,46).

Recent reviews have highlighted the ability of foodborne pathogens to internalize into a wide range of plant hosts in both roots and leafy tissue (18,30). A number of these studies have examined internalization of the bacteria in produce that has been harvested and processed (e.g., bagged salad leaves) (5,48,54). Although these studies are useful in indicating which parts of the plant tissue the bacteria can access and, as a consequence, have important implications for postharvest contamination control, the interactions between bacteria and actively growing plants are likely to be different. First, immune recognition of enteric bacteria has been demonstrated for *Arabidopsis*, *Medicago*, and *Lactuca* hosts, with some bacteria species-specific differences (33,38,49,50,52). Second, it is likely that there will be differences in how the bacteria adapt to living, preharvest plants compared with processed plant material. For example, several studies have shown that distinct subsets of *E. coli* O157:H7 or *Salmonella enterica* genes are expressed during interactions with plant material of various types (2,24,37,39). Finally, there may be differences in the native microflora between pre- and postharvest plants which may, in turn, affect the outcome of the potential for colonization by enterica bacteria (13,15).

Work on archetypal phytopathogen–plant model systems, such as *Pseudomonas syringae* and *Arabidopsis thaliana*, has demonstrated that the cross-talk between bacteria and plant hosts determines the outcome of bacterial colonization and is influenced by multiple plant and bacterial factors (34). With that in mind, the aim of this work was to determine the extent of internal colonization and the location of *E. coli* O157:H7 in growing plants, under conditions that reflect the interactions that occur prior to harvest. Two fresh produce plants that have been frequently associated with foodborne outbreaks were used to examine the interactions with living plants: spinach (*Spinacia oleracea*) and lettuce (*Lactuca sativa*). *E. coli* O157:H7 isolate Sakai (*stx*-) was used because of its previous association with plants (17,43). Numbers of internalized bacteria were quantified, together with a detailed microscopy examination of the pathway to and final location of bacteria within plant tissue. Comparisons were made with a closely related phytopathogen, *Pectobacterium atrosepticum*, and the laboratory strain *E. coli* K-12. Although roots of leafy vegetables are not eaten, it is important to understand the basis to the bacteria–root interactions because this niche is more likely to support higher numbers of bacteria than aerial parts of the plant under field conditions (10). Furthermore, infected roots

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may provide a source of contamination of leafy, edible tissue, either through migration of bacteria from roots to shoots or, potentially, from cross-contamination during harvest and processing and, therefore, still pose a potential health risk.

MATERIALS AND METHODS

Bacteria. *E. coli* O157:H7 isolate Sakai (*stx*-) (17), *E. coli* K-12 isolate MG1655 (9), and *P. atrosepticum* isolate SCRI_1043 (3) were routinely grown in Luria-Bertani (LB) media at 37 or 28°C, respectively, from long-term –80°C stocks stored in 20% glycerol. Growth media and media solidified with agar were supplemented with nalidixic acid (NA) (30 μg μl^{-1}) or chloramphenicol (CML) (25 μg μl^{-1}) as required, to select for antibiotic resistant bacteria or maintain plasmids, respectively. Spontaneous NA-resistant (Nal-R) variants of *E. coli* (Sakai and MG1655) were generated to determine the extent of internalized bacteria, which showed no phenotypic differences from the isogenic parent in terms of growth rate in LB media with aeration at 37 and 20°C, biofilm formation on polystyrene 96-well plates in LB media (static growth) at 37 and 20°C, and motility in LB media containing 0.4% agar at 37°C. The Nal-R variant was used to definitively recover the same bacterial strain used to inoculate plants, and Nal-R bacteria were never recovered from uninfected control plants.

Plants. Plant seeds of spinach (*S. oleracea* 'Matador') and lettuce (*L. sativa* 'Rosetta') were obtained from Suttons and Dobies (UK). *Nicotiana benthamiana* plants that express red fluorescent protein (RFP) fused to histone 2B (RFP-H2B) were kindly provided by M. M. Goodin (42). Seeds were surface sterilized in 0.2% $Ca(OCl)_2$ for 15 min, washed six times in sterile distilled water, and germinated on water agar (0.5% wt/vol). Germinated seedlings were transferred to plant culture tubes containing ≈10 ml of 0.5× Murashige Skoog liquid media (MS) (no sucrose) and grown in a cycle of 16 h of light and 8 h of darkness at 20°C for ≈14 days (spinach and lettuce) or ≈21 days (*N. benthamiana*) prior to bacterial inoculation. Micropropagated 'Estima' potato plants, obtained from GenTech Propagation Ltd., Dundee, UK), were transferred to large petri dishes containing 0.7% distilled water agar and grown at a 45° angle in a cycle of 16 h of light and 8 h of darkness at 21°C for 7 days prior to experimentation.

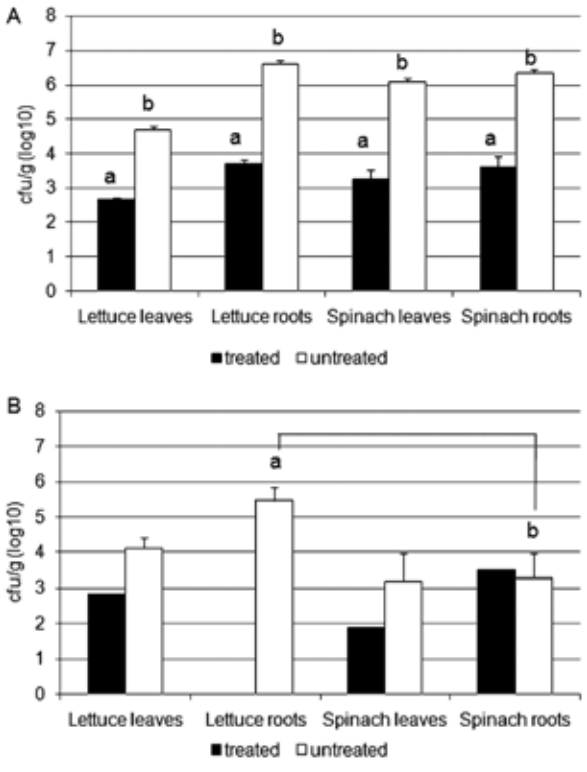

Fig. 1. Enumeration of *Escherichia coli* (Sakai and MG1655) recovered from lettuce and spinach plants. The bar chart shows the average number of bacteria (CFU g^{-1} $\log_{10}$) recovered from gentamicin-treated (black bars) and untreated plants (white bars) for **A,** *E. coli* Sakai and **B,** *E. coli* K-12 (MG1655). Bacteria were enumerated from between 11 and 13 plants per assay. Error bars represent the standard deviation and the different letters refer to data that differ significantly (with at least 95% confidence). Statistical analysis was not provided for *E. coli* MG1655 from gentamicin-treated lettuce or spinach because only single plants contained detectable bacteria in each case. Uninfected control plants did not contain any nalidixic-acid-resistant bacteria (not shown).

Plant inoculations. Bacteria were subcultured from LB media overnight cultures into 3-(N-morpholino)-propanesulfonic acid media supplemented with 0.2% glucose and amino acids (44) and grown with aeration at 18°C to an optical density at 600 nm of ≈2.5. Plant seedlings were inoculated at the roots by replacing the plant growth media with ≈10 ml of bacteria suspended in 0.5× MS liquid media and adjusted to a final concentration of $\approx 2 \times 10^7$ CFU ml^{-1}. A relatively high inoculum was used as standard to obtain reproducible experimental infections. For quantification of internal bacteria, spinach and lettuce plants were surface sterilized 10 days postinfection (dpi) by submersion of the whole plant in gentamicin solution (50 μg μl^{-1}) for 2 h at room temperature, followed by three washes in sterile distilled water and three washes in sterile phosphate-buffered saline (PBS). The method was similar to that used previously for surface sterilization of spinach leaves (48). Treatment of spinach with gentamicin at 200 μg/ml did not significantly change the numbers of bacteria recovered from roots, whereas the number of bacteria recovered from leaves was reduced by 1.7 ($\log_{10}$). Verification of the surface sterilization was obtained by placing the gentamicin-treated and washed plants on LB media solidified with agar for several minutes, to obtain plant "imprints": the data from any plants with imprints that produced colonies after incubation of the agar at 37°C for 18 h for *E. coli* was discarded from subsequent analysis. However, this was a relatively rare event, resulting in only one or two "discards". Plant tissue was separated below the crown (termed "roots") and just below the cotyledons (termed "leaves"), and macerated with a mortar and pestle in 1 ml of PBS buffer. Serial dilutions (10-fold) of the suspension were made in PBS buffer and incubated on LB-NA media solidified with agar at 37°C for 18 to 24 h for *E. coli*. Data were obtained from at least 10 untreated plants and processed as described above (except that they were submerged in water for 2 h instead of a gentamicin solution), and the assays were repeated between two and three times, on independent occasions. Resultant colony count data were analyzed with a two-sample *t* test with unequal variance (MS Excel) and significance measured at the 95% confidence level.

Microscopy. Bacteria were transformed with a plasmid containing a fluorescent (green fluorescent protein [GFP]) reporter: *loc8-egfp* for *E. coli* (Sakai and MG1655) (41) or *gyrA-gfp+* for *P. atrosepticum* (this study). The *gyrA* promoter was amplified by polymerase chain reaction (PCR) with forward (5′-GCTCTAG AGCAATATAGCCCAGACGCA) and reverse (5′-GCTCTAGAA TCCCTCTACTGTATCCC) primers, and cloned into pKC26 (32) using the *Xba*I cloning site. Spinach (Matador) plants were inoculated with *E. coli* Sakai transformed with the *loc8-egfp* reporter plasmid as described above, except that the inoculated plants were maintained in 0.5× MS (no sucrose) supplemented with CML, in order to maintain the *gfp*-reporter plasmid. For potato inoculations, *P. atrosepticum* containing the *gyrA-gfp* reporter plasmid was first grown in 10 ml of LB-CML at 27°C with aeration to stationary phase ($\approx 1.0 \times 10^9$ cells/ml) and then resuspended in 10 mM Mg SO_4 to a final concentration of $\approx 2 \times 10^7$ CFU ml^{-1}. Potato plants were infected at the roots by adding 200 μl of the

P. atrosepticum bacterial suspension into the 0.5× MS CML liquid plant growth media incubated at 21°C for 10 days.

To visualize spinach and potato roots by microscopy, the roots of intact plants were rinsed three times in sterile distilled water prior to being submerged in a solution of Texas Red (2 mg ml^{-1} of saturated stock solution diluted 1:500) or 5 μM CellTracker Red (both Invitrogen, now Life Technologies, Paisley, UK) for ≈18 h at room temperature. Texas Red accumulates in cell walls, often penetrating into the cortical layer, which results in the increased prominence of cortical cells (thicker walls) compared with the epidermal cells (thinner walls). CellTracker Red frequently accumulates in plant vacuoles in a reaction probably involving glutathione S-transferase. However, this reaction is less characterized and unpredictable in the roots; hence, we used double

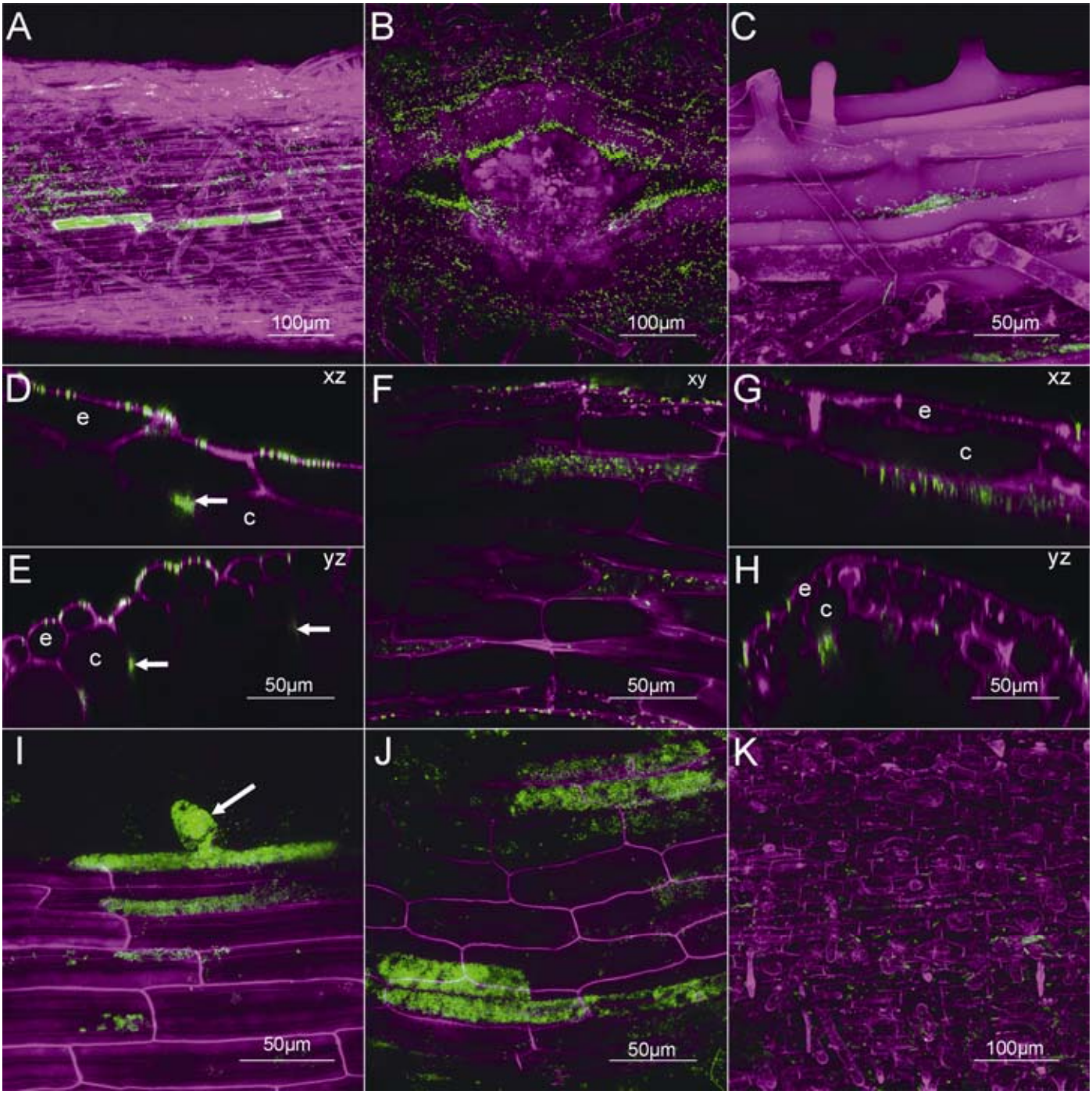

Fig. 2. A to H, Colonization of live roots following infection of spinach by *Escherichia coli* (Sakai); **I and J,** potato by *Pectobacterium atrosepticum* (SCRI-1043); and **K,** spinach by *E. coli* (MG1655). **A,** Spinach root labeled with Texas Red showing bacterial cells aligned with the position of epidermal cells 6 days postinfection (dpi) with *E. coli* O157:H7 (Sakai). **B,** At 9 dpi, *E. coli* (Sakai) bacteria have accumulated in epidermal cells surrounding an emerging lateral labeled with CellTracker Red and Texas Red, and **C,** at 10 dpi, *E. coli* (Sakai) bacteria have colonized an area between epidermal cells which have accumulated CellTracker Red in their vacuoles. **D and E,** *E. coli* (Sakai) can be seen at 10 dpi in the apoplast between epidermal and cortical cells labeled with Texas Red. **F to H,** In a 10-dpi root treated with both CellTracker Red and Texas Red, *E. coli* (Sakai) bacteria have accumulated in a large area within the cortical region. **I,** Potato root labeled with Texas Red 6 dpi with *P. atrosepticum* (SCRI-1043) showing bacteria colonizing a root hair and its subtending epidermal cell and **J,** other thin-walled epidermal cells overlying prominent cortical cell walls. **K,** Spinach root 10 dpi with *E. coli* (MG1655) with small numbers of bacteria associated with the surface of root hairs and epidermal cells. Labeled plant tissue is magenta and fluorescent bacteria are green. A to C and I to K are maximum-intensity projections of z-stacks, while D to H are single orthogonal slices in xy, xz, or yz axes. Uninfected plants did not contain any bacteria (not shown).

labeling to aid in identification of the plant cell positions. Plant tissue was either mounted in water or perfused with perfluorodecalin to improve the z-axis resolution of some plants during imaging (Aldrich, Germany) (40) prior to image capture using a Zeiss LSM710 confocal microscope (Zeiss, Germany) Samples were imaged using excitation at 561 nm and emission at 573 to 635 nm for Texas Red or CellTracker Red, emission at 578 to 613 for RFP, and with excitation at 488 nm and emission at 500 to 549 nm for bacteria expressing *gfp*. To obtain infected root tissue for embedding, whole roots were first screened by confocal laser-scanning microscopy (CLSM) to locate sections with fluorescent bacteria (no plant tissue stains were used). Segments, ≈80 mm in length, were excised, fixed, and embedded at low temperature in LR White resin, as described (35), and processed with a Leica EM AFS2. Semithin sections (≈1 μm) of roots were stained with 1% toluidine blue in 1% sodium tetraborate, examined under a light microscope, and imaged using the three-color transmission facility of the LSM710 to confirm the presence of infecting bacteria, before ultrathin sections were taken. Immunogold labeling was performed with a monoclonal O157-specific antibody (commonly used for in vitro diagnostics) (Mast Diagnostics, Bootle, UK) at 1:50 dilution and a secondary goat anti-rabbit antibody conjugated to 15 nm gold (GE Healthcare, Chalfont St. Giles, UK), followed by staining with uranyl acetate. Image capture was obtained with a JEOL JEM-1400 electron microscope (Tokyo, Japan).

RESULTS

To determine the extent of internal colonization of *E. coli* O157:H7 in fresh produce plants, spinach and lettuce plants were placed in liquid growth media containing a suspension of *E. coli* O157:H7 *stx*- (isolate Sakai). The number of bacteria was enumerated from surface-sterilized (gentamicin-treated) plants after 10 days, using a method similar to that reported by others (48). *E. coli* (Sakai and MG1655) is sensitive to gentamicin: incubation of bacteria on LB agar plates or in a solution containing gentamicin at 50 μg/ml did not result in any visible growth; therefore, bacteria that survive treatment are located within protected areas of the plant, including protected cavities (e.g., at the point of emergence of lateral roots and deeper locations with the plant tissue, where bacteria are considered to be "truly" internalized). Sucrose was omitted from the plant growth media at all times to ensure that any available carbon source was provided by the plants: no bacterial growth was observed in the media in the absence of plants. Gentamicin treatment of lettuce and spinach seedlings resulted in recovery of *E. coli* (Sakai) from both plant species and from both the roots and leafy tissue. The number of individual spinach plants supporting gentamicin-protected bacteria was higher than lettuce: 81 compared with 23%, respectively, for the leaves and 91 compared with 31%, respectively, for the roots (Fig. 1A). *E. coli* Sakai was recovered from 100% of untreated plants for both leaves and roots and both spinach and lettuce. The average number of internalized *E. coli* Sakai recovered from lettuce and spinach leaves was 2.66 and 3.24 CFU/g ($\log_{10}$), respectively, and from lettuce and spinach roots was 3.70 and 3.60 CFU/g ($\log_{10}$), respectively. These numbers approximated to 0.5% of the total population for each tissue type tested (Fig. 1A). In comparison, *E. coli* MG1655 was only recovered from single treated plants for lettuce ($n = 10$) and spinach ($n = 12$). The number of *E. coli* MG1655 on untreated spinach was significantly lower than the number recovered from untreated lettuce roots and approximately three orders of magnitude lower than that seen for *E. coli* Sakai (Fig. 1B).

E. coli (Sakai)–spinach root interactions were examined by CLSM on live, non-fixed tissue. Washed roots were not surface sterilized to allow identification of bacteria on both external and internal tissue. Six days after adding the bacterial inoculum, *E. coli* (Sakai) colonies were clearly associated with the root epidermal cells (Fig. 2A) and also observed surrounding emerging lateral roots (Fig. 2B). Staining the vacuole highlighted the formation of microcolonies between intact epidermal root cells (Fig. 2C). After 9 days, *E. coli* (Sakai) colonies had penetrated below the surface of the root and formed internal colonies in the apoplast (Fig. 2D). Furthermore, the extent of colony formation suggested that some colonies had formed within cells (Fig. 2E to H). Therefore, in order to identify whether bacteria were present within cells as well as in the apoplastic spaces, spinach root fragments that contained *gfp*-fluorescent bacteria, identified by CLSM, were fixed and embedded for high-resolution visualization of internalized bacteria. *E. coli* (Sakai) colonies were found within epidermal, root hair, and cortical cells and in the apoplast, in semithin sections (Fig. 3A to C). The identity of bacteria was confirmed as *E. coli* O157:H7 by immunogold labeling of ultrathin (≈90 nm) sections with the O157 O-antigen antibody (Fig. 3D to F). Some *E. coli* (Sakai) colonies appeared to form inside cells, internal to the cell wall but apparently outside the plasma membrane (Fig. 3B, D, and E).

Because *E. coli* O157:H7 shares ≈70% coding DNA sequence identity with the related phytopathogen *P. atrosepticum* (53), similarities in colonization between *E. coli* (Sakai) on spinach and *P. atrosepticum* (isolate SCRI_1043) on potato seedlings were examined. Colonization comparisons for the two different enterobacteria were carried out with the most relevant crop host for each: spinach for *E. coli* (Sakai) because there has been a major disease outbreak from infected spinach (23) and potato for *P. atrosepticum* because this is the main disease host for the phytopathogen (46). Cross-comparison was not carried out because the work focused on bacteria–plant interaction most relevant to food safety and agro-commerce and was not designed to compare host specificities. Nine days after infection, *P. atrosepticum* colonies were observed within epidermal cells, some with associated root hairs (Fig. 2I and J), and the pattern of colonization was very similar to that observed for *E. coli* (Sakai) on spinach. In contrast, only occasional cells or colonies of the lab strain *E. coli* K-12 (isolate MG1655) (9) were present on external tissue of spinach roots and not detected in the internal tissue (Fig. 2K).

To better determine the location of *E. coli* (Sakai) that appeared to be within plant cells (termed invasive), a fluorescently marked plant line of *N. benthamiana* was used (RFP-H2B), which contained an RFP fusion to histone 2B in the nuclei. Because transgenic plant lines with such markers are currently unavailable for spinach, a suitable surrogate was used. We have found that *E. coli* Sakai colonizes *N. benthamiana* roots to a level comparable with spinach (4.52 ± 0.66 $\log_{10}$ CFU/g). Ten days after inoculation of *N. benthamiana* RFP-H2B with *E. coli* (Sakai) transformed with p*loc8-egfp*, green fluorescent bacteria were observed within epidermal cells (Fig. 4A to C). In particular, some invasive colonies were found to be immediately adjacent to the plant nuclei, as determined from single "z-stack" image analysis (Fig. 4D to F), indicating that the bacteria had invaded the cytoplasm of plant cells.

DISCUSSION

The extent of internal *E. coli* (Sakai) has been determined for two important fresh produce species (lettuce and spinach), and the internal location has been determined in the roots of spinach and fluorescently tagged *N. benthamiana*. Surface sterilization of spinach and lettuce indicated that ≈0.5% of the total plant-associated *E. coli* (Sakai) population was found to be internalized within the tissue. Furthermore, the majority of the spinach plants tested contained gentamicin-protected bacteria. Quantification of internalized *P. atrosepticum* on potato plantlets was not possible because gentamicin treatment did not completely eradicate surface-associated bacteria (not shown). It is possible that *P. atrosepticum* produced protective biofilm that was not fully pene-

trated by the antibiotic. It is of note that, for a closely related *Dickeya* sp., colonies were not evident in epidermal or outer cortical cells following surface sterilization of potato roots with ethanol and hyperchlorite but, instead, the bacteria were associated with xylem tissue (16). Although the bacterial count for gentamicin-protected *E. coli* (Sakai) was lower than that reported for *S. enterica* on lettuce tissue (as determined after disinfection of plant surfaces with 70% ethanol), the number of plants containing internalized bacteria was similar (38). Studies with *E. coli* O157:H7-spiked soil have shown that a lower number of spinach roots contained internalized bacteria than observed in our hands (≈21% of the plants tested), which may reflect differences in the plant growth conditions as well as competition from other microorganisms that are present in the soil (51). Also, it is possible that the numbers of bacteria recovered from within tissue are an underestimate of the true number, because one study found that

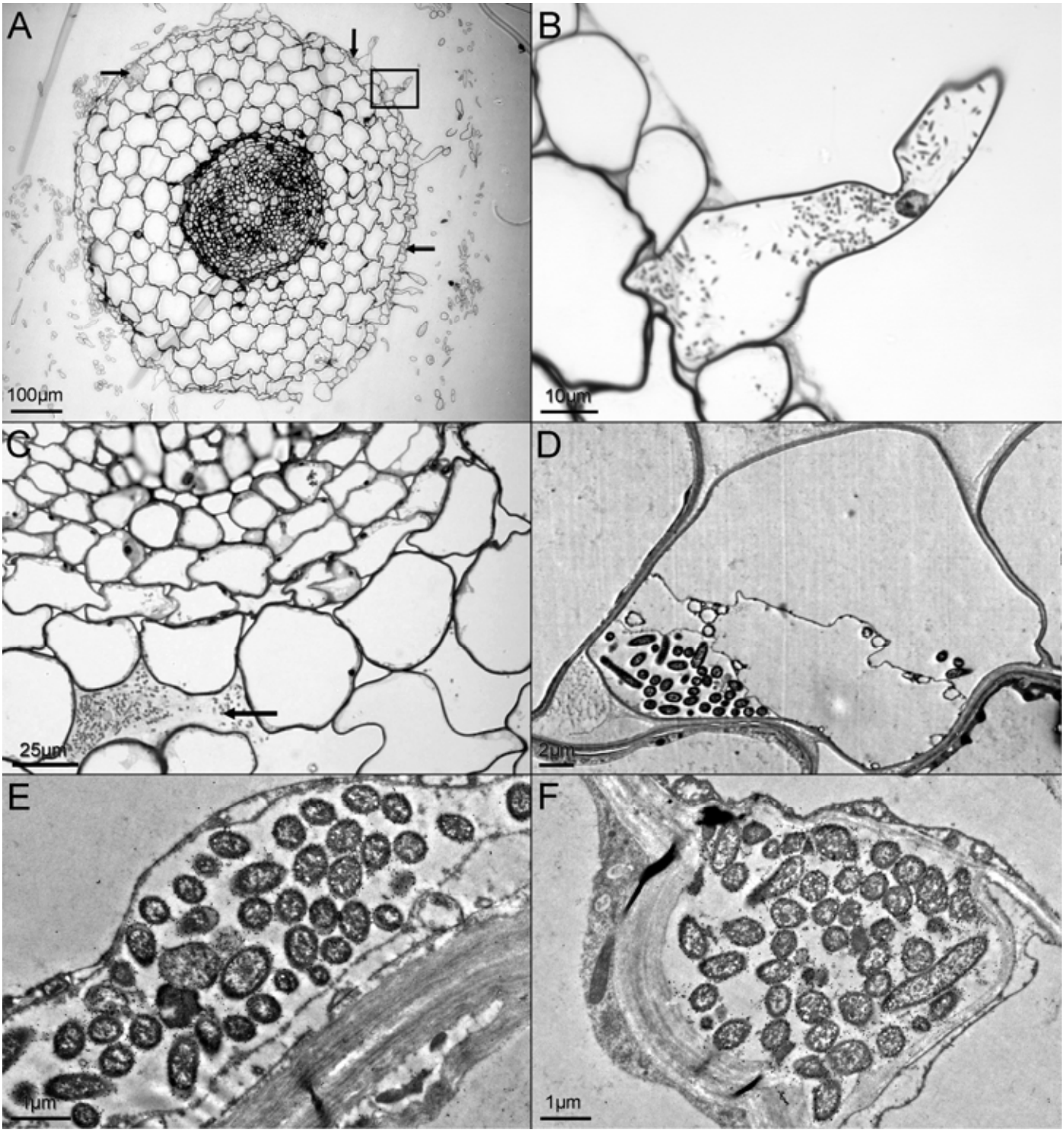

Fig. 3. A to C, Light microscopy (LM) and **D to F,** transmission electron microscopy (TEM) of infected spinach root sections at 10 days postinfection. **A,** LM of *Escherichia coli* (Sakai), at different magnifications in epidermal cells and **B,** a root hair and **C,** an apoplastic space. EM of *E. coli* (Sakai) in **D,** an epidermal cell; **E,** a cortical cell; and **F,** an apoplastic space. Black dots at the periphery of bacterial cells in E and F result from immunogold-labeling with O157 O-antigen-specific antisera followed by a secondary antibody conjugated to gold particles (15 nm). Negative controls without the primary antisera showed no gold labeling of bacterial cells (not shown). Observations were made using three-color transmission imaging on the Zeiss LSM710 (A to C) or a TEM (JEM-1400) (D to F).

direct enumeration of *S. enterica* serovar Typhimurium from parsley leaves resulted in recovery of as little of 1% of the initial inoculum, despite the fact that ≈40% of the initial inoculum was still viable on leaves. The low level of recovery was thought to have occurred as a result of changes in culturability, irreversible attachment to tissue, or cell death (36). For direct enumeration, maceration with a mortar and pestle (as used here) proved to be the most sensitive method.

Microscopy analysis showed that *E. coli* (Sakai) formed colonies on internal and external plant tissue, and that the shape of the *E. coli* (Sakai) colonies on spinach roots approximated the same shape as the plant epidermal cells, similar to that seen for *P. atrosepticum* on its host, potato (compare Fig. 2A and B with I and J). In addition, both species colonized sporadic epidermal cells rather than every cell. Furthermore, both species were found within root hairs. The pattern of colonization for the lab strain of *E. coli* K-12 on spinach appeared to be completely different, with few cells or colonies present. Microscopy examination of spinach infected by *E. coli* (Sakai) showed a pattern of colonization of the extracellular apoplast as well as an intracellular localization within epidermal and cortical cells, which was similar to other plant-associated endophytes, such as *Serratia* spp. (28). This raises the possibility that there are common mechanisms of plant colonization. Plant cells that contained invasive bacteria showed retraction of the cytoplasm, which may have occurred as a result of bacterial invasion. However, it cannot be ruled out that it was as a result of tissue processing during embedding. Alternatively, it may indicate that the plant cells were dead or senescing prior to colonization. Nevertheless, the observation that intracellular bacteria were able to penetrate *N. benthamiana* cells as far as their

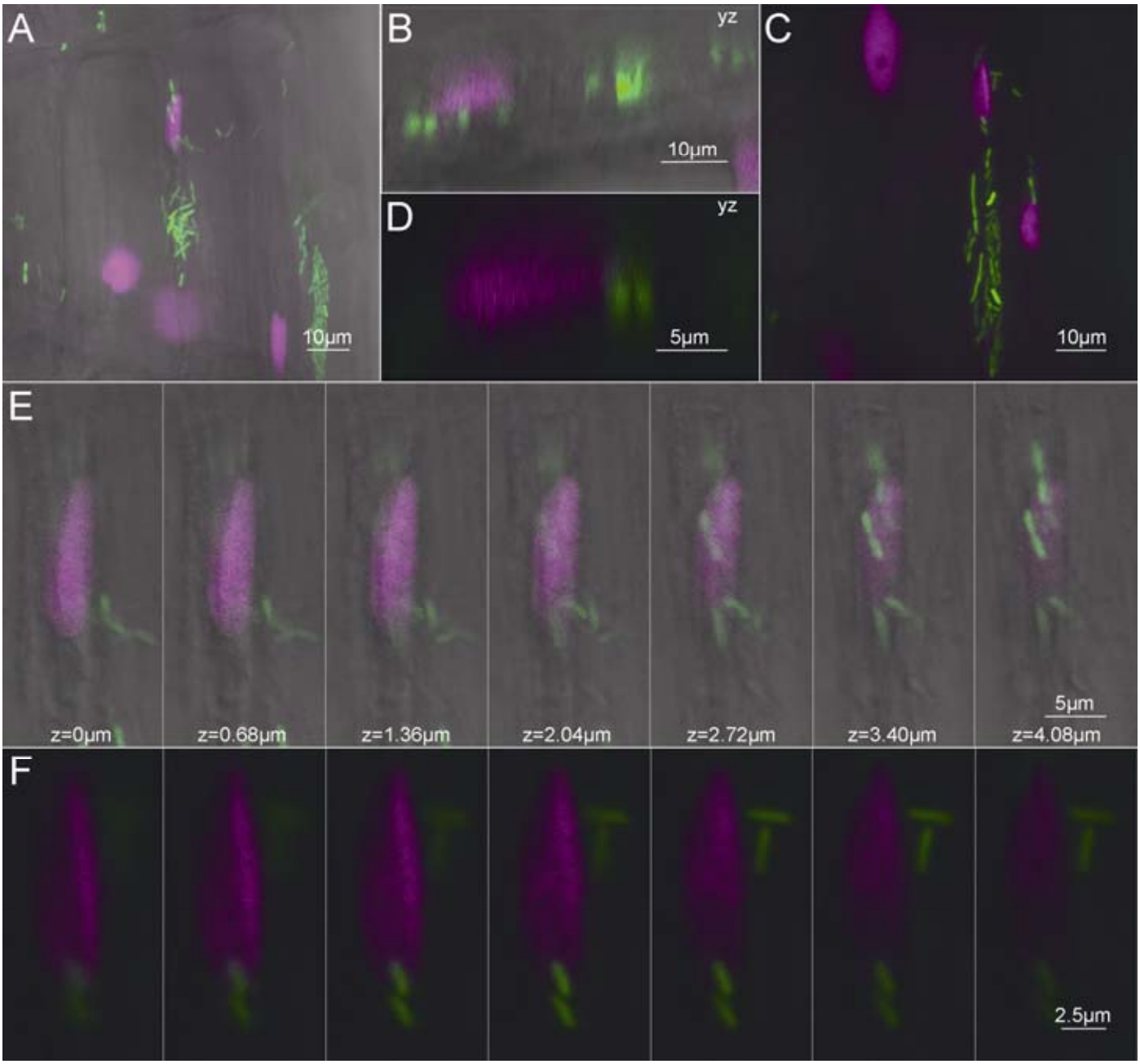

Fig. 4. Colonization of live roots of *Nicotiana benthamiana* RFP-H2B 10 days after infection by *Escherichia coli* (Sakai). Colonization of epidermal cells as observed in **A,** a maximum intensity projection and **B,** a single yz orthogonal slice showing bacteria adjacent to and underlying the nucleus of an epidermal cell. *E. coli* (Sakai) cells colonizing an epidermal cell observed in **C,** a maximum intensity projection and **D,** a single yz orthogonal slice. **E and F,** Individual xy sections of the nuclei illustrated in A and C, respectively, indicate the relative positions of the bacteria and nuclei, with the relative z-depths applying to both E and F. Fluorescence from red fluorescent protein is magenta and from bacteria is green.

fluorescent nuclei (Fig. 4) raises the possibility that the bacteria can enter live cells and are the cause of subsequent cell death.

Previous related studies have shown apoplastic colonization of VTEC within leafy tissue (18,19,48) and associated with root hairs in spinach (51) as well as apoplastic colonization of *S. enterica* in growing lettuce plants (38). However, this study shows bacterial accumulation within the root cells of living plants. Furthermore, we were able to unequivocally identify *E. coli* O157:H7 in the apoplast and within plant cells using O-antigen labeling. Intracellular invasion of mammalian cells by VTEC is considered to be relatively rare, although invasion has been quantified in bovine cells (20), where bacteria are contained in a membrane-bound vacuole within the cytoplasm (22).

Colonization of the roots by *E. coli* (Sakai) appears to follow a process similar to that of some endophytic bacteria, including *Klebsiella pneumoniae* and *Pectobacterium* spp., which exploit natural openings, such as emerging lateral roots (21,46). Some endophytes have been shown to reside within root intercellular spaces as well as within dead cortical cells and are able to translocate within the vascular system to the aerial parts of the plant (28,29,46). Whether such translocation of *E. coli* (Sakai) also occurs from the roots to aerial parts of the plant is the subject of ongoing research.

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