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Visualizing pathogen internalization pathways in fresh tomatoes using MicroCT and confocal laser scanning microscopy☆

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ABSTRACT

Pathogen contamination of fresh produce significantly impacts public health and consumer confidence in eating fresh fruits and vegetables. In tomato fruits, studies have shown that the stem scar plays an important role in pathogen infiltration. However, the exact mechanisms and pathways for pathogen movement inside tomatoes are not well documented. This study examined in detail the pathway of pathogen internalization in tomato fruit. Tomatoes were infused with potassium iodide and examined with Xradia Bio MicroCT at 0.5×~4×. Micro CT images revealed that infiltrated potassium iodide moved along the vascular bundles inside the fruit. In separate trials, tomato fruits were further infiltrated with red fluorescent microspheres and *E. coli* O157:H7 -pGFP. Stem scars and core tissues were subsequently excised after fruit surface disinfection and observed using a Zeiss 710 laser confocal microscope, or stomached, cultured, and enumerated for infiltrated *E. coli* O157:H7. Populations of internalized *E. coli* O157:H7 were confined in xylem vessels and concentrated immediately beneath the stem-scar, with a sharp decline in population with vertical distance from the stem-scar. Our observations suggest that 1) vascular bundles, especially the xylem vessels in tomato fruit, play a critical role in pathogen internalization, and 2) pathogen internalization is a passive, rather than active process, as the infiltration and movement of both microspheres (non-living) and *E. coli* O157:H7 cells (living) behaved similarly inside tomatoes. This study presents visual evidence of the critical role of vascular bundles in pathogen internalization in tomato fruit using Micro CT and Confocal Laser Microscopy. The finding is important for developing science-based food safety practices, interventions in controlling pathogen internalization, and new tools for probing pathogen-plant interactions.

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1. Introduction

Food-borne illness outbreaks associated with consumption of tomatoes contaminated with human pathogens negatively impact human health and beset the tomato industry with economic losses (Bennett, Littrell, Hill, Mahovic, & Behravesh, 2015; Ribera et al., 2012). Contamination of tomatoes may happen in the field via contaminated irrigation water (Greene et al., 2008; Hintz, Boyer, Ponder, Williams, & Rideout, 2010), contaminated pesticide solutions (Guan, Blank, & Holley, 2005), or contaminated wind-borne soil/manure particles (Kumar et al., 2017). Contamination of the blossoms may lead to contaminated tomato fruit (Zheng et al., 2013). However, postharvest handling may also result in contamination and cross-contamination of tomatoes with human pathogens (Bennett et al., 2015; Warning, Datta, & Bartz, 2016; Xia et al., 2012). Food-borne illness investigations associated with tomato fruit suggest that contaminated wash water in packing facilities is one of the main factors contributing to microbial contamination (Hedberg et al., 1999; Sivapalasingam, Friedman, Cohen, & Tauxe, 2004). It is well known that food-borne human pathogens can attach to produce surfaces, especially wrinkled surfaces and

☆ Mention of trade names or commercial products in this article is solely for the purpose of providing specific information, and does not imply recommendation or endorsement by the US Department of Agriculture.

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crevices, and internalize into inner tissues through wounds and natural openings (Barak and Liang, 2008; Barak, Liang, & Narm, 2008; Chen & Zhu, 2011; Takeuchi & Frank, 2000). Once internalized, removal or inactivation of the pathogen without compromising the produce quality becomes almost impossible (Warriner, Huber, Namvar, Fan, & Dunfield, 2009). Pathogens on tomatoes can further grow during post-harvest handling and storage (Zhou et al., 2014a).

Harvested tomatoes are often washed in the dump tank to remove soils and debris (Zhou et al., 2014b). The waxy skin of tomato fruits is relatively resistant to pathogen infection, but the stem scar is vulnerable to pathogen attachment, colonization, and internalization (Beuchat, Nail, Adler, & Clavero, 1998; Xia et al., 2012). Earlier studies on freshly harvested tomatoes have shown that a much higher percentage of microorganisms were isolated from the stem scar and the area directly beneath the stem scar, than from the locule and pericarp tissues (Samish & Etinger-Tulczynska, 1963a, 1963b, 1961; Xia et al., 2012). Earlier studies on the plant disease, soft rot, has shown that the plant pathogen, *Erwinia carotovora,* may have been internalized through water uptake via tomato stem scars (Bartz, Schneider, Sargent, & Felkey 2002; Smith, Scott, & Bartz, 2006; Smith, Scott, Bartz, & Sargent, 2008). However, no visual evidence has been presented to show that human pathogens such as *Salmonella* have been internalized through water uptake. Furthermore, no studies have shown whether pathogens have played an active or passive role during internalization.

Feldkamp, Goldstein, Parfitt, Jesioil, and Kleerekoper (1989) introduced MicroCT as a scanning technology to detect small structural defects in human cancellous bone. The material to be evaluated is scanned and a series of 2D slices are generated. A 3D-reconstruction array is created by stitching together the 2D images. With measures taken to provide contrast between vascular and background tissues, MicroCT scanning could be used as a non-destructive method to visualize pathways of pathogen infiltration in tomato fruit. Aqueous dye solutions, GFP-tagged bacteria, and florescent microbeads have been employed as indicators of bacterial infiltration into produce (Buchanan, Edelson, Miller, & Sapers, 1999; Niemira, 2007). Confocal laser scanning microscopy (CLSM) with fluorescent-labeled microspheres or bacteria has been utilized as a major optical tool for visualizing microbial internalization. CLSM is designed to obtain in-focus high-resolution images from selected depths through optical sectioning and can provide highly accurate images with minimum sample preparation, as well as improved lateral resolution. Kroupitski et al. (2009) used CLSM to observe GFP-tagged *Salmonella enterica* on iceberg lettuce leaves and reported that bacteria aggregated near open stomata and invaded the inner leaf tissue. Macarisin, Bauchan, and Fayer (2010) utilized techniques of CLSM and scanning electron microscopy to discover that leaf stomata were critical sites harboring Cryptosporidium parvum oocysts, and posed a potential threat for food safety. They (2013) also discovered using CLSM and scanning electron microscopy that surface roughness plays a major role in adhesion of *E. coli* to spinach leaf surfaces. Using CLSM, Burnett, Chen, and Beuchat (2000) found preferential cellular attachment of *E. coli* O157:H7 to discontinuities in the waxy cuticle of apple external surfaces, seeds, cartilaginous pericarp, and internal trichomes and to damaged tissues surrounding puncture wounds. Infiltration of the pathogen into apples was observed to occur through the floral tube. Warning et al. (2016), using the techniques of Micro CT and scanning electron microscope (SEM), found that there are many wide openings in the stem scar, and a porous, connected network exists from the openings into the tomato. The pore size of these openings is much larger than the dimension of bacterial cells, and could provide an initial access for water and bacteria to penetrate into the tomato.

This study reports the first use of high resolution imaging technologies to visualize the pathogen internalization and movement inside tomato fruits. Findings will answer two key questions: the primary route and pathway of pathogen internalizations in tomatoes; and 2) the role of bacteria during internalization, active vs. passive.

2. Materials and methods

2.1. Tomato variety and harvesting

Mature green tomatoes (*Solanum lycopersicum* L., cv. BHN961) were harvested with intact stems from a commercial producer in Beltsville, MD. Tomatoes were sorted to remove samples with visible defects or damage. Stems were manually removed prior to inoculation, and care was taken to ensure that no damage occurred to fruit during stem removal. All tomatoes were kept overnight at 32.2 °C to ensure uniform tomato fruit tissue temperature.

2.2. Inoculum preparation

E. coli O157:H7 strain (CDC B6914/pGFP, AmpR) was obtained from USDA, ARS, Environmental Microbial and Food Safety Laboratory collections, and grown overnight in trypticase soy broth (TSB, Becton Dickenson & Co., Sparks, MD) supplemented with 100 μg/ml ampicillin at 37 °C. Cells were washed once in phosphate buffered saline (PBS) before adding 2.3 mL of this culture to 7.0 L of distilled water to prepare inoculum with the targeted final concentration of 10^5 CFU/ml. Fluoresbrite® Polychromatic Red Microspheres (1.0 μm, 2.5% solids [wt/vol], 4.55×10^{10} particles/ml; Polysciences, Inc., Warrington, PA) were diluted 1:1000 in phosphate buffered saline (PBS).

2.3. E. coli O157:H7 internalization evaluation

Tomato temperatures were brought to 32.2 °C (90 °F) overnight to ensure uniform tomato pulp temperature. Sterile distilled water was maintained at 26.6 °C (80 °F) to generate a temperature difference of −5.6 °C (−10 °F) between tomato pulp temperature and the *E. coli* suspension. Four batches of five tomatoes each were sequentially submerged in the *E. coli* suspension at a depth of 12 cm for 30 min under a plastic net weighted down with a plastic Nalgene bottle filled with water. To ensure consistent testing conditions, care was taken to ensure that all tomatoes were immersed in the *E. coli* suspension with the stem scar facing downwards, and the submergence depth was equivalent to 2 layers of tomatoes. Fruits were removed from the bacterial suspension after 30 min and surface-sanitized with paper towels saturated with 70% ethanol, followed by excising the internal core tissues beneath the stem-scar. A longitudinal cut was made at the shoulder of the tomato to expose the internal tissue. This was followed by two latitudinal cuts initiating from the 1st cut surface, at approximately 2 mm and 27 mm below the stem-scar. A cylindrical plug of tomato core tissue beneath the stem scar was obtained from the slab of the tomato obtained by the 3 cuts described above, by pushing a cork borer from the less contaminated area towards the more contaminated stem scar. The core tissue plug inside the cork borer was then removed by pushing (with a sterile cotton swab) in the reverse direction. The core tissue was sequentially cut into 5 mm segments from the stem scar to the internal edge, making a total of 5 segments. In order to avoid any potential for contamination, all items that came into contact with tomatoes were pre-sterilized and a different identical blade was used for each cut to minimize the risk of contamination and experimental error. The internal tomato tissue samples obtained as described above were placed in a sterile

stomacher bag and mixed with gentamicin (50 μg/ml)-supplemented TSB at 10 times the sample weight. The samples were homogenized for 2 min using a stomacher blender (Seward 400 Biomaster, Brinkmann Seward, Ontario, Canada). The bacterial concentration in the filtrate was enumerated with a microplate-based 8-well most probable number (MPN) method as previously described (Nou & Luo, 2010) with modifications. In brief, four 2.0 ml aliquots were serially diluted in ampicillin (100 μg/ml)-supplemented TSB in a deep-well microplate and incubated at 37 °C overnight. This was followed by plating 3.0 μl droplets of all dilutions on ampicillin (100 μg/ml)-supplemented TSA media. Colonies were confirmed as pGFP by the presence of green fluorescence under UV light. Cell populations were determined using MPN software program available online (Curiale, 2004). The internalization incidence in each replication was calculated based on the number of confirmed positive samples over the total number of tomatoes.

2.4. Observation of dye infiltration into tomatoes

Tomatoes were held in 1% methylene blue (MB) dye solution for 30 min at a depth of 20 inches below the solution surface. The temperature of MB solution was 10 °C colder than tomato pulp temperature as described above. The tomatoes were then cut in half longitudinally, starting at the stem end and cutting across the stem scar using a sterile knife. Images of the cut surfaces were captured optically using a digital camera (Nikon MB-E5000; Nikon, Melville, N.Y.).

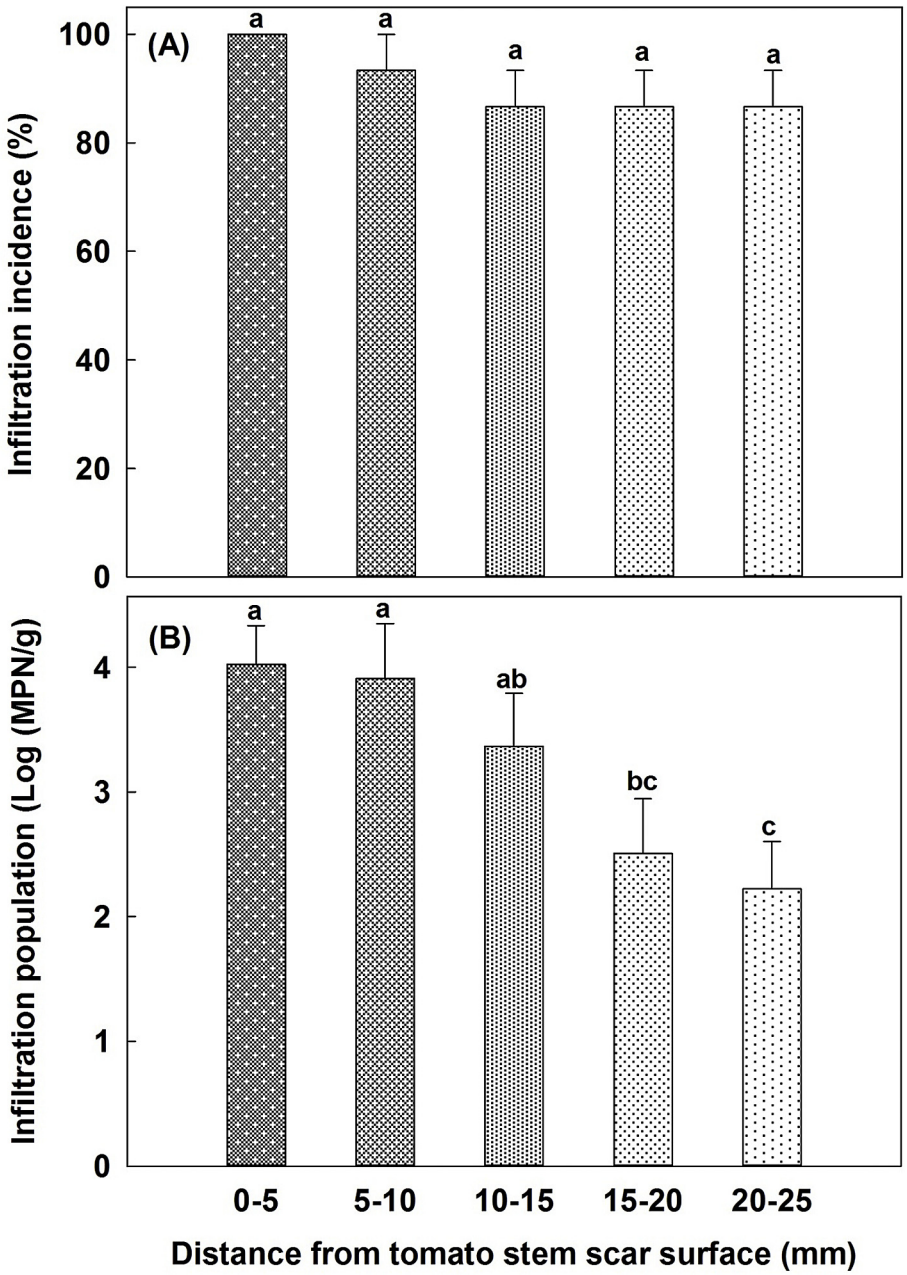

Fig. 1. Incidence (A) and population density (B) of *E. coli* O157: H7 inside tomatoes along with the core tissue segment underneath the stem-scar.

2.5. Non-destructive tissue visualization using Xradia Bio MicroCT

Tomato stem scars were infiltrated with 0.25 ml of 120 mg/mL potassium iodide and incubated for 1 h. A 10 × 30 mm segment around the stem scar was excised from the tomato fruit, inserted into polyimide tubing, and scanned by a three-dimensional (3D) Xradia Bio MicroCT (MicroXCT-400, Xradia Inc. Concord, CA) at 0.5×~4×. The objects were viewed in 3D using computed tomography (CT) with virtual slices spaced at 1 μm, which enabled illustrating bulk structure in nonhomogeneous tissues with density variations. An X-ray tube with a tungsten anode setting of 40 kV at 4 W was adopted for the transmission of X-ray images of the specimens. The 3D images were constructed using 720 images taken at 10 s integration time per image. By collecting a series of two-dimensional images through the depth of a sample, a highly accurate three-dimensional image of the entire sample was reconstructed using Amira 5.6.0 software (FEI Visualization Sciences Group, Bordeaux, France).

2.6. Visualizing internalization of polychromatic red microspheres and E. coli O157:H7 using confocal laser scanning microscopy (CLSM)

Stem scars were infiltrated with 250 μl of Fluoresbrite® Polychromatic red microspheres immediately after the stems were removed, and incubated at 4 °C for 1 h. A cube (11 mm × 11 mm × 11 mm) of tomato tissue containing the stem scar was excised and sliced using a sterile knife, either transversely or longitudinally into thin sections (thickness = 0.5–1 mm), and placed in PELCO® glass bottom dishes (Ted Pella, Inc., Redding, CA) containing enough glycerol to submerge the sample. The tomato slice was covered with a glass cover clip (Electron Microscopy Sciences, Hatfield, PA), and scanned using a Zeiss 710 CLSM system (Carl Zeiss MicroImaging, LLC, Thornwood) according to Macarisin et al. (2010; 2013). The images were observed using a Zeiss Axio Observer inverted microscope with Plan-Apochromatic objectives: 10 × 0.45 NA and 25 × 0.8 NA. Dual differential interference contrast (DIC) and fluorescent CLSM images were acquired. A photomultiplier tube captured the light emitted from a 488-nm argon laser with a 3.7-μm pin hole passing through an MBS 488 filter with limits set between 490 and 545 nm for detection of fluorescent microspheres and between 640 and 720 nm for detection of auto fluorescence from chloroplasts. Zeiss Zen 2008 software was used to obtain images with 512 × 512 pixel resolution, 6:1 zoom, and a *z* stack of 35–60 focal planes.

For visualizing microbial internalization, the stem scars were spot-inoculated with 250 μl each of *E. coli* O157:H7 inoculum immediately after the stems were removed, and held at 4 °C for 1 h. The cube (11 mm × 11 mm × 11 mm) with stem scar was taken from an inoculated tomato. Thin longitudinal sections (thickness = 0.5–1 mm) were cut from the tomato cubes and scanned under a Zeiss 710 CLSM system under the conditions described above.

2.7. Experimental design and statistical analysis

Randomized factorial designs were employed for infiltration experiments with 3 replications. The incidence (frequency) data were arcsine-transformed, and the MPN data log-transformed, to meet the requirement for normality and variance homogeneity. Statistical analyses for all experiments were performed using the Proc Mixed model (GLM) of SAS 9.3 (SAS Inst. Inc., Cary, NC.). When effects were statistically significant, mean comparisons were performed with Fisher's least-significant-difference test to maintain experiment-wise error (α) at $\leq$0.05.

3. Results and discussion

All segments of the tomato core tissues, excised from tomato fruit after immersion in *E. coli* O157:H7 contaminated water, exhibited *E. coli* contamination (Fig. 1A). Among all tomato fruit tested, the highest internalization incidence (100% of tomatoes sampled) occurred in the segment immediately beneath the stem-scar. Incidence declined to 86% and remained relatively constant at distances further (10–25 mm) from the stem scar. Although *E. coli* incidence was high in all core segments evaluated, the population density varied significantly as a function of distance from the stem scar. The density was highest (average 4.02 Log MPN/g) in the segment immediately beneath (0–5 mm) the stem scar (Fig. 1B) and declined to 2.22 Log MPN/g on samples taken 22–27 mm below the stem-scar. This is in agreement with the data from Xia et al. (2012) for *Salmonella* species, suggesting that the stem scar is a significant entry point for food-borne pathogens, irrespective of bacteria type, either *E. coli* O157:H7 or *Salmonella* spp.

Previous studies with post-harvest diseases also indicate that tomato stem-scars play an important role in the infiltration of plant pathogens. Reports from Samish and Etinger-Tulczynska (1963a) showed a high incidence of bacterial flora in the stem scar and in central core tissues beneath the stem scar. Many factors may impact bacterial infiltration. Hydrostatic pressures on tomato fruits submerged inside the water, and temperature differences between tomato pulp and water can promote absorption of dump tank water along with bacteria in the water. Numerous studies have shown that duration of fruit submergence, tomato variety, and time after stem removal can influence pathogen infiltration (Bartz & Showalter, 1981; Bartz, 1982; Xia et al., 2012; Yuk, Bartz, & Schneider, 2005; Zhuang, Beuchat, & Angulo, 1995). However, visual evidence that clearly depict microbial infiltration into tomato fruit was previously unavailable.

Fig. 2a and b are optical images showing the columella tissues and vascular bundles in tomato stem-scars, and the dye uptake into

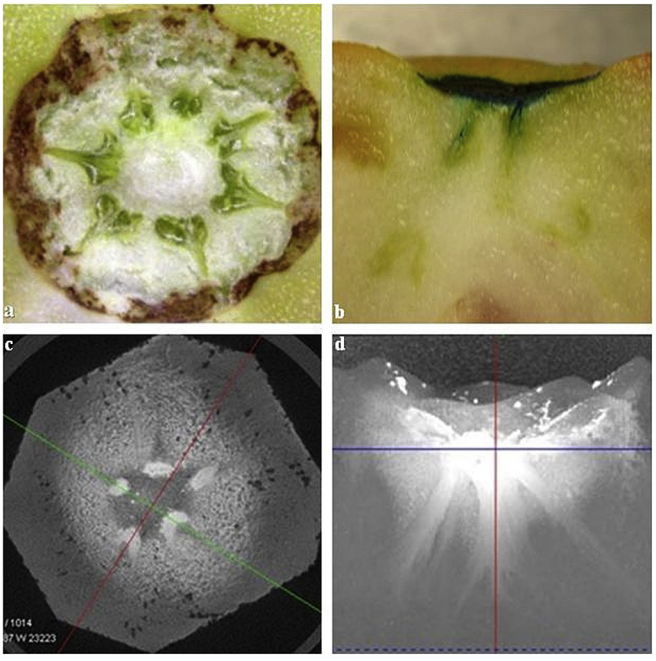

Fig. 2. Optical images of the tomato stem-scar, (a) transverse and (b) longitudinal views showing vascular bundles; Exradia Bio MicroCT 3D renderings of the infiltration of potassium iodide into tomato tissue through the stem scar, (c) transverse and (d) longitudinal views showing vascular bundles.

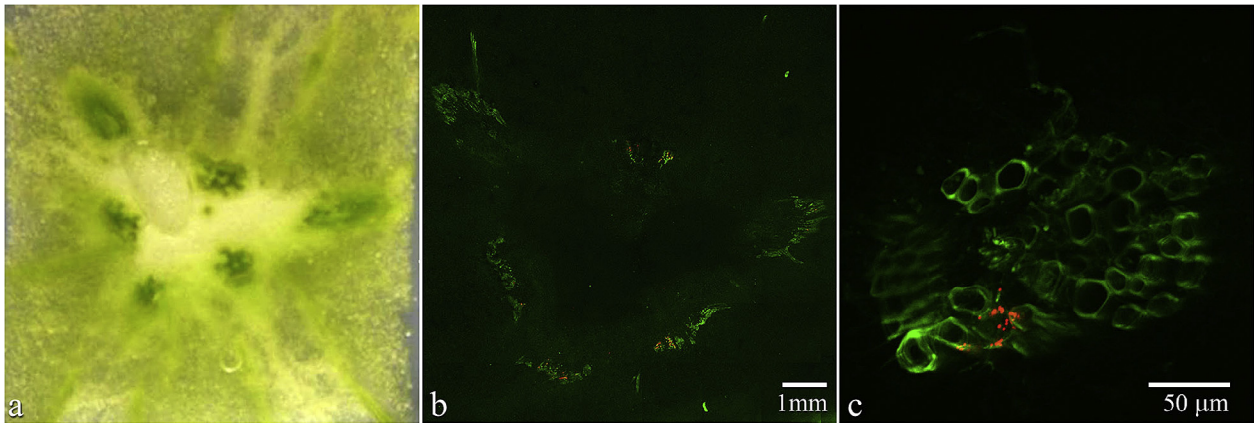

Fig. 3. Transverse-sectional observations on infiltration of red fluorescent microspheres into tomato tissues through the stem scar; (a)– optical image showing transverse-section of stem scar tissue; (b) CLSM image of transverse-sectioned stem scar tissue with red beads embedded, 10×; and (c) CLSM image of one transverse-sectioned vascular bundle tissue with red fluorescent microspheres embedded, 33×.

the vascular bundles inside the tomato fruit, respectively. Since dyes can only be viewed after cutting tomatoes, and cutting can drag and relocate the dyes, MicroCT imaging technology was thus employed to view the internal tissues non-destructively to avoid any artifacts caused by dye dragging. Potassium iodide infiltration of intact fruit was used to enhance the contrast of infiltrated tissues from the background. Fig. 2c and d are 3D transverse and longitudinal images, respectively, constructed from MicroCT scans of excised core tissues. As shown in these images, potassium iodide solution infiltrated tomato stem scar tissue along the vascular bundles. These images provided further evidence that vascular bundles are likely channels for any extrinsic substance, including human and plant pathogens in liquid matrix, to travel along inside tomato fruit.

Fig. 3a is an optical image of the transverse view of the tomato vascular bundles, and Fig. 3b and c are the same transverse section viewed under CLSM at different magnifications. As shown in Fig. 3b, red fluorescent microspheres infiltrated inside tomato fruit are located in the vascular bundle tissues. Under a higher magnification, Fig. 3c further revealed that these red microspheres were confined inside the xylem vessels. This seems reasonable as water flows through the xylem vessels in vascular bundles (Dimond, 1966). Longitudinal sections of the tomato fruit with optical image (Fig. 4a), and CLSM images at different magnifications (Fig. 4b

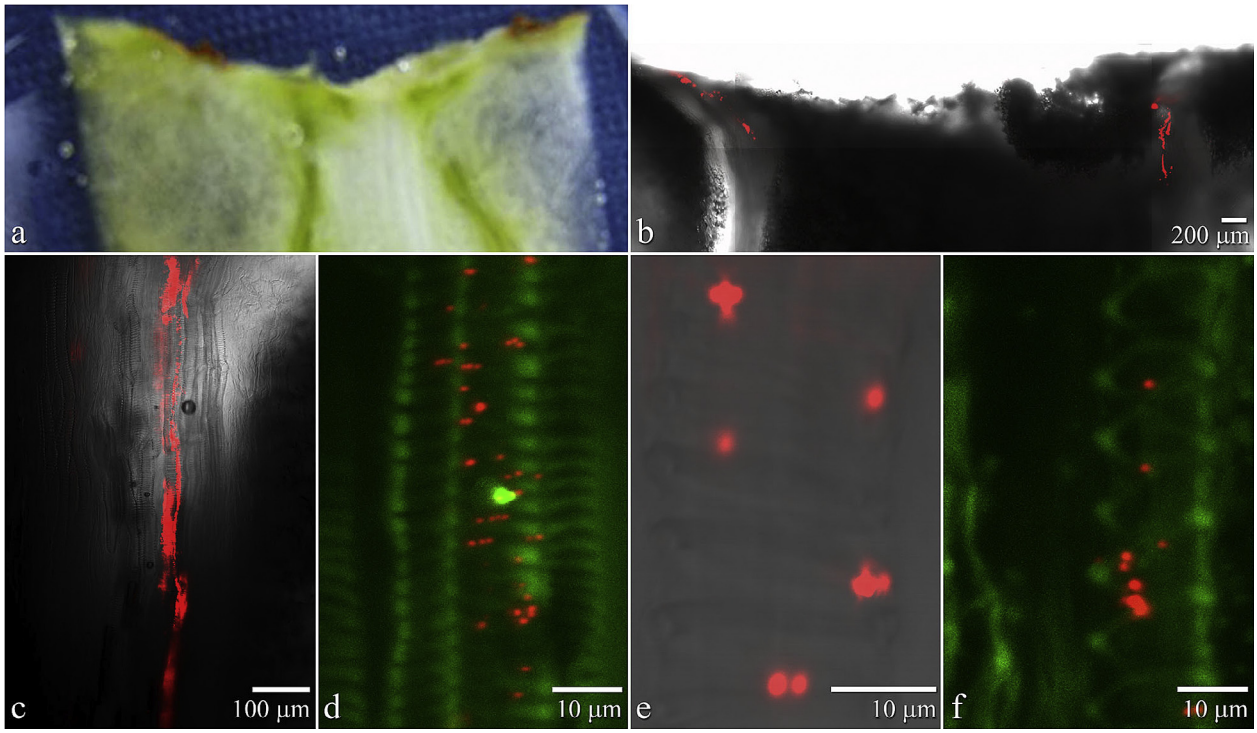

Fig. 4. Longitudinal-sectional observations on infiltration of red fluorescent microspheres into tomato tissues through the stem scar; (a) optical image of sectioned stem scar tissue; (b) CLSM overview including dual differential interference contrast (DIC) and fluorescent imaging of the stem scar showing red beads infiltration, 10×; (c) CLSM dual DIC and fluorescent image of xylem tissue with red beads embedded, 10×; (d) CLSM image of the xylem with red beads embedded, 87×; (e) CLSM dual DIC and fluorescent image of the xylem with red beads embedded, 130×; and (f) CLSM of the xylem with red fluorescent microspheres embedded 130×.

and c) further illustrate the distribution of red florescent microspheres inside the vascular bundles. Consistent with transverse views, longitudinal section views provide additional evidence that infiltrated red florescent microspheres are confined in the xylem vessels.

Fig. 5a–d shows the presence of *E. coli* O157:H7 inside the xylem vessels at different magnifications. These observations are in accordance with research on plant pathogen infiltration and mobilization in a variety of host plants. Utilizing tissue blot enzyme immunoassay, Harrison and Davis (1988) found evidence that *Clavibactor xyli* subsp. *xyli* colonized stalk internode vascular bundles of sugarcane. Vasse, Frey, and Trigalet (1995) described *Ralstonia. solanacaerum* colonization on the root surfaces of hydroponically grown tomato plants, infection of the vascular parenchyma, invasion of the xylem, and movement along the xylem vessels. *E. coli* O157:H7 was reported to move long distances through the vascular system of *Arabidopsis thaliana*, resulting in whole plant contamination in the absence of microbial competitors (Cooley, Miller, & Mandrell, 2003).

4. Conclusions

This study examined in detail the pathway of pathogen internalization in tomato fruit. The results provide clear visual evidence that vascular bundles, and specifically the xylem vessels, are major channels for pathogen movement through stem scars into the internal tissues of tomato fruit. The internalization process is passive, not active. Thus, to prevent pathogen internalization it is expedient to prevent pathogen contamination and attachment to the stem scars, and the uptake of contaminated water into tomato fruit.

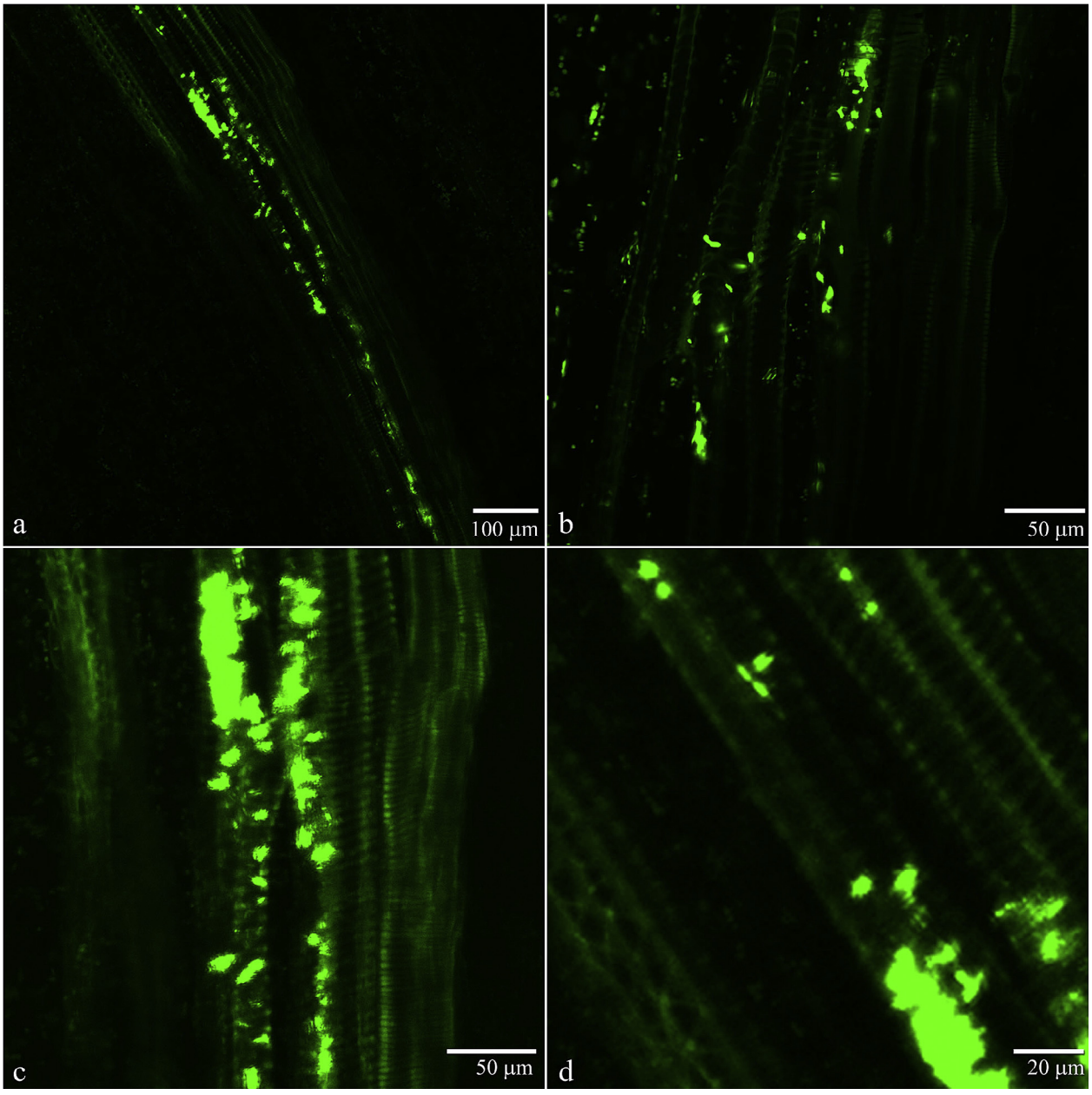

Fig. 5. Infiltration of *E. coli* O157:H7 into tomato tissues observed using CLSM. Vascular bundles with *E. coli* O 157:H7 cells embedded: (a) 10×; (b) 12×; (c) 25×; and (d) 87×.

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