

## Effect of Spinach Cultivar and Bacterial Adherence Factors on Survival of *Escherichia coli* O157:H7 on Spinach Leaves

DUMITRU MACARISIN,<sup>1</sup> JITENDRA PATEL,<sup>1\*</sup> GARY BAUCHAN,<sup>2</sup> JORGE A. GIRON,<sup>3</sup> AND SADHANA RAVISHANKAR<sup>4</sup>

<sup>1</sup>U.S. Department of Agriculture, Agricultural Research Service, Environmental Microbial & Food Safety Laboratory, 10300 Baltimore Avenue, Building 201, Beltsville Agricultural Research Center East, Beltsville, Maryland 20705; <sup>2</sup>U.S. Department of Agriculture, Agricultural Research Service, Electron & Confocal Microscopy Unit, 10300 Baltimore Avenue, Building 465, Beltsville Agricultural Research Center East, Beltsville, Maryland 20705; <sup>3</sup>Department of Molecular Genetics and Microbiology, Emerging Pathogens Institute, University of Florida, Gainesville, Florida 32610; and <sup>4</sup>Department of Veterinary Science and Microbiology, University of Arizona, 1117 East Lowell Street, Tucson, Arizona 85721, USA

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### ABSTRACT

Similar to phytopathogens, human bacterial pathogens have been shown to colonize the plant phylloplane. In addition to environmental factors, such as temperature, UV, relative humidity, etc., the plant cultivar and, specifically, the leaf blade morphological characteristics may affect the persistence of enteropathogens on leafy greens. This study was conducted to evaluate the effect of cultivar-dependent leaf topography and the role of strain phenotypic characteristics on *Escherichia coli* O157:H7 persistence on organic spinach. Spinach cultivars Emilia, Lazio, Space, and Waitiki were experimentally inoculated with the foodborne *E. coli* O157:H7 isolate EDL933 and its isogenic mutants deficient in cellulose, curli, or both curli and cellulose production. Leaves of 6-week-old plants were inoculated with 6.5 log CFU per leaf in a biosafety level 2 growth chamber. At 0, 1, 7, and 14 days, *E. coli* O157:H7 populations were determined by plating on selective medium and verified by laser scanning confocal microscopy. Leaf morphology (blade roughness and stoma density) was evaluated by low-temperature and variable-pressure scanning electron microscopy. *E. coli* O157:H7 persistence on spinach was significantly affected by cultivar and strain phenotypic characteristics, specifically, the expression of curli. Leaf blade roughness and stoma density influenced the persistence of *E. coli* O157:H7 on spinach. Cultivar Waitiki, which had the greatest leaf roughness, supported significantly higher *E. coli* O157:H7 populations than the other cultivars. These two morphological characteristics of spinach cultivars should be taken into consideration in developing intervention strategies to enhance the microbial safety of leafy greens.

The diversity and populations of phyllosphere bacteria are influenced by the environmental conditions (15, 29), plant species (3, 14), cultivar (26, 31), and developmental stage of plant and leaf (6, 7, 11, 17, 39). Seasonal temperature fluctuation, rainfall events, and solar activity have strongly affected bacterial communities on leaves of snap bean (10), maize (13), sugar beet (37), and olive tree (7). Differences in the morphology of the leaf surfaces, nutrient concentration, and secondary metabolites also affect populations of epiphytic bacteria among plant species. An extensive study of 47 plant species belonging to 27 botanical families showed that, irrespective of the species, phyllosphere bacteria were predominantly associated with leaf structures, including trichomes, veins, stomata, and the junctions between epidermal cell walls (3). In bean leaves experimentally inoculated with *Pseudomonas syringae*, bacterial aggregates were preferentially associated with glandular trichomes and veins (27). Comparative analysis of the phyllospheric microbial populations in four vegetable crops, red leaf mustard (*Brassica juncea*), Chinese mustard (*Brassica campestris*), endive (*Cichorium endivia*), and

spinach (*Spinacea oleracea*), revealed that the spinach phyllosphere had the highest bacterial population (32). The intraspecific and interspecific differences of leaf morphology among horticultural crops are important factors that affect the microbial community of the phyllosphere. For instance, cultivar differences were shown to affect the ability of *P. syringae* to colonize snap bean (10). Using a metagenomic approach based on terminal restriction fragment length polymorphism, Rasche et al. (31) showed that the cultivar type strongly affected the diversity of epiphytes colonizing sweet pepper plants.

In recent years, it became evident that, similar to phytopathogenic and phylloepiphytic bacteria, human enteric pathogens, such as *Salmonella enterica* and *Escherichia coli* O157:H7, are able to attach and even proliferate on plant surfaces (18, 21, 35). An overlap in host colonization mechanisms between zoonotic and phytopathogens was suggested to explain enteropathogen survival on plants (5). This phenomenon was also observed and described in several plant-human pathogen systems (1, 23, 30, 33, 43). Flagella and the type 3 secretion system were shown to be involved in *E. coli* O157:H7 colonization of the spinach leaf (33, 43). Extracellular cellulose, shown to be involved in bacterial attachment to cultured animal cells and

\* Author for correspondence. Tel: 301-504-7003; Fax: 301-504-8438; E-mail: jitu.patel@ars.usda.gov.

intestinal epithelium (34), was also suggested to play a role in enteric pathogen persistence on produce (1, 25). Prior studies showed that *E. coli* O157:H7 virulence factors associated with pathogenesis in humans, such as curli fimbriae, were involved in bacterial cell attachment and persistence on produce surfaces (12, 23, 30, 33, 38). Notably, Klerks et al. (16) demonstrated that, similar to epiphytic bacteria, *S. enterica* colonization on lettuce was strongly affected by the cultivar. A recent study (20) of the phyllosphere bacterial communities in spinach suggested that cultivar-specific leaf blade topography influenced the native microbial populations harbored on the plant surface. However, the effect of leaf blade topography on the persistence of foodborne pathogens on plant surfaces is yet to be studied.

We hypothesized that cultivar-specific leaf blade topography can affect the persistence of human enteric pathogens on spinach plants. The objective of the current study was to evaluate both the effect of the spinach cultivar as it relates to the leaf blade topography at the immature “baby” stage (commonly harvested for packaged salads) and the role of *E. coli* O157:H7 strains expressing extracellular virulence factors on bacterial persistence in the spinach phylloplane under preharvest conditions.

## MATERIALS AND METHODS

**Plant material.** Seeds of four organic spinach cultivars, Emilia, Waitiki, Lazio, and Space, were obtained from a commercial seed supplier (SeedWay, Elizabethtown, PA). The seeds were surface sterilized for 30 min with 1.0% (vol/vol) sodium hypochlorite, followed by five washes with sterile deionized water. To promote uniform and vigorous germination, the seeds were primed in a solution containing 30% (wt/vol) polyethylene glycol 8000 (Sigma Aldrich, Milwaukee, WI) for 72 h as recommended by Hart et al. (9). After priming, polyethylene glycol was removed from the seeds by washing, and they were planted in Miracle-Gro Organic Choice garden soil (Miracle-Gro, Marysville, OH) in 4-inch pots (Stuewe & Sons, Inc., Corvallis, OR). Germination and seedling growth were carried out in a growth chamber at 22°C with a relative humidity of 50 to 60% using a photoperiod consisting of 18 h of light ( $600 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) and 6 h of darkness. The spinach plants were soil drench irrigated as needed to maintain soil moisture and plant turgor. The USDA National Organic Program guidelines (41) for growing organic spinach plants were followed.

***E. coli* O157:H7 mutants and inoculum preparation.** *E. coli* O157:H7 strain EDL933, which originated from a human disease outbreak, and its isogenic mutants EDL933 $\Delta$ csGA, EDL933 $\Delta$ csGA/ $\Delta$ bcsA, and EDL933 $\Delta$ bcsA were available from previous studies (23). Strains were grown individually in 10 ml of Luria-Bertani (LB) broth (Acumedia, Lansing, MI) at 37°C and supplemented with the following antibiotics (Sigma-Aldrich) as required: kanamycin (50  $\mu\text{g/ml}$ ), chloramphenicol (30  $\mu\text{g/ml}$ ), and ampicillin (200  $\mu\text{g/ml}$ ). After 24 h of growth, 5 ml of bacterial culture of each strain was centrifuged at  $5,000 \times g$  for 15 min in an Avanti J-20 XPI centrifuge (Beckman Coulter, Palo Alto, CA). Pelleted cells were washed twice with phosphate-buffered saline (PBS; Acumedia) and resuspended in 100 ml of 0.1% (wt/vol) buffered peptone water (BPW; Acumedia) to obtain 7.5 log CFU/ml of each *E. coli* O157:H7 mutant.

**Plant inoculation.** Approximately 6 weeks after seedling emergence, the leaf area for each cultivar reached the baby leaf stage commonly harvested for packaged salads. Fresh bacterial suspensions of wild-type *E. coli* O157:H7 strain EDL933 and its isogenic mutants were prepared in 0.1% (wt/vol) BPW as described above. Leaves were inoculated with 100  $\mu\text{l}$  (as 5- $\mu\text{l}$  droplets, evenly spread) of the wild-type strain or the individual mutants on the adaxial surface of 4- to 6-cm-long leaves to obtain ca. 6.5 log CFU per leaf. Uninoculated plants were used as controls. Treated (contaminated) and control (uninoculated) plants continued to be soil drenched with water as required.

**Bacterial retention assays.** Immediately and on 1, 7, and 14 days after inoculation, 15 plants for each cultivar were sampled as follows. Five individual 4- to 6-cm-long leaves of each cultivar were collected from each of three plants previously inoculated by one of the *E. coli* O157:H7 EDL933 strain variants. Leaves were detached from the plants using scissors at the base of the petioles. The scissors were disinfected with ethanol before use and between the harvests of different plants. Aseptically harvested leaves were immediately weighed and transferred to sterile stomacher bags containing 20 ml of sterile 1% (wt/vol) BPW. Leaves were sonicated in sonication bath (Branson, Danbury, CT) for 1 min and then pummeled for 2 min in a Bagmixer (Interscience, St. Nom la Bretèche, France). The resulting BPW suspensions containing dislodged bacterial cells were spiral plated on MacConkey-sorbitol agar (Acumedia) supplemented with a corresponding antibiotic. After overnight incubation at 37°C, presumptive *E. coli* O157:H7 colonies were counted using a ProtoCOL colony counter (Microbiology International, Inc., Frederick, MD). An eight-tube most-probable-number enrichment procedure was followed when populations were undetectable by direct plating (30). Randomly selected colonies were confirmed for *E. coli* O157 lipopolysaccharide antigens by latex agglutination assay (Oxoid Ltd., Cambridge, UK).

**LSCM analysis.** On 1, 7, and 14 days postinoculation, leaves harvested as described above were fixed in  $-40^\circ\text{C}$  methanol for 5 min, followed by three washes with PBS and incubation for 30 min in PBS containing 2% (wt/vol) nonfat dry milk to block nonspecific immunoglobulin binding in subsequent steps. After blocking, the samples were incubated with rabbit anticurli polyclonal antibodies (34) (1:1,000 dilution) for 2 h at 37°C, followed by 2 h of incubation with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (Thermo Scientific, Rockford, IL) diluted 1:50 in PBS-Tween 20 (Sigma-Aldrich). All unbound antibodies were removed after each step by three washes with PBS-Tween 20. Laser scanning confocal microscopy (LSCM) analysis was conducted as described previously (23).

**LT-SEM analysis of leaf anatomical features.** Five individual 4- to 6-cm-long control (uninoculated) leaves from different plants of each cultivar were collected to determine the stoma density under low-temperature scanning electron microscopy (LT-SEM) using an S-4700 field emission SEM (Hitachi High Technologies America, Inc., Pleasanton, CA) equipped with a Polaron Polar Prep 2000 (Energy Beam Sciences, East Granby, CT) cryotransfer system. Leaf fragments were placed on copper plates (16 by 30 mm) that contained a thin layer of Tissue Tek (Ted Pella, Inc., Redding, CA) and flash frozen by placing the plates on a precooled ( $-96^\circ\text{C}$ ) brass bar whose lower half was submerged in liquid nitrogen. Frozen samples were analyzed as described previously (24). The numbers of stomata in high-resolution digital images of the adaxial surface of each leaf in five fields of approximately 1,270 by 960  $\mu\text{m}$  were manually counted.

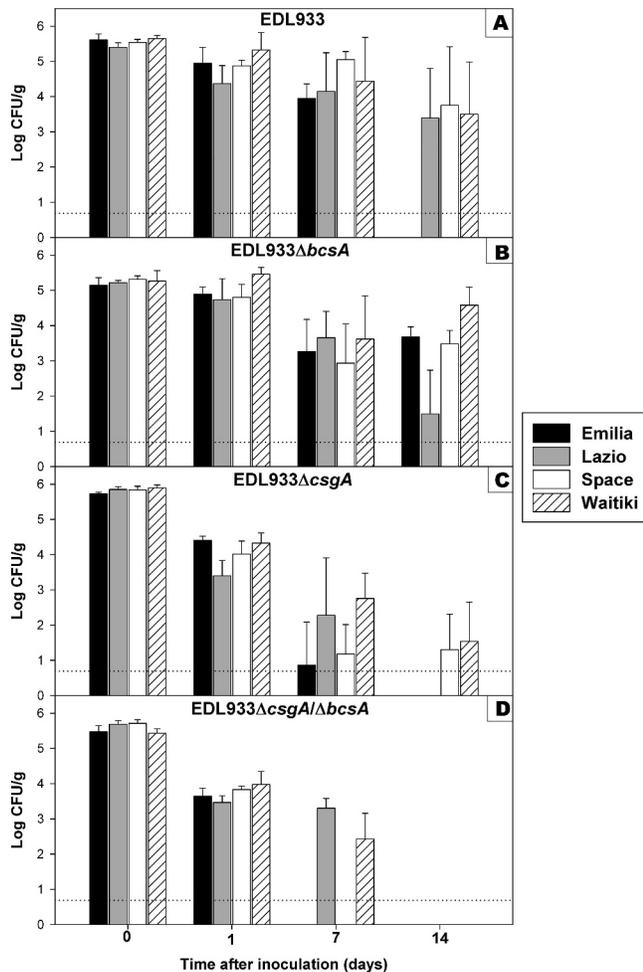


FIGURE 1. The effect of cultivar on *E. coli* O157:H7 populations persisting on spinach plants grown in a growth chamber. The persistence of (A) wild-type EDL933, (B) cellulose-deficient EDL933 $\Delta$ bcsA, (C) curli-deficient EDL933 $\Delta$ csgA, and (D) curli- and cellulose-deficient EDL933 $\Delta$ csgA/ $\Delta$ bcsA strains was evaluated for 14 days after leaves were spot inoculated with each individual mutant. The values (log CFU per gram of leaf) plotted are the averages of three replicates. Error bars represent standard errors of the means.

**VP-SEM analysis of leaf topography.** An S-3700 variable-pressure SEM (VP-SEM; Hitachi High Technologies America, Inc.) with a Deben Coolstage Peltier stage (Deben UK Ltd., Suffolk, UK) set at  $-25^{\circ}\text{C}$  was utilized. Twenty-millimeter-diameter discs of the fresh leaves of spinach cultivars Waitiki, Space, Lazio, and Emilia were placed directly onto SEM sample stubs (25-mm diameter) with double-sided sticky carbon tabs (Ted Pella, Inc.). Samples were analyzed at 15 kV accelerating voltage at a working distance of 12 mm. Topographical images were obtained using four backscatter detectors in the SEM which produced four images at discrete angles that were processed by 3D Image Viewer software (Hitachi High Technologies America, Inc., Pleasanton, CA) to develop the topographical images. All data were stored using Quartz PCI (Quartz Imaging Corp., Vancouver, Canada).

**Statistical analysis.** A randomized complete block design with three replicates per treatment was used. The populations of attached *E. coli* O157:H7 cells obtained at each sampling period were converted to log CFU per gram. The data obtained from three replicates were analyzed by two-way analysis of variance using

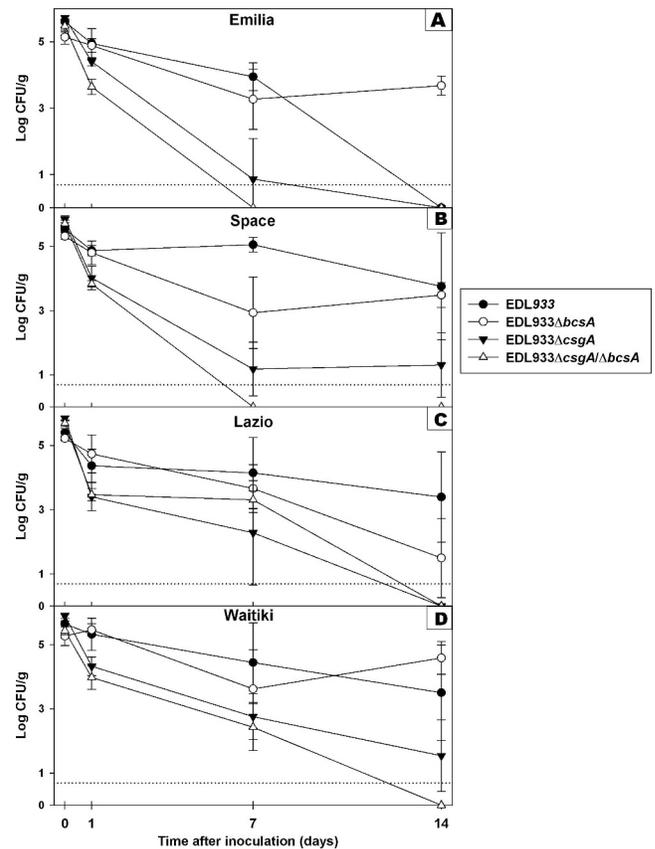


FIGURE 2. The effect of bacterial adherence factors on *E. coli* O157:H7 persistence on spinach plants grown in a growth chamber. *E. coli* O157:H7 populations on (A) Emilia, (B) Lazio, (C) Space, and (D) Waitiki leaves were evaluated for 14 days. The values (log CFU per gram of leaf) plotted are the averages of three replicates.

Proc Mixed (SAS 8.2, SAS Institute, Cary, NC) for interaction effects of the strain, cultivar, and sampling period. The results were considered statistically significant at  $P$  values of  $<0.05$ .

## RESULTS

### Attachment and persistence of *E. coli* O157:H7 cells on spinach leaves.

Immediately after inoculation (time 0), the numbers of attached bacteria were similar ( $P > 0.05$ ) among the *E. coli* O157:H7 strain variants, ranging from 5.1 to 5.9 log CFU/g (Fig. 1). The *E. coli* O157:H7 populations recovered from the leaves after 24 h varied significantly among spinach cultivars (Fig. 1). At 24 h, differences in populations among *E. coli* O157:H7 mutants were also observed (Fig. 2). The populations of the cellulose-deficient mutant EDL933 $\Delta$ bcsA (5.5 log CFU/g) and the wild-type EDL933 (5.3 log CFU/g) recovered from cultivar Waitiki were significantly higher than those of the noncurliated mutants EDL933 $\Delta$ csgA (4.3 log CFU/g) and EDL933 $\Delta$ csgA/ $\Delta$ bcsA (3.9 log CFU/g) (Fig. 2D). In general, the populations of the two curli-competent *E. coli* O157:H7 strains recovered from cultivars Space, Lazio, and Emilia after 24 h were significantly higher than those of curli-deficient cells (Fig. 2A through 2C). On the 7th day after inoculation, the cultivar effect became more pronounced in noncurliated mutants. The EDL933 $\Delta$ csgA populations on leaves of Lazio and Waitiki were 2.3 and

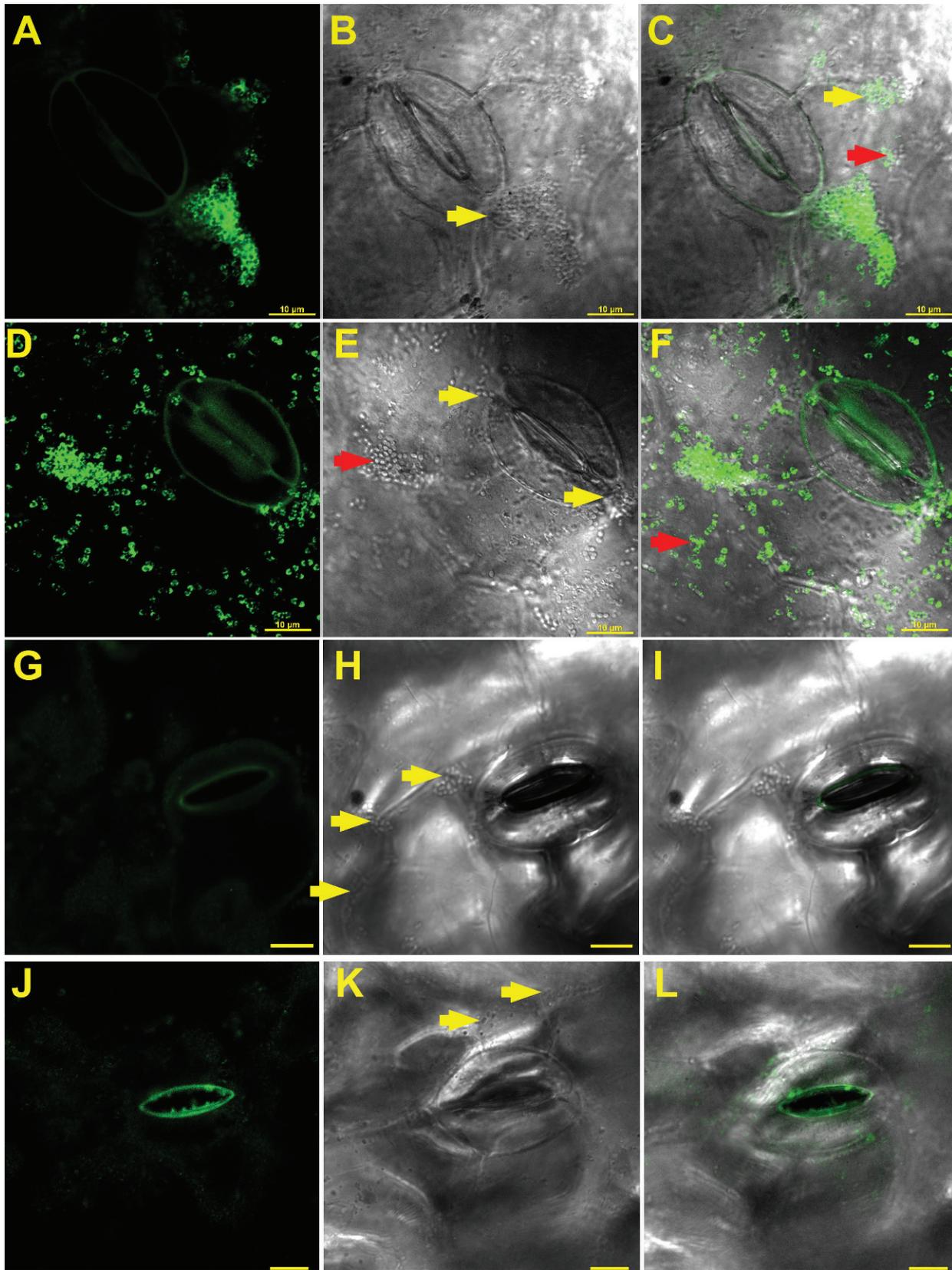


FIGURE 3. LSCM images of curli-producing *E. coli* O157:H7 wild-type strain EDL933 (A through C), cellulose-deficient mutant EDL933 $\Delta$ bcsA (D through F), and curli-deficient mutants EDL933 $\Delta$ csgA (G through I) and EDL933 $\Delta$ csgA/ $\Delta$ bcsA (J through L) attached to the surface of a leaf of cultivar Waitiki. Green fluorescence in single-channel fluorescence images (left column) indicates the expression of curli as recognized by anticurli polyclonal antibodies (A through F). Guard cells also fluoresce slightly in green as a result of cell wall autofluorescence. Differential interference contrast images (central column) illustrate *E. coli* O157:H7 cells predominantly attaching to the guard cells and junctions between epidermal cells, as highlighted by yellow arrows. Red arrows indicate bacteria attaching to smooth epithelium cuticle. The right column shows overlays of the differential interference contrast and fluorescence images. Scale bar = 10  $\mu$ m.

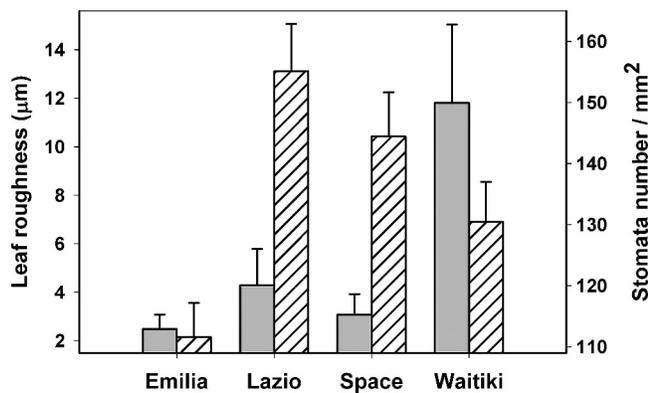


FIGURE 4. Topological and morphological features of the adaxial side of intact spinach leaf surfaces from four cultivars, Emilia, Lazio, Space, and Waitiki. Grey bars represent mean values for leaf roughness ( $n = 200$ ) on the left y axis, and hatched bars represent average values for number of stomata per square millimeter on the adaxial side of the leaf blade ( $n = 5$ ) on the right y axis. Error bars indicate the standard deviations for the numbers of replications indicated above.

2.8 log CFU/g, respectively, whereas on Space and Emilia, this Shiga toxin-producing *E. coli* mutant was detected at significantly lower levels (1.2 and 0.9 log CFU/g, respectively) (Fig. 1C). Similarly, the double mutant (cellulose- and curli-impaired) EDL933 $\Delta$ *csgA*/ $\Delta$ *bcsA* populations were 3.3 and 2.4 log CFU/g on Lazio and Waitiki leaves, respectively, while in cultivars Space and Emilia, they were undetectable (limit of detection, 0.69 log CFU/g) (Fig. 1D). The effect of the cultivar on populations of curli-expressing strains was not significant on day 7 (Fig. 1A and 1B).

Two weeks after inoculation, the lowest numbers of curli-expressing wild-type strain (EDL933) cells were recovered from the leaves of cultivar Emilia (Fig. 1A). The largest populations of the curli-negative EDL933*csgA* mutant were detected on leaves of cultivar Waitiki (1.5 log CFU/g), whereas this mutant was undetectable ( $<0.69$  log CFU/g) on Lazio and Emilia leaves (Fig. 1C). Similarly, the cellulose-deficient but curli-expressing Shiga toxin-producing *E. coli* mutant EDL933 $\Delta$ *bcsA* developed a stronger association with leaf surfaces of cultivar Waitiki (4.6 log CFU/g) than with the three other spinach cultivars used in the study (Fig. 1B). The curli- and cellulose-deficient mutant EDL933 $\Delta$ *csgA*/ $\Delta$ *bcsA*, however, was undetectable 2 weeks after inoculation (Fig. 1D). *E. coli* O157:H7 populations were detectable by the eight-tube most-probable-number procedure in most cases when they were undetectable by direct plating (data not shown).

**LSCM analysis of bacterial attachment on leaf blade.** The LSCM analysis of the inoculated spinach leaves, using rabbit anticurli polyclonal antibodies, revealed that curli fimbria-expressing strains attached in greater numbers to leaf surfaces. The highest densities of bacterial cells were observed at the stomata poles and at junctions between epidermal cell walls at all time periods after inoculation. Figure 3 shows leaf surfaces colonized by the *E. coli* O157:H7 wild-type parental strain EDL933 (Fig. 3A

through 3C) and the cellulose-deficient mutant EDL933 $\Delta$ *bcsA* (Fig. 3D through 3F), which both immunostained intensely for curli. The curli-impaired EDL933 $\Delta$ *csgA* (Fig. 3G through 3I) and EDL933 $\Delta$ *csgA*/ $\Delta$ *bcsA* (Fig. 3J through 3L) mutants were not recognized by anticurli polyclonal antibodies and persisted at significantly lower numbers ( $P < 0.01$ ) on leaf surfaces on day 7 after inoculation.

**SEM analysis of spinach leaf morphology.** SEM observation of the leaf blade in cultivars Emilia, Waitiki, Lazio, and Space at a stereomicroscopic scale ( $\times 100$ ) did not reveal significant differences in surface morphology among spinach genotypes (data not shown). Granular trichomes were not detected on the abaxial or adaxial surfaces of any cultivars (data not shown). Greater numbers of stomata, 155.1 and 144.4 stomata per mm<sup>2</sup>, respectively, were detected in cultivars Lazio and Space (Fig. 4). A lower number of stomata was observed in Waitiki (130.5 stomata per mm<sup>2</sup>), whereas Emilia had the fewest stomata (111.6 stomata per mm<sup>2</sup>). Visualization of the adaxial leaf surfaces at a higher magnification ( $\times 250$ ) provided more details in regard to the shape and curvature of the individual epidermal cells (Fig. 5). These observations suggested topographical differences among cultivars; however, their quantitative evaluation was difficult.

**VP-SEM determination of leaf topography.** To evaluate surface roughness, VP-SEM images of five different leaf areas per cultivar, with a surface area of 0.126 mm<sup>2</sup> each, were analyzed using 3D Image Viewer software. Figure 6 shows roughness contours for individual epidermal cells and cell junctions on representative pseudocolored VP-SEM images for cultivars Emilia, Waitiki, Lazio, and Space. For each of the five images per cultivar, 40 roughness contours were generated and then used to evaluate average leaf roughness. Among the four cultivars, Emilia had the smoothest surface with the lowest average roughness (*Ra*) value (arithmetic average of the absolute values of the surface height and depth deviations measured from the mean plane of the leaf blade surface),  $2.48 \pm 0.59$  µm (Fig. 4). A rougher surface topology was detected in cultivars Space and Lazio, which had *Ra* values of  $3.08 \pm 0.84$  and  $4.28 \pm 1.50$  µm, respectively. Cultivar Waitiki was found to have the roughest leaf blade surface, with an *Ra* value of  $11.81 \pm 3.23$  µm.

## DISCUSSION

Colonization of the phylloplane in horticultural crops by phytopathogens and epiphytes has been shown to be affected by the plant cultivar and, specifically, by the leaf blade morphological characteristics (20). An earlier report (26) suggests that cultivar-specific leaf surface morphology may play a role in the persistence and survival of enteropathogens on fresh produce surfaces. We used organic farming practices in this study; there is no published information on the effect of farming practices on spinach leaf structure or *E. coli* O157:H7 persistence on spinach leaves. The current study was conducted to evaluate the

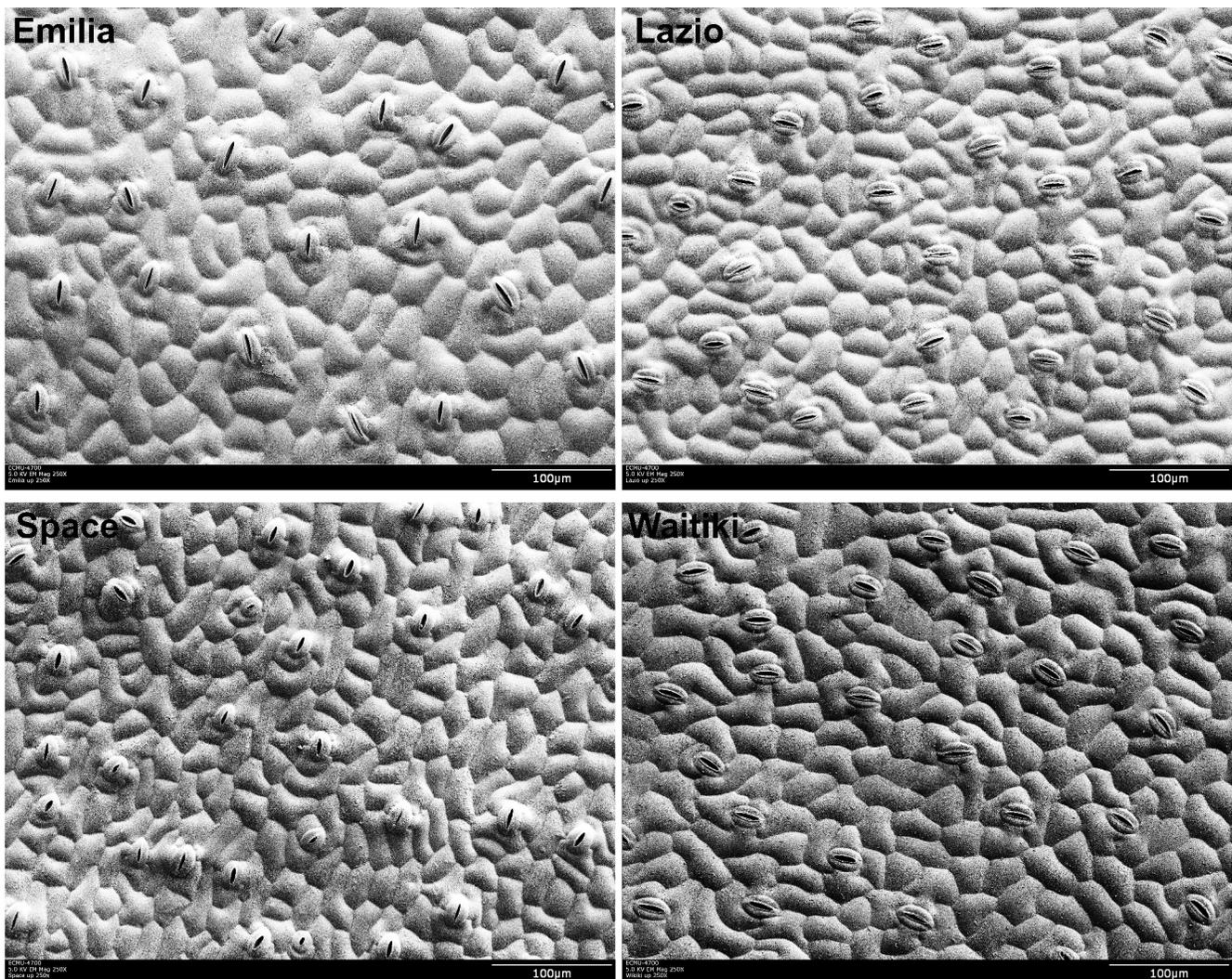


FIGURE 5. *LT-SEM* images of the surface topography of the intact adaxial leaf surface of four spinach cultivars, Emilia, Lazio, Space, and Waitiki. Scale bar = 100  $\mu$ m.

effect of cultivar-dependent leaf topography and the role of bacterial strain-dependent phenotype on *E. coli* O157:H7 persistence in the spinach phylloplane. The time-course dynamics of *E. coli* O157:H7 persistence associated with leaves of four spinach cultivars revealed differences in populations at discrete time intervals during the 2-week period after inoculation. *E. coli* O157:H7 cells were recovered at higher levels from cultivars Waitiki and Lazio than from cultivars Space and Emilia. Strain-dependent phenotypic characteristics of *E. coli* O157:H7 strains also significantly affected bacterial persistence on spinach. The average decline in the populations of curli-expressing *E. coli* O157:H7 strains was approximately 2.4 log CFU/g in the 2-week period after the initial inoculation. However, the populations of noncurliated EDL933 $\Delta$ *csgA* and EDL933 $\Delta$ *csgA*/ $\Delta$ *bcsA* strains decreased by 5.3 log CFU/g in the 2-week period. These data correlate well with earlier reports showing a greater persistence of curli-producing bacteria than of noncurliated bacteria on produce surfaces (12, 23, 30, 33).

Cultivar-dependent differences in the persistence of *E. coli* O157:H7 on leaves were more pronounced in curli-deficient strains than in those expressing curli. Likewise,

24 h after inoculation, the populations of the wild-type strain were ca. 0.4 log higher ( $P \leq 0.05$ ) on cultivar Waitiki than on other cultivars. On day 14, the wild-type-strain populations had declined to undetectable levels (by direct plating) on cultivar Emilia. The curli-competent but cellulose-deficient Shiga toxin-producing *E. coli* strain EDL933 $\Delta$ *bcsA* also associated in greater numbers with cultivar Waitiki, from which its levels of recovery were 0.6 and 1.0 log higher than from the rest of the cultivars on days 1 and 14, respectively. The effect of the cultivar on the recovery of the curli-impaired *E. coli* O157:H7 strain EDL933 $\Delta$ *csgA* was significant. For instance, the populations of strain EDL933 $\Delta$ *csgA* recovered from cultivars Lazio and Waitiki on day 7 exceeded by 2.5-fold the numbers recovered from Emilia and Space. Similarly, the populations of the curli- and cellulose-deficient strain EDL933 $\Delta$ *csgA*/ $\Delta$ *bcsA* were 3.3 and 2.4 log CFU/g on Lazio and Waitiki, respectively, whereas in Emilia and Space, these bacteria were undetectable. A possible reason for a less-obvious cultivar effect on curli-expressing strains could be the fact that curli are highly adhesive proteinaceous filaments specialized in bacterial attachment to biologic and

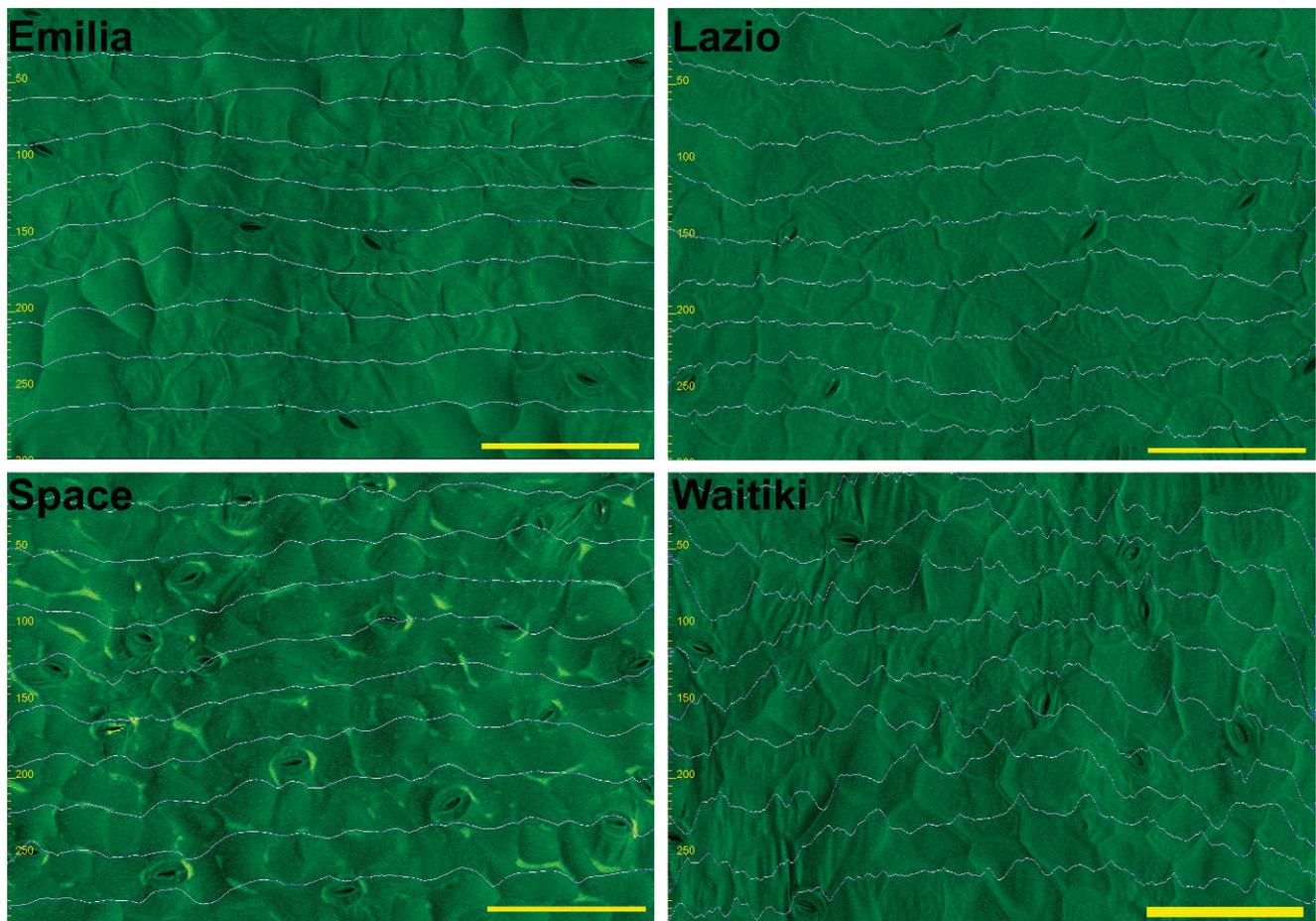


FIGURE 6. VP-SEM pseudocolored images of the adaxial side of the leaf blades of cultivars Emilia, Waitiki, Lazio, and Space. Nine roughness contours are applied on these representative images to provide visual differences in roughness among the cultivars studied. Scale bar = 100  $\mu\text{m}$ .

inanimate surfaces (34, 40). Therefore, cultivar-specific leaf morphology has a moderate impact on the attachment of curli-producing strains to spinach leaves. In contrast, noncurliated strains are more dependent on leaf surface characteristics that influence the retention and movement of water with bacterial suspensions, since they require longer times for stronger attachment to the plant surface (23). The current study showed that the leaf surface characteristics, as they differed by cultivar, significantly increased the persistence of the curli-deficient (EDL933 $\Delta\text{csgA}$  and EDL933 $\Delta\text{csgA}/\Delta\text{bcsA}$ ) compared with the curliated (wild-type EDL933 and EDL933 $\Delta\text{bcsA}$ ) strains.

LSCM data showed that the majority of *E. coli* O157:H7 cells were localized in the most-common sites of bacterial colonization (44), specifically, junctions among epithelial cells and depressions at the guard cell poles. This observation is consistent with other reports on LSCM analysis of *E. coli* O157:H7 distribution on the surface of spinach leaves (26). We observed that curli-deficient *E. coli* O157:H7 mutants were primarily detected at epithelial cell junctions or stoma poles, whereas populations of curli-expressing *E. coli* O157:H7 were also detected in other areas of the leaf surface (Fig. 3C, 3E, and 3F, red arrows). Because the microscopy data correlated well with the bacterial recovery results, we hypothesize that the persis-

tence of curli-deficient *E. coli* O157:H7 mutants on spinach leaves is more dependent on surface roughness. Because the expression of curli fimbriae by bacterial cells assures greater hydrophobicity and adhesiveness of bacterial cells, it may also result in conditioning rapid and stronger attachment to plant surfaces (23, 30).

Analysis of leaf blade morphology at the stereomicroscopic scale did not show significant differences in the leaf blade surface structure, except in the numbers of stomata per unit area of the leaf. Emilia had the lowest density of stomata on the adaxial side of the leaf, and all *E. coli* O157:H7 strains consistently showed the least recovery from this cultivar. These data might indicate a positive correlation between stoma density and *E. coli* O157:H7 persistence on spinach leaves. Likewise, Lopez-Velasco et al. (20) reported significantly higher recovery of epiphytic bacteria from field-grown spinach cultivars Menorca and Unipak than from Monza. Spinach cultivar Monza had the lowest stoma density when compared with cultivars Menorca and Unipak (20). The current study, however, found that bacteria associated in greater numbers with cultivar Waitiki, which had stoma densities higher than those of cultivar Emilia but lower than those of cultivars Lazio and Space. Together, the current data and previous reports suggested that stoma densities and some other leaf

morphological characteristics may affect *E. coli* O157:H7 persistence in the spinach phylloplane.

The surface roughness of biological and inanimate surfaces at the micrometer level has been suggested to play an important role in bacterial ability to attach and persist on those surfaces (2–4, 8, 19, 28, 36, 42). The results of the LT-SEM analysis of leaf surface indicated differences in the shape and curvature of epithelial cells among spinach cultivars; however, their quantitative comparison was difficult. A technique referred to as optical slicing has been suggested to evaluate the *Ra* of produce surfaces (42). Though this technique works well in assessing the roughness of inanimate surfaces, it has several limitations for use in wet biological samples. In the process of optical slicing, a laser beam penetrates through the sample to acquire a series of 2-dimensional images. Depending on the image resolution, speed of scanning, number of optical sections, and the length of the *z* axis (the thickness of the sample), the scanning process can be lengthy. The type of laser (blue, red, or green) used for scanning and its intensity, as well as the duration of scanning, determine the amount of energy to be delivered to the sample. Most of this energy is converted to heat, which dissipates, causing active dehydration, shrinking, deformation of the sample, and some other artifacts (22). As a result, a 3-dimensional reconstruction profile is not always sufficiently accurate for the detection of micrometer-scale differences. To reduce these effects, some investigators used critical-point drying, liquid substitution, and freeze-drying of the sample; however, these methods are invasive and destructive to some extent (42). Therefore, this study used VP-SEM as the least invasive technique, under which a fragment of the leaf was detached from the plant and immediately analyzed. The *Ra* values for the adaxial side of leaves of the four spinach cultivars were calculated. The *Ra* represents the arithmetic average of the absolute values of the surface height and depth deviations measured from the mean plane of the leaf blade surface. Waitiki had by far the highest *Ra* values ( $11.81 \pm 3.23 \mu\text{m}$ ) among the spinach cultivars used in the current study. The wild-type *E. coli* O157:H7 strain and its isogenic mutants were recovered in greater numbers from leaves of this cultivar, indicating that the leaf blade *Ra* correlated positively with the *E. coli* O157:H7 persistence on spinach. These results are also supported by a recent report (43) which demonstrated an increase in *E. coli* O157:H7 populations from 0.7 to 2.3 log CFU/g when the leaf surface roughness increased from 0.3 to 8.4  $\mu\text{m}$ . Significantly lower *E. coli* O157:H7 populations were recovered from Emilia, the cultivar with the lowest *Ra* values ( $2.48 \pm 0.59 \mu\text{m}$ ), supporting a positive relationship between leaf roughness and *E. coli* O157:H7 persistence on spinach. Lazio ( $4.28 \pm 1.50 \mu\text{m}$ ) and Space ( $3.08 \pm 0.84 \mu\text{m}$ ) had *Ra* values slightly higher than those of Emilia ( $2.48 \pm 0.59 \mu\text{m}$ ). Nevertheless, the *E. coli* O157:H7 populations recovered from these cultivars were significantly greater than the populations recovered from Emilia. Furthermore, the *E. coli* O157:H7 populations recovered on Lazio and Waitiki were similar in some cases. This phenomenon can be explained by the observation made with LSCM that *E.*

*coli* O157:H7 was often attached to the guard cells and at the stoma poles. The leaf surface in Lazio has a significantly higher stoma density (155.1 stomata per  $\text{mm}^2$ ) than the leaf surface in Waitiki (130.5 stomata per  $\text{mm}^2$ ). Larger numbers of stomata potentially compensate for the lower *Ra* values, thereby indicating that these two morphological features of the leaf blade surface constitute critical cultivar determinants that affect *E. coli* O157:H7 persistence on spinach.

In summary, *E. coli* O157:H7 persistence on spinach is significantly affected by cultivar and strain phenotypic characteristics, specifically, curli fimbriae. Leaf blade roughness and stoma density appear to be important factors determining the differential attachment and persistence of *E. coli* O157:H7 on spinach. These two morphological characteristics of the leaf should be taken into consideration by growers in selecting fresh-market spinach cultivars.

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