Journal of Food Protection, Vol. 65, No. 1, 2002, Pages 18–25

Association of *Escherichia coli* O157:H7 with Preharvest Leaf Lettuce upon Exposure to Contaminated Irrigation Water

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MS 01-159: Received 19 April 2001/Accepted 23 July 2001

ABSTRACT

Recent foodborne outbreaks have linked infection by enterohemorrhagic *Escherichia coli* (EHEC) serotype O157:H7 to the consumption of contaminated lettuce. Contamination via food handler error and on-the-farm contamination are thought to be responsible for several outbreaks. Though recent studies have examined the application of EHEC to store-bought lettuce, little is known about the attachment of EHEC to growing plants. We investigated the association of lettuce seedlings with EHEC O157:H7 strains implicated in lettuce or fruit outbreaks using hydroponic and soil model systems. EHEC strains that express the green fluorescent protein were observed by stereomicroscopy and confocal laser scanning microscopy to determine adherence patterns on growing lettuce seedlings. Bacteria adhered preferentially to plant roots in both model systems and to seed coats in the hydroponic system. Two of five nonpathogenic *E. coli* strains showed decreased adherence to seedling roots in the hydroponic system. EHEC was associated with plants in as few as 3 days in soil, and contamination levels were dose-dependent. EHEC levels associated with young plants inoculated with a low dose suggested that the bacteria had multiplied. These data suggest that preharvest crop contamination via contaminated irrigation water can occur through plant roots.

Within the last decade, 17 foodborne outbreaks have been linked to contaminated lettuce or salad. Eight of these outbreaks were attributed to contamination by enterohemorrhagic *Escherichia coli* (EHEC) serotype O157:H7, resulting in over 366 illnesses and several cases of severe hemolytic-uremic syndrome (see Internet: http://www.cspinet.org/reports/outbreak_alert/appendix_a.htm, http://www.cspinet.org/new/prodhark.html). Though the source of EHEC contamination is often unknown, on-the-farm contamination and cross-contamination via food handler error are thought to be responsible for several of these outbreaks *(15, 24).* Field contamination of EHEC O157:H7 may occur because of water runoff from nearby cow pastures, exposure to contaminated feces from wild animals, or utilization of improperly composted manure *(15, 22).*

The proximity of domestic or wild animals to irrigation water may serve as a vehicle for EHEC O157:H7 to gain access to produce growing in the field. The organism has been shown to survive in pure water and lake water to a lesser extent, with the greatest survival rates known to occur at low temperatures *(34).* Numerous examples exist of water-associated EHEC O157:H7 outbreaks, both from the consumption of contaminated water or ice as well as from accidental ingestion by swimming in a contaminated water source *(4, 5).* In addition, it was found that EHEC O157:H7 could survive, replicate, and move within soil, and that the presence of manure enhanced this survival rate *(11).*

Contaminated wash water in a farm-processing shed was thought to be a likely source for a 1996 EHEC O157:H7 lettuce outbreak that sickened 61 people, permitting shipment of contaminated produce to widely dispersed geographic areas. Though the pathogen was not recovered by onsite sampling, the farm in question allowed free-range chickens access to lettuce fields as well as to an adjacent cow pasture. Airborne carriage of contaminated cow manure to crops growing in close proximity was another risk factor. Additionally, pipes that supplied well water to the processing shed were connected to both cattle pastures and lettuce fields, allowing the possibility that contaminated water may have been used to irrigate the product *(15).*

Because EHEC O157:H7 lettuce outbreaks have been recognized only recently, little is known about this bacterial-plant interaction. Studies have shown that EHEC can survive on lettuce leaf surfaces for extended periods of time when applied in an aqueous solution and using bovine feces as a carrier *(1, 3).* The organism may attach inside stomatal pores or gain access to the interior tissue via cut leaf edges, thereby evading chlorine treatment in a processing facility *(26, 28, 30).* However, little is known about the initial association with, and consequent growth of, EHEC on seedlings or field plants. Colonization of produce by human pathogens is substantially increased in the presence of some plant pathogens *(35).* This may be because human pathogens lack appropriate pathogenicity genes that confer the capability to parasitize and grow within plant tissue *(2).*

Here, we investigated the initial association of lettuce seedlings with relevant EHEC O157:H7 strains implicated in lettuce or fruit outbreaks. Both aqueous and soil models were used to study bacterial adherence to young plants. EHEC strains that express the green fluorescent protein

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TABLE 1. *Enterohemorrhagic* Escherichia coli *and other* E. coli *strains used in this study*

Strain	Serotype, description[a]	Reference/Source
EDL933	O157:H7, clinical isolate, hamburger outbreak	*23*
F6460[b]	O157:H7, clinical isolate, NE iceberg lettuce outbreak	T. Barrett, Centers for Disease Control and Prevention (CDC); T. Safranek, N.E. Health and Human Services
H1827	O157:H7, Conn./Ill. mesclun lettuce outbreak	T. Barrett, CDC
HS	*E. coli* O9:H4, nonpathogenic, normal flora isolate	*9, 20*
KSU1	*E. coli* environmental isolate, cattle feedlot, nonpathogenic	J. Galland, D. Hyatt, Kansas State University, *18*
96A13466[b]	O157:H7, clinical isolate, apple juice outbreak	S. Abbott, M. Janda, Calif. State Department of Health Services, (DHS), Microbial Diseases Laboratory
96A-11901	*E. coli* normal flora isolate, nonpathogenic	S. Abbott, M. Janda, Calif. State DHS
98A-9918/2	*E. coli* normal flora isolate, nonpathogenic	S. Abbott, M. Janda, Calif. State DHS
97A-5984	*E. coli* normal flora isolate, nonpathogenic	S. Abbott, M. Janda, Calif. State DHS

[a] Strains are enterohemorrhagic *E. coli* unless otherwise indicated.

[b] Because these strains were recent patient isolates and previously unstudied, they were verified to be *eae* positive by polymerase chain reaction or Southern colony blot analysis using internal *eae* probes (data not shown).

(GFP) were used to visualize bacteria by confocal laser scanning microscopy with little sample disruption. To our knowledge, this is the first study of the association of EHEC O157:H7 with growing plants.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* isolates used in this study are listed in Table 1. Bacterial strains were stored at −80°C in Luria broth (LB; Difco Laboratories, Detroit, Mich.) containing 25% (vol/vol) glycerol. Strains were grown on LB agar plates at 37°C. When appropriate, ampicillin was added to media at a concentration of 100 μg/ml.

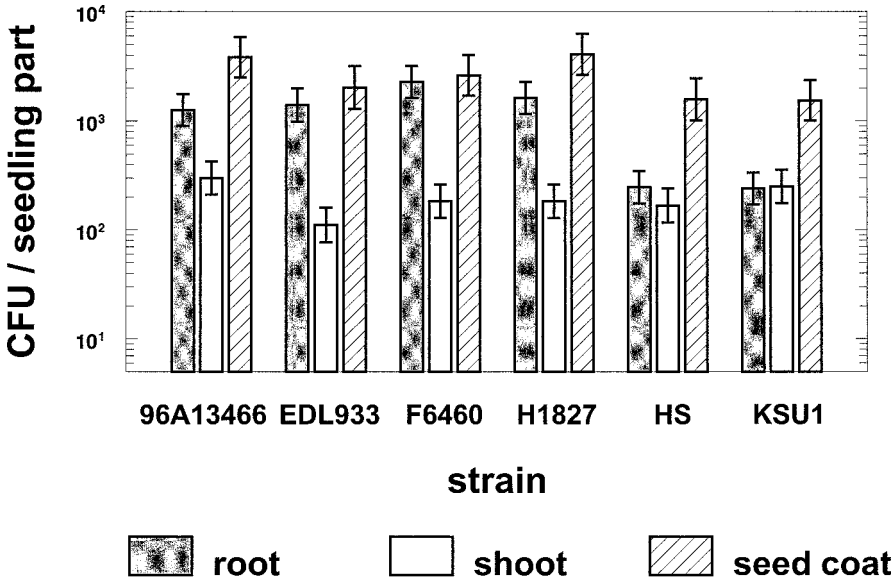
Generation of EHEC strains that express GFP. The QIAGEN Plasmid Mini Kit (QIAGEN Inc., Valencia, Calif.) was used to prepare plasmid DNA of pGFPuv (Clontech Laboratories, Inc., Palo Alto, Calif.) and pGreenTIR *(21)*. Electrocompetent EHEC strains were made according to Sizemore et al. *(27)* and were used immediately or frozen in a dry ice-ethanol bath and stored at −80°C for later use. Five hundred nanograms of DNA was electroporated into EHEC strains using a Gene Pulser II (Bio-Rad, Hercules, Calif.), and transformants were selected by growth on LB agar plates containing 100 μg/ml ampicillin (Sigma Chemical Co., St. Louis, Mo.). Transformants that expressed GFP to the greatest extent, as assessed by brief visualization under UV light, were purified three times on LB agar plates containing ampicillin. Purified strains were then stored at −80°C for later use. Growth curves of EHEC GFP strains grown in LB broth at 37°C were similar to that of the wild-type strains (data not shown).

Adherence assays utilizing a hydroponic model system. Ten Prizehead leaf lettuce seeds (Lilly Miller, The Garden Grow, Independence, Oreg.) were added to 20 ml of dH_2O in a 20-mm-tall petri dish and grown with gentle agitation for 48 h in a plant growth chamber (Conviron, Winnipeg, Manitoba, Canada; 16°C, 45% relative humidity night/20°C, 75% relative humidity day, 14-h day length, gradual maximum light = 200 μEinsteins m^{-2} s^{-1}). EHEC strains were grown overnight with aeration in LB broth containing the appropriate antibiotics at 37°C. For quantitative assays, bacteria were diluted in dH_2O water, added to seedlings at a concentration of ~10^6 CFU/ml, and incubated overnight with gentle agitation in the growth chamber. Seedlings were aseptically divided, rinsed once, and washed with 1 ml of phosphate-buffered saline using vigorous aspiration. Seedling sections were homogenized with a sterile pestle in 500 μl phosphate-buffered saline, and viable bacterial counts were determined by plating on sorbitol-MacConkey agar plates (Difco). Three samples were tested per experiment, and the experiment was repeated six times. Seedlings for visualization by microscopy were inoculated at a concentration of ~10^8 CFU/ml and incubated overnight with gentle agitation in the growth chamber. Seedlings were rinsed in dH_2O and observed under the microscope.

Adherence studies of plants grown in soil and inoculated via irrigation water. Commercial potting soil (Supersoil, Rod McLellan Co., San Mateo, Calif.) was placed into vented Magenta vessels (Sigma) to a depth of 3 cm. EHEC strains were grown overnight in LB broth containing 100 μg/ml ampicillin at 37°C and diluted in water to ~10^8, 10^6, 10^4, and 10^2 CFU/ml. The soil was watered until moist with 40 ml of the bacterial suspensions, and ~25 Salad Bowl leaf lettuce seeds (Garden Grow) were distributed evenly on the soil surface. Lettuce seeds were covered lightly with ~5 mm of soil and watered again with 10 ml of the bacterial suspensions. Plants were housed in a plant growth chamber (Conviron; 16°C, 45% relative humidity night/20°C, 75% relative humidity day, 14-h day length, gradual maximum light = 200 μEinsteins m^{-2} s^{-1}). Seedlings were aseptically removed from the Magenta boxes at the times indicated. Soil adhering to the roots was teased off, and plants were rinsed gently with dH_2O. Seedlings were dissected aseptically, placed in Eppendorf tubes containing 500 μl phosphate-buffered saline, homogenized, diluted, and plated onto MacConkey agar plates or LB agar plates containing 100 μg/ml ampicillin. Three samples were tested for each experiment, and the experiment was repeated three times.

Statistical analysis. Statistical analysis was performed using SAS Mixed Procedure (SAS OnLine Doc, Version 8, SAS Institute, Cary, N.C.) *(25)*. For each set of experiments, original counts were transformed by log base 10 to stabilize the variance. Since inocula could not be controlled precisely, statistical tests and predictions were adjusted to a common inoculum by fitting the measured value of inoculum as a covariate. In each case, designed treatments, such as strain, were modeled as fixed effects, and experiments, or replication of the experimental design, were mod-

FIGURE 1. *Quantitation of EHEC adherence to lettuce seedlings and seed coats. Data shown are the means and standard errors of seven experiments; three samples were tested in each experiment. Adherence of strains to roots, shoots, and seed coats was compared by a Student's* t *test adjusted by the Tukey-Kramer method. Adherence of the nonpathogenic* E. coli *strains HS and KSU1 to roots was statistically less than that of the pathogenic strains (*$P < 0.02$ *for all comparisons tested).*



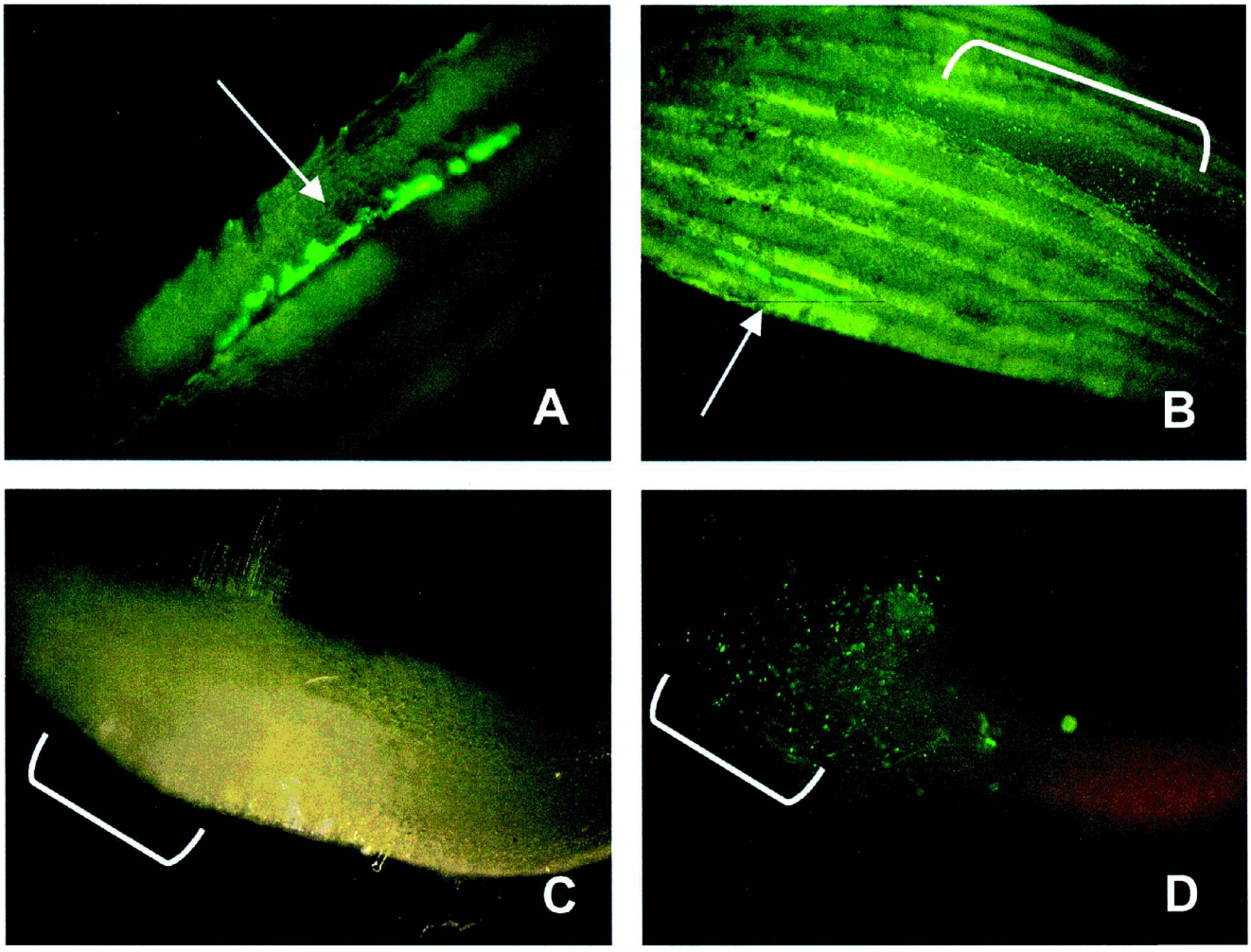


FIGURE 2. *Visualization of EHEC attachment to lettuce seed coats and seedlings. EHEC strain H1827 pGreenTIR was observed on lettuce seedlings and seed coats by fluorescence stereomicroscopy. Arrows and arcs indicate the site of EHEC attachment; EHEC appears green. (A) EHEC attachment within the deep grooves of a lettuce seed coat. (B) EHEC attachment to the split edges and grooves of the seed coat. (C) Brightfield image of the seedling root showing root hairs. (D) EHEC adherence along the root hairs of a seedling root (corresponding fluorescence image to panel C).*

eled as a random factor. Where appropriate, preplanned comparisons were estimated and tested with a *t* test. Where all pairwise comparisons were desired, the significance probability values were adjusted by Tukey's method of multiple range comparisons *(32)* with Kramer's extension for unbalanced data *(19)*. When comparisons to one parameter were desired, Dunnett's test was invoked *(8)*.

Microscopy. Seeds and seedlings were examined using a Leica MZ-FLIII fluorescence stereomicroscope (Leica Microsystems, Heidelberg, Germany) and a 41017 Endow GFP filter set (Chroma Technology Corp., Brattleboro, Vt.) for visualization of bacterial adherence. The stereomicroscope is capable of 20- to 256-fold magnification. Photographs were obtained with a Sony DKC-5000 digital camera (Sony Electronics, Inc., Tokyo, Japan) and imported into Adobe Photoshop 5.0 (Adobe Systems, Inc., San Jose, Calif.) from a Sony DKS-5000 workstation.

Confocal laser scanning microscopy was performed with a Leica TCS NT confocal laser scanning microscope equipped with Ar, Kr, and He/Ne lasers. Samples were stained, where indicated, for 1 h in 20 μM SYTO 59 prepared in water (Molecular Probes, Eugene, Oreg.). Slides were mounted with Aqua Poly/Mount (Polysciences, Inc., Warrington, Penn.), dried at room temperature, and stored at 4°C in the dark. Samples were viewed with a ×63 water objective using a FITC/TRITC/Cy5 filter. An Ar laser excited GFP (λ_{exc} = 488 nm), and an He/Ne laser was used to excite samples stained with SYTO 59 (λ_{exc} = 633 nm). GFP fluorescence was detected with a BP525/50 filter set and assigned the color green. Plant autofluorescence was detected with a BP600/30 filter set and assigned the color red. SYTO 59 fluorescence was detected with a LP645 filter set and assigned the color blue. Color images were obtained by overlaying images from the green, blue, and red channels. Images were collected using Leica TCS NT software version 1.6.551 and were modified using Paint Shop Pro 6 (Jasc Software, Eden Prairie, Minn.) and Adobe Photoshop 5.0. Images shown are representative of at least three independent experiments.

RESULTS

Variable EHEC adherence to lettuce seeds and seedlings in a hydroponic system. To examine EHEC adherence to growing plants, we developed a simple seed and seedling adherence model in the presence of water. We focused these seedling studies on several relevant strains involved in lettuce- or fruit-associated EHEC outbreaks and compared their adherence levels to that of a hamburger outbreak strain and several nonpathogenic *E. coli* strains.

Attachment levels were greatest to the roots and seed coats (Fig. 1). Adherence to lettuce shoots and seed coats was similar for all strains tested. However, the two nonpathogenic *E. coli* strains (HS and KSU1) displayed adherence to roots that was significantly less than that of the pathogens ($P < 0.02$ for all comparisons tested). Adherence of the nonpathogenic strains to roots was ~15% that of the pathogenic strains. To determine if decreased root adherence was a characteristic of other nonpathogenic *E. coli* strains, three additional normal flora isolates were tested in the seedling adherence assay (96A-11901, 98A-9918/2, and 97A-5984). These additional isolates displayed levels of adherence to roots, shoots, and seed coats that were not significantly different from those of the pathogenic strains (data not shown). Plating homogenates from uninoculated lettuce seedlings resulted in no growth on selective plates.

We wished to investigate whether EHEC adherence to seeds and seedlings was dependent on prior colonization by natural epiphytes. However, we found that lettuce seeds and seedlings typically contained very few bacteria (on average, <10 CFU per seed or seedling), as assessed by growth of homogenates on King's B media for 3 days at 25°C. In addition, few epiphytes were observed by microscopy after staining seedlings with SYTO 59, a cell-permeant nucleic acid stain. Adherence levels to seed coats sterilized with 0.2% calcium hypochlorite were similar to those on unsterilized seeds for all strains tested (data not shown).

To better understand EHEC association with plants, we observed bacterial adherence to growing seeds and seedlings by microscopy. EHEC strains were transformed with GFP plasmids, allowing the visualization of fluorescent bacteria under UV light in the absence of additional cofactors *(6)*. Examination of inoculated seeds revealed EHEC attached within the deep grooves and tips of seed coats (Fig. 2A). Emergence of the radicle during germination split the seed coat, allowing association of EHEC with the broken edges (Fig. 2B). In addition, the mature zone of seedling roots was demarcated by extensive association of EHEC along the root hairs (Fig. 2C and 2D). No or few EHEC was observed on other regions of the root. These attachment patterns were similar for a number of EHEC O157:H7 strains in several experiments.

EHEC association with soil-grown seedlings inoculated via irrigation water. To determine if irrigation with contaminated water could lead to bacterial association with produce, we sprouted lettuce seeds in soil in the presence of water that had been inoculated with various concentrations of EHEC O157:H7. Seedlings were allowed to germinate, and EHEC was permitted to associate with plants as it would in the field, without direct application of the pathogen to the plant. EHEC populations associated with the seedlings were quantitated after several days of growth.

Though a great deal of variability existed in these experiments, several trends remained consistent despite the complex growth environment. Bacterial-plant association was generally dose-dependent, with the highest inoculation doses resulting in the greatest association levels (Fig. 3A through 3C). An EHEC dose of 10^8 CFU/ml resulted in high numbers of EHEC found in plants, especially the roots (Fig. 3A). EHEC was associated with seedlings within 3 days, the first time point tested. This was the first time point that the plants were large enough to test. In one case (F6460, day 10), the high numbers of EHEC found associated with plants inoculated with 10^2 CFU/ml suggested that the bacterial population had multiplied, given the total number of bacteria added to the system (5.8×10^3 CFU associated with all parts of the seedlings combined versus ~5×10^3 total CFU added to the container). EHEC was associated with all plant parts at all time points tested, and generally, the bacterial numbers did not decrease with time. The 10^8 CFU/ml dose appeared to saturate bacterial binding

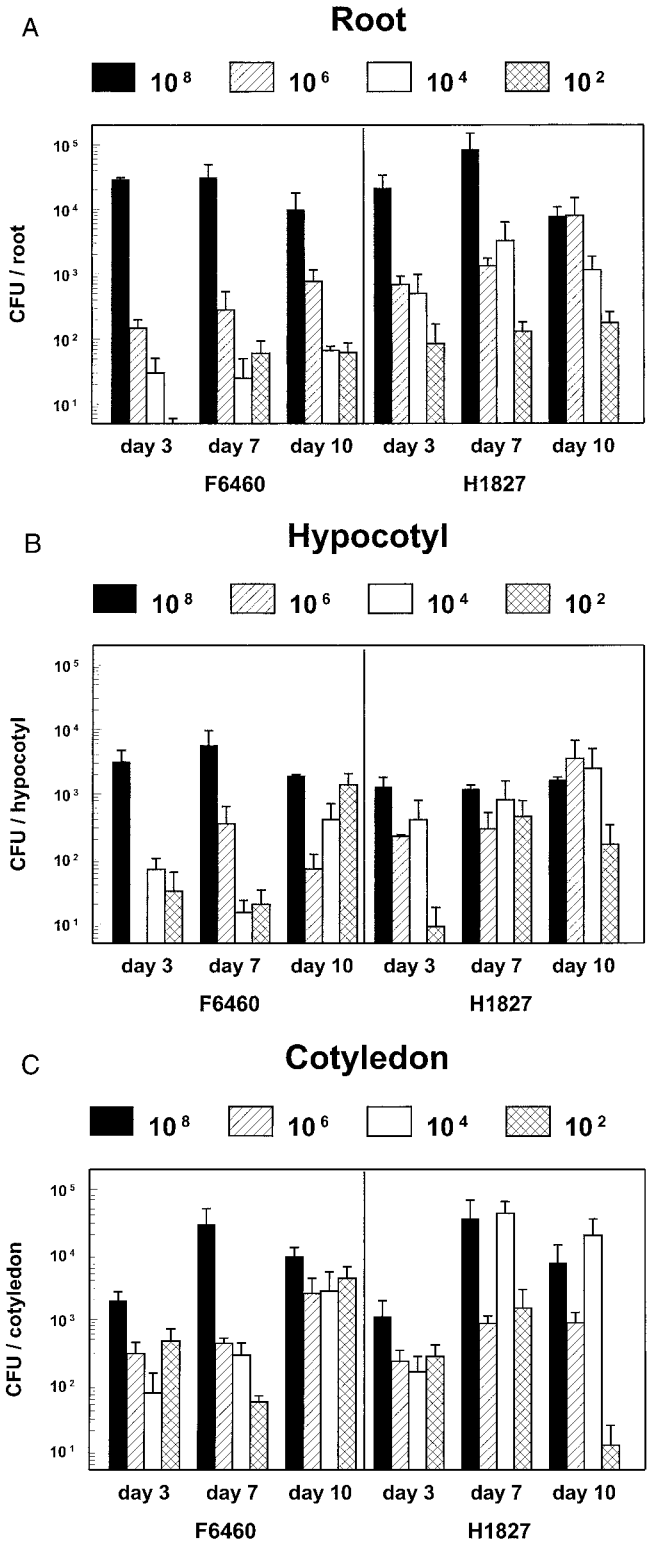


FIGURE 3. *EHEC association with soil-grown seedlings inoculated via contaminated irrigation water. Lettuce seedlings were grown in soil in the presence of water inoculated with various concentrations of EHEC strains F6460 or H1827 (CFU/ml of inoculum indicated). EHEC populations associated with the seedlings were quantitated after several days of growth. Data shown are the means and standard errors of three samples from one experiment. (A) Root; (B) hypocotyl; (C) cotyledon.*

sites, with the highest bacterial numbers reaching ~2 × 10^4 CFU in roots.

Observation of soil-grown seedlings by confocal laser scanning microscopy revealed the greatest numbers of EHEC associated with roots, in agreement with quantitative data. EHEC was found attached to roots both singly and in small aggregates (Fig. 4A and 4B). Pathogens were associated with cotyledons close to the petiole (Fig. 4C), as well as the leaf blade. EHEC was also observed associated with the cotyledon in close proximity to a stomatal pore (Fig. 4D). Attachment of EHEC to stomatal guard cells and pores has been observed by us and others and may be an entry point for bacterial internalization into the inner tissue *(26, 28, 33).* Remarkably, we observed fluorescent EHEC moving within the vasculature of a hypocotyl. The bacteria were most likely located within the xylem, since this is the only open vessel within the hypocotyl (data not shown).

DISCUSSION

Though many EHEC O157:H7 outbreaks have been associated with the consumption of contaminated hamburger, produce has recently become recognized as a foodborne source of this organism. The purpose of these studies was to examine EHEC adherence to growing plants to better understand how the organism attaches to plants in the field, potentially through such vehicles as contaminated runoff or irrigation water. Initially, we sought to develop a simple seed and seedling adherence model in the presence of water. This basic model mimics bacterial adherence in a hydroponic system, used for the growth of some commercial lettuces, such as butter lettuce. For the strains tested, we observed preferential EHEC adherence to lettuce roots and the deep grooves of seed coats in the hydroponic model system. In addition, significant adherence was observed to seedlings grown in soil in as little as 3 days. Though EHEC adherence was greatest to the roots, significant bacterial numbers were found associated with the edible portion of the plant, both singly and in aggregates. We and others have found that EHEC is capable of attachment to the interior of stomatal pores as well *(26, 28, 33).* These attachment sites and aggregative association patterns may cause bacterial resistance to physical methods of seed surface disinfection, as well as to traditional chemical interventions, such as chlorination *(29).* We currently have no evidence that EHEC bound to a specific location on plants. However, the aggregate seen on roots suggests that the bacteria bound as an aggregate, or that the bacteria grew after initial binding, potentially around a favorable nutrient source. Significant EHEC attachment to roots, though not a direct health risk in leaf vegetables, may cause contamination of edible root crops such as carrots, onions, and radishes as well. Thus, preharvest produce contamination can pose serious postharvest challenges.

These laboratory studies indicate that irrigation water contaminated with human pathogens can be a source for the inoculation of food crops. We have also observed the association of *E. coli* with field crops when plants were irrigated with water inadvertently contaminated with human sewage *(33).* Though sporadic releases of human sewage

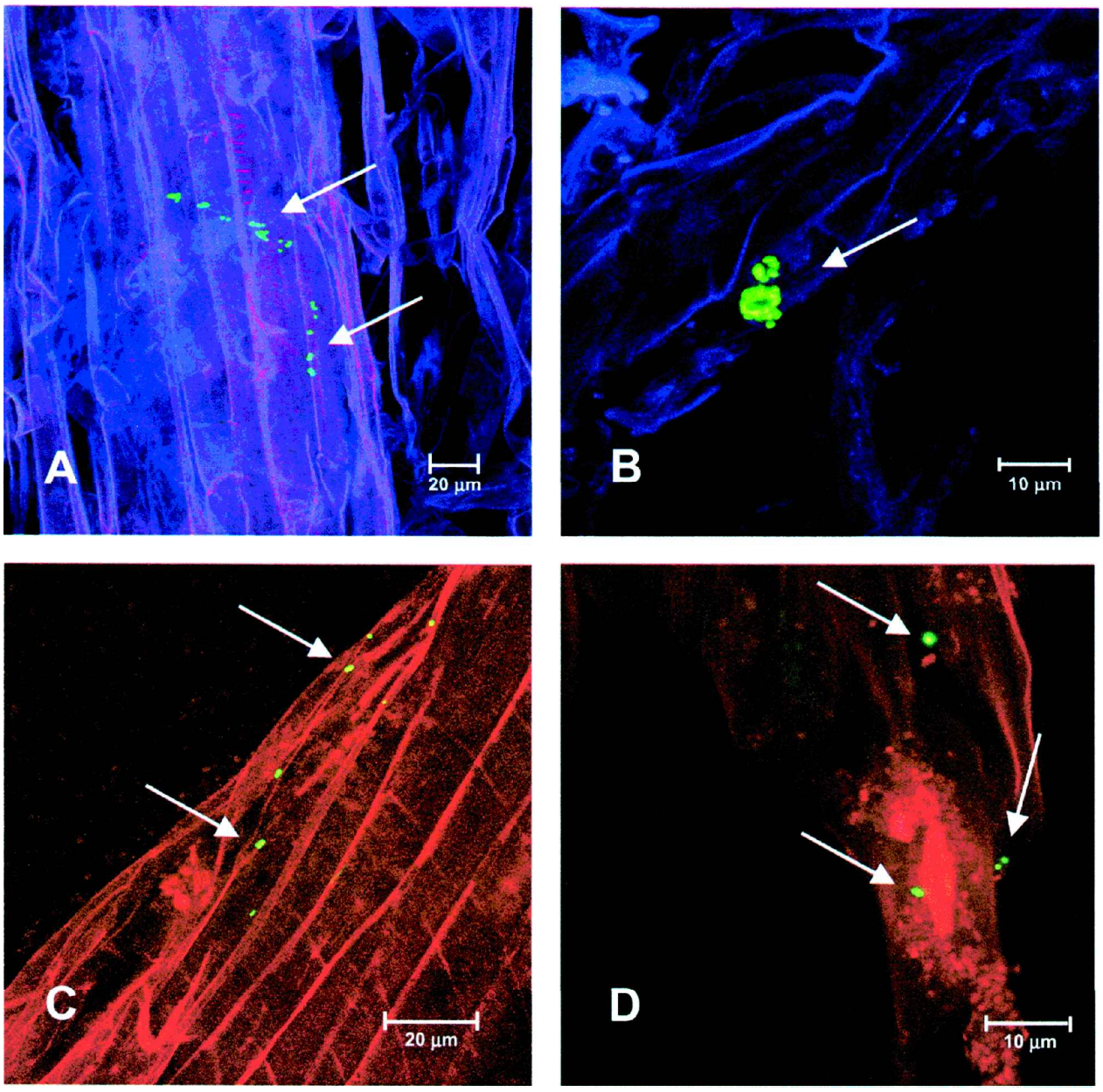


FIGURE 4. *Confocal laser scanning microscopy of seedlings grown in soil and inoculated via irrigation water. Lettuce seedlings were grown in soil for 5 days in the presence of water inoculated with EHEC strain 96A13466 pGFPuv. Arrows indicate the site of EHEC attachment; EHEC appears green. (A) Single EHEC cells associated with a growing lettuce root (pseudo-colored blue). Xylem are visible and extend longitudinally within the root (red). (B) Aggregate of EHEC associated with a lettuce root. (C) Single EHEC cells adhering to the lettuce cotyledon; the cotyledon autofluoresces red. (D) Single EHEC cells associated with the surface of a cotyledon in close proximity to a stomate.*

may contaminate irrigation water, runoff from animal manure is another mechanism of crop contamination. A recent study showed that the overall prevalence of EHEC O157:H7 in cattle is much higher than thought previously *(10).* Because the infectious dose for EHEC may be fewer than 50 organisms in some cases *(14, 31),* these observations stress the continuing need for intervention strategies to prevent contamination of water that may be exposed to bovine manure *(12).*

Our observation that EHEC adheres preferentially to lettuce roots and seed coats is not unique to this organism or this commodity. Recent studies indicate that both *Salmonella enterica* serovar Newport and an EHEC O157:H7 strain efficiently colonize the roots and seed coats of alfalfa, radish, and mung bean sprouts. However, adherence to sprout roots by the *Salmonella* strain was much greater than the EHEC strain *(7).* Our laboratory studies suggested that bacterial multiplication occurred in young plants irrigated with contaminated water. Human pathogens grow to significant levels in older plants only in the presence of plant pathogens *(35).* However, human pathogens are capable of growth on seedlings for the first few weeks of growth, depending on the plant type *(16).* This highlights the necessity for attention to food safety measures in the growth of trans-

plants used for food crops, including the quality of water and other amendments.

Three of five nonpathogenic *E. coli* strains tested displayed root adherence levels similar to pathogenic EHEC strains in the hydroponic adherence model. The reason for this strain variability requires further study but is striking, given the simple model system used. These nonpathogens do not possess intimin or bundle-forming pili, two well-characterized adherence factors of enterohemorrhagic and enteropathogenic *E. coli (13, 17).* This leaves open the possibility that some nonpathogens express factors that confer their attachment to produce. We are interested in characterizing such attachment factors further, as well as characterizing the attachment factors of pathogens. We are currently screening a variety of mutants in the laboratory to identify important components involved in the attachment process. We hope to use these data to develop new strategies to control EHEC colonization of lettuce. It is likely that this information will be applicable to the interactions between a variety of pathogenic organisms and other commodities as well.

ACKNOWLEDGMENTS

We thank Amy Charkowski and Lee-Shin Tsai for helpful discussions and critical reading of the manuscript and Michael Manoochehri, Staci Leffel, and Maria Brandl for technical assistance. We thank William Miller and Steve Lindow for the GFP plasmid pGreenTIR and Sharon Abbott, Mike Janda, Tim Barrett, Tom Safranek, Doreene Hyatt, and John Galland for EHEC and normal flora isolates. This work was funded by the U.S. Department of Agriculture, Agricultural Research Service CRIS project no. 5325-42000-022.

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