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Proliferation of *Escherichia coli* O157:H7 in Soil-Substitute and Hydroponic Microgreen Production Systems

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ABSTRACT

Radish (*Raphanus sativus* var. *longipinnatus*) microgreens were produced from seeds inoculated with *Escherichia coli* O157:H7 by using peat moss–based soil-substitute and hydroponic production systems. *E. coli* populations on the edible and inedible parts of harvested microgreen plants (7 days postseeding) and in growth medium were examined. *E. coli* O157:H7 was shown to survive and proliferate significantly during microgreen growth in both production systems, with a higher level in the hydroponic production system. At the initial seed inoculation level of 3.7 log CFU/g, *E. coli* O157:H7 populations on the edible part of microgreen plants reached 2.3 and 2.1 log CFU/g (overhead irrigation and bottom irrigation, respectively) for microgreens from the soil-substitute production system and reached 5.7 log CFU/g for those hydroponically grown. At a higher initial inoculation of 5.6 log CFU/g seeds, the corresponding *E. coli* O157:H7 populations on the edible parts of microgreens grown in these production systems were 3.4, 3.6, and 5.3 log CFU/g, respectively. Examination of the spatial distribution of bacterial cells on different parts of microgreen plants showed that contaminated seeds led to systematic contamination of whole plants, including both edible and inedible parts, and seed coats remained the focal point of *E. coli* O157:H7 survival and growth throughout the period of microgreen production.

Consumption of leafy greens has increased in recent years due to their health benefits *(11)*. However, leafy green vegetables have also been identified as sources of foodborne illnesses worldwide over the past decade. Enteric bacterial pathogens, such as *Escherichia coli* O157:H7 and *Salmonella enterica,* have been implicated in a number of food poisoning outbreaks associated with raw vegetable consumption *(1, 8)*.

Microgreens are a class of specialty fresh produce that have gained increasing popularity as a new culinary trend over the past few years *(15)*. They are young seedlings of vegetables, herbs, or other plants, with cotyledons fully developed and the first pair of true leaves emerged or partially expanded. Chemical analyses have shown microgreens contain high levels of bioactive compounds; therefore, they are nutritious for human diet and health *(19)*.

Although microgreens share many characteristics with sprouts as tender plants, an important distinction between sprouts and microgreens is the production process. For commercial production, sprouts typically germinate from seeds in rolling drums or sprouting bins under warm and humid conditions. In contrast, microgreens are grown hydroponically or in a shallow layer of soil or soil substitutes (such as peat moss, perlite, vermiculite, rock wool, or other fibrous materials) in green houses as real plants. Sprouts have long been recognized a class of produce prone to contamination by foodborne bacterial pathogens *(14),* and sprout consumption has been implicated in several high-profile foodborne illnesses outbreaks. From 1998 through 2010, there have been at least 33 foodborne disease outbreaks associated with sprout consumption and 1,330 cases of reported illness in the United States *(2)*. In 2011, a large outbreak of Shiga toxin–producing *E. coli* O104:H4 associated with fenugreek sprouts occurred in Germany and France, resulting in more than 4,000 illnesses and 50 deaths *(16)*. These outbreaks further heightened public health concerns over the safety of sprouts. Although it is likely that microgreens could also serve as vehicle of bacterial pathogens, to date, no foodborne outbreak associated with consumption of microgreens has been reported. The lack of food safety incidence could be attributed to the low production and consumption, high consumer geographic and demographic selectivity, or purported intrinsically safe characteristics of microgreens. Although the interactions of pathogens with sprouting seeds and leafy greens have been well studied, there is a knowledge gap with respect to microgreens. In addition, the similarity of microgreens and sprouts and rapid growth in microgreen production and consumption also led to increased concerns over the food safety of microgreens.

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Microgreen production was evolved from small individual or family-scale operations. As the microgreen industry develops, new production methods continue to emerge. Among the diverse current microgreen production methods, there are two leading production systems (soil and hydroponic production) in the United States, especially for commercial productions. Although the traditional soil or soil substitute–based production is simple and easy to operate, hydroponic production using state-of-the-art modern commercial facilities for microgreen production has gained increasing popularity. At the same time, family-scale production of microgreens by consumers, using traditional soil or hydroponic growth kits, is widely practiced.

Contaminated seeds are generally the source in most sprout-related outbreaks *(6)*. Similar to sprouts, microgreens are typically produced in enclosed facilities, and seeds could be the most important source of contamination *(13)*. Unlike sprouts, the current U.S. Food and Drug Administration regulation does not mandate seed sanitization treatment being used in microgreen production. Our previous study *(20)* showed that *E. coli* O157:H7 and O104:H4 on inoculated radish seeds proliferated significantly during the germination and growth of microgreens, although to a lesser extent than those during sprouting. However, the mechanisms related to human pathogen colonization and distribution on seeds and microgreen plants during growth were not explored yet. To extend our knowledge regarding initial pathogen-plant interactions, the distribution of green fluorescent protein (GFP)–labeled *E. coli* O157:H7 on artificially contaminated radish seeds and grown microgreens was investigated.

The primary objectives of this work were to (i) compare the nature of bacterial attachment, transmission, and proliferation of *E. coli* O157:H7 on microgreens grown in soil-substitute and hydroponic production systems and (ii) examine the distribution of *E. coli* O157:H7 on microgreen plants and growth media.

MATERIALS AND METHODS

Bacterial strains and inoculum preparation. *E. coli* O157:H7 strains ATCC 43888, ATCC 43895, and EC415 harboring a stable plasmid that encoded for GFP and ampicillin resistance (pGFP) *(3)* were described previously *(20)*. The stability of the pGFP plasmid in these strains was evaluated by three consecutive overnight subculturings in tryptic soy broth (TSB; BD Biosciences, Sparks, MD) in the absence of ampicillin, followed by plating on nonselective agar plates. All the colonies examined expressed GFP, indicating stable maintenance of the plasmid in the absence of selective pressure.

The three *E. coli* O157:H7 strains were grown individually in TSB containing 100 μg/ml ampicillin overnight at 37°C. Cells were harvested by centrifugation at 2,300 × *g* for 10 min at 4°C and washed once in sterile phosphate-buffered saline (PBS; pH = 7.4) solution. Cells were resuspended in sterile distilled water. Equal volumes of cell suspensions of the three *E. coli* O157:H7 strains were combined as a cocktail and further diluted in sterile distilled water to obtain desired cell concentrations for seed inoculation.

Seeds and inoculation. Daikon radish (*Raphanus sativus* L. var. *longipinnatus*) seeds were obtained from Living Whole Foods, Inc. (Springville, UT). All the seeds were sealed and stored in a refrigerator until use. Seeds were visually inspected prior to inoculation to purge those with visible defects. The inoculum suspensions were diluted to the levels of 10^5 to 10^6 and 10^7 to 10^8 CFU/ml, for achieving targeted microbial load at low (3 to 4 log CFU/g) and high (5 to 6 log CFU/g) levels on seeds, respectively. For inoculation, 200 g of seeds was immersed in 400 ml of inoculum suspension with gentle swirling for 5 min at room temperature. After draining, seeds were spread over sterile double-layer cheesecloth on top of a plastic tray and air dried overnight under a laminar flow biological safety hood at room temperature. Air-dried inoculated seeds were then stored at 4°C for 24 to 48 h before use. For the low and high inoculation levels, *E. coli* O157:H7 counts on radish seeds were 3.7 ± 0.2 log CFU/g (∼1.7 log CFU per seed) and 5.6 ± 0.1 log CFU/g (3.7 log CFU per seed), respectively.

Microgreen growth. Microgreens were grown either on Fafard Super Fine Germination Mix (Griffin Greenhouse Supplies, Inc., Bridgeton, NJ) as soil substitute or hydroponically by using Sure to Grow growing pads (Beachwood, OH) of polyethylene terephthalate (PET) fibers. The main ingredients of Fafard Super Fine Germination Mix includes Canadian sphagnum peat moss, perlite, vermiculite, dolomitic limestone, and wetting agent. Growth of radish microgreens on Fafard Super Fine Germination Mix as growth medium was carried out, as previously described *(20)*. For microgreen growth on Sure to Grow growing pads, 10 g of radish seeds was sown evenly on the PET fiber pad (25 cm wide by 51 cm long by 0.9 cm deep) sited in tandem of two standard 1020 flat plastic culture trays (28 cm wide by 54 cm long by 6 cm deep; Growers Supply, Dyersville, IA) with the top one perforated. Growing pads were moisturized by adding the sterile tap water to the bottom solid tray.

Seeds planted in trays of both germination mix and PET fiber pads were germinated and grown in a growth chamber with the temperature set at 25 and 18°C (day and night). During the first 3 days, trays were covered to allow seed germination in dark. On day 4, the seedlings were exposed to white fluorescent light (light irradiance = ∼150 μmol/s/m^2, determined by a LI-1000 datalogger, LI-COR, Lincoln, NE) with a 12-h photoperiod.

Radish seeds planted and microgreens grown in germination mix were daily irrigated either overhead to saturation with a fine sprayer or at the bottom by pouring water into the bottom tray until the growth medium was moisturized. Radish microgreens grown in growing pads were daily watered by bottom irrigation.

Harvesting and sampling. Microgreen plants were harvested and sampled for microbiological analyses on day 7 postseeding. For sampling, 25 randomly selected microgreen plants were carefully pulled out from growth medium and cut into parts according to the purpose of the experiments by using sterile scissors. For experiments examining the growth of *E. coli* O157:H7 in different production systems, each plant was cut into two sections: edible part (including cotyledon and hypocotyl, cut from 1 cm above the root and substrate surface) and inedible part (the remaining hypocotyl and root). For experiments examining the spacial distribution of *E. coli* O157:H7 on microgreen plants, each plant was dissected into four parts: cotyledons (cut from hypocotyl upper end), upper hypocotyl (cut from 1 cm above the root and substrate surface), lower hypocotyl (cut from the root and substrate surface), and roots (without effort to remove small amount of attached germination mix). For microgreens grown in hydroponic trays, roots were collected from the underside of the growth pad without effort to distinguish roots of individual plants. In addition,

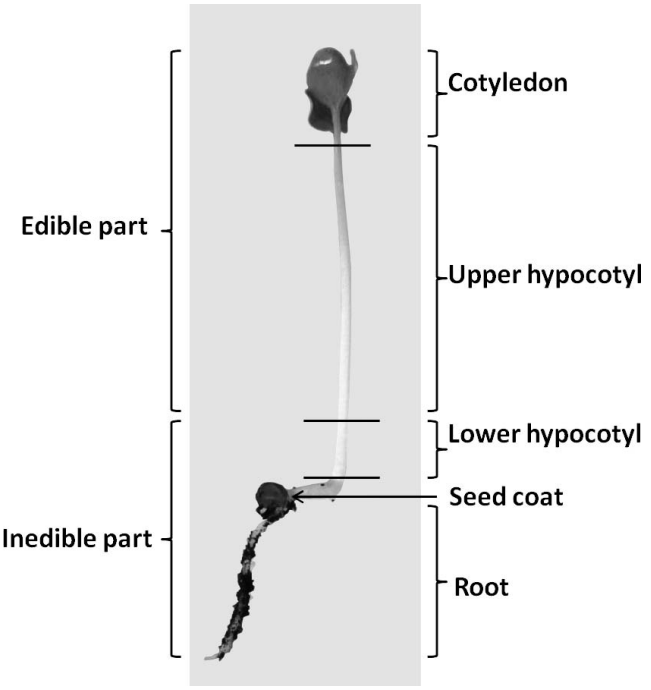

FIGURE. 1. *Dissection diagram of a 7-day-old radish microgreen plant. Straight lines across the stem indicate the typical positions of cutting during harvest and sampling. Plant tissue above the middle cutting line is referred to as edible part.*

seed coats of the corresponding plants were also collected (Fig. 1). Therefore, each sample (except root samples of hydroponic microgreens) was composed of specified sections of 25 individual plants from the same tray.

Microbial enumeration. The corresponding individual parts of 25 randomly selected microgreens from the same growth tray were collected as one sample. After weighing, each sample was pummeled with nine times the sterile PBS in filter bags for 2 min at the high level of the stomacher blender (model 80, Seward Medical, London, UK), and *E. coli* cells in the filtrate were enumerated by using a combination of spiral plating and most-probable-number methods described previously *(4)*. Sorbitol MacConkey agar supplemented with 200 μg/ml ampicillin was used for selective growth of *E. coli* O157:H7 (colorless colonies), and plates were also examined under UV light for positive confirmation of *E. coli* O157:H7 by green fluorescence. The filtrates of germination mix and of radish seeds were also plated on sorbitol MacConkey agar containing 200 μg/ml ampicillin to screen for the presence of bacteria in the germination mix and radish seeds that might form colonies indistinguishable from that of *E. coli* O157:H7. No such colonies were observed.

Confocal laser-scanning microscopy. Confocal laser-scanning microscopic examination was carried out in the Electron and Confocal Microscopy Unit of the U.S. Department of Agriculture (USDA), Agricultural Research Service, following the previously developed procedures *(5, 10)*. Plant tissues were placed in plastic petri dishes with glass bottoms (MatTek Corp., Ashland, MA) for observation. A Zeiss LSM710 confocal laser scanning microscopy system (Carl Zeiss, Inc., Thornwood, NY) was used, and the images were observed by using a Zeiss Axio Observer inverted microscope with 63 × 1.4 NA Plan-Apochromat oil immersion objectives. A 488-nm argon laser with a pinhole of 30 μm passing through a MBS 488 beam splitter filter, with limits set between 493 and 545 nm, was used. The Zeiss Zen 2012 (Carl Zeiss, Inc.) 64-bit software was used to obtain 6 to 30 images with 30 μm per frame z stack to produce three-dimensional renderings that were used to develop the two-dimensional maximum intensity projections for publication.

TABLE 1. E. coli *O157:H7 populations on edible and inedible parts of radish microgreen samples and in growth medium (germination mix or water) in different production systems* (n = *4)*

Production system	Samples	*E. coli* O157: H7 populations (log CFU/g)[a] Low inoculation level	High inoculation level
Soil-overhead	Edible parts	2.3 ± 0.6 AB[b]	3.4 ± 0.6 AB
	Inedible parts	3.9 ± 0.1 CD	4.6 ± 0.3 BCD
	Medium (soil)	1.5 ± 0.0 A	2.9 ± 0.4 A
Soil-bottom	Edible parts	2.1 ± 0.5 AB	3.6 ± 0.2 AB
	Inedible parts	4.8 ± 0.2 DE	5.7 ± 0.0 DE
	Medium (soil)	2.9 ± 0.2 BC	3.9 ± 0.6 ABC
Hydroponic	Edible parts	5.7 ± 0.0 EF	5.3 ± 0.1 CD
	Inedible parts	7.0 ± 0.1 F	7.1 ± 0.1 E
	Medium (water)	5.6 ± 0.1 E	5.7 ± 0.3 DE

[a] The initial inoculation levels of radish seeds were at the level of 3.7 ± 0.2 log CFU/g (low) and 5.6 ± 0.1 log CFU/g (high).
[b] Values in the same column followed by a common letter are not significantly different ($P > 0.05$).

Experimental design and statistical analysis. In this study, the experiment examining the growth of *E. coli* O157:H7 in different production systems and the experiment examining the spacial distribution of *E. coli* O157:H7 on microgreen plants were run separately, both of which were conducted in four replicates. Samples collected from the same growth tray were considered as one replicate. All the microbiological data were log transformed and reported as the mean value of four replicates ± standard error. Data were analyzed using SPSS 13.0 for Windows (SPSS Inc., Chicago, IL). One-way analysis of variance was conducted to check the normality and variance homogeneity of the linear models. The statistical significance of the data was determined by performing Tukey's honestly significant difference tests for post hoc multiple comparisons at an experiment-wise significance level of 0.05.

RESULTS AND DISCUSSION

Survival and proliferation of *E. coli* O157:H7 in soil and hydroponic microgreen production systems. In this study, microgreens were grown either in peat moss–based soil substitute or hydroponic system, and we compared the survival and proliferation of *E. coli* O157:H7 on microgreens. The *E. coli* O157:H7 populations on the edible and inedible parts of microgreens and in both growth media (germination mix for the soil system and water for hydroponic system) are shown in Table 1.

E. coli O157:H7 contamination on both edible and inedible parts of the young plants, as well as in growth media, were observed with both soil (soil substitute) and hydroponic production systems. In comparison to the soil-grown system, a significantly ($P < 0.05$) higher degree of

TABLE 2. E. coli *O157:H7 populations on different parts of radish microgreen plants grown in germination mix with bottom irrigation (*n = *4)*

Sample	*E. coli* O157: H7 population (log CFU/unit)[a]	
	Low inoculation level	High inoculation level
Leaves	2.7 ± 0.4 B[b]	3.9 ± 0.2 B
Upper hypocotyls	2.2 ± 0.1 AB	2.3 ± 0.3 A
Lower hypocotyls	1.1 ± 0.5 A	2.1 ± 0.3 A
Roots	2.8 ± 0.3 B	3.3 ± 0.1 B
Seed coats	3.5 ± 0.2 B	4.1 ± 0.1 B

[a] The initial inoculation levels of radish seeds were at the level of 3.5 ± 0.1 log CFU/g (1.7 ± 0.1 log CFU per seed) (low) and 5.6 ± 0.1 log CFU/g (3.7 ± 0.0 log CFU per seed) (high).

[b] Values in the same column followed by a common letter are not significantly different ($P > 0.05$).

bacteria survival and growth on radish microgreens was observed in hydroponic production system with an approximate range of 1.8 to 3.5 log, for both low and high inoculation levels of seeds. For the low seed contamination level (initial seed inoculation 3.7 ± 0.2 log CFU/g), the edible parts of radish microgreens grown in soil (overhead and bottom irrigation) and hydroponic systems carried *E. coli* O157:H7 population at the levels of 2.3 ± 0.6, 2.1 ± 0.5, and 5.7 ± 0.0 log CFU/g, respectively. A similar trend was seen in the case of microgreens grown from seeds inoculated at a higher level (initial seed inoculation 5.6 ± 0.1 log CFU/g), with *E. coli* O157:H7 population on the edible parts of radish microgreens grown in soil (overhead and bottom irrigation) and hydroponic systems reaching 3.4 ± 0.6, 3.6 ± 0.2, and 5.3 ± 0.1 log CFU/g, respectively.

Our observation was in agreement with previous findings by Warriner et al. *(18)* that *E. coli* interacted differently with spinach plants growing in hydroponic system and in soil. Such differences could be due to that hydroponic production system providing a more favorable condition for bacterial growth due to the presence of higher moisture and greater water availability. Also, the microgreen root system in the hydroponic model may have a greater accessibility for *E. coli* O157:H7 attachment and colonization, and the bacterial cells have increased mobility in hydroponic environments *(9, 18)*. Alternatively, the observed differences between soil and hydroponic systems could be due to the presence of natural microflora in soil, which could outcompete *E. coli* and exert an inhibitory effect on its colonization of roots. However, both soil-based and hydroponic systems for microgreen production vary greatly in practice. The data should be interpreted with caution to avoid generalization when production practice differs significantly from that used in this study.

Effect of irrigation method on the survival and proliferation of *E. coli* O157:H7. Overhead spraying is typically used in soil and soil substitute–based microgreen production systems, while hydroponic microgreen productions typically use periodic saturation of the growth substrata from the bottom. In this study, these two irrigation methods were applied on radish microgreens grown in the soil-substitute system (Table 1). For overhead irrigation, water was applied to seeds and plants by using a pressurized fine garden sprayer. For bottom irrigation, water was applied directly to the germination mix by pouring water into the bottom tray, as for the hydroponic growing system. In this case, water primarily contacted the soil and roots of growing plants, which should prevent any perturbation to the natural distribution of pathogens on edible portions of plants. For both low and high initial inoculation level on the seeds, *E. coli* O157:H7 populations on edible (2.3 versus 2.1 log CFU/g for low inoculation and 3.4 versus 3.6 log CFU/g for high inoculation) parts of the radish microgreens using these two irrigation methods was not significantly ($P > 0.05$) different. For the inedible parts, *E. coli* O157:H7 populations with bottom irrigation were about 1 log higher than those with overhead irrigation (4.8 versus 3.9 log CFU/g for low inoculation and 5.7 versus 4.6 log CFU/g for high inoculation), which were statistically insignificant differences. It appeared that the mechanical disturbance by overhead irrigation did not play a significant role in washing off or redistributing bacterial cells from the cotyledons and hypocotyls. This observation was in contrast to a previous study of irrigation methods for lettuce growth, which determined that the bottom irrigation greatly reduced the likelihood of crop contamination in comparison to overhead irrigation *(12)*. The contradiction was likely due to the experimental designs. Solomon et al. *(12)* directly used contaminated irrigation water, while the contamination source in this study was the seeds. The difference between productions in the outdoor lettuce farms and indoor microgreen growing environments could simply be the reason. The overhead spraying used in this study was fine and gentle and did not cause visible splash from the surface of the growth medium. It can be envisaged that overhead irrigation causing significant splashing could have resulted in different outcomes.

Spatial distribution of *E. coli* O157:H7 on soil-grown microgreens. To examine the spatial distribution of seed-originated *E. coli* O157:H7 on the microgreen plants and in the production environment, radish microgreens were grown on peat moss germination mix with bottom irrigation. This method allowed us to minimize the influence of irrigation on the spatial distribution of *E. coli* and to examine the relatively intact roots of individual plants. The *E. coli* O157:H7 counts on leaves, upper hypocotyls, lower hypocotyls, roots, and seed coats of 25 randomly selected microgreens plants were determined and expressed as log CFU per unit (here, unit refers to one specific section of a single microgreen plant; Table 2).

At a relatively low inoculation level of 3.5 log CFU/g of seeds (approximately 1.7 log CFU per seed), *E. coli* O157:H7 counts ranged from 1.1 to 3.5 log CFU per unit on different sections of the microgreen plant, and 2.7 log CFU/g in the germination mix at the time of harvest (7 days after seeding). The seed coats remained the most densely populated part of the microgreen plant by *E. coli* O157:H7, and the hypocotyls were the least populated, while the leaves and the roots had comparable levels of contamination. Similar trends were

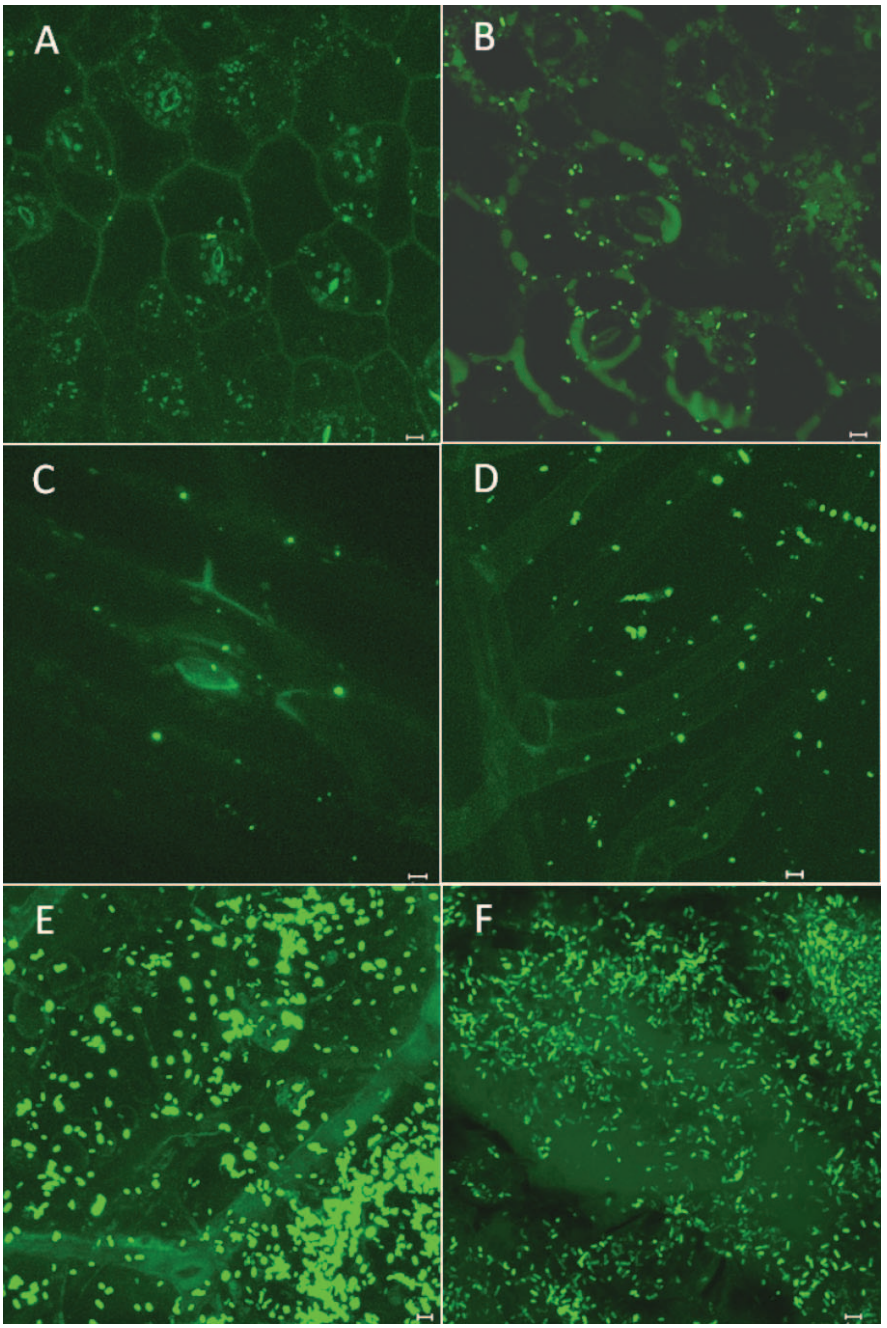

FIGURE. 2. *Presence of* E. coli *O157:H7 on different parts of microgreen plants. Confocal laser scanning microscopy images show representative views of the relative densities of GFP-labeled* E. coli *O157:H7 on (A) cotyledon, adaxial; (B) cotyledon, abaxial; (C) hypocotyl; (D) root; (E) seed coat on day 7; and (F) seed coat on day 0, respectively. Scale bar represents 5 μm.*

observed when the seeds were inoculated at high level (5.6 log CFU/g or approximately 3.7 log CFU per seed). However, *E. coli* O157:H7 counts on the seed coats did not increase proportionally, suggesting a growth limitation of the inoculated *E. coli* O157:H7 cells on the seed coat surface.

In this experiment, microgreen plants were irrigated by passively soaking the growth medium into water from the bottom tray; hence, there were no external factors that would have mechanically influenced the spatial distribution of *E. coli* O157:H7 (inoculated on the seeds) on the aerial part of the microgreen plants. Although the irrigation water could have served as a medium to mobilize bacterial cells from seed coats to soil matrix and the roots, the presence of *E. coli* O157:H7 cells on the leaves and the upper hypocotyls (edible parts) should be due to contamination occurring during natural germination of the seeds *(17)*.

On the scale of individual seeds or plants grown in the soil production system, the average combined *E. coli* O157:H7 counts on a microgreen plant at harvesting was approximately 2 log higher than that on a seed at sowing, at the inoculation level of 1.7 log CFU per seed. In contrast, the combined cell counts on the plants at harvest was only approximately 0.5 log higher than on the seeds at the initial inoculation level of 3.7 log CFU per seed. Although it is clear that *E. coli* O157:H7 contaminating the seeds is capable of significant proliferation during microgreen production, the relatively low altitude of *E. coli* O157:H7 increase at a higher inoculation level is suggestive that the growth of *E. coli* O157:H7 was approaching the limit. This limitation could be determined either by the amount of nutrient released by the germinating seeds or the abundance of competitive microflora present on the seed or in the

germination mix. Considering the higher proliferation in the hydroponic growth system previously described, it could be plausible that competitive microflora from the germination mix be a major contributor for controlling the growth of *E. coli* O157:H7.

Confocal imaging. The presence of GFP-labeled *E. coli* O157:H7 on different portions of radish microgreens was determined by confocal laser scanning microscopy (Fig. 2). GFP-labeled *E. coli* O157:H7 cells, which manifested as bright green rods on a subtly light green background of the microgreen tissues, were observed in all parts of radish microgreens grown from the contaminated seeds. On the cotyledons, *E. coli* cells were predominantly present on the abaxial side (Fig. 2B) instead of the adaxial side (Fig. 2A). It was previously reported that *E. coli* O157:H7 inoculated on lettuce leaves survived in greater populations on the abaxial side of the leaves than on the adaxial side *(21),* indicating that the environment of abaxial surface of leaves may be more conducive for pathogen survival. However, cotyledons are structurally and functionally different from the true leaves. Therefore, the predominant presence of *E. coli* O157:H7 cells on the abaxial might be a reflection that the abaxial was more accessible to the bacterial cells during seed germination. In comparison, the hypocotyls (Fig. 2C) were sparsely populated by *E. coli* cells at a level similar to that on the adaxial cotyledon, and the roots (Fig. 2D) were populated at a level similar to that on abaxial side of cotyledons. The *E. coli* cell density on seed coats was high (Fig. 2E). In comparison to those on the seed surface at the time of inoculation (Fig. 2F), there were no significant changes in bacterial populations on the seed coats by the time of microgreen harvest, indicating that seed coats remained the focal points of contamination throughout the production cycle.

This study demonstrated that *E. coli* O157:H7 can proliferate significantly during microgreen growth in both soil-substitute and hydroponic production systems from contaminated radish seeds. Compared with the hydroponic production system, the soil production system demonstrated less bacterial survival and proliferation on radish microgreens. Regardless of initial inoculation levels on seeds, overhead spraying and bottom irrigation did not show significant difference on the *E. coli* O157:H7 populations on edible parts of radish microgreens. Spatial distribution analyses revealed that the contamination was systematic, although the seed coats remained the focal point of bacterial persistence and growth.

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