Transmission of *Escherichia coli* O157:H7 to Internal Tissues and Its Survival on Flowering Heads of Wheat

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MS 14-298: Received 23 June 2014/Accepted 28 October 2014

ABSTRACT

*Escherichia coli* O157:H7 is a human pathogen that can cause bloody diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome. *E. coli* O157:H7 illnesses are mainly associated with undercooked beef; however, in recent years, outbreaks have been linked to fresh produce, such as spinach, lettuce, and sprouts. In 2009, flour was implicated as the contamination source in an outbreak involving consumption of raw cookie dough that resulted in 77 illnesses. The objectives of this research were to determine (i) whether *E. coli* O157:H7 could be translocated into the internal tissues of wheat (*Triticum aestivum*) seedlings from contaminated seed, soil, or irrigation water and (ii) whether the bacterium could survive on flowering wheat heads. The levels of contamination of kanamycin-resistant *E. coli* O157:H7 strains in seed, soil, and irrigation water were 6.88 log CFU/g, 6.60 log CFU/g, and 6.76 log CFU/ml, respectively. One hundred plants per treatment were sown in pot trays with 50 g of autoclaved soil or purposely contaminated soil, watered every day with 5 ml of water, and harvested 9 days postinoculation. In a fourth experiment, flowering wheat heads were spray inoculated with water containing 4.19 log CFU/ml *E. coli* O157:H7 and analyzed for survival after 15 days, near the harvest period. To detect low levels of internalization, enrichment procedures were performed and Biotecon real-time PCR detection assays were used to determine the presence of *E. coli* O157:H7 in the wheat, using a Roche Applied Science LightCycler 2.0 instrument. The results showed that internalization was possible using contaminated seed, soil, and irrigation water in wheat seedlings, with internalization rates of 2, 5, and 10%, respectively. Even though the rates were low, to our knowledge this is the first study to demonstrate the ability of this strain to reach the phylloplane in wheat. In the head contamination experiment, all samples tested positive, showing the ability of *E. coli* O157:H7 to survive on the wheat head.

*Escherichia coli* O157:H7 is a human pathogen that can cause a wide spectrum of diseases, such as bloody diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome. Due to the low dose of infection (between 10 and 50 cells) and the severity of the illness, the food industry is required to ensure the absence of pathogens in their final products (16, 42). Although beef has been the major vehicle of *E. coli* O157:H7 in the wheat, using a Roche Applied Science LightCycler 2.0 instrument. The results showed that internalization was possible using contaminated seed, soil, and irrigation water in wheat seedlings, with internalization rates of 2, 5, and 10%, respectively. Even though the rates were low, to our knowledge this is the first study to demonstrate the ability of this strain to reach the phylloplane in wheat. In the head contamination experiment, all samples tested positive, showing the ability of *E. coli* O157:H7 to survive on the wheat head.

*Escherichia coli* O157:H7 is a human pathogen that can cause a wide spectrum of diseases, such as bloody diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome. Due to the low dose of infection (between 10 and 50 cells) and the severity of the illness, the food industry is required to ensure the absence of pathogens in their final products (16, 42). Although beef has been the major vehicle of transmission, fresh produce has also been implicated as a carrier for this strain since 1990 (11, 21, 31). A large survey conducted by the U.S. Department of Agriculture (USDA) (43) analyzed fresh produce, including cantaloupe (*Cucumis melo*), celery (*Apium graveolens* var. *dulce*), leaf lettuce (*Lactuca sativa*), romaine lettuce (*L. sativa* var. *longifolia*), and tomatoes (*Lycopersicon esculentum*), and it was found that 0.62% of the produce was contaminated with *E. coli* that had virulence genes encoding at least one of the following: Shiga toxin (SLT-1, SLT-2, HlyA, intimin, and eae-gamma), heat-stable toxins types a and b (STa and STb), invasive characteristics (enteroaggregative *E. coli* [EAEC]), cytotoxic necrotizing factor (CNF-1, CNF-2), and capsular antigen (K1) (40). Even though this percentage is low, there is a high risk due to these products being consumed raw.

The transmission routes for produce have often been unclear, but research suggests that common contamination sources are soil, seed, and irrigation water (4, 8, 40). According to current regulations for organic production by the USDA (44), raw manure can be applied to crops providing there is at least 90 days from the application to the harvest period where the edible parts are not in contact with soil. In soil, the survival of *E. coli* O57:H7 can be extremely long, varying from 2 months in plain soil (37) to 6 months when temperatures are lower (4°C) (48) or even as long as 500 days when frozen soil conditions exist (17). In soil used for crop production, manure incorporation has been used as a common practice to increase water holding capacity and as a soil amendment; however, this also could increase the chance of contamination with *E. coli* O157:H7 (11, 23). Moreover, it has been reported that 0.7% of the wheat planted during 2006 was intentionally fertilized with manure, which may increase the risk of contamination with this bacterium (29). This strain has been shown to survive in this mixture for a period of 168 days and was present

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through the entire production cycle of carrots (Daucus carota) (23). Therefore, soil is considered an important source of contamination for the transmission of E. coli O157:H7 to raw agricultural products.

Water sources can be contaminated with E. coli O157:H7 through run-off from livestock operations during the rainy season (14). The survival of E. coli O157:H7 in water can be as long as 91 days at 8°C, 49 to 84 days at 25°C (49), and 12 to 18 days under farm conditions with a low inoculum level (3 log) (30). These survival periods would allow enough time for the organism to gain entry into the irrigation system, which would potentially become a vehicle of transmission.

Seeds are considered another important source of contamination of produce that is consumed raw (4). This is especially true in sprouts (Brassica oleracea), in which seeds historically have been an important vehicle of E. coli O157:H7 contamination. The organism’s survival in seeds could be as long as 1 year, and this strain can be recovered in high numbers (>3.5 log) when plants start to sprout again after 2 years (47). In 2011, an outbreak caused by consumption of sprouts (fenugreek) (Trigonella foenum-graecum) confirmed the possibility of transmission of pathogenic E. coli strains (O104:H4) from seeds to humans (15).

It has been demonstrated that E. coli O157:H7 can use the roots to translocate internally into plants, especially in the intercellular space (3). This contamination via the root system was shown to occur when manure, soil, or water contaminated with E. coli was used to grow lettuce (40, 41), alfalfa (Medicago sativa) sprouts (9), spinach (Spinacia oleracea) (38), and maize (Zea mays) (5). Other plant parts have been targeted for colonization by this bacterium as well, such as hypocotyls and cotyledons of radish (Raphanus sativus) (24) or leaves via stomata (26). E. coli O157:H7 has the ability to move within the Arabidopsis thaliana plant and apparently reach the flower and seeds (10).

Although previous research provides evidence of the ability of E. coli O157:H7 to internalize and translocate into certain types of plants, its ability to internalize into wheat plants has never been studied. One study showed that pathogenic bacteria, such as Salmonella enterica, were able to colonize the roots and be transferred into the shoot of barley (Hordeum vulgare) (27). As both barley and wheat belong to the grass family Poaceae, there is a possibility that a pathogen like E. coli O157:H7 could internalize in these plant species as well.

Wheat flour is an agricultural product usually sold without any pasteurization treatment. Therefore, flour could contain pathogenic bacteria when it is produced from contaminated grains. In a 1993 survey, it was determined that 12.8% of U.S. wheat flour was contaminated with E. coli (nonpathogenic strains) and 1.3% with Salmonella (36). In a study in Thrace in 2009 (5 years ago), the E. coli count in 72 (50.7%) of 142 wheat flour samples exceeded the legal limits of the Turkish Food Codex (1). Contamination would likely come from the environment in which these wheat kernels are produced, and potential sources would include seed, soil, or irrigation water or be encountered during postharvest handling interventions.

In 2009, flour was suspected as the contamination source in an outbreak involving the consumption of raw cookie dough resulting in 77 illness cases, and 10 people developed hemolytic uremic syndrome (34). According to the report, around 3.5 million packages of cookie dough and reformulated products were subjected to a recall (34). About 11% of patients declared that they ate the cookie dough raw and actually intended to eat it unbaked (34). This is the first time that flour was suspected as the vehicle of transmission of E. coli O157:H7. Other illnesses have also been associated with the consumption of raw flour. For example, Salmonella produced 66 cases of illness by the consumption of raw flour in New Zealand (12). Another outbreak involving cake batter ice cream contaminated with Salmonella occurred in 2005 in the United States (53). These outbreaks suggest that flour could be a route of contamination for humans when it is consumed raw. One survey suggests that Salmonella can be present in the range of 0.0 to 1.3% in flour samples in Australia and North America (18). If pathogens like E. coli O157:H7 have the ability to internalize into wheat, it could explain one possible source of infection in outbreaks linked to flour.

The objective of this study was to determine the probability of transmission of E. coli O157:H7 to the tissue or phylloplane of wheat from contaminated soil or seed or irrigation water in the early stage of wheat seedling growth, as well as the ability of this strain to survive on wheat heads for a 15-day period.

**MATERIALS AND METHODS**

**Bacterial strains and preparation of inoculum.** Five strains of E. coli O157:H7 with resistance to kanamycin (AU156 T2, AU1811 LEE3, AU726 LEE2, AU1809 LEE2, and AU1823), isolated from calf and cattle manure and human stools, were used in these experiments (51). A pure culture of each strain was stored in 10% glycerol at −80°C until needed. At the time of inoculum preparation, 100 µl of each stock culture was transferred to test tubes containing 9 ml of tryptic soy broth (TSB; Acumedia, Lansing, MI) with 50 µg/ml kanamycin (Fisher Scientific, Newark, DE) and incubated for 24 h at 37°C. After incubation, three consecutive transfers were performed in TSB as previously described. After the third transfer, E. coli O157:H7 cells were then harvested by centrifugation (Eppendorf 5810R, Eppendorf, Hauppauge, NY) at 5,500 × g for 10 min at 4°C in 50-ml conical tubes and finally resuspended in phosphate buffer solution to a final cell density of ≈10⁶ CFU/ml. Equal volumes (10 ml) of the five E. coli O157:H7 strain suspensions were combined into a single cocktail.

**Seeds and sanitization procedure.** Seeds of two wheat cultivars, Coker 9835 and VA04W-433, were obtained from the Department of Plant Pathology at the University of Nebraska–Lincoln. The seeds were originally provided by Dr. Carl Griffeys, Virginia Polytechnic Institute and State University. Coker 9835 is a soft red winter wheat with susceptibility to Fusarium head blight (FHB), whereas VA04W-433 is a soft red winter wheat with moderate resistance to FHB (19). FHB is caused by a fungal pathogen whose spores infect wheat heads during the early flowering growth stage. These cultivars were being used in a study...
on FHB and were chosen for use in these experiments because their seeds have been commercially grown by wheat farmers and were available. Both cultivars were used in the seed, soil, and irrigation contamination experiments. Seed sanitization consisted of submersion of the seeds in 70% ethanol (1 min), followed by submersion in 6% sodium hypochlorite (1 min), three rinses with sterile distilled water (1 min), and a final drying step lasting at least 2 h inside a biosafety cabinet (32). The seeds were then stored in an aseptic container to avoid recontamination, and the storage period was no longer than 1 day before use.

Soil preparation and sanitization. Steam-pasteurized soil mix (33% clay loam soil, 33% peat soil, 16.5% sand, and 16.5% vermiculite) was passed through a 4-mm-pore-size sieve. It was then autoclaved twice for 1 h at 121°C and placed in pot trays (50 g of soil per pot).

Seed inoculation. Two grams of sanitized wheat seeds was contaminated by submersion for 40 min into 40 ml of E. coli O157:H7 cocktail (population of >8 log CFU/ml), using pin-hole-perforated sterile aluminum pans and a sterile beaker. Seeds were placed into the cocktail (in a beaker) using the aluminum pan. After 40 min, seeds were drained and allowed to air dry on a sterile filter paper placed in a petri dish in a biosafety cabinet for 2 h. One gram of the seeds was retained, as a control, to determine the initial bacterial counts using tryptic soy agar (TSA; Acumedia) plus kanamycin (50 µl/g). The remaining inoculated seeds were sown in sterile soil.

Soil inoculation. Soil was placed in a tray at a depth of 2.5 cm and sprayed evenly with the five-strain bacterial cocktail using an atomizer to assure a population of 6 log CFU/g of soil of E. coli O157:H7. This procedure was performed inside a type II biosafety cabinet. The soil was then distributed into pot trays, where 50 g of soil was placed in each pot. Sanitized seeds were sown in these pot trays.

Irrigation water inoculation. For this experiment, sanitized seeds were planted in sterile soil. The initial contamination occurred when 5 ml of contaminated water (6.76 log CFU/ml) was added in a circular area (1 cm in diameter) around each seed during sowing. Using a pipet tip, water was added carefully into an area 1 cm in diameter from the center of the seed. This water then slowly infiltrated through the soil until it reached the seed.

Growth conditions. Fifty grams of soil (sterile or inoculated) was placed in each cell (5 by 4 by 5.7 cm). For the seed, soil, and irrigation water experiments, seeds were planted at a depth of 15 mm in the soil and watered daily with 5 ml of Butterfield’s solution. The ambient temperature ranged from 23.5 to 25.5°C for all experiments. The plants were grown by placing the cell trays in a serological water bath (model 148007, Boekel Industries, Feasterville, PA) retrofitted with a light supply (10 to 12 h of light per day).

Seed, soil, and irrigation experiments. After seeds germinated under the growth conditions described, wheat seedlings (approximately 50 seedlings from each wheat variety) were collected 9 days postinoculation. Seedlings from both cultivars were cleaved 1 cm above the soil surface in the cell tray pots using a sterile scalpel and forceps. Seedlings were sanitized following the protocol previously described by Mitra et al. (32). This protocol was used to eliminate E. coli O157:H7 that could potentially be present on the outside of the plants, as well as other bacteria on the surface, leaving only bacteria that might be internalized in the seedling. The cleaved and sanitized seedlings were cut into small pieces (1 cm) inside a sterile bag for the seed and soil experiments. For the irrigation water experiment, seedlings were macerated. In both preparations, the seedling fragments were diluted (approximately 1:10) using modified TSB plus novobiocin (Acumedia) as enrichment for E. coli O157:H7. The bags were then placed in a stomacher for 2 min and incubated for 48 h at 37°C. Qualitative detection of E. coli O157:H7 was performed on enriched samples by PCR.

E. coli O157:H7 detection by qPCR. Samples were analyzed with AOAC International–validated Foodproof E. coli O157 detection kits (hybridization probes LC 2.0 [for LightCycler], manufactured by Biotecno Diagnostics [Potsdam, Germany], using the LightCycler 2.0 real-time PCR (qPCR) system [Roche Applied Science, Penzberg, Germany]. To avoid false positives, the ethidium monoozide–loop-mediated isothermal amplification method was used to eliminate amplification of DNA from nonviable cells. The DNA extraction procedure was followed according to the manufacturer’s instructions (7). Controls were analyzed to confirm correct performance of the qPCR assays, including positive and matrix controls.

Head inoculation experiment. The wheat cultivar Wheaton was used for the head contamination experiments. This cultivar is a hard red spring wheat that is also susceptible to FHB (54). The cultivar was chosen for use in this experiment because it was being used in a study on FHB, its seed was available, and its seed does not require vernalization (exposure to cold temperature to trigger reproductive development) to induce flowering. Seeds were not sanitized and were planted with their natural microflora. Seeds were planted in 6-in. pots (four seeds per pot). Plants were allowed to grow in a plant pathology greenhouse room at the University of Nebraska–Lincoln for approximately 70 days until they reached the flowering stage. The temperature in the green house room ranged from 21°C (nighttime) to 27°C (maximum daytime). Plants were watered twice a day (morning and evening) and were fertilized with water-soluble 20-10-20, N-P-K fertilizer (Peter’s Professional Peat-Lite Special) at a concentration of 250 ppm, 5 days a week. Plants were transported to a biosafety level 2 laboratory for the inoculation experiments.

Using a laboratory atomizer to assure even distribution, the heads were sprayed with 5 ml of a culture cocktail of E. coli O157:H7 (the same used in previous experiment) with a population close to 4 log CFU/ml. Three heads per plant were contaminated with at least 3 log CFU per head E. coli O157:H7 per pot. Extra heads were inoculated as controls and analyzed after 24 h to determine whether the desired inoculation levels were achieved. After inoculation, plants were placed in a model 3759 Precision plant growth incubator (Thermo Fisher Scientific, Marietta, OH) with a 12-h photoperiod and a constant temperature of 23.7°C (maximum daytime). Plants were watered every 2 days with 60 ml of potable water.

At 15 days postcontamination, each head was removed, cleaved into small pieces inside a sterile bag, and diluted (1:100) with modified TSB plus novobiocin as enrichment. The bags were homogenized using a Stomacher 400 (Seward Ltd., London, UK) for 2 min. Enumeration (using TSA plus kanamycin) was performed to determine the bacterial population on each wheat head. Control heads were enumerated following the same procedure. Finally, the enrichments were incubated for 48 h at 37°C to confirm the presence of E. coli O157:H7 by qPCR.

Experimental and statistical design. In soil, seed, or water experiments, each cell tray was considered an independent experimental unit. The target number of units per experiment was 50 seedlings of each of the two wheat cultivars used. To
achieve 50 seedlings per variety, plantings were performed in two batches. From the seedlings that germinated in each batch, 25 units per cultivar were randomly selected and analyzed 9 days after planting. A total of 50 negative-control seedlings were analyzed along with the ones contaminated with *E. coli* O157:H7 to assess any potential cross-contamination in each of the three seedling experiments. Chi-square analysis was performed using SAS 9.2 software (SAS Institute Inc., Cary, NC) to determine differences among experiments in the rate of *E. coli* O157:H7 internalization.

For the wheat head inoculation experiment, 100 contaminated heads were targeted and analyzed in three batches. For each batch, three heads were contaminated per plant, and at least 12 plants were grown, along with control plants. In each batch, 33 or 34 heads were analyzed, along with 10 negative-control heads (without contamination).

### RESULTS AND DISCUSSION

**Recovery of internalized *E. coli* O157:H7 from wheat seedlings grown from inoculated seeds.** The population of *E. coli* O157:H7 on seeds was, on average, 6.68 ± 0.44 CFU/g in the four batches tested (two batches per wheat cultivar). Nine days postinoculation, cultivar Coker 9835 had 1 seedling of the 50 analyzed that was positive for *E. coli* O157:H7, indicating that this organism had become internalized into the plant. Similarly, the wheat cultivar VA04W-433 had 1 positive seedling from 46 seedlings tested over two batches. The results for the negative controls indicated that there was no cross-contamination during the analysis, and the results for the positive controls confirmed the performance of the qPCR kits. Therefore, according to the data, seed contamination resulted in internalization of *E. coli* O157:H7 in 2 of 96 seedlings tested.

**Recovery of internalized *E. coli* O157:H7 from wheat seedlings grown in inoculated soil.** The soil used in this experiment was contaminated on average with 6.60 ± 0.58 log CFU/g *E. coli* O157:H7, according to bacterial counts performed using TSA plus kanamycin. Qualitative analysis using qPCR revealed three seedlings positive for the presence of *E. coli* O157:H7, indicating the internalization of this organism into seedlings of cultivar Coker 9835. These positive seedlings were among 50 that were analyzed in two batches. For variety VA04W-433, 2 *E. coli* O157:H7–positive seedlings were detected among 50 tested. Therefore, 5 positive samples were detected out of 100 from both wheat cultivars. This trial detected a higher number of positive seedlings (arithmetically) than was found in the seed contamination experiments; however, there was no significant difference between results for the seed and soil contamination experiments (Table 1).

**Recovery of internalized *E. coli* O157:H7 from wheat seedlings grown using inoculated irrigation water.** The irrigation water used for contamination of soil and seed in this experiment had, on average, a population of 6.76 ± 0.11 log CFU/ml *E. coli* O157:H7. Qualitative qPCR revealed 5 of 50 seedlings positive for the presence of *E. coli* O157:H7 for each cultivar. These data suggest that internalization occurred in 10% of the cases when this route of transmission was used.

**Wheat internalization in seed, soil, and irrigation experiments.** For each of these experiments, the contents of 10 cells from the plastic trays (soil samples plus the roots of the cleaved plants) were analyzed to determine the presence of *E. coli* O157:H7 9 days postinoculation. In all cases, *E. coli* O157:H7 was recovered from the pots, confirming its presence at seedling sampling time. This result was expected, since the time period used during this experiment was relatively short. Fifty negative controls were also analyzed during the experiments, and as expected, all of them were negative for *E. coli* O157:H7.

Statistical analysis showed no difference in the internalization rates for any experiment (seed, soil, or irrigation water) due to the wheat variety (P values of 0.9525, 0.644, and 1, respectively); therefore, results were combined to increase the statistical power to determine the differences in internalization rates between experiments. The results from chi-square analysis showed the differences between experiments and controls (Table 1). It was shown that the results for irrigation water were statistically different from the results for the control, demonstrating a probability of plant internalization of *E. coli* O157:H7 in 10% of the cases. The results for the seed and soil experiments were not statistically different from the results for the controls (Table 1). Even though sanitation procedures were carefully followed to eliminate any external contamination, it is possible that a few bacterial cells could have escaped or survived the sanitation process in the wheat seedlings and, therefore, have contributed to the 10% rate observed. In the seed contamination experiment, the population of *E. coli* O157:H7 was 6.68 log CFU/g of seeds, and each cell received only one seed, which is equivalent to approximately 3.90 log CFU/g of soil. However, in the soil experiment, the soil was contaminated with 6.60 log CFU/g and each cell had 50 g of soil; therefore, the population per pot in this experiment was higher than the population used for the seed experiment. This difference may have influenced the rate of internalization, leading to a higher rate than was found in the seed experiment; however, no statistically significant difference was found. For the irrigation water experiments, as described above, 5 ml of contaminated water was placed in each of the cells containing 50 g of soil, therefore making an extra 10-fold dilution, bringing the count to 5.76 log ± 0.11 CFU/g in the soil. Moreover, other researchers have suggested that water serves to mobilize pathogens to the roots of plants in a more

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**TABLE 1. Internalization of *E. coli* O157:H7 in seedlings using different sources of contamination**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>No. of positive samples/ total no. of samples</th>
<th>Internalization rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seed contamination</td>
<td>2/96 b</td>
<td>2.1</td>
</tr>
<tr>
<td>Soil contamination</td>
<td>5/100 b</td>
<td>5.0</td>
</tr>
<tr>
<td>Irrigation water</td>
<td>10/100 A</td>
<td>10.0</td>
</tr>
<tr>
<td>Total controls</td>
<td>0/50 b</td>
<td>0.0</td>
</tr>
</tbody>
</table>

*Statistical significance was detected using chi-square analysis (P < 0.05). Values with different letters are significantly different.*
advantageous way than moist soil (13). Therefore, contaminated irrigation water may be the most likely route of internalization, since the rate was higher even when the population was lower.

Unfortunately, the exact location where E. coli O157:H7 entered the plant was not determined during these experiments. It has been suggested that E. coli could be internalized more efficiently in young plants due to low plant defenses that are not well developed during germination when seed breakage occurs (20). Habteselassie et al. (20) reported that contaminated irrigation water (6 log CFU/ml E. coli O157:H7) resulted in contaminated lettuce at 10 days postinoculation. In that research, E. coli O157:H7 was translocated to the leaves without direct contact with the irrigation water. Internalization of E. coli O157:H7 in lettuce was also studied, using low contamination levels (<4 log CFU/g) with manure or soil as the contamination source, and that research showed an internalization rate of 7% (33), similar to the rate reported here.

In previous studies, E. coli O157:H7 was able to internalize into A. thaliana (10), lettuce sprouts (20), and mung bean sprouts contaminated with E. coli P36 (50). Although our study is not the only one to report internalization of E. coli O157:H7 in plants, it is the first to report internalization of the bacterium in wheat. Seeds produced from contaminated Arabidopsis plants were also contaminated with E. coli O157:H7, with an internalization rate of around 26.7% (10). E. coli O157:H7 has also been found to be internalized in other plants, such as tall fescue (Festuca arundinacea Schreb) (175 days old), which was contaminated using slurry manure with 5 log CFU/g, with the results showing an internalization rate of 18% (28).

These experiments provide evidence that E. coli O157:H7 can become internalized and be translocated from a contaminated soil matrix environment into different parts of the plant in rare cases only. However, it is important to note that all studies available on internalization in plants have different internalization rates, procedures, and sampling time after exposure, which makes it difficult to conclude whether internalization is driven by a single factor. Researchers have investigated internalization and survival of E. coli O157:H7 over time via roots in leafy greens, and their results suggest that internalization decreases a short time (5 days) after exposure (13). Other investigators suggested that internalization could only occur after a short period of time during the initial transplantation procedure in cabbage under tropical conditions (35). Therefore, it seems unlikely that E. coli O157:H7 would be able to translocate itself from the root to the stem of the plants at the seedling stage and survive long enough to reach the harvesting period of wheat. However the situation is still unclear until internalization rate.

**Recovery of E. coli O157:H7 from inoculated wheat heads.** Heads of wheat cultivar Wheaton at the flowering growth stage (average weight of 1.23 ± 0.24 g) were sprayed with 5 ml of water contaminated with E. coli O157:H7 at a population of 4.19 ± 0.61 log CFU/ml. The absorption of the inoculum was measured from a range of 120 to 200 µl per head, which suggests a final contamination population of 3.37 ± 0.55 log CFU/g. Enumeration using TSA plus kanamycin after 24 h was performed to determine the population of E. coli O157:H7 on the heads after inoculation. The population of E. coli O157:H7 on the heads after this 24-h period was 6.51 ± 0.38 log CFU/g. This is higher than the expected population (3.37 ± 0.55 log CFU/g) or the inoculum population (4.19 ± 0.61 log CFU/ml). These results suggest that E. coli O157:H7 has the ability to multiply on wheat heads in the flowering stage, since no kanamycin-resistant bacteria were found in control plants. To verify whether these bacteria enumerated using TSA plus kanamycin were the strains inoculated on the heads, selected colonies were collected and streaked onto sorbitol MacConkey agar (Acumedia). All of the selected isolates grew well on the selective medium, indicating that these bacteria were indeed E. coli O157:H7.

Wheat head samples were analyzed using qPCR to determine the presence of E. coli O157:H7 at 15 days postinoculation. The results of the analysis showed that all samples were positive. Enumeration of the pathogen on the heads was also done to determine the population of E. coli O157:H7 after this time period. The results showed populations of 5.89 ± 0.61, 5.98 ± 0.38, and 5.69 ± 1.13 log CFU/g of heads for the three batches of plants (total of 100 heads analyzed). The ability of E. coli O157:H7 to survive on wheat heads for 15 days has not been reported before; at this point, wheat maturity has been reached and it could be close to harvest, depending on environmental conditions. This may indicate a safety risk, since wheat heads could become contaminated with the organism in the field and it could subsequently be carried through the wheat chain, eventually reaching products such as wheat flour.

Other studies have used water as a source of contamination by E. coli O157:H7 and determined its survival in produce. Field experiments have found that E. coli O157:H7 survived in the phylloplane on lettuce for more than 5 days when contaminated water was used for irrigation just before harvest (2). Investigators found that when lettuce was sprayed with fairly low populations (2 log CFU/ml) of E. coli O157:H7, the population could increase on the surface of plants with each watering (39). That study also showed that even with a low contamination level (2 log CFU/ml), E. coli O157:H7 could survive on the leaves of lettuce for at least 10 days after inoculation. Other studies showed longer survival times for E. coli O157:H7, as long as 77 days on lettuce and 177 days on parsley (Petroselinum crispum) (22).

An important aspect related to the survival of pathogenic E. coli on the surfaces of plants is that research
has shown that internalization may occur when bacterial cells are deposited on external surfaces of plants, such as leaves. In this case, inoculation levels play a key role in the internalization of *E. coli* O157:H7 in spinach (14). The results showed that immediately after contamination with low inoculation levels, there was no internalization of bacteria (2 log CFU/ml), but for high doses (6 log CFU/ml), the internalization rate was 25% of the leaf samples immediately after contamination. This study showed that attachment to the phylloplane can occur almost immediately when high doses are applied (14).

In the production of wheat, the peak of irrigation, according to Yonts et al. (52), is during the heading growth stage, which is the stage during which the head experiment reported here was performed. According to a USDA report in 2014, wheat production is located mainly in states like Kansas, North Dakota, Montana, Texas, and Washington (46), which also have abundant cattle operations (45). Therefore, potential contamination may occur if the irrigation water is contaminated with *E. coli* O157:H7. Wheat fields located close to livestock feedlots, where there is abundant manure, could increase the risk of *E. coli* O157:H7 contamination of wheat heads during rain storms or irrigation. Low inoculum levels can remain viable for a substantial period of time; therefore, the control of pathogenic bacteria in irrigation water is a key strategy in managing food safety risks.

In conclusion, the present study demonstrated for the first time that internalization of *E. coli* O157:H7 in wheat plants occurred under experimental conditions (sterile soil, absence of natural microflora, ambient lighting, temperature, etc.), but it was infrequent. These conditions may not simulate the environmental conditions present in real-world crop production, and hence, this information may not be directly transferable to the field. The most important finding of this study, however, is that irrigation of wheat plants at the flowering growth stage is the most likely route of contamination under real environmental conditions, since *E. coli* O157:H7 showed a high rate of survival on the wheat heads. Furthermore, this study provided important information for understanding the possible routes of contamination of wheat by *E. coli* O157:H7.

**ACKNOWLEDGMENT**

This study was supported by ConAgra Foods, Inc., Omaha, NE.

**REFERENCES**

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