Research Note

Internalization of Salmonella enterica Serovar Montevideo into Greenhouse Tomato Plants through Contaminated Irrigation Water or Seed Stock

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ABSTRACT

Tomatoes have been linked to outbreaks of salmonellosis, demonstrating the need to identify sources of contamination. Objectives of this study included determining the ability for Salmonella enterica serovar Montevideo to be internalized into tomatoes from contaminated irrigation water and seed stock, and establishing whether Salmonella Montevideo can survive in fertilizer solutions. Six treatment groups (five plants per group) were irrigated with 350 ml of 7 log CFU/ml of Salmonella Montevideo every 14 days for 70 days, each group receiving an increased number of contaminated water events progressively: group 1 received one contaminated watering at day 0, and group 6 received a total of six contaminated waterings. Group 7 was a control, and group 8 was grown from seeds soaked in 8 log CFU/ml of Salmonella Montevideo for 24 h. All plants were watered daily with uncontaminated water. Three replications were completed. Fruit from every plant, and roots, stems, and leaves of one plant per treatment were sampled. All tomatoes were negative for Salmonella Montevideo; five root samples tested positive. For fertilizer studies, a commercially available fertilizer, two custom mixed and 1.0% dilutions of each (total of six solutions), and sterile water were inoculated with 8 log CFU/ml of Salmonella Montevideo. Uptake of Salmonella Montevideo into plant tissues via other routes has also been identified. When tomato plant flowers were brushed with a five-strain cocktail of S. enterica (serovars Enteritidis, Hartford, Michigan, Montevideo, and Poona), 25% of the ripened fruit was contaminated (8). Additionally, plants inoculated by stem injection 5 cm below the flower base also produced contaminated fruit (8). Salmonella Montevideo was the most persistent serovar present on the fruit (8).

The increased number of foodborne outbreaks associated with tomatoes prompted the U.S. Food and Drug Administration to implement a multiyear initiative aimed at reducing tomato-related illnesses (15, 16). Goals of this initiative include identifying production practices or conditions that may lead to tomato contamination, as well as facilitating and promoting research on tomato safety (15, 16). The increasing number of outbreaks has raised concerns about the preharvest colonization of tomatoes with Salmonella enterica.

Irrigation of plants and seedlings with contaminated water could be a potential source of fruit contamination. Internalization of S. enterica into the hypocotyls and cotyledons, stems, and leaves of tomato seedlings occurred after 1, 5, and 9 day(s) of growth when grown hydroponically in contaminated nutrient solution (10). Uptake of S. enterica into plant tissues via other routes has also been identified. When tomato plant flowers were brushed with a five-strain cocktail of S. enterica (serovars Enteritidis, Hartford, Michigan, Montevideo, and Poona), 25% of the ripened fruit was contaminated (8). Additionally, plants inoculated by stem injection 5 cm below the flower base also produced contaminated fruit (8). Salmonella Montevideo was the most persistent serovar present on the fruit (8).

There is little research regarding whether S. enterica is capable of translocation through the vascular tissue of tomato plants, from root to fruit. Jablasone et al. (12) investigated the potential for Salmonella Enteritidis to transfer into tomatoes through irrigation water. In their study, no Salmonella Enteritidis was recovered from stems, leaves, or fruit of tomato plants; roots were not evaluated (12). Studies tracing potential colonization pathways of Salmonella Montevideo through the root system into tomato plants from seedlings to fruit have not been completed but should be, since this serovar is most persistent in/on tomatoes under experimental conditions. The objectives of this study were to determine the ability of Salmonella Montevideo to be internalized in tomato fruit and plant tissue from contaminated irrigation water and seed stock, and to establish the ability of the bacterium to survive in both commercial, custom mixed, and diluted fertilizer stock solutions.

MATERIALS AND METHODS

Bacterial cultures. Salmonella Montevideo, isolated from a tomato related outbreak, was provided by Dr. Larry Beuchat from the Center for Food Safety and Department of Food Science and Technology, University of Georgia, Griffin.

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Table 1. Schedule for the application of Salmonella Montevideo–contaminated water per treatment group; all plants received 350 ml of water all other days during growth

<table>
<thead>
<tr>
<th>Treatment group (n = 15)</th>
<th>Day</th>
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<tr>
<td></td>
<td>1</td>
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<tr>
<td>1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
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<tr>
<td>2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
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<td>3&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>4&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>5&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>6&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>–</td>
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<tr>
<td>8&lt;sup&gt;d&lt;/sup&gt;</td>
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<sup>a</sup> +, watered with 350 ml of Salmonella Montevideo–contaminated water; −, watered with 350 ml of sterile distilled water.

<sup>b</sup> Groups 1 through 6 were irrigated with Salmonella Montevideo at 14-day increments.

<sup>c</sup> Group 7 was the control—no Salmonella Montevideo irrigation.

<sup>d</sup> Group 8 comprised seeds soaked in 8 log CFU/ml of Salmonella Montevideo prior to germination—no Salmonella Montevideo irrigation.

Inoculum preparation. Bacterial cultures were stored in a 30% glycerol, tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD) solution at −80°C. Cells were activated by three successive 24-h incubations at 37°C in TSB. The suspension was then centrifuged for 20 minutes at 7,500 × g, and the cells in the pellet were resuspended in sterile distilled water. The culture was plated onto tryptic soy agar (Difco, Becton Dickinson), incubated for 24 h at 37°C, and confirmed by using the API 20E test kit (bioMérieux, l’Étoile, France).

To prepare watering inoculum, 18 ml of TSB was inoculated with Salmonella Montevideo and incubated for 24 h at 37°C. The 18-ml tubes were centrifuged at 7,500 × g for 20 min, and the cells in the pellet were resuspended in 18 ml of sterile 0.1% peptone water. This suspension was added to 1,782 ml of sterile distilled water to create a suspension containing 7 log CFU/ml. Each batch was divided into five 350-ml aliquots.

Tomato plant cultivation. Tomato seeds, cv. Trust (Coor Farm Supply, Smithfield, NC), were grown to seedling stage in 46-cm<sup>3</sup> plug trays containing commercial planting medium (uniform in size and color, pH adjusted to 5.5 with hydrated lime). Each tray contained 54 uninoculated seeds and 15 seeds inoculated with Salmonella Montevideo; plugs containing inoculated seeds were separated and placed in plastic saucers to collect any contaminated runoff water and to prevent contamination of other seedlings. Seedlings were grown in plug trays until the four true-leaf stage (<2 weeks). Thirty-five uninoculated seedlings and 10 seed-inoculated seedlings were selected for uniformity and transplanted to Dutch Bato Buckets (Coor Farm Supply, Smithfield, NC) 30 by 27 by 22 cm, containing aged lobolly pine bark medium (pH 4.2) mixed with 833 g/m<sup>2</sup> micronutrients (Micromax, Scotts Co., Marysville, OH) and 3.3 kg/m<sup>3</sup> dolomitic lime. Each bucket was placed in a separate 40-cm<sup>3</sup> plastic saucer to collect runoff.

Three complete growth replications were completed. Two were grown in university greenhouses on the Virginia Polytechnic Institute and State University, Blacksburg, campus (average temperature of 21.5 ± 5°C, relative humidity of 69%); a third repetition was grown in the university-owned Clover Hollow greenhouse in Newport, Virginia (average temperature of 22.1 ± 7°C, relative humidity of 71%). Both greenhouses were comparable in structure and environmental conditions. Greenhouses contained ridge vents for cooling, an evaporative cooler, and a gas heater.

Two custom fertilizers, recommended and donated by Coor Farm Supply, were mixed into separate batches. Fertilizer 1 (US-S) consisted of 11.3 kg of UltraSol (SQM Corp., Atlanta, GA), 4.53 kg of Epsom salts, and 2.27 kg of 0N-0P-43.2K fertilizer to 114 liters of water; fertilizer 2 (CN-S) consisted of 11.3 kg of calcium nitrate and 56.7 g of iron chelate to 114 liters of water. Each concentrated (stock) fertilizer was processed through a fertilizer injector (1:100, Dosatron International, Clearwater, FL) to create a 1.0% fertilizer water solution (CF-1.0, US-1.0, and CN-1.0). Plants were watered (350 ml) and fertilized daily, alternating fertilizers 1 and 2 every other day.

Preparation of fertilizer solutions for survivability experiments. Full strength (stock) and 1.0% diluted concentrations of one commercial (CF-S; 20N-4.4P-16.6K, Scotts Co.) and the two custom-mixed fertilizers (US-S and CN-S, previously described) were used to evaluate the survivability of Salmonella Montevideo in fertilizer solutions. Diluted fertilizers were created by using fertilizer injector, as described previously.

Inoculation of plants, seeds, and fertilizer. Eight treatment groups of plants were tested per replication. Six treatment groups, (five plants per group) were irrigated with one 350-ml aliquot of 7 log CFU/ml of Salmonella Montevideo every 14 days for 70 days, each group receiving an increasing number of contaminated water events: group 1 received one contaminated watering at day 0, and group 6 received a total of six contaminated aliquots, every 14 days (Table 1). The suspension was poured directly onto the pine bark medium and roots of the plant. Group 7 was the control, and group 8 plants were grown from seeds soaked in 8 log CFU/ml of Salmonella Montevideo prior to germination—no Salmonella Montevideo irrigation.

Microbiological analysis. All fruit from each plant, not damaged or diseased, was sampled. Tomatoes were harvested at the red-ripe stage (approximately 80 days posttransplant), and weighed prior to analysis (65.2 ± 20 g). Tomatoes were surface sanitized and rinsed according to methods previously described (10). Briefly, tomatoes were surface sanitized by completely immersing in a 70% ethanol solution for 2 min and stored under a laminar flow hood until dry. The tomatoes were then placed in a sterile stomacher bag containing 20 ml of 0.1% peptone water and hand rubbed for 2 min. The resulting wash water was streaked onto Hektoen enteric agar (Remel, Lenexa, KS), and incubated at 37°C for 24 h. The stem scar and pulp were dissected by using a sterile scalpel and placed into a sterile stomacher bag. Samples were homogenized for 2 min in 10 ml of 0.1% peptone. One milliliter of sample was enriched in 9 ml of selenite cysteine broth (Difco, Becton Dickinson) incubated for 24 h at 37°C and streaked onto Hektoen enteric agar for another 24-h incubation at 37°C. Salmonella was identified by typical colony formation (blue-green colonies with black centers, indicating H<sub>2</sub>S production).

The roots, stems, and leaves from one tomato plant per treatment group were sampled (eight plants per replication and three replications, for a total of 24 plants). The roots were washed in tap water and dipped in a 1% sodium hypochlorite solution for 2 min, re-rinsed with sterile tap water, disinfected with a 70% ethanol spray, and allowed to dry under a hood. The stems and leaves
were surface disinfected by spraying with a 70% ethanol solution and placing under a flow hood until dry. Surface disinfection treatments were confirmed in preliminary work by surface inoculating samples, allowing them to dry for 1 h under a flow hood, subjected to surface disinfection treatments mentioned previously, and plating homogenates (data not shown). After surface disinfection, stems were sampled in 3-cm portions, beginning at 20, 40, and 60 cm from the soil line. Each of the tissue samples were combined with 10 ml of 1 M MgSO₄ and stomached for 2 min. One milliliter of homogenate was used to inoculate 9 ml of selenite cysteine broth, incubated for 24 h at 37°C, and streaked onto Hektoen enteric agar for an additional 24-h incubation at 37°C. *Salmonella* Montevideo colonies were identified by typical colony formation.

Serial dilutions of each fertilizer sample were made and spread plated in duplicate onto Hektoen enteric at 0, 24, 48, and 72 h. Because enumeration procedures yielded no growth, sample CF-S was also enriched in selenite cysteine broth for 24 h at 37°C and streaked onto Hektoen enteric agar.

**Statistical analysis.** Three replications were completed. Each replicate contained five experimental units per treatment group (40 plants). No less than five tomatoes per plant were analyzed. In addition, roots, stems, and leaves from one plant per treatment group were sampled.

Differences in survival of *Salmonella* Montevideo (log CFU per milliliter) in seven fertilizer solutions (CF-S, CF-1.0, US-S, US-1.0, CN-S, CN-1.0, and control), and the effect of time (0, 24, 48, and 72 h) were analyzed by using the General Linear Model procedure in Statistical Analysis Software, version 9.1 (SAS Institute, Inc., Cary, NC). Means were separated for significance by using Tukey’s honestly significant difference test with a confidence level of $P \leq 0.05$.

**RESULTS AND DISCUSSION**

None of the tomatoes tested in all treatment groups was positive for the presence of *Salmonella* Montevideo in either the stem scar or the fruit pulp. Additionally, plants grown from contaminated seed (group 8) did not yield any fruit or plant tissues positive for *Salmonella* Montevideo.

One plant from each treatment group per replication (a total of three per treatment) was analyzed for stem, leaf, and root contamination. All 24 stem and leaf sections tested negative for *Salmonella* Montevideo. However, five root samples were positive: three from group 6, and one each from groups 4 and 5. Positive root samples from groups 4, 5, and 6 is most likely due to the greater number of contaminated watering events in combination with the relatively low persistence of *Salmonella*. The continuous inoculation of *Salmonella* Montevideo most likely replenished the levels or encouraged persistence of the bacterium in the pine bark medium over time; however, this was not investigated. *S. enterica* serovars (Newport, Enteritidis, Hartford, Michigan, Montevideo, Poona, and Typhimurium) are capable of persisting in potting mix (40% peat, 40% coco fibers, and 20% volcanic ash) for 4.7 to 10 weeks, and in soil (80% sand, 12% silt, and 8% clay; or 1 loam:1 perlite:1 moist peat) for at least 4 or 5 weeks, respectively (2, 3, 12).

Roots from tomato plants were sanitized prior to sampling to remove bacteria present on the exterior of the root. *Salmonella* Montevideo may have gained entry into the roots of some of the plants. Colonization of the interior of the root occurs passively via natural openings or wounds in the root (11). These openings can be natural breaks in the endodermis, which result during growth of the plant, or wounds that were caused during transplantation, providing access to microorganisms (11). Colonization of bacterial endophytes is thought to occur primarily at the points of secondary lateral root emergence during root development (6, 11). *S. enterica* concentrates at the root tips and lateral branches of the tomato, *Arabidopsis thaliana*, and is capable of invading the roots at lateral root junctions and surviving in the primary root (4). Additionally, there are strong correlations between rhizosphere colonization of alfalfa with *S. enterica* and presence of the pathogen into the aerial plant tissues (5). In this study, the *Salmonella* Montevideo present in the pine bark medium surrounding the root after inoculation may have initially colonized the root and entered via cracks present in some of the plants during either growth or transplantation.

The ultimate question is whether exposure of roots to *Salmonella* Montevideo results in *Salmonella* Montevideo–positive tomato fruit. Evidence that *S. enterica* serovars are able to enter tomato plant systems through contaminated irrigation water is inconsistent. The hypocotyls and cotyledons, stems, and leaves from 9-day-old tomato seedlings were contaminated with 4.02, 3.70, and 3.61 log CFU/ml of a *Salmonella* cocktail when grown hydroponically (10). However, Jablasone et al. (13) found that *Salmonella* Typhimurium did not persist in 9-day-old tomato seedlings after the bacterium was identified on germinated seeds at levels of 4.48 log CFU/g. Additional studies by Jablasone et al. (12) also found no contamination of stems, leaves, and fruit in patio tomato plants irrigated with *Salmonella* Enteritidis. This study confirmed their results, finding no evidence of *Salmonella* Montevideo survival in the stems, leaves, or fruit of the tomato plant.

*Salmonella* Montevideo was capable of surviving in all diluted fertilizer solutions, but was significantly ($P = 0.05$) reduced in undiluted fertilizers CF-S, US-S, and CN-S (Table 2). In commercial fertilizer CF-S, *Salmonella* Montevideo was not detectable via enrichment after 24 h. *Salmonella* Montevideo was capable of survival in diluted fertilizer solutions. After 72 h, no significant ($P > 0.05$) decreases in *Salmonella* Montevideo levels in fertilizer solutions CF-1.0, US-1.0, or CN-1.0 occurred (Table 2). Many farms use concentrated fertilizer solutions that can be diluted and pumped into irrigation water. A fertilizer injector combines the concentrated fertilizer stock solutions with water to create the desired dilution. The dilution value of fertilizer applied to the tomatoes in this experiment was 1.0%, a common value used in the industry.

According to recommendations outlined in *Commodity Specific Food Safety Guidelines for the Tomato Supply Chain* (14), any water used for irrigation must not be contaminated with animal or human feces and must meet the standard for *E. coli* in recreational water (40 CFR Part 131.41[c]). Additionally, water used for foliar application should be potable. Fertilizer stock solutions are often mixed in large batches and held for days. These batches could
become contaminated with Salmonella Montevideo if mixed with water from an unclean source, or through exposure to birds and reptiles. Results from this study indicate that Salmonella Montevideo is able to survive in some of these fertilizers. While most fertilizers are applied at the ground level (these solutions can still come in contact with tomato fruit hanging from the plant), pesticides are applied directly to the plant. Once contamination occurs, S. enterica is capable of survival and growth on the inside and outside of the tomato despite its acidic interior (pH 3.99 to 4.37) (1, 9, 17, 18). Salmonella is also capable of survival and growth in some commercial pesticide mixes (7). The results from this study, along with the study by Guan et al. (7), confirm the importance of using quality water to mix agricultural-use water.

ACKNOWLEDGMENT

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REFERENCES


TABLE 2. Survival of Salmonella Montevideo in three different fertilizer solutions and one control over a 72-h period, held at 25°C

<table>
<thead>
<tr>
<th>Fertilizer</th>
<th>pH</th>
<th>0</th>
<th>24</th>
<th>48</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td>CF-S</td>
<td>4.41</td>
<td>6.76 ± 0.37 A</td>
<td>0 ± 0.0 A B</td>
<td>0 ± 0.0 A B</td>
<td>0 ± 0.0 A B</td>
</tr>
<tr>
<td>CF-1.0</td>
<td>6.66</td>
<td>6.82 ± 0.30 a</td>
<td>7.19 ± 0.69 b</td>
<td>6.73 ± 0.12 b</td>
<td>6.65 ± 0.1 b</td>
</tr>
<tr>
<td>US-S</td>
<td>7.82</td>
<td>6.86 ± 0.10 a A</td>
<td>5.34 ± 0.63 c B</td>
<td>4.51 ± 0.21 c CD</td>
<td>3.96 ± 0.73 cd D</td>
</tr>
<tr>
<td>US-1.0</td>
<td>7.96</td>
<td>6.85 ± 0.14 a</td>
<td>6.98 ± 0.45 b</td>
<td>6.17 ± 1.02 b</td>
<td>6.69 ± 0.7 b</td>
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<tr>
<td>CN-S</td>
<td>5.70</td>
<td>6.74 ± 0.08 a A</td>
<td>5.52 ± 1.01 c B</td>
<td>2.06 ± 0.94 d B</td>
<td>2.22 ± 0.31 ad B</td>
</tr>
<tr>
<td>CN-1.0</td>
<td>6.53</td>
<td>6.71 ± 0.03 a</td>
<td>7.12 ± 0.43 b</td>
<td>6.90 ± 0.80 b</td>
<td>4.81 ± 3.30 bc</td>
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<tr>
<td>SDW</td>
<td>6.55</td>
<td>6.88 ± 0.27 a A</td>
<td>6.93 ± 0.30 b</td>
<td>6.55 ± 0.38 b</td>
<td>6.78 ± 0.45 b</td>
</tr>
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</table>

a Values are log CFU per milliliter ± standard deviation.
b S, stock solution; 1.0, 1.0% solution; SDW, sterile deionized water.
c Lowercase letters represent the significance of differences between values within a column. Uppercase letters after lowercase letters represent the significance of differences between values within a row. If uppercase letters are not provided, it is assumed that no significant differences occurred between values.