Research Note

Potential Interactions between *Salmonella enterica* and *Ralstonia solanacearum* in Tomato Plants

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ABSTRACT

Over the past decade, the Eastern Shore of Virginia (ESV) has been implicated in at least four outbreaks of salmonellosis associated with tomato, all originating from the same serovar, *Salmonella enterica* serovar Newport. In addition to *Salmonella* Newport contamination, the devastating plant disease bacterial wilt, caused by the phytopathogen *Ralstonia solanacearum*, threatens the sustainability of ESV tomato production. Bacterial wilt is present in most ESV tomato fields and causes devastating yield losses each year. Although the connection between bacterial wilt and tomato-related salmonellosis outbreaks in ESV is of interest, the relationship between the two pathogens has never been investigated. In this study, tomato plants were root dip inoculated with one of four treatments: (i) 8 log CFU of *Salmonella* Newport per ml, (ii) 5 log CFU of *R. solanacearum* per ml, (iii) a coinoculation of 8 log CFU of *Salmonella* Newport per ml plus 5 log CFU of *R. solanacearum* per ml, and (iv) sterile water as control. Leaf, stem, and fruit samples were collected at the early-green-fruit stage, and *S. enterica* contamination in the internal tissues was detected. *S. enterica* was recovered in 1.4 and 2.9% of leaf samples from plants inoculated with *Salmonella* Newport only and from plants coinoculated with *Salmonella* Newport plus *R. solanacearum*, respectively. *S. enterica* was recovered from 1.7 and 3.5% of fruit samples from plants inoculated with *Salmonella* Newport only and from plants coinoculated with *Salmonella* Newport plus *R. solanacearum*, respectively. There were significantly more stem samples from plants coinoculated with *Salmonella* Newport plus *R. solanacearum* that were positive for *S. enterica* (18.6%) than stem samples collected from plants inoculated with *Salmonella* Newport only (5.7%). Results suggested that *R. solanacearum* could influence *S. enterica* survival and transportation throughout the internal tissues of tomato plants.

Foodborne illnesses are estimated to cause over 183,000 hospitalizations, 47 million sicknesses, and 3,000 deaths annually in the United States (21, 23). *Salmonella enterica* is the leading cause of foodborne bacterial diseases in the United States (4, 5, 22). *S. enterica* causes 1.4 million cases of illness and 500 deaths in the United States every year, with total estimated costs of $3.4 billion per year (5). Fresh produce has gained notoriety in recent years as a vehicle for human salmonellosis. Between 1990 and 2005, contaminated produce yielded the greatest number of foodborne cases annually and the second-highest number of outbreaks (seafood being the highest) (3, 6). In the United States, diseases caused by the major human pathogens alone are estimated to cost up to $51 billion annually in medical costs and lost productivity (23).

The Eastern Shore of Virginia (ESV) is responsible for approximately 80% of Virginia’s tomato production. Since 2002, ESV has been implicated in at least four outbreaks of salmonellosis associated with tomatoes, and the sustainability of this industry is threatened (18). Not only does *Salmonella* Newport contamination threaten the fresh market tomato industry, but so does the devastating plant disease bacterial wilt, caused by the phytopathogen *Ralstonia solanacearum*. *R. solanacearum* is a soilborne pathogen that infects the roots of plants through natural openings or wounds. Bacterial wilt is the number one disease problem of tomato production on the ESV, causing devastating yield and economic losses each year. In some instances, growers have had to forfeit harvesting a field because the bacterial wilt infestation was so severe that it was not possible to make a profit from the harvestable fruit. Good agricultural practices guidelines urge growers not to harvest fruits from diseased plants in fear that the plant’s compromised immune system would make it more susceptible to human pathogens such as *S. enterica* (19). These recommendations are not enforced by law, and fruit from health-compromised plants do inevitably end up in our food system. Studies investigating the effects of plant pathogens on *S. enterica* populations on produce have been conducted. One such study was conducted by Wells and Butterfield (28), who investigated interactions between the causal agent of bacterial soft rot, *Erwinia* spp., and *S. enterica* in fresh fruits and vegetables. They discovered that *S. enterica* populations increased up to 10-fold when coinoculated with *Erwinia* spp. compared with the populations enumerated.

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when the fruits and vegetables were inoculated with *S. enterica* alone. A study conducted by Barak and colleagues (1) found that *S. enterica* populations on tomato plants were increased in the presence of *Xanthomonas campestris*, the causal agent of bacterial spot. Because previous research suggests that plant pathogens can cause an increase in *S. enterica* populations on plants (1, 9), the effect of *R. solanacearum* on tomato plant and fruit susceptibility to *S. enterica* is of interest.

In this study, a series of greenhouse experiments were conducted to assess the effect of *R. solanacearum* infection on *Salmonella* Newport uptake via the tomato plant root system. Understanding the interactions among these two pathogens can provide insight into the studies about *S. enterica* contamination within tomato fruit.

**MATERIALS AND METHODS**

**Bacterial strains and inoculum preparation.** *Salmonella* Newport strain J1892 was isolated from a tomato-related salmonellosis outbreak and originally obtained from the Centers for Disease Control and Prevention in Atlanta, GA. The strain was plated onto xylose lysine deoxycholate medium (BD, Franklin Lakes, NJ) and incubated for 24 h at 37°C for preliminary confirmation of *S. enterica* species. Colonies presumptively positive for *S. enterica* were red with black centers, indicating hydrogen sulfide production. Serotype identification was confirmed with the slide agglutination test (BD, Sparks, MD). The *R. solanacearum* isolate used for the studies was obtained from a local commercial tomato field on the ESV infected with bacterial wilt. The isolate was plated onto triphenyl tetrazolium chloride medium (BD, Franklin Lakes, NJ) and incubated overnight at 27°C, and the bacterium was confirmed to be *R. solanacearum* via the AgDia (Elkhart, IN) *R. solanacearum* Rapid Test kit. Both strains were stored at −80°C in glycerol stocks.

Before inoculation, *Salmonella* Newport was incubated overnight on xylose lysine Tergitol 4 medium (Dott Scientific Inc., Burton, MI) and suspended in sterile water to a level of 8 log CFU/ml for inoculation. *R. solanacearum* was grown on triphenyl tetrazolium chloride medium for 24 h at 27°C and suspended in sterile water to levels of 5 to 7 log CFU/ml for the susceptibility test and 5 log CFU/ml for interaction experiments. The bacterial level of the suspension was measured with a hemacytometer (Hausser Scientific, Horsham, PA) and an Olympus BX41 microscope (Olympus Imaging America Inc., Center Valley, PA).

**Tomato cultivar and plant growth.** Tomato seeds of the cultivar BHN602, a popular commercial cultivar in ESV, were planted in a 128-cell Styrofoam plug tray (Speedling Inc., Sun City, FL), with ProMix BX Mycorise Pro (Premier Horticulture Inc., Rivière-du-Loup, Quebec, Canada) used as a growing medium. The plants were grown in a biological safety level 2 greenhouse. Two weeks after the sowing of seeds, seedlings were transplanted into 24.6-liter plastic pots (Wetsel, Harrisonburg, VA) containing the same growing medium. The plants were irrigated with well water and fertilized with Miracle Gro (The Scotts Company LLC, Marysville, OH) as needed for optimal growth.

**Susceptibility test.** A pretest for the susceptibility of tomato cultivar BHN602 to *R. solanacearum* infection was conducted to determine specific experimental parameters optimal for *Salmonella* Newport–*R. solanacearum* interaction experiments. Three different *R. solanacearum* inoculum levels (5, 6, and 7 log CFU/ml) were used to determine the optimal sublethal dose of *R. solanacearum* to induce disease without plant death, and three different plant growth stages for inoculation timings (at the time of transplanting, 2 weeks old; preflowering, 4 to 6 weeks old; and flowering, 7 to 8 weeks old) were examined. Plant disease incidence was evaluated by measuring the percentage of infected plants (killed) after inoculation.

**Plant inoculation.** During the flowering stage, tomato plants were inoculated with one of four treatments: (i) 8 log CFU of *Salmonella* Newport per ml; (ii) 5 log CFU of *R. solanacearum* per ml; (iii) a coinoculation of 8 log CFU of *Salmonella* Newport and 5 log CFU of *R. solanacearum* per ml; and (iv) sterile water as control. The experiment was conducted in triplicate (experiments 1 to 3) by a completely randomized design in biosafety level 2 greenhouses. Experiment 1 consisted of 8 plants per treatment for a total of 32 plants, experiment 2 consisted of 12 plants per treatment for a total of 48 plants, and experiment 3 consisted of 15 plants per treatment for a total of 60 plants. There were a total of 35 plants per treatment including all experiments. Plants were inoculated by the root dip method for 2.5 min to mimic natural infection of *R. solanacearum*, which infects the plants through natural openings or through wounds in the roots (20). Plants were uprooted, the roots were submerged in water to remove potting debris, and then the roots were submerged in the designated bacterial suspension for 2.5 min followed by replanting. During the uprooting process, the roots were minimally damaged, which created minor wounds that aid in bacterial infection (27).

**Salmonella detection.** After inoculation, plants were staked and strung to ensure upright growth and irrigated as needed. Early green fruit were sampled from each plant when two or more green fruit were produced. In total, 235, 199, 202, and 284 fruit were harvested from plants treated with *Salmonella* Newport, *R. solanacearum*, *Salmonella* Newport plus *R. solanacearum*, and sterile water, respectively, and were analyzed for *Salmonella* Newport contamination inside the fruit pulp. In total, 70 leaf samples and 70 stem samples were collected from each treatment of the experiments. Leaf samples were collected from each plant from the first and fifth nodes, and stem samples were collected approximately 2.5 cm below the first and fifth nodes.

Plants were sampled aseptically with scissors sterilized by submersion in 70% ethanol for 30 s between each sample. Plant samples (stem and leaf) were surface sterilized with 70% ethanol until runoff. Samples were placed into individual labeled Whirl-Pak sterile sampling bags. Fruit samples were surface sterilized by submersion in a solution of 70% ethanol for ≥2 min and placed into individual labeled Whirl-Pak sterile sampling bags (10, 12, 16). All samples were homogenized, and the liquid exudates were collected in labeled, sterile 2-ml Eppendorf tubes (Sigma-Aldrich, St. Louis, MO). Three 100-μl aliquots of each sample were plated onto *Salmonella*-selective xylose lysine Tergitol 4 medium plates with the Eddy Jet 2 spiral plater (IUL Instruments, Barcelona, Spain) at mode c100 and incubated at 42°C for 24 h. After incubation, observations were made for presumptive (black) colonies. One putative colony of each plate was streaked onto another xylose lysine Tergitol 4 medium plate and was further confirmed to be *S. enterica* via PCR by utilizing primers INVA-1 (5′-ACA GTG CTC GTT TAC GAC CTG AAT-3′) and INVA-2 (5′-AGA CGA CTG GTA CTG GAT AAT-3′), which are specific for the *inVA* gene, followed by gel electrophoresis (on 1.5% agarose gels) as described previously (8).

**Statistical analyses.** JMP 9 statistical software (SAS Institute Inc., Cary, NC) was used to compute a one-way analysis of leaf,
TABLE 1. Percentage of S. enterica–positive samples from surface-sterilized leaf, stem, and fruit from inoculated tomato plants grown in a biosafety level 2 greenhouse

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Bacterial level (log CFU/ml)</th>
<th>% of S. enterica–positive leaf samples</th>
<th>% of S. enterica–positive stem samples</th>
<th>% of S. enterica–positive fruit samples/total no. of sampled fruit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salmonella Newport</td>
<td>8</td>
<td>1.43 A</td>
<td>5.71 b</td>
<td>2.13 ±/235</td>
</tr>
<tr>
<td>R. solanacearum</td>
<td>5</td>
<td>0 A</td>
<td>0 b</td>
<td>0.50 ±/199</td>
</tr>
<tr>
<td>SN + Rs</td>
<td>8 + 5</td>
<td>2.94 A</td>
<td>17.46 A</td>
<td>3.47 ±/202</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>0 A</td>
<td>0 b</td>
<td>0.70 ±/284</td>
</tr>
<tr>
<td>P value</td>
<td>0.2305</td>
<td>&lt;0.0001</td>
<td>0.0739</td>
<td></td>
</tr>
</tbody>
</table>

a One-way means comparison analysis of S. enterica–positive samples by Tukey-Kramer’s honestly significant difference was performed with JMP 9 statistical software. Values followed by the same letter are not significantly different (α = 0.05). Statistical findings compare results within columns.

b Bacterial level in 2.5-min root dip inoculation procedures.

c A total of 68 leaf samples subjected to Salmonella Newport plus R. solanacearum treatment were collected and analyzed for the presence of S. enterica. A total of 70 leaf samples were collected from each of the remaining treatments and analyzed for the presence of S. enterica.

d A total of 70 stem samples were collected from each of the Salmonella Newport and control treatments, and a total of 63 stem samples were collected from each of the R. solanacearum alone and Salmonella Newport plus R. solanacearum treatments.

e All fruit samples from each plant were collected and analyzed for the presence of S. enterica.

f SN + Rs, Salmonella Newport plus R. solanacearum treatment.

The P values reported are for comparisons within columns between all treatments.

RESULTS

Susceptibility of tomato cultivar BHN 602 to R. solanacearum. Inoculation of R. solanacearum at a level of 7 log CFU/ml killed all tomato plants at all three growth stages. R. solanacearum inoculation at a level of 6 log CFU/ml killed the plants at the transplanting and preflowering stages tested and killed 50% of the plants inoculated at the flowering stage. R. solanacearum inoculation at a level of 5 log CFU/ml killed the plants at the transplanting stage, 50% of the plants at the preflowering stage, and no plants at the flowering stage. The percent disease incidence of tomato plants decreased when inoculation was done at lower levels and/or at older plant growth stages (Fig. S1; supplementary figures available at http://filebox.vt.edu/users/mlpeyton/ supplementary%20figures.pdf). Based upon these results of susceptibility testing, inoculations of R. solanacearum in subsequent interaction experiments were applied at a level of 5 log CFU/ml at the flowering stage.

Interaction experiments. In experiment 1, S. enterica was isolated from one leaf sample on the fifth node (6.25%) from plants inoculated with Salmonella Newport and from two leaf samples (12.5%) from plants coinoculated with Salmonella Newport and R. solanacearum, one from the first node of a plant and one from the fifth node of a different plant. Salmonella was also isolated from three first-node stem samples (18.75%) from plants coinoculated with Salmonella Newport plus R. solanacearum. S. enterica was not recovered from any stem samples from plants inoculated with Salmonella Newport only. Among the fruit sampled from plants inoculated with Salmonella Newport only, 4.84% were contaminated by Salmonella; 6.09% of fruit sampled from plants coinoculated with Salmonella Newport plus R. solanacearum were Salmonella positive (Table 1).

In experiment 2, S. enterica was isolated from 16.67% of stem samples from plants inoculated with Salmonella Newport only and from 4.17% of stem samples from plants coinoculated with Salmonella Newport plus R. solanacearum (n = 24) (Table 1). S. enterica was not recovered from any leaf or fruit samples for this experiment.

In experiment 3, 30.43% of stem samples from plants coinoculated with Salmonella Newport plus R. solanacearum were Salmonella positive, while the bacterium was not recovered from any other samples (Table 1).

Statistical analysis revealed that there were significantly more S. enterica–positive stem samples from plants coinoculated with Salmonella Newport plus R. solanacearum than from plants subjected to other treatments (Table 1). No S. enterica cells were isolated from the plants treated with R. solanacearum only or from control plants. In all experiments, selected bacterial isolates were confirmed to be S. enterica via PCR and gel electrophoresis (Fig. S1).

DISCUSSION

In this study, S. enterica was recovered from surface-sterilized tomato leaves, stems, and fruit after root-dipping inoculation, which indicated that S. enterica may be able to enter the plant through the roots and move throughout the plant’s vascular system to contaminate internal leaf, stem, and fruit tissues. These results support previous research reporting S. enterica movement throughout, and colonization of, internal plant tissues. Several studies have reported S. enterica internalization within plants via flower brush inoculation (11), stomata entry (14, 15), and rhizosphere infection (29). This study focused on the interaction of R. solanacearum and Salmonella Newport in tomato plants and utilized a root dip inoculation method involving irrigation-based plant inoculation. Previous studies have
indicated that *S. enterica* can colonize produce via contaminated irrigation water as well as via contaminated soil (6, 9, 13, 24, 26). Previous studies have also indicated a possible interaction between plant pathogens and *S. enterica* whereby plant pathogens may enhance *S. enterica* colonization on the plant (1, 2, 9). However, although previous studies have indicated that internal plant colonization of *S. enterica* occurs, in this study the internal contamination ratio of *Salmonella* Newport within tomato plant and fruit samples from inoculated plants may indicate that plant tissues may not be an optimal environment for the internal colonization of the human foodborne pathogen *S. enterica*.

Due to the low internal contamination chance, it would be difficult to draw a definite conclusion about the interaction between *R. solanacearum* infection and *Salmonella* Newport contamination in tomato plants. However, these findings are in agreement with previous studies showing that there was a significantly higher chance of contamination by *S. enterica* in the stems as well as a relative higher ratio in sampled fruits treated with a combination of *Salmonella* Newport and *R. solanacearum*, which suggests that infection by a plant pathogen could increase the susceptibility of plants to *S. enterica* (1, 10). It is possible that significant differences in *S. enterica* recovery between plants inoculated with *Salmonella* Newport only and plants coinoculated with *Salmonella* Newport and *R. solanacearum* were observed only in stem samples because *R. solanacearum* clogs the vascular system of the plants, which causes the typical wilting symptoms seen in diseased plant and prevents significant bacterial transportation throughout the plant. The susceptibility test identified factors that might affect experiment success, including the *R. solanacearum* inoculum level and the optimal plant growth stage for inoculation. Based on this pretest, a sublethal inoculation level of *R. solanacearum* was identified (5 log CFU/ml), along with the optimal plant growth stage (the flowering stage) for inoculations. Besides these two factors, Gallegly and Walker’s study (7) showed an increase of bacterial wilt disease in tomato plants subjected to warmer soil (22 to 36°C) and air temperatures (16 to 28°C) and indicated that the inoculation of *R. solanacearum* at high temperature and early plant growth stages (before flowering) could kill the plants before fruit set. Previous research also showed that *S. enterica* could multiply more quickly at higher temperatures but persist longer at lower temperatures with a higher survival rate (17, 25). In addition, *S. enterica* has been observed to persist in tomato leaves and fruits but was not able to multiply (10).

In this study, the highest recovery rate was from experiment 1, while *S. enterica* was not recovered from the fruit or leaf samples from experiment 2 or 3. Experiment 1 was conducted in the fall of 2011 at the main campus of Virginia Tech in Blacksburg, and the second and third experiments were conducted in the late spring and summer of 2012 at Virginia Tech Eastern Shore Agricultural Research & Extension Center in Painter. The average temperatures in the greenhouses for experiments 1, 2, and 3 were approximately 20, 30, and 35°C, respectively, and the highest temperature in experiments 2 and 3 could reach 43°C. These findings suggested that the lower temperature in experiment 1 might have enhanced *S. enterica* colonization in the tomato plants, thus producing a greater *S. enterica* recovery rate, while high temperatures in experiments 2 and 3 may have enhanced bacterial wilt disease onset and deterred *S. enterica* colonization. The elevated bacterial wilt infection caused some plants to die before fruit set. Although these temperature conditions were not ideal for plant survival, the conditions most closely reflected field conditions on ESV.

In conclusion, *R. solanacearum* infection may influence *S. enterica* colonization and transportation throughout the internal tissues of tomato plants. Exploring factors that have an impact on *S. enterica* contamination on fresh produce is essential in determining the cause of contamination events. Further research is needed to better pinpoint how various factors contribute to *S. enterica* contamination of fresh produce. Further field studies with a larger sample size and an attenuated *Salmonella* strain could aid in determining the effect of *R. solanacearum* infection of tomato plant on its susceptibility to *S. enterica* contamination. In addition, further studies to evaluate the differences in bacterial population densities between the treatments could offer greater insight on possible effects of *R. solanacearum* infection.

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REFERENCES


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