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Evidence of Association of Salmonellae with Tomato Plants Grown Hydroponically in Inoculated Nutrient Solution

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The possibility of uptake of salmonellae by roots of hydroponically grown tomato plants was investigated. Within 1 day of exposure of plant roots to Hoagland nutrient solution containing 4.46 to 4.65 $\log_{10}$ CFU of salmonellae/ml, the sizes of the pathogen populations were 3.01 CFU/g of hypocotyls and cotyledons and 3.40 $\log_{10}$ CFU/g of stems for plants with intact root systems (control) and 2.55 $\log_{10}$ CFU/g of hypocotyls and cotyledons for plants from which portions of the roots had been removed. A population of $\geq$3.38 $\log_{10}$ CFU/g of hypocotyls-cotyledons, stems, and leaves of plants grown for 9 days was detected regardless of the root condition. Additional studies need to be done to unequivocally demonstrate that salmonellae can exist as endophytes in tomato plants grown under conditions that simulate commonly used agronomic practices.

Tomatoes, seed sprouts, and melons are among the raw produce repeatedly identified as potential vehicles of human salmonellosis (9, 20, 21, 29). The inner tissue of sound produce is generally considered to be sterile. However, endophytic bacteria are known to reside in a wide range of plant tissues (7, 16, 28), including those consumed as raw vegetables (11, 12, 19, 23, 25, 27, 31). Samish et al. (23) studied 10 fruits and vegetables and found that bacteria, mostly members of two families, the *Pseudomonadaceae* and the *Enterobacteriaceae*, were present in sound, raw cucumber and tomato fruits. *Ralstonia solanacearum*, which causes bacterial wilt of tomatoes, is known to enter the plant through the root, to penetrate the xylem, and to systemically colonize the stem (18, 22, 30). *Pseudomonas syringae* pathovar tomato, the causative agent of bacterial speck of tomato, inhabits leaf trichomes (24).

None of the chemical or physical treatments currently authorized by regulatory agencies in the United States for use as disinfectants for raw produce can be relied on to eliminate all types of pathogens (2). Devising successful intervention steps to reduce populations of human pathogens on and in fruits and vegetables eaten raw should be aided by information concerning the sources of contamination and the ecology of pathogens affected by agronomic and minimal processing practices (2, 3).

In a previous study (6), we observed that salmonellae inoculated onto flowers and into stems of tomatoes survived for at least 49 days and were recovered from ripened fruits. Contact of tomato fruits with soil containing salmonellae can result in infiltration of the pathogens into subsurface tissues (5). The objectives of the study reported here were to investigate the possibility of association of salmonellae with hypocotyls, cotyledons, stems, and leaves of young plants grown in a hydroponic nutrient solution inoculated with the pathogen.

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Bacterial cultures. Five serotypes of *Salmonella enterica* obtained from the Centers for Disease Control and Prevention, Atlanta, Ga., were used. *S. enterica* serotype Montevideo (serogroup C_1) was isolated from a patient in an outbreak of salmonellosis associated with consumption of raw tomatoes; *S. enterica* serotype Michigan (serogroup J) was isolated from cantaloupe; *S. enterica* serotype Poona (serogroup G) was isolated from a patient in an outbreak associated with consumption of cantaloupe; *S. enterica* serotype Hartford (serogroup C_1) was isolated from a patient in an outbreak associated with consumption of orange juice; and *S. enterica* serotype Enteritidis (serogroup D) was isolated from a patient in an outbreak associated with consumption of eggs. Cross-streaking of cultures on brain heart infusion (BHI) agar (BBL/Difco, Sparks, Md.) revealed that none of the serotypes inhibited the growth of the other serotypes.

Inoculum preparation. Transformation of the five *Salmonella* serotypes with an enhanced green fluorescent protein (EGFP) plasmid (Clonetech Laboratories, Inc., Palo Alto, Calif.) was achieved by using a Gene Pulser electroporator (Bio-Rad Laboratories, Hercules, Calif.) with the following parameters: 2.4 kV; field strength, 12.00 kV/cm; capacitance, 25 μF; resistance, 400 Ω; and time constant, 9.7 ms. This plasmid also codes for ampicillin resistance. The transformants were transferred to BHI agar supplemented with 100 μg of ampicillin (Sigma, St. Louis, Mo.) per ml (BHI/Amp agar), incubated at 37°C for 16 to 18 h, and examined under UV light (wavelength, 365 nm). Bright green fluorescent colonies were distinguished as colonies that were formed by *Salmonella* cells containing EGFP.

Stock cultures of the five *Salmonella* serotypes labeled with EGFP were maintained at −80°C. Cultures of *Salmonella* serotypes were individually prepared for use in the inoculum by transferring stock cultures to 50 ml of BHI broth (BBL/Difco) supplemented with 100 μg of ampicillin per ml (BHI/Amp broth) and incubated at 37°C. Cultures (one loopful) were transferred three times at 24-h intervals. Cells were harvested when the A_{600} of the broth reached ca. 1.0, which corre-

sponded to a concentration of 10^9 CFU/ml. Cells in each culture were harvested by centrifuging at 10,000 × *g* for 5 min, washed in 50 ml of 0.01 M sodium phosphate buffer (pH 7.2) containing 0.85% sodium chloride (phosphate-buffered saline) twice, and resuspended in 50 ml of sterile tap water. Equal volumes of the serotype cell suspensions were combined to form an inoculum.

Hoagland nutrient solution (10) was prepared by using ingredients purchased from Fisher Scientific (Pittsburgh, Pa.). The inoculum (4 ml) was added to 4 liters of half-strength modified Hoagland solution modified to contain ampicillin (100 μg/ml) in order to obtain a preparation containing 4.55 ± 0.10 $\log_{10}$ CFU of salmonellae/ml.

Tomato plants. Cultivar Better Boy tomato seeds were purchased from Park Seed Company, Greenwood, S.C. Sand (QUIKRETE Companies, Atlanta, Ga.) was used as a medium to germinate seeds and grow plants to a height of ca. 4 cm. The sand was moistened with deionized water and placed in a 3-cm layer in a polycarbonate tray (50 cm long by 30 cm wide by 15 cm deep; Nalge Company, Rochester, N.Y.). Tomato seeds were placed on the surface, covered with a 0.5-cm layer of sand, and kept in the dark at 25°C in a forced-air incubator for 7 days or until cotyledons emerged.

Hydroponic system. The hydroponic system was set up in a walk-in incubator in which the temperature was 25°C. Eight polycarbonate hydroponic trays (27 cm long by 21 cm wide by 14 cm deep), each designed to hold 12 plants, were used. The sides of the trays were covered with aluminum foil to shield the Hoagland solution from light. A sheet of Styrofoam (2.5 cm thick) containing holes (1 cm in diameter) was placed 1.5 cm above the surface of half-strength Hoagland solution (4 liters, 12 cm deep) in each hydroponic tray. Plants (4 cm high) from which sand was removed from the roots by washing with water were placed in the holes and tightly secured with nonadsorbant cotton so that the roots were below the Styrofoam and the hypocotyls, cotyledons, stems, and leaves were above the Styrofoam. Hoagland solution was aerated by using air stones connected to an aquarium air pump (Tetra/Secondnature, Blacksburg, Va.) with tubes and splitters to facilitate distribution of air bubbles. Sets of four trays were enclosed within perforated plastic film to minimize airflow. Wide-spectrum fluorescent bulbs (photosynthetic photon flux density [400 to 700 nm], ca. 80 μmol/m^2/s; General Electric Company, Cleveland, Ohio) were used as a light source for the tomato plants. A daily cycle consisting of 16 h of light and 8 h of darkness was used. The pH of Hoagland solution was monitored daily and was maintained at 5.5 to 6.5 by replenishing with new solution.

Uptake of salmonellae by roots. After plants were grown for 7 days in uninoculated Hoagland solution, the bottom portions (1 cm) of the root systems (5 cm long) of one-half of the plants were removed with a sterile scalpel. Plants with intact or cut roots were then transferred to trays containing 4 liters of Hoagland solution inoculated with a five-serotype mixture of EGFP-tagged salmonellae. Control plants were grown in Hoagland solution not inoculated with salmonellae. The plants were positioned in holes in a sheet of Styrofoam placed 1.5 cm above the inoculated Hoagland solution. After three true leaves emerged, the hypocotyls, cotyledons, stems, and leaves above the Styrofoam sheet were severed from the roots. Three severed portions (hypocotyls plus cotyledons, stem, leaves) from each plant were analyzed for populations and the presence of salmonellae.

Microbiological analysis. Three plants in each of three replicate experiments were analyzed for each set of experimental parameters. Samples (0.11 to 2.21 g) of hypocotyls plus cotyledons, stem, and leaves of each plant were placed individually in sterile plastic bags (16 by 10 cm) containing 5 ml of sterile 0.1% peptone water, macerated by hand, and mixed by hand agitation for 1 min. Samples of macerates were surface plated (0.25 ml in quadruplicate and 0.1 ml in duplicate) on BHI/Amp agar. The plates were incubated at 37°C for 24 h before they were examined for the presence of presumptive colonies of salmonellae. The remaining macerate of tissue and peptone water was enriched by adding 5 ml of universal preenrichment broth (BBL/Difco) and incubating the mixture at 37°C for 24 h. Cultures were streaked on BHI/Amp agar and incubated at 37°C for 24 h before they were examined for the presence of presumptive *Salmonella* colonies by using UV light. Five fluorescent colonies from each sample were randomly picked and subjected to serological testing and enterobacterial repetitive intergenic consensus PCR analysis. Serological identification was performed by using *Salmonella* antiserum for groups C_1, D_1, G, and J (BBL/Difco) according to the manufacturer's instructions.

Confirmation of presumptive *Salmonella* colonies by PCR. PCR fingerprinting was done to compare the serotypes of isolates obtained from tissues collected 9 days after the initial exposure to salmonellae to the serotypes in the inoculum. The primer used for PCR fingerprinting was 5′-AAG TAA GTG ACT GGG GTG AGC G-3′, based on a highly conserved, enterobacterial repetitive intergenic consensus sequence which consists of 126 bp and appears to be restricted to transcribed regions of the genome, either in intergenic regions of polycistronic operons or in untranslated regions upstream or downstream of open reading frames (13). Crude DNA was prepared by boiling 20-h cultures of isolates in BHI broth for 10 min. One milliliter of a 20-h culture grown in BHI broth at 37°C was centrifuged at 12,000 × *g* for 2 min. The pellets were resuspended in 200 μl of sterile distilled water, boiled for 10 min, and centrifuged at 12,000 × *g* for 2 min. A 5-μl sample was used as a template for each PCR. Each 50-μl PCR mixture contained PCR buffer, deoxynucleoside triphosphates (each at a concentration of 0.4 mM), primers (each at a concentration of 1 μM), *Taq* polymerase (1 U; Roche Diagnostics, Indianapolis, Ind.), and DNA template. The PCRs were performed in a DNA thermal cycler 480 (Perkin-Elmer, Norwalk, Conn.) by using one cycle of 94°C for 5 min, followed by 40 cycles of 92°C for 45 s, 25°C for 1 min, and 68°C for 10 min and a final extension at 72°C for 20 min. The PCR amplicons were analyzed by gel electrophoresis on a 1% agarose gel (GIBCO BRL, Rockville, Md.) in TBE buffer (0.089 M Tris-borate, 0.002 M EDTA; pH 8.0). The gel was stained with ethidium bromide and visualized by using a Gel Doc System 2000 (Bio-Rad Laboratories).

The size of the population of EGFP-tagged salmonellae in inoculated Hoagland solution was 4.46 to 4.65 $\log_{10}$ CFU/ml throughout the 9-day period that tomato plants with intact and cut roots were grown and analyzed for the presence and populations of the pathogens in hypocotyl-cotyledon, stem, and leaf portions. Salmonellae were not detected in plants grown in

Hoagland solution that was not inoculated with the pathogen. Within 1 day of exposure of plant roots to the inoculated solution, the sizes of the populations of salmonellae were 3.01 and 3.40 $\log_{10}$ CFU/g of hypocotyl-cotyledon and stem portions, respectively, of plants with intact root systems (control) and 2.55 $\log_{10}$ CFU/g of hypocotyl-cotyledon portions of plants from which portions of the roots had been removed (Table 1); salmonellae were also detected in enriched samples of stems of plants from which portions of the roots had been removed. The pathogens were not detected in leaves of plants grown in inoculated Hoagland solution for 1 day, regardless of root treatment. Except for the stem portion on day 3, salmonellae were detected in the enriched samples of hypocotyl-cotyledon, stem, and leaf portions of plants with intact roots on days 3 through 9. Salmonellae were detected by enrichment in leaves of plants with intact roots on day 3, but they were not detected in leaves of plants from which portions of the roots had been removed until day 5. In stems of plants from which portions of the roots had been severed, salmonellae were detected by enrichment on day 1 but not on day 3 or 5 and salmonellae were detected at a population of 1.14 $\log_{10}$ CFU/g on day 7. None of the hypocotyl-cotyledon, stem, or leaf samples was surface sterilized before analysis. Thus, while extreme caution was taken to prevent cross-contamination of aerial tissues with salmonellae in the inoculated hydroponic solution or on roots immersed in the solution, it is possible that capillary action could have resulted in movement of the pathogens from the solution to the surfaces of the aerial portions of plants during the 9-day monitoring period. However, the relatively high populations of salmonellae detected in some of the hypocotyl-cotyledon, stem, and leaf samples would not be expected to have originated via surface contamination.

Analysis of plant tissues by PCR (Fig. 1) revealed that of the five serotypes inoculated into Hoagland solution, *Salmonella* serotype Montevideo (8 of 10 isolates) and *Salmonella* serotype Michigan (2 of 10 isolates) were dominant. *Salmonella* serotype Montevideo and *Salmonella* serotype Michigan were identified as the serotypes of 9 of 10 and 1 of 10 isolates, respectively, from hypocotyl-cotyledon samples analyzed on day 9 of the experiment. *Salmonella* serotype Montevideo was also detected in stems and leaves on day 9. *Salmonella* serotype Enteritidis, *Salmonella* serotype Hartford, and *Salmonella* serotype Poona were not detected in tissues of any of the 9-day samples. PCR fingerprinting of isolates from tomato tissues revealed the same trend in survival of serotypes observed in a previous study, in which the prevalence of the same serotypes recovered from tomato fruits produced on plants inoculated at the time of flowering was observed (6). In both studies, *Salmonella* serotype Montevideo was the most persistent and dominant serotype isolated from tomato tissues.

Salmonellae survived in Hoagland nutrient solution supplemented with 100 μg of ampicillin per ml, which helped maintain selective pressure and thus stability and expression of EGFP. Within 24 h, apparent uptake of salmonellae by intact and cut roots was evident due to the presence of the organisms in the hypocotyls and cotyledons. As the 9-day growing period progressed, although the data were not consistent from one sampling time to the next and the populations varied, salmonellae were detected in stems and leaves of plants with either intact or cut roots. This observation supports the hypothesis

TABLE 1. Population sizes of salmonellae in tissue samples from hydroponically grown tomato plants after exposure of the plant roots to inoculated Hoagland solution for up to 9 days[a]

Root treatment	Tissue	Zero time		Day 1		Day 3		Day 5		Day 7		Day 9	
		Population size ($\log_{10}$ CFU/g)	Enriched sample	Population size ($\log_{10}$ CFU/g)	Enriched sample	Population size ($\log_{10}$ CFU/g)	Enriched sample	Population size ($\log_{10}$ CFU/g)	Enriched sample	Population size ($\log_{10}$ CFU/g)	Enriched sample	Population size ($\log_{10}$ CFU/g)	Enriched sample
Intact	Hypocotyls and cotyledons	ND[c]	–	3.01	+	2.70	+	2.48	+	4.37	+	4.02	+
	Stems	ND	–	3.40	+	<1.53	–	2.01	+	2.06	+	3.70	+
	Leaves	ND	–	<1.22	–	<1.28	+	<0.98	+	1.06	+	3.61	+
Cut	Hypocotyls and cotyledons	ND	–	2.55	+	1.89	+	1.44	+	4.12	+	4.29	+
	Stems	ND	–	<1.32	+	<1.30	–	<1.12	–	1.14	+	3.58	+
	Leaves	ND	–	<0.97	–	<0.97	–	1.08	+	<0.47	–	3.38	+

[a] Mean values are presented. The minimum limits of detection were different for different samples because the weights of the samples varied (0.11 to 2.21 g) while the volume of peptone water (5 ml) that was combined with samples to prepare macerates was constant.
[b] +, present; –, absent.
[c] ND, none detected in 0.11- to 2.21-g sample before roots of plants were immersed in Hoagland solution inoculated with salmonellae.

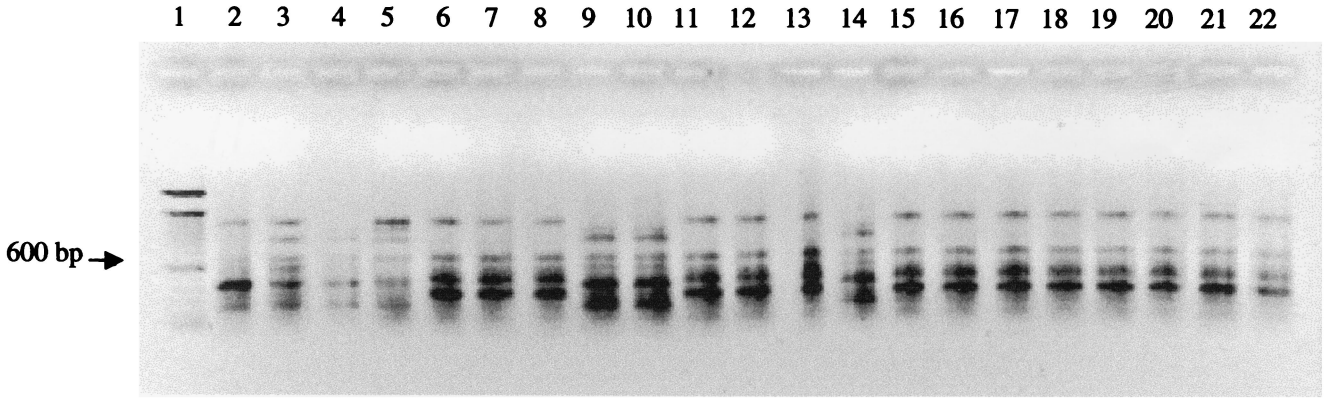

FIG. 1. DNA-based typing of presumptive *Salmonella* colonies isolated from tomato plants grown in Hoagland solution inoculated with a five-serotype mixture of pathogens. Lane 1, 100-bp DNA marker; lanes 2 through 6, DNA profiles of *Salmonella* serotype Enteriditis, *Salmonella* serotype Hartford, *Salmonella* serotype Michigan, *Salmonella* serotype Poona, and *Salmonella* serotype Montevideo, respectively; lanes 7 through 10, profiles of isolates from inoculated Hoagland solution; lanes 11 through 14, profiles of isolates from the hypocotyls and cotyledons of tomato plants on day 9; lanes 15 through 18, profiles of isolates from stems of tomato plants on day 9; lanes 19 through 22, profiles of isolates from leaves of tomato plants on day 9.

that the pathogen can be transported systemically in tomato plants.

The points of entry of bacteria into plants include stomata, hydrathodes, nectarthodes, lenticils, germinating radicles, and areas of emergence of lateral roots (7, 12, 28). Major points of entry appear to be wounds that naturally occur as a result of growth, through root hairs, at the root emergence zone, and at epidermal conjunctions (1, 15, 17, 26). Vasse et al. (30) characterized a three-phase process in which roots of hydroponically grown tomato plants become infected with *R. solanacearum*. Colonization of the root surface, followed by infection of the vascular parenchyma and invasion of the xylem, was described. The production of plant cell wall-degrading enzymes, which is not an absolute requirement for invasion (18), and the production of extracellular polysaccharide by *R. solanacearum* enhance its virulence (22). Whether the association or mode of entry of salmonellae into tomato roots is similar to that of *R. solanacearum* or other endophytic bacteria is not known. Factors that influence internalization of bacteria in tomato tissues may also be different in hydroponically grown plants and in plants grown in soil. Certainly, the macrostructures of the roots differ substantially in the two nutrient support systems, thus potentially affecting the behavior of endophytic bacteria.

Other researchers have reported systemic association of human pathogens in plants. Hydroponically grown radish sprouts have been epidemiologically implicated as a vehicle for transmitting *Escherichia coli* O157:H7 infection (32). Hara-Kudo et al. (8) studied the distribution of this pathogen on and in radish sprouts grown in inoculated water. Hypocotyls and cotyledons were externally contaminated with the pathogen when sprouts were grown from seeds soaked in water containing *E. coli* O157:H7 at populations of 3.0 to 3.1 $\log_{10}$ CFU/ml. The pathogen was detected in hypocotyls and cotyledons within 18 h of exposure of roots to inoculated water. Itoh et al. (14) detected *E. coli* O157:H7 in the inner tissues and stomata of cotyledons of radish sprouts grown in an aqueous suspension of the pathogen. Gandhi et al. (4) used *Salmonella* serotype Stanley tagged with green fluorescent protein to investigate survival and spatial location within alfalfa sprouts. Microscopy revealed the presence of salmonellae in subsurface areas of root, hypocotyl, and cotyledon tissues. More recently, association of green fluorescent protein-tagged *E. coli* O157:H7 with lettuce plants grown in soil inoculated with the pathogen (25) and in soil to which inoculated irrigation water was applied (31) has been reported.

The hydroponic system used in this study provided a controlled environment in which to study the possible association of salmonellae with aerial tissues, with minimal concerns about environmental contamination or temperature fluctuation. The results provide evidence that salmonellae can be transported from an inoculated nutrient solution to the hypocotyls-cotyledons, stems, and leaves of young tomato plants. It is not known if the same phenomenon would occur if roots of older plants (e.g., plants nearer the time of fruit maturation) were exposed to salmonellae. The potential for systemic association of salmonellae with plants grown in soil has likewise not been demonstrated. Additional studies need to be done to unequivocally demonstrate that salmonellae can exist as endophytes in tomato plants grown under conditions that simulate commonly used agronomic practices.

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