Salmonella and *Escherichia coli* O157:H7 Survival in Soil and Translocation into Leeks (*Allium porrum*) as Influenced by an Arbuscular Mycorrhizal Fungus (*Glomus intraradices*)

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A study was conducted to determine the influence of arbuscular mycorrhizal (AM) fungi on *Salmonella* and enterohemorrhagic *Escherichia coli* O157:H7 (EHEC) in autoclaved soil and translocation into leek plants. Six-week-old leek plants (with [Myc+] or without [Myc–] AM fungi) were inoculated with composite suspensions of *Salmonella* or EHEC at ca. 8.2 log CFU/plant into soil. Soil, root, and shoot samples were analyzed for pathogens on days 1, 8, 15, and 22 postinoculation. Initial populations (day 1) were ca. 3.1 and 2.1 log CFU/root, ca. 2.0 and 1.5 log CFU/shoot, and ca. 5.5 and 5.1 CFU/g of soil for *Salmonella* and EHEC, respectively. Enrichments indicated that at days 8 and 22, only 31% of root samples were positive for EHEC, versus 73% positive for *Salmonella*. The mean *Salmonella* level in soil was 3.4 log CFU/g at day 22, while EHEC populations dropped to ≤ 0.75 log CFU/g by day 15. Overall, *Salmonella* survived in a greater number of shoot, root, and soil samples, compared with the survival of EHEC. EHEC was not present in Myc– shoots after day 8 (0/16 samples positive); however, EHEC persisted in higher numbers ($P = 0.05$) in Myc+ shoots (4/16 positive) at days 15 and 22. *Salmonella*, likewise, survived in statistically higher numbers of Myc+ shoot samples (8/8) at day 8, compared with survival in Myc– shoots (i.e., only 4/8). These results suggest that AM fungi may potentially enhance the survival of *E. coli* O157:H7 and *Salmonella* in the stems of growing leek plants.

Food-borne illnesses associated with the consumption of contaminated fresh produce have escalated in recent years. Food-borne bacterial pathogens have been shown to exist on plant surfaces and exhibit the ability to internalize into plant tissues under certain conditions (1–15). Home preparation and consumption of leeks was recently associated with an 8-month-long food-borne illness outbreak of *Escherichia coli* O157:H7 phage type 8 in the United Kingdom. This outbreak affected at least 250 persons (100 under the age of 16), leading to 74 hospitalizations and one mortality between the months of December 2010 and July 2011 (16, 17). Although unique in its association with leeks, this outbreak is representative of similar multistate food-borne outbreaks recently occurring in the United States, attributed to the consumption of fresh produce such as cantaloupes, mangoes, sprouts, Romaine lettuce, spinach, papaya, peppers, and tomatoes (18).

Studies examining the internalization, translocation, and persistence of food-borne pathogens in produce, as well as in crop soil, have been reported (19–28). Jiang and Shepherd (29) stated that the survival and growth of enteric bacteria introduced from manure may be affected by organic compounds released via plant roots or from microorganisms within the rhizosphere, and suggested that the persistence of enteric pathogens in the rhizosphere is the result of interaction among pathogens, soil microbes, soil, and plant roots. Erickson et al. (19) found that *E. coli* O157:H7 persisted in soil for up to 76 days following application of contaminated irrigation water. Enterohemorrhagic *Escherichia coli* (EHEC) has also been known to survive in soil, following the application of contaminated water or compost, for 154 to 217 days (25). *Salmonella enterica* serovar Typhimurium, likewise, was shown to persist for up to 231 days in contaminated compost-

amended soil in which lettuce and parsley were grown; the pathogen was detected for up to 63 and 231 days on the surface of lettuce and parsley, respectively (24). Gagliardi and Karns (30) demonstrated by use of a soil core model that EHEC had the ability to survive for 25 to 47 days in fallow soil, 47 to 96 days on rye roots, and 92 days on alfalfa roots. Moreover, the authors found that the metabolic activity of microbial communities within soil microcosms was enhanced due to the presence of rye and alfalfa roots after 14 and 63 days, respectively. Klerks et al. (9) indicated that *Salmonella enterica* serotypes migrated toward the root exudates produced by the lettuce cultivar *Tamburo* through chemotaxis.

Intensively managed agrosystems may be environmentally unsustainable due to heavy application of herbicides, fungicides, phosphates, nitrates, fumigants, etc. Overuse of land, in this respect, may result in long-term environmental harm in some areas (31, 32). Consequently, modern agriculture is in need of alternatives to (i) reduce negative environmental impacts of conventional chemical inputs and (ii) enhance crop production efficiency. One proven biosustainable alternative is naturally occurring arbuscular mycorrhizal (AM) fungi, which are obligate symbionts that establish a mutualistic relationship with most crop plants (33–35). The fungus receives fixed carbon in the form of hexose from the

Received 17 September 2012 Accepted 19 December 2012

Published ahead of print 11 January 2013

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doi:10.1128/AEM.02855-12

host root and, in exchange, supplies the root with mineral nutrients, notably phosphorus, zinc, and copper. The extraradical phase of the fungus can extend 14 cm from the root surface, effectively increasing the efficiency of the root system to take up minerals that are immobile in soil solutions (36). Other benefits of AM fungi include enhanced disease and drought resistance (37). It is evident from these benefits that the utilization of AM fungal symbiosis could be an essential component of agricultural systems in which synthetic chemical inputs are minimized. Thus, it has been stated that “as the non-renewable sources of P rock are increasingly depleted, P fertilizers will become more expensive and all plant strategies that increase P uptake and use efficiencies will become increasingly valuable” (38).

The life cycle of AM fungi in soils of annual crops begins with the germination of fungal spores in the soil. Germ tube hyphae extend through the soil and branch in response to signals—strigolactones and other compounds—that are exuded from roots of potential host plants (39, 40). Once the growing hyphal tip contacts the root, penetration most often occurs through the epidermis (41). Further signaling and recognition events lead to the production of a “prepenetration apparatus” by the host cell (42), in addition to the fungal secretion of hydrolytic enzymes to loosen the plant cell wall (43, 44), leading to root penetration. Growth through the root hypodermis occurs through unsubsized cells known as passage cells (45). The hyphae continue their growth into the root, forming arbuscules within the layers of cortical cells closest to the vascular cylinder, although colonization of the vascular cylinder does not occur (46). The presence of the AM association, therefore, establishes a potential bacterial port of entry into the root from the rhizosphere. Further, the biocontrol fungus *Trichoderma harzianum* was recently demonstrated to gain entry into potato roots through the hyphae of an AM fungus (47).

While numerous studies have investigated internalization of human-pathogenic bacteria into plant tissues and survival in soil, many of these studies involved hydroponically grown plants and none of these trials were known to include plants colonized by AM fungi. Therefore, the impact of root penetration by AM fungi on internalization of human pathogens in plant tissues is unknown, but critical to understanding the ecology of human food-borne pathogens present in agricultural soils. The objectives of this study were to determine the influence of symbiotic AM fungi (*Glomus intraradices*) on translocation and persistence of *Salmonella enterica* and *E. coli* O157:H7 in a model vegetable plant—the leek (*Allium porrum*)—and on pathogen survival in cultivable soil.

MATERIALS AND METHODS

Preparation of soil. Soil used in this study was prepared in-house by combining a 0.75:1.0:1.0:0.75 (vol/vol) mixture of sterilized field soil, sand, vermiculite, and Turface calcined clay (Applied Industrial Materials, Corp., Deerfield, IL) (SSVT) for a final total carbon content of 0.6%. Soil sterilization was done primarily to inactivate naturally occurring mycorrhizal fungi for growth of nonmycorrhizal controls, as well as to allow for complete and exclusive colonization by our inoculated AM fungus spores.

Preparation of mycorrhizal fungi. The spores of *G. intraradices* Schenck and Smith (DAOM 181602) were produced *in vitro* in two-chambered, split petri plates with Ri T-DNA-transformed carrot roots, according to the method of St-Arnaud et al. (48). Medium in the distal half of one plate containing spores and hyphae (no root tissue) was removed and solubilized by blending for 45 s at high speed in a 10 mM solution of sodium citrate (pH 6.0) in a Waring blender (49). The result-

ing spore suspension was collected on a 38- μ m-pore-size mesh sieve and rinsed thoroughly before resuspending in 100 ml of water with magnetic stirring. Inocula used in this experiment, therefore, contained only AM fungus tissues and no other microorganisms typically contained in AM fungus inocula. This method precluded the need for the application of an “inoculum filtrate” to nonmycorrhizal control plants to equilibrate soil microflora.

Preparation of leek plants and inoculation with *G. intraradices*. Conical plastic pots (RLC-4 “Pine Cell,” Stuewe and Sons, Corvallis, OR) (66 cm³) were filled 50% full with SSVT. A water suspension (1 ml) containing 400 spores of *G. intraradices* was pipetted onto the surface of the potting mixture. Uninoculated controls (Myc−) received only water. The remainder of the conical pot was then filled with the potting mixture, and each pot received one leek (*A. porrum* L.) seedling. Plants were grown in a growth chamber with dark/light cycles of 16 h/8 h at temperatures of 25/18°C for 6 weeks prior to use in the experiments. During this period, plants received 3 to 4 applications of Hoagland’s solution without phosphorus (50). Immediately prior to use in the experiments, a subsample of 4 seedlings was destructively harvested for measurement of height, AM fungus colonization of roots, and shoot dry weight.

Root staining, microscopy, and determination of colonization by *G. intraradices*. Colonization of roots by AM fungi was determined after clearing in 10% KOH (wt/vol) and staining in trypan blue (51) via the gridline intersect method (52). Representative sections of AM fungus-colonized roots as well as uncolonized leek roots were selected for microscopic imaging. Excised root pieces, previously cleared and stained as described above, were immersed in glycerol on microscope slides and compressed slightly with a borosilicate coverslip. Roots were visually observed to determine colonization under a dissecting bright-field light microscope at $\times 20$ magnification. Micrographs were also digitally captured at $\times 25$ magnification under a Leica (Buffalo Grove, IL) MZ FLIII fluorescence stereo microscope using a Leica DFC420C 5.0-megapixel camera with a Peltier cooling system.

Preparation of bacterial inocula. Four serovars of *S. enterica* subsp. *enterica* (viz., Newport [U.S. Department of Agriculture {USDA}, Agricultural Research Service {ARS}, ERRC Wyndmoor collection], St. Paul 02-517-1 [food-borne outbreak strain isolated from cantaloupe], Typhimurium ATCC 14028, and Montevideo ATCC 8387) and three strains of *Escherichia coli* O157:H7 (ATCC 43894, ATCC 43895, and C9490 [all clinical food-borne outbreak isolates]) were chosen for this study. All four strains of *Salmonella* and three strains of *E. coli* O157:H7 were selected for spontaneous mutants resistant to 100 ppm nalidixic acid (MP Biomedicals, Solon, OH). Each isolate was stored at 4°C on tryptic soy agar (EMD, Gibbstown, NJ) slants with 100 ppm nalidixic acid (TSAN). Isolates were grown separately in borosilicate centrifuge tubes containing 10 ml of tryptic soy broth (TSB; EMD)–100 ppm nalidixic acid (TSBN) for 24 h at 37°C. The seven suspensions were centrifuged at 4,000 rpm (1,980 $\times$ g) for 10 min at 20°C, supernatant fluid was decanted, and each pellet was resuspended with 10 ml of 0.1% sterile peptone water (soy peptone; EMD) with a vortex procedure. All four strains of *Salmonella* were combined to make a 40-ml, four-strain composite of *Salmonella*, and all three strains of *E. coli* O157:H7 (EHEC) were combined to make a 30-ml, three-strain composite of EHEC. Composite inoculum populations were determined by plating onto TSAN with 0.1% sodium pyruvate (TSAPN; Alfa Aesar, Ward Hill, MA). Sixteen milliliters of each composite inoculum (*Salmonella* and EHEC, respectively) was added to separate 256-ml quantities of 0.1% sterile peptone water in preparation for inoculation of potted leek seedling soil.

Bacterial inoculation of leek potting soil. Soil of leek plants (mycorrhizal fungus positive [Myc+] and mycorrhizal fungus negative [Myc−]) was inoculated with either 8 ml of the diluted four-strain *Salmonella* composite or 8 ml of the three-strain EHEC composite by direct addition to soil in the 66-cm³ RLC-4 Pine Cell conical plastic pots. Myc+ and Myc− plants inoculated with 8 ml of sterile deionized (DI) water served as negative controls. Plants were placed in a modified Bactron IV biocontrol

chamber (Shel Lab, Cornelius, OR), illuminated by one bank of four 24-in. Agrobrite high-output HO-T5 fluorescent grow bulbs (Hydrofarm, Fairless Hills, PA) and one bank of two 24-in. conventional T12 fluorescent grow bulbs. Photosynthetically active radiation was measured with a LI-189 Quantum Radiometer/Photometer (LI-COR, Inc., Lincoln, NE) and determined to be 160 $\mu\text{mol}/\text{m}^2/\text{s}$, which was equivalent to 10% full sun. Lights were set with automatic timers for 16 h/8 h (light/dark) per day. All plants were watered three times weekly with 8 ml of sterile deionized (DI) water.

Leek sample preparation and microbiological analyses. Inoculated leeks were sampled in triplicate on days 1, 8, 15, and 22 postinoculation. One uninoculated Myc+ leek and one Myc− leek, on each day of sampling, served as negative controls. Leeks were rinsed with sterile DI water to remove soil and gross surface debris and then surface sterilized for 30 s in a 1% silver nitrate solution (ThermoFisher Scientific, FairLawn, NJ), followed by two consecutive rinses in sterile Dey-Engley (DE) neutralizing broth (BD Difco, Sparks, MD). Leek shoots were then aseptically separated from the roots with a flame-sterilized scalpel. Each of the two separated plant pieces were placed in a 7-oz. (ca. 200-ml), sterile filter stomacher bag (Whirl-Pak; Nasco, Fort Atkinson, WI) containing 9 ml of sterile DE neutralizing broth with 0.1% sodium pyruvate and 100 ppm nalidixic acid (DEPN), macerated on a hard surface, and pummeled in a stomacher (Compact Masticator; IUL Instruments, Barcelona, Spain) for 90 s. Homogenates were then plated in duplicate on TSAPN using a DW Whitley Wasp II Spiral Plater (Microbiology International, Frederick, MD) at the 50- μl setting. Undiluted homogenate samples (250 μl /plate) were also spread plated with sterile cell spreaders on TSAPN and incubated. All plates were incubated at 37°C for 24 h, and colonies were counted using the Symbiosis β COLyte Supercount automated colony counting system (Microbiology International).

Bacterial pre-enrichment, enrichment, and confirmation. The balance of root and stem homogenate in sample bags after plating was used as a nonselective pre-enrichment by incubating at 37°C for 24 h. Pre-enrichments (for *Salmonella* samples only) were then mixed thoroughly, and 1 ml was transferred into 9 ml of TT-Hajna broth (tetrathionate broth base [BD Difco], iodine [JT Baker, Phillipsburg, NJ], potassium iodide [La Motte, Chestertown, MD], brilliant green [Sigma])–0.1% sodium pyruvate–100 ppm nalidixic acid (TTPN) as a selective enrichment and incubated overnight at 42°C. The TTPN was subjected to a vortex procedure, and one 10- μl loop was streaked onto XLT4 agar (BD Difco)–0.1% sodium pyruvate–100 ppm nalidixic acid (XLT4PN). Random presumptively positive colonies were confirmed by serological agglutination (Difco *Salmonella* O Antiserum Poly A-I & Vi).

Pre-enrichments for *E. coli* O157:H7 samples were handled in the same manner as the *Salmonella* samples, with the following modifications. Selective enrichments (following 24 h of nonselective pre-enrichments) were performed by adding 1 ml of the pre-enrichment to 9 ml of a modified selective TSB containing 0.15% bile salts 3 (Difco), 0.00133% brilliant green, 0.0001% crystal violet, 0.1% sodium pyruvate, and 100 ppm nalidixic acid (TSB-select) and incubated for 18 to 24 h at 42°C. Enrichments were then streaked (10 μl) into MacConkey II agar with sorbitol, cefixime, and tellurite (tcSMAC) (Difco)–0.1% sodium pyruvate–100 ppm nalidixic acid (tcSMAC-PN) and incubated at 37°C for 24 h. Random presumptively positive colonies were confirmed by serological agglutination (Remel Rim *E. coli* O157:H7 latex test; Thermo Fisher).

Soil sample preparation and microbiological analyses. Soil was carefully separated from each leek root and (along with the balance of soil remaining in each conical plastic pot) added to sterile empty stomacher bags (Thermo Fisher), and bags were shaken thoroughly to mix soil. One gram of soil from each bag was then weighed and placed into a Whirl-Pak filter bag containing 9 ml of TTPN (*Salmonella* samples only) or TSB-select (EHEC samples only). Serial dilutions were made and then spiral plated in duplicate on XLT4PN (for *Salmonella* samples) or tcSMAC-PN (for EHEC samples) and incubated at 37°C for 24 h. Undiluted samples (250 μl /plate) were also spread plated and incubated accordingly. The

TABLE 1 Status of plants prior to inoculation with *E. coli* and *Salmonella*^a

Treatment	Shoot height (cm) ^b	Shoot dry wt (g) ^c	AM fungus colonization (% root length) ^d
Nonmycorrhizal	16.4 $\pm$ 1.1	0.056 $\pm$ 0.010	0.0 $\pm$ 0.0
Mycorrhizal	19.9 $\pm$ 3.4	0.062 $\pm$ 0.025	68.9 $\pm$ 4.7

^a Numbers represent the means of four observations $\pm$ standard errors of the means (SEM).

^b By ANOVA, probability of a greater F value ($\text{Pr} > F$), 0.3654; minimum significant difference (MSD), 8.8.

^c $\text{Pr} > F$, 0.8300; MSD, 0.066.

^d $\text{Pr} > F$, <0.0001; MSD, 11.5.

balance of soil sample suspensions in selective broth was incubated as selective enrichments at 42°C for 24 h. TTPN or TSB-select was then mixed thoroughly, and a 10- μl loop of selective enrichment was streaked onto XLT4PN and tcSMAC-PN for *Salmonella* and EHEC, respectively, and random colonies were confirmed by serological agglutination.

Statistical analysis. Three independent replicate experimental trials were conducted, samples from each trial were averaged, and means were $\log_{10}$ transformed to obtain normal distributions for statistical analyses. For root and shoot analyses, duplicate samples were measured at each sampling point for replicate 1, while triplicate samples were measured at each sampling point for replicates 2 and 3, to increase the statistical strength of the experimental design. Qualitative data for soil selective enrichment samples (presence/absence determinations) were also attained in all three repetitions; however, quantitative results were determined only during repetitions 2 and 3, with triplicate soil samples analyzed at each sampling time. Data for all quantitative results (shoot, root, and soil) were analyzed by nonlinear regression analysis using PROC NLIN of SAS software version 9.3. Nonlinear regression was executed by fitting the log-transformed data to the exponential decay model with asymptote (equation 1), as described by Gu et al. (22), as follows:

$$C_t = A + (M - A)e^{-Rt} + E_t \quad (1)$$

where C = *Salmonella* or EHEC populations [$\log$ (CFU/g)], A = asymptote [$\log$ (CFU/g)], M = initial bacterial concentration [$\log$ (CFU/g)], R = growth rate (day^{-1}), t = time (day), and E = error term. Parameter (i.e., A , M , and R) estimates and standard errors were used to compare nonlinear regression coefficients by analysis of homogeneity of nonlinear regression curves using Proc Mixed in SAS. Significant differences were determined by the Bonferroni least significant difference (LSD) technique ($P < 0.05$). Qualitative (presence/absence) data were subjected to contingency table analysis using PROC FREQ of SAS, and significant differences between *Salmonella* or EHEC populations were determined using either the chi-square test or Fisher's exact test ($P \leq 0.05$), when statistically necessary, based on limiting values within cells.

RESULTS

Leek roots were verified to be colonized with AM fungi (colonization mean value of 68.9% of root length) by staining and microscopic evaluation via the gridline intersect method (Table 1). No colonization was detected in uninoculated seedlings. Figure 1 presents micrographs of AM fungus-colonized and -uncolonized leek roots after staining. Note the dark-blue-stained intra- and extraradical fungal hyphae and vesicles in Fig. 1B, which may be a potential factor for internalization and survival of *Salmonella* and EHEC within the root. There were no statistically significant differences in growth between Myc+ and Myc− plants (*viz.*, shoot height and shoot dry weight) prior to inoculation with pathogenic bacteria (Table 1).

Exponential decay model. The exponential decay model used to describe survival of *Salmonella* or EHEC had mean square er-

FIG 1 Micrograph of an uncolonized (A) and an AM fungus-colonized (B) leek root after clearing in 10% (wt/vol) KOH and staining with trypan blue. The micrograph was taken at the microscopy facility of the USDA ARS Eastern Regional Research Center. The arrow denotes the location of trypan blue-stained fungal tissue in the root cortex.

rors ranging from 0.06 to 1.81, averaging ca. 0.48, and all regressions were significant at a P of <0.05 . The A parameter (asymptote) for Myc+ *Salmonella* was significantly higher for root ($A = 0.84$) than for shoot ($A = 0.00$). In soil samples, asymptote *Salmonella* values for Myc+ and Myc- ($A = 3.14$ and 3.19 , respectively) were significantly higher than those for EHEC ($A = 0.86$ and 0.82 , respectively). The initial bacterial concentration parameter (M) was significantly higher for *Salmonella* than for EHEC across all root, shoot, and mycorrhizal combinations. In addition, a mycorrhizal effect was observed for EHEC and shoot, with the Myc- samples having a significantly larger M value than Myc+ samples. There were root and shoot differences for *Salmonella*, with the root having larger values than the shoot for Myc+ and Myc- samples. There were no observed differences for M among soil samples. The only observed difference among the decay rate parameter values (R) was for the Myc+ EHEC, with a significantly larger value for the root ($R = 2.76$) than for the shoot ($R = 0.65$).

Survival of *Salmonella* and EHEC populations over time. Survival of both pathogens in leek shoot and root in all 8 Myc+ and Myc- treatments declined over time ($P < 0.05$), as determined by presence/absence tests and chi-square analysis (Table 2). For example, 24/24 (100%) of shoot samples were positive for both pathogens after 24 h, but only 4/24 (17%) of shoots were positive for either pathogen at day 22. While EHEC was also isolated less frequently over time in soil, *Salmonella*, in contrast, survived in soil and was isolated from 100% of samples at each sampling time throughout each 22-day trial, irrespective of the presence or absence of mycorrhizal fungi.

Comparison of differences between *Salmonella* and EHEC populations. Initial populations in diluted composite suspensions of EHEC and *Salmonella* (i.e., 16 ml of initial composite inoculum suspension diluted into 256 ml of 0.1% sterile peptone water) were the same (i.e., 7.36 and 7.29 log CFU/ml, respectively). Inoculating leek plant soil with 8 ml of diluted composite suspensions, therefore, effectively resulted in day 0 applications of 8.26 and 8.19 log CFU/plant for EHEC and *Salmonella*, respectively. Mean initial pathogen populations for Myc+ and Myc- tissues (24 h following inoculation) were 3.1 and 2.2 log CFU/root system and ca. 2.0 and 1.6 log CFU/shoot for *Salmonella* and EHEC, respectively (Fig. 2 and 3). Initial pathogen populations in

soil (24 h after inoculation) were ca. 5.5 and 5.1 log CFU/g for *Salmonella* and EHEC, respectively (Fig. 4).

Nonlinear regression analysis by the exponential decay model with asymptote determined that the survival of *Salmonella* was

TABLE 2 Presence of *E. coli* O157:H7 and *Salmonella enterica* in leek root, shoots, and soil with or without arbuscular mycorrhizal fungi as detected by selective enrichments

Sample	Treatment ^a	No. of positive samples/total no. of samples ^b			
		Day 1	Day 8	Day 15	Day 22
Shoot ^c	<i>Salmonella</i> Myc-	8/8	4/8	5/8	1/8
	<i>Salmonella</i> Myc+	8/8	8/8	5/8	2/8
	EHEC Myc-	8/8	7/8	0/8	0/8
	EHEC Myc+	8/8	5/8	3/8	1/8
	Negative control ^d	0/3	0/3	0/3	0/3
Root	<i>Salmonella</i> Myc-	8/8	8/8	5/8	5/8
	<i>Salmonella</i> Myc+	8/8	6/8	7/8	4/8
	EHEC Myc-	8/8	4/8	2/8	1/8
	EHEC Myc+	8/8	4/8	1/8	3/8
	Negative control ^d	0/3	0/3	0/3	0/3
Soil ^e	<i>Salmonella</i> Myc-	6/6	6/6	6/6	6/6
	<i>Salmonella</i> Myc+	6/6	6/6	6/6	6/6
	EHEC Myc-	6/6	6/6	5/6	2/6
	EHEC Myc+	6/6	6/6	5/6	4/6
	Negative control ^f	0/1	0/1	0/1	0/1

^a Myc+, treatment with arbuscular mycorrhizal fungi; Myc-, no treatment with arbuscular mycorrhizal fungi.
^b Numbers indicate samples positive for *Salmonella* or EHEC in three replicate experiments/total numbers of samples.
^c Shoot and root samples were pre-enriched for 24 h at 37°C in 9 ml of TSBN. One milliliter of pre-enrichment was then added to 9 ml of TTPN (*Salmonella*) or 9 ml of TSB-select (EHEC) and incubated at 42°C for 24 h before streaking on XLT4PN (*Salmonella*) or tcSMAC-PN (EHEC), incubating overnight at 37°C, and confirming presumptive positive colonies by serological agglutination.
^d One negative control sample was tested during each day of sampling for each of three replicate experiments.
^e Soil samples were enriched (1 g) into 9 ml of TTPN (*Salmonella*) or 9 ml of TSB-select (EHEC) for 24 h at 42°C before streaking on XLT4PN (*Salmonella*) or tcSMAC-PN (EHEC), incubating overnight at 37°C, and confirming presumptive positive colonies by serological agglutination.
^f Negative controls in soil were tested only in the third repetition of the study.

FIG 2 Survival of *E. coli* O157:H7 and *Salmonella enterica* in leek (*Allium porrum*) shoots as influenced by the presence of mycorrhizal fungi (*Glomus intraradices*) for up to 22 days. Predicted regression curves, based on the exponential decay model with asymptote, represent *Salmonella* MYC+ (filled squares), *Salmonella* MYC- (open squares), *E. coli* MYC+ (closed circles), and *E. coli* MYC- (open circles) strains. Nonlinear regression curves represent predicted values. Data points represent the means (log CFU) of the results of three independent replicate experiments with 2 or 3 observations per time point. Duplicate samples were analyzed for repetition 1 and triplicate samples for replicates 2 and 3.

significantly greater ($P < 0.05$) than that of EHEC in both Myc+ and Myc- soils, as a function of the asymptotic inactivation level (parameter A) in the tailing portion of the curves. Presence/absence results (Table 2) indicated that from day 8 through 22, only 31% of root samples were positive for EHEC, versus 73% of root samples which were positive for *Salmonella*. When data from Table 2 were statistically analyzed by contingency tables in SAS, *Salmonella* survived in more samples ($P < 0.05$) than EHEC, as follows (percent greater *Salmonella* than EHEC survival is listed in

FIG 3 Survival of *E. coli* O157:H7 and *Salmonella enterica* in leek (*Allium porrum*) roots as influenced by the presence of mycorrhizal fungi (*Glomus intraradices*) for up to 22 days. Predicted regression curves, based on the exponential decay model with asymptote, represent *Salmonella* MYC+ (filled squares), *Salmonella* MYC- (open squares), *E. coli* MYC+ (closed circles), and *E. coli* MYC- (open circles) strains. Nonlinear regression curves represent predicted values. Data points represent the means (log CFU) of the results of three independent replicate experiments with 2 or 3 observations per time point. Duplicate samples were analyzed for repetition 1 and triplicate samples for replicates 2 and 3.

FIG 4 Survival of *E. coli* O157:H7 and *Salmonella enterica* in soil as influenced by the presence of mycorrhizal fungi (*Glomus intraradices*) for up to 22 days. Predicted regression curves, based on the exponential decay model with asymptote, represent *Salmonella* MYC+ (filled squares), *Salmonella* MYC- (open squares), *E. coli* MYC+ (closed circles), and *E. coli* MYC- (open circles) strains. Nonlinear regression curves represent predicted values. Data points represent the means (log CFU) of the results of two independent replicate experiments with 3 observations per time point. Duplicate samples were analyzed for repetition 1 and triplicate samples for replicates 2 and 3.

parentheses): day 8/Myc-/root (+50%); day 15/Myc-/shoot (+63%); day 15/Myc+/root (+77%); day 22/Myc-/root (50%, where $P = 0.056$ by Fisher's exact test); and day 22/Myc-/soil (+67%). When the last two sampling days were composited, greater numbers of Myc- shoots were also positive for *Salmonella* (i.e., 38% more) than Myc- shoots that were positive for EHEC. Accordingly, significantly more ($P = 0.01$) Myc+ root as well as Myc- root samples (i.e., +44% and +44%, respectively) were positive for *Salmonella* than were positive for EHEC during the last two composited sampling times (i.e., days 15 and 22), as well more being positive (viz., +38% and +46% more, respectively) during the last three composited sampling times (i.e., days 8, 15, and 22). When data were composited for all four sampling times, *Salmonella* survived in a greater number of samples ($P < 0.05$) than did EHEC, as follows (percent greater *Salmonella* than EHEC survival is listed in parentheses): Myc+ root (+28%), Myc- root (+34%), and Myc- soil (+21%).

Pathogens behaved similarly in soil, where *Salmonella* persisted longer than EHEC. *Salmonella* levels in Myc+ and Myc- soil samples were still as high as 3.4 log CFU/g at day 22, while EHEC populations dropped to ≤ 0.75 log CFU/ml by day 15. When levels of pathogen presence in soil were compared qualitatively (Table 2), *Salmonella* was detected in all soil enrichment samples tested throughout the study, whereas, at the last two samplings, EHEC was not present in 25% of the Myc+ samples or 42% of the Myc- samples. Significantly fewer ($P < 0.05$) Myc- samples (i.e., 2/6) were positive for EHEC on day 22 in comparison with the 100% of samples (i.e., 6/6) which were positive for *Salmonella*. A greater proportion of Myc- soil samples were positive for *Salmonella* during the last three composited sampling periods (28% more, $P = 0.02$) than were positive for EHEC; however, there was no statistical difference between the results determined for the two pathogens among Myc+ soil samples in the same period. Overall, a clear trend emerged, reflecting the survival of *Salmonella* in a greater number of shoot, root, and soil samples compared with the survival of EHEC in the same samples.

Comparison of population differences between root and shoot samples. Results of nonlinear regression decay rates (parameter R) of $\log_{10}$ bacterial populations in the exponential decay model did not differ significantly between samples, with the exception of EHEC in Myc+ roots, which showed a higher decay rate ($P < 0.05$) than EHEC in Myc+ stems (Fig. 2 and 3). In similar fashion, the values for the asymptotic tailing effect (parameter A) within each of the pathogens did not differ, except for the values representing the survival of *Salmonella* in Myc+ roots, which had a smaller asymptote (i.e., inactivation within the right-handed tail of the regression curve) ($P < 0.05$), compared with the values for survival of *Salmonella* in Myc+ shoots. This finding reflects the ability of salmonellae to survive over time better within leek roots than within leek shoots.

Comparison of Myc+ and Myc- pathogen populations. There were no significant differences in either *Salmonella* or EHEC within soil, shoot, or root systems in comparisons of Myc+ and Myc- samples based on decay rate (R) or asymptotic tailing effect (A), as assessed by the nonlinear regression exponential decay model with asymptote. Nevertheless, although pathogen decay rates between mycorrhizal treatments were not statistically different between days 1 and 15 in Myc+ shoots, populations for both pathogens observationally declined in the Myc- shoots during the same period of time (Fig. 2). Furthermore, compared qualitatively for detection/nondetection of pathogens by selective enrichment (Table 2), EHEC was not present (0/16 samples positive) in Myc- shoots after day 8; however, EHEC persisted in higher numbers (4/16 positive) ($P = 0.05$, by Fisher's exact test) in Myc+ shoots at days 15 and 22. *Salmonella*, likewise, survived in statistically higher numbers ($P = 0.04$) in Myc+ shoot samples (8/8) at day 8 than in Myc- shoots (i.e., only 4/8).

DISCUSSION

It is hypothesized that *E. coli* O157:H7 may internalize only infrequently within the roots of lettuce plants in a transient manner for up to 7 days, and may not have the ability to internalize within the leafy portion of the vegetable via the vascular root system, following an initial contamination event (19, 26). In fact, Jablason et al. (8) suggested that vegetable seedlings may have a greater propensity for internalization of bacterial pathogens than more mature plants, and speculated that risks associated with human pathogens internalized in vegetables are low. However, those authors admitted that contamination of vegetables with human pathogens during developmental stages may improve the chances of bacterial pathogens surviving in the rhizosphere. Our present results with the leek model demonstrate the ability of *E. coli* O157:H7 inoculated into soil at 8 log CFU/plant to vascularly internalize and survive within leek roots only (as well as *Salmonella* within leek roots and shoots) for up to 22 days in the absence of mycorrhizal fungi. Although EHEC (Myc+ only) survived in leek shoots for up to 22 days, EHEC (unlike salmonellae) was not detected in the shoots after day 8 in the absence of AM fungi. These results suggest a potential role of mycorrhizal fungi in facilitating the longevity of an otherwise relatively short-lived food-borne pathogen in plant tissues. Because EHEC has occasionally demonstrated less desiccation resistance than *Salmonella* (53–56), a network of AM fungal hyphae present in soil could provide a harborage for bacterial cells that would otherwise die in dry periods, which might explain the survival of EHEC in shoots in the presence (but not absence) of AM fungi. As a means of comparison, Erickson et al. (20) reported

that spinach leaves, irrigated with EHEC-contaminated water, tested negative for surface or internalized contaminants 7 days after inoculum spraying with three different inoculation concentrations (10^2 , 10^4 , and 10^6 CFU/ml). However, at the highest inoculum level (10^8 CFU), internalization was detected in leaves for up to 14 days, demonstrating that EHEC can survive in plant shoots for extended periods of time when inoculated at a high level. Solomon et al. (28) also detected the migration of EHEC from contaminated manure into the plant's root system and into edible portions of lettuce by means of laser scanning confocal microscopy and epifluorescence microscopy. Others have noted that the fate of bacteria on plant leaves may partially be a function of rapid fluctuations in temperature and humidity, as well as limited nutrients present on the leaf, which could have applications for internalized food-borne bacterial pathogens (57).

Various studies have addressed the question of produce-internalized human pathogens, primarily with plants grown hydroponically or in conventional agricultural systems (10, 19, 20, 58), without acknowledging the potential influence of mycorrhizal fungi. Utilization of AM fungi is an important aspect of both organic and conventional farming, due to the formation of its symbiotic associations with crops (33). Several studies have established synergistic properties between AM fungi and biocontrol agents such as *Pseudomonas fluorescens*, *Burkholderia cepacia* Pal-leroni & Holmes, *Trichoderma harzianum* Rifai, and *Verticillium chlamydosporium* Kamyschko ex Barron (58). According to Dalpé and Monreal (58), these interactions suggest that AM fungi may affect plant and soil microbial activity by influencing the production of root exudates, hydroxyproline-rich glycoproteins, phenolic compounds, peroxidases, and phytoalexins. Moreover, activity of plant defense genes heightens the production of glucanases, chitinases, flavonoids, and phytoalexins—all of which have been detected during mycorrhizal growth—but these defense induction properties remain transitory (58, 59). Additionally, AM fungi are known to enhance the populations of endogenous soil bacteria, inhibiting the growth of plant pathogens and improving crop productivity (60). Mycorrhizal fungi are also capable of influencing root hydraulic conductivity, improving stomatal conductance, enhancing osmotic adjustment of the host, and increasing connections with soil particles through the binding mechanism of the hyphae, enabling water to be extracted from smaller pores within the soil (61).

All of the aforementioned processes may contribute to the fate of human bacterial pathogens in edible plants and the rhizosphere in which they are grown. Although AM fungi are environmentally ubiquitous and beneficial to the majority of vegetable crops, as previously described, conventional agricultural practices often disrupt or destroy this symbiotic relationship. Thus, studies regarding the influence of AM fungi on human food-borne pathogen survival may help determine the benefit or liability of promoting growth of AM fungi in crop-producing soils. While the current work was conducted in autoclaved soil (necessary for the elimination of indigenous mycorrhizae), soil containing background microbiota may elicit leek/pathogen responses that differ from what we report here.

Further unaddressed issues include determining at what point during the developmental stages of AM fungal symbiosis the initiation of pathogen internalization occurs and whether there are any major microbiological or chemical mechanisms that influence their interactions and responses. Though studies conducted in

relation to the translocation, internalization, and persistence of food-borne pathogens in produce grown in contaminated soil have been conducted (e.g., 19–21, 24–28), to our knowledge, no comparative analyses of pathogen contamination as influenced by AM fungi have been published.

While the present study was conducted in a growth chamber with leeks harvested at between 6 and 11 weeks of age, it is possible that results may differ in conventional agricultural settings with leeks grown to typical harvesting age (*viz.*, 100 days), revealing a role of either greater or lesser significance of AM fungi in survival of human pathogens. Validation of the phenomenon reported in this paper, if conducted in larger greenhouse or field studies, could establish the significance of potential risks under large-scale commercial growing conditions. The exploratory nature of these experiments precluded such scaled-up trials, which also have challenges due to the use of human-pathogenic enteric bacterial pathogens in open-field agricultural settings.

Results reported in this study emphasize the importance of good agriculture practices (GAP) and guidelines useful for mitigating diseases caused by human pathogens in crop production. Managing vegetable fields by incorporating knowledge of the land that is being used, adequately composting manure, testing or monitoring irrigation water sources for human pathogens, managing field workers via proper training, and application of GAPs, good manufacturing practices (GMPs), and best manufacturing practices (BMPs), as well as the potential use of AM fungi, may be useful to aid in reducing produce contamination by *E. coli* O157:H7 and *Salmonella*. Significant findings of this study include that (i) EHEC is capable of translocation and persistence within the leek shoot for up to 22 days in the presence of AM fungi but for only up to 8 days in the absence of AM fungi, (ii) the survival of *Salmonella* and EHEC in the leek model seems to be favored more in the presence of AM fungi than in its absence, and (iii) innate survival mechanisms may allow *Salmonella* to internalize and persist better than EHEC within the leek model. Future studies should address the influence of AM fungi on *Salmonella* and EHEC in other fresh produce commodities, and under larger-scale greenhouse and/or field conditions.

ACKNOWLEDGMENTS

We thank Rebecca Bailey, Lindsay Rogers, and Amanda Smelser for excellent technical laboratory assistance and Gary Richards and Andy Hwang for reviewing the manuscript and providing helpful feedback.

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