

Survival of *Escherichia coli* O157:H7 in soil and on lettuce after soil fumigation

A. Mark Ibekwe, Catherine M. Grieve, and Ching-Hong Yang

Abstract: Long-term survival of *Escherichia coli* O157:H7 in soil and in the rhizosphere of many crops after fumigation is relatively unknown. One of the critical concerns with food safety is the transfer of pathogens from contaminated soil to the edible portion of the plants. Multiplex fluorogenic polymerase chain reaction was used in conjunction with plate counts to quantify the survival of *E. coli* O157:H7 in soil after fumigation with methyl bromide and methyl iodide in growth chamber and microcosm laboratory experiments. Plants were grown at 20 °C in growth chambers during the first experiment and soils were irrigated with water contaminated with *E. coli* O157:H7. For the second experiment, soil microcosms were used in the laboratory without plants and were inoculated with *E. coli* O157:H7 and spiked with the two fumigants. Primers and probes were designed to amplify and quantify the Shiga-like toxin 1 (*stx1*) and 2 (*stx2*) genes and the intimin (*eae*) gene of *E. coli* O157:H7. Both fumigants were effective in reducing pathogen concentrations in soil, and when fumigated soils were compared with nonfumigated soils, pathogen concentrations were significantly higher in the nonfumigated soils throughout the study. This resulted in a longer survival of the pathogen on the leaf surface especially in sandy soil than observed in fumigated soils. Therefore, application of fumigant may play some roles in reducing the transfer of *E. coli* O157:H7 from soil to leaf. Regression models showed that survival of the pathogen in the growth chamber study followed a linear model while that of the microcosm followed a curvilinear model, suggesting long-term survival of the pathogen in soil. Both experiments showed that *E. coli* O157:H7 can survive in the environment for a long period of time, even under harsh conditions, and the pathogen can survive in soil for more than 90 days. This provides a very significant pathway for pathogen recontamination in the environment.

Key words: survival, *Escherichia coli* O157:H7, soil, rhizosphere, fumigant, contamination.

Résumé : La survie à long terme de *Escherichia coli* O157:H7 dans le sol et la rhizosphère de plusieurs récoltes après fumigation est relativement inconnue. Une des préoccupations principales dans un contexte de sécurité alimentaire est le transfert de pathogènes du sol contaminé aux parties comestibles des plantes. Un essai en réaction de la polymérase en chaîne multiplex fluorogène a été utilisé conjointement à des décomptes sur plaque pour quantifier la survie de *E. coli* O157:H7 dans le sol après fumigation au bromure de méthyle et à l'iodure de méthyle, en chambre de croissance et lors d'expériences en laboratoire en microcosme. Les plantes ont été cultivées à 20 °C dans des chambres de croissance lors de la première expérience, et les sols ont été irrigués avec de l'eau contaminée par *E. coli* O157:H7. Lors de la deuxième expérience, les microcosmes du sol ont été utilisés en laboratoire en absence de plantes, inoculés avec *E. coli* O157:H7 et traités aux deux agents de fumigation. Les amorces et les sondes ont été conçues pour amplifier et quantifier les gènes codant les toxines (ou analogues) Shiga 1 (*stx1*) et 2 (*stx2*), ainsi que le gène de l'intimine *eae* de *E. coli* O157:H7. Les deux agents de fumigation ont été efficaces dans la réduction de la concentration du pathogène dans le sol et lorsque les sols traités ont été comparés aux sols non-traités, les concentrations de pathogène étaient significativement plus élevées dans les sols non-traités tout au long de l'étude. Ceci a résulté en une survie plus longue du pathogène à la surface des feuilles, spécialement dans la terre sablonneuse, comparativement à ce que l'on a observé avec les sols soumis à la fumigation. Ainsi, l'application d'agents de fumigation peut jouer un certain rôle dans la réduction du transfert de *E. coli* O157:H7 du sol à la feuille. Des modèles de régression ont montré que la survie du pathogène dans les chambres de croissance suit un modèle linéaire alors que ceux des microcosmes suivent un modèle curviligne, suggérant une survie à long terme du pathogène dans le sol. Les deux expériences ont montré que *E. coli* O157:H7 peut survivre dans l'environnement pour une longue période de temps et même sous des conditions difficiles, le pathogène peut survivre dans le sol pendant plus de 90 jours. Ceci constitue une voie de recontamination pathogène significative dans l'environnement.

Mots-clés : survie, *Escherichia coli* O157:H7, sol, rhizosphère, agent de fumigation, contamination.

[Traduit par la Rédaction]

Received 16 August 2006. Revision received 21 November 2006. Accepted 24 November 2006. Published on the NRC Research Press Web site at cjm.nrc.ca on 22 June 2007.

A.M. Ibekwe¹ and C.M. Grieve. USDA-ARS-US Salinity Laboratory, 450 West Big Springs Road, Riverside, CA 92507, USA.

C.-H. Yang. Department of Biological Sciences, University of Wisconsin, Milwaukee, WI 53211, USA.

¹Corresponding author (e-mail: aibekwe@ussl.ars.usda.gov).

Introduction

Animal waste in the form of washwater, raw manure, or composted manure from cattle may be applied to the land as fertilizer or soil amendments before planting or near the locations where crops are grown. The steps in the production chain that have the greatest potential for pathogen contamination are soil preparation, which may include the use of uncomposted organic manures and planting and growing of plants with the use of irrigation water (Islam et al. 2004). A potential risk arising from the disposal of animal waste is the spread of enteric bacteria such as *Escherichia coli* O157:H7 (Pell 1997). There is little information regarding the behavior and metabolic status of *E. coli* O157:H7 in the environment after fumigation, although some reports in non-fumigated environments suggest the potential for considerable survival in cattle feces, soil, and water (Wang et al. 1996; Kudva et al. 1998; Fukushima et al. 1999; Jiang et al. 2002; Ibekwe et al. 2004).

Methyl bromide (MeBr) is a highly effective soil fumigant that has been used for many years for the control of insects, nematodes, weeds, and plant pathogens in many crops. MeBr is one of the chemicals that deplete the protective ozone layer. As a result, stringent regulations on MeBr consumption by the 1987 Montreal Protocol have stimulated research into alternative fumigants after MeBr is phased out. Since the main objective of fumigation is the control of plant pathogens, such as nematodes, soil-borne diseases, and weeds, there has been no study looking at the effects of fumigants on manure-borne human pathogens such as *E. coli* O157:H7. The immediate impact of fumigants may be the reduction of certain bacterial species in the soil and the development of new communities (Ibekwe et al. 2001; Dungan et al. 2003). High value cash crops such as strawberry and tomato are generally planted in the field 2 weeks after fumigation to control plant pathogens, nematodes, and weeds. In the semi-arid regions of the southwestern United States, the land is generally irrigated before fumigation and irrigated intermittently after fumigation.

Cross-contamination of produce from manure or improperly composted manure used as soil amendment may result in pathogen contamination during preharvest of high value cash crops, especially in developing nations. *Escherichia coli* O157:H7 outbreaks have been associated with lettuce and minimally processed fresh fruit juices (Ackers et al. 1998; Hilborn et al. 1999). Although many pathogens have been associated with fresh produce, *E. coli* O157:H7 is of particular concern because ingestion of relatively few cells can cause illness (Buchanan and Doyle 1997). *Escherichia coli* O157:H7 can survive for extended periods of time in water and in soil, and under dry and acidic conditions (Centers for Disease Control and Prevention 1999, 2001). Using laser scanning confocal microscopy, epifluorescence microscopy, and recovery of viable cells from the inner tissues of plants, the transmission of *E. coli* O157:H7 from manure-contaminated soil and irrigation water to lettuce plants was recently demonstrated by Solomon et al. (2002a, 2002b). The presence of *E. coli* O157:H7 in the edible portion of the plant was attributed to the direct migration through the conducting tissues of the root system. *Escherichia coli* O157:H7 may be introduced into crops by flood irrigation

with water contaminated with cattle feces or by contaminated surface runoff (O'Conner 2002).

The first study focused on determining the survival of *E. coli* O157:H7 in the rhizosphere, nonrhizosphere, and leaf surface of lettuce in a 5 week growth chamber study. A second study evaluated the survival of the pathogen in laboratory microcosms over a 3 month period. Both plate count and real-time polymerase chain reaction (PCR) approaches were used to determine the survival of *E. coli* O157:H7 in the two soils and plant tissues. The availability of real-time PCR has greatly aided the study of pathogens such as *E. coli* O157:H7 in the environment (Heid et al. 1996; Oberst et al. 1998). It has been used in studies for the detection and relative quantification of *E. coli* O157:H7 in food and clinical samples (Sharma et al. 1999; Bellin et al. 2001). Recently, naturally occurring *E. coli* O157:H7 was quantified in soil, cattle feces, manure, waste water, and rhizosphere and phyllosphere using multiplex real-time PCR (Ibekwe et al. 2002, 2004; Ibekwe and Grieve 2003). Our main objective was to determine the survival of *E. coli* O157:H7 cells in fumigated and nonfumigated soils artificially contaminated with the pathogen.

Materials and methods

Bacterial strain and growth conditions

An *E. coli* O157:H7 strain expressing green fluorescent protein from a plasmid (pGFP) (Fratamico et al. 1997) was used for this study. *Escherichia coli* O157:H7/pGFP was cultured at 37 °C overnight in modified Tryptic[®] soy broth (mTSB; Difco Laboratories, Cockeysville, Maryland) supplemented with ampicillin (50 µg/mL) (Sigma-Aldrich, St. Louis, Missouri). The specificity and sensitivity of the multiplex PCR was previously characterized (Ibekwe et al. 2002) with 33 different strains of *E. coli* O157:H7 and other related strains. *Escherichia coli* O157:H7 strain 72 harboring pGFP was inoculated into 100 mL of Tryptic[®] soy broth (TSB; Becton Dickinson, Sparks, Maryland) containing 50 µg/mL ampicillin (TSB-A) and was incubated at 37 °C for 12 h with agitation (200 r/min). The bacteria were harvested by centrifugation (7000g for 10 min), washed three times in 0.1% phosphate-buffered saline (PBS), and the cell pellet was resuspended in 0.1% PBS to a final optical density (OD 550 nm) of 1.78 (ca. 4.4×10^8 CFU/mL) prior to using this suspension for inoculating soils.

Soil preparation for planting

Clay soil (Willows silty clay, saline-alkaline) and sandy soil (Dello loamy sand) were collected from Mystic Lake dry bed and the Santa Ana River bed, respectively. The soils were sieved through a 4 mm sieve before planting, and the high salt content (electrical conductivity = 15 ds/m) in the clay soil was reclaimed as described by Ibekwe and Grieve (2004) to less than 1.5 ds/m. The clay soil had a bulk density of 1.51 Mg/m³ with 3.7% sand, 49.1% silt, and 47.2% clay. The sandy soil had a bulk density of 1.67 Mg/m³ with 99.1% sand, 0.20% silt, and 0.70% clay. The moisture content of the clay soil was 4.02% and that of sandy soil was 5.32% before both were increased to about 12% at the start of the experiment. The pH of sand was 6.85 and that of clay was 7.45. Soils were tested by culture and PCR methods to

make sure that they were *E. coli* O157:H7 negative by serial dilution in TSB and plating on cefixime–tellurite–sorbitol MacConkey agar (CT-SMAC) with BCIG (5-bromo-4-chloro-3-indoxyl- β -D-glucuronide) containing 0.05 mg/L of cefixime and 2.5 mg/L of tellurite (LAB M; IDG, Boston, Massachusetts).

Growth chamber experiment after fumigation

The fumigant methyl iodide (MeI, iodomethane, >99% purity) was purchased from Chem Service (West Chester, Pennsylvania) and MeBr (>99% purity) was obtained from Great Lakes Chemical company (West Lafayette, Indiana). Plastic trays (60 cm \times 45 cm \times 20 cm) were filled with approximately 40 kg of soil to a depth of 15 cm. The soils were irrigated and inoculated with approximately 4.2×10^8 CFU *E. coli* O157:H7 to bring soil moisture to about 12%. Bacteria were inoculated into the irrigation lines with a Cole-Parmer HPLC pump (Cole-Parmer, Chicago, Illinois) and delivered through polyvinylchloride pipes to each tray with five surface drip lines. After irrigation, soil samples were taken immediately for *E. coli* O157:H7(pGFP) concentrations. Cell concentrations within the top 10 cm of the soils immediately after inoculation were approximately 10^6 CFU by plate count 10^8 by real-time PCR.

After the initial sample collection, trays were manually taped with a virtually impermeable plastic film; 0.038 mm Hytibar[®] film (Klerk Plastics, Belgium) and fumigants were applied. Fumigation treatments were randomized with three replicates per treatment at each growth chamber. Fumigant rates and application methods were selected according to the recommended field application rate for each chemical (Table 1). To avoid the emission of fumigants to the growth chamber, syringes were used to inject fumigant (MeBr gas and MeI liquid) into the trays, and the injection ports covered immediately with duct tape and were left in the growth chamber for 10 days. After 10 days, trays were moved outside and the Hytibar film was removed. Trays remained opened and aerated outside in an area covered with barb wires for 2 days before they were moved back to the growth chamber for the continuation of the experiment. At this point, a total of 14 days had elapsed since fumigation was initiated; soil samples were collected for the second time for *E. coli* O157:H7(pGFP) concentration.

Growth conditions

Seeds of green Romaine lettuce *Lactuca sativa* L. 'Green Forest' were purchased from Johnny's Selected Seed Co. (Albion, Maine). The plants were grown at 20 °C with 70% relative humidity and a photoperiod consisting of 16 h of light and 8 h of darkness. Lettuce seedlings were sprouted in 50% Hoagland's solution (Hoagland and Arnon 1950) and transplanted into the soils in the two growth chambers. There were 10 plants in each tray at transplanting and one plant was harvested from each tray during the analysis described below. The experiment was completely randomized with three replications per fumigant level. Plants in both soils were irrigated with distilled water daily and received the nutrient solution weekly. The whole plant was harvested and the rhizosphere was cut at the soil level and placed in a sterile Petri dish. Survival of *E. coli* O157:H7 in the rhizosphere was measured at intervals after the planting of let-

tuce. Compared with the soil, population measurement in the rhizosphere started a week after planting because it took at least 7 days for the plants to have significant root mass. Since the population in the rhizosphere was expressed as CFU per gram of root, we measured the total population within the rhizosphere as it related to the root mass. We obtained the accurate mass since results needed to be normalized to remove root size as a variable and the population expressed as CFU per gram of root, as is typical (Calderwood et al. 1987; DeFreitas and Germida 1992; Kluepfel and Tonkyn 1992; Angle et al. 1995; Brandl et al. 2004). The leaves were placed in separate sterile Petri dishes or collection bags. All samples including bulk soil were collected weekly for 5 weeks for *E. coli* O157:H7. Leaf and root samples were placed in a stomacher bag and weighed. Nonrhizosphere soil (0–10 cm) was collected 30 cm from the plant stem using a 2 cm diameter sterile stainless steel soil probe. These samples were transferred to plastic bags and 10 g of sample was used for serial dilution. *Escherichia coli* O157:H7 was extracted from the rhizosphere and leaf surfaces by homogenization with 100 mL of PBS for 2 min at 260 r/min in a Seward Stomacher 400[™] circulator (Seward, London, UK). The homogenate was centrifuged at 3000g for 10 min, the pellet was resuspended in 2 mL of PBS, the suspension plated after serial dilution on TSA with ampicillin, and incubation was carried out at 37 °C overnight. *Escherichia coli* colonies were enumerated using a hand-held Spectroline[®] ultraviolet lamp (Spectronics Corporation, Westbury, New York). Total bacterial community DNA obtained from concentrated samples was used for quantification of *E. coli* O157:H7 by real-time PCR.

Microcosm experiment

A microcosm experiment was conducted to compare the survival of *E. coli* O157:H7 in microcosms to that in the growth chamber. The microcosms were made of glass containers with an aluminum cap and a small hole at the top for air circulation (Ibekwe et al. 2001). The main objectives were to examine pathogen survival over a longer period of time with decreasing soil moisture content than in the growth chamber and to determine pathogen survival at a fumigant concentration five times the recommended application rate. The soil (1.5 kg dry mass) was treated to obtain a moisture content of about 12% after the addition of *E. coli* O157:H7 or water. The experimental design consisted of two fumigants at three different concentrations and a control in three replicate microcosms. Fumigants were added as freshly prepared aqueous solutions or gases. Microcosms were sealed for 24 h after fumigant application and were vented continuously through a small opening in the cover for the remainder of the experiment as previously described (Ibekwe et al. 2001). Samples for *E. coli* O157:H7 concentrations in each microcosm soil (0–10 cm) were taken before fumigation and at weeks 2, 4, 8, and 12 after fumigant treatment.

DNA extraction and primer and probe design for real-time PCR

Genomic DNA was isolated from pure cultures of *E. coli* O157:H7/pGFP grown for 12 h at 37 °C and extracted with a QIAamp[®] DNA mini-kit (Qiagen, Valencia, California).

Table 1. Rate constant (*K*) of *Escherichia coli* O157:H7 persistence in soils, rhizosphere, and phyllosphere after 50 days in a growth chamber experiment irrigated with water contaminated with *E. coli* O157:H7.

Treatment and concentration*	Analysis technique	Soil			Root			Leaf		
		<i>K</i>	<i>P</i> > <i>F</i>	<i>R</i> ²	<i>K</i>	<i>P</i> > <i>F</i>	<i>R</i> ²	<i>K</i>	<i>P</i> > <i>F</i>	<i>R</i> ²
S-MeBr-0.5×+E	Plate count	49.5	0.0001	0.87	44.1	0.003	0.69	37.3	0.0028	0.69
	<i>stx1</i>	112.2	0.0136	0.47	123.9	0.0215	0.50	72.7	0.0040	0.67
	<i>stx2</i>	65	0.0008	0.69	59.2	0.0017	0.73	75.8	0.0690	0.36
	<i>eae</i>	90.9	0.0001	0.82	126.2	0.0291	0.50	57.6	0.0011	0.75
S-MeBr-1×+E	Plate count	48.5	0.0001	0.86	44.1	0.0028	0.69	37.2	0.0028	0.69
	<i>stx1</i>	116.8	0.0402	0.36	127.7	0.0663	0.36	66.1	0.0006	0.79
	<i>stx2</i>	65.7	0.0052	0.56	72.5	0.0250	0.50	56.3	0.0017	0.72
	<i>eae</i>	81.4	0.0001	0.82	148.8	0.0818	0.33	66.2	0.0021	0.71
S-MeI-0.5×+E	Plate count	41.2	0.0002	0.77	40.42	0.0024	0.70	37.3	0.0028	0.69
	<i>stx1</i>	98.1	0.0003	0.74	142.5	0.1585	0.23	68.5	0.0213	0.51
	<i>stx2</i>	58.6	0.0001	0.81	69.8	0.0239	0.50	64.8	0.0327	0.45
	<i>eae</i>	74.5	0.0001	0.97	124.5	0.2081	0.20	64.5	0.0082	0.60
S-MeI-1×+E	Plate count	44.0	0.0001	0.84	40.1	0.0017	0.73	37.3	0.0028	0.69
	<i>stx1</i>	148.0	0.1180	0.23	135.1	0.0663	0.36	72.2	0.0026	0.70
	<i>stx2</i>	75.4	0.0080	0.52	76.5	0.0224	0.50	82.4	0.1587	0.23
	<i>eae</i>	118.8	0.0001	0.86	127.1	0.0339	0.45	71.1	0.0022	0.71
S-0+E	Plate count	72.5	0.0026	0.70	48.3	0.0001	0.90	39	0.0037	0.67
	<i>stx1</i>	97.5	0.0017	0.64	212.9	0.2453	0.16	122.6	0.1256	0.28
	<i>stx2</i>	63.5	0.0012	0.67	121.9	0.2590	0.16	76.5	0.0973	0.31
	<i>eae</i>	106.6	0.0126	0.50	185.5	0.0522	0.39	84.5	0.0347	0.45
C-MeBr-0.5×+E	Plate count	47.0	0.0093	0.51	34.7	0.0030	0.74	37.3	0.0028	0.69
	<i>stx1</i>	109.4	0.0816	0.27	91.8	0.0056	0.64	67.2	0.0079	0.61
	<i>stx2</i>	60.4	0.1636	0.18	62.9	0.0015	0.74	57.9	0.0008	0.77
	<i>eae</i>	73.4	0.0459	0.34	75.5	0.0021	0.71	67.4	0.0023	0.71
C-MeBr-1×+E	Plate count	42.2	0.0019	0.68	35.2	0.0132	0.74	35.2	0.0132	0.74
	<i>stx1</i>	92.8	0.0300	0.40	102.5	0.0456	0.41	58.4	0.0093	0.70
	<i>stx2</i>	94.4	0.1714	0.18	60.7	0.0012	0.75	51.8	0.0093	0.70
	<i>eae</i>	62.4	0.0062	0.54	69.8	0.0050	0.65	63.5	0.0192	0.63
C-MeI-0.5×+E	Plate count	38.7	0.0236	0.45	35.0	0.0326	0.72	35.4	0.0054	0.75
	<i>stx1</i>	127.3	0.1089	0.24	65.3	0.0006	0.96	59.4	0.0159	0.80
	<i>stx2</i>	ND	ND	ND	57.6	0.0262	0.75	57.2	0.0150	0.80
	<i>eae</i>	82.6	0.0608	0.31	66.6	0.0007	0.96	71.8	0.0481	0.66
C-MeI-1×+E	Plate count	40.9	0.0002	0.77	37.8	0.0126	0.67	37.4	0.0059	0.68
	<i>stx1</i>	113.9	0.0871	0.26	94.6	0.0085	0.65	66.5	0.0163	0.53
	<i>stx2</i>	61.9	0.0143	0.50	66.2	0.0280	0.52	56.6	0.0031	0.69
	<i>eae</i>	88.5	0.0115	0.50	80.2	0.0109	0.63	67.6	0.0012	0.75
C-0+E	Plate count	45.3	0.0123	0.50	39.1	0.0047	0.65	37.3	0.0028	0.69
	<i>stx1</i>	91.6	0.0471	0.34	89.9	0.0031	0.69	69.8	0.0126	0.56
	<i>stx2</i>	80.6	0.0913	0.26	108.6	0.3601	0.11	86.7	0.2027	0.19
	<i>eae</i>	69.4	0.0204	0.43	75.1	0.0035	0.68	69.1	0.0056	0.64

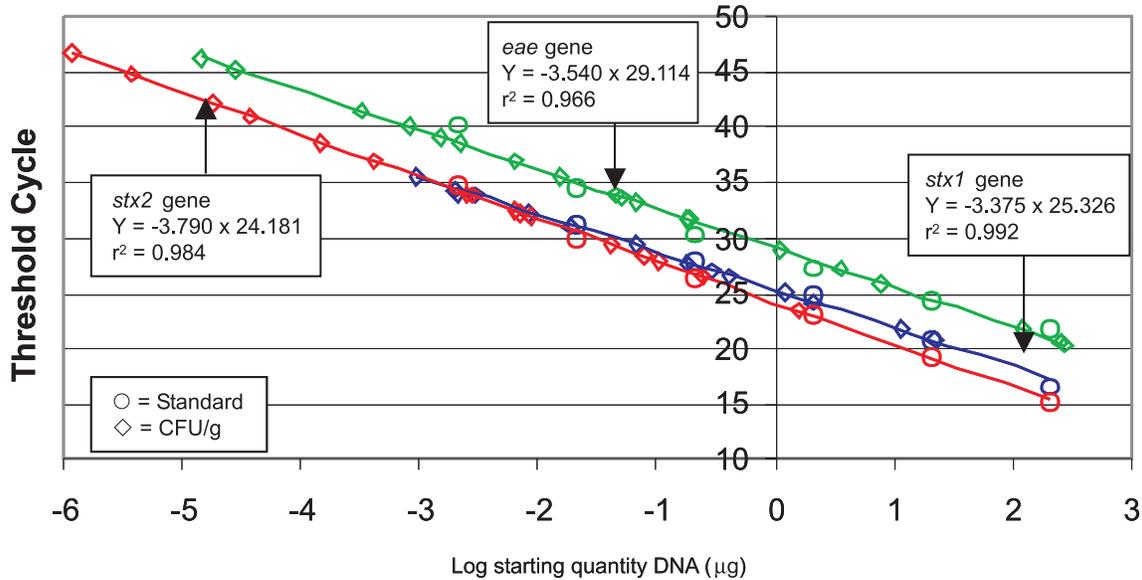
*S, sandy soil; C, clay soil; MeBr, methyl bromide; MeI, methyl iodide. Application rates for MeBr and MeI are indicated by 0.5×, 1×, and 5.0× representing MeBr at 250, 500, and 2500 g/kg soil, respectively, and MeI at 225, 450, and 2250 g/kg soil, respectively (i.e. at half, normal, and five times the recommended agricultural application rates, respectively). +E, *E. coli* O157:H7 applied to the samples. S-0 and C-0 denote control soil without fumigation.

DNA extracted from O157:H7/pGFP was used for the construction of a standard curve and for the determination of detection limits of the *E. coli* by real-time PCR. Total bacterial DNA was extracted from plant rhizospheres with the Ultra Clean Soil™ DNA kit (MoBio Laboratories, Solana Beach, California) and stored at -20 °C. Primers and probes used for the detection and quantification of the *E. coli* O157:H7 *stx1*, *stx2*, and *eae* genes were as described elsewhere (Ibekwe et al. 2002). Real-time, quantitative PCR was performed with the iCycler® iQ (Bio-Rad, Hercules, California) (Ibekwe et al. 2004).

Assay sensitivity and amplification efficiency

Standard curves generated from plotting the threshold cycle (*C*_T) versus log₁₀ of starting DNA quantities were used for determining the detection limit of the assay (Heid et al. 1996). Optimization of the multiplex assay was done as previously described (Ibekwe et al. 2002; Sharma 2002; Ibekwe and Grieve 2003). The standard curves were constructed by using known quantities of genomic DNA containing 1.6 × 10⁻² to 1.6 × 10⁸ CFU/mL of *E. coli* O157:H7. The concentration of the extracted DNA was measured by an Ultrospec® 4000 spectrophotometer with SwiftII™

Fig. 1. Standard curves from multiplex real-time polymerase chain reaction analysis of *Escherichia coli* O157:H7 in microcosm soil showing threshold cycle (C_T) versus log DNA starting quantity. The curve (as an example) was used to generate detection limits for quantification of *E. coli* O157:H7. The reaction efficiency for the *stx1* and *stx2* genes were better than that of *eae* gene, as seen by the closeness of the standard curves and with r^2 greater than 0.96. C_T values of known (standard) quantities (\circ) and unknown (\diamond) samples were plotted against corresponding DNA starting quantities.



application software (PharmaciaBiotech, Cambridge, England) and viable counts (CFU/mL) were determined by plating culture dilutions on mTSB. For a comparison of PCR amplification efficiency and detection sensitivity among different experiments, slopes of the standard curves were calculated by performing a linear regression analysis with the iCycler[®] iQ software. The concentrations of *E. coli* O157:H7 present in unknown samples were determined from the standard curves. The concentrations from the standard curves were subsequently related to the CFU per millilitre, as determined from the concentrations obtained by plating culture dilutions containing 1.6×10^{-2} to 1.6×10^8 CFU/mL of *E. coli* O157:H7 on TSB-A. Amplification efficiency (E) was estimated by using the slope of the standard curve and the following formula: $E = (10^{-1/\text{slope}}) - 1$. Reaction with 100% efficiency generated a slope of -3.32 .

Data analyses

Standardization of DNA quantities between known and unknown samples was accomplished by dividing total CFU per millilitre of *E. coli* O157:H7/pGFP by the mean starting DNA concentration of that CFU per millilitre from the instrument analysis as described previously (Ibekwe et al. 2002). Statistical analyses were done with the general linear model (GLM) procedure of the Statistical Analysis System (SAS Institute 1991). The population data were log-transformed to obtain a normal distribution. Comparisons between pairs of treatment means within each soil at any date were accomplished with the Tukey's test. The log-transform data of *E. coli* O157:H7 population size of all individual samples were plotted over time after inoculation, and analyzed by regression analysis. The linear regression model was as described by Brandl et al. (2004). The rate constant K was calculated from the regression line to estimate the survival of *E. coli* O157:H7 at various times, dif-

ferent treatments, and environmental conditions for the growth chamber study. In the microcosm experiment a polynomial model was used to describe the survival pattern in the soil after 90 days incubation. The model produced an equation as follows: $y = B_0 + B_1x + Bx^2$; where y represents the dependent variable (treatment) and x represents the independent variable (time) (Steel et al. 1997).

Results

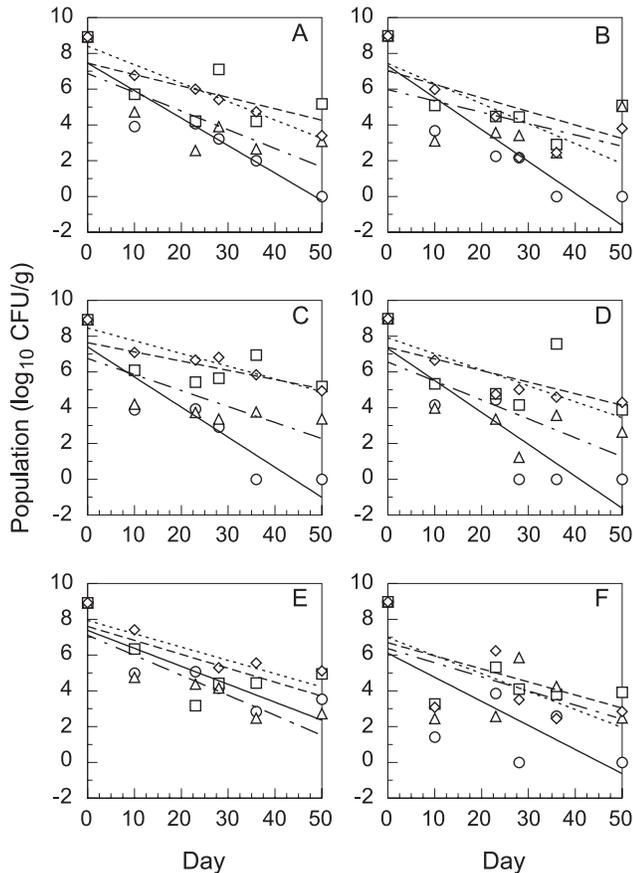
Sensitivity, standard curve, and amplification efficiency of the real-time PCR assay

In our previous study (Ibekwe et al. 2002), it was shown that the three genes used in the present study can distinguish *E. coli* O157:H7 from other serotypes of *E. coli* after analyzing 33 Shiga toxinogenic *E. coli* O157:H7 (STEC) and non-STEC *E. coli* strains. The detection sensitivity of the quantitative real-time PCR assay was determined by testing triplicate sets of genomic DNA prepared from serial dilutions (1.6×10^{-2} to 1.6×10^8) of *E. coli* strain 72 (Fig. 1). The results were reported as C_T numbers versus log starting quantities of DNA. Based on this approach, a correlation was observed between the C_T value and the starting quantity of *E. coli* O157:H7 DNA. A detection limit of 2.4×10^3 CFU/mL of starting DNA was determined. The ratio of added cell numbers to both soils (4.5×10^8 CFU/g) to cell concentrations at the surface of both soils immediately after spiking were approximately 10^6 CFU/g by plate count and 10^8 /g by real-time PCR.

Growth chamber study: soil

Linear regression of *E. coli* O157:H7 survival in soil after fumigation as a function of time was used to assess the survival pattern of *E. coli* O157:H7 in sandy and clay soil. We used linear regression to show the effects of fumigants on

Fig. 2. Linear regression curves showing predictive models for persistence of *Escherichia coli* O157:H7 in sand and clay soils. The following regression lines represent data from plate count (○); real-time polymerase chain reaction with *stx1* (□), *stx2* (△), and *eae* (◇) genes. Pesticide concentrations were at the normal agricultural application rate for (A) methyl bromide (MeBr) sandy soil, (B) MeBr clay soil, (C) methyl iodide (MeI) sandy soil, (D) MeI clay soil, (E) nonfumigated sandy soil, and (F) nonfumigated clay soil. All regression models (see Table 1) used log transformed data.



the pathogen because the number of cells inoculated into the different trays in the two growth chambers varied slightly; therefore, comparison of treatment means alone at each point may be misleading. As shown in Table 1, we also statistically compared the slopes of predictive lines and the x intercepts. This allowed us to predict the rate constant in which the population approached zero. The population of *E. coli* O157:H7 in sandy soil (Fig. 2A) and clay soil (Fig. 2B) at the normal application rate (Table 1) declined at a significant rate in the growth chamber study throughout the experiment. In the growth chamber soil, 10 days after fumigation, *E. coli* O157:H7 was significantly lower ($P = 0.0001$) in fumigated soils than the control at the normal application rate. During the rest of the study, there were no significant differences in the effect of the two fumigants on the pathogen, except on day 36 ($P = 0.046$) where the effects varied (data not shown). Real-time PCR analysis showed that 10 days after fumigation, *E. coli* O157:H7 concentrations in nonfumigated soils were significantly higher ($P = 0.002$) in sandy soil than in clay soil. There was no significant difference ($P = 0.56$) in pathogen concentration on

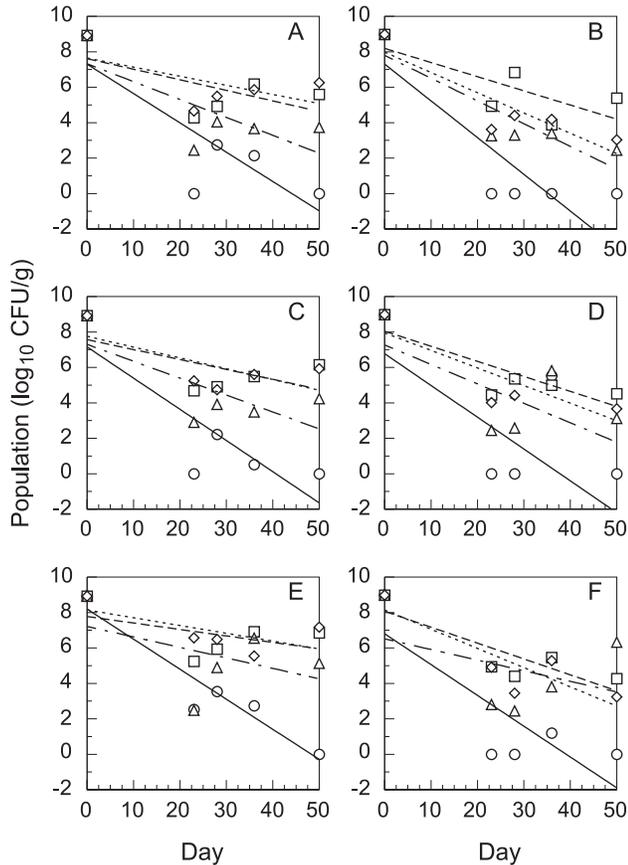
day 23 when real-time PCR was used for the analysis. The same effect was observed on days 36 and 50 (data not shown). In the sandy soil, the population was no longer detectable in soil by plate count after 48.5 days based on prediction by the regression equation (Fig. 2, Table 1), while the population in the clay soil was not detectable by plate count after 42.2 days (Fig. 2). We also tested the survival of *E. coli* O157:H7 at half the application rate. All models produced significant linear population reductions over the experimental period for sandy soil, whereas in the clay soil only the plate counts and the *eae* gene produced significant linear reduction (Table 1). The number of days that the pathogen persisted was slightly lower in clay soil than in sandy soil.

MeI was the second fumigant used in the study. *Escherichia coli* O157:H7 survival was longer in the nonfumigated soils than the soil with normal application rate of fumigant when survival was determined by plate count. Survival was also longer in sandy soil than clay soil. Mean comparison by day and method along the regression lines was used to determine differences between the date provided by real-time PCR and plate count. Direct comparison between MeBr and MeI within each soil showed that both had similar impact on *E. coli* O157:H7. *Escherichia coli* O157:H7 concentrations by plate count at the normal application rate of MeBr and MeI were significantly lower than the concentrations by real-time PCR except for *stx2* in all the treatments in both soils (data not shown). Linear regression curves showing predictive models for survival of *E. coli* O157:H7 in sandy and clay soils (Figs. 2C and 2D, Table 1) are shown for normal agricultural application rate for MeI. All models produced significant linear population reductions over the experimental period, except for the *stx1* gene with sandy soil and the *stx2* gene for clay soil. *Escherichia coli* O157:H7 population survived for a shorter number of days in the MeI-treated sandy soil (44 days) compared with MeBr-treated soil (48.5 days) based on the predictive model. Using real-time PCR, *E. coli* O157:H7 population was observed to persist longer in MeI-treated sandy soil than in MeBr-treated sandy soil according to the predictive model. For the clay soil, *E. coli* O157:H7 also survived for a shorter number of days for the MeI-treated soil at the normal application rate than in the MeBr-treated soil (Fig. 2D and Table 1). Results obtained from both sandy and clay soils at the 0.5 \times treatment of MeI showed a significant pathogen decline in plate counts and genes, except *stx1* in clay soil (Table 1). For MeBr, significant decline of the pathogen was obtained for all genes and in the plate counts, except for *stx1* and *stx2* in clay soil. Treatments without fumigants followed the same pattern of significant reduction as those with fumigants (Figs. 2E and 2F, Table 1).

Growth chamber study: rhizosphere

Escherichia coli O157:H7 populations in the rhizosphere of sandy and clay soils significantly declined as assessed by the plate count method throughout the growth chamber study (Figs. 3A and 3B). The population size in the sandy soil rhizosphere was no longer detectable after 44 days, while the population in the clay rhizosphere was not detectable after 35 days with treatments receiving the normal application rate of MeBr based on predictive models from the

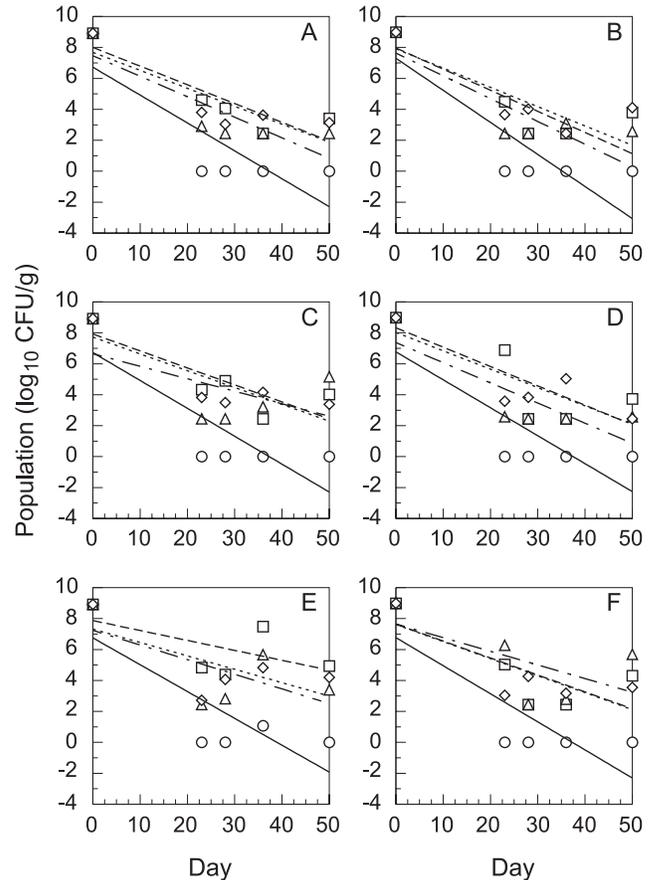
Fig. 3. Linear regression curves showing predictive models for persistence of *Escherichia coli* O157:H7 in lettuce rhizospheres grown in sandy and clay soils from the growth chamber experiment. The following regression lines represent data from plate count (○); real-time polymerase chain reaction with *stx1* (□), *stx2* (△), and *eae* (◇) genes. Pesticide concentrations were at the normal agricultural application rate for (A) methyl bromide (MeBr) sandy soil, (B) MeBr clay soil, (C) methyl iodide (MeI) sandy soil, (D) MeI clay soil, (E) nonfumigated sandy soil, and (F) nonfumigated clay soil. All regression models (see Table 1) used log transformed data.



regression equations. Real-time PCR quantification of the *stx1*, *stx2*, and *eae* genes in rhizosphere samples revealed that *E. coli* O157:H7 survived longer in clay soil than in sandy soil (Table 1, Figs. 3A and 3B). Mean comparison using Tukey's procedure ($P < 0.05$) at the different sampling points showed that real-time PCR data were significantly higher than plate count data, except on day 36 for all treatments and on day 50 in treatments where fumigants were not applied (data not shown). Survival of *E. coli* O157:H7 at half the application rate was also tested and all models produced significant linear population reductions in the rhizosphere over the experimental period for both soils (Table 1). From the regression equations, *E. coli* O157:H7 populations were estimated to survive longer in the sandy soil than in the clay soil at the $0.5\times$ application rate (Table 1).

Significant population reductions were observed over the experimental period for sandy soil fumigated with MeI at the normal agricultural application rate (Figs. 3C and 3D), except for the *stx1* gene. *Escherichia coli* O157:H7 popula-

Fig. 4. Linear regression curves showing predictive models for persistence of *Escherichia coli* O157:H7 in lettuce phyllosphere grown in sandy and clay soils from the growth chamber experiment. The following regression lines represent data from plate count (○); real-time polymerase chain reaction with *stx1* (□), *stx2* (△), and *eae* (◇) genes. Pesticide concentrations were at the normal agricultural application rate for (A) methyl bromide (MeBr) sandy soil, (B) MeBr clay soil, (C) methyl iodide (MeI) sandy soil, (D) MeI clay soil, (E) nonfumigated sandy soil, and (F) nonfumigated clay soil. All regression models (see Table 1) used log transformed data.

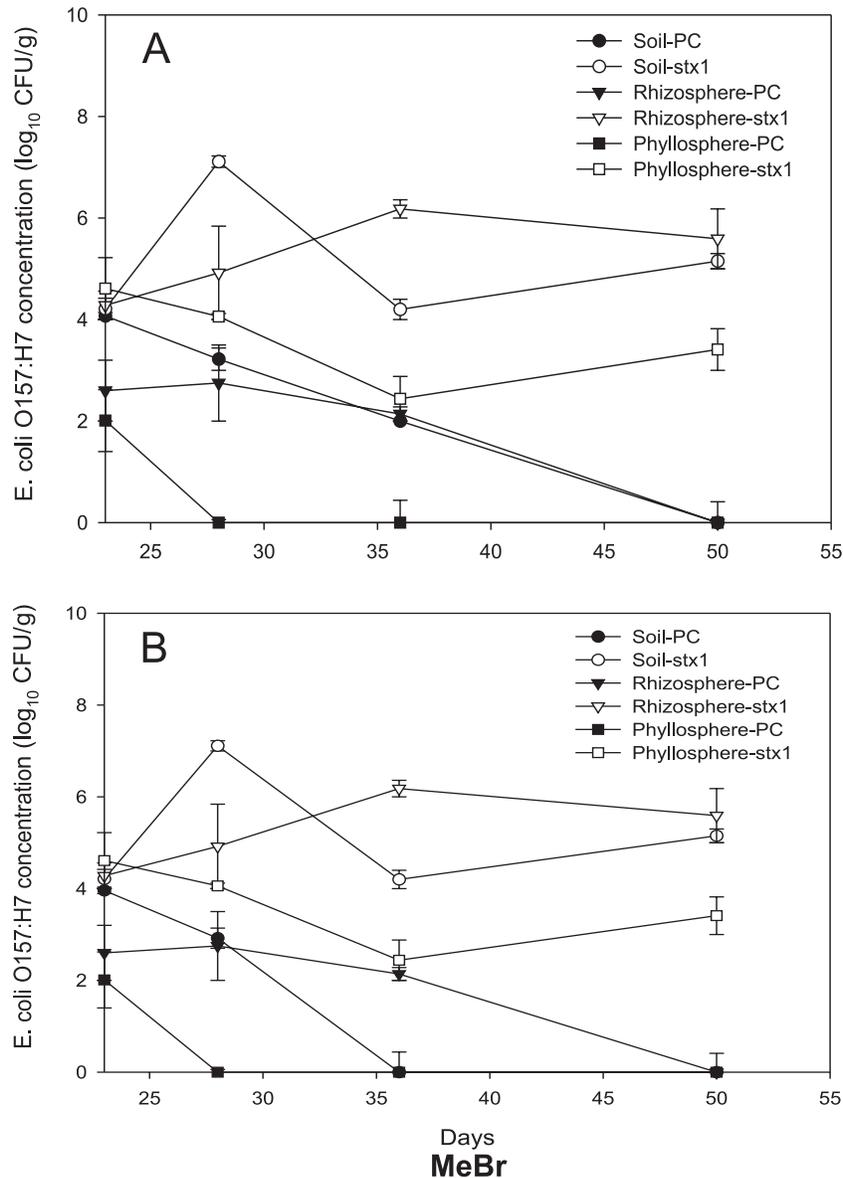


tions survived for a shorter number of days in MeI-treated rhizospheres in sandy soil (40 days) compared with MeBr-treated rhizosphere soil (44 days). Results obtained from plant rhizospheres in both sand and clay soils at a MeI treatment of $0.5\times$ treatment demonstrated a significant pathogen decline by plate count and via real-time PCR quantification of all the genes in both soils, except for *stx1* in clay soil (Table 1). In the nonfumigant treatment control, survival was longer in the sandy soil (48 days) compared with the clay soil (39 days) based on the x intercept on the regression curve with the plate counts. Real-time PCR revealed significant pathogen reductions for the *eae* gene in the sandy soil and for the *stx1* and *eae* genes in the clay soil, with the x intercepts for both genes at 90 and 75 days, respectively (Figs. 3E and 3F, Table 1).

Growth chamber study: leaf surface

In all the treatments where *E. coli* O157:H7 was added to the soil, the concentration of the pathogen recovered on leaf surfaces ranged from $1.5 \log_{10}$ CFU/g of leaf with the plate

Fig. 5. Concentration of *Escherichia coli* O157:H7 on plant surfaces compared with the populations in nonrhizosphere samples. Pesticide concentrations were at the normal agricultural application rate for (A) methyl bromide (MeBr) sandy soil, (B) MeBr clay soil, (C) methyl iodide (MeI) sandy soil, (D) MeI clay soil, (E) nonfumigated sandy soil, and (F) nonfumigated clay soil. Standard errors are from log transformed data of the means.



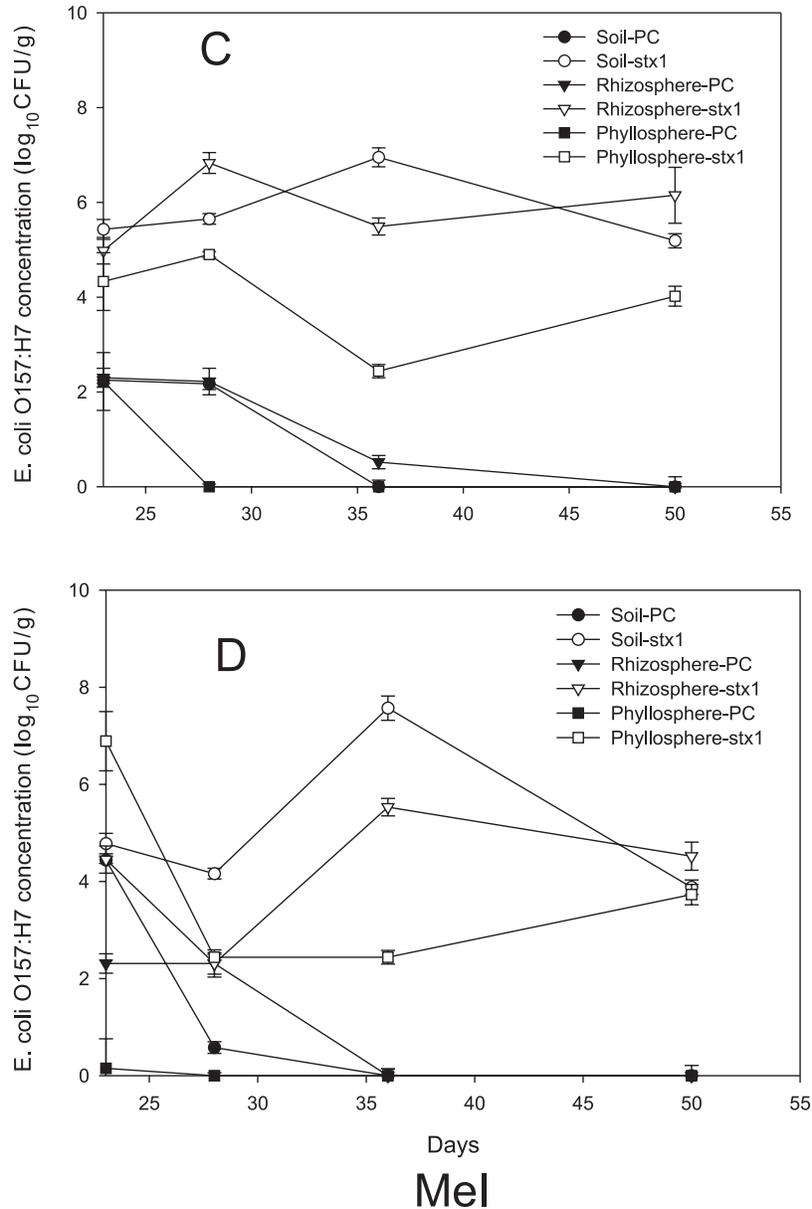
count method to $4.90 \log_{10}$ CFU/g with real-time PCR. The aim was to determine the effect of the presence of soil on the survival of *E. coli* O157:H7 on leaf surfaces with plant growth. *Escherichia coli* O157:H7 survived for 37.3 days on the leaf surface when measured by plate count and twice the number in some cases by real-time PCR (Table 1; Figs. 4A–4F). Thus, the population size of *E. coli* O157:H7 was significantly lower on leaves that were contaminated with soil during the early stages of growth than in the rhizosphere or bulk soil.

Survival of *E. coli* O157:H7 on root and leaf compared with survival in soil after fumigation

The survival of *E. coli* O157:H7 in plant materials (root and leaf) were compared with the survival in soils to deter-

mine if fumigation could reduce the incidence of pathogen contamination of edible plant parts during growth. All analyses were done at the normal application rate of fumigants to agricultural soil using data from plate count and the *stx1* gene as examples. Survival of *E. coli* O157:H7 on leaf surface was significantly lower ($P = 0.001$) in both clay and sandy soils with MeBr at the normal application rate than in soil and rhizosphere samples by the plate count method. By day 28, the plate-count-determined concentration on the leaf surface was reduced to an undetectable number, but survival was reduced to below detection in the soil and roots by day 50 (Figs. 5A and 5B). There were no differences in concentrations of *E. coli* O157:H7 on the root and in the soil during this period. Real-time PCR analysis showed no significant differences at day 23, but thereafter the concen-

Fig. 5 (continued).



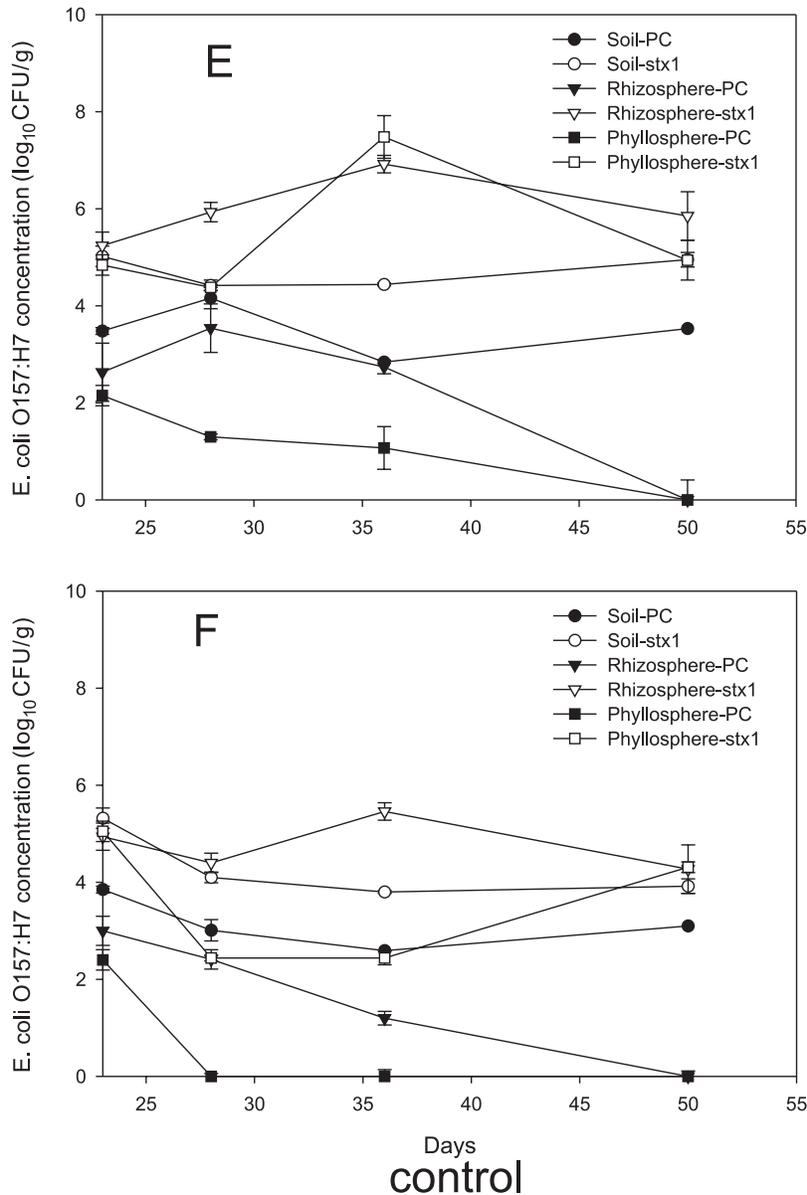
tration of the pathogen was significantly lower on the leaf surface than in either the soil or root (Figs. 5A and 5B). Also, real-time PCR indicated the possibility of bacterial regrowth on the leaf surface in both clay and sandy soils in the growth chamber study. In MeI-fumigated soils, survival of *E. coli* O157:H7 followed the same pattern as seen with MeBr (Figs. 5C and 5D). The pathogen survived longer in the sandy soil than in the clay soil as assessed by the plate count method, and the numbers of the pathogen on the leaf were significantly lower ($P = 0.004$) than those on the root and soil. In both soils, there was regrowth of *E. coli* O157:H7 on leaf surface to almost the same level as seen in both soil and root as determined by real-time PCR (Figs. 5C and 5D). In the control soils without fumigation, one drastic effect was observed. The target bacteria were detected in both soils throughout the study (Figs. 5E and 5F). This resulted in longer survival of the pathogen on the leaf surface,

especially in sandy soil, than was observed in fumigated soils. Real-time PCR analysis of samples indicated the possibility of long term survival in all the soil, root, and leaf samples. Therefore, application of fumigant may play some role(s) in reducing the transfer of *E. coli* O157:H7 from soil to leaf.

Microcosm study

The survival of *E. coli* O157:H7 was studied for 90 days in the microcosm because the relationship between time of survival (days) and population decline could be considered over a longer period than was used for lettuce growth. However, as storage time in the microcosm increased, survival of the pathogen continued throughout the experimental period, and a linear equation was inadequate to describe the relationship. A curvilinear model was used to describe the pathogen's survival pattern over an extended period of time. All

Fig. 5 (concluded).



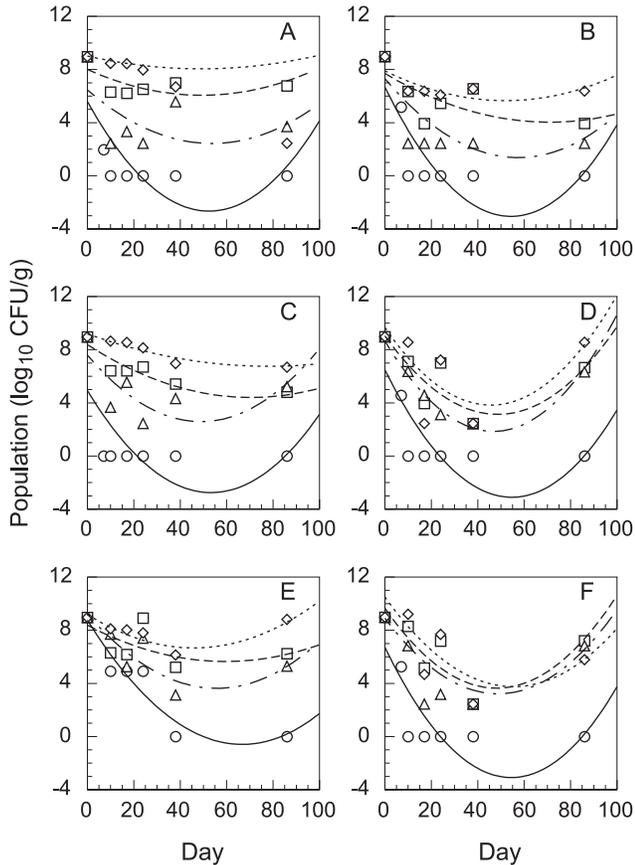
data used for the analysis were log transformed to obtain normal distribution. In the microcosm study, another treatment was added with five times the normal concentration of the two fumigants to determine if the fumigants could completely kill all the *E. coli* in the container after a certain number of days. In this treatment, there was also a significant curvilinear model that described the survival of pathogen (Figs. 6A–6F).

Discussion

Our study has revealed that based on real-time PCR analysis, *E. coli* O157:H7 can survive for extended periods of time in soil even after fumigation. During the microcosm experiment, *E. coli* O157:H7 populations survived for a longer period of time even with the addition of five times the normal application concentration of fumigant. In most of the tested cases, the slope of the second regression line rose

over time (Fig. 6), indicative of the long-term survival in these microcosms with the possibility that populations can increase over time. We also believe that if the plants had remained in the growth chamber soil for such a long period of time, *E. coli* O157:H7 would have survived for a similar extended period of time. Similar results had been found in fallow field plots and microcosm soil inoculated with *Pseudomonas chlororaphis* 3732RN-L11 (Gagliardi et al. 2001) and with *E. coli* O157:H7 (Artz et al. 2006). *Escherichia coli* O157:H7 is considered a casual or transient colonist of leaves compared with *Pseudomonas* and *Erwinia*, which are considered to be true residents of leaves (Leben 1965). Therefore, *E. coli* O157:H7 can grow and survive under a more limited set of environmental conditions, generally in response to wounds (Suslow 2002). It has also been shown that a variety of lettuce types can support the post-harvest multiplication of *E. coli* O157:H7 under conditions of permissive temperature and relative humidity, thus in-

Fig. 6. Curvilinear regression curves showing predictive models for persistence of *Escherichia coli* O157:H7 in microcosm sandy and clay soils during a laboratory experiment. The following regression lines represent data from plate count (○) and real-time polymerase chain reaction with *stx1* (□), *stx2* (△), and *eae* (◇) genes. Pesticide concentrations were at the normal agricultural application rate for (A) methyl bromide (MeBr) sandy soil, (B) MeBr clay soil, (C) methyl iodide (MeI) sandy soil, (D) MeI clay soil, (E) nonfumigated sandy soil, and (F) nonfumigated clay soil. All regression models used log transformed data.



creasing the risk of food-borne illnesses (Abdul Raouf et al. 1993). These authors have also shown the capacity for *E. coli* O157:H7 to multiply in a variety of undamaged or wounded produce. A variety of vegetables and low-acid fruits can support the multiplication of this pathogen at temperatures ranging from 15 to 25 °C, which are typically encountered during handling of produce (Suslow (2002). Our study was conducted inside two growth chambers with a temperature of 20 °C. At this temperature and in the absence of fumigation there was no significant reduction in *E. coli* O157:H7 concentrations in both soils as determined by both plate counting and real-time PCR (Figs. 5E and 5F). Even in the fumigated soils, real-time PCR was able to quantify the pathogen in leafy samples throughout the 50 days study (Figs. 5A–5F). Therefore, one-time fumigation may not be enough to reduce the presence of the pathogen in soil.

The present study verifies that *E. coli* O157:H7 is capable of survival for a long period. We also show that with long-term survival, linear regression alone may not accurately explain survival characteristics. In the microcosm study, our

data show that after a long storage period a curvilinear model provides the best-fitting technique for explaining the extended survival of *E. coli* O157:H7 in soil. This is in agreement with Maule (2000), who reported survival of *E. coli* O157:H7 for 130 days at 18 °C when inoculated into a laboratory-prepared soil and glass microcosm. *Escherichia coli* O157:H7 can also survive for more than 2 months in organic wastes, followed by a significant and gradual decline in bacterial numbers (Avery et al. 2005; Williams et al. 2005). However, in most cases, long-term storage can not be expected to completely eliminate *E. coli* O157:H7 from soil (Avery et al. 2005; Williams et al. 2005). Gagliardi and Karns (2002) showed that *E. coli* O157:H7 can persist for 25–41 days in fallow soils, for 47–92 days on rye roots, and for 92 days on alfalfa roots in a silty loam soil. Presently, *E. coli* O157 survival in microcosm soils indicates an increased risk of the organisms surviving in sufficient numbers to cause infection. Indeed, the pathogen can persist even in soils with a moisture content of less than 3%. This agrees with a study by Maule (1999) who showed that a range of *E. coli* O157 strains could survive in a desiccated state on stainless steel in excess of 60 days.

In contrast to the culture-based assay, *E. coli* O157:H7 DNA was detected by real-time PCR throughout the duration of the study in the soil, rhizosphere, and on the plant leaves. This is not surprising considering the fact that culture-based methods may not detect very low numbers of bacteria. In most studies where culture-based methods are used, enrichment is usually necessary (Gagliardi and Karns 2002; Jiang et al. 2002; Johannessen et al. 2005). Automated immunomagnetic separation in addition to enrichment can enhance the detection of *E. coli* O157:H7 in soils where culture-based methods could not detect the pathogen (Johannessen et al. 2005; Grewal et al. 2006). Significant differences among treatments were also observed when cell numbers were detected by real-time PCR and plate count (Artz et al. 2006). These authors also noted a 10% ± 6% decline in cell numbers detected by real-time PCR in soil close to wilting point compared with a decrease of 38% ± 1% in soil at near field capacity and a loss of 43% ± 7% of the originally added cells in slightly dry soil. They suggested that the differences must be due to the different fates of nonviable cells under different moisture regimes, considering that the potential luminescence per cell increased by only a maximum of one order of magnitude, whereas cell numbers detected by plate counts decreased by four orders of magnitude in all moisture regimes. We agree with these authors that the discrepancies between their and our studies may be partially explained by the translocation of the cells into the smaller soil pores in dry soil (Postma and van Veen 1990). It is likely that smaller pores in dry soils can allow for longer continued existence of nonviable cells that only nonculture method can detect.

Survival of *E. coli* O157:H7 is likely to be affected by a range of factors including solar radiation, desiccation, temperature, and contact with soil (Bolton et al. 1999). Thus, the long-term survival of *E. coli* O157 in these soils has implications for the subsequent transmission to crops and surrounding water bodies, and also to direct infection of humans and animals in contact with the contaminated soil (Maule 2000). Irrigation water provides a vehicle by which

E. coli O157:H7 may enter and become disseminated throughout the agricultural environment, thus posing a potential health risk. In many less developed countries, raw and treated sewage liquids are frequently used for the irrigation of crops (Scott et al. 2000). Some of our fresh produce including lettuce, carrot, cucumber, and spinach are imported from these countries. It is also a common practice for farmers to use washwater from milking cows that has been stored in holding ponds for the irrigation of pastures.

The curvilinear response observed in the microcosm experiment suggests that *E. coli* O157:H7 is capable of being translocated into smaller soil pores in dry soil during the long period that the experiment was conducted. This may increase the chances of survival of the pathogen. Long-term survival of *E. coli* O157:H7 in soil may be a significant factor in its contamination cycle that may result in recontamination of produce, surface water, and groundwater after the initial contamination event.

Acknowledgements

We thank Pamela M. Watt for technical help, Phyllis Nash for statistical help, and Jack Jobes for collecting soil samples from the Santa Ana River bed and Misty Lake. We also thank John Draper for building the irrigation system inside the growth chamber. This research was supported by the 206 Manure and Byproduct Utilization Project of the USDA-ARS. Mention of trademark or proprietary products in this manuscript does not constitute a guarantee or warranty of the property by the USDA and does not imply its approval to the exclusion of other products that may also be suitable.

References

- Abdul Raouf, U.M., Beuchat, L.R., and Ammar, M.S. 1993. Survival of *Escherichia coli* O157:H7 on salad vegetables. *Appl. Environ. Microbiol.* **59**: 1999–2006. PMID:8357240.
- Ackers, M.L., Mahon, B.E., Leahy, E., Goode, B., Damrow, T., Hayes, P.S., et al. 1998. An outbreak of *Escherichia coli* O157:H7 infections associated with leaf lettuce consumption. *J. Infect. Dis.* **177**: 1588–1593. PMID:9607837.
- Angle, J.S., Levin, M.A., Gagliardi, J.V., and McIntosh, M.S. 1995. Validation of microcosms for examining the survival of *Pseudomonas aureofaciens* (*lacZY*) in soil. *Appl. Environ. Microbiol.* **61**: 2835–2839. PMID:16535091.
- Artz, R.R., Avery, L.M., Jones, D.L., and Killham, K. 2006. Potential pitfalls in the quantitative molecular detection of *Escherichia coli* O157:H7 in environmental matrices. *Can. J. Microbiol.* **52**: 482–488. doi:10.1139/W05-149. PMID:16699574.
- Avery, L.M., Killham, K., and Jones, D.L. 2005. Survival of *E. coli* O157:H7 in organic wastes destined for land application. *J. Appl. Microbiol.* **98**: 814–822. doi:10.1111/j.1365-2672.2004.02524.x. PMID:15752326.
- Bellin, T., Pulz, M., Matusset, A., Hempen, H.-G., and Gunzer, F. 2001. Rapid detection of enterohemorrhagic *Escherichia coli* by real-time PCR with fluorescent hybridization probes. *J. Clin. Microbiol.* **39**: 370–374. doi:10.1128/JCM.39.1.370-374.2001.
- Bolton, D.J., Byrne, C.M., Sheridan, J.J., McDowell, D.A., and Blair, I.S. 1999. The survival characteristics of a non-toxicogenic strain of *Escherichia coli* O157:H7. *J. Appl. Microbiol.* **86**: 407–411. doi:10.1046/j.1365-2672.1999.00677.x. PMID:10196745.
- Brandl, M.T., Haxo, A.F., Bates, A.H., and Mandrell, R.E. 2004. Comparison of survival of *Campylobacter jejuni* in the phyllosphere with that in the rhizosphere of spinach and radish plants. *Appl. Environ. Microbiol.* **70**: 1182–1189. doi:10.1128/AEM.70.2.1182-1189.2004. PMID:14766604.
- Buchanan, R.L., and Doyle, M.P. 1997. Foodborne disease significance of *Escherichia coli* O157:H7 and other enterohemorrhagic *E. coli*. *Food Technol.* **51**: 69–76.
- Calderwood, S.B., Auclair, F., Donohue-Rolfe, A., Keusch, G.T., and Mekalanos, J.J. 1987. Nucleotide sequence of the Shiga-like toxin genes of *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* **84**: 4364–4368.
- Centers for Disease Control and Prevention. 1999. Outbreak of *Escherichia coli* O157:H7 and *Campylobacter* among attendees of the Washington county fair-New York. *Morb. Mort. Weekly*, **48**: 803–804.
- Centers for Disease Control and Prevention, National Center for Infectious Diseases, Division of Bacterial and Mycotic Diseases. 2001. *Escherichia coli* O157:H7: general information [online]. Available from www.cdc.gov/ncidod/dbmd/diseaseinfo/escherichiacolipg.htm [accessed 7 September 2003].
- DeFreitas, J.R., and Germida, J.J. 1992. Growth promotion of winter wheat by fluorescent pseudomonads under growth chamber conditions. *Soil Biol. Biochem.* **24**: 1127–1135. doi:10.1016/0038-0717(92)90063-4.
- Dungan, R.S., Ibekwe, A.M., and Yates, S.Y. 2003. Effect of propargyl bromide and 1,3-dichloropropene on microbial communities in an organically amended soil. *FEMS Microbiol. Ecol.* **43**: 75–87.
- Fenlon, D.R., Ogden, I.D., Vinten, A., and Svoboda, I. 2000. The fate of *Escherichia coli* and *E. coli* O157:H7 in cattle slurry after application to land. *J. Appl. Microbiol.* **88** (Suppl.): 149S–156S.
- Fratamico, P.M., Deng, M.Y., Strobaugh, T.P., and Palumbo, S.A. 1997. Construction and characterization of *Escherichia coli* O157:H7 strains expressing firefly luciferase and green fluorescent protein and their use in survival studies. *J. Food Prot.* **60**: 1167–1173.
- Fukushima, H., Hoshima, K., and Gomyoda, M. 1999. Long-term survival of shiga-toxin producing *Escherichia coli* O26, O11, and O157 in bovine feces. *Appl. Environ. Microbiol.* **65**: 5177–5181. PMID:10543842.
- Gagliardi, J.V., and Karns, J.S. 2002. Persistence of *Escherichia coli* O157:H7 in soil and on plant roots. *Environ. Microbiol.* **4**: 89–96. doi:10.1046/j.1462-2920.2002.00273.x. PMID:11972618.
- Gagliardi, J.V., Angle, J.S., Germida, J.J., Wyndham, R.R., Chanway, C.P., Watson, R.J., et al. 2001. Intact soil-core microcosms compared with multi-site field release testing of microbes in diverse soils and climates. *Can. J. Microbiol.* **47**: 237–252. doi:10.1139/cjm-47-3-237. PMID:11315115.
- Grewal, S.K., Rajeev, S., Sreevatsan, S., and Michel, F.C., Jr. 2006. Persistence of *Mycobacterium avium* subsp. *paratuberculosis* and other zoonotic pathogens during simulated composting, manure packing, and liquid storage of dairy manure. *Appl. Environ. Microbiol.* **72**: 565–574. doi:10.1128/AEM.72.1.565-574.2006. PMID:16391093.
- Heid, C.A., Stevens, J., Livak, K.J., and Williams, P.M. 1996. Real-time quantitative PCR. *Genome Res.* **6**: 986–994. PMID:8908518.
- Hilborn, E.D., Mermin, J.H., Mshar, P.A., Hadler, J.L., Voetsch, A., Wojtkunski, C., et al. 1999. A multistate outbreak of *Escherichia coli* O157:H7 infections associated with consumption of mesclun lettuce. *Arch. Intern. Med.* **159**: 1758–1764. doi:10.1001/archinte.159.15.1758. PMID:10448779.

- Hoagland, D.T., and Arnon, D.I. 1950. The water culture methods for growing plants without soil. University of California (Berkeley) Agriculture Experiment Station. Circulation 237 (revised).
- Ibekwe, A.M., and Grieve, C.M. 2003. Detection and quantification of *Escherichia coli* O157:H7 in environmental samples by real-time PCR. *J. Appl. Microbiol.* **94**: 421–431. doi:10.1046/j.1365-2672.2003.01848.x. PMID:12588551.
- Ibekwe, A.M., and Grieve, C.M. 2004. Changes in developing plant microbial community structure as affected by contaminated water. *FEMS Microbiol. Ecol.* **48**: 239–248.
- Ibekwe, A.M., Papiernick, S.K., Gan, J., Yates, S.R., Yang, C.-H., and Crowley, D.E. 2001. Impact of fumigants on soil microbial communities. *Appl. Environ. Microbiol.* **67**: 3245–3257. doi:10.1128/AEM.67.7.3245-3257.2001. PMID:11425748.
- Ibekwe, A.M., Watt, P.M., Grieve, C.M., Sharma, V.K., and Lyons, S.R. 2002. Multiplex fluorogenic real-time PCR for detection and quantification of *Escherichia coli* O157:H7 in dairy wastewater wetlands. *Appl. Environ. Microbiol.* **68**: 4853–4862. doi:10.1128/AEM.68.10.4853-4862.2002. PMID:12324331.
- Ibekwe, A.M., Watt, P.M., Shouse, P.J., and Grieve, C.M. 2004. Fate of *Escherichia coli* O157:H7 in irrigation water on soils and plants as validated by culture method and real-time PCR. *Can. J. Microbiol.* **50**: 1007–1014. doi:10.1139/w04-097. PMID:15714231.
- Islam, M., Doyle, M.P., Phatak, S.C., Millner, P., and Jiang, X.P. 2004. Persistence of enterohemorrhagic *Escherichia coli* O157:H7 in soil and on leaf lettuce and parsley grown in fields treated with contaminated manure composts or irrigation water. *J. Food Prot.* **67**: 1365–1370. PMID:15270487.
- Jiang, X., Morgan, J., and Doyle, M.P. 2002. Fate of *Escherichia coli* O157:H7 in manure-amended soil. *Appl. Environ. Microbiol.* **68**: 2605–2609. doi:10.1128/AEM.68.5.2605-2609.2002. PMID:11976144.
- Johannessen, G.S., Bengtsson, G.B., Heier, B.T., Bredholt, S., Wasetson, Y., and Rørvik, L.M. 2005. Potential uptake of *Escherichia coli* O157:H7 from organic manure into crisphead lettuce. *Appl. Environ. Microbiol.* **71**: 2221–2225. doi:10.1128/AEM.71.5.2221-2225.2005. PMID:15870303.
- Kluepfel, D.A., and Tonkyn, D.W. 1992. The ecology of genetically altered bacteria. In *Biological control of plant diseases*. Edited by E.S. Tjamos, G.C. Papavizas, and R.J. Cook. Plenum Press, New York. pp. 407–413.
- Kudva, I.T., Blanch, K., and Hovde, C.J. 1998. Analysis of *Escherichia coli* O157:H7 survival in ovine or bovine manure and manure slurry. *Appl. Environ. Microbiol.* **64**: 3166–3174. PMID:9726855.
- Leben, C. 1965. Epiphytic microorganisms in relation to plant diseases. *Annu. Rev. Phytopathol.* **3**: 209–230. doi:10.1146/annurev.py.03.090165.001233.
- Maule, A. 1999. Environmental aspects of *E. coli* O157. *Int. food. Hyg. Med.* **9**: 21–23.
- Maule, A. 2000. Survival of verocytotoxigenic *Escherichia coli* O157:H7 in soil, water and on surfaces. *J. Appl. Microbiol.* **88** (Suppl.): 71S–78S.
- Oberst, R.D., Hays, M.P., Bohra, L.K., Phebus, R.K., Yamashiro, C.T., Paszko-Kolva, C., et al. 1998. PCR-based DNA amplification and presumptive detection of *Escherichia coli* O157:H7 with internal fluorogenic probe and the 5' nuclease (TaqMan) assay. *Appl. Environ. Microbiol.* **64**: 3389–3396. PMID:9726887.
- O'Conner, D.R. 2002. Report of the Walkerton inquiry. The events of May 2000 and related issues [online]. www.walkertoninquiry.com. Queen's printer for Ontario.
- Pell, A.N. 1997. Manure and microbes: Public and animal health problem? *J. Dairy Sci.* **80**: 2673–2681. PMID:9361239.
- Postma, J., and van Veen, J.A. 1990. Habitable pore space and survival of *Rhizobium leguminosarum* biovar *trifolii* introduced into soil. *Microb. Ecol.* **19**: 149–161. doi:10.1007/BF02012096.
- SAS Institute Inc. 1991. SAS/STAT User's Guide, Release 6.03. Cary, N.C.
- Scott, C.A., Zarazua, J.A., and Levine, G. 2000. Urban wastewater reuse for crop production in the water-short Guanajuato River Basin, Mexico. International Water Management Institute Research Report 41. IWMI, Sri Lanka.
- Sharma, V.K. 2002. Detection and quantitation of enterohemorrhagic *Escherichia coli* O157, O111, and O26 in beef and bovine feces by real-time polymerase chain reaction. *J. Food Prot.* **65**: 1371–1380. PMID:12233845.
- Sharma, V.K., Dean-Nystrom, E.A., and Casey, T.A. 1999. Semi-automated fluorogenic PCR assays (TaqMan) for rapid detection of *Escherichia coli* O157:H7 and other Shiga toxinogenic *E. coli*. *Mol. Cell. Probes*, **13**: 291–302.
- Solomon, E.B., Yaron, S., and Matthews, K.R. 2002a. Transmission of *Escherichia coli* O157:H7 from contaminated manure and irrigation water to lettuce plant tissue and its subsequent internalization. *Appl. Environ. Microbiol.* **68**: 397–400. doi:10.1128/AEM.68.1.397-400.2002. PMID:11772650.
- Solomon, E.B., Potenski, C.J., and Matthews, K.R. 2002b. Effects of irrigation methods on transmission to and persistence of *Escherichia coli* O157:H7 on lettuce. *J. Food Prot.* **65**: 673–676. PMID:11952218.
- Steel, R.G.D., Torrie, J.H., and Dickey, D.A. 1997. Principles and procedures of statistics: a biometrical approach. McGraw Hill, New York.
- Suslow, T.V. 2002. Production practices affecting the potential for persistent contamination of plants by microbial foodborne pathogens. In *Phyllosphere microbiology*. Edited by S.E. Lindow, E.I. Hecht-Poinar, and V.J. Elliott. The American Phytopathological Society, St. Paul, Minnesota. pp. 241–256.
- Wang, G., Zhao, T., and Doyle, M.P. 1996. Fate of enterohemorrhagic *Escherichia coli* O157:H7 in bovine feces. *Appl. Environ. Microbiol.* **62**: 2567–2570. PMID:8779595.
- Williams, A.P., Avery, L.M., Killham, K., and Jones, D.L. 2005. Persistence of *Escherichia coli* O157 on farm surfaces under different environmental conditions. *J. Appl. Microbiol.* **98**: 1075–1083. doi:10.1111/j.1365-2672.2004.02530.x. PMID:15836476.