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Research Article

Quantification of Survival of *Escherichia coli* O157:H7 on Plants Affected by Contaminated Irrigation Water*

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Enterohemorrhagic *E. coli* O157: H7 (EHEC) is a major foodborne pathogen capable of causing diarrhea and vomiting, with further complications such as hemolytic-uremic syndrome (HUS). The aim of this study was to use the real-time PCR method to quantify the survival of *Escherichia coli* O157:H7/pGFP in phyllosphere (leaf surface), rhizosphere (volume of soil tightly held by plant roots), and non-rhizosphere soils (sand and clay) irrigated with contaminated water and compare the results obtained between real-time PCR method and conventional plate counts. The real-time PCR probe was designed to hybridize with the (*eae*) gene of *E. coli* O157:H7. The probe was incorporated into real-time PCR containing DNA extracted from the phyllosphere, rhizosphere, and non-rhizosphere soils irrigated with water artificially contaminated with *E. coli* O157:H7. The detection limit for *E. coli* O157:H7 quantification by real-time PCR was 2.3×10^3 in the rhizosphere and phyllosphere samples. *E. coli* O157:H7 survived longer in rhizosphere soil than the non-rhizosphere soil. The concentration of *E. coli* O157:H7/pGFP in rhizosphere soils was $\geq 10^4$ CFU/g in both soils at day 12 based on both plate count and real time PCR, with the clay soil significantly ($P = 0.05$) higher than the sandy soil. This data showed that *E. coli* O157:H7 can persist in the environment for more than 50 d, and this may pose some risk for both animal and human infection and provides a very significant pathway for pathogen recontamination in the environment.

Keywords: Bacteria, Contaminated water, PCR

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1 Introduction

Escherichia coli O157:H7 causes a wide spectrum of diseases in humans, ranging from mild to bloody diarrhea, hemorrhagic colitis, and complications including hemolytic uremic syndrome (HUS) and seizures that are particularly severe in children [1]. The life-threatening complication of HUS can result in acute kidney failure. It has been estimated that approximately 25,000 cases of foodborne illness are attributed to *E. coli* O157:H7 each year with an estimated 6 deaths [2]. *E. coli* O157:H7 outbreaks have been associated with ground beef,

raw milk, lettuce, and minimally processed fresh fruit juices [3,4]. Recently Solomon et al. [5,6] demonstrated the transmission of *E. coli* O157:H7 from manure-contaminated soil and irrigation water to lettuce plants using laser scanning confocal microscopy, epifluorescence microscopy and recovery of viable cells from the inner tissues of plants. They attributed the presence of *E. coli* O157:H7 in the edible portion of the plant to the direct migration through the conducting tissues of the root system.

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 [7]. *E. coli* O157:H7 can survive for extended periods of time in water and in soil, and under dry and acidic conditions [8]. One of the most common vehicles by which *E. coli* O157:H7 may be introduced into crops is by flood irrigation with water contaminated with cattle feces or by contaminated surface runoff [9]. Water contamination is becoming more common in rural areas of the United States, with up to 40% of tested drinking

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water wells contaminated with *E. coli* [10]. In Walkerton, Ontario, more than 1000 people fell ill and five died of *E. coli* O157:H7 infections following a storm [9]. Intensification of regional livestock enterprises was named as the likely cause of drinking water wells' contamination.

The recent availability of new technologies such as real-time PCR [11] has greatly aided the study of pathogens such as *E. coli* O157:H7 in the environment [12]. It has been used in studies for the detection and relative quantification of *E. coli* O157:H7 in food and clinical samples [13,14]. The recently described quantification of natural *E. coli* O157:H7 in soil, cattle feces, manure, waste water, rhizosphere and phyllosphere has used multiplex real-time PCR [15–17]. Little research has been done on the quantification of this pathogen in the rhizosphere and phyllosphere of plants. In this study, we used primers and probes specific for the intimin gene that have been developed to quantify *E. coli* O157:H7 in soil and fecal samples by real-time PCR [15]. Both plate count and real-time PCR approaches were applied to determine the survival of *E. coli* O157:H7 in the two soils and plant tissues. Our data from this study, therefore, points out the importance of using both PCR techniques and conventional methods in understanding pathogen survival in the environment.

The purpose of this study was to determine the survival of *E. coli* O157:H7 from contaminated irrigation water to the rhizosphere and phyllosphere of lettuce grown under flood irrigation.

2 Materials and Methods

2.1 Bacterial Strain, Growth Conditions, and Inoculum Preparation

E. coli O157:H7 strain 34 with green fluorescent protein (pGFP) was used for this study. Plasmid construction of the strain has previously been described [18]. *E. coli* O157:H7/pGFP was cultured at 37 °C overnight in modified Tryptic Soy broth (mTSB) (Difco Laboratories Inc., Cockeysville, MD) supplemented with 50 µg of ampicillin/mL (Sigma, St. Louis, MO). The cells were harvested by centrifugation at 3500 × g for 10 min and resuspended in PBS (Fisher Scientific, Pittsburgh, PA) to a concentration of ~10⁸ CFU/mL.

2.2 Irrigation of Plants, and Recovery of *E. coli* O157:H7/pGFP

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 clay soil had a bulk density of 1.51 mg/m³ and 3.7% sand, 49.1% silt, and 47.2% clay. The sandy soil had a bulk density of 1.67 mg/m³ and 99.1% sand, 0.20% silt, and 0.70% clay. The soils were sieved through a 4 mm sieve before planting, and the high salt content (EC = 15 ds/m) in the clay soil was reclaimed as described by Ibekwe and Grieve [19]. Seeds of green romaine lettuce (*Lactuca sativa* (L.) cv. Green Forest) were purchased from Johnny's Selected Seed Co. (Albion, ME). 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. Soils were tested to make sure that they are *Escherichia coli* O157:H7 negative.

Lettuce seedlings were sprouted in 50% Hoagland's solution [20] and transplanted into the soils in two growth chambers. The experiment was randomized with three replications. There were ten plants in each tray at transplanting and one plant was harvested from each tray during analysis as stated below. The first irrigation with contaminated water occurred at transplanting (day 1) for both soils and the second contamination event occurred 15 days later. Irrigation solutions were prepared in 1000-L reservoirs and pumped to provide irrigation to clay and sandy soil in polypropylene trays. The soils were irrigated with approx. 4.2 × 10⁸ *E. coli* O157:H7. Bacteria were inoculated into the irrigation lines with a Cole-Parmer HPLC pump (Cole-Parmer, Chicago, Illinois) and delivered through PVC pipes to each tray with five surface drip lines.

Soil samples were collected before and after inoculation for community analysis. After irrigation, soil samples were taken immediately for *E. coli* O157:H7(pGFP) concentrations, heterotrophic plate counts and total bacterial DNA. Cell concentrations at the surface of the soils (10 cm) immediately after inoculation were approx. 1.4 × 10⁶ by plate count and 2.3 × 10⁸ by real time PCR. Plants in the clay soil were irrigated with distilled water daily and received the nutrient solution weekly. The plants in the sandy soil received the above nutrient solution daily. Overflow irrigation solution was returned to the stainless steel containers, and by gravity flow, to a different reservoir that was subsequently decontaminated. After each contamination event, the irrigation system was completely decontaminated before use for the daily irrigation application. Microbiological analysis was performed to make sure that the irrigation system was truly decontaminated.

The effects of the native microflora on the survival of *E. coli* O157:H7 in clay and sandy soils was first determined in the two soils used for this study. This was done to determine the influence that indigenous microorganism may have on the survival of *E. coli* O157:H7 in both soils. Soil, rhizosphere and phyllosphere samples were harvested 3, 5, 9, 12, 15, 18, 25, 29, and 45 days after transplantation. Phyllosphere, rhizosphere and non-rhizosphere soils were collected in separate sterile Petri dishes or collection bags. Bacteria were recovered from the plant material by homogenization with 100 mL of PBS for 2 min at 260 rpm in a Seward Stomacher 400 Circulator (Seward Ltd., London, UK). The homogenate was centrifuged at 3000 × g for 10 min, the pellet was resuspended in 2 mL of PBS, plated on mTSA with ampicillin and incubated at 37 °C overnight. *E. coli* O157:H7 colonies were enumerated under a hand-held Spectroline ultra-violet lamp (Spectronics Corporation, Westbury, N.Y.). Concentrated samples were used for isolation of the genomic DNA used for quantification of *E. coli* O157:H7 by real-time PCR.

2.3 DNA Extraction and Real-Time PCR

Genomic DNA was isolated from a pure culture of *E. coli* O157:H7 and used for the construction of standard curves and for the determination of detection limits of *E. coli* O157:H7 by

real-time PCR. *E. coli* O157:H7 was grown for 8 h at 37 °C and DNA extracted with the Qiagen tissue kit (QIAamp DNA Mini Kit; Valencia, CA). Total bacterial DNA was extracted from plant samples with the Ultra Clean Soil DNA Kit (MoBio Laboratories, Solana Beach, CA) and stored at –20 °C. Primers and probe used for the detection and quantification of the *eae* gene in *E. coli* O157:H7 was as described by Ibekwe et al. [15]. The primers and probe sequences are as follow: *eae*-F GTA AGT TAC ACT ATA AAA GCA CCG TCG A; *eae*-R TCT GTG TGG ATG GTA ATA AAT TTT TG; and *eae*-Probe AAA TGG ACA TAG CAT CAG CAT AAT AGG CTT GCT. Fluorescent reporter dye, Texas Red (Sulforhodamine 101), was conjugated at the 5' end of the probe, and quencher dye, Black Hole Quencher (BHQ) II, at the 3' end (Black Hole Quencher dyes, Biosearch Technologies, Novato, CA). Real-time, quantitative PCR was performed with the iCycler iQ Real-Time PCR Detection System (BIO-RAD, Hercules, CA). The iCycler iQ Real Time PCR Detection System software was used for data analyses.

Standard curves generated from plotting the threshold cycle (C_T) versus $\log_{10}$ of starting DNA quantities (pg) were used for determining the detection limit of the assay. The standard curve was constructed from known quantities of genomic DNA extracted from *E. coli* O157: H7. The concentrations (CFU/mL) of *E. coli* O157:H7 present in unknown samples was determined from the standard curve. The slopes of the standard curves were calculated by performing linear regression analysis within the iCycler iQ software to compare the PCR amplification efficiency and detection sensitivity among different experiments. Amplification efficiency (E) was estimated by using the slope of the standard curve and the formula:

$$E = (10^{-1/\text{slope}}) - 1 \quad (1)$$

Reaction with 100 % efficiency generated a slope of –3.32. *E. coli* O157:H7/pGFP concentrations were converted to log CFU/g for regression analysis. Comparisons between pairs of treatment means at any date were accomplished with the Tukey's test. All calculations were performed using the general linear model (GLM) procedure of the Statistical Analysis System [21].

3 Results

3.1 Quantification of Heterotrophic Bacteria by Selective Media

The initial heterotrophic plate count in soil was 2.1×10^8 CFU/g. After storage at 20 °C in the growth chamber, the total aerobic plate counts decreased steadily from approx. 10^8 to approx. 10^6 CFU/g $\pm 10^2$ during the experimental period. There were no differences in the levels of background bacteria as determined by the heterotrophic plate count in the phyllosphere and rhizosphere soil during the first 12 d of the study (data not shown). The phyllosphere consistently showed lower numbers (10^4 to 10^5), while the rhizosphere popu-

lation was higher ($\geq 10^6$). The CFU/g values from the phyllosphere, rhizosphere, and non-rhizosphere soil samples were similar in both the sand and clay soils. This trend was also observed following the second contamination event where the numbers of heterotrophic plate counts followed the same pattern as observed from the first 12 days.

3.2 Real-Time PCR Analysis

The sensitivity of the Texas Red-labeled probe to specifically detect and quantify the *eae* gene was determined by plotting the log DNA starting quantities of *E. coli* O157:H7/pGFP. The mean C_T values were between 21 and 23 when 5 to 10 pg DNA/mL was used as a template. The detection sensitivity of the quantitative real-time PCR was determined to be between 10^3 to 10^8 CFU/mL of *E. coli* pGFP (see Fig. 1). The concentrations of *E. coli* O157:H7 in plant and soil samples were calculated from this standard curve. Based on this approach, a correlation was observed between the C_T and the CFU/mL of the starting quantity of *E. coli* O157:H7 DNA, with a detection limit of 2.3×10^3 CFU/mL. The efficiency of each assay, calculated from the slope of each standard curve, was above 99.9 %, with correlation coefficients of about 0.99 for each curve. Figs. 2, 3, and 4 show the quantification of *E. coli* O157:H7 detected over several weeks in the non-rhizosphere soil, rhizosphere, and phyllosphere samples by real-time PCR and plate count.

3.3 Survival of *E. coli* O157:H7 in the Soil, Rhizosphere and Phyllosphere

In this study, a higher concentration of pathogens was observed in the non-rhizosphere clay soil than in the sandy soil by both plate count and real time PCR methods (see Fig. 2A)

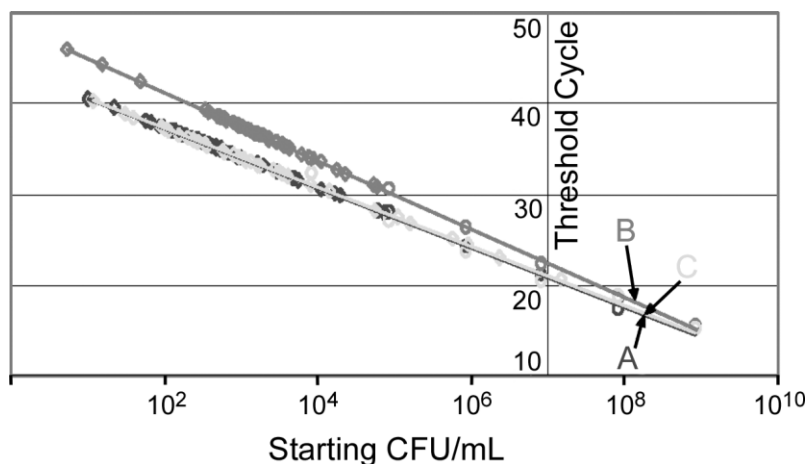


Figure 1. Standard curves for the real-time, quantitative PCR analysis of *E. coli* O157:H7 strain 34 pGFP. C_T values of known (○) and unknown (◇) samples were plotted against the corresponding CFU/mL of genomic DNA. The concentrations of *E. coli* O157:H7 were determined in plant and soil samples from 3 assays: (A) $r = 0.992$, slope = –3.314, $Y = -3.29X + 20.96$; (B) $r = 0.998$, slope = –3.779, $Y = -3.779X + 22.367$; (C) $r = 0.957$, slope = –3.234, $Y = -3.234X + 21.148$.

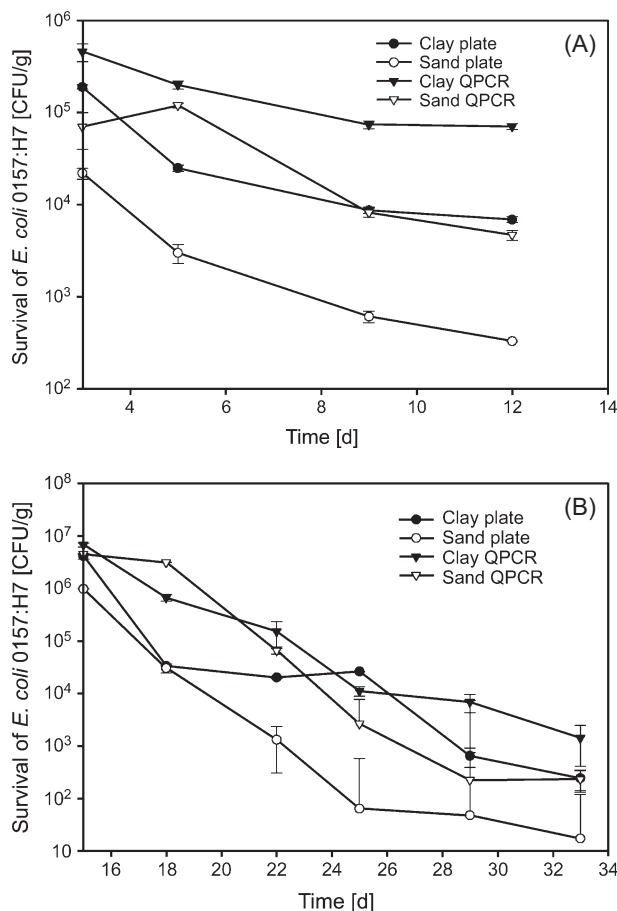


Figure 2. Quantification of *E. coli* O157:H7 in the non-rhizosphere soil after (A) 12 d and (B) from day 15 to 45. *E. coli* O157:H7 was enumerated from clay and sandy soil by plate count, from clay and sandy soil by real-time PCR.

during the first contamination event. This trend continued thirty days following the second contamination event (see Fig. 2B) with very little variations. While the decrease in the population of culturable *E. coli* O157:H7 cells on non-rhizosphere soil was apparent, the *E. coli* O157:H7 population decline rate constant for the two soils were insignificant ($r^2 = 0.35$ and $P = 0.42$).

The pathogen survived longer in rhizosphere soil than the non-rhizosphere soil. The concentration of *E. coli* O157:H7/pGFP in rhizosphere soils decreased from the original value of $\geq 10^6$ to $\geq 10^4$ CFU/g in both soils at day 12 based on both plate count and real time PCR (see Fig. 3A), with the clay soil significantly higher than the sandy soil. However, the trend in survival after the second contamination event (day 15) was different from the first contamination event (see Fig. 3B). Furthermore, the *E. coli* O157:H7/pGFP population decline rate constant was significantly different on both the clay and sandy soils ($r^2 = 0.99$ and $P = 0.0001$).

The concentrations of *E. coli* O157:H7 in the rhizosphere soils obtained by real-time PCR (see Fig. 3A and B) were very close to the numbers obtained by the traditional culture meth-

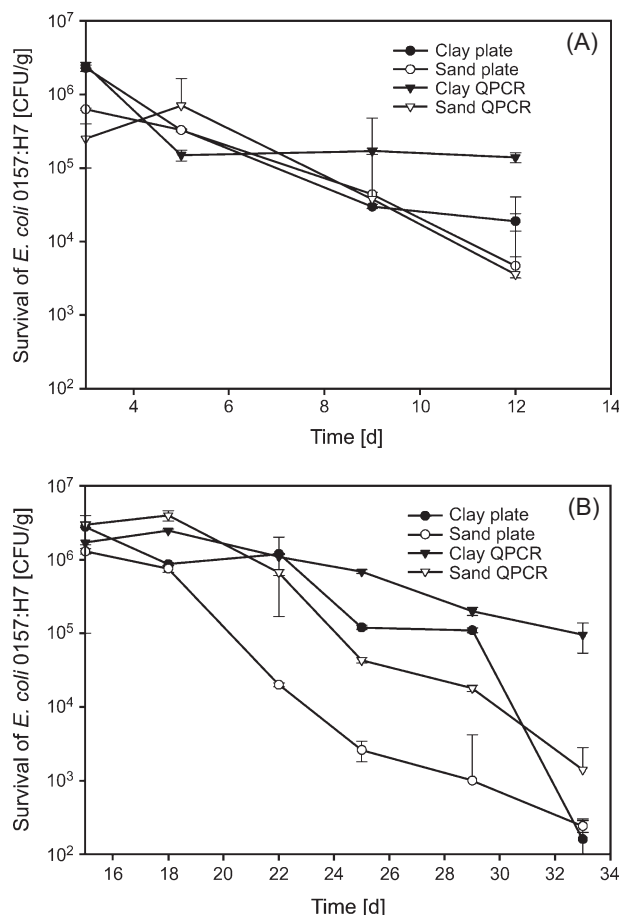


Figure 3. Quantification of *E. coli* O157:H7 in the rhizosphere after (A) 12 d and (B) from day 15 to 45. *E. coli* O157:H7 was enumerated from clay soil and sandy soil by plate count and from clay and sandy soil by real-time PCR.

ods on mTSA during the first three to five days of the first contamination event (see Fig. 3A), and days 15 to 18 of the second contamination event (see Fig. 3B). After this point, except in a few instances, the concentration by real-time PCR was higher by 1 to 3 logs. This was observed in clay soil on day 9, sand rhizosphere day 9, and 15. Our study shows that pGFP is highly stable for a few days, but it may not be a good marker for monitoring long-term survival of bacteria due to the degradation of the pGFP. The higher concentrations of bacteria determined by real-time PCR versus plate count, 9 days after inoculation in some instances, confirmed this observation. Real-time PCR also had the advantage of higher throughput, reproducibility, and less time required to screen a large volume of samples.

The survival of *E. coli* O157:H7/pGFP in the rhizosphere was 2 logs higher than on leaf surfaces. *E. coli* O157:H7 populations in the phyllosphere samples after the first contamination event decreased an average > 3 logs between day 3 and 12 in the clay soil and < 4 logs for the sandy soil (see Fig. 4A) by both plate count and real time PCR. On the average, about 1.7×10^2 CFU of *E. coli* O157:H7/g was recovered from lettuce

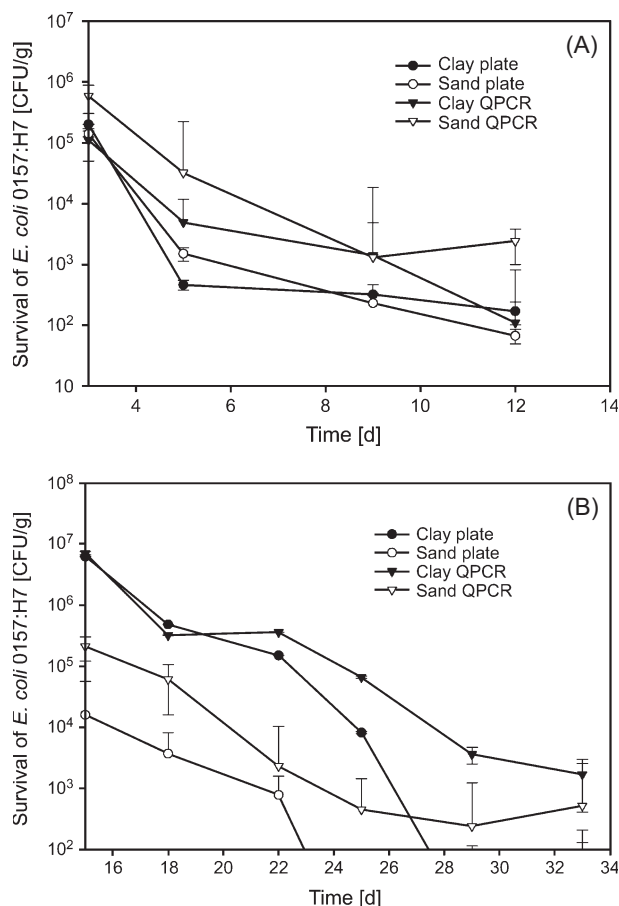


Figure 4. Quantification of *E. coli* O157:H7 in the phyllosphere after (A) 12 d and (B) from day 15 to 45. *E. coli* O157:H7 was enumerated from clay and sandy soil by plate count, from clay and sandy soil by real-time PCR.

phyllosphere grown on clay soil and about 0.67×10^2 CFU of *E. coli* O157:H7/g was recovered from lettuce phyllosphere grown on sandy soil after the first 12 days by plate count. Following the second contamination event, the concentration of the pathogen in the phyllosphere of both soils after 45 days dropped below the detection limit by plate count in both clay and sandy soils and $\leq 10^3$ CFU/g (compare Fig. 4B) by real time PCR. Throughout the 45-day study, the *E. coli* O157:H7 population decline was significantly linear in the phyllosphere with the plate count but insignificant with real time PCR technique. It is interesting to note that although the same number of *E. coli* O157:H7 was inoculated in both soils, the clay soil showed a 1.5 log higher survival after the second inoculation.

4 Discussion

This study revealed that *E. coli* O157:H7 can survive for extended periods of time in soil contaminated with the pathogen. Maule [22] reported the survival of *E. coli* O157:H7 for 130 days at 18 °C when inoculated into a laboratory-prepared soil and grass microcosm. *E. coli* O157:H7 was also able to

persist for more than 2 months in 21 of the 27 wastes tested [23, 24]. These authors concluded that long-term storage of organic waste led to a significant and gradual decline in *E. coli* O157:H7 numbers. However, in most cases, long-term storage cannot be expected to completely eliminate *E. coli* O157:H7 from soil. Gagliardi and Karns [25] confirmed the above observations in fallow soils where *E. coli* O157:H7 persisted for 25–41 days on rye roots, 47–92 days; and on alfalfa roots, in a silty loam soil, for 92 days.

This study was conducted in a controlled environment with a constant temperature (20 °C), photoperiod (12 h), and relative humidity (70 %) to minimize the influence of non-related soil variables on the survival and growth of *E. coli* O157:H7. The antagonistic effect of indigenous soil microorganisms may most likely play a significant role in the survival of *E. coli* O157:H7 in the non-rhizosphere soils as well as in the interaction of pathogen with clay particle which can influence the detection of the pathogen. Thus *E. coli* O157:H7/pGFP survived best and remained culturable for a longer period of time in the rhizosphere. This agreed with a recent report by Gagliardi and Karns [26], where *E. coli* O157:H7 persistence was enhanced in the rhizosphere of rye and legumes grown in clay soil. There was also suggested that the rhizosphere and soil types had significant influences on the persistence of *E. coli* O157:H7 in plants. The type of soil may also have a stronger influence on the persistence of the pathogen after many weeks compared to the first few days after the initial contamination event.

The leaf surface of any plant is a very harsh environment for bacterial growth, and the survival may be limited by competition from other epiphytic microorganisms and nutrient availability. The number of *E. coli* O157:H7 on leaves in this study may be due to cross contamination between the phyllosphere and soil irrigated with contaminated water during the early stages of growth, as pathogens may reside and survive between the leaves and the stems of the plants [27]. It may also be through direct transmission from the roots through the veins to the leaves as previously reported by Solomon et al [5, 6]. We did not examine this process microscopically as previously described by Solomon et al. [5, 6], since our objective was to determine the persistence of pathogen on the three matrices.

Previous work [15] showed the *eae* gene to be a much conserved marker for distinguishing *E. coli* O157:H7 from other serotypes of *E. coli* after analyzing 33 Shiga toxigenic *E. coli* (STEC) and non-STEC *E. coli* strains. Successful quantification of *E. coli* O157:H7 in soils, manure, and fecal samples in previous studies [15, 16] led to the evaluation of this pathogen in the rhizosphere and on leaf surfaces. These matrices are known to harbor different microbial communities [28, 29].

The ability to quantify *E. coli* O157:H7 in fresh produced and other food matrices without using culture methods will be very helpful for developing risk assessment models. Currently, most data demonstrating the risk of *E. coli* O157:H7 in food are dependent on culture techniques [30]. For example, these authors used a quantitative risk assessment of *E. coli* O157:H7 in apple cider with data from plate counts from scientific journals to develop probability distribution functions. Other investigators have looked at the detection of *E. coli* O157:H7 in food samples [31, 32]. The authors of this study introduced a new method, converting a fluorescent signal into target cell densi-

ties related directly to cell densities in the phyllosphere, rhizosphere, and non-rhizosphere soil samples by using a standard curve. This study suggests that *E. coli* O157:H7 has the capability to exploit the nutrient resources on leaves under conditions in which the physical environment does not limit their activities, and therefore can survive as part of the community. This observation was confirmed in a related study by Ibekwe and Grieve [19], which showed that microbial community development in lettuce, took about 7 to 12 days and that this may be the most likely period for maximum pathogen contamination in plants.

Real-time PCR is able to detect the pathogen, where culture-based methods could not eliminate the need for post incubation enrichment, unless it is for the purpose of identifying isolates. Significant differences among treatments were also observed when the cell numbers were detected by real-time PCR analysis and plate count [32]. These authors also noted a $10\% \pm 6\%$ decline in cell numbers detected by real-time PCR in soil close to the wilting point compared to a decrease of $38\% \pm 1\%$ in soil at near field capacity and a loss of $43\% \pm 7\%$ of the originally added cells in slightly dry soil. They suggested that the differences must be due to the different fate 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 agreed with these authors that the discrepancies observed in their study and our study may be partially explained by the translocation of the cells into the smaller soil pores in dry soil. It is therefore likely that smaller pores in dry soils can allow for longer continued existence of nonviable cells that only non-culture method can detect.

Our procedure is in contrast to other studies of *E. coli* O157:H7, where presumptive detection by TaqMan PCR was used to estimate the population size of *E. coli* O157:H7 [12, 13]. The strategy used in this study has also recently been applied to the detection and quantification of *E. coli* O157, O111, and O26 in beef and bovine feces [33]. The automated PCR amplification and detection of target gene amplicons described in this study is conducive for screening large numbers of samples in a single assay. The real-time PCR can be a useful method for processing plants to monitor the contamination of fresh produce for risk analysis before sending it to the consumers.

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