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ORIGINAL ARTICLE

Persistence of *Escherichia coli* O157:H7 on the rhizosphere and phyllosphere of lettuce

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Abstract

Aims: The major objective of this study was to determine the effects of low levels of *Escherichia coli* O157:H7 contamination on plant by monitoring the survival of the pathogen on the rhizosphere and leaf surfaces of lettuce during the growth process.

Methods and Results: Real-time PCR and plate counts were used to quantify the survival of *E. coli* O157:H7 in the rhizosphere and leaf surfaces after planting. Real-time PCR assays were designed to amplify the *stx*1, *stx*2 and the *eae* genes of *E. coli* O157:H7. The detection limit for *E. coli* O157:H7 quantification by real-time PCR was $2{\cdot}4 \times 10^3$ CFU g^{-1} of starting DNA in rhizosphere and phyllosphere samples and about 10^2 CFU g^{-1} by plate count. The time for pathogens to reach detection limits on the leaf surface by plate counts was 7 days after planting in comparison with 21 days in the rhizosphere. However, real-time PCR continued to detect *stx*1, *stx*2 and the *eae* genes throughout the experimental period.

Conclusion: *Escherichia coli* O157:H7 survived throughout the growth period as was determined by real-time PCR and by subsequent enrichment and immunomagnetic separation of edible part of plants.

Significance and impact of the Study: The potential presence of human pathogens in vegetables grown in soils contaminated with *E. coli* O157:H7 is a serious problem to our national food supply as the pathogen may survive on the leaf surface as they come in contact with contaminated soil during germination.

Introduction

Appropriate management of farm waste is critical in controlling the spread of pathogens such as *E. coli* O157:H7 to fresh produce. Soil fumigation is a common practice used for baby greens production in Salinas valley of California (Platts 2006). Soil fumigation reduces the survival of many soil pathogens, but very little has been done on the survival of *E. coli* O157:H7 in soil after fumigation. After fumigation, soils are commonly irrigated before planting, and contaminated irrigation water may be one of the main sources of *E. coli* O157:H7 outbreaks linked to the consumption of leafy vegetables. *Escherichia coli* O157:H7 is of particular concern to public health officials, because ingestion of relatively few cells can cause illness (Buchanan and Doyle 2000). The steps in the production chain that have the greatest potential for pathogen contamination are soil preparation and planting (Islam *et al.* 2004).

Recently, Solomon *et al.* (2002) 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. Also, Ibekwe *et al.* (2004) described quantification of *E. coli* O157:H7 rhizosphere and phyllosphere using multiplex real-time PCR for quantification of natural *E. coli* O157:H7. In this study, we monitored the survival of *E. coli* O157:H7 in lettuce rhizosphere and phyllosphere during the growth process. This provided us with the opportunity to study the production process from contaminated soil at planting to a full-grown plant at the harvest after 5 weeks.

Material and methods

Bacterial strain and growth conditions

Escherichia coli O157:H7 strain 72 was kindly provided by Dr Pina Fratamico of USDA-ARS. This strain produced Shiga-like toxin genes 1 and 11 (*Stx1, Stx2*) and pGFP-expressing green fluorescent protein (GFP) and ampicilin resistance. *Escherichia coli* O157:H7/pGFP was cultured at 37°C overnight in modified tryptic soy broth (mTSB) (Difco Laboratories Inc., Cockeysville, MD, USA) supplemented with 50 μg of ampicillin ml^{-1} (Sigma, St Louis, MO, USA). Cells were harvested by centrifugation at 5000 ***g*** for 10 min and resuspended in phosphate-buffered saline (PBS) (Fisher Scientific, Pittsburgh, PA, USA) at a concentration of *c.* 10^8 CFU ml^{-1}.

Growth chamber experiment and fumigation

The two soils used in the growth chamber experiment were clay soil (Willows silty clay, saline-alkaline) and sandy soil (Dello sand) collected from Mystic Lake dry bed and the Santa Ana River bed, respectively, in Riverside County, California. The soils were sieved through a 4- mm sieve before planting, and the high salt content (electrical conductivity = 15 ds m^{-1}) in the clay soil was reclaimed as described by Ibekwe and Grieve (2004). The fumigant methyl iodide [MeI, iodomethane, >99% purity was purchased from Chem Service (West Chester, PA, USA)] and methyl bromide (MeBr >99% purity) were obtained from Great Lakes Chemical company (West Lafayette, IN, USA).

Fumigation treatments were randomized with two replicates per treatment for each soil. Application rates for MeBr and MeI were *c.* 48 and 40 kg ha^{-1}, respectively. These rates were designated 1× to indicate approximate field application rates. Methyl bromide and MeI were also applied at half of this rate (0·5×), and nonfumigated controls (0×) were included. Plastic trays (58·2 × 43·2 × 18·5 cm) were filled with *c.* 40 kg of both soils and placed in both chambers with the sandy soil in one chamber and the clay soil in another chamber. To avoid the emission of fumigants to the growth chamber, syringes were used to inject 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 outside in an area covered with barb wires, opened and aerated for 2 days before they were moved back to the growth chamber for the continuation of the experiment. The concentrations of *E. coli* O157:H7 in the two soils were $<10^4$ CFU g^{-1} of soil at planting.

Soil inoculation

The soils were irrigated with *c.* $2{\cdot}2 \times 10^8$ *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 at the day of inoculation for *E. coli* O157:H7 concentrations. The concentrations of *E. coli* O157:H7 in the two soils were about 10^5 CFU g^{-1} of soil after irrigation and mixing of soil in each tray. After the initial sample collection, trays were manually tarped with a virtually impermeable plastic film; 0·038 mm Hytibar® film (Klerk Plastics, Belgium) and treated with fumigants as stated earlier.

Growth conditions

Seeds of green romaine lettuce *Lactuca sativa* (L.) cv. Green Forest were purchased from Johnny's Selected Seed Co. (Albion, ME, USA). 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 solution (Hoagland and Arnon (1950) for 3 days and planted in the two growth chambers. The experiment was a completely randomized design with two replications. Plants in the clay soil were irrigated with distilled water daily and received the nutrient solution weekly. Plants in the sandy soil received the same nutrient solution twice daily. There were ten plants in each tray at transplanting, and one plant was harvested from each tray during analysis.

Bacterial sampling and analysis

Plant phyllosphere and rhizosphere samples were collected weekly for 5 weeks (which was the growth period in the growth chambers) for *E. coli* O157:H7 analysis. The samples were collected in separate sterile petri dishes or collection bags. Negative controls were collected first. Phyllosphere samples were cut above the soil surface with sterile blade, placed in the stomacher bags and weighed.

The rhizosphere samples were collected after shaking loosely held soil on the roots into the stomacher bags and weighed. Total bacterial community DNA was recovered from the plant material by homogenization using 100 ml of PBS for 2 min at 260 rev min^{-1} in a Seward Stomacher 400 Circulator (Seward Ltd., London, UK). The homogenate was centrifuged at 5000 ***g*** for 10 min, and the pellet was resuspended in 2 ml of PBS. *E. coli* O157:H7 population was determined by plating samples on modified tryptic soy agar (TSA; Becton Dickinson) plates containing 100 μg of ampicillin ml^{-1} (TSA-A), and the gfp-expressing *E. coli* O157:H7 was enumerated under a Spectroline ultra-violet lamp (Spectronics Corporation, Westbury, NY, USA), and the result expressed as CFU g^{-1} soil or plant. Concentrated samples were used for isolation of the genomic DNA that was used for quantification of *E. coli* O157:H7 by real-time PCR. At the end of the study, samples were also enriched with a 10× volume of buffered peptone water (Lab M, Bury, United Kingdom) supplemented with vancomycin (8 mg l^{-1}) for 6 h at 42°C. This was followed by immunomagnetic separation (IMS) by plating onto Harlequin cefixime-tellurite sorbitol MacConkey (CT-SMAC) agar with BCIG (5-bromo-4-chloro-3-indoxyl-ß-D-glucuronide) containing 0·05 mg of cefixime l^{-1} and 2·5 mg of tellurite l^{-1} (LAB M; IDG).

To evaluate the number of *E. coli* O157:H7 cells that were located in internal tissues in lettuce leaves grown in soil contaminated by *E. coli* O157:H7, leaf samples were gently agitated in 2 g l^{-1} of tween 20 and 20 g l^{-}1of sodium hypochlorite for 3 min; rinsed it three times using sterile water; placed it in 20 ml of potassium phosphate buffer, pH 7, containing 10 g l^{-1} of peptone in 10 g l^{-1} of glycerin. Samples were stored at −20°C for 4 weeks as has been performed previously (Beattie and Marcell 2002; Sabaratnam and Beattie 2003). After 4 weeks, samples were rapidly thawed and homogenized. The leaf homogenate was transferred to plates of mTSA and CT-SMAC agar for *E. coli* O157:H7 enumeration as stated previously. DNA was also extracted from the homogenate and quantified by real-time PCR and IMS.

DNA extraction and real-time PCR analyses

Genomic DNA was isolated from pure culture of *E. coli* O157:H7 strain 72 pGFP, grown for 12 h at 37°C and extracted using the Qiagen tissue kit (QIAamp DNA Mini Kit; Valencia, CA, USA) and used for the construction of standard curve and for the determination of detection limits of the *E. coli* O157:H7 by real-time PCR. Total bacterial DNA was extracted from plant rhizosphere using the Ultra Clean Soil DNA Kit (MoBio Laboratories, Solana Beach, CA, USA) and stored at −20°C. Primers and probes used for the detection and quantification of the *stx*1, *stx*2 and the *eae* genes in *E. coli* O157:H7 were as described by Ibekwe *et al.* (2002). Real-time quantitative PCR was performed using the iCycler iQ (Bio-Rad, Hercules, CA, USA) as described by Ibekwe *et al.* 2002. Briefly, template DNA (2 μl) was added to 48 μl of the reaction mixture in a total volume of 50 μl containing 200 μmol l^{-1} of dNTPs, 2·5 U of AmpliTaq Gold polymerase, 5 μl of 10× TaqMan buffer (PE Applied Biosystems, Foster City, CA, USA), 0·3 μmol l^{-1} of each primer, 0·1 μmol l^{-1} of probe and 3·5 mmol l^{-1} of $MgCl_2$. *E. coli* O157:H7 concentrations in rhizosphere and leaf surfaces were confirmed quantitatively by assaying serial dilutions ($7{\cdot}9 \times 10^{0}$ to $7{\cdot}9 \times 10^{-9}$ pg ml^{-1}) of strain 72 pGFP DNA in the same plate. PCR was performed using the iCycle iQ thermal cycler (Bio-Rad, Hercules, CA, USA) with the following cycle conditions: denaturation at 95°C for 10 min, 50 cycles of 94°C for 20 s, 55°C for 30 s, 72°C for 40 s, followed by a 5- min extension at 72°C and a hold at 4°C.

Assay sensitivity, amplification efficiency and data analysis and standard curves were as described previously (Ibekwe *et al.* 2002; Sharma 2002). The detection limit for *E. coli* O157:H7 quantification by plate counts was 10^2 CFU g^{-1} and for real-time PCR was $2{\cdot}4 \times 10^3$ CFU g^{-1} of starting DNA in rhizosphere and phyllosphere samples, and the amplification efficiency (E) was estimated by using the slope of the standard curve and the formula $E = (10^{-1/\text{slope}}) - 1$. A reaction with 100% efficiency will generate a slope of−3·32.

Data analysis

Escherichia coli O157:H7 concentrations were converted to log CFU g^{-1} for regression analysis. Statistical analyses were performed with the general linear model (GLM) procedure of the Statistical Analysis System (SAS Institute Inc. 1991). The population data were log transformed to obtain a normal distribution of the data. Comparisons between pairs of treatment means at any date were accomplished with the Tukey's test.

Results

Survival of *E. coli* O157:H7 in rhizosphere was measured at intervals after planting of lettuce during the 5-week growth period. *E. coli* O157:H7 population in the rhizosphere of sandy and clay soils with normal concentration of methyl bromide declined by the plate count method at significant rates throughout the growth chamber study (Fig. 1a,b). *Escherichia coli* O157:H7 was no longer detectable after 21 days in sandy soil rhizosphere and after 7 days in clay soils rhizosphere by plate count

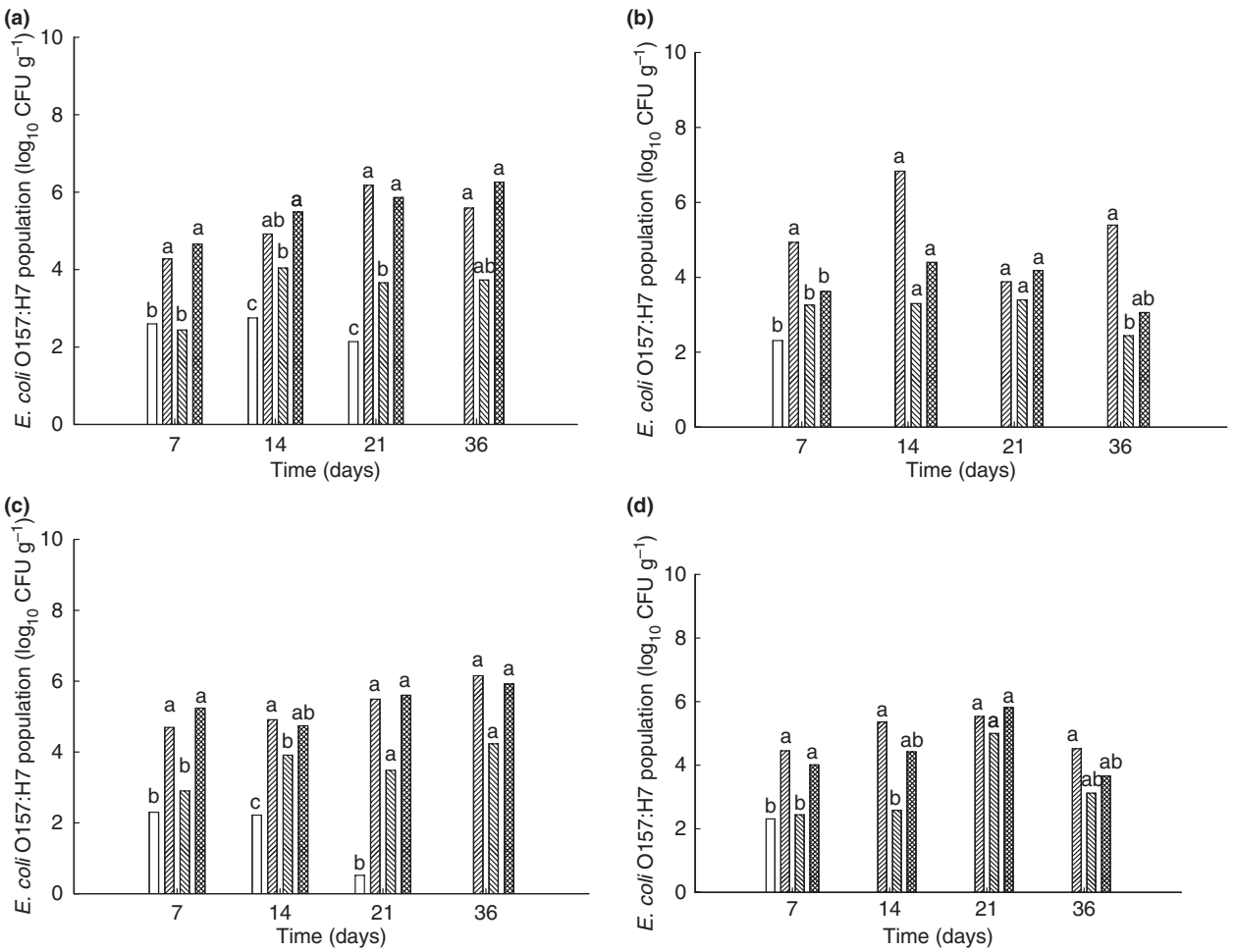

Figure 1 Survival of *Escherichia coli* O157:H7 in the lettuce rhizosphere grown in the growth chamber. The detection limit for plate counts was $\leq 10^2$ CFU g dw^{-1}, and for real-time PCR was *c.* $2{\cdot}4 \times 10^3$ CFU g^{-1}. *Escherichia coli* O157:H7 was enumerated from rhizosphere samples grown in sandy and clay soil by plate count (PC) (□) and by real-time PCR (RT-PCR) using *stx*1 (▨), *stx*2 (▧) and *eae* (▩) genes. *Escherichia coli* O157:H7 concentration in rhizosphere samples from sandy soil (a), clay soil (b), with normal application rate of methyl bromide; sandy soil (c), clay soil and (d), with normal application rate of methyl iodide. The letters a, b or c indicate significant differences if they are different from each other at $P \leq 0{\cdot}05$ using Tukey's studentized range test.

method with soils treated with methyl bromide at normal application rate. The same trend was seen with soils fumigated with methyl iodide as the pathogen was detected in lettuce rhizosphere up to 21 days in sandy soil and only 7 days in clay soil (Fig. 1c,d) with a detection limit of about 10^2 CFU gdw^{-1} using plate count method. However, the detection limit for *E. coli* O157:H7 quantification by real-time PCR was $2{\cdot}4 \times 10^3$ CFU g^{-1} of starting DNA in rhizosphere and phyllosphere samples. Quantification of rhizosphere samples using RT-PCR with *stx*1, *stx*2 and the *eae* genes showed no significant decline in *E. coli* O157:H7 populations in both soils (Fig. 1a–d). Mean comparison using Tukey's procedure ($P < 0{\cdot}05$) at the different sampling points showed that RT-PCR produced significant higher population than plate counts in 13 out of the 24 sampling points with methyl bromide and methyl iodide at normal application rate (Fig. 1). Enrichment of the samples with IMS showed that the pathogen was still present on the rhizosphere (data not shown) whereby confirming the accuracy of RT-PCR.

The concentration of the pathogen recovered on leaf surfaces ranged from 1·5 log10 CFU g of leaf with the plate count method to 4·90 log10 CFU per g with real-time PCR (Fig. 2). The aim was to determine the influence of the presence of soil on the survival of *E. coli* O157:H7 on leaf surfaces with plant growth. Leaf surfaces of lettuce plants were contaminated with soil in the growth chamber during the growth process as plants come in contact with soil. Pathogen was detected on the

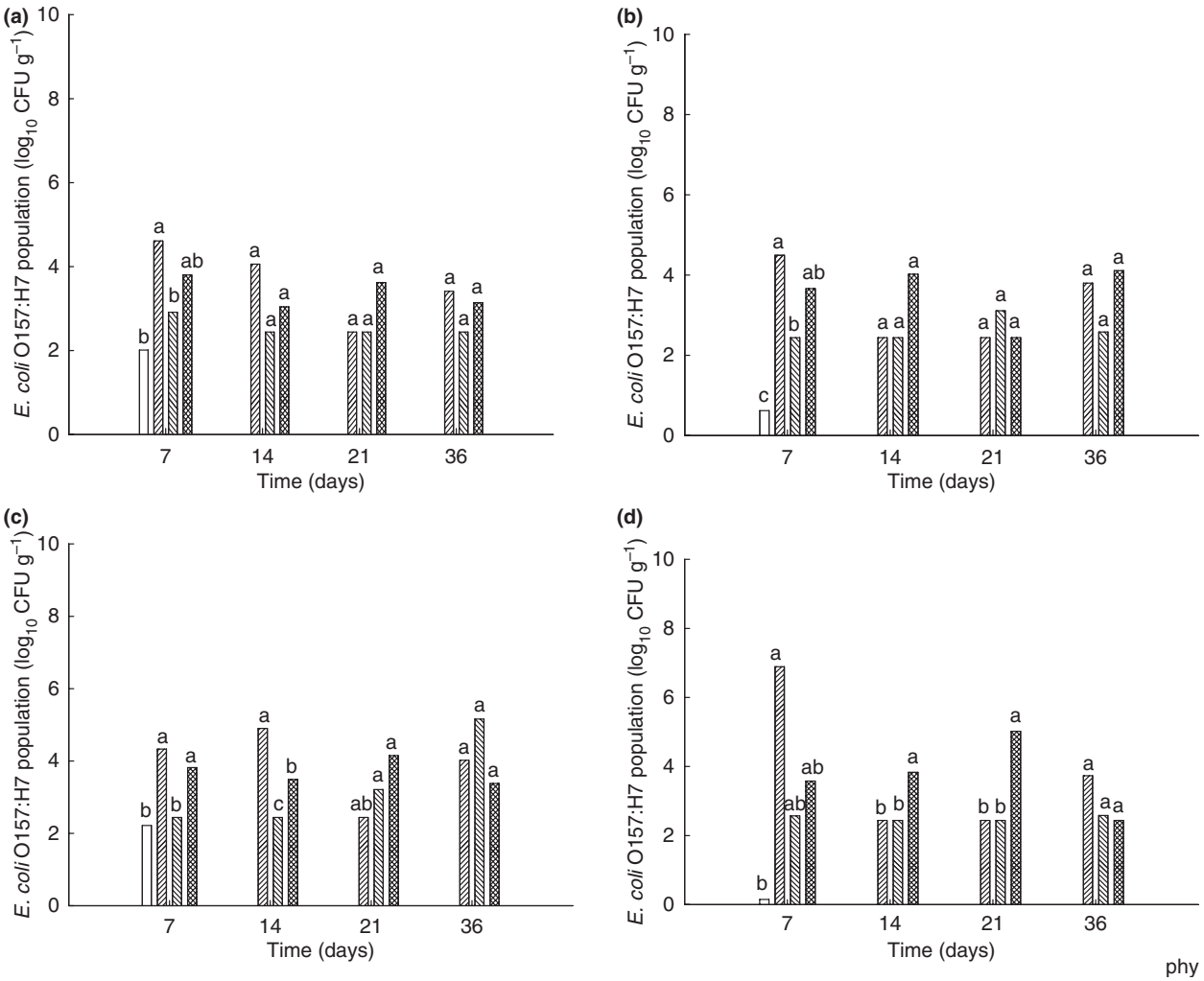

Figure 2 Survival of *Escherichia coli* O157:H7 in the lettuce phyllosphere grown in the growth chamber. The detection limit for plate counts was $\leq 10^2$ CFU g dw^{-1}, and for real-time PCR was *c.* $2{\cdot}4 \times 10^3$ CFU g^{-1}. *Escherichia coli* O157:H7 was enumerated from rhizosphere samples grown in sandy and clay soil by plate count (PC) (▩) and by real-time PCR (RT-PCR) using *stx*1 (▨), *stx*2 (▧) and *eae* (▩) genes. *Escherichia coli* O157:H7 concentration in rhizosphere samples from sandy soil (a), clay soil (b), with normal application rate of methyl bromide; sandy soil (c), clay soil and (d), with normal application rate of methyl iodide. The letters a, b or c indicate significant differences if they are different from each other at $P \leq 0{\cdot}05$ using Tukey's studentized range test.

leaf surfaces by plate count 7 days after planting (Fig. 2a–d). Pathogen was detected in the phyllosphere samples throughout the 5 weeks by RT-PCR; however, pathogen concentrations were smaller in the phyllosphere than in the rhizosphere. In the nonfumigated samples, pathogen was detected in lettuce rhizosphere up to 21 days in both soils (Fig. 3a,b). As observed in the fumigated soils, the same trend was seen in the nonfumigated soil where pathogen was detected in the lettuce phyllosphere during the first 7 days of planting (Fig 3c,d). Our results also showed that no *E. coli* O157:H7 cells were found in the internal leaf tissues, and this was confirmed by RT-PCR and IMS.

Discussion

In the present study, contamination of *E. coli* O157:H7 on the rhizosphere and phyllosphere via soil to the lettuce surface occurred, and the pathogens survived for at least 36 days as confirmed by RT-PCR and IMS. However, none of the samples was positive for the pathogen when internal leaf tissues were analysed. Therefore, our study showed that leaf surface was contaminated during the germination process as they come in contact with contaminated soil. The detection of the pathogen by IMS and by PCR suggests that very few cells were still viable on the plants at harvest but at numbers below the

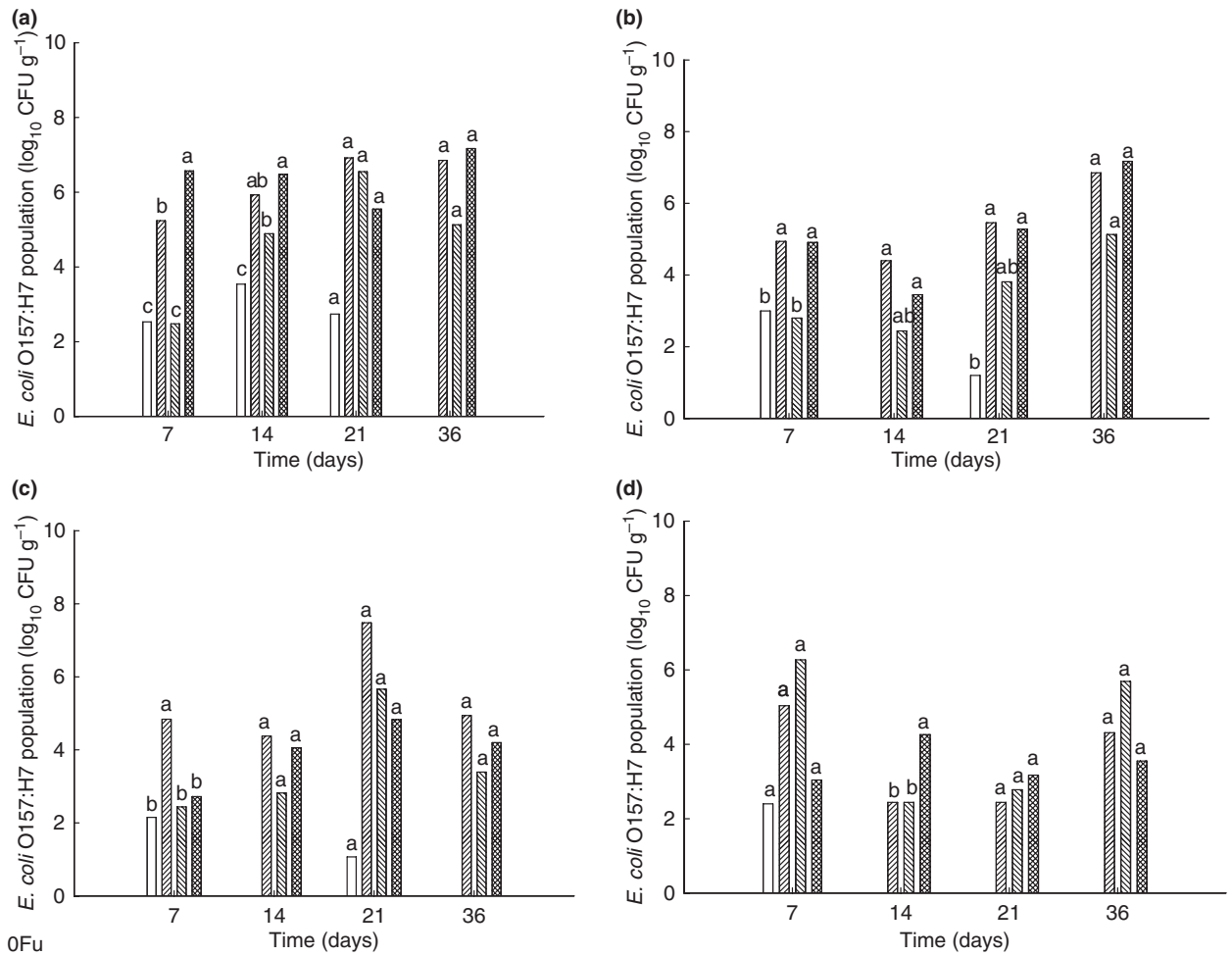

Figure 3 Survival of *E. coli* O157:H7 in the lettuce rhizosphere and phyllosphere grown in the growth chamber without fumigant treatments. The detection limit for plate counts was $\leq 10^2$ CFU g dw^{-1}, and for real-time PCR was c. $2{\cdot}4 \times 10^3$ CFU g^{-1}. *Escherichia coli* O157:H7 was enumerated from rhizosphere samples grown in sandy and clay soil by plate count (PC) (□) and by real-time PCR (RT-PCR) using *stx*1 (▨), *stx*2 (▧) and *eae* (▩) genes. *E. coli* O157:H7 concentration in rhizosphere samples from sandy soil (a), clay soil (b); phyllosphere sandy soil (c) and clay soil (d). The letters a, b or c indicate significant differences if they are different from each other at $P \leq 0{\cdot}05$ using Tukey's studentized range test.

detection limit of 10^2 CFU g^{-1}. This result may also be different if this study was performed in the field under the influence of UV light, which may kill most of the bacteria on the plants compared to the growth chamber in which this study was conducted. Therefore, the growth environment may have potential influence on *E. coli* O157:H7 survival on plants.

The potential presence of human pathogens in vegetables grown in soils contaminated with *E. coli* O157:H7 is a serious problem to our national food supply. In our study, the pathogen was introduced into the soil 2 weeks before planting. Seven days after planting, the first set of sampling was performed. At this time, the population in the soils (3·5 log10 CFU g^{-1}) may have been too low to be taken up by the internal plant tissues and transferred to the edible portion of the plants. In contrast, other studies that have investigated the risks of using manure as fertilizer on vegetables have found that transmission may occur under experimental conditions in the greenhouse through the internal organs and to the edible portion of the plant with higher cell counts of about 10^6 CFU g^{-1}(Natvig *et al.* 2002; Solomon *et al.* 2002). Our study used large amount of soil (≥40 kg) in large trays in the growth chamber to grow the plants. We did this to closely mimic the volume of soil that lettuce may grow in the field. Fields studies with avirulent strain of *E. coli* O157:H7 have shown the survival of the pathogen on lettuce, parsley, carrots and onions surface up to 77 and 177 days (Islam *et al.* 2004). These studies used a higher concentration of pathogens (10^7 CFU g^{-1}) in the

manure than the study by Johannessen *et al.* (2005) (10^4 CFU g^{-1}) which may suggest that the concentration of pathogens also plays a role in the contamination of vegetables from manure. The results from our study as well as results from other studies indicate that both the time of introduction and the concentration of pathogen present might influence the uptake of bacteria. Also, our study was conducted with green romaine lettuce, and other studies used different cultivars such as green ice lettuce (Solomon *et al.* 2002), iceberg lettuce (Franz *et al.* 2005), crisphead lettuce (Johannessen *et al.* 2005) and leaf lettuce (Islam *et al.* 2004).

In conclusion, *E. coli* O157:H7 DNA was detected by real-time PCR throughout the duration of the study and by IMS at the end of the study on the rhizosphere and on leaf surfaces. However, because of low concentrations of *E. coli* O157:H7 in soil (3·5 log10 CFU g^{-1}) at the time of planting, pathogen was not transferred through the inner tissues to the edible part of plants.

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