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Evaluation of post-contamination survival and persistence of applied attenuated *E. coli* O157:H7 and naturally-contaminating *E. coli* O157:H7 on spinach under field conditions and following postharvest handling

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ABSTRACT

This study determined the variability in population uniformity of an applied mixture of attenuated *E. coli* O157:H7 (*attEcO157)* on spinach leaves as impacted by sampling mass and detection technique over spatial and temporal conditions. Opportunistically, the survival and distribution of naturally contaminating pathogenic *E. coli* O157:H7 (*Ec*O157), in a single packaged lot following commercial postharvest handling and washing, was also evaluated. From the main study outcomes, differences in the applied inoculum dose of 100-fold, resulted in indistinguishable population densities of approximately Log 1.1 CFU g^{-1} by 14 days post-inoculation (DPI). Composite leaf samples of 150 g and the inclusion of the spinach petiole resulted in the greatest numerical sensitivity of detection of *attEcO157* when compared to 25 and 150 g samples without petioles ($P < 0.05$). Differences in population density and protected-site survival and potential leaf internalization were observed between growing seasons and locations in California ($P < 0.05$). A Double Weibull model best described and identified two distinct populations with different inactivation rates of the inoculated *attEcO157*. Linear die-off rates varied between 0.14 and 0.29 Log/Day irrespective of location. Detection of *Ec*O157- *stx*1-negative and *stx2*-positive, resulting from a natural contamination event, was observed in 11 of 26 quarantined commercial units of washed spinach by applying the 150 g sample mass protocol. The capacity to detect *Ec*O157 varied between commercial test kits and non-commercial qPCR. Our findings suggest the need for modifications to routine pathogen sampling protocols employed for lot acceptance of spinach and other leafy greens.

1. Introduction

The spatial distribution of culturable epiphytic microbiota is widely recognized to be extremely non-uniform, typically log-normally distributed across a plant population and among individual leaves, and even between opposing leaf pairs, within a single plant (Andrews et al., 1980; Andrews and Harris, 2000; Lindow and Brandl, 2003; Leveau, 2006). Differential physical, environmental, and competitive dynamics occurring within the phyllosphere have been documented to result in this lognormal distribution (Crosse, 1959; Andrews et al., 1980; Hirano et al., 1982; Kinkel, 1997; McGrath and Andrews, 2007). Consequently, within studies of resident and transient phyllosphere colonizers, the experimental design and sampling strategy significantly influence the outcomes and, subsequently, interpretation of the data analysis (Kinkel et al., 1995; Meyer and Leveau, 2012; Wiken Dees et al., 2015).

In open environments, deterministic temporal factors affect the population flux and degree of heterogeneity following deposition of both bacterial plant epiphytes and bacterial pathogens of food safety concern on horticultural food crops (Leveau, 2006; Meyer and Leveau, 2012; Rastogi et al., 2012). The greatest concern for post-contamination survival, understandably, is associated with perishable horticultural foods typically consumed in a raw state. These temporally variable and interacting factors include the source of contamination (aqueous planktonic cells or aggregates imbedded in suspended sediments or an organic matrix), regional climate and field-scale microclimate, as well as plant developmental and anatomical/physiological traits (Rastogi et al., 2012). Bacterial populations on leaf surfaces are normally estimated by using standardized sample units, including leaf segments,

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individual leaves, or the entire plant (Tyler and Triplett, 2008; Morris and Lucotte, 1993). Scaling within a sample unit; in which size or weight have a direct influence on estimating bacterial numbers and spatial distribution among samples from within a plant population, has been a major focus of descriptive and quantitative phyllosphere microbiology (Williams and Marco, 2015; Widder et al., 2016), since sample size and plant organ may impact probability of detection and recovery efficiency.

Variability in bacterial phyllosphere populations has been correlated with leaf position, age and nutrient availability as well as the accessibility or exposure of leaves to the deposition of airborne microbiota, microclimates, growing season, leaf orientation, plant structure and the physicochemical condition of leaves (Mew and Kennedy, 1982; Plummer et al., 1992; Jacques et al., 1995; Ong et al., 1999; Redford and Fierer 2009; Lindow and Brandl, 2003; Aruscavage et al., 2006; Monier and Lindow, 2005; Redford et al., 2010; Jackson and Denney, 2011; Kroupitski et al., 2011; Burch et al., 2016).

Rastogi et al. (2012) evaluated the extent and presumptive sources of variability on the phyllosphere bacterial community composition on Romaine lettuce as a function of time, spatial separation within and between commercial fields, and seasonal environmental and geospatial differences. It was determined that the distance between fields or the timing of a natural environmental phenomenon and not Romaine cultivar or irrigation method explained differences in bacterial community composition between different commercial fields; suggesting that the mechanisms of bacterial community composition within plants and their subsequent variability are a function of environmental factors as suggested by Wiken Dees et al. (2015), Franz (2008), and Leff and Fierer (2013).

Leafy greens have consistently been implicated in foodborne disease outbreaks over the past 44 years (Herman et al., 2015). One response to this fact has been the implementation of routine audit standards compliance and lot acceptance monitoring of fresh produce for the presence of fecal indicators and bacterial human pathogens. These testing programs have increased dramatically following three unrelated STEC outbreaks on leafy greens in 2006 (Gil et al., 2014) As sampling strategies were not standardized, a keen interest emerged to better characterize population variability within a production lot and to improve the statistical validity and predictive capacity of any sampling strategy as suggested by Callahan et al. (2016).

Past and some current rapid test kits including Reveal (Neogen, Lansing, MI), SDI RapidChek (Strategic Diagnostics, Newark, DE), BAX O157 and BAX O157:H7 MP (DuPont Qualicon, Wilmington, DE) FDA BAM method (1998) and Assurance GDS O157:H7 (BioControl Systems, Inc., Seattle, WA) specify a 1:10 (w:w) ratio with a wash buffer for enumeration or enrichment detection protocols (APHA, 2001; AOAC, 2007). Test kit accompanying technical instructions refer to validated or performance-tested protocols with 10–25 g sample mass for an array of foods over a defined pre-detection enrichment period. This approach had raised concerns that this sample mass unit and paired detection systems may not have been fully characterized or validated for pathogen detection in fresh produce (UFPA, 2010 and D'Lima and Suslow, 2009). To that end, recent efforts by Lopez-Velasco et al. (2015) and Fang and Patel (2017) highlight the importance of using composite or pooled sample unit sizes greater than 200 g to improve the probability of pathogen detection in controlled and open-field environments of tender greens cropping systems with high seedling densities like baby spinach.

This current study was initiated to investigate the variability in populations of applied *attEcO157* on the surface of spinach leaves and associated plant tissue under field conditions. Of special interest was to determine the post-inoculation variability of leaf surface populations from the same plant and among groups of plants with and without a 1% silver nitrate surface-disinfection treatment known to effectively inactivate human pathogens in laboratory studies. During the course of these controlled field studies and a single naturally-occurring contamination even with *E. coli* O157:H7 (*EcO157*) was detected by industry collaborators. With the information from the controlled studies, we sought to asses in this natural contamination event; 1- the variability among independent 'contamination' incidents in distribution of surviving, culturable *EcO157* and; 2- the extent of variation in extraction and detection efficiencies differing among selected methods and between known and uncharacterized *Ec*O157 serotypes. Such comparisons were viewed to provide insights into qualitative assessments of the prevalence of *Ec*O157 based on sample mass distribution in commercially washed and packaged spinach and among individual leaves from known positive pre-market retained samples.

2. Materials and methods

2.1. Field cultivation

Spinach seeds (*Spinacia oleracea* L. cv Barbados, Emilia and Blackhawk) were sown according to standard commercial practices during the months of May through November of 2008–2010 in Salinas (2008, 2010) and Soledad California (2009–2010), approximately 48 km separation north to south, respectively. Each cultivar was managed under standard fertilizer and pest management practices by cooperative growers at all locations. A total of 3 field trial experiments were performed at Soledad while 2 were performed at Salinas in parallel or separately. Growing conditions in Salinas: Plants were cultivated in a Chualar Loam soil composed of 14% Clay, 45% sand and 41% silt (Soilweb, 2017). Organic matter within the top 15 cm of the soil was determined to be 2.3% with an average pH of 7.2 and electrical conductivity (Ec) of 1.8 (dS/m). Nitrogen fertilization consisted of the following fertilizer program (6-00-20, 22-00-13 (liquid) and UN32 (urea and ammonium nitrate)) to achieve a final total applied N:NO3 of 225 kg/ha. Growing conditions in Soledad: Plants were cultivated in a Pico Fine Sandy Loam soil composed of 11% Clay, 72% sand and 17% silt (Soilweb, 2017). Organic matter within the top 15 cm of the soil was 1.2% with an average pH of 7.9 and Ec of 1.1 (dS/m). Nitrogen fertilization consisted of the following fertilizer program (ammonium nitrate and urea (UN32)) to achieve a final total applied N:NO3 of 180 kg/ha. At both growing locations plants were managed and harvested at dawn under standard commercial practices (Koike et al., 2011). Weather data at both growing locations was accessed through CIMIS (California Irrigation Management Information System) stations within 500 m from the position of each experimental setup and the monthly averages are provided in Fig. 1. Within each cultivated field plot dimension were 160 m long by 30 m wide. Each raised seed bed (n = 4), oriented west to east, was divided into 4 blocks (each 40 m long) across the length of the bed, each further divided into 2 additional subsections (each 10 m long). Inoculation and subsequent characterization of applied isolate persistence was conducted within this experimental design configuration (total 8 locations per bed). The remainder of the beds (total of 8) within the area selected were similarly managed to provide a sufficient cropping footprint, including buffer rows (Gutiérrez-Rodríguez et al., 2011). Inoculated leaves were combined from each block-bed zone for a total of 16 field replicates that were later combined into 8 replicates for laboratory analysis. Total average weight per replicate was 1.5 kg.

2.2. Inoculation studies

Plants at the 4–6 true leaf stage were spray-inoculated with an equal mixture of two *attEcO157* O157:H7 (PTVS 154 and PTVS 155 – *stx*1 and *stx*2 negative; collectively referred to in the remainder of this paper as *attEcO157*) in the afternoon just before dusk. This timing was selected based on previous studies under field conditions in Davis, CA where greater survival of the inoculated strains had been observed relative to applications prior to and spanning peak periods of solar UV flux (from CIMIS reference database; data not shown). Spray application