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Conditions at the time of inoculation influence survival of attenuated *Escherichia coli* O157:H7 on field-inoculated lettuce

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ABSTRACT

The impact of plant development, environmental conditions at the time of inoculation, and inoculum concentration on survival of attenuated BSL1 *Escherichia coli* O157:H7 strain ATCC 700728 on field-grown romaine lettuce was evaluated over 3 years. *E. coli* 700728 was inoculated onto 4- and 6-week-old romaine lettuce plants in the Salinas Valley, CA, at night or the next morning with either low (5 log) or high (7 log) cell numbers per plant to simulate a single aqueous contamination event. At night, when leaf wetness and humidity levels were high, *E. coli* cell numbers declined by 0.5 log CFU/plant over the first 8–10 h. When applied in the morning, *E. coli* populations declined up to 2 log CFU/plant within 2 h. However, similar numbers of *E. coli* were retrieved from lettuce plants at 2 and 7 days. *E. coli* cell numbers per plant were significantly lower ($P < 0.05$) 7 days after application onto 4-week-old compared to 6-week-old plants. *E. coli* 700728 could be recovered by plating or enrichment from a greater proportion of plants for longer times when inoculated at high compared with low initial concentrations and after inoculation of 6-week-old plants compared with 4-week-old plants, even at the low initial inoculum. A contamination event near harvest or when leaf wetness and humidity levels are high may enhance survivability, even when low numbers of *E. coli* are introduced.

1. Introduction

Escherichia coli O157:H7 gastroenteritis has been associated with consumption of many plant-based foods, and numerous foodborne outbreaks worldwide have been linked to the consumption of fresh produce (Wadamori et al., 2017). Leafy lettuce, in particular, has been implicated in multiple outbreaks associated with Shiga toxin–producing *E. coli* (Ackers et al., 1998; CDC, 2018, 2019; Friesema et al., 2008; Hilborn et al., 1999; Marder et al., 2014; Mikhail et al., 2018; Slayton et al., 2013; Söderström et al., 2008; Taylor et al., 2013; Turner et al., 2019). Some U.S. outbreaks were traced to fields in Arizona and California, the states where the majority of U.S. leafy greens are grown.

Before harvest, plants can be exposed to human pathogens through contact with contaminated water (irrigation or other foliar application), soil, airborne dusts, animals, or humans (Alegbeleye et al., 2018; Heaton and Jones, 2008; Park et al., 2012). Although nutrients are often limited in open field environments, *E. coli* O157:H7 can persist in soil, manure, and water for days and up to months (Islam et al., 2004, 2005; Ma et al., 2014). Controlled laboratory and field experiments have provided evidence to support the potential for water to be a route of contamination with pathogenic microorganisms (Fonseca et al., 2011; Wachtel et al., 2002). In 2018, two U.S. outbreaks of *E. coli* O157:H7 were linked to romaine lettuce. The outbreak strains were isolated from irrigation canal water in the growing region associated with an outbreak in the spring of 2018 (FDA, 2018) and in sediment from a water reservoir located on a farm associated with an outbreak in the fall of 2018 (FDA, 2019), leading to speculation that contaminated water might have been a route of contamination. In an attempt to reduce this risk, agricultural water testing and metrics for water quality are often provided for produce, especially when the water comes into direct contact with the harvestable crop (CA LGMA, 2019; Federal Register, 2015; WHO, 2001).

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Table 1
Dates and times of inoculation in the field trials for romaine lettuce.

Trial	Year	Date of seeding	Date of last sampling	Date of inoculation (H, L[a])	Time of sunset and sunrise [b]	Date of inoculation (H, L[a])	Time of sunset and sunrise	Time of inoculation initiation [b]			
								4 weeks		6 weeks	
				4 weeks		6 weeks		Night (p.m.)	Morning (a.m.)	Night (p.m.)	Morning (a.m.)
1	2010	May 4	July 5	June 7 (H)	5:48 a.m.	June 20 (H)	5:48 a.m.		7:30[c]		7:30[c]
2	2010	July 28	October 4	August 30 (H)	6:36 a.m.	September 13 (H)	6:47 a.m.		8:30[c]		7:30[c]
3	2011	May 23	July 20	June 28 (H, L)	8.29 p.m. 5:50 a.m.	July 12 (H, L)	8:27 p.m. 5:58 a.m.	9:30[d]	7:00[c]	9:00[c]	7:30[c]
4	2012	June 12	August 7	July 15 (H, L)	8:25 p.m. 6:00 a.m.	July 31 (H, L)	8:13 p.m. 6:12 a.m.	10:00[c]	9:00[c]	11:00[c]	9:00[c]
5	2012	August 14	October 25	September 19 (H, L)	7:07 p.m. 6:53 a.m.	October 10 (H, L)	6:36 p.m. 7:10 a.m.	9:30[c]	9:00[c]	10:00[c]	9:00[c]

[a] H and L refer to inoculation at high (7 log CFU/plant) and low (5 log CFU/plant) levels, respectively; inoculation at low level was applied only during night inoculation.
[b] Pacific Standard Time.
[c] Inoculation was accomplished in 1 h.
[d] Inoculation was accomplished in 2 h.

In our previous field experiments conducted in the Salinas Valley region of California, the behavior of attenuated *E. coli* O157:H7 ATCC 700728 (*E. coli* 700728) was monitored after simulating a single contamination event by spraying 2- or 4-week-old lettuce plants at 6 log CFU/plant with inoculated water (Moyne et al., 2011). *E. coli* 700728 could not be detected by enrichment of whole plants (240 plants) 4 weeks after inoculating 2-week-old plants. The organism was recovered at very low levels (fewer than 10 cells per plant), 4 weeks after inoculation of 4-week-old plants, in 0.5–27.5% of lettuce plants (out of 360–120 plants) in three trials. Dead and live cells were quantified with a combination of propidium monoazide (PMA) and real-time PCR, demonstrating that the rapid decline in culturable *E. coli* during the first hours after inoculation was not due to dispersal or an inability to recover the organism from the lettuce but to cell death (Moyne et al., 2013). The rapid initial die-off, followed by low-level persistence, was also recorded at two field sites in Canada for the same strain inoculated onto lettuce (Bezanson et al., 2012). Different predictive models, using multiple sets of data (including those reported above) collected in different geographical regions of the United States and Canada (California, USA; Georgia, USA; British Columbia, Canada; and Nova Scotia, Canada) were evaluated by McKellar et al. (2014) for describing *E. coli* O157:H7 survival after introduction onto field lettuce. The authors recommended the use of biphasic models, such as Weibull and Cerf distribution, to predict the fate of *E. coli* O157:H7 on field grown leafy greens. In a more recent field trial conducted in the northeastern United States, inoculated nonpathogenic *E. coli* die-off also followed a biphasic pattern (Weller et al., 2017). Die-off rates of pathogens are used in quantitative microbial risk assessment (QMRA) quantify the level of pathogen contamination and develop strategies to reduce the risk associated with exposure to pathogenic organisms in agricultural water (FDA – iRisk 4.0, https://irisk.foodrisk.org).

Numerous laboratory studies have been conducted to identify factors that enhance the ability of *E. coli* O157:H7 to persist or grow on plants (Brandl and Amundson, 2008; Franz et al., 2007; Patel et al., 2010; Quilliam et al., 2012; Seo and Matthews, 2014; Solomon et al., 2002b; Wachtel et al., 2002; Zhang et al., 2009). High humidity, free water on the leaf surface, and warm temperatures (28 °C) have been conducive to *E. coli* O157:H7 multiplication on lettuce plants under laboratory conditions (Brandl and Amundson, 2008). Laboratory conditions cannot simulate the complex environment encountered by bacteria on plants grown in an open field. Low solar radiation, high relative humidity, and the presence of moisture on the leaf are among the conditions generally assumed to be favorable for survival or growth of leaf-associated bacterial populations in the field (Aruscavage et al., 2006; Beattie, 2011; Beattie and Lindow, 1994).

In the Salinas Valley, higher humidities are encountered at night (Moyne et al., 2011) and water condenses on the lettuce leaf surfaces. During the day, higher temperatures and lower humidity, coupled with regular afternoon increases in wind speed, lead to drying of the leaf surfaces. The current study was conducted to assess the influence of the time of inoculation (night or morning), the plant age (4 or 6 weeks post seeding), and the inoculum level (5 or 7 log), on the behavior of *E. coli* O157:H7 in the lettuce phyllosphere under field conditions.

2. Materials and methods

2.1. Bacterial strains and culture conditions

An attenuated rifampicin-resistant variant of *E. coli* O157:H7 ATCC 700728 (*E. coli* 700728) was selected for field inoculation because it is classified as a BSL1 strain, does not produce Shiga toxin, and was used in our previous field trials (Moyne et al., 2011; Williams et al., 2013). The sequence for *E. coli* 700728 was deposited in Genbank under accession number GCA_000335055.2 (Project PRJNA68603). A stock culture of rifampicin-resistant *E. coli* 700728 was streaked onto tryptic soy agar supplemented with rifampicin (Gold Biotechnology, St. Louis, MO) at 50 mg/L (TSAR), and the plates were incubated overnight at 37 °C. A single isolated colony was inoculated into 2 ml of tryptic soy broth supplemented with rifampicin at 50 mg/L (TSBR), and the tubes were incubated at 37 °C, with shaking at 200 rpm, for 12 h. Aliquots (20 µl) of the bacterial liquid culture were plated with an automated spiral plater (Autoplate, Advance Instrument Co., Norwood, MA) onto TSAR and incubated at 37 °C for 12 h to produce a bacterial lawn. Five ml of 0.1% peptone was added to each plate, the lawn was loosened with a sterile spreader, and the suspended cells were collected. The resulting stock culture had a concentration of approximately 10 log CFU/ml and was diluted immediately after preparation for use in inoculations later that night or was held overnight at 4 °C and then diluted just before use the next morning.

2.2. Field experimental design

Field trials were conducted in the Salinas Valley twice in 2010 (trials 1 and 2), once in 2011 (trial 3), and twice in 2012 (trials 4 and 5) (Table 1). Permits and approvals for use of U.S.-owned land for the trials were granted by the U.S. Department of Agriculture. Romaine lettuce (*Lactuca sativa*) cv. Green Towers (2010 and 2011) or cv. Braveheart (2012) seeds were planted in two rows 30 cm apart on 1-m wide (60-cm bed top width) raised beds, according to standard commercial practice. Plots measured 40 m (trials 1, 2, 4, and 5) or 20 m (trial 3) in length. To evaluate the effects of plant development (all trials), time of inoculation (trials 3–5), and initial inoculum level (trials 3–5), fields were divided into 6 blocks with 9 beds per block in trials 1 and 2, 9 blocks with 10 beds per block in trial 3, or 4 blocks with 10 beds per block in trials 4 and 5. Trials 1 and 2 were established as previously described (Moyne et al., 2011), with 3 blocks irrigated with overhead sprinklers and 3 blocks irrigated with drip tape. A buffer of 10 unplanted beds was retained between the drip and overhead irrigation blocks to reduce drift from the overhead sprinkler irrigation. In trials 3 to 5, because fields were irrigated only with overhead sprinklers, the blocks were separated by one unfarmed bed.

Overhead sprinklers (Rainbird 20JH, Tucson, AZ) were spaced in a 9.1 m by 9.1 m grid pattern. Similar to commercial operations the first three irrigations of the field trials were with overhead sprinklers to germinate the seeded lettuce. After emergence, drip tape was installed in the drip plots in trials 1 and 2. One drip tape line was placed on the soil surface of each bed equidistant between the two rows of lettuce plants. The crop was irrigated two times per week. Drip-irrigated plots were watered 2–4 h (application of 0.7–1.3 cm) per irrigation, and sprinkler-irrigated plots were watered 1.5–2.5 h (application of 1–1.8 cm) per irrigation. The total amount of water applied to the crops varied from 15 to 31 cm, depending on the weather conditions.

After seeding, but before the first irrigation, a pre-emergent herbicide, pronamide (Kerb 50 W, Dow AgroSciences, Indianapolis, IN), was applied to all beds at the rate of 2.24 kg/ha. Plants were initially thinned at the four to six true-leaf stage so that remaining plants were 25 cm apart. The crop was thinned to a final population of 65,000 plants/ha, approximately 30 days after seeding. Plots were fertilized before, at, and after planting, for a total nitrogen application level (kg/ha) of 120 in trials 1 and 2, 98 in trial 3, 97 in trial 4, and 120 in trial 5.

2.3. Inoculation and sampling

Treatments of plant development, inoculation time, and inoculum level, were applied randomly on different beds within each block. Spray bottles were used to apply inoculum to individual lettuce plants; approximately 1 ml was delivered in a single spray directed from above the plant, at a height of approximately 20 cm, for maximum coverage of the plant leaves. A summary of field trial and inoculation dates and times is provided in Table 1. Plants were inoculated once either at night after sunset (initiated between 9 and 11 p.m. Pacific Standard Time [PST]) or the following morning after sunrise (initiated between 7 and 9 a.m. PST). The inoculum was adjusted to 10^7 CFU/ml (high, all trials) or 10^5 CFU/ml (low, night only [trials 3–5]). Inoculation was conducted at two separate intervals: on 4-week-old plants (4–28 g/plant; 4-week inoculation) or on separate 6-week-old plants (56–208 g/plant; 6-week inoculation) in all trials (Supplementary data, Table S1). Plants in one bed were inoculated only once during the season, and a buffer zone of one or two beds with non-inoculated plants was maintained between each treatment. To minimize dispersal of the inoculum, adjacent plants were protected with a hand-held screen. Each application was completed in 1 h except for trial 3 where the night inoculation on June 28th lasted 2 h. The concentration of the inoculum was verified before and after the inoculation by serial dilution in 0.1% peptone, plating onto TSAR, and incubating overnight at 37 °C.

To collect samples, lettuce heads were separated from the roots with a sterile scalpel, approximately 3 cm above the ground, and the heads were bagged individually into 710-ml or 1630-ml Whirl-Pak filter bags (Nasco, Modesto, CA) or zippered polyethylene bags (30.5 × 30.5 cm) (Bitran, Com-Pac International, Carbondale, IL) according to plant size. Samples were collected randomly among the inoculated lettuce beds immediately after inoculation on day 0 and up to 37 days after inoculation. The total number of samples collected at each time (10–180) is provided in Tables S2–S5 (Supplementary data). Non-inoculated lettuce heads (control) were collected randomly on day 0 just before the inoculum was applied and throughout the duration of each trial. Lettuce samples collected through the first 7 days after the 4-week inoculation and up to 14 days after the 6-week inoculation were brought from the field to the laboratory in a cooler containing ice, held at 4 °C, and processed within 24 h. Samples collected ≥14 days after the 4-week inoculation were transported, without cooling, to the laboratory for detection of *E. coli* 700728 through enrichment.

2.4. Inoculum recovery and quantification on lettuce plants

To recover the inoculated bacteria, individual whole or subdivided lettuce heads were placed in a sterile Whirl-Pak bag containing 0.1% peptone (50–200 ml), and sample bags were stomached with a Smasher (AES-BioMerieux, Durham, NC) for 1 min at the fast speed setting. Each sample comprised the entire lettuce head, which increased in weight throughout the field trial (Supplementary data, Table S1). Heads weighing more than 50 g were divided into multiple bags, with up to 50 g per bag, for further processing. The volume of media added to each sample bag was adjusted according to the weight of the lettuce: in general, a vol/wt ratio of 2:1 was used, with a minimum volume of 50 ml and a maximum of 200 ml per bag.

Indigenous bacteria on the control plants were retrieved as previously described by Williams et al. (2013), for trials 1 to 4. Briefly, the lettuce head was submerged in a vol/wt ratio of 4:1 with 0.1% peptone (50–250 ml) and sonicated for 7 min in a Branson 8510 Ultrasonicator water bath (Branson Ultrasonics Corp., Danbury, CT). For trial 5, control lettuce was processed as described above for the inoculated lettuce. For all trials, the entire head was processed when its weight was less than or equal to 50 g. For lettuce heads > 50 g, only the outer leaves (up to 50 g) were processed for bacterial enumeration.

E. coli 700728 numbers were determined by different methods at each sampling time for all inoculated lettuce samples (Table 2). Enumeration was carried out after spiral plating 50-µl and/or 250-µl aliquots of the undiluted prepared sample in duplicate on TSAR and incubating the plates overnight at 37 °C. For samples collected after inoculating 4-week-old plants, 5 or 10 ml, or the entire cell suspension was filtered onto disposable analytical filter units (0.45 µm; Nalgene, Rochester, NY) to lower the limit of detection. The filter membranes were removed and placed on plates of CHROMagar O157 (CHROMagar, Paris, France) supplemented with rifampicin at 50 mg/L, and plates were incubated overnight at 37 °C. For samples collected after inoculating 6-week-old plants, most-probable-number (MPN) methods (described in section 2.5) were employed instead of filtration because the membrane rapidly became blocked before a sufficient amount of cell suspension could be filtered. The plant material with the remaining cell suspension in the bag was enriched 1:5 (wt:vol) for lettuce to TSBR, and incubated for 18 h at 42 °C. To detect rifampicin-resistant *E. coli* 700728, the enrichment broth was spiral plated onto CHROMagar O157 with rifampicin and incubated overnight at 37 °C. For all samples collected after day 7, the entire lettuce heads were processed only by enrichment, as described above, with the exception of the last sampling time in trials 1 and 2 when only the outer leaves were processed.

To enumerate total, culturable bacteria present in the phyllosphere, serial dilutions of the cell suspension retrieved from control lettuce heads were plated on TSA, and plates were incubated at ambient temperature (22 °C) for 48 h. To enumerate generic *E. coli* and total coliforms, cell suspensions were plated on CHROMagar ECC

Table 2
Methods used to measure *E. coli* concentration or evaluate its presence at different sampling times.

Trial	Plant age (weeks post-seeding)	Inoculation time	Inoculation level	Plating	Filtration	MPN	Enrichment
1	4	Morning	High [a]	0–7 d	2 h to 7 d	ND[b]	2 d–28 d
	6	Morning	High	0–7 d	ND	ND	2 d–14 d
2	4	Morning	High	0–7 d	2 h to 7 d	ND	2 d–28 d
	6	Morning	High	0–14 d	ND	ND	2–21 d
3	4	Night	Low	0–2 d	2 h to 2 d	ND	1–7 d
		Night	High	0–2 d	1–2 d	ND	2–21 d
		Morning	High	0–2 d	1–2 d	ND	2–21 d
	6	Night	Low	0–10 h	ND	ND	2 h to 7 d
		Night	High	0–2 d	ND	2–7 d	7 d
		Morning	High	0–2 d	ND	2–7 d	7 d
4	4	Night	Low	0–12 h	10 h to 1 d	ND	12 h to 15 d
		Night	High	0–7 d	8 h to 7 d	ND	2–15 d
		Morning	High	0–7 d	2 h to 7 d	ND	7–15 d
	6	Night	Low	0 h	ND	6–16 h	1–7 d
		Night	High	0–7 d	ND	7 d	ND
		Morning	High	0–7 d	ND	6 h to 7 d	ND
5	4	Night	Low	0–1 d	9 h–21 h	2 d	13 h to 21 d
		Night	High	0–2 d	13 to 2 d	ND	2–21 d
		Morning	High	0–7 d	13 to 7 d	ND	7–21 d
	6	Night	Low	0–13 h	ND	10 h to 7 d	7–14 d
		Night	High	0–19 h	ND	19 h to 7 d	14 d
		Morning	High	0–8 h	ND	8 h to 7 d	14 d

[a] Lettuce plants were inoculated at high (7 log CFU/plant) and low (5 log CFU/plant) levels.

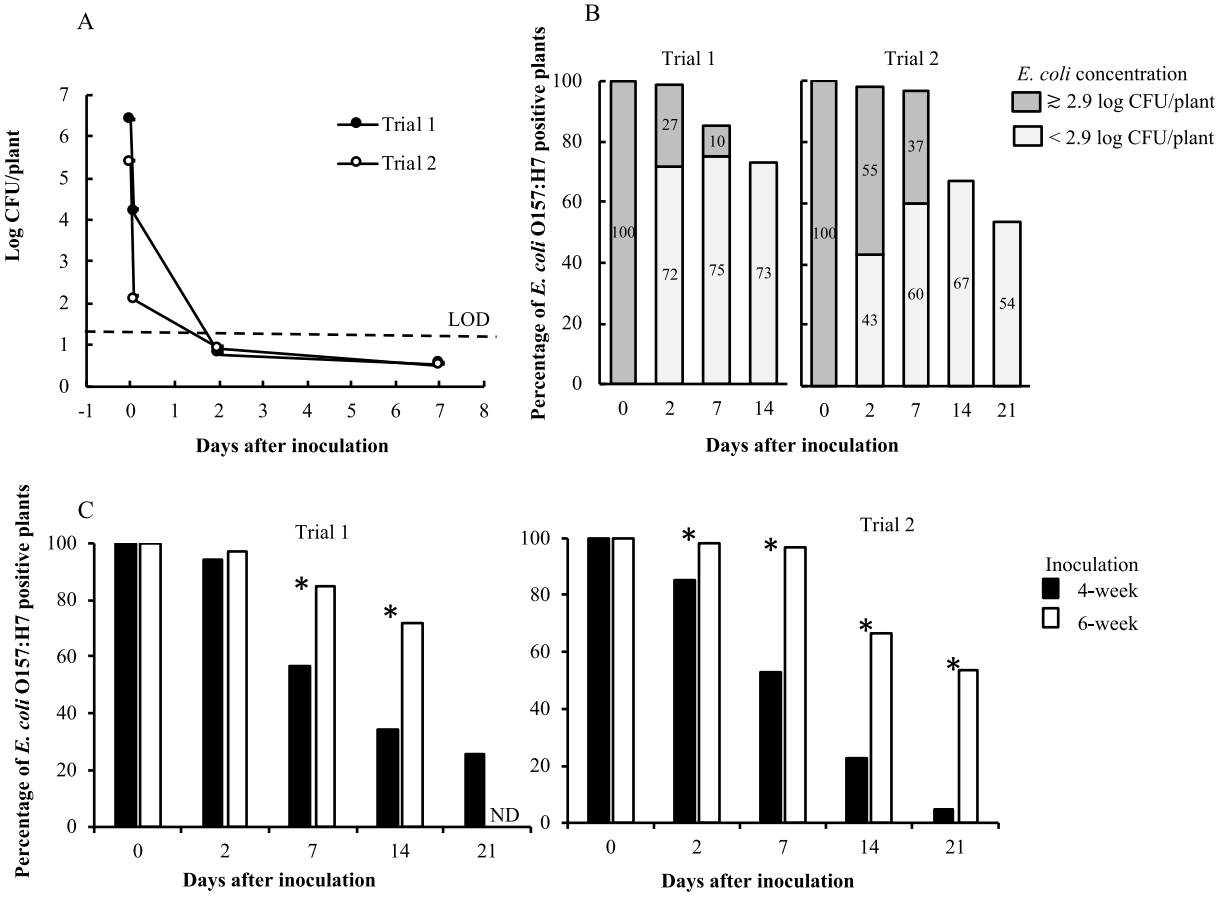

Fig. 1. Survival of *E. coli* 700728 after inoculation on 4-week-old (A) or 6-week-old (B) lettuce plants, and prevalence (C) in trials 1 and 2. *E. coli* 700728 was inoculated at the high initial level in the morning. In A, each point represents the average concentration ± SE; the limit of detection (LOD) was 20 (1.3 log) and 10 (1.0 log) CFU/plant for trial 1 and 2, respectively. In B, after inoculation of 6-week-old lettuce plants, the percentage of *E. coli* O157:H7–positive plants was divided in two categories of plants, based on a concentration of *E. coli* 700728 higher or lower than the LOD by plating (2.9 log CFU/plant). In C, the asterisk, *, indicates significant difference ($P < 0.05$) between inoculation of the 4- and 6-week-old plants within a trial, as determined by Pearson's chi-square test. ND: not done. $n = 60$ at day 0, and $n = 120$ from day 2–21 after inoculating 4-week-old lettuce; $n = 30$ at day 0, and $n = 60$ from day 2–21 after inoculating 6-week-old lettuce.

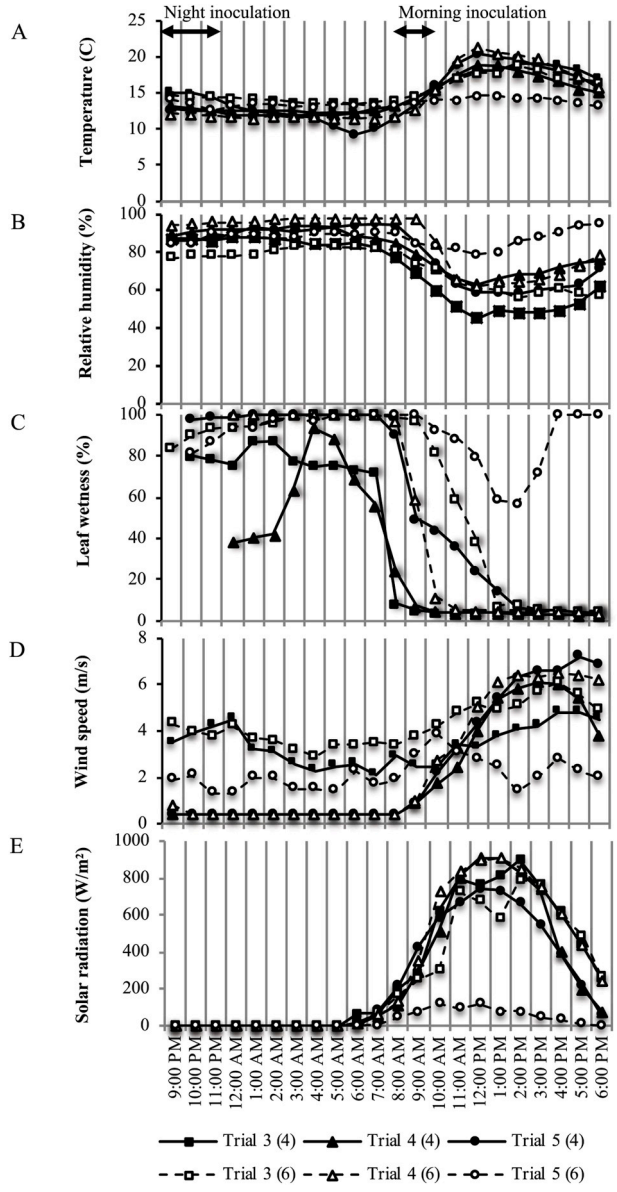

Fig. 2. Temperature (A), relative humidity (B), leaf wetness (C), wind speed (D), and solar radiation (E) recorded during the first day after inoculation of 4-week-old (solid line) or 6-week-old (dashed line) lettuce plants in field trials 3 to 5. Different plants were inoculated at night or in the morning of the next day (Table 1).

(CHROMagar) and incubated at 37 °C for 24 h.

2.5. MPN procedure

MPN methods were developed to quantify low bacterial levels when no counts were obtained by direct plating and sample filtration could not be used. Lettuce collected after the 6-week inoculation, during trials 3 to 5, was processed by stomaching in larger volumes of media (100–1000 ml), which generated considerable debris that clogged the filters. Since the limit of detection by plating was high (between 800 [2.9 log] to 4000 [3.6 log] CFU/plant), three different MPN protocols were used, which resulted in a better estimate of *E. coli* 700728 cell density and lowered the limit of detection to 1 CFU/plant. The cell concentration estimates ranged from 410 to 20,350 cells/100 ml with the first protocol, and from 1 to 2000 cells/100 ml with the second protocol. A third protocol was used to quantify cell concentrations lower than 1 cell per 100 ml when a volume > 100 ml was used to retrieve the inoculated bacteria on the entire lettuce head.

For the first protocol, each sample was serially diluted from 10^{-1} to 10^{-4} in a 96-well microtiter plate prefilled with 180 µl of TSBR per well. The first dilution was obtained by adding 20 µl of undiluted sample into each of the 12 wells in the first row. Ten-fold serial dilutions were made by transferring 20 µl from each well in the first row to the corresponding well in the second row with a multichannel pipette, and then mixing the well contents four times. The process of transferring 20 µl and mixing was repeated twice. Pipette tips were changed between each dilution. Two different samples were loaded per plate. The second protocol was an adaptation of the MPN method used in the Quanti-Tray system (Idexx, Westbrook, ME): 200-µl and 2-ml aliquots of sample were distributed with a repeater (Eppendorf, Hauppauge, NY) into 48 wells of a 96-well plate with volume capacities of 350 µl and 2.2 ml per well, respectively. Finally, the third protocol involved the distribution of the entire cell suspension in 2-ml aliquots per well of a 2.2-ml capacity 96-well plate.

All 96-well plates were sealed with microplate adhesive film, and plates were incubated at 42 °C for 24 h. After incubation, the adhesive film was removed, and the enrichment broth was transferred with a 96-pin sterile replicator (Phenix Research Products, Candler, NC) to a 96-well plate containing 100 µl per well of CHROMagar O157 supplemented with rifampicin at 50 mg/L; plates were sealed and incubated at 37 °C for 24 h. Positive wells (mauve color) were scored.

The Thomas approximation (Blodgett, 2010) was used to estimate *E. coli* populations for the first and second protocol:

$$\mathrm{MPN/ml} = \mathrm{P}/\sqrt{\mathrm{NT}}$$

where P is the number of positive wells, N is the total sample volume (ml) of all negative tubes, and T is the total sample volume (ml) in all tubes.

The Poisson formula was used to estimate *E. coli* 700728 populations for the third protocol:

$$\mathrm{MPN/ml} = -2.303/\mathrm{V}\ \log(\mathrm{S/N})$$

where V is the volume of sample tested (2 ml), S is the number of negative wells, and N is the total number of wells.

The accuracy of the MPN procedure was evaluated by comparing the population density estimated by MPN with the enumeration of diluted pure culture of *E. coli* 700728 in a preliminary experiment in the laboratory (results not shown) and on selected inoculated lettuce plants collected during trials 3 to 5 (Supplementary data, Table S6). Paired *t*-tests indicated that differences between calculated MPN and plate counts were not significant ($P > 0.05$).

2.6. Environmental factors

For the duration of each trial, leaf wetness, temperature, and humidity measurements were recorded every 15 min with a HOBO weather station data logger (Onset, Bourne, MA) located within the field. The leaf wetness smart sensor (Onset), and air temperature and relative humidity sensors were located at heights of 1.2, 1.0 and 0.80 m above the ground, respectively. Precipitation, wind speed, and solar radiation data were retrieved from the Salinas South weather station (California Irrigation Management Information System, CIMIS #89) through the University of California Integrated Pest Management website (www.ipm.ucdavis.edu/index.html).

2.7. Data analysis

The detection limit by direct plating varied with the lettuce head weight and the amount of 0.1% peptone used to recover the inoculated bacteria, from a minimum of 200 CFU/plant (2.3 log CFU/plant) to a maximum of 4800 CFU/plant (3.7 log CFU/plant). Filtration improved the limit of detection to 10, 20, or 40 CFU/plant for samples collected after the 4-week inoculation (Supplementary data, Tables S2, S3, S4,

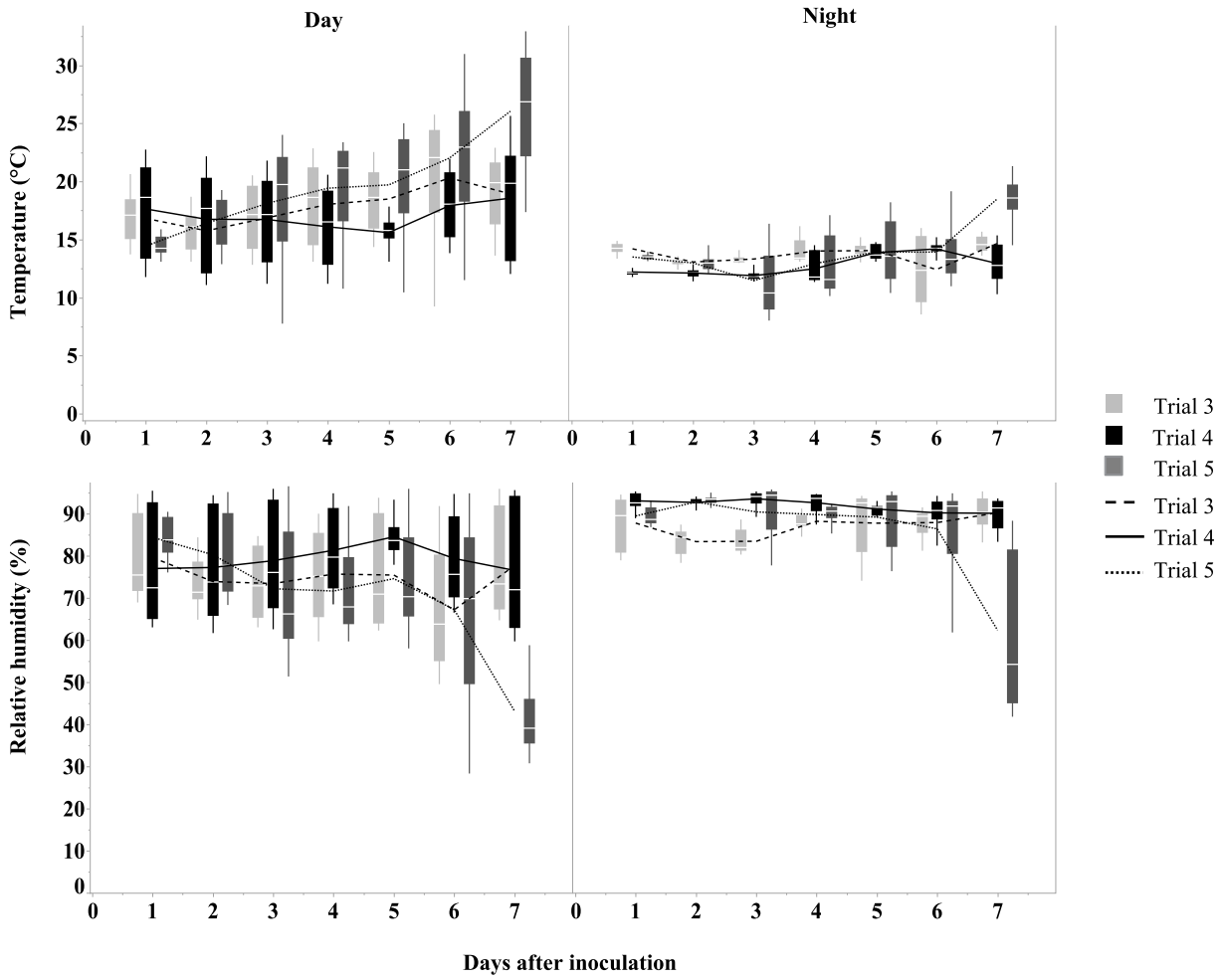

Fig. 3. Temperature and relative humidity data recorded for 8 days after inoculation of 6-week-old lettuce plants in field trials 3 to 5. Data are presented in a boxplot graph for day and night separately; the box denotes the 25th and 75th percentile and the whiskers denote the minimum and maximum values of all the data. The line connects the average temperature or relative humidity by day.

and S5). The MPN methods improved the limit of detection to 1 CFU/plant for samples collected after the 6-week inoculation, in trials 3 to 5. When *E. coli* 700728 was not detected by plating, filtration, MPN, or whole sample enrichment, the sample counts were treated statistically as zero. When *E. coli* 700728 was detected only by whole sample enrichment, a value just below the limit of detection was assigned for calculation of the mean. Microbial data (CFU/plant) were log transformed before statistical analysis with JMP software (SAS Institute Inc., Cary, NC). Analysis of variance was used to compare *E. coli* 700728 population size between inoculated plants that were irrigated by drip or overhead sprinkler at each time point during trials 1 and 2; population size was not significantly different ($P > 0.05$) and, therefore, counts from both irrigation treatments were combined and averaged. Pearson's chi-square test and two-tailed Fisher's exact test were performed to compare the distribution of plants that tested positive by enrichment after inoculation at night or in the morning and after the 4-week or 6-week inoculations. Relative humidity and temperatures recorded over the first week after the 6-week night inoculation in trials 3 to 5 were compared with a one-way ANOVA followed by Tukey's HSD.

3. Results

3.1. Influence of plant age on E. coli 700728 survival in trials 1 and 2

To compare the survival of *E. coli* 700728 on lettuce plants of different ages, in trials 1 and 2, the same number of bacteria (target 7 log CFU/plant) were inoculated in the morning onto separate individual plants at 4 or 6 weeks after seeding (Table 1). The average bacterial cell numbers, recovered immediately after inoculation, were 6.39 ± 0.03 (trial 1) and 5.35 ± 0.08 (trial 2) log CFU per 4-week-old plants, and 6.33 ± 0.04 (trial 1) and 6.97 ± 0.03 (trial 2) log CFU per 6-week-old plants (Supplementary data, Tables S2 and S3).

The limit of detection by plating was higher for the 6-week-old plants (800 [2.90 log] CFU/plant) than for the 4-week-old plants (20 [1.30 log] CFU/plant) because filtration could not be used for the more mature, larger heads of lettuce. Stomaching 6-week-old lettuce heads generated greater amounts of debris that clogged the filter. When the concentration of *E. coli* 700728 was under the limit of detection by plating or filtration, the remaining sample was enriched.

Two days after inoculating 4-week-old lettuce, the average *E. coli* 700728 populations were 0.79 ± 0.05 and 0.29 ± 0.07 log CFU/plant in trials 1 and 2, respectively (Fig. 1A). The *E. coli* 700728 concentration was at or above 2.90 log CFU/plant for only 1% (1/120; trial 1) and 3% (4/120; trial 2) of plants. In contrast, 2 days after inoculating 6-week-old plants, the *E. coli* 700728 concentration was at or above 2.90 log CFU/plant in 27% (16/60; trial 1) and 55% (33/60; trial 2) (Fig. 1B).

By day 14, the inoculated bacteria could only be recovered by enrichment. However, the percentage of *E. coli* 700728–positive plants was significantly higher ($P < 0.05$) among plants inoculated with the

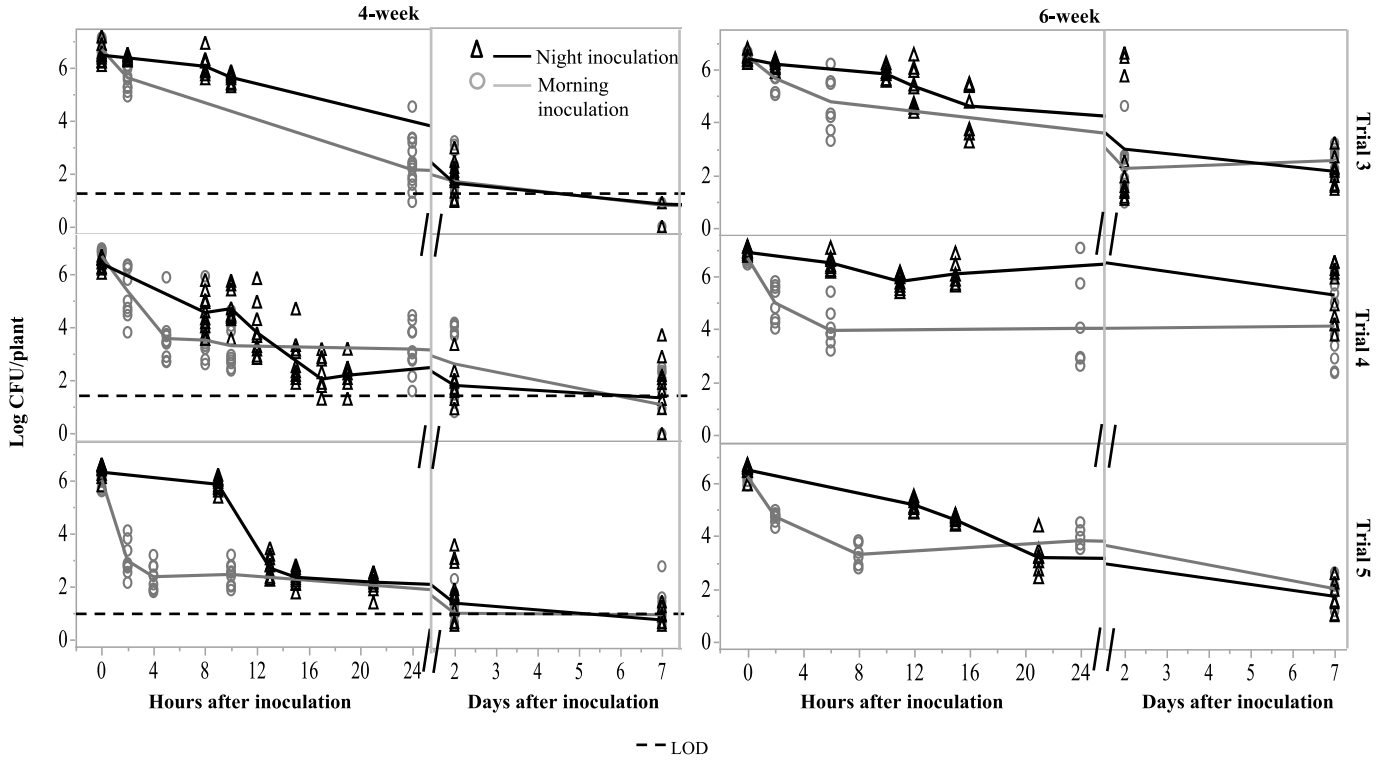

Fig. 4. Influence of the time of inoculation (night or morning) and lettuce age on *E. coli* 700728 survival in trials 3 to 5. *E. coli* 700728 was inoculated onto 4-week-old or 6-week-old lettuce plants at the high initial level at night (black line and circles) and in the following morning (grey line and triangles). Each symbol represents the concentration of *E. coli* 700728 determined for one sample. The line connects the mean concentration by time point. The limit of detection (LOD) for the inoculation of 4-week old plants was 1.3 (trial 3), 1.6 (trial 4), and 1.0 (trial 5) log CFU/plant; the LOD for inoculation of 6-week old plants was 1 MPN/plant in trials 3 to 5.

organism at 6 weeks than at 4 weeks after planting. Fourteen days after inoculation, 34% (trial 1) and 23% (trial 2) of the 4-week inoculated lettuce plants were positive, compared with 72% (trial 1) and 67% (trial 2) of the 6-week inoculated lettuce plants (Fig. 1C; Supplementary data, Table S7). At day 21, the percentage of positive plants was still high (54%) on plants inoculated at 6-weeks (trial 2). Corresponding data were not determined in trial 1.

3.2. Environmental factors

Temperature, relative humidity, leaf wetness, wind speed, and solar radiation were recorded for all trials throughout their duration. To compare the environmental conditions following night or morning inoculation, these measurements, over the first 20 h after inoculation in trials 3 to 5, are presented in Fig. 2. For all sampling times except after the 6-week inoculation in trial 5, temperatures were cooler (10–15 °C) (Fig. 2A) and relative humidity was higher (80–100%) (Fig. 2B) at night. In trial 5, after the 6-week inoculation the temperature and humidity remained constant from night to day and minimal solar radiation was recorded. During the day, temperature increased to a maximum of 19–21 °C and relative humidity decreased to a minimum that ranged from 45 to 62% between 1 and 2 p.m.

No rainfall was recorded after inoculation during any of the field trials. However, 5 mm of rain was recorded just before the 4-week night inoculation in trial 3; the lettuce was inoculated 1 h after the rain had stopped. For all other night inoculations, water was visible on the lettuce leaves at the time of inoculation, and leaf wetness was high throughout the night (Fig. 2C). Dew disappeared within a few hours after sunrise for all inoculations except in trial 5 where dew persisted throughout the day after the 6-week inoculation. Among all the environmental data recorded, humidity was the highest and temperature, wind speed, and solar radiation were the lowest during the day after the 6-week inoculation in trial 5 (Fig. 2). The wind speed typically increases during the afternoon in the Salinas Valley; maximum speeds were observed between 1 and 6 p.m. (Fig. 2D).

Temperature and relative humidity data recorded during the first week after the 6-week night inoculation in trials 3 to 5 are presented in Fig. 3. Temperatures and relative humidity levels, recorded during the first week after inoculating 6-week-old plants, were statistically different among the three trials (ANOVA, $P < 0.05$). Lower temperatures and higher relative humidity levels were recorded in trial 4 compared with trials 3 and 5 (Tukey's HSD test, $P < 0.05$).

3.3. Influence of environmental conditions and plant age at time of inoculation on survival of E. coli 700728

The survival of *E. coli* 700728 following night or morning inoculation of 4- or 6-week-old lettuce plants was compared in trials 3, 4, and 5. To overcome cell enumeration limitations encountered in trials 1 and 2, an MPN method was added to estimate numbers of surviving *E. coli* 700728 on plants inoculated 6 weeks after planting. To facilitate quantification, a high level (target 7 log CFU/plant) inoculum was applied. The average total population effectively delivered per plant, estimated by collecting lettuce heads immediately after spraying, ranged from 6.06 to 6.89 log CFU (Supplementary data, Tables S2, S3, S4). Because the lettuce weight increased with plant maturity (Supplementary data, Table S1), the population delivered ranged from 5.15 to 5.77 log CFU/g on the 4-week-old plants and from 4.20 to 4.62 log

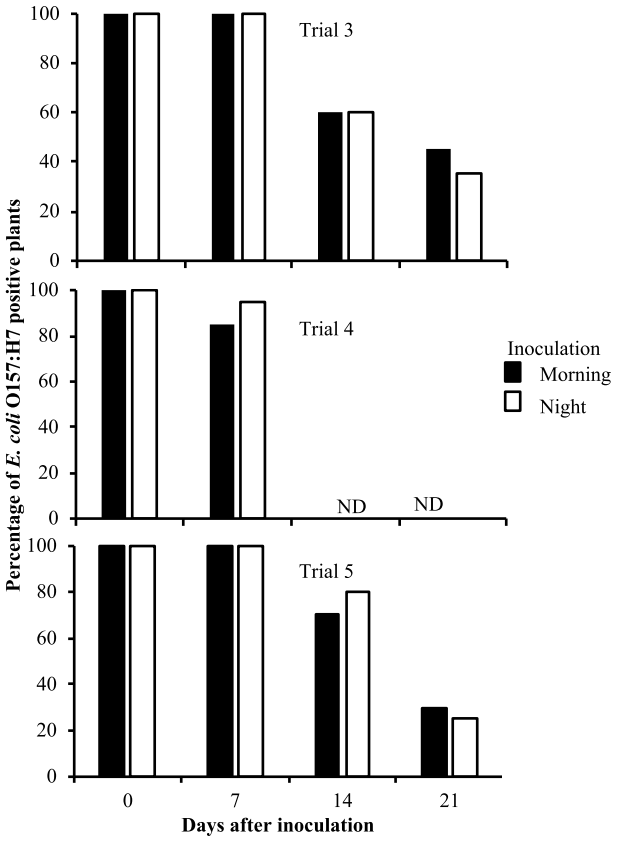

Fig. 5. Influence of time of inoculation (night or morning) on *E. coli* 700728 prevalence at 0, 7, 14, and 21 days post-inoculation in trials 3 to 5. Data are presented only for inoculation of *E. coli* 700728 at the high initial level onto 4-week old plants. (For the night or morning inoculation of 6-week old plants, all plants were positive for *E. coli* 700728 at days 7 and 14 for trials 3 to 5; see Supplemental data, Table S7). $n = 10$ at day 0, and $n = 20$ at days 7–21.

CFU/g on the 6-week-old plants.

When lettuce was inoculated at night, *E. coli* 700728 populations declined by 0.5–2 log CFU/plant in the first 8 h (Fig. 4; Supplementary data, Table S4). In contrast, when lettuce was inoculated in the morning, *E. coli* 700728 populations declined by 1–3 log CFU/plant in the first 2 h (Fig. 4; Supplementary data, Tables S2 and S3).

In trials 4 and 5, the number of sampling time points was increased on the first day after the morning inoculation to directly compare the die-off of the *E. coli* 700728 population between the freshly inoculated bacteria and the bacteria that survived the night inoculation. The daytime die-off was similar between these two populations, with the exception of the 6-week night inoculation in trial 4. For this trial, when 6-week-old plants were inoculated at night, no significant reductions in the *E. coli* 70028 population were observed during the day after inoculation, and numbers remained high when tested 7 days later (average of 5.29 and 4.12 log CFU/plant for night and morning inoculation, respectively) (Fig. 4). For trials 3 and 5, similar populations of *E. coli* 700728 were recovered 2 and 7 days after either night or morning inoculation (Fig. 4).

Overall, environmental conditions at the time of inoculation (e.g., night or morning) did not influence the long-term survival of *E. coli* 700728. The percentage of *E. coli* 700728–positive plants inoculated at night or in the morning was similar from 7 days post-inoculation through the end of the field trials (no significant differences as determined by Pearson's chi-square test) (Fig. 5; Supplementary data, Table S7). However, plant age did influence the survival of *E. coli* 700728. At day 7 after inoculating 4-week-old plants, the average *E. coli* 700728 population was under the limit of detection by plating and filtration, and *E. coli* 700728 was detected mainly by enrichment regardless of the time of inoculation (Fig. 4; Supplementary data, Tables S2 and S4). In comparison, average *E. coli* 700728 populations 7 days after application onto 6-week-old plants were 2.14 ± 0.16 (trial 3) and 1.74 ± 0.19 (trial 5) log CFU/plant for inoculations performed at night, and 2.55 ± 0.14 (trial 3) and 2.02 ± 0.15 (trial 5) log CFU/plant when *E. coli* 700728 was applied in the morning (Fig. 4; Supplementary data, Tables S3 and S4).

3.4. Influence of initial inoculum level on E. coli 700728 survival

Inoculum level was compared only for the night inoculation. *E. coli* 700728 was inoculated onto lettuce plants at low (5 log CFU/ml) and high (7 log CFU/ml) numbers in trials 3, 4, and 5 onto both 4- and 6-week-old plants (Table 1). The amount of inoculated bacteria recovered after inoculation at the low inoculum level ranged from 3.91 to 5.40 log CFU per plant (Fig. 6A; Supplementary data, Table S5). Because the lettuce weight increased with the plant development (Supplementary data, Table S1), the population delivered ranged from 2.81 to 3.45 log CFU/g on the 4-week-old plants and from 0.81 to 2.47 log CFU/g on the 6-week-old plants. As observed for the high initial inoculum level applied at night, smaller population declines occurred during the first 8 h after inoculation (0.13–1.11 log CFU/plant). This was followed by a more rapid decline during the subsequent 12 h of daylight (Fig. 6A). The average population remaining on the 4-week-old plants had reached the limit of detection by filtration (< 20 cells per plant) 21–32 h after inoculation for all trials. In contrast, on 6-week-old plants the average population was 2.40 ± 0.18 log CFU/plant at 36 h after inoculation in trial 5 (data not available for trials 3 and 4) (Fig. 6A).

At day 7 post-inoculation in trials 3 and 4, a significantly smaller percentage of plants inoculated 4 weeks after planting (0 and 12.5%, respectively) were positive for *E. coli* 700728 compared with those inoculated at 6 weeks (50 and 90%, respectively) (Fig. 6B). However, in trial 5, plant development had no effect on the percentage of plants that were positive for *E. coli* 700728 at 7 and 14 days post-inoculation (Fig. 6A). Overall, a higher percentage of *E. coli* 700728–positive plants at each time point was observed when plants were inoculated at the higher initial inoculum concentration (Figs. 5 and 6B, Supplementary data, Table S7).

3.5. Indigenous bacterial and coliform populations in the phyllosphere

Aerobic indigenous bacterial populations were quantified for all trials, but results are only presented for trials 3 to 5 since the results for trials 1 and 2 were published separately (Williams et al., 2013). Average aerobic bacteria populations on the lettuce ranged between 4.25 and 7.38 log CFU/g (Table 3), with lower counts observed in trials 3 and 4 than in trial 5. Total *E. coli* and coliforms were enumerated for lettuce samples collected in trials 4 and 5 only. *E. coli* was either not recovered or was under the limit of detection (1.99 log CFU/g) for samples collected during trial 5, and was isolated from 2 out of 30 lettuce samples (1.99 and 3.07 log CFU/g) during trial 4 (data not shown). Coliforms were detected in all lettuce samples from trial 5, with a range of 3.79–5.87 log CFU/g; whereas in trial 4, fewer plants with coliform populations higher than the limit of detection (1.99 log CFU/g) were detected but the number increased with lettuce maturation (Table 3).

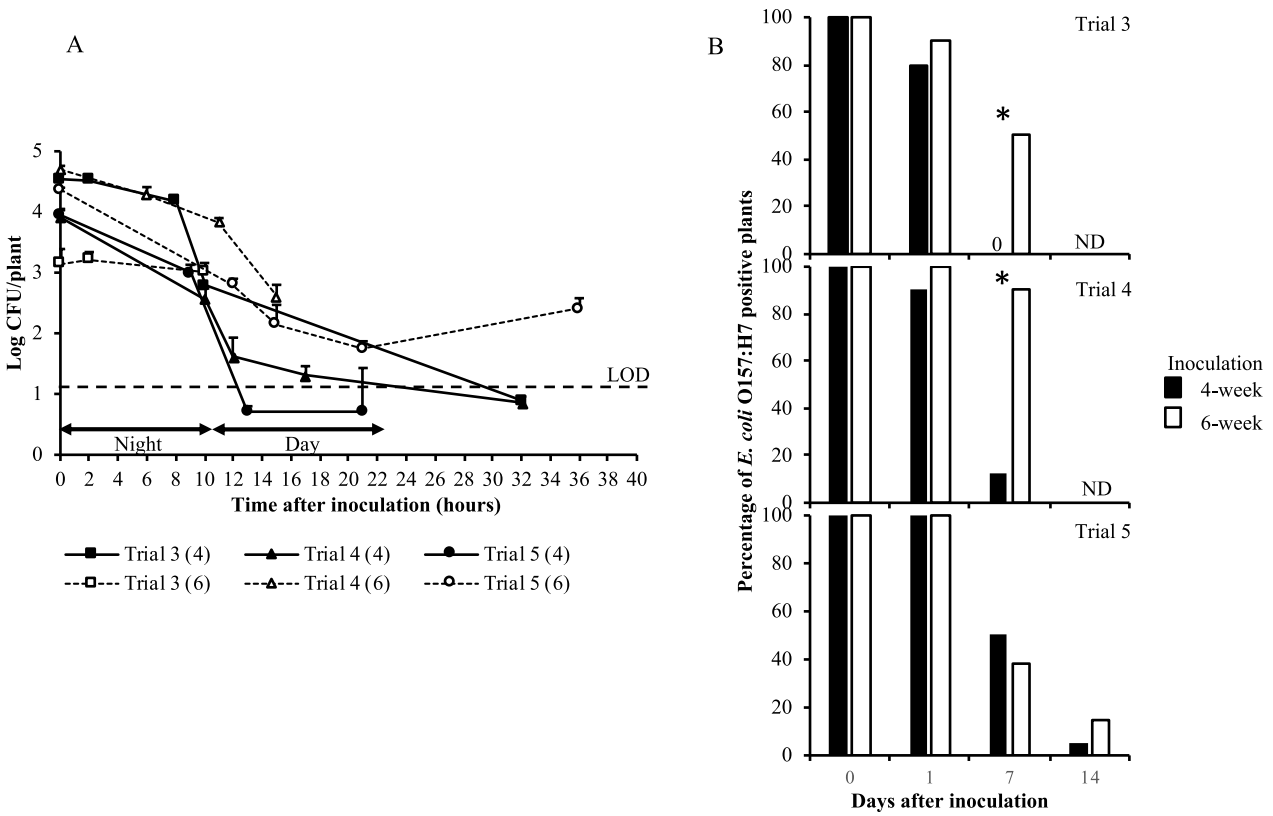

Fig. 6. Survival of *E. coli* 700728 on lettuce plants after inoculation at the low initial level, as measured by enumeration, filtration, and MPN up to 36 h after inoculation (A) and prevalence up to 14 days (B). *E. coli* 700728 was inoculated at night onto 4-week-old (solid line) or 6-week-old (dashed line) lettuce plants in field trials 3 to 5. In A, each data point represents the mean population of *E. coli* 700728 (log CFU/plant) ± SE; $n = 8$ or 10. The dotted line indicates limit of detection, LOD (20 [1.30 log] CFU/plant). In B, the asterisk, *, indicates a significant difference ($P < 0.05$) between 4-week-old and 6-week-old plants within a trial, as determined by Pearson's chi-square test. ND: not done. Sample numbers after inoculating 4-week-old lettuce were as follows: $n = 10$ at day 0, $n = 20$ at day 1, and $n = 40$ at day 7 for trial 3; $n = 10$ at days 0 and 1, and $n = 40$ at day 7 for trial 4; $n = 10$ at days 0 and 1, and $n = 20$ at days 7 and 14 for trial 5. Sample numbers after inoculating 6-week-old lettuce were as follows: $n = 8$ at days 0 and 1 for all trials; $n = 24$ (trial 3), 10 (trial 4), and 8 (trial 5) at day 7; $n = 20$ at day 14 for trial 5.

Table 3
Total aerobic bacteria and coliform counts in non-inoculated lettuce during trials 3 to 5.

Time post-inoculation	Trial 3	Trial 4			Trial 5	
	Aerobic bacteria (log CFU/g)[a]	Aerobic bacteria (log CFU/g)	No. of samples with coliforms	Coliforms (log CFU/g)[b]	Aerobic bacteria (log CFU/g)	Coliforms (log CFU/g)
0 h	4.73 ± 0.06	4.72 ± 0.06	ND[d]		5.28 ± 0.13	3.79 ± 0.13
2 d	4.34 ± 0.16	4.48 ± 0.14	0		ND	ND
6 or 7 d[c]	4.93 ± 0.19	4.25 ± 0.07	3	1.99, 1.99, 2.46	6.01 ± 0.14	5.01 ± 0.14
14 d	4.93 ± 0.19	4.61 ± 0.09	6	1.99, 3.11, 4.02, 2.92, 2.54, 2.49	6.10 ± 0.11	5.87 ± 0.11
21 d	5.29 ± 0.12	ND	ND		ND	ND
30 d	ND	ND	ND		7.38 ± 0.17	5.02 ± 0.17
37 d	ND	ND	ND		5.70 ± 0.22	5.20 ± 0.22

[a] Values represent mean population ± SE; $n = 8$ at day 0 and 2 during trial 4, and $n = 10$ for all other sampling times.
[b] Values represent the log CFU/g for each lettuce sample in which coliforms were found.
[c] Sampling was done at day 7 in trials 3 and 4, and at day 6 in trial 5.
[d] ND, not done.

4. Discussion

Lettuce is generally planted in the Salinas Valley from December through October, with harvests from March through November (Smith et al., 2010). Multiple studies support the conclusion that *E. coli* O157:H7 populations decline after introduction in the phyllosphere under field conditions but survive at very low levels (at the LOD [1 log CFU/g to 1 log CFU/100 g] or under the limit of detection by plating but detected by enrichment) for long periods of time (Bezanson et al., 2012; Erickson et al., 2010; Fonseca et al., 2011; Moyne et al., 2011; Solomon et al., 2002a; Wood et al., 2010). The data presented here provide evidence that, for short periods of time, a contamination event occurring under cool conditions with high humidity (e.g., at night), or onto older lettuce plants, can result in higher amounts (~5 log CFU/plant 7 days after inoculation at ~7 log CFU/plant or < 2 log CFU/plant decline) of *E. coli* O157:H7 recovered per plant and a higher incidence of contaminated plants over longer periods than previously reported, even when the inoculated bacteria are introduced at relatively

low initial levels.

Leaf surfaces represent a presumably hostile environment for bacteria due to the cyclic availability of free moisture and the limited access to nutrients (Lindow and Brandl, 2003). The rapid decline in populations observed when *E. coli* 700728 was inoculated in the morning might be due to an increase in sunlight radiation, which is known to have bactericidal properties, coupled with corresponding desiccation due to the observed decreases in relative humidity and increases in wind speed and temperature. Although most leafy green irrigation takes place during the day it may continue into the night. In the Salinas Valley most of the lettuce is drip irrigated after thinning, which would minimize contact of water with the leaves. However, drip tape needs appropriate maintenance. Damaged drip tape that allows localized release of water can lead to direct application of irrigation water to lettuce plants in close proximity. Holes in surface drip tape can result in forceful water sprays that can cause significant wetting of foliage. Water, used as a diluent, may also contact lettuce leaf surfaces through aerial or land-based spray application of crop protection chemicals.

Under laboratory-controlled conditions, *E. coli* O157:H7 grew on lettuce when temperatures were warm (28 °C) and free water was available on the plant (Brandl and Amundson, 2008). In the current study, favorable conditions for bacterial survival were recorded at night, when humidity was high, temperatures were low, and dew or rain events provided free water on the plant surfaces. The negative effects of solar radiation on bacterial survival have been reported for *E. coli* inoculated on spinach, where the populations were always higher on spinach grown under shaded screening than on spinach grown unprotected (Wood et al., 2010), and for inoculated *E. coli* O157:H7 on the abaxial side of lettuce leaves (Erickson et al., 2010). In the Salinas Valley, the dry season typically occurs between May to October and rainfall is rare during this period. All field trials in this study occurred during the dry season; however, rain did fall up to 1 h before the night inoculation of 4-week-old plants in trial 3. After this inoculation, *E. coli* O157:H7 populations remained stable, with a decline of 0.5 log CFU/plant within 8 h, regardless of the inoculum level.

Overall, *E. coli* 700728 populations declined slowly during the night when inoculated at high initial cell numbers, except in trial 4 when a decline of 1.8 log CFU/plant was measured at 8 h on 4-week-old plants. Despite very similar cool temperatures and high relative humidity during the night hours that followed the inoculations in trials 3, 4, and 5, leaf wetness was lowest (38%) after the 4-week inoculation in trial 4. For all other inoculations, free water was available on the plant surface throughout the night, with high leaf wetness (80–100%) recorded from 10 p.m. to 7 a.m. High wind speeds (4 m/s) recorded after the 4-week and 6-week inoculations in trial 3 did not impact the survival of *E. coli* 700728 during the night. The availability of water has been shown to be one of the most important factors for the establishment and maintenance of epiphytic bacterial populations (Beattie, 2011; Monier and Lindow, 2005), and in the current study *E. coli* 700728 populations remained stable during the night when free water was present on the leaf surfaces. However, the bacteria introduced during the night did not maintain high populations during the following day for most of the field trials. As a result, population sizes were very similar 2 days after being inoculated onto lettuce plants during the night or day, and the probability of detecting *E. coli* 700728 in the phyllosphere was similar at later sampling time points. The trial 4 results were unique, as *E. coli* 700728 populations remained high 7 days after the 6-week inoculation. Declines of 1.61 and 2.59 log CFU/plant between inoculation and day 7 were observed after night and morning inoculation, respectively, on plants inoculated with the high number of cells (7 log CFU/plant). Higher relative humidity levels and lower temperatures were observed during the first week after inoculation in trial 4 compared with trials 3 and 5.

To compare the population dynamics between inoculations under different conditions, *E. coli* 700728 was inoculated onto plants at levels not usually encountered in contaminated water; however, a lower inoculum level was also applied during the night inoculation. Under the environmental conditions experienced during our trials, *E. coli* 700728 populations did not increase during the night, at either inoculum level, in part because temperatures were lower than 15 °C. Although *E. coli* 700728 was detected for a longer period of time when inoculated at a high rather than at a low initial concentration, the inoculated bacteria were detected in 15% (3/20) of plants at 14 days after inoculation onto 6-week-old plants at the low inoculum level in trial 5. The percentage of *E. coli* 700728–positive plants was higher at all sampling time points after inoculating 6-week-old plants than after inoculating 4-week-old plants. Erickson et al. (2010) reported that *E. coli* O157:H7 was detected in spinach 7 days after inoculation only when the concentrations exceeded 6 log CFU/ml in the irrigation water (~3 log CFU/g of spinach); *E. coli* O157:H7 could not be retrieved by enrichment immediately after inoculation in spinach samples when the bacterial concentration in contaminated water was 2 log CFU/ml, and 6 out of 20 samples (30%) tested positive for *E. coli* O157:H7 when the bacterial concentration in contaminated water was 4 log CFU/ml. Hutchison et al. (2008) detected *E. coli* O157:H7 in lettuce and spinach 7 days after inoculation only when the contaminated water had an initial concentration of 10^5 CFU/ml. The inoculated bacteria were not recovered 7 days after inoculation when an initial inoculum of 10^2 CFU/ml was applied, although at 2 h post-inoculation the population was 4.1 log CFU/g of lettuce. In comparison, in trial 3 of the current study, *E. coli* 700728 was detected by enrichment in 50% of lettuce plants after the 6-week-old plants were inoculated with a low initial concentration of the bacteria (5 log CFU/plant or 0.85 log CFU/g).

The age of the lettuce plant had a greater influence than the time of day on the persistence of the inoculated pathogen in the field. Overall, plants inoculated closer to harvest sustained a higher *E. coli* 700728 population for at least 1 week compared with plants inoculated closer to planting, and a higher prevalence of positive plants at the end of the field trial. Four-week-old romaine lettuce plants typically have a rosette-shaped circular cluster of 8–12 leaves; by 6 weeks the plants have a denser center with larger overlapping leaves that allow for more free water to accumulate at the base. Bacteria are likely more exposed to environmental stresses (e.g., desiccation, UV light) on the leaves of 4-week-old plants than on the leaves of 6-week-old plants.

An increase in spinach leaf roughness was correlated with an increase in the ability of bacteria to attach to leaf surfaces, resulting in enhanced survival of *E. coli* O157:H7 (Macarisin et al., 2013). Similar results were reported for the adhesion of *Salmonella* Enteritidis to lettuce leaves (Lima et al., 2013). Limited nutrients or differences in leaf exudates could also play a role in the survival of the bacteria. Brandl and Amundson (2008) demonstrated that *E. coli* O157:H7 populations grew to higher levels on young (inner) leaves than on middle leaves of potted mature lettuce plants in the greenhouse or on harvested field-grown romaine lettuce heads; nitrogen was identified as a limiting factor of *E. coli* O157:H7 growth on middle leaves.

In previous studies that included trials 1 and 2 described here, bacterial populations and diversity in the lettuce phyllosphere were quantified and identified by culture, real-time PCR, and next-generation sequencing (Williams and Marco, 2014; Williams et al., 2013). Season, irrigation, and plant age influenced the diversity of indigenous bacterial populations in the lettuce phyllosphere (Williams et al., 2013), which may have also influenced the survival of inoculated *E. coli*.

The current study identified environmental conditions that increase the survival of *E. coli* O157:H7 under commercial lettuce field production practices in the Salinas Valley. High humidity and cooler temperatures at night favored the formation of free water on the lettuce leaves and may have contributed to the short-term enhanced survival of *E. coli* 700728 inoculated at night. The age of the lettuce had a more pronounced effect on the survival of a higher population of inoculated bacteria on plants, which resulted in a higher incidence of positive plants over a longer period. As lettuce heads reach maturity, a contamination event near harvest, or at night, or under conditions of high

humidity or rain, may represent a risk factor (enhanced survivability) even when low levels of pathogen are introduced in the lettuce phyllosphere.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.103274.

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