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Impact of relative humidity, inoculum carrier and size, and native microbiota on *Salmonella* ser. Typhimurium survival in baby lettuce

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ABSTRACT

The effects of relative humidity (RH), fluctuating climate conditions, inoculum size and carrier on the survival of *Salmonella enterica* serovar Typhimurium on baby lettuce in environmental test chambers were studied. Buffered peptone water (BPW), distilled water (DW), and irrigation water (IW) were compared as inoculum carriers. Additionally, survival of *Salmonella* in suspensions prepared using filtered and unfiltered IW was assessed. *Salmonella* Typhimurium survived better on baby lettuce plants at high RH independently of the inoculum size. When lettuce plants were grown under fluctuating environmental conditions, *Salmonella* survival was similar under both RH conditions. Regarding the inoculum carrier, the inoculated microorganism survived better on lettuce plants when BPW was used as carrier both at high and low RH. Survival rate of *Salmonella* in IW was affected by the presence of native microbiota. Native microbiota present in IW did not affect survival of *Salmonella* or the levels of mesophilic bacteria on the baby lettuce leaves. The information obtained in the present study contributes to the knowledge on the effect of environmental conditions on pathogenic bacteria survival on growing edible plants. These results are useful when selecting the methodology to carry out experimental studies on the survival of microbial pathogens under different pre-harvest conditions.

1. Introduction

Foodborne outbreaks linked to the presence of *Salmonella* in leafy vegetables have been reported in recent years in Europe (EFSA, 2013). Within the vectors that can facilitate contact of enteric bacteria with plants, water is probably the main vehicle for the transmission of pathogens from faeces to the environment and then to the crop (Barak and Schroeder, 2012). Once they get in contact with the plant tissue, enteric bacteria can attach and persist for significant periods of time (Solomon and Sharma, 2009). Behaviour of enteric pathogens in water used for irrigation is relevant (Pachepsky et al., 2011) and also the interaction with naturally occurring microbiota. For the standardization of protocols, differences in the inoculum carriers can impact the dissemination and survival of pathogens in the plant and in irrigation water (Van Der Linden et al., 2014a). Ideally, irrigation water should be used as a carrier for the inoculation of the pathogen, although variations in the water characteristics between different sources (channels, wells or ditch), locations and seasons make difficult to avoid variability to understand pathogen behaviour (Harris et al., 2012).

A better understanding of the impact of different climatic conditions that occur in greenhouse production, including temperature and relative humidity (RH), on the survival of foodborne pathogens is needed to determine the risk and if possible to reduce or prevent microbial growth during production (Liu et al., 2013). In a controlled laboratory experiment, Devleesschauwer et al. (2017) identified that tomatoes that were incubated at higher humidity the week prior to harvest supported significantly lower postharvest *Salmonella* proliferation than tomatoes that were incubated at lower RH.

The performance of laboratory experiments in environmental test chambers also called climate chambers has the advantage of mimicking greenhouse growing conditions. The use of pathogenic strains is possible in such environmental test chambers. Thus, plants can be artificially contaminated for a better understanding of the survival of pathogenic microorganisms under exposure to individual climate variables.

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In this study, the survival of *Salmonella* Typhimurium was assessed on baby lettuce exposed to different climate conditions and experimental choices (inoculum size, inoculum carrier) using environmental test chambers. Survival of *Salmonella* in irrigation water and the influence of native epiphytic microbiota was also studied.

2. Materials and methods

2.1. Inoculum preparation

Salmonella enterica ser. Typhimurium NCTC 12023, which contained plasmid pFVP25 providing resistance to ampicillin and green fluorescent protein expression, was kindly provided by Dr Beuzón (Beuzón et al., 2002). This strain was originally isolated from bovine septicemic liver. The strain was grown in brain heart infusion broth (BHI, Oxoid, Basingstoke, UK) containing 80 μg/mL ampicillin (Sigma-Aldrich, Saint Louis, US) at 37 °C for 20 h. The inoculum was centrifuged at 3200 *g* 10 min and washed twice using the inoculum carrier before diluting (if needed) and re-suspending in the inoculum carrier. Distilled water (DW), 0.1% buffered peptone water (BPW, Scharlab, Barcelona, Spain), and irrigation water (IW) were tested as inoculum carriers. Irrigation water was obtained from an irrigation ditch located in the village of Santa Cruz (38°01′N-01°03′W, Murcia, Spain), transported in refrigerated conditions to the lab, and used within 24 h of collection.

2.2. Plant material and inoculation

Three-week old greenhouse-grown baby romaine lettuce plants cultivated in alveolus trays (290 plants per tray) obtained from a local plant nursery (Baby Plant S.L., Santomera, Spain) were used as plant material. Trays were divided in eight portions of approximately thirty-six plants, which each constituted a replicate. Plants were inoculated by spraying with contaminated water to simulate overhead irrigation. A volume of 500 mL obtained by mixing re-suspended inoculum with inoculum carrier was poured in a 3.5 L pressure sprayer (Geolia, Lezennes, France). Preliminary trials were made to standardize the distance and the speed of movement of the spray nozzle over the plants. These preparatory trials showed that spraying for 45 s at a distance of approximately 20 cm resulted in a homogeneous distribution of the inoculum (data not shown). Two environmental chambers (model MLR-352H, Panasonic, Gunma, Japan) were used simultaneously in each test, allowing the assessment of two different environmental conditions. Three inoculated trays and one control non-inoculated tray (sprayed with non-inoculated carrier), were placed on the shelves of each environmental test chamber. Each test was executed in duplicate.

2.3. Plant growing conditions and survival test

During the experiments, plants were watered by adding water to the bottom of the trays to avoid washing the inoculated bacteria from the leaves. The volume of water added depended on the environmental conditions set in the chambers, being 200 and 300 mL/day per tray approx. for high and low RH conditions, respectively. Samples were taken the day of inoculation (zero days) and one, two, three, six, and seven days after inoculation. Three inoculated replicates (one from each inoculated tray) and two non-inoculated replicates (from the control tray) were taken from the same environmental chamber each day of analysis. Each replicate was formed of three to five plants (total weight 5–10 g) randomly and aseptically taken from the trays into sterile stomacher bags (Seward, Worthing, UK). Samples were diluted 1:10 in 0.1% BPW and homogenized for 3 min at normal speed in a stomacher (AES chemunex, Combourg, France). Serial dilutions in BPW were prepared as needed. Preliminary trials indicated that non-selective media supplemented with ampicillin (80 μg/mL) should not be used for plating due to the presence of false positives (data not shown). Salmonella-Shigella agar (Scharlab, Barcelona, Spain) supplemented with sodium pyruvate (1 g/L, Sigma-aldrich, St. Louis, US) and ampicillin (80 μg/mL) was used for plating. One mL of the appropriate dilution was plated in the selective media. The initial level of *Salmonella* in the inoculated baby lettuce was 7 log cfu/g approximately, except in the case of experiments performed to assess the effect of inoculum size where the plants were inoculated with an initial level of 4 log cfu/g. Plates were incubated at 37 °C for 24 h before counting *Salmonella* colonies. For assessment of aerobic mesophilic count, samples were plated on plate count agar (PCA, Scharlab, Barcelona, Spain) and incubated 36–48 h at 30 °C before counting.

In a first set of experiments, the effect of two RH (85% as high RH and 60% as low RH) on the survival of *Salmonella* was assessed using BPW as carrier. Conditions applied in these experiments are shown in Table 1. Experiments lasted seven days and were performed in duplicate. To assess the effect of inoculum size, plants were inoculated with 4 and 7 log cfu/g of initial inoculum levels.

In a second set of experiments, environmental conditions were arranged similarly to the meteorological data corresponding to the month of March in Murcia (37°9′ N-1°1′ W, Spain) as one of the most popular areas for the cultivation of leafy vegetables in Europe. Meteorological data were obtained from the Agricultural Information System of Murcia (SIAM). Along the 24 h, changes in the RH, temperature and lighting conditions were applied to simulate the fluctuating environmental conditions that occur in a greenhouse (Table 2).

To elucidate the effect of inoculum carrier on the survival of *Salmonella* on lettuce plants, IW, DW and BPW were compared under the environmental conditions described in Table 1. In these tests, in addition to *Salmonella*, level of aerobic mesophilic bacteria was also measured. Physicochemical characteristics of the inoculum carriers such as pH, conductivity, turbidity, oxidation reduction potential (ORP), total dissolved solids (TDS), total organic carbon (TOC), inorganic carbon (IC), and nitrogen content were also analysed. Values of ORP and pH were measured using a multimeter (pH & Redox 26, Crison, Barcelona, Spain). Conductivity was determined by means of a conductivity meter (model CM35, Crison, Barcelona, Spain). Turbidity was measured with a turbidimeter (model Turbiquant 3000IR, Merck, Darmstadt, Germany). Total dissolved solids were quantified following the standard method 2540 C from APHA (1998). Values for TOC, IC and N were assessed by means of a multi N/C 3100 analyzer (Analytik Jena, Jena, Germany).

2.4. Irrigation water and survival tests

Inoculum of *Salmonella* was prepared as described before. Survival was assessed in unfiltered and filtered irrigation water. Two pore size filters were compared, 5 μm (IW5) and 0.22 μm (IW0.22)

Table 1
Relative humidity (RH), exposure time (h), temperature (T) and lighting conditions (Photosynthetic Photon Flux Density: PPFD) in tests performed to assess the effect of RH on the survival of *Salmonella* Typhimurium on baby lettuce.

Treatment	RH (%)	Time (h)	T (°C)	PPFD (μmol/m^2·s)
High RH	85	12	23	275
		12	18	0
Low RH	60	12	23	275
		12	18	0