Food Microbiology 46 (2015) 139–144

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Effect of the irrigation regime on the susceptibility of pepper and tomato to post-harvest proliferation of *Salmonella enterica*

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ARTICLE INFO

Article history:
Received 25 September 2013
Received in revised form
15 July 2014
Accepted 17 July 2014
Available online 8 August 2014

Keywords:
Produce safety
Human pathogens
Irrigation regime

ABSTRACT

Raw produce is increasingly recognized as a vehicle of human gastroenteritis. Non-typhoidal *Salmonella*, pathogenic *Escherichia coli*, and other human pathogens have been isolated from fruits and vegetables in the field and in the marketplace, which led to the hypothesis that these microbes can use plants as alternate hosts. However, environmental and physiological factors that facilitate persistence of these bacteria in the crop production environment and make produce more vulnerable to post-harvest contamination have not been fully delineated. This study tested the effect of irrigation regimes on the susceptibility of peppers and tomatoes to post-harvest proliferation of *Salmonella*. The experiments were carried out over three experimental seasons in two locations using seven strains of *Salmonella*. The irrigation regime *per se* did not affect susceptibility of tomatoes and peppers to post-harvest proliferation of *Salmonella*; however, in some of the seasons, irrigation regime-dependent differences were observed. Red peppers and tomatoes were more conducive to proliferation of *Salmonella* than green fruit in all seasons. Inter-seasonal differences were the strongest factors affecting proliferation of *Salmonella* in peppers.

1. Introduction

Although relatively rare, *Salmonella* and pathogenic *Escherichia coli* have been isolated from agricultural fields, irrigation water, and field-grown produce. The presence of these human pathogens was also reported on fruits and vegetables (including tomatoes and peppers) in the market place (Arthur et al., 2007; Benjamin et al., 2013; Gallegos-Robles et al., 2008; Greene et al., 2008; Guchi and Ashenafi, 2010; Micallef et al., 2012; Weber et al., 2006; Wells and Butterfield, 1997, 1999). Even though human pathogens are fairly uncommon in the crop production environment, fresh produce has been implicated in at least 130 outbreaks of gastroenteritis in the U.S. since 1996. Sixty five percent of these outbreaks were caused by strains of non-typhoidal *Salmonella* (Center for Disease Control and Prevention, 2013). After sprouts and leafy greens, tomatoes have been linked to the greatest number of outbreaks of gastroenteritis, and both tomatoes and hot peppers were implicated in the *Salmonella* Saintpaul outbreak resulting in over 1500 documented illnesses (Barton Behravesh et al., 2011; Center for Disease Control and Prevention, 2013). While plants have been suggested as alternate hosts for human enteric pathogens, outbreaks of produce-associated gastroenteritis have been sporadic, suggesting that a number of factors must converge pre- and post-harvest to lead to an outbreak. Presence of sources of pathogens and their vectors, genotype and physiological status of the crop and the pathogen, as well as native plant microbiota capable of promoting or inhibiting human pathogens are all considered as parts of such a "perfect storm" scenario of an outbreak (Mandrell, 2009). How these factors interact and to what extent they contribute to the "perfect storm" is not clear. A better understanding of the role of crop production practices that affect susceptibility of crops to human pathogens pre- and post-harvest may eventually result in a significant reduction of the number and/or severity of the produce-associated outbreaks.

Therefore, with this study we focused on the effects of irrigation practices on susceptibility of bell peppers and tomatoes to post-harvest proliferation of *Salmonella*. The effect of irrigation on the susceptibility of these fruits to *Salmonella* could be direct or indirect. For example, excessive water within tissues may compromise a fruit's basal defenses against microbes and opportunists (such as

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http://dx.doi.org/10.1016/j.fm.2014.07.014
0740-0020/

Salmonella and/or *E. coli*) would be able to proliferate to higher numbers. Over-irrigation can promote phytophathogens, and this indirectly may favor proliferation of human pathogens. In fact, marketplace surveys indicate that the presence of soft rots or fungal molds correlate with a higher rate of isolation of *Salmonella* from tomatoes and peppers (Wells and Butterfield, 1997, 1999). Conversely, types of irrigation regimes (overhead vs drip) have been shown to affect composition and the diversity of epimicrobiota of field-grown vegetables (Poza-Carrion et al., 2013; Williams et al., 2013). The ability of human pathogens, like *Salmonella* and *E. coli* O157:H7, to colonize and persist on plant surfaces under the field conditions correlated with the diversity of the epiphytic microbiota (Poza-Carrion et al., 2013; Williams et al., 2013). Therefore, optimization of crop production practices, like irrigation regime, has the potential to reduce the availability of the favorable niches where pathogens can persist and multiply under the field conditions and post-harvest.

2. Materials and Methods

2.1. *Field production conditions and design.* Seeds of pepper (cv. Aristotle) and tomatoes (cvs. Florida-47, Solar Fire, Bonny Best) were purchased from Siegers Seed Co. (Holland, MI) and Harris Co. (Rochester, NY). Transplants were produced in an environmental chamber and then planted in the field (in Spring 2011 and Spring 2012 in Live Oak, FL; in Fall 2012 in Citra, FL).

General recommended practices for Florida tomato production were used for this research (Olson et al., 2012). At both production sites, the soil tested high in phosphorus (P) and low in potassium (K) by the Mehlich-1 soil testing method. Site preparation for the tomato experiments was as described earlier for the parallel experiments (Marvasi et al., 2013 and Marvasi et al., in press). Peppers were planted in two rows on each bed; the rows were spaced 0.3 m apart and the plants were spaced 0.3 m apart within rows. A fertilizer injection system was set up to apply soluble fertilizer (N and K) in bi-weekly amounts to supplement the pre-plant fertilizer. Total N amount was 225 kg/ha and total season K amount was 208 kg/ha. Irrigation was applied to maintain volumetric water content (measured by time domain reflectometry) at 10% (Munoz-Carpena, 2012). Early in the season one irrigation event of 30 min per day was satisfactory to maintain optimal soil moisture. Irrigation frequency was increased to two 30-min runs per days as the crop developed and then finally to three 30-min runs per day as the fruit matured. Two weeks prior to the onset of harvesting, additional drip tubes were placed in the beds to apply the irrigation treatments. New drip irrigation tubing was threaded under the mulch with a string. One tube was used for the driest water level, 2 tubes for the medium level, and three tubes for the wettest level. Three irrigation sessions of 30 min each were applied each day. The soil moisture targets were 6%, 10%, and 12% volumetric water content.

2.1. Inoculations with Salmonella

Tomatoes were harvested on 6/20/2011, 6/21/2011, 6/2/2012, 6/14/2012, 10/25/2012 and 10/29/2012. Peppers were harvested on 6/21/2011, 6/2/2012, 6/22/2012, 10/25/2012 and 10/29/2012. At each harvest, at least 10 tomatoes and at least 8 peppers of each maturity were harvested per treatment from each treatment plot. At least 100 tomatoes and 35 peppers were inoculated at each harvest. Fruits that developed rots during the experiment were discarded. Inoculation with *Salmonella* was conducted in the laboratory post-harvest within 2–24 h of harvest. The inoculation procedure was chosen to mimic likely routes of post-harvest contamination (through wounding and depositing *Salmonella* on wounded surfaces). For the inocula, the type strain *Salmonella enterica* sv Typhimurium ATCC14028 or strains of (*S.* Javiana ATCC BAA-1593, *S.* Montevideo LJH519, *S.* Newport C6.3, *S.* Braenderup 04E01347, 04E00783, 04E01556) linked to the human outbreaks of salmonellosis were individually grown overnight at 37 °C in LB with 200 rpm shake cultures. They were then washed twice in phosphate-buffered saline, and the strains from the outbreaks were combined into a six-strain "cocktail" containing an approximately equal number of cells of each strain. The washed culture of *S.* Typhimurium 14028 and the outbreak cocktail were then further diluted in sterile water. Shallow (~1 mm) wounds were made in the fruit epidermis with a blunt end of a paper clip, there were three wounds in each fruit. Three microliters of the *Salmonella* inoculum suspension (containing between 100 and 1000 CFU) were spotted onto each wound. For each inoculation, the dose of *Salmonella* was calculated based on the results of dilution plating. Infected fruits were incubated at room temperature (22 °C) for a week. Tomato ripeness was assessed using USDA maturity chart (http://postharvest.ucdavis.edu/pfvegetable/TomatoPhotos/?repository=30014&a=83755): tomatoes that were at stage 6 at field harvest were considered "ripe", those that were harvested at stage 5 or below and then ripened during the experiment, were still considered "ripe", and those that were harvested at stage 3 or below and did not ripen beyond stage 5 during the experiment were considered "unripe". Upon completion of the incubation, peppers and tomatoes were macerated in an equal volume of PBS using a stomacher (Sevard) (200 rpm for 1 min) and aliquots of the suspension were plated onto a Xylose Lysine Deoxylate (XLD) agar and incubated at 37 °C over night. Proliferation was calculated by dividing the final CFU by the CFU in the inoculum and the ratios were further subjected to $\log_{10}$ transformation; this unit-less measure of proliferation was adopted to reflect the fact that *Salmonella* growth in tomatoes or peppers is not uniform and is confined to the site of the inoculation (Fig. S1). The XLD plates on which there were no *Salmonella* colonies upon completion of the incubation were treated based on the rules of the Most Probable Number (MPN) analysis and assessed as LS Means as described below and previously (Marvasi et al., 2013).

2.2. Data analysis

The experiment was run as a four-factor split plot design, with three irrigation treatments as the whole-plot treatments according to a randomized complete block design with three blocks, and four strain by maturity treatments as the split-plot treatments randomized within the whole plots. Split plot with repeated measures statistical design was used to analyze the data. Only main effects and two-way interaction effects were included in the model. The significance of the main effects and the two-way interaction effects were tested using the partial (type III) F-tests for the fixed effects. Since the random effects of the blocks were not significant, we refitted the model by excluding the random effects associated with the blocks. Mean separation for each significant fixed effect in the model was performed using Tukey's multiple comparison testing procedure. Goodness of fit tests for the studentized residuals were performed in order to validate the normality assumption of the mixed effects model.

Data analysis was performed using SAS software. Specifically, we fitted the following linear mixed effects model for the split plot statistical design with repeated measures over seasons:

$$Y_{ijklst} = \mu + \tau_i + (\tau\beta)_{ij} + \gamma_k + (\tau\gamma)_{ik} + \eta_l + (\tau\eta)_{il} + (\gamma\eta)_{kl} + \delta_{ijkl} + \nu_t + (\tau\nu)_{it} + (\gamma\nu)_{kt} + (\eta\nu)_{lt} + \varepsilon_{ijklst} \ ,$$

Table 1
Analysis of Variance (ANOVA) with the (type III) F-tests for the main effects and the two-way interaction effects of the factors Irrigation, *Salmonella* Strain, Pepper maturity, and Time of Harvest on susceptibility of the crop to proliferation of *Salmonella*.

Effect	*F* Value	Prob > *F*
Irrigation	0.55	0.600
Strain	0.15	0.704
Irrigation**Salmonella* Strain	0.02	0.984
Time of Harvest	25.08	0.001*
Irrigation*Time of Harvest	2.78	0.012*
Salmonella strain*Time of Harvest	1.64	0.181
Maturity	36.97	0.001*
Maturity*Irrigation	1.70	0.204
Maturity**Salmonella* Strain	0.11	0.743
Maturity*Time of Harvest	4.21	0.006*

F value represents the value of the *F* test and Prob > *F* is the *p*-value of the F test for the corresponding effect. An asterisk (*) indicates statistically significant effects at 0.05 nominal level.

where Y_{ijklst} is the response, μ is the overall mean, τ_i, γ_k, η_l, and ν_t are the main effects of irrigation, strain, maturity, and time of harvest, respectively, $(\tau\beta)_{ij}$ and δ_{ijkl} are the random effects of the whole plot and split plot errors, and $(\tau\gamma)_{ik}$, $(\tau\eta)_{il}$, $(\gamma\eta)_{kl}$, $(\tau\nu)_{it}$, $(\gamma\nu)_{kt}$, and $(\eta\nu)_{lt}$ are the two-way interaction effects, with $(\tau\beta)_{ij} \sim N(0, \sigma^2_{\tau\beta})$, $\delta_{ijkl} \sim N(0, \sigma^2_{\delta})$, and $\varepsilon_{ijklst} \sim N(0, \sigma^2_{\varepsilon})$ are the independent random effects and the observational errors, respectively. Note that *s* is an index associated with the subsampling units (in each block, multiple tomatoes are randomly selected and inoculated with *Salmonella* and the maturity level recorded).

The mixed effects linear model was fitted and analyzed using SAS/GLIMMIX software, Version 9.3 of the SAS system for Windows, 2013, SAS Institute Inc. We first carried out partial F-tests and identified the significant effects using the nominal level of 0.05. Then, we carried out Tukey's multiple comparison procedure (including the lines display) to separate the predicted marginal means (also known as LS Means) for the significant effects in the model. This analysis enabled us not only to identify significant differences between various treatment means, but also to assess the magnitude of the effects of the significant treatments. Appropriateness of the fitted model was conducted in SAS/UNIVARIATE by carrying out goodness of fit tests to test the normality of the studentized residuals, such as the Kolmogorov–Smirnov and Cramer-von Mises tests, among others. Although significant at the 0.05 nominal level, there was not a strong evidence against the normality of the residuals; e.g., the *p*-value of the Cramer-von Mises test was 0.046. Thus, we can confidently state that the statistical conclusions reported in this research are highly accurate and precise.

3. Results and discussion

3.1. Production conditions and susceptibility of peppers to *Salmonella*: general observations

As shown by the ANOVA table with the type III F tests for the main and interaction effects displayed in Table 1, although the irrigation regime itself did not affect susceptibility of peppers to proliferation of *Salmonella* (i.e., the main effects of irrigation were not significant), strong statistically significant interactions were observed between the irrigation regimes and seasons (harvest occasions) in which the crop was produced (Table 1, Fig. 1). However, it is important to note that peppers that were the most and least conducive to *Salmonella* proliferation were harvested from the treatments receiving the least water; similar variability was also observed in the treatments that received excess water (Table 2, Fig. 2). Peppers harvested from the plots receiving optimal levels of irrigation tended to support more consistent populations of *Salmonella* (Table 2, Fig. 2); however these populations were not significantly different from either extreme. Fruit maturity and time of harvest had an overall strong effect on the susceptibility of

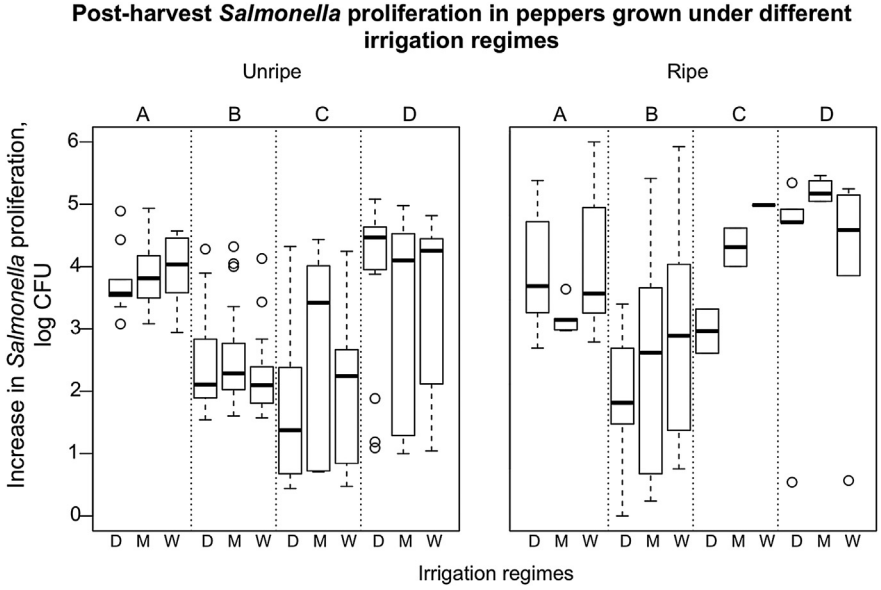

Fig. 1. Post–harvest proliferation of *Salmonella* in ripe and un–ripe peppers grown under different irrigation regimes. Peppers (cultivar Aristotle) were grown in three field seasons (Spring 2011, Live Oak, FL; Spring 2012, Live Oak, FL; Fall 2012, Citra, FL) under different irrigation regimes: D = 6%, M = 10% (recommended for tomato production), *W* = 12% volumetric soil moisture content, which were imposed within two weeks of the first harvest. Four independent samplings (A, B, C, D) were conducted: in Spring 2011, in Spring 2012, 3rd and 4th week of October during Fall 2012, respectively. An increase in proliferation was calculated by using the following formula Log_{10} (total CFU $_{\text{HARVEST}}$/total CFU $_{\text{INOCULUM}}$). Using the ratio allows to account for differences in sizes of fruits, and log transformation decompresses the scale. Data for inoculations with both types of the inoculum (type strain of *Salmonella* Typhimurium and a six-strain cocktail of the outbreak strains) are shown. The box-plots encompass the lower and upper quartiles, thick lines within the box are the median values, and the whiskers indicate the degree of dispersion of the data. Outliers are shown as dots. .

Table 2
Predicted means (LSMEANS) and Tukey mean separation (with the letter grouping display) of the two-way interaction effects for factors Irrigation and Time of Harvest with respect to susceptibility of peppers to proliferation of *Salmonella*.

Effect (Irrigation[a] x time of harvest, location)	LSMEANS	Grouping
D x Late Fall 2012, Citra, FL	4.328	A
W x Spring 2011, Live Oak, FL	4.173	A
D x Spring 2011, Live Oak, FL	4.943	BA
M x Early Fall 2012, Citra, FL	4.001	BAC
W x Late Fall 2012, Citra, FL	3.952	BAC
M x Late Fall 2012, Citra, FL	3.864	BAC
M x Spring 2011, Live Oak, FL	3.858	BAC
W x Early Fall 2012, Citra, FL	3.537	BAC
W x Spring 2012, Live Oak, FL	2.888	BC
M x Spring 2012, Live Oak, FL	2.854	BC
D x Early Fall 2012, Citra, FL	2.601	BC
D x Spring 2012, Live Oak, FL	2.586	C

[a] The factor Irrigation has three levels: D for 6% volumetric soil moisture content, M for 10% volumetric soil moisture content, and W for 12% volumetric soil moisture content, which were imposed within two weeks of the first harvest.

peppers to *Salmonella* (Table 1). These results are discussed below in detail.

3.2. Fruit maturity stage and Salmonella proliferation

In red peppers (cv. Aristotle), *Salmonella* was able to reach up to ~$10^{6.5}$ cfu/fruit within a week after inoculation with as few as ~20 cells per fruit, and in green peppers, *Salmonella* was able to reach up to ~$10^{5.4}$ cfu/fruit within a week. On average, the increase in *Salmonella* cell numbers over a 7-day incubation at 22 °C was 2.996 ± 0.12 $\log_{10}$ for the mature green bell peppers, and 4.12 ± 0.183 $\log_{10}$ for the red bell peppers. In every harvest the proliferation of *Salmonella* was significantly higher in red pepper compared to green peppers, with the *p*-values of the F-tests for the effect of Maturity for each harvest equal to 0.409, 0.001, 0.001, and 0.002 for the harvests A, B, C, and *D*, respectively (Fig. 3). These observations are generally in line with the reports that red tomatoes were significantly more conducive to proliferation of *Salmonella* than immature tomatoes (Shi et al., 2007; Marvasi et al., 2013). The immediate implication of these results for the industry

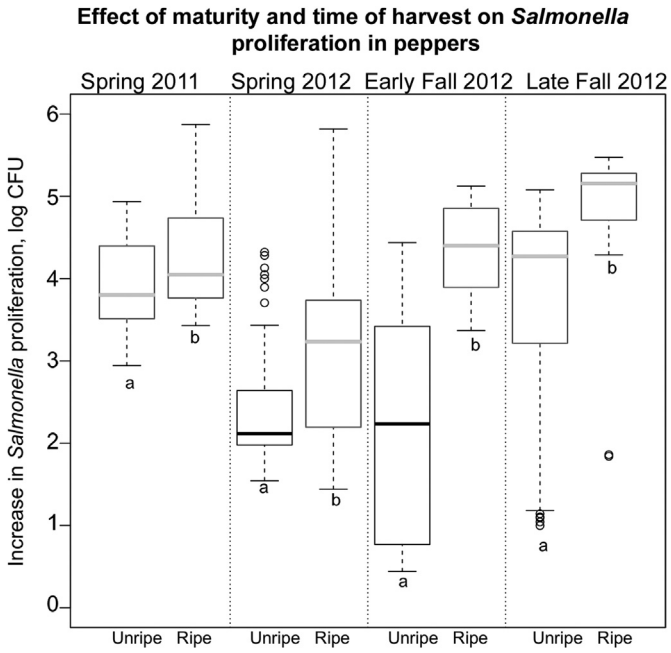

Fig. 3. Effect of pepper maturity on *Salmonella* proliferation in fruits harvested in different seasons. Post-harvest proliferation of *Salmonella* in green and red peppers is represented by box plots, boxes encompass the lower and upper quartiles, thick lines within the box are medians, and the whiskers indicate the degree of data dispersion. Outliers are shown as dots. Lower case letters indicate statistically significant differences ($p = 0.05$) for each harvest.

is not clear: unlike tomatoes that are sold green only for specialty uses, bell peppers are marketed as both green and red.

3.3. Responses of pepper and tomatoes to different irrigation regimes

Differences in the patterns with which *Salmonella* colonizes different crops and varieties of the same crop have been previously documented (Barak et al., 2011, 2008; Klerks et al., 2007). Therefore,

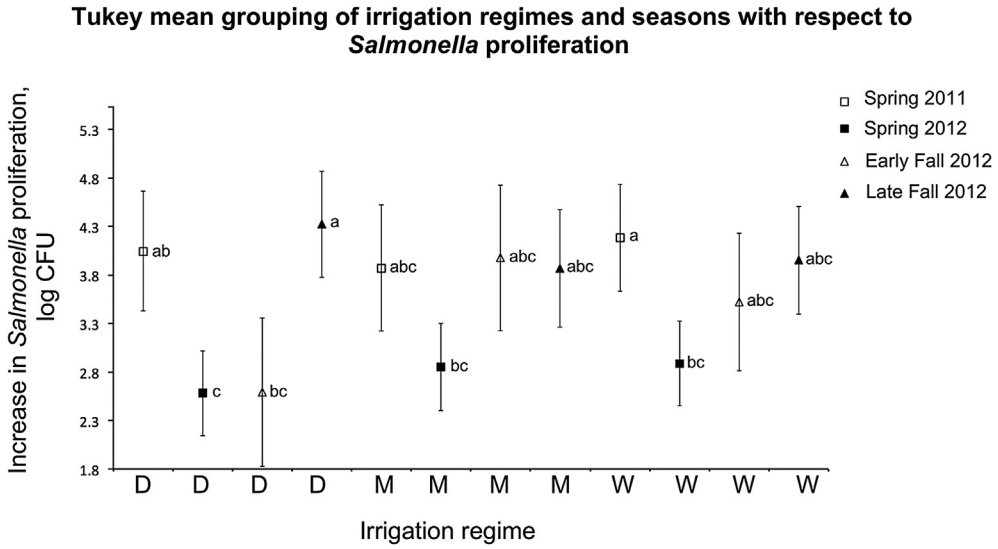

Fig. 2. The effect of the irrigation regime on proliferation of *Salmonella* in peppers in different production seasons "D" refers to the driest treatment (6%, volumetric soil moisture content); "M" refers to the moderate level of moisture (10% volumetric content, recommended for tomato production), and "W" represents the wettest treatment, at 12% volumetric soil moisture content. Means are plotted, whiskers are standard errors. Small letters represent ANOVA groups.

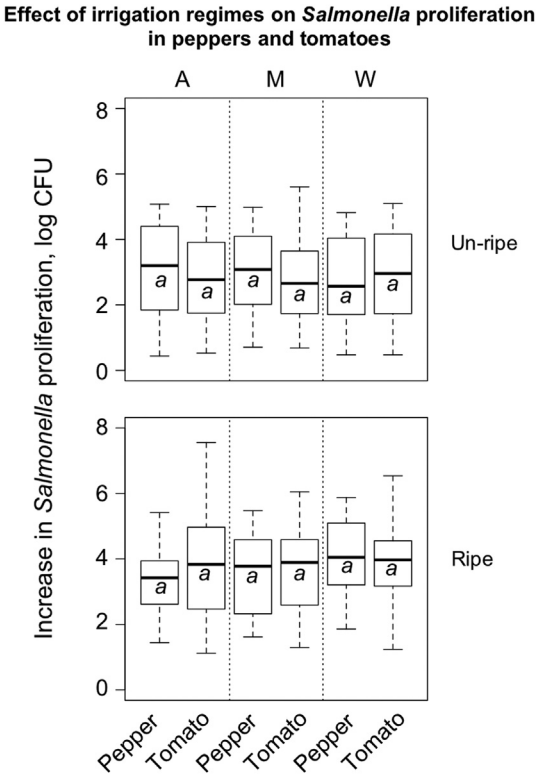

Fig. 4. Effect of the irrigation regime on the proliferation of *Salmonella* in tomatoes and peppers. Tomatoes (cultivars Bonny Best, Solar Fire and Florida-47) and pepper (cv. Aristotle) were harvested in the field as for a commercial harvest, inoculated with 10^2 CFU of *Salmonella*, and incubated for a week. Box-plots with the increase in the log-scale of *Salmonella* proliferation relative to the initial inoculum, grouped by the irrigation regimes ("D" low volumetric water content, "M" medium and "W" high volumetric water content) and type (pepper versus tomatoes) are displayed. The box-plots show the lower and upper quartiles (determined by the boxes), thick lines within the box are the median values, and the whiskers indicate the degree of dispersion of the data. The letters within the box-plot indicate the letter grouping.

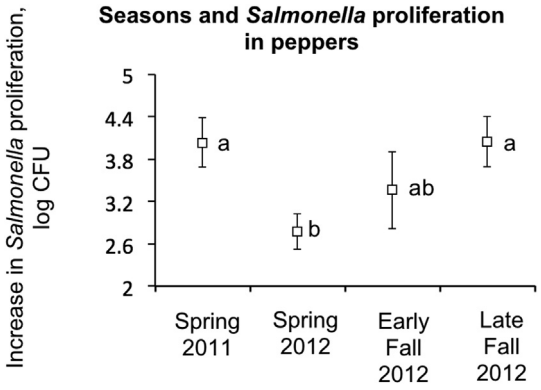

Fig. 5. Seasonal effects on the proliferation of *Salmonella* in peppers. Post-harvest proliferation of *Salmonella* in red and green peppers over four samplings. Predicted means, confidence limits, and letter grouping of Tukey's pairwise comparisons of the Season × Irrigation interaction effects.

we tested whether significant differences can be observed in the ability of *Salmonella* to multiply within fruit tissues of bell pepper (cv. Aristotle) and tomatoes (cvs. Florida-47, Bonny Best and Solar Fire). All data for tomatoes of the three varieties were combined to serve as a broader basis for comparison, while a more comprehensive analysis of the responses of different tomato varieties to different irrigation regimes is reported elsewhere (Marvasi et al., 2013). As shown in Fig. 4, the increase in proliferation of *Salmonella* within red and green peppers was similar to its proliferation within red and green tomatoes. The effects of the irrigation regimes on the susceptibility of green and red peppers to *Salmonella* mirrored those of green and red tomatoes.

3.3.1. Seasonal effects

Aside from maturity, seasonal effects were very strong (Table 1, Fig. 5). Strong seasonal variability was also noted in the field studies with lettuce epimicrobiota and the persistence of *E. coli* O157:H7 (Gutierrez-Rodriguez et al., 2012; Williams et al., 2013). *Salmonella* proliferation was the highest in fruits harvested in during the sampling Spring 2011 (Live Oak, FL) and was the lowest for the fruits harvested in Spring 2012 at the same location. Intermediate levels of the susceptibility of peppers to *Salmonella* were observed in the Fall samplings in Citra, FL in 2012 (Fig. 5). Weather conditions within a month prior to harvests were different in each of the three experimental seasons. Average daily temperatures in Spring 2011, Spring 2012, and Fall 2012 were 26.5 °C, 25.1 °C, and 21.8 °C, and average relative humidity levels during these production seasons were 71.9%, 79.6.%, and 82.1%, respectively. Average precipitation during each production season was 11.26, 67.8, and 14.32 (cm m^{-2}), and average total radiant flux levels were 21.51, 17.72, and 13.92 (MJ m^{-2}), respectively. The second harvest in Fall 2012 was immediately preceded by a temperature drop to 1.63 °C. Therefore, the season in which the crops were the most susceptible to proliferation of *Salmonella* was the driest, with the least cloud cover, and fewer precipitation events. A chilling injury during the last harvest in Fall 2012 also likely pre-disposed tomatoes to proliferation of *Salmonella*. Peppers were the least conducive to proliferation of *Salmonella* in the second production season. As shown in Fig. 3B, varying irrigation regimes did not alleviate or exacerbate susceptibility of peppers to *Salmonella* proliferation within each sampling (Fig. 3B).

4. Conclusions

The subtle differences in the irrigation regimes imposed within a 2–4 weeks of harvest had modest, but statistically significant effects on the susceptibility of bell peppers to post-harvest proliferation of *Salmonella* in some production seasons. The difference in maturity at harvest was the strongest effect related to susceptibility of peppers to *Salmonella* (with the corresponding partial F value of 51.1) followed by seasonal variability (with the corresponding partial F value of 25.1). In general, proliferation of *Salmonella* was higher in red peppers than in green fruit. Interestingly, peppers harvested in the driest, sunniest of the seasons were the most conducive to *Salmonella* proliferation, and the ability of *Salmonella* to multiply in peppers and tomatoes was similar.

Acknowledgments

This research was supported by funding provided by the Center for Produce Safety through UCANR/USDA NIFA grant #2010-34608-20768 (SA7660) and the Florida Specialty Crops Foundation. A.E. George acknowledges support from the McKnight Fellowship Program. We thank C. Chen, M. Carrazana, E. Gause, M. Farias, A. Aruca, M.H. Moraes, K. Jenkins, and D. Lucas for their help with pepper processing during harvest.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fm.2014.07.014.

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