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Impact of phytopathogen infection and extreme weather stress on internalization of *Salmonella* Typhimurium in lettuce

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ABSTRACT

Internalization of human pathogens, common in many types of fresh produce, is a threat to human health since the internalized pathogens cannot be fully inactivated/removed by washing with water or sanitizers. Given that pathogen internalization can be affected by many environmental factors, this study was conducted to investigate the influence of two types of plant stress on the internalization of *Salmonella* Typhimurium in iceberg lettuce during pre-harvest. The stresses were: abiotic (water stress induced by extreme weather events) and biotic (phytopathogen infection by lettuce mosaic virus [LMV]). Lettuce with and without LMV infection were purposefully contaminated with green fluorescence protein-labeled *S.* Typhimurium on the leaf surfaces. Lettuce was also subjected to water stress conditions (drought and storm) which were simulated by irrigating with different amounts of water. The internalized *S.* Typhimurium in the different parts of the lettuce were quantified by plate count and real-time quantitative PCR and confirmed with a laser scanning confocal microscope. *Salmonella* internalization occurred under the conditions outlined above; however internalization levels were not significantly affected by water stress alone. In contrast, the extent of culturable *S.* Typhimurium internalized in the leafy part of the lettuce decreased when infected with LMV under water stress conditions and contaminated with high levels of *S.* Typhimurium. On the other hand, LMV-infected lettuce showed a significant increase in the levels of culturable bacteria in the roots. In conclusion, internalization was observed under all experimental conditions when the lettuce surface was contaminated with *S.* Typhimurium. However, the extent of internalization was only affected by water stress when lettuce was infected with LMV.

1. Introduction

Foodborne illness outbreaks associated with fresh produce have increased over the last several decades due to the increased consumption of raw fruits and vegetables. This phenomenon has made fresh produce one of the leading causes of foodborne illness in the United States (Centers for Disease Control and Prevention, 2011; Lynch et al., 2009). The produce can be contaminated with pathogens at any stage in the food supply chain, including cultivation, harvesting, handling, and processing (Tournas, 2005). Many human pathogens, such as *Salmonella* and *Escherichia coli* O157:H7, are able to attach to the surface and/or roots of the produce and enter into the plant tissues, a process called bacterial internalization (Bernstein et al., 2007; Franz et al., 2007; Gautam et al., 2011; Warriner and Namvar, 2010). The internalization of human pathogens reduces the efficacy of conventional washing methods which may employ chemical disinfectants such as chlorine. However, the disinfectant cannot effectively access pathogens inside the fresh produce. Therefore, the internalized pathogens can pose risks for humans to foodborne illness when consumed raw (Nyachuba, 2010; Sapers, 2001).

The level of human pathogen internalization depends on microbial species, plant types, contamination pathways, and contamination levels. It is also influenced by environmental factors during cultivation (Ge et al., 2012; Mitra et al., 2009; Pu et al., 2009). Environmental changes have facilitated microbial evolution, including human pathogens, to enable them to adapt to environmental stress and survive longer. As climate change is becoming more evident and the frequency of extreme weather events is increasing, it is imperative to understand their impact on pathogen internalization in fresh produce during pre-harvest. However, few studies have been conducted and a prodigious knowledge gap exists on this topic. In addition, Melotto et al. (2006) reported that phytopathogen infections, which occur frequently during field cultivation, could affect the interaction between human pathogens and plants. Thus, it is worthwhile to investigate the combined effects of phytopathogen infection together with extreme weather events on internalization of human pathogens in fresh produce.

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In this study, human pathogen (*Salmonella enterica* serovar Typhimurium) internalization was investigated in leafy green (iceberg lettuce) following surface contamination and growing under stressful conditions. Water stress (drought and storm) which could be induced by extreme weather events were simulated by varying the level of irrigation in order to cause an appropriate abiotic stress. To simulate biotic stress during plant growth, the lettuce was infected with lettuce mosaic virus (LMV), which causes a common disease in lettuce crops around the world. To our knowledge, this is the first study that evaluates pre-harvest human pathogen internalization under the combined impact of extreme weather conditions and plant pathogen infection.

2. Materials and methods

2.1. Cultivation of lettuce

Iceberg lettuce (*Lactuca sativa var. capitata*) seeds were purchased from Burpee & Co. (Warmister, PA, USA). All of the plants were grown in a greenhouse at the Department of Horticulture and Crop Science, The Ohio State University (Columbus, OH, USA) for three weeks (for the experiments with LMV infection) or four weeks (for those without LMV infection). The plants were transferred to a growth chamber in our laboratory for *S.* Typhimurium inoculation. All growth conditions in the greenhouse and laboratory were the same as those described in our previous work (Ge et al., 2012). The growing conditions in the greenhouse were: 25–30 °C, 40–50% relative humidity, and an irrigation volume of 250 mL per pot per day. The growing conditions in the laboratory growing chamber were: 24–27 °C, 35–40% relative humidity, 16 h lighting per day, and the irrigation volume varied depending on the irrigation methods.

2.2. Preparation of S. Typhimurium

Green fluorescence protein (GFP)-labeled *S.* Typhimurium (ATCC 19585) was constructed in our previous study and the associated incubation and bacterial recovery were also following the procedure described in Ge et al. (2012).

2.3. Growing lettuce under stress conditions and inoculation of lettuce with S. Typhimurium and LMV

The entire experimental design is summarized in Fig. 1. In order to generate biotic stress, the three-week-old lettuce was infected with LMV (ATCC PV-63, ATCC, Manassas, VA, USA). For the viral inoculation procedure, we followed the plant virus revival method provided by ATCC (https://www.atcc.org/How_to_Revive_Cultures.aspx#plant).

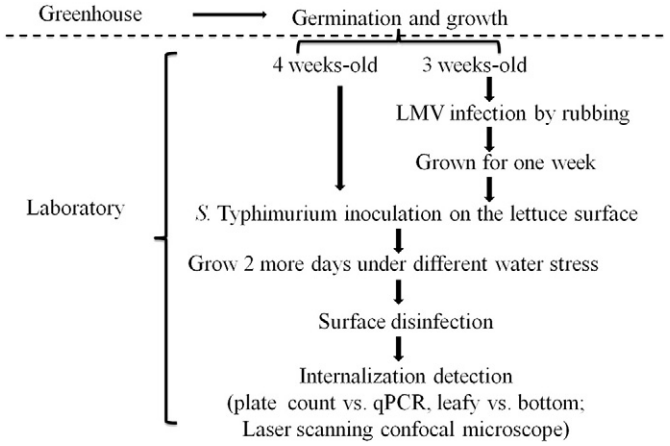

Fig. 1. Summary of experimental procedures used in this study.

The viral RNA copies were determined using reverse transcription-quantitative PCR (RT-qPCR), as described in the following Section 2.6. Briefly, 0.1 g of LMV-infected-lettuce tissue (purchased from ATCC) was mixed with 3 mL of phosphate buffered saline (PBS, pH 7.4, Fisher Scientific, Fair Lawn, NJ, USA) and then blended using a mortar and pestle. About 20 μL (~2×10^{10} RNA copies/mL) of LMV suspension was applied to two leaves of each lettuce sample by rubbing them with a cotton swab and 1% of carborundum powder (400 grit, Fisher Science Education, Hanover Park, IL, USA). The infected leaves were rinsed with deionized water after inoculation to remove the salts and carborundum powder from the leaf surfaces. All of the infected lettuce grew for one more week in the growth chamber until obvious symptoms of LMV infection (yellow circular spots) appeared on the leaves. At this point, they were inoculated with *S.* Typhimurium. At the same moment, the four-week-old lettuce (without LMV infection) was inoculated with *S.* Typhimurium immediately being transported to the laboratory. Thus, all batches of lettuce, with or without LMV infection, were inoculated with *S.* Typhimurium at the same age, i.e., the fourth week (Fig. 1).

In our preliminary experiments, no culturable internalized *S.* Typhimurium was detected using the plate count method, regardless of LMV infection or watering condition when the *S.* Typhimurium concentration was 10 colony forming units (CFU)/mL. Therefore, three concentrations above 10 CFU/mL (10^2, 10^4 and 10^6 CFU/mL) of *S.* Typhimurium, were chosen for the leaf surface inoculations. Different concentrations of *S.* Typhimurium solutions were inoculated onto all the leaves on each head of lettuce using a pipette with a 10 μL tip, and the droplets on each leaf were gently spread uniformly on the surface using the same tip. For preventing contamination of the soil during the inoculation step, the soil was covered with layers of tissues (Kimwipes, Kimberly-Clark, Roswell, GA, USA). After the inoculation step, all the tissues were removed. In order to minimize variation, lettuce leaves of similar size were selected (the estimated mass of one head of four-week-old lettuce was 6.28 ± 2.74 g, each lettuce had 6–8 leaves) and the inoculum volume for each head of lettuce was 1.5 ± 0.2 mL. Thus, the resultant *S.* Typhimurium levels on each lettuce sample could be converted to 2.30 ± 0.06 log CFU/lettuce, 4.30 ± 0.25 log CFU/lettuce, and 6.30 ± 0.13 log CFU/lettuce when each experimental group was inoculated with 2 log, 4 log, or 6 log CFU/mL of *S.* Typhimurium suspension, respectively. For convenience, the three different inoculation levels of *S.* Typhimurium are referred as low (2 log CFU/mL), medium (4 log CFU/mL) and high (6 log CFU/mL) throughout this study. The shoots contaminated with the same concentration of *S.* Typhimurium were divided into three subgroups (two heads of lettuce per subgroup) and subjected to three different irrigation levels to simulate growth under optimal irrigation or water stress conditions. Each pot contained ~360 g soil, and the optimal irrigation group was watered with 250 mL/pot per day; the storm (excessive water) group was watered with 750 mL/pot per day; no irrigation was conducted for the drought group. Soil moisture content was measured using the gravimetric method to evaluate the actual water changes in the soil (International Organization for Standardization, 1993). All samples were kept in the growth chamber for two days after the *Salmonella* inoculation (Fig. 1). A total of six groups of lettuce without *S.* Typhimurium inoculation was used as negative control groups and were cultivated in the same growth chamber. Three groups of four-week-old lettuce were not infected with LMV and were grown under the three different irrigation conditions. Three other groups were infected with LMV at week 3 and each group was irrigated differently at week 4. Each control group contained 2 heads of lettuce, which were collected at the same day as the *S.* Typhimurium inoculated plants. The entire experiment was repeated 3 times.

2.4. Lettuce sample processing

In order to remove and inactivate the bacteria that survived on the lettuce surface, the lettuce was immersed in 80% ethanol for 10 s, then

1% $AgNO_3$ for 5 min, and finally rinsed with tap water and distilled water (modified from Franz et al., 2007).

For the LMV-infected leaves, "sick leaves" were defined as those showing obvious symptoms (mosaic pattern, each head of LMV-infected lettuce had two sick leaves). The leaves that were not directly infected by and did not show apparent signs of LMV infection were considered "healthy leaves" throughout this study.

For the lettuce being infected with LMV, the sick leaves were separated out for determining LMV concentration before surface disinfection. The rest of the lettuce was cut into two parts after surface disinfection, healthy leaves and roots, to determine the internalized *S.* Typhimurium and LMV concentrations. Each part (healthy leaves or roots) was weighed and then blended for 2 min in a stomacher (Stomacher® 80, Seward, UK) in 30 mL of 0.1% peptone water.

2.5. Quantification of the internalized S. Typhimurium using plate counts and TaqMan® real-time quantitative PCR (qPCR)

The blended lettuce suspension (100 μL, pH ~7) was plated in duplicate onto LB agar supplemented with ampicillin (final concentration, 100 μg/mL) and all agar plates were then incubated at 37 °C for 18 h. Prior to counting, colonies on the plates were examined under UV light at 365 nm and those showing green fluorescence were counted.

For qPCR detection, 2 mL of the homogenized plant suspension was used for total genomic DNA extraction using a QIAamp® DNA Mini Kit (Qiagen, Valencia, CA, USA). The final DNA product was eluted with 100 μL of deionized water. The qPCR was performed as described by Chen et al. (2010) using the same primers, probe (Applied Biosystems, Foster City, CA, USA), and cycling conditions.

In order to determine if the disinfection method was sufficient to inactivate the bacteria on the leaf surface and eliminate the bacterial DNA to avoid false-positive results both in plate count and qPCR, 6 log CFU/mL of *S.* Typhimurium was sprayed onto the surface of lettuce. The lettuce was then immediately disinfected as described above followed by rapid drying (~15 min) in a laminar flow biosafety cabinet (Thermo Scientific 1300 Series A2 Class II, Asheville, NC, USA). A total of 20 heads of lettuce were used for verification of surface disinfection.

2.6. Quantification of LMV using real-time quantitative reverse transcription-PCR (qRT-PCR)

In order to determine the concentration of LMV in the lettuce, RT-qPCR was used to construct a standard curve. The LMV-infected lettuce (0.1 g, purchased from ATCC) was ground using a mortar and pestle after 3 mL of PBS was added (pH 7.4, Fisher Scientific, Fair Lawn, NJ, USA). The LMV RNA was extracted from 0.1 mL of the lettuce tissue suspension using an RNeasy® Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instruction. The cDNA was synthesized and amplified using a QIAGEN® OneStep RT-PCR Kit (Qiagen, Valencia, CA, USA). Each 20 μL-reaction contained 400 nM of primers (forward and reverse) and 10 μM dNTPs (Invitrogen, Grand Island, NY, USA), and 3 μL of RNA as a template. The sequences of primers were as follows: forward primer-5′CCGTACATAGCIGARTGTGCT3′, reverse primer-5′GCGTTDATGTCGTC GTCYTT3′ to amplify LMV genomic fragment encoding of LMV nuclear inclusion B and capsid protein (Revers et al., 1999). The one-step RT-PCR was performed using a MultiGene Gradient PCR cycler (Labnet International Inc., Edison, NY, USA) with the conditions including reverse transcription at 50 °C for 30 min, then denaturation at 95 °C for 10 min, 30 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 40 s, and a final extension at 72 °C for 10 min. The RT-PCR product (277 bp) was examined by 2% agarose gel electrophoresis and then purified using a QIAquick® PCR Purification Kit (Qiagen, Valencia, CA, USA) according to manufacturer's instruction. The purified LMV cDNA fragment was inserted into pGEM®-T vector (Promega, Madison, WI, USA) and then transferred into *E. coli* DH5α competent cells using a MicroPulser Electroporator (25 μF capacitance, 1.25 kV, and 200 Ω resistance, Bio-Rad, Hercules, CA, USA). The recombinant *E. coli* was mixed with 100 μL of X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside) (2%, w/v) and 20 μL of isopropyl β-D-1-thiogalactopyranoside (IPTG, 20%, w/v), then spread on LB agar plates supplemented with ampicillin (final concentration, 100 μg/mL). They were then incubated overnight at 37 °C. White colonies were selected and further cultured in tubes with 3 mL of LB media supplemented with ampicillin (final concentration, 100 μg/mL) at 37 °C for 18 h. The plasmid DNA was extracted using a QIAprep® Spin Miniprep Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instruction and confirmed by PCR using the same LMV primers and PCR conditions described above. The recombinant plasmid DNA concentration (ng/μL) was measured using a NanoDrop system (NanoDrop Technologies, Wilmington, DE, USA), and the plasmid copy numbers were calculated using the method available on the website of The University of Rhode Island Genomics & Sequencing Center (http://www.uri.edu/research/gsc/resources/cndna.html). The plasmids were serially diluted from 10^{11} to 10 double-stranded DNA copies/g, and 2 μL of each dilution was used as the qPCR template to construct a standard curve measuring the cycle threshold (Ct) value against ds DNA copies/g. The final LMV concentration (RNA copies/g) was 2 times greater than the ds DNA copies since LMV is a single-stranded RNA virus. Each 20 μL-reaction contained 10 μL of SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and 400 nM of each primer. Thermal cycling consisted of initial cycles of 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 56 °C for 1 min. After amplification, melting curve analysis was performed by heating the samples to 95 °C for 15 s, cooling to 56 °C for 1 min, and then heating the samples at 1.0 °C/s to 95 °C (Dhar et al., 2002; Revers et al., 1999).

RNA was extracted from the sick leaves and healthy leaves and roots, respectively, of each lettuce sample as described above. Lettuce without LMV infection was used as a negative control. The first strand of cDNA was synthesized using an iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Briefly, each 20 μL-reaction contained 1 μL of reverse transcriptase, 4 μL of 5× reaction mix, and 10 μL of total RNA as the template and 5 μL of RNase free water. The cycling conditions were 25 °C for 5 min, 42 °C for 30 min and 85 °C for 5 min, ending at 4 °C. The cDNA product from RT-PCR was used as the DNA template in the next step of SYBR® Green qPCR with the same primers and qPCR conditions described above (to construct the standard curve).

2.7. Laser scanning confocal microscopy (LSCM)

After the lettuce surfaces were disinfected, small pieces of lettuce were excised from the leafy parts and roots, stained with 10 μg/mL of propidium iodide for 30 min, washed twice with distilled water, and then mounted on glass microscope slides. The slides were examined with an Olympus FluoView FV1000-LSCM equipped with a 100× oil objective (Olympus, Tokyo, Japan). The GFP-labeled *S.* Typhimurium

Table 1
Soil moisture content after 3 types of water stress scenarios (n = 6)[a].

	Initial (g/g dry soil)	Irrigation[b] (g/g dry soil)		
		Optimal	Drought	Storm
LMV(−)[c]	2.14 ± 0.28	2.17 ± 0.23	1.15 ± 0.11	3.84 ± 0.41
LMV(+)[c]	2.22 ± 0.32	2.42 ± 0.19	1.20 ± 0.14	3.98 ± 0.27

[a] The results are presented as arithmetic mean ± standard deviation.
[b] Soil moisture content was measured at the end of Day-2 after *S.* Typhimurium contamination.
[c] The lettuce group without LMV infection was marked as LMV(−) and the group infected with LMV was marked as LMV(+).

was excited with a 405 nm laser line and emission was detected at 513 nm. The propidium iodide-stained plant tissue was excited using a 543 nm laser line and emission was detected at 603 nm. The confocal images were captured and a 3D demonstration was constructed using FV10-ASW 2.0 Viewer software (Olympus, Tokyo, Japan).

2.8. Statistical analysis

The statistical analysis was carried out with SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA). Arithmetic means and standard deviations of the data were determined, including: the plate count and qPCR for detection of the internalized *S.* Typhimurium, the RT-qPCR results for LMV quantification, and the soil moisture content. Analysis of variance and Dunnett's test were applied to evaluate the differences, and the results were considered significant at $P < 0.05$.

3. Results

3.1. S. Typhimurium internalization under abiotic factors (water stress)

The soil moisture content was measured before the *S.* Typhimurium inoculation and after the lettuce harvest to evaluate the actual changes in moisture content of the soil (Table 1). The moisture content of the soil was not significantly changed under optimal irrigation conditions ($P > 0.05$). However, it changed significantly under drought (46.3% decrease) and storm (79.4% increase) conditions ($P < 0.05$).

The level of internalized *S.* Typhimurium in different parts of the lettuce (upper leafy versus root) is summarized in Fig. 2. Although culturable *S.* Typhimurium in 20 lettuce samples (used as verification of surface disinfection, see Section 2.5) was undetectable using plate count, the qPCR result showed that the *S.* Typhimurium concentration was 1.37 ± 0.36 log CFU/lettuce, this was significantly lower than all the PCR results in the internalization tests for the lettuce leaves (e.g. 2.7–5.0 log CFU/g varying in contamination levels) according to statistical analysis ($P < 0.05$). This implies that any *S.* Typhimurium DNA originating from *S.* Typhimurium remaining on the surface would not significantly affect the measurement of internalization levels in the tested samples.

From the plate count results, culturable internalized *S.* Typhimurium was not found in any root samples (Fig. 2B), but was found in the leafy parts, regardless of irrigation type (Fig. 2A). The mean values of internalized *S.* Typhimurium ranged from 2.73 to 3.41 log CFU/lettuce depending upon the inoculation level (low, medium or high). Levels of internalization under water stress conditions (drought or storm) were not significantly different when compared to groups grown under optimal conditions ($P > 0.05$).

Results of qPCR show that the level of internalized *S.* Typhimurium was around 4 log CFU/lettuce in most samples. The mean values were

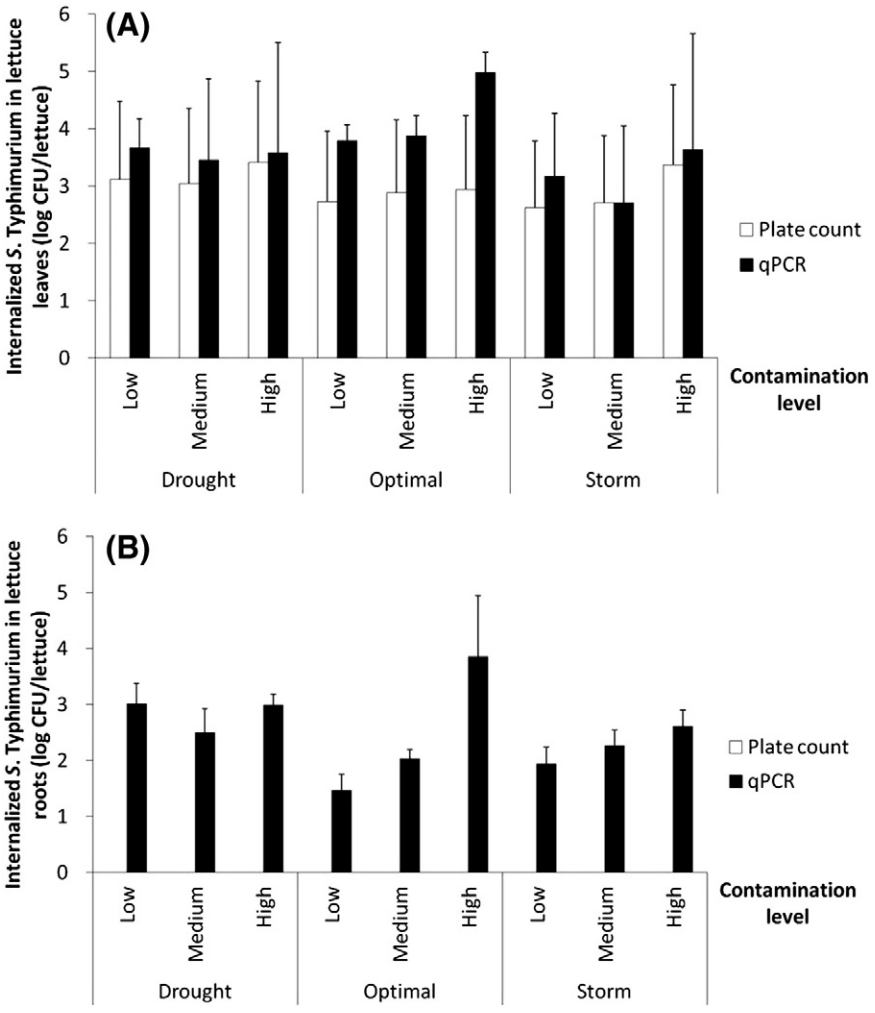

Fig. 2. Levels of internalized *S.* Typhimurium in the lettuce without LMV infection through surface contamination and grown under drought and heavy rain determined with plate count and qPCR ($n = 6$). *S.* Typhimurium was not detected in the control groups. The detection limit of both methods was 1.00 log CFU/lettuce. When the sample yielded a negative result, the number was assigned as 0.00. (A) *S.* Typhimurium internalization levels in lettuce leaves detected using plate count and qPCR; (B) *S.* Typhimurium internalization levels in lettuce roots detected using plate count and qPCR.

Table 2
Quantification of LMV in infected lettuce leaves using real-time RT-PCR[a] (n = 18)[a].

Lettuce part	Water stress	Before *S.* Typhimurium inoculation (log RNA copies/g of lettuce)[b]	After *S.* Typhimurium inoculation (log RNA copies/g of lettuce)[b,c]
Healthy leaf	Optimal	0.00 ± 0.00	0.58 ± 0.83
	Drought	0.00 ± 0.00	0.00 ± 0.00
	Storm	0.00 ± 0.00	0.00 ± 0.00
Sick leaf	Optimal	6.51 ± 0.85	6.98 ± 0.49
	Drought	6.88 ± 0.06	6.99 ± 0.32
	Storm	7.00 ± 0.26	7.46 ± 0.91
Root	Optimal	3.19 ± 3.17	3.10 ± 4.26
	Drought	2.32 ± 4.30	3.45 ± 3.27
	Storm	3.76 ± 1.91	3.04 ± 3.86

[a] The results are presented as arithmetic mean ± standard deviation.
[b] The detection limit was 1 log copy numbers/g of lettuce. When the sample yielded a negative result, the number was assigned as 0.00. LMV was under the detection limit in all samples without LMV infection, which were the control groups.
[c] The LMV concentration did not vary depending on the *S.* Typhimurium contamination levels, so the results of each irrigation group (containing 18 heads of lettuce, 6 heads for each contamination level) were combined.

dependent on the initial inoculation level (low, medium, or high), except for the drought group, but the difference was not significant ($P > 0.05$) (Fig. 2A). At the same inoculation level, the amounts of internalized bacteria detected with qPCR in the leafy part were not significantly different from the plate count measurements (Fig. 2A) ($P > 0.05$). The internalized *S.* Typhimurium in the roots was detectable by qPCR and its internalization level was similar to that found in the leafy parts (Fig. 2) ($P > 0.05$). Similarly, no significant difference in *S.* Typhimurium internalization was found between the water stress groups and the optimal groups, in either the leafy parts or the roots ($P > 0.05$).

3.2. *Salmonella* Typhimurium internalization under LMV infection (biotic factor) and water stress (abiotic factor)

The soil moisture content was significantly changed when the groups underwent drought and storm conditions ($P < 0.05$) (Table 1). The LMV concentration in the leaves and roots was quantified after the lettuce was harvested, as shown in Table 2. The LMV concentration was ~7 log RNA copies/g in the sick leaves, ~3 log RNA copies/g in the roots, and was under the detection limit in healthy leaves, except for the optimal group (0.58 ± 0.83 log RNA copies/g). The LMV concentration in each part of the lettuce (healthy leaf, sick leaf, or root) was not significantly affected by the irrigation conditions or *Salmonella* contamination levels ($P > 0.05$).

The internalized *S.* Typhimurium in the LMV-infected lettuce was detected using both plate count and qPCR methods (Fig. 3). The plate count results showed that the mean value of the internalization levels in each irrigation group (except the storm groups) followed a dose-

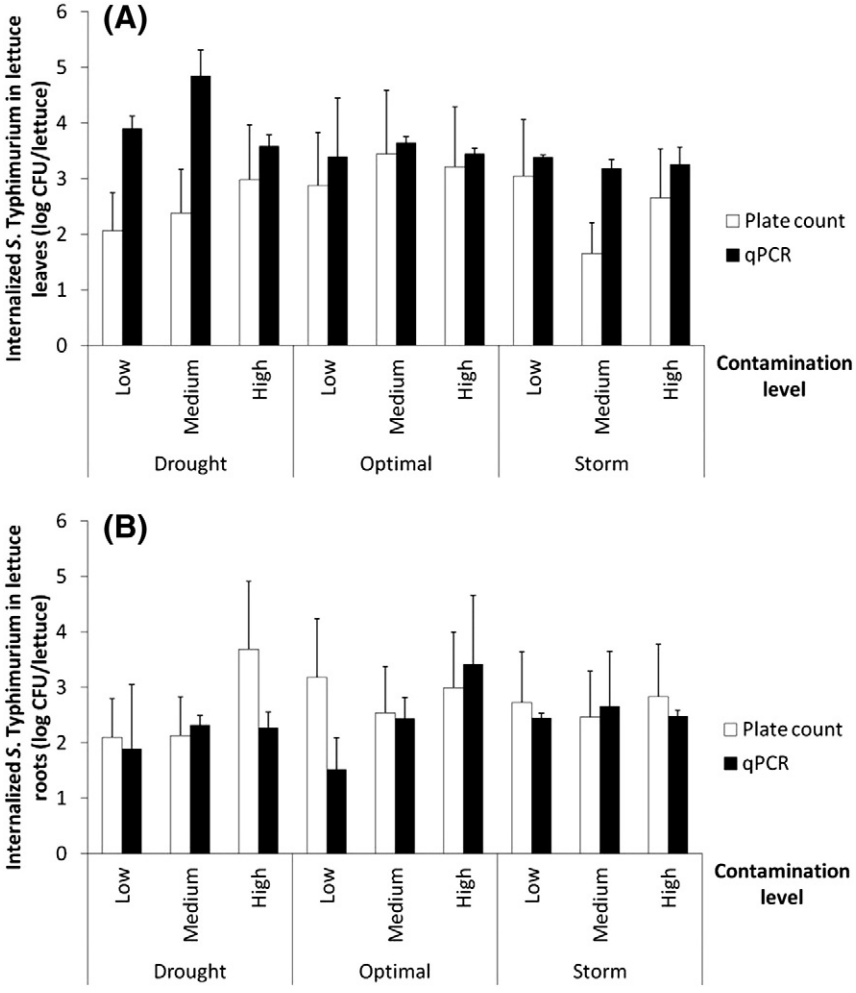

Fig. 3. Levels of internalized *S.* Typhimurium in lettuce infected with LMV through surface contamination and grown under drought and heavy rain determined with plate count and qPCR (n = 6). The lettuce samples were infected with LMV and grown for another week before being contaminated with *S.* Typhimurium. *S.* Typhimurium was not detected in the control groups. The detection limit of both methods was 1.00 log CFU/lettuce. When the sample yielded a negative result, the number was assigned as 0.00. (A) *S.* Typhimurium internalization levels in lettuce leaves detected using plate count and qPCR; (B) *S.* Typhimurium internalization levels in lettuce roots detected using plate count and qPCR.

dependent pattern, but the results were not significantly different ($P > 0.05$). As shown in Fig. 3, culturable *S.* Typhimurium was found in both leaves and roots of lettuce after LMV infection, but the *S.* Typhimurium concentrations were not significantly different ($P > 0.05$).

The internalization levels of all the groups examined by qPCR are shown in Fig. 3. The *S.* Typhimurium internalization levels were similar (~4 log CFU/lettuce) in most of the lettuce samples regardless of irrigation type ($P > 0.05$). The mean levels of internalized *S.* Typhimurium in the leafy parts were higher than those in the roots in all cases. However, three groups (medium contamination under optimal irrigation, high contamination under drought, and low contamination under storm conditions) showed statistical difference in *S.* Typhimurium internalization between the two plant locations ($P < 0.05$). In addition, the internalized *S.* Typhimurium detected with qPCR in the leafy parts was significantly higher than the plate counts when the contamination levels were medium and high under water stress conditions ($P < 0.05$).

3.3. Visualization of the internalized S. Typhimurium in lettuce tissue

The internalization of GFP-labeled *S.* Typhimurium beneath the lettuce surface was examined using LSCM. The confocal microscopic images captured at different depths below the lettuce surface and z-series demonstration are shown in Fig. 4A and B, respectively. The 3-D pictures were reconstructed using the z-series images (Fig. 4C and D). Both z-series and 3-D pictures clearly displayed the presence of *S.* Typhimurium in the leaves and roots of the lettuce samples. The internalized *S.* Typhimurium (green fluorescence) was localized at the intercellular region inside of the root samples, and was also observed around the stomata on the leaf and within the parenchyma (Fig. 4) (Kroupitski et al., 2009a). The red fluorescence is caused by propidium iodide staining. The yellow fluorescence is the overlap of green and red fluorescence, representing bacteria and chloroplasts, respectively.

4. Discussion

Salmonella internalization in fresh produce via leaf surface contamination has been observed in numerous studies. Internalized *Salmonella* was found in 25% of ripened tomatoes after the plant flowers were brushed with a five-strain cocktail of *S. enterica* (Guo et al., 2001). Golberg et al. (2011) also examined *Salmonella* internalization in various fresh produce by inoculating 10^8 CFU/mL of *S.* Typhimurium onto the leaf surfaces. They reported that 46% of fresh basil, and over 80% of arugula leaves and iceberg lettuce were internalized with *S.* Typhimurium. In the current study, *S.* Typhimurium was able to internalize in the edible parts of the iceberg lettuce even when the contamination level on the surface was low (2 log CFU/mL) (Figs. 2 and 3). Human pathogens could be introduced onto the surface of fresh produce if sprayed with contaminated irrigation water, which may occur during the pre-harvest stage. Pathogens could also be splashed onto the surface due to heavy rain or water gun irrigation if the soil or manure fertilizer contained human pathogenic bacteria (Heaton and Jones, 2008). Wildlife could also potentially spread pathogens to the aerial parts of plants by biting or through their droppings (Refsum et al., 2002; Talley et al., 2009).

The confocal microscopic images confirmed that *S.* Typhimurium internalized in the intercellular spaces of underlying parenchyma cells and the region around stomata of edible lettuce parts (Fig. 4A and C), which is consistent with other studies (Kroupitski et al., 2009a, 2009b). Based on the findings using LSCM and scanning electron microscopy (SEM), a potential route of *Salmonella* penetration and internalization in the plant tissue was proposed by Kroupitski et al. (2009a): *S.* Typhimurium can be stimulated by chemotaxis, move toward and aggregate around the stomata, and then penetrate into the plant tissue through the open stomata, which are regulated by light. Erickson et al. (2010) also found that more *E. coli* O157:H7 was internalized in lettuce when the leaves were contaminated at the abaxial (lower) side of the leaves rather than at the adaxial (upper) side due to a greater stomata density on the former. Quilliam et al. (2012) observed that when *E. coli* O157 firmly attached to lettuce leaves or became internalized, the metabolic activity of bacteria was greater than the bacteria loosely staying at the phyllosphere, which indicated that bacteria were able to actively utilize the nutrients in the lettuce leaves. In addition, bacterial endophytic colonization is also affected by lettuce cultivars.

In the current study, when the lettuce was divided into two parts, culturable internalized *S.* Typhimurium was detected in the edible

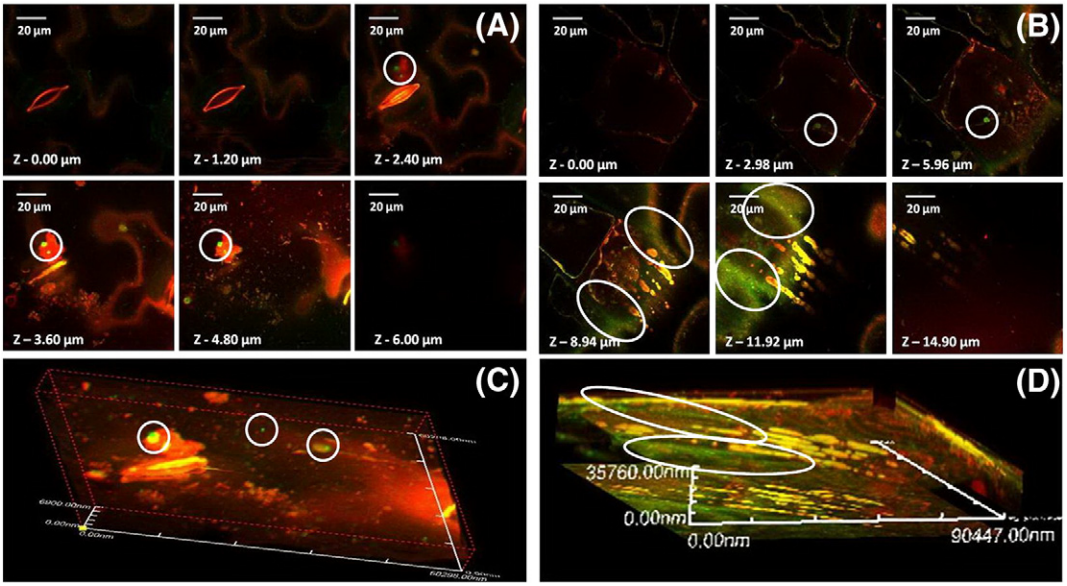

Fig. 4. Optical thin sections of lettuce scanned by laser scanning confocal microscopy (A and B) and 3-D construction (C and D). Lettuce leaves were contaminated with 6 log CFU/mL of GFP-labeled *S.* Typhimurium suspension. The leaf sample was sliced from lettuce that had no LMV infection and the root sample was taken from lettuce with LMV infection. The GFP-labeled *S.* Typhimurium is (green) embedded in plant tissue and marked in white circles/ovals. (A) Leaf, each successive image progressed by1.20 µm, deeper into the lettuce leaf; (B) root, each successive image progressed by 2.98 µm, deeper into the green lettuce root; (C) leaf; (D) root.

parts (the leafy parts) but not in the roots (Fig. 2). However, DNA of internalized *S.* Typhimurium was detected in both roots and leafy parts at all contamination levels when determined by qPCR. Considering that the qPCR method targets specific bacterial DNA sequences, the results generated by qPCR represent the total amount of internalized *S.* Typhimurium, whereas the plate count data reflect the cultivable internalized bacteria in the lettuce (Josephson et al., 1993). For the validation of surface disinfection efficiency, the surface *S.* Typhimurium was removed right after inoculation to avoid bacterial internalization because it could affect final determination of bacterial internalization. However, there may be a concern that the disinfection efficiency might be altered 2 days after the inoculation because some human pathogens (e.g. *E. coli* O157) have been shown to attach firmly to the leaf surface and became more resistant to hostile environment (Garrood et al., 2004; Kyle et al., 2010). This issue may need to be more discussed in a future study.

At low contamination levels, the extent of internalization, detected either by plate count or qPCR, was higher than the levels of bacteria in the initial inocula (Figs. 2 and 3). This indicates that *S.* Typhimurium is able to penetrate and multiply in the leaves, and be disseminated to the lower parts (roots). However, some of the internalized *S.* Typhimurium in the leaves and all in the roots, lose their culturability after 2 days. A similar phenomenon was observed in our previous study (Ge et al., 2012); all culturable internalized *S.* Typhimurium were detected only in the leaves, even though *S.* Typhimurium was inoculated in the rhizosphere soil. This could be attributed to plant defenses triggered by bacterial invasion (Schikora et al., 2009; Warriner and Namvar, 2010). However, the exact mechanism is unclear. Additionally, a reasonable explanation for the absence of culturable internalized *S.* Typhimurium in the roots is that the leafy part of the lettuce is more favorable for bacterial survival. This may be due to the presence of more nutrients in the leaves that are produced from photosynthesis and are available for bacterial utilization (Kroupitski et al., 2009a).

To simulate the water stress conditions, different volumes of irrigation water were used in this study. Application of drought and storm conditions caused significant changes in the moisture content of the soil compared to the optimal irrigation conditions (Table 1). Environmental changes induced by extreme weather are actually able to alter the plant's physiological activities. Under drought conditions, the production of a plant hormone called cytokinin is reduced, which consequently favors stomatal closure to prevent foliar water loss (Davies et al., 2005; Hare et al., 1997). On the other hand, in a field study, heavy rain was reported to trigger plant wound response and then promote stomatal opening, which can facilitate bacterial infiltration into the leaves (Hirano and Upper, 1999; Melotto et al., 2006). Therefore, either drought or storm could possibly result in changes in the penetration and internalization of *S.* Typhimurium in lettuce through the regulation of the stomata. However, in the current study, no statistically significant change in internalization was observed from the results of the plate count and qPCR ($P>0.05$). This suggests that the level of *S.* Typhimurium internalization in the lettuce tissue was not significantly changed by water stress when *S.* Typhimurium contamination occurred on the lettuce surface.

LMV was used to mimic plant stress caused by phytopathogen infection during field growth and to explore whether such stress can affect *S.* Typhimurium internalization under various irrigation conditions. As shown in Table 2, the concentration of LMV was relatively consistent in the infected leaves ($P > 0.05$). Microbial community and diversity could vary in lettuce species due to their differences in morphological and physiochemical characteristics. Studies about microbial interaction showed that competition among different microorganisms may be beneficial to the plants, and some microorganism can even suppress the growth of human pathogens (Hunter et al., 2010; Rosenblueth and Martinez-Romero, 2006). For example, the presence of *Enterobacter asburiae* was able to suppress the growth of *E. coli* O157:H7 or *S. enterica* on the seeds of *Arabidopsis thaliana* (Cooley et al., 2003). Research focusing on co-inoculation of phytopathogens and human pathogens on plants implied that phytopathogens may affect human pathogen internalization: stomatal closure is considered as an innate immunity response to inhibit foreign material invasion, but in the case of *Pseudomonas syringae pv. Tomato*, a phytopathogen, it can reopen the stomata in the *Arabidopsis* leaf epidermis. This would allow human pathogens to penetrate into the plant tissues (Melotto et al., 2006). Higher incidences of *Salmonella* Poona internalization were observed in cantaloupe when the fruits were co-inoculated with *Erwinia tracheiphila*, a common cucurbit wilt bacteria (Gautam et al., 2011). However, to our knowledge, internalization of human pathogens affected by plant viruses under extreme weather conditions had not been investigated.

In the study presented here, no significant difference was observed between the LMV-infected group and non-LMV-infected sample groups (Figs. 2 and 3). However, within the LMV-infected group, the culturable internalized *S.* Typhimurium in the drought and storm groups was significantly lower than the total internalization level (detected with qPCR), when the lettuce was contaminated with medium and high levels of *S.* Typhimurium. This was not observed in the non-LMV-infected groups (Fig. 2B). Several alleles in lettuce have been identified that combat or tolerate LMV infection and studies have revealed that the systemic movement of LMV to the non-inoculated leaves could be inhibited due to blockage of entry into the internal phloem (German-Retana et al., 2008; Pink et al., 1992). This may explain why the viruses in most of the healthy leaves were below detection level (Table 2); i.e., the lettuce's systemic defense was combating LMV dispersion to the non-infected leaves. The culturability of internalized *S.* Typhimurium in leaves could also be affected by the systemic LMV-induced defensive reactions, especially when the contamination level was medium or high. Different from the leafy parts, LMV infection seemed to be beneficial to the survival of internalized *S.* Typhimurium in the roots, regardless of water stress ($P<0.05$) (Figs. 2B and 3B).

In summary, this study showed that *S.* Typhimurium can be internalized in lettuce via contamination of leaf surfaces. The level of culturable internalized *S.* Typhimurium in lettuce was not affected by water stress conditions (abiotic stress) alone. Culturable internalized *S.* Typhimurium was lower in leaves after LMV infection (biotic stress) under water stress conditions when the inoculation level was medium or high. Culturable internalized *S.* Typhimurium was detectable in lettuce roots when the leaves were infected with LMV, whereas *S.* Typhimurium was undetectable without LMV infection. The present study indicates that the levels of pathogen internalization in fresh produce are influenced by stress factors frequently observed in the natural environment (e.g. phytopathogen infection). However, some environmental factors (e.g. water stress) may not significantly change *S.* Typhimurium internalization levels during the pre-harvest stage when the pathogens are presented to the plant surface.

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