CENTER for PRODUCE SAFETY

CPS 2010 RFP
FINAL PROJECT REPORT

Project Title
Risk assessment of *Salmonella* preharvest internalization in relation to irrigation water quality standards for melons and other cucurbits

Project Period
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Objectives
1. Conduct expanded assays of internalization with multiple Salmonella serovars to determine whether the observed absence of uptake is isolate or serovar specific.
2. Verify preliminary data of systemic internalization of pathogenic bacteria into melon vines by repeating select greenhouse studies with larger populations of plants.
3. Determine whether an internalization threshold can be observed for Salmonella into cantaloupe and honeydew fruit under greenhouse conditions.
4. Determine the potential for root uptake of Salmonella to vines and fruit under worst case field conditions.
5. Expand the delineation of irrigation water contamination thresholds for Salmonella to a broader group of melons and other cucurbits.

FINAL REPORT

1. Abstract

Internalization of *Salmonella enterica* from root uptake and transport within the vascular system of vines of various cucurbits was evaluated. Contaminated water and soil were utilized as the mode of delivery to determine whether previously characterized root uptake and limited aerial vine internalization of the applied bacterium may result in transference to fruit. Experiments using growth chamber, greenhouse and field studies were performed to evaluate the effect of a) different melon and cucurbit varieties, b) diverse *Salmonella enterica* serovars, c) variable dose of inoculum in water, d) time of plant exposure to contaminated water and soil, e) water deprivation on the ability of internalization of *Salmonella* for root uptake in melon and other cucurbits. Evaluation of *Salmonella* internalization into vines through root system, was assessed after application of pathogenic strains to the soil using contaminated water at two different inoculum doses (log 2 and 6 CFU/mL). Aerial sections of vines were excised and surface sterilized with silver nitrate after 2 and/or 7 days of inoculation and further analyzed through sample enrichment in selective media. Results indicate that the rate of internalization into vines is highly variable among a population of individuals and across replicated planting dates within a single seed source of a given cucurbit cultivar. Evidence for internalization is preferentially observed within the first vine inter-nodal region as a greater percentage of recovery was determined closer to the root:hypocotyl region as compared to the second vine inter-nodal segment. This repeated result, shown to be highly dose dependent, indicates a limited migration of the applied bacterium through the vascular xylem elements. In addition, the percentage of recoverable internal populations of *Salmonella* in these same vine segments decreases with time post- exposure which may also indicate low survivability or transition to a viable but not culturable (VBNC) cell condition within the vine. Comparison among different cultivars for the same cucurbit internalization assay was strongly dose dependent with results ranging from non-detection to ≤10% internalization at an initial inoculum dose of log 2 CFU/mL in contrast to ≥25% internalization within vines inoculated with log 6 CFU/mL. Field experiments utilizing virulence-attenuated *Salmonella enterica* sv Typhimurium applied through a drip irrigation line, demonstrated that although the soil rhizosphere was heavily contaminated with the bacterium, lack of detectable internalization into fruit and adjacent vine tissue of various cucurbits, including cucumber, zucchini, watermelon and squash, indicates that internalization is a highly uncommon and unlikely event under reasonable irrigation water quality parameters. In contrast, delivery of the attenuated *Salmonella* directly into the peduncle (point of fruit attachment to the main vine) of cantaloupe after a minor tissue wounding approximately 2 cm from the fruit abscission zone resulted in bidirectional movement in the vine and detection of the applied bacterium in the sub-rind tissue of the fruit. We conclude that internalization of *Salmonella enterica* delivered through irrigation water will result in soil contamination however root uptake and systemic transfer of the bacterium is highly limited and systemic transfer to the edible portion of cucurbits is of low risk concern. Furthermore, preliminary results support prior associative observations that common crop management practices including hardening-off of transplants and deficit irrigation of young established vines, prior to in-season cultivation, results in plant-based limitations on vascular mobility in the vine. Regardless, surface contamination should remain a consideration in food safety system design and assessment of irrigation water sources due to the potential for risk to consumer's health. Within current practices for cucurbit production in arid regions, the acceptability of low pathogen presence thresholds in source water may be used in defining appropriate microbiological standards for cucurbit irrigation provided crop management and other hurdles assure mechanisms to prevent foliar or fruit contact with applied water.

2. Background

Melons, including cantaloupe, honeydew, watermelon, and various mixed specialty melons (i.e. casaba, crenshaw, Galia, Juan Canary) is a major horticultural specialty crop in the United States for domestic production and overall consumption. California is responsible for approximately 58% of the national production and the State ranks number one in production acreage of honeydew and cantaloupe (NASS, 2011), while Florida leads the fresh market production of cucumber, squash and watermelon (Cantliffe et al. 2007). Over the past decade, melons produced internationally and domestically have been implicated in outbreaks of foodborne illness as well as multiple recalls due to positive pathogen detection, most typically due to presumptive or confirmed *Salmonella enterica* mostly on cantaloupes (Bowen et al, 2006; CDC, 1991; CDC 2002a, 2002b; Mohle-Boetani et al., 1999; Munnoch et al, 2009; Steele et al., 2005; Powell, 2011). As result, cantaloupes have been classified as a produce item of concern and their marketing has drawn particular focus for attention by the Food and Drug Administration (FDA) as it relates to microbiological food safety. Although cantaloupes are the central concern, in recent years, outbreaks and recalls caused by *Salmonella* have been associated with consumption of other cucurbits including fresh raw cucumber and watermelon (Food Safety News 2011, 2012). Commodity Specific Food Safety Guidelines for the Melon Supply Chain (PMA and United Fresh, 2005) and FDA Guide to Minimize Food Safety Hazards of Melons (FDA 2009) are evolving documents describing the result of hazard analysis and practice-based risk identification upon which both general and specific standards and audit criteria continue to be refined. One clear knowledge gap the CA melon industry has identified is the development of practical irrigation water quality standards, mainly in relation to the potential for systemic uptake and internalization of pathogens from applied water via soil contamination around the root/rhizosphere. Given aspects of common agricultural practices utilized for the production of most cucurbits, the extension of this research area to other cucurbits is an essential part of risk assessment to contribute to the development commodity specific standards that favor the food safety efforts in the produce supply chain.

Pathogen internalization into produce edible portions has been speculatively identified as a major risk as once microorganisms reach internal spaces or tissues the produce itself becomes a protective barrier against postharvest interventions applicable to fresh product handling, such as a wash-disinfection during packing, fresh processing, or consumer food preparation. Early studies suggested that *S. enterica* and *E. coli* could be transported to edible portions of plants through root systems (Bernstein et al., 2007; Solomon et al., 2002) in model systems, however recent studies have demonstrated that pathogen internalization is rather a rare event and highly dose dependent (Erickson et al., 2010 a, 2010b; Miles et al., 2009; Zhang et al., 2009). In the particular case of non-foliar contact water, water testing or treatment are recommended as routine performance criteria, but there are no accurate standards that establish a threshold dose associated with the likelihood of pathogenic bacteria to contaminate plants during melon production and the potential for fruit internalization.

3. Methodology description

Experimental design will be discussed individually in the research result section, thus technical information regarding the methodology is described in this section.

Strains utilized in this study.

a. Culture of *Salmonella* strains

All bacterial strains were cultured at 37°C for 18 hours on tryptic soy agar TSA supplemented with either kanamycin or rifampicin (TSA/kan and TSA/rif respectively). Approximately five colonies were re-suspended in 5 mL of Butterfield's Phosphate Buffered Saline (BPBS). A total of 100 μL were spread onto selective media and incubated for 18 h at 37 °C to allow the formation of a uniform lawn. In various studies, agar grown cells were found to be more tolerant to acute desiccation death associated with field inoculation (data not shown). In addition, during prior related studies and repeated here, better internalization rates were observed in vines inoculated with *Salmonella* that was previously cultured as a lawn on an agar-surface rather than in planktonic broth growth (Table 1). After incubation, cells were harvested by adding a small aliquot of BPBS and gently scraping the agar surface with a sterile rubber spatula to liberate cells and subsequently cells were more fully suspended in a target volume of BPBS. The resultant bacterial suspension was centrifuged at 1,500 x g for 10 min. The pellet was washed twice in BPBS and re-suspended in BPBS to adjust the optical density at 600 nm (approximately 0.750 absorbance) which corresponds to log 9 CFU/mL. The inoculum was then diluted to the desired concentration for inoculation of irrigation water and addition to soil. The final applied inoculum was serially diluted and plated on the corresponding selective media to determine the nominal concentration of inoculum.

Table 1. Comparison of melon vine internalization of *S. enterica* sv. Poona:Ina;kan^R+GFP (PTVS 151) previously grown on tryptic soy agar or tryptic soy broth culture.

Inoculation level (CFU/mL)	**Log 6**		**Log 2**	
Type of inoculum	Lawn Total positives (% of positives)	Planktonic Total positives (% of positives)	Lawn Total positives (% of positives)	Planktonic Total positives (% of positives)
Node 1	17 (29.8)	5 (8.77)	20 (35.1)	0 (0.00)
Node 2	2 (3.50)	1 (1.75)	4 (7.01)	0 (0.00)

[A] Recovery on BPW/Kan 48 h after vine inoculation. Results represent the number of positive samples by tube-nucleation assay screening after confirmation of the positive tubes on TSA/Kan (n=57 vines per treatment)

a.1 Culture and detection of *Salmonella enterica* with ice nucleation activity

Salmonella enterica sv. Poona Ina/Kan^R+GFP (strain PTVS 151) was previously selected as a transformant in Suslow laboratory to facilitate screening of large number of vines by utilizing the conferred trait to produce ice nucleation events after incubation in a screening assay at temperatures from -2 to -6 °C. Excised vine tissue samples, from plants previously root/soil inoculated with this isolate or irrigated with Salmonella-free water, were first enriched in 10 mL buffered peptone water supplemented with 50 mg/L of kanamycin (BPW/Kan) at 37 °C for 18 h. All new tubes with sterile BPW/Kan are pre-screened to -6°C before use in enrichments and any tubes that freeze at this temperature, often due to defects in the glass, are discarded. After enrichment, samples were removed from incubation and cooled in racks for 2 h at 2.5°C. Lowering temperatures following incubation at 37°C is essential to allow the outer-membrane proteins to fully aggregate and assemble into an ice crystal template before screening. Ice nucleation activity (Ina+) was screened by placing tubes with the enrichment culture in a 70% ethanol bath adjusted to 0°C; decreases in temperature at 0.5°C increments was done every 15

min until a bath temperature of -6°C were reached. Vines free of ice-nucleators will supercool to at least -5°C before spontaneous nucleation from other sources is likely. Between each incremental temperature drop each tube was observed to determine if ice formation had occurred. Samples with positive Ina+ events were plated on Tryptic Soy Agar amended with 50 mg/L of kanamycin (TSA/Kan) to confirm the presence of the applied PTVS 151. Representative tubes not displaying ice-nucleation above -4.5°C were also selected and tested for presence/absence of Kan^R and Kan^R+GFP as controls. Resultant colonies were screened for characteristic GFP fluorescence and PCR amplification of the *invA* as described by Ziemer et al. (2003).

a.2 Culture and detection of *Salmonella enterica* with rifampicin resistance

Salmonella enterica including serovars Poona (PTVS26), Gaminara (PTVS41), Enteriditis (PTVS44), Montevideo (PTVS45), Michigan (PTVS42), Agona (PTVS43), Newport (PTVS073), Typhimurium (aPTVS177), are derivative isolates which have rifampicin-resistance for facilitated recovery that were selected via spontaneous mutation for tolerance to 80 mg/L. Rifampicin is routinely used to selectively facilitate detection and recovery by minimizing interference from background bacteria, especially during greenhouse and field trials conducted in this study. Samples inoculated with these strains were first enriched in buffered peptone water supplemented with 80 mg/L of rifampicin (BPW/rif) at 37°C for 18 h. Enrichments were re-streaked onto *Salmonella* differential and selective agar Xylose Lactose Tergitol 4 (XLT-4) supplemented with 80 mg/L of rifampicin (XLT-4/rif) and incubated up to 48 h at 37°C; colonies were additionally evaluated by PCR amplification screening for *invA* as described above for confirmation. For field and greenhouse studies only strain aPTVS177 (virulence attenuated) was utilized. The strain lacks adenylate cyclase and cyclic AMP receptor protein rendering it avirulent yet still immunogenic. A derivative isolate, aPTVS177, is a rifampicin-resistant strain from aPTVS150 selected via spontaneous mutation for tolerance to 80 mg/L. Lab studies verified that aPTVS177 had an *in vitro* growth rate in several media indistinguishable from the parent strain and a plating efficiency exceeding 85% on selective and differential media (XLT-4/rif) following 20 generations without rifampicin amendment in the growth media or on plant surfaces (data not shown). The use of aPTVS177 in both, greenhouse and field studies was approved by the Office of Environmental Health and Safety (EH&S) and the Institutional Biosafety committee of the University of California, Davis.

b. Growth chamber studies and establishment of cucurbit vines

Melon and other cucurbits were planted in UC mix (33% peat, 25% sand, 42% fir bark) in the research greenhouse of University of California, Davis. After seeding, pots were watered every other day and fertilized every fourth day with 50% Hoagland's solution. When cucurbit vines developed up to three internodes, plants were transferred to the laboratory and placed in a plant growth-light outfitted growth chamber adjusted to 25°C. Plants were inoculated with 5 mL of inoculum of pathogenic strains of *Salmonella* (Section a), always making sure plants were watered to field capacity the day before of inoculation. Depending on the sub-objectives of each study (see Results section), inoculated vines were inoculated in the growth chamber and held for 48 h for vine internalization screening and up to 7 days with a light/dark cycle of 16/8 h. If vines were held more than 2 d, plants were irrigated every other day. The total plant populations for each of the trials performed is indicated in the Result section.

For vine analysis, vines were cut with a sterile scalpel above the soil-line and organized in groups of 5 vines; the group-cluster was secured with tape and the excision-ends were

covered with Parafilm™. Vines were surface sterilized by soaking them in a solution of 1% silver nitrate for 1 min and then rinsed in sterile distilled water for 1 min. The section covered with Parafilm™ was discarded and vines were cut from the first node to the area just below the second node junction and then, in sequence, from the second node to the area below the third node junction. Excised vines segments were cut transversally with a sterile scalpel and deposited into a test tube containing 10 mL of selective broth (BPWkan or rif) and enriched to screen for either ice nucleation or rifampicin resistance as described above.

c. Greenhouse trials

In a first trial, three cultivars of melon seeds (Cantaloupe “Oro Rico” F1 –OR and “Top Mark” –TM; Honeydew “Summer Dew” HMX 4593 –SD) were planted in UC mix watered daily and fertilized as needed with 50% Hoagland's Solution following standard practices in the research greenhouse of University of California, Davis. A total of 66 plants were established in an effort to produce fruit-bearing vines on a trellis-support system (22 plants per cultivar). At the stage of first male flowers, each vine root-mass was inoculated with 400 mL of log 7 CFU/mL of aPTVS177. After the first inoculation event, plants were inoculated every week with the same population of aPTVS177, until a total of 4 inoculations were completed. After 15 and 49 days from first inoculation, a total of 6 vines were excised just above the soil line. Vines were surface sterilized by soaking them into a solution of 1% silver nitrate for 1 min and then rinsed in sterile distilled water for 1 min. First and second internode sections of vines were cut transversally with a sterile scalpel and deposited into a sterile bag. Samples were covered with buffered peptone water (BPW/rif) and screened for rifampicin resistance and further colony confirmation of positives as described above.

To determine whether internalization of *Salmonella* occurred, mature melons were first surface sterilized and then analyzed to determine the presence of the bacterium after enrichment. Briefly, 2 sterile paper towels were saturated with 1% silver nitrate and placed on top of the melon rind surface, including the stem-scar area, for 10 min at room temperature. After paper sheets appeared dry these were removed and two new sterile paper towels saturated with 80% ethanol were draped on the rind for an additional 10 min. A sharpened cylindrical coring tool (1.5 cm diameter) was disinfected with 70 % ethanol and utilized to cut and extract the melon core (stem scar and subtending tissue to approximately 12-15 cm). The melon core sample was divided in two portions by using a sterile scalpel, the stem scar and the sub-rind melon mesocarp flesh, and analyzed separately. Samples were placed in individual sterile bags, covered with BPW/rif and screened for rifampicin resistance and colony confirmation of *Salmonella* as described above.

Fig 1. Honeydew production under greenhouse conditions

A second trial was attempted during the project period, however due to high powdery mildew pressure on most plants only honeydew vines were suitable for experiments. For this set of plants, immature fruits were inoculated at the peduncle with a drop of approximately 20 μL of log 6 CFU/mL of aPTVS177. After the drop was deposited on the peduncle, the vine tissue was slightly injured with a sterile needle right at the point where the inoculum was deposited to attempt to ensure entrance of the applied *Salmonella* to the plant vascular system with the least tissue disruption necessary. Melons, associated peduncles, and adjacent acropetal and basipetal vines were collected after 6 and up to 33 days of inoculation and transported in individual sterile bags to the laboratory. In the laboratory, adjacent vines and peduncles were detached from mature melons. Melons were surface sterilized prior to fruit and stem scar excision and enrichment, as previously described, to evaluate the presence of *Salmonella* in sub-rind fruit tissues. Additionally, adjacent vines were disinfected with 95% ethanol and then transversally cut in half for enrichment; peduncle and peduncle basal tissue were also enriched and *Salmonella* confirmation was performed as described above. For this particular trial, we noticed that for all collected melons from day 6 through day 18, detection of applied aPTVS177 was positive only at the peduncle but not in any adjacent tissues including the fruit, thus after 18 days of inoculation a second puncture without with a sterile needle (without further application of inoculum) was performed as it was suspected that the depth of the wound was insufficient to cause the bacterium access to vascular system.

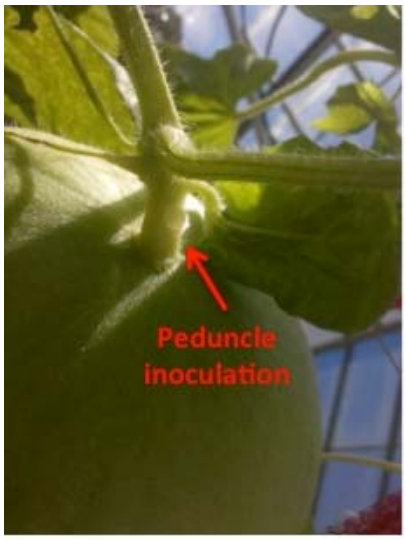


Fig 2. Honeydew inoculation at the peduncle

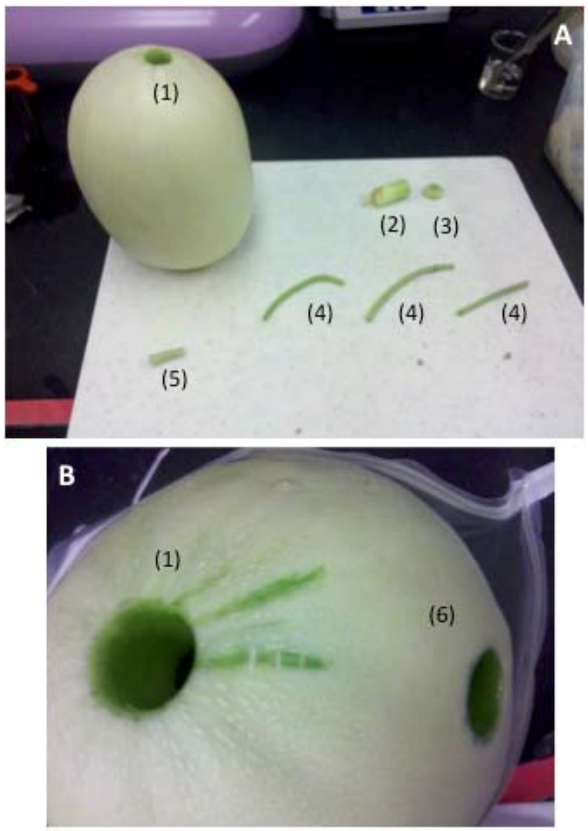


Fig 3. Melon collection and analysis for detection of *Salmonella enterica* (A) Analyzed melon sections (1) melon core (2) fruit from melon core) (3) stem scar (4) adjacent vines (5) peduncle (B) Melon fruit and skin collected around melon equator (6).

d. Field trials performed at UC Davis Research Farm facility

d.1 Root uptake of attenuated *Salmonella* Typhimurium and systemic transfer to cucurbit vines and fruit through sub-surface drip-emitter irrigation.

During this project period, cucurbits including melons, cucumbers, squash and watermelon were transplanted to the field. A total of 36 seedlings per bed were transplanted with an in-row spacing of 77 cm with 18 vines of each variety randomized to have a total of two replicated beds, having a total of 5 planted rows. A pressurized-tank of 1000 L of water, that fed the main drip manifold lines, was inoculated with 500 mL of log 9 CFU/mL of aPTVS177. Water was mixed in the tank by re-circulation and internal agitation prior to release to the drip lay-flat manifold lines. Pumping from the tank continued until the tank was fully discharged. After 2 h, water samples were collected at each bed at the end of the drip line to confirm distribution along the full length and to determine the resultant average concentration of the surrogate pathogen in the water by plating 100 μL on TSA/rif. A total of 12 vines with immature fruits of the cucurbits were collected after 10d of the application of aPTVS177, vines and immature fruits were analyzed for rifampicin resistance to determine the presence of attenuated *Salmonella*.

Figure 4. Appearance of cucurbit plant with immature fruit after surface sterilization.

d.2 Survival of attenuated *Salmonella* on melon surfaces

During the 2011 field trial, the study was focused on the survival of *S. enterica* on cantaloupe surfaces and in the evaluation of the likelihood of internalization to fruit assuming

that a large population *Salmonella* could reach the peduncle and fruit adjacent vine tissue. The setup of the field described for furrow-irrigated melons was utilized for this trial, but only 3 beds were planted. The melon field was divided in three sub-plots and randomly selected mature melons (n=30) from each subplot were tagged and marked with indelible ink with a circumference of about 10 cm of diameter. Marked cantaloupes from each subplot were inoculated with log 4, 6 or 8 CFU/mL of aPTVS177 with a spray bottle that was previously calibrated to release 2 mL of inoculum on the marked area. Cantaloupes were harvested after 48 h and 10 days of inoculation to determine the recoverable population of aPTVS177.

Collected melons were analyzed by aseptically removing the rind from the inoculated marked area with a sterilized knife. Inoculated melon rind was transferred to a sterile bag containing sterile potassium phosphate buffer (3.9 mM KH_2PO_4 and 6.1 mM K_2HPO_4) supplemented with 0.05% Tween 20. The rind was vigorously rubbed to detach bacteria and the suspension was plated on TSA/rif supplemented with 1% pyruvate acid (to promote recovery from sub-lethal environmental stress) to determine the population of aPTVS177. After plating, the remaining cell suspension was enriched with 10 mL of double strength BPW/rif and incubated at 37 °C for up to 24 h. Colony confirmation from enrichments was performed as described above.

d. 3 Internalization of attenuated Salmonella

A second set of randomly selected melons (n=60) from each subplot were tagged and the peduncle of each melon was inoculated with 20µL of either log 4, 6 or 8 CFU/mL of aPTVS177. After the droplet was deposited on the peduncle, the vine tissue was slightly injured with a sterile needle right at the point where the inoculum was deposited to ensure entrance of the applied *Salmonella* to the plant vascular system. Melons, associated peduncles, and adjacent acropetal and basipetal vines were collected after 9 days of inoculation and transported in individual sterile bags to the laboratory and were processed as described in section c.

4. Results

A set of consecutive trials were performed to determine the rate of internalization of *S. enterica* in a single cultivar of cantaloupe, an average of 23% of positive internalization was determined within the first node and right below the second node and approximately 10% for the second node up to the third node (Table 2). The reduction of almost half of the number of positives found indicates that the bacterium, although able to be transferred from roots within the vascular system, it appears likely that its movement through the vine xylem elements is very limited. Previous work funded by the California Melon Research Board, showed lack of internalization after irrigation with water contaminated with attenuated *Salmonella*, it is likely that although *Salmonella* can be taken through the root system the bacterium cannot reach the edible portion due to attachment events, limited mobility, and die-off during their residence in the vascular system. Additionally, variation among trials was also observed, which were empirically associated with the size and succulence of the plant. Trials 1 through 3 were done consecutively within one week and 2 days apart respectively, although the first two nodes had already appeared when first trial was performed, plants were about 2-3 cm smaller than those collected from trials 2 and 3, which suggests that internalization is associated with plant growth parameters of the vine.

Table 2. Trial variability influence on the rate of detectable internalization of *S. enterica* sv. Poona (PTVS151) in vines of cantaloupe variety Oro Rico

	Percentage of positive ice nucleation activity and *Salmonella* colony confirmation[A]		
Trial	First node	Second node	N
1	41.4	18.6	70
2	14	8	54
3	12	4	54
4	27	12	57
Mean± standard deviation	23.6 ± 13.6	10.6 ± 6.2	

[A] Recovery on BPW supplemented with kanamycin 48 h after vine inoculation. Results represent the number of positives samples after screening ice-nucleation positive tubes on TSA/kan

N represents the total number of inoculated vines with log 6 CFU/mL

The effect of dose and melon cultivar was determined, which showed a marked difference between the two applied doses (log 2 and 6 CFU/mL). The lower dose revealed up to 8% of the plants were positive for ice nucleation activity for both trials. In contrast the higher dose applied produced an increased frequency of internalization that, in some cases, represented 100% of analyzed plants being positive (Table 3). Non-inoculated plants and individual samples that did not show Ina+ above -4.5^{o}C were confirmed to be negative by culture plating of enrichments, as described above. Comparison among cultivars, showed different rates of internalization among cultivars, however this outcome was not consistent among trials. Though difficult to draw absolute conclusions, variations were found mostly among species (cantaloupe and honeydew), but not within cultivars of the same species. As mentioned for results in Table 2, these trials were done consecutively within one week, thus plants from Trial 2 were one week older than those for Trial 1, which may support that conclusion that the distance migrated for the bacterium is developmentally limited by plant age and growth traits.

Based on these outcomes and observations, a separate trial was initiated to explore the relationship between various presumptive physiological insults and the occurrence and rate of internalization of *Salmonella* into the two primary melon cultivars, Oro Rico (Cantaloupe) and Summer Dew (Honey Dew). A preliminary trial monitored the internalization, movement and survival of an ampicillin resistant strain of *S.enterica* ser. Montevideo into both melon cultivars that were subjected to various water availability conditions simulating flood and drought events. Five weeks after emergence, samples were inoculated with 5mL of log 8 CFU/mL *S. enterica* ser. Montevideo; their survival and distribution were determined by selective enrichment as described above. Outcomes of these trials appear to support the earlier observations that stressed plants, such as during hardening-off for transplanting or irrigation restrictions prior to early in-season cultivation may break continuity of the vascular elements and restrict internal movement of introduced bacteria such as *Salmonella*. In a subsequent trial, both cultivars were irrigated to exceed soil field capacity conditions under various mineral nutrient availabilities, specifically available nitrogen. Samples were similarly inoculated five weeks after emergence with a 5mL cocktail of Rifampicin resistant *S. enterica* ser. Poona and Montevideo (log 8 CFU/mL); their uptake, survival and distribution were determined by recovery in selective

enrichment media. The results of this ongoing project indicate that *Salmonella* species are more likely to internalize into both cultivars under flood-like water conditions irrespective of prior nitrogen availability.

Table 3. Effect of dose and melon variety on internalization of *S. enterica* sv Poona (PTVS151) in vines.

		Percentage of positive ice nucleation activity and *Salmonella* colony confirmation[A]			
		Trial 1		**Trial 2**	
	Inoculum dose (CFU/mL)	**2**	**6**	**2**	**6**
Cantaloupe varieties	Oro Rico	0	83	8	8
	Gold Express	8	100	0	25
	Durango	25	100	0	8
	Trinity	9	72.7	0	42
	Yosemite	0	17	0	42
	Olympic Gold	8	42	0	25
	Mean± standard deviation	8.3 ± 9.1	69.1 ± 33.3	1.3 ± 3.2	25.0 ± 15.2
Honeydew varieties	Summer Dew	8	33	0	8
	Honey Dew H2196	0	42	8	0
	Saturno	0	25	0	25
	Mean± standard deviation	2.0 ± 4.0	33.3 ± 8.5	2.0 ± 4.0	14.5 ± 12.5

[A] Enrichment in BPW/kan 48 h after vine inoculation. Results represent the number of positives samples form ice nucleation after screening the tubes on TSA/Kan (n=12 vines per treatment)

Internalization frequency of different *Salmonella* serovars was observed (Tables 4A and 4B). In this experiment two independent trials were performed. The first trial consisted of screening seven different serotypes with a single popular cantaloupe cultivar, Oro Rico. The percentage of internalization detected was observed to be variable depending on the serovar, particularly at the level of the second node, suggesting that serovars might have different potential for internalization and subsequent movement to upper levels of the melon vines. In this case, sevorars Poona and Enteriditis, had the greater percentage of internalization in both first and second node, followed by Newport, Michigan, Agona and Montevideo and a lower rate for *S. enterica* serovar Typhimurium under the test conditions (Table 3).

After the first trial, a second trial using *Salmonella* cocktails of the different serovars was done by grouping the serovars by their rates of internalization: Poona and Enteriditis, Michigan and Gaminara, Montevideo and Agona. As occurred before, lower rates of inoculation were observed, this also corresponded with younger plants for the first trial (31 days) and slightly

older plants for the second trial (40 days). After 7 days of inoculation, remaining vines that were held in the growth chamber were analyzed (n=10) but *Salmonella* was not detected, suggesting a die off of the bacterium or transition to VBNC state during this time interval.

For this second trial, cocktails of Poona and Enteriditis and Montevideo and Agona, showed greater percentages of internalization as compared to the Michigan and Gaminara cocktail, which corresponded with the observations in the first trial; all outcomes supporting that *Salmonella* serovar likely influence the potential for internalization and transference.

Table 4A. Comparison of melon vine internalization (cantaloupe Oro Rico) by different *Salmonella enterica* serovars.

		Percentage of positive detection of *S. enterica* after vine enrichment[A]	
Strain ID	**Serovar**	**First node**	**Second node**
PTVS177	Typhimurium	22	66
PTVS041	Gaminara	89	11
PTVS042	Michigan	89	28
PTVS045	Montevideo	94	11
PTVS043	Agona	94	22
PTVS073	Newport	94	39
PTVS026	Poona	100	50
PTVS044	Enteriditis	100	56

[A] Enrichment in BPW supplemented with rifampicin 48 h after vine inoculation. (n=18 vines per treatment). Initial concentration of inoculum was 5 mL of log 6 CFU/mL

Table 4B. Comparison of melon vine internalization by different *Salmonella enterica* serovar cocktails.

		Percentage of positive detection of *S. enterica* after vine enrichment[A]	
Cocktail	**Serovar**	**First node**	**Second node**
A	Michigan Gaminara	6	0
B	Poona Enteriditis	28	6
C	Montevideo Agona	39	6

[A] Enrichment in BPW supplemented with rifampicin 48 h after vine inoculation (n=18 vines per treatment). Initial concentration of inoculum was 5 mL of log 6 CFU/mL

Evaluation of internalization of *Salmonella* into vines of different cucurbits was determined. As showed in previous experiments, for all cultivars, the effect of inoculum dose was strongly correlation with the frequency of detectable internalization; greater numbers of positives were determined for those inoculated with log 6 CFU/mL than those with log 3 CFU/mL (Table 5). Differences among cultivars were also observed; in particularly squash had a greater rate of internalization at both nodes than the remainder of the cucurbits tested, while cucumber had the least rate of detected internalization (Table 5). In contrast to outcomes observed for melons, squash and cucumbers were found to have a greater percentage of

internalization after 7 days of inoculation than after 2 days (Table 6). It is being hypothesized that melon vines might cause die-off of *Salmonella* which could be associated with preformed, colonization-induced, or wound-induced (during excision for detection assays) constituents present in the vascular system. This characteristic could be related with the plant species, thus if there is not a limiting factor within the vascular system the internalized bacterium could remain viable or culturally recoverable for a longer time within the vine system.

Table 5. Effect of dose and cucurbit variety on the internalization of *S. enterica* into different cucurbit vines.

	Percentage of positive detection of *S. enterica* in cucurbit vines after enrichment[A]			
	First node		**Second node**	
Inoculum dose (log CFU/mL)	**2**	**6**	**2**	**6**
Grisson Pattison Squash F1	0	94.4	0	27.8
Squash OFEQF1	11	100	0	66.7
Melon H2DF1	0	66.7	0	11.1
Melon OroRico	0	68.8	0	6.3
Melon 8HD2d2F1	0	72.2	0	11.1
Melon 8H68bF1	0	38.9	0	0
Watermelon 8402F1	0	61.1	0	11.1
Mini-watermelon EDOMF1	0	72.2	0	11.1
Cucumber ADAF1	0	33.3	0	5.6

[A] Enrichment in BPW/rif 48 h after vine inoculation (n=18 vines per treatment). Initial concentration of inoculum was 5 mL of the indicated dose of a *Salmonella enterica* cocktail that was composed of equal number of cells of the serovars Poona, Newport and Michigan

Table 6. Effect of time of vine exposure to *Salmonella enterica* on its internalization into different cucurbit vines.

	Percentage of positive detection of *S. enterica* in cucurbit vines after enrichment[A]			
	First node		**Second node**	
Time of exposure to inoculum (days)	**2**	**7**	**2**	**7**
Grisson Pattison Squash F1	33.3	69.2	16.7	23.1
Squash OFEQF1	61.5	85.7	23	50
Melon H2DF1	60.0	0	0	6.7
Melon OroRico	33.3	0	6.7	0
Melon 8HD2d2F1	62.5	0	18.8	0
Melon 8H68bF1	55.6	5.6	0	0
Watermelon 8402F1	33.3	8.3	0	0
Mini-watermelon EDOMF1	26.7	21.4	6.7	0
Cucumber ADAF1	5.6	0	0	5.9

[A] Enrichment in BPW rif 48 h after vine inoculation (n=18 vines per treatment). Initial concentration of inoculum was 5 mL of log 6 CFU/mL of *Salmonella enterica* cocktail that was composed of equal number of cells of the serovars Poona, Newport and Michigan

Similarly, field trials performed to determine whether internalization into vines and fruit of different cucurbits showed that after application of the inoculum through sub-surface drip irrigation lines, detection of applied *Salmonella* was negative for both immature fruits and adjacent vines, although the plant rhizosphere was positive (Table 7). This indicates that although the root system was in contact with the applied microorganism, and with previous demonstration that vines can uptake the bacterium under greenhouse conditions, the bacteria may not be internalized under field soil conditions, is not mobile in the vascular system, or likely does not survive for extended periods of time and thus does not reach edible portion or fruit adjacent vines, which has also been thoroughly studied for melon fruits under similar conditions.

Table 7. Detection of attenuated *S. enterica* in different immature cucurbit fruits and after 10 d of exposure to contaminated water through drip irrigation during field production

	Samples with positive detection of aPTVS177/total number of analyzed samples		
	Fruit	**Adjacent vines**	**Soil rhizosphere**
Cucumber	0/6	0/12	3/3
Zucchini	0/6	0/12	3/3
Small cucumber	0/6	0/12	3/3
Watermelon	0/6	0/12	3/3
Squash	0/6	0/12	3/3

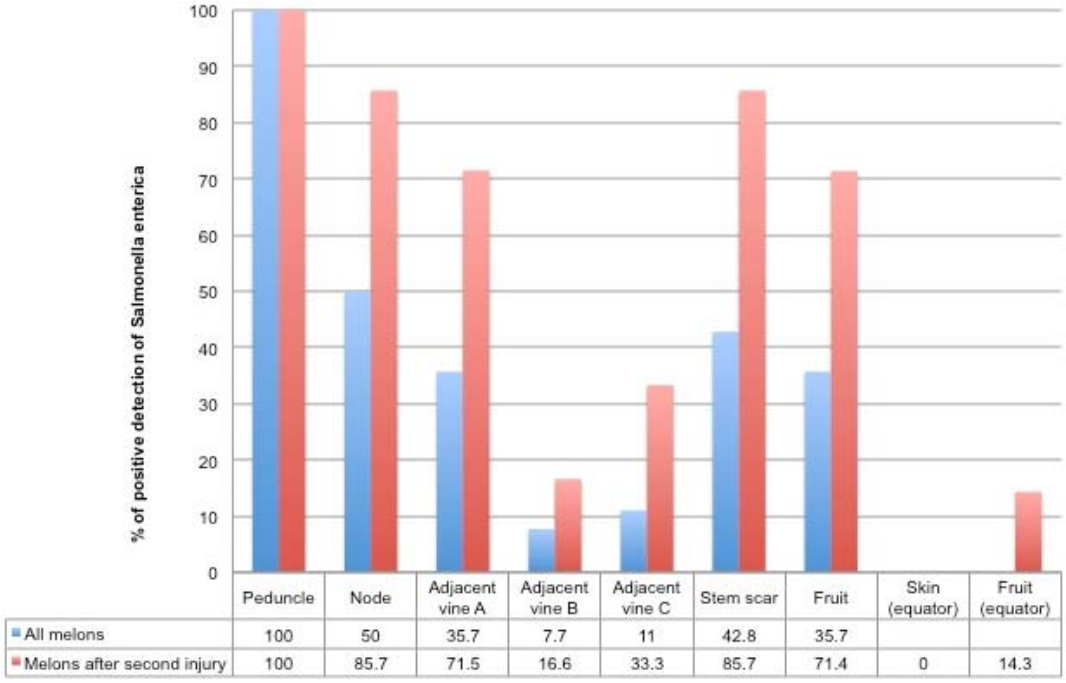
Water concentration after first irrigation through drip lines was approximately log 5 CFU/mL

Given the limited migration of *Salmonella* observed within cucurbit vines after the exposure opportunity for uptake from the root system, a set of greenhouse and field studies were performed to determine whether internalization of this bacterium would occur if it could reach sections of the vines closer to the edible portion. Inoculation at the peduncle was performed first under greenhouse conditions by applying a single dose of log 6 CFU/mL which was then introduced by creating a minor wound in the inoculated area. The purpose was to answer the question, “Can *Salmonella* cross the fruit abscission zone and reach sub-rind tissue?”. The approach was to allow the internalization of the bacterium to the vascular system and then conducted destructive sampling of melon and adjacent vines after 6 days of inoculation by collecting the honeydews as they were maturing. From day 6 up to day 18, all melons were only positive at the peduncle section were inoculum was directly applied however the remainder of the analyzed tissues were negative. Therefore, with the limited fruit availability, a second wound was performed without adding inoculum to encourage entry and internalization of the remaining viable *Salmonella* at the site of previous inoculation into vascular system, after 18 days. The frequency of positive detection increased within most analyzed tissues in a bidirectional orientation (above and below the wound site); adjacent vines and the associated fruit were both positive, indicating that *Salmonella* can potentially persist in wounded melon vine tissue and be mobilized within the vine. These results further suggest that if *Salmonella* were able to reach the vascular system in vine tissue close to the edible fruit, it could be transferred to the sub-rind fruit tissue (Fig. 5).

One outcome worth noting, but as a sole data point deserves limited assignment of significance, was the detection of a single fruit with confirmed sub-rind presence of the applied Salmonella removed from the stem scar region (Fig. 5 – Fruit equator). We are confident that all protocols and precautions to prevent and preclude laboratory cross-contamination were taken in the handling and processing of samples. However, as a rare outlier event of detection it is not

possible to prove such an event was not responsible for *Salmonella* in this fruit tissue. We feel the field inoculation data are sufficiently compelling to support the potential that some transference of inoculum to sub-rind tissue is possible under speculative conditions of vine wounding and elevated cell densities (Fig. 6).

A similar experiment was repeated in the field, but utilizing different inoculum dose concentrations and methods ensuring that the wounding was deep enough to reach the vascular system, a total of three dose levels were applied to the peduncle; log 4, 6 and 8 CFU/mL and melons were collected after 9 days of inoculum application. Internalization into fruit and adjacent vines did not occur in a strictly dose-dependent manner, but higher frequencies among the number of individual fruit tested at the higher doses suggests that, developmentally, the vascular system is sufficiently open and suitable environment for *Salmonella* to allow low number of cells to be transferred short distances (Fig 6; Table 8). Based on the limited number of replicates, in this preliminary evaluation under field conditions, once within the fruit mesocarp tissue (edible flesh) conditions may allow for localized accumulation of contamination on an infrequent basis; one of twenty fruit (Table 8 – log 4 CFU/ml)



	Peduncle	Node	Adjacent vine A	Adjacent vine B	Adjacent vine C	Stem scar	Fruit	Skin (equator)	Fruit (equator)
All melons	100	50	35.7	7.7	11	42.8	35.7		
Melons after second injury	100	85.7	71.5	16.6	33.3	85.7	71.4	0	14.3

Figure 5. Percentage of positive detection of *Salmonella enterica* sv Typhimurium (PTVS 177) in different tissues adjacent to the area of inoculation, after inoculation at the peduncle. (All melons data –blue- include melons collected from day 6 to day 18, while melons after second injury –red- corresponds to the data collected after 18 up to 33 days from the first inoculation and after a second wounding was created)

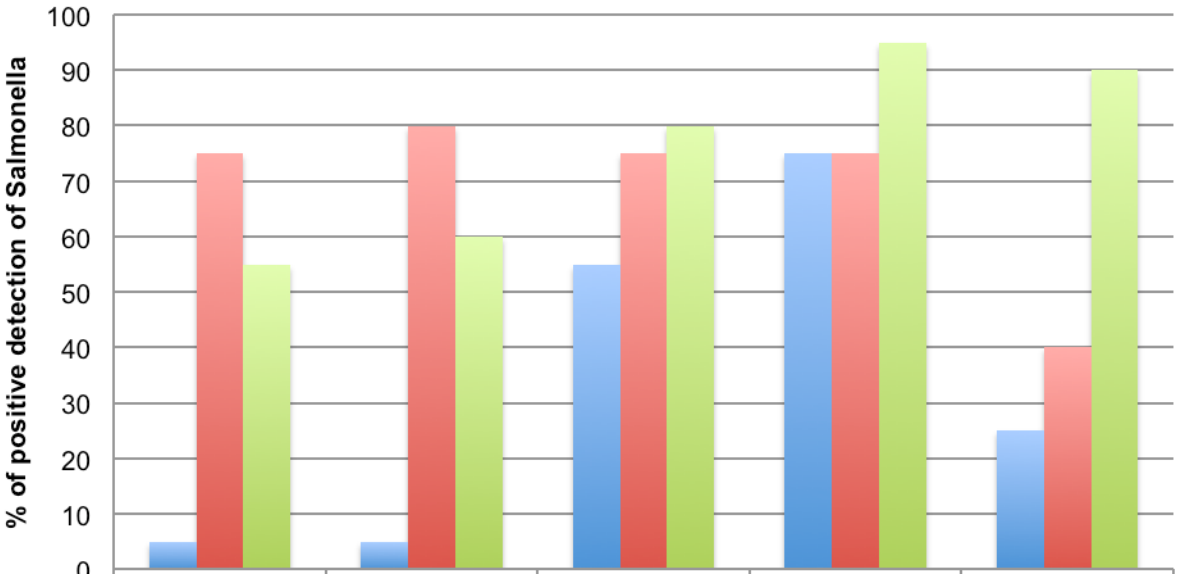


Figure 6. Dose dependent internalization of *S. enterica* to melon fruit and associated tissues after 9 days of inoculation in melon peduncle during field production

Table 8. Quantitative recovery of peduncle-inoculated attenuated *Salmonella enterica* from sub-rind tissue at the stem scar region following surface disinfection with 1% silver nitrate

Applied dose (log CFU/mL)	**4**	**6**	**8**
Direct recovery of Salmonella in mesocarp tissue (aPTVS177) [log CFU/g]	5.09 ± 0.00	2.96 ± 0.85	4.07 ± 0.96
Total number of positives by direct plating[A]	1	10	5
Total number of positives [B]	1	16	10

[A] Number of positives where quantification of the applied Salmonella was feasible by direct plating (n=20 total number of samples)
[B] Number of positive samples including both; direct quantification by plating and after enrichment (n=20 total number of samples)

Conclusions

Internalization of *Salmonella* into vines by root uptake and subsequent transference within the vascular system of the *Cucurbitaceae* appears to be influenced by the plant species, the *Salmonella* serovar, and, more definitively, by the dose of *Salmonella* applied. Our outcomes provide evidence in support of the potential for uptake of the pathogen through the root system, however this internal acquisition of an enteric human pathogen seems to be transient in nature as only limited mobility and persistence in time and distance within the vine was observed. Thus, although internalization of *Salmonella* delivered through irrigation water can result in soil contamination the likelihood of internalization to edible plant portions of the different cucurbits analyzed appears to be a very low risk-concern priority. Similarly, it may be that sub-surface soil contamination in general, at low pathogen cell densities by other inputs or unintended pre-seeding or pre-transplanting contamination, may have a very low probability of resulting in root uptake and transfer to developing and maturing fruit. The extended post-wound inoculation survival and apparent capacity for bidirectional movement in melon vine tissue, under experimental conditions, would seem to negate the belief that bacteria such as *Salmonella* cannot cross anatomical barriers in the abscission zone at the point of fruit attachment. The establishment of irrigation water standards for this commodity group should consider these outcomes as a baseline of evidence to support that a high indicator threshold may be used for non-foliar contact water. Regardless, irrigation water quality is not to be summarily dismissed in any site-specific hazard analysis. Careful consideration of water quality influences and proactive design of preventive controls should be taken to address the potential for surface contamination of melons and related cucurbits, which remain an area of concern related to irrigation source, mode of application, and transfer from soil.

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6. Outcomes and Accomplishments

The key outcome was the demonstration of the possibility of root uptake and transport of *Salmonella* into cucurbit vine tissue but the absence of practical concern, in illness and outbreak prevention, for the role of movement of bacterial contaminants in the vascular system to fruit. We have accomplished documenting combined controlled environment and open-fled environment evidence that microbiological irrigation water standards for melon production, which typically involve limited or no foliar contact, may be considerably less stringent than in some other crop management schemes. There was

no evidence observed within the experimental assessments to suggest a significantly greater risk of internal persistence and transfer to fruit among the cucurbits evaluated by species or cultivar.

7. Summary of Findings and Recommendations

Uptake of enteric bacterial pathogens via roots, from mildly contaminated field soil or non-foliar contact irrigation should not be a preoccupying concern for the industry or public health regulators relative to other sources of risk to public health. While several highly intriguing research questions result from the investigation outcomes regarding the variable capacity of *Salmonella* to survive as an endophyte in melon vine tissue and the role for melon vine constituents to limit persistence of internalized cells, this does not seem a priority area for future funding by CPS or the melon industry.

APPENDICES

Publications and Presentations (required)

- "Characterization of root uptake and systemic transport of *Salmonella* enterica into melon vines and fruits" Lopez-Velasco, G., Sbodio, A., Hup T., Wei, P., Maeda S., Suslow, T.V. International Association for Food Protection, Milwaukee, WI, August 2010.
- Overview of Melon Food Safety Research. Oral presentation by T. Suslow. CA Melon Research Board Annual Meeting. Jan 2011 and 2012. San Diego, CA.
- "Risk assessment of root uptake and systemic vine-transport of *Salmonella enterica* sv. Typhymurium by melon (*Cucumis melo*) during field production." G. Lopez-Velasco, A. Sbodio, A. Tomás-Callejas, P. Wei, K. H. Tan, and T. V. Suslow. Submitted to Int. J. of Food Microbiol.

Budget Summary (required)

All funds allocated for salary and benefits, greenhouse and field research space charges, travel, supplies, and biohazardous waste disposal were expended to execute this project during the funding period and approved extension.

My greatest disappointment and regret relative to this project and the expenditure of allocated funds was near complete failure to establish a useable stand of melons and other cucurbits in our field trials. Highly successful cantaloupe and honeydew trials were completed in 2009 and 2010 at the same Experimental Farm but it is not an exaggeration to identify 2011 as a substantial crop establishment disaster. Without dwelling on the details of weather as a key barrier compounded by worker proficiency shortfalls outside of our control as the key contributors for these lost opportunities, it is sufficient to say that the lack of planning and communication by technical staff was not the reason for failing to fully meet the planned objectives. In the same regard, the Plant Sciences farm manager and staff, recognizing the dual shortfall in crop establishment, were highly responsive and helpful in defining alternative melon field access which allowed us to complete many of the planned objectives, though in a truncated form.

Suggestions to CPS (optional)

Appendix to Final Report CPS-013112

Project Title:
Risk assessment of *Salmonella* preharvest internalization in relation to irrigation water quality standards for melons and other cucurbits

Appendix title: Internalization of *Salmonella enterica* through root uptake in various cucurbits during experimental field production.

Principal investigator: Trevor V. Suslow tvsuslow@ucdavis.edu

Research staff: Gabriela Lopez-Velasco
Adrian O. Sbodio
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Polly Wei
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The assessment of root uptake and systemic vine transport of *Salmonella enterica* has been evaluated in greenhouse conditions with melon vines of *Cucumis melo* (cantaloupe and honeydew) as well as other *Cucurbitacea* vines including watermelon, cucumber and squash. The final report was submitted to the Center for Produce Safety in early 2012 but due to field production issues during the main project period some objectives were not fully completed. To complement the Final Report results, field trials were conducted during 2012 under research gift funding. Results of the earlier study showed that internalization of *Salmonella* into vines was predominantly dependent on the inoculation dose, strain of *Salmonella* and cucurbit cultivar. The same study also showed that the levels of internalization into vines was limited to the initial inoculum exposure event into the plant root system and thus the percentage of detectable internalization in vines declined rapidly over a two week period.

To extend these field observations, during late spring and summer of 2012, populations of various cucurbits were transplanted into the UCD Plant Sciences Research Farm, at a location approved by the Institutional Biosafety Committee, to:

1. Determine the potential for root uptake of Salmonella to vines and fruit under worst- case field contamination conditions.
2. Expand the delineation of irrigation water contamination thresholds for Salmonella to a broader group of melons and other selected cucurbits.

Both objectives had been previously evaluated for honeydew and cantaloupe fruits in which, the rate of internalization of Salmonella into mature fruit was evaluated after application of contaminated water through furrow or drip delivery systems. The study showed that under worst-case scenario, *Salmonella* was able to survive in soil which in turn served as vehicle to contaminate the outer rinds of mature product, however internalization was never detected (Lopez-Velasco et al. 2012).

In the present study, the ability of *Salmonella* to internalize cucurbit vines, once the rhizosphere has been contaminated, was evaluated during field production.

Materials and methods

Bacterial strains and inoculum preparation

For field studies *Salmonella* enterica *sv* Typhimurium (*aPTVS177)* was utilized. The strain lacks adenylate cyclase and cyclic AMP receptor protein rendering it avirulent yet still immunogenic. A derivative isolate, aPTVS177, is a rifampicin-resistant strain from aPTVS150 selected via spontaneous mutation for tolerance to 80 mg/L. Lab studies verified that aPTVS177 had an *in vitro* growth rate in several media indistinguishable from the parent strain and a plating efficiency exceeding 85% following 20 generations without rifampicin amendment in the growth media or on plant surfaces. The use of aPTVS177 in field studies was approved by the Office of Environmental Health and

Safety (EH&S) and the Institutional Biosafety committee of the University of California, Davis.

A pure colony of aPTVS177 was cultured at 37 °C for 18 hours on tryptic soy agar TSA supplemented with 80 mg/L of rifampicin (TSA/rif). Approximately five colonies were re-suspended in 5 mL of Butterfield's Phosphate Buffered Saline (BPBS). A total of 100 μL were spread onto selective media and incubated for 18 h at 37 °C to allow the formation of a uniform lawn. After incubation, cells were harvested by gently scraping the agar surface with a sterile rubber spatula and suspended in BPBS. The resultant bacterial suspension was centrifuged at 1,500 x g for 10 min. The pellet was washed twice in BPBS and re-suspended in BPBS to adjust the optical density at 600 nm, approximately 0.750 absorbance, which corresponds to log 9 CFU/mL. The inoculum was then diluted to log 6 CFU/mL. Final inoculum was serially diluted and plated on the corresponding selective media to determine the best estimate of actual delivered inoculum concentration.

Field study

A total of eight beds of 200 ft each were utilized for this experiment. Each bed was divided in half, having a total of 16 blocks that were randomized to have a total of two repeated beds (Fig. 1). Transplants of eight cucurbits (cucumber, squash -2 varieties-, melons -3 varieties-, mini-watermelon and watermelon) were transplanted to the field. Two rows of 36 seedlings each were transplanted with an in-row spacing of 35 cm in each bed. Transplants were initially irrigated with sprinklers for establishment and approximately 2 weeks later irrigation was switched to a furrow delivery.

Inoculation of the plants was performed approximately four weeks after seedlings were transplanted. For watermelons, cucumbers and melons approximately 5 to 8 leaf-nodes had been already developed and immature fruits were present. In the

case of squash, the growth was faster and succulent vegetative growth and fruit closer to harvestable maturity were present. The inoculation was done with 20 mL of log 6 CFU/mL that were directly deposited in the root zone at approximately 15 cm from the origin of the vine (Fig. 2). Each plant was individually inoculated. After inoculation, irrigation through furrow delivery was performed to allow the movement of the inoculum within soil pore spaces to the rhizosphere.

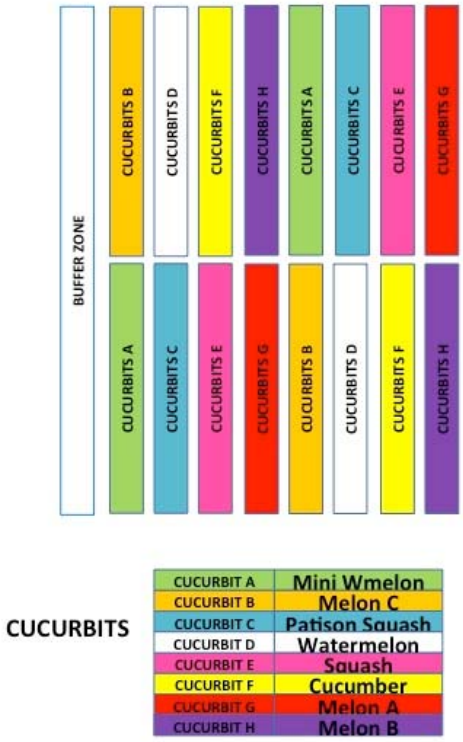


Figure 1. Experimental design and bed distribution for cucurbit production

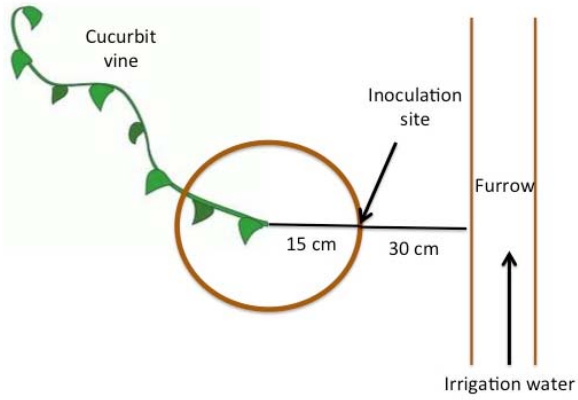


Figure 2. Schematic diagram of vine inoculation

Sample processing and *Salmonella* recovery

Sample collection was done after 3 and 7 days of the inoculation. The sample size ranged between 15 to 60 plants depending of the stage of maturity and healthy plant availability. The entire plant (including fruit if present and root-rhizoplane mass) were individually placed in a collection bag and brought to the laboratory. The root area was removed and processed separately. For vine analysis, sections approximately 20 cm from the hypocotyl region above the soil were collected. All exposed tissue was covered with parafilm and plants were first rinsed with water and then submerged in 1% silver nitrate for 1 min and rinsed twice with sterile water. For the fruit, when present, the peduncle was severed from the plant, covered with parafilm and surface disinfected with 1% silver nitrate as described above.

The first two to three nodes of the vines (approximately 5-10 cm) were transversally sliced along the plant with a sterile scalpel and placed in a test tube containing 10 mL of buffered peptone water amended with 80 mg/L of rifampicin (BPW/rif). Tubes were incubated at 22^{o}C for 2 h and moved to 37^{o}C for 18 h for enrichment. Enrichment cultures were re-streaked onto *Salmonella* differential and selective agar Xylose Lactose Tergitol 4 (XLT-4) supplemented with 80 mg/L of rifampicin (XLT-4/rif) and incubated up to 48 h at 37°C for colony confirmation.

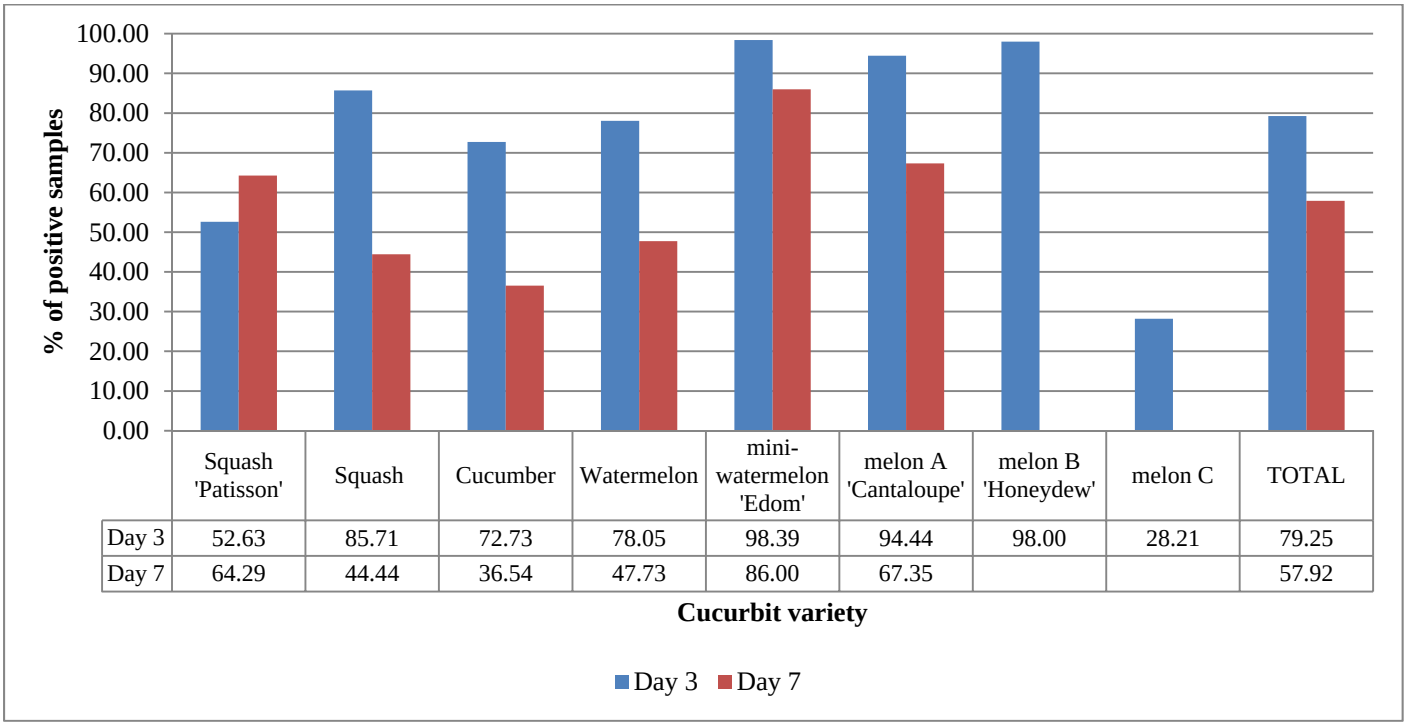
The peduncle of the surface disinfected fruit was discarded and fruits were sliced transversally to expose internal tissue. Sliced fruits were placed in a sterile collection bag and sufficient BPW/rif added until the entire sample was covered. Samples were enriched and colony confirmation was performed as described for vines.

The roots were directly placed in a sterile collection bag and 75 mL of BPW/rif were added. Samples were enriched and colony confirmation was performed as described for vines. Roots were not washed to demonstrate that the inoculated bacterium was able to reach the root-rhizoplane surface.

Results and discussion:

After three days of inoculation and the immediate irrigation event, the deposited inoculum was able to reach the root system of most cucurbits. The percentage of detection in the samples seemed to vary by cultivar, however with exception of Squash var. Pattison and one variety of cantaloupe where only 50 and 28% of the roots were contaminated with *Salmonella*, the applied microorganism was detected in 70 to 98% of the analyzed roots (n=294) with an average of 80% for all varieties (Fig. 1). As previously mentioned, irrigation was applied through the furrows to allow lateral water movement across the transplant bed profile and, presumably, inoculum distribution within the root zone. During the irrigation, the applied water was always below the bed shoulder, never crossing the bed surface, thus inoculum distribution could be attributed to sub-surface lateral water movement from the furrows to the beds. Although it would be unlikely to have a concentration of a million cells of *Salmonella* in the soil, the results suggests that such a focal point source, similar to fecal matter deposit, on the soil could contaminate a localized root zone area. Irrigation could provide a means for wider distribution of pathogens around root area and down a furrow to cause surface contamination.

The prevalence of *Salmonella* in the root system declined to an average of 57% after seven days of inoculation which could be associated with a reduction in detectable recovery of these populations, as was observed in earlier greenhouse studies (Fig. 3). However, detection in more than 50% of the samples supports the findings of other studies where *Salmonella* can survive for long periods of time in the soil and around the rhizosphere (revised by Jacobsen and Bech, 2012).

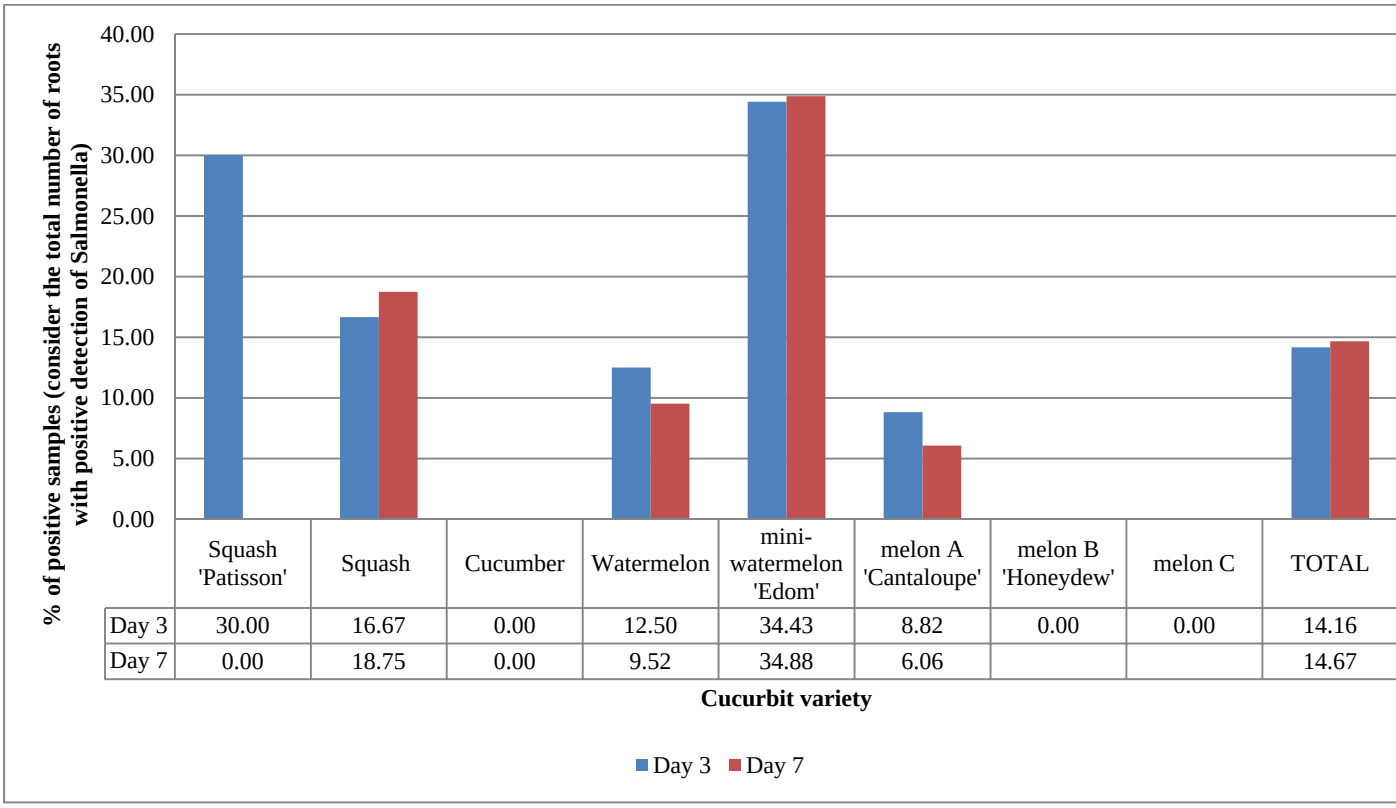


	Squash 'Patisson'	Squash	Cucumber	Watermelon	mini-watermelon 'Edom'	melon A 'Cantaloupe'	melon B 'Honeydew'	melon C	TOTAL
Day 3	52.63	85.71	72.73	78.05	98.39	94.44	98.00	28.21	79.25
Day 7	64.29	44.44	36.54	47.73	86.00	67.35			57.92

Figure 3. Detection of *Salmonella* in the root rhizoplane system

Internalization of *Salmonella* was assessed in vines and immature fruits after surface sterilization with silver nitrate. *Salmonella* in vines (up to second to third node) was detected in watermelon, squash, mini-watermelon and one variety of cantaloupe (Fig 4). The rate of internalization ranged from 12 to 34% with the highest value corresponding to mini-watermelon samples and an average of 14% for all samples (n=294). The applied microorganism was not detected in cucumber or honeydew vines

tested in this trial. The tendency of these results is similar to greenhouse studies (Table 1A-B for reference) which strongly suggests the influence of cultivar in the internalization of *Salmonella.* Similarly to previous studies performed under greenhouse conditions, after 7 days the percentage of detectable internalization declined, which could be associated to a reduction of the population in the soil and also to the limited endophytic fitness of the inoculated *Salmonella* Typhimurium isolate within the vine.



	Squash 'Patisson'	Squash	Cucumber	Watermelon	mini-watermelon 'Edom'	melon A 'Cantaloupe'	melon B 'Honeydew'	melon C	TOTAL
Day 3	30.00	16.67	0.00	12.50	34.43	8.82	0.00	0.00	14.16
Day 7	0.00	18.75	0.00	9.52	34.88	6.06			14.67

Figure 3. Detection of *Salmonella* in the vine system after surface sterilization. (Melon B and C were not analyzed after 7 days of inoculation)

During the first collection timepoint (day 3), there were a number of squash (zucchini type) that were close to harvestable maturity; a total of 12 fruit were collected and surface sterilized and *Salmonella* was present in 50% of the analyzed fruits (data not shown). After seven days, there were sufficient immature fruit developing in other

cucurbits, thus a total of 90 samples were collected. Detection of *Salmonella* after surface sterilization was, however, present in 1 out of 20 samples of mini-watermelon, which corresponded to 1% of internalization from the total sample collection, and the detection was negative for all other immature cucurbit (data not shown).

The results support our previous findings with large number of analyzed cantaloupe and honeydews, in which although it is possible that *Salmonella* can reach the vine system from the root, it does not appear to move systemically beyond the initial few nodal junctions or it dies off (Lopez-Velasco et al. 2012). Further studies to examine whether endophytic traits are expressed in this environment could certainly help to elucidate the actual risk of *Salmonella* internalization. It is important to point out that in comparison with other cucurbits, the development of the fruit in compact squash plants occurs in close proximity the root system thus the high percentage of internalization at day three may be a function of differential anatomy of the vascular system and degree of openness to bacterial movement.

Table 1A. Effect of dose and cucurbit variety on the internalization of *S. enterica* into different cucurbit vines during greenhouse trials.

	Percentage of positive detection of *S. enterica* in cucurbit vines after enrichment[A]			
	First node		**Second node**	
Inoculum dose (log CFU/mL)	**2**	**6**	**2**	**6**
Grisson Pattison Squash F1	0	94.4	0	27.8
Squash OFEQF1	11	100	0	66.7
Melon H2DF1	0	66.7	0	11.1
Melon OroRico	0	68.8	0	6.3
Melon 8HD2d2F1	0	72.2	0	11.1
Melon 8H68bF1	0	38.9	0	0
Watermelon 8402F1	0	61.1	0	11.1
Mini-watermelon EDOMF1	0	72.2	0	11.1
Cucumber ADAF1	0	33.3	0	5.6

[A] Enrichment in BPW/rif 48 h after vine inoculation (n=18 vines per treatment). Initial concentration of inoculum was 5 mL of the indicated dose of a *Salmonella enterica* cocktail that was composed of equal number of cells of the serovars Poona, Newport and Michigan

Table 1B. Effect of time of vine exposure to *Salmonella enterica* on its internalization into different cucurbit vines during greenhouse trials.

	Percentage of positive detection of *S. enterica* in cucurbit vines after enrichment[A]			
	First node		**Second node**	
Time of exposure to inoculum (days)	**2**	**7**	**2**	**7**
Grisson Pattison Squash F1	33.3	69.2	16.7	23.1
Squash OFEQF1	61.5	85.7	23	50
Melon H2DF1	60.0	0	0	6.7
Melon Oro Rico	33.3	0	6.7	0
Melon 8HD2d2F1	62.5	0	18.8	0
Melon 8H68bF1	55.6	5.6	0	0
Watermelon 8402F1	33.3	8.3	0	0
Mini-watermelon EDOMF1	26.7	21.4	6.7	0
Cucumber ADAF1	5.6	0	0	5.9

[A] Enrichment in BPW rif 48 h after vine inoculation (n=18 vines per treatment). Initial concentration of inoculum was 5 mL of log 6 CFU/mL of *Salmonella enterica* cocktail that was composed of equal number of cells of the serovars Poona, Newport and Michigan

Conclusions:

Internalization of *Salmonella* into vines and vascular system of the *Cucurbitaceae* is influenced by the plant species; although uptake of *Salmonella* may occur through the root system the event appears to be transient and limited within the vine system. Thus, although internalization of *Salmonella* through contaminated soil and root uptake can occur, the risk of internalization from sub-surface soil contamination to edible plant portions of the different cucurbits appears low.

Cited references:

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