CENTER for PRODUCE SAFETY

CPS 2014 RFP
FINAL PROJECT REPORT

Project Title
Towards preventing internalization and persistence of human bacterial pathogens in fresh produce

Project Period
January 1, 2015 – December 31, 2015

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Objectives

1. Assess the contribution of stomate-based defense to prevent colonization of leafy vegetables by S. enterica serovar Typhimurium SL1344 and E. coli O157:H7.

2. Elucidate the effect of air relative humidity on the effectiveness of stomatal defense in leafy vegetables.

Funding for this project provided by the Center for Produce Safety through:
CPS Campaign for Research

FINAL REPORT

Abstract

Human pathogens can internalize and persist in crop plants leading to foodborne outbreaks. Pathogenic bacteria might use natural openings on the plant surface, such as the stomatal pores, to penetrate the leaf interior and colonize the intercellular space. Once internalized, these pathogens often escape current sanitation procedures that are efficient to clean mostly the plant surface. Plants have evolved mechanisms to quickly perceive the presence of bacteria and close the stomatal pores, potentially diminishing leaf contamination. This phenomenon is recognized as stomatal immunity. In this study, we assessed the ability of several fresh leafy vegetables in mounting stomatal immunity against *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium SL1344, and determined the influence of two levels of air relative humidity on the effectiveness of the stomatal response. Butter lettuce, Romaine, Basil, Cilantro and Spinach showed strong stomatal closure in response to O157:H7 when compared with the water control. *Salmonella* induced a transient stomatal closure in Butter lettuce, Romaine, and Basil. However, Basil stomata exposed to *Salmonella* did not fully open to the level of the water control. Unlike the other plants tested, Cilantro and Spinach stomata remained closed in response to *Salmonella* for the duration of the experiment. Furthermore, air relative humidity did not affect the stomatal response to these bacteria but often favored *Salmonella* population survival in plant tissue, which also varied depending on the plant type. In conclusion, the ability of *Salmonella* to overcome stomatal immunity depends on the plant species, which provides opportunities to discovering the genetic basis for this variability and to proposing additional plant-specific control measures to reduce pathogen load on/in leafy greens.

Background

Consumption of fresh produce has increased over the past two decades as more people are willing to adapt to healthy eating habits. Promotion of 'healthy eating' advice by doctors, media, magazines, and blogs have increased not only the market for fresh produce but also the availability throughout the year. Fresh produce safety has been threatened by contamination with human pathogens, including bacteria, viruses, and parasites. As ready-to-eat fresh produce is minimally processed, contamination of these products by human pathogens can epitomize significant health risk. Foodborne illness and outbreaks associated with fresh produce have increased rapidly in recent years (Warriner et al., 2009; Scharff 2010) and contamination of human pathogens can occur at any step of the food production chain (World Health Organization, 2008). According to one report, 20 million illnesses costing US$38.6 billion every year were associated with produce (fresh, processed, or canned) (Sivapalasingam et al., 2004). After finding lettuce and greens-based salads to be one of the most common items associated with foodborne outbreaks, the World Health Organization categorized leafy green vegetables as the highest priority in terms of fresh produce safety (World Health Organization, 2008).

Escherichia coli O157:H7 and several *Salmonella enterica* serovars have been found to be associated with stomata (Fig. 1), naturally occurring lesions, and wounds, indicating that these are possible entry ports into plant tissues (Brandl et al., 2002; Duffy et al., 2005). We have previously found that Arabidopsis and lettuce respond actively against two human pathogenic strains, *S. enterica* serovar Typhimurium SL1344 (hereafter referred as SL1344) and *E. coli* O157:H7, to fight against endophytic colonization (Roy et al., 2013). In particular, *E. coli* O157:H7 induces a strong and lasting stomatal closure in both Arabidopsis and lettuce plants, whereas SL1344 induces a transient stomatal closure in both plants and is able to move towards open stomata (Melotto et al., 2006; Kroupitski et al., 2009; Roy et al., 2013). *Salmonella*

may be adapted to avoid and/or overcome plant's immunity and be able to colonize the leaf intercellular space (Kroupitski et al., 2009; Melotto et al., 2014).

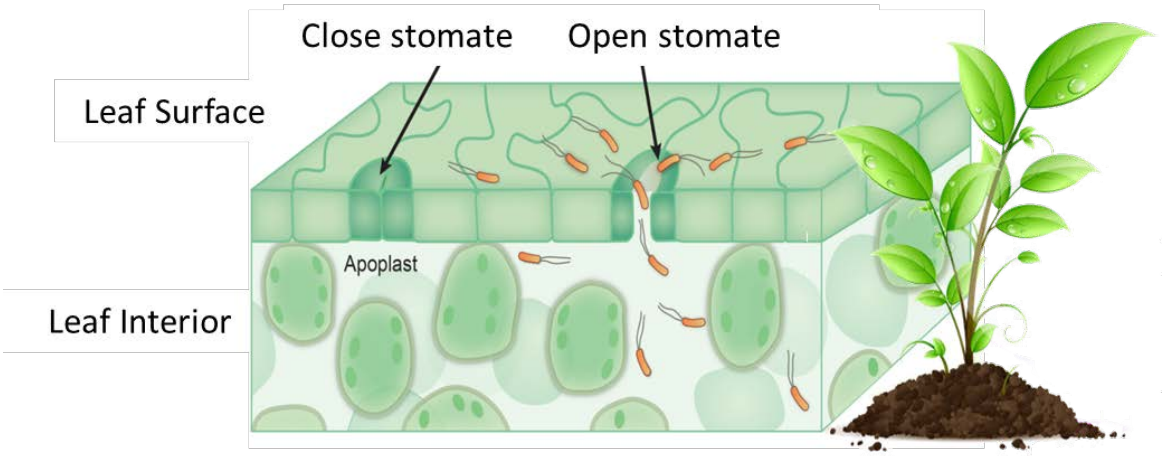

Fig. 1. Diagram of a plant leaf cross-section showing stomata in the epidermis and the apoplast space inside the leaf. The apoplast provides a niche for human pathogen survival and persistence.

Contamination of plants with human pathogens depends on several factors, such as produce type, cultivar, bacterial strain, and physiological state of both plant and pathogen, which can impact a pathogen's internalization, survival, and multiplication (Melotto et al., 2014). Plant genetic resistance to human pathogens has been understudied primarily because normally these pathogens do not cause visual symptoms in plants and, until recently, it was not known that plants may be able to fight against colonization by these bacterial strains. Therefore, our specific objectives were guided by two questions:

1) *Do all plants have the genetic capability to mount stomatal immunity against human pathogenic bacteria?*
2) *If so, how robust is this response under variable air relative humidity conditions?*

In this study, we assessed the potential variability in pathogen persistence and stomatal defense among several types of produce (Butter lettuce, Romaine, Basil, Cilantro, and Spinach) under pre-harvest and post-harvest environmental conditions.

Research Methods and Results

Experimental Procedures

Bacterial strains, culturing conditions, and inoculum preparation. Bacterial cells, *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium SL1344 (hereafter referred to as O157:H7 and SL1344, respectively), were grown in Luria-Bertani medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, pH=7.0) at 30°C for all experiments. Cells were streaked on solid medium from frozen glycerol stocks for inoculum preparation. Medium was supplemented with spectinomycin (100 µg/mL) to grow SL1344. Cells were cultured until an optical density value at 600 nm (OD_{600}) of 0.9 to 1 was reached. Bacterial cells were collected by centrifugation and re-suspended in water to the final concentration of 1×10^8 CFU/mL.

Plant material. For experiments to study plant stomatal response against SL1344 and O157:H7 under pre-harvest environmental conditions we used Romaine and Spinach. Plants were

obtained from a grower in the Salinas Valley (CA), placed in pots (Fig. 2), and transported to our laboratory. Experiments were performed on the very next day. Potted plants were kept inside an environmental chamber set at the same temperature and relative humidity (RH) recorded in the field when they were uprooted.

Spinach Romaine

Fig. 2. Uprooted plants from the field were transported to the laboratory in pots; environmental conditions were recorded in the field and the plants were maintained at the same conditions until the end of the experiment.

For experiments to study plant stomatal response against SL1344 and O157:H7 under post-harvest environmental conditions (light intensity, RH, and temperature) we bought fresh produce from a local store (Safeway, Davis, CA). Plants used were hydroponically grown naturally pest-free Butter lettuce, Romaine, Basil, Cilantro, and Spinach (Fig. 3), and stored in a refrigerator (4°C). The night before the experiment, plants were moved to an environmental chamber set at the conditions used for the stomatal assays or bacterial inoculation assay.

Butter Lettuce Romaine Basil Cilantro Spinach

Fig. 3. Store-bought produce was maintained in the refrigerator (4°C in darkness, similar to common households) until the end of the experiment.

Bacterial inoculations. For dip-inoculation of whole plants, 0.03% Silwet L-77 (Lehle Seeds Co., Round Rock, TX) was added to the inoculum. Inoculum for syringe-infiltration of plants did not contain Silwet. Mock-inoculation was included as a control. Inoculated plants were immediately incubated under the specific environmental conditions as detailed below for each assay for the duration of the experiment. Highly humid conditions were obtained by keeping well-watered plants covered with plastic domes sprayed with water and inside controlled environmental chambers. The level of humidity was monitored with a digital hygrometer (Traceable, VWR). Bacterial populations in the plant apoplast were determined as previously described (Roy et al., 2013). All experiments reported here were repeated at least three times with similar results**.**

Stomatal assay. Stomatal movement in leafy greens infected with O157:H7 and SL1344 was evaluated as previously described (Roy et al., 2013). Briefly, stomatal aperture width (>60 stomata per sample) of inoculated leaves was measured under a light microscope (Eclipse Ni-

U, Nikon Corp., Tokyo, Japan) equipped with long-distance objectives. Measurements were taken at 2 and 4 hours post inoculation (hpi) using the NIS Element software (Nikon).

Thermoimaging. Plants dip-inoculated with individual bacterial strains and a mock control were monitored for leaf surface temperature at 2 and 4 hpi. Thermo-images were taken with an infrared camera (T-420, FLIR, Sweden) and analyzed with the FLIR software associated with the camera. Stomatal aperture size is directly correlated with the leaf surface temperature (Mustilli et al., 2002). Plants with mostly open stomata have colder leaf surfaces due to the evaporative cooling effect.

Statistical analysis. All experiments were repeated at least three times (biological replicates) using a minimum of six technical replicates each time. Statistical significance of data from the stomatal assay and bacterial population counts in the apoplast was calculated using 2-tailed Student's *t*-test for comparison of the mean values.

Research Results

Testing Objective 1 and 2 under field conditions:

Romaine and Spinach plants from the field were tested under 22°C, 12 hours of light daily at 100 µmol/m/s, and two RH levels (low: 60% ± 5%; high: 93% ± 5%) to simulate field conditions.

Assay 1: At the low RH level, O157:H7 closed the stomatal pores of both Romaine and Spinach, whereas SL1344 was able to re-open the stomatal pores of Romaine but not Spinach (Fig. 4). At high RH, O157:H7 could still trigger stomatal immunity in both plants, however this closure was less pronounced in Romaine; SL1344-induced stomatal closure was also less pronounced in Romaine under high RH (Fig. 4A). While there was no effect of RH on O157:H7-induced stomatal immunity in Spinach, high RH partially affected the ability of SL1344 to close Spinach stomatal pores (Fig. 4B).

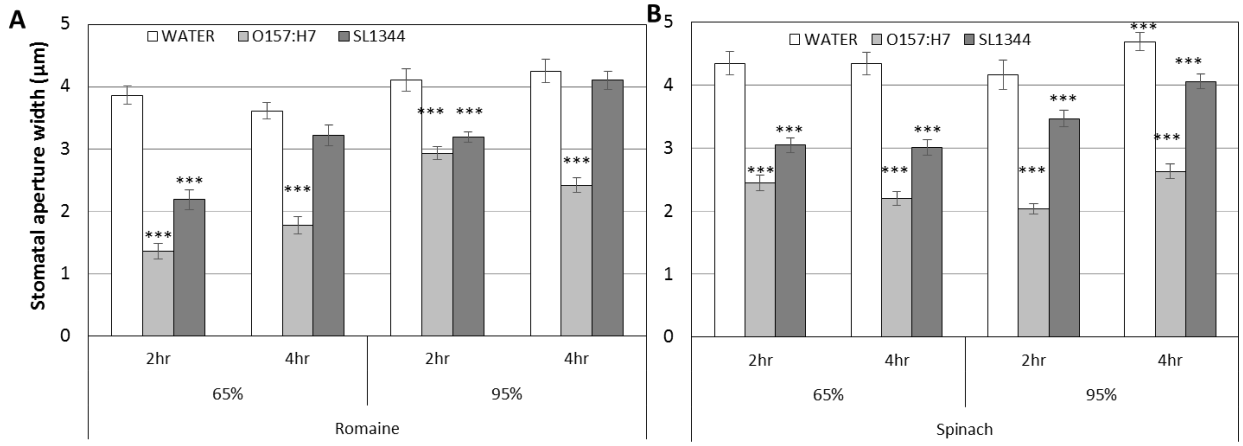

Fig. 4. Stomatal movement in field-grown plants in response to O157:H7 and SL1344 infection of Romaine (A) and Spinach (B). Stomatal assays were performed at 60% ± 5% or 93% ± 5% RH. Results are shown as mean of stomatal aperture width (n= 50 to 70) ± standard error. Statistical significance of the difference between the means (mock versus bacterium treatment) was detected with two-tailed Student's *t* -test (***P < 0.001.)

Assay 2: Thermo-imaging of Spinach plants showed that the water-treated control plant is colder (as indicated by the dark purple color) than the plants inoculated with O157:H7 or SL1344 at 2 hpi (Fig. 5, top row of images). At 4 hpi, the mock-inoculated plant remained colder, O157:H7-inoculated plant remained hotter, and SL1344-inoculated plant became colder than at 2 hpi (Fig. 5, bottom row of images). This result demonstrated a whole-plant response that correlates with the quantitative stomatal assay (Assay 1 above).

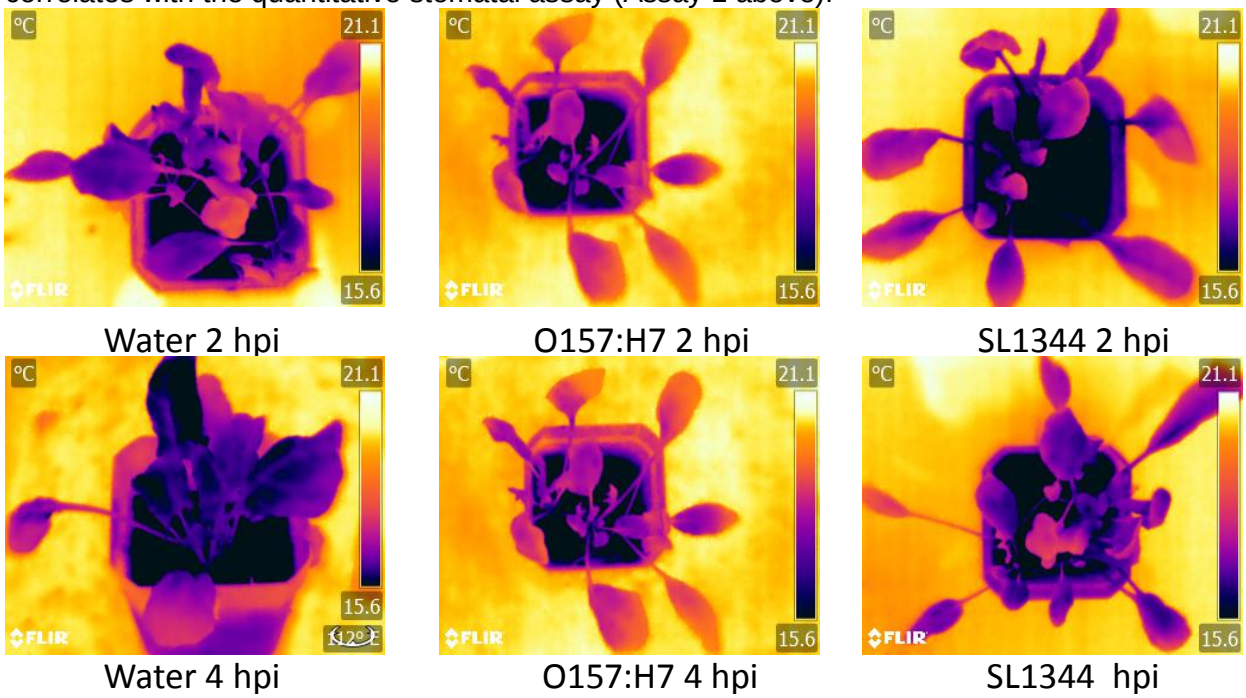

Fig. 5. Thermo-images indicating the leaf surface temperatures of Spinach plants 2 and 4 hours post inoculation (hpi) with water (mock), O157:H7, or SL1344.

Assay 3: Although there was a significant decline of both O157:H7 and SL1344 populations inside the apoplast of both Romaine and Spinach leaves, these bacteria were detected for 7 days under both RH conditions tested. High RH (93% ± 5%) only favored the SL1344 population in Romaine and Spinach (Fig. 6). The O157:H7 population was not significantly affected by RH. Therefore, RH as high as 93% ± 5% significantly favors SL1344 but not O157:H7 survival.

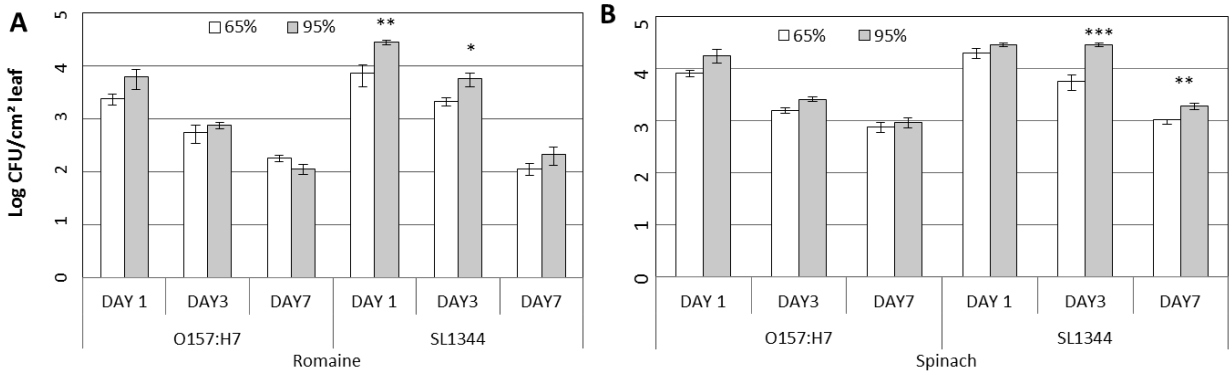

Fig. 6. Population counts of O157:H7 and SL1344 inside the leaf apoplast of field-grown plants of Romaine (A) and Spinach (B). Populations were enumerated at several days after dip-inoculation with O157:H7 or SL1344 under 60% ± 5% and 93% ± 5% RH. Results are shown as the mean (n=6) ± standard error. Statistical significance between the means (60% versus >95% RH at each time point) was detected with two-tailed Student's *t*-test (* P < 0.05, **P < 0.01 and *** P < 0.001).

Assay 4: Syringe-infiltrated Romaine and Spinach showed significantly more bacterial titers inside the leaf apoplast as compared with the dip-inoculated plants (Fig. 7). Bacterial cells may be dying on the leaf surface and/or are not efficiently penetrating leaf surfaces to find protection within. These possibilities are not mutually exclusive and need to be investigated further.

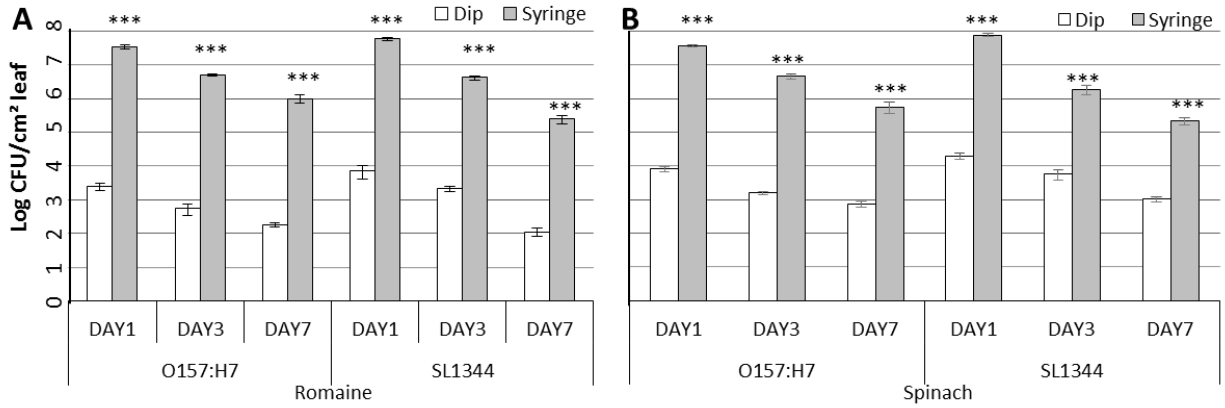

Fig. 7. Bacterial enumeration in the leaf apoplast of Romaine (A) and Spinach (B**)** at different days after dip- or syringe-infiltration with O157:H7 or SL1344. Results are shown as the mean (n=6) ± standard error. Statistical significance of the difference between the means (dip-inoculation versus syringe-infiltration at each time) was detected with two-tailed Student's *t*-test (*** P < 0.001).

Testing Objective 1 and 2 under storage conditions:

Store-bought fresh produce (Butter lettuce, Romaine, Basil, Cilantro and Spinach) was tested under 25°C, 12 hours of light daily at 100 µmol/m/s, and 60% ± 5% RH to follow the same experimental conditions as previously published (Roy et al., 2013). However, as harvested leafy vegetables must be stored at a low temperature, and usually in the dark and high RH, these leafy greens were also tested at 4°C, in the dark and at 60% ± 5% and 93% ± 5% RH.

Assay 5: O157:H7 and SL1344 closed the stomatal pores of all produce tested at 25°C, light, 60% ± 5% RH (Fig. 8). However at 4 hpi, SL1344 was able to re-open the stomatal pores of Butter lettuce, Romaine, and Basil, but not Cilantro, suggesting that stomatal response to SL1344 is plant-specific.

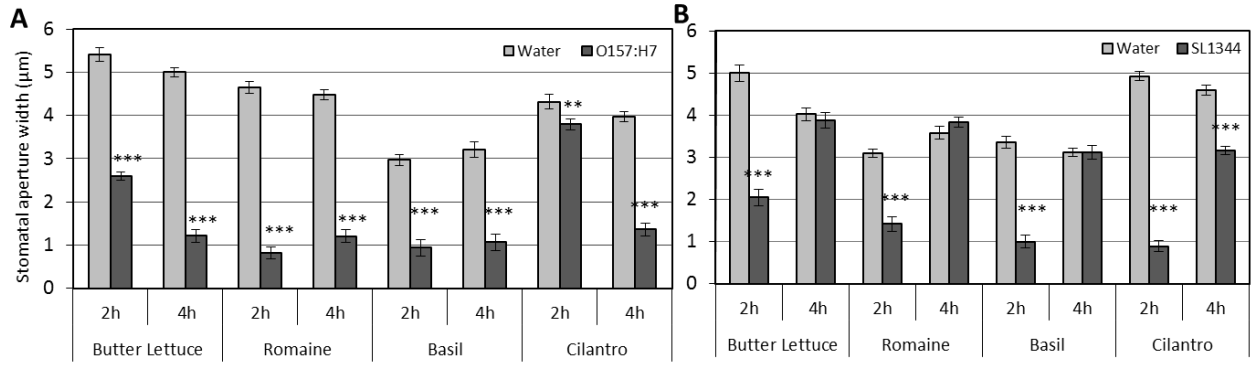

Fig. 8. Stomatal movement in store-bought plants in response to O157:H7 (A) and SL1344 (B) infection. Stomatal assay was performed under 25°C, light, and 60% ± 5% RH. Results are shown as mean of stomatal aperture width (n= 50 to 70) ± standard error. Statistical significance of the difference between means (mock versus bacterium treatment) was detected with two-tailed Student's *t* -test (***P < 0.001).

Assay 6: Bacterial internalization and persistence in store-bought Butter lettuce was tested under 25°C, 12 hours of light daily at 100 µmol/m/s, and 60% ± 5% RH. Although both O157:H7 and SL1344 populations declined significantly over time, they persisted at detectable levels for up to 21 days post dip-inoculation (Fig. 9).

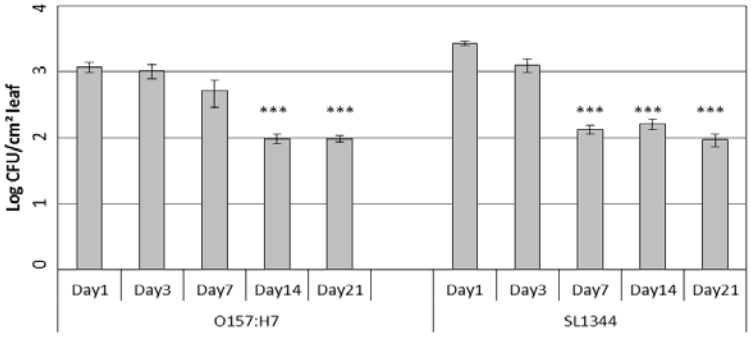

Fig. 9. O157:H7 and SL1344 populations in Butter lettuce leaves at 25°C, 12 h light daily, and 60% ± 5% RH. Results are shown as the mean (n=6) ± standard error. Statistical significance of the difference between means (Day 1 compared to other days after inoculation) was detected with two-tailed Student's *t* -test (***P < 0.001).

Assay 7: Under 4°C and darkness, O157:H7 infection triggered prolonged stomatal closure in all store-bought produce types irrespective of humidity (65% ± 3% or 93% ± 5% RH) (Fig. 10A). SL1344 could re-open stomata in Butter lettuce and Romaine, but not in Basil, Cilantro, and Spinach (Fig. 10B). Thus SL1344 is not adapted to overcome the basic stomatal immune response of all plants and RH has no apparent effect on stomatal response to these bacteria.

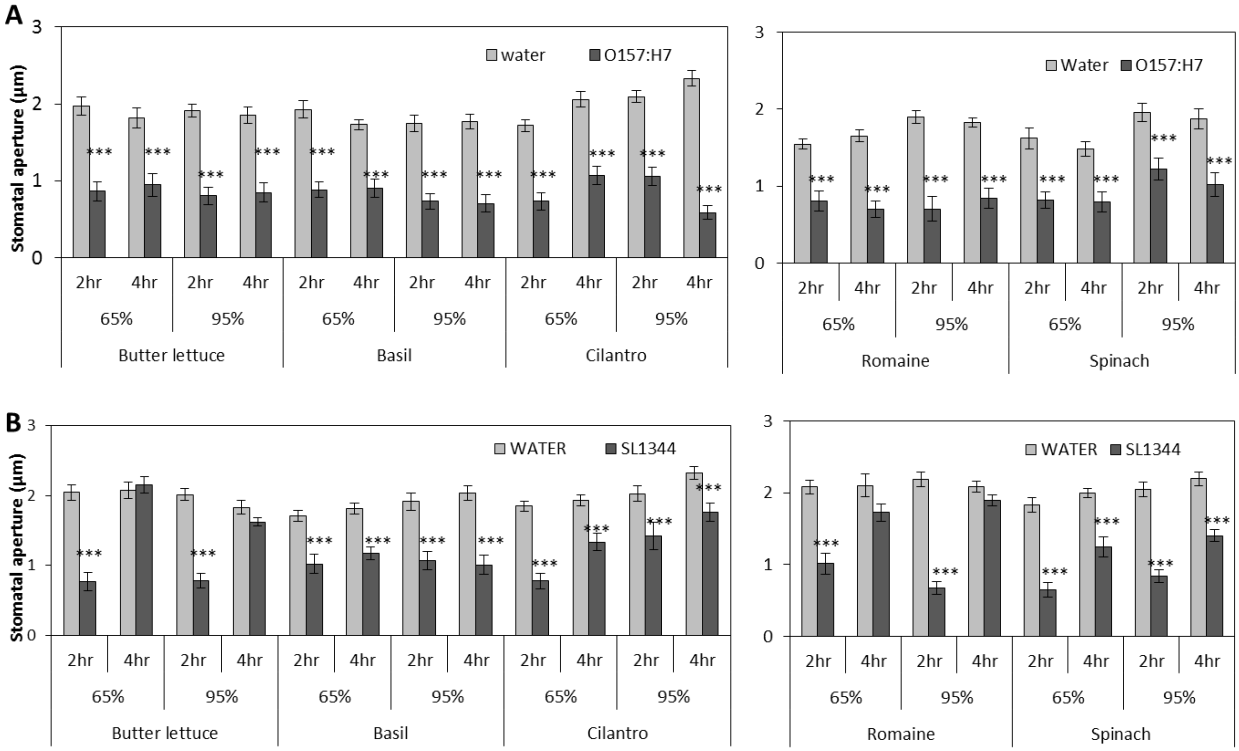

Fig. 10. Stomatal movement of store-bought plants in response to O157:H7 (A) and SL1344 (B) at 4°C, darkness, and 60% ± 5% or 93% ± 5% RH. Results are shown as mean of stomatal aperture width (n= 50 to 70) ± standard error. Statistical significance of the difference in the means (mock versus bacterium treatment) was detected with two-tailed Student's *t* -test (*** P < 0.001).

Assay 8: SL1344 and O157:H7 populations in store-bought, dip-inoculated produce (Butter lettuce, Romaine, Basil, Cilantro, and Spinach) were monitored under storage conditions (4°C, darkness, and 60% ± 5% and 93% ± 5% RH). SL1344 and O157:H7 populations declined in all produce but could be detected up to 7 days post inoculation under both RH conditions (Fig. 11). High RH favored SL1344 survival in all produce tested, whereas high RH only affected O157:H7 survival in Romaine and Spinach leaves (Fig. 11). Results suggest that the effect of humidity on O157:H7 survival was dependent on the plant genotype tested.

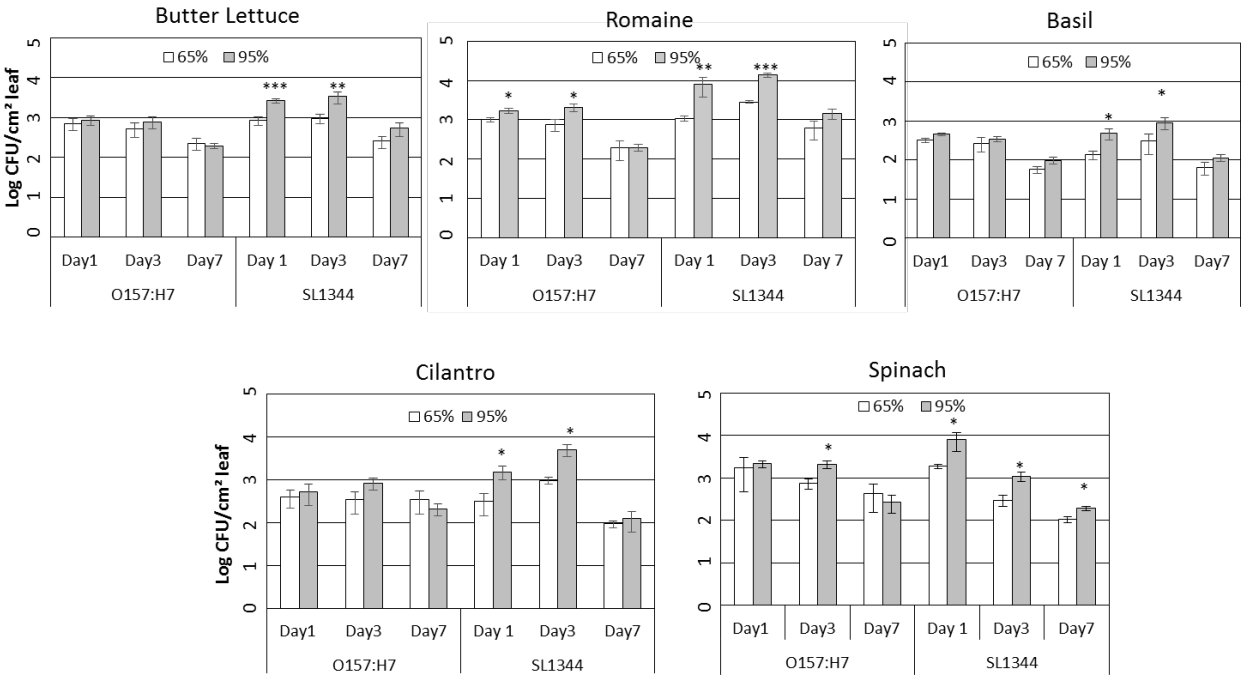

Fig. 11. O157:H7 and SL1344 population counts in store-bought leafy greens at different days after dip-inoculation. Assay was performed under storage conditions (4°C, darkness, and 60% ± 5% and 93% ± 5% RH). Results are shown as the mean (n=6) ± standard error. Statistical significance of the difference between the means (low versus high RH at each time point) was detected with two-tailed Student's *t*-test (*P < 0.05, **P < 0.01, and ***P < 0.001).

Outcomes and Accomplishments

Previous research on plant defenses against *E. coli* O157:H7 and *S. enterica* has been conducted with model plant species (Melotto et al. 2006; Kroupitski et al., 2009; Roy et al., 2013). Thus, this project was designed as a proof-of-concept to determine whether: (a) freshly consumed leafy vegetables also employ immune responses towards these human pathogens and (b) environmental conditions affect these plant responses. We have completed a series of experiments to address these possibilities and reached the following overall conclusions:

1) *E. coli* O157:H7 induces strong stomatal immunity in all plant species tested, which is not affected by the level of air relative humidity.

2) SL1344 induces transient stomatal closure in Butter lettuce and Romaine, but induces a lasting stomatal closure in Basil, Cilantro, and Spinach. However, the Basil response to SL1344 was dependent on temperature (at 25°C, SL1344 was able to re-open Basil

stomata). The level of RH does not or only partially affects this response and depends on the plant species.

3) High RH favors increases in internal SL1344 populations in all produce types (field or store-bought), whereas high RH only favors increased O157:H7 populations in Butter lettuce and Romaine at post-harvest conditions.

4) Neither O157:H7 nor SL1344 multiply inside these plants or cause any visual symptoms.

5) Both field-uprooted and store-bought Romaine and Spinach showed very similar stomatal response towards O157:H7 and SL1344 bacteria, although the size of the stomatal aperture was smaller under post-harvest conditions. The bacterial population counts in these plants showed a similar trend; however, field-grown plants supported slightly larger populations.

Summary of Findings and Recommendations

In this study we conducted a survey to characterize several plant-bacterium interactions and identify variations for future genetic-based analysis and control measures to alleviate pathogen load in fresh produce.

We used two clinical strains of highly virulent bacterial pathogens of humans: *Salmonella enterica* serovar Typhimurium SL1344 (SL 1344) and *E. coli* O157:H7. Both strains did not cause any visual symptoms on the leaves of any plant tested, making it impossible to determine their presence in the leaf without analytical tools. However, these bacteria induce stomatal immune response in all plants that we have tested thus far, indicating that physiological changes occur in contaminated leaves at the molecular level. This finding creates an opportunity to develop qPCR-based and plant-specific molecular markers as an indirect method of detection of contaminated leaves. In the long-term, this approach may be added as a tool for quality control of fresh produce.

The SL1344 strain was not able to fully open the stomatal pores of basil, cilantro, and spinach. This result indicates that SL1344 is not adapted to overcome the basic stomatal immune response of all plants. In the long-term, the genetic basis of specific SL1344-plant interactions could be explored to facilitate the development of plants that resist SL1344 contamination.

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APPENDICES

Publications and Presentations

Poster Presentations

Melotto M., Roy D., 2015. Towards preventing internalization and persistence of human bacterial pathogens in fresh produce. Produce Research Symposium, June 23-24. Atlanta, GA, USA.

Roy D., Melotto M. 2015. Internalization and persistence of human bacterial pathogens in fresh produce. III International Conference on Fresh-cut Produce – ISHS, Davis, CA, USA.

Budget Summary

The approved budget was sufficient to hire a part-time technician (50%) to carry out all the required experiments. Some adjustments were made among the transaction categories to cover payroll benefits and travel expenses. The balance carried over will be used to cover travel expenses for the PI to attend the 2016 CPS Research Symposium. The budget breakdown and expenditures are shown in the table below:

Transaction Category	Credit	Expend	Balance
General Assistance	$25,000	$26,206.70	-$1,206.70
Leave Assessment	$0	$1,412.40	-$1,412.40
Supplies and expenses	$11,575	$6,873.89	$4,624.46
Travel	$950	$1,392.59	-$442.59
Employee benefits	$2500	$2850.93	-$350.93
Total	$40,025	$36,171.51	$1,211.84