RESEARCH COMPLETED

Towards Preventing Internalization and Persistence of Human Bacterial Pathogens in Fresh Produce

SUMMARY

Human pathogens can internalize and persist inside crop plants leading to foodborne outbreaks. Pathogenic bacteria might use natural openings on the plant surface, such as the stomatal pore, 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. Thus, this project was designed as a proof-of-concept to determine whether: (a) freshly consumed leafy vegetables also employ immune responses towards *Salmonella enterica* serovar Typhimurium SL1344 and *Escherichia coli* O157:H7, and (b) environmental conditions affect these plant responses. We observed the existence of genetic variability among the plant-bacterium interactions studied, which provides an opportunity to breed for plant genotypes that can effectively resist contamination by bacterial pathogens of humans.

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.

METHODS

Fresh produce (leafy greens) was purchased from a local store or uprooted from the field. The night before the experiment, plants were moved to an environmental chamber set at the conditions used for the experiments. Bacterial strains were cultured at 30°C in liquid Luria-Bertani medium supplemented with appropriate antibiotics. Plants were inoculated with $1x10^8$ CFU.mL^{-1} or a mock control. Apoplastic bacterial population counts inside the various produce and stomatal assays were performed as previously described (Katagiri et al., 2002; Chitrakar and Melotto, 2010). 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 assays and bacterial population counts in the apoplast was calculated using two-tailed Student's *t*-test for comparison of the mean values.

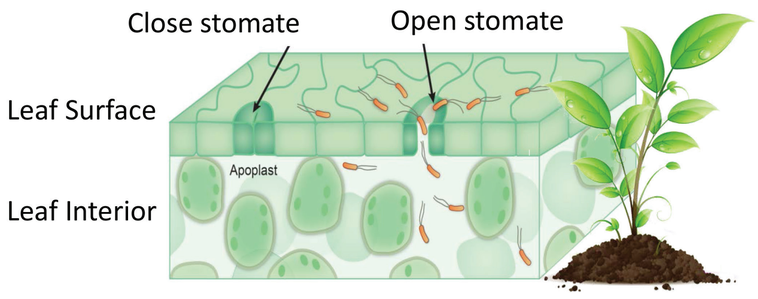

Figure 1. A diagram of a plant leaf cross-section showing stomata in the epidermis and the apoplast space inside the leaf. The apoplast provides a niche for human pathogen survival and persistence.

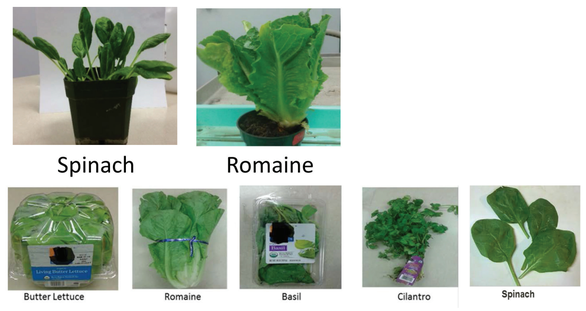

Figure 2. Top Row: Uprooted plants from the field were transported to the laboratory in pots. Environmental conditions were recorded in the field and the plants were maintained in the same conditions until the end of the experiment. **Bottom Row:** Store-bought produce were maintained in the refrigerator (4°C in the dark, similar to common households) until the end of the experiment.

RESULTS TO DATE

We have completed this proof-of-concept project and reached the following overall conclusions:

1. O157:H7 induces strong stomatal immunity in all plant species tested, which is not affected by the level of air relative humidity (RH).
2. SL1344 induces transient stomatal closure in butter lettuce and romaine, but induces a lasting stomatal closure in basil, cilantro, and spinach. The level of RH does not or partially affects this response depending on the plant species.
3. High RH increases the internal population of SL1344 in all produce types from field or local store, whereas high RH only favors increased O157:H7 population in Butter Lettuce and Romaine at post-harvest conditions.
4. Both field-uprooted and store-bought romaine and spinach showed very similar stomatal response towards these two bacteria, although the size of the stomatal aperture was smaller under post-harvest conditions. In addition, the bacterial population counts inside these plants showed similar trends; however, field-grown plants supported slightly larger populations.

BENEFITS TO THE INDUSTRY

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. Both of these strains do 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 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 is 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.

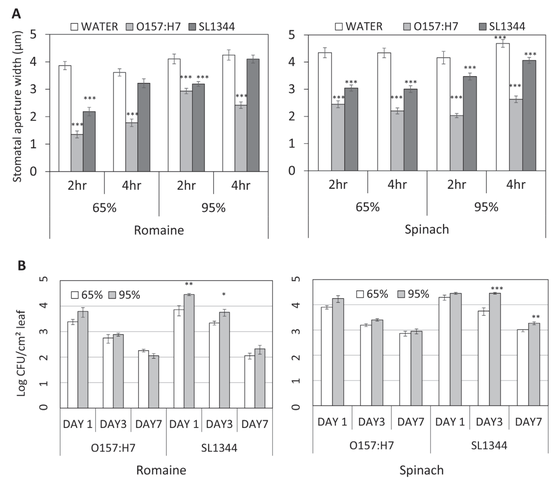

Figure 3. (A) Stomatal movement in **field-grown plants** in response to O157:H7 and SL1344 infection. Stomatal assay was performed under 60±5% or 93±5% RH. Results are shown as mean of stomatal aperature width (n= 50 to 70) ± standard error. Statistical significance of the difference between the means (mock versus bacterium treatment) was detected with two-tailed Student's *t*-test (***p < 0.001.). **(B)** Population counts of O157:H7 and SL1344 inside the leaf apoplast of field-grown plants. Bacterial population was enumerated at several days after dip-inoculation with O157:H7 or SL1344 under various RH (60±5% or 93±5%). Results are shown as the mean (n=6) ± standard error. Statistical significance between the means (65% 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).

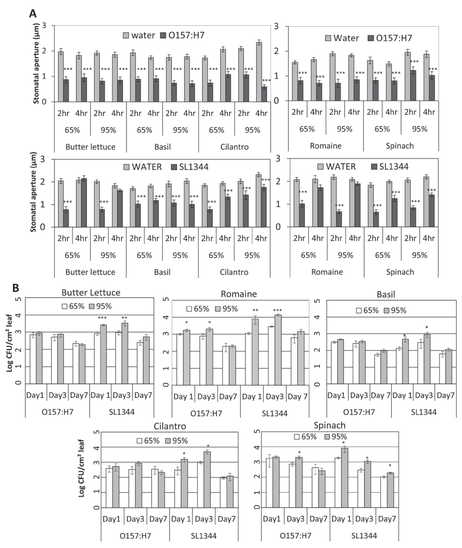

Figure 4. (A) Stomatal movement in **store-bought plants** in response to O157:H7 **(top)** and SL1344 **(bottom)**. Stomatal response was tested at 4°C, darkness and 60±5% or 93±5% RH. Results are shown as mean of stomatal aperture width (n= 50 to 70) ± standard error. Statistical significance of the difference between the means (mock versus bacterium treatment) was detected with two-tailed Student's *t*-test (***p < 0.001.). **(B)** O157:H7 and SL1344 population counts inside of leaf apoplast of **store-bought leafy greens** at different days after dip-inoculation. Assay was performed under various storage conditions (4°C in darkness; 60±5% and 93±5% RH). Results are shown as the mean (n=6) ± standard error. Statistical significance of the difference between the means (65% 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).

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