SUPPRESSION OF *LACTUCA SATIVA* INNATE IMMUNE RESPONSE BY *SALMONELLA ENTERICA*

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Plant and Soil Sciences

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Finally, if my family and Ernesto Vladimir Johnson had not pushed me to pursue a higher academic degree I wouldn't be here anyway, Thank You.

This Thesis is dedicated to Dorothy Johnson.

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ABSTRACT

Plants interact with millions of different organisms in each growing season. The majority of these have no adverse impact on the plant, but certain organisms can be beneficial or detrimental to plant health. When a plant is consumed these bacteria can be taken along with the harvested plant organs and may be ingested or transferred to other un-infested plants. Human enteric pathogens can be also recovered from these contaminated foods, in this thesis we have researched one such bacteria, *Salmonella enterica* serovar Typhimurium. This common food borne pathogen has been documented as producing disease like symptoms in model and agricultural plants. To evaluate *S.* Typhimurium on lettuce plants several procedures were used, normal stomatal features were imaged to evaluate *S.* Typhimurium's adverse effects on plant innate immunities, and to find the degree that *S.* Typhimurium can ingress in lettuce possibly prolonging its persistence. We then hypothesized that *S.* Typhimurium was actively suppressing the innate immunity with SPI1 and or SPI2 when mutants of either failed to suppress plant immunity. Gene expression showed that the ABA pathways were disrupted by unknown proteins in *S.* Typhimurium. These adverse effects were negated by addition of a root associated plant growth promoting rhizobacteriu, which through plant contact can mediate and strengthen plant immune responses to disease and non-pathogenic organisms. Our finding impact issues of contamination in raw leafy greens and novel uses of safe biocontrol techniques that do not require chemicals.

Chapter 1

LITERATURE REVIEW

Introduction:

Reducing the risk of foodborne illness associated with fresh produce is a task which the industry and academic researchers have been struggling with for many years, with greater focus since the 2006 outbreak of gastroenteritis associated with fresh spinach caused by *Escherichia coli* O157:H7. A similar outbreak occurred last year (2018) with *E. coli* O157:H7 contamination of romaine lettuce grown in the Yuma region of Arizona. These outbreaks continue to put pressure on our need to better understand how bacterial pathogens interact with plants and evade our control mechanisms. The plant microbiome is a rich environment with many unknown and known organisms, of these microbes some may be harmful to the plant, or harmful to other life which in this case will be referring to food borne pathogens. Plants may encounter foodborne bacterial pathogens under both pre- and post-harvest conditions when conditions and viable bacteria are present. A number of foodborne pathogens use plants as temporary hosts, and the defense and innate immune system in plants exposed to these pathogens is not very well defined. This review will address the importance of plant immunity in regard to food borne pathogens, as well as why some bacteria have adapted to better colonize the epiphytic and endophytic spaces. Finally, we will address plant growth promoting rhizobacteria (PGPR) as a viable and safe food safety control.

The plant immune system:

The phyllosphere is depended on for air exchange, sugar production, hormone production, and plant defense (Vacher, et al. 2016). A key organ for proper plant physiological function is the stomata, which are microscopic pores arranged on the

abaxial and adaxial leaf surface (Melotto, et al. 2017; Vacher, et al. 2016). Stomata maintain proper evapotranspiration and gas exchange when peripheral cells called guard cells are turgid and the stomata aperture is open. Stomatal function is controlled by relative ion concentrations in the guard cells, which in turn results in an influx of water via osmosis resulting in turgid cells and a wider stoma (Israelsson, et al. 2006; Hosy, et al. 2003). Regulated closure of the stoma occurs in the dark, during drought stress, and biotic stress. Stomatal closure can be induced using a variety of substances such as salicylic acid (SA) and abscisic acid (ABA) (Acharya and Assmann. 2009; Merilo, et al. 2015). Both SA and ABA are crucial to defense and growth through which the homeostasis of a plant is maintained (Melotto, et al. 2017). The initial plant responses to pathogens as well as non-pathogenic organisms rely on proper SA and ABA function to quickly shut stomata and prevent ingression from the leaf surface into mesophilic spaces.

In response to non-pathogenic threats, basal plant immunities produce an array of physical responses and chemical secretions. Initial response to foreign organisms is induction of PAMP triggered immunity (PTI) via transmembrane receptors that recognize conserved microbial components called microbe associated molecular patterns or MAMPS (Jones and Dangl. 2006). Induction of PTI induces reactive oxygen species (ROS) production in the leaves and results in a changed ion concentration around guard cells, thus causing an efflux of water finally resulting in closure of stomatal pores. PTI is called basal immunity because it is response to highly conserved parts of bacteria, fungi, nematodes, and the plant itself.

A more robust and pathogen dependent immune response called effector triggered immunity (ETI) requires a resistant plant and a plant pathogen. ETI is solely dependent on only a few genes at most called R-genes, short for resistance (Jones and Dangl, 2006). R-genes usually have a nucleotide binding site and a kinase domain, which can effectively activate when the correct effector from a pathogen is present at the binding site, thus triggering a kinase cascade which activates PATHOGENISIS RELATED-1(PR-1) and downstream products(Jones and Dangl, 2006). ETI results in

longer stomatal closure than PTI, as well as a stronger ROS presence for signaling and antimicrobial action. Not present during PTI is a systematic cell death called patterned cell death or a hypersensitive response (HR). ETI is a strong defense against pathogens, but it is eventually overcome by pathogen evolution and new effectors (Jones and Dangl. 2006). In general, all non-pathogenic organisms induce average levels of PTI and lack the machinery and proteins to induce PR-1 driven responses.

PTI induction causes swift stomatal closure that does not persist and is one of many initial reactions to prevent ingression of an organism into the plant. Functional stomata are vital in the response to both plant pathogens and benign organisms. Ample evidence has been generated that proves some phytopathogens have evolved mechanism to subvert the immune system and change stomatal phenotypes to allow ingression. Using a model system, *Arabidopsis thaliana* and *Pseudomonas syringae* DC3000, it was proven that *Ps. syringae* DC3000 will override plant innate defenses through production of a compound named coronatine which causes stomata to reopen thus allowing ingression through this route (Melotto, et al. 2006). Coronatine, a JA mimic prevents stomatal closure by allowing JA to accumulate thus counteracting and suppressing SA dependent functions, such as those present during PTI. A second pathogen, *Ps. syringae* pv. *syringae,* contains a virulence factor for stopping closure or causing stomata to reopen through an entirely different process. Syringolin A, a proteasome inhibitor reverses stomatal closure in both *A. thaliana* and *Phaseolus vulgaris* (Schellenberg, et al. 2010). In both Melotto et al. (2006) and Schellenber et al. (2010) phytopathogens lacking a functional virulence gene resulted in less disease or enhanced stomatal closure post inoculation. The adaptations in *Ps. syringae* strains are only two examples of reversed stomatal closure (Melotto, et al. 2017). Induction of PTI is not limited to plant pathogenic bacteria, aLmost all bacteria can induce some amount of stomatal closure and PTI, the importance of this will be discussed below with regards to *Salmonella enterica* a common causal agent of food poisoning and a bacterium isolated from outbreaks caused by consumption of contaminated produce such as spinach and lettuce.

Food borne pathogens in the plant system:

Contaminated foods such as produce, dairy, and meat products will cause upwards of half a billion documented cases of food borne illness every year. Of the various organisms and viral agents considered food borne pathogens the gram-negative rod-shaped bacteria *Salmonella enterica* is a frequent etiological agent recovered from contaminated food and is the most common bacteria isolated from contaminated produce (Bean, et al. 2015). *S. enterica* is an obligate host to many mammals but infections tend to be minor and self-limiting and symptoms attributed to *S. enterica* infection most often involve gastroenteritis, diarrhea, and nausea, akin to what is normally called food poisoning. *Salmonella* infections may result in systemic infections but rarely leading to death. The large number of cases each year result in a relatively large mortality number even if the actual percentage of fatal salmonellosis is still near or less than 1% (F1000 Research Ltd. 2015). Typhus and typhoid fever will not be discussed in this review.

Despite being an obligate human and animal pathogen, in the last few decades both reviews in food safety and research articles have been continually stating that *S. enterica* is not as simple as once thought and portrays facultatively phytopathogenic behavior in several plant genera. *S. enterica* can enter plants through several routes both in the rhizosphere and from the phyllosphere, the species of plant will thereby relate to the potential risk that a contaminated portion will be consumed following harvest.

Soil contamination and root colonization of *S. enterica:*

Contamination of produce is often linked to direct and indirect sources (Monaghan and. Hutchison. 2012). Sources of inoculum can be a result of contaminated irrigation water, soil, and pesticide or fertilizer diluent which can be the result of uncontrollable situations such as animal waste from wildlife, including insects, or birds. *S. enterica* is often found in domestic animal waste which is still used as an organic nitrogen and carbon source. Microbes including food borne pathogens

can enter the plant corpus through or attach to hydathodes, flower parts, roots, stomata, trichomes, and wound sites be they natural or man-made via transplanting and field work (Kroupitski, et al. 2009; Barak, et al. 2002; Gu, et al. 2013; Zheng, et al. 2013).

In the rhizosphere *S. enterica* serovar Enteritidis is shown to require human virulence genes for attachment to alfalfa sprouts, from a pool of 6000 mutants 20 were shown to have reduced attachment, of these genes *agfB* a surface-exposed aggregative fimbria (curli) nucleator mutant was most reduced in attachment to alfalfa sprouts (Barak, et al. 2005). The same study showed that *agfA* did not have reduced attachment, therefore concluding *agfB* was essentially required for attachment to the alfalfa sprouts as it had to most decreased attachment when removed (Barak, et al. 2005). Earlier work examined the adhering capability of multiple *S. enterica* serovars that were shown to adhere better than *Escherichia coli* O15:H7 to alfalfa sprouts (Barak, et al. 2002). Attached bacteria may survive post-harvest sanitation treatment, thus leading to out-breaks such as one in 1995, where contaminated sprouts caused 242 *S.* Stanley infections in 17 States (USA) and Finland (Mahon, et al. 1997).

Recaptured water such as treated urban wastewater can result in contaminated crops, and can further contaminate rivers, and irrigation canals which remain important water sources in many agricultural areas (Steele and Odumeru. 2004; Forslund, et al. 2010). Soil contamination remains an important source of study, as are direct means of contamination through overhead irrigation, plant handling, and foliar or fruit contact with bacteria; these methods are simulated in more controlled laboratory experiments and have led to many advances in our understanding of the plant micro-biome and the facultative pathogenicity of several *S. enterica* serovars.

S. enterica phyllosphere interaction and suppression of plant defense:

Many studies use *A.s thaliana* as a model organism for viewing responses and interaction between plant and pest. The *A. thaliana* and *S. enterica* model was used to observe disease like symptoms resulting from infection by *S. enterica* (Schikora, et al. 2008). The same methods were used to show facultative pathogenicity of

Pseudomonas aeruginosa (Walker, et al. 2004; Plotnikova, et al. 2000). Despite the conclusions from Schikora et al. (2008) replication of the experimental conditions were not observed in a following paper where *A. thaliana* mounted a PAMP triggered immune response (Garcia, et al. 2014a). While Garcia et al. (2014) showed a lack of disease after inoculation of *S. enterica* serovar Typhimurium 14028s, a T3SS mutant -*prgh* a factor in the needle complex of the SPI1 T3SS did produce a greater immune response than the wild type which indicated reliance on a T3SS for translocation of unknown effector like proteins. In addition to -*prgh* other isogenic mutants of the T3SS maintained lower populations than *S. enterica* 14028s, the mutants -*invA* part of the export apparatus (ecoded by SPI1) and *sseJ* (encoded by SPI2) lead to the speculation that *S. enterica* 14028s needs two functional T3SS for normal plant colonization (Schikora et al., 2011).

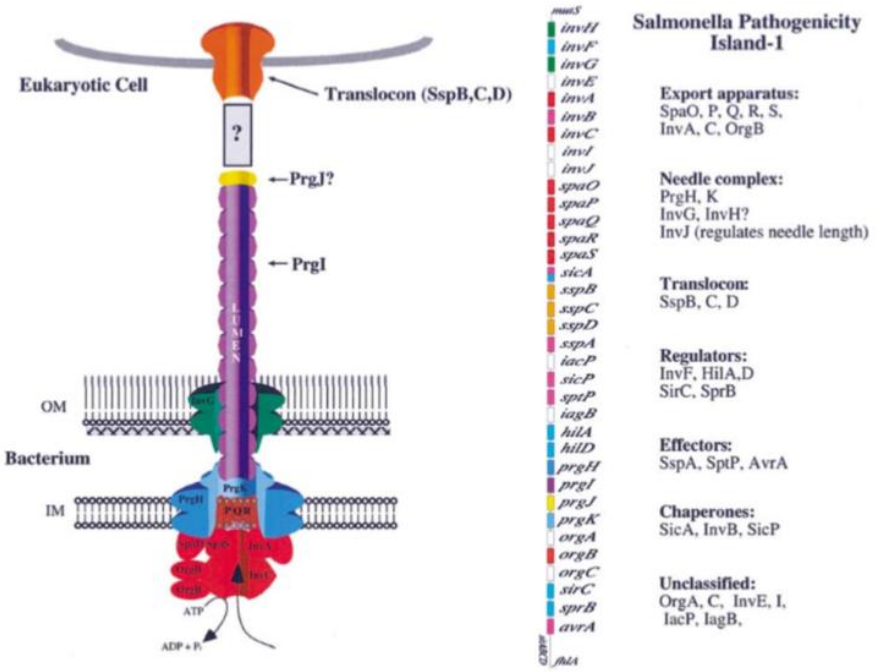

Figure 1.1 The Type Three Secretion System of *S. enterica* encodes the necessary effectors for pathogenesis in human hosts, effectors are translocated through the needle complex across two membrane layers into the eukaryotic cell. T3SS lacking factors from this needle complex or the base structure will have reduced pathogenicity, which has been shown in both plants as well as the mammalian host. Figure used from (Kimbrough and Miller, 2002)

Intercellular *S.* Typhimurium 14028s was observed on the roots system but it was not shown to be able to mobilize in the xylem, these epidermal cells may have been wounded and thus were colonized opportunistically by *S.* Typhimurium (Schikora, et al. 2008). In the phyllosphere *S. enterica* use chemical clues from active photosynthesis to locate and move toward open stomata in iceberg lettuce (*Lactuca sativa*) (Kroupitski, et al. 2009). Evidence of this has not yet been shown in other plant genera; chemical signals do attract both beneficial and antagonist (pathogenic) organisms to the plant root system.

Further modeling of *S. enterica* the opportunistic pathogen was conducted in a model solanaceous plant *Nicotiana benthamiana* where *S. enterica* serovar Typhimurium induced PTI and prevented subsequent cell death induced by *Ps. syringae* pv. Tomato DC3000 in a cell death suppression assay (Meng, et al. 2013). *S. enterica* flagellin mutants, *fliC,fliB* a double mutant was unable to induce PTI, but *fliC* did successfully induce PTI, thus confirming the partial role of flagellin being required for proper induction of PTI through FLS genes (Meng, et al. 2013). Plants with silenced FLS2 and BAK1 presented less PTI induction and suppression of cell death produced by DC3000 (Meng, et al. 2013). Unlike *S. enterica*'s facultative plant pathogenicity in *A. thaliana,* no signs of pathogenicity were observed in *N. benthamiana* and at the very least a partially functional flagella were required for PTI induction.

In another solanaceous plant tomato *S. enterica* strains will attach at a higher rate numbers to *Solanum lycopersicum* rather than *S. pimpinellifolium* where they avoided the stomata but did attach to type one trichomes (Barak, et al. 2011). Inoculation of a 5-strain cocktail to the rhizosphere, leaves, and blossom of tomato showed that *S. enterica* serovars were deemed to survive better in certain location (Zheng, et al. 2013). *S.* Typhimurium was not recovered from any sampled tissue, *S.* Montevideo was isolated from the blossoms for at least 6 days after inoculation, and *S.* Newport and *S.* Montevideo persisted on the leaves for 23 days (Zheng, et al. 2013). In trials conducted with contaminated soils, lettuce and parsley held viable bacteria for

up to 231 days (Islam, et al. 2004). In field trials it was also shown that more than a single crop set can be contaminated through surviving inoculum in plant debris, and plant disease can influence potential contamination amounts as observed in two independent research articles (Barak and Liang. 2008; Meng, et al. 2013).

***S. enterica* interactions with leafy greens:**

Crops in which green material is consumed such as sprouts, greens, and herbs pose a higher risk for contamination and distribution when sanitary procedures do not remove all pathogens. Leafy greens should be considered a large source of contaminated food, due to ready to eat foods, proximity to the soil, and risk of post-harvest contamination. A common species grown with a plethora of cultivars and attractive qualities is *Lactuca sativa* (lettuce) which has been implicated several times as a secondary host for *S. enterica, E. coli,* and *L. innocua* a surrogate for *L. monocytogenes*.

Comparatively little work has been completed as to the physiological effects of lettuce when it interacts with *S. enterica.* In leaves contaminated with pathogens stomatal apertures are often used as a measure of plant immunity, this being true for *A. thaliana* as well leafy greens like spinach and lettuce (Roy and Melotto. 2019; Melotto, et al. 2006). Environmental and experimental conditions may change overall stomatal size and closure rate, such as humidity, light intensity, and cultivar but normal responses to PTI remain observable to a point. In leafy green lettuce, *S. enterica* Strain SL44 caused less closure in lettuce at both 65% and 95% relative humidity, while *E. coli* O157:H7 caused more closure at identical humidifies and time points (Roy and Melotto. 2019). In spinach, *S. enterica* SL44 and *S.* Newport was observed to cause similar closure as *E. coli* O157:H7 or *L. innocua* (Roy and Melotto. 2019; Markland, et al. 2015). On lettuce plants it was observed that *S. enterica* may locate stomata though chemicals produced by the plant and therefore move to the stomata through chemotaxis, furthermore plants that are actively photosynthesizing (therefore open) promoted both attachment and sub-stomal colonization (Kroupitski, et al. 2009).

More work must be completed in the subject to determine methods that *S. enterica* either influences the plant immune system or escapes notice. It is well known that portions of the bacteria other than flagellin can induce PTI, for example lipopolysaccharides and culture filtrates containing unknown bacterial constituents.

How *S. enterica* translocates effectors or proteins into the plant cell is yet to be determined. A T3SS is the logical source, plant pathogens use the same organelles for translocation of effectors that can suppress and override plant immunities. This subject will require further research into how the *S. enterica* T3SS penetrates the enlarged cell barriers that make up the cell wall in plants.

To prevent incursion and defense suppression either a chemical or biological approach is required, due to the erratic behavior of outbreaks a cost-effective pre-harvest approach may yield a better option than chemical treatments and increased post-harvest costs. In this case the use of beneficial soil bacteria called plant growth promoting rhizobacteria (PGPR) will be analyzed as a potential method for plant protection against opportunistic food borne pathogens like *S. enterica*

Beneficial bacteria in relationship to food borne pathogens:

Despite the lack of a union between plant and food science/ food safety ample work has contributed to the latter in methods of inactivation and post-harvest sanitary steps. Some of these treatments can be very effective, but can be impractical or expensive such as x-ray and radiation treatments of respectively low-priced foods. Among common post-harvest wash steps is a sodium hypochlorite and water mix, even though bleach is a normal disinfectant in homes, hospitals, and restaurants it can leave some cells attached and viable when used at safe concentrations. Bacteria that have internalized to the mesophilic regions of the leaf are even more likely to survive. Other treatments such as acidic chlorinated water, acidic electrolyzed water, x-rays, as well oils with antimicrobial behavior such as that from oregano are shown to reduce bacterial loads with mixed results (Stopforth, et al. 2008; Mahmoud, et al. 2010; Moore-Neibel, et al. 2013). To ensure food safety many steps must be climbed to ensure both preharvest and post-harvest cleanliness, which is why many researchers

are now looking at novel uses for bio-controls or bio-inoculum as unconventional control schemes.

Beneficial microbes called plant growth promoting rhizobacteria (PGPR) have been employed for improving crop health for well over two decades. Usually consisting of two or more bacteria in a "cocktail" of PGPR bacteria can improve plant biomass, drought tolerance, root nodulation, and disease resistance (Bais, et al. 2014; Zheng, et al. 2018; Rosier, et al. 2018; Ramamoorthy, et al. 2001). Recent papers have also addressed its uses for reducing contamination from food borne pathogens including *S. enterica* and *L. innocua* (Markland, et al. 2015; Hsu and Micallef, 2017a). More compelling is the use of PGPR's against plant pathogens, which plant disease is shown to increase contamination rates of *S. enterica* by weakening the host plants immune system (Potnis, et al. 2014).

PGPRs have been experimentally proven to have varied effects on the plant immune system. Colonization is shown to briefly suppress defense related genes in *A. thaliana* during initial attachment (Bais, et al. 2004). Once attached *B. subtilis* must form a biofilm to convey proper protection to the plant, and once established antimicrobial compounds such as surfactin are secreted and actively are harmful against bacteria pathogens such as *Ps. syringae* pv Tomato DC3000 (Bais, et al. 2004). Pathogen presence on the roots or shoots ad promotes root biofilm formation (Rudrappa, et al. 2008). Following biofilm formation and presence of a pathogen a systemic disease resistance is triggered known as Induced Systemic Resistance (ISR), which although similar to Systemic Acquired Resistance (SAR), is dependent on the presence of the PGPR biofilm. ISR induces broad spectrum resistance to plant pathogens and has been studied in conjunction with *Pst.* DC3000, *Xanthomonas oryzae*, *Phytopthora capsica*, and others (Bais, et al. 2004; Udayashankar, et al. 2011; Zhang, et al. 2010). PGPRs are an important part of future agricultural programs, due to increasing pathogen resistance against increasingly harmful pesticides as well as climate shifts spreading pathogens to new regions.

In recent years food-borne pathogens have also been tested against PGPR. The PGPR *B. subtilis* UD1022 (formerly FB17) has been proven to reduce *S. enterica* Newport populations in both lettuce and spinach as well as many plant pathogens. In addition to *B. subtilis, Pseudomonas* spp. shows possible use in protection against food borne pathogens in tomato and spinach (Hsu and Micallef. 2017b).

Food safety is of utmost importance as the human population burgeons in all parts of the world. By using PGPRs as potential devices for ensuring safer, cleaner, more productive plants the agricultural communities will have another method for avoiding recalls and the costly as well as sometimes deadly outbreaks.

Thesis direction:

S. enterica is shown to be behave like a facultative pathogen for lettuce. In the following research *S. enterica* is shown to suppress the plant immune system of *Lactuca sativa* cultivar Black Seeded Simpson. We used a microscopy, genetic, and classical colony counts to determine how *S. enterica* effects the plant immune system. Confocal microscopy of stomata was used to find what effects *S. enterica* and isogenic mutants donated by Dr. Altier of Cornell university had on innate immunities. Microscopy took place at the Delaware Biotechnical institutes Bioimaging Center, but all imaging was completed by Nicholas Johnson except cryo-SEM which required supervision by an employee at the Bioimaging Center. Several genetic markers for immune response and stomatal function is examined for the changes in the basal immune responses using gene expressions. Overall population sustainability of *Salmonella* on lettuce is calculated using standard plate counts as well as a modified most-probable numbers (MPN) method for internalized cells these experiments were completed by Dr. Kaur-Litt of Kniel Lab in the Animal and Food Science Depart of the College of Agriculture and Natural Resources both associated with the University of Delaware experiments were designed by both Nicholas Johnson and Dr. Kaur-Litt. By comparing lettuce responses to wildtype Salmonella and several pathogenicity mutants we will explain why and how *S. enterica* influences plant physiology and defense, thus allowing it to persist for long periods of time.

Further evidence that plant growth promoting rhizobacteria can be used for food borne pathogen control will be implemented through use of *Bacillus subtilis* strain UD1022 as a preharvest inoculant in both a contained hydroponic and soilless media growth setting.

Chapter 2

EVASION OF PLANT INNATE DEFENSE RESPONSE BY *SALMONELLA* ON LETTUCE[1]

Abstract

When bacteria interact with higher organisms, such as plants and humans, some cause disease but others establish a symbiosis that benefits the host. To establish host association, the innate immune system, which is one of the first lines of defense against infectious disease, must be circumvented. Plants encounter enteric foodborne bacterial pathogens under both pre- and post-harvest conditions. Human enteric foodborne pathogens use plants as temporary hosts. This unique interaction leads to outbreaks and recalls in raw agricultural commodities. There are reports which suggest that opportunistic human pathogens may bypass plant innate defense for ingression. The purpose of this study was to determine if *Salmonella enterica* Typhimurium applied to lettuce leaves is able to suppress innate stomatal defense in lettuce. We also conceptualized if stomatal ingression by *S.* Typhimurium prolongs its persistence in planta. *S.* Typhimurium applied to the leaves of lettuce prolonged stomatal opening, suggesting *S.* Typhimurium can suppress innate stomatal defense. Interestingly, *S.* Typhimurium SPI1 and SPI2 mutants showed inability to reopen stomates in lettuce suggesting the involvement of key T3SS components in suppression of innate response in plants. Gene expression analysis suggests that *S.* Typhimurium may perturb the ABA biosynthesis in lettuce to override the stomatal defense response. These findings impact issues of contamination related to plant performance and innate defense responses for plants grown hydroponically and in soil.

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[1] Portions of this chapter have been submitted for publication in Frontiers in Microbiology.

Introduction:

Reducing the risk of foodborne illness associated with fresh produce is a task which the fresh produce industry and academic researchers have been struggling with for many years, in particular with great focus since the 2006 outbreak of gastroenteritis associated with fresh spinach caused by *Escherichia coli* O157:H7, a turning point for the industry. Over 600 million cases of foodborne illness are estimated to occur each year, although a large percentage of these occur in developing countries nearly 9.3 million cases occur annually in the United States alone (Gould, et al. 2013; Bean, et al. 2015). Microbial contamination can originate from countless areas along the farm to fork continuum. At greatest risk are those aspects of contamination that can occur within the pre-harvest environment, whereby microbial contamination can come in contact with plant foliar tissues from water, soil amendments, wind, birds, insects, animals, and other fomites (Berger, et al. 2010; Beuchat, 2004; Brandl, 2006). Risk of contamination of plants in the field likely occurs *via* direct and indirect mechanisms (Monaghan and Hutchison, 2012). Laboratory studies suggest that bacterial pathogens on plants in the field decrease quickly over the first 3 days, but low numbers continue to persist for several weeks, which may cause human health issues (Erickson., 2012). *Salmonella* isolates are documented to be in the top three most common etiological agents isolated from people who contracted food borne illness, with norovirus and *Campylobacter jejuni* the first and second most common etiological agents (F1000 Research Ltd. 2015; Gould, et al. 2013). To better understand the plant anatomy and physiology required to facilitate a successful foodborne pathogen infestation we must delve into interactions not usually observed between plants and plant benign bacteria, which in this case happens to be human pathogens.

The Centers for Disease Control and Prevention attributed 46% of illnesses to fresh produce and in particular leafy greens (Painter, et al. 2013). Indicating that greater and more sophisticated efforts are needed to prevent contamination on these commodities that cause foodborne illness. Plants grow in close association with large

communities of microbes, yet comparatively little is known about the diversity of microbes that associate with plants, and their interactions and effects on performance, crop yields and plant protection. The fields of plant science and food microbiology have been merging over the past few years in the best interest of produce safety; however, critical knowledge gaps remain. Most importantly, there is very limited knowledge about the modes of entry human bacterial pathogens may utilize for plant ingression, which may occur through several routes, including openings in roots, on the cortex, or epidermis (Deering, et al. 2012; Erickson. 2012; Hirneisen, et al. 2012). In addition, the knowledge pertaining to how human enteric pathogens evade or suppress plant defense for ingression is also not known. Therefore, novel strategies to control human pathogens merit attention and specifically efforts are needed to better understand its plant association.

Several reports have shown that human pathogens that infect mammals and other higher animals are able to infect plants (Rahme, et al. 2000). Research involving *Pseudomonas aeruginosa* resulted in the conclusion that conserved virulence factors can infect multiple hosts including plants (Rahme, et al. 2000). Like *Ps. aeruginosa*, *Salmonella enterica* also uses plants as alternative hosts to humans and other animals. It is shown that these bacteria are able to adhere to plant surfaces and actively infect the interior of plants. Using *Arabidopsis thaliana* as a model system, it was shown that, *S.* Typhimurium suppresses plant defense responses mediated by a type III secretion mechanism (Schikora, et al. 2008). In addition, it was also shown that several *Salmonella* serovars exhibit variations in pathogenicity, on different plant species revealing different innate defense response towards these bacteria (Schikora, et al. 2012; Schikora, et al. 2011) An important feature of *Salmonella* infections in plants is its ability to adhere to plant surface. Interestingly, it was shown that several *S. enterica* serovars adhere to plant surfaces better then pathogenic *E. coli* strain O157:H7 (Barak, et al. 2002). A mutant screen identified over 20 mutants impaired in surface attachment in *S.* Enteritis on *Medicago sativa* (alfalfa) sprouts. Less adherence was observed in mutants of a surface-exposed aggregative fimbria nucleator curli

(agfB) and the global stress regulator rpoS which regulates the production of curli, cellulose and other adhesins (Barak, et al. 2005). Contrastingly, not much is known about how *Salmonella* spp. infect and ingress in leafy green plants. In mammalian systems, *S. enterica* uses both pathogenicity islands 1 and 2 (SPI1, SPI2) to invade the epithelial lining and reproduce in the *Salmonella* containing vacuole (Coburn, et al. 2007). Primary function of the SPI1 is cellular invasion, while the SPI2 is required for cellular survival and reproduction in the *Salmonella* containing vacuole (SCV) (Ohl and Miller. 2001; Löber, et al. 2006). SPI1 virulence island is controlled via several genes; HilC, HilD and SirA/BarA that have been identified as regulators of HilA and downstream products in the SPI1 (Ellermeier, et al. 2005). Regulation of HilA controls all SPI1 functions, including the T3SS. It is thus evident that the genetic equipment of *Salmonella*, previously thought to be animal-infection specific, plays an important role in the infection of animals and plants alike.

Here, we developed a model patho-system with lettuce as a plant system to evaluate the ingression and persistence of *S. enterica* in a phytosystem. We observed that *S.* Typhimurium may bypass the early stomatal closure defense response in lettuce. Our observation shows that *S.* Typhimurium subverts the immune system and prevents stomatal closure at times when it normally would be closed as part of the innate immune response. Interestingly, few key T3SS components in *S.* Typhimurium were involved in overriding stomatal defense response in Lettuce for ingression. We also show that the T3SS in *S.* Typhimurium plays a critical role in persistence of *S.* Typhimurium in planta. Gene expression analysis shows that *S.* Typhimurium may perturb the ABA biosynthesis pathway to subvert stomatal defense. Our findings show the evidence and the adaption of a pathosystem involving a leafy green species with *S.* Typhimurium. The model system provides many possibilities of understanding molecular, biochemical and physiological networks that underpin this unique plant-human pathogen interaction.

Materials and Methods.

Plant Growth Methods:

Lectica sativa (Family: Asteraceae) var. Black Seeded Simpson was purchased from Johnny's Select Seed. Before cultivation all seeds were stratified for 48 hours on a damp paper towel in a conical tube at 4℃, the seeds were then soaked in 50% bleach for 8 minutes in the same 50 mL conical tube before being washed thrice with a minimum of 25 mL sterile water each time. This cleaning method resulted in only one plant in two years that had a mold presence and no loss of seed viability or germination rate. The clean seeds were placed on MS agar with 1% sucrose and grown under a 1750 (PAR= 200-230) lumen grow light grid at room temperature (25±3 ^{o}C) for 2 weeks with a 12h photoperiod. For persistence internalization assays: *Lactuca sativa* var. Black Seeded Simpson was planted in a sterile pro-mix made up of 85% Canadian sphagnum peat moss with perlite, vermiculite, dolomitic and calcitic lime, a wetting agent, and mycorrhizae (Premier Tech Horticulture, Quakertown, PA) in a seed tray with holes at the bottom (4 cm· 3.5 cm · 4.5 cm in dimension; T.O. Plastics, Ontario, Canada) and placed in another plastic container. Trays were maintained in Biosafety Level 2 growth chamber (Percival Scientific, Boon, IA) at 20°C with 12 h photoperiod and at a constant relative humidity of 60% (Markland et al. 2015). Plants were irrigated by pouring water into the bottom plastic container to saturation of the soil.

Bacteriological procedures:

All bacterial strains were kept at -80℃ freezer in 20% glycerol for long term storage. Prior to use each bacterial strain was streaked onto a complex-media (Tryptic Soy or Luria-Bertani) containing necessary antibiotics where applicable (SOM Table 1). Each plate was incubated for 16-24 hours at 30 $^{\circ}$C, and re-streaked from the glycerol stocks as needed. Before experimental use, a single colony from solid media was moved into liquid media via sterile loop technique and incubated at 30 $^{\circ}$C overnight on an orbital shaker at 200 rpm. Following incubation liquid bacterial cultures were

aliquoted into conical tubes and centrifuged for 15 minutes at 4000 rpm and washed twice with 25 mL PBS buffered to a pH of 7.4, followed by a final suspension in PBS. The optical density was measured at 600 nm with a Biorad SmartSpec+ and adjusted to the working concentration of 10^7 CFU mL^{-1} in sterile DI water in a new sterile flask or test tube.

Table 2.1 Bacterial isolates used in this study

Strains	Description	Antibiotic	Source:
S. enterica Typhimurium 14028s	Wild type	None	C. Altier
S. enterica Typhimurium *hilD*	*Salmonella* Pathogenicity Island-1 (SPI-1) transcriptional regulator hilD mutant	Chloramphenicol	C. Altier
S. enterica Typhimurium *hilA*	*Salmonella* Pathogenicity Island-1 (SPI-1) transcriptional regulator hilA mutant	Kanamycin	C. Altier
S. enterica Typhimurium *sseB*	T3SS mutant (SPI2) sseB Chaperone protein mutant	Tetracycline	C. Altier
S. enterica Typhimurium *sseA*	T3SS mutant (SPI2)	Tetracycline	C. Altier
S. enterica Typhimurium *fliC*	Phase 1 flagellin gene *fliC* mutant	Chloramphenicol	C. Altier
S. enterica Typhimurium *fljB*	Phage 2 flagellin gene *fliB* mutant	Kanamycin	C. Altier
S. enterica Typhimurium *invA*	SPI-1 regulatory mutant	Kanamycin	C. Altier
S. enterica Typhimurium *prgh*	SPI-1 T3SS effector mutant	Tetracycline	C. Altier
S. enterica Typhimurium *sirA*	Global SPI regulatory mutant	Tetracycline	C. Altier
S.enterica Newport	Wild type	Rifampicin	K. Kniel
L. monocytogenes-GFP	Nonmutant- GFP tagged	Erythromycin	L. Gorski

Bacterial culture filtrate (CFL) preparation:

Salmonella enterica serovar Typhimurium 14028s (henceforth *S. enterica*), *sseB,* or *hilD* were grown in 50 mL M9 media with 2% dextrose for 24 hours at 30^{o}C, the resulting suspension was measured with a BioRad SmartSpec+ to check for similar cell density. The culture was then centrifuged for 15 minutes at 4000 rpm and filter sterilized through a 0.22 μm filter. Heat treated CFL was prepared in an identical fashion followed by 3h incubation in a 65 $^{\circ}$C water bath before use. Contamination was checked for via plating 100uL onto LB agar; no contamination was ever observed. CFLs were only used the day they were prepared.

Stomatal assay:

Light adapted 2-week-old lettuce plants grown on MS agar were brushed with sterilized water, a suspension of 10^7 cfu mL^{-1} of the bacteria listed in Table 1, various MAMPs [S. *enterica* Typhimurium LPS 10μg mL^{-1}, *Pseudomonas aeruginosa* LPS 10μg mL^{-1}, FLG22 peptide at 10μg mL^{-1}] (All purchased from Thermo-Fischer), or plant growth hormones [5μM Salicylic Acid (SA), or 20 μM (+-)-Abscisic Acid (ABA)] (Both purchased from Sigma). Co-inoculation of bacteria and a plant growth hormone [5μM (SA), or 20 μM (ABA)] occurred by brushing the plants first with ABA or SA followed by bacteria with a separate sterilized brush to avoid cross contamination. Following inoculation, the plants were incubated at room temperature under the previously described grow light grid for 3-12 hours, stomatal aperture were monitored at 3, 6, or 12 hours post inoculation. For image analysis inoculated leaves were excised with alcohol sterilized forceps. From the excised leaf a small circle was removed with a potato corer. This subsample was stained with propidium iodide for 8 minutes followed by a light rinse with deionized water and placed abaxial side up under a glass block in chambered cover glass (NUNC/ VWR). For each treatment one leaf subsample was taken from three separate plants, replications were completed on non-adjacent days one replication for each treatment is present in the results.

Confocal imaging parameters:

Imaging was performed using a Zeiss LSM710 inverted laser scanning confocal microscope located in the University of Delaware Bio-Imaging Center, samples were imaged at 0, 3, 6, and 12 hours post inoculation. Propidium iodide was excited with a 561 nm laser through a 488/561 bandpass filter with the emission spectra set to 580-640 nm to avoid fluorescence from chlorophyll. Images were captured with 2048 x 2048 pixels per frame and 20 x magnification + 1x digital zoom for 425.1 μm per frame. Settings were consistent over all samples with exception to, digital gain, and the aerie units for poorly stained samples. In addition to PI, the phase image in grayscale was retrieved for every image. Stomatal aperture quantification was completed by measuring each stomate at its widest point using ImageJ software calibrated to 2048x2048 pixel images with 425.1 μm per frame, for a scale of 4.8711 pixels/μm. For each sample 40-60 stomata were measured to record the average aperture size and standard deviation.

Cryo-SEM imaging:

Light adapted 2-week-old lettuce was brushed with a suspension of either *S.* Typhimurium, hilD, *sseB*, or left uninoculated (control). To prepare leaves for SEM small holes were removed from each sample leaf with a potato corer (1 per leaf) to retrieve symmetrical circles for imaging, each leaf circle was placed adaxial side up on a gold block with tissue mounting fluid and carbon-black. After mounting, the leaves were flash frozen by being plunged into liquid nitrogen and contained under a vacuum, the block was then transferred to the chamber of a Hitachi SM4700 scanning electron microscope and brought to -120 $^{\circ}$C, the leaves were then sublimated at -90^{o}C to remove ice-films on the surface, and finally sputter coated with gold and palladium before imaging took place at -120^{o}C. Leaves treated with *S.* Typhimurium, *sseB, hilD* were imaged at 3 and 6 h post inoculation, with a references plant sample left uninoculated (0 h post inoculation). Each leaf subsample was imaged at a magnification of 500X. All images were processed in the same fashion as those from confocal based leaf assays, albeit with an adjusted scale.

Gene expression studies:

Light adapted 2-week-old lettuce was brushed with either water or *S.* Typhimurium in the identical fashion to the above stomatal assay. At 0 h post inoculation, 3 h post inoculation, and 6 h post inoculation samples were flash frozen in liquid nitrogen followed by RNA extraction following the Qiagen RNeasy protocol. In order to quantify the expression levels within *S enterica* treated lettuce the RNA was converted to complementary DNA via reverse transcription PCR. Rt-PCR was completed with Multiscribe® Reverse Transcriptase (Thermo-Fischer), for each reaction 1000 ng of RNA was used. The master mix was made as follows; 2 µL RT-Buffer, 2µL RT-random Primers, .8µL dNTP- Nucleotides, 1 µL Multiscribe Reverse Transcriptase, 1000ng RNA, and enough nuclease free water to bring the reaction volume to 20 µl. RtPCR parameters were as follows for every sample: 25℃ for 10 minutes, 37℃ for 2 hours, 85℃ for 5 mins, and 4℃ until samples were moved to a -20℃ freezer for long term storage. A semi-quantitative PCR technique using a DreamTaq Green Polymerase and protocol (Thermo-Fischer) was employed to check cDNA viability and relative expression of genes. The primers and PCR conditions used can be found in Table 2.2 and Table 2.3.

Table 2.2 Conditions for polymerase chain reactions (PCR) described in the present study

Genes	Hotstart (95ºC)	Annealing	Binding		Cycles	Curing
ACTIN	5 minutes	94ºC/ 15 s	54ºC/ 30s	72ºC/ 30 s	40	72ºC/ 5 min
PR1	5 Minutes	94ºC/ 15 s	57ºC/ 30s	72ºC/ 30 s	35	72ºC/ 5 min
NPR1	5 Minutes	94ºC/ 15 s	53.5ºC/ 30	72ºC/ 30 s	40	72ºC/ 5 min
PDF1.2	5 Minutes	94ºC/ 15 s	54.5ºC/ 30s	72ºC/ 30 s	40	72ºC/ 5 min
NCED1	5 Minutes	94ºC/ 30 s	55ºC/ 30s	72ºC/ 30s	40	72ºC/ 5 min
NCED2	5 minutes	94ºC/ 15 s	57ºC/ 30s	72ºC/ 30 s	30	72ºC/ 5 min
ABA3	5 minutes	94ºC/ 15 s	56ºC/ 30s	72ºC/ 30 s	40	72ºC/ 5 min
NCED3	5 Minutes	94ºC/ 15 s	57ºC/ 30s	72ºC/ 30 s	35	72ºC/ 5 min
ZEP1	5 Minutes	94ºC/ 15 s	57ºC/ 30	72ºC/ 30 s	40	72ºC/ 5 min

Table 2.3 Primers used and described in the present study

	Oligo-DNA Primers		
Gene	**Forward Primer**	**Reverse Primer**	**Reference**
ACTIN	CAAGCCGTTCTTTCCCTGTA	TCCTGCTGAGGTCGTGAATG	(Markland et al. 2017)
PR1	GAAGGGTTGGGTGTGCTAGA	CACAAGAAACAAGGGCGTAG	(Markland et al. 2017)
NPR1	TCGATCGTCTATCGGAAACC	TCACATTGCGATTCTTGTCC	(Markland et al. 2017)
PDF1.2	GCCATCTTCTCTGCTTTTGAA	ACACAAGACACTGCGACGAC	(Markland et al. 2017)
NCED2	TCGACTCTTTGTGCACTTCATACTC	CAACGGGACAAGTTGAGGTTTTA	(Huo et al. 2013)
NCED1	CGCAATCACCGAGAACTTTGT	GGCGATCCTCCTTTTATCATTTC	(Huo et al. 2013)
NCED3	AGCTCAGCTTGGTTCCCTGTTATA	CTTCACAAACTGGCTGAAAACGTAT	(Huo et al. 2013)
ABA3	CAGCTCTAGCTTGACCTCATCA	CCAAGGACACTGTTGTGGTTCT	(Argyris et al. 2008)
ZEP1	GGGCTACACTTGTTACACTGGGATA	GCCCCAAGAACACCCGATA	(Huo et al. 2013)

Persistence of *Salmonella* T3SS mutants on lettuce surface:

2-week-old lettuce plant leaves were spot-inoculated randomly with 120 µl (6 droplets) of respective *Salmonella* culture on the leaf surface. Inoculated leaves were placed in the growth chamber for 2 h, to facilitate bacterial attachment and leaves. Samples were

collected for each treatment group separately and processed to enumerate surviving *Salmonella* populations on day 0, 1, 3, 5, and 7. A sample of 6 plants (total of 12 leaves in each) was collected and split into two equal sections for bacterial enumeration and pathogen internalization assay. To enumerate, leaf samples (6 leaves) were weighed in individual Whirl-Pak™ bags (Nasco, Fort Atkison, WI, USA) and submerged in 0.1% buffered peptone water (BPW; Oxoid Ltd., Basingstoke, Hampshire, England) in a 1:9 ratio. The sample was mixed for 2 min at 230 rpm in a stomacher (BagMixer® 400 S, Interscience), the resulting mix was serially diluted in 0.1% BPW and plated on TSA with antibiotic (Table 2), or by a mini-MPN method (Sharma et al., 2016). *Salmonella* colonies counted after 22-24 h of incubation at 37ºC. Persistence assays completed by Kniel Lab at the University of Delaware

Internalization assay:

To detect pathogen internalization in lettuce, leaves were surface disinfected by immersing them in 80% ethanol for 10s, followed by 10 min dip in 0.1% mercuric chloride (Markland et al. 2015; Zhang et al. 2009). Leaves were washed with 10 mL sterile water and grounded using a rubber mallet. Samples were re-suspended in 0.1% BPW and homogenized for 2 min at 230 rpm in a stomacher and 1 mL of the resulting mix was transferred to 9 mL TSB. Inoculated TSB was incubated for 18-24 h at 37°C. After incubation, a loop full sample from each tube was streaked onto TSA plates and incubated for 18 h at 37°C. Plates were observed for CFU counts. Internalization assays were completed by Kniel Lab at the University of Delaware

Statistical analysis:

All the experiments were repeated 3 times. Surviving *S.* Typhimurium populations, recovered at each sampling point, were converted to $\log_{10}$ CFU/g or MPN/g and mean values of the three replicates obtained. The limit of detection was 1 log CFU/g. Data were analyzed using one-way analysis of variance to determine the effect of *Salmonella* mutants on survival. A Student's t-test was performed to compare the means of bacterial populations over time by using JMP (JMP v.14 software; SAS Institute Inc., Cary, NC) at a significance level of $p<0.05$.

Results.

Salmonella enterica treatment modifies stomatal aperture in Lettuce:

Previous studies have shown that stomata act as an entry points for various plant and human pathogens to ingress in plants (Kumar et al. 2012; Markland et al. 2015). To determine if different opportunistic pathogens such as *S.* Typhimurium and *L. monocytogenes* modulate stomatal aperture in lettuce, we treated lettuce (2-weeks-old) with *S.* Typhimurium or *L. monocytogenes*. We evaluated two methods to measure stomatal aperture: confocal microscopy of PI stained leaf subsamples and cryo-scanning electron microscopy (cryo-SEM) of the frozen leaf subsamples (Van Gardingen, et al. 1989). Cryo-SEM was chosen as the validation method because it was possible to examine and capture the effect of the signals generated during the early time points of tritrophic interactions in the context of the entire plant. *S.* Typhimurium leaf inoculation didn't change or reduce the average stomatal aperture size at both 3 and 12 h post inoculation compared to the mock inoculation that being sterilized nanowater (Figure 2.1). Both confocal microscopy and Cryo-SEM showed similar aperture profile under *S.* Typhimurium treatment. Using images obtained through the confocal imaging and cryo-SEM technique the stomatal width taken at the widest point was averaged for 40-60 stomata per leaf after which the average of all samples in set were averaged (n=3) (Fig. 2.1 and Fig. 2.3). Treatment of both *S.* Typhimurium and *L. monocytogenes* showed stomatal aperture reduction at 6 h post inoculation, but only *L. monocytogenes* showed continued reduction at 12 h post inoculation compared to the control and *S.* Typhimurium treatment (Fig. 2.1). *S.* Typhimurium presented a phenotype similar to that of the mock after 3 hours and was not different from the control. Both the control and *S.* Typhimurium were significantly

different ($p<.05$) from the reduced stomatal aperture observed in *L. monocytogenes* treated plants. Further analysis showed that *L. monocytogenes* leaf inoculation significantly increased the percentage of closed stomata on the abaxial leaf surface at both 3 and 6 h post inoculation compared to the control and *S.* Typhimurium treatments (data not shown). Taken together, these data showed that leaf inoculation of *S.* Typhimurium can modulate stomatal aperture to keep stomates open post inoculation thus potentially increasing the likely hood of internalization.

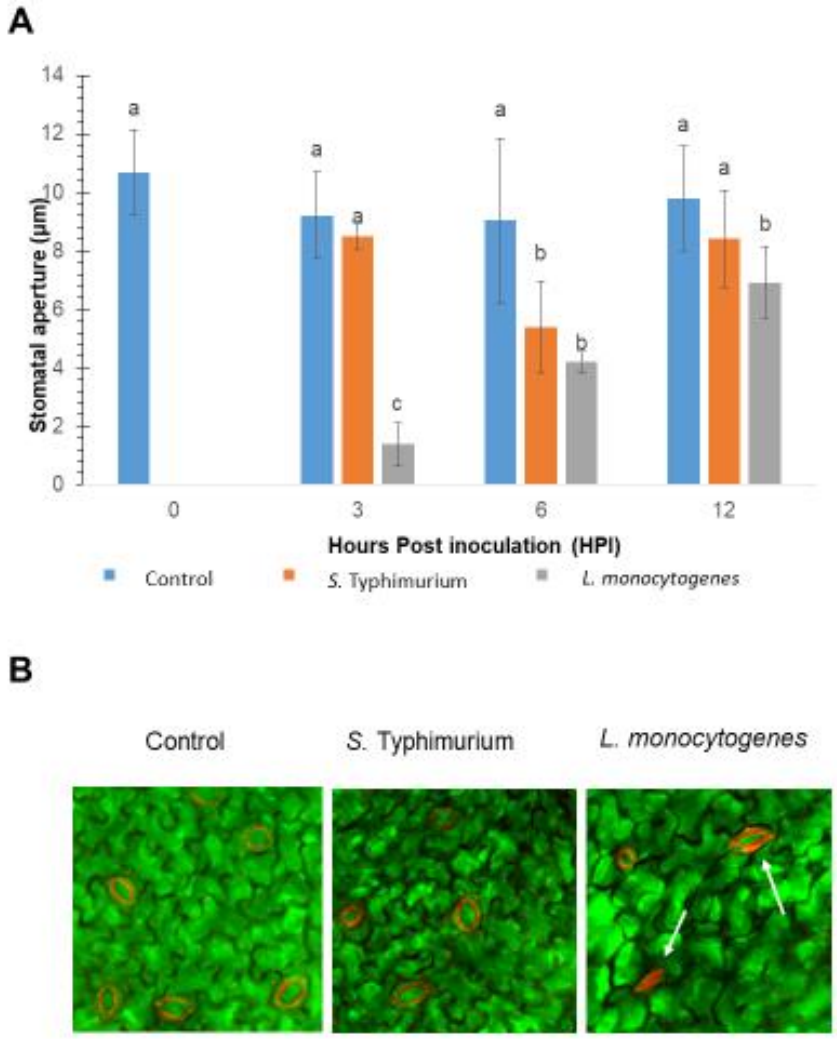

Figure 2.1 A: Comparison of innate immune response in terms of stomatal closure by *L. monocytogenes* and *S.* Typhimurium on lettuce. Different letters signify a difference at $p<.05$. Average stomatal aperture was found by measuring 40-60 stomates from three independent plants, error bars represent the standard deviation between three biological replicates. B: Confocal images of open stomata phenotype observed with a water control (left), *S.* Typhimurium (middle), and closed phenotype observed with *L. monocytogenes* (right) all images taken at 3hpi. Arrows indicate reduction in stomatal aperture. Scale bar= 30µm.

***Salmonella enterica* culture filtrate (CFL) and microbe-associated molecular patterns (MAMPs) modifies stomatal aperture in lettuce:**

Since both *S.* Typhimurium treatments to lettuce leaves showed no change of stomatal aperture size post-inoculation, we argued that *S.* Typhimurium or *S.* Typhimurium - derived products may modify plant innate defense response to keep the stomata open. To test the products derived from *S.* Typhimurium on stomatal closure, a cell free culture filtrate (CFL) was prepared and stomatal apertures were subsequently measured at 3 h post inoculation. Interestingly, the CFL from *S.* Typhimurium led to closure of stomates compared to the untreated control and *S.* Typhimurium post-three hours off inoculation suggesting proteins from *S.* Typhimurium can close stomata most likely plagellin and contents from cells lysed during centrifugation (Fig. 2.4). In contrast, the heat killed CFL (HK CFL) prepared from *S.* Typhimurium showed the stomatal phenotype similar to *S.* Typhimurium, suggesting that the same proteins that triggered stomatal closure were destroyed during the heating process and are therefore not stable (Fig. 2.4). It is known that MAMPs derived from bacterial plant pathogens trigger plant innate defense response to close stomates (Melotto et al. 2006; Kumar et al. 2012). MAMPs such as *S.* Typhimurium serovar Typhimurium LPS, a nonspecific LPS (*Pseudomonas aeruginosa*) and Flg22 (10 µg mL^{-1}) were applied to lettuce leaves and stomatal apertures were monitored and measured post 3 h of inoculation with confocal microscopy. Each MAMP caused closure statistically similar to *S.* Typhimurium CFL-challenged lettuce, except flg22 which caused 50% more closure than *S.* Typhimurium CFL (Fig. 2.4). The stomatal closure induced by the MAMPs was 60% to 25% smaller than the aperture means gathered in the control and live *S.* Typhimurium trials. The data suggests that *S.* Typhimurium but not the secreted cell free lysate from *S.* Typhimurium or LPS from two separate and known human pathogens can regulate stomatal closure by suppressing the immune system of lettuce.

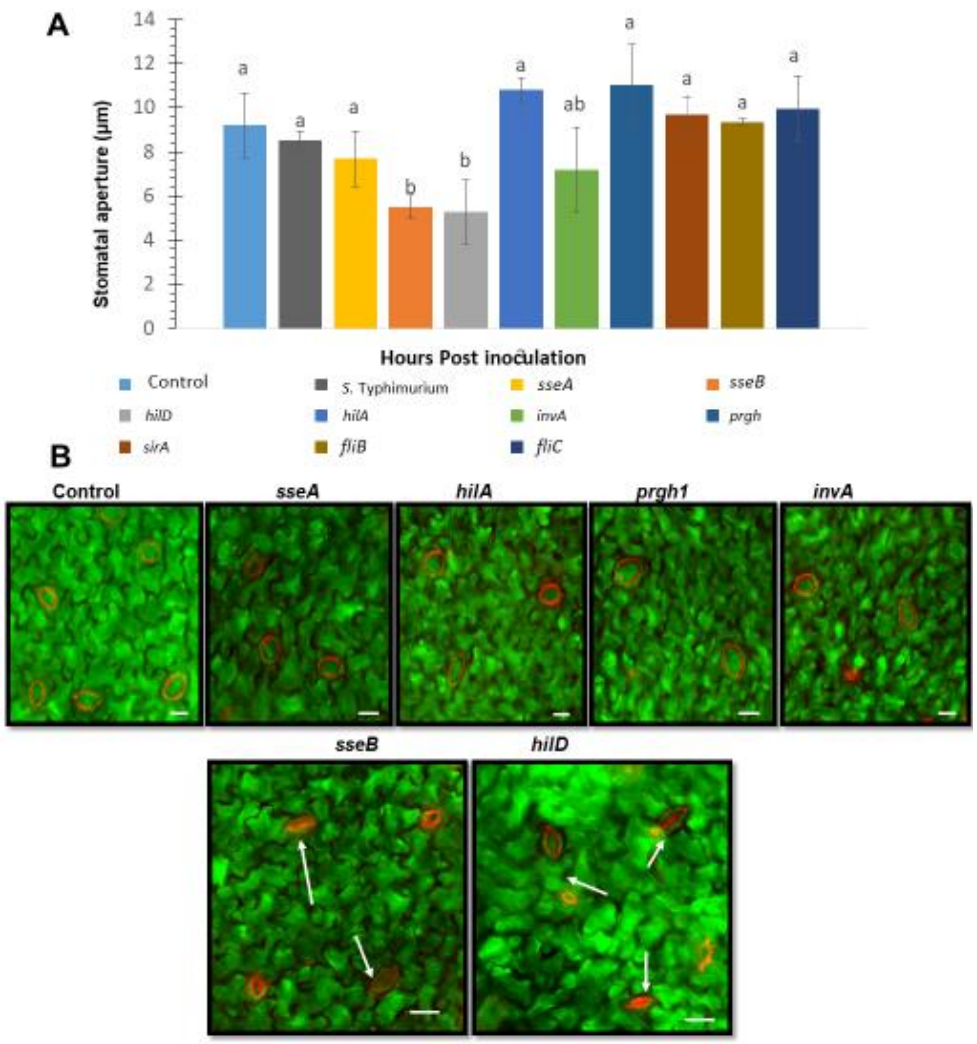

Figure 2.2 A: Stomatal closure was observed at 3 HPI in both *S.* Typhimurium, *sseB* and, *hilD* with significantly smaller stomatal apertures compared to the untreated control ($p<.01$). Different letters signify a difference at $p<.05$. Average stomatal aperture was found by measuring 40-60 stomates from three independent plants. B: Representative confocal images of stomatal apertures in lettuce, in order from top left-right, Water control, *S.* Typhimurium *sseA*, *hilA*, *prgh1*, *invA*. Bottom row: *sseB*, *hilD* Arrows point to partially closed and fully closed stomata. Images captured with 20x Objective lens. Scale Bar= 15µm

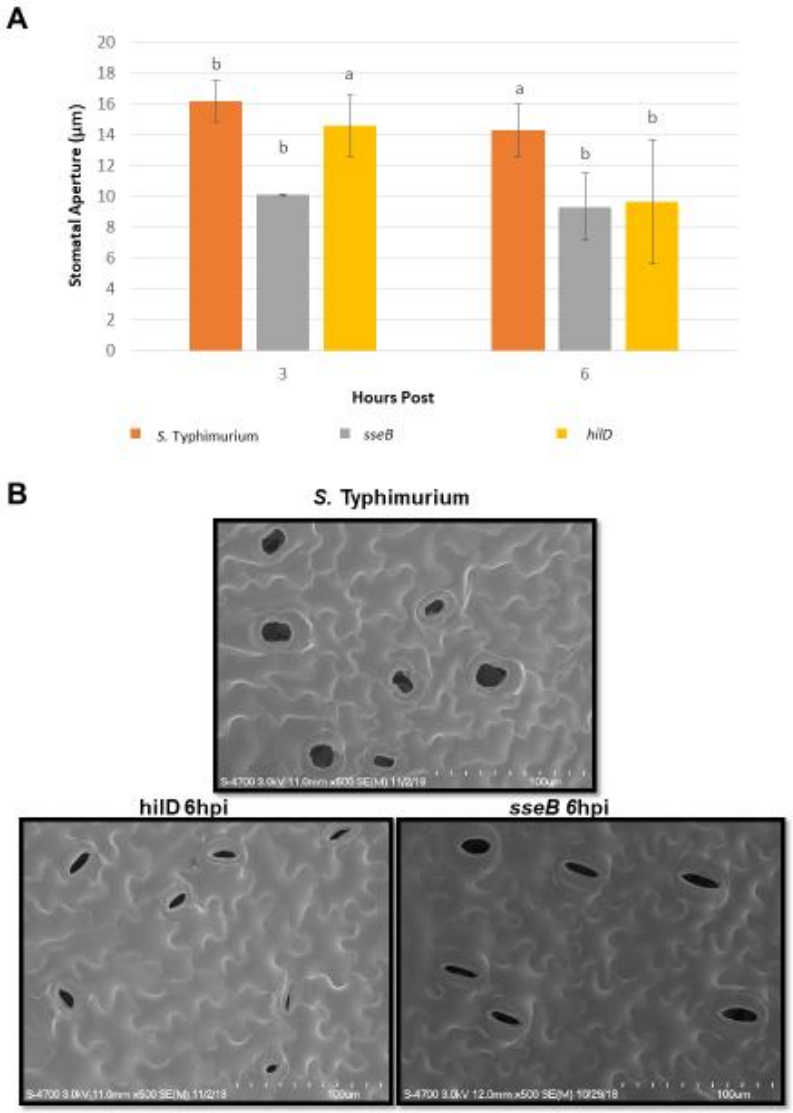

Figure 2.3 A. Lettuce inoculated with either *S.* Typhimurium, *sseB, hilD* was imaged after cryogenic preservation and sputter coating with gold-palladium. Closure or stomatal aperture reduction was observed at 6hpi on both *sseB* and *hilD* treated leaves but was not observed at 6hpi on any leaves treated with *S.* Typhimurium. Asterisks represent significant differences between *S.* Typhimurium, *sseB* and *hilD*. B: The panel B shows representative images from cryo-SEM experiments. The images were captured 6 hours post inoculation Top line: *S.* Typhimurium WT 6hpi. Bottom line: *hilD* 6 hpi, *sseB* 6hpi. 500X magnified.

S. Typhimurium mutants lacking Type-III secretion system (T3SS) virulence factors show reduced suppression of stomatal defense response:

Various studies have shown that *S.* Typhimurium possesses PAMPs that are recognized in plants (Garcia and Heribert Hirt. 2014; Shirron and Yaron. 2011; Schikora, et al. 2011). The experiments involving CFL from *S.* Typhimurium showed that plants recognize secreted components from *S.* Typhimurium to launch a PAMP triggered immunity to close stomata, but that these are destroyed by a heat treatment. Furthermore, inoculation of *S.* Typhimurium serovars on *Arabidopsis thaliana* seedlings triggered MAPK activation and defense gene expression to a similar extent as that provoked by *Ps. syringae* inoculation (Schikora, et al. 2008; Schikora, et al. 2011; Garcia and Hirt. 2014) . We also showed that classical MAMPs such as flg22 and two different LPS induce PTI-stomatal closure in lettuce. We hypothesized that factors governed through the T3SS system in *S.* Typhimurium may modify stomatal defense response differently in lettuce. To this end, we used various T3SS mutants belonging to both SP1 and SP2 system in *S.* Typhimurium for modification of stomatal defense response in lettuce. Recently, functional *S.* Typhimurium T3SSs and effectors were proposed to contribute to the plant colonization process (Garcia and Heribert Hirt. 2014). *S.* Typhimurium mutants in T3SS-1 and T3SS-2 induced PTI and reduced proliferation in *Arabidopsis* leaves (Schikora et al. 2011). We tested all the *S.* Typhimurium mutants listed in Table 1 by applying them to light adapted lettuce leaves and the stomatal aperture 3 hours post inoculation was recorded. Of the nine mutants applied to the stomatal assay only two resulted in consistent stomatal closure like that seen on plants treated with *S.* Typhimurium CFL (Fig. 2.2 and Fig. 2.4). A majority of T3SS mutants caused no apparent stomatal closure at 3 h post inoculation. Most *S. enterica* serovars carry two flagellin-encoding genes, *fliC* and *fljB* (Silverman and Simon. 1980). The mutants lacking flagellar genes (fliB and fliC) in *S.* Typhimurium also showed lack of stomatal closure (Fig. 2.2). It is shown that *S. enterica* flagellin mutants triggered reduced defense responses in *Arabidopsis* and

tomato (Iniguez, et al. 2005; Garcia, et al. 2014a). The T3SS mutants *hilD* and *sseB* caused stomatal closure that was significantly different ($p<0.05$) then the mock or live *S.* Typhimurium (Fig. 2.2). HilD is a transcription regulator for the master control of SPI1 and SseB is an element in the translocon of SPI2 (Ellermeier, et al. 2005; Ruiz-Albert, et al. 2003). The mutant *invA* was observed to be partially able to induce stomatal closure, this may be due to *invA*'s function in the T3SS and SPI1.

Validation of *hilD* and *sseB* stomatal phenotype was completed using cryo-SEM; stomatal apertures of plants treated with *sseB* showed significant closure compared to *S.* Typhimurium at both 3 and 6 h post inoculation. *HilD* challenged plants showed a significant change in stomatal aperture at only 6 h post inoculation (Fig. 2.3A-B). We also evaluated if host sensing by lettuce is mediated through T3SS, to this end, the CFL from the two T3SS mutants (*hilD* and *sseB*) were added to lettuce leaves and compared with live *sseB* and *hilD* for stomatal apertures (Fig 2.4B). The CFL from both T3SS mutants led to stomatal closure as seen with WT CFL and live *sseB* or *hilD* cells, suggesting that the host sensing by lettuce is independent of T3SS and T3SS may be required to bypass stomatal defense in plants.

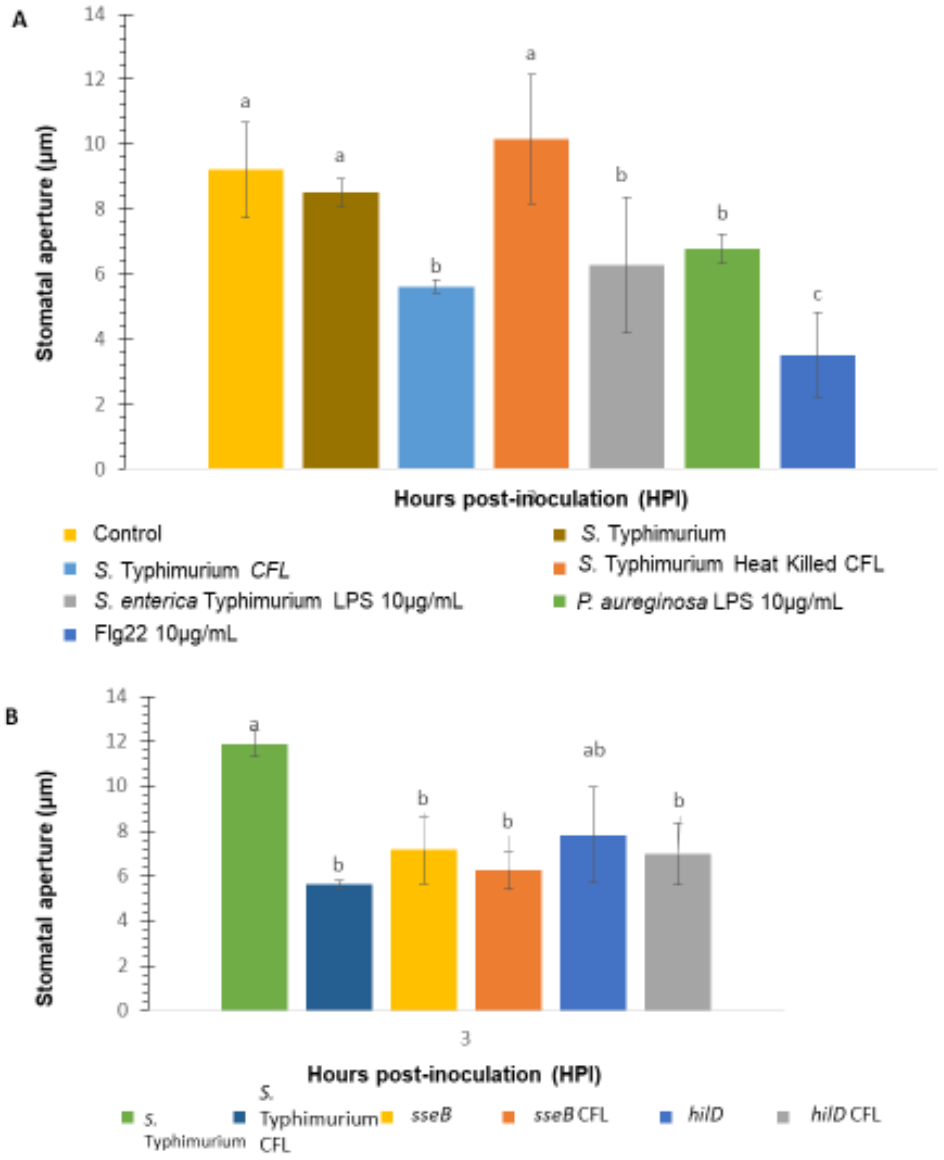

Figure 2.4 A. Stomatal apertures from *S.* Typhimurium CFL and heat treated CFL along with known MAMPS were recorded after inoculation. Different letters signify a difference at p<.05. Average stomatal aperture was found by measuring 40-60 stomates from three independent plants. B. Stomatal apertures from plants treated with CFL from *sseB* and *hilD*. Different letters signify a difference at p<.05. Average stomatal aperture was found by measuring 40-60 stomates from three independent plants.

Persistence and internalization of *S. enterica* mutants lacking Type-III secretion system (T3SS) virulence factors in lettuce grown under greenhouse conditions:

S. Typhimurium populations were significantly reduced ($p<0.05$) in plants inoculated with T3SS mutants (*hilD* and *sseB*) by day 3 compared to wild type control (*S.* Typhimurium 14028s) (Fig. 2.5). Mutants *hilD* and *sseB* populations were reduced to 2.1 and 2.3 MPN logs g^{-1} on day 3 compared to day 0 (4.1 and 4.6 log CFU g^{-1}), respectively. *Salmonella* populations were further reduced to undetectable levels for *hilD* and to 0.7 log MPN g^{-1} for *sseB* mutants, by day 5. Internalization assay results showed that *Salmonella* internalization was not detected in plants inoculated with *hilD* and *sseB* mutants, suggesting that these genes are vital for *S.* Typhimurium internalization in lettuce (Table 1.4). The mutant *invA* appeared to be marginally better at surviving on the leaf and was not recovered on day 5 with slight recovery by day 7 (Fig. 2.5). However, in the wild type control, no significant ($p<0.05$) change in pathogen populations (5.5-5.6 logs CFU $MPN^{-1}g^{-1}$) was observed on lettuce surface, during 7-day trial. It has been shown that *Salmonella* T3SS genes play different roles during plant-bacteria interactions based on plant species (Brandl et al., 2013). Iniguez et al. (2005) observed hyper-colonization of *Salmonella* T3SS SPI mutants than the wild type in alfalfa sprouts and *Arabidopsis thaliana,* On the contrary, in the current study; significantly low colonization was recorded in SPI mutants compared to the wild type. It is noteworthy that previous study tested SPI- structural gene (s*ipB*) mutant, while the current study analyzed the effects of SPI-1 transcriptional regulator (*hilD*) and SPI-2 (*sseB*) mutants on survival. This could explain the differences in survival and internalization of mutants and suggests that these genes could affect *Salmonella* survival and persistence in lettuce leaves.

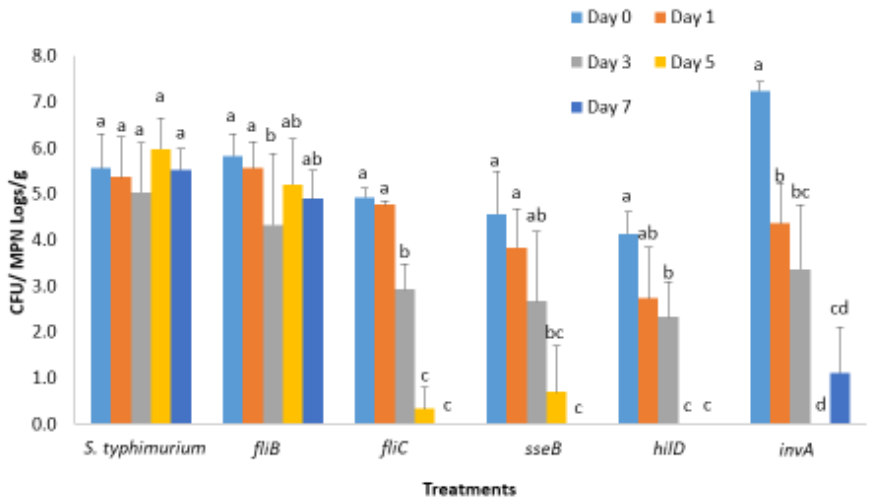

Figure 2.5 Persistence of *S.* Typhimurium populations on lettuce over a 7-day period. *Values represent the average of three replications. Standard deviation (±) for surviving S. Typhimurium population (Log10 CFU or MPN/g) follows mean value. Letters a, b, c shows statistically significant differences ($p < 0.05$) between sampling days for the same strain. Persistence data was gathered for this thesis by Dr. Kaur-Litt

Table 2.4 Internalization of *S. enterica* and T3SS mutants in lettuce plants grown under greenhouse conditions

Treatments	Day 0	Day 1	Day 3	Day 5	Day 7
sseB	-	-	-	-	-
hilD	-	-	-	-	-
FliB	+	+	+	+	+
FliC	+	+	+	+	-
invA	+	+	-	-	-
S. Typhimurium WT	+	+	+	+	+

Exogenous hormones SA and ABA induce differing stomatal phenotypes in lettuce treated with *S. enterica*:

Previous studies have shown that defense hormones play a critical role in the interaction between *S. enterica* and plants (Garcia and Hirt, 2014) Growth regulators such as SA, JA and ET signaling pathways regulate *S. enterica* colonization in plants (Iniguez et al., 2005; Schikora et al., 2008). Studies have also shown that plants treated with *S. enterica* Typhimurium biosynthesize SA and the induction of several marker genes of the SA pathway (Garcia et al., 2014). To this end, we evaluated if addition of exogenous SA and ABA, another key growth regulator which plays a critical role in stomatal physiology (Kumar et al. 2012) plays a role in co-inoculation with *S. enterica* in lettuce. Plants were inoculated with SA/ABA alone or co-inoculated with live *S.* Typhimurium and stomatal apertures were measured post 3-12 h post inoculation. When compared to other PTI induced plants like those treated with CFL and flg22, SA was similar in percentage and duration (Fig. 2.6A). At 3 h post inoculation stomatal closure was apparent and different from the mock, and by 12 h post inoculation SA-challenged plants had returned to a normal stomata phenotype (Fig. 2.6A). What was interesting is that exogenous SA in co-inoculation assays caused stomata to remain closed for longer than when just SA was applied. In fact, the stomatal stays closed twice as long (out to 12 hpi) when both SA and *S.* Typhimurium was present. The reasoning for this is that the SA pathways was not affected by *S.* Typhimurium's phytopathogenic behavior therefore the presence of exogenous SA triggered PTI independently from *S.* Typhimurium. Despite these interesting observations, no hypersensitive response (patterned cell death) was observed on any plants, which may only be due to the 12-hour length of the experiments. Some cell death was observed in research with *S. enterica* applied to *Tabacum* and *Lycopersicum* (Family: Solanaceae). (Meng, et al. 2013)

We also evaluated the role of ABA in co-inoculation with *S.* Typhimurium in lettuce. It is shown that ABA plays a critical role in stomatal physiology under various biotic and abiotic conditions and is shown to be both synergistic and antagonistic with

other plant hormones (Merilo, et al. 2015; Anderson, et al. 2004). When ABA was exogenously applied to the lettuce leaves, stomatal closure was observed just like on SA-challenged, plants (Fig. 2.6B). Unlike SA, ABA caused closure at 3 h post inoculation and 6 h post inoculation but at 12 h post inoculation, when most plants in previous tests had reopened stomata, almost 100% of the stomata had closed (Fig. 2.6B). The ABA induced closure significantly different ($p<.01$) from both SA and SA co-inoculated tests at 12 h post inoculation (Fig. 2.6A-B). The co-inoculation with ABA and *S.* Typhimurium was set up by exogenously adding ABA (20 µM) and *S.* Typhimurium together. The stomatal apertures using confocal imaging were performed at 3, 6 and 12 h post inoculation. ABA co-inoculation with *S.* Typhimurium was performed with identical parameters and concentrations. The expected results with the co-inoculation of ABA with *S.* Typhimurium were to see stomatal closure from 3 until 12 h post inoculation as before; oddly enough the resulting stomatal phenotype was most similar to *S.* Typhimurium -challenged plants (Fig. 2.1A and 2.6B). The data clearly showed that *S.* Typhimurium mediated stomatal opening was not actively closed by ABA treatment under co-inoculation, suggesting that *S. enterica* may disrupt ABA biosynthesis and signaling *in planta* as a roundabout way of preventing stomatal closure. *SseB* and *hilD* were also tested in a co-inoculation experiment with ABA. At three h post inoculation, *sseB* showed stomatal closures similar to ABA treatment (Fig. 2.6C) Co-inoculation of ABA with *sseB* led to stomatal reopening after 3 h post inoculation compared to *hilD* which at first had a phenotype more like that of *S.* Typhimurium but by 12 hpi it had closed to that of the 20 µM ABA treated plants (Fig. 2.6C) The later time point (6 and 12 h post inoculation) showed no reopening of stomates under a co-inoculation experiment with ABA and *hilD* , suggesting, the involvement of T3SS in suppressing ABA-mediated stomatal defense may occur early in plants interaction with *S.* Typhimurium.

To analyze if addition of ABA later to *S.* Typhimurium supplementation in lettuce leaves changes stomatal physiology, ABA was added 3 h post *S.* Typhimurium supplementation. As shown previously, supplementation of ABA rapidly closes

stomates in multiple plants (Kriedemann, et al. 1972; Melotto, et al. 2006). In comparison to co-inoculation experiment when ABA was added as a delayed application 3 h post inoculation post *S.* Typhimurium treatment, the stomatal re-opening with *S.* Typhimurium was seen post 6 h post inoculation (12h time point), though the reopening of stomates was not as prominent as co-inoculation experiment (Fig. 2.6B and Fig2.6C).

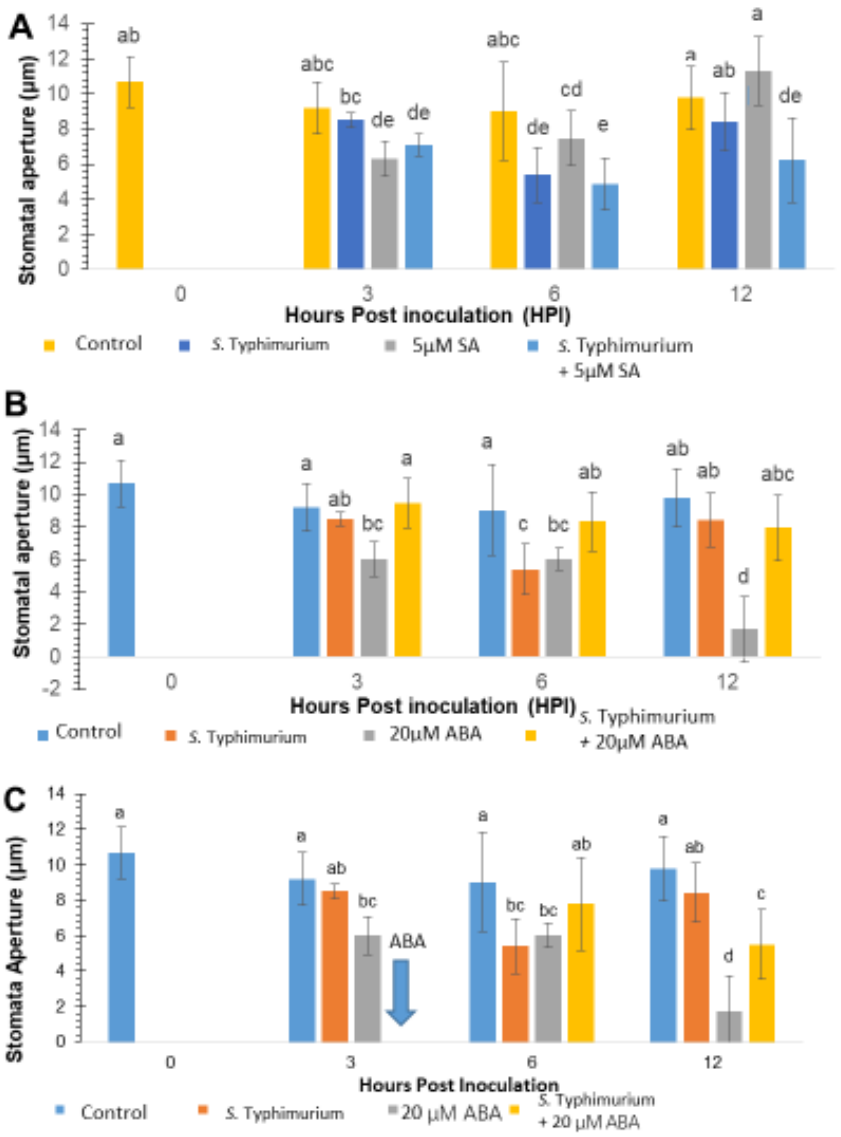

Figure 2.6 A. Treatment and co-inoculation of salicylic acid with *S.* Typhimurium caused more stomatal closure than *S.* Typhimurium. Different letters signify a difference at $p<.05$. Average stomatal aperture was found by measuring 40-60 stomates from three independent plants. B. Treatment and co-inoculation with abscisic acid with *S.* Typhimurium resulted in significantly different stomatal closure t 3 and 12 hpi. Different letters signify a difference at $p<.05$. Average stomatal aperture was found by measuring 40-60 stomates from three independent plants. C. Delayed application of ABA in co-inoculation resulted in stomatal closure by 12 hpi but not before. Different letters signify a difference at $p<.05$. Average stomatal aperture was found by measuring 40-60 stomates from three independent plants

***S. enterica* affects genes in the ABA biosynthesis and translocation pathway**: ABA regulates stomatal closure (Acharya and Assmann, 2009). The precise mechanism through which *S.* Typhimurium suppresses or blocks ABA effects on stomates remains to be shown. By examining transcript levels of ABA biosynthetic genes, (ZEP1, NCED3 and ABA3) in lettuce plants treated with *S.* Typhimurium we showed that two genes were down regulated by 6 hours post inoculation in the roots, but not the leaves (Fig. 2.7). These genes ABA3 and NCED3 are required for starting biosynthesis of the hormones.

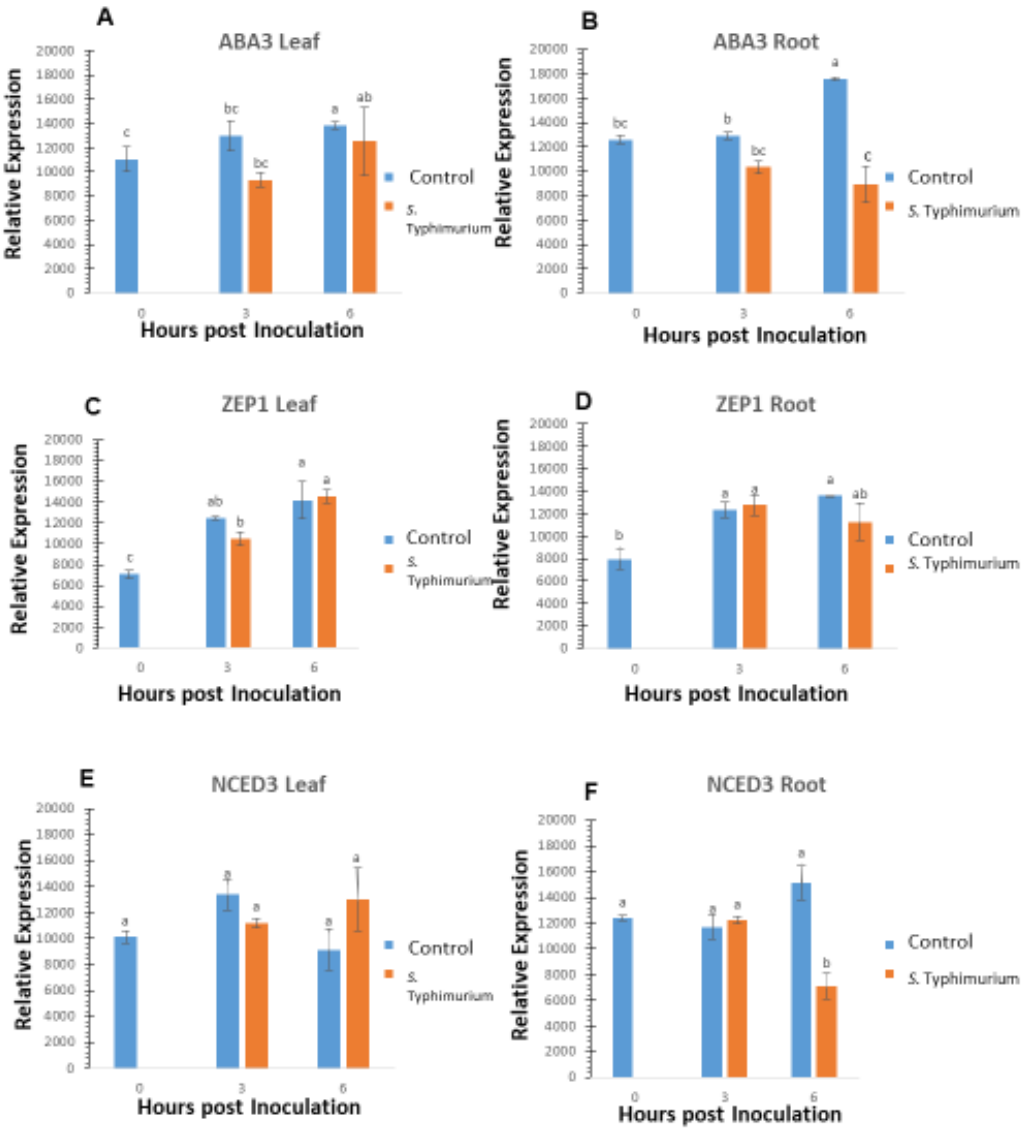

Figure 2.7 A-F. Semiquantitative gene expression analysis of various ABA biosynthesis genes in roots and leaves of lettuce plants treated with *S.* Typhimurium. Different letters signify a difference at $p<0.05$. For each time point n= 3 plants.

Discussion.

It is shown that few plant pathogens have an ability to modify stomatal physiology to ingress and cause infection (Melotto et al. 2006). Bacterial pathogens such as *Ps. syringae* DC3000 exploits a polyketide (coronatine) to reopen stomata in *A. thaliana* to cause infection (Melotto et al. 2006; Kumar et al. 2012). On the contrary, plants get exposed to various human pathogens such as *Ps. aeruginosa* and *Enterococcus faecalis* and shown classical infection as seen previously with plant pathogens (Jha, et al. 2005; He, et al. 2004). Along the same lines, few opportunistic human pathogens such as *E. coli* serovars, *L. monocytogenes* and *Salmonella* serovars also interact with plants and cause ingression and contamination (Gorski, et al. 2009; Deering, et al. 2012). The mechanism by which opportunistic human pathogens such as *Salmonella* ingress plants are not known. It is often speculated that opportunistic human pathogens such as *Salmonella* may use natural entry points (stomata) or mechanical injuries to ingress in planta (Zheng, et al. 2013; Barak, et al. 2011; Deering, et al. 2012; Melotto, et al. 2006). In this study, we report that one of the *S. enterica* serovar Typhimurium 14028s may bypass the plant innate immune response through suppression of stomatal defense a possible adaption to improve ingression and survival. We also show that *S. enterica* uses key T3SS factors to overcome stomatal defense for ingression. This revealed that plants treated with other opportunistic pathogens such as *L. monocytogenes* can induce stomatal closure, but only *S.* Typhimurium treatments kept stomata open. We also showed that ability of *S.* Typhimurium to overcome stomatal defense was T3SS dependent. In addition, the SPI1 and SPI2 mutants of T3SS mutants revealed poor fitness and persistence under realistic growth conditions in lettuce. Using exogenous application of SA and ABA, we showed that *S.* Typhimurium may suppress ABA response in lettuce to keep the stomates open for longer durations. Using the gene expressions for ABA specific biosynthetic genes, we discovered that the addition of the *S.* Typhimurium, was suppressing stomatal closure through suppression of the ABA biosynthetic pathway.

This study shows that the frequent association of opportunistic human pathogen such as *S.* Typhimurium with plants may lead to development of key strategies by *S.* Typhimurium to invade plants and cause contamination.

S. enterica live cells and cell free lysate treatment indicate the critical role of stomatal defense in identifying microbial-derived factors regulating innate response:

Previous studies showed that application of bacteria to isolated epidermal peels from *A. thaliana* induced a MAMP-triggered immune response that closed stomata (Melotto et al. 2006; Zeng et al. 2010). Our data show that leaf inoculation of bacteria such as *L. monocytogenes* closed stomata post 3 h post inoculation in contrast, *S.* Typhimurium treatments kept the stomata until 12 h post inoculation. The rapid closure of stomata with *L. monocytogenes* appeared to be a classical innate defense response to close stomata suggests that the response was most likely related to a MAMP-triggered immune response (Zeng et al. 2010). A similar response was observed when known MAMPs, such as LPS and flg22 were added to the lettuce leaves. Melotto et al. (2006) and Roy et al. (2013) showed that flg22 and LPS close stomata in the isolated leaf epidermal peels of Col-0 and lettuce plants, similarly, our data showed that supplementation of MAMPs such as LPS and flg22 closed stomata in whole lettuce leaves. The stomatal closure by MAMPs was contrastingly opposite in case of the *S.* Typhimurium treatment where no stomatal closure was observed 3 and 12 h post inoculation. The data by Roy and Melotto (2019) showed treatment of *S. enterica* SL1344 negated stomatal closure in Romaine at 4 h post inoculation under both 65 and 95% relative humidity. In contrast, cell free lysate (CFL) from *S.* Typhimurium induced a classical MAMP based stomatal closure compared to live cell treatments, suggesting that cell free or secreted components from *S.* Typhimurium are recognized by plants to trigger innate response. The host sensing of cell free lysate from *S.* Typhimurium was monitored and shown with human cells, wherein cell free lysates from *Salmonella* were recognized by the hosts leading to caspase-1-mediated proteolytic cell death contributing to pathogen clearance (Shivcharan et al. 2018).

Interestingly, the heat-killed CFL treatment showed similar stomatal aperture as seen previously with live *S.* Typhimurium cells, suggesting that host sensing for innate stomatal defense requires secreted exo-metabolites which were not thermally stable. The studies pertaining to how *S. enterica* suppresses plant innate stomatal defense response is not known. The recent study has shown that under high relative humidity conditions *S. enterica* SL1344 but not E. coli O157:H7 suppresses stomatal defense response in Romaine compared to Spinach (Roy and Melotto, 2019). However, through the current study it is evident that the host defense suppression and host sensing are two different layers of innate immune response that follow invasion of *S. enterica* in lettuce.

***S. enterica* T3SS components regulate both host sensing and innate stomatal defense response in lettuce:**

To further test the hypothesis that *S.* Typhimurium can modulate the plant innate defense response several T3SS mutants of *S.* Typhimurium were tested with the stomatal assay. Our results showed the dependency of both SPI1 and the T3SS of SPI2 (*hilD* and *sseB*) in suppressing stomatal defense response at early time points of the exposure of *S.* Typhimurium. The mutants (*hilD* and *sseB*) are defective in SPI1, and an export product of SPI2 respectively. A lack of *hilD* lead to stomatal closure at time points consistent with that observed in *L. monocytogenes*-treated plants with confocal microscopy. SseB is the chaperone target of SseA, *sseA* was not observed to cause stomatal closure like that of *sseB* which induced strong stomatal closure at 3 h post inoculation. The data shows that *S. enterica* may need both SPI1 and exported products of the SPI2 to fully subvert the basal immunities of *L. sativa*. Internalization assays showed that while *S.* Typhimurium was very capable at internalizing and surviving on the plant surface concurrently: *fliC*, *invA*, *hilD* and *sseB* did not persist on leaf and no internalized cells were isolated within the 7-day long trial after day 0 sampling. Intercellular transportation and colonization by Salmonella is a pathogenic trait that allows rapid spread and infection in mammalian hosts and is mediated by SPI2 (Löber, et al. 2006). Previous work has shown that *Salmonella* ingress in plant

tissues using natural openings as stomata, trichomes or injured roots (Deering, et al. 2012; Barak, et al. 2011; Barak, et al. 2002). Our studies and that of others showed *S. enterica* T3SS mutants have high morbidity rates on and in plants, suggesting that both SPI-1- and SPI-2-encoded apparatuses are necessary to establish robust proliferation and ingression in plants (Schikora et al. 2011). Using a brush inoculation technique, we didn't observe any hypersensitive response or lesion formation with both WT and T3SS mutants in plants. Schikora et al. (2011), showed a classical HR and lesion formation followed with chlorosis using Salmonella WT and T3SS mutants by using blunt injection technique. It is to be mentioned that a blunt injection technique may bypass both leaf and stomatal defense response in plants. A similar study used tobacco as a model system and showed that wild type Salmonella, but not the T3SS mutant *invA*$^{-}$, were able to suppress the oxidative burst and the increase of extracellular pH after inoculation, suggesting that *Salmonella* actively suppresses plant defense mechanisms using the SPI-1 encoded T3SS (Shirron and Sima Yaron. 2011). Our data showed that treatment with *S.* Typhimurium may keep the stomates open for possible ingression, similarly, Roy et al. (2013), revealed that *S.* Typhimurium treatment of *Arabidopsis* and lettuce leaves triggered reduced stomatal closure as compared with *Escherichia coli* (Roy et al. 2013). Similar to the results presented here, *Salmonella* treated leaves showed stronger stomatal reopening 4 h after bacterial inoculation (Roy et al. 2013, Roy and Melotto, 2019). Our studies show that *Salmonella* may keep the stomates open for longer duration and the suppression of stomatal defense is partly dependent on SPI1 triggered T3SS during the early onset of *Salmonella* exposure with lettuce plants.

ABA has a central role in *Salmonella's* suppression of stomatal defense for ingression:

Stomatal regulation and function are affected by abiotic, biotic and hormonal interactions, and typically, ABA plays an overriding role during stomatal closure (Acharya and Assmann, 2009). Previously, it was shown that beneficial soil microbes may mediate stomatal closure mediated through ABA (Kumar et al. 2012).

Suppression of ABA biosynthetic pathway was critical for Salmonella's suppression of stomatal defense. Supplementation of *Salmonella* with ABA in a co-inoculation experiment showed that *S.* Typhimurium may override ABA's effect to close stomates. In addition, *S.* Typhimurium inoculated plants showed the downregulation of transcript levels of ABA biosynthetic genes. Specifically, biosynthetic genes such as NCED3, and ABA3 were all downregulated post *S.* Typhimurium treatment at 6 h post inoculation. Both ZEP1 and NCED are involved in early steps of ABA biosynthesis (Xiong and Zhu. 2003). ZEP1 regulates the conversion of zeaxanthin to neoxanthin and is reported to be regulated by circadian rhythm (Taylor, et al. 2000), in contrast, the regulation of ZEP1 in roots is regulated under drought conditions (Taylor, et al. 2000). Similarly, the expression of NCED is highly regulated under both biotic and abiotic stress regimes (Xiong and Zhu, 2003). It is also shown that the expression of NCED is fairly quick under abiotic/biotic stress regime (Qin and Zeevaart, 1999). The *S.* Typhimurium treatment to leaves of lettuce led to downregulation of NCED3, but not ZEP1, post 3 and 6 h post inoculation in roots suggesting the inducible nature of some ABA biosynthetic genes. The data suggests that both ABA biosynthesis and translocation may be modulated by *S.* Typhimurium treatment. The ABA modulation by *S.* Typhimurium was independent of T3SS apparatus. Study with an ABA overproducer transgenic line in tomato (Sp5) showed that the decline rate of *Salmonella* on the leaf surface of Sp5 was significantly higher than that of its wild type "Ailsa Craig" (Gu, et al. 2013). In contrast, there was no significant difference for the internal decline rate of Salmonella in between Sp5 and the parental line (Gu, et al. 2013). How *S.* Typhimurium modulates temporal production and translocation of ABA *in planta* needs to be elucidated. There is a tempting possibility that ABA directly may impact the growth of Salmonella in planta, which needs to be evaluated.

Taken together, these observations suggest that *Salmonella* uses T3SS-delivered effector proteins to suppress the immune stomatal defense system. The two-tier system of stomatal innate defense response and host sensing was shown using a CFL from *S.*

Typhimurium (Fig. 2.4A). The study suggests that exo-metabolites from *S. enterica* may be sensed by plants to trigger stomatal defense response.

How *Salmonella* achieves the delivery of effectors across plant cell walls and plant plasma membranes remains unclear. However, numerous phytopathogenic bacteria (like *Pseudomonas, Erwinia* and *Xanthomonas spp.*) are known to utilize T3SS to deliver effector proteins across plant cell wall, indicating that the plant cell wall is not a sufficient barrier to prevent bacteria from effector delivery (Schikora et al. 2011). *S. enterica* remains a very potent foodborne pathogen and food safety hazard for both pre- and post-harvest conditions, and knowledge pertaining to why it is so effective non-host survival can aid growers in producing cleaner and safer food.

Chapter 3

BACILLUS SUBTILIS RETURNS SALMONELLA ENTERICA COMPROMISED LACTUCA SATIVA IMMUNE RESPONSES[2]

Abstract

As climates shift and our population burgeons; new technologies must be employed to keep our food safe and our crops ever more productive. *S. enterica* the third most common food borne pathogen is known to use plants as a vector to ingress and cause food contamination, specifically leafy greens such as lettuce. Over 10 million cases of food borne illness are documented in the United States annually. By utilizing novel biotechnology in the field and at each point in the farm to fork continuum the occurrences of food contamination can be lessened. We have analyzed the efficacy of a Gram-positive *Bacillus subtilis* UD1022 and its ability to circumvent the defenses of the host, that may change *S. enterica*'s ability to ingress in plants. UD1022 promote stomatal closure in lettuce and reduce pathogen ingression and persistence compared to uninoculated lettuce while leaving normal physiological functions unchanged. The next step in our research would be implement field trials on a larger scale to check the effect of UD1022 in the unpredictable environmental conditions presents in a field setting.

[2] Portions of this chapter have been submitted for publishing in Frontiers in Microbiology.

Introduction:

Microbes are of vital importance to all pieces of life, plants and humans alike depend on the nutrients and secondary metabolites provided by many thousands of bacteria genera. Among bacteria are those that are plant pathogenic, destructive diseases that reduce crop worth and food production. New advances in crop production and widespread use of fungicides, pesticides, and organicides to combat the more negatively influencing bacteria plant pathogens has increased disease prevalence by selecting for strains insensitive to the applied chemicals. In the wake of disease, droughts, climate change and phytopathogen chemical resistance some highly destructive bacteria can slip through under noticed and under studied, these bacteria while not phytopathogenic do cause disease in humans and are vectored by plants and animals. Highly prevalent in produce and a common cause of food poisoning is *Salmonella enterica*, a gram-negative rod-shaped bacterium. Annually *S. enterica* can cause 25% of all documented food borne poisoning, and well as a large percentage of outbreaks (Bean, et al. 2015; Dewey-Mattia, et al. 2018). One such outbreak in 1995 resulted in 242 *S. enterica* serovar Stanley infection in seventeen states and Finland, the food found to be contaminated was radish sprouts (Mahon, et al. 1997). In recent years *S. enterica* has caused several outbreaks from papaya, sprouts, and melons but can contaminate many fruits and leafy greens as well.

Food borne pathogens can be transmitted to plants through diverse routes such as root contact or leaf contact, or indirectly through irrigation water, pesticide diluent, and biosolids such as manure and treated waste products. (Berger, et al. 2010; Monaghan and Hutchison. 2012). Populations can survive in the soil and therefore may infest more than a single crop (Barak and Anita S. Liang. 2008). *S. enterica* itself can be located both as an inhabitant of the rhizosphere and or an inhabitant of the phyllosphere (Deering, et al. 2012; Barak, et al. 2002; Zheng, et al. 2013; Jablasone, et al. 2005). Research into the mechanism of survival and infestation is ongoing and contains a relatively new data that portrays *S. enterica* as facultatively plant pathogenic.

The phytopathogenic ability of *S. enterica* is not constant and may depend on strain variability and experimental procedure. *S. enterica* was shown to induce pathogenic like symptoms in *A. thaliana* and *N. benthamiana*, but also at different times positively induced the immune system of both plants (Shirron and Sima Yaron. 2011; Schikora, et al. 2008; Meng, et al. 2013; Garcia, et al. 2014b). Pathogenic traits were also observed in leafy greens, specifically lettuce, but with no establishment of symptoms normally observed (chlorosis, wilting) (Markland, et al. 2015). Plant genotype and permissible conditions appear to influence *S. enterica* pathogenesis, secondly some plants that are commonly infested (tomato, melon) have not been examined for susceptibility to *S. enterica* induced disease. As a pathogen direct suppression of the immune system could improve *S. enterica*'s ability to persist both epiphytically and endophytically.

We now know *S. enterica* relies on light and chemotaxis to find ingression points such as stomata (Kroupitski, et al. 2009). Wild type *S. enterica* can both suppress stomatal closure as well as general immune responses in model organisms, and at the same time this suppression allows increased endophytic populations (Shirron and Yaron. 2011; Schikora, et al. 2011; Garcia, et al. 2014b; Garcia and Hirt. 2014). To combat growing concerns regarding the safety of fresh and healthier produce foods, non-conventional methods have been researched but are yet to be deployed in the field. Once such device is using beneficial bacteria called PGPRs as a middleman for the plant immune system.

PGPRs have been used for disease resistance and plant growth promotion and are sold as consortiums of one or several strains possibly in conjunction with a Rhizobium species. The PGPR UD1022 is one of these commercially available strains (Bais, et al. 2014). UD1022 has been previously shown to reduce *S. enterica* and *L. innocua* populations (Markland, et al. 2015). This report strengthens evidence for its use as a potential preharvest treatment. UD1022 does not cause stress to the plant after initial association, and can promote both stomatal closure in the presence of both plant

diseases and food borne pathogens at earlier time points (Kumar, et al. 2012; Markland, et al. 2015).

This chapter will illustrate a model for using PGPRs as a food safety initiative to lower rates of contamination but will require further research into how it can be applied in the field. *S. enterica* is presented as a model organism because of its abilities to use plant hosts as vectors. The procedure of using benign microbes as a biocontrol can be applied to any food borne pathogen that is plant associated, most important of these is *E. coli* O157:H7 which was isolated in multistate outbreaks in 2018 years as well as the highly fatal *Listeria monocytogenes* which caused a fatal outbreak in 2012 (The Center for Disease Control. 2018; the Center for Disease Control. 2012).

Methods.

Plant growth methods:

Lactuca sativa Variety Black Seeded Simpson was used for all experiments. Plants used for microcopy and stress testing were grown in aseptic magenta boxes containing autoclaved hydroponic clay and a modified Sonneveld Solution (Mattson and CARI Peters. 2014). Before cultivation lettuce seeds were stratified for 48 hours at 4 ℃ on a damp paper towel, the seeds were then washed in 25 mL 50% bleach for 8 minutes and not longer and then rinsed with 3 aliquots of 25 mL sterilized nano water. The plants were placed in a reach in growth chamber at 25 ℃ for 12 hours and an average PAR of 200-230 followed by a 12-hour dark period at 20℃. The plants were grown for 2 weeks before being moved under a 300-350 PAR light grid at room temp for 24 hours to fully open stomata.

Bacterial culture methods:

All bacterial strains were kept at -80℃ freezer in 20% glycerol for long term storage. Prior to use each bacterial strain was streaked onto a complex-media (Tryptic Soy or Luria-Bertani) containing necessary antibiotics where applicable (Table 2.1). Each plate was incubated for 16-24 hours at 30 $^{\circ}$C, and re-streaked from the glycerol stocks as needed. Before experimental use, a single colony from solid media was moved into liquid media via sterile loop technique and incubated at 30 $^{\circ}$C overnight on an orbital shaker at 200 rpm. Following incubation liquid bacterial cultures were aliquoted into conical tubes and centrifuged for 15 minutes at 4000 rpm and washed twice with 25 mL PBS buffered to a pH of 7.4, followed by a final suspension in PBS. The optical density was measured at 600 nm with a Biorad SmartSpec+ and adjusted to the working concentration of 10^7 CFU mL^{-1} in sterile DI water in a new sterile flask or test tube.

Stomatal assay: Aseptic lettuce plants contained in magenta boxes were inoculated with 10 mL of 10^5 CFU/mL UD1022 in nano water for a volumetric count of 20000 CFU/mL media. Inoculated magenta boxes were returned to the growth chamber for 1

day followed by a 24 hour photoperiod under grow lights at room temp (PAR=300-350) Following the light adaptation phase the most recently mature leaf from each plant was brushed with a suspension of *S.* Typhimurium, *sseB, hilD*, or water. Leaves were imaged at 0 hours, 3 hours, 6 hours and 12 hours post inoculation. For each time point 3 plants were used for imaging and 40-60 stomata were measured per leaf.

Imaging parameters:

Before imaging each leaf was staining with 5 nM propidium iodide for 5 minutes, from the stained leaf, two small circles were removed with a potato corer. The stained leaf cuts were placed under a glass block adaxial side down on chambered cover glass. The leaf subsamples were imaged directly following this step and no set of leaves were used after an hour. At each time point 3 plants were used for imaging. Experiments were replicated when needed on non-adjacent days with an entirely new sets of plants.

Persistence and internalization of *S. enterica* mutants lacking Type-III secretion system (T3SS) virulence factors and flagellar genes in lettuce grown under greenhouse conditions.:

2-week-old lettuce plant leaves were spot-inoculated randomly with 120 µl (6 droplets) of respective *S. enterica* culture on the leaf surface. Inoculated leaves were placed in the growth chamber for 2 h, to facilitate bacterial attachment to leaves. Samples were collected for each treatment group separately and processed to enumerate surviving *S. enterica* populations on day 0, 1, 3, 5, and 7. A sample of 6 plants (total of 12 leaves in each) was collected and split into two equal sections for bacterial enumeration and pathogen internalization assay. To enumerate, leaf samples (6 leaves) were weighed in individual Whirl-Pak™ bags (Nasco, Fort Atkison, WI, USA) and submerged in 0.1% buffered peptone water (BPW; Oxoid Ltd., Basingstoke, Hampshire, England) in a 1:9 ratio. The sample was mixed for 2 min at 230 rpm in a stomacher (BagMixer® 400 S, Interscience), the resulting mix was serially diluted in 0.1% BPW and plated on TSA with antibiotic (Table 2.1), or by a mini-MPN method (Sharma et al. 2016). *S. enterica* colonies were counted after 22-24

h of incubation at 37ºC. Persistence and Internalization data gathered by Kniel Lab at the University of Delaware.

Results.

UD1022 improves defense responses in the presence of *S. enterica* serovar Typhimurium and its mutants:

To quantify the defense response to a leaf inoculated food borne pathogen we first inoculated UD1022 48 hours prior to the pathogen, thus mimicking a transplant water inoculation occasionally used for bioinoculants. A 48-hour window was provided to allow UD1022 to associate with the roots and for stomatal to reopen after brief closure in response to initial UD1022 associated, a time frame that was determined via trial and error as well as followed by a 72 hour UD1022 leaf assay that showed initial closure was corrected by 48 hours, this was also observed in Kumar et al., (2012) (Fig. 3.1).

After the 48-hour time period, which included 24 hours to light adapt the plants either *S.* Typhimurium, *sseB,* or *hilD* was brushed on a leaf at a rate of 10^6 CFU/mL. For imaging two circles were removed from the inoculated leaves and stained in propidium iodine before confocal microscopy. As expected, stomata of UD1022 treated plants did not open 3 hours post inoculation and remained closed for up to 12 hours (Fig. 3.1). An identical response was observed for both *sseB* and *hilD*, the strongest response was measured on those plants treated with *hilD* (Fig. 3.1) Nonetheless all three treatments were statically similar, and different by from the control water inoculated UD1022 treated plants and plants not inoculated with UD1022.

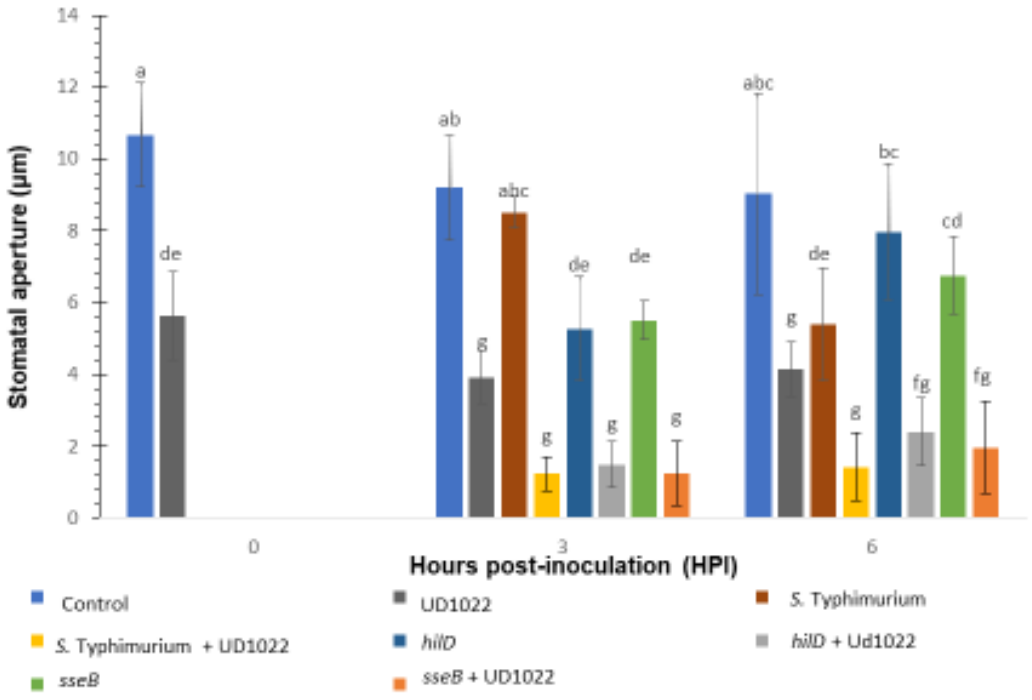

Figure 3.1 Stomatal aperture response in lettuce roots treated with UD1022 48 hours or untreated before inoculation with water (control), *S.* Typhimurium WT, and its mutants on the leaves. Different letters signify a difference at $p<.05$. Average stomatal aperture was found by measuring 40-60 stomates from three independent plants.

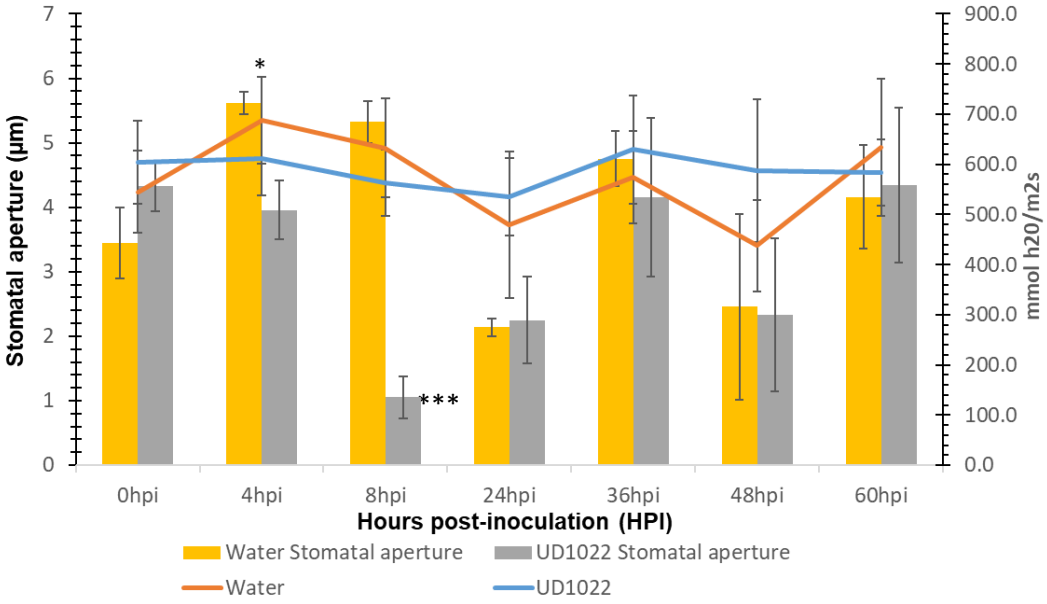

Figure 3.2 Graph showing stomatal aperture width under relative stomatal conductivity of UD1022 treated roots or water treated roots. Differences were only observed at early time points 4 hpi and 8 hpi for stomatal aperture width. Only 4 hpi showed a difference in stomatal conductivity. (*=p<.05, ***=p<.001).

UD1022 inoculation does not cause physiology synonymous with stress in lettuce: By using a combination of stomatal measurements, leaf porometer measurements it was concluded that UD1022 did not adversely change the plants biochemistry and efficiency. As previously shown by (Kumar, et al. 2012) UD1022 causes closure of stomatal for up to 36 to 48 hours but the plants remain attuned to the circadian rhythm and stomata close further during a dark cycle (Fig. 3.2). Only at early time points (8 hours post inoculation) was a significant difference observed between the controls and UD1022 treated plants. An effect that was corrected by the following days light cycle (36 hours post inoculation) A similar change occurs with regards to stomatal conductivity which is used to extrapolate total transpiration with minimally damaging a plant. Plant stress can cause reduced growth, less nutritious food and makes a plant more susceptible to disease.

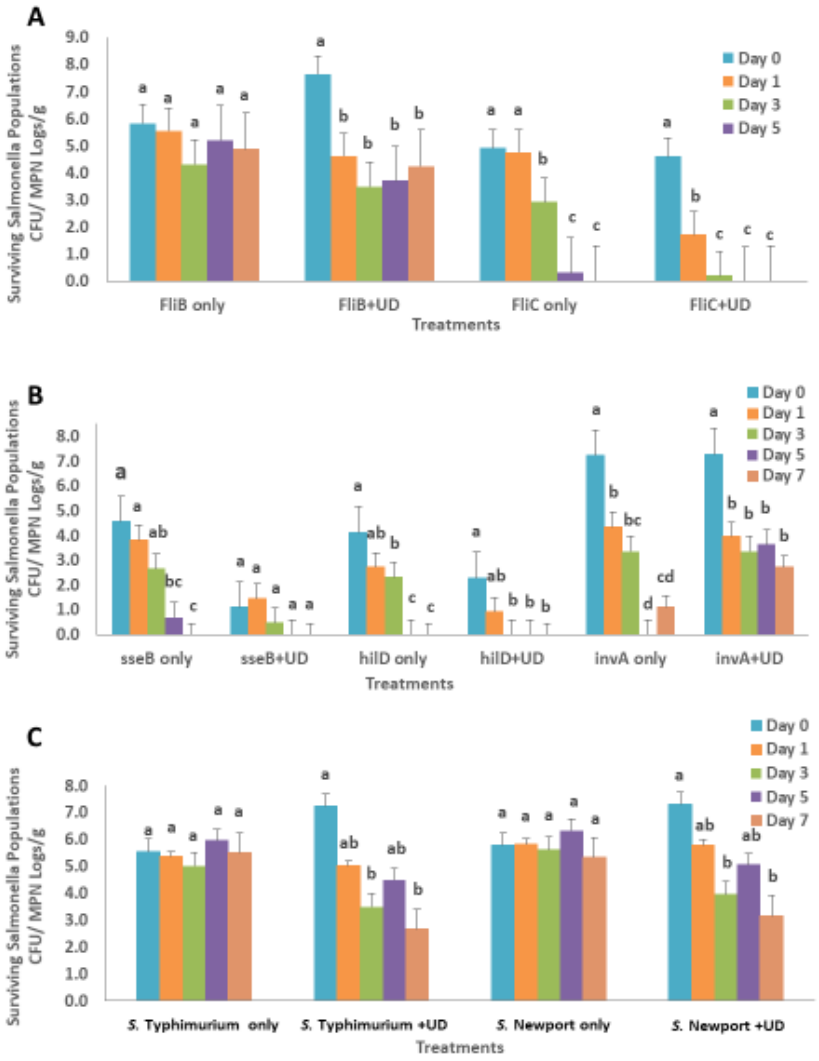

Figure 3.3 (a-c). *Salmonella* Typhimurium survival and persistence on lettuce surface with or without UD1022 (a) flagella gene mutants (b) pathogenicity island genes mutant and (c) wild type and S. Newport controls *Values represent the average of three replications. Standard deviation (±) for surviving S. Typhimurium population (Log10 CFU or MPN/g) follows mean value. Letters a, b, c show statistically significant differences ($p < 0.05$) between sampling days for the same strain.

UD1022 lowered *S. enterica* survival and prevented internalization:

Overall *Salmonella* populations were significantly ($p<0.05$) reduced in all UD1022 treated groups by day 3 except *fliB* and *invA*, compared to the wild type (Fig. 10A-C). The *fliB* and *invA* showed significant ($p<0.05$) decrease in pathogen survival on day 1 (4.6 and 4.0 logs CFU/g, respectively) compared to day 0 (7.7 and 7.2 CFU/g, respectively). Other mutant's *fliC, hilD,* and *sseB* were reduced to undetectable levels in UD1022 treated plants compared to wild type strain (3.5 logs CFU/g), by day 3 (Fig 3.3A and 3.3B). It is noteworthy that in *hilD,* and *sseB* groups had significantly ($p<0.05$) lower (2.3-1.1 logs CFU/g) levels of mutant's colonization compared to the wild type (7.2 logs CFU/g) strain, on day 0 (Fig 3.3B and 3.3C). Whereas the same mutants (*hilD,* and *sseB*) showed higher colonization on day 0 in the lettuce plants which were not treated with UD1022, suggesting an interaction between UD1022, these mutants and plant itself through a signal exchange to prevent bacterial attachment and internalization. In control groups, wild type *S.* Typhimurium and *S.* Newport populations decreased significantly ($p<0.05$) to 3.5 and 3.9 logs CFU/g, respectively, by day 3, compared to day 0 (7.2 and 7.3 logs CFU/g, respectively) (Fig 3.3C).

A study showed that plant growth promoting rhizobacteria *Bacillus amyloliquefaciens* (SN13) can effectively control a pathogenic plant fungus i.e., *Rhizoctonia solani* in-vitro and in Rice plant (Srivastava et al., 2016). Authors suggested that the rhizobacteria can elicit immune response in plant by modulating metabolic and biochemical pathways, which could play an important role in protecting plant against infection and pathogens. Similarly, in the current, plants primed/ inoculated with UD1022 displayed decreased internalization incidence in *Salmonella* mutants and controls, suggesting that rhizobacteria could be of importance to plant against human pathogen. Moreover, low levels of bacterial colonization observed in SPI mutants (*hilD,* and *sseB*) inoculated plants along with UD1022 indicates that these genes may play a critical role to override signal transmission in plant against *Salmonella*. In the absence of these genes, the plant growth promoting rhizobacteria

UD1022 was able to express signals and plant immune response with decreased internalization and surface colonization. A study by Hsu and Micallef (2017) showed a similar result, where the plant growth promoting rhizobacteria i.e., *Pseudomonas* strain S2 and S4 significantly reduced *S.* Newport population on spinach, lettuce and tomato surface. The authors evaluated the wild type strain and suggested that increased leaf nitrogen content might have limited pathogen survival on the surface. Whereas, the current study revealed the effect of *Salmonella* virulence genes and their interactions with rhizobacteria on its survival and persistence on lettuce surface.

Discussion.

Biotechnology continues to create novel uses for bacteria, fungus, and similar biological entities. Bacteria referred to as Plant Growth Promoting Rhizobacteria can be used to improve drought tolerance, disease resistance, over-all growth, and/or hormone synthesis (Zheng, et al. 2018; Bhattacharyya and Jha. 2012; Bais, et al. 2014; Kumar, et al. 2012). In this study a novel use for one such bacteria were researched for use in preventing or reducing the presence of the food borne pathogen *Salmonella enterica.* These Gram-negative bacteria causes 93.7 million documented cases of food poisoning every year worldwide (Majowicz, et al. 2010).

As discussed in chapter two of this text *S.* Typhimurium has exhibited plant pathological behaviors which consists primarily suspension of the easily observed stomatal closure which is triggered by various MAMPS and PAMPS (Fig. 2.1, Fig. 2.3A). Stomatal closure can prevent ingression through the leaf and is only the first step in a cascade of physiological events that both kill off "invaders" and can trigger patterned cell death (PCD) that restricts further movement in the plant. Initial closure of stomata is not observed in lettuce treated with *S.* Typhimurium but is observed when lettuce was treated with either SPI1 or SPI2 mutants *hilD* and *sseB* respectively (Fig. 2.1, Fig. 2.2). Stomatal closure induced by PTI is only transient, and stomata reopened by 12 hours post inoculation.

To further analyze nonconventional uses for UD1022 we hypothesized that UD1022 could be utilized to prevent stomatal response suppression and can mediate stomatal closure following a *S.* Typhimurium inoculation, thus either slowing or preventing both *S.* Typhimurium ingression and epiphytic survival. An application of UD1022 48 hours prior to pathogen exposure allowed plants to fully associate with the PGPR and then recover from transient closure observed in this work and Kumar et al. (2012). Following the 48-incubation period leaf inoculated *S.* Typhimurium resulted in prolonged and rapid stomatal closure for plants treated with WT, *hilD*, or *sseB*. UD1022 mediated closure resulted in tightly shut stomata (aperture width near 0

microns), stomatal closure was observed until 12 hpi when the experiments were stopped.

The improved responses by UD1022 treated plants indicate that UD1022 can mediate stomatal closure through multiple pathways, such as SA or ethylene to improve resistances. We know this WT *S.* Typhimurium can target the ABA biosynthesis pathway which is crucial for stomatal closure after PTI induction (Fig. 2.6 and Fig. 2.7).

Salmonella can survive for several weeks as both an epiphyte and endophyte and the benefits of adding UD1022 were examined for overall survivability and internalization of *S.* Typhimurium, *S.* Newport, and *S.* Typhimurium mutants. Following inoculation, the population of *S.* Typhimurium dropped by 2 log CFU/gram one day after inoculation, and populations were significantly different from day zero on days three and seven. A similar trend is present on plants treated with *S.* Newport and UD1022. UD1022 doped plants treated with *S.* Typhimurium 14028s mutants *hilD* and *sseB* had very low persistence on the leaf surface, with no recovered bacteria on days five and seven (Fig. 3.3B). We see that UD1022 will reduce overall population of *S. enterica* strains and mutants. One odd occurrence is the improved survival rate of *invA* in UD1022 primed plants, we can speculate that despite lacking this gene another is influencing the overall immune system in lettuce which returns it to a near WT response. This was not observed in non UD1022 primed plants, which also responded by having a middle amount of stomatal closure (Fig 2.4), this indicated that InvA may be responsible for some amount of immune suppression, but *S. enterica* relies on more than one exported product to the plant for total immune suppression.

The dual validation that UD1022 both effectively closes stomata in response to *S. enterica* and reduces total external populations on lettuce by 3 days supports that it could potentially be used as a biocontrol for preharvest protection against food borne pathogens. Effectiveness against food borne pathogens infesting a plant from the root zone is not determined and will be examined in the future.

Chapter 4

SUMMARY

Food borne illness can cause up to half a billion documented cases of illness each year, in the United States this number is 9.4 million, which will no doubt increase as the global population burgeons past sustainable levels (F1000 Research Ltd, 2015; Scallan et al. 2011). The Gram-negative bacteria, *Salmonella enterica* is a well-adapted enteric pathogen. The strain used in the present study *Salmonella enterica* serovar Typhimurium 14028s is shown to overturn plants innate response by keeping the stomates open compared to another opportunistic pathogen such as *Listeria monocytogenes*. By applying *S.* Typhimurium mutants *hilD* and *sseB* to the identical stomatal assay we showed that *S.* Typhimurium required both functional pathogenicity islands (SPI-I and II) in order to fully suppress the immune system in lettuce. A single other mutant *invA* induced a level of stomatal closure between WT *S.* Typhimurium and *S.* Typhimurium mutants *hilD* and *sseB*. In persistence and internalization assays *S.* Typhimurium 14028s was proven to survive better than both *hilD* and *sseB*, and only *S.* Typhimurium and a single flagellin mutant *fliB* could penetrate the leaf epidermis and persist internally but did not have any quantifiable reproductive ability. *S. enterica* being a human pathogen has not shown to be able to inject effectors through the considerably thicker plant cell membrane and cell wall.

Despite lack of evidence that *S. enterica* can inject effectors into the plant cell, we speculate with reasonable assurance that *S. enterica* disrupts certain plant functions and requires both *Salmonella* pathogenicity islands (SPI-I and II) in order to completely suppress the basal immune system of lettuce. To evaluate what genetic and hormone pathways were disrupted *S.* Typhimurium was co-inoculated with either salicylic acid (SA) or abscisic acid (ABA). Plants treated with both ABA and *S.* Typhimurium did not have positive stomatal closure that those co-inoculated with SA. The stomatal closure in SA co-inoculated was statistically greater at 6 and 12 hpi compared to SA alone. This suggests a positive PTI and or systemic required

resistance leading to long term protection to plants that are attacked repeatedly by the same pathogen in this case *S.* Typhimurium. Further evidence that the ABA pathway is disrupted was evaluated through gene expressions. The gene expression data showed that two key ABA biosynthesis genes ABA3 and NCED3 were downregulated at 6 hours post inoculation.

Future goals will be to research the efficacy of UD1022 cellular biproducts in the form of a culture filtrate which would remove the need for live bacteria in a hydroponic or controlled environmental system such as vertical farming.

Future goals regarding *S. enterica* physiology and plant pathogenicity will be to locate and identify the effectors causing immune suppression in terms of reduced stomatal defense response. Identification of traits such as stomatal defense response may lead to further advances in understanding how food borne pathogens ingress in non-host plants like lettuce and spinach. The use of reporter cell lines and more resolved imaging could lead to incite as to the functionality of the T3SS in opportunistic food borne pathogens to invade the non-host plants.

The implications to food safety of the above discussion is exacerbated by the increasing rate of fresh food consumption. To facilitate plant protection against these food safety threats we proposed a relatively novel use for plant growth promoting rhizobacterium, specifically the *Bacillus subtilis* strain UD1022. The benefits of this and other PGPR strains has been shown to work efficiently against plant pathogens as a biocontrol. PGPRs after colonization of the roots and biofilm formation can provide broad spectrum protection to disease and improve abiotic stress management as well. The strain in used for this study, UD1022, can be utilized as a seed treatment or soil bioinoculant, and we have shown it could also potentially be used in hydroponic systems. For the work completed here a soil amendment and hydroponic system addition was used. The results were all positive, as an increased immune response was observed through confocal microscopy in plants grown hydroponically and inoculated with UD1022 and subjected to a leaf inoculated *S.* Typhimurium. In a soilless media plants that were inoculated with UD1022 were better able to reduce the external

populations of *S.* Typhimurium compared to control treatments with *S.* Typhimurium that maintained populations of 5 log cfu/g over 7 days. In these experiments no internal populations of *S.* Typhimurium were observed, which has better implications for food safety as epiphytic cells are much simpler to remove with proper sanitation steps. UD1022 shows promise as a preharvest preventative measure against general *S. enterica* and potentially as well as other food borne pathogens.

Future goals will be to research the efficacy of UD1022 cellular biproducts in the form of a culture filtrate which would remove the need for live bacteria in a hydroponic or controlled environmental system such as vertical farming.

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