PLANT IMMUNE DEFENSES AGAINST THE HUMAN PATHOGENS *ESCHERICHIA COLI* O157:H7 AND *SALMONELLA ENTERICA*

by

DEBANJANA ROY

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Abstract

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Debanjana Roy, PhD

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Supervising Professor: Maeli Melotto

Several pathogenic and non-pathogenic microorganisms reside on plant leaf surfaces while bacteria being the most abundant of them all. Often bacteria not only use the plant leaf surface as their habitat but also penetrate through stomatal pore on leaf epidermis to survive in the plant's intercellular space also known as the apoplast. To fight against infection caused by bacteria plants rely on their innate immunity system which consists of pathogen-triggered immunity (PTI) and effector-triggered immunity (ETI). PTI is considered to be the first line of defense response and it is effective against a broad range of pathogens. Nonetheless, some microbial species are able to overcome and /or avoid plant defenses and colonize the apoplast. In particular, there have been frequent cases of association of human pathogens, such as *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium SL1344, with fresh fruits and vegetables that can cause illness or death among consumers who eat contaminated produce. The economic consequences of produce-associated outbreaks including medical costs, damage control cost, produce recall cost, and production time are substantial. At this point the underlying mechanism of this intriguing phenomenon of human pathogens and plant interaction is not well understood and this is the area of my study. In the first chapter of my dissertation I introduce the newly developed field of human pathogen on plants following innate immunity of plant against human pathogens in the second chapter.

In the third chapter, the defense pathway(s) playing significant role in diminishing human pathogens populations in plant tissue is explored. Laying emphasis on these puzzling areas of plant pathology, my study will contribute to understanding the initial contamination process, effect of environmental factors on human pathogen infection of plants, and important defense pathway(s) in plants which are paramount in preventing food borne diseases.

Table of Contents

List of Illustrations

Chapter 1

Introduction to Human pathogen and plant interactions

Chapter 1 was formatted and published in Frontiers of Microbiology (Melotto, M., Panchal, S., and Roy, D. 2014. Plant innate immunity against human bacterial pathogens. Front. Microbiol. 5:411. doi: 10.3389/fmicb.2014.00411).

1.1 Overview

Resent concern about microbial contamination of food and fresh produce which results in foodborne illness is the main focus point of chapter1. The consistent rise in the number of foodborne illness outbreaks which are linked to fresh fruits and vegetables are new challenges indicating enteric pathogens association with plants. Several reports have showed that though not known as plant pathogen, few human pathogens can colonize plants at pre and post-harvest stages. These findings have encouraged researches, new collaborations and connections between the scientific communities of plant pathology and food safety. This chapter will provide a comprehensible overview about the current interest of so-called human pathogens on plants (HPOPs). In this part of my thesis I will be focusing on human pathogens such as *Salmonella enterica* and *Escherichia coli*, which are not recognized as plant pathogens (Barak and Schroeder, 2012; Meng et al., 2013) but still major threats to fresh produce quality and human health.

1.1.1 *Arabidopsis is an established model plant for studying plant-pathogen interaction*

Usage of *Arabidopsis thaliana,* which is a member of cruciferous (mustard) family, is very common in plant pathology. This is considered to be a powerful tool because of its small size, short generation time, high efficiency for transformation (Meyerowitz and Somerville, 1994) and completely sequenced genome (Arabidopsis Genome Initiative, 2000) which facilitates positional cloning and reverse genetics.

Additionally, sequenced-indexed T-DNA knockout collections, several published mutants, DNA and large-scale gene profiling analysis data availability (http://www.arabidopsis.org) provide unparalleled advantages for using this plant as a model to study various basic cellular pathways.

After Arabidopsis was established as a model plant to study host-pathogen interactions in the late 1980s (Whalen et al., 1991; Dong et al., 1991; Debener et al., 1991), the molecular mechanism underlying plant immunity against pathogens was intensely studied. Due to all that above mentioned reasons, this plant is a good model to study plant-human pathogen interaction which is extremely crucial to prevent food borne diseases and its long term consequences.

1.1.2 *Salmonella enterica and Escherichia coli can be used to study plant-human pathogen interaction.*

Salmonella enterica is a Gram-negative pathogen which is known to cause infection in animals and humans. *S. enterica* serovars are divided into typhoidal and non-typhoidal and the *S. enterica* serovar Typhimurium used in this study is non-typhoidal and causes abdominal pain, gastroenteritis, vomiting and inflammatory diarrhea in humans and animals. Consumption of contaminated food or water leads to the *Salmonella* related infection.

E. coli is also a short Gram-negative, rod-shaped bacterium that is a normal inhabitant of the lower gastrointestinal tract of warm-blooded animals. In addition to thriving in the colon, *E. coli* can also survive outside the body and can be spread through feces. Being a single cell organism which is able to grow very rapidly and variable growth conditions this organism was also found to be strive inside plant tissues.

Plant pathology was mainly the study of plant diseases, host plant defense system and disease management strategies. The focus is mainly to minimize plant death due to diseases and its impact on global economy. Recently along with plant pathogens, plant commensals and plant beneficial bacteria, these bacteria also found to be associated with plants although plants are not natural hosts for human enteric pathogens. It's still unknown if plants are host, vector or reservoir for human pathogens. But recurring occurrence of outbreaks leading to human illness and death have broadened the research opportunities of plant pathology where HPOP is being studied for a comprehensive knowledge about these human enteric pathogens and plant interaction.

Fruits, vegetables, fresh salad are sold in market labeled ''pre-washed'' or ''ready to eat'' and are often eaten uncooked. Enteric pathogens like Shiga-toxin producing *E. coli* and *Salmonella* spp. can spoil those foods by silently being present inside the plant tissue. Between year of 2000 to 2008 *Salmonella* spp. and norovirus contributed to 11% and 58% foodborne illness respectively in the United Sates (Scallan et al., 2011). Non-typhoidal *Salmonella* alone contributed to 35% hospitalization and 28% deaths and was ranked to be the topmost bacterial pathogen contributing to human illness (Scallan et al., 2011). Not only direct effects on human health, food contamination by enteric pathogens leads to enormous economic losses due to compromised food quality, less demand of fresh produce, food recall from retail stores state wise. So, this should be understood that this is not only loss on individual scale but also on a larger scale where numerous growers, workers and distributers get affected. Furthermore, skepticism of general public towards particular foods and less demand of those foods can lead to loss for the food industry as well. Statistics has proved that money spent on foodborne outbreak prevention by producers is much less than the cost incurred after the outbreak (Ribera et al., 2012).

Enteric bacterial pathogens which are found to be commonly transmitted through foods are well adapted to sustain life inside vertebrate host gut. Few have humans as their primary or sole host while others are known to sustain in animal population. Those bacteria which are adapted in animal guts affect human and plants only incidentally. This is why the term Human Pathogen On Plants (HPOP) is a newly coined term to describe cases when these enteric pathogens inhabit, colonize, and interact with plants.

According to several studies and reports food contamination can occur from 'farm to fork' anytime. Pre-harvest and post –harvest steps are equally prone to enteric pathogen contamination and the main causes include contaminated irrigation water, improper personal sanitization of workers, fecal contamination by farm animals (Lynch et al., 2009; Barak and Schroeder, 2012). Post-harvest contamination can occur due to or at some stage of unclean transportation, processing as well as bagging (Lynch et al., 2009). Additionally mechanical damage during harvest or transport can increase the probability of human pathogen entry inside edible parts of plants noticeably (Aruscavage et al., 2008). Food Safety Modernization Act (US food and drug administration) and Hazard Analysis and Critical Control Point system (HACCP) defined control measures to decrease the pathogen load on plant surfaces. US Department of Agriculture (USDA) approved the use of chlorine for post-harvest crop handling though few studies suggested internalized human pathogens can escape sanitization and chlorine treatment (Seo and Frank, 1999; Saldana et al., 2011). Therefore understanding the biology behind human pathogen and plant interaction is essential in order to prevent human pathogen colonization or survival in/on plants, and to integrate further, complementing measures to control food borne outbreaks. As plants are recognized vectors for these pathogens, enhancing the plant immune system against human pathogens increases a unique opportunity to interrupt the pathogen cycle.

1.1.3 *Plant surface is the first barrier for bacterial invaders*

The plant leaf surface thought to be a hostile environment for bacteria as it is exposed to rapidly changing temperature, humidity, UV radiation (Lindow and Brandl, 2003). Pathogens adapted in animal gut are not used to this type of fluctuation in their microenvironment however, the increasing incidence of *S. enterica* and *E. coli* O157:H7 on fresh produce, vegetables, and sprouts indicate a certain level of human pathogen fitness in/on the leaf.

Plants lack an adaptive immune system like other animals but are known to have multifaceted defense mechanisms that protect plants from infection by a diverse population of potential pathogens. The plant surface acts as the first barrier to bacterial invaders by the presence of cuticle, wax, cell wall, trichomes and stomata. On the other hand several bacteria are capable of enduring on leaf surface and eventually penetrate within the plant interior. Nevertheless plant leaf architecture is non-uniform and provides different micro environmental condition as there are bulges and troughs, leaf hair or trichomes, stomata and hydathodes. All these structures form micro sites for bacterial survival on leaf surface with sufficient water and nutrient availability and protection from temperature and UV radiation (Leveau and Lindow, 2001; Miller et al., 2001; Brandl and Amundson, 2008; Kroupitski et al., 2009; Barak et al., 2011). Aggregates of *S. enterica* were found on cilantro leaf surfaces near vein region (Brandl and Mandrell, 2002). *S. enterica* also demonstrated affinity towards abaxial surface of lettuce which showed technique adapted by this bacteria to avoid UV (Kroupitski et al., 2011). Even conversion of *E. coli* O157:H7 cells to viable but non-culturable (VNBC) on lettuce leaves might also be a strategy to escape hostile environmental conditions on leaf surface (Dinu and Bach, 2011). All these strategies of human pathogens to localize to favorable micro sites on leaf surface, escaping harsh environment, survival by

aggregation or conversion to non-culturable state indicate their suitability to survive and at times multiply on leaf surface.

Stomata, abundant natural pores on plant leaf surfaces and embedded in plant leaf epidermis serve as entrance points for several bacteria to colonize the leaf interior such as apoplast, xylem, and phloem. Populations of *E. coli* O157:H7 and *S. enterica* are found to be as large as 4logs per cm^2 of leaf inside plant leaf apoplast of Arabidopsis after surface inoculation under 60% relative humidity (Roy et al., 2013) suggesting that those bacteria are capable of entering plant interior through intact leaf as well. Other microscopy studies also showed *S. enterica* serovar Typhimurium SL1344 internalizing iceberg lettuce and arugula through stomata which indicated and demonstrated association of pathogens on or near stomata specifically guard cells (Golberg et al., 2011). Cells of *S. enterica* serovar Typhimurium MAE110 (Gu et al., 2011), enteroaggregative *E. coli* (Berger et al., 2009b), and *E. coli* O157:H7 (Saldaña et al., 2011) were found to be associated with stomata in tomato, arugula leaves, and baby spinach leaves, respectively. *E. coli* O157:H7 and *Salmonella* serovar Typhimurium were even found in the stem hypocotyl as well as in other stem tissues such as epidermis, cortex, vascular bundles and pith when seedlings were germinated from contaminated seeds (Deering et al., 2011a, b). Not only leaf and stem, nutritionally enriched root exudates was seen to attract *S. enterica* to lettuce roots (Klerks et al., 2007). Although *S. enterica* and *E. coli* O157:H7 cannot directly penetrate through root cells, root cracks and sites at the lateral root emergence provide ports of entry for bacteria into root tissues (Cooley et al., 2003; Dong et al., 2003; Klerks et al,. 2007b; Tyler and Triplett, 2008), sometimes between epidermal cells and often *S. enterica* in the root- shoot transition area (Klerks et al., 2007 b). Once internalized both bacterial pathogens are found to be in the intercellular space of root outer cortex of *Medicago*

truncatula (Jayaramna et al., 2014) and *S. enterica* alone was found to be present in the parenchyma, endodermis, pericycle, and vascular system of lettuce roots (Klerks et al., 2006) as well as inner root cortex of barley (Kutter et al., 2006). An extensive study on *E. coli* localization in plant root showed this bacterium can colonize plant cell wall, apoplast and cytoplasm (Wright et al., 2013). Surprisingly most studies demonstrated *E. coli* O157:H7 to be better capable of localizing the intercellular region inside plants rather than intracellular. These observations lead to a speculation that bacterial transmission from roots to the phyllosphere may be a result of either bacterial migration on the plant surface in a flagellum-dependent manner (Cooley et al., 2003) or through the vasculature (Itoh et al., 1998). However the mechanism of enteric pathogen movement from root cortex to the root vascular system through the endodermis and casparian strips and their movement from roots to phyllosphere through vasculature is yet to be demonstrated.

Numerous outbreaks of *S. enterica* had also been associated with bacterial contamination of fruits though *S. enterica* is tested to be unlikely to survive on the surface of intact fruit (Wei et al., 1995). So, the routes of this bacterium inside fruit are still a matter of question. Nevertheless is was suggested that *Salmonella spp.* can travel from inoculated leaves (Barak et al., 2011), stems and flowers (Guo et al., 2001) to tomato fruits. The phloem was predicted to be the probable route of movement of bacteria to non-infected or non-inoculated parts of the plants as microscopy successfully detected bacterial cells in those parts (Gu et al., 2011).

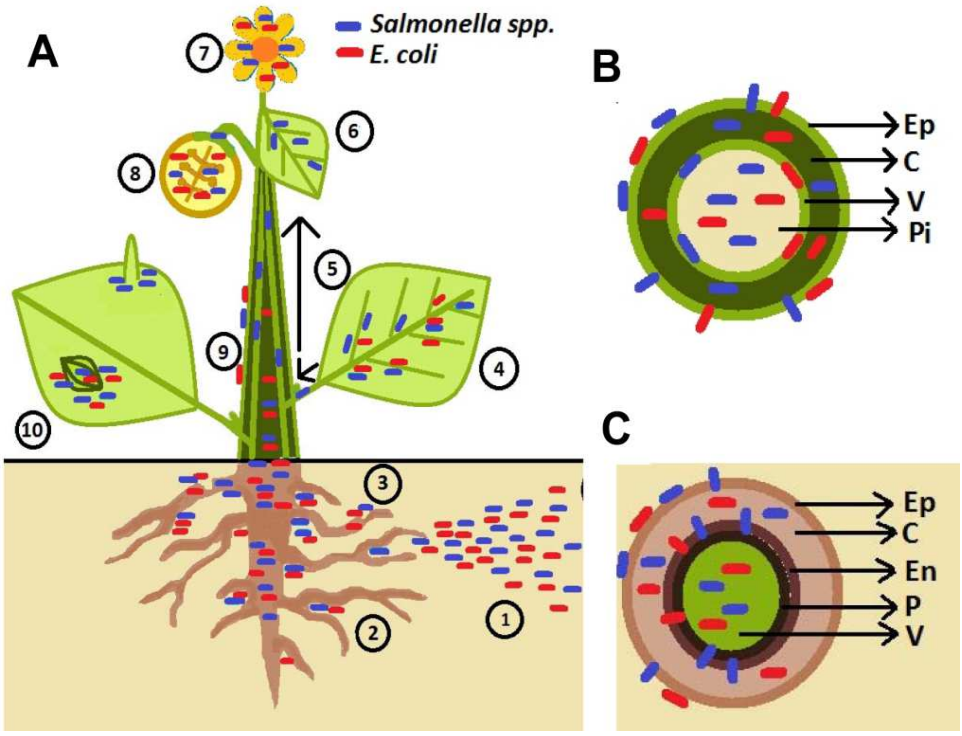

Figure 1.1 Diagrammatic representations of human pathogen (HP) interaction and colonization in plants. **A**. Pathogen source to soil is through contaminated irrigation water, fertilizers, pesticides and manure (1). HPs are attracted towards rhizosphere (**2**; Klerks et al., 2007a) and use the sites of lateral root emergence, root cracks and root-shoot transition area to infiltrate root, (**3**;Cooley et al., 2003; Dong et al., 2003; Klerks et al., 2007b;Tyler and Triplett,2008). HPs were observed to survive on the leaf surface close to veins (Brandl and Mandrell, 2002), in the leaf apoplast (intercellular space)(Brandl and Mandrell, 2002; Solomon et al., 2002; Niemira, 2007; Kroupitski et al., 2009; Barak et al., 2011; Dinu and Bach, 2011; Gu et al., 2011; Roy et al., 2013), and at times their affinity for abaxial side of leaf was also noticed (e.g., *S. enterica*; (Kroupitski et al., 2011) (**4**). *Salmonella enterica* Typhimurium can penetrate tomato plants *via* leaves and move through vascular bundles (petioles and stems) (**5**) into non-inoculated leaves (**6**) and fruits (**8**) (Gu et al., 2011). Association of HPs with flower was also found (**7**; Guo et al., 2001; Cooley et al., 2003). *Salmonella* showed the ability to travel from infected leaves (**4**), stems (**5**), and flowers (**7**) to colonize the fruit interior (the diagram represents across-section of a fruit)and fruit calyx (**8**) Guo et al., 2001; Janes et al., 2005; Barak et al., 2011. *Escherichia coli* O157:H7 has also been observed in the internal parts of the apple and the seeds following contamination of the flower (**8**) (Burnett et al., 2000). Movement on the plant surface has also been observed (**9**) (Cooley et al., 2003).

Epiphytic *Salmonella* and *E. coli* O157:H7can aggregate near stomata and sub-stomatal space (**10**; Shaw et al., 2008; Berger et al., 2009a,b; Golberg et al., 2011; Guetal., 2011; Saldaña et al., 2011), reach the sub-stomatal cavity and survive/colonize in the spongy mesophyll (Solomon et al., 2002; Wachtel et al., 2002; Warriner et al., 2003; Jablasone et al., 2005; Franz et al., 2007). *Salmonella* cells were observed near trichomes (**10**; Barak et al., 2011; Gu et al., 2011). **(B)** Stem cross-section showing bacteria located in different tissues (Ep, epidermis; C, cortex; V, vascular tissue; Pi, pith) (Deering et al., 2011a,b). **(C)** Root cross-section showing bacteria on the root surface, internalizing between the epidermal cells, and colonizing root outer and inner cortex, endodermis (En), pericycle (P) and vascular system (Kutter et al., 2006; Klerks et al., 2007a,b; Jayaraman et al., 2014).

1.1.4 *Plant immune system and perception of human pathogens*

As mentioned earlier, instead of adaptive immune system like advanced eukaryotes, plants possess complex and efficient innate immune system to fight against microbial attack and infection (Jones and Dangl, 2006). Plants are capable of mounting a generalized basic defense response that is triggered by modified/ degraded plant products or conserved pathogen molecules called damage or pathogen associated molecular patterns (DAMP/PAMP) respectively. Mostly conserved PAMPs are components of cell walls or surface structures like flagellin, lipopolysaccharides, chitin (Zeng et al., 2010) and also intracellular PAMPs like elongation factor EF-Tu (Kunze et al., 2004). Plants are equipped with diverse set of extracellular receptors known as pattern-recognition receptors (PRRs) that convey intracellular signals initiating an army of defense molecules to prevent the pathogenic invasion. Most described PRRs can activate array of signaling events upon external molecular recognition (Monaghan and Zipfel, 2012). This specific branch of immunity in plants is known as pathogen-triggered immunity (PTI) and it is the first line of active defense against pathogens. Human pathogens on plants being an emerging field, caught attention of plant biologists and phytopathologists pretty recently and in last 5-10 years the focus was mainly on most studied PAMPs flagellin and lipopolysaccharide (LPS).

Flagellin is the structural component of flagellum in bacteria involved in the attachment and motility on the plant (Cooley et al., 2003). Plant PRR, FLS2 (Flagellin Sensitive 2) can recognize a well-studied PTI elicitor in flagellin known as flg22 (Felix et al., 1999). The flg22 epitope in *S. enterica* serovar Typhimurium 14028 was found to be an effective PAMP as well as elicitor of downstream immune response in Arabidopsis (Garcia et al., 2014), tomato and tobacco plants (Meng et al., 2013). *S. enterica* serovar Typhimurium mutant lacking flagellum are found to be better colonizers of alfalfa, wheat and Arabidopsis roots as compared to wild type bacteria (Iniguez et al., 2005) suggesting further that flagellum induces plant defense that might play a very crucial role in restricting bacterial survival inside several plant organs. However the *Salmonella* flg22 peptide is not the sole PAMP for elicitation of plant immune system as *fls2* mutant of Arabidopsis still showed a low level of PTI activation in response to this PAMP (Garcia et al., 2014).

Flagellin deficient mutant of O157:H7 has been tested and was not found to activate the salicylic acid-dependent BGL2 gene promoter compared to the wild type strain and also showed greater population in Arabidopsis than the wild type strain (Seo and Matthews, 2012). These results suggested that bacterial surface structures of this pathogen are perceived by the plants.

The variation in plant responses towards human pathogens could be attributed to the presence of other signature elicitors present in microbes. Differences in plant responses to *S. enterica* flagellin might be a reason of host-strain specificity as well. Although flagellin sequences from bacterial strain are known to be highly conserved, even a very minor change of five amino acids in the flg22 epitope resulted in establishment of a comparatively reduced activation of PTI in Arabidopsis, tobacco and tomato plants (Garcia et al., 2014). Brassicaceae and Solanoeceae plants have shown

to recognize specific flagellin (Robatzek et al., 2007; Clarke et al., 2013). Therefore evolving variation in flagellin sequences might be considered as the strategic move by the pathogens to trick plant recognition which eventually leads to the defense response in plant. Several studies have also demonstrated other important roles of bacterial flagella as far as bacterial behavior on and inside plants are concerned such as attachment to the leaf surfaces and movement on plant surfaces (Berger et al., 2009a, b; Xicohtencatl-Cortes et al., 2009; Saldaña et al., 2011; Shaw et al., 2011).

Another important aspect of plant recognition of pathogens is LPS perception. Lipopolysaccharide (LPS) is an element of the cell wall of Gram-negative pathogens of plants and animals. In case of animal hosts LPS is a well characterized PAMP which is recognized by the host Toll-Like receptor 4 (de Jong et al., 2012). However, in plants, receptor of LPS is yet to be discovered but the current studies producing evidences showed that human-pathogen derived LPS can be received and recognized by plants resulting in PTI response. For instance, on the leaf surface purified LPS from *Pseudomonas aeruginosa*, *S. Minnesota* R595, and *E. coli* O55:B5 induces strong stomatal closure in Arabidopsis (Melotto et al., 2006). Purified LPS from *Salmonella* also elicits extracellular alkalinization, ROS production in tobacco cell suspension (Shirron and Yaron, 2011) but failed to do the same in tomato leaves (Meng et al., 2013) indicating that LPS perception may either be on experimental scenario or variable among plant species. Other genetic evidences suggested that high activity of SA-dependent BGL2 gene promoter in Arabidopsis is dependent on *E. coli* LPS as LPS mutant generated lower activity of this promoter than the wild type bacteria (Seo and Mattews, 2012). Live *S. typhimurium* also do not induce ROS production in the epidermal tissue of tobacco plants (Shirron and Yaron, 2011) indicating that at least *Salmonella* is capable of suppressing LPS-induced ROS generation and extracellular

alkalinization. Like flagellin, the O-antigen moiety of LPS is important for plant perception of bacteria as well as bacterial attachment, fitness and survival on plants (Barak et al., 2007; Berger et al., 2011; Marvasi et al., 2013).

After perception of bacterial cells the very first PTI response in plants is stomatal closure that is proved to decrease pathogen entry into plants interior. Molecular components of PTI like flagellin, LPS perception and hormone perception and signaling contribute largely in this response (Melotto et al., 2006, 2008; Zeng and He, 2010; Sawinski et al., 2013). Human pathogen *S. enterica* serovar Typhimurium SL1344 and *E. coli* O157:H7 induced stomatal immunity in plants (Melotto et al., 2006; Kroupitski et al., 2009; Roy et al., 2013) at various level such as O157:H7 induces a strong stomatal immunity whereas SL1344 triggers only a temporary stomatal closure both in Arabidopsis (Melotto et al., 2006; Roy et al., 2013) and lettuce (Kroupitski et al., 2009; Roy et al., 2013). These inferences signified that strain SL1344 can either induce weaker stomatal immunity or can weaken stomata-based defense. SL1344 failing to re-open dark-closed stomata indicated that active suppression of stomatal closure by this strain might be unlikely (Roy et al., 2013), although it is probable that signaling pathways underlying dark-induced stomatal closure and bacterium-triggered stomatal closure are not entirely overlapping and SL1344 acts on immunity-specific signaling to subvert stomatal immunity.

1.1.5 *Plant intracellular responses to human pathogen infection*

Perception of PAMPs by PRRs gives rise to numerous complex cellular defense responses that are classified based on the timing of the response upon bacterial recognition. Early responses in plant upon bacterial recognition occur within seconds to

minutes, which include ion influxes, extracellular alkalinization and oxidative burst. Intermediate responses are seen to be activated within minutes to hours including stomatal closure, ethylene production, mitogen-activated protein-kinase (MAPK) signaling and transcriptional reprogramming while late responses consist of callose deposition, salicylic acid accumulation and defense gene transcription which happen after hours and days of infection.

These entire hallmarks of plant defense have also been tested for human pathogens *E. coli* and *S. enterica*. *S. enterica* infection specifically showed induction of *MPK3/MPK6* kinase activity and plant defense-related genes *PDF1.2, PR1, PR2* in Arabidopsis leaves (Schikora et al. 2008) and *PR1, PR4*, and *PR5* in lettuce (Klerks et al., 2007b). Activation of MPK6 being independent of FLS2 (Schikora et al., 2008) indicated presence of active PAMPs other than flagellin in case of *Salmonella*. Plant response to those other PAMPs may converge at MAPK signaling. Direct comparison of *PRI* gene expression level upon *E. coli* and *Salmonella* infections in Arabidopsis expressed that both O157:H7 and SL1344 are able to trigger defense marker gene though at different expression level. SL1344 infection induced lower *PR1* gene expression which indicates either weaker immune response or suppression of immune system by *Salmonella* (Roy et al 2013).

Plant hormones also play substantial role in plant defense against pathogenic bacteria. Ethylene being a very important plant hormone is well studied from the view point of its role in plant defense mechanism. Ethylene- insensitive mutant of Arabidopsis, *ein2*, supports higher *Salmonella* 14028 population inside whole seedling than the wild type plants (Schikora et al., 2008). Addition of 1-methylcyclopropene (1-MCP) to the growth medium, which is an inhibitor of ethylene, mediated signaling,

resulted in increased *S. enterica* 14028 titer inside *Medicago truncatula* but not *M. sativum* roots or hypocotyls (Iniguez et al., 2005) suggesting the role of endogenous ethylene signaling might be plant-bacterium specific . Surprisingly, ethylene signaling played contrasting role during fruit contamination as tomato mutants with defective ethylene synthesis, perception and signal transduction show noticeably less *Salmonella* proliferation within their fruits as compared to the wild type (Marvasi et al., 2014).

Similar to *ein2* mutant, the coronatine-insensitive mutant of Arabidopsis, *coi1-16* also favors high *Salmonella* 14028 inside seedlings (Schikora et al., 2008). In the same study it was also suggested that jasmonate signaling is also a significant component to prevent *Salmonella* infection in Arabidopsis though *coi1* mutants are well established to have increased resistant to several bacterial plant pathogens (Feys et al., 1994; Kloek et al., 2001).

Another critically important plant hormone which has direct role in defense mechanism against invading pathogens is Salicylic acid (SA). Two genetic lines of Arabidopsis, *nah-G* and *npr1* had been used extensively to resolve the role of this hormone in plant defense against phytopathogens. *nahG* plants are unable to accumulate SA (Friedrich et al., 1995) and *npr-1* has disrupted SA-dependent and independent defense responses (Ton et al., 2002). Both these lines support elevated population quantity of *Salmonella* 14028 inside roots (Iniguez et al., 2005) and seedlings (Schikora et al., 2008) when compared to the control wild type plants. *NPR1* was found to be important in reducing bacterial population of curli-negative *E.coli* O157:H7 43895 but not curli-positive strain 86-24 in Arabidopsis leaves (Seo and Matthews, 2012). Though tested with few strains of these two human pathogens, there is a promising

pattern which is indicating SA itself and activation of SA-signaling can potentially restrict HPOP.

Global analyses of transcriptome of plant in response to human bacterial pathogens have been analyzed in attempts to comprehend the overall cellular transcriptional response. *E. coli* O157:H7 regulates PTI-associated genes in Arabidopsis in flagellin dependent manner. Medium-grown Arabidopsis seedlings were used to conduct a similar transcriptomic analysis where after 2hr of inoculation with *S. enterica* serovar Typhimurium 14028, *E. coli* K-12, and *P. syringae* pv. *tomato* DC3000 displayed strong overlap among genes responsive to each bacterial infection. This indicates a common mechanism of plant basal defense response against bacteria (Schikora et al., 2011). Gene expression analysis of *Medicago truncatula* seedlings where seedling root was inoculated with two bacterial cells per plant showed 83 gene probes were commonly controlled in response to *S. enterica* and *E. coli* (Jayaraman et al., 2014). In a nutshell all studies regarding HPOP suggested that each human pathogenic bacterium can modulate different plant genes though there is a common basal defense mechanism.

1.1.6 *Are human pathogens able to induce plant ETI?*

Plants are well known to defend themselves from a wide array of pathogens using the innate immune system. But successful virulent plant pathogens have effectively evolved mechanisms to defeat this defense force by mounting its own set of artillery, like type three secretion system (T3SS) effectors and phytotoxins and produce disease in the targeted host plant (Melotto and Kunkel, 2013; Xin and He, 2013). In case of incompatible interactions where bacteria show a very low level of plant colonization

and no disease symptom on leaves, the host plant's R proteins recognize bacterial effectors and employ specific defense response. This is called effector triggered immunity or ETI. As type three secretion system is an established bacterial virulence strategy against both plant and animals, it is reasonable to predict that this system might be important for HPOP as well. Because of the presence of the cell wall, plant cells are impenetrable by the secretion needle of extracellular human pathogens like *E. coli* and *Salmonella (*He et al., 2004). This give rise to many questions including how these effectors reach plant cytoplasm and interfere with plant defense. Till date evidences are lacking which can shed light on human pathogens and its trick to inject effectors inside plant cells. A probable explanation of this might be that T3SS is still active on the cell surface and those effectors are secreted into the apoplastic region of plants. In that case plant cell surface receptors would be a necessary tool to recognize the effectors and trigger plant cellular response. It has been observed that *E. coli* O157:H7 T3SS mutant, *escN,* showed reduced capability to attach and colonize baby spinach leaves (Saldana et al., 2011). Even T3SS mutants of *S. enterica* serovar Typhimurium 14028 (*invA*, *prgH*, *ssaV*, and *ssaJ*) showed lesser population inside Arabidopsis leaves compared to wild type strain of the above mentioned (Schikora et al., 2011). Even defense related genes are noticed to be up regulated for prolonged time by the mutant *Salmonella* than wild type in Arabidopsis seedlings (Garcia et al., 2014). More studies have to be conducted to infer if T3SS of human pathogens can also be considered as ''recognizable'' surface structure like flagellin and delivers effectors in plant tissues which further trigger ETI.

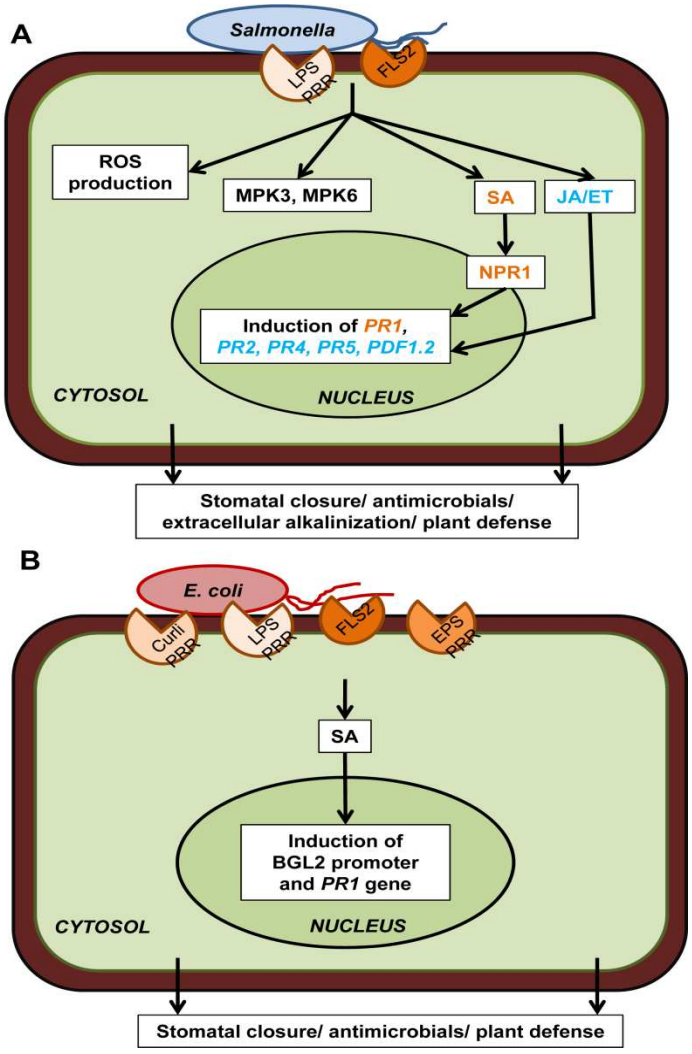

Figure 1.2 Plant cellular defense responses against human pathogens. **(A)** After PAMP perception such as flagellin, LPS through PRR (FLS2and putatively others), *Salmonella* spp. was found to trigger downstream plant defense responses including ROS production, MPK3/6, salicylic acid signaling through NPR1, jasmonic acid (JA) and ethylene (ET) signaling, defense-associated gene induction, and extracellular alkalinization. These cellular procedures eventually lead to stomatal closure, antimicrobial activity, and plant defense. **(B)** Curli, LPS, flagellin, EPS of *Escherichia coli* are also perceived by PRRs (FLS2and putatively others) present on plant cell surface which generates the induction of the SA-dependent BGL2 promoter activity and *PR1* gene expression. Plant defense responses in case of both these human pathogens are strain specific as well as plant cultivar specific. However this diagram shows components that have been directly demonstrated by experiments.

Interestingly T3SS and effectors of *P. syringae* pv. *syringae* shown to be involved in ETI induction as well as bacterial fitness on plant surface (Lee et al., 2012) like filamentous T3SS EspA protein in *E. coli* O157:H7, which is required for attachment to arugula leaves (Shaw et al., 2008). Structural mutant *invA,* which has defects in T3SS-1 system-associated phenotypes were able to induce high level of ROS production and extracellular alkalinization in tobacco BY-2 cell suspension and hypersensitive reaction (HR) in tobacco leaves when evaluated against wild type (Shirron and Yaron, 2011). This suggested T3SS to be a major component in bacteria for immunity suppression in plants although other reports claimed plant reaction to the regulatory mutant *phoP* that can modulate expression of several effectors and membrane components (Shirron and Yaron 2011; Dalebroux and Miller, 2014) which is no different from wild type bacterium. After all these researches question still remains that if the phenotypes monitored is due to the T3SS structure or due to translocated effectors. A new study has demonstrated HR elicitation due to transient expression of the type three effectors of *Salmonella* 14028 SseF in tobacco plants; and this response was reliant on the SGT1 protein (Ustun et al., 2012) indicating SseF can trigger resistant-like response in plants as well as demands for R protein signaling components. *Salmonella* 14028, which was claimed to transport the SseF effector, are not capable of inducing HR or any other disease symptoms when tested with tobacco leaves (Üstün et al., 2012; Shirron and Yaron, 2011). Hence it is still a matter of more intense study to determine how ETI is important in the plant and human pathogens interaction.

1.1.7 *Plant- Salmonella and plant- E. coli interactions depends on genotypic variability of plant and bacteria*

Although *S. enterica* and *E. coli* O157:H7 have never been conventionally known to be plant pathogens or plant invaders and alter plant physiology, all the studies, reports and evidences claim otherwise. An offense-defense evolution is observed in both plant and the human pathogens which were expected. Several studies have addressed the probability of genetic variability amongst plant species or within the same species (such as cultivars, varieties and ecotypes) to be the potential reason for differential bacterial behavior and/or colonization of plants. Barak et al., (2011) have explained that different cultivars of tomato plants can support different levels of *S. enterica* population after bacterial inoculation of those plants using sprinkler water suggesting plant factors may control bacterial success in terms of colonizing the phyllosphere. It was also shown in the same study that the cultivar with least *S. enterica* population had lowest number of speck lesions when infected with tomato pathogens *Pst* DC3000 which indicates the presence of strong basal defense in this plant cultivar that may have accounted for the low bacterial colonization. Same group of scientists presented another interesting result when they performed a comparative study of *S. enterica* infection of several crop species and found out that seedlings from Brassicaceae family have higher contamination compared to carrot, tomato and lettuce when all of those plants were grown in the same contaminated soil. Seedling contamination corresponds with *Salmonella* population in crop plant's phyllosphere except tomato. Variation in the internalization of *Salmonella* SL1344 in diverse leafy vegetables and fresh herbs were reported by Golberg et al., (2011) using confocal microscopy. Internalization incidence (% of microscopic fields containing bacterial cells) was more in case of iceberg lettuce and arugula, intermediate in romaine lettuce, red lettuce, and basil and low in parsley and tomato. Bacteria showed attraction towards

the stomata of Iceberg lettuce and basil but did not show the same in arugula, parsley, and tomato. The age of romaine lettuce and the population size of human pathogenic bacteria *E. coli* O157:H7 and *S. enterica* Thompson survived on plant leaves correlates (Brandl and Amundson, 2008). Young lettuce leaves (inner leaves) harbor larger bacterial titer than middle aged leaves. This study also reported exudates on the surfaces of young leaves contains higher nitrogen than middle aged leaves which might be a reason of higher number of bacterial cells survival on young leaves. Thus, it is tempting to hypothesize that the genetic variability existing among plant genotypes regarding the chemical composition of their organ exudates may be a determinant for human pathogen behavior (such as chemotaxis and tropism toward stomata and roots) and capability to inhabit plants. Mitra et al., in 2009 studied different methods of inoculations of bacteria in three cultivars of Spinach and its effect on the internalization and survival of *E. coli* O157:H7. The Spinach phyllosphere and stem provided the most and the least favorable niche for this bacterium respectively. Although the leaf surface was best region for *E. coli* to survive on plant but different leaf morphologies of different cultivars have affected the ability of this pathogen to survive.

1.1.8 *Future perspectives*

All these studies collectively point out that plant age, genotype, leaf morphology, chemical composition of leaf exudates and the primary infected organ influence the outcome of bacterial infection and colonization of plants. This whole process does not seem to be feasible for any generalization as this human pathogen and plant interaction is constantly changing and reshaping.

The basic knowledge of plant and human pathogen association that do not result in visual or macroscopic symptoms in affected plants, but yet major threats for food contamination is in its infancy. Both bacterial and plant factors are crucial for this cross-kingdom communication and rising evidences suggest a possible overlap between plant molecular response to human as well as plant pathogens. In future, challenge will be to figure out how these interactions are different from each other. Being a relatively new field of research, differences in conclusion from different studies regarding multiplication vs. decline in bacterial population overtime and disease-like symptoms vs. HR on inoculated plants have been observed. This is mainly due to the use of different methods for inoculation, different bacterial strains, inoculums concentration, plant cultivars or age etc. Standard protocols, consensus and collaborations among food scientists, microbiologists, plant pathologists and molecular biologists might help in solving numerous unanswered puzzles. Elucidating the specificity of each plant-human pathogen interaction and avoiding making generalized conclusion might help to know this fast evolving incident of cross-kingdom interaction a little better. Major point still needs to be resolved is, whether plant defense observed against *Salmonella* and it's PAMPs is due to weak recognition of bacteria by plant or active suppression of plant defense by bacteria. In case of active suppression of plant defense mechanism by bacteria a question still remains: what are the factor(s) responsible for that? This line of research might lead to a whole new paradigm that otherwise could not be revealed by only studying plant associations with its own natural pathogens.

1.1.9 *Research goals*

The association of plant and human pathogen being relatively new, it is being speculated that these organisms are using plants as a vector or temporary reservoir to be

able to infect animal hosts and maintain their life cycle (Barak and Schroeder, 2012*).* The main goal of my research was to study plant and human pathogen interaction to reveal how efficiently the plant defense system acts in the preventing human pathogens penetration and colonization of internal tissues. In chapter 2, Arabidopsis defense response modulation was studied during *S. enterica* serovar Typhimurium SL1344 and *E. coli* O157:H7 infection putting emphasis on plant innate immune response and defense gene response in early stages of infection. Primarily the stomatal defense response in Arabidopsis leaf was examined against SL1344 and O157:H7 infection under high humidity as well as low humidity. To extend the study to edible fresh produce along with model plant Arabidopsis, hydroponically grown lettuce were also used as plant model. In Chapter 3, I discussed the requirement of known Salicylic Acid and PAMP-signaling component involved in Arabidopsis innate immune response against SL1344 and O157:H7. In the same chapter the mode and ability of human pathogenic motility *in vitro* was also described. All these studies are important to know why plants that are not a natural host are still unable to clear human pathogen infection. This might help disrupting the survival of human pathogen on/in plant which might be an effective strategy to reduce food-borne outbreaks.

Chapter 2

Salmonella enterica serovar Typhimurium induces weaker immunity in plants compared to *Escherichia coli* O157:H7

Chapter 2 was formatted and published in Phytopathology, focus issueon Human Pathogens on Plants. (Roy, D., Panchal, S., Rosa, B. A., and Melotto, M. 2013. Escherichia coli O157:H7 induces stronger plant immunity than Salmonella enterica Typhimurium SL1344. Phytopathology. 103:326-32. doi: 10.1094/PHYTO-09-12-0230-FI).

2.1. Abstract

As discussed in the previous chapter the emergence of foodborne outbreaks associated with fresh produce, fruits, vegetables have stimulated interest and new research. All these foodborne illnesses are a burden on public health and contribute significantly to not only public health but also cost of health care, nation's economics and so on. A large number of these outbreaks are due to two major human enteric pathogens, enterohemorrhagic *Escherichia coli* and *Salmonella enterica*. In this study my attempt was to understand how plant innate immunity is manipulated by human pathogenic bacteria *E. coli* O157:H7 and *S. enterica* serovar Typhimurium SL1344. Plant defense was assessed when it was induced by fully pathogenic bacteria O157:H7 and SL1344 in both *Arabidopsis thaliana* and lettuce (*Lactuca sativa*). It was observed that *E .coli* O157:H7 induces a stronger plant immune response than SL1344 at both pre-invasion and post-invasion steps of infection indicating differential plant and human pathogen interactions. For instance O157:H7 triggered strong stomatal immunity even under high humidity which is an environmental condition known to weaken plant stomatal defense against bacteria. Interestingly SL1344 only could induce a transient stomatal immunity in both plants. It was also observed that plant defense related marker gene *PR1* showed

significantly higher level of expression in Arabidopsis leaves when infected with O157:H7 compared to SL1344. These observations suggested that plants may recognize and respond to different human pathogens differently being more efficient in case of few than other bacteria. Moreover, stomatal immunity can lessen the penetration of human pathogens through stomatal pore resulting in less bacterial population inside plant tissue. Better understanding of plant responses to diminish harmful human pathogenic bacterial contamination can be a paramount in preventing foodborne related outbreaks and improve food quality.

2.2 Introduction

Rising cases of illness, hospitalization and death indicate that something in the food safety system requires to be improved. The whole chain of food safety system which includes production, processing, packing, distribution or transportation, storage, preparation and selling is prone to contamination by undesired organisms such as bacteria. The recent cases of human pathogenic bacteria association with fresh produce, fruits and vegetables indicated emerging pathogens which is a threat to our food quality at a great extent. Research and other preventive measures have been going on to control these outbreaks and to learn to prevent similar cases in future. Many factors are there which determine a proper safe condition of food. Production, handling, preparation, storage practices are few of those. Food contamination can occur at any point of that whole process. Most of the time the contamination is not detected in food when purchased or consumed but presence of harmful microbial human pathogens eventually lead to illness in humans. Many of these microbes are known to be normal flora in healthy animal gut like cattle. These intestinal microorganisms can survive within the animal gut not harming the host but they proved to be dangerous when entered in human

gut through contaminated food which is mainly known as food borne illness. There are several causes of contamination that may introduce enteric pathogens to the agricultural field and initiate food contamination. Constant interaction of human and animal carriers with environment leads to the predisposition of these pathogens to an unconventional niche that has non-animal hosts. Additionally use of contaminated, improperly prepared manure as plant fertilizer and pesticides, irrigation water contaminated with enteric pathogens, poor hygiene practices by field-workers, lack of on-site sanitation services are few of those factors which can increase the risk of produce contamination in field after pathogen predisposition. It is still unknown that if airborne transmission of enteric pathogens also contributes in field contamination.

It is estimated that approximately 48 million Americans get sick, 12, 8000 millions are hospitalized and more than 3000 people die of foodborne diseases each year only in United States (CDC 2011). Enterohemorrhagic *S. enterica* and *E. coli* being two of the most common causal agents of illness in human associated with consumption of contaminated fresh produce, fruits or vegetables; are being studied extensively to uncover bacterial strategies to survive on plant and plants mechanism to defend or favor bacteria. *Salmonella* is known to cause diarrhea, abdominal cramps and fever and enterohemorrhagic *E. coli* O157:H7 causes hemolytic uremic syndrome and bloody diarrhea. Surprisingly it is not that long when it became evident that like plant pathogens human pathogens also have evolved mechanisms to successfully colonize and exploit respective hosts. These are the primary reason why human pathogens are being studied exclusively for their mechanism to interact with plants. Notably, mostly all of these studies were performed under laboratory set up which helped to understand this cross-kingdom interaction better but the rising incidence of food contamination of plant origin by these human pathogens in the natural environment remain unknown.

Few human pathogens can not only survive on plant surfaces but also can penetrate inside and maintain their population in the plant interior causing food borne illness when these plant parts are consumed by humans. Surface contamination and interior contamination of leaves are both equally dangerous but internal contamination is difficult, if not impossible to remove by standard disinfection methods. Hence a comprehensive understanding of the initial process of contamination is crucial to inhibit human pathogen related outbreaks.

Several studies showing association of *Salmonella* spp. and O157:H7 with stomata, naturally occurring lesions and wounds have indicated use of these entry ports by above mentioned pathogens to internalize plant interior (Brandl and Mandrell, 2002; Duffy and Erickson, 2008; Erickson, 2012; Itoh et al., 2005; Krouptiski et al., 2009). Although it is still unresolved that in the absence of wounding how these pathogens internalize plant tissue and deal with plant's active immune system.

Pathogen Triggered Immunity (PTI) and Effector Triggered Immunity (ETI) are two main branches of plant immune system to fight against invading pathogens (Jones and Dangl, 2006). In case of PTI, PAMPs are recognized by the surface receptors of plants like PRRs which leads to activation of defense related signaling pathways including plant hormone dependent and independent pathways (Denoux et al., 2008). The plant hormones salicylic acid, jasmonate, and ethylene are particularly important for plant defense (Grant and Jones, 2009). Successful virulent pathogens have seen to have overcome the first defense response of plant or PTI by different virulence strategies such as production of T3SS effectors and phytotoxins (Melotto and Kunkel, 2013). Further in non-compatible interactions pathogens released effectors, recognition of which by plant proteins initiates ETI, a very strong response leading to hypersensitive response limiting pathogen spread inside plant tissues.

Stomatal immunity being a part of plant's PTI works as an important component in initial defense against pathogens by diminishing bacterial entry through stomatal pore. This pore is used for gas exchange, photosynthesis and respiration but also proved to be a major route for bacterial entry into plan tissues. Melotto and collaborators showed that stomata can function as active innate immunity gates against pathogen invasion into Arabidopsis leaves when stomatal closure happens upon sensing presence of bacteria in the surrounding area (Melotto et al., 2006). O157:H7 was demonstrated to be incapable of overcoming stomatal defense resulting in prolonged activation of stomatal immune response (Melotto et al., 2006). Surprisingly a recent study provoked many questions when *S. enterica* serovar Typhimurium SL1344 was reported to migrate towards stomata and entered plant tissue without inducing stomatal immune response (Krouptiski et al., 2009). This unexpected yet exciting observation indicated human pathogens ability to subvert stomatal defense to colonize plant. However, it is equally possible that this bacterium is able to evade recognition by the plant immune system. Nonetheless plants response towards O157:H7 seem to be different that its response towards SL1344 infection. Here in this study I have provided evidences that SL1344 might have induced weaker response compared to O157:H7.

2.3 Materials and Methods

2.3.1 Plant material and growth conditions

Arabidopsis thaliana (L. Heyhn.) seed were sown in a 1:1:1 (vol/vol/vol) mixture of growing medium (Redi-earth plug and seedling mix; Sun Gro), fine vermiculite, and perlite. Plants were grown in controlled environmental chambers equipped with humidity control (Percival H2X Two Atomizer Humidifiers [Au: Please provide location?]) at 22°C,

60±5% relative humidity (RH), and a 12-h photoperiod under light intensity of 100 µmol m^{-2} s^{-1}. For all experiments, 4- to 5-week old plants were used. The ecotype Columbia (Col-0; ABRC stock CS60000) was used as a wild-type plant. The Arabidopsis mutant *ost1-2* (Mustilli et al., 2002)) and its wild-type Landsberg *erecta* (Ler) were a kind gift from Dr. J. Leung aavt CNRS, France. Hydroponically grown, naturally pest-free lettuce plants (*Lactuca sativa*, 'Live Gourmet' and 'Butter Lettuce') were obtained from a local grocery store and kept at 4°C until prior to experiments.

2.3.2 Bacterial strains and growth conditions

Wild-type bacterial cells of *E. coli* O157:H7 strain 86-24 (Sperandio et al., 2001) and *S. enterica* serovar Typhimurium SL1344 (Kroupitski et al., 2009) were grown in Luria-Bertani medium (tryptone at 10 g/liter, yeast extract at 5 g/liter, and NaCl at 5 g/liter; pH 7.0) at 30°C for all experiments. Cells were streaked on solid medium from frozen glycerol stocks for inoculum preparation. Medium was supplemented with spectinomycin (100 µg.ml^{-1}) to grow SL1344.

2.3.3 *Stomatal assay*

To evaluate the effect of relative humidity (RH) on stomatal immunity, plants were acclimatized under varying RH of 60±5% and ≥ 95% for 12 hour under 100µmol m^{-2} s^{-1} light, 25ºC temperature inside a growth chamber equipped with humidity control (Percival H2X Two Atomizer Humidifiers). The level humidity was monitored with a digital hygrometer (Traceble®; VWR). Plants acclimatized under each humidity condition were dip-inoculated in bacterial inoculums in the morning (3-4 hours after the lights were turned on). Stomatal assays were performed as described previously (Chitrakar and Melotto, 2010), except that unstained, whole leaves were imaged with a fluorescent

microscope Nikon Eclipse 80i (Nikon Corporations, Shinagawa-ku, Tokyo) equipped with DIC and long-distance objectives to measure the stomatal aperture width and to avoid the use of cover slip. For stomatal assays conducted with dark-closed stomata, plants were inoculated in the morning prior to turning on the lights and kept in the dark for the duration of the experiment. All the stomatal assays were completed before 3pm.

2.3.4 Bacterial inoculations

To examine the bacterial populations inside the leaf tissue, plants were inoculated with bacterial inoculums and bacterial pathogenesis assay was carried out. Bacterial strains were cultured at 30°C in liquid Luria-Bertani medium supplemented with appropriate antibiotics until an optical density at 600 nm of 0.9 to 1.0 was reached. Bacteria were collected by centrifugation and resuspended in water to the final concentration of 10^8 CFU ml–1 containing 0.03% Silwet L-77 (Lehle Seeds Co., Round Rock, TX) for dip inoculation of plants. For experiments under varying humidity level, plants were incubated under 60±5% or ≥ 95% for 12 hour under 100 µmol $m^{-2}.s^{-1}$ light, 25ºC temperature inside a growth chamber. Highly humid conditions were obtained by keeping well-watered plants covered with plastic domes in controlled environmental chambers. The level of humidity was monitored with a digital hygrometer (Traceable; VWR). Inoculated plants were immediately incubated under the following conditions: 25°C, 60 ± 5% or >95% RH, and 12 h of daily light (100 µmol.$m^{-2}.s^{-1}$) and kept there for the duration of the experiment. Leaves were surface sterilized in 70% ethanol for 2 min and bacterial population in the plant apoplast was determined as previously described (Katagiri et al., 2002).

2.3.5 Gene expression analysis

The qPCR experiment was done by Shweta Panchal and is included here with her permission.

Arabidopsis were acclimatized under unvarying humid conditions for 12hr under above mentioned temperature. To monitor the effect of human pathogen infection on plant defense related marker gene *PR1*, plants were infected with O157:H7 and SL1344 inoculum and leaf tissues were collected after certain times allowing bacterial infection. Plants from different pots were selected to collect leaf tissue to avoid touch or movement induction in genes. Total RNA was extracted from leaves using the RNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA) and quantified using a NanoDrop spectrophotometer (Thermo Scientific, Rockford, IL). Total RNA (1 µg) was synthesized into cDNA using the Takara RNA polymerase chain reaction (PCR) kit (AMV) (Clontech, Mountain View, CA) and diluted to a final concentration of 50 ng μl^{-1}. Quantitative PCR (qPCR) reaction (20µl) was performed with 10µl of iTaq SYBR Green Supermix (Bio-Rad, Hercules, CA), 2µl of cDNA template from the reverse transcriptase reaction described above, and 200nM reverse and forward gene-specific primers. Reactions were carried out in an Applied Biosystems 7300 thermocycler (Applied Biosystems, Foster City, CA) using the following cycling parameter: 1 cycle of 95°C for 5 min and 40 cycles of 95°C for 10 s and 60°C for 30 s. *PATHOGENESIS-RELATED 1* (*PR1,* At2g14610) gene expression levels relative to the control samples were calculated using the ΔΔ cycle threshold method (Livak and Schmittgen, 2001) considering the expression of the housekeeping gene *ACT8* (At1g49240) as an internal control. *ACT8* primers used were 5′-TTCCGGTTACAGCGTTTGGAGAGA-3′ (forward) and 5′AACGCGGATTAGTGCCTCAGGTAA-3′ (reverse) and *PR1* primers were 5′-CTTGTTCTTCCCTCGAAAGCTCAAGATAGC-3′ (forward) and

5vGAGCATAGGCTGCAACCCTCTC-3′ (reverse). Two biological replicates and three technical replicates were performed.

2.3.6 Statistical analysis

Statistical significance of each experiment was performed using the two-tailed Student's *t* test. All experiments reported were repeated at least two times for biological replicates using minimum of three technical replicates.

2.4 Results

2.4.1. Human pathogens trigger unique stomatal movements

The ability of Arabidopsis and lettuce to mount stomatal immunity against O157:H7 and SL1344, were tested under varying RH conditions (Fig 2.1). As leafy vegetables like lettuce are normally maintained at high RH prior to consumption, checking stomatal immunity under high RH would be suggestive about lettuce innate immune system against human pathogens. It was observed that the average stomatal aperture width in mock-inoculated plants were wider under >95% RH in comparison to 60% RH. Nevertheless live O157:H7 was observed to induce strong stomatal closure under both RH levels in both plants used and most stomata was found to be closed for the duration of the experiment (4hr). Interestingly SL1344 induced only transient stomatal immunity when stomata were monitored to be closed at 2hr post inoculation and stomata width returned to measurements similar to the width of stomatal aperture in mock inoculated plants under both RH at 4hr post inoculation.

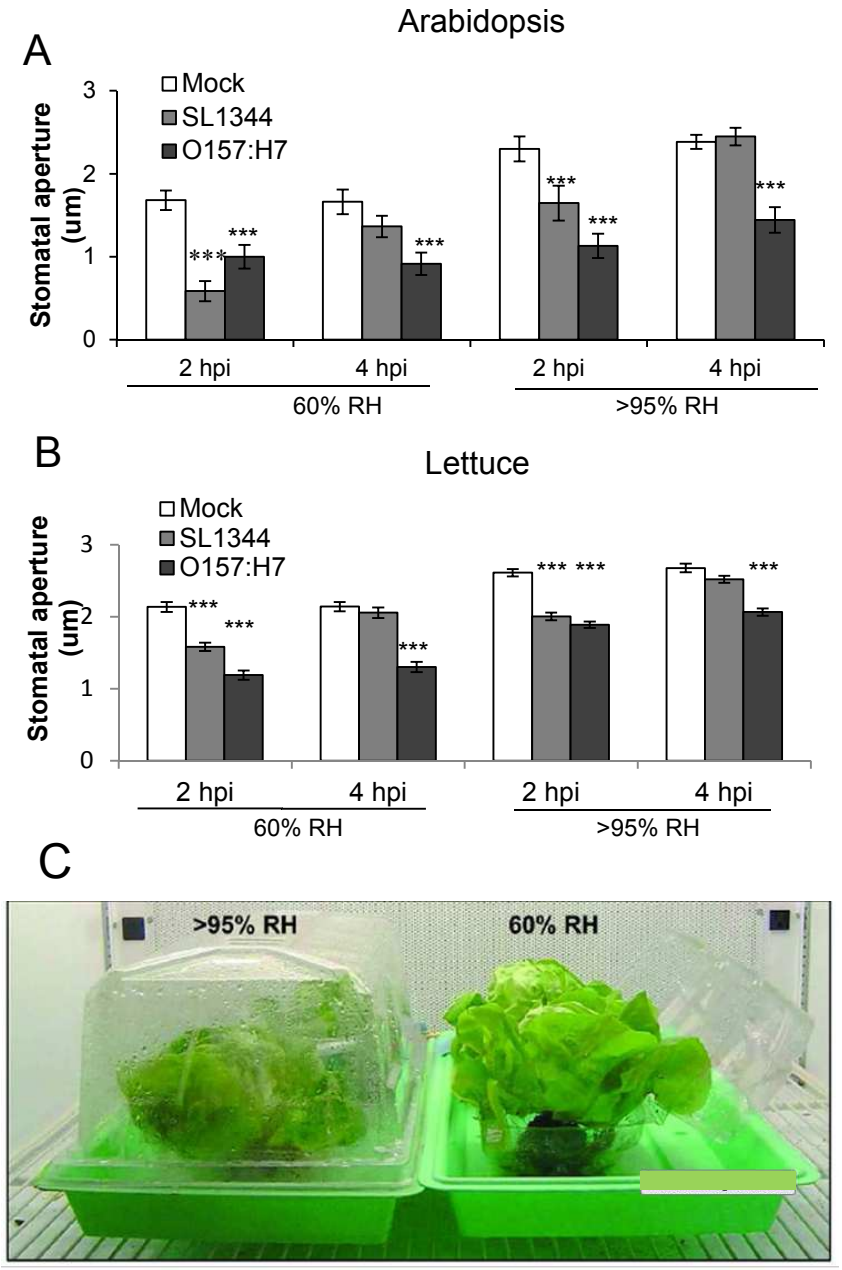

Figure 2.1 Human pathogens trigger unique stomatal movement. **A**. Arabidopsis and **B**. Lettuce heads were incubated with O157:H7 or SL1344 under different RH. Results are demonstrated as mean of stomatal aperture width (n=50-70) ± standard error. Statistical

significance of the difference in the means (mock versus bacteria treatment) was noticed with two-tailed Student's *t* test (*** indicates *P*<0.001). **C**. Hydroponically grown lettuce heads incubated at low (60%) and high (>95%) RH while the roots of the plants were kept moist for the duration of the experiments. Note: Reprinted from Roy et al (2013) with publisher's permission.

Next bacterial titers in the apoplast of dip-inoculated leaves of Arabidopsis and lettuce were determined. Dip-inoculation of plants allows bacterial penetration through the stomata closely imitating the natural way of bacterial infection in field. Both O157:H7 and SL1344 did not proliferate inside Arabidopsis apoplast and the titers decline as the plant started to senesce. However O157:H7 population reduced drastically (100 fold) approximately 15 days after inoculation. But SL1344 population declined 10-fold in the same time period (Fig 2.2). Failure of these human pathogens to proliferate aggressively inside plant apoplast and a very similar kinetics of stomatal movement under both relative humid conditions, it was tempting to infer that apoplastic bacterial enumeration would be also very similar and independent of RH. Though this hypothesis was true for O157:H7, but high RH surprisingly facilitates SL1344 to produce significantly larger ($P < 0.05$) apoplastic population in Arabidopsis. Lettuce plants were only maintained for 24h under 60% RH as maintaining lettuce under low RH was not possible. Nonetheless, lettuce also showed the same trend in human pathogen population counts in response to RH variation.

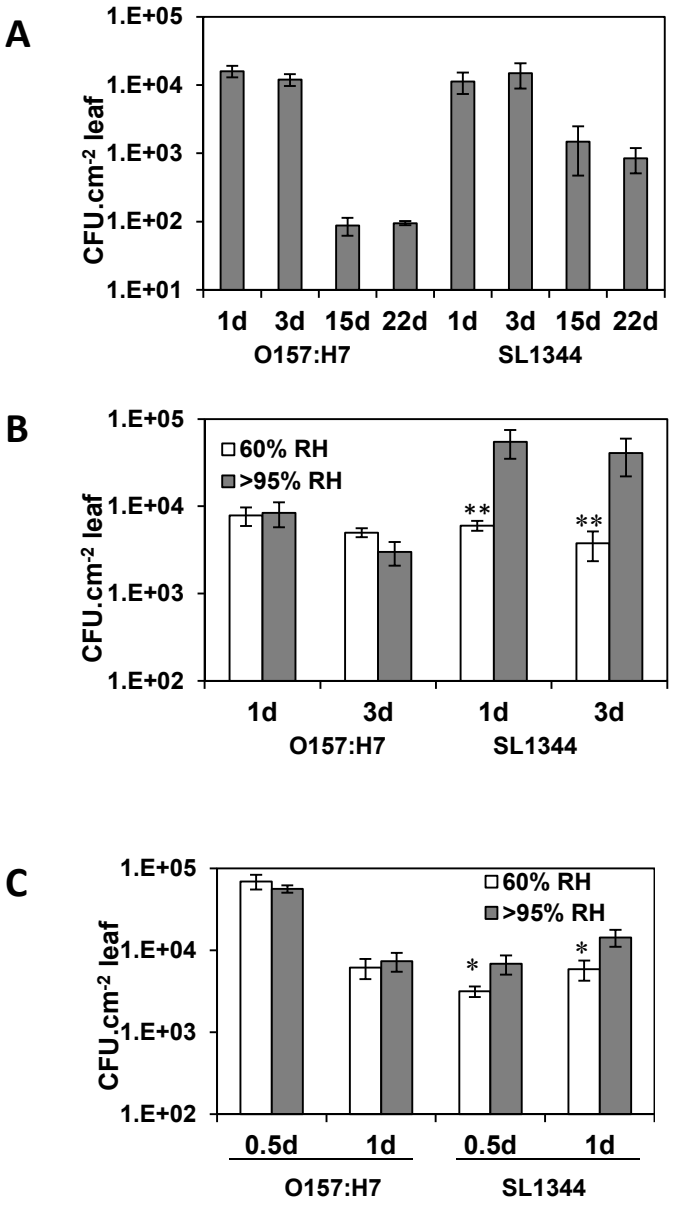

Figure 2.2 High RH supports penetration as well as survival of SL1344 but not O157:H7. **A** and **B**, Arabidopsis and **C**. Lettuce. Bacterial multiplication in the leaf apoplast at different day(s) after dip inoculation with SL1344 or O157:H7 under varying RH. Results shown here is the mea*n (n=12)* ± standard error. Statistical significance of the difference in the means (60% versus >95% RH at each time point) was observed with two tailed

Student's *t* test (*and ** indicates *P*<0.05 and 0.01 respectively). Note: reprinted from Roy et al (2013) with the permission of the publisher.

2.4.2 SL1344 cannot actively open stomata

The observations mentioned above instigate a hypothesis that SL1344 might be able to overcome stomatal immunity and actively open the pores like phytopathogenic bacteria *Pseudomonas syringae* (Melotto and Kunkel, 2013; Melotto et al., 2006) and *Xanthomonas campestris (*Gudesblat et al., 2009*).* The hypothesis was tested by conducting a stomatal assay under darkness at 60% RH. SL1344 was unable to open dark closed stomata (Fig 2.3) hence helped in reasoning that this bacterium triggers a weak immunity in the plant and the light stimulus is quickly prioritized by the guard cells which results in reopening of stomata. Contradictorily O157:H7 induces strong stomatal immune response that can't be overcome by light (Fig 2.1).

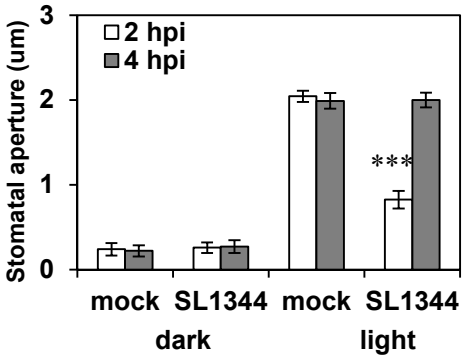

Figure 2.3 SL1344 cannot actively open stomata. Arabidopsis plants were dip inoculated with SL1344 and maintained at 60% relative humidity in the dark or under light for the duration of the experiment; hpi = h post inoculation. Results are shown as mean of stomatal aperture width (*n* = 50 to 70) ± standard error. Statistical significance of the difference in the means (mock versus bacterium treatment at specific time points) was detected with two-tailed Student's *t* test (*** indicates *P* < 0.001). Note: reprinted from Roy et al (2013) with the permission of the publisher.

To further strengthen the idea that guard cells can prioritize their response when challenged by biotic and abiotic stresses stomatal assay with flg22 was performed. Flg22 is a conserved 22-amino acid peptide of the flagellin subunit of bacteria flagella and a general inducer of plant's immune response (Zipfel et al., 2004). On the other hand it was noticed that high relative humidity diminished the flg22 effect on plants' immunity at a low concentration (2μM) although increasing the concentration to 10μM can cause stomata to close to the same extent independent of the RH level (Fig 2.4). All these results suggested that strong PTI overcomes the high RH effect in opening stomata, and guard cells prioritize their response to different external stimuli showing several degrees of stomatal innate immunity. Henceforth, it is possible that the reopening of stomata during SL1344 infection is a reflection of a weak stomatal response to SL1344 infection.

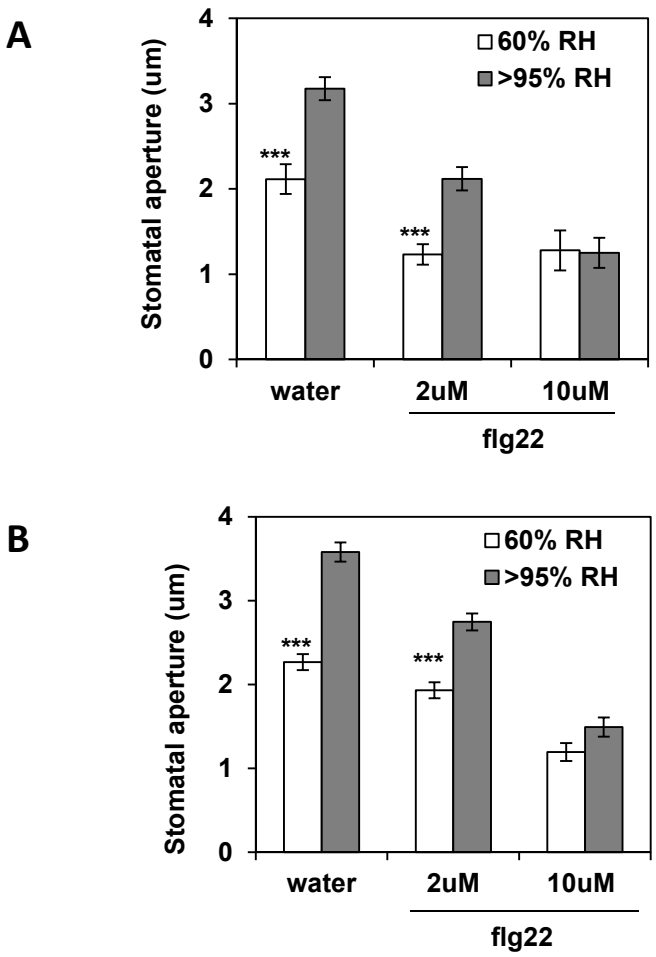

Figure 2.4 Strong pathogen-triggered immunity overrides the effect of high relative humidity (RH) in opening stomata. Stomatal response to different concentration of flg22 under varying RH and constant light. **A,** Arabidopsis or **B,** Lettuce leaves were floated on either water or flg22 solution and stomatal aperture was measured 4 h post incubation. Results are shown as mean of stomatal aperture width (n = 50 to 70) ± standard error. Statistical significance of the difference in the means (60% versus >95% RH) was detected with two tailed Student's t test (*** indicates $P < 0.001$). Note: This diagram was reprinted from Roy et al. (2013) with the publisher's permission.

2.4.3. Stomatal immunity diminish human pathogen penetration into leaves

As O157:H7 triggers strong stomatal closure in Lettuce and Arabidopsis under high RH, it was not feasible to determine if this layer of defense is successful in diminishing human pathogen contamination in plant interior using only wild type plants. To assess this hypothesis stomatal assay and pathogenesis assays were conducted with Arabidopsis mutant plant *ost1-2* that is unable to close stomata in response to bacterial PAMPs (Melotto et al., 2006). Above mentioned experiments were performed under moderate RH (60%) which environmental condition is not known for favoring bacterial penetration into plant tissue (Fig 2.2). Because *ost1-2* naturally have mostly open stomata, first it was determined if these mutant plants can be maintained under 60% RH without wilting. Stomatal assay results showed that stomata of *ost1-2* do not close even in the presence of O157:H7 and SL1344 on their phyllosphere (Fig 2.5 A) and pathogenesis assay demonstrated higher bacterial titer in mutant plants apoplast compared to the wild type plants *Ler* (Fig 2.5 B). Taken together these findings provide direct genetic evidence to support the hypothesis.

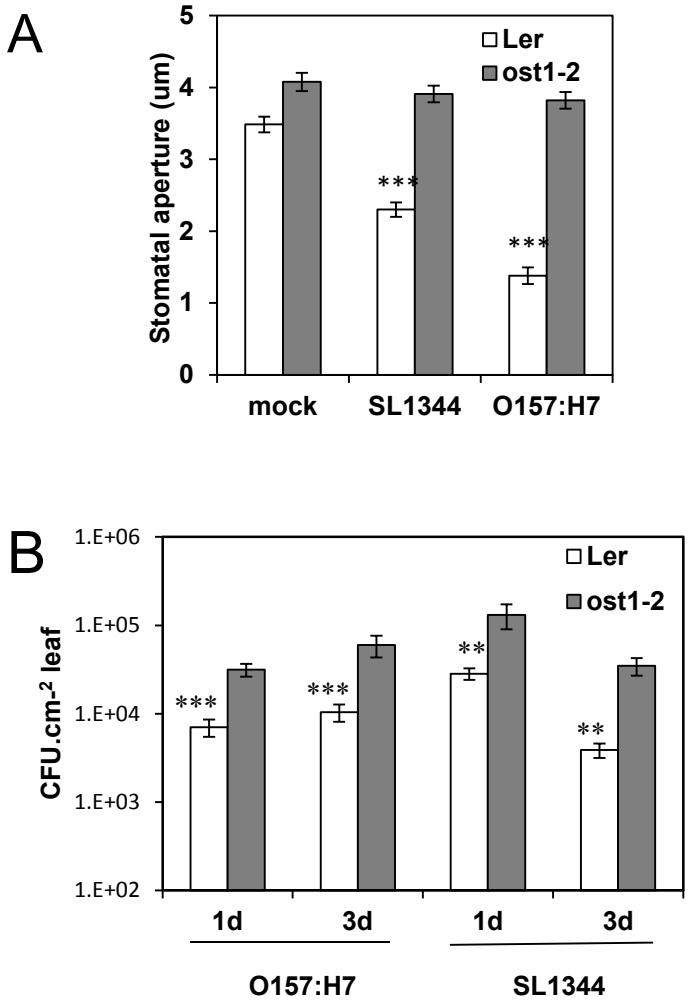

Figure 2.5 Stomatal immunity reduces penetration of human pathogens through the leaf epidermis. The *ost1-2* and wild-type Landsberg *erecta* (L*er*) plants were dip-inoculated with O157:H7 or SL1344. **A** Stomatal aperture width was measured 2 h after inoculation with bacteria or water control. Results are shown as mean of stomatal aperture width *(n = 50 to 70)* ± standard error (SE*).***B*** Bacterial enumeration in the leaf apoplast at different days (d) after inoculation. Results are shown as the mean (n = 18) ± SE. Statistical significance of the difference in the means (L*er* versus *ost1-2*) was detected with two-tailed Student's *t* test (** and *** indicate *P* < 0.01 and 0.001, respectively). Note: Reprinted from Roy et al. (2013) with publisher's permission.

2.4.4. O157:H7 induces higher PR1 gene expression than SL1344

The level of PR1 gene expression was monitored in Arabidopsis to assess if human pathogen infection can also modulate defense responses in whole leaves of this plant. Arabidopsis *PR1* (At2g14610) gene is a marker gene that is associated with immunity against bacteria. Reverse transcription qPCR analysis indicated that both SL1344 and O157:H7 induce an early expression *PR1* gene although O157:H7 infection showed high level of *PR1* gene expression than SL1344 (Fig 2.8) suggesting more active plant defense response against O157:H7 than SL1344. These results may also indicate at least in part, the fact that SL1344 titer persists more than O157:H7 population in plant apoplast of Arabidopsis at 15 and 22 days after infection even though the concentration of inoculums for both the bacteria were the same (1 x 10^8 CFU/ml).

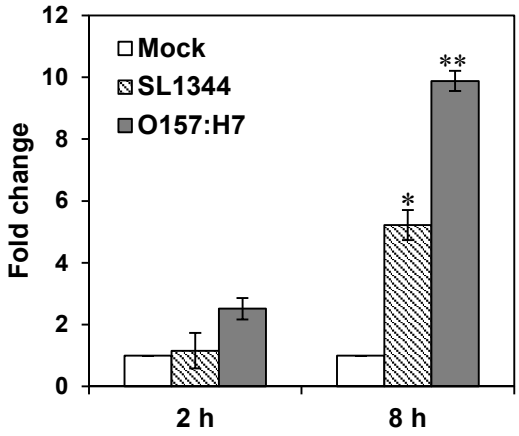

Figure 2.6 O157:H7 triggers higher level of *PR1* gene expression than SL1344. PR1 gene (At2g14610) expression at 2h and 8h post inoculation with bacteria relative to mock control was determined by quantitative polymerase chain reaction. Statistical significance of the difference in the mean expression of *PR1* in response to SL1344 and O157:H7 relative to that in the mock control was detected with two-tailed Student's *t* test (* and ** indicate $P < 0.05$ and 0.01, respectively). Note: Reprinted from Roy et al (2013) with publisher's permission.

2.5 Discussion

Outbreak associated cases are rising with consumption of fresh produce contaminated with enteric pathogens harmful for humans. However, there is no simple or single solution to this problem. But a very crucial aspect is to understand how these human pathogens can penetrate inside plant and maintain bacterial population for quite a long time. In this study my aim was to address plant innate immune response towards two well-known human bacterial pathogenic strains O157:H7 and SL1344 both at pre-invasion (such as stomatal immunity) and post-invasion stages of the infection.

Previously it was demonstrated that O157:H7 triggers a strong stomatal immunity in Arabidopsis when stomata were found to be closed until 8h post incubation (Melotto et al., 2006). Kroupitski and collaborators have shown recently SL1344 does not induce strong stomatal closure in lettuce compared to *P. syringae* pv. *tomato* DC300 but they have used saline bacterial suspension to submerged lettuce leaves and that is why it is probable that their finding that SL1344 does not close stomata was mainly due to the extreme wet conditions which would favor stomata to stay open. Therefore, stomatal response under varying RH condition were checked (Fig 2.1) High RH showed a positive influence on stomatal opening as shown with the water-treated plant's stomatal aperture. O157:H7 induced significant stomatal immunity but SL1344 was seen to induce a transient stomatal closure indicating weak stomatal immunity of plants against this bacterium irrespective of the RH condition.

The unique stomatal movement in response to these pathogens raised question about SL1344 having evolved mechanisms to overcome stomatal immunity. Questions were raised about plant being unequally efficient in recognizing O157:H7 and SL1344 as well. Three pieces of evidences were shown in this study to support the hypothesis that stomatal reopening during SL1344 infection was a result of weak induction of PTI. First

result shows SL1344 cannot open dark closed stomata. Therefore it is likely that reopening of stomata under light is a consequence of a weak stomatal immunity against SL1344 and stomata were able to return to the previous state quickly after the immune response phases out. Second, it was reported by Melotto et al. (Melotto et al., 2006) that increasing concentration of lipopolysaccharide (LPS) from *Escherichia, Salmonella*, and *Pseudomonas* spp. corresponds with increasing intensity if stomatal immunity. Denoux and collaborators showed in a study regarding the overall transcriptional changes caused by flg22 in Arabidopsis and inferred that this immune response elicitors alters the individual gene expression in a dose dependent manner and the transcriptional response revert back to basal level within 24h post inoculation (Denoux et al., 2008). In my study it was demonstrated that 2μM of flg22 (low concentration) at >95% RH is not as efficient as 10μM flg22 (high concentration) in inducing strong stomatal immunity in plants (Fig 2.4). This result indicates that environmental conditions facilitating stomatal opening such as high humid condition and light can overcome weak PTI in plant. O157:H7 penetration and survival inside leaf apoplast of Arabidopsis and lettuce, which trigger strong response, was not influenced by the variable RH (Fig 2.2 B and C) but unlike O157:H7, high RH supported both SL1344 entry and sustainability in both plants (Fig 2.2 B and C) which indicates effect of high RH in quickly diminishing the effect of a weak PTI caused by SL1344. Third, SL1344 was not found to induce the *PR1* gene expression (a hallmark of plant immune response) to the same level as O157:H7 (Fig 2.6). Altogether, these findings support the idea that SL1344 is weakly perceived by Arabidopsis and lettuce unlike O157:H7. Immune response in both guard cell and mesophyll are transient. To reject the alternative hypothesis completely that SL1344 have evolved mechanisms to defeat plant immunity, it still remains to be determined if SL1344 can down-regulates genes and metabolic pathways in plant defense response.

It was shown in this study that like Arabidopsis, tomato, tobacco, soybean and common bean lettuce is also to employ stomatal immunity against bacteria (Gudesblat et al., 2009; MacDonald and Cahill, 1999; Melotto et al., 2006; Schellenberg et al., 2010). This enforce that stomatal immunity is a widespread mechanism of defense among plants extending to both plant and human pathogens. Surprisingly, it gives the impression that the molecular elements involved in plant defense mechanism against plant and human pathogens are essentially similar. For example, OST1 kinase, the gurad-cell specific component is essential for the stomatal closure upon perception of pathogens (Melotto et al., 2006; this study). Similarly, mitogen-activated protein kinases (MPK3 and MPK6) are crucial for stomatal immunity in Arabidopsis against *X. campestris* pv. *campestris* (Gudesblat et al., 2009) and mesophyll immunity against *S. enterica* serovar Typhimurium 14028 (Schikora et al., 2008). This unique characteristic of plant immunity can be further explored to improve plant resistance against pathogen infiltration and consequent contamination and infection of plant interior.

A latest study has explained that populations of several strains of O157:H7 lacking cell structures are significantly more inside the wild type Arabidopsis and trigger less *PR* gene expression than those strains of O157:H7 with cell surface structures (Seo and Matthews, 2012). Lack of flagellin production or T3SS apparatus in *S. enterica* serovar Typhimurium 14028 increased alfalfa root endophytic colonization (Iniguez et al., 2005) and the O antigen moiety specially O: 1, 3, 19 in the LPS of *S. enterica* LPS moiety was found to be associated with wilting of Arabidopsis leaf. But strains which lack the O antigen or belong to different serogroups like SL1344 cause no noticeable reaction on leaves (Berger et al., 2011). Thus it is possible that few bacterial species may induce weaker immunity in plants than others and this phenomenon depends on how effectively plant can recognize bacterial MAMPs and induce defense responses. All together the

information also instigate the idea that SL1344 might have evolved mechanisms to escape plant defense and is capable of penetration and survival inside plant tissue as an endophyte more successfully than O157:H7.

In conclusion, as mentioned above, high RH favored penetration and survival of SL1344 but not O157:H7and it was reasoned that induction of strong PTI by O157:H7 may supersede the effect of high relative humidity on stomatal opening. However it was not ruled out that other factors that contribute to successful entry of SL1344 into plant tissue are ability to move on the leaf surface, chemotaxis towards stomatal pore due to nutrient secretion and overall fitness of SL1344 in the plant environment. Certainly plant's active immune system against human pathogens and active perception of pathogens also add to the outcome and intensity of plant infection by these human pathogens.

Chapter -3

Assessing molecular components responsible for defense mechanism of plants against human pathogens

Chapter 3 is in preparation to be submitted to a scientific journal for publication.

3.1 Abstract

Enterohemorrhagic *Escherichia coli* and *Salmonella enterica* are causative agents of the majority of food-borne bacterial infections and are responsible for more than 100 million food borne illnesses annually. Recently, studies have shown active survival mechanism by specific strains of these two above mentioned human pathogens on/in plants. In the previous chapter it was shown how stomatal immunity and apoplastic defenses are modulated in a model plant *Arabidopsis thaliana* and lettuce after *Salmonella* and *E. coli* infection. However, the molecular mechanism controlling this interaction is still largely unclear. In this chapter, bacterial strategies for their association with plants were studied along with plant's defense strategies. This study was focused on molecular level plant bacterial interaction to reveal whether well-studied molecular components in plant defense signaling pathways against plant pathogens still hold importance in defense responses against human pathogens mentioned above. Comparison of immune responses between mutant and wild type *Arabidopsis* plants revealed that FLS2 is a vital receptor even for human pathogen perception. Similar to their role in defense against plant pathogens, components of salicylic acid pathway, NPR1 and SID2 were found to be significant in restricting human pathogenic population inside plant apoplast. All these results highlight the possibility that plant might use similar signaling pathway(s) to prevent plant pathogen and human pathogen penetration and infection. This study contributes towards the demand to understand the interactions

between plants and human pathogens which further can be used to aid prevention of food-borne related illness at the initial stages.

3.2 Introduction

With the world population being more than 7 billion currently and being projected to reach 9 billion by 2050, the current main concern is how to achieve human health and sustainability goals accurately for a huge population. Food being one of the very basic needs, food safety is one of the pressing issues. With changing climate, newly emerging pathogens and increasing population, food safety is a major challenge nowadays. Pathogens featured frequently in today's headlines, such as *E. coli* O157:H7 and *Salmonella* spp. were not identified as major causes of food-borne illnesses 20 years back. In developing countries, 2.1 million deaths are reported annually due to food-borne diseases. Significant under-reporting leads experts to estimate that food-borne diseases may account for a substantially higher number of cases than reported worldwide (CDC, 2013).

Many organisms including bacteria, fungi, protists, insects are found to be associated with plants for their nutrient source. Not only microorganisms, humans are also dependent largely on plants for an exclusive source of food and other things. Plant diseases can be devastating for human health both directly, if plant is contaminated with harmful microorganisms, and indirectly, when plant diseases results in crop loss and subsequent malnutrition.

More and more cases of food contamination are reported as microorganisms which are pathogenic to humans are found to thrive on plants which ultimately lead to food-borne illness. This has given rise to a whole new niche of research where the main goal is to find out the reason and the mechanism responsible for this incident of human

pathogen association with edible plants. Several solutions for prevention of contamination of edible plant sources has been proposed and executed, although studies have demonstrated human pathogens are capable of escaping the effect of sanitization by colonizing plant interior (Seo and Frank, 1999; Saldana et al., 2011). In the previous chapter I have discussed about my findings regarding differential plant immune responses against human enteric pathogens. Yet, it is still unknown that what component(s) of plant defense system is/are responsible for immunity against enteric pathogens. Knowing that more clearly might help us to understand this newly developed cross-kingdom interaction better, which will in the longer run, allow targeted solutions to increase the safety of fresh produces.

3.2.1 *Plant's general immune system against bacteria*

To prevent invasion of pathogens, plants use their strong array of structural, chemical as well as protein based innate immune defense system. Pathogen entry inside host plant is a key initial phase of infection. Fortunately, stomatal closure was found to be effective as an innate immune system against bacteria in plants (Melotto at al., 2006). Bacterium-induced stomatal closure was found to require PAMP signaling and SA (salicylic acid) homeostasis (Melotto et al., 2006). Few plant genes which are found to be crucial for pathogen perception and apoplastic immunity are FLS2 and SID2/EDS16, NPR1 of SA bio-signaling pathway. This study will explore the role of all these defense related components in plants against *Salmonella enterica* serovar Typhimurium SL1344 (SL1344 henceforth) and *Escherichia coli* O157:H7 (O157:H7 hereafter).

3.2.2 *Human enteric pathogens on phyllosphere*

. Plants are continually exposed to microorganisms. To establish themselves as pathogens, microorganisms, most of the time must access the plant interior, either entering through wounds or natural openings like stomata, hydathodes etc or by penetrating leaf or root surfaces directly. Several studies have shown association of *Salmonella* spp. and *E. coli* O157:H7 with stomata and naturally occurring lesions (Brandl and Mandrell, 2002; Duffy et al., 2005; Itoh et al., 1998; Kroupitski et al., 2001). Wounds also provide a nutrient-rich path towards the plant interior (Erickson, 2012). It is generally assumed that successful penetration through leaf or interaction of bacteria with plant preceded by proliferation of the bacteria in the phyllosphere of the host plant. Human pathogenic bacteria are shown to be attracted to different niches on leaf surfaces like bulges, troughs formed by veins, leaf hair on trichomes, stomata, and hydathodes that create space for bacterial survival with increased availability of nutrients and water as well as protection from harsh environmental situations (Leveau and Lindow, 2001; Miller et al., 2001; Brandl and Amundson, 2008; Kroupitski et al., 2009; Barak et al., 2011) Aggregates of *S. enterica* were found on cilantro leaf in the vein regions (Brandl and Mandrell, 2002) and in addition this bacterium showed affinity towards abaxial side of lettuce leaf (Kroupitski et al., 2011). Since a lot of pathogenic bacteria carry complex machinery for motility and chemotaxis, bacterial motility, either chemotactic or random might be playing important role in this early stage of interaction too. Kroupitski and group also monitored that *S. enterica* serovar Typhimurium can move on the leaf surface toward stomata and this movement is mostly chemotactic and induced in the presence of light. Not only that, mutations affecting *Salmonella* motility and chemotaxis significantly inhibited bacterial internalization (Kroupitski et al., 2011). All these suggest this bacterium is attracted to nutrients produced *de novo* by photosynthetically active cells and use

chemotactic movement as their means of motility. But it is still unknown whether SL1344 and O157:H7 also can use chemotactic movement or other motility mechanism to reach stomata which can eventually be used by them as the port of entry to access plant interior. In this study the *in vitro* motility mechanism of these two bacteria using swimming assay and chemotactic assay techniques were monitored.

3.2.3 *FLS2 receptor is important for bacterial perception before infection*

Salmonella and *E. coli* association with plant stomata is not a very rare finding. Stomata were found to be utilized by *Salmonella* and *E. coli* to gain entry into internal leaf compartments, which provide them with a more favorable environment (Kroupitski et al., 2011; Roy et al., 2013). Stomatal innate immunity is found to be responsive to the attempt of most pathogens to enter inside plant tissue by initially triggering stomatal closure. Surprisingly when *S. enterica* was inoculated on lettuce leaves, it did not trigger extensive stomatal closure (Krouptiski et al., 2011; Roy et al., 2013). This weak plant immunity against *Salmonella* may imply poor recognition of the bacteria associated Pathogen Associated Molecular Patterns (PAMPs) as one of the possible reasons.

Innate immune system in plants has evolved several unique strategies for recognition of pathogen PAMPs which is based on several pattern recognition receptors (PRRs) which are transmembrane proteins and can perceive characteristic molecular structures present on large groups of microorganisms (Zipfel, 2008). This perception of microorganisms generates signals and the PRRs either induce or suppress an immune system depending upon the type of these signals (Medzhitov and Janeway, 2002). Arabidopsis receptor-like kinases (RLKs) FLS2 is one of the best studied receptors which recognizes the conserved 22 amino acid epitope of bacterial flagellin, flg22 (Gomez-Gomez and Boller, 2000). *AtFLS2* orthologue have been recently found in other plants as

well, such as tomato, tobacco depending on the requirement for flg22 perception and sequence homology (Robatzek et al., 2007; Hann and Rathjen 2007).

Upon PAMP perception, many different molecular, physiological and pathogenesis related cellular changes occur in plant cells which together is known as Pathogen Triggered Immunity (PTI). Seconds to minute after bacterial recognition ion-flux is noticed across the plasma membrane. Not only that, increased amount of Ca^{+} concentration, oxidative burst, MAP kinase (MAPK) activation, phosphorylation of protein, endocytosis of receptors, protein-protein interaction and an array of complex signal transduction are also reported after pathogen perception in plant through receptors (Nurnberger et al., 2004; Altenbach et al., 2007). Within 30mins transcriptional changes in Arabidopsis was demonstrated (Navaro et al., 2004). Other responses like induction on salicylic acid signaling (Sato et al., 2007), enhancement of ethylene biosynthesis, callose deposition and mainly stomatal closure are also prevalent in PTI (Altenbach and Robatzek, 2007). All these imply that when a plant is attacked by a pathogen, most of the time it can ward off the infection by mounting a wide range of defense responses (Yang et al., 1997) but for that, perception of the pathogen through surface receptors like FLS2 is crucial. A specific molecular pattern in flagella of phytopathogenic bacteria known as flagellin is recognized by the plant cells (Felix et al., 1999). Therefore recognition of flagella by plant receptors sends a signal of presence of potentially pathogenic bacteria as both virulent and avirulent pathogenic bacteria induce a general defense response in plant (Hutcheson, 1997).

Here in this chapter one of the main concerns of the study was to check if perception of flagellin in SL1344 and O157:H7 flagellum by FLS2 receptors is also crucial to initiate a battery of downstream defense responses to reduce bacterial penetration and multiplication in the apoplast of infected plant. Evidences are there which indicate *S.*

enterica flg22 can be detected by plants which activate hallmarks of defense responses or Pathogen Triggered Immunity (Garcia et al., 2014). Furthermore, inoculation of *S. enterica* serovars to *Arabidopsis thaliana* seedlings triggered MAPK activation and defense gene expression to a similar extent as that provoked by *P. syringae* inoculation (Schikora et al., 2008, 2011; Garcia et al., 2013). As human pathogen and plant interaction was several times found to be strain and cultivar specific respectively, in this chapter the stomatal and apoplastic immunity of mutant Arabidopsis plant without the FLS2 receptor will be monitored against O157:H7 and SL1344 in comparison to wild type Arabidopsis plants.

3.2.4 *Salicylic acid is crucial for plant defense against bacterial infection*

Host and their potential pathogens are occupied in a constant co evolutionary battle for dominance in nature. To protect themselves from harmful bacterial infection plants have evolved highly complex and efficient innate immune system. PAMP perception via receptors located on plant surface triggers the first level of defense known as PTI which is sufficient to fend off most invading microbes (Zipfel, 2008). To suppress this PTI against invading pathogens, invaders secrete and deliver effector proteins to host cells (Jones and Dangl, 2006; Zipfel, 2008). Plants also have evolved Effectors Triggered Immunity (ETI) which eventually leads to robust disease resistance that often includes localized host cell death or a hypersensitive response (HR) (Dodds and Rathjen, 2010; Jones and Dangl, 2006). Induction of defense responses is not always restricted only at sites of pathogen reception but also in distal areas of the infected plant which is known as systemic acquired resistance (SAR). SAR is shown to be an effective innate immune response that helps plants to prevent broad range biotrophic pathogens and can also be induced by Salicylic acid (SA) treatment of plants (An and Mou, 2011; Dong

2004; Durrant and Dong, 2004; Fu and Dong, 2013). SAR is a consequence of cumulative actions of ETI and PTI triggered transcriptional changes, increased concentration of cellular SA, induction of multiple downstream signaling cascades along with production of antimicrobial peptides like pathogensis-related (PR) proteins (Mukhtar et al., 2009; Wang et al., 2006). Arabidopsis regulates SA signals through the central immune regulator Nonexpressor of PR genes 1 or NPR1 (Dong, 2004). NPR1 is known to be associated with diverse immune signaling pathways like basal defense in plant, SAR, induced systemic resistance and ETI. It is also reported to mediate crosstalk between SA and other plant hormones (Dong, 2004; Canet et al., 2010; Rate and Greenberg, 2001; Moreau et al., 2012).

Plants lacking functional NPR1 are noticed to be impaired in their capacity to express *PR* genes and are almost fully defective in employing SAR response after pathogen infection (Durrant and Dong, 2004). Plant has been modified with orthologues of NPR1 for crop improvement as it was shown in studies that over expression of *AtNPR1* enhances resistance against root-knot nematode infection (Lui et al., 2002). Likewise over expression of *AtNPR1* induces expression of several defense genes and produced broad spectrum resistance to both biotrophic and necrotrophic pathogens (Wally et al., 2009) and so on. All these reports undoubtedly suggest that NPR1 is a central player in plant immunity and holds significant importance in crop plant protection against pathogens. Over expression of Arabidopsis NPR1 in several crops gives rise to various levels of disease resistance against diverse pathogens.

Significant progress has been made in understanding SA-mediated defense signaling networks which includes functional analysis of a large number of genes engaged in SA biosynthesis, regulation and signal transduction. Studies in various plant species have revealed that pathogen infection leads to SA accumulation in infected as

well as uninfected leaves that generates SAR (Malamy et al., 1990; M'etraux et al., 1990). This accumulation of SA in response to infection also activates expression of *PR* genes and resistance against invading bacteria (Malamy and Klessig, 1992). Also, mutation or application of inhibitor of enzyme responsible for SA biosynthesis has been shown to enhance plant susceptibility to pathogen but the resistance was shown to be possibly restored through exogenous SA (Mauch-Mani and Slusarenko, 1996; Nawrath and Metraux, 1999; Nawrath et al., 2002). After several biochemical analyses two distinct enzymatic pathways for SA biosynthesis have been identified (Lee et al., 1995; Chen et al., 2009). One is phenylalanine ammonia lyase (PAL)-mediated phenylalanine pathway, and the second is isochorismate synthase (ICS) mediated isochorismate pathway. Both pathways are common as per the requirement of the primary metabolite chorismate is concerned which is an intermediate of plant phenylpropanoid pathway (An and Mou, 2011). The well-studied Arabidopsis *SA INDUCTION-DEFICIENT 2 (SID2)* gene is reported to encode for isochorismate synthase, which converts chorismate to isochorismate which is a crucial stage in SA biosynthesis. In *sid2* mutants in addition to reduced amount of SA accumulation, enhanced disease susceptibility to various pathogens was observed which was demonstrated to be rescued by SA treatment (Nawrath et al., 1999; Wildermuth et al., 2001). *sid2* mutants have very low levels of SA after infection by virulent or avirulent bacterial and fungal pathogens. This implies that isochorismate pathway is presumably the main source of SA accumulation during plant-pathogen interaction in case of Arabidopsis (Wildermuth et al., 2001). Not only those *sid2* mutants have impaired SAR (systemic acquired resistance) response, they showed reduced *PR1* gene expression also. (Nawrath and Metraux, 1999).

3.2.5 *Research Goals*

Pathogen perception and induction of defense signaling against invading pathogens are two most important steps of plant immunity against microbial pathogens. Factors contributing in the interaction between plant pathogenic bacteria and plants have been studied extensively. Although it is not known if plant and human pathogens have a common pathogenicity strategy, the idea based on available literature is, plant can actively recognize the presence of enteric pathogens and can ward them off. However, the big question remains unanswered, whether plants also have common defense mechanisms to fight against plant pathogens and enteric pathogens. As human pathogen infection of plants is a comparatively new field of study, to test this hypothesis, here in this chapter my research goals are,

1. To assess the importance of plant surface receptor FLS2 in stomatal as well as apoplastic immunity of plants against SL1344 and O157:H7.

Additionally, Salicylic acid being one of the most crucial downstream signaling molecules contributing enormously in plant defense mechanism upon pathogen perception, my next goal is,

2. To study and understand the role of few popular key players of SA pathway, such as NPR1 and SID2 in plant defense against SL1344 and O157:H7 infections.

3.3 Methods

To further explore molecular mechanism of plant stomatal and apoplastic immunity against these human pathogens and the human pathogenic bacterial behavior *in vitro* we will be assessing bacterial survival and behavior and *in vivo* and *in vitro* conditions respectively.

3.3.1 Stomatal assay

To assess the response of guard cells to both O157:H7 and SL1344 infections, the mutant plants under study were dip-inoculated with bacterial suspension (10^8 CFU.ml^{-1}) and water (mock) using 0.03% Silwet L-77, and incubated at 25^{o}C for the duration of the experiment. The experiment was conducted after plants were under 100 µmol.m^{-2}.s^{-1} light for at least 3 hours in the morning to have the stomata all naturally open before infection. Leaves were plucked from water and bacteria treated plants at regular interval and the abaxial side was imaged under Nikon Eclipse 80i fluorescent microscope. Each time point data consists of 50-70 stomata and stomatal aperture was measured using Nikon NIS Elements imaging software. The experiment was repeated three times.

3.3.2 Pathogenesis assay

To assess correlation of mutant plant stomatal defense and bacterial survival inside apoplast, plants were dip-inoculated with bacterial suspension (1 x 10^8 CFU.ml^{-1}) or water (mock) supplemented with 0.03% Silwet L-77. Arabidopsis ecotype Col-0 was used as control plants for these experiments. The bacterial populations in the plant apoplast were measured as described in Chapter 2.

3.3.3 Swarming assay

Salmonella enterica serovar Typhimurium SL1344 and *Escherichia coli* O157:H7 were tested for their motility on a plate designed for evaluating swarming motility. Media for swarming assay was made with 5g peptone, 3g yeast and 4g agar dissolved in 1L water. Small plates were poured fresh with media just before the experiment. Bacteria were grown in liquid low salt Luria Bertani (LB) medium with proper antibiotic in a shaker at 25ºC to the desired optical density (OD_{600}-0.8-1.1). A dense culture of bacteria having

approximately 1.0 OD was then centrifuge and re-suspended in appropriate amount of water to get 10^7 cells per 10µl. Small discs of regular paper towel with fixed diameter were cut with ethanol sterilized hole-punch and placed on the swarming plates using ethanol-sterilized forceps. 10µl of bacterial inoculum was added on the disc and the plates were covered and incubated under different temperatures (22ºC, 25ºC and 30ºC).All the plates were allowed to sit undisturbed for 30minutes. Later the motility of the bacteria was monitored and photographed.

3.3.4 Chemotaxis assay

Bacterial cultures were grown overnight at 25ºC or RT on shaker to reach desired OD ~0.8-1.1. OD was diluted to 0.3-0.4 approximately in 40ml (volume required for two plates) of culture and then it was centrifuged at 2600 x g for 20 minutes. Pellet was then suspended in 5ml of chemotaxis buffer (30.75ml of 1M K_2HPO_4, 19.25ml of 1M K_2HPO_4 and 20µl of 0.5M EDTA in 500ml double distilled water) and centrifuged again at 2600 x g for 10 minutes. After removing supernatant, re-suspension of pellet was done in 12ml of chemotaxis buffer. 1% hydroxypropylmethylcellulose was added to make a final volume of 15 ml. Using serological pipette, 7.5 ml of viscous bacteria mix was dispensed into petri dishes with identical dimensions. 10µl of testing agent, either plant crude extract or chemotaxis buffer was added in the center of the plate. As a control, drop of 10µl chemotaxis buffer was added in one plate. Arabidopsis leaf extract was used as testing chemical to determine bacterial chemotaxis activity towards or away from the leaf exudates. Glycerol was used as positive control. Plates were kept undisturbed under desired temperature. Bacterial motility was monitored under different temperature conditions such as 22ºC, 25ºC and 30ºC. Visibility for chemotaxis was checked at several time points by checking the radial migration of bacteria towards the testing agent in the

center and was photographed. Photographs were taken under UV light and normal light to monitor bacterial motility.

3.3.5 Satistical analysis

Statistical significance of data from the stomatal assay, bacterial counts in the apoplast was calculated using 2-tailed Student's *t*-test. All experiments reported here were repeated at least two times (biological replicates) using a minimum of three technical replicates.

3.4 Results

3.4.1 FLS2 *is an essential part of plant immunity against human pathogens*

As early mentioned, plant defense system components are important to ward off pathogens. Human pathogens are also recognized by plants in a similar way plant pathogens are detected. For example, flagellin was showed to be an important PAMP of *S. enterica* (Garcia et al., 2013; Meng et al., 2013) and *E. coli* (Seo and Mathews, 2012) which can be detected by plant receptor FLS2. Detection of pathogens helps to trigger plant defense such as stomatal closure in Arabidopsis (Melotto et al., 2006). Because stomatal immunity is a very crucial initial part of plant innate immunity against pathogen attack, to observe the importance of flagellin perception through FLS2 for stomatal response against human pathogens, *fls2* mutant plants were used. As shown in Fig 3.1, O157:H7 induced stronger stomatal closure in wild type as well as mutant plants. Stomatal immunity was noticed up to 4hrs post inoculation of wild type and mutant plants inoculated with O157:H7. But interestingly SL1344 also induced noticeably strong stomatal immunity in mutant plants than wild type Col-0, even after 4hours of inoculation

stomatal closure was noticed to be effective. Reopening of stomata in case of SL1344 infection did not happen in *fls2* mutant plants like wild type plants after 4hours of inoculation. From all these results it can be concluded that O157:H7 and SL1344 trigger differential stomatal immunity in mutant plants than wild type plants.

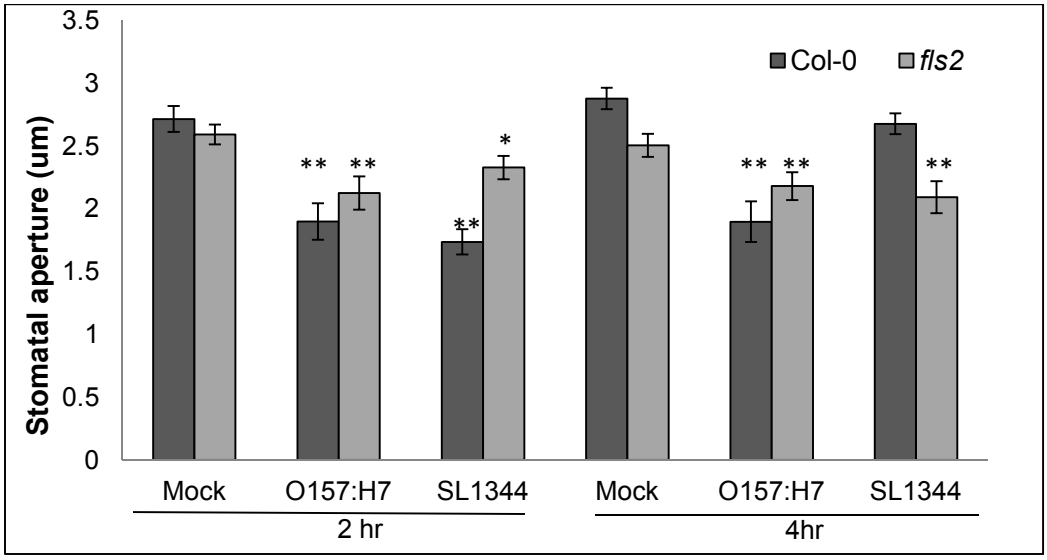

Figure 3.1 Stomatal response in *fls2* plants at 2hr and 4hr post inoculation. The unique stomatal immunity are shown as mean of stomatal aperture width (n=50–70) ± SE. Statistical significance of the difference in the means (mock versus bacterium treatment) was detected with two-tailed Student's *t*-test (*= p<0.05, **=p<0.01*** = p<0.001)

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To correlate stomatal response with bacterial penetration, next the role of FLS2 receptor in apoplastic immunity was assessed during enteric pathogenic strain infection using same mutant plants. Bacterial titers were measured inside dip inoculated mutant plants up to 14 days post infection, which allowed bacterial penetration through stomata. Population inside mutant plant tissue was then compared with population inside wild type plant that same day. Early days of infection of plants with both SL1344 and O157:H7 showed bacteria surviving well inside mutant plants lacking FLS2 receptor (Fig 3.2)

compared to wild type plants. SL1344 demonstrated enhanced population inside mutant plants in early days of infection indicating, perception of SL1344 by FLS2 and associated signaling pathway(s) might play significant role in diminishing SL1344 survival in plant apoplast. In contrary, O157:H7 apoplastic population is found to be significantly more inside plant apoplast during only the initial part of infection. Three days post infection, O157:H7 titer was not found to be significantly higher inside mutant plant than the wild type counterpart. But comparatively better O157:H7 survival inside mutant plants was observed throughout the experiment. Bacterial titers did not multiply inside plant tissue, and very similar kinetics of population survival was observed inside mutant as well as wild type plants. Both bacterial population either declined or remains static over the experimental time range (14days).

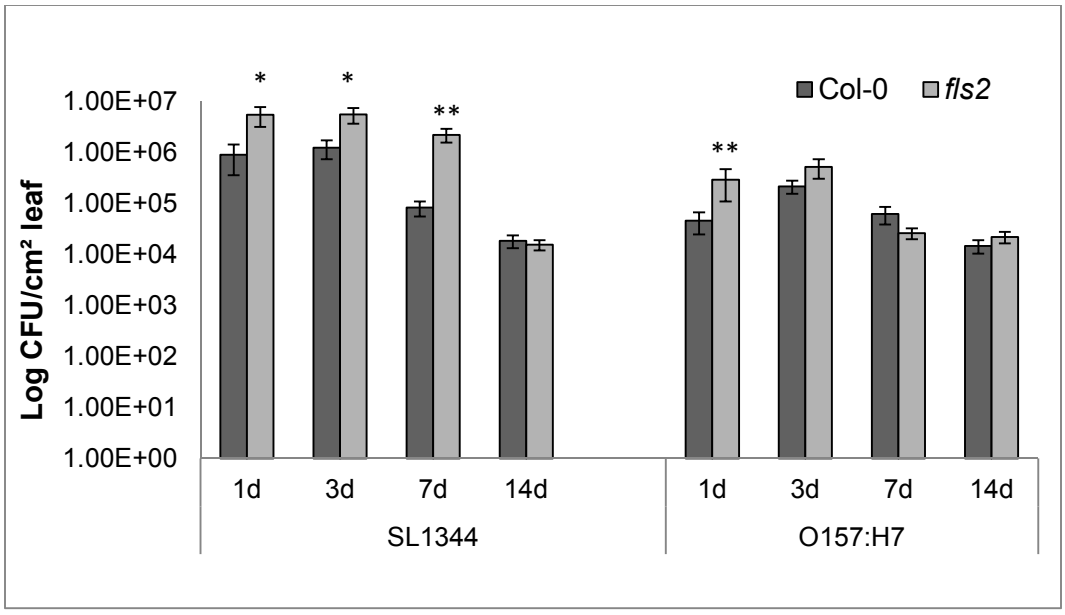

Figure 3.2 Bacterial enumeration in the leaf apoplast at different days (d) after dip-inoculation of Col-0 and fls2 mutant plants with SL1344 or O157:H7. Results are shown as the mean (n=12) ± SE. Statistical significance of the difference in the means between mutant plant and Col-0 was detected with two-tailed Student's *t*-test (* = $p<0.05$, ** = $p<0.01$).

3.4.2 Salicylic acid might be an important factor in plant immunity against SL1344 and O157:H7

When stomatal assay and bacterial pathogenesis assay were performed in *sid2* mutants, it was observed that *SID2* is unimportant for SL1344-trigerred closure as well as subsequent opening (experiment performed by Shweta Panchal and is explained in her dissertation). Re-opening of stomata was also observed in mutant plants infected with SL1344 just like Col-0, the wild type plant. Inoculation of mutant plants with O157:H7 also revealed no difference in stomatal response from Col-0 stomatal response. The ability of *sid2* mutant plants to enforce stomatal closure against SL1344 and O157:H7 negates direct involvement of SID2 in stomatal immunity against these human pathogenic strains.

To further assess whether *SID2* is important for apoplastic immunity against human pathogens, population of SL1344 and O157:H7 inside mutant leaf apoplast was determined over time. Salicylic acid being one of the crucial plant hormones associated with plant defense it was expected that *sid2* mutant plants would support much higher bacterial population inside plant apoplast than wild type plants. However, SL1344 failed to take advantage of the absence of induced production of SA during the early days of infection. SL1344 population was not reasonably different during first few days of infection inside mutant plant apoplast than wild type plant interior. But interestingly this strain shows significantly more survival inside *sid2* plant interior through later days of infection. On the other hand, *E. coli* O157:H7 survived inside *sid2* a lot more than Col-0 plants from the beginning of the infection and the bacterium maintained significantly more population than population inside wild type plants till later days of infection as well (Fig 3.3). As plants are dip-inoculated, the bacterial numbers obtained here depicts the number of bacteria entered naturally into the leaf apoplast. However similar to my previous results none of the tested bacteria showed aggressive growth/multiplication inside any plant tissue and the populations remain static or declined overtime during later days of experiment.

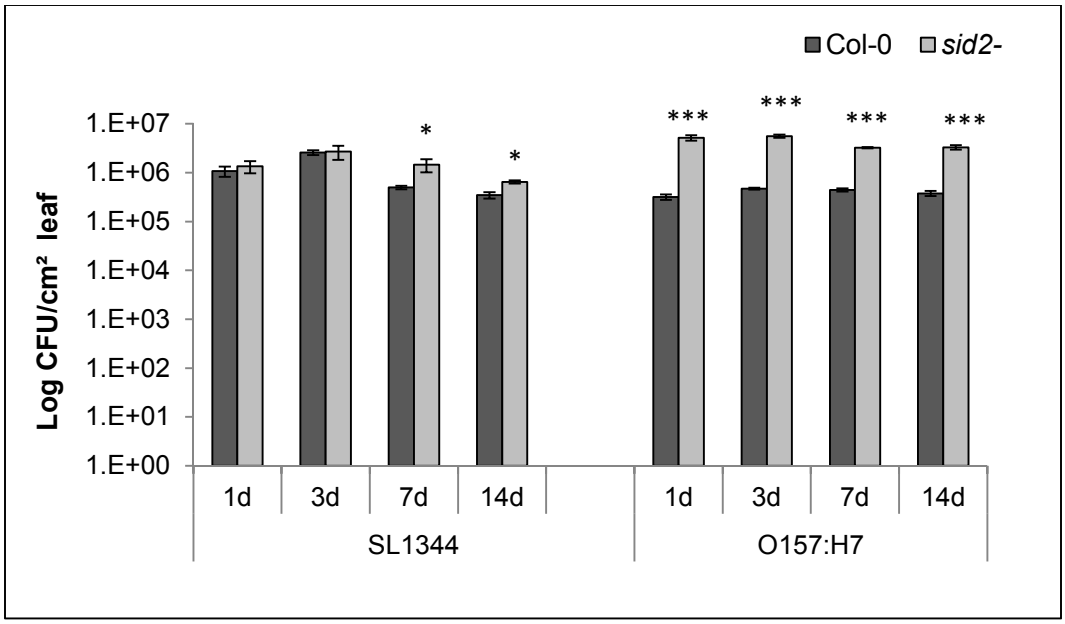

Figure 3.3 Mutant *sid2* plant apoplast favors better survival of human pathogenic strains than wild type plants. Results are shown as the mean (n=12) ± SE. Note that some error bars are too small and do not appear in a log scale bar graph. Statistical significance of the difference in the means of bacterial titer between mutant and wild type plants was detected with two-tailed Student's *t*-test (* = $p<0.05$, *** = $p<0.001$).

3.4.3 NPR1 contributes to controlling bacterial population inside plant apoplast

In this study mutant plant *npr1-1* and *npr1-2* and their response towards SL1344 and O157:H7 infection was examined to assess the role of *NPR1* gene in defense against these specific strains of human pathogens.

First, stomatal immunity of *npr1* mutant plants was tested. Both *npr1-1* and *npr1-2* mutant plants showed similar kinetics of stomatal immunity like wild type plants against SL1344 and O157:H7. Stomatal closure and following re-opening is seen in both mutants in response to SL1344. Persistent stomatal closure is seen in response to O157:H7 for 4h. This is similar to wild-type plant response. As *NPR1* is a downstream component of

defense related SA signaling pathway, similar results compared to wild type plants revealed, mounting plant stomatal immunity against human pathogen is not directly dependent on the activity of NPR1. This data was produced by Shweta Panchal and was included in her thesis.

Next the bacterial titer inside plant leaf tissue was determined which can shed some lights on the significance of NPR1's role in plant susceptibility or resistance against SL1344 and O157:H7. Similar to the *fls2* mutant plants, *npr1* mutant plants showed a trend of supporting more human pathogenic bacteria inside than the wild type Col-0. Although these two allelic mutant plants showed a little difference in the population dynamics of SL1344 compared to the wild type plant, in general SL1344 survived better inside mutant plants (Fig 3.4 A and B). *npr1-1* favored larger SL1344 population inside plant leaf tissue while the difference in population size was significantly more during later days of infection period monitored. But this difference in bacterial population between wild type and mutant plants was not detected by T test in case of *npr1-2* plants. If exposed to O157:H7, *npr1-1* plants supported better survival of this bacterium after or from third day of infection. O157:H7 was found to grow significantly more inside *npr1-1* mutant plants till fourteenth day of infection. Although this trend was to some extend similar for O157:H7 population survival inside the other mutant, *npr1-2,* but the result was not replicable exactly in this mutant like *npr1-1*. Both strains of bacteria were not noted to multiply vigorously in mutant plant apoplastic region over the period of time. However, even wild type plants did not show drastic multiplication of bacteria inside plant tissue and bacterial population declined after a while.

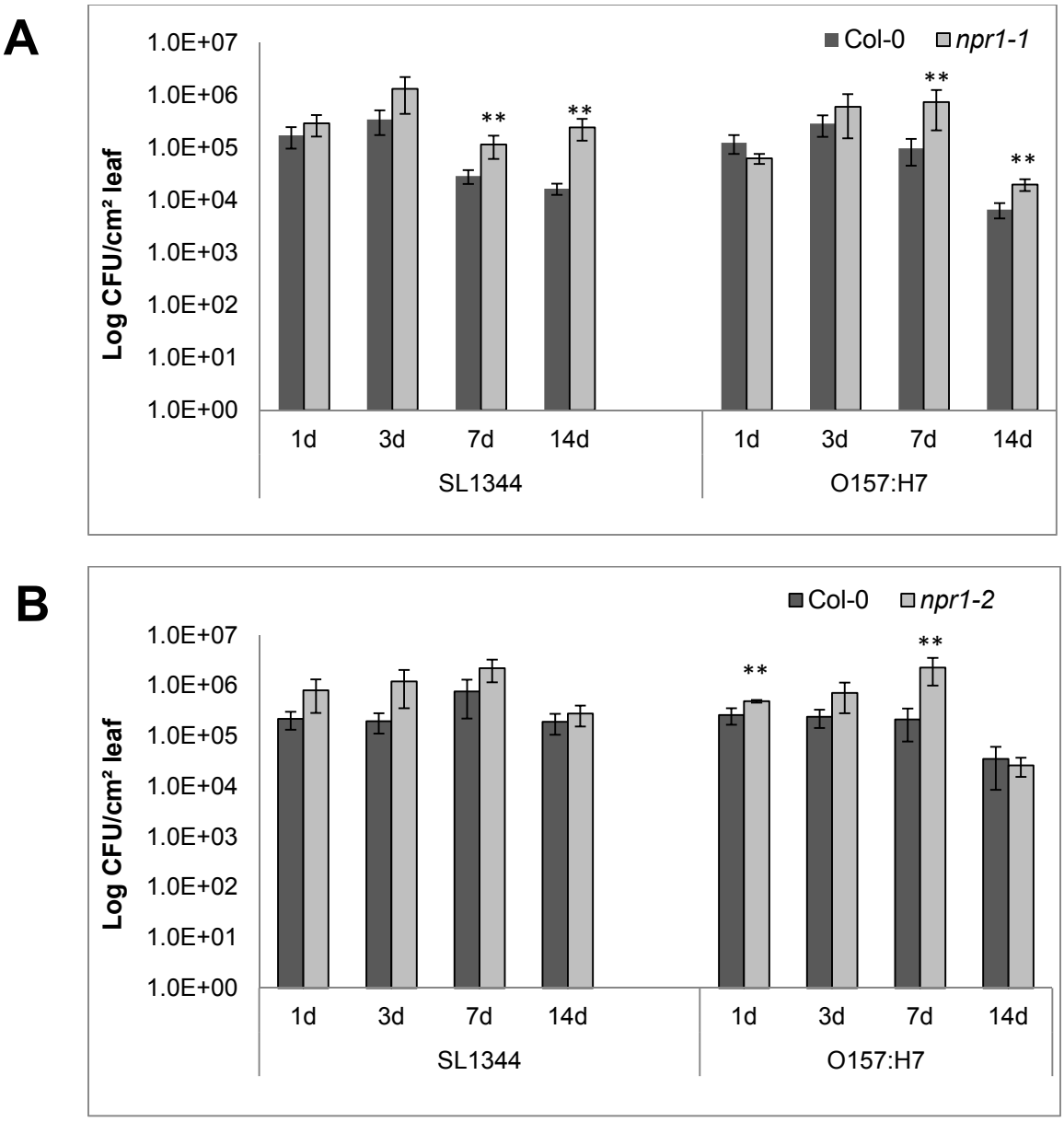

Figure 3.4 Bacterial enumeration in the leaf apoplast at different days (d) after inoculation of Col-0 and *npr1* mutants. **A.** The graph shows bacterial population inside mutant plant *npr1-1* in comparison with wild type Col-0. **B**. This diagram illustrates bacterial titer inside *npr1-2* mutant plants. Results are shown as the mean (n=12) ± SE. Statistical significance of the difference in the means was detected with two-tailed Student's *t*-test (* = $p<0.05$, ** = $p<0.01$).

3.4.4 SL1344 and O157:H7 show efficient swarming motility at 30ºC

To assess the mode of bacterial motility, the abovementioned strains of *Salmonella* and *E. coli* were monitored for swarming efficiency under 22ºC, 25ºC and 30ºC. The strains were able to swarm across the agar surface and showed better motility under higher temperature such as 25ºC and 30ºC, while bacterial motility on the agar surface seemed to be compromised under relatively low temperature like 22ºC (Fig 3.1). These results indicate that these human pathogens being flagellated might move towards favorable location on leaf surface using their flagella and this mobility might be temperature dependent. This conclusion requires more assessment which can decode the motility mechanism of these pathogens in more details and *in vivo* condition and in quantitative manner for proper comparison as well.

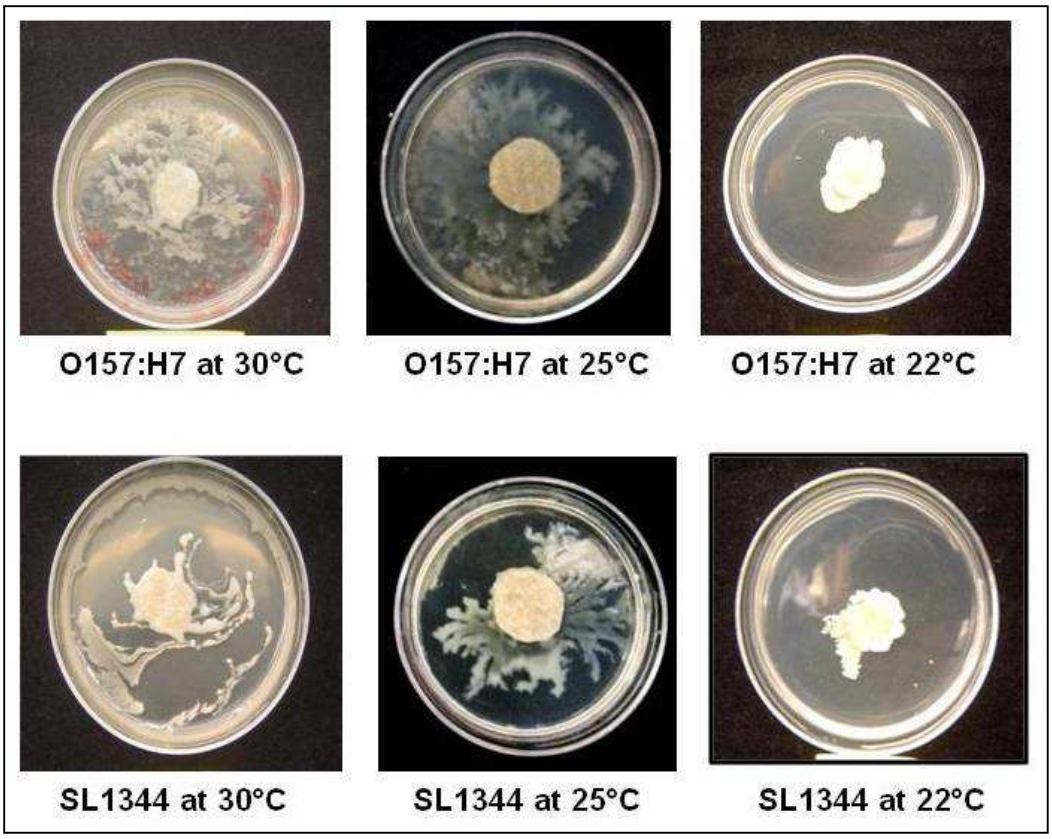

Figure 3.5 SL1344 (**B)** and O157:H7 (**A)** swarm on agar surface. Note high swarming activity at 30ºC and 25ºC as compared to 22ºC

3.4.5. Human pathogenic strain SL1344 and O157:H7 showed chemotatic ability towards plant extract

To get an idea if SL1344 and O157:H7 could sense plant exudates to be a chemical signal on plant leaf surface and if both are capable of travelling towards signal perceived, chemotactic ability of these strains are checked next. As per the hypothesis, both the test strains showed chemotactic movements *in vitro* towards plant leaf extract showing their affinity towards chemical components found in the same. In this study, both the human pathogenic strains were tested for chemotaxis at 5, 10, 15, and 30min after adding specific chemical attractants in the plates containing bacterial inoculum. Both

strains under scrutiny showed maximum chemotactic movement in the range of 5mins to 30mins post inoculation. After 5mins of incubation, a radial migration of bacteria towards the center was promoted and observed in case of both SL1344 and O157:H7 when plant extract was added in the center of the chemotactic plate as a chemoattractant (Fig 3.6 and Fig 3.7). No such cloudy appearance indicating migration in case of buffer plate suggested that the response was due to the presence of specific chemicals of plant extract in the medium. SL1344 and O157:H7 also showed positive chemotactic movements towards positive control, glycerol (Fig 3.6). Same experiment was performed again where the pictures were taken using UV light for confirmation. Similar movements were observed in the presence of plant extract for over 30mins after incubation of plates and this chemotactic movement was not detected after 30mins of incubation time (Fig 3.7). These observations indicate the capability of these pathogens to sense chemical signal such as plant crude extract and move towards it. Though this experiment does not vouch for the same mechanism happening on leaf surface but these results are strong indicator of similar mechanism occurring on phyllosphere. This will be followed up in my next study for more specific answer about human pathogen behavior on plant leaf surface by an *in vivo* study of bacterial chemotaxis.

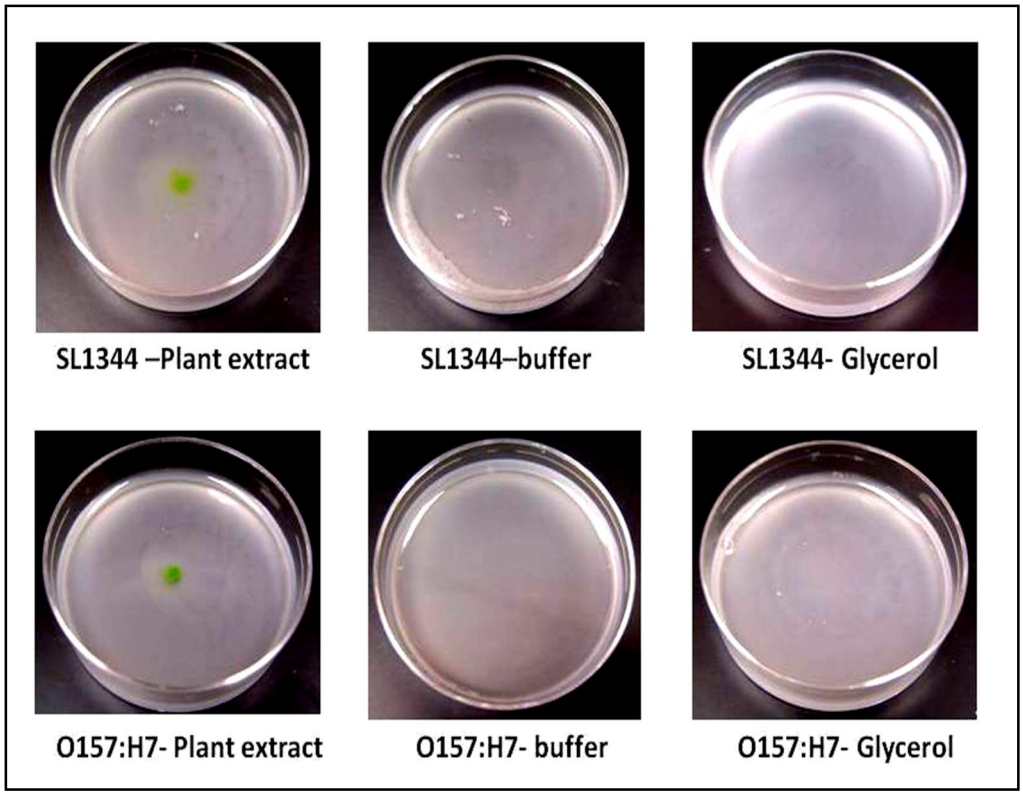

Figure 3.6 Chemotactic movement of SL1344 and O157:H7 at 15mins post inoculation on swim agar plate. **A**. Chemotactic behavior of SL1344 in the presence of Arabidopsis plant extract, control buffer and positive control glycerol. **B**. Chemotaxis of O157:H7 in the presence of similar plant extract, control buffer and glycerol.

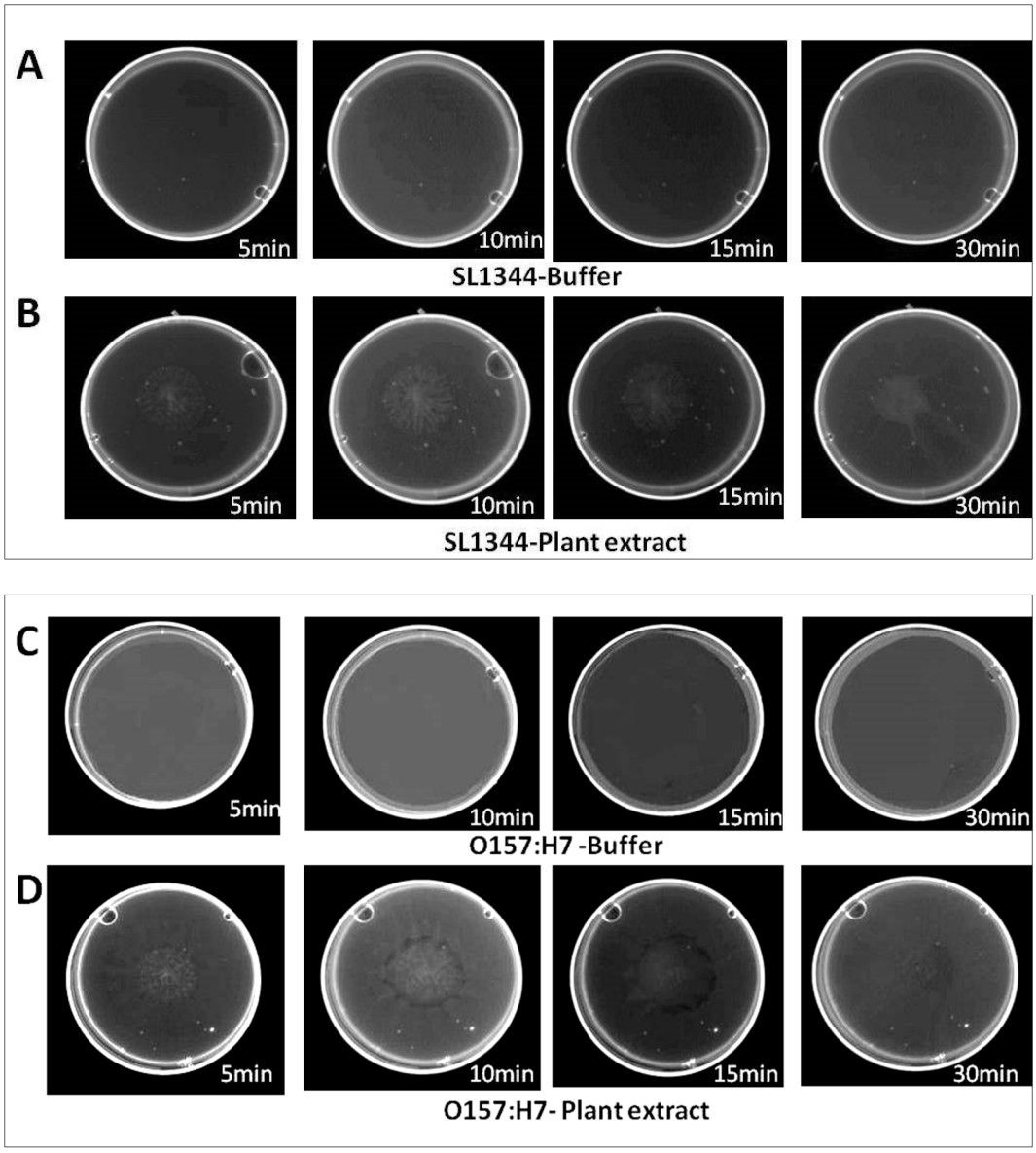

Figure 3.7 Chemotaxis of SL1344 and O157:H7**. A.** and **B** showing SL1344's chemotactic motility towards buffer and plant extract respectively at different time point. **C** and **D** represent chemotaxis of O157:H7 in the presence of buffer and plant juice in vicinity. Pictures were taken under ultra violet light for good imaging.

3.5 Discussion

Human pathogenic bacteria that are able to persist and proliferate outside animal hosts might interact directly with plants to exploit them as alternative hosts. The interaction of plant with human pathogens is pretty complex which involves adaptive processes for both the plant and the bacterium. Experimental evidences have been growing over times which show functional roles for several bacterial as well as plant factors. Moreover, plants are able to respond to bacterial infection and mount effective response. But not only plant defense, but also bacterial behavior is a major unexplored area of research which can help understanding initial incidents which eventually lead to contamination of plants. Knowing enteric pathogen's behavior outside their normal habitat and their survival on/in plants along with plants strategies to defend them might answer many questions and help protect plants from further contamination.

Stomatal immunity being one of the most important first lines of defense responses against pathogenic bacteria upon bacterial attack, perception of bacteria through FLS2 receptor is one of the most important factors in plant defense (Melotto et al., 2006). Innate immunity which is triggered through the action of the transmembrane receptors also includes activity of endogenous salicylic acid (SA) such as, metabolic and physiological responses in plants involved in plant defense against plant pathogenic bacteria (Lu, 2009; Chen et al., 2009; Vincent and Plasencia, 2011). It is still unknown if crucial components of stomatal immunity and SA biosynthetic pathway hold equal relevance in plant defense against all human pathogens and whether the observed bacterium- and PAMP-induced stomatal closure suffice for restriction of bacterial entry through the epidermis. To address this important question directly, this study was performed using mutant plants either with absence of FLS2 receptors or impaired in SA biosynthesis upon human pathogen attack. This will shed light on signal transduction

pathway involved in stomata-based defense during plant and human pathogen interaction. Plant and human pathogen association being very cultivar and strain specific this study will contribute to that filed answering question about plant and SL1344 and O157:H7 interaction specifically. Hopefully that will provide new perspectives that will aid the combat against human pathogens and improve our knowledge about plant defense against pathogens like *Salmonella* and *E. coli*.

Initial communication of plant and pathogens is mostly followed by plant perception of these non-plant pathogenic bacteria which further triggers innate immune response in plant making them resistant to the bacteria. The FLS2-mediated resistance was shown to be effective against bacteria inoculated onto the leaf surface, which mimics natural infection (Zipfel et al., 2004; Kim et al., 2005). However few recent studies have enabled the detection of *S. enterica* and *E. coli* O157:H7 inside the plant tissue after surface inoculation (Gu et al., 2011; Golberg et al., 2011; Barak et al., 2011; Roy et al., 2013). So assessing the importance of FLS2 receptor for plant immunity against human pathogenic strains was essential to see how stomata based defense is linked to this receptor. My study showed, inoculation with both SL344 and O157:H7 triggered strong stomatal immunity in mutant *fls2* plants. Unlike mutant plants, SL1344 infected Col-0 plants did not show persistent stomatal immunity and after 4 hour post inoculation stomata reopening occurred. Whereas O157:H7 could induce a persistent stomatal immunity in wild type Col-0 plants (Fig 3.1). These results indicate existence of stomatal immunity in Arabidopsis even in the absence of the most studied PAMP receptor, FLS2. This supports the idea that though FLS2 is a well-studied receptor of bacterial PAMPs which helps in mounting stomatal immunity, there are probabilities of other mechanism by which plant can still perceive human pathogens and trigger stomatal closure. Or in other words stomatal immunity is not solely dependent on FLS2 receptor only. Contradictory

result in SL1344 infection in mutant *fls2* plants which shows no stomatal reopening after 4hr of inoculation like wild type Col-0 plants also indicates that FLS2 may be required for stomatal re-opening in the presence of SL1344.

Dip inoculation of *fls2* plants to correlate stomatal immunity with bacterial penetration showed higher bacterial count inside plant apoplast which indicates FLS2 to be one of the most important receptor for bacterial PAMPs. SL1344 population count in mutant plant apoplast was found to be significantly higher than the counts in wild type plants in the early as well as late days of infection. This indicate active participation of FLS2 receptor towards downstream defense response to diminish bacterial survival inside plant apoplast. Even though FLS2 is the most well-known receptor in Arabidopsis, absence of this receptor did not show statistically significant difference in O157:H7 population inside mutant plants than wild type plants during the later stages of infection. However significantly more O157:H7 pathogens inside *fls2* on the first day of inoculation of plants helped to conclude that FLS2 might be one of the prime factors to inhibit O157:H7 penetration inside plant interior (Fig 3.2). It is tempting to predict that absence of FLS2 might not disturb the PAMP perception significantly but can affect overall plant immunity by making the interior of mutant plants more favorable for enteric bacterial survival. It is likely that perception of bacterial PAMPs through FLS2 receptor might induce specific signal transduction pathway(s) which makes plant apoplast less favorable place for human pathogens to sustain their life. Though it was not possible to rule out the presence of other receptors for human pathogen perception with my study but it was reasonable to conclude that enteric pathogen perception through FLS2 trigger signaling pathway which helps in reducing bacterial penetration and survival inside plant interior.

Similar to *fls2* mutant plants, *sid2* plants also favored higher number of O157:H7 and SL1344 population suggesting Salicylic acid to be a significant part of plant defense

mechanism against these strains of human pathogens. Although the counts for SL1344 population was only significantly more inside mutant plant after 3 days of infection, a trend was observed with slightly higher counts of SL1344 inside *sid2* plants during early infection time as well. On the other hand, significantly large O157:H7 population inside mutant plants was noticed throughout the test period (Fig 3.3). These results cumulatively explain the involvement of SA in defense signaling pathway even during these human pathogenic infections of plants. It was also proved earlier that both salicylic acid dependent and independent pathways are induced by *S. enterica* 14028 in Arabidopsis seedlings (Iniguez et al., 2005). But adult Arabidopsis leaves showed requirement of jasmonate and ethylene signaling also for defense against the same strain (Schikora et al., 2008). So it might also be true that, for SL1344 induced defense mechanism plants require more than just SA pathway. This conclusion requires follow up with other SA mutants along with mutant plants impaired in other hormonal pathways.

Other than *sid2* numerous Arabidopsis mutants with defects in SA signaling have been characterized and one of them is *npr1* (for nonexpresser of *PR* genes) (Pieterse et al., 1998; Cao et al., 1994). Zhang *et al.* provided evidence that, upon initiation of SAR, NPR1 activates *PR-1*gene expression by physically interacting with a leucine transcription factors that bind to promoter sequences required for SA-inducible *PR* gene expression. In my study, bacterial population inside two mutant plants *npr1-1* and *npr1-2* were tested to evaluate the importance of NPR1 during plant defense against enteric pathogens. Both mutant plants exhibited a trend of higher population counts of both the bacterial strains tested inside mutant plant tissue than wild type plants. But the kinetics of bacterial population survival inside plants varied widely. Like *sid2* mutant plants, *npr1-1* mutants supported comparatively higher number of SL1344 after few days post inoculation indication participation of SID2 and NPR1 in late defense response of plants

towards SL1344. Though O157:H7 showed more sustainability inside mutant plant *npr1-1* and *npr1-2*, and the population dynamics is similar in both mutant plants this study failed to claim exact significant difference in bacterial population inside both mutants compared to the wild type plans. From this study it was apparent that immunity against these enteric pathogens is compromised in the absence of the NPR1 protein which indeed suggests NPR1 to be an important component in plant defense pathway against SL1344 and O157:H7.

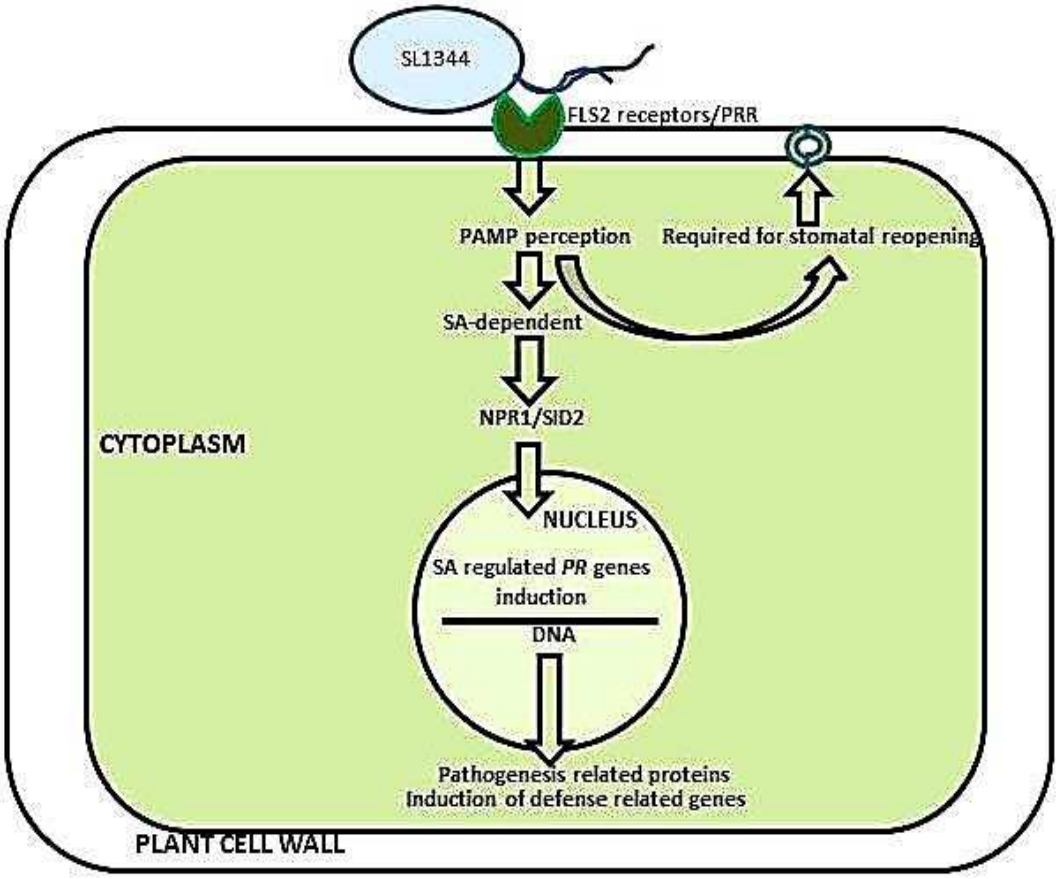

Figure 3.9 Indicated importance of FLS2, SID2 and NPR1 in plant defense against SL1344 infection

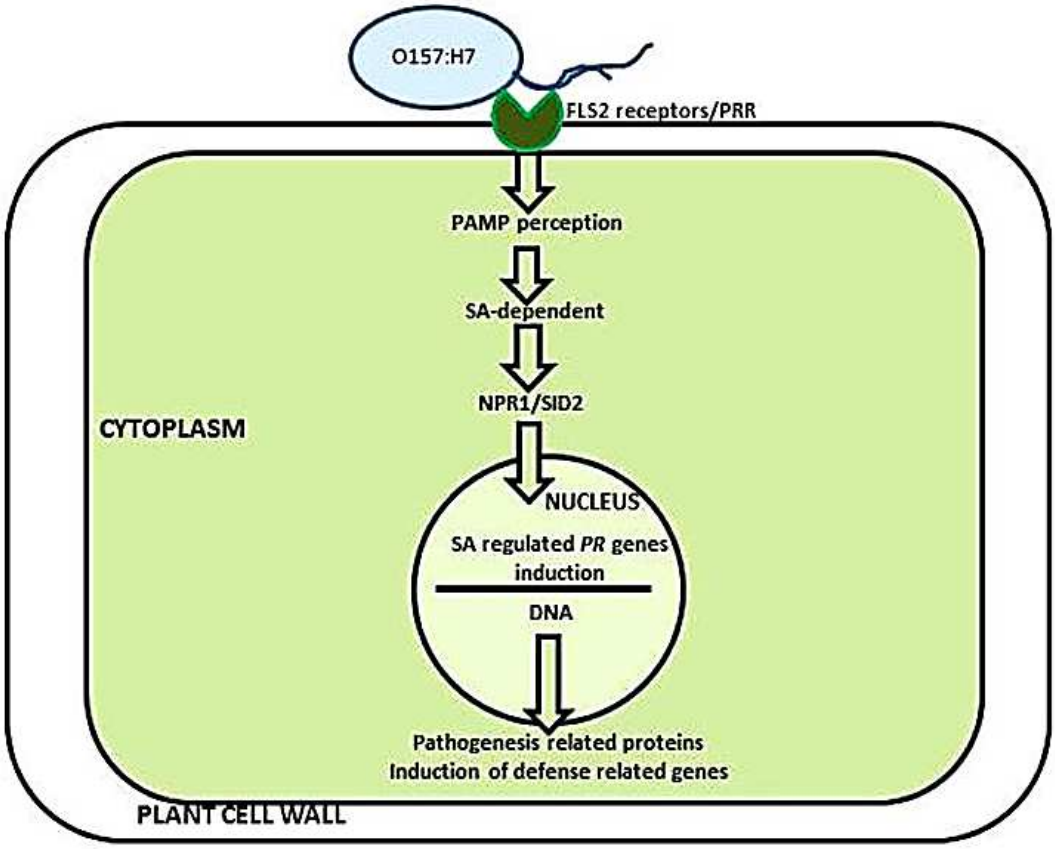

Figure 3.10 Indicated importance of FLS2, SID2 and NPR1 in plant defense against O157:H7 infection

Not only inside plant apoplast, evidences suggest human pathogens can also adapt on phyllosphere and one of the main factor helping in this process might be the presence of flagella (Kroupitski et al., 2009). A specific motility was discovered in well-characterized bacteria *E. coli* and *S. enterica* serovar Typhimurium which is known as swarming motility (Harshey and Matsuyama, 1994) which might be the tactic these human pathogens use to reach to stomata, wound, lesions. The swarming motility tested and described in this chapter confirms that both my testing strains of human pathogens are motile and motility is enhanced under 25ºC and 30ºC rather than 22ºC (Fig 3.5). This

gave an idea that the discrepancy in the survived population of SL1344 and O157:H7 inside plant tissue described in previous chapter (Fig 2.2) might not be a result of differential swarming activity of bacteria towards stomata (because plants were inoculated with same concentration of bacterial inoculums of SL1344 and O157:H7 and kept under similar environmental conditions) like Krouptiski and group showed with their research (Krouptiski et al. 2009). Though not quantitatively assessed, motility of SL1344 and O157:H7 at 25ºC, (which was the temperature maintained for stomatal and pathogenesis assay) did not seem to be very different. Lower swarming activity at 22ºC also indicated requirement of higher temperature for proper swarming of bacteria *in vitro* condition. In my future studies these strains will be tested for their *in vivo* swarming capability which might give a better idea of bacterial swarming behavior on phyllosphere.

Several *Salmonella* and *E. coli* strains have been studied for their chemotactic characteristics also. Here in this study, both the test organisms were analyzed with respect to their chemotactic pattern *in vitro* using plant extract as the chemical attractant. This would help to predict if these pathogenic strains can sense photosynthates and other sugars or amino acids leaching out from stomata and wound on plant surface and move towards those chemical signals using chemotaxis and eventually reach entry port like stomata or wound to penetrate into plant tissue. Both pathogenic bacteria tested showed chemotactic movement towards plant crude extract as depicted in Fig 3.6 and 3.7. Bacterial movement towards plant extract was clear by a radial migration of bacteria in plate which finally formed a dense cloudy appearance surrounding the extract. Disappearance of this signal dependent activity was noted after several minutes of incubation, when no such dense ring formation was found anymore in plates. This activity indicated efficiency of these strains to sense chemicals in their vicinity and their ability to quickly act towards it. This activity will also be assessed *in vivo* condition in my

upcoming studies to get a clear knowledge about bacterial modulation of chemotaxis on plant leaf surface.

All these results might explain the affinity of human pathogens for specific niche of leaf surface which protects them from UV, harsh sunlight, fluctuating temperature etc. Mobility of bacteria might also clarify the incidence of *S. enterica* near stomata (Krouptiski et al., 2009), naturally occurring wound, lesions, penetration through stomata, and travel of pathogens to un-inoculated plant parts from inoculated parts. Future *in vivo* study with human pathogenic strains of bacteria might be useful to learn the interaction of bacteria and plant as well as bacterial behavior on the plant surface.

In the literature evidences are there mentioning the importance of plants perception of bacterial flagellin through a PRR known as FLS2 which eventually mounts multifaceted downstream defense responses (Boller and Felix, 2009). Not only that, the importance of SID2 and NPR1 in mounting resistance against plant pathogens also have been discussed widely (Tsuda et al., 2008; Tsuda et al., 2009; Pieterse et al., 2012; Durrant and Dong, 2004). Absence of FLS2 shows necessity of this transmembrane protein in stomatal reopening during SL1344 infection. Contradictorily, absence of SID2 and NPR1 did not show any influence on stomatal immunity. But absence of all these proteins helped enteric bacterial survival inside plant tissue making plants more susceptible for infection.

The study was directed to answer a question, do plants use similar modes of defense strategies against plant pathogens and human pathogens? Taken together, the results from this study supported the idea that molecular components important for plant defense against plant pathogens are still being used by plants to mount apoplastic immunity against enteric pathogens as well. But as more researches are being conducted to reveal the mystery behind this cross-kingdom interaction, it is becoming clearer that it

is more complicated and specific than previously thought. Research with individual plant cultivar and bacterial strain might build a good foundation for understanding enteric pathogen association with plants.

3.6 Conclusion

Till date it is known that plant can defend themselves against most microbial pathogens by a basal immune system known as innate immunity. A lot have been reported regarding plant defense against plant pathogens. Perception of pathogen or microbial associated molecular patterns (PAMPs or MAMPs) by host encoded pattern recognition receptors (PRRs), PAMP triggered immunity (PTI), hormonal crosstalk are few of the most important strategies used by plants as their defense mechanisms. This study was conducted to accumulate information about the role of various important signaling pathways in plant against *Salnomella enterica* and *E.coli* to fight endophytic colonization. Plant immunity was tested at both Pre-internalization and post-internalization stages of human pathogen infection. The inclusive plant cellular responses to this biotic stress indicate that several components of plant defense pathways play similar roles against human pathogenic infection as they were found to against plant pathogens. This study indicates that, perception of pathogens through plant receptor and induced salicylic acid play vital role in immunity against human pathogenic strains. More studies will be persuaded in future to fill the rest of the puzzle to make a broad picture of plant signaling components/pathways working against or with human pathogens. I believe this will definitely provide important research-based information for specific design of prevention measures of foodborne diseases due to this relatively new incident of human pathogens and plant interaction. This eventually will help to reduce foodborne

outbreaks and will improve quality, safety and marketability of fresh produce or edible plants.

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Biographical Information

Debanjana Roy completed her Bachelor degree in Botany from Presidency University, Kolkata, India. She did her Master degree from Calcutta University, India. She enrolled as a doctoral student at the University of Texas at Arlington in 2010 and completed her degree in May 2015 under the guidance of Dr. Maeli Melotto. Debanjana worked as a graduate teaching assistant while completing her doctoral degree. In the future she wants to pursue research and work related to food microbiology.