INVESTIGATING BEHAVIORS OF ZOONOTIC AND HUMAN PATHOGENS ON PLANTS

by

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ABSTRACT

The Centers for Disease Control and Prevention (CDC) estimates the incidence of foodborne illness attributed to fruit and vegetable consumption at three million cases in the U.S. annually (Painter *et al.,* 2013). The means by which food crops become contaminated with foodborne pathogens as well as how these organisms persist within the phyllosphere and rhizosphere of these plants is an extremely complex issue. In addition, the CDC estimates that unspecified agents are responsible for 38.4 million out of the 48 million (80 %) cases of foodborne illnesses. It is hypothesized that environmental *E. coli* not typically associated with the ability to cause disease in humans could potentially be responsible for some of these cases. The role of avian pathogenic *E. coli* (APEC) in human disease remains questionable, although genomic evaluations of these isolates suggest the ability of these extraintestinal pathogenic *E. coli* (EXPEC) to cause intestinal illnesses as well as foodborne urinary tract infections (FUTIs) in humans. In these studies we demonstrate the enhanced ability of avian pathogenic *E. coli* (APEC) to persist on leafy greens indicating enhanced environmental fitness in these isolates and potential risks to human health.

Outbreaks associated with food crops continue to occur and it is clear that different approaches are critical to enhance the safety of these foods. Within the last few years the fields of food safety and plant science have begun to merge to more efficiently address some of the knowledge gaps involving the mechanisms by which

human pathogens contaminate plants. We currently know a great deal about the mechanisms by which plant pathogens are able to cause disease in plant crops as well as the immune response of these plants to these pathogens. By studying the relationships between plant pathogens and the plant immune response, scientists have been able to successfully develop biocontrol strategies to reduce crop damage attributed to plant disease. In these studies we also demonstrate the benefits of a plant growth promoting rhizobacteria (PGPR), *Bacillus subtilis* UD1022, and its ability to increase the plant immune response to reduce the persistence of *Listeria* on lettuce plants as well as its inhibitory properties towards *L. monocytogenes* on cantaloupe rind.

In addition to studying plant-microbe interactions between plants and human bacterial pathogens, it is also important to address plant associations with human viruses. Human norovirus is the leading cause of foodborne illness worldwide with the majority of outbreaks linked to fresh produce and leafy greens. It is essential that we also thoroughly understand the type of relationship and interactions that take place between plants and human norovirus in order to better utilize control strategies to reduce transmission of norovirus in the field onto plants harvested for human consumption. In these studies the expression of gene markers for the salicylic acid and jasmonic acid plant defense pathways were measured and compared in romaine lettuce (*Lactuca sativa*) and *Arabidopsis thaliana* Col-0 plants were inoculated with either MNV, TV, human norovirus GII.4 or HBSS (control). The results of these studies suggest that the jasmonic acid pathway of plant defense is likely involved in the plant immune response to human norovirus. This research provides the first pieces of

information regarding how foodborne viruses interact with plants in the pre-harvest environment.

PART I: **ASSOCIATIONS BETWEEN AVIAN PATHOGENIC *ESCHERICHIA COLI*, HUMAN DISEASE AND SURVIVAL ON PLANTS**

Chapter 1

OLD FRIENDS IN NEW PLACES: EXPLORING THE ROLE OF EXTRAINTESTINAL *ESCHERICHIA COLI* IN INTESTINAL DISEASE AND FOODBORNE ILLNESS

1.1 Abstract

The emergence of new antibiotic-resistant *Escherichia coli* pathotypes associated with human disease has led to an investigation in terms of the origins of these pathogens. According to the Centers for Disease Control and Prevention, unspecified agents are responsible for 38.4 million out of the 48 million (80 %) cases of foodborne illnesses each year in the United States. It is hypothesized that environmental *E. coli* not typically associated with the ability to cause disease in humans could potentially be responsible for some of these cases. In order for an environmental *E. coli* isolate to have the ability to cause foodborne illness, it must be able to utilize the same attachment and virulence mechanisms utilized by other human pathogenic *E. coli*. Recent research has shown that many avian pathogenic *E. coli* (APEC) isolated from poultry harbor attachment and virulence genes also currently found in human pathogenic *E. coli* isolates. Research also suggests that, in addition to the ability to cause gastrointestinal illnesses, APEC may also be an etiological agent of foodborne urinary tract infections (FUTIs). The purpose of this article is to evaluate the evidence pertaining to the ability of APEC to cause disease in humans, their potential for zoonotic transfer along with discussion on the types of illnesses that may be associated with these pathogens.

1.2 Introduction

There is currently some speculation as to whether atypical *E. coli*, not currently identified as having the ability to cause gastrointestinal illness, known as extraintestinal pathogenic *E. coli* (ExPEC), could actually be classified as foodborne pathogens. Subgroups of ExPEC include uropathogenic *E. coli* (UPEC) avian pathogenic *E. coli* (APEC) and neonatal meningitis *E. coli* (NMEC). Given the history of foodborne outbreaks associated with *E. coli* and the recent recognition of new pathotypes, it is important that ExPEC be evaluated for their ability to cause disease in humans, attach to food surfaces, and for their ability to persist in the agricultural environments. Evaluating these factors will assess the potential for zoonotic transfer as well as what types of illnesses are associated with these pathogens.

After the 2006 spinach outbreak, as more environmental testing for the presence of non-pathogenic and/or enterohemorrhagic *E. coli* (EHEC) in produce-growing or pre-harvest environments increased, scientists began to investigate further the virulence characteristics of environmental *E. coli*. These *E. coli* include a subset of the ExPEC - avian pathogenic *E. coli* (APEC) which may play a role in human disease (Figures 1 and 2). In the past an assumption was made that APEC strains do not possess zoonotic potential however; research has shown that APEC strains and their plasmids may be transmitted to human hosts (Levy *et al.,* 1976; Saif, 2008*).* In

this regard it is important to evaluate the genotypic profile of other environmental *E. coli*. Specifically, APEC and other *E. coli* isolated from environments be assessed for their potential to transfer from agricultural environments to humans.

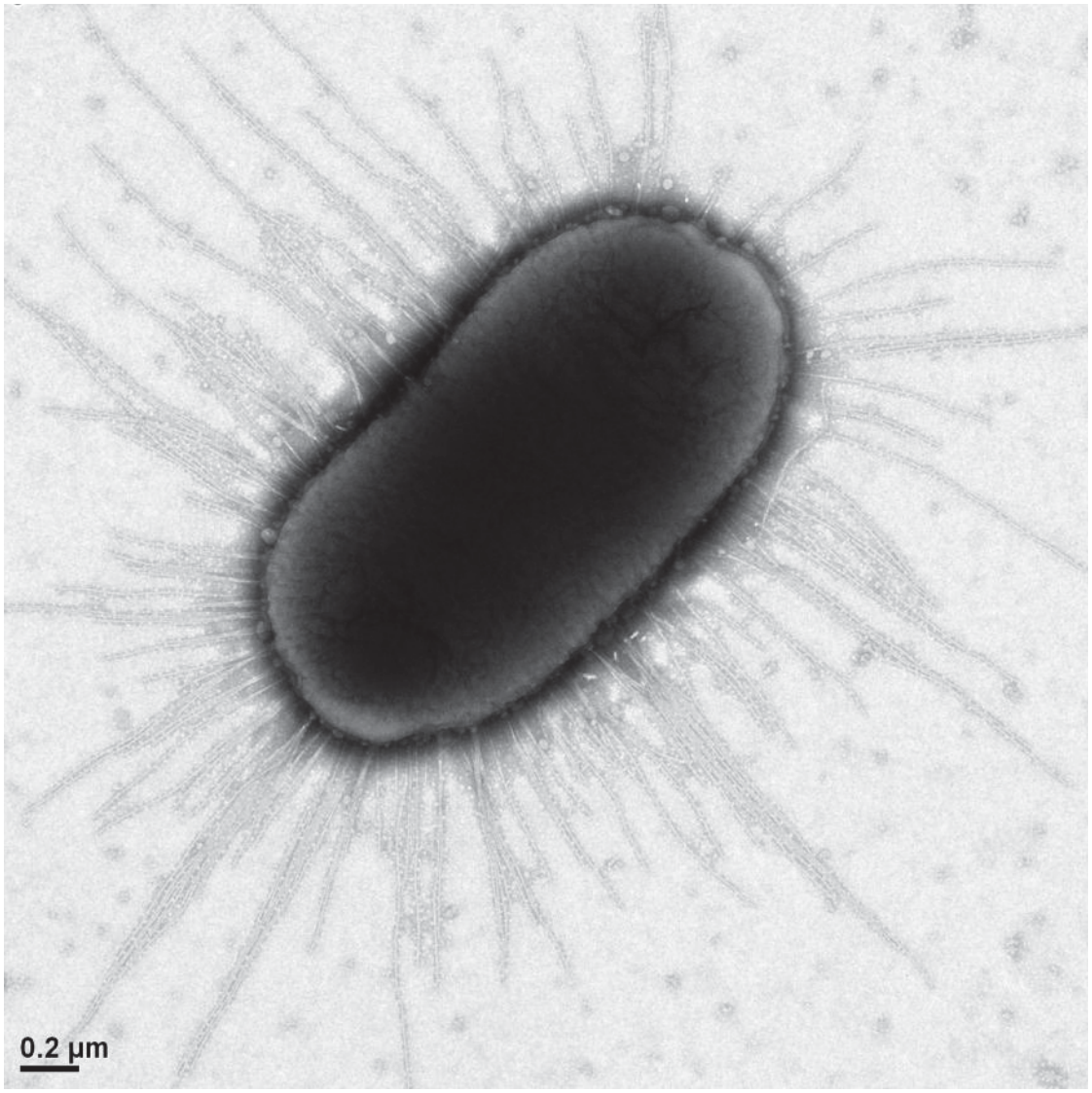

Figure 1: Transmission electron microscopy image of an avian pathogenic *E. coli* (APEC) isolated from the Delmarva Peninsula. Image taken at the Delaware Biotechnology Institute (Newark, DE).

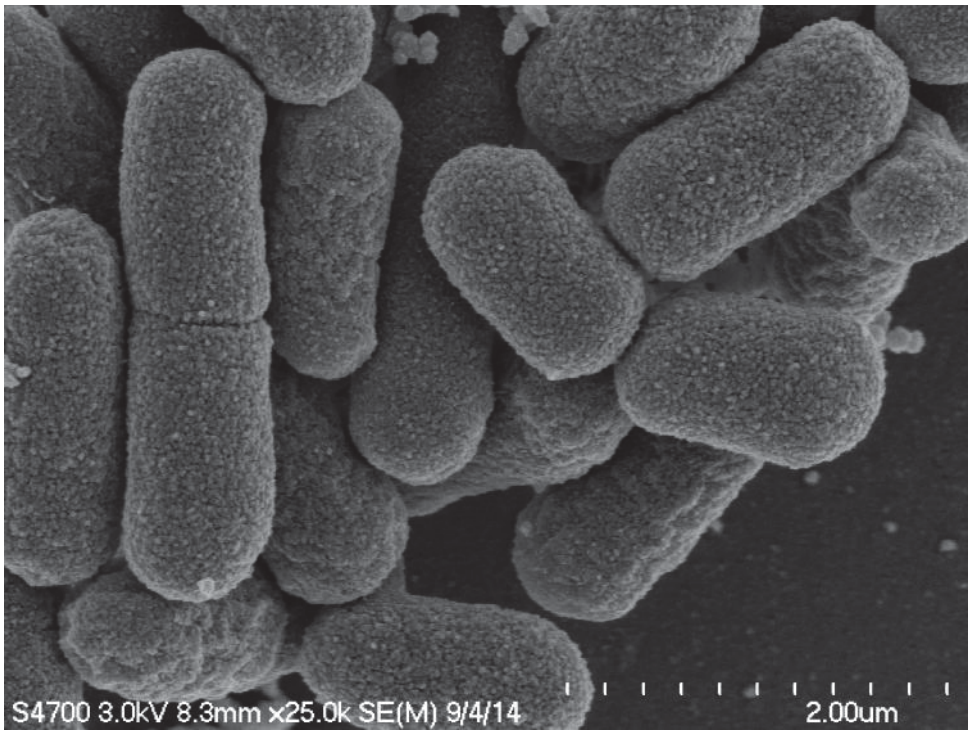

Figure 2: Scanning electron microscopy image of a cluster of avian pathogenic *E. coli* (APEC) cells isolated on the Delmarva Peninsula. Image take at the Delaware Biotechnology Institute (Newark, DE).

1.3 Zoonotic Potential

E. coli were previously organized into different phylogenic groups according to Clermont *et al*. (2000), including groups A, B1, B2, and D. Groups A and B1 are composed mostly of commensal strains, but some may harbor virulence genes. Groups B2 and D are composed of pathogenic strains with human pathogenic strains belonging majorly to group B2, and animal pathogenic strains belonging to group D (Clermont *et al.,* 2000; Johnson *et al*., 2008). One study in which over 1,000 ExPEC isolates were classified into phylogenic groups and screened for a variety of virulence-associated genes found that bacterial acquisition of the ColV virulence plasmid by bacteria in the B2 phylogenic group resulted in a mixed genotypic cluster (Johnson *et al.,* 2008). The strains from this mixed genotypic cluster exhibited enhanced virulence and the ability to survive in a variety of environments under multiple pressures, indicating increased potential for zoonoses (Johnson *et al*., 2008). It is possible that environmental *E. coli* included in this "mixed genotype" could contaminate our food sources, attributing to a potential foodborne illness. Recently Clermont *et al*., (2013) proposed the use of a PCR method based on large amounts of genomic sequence data that can be used to distinguish *E. coli* into one of 8 groups which include A, B1, B2, C, D, E, F or cryptic clade I. Phylo-group E includes a formerly small set of unassigned strains of which *E. coli* O157:H7 belongs (Tenaillon *et al*., 2010). Phylo-group F represents a pool of strains considered to be a sister group to those in phylo-group B2 (Jaureguy *et al.,* 2008; Clermont *et al*., 2011). Phylo-group C represents strains very similar to, but distinct from those in phylo-group B1 (Moissenet *et al.,*

2010, Clermont *et al*., 2011). It was proposed that *Escherichia* clade I also be considered a phylo-group due to the high occurrence of genetic recombination detected between strains belonging to clade I and *E. coli* (Luo *et al.,* 2011).

Another study by Stacy *et al.* (2014) showed for the first time prevalence of the ECP (*E. coli* common pilus) found in APEC strains as well as the potential role the ECP plays in the virulence of APEC isolates. The ECP is a newly associated member of the chaperone usher (CU) fimbriae family and is comprised of polymerized EcpD tip adhesion as well as a shaft major pilin EcpA (Garnett *et al.,* 2012). ECP has been shown to play a role in biofilm formation of bacteria as well as the ability for human pathogenic *E. coli* to recognize host cells (Avelino *et al.,* 2010; Lehti *et al*., 2010; Saldana *et al.,* 2009). The authors of this study found that 76 % (127/167) of the clinical APEC isolates screened were positive for *ecp*A by PCR. Deletion of the *ecp*A gene decreased biofilm formation and bacterial swimming *in vitro* in HeLa cells and decreased virulence in day old chicks including decreased colonization of bacteria in the bloodstream of these chicks (Stacy *et al.,* 2014). The authors concluded that APEC share virulence genes with human pathogenic *E. coli*, specifically the *ecp* genes which is commonly found in intestinal and extraintestinal *E. coli*, indicating the potential ability of APEC isolates to cause disease in humans (Stacy *et al.,* 2014).

Because poultry serve as the main host for APEC, there is potential for zoonotic transmission where humans may become infected with APEC through consumption of undercooked poultry. Raw produce that was fertilized with poultry litter may also serve as a means for zoonotic transmission. Poultry litter is a valuable soil

amendment, but has been shown to harbor a large number of enteric bacteria, including potentially pathogenic *E. coli* and *Enterococcus* species (Diarra *et al*., 2007; Diarrassouba *et al*., 2007; Lu *et al*., 2003) which could subsequently be transferred to growing plants in the field. Poultry litter may include a mixture of bedding, feces, feathers, insects, and other small invertebrates (Brye *et al.,* 2005*)*. Another more recent study by Park *et al*. (2013*)* reported that spinach crops were significantly (Odds Ratio = 172.1) more likely to be contaminated with generic *E. coli* when located within 10 miles proximity to a poultry farm than spinach planted a further distance away from poultry farms. In addition, the authors of this study reported that spinach plants grown in soil mixed with poultry manure were significantly more likely to be contaminated with generic *E. coli* compared to plants grown in soil mixed with dairy manure. Virulence and antibiotic resistance elements have been shown to be highly transmissible among *E. coli* strains and between the 4 phylogenic groups of bacteria discussed here.

Raw poultry and fresh produce could become contaminated with APEC through a variety of mechanisms. Poultry could become contaminated during processing, especially during evisceration, and by cross-contamination in the chiller. Produce could become contaminated by fecal dissemination from wild birds or through the presence of contaminated poultry litter on farms. In a study from Japan, *E. coli* strains were isolated from wild birds and screened for intimin (*eae*) and Shiga toxins (*stx1*, *stx2*), two virulence properties of EHEC (Kobayashi *et al.,* 2009). Although intimin was found in 25% of the strains, and Shiga toxin was found in 5% of the strains, only

a few of the strains screened in this study were determined to contain both virulence factors to be classified EHEC (Kobayashi *et al.,* 2009). The presence of these significant virulence genes within atypical enteropathogenic *E. coli* strains suggests that APEC in wild and/or domesticated birds could be a source of contamination in our food supply.

An interesting nationwide study was performed to determine the prevalence of bacteria found on chicken breasts purchased at various types of food retailers in 26 states which appeared in a non-peer reviewed publication (Morran, 2013). The authors screened 316 chicken breasts from conventional, antibiotic-free, and organically labelled brands. According to the article 65 % of the chicken breasts tested were positive for generic *E. coli*. Of the 65 % positive samples, 17.5 % were found to be ExPEC isolates typically associated with urinary tract infections. The presence of EXPEC and UPEC isolates in retail poultry further supports the theory UPEC can be introduced to humans through consumption of foods (Morran, 2013). About half (49.7 %) of the samples that tested positive for bacteria were multi-drug resistant and 11.5 % of these samples carried more than one multi-drug resistant bacteria. This article and the many subsequent discussions in social media highlight the fact that this topic is already regarded as an important issue by the media.

1.4 Antibiotic Resistance

The use of antibiotics in agricultural production has led to an increased incidence of antibiotic resistant bacteria, which presents major risks toward animal and human

health (Mulvey and Simor, 2009; Witte, 2000). Recent surveillance data from the 2000s indicates that antibiotic resistance to first line antibiotics among ExPEC has increased including cephalosporins, fluoroquinolones and trimethoprim-sulfamethoxazole (Pitout, 2012). Antibiotics commonly used in the field of veterinary medicine, including *B*- lactamases and tetracycline, have become compromised due to the increase of antibiotic resistant bacteria on the farm (Hornish and Kotarski, 2002; Chopra and Roberts, 2001; Diarra *et al.,* 2009). Furthermore, the emergence of *E. coli* O25:H4 belonging to ST (sequence type) 131, which is most commonly resistant to fluroquinolones and extended spectrum cephalosporins, has severely complicated treatment of bloodborne and urinary tract infections in the U.S. (Johnson et al., 2010). One survey of clinical antibiotic resistance isolates has indicated that this lineage (ST 131) of *E. coli* is responsible for approximately 70% of clinical *E. coli* infections resistant to fluoroquinolones, and 55% resistant to both fluoroquinolones and trimethoprim-sulfamethoxazole (Johnson *et al*., 2010). Further testing of *E. coli* in the ST 131 lineage has revealed that many isolates contain ExPEC virulence factors, including fimbrial (*papC, fimH,* and *tsh*) and toxin (*sat* – secreted autotransporter genes) genes (Johnson *et al*., 2010; Petty *et al*., 2013).

Pathogenic *E. coli* strains can harbor virulence and antibiotic resistance genes on the same genetic elements (Johnson *et al*., 2007; Saldana *et al*., 2009) and are easily shared between isolates. Antibiotic resistant APEC, can presumably contaminate crops through the use of untreated animal manure as other *E. coli* have contaminated crops. Antibiotic resistant bacteria are reportedly isolated in significantly higher numbers

from soil samples on farm environments compared to non-farm environments (Yang *et al.,* 2010). A study by Merchant *et al.* (2012) reported 77.6 % of the *E. coli* isolates recovered from soil fertilized with litter of broiler chickens fed antimicrobial supplemented diets were found to be resistant to at least one antibiotic as determined by an antimicrobial susceptibility assay. The highest percentage of resistance in this study was against tetracycline (75 %), ampicillin (71 %) and amoxicillin (66 %). In addition, of the 229 *E. coli* isolates found to be resistant to at least one antibiotic, 97 % of those isolates harbored at least one antibiotic resistant gene as determined by PCR. It is important to determine the antibiotic resistant determinants that characterize environmental *E. coli*, specifically those isolates found on farms where animal manure is used as a fertilizer.

1.5 Foodborne Urinary Tract Infections (FUTI)

Urinary tract infections (UTI) are the number one cause of bacterial infections in the developed world accounting for more than a million office visits each year and 500,000 emergency room visits per year (Spencer *et al.,* 2010). There are approximately 6-8 million uncomplicated UTI cases annually in the US – the majority of which are caused by *E. coli* (Russo and Johnson, 2003). The economic burden associated with UTIs is estimated to be approximately $1.5 billion in the US annually (Foxman, 2003). Several studies have provided evidence supporting the hypothesis that *E. coli* can cause UTIs through the ingestion of contaminated food however; the mechanism by which bacteria travel from the gastrointestinal tract to the urogentical

tract remains controversial. A study by Vincent *et al.* (2010) explored food as a reservoir for ExPEC by gathering isolates from women with UTIs, retail meat, and ready-to-eat foods from the same time period and geographic area. Two *E. coli* isolates from retail chicken and one isolate from honeydew melon were genotypically similar to certain strains causing human UTIs, providing supporting evidence for the ability of food to serve as a vehicle for ExPEC infections (Vincent *et al.,* 2010). In another study, 737 samples of retail beef, pork and chicken were screened for the presence of ExPEC, in which 41 isolates were recovered (Bergeron *et al.,* 2012). Of the 41 ExPEC isolates recovered, 71 % of them were isolated from retail chicken samples (Bergeron *et al.,* 2012). These results suggest that ExPEC involved in UTIs may be transmitted from food-animal sources, and that APEC may be of particular concern because of the strong indication for poultry to serve as a reservoir (Bergeron *et al.,* 2012).

According to Nordstrom *et al.* (2013) the term FUTI (foodborne urinary tract infection) has been adopted to provide a narrower definition for those UTIs caused by a contaminated food vehicle. According to the authors, FUTIs do not involve the traditional etiology for infection and colonization of the gastrointestinal tract. Instead, a FUTI occurs in two steps: 1) a susceptible host ingests the uropathogen and 2) an infectious dose of the uropathogen is transferred from the host's gastrointestinal tract to his or her urinary tract (Nordstrom et al., 2013). The rate limiting step is suggested to occur during the transfer of the pathogen from the gastrointestinal tract to the

urogenital tract. The virulence factors which support the ability of *E. coli* to transfer from the gastrointestinal tract to the urogenital tract have not yet been determined.

1.6 Conclusions and Research Needs

Environmental *E. coli*, including APEC, have been shown to harbor virulence genes contributing to antibiotic resistance and zoonotic transmission. With the majority of foodborne illnesses being of unknown origin, it is important to evaluate the ability of environmental and agricultural bacterial species to cause foodborne illness and determine if they should be recognized as emerging pathogens. APEC are of particular concern in raw poultry products and raw poultry manure that is used as a crop fertilizer. These bacteria can also harbor virulence and antibiotic resistance genes similar to other human pathogenic *E. coli*, indicating their ability to cause disease in humans. The emergence of multi-drug resistant pathogens necessitates the need for development of alternative means to treat bacterial infections such as the use of anti-adhesion therapy as suggested by Krachler and Orth (2013), which may potentially eliminate the selective pressure on the pathogen to develop resistance.

Future research will generate valuable information regarding the potential of environmental *E. coli* to cause foodborne illness and subsequently help identify critical control points along the production chain where such bacteria could come into contact with food. In addition, this research could help identify unrecognized food vehicles as causes of foodborne illness where unidentified emerging pathogenic *E. coli* species cause contamination. This could include crops commonly fertilized with

animal-based fertilizers such as poultry litter. In regards to the burden of unattributable foodborne illnesses, future research will reduce the burden of foodborne illness by identifying emerging foodborne pathogens and food vehicles. Future studies should be used to develop mitigation strategies including specific on-farm prevention strategies and Good Agricultural Practices (GAPs) that will prevent crop contamination of environmental *E. coli*. Consumer education programs regarding the handling of raw poultry products and spread of bacteria should also be considered.

In generating the data and review material that was used in writing this article, the specific question of the potential role of APEC and ExPEC was addressed through identified relevant work from experts in the field. We assessed the quality of studies relevant to the question and included a variety of sources and authors. We should note that there are limited resources available on this subject at this time. Sources were pulled from clinical and environmental studies published in peer-reviewed journals. The study conducted and published in *Consumer Reports* was included here due to the high impact and media attention; while it is not peer-reviewed the science presented was acquired through validated methodology (personal communication). The evidence was summarized and findings interpreted relative to our initial question. We generally followed these steps that are outlined as steps in conducting a systematic review (Khan *et al.,* 2003)

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Chapter 2

SURVIVAL OF PATHOGENIC *ESCHERICHIA COLI* ON BASIL, LETTUCE, AND SPINACH.

2.1 Abstract

The contamination of lettuce, spinach and basil with pathogenic *E. coli* has caused numerous illnesses over the past decade. *E. coli* O157:H7, *E. coli* O104:H4 and avian pathogenic *E. coli* (APECstx- and APECstx+) were inoculated on basil plants and in promix substrate using drip and overhead irrigation. When overhead-inoculated with 7 log CFU/ml of each strain, *E. coli* populations were significantly (p=0.03) higher on overhead-irrigated plants than on drip-irrigated plants. APEC*stx*-, *E. coli* O104:H4 and APEC*stx*+ populations were recovered on plants at 3.6, 2.3, and 3.1 log CFU/g at 10 dpi (days post-inoculation), respectively. *E. coli* O157:H7 was not detected on basil after 4 dpi. The persistence of *E. coli* O157:H7 and APECstx- were similar when co-inoculated on lettuce and spinach plants. On spinach and lettuce, *E. coli* O157:H7 and APEC populations declined from 5.7 - 6.1 log CFU/g and 4.5 log CFU/g, to undetectable at 3 dpi and 0.6-1.6 log CFU/g at 7 dpi, respectively. The detection of low populations of APEC and *E. coli* O104:H4 strains 10 dpi indicates these strains may be more adapted to environmental conditions than *E. coli* O157:H7. This is the first reported study of *E. coli* O104:H4 on a produce commodity.

2.2 Introduction

Fresh fruits and vegetables are increasingly recognized as significant sources of foodborne illness outbreaks, causing about 5 to 23 % of confirmed cases of foodborne illness in many countries including the US (Little and Gillespie, 2008; Tyler and Triplett, 2008). Fruit and vegetable products can become contaminated in the pre-harvest environment through a variety of routes including improperly composted manure, contaminated irrigation water, wild and domestic animals, and soil (Beuchat, 2002). Leafy greens are most commonly eaten raw without processing steps that would reduce or eliminate pathogenic bacterial populations. Within the last few years, outbreaks associated with fresh produce, including leafy greens, have been linked to the emergence of shiga-toxin producing non-O157 *E. coli* serotypes still considered enterohemorrhagic *E. coli* (EHEC). One notable outbreak in Germany in 2011 was caused by *E. coli* O104:H4, a shiga-toxin produce *E. coli* (STEC) strain linked to sprouted contaminated Fenugreek seeds that sickened thousands of people in 2011 (CDC, 2011). This particular *E. coli* isolate is an enteroaggragative E. coli (EAggEC) that also produces shiga-toxin, characteristic of EHEC strains; which classified the *E. coli* O104:H4 isolate as an STEC (Wu *et al*., 2011). A multistate outbreak of *E. coli* O145 in the US linked to shredded romaine lettuce from a single processing facility occurred in May of 2010 (CDC, 2012). More recently, a multistate outbreak of *E. coli* O26 linked to raw clover sprouts occurred in February and April of 2012 (CDC, 2012). The emergence of these highly virulent non-O157 STEC species associated with produce outbreaks poses a threat to public health. Little is known about the fitness and survival of these pathogens on leafy greens in comparison to

known *E. coli* O157:H7 strains, and whether they endure environmental stresses of pre-harvest environments better than O157-serotypes.

The versatile behavior of *E. coli* species throughout the environment can be explained in part by the genomic diversity of strains within this species. The genomes of *E. coli* strains can differ by up to 20% (Liu *et al.,* 1999). The virulence gene for shiga-toxin production (*stx*) in STEC have been found to be transferable to non-pathogenic *E. coli* strains through transduction, allowing for enhanced virulence of these previously non-pathogenic strains (Yaron *et al.,* 2000; Herold *et al.,* 2004). Until recently, the *E. coli* serotype most commonly associated with foodborne illnesses was thought to be shiga-toxin producing *E. coli* O157:H7. In 2011, Scallan *et al.* reported that annually in the US, there are approximately 63, 153 domestically acquired illnesses associated with O157 STEC (*E. coli* O157:H7), and approximately 112, 752 domestically acquired illnesses associated with non-O157 STEC serotypes. Therefore, the examination of the survival of non-O157 STEC in pre-harvest, produce growing environments is needed.

APEC (avian pathogenic *E. coli*) are widespread in poultry and causes colibacillosis in birds raised for meat and eggs, resulting in millions of dollars in losses in the poultry industry (Barnes *et al.,* 2008). APEC can also cause extraintestinal infections in humans. APEC strains are considered members of the extraintestinal *E. coli* (ExPEC), a pathogenic group of *E. coli* that also includes human uropathogenic *E. coli* (UPEC) and *E. coli* causing neonatal meningitis and septicemia (Johnson and Stell, 2000; Kaper *et al.,* 2004). Mellata *et al.* (2009) observed that virulence genes associated with plasmids in APEC strains, including pAPEC-1, particularly those involved in iron acquisition, are also prevalent in human ExPEC

indicating the zoonotic risk to humans of APEC strains. Poultry consumption has been found to serve as a source of human acquired ExPEC (Johnson *et al.,* 2005). And while no APEC isolates from poultry have been classified as EHEC or STEC, in a survey of *E. coli* isolates from wild birds, 5% and 25% of isolates were positive for *stx* and *eae*, respectively (Kobayashi *et al.,* 2009). The virulence factor *eae* is a determinant virulence factor in EPEC and EHEC strains. Since these virulence factors are responsible for the ability to cause gastrointestinal infections in humans, potential APEC strains which contain these virulence factors could contribute to the burden of foodborne illness currently unattributable to an etiological agent (Scallan *et al.,* 2011) as well. Understanding the persistence of these strains on herbs, leafy green commodities, and in pre-harvest environments may also help determine the risk to public health from both APEC and non-O157 STEC strains.

The increasing evidence of zoonotic transfer of APEC to humans indicates the need for the study of APEC alongside more established pathogenic serotypes of *E. coli* in high risk food systems such as fresh produce and leafy greens. The purpose of this study was to determine the survival of APEC and pathogenic strains of O157 and non-O157 *E. coli* on leafy greens including basil, lettuce, and spinach. The survival of APEC strains and non-O157 *E. coli* strains were assessed individually and as a multi-strain inoculum on plants.

2.3 Materials and Methods

2.3.1 Strains and Irrigation Solution Preparation

For basil plants, the survivals of four different *E. coli* types were individually assessed. An inoculum consisting of two APEC isolates lacking the *stx*2 and *eae*

virulence genes, an APEC isolate containing the *stx2* and *eae* genes, *E. coli* O104:H4 (German outbreak strain, ATCC TY2482), *E. coli* O157:H7 strain RM-4407-GFP-Nal was described previously (Sharma *et al*., 2009), and originally isolated from the 2006 US spinach outbreak. The two APEC isolates lacking the *stx*2 and *eae* genes included: one *E. coli* O157 serotype (APEC 07-1707); one *E.coli* O8 serotype (APEC 07-1307) and were labeled as APEC*stx*-. A rare APEC isolate containing the *stx2* and *eae* virulence genes is an *E. coli* O13 serotype (APEC 07-5668) and was labeled as APEC*stx*+. All APEC strains were isolated from poultry broilers within the Delmarva (Delaware, Maryland, and Virginia) area. All strains used in survival studies on basil plants were previously resistant to 50 µg/ml nalidixic acid (Sigma-Aldrich, St. Louis, MO). Cultures of each isolate were grown in modified EHEC broth (Biocontrol, Bellevue, WA) with 50 µg/ml nalidixic acid (mEHECN) at 37°C while shaking (New Brunswick Scientific, New Brunswick, NJ) at 200 rpm for 24 h. Cultures were then diluted 10-fold into autoclaved dairy manure slurry prior to inoculation to obtain a concentration of ~10^6 CFU/ml as confirmed by enumeration on sorbitol MacConkey agar (SMAC) (Oxoid Ltd., Basingstoke, Hampshire, England) supplemented with 50 µg/ml nalidixic acid (SMACN). The method used to create the dairy manure slurry was similar to that of Ingram *et al.* (2011). In brief, fresh manure was collected from the University of Delaware dairy farm (Newark, DE) and centrifuged in 50 mL tubes at 2500 x g (Beckman Coulter, Fullerton, CA) for 10 min to separate the solid from the liquid portion of the manure. The liquid portion of the manure was autoclaved for sterilization prior to inoculation and was used as the irrigation solution.

For lettuce and spinach plants, the APEC inoculum consisted of different strains than those used with basil. Four APEC strains which did not contain the *stx*2

gene – 07-0717, 07-6098, 05-2848, and 05-2737- were received from the University of Delaware collection and labeled as APEC*stx*-, were used in these studies. Strains 05-2848 and 05-2737 were made resistant to 50 μg/ml nalidixic acid by selecting a spontaneous mutation resistant to nalidixic acid. Strains 07-0717 and 07-6098 were previously resistant to 50 μg/ml nalidixic acid. Strains were struck from frozen stock on to SMACN. *E. coli* O157:H7 strain RM-4407-GFP-Nal (Sharma *et al.,* 2009) was used in these experiments as well. Each of the five strains were inoculated separately in 10 ml of sterile PLE (poultry litter extract) and incubated at 37°C for 48 h. PLE was obtained by obtaining poultry litter from the University of Maryland Eastern Shore (UMES), diluted 1:10 in sterile deionized water, and then stirred for 5 min before filtering through a double-layer of cheese cloth. The extract was then diluted 1:1 with deionized water and sterilized to prepare PLE. After incubation at 37°C in PLE, *E. coli* strains were placed on ice and stored at 4°C for 24 h to stabilize bacterial populations. Populations of individual strains were enumerated on SMACN. Cultures of APEC and *E. coli* O157:H7 strains were then combined in a single inoculum which contained 1.3 x10^5 CFU/ml of each APEC strain and 5 x 10^5 CFU/ml *E. coli* O157:H7 for a total inoculum at 1 x 10^6 CFU/ml to be applied to spinach or lettuce plants.

2.3.2 Basil cultivation

Thai basil (*Ocimum basilicum)* seeds (Johnny's Selected Seeds, Waterville, ME) were disinfected by soaking in a 10 % bleach solution for 2 min when the outer coat of the seed began to turn white. Seeds were then planted in a sterile pro-mix made up of 85% Canadian sphagnum peat moss with perlite, vermiculite, dolomitic and calcitic Lime, a wetting agent and Mycorrhizae (Premier Tech Horticulture, Quakertown, PA) and maintained in a Biosafety Level 2 (BSL-2) growth chamber

(Percival Scientific, Boon, IA) at the University of Delaware Fischer Greenhouse (Newark, DE). Growth conditions used included 30°C temperature with a 12-hour photoperiod until 2 two leaves were present on each plant (10-14 days). Plants were then transplanted to individual containers containing sterile promix (square containers 4 cm x 3.5 cm x 4.5 cm in dimension; T.O. Plastics, Ontario, Canada). Holes were created in the bottom of each individual container which was then placed into plastic bins inside the growth chamber. Throughout the experiment water was poured into the bottom of each plastic container to prevent influence of bacterial movement on plants and promix by direct watering. The growth chamber temperature conditions were monitored daily and temperature was maintained at 30 ± 0.30ºC, relative humidity was 44.3 ± 2.08 % and the water activity (A_w) of the promix was maintained at 1.046 ± 0.04.

2.3.3 Lettuce and spinach cultivation

Plants were grown in the Biosafety Level 2 (BSL-2) growth chamber (Conviron 4030, Winnipeg, Canada) at the Environmental Microbial and Food Safety Laboratory USDA ARS facility in Beltsville MD. Both spinach and lettuce plants were grown in sterilized, fine sandy loam (Keyport-Matawan) soil obtained from the ARS Beltsville Agricultural Research Center (BARC) north farm. For spinach, autoclaved soil was placed in sterile 'conetainers' (cone-shaped plastic vessels, model SC10, 164 ml, Stuewe & Sons, Inc. Tangent, OR.), each suitable for growing a single spinach plant. Spinach (*Spinacia oleracea*) cultivar 'Blackhawk' (Seminis, Oxnard, CA) was planted in these conetainers. During experiments, each plant was irrigated once a week with 20 ml of sterile water supplemented with 1.32 g / L Jack's Classic All Purpose 20-20-20 fertilizer (J.R. Peters, Inc., Allentown, PA). For Romaine

lettuce (*Lactuca sativa*), cultivar 'Fresh Heart Paragon' (Seminis) was grown in square containers. Both lettuce and spinach were grown under conditions set to 70% relative humidity for a 14 h photoperiod at 20ºC and 10 h dark period at 15ºC.

2.3.4 Plant and promix inoculation

Basil plants were inoculated when two true leaves were present on the plant (~14 day old plants). Basil plants were inoculated with each strain individually by either drip or overhead irrigation to simulate a one-time contamination event. For drip irrigation, 3 ml of irrigation solution was inoculated directly onto the promix of plants by pipette with no or minimal splash. For overhead irrigation, plants were sprayed directly with irrigation solution using an air brush (model 200, Badger Air Brush Company, Franklin Park, IL) for 15 s (3 ml). Plant and promix samples were collected on days 0, 1, 4, 7 and 10. Plants were inoculated in a biosafety cabinet prior to being placed in the growth chamber.

Lettuce and spinach plants were inoculated directly on the foliar surface at 30 and 28 days of age, respectively. . For each plant, 100 µl of the inoculum containing a multi-strain inoculum of APEC*stx*$^-$ and *E. coli* O157:H7 was added to each plant by adding 20 droplets (5 µl / droplet) using a micropipette to foliar tissue. Five droplets /leaf were added to each plant. Droplets were allowed to dry for 30 min in growth chambers before harvesting and enumeration.

2.3.5 Recovery and Enumeration

Basil plant and promix samples were pooled into 3 plants per sample (~0.46 g/plant) or 3 promix containers per sample (~13.07 g/container). Plants were cut 1 cm from the promix surface with sterile scissors and placed into sterile stomacher bags

while the entire container of promix was placed into stomacher bags. All samples were mixed with 40 ml of mEHEC broth supplemented with 50 mg/ml nalidixic acid (mEHECN) and stomached for 2 min. Enumeration was performed by standard plate count on SMACN. For lettuce and spinach plants, plants were harvested by using sterile scissors to cut the shoot tissue above the promix surface from from each spinach or lettuce plant. The average weight of lettuce and spinach plants were 5 and 3 g, respectively. Leaves were then deposited into a sterile stomacher bags, and 40 ml of mEHECN was added to each bag. Samples were then stomached (Bagmixer, Interscience, St. Nom, France) for 2 min. The resulting homogenate was then either plated on SMACN (Day 0) or used for MPN (Most Probable Number) determination (remaining days). A three-tube MPN assay was performed by adding 10, 1, or 0.1 ml of homongenate to 0, 9, or 9.9 ml mEHECN and incubated at 37°C for 24 h. A loopful (10 µl) from tubes which displayed turbidity was then struck for isolation SMACN plates. Colonies which did not show sorbitol fermentation (pale colonies) were determined to be *E. coli* O157:H7; colonies which evidenced fermentation of sorbitol were determined to be APEC*stx*- . For each plant, MPN assays were performed in triplicate. On each day of analysis, either 6 spinach plants or 6 lettuce plants were harvested and microbiologically analyzed. When MPN assays yielded undetectable numbers of *E. coli*, plant material in stomacher bags was enriched with 40 ml of mEHECN and incubated at 37ºC for 24 h. Enriched samples were then streaked for isolation to determine the presence of *E. coli* O157:H7 or APEC.

2.3.6 Statistical Analysis

Experiments were performed in duplicate for at least 2 trials per strain with each trial performed at different times. Results are reported as the means and standard

deviations as log CFU/g plant or promix. A one way ANOVA analysis was performed to compare means within the data set and a linear regression model was generated using JMP 9 software (SAS Institute Inc., Cary, NC). All p values less than 0.05 were considered significant ($\alpha = 0.05$). For lettuce and spinach MPN determination, the freeware calculator (VB6, www.i2workout.com/mcuriale/mpn/index.html) was used. Results were expressed as MPN/g.

2.4 Results

2.4.1 Individual strain survival on basil plants and promix

Overall, *E. coli* persisted at higher populations for longer periods of time in promix substrate than on plants ($p < 0.0001$). The greatest survival of *E. coli* from promix was for samples drip irrigated with APEC*stx*+ with 4.36 ± 0.04 log CFU/g recovered after 10 days (Figure 6). Basil plants which were spray-irrigated with APEC*stx*- had higher populations (3.60 ±0.06 log CFU/g) compared to other spray-irrigated *E. coli* (Figure 3). Recovery of *E. coli* O157:H7 populations was lower than that of other *E. coli* (APEC*stx*-, APEC*stx*+, or *E. coli* O104:H4) from both promix and plants regardless of the irrigation method used; however, differences in survival of *E. coli* populations was statistically insignificant ($p = 0.80$). For this particular isolate, no bacteria were detected after day 4 on plants that were spray irrigated or after day 1 on plants that were drip irrigated (Figures 3 and 4). *E. coli* O157:H7 was recovered from promix at populations of 3.31 ± 0.12 and 2.64 ± 0.65 log CFU/g when overhead- and drip- irrigated, respectively, on day 10. (Figures 7 and 8).

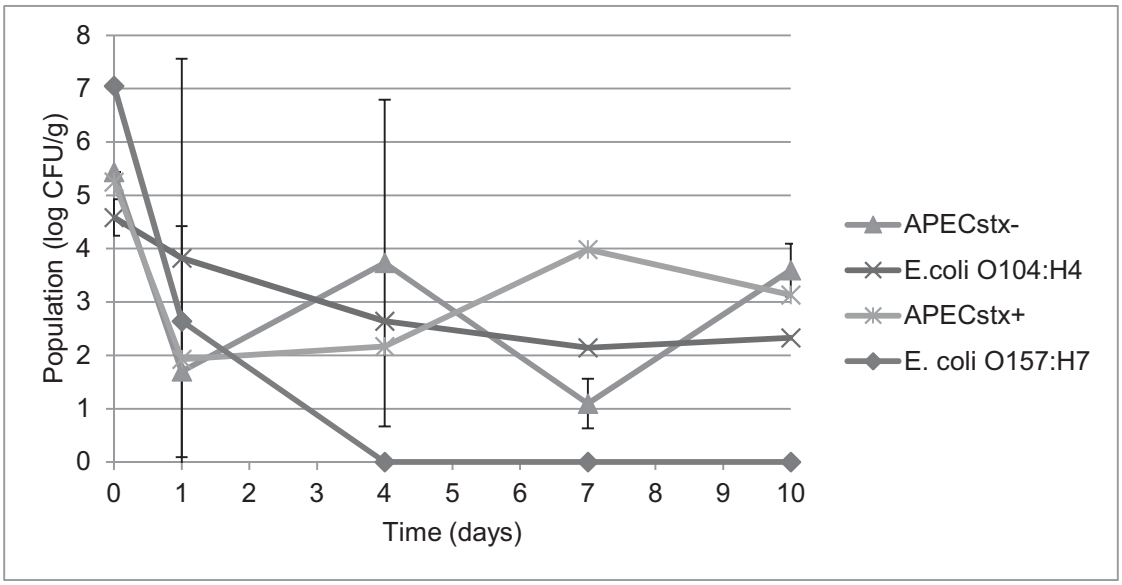

Figure 3: Survival of a multi-strain inoculum of avian pathogenic *E. coli* (APEC) not containing the *stx*2 gene (APEC*stx*-), an APEC strain containing the *stx*2 gene (APEC*stx*+), *E. coli* O104:H4, and *E. coli* O157:H7 inoculated individually onto basil plants by overhead irrigation over 10 days.

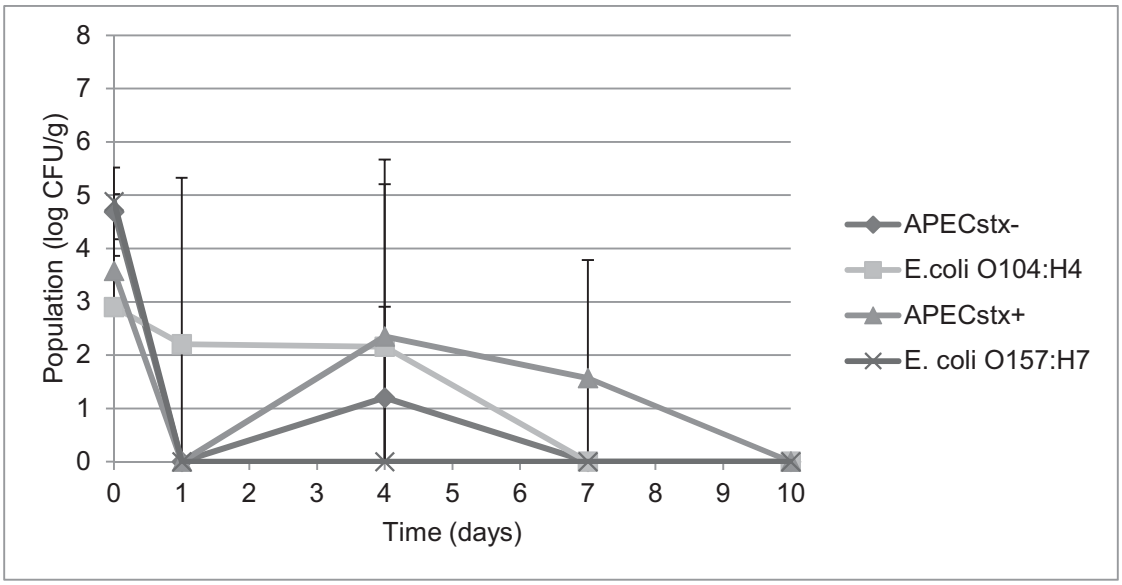

Figure 4: Survival of a multi-strain inoculum of avian pathogenic *E. coli* (APEC) not containing the *stx*2 gene (APEC*stx*-), an APEC strain containing the *stx*2 gene (APEC*stx*+), *E. coli* O104:H4, and *E. coli* O157:H7 inoculated individually onto basil plants by drip irrigation over 10 days.

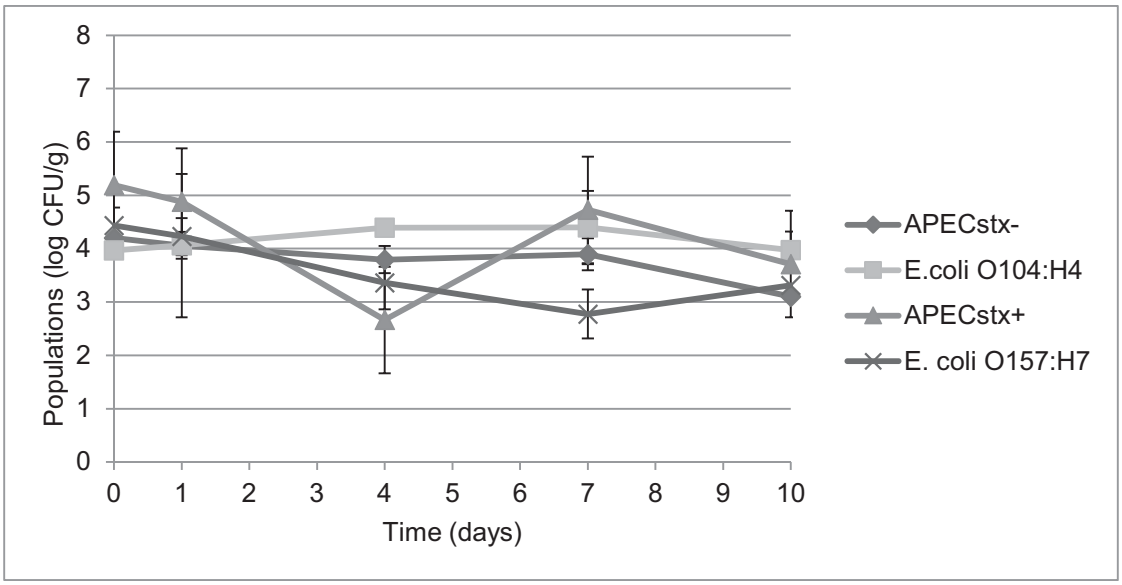

Figure 5: Survival of a multi-strain inoculum of avian pathogenic *E. coli* (APEC) not containing the *stx*2 gene (APEC*stx*-), an APEC strain containing the *stx*2 gene (APEC*stx*+), *E. coli* O104:H4, and *E. coli* O157:H7 inoculated individually onto promix of basil plants by overhead irrigation over 10 days.

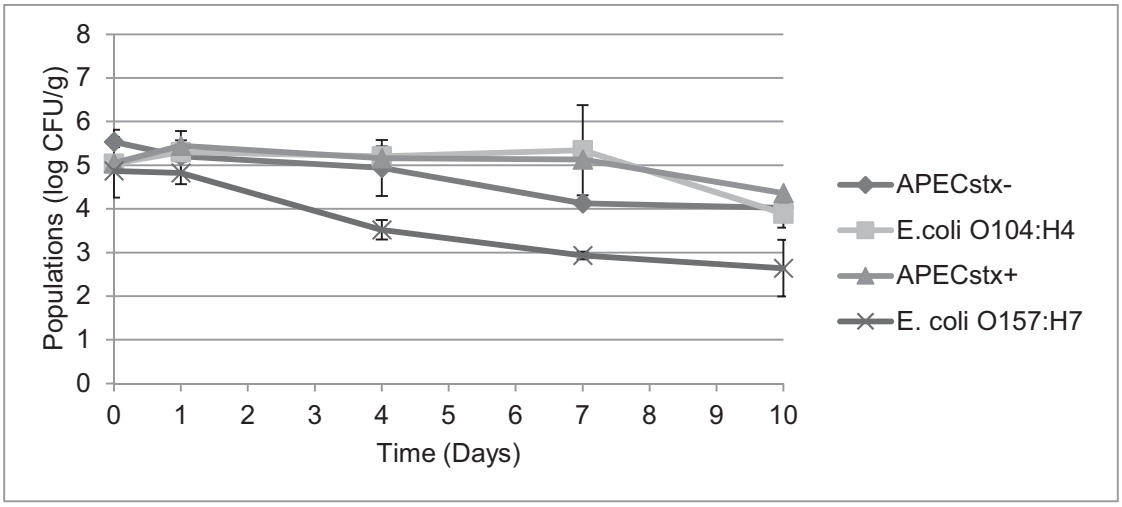

Figure 6: Survival of a multi-strain inoculum of avian pathogenic *E. coli* (APEC) not containing the *stx*2 gene (APEC*stx*-), an APEC strain containing the *stx*2 gene (APEC*stx*+), *E. coli* O104:H4, and *E. coli* O157:H7 inoculated individually onto promix of basil plants by drip irrigation over 10 days.

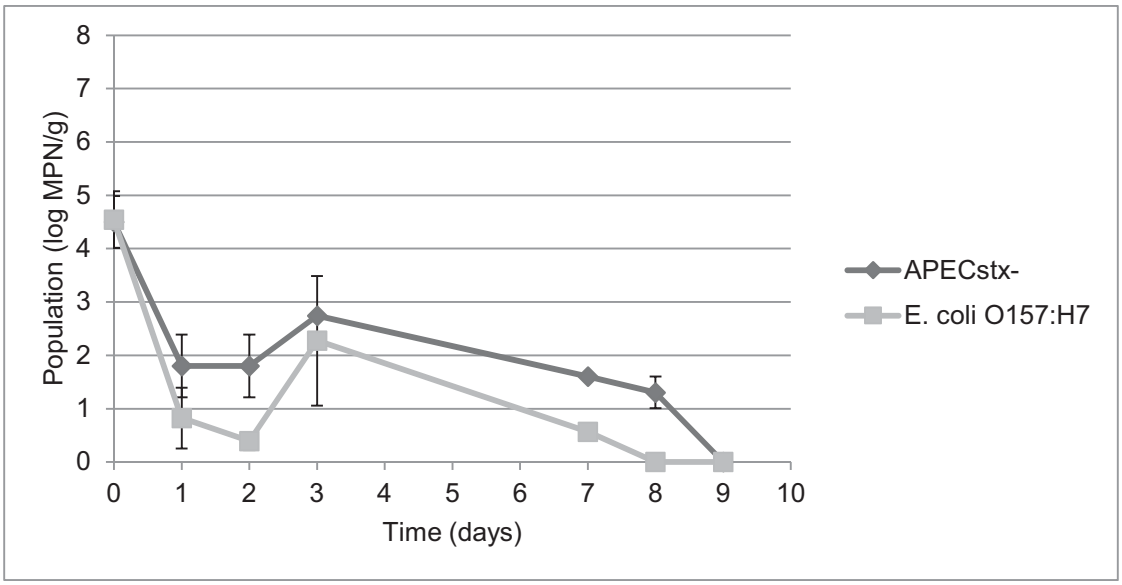

Figure 7: Survival of a multi-strain inoculum of Avian pathogenic *E. coli* (APEC) without *stx*2 gene (APEC*stx*-) and *E. coli* O157:H7 co-inoculated on spinach plants over 17 days.

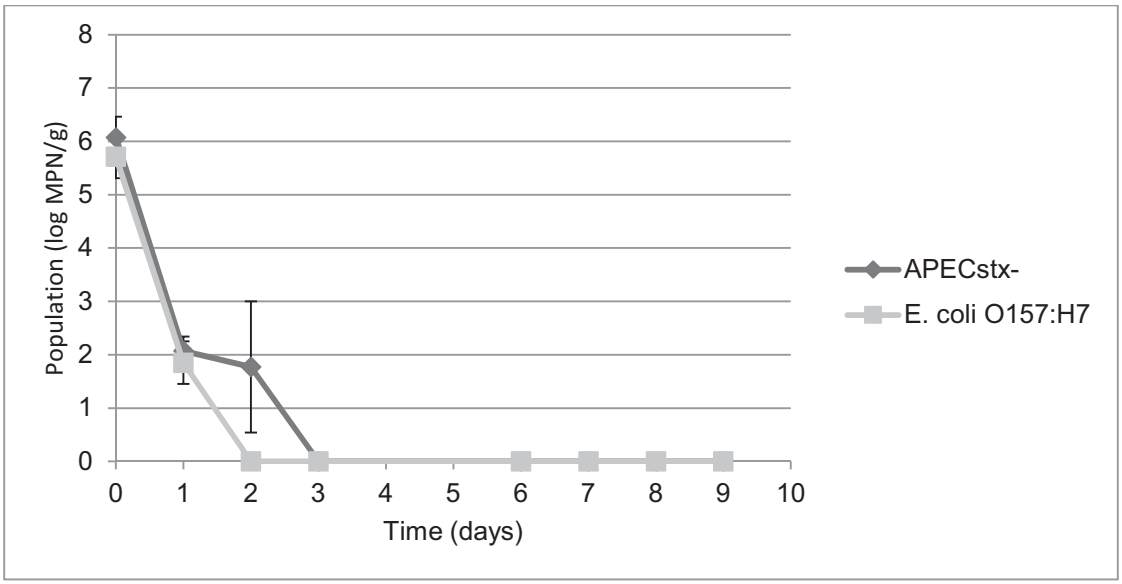

Figure 8: Survival of a multi-strain inoculum of Avian pathogenic *E. coli* (APEC) without *stx*2 gene (APEC*stx*-) and *E. coli* O157:H7 co-inoculated on lettuce plants over 6 days.

2.4.2 Simultaneous survival on lettuce and spinach

APEC and E. coli O157:H7 were co-inoculated simultaneously on lettuce and spinach plants. Initial populations (day 0) of APEC on spinach and lettuce were 6.1 and 4.5 log CFU/g, respectively. Initial populations (day 0) of E. coli O157:H7 on spinach and lettuce were 5.7 and 4.5 log CFU/g respectively (Figures 7 and 8). Populations of both types of E. coli declined rapidly on both commodities by day 1, as APEC populations declined by 4 log MPN/g on spinach and by 2.8 log MPN/g on lettuce. Similar declines were observed with respect to E. coli O157:H7 on both leafy green commodities; population declined by 3.9 log MPN/g on spinach and 3.7 log MPN/g on lettuce. E. coli populations persisted for shorter durations on spinach compared to lettuce; by day 3, no APEC or E. coli O157:H7 were detected by enrichment on spinach plants. On lettuce, however, low levels of APEC strains were detectable by MPN eight days after inoculation. Both APEC and E. coli O157:H7 were detectable by enrichment 14 and 17 days after inoculation. APEC survived at higher levels than E. coli O157:H7 on 7 and 8 days post-inoculation on lettuce plants.

2.5 Discussion

2.5.1 Individual survival on basil plants and promix

This study utilizes different methodologies to inoculate pathogenic *E. coli* onto three different produce commodities and determines that persistence of *E. coli* may be influenced by use of different plants and bacterial strains. This study also utilized a rare APEC isolate (*E. coli* O13) which contains boths *stx* and *eae* genes, classifying it as an EHEC strain. The isolation of an APEC strain with EHEC virulence factors shows importance of evaluating APEC strains on produce for their potential ability to cause human illness. Even among the different inoculum methods and strains used,

some overall conclusions can be reached. *E. coli* persisted at significantly ($p = 0.03$) higher populations on plants which were overhead irrigated compared to those which were drip irrigated after 10 d. These results imply that, when irrigation is necessary, basil should be irrigated using a drip irrigation system which prevents direct application of irrigation water onto the foliar surface of plants. However, this study also shows that even when careful application of contaminated water was applied directly to promix, *E.coli* was still detected on plants on day 0. The detection of *E. coli* on plants that were drip irrigated could have been due to unintentional splash from the promix substrate onto plants. Recent studies have demonstrated that splash that occurs during irrigation can propel *E. coli* on promix to heights up to 20 cm and horizontal distances of 25 cm (Monaghan and Hutchinson, 2012).

2.5.2 Simultaneous survival on lettuce and spinach

The lack of simultaneous survival of both APEC and *E. coli* O157:H7 strains on spinach plants compared to lettuce was surprising, given than inoculation methods were the same for both leafy green commodities. It is unclear why spinach plants supported a shorter duration of survival than lettuce plants for both APEC and *E. coli* O157:H7 strains. The exact role of epiphytic bacterial populations on enteric bacterial survival on foliar surfaces is unclear (Patel et al., 2009). It is possible that the epiphytic bacteria population on spinach leaves were more antagonistic towards *E. coli* than epiphytes on lettuce (Cooley et al, 2006). However, this interaction could have extended the duration of survival by protecting *E. coli* from ultra-violet stress, and other antagonistic stresses encountered by enteric bacteria on foliar surfaces. Other investigators have shown that bacterial cells introduced to a leaf surface have a better chance of surviving when they are deposited on or in aggregates of other

bacteria (Monier and Lindow, 2005). These aggregates are characterized by an exopolysaccharide matrix which contains a dense population of bacterial cells. The interaction of the APECs and *E. coli* O157:H7 with these aggregates may also partially explain their extended survival on the foliar tissue of lettuce plants; conversely, the potential lack of interaction of these aggregates on spinach surfaces may have also led to the more rapid decline of *E. coli* O157:H7 populations compared to on lettuce surfaces. It is also possible that lower recovery of both APEC and *E. coli* O157:H7 from lettuce plants on day 0 compared to spinach plants indicates that *E. coli* attached more tightly to lettuce tissue compared to spinach tissue, and fewer bacteria were able to be dislodged or were agitated from the lettuce tissue during homogenization, leading to lowered recovered populations detected by MPN. Previous work has shown that *Salmonella* Tennessee exhibited stronger attachment to intact lettuce than to intact cabbage leaves (Patel and Sharma, 2010). In this same work, different serovars (*S.* Tennessee and *S.* Negev) showed different strengths of attachment to the same types of leaves (lettuce). These findings indicate the variations in attachment, which occurred among closely related *Salmonella* serovars, may have also occurred in our study with *E. coli* strains and affected recovery and enumeration from the leafy green tissue. In addition, it should be noted that plants were inoculated during the daytime in a biosafety cabinet, where light intensity may have been different from that of the growth chamber. Because plant stomata respond to changes in light (Zieger, 1983) this light difference may have affected stomatal closure or opening and therefore; entry of the pathogens into the plant.. Plants were held within the biosafety cabinet for inoculation for <30 min. These experiments were performed in a controlled environment within a growth chamber inside greenhouse facilities. It

should be acknowledged that the results of these experiments may not reflect exact environmental conditions that plants experience in the field, such as animals, insects, wind, etc. However; due to governmental regulations restricting the use of pathogens in field studies, growth chamber studies must be utilized in place of field studies.

2.5.3 Plant survive at similar rates on plants individually and simultaneously

In general APEC survived at populations ~ 1 log MPN/g higher than *E. coli* O157:H7 on lettuce on days 1, 2, 7, and 8. Whether this difference in survival is related to the potential enhanced environmental fitness of APEC strains compared to *E. coli* O157:H7 is unclear. Since the APEC inoculum consisted of four strains compared to one strain for the *E. coli* O157:H7 inoculum, it is possible that the strain diversity in the APEC inoculum contained one or more strains which were more persistent than the *E. coli* O157:H7 outbreak strain used in this work. APEC strain 07-1707 is an *E. coli* O157 serotype and was used in both individual inoculation studies on basil and in simultaneous inoculation studies on spinach and lettuce. The potential survival of this strain compared to *E. coli* O157:H7 on all three commodities may indicate that persistence on foliar surfaces is less a function of serotype and more dependent on source of isolation or previous environmental exposure of the strain. Previous studies demonstrated that when co-inoculated on to spinach foliar surfaces, non-pathogenic *E. coli* isolates from produce commodities survived at higher populations for up to 28 days, compared to *E. coli* O157:H7 strains from produce outbreaks, which only survived for 7 days (Patel et al., 2009). Other investigators have shown that a non-pathogenic *E. coli* isolates from soil had significantly higher levels of persistence on spinach plantlets and in potting mix than *E. coli* isolates from irrigation water or Romaine lettuce (Guiterrez-Rodriguez et al., 2011). These authors

hypothesize that the specific geospatial location of the origin of the isolate may affect its environmental fitness. Our findings may indicate that *E. coli* O157:H7 strains from produce outbreaks may not survive as well in non-host environments (on foliar surfaces, in soilin water) as *E. coli* isolated from environmental sources, where they have had more of an opportunity to adapt to stresses in pre-harvest, leafy green growing environments. APEC are extraintestinal pathogenic *E. coli*; responsible for a variety of infections in humans and domestic animals using diverse virulence factors (Rogers et al., 2011). The high prevalence of faecal carriage of APEC in livestock is thought to lead to dissemination in the environment and foodborne transmission to humans (Vincent et al., 2010; Rogers et al., 2011). These findings again support the hypothesis that although highly pathogenic, *E. coli* O157:H7 outbreak strain may not possess the environmental fitness of other environmentally-isolated *E. coli* isolates.

Though different inoculation methods were used for both of these studies, similar patterns in the survival of APEC and *E. coli* on basil, lettuce, and spinach were observed. Whether APEC strains were inoculated individually or simultaneously with *E.coli* O157:H7 on plants, APEC persisted at greater populations compared to *E. coli* O157:H7. In addition, on all three plant commodities, *E. coli* O157:H7 declined rapidly initially and could not be detected after 4 days on basil or after 2 days on spinach. This is similar to the rapid decline of *E.coli* O157:H7 observed in previous studies examining inoculated spinach. In that study, more than one isolate of *E. coli* O157:H7 applied to spinach plants with an airbrush declined by 2-3 log MPN/plant between day 0 and day 1 (Ingram et al., 2011). In our study, *E. coli* O157:H7 survived up to 8 days on lettuce when simultaneously inoculated with other APEC strains.

To our knowledge, this was the first study assessing *E. coli* O104:H4 persistence on produce. Interestingly, *E. coli* O104:H4 and APEC populations survived at similar levels on basil plants. These strains may have enhanced environmental fitness compared to the *E. coli* O157:H7 strain used in this study. This addresses the hypotheses that some *E. coli* strains have evolved towards an enhanced fitness in open environments (van Elsas *et al.*, 2011) such as on plants and in soil, and may indicate that these strains have been present in these environments for durations long enough to adapt to these conditions. Much of this hypothesis was applied to the *E. coli* O104:H4 German outbreak strain linked to contaminated fenugreek seeds. A recent study by Safadi *et al.* (2012) reported that *E. coli* O104:H4 has enhanced abilities to form biofilms *in vivo*, enabling them to survive in a complex host environment. The ability of *E. coli* O104:H4 strain to form biofilms may explain its enhanced persistence on basil compared to *E. coli* O157:H7. However; the *E. coli* O157:H7 isolate used in this study was from the 2006 spinach outbreak that caused over 200 illnesses and 3 deaths in the US was also found to contain a gene (*norV,* nitric oxide reductase) potentially correlated to increase its ability to cause hemolytic uremic syndrome (HUS) and enhance the strain's virulence compared to other *E.coli* O157:H7 isolates (Kulesekara *et al.,* 2009). Similarly, the *E.coli* O104:H4 German outbreak strain caused a higher perecentage of HUS cases (19.8%) among victims of this outbreak than any other STEC outbreak previously recorded. (EFSA, 2011; Safadi *et al.* 2012). The emergence of these exceptionally virulent pathogenic *E. coli* which show the ability to persist on produce surfaces, poses a major threat for public health and consumption of raw produce.

This study demonstrated that avian pathogenic *E. coli* (APEC) isolates survived at higher populations and for longer durations when individually inoculated onto basil plants or co-inoculated on lettuce and spinach plants compared to *E. coli* O157:H7 survival. Duration of survival was observed to be dependent upon the method of irrigation and plant type, which included differences in epiphytic bacteria, leaf structure and topography. On basil, *E. coli* O104:H4 survived similarly to APEC strains for 10 days, indicating enhanced environmental fitness for these strains. The survival of APEC strains on basil and leafy green commodities, and their potential contribution to the burden of foodborne illness worldwide, indicates a potential public health risk associated with contaminated produce. The results of this study emphasizes the need for future studies assessing the survival of environmental and zoonotic *E. coli* isolates compared to known pathogenic *E. coli* isolates on produce crops.

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Part II: HUMAN PATHOGENS ON PLANTS

Chapter 3

HUMAN PATHOGEN-PLANT INTERACTIONS: CONCERNS FOR FOOD SAFETY

3.1 Abstract

The Centers for Disease Control and Prevention (CDC) estimate the incidence of foodborne illness attributed to fruit and vegetable consumption at three million cases in the U.S. annually (Painter *et al.,* 2013). The means by which food crops become contaminated with foodborne pathogens as well as how these organisms persist within the phyllosphere and rhizosphere of these plants is an extremely complex issue. Outbreaks associated with food crops continue to occur and it is clear that different approaches are critical to enhance the safety of these foods. Within the last few years the fields of food safety and plant science have begun to merge to more efficiently address some of the knowledge gaps involving the mechanisms by which human pathogens contaminate plants. We currently know a great deal about the mechanisms by which plant pathogens are able to cause disease in plant crops as well as the immune response of these plants to these pathogens. By studying the relationships between plant pathogens and the plant immune response, scientists have been able to successfully develop biocontrol strategies to reduce crop damage attributed to plant disease. Potential control strategies for human pathogens on plants in the pre-harvest environment include the use of plant growth promoting bacteria and N-acyl-homoserine lactone-producing bacteria. By studying comparable relationships

between foodborne pathogens and plants, we may be able to develop similar strategies to reduce the number of foodborne illnesses associated with fresh produce.

3.2 Merging Plant Science and Food Science to Address Food Safety

The CDC estimates the incidence of foodborne illness attributed to fruit and vegetable consumption at three million cases in the U.S. annually (Painter *et al.,* 2013). The means by which food crops become contaminated with foodborne pathogens as well as how these organisms persist within the phyllosphere and rhizosphere of these plants is a complex issue. Outbreaks continue to occur with food crops and it is clear that different approaches must be taken in order to enhance the safety of these foods. While the route of contamination may vary and can occur anywhere along the "seed to fork" continuum, in some cases the food crops were likely contaminated in the field (Table 1).

Table 1: List of multistate outbreaks associated with fresh produce where pre-harvest contamination was suspected from 2006-2014. Source: CDC, 2014 (http://www.cdc.gov/ foodsafety/outbreaks/multistate-outbreaks/outbreaks-list.html).

Multistate Outbreaks in the US Associated with Produce Where Pre-Harvest Contamination was Suspected from 2006-2014		
Year	Commodity	Pathogen
2014	Cucumbers	*Salmonella* Newport
2014	Bean Sprouts	*Salmonella* Enteritidis
2014	Cilantro	*Cyclospora cayetanensis*
2014	Clover Sprouts	*E. coli* O121
2013	Salad Mix	*Cyclospora cayetanensis*
2013	Cucumbers	*Salmonella* Saintpaul
2012	Spinach and Spring Mix	*E. coli* O157:H7
2012	Mangoes	*Salmonella* Branderup
2012	Cantaloupe	*Salmonella* Typhimurium and Newport
2011	Cantaloupe	*Listeria monocytogenes*
2011	Tomatoes	*Salmonella* Newport (061 PFGE Pattern)
2011	Romaine Lettuce	*E. coli* O157:H7
2011	Papayas	*Salmonella* Agona
2011	Alfalfa and Spicy Sprouts	*Salmonella* Enteritidis
2011	Cantaloupe	*Salmonella* Panama
2010	Tomatoes	*Salmonella* Newport (061 PFGE Pattern)
2010	Alfalfa Sprouts	*Salmonella* I 4, [5], 12:i:- (PFGE Pattern)
2010	Alfalfa Sprouts	*Salmonella* Newport
2009	Tomatoes	*Salmonella* Newport (061 PFGE Pattern)
2009	Alfalfa Sprouts	*Salmonella* Saintpaul

2008	Tomatoes	*Salmonella* Newport (061 PFGE Pattern)
2008	Jalapeño Peppers, Serrano Peppers, Tomatoes	*Salmonella* Saintpaul
2008	Cantaloupe	*Salmonella* Litchfield
2007	Tomatoes	*Salmonella* Newport (061 PFGE Pattern)
2006	Tamatoes	*Salmonella* Newport (061 PFGE Pattern)
2006	Tomatoes	*Salmonella* Typhimurium
2006	Fresh Spinach	*E. coli* O157:H7

Within the last few years the fields of food safety and plant science have begun to merge in order to more efficiently address some of the knowledge gaps involving the mechanisms by which human pathogens contaminate plants. We currently know a great deal about the mechanisms by which plant pathogens are able to cause disease in plant crops as well as the immune response of these plants to these pathogens. There is very little information available regarding the way plants respond to human pathogens. By studying the relationships between plant pathogens and the plant immune response, scientists have been able to successfully develop biocontrol strategies to reduce crop damage attributed to plant disease. This chapter addresses the mechanisms by which human pathogens interact with plants in the pre-harvest environment and the impact these interactions ultimately have on food safety and public health.

3.3 Human Pathogens on Plants

In general, many species of bacteria have close relationships with plants and in some cases promote plant growth and nitrogen uptake. Similar relationships exist with plant viruses; however, these relationships tend to be more detrimental to the plant when plant disease occurs along with large economic losses and threats to food security and public health. Alternatively, crops may become contaminated with bacterial and viral pathogens that are a threat to human health but not necessarily to plant health. Research has shown that plants can become contaminated with human pathogens in the pre-harvest environment through a variety of outlets including soil, feces, irrigation water, or water used to apply pesticides and fertilizers, dust, insects, land-applied manures and biosolids and directly from wild or domestic animals (Beuchat, 2002). Leafy greens are considered a high-risk food crop because they have been epidemiologically linked to foodborne illness (Tabana and Halkmanb, 2011), they are commonly consumed in their raw state, where little or no processing takes place to reduce contaminants. Leafy greens are not all the same when it comes to potential contamination. For example, spinach forms a canopy that may serve as a trap for zoonotic pathogens due to protection from environmental stressors, such as UV, if the plants become contaminated. Outer leaves of lettuce plants may provide a reservoir for pathogens but this route of contamination may not lead to illness. Addressing safer ways to manage crop growth and harvest is essential to minimizing microbial contamination (Sobsey *et al.,* 1980). In order to address these issues we must first develop a better understanding of how human pathogenic bacteria and viruses interact with plants.

It has been previously demonstrated that human pathogenic viruses, such as human norovirus, can be internalized into leafy greens through the roots and foliar

surfaces of lettuce plants (Wei *et al*., 2010, 2011; Hirneisen *et al.,* 2012, Hirneisen and Kniel, 2013; Wang and Kniel 2015) although it is not known how often this naturally occurs in the field and how internalization physically affects the plant. It is important to determine the type of relationships that human pathogens have with plants, whether they be symbiotic, endophytic or antagonistic. For example, it was suggested by Schikora *et al.* (2008) that human pathogenic *Salmonella typhimurium* infect and intracellularly proliferate within tissues of *Arabidopsis thaliana* through both the root and shoot of the plant. *Salmonella* infection also yielded disease symptoms in the plants including wilting, chlorosis and death of infected plant organs compared to non-inoculated controls (Schikora *et al.,* 2008). However; in these experiments *Salmonella* was infiltrated into plants, an event that does not occur in nature. *Arabidopsis* plants responded immunologically similarly to *Salmonella* as they would to the plant pathogen *Pseudomonas syringae* (DC3000) by induction of the mitogen activated protein kinase (MAPK) cascades as well as by enhanced expression of pathogenesis related (*PR*) genes (Schikora *et al.,* 2008). There are also studies demonstrating that bacteria normally pathogenic to humans and other mammals can induce immune response in plants including *Salmonella enterica*, *Pseudomonas aeruginosa*, *Burkholderia cepacia*, *Erwinia* spp., *Staphylococcus aureus*, *E. coli* O157:H7, and *Listeria monocytogenes* (Haapalainen *et al.* 2009; Holden *et al.* 2009; Plotnikova *et al.* 2000; Prithiviraj *et al*. 2005; Milillo *et al*., 2008).

Most of the studies described above have been performed on *Arabidopsis thaliana, which* according to the National Institutes of Health (NIH), is a small flowering plant that is widely used as a model organism in plant biology (NIH, 2015). *Arabidopsis* is a member of the mustard (*Brassicaceae*) family that includes crop

plants such as cabbage, cauliflower and broccoli. It is an important plant for genetic and biological research of crop plants although it has no importance in agriculture itself. Approximately 115 Mb of the 125 Mb genome has been sequenced and annotated and there are extensive genetic and physical maps available of all 5 chromosomes (Arabidopsis Genome Initiative, 2000). The life cycle of *A. thaliana* is short compared to most crop plants (only about 6 weeks) and can easily be cultivated in small spaces such as the laboratory or within a biochamber. There are a number of genetic mutant lines and genomic resources available to the scientific community, making *A. thaliana* a good candidate for multinational research in academia, industry and government. Because of all the above listed reasons, it can also be concluded that *Arabidopsis* serves as a good model for studying human foodborne pathogen – plant interactions.

Studies of the interactions between plants and plant pathogens, including the plant immune response, have led to the development of control measures for many devastating plant diseases. Through the study of the way plants respond to and effectively fight off plant pathogens, scientists have been able to develop plant breeding programs for disease resistant varieties. Many of the current control and prevention strategies for human pathogens on plants are at the post-harvest level - once the pathogens have already come into contact with or possibly penetrated the plant tissues. Alternative intervention measures target prevention of the initial contamination event (Cooley *et al.,* 2003). For effective measures to be developed for pre-harvest interventions, a better understanding of the mechanisms by which human pathogens colonize plants as well as how and if the plants are able to recognize these pathogens is needed (Cooley *et al.,* 2003).

The mechanism by which the plant immune system operates is through the detection of conserved microbial components (Jones and Dangl 2006). The plant physiological responses to plant infection can be due to activation of the induced systemic resistance (ISR) or systemic acquired resistance pathway (SAR). The SAR pathway is induced if the pathogen is able to elicit a hypersensitive reaction (HR) (Durrant and Dong, 2004; Enyedi *et al.* 1992), which causes apoptosis in the plant in order to rid itself of the infectious agent. These events may enable the plant to become more resistant to future attacks by the pathogen (Ross, 1961; Madamanchi and Kuc, 1991). When a plant is invaded by a pathogen, one of two defense signaling pathways are activated: a salicylic acid (SA) dependent pathway or an SA-independent pathway involving jasmonic acid (JA) or ethylene (ET) signaling molecules (Kunkel and Brooks, 2002). JA-dependent and ET-dependent plant defenses are generally activated by necrotrophic pathogens and chewing insects, whereas SA-dependent defenses are often triggered by biotrophic pathogens (Pandey and Somssich, 2009). JA and SA signaling usually act antagonistically, but synergism between these two molecules has also been observed (Mur *et al.*, 2006). The interactions among SA-, JA- and ethylene-dependent pathways are extremely complex (Katagiri *et al.,* 2002). For example, during an immune response of *A. thaliana* to plant pathogen *P. syringae* DC3000, activation of the JA- pathway has been observed to suppress the SA-pathway and reduce the plants general resistance to the pathogen *P. syrinage*, among which most plants use the SA-pathway (Katagiri *et al.,* 2002). It is also hypothesized that some virulent strains of *P. syringae* take advantage of this antagonistic interaction in order to suppress the *Arabidopsis* defensive response (Katagiri *et al.,* 2002).

3.4 Human Pathogen Interactions with Phytobacteria and Rhizobacteria

There is a substantial amount of evidence in the literature that shows the ability of human pathogens to survive in the pre-harvest environment such as in water, on plants, in manure or in fertilizers. While human enteric pathogens are not typically considered to be part of the epiphytic microbial population, this is being considered further as the fields of plant science and food safety begin to merge to address important food safety issues (Lim *et al.,* 2014). The ability of these pathogens to survive on plants is dependent upon many environmental factors such as UV exposure, humidity, weather and presence of epiphytic organisms. Results from studies investigating the ability of human pathogens to survive on plants varies considerably depending on the experimental design including whether the experiment was carried out in the field or in a greenhouse environment. It is also possible for human pathogens to become internalized in plants thorough different sources of contamination and may involve diverse plant entry mechanisms (Martiez-Vaz *et al.,* 2014). Internalization can occur via uptake through the root system along with water, via entry through natural plant openings such as the stomata or via would sites on the plant (Martiez-Vaz *et al.,* 2014). While it is possible for internalization to occur in leafy greens, this phenomenon most likely does not occur frequently with the population levels of bacteria normally found in soil or on plants (Sharma *et al.,* 2009; Zhang *et al.,* 2009; Erickson *et al.,* 2010).

Other factors that influence the colonization of human pathogens on plants include the presence of resident phytobacteria. Presence of plant-associated microbiota can either promote or inhibit the establishment of human enteric pathogens on plants (Lim *et al.,* 2014). One supermarket survey demonstrated that 60 % of produce showing symptoms of soft rot also tested positive for the presence of *Salmonella*

(Wells and Butterfield, 1997). Other laboratory studies have shown that the presence of other pectinolytic plant pathogens including *Erwinia chrysanthemi* and *Pectobacterium carotovorum* that positively influenced the growth of *S*. Typhimurium and *E. coli* O157:H7 to levels 10 times higher than that on healthy plants (Noel *et al.,* 2010; Yamazaki *et al.,* 2011). Similar studies have shown that this sudden increase of pathogens on plants could be directly correlated with the presence of soft rot symptoms (Brandl, 2008; Goudeu *et al.,* 2013). The diverse epiphytic microbial community on plant tissues varies functionally and structurally depending on the species of the plant (Brandl *et al.*, 2013). Plant pathogens including erwinias, xanthomonads and pseudomonads reside closely within the phyllosphere with human pathogens including proteobacteria, firmicutes, bacteroidetes and actinobacteria (Lopez-Velasco *et al.,* 2012; Rastogi *et al.,* 2012). It was also recently observed that colonization of plant pathogen *Xanthomonas perforans* positively influences the persistence of *S. enterica* on tomatoes (Potnis *et al.,* 2014). More recently, downy mildew disease caused by the oomycete *Bremia lactucae* was shown to promote the colonization of *E. coli* O157:H7 and *S*. Typhimurium on romaine lettuce (Simko *et al.,* 2015).

These data suggest the complexity of relationships between plants, resident plant microbiota and human pathogens. Perhaps even more complicated is the less studied interactions of plants, human pathogens and resident rhizobacteria. Human bacterial pathogens have been shown to be able to persist in soils for much longer time periods compared to on plants. *S*. Typhimurium and *E. coli* O157:H7 have been shown to persist in soil amended with contaminated compost for over 200 days (Islam *et al.,* 2004a; Islam *et al,* 2004b). Franz *et al.,* (2011) showed the ability of E. coli O157:H7

to survive for 211 days in manure-amended soil. In another study, *S*. Typhimurium was shown to be able to survive for up to 332 days in manure-amended soil (You *et al.,* 2006). Discrepancies between these survival studies in soil have been attributed to different environmental conditions, inoculation methods, and microbial enumeration methods (Martínez-Vaz *et al.,* 2014). Environmental factors, such as soil topography, moisture and proximity to water sources have also been shown to increase the frequency of isolating enteric bacteria from vegetable farms (Strawn *et al.,* 2013). Presence of protozoa in soils and subsequent encapsulation of pathogens is hypothesized to have a protective effect that allows for prolonged survival of enteric bacteria in contaminated environments such as manure-amended soils (Brandl *et al.,* 2013). Although there are many factors that affect the persistence of human pathogens on plants or within the soil, there is enough evidence to conclude that human pathogens can survive for long enough periods that allow them to contaminate plant roots, leaves, and fruit to allow them to gain access to the human host (Martínez-Vaz *et al.,* 2014). This further supports the theory that plants are used as a vector by human pathogens to ultimately reach their host.

Rhizobacteria are also able to interact with pathogens within the phyllosphere of plants through complex indirect plant-microbe interactions. Studies suggest that upon detection of a pathogen a plant is able to recruit bacteria to colonize the root surface, triggering induced systemic resistance (ISR) in aerial portions of the plant (Ryu *et al.* 2004; Lakshmanan *et al.,* 2012) and ultimately results in the closure of plant stomata (Kumar *et al*., 2012). Specifically in 2008, Rudrappa *et al.* found that plants can recruit specific *Bacillus subtilis* spp. to their roots via secretion of malic acid when aerial tissues come into contact with *Pst* DC3000. It has been demonstrated

that this specific plant growth promoting rhizobacteria (PGPR) are able to induce stomatal closure when the root is colonized (Kumar *et al*., 2012).

Interestingly, PGPR have been shown to have the ability to induce stomatal closure in romaine lettuce and spinach plants (Markland *et al.,* 2014 (Figures 8 and 9).

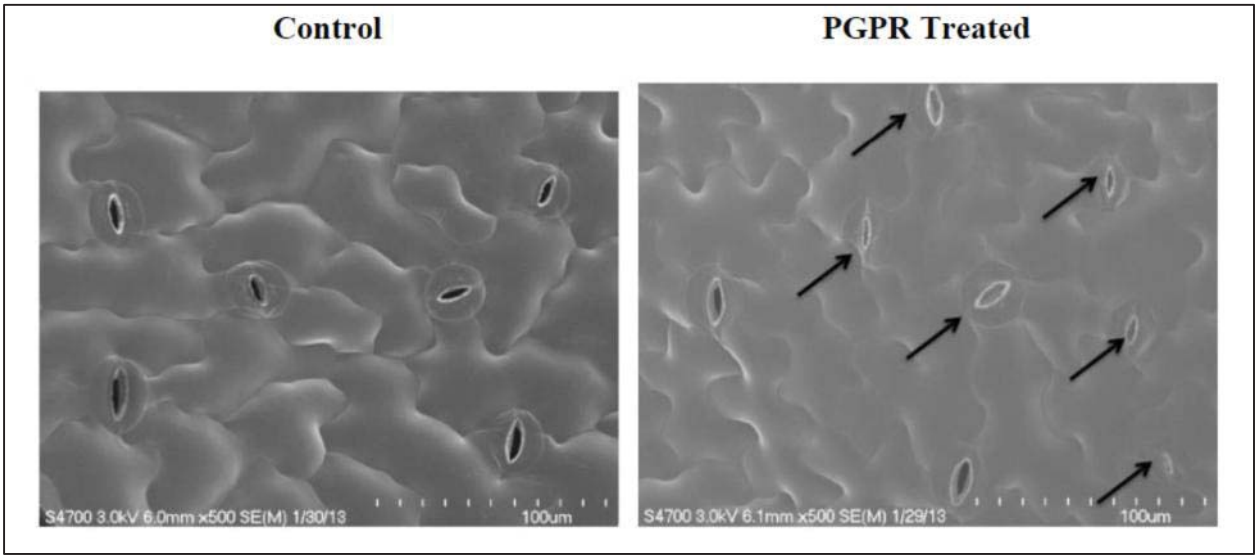

Figure 9: Cryo-scanning electron microscopy images of romaine lettuce leaf sections of PGPR treated and non-treated plants. Showing the effects of PGPR *Bacillus subtilis* UD1022 inoculation on the roots of romaine lettuce plants at 3 hours post inoculation. At 3 hours post inoculation, more stomata on PGPR treated plants are closed compared to non-treated plants (controls). Closed stomata are shown by arrows. Images taken at the University of Delaware Biotechnology Institute Bioimaging Center (Markland *et al*., 2014).

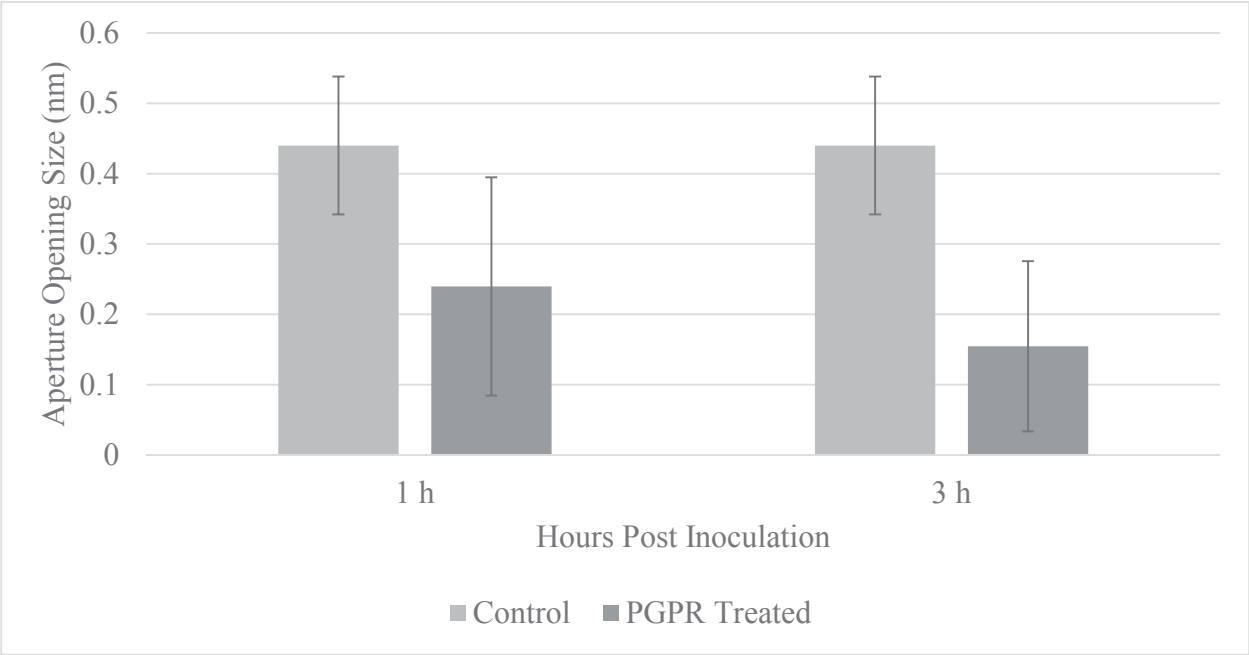

Figure 10: Showing the difference in the aperture opening size of stomata of romaine lettuce where roots were inoculated with water (light) or with PGPR (dark). Results show that stomata are significantly smaller at 3 hpi when roots were treated with PGPR compared to controls. Data was collected by measuring stomata aperture openings of SEM images using ImageJ Software (National Institutes of Health, Bethesda MD) (Markland *et al.,* 2014).

Over a 5 day study, addition of the same PGPR to the soil of lettuce plants was shown to significantly inhibit the persistence of *L. monocytogenes* on the leaves of lettuce plants (Markland *et al*., 2014). While this study only scratches the surface for the use of PGPR as a biocontrol agent to reduce the risk of human pathogens on plants in the pre-harvest environment, there is great potential for optimization and use. The use of PGPR has the potential to protect plants from infection by plant pathogens as well as from human pathogens. PGPR can also increase crop yield and may be an economical and sustainable means for protecting public health and crops. Further research in this field may enable the development of new technology that utilizes the natural association between rhizobacteria and plants to minimize contamination by human pathogens in food crops.

3.5 Relationships between Plant Stomata, Circadian Rhythm and Human Pathogens

A plant stoma is a pore located in the epidermis of the leaf and the stem that functions in gas exchange. A pair of parenchyma cells, also known as guard cells, borders the stoma and controls the size of the pore and the opening and closing of the pore. Carbon dioxide (CO_2) and oxygen (O_2) enter the plant through the stomata and are used in photosynthesis and respiration. The stomata also function in transpiration via the release of water vapor from the plant. Stomata are also known to be important global regulators of the atmospheric environment (Hetherington and Woodward, 2003). Much is known and has been studied involving the anatomy of the stomatal valve, but there is a lack of knowledge as to how exactly stomata sense and respond to their external stimuli (Brodribb and McAdam, 2011), specifically bacterial pathogens. These openings can also serve as a portal of entry for plant pathogens and the opening

and closing of the stomata is actually a triggered innate immune response (Melotto *et al.*, 2006). Although it has been known for quite some time that the phyllosphere is a common place for the colonization of plant and human pathogens (Melotto *et al.,* 2006), there is not enough data supporting the mechanism of entry of these pathogens into the plant host and the host immune response involved. Unlike fungal pathogens, bacteria do not have the ability to penetrate the plant epidermis and rely on plant openings or wounds to internalize into plant tissues (Melotto *et al.*, 2006).

For the first time in 2006, Melotto *et al.* demonstrated that the small pores located on the surface of leaves functioning in gas exchange actually close in response to microbial contact. The authors of this study also show that, in turn, human pathogens have the ability to reopen the stomata in order to gain subsequent entry into the plant. The authors used *Arabidopsis* as a plant model and observed closure of the stomata within 2 hours of inoculation with both the plant pathogen *Pseudomonas syringae* DC3000 and the human pathogen *E. coli* O157:H7. However; after 3 hours of incubation with *Pst* DC3000 stomata reopened, whereas, stomata on plants inoculated with *E. coli* O157:H7 remained closed throughout the experiment (8 h).

Although human pathogens were not be able to elicit reopening of plant stomata in this study, stomatal openings are still believed to play a role in contamination of leafy greens. Pathogens have previously been shown to migrate towards stomatal openings under certain conditions and can internalize into plants (Erickson, 2012). A recent study by Markland et al. (2014) also showed that *Salmonella* Newport and *Listeria innocua* were able to reopen stomata on spinach and lettuce plants, respectively, after 3 hours post inoculation on plant leaves. Intrinsic and extrinsic factors including circadian rhythm and photosynthesis (light) are thought to

play a role in stomatal opening and closing. Studies have shown that there are direct effects of light on both the defense response in the host plant and on the virulence of the attacking pathogen (Oberpichler *et al*. 2008). It is believed that plant defenses are heightened in response to light; however; some plant defense responses occur independently of light, such as jasmonic acid production (Zeier *et al.,* 2004). Light has been shown to play a particularly important role in salicylic acid (SA)-mediated defense responses (Roden and Ingle, 2009). Light is also required for the hypersensitive response (HR), a form of localized programmed cell death at the site of infection, activated during effector-triggered immunity (ETI) (Roden and Ingle, 2009). Because plant defense is heightened in the light, virulence of pathogens tends to be heightened in the dark. Oberpichler *et al.* (2008) showed that there is a link between light perception and virulence involving cell motility. The authors of this study showed that bacteria cultured in light had a reduced number of flagella (one or two compared to three to five in the dark) and exhibited reduced motility in colony assays. Virulence was also shown to be directly affected by light where reduced root attachment was observed in tomato and smaller tumor formation in cucumber in presence of light compared to darkness (Oberpichler *et al.,* 2008). Experiments have demonstrated that having a functional circadian oscillator with the same period as Earth's rotation gives plants an adaptive advantage and increased fitness (Dodd *et al*., 2005). It is important to further understand the role of circadian rhythm in plant defenses to human enteric pathogens in order to develop better crop management practices to reduce contamination of leafy greens. By understanding how foodborne pathogens utilize plant stomata to internalize and persist on plants, we may also be able to develop biocontrol strategies that control stomtal opening and closing.

3.6 Control and Prevention Strategies: Biocontrol and Plant "Probiotics"

In the pre-harvest environment there are many control and prevention strategies currently used by produce growers that help protect plants from infection by plant pathogens and pests that can cause damage to crops. There are other guidelines, including Good Agricultural Practices (GAPs), which provide information for growers on how to prevent their crops from becoming contaminated with human pathogens (FDA, 1998); however, addressing this issue is much more complex in that we do not know exactly how or why human pathogens interact with plants. It has been hypothesized that the ability for human pathogens to colonize edible plants may be an effective survival strategy that provides a direct route from its excretion in the environment back to its numerous herbivorous and omnivorous hosts (Brandl *et al.,* 2013). The implementation of the Food Safety Modernization Act (FSMA) in 2011 and introduction of the Produce Safety Rules will now require qualified growers to follow more stringent food safety practices that could increase the cost of food production and subsequently raise the cost of food. In addition, food trends including the organic and non-GMO campaigns have led to a decrease in the use of fertilizers previously used to reduce crop loss as well as higher food prices for these commodities.

It would be ideal, to innovate technologies that could reduce contamination of crops by plant and human pathogens simultaneously. One potential solution includes the use of plant growth promoting rhizobacteria (PGPR) which can act as plant "probiotics". PGPR are considered part of the natural microflora of plants as well as important contributors to plant health through plant growth promotion or biological disease control. They are often used to induce suppressiveness of plant pathogens by altering the diversity of microorganisms in the rhizosphere (Kloepper *et al*., 1999). In

most cases, biological control by PGPR results from bacterial production of metabolites that directly inhibit the pathogen such as antibiotics, hydrogen cyanide, iron-chelating siderophores, and cell-wall degrading enzymes (Kloepper *et al.,* 1999). Plant growth promotion is considered an indirect mechanism of disease control as the time a plant is in a susceptible state is shortened, allowing for the plant to escape infection (Kloepper *et al.,* 1999). Use of PGPR have also been used to reduce plant contamination by foliar pathogens where PGPR control involves induction of plant host defenses (Kloepper *et al*., 1999). As discussed in section 4 of this chapter, recent studies have demonstrated the ability of PGPR Bacillus Subtilis to induce stomatal closure in lettuce and spinach as well as reduce the persistence of *L. monocytogenes* on these plants. Through a series of interactions between the human pathogen, the plant and the PGPR, the plant recruits the PGPR by secretion of malic acid to form biofilms on the roots of the plants which induces plant defenses and ultimately somtata closure (Figure 10).

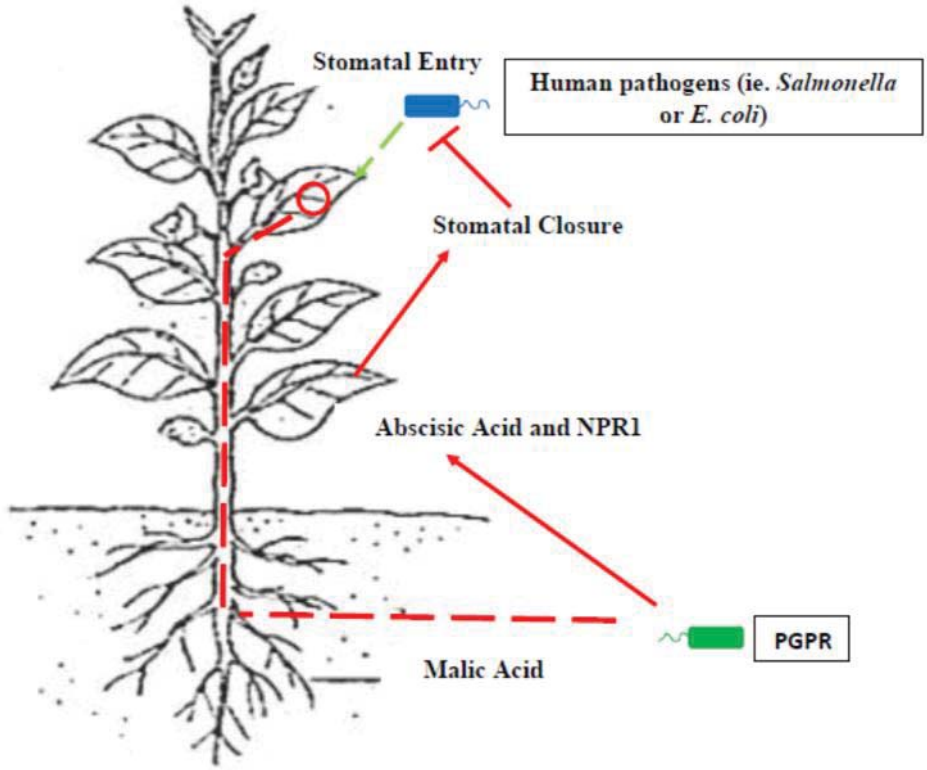

Figure 11: Showing a schematic of the mechanism by which plant growth promoting rhizobacteria (PGPR) induces induced systemic response (ISR) in plants. 1. Pathogen lands on aerial portions on the leaf of the plant 2. The plant detects pathogen associated molecular patterns (PAMPs), such as flagella which triggers an "SOS" signal to initiate plant defenses and secretes malic acid into the soil. 3. The malic acid signal recruits PGPR to the roots. 4. Systemic response is induced in the plant as well as stomatal closure via SA, NPR1 and ETH to protect plant from pathogen invasion. Figure adapted from Lakshmanan *et al.,* 2012.

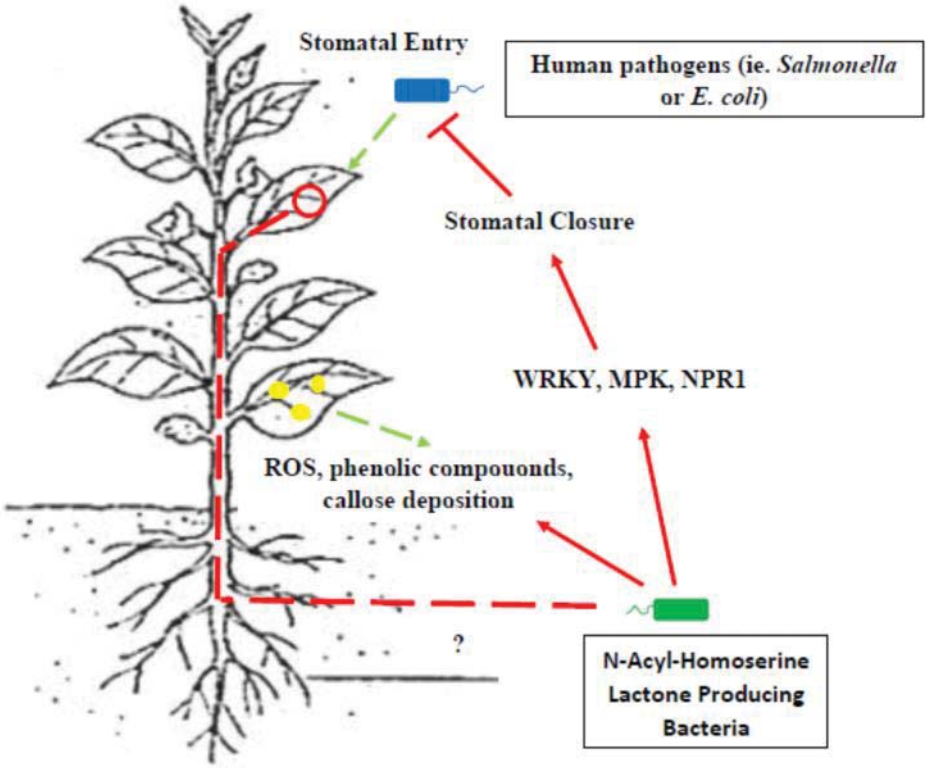

Figure 12: Showing a schematic of the mechanism by which N-acyl-homoserine lactone producing bacteria induces stomata closure in plants. 1. N-acyl-homoserine lactone producing bacteria activates MAPKs and enhances the expression of WRKY transcription factors. 2. Accumulation of ROS, phenolic compounds, and collose in the cell walls. 3. Elevated production of phytohormones oxylipin and salicylic acid. Induction of callose deposition and enhanced stomatal closure. Figure adapted from Schnek and Schikora 2014.

It was also demonstrated that plants have the ability to detect N-acyl-homoserine lactones (AHLs), used by gram negative bacteria for quorum sensing behavior, and are subsequently able to induce system resistance through induction of WRKY and PR-1 genes (Schikora *et al.,* 2011). It has been proposed that N-3-oxo-tetradecanoyl-l-homoserine lactone (oxo-C14-HSL) primes plants for enhanced callose deposition, accumulation of phenolic compounds, lignification of cell walls and increases levels of oxylipins and salicylic acid which favors closure of plant stomata (Schenk *et al*., 2014) (Figure 11). The use of PGPR, N-acyl-homoserine lactone-producing bacteria as plant probiotics and biocontrol agents may help provide a sustainable means for growers, including those raising organic crops, to reduce environmental and human health risks associated with growing produce as well as improving productivity and crop yield thus; reducing cost of production and increasing net farm income. Biocontrol and manipulation of pathogens on plants can reduce the initial contamination that may be unavoidable, at times, and spread by cross-contamination during washing or during packaging of produce.

3.7 Attachment and Attraction Mechanisms of Human Pathogens to Plants

The mechanisms by which human pathogens attach to plants are complex and vary greatly between different types of plant-pathogen interactions. There are four main types of interactions that involve attachment of human pathogens to plants including cell surface structures, virulence, motility and biofilm formation (Martínez-Vaz *et al.,* 2014). For example, *S*. Tennessee was previously found to be able to adhere more efficiently to lettuce and cabbage surfaces compared to other *Salmonella* serotypes likely due to its ability to form biofilms (Patel and Sharma, 2010). The components of the Salmonella extracellular matrix are involved in attachment to plant

surfaces including cellulose, capsule, and fimbriae (Lim *et al.,* 2014). It has also been confirmed that the gene for global stress regulation (*rpoS*) plants an important role in biofilm formation and adhesion regulation (Raina *et al.,* 1993; Prigent-Combaret *et al.,* 2001) and is required for initial attachment to plant surfaces (Lim *et al.,* 2014). A transcriptional regulator of the LuxR superfamily in *Salmonella* (agfD) regulates curli and cellulose production as well as the O-capsular antigen (Römling *et al.,* 1998). When *S.* Typhimurium presents the rdar morphotype, which is positively regulated by AgfD, it demonstrates improved attachment on tomato plants (Gu *et al.,* 2011). Motility and chemotaxis genes have also been shown to be required for early attachment of Salmonella on leafy greens. *Salmonella* utilizes flagellar motility and demonstrates chemotactic behavior toward leaf exudates for efficient attachment and internalization on lettuce (Kroupitski *et al.,* 2009). However; motility and chemotaxis characteristics among Salmonella species may vary depending on bacterial genotypes and environmental conditions (Martínez-Vaz *et al.,* 2014).

Virulence factors that have been shown to play a role in the attachment of *E. coli* O157:H7 to plant surfaces include curli fibers (Macarisin *et al*., 2012), the type III secretion system (Xicohtencatl-Cortes *et al*., 2009; Kenny *et al.,* 1995; Saldana *et al.,* 2009), and capsular production (Hassan and Frank, 2004; Rahn *et al.,* 2003). In *E. coli* and *Salmonella*, the yfcR gene, involved in tolerance to multiple stresses as well as biofilm formation, has been shown to plant a critical role for attachment to vegetable tissues (Fink *et al*., 2012; Deng *et al.,* 2011). In addition, the temperature and environmental growing conditions under which plants are grown can influence the regulation of certain virulence and attachment genes. Transcriptome analysis of human pathogens in close association with plant surfaces, specifically *E. coli* and *Salmonella*,

have shown that distinct changes occur in gene expression when pathogens are in association with damaged or intact plant surfaces (Martínez-Vaz *et al.,* 2014). Findings from these studies suggest that mechanisms for attachment of human pathogens in the phyllosphere or the rhizosphere may be species-specific; therefore, caution should be used when developing prevention and control strategies for human pathogens on plants in the pre-harvest environment because strains of the same bacterial species may respond differently to sanitation treatments (Martínez-Vaz *et al.,* 2014). Resident plant bacteria as well as plant pathogens also utilize curli, fimbriae and cellulose to adhere to plant surfaces (Figure 12) (Teplitski *et al.,* 2009). This shows that human pathogens use attachment mechanisms similar to those of plant pathogens in order to colonize plants (Martínez-Vaz *et al.,* 2014).

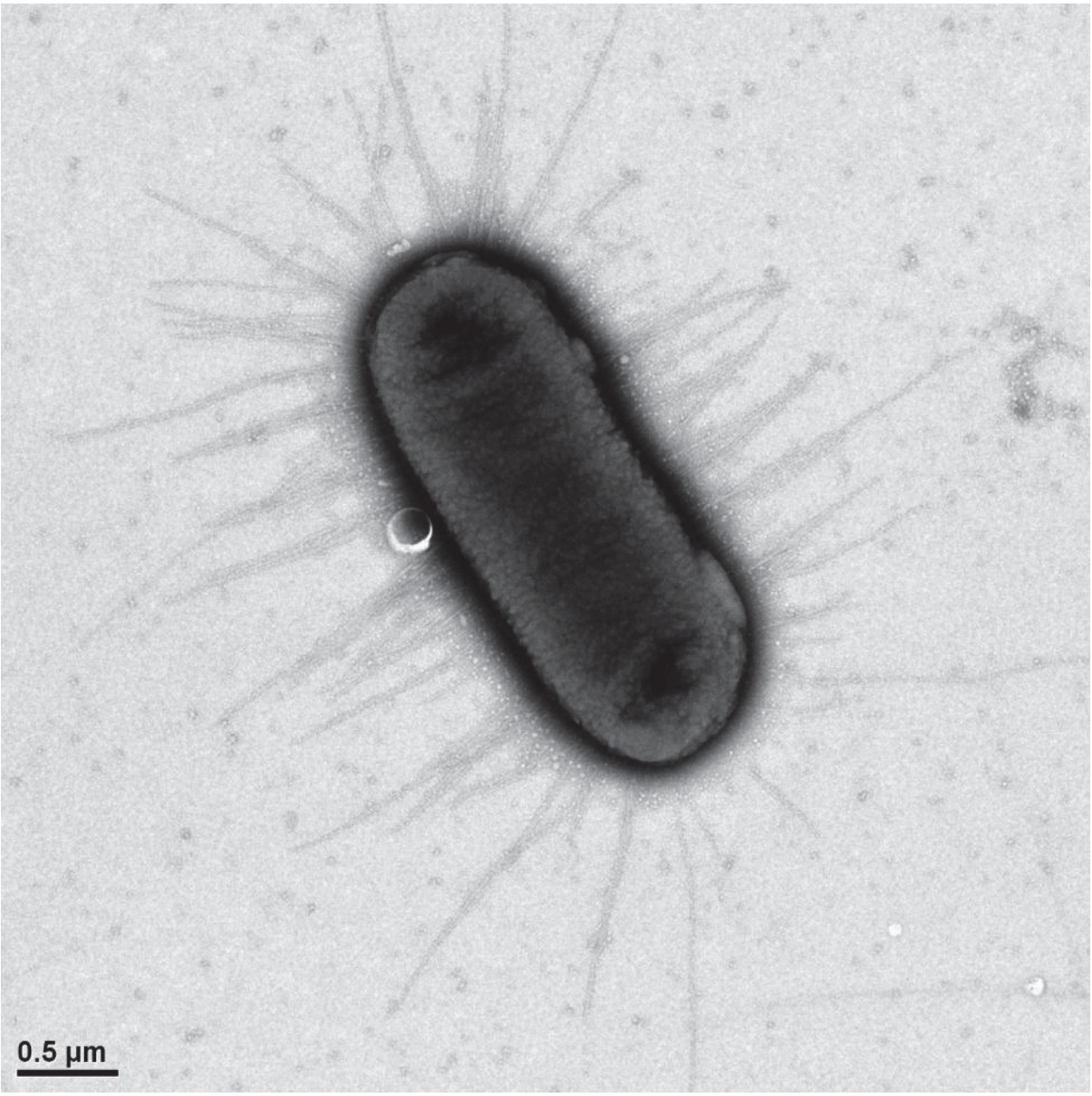

Figure 13: Transmission electron microscopy (TEM) image of an *E. coli* cell showing long fimbriae appendages extending from the bacterial capsule. Image taken at the University of Delaware Biotechnology Institute Bioimaging Center.

3.8 Research Needs and Conclusions

The study of human pathogens on plants has opened our minds to complicated interactions among bacteria, viruses, and plants. There is more to the survival of human pathogens on plant leaf surfaces and in the soil; much more that has yet to be uncovered. Future research in this area will generate fundamental information regarding the physical and molecular mechanisms that enable human pathogens to attach, internalize, grow and survive in and on fresh produce, specifically leafy greens. Additionally this work will generate information on how plants interact with foodborne viruses, specifically norovirus and hepatitis A virus, and if these associations affect the attachment and fate of human pathogens on fresh produce. There are many knowledge gaps involving how plants respond to norovirus contamination which is the number one cause of foodborne illness in the US, with a majority of illnesses involving leafy greens. Information concerning viral persistence and survival will impact growing and irrigation practices. Information gained by studying the plant defense response in relation to colonization by noroviruses may also impact pre-harvest growing practices.

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Chapter 4

INTERACTION OF GROWTH PROMOTING RHIZOBACTERIA WITH HUMAN PATHOGENS ON LEAFY GREENS

4.1 Abstract

Plant growth promoting rhizobacterium (PGPR) *Bacillus subtilis* UD1022 has been shown to trigger an induced systemic response (ISR) in *Arabidopsis thaliana*. This interaction causes plant stomata to close, protecting the plant from infection by plant pathogens in addition to increasing crop yield. The purpose of this study was to determine if UD1022 is able to induce stomata closure in leafy greens as well as influence the persistence of human pathogens (*Listeria* and *Salmonella*) on plants. UD1022 induced stomata closure in the presence of human pathogens on both lettuce and spinach 3 hpi ($p<0.0001$). Results were confirmed by root inoculation with heat-killed UD1022, which did not induce stomata closure. Presence of UD1022 on lettuce roots significantly reduced the persistence of *Listeria* on plants after 3 dpi ($p=0.02$) but had less of an effect on the persistence of *Salmonella*. The results of this study indicate that plant growth promoting rhizobacteria *B. subtilis* UD1022 may be able to prevent contamination by plant pathogens as well as by some human pathogens. This is the first study of its kind to assess the effects of a plant growth promoting biocontrol agent and human pathogens on plants in terms of plant immune response through stomata.

4.2 Introduction

Recently the Centers for Disease Control and Prevention attributed 46% of foodborne illnesses to fresh produce (Painter *et al.,* 2013), indicating the need for more sophisticated efforts to reduce contamination of human pathogens on plants. Plants grow in close association with large communities of microbes, yet comparatively little is known about the diversity of microbes that associate with plants in the pre-harvest environment as well as their interactions and effects on performance, crop yields and plant protection. The fields of plant science and food microbiology have been merging over the past few years in the best interest of produce safety; however, critical knowledge gaps remain. Therefore, novel strategies to control pathogens merit attention and specifically efforts are needed to better understand plant-microbe interactions within the environment. To date, there is limited utility of biocontrol strategies against human pathogens or other opportunistic pathogens of fresh produce crops. Biocontrol and manipulation of pathogens on plants can reduce initial contamination events that may be unavoidable due to various environmental pressures and subsequently may spread by cross-contamination during washing or packaging.

A recent turning point for the produce industry occurred this past year when the Jensen brothers received criminal charges for growing produce that was contaminated with *Listeria monocytogenes* and was associated with the an outbreak resulting in the deaths of 34 individuals (CDC, 2012). Microbial contamination can originate from countless points along the farm to fork continuum. At greatest risk are those aspects of contamination that can occur within the pre-harvest environment,

whereby microbial contamination can come in contact with plant tissues from water, soil amendments, wind, birds, insects, animals, and other fomites (Beuchat, 2002). While a wide array of research has been conducted on ways to reduce microbial contamination through better sanitation and use of safe irrigation water and soil amendments, contamination events still occur.

Bacteria found in association with the rhizosphere or the phyllosphere of plants can be classified into three groups based on the ways they affect plant growth; firstly, plant growth-promoting rhizobacteria (PGPR), secondly, pathogenic/deleterious or thirdly, neutral (Dobbelaere *et al.,* 2003). PGPR can affect plant growth either directly or indirectly (Beneduzi *et al*., 2012). We have recently learned that upon detection of a pathogen a plant is able to recruit PGPR to colonize the root surface, triggering induced systemic resistance (ISR) in aerial portions of the plant (Lakshmanan *et al.,* 2012; Potnis *et al.,* 2014) and ultimately closure of plant stomata (Rudrappa *et al.,* 2008). Specifically in 2008, Rudrappa *et al.* (Rudrappa *et al.,* 2008) found that plants can recruit specific *Bacillus subtilis* spp. to their roots via secretion of malic acid when aerial tissues come into contact with *Pseudomonas syringae* DC3000 pv tomato (*Pst* DC3000). It has been demonstrated that this specific PGPR (*Bacillus subtilis* UD1022) is able to induce stomata closure when the root is colonized (Rudrappa *et al.,* 2008). This specific strain serves as a biocontrol agent by restricting the stomata-mediated pathogen entry of *Pst* DC3000 in the laboratory plant model *Arabidopsis thaliana* (Rudrappa *et al.,* 2008).

While there is a great deal of information available on the mechanisms by which plants respond to plant pathogens, there is very little data available regarding the ways plants respond to human pathogens including *Escherichia coli*, *Salmonella*

and *Listeria*. By studying the relationships between plant pathogens and the plant immune response, scientists have been better positioned to successfully develop biocontrol strategies to reduce crop damage attributed to plant disease. Novel biocontol isolates not only increase plant crop yields and reduce infection from plant pathogens, but may also help prevent the spread of human disease by reducing the ability of human pathogens to persist on plants. The purpose of this study was to determine if UD1022 could potentially serve as a novel biocontrol agent by eliciting stomata closure in leafy greens via root colonization and affect the persistence of human pathogens on plants.

4.3 Materials and Methods

4.3.1 Romaine lettuce and spinach cultivation

Romaine lettuce (*Lactuca sativa L.* var. longifolia*)* and spinach seeds (*Spinacia oleracea)* (Johnny's Selected Seeds, Waterville, ME) were planted in a sterile pro-mix made up of 85% Canadian sphagnum peat moss with perlite, vermiculite, dolomitic and calcitic Lime, a wetting agent and Mycorrhizae (Premier Tech Horticulture, Quakertown, PA) and maintained in a Biosafety Level 2 (BSL-2) growth chamber (Percival Scientific, Boon, IA) at the University of Delaware Fischer Greenhouse (Newark, DE). Growth conditions used included 19°C temperature with a 12-hour photoperiod until 4 leaves were present on each plant (3-4 weeks). Plants were then transplanted to individual containers containing sterile promix (square containers 4 cm x 3.5 cm x 4.5 cm in dimension; T.O. Plastics, Ontario, Canada). Holes were created in the bottom of each individual container which was then placed into plastic bins inside the growth chamber. Throughout the experiment water was poured into the

bottom of each plastic container to prevent influence of bacterial movement on plants and soil by direct watering. The growth chamber temperature conditions were monitored daily and temperature was maintained at 19 ± 0.30ºC, relative humidity was 60.0 ± 2.08 %.

4.3.2 Bacterial growth and enumeration

The proposed biocontrol agent *Bacillus subtilis* UD1022 (previously known as *B. subtilis* FB17) as well as plant pathogen *Pseudomonas syringae* DC3000 were provided by Dr. Harsh Bais at the University of Delaware (Lakshmanan *et al.,* 2012; Rudrappa *et al.,* 2008) *Listeria innocua* (University of Delaware Culture Library) was used as a surrogate for the human pathogen *Listeria monocytogenes* for the safety of a pregnant female employee working in the laboratories during the time of these experiments. Human pathogen *Salmonella* Newport was provided by Dr. Michele Danyluk at the University of Florida (McEgan *et al*., 2013). *L. innocua* and *S. Newport* were grown with shaking at 37°C in Tryptic soy broth (Thermo Fisher Scientific, Inc. Fairlawn, NJ) overnight and cultures pelleted and resuspended in peptone water before application to plants. *Pst* DC3000 was grown for with shaking in Kings Broth at 28°C and cultures also pelleted and resuspended in peptone water before application to plants. Human and plant bacterial pathogens were applied to plants by brushing 1 ml of live culture directly onto the leaves of the plant using a paint brush (Hirneisen and Kniel, 2013). UD1022 (3 ml) was applied directly to the soil of plants (~1 cm from stem) directly following foliar inoculation. Bacteria were recovered from plants using selective media for specific pathogens, including Remel XLT-4 (Thermo Fisher Scientific, Inc. Fairlawn, NJ) for *Salmonella* species, and

Oxoid Brilliant Listeria Agar (Thermo Fisher Scientific, Inc. Fairlawn, NJ) for *L. innocua.*

4.3.3 Direct growth inhibition assays

In order to determine if UD1022 had any direct effects on the growth inhibition of human pathogens, growth curves of *L. innocua* (used as a surrogate for *L. monocytogenes*) and *S*. Newport were performed in the presence of varying concentrations (0.022 – 0.1776 mg/µl) of UD1022 lyophilized supernatant.

4.3.4 Cryo-scanning electron microscopy

Romaine lettuce and spinach plants were grown in a controlled biochamber as described above**.** Plants were primed with constant lighting for 24 hours prior to inoculation to ensure stomates were open. Human bacterial pathogens (*L. innocua* and *S*. Newport), plant pathogen *Pst* DC3000 or sterile water (control) were applied to plants by brushing 1 ml of live culture (10^7 CFU/ml) directly onto the leaves of the plant using a paint brush as described above. Roots of plants were inoculated with 3 ml UD1022 (10^8 CFU/ml) live culture, 3 ml UD1022 culture that had been heat treated at 65°C for 18 h in a water bath, or 3 ml sterile water (control) by pipette applied directly onto the soil. Following inoculation, small sections from 2 leaves of each plant were collected at 1 and 3 hours post inoculation and fixed to a stage using cryo-freezing medium mixed with carbon black and frozen in liquid nitrogen. Sections were examined by cryo-scanning electron microscopy at the Delaware Biotechnology Institute (Newark, DE). A total of 20 images were captured per leaf section at three different resolutions (200X, 400X and 1500X) in duplicate (n=40) for each trial performed. Images of individual stomata were captured using the highest resolution

(1500X) and the size (µm) of each individual stomata aperture opening was measured using ImageJ Software (National Institutes of Health, Bethesda MD) at 1 and 3 hours post inoculation.

4.3.5 External Persistence Assays

Methods are all similar to those for data described above. Plant leaves were inoculated with 1 ml (~10^8 CFU/ml) *L. innocua*, *S. Newport, P.syringae* DC3000 or water (control) using a paint brush. *L. innocua* was used as a surrogate for *L. monocytogenes* to maintain consistency with SEM studies. *Listeria innocua* and *S.* Newport were inoculated on leaves alone and in combination with FB17 treatment of plant roots. Water treatment was used as a control. Samples were collected at 0, 0+ (immediately after inoculation), 1, 3, 5, 7 and 10 days post inoculation. Leaves (4) were collected from 4 random plants pooled into one sample. One sample was processed for internalization of pathogens and one sample was processed for external persistence of pathogens on leaves for each sample collection time and treatment group. For external persistence, pooled leaf samples were collected at each sampling time into sterile stomacher bags containing 10 ml of buffered peptone water (BPW) (Thermo Fisher Scientific, Fairlawn NJ) and gently massaged by hand for 1 min. Serial dilutions were performed in BPW and bacterial enumeration for either *L.innocua* or *S.* Newport was performed on selective media as described above.

4.3.6 Internalization Assays

Internalized pathogens were detected by first disinfecting the surface of pooled leaf samples in sterile stomacher bags with 80% ethanol and 0.1% mercuric chloride as previously described by Ericksen *et al.,* 2010. Samples were washed by adding and

discarding 10 ml sterile water to and from each bag. Leaf samples were then ground up using a rubber mallet, resuspended in 10 ml BPW then stomached for 2 min. Aliquots (1 ml) from each bag were then placed into a test tube containing 9 ml Universal Enrichment Broth (Thermo Fisher Scientific, Fairlawn, NJ) and incubated for 18 h at 37°C. Using a sterile inoculation loop, samples from each tube were streaked onto selective medium and incubated again for 18 h then examined for presence or absence of internalized pathogens.

4.3.7 Interpretation and Analysis of Results

For all experimental methods, experiments were performed in duplicate with different trials performed at different times. Bacterial results are reported as log CFU/g plant with 4 leaves equaling one plant. A one-way ANOVA was performed to compare means within the data set and a linear regression model was generated using JMP software (SAS Institute Inc., Cary, NC). To determine the level of influence of the independent variables on the dependent variable (r), generation of a multivariate correlation matrix were also be performed. All p values at < 0.05 are considered significant.

4.4 Results

4.4.1 Direct Growth Inhibition Assays

Growth curve inhibition assays were performed to determine if UD1022 has any direct inhibitory effects towards *L. innocua* or *S*. Newport. The data demonstrated that UD1022 was able to inhibit the growth of *L. innocua* in a dose dependent manner with the most significant reduction occurring at 4 hpi with when *Listeria* was incubated with a UD1022 concentration of 0.044 mg/µl ($p=0.02$) (Figure 13). The

same direct inhibitory effects were not observed in the case of *S*. Newport where UD1022 did not seem to have a significant effect on growth inhibition (Figure 14).

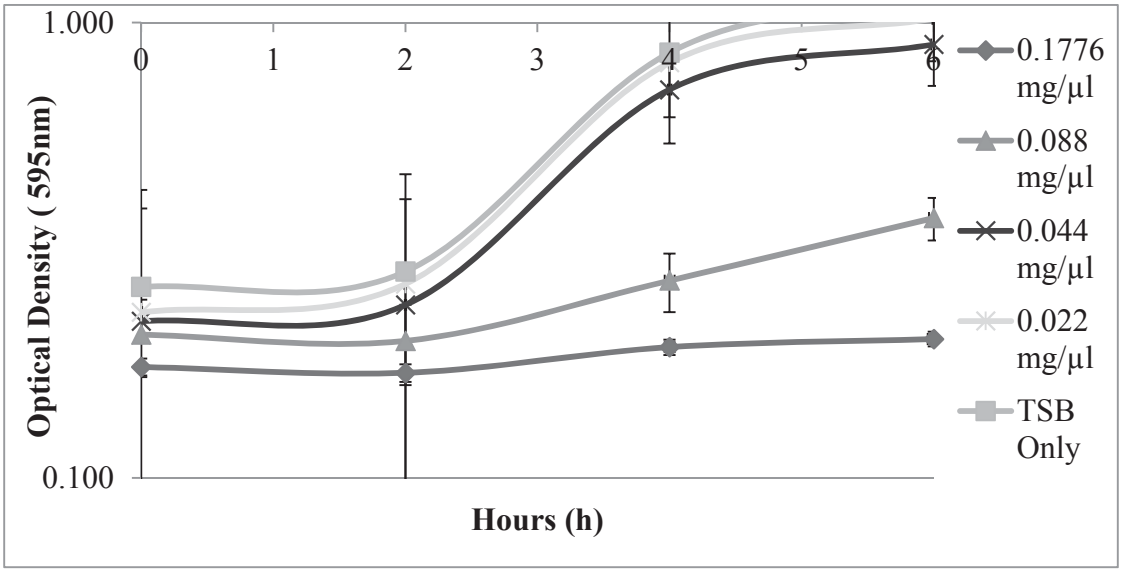

Figure 14: Growth inhibition of *L. innocua* by varying concentrations of lyophilized UD1022 supernatant (0.022 – 0.1776 mg/µl) resuspended inTSB. Growth inhibition was significantly reduced in a dose dependent manner with the most significant reduction occurring with a concentration of 0.044 mg/µl after 4 hours post inoculation (p=0.044) (n=6).

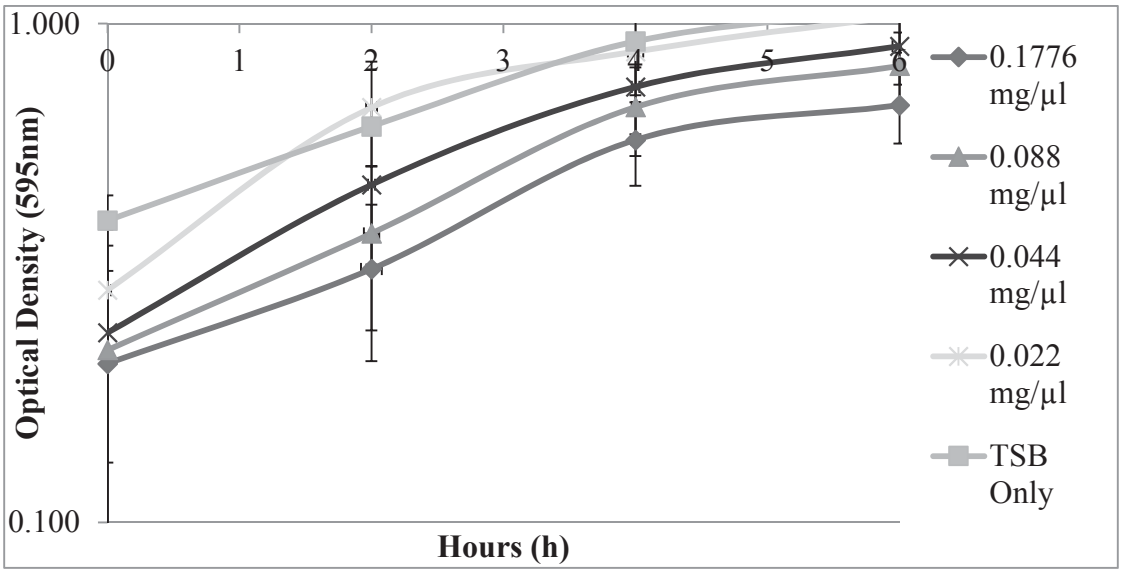

Figure 15: Growth inhibition of *S.* Newport by varying concentrations of lyophilized UD1022 supernatant (0.022 – 0.1776 mg/µl) resuspended in TSB. UD1022 did not effectively reduce levels of *Salmonella* over the 6 hour growth curve despite the level of UD1022 present (n=6).

4.4.2 Cryo-SEM

Significant differences in plant response were indicated between plant species and pathogen interactions ($p<0.0001$). UD1022 was able to significantly reduce the aperture size opening of stomata in lettuce and spinach after 3 hpi as determined by cryo -SEM (Figures 3-8). Results where UD1022 induced stomata closure and ISR in both lettuce and spinach plants were confirmed by inoculating roots of plants with heat killed (HK) UD1022, which did not induce stomata closure. Stomata aperture size was not significantly different from controls in lettuce plants inoculated with *L. innocua* on the leaves and UD1022 on the roots at 1 hpi ($p=0.21$) however; UD1022 inoculated onto the roots of lettuce plants with *L. innocua* simultaneously inoculated onto the leaves significantly reduced the stomata aperture opening size at 3 hpi in these plants indicating ISR was induced ($p = 0.001$) (Figures 15-17).

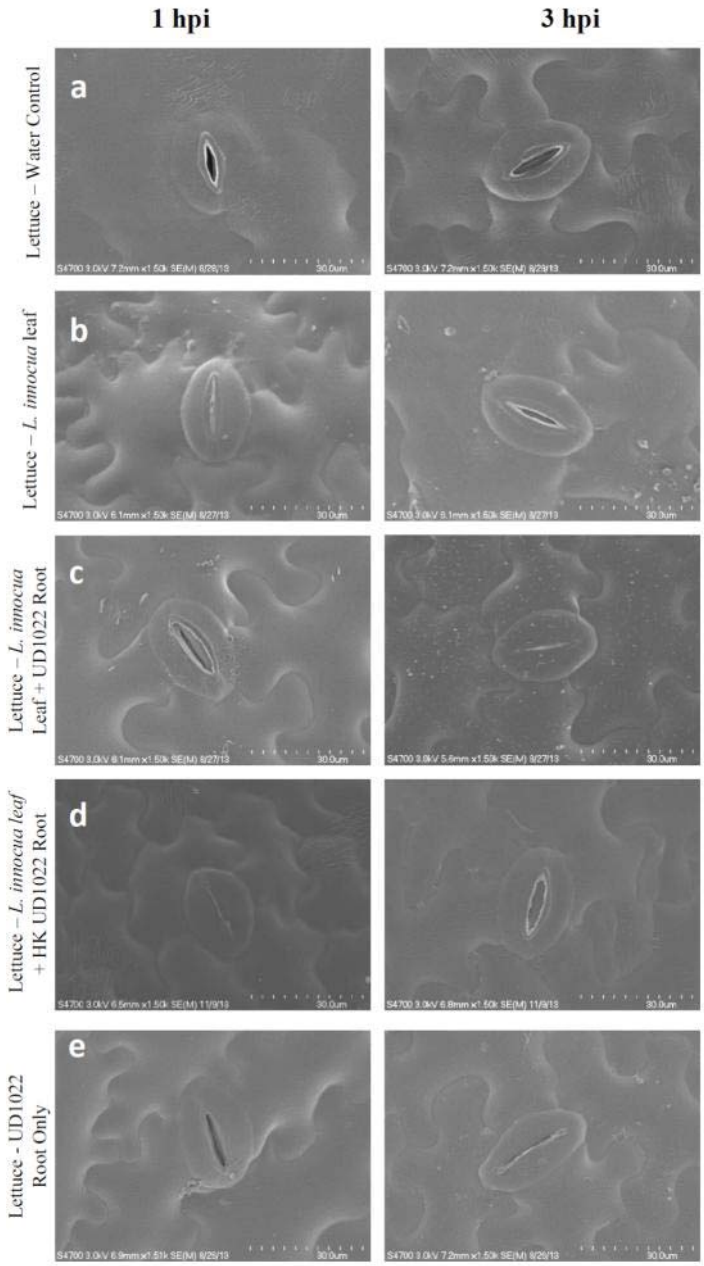

Figure 16: Cryopreservation scanning electron microscopy images (cry-SEM) of individual Romaine lettuce leaf stomata taken at 1.5X resolution. Images show individual leaf stomata after 1 and 3 hpi with the following treatments: (A) Water (control), (B) *L. innnocua* on leaves only, (C) *L. innocua* on leaves + UD1022 on roots, (D) *L. innocua* on leaves + heat killed (HK) UD1022 on roots and (E) UD1022 on roots only**.** A total of 40 stomata were analyzed per treatment (n=40). Scale bar = 30.0 µm

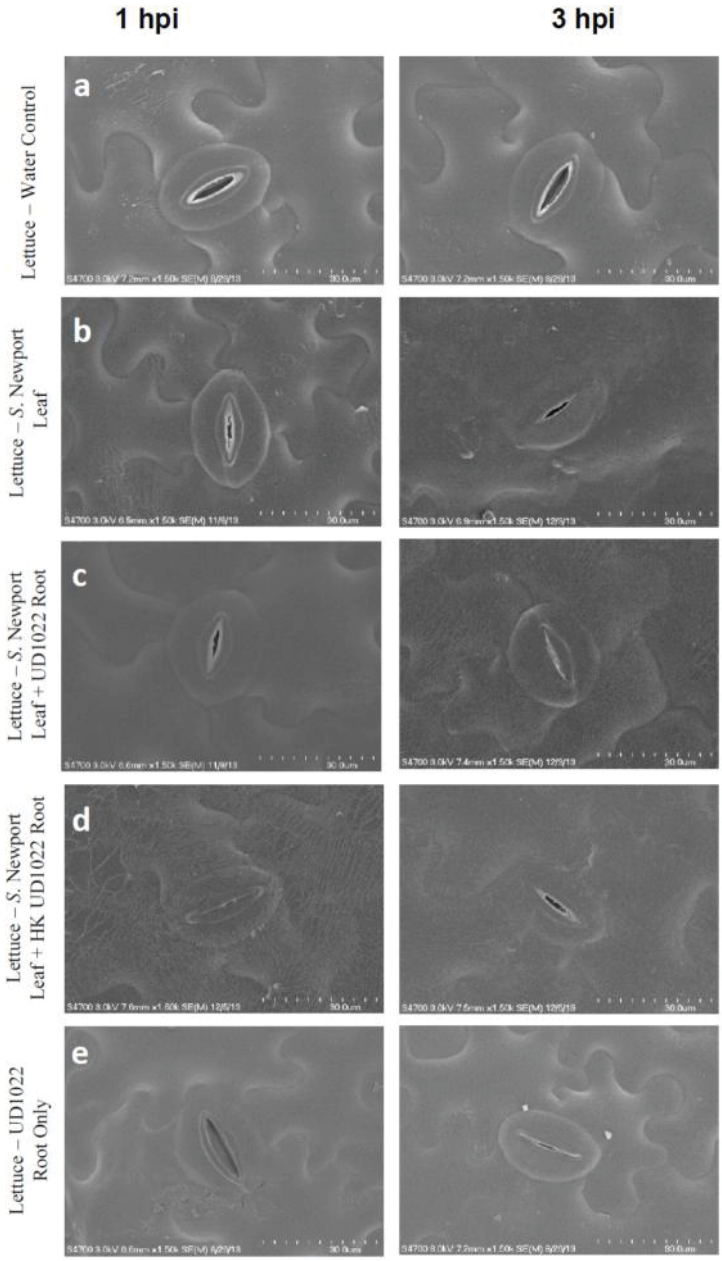

Figure 17: Cryopreservation scanning electron microscopy images (cry-SEM) of individual Romaine lettuce leaf stomata taken at 1.5X resolution. Images show individual leaf stomata after 1 and 3 hpi with the following treatments: (A) Water (control), (B) *S.* Newport on leaves only, (C) *S.* Newport on leaves + UD1022 on roots, (D) *S.* Newport on leaves + heat killed (HK) UD1022 on roots and (E) UD1022 on roots only. A total of 40 stomata were analyzed per treatment (n=40). Scale bar = 30.0 µm.

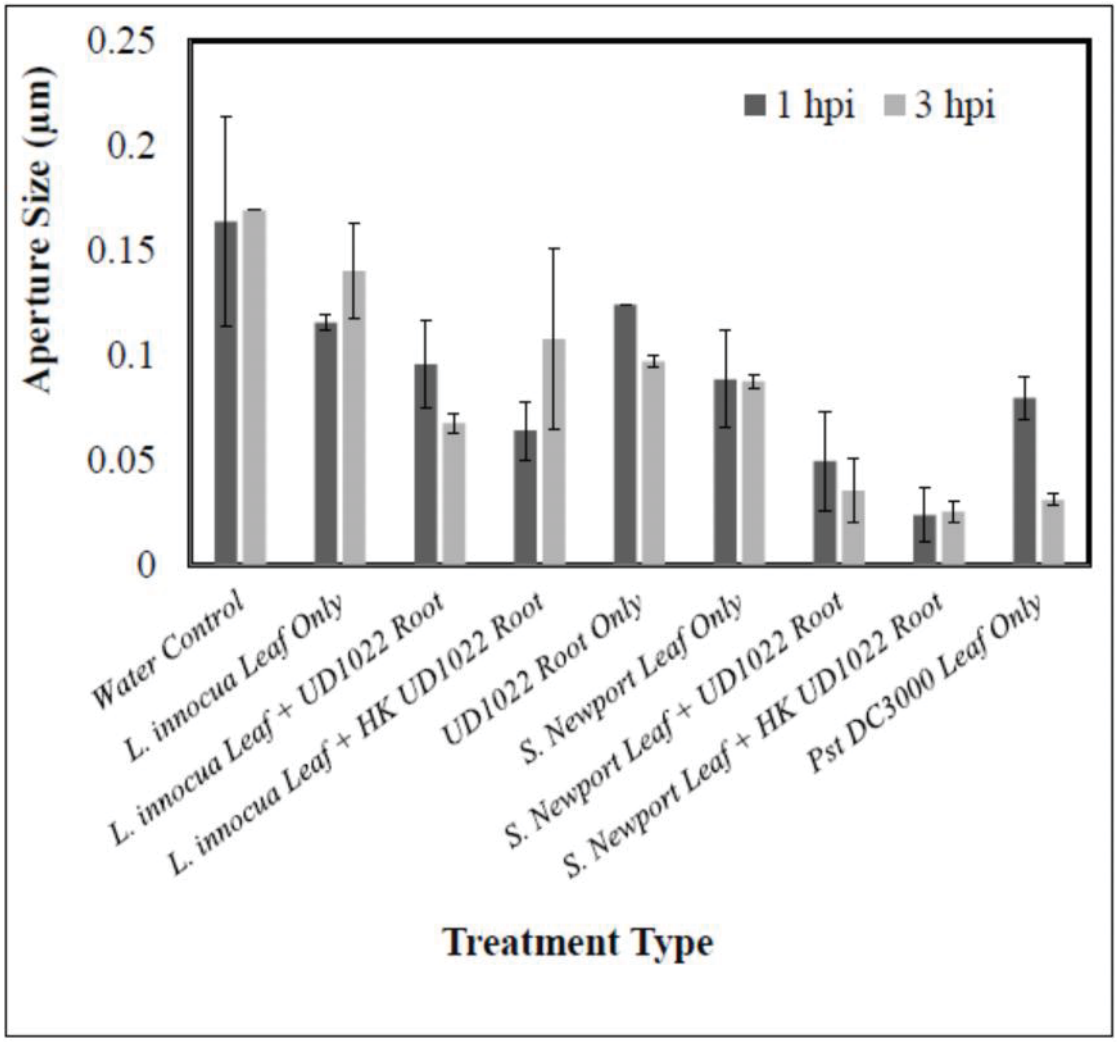

Figure 18: Average Romaine lettuce stomata aperture opening size following treatment at 1 and 3 hpi with water (control), *L. innocua* leaf only, *L. innocua* leaf + UD1022 root, *L. innocua* leaf + HK (heat-killed) UD1022 root, UD1022 root only, *S.* Newport leaf only, *S.* Newport leaf +UD1022 root, *S.* Newport leaf + HK UD1022 root or *Pst* DC3000 leaf only. Aperture opening size was measured using imageJ software of cryo-SEM images taken at 1.5 X resolution. UD1022 inoculated onto the roots of lettuce plants with *L. innocua* simultaneously inoculated onto the leaves significantly reduced the stomata aperture opening size at 3 hpi in these plants indicating ISR was induced ($p = 0.001$) (n=40).

In spinach, for all UD1022 treatments at 1 and 3 hpi stomata aperture openings were significantly smaller compared to controls ($p < 0.0001$) (Figures 18-20). Interestingly, in lettuce plants inoculated with *L. innocua* onto the leaves without UD1022 on the roots, stomata aperture size increased after 3 hpi indicating that *Listeria* may be recognized as a plant pathogen by lettuce (Figure 17). The observation also indicates that *Listeria*, like other plant pathogens may have capability to reopen stomata for ingression. When UD1022 was applied to the roots stomata were more closed at 3 hpi indicating that UD1022 was able to induce ISR in these plants potentially helping to prevent internalization of *L. innocua* into the leaves. Addition of heat killed UD1022 did not have this effect on the reduction of stomata aperture size. This occurred similarly in the case of *S*. Newport on lettuce leaves, but to a lesser extent (Figures 4-5). In spinach, stomata apertures were overall smaller in size compared to those of lettuce ($p<0.0001$) (Figure 20). Inoculation of *L. innocua* onto the leaves of spinach completely closed stomata 3 hpi (Figure 20). Spinach plants seemed to be less sensitive to the presence of *L. innocua* on the leaves when UD1022 was inoculated onto the roots as stomata aperture size was overall much larger than when *L. innocua* was inoculated alone onto lettuce plants. However; *S. Newport* seemed to act as a plant pathogen on spinach as *S*. Newport seemed to be able to reopen stomata in spinach plants 3 hpi (Figures 19-20). This was reversed when UD1022 was added to the roots of the spinach plants where stomatal aperture size was significantly smaller at 3 hpi ($p<0.001$) indicating ISR was triggered by UD1022 and able to close stomata (Figures 19-20). Addition of heat killed UD1022 to the roots of spinach plants did not reduce stomata aperture size (Figures 19-20), suggesting that live culture must be applied to induce stomata closure. In contrast, *Pst* DC3000 a plant

pathogen couldn’t reopen stomates in both spinach and lettuce (Figure 21), suggesting that coronatine may not play a role in stomatal opening in non-host plants.

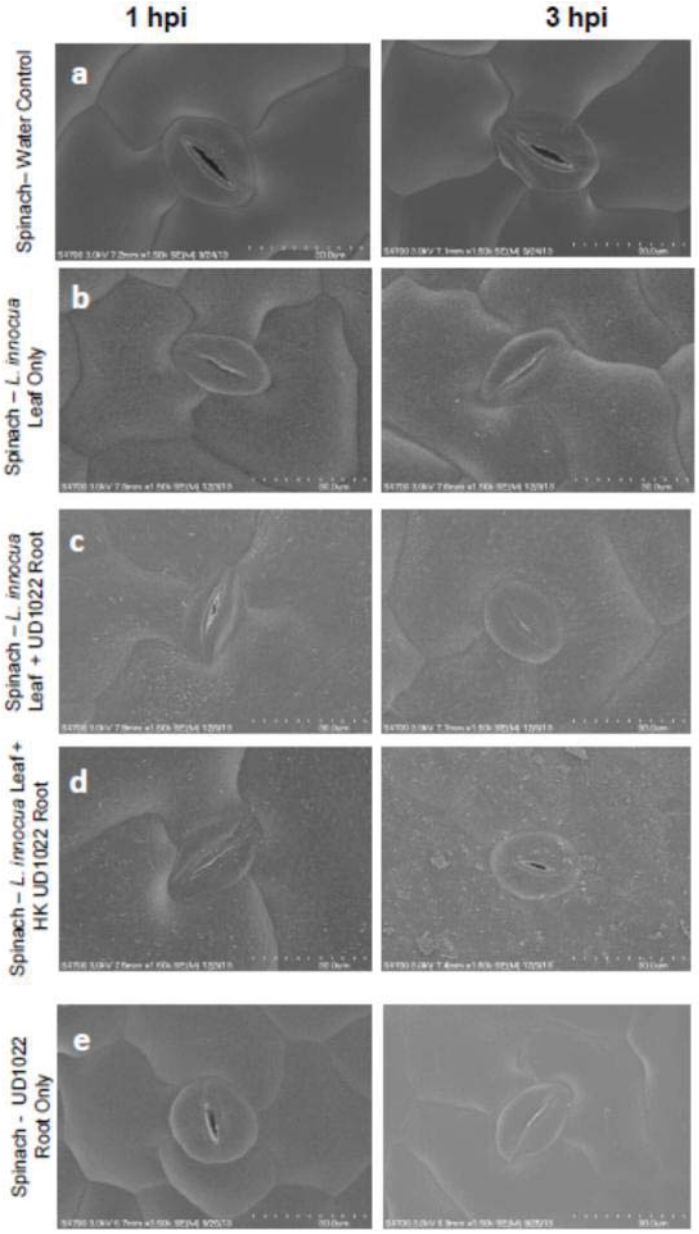

Figure 19: Cryopreservation scanning electron microscopy images (cry-SEM) of individual spinach leaf stomata taken at 1.5X resolution. Images show individual leaf stomata after 1 and 3 hpi with following treatments: (A) Water (control), (B) *L. innnocua* on leaves only, (C) *L. innocua* on leaves + UD1022 on roots, (D) *L. innocua* on leaves + heat killed (HK) UD1022 on roots and (E) UD1022 on roots only**.** A total of 40 stomata were analyzed per treatment (n=40). Scale bar = 30.0 µm.

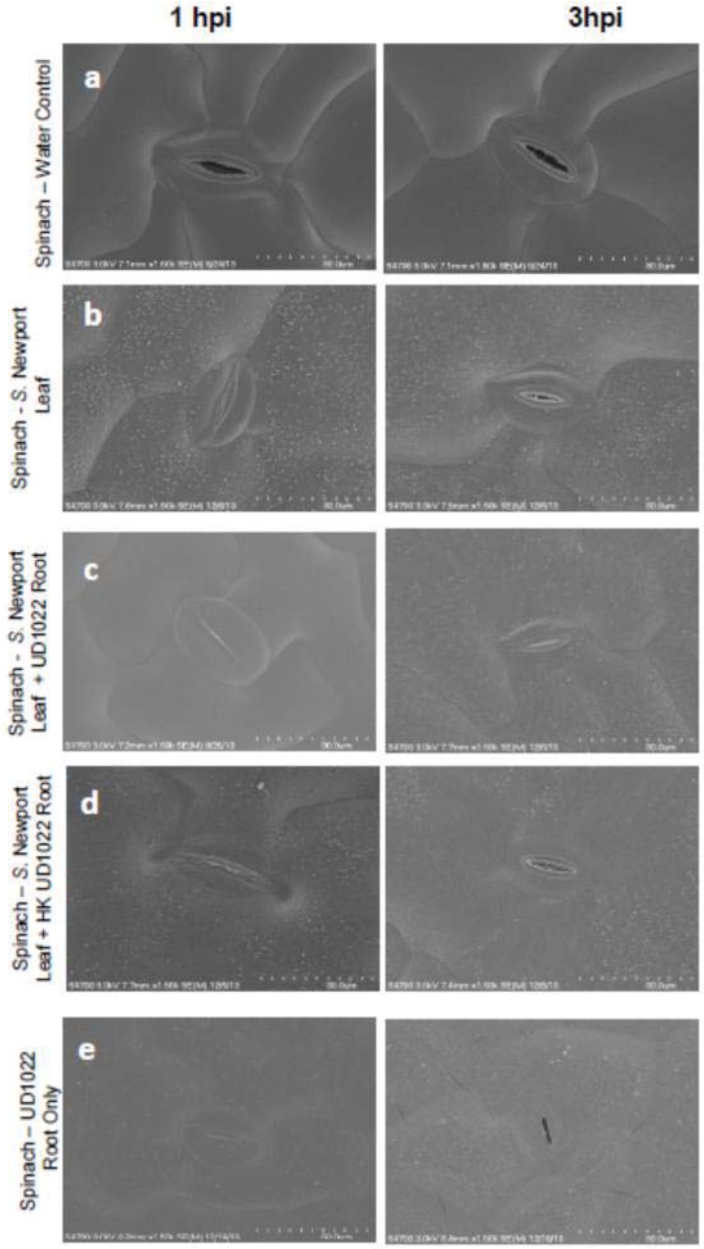

Figure 20: Cryopreservation scanning electron microscopy images (cry-SEM) of individual spinach leaf stomata taken at 1.5X resolution. Images show individual leaf stomata after 1 and 3 hpi with following treatments: (A) Water (control), (B) *S.* Newport on leaves only, (C) *S.* Newport on leaves + UD1022 on roots, (D) *S.* Newport on leaves + heat killed (HK) UD1022 on roots and (E) UD1022 on roots only. A total of 40 stomata were analyzed per treatment (n=40). Scale bar = 30.0 µm.

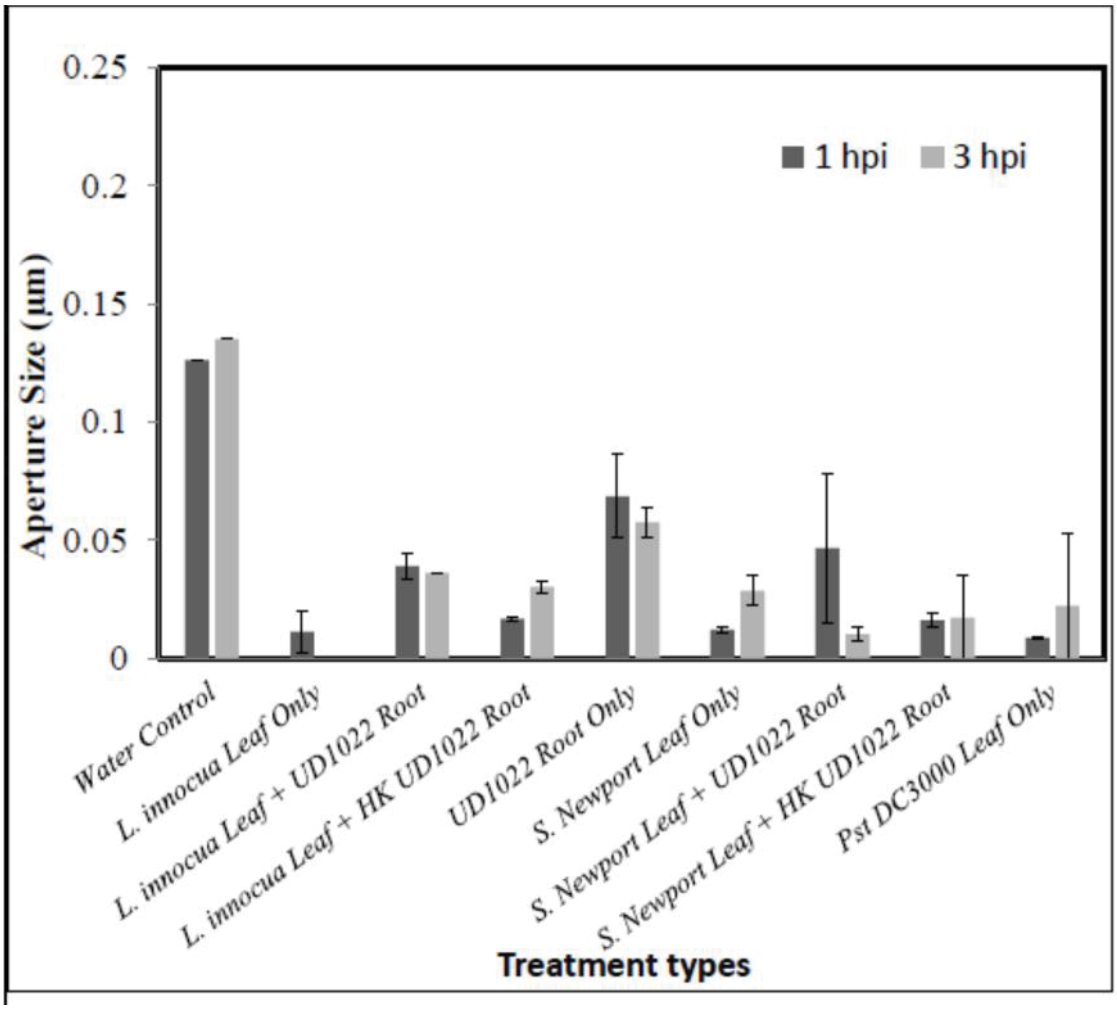

Figure 21: Average spinach stomata aperture opening size following treatment at 1 and 3 hpi with water (control), *L. innocua* leaf only, *L. innocua* leaf + UD1022 root, *L. innocua* leaf + HK (heat-killed) UD1022 root, UD1022 root only, *S.* Newport leaf only, *S.* Newport leaf +UD1022 root, *S.* Newport leaf + HK UD1022 root or *Pst* DC3000 leaf only. Aperture opening size was measured using imageJ software of cryo-SEM images taken at 1.5 X resolution. UD1022 was added to the roots of the spinach plants where stomatal aperture size was significantly smaller at 3 hpi ($p<0.001$) (n=40).

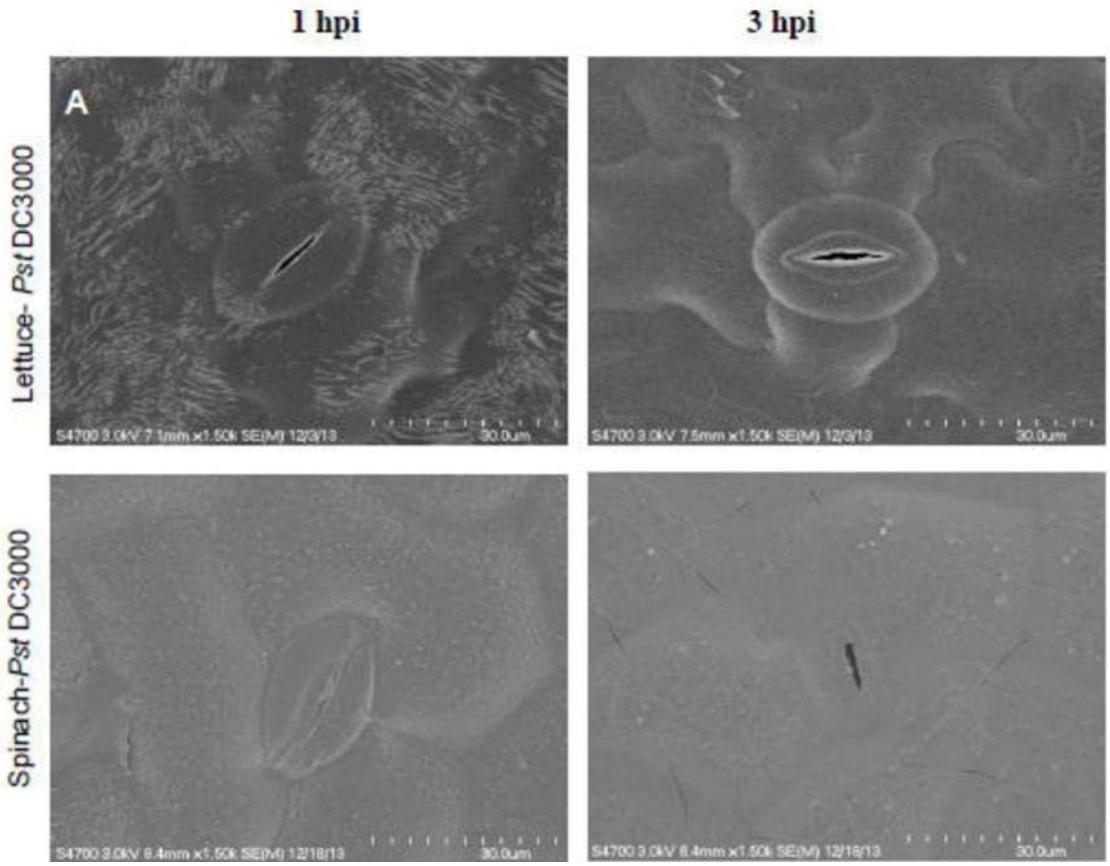

Figure 22: Cryopreservation scanning electron microscopy images (cry-SEM) of individual Romaine lettuce and spinach leaf stomata taken at 1.5X resolution. Images show individual leaf stomata after 1 and 3 hpi with following treatments with *Pst* DC3000 on leaves only. A total of 40 shots per treatment (n=40) were taken to get the representative shots. Scale bar = 30.0 µm

4.4.3 External Persistence and Internalization

Overall, concentrations of *L. innocua* and *S.* Newport on lettuce leaves decreased over the 10 day period. Concentration of *L. innocua* was highest immediately after inoculation (0+) with an average of 2.80 log CFU/plant and on day 1 post inoculation (dpi) with an average concentration of 1.94 log CFU/plant (Figure 10). The most significant decrease in *L. innocua* occurred after 5 dpi with a 1 log CFU/plant decrease. *L. innocua* was significantly reduced on lettuce leaves containing UD1022 on the roots compared to controls at 3 ($p=0.02$) and 5 ($p=0.003$) dpi (Figure 22).

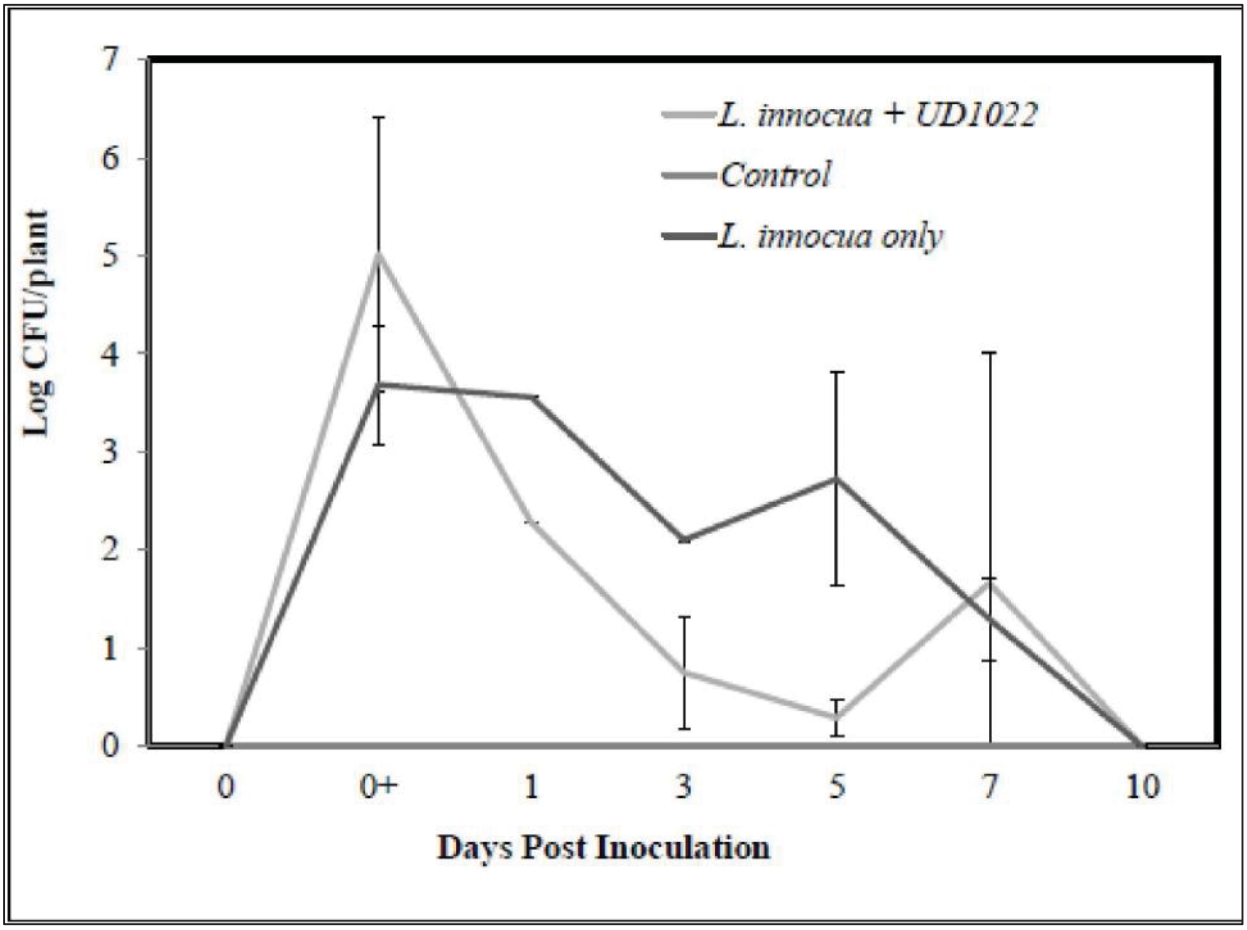

Figure 23: Persistence of either *L. innocua* inoculated onto leaves of Romaine lettuce plants with UD1022 simultaneously inoculated onto roots, water (control) only, or *L. innocua* on leaves only over a 10 day period. *L. innocua* was significantly reduced on lettuce leaves containing UD1022 on the roots compared to controls at 3 (p=0.02) and 5 (p=0.003) dpi (n= 8).

Concentrations of *L. innocua* on leaves of plants inoculated with UD1022 on the roots were reduced on days 7 and 10 to levels of those plants not treated with UD1022 (Figure 9). Presence of UD1022 on plants was more greatly correlated with a reduction in *L. innocua* on plants ($r = 0.52$) compared to the variable of time ($r = 0.47$). Concentration of *S*. Newport was not significantly reduced on lettuce plants treated with UD1022 compared to controls (p=0.15) (Figure 23).

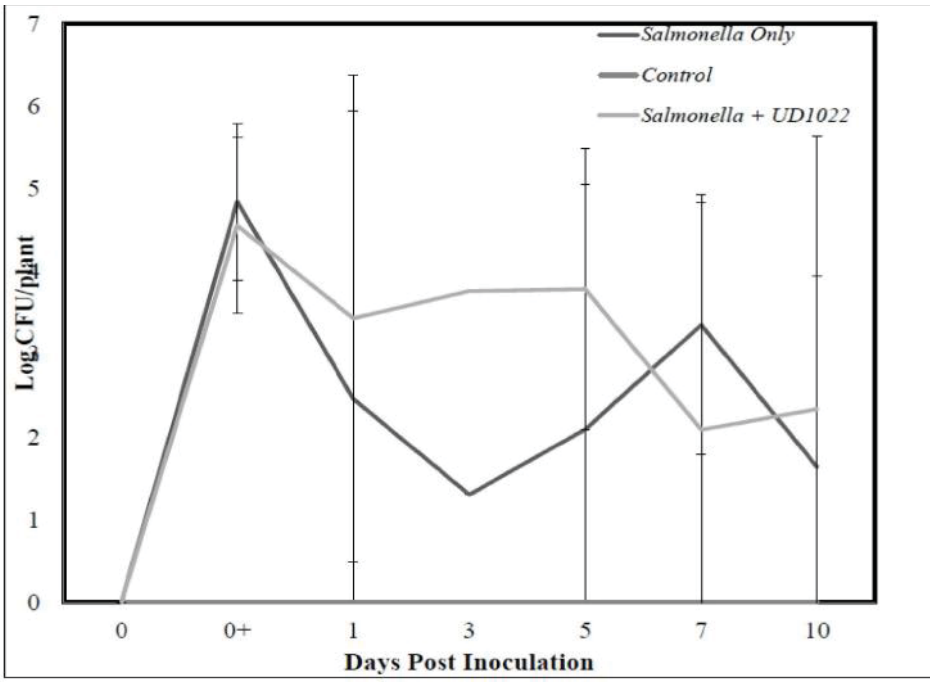

Figure 24: Persistence of either *S.* Newport inoculated onto leaves of Romaine lettuce plants with UD1022 simultaneously inoculated onto roots, water (control) only, or *S.* Newport on leaves only over a 10 day period. Concentration of *S*. Newport was not significantly reduced on lettuce plants treated with UD1022 compared to controls (p=0.15) (n=8)Interestingly on some days, plants treated with UD1022 contained higher levels of *S*. Newport, however; these plants appeared to be much healthier and showed fewer signs of stress compared to those plants treated with *S*. Newport alone (Figure 12). Plants inoculated with either *L. innocua* or *S*. Newport on the leaves and UD1022 on the roots were visibly more erect, slightly larger in size and showing fewer chlorosis lesions compared to plants not treated with UD1022 (Figure 12). There was a stronger correlation associated with the reduction of *L. innocua* on lettuce by UD1022 (r = 0.52) compared to *Salmonella* (r = 0.49). Neither *L. innocua n*or *S*. Newport were found to be internalized in lettuce plants throughout the 10 day experiment except in one of the trials on day 3, *L. innocua* was detected to be internalized in lettuce plant leaves where roots were not inoculated with UD1022 (data not shown).

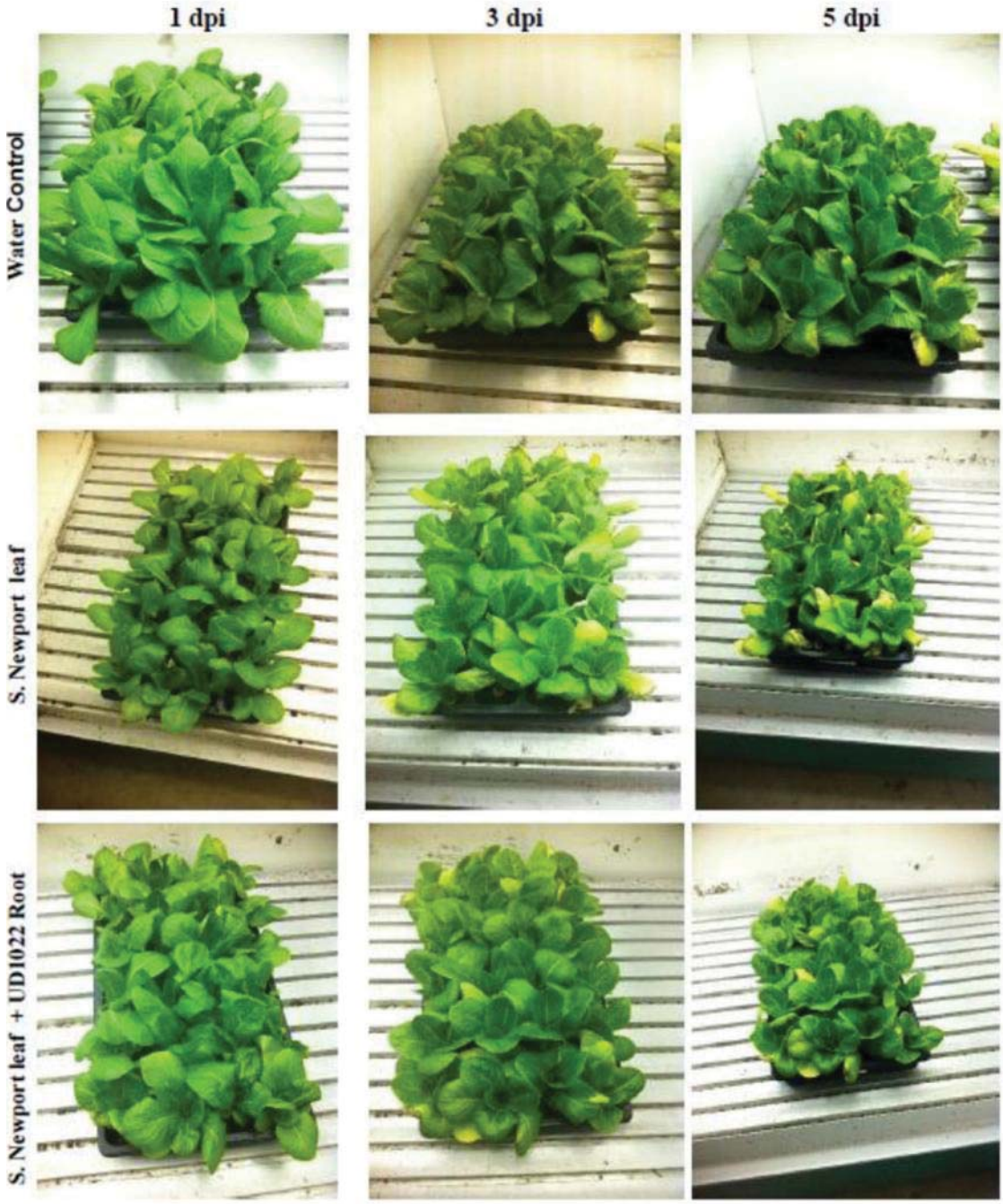

Figure 25: Showing symptoms of stress and chlorosis lesions of Romaine lettuce plants at 1, 3 and 5 dpi following inoculation with either water (control), *S*. Newport leaf only, or *S*. Newport leaf + UD1022 root.

4.5 Discussion

There has been an increasing amount of research involving the merge of the fields of food science and plant science to further investigate the plant-microbe interactions associated with edible plants and human pathogens. Such studies have led to the development of methods for improved study of the ways in which human pathogens interact with plants; including the ways plants respond to external persistence and internalization of human pathogens. Schikora *et al* observed the ability of *Salmonella* to overcome plant defense mechanisms in *Arabidopsis thaliana* as well as the ability for *Salmonella* to proliferate into plant tissues causing disease symptoms including wilting and chlorosis. The authors also showed the ability of *S. typhimurium* to trigger activation of plant immune responses including enhanced transcription of *PR* (pathogenesis related) genes concluding that *Salmonella* is pathogenic in *Arabidopsis*. This was one of the first studies suggesting that *Salmonella* triggers responses as a plant pathogen. In this study, *L. innocua* was also able to trigger responses in lettuce indicating its potential to serve as a plant pathogen. Most plant pathogens are able to overcome plant defenses in order to cause infection. For example, plant pathogen *Pst* DC3000 is able to reopen stomata in *A. thaliana* plants due to the plant pathogen's ability to secrete coronatine (Mittal and Davis, 1995). There is also some evidence that presence of plant pathogens influences growth of *Salmonella* on plants. A study by Potnis *et al.* demonstrated that presence of plant pathogen *Xanthomonas perforans*, which establishes disease by suppressing pathogen associated molecular pattern (PAMP)-triggered immunity that allows *S. enterica* to persist at levels 10 times higher on tomato plants compared to plants where *S. enterica* was applied alone.

Studies have also shown the ability of specific human pathogens to persist and internalize into plants under certain conditions. While the nonpathogenic *L. innocua*

was unable to persist on lettuce for longer than 10 days in the current study, *L. moncytogenes* was previously demonstrated to have the ability to persist on hydroponically grown lettuce for up to 49 days (Jablasone *et al.,* 2005). In the current study *S*. Newport was able to persist on lettuce plants for 10 days however; field studies have shown the ability of *S. typhimurium* to persist for up to 3 months on the edible portions of lettuce and parsley plants (Islam *et al.,* 2004). It is hypothesized that *Salmonella* may selectively colonize edible plants as a survival strategy that provides a direct route from bacterial excretion in the environment back to its mammalian host (Lynch *et al.,* 2009). This theory may also apply to other human pathogens such as *L. monocytogenes* however; there is currently no other published data in the literature to support this.

The use of plant growth promoting rhizobacteria (PGPR) to both promote plant growth and control plant pathogens has been studied for at least the last few decades. Because rhizobacteria are part of the natural microflora of healthy plants, they may be considered to be important contributors to plant health due to their diverse interactions with plant roots (Kloepper *et al.,* 1999). This is one of the first studies that addresses the use of a PGPR as a biocontrol agent to control human pathogens on plants with the goal of reducing the risk of human pathogen contamination of plants in the pre-harvest environment. It is possible that such PGPR could be identified as soil amendments to help farmers increase crop yield as well as increase the safety of their crops for human consumption. It has already been shown that UD1022 acts as a PGPR in *Arabidopsis thaliana* plants as it increases overall plant mass as well as protects plants from infection by *P. syringae* DC300 though triggering ISR which closes plant stomata (Kumar *et al,* 2012). In this study we show that UD1022 also has the ability to close

plant stomata in lettuce and spinach plants in which the effectiveness was supported by showing that application of heat killed UD1022 did not effectively close plant stomata. In addition when UD1022 was applied to the roots of plants containing *Listeria* or *Salmonella* on the leaves of lettuce or spinach plants, stomata apertures were smaller at 3 hpi compared to plants not treated with UD1022. This was especially significant in the cases of *L. innocua* on lettuce and *S*. Newport on spinach where both pathogens seemed to be able to reopen stomata at 3 hpi.

UD1022 was more effective at reducing the external persistence of *L. innocua* on lettuce than *S*. Newport where UD1022 was able to significantly reduce levels of *L. innocua* on lettuce leaves after only 3 days post inoculation. Interestingly, despite the ability of UD1022 to reduce *S*. Newport on lettuce leaves over the 10 day period, plants that were treated with UD1022 appeared visually healthier and showed fewer signs of stress (*ie*. chlorosis lesions) compared to plants treated with *S*. Newport alone (Figure 24). This observation may provide further support that UD1022 behaves as a beneficial PGPR in lettuce. It is possible that UD1022 may be more effective at controlling the persistence of *Listeria* and *Salmonella* on plants if applied to the seed prior to cultivation, if applied to the soil more frequently or during different stages of the particular plant's growth cycle. Overall direct inhibition and indirect inhibition assays demonstrated that UD1022 was more effective at reducing the persistence of *Listeria*.

4.6 Conclusions

Studying the plant-microbe associated interactions between human pathogens and plants may help increase the potential for the development of plant breeding programs for resistance to colonization by plant pathogens as well as human

pathogens (Brandl *et al.,* 2013). To date, a limitation of control of human pathogens on plants is the lack of understanding of basic interactions. This is the first study to assess the effects of a biocontrol agent for the reduction of contamination of human pathogens on plants in terms of plant immune response through stomata. Here UD1022 was useful to study a basic plant response to two different human pathogenic bacteria.

In 2011, 147 individuals were infected with five outbreak-associated subtypes of *Listeria monocytgenes* (McCollum *et al.,* 2013). The individuals lived in 28 states across the United States and all consumed cantaloupes contaminated with *L. moncytogenes*. Thirty-three deaths were confirmed from outbreak-associated cases of listeriosis. Prior to this outbreak there was little concern for *Listeria* on produce crops; however, we now recognize the need to understand how *Listeria* interacts with produce is imperative to future food safety policy and public health. Here we suggest that *Listera* may be recognized by the plant differently compared to Salmonella. This is an important first step into a more thorough understanding of the survival and persistence of *Listeria* in the complex pre-harvest environment, which has more recently been detected on celery (Gaul *et al*., 2013) and on stone fruit (Beach, 2014).

While this study only scratches the surface for the use of UD1022 as a biocontrol agent to reduce the risk of human pathogens on plants in the pre-harvest environment, there is great potential for optimization and use. UD1022 has the potential to protect plants from infection by plant pathogens as well as human pathogens helping to increase crop yield as well as protecting human health proving an economical means for farmers to protect their crops (Figure 25).

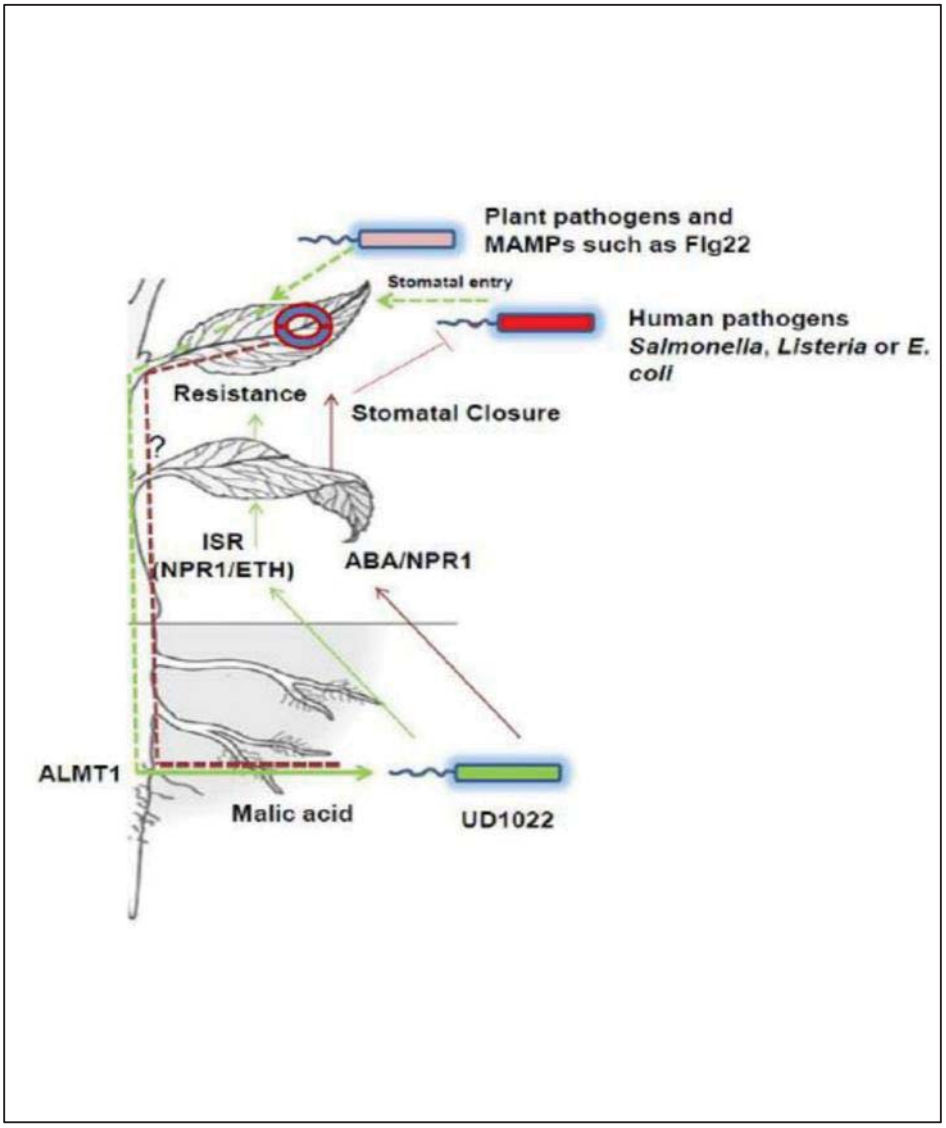

Figure 26: Showing a schematic of the mechanism by which UD1022 induces ISR. 1. Pathogen lands on aerial portions on the leaf of the plant 2. The plant detects pathogen associated molecular patterns (PAMPs), such as flagella which triggers an "SOS" signal to initiate plant defenses and secretes malic acid into the soil. 3. The malic acid signal recruits *B. subtilis* UD1022 and up regulates biofilm operons in UD1022. 4. UD1022 forms a biofilm on the roots of the plant and initiates an induced systemic response and stomatal closure in the plant via SA, NPR1 and ETH to protect plant from pathogen invasion. Figure adapted from Lakshmanan *et al.,* 2012.

This research will enable the development of new technology that can take advantage of the natural association between rhizobacteria and plants, and provide scientific evidence for the advancement to minimize contamination by human pathogens in food crops.

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Chapter 5

BACILLUS SUBTILIS REDUCES RISK OF *LISTERIA MONOCYTOGENES* CONTAMINATION ON CANTALOUPES

5.1 Abstract

Cantaloupes serve as one of the major crops in the state of Delaware accounting for approximately $850,000 in estimated annual sales. Preliminary data has shown the ability of *Bacillus subtilis* UD1022 to help increase crop yield through complex plant-microbe interactions that increase plant defenses toward plant pathogens. The purpose of this project is to evaluate the use of a plant growth promoting rhizobacteria, *Bacillus subtilis* UD1022, to reduce the contamination of cantaloupes by *L. monocytogenes* in the pre-harvest environment as well as in the packinghouse environment. In this study, pieces of cantaloupe rind (3.20 cm^2 x 2.50 cm^2) were treated with UD1022 cell-free supernatant or sterile water (control) immediately following inoculation with *L. monocytogenes* or after an 8 hour incubation at 37°C, 22°C or 4°C. Three cantaloupe rind pieces were pooled into one sample (n=6 samples). Bacterial enumeration was performed by plating samples onto Brilliant Listeria Agar. At 22°C, recovery of *L. monocytogenes* was 2.5 log lower when treated with UD1022 supernatant at 8 hours post-inoculation (hpi) and 3 log lower at 24 hpi compared to controls, which was statistically significant (p=0.0024). Recovery of *L. monocytogenes* was also 2.5 log lower at 22°C when UD1022 supernatant was applied at 8 hpi and *L. monocytogenes* was enumerated at 24 hpi. Although recovery of *L. monocytogenes* was lowest for all temperatures when treated with UD1022, this reduction was not significant at 37°C or 4°C. UD1022 did not affect the persistence of *L. monocytogenes* on cantaloupe seeds after 30 d of storage. These results indicate that UD1022 may be best suited for use as a natural biocontrol

agent to reduce the risk of contamination by *L. monocytogenes* on cantaloupe rind during times of temperature abuse in the packinghouse, storage or transport.

5.2 Introduction

Recently the Centers for Disease Control and Prevention attributed 46% of foodborne illnesses to fresh produce (Painter *et al.,* 2013), indicating that greater and more sophisticated efforts are needed to prevent contamination on these commodities. Plants grow in close association with large communities of microbes, yet comparatively little is known about the diversity of microbes that associate with plants, and their interactions and effects on performance, crop yields and plant protection. The fields of plant science and food microbiology have been merging over the past few years in the best interest of produce safety; however, critical knowledge gaps remain. Therefore, novel strategies to control pathogens merit attention and specific efforts are needed to better understand plant association. To date, there is limited utility of biocontrol strategies against human pathogens or other opportunistic pathogens of fresh produce crops.

A recent turning point for the produce industry occurred this past year when a cantaloupe growing company received criminal charges for growing produce that was contaminated with *Listeria monocytogenes* which associated with the an outbreak resulting in the deaths of 34 individuals. According to a recent report released by the Interagancy Food Safety Analytics Collaboration (IFSAC) Project, *L. monocytogenes* is considered a high priority pathogen due to the frequency and severity of the illness it causes as well as its susceptibility to targeted interventions (IFSAC, 2015). It is estimated to be the cause of 94 % and 15.9 % of hospitalizations and mortalities, respectively, linked to foodborne illness (Scallan *et al.,* 2011). Because of the 2011 *L.*

monocytogenes outbreak has had such an impact on attribution estimates, the IFSAC model now estimates that 50% of *L.monocytogenes* outbreaks are linked to fresh fruits with an average outbreak size of 147 illnesses (IFSAC, 2015).

Microbial contamination can originate from countless areas along the farm to fork continuum. At greatest risk are those aspects of contamination that can occur within the pre-harvest environment, whereby microbial contamination can come in contact with plant tissues from water, soil amendments, wind, birds, insects, animals, and other fomites (Beuchat, 2002). While a wide array of research has been conducted on ways to reduce microbial contamination through better sanitation and use of safe irrigation water and soil amendments, nevertheless contamination events still occur.

Recent field trials conducted at the University of Delaware and elsewhere provide evidence that rainfall significantly contributes to bacterial contamination of leaves (Park *et al.,* 2014; Spanninger *et al*., 2013). Likewise aerosol contamination from airborne transmission of pathogens in applied manure or other soil amendments has great potential for contamination (Millner, 2009). These are examples of contamination that cannot necessarily be explained or controlled; however, a biocontrol agent would be useful under these conditions to reduce contamination in the field. In higher-risk crops like cantaloupe, contamination is believed to occur in the field or in the packing house along distribution. The FDA is currently performing unannounced visits to cantaloupe packing houses across the US as a means of risk mitigation to identify risk-based behaviors. These facts serve as evidence for the need for a control measure for conventional and organic growers to reduce the chance of contamination of cantaloupe. Previous studies have demonstrated the ability of UD1022 to reduce the persistence of *Listeria* species in direct culture as well as on

lettuce plants (Markland *et al.,* 2014). This novel application of UD1022 would be particularly useful to safeguard cantaloupe which is one of the most popular fruits sold in the United States, and fresh-cut cantaloupe is a major value-added product made from the peeled fruit (Fang *et al.,* 2013).

The purpose of this project was to determine if *Bacillus subtilis* UD1022 is able to help reduce the contamination of cantaloupes (*Cucumis melo spp. Melo var. cantaloupensis*) by *Listeria monocytogenes* in the pre-harvest environment as well as the packing house environment. The use of UD1022 as a biocontrol agent will help provide a sustainable means for growers, including those raising organic crops, to reduce environmental and human health risks associated with growing cantaloupes as well as improving productivity and crop yield thus; reducing cost of production and increasing net farm income. Biocontrol and manipulation of pathogens on plants can reduce the initial contamination that may be unavoidable, at times, and spread by cross-contamination during washing or during packaging.

5.3 Materials and Methods

5.3.1 Bacterial Isolates, Growth and Enumeration

A *Listeria* outbreak strain isolated from a cantaloupe packing house was used in this study (*Listeria monocytogenes* 390-1) and was kindly provided by Dr. Jeff Chandler at Colorado State University. Bacterial cultures were grown in tryptic soy broth (TSB) (Thermo Fisher Scientific, Waltham, MA until exponential growth phase was reached (5 h), then 1 ml of culture was inoculated onto tryotic soy agar (TSA). Plates were incubated overnight at 37°C. The bacterial lawn was harvested from the plate by applying 15 ml of buffered peptone water (BPW) to the plate and scraping the

cells from the plate with a sterile inoculation loop. The culture was then pipetted into a sterile 15 ml tube and diluted to a concentration of 5.57±0.98 log CFU/ml prior to inoculation on cantaloupe rind (3g) or seeds (1g). For all experiments, bacterial enumeration was performed on Brilliance Listeria Agar (Figure 26) (Thermo Fisher Scientific Oxoid, Waltham, MA) at 37°C for 48 h.

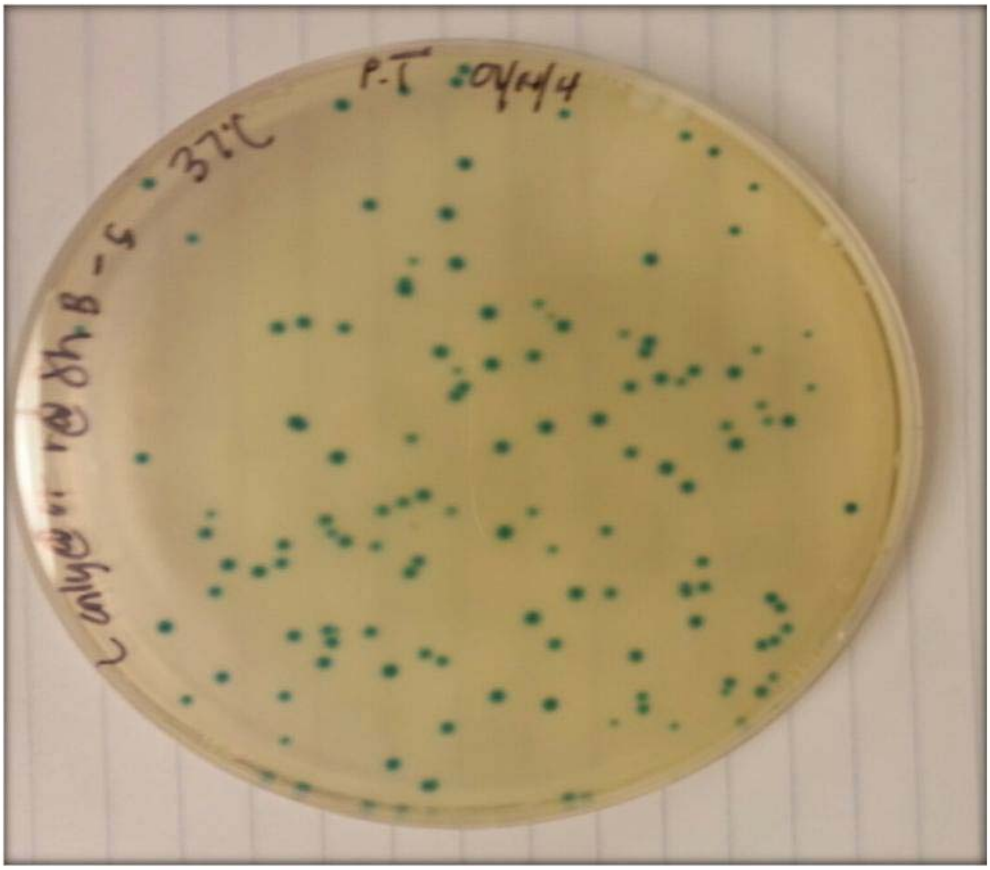

Figure 27: Cantaloupe rind pieces were pooled so that 3 pieces were equal to one sample. Samples were collected into sterile stomacher bags, suspended in 10 ml of BPW, and stomached for 2 minutes. Serial dilutions were performed and plated onto Brilliant Listeria agar. To make the agar more selective, 2 supplements were added to it in order to distinguish *Listeria monocytogenes* (blue colonies with clear halo) from *Listeria innocua* (blue colonies without halo) and other organisms.

5.3.2 Preparation of UD1022 Supernatant

Bacillus subtilis UD1022 was provided by Dr. Harsh Bais from the Department of Plant and Soil Sciences at the University of Delaware. UD1022 cultures were grown overnight in tryptic soy broth to a concentration of 7.99±0.75 log CFU/ml at 37°C the centrifuged at 2500 rpm for 10 min. Supernatant was collected and passed through a 0.22 µm filter (Sartorius Stedim North America, Bohemia, NY) by vacuum filtration. Filter sterilized supernatant was then utilized for further cantaloupe rind and seed storage experiments.

5.3.3 Inoculation of Cantaloupe Rind and Application of UD1022 Supernatant

Cantaloupes were purchased from a local grocery store (Shoprite, Newark, DE). Cantaloupe rind was separated from the fruit and cut into small square pieces weighing ~ 1 g (3.20 cm x 2.50 cm). Each individual piece was placed into the well of a 6-well plate. *L. monocytogenes* (100 µl) was inoculated by pipette onto each piece and spread across cantaloupe pieces by paint brush to allow for even distribution across the canopy. Rind pieces were allowed to dry within a laminar flow hood for 1 hour. Whole cantaloupe pieces were then dipped into either UD1022 supernatant or sterile water (control) immediately following the drying period or allowed to incubate at either 37, 22 (ambient) or 4°C for 8 h. Three cantaloupe rind pieces were pooled into one sample (n=6 samples/treatment). Cantaloupe rind pieces were placed in a sterile bender bag (Thermo Fisher Scientific, Waltham, MA) with 10 ml of BPW and stomached for 2 min. An aliquot of 1 ml was pulled from the bag and serial dilutions were performed prior to plating on Brilliant *Listeria* Agar for bacterial enumeration. All results are reported as log CFU/g of cantaloupe rind.

5.3.4 Inoculation of Cantaloupe Seeds and Application of UD1022 Supernatant

Cantaloupe seeds (1 g) were placed in to a sterile 15 ml conical tube (Thermo Fisher Scientific, Waltham, MA). Seeds were sterilized by adding 1 ml of 75 % ethanol and vortexing for 30 s. Seeds were then treated with 1 ml of a 10 % bleach solution and allowed to sit for 20 min before being rinsed with sterile water three times. *L. monocytogenes* culture (200 µl) was added to the seeds (~4 log CFU/g) and allowed to dry in a laminar flow hood for 2 hours. After drying either 200 µl of UD1022 supernatant, Phosphate Buffered Saline (PBS) (control) or sterile water (control) was added and allowed to dry in a laminar flow hood for another 2 h. Seeds were incubated at ambient temperature on the bench top and samples were collected at 0, 2, 5, 10, 15, 20 and 30 days. For each sample collected, 1 ml of PBS was added and vortexed for 1 min. The 1 ml sample was then pulled from the tube and serial dilutions were performed prior to bacterial enumeration on Brilliant Listeria Agar.

5.3.5 Statistical Analysis and Interpretation of Results

For all experimental methods, experiments were performed in duplicate with different trials performed at different times. Bacterial results are reported as log CFU/sample with 3 cantaloupe pieces equaling one sample or log CFU/g cantaloupe seeds. A one-way ANOVA was performed to compare means within the data set and a linear regression model was generated using JMP software (SAS Institute Inc., Cary, NC). To determine the level of influence of the independent variables on the dependent variable (r), generation of a multivariate correlation matrix were also performed. All p values at < 0.05 are considered significant.

5.4 Results

5.4.1 Use of UD1022 to Reduce the Persistence of *Listeria monocytogenes* on Cantaloupe Rind

Overall, growth of *L. monocytogenes* was highest on cantaloupe rind pieces when incubated at 37°C and lowest at 4°C (Figure 27). According to multivariate correlation analysis, incubation temperature had a moderately strong effect on the persistence of *Listeria* on cantaloupe rind (r=0.51). There was no significant difference in the overall growth of *L. monocytogenes* between 37°C and 22°C regardless of the treatment type or incubation period (p=0.057). Growth at 4°C was significantly lower compared to 37°C (p=<0.0001) and 22°C (p=0.02).

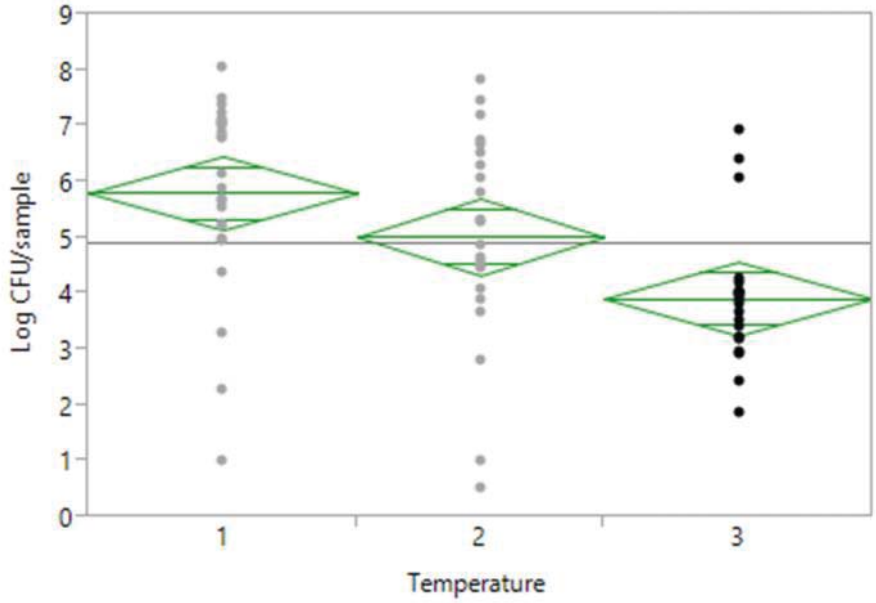

Figure 28: Showing data output from JMP (SAS, SAS Institute Inc., Cary, NC) Student's t test for comparison of means across the entire data set for the persistence of *Listeria monocytogenes* on cantaloupe rind treated with biocontrol agent *Bacillus subtilis* UD1022. This graph shows that growth of *L. monocytogenes* was highest at 37°C (1) and lowest at 4°C (3) regardless of the treatment and incubation period used. 2=22°.

After 8 h of incubation at 37°C, the concentration of *L. monocytogenes* present on cantaloupe pieces increased from 5.57 log CFU/g to 6.42 ± 0.35 log CFU/sample and to 6.03 ± 1.85 log CFU/g after 24 h incubation. At 22°C bacterial concentrations increased to 4.7 ± 0.52 log CFU/g after 8 h incubation and to 6.96 ± 0.88 log CFU/sample after 24 h incubation. At 4°C, bacterial populations persisted at 3.48±0.53 log CFU/g after 8 h incubation and at 3.55±0.45 log CFU/g after 24 h incubation. At all incubation temperatures (4, 22 and 37°C) *L. monocytogenes* growth was lowest when cantaloupe pieces were treated with UD1022 after 8 ($p=0.01$) or 24 h ($p=0.008$) incubation supernatant compared to controls (Figure 28).

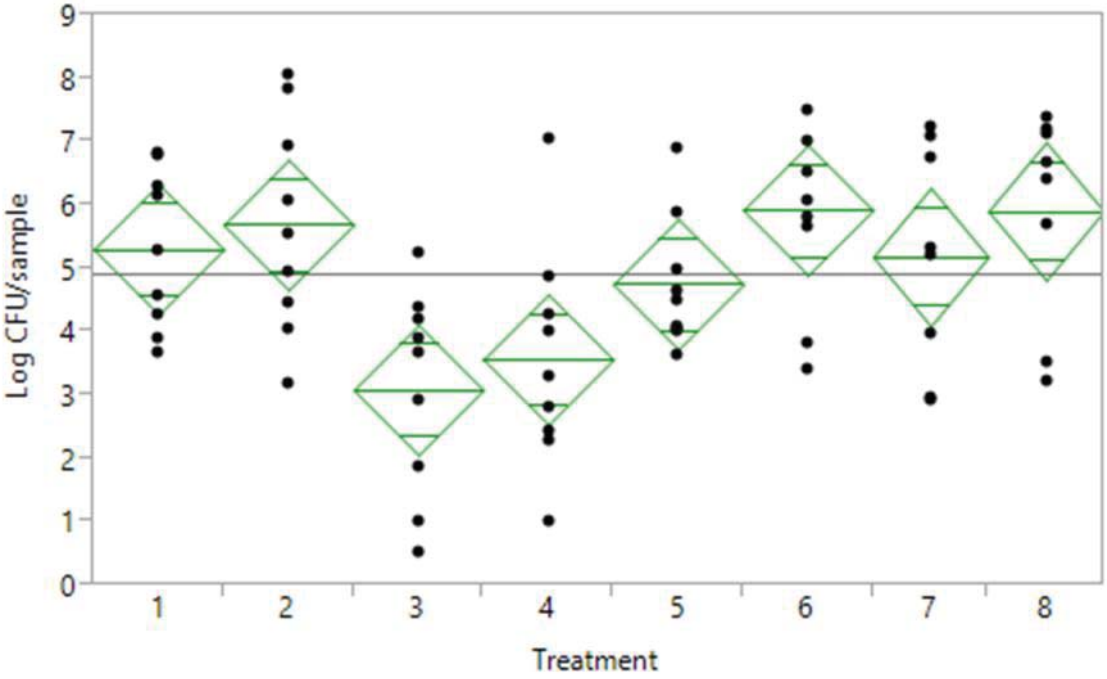

Figure 29: Showing data output from JMP (SAS, SAS Institute Inc., Cary, NC) for oneway analysis of variance (ANOVA) across the entire data set for the persistence of *Listeria monocytogenes* on cantaloupe rind treated with biocontrol agent *Bacillus subtilis* UD1022. This graph shows that growth of *Listeria* was lowest for all treatments where cantaloupe rind pieces were dipped in UD1022 (3 and 4) compared to *Listeria* alone (1 and 2) (positive control) or water (5 and 6) (negative control). Treatment 1=*Listeria* alone incubated for 8 h; 2=*Listeria* alone incubated for 24 h; 3=*Listeria* + UD1022 dip at 0 h incubated for 8 h; 4=*Listeria* + UD1022 dip at 0 h incubated for 24 h; 5=*Listeria* + H2O dip at 0 h incubated for 8 h; 6=*Listeria* + H2O dip at 0 h incubated for 8 h; 7=*Listeria* + UD1022 dip at 8 h incubated for 24 h; 8=*Listeria* + H2O dip at 8 h incubated for 24 h.

This inhibition was not statistically significant for cantaloupe pieces incubated at 37°C or 4°C (Figures 29-30). Inhibition, however, was statistically significant at ambient temperature (22°C) regardless of the duration of the incubation period or the time at which UD1022 was applied (p=0.002) (Figures 29-30). UD1022 was able to significantly decrease the persistence of *L. monocytogenes* on cantaloupe rind dipped in UD1022 supernatant immediately following *Listeria* inoculation (0+) and incubated for 8 h compared to nontreated (p=0.02) and water treated controls (p=0.05). Similarly, UD1022 was able to decrease bacterial persistence on *Listeria* on cantaloupe rind following 24 h incubation compared to non-treated controls (p=0.003) and those dipped in water (p=0.008). Additionally, UD1022 was able to significantly inhibit the growth of *Listeria* when applied after 8 h of incubation compared to non-treated (p=0.02) and water treated controls (p=0.046) (Figure 31).

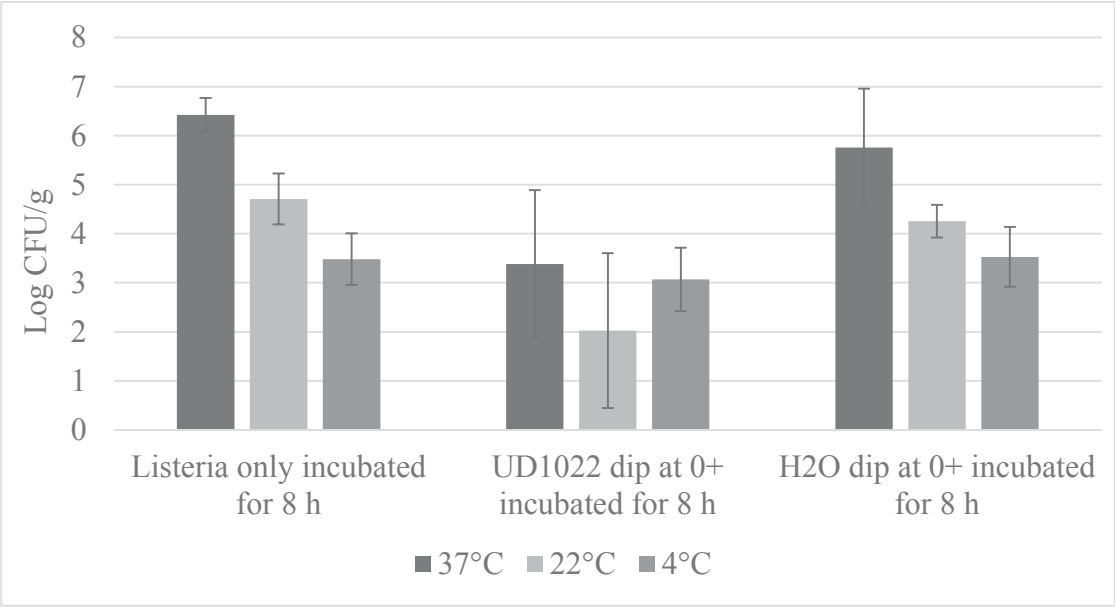

Figure 30: Growth of *L. monocytogenes* on cantaloupe rind after dipping in H_20 or UD1022 after 8 h incubation.

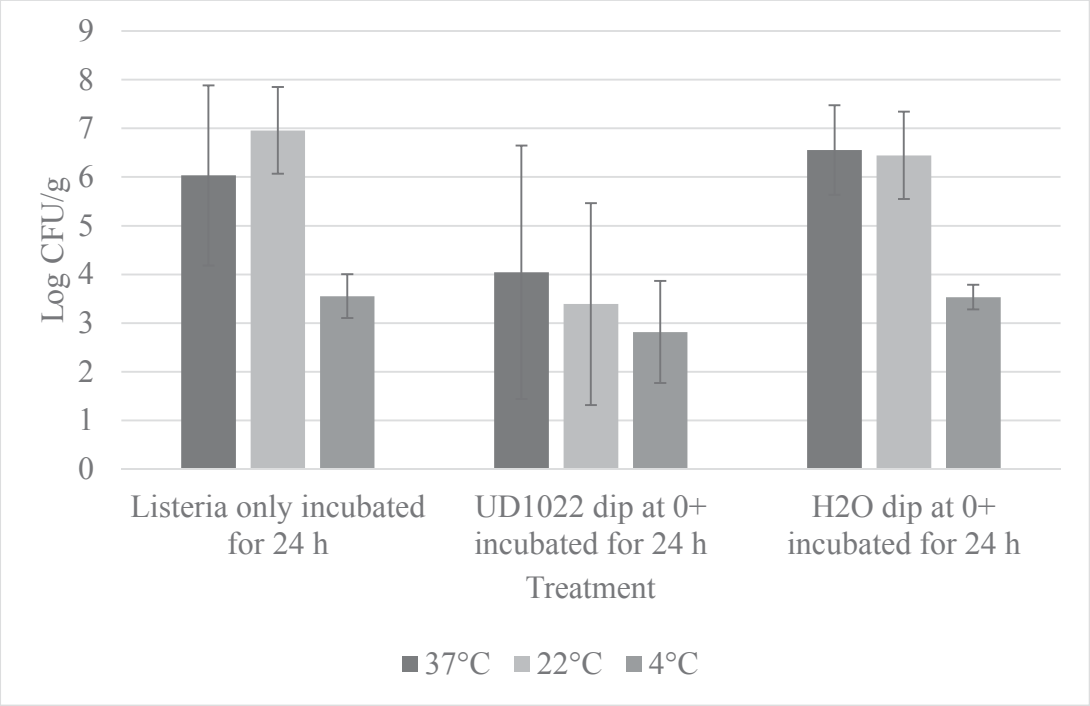

Figure 31: Growth of *L. monocytogenes* on cantaloupe rind after dipping in H_20 or UD1022 followed by 24 h incubation**.**

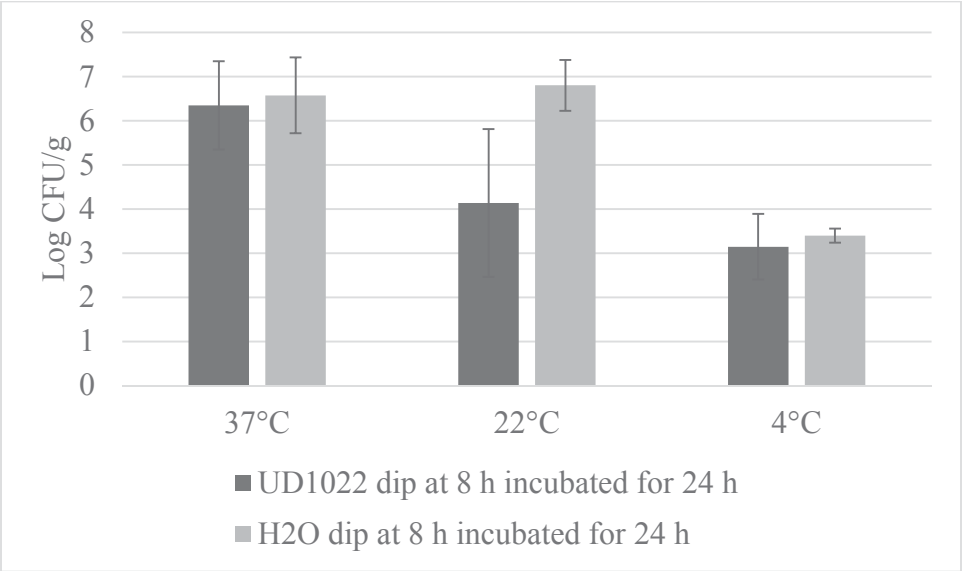

Figure 32: Growth of *L. monocytogenes* on cantaloupe rind after dipping in H20 or UD1022 at 8 h followed by 24 h incubation.

5.4.2 Effects of UD1022 on the Persistence of *Listeria monocytogenes* During Cantaloupe Seed Storage

After only a 48 h storage period, *Listeria* increased from 0.73±0.37 log CFU/g to 5.38±0.98 log CFU/g on cantaloupe seeds treated with water only and from 1.47±0.14 log CFU/g to 5.22±1.85 log CFU/g on seeds treated with PBS. Overall concentrations of *Listeria* were highest on day 10 (6.23 log CFU/g) and lowest immediately following inoculation on day 0+ (1.28 log CFU/g). UD1022 had no significant effect on the persistence of *L. monocytogenes* on cantaloupe seeds after the 30 day storage period compared to controls (p=0.74) (Figure 32).

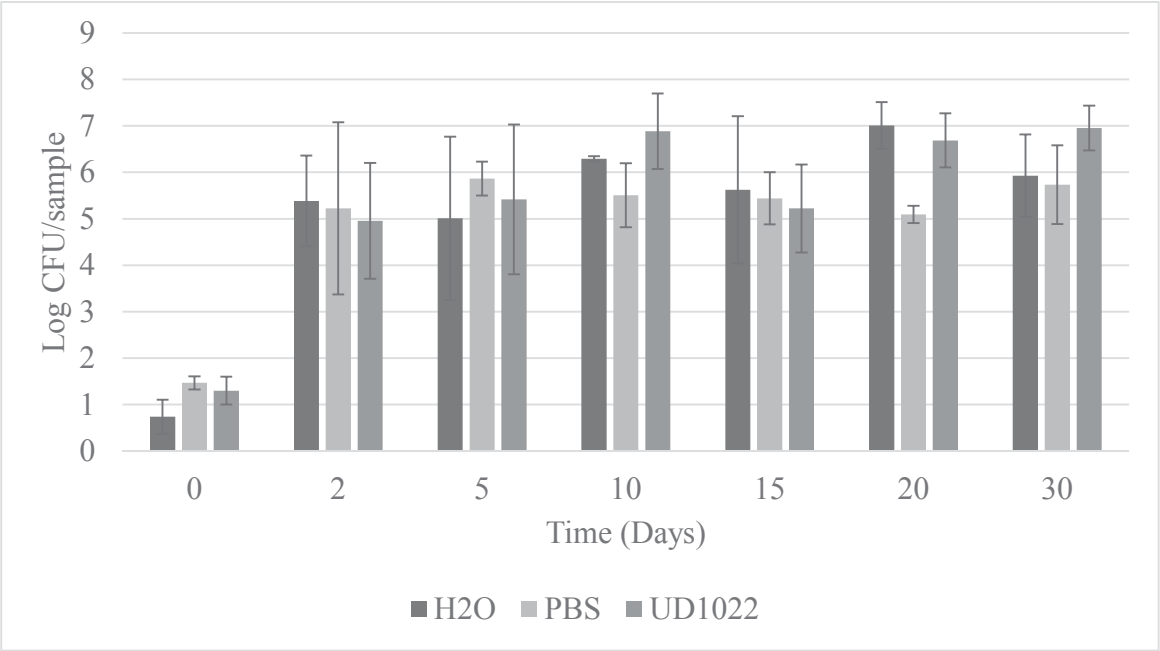

Figure 33: Showing persistence (log CFU/g) of *Listeria monocytogenes* on cantaloupe seeds treated with either sterile water (control), peptone buffered saline (PBS) or UD1022 supernatant after incubation at 0, 2, 5, 10, 15, 20 and 30 days post inoculation.

5.5 Discussion

The 2011 *Listeria* outbreak related to contaminated produce was named the fourth-deadliest outbreak in US history by Food Safety News (Flynn, 2015). In total, 33 deaths occurred from this outbreak and one woman suffered a miscarriage (CDC, 2012). Cantaloupes are currently considered a high risk commodity and safeguarding these products and the consumers from exposure to *Listeria* on fresh cut and RTE products is on the forefront of necessity (Raede, 2015). Because there is no "kill step" for such commodities, it is important to implement prevention measures that can help prevent initial contamination of the product that may occur in the field or in the packing house. In this study, *L. monocytogenes* was able to survive and even multiply on cantaloupe rind after 24 h incubation at various temperatures as well as cantaloupe seeds after 30 days of storage demonstrating the ability of this pathogen to persist on produce and seeds as well as in the environment. Other studies have shown that *L. monocytogenes* was able to persist on cantaloupe rind at 4°C and 20°C for up to 15 days (Ukuku and Fett, 2002). In another study *L. monocytogenes* was found to be able to persist on whole cantaloupe at 5°C with no significant decline in bacterial populations after 7 days of storage (Ukuku *et al.,* 2005).

The biocontrol agent used in this study was previously demonstrated to be able to reduce the persistence of *L. innocua* on lettuce plants over a 10 day period (Markland *et al.,* 2014). UD1022 was also shown to be able to inhibit the growth if *L. monocytogenes* in culture in a dose-dependent manner (Markland *et al.,* 2014). In the current study we show that UD1022 is also able to significantly inhibit the growth of *L. monocytogenes* on cantaloupe rind at ambient temperature when applied immediately following inoculation or after 8 h of incubation. These findings are significant considering the amount of bacterial contamination in the field or the

packinghouse is probably much lower than those used in this study. Application of UD1022 to whole cantaloupes may be particularly beneficial as a hurdle technology to help reduce contamination or inhibit the growth of *Listeria* where temperature abuse may occur, such as during transport. Determining the timing of application to the cantaloupes as well as the appropriate dosage are also necessary in order to optimize the use of UD1022 as a biocontrol agent for *L. monocytogenes* on whole cantaloupes.

There is currently a lack of knowledge regarding the attachment and colonization of *L. monocytogenes* on seeds. This study shows that *L. monocytogenes* is able to survive at high levels and may even be able to multiply during seed storage at ambient temperature. While UD1022 was not able to inhibit growth of *L. monocytogenes* in this study, optimization of this technology by determining the appropriate dosage as well as methods for concentrating UD1022 would be ideal. More importantly, it will be important to determine if UD1022 treatment of contaminated seeds can prevent the subsequent transfer of bacteria from the seed to the plant and then to the fruit.

5.6 Conclusions

The inability of UD1022 supernatant to significantly inhibit the growth of *L.monocytogenes* at 37°C or 4°C suggests that *Listeria* can overcome the inhibitory effects of UD1022 under certain conditions which should be further characterized for optimization. These results indicate that UD1022 may be used as a natural biocontrol agent to reduce the risk of contamination by *L. monocytogenes* on cantaloupe rind during times of temperature abuse in the packinghouse, during storage or transport. This is the first biocontrol agent identified to protect cantaloupe crops from plant

pathogens, increasing crop yields, as well as reducing risk for contamination by *L. monocytogenes* therefore; also protecting human health.

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Chapter 6

HUMAN NOROVIRUS AND ITS SURROGATES INDUCE PLANT IMMUNE RESPONSE IN *ARABIDOPSIS THALIANA* AND *LACTUCA SATIVA*

6.1 Abstract

Norovirus is the leading cause of foodborne illness worldwide with the majority of outbreaks linked to fresh produce and leafy greens. It is essential that we thoroughly understand the type of relationship and interactions that take place between plants and human norovirus in order to better utilize control strategies to reduce transmission of norovirus in the field onto plants harvested for human consumption. In this study the expression of gene markers for the salicylic acid and jasmonic acid plant defense pathways were measured and compared in romaine lettuce (*Lactuca sativa*) and *Arabidopsis thaliana* Col-0 plants were inoculated with either MNV, TV, human norovirus GII.4 or HBSS (control). Genes involving both the salicylic acid and jasmonic acid pathways were expressed in both romaine lettuce and *A. thaliana* for all three viruses, studies including gene expression of SA- and JA-deficient *A. thaliana* mutant lines suggest that the jasmonic acid pathway is more likely involved in the plant immune response to human norovirus. This research provides the first pieces of information regarding how foodborne viruses interact with plants in the pre-harvest environment.

6.2 Introduction

Human norovirus, with a highly infectious single-stranded RNA genome, is a leading cause of foodborne illness worldwide. Human norovirus may be spread by food (including fresh produce), water, person to person contact, or even by aerosolized vomitus droplets. Due to the high incidence and difficulty of testing and surveillance of the virus it is crucial that prevention of product contamination be a high priority to food producers. Some of the foods most commonly associated with norovirus outbreaks include ready to eat (RTE) foods such as fresh produce and leafy greens. It is estimated that only 18 virus particles are necessary to induce illness involving symptoms such as diarrhea, vomiting, nausea, fever, and fatigue (Teunis *et al.,* 2008), indicating that even a small amount of contamination has the potential to be a public health threat.

Noroviruses account for 60 % of all leafy green associated foodborne outbreaks (Herman *et al.,* 2008; DeWaal and Bhuiya, 2007) and are the number one cause of foodborne outbreaks related to leafy greens (Painter *et al.,* 2013). While it is recognized that many outbreaks are caused by contaminated food handlers, it is also recognized that contamination can occur on the farm. One major route of contamination of foods with human enteric viruses is through fruits and vegetables irrigated with fecally contaminated water or by direct contact with fecal material through the use of manure and biosolids in the field (Sair *et al.,* 2002). Viruses can be present in manures and municipal biosolids that are applied to agricultural crops which can contaminate water resources and the crops to which they are applied. Noroviruses of the human genotype were found to be shed by cattle and swine, thus increasing the likelihood for zoonotic transmission of norovirus (Mattison *et al.,* 2007). It has been estimated that nearly 80 million dry tons of solid manure are generated annually by the

beef, dairy, swine and poultry industries in the US (Edwards and Someshwar, 2000). Land application remains the most common and economic method to dispose and recycle animal manure.

Transmission of human pathogens to plants can also occur through contaminated irrigation water and contaminated hands, which have been documented under both laboratory and field conditions (Stine *et al.,* 2005; Endley *et al.,* 2003). Norovirus has been associated with contaminated groundwater, surface water, and recreational water and can survive in groundwater for more than 18 months (Cheong *et al.,* 2009). It has been previously demonstrated that norovirus can be internalized into leafy greens through the roots and foliar surfaces of lettuce plants (Wei *et al*., 2010a, 2010b; Hirneisen and Kniel, 2013; Wang and Kniel 2015) although it is not known how often this naturally occurs in the field and how internalization physically affects the plant or the viruses themselves. It is essential that we thoroughly understand the type of relationship and interactions that take place between plants and human norovirus in order to better utilize control strategies to reduce transmission of norovirus in the field onto plants harvested for human consumption.

It is also important to determine the type of relationship that human pathogens have with plants, whether it be endophytic or antagonistic. For example, it was recently suggested by Schikora *et al.* (2008) that human pathogenic *Salmonella typhimurium* can infect and intracellularly proliferate tissues of *Arabidopsis thaliana* through both the root and shoot of the plant. *Salmonella* infection also led to disease symptoms in the plants including wilting, chlorosis and death of infected plant organs (Schikora *et al.,* 2008). *Arabidopsis* plants responded immunologically similarly to *Salmonella* as they would to plant pathogen *Pseudomonas syringae* (DC3000) by

induction of the mitogen activated protein kinase (MAPK) cascades as well as enhanced expression of pathogenesis related (*PR*) genes (Schikora *et al.,* 2008). There are also studies demonstrating that bacteria normally pathogenic to humans and other mammals can also infect plants including *Salmonella enterica*, *Pseudomonas aeruginosa*, *Burkholderia cepacia*, *Erwinia* spp., *Staphylococcus aureus*, *E. coli* O157:H7, and *Listeria monocytogenes* (Haapalainen *et al.* 2009; Holden *et al.* 2009; Plotnikova *et al.* 2000; Prithiviraj *et al.* 2005; Milillo *et al.,* 2008). Most of the studies described above have been performed on *Arabidopsis thaliana* because the entire genome of the plant has been sequenced and much is known in regards to how this plant responds to plant pathogens physically and immunologically. However; questions remain regarding whether or not *A. thaliana* serves as a good model for the study of human pathogen-plant interactions. Another study by Klerks *et al.,* (2007) found that lettuce plants responded to the presence of *S. enterica* serovar Dublin at physiological and molecular levels shown through stunted plant growth and the expression of pathogenicity-related genes. Other studies have found that *E. coli* O157:H7 induced significantly higher gene expression in A. thaliana plants compared to *S. enterica* serovar Typhimurium suggesting that plants may recognize and respond to some human pathogens more effectively than others (Roy *et al.,* 2012).

While there are very few studies looking at the plant defense response of human bacterial pathogens, there are none regarding human pathogenic viruses. The mechanism by which the plant immune system operates is through the detection of conserved microbial components (Jones and Dangl 2006) referred to as microbe associated molecular patterns (MAMPs) or pathogen associated molecular pathogens (PAMPs). The plant physiological responses to plant infection can be due to activation

of the induced systemic resistance (ISR) or systemic acquired resistance pathway (SAR). The SAR pathway is induced if the pathogen is able to elicit a hypersensitive reaction (HR) (Durrant and Dong, 2004; Enyedi *et al.* 1992), which causes apoptosis in the plant in order to rid itself of the infectious agent. These events may enable the plant to become more resistant to future attacks by the pathogen (Ross, 1961; Madamanchi and Kuc, 1991). When a plant is invaded by a pathogen, one of two defense signaling pathways are activated: a salicylic acid (SA) dependent pathway or an SA-independent pathway involving jasmonic acid (JA) or ethylene (ET) signaling molecules (Kunkel and Brooks, 2002). JA-dependent and ET-dependent plant defenses are generally activated by necrotrophic pathogens and chewing insects, whereas SA-dependent defenses are often triggered by biotrophic pathogens (Pandey and Somssich, 2009). JA and SA signaling usually act antagonistically, but synergism between these two molecules has also been observed (Mur *et al.*, 2006).

The purpose of this study was to determine how the human enteric pathogen norovirus interacts with plants in the pre-harvest environment. We also sought to determine if plants respond differently to human norovirus as well as its widely used surrogates (murine norovirus and Tulane virus). In addition, we wanted to determine if plant model *Arabidopsis thaliana* and Romaine lettuce (*Lactuca sativa*) elicited an immune response toward human noroviruses and if so, which plant defense pathways were utilized by the plants.

6.3 Materials and Methods

6.3.1 Seed germination and cultivation

Arabidopsis thaliana, in addition to romaine lettuce, was used as a model for plant defense studies toward norovirus. *Arabidopsis thaliana* seeds of the wild type Colombiana (Col-0) as well as mutant lines *nahg* and *jin1* were generously provided by Dr. Harsh Bais (University of Delaware, Newark, DE). Mutant *nahg* plants are deficient in the ability to induce production of salicylic acid. Mutant *jin1* plants are insensitive to the production of jasmonic acid. *A. thaliana* and romaine lettuce (Paris Island cultivar) (Johnny's Selected Seeds, Waterville, ME) seeds were sterilized in a 50 % bleach solution and vortexed for 1 min then rinsed three times with sterile deionized water. Following sterilization, seeds were planted in individual containers containing sterile promix soil (square containers 4 cm x 3.5 cm x 4.5 cm in dimension; T.O. Plastics, Ontario, Canada). Holes were created in the bottom of each individual container which was then placed into plastic bins inside a growth chamber. The growth chamber temperature conditions were monitored daily and temperature was maintained at 20 ± 0.30^{o}C, relative humidity was 60 ± 2.08 % and the water activity (A_w) of the promix was maintained at 1.046 ± 0.04. Romaine lettuce plants were utilized for all experiments once they reached 4 weeks of age and *A. thaliana* plants were used at 2 weeks of age.

6.3.2 Virus inoculation and sample collection

Experiments were performed by inoculating romaine lettuce, *A. thaliana* Col-0, *nah*, as well as *jin1* mutant plants with either human norovirus surrogates including murine norovirus-1 (MNV) and Tulane virus (TV), or human norovirus GII.4. *A. thaliana nahg* mutant plant lines are salicylic acid deficient and were used to evaluate

the role of *PR-1* as a genetic marker for the salicylic acid pathway in the immune response of *A. thaliana* Col-0 to human norovirus and its surrogates. *A. thaliana jin1* mutant lines are jasmonic acid deficient and were used to evaluate the role of *PDF1.2 and VSP2a* as genetic markers for the jasmonic acid pathway in the immune response of *A. thaliana* Col-0 to human norovirus and its surrogates. Plants were also inoculated with Hank's Balanced Salt Solution (HBSS) as controls. HBSS was selected as a control as viral stocks inoculated onto plants were suspended in HBSS (Hyclone, Logan, UT). Plant viruses are known to enter plant cells through cellular damage (Mandadi and Scholthof, 2013), lesions were made on leaves of plants prior to inoculation using a sterile pipette tip. Plants with lesions only (no virus) were used as controls and for normalization of gene expression data. Concentrated virus in aliquots of 50 µl (10^7 log/ml MNV; 10^4 log/ml TV; 10^9 log/ml GII.4) was applied onto plant leaves where lesions were made using a sterilized paint brush in order to avoid runoff of inoculum. For experiments including romaine lettuce, 3 leaves per plant were pooled into one sample. Due to their small size, the leaves of 3 whole *A. thaliana* plants were also pooled into one sample. Each experiment was performed in triplicate and replicated at least twice (n=6). Samples were collected immediately following viral inoculation (0+) or at 6, 12 or 24 hours post inoculation (hpi). Plant tissues were immediately frozen in liquid nitrogen and ground up using a mortar and pestle. Samples were stored at -80°C until further analysis was performed.

6.3.3 Plant RNA extraction and RT-PCR

Plant RNA was extracted from frozen plant tissues using the ZR Plant RNA MiniPrep kit (Zymogen Research, Irvine, CA). An in-column DNase digestion was performed using DNAse I (Zymogen Research, Irvine, CA) according to the

manufacturer's instructions. Prior to cDNA synthesis, RNA was diluted to 500ng in a 10µl total volume. Synthesis of cDNA was performed using the Life Technologies High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA). RT-PCR master mix was prepared according to the manufacturer's instructions. Thermal cycling parameters as follows: 25°C for 10 min, 37°C for 120 min and 85°C for 5 min followed by a hold at 4°C using the Eppendorf Mastercycler (Eppendorf, NY).

6.3.4 Primer Design for Plant Defense Genes in Romaine Lettuce

Primers were designed for romaine lettuce gene markers involved in the plant defense pathways. Sequence homologues for *PR-1* and *NPR-1* primers in romaine lettuce were designed from known *A. thaliana* nucleotide sequences using the NCBI Nucletiode BLAST database (tbastx) (NCBI, NIH) (Table 2). *PR-1* and *NPR-1* served gene markers involved in the salicylic acid pathway of plant defense. Primers for genes involved in defense pathways in *A. thaliana* included *PR-1*, *PDF1.2* and *VSP2a* (Table 3). *PDF1.2* and *VSP2a* served as gene markers for the jasmonic acid/ethylene pathway of plant defense. *A. thaliana* primers were generously provided by Dr. Harsh Bais (University of Delaware, Newark, DE).

Table 2: Primer sequences for qPCR of plant genes in romaine lettuce homologous to genes in *A. thaliana*.

Primer	**Sequence (5'-3')**	**Gene homology in *A. thaliana***	**Plant defense pathway involved**	**Accession No. ***
LACT fwd	CAAGCCGTTCTTTCCCTGTA	Actin	-	KF976789
LACT rev	TTCTGCTGAGGTCGTGAATG		-	
LPR-1 fwd	GAAGGGTTGGGTGTGCTAGA	Pathogenesis-related protein-1 (PR-1)	SA	AY193547
LPR-1 rev	CACAAGAAACAAGGGCGTAG		SA	
LNPR-1 fwd	TCGATCGTCTATCGGAAACC	Non-pathogenesis related protein-1 (NPR-1)	SA	AF113953
LNPR-1 rev	TCACATTGCGATTCTTGTCC		SA	

* Accession numbers of sequences from GenBank via NCBI nucleotide BLAST

Table 3: Primer sequences for qPCR of plant genes in *A. thaliana*

Primer	**Sequence (5'-3')**	**Gene**	**Plant defense pathway involved**	**Accession No. ***
UBQ fwd	TCGTAAGTACAATCAGGATAAGATG	Ubiquitin	-	NM_115119
UBQ rev	CACTGAAACAAGAAAAACAAACCCT			
PR1 fwd	TTCTTCCCTCGAAAGCTCAA	Pathogenesis-related protein-1	SA	NM_127025
PR1 rev	CGTTCACATAATTCCCACGA			
PDF1.2 fwd	GGTGGACGCACAGAAGTTGT	Plant defensing 1.2	JA/ET	AY133787
PDF1.2 rev	AATACACACGATTTAGCACC			
VSP2a fwd	ACCGTTGGAAGTTGTGGAAG	Vegetative storage protein 2a	JA	NM_122386
VSP2a rev	CCAAATCAGCCCATTGATCT			

* Accession numbers of sequences from GenBank via NCBI nucleotide BLAST

6.3.5 Quantitative PCR and Calculation of Relative Gene Expression

The qPCR master mix was made using 10 µl of 2x QuantiTect SYBR Green PCR Master Mix, 2 µl of forward and reverse primers (10 mM), 4 µl of RNase-free water and 2 µl of cDNA template to each sample. Cycling parameters were as follows: PCR initial activation at 95°C for 15 min followed by 40 cycles of 94°C for 15s, 60°C for 30 s, and 72°C for 30 s. Data was acquired from the SYBR Green channel at the extension step (72°C). The ΔΔ Cq calucation method was utilized to determine relative gene expression as described by Haimes and Kelley (2014) using actin (*ACT*) as an endogenous reference gene in lettuce and ubiquitin (*UBQ*) as an endogenous reference gene in *A. thaliana*. Expression levels of target genes from plants induced with lesions only (no virus) were used for normalization of the gene targets.

6.3.6 Statistical Analysis and Interpretation of Results

For all experimental methods, experiments were performed in triplicate with two trials performed at different times. A one-way ANOVA was performed to compare means within the data set (student's t-test) using JMP software (SAS Institute Inc., Cary, NC). All p values ≤ 0.05 (α=0.05; CI=95%) were considered statistically significant.

6.4 Results

6.4.1 Romaine lettuce and *A. thaliana* elicits increase plant defenses in the presence of MNV, TV and GII.4

The relative gene expression of plant defense genes in romaine lettuce and *A. thaliana* plants inoculated with either MNV, TV or GII.4 was compared to that of plants inoculated with HBSS. In romaine lettuce, MNV-1 did not significantly induce expression of *PR-1*; however MNV did significantly induce the expression of *NPR-1*

at 6, 12 and 24 hpi compared to HBSS inoculated controls (Figures 33-34). This indicates that romaine lettuce plants elicit an immune response toward MNV via induction of *NPR-1* and the salicylic acid pathway of defense. Inoculation of TV onto romaine lettuce significantly induced *PR-1* and *NPR-1* at 6 hpi compared to controls (Figures 35-36). Human norovirus GII.4 significantly induced *PR-1* in romaine lettuce at 12 and 24 hpi and *NPR-1* was significantly induced at 6, 12 and 24 hpi (Figures 37-38). Results indicate that human norovirus, as well as its surrogates, were able to induce an immune response in romaine lettuce through the induction of the salicylic acid pathway through the increased expression of *PR-1* and *NPR-1* compared to controls. For all viruses, *NPR-1* was expressed significantly higher than *PR-1* in romaine lettuce (p=0.05). The highest expression levels were observed in lettuce plants inoculated with human norovirus GII.4, although this was not statistically significant.

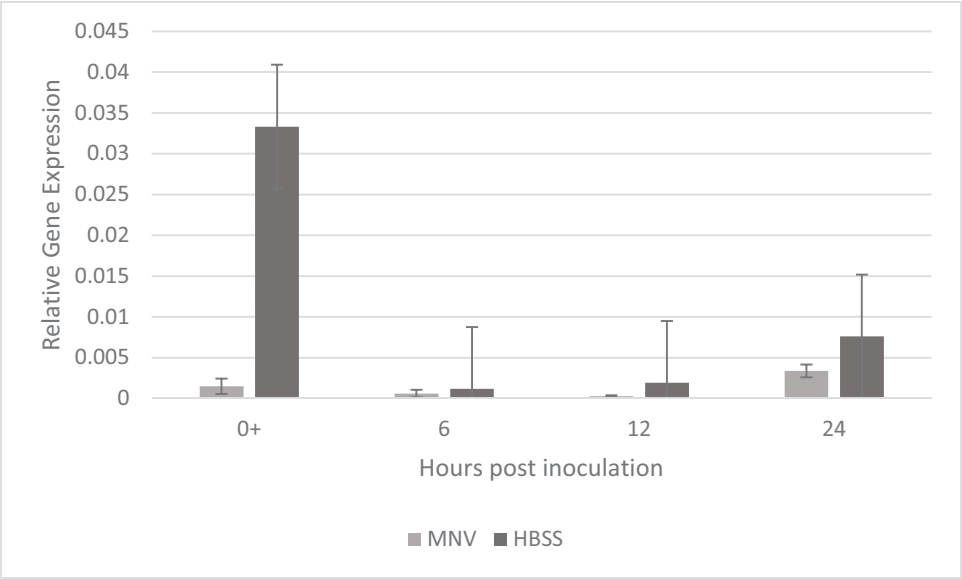

Figure 34: Relative gene expression of *PR-1* in romaine lettuce inoculated with MNV or HBSS

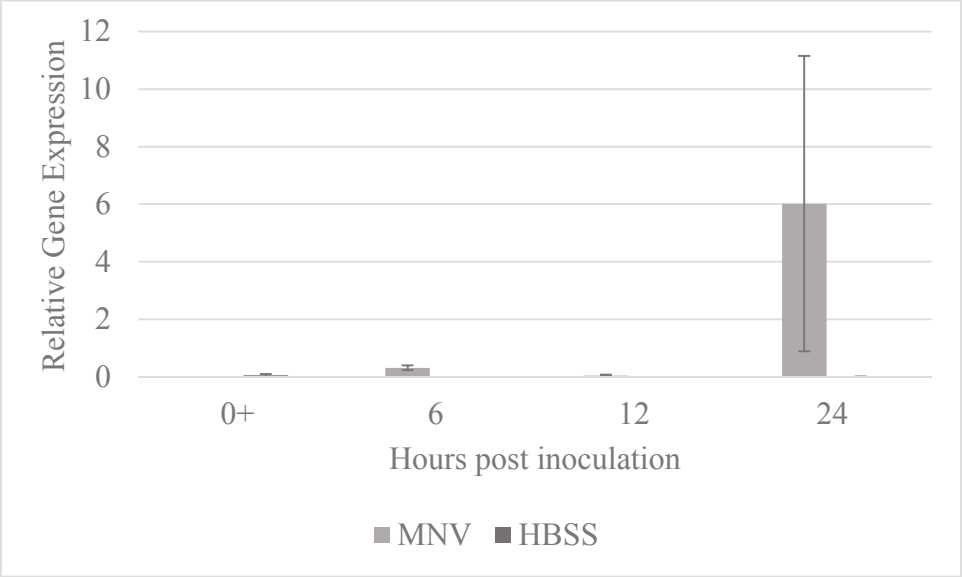

Figure 35: Relative gene expression of *NPR-1* in romaine lettuce inoculated with MNV or HBSS

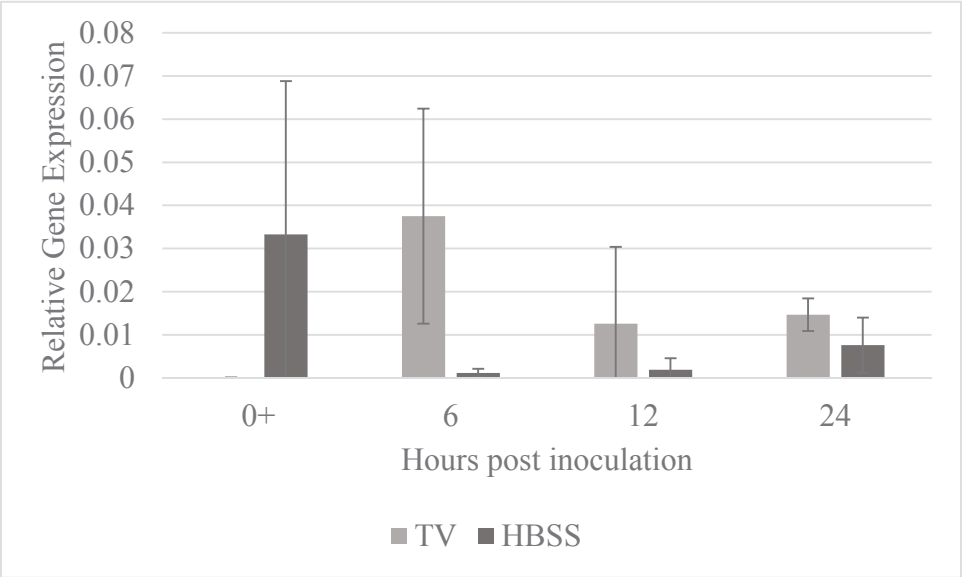

Figure 36: Relative gene expression of *PR-1* in romaine lettuce inoculated with TV or HBSS

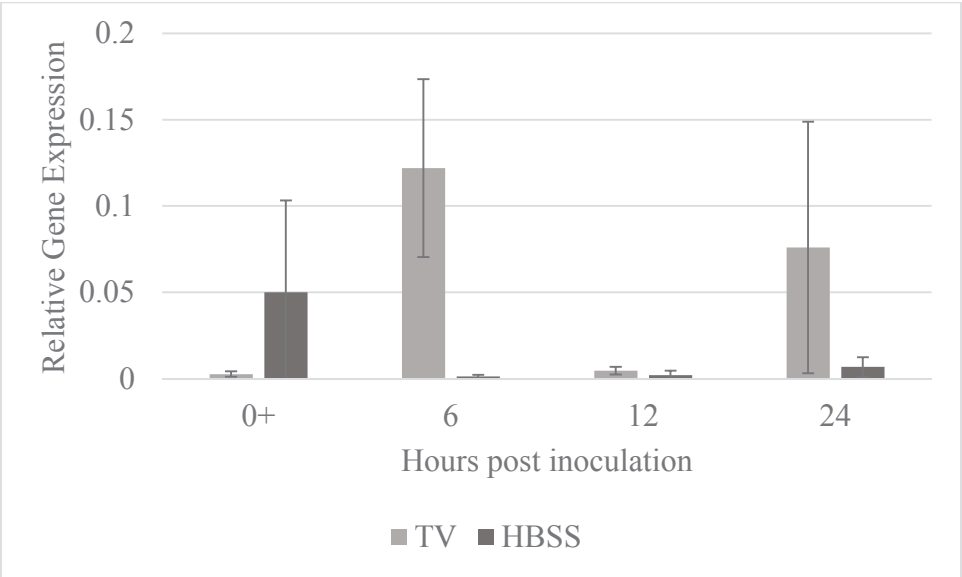

Figure 37: Relative gene expression of *NPR-1* in romaine lettuce inoculated with TV or HBSS

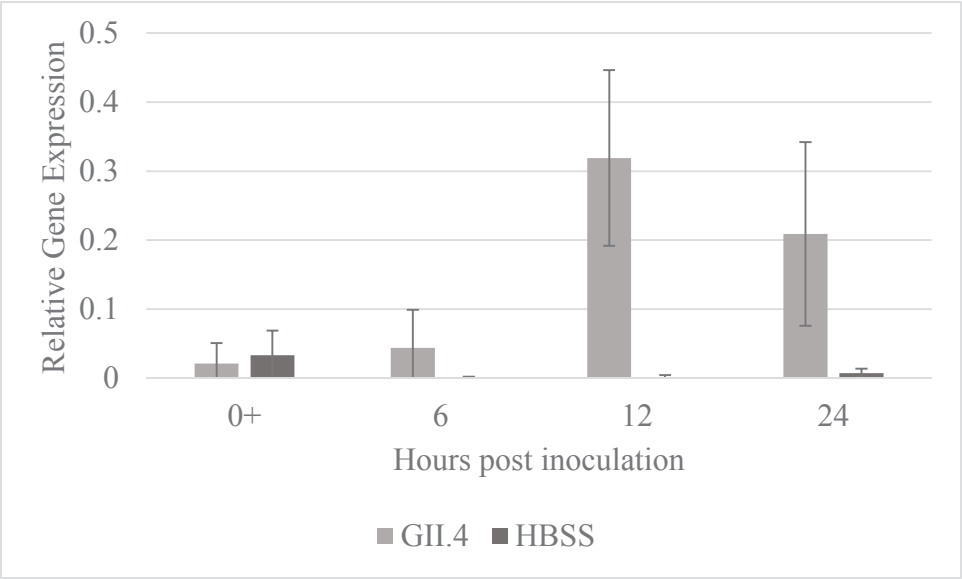

Figure 38: Relative gene expression of *PR-1* in romaine lettuce inoculated with human norovirus GII.4 or HBSS

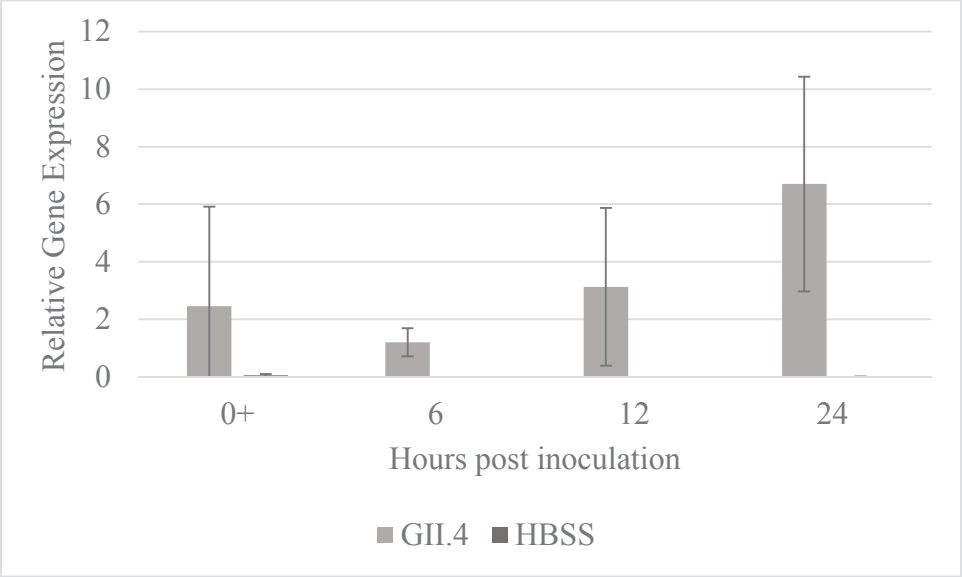

Figure 39: Relative gene expression of *NPR-1* in romaine lettuce inoculated with human norovirus or HBSS.

In *A. thaliana* Col-0 plants, MNV significantly induced the expression of *PR-1* and *PDF1.2* at 6 and 24 hpi, respectively (Figures 39-40). *VSP2a* was significantly induced at 0+, 6 and 12 hpi (Figure 41). Unlike in romaine lettuce, TV was not able to significantly induce expression for any of the genetic markers in *A. thaliana* (Figure 42-44). Human norovirus GII.4 was able to induce *PR-1* expression in *A. thaliana* at 0+, 12 and 24 hpi, similar to that which was observed in romaine lettuce (Figure 45). Human norovirus also significantly induced expression of *PDF1.2* at 0+ and 12 hpi and *VSP2a* at 12 hpi (Figure 46-47). These results indicate that only MNV and human norovirus were able to significantly induce an immune response in *A. thaliana*. In both cases, gene markers in both the salicylic acid and jasmonic acid pathways were significantly induced compared to controls. In *A. thaliana*, human norovirus GII.4 induced significantly higher levels of expression compared to MNV (p=0.02) and TV (p=0.01).

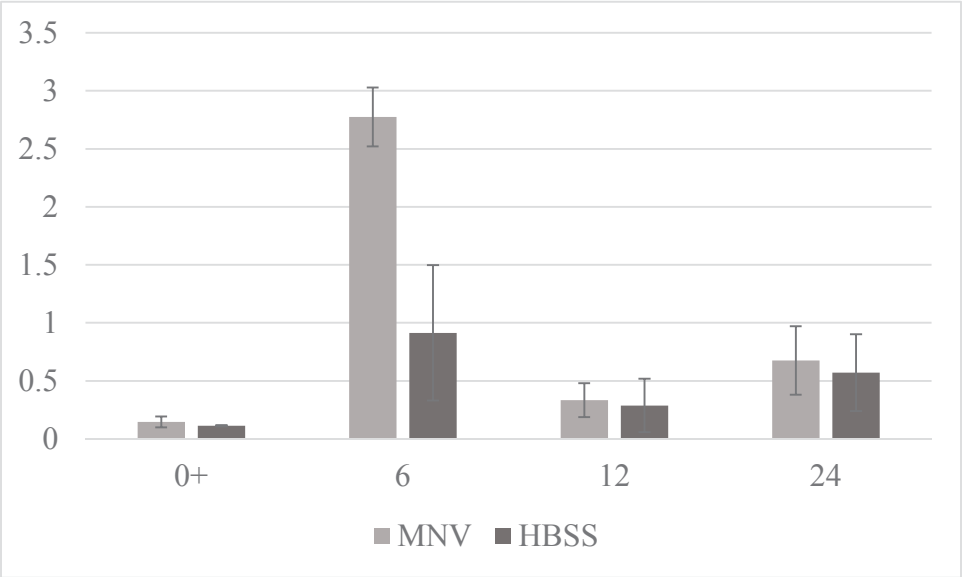

Figure 40: Relative gene expression of *PR-1* in *A. thaliana* Col-0 with MNV or HBSS

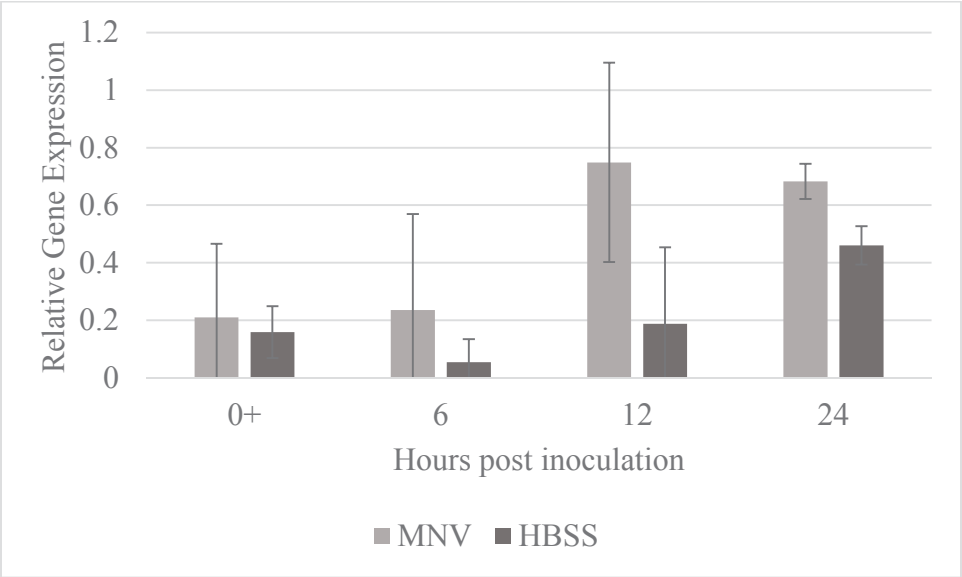

Figure 41: Relative gene expression of *PDF1.2* in *A. thaliana* Col-0 inoculated with MNV or HBSS

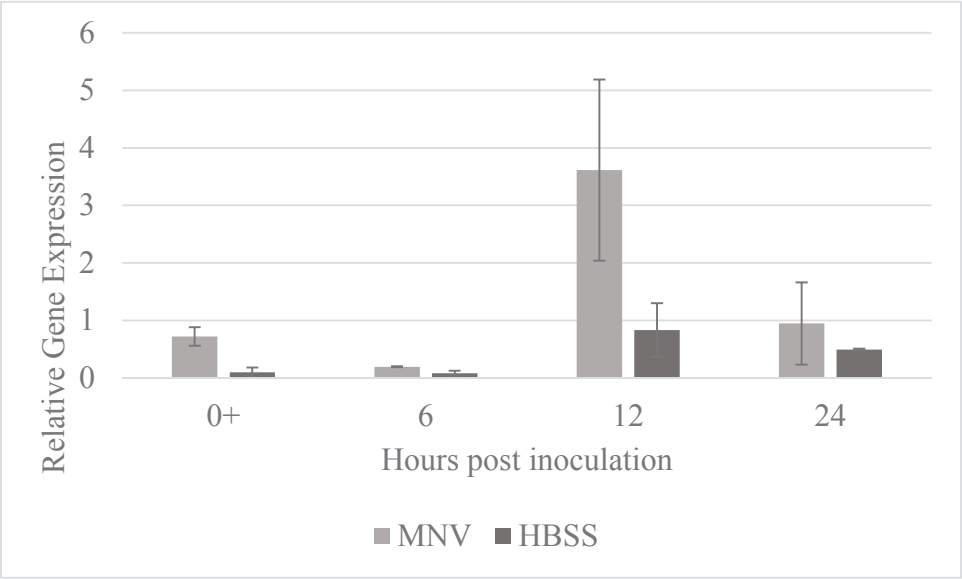

Figure 42: Relative gene expression of *VSP2a* in *A. thaliana* Col-0 inoculated with MNV or HBSS

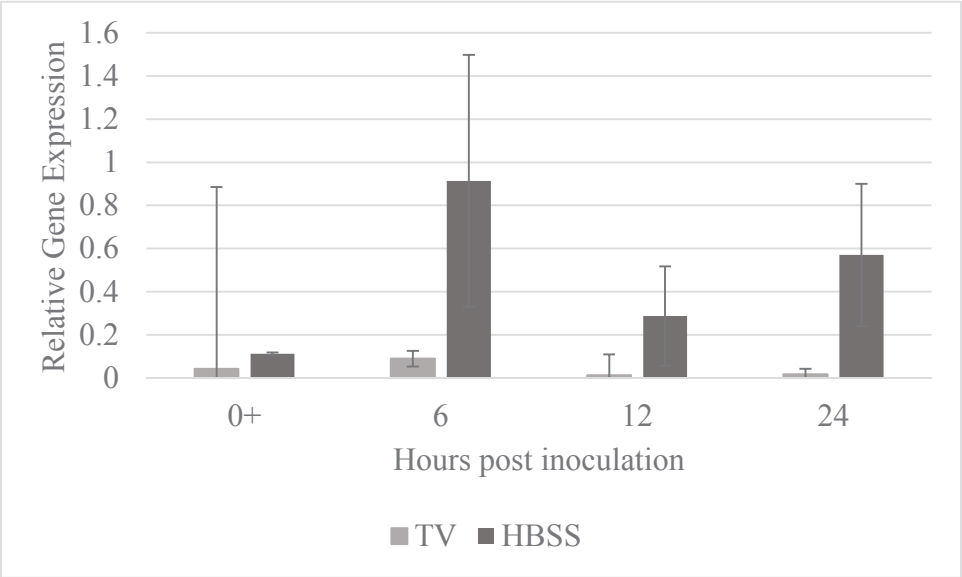

Figure 43: Relative gene expression of *PR-1* in *A. thaliana* Col- inoculated with TV or HBSS

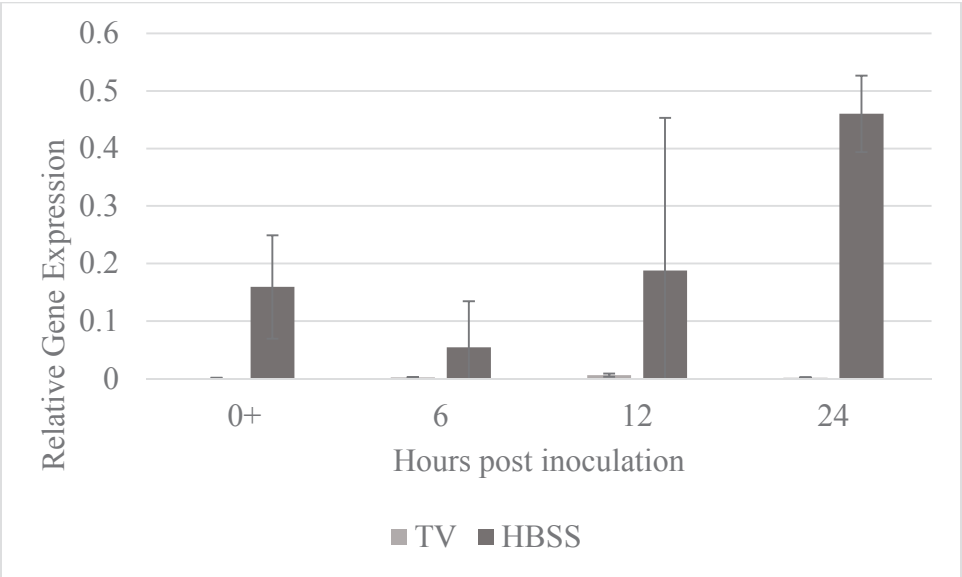

Figure 44: Relative gene expression of *PDF1.2* in *A. thaliana* Col-0 inoculated with TV or HBSS

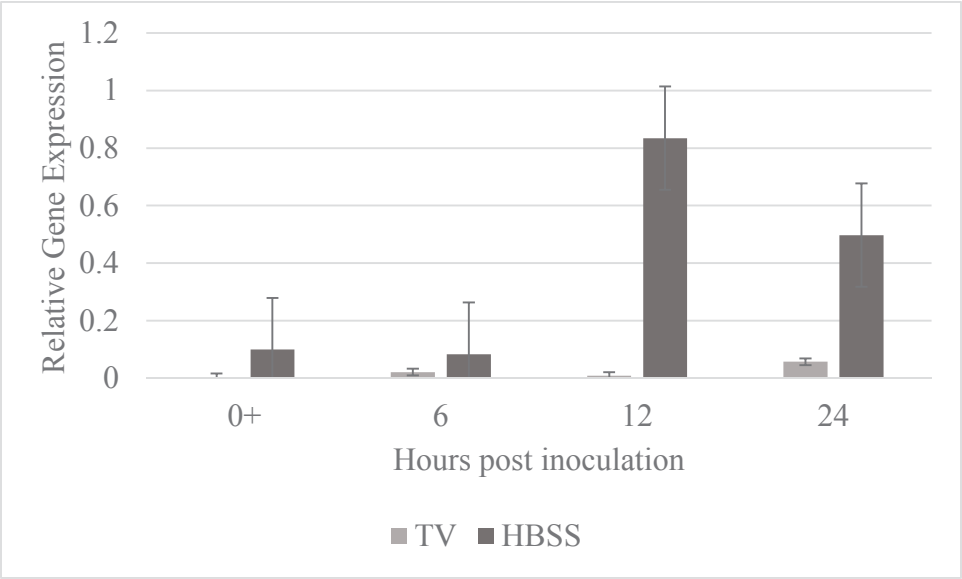

Figure 45: Relative gene expression of *VSP2a* in *A. thaliana* Col-0 inoculated with TV or HBSS

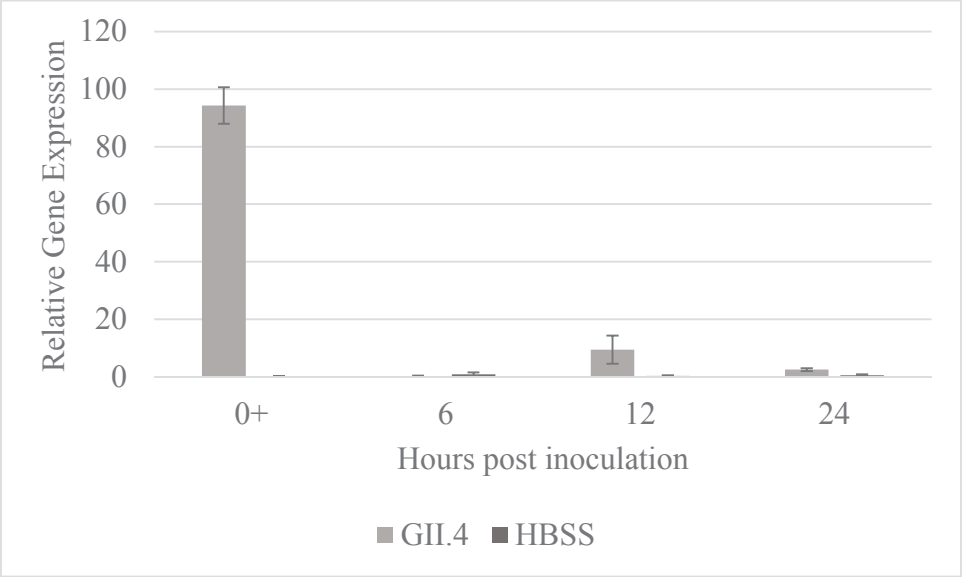

Figure 46: Relative gene expression of *PR-1* in *A. thaliana* Col-0 inoculated with human norovirus GII.4 or HBSS

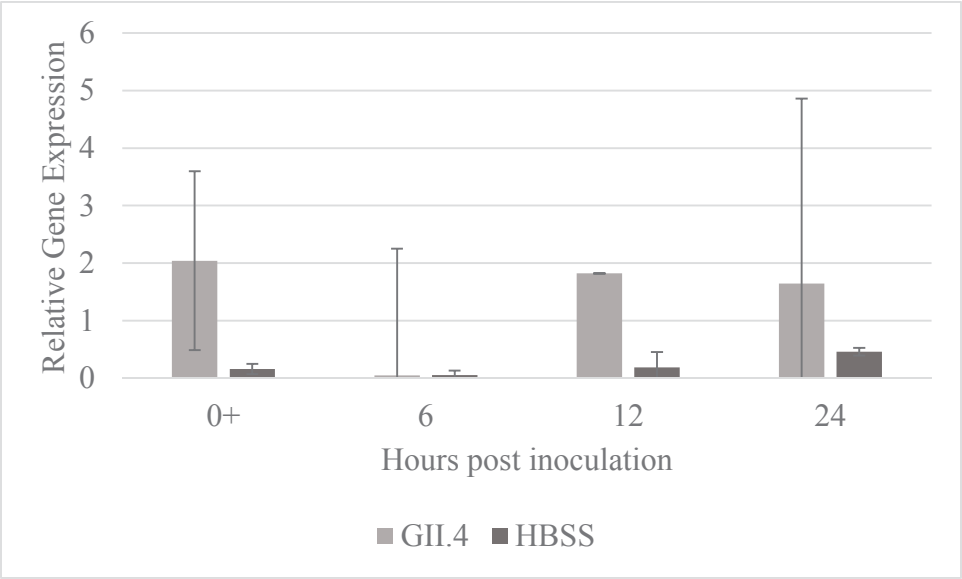

Figure 47: Relative gene expression of *PDF1.2* in *A. thaliana* Col-0 inoculated with human norovirus GII.4 or HBSS

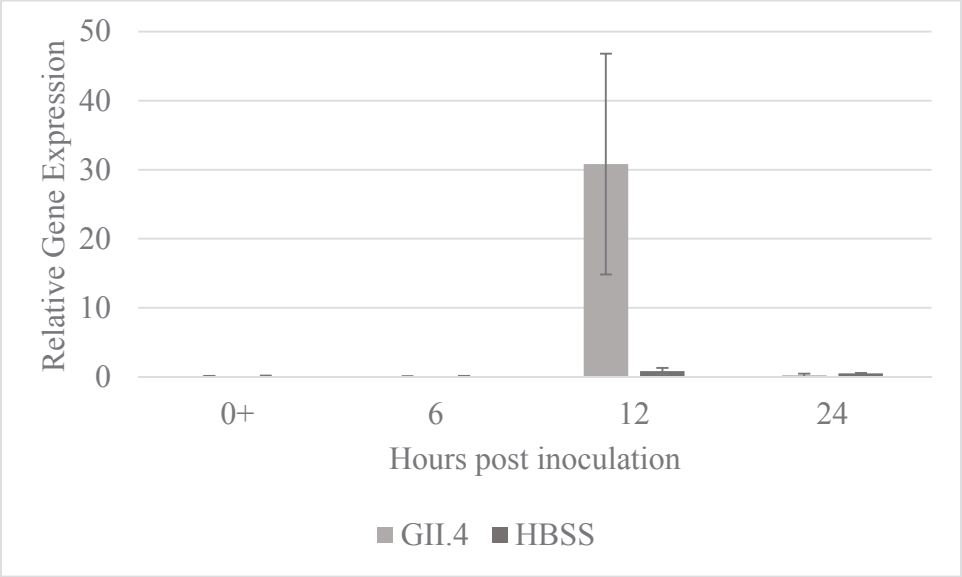

Figure 48: Relative gene expression of *VSP2a* in *A. thaliana* Col-0 inoculated with human norovirus GII.4 or HBSS

6.4.2 Gene expression in *A. thaliana* mutants confirms expression of plant defense genes in Col-0

The relative gene expression of *PR-1* was determined in *nahg* mutant plants and compared to expression levels in wild type (Col-0) plants to determine the role of *PR-1* and the salicylic acid pathway in the plant immune response of *A. thaliana* to human norovirus and its surrogates. Wild type plants (Col-0) inoculated with MNV and TV showed significant expression of *PR-1* at 6 hpi compared to expression in mutant plants. There was no significant difference in the expression of *PR-1* in wild type and *nahg* mutant plants inoculated with human norovirus GII.4. Wild type plants inoculated with MNV and TV also showed similar expression patterns of *PDF1.2* where significant expression was observed at 24 hpi for both viruses in wild type plants compared to *jin1* mutant plants. MNV and TV significantly induced *VSP2a* in wild type plants at 6 and 12 hpi compared to *jin1* mutant plants. Human norovirus GII.4 significantly induced expression of *VSP2a* in wild type plants at 12 hpi compared to expression in *jin1* mutant plants. Results are summarized in Table 4.

Table 4: Showing time points (hpi) where human norovirus GII.4 and its surrogates (MNV and TV) induced gene expression in *A. thaliana* wild type (Col-0) plants at statistically higher levels ($p \leq 0.05$) compared to mutant plant lines (*nahg* or *jin1*). *Gene expression significantly higher in wild type as indicated by (+).

	MNV			**TV**			**GII.4**		
hpi	*PR-1*	*PDF1.2*	*VSP2a*	*PR-1*	*PDF1.2*	*VSP2a*	*PR-1*	*PDF1.2*	*VSP2a*
0+	-	-	-	-	-	-	-	-	-
6	+	-	+	+	-	+	-	-	-
12	-	-	+	-	-	+	-	-	+
24	-	+	+	-	+	-	-	-	-

6.5 Discussion

In romaine lettuce, MNV was not able to significantly induce *PR-1* however; romaine lettuce plants inoculated with MNV demonstrated significant expression of *NPR-1* at 6, 12 and 24 hpi compared to controls. In *A. thaliana*, plants inoculated with MNV demonstrated early expression of *PR-1* at 6 hpi showing differences of expression patterns of gene markers related to the salicylic acid pathway between romaine lettuce and *A. thaliana*. It is somewhat surprising that *PR-1* was also not significantly induced by MNV in romaine lettuce because both *PR-1* and *NPR-1* are involved in the salicylic acid defense pathway. NPR-1 protein functions downstream of SA to mediate changes in expression of defense genes (Dong, 2004). However; depending on the pathogen type and stage of infection, *NPR-1* also interacts with components of jasmonic acid signaling (Mandadi and Sholthof, 2013). It is possible that the increased expression of *NPR-1* in romaine lettuce could be due to its interaction with the jasmonic acid pathway. This can be further supported by the fact that wild type *A. thaliana* plants demonstrated similar expression levels of gene markers related to the jasmonic acid pathway (*PDF1.2* and *VSP2a*). Compared to the jasmonic acid deficient plant mutants (*jin1*), *PDF1.2* (24 hpi) and *VSP2a* (6, 12, 24 hpi) expression levels were significantly higher in wild type plants.

TV significantly induced *PR-1* and *NPR-1* expression in romaine lettuce plants at 6 hpi compared to controls. In contrast, no significant levels of expression for any of the gene markers were observed for *A. thaliana* plants inoculated with TV. According to *A. thaliana* mutant experiments, expression patterns are almost identical to that of MNV. It is suspected that gene expression levels are lower from plants inoculated with TV compared to MNV and GII.4 because of the low titer level of TV stocks. Plants were inoculated with 10^4 log/ml TV compared to 10^7 log/ml MNV and 10^9 log/ml. The

high titer levels of human norovirus GII.4 inoculum may also explain the increased gene expression in romaine lettuce.

PR-1 expression in romaine lettuce plants inoculated with human norovirus was significantly higher than controls at 12 and 24 hpi, where *NPR-1* expression was significant at 6, 12 and 24 hpi. Results were similar for *PR-1* expression in *A. thaliana* Col-0 where expression was significantly higher than controls at 0+, 12 and 24 hpi. Expression of *PDF1.2* was significant at 12 and 24 hpi and *VSP2a* expression was significant at 12 hpi for *A. thaliana* Col-0 plants inoculated with human norovirus GII.4. Although *PR-1* and *NPR-1* expression was significant in both romaine lettuce and *A. thaliana* Col-0, according to *A. thaliana* mutant studies only *VSP2a* was significantly expressed in wild type plants compared to *jin1* mutants indicating the involvement of the jasmonic acid pathway in the plant immune response to human norovirus.

It is very interesting that both romaine lettuce and *A. thaliana* plants responded differently to the three viruses. By definition, viruses do not possess conserved P/MAMP-like features such as flagellin or chitin (Mandadi and Scholthof, 2013). However; structures including, but not limited to, the viral capsid (virion), viral ribonucleoprotein complexes, and viral-encoded glycoproteins emdedded on the host derived lipid membranes of plant rhabdoviruses (Goldberg *et al.,* 1991; Jackson *et al.,* 1995) are analogous to P/MAMPs (Mandadi and Scholthof, 2013). These viral structures are conserved among members of related virus taxa (Mandadi and Scholthof, 2013). Thus, it is hypothesized that such viral patterns are analogous to P/MAMPs and are accessible for recognition by membrane-bound receptor-like protetins to trigger immune responses in plants (Mandadi and Scholthof, 2013). It is

possible that these structures vary between MNV, TV and GII.4 explaining the differences in expression levels for the gene markers tested.

MNV and TV demonstrated different expression patterns in romaine lettuce and *A. thaliana*; however gene expression patterns in *A. thaliana* mutant line studies were nearly identical. A similar pattern was observed with human norovirus GII.4 in romaine lettuce and *A. thaliana*. *PR-1* was expressed in both romaine lettuce and *A. thaliana* Col-0, however; mutant line studies indicated that the only significant gene expression was related to *VSP2a* and the jasmonic acid pathway. While there is some argument in the field of food safety that *A. thaliana* does not serve as a good model to study the interactions between food plants and human pathogens, the results of this study indicate that *A. thaliana* should be used along with food plants in order to more clearly understand these interactions and how they can affect food safety and human health.

PR-1 genes are also known for expression of pathogen related (PR) proteins, which are recognized by plant pathogen receptors (PRRs) and are involved in the salicylic acid pathway of plant defense and leads to a hypersensitive reaction (HR) characterized by cell apoptosis and the development of chlorosis lesions during a plant systemic acquired response (SAR) to a plant pathogen. *PDF1.2* genes are also known as plant defensin genes involved in the jasmonic acid pathway of defense and a plant induced systemic response (ISR). *VSP2a* is known for expression of vegetative storage protein that is often used as a marker for the induction of the jasmonic acid pathway. Results from this study indicate that both romaine lettuce and *A. thaliana* demonstrate plant defense responses to human norovirus and its surrogates through both salicylic acid and jasmonic acid mediated pathways. Studies including gene expression of SA-

and JA-deficient *A. thaliana* mutant lines suggest that the jasmonic acid pathway is more likely involved in the plant immune response to human norovirus. It is likely that the plants are not recognizing noroviruses as plant pathogens and are instead recognizing conserved structural viral motifs that elicit a basal level of response through the jasmonic acid pathway and induction of a systemic induced response (ISR).

6.6 Conclusions

Romaine lettuce and *A. thaliana* demonstrated different expression levels of genetic markers involved in plant immune response to human norovirus and its surrogates (MNV and TV). While genes involving both the salicylic acid and jasmonic acid pathways were expressed in both romaine lettuce and *A. thaliana* for all three viruses, studies including gene expression of SA- and JA-deficient *A. thaliana* mutant lines suggest that the jasmonic acid pathway is more likely involved in the plant immune response to human norovirus. This research provides the first piece of information regarding how foodborne viruses interact with plants in the pre-harvest environment.

There are many knowledge gaps involving how plants respond to norovirus contamination which is the number one cause of foodborne illness in the US, with the majority of outbreaks involving leafy greens. Future studies should address the effects of the plant immune response on the persistence of human norovirus on plants. Such studies could aid in the development of pre-harvest interventions such as the development of plant breeding programs that will lead to varieties with enhanced plant defense mechanisms. Such interventions would provide sustainable and economically

feasible methods for reducing contamination of plants by plant pathogens as well as human pathogens in the pre-harvest environment including human viruses.

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