ABSTRACT

Title of Document: **SALMONELLA-INDUCED SYSTEMIC ACQUIRED RESISTANCE IN TOMATO AND ITS IMPACT ON SALMONELLA COLONIZATION OF TOMATO LEAVES**

Tommy Phannareth

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Directed By: Dr. Shirley A. Micallef

Plant Science and Landscape Architecture

Center for Food Safety and Security Systems

Salmonella enterica is an enteric human pathogen that lives in gastrointestinal tract; however, *Salmonella* are able to survive in plants. Thus, vegetables such as tomato are vectors for *Salmonella*. Evidence suggests that *Salmonella* induces PAMP-triggered immunity (PTI) in plants, however, plant systemic acquired resistance (SAR), which may act to suppress *Salmonella* populations, has not been explored. This research investigates whether *Salmonella* triggers SAR in tomato, and whether SAR activation restricts epiphytic *Salmonella* populations. Inoculation of tomato leaves with *Salmonella* increased SAR marker gene expression in distal tomato leaves, but did not reduce populations of the phytopathogen *Pseudomonas syringae* or *Salmonella* on distal leaves, even following treatment with chemical SAR activators. *NahG* plants, which are deficient in SAR signaling, supported higher *Salmonella* populations, and nitric oxide depletion on leaf surfaces favored *Salmonella* growth, suggesting that SAR is involved. SAR alone is insufficient to restrict *Salmonella* growth on tomato, despite being triggered.

THE INTERACTION BETWEEN TOMATO SYSTEMIC ACQUIRED RESISTANCE AND SALMONELLA ENTERICA

By

Tommy Phannareth

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Advisory Committee:
Dr. Shirley A. Micallef, Chair
Dr. Wendy Peer
Dr. Shunyuan Xiao

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Table of Contents

List of Figures

List of Tables

Table of Abbreviations

ASM	Acibenzolar-*s*-methyl
BABA	*B*-aminobutyric Acid
cPTIO	2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
DAB	3, 3'-diaminobenzidine
HA	Hexanoic Acid
NO	Nitric Oxide
PAMP	Pathogen-associated molecular pattern
Pst	*Pseudomonas syringae* pv. tomato DC3000
PTI	PAMP-triggered immunity
ROS	Reactive oxygen species
SA	Salicylic acid
SAR	Systemic acquired resistance
SeN	*Salmonella enterica* subsp. *enterica* Newport

Chapter 1: Introduction

1.1 Background and Literature Review

1.1.1 Salmonellosis outbreaks associated with fresh produce

Salmonella enterica is the causative agent of salmonellosis. As a zoopathogen, transmission has been traditionally associated with the consumption of contaminated eggs, poultry, and other animal food stuffs, as well as contact with animals and the excretions of infected persons (Mermin et al., 1997, Lynch et al., 2009). However, salmonellosis infections due to the consumption of fresh produce have become increasingly common. In 2008, *Salmonella enterica* on vine-stalk vegetables, fruits, and nuts was the most common cause of outbreak-related illnesses (CDC, 2014). Fresh tomatoes in particular have been linked to at least twelve outbreaks, seven of which were multistate outbreaks, since 1998 (Bernstein et al., 2007, Greene et al., 2008). This pathogen-commodity pair is second in a ranking of foodborne illness risk prioritization among fresh produce commodities (Anderson et al., 2011). These outbreak events seem to point to a relationship between tomato and *Salmonella* which sustain this pathogen-commodity association.

1.1.2 *Salmonella* adaptations for plant colonization

In a review of outbreak-associated *Salmonella* serovars, there appears to be serovar-commodity associations which also show grouping along meat/vegetable lines

(Jackson et al., 2013). This suggests that certain serotypes have acquired traits that confer the capacity to successfully colonize the plant phyllosphere. The majority of serotypes associated with multiple food commodities showed preference for either animal or plant contamination with two major exceptions: Typhimurium and Newport (Jackson et al., 2013). The Typhimurium serovar is associated with the most foodborne outbreaks of salmonellosis, and shows long-term environmental stability in comparison with other serovars. This environmental fitness does appear to manifest in crop field conditions, however, which suggests that *S.* Typhimurium contamination events occur post-harvest (Zheng et al., 2013). In the case of Newport, the cross-kingdom commodity contamination appears to be attributable to genetic differences within the serovar rather than a single distinguishing feature of the serovar as a whole (Jackson et al., 2013). There are several distinct genetic clades in the Newport serovar, making it comparatively genetically diverse when compared to other serovars (Jackson et al., 2013). They differ among each other for traits such as antimicrobial drug resistance, or association with reptiles and amphibians; this diversity may also explain the breadth of commodities with which this pathogen has been associated (Jackson et al., 2013)

Outside of these two major exceptions, serovars appear to be unable to successfully contaminate commodities outside of their outbreak association. In experiments with spinach, produce-associated strains were compared with poultry-associated strains for survivability in the phyllosphere; the poultry-associated strains were not detectable on the plants seven days post-inoculation (Patel et al., 2013). The main commonality of the produce-associated strains is the “rough, dry, and red” colony

morphology, which is associated with stronger biofilm formation and increase curli formation (Patel et al., 2013).

The ability to form curli appears to have a significant impact on *Salmonella* outcomes in different environments. When compared to the wild type, curli-deficient mutants showed a one log reduction in phyllosphere colonization but a one log increase in soil colonization (Lapidot and Yaron, 2009). Mutation of a *Salmonella* curli-nucleation gene reduced *Salmonella* attachment on alfalfa; however, mutation of a curli subunit *agfA* did not show differential attachment (Barak et al., 2005).

1.1.3 The tomato phyllosphere as a habitat for *Salmonella* colonization

In its host environment, the gastrointestinal tract, *Salmonella* is constantly receiving an influx of nutrients, as well as moisture and warmth. In the plant phyllosphere, however, conditions are much harsher: there are fewer available nutrients, and the bacteria are now exposed to fluctuations in temperature and moisture, as well as solar radiation.

Phytopathogens tend to congregate around plant stomata during initial infection (Nicaise et al., 2009). These openings allow the pathogens access to the apoplastic spaces within the plant tissue, which can provide a source of nutrients, as well as protect the microorganism from solar radiation and desiccation. *Salmonella* inoculated onto tomato, however, does not aggregate around the stomatal openings and instead prefers to colonize the base of glandular trichomes, (Barak et al., 2011). Glandular trichomes are hair-like epidermal structures which serve to ward off insect herbivory and exude exudates (Barak

et al., 2011). Trichome exudates can serve a wide variety of functions, depending on species and cultivar, such as defense against herbivory via toxicity, or as a nutrient source for bacteria (Wagner et al., 2004). *Salmonella* may be able to use trichome exudates as a food source and as source of moisture (Barak et al., 2011).

Salmonella colonization of the phyllosphere is also influenced by organ and cultivar dependent effects (Barak et al., 2011, Han and Micallef, 2014). For example, among the cultivars studied, Han and Micallef observed that fruits and leaves of different cultivars exhibited different susceptibilities to colonization, with *S.* Newport achieving highest on-fruit populations on tomato cv. 'Nyagous', and the highest on-leaf populations on cv. 'Virginia Sweets' (Han and Micallef, 2014).

1.1.4 Plant pathogen-defense mechanisms

1.1.4.1 Pathogen-Associated Molecular Pattern-Triggered Immunity

In order for an organism to defend itself against pathogens, it must first be able to recognize pathogenic agents with which it comes into contact. In plants, pathogen recognition functionality is distributed to each cell via membrane proteins called pattern recognition receptors, which recognize pathogen-associated molecular patterns (PAMPS) (Jones and Dangl, 2006). PAMPs are highly conserved molecular patterns found among pathogens, such as flagellin protein subunits.

Recognition of a PAMP by a plant typically results in an array of responses known as PAMP-triggered immunity (PTI) which include the following events: mitogen-activated protein kinase (MAPK) signaling, callose deposition, oxidative burst, stomatal closure, hormone production, ion fluxes, and gene silencing (Nicaise et al., 2009).

Oxidative burst refers to the production of reactive oxygen species (ROS) shortly after pathogen challenge. ROS are toxic, chemically active compounds containing oxygen, such as hydrogen peroxide. These compounds act as signals which up-regulate defense gene transcription, trigger cell hypersensitive response (HR), protect HR-adjacent cells from apoptosis, and initiate callose deposition (O'Brien et al., 2012).

Successful establishment of bacterial plant pathogens requires entry into the host tissue, and stomata present one possible point of entry (Melotto et al., 2008). *Pseuomonas syringae pv.*tomato (*Pst*) triggers initial stomatal closure in *Arabidopsis*, however the stomata re-open after four hours due to *Pst* generation of the phytotoxin coronatine, thus allowing infiltration into the tissue by the pathogen (Melotto et al., 2008). When coronatine-deficient *Pst* mutants are applied, however, the stomata stay closed, and pathogen populations are reduced when compared to the wild type. This further underscores the importance of stomatal closure to pathogen defense (Melotto et al., 2008).

1.1.4.2 Systemic Acquired Resistance

In 1961, in was demonstrated that tobacco plants inoculated with tobacco mosaic virus showed increased resistance to the pathogen in their distal tissues (Durrant and

Dong, 2004). This capacity for plants to develop resistance in distal tissues in response to local infection was called systemic acquired resistance (SAR).

SAR functionality occurs broadly in three steps: infection of local tissue, dissemination of a mobile signal to from the infected tissue to the distal tissue, and SAR activation in distal tissue. An overview of the SAR signaling network is illustrated in Figure 1.1.

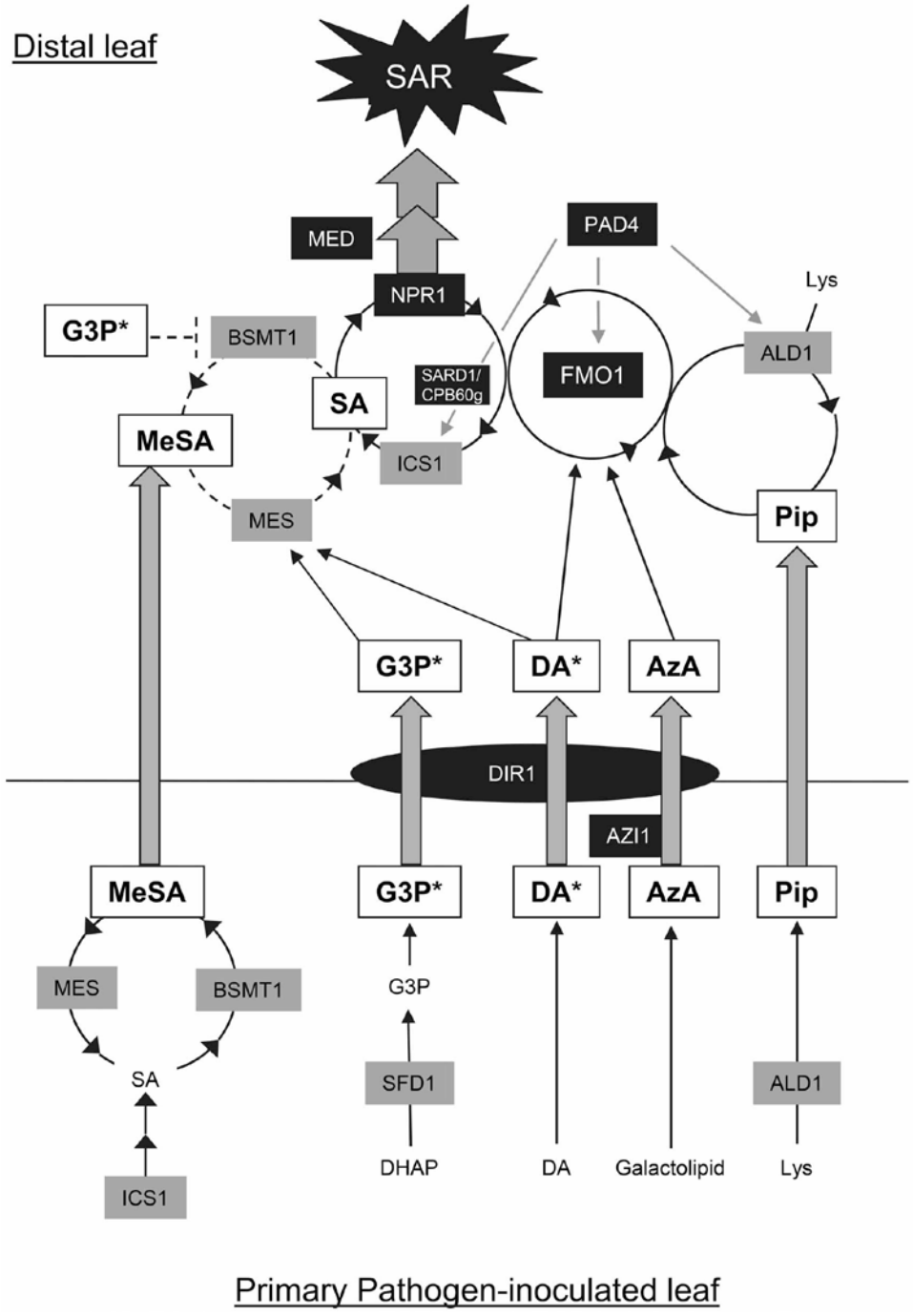


Figure 1.1 SAR circuitry involving a network of signaling molecules. (Shah and Zeier, 2013)

The central hormone associated with SAR is salicylic acid (SA), which acts to trigger defense responses in infected tissues, and signals elevation of the expression of SA-responsive *pathogenesis-related* (PR) genes in un-infected tissues (Shah and Zeier, 2013).

The function of the major marker gene for SAR, PR1, has not yet been fully characterized. Other genes in the PR family appear to have antimicrobial activity, acting as chitinases and glucanases (Ebrahim et al., 2011). PR gene expression is controlled by the nonexpressor of PR genes (NPR1) (Zhang et al., 1999). NPR1 exists in the cytoplasm as an oligomer; when it is converted into its monomeric form by SA induction it acts as a co-activator for PR1 (Tada et al., 2008).

There also exist several alternative pathways for PR gene expression which are independent of NPR1 control and SAR activation. Signals associated with HR have been shown to increase expression of PR1 and PR5 in NPR1 and SA-synthesis deficient mutants (Zhang and Shapiro, 2002) (Figure 1.2).

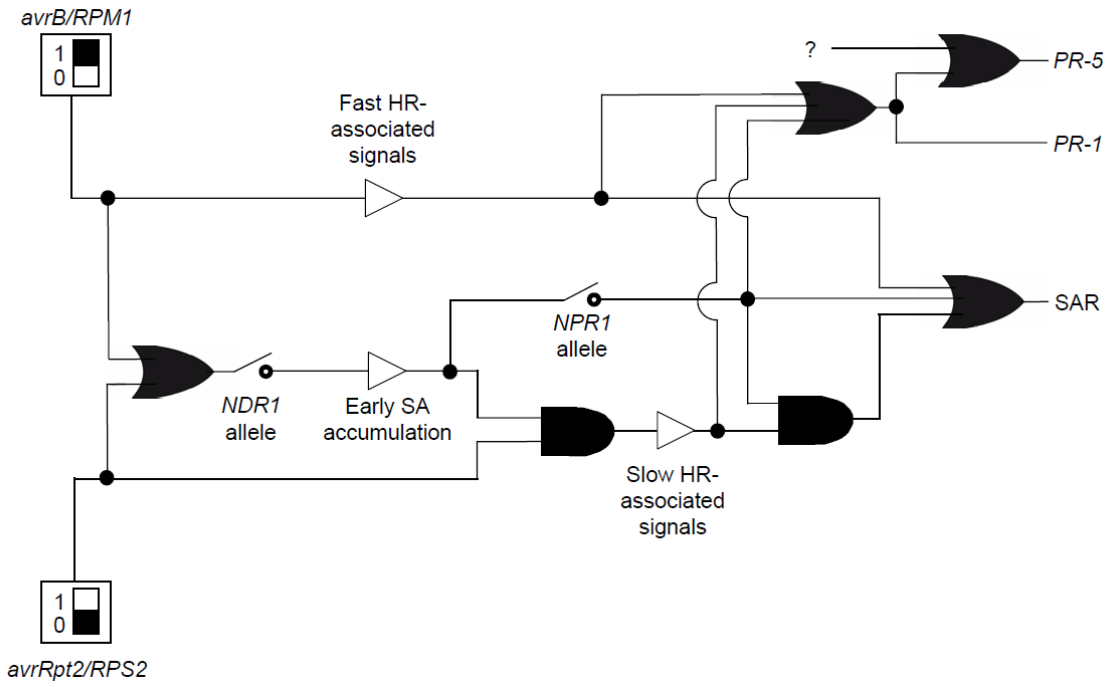


Figure 1.2 Boolean representation of SAR, PR1, and PR5 network. Filled circles indicate signal branching points. Bullets with flat left sides indicate "and" gates and require both inputs, bullets with concave left sides indicate "or" gates, which trigger with a single input. (Zhang and Shapiro, 2002)

As a critical system necessary for plant survival, SAR appears to have multiple, durable, and independent mechanisms for signaling distal tissues from an infection site. Methyl salicylate (MeSA) and azelaic acid have both been proposed as the mobile signal for SAR.

SA accumulated in infected tissues is inactivated by SA carboxyl methyltransferase (SAMT) and converted to MeSA, which is translocated to uninfected tissues and re-converted into SA by salicylic acid binding protein 2 (SABT) (Park et al., 2007). However, SAMT deficient *Arabidopsis* mutants appear to be able to continue to exhibit SAR capability (Attaran et al., 2009). Investigations in tobacco have revealed that

MeSA is necessary for SAR signaling in dark conditions, and that at least 3.5 hours of light exposure after pathogen infection was sufficient to make MeSA dispensable for triggering SAR (Liu et al., 2011).

The *AZELAIC ACID INDUCED 1* (AZI1) gene has been shown to be necessary for distal accumulation of SA and SAR signaling in *Arabidopsis* (Jung et al., 2009). *Arabidopsis azi1* mutants exhibited no change in local resistance, but were compromised in their ability to develop SAR (Jung et al., 2009). AZI1 expression is induced by azelaic acid applications, which suggests that azelaic acid may also be one of the signals for SAR (Jung et al., 2009).

Free radical compounds, such as reactive oxygen species (ROS) and nitric oxide (NO), also have a role to play in SAR expression, independent of PR-1 (Wang et al., 2014). Exogenous application of nitric oxide donors or H_2O_2 onto *Arabidopsis* resulted in reduced *Pseudomonas syringae* pv. tomato populations on distal tissues when applied 24 h before inoculation, but did not significantly increase PR-1 expression (Wang et al., 2014). Furthermore, *Arabidopsis* double mutants of nitrous oxide reductase and nitrous oxide-associated protein were compromised in their ability to mount SAR phenotype response after *Pst* challenge (Wang et al., 2014). NO and ROS appear to be on the same pathway for SAR induction. Application of ROS on NO-deficient mutants restored the SAR phenotype, but application of NO donors on ROS-deficient mutants did not, indicating that ROS functions downstream of NO (Wang et al., 2014).

SA and NO/ROS appear to be two branches of SAR. Their independence from each other was confirmed by exogenous applications of one compound on mutants deficient in the other compound (SA onto NO-mutants or ROS-mutants, NO or ROS onto

SA-mutants); in both cases, the exogenous application was insufficient to restore the SAR phenotype (Wang et al., 2014) (Figure 1.3).

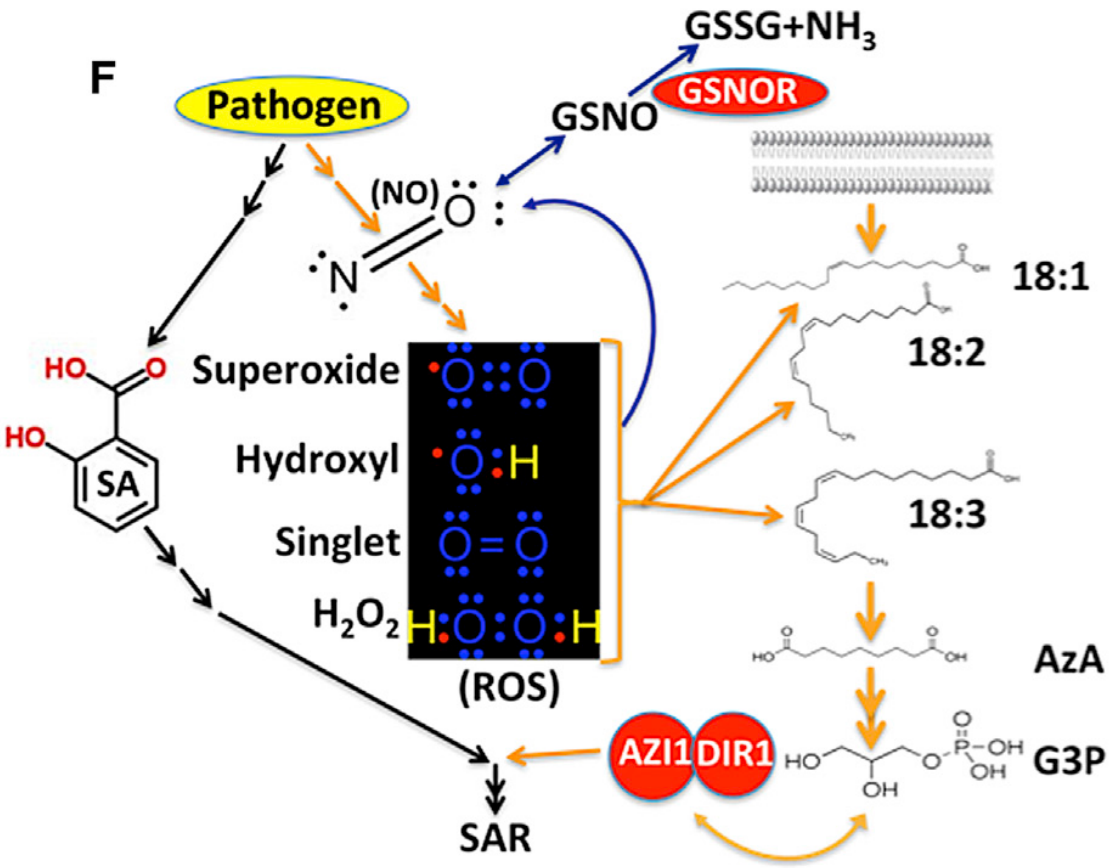


Figure 1.3 Simplified model illustrating NO-ROS signaling in SAR (Wang et al., 2014)

1.1.5 Chemical activators of SAR

1.1.5.1 Acibenzolar-*s*-methyl

Acibenzolar-*s*-methyl (ASM) is a functional SA analogue, and is one of the first commercially marketed chemical SAR activators, under the name BION® in Europe and ACTIGARD® (Syngenta) in North America (Walters et al., 2005). It has a

broad range of action, and is capable of activating SAR in a wide variety of crops against many classes of diseases (Table 1.1).

Crop	Bacteria	Viruses	Fungi	Nematodes	Insects
Cereals			+		
Rice	+		+		
Potato		+			+
Tobacco	+	+	+		
Tomato	+	+	+		+
Vegetables	+	+	+		+
Mango	+		+		
Citrus	+	+	+		
Grapes			+	+	
Banana			+	+	
Stone fruits	+				
Pome fruits	+		+		

Table 1.1 “ASM Activity in Important Crop Plants Against Various Classes of Pathogen” Adapted from (Oostendorp et al., 2001). “+” indicates pathogen resistance.

The mechanism of ASM is still undergoing investigation. It is readily translocated throughout the plant from local tissue application, and degrades below detectable levels in tobacco tissues after 72 hours (Tripathi, 2010). The protective effect of ASM is strongest at variable periods after application: ranging from 3 days in cauliflower and tomato, to nearly a week after application in cocoa (Baysal et al., 2003). The appearance of the protective effect after degradation in the tissue suggests that ASM is converted *in planta* to some other SAR-related compound. The protein SABP2 converts MeSA into SA to induce SAR in distal tissues (Tripathi, 2010). Conversion of ASM *in planta* to acibenzolar by SABP2 appears to be necessary for SAR protection against tobacco mosaic virus (TMV) in tobacco (Tripathi, 2010). SABP2-silenced tobacco fails to express the SAR marker gene PR1 after treatment with ASM, and does not reduce TMV

symptoms compared to wild type plants treated with ASM (Tripathi et al., 2010). However SABP2-silencing does not impact SAR efficacy against the bacterium *Pseudomonas syringae* on tobacco (Tripathi, 2010), indicating that the compound may influence multiple SAR pathways.

1.1.5.2 Hexanoic Acid

Hexanoic acid (HA) is a SAR activator known to induce resistance in tomato to *Botrytis cinerea* and *Pseudomonas syringae* (Scalschi et al., 2013). This effect was initially attributed to the antimicrobial action of HA; however, the protective effect of HA application is present even at sub-antimicrobial levels. HA primes different kinds of plant responses depending on the type of pathogen challenge; for example, it promotes callose deposition during *B. cinerea* challenge, but does not do so during *Pst* challenge (Scalschi et al., 2013). One of the main functions of HA appears to be inhibiting the influence of pathogenesis effectors. *Pst* up-regulates the synthesis of jasmonyl-isoleucine, a bioactive molecule which interferes with SA-responsive gene expression *(Scalschi et al., 2013)*. *Pst* also produces the phytotoxin coronatine, a jasmonyl-isoleucine mimic which also acts to suppress SA-dependent defenses and initiates opening of the stomata, allowing *Pst* to enter the apoplast (Scalschi et al., 2013). Jasmonyl-isoleucine is reduced in HA treated plants after *Pst* infection when compared to untreated, infected plants (Scalschi et al., 2013). HA treated plants were also able to express PR1 and close their stomata after exposure to coronatine, responses which were reduced in coronatine-exposed plants that were not treated with HA (Scalschi et al., 2013). Additionally, the growth of *Pseudomonas syringae Cma* mutants, which do not synthesize coronatine, was not

significantly inhibited (although these mutants exhibited overall less fitness in the phyllosphere compared to the coronatine-synthesizeing wild type) (Scalschi et al., 2013).

1.1.5.3 *B*-aminobutyric Acid

B-aminobutyric acid (BABA) is an activator of SAR and has been demonstrated to be efficacious a in wide variety of crops such as potato, tobacco, cucumber, melon, cauliflower, and more (Cohen, 2002). BABA application on tomato confers resistance to a broad spectrum of diseases, such as *Pseudomonas syringae*, *Fusarium oxysporum*, *Ralstonia solanacearum*, *Xanthomonas vesicatoria*, and *Clavibacter michiganensis*, among others (Hassan and Abo-Elyousr, 2013). Levels of PR protein accumulation differ after BABA treatment depending on the method of application: in *Arabidopsis* plants unchallenged by a pathogen, foliar application of BABA increased the accumulation of PR1, PR2, and PR5 proteins, whereas soil drench application did not (Cohen, 2002). Both methods of application, however, resulted in acquired resistance across several pathosystems, and soil drench-application plants quickly accumulated PR proteins after pathogen challenge (Cohen, 2002). PR protein accumulation after BABA application also shows family-dependent effects: members of Solanaceae, such as tomato and pepper, accumulate PR proteins regardless of method, whereas Brassicaeae plants appear to only accumulate PR proteins after pathogen challenge or with direct application of BABA to the tissue (Cohen, 2002). BABA treated plants also exhibit increased sensitivity to PAMPs: untreated tomato plants only exhibit HR after inoculation with live *Phytophthora infestans*, while BABA treated plants developed HR after inoculation with living and dead *P. infestans* (Cohen, 2002).

1.1.5 Plant defense and *Salmonella* interactions

Although *Salmonella* is not a phytopathogen, it is able to trigger an array of PTI-related responses in plants. In *Arabidopsis*, inoculation with *Salmonella* activates the MAPK signaling cascade, and triggers defense gene expression similar to that with challenge by the pathogen *Pseudomonas syringae* (Schikora et al., 2011). In tobacco cell cultures, ROS accumulation was observed following inoculation with heat inactivated *Salmonella* cells, indicating that the response trigger was some passive factor of *Salmonella*, such as a PAMP (Shirron and Yaron, 2011). *In vivo* application of live *Salmonella* also caused cell death on tobacco leaves, and purified *Salmonella* flagellin protein triggered ROS production, callose deposition, and growth reduction consistent with challenge by a pathogen (Meng et al., 2013).

Salmonella with mutated flagellin proteins induced a much weaker PTI response in tobacco (Meng et al., 2013). Decreased PTI was also demonstrated in *Arabidopsis* and tobacco mutants with silenced or mutated *FLS2*, which codes for a flagellin receptor, that were inoculated with wild type *Salmonella* (Meng et al., 2013). *Salmonella* flagellin mutants were able to colonize *Medicago* spp. to a greater degree compared to the wild type (Iniguez et al., 2005).

The reduction, rather than elimination, of PTI in the absence of flagellin indicates that there may be other *Salmonella* PAMPs which may be recognizable by plants. In *Arabidopsis*, the O antigen of *Salmonella* Senftenberg induces leaf chlorosis and wilting, indicative of the hypersensitive response (Berger et al., 2011). Synthetic *Salmonella* cold shock proteins trigger low levels of ROS in tobacco (Meng et al., 2013).

When PTI is triggered by a PAMP (rather than a whole pathogen) this trigger can act to restrict the success of subsequent pathogen establishment (Meng et al., 2013). Tobacco leaves treated with *Salmonella* derived flg22 protein showed significantly reduced *Pst* populations when compared to un-treated leaves (Meng et al., 2013).

As mentioned before, bacterial plant pathogens such as *Pst* typically trigger initial stomatal closure within the first hour of infection (Melotto et al., 2008). In *Arabidopsis*¸ the initial stomatal closure due to *Pst* infection is followed by re-opening of the stomata after four hours due to pathogen-emitted phytotoxins coronatine, which serves to help the pathogen infiltrate the tissue (Melotto et al., 2008). *E. coli*, which is not a plant pathogen, also triggers stomatal closure in *Arabidopsis*, but this closure is not subsequently followed by stomatal re-opening(Melotto et al., 2008). This closure is likely due to flagellin on *E. coli* being recognized by *Arabidopsis* as a PAMP. *E. coli* triggers the stomatal closure on lettuce as well (Roy et al., 2013). *Salmonella*, however triggers little to no stomatal movement in lettuce (Roy et al., 2013), which may indicate that *Salmonella* PAMPs do not trigger PTI in lettuce, or that *Salmonella* has a means of actively suppressing stomatal closure.

Salmonella effectors may contribute to *Salmonella*-plant interactions. Bacterial type III secretion systems (T3SS) are employed by zoopathogens and phytopathogens to deliver effectors to the host cell in order to enhance their own pathogenicity. Inoculation of *Arabidopsis* with *Salmonella* T3SS mutants caused chlorosis and a stronger cell death response than inoculation by the wild type, and exhibited reduced phyllosphere survival (Schikora et al., 2011). *Salmonella* T3SS mutants were also shown to trigger higher levels of plant defense gene expression in *Arabidopsis* when compared to the wild type

(Schikora et al., 2011, Garcia et al., 2014). However, T3SS deficient *Salmonella* Typhimurium mutants showed increased populations on *Medicago* sp. when compared to the wild type (Iniguez et al., 2005). Mutation of the T3SS had no impact on *Salmonella* outcomes during colonization of tomato (Meng et al., 2013).

It has not yet been established whether or not *Salmonella* is capable of delivering effectors *in planta*, however there is some evidence that *Salmonella* effectors could theoretically contribute to the *Salmonella*-plant interaction. Transient constitutive expression of *Salmonella* effector SSef in tobacco with a viral trigger caused necrotic lesions which may indicate a hypersensitive response (Ustun et al., 2012). *Xanothomonas campestris* (a non-pathogen of tobacco which itself employs a T3SS in other systems) was transformed to produce SSef, and the SSef mutants were also able to trigger a similar necrotic response in tobacco (Ustun et al., 2012)

1.2 Rationale and Significance

Salmonella enterica is a significant foodborne pathogen, with an estimated 1,000,000 cases of salmonellosis occurring each year in the United States (CDC, 2014). These cases cost the nation $3.7 million in medical expenses and productivity losses, and result in an estimated 370 deaths (United States Department of Agriculture Economic Research Service, 2014). Infections due to the consumption of fresh tomatoes have become increasingly common, and this pathogen-commodity pair is second in a ranking of foodborne illness risk prioritization (Anderson et al., 2011, Painter et al., 2013).

Management of this emerging vector is vital to maintaining public health, as well as the public's trust in fresh produce.

Traditionally, enteric pathogens such as *Salmonella* were believed to not have much fitness outside of the animal host, due to stark differences between the warm, moist, nutrient rich conditions of the gastrointestinal tract and the constantly fluctuating conditions of the outside environment; however there is an increasing amount of evidence that *Salmonella* (and other enteric pathogens) have adaptations that allow them to persist in the environment, and even form specific ecological relationships with plants. For example, produce-associated strains of *Salmonella* survive longer in the phyllosphere than poultry-associated strains, and develop different kinds of biofilms (Patel et al., 2013). Furthermore, these produce-associated strains have only been associated with outbreaks from particular produce commodities (such as tomato) suggesting that there is some plant contribution to *Salmonella* outcomes in the environment.

Salmonella appears to show some level of interaction with plant PAMP triggered immunity (PTI). Inoculation with *Salmonella* or *Salmonella*-derived flg22 protein triggers the MAPK signaling cascade, reactive oxygen species accumulation, PTI-associated gene expression, and is capable of suppressing *Pst* populations when applied as a pre-treatment to the leaves of *Arbidopsis* (Garcia and Hirt, 2014). Further, it has been demonstrated that *Salmonella* mutants with deficient type III secretion systems, which are compromised in their ability to infect animals, exhibit reduced phyllosphere survival in *Arabidopsis* (Schikora et al., 2011). This suggests that there may be an effector component to *Salmonella* phyllosphere outcomes impairing plant defense against it (Schikora et al., 2011). Effector triggered immunity (ETI) and PTI both confer increased

resistance to pathogen attack in distal tissues through the systemic acquired resistance mechanism (SAR) (Mishina and Zeier, 2007). However, the impact of SAR activation on *Salmonella* has not yet been fully explored. SAR activation may provide another tool in managing this food safety issue.

1.3 Hypotheses and objectives

To fill this data gap, this study investigated the SAR component of *Salmonella*-tomato interactions by testing two hypotheses:

1. *Salmonella* triggers PTI which may subsequently induce SAR.
2. *Salmonella* survival is hindered by tomato plants in which SAR has been triggered.

1.3.1 *Salmonella* triggers PTI which may subsequently induce SAR

To evaluate whether *Salmonella* is capable of inducing SAR on tomato, the following objectives were carried out:

Objective 1: Assess phenotypic expression of systemic acquired resistance by assaying *Pseudomonas syringae* populations on distal tomato leaf tissues after inoculation with *Salmonella.*

Objective 2: Assess phenotypic expression of plant defense response by assaying reactive oxygen species production after *Salmonella* inoculation.

Objective 3: Assess defense gene transcription in tomato after inoculation with *Salmonella*.

1.3.2 *Salmonella* survival is hindered by tomato plants in which SAR has been triggered.

This hypothesis was tested by assessing *Salmonella* populations on plants in which SAR was activated, and by assessing gene expression of SAR genetic markers. The following objectives were performed:

Objective 1: Assess *Salmonella* survival on plants which have been treated with chemical activators of SAR.

Objective 2: Assess defense gene transcription in tomato treated with chemical activators of SAR after inoculation with *Salmonella* to determine whether *Salmonella* triggers SAR in distal tissues.

1.4 Approach

Salmonella enterica subsp. *enterica* serovar Newport (*SeN*) was chosen as the primary strain used in this study as it is an outbreak strain associated with tomatoes (Greene et al., 2008). Tomato cv. 'Primo Red' was used in the majority of experiments due to its relative compactness, and determinate nature. Tomato cv. 'Heinz' and 'Nyagous' were additionally used in some experiments due to their cultivar-based influence on *Salmonella* outcomes (Han and Micallef, 2014). Tomato cv. 'Moneymaker'

was used due to the availability of a *NahG* line of 'Moneymaker', which facilitated investigations into the influence of salicylic acid accumulation on *Salmonella*. The *NahG* tomato line contains the bacterial transgene *NahG* encoding salicylic acid hydroxylase, which degrades salicylic acid to catechol, thus blocking SA defense signaling in the plant.

Bacterial inoculation was carried out by either needleless syringe or tissue dip into bacterial suspension. Syringe inoculation allowed for precise determination of initial inoculum load, and allowed for controlled application of the inoculum. Tissue dipping was employed in order to more closely match real bacterial exposure events.

Assays were performed on distal tissues, as the SAR response is characterized by resistance in tissues distal to the initial site of infection.

Chemical SAR activators were employed to trigger SAR due to the consistency of their SAR activation efficacy in other systems compared to pathogen challenge, and further because their influence on *Salmonella* populations had not yet been investigated.

To further confirm SAR activation, expression levels of the genes PR1, PR5, and SAMT were assessed. PR1 expression is the primary molecular marker for SAR activation (Durrant and Dong, 2004). PR5 expression is associated with alternative pathways of SAR signaling (Zhang and Shapiro, 2002). SAMT expression occurs downstream of SA synthesis, and SA accumulation is a fundamental component of the SAR response (Park et al., 2007).

Chapter 2: Assessing the Hypersensitive Response and Systemic Acquired Resistance in tomato in response to *Salmonella*

2.1 Introduction

The Salmonella-tomato pathogen-commodity pair ranked second among fresh produce commodities in a compilation of foodborne illness risk prioritizations (Anderson et al., 2011). Fresh tomatoes have been associated with several multistate outbreaks since 1998, some of which have been traced back to pre-harvest contamination in the field (Bernstein et al., 2007, Greene et al., 2008) The close association between tomatoes and *Salmonella* outbreaks suggests that there may be some underlying mechanism in this particular crop which supports *Salmonella* populations.

Plant detection of microbes is modulated by recognition of microbe-associated molecular patterns (MAMPs) by cell membrane-bound pattern recognition receptors (PRR) (Jones and Dangl, 2006). MAMPs common to phytopathogens are termed pathogen-associated molecular patterns, or PAMPs (Jones and Dangl, 2006). Recognition of PAMPs by the plant initiates an array of basal defense responses known was PAMP-triggered immunity (PTI) (Jones and Dangl, 2006). PTI) These defense responses include mitogen-activated protein kinase (MAPK) signaling, callose deposition, oxidative burst, programmed cell death (hypersensitive response), stomatal closure, hormone production, ion fluxes, and gene silencing (Nicaise et al., 2009).

Phytopathogen defense does not just occur at the site of infection; plants under phytopathogen challenge are also capable of mounting systemic acquired resistance (SAR) to subsequent phytopathogen infection (Durrant and Dong, 2004). Plants in which SAR has been induced are able to more quickly mount their phytopathogen defenses and thus interfere with pathogen colonization before critical pathogenesis population thresholds are met (Tripathi et al., 2010, Scalschi et al., 2013).

PTI and SAR responses are closely interconnected – SAR typically only manifests after pathogen recognition and initiation of PTI defenses (Durrant and Dong, 2004). Control of modulation of the PTI hypersensitive response is managed by free radical compounds such as nitric oxide (NO) and reactive oxygen species (ROS) (O'Brien et al., 2012, Wang et al., 2014). These compounds participate in parallel with salicylic acid to initiate SAR (Wang et al., 2014).

Salmonella is an enteric animal pathogen and is unable to cause infection in plants. However it has been shown to be capable of triggering an array of PTI-related responses in plants. In *Arabidopsis,* inoculation with *Salmonella* activates the MAPK signaling cascade, and triggers defense gene expression similar to that with challenge by the phytopathogen *Pseudomonas syringae* (Schikora et al., 2011). Silencing the *FLS2* gene in *Arabidopsis*, which encodes for a flagellin-specific PRR, reduced PTI expression after *Salmonella* inoculation when compared to wild type *Arabidopsis* (Meng et al., 2013). In tobacco, inoculation with *Salmonella*-derived flagellin resulted in PTI responses such as reactive oxygen species production, hypersensitive response, and callose deposition (Meng et al., 2013). *Salmonella*-derived flagellin also reduced the population counts of subsequently applied *Pseudomonas syringae* and *Salmonella* (Meng

et al., 2013). This combination of results suggests that *Salmonella* PAMPs are recognized by plants, and that PTI may act to inhibit *Salmonella* survival.

It is not presently known if the PTI response to *Salmonella* is sufficient to also initiate SAR in plant tissues, nor is it known the extent to which PTI or SAR are able to influence *Salmonella* outcomes in the phyllosphere. To better elucidate whether SAR is activated in tomato in response to *Salmonella*, and whether SAR activation would impair *Salmonella* colonization, this study aimed to determine whether:

1) Tomato detects *Salmonella* and responds by producing ROS.

2) Local *Salmonella* inoculation of tomato leaves would reduce the population of the plant pathogen *Pseudomonas syringae* on distal leaves, in a manner consistent with SAR.

3) Local *Salmonella* inoculation of tomato leaves would influence the population of *Salmonella* on distal tissues, in a manner consistent with SAR impact on plant pathogens.

4) Compromising plant defense signaling would increase *Salmonella* populations.

2.2 Materials and methods

2.2.1 Plant material

The following tomato (*Solanum lycopersicum*) cultivars were used in this study: ‘Heinz’, ‘Primo Red’, ‘Nyagous’, ‘Moneymaker’, and *NahG* lines in ‘Moneymaker’ background. ‘Heinz’ and ‘Primo Red’ seeds were purchased from Harris Seeds (Rochester, NY). ‘Nyagous’ seed was obtained from the Tomato Genetics Resource

Center, University of California-Davis, CA. 'Moneymaker' and its *NahG* line seeds were provided by the Dr. Ann Powell at University of California-Davis.

Seeds were started in 5 x 10 cell trays with potting mix, then transplanted to 6" x 6" pots with potting mix after the first true leaves emerged. Plants were grown for 4 weeks in greenhouse conditions (16L; 8D, 28°C/18°C, relative humidity 75%), then covered with black plastic and transferred to a growth chamber (16L; 8D, 28°C/18°C, relative humidity 80%) and allowed to acclimate for 3 days before conducting experiments.

2.2.2 Bacterial strains

Salmonella enterica subsp. *enterica* serovar Newport, a tomato outbreak strain (Greene et al., 2008) (adapted for rifampicin resistance) and *S.* Typhimurium LT2 (ATCC® 700720) were grown in trypticase soy agar (TSA) with 50 µg rifampicin/mL, and incubated at 37°C for 18 h. *Salmonella* Senftenberg (ATCC® 8400) was grown on TSA, and incubated at 37°C for 18 h.

Pseudomonas syringae pv. tomato DC3000 (*Pst*) (ATCC® BAA-871™) was kindly provided by Dr. Shunyuan Xiao. It was grown in Pseudomonas Agar F (PAF) with 50 µg rifampicin/mL, and incubated at 30°C for 48 h.

2.2.3 *Salmonella* multiple inoculation assay

Salmonella Newport (*SeN)* suspensions were prepared by flooding plates with sterile phosphate buffered saline (PBS), then lightly shaking for 30 s. The suspension was then poured off the plate, and diluted with additional PBS to achieve an optical density of 0.5 at 600 nm, which corresponds to ~8 log CFU/mL.

Four-week-old ‘Heinz’ and ‘Primo Red’ tomato plants were transported to the growth chamber from the green house three day before the start of the experiment. Four plants per treatment were inoculated via needleless syringe on the adaxial leaf surface with 100 µL of *Salmonella* suspension as scheduled on Table 2.1.

	Multiple Inoculation	Single Inoculation
Day 0	Inoculate Leaf 1	
Day 1		
Day 2	Inoculate Leaf 2	
Day 3		
Day 4	Inoculate Leaf 3	Inoculate Leaf 3
Day 5	Enumerate Leaf 3	Enumerate Leaf 3

Table 2.1: *Salmonella* Multiple Inoculation Schedule.

Inoculation sites were excised from enumerated leaves via a flame sterilized 17 mm cork borer. Leaf excisions were placed in sterile culture tubes with 2 mL of PBS, then shaken for 60 min at 100 rpm. The tubes were then briefly votexed at maximum speed, and serial dilutions of the rinsate with PBS plated on TSA + 50 µg rifampicin/mL and incubated at 37°C for 24 h.

2.2.4 Pre-inoculation assay

SeN suspension was prepared as in Section 2.2.3. *Pst* suspension was prepared as in Section 2.2.3, except that the flood solution was diluted to an optical density of 0.2 at 600 nm, and additionally diluted by 1 log to achieve ~8 log CFU/mL. A 100 mL aliquot of the *Pst* suspension was autoclaved for 40 minutes to use as an inactivated *Pst* inoculum.

Four week old ‘Primo Red’ tomato plants were transported to the growth chamber from the green house three days before the start of the experiment. Four plants per treatment were treated by dipping the first and second leaves above the cotyledon in bacterial suspensions of either *SeN*, *Pst*, heat inactivated *Pst*, or sterile PBS. Three days after treatment, the plants were inoculated with *Pst* by dipping the third and fourth leaves above the cotyledon. Three days post inoculation, the third and fourth leaves were excised, then placed in sample cups and suspended in 20 mL of PBS per gram of plant tissue. Samples were shaken at 100 rpm for 1 h, then briefly vortexed at maximum speed. Serial dilutions of the rinsate with PBS were plated on PAF + 50 µg rifampicin/mL and incubated at 30°C for 48 h for enumeration.

2.2.5 cPTIO Treatment

The third leaves of Primo Red plants were sprayed by spray bottle with 0.2 mM 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) or distilled H_2O, as previously described (Liao et al., 2013).

Twelve h after chemical treatment, treated leaves were inoculated via needleless syringe on the adaxial surface with 100 µL of *Salmonella* suspension.

Three days after inoculation, bacteria were enumerated as described in Section 3.2.4.

2.2.6 3, 3'-diaminobenzidine (DAB) Stain for ROS

Bacterial suspensions of *Salmonella* Newport, *Salmonella* LT2, *Salmonella* Senftenberg, and *Pst* were prepared as above, substituting sterile H_2O for PBS.

One mg/mL 3, 3'-diaminobenzidine (DAB) solution was prepared fresh before staining and adjusted to pH 3.6 with 10 mM sodium diphosphate.

'Primo Red' tomato leaflets were treated by vacuum infiltration of bacterial suspension for 20 minutes, followed by 6 h of shaking at 100 rpm while submerged. After treatment, the leaflets were placed in sample cups with sufficient DAB solution to submerge them. The samples were again vacuum infiltrated for 20 minutes, and then covered and shaken at 100 rpm for 4 h.

Stained leaves were then dipped in 3:1:1 ethanol:acetic acid:glycerol solution heated to 95° C for 15 minutes to remove chlorophyll. Images were taken with an iPhone 5c (Apple, USA). Protocol adapted from Daudi and O'Brien (Daudi and O'Brien, 2012).

2.2.7 Statistical Analysis

Statistical analysis was performed with JMP Pro 11.1 software. Bacterial CFU counts were log transformed to correct for the positive skew of microbial population densities per unit volume, to meet normality assumptions for statistical analysis, and to generate more stable estimators due to the high variances in the arithmetic means when compared to the mean log values. Treatments were compared using Student's t-test or one-way analysis of variance (ANOVA) with Tukey's HSD *post hoc* test, as appropriate. Statistical significance was evaluated at $p < 0.05$.

2.3 Results

2.3.1 *Salmonella* influence on *Pst* survival on distal leaves

To determine whether *Salmonella* is capable of inducing SAR in tomato and protect the plant against subsequent phytopathogen encounter, *Salmonella* effectiveness as a SAR trigger was evaluated by enumerating *Pseudomonas syringae* pv. tomato on tissues distal to *Salmonella* inoculation. Recoverable *Pst* in Log CFU/mL on tomato cv. 'Primo Red' plants treated with *SeN* are shown in Figure 2.1. No significant differences in *Pst* levels compared to the control were observed in plants pre-inoculated with *SeN*, live *Pst*, or heat inactivated *Pst*.

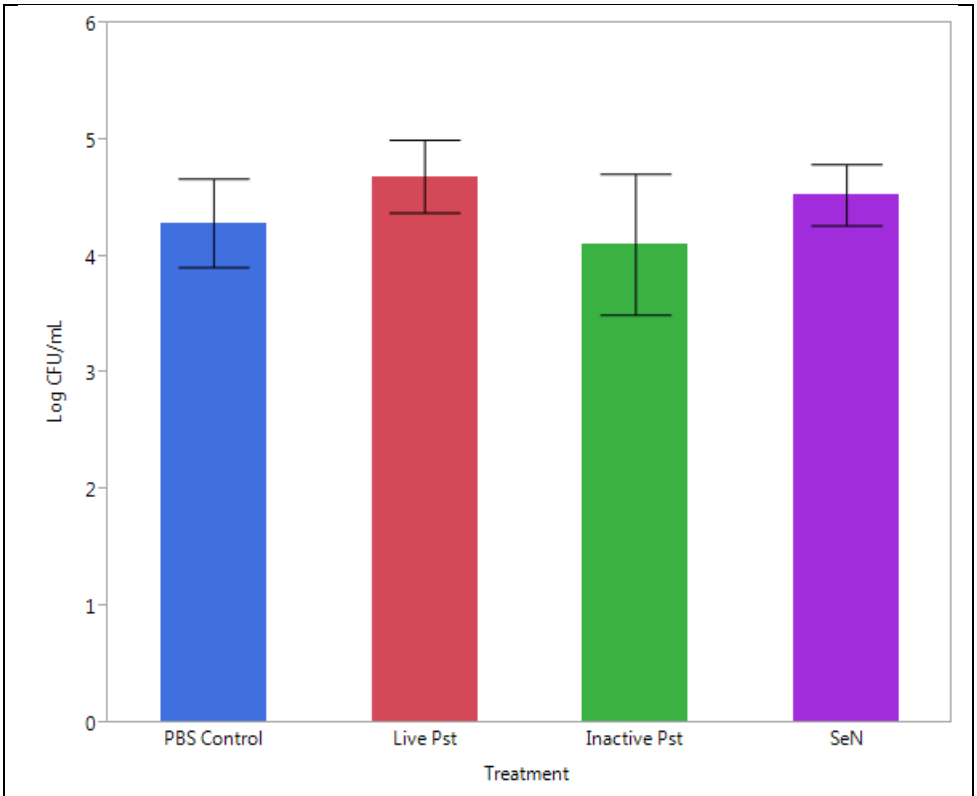


Figure 2.1 *Pseudomonas syringae* pv. tomato counts three days post inoculation on tomato cv. 'Primo Red' plants pre-treated with bacterial suspensions. No significant difference was detected between treatments by one-way ANOVA. Error bars indicate one standard error. Results are pooled from two independent experiments, each with four biological replicates per treatment.

2.3.2 *Salmonella* influence on *Salmonella* survival on distal leaves

Treatment of tomato cv. 'Primo Red' and 'Heinz' plants with *SeN* did not significantly influence *Salmonella* outcomes on distal tissues, as shown in Figure 2.2. Although *Salmonella* log reductions were slightly higher in tomatoes that were inoculated multiple times, the effects were not significant ('Heinz' p=0.184, 'Primo Red' p=0.589). Reductions were more pronounced in 'Primo Red' compared to 'Heinz' (p=0.008).

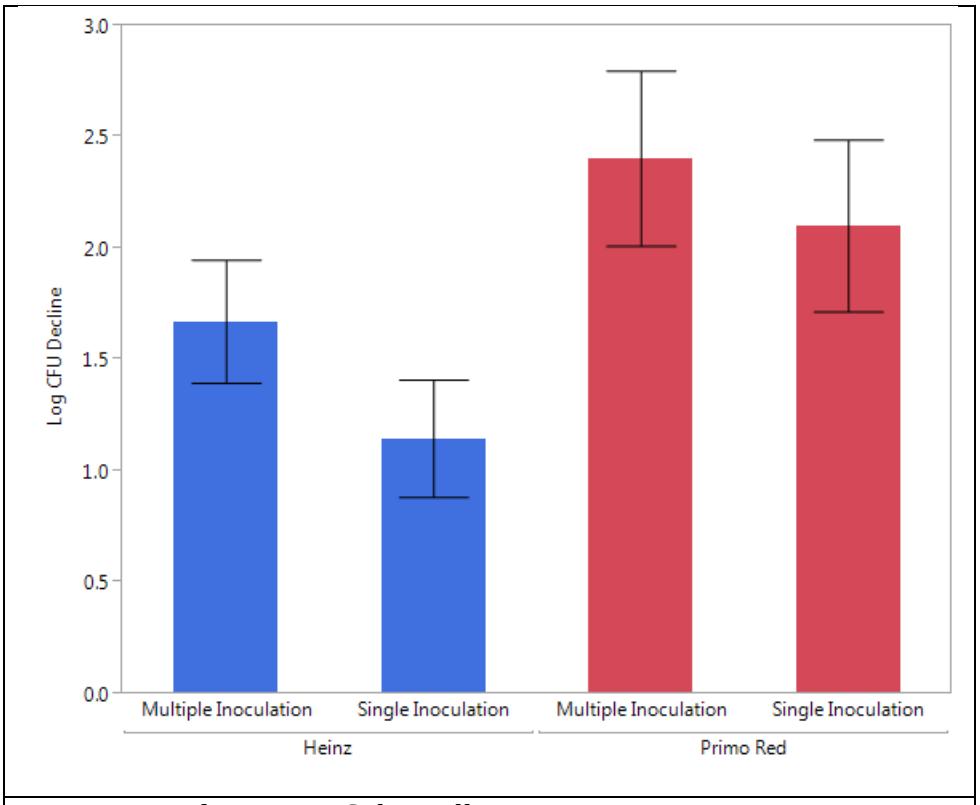


Figure 2.2 Reductions in *Salmonella* Newport counts on Tomato cv. 'Heinz' and 'Primo Red' after 24 hours. No significant differences between treatments using Student's t-test were observed. Error bars indicate one standard error. 'Heinz' results are pooled from two independent experiments with four biological replicates per treatment. 'Primo Red' results are from one experiment with four biological replicates, which was repeated with equivalent results.

2.3.3 Influence of salicylic acid on *Salmonella* survival

In order to determine the influence of the SAR hormone salicylic acid on *SeN*, an inoculation study was conducted on tomato cv. 'Moneymaker' and a 'Moneymaker' line which expressed *NahG*, a salicylate hydroxylase derived from *Pseudomonas* (Figure 2.6). *NahG* plants were able to support 0.47 log more CFU/mL than the wild type 'Moneymaker' (p = 0.041) (Figure 2.3).

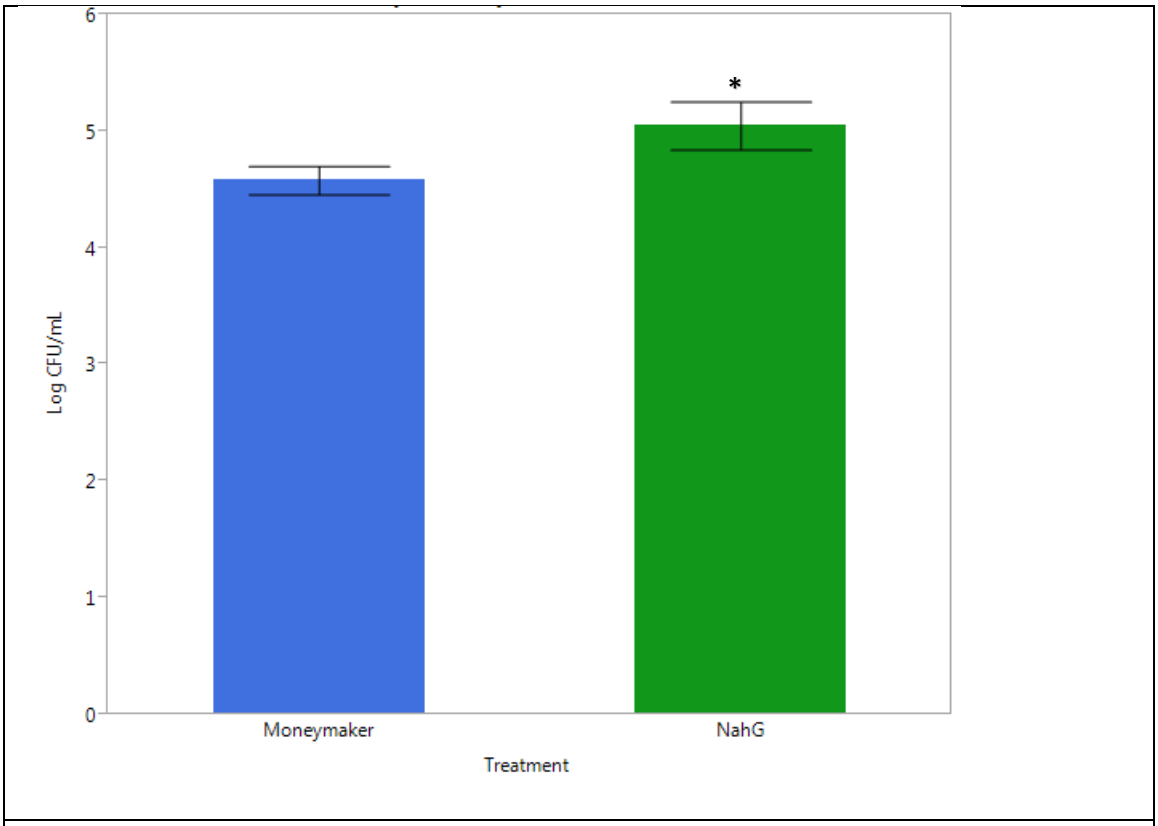


Figure 2.3 Mean *Salmonella* Newport counts three days post inoculation on tomato cv. 'Moneymaker' or a *NahG* line of 'Moneymaker'. The *NahG* plants supported higher levels of *SeN* colonization compared to the wild type (p = 0.041)

2.3.4 Influence of nitric oxide on *Salmonella* survival

Nitric oxide modulates HR, and also acts as one of the SAR signals (Ling et al., 2012, Wang et al., 2014). To investigate how these effects influence *SeN* population outcomes, the nitric oxide scavenger cPTIO was applied to reduce endogenous nitric oxide levels. *SeN* populations on cPTIO treated plants were 0.6 Log CFU/mL higher than populations on control plants, and this effect was significant ($p = 0.052$) (Figure 2.4).

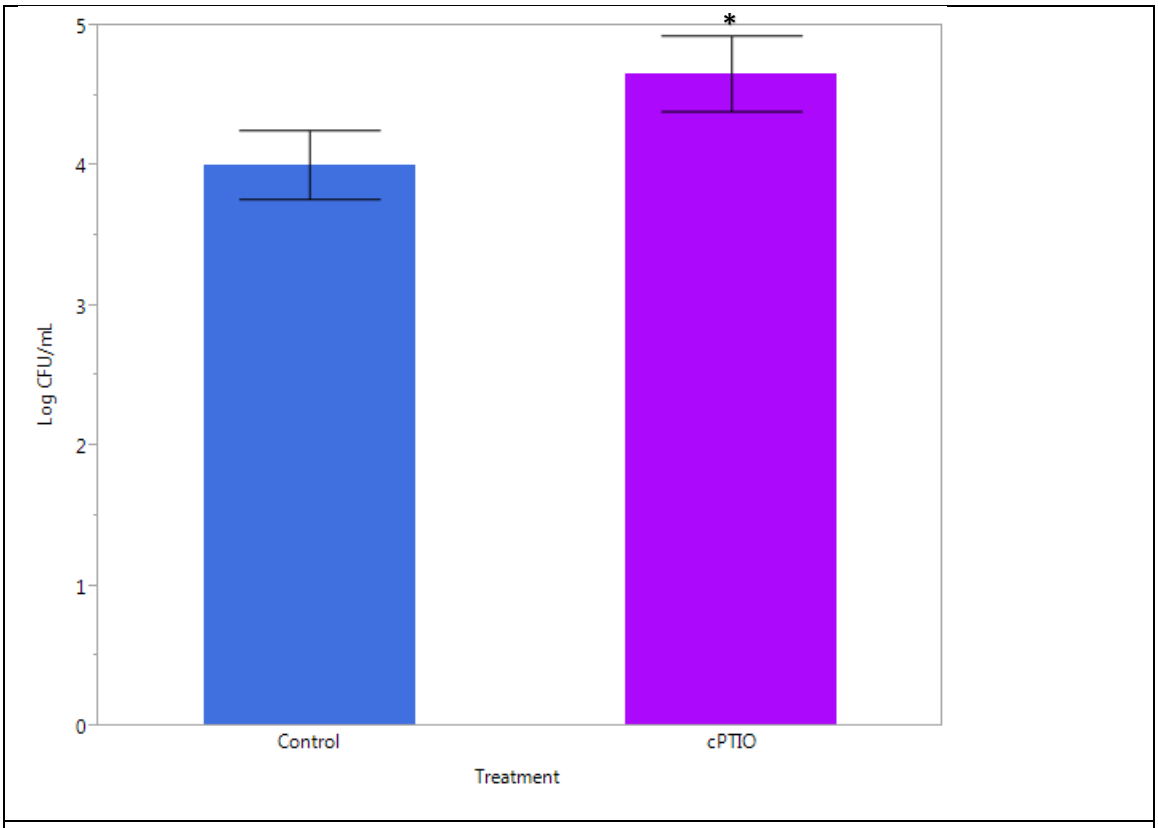


Figure 2.4 *Salmonella* Newport counts three days post inoculation on tomato cv. 'Primo Red' after spray treatment with H_2O (control) or cPTIO. The asterisk denotes a significant difference from the control ($p = 0.052$). Error bars indicate one standard error. Results are pooled from two independent experiments, each with three biological replicates per treatment.

2.3.5 *Salmonella* and reactive oxygen species

DAB staining was used to evaluate ROS production on tomato cv. 'Primo Red' leaves 6 h post inoculation with *Salmonella* LT2, *S.* Newport, *S.* Senftenberg, *Pst*, or sterile H_2O (Figure 2.5). DAB reacts with H_2O_2 in the presence of peroxidase to form a brown precipitate (Daudi and O'Brien, 2012). The experiment was conducted twice, first with four leaflets per treatment, then with five leaflets per treatment, for a combined total of nine leaflets per treatment. The degree of staining was inconsistent within treatment across all treatments. Staining was scored as "dark", "light", or "no staining". H_2O treated leaves had light staining in 2 leaflets, and no staining in 7 leaflets. *Pst* caused dark staining in 4 leaflets, light staining in 4 leaflets, and no staining on one leaflet. LT2 samples were darkly stained in 4 leaflets, lightly stained on 3 leaflets, and had no staining on 2 leaflets. *S.* Newport samples were lightly stained on 6 leaflets, and had no staining on 3 leaflets. *S.* Senftenberg samples were darkly stained on 1 leaflet, lightly stained on 7 leaflets, and not stained on 1 leaflet. Significant associations between staining and treatment were observed, Pearson chi-square score = 22.11 (8), $p = 0.0047$.

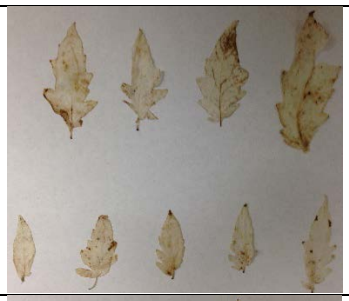
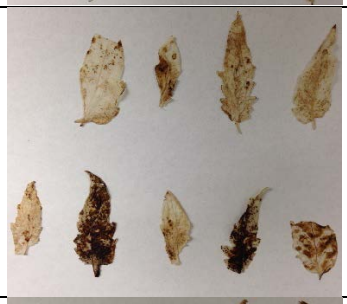
H_2O	
Pseudomonas syringae	
Salmonella LT2	
Salmonella Newport	
Salmonella Senftenberg	

Figure 2.5 DAB staining of tomato cv. 'Primo Red' leaflets 6 hpi. Results of two experiments, split by row (n = 4, n = 5).

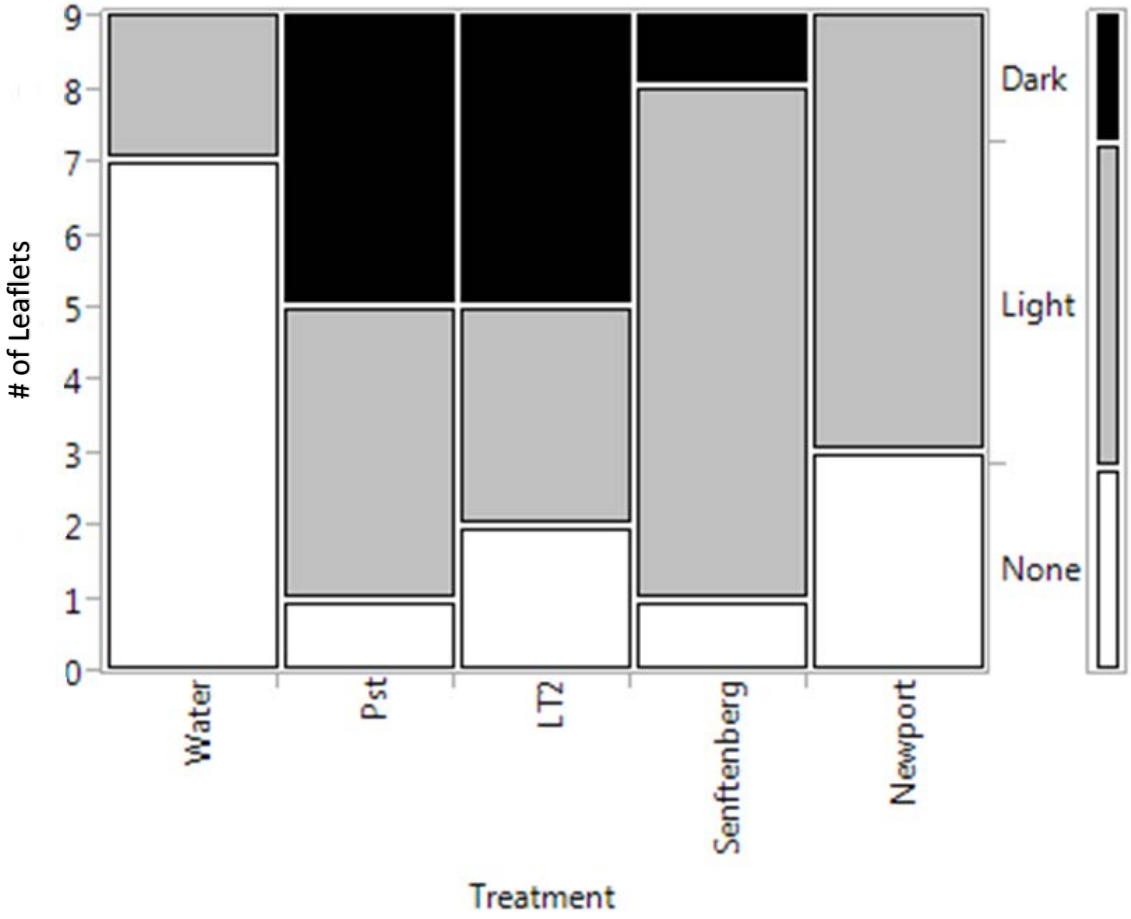


Figure 2.6 Mosaic plot of DAB stain scoring of tomato cv. 'Primo Red' leaflets. Pooled results of two experiments (n = 4, n = 5)

2.4 Discussion

This study attempted to determine whether tomato colonized by *Salmonella* could initiate SAR, hence augmenting defenses against subsequent *Salmonella*, or other phytopathogen encounters. Although there is a great deal of evidence which suggests that *Salmonella* triggers PTI, we were unable to observe significant reductions in *Pst* or *SeN* population levels in distal tissue after challenge with *SeN*.

Tomato *NahG* plants showed an increase in *SeN* populations of 0.47 log CFU/mL when compared to the wild type. Schikora et al. observed a modest increase of less than 1 Log CFU/mg plant tissue in *Arabdopsis NahG* plants when compared to the wild-type (Schikora et al., 2008). However, they also observed significantly higher increases on mutants which were compromised in their ethylene or jasmonic acid signaling pathways (Schikora et al., 2008). This combination of results suggests that SA accumulation plays a small role in *Salmonella* outcomes, and that the contribution of ethylene or jasmonic acid signaling to tomato-*Salmonella* interactions should be investigated further.

Han & Micallef observed up-regulation of genes in the nitrosative resistance operon in *Salmonella* inhabiting tomato leaves and roots (Han & Micallef, unpublished). cPTIO treatment, which should scavenge endogenous NO, resulted in higher populations of *SeN* when compared to the mock treatment, suggesting that NO production, or its downstream impacts on ROS production and SAR signaling capability, plays a role in the plant's response to *Salmonella* in the tomato phyllosphere.

Although no necrotic lesions were observed on plants inoculated with *Salmonella*, this pathogen triggered sporadic ROS production at the inoculation sites, although to a lesser degree than the phytopathogen *Pst.* This was observed for all three serotypes investigated, but ROS triggering varied between serovars. *S.* Newport appeared to trigger the weakest ROS response, followed by *S.* Senftenberg, then *S.* LT2. The *S.* Newport strain used in this study was the causal agent of two multi-state outbreaks associated with tomatoes in 2002 and 2005 (Greene et al., 2008). *S.* Senftenberg is the most commonly isolated serotype from turkeys and chickens, but has only been the cause of non-poultry associated outbreaks (Jackson et al., 2013). *S.* LT2 is an attenuated strain of *S.* Typhimurium with a mutation in the stress response gene *RpoS* (Jackson et al., 2013). The low ROS production following *S.* Newport inoculation, coupled with the strain's close outbreak association with tomatoes, may indicate adaptation by the strain to evade PTI. Although outbreaks associated with *S.* Senftenberg have all been with plant-commodities, the serovar is associated with overall fewer outbreaks (Jackson et al., 2013). Furthermore, there is evidence that the O-antigen of *S.* Senftenberg also acts as a PAMP which is recognized by *Arabidopsis* (Berger et al., 2011). *S.* Typhimurium is the second most common outbreak-associated serovar, and was the cause of 14% of all *Salmonella* outbreaks between 1998 and 2008 (Jackson et al., 2013). These outbreaks were associated with both plant and animal commodities (Jackson et al., 2013). However, although *S.* Typhimurium has been associated with plant-source salmonellosis outbreaks, it shows poor fitness under crop field conditions, which suggests that contamination events occur post-harvest (Zheng et al., 2013). Although *S.* LT2 is an attenuated strain of

S. Typhimurium, the strong ROS induced by *S.* LT2 suggests that *S.* Typhimurium might be unable to attenuate the tomato PTI.

It appears that tomato plants do recognize *Salmonella*, and that evasion of this recognition may be important to persistent *Salmonella* populations in the phyllosphere. Disabling basal plant defense signals, such as NO and SA signaling, increased *Salmonella* populations. However, inoculation with plant-adapted *S.* Newport does not appear sufficient to trigger SAR that was sufficient to impede *Salmonella* or *Pst* growth on distal tissues. It would be fruitful to examine whether or not *Salmonella* serovars which are not plant-adapted were capable of triggering PTI and subsequent SAR, and further whether their populations are negatively influenced by PTI and SAR.

Chapter 3: Influence of SAR activation on *Salmonella* colonization of tomato

3.1 Introduction

Chemical activators of systemic acquired resistance (SAR) are compounds that protect plants from pathogens not through direct antimicrobial action, but instead by priming the natural defenses plants have against pathogens (Tripathi, 2010). This mode of protection is theorized to be more durable than systemic fungicides and antimicrobials as it depends on an array of plant-initiated protective activities, rather than targeting a specific, narrow aspect of pathogen biology (Tripathi, 2010). Commercial use has been hampered by the inefficiency of these compounds when compared with fungicides and other antimicrobials, however they have also inspired research into more efficacious versions of these agents (Morton and Staub, 2008).

Natural plant defenses against pathogens take a variety of forms, and include release of antimicrobial compounds, stomatal closure to restrict access to apoplastic spaces, and denial of nutrition from plant tissues via hypersensitive cell death, among other responses (Melotto et al., 2008, Nicaise et al., 2009). This broad array of anti-pathogen responses may be able to be employed to restrict the colonization fitness of other, non-pathogenic plant-colonizing microbes. There are currently conflicting reports on the influence of SAR on plant-associated bacterial communities. Hein et. al reported a difference in rhizosphere microbe community structure associated with SAR constitutive

and SAR non-inducible *Arabidopsis* mutants, although they did not detect a loss of diversity in SAR constitutive mutants (Hein et al., 2008). However, Doornbos et. al examined rhizosphere bacterial communities in *Arabidopsis* plants treated with salicylic acid and benzothiadiazole, and were unable to detect any change in community density or structure (Doornbos et al., 2010).

Human pathogens on plants (HPOPs), such as *Salmonella enterica* and *E. coli* O157:H7, have been responsible for an increasing number of foodborne illness outbreaks associated with fresh produce (Anderson et al., 2011). If plant pathogen defenses can influence the colonization outcomes of non-phytopathogens, then chemical SAR activators may present another strategy for improving the safety of fresh produce crops.

Chemical activators of SAR have not been evaluated for their efficacy against human pathogens on plants. To explore whether chemical SAR activators can be employed to reduce food safety risk of fresh produce against the enteric pathogen *Salmonella enterica* subsp. *enterica*, we set out to determine whether *Salmonella* influences gene transcription in distal tissues consistent with the SAR response, and how this distal tissue gene expression is influenced by chemical activators of SAR. Further, the fate of *Salmonella* populations on plants treated with chemical activators of SAR, in a manner consistent with the influence of SAR activators on phytopathogens such as *Pst*, was assessed.

For this study, three chemical activators of SAR were used: acibenzolar-*s*-methyl (ASM), hexanoic acid (HA), and *B*-aminobutyric acid (BABA). ASM is a functional SA analogue, and is suggested to function after conversion to acibenzolar *in planta* by salicylic acid binding protein 2 (SABP2) (Tripathi et al., 2010). HA inhibits jasmonyl-

isoleucine synthesis, which acts antagonistically with genes which respond to the SAR hormone salicylic acid (Scalschi et al., 2013). It further inhibits the action of the phytopathogen emitted phytotoxin coronatine, which is a jasmonyl-isoleucine mimic (Scalschi et al., 2013). BABA up-regulates constitutive expression of pathogenesis related (PR) genes in Solanaceae plants. The PR gene family is closely related to SAR functionality, and some members encode for anti-microbial compounds such as chitinases and glucanases.

3.2 Materials and Methods

3.2.1 Plant Material

The following tomato (*S. lycopersicum*) cultivars were used in this study: 'Heinz', 'Primo Red', and 'Nyagous'. 'Heinz' and 'Primo Red' seeds were purchased from Harris Seeds (Rochester, NY). 'Nyagous' seed was obtained from the Tomato Genetics Resource Center, University of California Davis, CA.

Seeds were started in 5 x 10 cell trays with potting mix, then transplanted to 6" x 6" pots with potting mix after the first true leaves emerged. Plants were grown for 4 weeks in greenhouse conditions (16L; 8D, 28°C/18°C, relative humidity 75%), then covered with black plastic and transferred to a growth chamber (16L; 8D, 28°C/18°C, relative humidity 80%) and allowed to acclimate for 3 days before conducting experiments.

3.2.2 Bacterial strains

Salmonella enterica subsp. *enterica* serovar Newport, a tomato outbreak strain (Greene et al., 2008) (adapted for rifampicin resistance) was grown in trypticase soy agar (TSA) with 50 µg rifampicin/mL, and incubated at 37°C for 18 h.

Pseudomonas syringae pv. tomato DC3000 (*Pst*) was kindly provided by Dr. Shunyuan Xiao. It was grown in Pseudomonas Agar F (PAF) with 50 µg rifampicin/mL, and incubated at 30°C for 48 h.

3.2.3 Bacterial inoculum preparation

Salmonella Newport (*SeN)* suspensions were prepared by flooding plates with phosphate buffered saline (PBS), then lightly shaking for 30 seconds. The suspension was then poured off the plate, and diluted with additional PBS to achieve an optical density of 0.5 at 600 nm, which corresponds to 8 log CFU/mL. *Pst* suspensions were prepared in the manner, except that the flood solution was diluted to an optical density of 0.2 at 600 nm, and additionally diluted by 10x to achieve 8 log CFU/mL.

3.2.4 Bacterial Retrieval

Inoculated leaves were excised from the plant and placed in sample cups and suspended with 20 mL of PBS per gram of plant tissue. Samples were shaken at 100 rpm for 1 h, then briefly vortexed at maximum speed. Serial dilutions of the *Pst* inoculated plant rinsate were plated on PAF + 50 µg rifampicin/mL and incubated at 30°C for 48 h for enumeration. *Sen* inoculated plant rinsate dilutions were plated on TSA + 50 µg rifampicin/mL and incubated at 37°C for 24 h for enumeration.

3.2.5 Acibenzolar-*s*-methyl treatment

The first and second leaves above the cotyledon of four week old tomato cv. 'Primo Red', 'Heinz', and 'Nyagous' were dipped in 100 µg ASM (Sigma-Alrdrich) per mL H_2O or in de-ionized H_2O (Ishii et al., 1999). Three days after treatement, the third and fourth leaves were inoculated by dipping in bacterial suspensions of *SeN* or *Pst*, or in sterile PBS.

Three days after inoculation, bacteria were enumerated as described in Section 3.2.4.

3.2.6 Hexanoic acid treatment

Four week old tomato cv. 'Primo Red', 'Heinz', and 'Nyagous' were irrigated with 0.6 mM hexanoic acid (Sigma-Alrdrich) or distilled water until the soil was saturated, as previously described (Scalschi et al., 2013). Three days after treatement, the third and fourth leaves inoculated by dipping in bacterial suspensions of *SeN*, *Pst*, or in sterile PBS.

Three days after inoculation, bacteria were enumerated as described in Section 3.2.4.

3.2.7 *B*-aminobutyric acid treatment

Four week old tomato cv. 'Primo Red' plants were irrigated with 2.5 mM BABA (Sigma-Alrdrich) (Bengtsson et al., 2014) or distilled water, until the soil was saturated. Two days after treatment, the third and fourth leaves were inoculated by dipping in bacterial suspensions of *SeN*, *Pst*, or in sterile PBS.

Three days after inoculation, bacteria were enumerated as described in Section 3.2.4.

3.2.8 Analysis of gene expression by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

Gene expression levels of SAR markers were used to assess whether *Salmonella* triggers SAR with or without SAR chemical activators. The genes used were PR1 (*Pathogenesis Related 1*), PR5 (*Pathogenesis Related 5*), SAMT (*salicylic acid carboxyl methyltransferase*), and UBI (*ubiquitin*) as the reference gene. Leaf tissue was collected from all experiments from the next most apical leaf from the inoculated leaf, flash frozen in liquid nitrogen, and stored at -80°C.

Tomato leaves above and adjacent inoculated leaves were collected at the time of harvesting for bacterial enumration, and flash frozen in liquid nitrogen prior to collecting the inoculated leaves for bacterial enumeration. Thus, the leaves collected from plants that did not receive chemical SAR activators were collected 24 hpi, whereas the leaves collected from the plants that did receive chemical SAR activators were collected 72 hpi. The frozen leaves were finely ground with disposable Kontes™ pestles (Kimble-Chase), the RNA was extracted with the E. Z. N. A. Plant RNA Kit (Omega Bio-Tek), then stored at -80°C. cDNA was synthesized using the qScript cDNA Supermix kit (Quanta Biosceinces) and the Verso cDNA kit (Thermo Scientific) from 40 ng RNA/µL stock. qPCR was performed with the StepOne device (Applied Biosystems) using PerfeCta™ SYBR® Green SuperMix with ROX™ (Quanta Biosciences). The primers used to target the genes of interest and the endogenous control gene were all previously described, as shown in Table 3.2. The qPCR reaction mix and conditions are described in Table 3.3. Triplicate analyses was performed on all reactions using cDNA samples from two independent experiments. To assess differential gene expression, a comparitive cycle

theshold (cT) method was used (Schmittgen and Livak, 2008). The expression detected from tomato *ubiquitin* (*UBI)* genes was used as an endogenous control. Relative quantities (RQs) or fold changes in gene expression were calculated as:

$$\Delta Ct = Ct_{\text{gene of interest}} - Ct_{UBI}$$

$$\Delta\Delta Ct = \Delta Ct_{\text{reference sample}} - \Delta Ct_{\text{treated sample}}$$

$$RQ = 2^{(\Delta\Delta Ct)}$$

ΔCt values in samples where the gene of interest failed to amplify were evaluated as if the $Ct_{\text{gene of interest}} = 35$, the maximum reliable threshold value.

Gene Name	Primer	Reference
UBI	F: 5'-TCGTAAGGAGTGCCCTAATGCTGA-3' R: 5'-CAATCGCCTCCAGCCTTGTTGTAA-3'	(Scalschi et al., 2013)
PR1	F: 5'-CCGTGCAATTGTGGGTGTC-3' R: 5'-GAGTTGCGCCAGACTACTTGAGT-3'	(Scalschi et al., 2013)
PR5	F: 5'-AATTGCAATTTTAATGGTGC-3' R: 5'-TAGCAGACCGTTTAAGATGC-3'	(Zhao et al., 2013)
SAMT	F: 5'-TCAATATACACCATCACAAGGAGAAG-3' R: 5'-GCTCTCATGCACTTTGACACATTG-3'	(Scalschi et al., 2013)

Table 3.2 qRT-PCR Primers

qRT-PCR Reaction Mix			
Reagent	**Stock Concentration**	**Reaction Mix Concentration**	**Reagent Volume (µL)**
PerfeCta™ SYBR® Green SuperMix with ROX™	2x	1x	7.50
Forward Primer	10 µM	0.5 µM	0.75
Reverse Primer	10 µM	0.5 µM	0.75
Sample cDNA	20 ng RNA/µL	2.0 ng/µL	1.50
Water	N/A	N/A	4.50
Total Volume			**15.00**
qRT-PCR Run Method			
	# of Cycles	**Time (mm:ss)**	**Temperature**
Initial Denaturation	1	3:00	95°C
PCR Cycling	40	0:15	95°C

		0:45	60°C
Melt Curve	1	0:15	95°C
	1	1:00	60°C
	117	0:15	+0.3°C

Table 3.3 qRT-PCR conditions

3.2.9 Statistical Analysis

Statistical analysis was performed with JMP Pro 11.1 software.

Bacterial CFU counts were log transformed to correct for the positive skew of microbial population densities per unit volume, to meet normality assumptions for statistical analysis, and to generate more stable estimators due to the high variances in the arithmetic means when compared to the mean log values. Treatments were compared using Student's t-test or one-way analysis of variance as appropriate. Statistical significance was evaluated at $p < 0.05$.

Gene expression statistics were evaluated using ΔΔCt rather than RQ in order to meet normality assumptions for statistical analysis and generate more robust estimators due to high variances in arithmetic means of the RQ when compared to the ΔΔCt. Treatments were compared using one-way analysis of variance, and Tukey's HSD *post hoc* test. Statistical significance was evaluated at $p < 0.5$.

3.3 Results

3.3.1 Chemical SAR activators and *Pst* survival

Before evaluating the efficacy of SAR activators on *SeN*, it was necessary to ensure the activators were capable of priming a SAR response to phytopathogen challenge. Each of the chemical activators of SAR had a significant effect on recoverable *Pst* (Figure 3.1). Treatment with HA resulted in a reduction of 0.49 Log CFU/mL *Pst* ($p = 0.036$) relative to plants treated with a distilled water control. Treatment with BABA resulted in a reduction of 0.98 Log CFU/mL *Pst* ($p = 0.027$) compared to the control. ASM treatment had the greatest impact on *Pst* populations, with recoverable *Pst* below the limit of quantification ($p < 0.0001$).

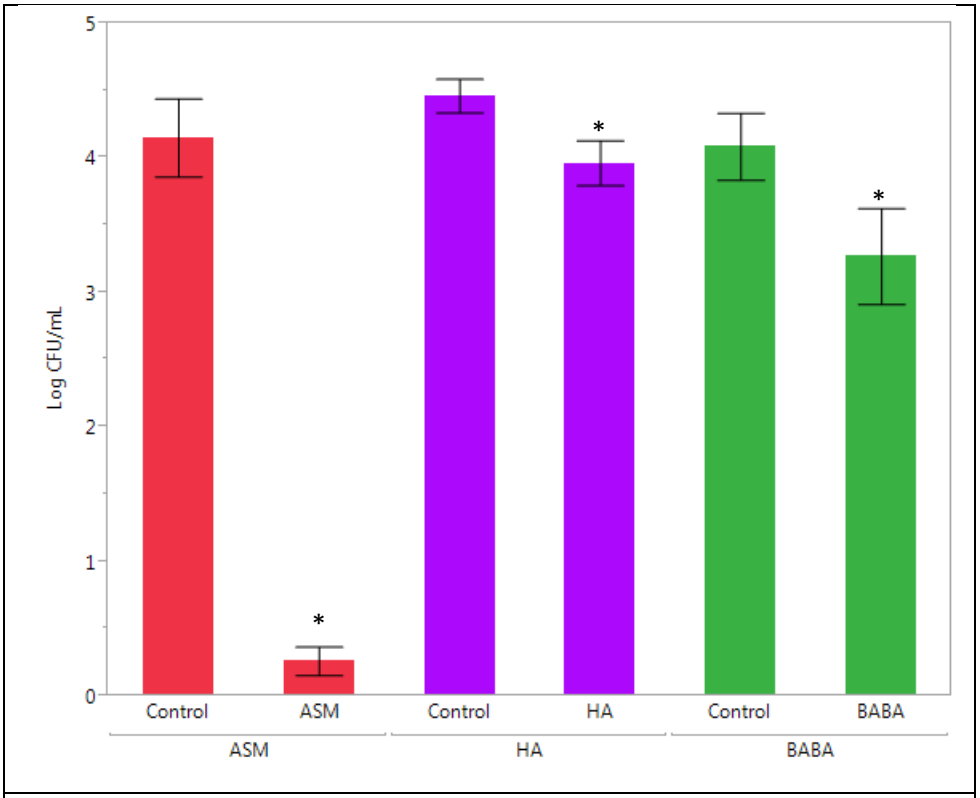


Figure 3.1 *Pseudomonas syringae* pv. tomato counts three days post inoculation on tomato cv.'Primo Red' plants treated with chemical SAR activators. Each treatment was significantly different from its associated control when compared with Student's t-test, $p < 0.05$.

3.3.2 Chemical SAR activators and *Salmonella* survival

Mean *SeN* survival in Log CFU/mL on tomato cv. 'Primo Red' plants treated with the three tested chemical SAR activators is shown in Figure 3.2. None of the chemical activators of SAR had any statistically significant impact on *SeN* populations on 'Primo Red'.

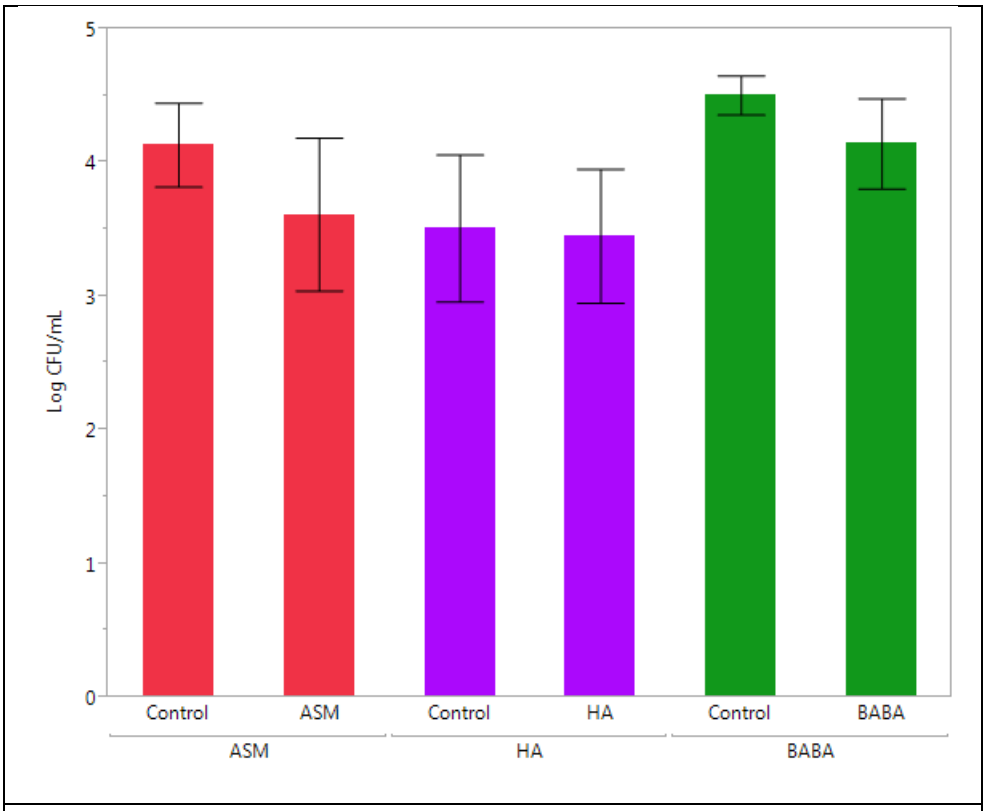


Figure 3.2 *Salmonella* Newport counts three days post inoculation on Tomato cv. 'Primo Red' plants treated with chemical SAR activators. No significant difference between treatments using Student's t-test. Error bars indicate one standard error.

To determine whether other cultivars might exhibit a different response to SAR activation, two other tomato cultivars, 'Heinz' and 'Nyagous', we also evaluated. In a previous study assessing 13 tomato cultivars for fruit susceptibility to *Salmonella* Newport colonization, cv. 'Heinz' was the least colonized, while 'Nyagous' harbored the

highest *Salmonella* populations (Han and Micallef, 2014). As observed with 'Primo Red', no statistically significant reductions in *SeN* populations were observed, relative to activator-untreated plants (Figure 3.3).

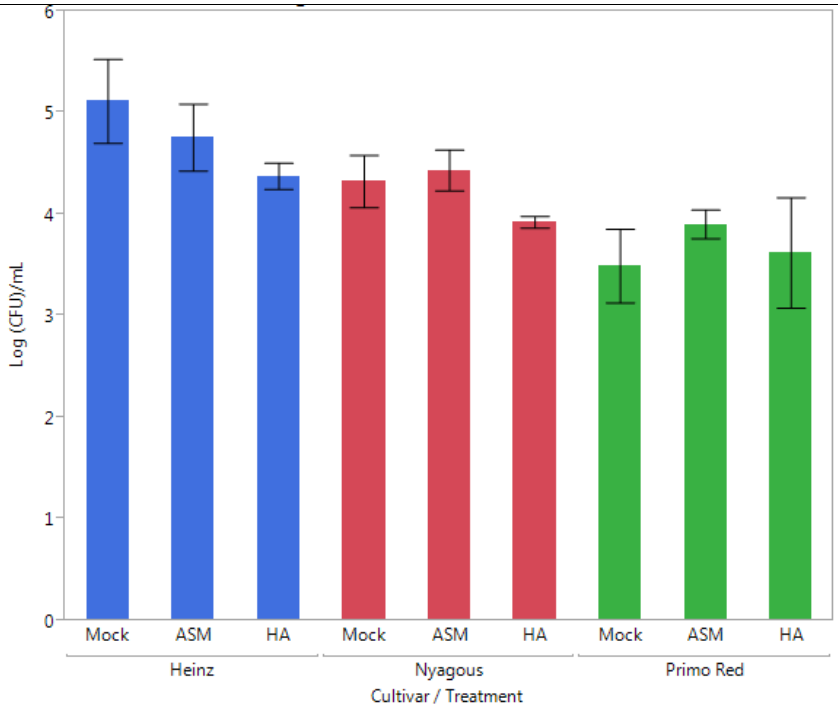


Figure 3.3 *Salmonella* Newport counts three days post inoculation on three tomato cultivars after treatment with chemical SAR activators. 'Primo Red' mock treatment bar representative of independent ASM and HA experiments. Error bars indicate one standard error.

3.3.3 SAR-related Gene Expression on Distal Tissues

To further examine the possibility to *SeN*-derived SAR, the expression levels of PR1, PR5, and SAMT in 'Primo Red' tissues distal from the inoculation sites were determined.

PR1 is routinely used as molecular marker for SAR, and is highly dependent on NPR1 functionality for expression (Scalschi et al., 2013, Shah and Zeier, 2013). However, weak PR1 activation may occur from other, non-SAR related signals, such as those associated with hypersensitive response (Zhang and Shapiro, 2002). In the absence of chemical activation, there was no significant difference between plants inoculated with *Pst*, *SeN*, or sterile PBS (Table 3.2). On the other hand, chemical activation by all three activators resulted in significant changes in PR1 expression in distal tissues when challenged by *Pst* or *SeN*. PR1 expression in ASM treated plants increased by 50-fold when the plants were inoculated with *Pst*, and by 9-fold in plants inoculated with *SeN* ($p < 0.05$). In HA and BABA treated plants, however, *SeN* inoculation generated the stronger distal tissue response, with a 300-fold and 2-fold increase in relative expression to the unchallenged plants, respectively. Interestingly, *Pst* down-regulated distal PR1 expression in BABA treated plants by 2-fold when compared to plants which had received no inoculum (Table 3.3).

		PR1			
Treatment	**Inoculum**	**RQ**	**RQ Min**	**RQ Max**	
Mock	Mock	1.00	0.73	1.37	a
	Pst	1.44	1.06	1.95	a
	SeN	1.50	1.15	1.95	a
ASM	Mock	1.00	0.82	1.23	a
	Pst	51.53	37.95	69.98	c
	SeN	9.27	5.00	17.20	b
HA	Mock	1.00	0.73	1.37	a
	Pst	46.90	33.74	65.20	b
	SeN	301.25	238.89	379.90	c
BABA	Mock	1.00	0.89	1.12	b
	Pst	0.58	0.50	0.67	a
	SeN	2.15	1.84	2.52	c

Table 3.3. PR1 expression relative to the mock inoculum for each chemical treatment. Rows with the same letter within each treatment are not significantly different from each other when comparing ΔΔCt by one-way ANOVA with Tukey's HSD *post hoc* test ($p < 0.05$).

PR5 is expressed independently of NPR1 in an SA-dependent fashion, and is also activated by cell-death related signals (Zhang and Shapiro, 2002). In this experiment, PR5 expression was below the limit of quantification in the mock control sample as well as the mock inoculated plants treated with HA (Table 3.4). For the purposes of relative quantification, ΔCt was calculated as if the cT of the missing PR5 amplification cycle threshold was 35. In plants which had not been chemically treated with SAR activators, inoculation with *SeN* or *Pst* was sufficient to trigger strong PR5 expression to quantifiable levels, 70-fold and 17-fold relative to the imputed control, respectively ($p < 0.05$). PR5 expression was detectable in ASM and BABA treated plants, but there was no significant difference observed between inoculated and un-inoculated plants, regardless of the inoculum. Expression of PR5 in HA treated, mock inoculated plants was so low as to be indistinguishable from expression levels at the limit of quantification. Bacterial inoculation did not significantly induce PR5 expression.

		PR5			
Treatment	**Inoculum**	**RQ**	**RQ Min**	**RQ Max**	
Mock	Mock*	1.00	0.46	2.19	a
	Pst	69.57	32.80	147.54	b
	SeN	16.89	4.49	63.50	b
ASM	Mock	1.00	0.45	2.23	a
	Pst	2.40	0.88	6.56	a
	SeN	0.68	0.44	1.05	a
HA	Mock*	1.00	0.70	1.42	a
	Pst	2.19	1.32	3.61	a
	SeN	1.29	0.92	1.80	a

	Mock	1.00	0.56	1.80	a
BABA	Pst	0.80	0.60	1.06	a
	SeN	1.05	0.51	2.16	a

Table 3.4 PR5 expression relative to the mock inoculum for each chemical treatment. Rows marked with an asterisk failed to amplify PR5, and ΔcT was calculated with $Ct_{gene\ of\ interest}$ = 35. Rows with the same letter within each treatment are not significantly different from each other when comparing ΔΔCt by one-way ANOVA with Tukey's HSD *post hoc* test.

SAMT encodes for salicylic acid carboxyl methyltransferase, which acts downstream of SA synthesis to convert SA into methyl salicylate, a mobile signal for SAR that is dispensable in light, but required for dark activation of SAR (Park et al., 2007, Liu et al., 2011).

SAMT expression was greatly increased in distal tissues following *Pst* and *SeN* inoculation, and significantly different compared to the mock, with a 51-fold and 7-fold up-regulation, respectively (Table 3.5). In plants that had been treated with ASM, inoculation with *Pst* or *SeN* resulted in decreased expression of SAMT when compared to the uninoculated, ASM treated plants ($p < 0.05$). There was no change in distal tissue expression of SAMT in HA treated plants inoculated with *Pst*; however, inoculation with *SeN* resulted in a 14-fold increased SAMT expression. No difference in gene expression due to bacterial inoculation was observed in BABA treated plants.

		SAMT			
Treatment	**Inoculum**	**RQ**	**RQ Min**	**RQ Max**	
	Mock	1	0.4323	2.313	a
Mock	Pst	51.2	20.985	125.08	c
	SeN	6.93	3.8757	12.386	b
	Mock	1.00	0.85	1.17	c
ASM	Pst	0.04	0.03	0.05	a
	SeN	0.11	0.05	0.22	b
	Mock	1.00	0.70	1.42	a
Hex	Pst	1.24	0.45	3.47	a
	SeN	14.22	10.99	18.40	b
BABA	Mock	1.00	0.88	1.14	a

	Pst	1.78	0.49	6.51	a
	SeN	0.70	0.45	1.08	a

Table 3.5 SAMT expression relative to the mock inoculum for each treatment. Rows with the same letter within each treatment are not significantly different from each other when comparing ΔΔCt by one-way ANOVA with Tukey's HSD *post hoc* test.

Gene expression was also measured in distal tissues of plants which had been treated with cPTIO and challenged with *SeN* (Table 3.6). Treatment with cPTIO suppressed the expression of PR1 and SAMT in distal leaves of *SeN* inoculated plants, but resulted in increased expression of PR5.

Treatment	Gene	RQ	RQ min	RQ Max	
Mock	PR1	1.00	0.81	1.24	a
cPTIO	PR1	0.29	0.27	0.32	b
Mock	PR5	1.00	0.67	1.49	a
cPTIO	PR5	4.36	3.63	5.24	b
Mock	SAMT	1.00	0.62	1.60	a
cPTIO	SAMT	0.20	0.12	0.31	b

Table 3.6 Gene expression of tomato cv. 'Primo Red' treated with cPTIO as a relative quantity to plants treated with a mock treatment (H_2O). Statistics performed on raw ΔΔCt values. Statistics performed on raw ΔΔCt values. Difference letters denote expression levels significantly different from each other within each gene when compared by Student's t-test.

3.4 Discussion

Although each of the SAR activators employed in this study had significant impact on the microbial populations of *Pst*, SAR activation in tomato has no impact on *SeN* populations on tomato leaves. The mechanisms that tomato employs during SAR act to inhibit internalization of pathogens, and also act to neutralize internalized pathogens through the use of antimicrobial compounds (Durrant and Dong, 2004). It is unlikely that

Salmonella is internalized in the phyllosphere, and instead appears to preferentially colonize the trichomes on the leaf surface (Barak et al., 2011). Thus, application of SAR activators is unlikely to provide any food safety benefit.

Salmonella alone did not trigger PR1 expression in distal tissue on tomato at three days post inoculation. Schikora et al. observed increases in PR1 expression after *Salmonella* Typhimurium inoculation of *Arabidopsis*; however, in these experiments the whole plant was inoculated, and the increase in expression was only observable in the wild type within 6 h of inoculation (Schikora et al., 2008). In ethylene insensitive mutants of *Arabidopsis*, PR1 expression occurred over the 24 h period of the experiment, with maximum expression occurring at the last measured time point, 24 h (Schikora et al., 2008). It may be that *SeN* triggered PR1 expression is diminished over time by an ethylene-related signaling pathway.

In this study no change in PR1 expression was observed after *Pst* inoculation, however Scalschi et al. observed a 3-fold change in PR1 expression 72 h post-*Pst* inoculation in tomato cv. 'Ailsa Craig', and Song et al. report a 5 Log-fold change in PR1 expression 72 h post-inoculation with *Alternaria solani* (Song et al., 2011, Scalschi et al., 2013). PR1 expression change in tomato after *Pst* inoculation appears to be a relatively small effect compared with other plant pathogens, and an expression change might have been observable in our experimental system with additional biological replicates. It may also be that the effectors released by the virulent *Pst* strain used in this study were sufficient to suppress PR1 up-regulation in distal tissues.

Although PR1 expression is strongly regulated by NPR1 in an SA-dependent manner, there exists an alternative SA-independent pathway which up-regulates PR1

expression via HR-associated signals (Zhang and Shapiro, 2002). Although this alternative pathway can be used to trigger SAR in *npr1* mutants of *Arabidopsis*, it is also capable of up-regulating PR1 in a SAR-independent manner (Zhang and Shapiro, 2002).

SeN up-regulated PR5 expression in distal tissues. Expression of PR5 has also been observed in lettuce inoculated with *Salmonella* Dublin, although not necessarily on distal tissue (Klerks et al., 2007). PR5 regulation appears to be controlled by the same SA-independent pathways that regulate PR1 expression, as well as additional SA-independent pathways which do not influence PR1 expression (Zhang and Shapiro, 2002). The *SeN* up-regulation of PR5 in distal tissues independent of PR1 up-regulation may be due to activation of these alternative related pathways.

SAR's protective action is a function of response priming; that is to say, in plants undergoing SAR the primary difference in defense response is the rapidity by which plant cells are able to mount their defenses after detecting a pathogen (Tripathi, 2010). One possibility for SAR's inability to control *SeN* populations may be that plants simply do not detect *SeN*, or interpret its presence in a pathogenic manner. However, the distal gene expression observed seems to contradict this theory.

Treatment with *SeN* or *Pst* both significantly up-regulated SAMT expression in distal tissues, which is a downstream indicator of SA synthesis. *SeN* impact on distal SAMT expression was greatly magnified in plants that had been treated with HA. Although in this study HA had no impact on SAMT expression 72 hours after challenge with *Pst*, Scalschi et. al observed a distinct increase in SAMT expression in HA-treated tomato plants 48 hours after *Pst* challenge when compared to plants treated with just HA, just *Pst*, or an un-treated control (Scalschi et al., 2013). *SeN* and *Pst* also both acted to

increase PR5 expression in the absence of chemical activation, as well as SAR marker gene PR1 expression in plants treated with HA and ASM. These parallel trends in distal gene expression suggest that plants do in fact detect the presence of *SeN* during SAR activation and thus mount a defensive response. However, as stated earlier, the defense responses mounted by SAR are likely to be ineffective at controlling *SeN* populations. *SeN* colonizes the trichomes on the leaf surface, and is thus not hindered by anti-internalization defenses (stomatal closure, callose deposition) nor internally disseminated antimicrobial compounds.

Both *Pst* and *SeN* initiated down-regulation of SAMT in the presence of ASM. It has been proposed that ASM activation is due to its conversion to acibenzolar by the de-methylating enzyme SABP2 (Tripathi, 2010). In SAR events without chemical activators, SAMT acts to convert SA into the mobile signal MeSA by methylation, and SABP2 acts to convert MeSA back into SA in distal tissues (Park et al., 2007). As both enzymes act to methylate and demethylate SA, and SABP2 acts to demethylate ASM, it may be possible for SAMT to methylate acibenzolar back into ASM. If acibenzolar is the active SAR promoting state of ASM, then plants may reduce their SAMT activity during pathogen challenge to preserve acibenzolar levels in their tissues, or because levels of SA in distal tissues are elevated. Additionally, SAR signals such as the glycerol-3-phosphate derived factor act to down-regulate SAMT synthesis in distal tissues to preserve SA levels, and the priming action of ASM might also act to up-regulate these signals (Shah and Zeier, 2013).

The lack of difference of PR5 expression in BABA treated plants is likely due to BABA's method of action in Solanaceous crops: the activator increases accumulation of

PR proteins even in the absence of pathogen challenge, and thus inoculation with *SeN* and *Pst* may not significantly increase the already elevated expression levels (Cohen, 2002). *Pst* down-regulated PR1 expression in distal tissues of plants treated with BABA; this may be due to *Pst* employing effectors to interfere with distal SAR signaling and further down-regulate PR1 expression. This effect would not necessarily be observed in plants treated with other chemical activators as those activators do not cause the plant to constitutively increase PR gene expression, and so any PR1 expression suppression by *Pst* is counterbalanced by the primed increased of PR1 expression by these activators after pathogen challenge.

Inoculation with *SeN* onto cPTIO-treated leaves resulted in reduced SAMT and PR1 expression in the distal tissues. Although NO-based SAR signaling is SA-independent, distal tissues may be employing SA-dependent signaling to continue SAR signal transmission. cPTIO treatment did not suppress, and instead increased expression of PR5 in the distal tissues of *SeN* inoculated plants.

In conclusion, it appears that tomato plants do recognize *Salmonella* and are able to influence the expression of SAR-inducible genes distal to the site of *Salmonella* inoculation. Moreover, SAR chemical activation does appear to prime plants to react more strongly to *SeN* colonization. However, due to *Salmonella* colonization behavior in the phyllosphere, SAR activation of tomato does not appear sufficient to influence *SeN* population levels.

Chapter 4: Summary of conclusions, reflections, and future research

4.1 Summary of main conclusions

In contrast to the *Salmonella*-triggered PTI defense responses observed in other studies (see Section 1.1.5), local *Salmonella* colonization does not appear to influence population levels of subsequent *Salmonella* colonization on distal tissues, nor those of the phytopathogen *Pseudomonas syringae* on distal tissues in a fashion consistent with the SAR phenotype. However, in plants unable to accumulate the SAR-related compounds SA or NO there was a mild increase in *Salmonella* populations, which suggests that functional plant defenses do act to inhibit *Salmonella* populations.

Additionally, although no significant SAR phenotype was observed, gene expression consistent with SAR activation was observed after *Salmonella* inoculation. Some of this gene expression was further enhanced in plants which had been chemically primed for SAR activation. This suggests that *Salmonella* inoculation triggers some initial SAR signaling, if not expression of the SAR phenotype.

4.2 Summary of additional conclusions

The initial hypothesis to be tested by the ROS assay was whether or not *Salmonella* triggered ROS accumulation, which would be consistent with both PTI

expression and SAR signaling. Comparison of the ROS accumulation triggered by different serovars, however, suggests the following inference: that plant-associated *Salmonella* serovars trigger less ROS accumulation, and thus weaker plant defense responses, than serovars not associated with plants.

Gene expression was analyzed in order to further confirm SAR activation in distal tissues, and to determine whether *Salmonella* triggered SAR-gene expression in a similar fashion to *Pseudomonas syringae*. Here we observed that in ASM treated plants, there is a down-regulation of SAMT after inoculation with either *Salmonella* or *Pseudomonas*. The mechanism by which ASM activates SAR is still under investigation, although recent studies suggest that demethylation of ASM to acibenzolar by SABP2 is a necessary component. SABP2 and SAMT have opposing functions: the former de-methylates MeSA, and the latter methylates SA. If acibenzolar is the bioactive form of ASM, then down-regulation of SAMT in ASM treated plants after pathogen challenge may be a plant response to preserve acibenzolar levels in the tissue.

4.3 Reflections and future directions

4.3.1 Reflections on experimental methods and materials

In this study, the *Salmonella* serovar Newport was used due to its association with tomato-sourced outbreaks. However, in order to get a fuller picture of the possible inhibitory contribution of SAR, it would have been useful to employ serovars associated with both different plant commodities, and serovars associated with animal commodities.

In preliminary experiments conducted to determine inoculation and enumeration methods, the attenuated laboratory strain *S.* LT2 was used. This serovar was not used in the primary experiments of the study, however, due to the frequency that populations would fail to establish or completely decline in the phyllosphere, which presented statistical analysis issues. Furthermore, one of the main motivations of this study was to determine whether or not SAR activators could be employed to improve the food safety of tomatoes. As this serovar was not associated with any outbreaks, it did not seem worthwhile at the time to pursue it for further investigation. On reflection, it may have served as an ideal contrast to the plant-adapted *S.* Newport: if plants act to inhibit colonization of *Salmonella*, it would be useful to examine the cases where that inhibitory action actually occurs rather than the cases where *Salmonella* is capable of evading or withstanding that response. Additional candidates for study include wild type *S.* Typhimurium (the strain from which *S.* LT2 is derived), as well as the poultry associated strains *S.* Enteriditis, and *S.* Heidelberg.

For the assessment of the SAR phenotype, it may have also been useful to employ an avirulent strain of *Pst*, as the fully virulent strain may have been capable of suppressing SAR in our system, or may have been competent enough to overcome any attenuated SAR response triggered by *Salmonella* inoculation.

The plant materials for this study were grown in greenhouse conditions, and were thus exposed to any errant environment which may have been carried in by other greenhouse users. Various disease symptoms (leaf mold, powdery mildew) appeared sporadically within the greenhouse, although plants which showed signs of disease were immediately destroyed upon detection. Furthermore, there were sporadic outbreaks of

whitefly infestation. However, for this study, it was determined that sterile propagation of plants was undesirable due to the impact this method might have on plant defense competence. For future studies, propagation in growth chamber conditions may provide an appropriate compromise.

In the experiments where non-accumulation of SAR signaling compounds was relevant (*NahG* non-accumulation of SA plants, NO scavenging by cPTIO), it would have been useful to confirm the compound deficiency by high performance liquid chromatography for SA, or difluoroflurescein diacetate fluorescence for NO.

Accumulation of ROS was assayed by DAB stain, which generates a dark brown precipitate in the presence of ROS and peroxidase. However, this method does not lend itself to quantitative analysis. The stain intensity does not proceed stoichiometrically from the reaction; the precipitate produced from the DAB reaction is a light scatterer rather than absorber, and thus does not follow the Beer-Lambert law. DAB does have the advantage of being able to detect low levels of ROS production over time, however, as the precipitate continues to accumulate with continued exposure to ROS. In future iterations of this study, the DAB stain should be coupled with quantifiable ROS assays such as luminol fluorescence over a series of time points.

In this study, qPCR was constrained by only having access to a 48-well device. This obligated narrower comparisons in order to account for plate-to-plate variation between qPCR experiments. With a larger system, a robust randomized block design could be implemented between qPCR plates to allow for direct comparison of a broader array of treatments.

4.3.2 Future directions

This study was conducted to determine if there was a SAR contribution to the pathogen-commodity interaction between *Salmonella* and tomato; however, it may prove to be more useful to examine the narrower scope of serovar-commodity associations. Constraining the question this way would highlight if defense responses serve to inhibit non-plant adapted *Salmonella* serovars, and also if plant-adapted *Salmonella* serovars have adaptations which allow them to evade, disable, or withstand plant defenses.

Although *Salmonella* is not typically found in the phyllosphere microbiome, the results of this study may inform future studies into the SAR defense effects on the microbial communities associated with plants. Presently there exists conflicting information on the contribution that SAR has on plant-associated microbial communities. However in these studies SAR has merely been activated and the existing microbial community analyzed; it would be illustrative to see if SAR acts to shape communities when the community is changed by the introduction of a new species.

Appendix 1: Bacterial Counts

Treatment	Iteration	Log CFU/0.05g
SeN	1	3.00
SeN	1	4.91
SeN	1	4.54
SeN	1	4.67
Live Pst	1	3.64
Live Pst	1	4.91
Live Pst	1	3.82
Live Pst	1	5.08
Control	1	4.08
Control	1	4.52
Control	1	4.33
Control	1	1.96
SeN	2	4.19
SeN	2	5.28
SeN	2	4.31
SeN	2	5.32
Live Pst	2	3.75
Live Pst	2	4.87
Live Pst	2	6.16
Live Pst	2	5.26
Control	2	4.14
Control	2	5.33
Control	2	4.47
Control	2	5.48
Inactive Pst	2	4.17
Inactive Pst	2	5.36
Inactive Pst	2	4.42
Inactive Pst	2	2.46

Table A1 – *Pst* counts on pre-inoculated tomato cv. ‘Primo Red’

Treatment	Cultivar	Iteration	Initial Log CFU/leaf	Final Log CFU/leaf	Log CFU decline/Leaf
Multi	Heinz	1	7.44	6.17	1.27
Multi	Heinz	1	7.44	5.18	2.26
Multi	Heinz	1	7.44	4.97	2.47
Multi	Heinz	1	7.44	4.95	2.49
Multi	Heinz	1	7.44	4.20	3.24
Single	Heinz	1	7.44	6.76	0.68
Single	Heinz	1	7.44	6.49	0.95
Single	Heinz	1	7.44	6.41	1.03
Single	Heinz	1	7.44	6.26	1.18
Single	Heinz	1	7.44	5.27	2.17
Multi	Heinz	2	7.61	7.23	0.38
Multi	Heinz	2	7.61	6.95	0.66
Multi	Heinz	2	7.61	6.83	0.78
Multi	Heinz	2	7.61	6.62	0.99
Multi	Heinz	2	7.61	5.85	1.76
Multi	Heinz	2	7.61	5.50	2.11
Single	Heinz	2	7.61	7.44	0.17
Single	Heinz	2	7.61	7.38	0.23
Single	Heinz	2	7.61	7.13	0.48
Single	Heinz	2	7.61	6.88	0.73
Single	Heinz	2	7.61	5.43	2.18
Single	Heinz	2	7.61	4.78	2.83
Multi	Primo Red	1	7.53	6.78	0.75
Multi	Primo Red	1	7.53	6.54	0.99
Multi	Primo Red	1	7.53	6.13	1.40
Multi	Primo Red	1	7.53	5.79	1.74
Multi	Primo Red	1	7.53	5.37	2.16
Multi	Primo Red	1	7.53	5.16	2.37
Single	Primo Red	1	7.53	6.95	0.58
Single	Primo Red	1	7.53	6.87	0.66
Single	Primo Red	1	7.53	6.48	1.05
Single	Primo Red	1	7.53	6.47	1.06
Single	Primo Red	1	7.53	5.30	2.23
Single	Primo Red	1	7.53	4.71	2.82
Multi	Primo Red	2	8.30	5.18	3.12
Multi	Primo Red	2	8.30	5.18	3.12
Multi	Primo Red	2	8.30	4.46	3.84
Multi	Primo Red	2	8.30	3.75	4.55
Single	Primo Red	2	8.30	5.38	2.92
Single	Primo Red	2	8.30	4.97	3.32
Single	Primo Red	2	8.30	4.17	4.13
Single	Primo Red	2	8.30	6.05	2.24

Table A2–*SeN* counts on tomato cv. ‘Primo Red’folowing single or multiple inoculations with *SeN*

Treatment	Log CFU/0.05g
NahG	5.52
NahG	5.59
NahG	5.27
NahG	4.44
NahG	4.47
NahG	5.00
Moneymaker	4.94
Moneymaker	4.81
Moneymaker	4.76
Moneymaker	4.27
Moneymaker	4.40
Moneymaker	4.28

Table A3 – *SeN* counts on tomato cv. 'Moneymaker' or *NahG* plants in the 'Moneymaker' background.

Treatment	Log CFU/leaf
Control	3.02
Control	4.41
Control	3.95
Control	3.89
Control	3.96
Control	4.81
cPTIO	3.95
cPTIO	4.11
cPTIO	4.33
cPTIO	5.19
cPTIO	4.71
cPTIO	5.66

Table A4 – *SeN* counts on tomato cv. 'Primo Red' treated with cPTIO

Treatment	Iteration	Experiment	Log CFU/0.05g
HA	1	HA	3.93
HA	1	HA	4.75
HA	1	HA	3.91
HA	2	HA	4.18
HA	2	HA	3.48
HA	2	HA	4.04
HA	2	HA	3.45
Control	1	HA	4.48
Control	1	HA	3.81
Control	1	HA	4.54
Control	2	HA	4.48
Control	2	HA	4.54
Control	2	HA	4.43
Control	2	HA	4.94
BABA	1	BABA	3.19
BABA	1	BABA	2.76
BABA	1	BABA	4.33
BABA	1	BABA	3.51
BABA	1	BABA	3.94
BABA	1	BABA	1.89
Control	1	BABA	4.59
Control	1	BABA	4.41
Control	1	BABA	4.88
Control	1	BABA	3.58
Control	1	BABA	3.46
Control	1	BABA	3.59
ASM	1	ASM	0.00
ASM	1	ASM	0.60
ASM	1	ASM	0.00
ASM	1	ASM	0.30
ASM	2	ASM	0.48
ASM	2	ASM	0.00
ASM	2	ASM	0.70
ASM	2	ASM	0.00
Control	1	ASM	4.92
Control	1	ASM	4.54
Control	1	ASM	3.48
Control	1	ASM	4.03
Control	2	ASM	2.59
Control	2	ASM	4.64
Control	2	ASM	5.03
Control	2	ASM	3.96

Table A5 – *Pst* counts on tomato cv. ‘Primo Red’ treated with chemical SAR activators

Treatment	Iteration	Experiment	Log CFU/0.05g
ASM	1	ASM	3.053462605
ASM	1	ASM	4.491375703
ASM	1	ASM	0
ASM	1	ASM	4.5185271
ASM	2	ASM	4.5185271
ASM	2	ASM	3.075911762
ASM	2	ASM	4.309651456
ASM	2	ASM	4.944487607
Control	1	ASM	4.612794449
Control	1	ASM	4.633478555
Control	1	ASM	2.303196057
Control	1	ASM	4.568213462
Control	2	ASM	4.857338528
Control	2	ASM	4.819550516
Control	2	ASM	3.612889769
Control	2	ASM	3.662852233
HA	1	HA	3.785401025
HA	1	HA	3.986816505
HA	1	HA	3.662852233
HA	1	HA	3.716086854
HA	2	HA	4.004364371
HA	2	HA	4.5185271
HA	2	HA	3.939569169
HA	2	HA	0
Control	1	HA	3.934548948
Control	1	HA	4.748195782
Control	1	HA	3.908538632
Control	1	HA	2.778874472
Control	2	HA	3.568319085
Control	2	HA	4.544080453
Control	2	HA	4.591075743
Control	2	HA	0
BABA	1	BABA	4.662757832
BABA	2	BABA	4.103803721
BABA	3	BABA	4.195899652
BABA	4	BABA	4.591064607
BABA	5	BABA	2.544068044
BABA	6	BABA	4.763427994
Control	1	BABA	4.716003344
Control	2	BABA	4.73239376
Control	3	BABA	4.568201724
Control	4	BABA	4.053078444
Control	5	BABA	4.071882007
Control	6	BABA	4.886490725

Table A6 – *SeN* counts on tomato cv. ‘Primo Red’ treated with chemical SAR activators

Treatment	Cultivar	Log CFU/0.05g
ASM	Heinz	5.74
ASM	Heinz	4.52
ASM	Heinz	4.45
ASM	Heinz	4.33
HA	Heinz	4.36
HA	Heinz	4.03
HA	Heinz	4.52
HA	Heinz	4.61
Mock	Heinz	4.02
Mock	Heinz	5.99
Mock	Heinz	5.05
Mock	Heinz	5.40
ASM	Nyagous	4.23
ASM	Nyagous	5.04
ASM	Nyagous	4.21
ASM	Nyagous	4.27
HA	Nyagous	3.88
HA	Nyagous	3.84
HA	Nyagous	4.10
HA	Nyagous	3.89
Mock	Nyagous	5.04
Mock	Nyagous	3.94
Mock	Nyagous	3.95
Mock	Nyagous	4.38

Table A7 – *SeN* counts on tomato cv. ‘Heinz’ and ‘Nyagous’ treated with chemical SAR activators

Appendix 2: qPCR Data

Treatment	Inoculum	ΔΔCt
ASM	Mock	-0.12923
ASM	Mock	0.18088
ASM	Mock	-0.28683
ASM	Mock	0.28252
ASM	Mock	-0.04734
ASM	Mock	0.01868
ASM	Pst	4.52501
ASM	Pst	5.28246
ASM	Pst	5.28755
ASM	Pst	4.21966
ASM	Pst	4.70129
ASM	Pst	4.80319
ASM	SeN	4.35319
ASM	SeN	2.60648
ASM	SeN	4.15029
ASM	SeN	1.76708
ASM	SeN	3.96844
ASM	SeN	2.58273
BABA	Mock	-0.09896
BABA	Mock	-0.20484
BABA	Mock	0.21543
BABA	Mock	0.08836
BABA	Mock	-0.05824
BABA	Mock	0.07860

BABA	Pst	-3.59147
BABA	Pst	-0.19131
BABA	Pst	-0.11249
BABA	Pst	0.57605
BABA	Pst	0.19237
BABA	Pst	0.03994
BABA	SeN	1.42382
BABA	SeN	0.31099
BABA	SeN	0.34528
BABA	SeN	-0.01332
BABA	SeN	0.14883
BABA	SeN	0.24706
HA	Mock	-0.74888
HA	Mock	0.58390
HA	Mock	0.24288
HA	Mock	-0.23526
HA	Mock	0.15737
HA	Mock	-0.01456
HA	Pst	0.83329
HA	Pst	-1.95984
HA	Pst	1.03672
HA	Pst	-1.16289
HA	Pst	-1.56137
HA	Pst	-0.26233
HA	SeN	-4.02205
HA	SeN	-3.72667
HA	SeN	-3.12432

HA	SeN	-3.91970
HA	SeN	-4.07378
HA	SeN	-4.11098
Mock	Mock	0.26911
Mock	Mock	1.34076
Mock	Mock	0.90467
Mock	Mock	-1.30141
Mock	Mock	-1.21313
Mock	Mock	0.01535
Mock	Pst	-7.07035
Mock	Pst	-4.03494
Mock	Pst	-5.41547
Mock	Pst	-6.19516
Mock	Pst	-5.11505
Mock	Pst	-5.26526
Mock	SeN	-2.13097
Mock	SeN	-2.51166
Mock	SeN	-3.73499
Mock	SeN	-2.32132
Mock	SeN	-3.12332
Mock	SeN	-2.93298
cPTIO	SeN	1.60916
cPTIO	SeN	0.69977
cPTIO	SeN	1.20253
cPTIO	SeN	2.56137
cPTIO	SeN	1.40584
cPTIO	SeN	1.63057

Table A8 – SAMT ΔΔCt values

Treatment	Inoculum	ΔΔCt
ASM	Mock	0.472364
ASM	Mock	0.028641
ASM	Mock	-0.19764
ASM	Mock	-0.01819
ASM	Mock	-0.28518
ASM	Mock	0.01158
ASM	Pst	-6.08467
ASM	Pst	-5.05125
ASM	Pst	-6.10176
ASM	Pst	-5.91219
ASM	Pst	-5.72115
ASM	Pst	-5.2531
ASM	SeN	-1.66038
ASM	SeN	-3.20799
ASM	SeN	-4.11859
ASM	SeN	-4.00716
ASM	SeN	-3.37008
ASM	SeN	-2.91216
BABA	Mock	0.039303
BABA	Mock	-0.07251
BABA	Mock	-0.1725
BABA	Mock	0.205708
BABA	Mock	-0.0666
BABA	Mock	0.066597
BABA	Pst	0.776734
BABA	Pst	0.78851

BABA	Pst	0.553455
BABA	Pst	1.070335
BABA	Pst	0.665094
BABA	Pst	0.929422
BABA	SeN	-1.28767
BABA	SeN	-1.31814
BABA	SeN	-0.88056
BABA	SeN	-0.93885
BABA	SeN	-1.08412
BABA	SeN	-1.12849
HA	Mock	2.097391
HA	Mock	1.743196
HA	Mock	1.106079
HA	Mock	1.179408
HA	Mock	1.117527
HA	Mock	1.44872
HA	Pst	-3.89222
HA	Pst	-4.78065
HA	Pst	-3.6899
HA	Pst	-4.04867
HA	Pst	-3.79106
HA	Pst	-4.41466
HA	SeN	-7.06807
HA	SeN	-7.21582
HA	SeN	-6.8821
HA	SeN	-6.34109
HA	SeN	-6.71147

HA	SeN	-6.49815
Mock	Mock	-0.20898
Mock	Mock	0.653109
Mock	Mock	-0.28181
Mock	Mock	0.280575
Mock	Mock	-0.44289
Mock	Mock	0.03051
Mock	Pst	-0.63645
Mock	Pst	-0.29569
Mock	Pst	-1.08372
Mock	Pst	-0.06929
Mock	Pst	-0.86008
Mock	Pst	-0.18249
Mock	SeN	-0.18632
Mock	SeN	-0.61131
Mock	SeN	-0.9445
Mock	SeN	-0.39881
Mock	SeN	-0.7779
Mock	SeN	-0.56541
cPTIO	SeN	1.06279
cPTIO	SeN	1.30354
cPTIO	SeN	1.28921
cPTIO	SeN	1.04128
cPTIO	SeN	1.17241
cPTIO	SeN	1.23081

Table A9 – PR1 ΔΔCt values

Treatment	Inoculum	ΔΔCt
ASM	Mock	1.30731
ASM	Mock	-0.87966
ASM	Mock	-0.42765
ASM	Mock	0.21382
ASM	Mock	-0.65365
ASM	Mock	0.43983
ASM	Pst	-0.19070
ASM	Pst	-2.96019
ASM	Pst	-1.97003
ASM	Pst	0.07470
ASM	Pst	-1.08036
ASM	Pst	-1.44275
ASM	SeN	1.21718
ASM	SeN	-0.02202
ASM	SeN	0.47728
ASM	SeN	0.59758
ASM	SeN	0.22763
ASM	SeN	0.84723
BABA	Mock	0.16687
BABA	Mock	-0.40536
BABA	Mock	-0.86178
BABA	Mock	1.10027
BABA	Mock	-0.34746
BABA	Mock	0.34746
BABA	Pst	-0.00145
BABA	Pst	0.22597

BABA	Pst	0.91318
BABA	Pst	0.15186
BABA	Pst	0.45586
BABA	Pst	0.18891
BABA	SeN	-0.35856
BABA	SeN	1.16270
BABA	SeN	-1.31482
BABA	SeN	0.20470
BABA	SeN	-0.83669
BABA	SeN	0.68370
HA	Mock	0.00100
HA	Mock	-0.64605
HA	Mock	0.05656
HA	Mock	0.58850
HA	Mock	-0.29475
HA	Mock	0.32253
HA	Pst	-0.60402
HA	Pst	-1.95527
HA	Pst	-0.82461
HA	Pst	-1.27964
HA	Pst	-1.38994
HA	Pst	-0.71431
HA	SeN	-1.02762
HA	SeN	-0.39161
HA	SeN	-0.09855
HA	SeN	0.06184
HA	SeN	-0.56309

HA	SeN	-0.16489
Mock	Mock	-0.45576
Mock	Mock	0.66243
Mock	Mock	-0.69185
Mock	Mock	-1.15246
Mock	Mock	1.63764
Mock	Mock	0.06215
Mock	Pst	-7.12370
Mock	Pst	-6.03448
Mock	Pst	-4.63828
Mock	Pst	-6.68496
Mock	Pst	-5.88099
Mock	Pst	-6.35972
Mock	SeN	-2.48561
Mock	SeN	-3.48601
Mock	SeN	-3.48601
Mock	SeN	-6.85536
Mock	SeN	-2.98581
Mock	SeN	-3.15255
cPTIO	SeN	-4.85317
cPTIO	SeN	-5.50062
cPTIO	SeN	-5.50062
cPTIO	SeN	-5.25702
cPTIO	SeN	-5.37882
cPTIO	SeN	-5.43972

Table A10 – PR5 ΔΔCt values

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