Investigation of SAR-associated small molecules as inducers of resistance in cucumber and biofilm formation by *Pseudomonas syringae* pv. *tomato* in *Arabidopsis*

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A Thesis Submitted to the School of Graduate Studies in Partial Fulfillment of the Requirements for the Degree Master of Science

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Title: Investigation of SAR-associated small molecules as inducers of resistance in cucumber and biofilm formation by *Pseudomonas syringae* pv. *tomato* in *Arabidopsis*

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Abstract

Greenhouse environments often promote bacterial and fungal infections in important crop plants. Exogenous application of chemical inducers could help reduce the severity of infection, or even prevent infection. Small molecules such as glycerol, azelaic acid and pipecolic acid have been implicated as being important signaling molecules during Systemic Acquired Resistance (SAR). To examine if these small molecules could be used to induce resistance in crop plants, exogenous treatment assays were developed in cucumber. Glycerol spray and azelaic acid infiltration induced modest resistance at locally treated leaves. Pipecolic acid soil treatment induced modest resistance in aerial tissue of cucumber plants, and strong resistance when plants were treated weekly. This knowledge may be useful in promoting the commercialization of SAR-associated compounds to protect important crop plants against disease.

Plants possess multiple defense pathways that include an SA signaling component to initiate resistance to microbial pathogens. However, during Age-Related Resistance (ARR) in Arabidopsis, a number of studies support that SA acts as an anti-microbial and anti-biofilm agent against *Pseudomonas syringae* pv. *tomato* (*Pst*) in the plant intercellular space. Little is known about the role of *Pst* biofilm formation during infection of young plants or if other defense responses act to suppress bacterial biofilm formation. Therefore *Pst* biofilm formation and the effect of PAMP Triggered Immunity (PTI) on bacterial biofilm formation was examined. PTI was induced with flg22 in wild-type Col-0, *fls*2, *bak1-3* (PTI mutants) and *sid2-2* (SA biosynthesis mutant). *In vivo* bacterial biofilm-like aggregate formation was monitored using *Pst* DC3000 PDSK-GFPuv and epifluorescence microscopy. *Pst* aggregate occurrence and size were positively correlated with bacterial success in susceptible plants (wild-type Col-0, *fls2, bak1-3*, *sid2-2)*, while fewer and smaller bacterial aggregates were observed in Col-0 undergoing PTI. To determine if the extracellular polysaccharide, alginate was a major contributor to

biofilm formation, *in vivo* bacterial aggregate formation was monitored using alginate deficient *Pst*-GFP. Alginate deficient *Pst*-GFP and wild-type *Pst* grew to similar levels in wild-type plants suggesting that the ability to produce alginate was not necessary for *Pst* pathogenicity and success in planta. Fewer alginate-deficient *Pst* aggregates were observed compared to wild-type *Pst* in inoculated plants, suggesting that the ability to produce alginate was modestly important for aggregate formation. These data provide novel insights into how biofilms form *in planta*, the association between pathogen virulence and biofilm formation, and how plant defense responses such as PTI not only reduce bacterial growth, but also target biofilms.

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List of Abbreviations

μl	microliter
μm	micrometer
μM	micromolar
ALD1	AGD2-like defense response
AlgA	Alginate biosynthesis protein AlgA
AlgD	GDP-mannose 6-dehydrogenase
AlgU	RNA polymerase sigma-H factor
ANOVA	Analysis of variance
ARR	Age-related resistance
ATP	Adenosine triphosphate
Aza	Azelaic acid
AZI1	Azelaic acid induced 1
BAK1	Brassinosteroid insensitive 1-associated receptor kinase 1
BiFC	Bimolecular fluorescence complementation
BTH	Benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester
Ca^{2+}	Calcium ion
cfu	colony forming units
Col-0	Columbia-0
ConA	Concanavalin A
COR	Coronatine
DAPI	4′,6-diamidino-2-phenylindole
DDE2	Delayed dehiscence 2
DIR1	Defective in induced resistance 1
eDNA	extracellular DNA
EIN2	Ethylene-insensitive protein 2
Elf18	Elongation factor 18
EPS	Extracellular polymeric substances
ET	Ethylene
ETI	Effector-triggered immunity
ETS	Effector-triggered susceptibility
FLC	Flowering Locus C
FLS2	Flagellin sensing 2
Flg22	Flagellin 22
FMO1	Flavin-dependent Monooxygenase 1
FOV	Fields of view
G3P	Glycerol-3-phosphate
GDP	Guanosine diphosphate
GFP	Green fluorescent protein

GLI	Glycerol insensitive
HIM	Hrp-inducing minimal medium
hpi	hours post-inoculation
HR	Hypersensitive response
Hrp	Hypersensitive response and pathogenicity
HSD	Honestly significant difference
ICS1	Iscochorismate synthase 1
JA	Jasmonic acid
ld	leaf disc
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
MES	2-(N-morpholino)-ethanesulfonic acid
MeSa	Methyl salicylate
$MgCl_2$	Magnesium chloride
MPK3	Mitogen-activated protein kinase 3
MPK 6	Mitogen-activated protein kinase 6
MucA A	Sigma factor AlgU negative regulatory protein
MucB B	Sigma factor AlgU negative regulatory protein
Mb	Mega base pairs
ml	milliliter
mM	millimolar
nm	nanometers
NHO1	Non-host resistance
N-OH-Pip	N-hydroxy-pipecolic acid
NPR1	Nonexpressor of PR genes 1
NPR3	Nonexpressor of PR genes 3
NPR4	Nonexpressor of PR genes 4
OD	Optical density
PAD4	Phytoalexin deficient 4
PAMP	Pathogen-Associated Molecular Pattern
Pip	Pipecolic acid
PR	Pathogenesis-Related Proteins
PRR	Pattern recognition receptor
Psg	*Pseudomonas syringae* pv. *glycinea*
Psl	*Pseudomonas syringae* pv. *lachrymans*
Psm	*Pseudomonas syringae* pv. *maculicola*
Pss	*Pseudomonas syringae* pv. *syringae*
Pst	*Pseudomonas syringae* pv. *tomato*
Pta	*P. syringae* pv. *tabaci*
PTI	Pattern-triggered immunity

R	Resistance
RLK	Receptor-like kinases
ROS	Reactive oxygen species
SA	Salicylic acid
SAR	Systemic Acquire Resistance
SID2	Salicylic acid induction deficient
SVAM	Snake venom agglutinin
TTSS	Type III Secretion System
TMV	Tobacco Mosaic Virus
Ws	Wassilewskija
Xcc	*Xanthomonas campestris* pv. *campestris*

Declaration of Academic Achievement

This thesis was written with the help and guidance of Dr. Robin K. Cameron. Cucumber experiments were developed by Robin Cameron, Manreet Dhaliwal, Natalie Belu, and myself. Experiments were performed by Christine Kempthorne, Manreet Dhaliwal, Ramzy Badrous, Garrett Nunn, Matei Dan-Dobre, Natalie Belu, and myself. PTI experiments were conceived of and developed by Robin Cameron, Noah Xiao, and myself. The majority of experiments were performed by Noah Xiao and myself with help from Garrett Nunn, and Abdul Halim performed the aggregate size analysis.

I declare this thesis to be an original report of our research, except where indicated by referencing. No part of this work has been submitted, in whole or in part, in any previous applications or publications for a degree at another institution. A portion of the research conducted in this thesis will be prepared as a manuscript for submission to a peer-reviewed publication.

Chapter 1—Introduction

1.1 Plant responses to the environment

Plants are immobile organisms and are therefore unable to move to escape stressors. Plants have evolved many mechanisms and strategies that allow them to rapidly detect and adapt to changes in environmental conditions. Potential stressors include both abiotic and biotic factors. Abiotic factors include light quality and quantity, nutrient and water availability, as well as temperature. Biotic stressors include viral and microbial pathogens (bacteria, fungi) in addition to pests and herbivores (insects and mammals). An example of the devastating effect of plant pathogens occurred in Ireland during the late 1840s when the entire potato crop was lost to the oomycete pathogen, *Phytophtora infestans,* leading to mass starvation and migration, now known as the Irish potato famine, (Bourke 1964). Currently, apple scab, caused by the fungal pathogen *Venturia inaequalis*, is a serious disease in Ontario orchards as it affects leaves, blossoms, and fruit in apple and pear trees. In severe cases, diseased trees become defoliated and fruits are unmarketable (OMAFRA 2011).

1.2 Plant responses to microbial pathogens

Understanding plant defense pathways at the molecular, cellular, and biochemical levels will contribute to the development of more effective methods of combating plant disease such as enhancing crop management. This includes the use of preventative as well as after-infection fungicides (OMAFRA 2011) and generating disease-resistant varieties through breeding or through genetic modification.

Plants possess many constitutive defense mechanisms that prevent pathogen entry, such as waxy cuticles or the presence of antimicrobial phytoalexins, for example glucosinolates that reduce microbial multiplication in plant intercellular spaces (Senthil-Kumar and Mysore 2013, Fan *et al.* 2011). Plants also have other defense mechanisms that are activated in the presence of pathogens. These induced disease resistance responses include PAMP-triggered immunity

(PTI), Effector-triggered immunity (ETI) and Systemic Acquired Resistance (SAR). These mechanisms will be further discussed below.

1.2.1 Bacterial infection Mechanisms

Pathogens possess pathogenicity genes that are essential for causing disease while virulence is defined as the degree of pathogenicity of a given pathogen (Agrios, 2005). To infect, grow and reproduce in plants, bacterial pathogens use multiple pathogenicity and virulence strategies. The highly studied *Arabidopsis thaliana-Pseudomonas syringae* pathosytem has provided many insights into bacterial pathogenicity and virulence mechanisms.

The phytotoxin coronatine (COR) is an important virulence factor synthesized by *Pseudomonas syringae* pv. *tomato* (*Pst*)*.* COR stimulates the jasmonic acid signaling pathways (Geng *et al.* 2014), which antagonizes salicylic acid (SA)-mediated defense signaling. Lee *et al.* (2013) demonstrated that when *P. syringae* pv. *tabaci (Pta),* which does not produce COR, was applied to the epidermis of tomato, stomata remained closed. However, when COR was added to the *Pta* suspension, most stomata remained open (Lee *et al.* 2013). COR also delays non-host hypersensitive response (HR) cell death (Lee *et al.* 2013). Mutant *Pst* DB29 that does not produce COR and wild-type *Pst* DC3000 were infiltrated into *Nicotiana benthamiana* (non-host). After 24 hours, the level of non-host HR cell death was significantly higher in COR^- *Pst* compared to wild-type *Pst* (Lee *et al.* 2013).

Once *Pst* cells have entered through the stomata, they live within the intercellular space or apoplast and cannot enter the cell. To overcome this, they have evolved the Type-III secretion system (TTSS), which allows them to secrete virulence effectors into the cell via pilus (Costa *et al.* 2015). Effectors act as virulence factors to suppress plant immunity and manipulate the plant metabolism to make the environment more hospitable to the pathogen (Boeller and He 2009). This will be discussed further in Effector-Triggered Susceptibility (ETS).

The ability to form biofilms has been found to be a pathogenicity factor for some bacteria, including *Pseudomonas aeruginosa* and *Pseudomonas syringae* (Aslam *et al.* 2008). Biofilms are

described as surface-adherent aggregates of microbial cells embedded in an extracellular matrix (Karatan and Watnick 2009). Extracellular polymeric substances (EPS) are composed of extracellular DNA (eDNA) and carbohydrate polymers produced by bacteria, which are secreted outside the cell and form a loosely associated extracellular slime (Whitfield *et al.* 1988; Karatan and Watnick 2009). EPS have been shown to provide a selective advantage to a number of bacterial species including *Xanthomonas oryzae, P. syringae* pv*. glycinea (Psg)* and *P. aeruginosa.*

1.2.1.1 Biofilm formation

In animal pathogens *P. aeruginosa* and Staphylococcal species (reviewed by Joo and Otto, 2013), biofilms are found in medical devices as well as open wounds, dental plaques, and in the lungs of cystic fibrosis patients. Biofilm formation is considered to occur in three main stages: (i) attachment to the device or cell surface, (ii) proliferation and formation of the characteristic mature biofilm structure, and finally (iii) detachment or dispersal (O'Toole *et al.* 2000). Attachment can occur passively in non-motile bacteria such as Staphlococcal species or actively in motile bacteria such as *P. aeruginosa* (Joo and Otto, 2013). Attachment is based on protein-protein interactions between the bacterial surface and human/animal matrix proteins (Clarke and Foster, 2006). Proliferation and maturation depend on adhesive factors such as exopolysaccharides, eDNA, and proteins (Joo and Otto, 2013). Detachment occurs when cell-cell disruptive factors, such as surfactants, leads to dispersal of bacterial cells (O'Toole *et al.* 2000). Biofilms also provide bacteria with an increased capacity to resist antibiotic treatment and host produced antimicrobial agents (Costerton *et al.* 1999). Based on *in vitro* studies, it is believed that the biofilm matrix may be a diffusion barrier for antibiotics and may minimize sensitivity to antibiotics that target active cell processes.

Bacterial biofilm formation during plant-bacteria associations has also been studied in recent years (reviewed by Bogino *et al.* 2013). Auto-aggregation or clumping of cells in liquid culture has been observed in *Escherichia coli* and *Sinorhizobium meliloti.* Lipopolysaccharides (LPSs) and surface bacterial components such as flagella, pilli and fimbriae are important during the

initial stages of biofilm attachment to surfaces of *Xylella fastidosa* and Rhizobacteria (Clifford *et al.* 2013; Cava *et al.* 1989; Garciá de los Santos *et al.* 1997), while growth and maturation are dependent on eDNA, exopolysaccharides, and proteins. These polymers promote or provide immobilization of bacterial cells into the matrix, mechanical stability of the biofilm structure, cohesive interaction with the interface, and the architecture and functionality of the encased microbial community (Flemming and Wingender, 2010).

1.2.1.2 *Pseudomonas syringae* biofilm formation may contribute to pathogenicity and virulence

Pseudomonas syringae pv. *glycinea (Psg)* produces two exopolysaccharides: levan, a polymer of fructofuranan and alginate, a co-polymer of O-acetylated β-1,4-linked D-mannuronic acid and L-guluronic acid (Fett *et al*. 1986; Gross and Rudolph 1987). Fett and Dunn (1989) extracted and purified bacterial exopolysaccharides from pathogens (*P. syringae* pathovars *phaseolicola, lachrymans*, and *tomato*) that when infiltrated into host leaves induced lesions (bean, cucumber, and tomato, respectively). They found that *in vivo,* most *P. syringae* pathovars produced alginate, and none produced levan (Fett and Dunn, 1989). Furthermore, a positive correlation was observed between growth of *P. syringae* pvs. *glycinea* and *phaseolicola,* and production of alginate *in planta* (Osman *et al.* 1986; Gross and Rudolph 1987).

Bean plants infected with *P.s.* pv. *syringae* (*Pss)* mutants deficient in alginate production displayed a ~100-fold reduction in the ability to multiply and produce disease symptoms on bean plants compared to wild-type *Pss*, suggesting that the production of alginate is important for *Pss* success and pathogenicity in bean leaves (Yu *et al.* 1999).

During infection of *Arabidopsis* leaves, *Pst* was shown to express *AlgA* and *AlgD* (Boch *et al.* 2002; Keith *et al.* 2003), genes involved in the biosynthesis of alginate. When *algD Pst* or wild-type strains were inoculated into *Arabidopsis* leaves, reduced growth of the *algD* mutant by about 10-fold compared to wild-type *Pst* was observed (Aslam *et al.* 2008). Given that alginate

has been shown to be a component of *P. aeruginosa* biofilms, reduced growth of *algD Pst* in Arabidopsis may suggest that biofilm formation contributes to *Pst* virulence (Aslam *et al.* 2008).

Alginate biosynthesis has been thoroughly studied in *P. aeruginosa* (Fialho *et al.* 1990; Fett *et al*. 1992; Peñaloza-Vázquez *et al.* 1997) (Figure 1.1). In *P. aeruginosa,* the alternative sigma factor AlgU regulates its own expression as well as the expression of the *AlgD* gene, which encodes GDP-mannose dehydrogenase, which catalyzes the oxidation of GDP-mannose to produce the precursor for alginate polymerization. MucA and MucB are anti-sigma factors that interact with AlgU to negatively regulate *AlgU* expression. Total genomic DNA of thirteen Pseudomonads was screened for sequences homologous to four *P. aeruginosa* alginate genes and it was discovered that *P. syringae* contains DNA homologous to *AlgA and AlgD* (Fett *et al.* 1992). Furthermore, DNA hybridization and gene mapping revealed that the spatial organization of key alginate biosynthesis genes was the same in both *P. syringae* and *P. aeruginosa* (Peñaloza-Vázquez *et al.* 1997)*.* These data suggest that alginate biosynthesis in *P. syringae* may be similar to *P. aeruginosa*. In cystic-fibrosis infected lungs, chronic infection and the exclusive association with mucoid (alginate-producing) as opposed to non-mucoidal *P. aeruginosa* suggest that the ability to produce alginate provides a selective advantage (May *et al.* 1991) and may protect the bacteria from the host immune system, antibiotic treatment and desiccation (Shankar *et al.* 1995). These data suggest that alginate production is important in *P. aeruginosa* and may be important in *P. syringae.*

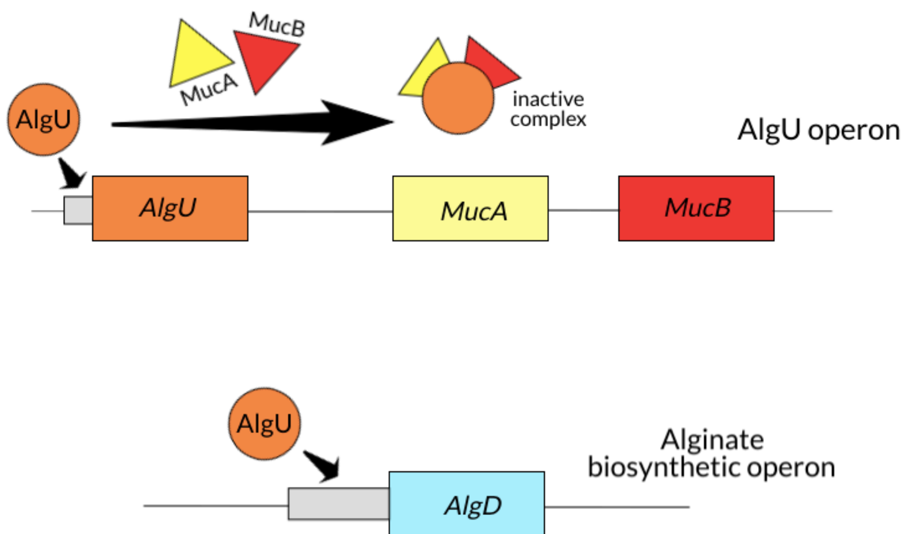


Figure 1.1. Genes involved in alginate synthesis. The AlgU alternative sigma factor directly regulates expression of itself, of the alginate biosynthetic operon, and of several other regulatory genes. The figure depicts events leading to transcriptional regulation of the *AlgD* gene (encoding GDP-mannose dehydrogenase), which lies at the 5 end of the alginate biosynthetic operon. The grey box represents the promoter region. The activity of the AlgU transcription factor is negatively regulated by the two anti-sigma factors MucA and MucB. Image modified from Lyczak *et al*. 2002.

1.2.1.3 Arabidopsis-*Pst* model system

Arabidopsis thaliana is a member of the *Brassicaceae* (Mustard) family and has become a model organism for plant biology. It has a small, fully sequenced genome (120Mb) (Theologis *et al.* 2000), fast generation time (8 to 12 weeks), small size, is able to self-fertilize, is easily transformable with many useful genetic tools, making it ideal for molecular and genetic research.

Pseudomonas syringae is a gram-negative bacterium that lives as an epiphyte on plant leaf surfaces before entering through wounds or stomata where it multiplies in the intercellular space (Katagiri *et al.* 2002). *P. syringae* proliferates as a biotroph in living plant tissue but later in its life cycle, switches to a necrotrophic form of growth, killing plant cells and escaping to the leaf surface for dissemination (Xin and He 2013) and is therefore considered to be a hemi-

biotrophic pathogen. *Psesudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) (Whalen *et al.*, 1991) is widely used to study plant-pathogen interactions including PAMP-triggered immunity in *Arabidopsis*. The complete genome of *Pst* was sequence in 2003 (Buell *et al.* 2003) leading to numerous studies of the roles of coronatine and the TTSS (reviewed in Xin and He 2013) as pathogenicity and virulence factors.

1.2.2 Local Resistance

1.2.2.1 Pattern-Triggered Immunity

Most Pattern-triggered Immunity (PTI) research has been done in *Arabidopsis. Arabidopsis* has many plasma membrane-associated receptor-like kinases and receptor-like proteins, many of which function as pattern recognition receptors (PRRs) (Jones and Dangl, 2006). These PRRs recognize pathogen-associated molecular patterns (PAMPs) such as bacterial flagellin (Gómez-Gómez 2004; Zipfel *et al.* 2006). PAMP perception by PRRs initiates mitogen-activated protein kinase (MAPK) signalling cascades, resulting in changes in gene expression, the accumulation of the defense hormone salicylic acid (SA). During PTI SA-dependent and independent signaling results in the production of antimicrobial phytoalexins and pathogenesis-related (PR) proteins, as well as extensive cell wall modifications (callose deposition), all of which contribute to suppression of pathogen growth (reviewed in Bigeard *et al.* 2015).

A well-studied example of PAMP perception in plants is the recognition of bacterial flagellin by the FLAGELLIN SENSING 2 (FLS2) receptor, which binds to a core flg22-sequence (RINSAKDD) using an extracellular leucine-rich repeat (LRR) receptor domain (reviewed in Robatzek and Wirthmueller 2013). A study by Tsuda *et al.* (2008) demonstrated that PTI is significantly induced 3-24 hours after flg22 treatment and peaked at 9 hours. SA levels in treated plants ranged from 2 to 14 times the levels of mock-treated plants (Tsuda *et al.* 2008). The expression of leucine-rich repeat receptor-like kinases (LRR-RLKs) like FLS2 are induced by PAMPs, so a collection of insertion mutants was examined to identify other LRR-RLKs that are important during PAMP signaling and perception (Chinchilla *et al.* 2007). Seedlings with mutations in the

At4g33430 locus had reduced sensitivity to flg22, but normal sensitivity to the PAMP elf18 compared to wild-type seedlings. This locus was found to encode the LRR-RLK, BAK1 (BRI1-Associated Kinase 1), suggesting that BAK1 is another PRR involved in flg22 perception (Chinchilla *et al.* 2007). This study also demonstrated that receptor-PAMP binding induces a signalling cascade that includes the BAK1 co-receptor (Chinchilla et al.2007), resulting in the activation of PTI. A study by Tsuda *et al.* (2009) provided evidence that in addition to SA signaling, both jasmonic acid (JA) and ethylene (ET) signaling also contribute to PTI. In this study, quadruple mutants in JA (*delayed-dehiscence2-2*, *dde)* and SA biosynthesis (*sid2*), as well as insensitive to ethylene (*ethylene-insensitive protein 2*, ein2) and deficient in SA signaling (*phytoalexin deficient 4, pad4*), were treated with either flg22 or another PAMP, elf18. Both flg22- and elf18-treated quadruple mutants were compromised for PTI by 50% compared to wild-type plants. This suggests that the PTI response induced by flg22 and elf18 is dependent on all four genes and therefore dependent on the contributions of the SA, JA, and ET pathways.

1.2.2.2 Effector-induced Susceptibility

Plant pathogens must overcome constitutive and PTI defenses to successfully infect a potential host. To accomplish this, phytopathogens use a repertoire of effector proteins to manipulate host cells. Effectors have been implicated in transcriptional manipulation of plant host genes, blocking the protein secretory pathway, as well as manipulating hormone signaling by modifying or degrading hormones such as SA (reviewed in Dou and Zhou 2012; Giraldo and Valent 2013; Lee *et al.* 2013). The action of these effectors renders the host plant susceptible to infection, resulting in effector-triggered susceptibility (ETS) (Jones and Dangl 2006). For many plant pathogenic bacteria, these effectors are delivered into host cells using the TTSS (Costa *et al.* 2015).

1.2.2.3 Effector-triggered Immunity

In order to combat pathogen effectors, plants evolved receptors known as Resistance (R) proteins (Jones and Dangl 2006). R proteins recognize pathogen effectors directly or indirectly such that R proteins become activated and initiate signalling for a disease-resistance response

termed Effector-Triggered Immunity (ETI) or R gene-mediated resistance (Jones and Dangl 2006). ETI shares many characteristics with the PTI response, but ETI is typically faster and stronger (Cui *et al.* 2015, Gassmann and Bhattacharjee 2012). Another difference between PTI and ETI is that unlike PTI, ETI is often associated with a form of programmed cell death known as the hypersensitive response (HR). The HR is thought to prevent the growth of biotrophic or hemi-biotrophic pathogens such as *P. syringae*, which rely on living host cells to obtain nutrients for at least part of their life cycle (Glazebrook 2005). More recent studies suggest that inhibiting HR cell death does not always affect disease resistance. The potato *Rx* and barley *Rrs1* genes control separate resistance and cell death responses (reviewed in Coll *et al. 2011*). This suggests that cell death itself is not the only cause of resistance, but rather it is the build-up of toxic defense compounds that cause cell death (reviewed in Coll *et al.* 2011).

1.2.2.4 Age-Related Resistance

Age-related resistance (ARR) is a form of developmentally regulated resistance in which plants become resistant to a particular pathogen as they age (reviewed in Carella *et al.* 2015). In *Arabidopsis*, mature plants inoculated with *Pst* support less bacterial growth and fewer symptoms compared to young plants (Kus *et al.* 2002). SA is required for ARR to *P. syringae*, as mutants and transgenics that are unable to accumulate SA are also ARR-defective (Cameron and Zaton, 2004; Carviel *et al.* 2009; Kus *et al.* 2002). SA signaling does not appear to be required for ARR, as the *npr1-1* SA signaling mutant is ARR-competent (Kus *et al.* 2002), which suggests that SA accumulation contributes to ARR in other ways.

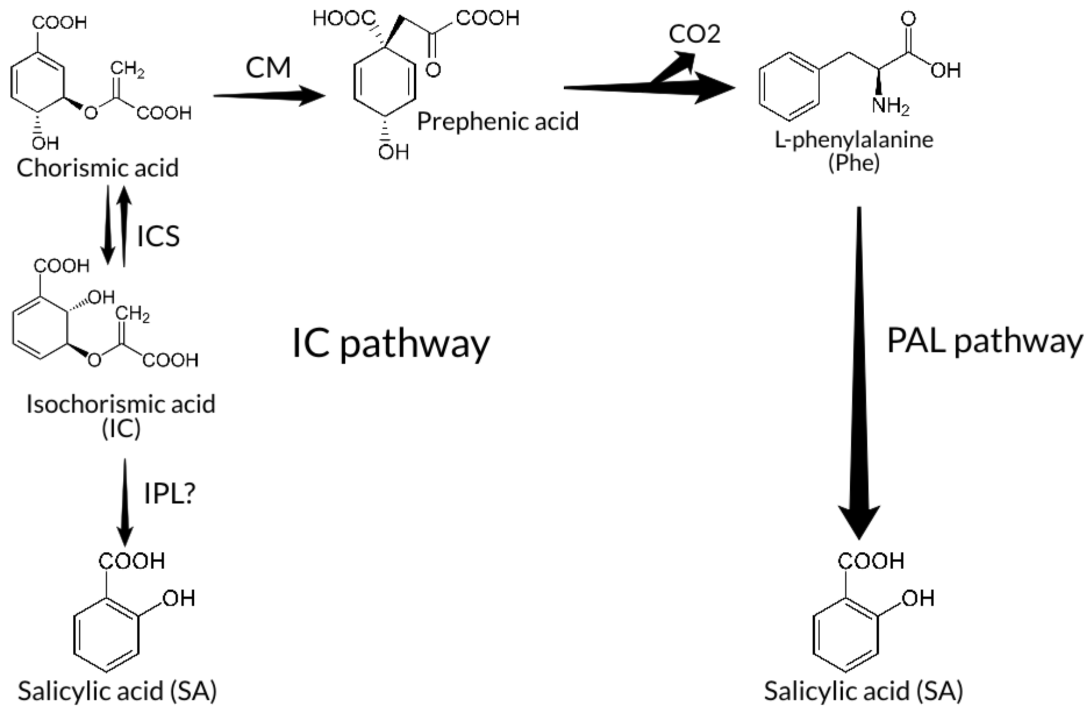


Figure 1.2. SA biosynthesis pathways. Plants have two pathways for SA production, the isochorismate (IC) pathway and the phenylalanine ammonia-lyase (PAL) pathway. Enzymes involved in SA biosynthesis are abbreviated as follows: CM (chorismate mutase), ICS (isochorismate synthase), and IPL (isochorismate pyruvate lyase). Question marks indicate that the enzyme responsible for the indicated conversion has not been identified. Figure modified from Dempsey et al. 2017.

1.2.2.4.1 SA as an antimicrobial and antibiofilm agent

Salicylic acid (SA) is a phytohormone that influences a wide variety of plant processes including development (Vicente and Plasencia 2011) and responses to abiotic stresses (Khan *et al.* 2015). SA also plays a key role in plant responses to pathogens (Vlot *et al.* 2009). In *Arabidopsis*, SA is synthesized in response to pathogens including *P. syringae*, primarily via the isochorismate (ICS) pathway (Nawrath and Mètraux 1999; Wildermuth *et al.* 2001) (Figure 1.2) ICS1 (ISOCHORISMATE SYNTHASE 1) is the major contributor to SA biosynthesis. *Salicylic induction deficient 2* (*sid2)* mutants accumulate little SA in response to foliar pathogens due to a mutation in the ICS1 gene.

Multiple studies have shown that SA exhibits antimicrobial activity and is able to suppress phytopathogen growth *in vitro* (Amborabé *et al.* 2002; Brown *et al.* 2007; Cameron and Zaton 2004; El-Mougy 2002; Georgiou *et al.*2000; Martín *et al.* 2010; Prithiviraj *et al.* 1997; Lowe-Power *et al.* 2016; Yuan *et al.* 2007). However, a large range of effective concentrations was observed, likely due to various experimental conditions and pathogens. A recent study found that incubation of *Pst* in media with 100 to 200 μM SA had approximately 10-fold greater antibacterial activity in hrp-inducing minimal (HIM) medium compared with rich media (Wilson *et al.* 2017). HIM medium mimics the conditions of the intercellular space with a low pH of 5.7 (Jia and Davies 2007) and minimal nutrients. It has been suggested that a low pH may enhance the ability of SA to cross bacterial cell membranes, an ability that may be required for its antimicrobial activity (Amborabé *et al.* 2002). The mechanisms behind the antimicrobial properties of SA are not fully understood, but some studies suggest that it may dissipate the transmembrane proton gradient required for ATP production (Gutknecht 1990; Jörgensen *et al.* 1976; Smith 1959; Normal *et al.* 2004; Stenlid and Saddik 1962) or inhibit respiration (Norma *et al.* 2004) or catalase activity (Chen *et al.* 1993).

Wilson *et al.* (2017) also discovered that *Pst* biofilm formation was reduced *in vitro* at SA concentrations of 2 to 10 μM, whereas antimicrobial activity as determined by *Pst* growth inhibition was observed to occur at higher SA concentrations of 50 to 100 μM. Concentrations of 2 to 10 μM SA were estimated to exist in the intercellular space of mature wild-type plants during ARR, suggesting that intercellular SA accumulation may also have antibiofilm properties. Experiments were also conducted to examine biofilm formation in planta in young and mature plants inoculated with GFP-expressing *Pst.* Aggregate formation defined as a group of tightly packed and immobile cells, was monitored, as well as intercellular SA accumulation (Wilson *et al.* 2017). A reduction in bacterial aggregate formation was observed in mature ARR-competent wild-type plants that suppressed *Pst* growth compared with young susceptible plants, which also accumulated lower levels of intercellular SA. These data suggest that SA accumulation in the intercellular space reduces either *Pst* growth, biofilm formation, or both during ARR (Wilson *et al.* 2017). The antibiofilm properties of SA have also been studied in animal pathogens. *In*

vitro biofilm assays in the presence of SA with animal pathogens such as *P. aeruginosa* and *Staphylococcus aureus* (Prithiviraj *et al.* 2005a and b; Yang *et al.* 2009) indicated that lower concentrations of SA (0.01 to 5 mM SA) reduced bacterial biofilm formation suggesting that lower concentrations of SA have antibiofilm properties.

1.2.3 Systemic Acquired Resistance

Systemic Acquired Resistance (SAR) is a plant defense response induced by an initial priming infection in one part of the plant that leads to broad-spectrum resistance to normally virulent pathogens in distant naïve tissues (Ross 1961). This resistance is long-lasting and can prime the whole plant, (Luna and Ton 2012; Luna *et al.* 2012; Slaughter *et al.* 2012) providing rapid defence upon a secondary infection. Studies using tobacco, cucumber and *Arabidopsis* indicate that SAR occurs in four distinct stages as shown in Figure 1.3: induction, propagation of the long-distance signal, establishment, and manifestation (Champigny and Cameron 2009).

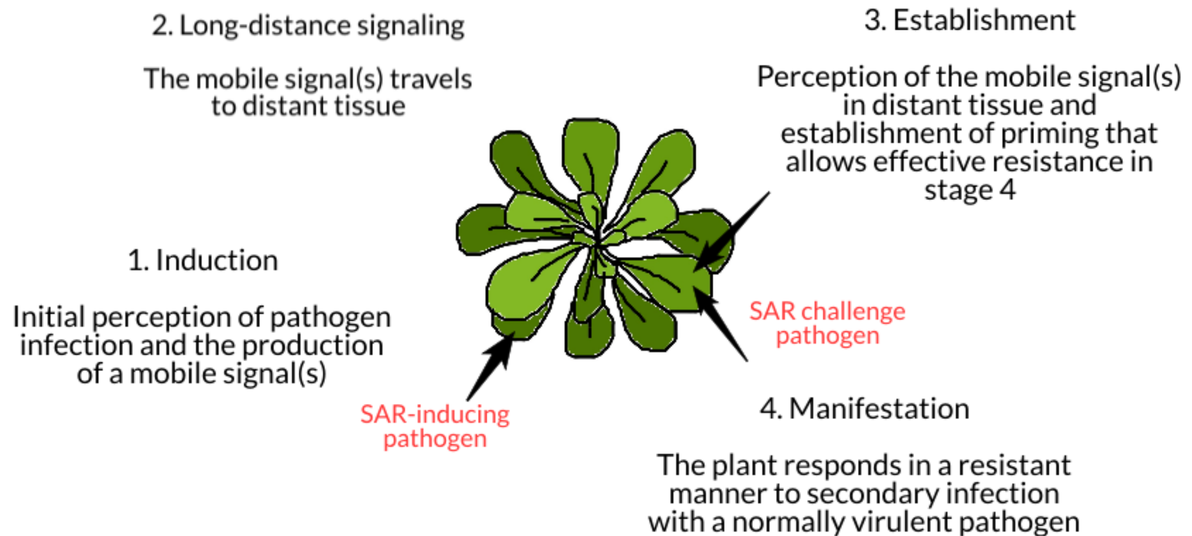


Figure 1.3. The four stages of SAR in Arabidopsis.

1.2.3.1 SAR induction

The SAR induction stage is initiated by pathogen-induced cell death, either caused by ETI-associated HR or by virulent infections that cause necrosis (Reviewed in Kuc 1982; Dong and Durrant 2004). Originally, it was believed that SAR was initiated only in response to pathogen-induced cell death. Since then, there has been evidence that PAMPs elevate SA levels as well as PR gene expression and SAR marker gene *FMO1* expression in distant leaves in the absence of cell death (Mishina and Zeier 2007). However, microscopic cell death was not monitored during this study, therefore it is possible that cell death is still essential for SAR induction. A more recent study demonstrated that fluorescence-labeled flg22 travels to distal tissues, and radiolabeled flg22 is transported via vascular tissue (Jelenska *et al.* 2017), suggesting that the resistance observed in response to flg22 treatment is due to movement of the flg22 PAMP to distant leaves where it initiates PTI, not SAR. During perception of a SAR-inducing pathogen is quickly followed by the accumulation of SA and expression of PR proteins. The degree of SA accumulation and PR gene expression depends on the pathogen used. Some virulent pathogens that suppress plant defense induce less SA accumulation and PR gene expression compared to avirulent pathogens, but SAR is induced in response to both pathogen types (Reviewed in Dong and Durrant 2004). SAR long-distance signals are produced and/or activated and are transported to distant leaf tissue.

1.2.3.2 Propagation of SAR long distance signals

During propagation, mobile signals travel to distant tissue. The *Arabidopsis-Pst* (Cameron *et al.* 1994), tobacco-*Tobacco mosaic virus (TMV)* (van Loon and Dijkstra 1976) and cucumber-*Pseudomonas syringae* pv. *syringae (Pss)* (Smith *et al.* 1991b) models have served as systems for the investigation of SAR mobile signals. Studies conducted by Jenns and Kuc (1979) showed that cucumber contains long-distance graft-transmissible signals that travel from SAR-induced rootstocks (roots) into grafted scions (shoots) to initiate SAR (Jenns and Kuc 1979). The SAR signals likely move through the phloem and/or cell-to-cell. This is supported by experiments in tobacco and cucumber where phloem movement was reduced using a girdling technique, which resulted in a reduced SAR response (Guedes *et al.* 1980; Tuzun and Kuc 1985). Cucumber,

tobacco, and *Arabidopsis* SAR signal movement may take different routes as it takes different lengths of time for SAR signals to travel to distant tissues. For example, cucumber SAR signals are transported very rapidly to distant tissues as demonstrated by cucumber leaf detachment experiments in which SAR signals left SAR-induced local tissues 4 hours after SAR induction and SAR was established by 24 hours in distant tissue (Smith *et al.* 1991a). Whereas in *Arabidopsis*, SAR signals move out of induced leaves within 4-6 hours (Truman *et al.* 2007; Chaturvedi *et al.* 2012) and it takes 36-48 hours to establish the primed state in distant tissues (Cameron *et al.* 1994). These experiments suggest that cucumber SAR signals move rapidly via the phloem while *Arabidopsis* uses a combination of phloem and cell-to-cell movement of SAR signals.

1.2.3.3 SAR establishment in distant tissues

Establishment of SAR involves perception of SAR mobile signals in distant tissue by an unknown receptor which interacts with mobile signals to initiate SAR establishment (Champigny and Cameron 2009). This leads to priming in which the plant responds rapidly and effectively to future pathogen attacks. The mechanisms of priming or the molecular memory of the initial attack are thought to include the accumulation of inactive map kinases MPK3/MPK6 in the cytosol as well as chromatin remodelling at the promoters of defense-related genes (Conrath *et al.* 2015) and modest levels of SA, leading to PR gene expression. This allows for rapid signal amplification and transcription of defense genes upon secondary infection during the manifestation stage.

1.2.3.4 Manifestation of SAR

In the final step, SAR is manifested when the primed plant responds to a secondary infection with a normally virulent pathogen by rapidly initiating SA-mediated signaling in which the transcription factor NPR1 is a critical player. Evidence suggests that SA may bind directly to NPR1 (Wu *et al.* 2012) and its paralogs (Fu *et al.* 2012; Wu *et al.* 2012) to stabilize and help activate NPR1 during SAR. A recent study found that NPR1 paralogs NPR3 and NPR4 work as transcriptional co-regulators to suppress expression of SA-responsive genes (Ding *et al.* 2018). Furthermore, as SA levels increase, SA binds to NPR3/NPR4 resulting in alleviation of defense

gene repression (Ding *et al.* 2018). To confirm if NPR1 is a SA receptor, the SA-binding domain of NPR1 was disrupted and found to no longer promote SA-induced defense gene expression (Ding *et al.* 2018). A number of studies demonstrate that NPR1 exists as a cytosolic oligomer held together by disulfide bonds in healthy uninfected plants. During SAR induction, active NPR1 monomers are released, allowing for NPR1 accumulation and signaling in the nucleus (Tada *et al.* 2008). NPR1 interacts with TGACG Motif-Binding Factor (TG) to promote transcriptional reprogramming (Zhang *et al.* 1999). The targets of NPR1 include PR proteins (14-30 kDa) generally destined for the secretory system for secretion into the apoplast during infection, where they may act as antimicrobial compounds (Wang *et al.* 2005). SAR induces broad-spectrum resistance and can protect the plant against pathogens that are the same or different from the initial pathogen.

1.2.3.5 Cucumber-*Psl* SAR model system

Several pathogens induce SAR in cucumber including *Pseudomonas syringae* pv. *lachrymans* (Caruso and Kuc 1979), *Pseudomonas syringae* pv. *syringae* (*Pss*) (Smith *et al.*, 1991a), *Colletotrichum lagenarium* (Caruso and Kuc, 1979) and tobacco necrosis virus (Jenns and Kuc, 1979). Studies conducted with cucumber demonstrated that SAR long-distance signals were graft transmissible (Jenns and Kuc, 1979). The cucumber-SAR model is also advantageous as concentrated phloem sap can be collected directly from the cut petiole and grafting is much faster and easier due to its large size compared to *Arabidopsis*. Few SAR studies in cucumber were conducted after the *Arabidopsis*-SAR model was developed because cucumber's genome was not sequenced until recently (Huang *et al.* 2009). However, with the newly available genome, the cucumber-SAR model can be further used to study resistance in one of Ontario's popular greenhouse crops (Foodland Ontario, 2019).

1.3 SAR mobile signals

1.3.1 Defective in Induced Resistance 1 (DIR1)

In a genetic screen of a T-DNA-tagged population of Wassilewskija (Ws) *Arabidopsis* plants, *dir1-1 (defective in induced resistance 1-1)* was identified (Maldonado *et al.* 2002). The *dir1-1* mutant was SAR-defective, but still competent in local defense responses such as PTI and ETI, making it the first mutant to be identified that was specifically compromised in SAR (Maldonado *et al.* 2002). The *dir1-1* mutant is competent for SAR induction as shown through an experiment where local accumulation of important defense genes *PR1* and *PR5* were expressed in similar amounts in both the *dir1-1* mutant and wild-type Ws. However, the absence of *PR1* expression in distant leaves suggests that the *dir1-1* mutation negatively affects SAR establishment and that DIR1 may be involved in long-distance signalling or signal perception (Maldonado *et al.* 2002). Furthermore, petiole exudates collected from SAR-induced wild-type plants induced PR1 gene expression when applied to leaves of *dir1-1*, suggesting that *dir1-1* is competent in establishment of SAR in distant tissues when phloem mobile signals are provided (Maldonado *et al.* 2002). These data suggest that DIR1 is involved in SAR long-distance signalling. However, the presence of DIR1 is not sufficient to activate SAR, as transgenic plants expressing high levels of DIR1 do not display enhanced resistance without pathogen exposure (Maldonado *et al.* 2002). This suggests that pathogen exposure is essential for DIR1 activation during SAR (Maldonado *et al.* 2002). DIR1 movement was monitored by probing phloem sap-enriched exudates of local or distant leaves with a DIR1 antibody. Protein gel blot analysis demonstrated that DIR1 accumulates in phloem sap-enriched petiole exudates collected from SAR-induced wild-type, but not mock-induced wild-type or *dir1-1* leaves (Champigny *et al.* 2013), suggesting that DIR1 moves to distant leaves during the induction of SAR. Furthermore, DIR1 was detected in petiole exudates of SAR-induced estrogen-inducible transgenic DIR1-EGFP lines using DIR1 antibody as well as GFP antibody, confirming that DIR1-GFP proteins are moving from the induced leaf to distant leaves during the induction and long-distance signaling stages of SAR (Champigny *et al.* 2013).

1.3.2 The role of SA during SAR

The phytohormone SA is required for the SAR response as SA-deficient mutants such as *sid2* and *NahG* are SAR-defective (Gaffney *et al.* 1993; Delaney *et al.* 1994; Vernooij *et al*. 1994; Lawton *et al*. 1995). Initially, SA was considered to be a candidate for the cucumber and tobacco SAR mobile signal (Malamy *et al*. 1990; Metraux *et al.* 1990). However, tobacco grafting experiments demonstrated that SA-deficient *NahG* roots that were induced for SAR produced mobile signals and SAR was observed in grafted wild-type stems, suggesting that SA is not required for the production or propagation of SAR mobile signals (Vernooij *et al.* 1994). Furthermore, in cucumber experiments, phloem sap was collected from SAR-induced leaves at various time-points (Rasmussen *et al.* 1991) and SA was not detected until 8 hours post-inoculation, while plants whose SAR-induced leaves were severed after only 6 hours established SAR in upper leaves (Rasmussen *et al.* 1991). These results suggest that SAR signals move out of induced leaves before SA does, suggesting that although SA accumulates in induced leaves (Gaffney *et al.* 1993; Delaney *et al.* 1994; Vernooij *et al.* 1994; Lawton *et al.* 1995) and in phloem sap of induced leaves (Maldonado *et al.* 2002 , Vernooij *et al.* 1994), SA is probably not a SAR mobile signal (Rasmussen *et al.* 1991, Vernooij et al., 1994).

Since then, several SAR mobile signal candidates have been proposed, including DIR1 (Maldonado *et al.* 2002), methyl salicylate (MeSA) (Park *et al*. 2007; Vlot *et al.* 2008; Liu *et al.* 2011), a lipid derived glycerol-3-phosphate (G3P) (Chaturvedi *et al.* 2008; Chanda *et al.* 2011), azelaic acid (Aza) (Jung *et al.* 2009), and pipecolic acid (Pip) (Návárová *et al.* 2012).

1.3.3 G3P and glycerol

Glycerol-3-phosphate (G3P) is a precursor molecule in plastid glycerolipid synthesis. Studies with G3P indicate that when mixed with phloem sap-enriched exudates collected from mock-induced and SAR-induced Arabidopsis plants, exudates from wild-type induced resistance in G3P mutants, whereas exudates from glycerol insensitive *gli/nho1* mutants did not induce resistance in wild-type or mutant plants (Chanda *et al.* 2011). This suggests that G3P present in phloem sap is required for SAR in *Arabidopsis*. Additional studies demonstrated that exogenous

application of G3P also induced resistance in soybean (Chanda *et al.* 2011). When total protein extracts from the phloem sap-enriched exudates of mock-induced or SAR-induced *Arabidopsis* were mixed with G3P and infiltrated into wild-type and *dir1-1* mutants, induced leaf exudates with G3P were unable to induce SAR in *dir1-1* plants, whereas induced leaf exudates with G3P were able to induce SAR in wild-type plants, suggesting that SAR induction by G3P requires DIR1 (Chanda *et al*. 2011). To test if G3P movement required DIR1, a study was conducted where exogenously applied radiolabeled G3P did not move on its own but required co-infiltration with recombinant DIR1 protein (Chanda *et al.* 2011). However, recombinant DIR1 protein was produced in *E. coli* in which DIR1 could not obtain its 4 disulfide bonds to create its hydrophobic cavity making it impossible to conclude that G3P requires DIR1 for movement. Additionally, the level of recombinant DIR1 protein applied may have induced cell death, which could have induced SAR. Additional evidence is required to determine if G3P moves to distant tissue and if this movement is dependent on DIR1. Based on these studies, G3P is hypothesized to be a mobile SAR signal (Chanda *et al.* 2011).

1.3.4 Azelaic acid

A study was conducted in which phloem sap-enriched exudates of mock- and SAR-induced wild-type *Arabidopsis* were subjected to gas chromatography and mass spectrometry analysis to identify small molecules involved in SAR. The study revealed that azelaic acid (Aza) was enriched 6-fold in exudates collected from SAR-induced leaves compared to mock-induced leaves (Jung *et al.* 2009). Furthermore, when Aza was sprayed onto plant leaves at concentrations greater than 1 mM, this elicited enhanced resistance to virulent *Pseudomonas syringae* pv. *maculicola DG6* (*Psm*) in both local and distant leaves (Jung *et al.* 2009). To determine if Aza treatment had an effect on defense gene expression, the expression of 464 defense related genes were examined. *AZELAIC ACID INDUCED 1* (*AZI1*) was significantly upregulated in phloem sap enriched exudates of Aza-treated plants. This suggested that *AZI1* could be an important SAR-related gene. In addition, the *azi1* mutant was defective in establishing resistance in response to Aza application, suggesting that *AZI1* function was required for Aza-induced resistance (Jung *et al.* 2009). The *azi1* mutant was defective in SAR but

responded like wild-type to local infections. To determine where in the SAR pathway *AZI1* functions, phloem sap-enriched exudates collected from SAR-induced *azi1* were applied to wild-type Col-0 and SAR was not induced, however SAR-induced wild-type exudates applied to *azi1* induced SAR. Therefore, the *azi1* mutant was competent for SAR induction, but defective in establishing SAR in distant tissues in response to pathogen infection. This suggests that AZI1 is involved in production or translocation of SAR long-distance signals. To determine if AZI1 interacts with potential mobile SAR signal DIR1, bimolecular fluorescence complementation (BiFC) and co-immunoprecipitation experiments were performed and determined that DIR1 and AZI1 form homo and heterodimers in *Nicotiana benthamiana* (Yu *et al.* 2013). Furthermore, overexpression of *DIR1* in *azi1* and overexpression of *AZI1* in *dir1-1* complemented the SAR-defective phenotypes of these mutants (Yu *et al.* 2013). This suggests that DIR1 and AZI1 interaction is needed for the production or translocation of the SAR signal.

1.3.5 Pipecolic acid

A study comparing changes in free amino acid content in SAR-induced *Arabidopsis* leaves revealed an accumulation of lysine, as well as a 70-fold increase in the lysine catabolite, pipecolic acid (Pip) (Návárová *et al.* 2012). Pip levels were also elevated in leaves inoculated with avirulent *Psm ES4326* or the PAMP flg22, suggesting that Pip levels are elevated in response to pathogen perception. Furthermore, this study demonstrated that Pip accumulated in exudates collected from SAR-induced leaves, but not mock-induced leaves. Pip levels were also 10-fold higher in distant leaves of SAR-induced versus mock-induced plants. This suggests that Pip accumulates in induced leaves and is transported to distant leaves during SAR. Since these experiments found that endogenous Pip is important during SAR, Návárová *et al.* (2012) decided to examine if exogenous application of Pip would induce resistance. Watering plants with Pip resulted in resistance to virulent *Psm* in leaves. Pip application did not enhance resistance in *sid2-1* and *npr1-2* mutants, suggesting that Pip functions upstream of SA and NPR1 during SAR establishment and manifestation (Návárová *et al.* 2012). To further understand the role of Pip accumulation during SAR, a Pip-deficient mutant was identified (Návárová *et al.*

2012). The SAR-defective mutant *agd2-like defense response protein 1 (ald1)* produced little Pip in untreated or SAR-induced leaves. The ALD1 aminotransferase is known to convert lysine to Pip *in vitro* and it is hypothesized that ALD1 is responsible for converting lysine to Pip during SAR. Treatment of *ald1* mutants with Pip led to the restoration of the SAR response to *Psm*, and Pip was detected in distant tissue (Návárová *et al.* 2012). This suggests that Pip may be a mobile SAR long-distance signal.

Recent studies have shown that FLAVIN- DEPENDENT MONOOXYGENASE 1 (FMO1) catalyzes the reaction in which Pip is converted to N-hydroxy-pipecolic acid (N-OH-Pip) (Chen *et al.* 2018, Hartmann *et al.* 2018). To test if like Pip, N-OH-Pip is sufficient to induce SAR, SAR-deficient *fmo1* mutants and wild-type Col-0 were treated with 1 mM Pip, 1 mM N-OH-Pip or mock-treated (Chen *et al.* 2018). When challenge-inoculated with *Psm*, both *fmo1* and Col-0 plants treated with N-OH-Pip displayed SAR as demonstrated by reduced bacterial growth compared to mock-treated or Pip-treated plants. This suggests that N-OH-Pip, and not Pip itself, may be the key to Pip-induced SAR, and FMO1 is required.

Hypothesis and objectives

Chapter 2 – Investigation of SAR-associated small molecules as inducers of resistance in cucumber

Hypotheses

1) Small molecules involved in the SAR pathway such as G3P, azelaic acid, and pipecolic acid induce resistance in cucumber

Objectives

1) Develop assays to examine chemical induction of resistance in cucumber using glycerol, azelaic acid, pipecolic acid
2) Investigate which pathways are involved in chemical induction by looking at SAR-associated gene expression
3) Optimize treatment methods to enhance chemically-induced resistance in cucumber (strength and length of response)

Chapter 3 – Investigation of biofilm formation by *Pseudomonas syringae* pv. *tomato* in *Arabidopsis*

Hypotheses

1) The ability to form biofilms contributes to successful infection by *Pst*
2) SA accumulation in intercellular spaces contributes to suppression of biofilm-like aggregate formation of *Pst* during the PTI response

Objectives

1) Investigate if *Pst* aggregates contribute to pathogenicity and bacterial success by examining aggregate size and number
2) Investigate if aggregates are biofilms
3) Examine if PTI-induced intercellular SA acts to limit pathogen growth and biofilm formation

Chapter 2—Investigation of SAR-associated small molecules as inducers of resistance in cucumber

2.1 Preface

Several SAR-associated small molecules have been identified and studied as potential inducers of SAR in *Arabidopsis* (Jung *et al.* 2009; Návárová *et al.* 2012; Chanda *et al.* 2007). Cucumber is an important crop in Ontario, and as such, it is important to minimize crop loss due to bacterial and fungal pathogens. Exogenous application of SAR-associated small molecules such as glycerol, azelaic acid and pipecolic acid could potentially induce whole-plant broad-spectrum resistance. In this study, assays were developed to test glycerol, azelaic acid, and pipecolic acid as inducers of local and systemic resistance in cucumber cultivar Wisconsin SMR 58, and the pipecolic acid assay was further optimized to induce a strong resistance response.

2.2 Author contributions

Angela Fufeng (AF), Christine Kempthorne (CK), Manreet Dhaliwal (MD), Ramzy Badrous (RB), Garrett Nunn (GN), Matei Dan-Dobre (MDD) and Natalie Belu (NB) performed the experiments. Experiments were conceived of and developed by Robin Cameron (RC), AF, GN and MD. CK performed biological SAR and glycerol experiments shown in Figures 2.1, 2.2, and 2.3. MD, RB, and AF performed glycerol and some pipecolic acid experiments shown in Figures 2.3, 2.5, and 2.6. MD performed azelaic acid experiments shown in Figure 2.4. GN and MDD performed pipecolic acid experiments shown in Figure 2.7. NB performed pipecolic acid experiments shown in Figure 2.8 and 9.

2.3 Development of biologically-induced cucumber SAR assays

Researchers began studying SAR in cucumber in the early ‘90s and developed a cucumber SAR model in which inoculation with *Pss D20* in local leaves induced resistance and SAR-associated gene expression in distant leaves to normally virulent *Colletotrichum* fungal pathogens (Smith *et al.* 1991). Therefore, *Pss D20* was used along with virulent *Psl* 8003 to

develop a cucumber bacterial SAR model in the Cameron lab. Cucumber plants were inoculated with avirulent *Pss D20 (*5x10^{7} cfu/ml), virulent *Psl 8003* (10^{6} cfu/ml) or mock-induced with 10 mM $MgCl_2$ on 2 lower leaves. At 48 hours post-induction (hpi), 4 to 5 upper leaves were challenge-inoculated with virulent *Psl 8003* (10^{6} cfu/ml). *In planta Psl* levels were quantified 72 hours after challenge inoculation in distant leaves. A significant 3-fold reduction in bacterial levels was observed in plants induced with *Psl* compared to mock-induced plants, while a significant 11-fold reduction in bacterial levels was observed in plants induced with *Pss* compared to mock-induced plants (Figure 2.1). This experiment was repeated 2 additional times with similar results observed (Figure A1) indicating that systemic resistance was induced by both *Psl 8003* and *Pss D20*.

A challenge inoculum dose of 10^{6} cfu/ml *Psl 8003* could overwhelm the SAR response, therefore a number of experiments were performed to determine the optimum challenge inoculum dose. A 2.5-fold reduction in bacterial levels was observed in plants challenge-inoculated with a high dose of 10^{6} cfu/ml *Psl* compared to mock-induced plants (Figure 2.2), while a 7-fold reduction in bacterial levels was observed when plants were challenge-inoculated with a low dose of 10^{5} cfu/ml *Psl* compared to mock-induced plants (Figure 2.2). Based on these experiments, the lower 10^{5} cfu/ml inoculum dose was used in subsequent experiments.

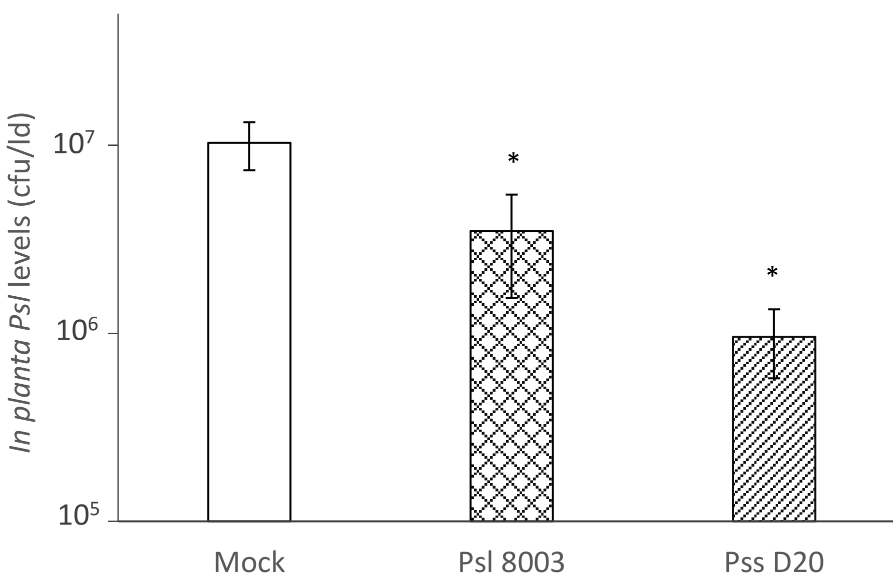


Figure 2.1. Cucumber biological SAR-inducers. Cucumber plants were induced by inoculating with 5×10^7 cfu/ml *Pss D20* or 1×10^6 *Psl 8003* or mock-induced with 10 mM $MgCl_2$ and then challenge-inoculated in distant leaves with 1×10^6 cfu/ml of *Psl 8003* 48 hpi. Bacterial levels were quantified 72 hours after challenge inoculation. * $p < 0.05$, Student's t-test. Repeated with similar results in 2 additional experiments (Figure A1).

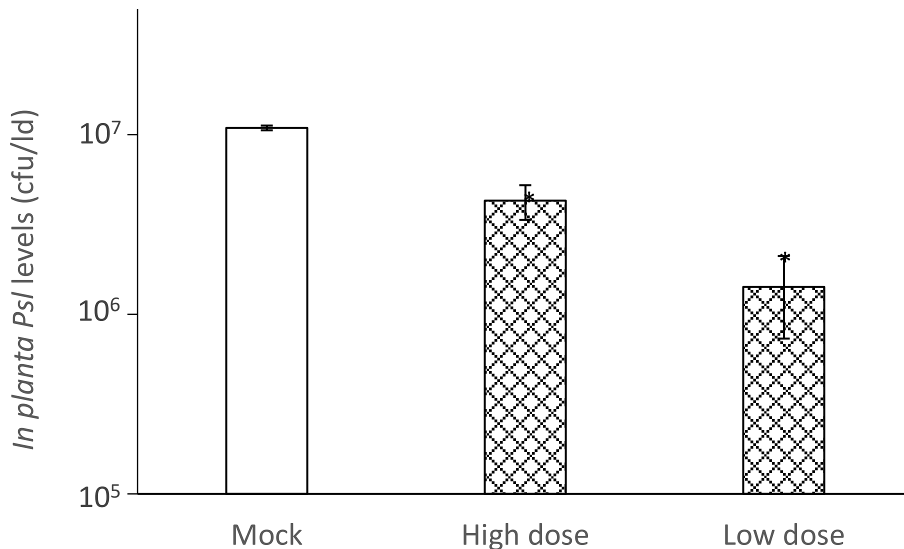


Figure 2.2. High dose and low dose challenge-inoculation with *Psl 8003.* Cucumber plants were induced by inoculating with 10^6 *Psl 8003* or mock-induced with 10 mM $MgCl_2$, followed by challenge-inoculation with 10^6 cfu/ml (high dose) of *Psl 8003* or 10^5 cfu/ml (low dose) of *Psl 8003* 48 hpi. Bacterial levels were quantified 72 hours after challenge. * $p < 0.05$, Student's t-test.

2.4 Glycerol treatment induces resistance in cucumber

After optimizing the bacterial biological SAR assay in cucumber, it was now possible to examine the ability of SAR-associated small molecules to induce SAR in cucumber. The ability of these molecules to induce resistance when applied directly into leaves or sprayed on one or two leaves with subsequent challenge inoculation in distant naïve leaves, was determined. In terms of protecting crops from disease in the future, identifying small molecules that induce resistance in systemic tissues would be beneficial.

Experiments with cocoa plants demonstrated that spray application of glycerol onto cacao leaves for three consecutive days induced resistance in sprayed leaves to *Phytophthora capsica* (Zhang *et al.* 2015). Spraying is a popular delivery system and may be more feasible than infiltration for crops grown in greenhouse environments (University of Massachusetts Amherst, 2019). Experiments similar to those conducted by Zhang *et al.* (2015) were performed to determine if glycerol treatment initiates resistance in cucumber leaves. Cucumber plants were treated with various concentrations of glycerol by spraying leaves on 3 consecutive days prior to challenge-inoculating treated leaves with *Psl 8003* (10^5 cfu/ml). This experiment was repeated 7 times between February and June 2016 and in 6 of 7 experiments, glycerol treatment induced resistance in treated leaves (Figure A2). An example is presented in Figure 3A in which plants were sprayed with 50 mg/l of benzothiadiazole (BTH), a strong SAR inducer (Lawton *et al.* 1996) to act as a control for resistance, or mock-sprayed with water as a control for disease, followed by challenge-inoculation as described as follows. Experimental plants were sprayed with 100 mM glycerol or mock-sprayed with water for 3 consecutive days prior to challenge with 10^5 cfu/ml *Psl.* Glycerol-treated plants displayed a 2-fold reduction in bacterial levels compared to mock-treated plants, and BTH- treated plants supported 26-fold fewer bacteria compared to mock-treated plants (Figure 2.3A). Higher glycerol concentrations were used to determine if the strength of the induced resistance response could be enhanced. Cucumber plants were sprayed with glycerol (100, 150, 200 mM) or mock-sprayed with water for 3 consecutive days prior to challenge inoculation with 10^5 cfu/ml *Psl*. A three- and six-fold reduction in bacterial levels was observed in plants treated with 100 or 150 mM glycerol, respectively, compared to mock-treated plants (Figure 2.3B). Glycerol treatments with 200 mM glycerol initiated modest resistance in 2 of 3 experiments (≤2.4-fold compared to mock-treated plants) (Figure A2). Considered together, these experiments indicated that 100 mM glycerol treatments were sufficient to induce resistance in treated leaves and a higher glycerol concentration did not enhance the level of resistance observed.

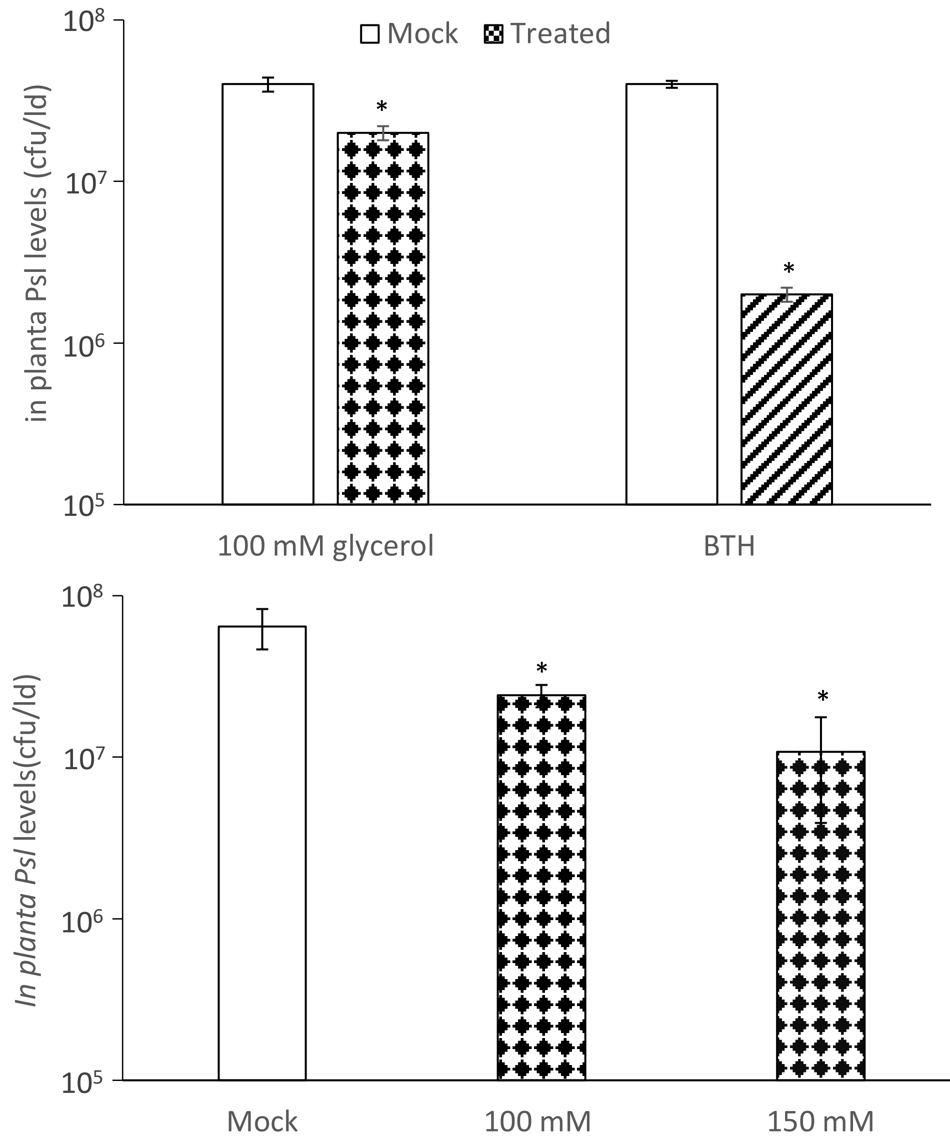


Figure 2.3. Spray application of glycerol induces resistance in treated leaves. A) Cucumber leaves were sprayed with 100 mM glycerol or mock-sprayed with water for 3 consecutive days prior to challenge-inoculation with 10^5 cfu/ml *Psl 8003* or sprayed with 50 mg/l BTH or water 1 day prior to challenge-inoculation with 10^5 cfu/ml *Psl 8003.* B) Cucumber leaves were sprayed with 100 mM or 150 mM glycerol or mock-sprayed with water for 3 consecutive days prior to challenge with 10^5 cfu/ml *Psl 8003*. * $p < 0.05$, Student's t-test. These experiments were repeated 5 additional times (Figure A2).

In planta, glycerol is phosphorylated to create G3P, which has been identified as a potential long-distance SAR signal and inducer of SAR (Chanda *et al.* 2011). Therefore, application of glycerol into local leaves could induce resistance in systemic naïve leaves. To

address this question, individual cucumber leaves were sprayed once or twice on two consecutive days prior to challenge-inoculation of distant naive leaves with 10^5 cfu/ml *Psl*. Spraying with glycerol one time did not induce resistance (Figure A3), while two consecutive sprays with glycerol induced resistance in distant leaves in 1 of 4 experiments (Figure A4). These data indicated that spray applications of glycerol rarely induced systemic resistance in cucumber.

2.5 Azelaic acid treatment induces resistance in cucumber

Azelaic acid (Aza) accumulates in phloem-sap-enriched exudates collected from SAR-induced plants, leading the authors of this study to hypothesize that Aza is an important long-distance signal during SAR (Jung *et al.* 2009). Furthermore, application of Aza to local leaves induced resistance in both treated local and naïve systemic leaves in *Arabidopsis* (Jung *et al.* 2009). To examine if Aza application also induces resistance in cucumber plants, 0.1 mM Aza dissolved in 5 mM MES or 5 mM MES (mock-induced control) was infiltrated into cucumber leaves one day prior to challenge-inoculation of the same leaves with 10^5 *Psl*. A 4-fold reduction in bacterial levels was observed in plants treated with Aza compared to mock-induced plants (Figure 2.4A). To test if higher concentrations of Aza enhanced the strength of the resistance response, leaves were infiltrated with 1 mM, 2 mM Aza, or 5 mM MES and a 2-fold reduction in bacterial levels was observed in Aza-treated versus mock-induced plants for all concentrations (Figure 2.4B) indicating that higher concentrations of Aza treatments did not enhance resistance. Seven similar experiments using varying concentrations of Aza were performed. Aza treatment induced resistance in treated leaves by 1.5- to 10-fold compared to mock-induced in 4 of 7 experiments (Figure A5) indicating that Aza treatment enhanced resistance to varying degrees in cucumber plants.

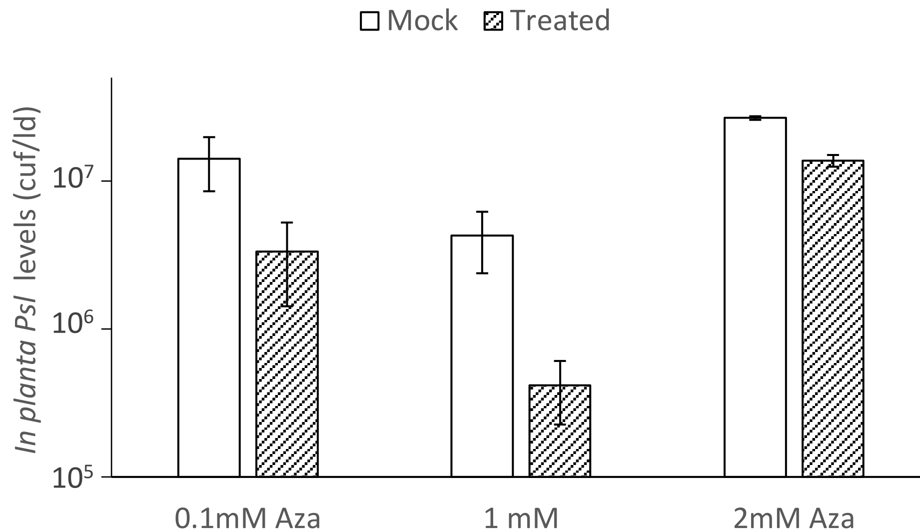


Figure 2.4. Resistance induced in leaves treated with azelaic acid. Cucumber plants were infiltrated with 0.1 mM, 1 mM, 2mM Aza or 5 mM MES (mock treatment), 24 hours prior to challenge inoculation with10^5 cfu/ml *Psl 8003* in 3 separate experiments.* $p < 0.05$, Student's t-test. These experiments were repeated 4 additional times (Figure A5).

Given that a number of experiments provide evidence that Aza may be a SAR mobile signal (Jung *et al.* 2009, Yu *et al.* 2013), an experiment was conducted to investigate if application of Aza to lower leaves leads to systemic resistance in upper naïve leaves challenged with *Psl*. Bacterial levels were similar in distant leaves in plants whose lower leaves were treated with Aza compared to mock-treated plants (Figure A6) indicating that Aza treatment of local leaves did not induce resistance in systemic naïve leaves of cucumber.

Pesticides are often delivered via spraying in the greenhouse industry (University of Massachusetts Amherst, 2019), therefore Aza spray treatments were performed to determine if this method was able to induce resistance in cucumber. In two separate experiment, resistance was not induced when the entire plant or only a lower leaf was sprayed with 1 mM or 2 mM Aza compared to mock-treated plants (Figure A7).

2.6 Pipecolic acid (Pip) treatment induces systemic resistance in cucumber

Studies with Arabidopsis demonstrated that Pip-deficient *ald1* mutants were defective for the SAR response and exogenous application of Pip restored SAR in these mutants (Návárová *et al.* 2012). Furthermore, exogenous application of Pip to roots greatly enhanced resistance in the leaves of wild-type Arabidopsis (Návárová *et al.* 2012). Based on these SAR studies in *Arabidopsis*, Pip treatment of cucumber plants may induce resistance in cucumber. Cucumber plants were treated with pipecolic acid by soil-drenching or mock-drenching with water. In Figure 2.5A, plants were treated with 60 ml of 10 mM pipecolic acid 24 hours prior to challenge-inoculation with *Psl* (10^5 cfu/ml). Bacterial levels in Pip-treated plants were reduced by 5-fold compared to mock-treated plants. Plants treated with 60 ml of 20 mM, 50 mM, or 100 mM pipecolic acid or water, supported a 6-, 5-, and 4.5-fold reduction in bacterial levels respectively, compared to mock-treated plants (Figure 2.5B). Variations of this experiment were repeated 11 times from March 2015 to February 2018 and Pip treatment induced resistance in 6 of 11 experiments (Figure A8). Based on these data 10 mM Pip treatment was sufficient to induce resistance and higher concentrations did not enhance the level of resistance observed.

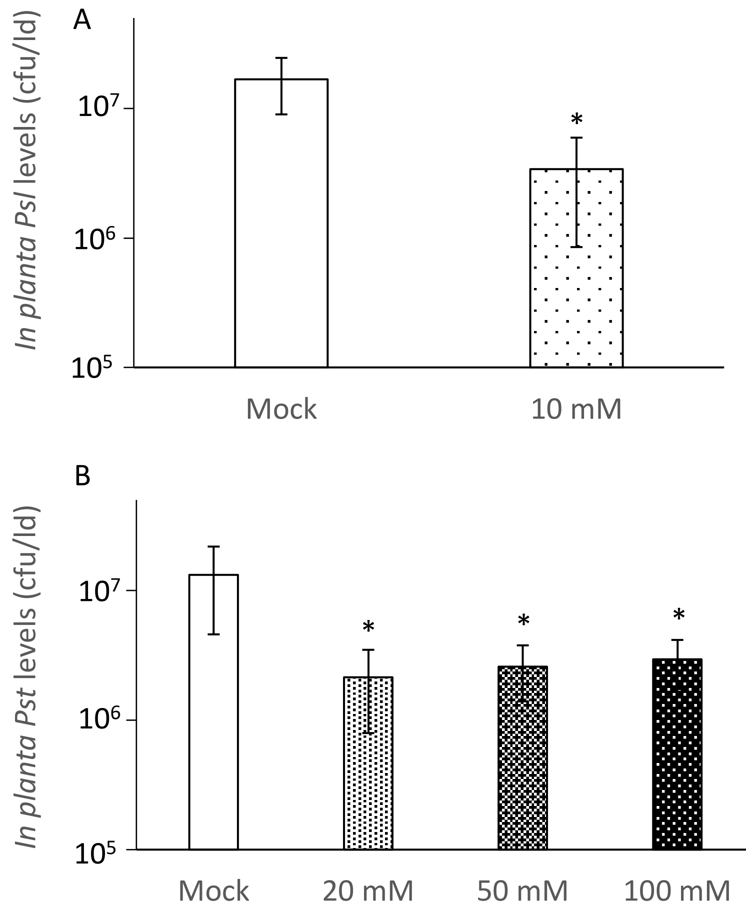


Figure 2.5. Pipecolic acid treatment induces resistance in leaves.
Plants were treated with Pip by soil-drenching with A) 60 ml of 10 mM pipecolic acid or water (mock-treatment) or B) 20, 50, 100 mM pipecolic acid or water. * $p < 0.05$ (Student's t-test). These experiments were repeated 9 additional times (Figure A8).

To determine if a stronger resistance response could be induced by saturating the soil with Pip solution, plants were soil-drenched with Pip until the solution was leaking from the bottom of the pots. Plants were watered to saturation with 100 mM Pip or water, 24 hours prior to challenge-inoculation with *Psl* (10^5 cfu/ml). Bacterial levels were reduced by 7-fold in Pip- compared to mock-treated plants (Figure 2.6A). In an additional experiment, plants were watered to saturation with 20 mM or 100 mM Pip or water, 24 hours prior to challenge with *Psl* and a 3- and 2-fold reduction in bacterial levels was observed in Pip- compared to mock-treated plants (Figure 2.6B). Variations of this experiment were repeated another 7 times between July

and November 2017. In 4 of 7 experiments, Pip single saturation treatments enhanced resistance by 2- to 7-fold (Figure A9). Taken together these data indicate that the lowest concentration of 20 mM was sufficient to induce resistance and treatment with higher concentrations did not further enhance resistance. Additionally, the level of resistance observed in plants treated to saturation or with 60 ml of Pip solution was similar indicating that both methods induce resistance. Moreover, increasing the volume of Pip solution during treatment of the roots by soil-drenching did not induce a stronger resistance response.

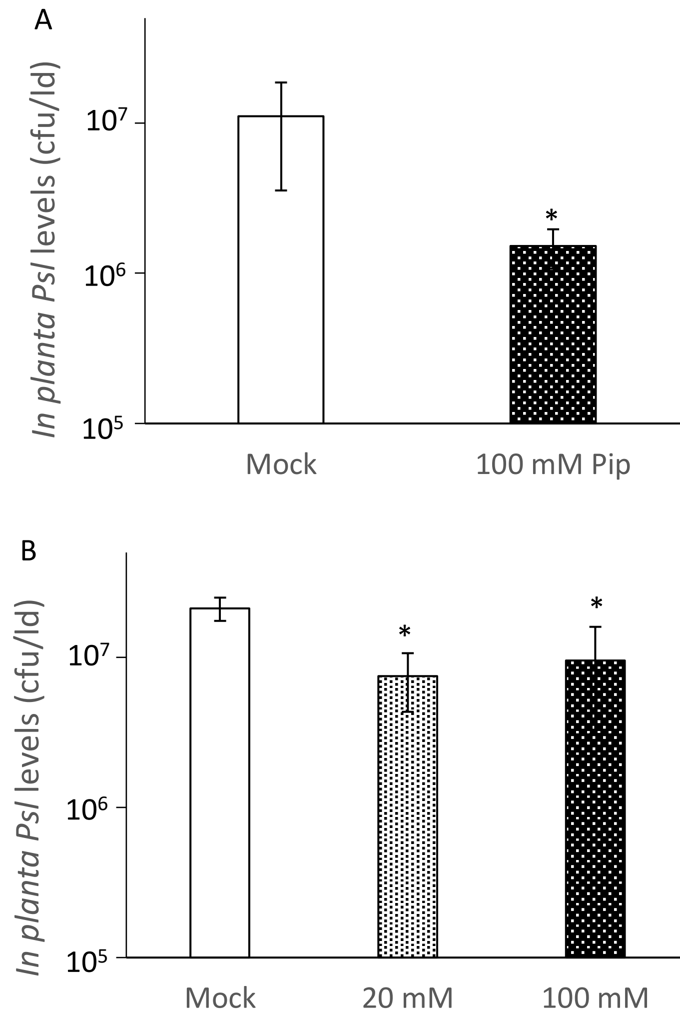


Figure 2.6. Pipecolic acid single saturation treatment of roots induces resistance in cucumber leaves. A) Plants were watered to saturation with 100 mM Pip or water or B) with 20, 100 mM Pip or water. * $p < 0.05$ (Student's t-test). These experiments were repeated 5 additional times (Figure A9).

2.6.1 Duration of Pip-induced resistance

To optimize when the treatment should be applied, or how often plants should be treated in a greenhouse, it is important to determine the duration of Pip-induced resistance. To investigate this, cucumber plants were treated by soil-drenching, 1, 3 or 7 days prior to challenge-inoculation with *Psl* (10^5 cfu/ml). Resistance was observed in 2 of 5 experiments performed between May 2017 and March 2018, in which Pip treatment occurred 1 day prior to challenge inoculation or when treatment occurred 3 or 7 days prior to challenge (Figure A10). In Figure 2.7, plants were treated with 20 mM Pip 1, 3, or 7 days prior to challenge. There was a significant 2.5-fold reduction in bacterial levels in plants treated 1 day prior to challenge compared to mock-treated plants. There was also a significant 7.5-fold reduction in bacterial levels in plants treated 3 days prior to challenge compared to mock-treated plants (Figure 2.7A). These data (Figure 2.7, Figure A10) indicate that in some experiments Pip-induced resistance lasted 3 days and in others it lasted 7 days. Although it sometimes appeared that Pip did not induce resistance, a closer look at individual plants revealed that there was variation in response to treatment between plants. Although it appeared that the 7-day induction treatment did not induce resistance (Fig 2.7A), when looked at individually, there was a significant reduction in one of the Pip-treated plants compared to the mock control (Fig. 2.7B). This suggests that Pip treatment induced resistance in some plants and not in others.

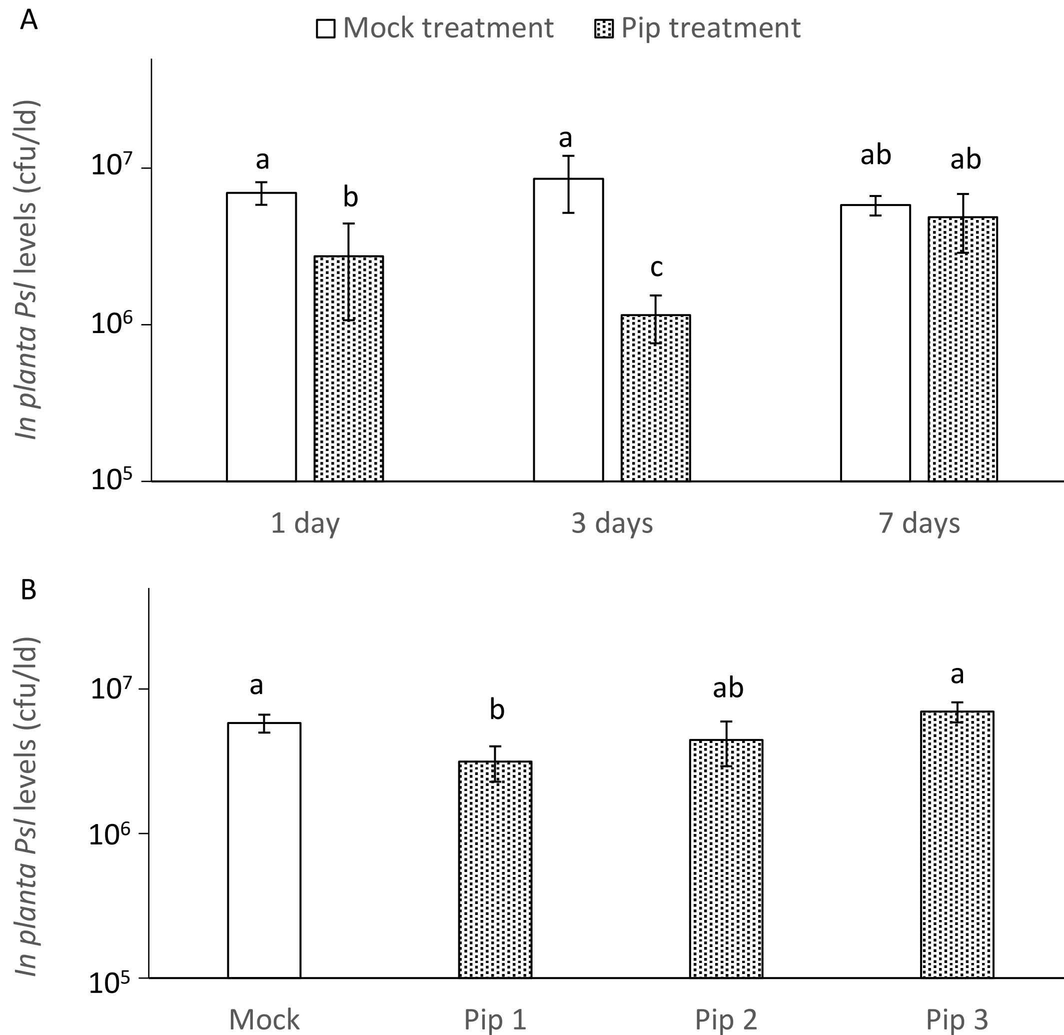


Figure 2.7. Pip-induced resistance 1, 3, or 7 days after treatment. A) Cucumber plants were watered with 20 mM Pip or water as a mock 1 day, 3 days, or 7 days prior to challenge with 1x10^5 cfu/ml *Psl 8003*. B) Cucumber plants treated with Pip or water 7 days prior to challenge presented as individual plants. ANOVA, Tukey's HSD. This experiment was repeated 6 additional times (Figure A10).

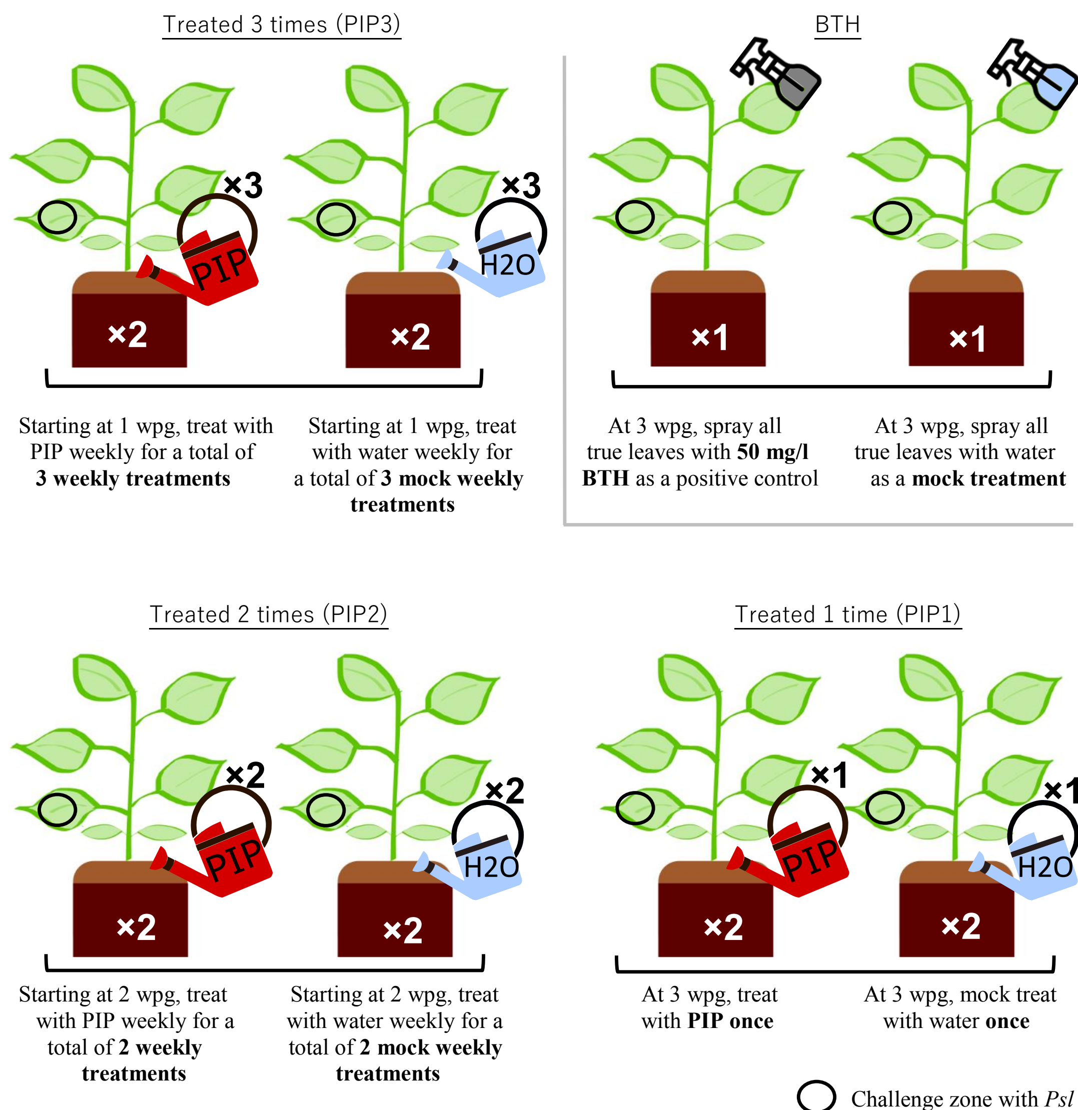


Figure 2.8. Pip-induced resistance assay to investigate the strength of resistance induced by 1, 2 or 3 weekly treatments. The PIP3 group was treated with PIP once per week for 3 weeks, the PIP2 group was treated twice per week for two weeks, and the PIP1 group was treated once per week for once week. Mock treatments consisted of 60 ml of ultrapure water applied on the same schedule as the corresponding PIP treatment. As a positive control, the adaxial and abaxial surfaces of all true leaves were sprayed with a solution of 50 mg/l BTH in ultrapure water 1 day prior to challenge (BTH plant). The first true leaf of all plants was inoculated with 10^5 cfu/ml *Psl* 8003 1 day after the final treatments. Image from Belu 2019 (unpublished).

2.6.2 Investigation of multiple Pip treatments

It was hypothesized that multiple Pip treatments induce a stronger systemic resistance response in cucumber leaves. Plants were watered to saturation or with 60 ml of Pip for 3 consecutive days prior to challenge inoculation with *Psl* (10^5 cfu/ml). Bacterial levels in plants treated with Pip were similar to mock-treated plants (Figure A11) indicating that in this experiment, consecutive treatments over three days did not induce resistance. It is possible that 3 consecutive days of Pip treatment led to root waterlogging stress and an inability to initiate Pip-induced resistance. To prevent waterlogging of the soil, plants were treated once per week over three weeks. Plants were watered with 60 ml of Pip once a week for 1, 2 or 3 weeks, followed by challenge inoculation with *Psl* (10^5 cfu/m) one day after the weekly treatment (see diagram in Figure 2.8). In Figure 2.9A, a positive control for resistance was included by spaying plants with BTH and water 24 hours prior to challenge, as a negative control for disease. Plants were watered with 200 mM Pip once per week for 1, 2 or 3 weeks and challenge-inoculated with *Psl* one day post Pip treatment. BTH treatment induced a 21-fold reduction in bacterial levels compared to plants sprayed with water. Plants treated once with Pip supported similar levels of bacteria, while bacterial levels were reduced by 3- and 6-fold in plants treated weekly with Pip two or three times, respectively, compared to the respective mock-treated plants. Variation in resistance in plants subjected to weekly treatments two or three times was examined by looking at the levels of resistance in individual plants in this experiment. The Pip 2 plant displayed a ~28-fold reduction in bacterial levels compared to Mock 1 or 2 plants, while the Pip 1 plant supported similarly high *Psl* growth as Mock 1 or 2 plants (Figure 2.9B), indicating variation among cucumber plants in terms of their ability to display Pip-induced resistance.

This experiment was repeated 6 times (Figure A12) during the summer and fall of 2018 with similar results. It is interesting to note that treatment followed the next day by challenge with *Psl,* was never observed to induce resistance in the 2018 experiments, whereas resistance was induced in experiments in 2017 (Figures 2.5 and 2.6). This suggests that plants respond differently year to year due to changing environmental conditions.

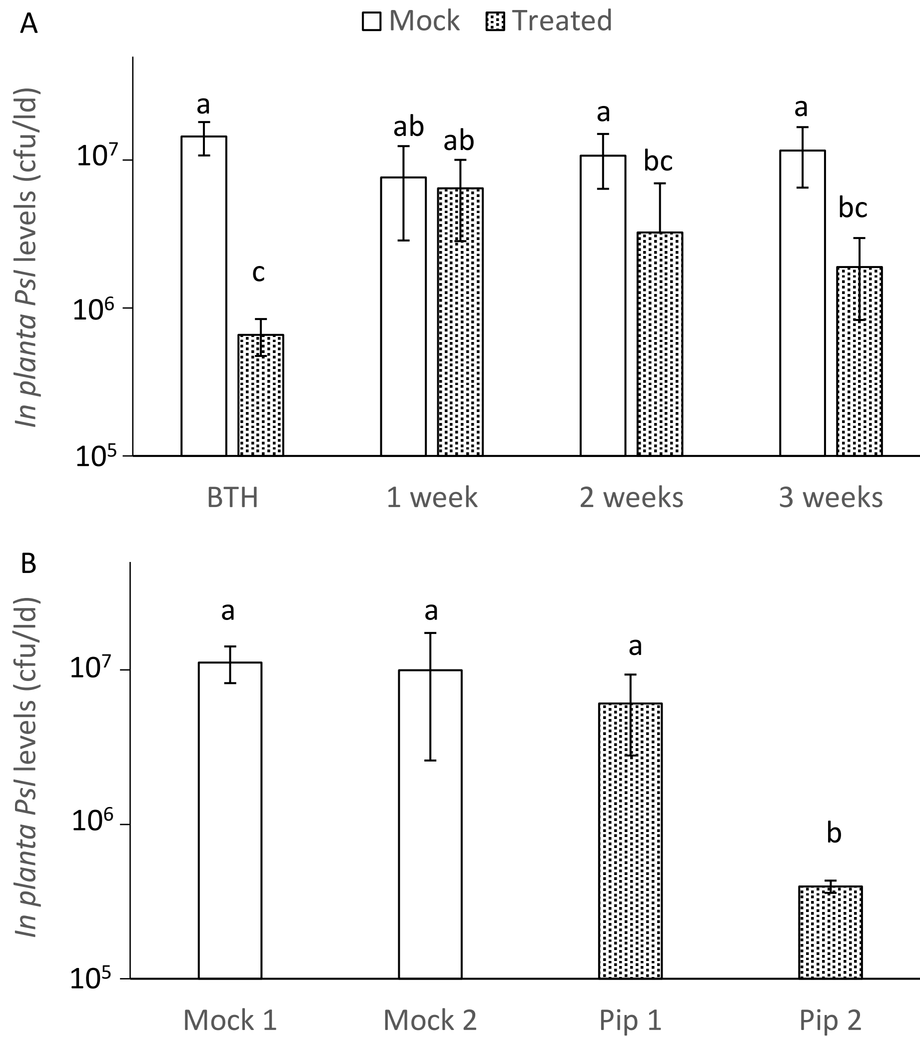


Figure 2.9. Multiple weekly Pip treatments induce resistance in cucumber. A) Cucumber plants were sprayed with 50 mg/l BTH or water 24 hours prior to challenge or watered with 200 mM Pip for 1, 2, or 3 weeks prior to challenge with 10^5 cfu/ml *Psl 8003*. B) Cucumber plants treated with Pip (Pip1, Pip2) or water (Mock 1, Mock2) for 2 weeks prior to challenge, presented as individual plants. This experiment was repeated 5 additional times (Figure A12).

Chapter 3

Investigation of biofilm formation by *Pseudomonas syringae* pv. *tomato* in *Arabidopsis*

3.1 Preface

A number of plant species including Arabidopsis display Age-Related Resistance (ARR) such that mature plants become highly resistant to certain pathogens that they were susceptible to when young. *In vitro* and *in vivo* studies suggest that SA accumulates in the intercellular space where it acts as an antibiofilm agent against *Pseudomonas syringae* pv. *tomato* during ARR (Carviel *et al.* 2014, Wilson *et al.* 2017). Although understudied in plant-bacteria interactions, there is some evidence to support the idea that biofilm formation is important for successful colonization of the plant intercellular space (Dow *et al.* 2003, Aslam *et al.* 2008, Schenk *et al.* 2008, Yu *et al.* 2009). These studies led us to investigate if *Pst* forms biofilm-like aggregates during infection of young susceptible *Arabidopsis* and if the PTI disease resistance response negatively impacts Pst biofilm formation and pathogenicity.

3.2 Author contributions

AF, Noah Xiao (NX), GN, and Abdul Halim (AH) performed the experiments. AF and NX performed all bacterial growth and aggregation formation experiments shown in Figures 3.1, 3.3, 3.4, 3.6 and 3.7. NX transformed GFP-expressing alginate mutant *Pst*. AH conducted aggregate size analysis on ImageJ, which was used for Figure 3.5. Experiments conceived and developed by RC, AF and NX.

3.3 Bacterial aggregate formation is associated with successful infection by *Pst*

There is some evidence supporting the idea that *Xanthamonas* and *Pseudomonas* species use biofilm formation as a tool for infection of host plants (Dow *et al.* 2003, Aslam *et al.* 2008, Schenk *et al.* 2008, Yu *et al.* 2009). Therefore, the contribution of biofilm formation to successful infection by *Pst* was examined using a GFP-expressing *Pst* strain to allow visualization of bacterial cells during infection of plants. Leaves were infiltrated with a 100 mM flg22 peptide solution to induce the PTI resistance response or mock-induced with water to examine the

susceptible interaction. One day later, these same leaves were inoculated with virulent GFP-expressing *Pst,* followed by determination of bacterial levels 3 days later in susceptible (mock-induced) and PTI-induced (flg22-induced) leaves. A 52-fold reduction in bacterial levels was observed in PTI- compared to mock-induced plants (Figure 3.1A) indicating that PTI was successfully established in this experiment. Bacterial behaviour was also monitored in this experiment by microscopic examination of GFP fluorescent bacteria in leaf intercellular spaces using epifluorescence microscopy. Bacterial cells were classified as aggregates (biofilm-like), defined as immobile and tightly grouped cells or planktonic defined as single free-swimming cells. Each microscopic field was viewed and classified as containing no bacteria, planktonic bacteria, bacterial aggregates, or both planktonic and bacterial aggregates, examples can be seen in Figure 3.2. In susceptible mock-induced plants, 20% of the fields of view contained planktonic bacteria and 80% had both planktonic and aggregated bacteria. In PTI-responding plants (flg22-induced), bacterial cells were not observed (Figure 3.1B). This was repeated 8 additional times with similar results (Figure A13). In 5 of these 9 experiments, aggregates were occasionally observed in PTI-responding leaves, suggesting that the strength of the PTI response varies between experiments. However, when aggregates were observed in PTI-responding leaves, these aggregates appeared to be smaller in size than aggregates found in susceptible plants (Figure 3.2), suggesting that the PTI response reduces the size of aggregates in addition to reducing aggregate formation. Overall, these data indicate that a successful infection by *Pst* (high bacterial levels) is associated with the formation of aggregates, and in PTI-responding leaves, *Pst* forms fewer aggregates.

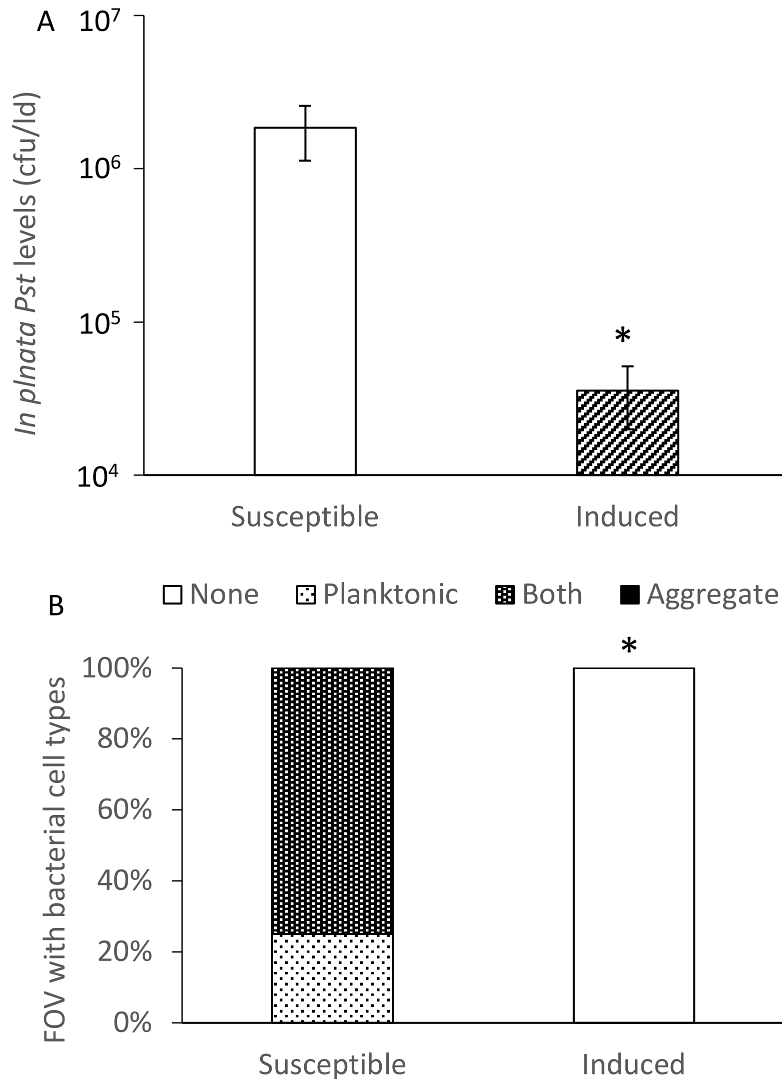


Figure 3.1. *Pst* aggregate formation in susceptible and PTI-responding plants. Leaves were pressure infiltrated with 1 μM flg22 (induced) or mock-treated with water. 24 hours later, the same leaves were inoculated with virulent GFP-expressing *Pst DC3000.* A) *In planta* bacterial quantitation of susceptible (mock-treated) and induced (flg22-treated) plants. * $p < 0.05$ (Student's t-test). B) Aggregate formation was monitored by categorizing each microscopic field of view (40 FOV per treatment) as containing no bacteria, only planktonic bacteria, only bacterial aggregates, or both planktonic and bacterial aggregates. * $p < 0.05$ (Kruskal-Wallis test). This experiment was repeated 8 additional times with similar results (Figure A13).

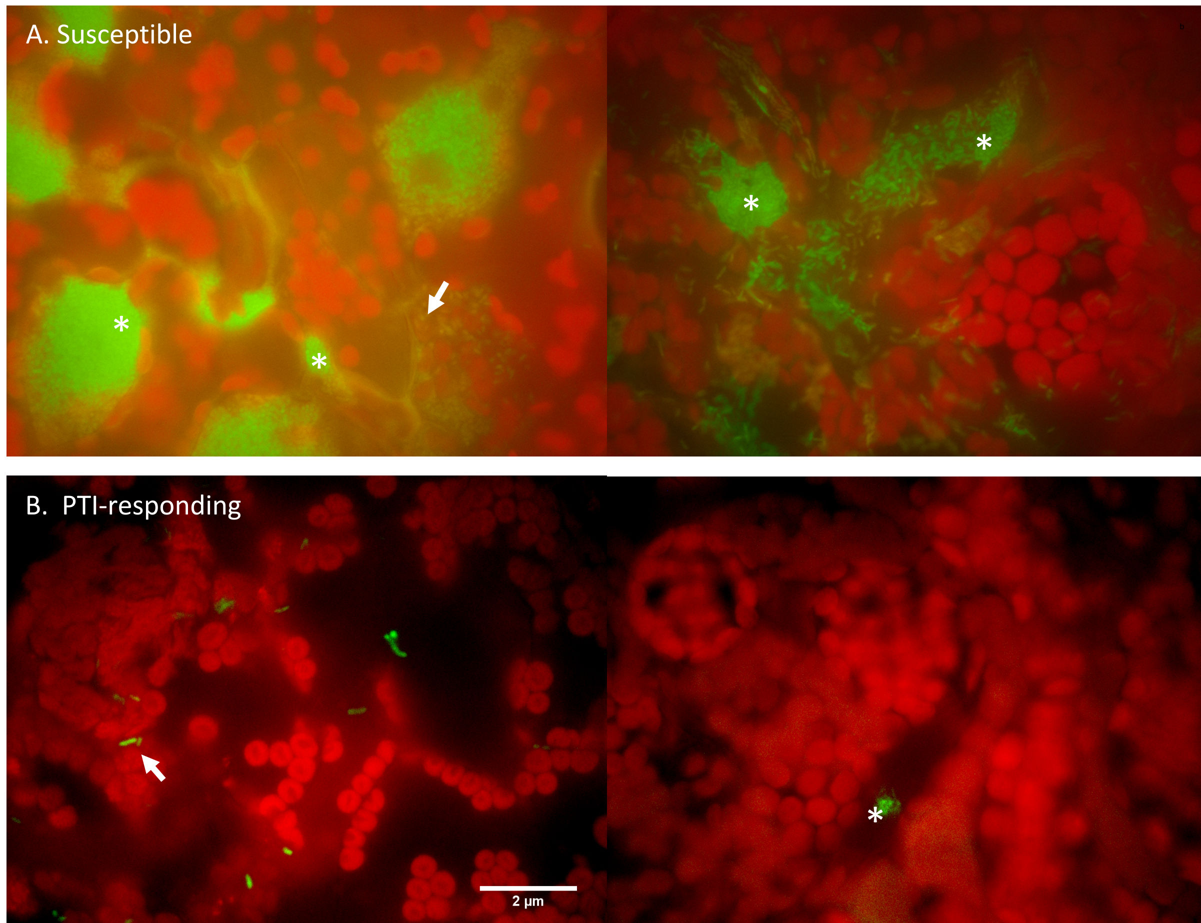


Figure 3.2. Visualization of GFP-expressing *Pst* in susceptible and PTI-responding leaves. Leaves and bacteria were viewed at 1000X magnification under epifluorescence in A) susceptible mock-induced plants and B) PTI-responding plants. Arrows mark examples of planktonic bacteria, asterisks mark examples of aggregated bacteria.

3.4 Aggregate formation is correlated with successful infection by *Pst*

An association between aggregate formation and bacterial success was observed above in Figures 3.1 and 3.2 such that leaves that supported high bacterial levels also contained many bacterial aggregates. To obtain quantitative evidence to support the idea that biofilm-like aggregates are associated with bacterial success, a statistical correlation analysis was performed. Bacterial levels and aggregate formation were examined at 24, 48, and 72 hours post-inoculation with *Pst* using wild-type Col-0, the SA biosynthesis mutant *sid2-2*, and PTI mutants *bak1* and *fls2*, in eight separate experiments. Pooled data from these experiments was

used in the correlation analysis shown in Figure 3.3, where the y-axis corresponds to the number of fields of view with aggregates divided by the total fields of view and the x-axis represents *in planta* bacterial levels (100 to 1 million cfu/ld). A positive correlation was observed between aggregation and bacterial levels as demonstrated by an increase in bacterial aggregation when bacteria reached high densities in planta, an indicator of bacterial success. The R-squared value of this correlation was ~0.42, indicating that the model explains ~42% of the variation around the mean, suggesting that there is a correlation between aggregation and bacterial levels. These data provide evidence that the ability to form aggregates is important for bacterial success.

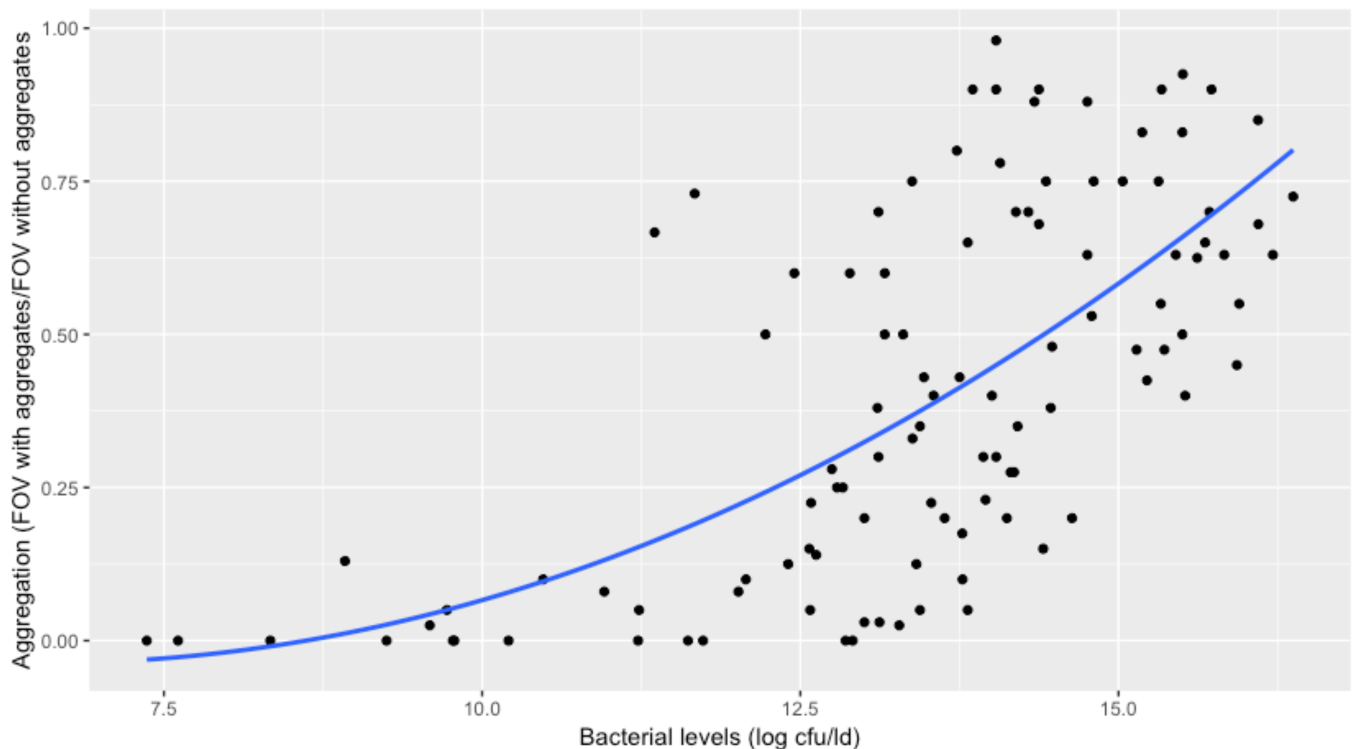
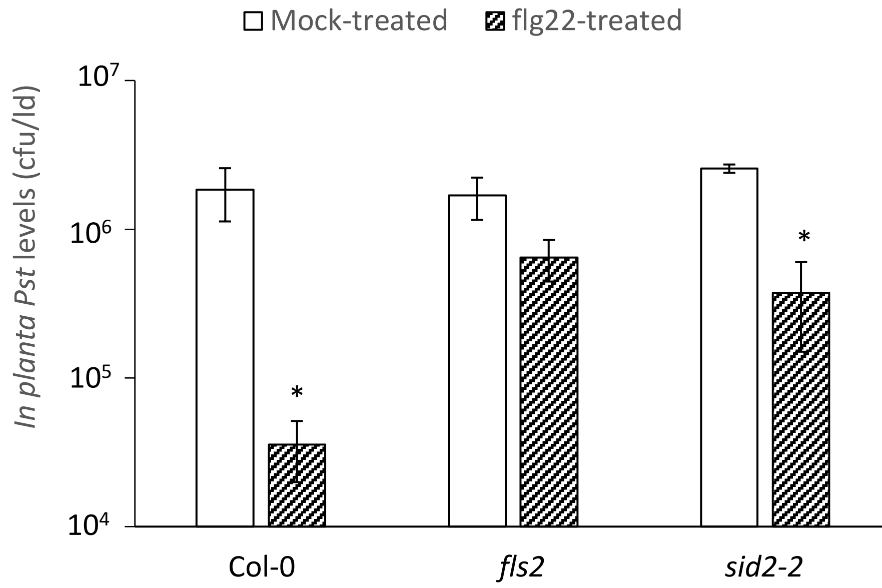


Figure 3.3. Quadratic correlation between bacterial levels and aggregation. Bacterial levels and aggregation across 8 experiments, 4 genotypes, and 3 time points were used. $y = 0.4059 + 2.0257x + 0.4548x^2$. Adjusted R-squared = 0.4197, $p = 2.129 \times 10^{-14}$.

3.5 The PTI response is associated with reduced bacterial aggregate formation

Since the data in Figures 3.1, 3.2, and 3.3 provided evidence that aggregate formation contributes to successful infection and *Pst* aggregate formation was suppressed by the plant PTI response, the effect of PTI on *Pst* aggregate formation was investigated in a quantitative manner. During this experiment the role of PTI-associated SA accumulation in suppression of *Pst* aggregate formation was also examined. *Pst* aggregate formation was compared in PTI-responding and susceptible wild-type Col-0, *fls2* (PTI mutant) and *sid2-2* (SA accumulation mutant) (Figure 3.4A). Col-0 plants treated with flg22 mounted a strong PTI response with a 53-fold reduction in bacterial levels compared to mock-treated susceptible Col-0. Bacterial levels in flg22-treated and mock-treated *fls2* were similarly high indicating PTI was not induced. A significant 7-fold reduction in bacterial levels in flg22-treated compared to mock-treated *sid2-2* was observed indicating that a modest or partial PTI response occurred in *sid2-2* (Figure 3.4A). This suggests that the PTI response is mostly SA-dependent, but SA-independent pathways are also involved. In terms of aggregate formation in Col-0 and *sid2-2*, (Figure 3.4B), fewer fields of view contained aggregates in PTI-responding (flg22-treated) versus susceptible (mock-treated) leaves. However, in *fls2* plants, FOV with aggregates was similar in both mock- and flg22-induced plants. This experiment was repeated 7 additional times, with similar results (Figure A14). Taken together these experiments demonstrate that suppression of bacterial aggregate formation is associated with the PTI response in an SA-dependent and SA-independent manner.



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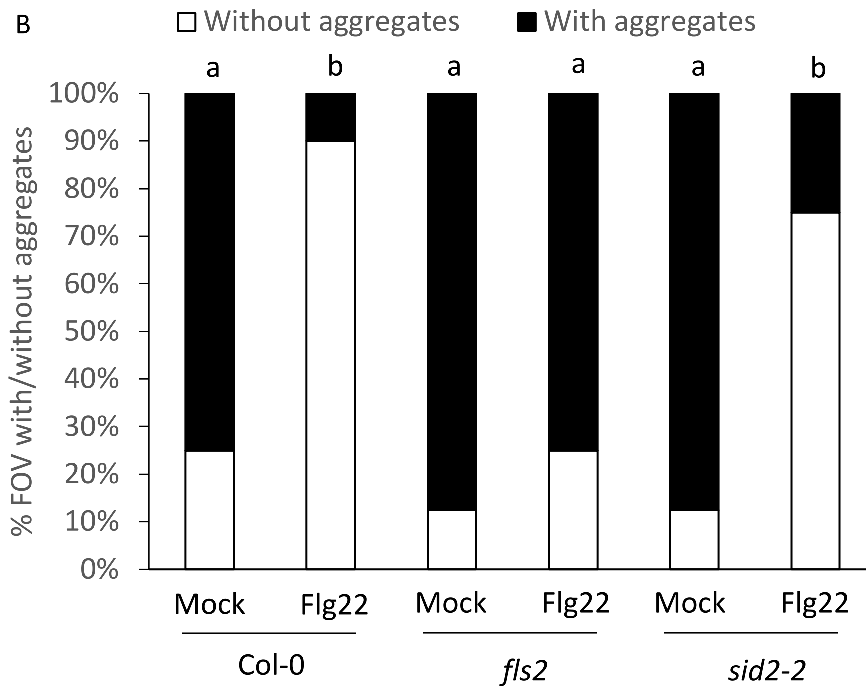
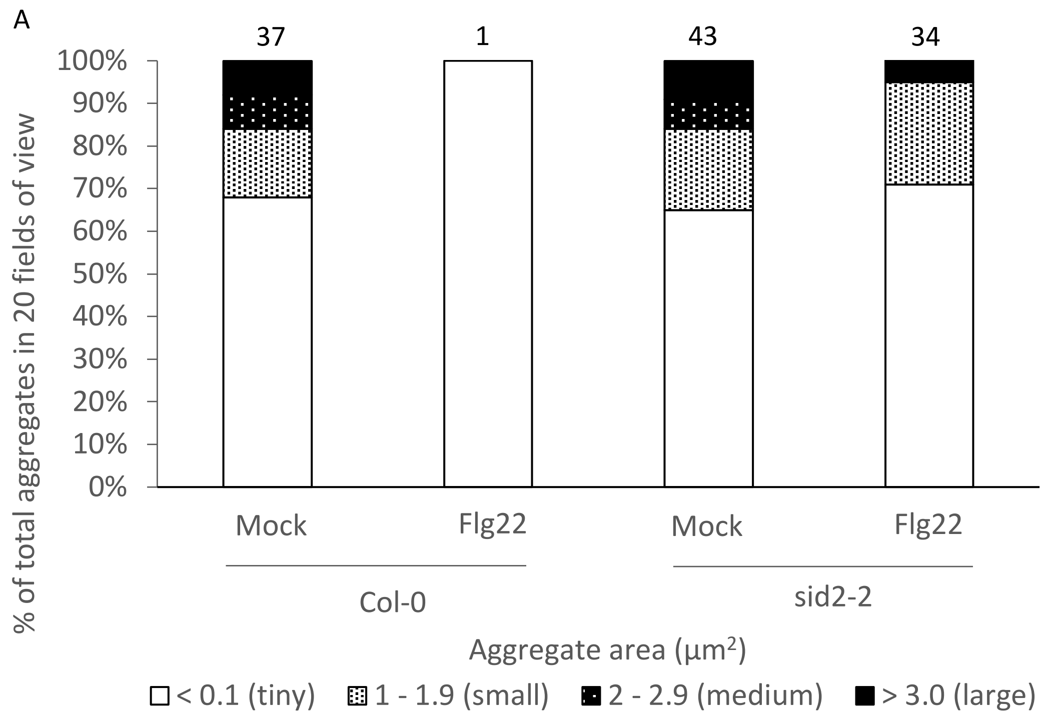


Figure 3.4. Aggregate formation of *Pst* in mock-induced and PTI-responding plants. Leaves were pressure infiltrated with 1 μM flg22 (flg22-treated) or mock-treated with water. 24 hours later, the same leaves were inoculated with virulent GFP-expressing *Pst DC3000.* A) *In planta* bacterial quantitation of mock-treated and flg22-treated Col-0, fls2, and sid2-2. * $p < 0.05$ (Student's t-test) B) Aggregate formation was monitored by categorizing each microscopic field of view (40 FOV per treatment) as with or without aggregates in mock-treated and flg22-treated Col-0, fls2, and sid2-2. Kruskal-Wallis test. This experiment was repeated 7 additional times with similar results (Figure A14).

3.6 Reduction in bacterial aggregate size in PTI-responding plants

As described above in Figure 3.2, bacterial aggregates in PTI-responding plants were observed to be smaller compared to aggregates in susceptible plants. To obtain quantitative data to confirm this observation, the area of each aggregate in 20 fields of view from the experiment in Figure 3.1 was obtained using ImageJ and categorized as tiny (< 1 μm^2), small (1-1.9 μm^2), medium (2-2.9 μm^2) or large (>3.0 μm^2). In susceptible plants (mock-treated Col-0, *sid2-2*), ~65% of the bacterial aggregates were tiny in size (< 1 μm^2), ~15% were small (1-1.9 μm^2), ~10% were of medium size (2-2.9 μm^2,) and ~10% were categorized as large (3 to 10 μm^2) (Figure 3.5A). In contrast, only 1 tiny (< 1 μm^2) bacterial aggregate was observed in 20 fields of view in PTI-responding flg22-induced Col-0. Flg22-treated *sid2-2* plants which displayed a modest PTI response were observed to have small aggregates (> 1 μm^2) in ~20% of the fields of view and medium sized aggregates (2 to 2.9 μm^2) in ~10% of the fields of view (Figure 3.5A). The reduction in aggregate size of flg22-treated *sid2-2* plants suggests that SA-independent pathways involved in PTI response contribute to the reduction of aggregate size. Also, the presence of small and medium-sized aggregates in flg22-treated *sid2-2* compared to only tiny-sized aggregates in flg22-treated Col-0 indicates that SA-dependent pathways still play the most important role in aggregate size reduction associated with PTI. This suggests that the PTI response not only suppresses the number of bacterial aggregates that form, but if aggregates form their size is also suppressed in an SA-dependent manner.



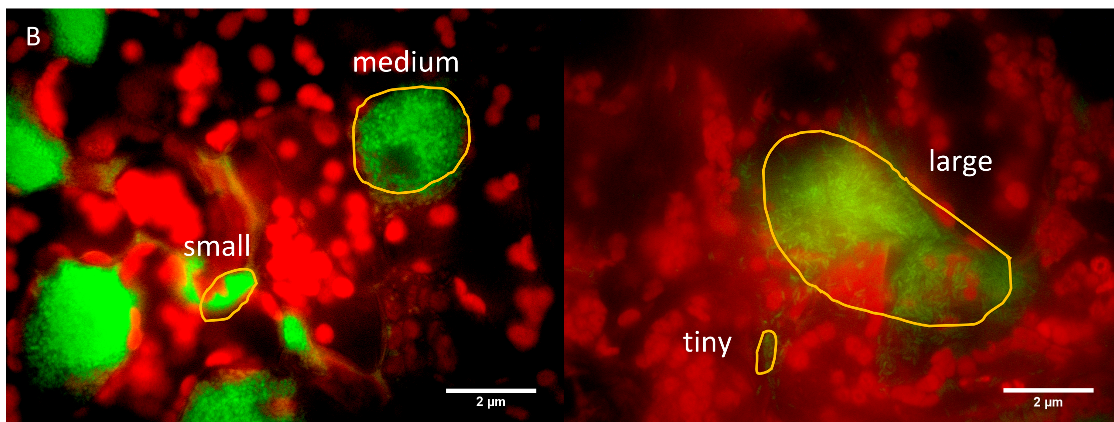


Figure 3.5. Aggregate size in mock-induced and flg22-induced Col-0 and sid2-2. ImageJ was used to process the area of all aggregates present in 20 fields of view. A) Aggregate area was categorized into < 1.0 μm^2 (tiny), 1-1.9 μm^2 (small), 2-2.9 μm^2 (medium), or > 3.0 μm^2 (large). The percent of aggregates in each size category was calculated relative to the total number of aggregates. The total number of aggregates in 20 fields of view is stated above each column. B) Examples of aggregate sizes: tiny, small, medium and large, as seen using fluorescence microscopy.

3.7 Modest reduction in the ability of *Pst algD* mutants to form biofilm-like aggregates

In vitro studies demonstrated that alginate is the major extracellular polysaccharide (EPS) present in biofilms of several species of *Pseudomonas* (Rudolph *et al.* 2004; Aslam *et al.* 2008). Additionally, alginate is one of two EPSs that are known to be produced by *Pseudomonas syringae* pv. *glycinea in vitro* (Laue *et al.* 2006). To investigate if alginate is a major component of *Pst* biofilm-like aggregates, bacterial success was examined by determining bacterial levels and aggregate formation in wild-type *Pst* and *Pst algD* alginate biosynthesis mutants. If alginate is a major component of *Pst* biofilm-like aggregates, the *algD* mutant will be less successful in colonizing plants than wild-type *Pst* in terms of bacterial numbers and aggregate formation. Both Col-0 and *sid2-2* plants inoculated with wild-type *Pst* and *algD Pst* supported similar bacterial levels suggesting that bacterial success was not affected by the absence of alginate (Figure 3.6A). In Col-0 leaves inoculated with *algD Pst*, 60% of the fields of view contained aggregates compared to 80% of the fields of view in leaves inoculated with wild-type *Pst*. In Col-0 plants inoculated with *algD Pst*, 5% of fields of view had no visible bacteria (Figure 3.6B). In *sid2-2* leaves, a similar result was seen, where in leaves inoculated with *algD Pst*, 50% of fields of view contained aggregates, whereas in *sid2-2* plants inoculated with wild-type *Pst*, 90% of fields of view contained aggregates. In *sid2-2* plants inoculated with *algD Pst,* 20% of fields of view had no visible bacteria (Figure 3.6B). This experiment was repeated 2 additional times with similar results (Figure A15). Overall, these data indicate that an inability to produce alginate modestly reduced the number of bacterial aggregates but did not impact the ability of *algD Pst* to colonize the plant intercellular space.

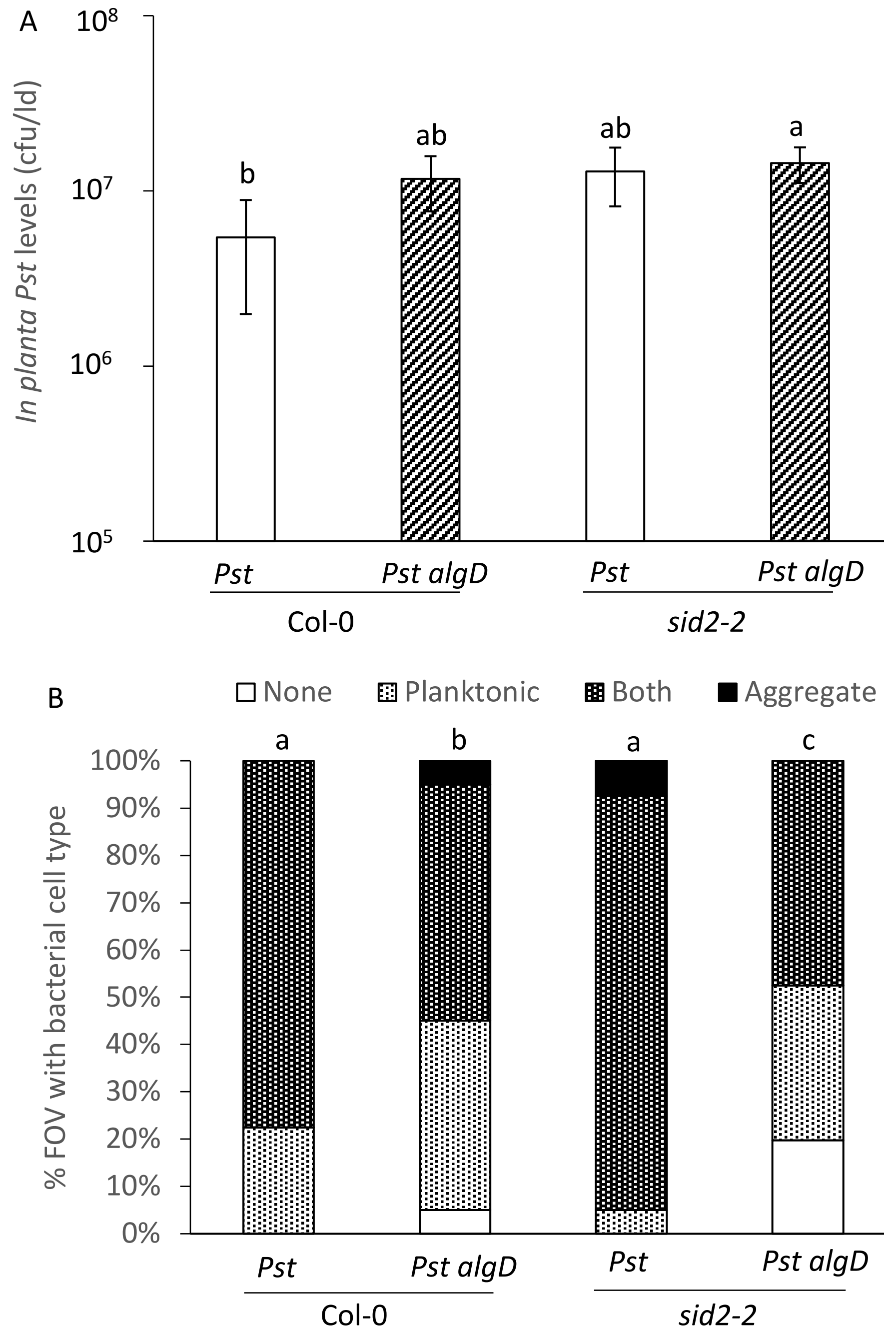
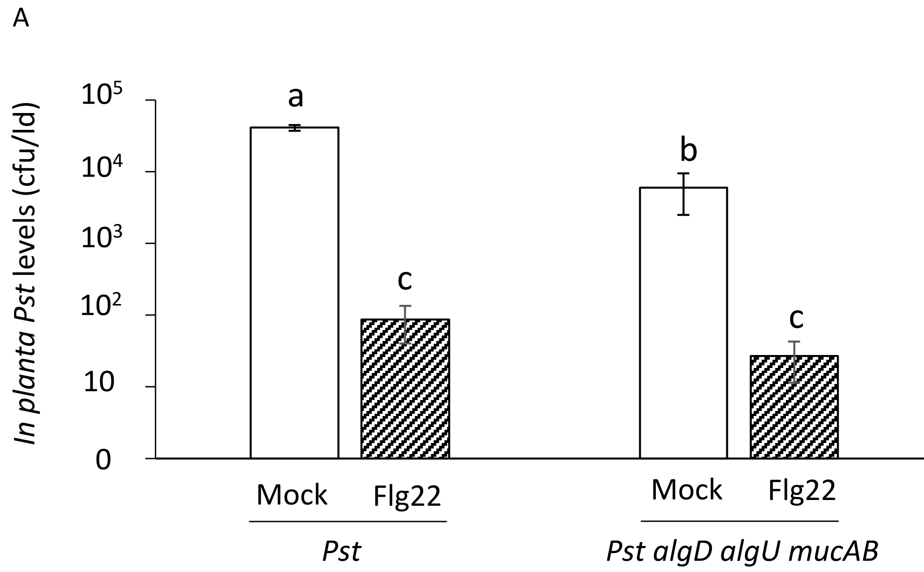


Figure 3.6. The effect of alginate production on aggregate formation of *Pst* in Col-0 and *sid2-2.* Leaves were inoculated with GFP-expressing wild-type virulent *Pst DC3000* or GFP-expressing alginate biosynthesis mutant *Pst algD.* A) *In planta* bacterial quantitation of wild-type *Pst*-inoculated and *algD*-inoculated Col-0 and sid2-2. Two-way ANOVA (Tukey's HSD) B) Aggregate formation was monitored by categorizing each microscopic field of view (40 FOV per treatment) as containing no bacteria, only planktonic bacteria, only bacterial aggregates, or both planktonic and bacterial aggregates. Kruskal-Wallis test. This experiment was repeated 2 additional times with similar results (Figure A15).

3.8 Bacterial virulence is associated with the ability to form biofilm-like aggregates

The *Pst* quadruple mutant *algD algU mucAB* was modestly impaired (~ 5-fold) in its ability to proliferate in the intercellular space of tomato plants and therefore exhibited reduced virulence (Markel *et al.* 2016). *AlgU* encodes a sigma factor that positively regulates the expression of *AlgD* as well as 38% of HrpL upregulated genes (Markel *et al.* 2016). AlgU may regulate structural genes for the TTSS apparatus and suppresses expression of 269 genes, including those that encode flagellin protein. MucA and MucB proteins form an anti-sigma factor that negatively regulates *AlgU* transcription (Wood and Ohman 2009) (Figure 1.1). To investigate the impact of the mutations in the *Pst algD algU mucAB* strain on its ability to form biofilm-like aggregates, this mutant strain was examined for its ability to grow and form aggregates in the plant intercellular space. Uninduced susceptible plants inoculated with the quadruple mutant supported 200 to 400-fold lower bacterial levels than plants inoculated with wild-type *Pst* (Figure 3.7A) confirming Markel *et al.*'s work (2016) that *Pst algD algU mucAB* mutants exhibit reduced virulence. In PTI-responding plants, bacterial levels were reduced in both wild-type *Pst*-as well as *Pst algD*-inoculated plants (Figure 3.7A), suggesting that PTI suppresses the growth of both strains. Comparing bacterial levels to aggregate formation (Figure 3.7B), a significant ~2-fold reduction in aggregate formation in plants inoculated with the quadruple mutant strain compared to the wild-type strain, was observed. This suggests that a strain with reduced virulence also formed fewer aggregates.

A



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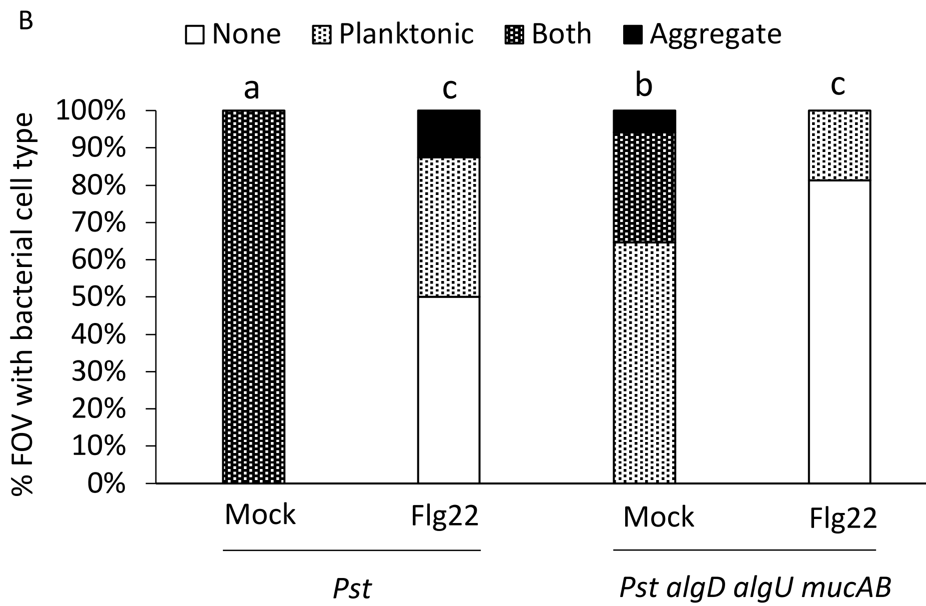

Figure 3.7. Aggregate formation of wild-type *Pst* or mutant *algD algU mucAB* in susceptible or PTI-responding Col-0. Leaves were pressure infiltrated with 1 μM flg22 (flg22-treated) to initiate PTI or mock-treated with water (susceptible). These same leaves were inoculated 24 hours later with GFP-expressing wild-type virulent *Pst DC3000* or GFP-expressing mutant *Pst algD algU mucAB.* A) *In planta* bacterial quantitation of wild-type *Pst*-inoculated and *algD algU mucAB*-inoculated Col-0 at 48 hpi. Two-way ANOVA (Tukey's HSD) B) Aggregate formation was monitored by categorizing each microscopic field of view (40 FOV per treatment) as containing no bacteria, only planktonic bacteria, only bacterial aggregates, or both planktonic and bacterial aggregates. Significant differences were identified by the Kruskal-Wallis test.

Chapter 4: Discussion and Conclusions

4.1 Glycerol as an inducer of resistance in cucumber

Numerous glycerol assays performed during the course of this thesis indicated that 100 mM glycerol foliar spray was sufficient to induce a modest 2 to 4-fold level of local resistance compared to mock-treated plants in 7 of 11 total experiments. Zhang *et al.* (2015) demonstrated glycerol foliar spray application led to enhanced resistance to the oomycete pathogen *Phytophthora capsica* in cacao plants. They found that 100 mM glycerol treatment resulted in little G3P accumulation, whereas treatment with 500 mM glycerol resulted in a 100-fold increase in G3P levels. We found that concentrations of 100 mM to 200 mM when applied to one or two leaves, did not induce systemic resistance in distant naïve leaves. Given that cacao plants treated with 100 mM glycerol did not result in G3P accumulation (Zhang *et al.* 2015), it is possible that in cucumber plants, 200 mM treatments did not result in significant G3P accumulation. Furthermore, they found that 100 mM glycerol was sufficient to induce local disease resistance against *P. capsica*, suggesting that G3P accumulation is not involved in local glycerol-mediated resistance (Zhang *et al.* 2015).

GLI/NHO1 phosphorylates glycerol to G3P in *Arabidopsis*, and the *gli/nho1* mutant is SAR-deficient, providing evidence that G3P is an important component of the SAR response (Chanda *et al.* 2011). Together, this suggests that a higher concentration of glycerol treatment may be needed to result in G3P levels that are sufficient to induce a SAR or systemic response in cucumber. It is possible that glycerol treatments cause osmotic dehydration when applied to plants (reviewed in Muñiz-Becerá *et al.* 2017). However, in an experiment in which Yacon root discs were incubated in 30% glycerol (w/v) at 30 °C, a significant loss of mass was not observed and the discs maintained a moisture content of 80% after 6 hours (Brochier *et al.* 2015). In this thesis, glycerol was applied at 100 mM or < 1% (w/v) and Zhang et al. (2015) sprayed cacao leaves with 500 mM (~4.5% w/v). Based on all of the above, glycerol treatments of 100 to 500 mM (1 to 4.5% w/v) are unlikely to cause osmotic dehydration.

4.2 Azelaic acid as an inducer of resistance in cucumber

Our findings suggest that treatment with azelaic acid leaf infiltration induced a modest 1.5 to 10-fold level of local resistance in cucumber at concentrations 0.1 to 2 mM in 4 of 7 experiments over several months (October 2016 to March 2017). However, Aza treatment did not induce systemic resistance in the one experiment that was conducted. We were initially interested in testing Aza as an inducer of SAR in cucumber because of the work of Jung *et al.* (2009) in which they discovered that Aza accumulated in petiole exudates of SAR-induced plants at 6 to 7-fold higher levels than mock-induced plants. They also demonstrated that exogenous application of Aza induced local and systemic resistance in *Arabidopsis.* Furthermore, a mutation in the Aza-inducible *AZI1* gene resulted in the loss of systemic immunity triggered by pathogen inoculation or Aza treatment, suggesting that Aza plays a key role in SAR induction (Jung *et al.* 2009). In one experiment, Aza treatment of one or two leaves did not induce systemic resistance in distant naïve leaves of cucumber, and local resistance was modestly induced and variable in terms of resistance outcome (4 out of 7 experiments). It is interesting to note that in additional studies on Aza's involvement in SAR, Návarová *et al.* (2012) did not observe accumulation of Aza in phloem sap-enriched exudates collected from SAR-induced *Arabidopsis* leaves. In fact, Zoeller *et al.* (2012) and Vicente *et al.* (2012) found that exogenous treatment with Aza did not significantly suppress growth of *Pst DC3000* in treated or distant *Arabidopsis* leaves. Ádám *et al.* (2018) did a series of experiments to examine the effect of exogenous application of Aza in enhancing resistance in *Nicotiana tabacum*. Local application of Aza (0.2-1.0 mM) did not affect lesion size of TMV (tobacco mosaic virus)-infected or systemic leaves. Furthermore, Aza treatment had no significant local or systemic effect on symptom development or multiplication of incompatible *Pst* or compatible *P. syringae* pv. *tabaci*.

Taken together, these findings indicate that Aza treatment rarely induces systemic resistance in *Arabidopsis* and tobacco. However, this could have been affected by variation in growth conditions between studies. Zoeller *et al.* (2012) grew their *Arabidopsis* plants in 9 hours of light (120 $\mu E\ m^{-2}\ s^{-1}$) and 65% humidity, and Vicente *et al.* (2012) grew their *Arabidopsis* plants with 16 hours of light and a much higher light intensity (250 $\mu E\ m^{-2}\ s^{-1}$). Ádám *et al.* (2018) grew

their tobacco plants under 14 hours of light, with no specifications on light intensity or humidity. Light intensity and humidity could play a potential role in the uptake of Aza, particularly when used as a foliar spray, as both light and humidity affect stomatal opening (Mott *et al.* 1999).

4.3 Pipecolic acid as an inducer of resistance in cucumber

Exogenous application of Pip via the root system was sufficient to induce a modest 4 to 6-fold systemic response with one application, and a strong response (up to 28-fold) with multiple applications. This is consistent with findings that *ald1 Arabidopsis* mutants that cannot synthesize Pip are SAR-defective and can be rescued with exogenous application of Pip (Návarová *et al*. 2012). The same study found that Pip accumulates in phloem sap-enriched exudates of SAR-induced leaves (Návarová *et al.* 2012). Furthermore, exogenous application of Pip was sufficient to induce a SAR-like response in wild-type *Arabidopsis* against virulent bacteria (Návarová *et al.* 2012). The enhanced strength of resistance observed (8 to 28-fold) with multiple treatments could be due to increased availability of Pip in the soil, leading to enhanced uptake of Pip by cucumber roots. Variation in Pip-induced resistance was observed between individual plants perhaps due to differences in root uptake of Pip from the soil.

4.4 Seasonal effect on induced resistance in cucumber

Variation in Pip-induced resistance was observed from year-to-year as discussed in Chapter 3. Moreover, weaker Pip-induced (2-fold) and PTI (2 to 8-fold) resistance responses were observed during the winter months of November to April (Figures A16-A21). For example, in 1 of 5 experiments, glycerol treatment did not induce resistance in the winter (Figure A16). In 4 of 4 experiments performed in the winter, Aza treatment did not induce resistance (Figure A17), and in 8 of 14 experiments performed in the winter, Pip treatment did not induce resistance (Figures A18-A21). In looking at the Pip experiments done in 2016-2017 (Figures A18-A20), Pip treatment did not induce resistance in 8 of 12 experiments performed in the winter. This suggests that there may be some kind of seasonal effect on SAR-associated molecule induced resistance responses. During these experiments, cucumber plants were primarily grown on growth stands with T5 fluorescent lights in rooms with windows and this may explain why these

plants responded differentially to spring/summer and fall/winter seasons. During late fall and winter, both cucumber and *Arabidopsis* plants grow slowly and are smaller compared to plants grown in the spring, summer and early fall. Plants grown in the late fall and winter display no resistance or modest resistance responses. This suggests that plants can sense the changing seasons, and this affects plant growth and development as well as resistance to pathogens.

4.5 Biofilm-like aggregate formation is important for successful *Pst* infection

AlgD encodes GDP-mannose dehydrogenase, which catalyzes alginate biosynthesis. *AlgD* is the first gene of a putative 12-gene operon that contains other alginate biosynthetic genes (Markel *et al.* 2016). The *algD* mutant cannot produce alginate. *AlgU* encodes the alternative sigma factor AlgU, which regulates its own expression, the alginate biosynthetic operon, as well as other regulatory genes. MucA and MucB are anti-sigma factors that form a complex with AlgU to inactivate AlgU. Evidence has been found to support that AlgU contributes to *in planta* growth and disease (Markel *et al.* 2016). Expression analysis indicates that AlgU regulates the expression of 38% of genes upregulated by HrpL, as well as genes associated with the synthesis of flagellin protein (Markel *et al.* 2016).

In this study, we demonstrated that the mutant strain *algD algU mucAB* which has reduced virulence, also has fewer aggregates *in planta*. Col-0 plants infected with wild-type *Pst* had significantly higher bacterial levels and 100% of fields of view had aggregates compared to Col-0 plants infected with *algD algU mucAB Pst,* in which 65% of fields of view had only planktonic bacteria. This suggests that genes that regulate virulence may overlap with genes that regulate aggregate or biofilm formation. This is supported by evidence that AlgU, which regulates alginate synthesis, also regulates virulence-associated genes (Markel *et al.* 2016).

In tomato plants, plants infected with *algD algU mucAB Pst* had lower bacterial levels than those infected with wild-type *Pst* or *algD Pst*. Furthermore, tomato plants infected with *algD algU mucAB Pst* with an AlgU expression vector supported similar bacterial levels as wild-type *Pst* (Markel *et al.* 2016). It was then confirmed that AlgU upregulates a number of genes in the

Hrp system, including structural genes for the TTSS apparatus, pilus protein gene, and effectors (Markel *et al.* 2016). Furthermore, AlgU may be important in genes related to coronatine biosynthesis (Ishiga *et al.* 2018). *Arabidopsis* and tomato plants infected with *algU* mutant *Pst* supported lower bacterial population compared to plants infected with wild-type *Pst.* Application of coronatine was able to restore the ability of the *algU Pst* to reopen stomata, suggesting that AlgU contributes to regulation of coronatine production and contributes to virulence by suppressing stomatal-based defense in the early stages of infection (Ishiga *et al.* 2018) as well as SA-mediated defence (Zheng *et al.* 2012).

4.6 Alginate and EPS in biofilm formation

Wild-type *Arabidopsis* infected with *algD Pst* supported similar bacterial levels as plants infected with wild-type *Pst*, suggesting that the ability to produce alginate is not required for successful *Pst* infection or pathogenicity. Furthermore, *in planta* biolfim-like aggregate formation by *algD Pst* was reduced but not eliminated compared to plants infected with wild-type *Pst.* In both Col-0 and *sid2-2*, *algD Pst* formed fewer aggregates with 50% of the fields of view (FOV) with aggregates, compared to Col-0 (78% FOV) and *sid2-2* (95% FOV) inoculated with wild-type *Pst.* Similarly, Ishiga *et al.* (2018) found that *Arabidopsis* and tomato plants inoculated with *algD* or wild-type *Pst* supported similar bacterial levels, however biofilm formation was not examined. Laue *et al.* (2006) investigated the roles of alginate and levan in *Pseudomonas syringae* pv. *glycinea (Psg)* biofilm formation *in vitro*. Strains deficient in the production of the extracellular polysaccharide alginate or the exopolysaccharide levan, or deficient in both, formed biofilms on polystyrene surfaces (Laue et al, 2006), providing evidence that neither alginate nor levan are major contributors to *in vitro Psg* biofilm formation. This study also demonstrated that other polysaccharides such as lectins bind to *Psg* biofilms *in vitro* and may be a component of *Psg* biofilms (Laue *et al.,*2006), however this study was performed in rich media that does not mimic the plant intercellular space. The evidence from this thesis and Laue et al (2006) supports the hypothesis that alginate is not a major contributor to biofilm formation in *P. syringae*.

In Aslam *et al.* (2008), they showed that *Arabidopsis* infected with *algD Pst* supported 10-fold reduction in bacterial levels compared to plants infected with wild-type *Pst.* However, in that study, insertional inactivation was used to create the *Pst algD* mutation whereas in Markel *et al.* (2016), a deletion construct was used to delete the *algD* gene. The strain used in this thesis was the mutant strain produced by Markel *et al.* (2016). Markel *et al.* (2016) also used DNA sequencing to confirm all plasmids and mutant clones, whereas Aslam *et al.* (2008) did not describe any kind of confirmation. This could mean that the suggestion that alginate was an important virulence factor for *Pst* (Aslam *et al.* 2008) could be incorrect.

However, the pathogen used during this work (*Pst* DC3000) produces corontine a phytotoxin that acts to suppress plant defense including SA accumulation (Zheng *et al.* 2012). Additionally, virulence effectors secreted into the plant cytosol suppress SA synthesis and accumulation, resulting in a reduced PTI response (reviewed in Tanaka *et al.* 2015). Evidence from the Cameron lab supports this idea as *Arabidopsis* plants infected with virulent *Pst* that produces coronatine and secretes effectors, accumulates 10-fold less intercellular SA compared to plants responding with ETI to avirulent *Pst* (Carviel *et al.* 2014). It is possible to speculate that during a successful PTI response in which SA is produced, alginate as a component of the *Pst* biofilm is important for *Pst* success and pathogenicity.

4.7 Biofilm formation and visualization *in vivo*

Autoaggregation is defined as a tendency to congregate or aggregate based on adhesive interactions among bacteria (Bogino *et al.* 2013). Autoaggregative behaviour has been associated with biofilm formation ability in beneficial plant-rhizobacterium associations. *In vitro* autoaggregation and biofilm formation assays of several strains of *Sinorhizobium melliloti* revealed that there was a positive correlation between autoaggregation (percent) and biofilm formation ability (OD_{560}/OD_{600}) (Sorroche *et al.* 2012). This suggests that autoaggregation and biofilm formation depend on the same adhesive forces. To prove that the *Pst* biofilm-like aggregates observed in Arabidopsis are actually biofilms, it will necessary to determine if these aggregates are encased in a matrix of polysaccharides, proteins and eDNA. Extracellular DNA

(eDNA) has been observed in bacterial biofilms, including *P. aeruginosa* biofilms (reviewed in Flemming and Wingender 2010). DAPI staining was used to visualize eDNA in plants inoculated with *Pst* and eDNA was observed to surround biofilm-like aggregates (data not shown), suggesting that the *Pst* aggregates observed in this thesis are biofilms. Biofilm formation by bacterial pathogens has been primarily studied in the human pathogens, *P. aeruginosa* and *Staphylococcus* species (reviewed by Joo and Otto, 2013). Biofilm formation during plant-bacterial associations has been studied in recent years (reviewed by Bogino *et al.* 2013). Several studies examined factors affecting biofilm formation *in vitro* (Laue *et al.* 2006, Markel *et al.* 2016, Sorroche *et al.* 2018), and biofilms have been visualized *in vitro* (Rasamiravaka *et al.* 2014). A few studies have visualized biofilm formation *in vivo* (Godfrey *et al.* 2010; Gottig *et al.* 2009; Guilhabert and Kirkpatrick 2005; Penaloza-Vasquez *et al.* 2010; Bais *et al.* 2004; Rudrappa *et al.* 2007; Walker *et al.* 2004). These have primarily examined biofilms formed during bacteria-root surface interactions with *Arabidopsis* (Bais *et al.* 2004; Rudrappa *et al.* 2007; Walker *et al.* 2004). Godfrey *et al.* (2010) observed a reduction in GFP-expressing *P.s. phaseolicola* colony formation in bean leaves during the HR compared to healthy tissue. They examined 1 leaf per treatment, 3 sections per leaf, and 12 FOV per section. To quantify aggregate formation, ImageJ was used to quantify fluorescent pixels. However, quantification of fluorescent pixels would not only include aggregate bacteria, but also planktonic bacteria. Therefore, their analysis may not be an accurate representation of aggregate formation. Penaloza-Vasquez *et al.* (2010) found that HrpM and AlgD are both required for *P.s. syringae* to form aggregates in pear leaves. They performed 2 replicate experiments and examined 6 leaves per strain and 6 FOV per leaf. However, there was no quantitation of aggregate number or size. In the experiments conducted in this thesis, we examined aggregate formation by quantifying number of FOV with aggregates, which kinds of bacterial cell types were observed, as well as aggregate size.

4.8 SA-mediated suppression of biofilm formation

In this thesis, suppression of aggregate formation during PTI was observed leading to the idea that the PTI response includes pathogen biofilm suppression. Multiple studies suggest that

biofilm formation is an important virulence mechanism for plant pathogens (Markel *et al.* 2016, Ishiga *et al.* 2018, Rudolph *et al.* 1994), therefore it makes sense that plant defense pathways such as ARR (Wilson *et al.* 2017) and PTI (this thesis) suppress biofilm formation. Furthermore, not only was suppression of aggregate formation observed during PTI, but a reduction in aggregate size was also observed. These observations suggest that the PTI response includes the prevention of or disruption of biofilm formation. Given the evidence that supports the idea that intercellular SA accumulation contributes to ARR by suppressing bacterial biofilm formation (Wilson *et al.* 2017), perhaps the same is true during PTI. We have evidence supporting that during flg22-triggered PTI, *sid2-2* plants support more, and larger aggregates compared to Col-0. This also suggests that SA plays some role in suppressing aggregate formation as well as reducing aggregate size during PTI.

There is some evidence that SA may act on biofilms by mitigating EPS-related Ca^{2+} depletion. The EPS, alginate from *P. aeruginosa* and EPS, xanthan from *Xanthomonas campestris* pv. *campestris* (*Xcc*) have been shown to bind calcium ions *in vitro,* leading to the idea that bacterial EPS depletes plant apoplastic Ca^{2+} during infection (Aslam *et al.* 2008). Therefore, biofilm-associated EPS depletion of apoplastic Ca^{2+} may impact cytosolic Ca^{2+} and reduce the ability of $Ca^{2+}$to act as a second messenger during plant defense (Aslam *et al.* 2008). Evidence to support this idea comes from experiments in which *Arabidopsis* inoculated with wild-type *Xcc* were observed to have a lower influx of Ca^{2+} into the cytosol compared to plants inoculated with EPS-deficient *Xcc* (Aslam *et al.* 2008). This suggests that bacterial associated EPS suppress calcium influx. Kawano *et al.* (1998) found that addition of SA to tobacco suspension cells induced a transient increase in cytosolic Ca^{2+} concentration. Based on these observations it is possible to speculate that intercellular SA accumulation during defense responses like ARR act to suppress *Pst* biofilm formation by enhancing cytosolic Ca^{2+} influx and defense signaling in order to overcome *Pst* EPS-related Ca^{2+} depletion.

In addition to suppressing bacterial biofilm formation, SA has also been shown to act as an antimicrobial agent that limits *Pst* growth in the intercellular space during ARR (Wilson *et al.*

2017) and in this thesis during flg22-induced PTI. The mechanism of action of SA antimicrobial activity is not well understood. Several *in vitro* experiments suggest that SA may disrupt the transmembrane proton gradient required for ATP production in animals (Gutknecht 1990, Jörgensen *et al.* 1976, Smith 1959) and in plants (Norman *et al.* 2004, Stenlid and Saddik 1962) or inhibit respiration (Norman *et al.* 2004) or catalase activity (Chen *et al.* 1993) in plant tissue. Previous studies in the Cameron lab demonstrated that *Pst* grown in apoplast mimicking media (hrp-inducing minimal medium) displayed reduced growth at SA concentrations as low as 100-200 µM. Wilson et al (2017) estimated the SA concentration in the intercellular space during the ARR response to be in the range of 50 to 100 mM suggesting that SA may act as an antimicrobial agent *in planta*.

4.9 Recommendations for future experiments

1. To determine if glycerol, Aza and Pip induce SAR in cucumber, examine cucumber-related SAR markers, such as peroxidase, in treated leaves.
2. Given that Pip is thought to enter roots and move to the aerial parts of Arabidopsis by the xylem (Endo *et al.* 2018), it would be useful to examine if this is true in aerial parts of cucumber by examining Pip accumulation in Vaseline-dipped leaves (inhibits xylem transport)
3. It is also possible that Pip accumulation in roots initiates the movement of SAR long-distance signals such as DIR1. This could be examined using the estrogen inducible DIR1-EGFP/*dir1-1* line to observe if DIR1-EGFP expressed in roots moves to distant leaves upon SAR induction.
4. It appears that Pip is not always taken up by the roots, therefore future experiments should incorporate adjuvants that enhance the uptake of chemicals (Tu *et al.* 2001) by plant roots such as Tween 80, Xiameter OFX307, and Crodamol GTCC.
5. Evidence found in this thesis suggests that *Pst* aggregate formation was suppressed in an SA-dependent manner. To determine if SA accumulates in the intercellular space to act as an antibiofilm agent during PTI, SA levels must be determined in intercellular washing fluids after flg22 treatment but *before Pst* inoculation as well for several time

points after *Pst* inoculation to examine both the accumulation of SA due to flg22 treatment as well as SA accumulation in response to *Pst*.

6. Much of the aggregate data collected has not been analyzed. There are many factors we are interested in: total number of aggregates, aggregate size, number of aggregates in each field of view, bacterial growth, and how all of these may intersect. We need to think about how this data can be analyzed to further contribute to our findings.
7. Virulent *Pst* DC3000 can suppress SA accumulation, making it difficult to state that alginate is not important during biofilm formation. To determine if alginate is important for biofilm formation when SA accumulation is not suppressed, hrpA$^-$ *Pst* should be used to compare with wild-type *Pst*.
8. Determine if *Pst* aggregates are biofilms by determining if biofilm components (EPS, eDNA, proteins) are associated with aggregate formation in planta by developing *in vivo* methods to stain for EPS or DAPI.

4.10 Conclusions

The work in this thesis provides evidence that glycerol and azelaic acid treatments of cucumber leaves induced modest 2 to 5-fold resistance in treated leaves in 7 of 12 experiments and 4 of 9 experiments, respectively. Pipecolic acid root treatments induced modest 2 to 5-fold resistance in leaves when treated once in 13 of 25 experiments and when treated 2 to 3 times on a weekly basis led to a stronger 5 to 28-fold level of resistance in 5 of 6 experiments. Based on these experiments, weekly pipecolic acid soil application may be a useful and easy method to enhance disease in greenhouse-grown cucumber plants. Evidence was also obtained that demonstrated that the ability to form biofilm-like aggregates is important for successful infection by *P. syringae* pv. *tomato*. Unlike *Pseudomonas aeruginosa*, data obtained in this thesis demonstrated that alginate is not a major component of *Pst* biofilm-like aggregates. Compelling evidence was obtained demonstrating that the PTI response includes the ability to suppress *Pst* biofilm-like aggregates in terms of aggregate number and size.

Chapter 5—Materials and Methods

5.1 *Arabidopsis* plant lines and growth conditions

All plant lines used are in the Col-0 background and have been described previously: *sid2-2* (Nawrath and Metraux 1999), *fls2* (Zipfel *et al.* 2004), *bak1-3* (Chinchilla *et al.* 2007). Seeds were surface-sterilized, were stratified for 2 days in darkness at 4°C and were then plated on Murashige and Skoog medium. Approximately 1 week later, cotyledon-stage seedlings were transplanted to soil (Sunshine Mix #1 or JVK Agro Mix G5) moistened with 1 g of all-purpose 20-20-20 fertilizer per liter. Growth conditions were 22 ± 2C, 80% ± 10% relative humidity, and 9 h of light (mixed fluorescent and incandescent, 120 to 150 μE/m^2/s).

5.2 *C. sativus* growth conditions

Wisconsin SMR 58 cucumber seeds were planted at a depth of approximately 1 cm in soil (Sunshine Mix) previously moistened with 200 ml of 1 g/l of 20-20-20 fertilizer per pot. Pots were enclosed under a lid to maintain high humidity for several days until seedling emergence. Plants were grown at room temperature in 16 hours light (fluorescent, 110-230 μE/m^2/s) and watered when the top of the soil was visibly dry.

5.3 Bacterial transformation

Mutant *Pst* strains *algD Pst* (PS392) and *algU mucAB algD Pst* (PS519) were transformed with pDSK-GFPuv (Wang et al. 2007) using the triparental mating method. First, *E. coli* DH5 harboring the GFP plasmid, helper strain of *E. coli* RK600 and recipient strains of *Pst* were grown overnight. Then, the bacterial cultures were centrifuged for 7min at 1000g and the supernatant was discarded. Cell pellets were resuspended with 20 ml of 10 mM $MgCl_2$. Washing was repeated twice to remove the antibiotic residue from the overnight cultures. Then resuspended cell cultures (50 μl per strain) were mixed in 1ml of KB media. One hour was given for mating to occur under room temperature (22°C) and 50 μl of the mating mixture was transferred onto a LB plate with rifampicin (for *Pst*) and kanamycin (for pDSK-GFPuv) to select for successful trans-

conjugants. The presence of captured GFP plasmid was verified by exposing colonies under a UV lamp (365nm) for GFP emission.

5.4 Disease resistance assays

Overnight cultures of wild-type *P. syringae* pv. *tomato DC3000*, alginate deficient *algD Pst* (PS392) and *algU mucAB algD Pst* (PS519) were grown in KB medium to exponential phase. Cells were collected by centrifugation, were resuspended in 10 mM $MgCl_2$ to 10^6 cfu/ml, and were pressure-infiltrated into fully expanded leaves using a needleless syringe. *Pseudomonas syringae* pv. *lachrymans 8003* (*Psl*) was used in cucumbers. *Psl* cultures were grown overnight with agitation at room temperature in KB medium containing 50 μg/ml of rifampicin. Cells were collected by centrifugation, were resuspended in 10 mM $MgCl_2$ to 10^5 cfu/ml, and were pressure-infiltrated into fully expanded leaves using a needleless syringe.

For quantification of *in-planta* bacterial levels, three sets of eight leaf disks (4 mm diameter) were collected and shaken at 200 rpm for 1 h in 10 mM $MgCl_2$ with 0.1% Silwet L-77. Serial dilutions were plated on KB medium with kanamycin (50 μg/ml) and rifampicin (100 μg/ml) for *Pst* and only rifampicin (100 μg/ml) for *Psl*. Plates were incubated at room temperature (Pst) or 30°C (Psl) for 2 days before colonies were counted.

5.5 Chemical preparation

Pipecolic acid solution was freshly prepared immediately before treating plants at concentrations of 10, 20, 50 and 100 mM. Pipecolinic acid (Sigma Aldrich P45850), which is a mixture of equal of the L- and D-stereoisomers of pipecolic acid, was dissolved in sterile water with a magnetic stirrer until all visible precipitate was dissolved. Azelaic acid solution was prepared one day prior to treating plants at a concentration of 1 mM. Azelaic acid (Sigma Aldrich 246379) was dissolved in 5 mM MES (2-(N-morpholino)-ethanesulfonic acid) with a magnetic stirrer for approximately 30 minutes or until fully dissolved. Glycerol (BioBasic GB0232) was prepared immediately before treating plants and was diluted to the appropriate concentrations using sterile water and shaken until fully mixed. 50 mg/l benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH Actigard 50WG) was dissolved in water.

5.6 Imaging of *P. syringae* pv. *tomato* in the intercellular space by epifluorescence microscopy

Plants were inoculated with 1μM flg22 peptide (PhytoTech Labs #P6622) or mock-treated with sterile water. Twenty-four hours later the same leaves were inoculated with a 10^6 cfu/ml solution of *P. syringae* pv. *tomato* carrying pDSK-GFPuv (Wang *et al.* 2007). After 24, 48, and 72 hours, leaves were cut at the petiole and sections of the lower epidermis were removed using invisible tape. Sections without the lower epidermis were isolated using a razor blade and were mounted in water on a glass slide with coverslip with the epidermis-less surface facing upwards. Slides were imaged immediately, using a Nikon Eclipse E800 microscope fitted with a Nikon DS-Fi1 camera head and the DS-U3 control unit using 100× oil immersion lenses and a B-2A filter cube. For comparing the proportion of cell types between flg22-treated and mock-treated plants of different genotypes, tissue preparation and imaging were performed by different individuals so that the scoring was blind. Aggregate size was measured using ImageJ.

5.7 Statistical tests

Statistical significance was determined either using a student's t-test, an analysis of variance (ANOVA), or Kruskal-Wallis test as indicated. For the student's t-test, a two-tailed test for either equal or unequal variance was performed where $p < 0.05$. Single variable ANOVA analysis was performed with $p < 0.05$ and followed up with a Post HOC test, Tukey's HSD. Kruskal-Wallis test was performed with $p < 0.05$ and followed by Dunn's test.

Appendix

Experiment	*Psl 8003*	*Pss D20*
1	3	11
2	2.5	NS
3	2.5	NS

Figure A1. Summary of bacterial fold difference in cucumber plants SAR-induced with *Psl 8003* or *Pss D20* compared to mock-induced plants. Three replicate experiments were conducted where cucumber plants were induced by inoculating with $5x10^7$ cfu/ml *Pss D20* or $1x10^6$ *Psl 8003* or mock-induced with 10 mM $MgCl_2$ and then challenge-inoculated with $1x10^6$ cfu/ml of *Psl 8003* 48 hpi. Bacterial levels were quantified 72 hours after challenge inoculation. Statistical significance was determined with Student's t-test when $p < 0.05$. NS = no statistical difference.

Glycerol concentration	SAR	Level of Resistance observed
100 mM	2/2	2-3
150 mM	2/3	2-5
200 mM	3/3	1.03-2

Figure A2. Summary of bacterial fold difference in cucumber plants treated with glycerol as an inducer of resistance in cucumber when sprayed for 3 consecutive days. Cucumber plants were sprayed with 100 mM, 150 mM, 200 mM glycerol or mock-sprayed with water for 3 consecutive days prior to challenge with 10^5 cfu/ml *Psl 8003.* Induction rate was determined by the number of experiments where glycerol induced resistance out of the total number of experiments. The fold difference represents the statistically significant difference in bacterial growth in glycerol-sprayed plants compared to mock-sprayed plants. A total of 7 experiments were conducted. Statistical significance was determined with Student's t-test when $p < 0.05$.

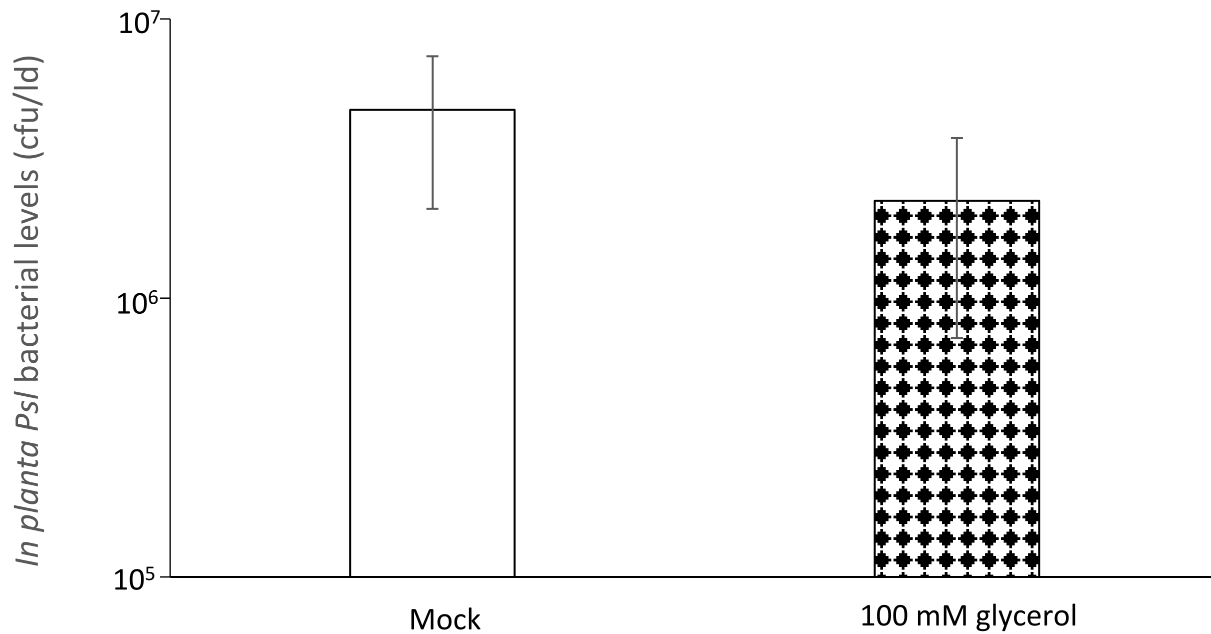

Figure A3. Spray application of glycerol does not induce resistance in distal leaves. One lower leaf per plant was sprayed with 100 mM glycerol or mock-sprayed with water for 3 consecutive days prior to challenge-inoculation of a distal leaf with 10^5 cfu/ml *Psl 8003.* This experiment was not repeated.

Experiment	Fold difference
1	2
2	NS
3	NS
4	NS

Figure A4. Summary of bacterial fold difference in cucumber plants treated with glycerol as an inducer of resistance in cucumber when sprayed on 2 consecutive days. Cucumber plants were sprayed with 150 mM gycerol or mock-sprayed with water for 2 consecutive days prior to challenge with 10^5 cfu/ml *Psl 8003.* The fold difference represents the statistically significant difference in bacterial growth in glycerol-sprayed plants compared to mock-sprayed plants. A total of 4 experiments were conducted. Statistical significance was determined with Student's t-test when $p < 0.05$. NS = no statistical difference.

Aza concentration	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6	Exp. 7
0.1 mM					4		
1 mM	10	NS		NS	NS	1.5	NS
2 mM		NS	2		NS		

Figure A5. Summary of bacterial fold difference in cucumber plants treated with azelaic acid as an inducer of local resistance in cucumber. Cucumber plants were vacuum infiltrated with 0.1 mM, 1 mM, 2 mM Aza, or 5 mM MES 24 hours prior to challenge-inoculation with10^5 cfu/ml *Psl 8003.* The fold difference represents the statistically significant difference in bacterial growth in Aza-infiltrated plants compared to MES-infiltrated plants. A total of 7 experiments were conducted. Statistical significance was determined with Student's t-test when $p < 0.05$. NS = no statistical difference.

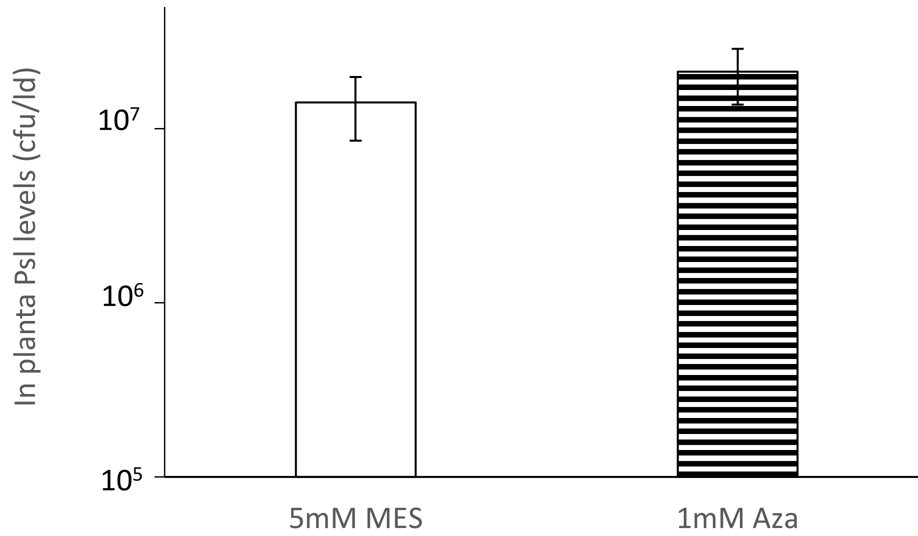

Figure A6. Infiltration of azelaic acid does not induce systemic resistance in cucumber. Cucumber lower leaves were infiltrated with 1 mM Aza or 5 mM MES 24 hours prior to challenge-inoculation of a distal leaf with 10^5 cfu/ml *Psl 8003.* This experiment was not repeated.

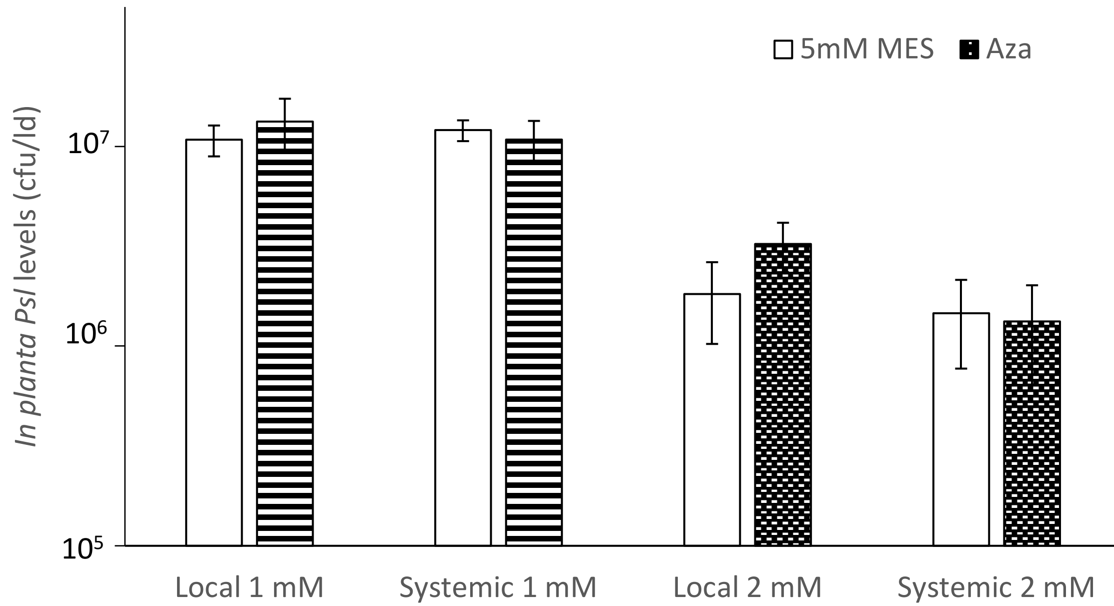

Figure A7. Spray application of azelaic acid does not induce local or systemic resistance in cucumber. Cucumber lower leaves were sprayed with 1 mM or 2 mM Aza, in separate experiments, or 5 mM MES 24 hours prior to challenge-inoculation of the same or a distal leaf with 10^5 cfu/ml *Psl 8003.* Only one experiment for each concentration was conducted.

Pip (mM)	Ex 1	Ex 2	Ex 3	Ex 4	Ex 5	Ex 6	Ex 7	Ex 8	Ex 9	Ex 10	Ex 11
1	2 (10 ml)										
10			NS (20ml) NS (40ml) 10 (60ml)								
20		6	NS (20ml)	2.5	NS	NS	NS	2.5	10	NS	NS
50		5.5	NS (20ml)								
100		5	2 (20 ml)								

Figure A8. Summary of bacterial fold difference in cucumber plants treated with pipecolic acid as an inducer of systemic resistance in cucumber plants treated once with a fixed volume. Cucumber plants were treated with Pip by soil-drenching with 10, 20, 40, or 60 ml of 1 mM, 10 mM, 20 mM, 50 mM, 100 mM pipecolic acid or water (mock-treatment) 24 hours prior to challenge-inoculation with 10^5 cfu/ml *Psl 8003* .Treatment volume is 60 ml unless specified otherwise in table. The fold difference represents the statistically significant difference in bacterial growth in Pip-treated plants compared to mock-treated plants. A total of 7 experiments were conducted. Statistical significance was determined with Student's t-test when $p < 0.05$.A total of 11 experiments were conducted. NS = no statistical difference.

Pipecolic acid concentration	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6	Exp. 7
20 mM	NS		3	4			NS
50 mM						2.5	
100 mM	NS	10	2	4.5	NS	5.5	

Figure A9. Summary of bacterial fold difference in cucumber plants treated with pipecolic acid as an inducer of systemic resistance in cucumber plants induced with a single saturation treatment. Cucumber plants were treated with Pip by soil-drenching to saturation with 20 mM, 50 mM, 100 mM pipecolic acid or water (mock-treatment) 24 hours prior to challenge-inoculation with 10^5 cfu/ml *Psl 8003*. The fold difference represents the statistically significant difference in bacterial growth in Pip-treated plants compared to mock-treated plants. A total of 7 experiments were conducted. Statistical significance was determined with Student's t-test when $p < 0.05$.A total of 7 experiments were conducted. NS = no statistical difference.

Pipecolic acid concentration	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6	Exp. 7
1 day prior	NS	2.5	10	NS		NS	NS
3 days prior	NS	7.5	NS		NS	NS	NS
7 days prior	NS	NS	NS	40		NS	NS

Figure A10. Summary of bacterial fold difference in cucumber plants treated with pipecolic acid as an inducer of systemic resistance in cucumber plants treated once 1, 3 or 7 days prior to challenge. Cucumber plants were treated with Pip by soil-drenching to saturation with 20 mM pipecolic acid or water (mock treatment) 1, 3, or 7 days prior to challenge-inoculation with 10^5 cfu/ml *Psl 8003*. The fold difference represents the statistically significant difference in bacterial growth in Pip-treated plants compared to mock-treated plants. A total of 7 experiments were conducted. Statistical significance was determined with Student's t-test when $p < 0.05$. A total of 7 experiments were conducted. NS = no statistical difference.

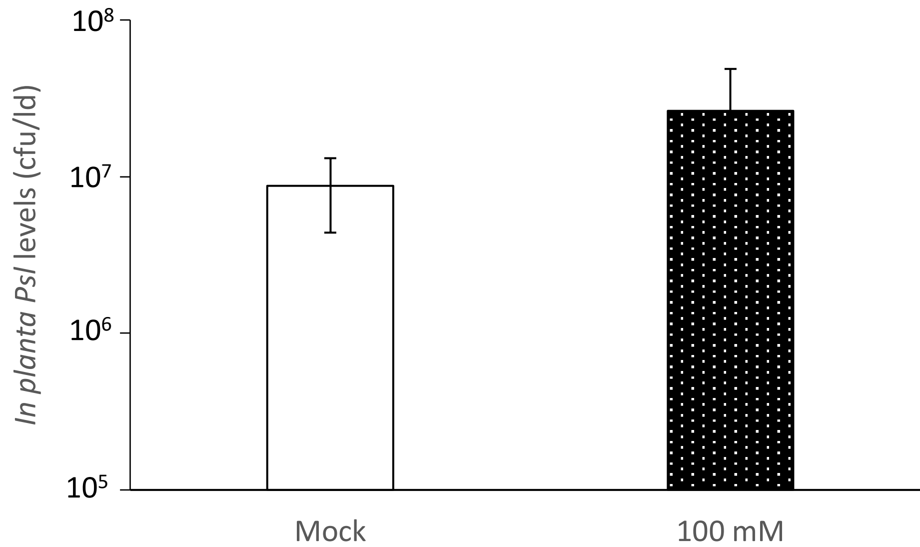

Figure A11. Pipecolic acid is not an inducer of systemic resistance in cucumber plants saturated for 3 consecutive days prior to challenge. Cucumber plants were treated with Pip by soil-drenching to saturation with 100 mM pipecolic acid or water (mock-treatment) for 3 consecutive days prior to challenge-inoculation with 10^5 cfu/ml *Psl 8003*.This experiment was not repeated.

Treatment	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6
1 week	NS	NS	NS	NS		
2 weeks	7.5	NS	Up to 26.5	NS	5	
3 weeks	NS	NS	Up to 12.5	Up to 15	NS	Up to 8.5

Figure A12. Summary of bacterial fold difference in cucumber plants treated with pipecolic as an inducer of systemic resistance in cucumber plants treated once weekly for 1, 2, or 3 weeks. Cucumber plants were soil-drenched with 50 mM or 200 mM Pip or water (mock-treatment) for 1, 2, or 3 weeks prior to challenge with 10^5 cfu/ml *Psl 8003*. The fold difference represents the statistically significant difference in bacterial growth in Pip-treated plants compared to mock-treated plants. A total of 7 experiments were conducted. Statistical significance was determined with an ANOVA and Tukey's HSD when $p < 0.05$. A total of 6 experiments were conducted. NS = no statistical difference.

	Exp. 1	**Exp. 2**	**Exp. 3**	**Exp. 4**	**Exp. 5**	**Exp. 6**	**Exp. 7**	**Exp. 8**	**Exp. 9**
Induced	0	25	0	0	15	5	3	0	10
Susceptible	75	80	40	60	50	40	40	20	55

Figure A13. Percent aggregation in PTI-induced and susceptible plants. Leaves of wild-type Col-0 were pressure infiltrated with 1 μM flg22 (induced) or mock-treated with water. 24 hours later, the same leaves were inoculated with virulent GFP-expressing *Pst DC3000.* Percent aggregation was monitored by categorizing each microscopic field of view as with or without aggregates. This experiment was conducted a total of 9 times.

Genotype	Treatment	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6	Exp. 7	Exp. 8
Col-0	Mock	75	80	40	60	40	40	20	55
	Flg22	0	25	0	0	5	3	0	10
sid2-2	Mock	90	90	90	60	70	60	20	50
	Flg22	25	40	50	20	45	20	20	5
fls2	Mock	90	90	70	75	50	70	30	40
	Flg22	75	90	30	80	90	80	3	45

Figure A14. Percent aggregation flg22-treated and mock-treated plants. Leaves of wild-type Col-0, sid2-2, and fls2 mutants were pressure infiltrated with 1 μM flg22 (induced) or mock-treated with water. 24 hours later, the same leaves were inoculated with virulent GFP-expressing *Pst DC3000.* Percent aggregation was monitored by categorizing each microscopic field of view as with or without aggregates. This experiment was conducted a total of 8 times.

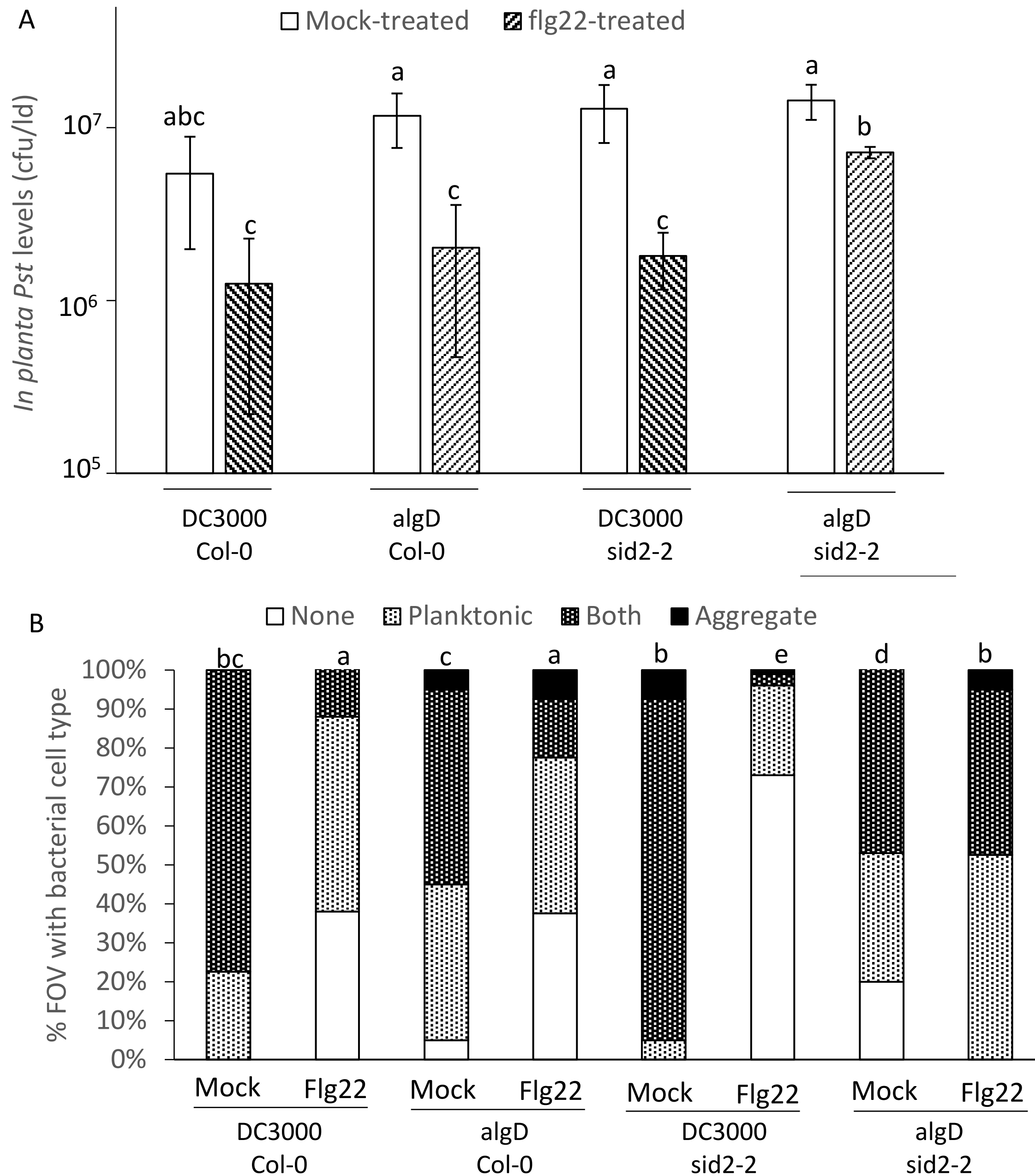

Figure A15. Aggregate formation of wild-type *Pst DC3000* or alginate mutant *algD* in Col-0 and *sid2-2.* Plants were pressure infiltrated with 1 μM flg22 (induced) or mock-treated with water. 24 hours later, the same leaves were inoculated with virulent GFP-expressing *Pst DC3000* or GFP-expressing alginate biosynthesis mutant *Pst algD.* A) *In planta* bacterial quantitation of wild-type *Pst*-inoculated and *algD*-inoculated Col-0 and sid2-2. Two-way ANOVA (Tukey's HSD). B) Aggregate formation was monitored by categorizing each microscopic field of view as containing no bacteria, only planktonic bacteria, only bacterial aggregates, or both planktonic and bacterial aggregates. Kruskal-Wallis test.

Glycerol	**3 sprays (local)**							**2 sprays (local)**				**systemic**
Experiment:	1	2	3	4	5	6	7	8	9	10	11	12
May-October				Y	Y	Y	Y	Y	N	N	N	N
November-April	Y	Y	N									

Figure A16. Summary of the effects of season on glycerol-induced resistance. N = no resistance induced. Y = resistance induced.

Aza	Vacuum infiltrated (local)							Spray (local)	Systemic
Experiment:	1	2	3	4	5	6	7	8	9
May-October						Y			
November-April	Y	Y	Y	Y	N		N	N	N

Figure A17. Summary of the effects of season on azelaic acid-induced resistance. N = no resistance induced. Y = resistance induced.

Pip	**Once, 60 ml**										
Experiment	1	2	3	4	5	6	7	8	9	10	11
May-October		Y	Y				N	Y	Y		
November-April	Y			Y	N	N				N	N

Figure A18. Summary of the effects of season on pipecolic acid-induced resistance when cucumber plants are treated once with a fixed volume. N = no resistance induced. Y = resistance induced.

Pip	Once, saturated						
Experiment	1	2	3	4	5	6	7
May-October	N	Y	Y	Y	N	Y	
November-April							N

Figure A19. Summary of the effects of season on pipecolic acid-induced resistance when cucumber plants are treated once to saturation. N = no resistance induced. Y = resistance induced.

Pip	**1, 3 and 7 days**						
Experiment	1	2	3	4	5	6	7
May-October	N	Y	Y	Y			
November-April					N	N	N

Figure A20. Summary of the effects of season on pipecolic acid-induced resistance when cucumber plants are treated 1, 3 or 7 days prior to challenge-inoculation. N = no resistance induced. Y = resistance induced.

Pip	**3 consecutive days**	**Weekly treatments**				
Experiment	1	2	3	4	5	6	7
May-October	N	Y	N	Y	Y	Y	
November-April							Y

Figure A21. Summary of the effects of season on pipecolic acid-induced resistance when cucumber plants are treated multiple times. N = no resistance induced. Y = resistance induced.

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