Getting to the Root of Infection: Using a Novel Model to Study Innate Immune Responses in Canola

by

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ABSTRACT

Agriculture is important towards the world's population survival but is challenged by plant pathogens affecting crops worldwide. Canola is one of the world's most important oilseed crops. *Pseudomonas aeruginosa* is an opportunistic pathogen with a broad host range and causes disease plants. In this study, we developed a novel root infection model of *P. aeruginosa* (strain PA14) in canola seedlings to study plant host immunity and bacterial pathogenesis. We showed that *P. aeruginosa* infection of seedlings caused dramatic weight loss, and that the quorum sensing system (specifically LasR) in *P. aeruginosa* was required for virulence. We also showed that genes involved in the phytorhomone signalling pathways: salicylic acid, jasmonic acid and ethylene were up regulated throughout the infection. We discovered *P. aeruginosa* produced coronatine, a jasmonic acid mimic using metabolomics. Finally, we limited the pathogen-induced stress ethylene in canola seedlings, and these plants were less susceptible to *P. aeruginosa* infection.

LIST OF ABBREVIATIONS USED

ABA	Abscisic acid
ACC	1-aminocyclopropane-a-carboxylic acid
ACCD	1-aminocyclopropane-a-carboxylic acid deaminase
ACS	ACC synthase
AHL	N-Acyl homoserine lactone
ANOVA	Analysis of variance
AVG	2-aminoethoxyvinyl glycine
BR	Brassinosteroid
BLAST	Basic local alignment search tool
CF	Cystic fibrosis
CFTR	Cystic fibrosis conductance regulator
CFU	Colony forming units
CK	Cytokinins
COR	Coronatine
DNA	Deoxyribonucleic acid
ET	Ethylene
ETI	Effector-triggered immunity
ETS	Effector-trigger susceptibility
GAC	Global activator of antibiotic and cyanide synthesis
GC	Gas chromatography
gDNA	Genomic DNA
GDP	Gross domestic product

GO	Gene ontology
Hcp	Homologous to Xcp
HHQ	2-heptyl-4-quinolone
HR	Hypersensitive response
HRMS	High-resolution mass spectrometer
I3G	Methoxy-indole-3-glucosinolate
IAA	Indole-3-acetic acid
IAN	3-indolylacetonitrile
ICN	Iodine cyanide
ISR	Induced systemic resistance
JA	Jasmonic acid
LadS	Lost adherence sensory
LC	Liquid chromatography
LRR	Leucine rich repeat
MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
MS	Murashige and Skoog
NB	Nucleotide binding
N/A	Not available
N/D	Not detected
OD	Optical density
PAMP	Pathogen-associated molecular pattern

PCR	Polymerase chain reaction
PGPB	Plant growth promoting bacteria
PRR	Pattern recognition receptor
Pst	*Pseudomonas syrinage* pathovar tomato strain DC3000
PTI	Pattern-triggered immunity
QS	Quorum sensing
RetS	Regulator of exopolysaccharide and type III secretion
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	rotations per minute
Rt	Retention time
SA	Salicylic acid
SAR	Systemic acquired resistance
T2SS	Type II secretion system
T3SS	Type III secretion system
T6SS	Type VI secretion system
TMV	Tobacco mosaic virus
WHO	World Health Organization
WT	Wild type
Xcp	Extracellular protein

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CHAPTER 1. INTRODUCTION

1.1 Importance of Agriculture

Agriculture is incredibly important towards the survival of the human race. With an ever-expanding population, we need to secure a sustainable amount of food for exponential population growth (Gerland *et al.*, 2014; Balatsky, Balatsky & Borysov, 2015). Plants (e.g. fruits and vegetables) and plant-based products (e.g. grains) make up half of Canada's food group guide (Eating Well with Canada's Food Guide, 2011), and even non-essential items in our diet, such as chocolate and candy are either derived from plants or made up of plant-based products, respectively. In Canada, the agricultural sector contributes over 110 billion dollars to the gross domestic product (GDP), and employs around 2.3 million Canadians (Agriculture & Agri-Food Canada, 2017). Canada largely produces oilseed and grain crops, which are primarily grown in the Prairie provinces and Ontario (Agriculture & Agri-Food Canada, 2017). Canada is the largest exporter of flaxseed, canola, pulses and durum wheat, and also produces around 75% of the world's maple syrup (Agriculture & Agri-Food Canada, 2017).

1.2 Threats to Agriculture

Importantly, before we can save our agriculture plants we must specifically identify their threats. It is well known, and easy to spot when insects are destroying crops because insects are large enough to spot by eye. In Canada there are many insects such as flea beetles that affect newly germinated canola plants (Insect Pests, 2015). Insects cause yield losses in Canada between 5 – 25% for cereal grains, and about 5 – 10% for canola alone (Insect Pests, 2015). Insects are becoming a huge threat to agriculture due to emergence of

insecticide resistant insects, perhaps more concerning is the environmental contamination from pesticides (Orzech & Nichter, 2008; Gavrilescu *et al.*, 2015). However, of larger concern is the microbial pathogens that we cannot see by eye, which are bacteria, viruses and fungi. Traditionally, it has been difficult to identify these pathogens using standard culture techniques and biochemical test. However, with the advancements in sequencing over the past few years it has been easier to identify agricultural pest (Hartmann *et al.*, 2015). A major pathogen of concern in Canada, *Plasmodiophora brassicae* is a fungus that infects canola plants primarily within the prairie provinces (Hwang *et al.*, 2011). *Plasmodiophora brassicae* causes clubroot in canola, which prevents the plant roots from taking up water and nutrients, ultimately leading to plant death, and major crop yield loss (Schwelm *et al.*, 2015). To date, in order to combat this disease farmers have been using clubroot resistant canola plants (Zhan *et al*., 2017), and also employing crop rotations in order to limit the spread of *P. brassicae*. There also exist many bacteria that are pathogenic to plants. Bacteria belonging to the genus of *Pseudomonas, Xanthomonas* and *Erwinia* are of particular threat to farmers due to the number of agriculturally important crops they infect (Mansfield *et al*., 2012). *Pseudomonas syringae* pathovars cause bacterial speck of tomato plants; *Xanthomonas campestris* pathovars cause black rot of crucifiers that affect all cultivated brassicas; while *Erwinia carotova* causes soft rot in potato plants (Mansfield *et al*., 2012). As for viruses, there is no better example than the agricultural losses caused by tobacco mosaic virus (TMV). TMV is a broad host range virus capable of infecting tobacco, tomato, potato and cucumber plants (Scholthof et al., 2011). In the United States, TMV is estimated to account for an average loss of 40 million pounds of tobacco annually (Scholthof, 2004). Luckily there are alternative agricultural methods that can protect plants,

such as genetically modifying crops so that they are resistant to such threats, or by the addition of beneficial microbes that can fight off potential pathogens and reduce stress within plants.

1.3 Bacterial-Plant Interactions

In nature, it is possible to observe three types of interactions between two different organisms: mutualistic, pathogenic and commensal. Mutualistic relationships occur when both organisms benefit from the interaction. Pathogenic relationships occur when one organism benefits from the interaction, while it is harming the other partner. Commensal relationships occur when one partner benefits from the interaction and the other partner is not harmed. In regard to bacterial-plant interactions, we can observe all three interactions within a plant's rhizosphere, which is a narrow region of soil directly associated with plant roots (Bais *et al.,* 2006). The rhizosphere is considered to be one of the most diverse environments on earth (Van Der Heijden, Bardgett & van Straalen, 2008).

One of the best-studied mutualistic interactions is between plant growth promoting bacteria (PGPB) and plants. PGPB are beneficial to plants, because they can reduce abiotic plant stress, fix atmospheric nitrogen, and protect plants against pathogens (Lugtenburg & Kamilova, 2009). An example of how PGPB can reduce plant stress is through the production of 1-aminocyclopropane-a-carboxylic acid (ACC) deaminase (ACCD), which reduces the amount ACC within plant cells, which in turn limits ethylene (ET) production in plants (Glick, 2014). ET is a major stress molecule in plants, and if it builds up the plants will die (Li *et al*., 2013). PGPB can also induce the formation of root nodules using their type III secretion systems (T3SS) (Okazaki *et al.*, 2013). Within the root nodules are sites

in which PGPB can fix atmospheric nitrogen, therefore converting nitrogen into ammonium or nitrate that plants can use (Masson-Boivin *et al.,* 2009). Finally, PGPB produce antimicrobial compounds that kill pathogenic fungi and bacteria, which ultimately provide protection for plants (Lugtenburg & Kamilova, 2009). In return, PGPB get compensated with nutrients that are provided by the plant (Haichar *et al.*, 2008).

Plants make excellent host for bacterial pathogens because they are rich in nutrients. Plants are host to many bacterial pathogens; the most common genera of bacterial pathogens are *Pseudomonas*, *Erwinia* and *Xanthomonas* (Mansfield *et al.,* 2012). These three bacterial genera are agricultural threats because they infect many agriculturally important crops (Mansfield *et al.,* 2012). An example of a pathogenic interaction is *P. syringae* infecting tomato plants. *Pseudomonas syringae* pathovar tomato (Pst) strain DC3000 usually enters through tomato plants leaf stomata, multiplies within the intercellular space and produces necrotic lesions on both the plants leafs and fruiting bodies (Buell *et al.*, 2003). Pst DC3000 can successfully infect host plants through the use of its T3SS (described in more detail below), which ultimately leads to plant death (Kvitko *et al*., 2009).

Commensal interactions occur all the time within the plant's rhizosphere. It is a dynamic environment in the sense that bacterial-plant interactions are constantly changing (Berendsen, Pieterse & Bakker, 2012). There are some bacteria within the rhizosphere where at different times can be both pathogenic and mutualistic (Berendsen, Pieterse & Bakker, 2012). There are likely bacteria that do not fit into either of those two roles, and they are simply present within the rhizosphere because it is a nutrient rich environment (Berendsen, Pieterse & Bakker, 2012). A specific commensal interaction example is

lacking; however, any bacteria present within the rhizosphere that neither harms nor benefits the plant could be considered commensal (Berendsen, Pieterse & Bakker, 2012).

1.4 Plant Innate Immune Responses

Plants are similar to mammals in the sense that they possess an immune system; however, the immune responses are quite different (Ausubel, 2005). Mammals have both adaptive and innate immune responses, whereas plants only have an innate immune response (Ausubel, 2005). Plants also lack mobile defender cells; instead they rely on each cell being able to mount a successful immune response against invading pathogens (Jones & Dangl, 2006). Regardless, plants are still quite successful at fighting off invading pathogens (Jones & Dangl, 2006). Plants can recognize invading pathogens in two ways, pattern-triggered immunity (PTI) and effector-triggered immunity (ETI) (Jones & Dangl, 2006).

All microbes that interact with plants have extracellular proteins (e.g. flagellin) or pathogen-associated molecular patterns (PAMPs) that can be recognized by plant cells (Millet *et al*., 2010). Once these PAMPs are recognized by transmembrane pattern recognition receptors (PRRs), this triggers a cascade of immune signalling events (Jones & Dangl, 2005). Detection of the synthetic peptide Flg22 (flagellin peptide from *P. aeruginosa*) leads to the activation of a mitogen-activated protein kinase (MAPK) cascade consisting of MEKK1 [mitogen-activated protein kinase kinase kinase (MAPKKK)], MKK4/5 [mitogen-activated protein kinase kinase (MAPKK)], and MPK3/6 (MAPK) (Asai *et al*., 2002; Cheng *et al*., 2015). There is also a second signalling cascade that involves MEKK1. MEKK1 functions in the MEKK1-MKK1/2-MPK4 cascade, which is

also activated by recognition of Flg22 (Suarez-Rodriguez *et al.*, 2006; Qui *et al.*, 2008). The MAPK cascades are key parts of the PTI response in plants (Pieterse *et al.*, 2009) (Figure 1a).

On the other hand, bacterial pathogens have evolved over time to evade recognition by the plants immune system. Over evolutionary time, bacterial pathogens have acquired genes that can suppress PTI, which is called effector-trigger susceptibility (ETS) (Pieterse *et al.*, 2009) (Figure 1b). Pst DC3000 secretes 28 effectors via its T3SS to supress the PTI response in plants (Jones & Dangl, 2006). Two of these effectors, AvrRpm1 and AvrB induce phosphorylation of RIN4 (a PRR) (Mackey *et al.*, 2002). Whereas, the third effector AvrRpt2 is a cysteine protease, which cleaves RIN4 at two sites, thus inactivating this PRR (Axtell *et al.*, 2003; Axtell & Staskawicz, 2003; Coaker, Falick & Staskawicz, 2005; Kim *et al.*, 2005). Altogether, these effectors supress the plant innate immune response. *Pseudomonas syringae* also encodes other effectors that cause chlorosis (HopG1), lesion formation (HopAM1-1), and promote bacterial growth and symptom production within plants (HopM1, HopE1, AvrE, HopAA1-1 and HopN1) (Cunnac *et al*., 2011).

In some cases, these effectors allow the bacterial pathogen to suppress the PTI response; however, these effectors can also be recognized and trigger an immune response (Tsuda & Katagiri, 2010) (Figure 1c). In the ETI response, bacterial effectors are recognized by specific disease resistance (*R*) genes found within the plant (Chisholm *et al.*, 2006). Most *R* genes encode nucleotide binding (NB) and leucine rich repeat (LRR) domain proteins (Chisholm *et al.*, 2006). Little is known about the precise signalling events that occur during ETI; however, it is believed to be similar to the PTI response (Tsuda & Katagiri, 2010). The ETI response in plants is generally quicker and longer lasting

compared to PTI (Tsuda & Katagiri, 2010). ETI generally results in a hypersensitive response (HR), a localized defence response, in which cells infected with bacterial effectors will die in order to limit the infection (Jones & Dangl, 2006).Callose (a plant cell wall polysaccharide) will also be deposited by neighbouring cells in order to strengthen their cell walls and limit the spread of the infection (Luna *et al.*, 2011). A similar defense response occurs when plants recognize invading viruses, in order to limit the spread of the virus (Mandadi & Scholthof, 2013).

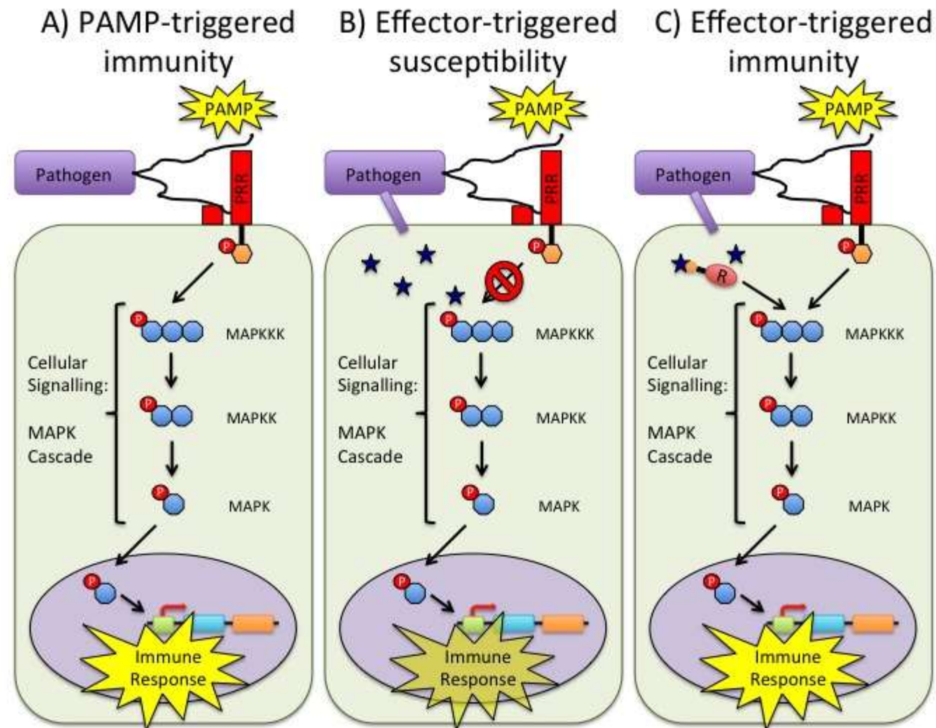

Figure 1.1: Simplified drawing representing plant innate immunity. **A,** Once a pathogen attacks plant cells, pathogen-associated molecular patterns (PAMPs) activate pattern recognition receptors (PRRs), which lead to downstream signalling cascade activating pattern-triggered immunity (PTI). **B,** Successful plant pathogens have acquired effectors (purple stars) over evolutionary time that can supress PTI, resulting in effector triggered susceptibility (ETS). **C,** However, plants have also acquired resistance (R) proteins that can recognize specific effector molecules, resulting in a secondary immune response called effector triggered immunity (ETI). Adapted from Pieterse *et al.*, 2009.

1.4.1 Reactive Oxygen Species Production and their Role in Plant Immunity

One of the earliest immune response in plants is the production of reactive oxygen species (ROS) (Bailey-Serres & Mittler, 2006). ROS is produced following the MAPK signalling cascade, which as previously mentioned follows recognition of the pathogen by PTI or ETI (Tsuda & Katagiri, 2010). ROS production has two functions; ROS can be a signalling molecule and is also a form of antimicrobial protection (Bailey-Serres & Mittler, 2006). ROS production damages bacterial cell lipids, proteins and DNA (Sewelam, Kazan & Schenk, 2016). Unsurprisingly, ROS production damages host cells if not anti-oxidized (Sewelam, Kazan & Schenk, 2016). The host plant normally encodes antioxidant enzymes, such as SOD (converts O_2^- to H_2O_2), catalases and peroxidase (which remove H_2O_2) (Sewelam, Kazan & Schenk, 2016). In terms of signalling, ROS production was shown to induce genes involved in the HR, stomata closure, and the induction of WRKY transcription factors (Jones & Dangl, 2006; Sewelam, Kazan & Schenk, 2016).

1.4.2 Phytohormones and their Role in Plant Immunity

Plants use many phytohormones to regulate diverse biological processes within cells. Three main phytohormones: salicylic acid (SA), jasmonates and ET are involved in regulating the defence response in plants (Pieterse *et al.*, 2009). However, more recently, researchers have discovered that phytohormones involved in growth and development (Auxin, abscisic acid, gibberellin, cytokinin, brassinosteroids and peptide hormones) may also play a role in plant defence (Denancé *et al.*, 2013). Therefore, the innate immune response in plants is quite complex and involves many phytohormones.

SA plays an important role in plant immunity. SA production activates genes involved in the defence response against biotrophic (derive energy from living tissues) and hemi-biotrophic (derive energy from living tissues but can also live in dead tissue) pathogens (Bari & Jones, 2009; Groen *et al.*, 2013). SA production also induces genes involved in systemic acquired resistance (SAR), which is a whole plant defence response following detection of a pathogen (Tsuda *et al.*, 2008). Whereas, Jasmonic acid (JA) and ET are normally associated with defence against necrotrophic (derive energy from dead tissue) pathogens (Groen *et al.*, 2013). JA and ET production induces genes involved in induced systemic reistance (ISR), which trigger a localized immune response toward invading pathogens (Pieterse *et al.*, 2014). JA's roles in seed germination, root growth, tuber formation, fruit ripening, leaf senescence and stomatal opening are well characterized (Bari & Jones, 2009). JA accumulates during an active infection and has an impact on the expression of genes involved in the innate immune response (Hickman *et al.*, 2017). JA has been shown to be involved in long distance information transfer during an active infection, which suggest JA may be involved in induced SAR (Truman *et al.*, 2007). Interestingly, there are synergistic and antagonistic interactions that exist among these three phytohormones: SA production is antagonistic to JA production, whereas JA and ET production is synergistic (Pieterse *et al.*, 2009). It was proposed that plants could modulate the abundance of SA, JA and ET levels to modify the expression of genes involved in the defence response, as well as coordinate interactions between complex defence signalling pathways to generate an effective defence response against invading pathogens (Bari & Jones, 2009).

There exists a complex interaction between phytohormones involved in the innate immune response and plant growth and development. Some phytohormones involved in plant growth and development have been shown to play a role in innate immunity (Denancé *et al.*, 2013). Auxin has typically been studied for its role in root development; auxin production activates the expression of expansins, which loosen plant cell walls (Ding *et al.*, 2008). This could play an important role in innate immunity because the loosening of the cell wall could be exploited by potential pathogens (Ding *et al.*, 2008). Studies have shown that auxin production is down regulated in tissue after the induction of SAR (Wang *et al.*, 2007).

Abscisic acid (ABA) has been shown to be involved in many growth and development responses, including seed germination, embryo maturation, leaf senescence, stomatal aperture and adaptation to biotic and abiotic stresses (Bari & Jones, 2009). However, ABA's role in plant defence is quite complex and varies from species to species (Denancé *et al.*, 2013). ABA has been shown to activate stomatal closure, which acts as a barrier for invading pathogens (Takahashi *et al.*, 2013). ABA production has been shown to both positively and negatively regulate the plant defence response (Mauch-Mani & Mauch, 2005; Asselbergh *et al.*, 2008; Ton *et al.*, 2009). ABA plays an important role in activating genes involved in plant defence through transcriptionally reprogramming the metabolism of plant cells (Adie *et al.*, 2007). Moreover, ABA has been shown to be required for JA biosynthesis, and the deposition of callose (Adie *et al.*, 2007).

Gibberellin (GA) promotes plant growth by stimulating the degradation of DELLA proteins, which are negative regulators of plant growth (De Bruyne, Höfte & De

Vleesshauwer, 2014). Previously it was also shown that DELLA proteins could modulate plant innate immune responses by controlling SA and JA dependent defence responses (Navarro *et al.*, 2008). DELLA proteins promote resistance to necrotrophs by activating JA/ET-dependent defence responses; however, this makes plants susceptible to biotrophs by repressing SA-dependent defence responses (Navarro *et al.*, 2008). DELLA proteins are also involved in regulating ROS levels after biotic or abiotic stress by promoting the expression of ROS detoxification enzymes (Achard *et al.*, 2008).

Cytokinins (CK) are plant hormones involved in diverse processes, which include stem-cell differentiation, vascular differentiation, chloroplast biogenesis, seed development, leaf senescence, nutrient balance, stress tolerance, and growth and branching of root, shoot and inflorescence (Bari & Jones, 2009). CK's role in plant defence is poorly understood; however, CK has been shown to play an important role in the development of clubroot disease caused by *P. brassicae* in *Arabidopsis* (Siemens *et al.*, 2006).

Brassinosteroids (BRs) are plant hormones involved in the regulation of growth and development, which include seed germination, cell division, cell elongation, flowering, reproductive development, senescence, and abiotic stress response. However, very little is known about their involvement in plant innate immunity. BR has been shown to induce genes involved in the biosynthesis of ET (ACC synthases) (Yi *et al.*, 1999).

Finally, peptide hormones (the newest class of hormones) have been shown to be involved in plant growth and development as well as defence responses to attacking pathogens (Matsubayashi & Sakagami, 2006). These peptides are typically 10 amino acids in length and are processed from wound- and JA-inducible precursor proteins

(Matsubayashi, 2014). Peptide hormones have been shown to be involved in both local and distant defence responses in plants (Matsubayashi, 2014).

1.5 *Pseudomonas aeruginosa* Background and Clinical Concerns

Pseudomonas aeruginosa is a Gram-negative rod-shaped bacterium that belongs to the Pseudomonadacea family of bacteria. *Pseudomonas aeruginosa* is a ubiquitous, opportunistic pathogen with a broad host range. It infects many model organisms such as *Danio rerio*, *Mus musculus*, *Drosophila melanogaster*, *Caenorhabditis elegans* and *Arabidopsis thaliana* (Lee & Zhang, 2014). Since *P. aeruginosa* is found ubiquitously within the environment, it is a frequent colonizer of our skin's microflora (Lyczak, Cannon & Pier, 2000). However, due to its opportunistic nature, it rarely causes infections in immunocompetent humans (Clatworthy *et al.,* 2009). However, it is the most common cause of infections in burn patients (e.g. septic shock, hemorrhage and necrosis) and the outer ear (e.g. Swimmers ear) (Mesaros *et al.*, 2007). *Pseudomonas aeruginosa* can be spread to immunocompromised patients in hospitals due to improper hand washing by medical staff and through medical devices (e.g. catheters, in which it causes urinary tract infections) (Engelhart *et al.,* 2002; Girou *et al.,* 2004). *Pseudomonas aeruginosa* is responsible for approximately ten percent of all hospital acquired infections (EHA Consulting Group, 2018), and is the most common pathogen isolated from patients that have been hospitalized for longer than one week (Medscape, 2017). *Pseudomonas aeruginosa* is capable of infecting many tissues within our body (Table 1). Most notably, *P. aeruginosa* infections in humans are often associated with people living with cystic fibrosis (CF) (Oliver *et al*., 2000).

Table 1.1: Tissue specific diseases caused by *P. aeruginosa*.

Body System	Disease	Reference
Respiratory tract	Pneumonia	Marion *et al.,* 2016
Bloodstream	Bacteremia	Buehrle *et al.,* 2017
Heart	Endocarditis	Hagiya *et al.*, 2016
Central Nervous System	Meningitis,	Pai *et al.,* 2016
Ear	Otitis Externa	Lutz & Lee, 2011
Eye	Bacterial Keratitis	Sharma *et al.,* 2018
Bones and Joints	Osteomyelitis	Krajewski *et al.,* 2014
Gastrointestinal Tract	Diarrhea, Enteritis, Enterocolitis	Chuang *et al.,* 2014
Urinary Tract	Urinary Tract Infection	Carmeli *et al.*, 2016
Skin	Ecthyma Gangrenosum	Vaiman et al., 2015

1.5.1 Troubles with Treating a *P. aeruginosa* Infection

Pseudomonas aeruginosa clinical infections are typically very difficult to treat, because this bacterium forms intricate biofilms (Wozniak *et al.*, 2003). Biofilms are groups of bacteria composed of extracellular polymeric substances, such as proteins, DNA, polysaccharides (e.g. alginate) and RNA (Nadell *et al.*, 2015). Bacteria typically form biofilms on wet/moist surfaces, which prevent the colonies from drying out (Nadell *et al.*, 2015). Bacterial colonies produce biofilms in order to protect them from external factors, such as antibiotics (Wozniak *et al.*, 2003; Nadell *et al.*, 2015). Antibiotics have difficulty penetrating the layers of the biofilm, therefore making it harder to treat infections caused by biofilm producing bacteria (Wozniak *et al.*, 2003). This is especially troublesome for patients with CF, when *P. aeruginosa* enters the lungs of people with CF; it is incredibly hard for clinicians to treat this infection (Wozniak *et al.*, 2003). CF is caused by a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which leads to the production of thick mucus (Cutting, 2015). The overproduction of thick mucus makes it extremely difficult for the patients to clear bacterial infections by their own immune system (Cutting, 2015). With clinical isolates of *P. aeruginosa* producing biofilms, it is nearly impossible to eradicate these infections using antibiotics (Wozniak *et al.*, 2003).

Adding to the problem, many *P. aeruginosa* clinical isolates carry antibiotic resistance genes (Rizek *et al.*, 2014), and even when these genes are not present within its genome, *P. aeruginosa* usually persists through antibiotic treatment due to biofilm formation and its large number of efflux pumps (Flemming *et al*., 2016; Chalhoub *et al.*, 2017). The world health organization (WHO) has recently listed carbapenem-resistant *P.*

aeruginosa as one of three bacterial species in which there is a critical need for the development of new antibiotics to treat infections (World Health Organization, 2017).

1.5.2 *Pseudomonas aeruginosa* Environmental Concerns

As previously mentioned, *P. aeruginosa* has also been shown to infect plants. The earliest described *P. aeruginosa* infection actually occurred in tobacco plants in the Philippines in 1930 (Clara, 1930). In tobacco plants, *P. aeruginosa* causes severe leaf spotting and necrosis, as well as soft stem rot in seedlings (Clara, 1930). From this point onwards, *P. aeruginosa* was studied primarily as an animal pathogen (Elrod & Braun, 1941), and to a lesser extent as a plant pathogen (Elrod & Braun, 1942). Kominos *et al*. (1972), found that *P. aeruginosa* was being introduced into hospitals by contaminated vegetables. They isolated *P. aeruginosa* from tomatoes, radishes, celery, carrots, endive, cabbage, cucumbers, onions and lettuce, and it was found that a patient consuming a tomato salad might ingest as many as 5×10^5 colony forming units (CFU) of bacteria (Kominos *et al*., 1972). More recently, proteins secreted by *P. aeruginosa* were tested as potential virulence factors in *Arabidopsis* (Rhame *et al.*, 1995; Rhame *et al.*, 1997; Rhame *et al.*, 2000). Cheng *et al.* (2015) examined the innate immune response against *P. aeruginosa* in *Arabidopsis and* found that *P. aeruginosa* secretes an effector protein (Protease IV) that can cleave PRRs in *Arabidopsis*, which gave us a better understanding of how this pathogen can evaded immune responses.

1.6 *Pseudomonas aeruginosa* Secreted Virulence Factors

Pseudomonas aeruginosa is a versatile opportunistic pathogen that possesses a wealth of pathogenic weapons, which allow it to survive in harsh environments (Bleves *et al.*, 2010). These environments include tissues within the human body (e.g. lungs), in which *P. aeruginosa* needs to fight against the human immune system in order to proliferate and avoid being cleared by an immune response (Gellatly & Hancock, 2013). *Pseudomonas aeruginosa* can also proliferate within one of the most complex environments on earth, the rhizosphere (Pandey *et al*., 2005). This environment is full of not only microorganism (e.g. bacteria and fungi) but also eukaryotic organism (e.g. worms), which *P. aeruginosa* must compete against in order to survive. The main secretion systems will be discussed below.

1.6.1 *Pseudomonas aeruginosa* Type II Secretion Systems

Type II secretions systems (T2SS) are highly versatile because they allow bacteria to secrete large multimeric exoproteins that are already folded in the periplasm (Thomassin *et al.*, 2017). *Pseudomonas aeruginosa*'s genome encodes two T2SS, Xcp (extracellular protein) and Hxc (homologous to Xcp) (Ball *et al.*, 2002). The organisation of these two secretion systems is highly similar; the main difference is that Xcp T2SS secretes multiple substrates, whereas the Hxc T2SS secretes only one exoprotein, an alkaline phosphatase (Table 2) (Bleves *et al.*, 2010). The structure of the Xcp T2SS in *P. aeruginosa* is a protein platform set up on the inner membrane (Proteins XcpAPSYZ), and a large channel embedded in the outer membrane (the secretin XcpQ) (Bleves, Lazdunski & Filloux, 1996; Gérard-Vincent *et al.*, 2002; Michel, Durand & Filloux, 2007; Bleves *et al.*, 2010). Attached to the secretion apparatus on the inner membrane is an adenosine triphosphatase

motor (XcpR), which uses adenosine triphosphate to secrete proteins through the channel (Robert, Filloux & Michel, 2005). Finally, there is a fimbrillar structure called the pseudopilus, which is formed by the assembly of the major pseudopilin (XcpT) and then capped with the minor pseudopilins XcpU-X (Durand *et al.*, 2003; Alphonse *et al.*, 2010). The pseudopilus functions as a piston, which pushes the exoproteins through the secretion channel and out of the bacterial cell (Durand *et al.*, 2005; Douzi *et al.,* 2009). The Xcp T2SS secretes many exoproteins, including elastase, staphylolysin, aminopeptidase and proteases (Table 2). As discussed below, the T2SSs are regulated by the quorum sensing (QS) systems in *P. aeruginosa* (Bleves *et al.*, 2010).

Table 1.2: Exoproteins secreted by *P. aeruginosa.*

Secretion System	Secreted Protein	Function	Reference
T2SS (XcP)	CbpD	Chitin binding protein	Folders *et al.*, 2000
	LasB	Protease-elastase	Braun *et al.*, 1998
	LasA	Protease-elastase	Braun *et al.*, 1998
	PlcH	Hemolytic phospholipase	Barker *et al.*, 2004
	PlcN	Phospholipase	Voulhoux *et al.*, 2001
	PlcB	Phospholipase	Voulhoux *et al.*, 2001
	LoxA	Lipoxygenase	Vance *et al.*, 2004
	ToxA	Exotoxin A-ADP ribosyltransferase	Lu *et al.*, 1993
	PmpA	Putative protease	Bleves *et al.*, 2010
	PrpL	Protease	Fox *et al.*, 2008
	LipA	Lipase	Jaeger *et al.*, 1994
	LipC	Lipase	Martinez *et al.*, 1999
	PhoA	Alkaline phosphatase	Filloux *et al.*, 1988
	PaAP	Aminopeptidase	Braun *et al.*, 1998
T2SS (Hxc)	LapA	Low-molecular weight alkaline phosphatase	Ball *et al.*, 2002
T3SS	ExoS	GAP* and ADRPT**	Yahr *et al.*, 1997
	ExoT	GAP* and ADRPT**	Yahr *et al.*, 1997
	ExoU	Patatin-like phospholipase	He *et al.*, 2004
	ExoY	Adenylate cyclase	Yahr *et al.*, 1998
T6SS	Tse1	Cell wall degrading	Hood *et al.*, 2010
	Tse2	Cell wall degrading	Hood *et al.*, 2010
	Tse3	Cell wall degrading	Hood *et al.*, 2010
	Tle1	Phospholipase	Russell *et al.*, 2013
	Tle2	Phospholipase	Russell *et al.*, 2013
	Tle3	Phospholipase	Russell *et al.*, 2013
	Tle4	Phospholipase	Russell *et al.*, 2013
	Tle5	Phospholipase	Russell *et al.*, 2013

* GTPase activating protein (GAP)
** Adenosine diphosphate ribosyltransferase domain (ADPRT)

1.6.2 *Pseudomonas aeruginosa* Type III Secretion System

Wide ranges of pathogenic (both human and plant) Gram-negative bacteria possess a T3SS (Coburn, Sekirov & Finlay, 2007). Strikingly, it has been recently discovered that plant growth promoting rhizobacteria also encode a T3SS, which is thought to be used for promoting root nodule formation (a site for fixing atmospheric nitrogen) (Masson-Boivin *et al.*, 2009). T3SSs are needle-like apparatuses, which translocate bacterial effectors across the eukaryotic plasma membrane (Radics, Königsmaier & Marlovits, 2014). In *P. aeruginosa*, the T3SS is straight hollow tube measuring about 60-80 nm long and 7 nm wide, which is made up of PscF subunits (Pastor *et al.*, 2005; Soscia *et al.*, 2007). PscE and PscG are cytoplasmic chaperones, which prevent premature polymerization of PscF (Quinaud *et al.*, 2005). PscP is considered to be a molecular ruler, which controls the length of the T3SS (Journet *et al.*, 2003). Not a lot is known about the proteins involved in forming the base of the T3SS in *P. aeruginosa* (Bleves *et al.*, 2010). It has been inferred that PscN is likely the ATPase powering the system, PscI is and anchoring protein (Monlezun *et al.*, 2015) and PscJ is thought to the major component of the base of the T3SS since they share similarities with proteins found in the *Yersinia enterocolitica* T3SS (Bleves *et al.*, 2010). PscC is a secretin that polymerizes in the presence of the PscW pilotin, which form a channel through the bacterial outer membrane and allows the passage of the T3SS (Koster *et al.*, 1997; Burghout *et al*., 2004).

Once the needle-like structure is assembled, the translocators PopB and PopD, and PcrV are the first proteins secreted through the T3SS (Sawa *et al*., 1999; Dacheux *et al*., 2001; Sundin *et al*., 2002). PcrV is localized at the tip of the T3SS, whereas PopB and PopD are secreted through the needle to form a pore in the eukaryotic plasma membrane

(Schoehn *et al.*, 2003). Importantly, the PopB/D translocon proteins share a chaperone, PcrH, which prevents premature interactions with bacterial membranes (Schoehn *et al.*, 2003). The T3SS in *P. aeruginosa* encodes four effector proteins ExoS, ExoT, ExoU and ExoY (Table 2) (Wolfgang et al., 2003). ExoS and ExoT share a chaperone SpcS (Shen *et al.*, 2008), whereas the chaperone for ExoU is SpcU (Finck-Barbançon, Yahr & Frank, 1998). The T3SS regulon is largely under the control of 2-component regulatory systems RetS/LadS/Gac-Rsm and is co-regulated by the QS system in *P. aeruginosa* (Bleves *et al.*, 2010).

1.6.3 *Pseudomonas aeruginosa* Type VI Secretion Systems

The type VI secretion system (T6SS) is one of the most recently described secretion system in *P aeruginosa*. It is believed that *P. aeruginosa*'s genome encodes three such systems, H1-T6SS to H3-T6SS (Silverman *et al*., 2012). In general, T6SS encode 13 conserved genes, which are important for its functionality (Silverman *et al*., 2012). There are three membrane-associated proteins; TssL and TssM are integral membrane proteins, whereas TssJ is a lipoprotein (Zoued *et al.*, 2016). The 'harpoon' or bacteriophage tail components of the T6SS are TssE, TssF, TssG and TssK proteins that form the baseplate of the T6SS; TssB and TssC are proteins that form the sheath of T6SS; Hcp (inner tube) and VgrG (spike) are components of the bacteriophage tail; and TssA is the cap protein of the bacteriophage tail (Zoued *et al.*, 2016; Zoued *et al.*, 2017). In *P. aeruginosa* five effector proteins have been described, and they are secreted through the T6SS once the VgrG spike falls off the bacteriophage tail in the foreign bacterial cell (Silverman *et al*., 2012). The effectors include Tse1 and Tse3, which act on bacterial peptidoglycan; and Tle1-5, which

act on bacterial cell membranes (Table 2) (Russell, Peterson & Mougous, 2014). Tse1 cleaves the peptide crosslinks of peptidoglycan, whereas Tse3 cleaves the glycan backbone of peptidoglycan (Russell, Peterson & Mougous, 2014). Tle1-5 are phospholipases that target the lipid components of bacterial cell membranes (Russell, Peterson & Mougous, 2014). The T6SS is regulated through environmental signals and the QS system in *P. aeruginosa* (Silverman *et al*., 2012).

1.7 Regulation of Virulence Factors in *P. aeruginosa*

Cell-to-cell communications using diffusible signalling molecules give individual bacterial cells the ability to communicate with one another. In Gram-negative bacteria, the majority of these diffusible signalling molecules are *N*-Acyl homoserine lactones (AHLs) (Whitehead *et al.,* 2001). AHLs consist of fatty acids of variable length and substitution, which are linked via a peptide bond to a homoserine lactone (Nadal Jimenez *et al.,* 2012). These AHL molecules allow bacterial cells to trigger responses and perform activities at a community level (Whitehead *et al.,* 2001). These triggered responses include localization signals, biofilm production and the regulations of many virulence factors in *P. aeruginosa* (Schuster & Greenberg, 2006). The signalling network in *P. aeruginosa* is particularly complex; however, remains to be one of the best studied signalling networks among all microbial systems (Nadal Jimenez *et al.,* 2012).

1.7.1 Regulation of the QS System in *P. aeruginosa*

In *P. aeruginosa*, two different AHL systems control QS, which are the Las and Rhl systems (Nadal Jimenez *et al.,* 2012). These two systems are quite different in the sense

that they respond to different AHL signalling molecules (Nadal Jimenez *et al.,* 2012). The Las system produces and responds to *N*-3-oxo-dodecanoyl homoserine lactone (3-oxo-C_{12}-HSL), which is produced by the LasI synthase and recognized by the transcriptional regulator LasR (Passador *et al.*, 1993; Pearson *et al.*, 1994). The Las systems regulates the production of many virulence factors that are involved in acute infections, which include the T2SS effectors; LasA and LasB elastases and exotoxin A (Gambello, Kaye & Iglewski, 1993; Jones *et al.*, 1993; Passador *et al.*, 1993).

The Rhl AHL systems produce and respond to *N*-butanoyl homoserine lactone (C_4-HSL), which is produced by the RhlI synthase and sensed by the transcriptional regulator RhlR (Pearson *et al.*, 1995). The Rhl system regulates the production of rhamnolipids and can represses genes involved in the assembly of the T3SS (Bleves et al., 2005). Interestingly, there is a hierarchical relationship between the two QS systems, in which the Las system controls the Rhl system (Latifi *et al.*, 1996). The 3-oxo-C_{12}-HSL-LasR complex formed during the Las system QS, directly upregulates *rhlR* transcription (Figure 2) (Nadal Jimenez *et al.,* 2012).

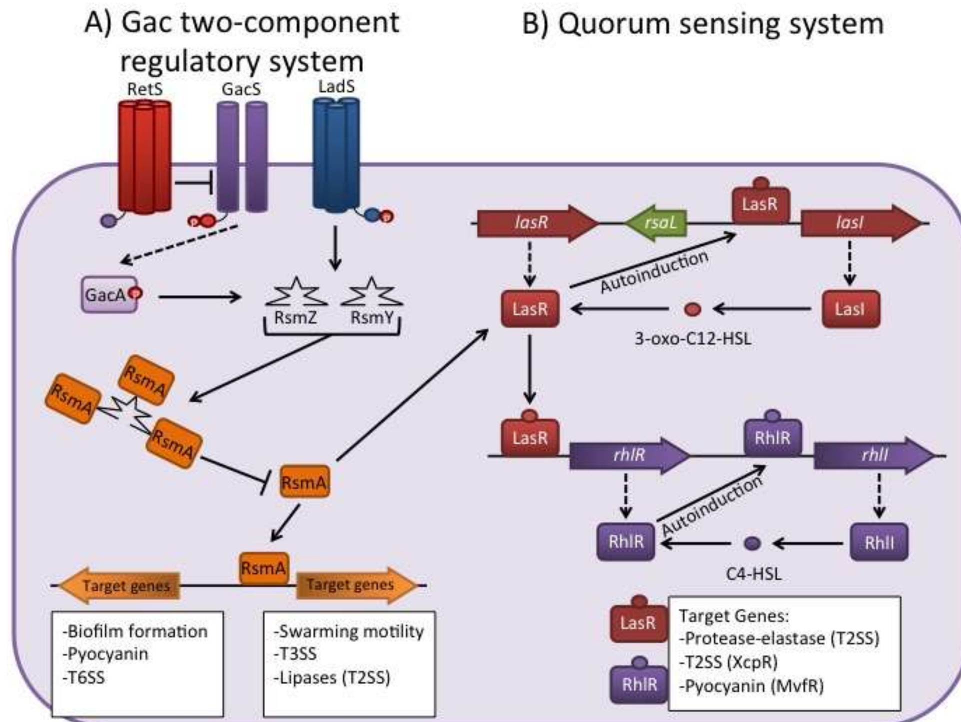

Figure 1.2: The hierarchical regulation of virulence genes in *P. aeruginosa*. A, When free, RsmA can bind to the promoters of bacterial motility and acute virulence genes, which in turn activates the expression of these genes. RsmA also controls the expression of genes involved in chronic infections. However, the phosphorylation of GacA by GacS stimulates the production of small RNAs RsmZ and RsmY, which bind RsmA and switch the repression of chronic infection genes to acute infection genes. LadS works together with GacS to activate the production of RsmZ and RsmY, while RetS works in the opposite manner and Represses the production of RsmZ and RsmY. **B,** Upon detection of certain threshold concentration of 3-oxo-C_{12} HSL, the 3-oxo-C_{12} HSL – LasR complex binds to the promoter of multiple genes, which either activates or represses transcription. One of the genes activated by this complex is *lasI*, which enhances the production of 3-oxo-C_{12} HSL (autoinduction), and *rhlR,* which increases the production of RhlR. RhlR can then activate the second AHL pathway Rhl. The expression of many virulence genes is controlled by these two QS systems in *P. aeruginosa*. Adapted from Nadal Jimenez *et al.*, 2012.

1.7.2 Quinolone Signalling in *P. aeruginosa*

The quinolone signalling system in *P. aeruginosa* is a third QS system that is governed by the transcriptional regulator, MvfR (Gallagher *et al*., 2002; Déziel *et al.,* 2004). It should be noted that this QS system is also hierarchically regulated by the Las system (Hentzer *et al.,* 2003; Schuster *et al.,* 2003). The signalling molecule 2-heptyl-3-hydroxy-4-quinolone (PQS) is synthesized in a multistep pathway, where anthranilate (produced by the *phn* biosynthesis genes *phnAB*) and a α-keto-fatty acid (produced by the *pqs* biosynthesis genes *pqsABCD*) are converted into 2-heptyl-4-quinolone (HHQ) (Bredenbruch *et al*., 2005; Farrow & Pesci, 2007). PqsH finally converts HHQ into PQS (Nadal Jimenez *et al.,* 2012). This PQS biosynthesis system works as a positive feedback loop: when PQS reaches a certain threshold in the extracellular media, it binds to MvfR, which activates expression of *pqsABCDE* and *phnAB* operons, increasing the production of PQS and the virulence factor pyocyanin (*phzA1*-*phzG1*) (Cao *et al*., 2001; Diggle *et al*., 2003; Déziel *et al*., 2005; Xiao *et al*., 2006).

1.7.3 The GAC System: A Two-Component Transduction system in *P. aeruginosa*

In addition to the two QS systems previously mentioned, *P. aeruginosa* also controls its lifestyle (free-living vs. biofilm) and the production of virulence factors using two-component signal transduction systems (Nadal Jimenez *et al.,* 2012). These systems act through phosphorylation cascades, which induce conformational changes in regulatory proteins that ultimately result in changes in gene expression profiles in *P. aeruginosa* (Heeb & Haas, 2001). *Pseudomonas aeruginosa*'s genome possesses over 60 two-component regulatory systems. One of the major two-component signalling module is the GAC system

(global activator of antibiotic and cyanide synthesis) (Nadal Jimenez *et al.,* 2012). The GAC two-component regulatory system is interesting due to its implications in the transition from acute to chronic infections. The GAC systems consists of a transmembrane sensor kinase, GacS, which when autophosphorylated, transfers a phosphate group to its regulator, GacA, which upregulates the expression of small regulatory RNAs, RsmZ and RsmY (Mulcahy *et al.*, 2008). The binding of RsmZ and RsmY to the small RNA-binding protein RsmA upregulates genes involved in biofilm formation and downregulates genes involved in acute virulence and motility (Mulcahy *et al.*, 2008).

Two other sensor kinases have been identified in this system as well, LadS (lost adherence sensory) and RetS (regulator of exopolysaccharide and type III secretion). LadS functions similarly to GacS, that is it positively controls the expression of the *pel* operon, which increase biofilm production, and represses genes involved in the T3SS (Ventre *et al.*, 2006). Whereas, RetS controls GacA in an opposite manner to the previously mentioned sensor kinases (GacS and LadS). RetS promotes acute infection (T3SS) and represses genes involved in biofilm formation (Ventre *et al.*, 2006).

1.8 Research Objectives

Currently, a major research gap in the field of bacterial plant interactions is the detailed understanding of the roles that phytohormones play in orchestrating a defence against microbial pathogens. As previously mentioned, it is well known that SA production is generally upregulated when plants are infected with biotrophs and hemibitrophs, whereas JA and ET production are thought to be upregulated during a necrotrophic infection (Jones & Dangl, 2006). However, the roles of other phytohormones (e.g. auxin, gibberellin and

peptide hormones) during a defence response are less well known. In regard to *P. aeruginosa*, its route of infection still remains elusive. Researchers have used a leaf infection model (Cheng *et al.*, 2015) to study *P. aeruginosa* pathogenesis in plants. However, since *P. aeruginosa* is found ubiquitously within the soil, it may be taken up by the roots and able to cause a systemic infection. It is also not known which secretion system *P. aeruginosa* primarily uses to infect host plants. Due to the number and diversity of effectors secreted by the T2SS, one would believe this is the main secretion apparatus; however, this has not been confirmed. We also largely do not understand specific plant defence responses towards *P. aeruginosa*.

The primary research object for this project was to develop a novel plant infection model using *P. aeruginosa* PA14. We chose to study *P. aeruginosa* PA14 as our pathogen of interest since our lab has extensive genetic resources (non-redundant transposon mutant library) readily available for this bacterium. We chose *Brassica napus* (canola) as our plant of interest for this infection model, because it is an agriculturally important crop in Canada. Using our novel infection model, we were able to measure plant health (e.g. tissue weight loss) and examine symptoms of disease (e.g. number of black spots on the plant leaves) during an infection. Secondly, we examined tissue specific global transcriptomic changes in canola over the course of infection. Finally, we investigated the profiles of the secondary metabolites exuded by canola roots during an infection and the role of ET production in our infection model.

CHAPTER 2. MATERIAL AND METHODS

2.1 Plant Growth

Brassica napus (canola) seeds were surface sterilized using a 20% solution of commercial bleach (LAVO; Cat. No. 44034) and two drops of Tween™ 20 (Fisher Bioreagents™; Cat. No. BP337500), then washed three times using sterile water and planted on MS (Murashige and Skoog basal medium with vitamins from Phytotechnology Laboratories supplemented with 0.5 g L^{-1} MES hydrate and 0.5% sucrose at pH 5.7) agar plates. After seven days, seedlings were transferred to 50 mL conical tubes (Falcon) containing 5 mL of MS liquid media. The tops of the conical tubes were sealed with Micropore tape and placed in a tube holder on a growth light stand (Hydrofarms) for three days at 22 °C, under 16 hours of daylight (750 lumens) before infection.

2.2 Bacterial Growth

P. aeruginosa PA14 is a primary clinical isolate from a burn patient and is highly virulent in a variety of host (plants, insect, mice) (Lee *et al.*, 2006; Liberati *et al.*, 2006). *P. aeruginosa* PA14 wild type (WT) and various mutants (Table 2.1) were grown overnight in LB media for 16 hours at 37 °C. Overnight cultures were spun down at 9,000 rpm for five minutes, washed twice with sterile 10 mM $MgSO_4$ and were resuspended in MS liquid media. Bacterial optical densities (OD) were adjusted to 0.1 for all infections.

Table 2.1: *Pseudomonas aeruginosa* PA14 mutants used in this study.

Bacterial Strain	Gene Description/ Function	Reference
P. aeruginosa PA14 *gacA*::tn	Global activator of gene expression (GAC)	Liberati *et al.*, 2006
P. aeruginosa PA14 Δ*lasI*	Acyl-homoserine lactone synthase (QS)	Haller *et al.*, 2018
P. aeruginosa PA14 Δ*lasR*	Transcriptional regulator (QS)	Haller *et al.*, 2018
P. aeruginosa PA14 Δ*rhlI*	Acyl-homoserine lactone synthase (QS)	Haller *et al.*, 2018
P. aeruginosa PA14 Δ*rhlR*	Transcriptional regulator (QS)	Haller *et al.*, 2018
P. aeruginosa PA14 Δ*mvfR*	Transcriptional regulator (QS)	Djonović *et al.*, 2013
P. aeruginosa PA14 Δ*lasR/rhlR*	Transcriptional regulators (QS)	Received from Dr. Frederick Ausubel
P. aeruginosa PA14 Δ*lasR/mvfR*	Transcriptional regulators (QS)	Same as above
P. aeruginosa PA14 Δ*rhlR/mvfR*	Transcriptional regulators (QS)	Same as above
P. aeruginosa PA14 Δ*lasR/rhlR/mvfR*	Transcriptional regulators (QS)	Same as above
P. aeruginosa PA14 *xcpR*::tn	Type II Secretion System ATPase (T2SS)	Liberati *et al.*, 2006
P. aeruginosa PA14 Δ*pscD*	Type III Secretion System basal body protein (T3SS)	Miyata *et al.*, 2003
P. aeruginosa PA14 ΔHSI-II	Type VI Secretion System (T6SS)	Lesic *et al.*, 2009
P. aeruginosa PA14 ΔHSI-III	Type VI Secretion System (T6SS)	Lesic *et al.*, 2009

2.3 Canola Infection: Testing the Infection Model

Ten-day-old canola seedlings had their roots damaged (poked with a pipette tip) or undamaged and half of the damaged and undamaged canola seedlings were infected by adding *P. aeruginosa* PA14 WT at a final OD of 0.1. The undamaged seedlings and the other half of the damaged seedlings were used as controls. All of the following measurements were done on days zero (two hours post infection), one, three and five post infections. The canola seedlings were photographed, and their tissues (root and shoot) were separated before being weighed. The numbers of black spots (cell death) on the leaves were counted as an indicator of disease severity. After the roots were weighed, the roots were put into separate 1.5 mL microcentrifuge tubes containing 200 µL of 10 mM $MgSO_4$. After the shoot was weighed, the leaves were cut-off and weighed independently. The leaves were then separated into 1.5 mL microcentrifuge tubes containing 200 µL of 10 mM $MgSO_4$. The tissues (roots and leaves) were then grinded using a tissue homogenizer, and then transferred into a 96 well plate. Once in the 96 well plate, the samples were serially diluted 10-fold from $10^0 – 10^{-7}$, and then plated on a rectangular LB + Rif15 (Rifampicin 15 µg/µL) plate. The plates were incubated at root temperature (22 °C) for two days. Colony forming units were then counted to look at bacterial colonization of the different plant tissues.

2.4 Canola Infection: Changing Infected MS Media Two Hours Post Infection

Ten-day-old canola seedlings had their roots damaged (poked with a pipette tip) and half of the damaged canola seedlings were infected by adding *P. aeruginosa* PA14 WT at a final OD of 0.1. The other half of the damaged seedlings were used as controls. Two

hours post infection, the MS media was changed to new MS media to ensure the bacteria that colonized the plants tissue were causing the infection, and that the infection was not due to free-living bacteria in the media. All of the plant health, symptoms of disease, and CFU measurements were repeated for this infection (outlined in section 2.3).

2.5 Canola Infection: Testing *P. aeruginosa* Virulence Factors

Ten-day-old canola seedlings had their roots damaged (poked with a pipette tip) and were infected with *P. aeruginosa* PA14 WT and various *P. aeruginosa* PA14 mutants (Table 2.1). Uninfected damaged seedlings were used as controls. All of the plant health, symptoms of disease, and CFU measurements were repeated for this infection (outlined in section 2.3).

2.6 Canola Infection: Transcriptomic Profile of Canola Seedlings infected with *P. aeruginosa*

Ten-day-old canola seedlings had their roots damaged (poked with a pipette tip) and were infected with *P. aeruginosa* PA14 WT. Uninfected damaged seedlings were used as controls. The canola seedlings had their root and shoot tissues separated before RNA extraction. RNA was extracted from the root and shoot of canola seedlings on days one, three and five.

2.6.1 Primer Specificity and Efficiency

Integrated DNA Technologies synthesized all primers used in this study. Primers were diluted to stock concentration of 100 μM, and then further diluted 10-fold to make 10

μM working stock primers. All primers were diluted using UltraPure™ water (Invitrogen™; Cat. No. 10977015). Primer specificity was assessed by running an endpoint polymerase chain reaction (PCR) on genomic DNA (gDNA) extracted from whole canola seedlings using a Wizard® Genomic DNA Purification Kit (Promega; Cat. No. A1120). Briefly, the PCR reaction contained 10 μL of GoTaq® Green Master Mix (Promega; Cat. No. M7122), 1 μL of forward and reverse primers, 4 μL of sterile water and 5 μL of gDNA template (450 ng/μL). PCR reactions were performed using T100™ Thermo Cycler (Bio-Rad; Cat. No. 1861096) with the following parameters: 95 °C for three minutes followed by 30 cycles of 95 °C for 15 seconds, 55 °C for 30 seconds and 72 °C for one minute, followed by a final extension of 72 °C for five minutes. PCR products were then run on 1.5 % agarose gel and imaged using a ChemiDoc MP (Bio-Rad; Cat. No. 12003154), only single producst were observed (Figure 2.1A).

Finally, the primers efficiency was then tested using canola gDNA (450 ng/μL) as a template, where it was serially diluted 10-fold from $10^0 - 10^{-4}$. Briefly, the qPCR reaction contained 10 μL of SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad; Cat. No. 1725275), 5 μL of forward and reverse primers (1.2 μM working stock) and 5 μL of cDNA template (1 μg/μL). PCR reactions were performed as follows: 95 °C for three minutes followed by 40 cycles of 95 °C for 10 seconds and 55 °C for 30 seconds. Primer efficiency was calculated using this formula:

$$\text{Amplification efficiency} = [10(-1/\text{m})] - 1 \qquad \text{(eq. 2.1)}$$

where m is the slope of the trend line (Figure 2.1B).

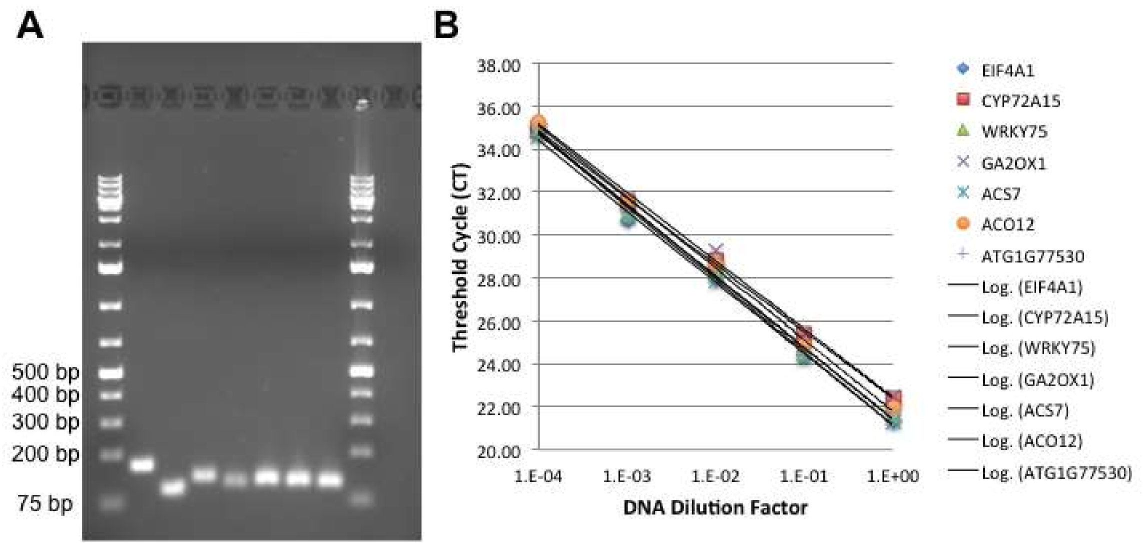

Figure 2.1: Testing the specificity and efficiency of primers used for RT-qPCR. A, Endpoint PCR products matching the correct size for each primer used for RT-qPCR. Lanes **1** and 9 contain the GeneRuler 1 kb Plus DNA ladder (Thermo Scientific™; Cat. No. SM1331), Lane 2 contains PCR product from *EIF4A1* primers, Lane 3 contains PCR product from *CYP72A15* primers, Lane 4 contains PCR product from *WRKY75* primers, Lane 5 contains PCR product from *GA2OX1* primers, Lane 6 contains PCR product from *ACS7* primers, Lane 7 contains PCR product from *ACO12* primers, Lane 8 contains PCR product from *AT1G77530* primers, and lane 11 is the negative control. **B**, Standard curve generated from qPCR to calculate primer efficiency.

2.6.2 RNA Isolation and RT-qPCR Analysis

Total plant RNA was isolated according to the manufacturers instructions using a RNeasy Plant Mini Kit (Qiagen; Cat. No. 74904). DNA was removed from all of the RNA samples using the DNA-free kit (Invitrogen™; Cat. No. AM1906), and reverse transcription reactions were performed using an iScript cDNA synthesis kit (Bio-Rad; Cat. No. 1708891). Complementary DNA (cDNA) concentrations were measured using a Nano-drop instrument (Thermo Scientific™; Cat. No. ND-ONEC-W). RT-qPCR reactions were performed using CFX96 real-time PCR machine (Bio-Rad; Cat. No. 1855201) using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad; Cat. No. 1725275). Briefly, the RT-qPCR reaction contained 10 μL of SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad; Cat. No. 1725275), 5 μL of forward and reverse primers (1.2 μM working stock) and 5 μL of cDNA template (1 μg/μL). PCR reactions were performed as follows: 95 °C for three minutes followed by 40 cycles of 95 °C for 10 seconds and 55 °C for 30 seconds. Fold change was calculated relative to plants treated with sterile water. Gene induction values represent the mean ± standard deviation, n = 3. Gene expression values were normalized to the eukaryotic translational initiation factor 4A1 (*EIF4A1*). Primers used in this study can be found in Table 2.2.

Table 2.2: *Brassica napus* primers used for RT-qPCR.

Name	Primer sequences (5' → 3')	Product size (bp)	Amplification Efficiency (%)*
***EIF4A1*-F**	CATGCTTGTGTTGGTGGAAC	160	97.15
***EIF4A1*-R**	CATCAGCTTCGTCAAGGACA		
***WRKY75*-F**	AGGTGCACACATGGAGGATG	101	93.27
***WRKY75*-R**	GGATGCGAGTGGACTCCTTC		
***CYP72A15*-F**	TGTGTCCTGGTGGGTATGGA	131	109.51
***CYP72A15*-R**	AGTTCCTCTTCAAGTCGCCG		
***ACS7*-F**	TGTCGAGTTTCACGCTTGTC	115	99.88
***ACS7*-R**	CGTCTCCTAAGCCTTTCACG		
***ACO12*-F**	CGACGAGACGAAAGAAGGAG	121	91.72
***ACO12*-R**	CGCTAGGTCTGGAACAGAGG		
***GA2OX1*-F**	CCAACTCGCAGGTTATCCGT	122	109.29
***GA2OX1*-R**	CTGGGAATGGAACCGAACCA		
***AT1G77530*-F**	AGCCCTTACCCACTTACCCT	116	99.54
***AT1G77530*-R**	AACCATGGGGAAAGCTACGG		

*Amplification efficiency = [10(-1/m)] - 1

2.6.3 RNA Sequencing

Total plant RNA (separated root and shoot tissue) was isolated according to the manufacturers instructions using a RNeasy Plant Mini Kit (Qiagen; Cat. No. 74904). DNA was removed from all of the RNA samples using the DNA-free kit (Invitrogen™; Cat. No. AM1906). Total RNA (1µg) was used for preparing the library with TrueSeq RNA sample Prep Kit v2 (Illumina®; Cat. No. RS-122-2001) according to manufacturer's guidelines with 24 different barcodes (duplicate biological replicates). The libraries were sequenced for 50 cycles on a HiSeq 2500 (Illumina) rapid mode using two lanes of a flow cell. The sequencing was performed at the MGH Next Generation Sequencing Core facility (Boston, USA). Fastq files, downloaded from the core facility, were used for data analysis. The quality of each sequencing library was assessed by examining fastq files with FASTQC.

2.6.4 RNA Sequencing Analysis

Raw single-end reads were mapped to the *Brassica napus* assembly (AST_PRJEB5043_v1) using bowtie2 (v2.24; Langmead and Salzberg, 2012) with default options. To circumvent the issue of reads mapping to duplicate genes on homologous chromosomes we quantified *B. napus* gene expression levels with mmquant (v1.2; Zytnicki, 2017), which collapses genes mapped by multiple reads into single categories. Gffread (v0.9.9; https://github.com/gpertea/gffread) was used to convert the annotation file format from GFF3 to GTF. Mmquant was used to run our custom scripts to parse the output described below in parallel with GNU parallel (version 20170722; Tange, 2011). Homologs of all *B. napus* genes in *Arabidopsis thaliana* by were identified running BLASTn (v2.2.31+; Altschul et al., 1990) on each *B. napus* gene against all *A. thaliana* genes. The

top matching *A. thaliana* genes were identified as homologs of *B. napus* genes if their E-values < 0.0001. Of the 101,040 genes in *B. napus*, 64,996 (64.33%) matched homologs in *A. thaliana*. The gene expression levels of these *A. thaliana* homologs were inferred by summing the number of reads mapped to *B. napus* genes with the same homolog. Next, DESeq2 (v1.16.1; Love et al., 2014) was run in an R environment (v3.4.3; R Core Team, 2017) to identify differentially expressed homologs between control and infected samples. Default options were used, and log-fold change shrinkage was performed with the "lfcShrink" function in R. DESeq2 was also ran to identify genes that are core markers of immune response by identifying differentially expressed homologs after controlling for day and tissue. Venn diagrams of the number of genes called as differentially expressed overlapping across days were generated with the VennDiagram (v1.6.17) R package. The $\log_2$-ratios of gene expression levels of all homologs between control and infected samples were read in Cluster3 (v3.0; de Hoon et al. 2004). Homologs with less than an absolute $\log_2$-ratio of 1 in at least 2 samples were filtered out, which resulted in 7,575/19,286 genes retained. Gene set enrichment analyses for biological processes were performed using AgriGO analysis tools (Zhou *et al.*, 2010). Finally, the homologs were clustered with the correlation (centered) similarity metric and the centroid linkage clustering method. All Python and R scripts run for the custom bioinformatics methods are available here: https://github.com/gavinmdouglas/canola_pseudomonas_RNAseq.

2.7 Metabolomics Profile of Infected Canola Seedlings

Ten-day-old canola seedlings had their roots damaged (poked with a pipette tip) and were infected with *P. aeruginosa* PA14 WT. Uninfected damaged seedlings were used

as controls. *P. aeruginosa* PA14 WT was also inoculated in MS media and also used as a control. MS media from infected and uninfected seedlings was collected on days one, three and five. The *P. aeruginosa* PA14 WT inoculated MS media was also collected on days one, three and five. All of the samples were spun down at 5,000 x g for ten minutes to remove bacteria from the samples (infected plants and bacterial control). The supernatant (MS media) was removed, and stored at -20°C.

2.7.1 Metabolite Elution

Bacterial and plant metabolites were eluted from the MS media using a Bond Elut C18 column (Agilent; Cat. No. 12102028). The columns were attached to vacElut Cartridge Manifold (Agilent; Cat. No. 5982-9117) in order to apply slight vacuum pressure (>10 psi) to speed up sample processing. Briefly, the columns were conditioned, equilibrated and washed using 100% methanol (Thermo Scientific™; Cat. No. A412-4), 2% formic acid (Sigma-Aldrich®; Cat. No. 106526) in methanol and filter sterilized water, respectively. The samples were then run through the columns and washed using filter sterilized water. The metabolites were eluted from the columns using 2% formic acid in methanol, and the samples were dried using a centrifugal evaporator (Genevac; Cat. No. EZ-2 Elite).

2.7.2 Metabolomic Profiling using Liquid Chromatography with Tandem Mass Spectrometry

The dried metabolomics samples were resolubilized in 100% methanol containing 1 µM of reserpine Sigma-Aldrich®; Cat. No. R0875) as an internal standard. The samples were analyzed using Exactive benchtop Orbitrap high-resolution mass spectrometer

(HRMS) (Thermo Scientific™; Cat. No. IQLAAEGAAPFALGMBDK) with separation carried out on Acquity HSS T3 column (Waters; Cat. No. 186003539) and eluted with acetonitrile and water gradient. Duplicated injections were used for each sample. HRMS data from both positive and negative modes were acquired.

2.7.3 Metabolomic Data Analysis

Liquid Chromatography (LC)-HRMS raw data file was converted into netCDF files using a built-in software module XCalibur, and then mzMine 2 (Pluskal *et al.*, 2010) was used for preprocessing, including mass detection, chromatogram building, deisotoping, and joint alignment. The list of buckets defined by a retention time (Rt) and mass to charge ratio (m/z) was compared between sample datasets in order to highlight key metabolites with higher level of differences. Tentative identification of these metabolites was done based on HRMS data and literature search.

2.8 Canola Infection: Chemically Inhibiting Ethylene production using 2-aminoethoxyvinyl glycine

Ten-day-old canola seedlings had their roots damaged (poked with a pipette tip) and had their media MS media changed, half received new sterile MS media, and the other half received fresh MS media supplement with 0.05 mM of 2-aminoethoxyvinyl glycine (AVG) (Cayman Chemical; Cat. No. 15546) a chemical inhibitor of ethylene production, for the duration of the infection, and the other half were used as no AVG controls. Then, half of the seedlings were infected with *P. aeruginosa* PA14 WT. All of the plant health,

symptoms of disease, and CFU measurements were repeated for this infection (outlined in section 2.3).

2.9 Canola Infection: Inhibiting Ethylene Production in Seedlings using Transgenic Canola expressing 1-aminocyclopropane-1-carboxylate deaminase

Ten-day-old canola seedlings [*B. napus* cv. Westar or transgenic *B. napus* cv. Westar ($ACCD^+$)] had their roots damaged (poked with a pipette tip). The transgenic *B. napus* cv. Westar ($ACCD^+$) was established by cloning the ACCD (*acdS*) gene from *Pseudomonas spp.* UW4 in plasmid pKYLX71.1 under the transcriptional control of the *rolD* promoter from *Agrobacterium rhizogenes* (Sergeeva, Shah & Glick, 2006). The plasmid was then mobilized into *Agrobacterium tumefacians* C58 using triparental mating using plasmid pRK2013 (Sergeeva, Shah & Glick, 2006). Transformed *A. tumefacians* C58 was then used to transform *B. napus* cv. Westar cotyledons, and transformants were selected using kanamycin plates (Sergeeva, Shah & Glick, 2006). Further, half of the seedlings were infected with *P. aeruginosa* PA14 WT. All of the plant health, symptoms of disease, and CFU measurements were repeated for this infection (outlined in section 2.3).

2.10 Statistical Analysis

All statistical analysis reported in Chapter 3 (Results) were conducted using GraphPad Prism 6.0 software using the recommended parameters. Multiple grouped sample means were analyzed using a two-way analysis of variance (ANOVA) with a post-hoc (Sidak's multiple comparisons test) test to determine differences between samples.

Whereas, independent sample means were analyzed using an ANOVA with a post-hoc (Tukey's multiple comparisons test) test to determine differences between samples. Significance was measured at $p < 0.05$, and significant differences were reported as different letters.

CHAPTER 3. RESULTS

3.1 Wild Type *P. aeruginosa* PA14 Reduces Canola's health and Promotes Symptoms of Disease.

It has been previously shown that *P. aeruginosa* PA14 infection can cause symptoms of disease in *Arabidopsis thaliana* (Plotnikova, Rahme & Ausubel, 2000; He *et al.*, 2004; Cheng *et al.,* 2015); however, little work has been done to show its pathogenicity in agriculturally important crops such as canola. Traditionally, *P. aeruginosa* PA14 culture has been infiltrated into the plant leaves in order to study its pathogenicity, since this was the infection model developed for the well-described plant pathogen *P. syringae*. However, since *P. aeruginosa* is found ubiquitously within the environment, we believe that *P. aeruginosa* can enter the seedlings via damages on the surface of the plant's roots. Therefore, in order to test our hypothesis, we had to develop a novel infection model for *P. aeruginosa* PA14 in canola seedlings.

As described in the material and methods section above, plant roots were artificially damaged using a pipette tip prior to each infection. Importantly, we did not see a significant difference between the undamaged-control seedlings and our damaged-control seedlings on any parameters (tissue weight, leaf spotting, and CFU) measured during the infection (Figure 3.1 A, B; Figure 3.2 A, B; Figure 3.3). However, large differences were observed when comparing the damaged-control vs. damaged-infected seedlings with *P. aeruginosa* PA14. There was no significant difference in root weight observed between damaged-control and damaged-infected seedlings on day zero (two-hours post infection) (Figure 3.1 A). However, on days one, three and five there were decreases in root weight between damaged-control and damaged-infected of 41%, 66% and 82%, respectively (Figure 3.1

A). There was no significant difference in root weight observed between undamaged-control and undamaged-infected seedlings on days zero (two-hours post infection) and one (Figure 3.1 A). However, on days three and five there were decreases of in root weight between undamaged-control and undamaged-infected of 63% and 67%, respectively (Figure 3.1 A). Interestingly, there was no significant differences observed in root weight between undamaged-infected and damaged-infected seedlings on any days measured.

Similarly, there was no significant difference in shoot weight observed between damaged-control and damaged-infected seedlings or undamaged-control and undamaged-infected on days zero (two-hours post infection) and one (Figure 3.1 B). However, on days three and five there were decreases in shoot weight between damaged-control and damaged-infected of 46% and 56%, respectively (Figure 3.1 B). On days three and five there were decreases of in shoot weight between undamaged-control and undamaged-infected of 29% and 42%, respectively (Figure 3.1 B). Interestingly, on days three and five there were significant decreases in shoot weight between undamaged-infected and damaged-infected of 21% and 22%, respectively (Figure 3.1 B).

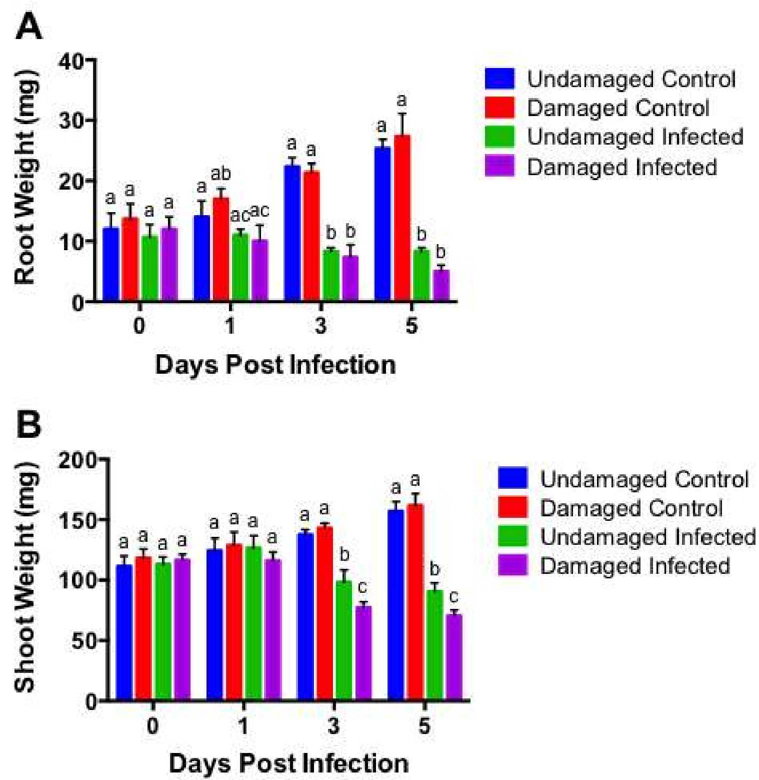

Figure 3.1: Canola tissue weight recorded during a five-day infection with *P. aeruginosa* PA14. A, Canola root weight measured on days zero (two-hours post infection), one, three and five. **B,** Canola shoot weight measured on days zero (two-hours post infection), one, three and five. Error bars represent standard deviation of three independent biological replicates. Statistical significance was measured at the $p \leq 0.05$ probability level; the same letters indicate non-statistically significant differences between groups, whereas different letters indicate statistically significant difference between groups.

Pseudomonas aeruginosa PA14 root CFU measured from damaged-infected was significantly higher compared to undamaged-infected ranging from 0.7-fold to 1.8-fold throughout the five-day infection (Figure 3.2 A). There was no significant difference in *P. aeruginosa* PA14 leaf CFU measured from damaged-infected and undamaged-infected on days zero (two-hours post infection) and five (Figure 3.2 B). However, *P. aeruginosa* PA14 leaf CFU measured from damaged-infected was significantly higher compared to undamaged-infected by 180% and 166%, on days one and three, respectively (Figure 3.2 B).

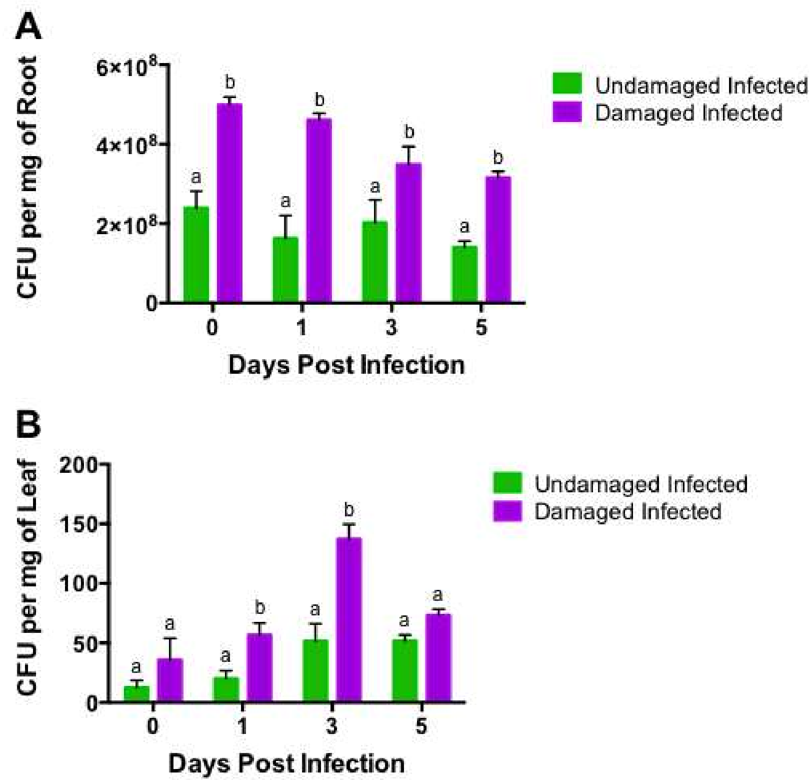

Figure 3.2: *Pseudomonas aeruginosa* PA14 colony forming units (CFU) measured on canola roots and within canola leaves. **A,** *Pseudomonas aeruginosa* PA14 root colonization measured on days zero (two-hours post infection), one, three and five. **B,** *Pseudomonas aeruginosa* PA14 leaf colonization measured on days zero (two-hours post infection), one, three and five. Error bars represent standard deviation of three independent biological replicates. Statistical significance was measured at the $p \leq 0.05$ probability level; the same letters indicate non-statistically significant differences between groups, whereas different letters indicate statistically significant difference between groups.

Plants undergo localized cell death in order to limit the spread of infection to other areas within the leaf tissue. The number of black spots on plant leaves is indicative of cell death within this region, which could be a sign of bacterial infection. The number of spots on the leaves of canola seedlings infected with *P. aeruginosa* PA14 increased throughout the infection (Figure 3.3). There were no black spots observed on canola seedling leaves on day zero (two-hours post infection) and one of the infection (Figure 3.3). There were significantly more black spots on damaged-infected compared to undamaged-infected canola seedlings by 3.6-fold and 2.8-fold, on days three and five, respectively (Figure 3.3).

Based on all parameters measured there was no significant differences caused by manually damaging plant roots. However, it was observed that damaging canola seedling roots speeds up the course of infection. Therefore, herein we chose to only use the damaged plants for infection. Next, we wanted to test whether canola's health and symptoms of disease were most affected by *P. aeruginosa* PA14 cells that had colonized the root tissue vs. free-living bacterial cells. Therefore, the MS media was removed two hours post infection and replaced with new sterile MS media. Importantly, we did not notice a difference in progression of the infection when the MS media was changed two hours post infection (Appendix A). Therefore, all of the following infections were done without changing the MS media.

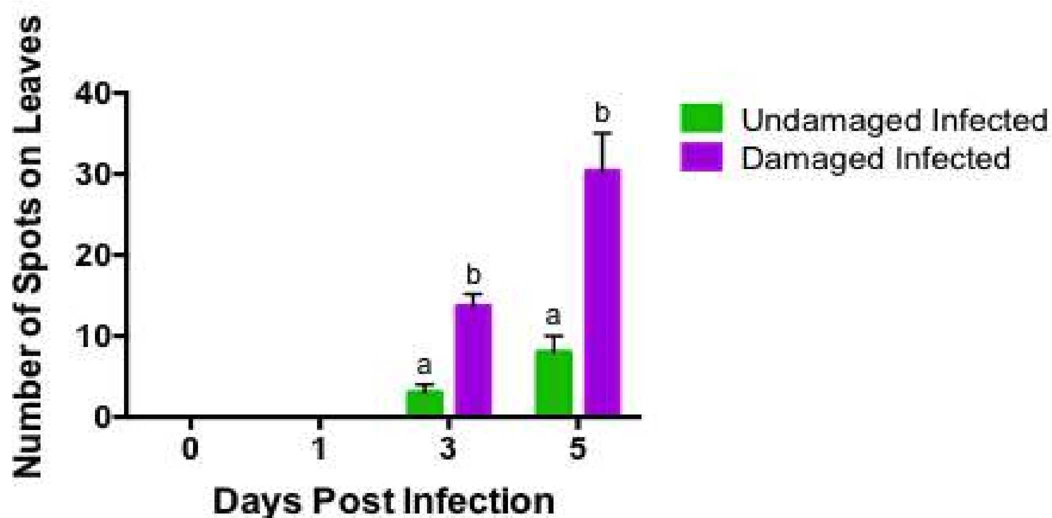

Figure 3.3: Number of black spots on canola seedling's leaves during a five-day infection with *P. aeruginosa* PA14. The number of black spots on the leaves was recorded on days zero (two-hours post infection), one, three and five. Error bars represent standard deviation of three independent biological replicates. Statistical significance was measured at the $p \leq 0.05$ probability level; the same letters indicate non-statistically significant differences between groups, whereas different letters indicate statistically significant difference between groups.

3.2 Canola Seedlings Infected with QS Mutants of *P. aeruginosa* PA14 are Healthier and Show Fewer Symptoms of Disease Compared to Wild Type.

Pseudomonas aeruginosa PA14 has many virulence weapons encoded by its large genome. We sought to test the virulence of various PA14 mutants in canola seedlings with specific virulence factors knocked out. *Pseudomonas aeruginosa* has three QS system: Las, Rhl and PQS (Table 2.1). It is well known that the QS systems in PA14 control the expression of its virulence factors (Nadal Jimenez *et al.,* 2012), therefore we tested single, double and triple mutants of genes involved in QS. We also tested a mutant (*gacA*::Tn) of the GAC two component regulatory system. Finally, we tested mutants for T2SS, T3SS, as well as the T6SS. There was no significant difference in tissue weight or colonization between WT PA14 and *gacA*::Tn or any of the secretion system (*xcpR*::tn, Δ*pscD*, ΔHSI-II and ΔHSI-III) mutants (Appendix B). There was a significant difference in the number of spots on the leaves of canola seedlings between *P. aeruginosa* PA14 WT and all of the secretion system mutants (*xcpR*::tn, Δ*pscD*, ΔHSI-II and ΔHSI-III) five days post infection (Appendix B). There were significant differences between WT PA14 and the double and triple QS mutants (Figure 3.4 C, D; 3.5 C, D; Figure 3.6 B; Figure 3.7 B, C; Figure 3.8 A, B).

There was no significant difference observed between control, WT or mutant infected seedlings on days zero (two-hours post infection) and one for root weight loss (Figure 3.4 A, B). However, on day three there were significant decreases in root weight between control, and WT, Δ*lasI,* Δ*lasR,* Δ*rhlI,* Δ*rhlR,* Δ*mvfR*, Δ*lasR/rhlR*, Δ*lasR/mvfR*, Δ*rhlR/mvfR* and Δ*lasR/rhlR/mvfR* ranging from 45% to 63% (Figure 3.4 C). On day five there were significant decreases in root weight between control, and WT, Δ*lasI,* Δ*lasR,*

Δ*rhlI,* Δ*rhlR,* Δ*mvfR*, Δ*lasR/rhlR*, Δ*lasR/mvfR*, Δ*rhlR/mvfR* and Δ*lasR/rhlR/mvfR* ranging from 43% to 70% (Figure 3.4 D).

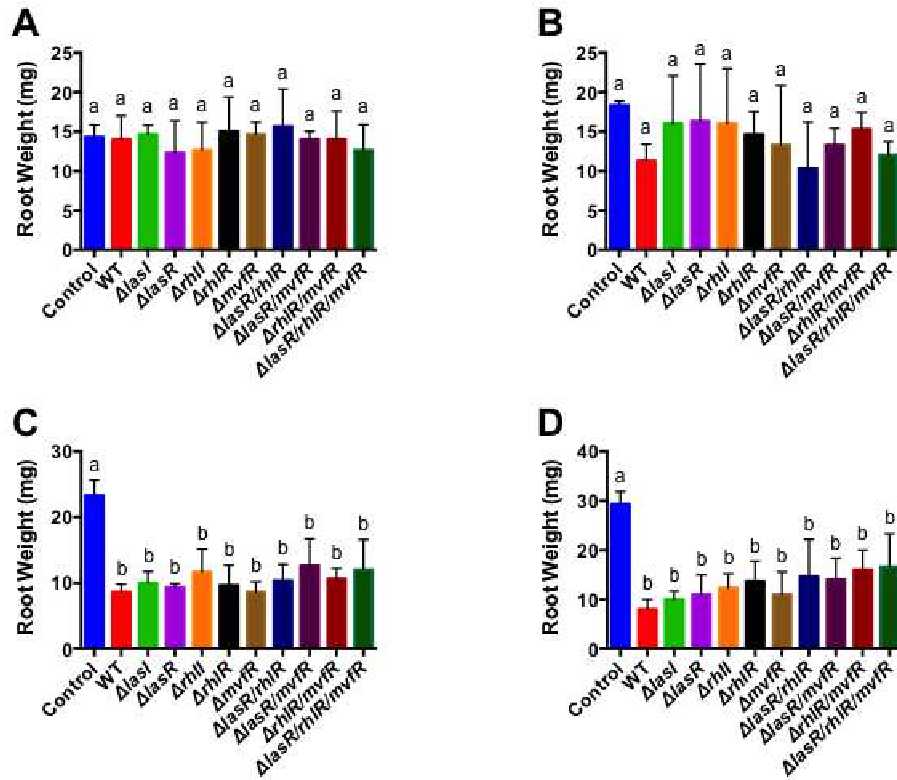

Figure 3.4: Canola root weight recorded during a five-day infection with *P. aeruginosa* PA14 wild type or QS mutants. **A,** Canola root weight measured on day zero (two-hours post infection). **B,** Canola root weight measured on day one. **C,** Canola root weight measured on day three. **D,** Canola root weight measured on day five. Error bars represent standard deviation of three independent biological replicates. Statistical significance was measured at the $p \leq 0.05$ probability level; the same letters indicate non-statistically significant differences between groups, whereas different letters indicate statistically significant difference between groups.

Similarly, there was no significant difference observed between control, WT or QS mutant infected seedlings on day zero (two-hours post infection) for shoot weight loss (Figure 3.5 A). However, on day one there were significant decreases in shoot weight between control, Δ*lasR* and Δ*mvfR* of 21% and 24%, respectively (Figure 3.5 B). On day three there were significant decreases of in root weight between control, and WT, Δ*lasI,* Δ*lasR,* Δ*rhlI,* Δ*rhlR,* Δ*mvfR*, Δ*lasR/rhlR*, Δ*lasR/mvfR*, Δ*rhlR/mvfR* and Δ*lasR/rhlR/mvfR* ranging from 21% to 45% (Figure 3.5 C). Interestingly, canola seedlings infected with Δ*lasR/rhlR*, Δ*lasR/mvfR* and Δ*lasR/rhlR/mvfR* weighed more compared to WT by 35%, 39% and 44%, respectively (Figure 3.4 C). On day five there were significant decreases in root weight between control, WT, Δ*lasI,* Δ*lasR,* Δ*rhlI,* Δ*rhlR,* Δ*mvfR*, Δ*lasR/rhlR*, Δ*lasR/mvfR*, Δ*rhlR/mvfR* and Δ*lasR/rhlR/mvfR* ranging from 22% to 63% (Figure 3.5 D). Again, canola seedlings infected with Δ*lasR/rhlR*, Δ*lasR/mvfR* and Δ*lasR/rhlR/mvfR* weighed more compared to WT by 87%, 70% and 113%, respectively (Figure 3.5 D).

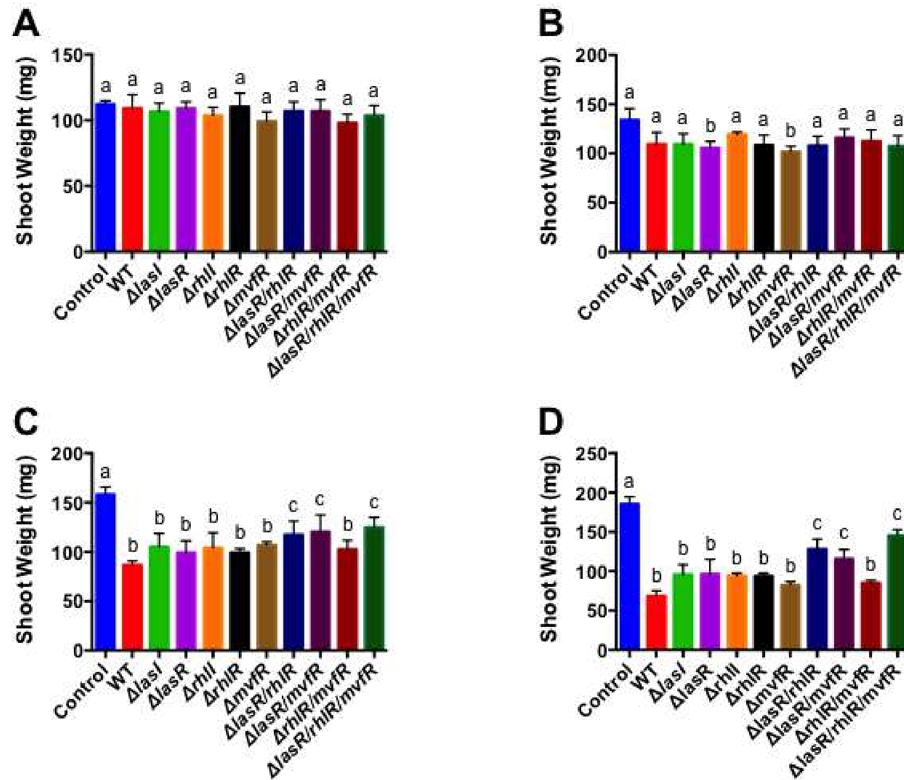

Figure 3.5: Canola shoot weight recorded during a five-day infection with *P. aeruginosa* PA14 wild type or QS mutants. **A,** Canola shoot weight measured on day zero (two-hours post infection). **B,** Canola shoot weight measured on day one. **C,** Canola shoot weight measured on day three. **D,** Canola shoot weight measured on day five. Error bars represent standard deviation of three independent biological replicates. Statistical significance was measured at the $p \leq 0.05$ probability level; the same letters indicate non-statistically significant differences between groups, whereas different letters indicate statistically significant difference between groups.

Next, we compared colonization of plant tissues by both *P. aeruginosa* PA14 WT and QS mutants. On days zero (two-hours post infection), three and five there was no significant difference in WT's ability to colonize canola roots compared to any of the QS mutants (Figure 3.6 A, C, D). However, on day one there were more WT bacteria colonizing the roots compared to Δ*lasI*, Δ*lasR,* Δ*lasR/mvfR*, Δ*rhlR/mvfR* and Δ*lasR/rhlR/mvfR* ranging from 1.2-fold to 1.4-fold (Figure 3.6 B).

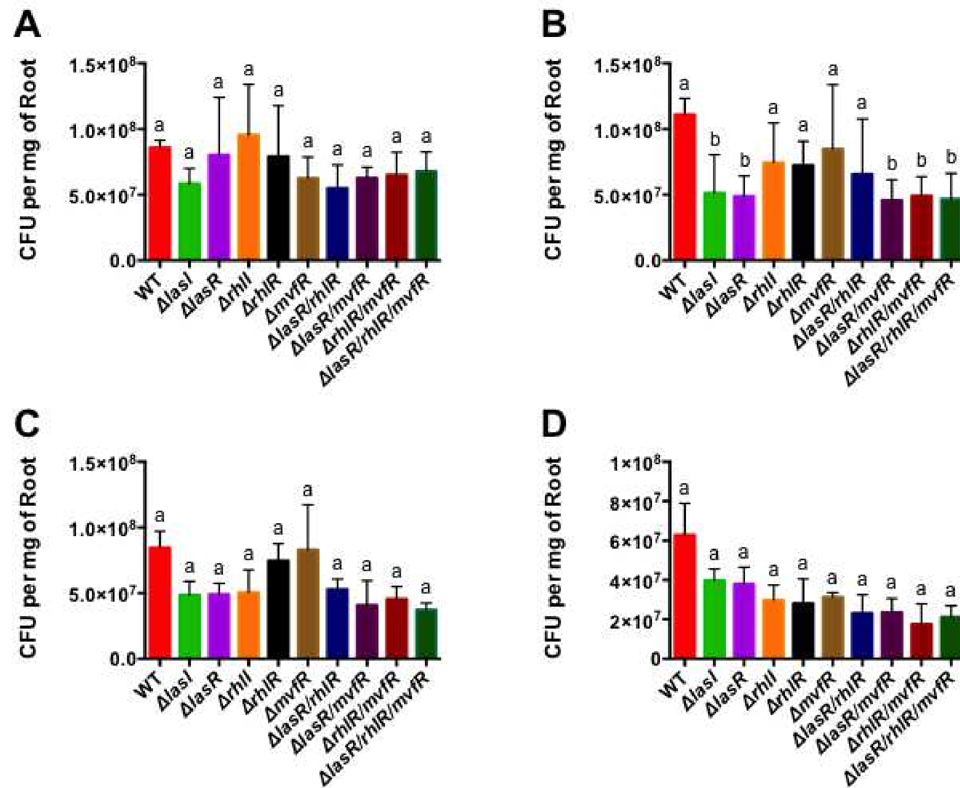

Figure 3.6: *Pseudomonas aeruginosa* PA14 wild type or QS mutants colony forming units (CFU) measured on canola roots during a five-day infection. **A,** *Pseudomonas aeruginosa* PA14 root colonization measured on day zero (two-hours post infection). **B,** *Pseudomonas aeruginosa* PA14 root colonization measured on day one. **C,** *Pseudomonas aeruginosa* PA14 root colonization measured on day three. **D,** *Pseudomonas aeruginosa* PA14 root colonization measured on day five. Error bars represent standard deviation of three independent biological replicates. Statistical significance was measured at the $p \leq 0.05$ probability level; the same letters indicate non-statistically significant differences between groups, whereas different letters indicate statistically significant difference between groups.

Similarly, on days zero (two-hours post infection) and five, there was no significant difference in WT's ability to colonize canola leaves compared to any of the QS mutants (Figure 3.7 A, D). However, on day one there were more WT bacteria colonizing the leaves compared to Δ*lasI,* Δ*rhlI*, Δ*rhlR*, Δ*lasR/rhlR*, Δ*lasR/mvfR*, Δ*rhlR/mvfR* and Δ*lasR/rhlR/mvfR* ranging from 1.9-fold to 5.2-fold (Figure 3.7 B). On day three there were more WT bacteria colonizing the leaves compared to Δ*lasI,* Δ*rhlI*, Δ*rhlR*, Δ*lasR/rhlR*, Δ*lasR/mvfR*, Δ*rhlR/mvfR* and Δ*lasR/rhlR/mvfR* ranging 1.2-fold to 3.8-fold (Figure 3.7 C).

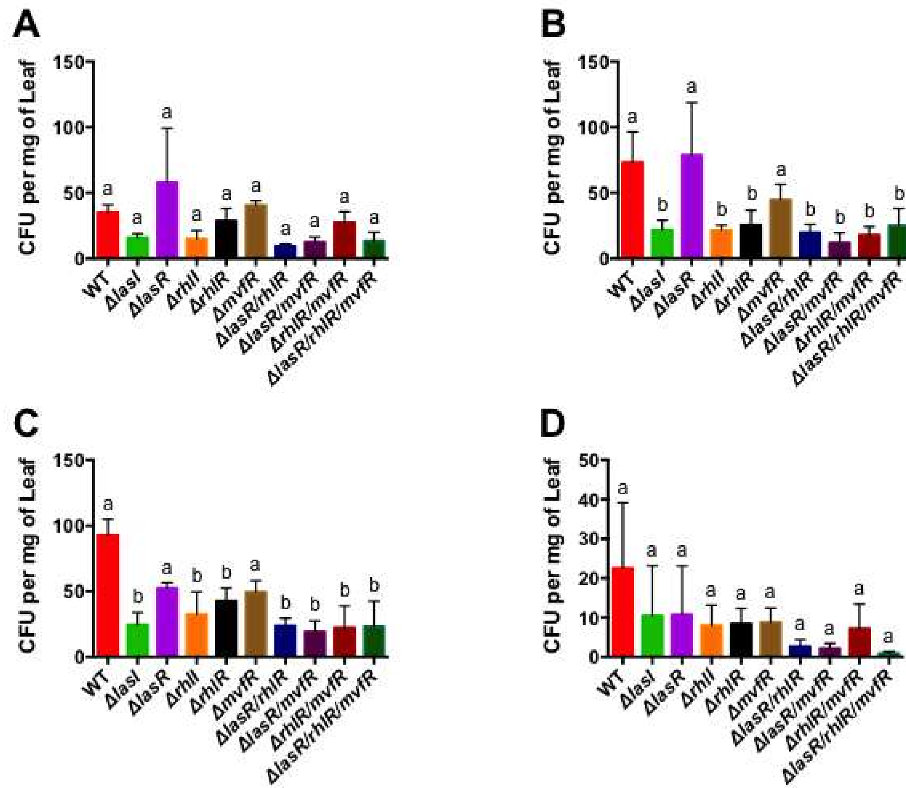

Figure 3.7: *Pseudomonas aeruginosa* PA14 wild type or QS mutants colony forming units (CFU) measured in canola leaves during a five-day infection. **A,** *Pseudomonas aeruginosa* PA14 leaf colonization measured on day zero (two-hours post infection). **B,** *Pseudomonas aeruginosa* PA14 leaf colonization measured on day one. **C,** *Pseudomonas aeruginosa* PA14 leaf colonization measured on day three. **D,** *Pseudomonas aeruginosa* PA14 leaf colonization measured on day five. Error bars represent standard deviation of three independent biological replicates. Statistical significance was measured at the $p \leq 0.05$ probability level; the same letters indicate non-statistically significant differences between groups, whereas different letters indicate statistically significant difference between groups.

The number of spots on the leaves of canola seedlings infected with *P. aeruginosa* PA14 WT or the QS mutants increased throughout the infection (Figure 3.8). There were no black spots observed on canola seedling leaves on days zero (two-hours post infection) and one of the infection (Figure 3.8). On day three there were significantly more black spots on canola seedlings infected with WT compared to Δ*lasI*, Δ*lasR*, Δ*rhlI*, Δ*rhlR*, Δ*mvfR*, Δ*lasR/rhlR*, Δ*lasR/mvfR*, Δ*rhlR/mvfR* and Δ*lasR/rhlR/mvfR* ranging from 1.7-fold to 12.7-fold (Figure 3.8 A). On day five there were significant more black spots on canola seedlings infected with WT compared to Δ*lasI*, Δ*lasR*, Δ*rhlI*, Δ*rhlR*, Δ*mvfR*, Δ*lasR/rhlR*, Δ*lasR/mvfR*, Δ*rhlR/mvfR* and Δ*lasR/rhlR/mvfR* ranging from 2.8-fold to 26.8-fold (Figure 3.8 B).

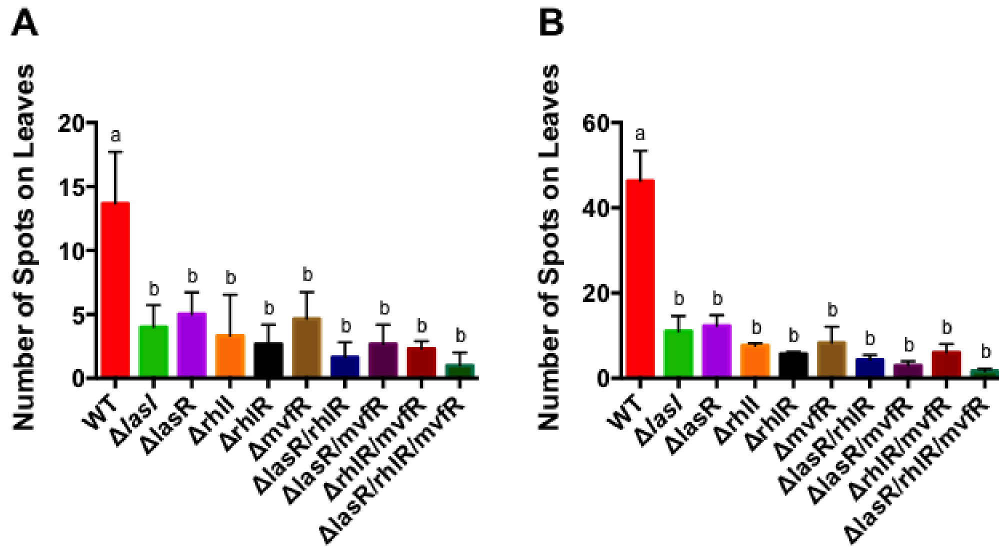

Figure 3.8: Number of black spots on canola seedling's leaves during a five-day infection with *P. aeruginosa* PA14 wild type or double and triple QS mutants. A, The number of black spots on the leaves was recorded on day three. **B,** The number of black spots on the leaves was recorded on day five. Error bars represent standard deviation of three independent biological replicates. Statistical significance was measured at the $p \leq 0.05$ probability level; the same letters indicate non-statistically significant differences between groups, whereas different letters indicate statistically significant difference between groups.

3.3 *Pseudomonas aeruginosa* PA14 infection of canola up-regulates genes involved in plant innate immunity and cell death, and down-regulates genes involved in plant growth and development.

Canola seedlings (*Brassica* napus) were either uninfected as the control or infected with *P. aeruginosa* PA14 WT, to determine plant transcriptomic changes in response to *P. aeruginosa* infection. RNA was extracted on days one, three and five, and the extracted RNA was then DNase treated and an RNA sequencing library was created. The number of reads, and percentage of mapped reads can be found in Table 3.1. Since *B*. napus is not a model organism, and therefore lacks gene annotation information, we mapped all of our RNA sequencing reads back to *Arabidopsis thaliana*, a closely related model organism. We successfully mapped 19,210 genes from *B. napus* with significant hits ($p \leq 0.05$) to *A. thaliana*'s genome. From these 19,210 genes, there were 4,275 genes with a $\log_2$fold change greater than 2, which is indicative that these genes are either being highly up regulated or strongly down regulated (Figure 3.9).

Table 3.1: RNA sequencing number of reads, percentage of reads mapped to *B. napus'* genome and percentage of reads that mapped to homologous genes in *A. thaliana*.

Day	Sample	Number of mapped reads	Percentage of reads mapped to *Brassica napus* genes	Percentage of reads mapped to *Arabidopsis thaliana* homologues
1	Shoot Control 1	7,868,533	90%	94%
	Shoot Control 2	9,554,932	92%	95%
	Root Control 1	7,693,745	89%	93%
	Root Control 2	9,343,498	88%	93%
	Shoot Infected 1	10,705,948	90%	93%
	Shoot Infected 2	10,556,085	90%	93%
	Root Infected 1	13,087,136	88%	92%
	Root Infected 2	6,304,352	88%	92%
3	Shoot Control 1	45,159,500	91%	94%
	Shoot Control 2	32,664,511	91%	94%
	Root Control 1	9,325,470	80%	91%
	Root Control 2	9,964,265	85%	91%
	Shoot Infected 1	6,290,422	89%	93%
	Shoot Infected 2	7,206,482	88%	93%
	Root Infected 1	5,504,582	83%	90%
	Root Infected 2	7,445,814	81%	90%
5	Shoot Control 1	9,446,304	91%	94%
	Shoot Control 2	9,817,663	91%	94%
	Root Control 1	8,454,463	87%	92%
	Root Control 2	7,082,782	85%	91%
	Shoot Infected 1	11,025,423	90%	91%
	Shoot Infected 2	8,366,129	90%	91%
	Root Infected 1	7,013,881	88%	91%
	Root Infected 2	4,300,104	85%	92%

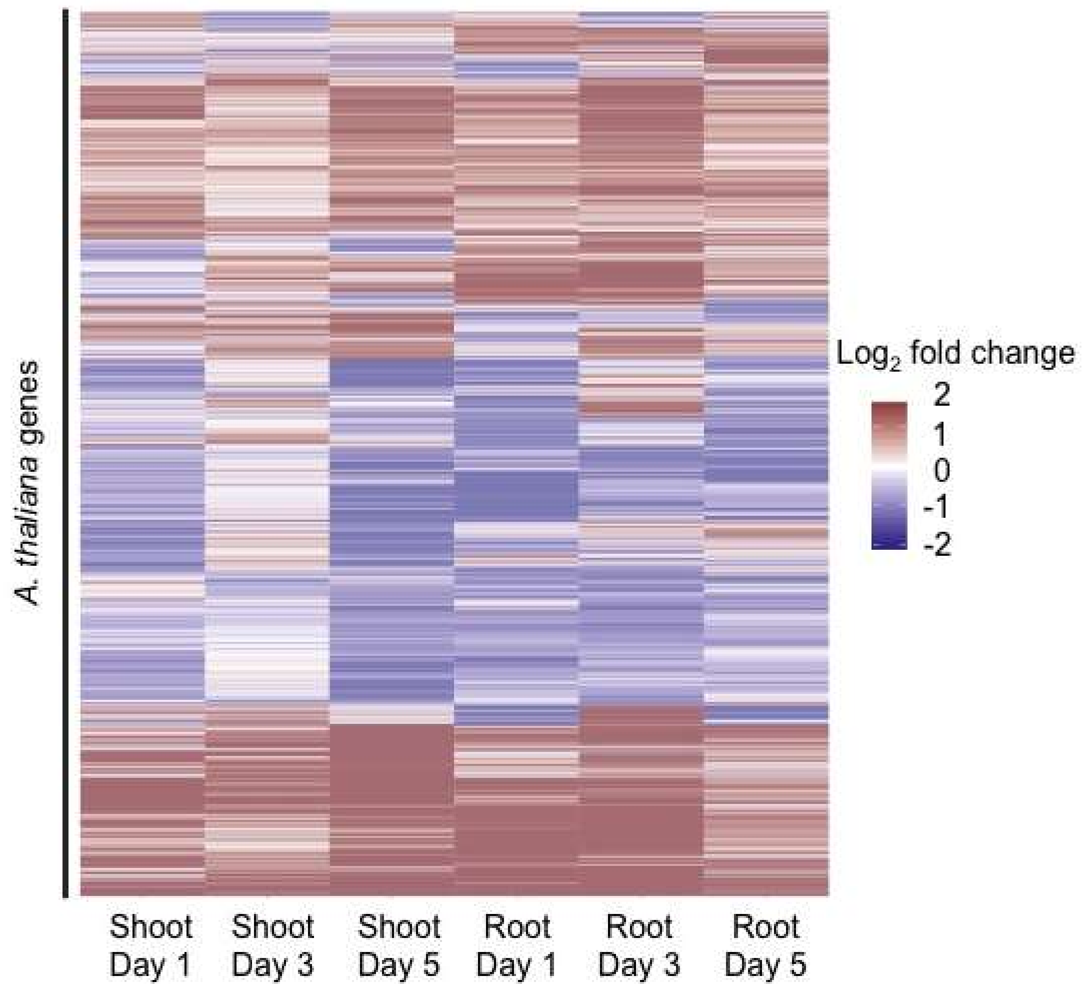

Figure 3.9: Heat map of differentially expressed genes in *B. napus* during a five-day infection with *P. aeruginosa* PA14. *B. napus* genes were BLASTed against *Arabidopsis thaliana* genes to obtain functional gene annotations. Gene expression of infected canola seedlings was calculated relative to uninfected controls, with two independent biological replicates for each condition.

The number of differentially expressed genes was greater in the root tissue (2,705) compared to shoot tissue (998) (Figure 3.10). This is not surprising since the roots are the first site of infection. The greatest number of differentially expressed genes occurs in the roots at day three of the infection, whereas the highest number of differentially expressed genes in the shoot occurs on day five of the infection (Figure 3.10).

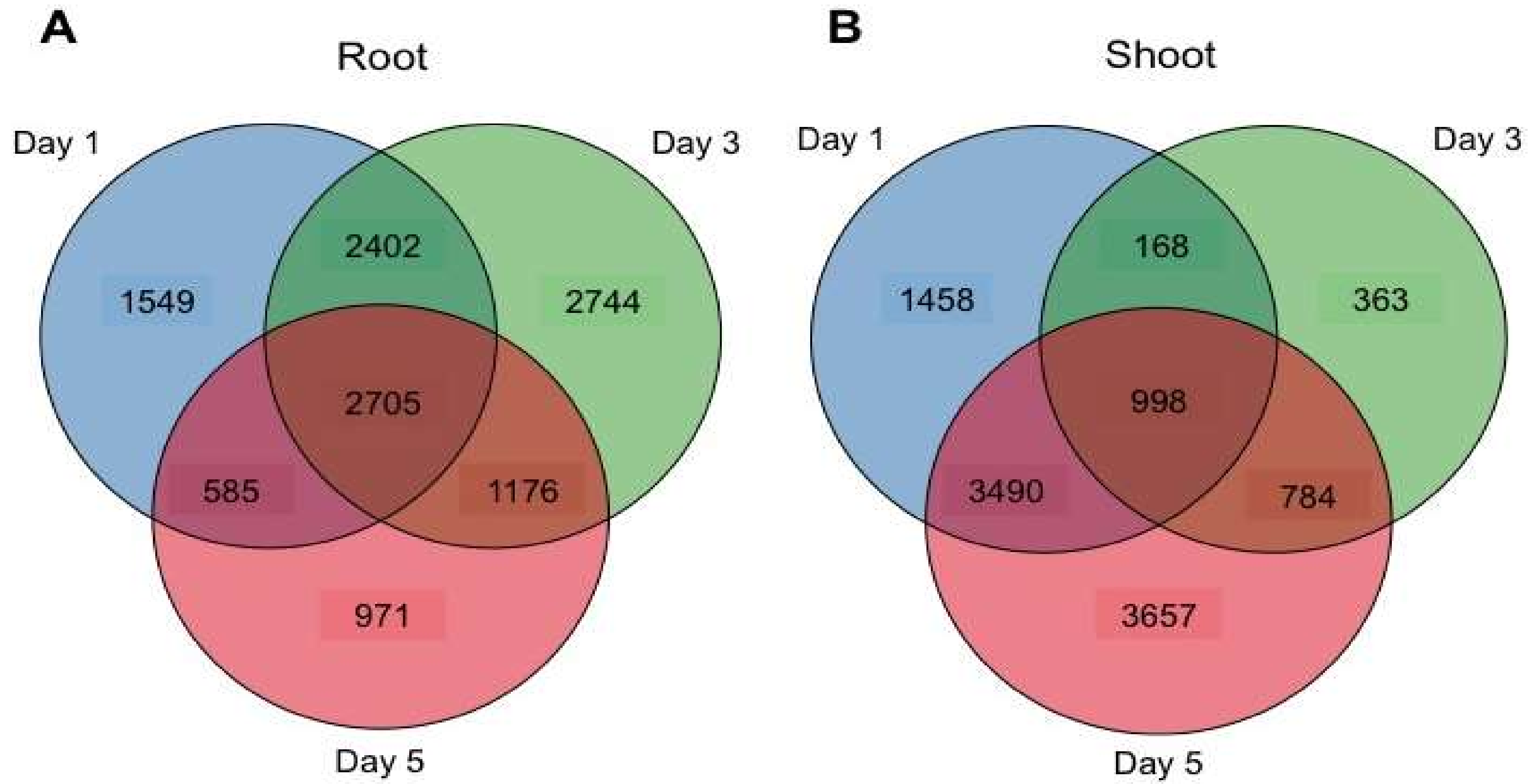

Figure 3.10: Number of differentially expressed genes in *Brassica napus* tissues (roots and shoot) during a five-day infection with *P. aeruginosa* PA14. A, Differentially expressed genes in the roots of canola seedlings. **B,** Differentially expressed genes in the shoot of canola seedlings.

The genes that were differentially expressed in canola seedlings throughout the infection (days one-five) were analysed using AGRIgo (Du *et al.*, 2010), a gene ontology (GO) analysis for agricultural crops. These analyses allowed us to identify biological responses that were either up or down regulated in canola seedlings throughout our infection with *P. aeruginosa* PA14. In the roots, genes involved in plant cell death ($p = 0.00017$), response to stimulus ($p = 1.6e^{-10}$), and immune system processes ($p = 2e^{-6}$) were significantly up regulated during the infection (Figure 3.11). Whereas, genes involved in cellular processes ($p = 9.8e^{-9}$), and metabolic processes ($p=1.9e^{-8}$) were significantly down regulated during the infection (Figure 3.11). Similarly, in the shoot, genes involved in response to stimulus ($p = 1.6e^{-13}$), and immune system processes ($p = 0.00026$) were significantly up regulated during the infection (Figure 3.12). There were no biological processes that were significantly down regulated in the shoots of infected plants (Figure 3.12).

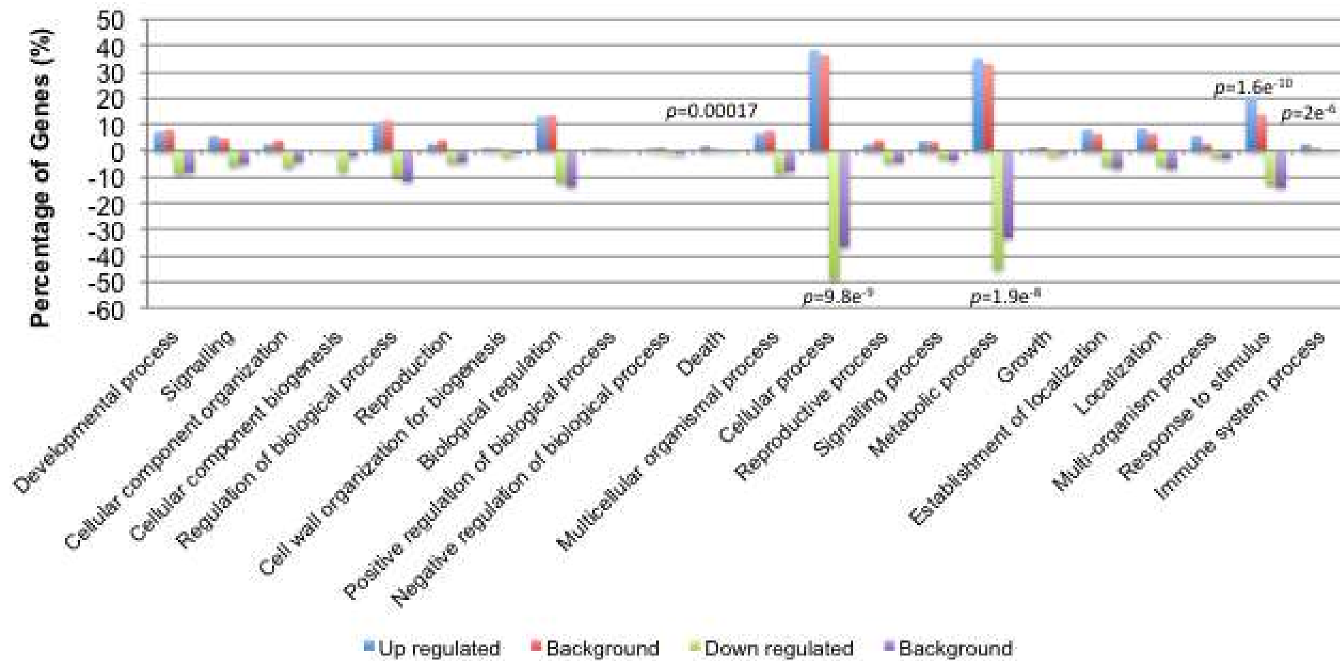

Figure 3.11: GO analysis of biological functions occurring in the roots of *B. napus* during a five-day infection with *P. aeruginosa* PA14. Bars represent the percentage of genes involved in GO biological processes that were up or down regulated throughout the infection compared to background gene expression for that GO category.

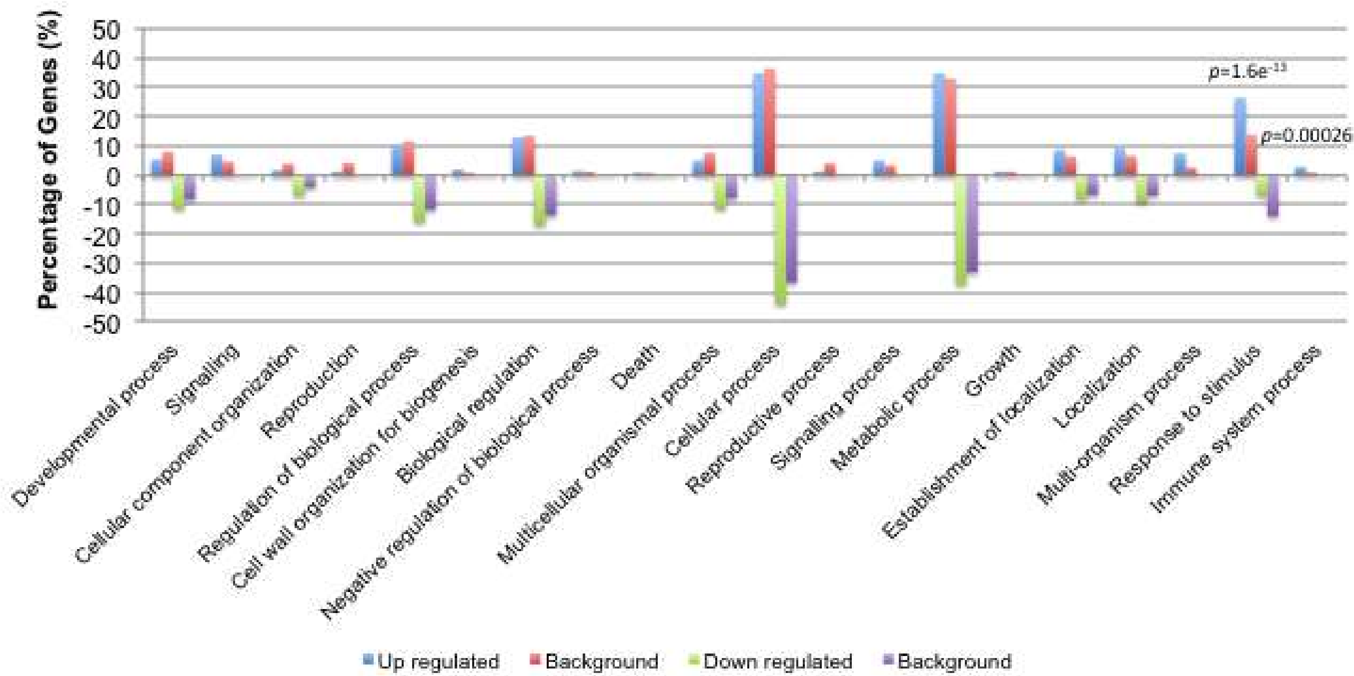

Figure 3.12: GO analysis of biological functions occurring in the shoot of *B. napus* during a five-day infection with *P. aeruginosa* PA14. Bars represent the percentage of genes involved in GO biological processes that were up or down regulated throughout the infection compared to background gene expression for that GO category.

Using the GO analysis data, we were able to create various categories of smaller groups of heat maps based on specific biological responses. Genes involved in the innate immune response in canola seedlings were highly up regulated during the five-day infection with *P. aeruginosa* PA14 (Figure 3.13). Similarly, genes involved in secondary metabolism in canola seedlings were highly up regulated during the five-day infection with *P. aeruginosa* PA14 (Figure 3.14). Secondary metabolites are not required for plant survival; however, can be antimicrobial compounds (Dixon, 2001). Again, genes involved in plant cell death were highly up regulated during the five-day infection with *P. aeruginosa* PA14 (Figure 3.15). Whereas, genes involved in primary metabolism in canola seedlings were highly down regulated during the five-day infection with *P. aeruginosa* PA14 (Figure 3.16). Finally, genes involved in plant growth in canola seedlings were highly down regulated during the five-day infection with *P. aeruginosa* PA14 (Figure 3.17).

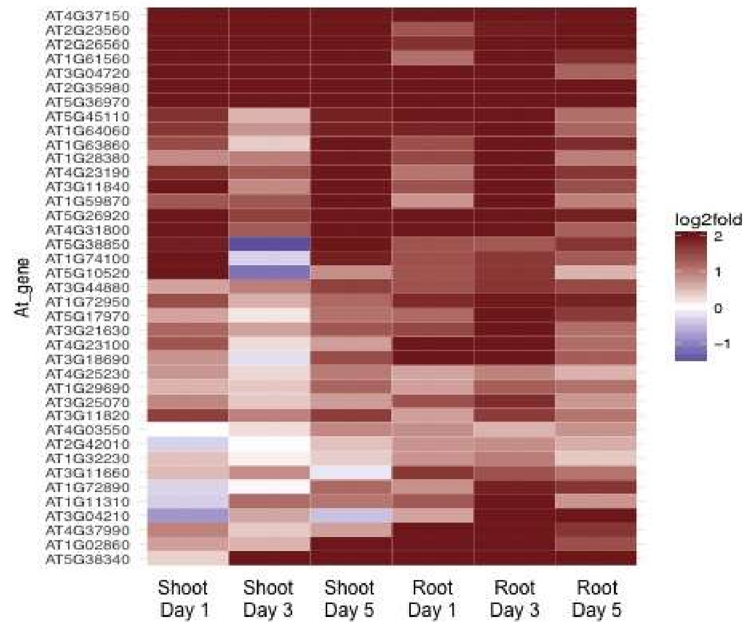

Figure 3.13: Genes involved in the innate immune response in *Brassica napus* are up regulated during a five-day infection with *P. aeruginosa* PA14. *B. napus* genes were BLASTed against *Arabidopsis thaliana* genes to obtain functional gene annotations. Gene expression of infected canola seedlings was calculated relative to uninfected controls, with two independent biological replicates for each condition.

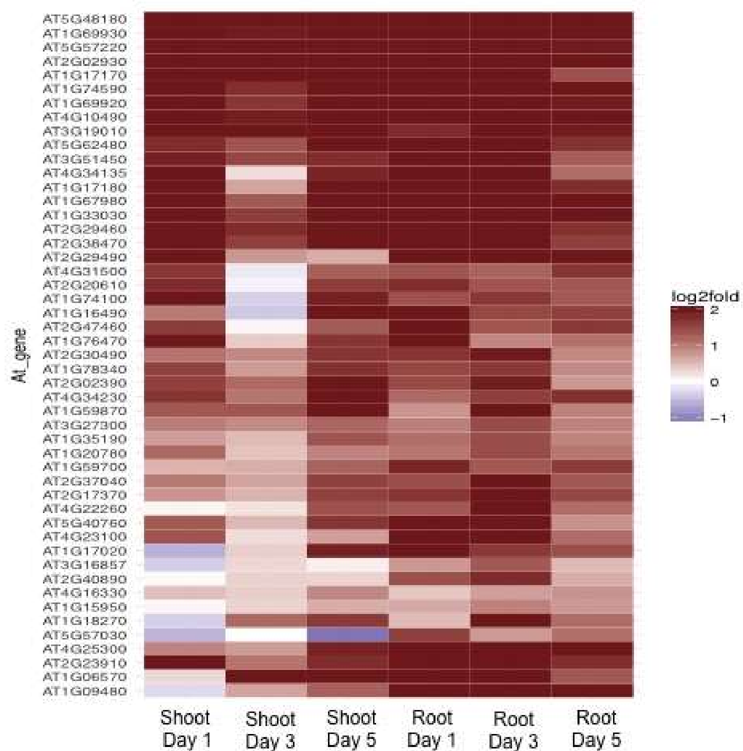

Figure 3.14: Genes involved in secondary metabolism in *Brassica napus* are up regulated during a five-day infection with *P. aeruginosa* PA14. *B. napus* genes were BLASTed against *Arabidopsis thaliana* genes to obtain functional gene annotations. Gene expression of infected canola seedlings was calculated relative to uninfected controls, with two independent biological replicates for each condition.

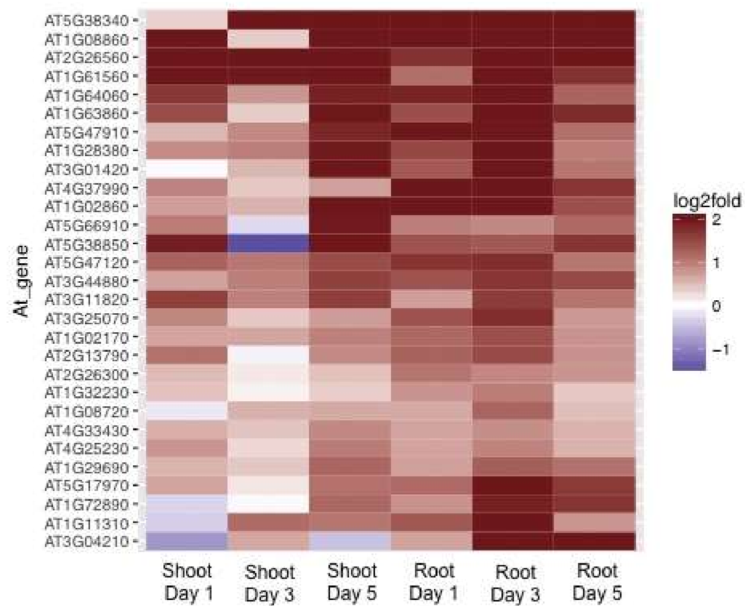

Figure 3.15: Genes involved in plant cell death in *Brassica napus* are up regulated during a five-day infection with *P. aeruginosa* PA14. *B. napus* genes were BLASTed against *Arabidopsis thaliana* genes to obtain functional gene annotations. Gene expression of infected canola seedlings was calculated relative to uninfected controls, with two independent biological replicates for each condition.

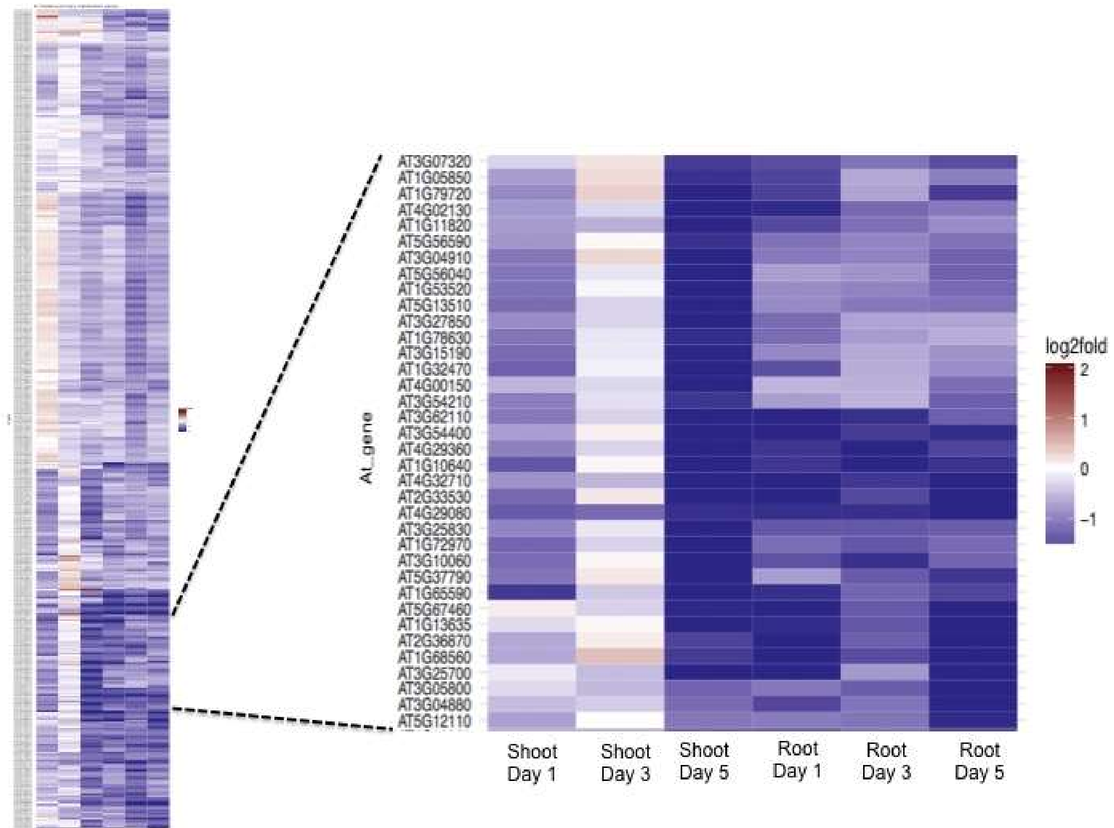

Figure 3.16: Genes involved in primary metabolism in *Brassica napus* are down regulated during a five-day infection with *P. aeruginosa* PA14. *B. napus* genes were BLASTed against *Arabidopsis thaliana* genes to obtain functional gene annotations. Gene expression of infected canola seedlings was calculated relative to uninfected controls, with two independent biological replicates for each condition.

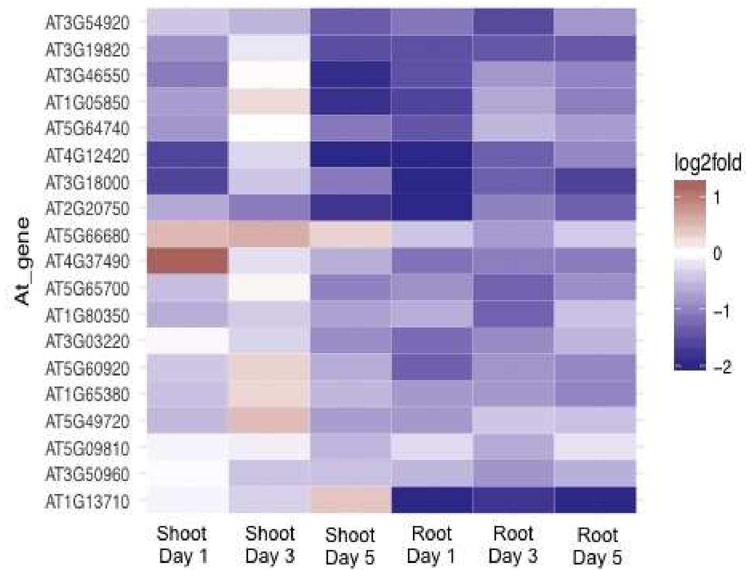

Figure 3.17: Genes involved in plant growth in *Brassica napus* are down regulated during a five-day infection with *P. aeruginosa* PA14. *B. napus* genes were BLASTed against *Arabidopsis thaliana* genes to obtain functional gene annotations. Gene expression of infected canola seedlings was calculated relative to uninfected controls, with two independent biological replicates for each condition.

3.3.1 Validation of RNA Sequencing using RT-qPCR.

In order to validate the RNA sequencing we used RT-qPCR to measure the expression changes in selected genes involved in innate immunity and plant growth and development. Similar trends were observed when comparing the $\log_2$ relative expression RNA sequencing with the values obtained using RT-qPCR. The $\log_2$ relative expression for all genes measured was greater when measured with RT-qPCR compared to RNA sequencing. Some differences were *ACS7* relative expression measured using RNA sequencing was down regulated in the shoot, whereas the RT-qPCR showed expression was upregulated in both tissue types (Figure 3.19 A). Similarly, the relative expression of *GA2OX1* was down regulated in the RNA sequencing for both tissue types, whereas the RT-qPCR showed that expression was up regulated in the root tissue (Figure 3.20 A). Finally, the expression of *AT1G77530* was strongly down regulated in the RNA sequencing, whereas the RT-qPCR showed that expression for the most part was up regulated (Figure 3.20 B).

The expression of *WRKY75* remained constant (8.6-$\log_2$fold) within the shoot tissue of canola seedlings throughout the five-infection with *P. aeruginosa* PA14, whereas in the roots the expression increased from day one to three by 10% and then decreased from day three to five by 27% (Figure 3.18 A). The expression of *CYP72A15* increased within the shoot from day one to three by 188% and from day three to five by 42%, whereas in the roots the expression increased from day one to three by 15% and then decreased from day three to five by 45% (Figure 3.18 B). The expression of *ACS7* increased within the shoot tissue from day one to three by 15% and decreased from day three to five by 12%, whereas in the roots the expression decreases from day one to three by 22% and from day three to

five by 43% (Figure 3.19 A). The expression of *ACO12* remained constant within the shoot during the five-day infection (3.3-log_2fold) whereas in the roots the expression increased from day one to three by 39% and then remained constant from day three to five (2.26-log_2fold) (Figure 3.19 B). The expression of *GA2OX1* remained constant within the shoot from day one to three (-1.0-log_2fold) and increased from day three to five by 79%, whereas in the roots the expression remained constant throughout the five-day infection (2.4-log_2fold) (Figure 3.20 A). The expression of *AT1G77530* remained constant within the shoot from day one to three (9.74-log_2fold) and increased from day three to five by 34%, whereas in the roots the expression decreased from day one to three by 21% and then decreased from day three to five by 121% (Figure 3.20 B).

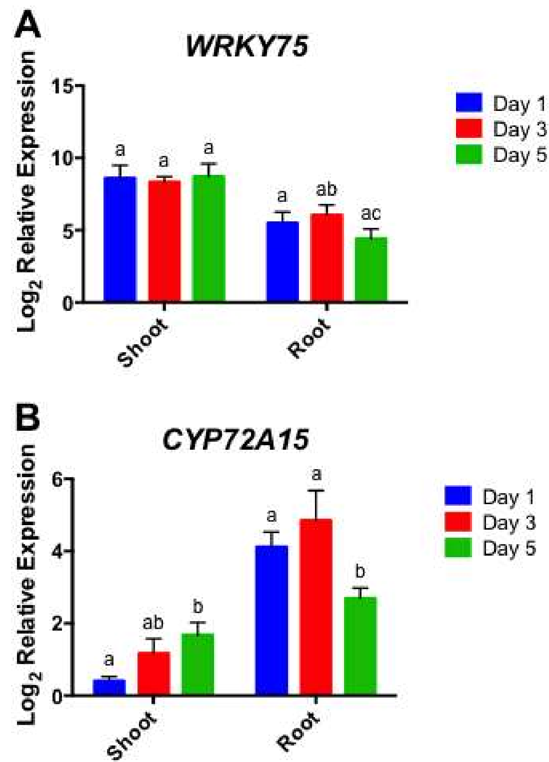

Figure 3.18: Relative expression (log_2) of genes involved in the innate immune response in *B. napus* during a five day infection with *P. aeruignosa* PA14 WT. A, Relative expression of *WRK75* measured on days one, three and five. **B,** Relative expression of *Cyp72A15* measured on days one, three and five. Error bars represent standard deviation of three independent biological replicates. Statistical significance was measured at the $p \leq 0.05$ probability level; the same letters indicate non-statistically significant differences between groups, whereas different letters indicate statistically significant difference between groups.

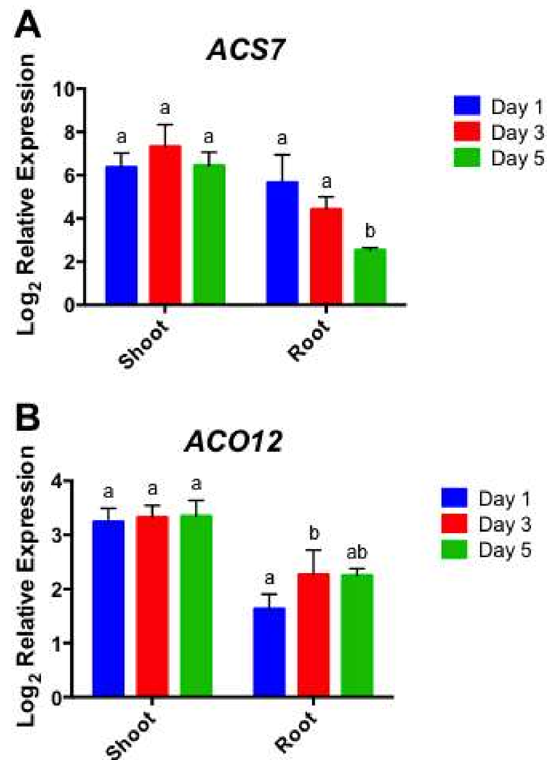

Figure 3.19: Relative expression (log_2) of genes involved in ET production in *B. napus* during a five day infection with *P. aeruignosa* PA14 WT. A, Relative expression of *ACS7* measured on days one, three and five. **B,** Relative expression of *ACO12* measured on days one, three and five. Error bars represent standard deviation of three independent biological replicates. Error bars represent standard deviation of three independent biological replicates. Statistical significance was measured at the $p \leq 0.05$ probability level; the same letters indicate non-statistically significant differences between groups, whereas different letters indicate statistically significant difference between groups.

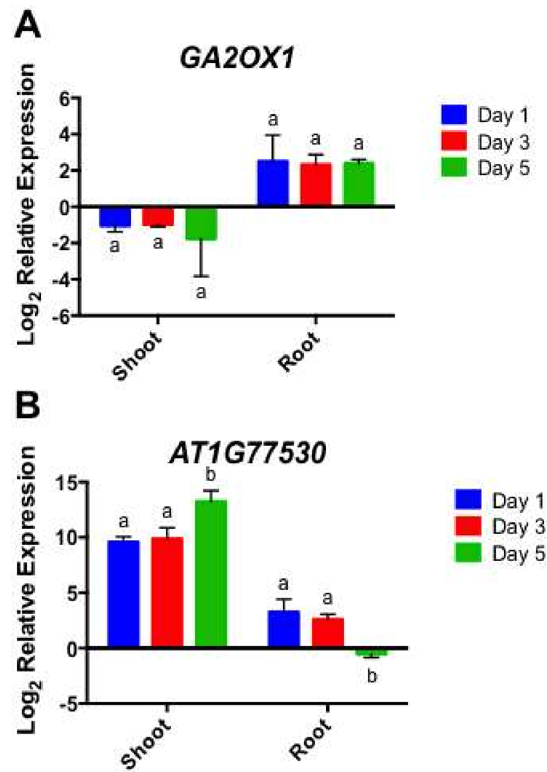

Figure 3.20: Relative expression ($\log_2$) of genes involved in plant growth in *B. napus* during a five day infection with *P. aeruignosa* PA14 WT. A, Relative expression of *GA2OX1* measured on days one, three and five. **B,** Relative expression of *AT1G77530* measured on days one, three and five. Error bars represent standard deviation of three independent biological replicates. Error bars represent standard deviation of three independent biological replicates. Statistical significance was measured at the $p \leq 0.05$ probability level; the same letters indicate non-statistically significant differences between groups, whereas different letters indicate statistically significant difference between groups.

3.4 *Pseudomonas aeruginosa* Infection Perturbs the Homeostasis of Plant Hormones in Canola, and Promotes the Production of Antimicrobial Compounds

It has been well studied in the literature that infecting plants with pathogens will cause a shift in the concentration of hormones found in plants (Pieterse *et al.*, 2009). Production of hormones involved in plant innate immunity will be up regulated, whereas hormones involved in growth and development will become down regulated (Pieterse *et al.*, 2009). Phytohormone concentrations will vary from plant to plant and are also specific to the pathogen that is infecting the plant. Similarly, the production of antimicrobial compounds (metabolites) generally increases when plants come into contact with pathogens (Dixon, 2001). Therefore, we infected canola seedlings with *Pseudomonas aeruginosa* PA14 WT in order to study the production of plant hormones, and antimicrobial compounds produced during an infection. The high-resolution LC/MS traces for days one, three and five showing biological replicate reproducibility (n=4) can be found in Appendix C. Whereas, the high-resolution LC/MS traces for days one, three and five showing comparisons between sample types (Infected plants, Control plants and Control bacteria) can be found in Appendix D.

3.4.1 Targeted Identification of Antimicrobial Compounds Produced by Canola During a *P. aeruginosa* Infection.

In order to test our metabolomics samples for the presence of antimicrobial compounds, we first did a literature search to find common metabolites produced by plants for defense from pathogens. We also searched within our RNA sequencing data to find

secondary metabolism genes that were upregulated during the infection. From our literature search and our analysis of RNA sequencing data we complied a list of 32 metabolites known to be involved in plant innate immunity. From that list, we identified 13 common antimicrobial compounds produced by canola seedlings during a *P. aeruginosa* PA14 WT infection (Table 3.2). Of particularly interest are 5 tentative metabolites; coumarin, rishitin, benzoxazione, iodine cyanide (ICN) and indole-3-carboxylic acid methyl ester since they are present at least 2-fold more in infected seedlings vs. control seedlings (Table 3.2). We also identified four antimicrobial compounds found only in infected plant samples namely camalexin, methoxy-I3G, medicarpin and 4-hydroxyindole-3-carbonyl nitrile (Table 3.2).

Table 3.2: Targeted identification of antimicrobial metabolites produced by canola seedlings during a *P. aeruginosa* PA14 WT infection.

Tentative Metabolites*	Chemical Formula	Protonated Compound $[M+H]^+$	Peak Area Ratio (Infected Plant/Plant Control)		
			Day 1	Day 3	Day 5
Camalexin$^{\Psi}$	$C_{11}H_8N_{25}$	201.04810	N/A	N/A	N/A
Methoxy-I3G$^{\Psi}$	$C_{17}H_{22}N_2O_{10}S_2$	479.07886	N/A	N/A	N/A
Coumarin	$C_9H_6O_2$	147.04406	2.8	6.0	4.2
Rishitin	$C_{14}H_{22}O_2$	223.16926	2.7	3.0	1.6
Momilactone A	$C_{20}H_{26}O_3$	315.19547	0.9	1.1	0.8
Resveratrol	$C_{14}H_{12}O_3$	229.08592	1.3	1.5	0.8
Benzoxazinone	$C_9H_9NO_3$	180.06552	10.8	9.1	4.2
Indole-3-carboxylic acid methyl ester	$C_{10}H_9NO_2$	176.07061	1.1	2.0	1.5
Medicarpin$^{\Psi}$	$C_{16}H_{14}O_4$	271.09649	N/A	N/A	N/A
4-hydroxyindole-3-carbonyl nitrile$^{\Psi}$	$C_{10}H_6N_2O_2$	187.05020	N/A	N/A	N/A
Batatasin IV	$C_{15}H_{16}O_3$	245.11722	0.5	0.3	0.3
ICN	$C_{10}H_6N_2O$	171.05529	1.1	2.4	2.2
ICA methyl ester	$C_{10}H_9NO_2$	176.07061	1.1	2.0	1.5

*Not confirmed with standards, identified solely based on accurate masses.
$^{\Psi}$Only detected in infected plant samples.

3.4.2 Untargeted Identification of Plant Hormones, and Amino Acids Produced by Canola during a *P. aeruginosa* infection.

We were also hoping to identify novel antimicrobial metabolites being produced by canola seedlings during an infection with *P. aeruginosa* PA14; therefore, we adopted an untargeted approach for metabolite identification. We identified plant hormones and amino acids by looking at differences between the LC/MS traces comparing infected plant with control plant samples. Peaks that were different between the infected plant and the control plant samples, and had a high magnitude were identified using SciFinder (American Chemical Society, 2018). The plant hormone metabolites that changed greater than 2-fold in infected plants vs. control plants were indole-3-acetic acid (IAA; auxin), indole-3-acetaldehyde (auxin), and cyclomethane (JA-derivative) (Table 3.3). Cucurbic acid (JA-derivative) levels decreased over the infection (Table 3.3). Whereas, all of the other plant hormone metabolites including methyl salicylate, JA, indole-3-carboxylic acid, indole-3-acetic acid methyl ester, indolyl-3-methanol and ACC were only detected in infected plant samples (Table 3.3). Similarly, the levels of amino acids (isoleucine, phenylalanine and tryptophan) detected within the media increased over the infection when comparing infected plants with control plants (Table 3.4). Valine was only present within infected plant samples (Table 3.4).

Table 3.3: Untargeted identification of plant hormones produced by canola seedlings during a *P. aeruginosa* PA14 WT infection.

Tentative Metabolites*	Chemical Formula	Plant Control (Peak Area)			Infected Plant (Peak Area)			Peak Area Ratio (Infected Plant/Plant Control)		
		Day 1	**Day 3**	**Day 5**	**Day 1**	**Day 3**	**Day 5**	**Day 1**	**Day 3**	**Day 5**
Methyl salicylate$^{\Psi}$	$C_8H_8O_3$	N/D	N/D	N/D	$1.30e^{6}$	$2.60e^{6}$	$2.01e^{6}$	N/A	N/A	N/A
Jasmonic acid$^{\Psi}$	$C_{12}H_{18}O_3$	N/D	N/D	N/D	$5.06e^{6}$	$9.48e^{6}$	$1.38e^{7}$	N/A	N/A	N/A
Cucurbic acid	$C_{12}H_{20}O_3$	$9.16e^{6}$	$3.50e^{6}$	$6.31e^{6}$	$1.01e^{6}$	$7.24e^{5}$	$1.82e^{5}$	0.11	0.21	0.029
Indole-3-acetic acid	$C_{10}H_9NO_2$	$9.90e^{6}$	$9.19e^{6}$	$1.79e^{7}$	$1.07e^{7}$	$1.90e^{7}$	$2.83e^{7}$	1.1	2.1	1.6
Indole-3-acetaldehyde	$C_{10}H_9NO$	$4.23e^{7}$	$4.54e^{7}$	$6.57e^{7}$	$1.05e^{8}$	$2.01e^{8}$	$2.97e^{8}$	2.5	4.4	4.5
Indole-3-carboxylic acid$^{\Psi}$	$C_9H_7NO_2$	N/D	N/D	N/D	$1.31e^{6}$	$2.75e^{6}$	$4.11e^{6}$	N/A	N/A	N/A
Indole-3-acetic acid methyl ester$^{\Psi}$	$C_{11}H_{11}NO_2$	N/D	N/D	N/D	$4.07e^{5}$	$4.75e^{6}$	$6.94e^{6}$	N/A	N/A	N/A
Indolyl-3-methanol$^{\Psi}$	C_9H_9NO	N/D	N/D	N/D	$5.24e^{5}$	$1.01e^{6}$	$7.26e^{5}$	N/A	N/A	N/A
1-Aminocyclopropane -1-carboxylic acid$^{\Psi}$	$C_4H_7NO_2$	N/D	N/D	N/D	$8.54e^{5}$	$1.50e^{6}$	$1.90e^{6}$	N/A	N/A	N/A

*Not confirmed with standards, identified solely based on accurate masses.
$^{\Psi}$Only detected in infected plant samples.

Table 3.4: Untargeted identification of amino acids produced by canola seedlings during a *P. aeruginosa* PA14 WT infection.

Tentative Metabolites*	Chemical Formula	Plant Control (Peak Area)			Infected Plant (Peak Area)			Peak Area Ratio (Infected Plant/Plant Control)		
		Day 1	Day 3	Day 5	Day 1	Day 3	Day 5	Day 1	Day 3	Day 5
Valine$^{\Psi}$	$C_5H_{11}NO_2$	N/D	N/D	N/D	$3.66e^{6}$	$5.70e^{6}$	$5.15e^{6}$	N/A	N/A	N/A
Isoleucine	$C_6H_{13}NO_2$	N/D	$1.07e^{6}$	$1.25e^{6}$	$2.24e^{6}$	$5.64e^{6}$	$7.20e^{6}$	N/A	5.3	5.8
Phenylalanine	$C_9H_{11}NO_2$	$3.54e^{6}$	$2.55e^{6}$	$6.24e^{6}$	$8.03e^{6}$	$8.29e^{6}$	$2.56e^{7}$	2.3	3.3	4.1
Tryptophan	$C_{11}H_{12}N_2O_2$	$5.27e^{6}$	$3.59e^{6}$	$7.59e^{6}$	$7.23e^{6}$	$9.46e^{6}$	$2.05e^{7}$	1.4	2.6	2.7

*Not confirmed with standards, identified solely based on accurate masses.

$^{\Psi}$Only detected in infected plant samples.

3.4.3 Untargeted Identification of Antimicrobial Compounds and Phytoalexins Produced by Canola During a *P. aeruginosa* Infection.

We identified antimicrobial compounds and phytoalexins by looking at differences between the LC/MS traces comparing infected plant with control plant samples. Peaks that were different between the infected plant and the control plant samples, and had a high magnitude were identified using SciFinder (American Chemical Society, 2018). Syringaldehyde and 1-methoxy-indole-3-carboxylic acid levels increased during the five-day infection of canola seedlings with *P. aeruginosa* PA14 WT (Table 3.5). Whereas, homovanillic alcohol and polyoxin B levels increased in canola seedlings with *P. aeruginosa* PA14 WT; however, their levels were on a downward trend throughout the five-day infection (Table 3.5). The other antimicrobial compounds were only found the infected plant samples (Table 3.5). The phytoalexins, isalexin, 1-acetylindole-3-carboxaldehyde, formamide and spirobrassinin all increased during the five-day infection of canola seedlings with *P. aeruginosa* PA14 WT (Table 3.6). S-methyl dithiocarbamate and brassilexin were only found the infected plant samples (Table 3.6).

Table 3.5: Untargeted identification of antimicrobial compounds produced by canola seedlings during a *P. aeruginosa* PA14 WT infection.

Tentative Metabolites*	Chemical Formula	Plant Control (Peak Area)			Infected Plant (Peak Area)			Peak Area Ratio (Infected Plant/Plant Control)		
		Day 1	Day 3	Day 5	Day 1	Day 3	Day 5	Day 1	Day 3	Day 5
GABA$^{\Psi}$	$C_4H_9NO_2$	N/D	N/D	N/D	$3.62e^{6}$	$1.35e^{7}$	$1.57e^{7}$	N/A	N/A	N/A
Phenylethylamine$^{\Psi}$	$C_8H_{11}N$	N/D	N/D	N/D	$1.45e^{6}$	$1.68e^{6}$	$7.73e^{6}$	N/A	N/A	N/A
Goitrin$^{\Psi}$	$C_5H_7NO_5$	N/D	N/D	N/D	$7.74e^{5}$	$6.36e^{6}$	$2.00e^{7}$	N/A	N/A	N/A
Hypoxanthine$^{\Psi}$	$C_5H_4N_4O$	N/D	N/D	N/D	N/D	$4.10e^{5}$	$4.30e^{5}$	N/A	N/A	N/A
Coumaric Acid$^{\Psi}$	$C_9H_8O_3$	N/D	N/D	N/D	$3.06e^{5}$	$7.10e^{5}$	$1.19e^{5}$	N/A	N/A	N/A
Homovanillic alcohol	$C_9H_{12}O_3$	$8.28e^{5}$	N/D	$5.31e^{5}$	$3.67e^{6}$	$5.83e^{5}$	$2.00e^{6}$	4.4	N/A	3.8
Syringaldehyde	$C_9H_{10}O_4$	$1.44e^{5}$	$1.81e^{5}$	$5.03e^{5}$	$6.01e^{6}$	$1.47e^{6}$	$2.59e^{6}$	4.2	8.1	5.1
4-hydroxy-alpha-cyanocinnamic acid$^{\Psi}$	$C_{10}H_7NO_3$	N/D	N/D	N/D	$2.50e^{6}$	$2.26e^{6}$	$2.26e^{6}$	N/A	N/A	N/A
Brassicanal A$^{\Psi}$	$C_{10}H_9NOS$	N/D	N/D	N/D	$5.70e^{5}$	$4.03e^{7}$	$2.18e^{8}$	N/A	N/A	N/A
1-methoxy-indole-3-carboxylic acid	$C_{10}H_9NO_3$	$5.29e^{6}$	$1.64e^{6}$	$4.17e^{6}$	$3.20e^{7}$	$3.64e^{7}$	$2.35e^{7}$	6.0	22.2	5.6
Pantothenic acid$^{\Psi}$	$C_9H_{17}NO_5$	N/D	N/D	N/D	$1.74e^{7}$	$2.50e^{7}$	$4.13e^{7}$	N/A	N/A	N/A
Traumatic acid$^{\Psi}$	$C_{12}H_{20}O_4$	N/D	N/D	N/D	$8.09e^{6}$	$1.58e^{7}$	$1.92e^{7}$	N/A	N/A	N/A
Polyoxin B$^{\Psi}$	$C_{17}H_{25}N_5O_{13}$	N/D	N/D	N/D	$8.09e^{6}$	$1.58e^{7}$	$1.92e^{7}$	50.2	11.9	15.4

*Not confirmed with standards, identified solely based on accurate masses.

$^{\Psi}$Only detected in infected plant sample

Table 3.6: Untargeted identification of phytoalexins produced by canola seedlings during a *P. aeruginosa* PA14 WT infection.

Tentative Metabolites*	Chemical Formula	Plant Control (Peak Area)			Infected Plant (Peak Area)			Peak Area Ratio (Infected Plant/Plant Control)		
		Day 1	Day 3	Day 5	Day 1	Day 3	Day 5	Day 1	Day 3	Day 5
S-methyl dithiocarbamate$^{\Psi}$	$C_2H_5NS_2$	N/D	N/D	N/D	N/D	$1.12e^{6}$	$1.79e^{6}$	N/A	N/A	N/A
Isalexin	$C_9H_7NO_3$	$7.28e^{5}$	$1.01e^{5}$	N/D	$1.42e^{6}$	$2.49e^{6}$	$3.90e^{6}$	2.0	24.5	N/A
Brassilexin$^{\Psi}$	$C_9H_6N_2S$	N/D	N/D	N/D	N/D	$1.46e^{7}$	$8.35e^{7}$	N/A	N/A	N/A
Formamide	$C_{10}H_{10}O_2N_2$	N/D	N/D	$4.89e^{4}$	$4.33e^{7}$	$6.85e^{7}$	$5.89e^{7}$	N/A	N/A	1204.4
1-acetylindole-3-carboxaldehyde	$C_{11}H_9NO_2$	$2.24e^{6}$	$1.55e^{6}$	$3.30e^{6}$	$3.11e^{6}$	$4.03e^{6}$	$9.18e^{6}$	1.4	2.6	2.8
Spirobrassinin	$C_{11}H_{10}N_2OS_2$	$8.03e^{5}$	$1.99e^{5}$	$9.12e^{4}$	$1.07e^{6}$	$2.64e^{7}$	$1.35e^{8}$	1.3	132.3	1475.6

*Not confirmed with standards, identified solely based on accurate masses.

$^{\Psi}$Only detected in infected plant samples.

3.4.4 Untargeted Identification of Metabolites Produced by *P. aeruginosa* During a Canola infection.

We identified metabolites specifically produced by *P. aeruginosa* PA14 during the infection of canola seedlings by looking at differences between the LC/MS traces comparing infected plant with control bacterial samples. Peaks that were different between the infected plant and the control plant samples, and had a high magnitude were identified using SciFinder (American Chemical Society, 2018). Interestingly, the production of QS (N- butanoyl-homoserine lactone, 2-heptyl-4-quinolone and N-oxo-2-heptyl-4-hydroxyquinoline) molecules by *P. aeruginosa* PA14 levels increased during infection of canola seedlings; however, their levels were on a downward trend throughout the five-day infection (Table 3.7). Similarly, major virulence factors (pyocyanine, rhamnolipid RL1 and coronatine) produced by PA14 decreased throughout the infection (Table 3.7). The production of the phenazines, 1-hydroxyphenazine by PA14 increased throughout the infection (Table 3.7). Whereas, the production of 1-phenazinecarboxamide by PA14 decreased throughout the infection (Table 3.7).

Table 3.7: Untargeted identification of metabolites produced by *P. aeruginosa* PA14 WT infection of canola seedlings.

Tentative Metabolites*	Chemical Formula	Bacterial Control (Peak Area)			Infected Plant (Peak Area)			Peak Area Ratio (Infected Plant/Bacteria Control)		
		Day 1	Day 3	Day 5	Day 1	Day 3	Day 5	Day 1	Day 3	Day 5
N-butanoyl-homoserine lactone	$C_8H_{13}NO_3$	$1.99e^{6}$	$3.91e^{6}$	$3.02e^{7}$	$1.67e^{7}$	$2.82e^{7}$	$3.60e^{7}$	8.4	7.2	1.2
1-hydroxyphenazine$^{\Psi}$	$C_{12}H_8N_2O$	N/D	N/D	N/D	$1.47e^{7}$	$2.40e^{7}$	$3.29e^{7}$	N/A	N/A	N/A
Pyocyanine	$C_{13}H_{10}N_2O$	N/D	N/D	$6.12e^{5}$	$3.11e^{7}$	$3.51e^{7}$	$2.60e^{7}$	N/A	N/A	42.5
2-heptyl-4-quinolone	$C_{16}H_{21}NO$	$9.19e^{7}$	$1.12e^{8}$	$2.17e^{8}$	$1.55e^{8}$	$1.08e^{8}$	$1.87e^{8}$	1.7	0.96	0.86
Rhamnolipid RL1	$C_{26}H_{48}O_9$	$3.82e^{6}$	$2.89e^{6}$	$3.07e^{6}$	$9.91e^{7}$	$6.21e^{7}$	$7.06e^{7}$	26.0	21.5	23.0
Coronatine	$C_{18}H_{25}O_4N$	$2.00e^{7}$	$4.03e^{7}$	$7.35e^{7}$	$1.31e^{7}$	$1.56e^{7}$	$1.47e^{7}$	0.65	0.39	0.20
1-phenazinecarboxamide	$C_{13}H_9N_3O$	$9.46e^{5}$	$1.17e^{6}$	$1.74e^{7}$	$3.61e^{7}$	$3.53e^{7}$	$2.60e^{7}$	38.2	30.2	1.5
N-oxo-2-heptyl-4-hydroxyquinoline	$C_{16}H_{21}NO_2$	$2.24e^{7}$	$2.42e^{7}$	$1.24e^{7}$	$9.94e^{7}$	$7.68e^{7}$	$1.22e^{8}$	4.4	3.2	9.8

*Not confirmed with standards, identified solely based on accurate masses

$^{\Psi}$Only detected in infected plant samples.

3.5 Inhibition of ET Production Increases Plant Health and Decreases Symptoms of Disease in Canola During *P. aeruginosa* Infection.

ET is major phytohormone involved in regulating plant's innate immune response towards invading pathogens. ET has been shown to primarily play a role in defense responses towards necrotrophic plant pathogens (Groen *et al.*, 2013). ET is also known to be an antagonist for SA in regard to phytohormone signalling during an immune response in plants (Pieterse *et al.*, 2009). However, during our transcriptomic analysis of canola seedlings infected with *P. aeruginosa* PA14 we found that many genes involved in ET biosynthesis and ET signalling were up regulated (Figure 3.19 A, B). Therefore, we wanted to investigate ET role in canola's immune response towards *P. aeruginosa* PA14. We hypothesized that reducing ET levels in canola seedlings would lead to a more robust immune response since SA immune signalling would not longer to antagonized. ET levels were reduced in canola seedings using AVG; a chemical compound that inhibits ACC synthase, therefore, reducing the level of ACC within plant cells, which results in the reduction of ET.

3.5.1 Inhibition of ET Production by AVG Increases Plant Health and Decreases Symptoms of Disease in Canola During *P. aeruginosa* Infection.

Importantly, we did not see a significant difference between the untreated-control seedlings and our treated-control (AVG) seedlings on any parameters (tissue weight, leaf spotting, and CFU) measured during the infection (Figure 3.21 A, B; Figure 3.22 A, B; Figure 3.23). However, large differences were observed when comparing the untreated-control vs. untreated-infected, or the treated-control (AVG) vs. treated-infected (AVG)

seedlings with *P. aeruginosa* PA14. There was no significant difference in root weight observed between untreated-control and untreated-infected seedlings on day zero (two-hours post infection) (Figure 3.21 A). Similarly, there was no significant difference in root weight observed between treated-control (AVG) and treated-infected (AVG) seedlings on day zero (two-hours post infection) (Figure 3.21 A). However, on days one, three and five there were decreases in root weight between untreated-control and untreated-infected of 47%, 72% and 80%, respectively (Figure 3.21 A). There was no significant difference in root weight observed between treated control and treated-infected (AVG) seedlings on days zero (two-hours post infection) (Figure 3.21 A). However, on days one, three and five there were decreases of in root weight between untreated-control and untreated-infected of 46%, 44% and 73%, respectively (Figure 3.21 A). Interestingly, there was a significant increase in root weight between untreated-infected and treated-infected (AVG) seedlings of 78% on day three (Figure 3.21 A).

Similarly, there was no significant difference in shoot weight observed between untreated-control and untreated-infected seedlings or treated-control (AVG) and treated-infected (AVG) seedlings on day zero (two-hours post infection) (Figure 3.21 B). However, on days one, three and five there were decreases in shoot weight between untreated-control and untreated-infected of 18%, 42% and 55%, respectively (Figure 3.21 B). Similarly, on days one, three and five there were decreases of in shoot weight between treated-control (AVG) and treated-infected (AVG) of 14%, 36% and 29%, respectively (Figure 3.21 B). Interestingly, on day five there were significant increase in shoot weight between untreated-infected and treated-infected (AVG) of 51% (Figure 3.21 B).

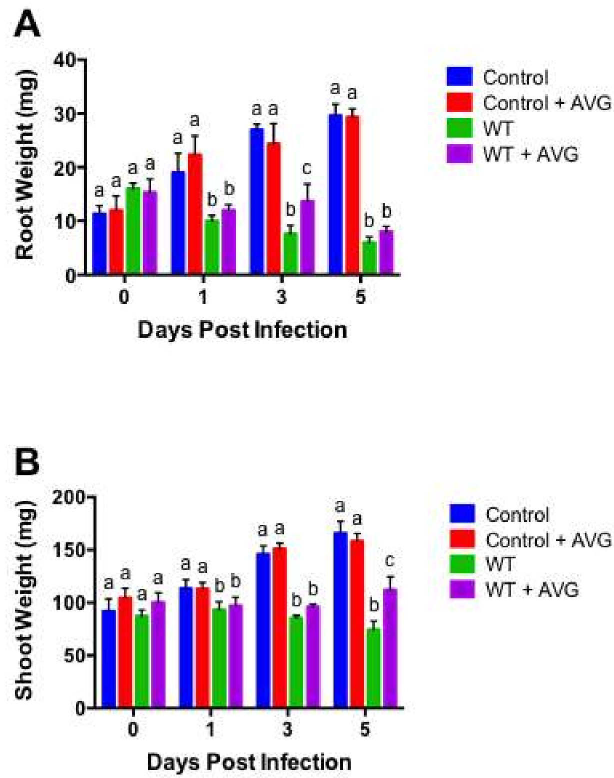

Figure 3.21: Canola seedlings untreated or treated (AVG) tissue weight recorded during a five-day infection with *P. aeruginosa* PA14. A, Canola root weight measured on days zero (two-hours post infection), one, three and five. **B,** Canola shoot weight measured on days zero (two-hours post infection), one, three and five. Error bars represent standard deviation of three independent biological replicates. Statistical significance was measured at the $p \leq 0.05$ probability level; the same letters indicate non-statistically significant differences between groups, whereas different letters indicate statistically significant difference between groups.

There was no significant difference in *P. aeruginosa* PA14 root CFU measured from untreated-infected and treated-infected (AVG) canola seedlings on days zero (two-hours post infection), one, three and five (Figure 3.22 A). Therefore, tissue weight loss cannot be explained by *P. aeruginosa* PA14's colonization of canola seedlings treated with AVG. However, *P. aeruginosa* PA14 leaf CFU measured from untreated-infected was significantly higher compared to treated-infected (AVG) by 3.9-fold and 2.9-fold, on days zero (two-hours post infection) and one, respectively (Figure 3.22 B). There was no significant difference in *P. aeruginosa* PA14 leaf CFU measured from damaged-infected and undamaged-infected on days three and five (Figure 3.22 B).

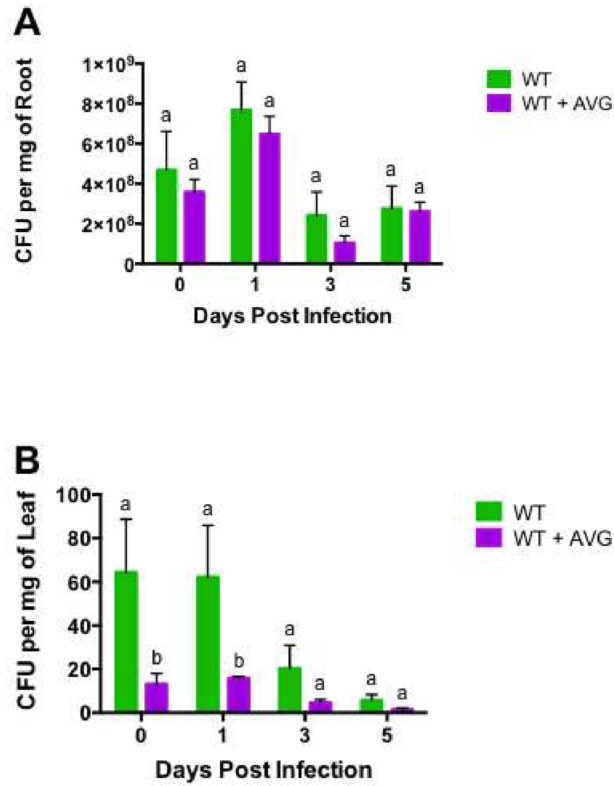

Figure 3.22: *Pseudomonas aeruginosa* PA14 colony forming units (CFU) measured on canola roots and within canola leaves of untreated and treated (AVG) seedlings. **A,** *Pseudomonas aeruginosa* PA14 root colonization measured on days zero (two-hours post infection), one, three and five. **B,** *Pseudomonas aeruginosa* PA14 leaf colonization measured on days zero (two-hours post infection), one, three and five. Error bars represent standard deviation of three independent biological replicates. Statistical significance was measured at the $p \leq 0.05$ probability level; the same letters indicate non-statistically significant differences between groups, whereas different letters indicate statistically significant difference between groups.

The number of spots on the leaves of both untreated and treated (AVG) canola seedlings infected with *P. aeruginosa* PA14 increased throughout the infection (Figure 3.23). There were no black spots observed on both untreated and treated (AVG) canola seedling leaves on day zero (two-hours post infection) (Figure 3.23). There was no significant difference in the number of black spots on untreated-infected compared to treated-infected (AVG) canola seedlings on days one and three (Figure 3.23). However, there were significantly more black spots on treated-infected (AVG) compared to untreated-infected canola seedlings by 37% on day 5 (Figure 3.23). This is likely due to the treated (AVG) canola seedlings being healthier, making them more fit to fight off *P. aeruginosa* PA14.

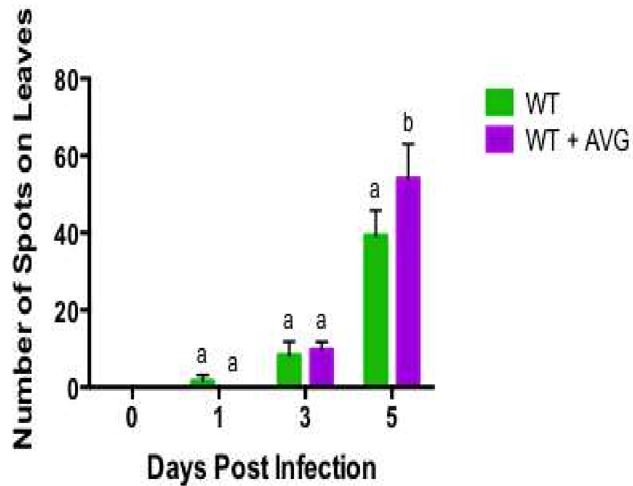

Figure 3.23: Number of black spots on untreated and treated (AVG) canola seedling's leaves during a five-day infection with *P. aeruginosa* PA14. The number of black spots on the leaves was recorded on days zero (two-hours post infection), one, three and five. Error bars represent standard deviation of three independent biological replicates. Statistical significance was measured at the $p \leq 0.05$ probability level; the same letters indicate non-statistically significant differences between groups, whereas different letters indicate statistically significant difference between groups.

3.5.2 Inhibition of ET Production by ACC Deaminase Increases Plant Health and Decreases Symptoms of Disease in Canola During *P. aeruginosa* Infection.

To provide further support for our hypothesis, we perfomed the same infection using transgenic canola seedlings expressing ACCD. ACCD limits the amount of ET by breaking down ACC, the precurosor molecule of ET in plants. Interestingly, there were significant increase in root weight between *B. napus* cv. Westar control and *B. napus* cv. Westar ($ACCD^+$) control of 40% and 33% on days three and five, respectively (Figure 3.24 A). Large differences were observed when comparing the *B. napus* cv. Westar control vs. infected, or the *B. napus* cv. Westar ($ACCD^+$) control vs. infected seedlings with *P. aeruginosa* PA14. There was no significant difference in root weight observed between *B. napus* cv. Westar control and infected seedlings on day zero (two-hours post infection) (Figure 3.24 A). However, on days one, three and five there were decreases in root weight between *B. napus* cv. Westar control and infected of 41%, 53% and 77%, respectively (Figure 3.24 A). Similalrly, on days zero (two-hours post infection), one, three and five there were decreases of in root weight between *B. napus* cv. Westar ($ACCD^+$) control and infected ranging from 30% to 79% (Figure 3.24 A). Interestingly, there were no significant differences in root weight between *B. napus* cv. Westar infected and *B. napus* cv. Westar ($ACCD^+$) infected seedlings (Figure 3.24 A).

Similarly, there were significant increase in shoot weight between *B. napus* cv. Westar control and *B. napus* cv. Westar ($ACCD^+$) control of 16% and 25% on days zero (two-hours post infection) and five, respectively (Figure 3.24 B). There was no significant difference in shoot weight observed between *B. napus* cv. Westar control control and infected seedlings or *B. napus* cv. Westar ($ACCD^+$) control and infected seedlings on days

zero (two-hours post infection) and one (Figure 3.24 B). However, on days three and five there were decreases in shoot weight between *B. napus* cv. Westar control and infected of 42% and 60%, respectively (Figure 3.24 B). Similarly, on days three and five there were decreases of in shoot weight between *B. napus* cv. Westar control and infected of 33% and 58%, respectively (Figure 3.24 B). Interestingly, there were significant increase in shoot weight between *B. napus* cv. Westar infected and *B. napus* cv. Westar ($ACCD^{+}$) infected of 25% and 32% on days three and five, respectively (Figure 3.24 B).

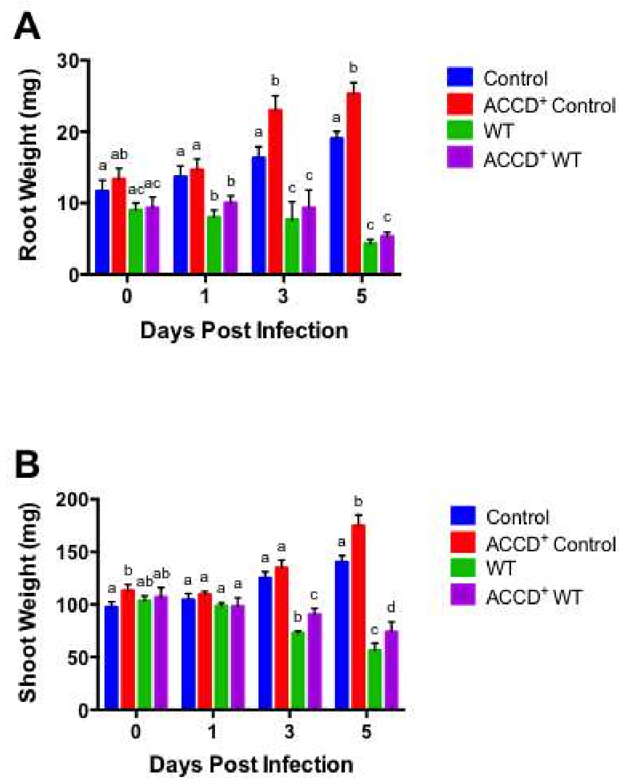

Figure 3.24: *B. napus* cv. Westar and *B. napus* cv. Westar (ACCD^{+}) seedlings tissue weight recorded during a five-day infection with *P. aeruginosa* PA14. A, Canola root weight measured on days zero (two-hours post infection), one, three and five. **B,** Canola shoot weight measured on days zero (two-hours post infection), one, three and five. Error bars represent standard deviation of three independent biological replicates. Statistical significance was measured at the $p \leq 0.05$ probability level; the same letters indicate non-statistically significant differences between groups, whereas different letters indicate statistically significant difference between groups.

There was no significant difference in *P. aeruginosa* PA14 root CFU measured from *B. napus* cv. Westar infected and *B. napus* cv. Westar ($ACCD^+$) infected canola seedlings on days zero (two-hours post infection), one, three and five (Figure 3.25 A). Therefore, tissue weight loss cannot be explained by *P. aeruginosa* PA14's colonization of *B. napus* cv. Westar ($ACCD^+$) seedlings. However, *P. aeruginosa* PA14 leaf CFU measured from *B. napus* cv. Westar infected was significantly higher compared to *B. napus* cv. Westar ($ACCD^+$) infected by 56.6-fold, 2.2-fold, and 3.2-fold, on days zero (two-hours post infection), one and three, respectively (Figure 3.25 B). There was no significant difference in *P. aeruginosa* PA14 leaf CFU measured from damaged-infected and undamaged-infected on day five (Figure 3.25 B).

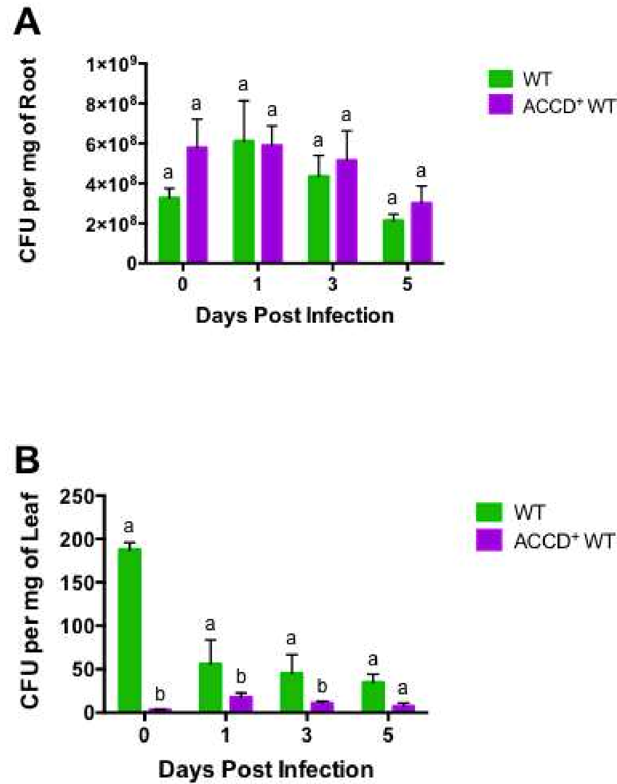

Figure 3.25: *Pseudomonas aeruginosa* PA14 colony forming units (CFU) measured on canola roots and within canola leaves of *B. napus* cv. Westar and *B. napus* cv. Westar (ACCD$^+$) seedlings. A, *Pseudomonas aeruginosa* PA14 root colonization measured on days zero (two-hours post infection), one, three and five. **B,** *Pseudomonas aeruginosa* PA14 leaf colonization measured on days zero (two-hours post infection), one, three and five. Error bars represent standard deviation of three independent biological replicates. Statistical significance was measured at the $p \leq 0.05$ probability level; the same letters indicate non-statistically significant differences between groups, whereas different letters indicate statistically significant difference between groups.

The number of spots on the leaves of both *B. napus* cv. Westar infected and *B. napus* cv. Westar ($ACCD^+$) seedlings infected with *P. aeruginosa* PA14 increased throughout the infection (Figure 3.26). There were no black spots observed on both untreated and treated (AVG) canola seedling leaves on days zero (two-hours post infection) and one (Figure 3.26). There were significantly more black spots on *B. napus* cv. Westar ($ACCD^+$) infected compared to *B. napus* cv. Westar seedlings by 3.9-fold and 0.4-fold on days three and five, respectively (Figure 3.26). This is likely due to the *B. napus* cv. Westar ($ACCD^+$) seedlings being healthier, making them more fit to fight off *P. aeruginosa* PA14.

Based on all parameters measured, canola seedlings with reduced ET levels are healthier compared to canola seedlings with normal levels of ET.

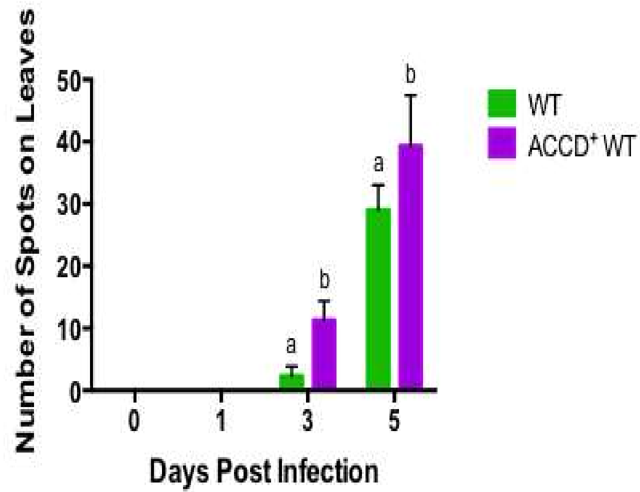

Figure 3.26: Number of black spots on *B. napus* cv. Westar and *B. napus* cv. Westar (ACCD$^+$) seedling's leaves during a five-day infection with *P. aeruginosa* PA14. The number of black spots on the leaves was recorded on days zero (two-hours post infection), one, three and five. Error bars represent standard deviation of three independent biological replicates. Statistical significance was measured at the $p \leq 0.05$ probability level; the same letters indicate non-statistically significant differences between groups, whereas different letters indicate statistically significant difference between groups.

CHAPTER 4. DISCUSSION

4.1 *Pseudomonas aeruginosa* is an Opportunistic Plant Pathogen

It has been known for quite a while now that *P. aeruginosa* can colonize and infect plant tissue (Clara, 1930). However, *P. aeruginosa* is predominantly studied mainly in the context of human infections. Characteristically, *P. aeruginosa* infections in plants are noted by soft stem rot in seedlings, as well as necrosis and spotting of the leaves (Plotnikova, Rahme & Ausubel, 2000). This phenotype has been observed in many plants, which include tomato, lettuce, onion and tobacco (Elrod & Braun, 1942; Burkholder, 1950; Kominos *et al.*, 1972). In this study, we also observed a similar phenotype in canola seedlings using a root infection model. Typically, in the past, *P. aeruginosa* infections in plants were studied using a leaf infiltration infection model (Rahme *et al*., 1995). Plants would be grown in growth chambers, and few days prior to the infection the humidity would be increased (>70% humidity) in order for the plants to open their stomata, then bacterial culture (10^3 cfu/cm^2 leaf area) would be infiltrated into the leaves using a blunt end syringe (Rahme *et al*., 1995). This is a widely-used infection model; however, like any good model it has limitations. First of all, it only allows us to study a localized infection within the leaves. Secondly, since *P. aeruginosa* is found ubiquitously within the environment, we believe that *P. aeruginosa* may naturally enter plants through wounds in their roots. Therefore, we established a novel root infection model for *P. aeruginosa* using canola seedlings.

A distinct feature about *P. aeruginosa* PA14's infection of *Arabidopsis* leaves is that the bacterial cells attach perpendicularly to plant mesophyll cell walls (Plotnikova, Rahme & Ausubel, 2000). In the study by Plotnikova, Rahme & Ausubel (2000), they showed that in a leaf infection model, *P. aeruginosa* PA14 moves rapidly towards stomatal

opening, and once inside the substomatal cavity, bacterial cells multiplied rapidly and spread throughout the leaf's mesophyll. It is also known that *P. aeruginosa* PA14 forms vast biofilms on the roots of *Arabidopsis* and sweet basil plants (Walker *et al.*, 2004). Interestingly, it has been shown that *P. aeruginosa* PA14 can also perforate holes in the cell walls of mesophyll cells in *Arabidopsis*. *Pseudomonas aeruginosa* PA14 secretes hydrolytic enzymes that allow it to digest the mesophyll cell walls, creating permanent holes within the plant (Plotnikova, Rahme & Ausubel, 2000). Therefore, *P. aeruginosa* PA14 can also generate its own entry into plant cells when necessary.

In our infection model, canola seedlings were germinated on MS agar for seven days prior to be transferred to a conical tube containing MS liquid media, and three days later the seedlings were infected with *P. aeruginosa* by adding diluted bacterial culture to the liquid MS media. The seedling roots were damaged prior to the infection with *P. aeruginosa*. We believe this represent a more natural infection, since *P. aeruginosa* gains access to the seedlings via their roots and can spread throughout the whole plant. In nature, plant roots can be damaged due to the roots rotting, pathogens and pests. We do acknowledge that ultimately a similar infection model in soil would be best; however, this would create limitations to our study. A soil infection model would have made it significantly more difficult to separate root and shoot tissue for RNA sequencing and RT-qPCR, and would have complicated our root metabolomics profile of canola seedlings.

We decided to damage the roots of canola seedlings prior to infection because it increased the severity of the symptoms of disease (Figure 3.1 A, B; Figure 3.3), while also shortening the duration of the infection. A similar infection model was employed by Walker *et al*. (2004), where they cut the tips of both *Arabidopsis* and sweet basil plants and then

infected these plants with either *P. aeruginosa* PA14 or PAO1 strains. However, they found no difference in severity of disease symptoms between plants with cut root tips and uncut root tips (Walker *et al*., 2004). It should be noted that symptoms of disease were simply examined visually, whereas for all of our experiments we quantified symptoms of disease visually (i.e. number of spots per leaves), as well as quantified it by measuring tissue weight. We observed a significant difference in symptoms between canola seedlings with damaged roots vs. undamaged roots (Figure 3.1 A, B; Figure 3.3).

Finally, since bacteria were added directly to the plant liquid media in our infection model, we wanted to ensure that it was the bacteria that successfully colonize the canola seedlings that caused the symptoms of disease. Therefore, we infected the canola seedlings by adding *P. aeruginosa* PA14 directly into the MS media, and two hours post infection we changed the MS media (containing bacteria) for new sterile media. However, we observed similar progression in symptoms of disease (tissue weight loss and leaf spotting) compared to not changing the MS media (Appendix A). Therefore, we concluded that the symptoms of disease in canola seedling were caused by *P. aeruginosa* PA14 that successfully colonized the plants tissues vs. planktonic bacteria in the MS media.

Our metabolomic profile of the contents of the MS media containing infected canola seedlings revealed the production of many of *P. aeruginosa* PA14 virulence factors. These virulence factors are QS molecules: N-butanoyl-homoserine lactone (Rhl; Kay *et al.*, 2006), 2-heptyl-4-quinolone (Pqs; Diggle *et al.*, 2007) and N-oxo-2-heptyl-4-hydroxyquinoline (Pqs; Déziel *et al.*, 2004). Interestingly, the amount of QS molecules detected is increasing over the duration of the infection, suggesting both an increase in bacterial number and/or an increase in virulence factor production (Table 3.6). Other virulence factors detected are:

pyocyanin, rhamnolipid RL1, coronatine (COR) (discussed below), 1-hydroxyphenazine and 1-phenazinecarboxamide. Similarly, the amount of virulence factors is also increasing over the duration of the infection (Table 3.6). Pyocyanin production inhibits primary root growth and promotes lateral root and root hair formation (Ortiz-Castro *et al.*, 2014). Interestingly, Ortiz-Castro *et al.* (2014) found that pyocyanin modulation of root growth was likely independent of auxin, CK and ABA but required ET production. *Arabidopsis* mutants lacking *etr1-1*, *ein2-1* and *ein3-1* were less sensitive to pyocyanin-induce root modulation. Rhamnolipids are involved in bacterial surface movement as well as biofilm formation (Sanchez *et al*., 2012); however, have been shown to have antifungal properties, such as inhibiting spore germination and mycelium growth of *Botrytis cinerea* (Varnier *et al.*, 2009). Both 1-hydroxyphenazine and 1-phenazinecarboxamide have been shown to be important biocontrol agents, which limit fungal growth (Kerr *et al.*, 1999; Chen *et al*., 2015).

Altogether, our data suggest that *P. aeruginosa* PA14 is a highly successfully opportunistic pathogen of canola seedlings. Many other studies have also confirmed that *P. aeruginosa* PA14 is an opportunistic plant pathogen (Rahme *et al*., 1995; Plotnikova *et al.*, 2000; Djonović *et al*., 2013; Cheng *et al.*, 2015). For example, *P. aeruginosa* PA14 was found to be highly pathogenic in both lettuce and *Arabidopsis* leaves (Rahme *et al.*, 1997). *Pseudomonas aeruginosa* PA14 genome encodes two pathogenicity island (PAPI-1 and PAPI-2) that encode homologous genes involved in pathogenicity in two well characterized plant pathogens, Pst DC3000 and *Xanthomonas axonopodis* pv. *citri* 306 (He *et al.*, 2004), thus providing more support for *P. aeruginosa*'s role as a plant pathogen. A study by Green *et al*. (1974) found that *P. aeruginosa* could be isolated from the leaves of both tomato and

celery plants, and could also be isolated from soil near roots of tomato, celery, corn and cotton. Interestingly, some of the soil isolates were also resistant to carbenicillin. Therefore, they suggested that agricultural plants act as a reservoir for the opportunistic human pathogen *P. aeruginosa* (Green *et al*., 1974).

4.2 Elimination of QS in *P. aeruginosa* Significantly Decreases its Virulence in Canola Seedlings

Many Gram-negative bacteria rely on QS to communicate with one another (Whitehead *et al.,* 2001). *Pseudomonas aeruginosa* not only relies on QS for cell-to-cell communication but the three QS systems (Las, Rhl and Pqs) also regulate the expression of many virulence genes (Nadal Jimenez *et al.,* 2012). Due to the hierarchical regulation of the Las and Rhl QS systems, there is a lot of overlap in regulation of gene expression between the two systems. The Las and Rhl QS systems regulate the expression of LasA and LasB elastases, exotoxin A, alkaline protease, as well as the expression of both the T2SS and T3SS systems (Gambello, Kaye & Iglewski, 1993; Jones *et al.*, 1993; Passador *et al.*, 1993; Bleves et al., 2005). The Las QS system also hierarchically regulates the expression of the Pqs QS system, through the transcriptional regulator MvfR (Hentzer *et al.,* 2003; Schuster *et al.,* 2003). The Pqs QS system regulates the expression of pyocyanin and hydrogen cyanide production (Rampioni *et al.*, 2007). Therefore, we hypothesized that deletion of regulatory genes within the QS system in *P. aeruginosa* PA14 would decrease its virulence in our canola seedling infection model.

Interestingly, in our study, there was only a significant difference in canola seedling shoot weight loss between *P. aeruginosa* PA14 WT and Δ*lasR*/*rhlR*, Δ*lasR*/*mvfR* and

Δ*lasR*/*rhlR*/*mvfR* five days post infection (Figure 3.6 D). However, there was a significant difference in the number of spots on the leaves of canola seedlings between *P. aeruginosa* PA14 WT and all of the QS mutants on days three and five post infection (Figure 3.8 A, B). A similar study by Walker *et al*. (2004), showed that *Arabidopsis* plants were susceptible to all *P. aeruginosa* PAO1 QS mutants tested (Δ*lasI*, Δ*rhlI* and Δ*lasI*/*rhlI*) and succumbed to the infection seven days post infection.

Both weight loss in canola seedling tissues and the spotting on the leaves are symptoms of disease in plants. Interestingly, in all measurements deletion of *lasR* (alone, and especially in double or triple mutants) is playing a key role in reducing symptoms of disease in canola seedlings, which is not surprising since it regulates the expression of many key virulence factors in *P. aeruginosa* (Nadal Jimenez *et al.,* 2012). LasR is also the hierarchical regulator of the other two QS system (Rhl and Pqs) (Hentzer *et al.,* 2003; Schuster *et al.,* 2003). Many studies using nematodes and mice models have also shown that deletion of *lasR* reduces the virulence of *P. aeruginosa* (Tan *et al*., 1999; Gallagher *et al*., 2002; Evans, Kawli & Tan, 2008; Feinbaum *et al*., 2012).

Many other studies have also demonstrated the importance of the QS system in *P. aeruginosa* towards virulence in other host. Tan *et al*. (1999) reported that deletion of *lasR* in *P. aeruginosa* PA14 lead to less than 30% killing in *Caenorhabditis elegans* after 90 hours, whereas *P. aeruginosa* PA14 WT killed 50% of *C. elegans* in less than 40 hours. Similarly, deletion of *lasR* in *P. aeruginosa* PA14 lead to 50% mouse mortality compared 100% mortality in mice infected with *P. aeruginosa* PA14 WT (Tan *et al*., 1999). Deletion of *lasI* in *P. aeruginosa* PAO1 led to a decrease in virulence (>10% mortality in mice) compared to *P. aeruginosa* PAO1 WT (21% mortality in mice) (Pearson *et al*., 2000).

Similarly, a study by Mukherjee *et al*. (2017) found that deletion of *rhlR* in *P. aeruginosa* PA14 lead the bacteria to be avirulent in both mice and nematode infection models. However, deletion of *rhlI* in *P. aeruginosa* PA14 had no effect on bacterial virulence in mice and nematode infection models. MvfR also plays a key role in *P. aeruginosa* PA14 virulence, Xiao *et al.* (2006) found that bacterial cells lacking MvfR showed reduced virulence in a mouse infection model.

In regard to colonization of the canola seedlings roots, there was no significant difference between *P. aeruginosa* PA14 WT and the QS mutants on days zero (two-hours post infection), three and five post infection (Figure 3.6 A, C, D). There significantly more *P. aeruginosa* PA14 WT colonizing canola seedling roots compared to Δ*lasI*, Δ*lasR*, Δ*lasR*/*mvfR*, Δ*rhlR*/*mvfR* and Δ*lasR*/*rhlR*/*mvfR* on day one post infection (Figure 3.6 B). We expected there to be more significant differences between colonization of *P. aeruginosa* PA14 WT and the QS mutants, therefore this was a bit surprising, but is likely due to the high variability of CFU measured between biological replicates. However, this adds support to reduced virulence between *P. aeruginosa* PA14 WT and Δ*lasR*/*rhlR*, Δ*lasR*/*mvfR* and Δ*lasR*/*rhlR*/*mvfR* since it cannot be due to reduced colonization by the QS mutants. Similarly, there was no significant difference in CFU within the leaves of infected canola seedlings on days zero and five post infection when comparing *P. aeruginosa* PA14 WT and any of the single, double or triple QS mutants (Figure 3.7 A, D). However, theres significantly more *P. aeruginosa* PA14 WT colonizing canola seedling leaves compared to Δ*lasI*, Δ*rhlI,* Δ*rhlR*, Δ*lasR*/*rhlR*, Δ*lasR*/*mvfR*, Δ*rhlR*/*mvfR* and Δ*lasR*/*rhlR*/*mvfR* on days one and three post infection (Figure 3.7 B, C). Interestingly, a study by Gopalan and Ausubel (2011), showed that *Arabidopsis* seedling's leaves infected with *P. aeruginosa*

PA14Δ*lasR* had a higher bacterial load compared to seedlings treated with *P. aeruginosa* WT measured using a luciferase-based assay. However, the study by Walker *et al*. (2004), showed that there was a reduction in colonization and biofilm formation between *P. aeruginosa* PAO1 and the QS mutants tested (Δ*lasI*, Δ*rhlI* and Δ*lasI*/*rhlI*). Therefore, it seems that there is quite a bit of variation between mutant colonization of plant tissues. We think that the differences between our study and previously published work by Walker *et al*. (2004) could be due to differences in the strain of *P. aeruginosa* and also differences in host plant.

4.3 Elimination of the GAC Two-Component Regulatory System, T2SS, T3SS and T6SS in *P. aeruginosa* does not Decreases its Virulence in Canola Seedlings

The GAC two-component regulatory system is highly important in virulence because it regulates the expression of numerous pathogenic weapons within *P. aeruginosa*. These pathogenic weapons include many of *P. aeruignosa*'s secretion systems (T2SS, T3SS and T6SS) (Ventre *et al.*, 2006). The GAC system also regulates the expression of QS systems (LasR and RhlR) in *P. aeruginosa*, which as previously mentioned also regulates the expression of multiple virulence factors (Reimman *et al.*, 1997). Therefore, we hypothesized that deletion of *gacA* in *P. aeruginosa* PA14 would decrease its virulence in our canola seedling infection model. Since GacA regulates the expression of T2SS, T3SS and T6SS in *P. aeruginosa*, we also tested knockouts of these secretion systems for reduced virulence in canola seedlings.

All of the *P. aeruginosa* PA14 mutants (*gacA*::tn, *xcpR*::tn, Δ*pscD*, ΔHSI-II and ΔHSI-III) caused significant root weight loss on days three and five compared to the

uninfected control in canola seedlings (Appendix B). However, when comparing root weight of the infected seedlings there was no significant difference in weight loss between seedlings infected with *P. aeruginosa* PA14 WT and mutants (*gacA*::tn, *xcpR*::tn, Δ*pscD*, ΔHSI-II and ΔHSI-III) (Appendix B). Similarly, all of the *P. aeruginosa* PA14 mutants (*gacA*::tn, *xcpR*::tn, Δ*pscD*, ΔHSI-II and ΔHSI-III) caused significant shoot weight loss on days three and five compared to the uninfected control in canola seedlings (Appendix B). However, when comparing shoot weight of the infected seedlings there was no significant difference in weight loss between seedlings infected with *P. aeruginosa* PA14 WT and mutants (*gacA*::tn, *xcpR*::tn, Δ*pscD*, ΔHSI-II and ΔHSI-III) (Appendix B). There was a significant difference in the number of spots on the leaves of canola seedlings between *P. aeruginosa* PA14 WT and all of the secretion system mutants (*xcpR*::tn, Δ*pscD*, ΔHSI-II and ΔHSI-III) five days post infection (Appendix B). There was no significant difference in tissue (root or leaves) colonization between seedlings infected with *P. aeruginosa* PA14 WT and mutants (*gacA*::tn, *xcpR*::tn, Δ*pscD*, ΔHSI-II and ΔHSI-III) on any days post infection (Appendix B).

Altogether these results suggest that the deletion of the regulator (Δ*gacA*) of the GAC two-component regulatory system, and the single deletion of the secretion systems do not drastically affect *P. aeruginosa* PA14 virulence towards canola seedlings. This finding was not terribly surprising due to the wealth of pathogenic weapons *P. aeruginosa* PA14's genome encodes. A study by Gopalan and Ausubel (2011) demonstrated that GacA in *P. aeruginosa* PA14 is not required for virulence in *Arabidopsis* leaves. However, earlier studies have suggested that GacA is important for *P. aeruginosa* PA14 virulence in *Arabidopsis* and mice (Rahme *et al.*, 1995; Rahme *et al.*, 1997). Similarly, to our study, the

T3SS (Δ*pscD*) was found to not be essential for virulence in *C. elegans* and *Arabidopsis* model systems (Miyata *et al.*, 2003). A study by Jyot *et al*. (2011), found that the T2SS (Δ*xcpQ*) in *P. aeruginosa* PAK was not essential for virulence in WT mice. However, a double mutant lacking both the T2SS (Δ*xcpQ*) and T3SS (Δ*pscF*) was avirulent in WT mice. Interestingly, the T6SS loci in *P. aeruginosa* PA14 were also implicated in virulence in plant (*Arabidopsis*) infections, and lung and burn infections in mice. *P. aeruginosa* PA14 with knockouts in T6SS loci had decreased virulence in the previously mentioned infection models (Lesic *et al*., 2009). Differences between our study and previously published work by Rahme *et al.* (1995) could be explained by different infection models, we used a root infection model, whereas they used a leaf infiltration system. Rahme *et al.* (1995), also used a different host plant (*Arabidopsis*), whereas we used canola.

4.4 Transcriptomic and Metabolomic Changes in Canola Seedlings during an Infection with *P. aeruginosa* PA14

Profiling the transcriptome of canola seedlings during an infection with *P. aeruginosa* PA14 gave us many insights into how plants can fight off bacterial infections. We employed both RNA sequencing and RT-qPCR to study transcriptomic changes within canola seedlings during a *P. aeruginosa* PA14 infection. Extracting RNA on days one, three and five allowed us to study transcriptomic responses over a broad range of time points, representing early, middle and late stages of an infection (Figure 3.9). Similarly, separating plant tissue (root and shoot) allows us to look at tissue specific responses in canola seedlings during a *P. aeruginosa* PA14 infection (Figure 3.9).

As previously mentioned, studying transcriptomic responses in a non-model organism has its own challenges. Even though canola's genome has been sequenced, many genes are lacking functional annotation. Therefore, we had to map our transcriptomic reads from canola to *A. thaliana*, a closely related model organism (Table 3.1). This proved to be challenging since canola arose from the hybridization of *B. rapa* and *B. oleracea* and then its genome doubled, making canola allopolyploid. Canola's genome is about ten times larger compared to *A. thaliana* (1,130 Mbp vs. 135 Mbp). Canola's genome encodes about three times more genes compared to *A. thaliana* (101,040 vs. 27,655). Also, canola's genome can encode multiple copies of a gene found as a single copy within *A. thaliana*'s genome. We found that there were 4,275 genes with a log_2fold change greater than 2, which is indicative that these genes are either being highly up regulated or strongly down regulated (Figure 3.9). There was a greater number of genes differentially expressed in root tissue compared to shoot tissue during canola's infection with *P. aeruginosa* PA14 (Figure 3.10). This is not surprising since in our infection model, the canola's roots are the primary contact location for *P. aeruginosa* PA14. Similarly, a study looking at transcriptional response of tomato plants towards the tomato spotted wilt virus revealed a greater number of differentially expressed genes in the leaves of tomato plants, which was the primary site of infection (Catoni *et al*., 2009).

To gain insight into the functionality of genes responsive to *P. aeruginosa* PA14, we performed GO term enrichment analysis using agriGO (Zhou *et al.*, 2010). As expected genes involved in canola's innate immune response, secondary metabolism and death were significantly upregulated throughout the five-day infection (specifically on day three in root tissue, and day five in shoot tissue) with *P. aeruginosa* PA14 (Figure 3.11; Figure 3.12;

Figure 3.13; Figure 3.14; Figure 3.15). Whereas genes involved in primary metabolism and plant growth were significantly downregulated throughout the five-day infection (specifically on day one in root tissue, and day 5 in shoot tissue) with *P. aeruginosa* PA14 (Figure 3.11; Figure 3.16; Figure 3.17).

In order to confirm our RNA sequencing results, we chose genes that were highly induced [*WRKY75* (root and shoot), *CYP72A15* (root and shoot), *ACS7* (shoot), *ACO12* (root and shoot) and *AT1G77530* (shoot)] and genes that were repressed [*GA2OX1* (root and shoot), *ACS7* (root) and *AT1G77530* (root)] and verified their expression using RT-qPCR. The induced expression of *WRKY75, CYP72A15* and *ACO12* in both root and shoot tissue were confirmed using RT-qPCR (Figure 3.18 A, B; Figure 3.19 B). The expression of *ACS7*, *GA2OX1* and *AT1G77530* in shoot tissue was also confirmed using RT-qPCR (Figure 3.19 A; Figure 3.20 A, B). However, the expression of *ACS7, GA2OX1* and *AT1G77530* had variable results when comparing RNA sequencing and RT-qPCR. *ACS7* relative expression measured using RNA sequencing was down regulated in the shoot, whereas the RT-qPCR showed expression was upregulated in both tissue types (Figure 3.20 A). Similarly, the relative expression of *GA2OX1* was down regulated in the RNA sequencing for both tissue types, whereas the RT-qPCR showed that expression was up regulated in the root tissue (Figure 3.21 A). Finally, the expression of *AT1G77530* was strongly down regulated in the RNA sequencing, whereas the RT-qPCR showed that expression for the most part was up regulated (Figure 3.21 B). Some possible explanations for this could be: RNA sequencing biases may have occurred when mapping genes from canola to *Arabidopsis* occurred since canola has multiple copies of some genes found as a single copy in *Arabidopsis*. Perhaps not all copies of the gene measured were up or down

regulated at the same time. Similarly, for RT-qPCR, the primers used may not have been specific enough to measure the same gene variant detected using RNA sequencing. Therefore, qPCR products should be sent for Sanger sequencing to ensure the proper variant of the gene of interest was amplified.

4.4.1 Upstream Signalling Events in Canola Seedlings during an Infection with *P. aeruginosa*

A number of genes responsive to plant pathogens during the early stages of infection were identified in our study. For instance, *MEKK1*, a MAPKKK involved in the MAPK phosphorylation cascade was strongly upregulated (Asai *et al.*, 2002), as well as many *WRKY* transcription factors (*WRKY6/15/18/25/33/48/75*), which are known to be responsible for salicylic acid production (Phukan, Jeena & Shukla, 2016). We also identified many oxidase (e.g. *AOX1A*) and peroxidases (e.g. *PRXCB*) that generate ROS, which can act as both a signalling molecule as well as having antimicrobial properties (Bailey-Serres & Mittler, 2006). All are key players towards the initiation of an early innate immune response in plants (Jones & Dangl, 2006).

4.4.2 *Pseudomonas aeruginosa* Infection of Canola Seedlings Up-Regulates the Production of SA, JA, ET, ABA, Auxin, BR and CK, and Down-Regulates the Production of GA

We identified a number of genes responsible for the production of many phytohormones in plants. There was an increase in expression in canola seedling roots for genes involved in the signalling pathways for the three main phytohormones involved in

plant innate immunity: SA (*NHL25* and *SYP121*), JA (*ANAC055*, *SYP121*, *RCD1* and *JAZ1*) and ET (*EIL3*, *EIN3*, *ERF1*, *RCD1* and *MBF1C*). We also observed an increase in expression for genes involved in the signalling pathways of other phytohormones shown to play a role in plant innate immunity: auxin (e.g. *VIK*), ABA (*ATRBOH F*, *CBL1* and *ATCDPK1*), BR (*BSK1*, *BAK1*, *VIK*, *ARL*) and CK (*ARR1*). However, we only observed increases of expression in canola seedling shoots in the phytohormones: SA (*NHL25* and *SYP121*, *WRKY38/62*), JA (*ANAC055* and *SYP121*), ET (*MBF1C*, *ORA59* and *ERF2*), ABA (*LECRKA42*, *ATCDPK1*, *AFP1*, *CPK6*, *LTI65* and *ATHB-7*) and CK (*AHP1*).

We also identified the production and secretion of phytohormones by canola seedlings during an infection with *P. aeruginosa* PA14 using mass spectrometry. We identified the following phytohormones in the MS media of infected plants: methyl salicylate (SA), JA, cucurbic acid (JA derivative), indole-derivatives (auxin) and ACC (ET precursor molecule) (Table 3.3). A study by Schmelz *et al*. (2003) found that infection of *Arabidopsis* plants by the phytopathogen Pst DC3000 led to increased production of the phytohormones salicylic acid, jasmonic acid, abscisic acid and brassinosteroids.

SA is an important phytohormone involved in the defence response against biotrophs and hemi-biotrophs (Bari & Jones, 2009; Groen *et al.*, 2013). SA production is required for both localized defense response and SAR (Zhang *et al.*, 2010). ET is an important phytohormone known to play a role in defenses towards necrotrophic fungi (Diaz, ten Have & van Kan, 2002). ET production is required for ISR (Pieterse *et al.*, 1998), and ISR was abolished in ET-insensitive mutants (*ETR1-1, EIN2* and *EIN7*) in *Arabidopsis* (Knoester *et al*., 1999). Nandi *et al.* (2003) found that ET and JA signalling affects the expression of defenses genes (e.g. *PDF1.2*) in *Arabidopsis*. It is known that SA and ET

production act as positive regulators of cell death propagation in plants (O'Donnel *et al.*, 2001; Bouchez *et al.*, 2007). *Pseudomonas aeruginosa* PA14 is inducing the production of JA and ET (antagonist of SA), which are known to supress SA-dependent defense responses (Pieterse *et al.*, 2009).

Interestingly, we identified the production of COR in the MS media of infected plants (Table 3.7). COR is a well-studied phytotoxin in Pst DC3000, which mimics the plant hormone JA-isoleucine and promotes opening of stomata for bacterial entry, bacterial growth in the apoplast, systemic susceptibility, and disease symptoms (Schmels *et al.*, 2003; Cui *et al.*, 2005 Zheng *et al.*, 2012). Therefore, we believe that *P. aeruginosa* PA14 is producing COR in order to manipulate canola seedlings innate immune response, specifically suppress MAMP-induced callose deposition in the cell walls of roots, which was found to occur in *P. syringae* (Millet *et al.*, 2010; Geng *et al*., 2014).

The production of SA and auxins are known to act both as signalling molecules and antimicrobial compounds. A study by Prithiviraj *et al.* (2005) showed that the production of SA or its derivatives (methyl SA, acetyl SA and salicylamide) and benzoic acid (precursor of SA) decreases the production of virulence factors including pyocyanin, total protease and elastase in *P. aeruginosa* PA14. Elevated concentrations of SA also significantly affected *P. aeruginosa* PA14 virulence in *Arabidopsis*. Indole derivatives [3-indolylacetonitrile (IAN), and indole-3-carboxyaldehyde] inhibited biofilm formation, and IAN also decreased the production of virulence factors including PQS, pyocyanin and pyoverdine in *P. aeruginosa* PAO1 (Lee, Cho & Lee, 2011). Indole and 7 hydroxyindole also decreased the production of virulence factors including pyocyanin, rhamnolipid, PQS and

pyoverdine in *P. aeruginosa* PAO1 (Lee *et al.*, 2009). Both studies indicated that indole derivatives could successfully inhibit *P. aeruginosa* virulence. Studies have also indicated that many *P. syrinage* pathovars can produce IAA in the presence of tryptophan (Gardan *et al.*, 1992), which may decrease the plants defense response toward invading pathogens (Glickmann *et al.*, 1998). Therefore, the production of auxins (indole-3-acetic acid, indole-3-acetaldehyde, indole-3-carboxilic acid, indole-3-acetic acid methyl ester, indolyl-3-methanol and indole-3-carboxylic acid methyl ester) is likely an antimicrobial response by the plant (Table 3.2; Table 3.3; Table 3.5). However, a study by Chen *et al.* (2007) showed that AvrRpt2 in Pst DC3000 increased the free levels of IAA, which promoted bacterial virulence and disease symptoms in *Arabidopsis*. Altogether, the production of auxins by canola seedlings is likely in response to *P. aeruginosa* PA14 and could be either antimicrobial or perhaps *P. aeruginosa* PA14 is disrupting phytohormone signalling, leading to a reduced immune response.

4.4.3 ***Pseudomonas aeruginosa*** **Infection of Canola Seedlings Increase the Production of the Amino Acids: Valine, Isoleucine, Tryptophan and Phenylalanine**

We identified the production of amino acids by canola seedlings during *P. aeruginosa* PA14 infection of canola seedlings using mass spectrometry. The amino acids identified were valine, isoleucine, tryptophan and phenylalanine, and their levels were increasing over the course of the infection (Table 3.4). There was also an up regulation in the genes involved in tryptophan (*TSB1*) and phenylalanine (*GP*

ALPHA 1) biosynthesis. Similarly, a study by Ward *et al.* (2010) showed an increase in levels valine, leucine, isoleucine, threonine, alanine, phenylalanine, tyrosine and glutamine in *A. thaliana* following infection with Pst DC3000. We believe that these amino acids are being synthesized by canola seedlings to make JA and auxin derivatives (e.g. jasmonate-isoleucine and IAA) (Westfall, Muehler & Jez, 2013), or as precursor molecules for antimicrobial compounds. Glucosinolates are defense chemicals produced by plants which can be stored in plant tissues or mobilized to pathogen challenge sites, which get broken down into biologically active compounds by plant myrosinases (Bednarek & Osbourn, 2009). Glucosinolates can be derived from many amino acids including, isoleucine, valine, tryptophan and phenylalanine, which allows this class of antimicrobial compounds to have great structural diversity (Fahey, Zalcmann & Talalay, 2001; Agerbirk & Olsen, 2012; Piasecka, Jedrzejczak-Rey & Bednarek 2015). Cytochromes P450 catalyze the conversion of amino acids to aldoximes (Hull, Vij & Celenza, 2000; Mikkelsen *et al.*, 2000), which are then converted to glucosinolates through many biochemical steps (Dixon, 2001; Fahey, Zalcmann & Talalay, 2001).

4.4.4 *Pseudomonas aeruginosa* Infection of Canola Seedlings Increase the Production of Antimicrobial Compounds

We identified the production of many antimicrobial compounds in canola seedlings in response to infection with *P. aeruginosa* PA14. Some of these compounds were identified using a targeted approach based on well-known antimicrobial compounds or antimicrobial compounds whose expression was induced during RNA sequencing analysis

(Table 3.2). Levels of coumarin increased drastically over the course of infection. Consistent with this, a gene involved in coumarin biosynthesis (*CYP98A3*) was also up regulated in canola seedlings during the infection with *P. aeruginosa* PA14 (Table 3.2). A study by Cottiglia *et al.* (2001) found that the coumarin, daphnetin at 50 µg/mL, inhibited the growth of *P. aeruginosa* CA2.

During our mass spectrometry data analysis, we identified methoxy-indole-3-glucosinolate (I3G); however, it was only present at three days post infection (Table 3.2). We also observed a number of genes involved in glucosinolate biosynthesis (*SOT16*, *CYP83B1*, *SUR1* and *CSH1*) being up regulated in canola seedlings during the infection with *P. aeruginosa* PA14. Glucosinolates are not considered to be toxic; however, when broken down by myrosinases, the products of these reactions have been shown to be toxic to insects, fungi and bacteria (Brader, Tas & Palva, 2001; Shroff *et al.*, 2008; Bednarek *et al.*, 2009). Interestingly, a study by Clay *et al.* (2009) found that *PEN2* and *PEN3* are glucosinolate-activating enzymes, and 4-methoxy-I3G was required for callose deposition in *Arabidopsis*. Our study identified that genes involved in callose deposition (*CYP83B1*, *NSL1*, *ATRBOH F*, *CYP81F2*, *PEN3*, *GSHP* and *ATG5LOS*) were upregulated in canola seedlings during the infection with *P. aeruginosa* PA14. Callose serves as matrix in which antimicrobial compounds can be deposited and its deposition is an important immune response in plants (Luna *et al.*, 2011).

The majority of the antimicrobial compounds identified in this study were done using an untargeted approach (Table 3.5 and 3.6). Interestingly, the antimicrobial compound with the highest level on day one of the infection of canola seedlings with *P.*

aeruginosa was polyoxin B (Table 3.5). Polyoxins are a class of antimicrobial peptides that inhibit the enzyme chitin synthase, preventing the biosynthesis of chitin (Keymanesh, Soltani & Sardari, 2009). Bacterial cell walls are not composed of chitin; however, fungal cell walls and insect carcases are composed of chitin. A possible explanation for this would be early on during plant infections; plants produce a broad (non-specific) range of antimicrobial compounds to limit the infection (Chisholm *et al*., 2006).

We also identified a number of phytoalexins being produced by canola seedlings during the infection with *P. aeruginosa* PA14. Isalexin and spirobrassinin levels were high throughout the infection (Table 3.6). Isalexin was shown to inhibit the growth of 48% *Phoma lingam* at a concentration of 0.5 mM (Pedras, Montaut & Suchy, 2004). Whereas, spirobrassinin only weakly inhibited the growth of *Bipolaris leersiae* at a concentration of 1 mM. Camalexin was identified; however, it was only present in 2 samples at five days post infection (Table 3.2). Camalexin is an antimicrobial compound that damages the cell wall of invading pathogens (Nafisi *et al.*, 2007). *WRKY33* a gene involved in camalexin biosynthesis was also up regulated in canola seedlings infected with *P. aeruginosa* PA14. Moreover, we identified 1-methoxy-indole-3-carboxylic acid and 1-acetylindole-3-carboxaldehyde at high levels, which is a precursor molecule for camalexin biosynthesis (Böttcher *et al.*, 2014), throughout the infection (Table 3.5).

4.5 *Pseudomonas aeruginosa* Infection is Less Virulent in Canola Seedlings with Reduced Levels of ET

In our study we found that *P. aeruginosa* PA14 infection of canola seedlings led to up regulation in genes involved in ET production, as well as genes involved in ET's

signaling pathway. We also found that ACC, the precursor molecule for ET production was present within the media of infected seedlings, and ACC's levels were increasing throughout the infection. Altogether, our results suggest that ET is an important modulator of an immune response towards *P. aeruginosa* PA14. Therefore, we employed two strategies to limit the production of ET in canola seedlings: first we chemically blocked the ACC synthase (*ACS*) for ET's precursor molecule ACC using AVG; secondly, we used *B. napus* cv. Westar transformed with the ACC deaminase gene from *Pseudomonas spp.* UW4, which breaks down ACC. In both cases, infected canola seedlings with inhibited ET levels weighed significantly more and produced more spots on their leaves compared to uninhibited seedlings (Figure 3.21 A, B; Figure 3.24 A, B; Figure 3.23; Figure 3.26). Root colonization was similar between canola seedlings with inhibited and uninhibited ET levels (Figure 3.22 A; Figure 3.25 A). However, there were significantly fewer bacterial cells in the leaves of canola seedlings with inhibited ET levels compared to uninhibited seedlings (Figure 3.22 B; Figure 3.25 B). All of these results suggest that ET production in canola seedlings exacerbate symptoms of disease.

ET production in plants is thought to occur in two peaks; the first peak is thought to be responsible for initiation of genes that encode plant defense/protective proteins (Robison *et al.*, 2001a), whereas the second peak is detrimental to plant growth, and is involved in the initiation of senescence, chlorosis and leaf abscission (Pierik *et al.*, 2006; Van Loon *et al.*, 2006; Glick *et al.*, 2007). Therefore, alleviation of ET levels in plants by PGPB (expressing ACCD; Glick *et al.*, 2007) or in our case chemically (e.g. AVG; Yu & Yang, 1979), and using transgenic canola seedlings expressing ACCD (Sergeeva, Shah & Glick, 2006) reduce weight loss in canola seedlings. The study by Sergeeva, Shah and Glick

(2006) showed that *B. napus* cv. Westar transformed with ACCD from *Pseudomonas spp.* UW4 increased the plants tolerance up to 200 mM NaCl, suggesting that ACCD provides tolerance to abiotic stresses. Similarly, previous studies have shown that transgenic tomato plants expressing bacterial ACCD lowered ET levels, which extended the shelf life of tomatoes (Klee *et al*., 1991), and partially protected plants against growth inhibition by metals, plant pathogens and flooding (Grichko, Filby & Glick, 2000; Grichko & Glick, 2001; Robison *et al*., 2001b). Interestingly, it has been shown that reduced ET production in *Arabidopsis* and tomato decreased *P. syrinage* DC3000 virulence (Bent *et al.*, 1992; Lund, Stall & Klee, 1998). Cohn and Martin (2005) have shown that Pst DC3000 effectors AvrPto and AvrPtoB induced a set of host (tomato) genes involved in ET biosynthesis, and in particular regulated the expression of *LeACO1* and *LeACO2,* which encode ACC oxidase. Therefore, there is a clear link between Pst DC3000 virulence and ET production in hosts.

4.6 Future Research Directions

In our study, we identified that the QS system plays an important role in *P. aeruginosa* PA14 virulence in canola seedlings in a root infection model. We identified that double (Δ*lasR*/*rhlR* and Δ*lasR*/*mvfR*) and triple (Δ*lasR*/*rhlR*/*mvfR*) QS mutants had the most drastic decrease in virulence. We could quantify the expression of virulence factors regulated by the QS system in *P. aeruginosa* PA14 using RT-qPCR. Also, since we did not see a significant difference between *P. aeruginosa* PA14 WT and Δ*xcpR* or Δ*pscD*, we should test a double mutant (e.g. Δ*xcpR/pscD*) to both demonstrate their role in virulence

and to also confirm that when one secretion system is knocked out another system can compensate for it (Jyot *et al*., 2011).

In order to confirm our RNA sequencing results, we should pick a few more genes that were down regulated during RNA sequencing analysis, and confirm their expression using RT-qPCR.

We also identified a number of phytohormones derivatives and antimicrobial compound using LC/MS mass spectrometry. However, these compounds were identified based on accurate masses and have not been confirmed using corresponding standards. Therefore, an upcoming experiment would be to confirm the identity of our compounds of interest using pure standard chemicals. We also need to confirm that some of the phytohormones derivatives (e.g. IAA) are being produced by the canola seedlings and not *P. aeruginosa* PA14. It has been shown in the literature that two closely related *Pseudomonas spp.* (UW4 and GR12-2) are capable of synthesizing IAA from tryptophan (Saleh & Glick, 2001; Patten & Glick, 2002). Similarly, it has been shown that *P. syringae* pathovars are capable of synthesizing IAA (Glickman *et al.*, 1998), likely to compromise plant defense responses. Therefore, we should test whether *P. aeruginosa* PA14 can synthesize IAA. A quick BLAST search showed that *P. aeruginosa* PA14 is lacking the typical genes (*ipdc*, *iaaH* and *iaaM*) required for IAA synthesis; however, we will measure possible bacterial IAA production based on methods outlined in Patten and Glick (2002).

Finally, in order to confirm that ET production in canola seedlings make them more susceptible to *P. aeruginosa* PA14, we will measure the amount of ET being produced during an infection using gas chromatography (GC). We should also confirm that both

AVG treatment of canola seedlings and our transgenic canola seedlings expressing ACC deaminase (*B. napus* cv. Westar ACCD$^+$) leads to reduced levels of ET using GC.

4.7 Concluding Remarks

The primary goal of this thesis was to establish a novel root infection model for *P. aeruginosa* PA14 in canola seedlings. Using this infection model, we demonstrated that the deletion of *lasR* in *P. aeruginosa* PA14 was essential for pathogenesis in the root infection of canola seedlings. We also identified the production of COR by *P. aeruginosa* PA14. COR is a mimic of phytohormone JA and is produced by bona fide phytopathogens to manipulate plants' defense responses. We also showed that *P. aeruginosa* PA14 produced other metabolites, including virulence factors pyocyanin, rhamnolipid RL1, 1-hydroxyphenazine and 1-phenazinecarboxamide, as well as QS molecules N-butanoyl-homoserine lactone (Rhl; Kay *et al.*, 2006), 2-heptyl-4-quinolone (Pqs; Diggle *et al.*, 2007) and N-oxo-2-heptyl-4-hydroxyquinoline (Pqs; Déziel *et al.*, 2004), during the infection of canola seedlings.

Using this infection model, which allows us to easily separate root and shoot of infected plants and collect metabolites produced and secreted, we characterized canola's defense response towards *P. aeruginosa* PA14 both at the transcriptomic level using RNA sequencing as well as by investigating metabolite profiles using mass spectrometry. We showed an up regulation in both root and shoot of genes involved in the signaling pathways of primary phytohormones (SA, JA, ET and auxin) in plants. This is strongly supported by our metabolomic profiling of the MS media from infected plants that showed the increases

in methyl salicylate (SA), JA, cucurbic acid (JA derivative) ACC (ET) and auxin-derivatives.

Finally, we demonstrated the important role of one of the plant defense hormone ET in canola seedlings in response to *P. aeruginosa* pathogenesis. Many genes involved in ET biosynthesis and signalling pathway were up regulated in canola seedlings infected with *P. aeruginosa* PA14. When ET production was decreased both chemically and genetically we observed that infected canola seedling's root and shoot weighed more compared to seedlings with "normal" ET levels. Similarly, the leaves of infected canola seedlings with reduced ET levels had reduced bacterial load in their leaves compared to seedlings with "normal" ET levels. Therefore, low levels of ET are responsible for initiating genes involved in plant defense/protective proteins, whereas high ET levels are detrimental to plant growth.

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APPENDIX A: Changing MS Media Two-Hours Post-Infection does not Improve Canola's Health or Reduced Symptoms of Disease.

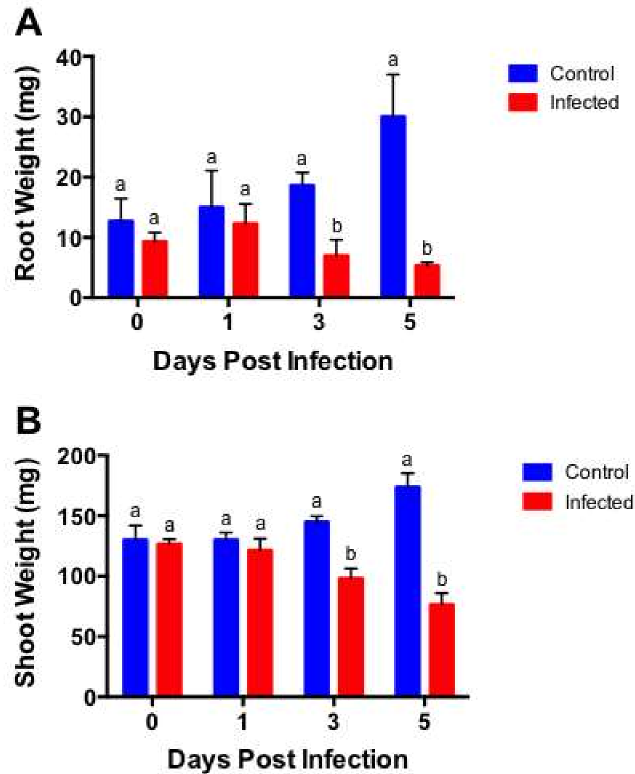

Figure A.1: Canola tissue weight recorded during a five-day infection with *P. aeruginosa* PA14, MS media was changed two hours post infection. A, Canola root weight measured on days zero (two-hours post infection), one, three and five. **B,** Canola shoot weight measured on days zero (two-hours post infection), one, three and five. Error bars represent standard deviation of three independent biological replicates. Statistical significance was measured at the $p \leq 0.05$ probability level; the same letters indicate non-statistically significant differences between groups, whereas different letters indicate statistically significant difference between groups.

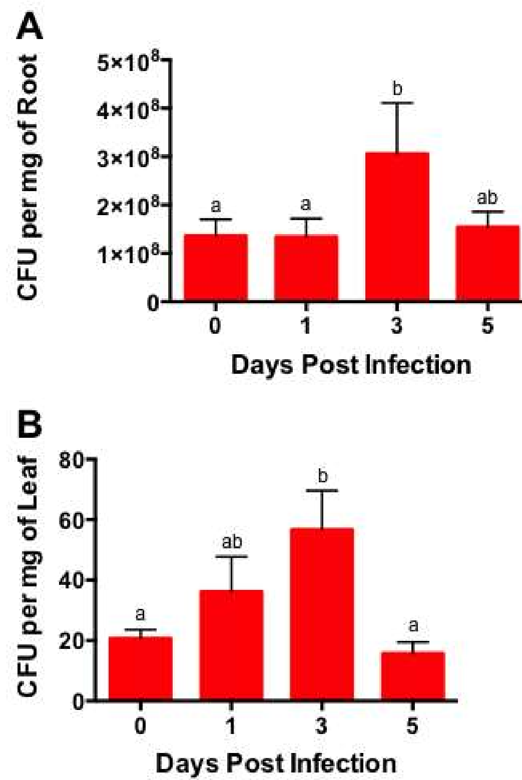

Figure A.2: *Pseudomonas aeruginosa* PA14 colony forming units (CFU) measured on canola roots and within canola leaves, MS media was changed two hours post infection. **A,** *Pseudomonas aeruginosa* PA14 root colonization measured on days zero (two-hours post infection), one, three and five. **B,** *Pseudomonas aeruginosa* PA14 leaf colonization measured on days zero (two-hours post infection), one, three and five. Error bars represent standard deviation of three independent biological replicates. Statistical significance was measured at the $p \leq 0.05$ probability level; the same letters indicate non-statistically significant differences between groups, whereas different letters indicate statistically significant difference between groups.

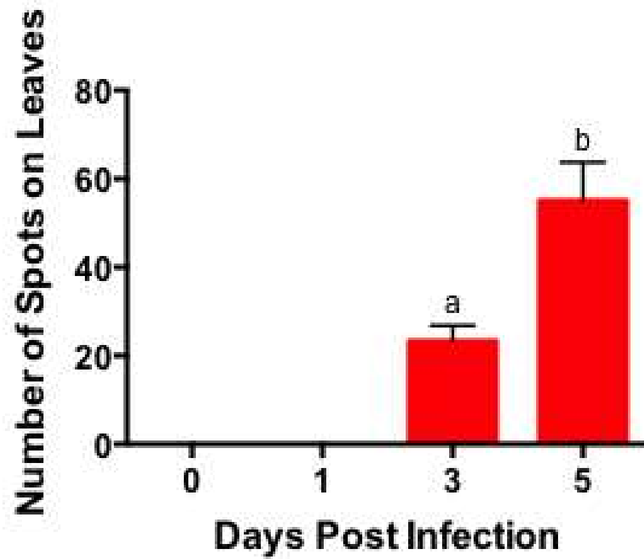

Figure A.3: Number of black spots on canola seedling's leaves during a five-day infection with *P. aeruginosa* PA14, MS media was changed two hours post infection. The number of black spots on the leaves was recorded on days zero (two-hours post infection), one, three and five. Error bars represent standard deviation of three independent biological replicates. Statistical significance was measured at the $p \leq 0.05$ probability level; the same letters indicate non-statistically significant differences between groups, whereas different letters indicate statistically significant difference between groups.

APPENDIX B: Elimination of the GAC Two-Component Regulatory System, T2SS, T3SS and T6SS in *P. aeruginosa* does not Decreases its Virulence in Canola Seedlings

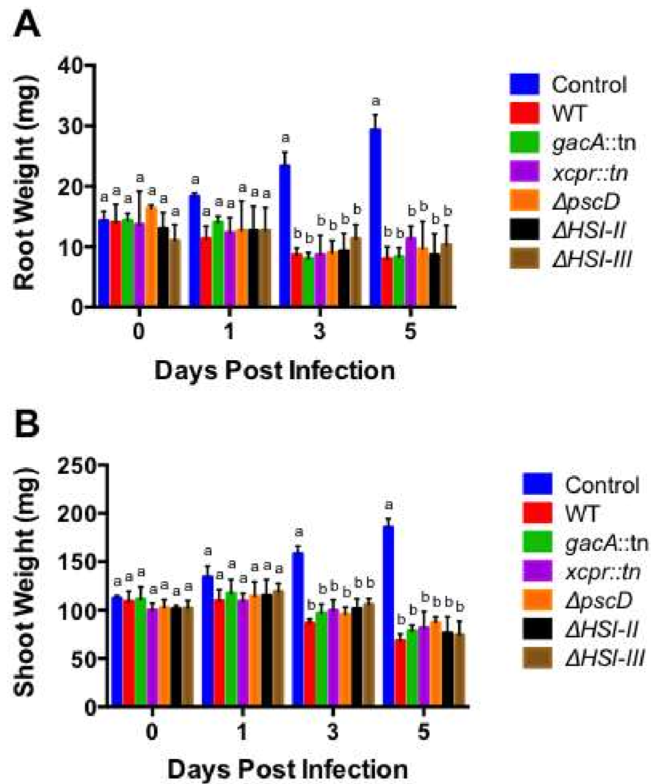

Figure B1: *Pseudomonas aeruginosa* PA14 wild type, *gacA::tn* or secretion system mutants tissue weight measured on canola roots during a five-day infection. **A,** *Pseudomonas aeruginosa* PA14 root weight measured on day zero (two-hours post infection), one, three and five. **B,** *Pseudomonas aeruginosa* PA14 shoot weight measured on day zero (two-hours post infection), one, three and five. Error bars represent standard deviation of three independent biological replicates. Statistical significance was measured at the $p \leq 0.05$ probability level; the same letters indicate non-statistically significant differences between groups, whereas different letters indicate statistically significant difference between groups.

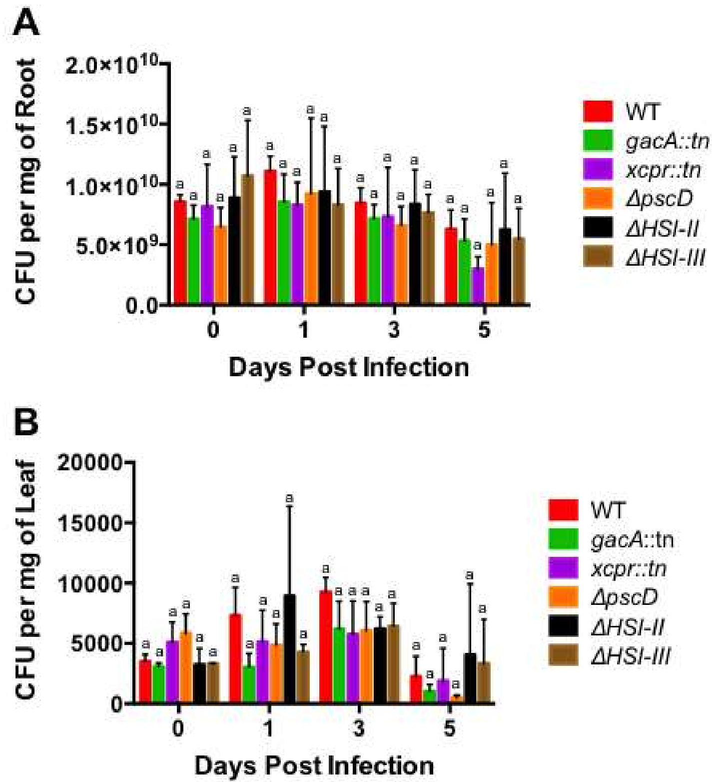

Figure B2: *Pseudomonas aeruginosa* PA14 wild type, *gacA::tn* or secretion system mutants colony forming units (CFU) measured on canola tissues during a five-day infection. **A,** *Pseudomonas aeruginosa* PA14 root colonization measured on day zero (two-hours post infection), one, three and five. **B,** *Pseudomonas aeruginosa* PA14 leaf colonization measured on day zero (two-hours post infection), one, three and five. Error bars represent standard deviation of three independent biological replicates. Statistical significance was measured at the $p \leq 0.05$ probability level; the same letters indicate non-statistically significant differences between groups, whereas different letters indicate statistically significant difference between groups.

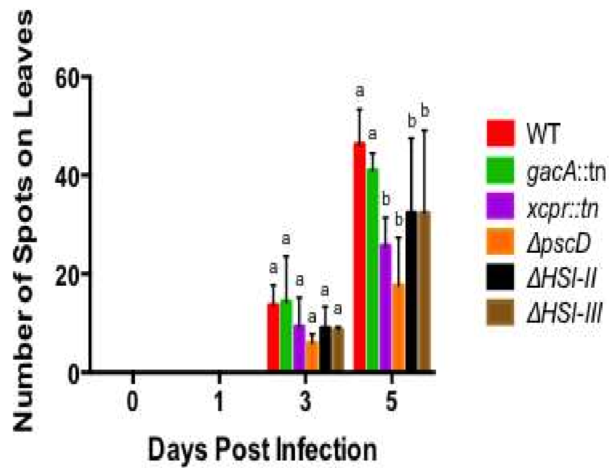

Figure B3: Number of black spots on canola seedling's leaves during a five-day infection with *P. aeruginosa* PA14 wild type, *gacA::tn* or secretion system mutants. Error bars represent standard deviation of three independent biological replicates. Statistical significance was measured at the $p \leq 0.05$ probability level; the same letters indicate non-statistically significant differences between groups, whereas different letters indicate statistically significant difference between groups.

APPENDIX C: High-Resolution LC/MS Traces Showing Biological Replicate Reproducibility

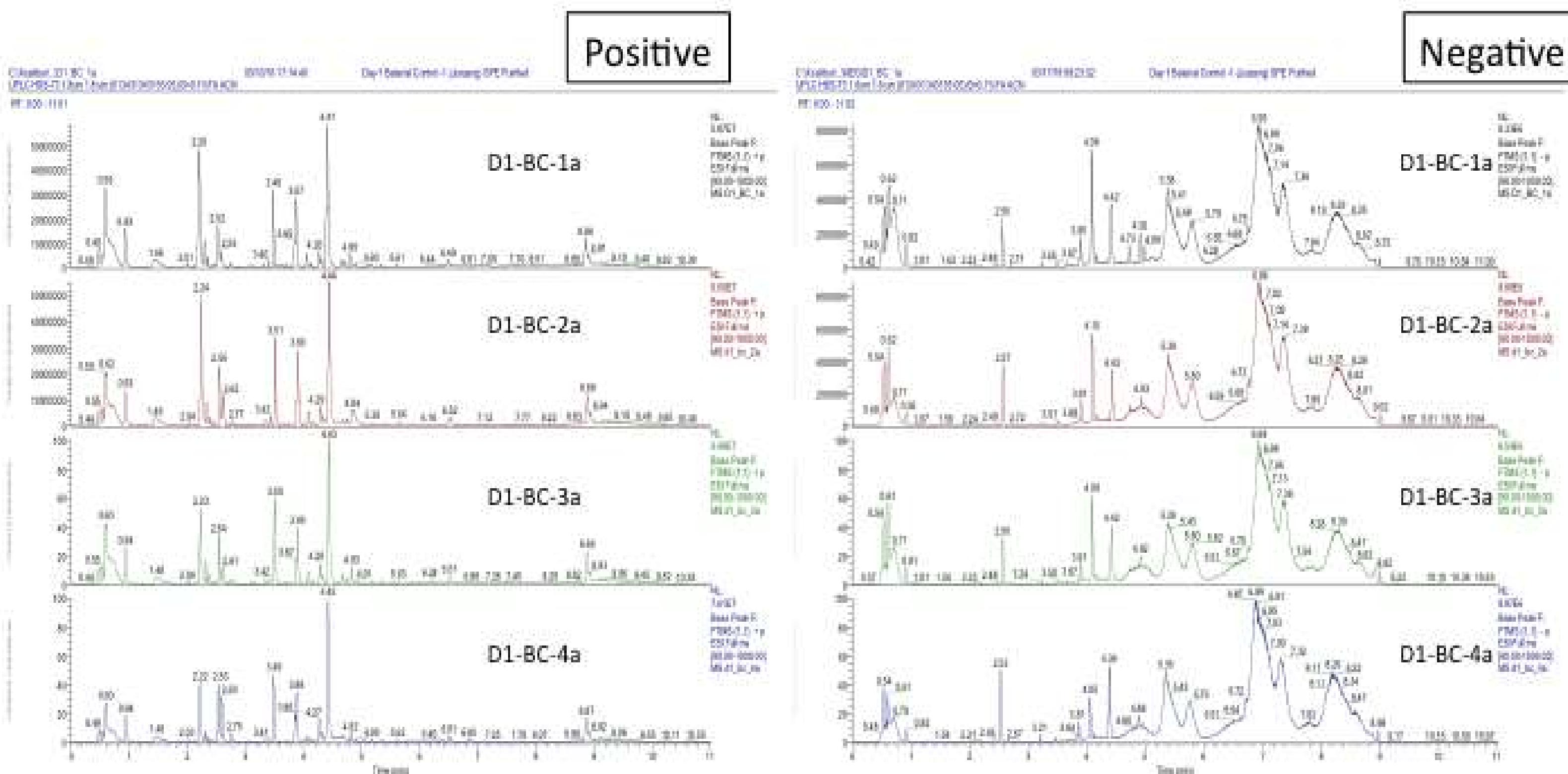

Figure C1: High-Resolution LC/MS traces for bacterial control (BC) samples taken on day one. *Pseudomonas aeruginosa* PA14 was grown in MS media for one day.

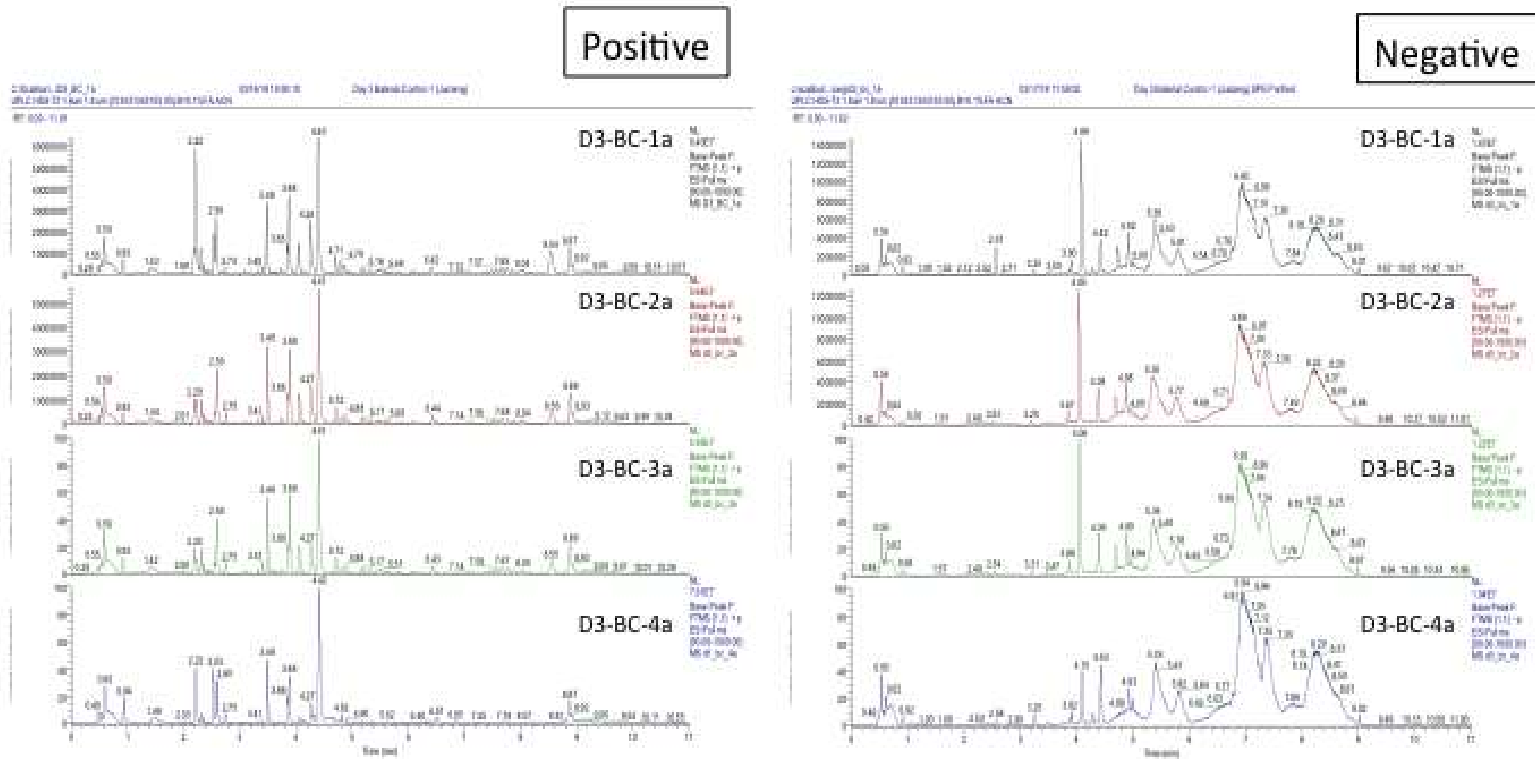

Figure C2: High-Resolution LC/MS traces for bacterial control (BC) samples taken on day three. *Pseudomonas aeruginosa* PA14 was grown in MS media for three days.

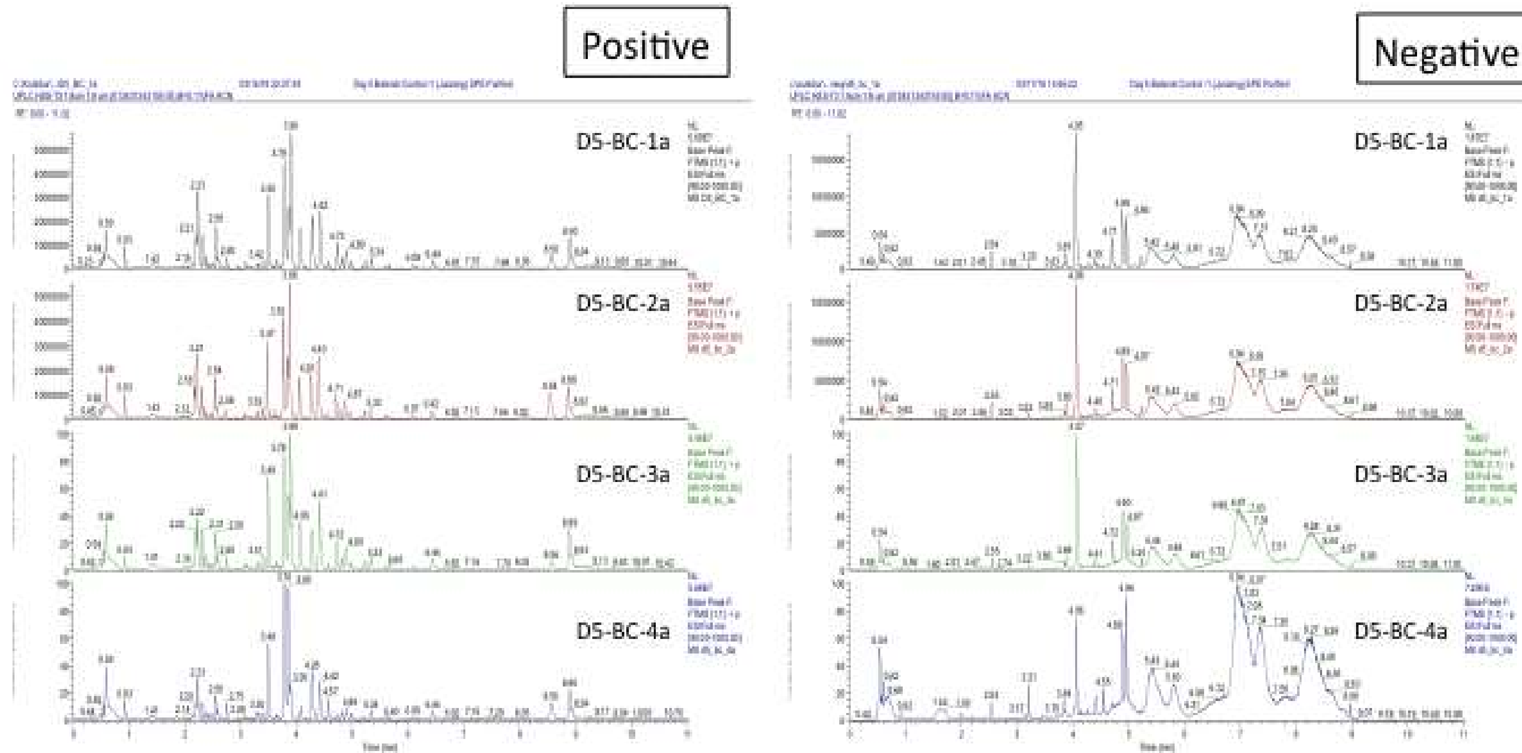

Figure C3: High-Resolution LC/MS traces for bacterial control (BC) samples taken on day five. *Pseudomonas aeruginosa* PA14 was grown in MS media for five days.

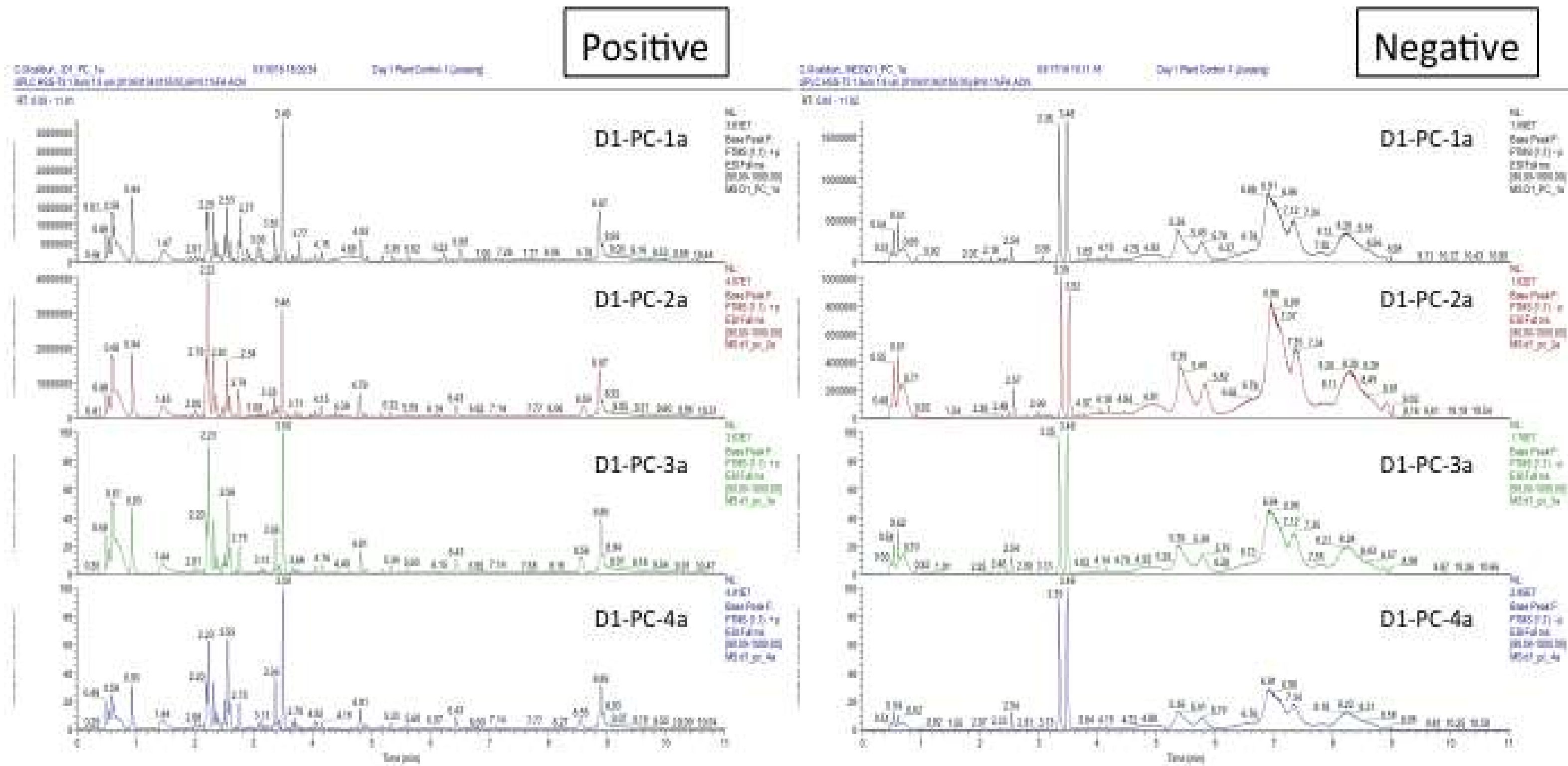

Figure C4: High-Resolution LC/MS traces for plant control (PC) samples taken on day one. Canola seedlings were grown in MS media for one day.

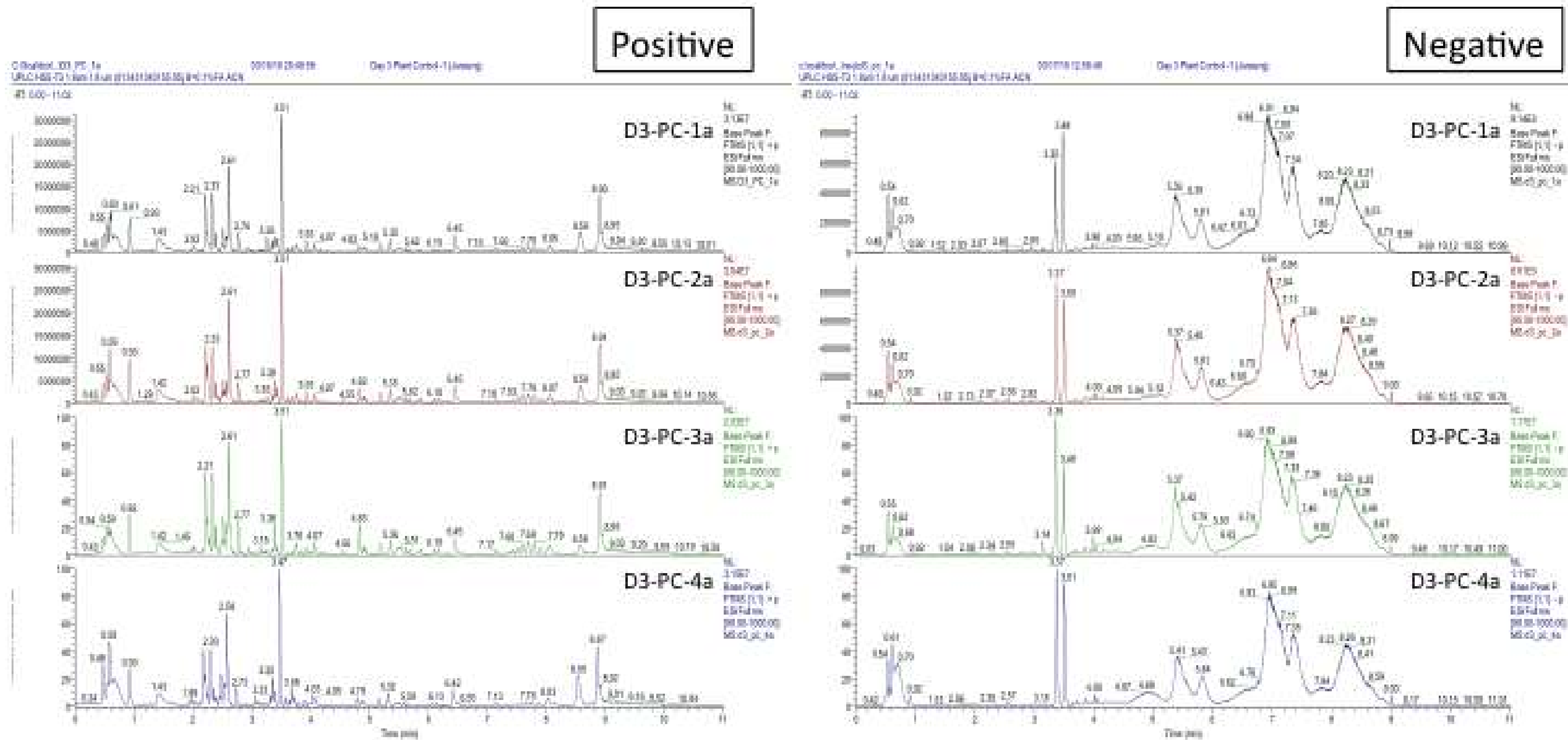

Figure C5: High-Resolution LC/MS traces for plant control (PC) samples taken on day three. Canola seedlings were grown in MS media for three days.

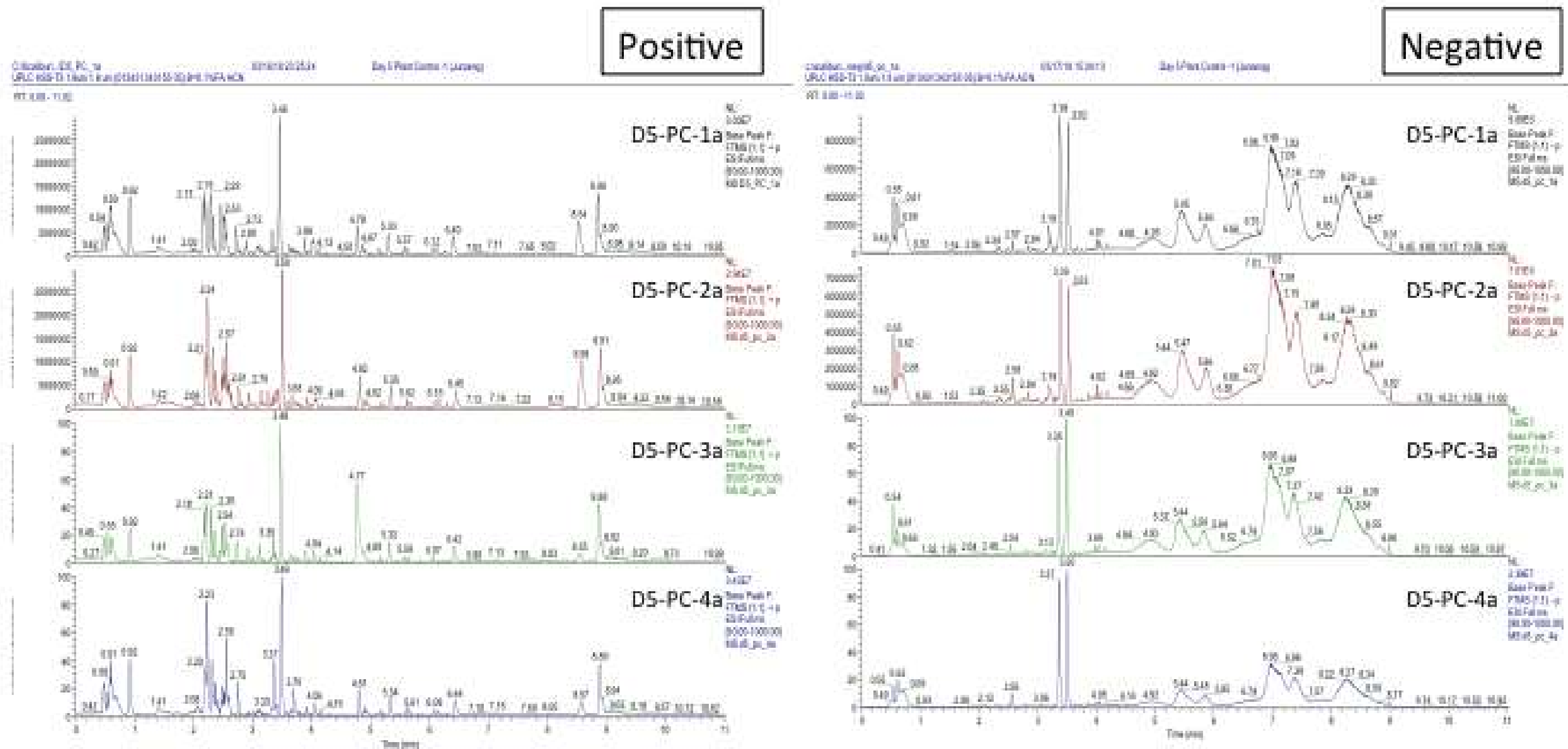

Figure C6: High-Resolution LC/MS traces for plant control (PC) samples taken on day five. Canola seedlings were grown in MS media for five days.

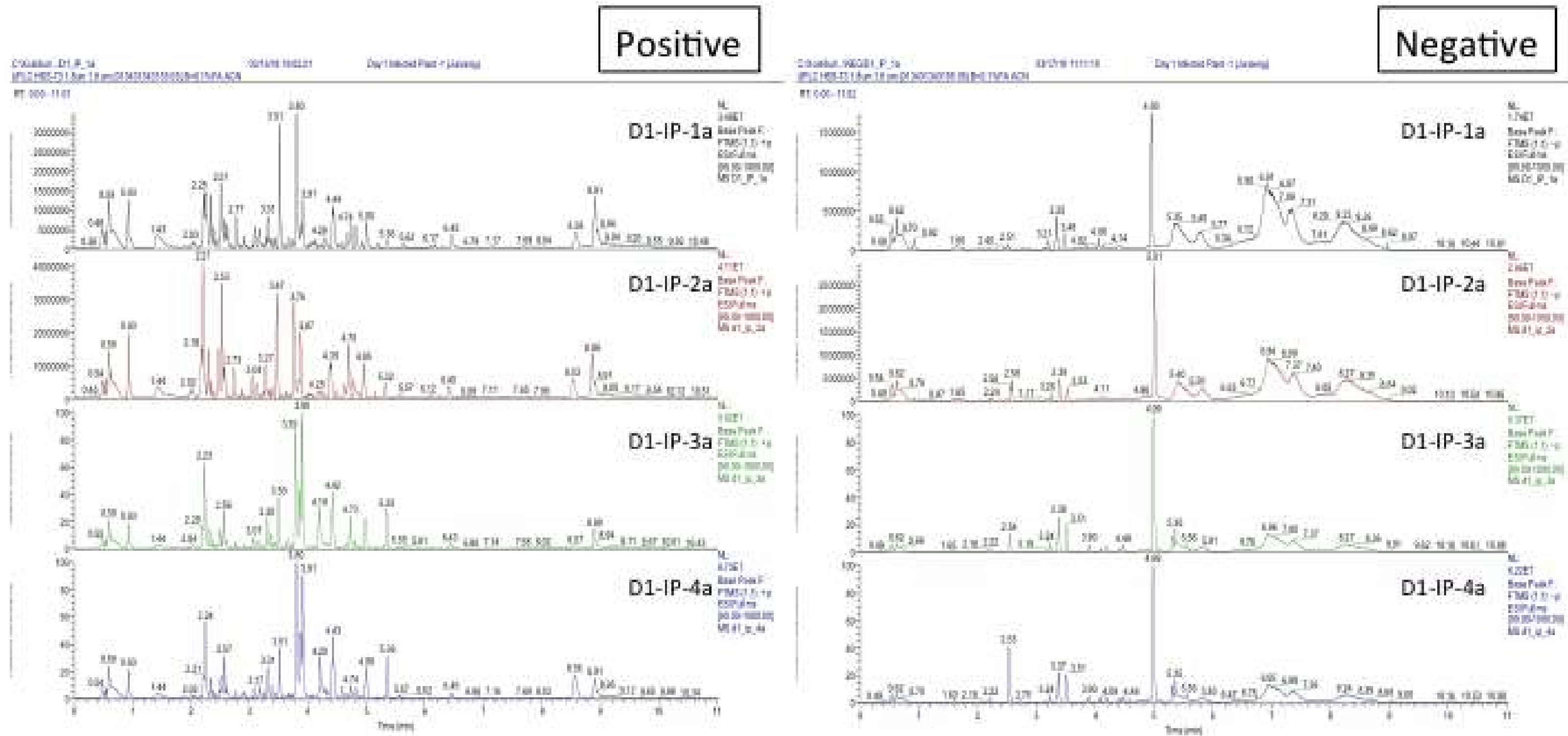

Figure C7: High-Resolution LC/MS traces for infected plant (IP) samples taken on day one. Canola seedlings were infected with *P. aeruginosa* PA14 in MS media for one day.

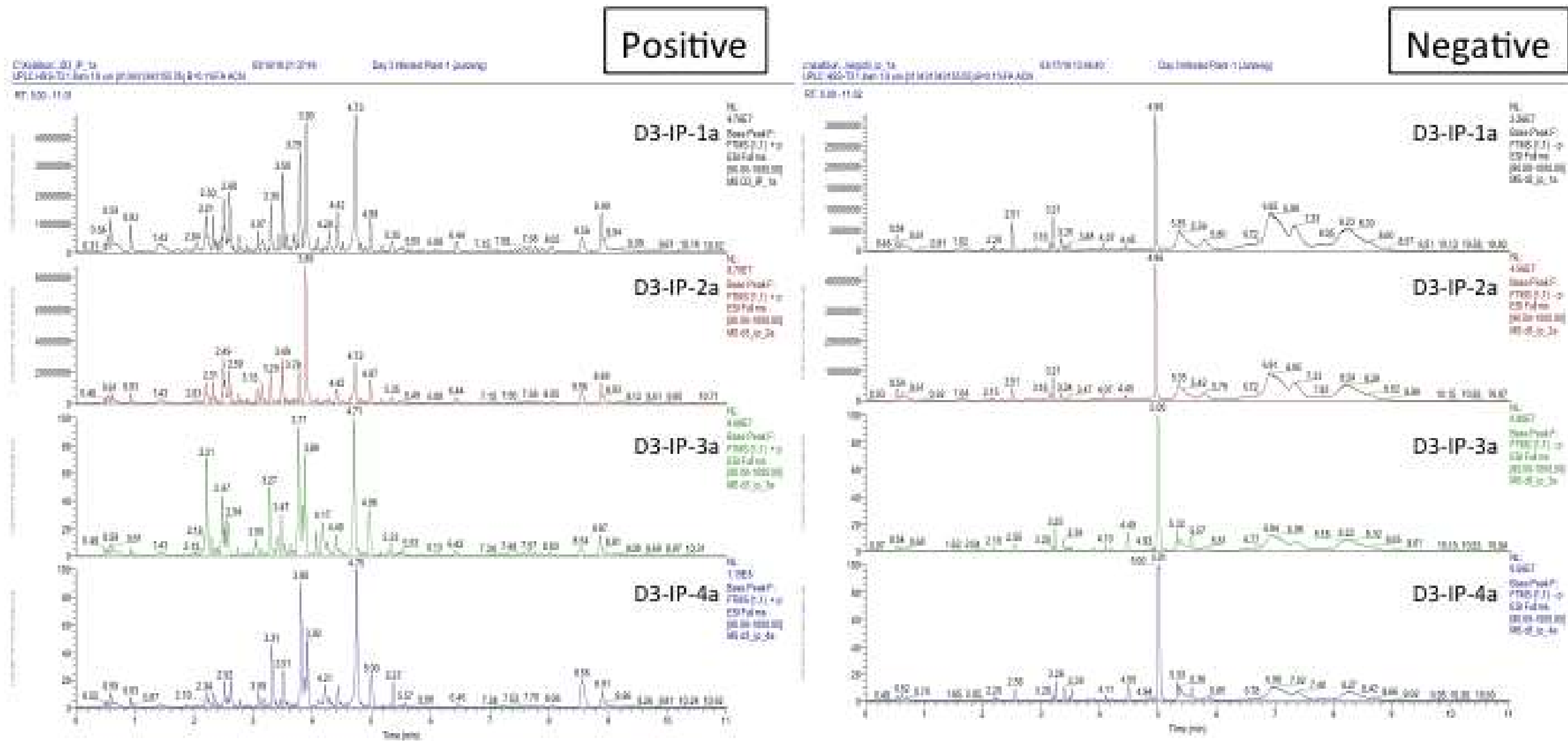

Figure C8: High-Resolution LC/MS traces for infected plant (IP) samples taken on day three. Canola seedlings were infected with *P. aeruginosa* PA14 in MS media for three days.

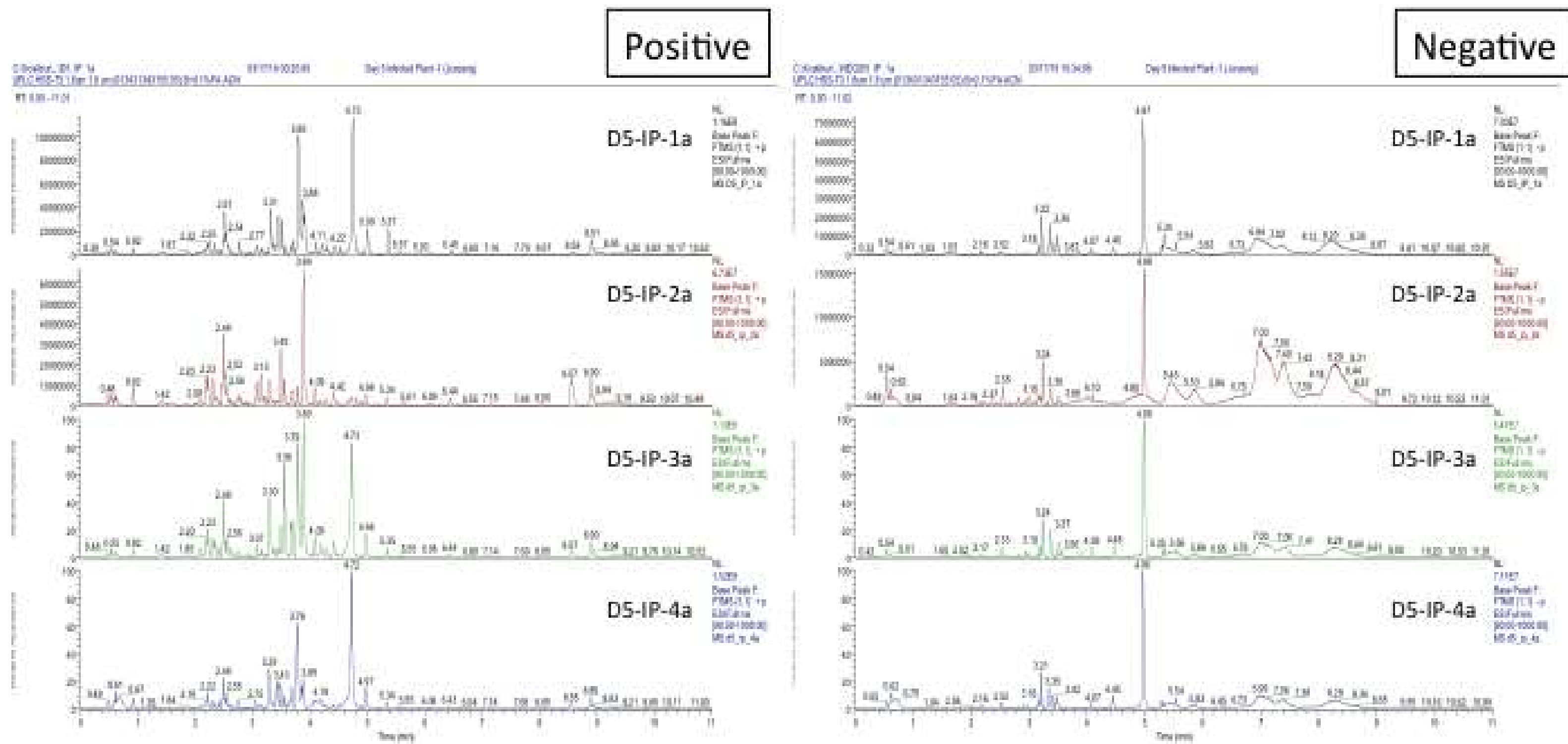

Figure C9: High-Resolution LC/MS traces for infected plant (IP) samples taken on day five. Canola seedlings were infected with *P. aeruginosa* PA14 in MS media for five days.

APPENDIX D: High-Resolution LC/MS Traces Showing Comparisons Between Samples

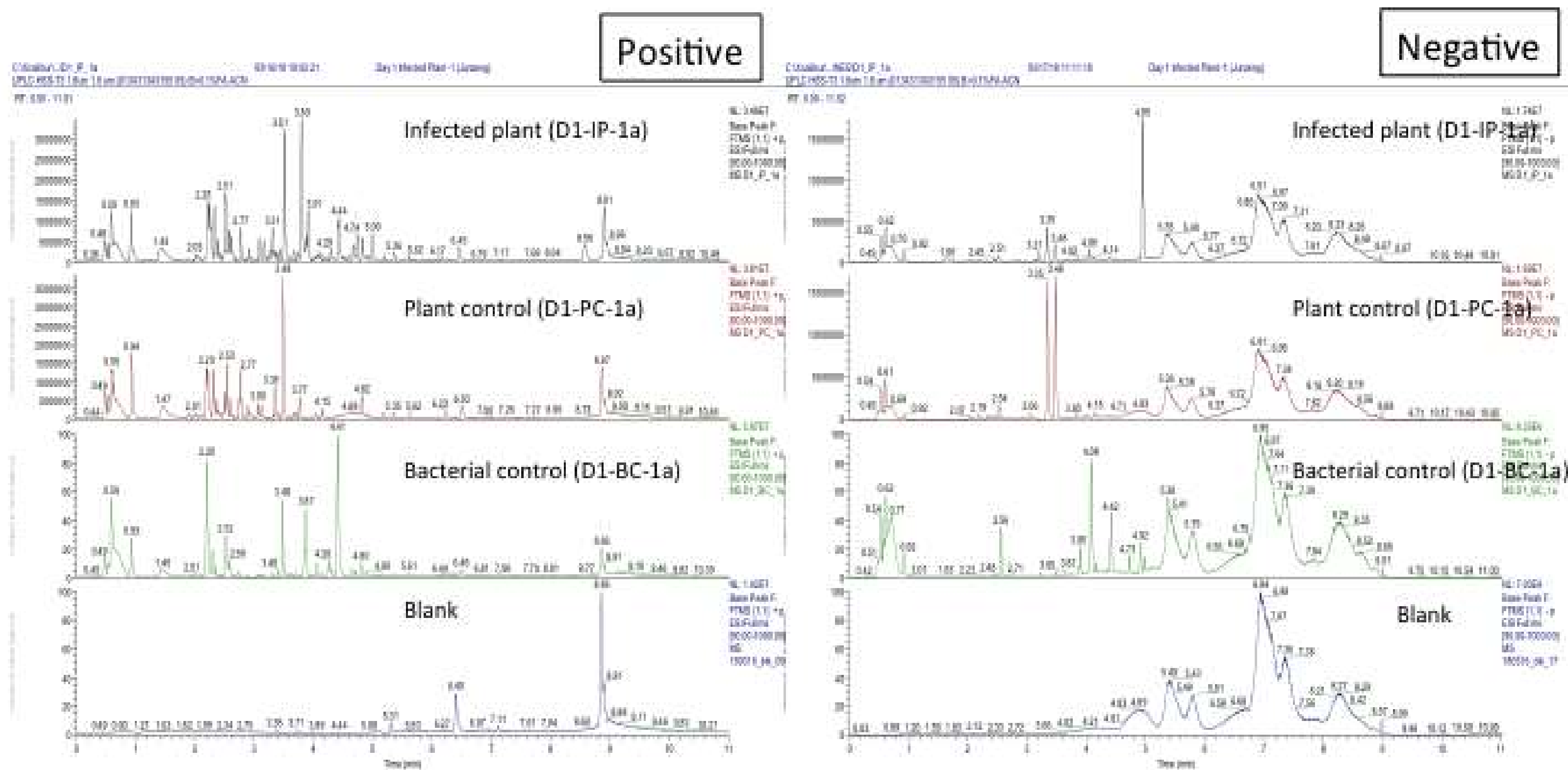

Figure D1: High-Resolution LC/MS traces for infected plant (IP), plant control (PC) and bacterial control (BC) samples taken on day one.

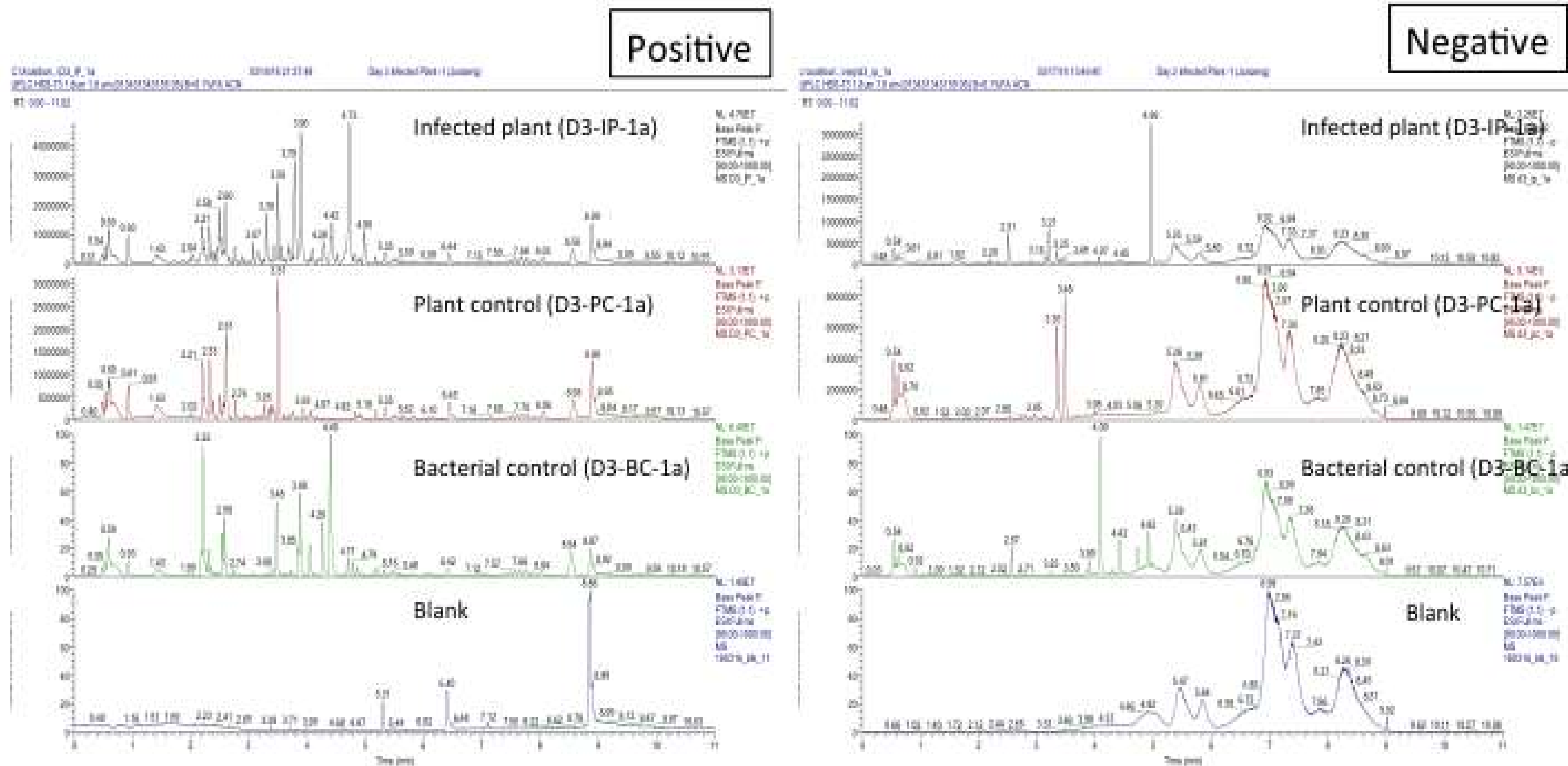

Figure D2: High-Resolution LC/MS traces for infected plant (IP), plant control (PC) and bacterial control (BC) samples taken on day three.

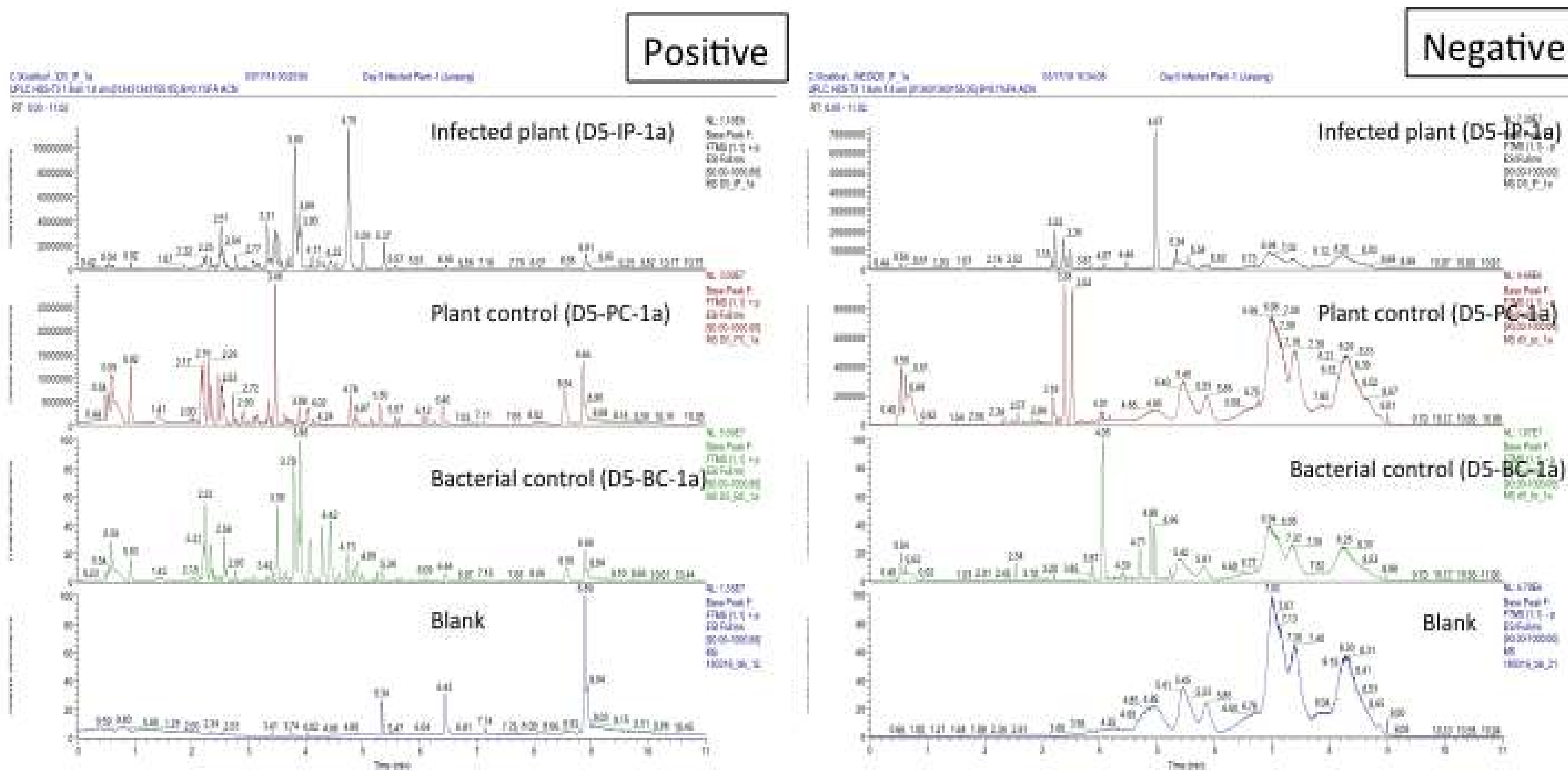

Figure D3: High-Resolution LC/MS traces for infected plant (IP), plant control (PC) and bacterial control (BC) samples taken on day five.