

**Influence of Endosymbionts in Mediating Plant Defense Responses to Herbivory by the Pea  
Aphid, *Acyrthosiphon pisum***

By

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A thesis submitted to the Graduate Faculty of  
Auburn University  
in partial fulfillment of the  
requirements for the Degree of  
Master of Science

Auburn, Alabama  
May 3, 2014

**Key words:** *Acyrthosiphon pisum*, *Regiella insecticola*, endosymbionts, insect oral secretions, phytohormone-dependent defense signaling, plant enzymatic activity

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## **Abstract**

Growing evidence suggests that symbiotic microbes that colonize different tissues and organs, known as endosymbionts, may influence the behavioral and physiological traits exhibited by insects as they interact with plants. Endosymbionts and insect hosts share a mutual interest in combating plant defenses, and research is demonstrating that endosymbionts can manipulate phytohormone-dependent signaling pathways that regulate a variety of defenses against herbivorous insects. Using the pea aphid, *Acyrthosiphon pisum*, and the aphid endosymbiont, *Regiella insecticola*, this study tested the hypothesis that endosymbionts can modify pea plant defenses to the benefit of aphid hosts. Defense-related activities associated with major molecular-signaling pathways were examined to determine the effects of a *R. insecticola*-aphid unification in plant enzymatic activity and gene expression after a period of aphid infestation. Later, aphid nymph and adult stages were assessed to compare parameters of survival and fecundity between aphids with and without *R. insecticola*. Interestingly, plant enzymatic activity and gene expression levels were always highest in response to infestation by aphids lacking *R. insecticola* and lowest after infestation by aphids harboring *R. insecticola*. However, benefits to aphid survivability and reproduction on plants with defenses suppressed were not as clear, and although in some cases aphids harboring *R. insecticola* outperformed other aphid treatments, the results were never statistically significant. Nevertheless, these results suggest that *R. insecticola*, through unidentified mechanisms, suppress pea plant defenses intended to deter *A. pisum*. While it can only be speculated, other underlying mechanisms may be responsible for aphid recovery from plant defenses or more elusive traits of aphids may be promoted by *R. insecticola* which may explain the unremarkable effect of suppressed plant defenses in aphid performance.

## **Acknowledgements**

I would like to thank my advisor, Dr. Henry Fadamiro, for providing me with the opportunity to obtain a graduate level education and to research an area of personal fascination which will certainly be of value in my future endeavors. Also, I owe a tremendous thanks to many great people for their guidance, generosity, and support that my research would not have been as rewarding or even possible without. First, Dr. Simon Zebelo, by lending your expertise and patient demeanor as I unskillfully began to practice a variety of techniques under your guidance, I give you major credit towards my improved competencies in molecular experimental methodologies. Furthermore, your friendship and zeal for research were consistent sources of amusement and encouragement throughout my experience at Auburn. Also, thanks to Dr. Nicole Gerardo of Emory University for her willingness to provide me with different pea aphid colonies, a contribution that *literally* made this research possible within such a limited time frame. I would also like to express my gratitude to Dr. David Held for contributing his vital resources that were necessary for completing my research. Your generosity was so flexible that at one point I had each of your growth chambers completely filled with plants and aphid colonies, and it was your contribution that put an end to my early failures as an entomologist-in-training at keeping my aphids alive. Also, a big heart-felt thank you goes out to my mom. Although I live many states away, you still manage to provide me with a figurative shoulder to lean on and an ear to vent to, and our many conversations via phone or Skype have been an invaluable source of comfort during times of stress. Finally, I would like to thank my

grandmother not only for introducing me to the world of art and creative thinking responsible for many of my skills and interests, but also for being one of few genuine people I know that share my “no B.S.” approach to life. Your enduring strength through recent health battles will forever amaze and inspire me to keep on fighting even when confronted by life’s most challenging hardships.

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## **Chapter 1: Introduction and Review of Literature**

### **1.1 Plants Defenses against Insect Herbivores**

The evolution of defensive strategies has enabled plants to persist in dynamic environments where they encounter various abiotic and biotic stressors on a continual basis. Defense mechanisms can protect plants in either a direct or indirect manner. Direct defenses include any plant traits that single-handedly impact the performance of herbivores and pathogens and the susceptibility of plants to their attackers. They include plant secondary metabolites which are not directly involved in growth and development, but instead can be toxic defenses to insects and can deter insect feeding (Bennett & Walls, 1994). However, plants can indirectly utilize secondary metabolites by emitting them as herbivore-induced plant volatiles (HIPVs) that natural enemies may use as cues to locate insect prey (Frost *et. al.*, 2008). A suite of defenses can be constitutively expressed by plants or they can be induced by cues associated with different forms of stress. Both constitutive and inducible defenses provide plants with protection from damage by abiotic stressors, such as nutrient deficiency, drought, and pollution, and biotic stressors, including pathogens and herbivores. As damage typically accumulates in plants from multiple, simultaneous forms of stress, highly sophisticated molecular mechanisms have evolved in plants which communicate through a network to enact a broad-spectrum response to combat stress (Atkinson & Urwin, 2012).

Defenses that are always present in the plant are considered constitutive and include protective barriers, such as bark, waxy epidermal cuticles, and cell walls, which provide structural support in addition to preventing water loss and microbial invasion. Constitutive emission of VOCs occurs from the plant surface and/or from storage structures in the leaf and often include

monoterpenes, sesquiterpenes, and aromatics (Paré & Tumilson, 1999). Secondary plant metabolites, which generally consist of terpenoids, isoflavanoids, and alkaloids, that can significantly impact herbivore performance and survival can be also be present in constitutive levels within plants. However, as production and maintenance of these chemicals are associated with high energy costs and nutrient requirements, levels of defensive chemicals in plants can vary as a function of biotic pressure (Wittstock & Gershenson *et. al.*, 2002). Plants in lower-risk environments where they are less exposed to major stressors may benefit from induced defenses (Agrawal, 1998). Inconsistent exposure of plants to stress may not warrant the sustained production of particular defensive chemicals, and plants may instead conserve resources until necessary to ward off enemy attack.

Defenses deployed by plants in response to herbivory, pathogen attack, and various abiotic elicitors, including heavy metals, are considered induced defenses. Benefits of induced defenses include phenotypic plasticity which may impede the ability of enemies to adapt to and override plant defenses. Although induced defenses reduce energy costs associated with implementing defenses in the absence of enemies, plants are left susceptible to attack. Once attack occurs, they may suffer considerable damage during the time period required to mount a defense response (Frost *et. al.*, 2008). In order to overcome this vulnerability, many plants exhibit a physiological phenomenon known as priming. Any environmental cues that reliably indicate the presence of an herbivore and, therefore, the threat of potential attack can serve as signals capable of inducing priming (Frost *et. al.*, 2008). Herbivore-induced plant volatiles (HIPVs), a subset of VOCs that are released upon herbivore attack, are obvious priming agents that are sensed by neighboring plants or different parts of the same plant. Plant defenses that are primed are conditioned to

readily respond to future abiotic and biotic stressors without incurring costs associated with full implementation of an induced defense response. However, plant defenses may be primed for prolonged periods during which minimal stress is encountered, and this may offset benefit of primed defenses that become costly to maintain (van Hulten *et. al.*, 2006).

Highly effective induced plant defenses also include the production of other secondary plant metabolites, most commonly phenols. For instance, lignin, a phenolic heteropolymer, plays a pivotal role in plant defenses against herbivores by increasing leaf toughness and decreasing leaf nutritional quality, thereby reducing herbivore feeding and performance (Frost *et. al.*, 2008). Oxidation of phenols into quinones catalyzed by polyphenol oxidase (PPO) and peroxidase (POD) provides plants with an important defense mechanism against herbivores through production of quinone products that inhibit the digestibility of plant material and can be directly toxic to insects.

Plant enzymes, such as PPO and POD, are extremely important as antinutritive defenses against herbivores (Bhonwong *et. al.*, 2009). A number of processes that directly or indirectly play a role in plant defenses are regulated by these enzymes, which have major impacts on herbivore feeding, growth, and development on plants. Through the formation of reactive o-quinones, which limit the availability of essential amino acids for the insects, PPO and POD inhibit insect digestion. Quinone products generated by PPO can reduce plant nutritional quality, can create oxidative stress in the insect gut lumen, and can be toxic to herbivores. Additionally, reactive oxygen species (ROS) resulting from phenolic oxidation by PPO can also be ingested during insect feeding and have toxic effects. POD, in addition to reducing plant nutritional quality and

herbivore performance, scavenges ROS, such as hydrogen peroxide ( $H_2O_2$ ). Production of  $H_2O_2$  and other ROS occurs as an early plant defense response initiated by insect feeding damage that leads to oxidative destruction of plant cells and can effectively deter herbivores from feeding.

There are other key signaling hormones responsible for the induction of defense networks that involve a variety of plant responses to numerous biotic and abiotic stressors. These hormones can act synergistically, antagonistically, or individually through so called “cross-talk” (Kunkel & Brooks, 2002). This network communication may allow for responses of plants to be fine-tuned to specific, and often simultaneous, attackers (Koornneef & Pieterse, 2008). Defenses against chewing insects and necrotrophic pathogens are largely dependent on the jasmonic acid (JA) signaling cascade, while resistance to biotrophic pathogens and some herbivores is regulated by another vital phytohormone, salicylic acid (SA). Local and systemic accumulation of SA serves as a trigger for a plant defensive state known as systemic acquired resistance (SAR). A plant with activated SAR achieves a long lasting state of resistance that confers broad-based protection against numerous different pathogens, including viruses, bacteria, and fungi (Yang *et. al.*, 1997). During this state a plant is induced to express a large set of SAR genes, some of which code for pathogenesis-related (PR) proteins. Interestingly, aphid feeding has been shown to induce local and systemic increases in mRNA transcription or enzymatic activity of several SA-associated PR proteins, such as PODs (War *et. al.*, 2012). Aphids can also induce JA-associated defenses, and it has been hypothesized that by promoting SA- defenses, aphids have evolved a mechanism to exploit antagonistic cross-talk between the signaling pathways in order to inhibit more efficient JA defenses (Giordanengo *et. al.*, 2010).

The mechanisms by which plants perceive and enact sophisticated defense signaling cascades in response to different attackers are only beginning to be understood (Maffei *et. al.*, 2007; Zebelo *et. al.*, 2012). Recent evidence has demonstrated that compounds present within the oral secretions (OS) and regurgitant of insects, known as herbivore-associated molecular patterns (HAMPs), are essential for the plant recognition and response process (Schafer *et. al.*, 2011). Despite the unpredictability of HAMP-induced plant responses, regulation of plant defenses by JA appears to be a highly conserved trait across several plant families (Schmelz *et. al.*, 2009). While many phytohormones are essential for plant defense responses, JA is recognized as the most important phytohormone in defenses against herbivores (Reymond & Farmer, 1998). Signaling cascades ultimately leading to JA production are extremely complex. Nevertheless, JA can reach detectable levels within plant tissues only a few minutes after herbivore damage begins. After being released as components of insect OS into damaged plant tissues, HAMPs activate peptide phytohormones, such as systemin in the family Solanaceae, that are involved in the orchestration of early defense responses of plants to herbivory.

Some HAMPs are enzymes introduced by feeding insects to the molecular defense signaling interface which coerce plants to attain physiological states appropriate for the execution of wound signaling cascades. Prior to the occurrence of key events that characterize major pathways of signal transducing networks, initial detection of insect-associated elicitors instigates the synthesis of mature peptide phytohormones from plant-derived precursors (León *et. al.*, 2001). Once they are manufactured, systemin or similar phytohormones are released from vascular bundles into the cytosol to locate attachment sites on plasma membrane-bound receptor proteins. The resulting hormone-receptor complex elicits a series of virtually coupled

biochemical transformations that facilitate mobilization of the signal through a cohesive network of pathways connected by extensive branching complexes.

At any given moment, plants are capable of integrating multiple signals from a range of biological and physical elements of the surrounding environment. A surge of intracellular calcium that emerges during a response to an isolated stimulus may only be a fraction of a much broader, integrated signal (Cheng *et. al.*, 2002). Transient fluctuations in free calcium concentration triggered by multiple stimuli may induce calcium-related signaling events of parallel or diverging pathways. Oscillations in calcium concentration generate encrypted signals that may be decoded by calcium sensory proteins, such as calmodulin, calmodulin binding proteins, and calcium-dependent protein kinases (CDPKs).

These proteins are each important promoters of signal transduction, but CDPKs are highly influential in mechanisms associated with the broad resistance of plants to various biotic and abiotic stressors. Transduction of CDPK-dependent pathways can also regulate the activity associated with another stress-induced regulatory protein, mitogen-activated protein kinases (MAPKs). Partial overlap of parallel activated CDPK and MAPK cascades can promote specificity of plant defenses by providing a context for reciprocal modification of pathways during signal transduction (Ludwig *et. al.*, 2004). Cross-talk between these pathways can ultimately lead to cascades which cause the release  $\alpha$ -linoleic acid from the plasma membrane. Through the consecutive activity of plastid-localized lipoxygenase (LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC), in the remaining portion of what is known as the octadecanoid pathway, linoleic acid will be converted into JA (Wasternack, 2007). Genes

subsequently activated by JA include those that are involved in aspects of plant development and mediate numerous plant defenses, including the production of proteinase inhibitors that prevent the proteolytic activity of insect digestive enzymes, defense-related enzymes like POD and PPO, and VOC emissions.

## **1.2 The Pea Aphid, *Acyrtosiphon pisum*, and its Key Features as a Plant Pest**

Aphids are major agricultural pests serving as vectors of about 275 different plant viruses (Powell, 2005), and annual worldwide yield losses resulting from aphids are estimated at hundreds of millions of dollars. The pea aphid, *Acyrtosiphon pisum*, infests legume crops including alfalfa, pea, and clover, and ranks among pests of economic significance by causing considerable yield reductions of several important crop plants. Aphids feed upon phloem sap, which they ingest through stylets used to probe plant tissue, a mode of feeding that causes little damage to plants. Rather, it is the viruses transmitted by aphids during feeding that greatly impact plant vigor.

Aphids are considered piercing-sucking insects that use specialized stylets to puncture sieve tubes and establish feeding sites within phloem. Stylets are inserted between two epidermal cells and continue on an apoplastic pathway between primary and secondary cell wall layers. During penetration, aphids continuously secrete gelling saliva which forms a lubricated and hardened sheath around stylets. Once a sieve element is reached, aphids secrete a second type of watery saliva directly into the phloem which is implicated in suppressing early plant defense responses. Although this mode of feeding causes little mechanical damage to plant tissue, through their oral secretions (OSs) aphids can transmit numerous debilitating plant viruses which cause

developmental malformations and stunt growth (Brisson & Stern, 2006).

Pea aphids are the primary vector of the pea enation mosaic virus (PEMV), which infects numerous legume plants worldwide and has a major economic impact by causing severe crop losses in beans and peas (Hodge & Powell 2010). Interestingly, PEMV is composed of two obligately-associated symbiotic RNA viruses, PEMV-1 and PEMV-2, which belong to the genera *Enamovirus* and *Umbravirus*, respectively. PEMV-2 enables mechanical transmissibility of PEMV-1 among plants and systemic infection of all host plant tissues; PEMV-2 relies on PEMV-1 for structural proteins necessary for encapsidation and aphid transmission. This virus is transmitted by *A. pisum* in a persistent-circulative manner which entails ingestion of virions by aphids during sustained phloem-feeding. Virions move from the gut lumen of aphid vectors into the hemolymph or other tissues. When in the hemolymph, virions appear to benefit from the obligate aphid gut-endosymbiont, *Buchnera aphidicola* which has an ancient evolutionary history with numerous aphid species. The bacteria produce a chaperonin protein, which enables the correct folding of proteins and, thus, prevents protein aggregation, called GroEL. The chaperonin is hypothesized to bind to virions in the hemolymph, thereby protecting virions from degradation by hemolymph enzymes (Bouvaine *et. al.*, 2011). Virions reside in salivary glands, and infected aphids subsequently transmit them to new host plants via salivation while feeding.

The composition of gelling salivary is thought to be consistent across different aphid species and is primarily composed of proteins (such as phenoloxidases, peroxidases, pectinases, and  $\beta$ -glucosidases), phospholipids, and conjugated carbohydrates (Giordanengo *et. al.*, 2010). Watery saliva, on the other hand, differs between and within aphid species and contains a more complex

enzymatic mixture including pectinase, pectinmethyl esterase, polygalacturonase, cellulase, and other components (Giordanengo *et. al.*, 2010). Watery saliva is composed of numerous hydrolytic enzymes capable of modulating differential plant defensive activities. For instance, PPO, can serve as an elicitor that activates plant defenses while certain calcium-binding salivary components can suppress responses, including those involved in the occlusion of sieve plates.

These calcium-binding components of aphid watery salivary secretions provide a means by which aphids can circumvent calcium-dependent defense mechanisms of plants (Will *et. al.*, 2007). For instance, a regucalcin-like protein has been identified which is capable of sequestering calcium ions in phloem sap (Carolan *et. al.*, 2009). By binding calcium, this protein prevents events that would lead to the occlusion of sieve tubes, a process that would normally hinder aphid feeding by preventing continued leakage of phloem through puncture wounds of aphid feeding sites. The ability of aphids to establish feeding sites within sieve elements, may also be impacted by a salivary enzyme called C002. It was demonstrated that suppression of *c002* transcripts by RNA interference (RNAi) prevented sustained feeding of aphids from sites established within sieve elements and *c002*-knockdown aphids had shorter lifespans than aphids with intact *c002* genetic mechanisms (Mutti *et. al.*, 2008).

Elicitors within aphid watery saliva can also serve as signals recognized by plants as a threat-potential, initiating appropriate defense responses (De Vos & Jander, 2009). Polyphenol oxidase (PPO) within aphid salivary secretions elicits defenses associated with the jasmonic acid (JA) signaling pathway. After treating wheat seedlings with grain aphid saliva and PPO at a concentration consistent with aphid saliva, Ma *et. al.* (2010) found a significant induction of

gene *aos*. As this gene encodes the key enzyme, allene oxide synthase, in the JA defense signal pathway, it was inferred that PPO serves as the main elicitor of JA-mediated defense responses in wheat.

Providing further proof of the ubiquitous relevance of insect OSs in plant defense responses, but with novel information, a recent study utilized a different model insect system to demonstrate that other components within insect OSs may modulate plant defenses, as well. Chung *et. al.* (2013) found that antibiotic-fed larvae of the Colorado potato beetle, *Leptinotarsa decemlineata*, were unable to suppress JA-mediated defense in tomato plants while larvae that were fed a diet free of antibiotics suppressed JA-defenses, but induced plant defenses associated with the phytohormone salicylic acid (SA). After analyzing larval salivary secretions, three microbial symbionts were implicated in the modification of plant defenses that resulted in enhanced larval performance. These orally secreted bacteria may be exploited by *L. decemlineata* to promote the induction of defenses that are mild in comparison to the highly deterrent JA-mediated defenses. Many insect species have a strong coevolutionary history with symbiotic microbes, which confer substantial nutritional benefits and several important ecological traits. Pea aphids harbor a well-documented microbiome that include strains of bacterial species *Regiella insecticola*, *Hamiltonella defensa* and *Serratia symbiotica* that confer numerous benefits impacting their host's physiology. Studies have yet to examine whether this suite of defensive endosymbionts are associated with modified plant defense responses similar to those recently documented in *L. decemlineata*. However, interest in this area is rapidly growing, especially since the supporting information provided by Chung *et. al.* (2013) and other recent studies (Casteel *et. al.*, 2012; Frago *et. al.*, 2012. Information provided by these studies is revealing the ubiquity of

endosymbionts in insects and there relevance in the physiological traits of both insects and plants. The endo-microbial ecology within aphids has already been well-documented, but evidence of recent studies necessitates that more research of aphid endosymbionts is needed, but from the additional perspective considering plant defense mechanisms, as well.

### **1.3 Endosymbiotic Bacteria: Equipping Aphids in an Evolutionary Arms Race**

Aphids harbor a variety of endosymbiotic microbial species within different organs and tissues. The endosymbionts may have obligate roles tightly integrated within their host's physiology, or other non-essential endosymbionts may provide several physiological benefits depending on conditions of their host's environment (Laughton et. al., 2014). Obligate, or primary, endosymbionts are essential for host survival, are stored in specialized cells (bacteriocytes) that may form organs called bacteriomes, and contain highly reduced, AT-biased genomes as a result of the elimination of genes that may be considered superfluous from the context of insect-microbe association (McCutcheon & Moran, 2011). Obligate status of endosymbionts can be attained when neither insect nor microbe has the physiological capacity to reliably survive for prolonged periods alone and generally transpire within associations with a deep evolutionary history. The obligate endosymbiont *B. aphidicola* found in nearly all aphid is estimated to have emerged hundreds of millions of years ago (Moran et. al., 1993). Aphid hosts benefit from their obligate endosymbionts which, in exchange for the more secure internal environment within aphids, promote quality of aphid nutrition through synthesis of essential amino acids lacking in their phloem-sap diet. Also, presence of obligate endosymbionts like through subsequent generations of its host is maintained by its vertical mechanism of inheritance via trans-ovarial transmission, where developing embryos of aphid clones are colonized within the maternal body

(Kikuchi, 2009).

Association of aphids with an assemblage of facultative, or secondary, endosymbionts differs from the host-obligate partnership in several characteristics. For instance, facultative endosymbionts of aphids are neither necessary for host survival nor confined to bacteriocytes, but are generally present extracellularly in the hemolymph of aphid body cavities which may indicate their more novel colonization of aphids compared to *B. aphidicola* due to lack of specialized structures required to sustain the association. Further evidence supporting a more recent establishment is demonstrated by multiples modes of progeny inheritance. Facultative endosymbionts can be transmitted to newer generations via the vertical, trans-ovarial mechanism and a horizontal transmission mechanism. Environmental acquisition of symbiotic microbes may reflect the early events that initiate associations which can evolve to more intricate levels characteristic of obligate symbioses (Oliver *et. al.*, 2010). Although it is predicted that facultative insect-microbe symbioses are more recently established than obligate associations, this does not undermined the relevance of these microbes in diverse assemblages of insect species beginning at the host molecular scale up to the ecological interactions of the host with its dynamic external environment.

Facultative endosymbionts mediate several well-documented ecological traits of aphid hosts by promoting traits attributed to increasing their thermal tolerance (Montllor *et. al.*, 2002), broadening their range of suitable food plants (Oliver *et. al.*, 2010), or enhancing resistance to parasitoids or other natural enemies (Moran *et. al.*, 2008; Oliver *et. al.*, 2012). For example, the facultative endosymbiont *H. defensa* confers resistance to parasitism by inhibiting the

development of wasp larvae and thereby rescuing the aphid host (Degnan *et. al.*, 2009). Similarly, a study using *Myzus persicae* found that clones harboring a strain of *R. insecticola* were entirely resistant to two common parasitoids, *Aphidius colemani* and *Diaeretiella rapae* (von Burg *et. al.*, 2008). A strain of *R. insecticola* has also been found to protect aphids against fungal pathogens (Scarborough *et. al.*, 2005).

Facultative endosymbiosis confers advantages to aphids by modifying their inherent physiological capacities to more traits better suited to tolerate a wider-range of certain abiotic environmental conditions. While the exact mechanism is still unclear, the facultative endosymbiont *Serratia symbiotica* has been demonstrated to confer heat tolerance in the pea aphid, *A. pisum* (Chen *et. al.* 2000). Of related interest, recent studies have indicated that *B. aphidicola* appears to be losing its symbiotic capacity and until its tentative replacement by *S. symbiotica* as the obligate endosymbiont. Lamelas *et. al.* (2011) used cedar aphids (*Cinara cedri*), which contain the most reduced *B. aphidicola* genome, to demonstrate that a strain of *S. symbiotica* associated with these aphids may be in a stage of genome reduction similar to that expected during development of an obligate endosymbiosis. Genome sequencing has revealed an inability of *B. aphidicola* to synthesize both tryptophan and riboflavin, signifying a loss of some of its vital symbiotic function. Remarkably, *S. symbiotica* can assume a nutritional role atypical of facultative endosymbionts that partially fulfills the deficiency by synthesizing tryptophan (Lamelas *et. al.*, 2011).

The significance of the benefits bestowed by insect endosymbionts continues to expand. Studies are beginning to demonstrate that endosymbionts may also advance their insect hosts by altering

plant defense signaling pathways (Zhang *et. al.*, 2011). A recent study using the tomato psyllid, *Bactericera cockerelli* (Sulc), a vector of *Candidatus Liberibacter psyllaurous* (*Lps*), found that the endosymbiont has the ability to manipulate plant signaling activity that is accompanied by the activation of certain plant defense responses (Casteel *et. al.*, 2012). Different life stages of *B. cockerelli* infected with *Lps* were used, and it was found that younger life stages had lower *Lps* titers than older life stages. Nymphs induced the expression of genes regulated by JA and SA while adults containing higher *Lps* titers appeared to suppress these defense pathways. Although ampicillin treatment failed to cure psyllids of *Lps* and, therefore, prevented comparison of the performance of *Lps* infected and uninfected individuals, the outcome of the study serves as evidence that an asset of endosymbiont infection of an insects can be affiliated with a suite of modified defenses generated by host-infested plants.

Further information about the significance of endosymbionts in plant defenses was provided by Davis *et. al.* (2012) also with *B. cockerelli* as a model insect, but with focus on one of its other endosymbionts, *Candidatus Liberibacter solanacearum*. The results of this study indicate the occurrence of an endosymbiont-mediated mechanism for suppression defensive events preceding volatile emissions in potato plants. Volatile profiles of infested plants were found to be more similar to one another than to control plants regardless of psyllid endosymbiont-infection status. However, they were found to be statistically distinct in terms of the abundance of particular compounds. The differential abundance of compounds across volatile profiles of psyllid-infested plants is likely attributed to endosymbiont-imposed limitations on the molecular manufacturing processes responsible for emission of defensive volatile products.

Inhibition of plant defenses by endosymbionts may also occur as mechanisms for detoxification of plant secondary metabolites, relieving insect hosts from toxic effects. Lauzon *et. al.* (2003) found that phloridzin, a plant-derived compound that is toxic to the apple maggot fly, can be degraded and detoxified by *Enterobac agglomerans*, a phylloplane and gut microorganism. It is possible that gut microorganisms use plant defense compounds as a nutrient source that may reduce their potency. Endosymbiont detoxification may not be limited to noxious plant compounds. Werren (2012) demonstrated that stink bugs experimentally infected with pesticide-degrading *Burkholderia* have significantly increased resistance to pesticide treatment. In their natural environment, stink bugs acquire the bacteria from soil and store them in a specialized midgut structure. *Burkholderia* can metabolize organophosphorous pesticides exposed to insect hosts into usable sources of carbon, nitrogen, and phosphorous.

Endosymbiotic relationships are fascinating artifacts of coevolution, and a surge of studies in the past few decades has uncovered many fundamental aspects of endosymbiosis currently fueling the expansive research of symbiotic interactions viewed from novel angles, such as those relevant in to each organism of multitrophic ecological complexes. Very rapidly accumulating information has also prompted novel studies of the practicality of exploiting endosymbioses affiliated with different insect pests as an agricultural biocontrol tactic. Advanced molecular procedures have supplied the tools necessary to characterize many traits of endosymbiotic interactions, providing the frame-work necessary to develop management strategies. One approach entails introduction of modified endosymbionts through techniques generating the genetic drive required to replace native endosymbiont species with genetically modified ones (Ahantarig & Kittayapong, 2011). The introduction of GMO endosymbionts into populations

may generate microbial species that antagonize host insects and no longer perform beneficial physiological functions.

Another feature of these associations is also prompting research of endosymbiont derived biocontrol mechanisms of insect pests. Many of the deleterious plant viruses transmitted by aphids appear to be exploiting beneficial properties of these microorganisms. Virions of the Potato Leaf-Roll virus (PLRV) display a high affinity for a transporter protein (GroEL) produced by the aphid obligate endosymbiont, *B. aphidicola*. Studies generating aposymbiotic aphids lacking *B. aphidicola* via antibiotic-treatment demonstrated the reduced capacity of aposymbiotic aphids to transmit virions relative to aphids with intact microbiomes. The result was not only attributed to a reduction in aphid performance in absence of *B. aphidicola*, but also to the degradation of PLRV proteins likely more vulnerable to aphid enzymatic properties in absence of the protection supplied by GRoEL (Van den Heuvel *et. al.*, 1994).

Endosymbiotic bacteria of insects cannot be cultured outside the host, imposing a several limitation in our ability to clearly define molecular mechanisms underlying host-symbiont interactions. However, cultures of lesser known species of aphid facultative endosymbionts, *Adiaceo aphidicola* and *Consessoris aphidicola*, have been established in three insect (two dipteran and one lepidopteran) cell lines (Darby *et. al.*, 2005). By identifying similar culture techniques, research may soon uncover advanced methods which retain several of the important characteristics of *in vivo* host-microbe interactions. However, studies utilizing culture-independent molecular techniques based primarily on PCR and molecular cloning of small subunit 16S ribosomal RNA sequences have also proven informative sources regarding

endosymbiont-related traits of host's fitness (Chen *et. al.*, 2000), their physiological influence in single organisms or multitrophic ecological complexes (Laughton *et. al.*, 2011), and the various defensive qualities they afford to a variety of hosts (Casteel *et. al.*, 2012).

Nevertheless, it is becoming apparent that considerable amounts of information regarding the relevance of insect endosymbionts has yet to be revealed. Although, the swell of recent interest in this research area may accelerate the exposure of prominent traits of different insect endosymbioses. For instance, identification of secondary hosts of these insect endosymbionts, such as an insect host plant serving as a microbial reservoir that introduces the endosymbionts to new insect hosts as they feed, may be discovered. Of course, this is all speculation, but progressive research using molecular methods and genomics will develop advanced methodologies for building a more comprehensive picture of the relevance of microbes in insect ecology.

#### **1.4 Model System**

This multitrophic study uses the pea aphid (*Acyrtosiphon pisum*) and its facultative endosymbiont, *Regiella insecticola*, to determine whether the presence of these endosymbionts correlates with manipulated defenses of snow peas (*Pisum sativum*). *Acyrtosiphon pisum* is considered a major pest of economically important legume crops due to its ability to transmit numerous damaging plant viruses. Annually, pea aphids cause severe yield reductions in crops, such as peas and beans. Several studies have used *A. pisum* as a model organism for research investigating the functional roles of symbiotic bacteria within insect physiological systems (Gerardo *et. al.*, 2010; Montllor *et. al.*, 2002; Chen *et. al.*, 2000). These aphids are known to

harbor a range of facultative endosymbionts that affect ecologically important traits having significant impacts on their immunity and physiological tolerance. Sequencing of the whole pea aphid genome (International Aphid Genomics Consortium, 2010) has provided a foundation for post-genomic research seeking to enhance our understanding of these biologically complex organisms and traits mediated by their remarkable endosymbiont inhabitants. Furthermore, *R. insecticola* is known to enhance the defensive capabilities of their aphid hosts, and it is likely that the protective properties of these microbes may extend well beyond what is currently understood.

## **1.5 Justification for the Study**

The defensive benefits incurred by pea aphids harboring various endosymbiotic bacteria have been well documented (Scarborough *et. al.*, 2005; Oliver *et. al.*, 2003; Oliver *et. al.*, 2009). Studies using other insects have demonstrated that endosymbionts may enable their hosts to manipulate plant defenses for their own benefit (Casteel *et. al.*, 2012; Robert *et. al.*, 2013). However, the influence of the well-documented consortium of aphid endosymbionts in plant defenses has yet to be examined. Also, viral affinity for endosymbiont-derived proteins suggests that aphid endosymbionts may play a pivotal role in the transmission of many injurious plant viruses vectored by aphids. Each year aphids, within the Hemipteran suborder Sternorrhyncha, as a whole cause hundreds of millions of dollars in damage to crops (International Aphid Genomics Consortium, 2010). Pea aphids are considered legume crop pests of major economic importance that severely reduce crop production primarily by infecting plants with deleterious viruses.

The involvement of insect endosymbiotic bacteria in the transmission of plant viruses has been demonstrated in several different insects (Van den Heuvel *et. al.*, 1994; Bouvaine *et. al.*, 2011; Morin *et. al.*, 1999). In pea aphids, the obligate endosymbiont, *Buchnera aphidicola*, and its potential successor, *Serratia symbiotica*, are known to produce chaperone proteins that bind to virions, thereby protecting them within the aphid host's hemolymph. Studies using antibiotics to eliminate *B. aphidicola* from aphids found that absence of the obligate endosymbiont not only attenuates aphid development (Douglas 1998), but also results in poor virus transmission (Van den Heuvel *et. al.*, 1994).

Aphids, as well as all other insects, can engender or, as recent evidence supports, modulate plant defenses that are activated in response to damage caused by their feeding on different types of plant tissue, the mode of feeding utilized (i.e. chewing, piercing-sucking, etc.) by the insect attacker, and to the constituents within an insects oral secretions (i.e. defense elicitors or suppressors). Generation of plant defenses involves highly complex hormone-regulated signaling pathways that network at points of overlap of molecular reactions. Through cross-communication, signaling molecules may influence ultimate defenses to suitably respond to an attacking herbivore. Recent studies have provided evidence that insect endosymbiotic bacteria may also inhibit plant defensive quality by facilitating the detoxification of harmful plant compounds (Lauzon *et. al.*, 2003), interfering with enzymatic activity (Casteel *et. al.*, 2012), and altering plant volatile emissions (Davis *et. al.*, 2012).

The current lack of knowledge, but recent supporting evidence regarding the relevance of endosymbiotic bacteria in plant-insect interactions merits the research objectives defined in this

study. The likelihood that endosymbionts are vital factors overlooked in strategies currently used to control insect populations has prompted inquiries of implications for pest management. This research will contribute information that may be used to assess the importance and feasibility of incorporating endosymbiont manipulation tactics into methods used to control problem-insects in agriculture.

## **1.6 Thesis Goal and Outline**

Through this research, we hope to expand our understanding of the defensive capabilities of aphid endosymbiotic bacteria. Specifically, this study investigates whether the advantages conferred to pea aphids infected with either of two different strains of the facultative endosymbiont, *Regiella insecticola*, may extend beyond the host insect-plant interface. The intended purpose of this research is to provide information that can assist in answering the following questions: 1) may the activities of enzymes involved in defense signaling be altered by aphid *R. insecticola*? 2) are the expression of genes associated with defenses against aphids suppressed by aphid *R. insecticola*? 3) does pea aphid performance on pea plants vary according to *R. insecticola* infection status?

Enzymatic activities and transcript levels associated with plant defenses were generally highest in response to infestation with R<sup>-</sup> aphids and lowest after infestation by R<sup>+</sup> aphids. The results were repeatable and indicative of a suppressive role of *R. insecticola* in pea plant defenses activated during aphid feeding. It is likely that metabolic products of the endosymbiont are introduced to the plant through aphid saliva which is known to contain products that can adjust plant defenses to facilitate aphid feeding. Additionally, the outcomes of these objectives propose

that *R. insecticola* can enhance aspects of aphid performance (i.e. survival, fecundity, etc.) by manipulating defenses generated by plants to deter aphids which may allow longer feeding periods.

However, benefits of suppressed plant defenses to aphid survival and fecundity were not as clear as anticipated. Parameters were chosen to indicate the quality of aphid performance as they developed on pea plants. These included the following: 1) percent of initial colony of nymphs surviving to adulthood, 2) number of days to reach adulthood, and 3) average number of days at which 50% mortality and 100% mortality of the adult colony was reached. Although a higher percentage of nymphs from both *R. insecticola*-infected lines survived until the adult stage, the result was not significant. Likewise, there was no significant difference in adult reproduction and survival between R<sup>+</sup> and R<sup>-</sup> aphids.

Although the results of the aphid performance experiments appear to contrast with the results from the initial experiments showing significant suppression of plant defenses, they may reflect the highly unpredictable nature of deceptively simple microorganisms. The complexity of microbes readily becomes apparent while they interact with microscopic components destined for highly specified objectives within the molecular environment of higher-order organisms. Hosts are often infected with multiple species of bacteria and fungi that likely interact to cause complex effects in host physiology and external ecology. Thus, among other plausible explanations, underlying mechanisms may be responsible for relieving aphids from plant defensive activities.

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## **Chapter 2**

### **A Facultative Endosymbiont in *Acyrthosiphon pisum* Corresponds with Suppressed Plant Defenses, but Unclear Effects on the Performance of Pea Aphid Hosts**

#### **2.1 Introduction**

Differential feeding mechanisms employed by herbivorous insects have arisen from a myriad of interactions with host plants which have reciprocated advancements in insect defenses systems by adapting their own traits to countermand them. Through these historical coevolutionary events plants have acquired a dynamic network of interconnected molecular defense pathways regulated by various signaling elements. These molecular signaling networks are responsible for an enumerable range of plant traits in addition to their phenotypic defenses. In response to insect attack, plants can display generalized traits capable of hindering a variety of insect attackers or their defenses can be very specific for a limited group of insects. Different defense signaling pathways activated concurrently can modulate one another through promotional or antagonistic interactions. Cross-communication between signaling pathways is believed to provide a mechanism for plants to adjust their regulatory systems to ensure the efficiency of the generated defense response (Kunkel & Brooks, 2002).

Evolution of highly intricate plant defense systems has been driven in part by an abundance of insect species accounting for a large proportion of all biodiversity. Various ecological mechanisms have permitted copious herbivorous insects to coexist within mutual environments by minimizing niche overlap and competition among different species. Resiliency is a major character of insects that successfully occupy niches and maintain the integrity of their ecological status. Further, partitioning of plant food resources required insects to evolve morphologically

specialized mouthparts with distinct mechanisms for consuming plant tissue. In general, insect mouthparts predominately tear into and grind plant tissue, or puncture tissue to imbibe phloem (Fürstenberg-Hägg *et. al.*, 2013). The structure of insect mouthparts influences which plants and specific tissues an insect will feed on, and the degree of damage inflicted upon plants.

The magnitude of physical damage and various events at the cellular level influence the nature of plant defenses deployed to deter insect with different feeding strategies. Molecular reflexes of plants initiate the production of specific or generalized plant defenses depending on information conveyed by plant recognition systems about the attacker (Ali & Agrawal, 2012). Some insects employ more discreet mechanisms that cause minimal tissue damage, but still deliver serious problems to plants. Aphids, amidst the most challenging phloem-feeding agricultural pests, utilize mouthparts equipped with stylets to puncture sieve elements and imbibe phloem. Stylets may cause moderate injury to plants as they perforate tissues, en route to feeding sites (Walling, 2008). However, their reputation as problematic pests is attributed to the numerous injurious plant viruses they deliver to plants as they feed.

To perforate plant tissues, stylets are injected at the tissue surface between two epidermal cells and proceed down an apoplastic route between primary and secondary cell wall layers. While inserting their stylets into tissue, aphids secrete a gelling saliva that forms a protective sheath to prevent stylets from contacting plants to avoid triggering defensive reactions (Nicholson *et. al.*, 2012). Aphids feeding sites are reached within sieve elements where aphids secrete a watery saliva directly into the phloem which is implicated in the suppression of early plant defenses.

Though watery saliva aphids may transmit not only numerous injurious plant viruses, but also various hydrolytic enzymes and metabolites capable of modulating plant defensive activity.

Interestingly, recent evidence has identified compounds in insect oral secretions (OSs) that are attributed to microbes living within insects (Chung *et. al.*, 2013). Products from insect gut microbes, also known as endosymbionts, correlated with altered plant defense activity. The abundance of endosymbionts may also affect the intensity of plant defense activation (Casteel *et. al.*, 2010). The relevance of OSs from insects in the induction of plant defense has been demonstrated by many recent studies (Weech *et. al.*, 2008; Takabayashi *et. al.*, 2000, Zarate *et. al.*, 2007). Enzymatic and nonproteinaceous components of insect OSs that include microbial-derived compounds elicit a suite of plant defense reactions (Chung *et. al.*, 2013). Other compounds in OSs, conversely, can suppress defenses. Aphid OSs are known to contain effectors that are essential components facilitating the aphid feeding process.

Enzymatic components in aphid OSs can circumvent plant defenses by harnessing a molecular affinity for ions necessary for many physiological processes. This provides a molecular strategy to exploit vital calcium-dependent mechanisms vulnerable to the manipulation of ion concentration gradients (Will *et. al.*, 2007). Limiting the availability of free calcium inhibits plant defenses that prevent continued leakage of phloem through puncture wounds of aphid feeding sites. By preventing the occlusion of sieve tubes, OS components can prolong aphid feeding periods. Other protein effectors within aphid OSs have been implicated that promote aphid feeding. Though the mechanism is still unclear, C002 appears to play a vital role in the feeding process as *c002*-knockdown aphids could not sustain feed and had shorter lifespans than

aphids with intact *c002* genetic mechanisms (Mutti et. al., 2008).

Constituents present in aphid OS can also serve plant recognition systems by providing signals indicative of a potential threat. Detection of specific elicitors instigates a complex series of biochemical transformations that expedite the mobilization of the signal through an intricate molecular network. There, the signal is integrated and directed down pathways destined for the activation of necessary defenses. Defense signaling activities elicited by polyphenol oxidases (PPO) within aphid OSs are regulated by an important phytohormone jasmonic acid (JA) (Moran & Thompson, 2001). Ma *et. al.* (2010) demonstrated high expression levels of a gene (*aos*) encoding a key JA-associated enzyme in plants treated with OSs from grain aphid and PPO at concentrations consistent with aphid OSs. Several important defenses are attributed to JA activity, including production of proteinase inhibitors, such as peroxidases (POD) that target specific insect metabolic enzymes. Polyphenol oxidases are also major plant enzymes with similar functioning to POD, and which reduce plant nutritive quality, digestibility, and palatability to herbivorous insects (Bhonwong *et. al.*, 2013).

Biosynthesis of defense pathways regulated by JA are primarily generated in response to insect feeding and necrotrophic pathogens. Salicylic acid (SA) is another important defense signaling phytohormone activated by biotrophic pathogens and piercing-sucking insects like aphids. These different phytohormone signaling pathways do not function independently, but instead function simultaneously and modulate one another through antagonistic interactions. There are numerous reports of OSs from insects which appear to exploit negative JA/SA crosstalk. Glucose-oxidase within aphid OSs is a strong inhibitor of lipoxygenase activity leading to production of JA

(Musser *et. al.*, 2005; Giordanengo *et. al.*, 2010). By inhibiting challenging JA-mediated defenses, this enzyme ameliorates aphid stress and will promote less efficient SA-regulated defenses. Also, by presenting SA-eliciting symbiotic microbes via OSs, insects may employ a decoy strategy to reroute plant responses away from JA-signaling.

Advancements in technology have afforded researchers the tools to identify elements engaged in molecular processes, and gradually studies are linking the fragments of pathways building an elaborate signaling network. The erratic behavior of elements functioning within different pathways has limited the progress of research attempting to characterize them. There is still a lack of clarity about the origin of many molecular elements that influence different physiological processes. Novel information regarding defenses in plant-insect systems has traced to the source of molecules inhibiting plant activity to microbes housed within the gut of an insect (Chung *et. al.*, 2013), and a growing body of evidence is indicating that plant defensive signal transduction can be under the regulation of intimately associated endosymbionts of insects (Barr *et. al.*, 2010; Spiteller, Dettner, & Boland, 2000; Casteel *et. al.*, 2012). Nearly all insects contain a consortium of symbiotic microbes within different organs and tissues, and aphids have a very well-documented collection of these endosymbionts that confer many physiological benefits.

Endosymbiotic microbes from *Enterobacteriaceae* provide aphids hosts enduring stress with several protective benefits, including increased resistance to parasitism (Oliver, Moran, & Hunter, 2005) and elevated thermal tolerance (Dunbar *et. al.*, 2007), and can also facilitate host plant use (McLean *et. al.*, 2011). Ancient associations have resulted in mutual dependency of aphids and an obligate endosymbiont *Buchnera aphidicola* that fulfills an aphid nutritional

deficiency. However, a functional role of endosymbionts within aphid-plant interactions has not yet been defined.

Closely associated endosymbionts share a mutual interest upon the quality of the internal conditions of insect hosts for survival. Therefore, it is likely that their influential mechanisms have evolved to operate beyond the plant-insect interface where there are processes working to inflict harm to insect hosts. Microbes or microbial-derived components may be delivered to plants through host OSs during feeding to moderate the activity of harmful plant defenses. Using the pea aphid (*Acyrthosiphon pisum*), this study identifies whether endosymbiont infection of aphids correlates with differential plant defense activity. Plants were infested with aphids differing in their infection status with the facultative endosymbiont *Regiella insecticola* which is known to enhance host defenses, especially aphid parasitoid resistance. Comparisons were made of the levels activity of defense related enzymes and transcripts of defense-related genes in pea plants receiving different aphid infestation treatments. Experiments were also conducted to assess different aspects of aphid development and survival to identify whether *R. insecticola*-infection status related to differential aphid development.

## 2.2 Materials and Methods

**2.2.1 Insects.** Colonies of *Acyrthosiphon pisum* of the 5A genotype were obtained from the laboratory of Dr. Nicole Gerardo at Emory University (Atlanta, GA). Aphids were reared on fava bean plants in a growth chamber maintained at  $20\pm1^{\circ}\text{C}$  and 14:10 L:D photoperiod. Three aphid colonies were maintained, two harboring one of two different strains of *Regiella insecticola* ( $\text{R}^+$  aphids; 5A-Ri or 5AU-2) and one colony with aphids lacking these facultative

endosymbionts ( $R^-$  aphids; 5A0). The colonies with facultative endosymbionts were established from 5A0 aphids through experimental infection with a single strain of endosymbiotic bacteria. To confirm endosymbiont presence or absence, DNA extraction from single, whole insects was conducted using a QIAamp mini kit (QIAGEN) and diagnostic PCR was performed on both  $R^+$  and  $R^-$  aphids. Aphids were also screened for possible contamination with other genera of facultative endosymbionts (i.e. *Serratia symbiotica* and *Hamiltonella defensa*). Primers specific to the 16S ribosomal RNA genes of each bacterial species and a temperature profile of 94°C for 2 min, followed by 30 cycles consisting of 94°C for 1 min, 55°C for 1 min, and 70°C for 2 min was used. Agarose gel electrophoresis was performed to analyze each of the PCR products.

**2.2.2 Enzyme Assays** Enzymes are generated by plants during their responses to numerous stressors and provide effective defensive strategies. The oxidative enzymes examined in this study, polyphenol oxidase (PPO) and peroxidase (POD), are components of plant defense strategies to deter herbivorous insects, including those inhibiting insect digestion. By oxidizing phenols, these enzymes generate reactive o-quinones that polymerize with the nucleophilic groups of proteins. This process limits the availability of free amino acids to be utilized during the insect digestion process. The treatments used in the study were as follows: 1) pea plants infested with *R. insecticola* strain 5AU-infected aphids, 2) pea plants infested with *R. insecticola* strain 5A-Ri-infected aphids, 3) pea plants infested with aphids lacking *R. insecticola* infection, and 4) uninfested pea plants as controls. Both aphid lines infected with either *R. insecticola* strain are referred to as  $R^+$  aphids and the aphids lacking *R. insecticola* infection are called  $R^-$  aphids.

Snow pea plants were infested with R<sup>+</sup> or R<sup>-</sup> aphids for a 48-hour feeding period after which all of the aphids were removed. Then, at 0h, 24h, and 48h hours after aphid removal, plant tissue was sampled, frozen in liquid nitrogen, and kept -80°F until use. Leaves were ground in liquid nitrogen and homogenized in 2.0 ml ice cold 0.05 M phosphate buffer (pH 7.2 for POD, pH 7.8 for PPO) containing 1% (w/v) polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 12,000 g for 45 min at 4°C. The supernatant was collected and used for assaying the activities of POD and PPO by using spectrophotometer. Four replicates were performed for each treatment at each time point.

**2.2.3 Total RNA Isolation and cDNA Synthesis.** To obtain RNA samples from plants that will be used to assess their defense-related genetic activity, snow pea plants were infested with R<sup>+</sup> or R<sup>-</sup> aphids for a 48-hour feeding period after which all of the aphids were removed. Thereafter, plant tissue was sampled, frozen in liquid nitrogen, and kept -80°F until use. Three replicates of each of the 4 treatments described earlier were performed. Frozen tissue samples were ground to a fine powder in liquid nitrogen with a pestle and mortar. Total RNA was extracted from 100 mg of each plant sample using Spectrum<sup>TM</sup> plant total RNA kit (Sigma Aldrich USA), according to the manufacturer's instructions. RNA concentration and purity was determined using a NanoDrop<sup>TM</sup> Spectrophotometer ND-2000 (Thermo Scientific, Wilmington, USA). First stand cDNA will be synthesized from 200 ng RNA using a First-Strand cDNA Synthesis by using Goscrypt<sup>TM</sup> reverse transcription system Kit (Promega USA) according to the manufacturer's instructions.

**2.2.4 Real-Time PCR.** To quantify the expression of genes regulated by the phytohormones jasmonic acid (JA) and salicylic acid (SA) (*Proteinase inhibitor 1*, *Pathogenesis-related protein 1*, *Allene oxide synthase*, and *Phenylalanine ammonia-lyase 1*) in plants infested with either R<sup>+</sup> or R<sup>-</sup> aphids, real-time quantitative RT-PCR was performed. The real-time PCR was carried out on an ABI 7500 Real Time PCP System (Applied Biosystems) with a 96-well rotor. The amplification reactions were performed with 25 µl of mixture consisting of 12.5 µl of PerfeCTA® SYBR® Green Fastmix®, ROX qPCR Master Mix (Quanta biosciences, Inc, USA), 0.5 µl of cDNA and 100 nM primers (Integrated DNA Technologies, Coralville, IA, US). Primers were designed by using previously cloned template genes from NCBI and designed with help of Primer3 software. Relative RNA levels were calibrated and normalized with the level housekeeping genes for Actin ribosomal mRNA. PCR conditions were determined by comparing threshold values in a dilution series of the RT product, followed by non-template control for each primer pair. Relative expression levels of genes were calculated by using the Pfaffl method, and a suitable melt curve analysis was performed.

**2.2.5 Aphid Fitness.** Experiments were conducted to assess different parameters of aphid development to characterize their performance and make comparisons between R<sup>+</sup> and R<sup>-</sup> aphids to implicate *Regiella insecticola* as a potential contributor to aphid performance. Each experiment was performed in a growth chamber maintained at 20±1°C and 14:10 L:D photoperiod. Three adult pea aphids from either the R<sup>+</sup> or R<sup>-</sup> colony were placed on a single pea plant for a 24-hour period of reproduction, after which the adult aphids were removed. All clones were left on the plant and were checked daily to record information regarding their development. Due to the fact that the 5A-Ri aphid line lost its infection with *R. insecticola*, we

were only able to use the 5AU aphid line to represent the R<sup>+</sup> aphids for these experiments. Certain parameters were chosen to serve as reliable indicators of the quality of aphid development. These included the following: 1) percent of initial colony of nymphs surviving to adulthood, 2) number of days to reach adulthood, and 3) average number of days at which 50% mortality and 100% mortality of the adult colony was reached. Each individually treated plant was considered a single biological replicate. Four replicates were performed.

**2.2.6 Data Analysis.** A one-way Analysis of Variance (ANOVA) was performed using SigmaPlot software with a statistical significance of  $P<0.5$ . Data that did not meet the requirement of normality were log-transformed. This was followed by a Tukey-Kramer HSD multiple comparison test.

## 2.3 Results

**2.3.1 Plant Enzymatic Activity.** The activity levels of polyphenol oxidase (PPO) and peroxidase (POD) in pea plants were typically highest after feeding damage by R<sup>-</sup> aphids (lacking the endosymbiont; 5A0) (Fig. 1A & B). At the 24 and 48 hour sampling periods, the activity of PPO (Fig. 1A) was significantly higher in plants infested with R<sup>-</sup> aphids than uninfested and R<sup>+</sup> aphid (with the endosymbiont; 5A-Ri and 5AU-2) infested plants ( $P <0.001$ ). Similarly, activity of POD (Fig. 1B) was significantly higher in plants infested with R<sup>-</sup> aphids at both the 24 and 48 hour sampling periods than all other treatments ( $P <0.001$ ).

**2.3.2 Transcript Levels of Defense-Related Genes.** In general, transcript levels of defense-related genes related to either JA or SA signaling were significantly higher in plants fed

on by R<sup>-</sup> aphids than in plants infested with R<sup>+</sup> aphids (Figure 2;  $P < 0.001$ ). Furthermore, feeding by R<sup>+</sup> aphids generally resulted in significant genetic suppression to levels below those of uninfested plants likely representative of constitutive expression, and below levels following infestation with R<sup>-</sup> aphids. These results are supportive of our hypothesis that *R. insecticola* has adapted strategies to modify plant defenses, likely in order to alleviate stress experienced by aphid hosts.

**2.3.3 Aphid Survival and Fecundity as Measures of Performance.** To determine if there were any effects of the suppression of defense-related plant activities on aphid performance, four parameters were chosen to describe the development and fecundity of aphids throughout all life stages. Although there were more R<sup>+</sup> nymphs that survived to the adult life stage than nymphs from R<sup>-</sup> aphids, the result was not significant (Figure 3;  $P=0.515$ ). Similarly, there was no significant effect of *R. insecticola* infection in the average total number of clones produced by aphids (Figure 4;  $P = 0.469$ ) and there were no differences in the survivability of R<sup>+</sup> and R<sup>-</sup> aphids as measured by the average number of days until 50% mortality (Figure 5;  $P=0.377$ ) and 100% mortality (Figure 5;  $P=0.786$ ) of each adult aphid colony.

## 2.4 Discussion

A growing body of evidence has revealed that metabolites or proteinaceous components of oral secretions (OSs) introduced to plant molecular systems by insects as they consume their tissue, can elicit, redirect, or suppress molecular signaling of plants. Furthermore, certain constituents present in OS originate from endosymbiotic microbes often colonizing their gut (Chung *et. al.*, 2013). Our results are the first to provide support of a contributing function of an aphid

endosymbiont in defense reactions occurring within an aphid host plant. Overall, infestation of pea plants with aphids lacking the facultative endosymbiotic bacteria, *Regiella insecticola* (hereon R<sup>-</sup> aphids) exhibited the most pronounced defensive activities compared to those from plants that received all other treatments involving infected aphids or uninfected plants. Conversely, infestation with *R. insecticola*-infected aphids (hereon R<sup>+</sup> aphids) resulted in debilitated plant defenses, as evidenced by the inert behavior of defenses that performed beneath levels expressed in uninfested plants. The consistency and repeatability of the observed molecular activities in the uninfected plants that were not exposed to aphid feeding damage suggests that the levels observed in these plants may represent constitutively expressed traits. However, no testing was done to confirm this.

After a few hours of aphid feeding the activities of pea plant the defense-related enzymes, polyphenol oxidase (PPO) and peroxidase (POD), were most elevated in plants treated with R<sup>-</sup> aphids. As time progressed, significant differences in the behavior of enzymes transpired as activity levels grew in plants treated with R<sup>-</sup> aphids yet remained at or below activities exhibited by uninfested plants in R<sup>+</sup> aphid-treated plants. Furthermore, with the exception of *Pathogenesis-related protein 1 (PR-1)*, the abundance of defense gene transcripts were significantly different between each treatment, and the highest levels of gene expression occurred in plants treated with uninfected aphids. Interestingly, for *PR-1* the only treatment combination to produce significantly different transcript levels was R<sup>-</sup> aphids vs. 5AU, R<sup>+</sup> aphids. However, because defense induction was overall significantly lower in plants R<sup>+</sup> aphids compared to all other treatments, the results suggest a suppressive role for *R. insecticola* in pea plant defense responses to pea aphid activity.

The endosymbiont may interfere with plant defenses either directly by delivering its molecular byproducts through aphid host OSs, or defenses can be altered indirectly as products resulting from *R. insecticola*'s interactions with other microbes present within the host. The former explanation was recently supported by a very recent study on the gut microbes that used a well-rounded set of experiments to clearly implicate orally secreted microbes as major regulators of plant-insect defensive interactions. Chung *et. al.*, 2013 found bacteria from three genera (*Stenotrophomonas* sp., *Pseudomonas* sp., and *Enterobacter* sp.) suppressed plant defenses among the microbial assemblage found in Colorado potato beetle larval OSs. Specifically, *Pseudomonas* sp. produces flagellin as an effector of JA- mediated defenses involved in inhibiting PPO activity and down regulating expression levels of *PPOF*. However, expression of SA-regulated *Pathogenesis Related Protein-1*(*PR-1*) was increased, a trend recognized in previous studies of OSs from many different insects (Weech *et. al.*, 2008; Takabayashi *et. al.*, 2000, Zarate *et. al.*, 2007).

Gene expression in this study is did not clearly indicate an aphid strategy of exploiting negative JA/SA cross-talk in plants. Transcripts of the SA-regulated *PR-1* were lower than the other genes regulated by JA-signaling across all treatments. While lower *PR-1* expression and higher JA-defense activation could be expected in R<sup>-</sup> aphids, the inverse behavior, where SA defenses are higher than JA defenses, was not present in R<sup>+</sup> aphids, and, thus, does not reflect cross-talk manipulation. There are countless explanations for the discrepancy and confirmation of the results by repeated experiments that were not possible in this study due to time constraints and limitation of resources, would build confidence in these results. Other than experimental error,

inherent differences in the model insect-microbe system could account for the opposing trend. However, aphids, and piercing-sucking insects in general, have been associated with SA-related plant defenses and a weaker induction of defenses regulated by JA; thus, it is likely that higher expression of genes related to JA signaling and lower levels of SA-related gene expression found in this study are due to human or mechanical error.

More inconsistencies with previous studies is presented by the higher gene expression of SA-regulated *PR-1* in plants fed on by R<sup>-</sup> aphids lacking the endosymbiont than levels produced in plants treated with R<sup>+</sup> aphids. There, again, are multiple explanations for this outcome, including experimental error or influence from the obligate endosymbiont *Buchnera aphidicola* or other unidentified microbes within aphids. *B. aphidicola* is not eliminated by the antibiotic-treatment used to clear facultative endosymbionts to establish the 5A0 line, and the case may be the same for unknown microbes that may colonize these aphids. When establishing infected colonies, introduction of *R. insecticola* likely impacts the microbial ecology of aphid endosymbionts that may antagonize or promote one another. As demonstrated by the results obtained in this study, *R. insecticola* may antagonistically effect the SA-inducing microbial population, and the intensity of the negative impact may be strain-dependent. However, despite obvious discrepancies in gene expression, this study does indicate an influence from *R. insecticola* in the defensive interactions of aphids and plants.

Our performance experiments, however, did not significantly correlate greater aphid survival or fecundity to suppressed plant defenses. This is may be an effect of other mechanisms not tightly coupled with *R. insecticola* that play a larger role in physiological processes regulating important

aspects of aphid health. Perhaps major effects could be tied back to nutritional benefits provided by *B. aphidicola*, and although *R. insecticola* may suppress plant defenses there are other intervening mechanisms operating to support aphid health. Although aphids infected with *R. insecticola* performed better than uninfected aphids in nymph survival experiments, the differences were not significant. Similarly, adult survival and fecundity were not significantly linked to *R. insecticola*.

It is important to note that no studies have yet to identify a nutritional role or major physiological function for *R. insecticola* in aphids. Preliminary studies correlating *R. insecticola* and down-regulated gene expression merited using aphids infected with this particular facultative endosymbiont. Infected aphids receive major advantages in their ability to manage stress inflicted by natural enemies, and when exposed to certain parasitoid wasps or fungal pathogens, infected aphids perform significantly better than aphids without *R. insecticola*. In the absence of stress, endosymbionts impose costs to hosts that must allocate resources to processes maintaining microbial abundances, and this may reflect the sporadic loss of facultative endosymbionts observed in aphid populations (Laughton *et. al.*, 2014).

There were flaws in the methodology which likely contributed to the experimental outcome. Experiments did not begin with equal numbers of aphid nymphs which may have presented artefacts. However, additional experiments performed to account for this error also lacked significant results. These experiments were not included in the study because they used aphids from a different stock of the 5AU line aphids with moderately different performance than the original colony used in previous experiments that lost its *R. insecticola* infection. Perhaps, there

is also a strain-dependent effect on aphids and the 5A-Ri line that eradicated *R. insecticola* before performance experiments began, may have performed differently. Finally, although pea aphids are legume specialists, use of pea plants in this study may have masked the effects of *R. insecticola* in aphids that have been maintained on fava plants since 2003. Restricted resources prevented the option of designing primers which were not available for fava, but were for pea plants.

## 2.5 Conclusion

Symbiotic microbes assume a vague presence in the environment experienced at most biological levels, but their elusive contributions at the molecular scale are ubiquitous among cellular processes of most life forms. Many physiological traits of higher-order organisms can be attributed to different bacteria, fungi, or viruses that colonize a variety of organs and tissues. Although microorganisms in general are notorious for causing debilitating diseases, mutualistic relations between hosts and endosymbionts is very common.

Insect endosymbioses are prevalent in nature, but the implications of these associations in multitrophic ecology are only beginning to be understood. Aphids arguably have the best-studied collection of endosymbiotic bacteria that have evolved obligate roles in nutrition or traits to promote tolerance of aphids to commonly-encountered forms of stress. A growing body of evidence has revealed that endosymbionts influentially moderate plant defenses to herbivory by delivering elicitor or effector molecules through their host's oral secretions. As OSs of aphids are known to play a critical role in aphid colonization of host plants, it is very likely that aphid gelling and/or watery saliva may contain endosymbiont-derived molecules involved in the

modulation of plant defenses.

Although time and resource restrictions limited the depth of our experiments in order to clearly implicate *R. insecticola* in plant defense manipulation, this study found a definite relationship between presence of the endosymbiont and the suppression of plant defenses. These findings will supplement future experiments which isolate samples of aphid oral secretions to apply to wounded plant tissue and diagnose microbial presence within samples. Furthermore, identification of the particular pathways involved in the plant defense responses would eliminate some of the suspected discrepancies of this study and previous work in regards to aphid exploitation of phytohormone-signaling cross-talk.

## **2.6 Acknowledgments**

This study would not have taken place without some generous contributions. I would like to thank Dr. Nicole Gerardo of Emory University, Atlanta, GA for contributing the aphids used in this study. Also, thank you Dr. David Held of Auburn University, AL for providing ample space in your growth chambers to rear aphids and perform the experiments. Finally, Dr. Simon Zebelo, I am greatly indebted to you for your eagerness to teach me about molecular biology so I could properly conduct each of the experiments in this study. Your incredible work ethic and your genuine passion for research were really appreciated and encouraging.

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## 2.8 Figure Legends

**Figure 1.** Activity of PPO (A) and POD (B) was assessed in pea plants after a 48-hour period of infestation with aphids harboring ( $R^+$  aphids; 5A-Ri; 5AU) or lacking ( $R^-$  aphids; 5A0) the endosymbiont, *Regiella insecticola*. Samples were collected from different plants at 0, 24, and 48 hours after aphid infestation. At the 24 and 48 hour sampling periods, the activity of PPO was significantly higher in plants infested with  $R^-$  aphids than uninfested and  $R^+$  aphid (with the endosymbiont; 5A-Ri and 5AU-2) treatments ( $P < 0.001$ ). Similarly, activity of POD was significantly higher in plants infested with  $R^-$  aphids at both the 24 and 48 hour sampling periods than all other treatments ( $P < 0.001$ ).

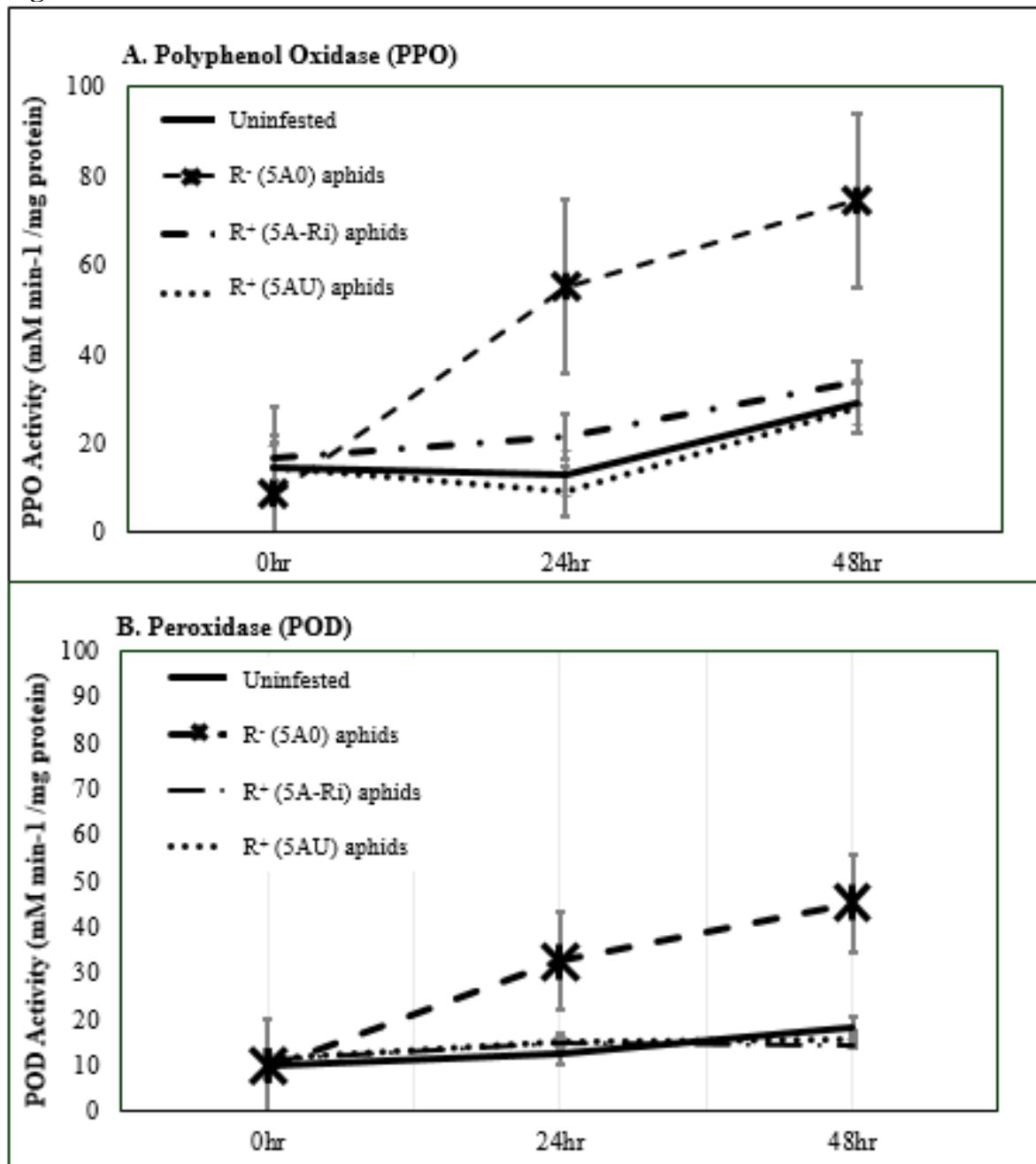
**Figure 2.** Transcript levels of defense-related genes in pea plants infested for 48 hours with aphids harboring ( $R^+$  aphids; 5A-Ri; 5AU) or lacking ( $R^-$  aphids; 5A0) the endosymbiont *Regiella insecticola*. Samples were collected 48 hours after the removal of aphids. Transcript levels of defense-related genes associated with either the JA (*PIN-1*, *AOS*, and *PAL-1*) or SA (*PR-1*) molecular signaling pathways were examined. Expression of *PIN-1* was significantly higher in plants after  $R^-$  aphid infestation compared to all other treatments and significantly lower following  $R^+$  aphid treatment ( $P < 0.001$ ). Similarly, *AOS* expression was significantly different between most treatment combinations ( $P < 0.001$ ), but not for uninfested vs. 5A-Ri,  $R^+$  aphid

treatments. Interestingly, for *PR-1* the only treatment combination to produce significantly different transcript levels was R<sup>-</sup> aphids vs. 5AU, R<sup>+</sup> aphids ( $P<0.038$ ). Feeding by R<sup>+</sup> aphids significantly reduced *PAL-1* expression to levels below those in plants following R<sup>-</sup> aphid infestation ( $P<0.001$ ), but there was no significant difference between uninfested and R<sup>+</sup> aphid treatments.

**Figure 3.** Aphid performance at different life stages as measured by averaging the number of nymphs surviving to adulthood (A) and the colony size generated by the surviving adult aphids throughout their lifetime (B). Adult survivability was determined as the number of days until 50% of aphids had deceased and 100% of adults had deceased (C). Although there were more R<sup>+</sup> nymphs that survived to the adult life stage than nymphs from R<sup>-</sup> aphids, the result was not significant (A;  $P=0.515$ ). Similarly, there was no significant effect of *R. insecticola* infection in the average total number of clones produced by aphids (B;  $P = 0.469$ ), and there were no significant differences in the survivability of R<sup>+</sup> and R<sup>-</sup> aphids as measured by the average number of days until 50% mortality (C;  $P=0.377$ ) and 100% mortality ( $P=0.786$ ) of each adult aphid colony.

## 2.9 Figures

Figure 1.



**Figure 2.**

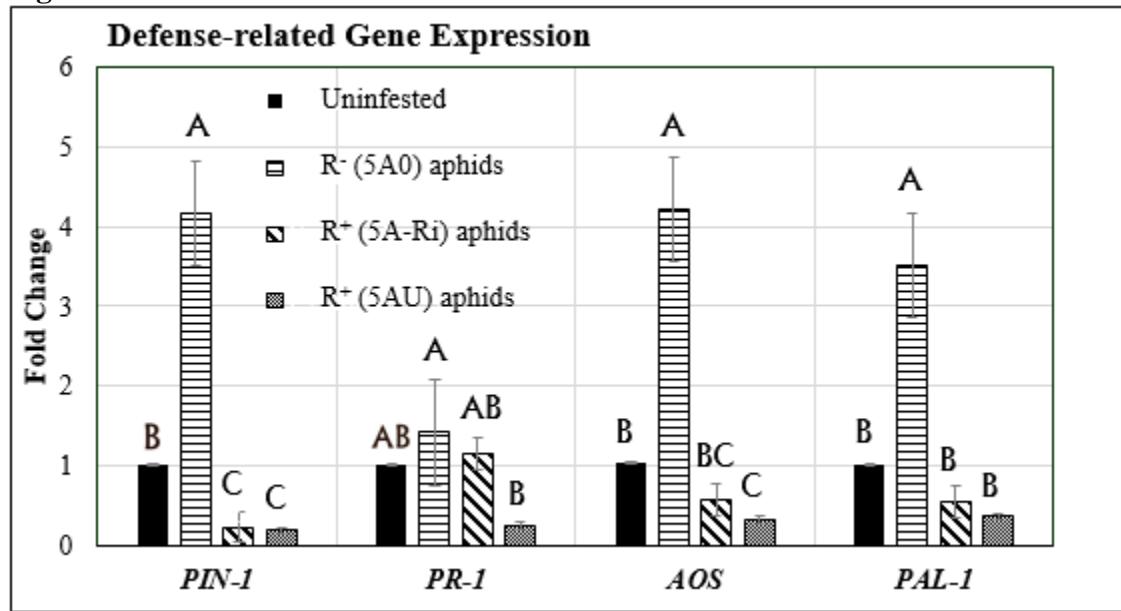


Figure 3.

