THE RELATIONSHIP BETWEEN ONION THRIPS (*THRIPS TABACI*) AND THE BACTERIAL PATHOGENS *PANTOEA AGGLOMERANS* AND *PANTOEA ANANATIS*

By

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ABSTRACT

THE RELATIONSHIP BETWEEN ONION THRIPS (*THRIPS TABACI*) AND THE BACTERIAL PATHOGENS *PANTOEA AGGLOMERANS* AND *PANTOEA ANANATIS*

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Onion thrips (*Thrips tabaci*), a cosmopolitan pest of onion, are known vectors of *Pantoea agglomerans*, causal agent of bacterial leaf blight, and *P. ananatis*, causal agent of bacterial center rot. The objectives of this thesis were to determine the relationship between onion thrips and *P. agglomerans* under field conditions, investigate the effectiveness of combining insecticides and bactericides for managing both onion thrips and bacterial leaf blight and determine the role of feeding injury by onion thrips in influencing the pathogenicity of *P. ananatis*. In the first study, positive correlations between thrips population density, proportion of thrips positive for *P. agglomerans* and bacterial leaf blight incidence in onion fields were determined. In the pesticide trial conducted in 2015 and 2016, insecticides reduced both onion thrips abundance and bacterial leaf blight incidence, while bactericides showed no effect on reducing bacterial leaf blight symptoms. In the laboratory study significant positive correlations were determined between the number of thrips per plant and the severity of leaf blight symptoms as well as the percentage of feeding damage and the severity of leaf blight symptoms. Lastly, by utilizing a GFP-tagged strain of *P. ananatis*, the colonization of onion thrips feeding injury by *P. ananatis* was observed using fluorescence microscopy. These studies suggests that onion thrips play a significant role in the development of bacterial leaf blight and center rot in onions. Results from the pesticide trial indicate that thrips feeding damage is positively correlated with bacterial leaf blight incidence. Therefore, in order to improve bacterial disease suppression in onions, management efforts should focus on reducing onion thrips populations.

This thesis is dedicated to my mother and father
Thank you for your unconditional support

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Introduction

The importance of onion (*Allium cepa* L.), commonly known for causing tears in the kitchen, as a food source for humans has been evident throughout the ages. First cultivated in ancient Egypt (ca. 3000 BCE), onions symbolized eternity and were often found buried in the tombs of Pharaohs (Fritsch and Friesen 2002). During modern times, onions are grown across the world and are a staple vegetable used as an ingredient in dishes across a wide diversity of cultures (Griffiths et al. 2002). Dry bulb onions became a staple food partly due to their long-term storage characteristics and durability during the shipping process (Griffiths et al. 2002).

Onions are grown internationally by approximately 170 countries, the leaders being China (~33% global production), India (~13%), United States (~5%), Turkey (~3%) and Pakistan (~3%) (FAOSTAT 2014). In the United States this industry is valued at $1 billion dollars per year (USDA/NASS 2015). The leading producers of onions in the United States are Washington, Idaho - Eastern Oregon and California, which combined represent approximately 50% of United States onion production (National Onion Association 2017). Michigan's onion industry is valued at approximately $10 million per year accounting for 1% of national onion production (USDA/NASS 2015). In terms of acreage, Michigan currently ranks 11th in terms of acres harvested; however, this number has been decreasing annually, falling from 5,067 acres in 1997 to 2,400 acres in 2016 (USDA/NASS 1997, 2016).

Onions vary in color, flavor profile, size and seasonal availability. Yellow onions make up over 87% of the US crop while the other 13% is comprised of red and white onions (National Onion Association 2017). Onion varieties can also be regionally specific; for example, Vidalia onions are grown on only 6,000 hectares in Georgia but represent 40% of the spring onion production value of the United States (Boyhan and Torrance 2002). Most of Michigan's 45 million kilograms of commercial onions produced per year are for dry bulbs stored through the winter, and are direct seeded.

Onion pests

Onions are attacked by a variety of viruses, bacterial and fungal diseases and insect pests. The most common virus that affects onion is Iris Yellow Spot Virus (*Bunyaviridae, Tospovirus* spp.; IYSV). IYSV was originally identified in Brazil (Pozzer et al. 1994) but has spread to other major onion producing areas of the world (Hall et al. 1993, Gent et al. 2006). Symptoms of IYSV appear as lesions along the edges of leaves (Gent et al. 2006) which reduce bulb size (Gent et al. 2004) and cause yield loss (Pozzer et al. 1999). The only confirmed vector of this pathogen is the onion thrips (*Thrips tabaci* Lindeman, Thysanoptera: Thripidae), an economically important indirect pest of onion (Kritzman et al. 2001).

Many fungal pathogens cause onion diseases which affect different portions of the plant. Pink root, caused by *Setophoma terrestris* (H.N. Hansen) Gruyter, Aveskamp and Verkley, is characterized by the pink color of infected roots, and causes the shrinking and withering of onion roots (Sumner 1997). This leads to earlier development of onion bulbs which results in a reduction of bulb size (Sumner 1997). Purple blotch is a foliar disease of onion caused by the fungal pathogen *Alternaria porri* (Ellis) which enters the plant through openings in the leaves

(Miller 1983). Purple blotch infection can lead to premature defoliation and can compromise bulb quality. The feeding damage of onion thrips has been linked with purple blotch incidence, providing alternative entry points for *A. porri* (McKenzie et al. 1993). Downy mildew, caused by the fungal pathogen *Peronospora destructor*, causes premature death of onion leaves and a reduction in bulb size, it can also indirectly cause storage rot (Scholten et al. 2007).

Bacterial diseases of onion cause variable levels of yield losses, and can be especially severe when weather conditions favor the growth of pathogens. Xanthomonas leaf blight is a common foliar disease of onion, caused by *Xanthomonas axonopodis* pv. *allii*. Xanthomonas leaf blight favors conditions of high humidity and high temperatures (> 26 ºC) (Alvarez et al. 1978). This disease reduces photosynthetic areas leading to a reduction in bulb size and severe yield losses (> 20%) (Sanders et al. 2003). Two species belonging to the genus of *Pantoea* are causal agents of bacterial leaf blight (*Pantoea agglomerans*) and bacterial center rot (*Pantoea ananatis*), which; can cause foliar damage as well as bulb rot during long-term storage (Gitaitis and Gay 1997, Edens et al. 2006). The symptoms of center rot are white necrotic streaks that run along the length of the leaf which can progress into the bulb and predispose it to rotting caused by secondary microbes (Gitaitis et al. 2003, Edens et al. 2006). Bacterial diseases are spread around fields during wind and rain events (Wiriyajitsomboon et al. 2014), and often enter plant tissue through wounds caused by extreme weather, machinery and field workers (Gitaitis et al. 1978). Onion thrips can also transmit the bacterial pathogens, *P. ananatis* and *P. agglomerans*, to onion through feces (Dutta et al. 2014).

Onions have a variety of insect pests, both direct and indirect, that cause damage to different areas of the plant. In the United States, the two major insect pests of onion are the onion maggot (*Delia antiqua* Meigen, Diptera: Anthomyiidae), which causes damage to the bulb and

seedlings, in the larval stage (Whitfield et al. 1985), and onion thrips, which cause damage to leaf tissue as both larvae and adults (Gill et al. 2015). *Delia antiqua* is a direct pest of alliums since it attacks the marketable portion of the crop, the bulb (Ellis and Eckenrode 1979). It has multiple generations in Michigan but first generation are of most concern because they cause severe damage early in the season (Hoepting et al. 2004). Eggs, which are laid in the soil surrounding the bulbs, hatch and larvae feed continuously on developing seedlings and bulbs (2-3 weeks) until they pupate into adults (Loosjes 1976). Feeding damage from *D. antiqua* varies depending on the stage of the onion. In the seedling stage feeding damage results in the death of the seedling which can reduce stands in the early season. If feeding occurs in the mid or late season the damage results in a rotting bulb making onions unmarketable (Hausmann and Miller 1989). Since seed treatments are currently an effective way to manage *D. antiqua*, in Michigan, onion thrips are the most important insect pest of onions requiring frequent insecticide applications to maintain their populations below an economic threshold (Fournier et al. 1995). Onion thrips has also been linked to the transmission of plant pathogens as both a direct and an indirect vector which will be the focus of this thesis.

Onion thrips

Biology. Since its first description in 1888 (Lindeman 1889), onion thrips have been the subject of considerable research and extension. Onion thrips possess unique asymmetrical mouthparts made up of a single mandibular stylet and paired maxillary stylets. They use this single mandible to pierce through the cell membrane upon which they retract the mandible and insert their maxillary stylets (Chisholm and Lewis 1984). Pre-digestion substances are released into the plant which help break down leaf tissue (Boateng et al. 2014). Onion thrips use a tube formed by the

maxillary stylets to draw up and consume mesophyll cells by cibarial pumping (Chisholm and Lewis 1984). This unique feeding style is often referred to as "punch and suck" (Lewis 1991). Thrips damage can be characterized by silvery leaf spots that turn into white blotches along the leaves, a result of the loss of chlorophyll from feeding (Bailey 1938). Thrips feeding stresses onion plants through the destruction of the leaf tissue ultimately reducing the photosynthetic ability of the plant (Molenaar 1984, Parrella and Lewis 1997). In severe cases damage by onion thrips has caused yield reductions of up to 60% in bulb weight (Waiganjo et al. 2008, Kendall and Capinera 1987, Fournier et al. 1995, Rueda et al. 2007, Diaz-Montano et al. 2010). The feeding damage caused by onion thrips can also create an entry point for plant pathogens (Orloff et al. 2008).

Life history. Onion thrips are holometabolous insects with a lifecycle consisting of an egg, first and second instar stages, prepupa, pupa, and adult (Ghabn 1948, Nakahara 1991, Diaz-Montano et al. 2011). They feed on plant tissue as first and second larval instars as well as adults. Eggs are laid singly and inserted directly into the leaf tissue. They are microscopic, averaging 0.23mm long and 0.08mm wide (Patel et al. 2013) and incubate for a period of 4 to 5 days inside the leaf before hatching (Fekrat et al. 2009). Larvae are semitransparent and dull white as first instars later becoming more yellow as second instars (Patel et al. 2013, Gill et al. 2015). The average length of first and second instars is 0.35-0.38mm and 0.7-0.9mm, respectively (Gill et al. 2015), and they spend 2 to 3 days in first instar stage and 3 to 4 days in the second instar stage (Pourain et al. 2009).

During the prepupal and pupal stage onion thrips do not feed. Pupation normally occurs at the base of the onion's apical meristem or within the soil (Rueda and Shelton 1995). The entire

pupal period lasts between 3 to 10 days (Patel et al. 2013), with temperature causing variation in pupation time (Diaz-Montano et al. 2011). Adults are generally 1.0-1.3mm in length (Orloff et al. 2008), and have a longevity varying between 28-30 days on onion (Patel et al. 2013). The adult's body color varies with temperature from yellow to brown (Murai and Toda 2002), and adults possess fringed forewings and hindwings making them more mobile than larval and pupal stages (Gill et al. 2015). Adult onion thrips are able to disperse various distances ranging from trivial dispersal (short-range movement between plant hosts) to long-distance dispersal (long-range flight away from immediate plant hosts). Onion thrips flight tends to occur when daylight is present, temperatures are above 17 ºC and wind speeds are low (Smith et al. 2016). Long-distance movement often occurs in the late season when insecticide applications are reduced which can lead to the spread of plant pathogens or insecticide-resistant alleles (Smith et al. 2015).

Reproduction. Onion thrips have the ability to reproduce asexually through parthenogenesis. Most commonly onion thrips reproduce through a mode of parthenogenesis in which females are produced from unfertilized eggs, called thelytoky. An alternative mode of parthenogenic reproduction, arrhenotoky, in which males are produced from unfertilized eggs, is much less common (Gill et al. 2015). Consequently male onion thrips are much less common in most areas of the world (Lewis 1973, Kendall and Capinera 1990). Asexual reproduction leads to multiple problems: (1) thelytokous individuals do not need to find a mate and therefore reproduction is not mate-limited and, (2) if the adult female is resistant to an insecticide, her offspring will be resistant as well (Diaz-Montano et al. 2011). This becomes problematic to onion growers because it can result in large resistant populations and major/significant economic yield losses.

Onion thrips development and reproduction is dependent on environmental conditions, such as temperature. Six to eight generations per growing season have been estimated for New York (Hoffmann et al. 1996), and similar generation numbers were observed in Kentucky and North Carolina (Bessin 2004 and Carter and Sorenson 2013). Females begin laying eggs as soon as temperature increases during the spring (Sites and Chambers 1990), and a 1 week preoviposition period is followed by up to 3 weeks of egg laying (Gill et al. 2015). Higher temperatures lead to shorter generation times (17 days at 30 ºC) and increased oviposition rates (8 eggs per day at 25 ºC); however, the survival rate of eggs decreases at high temperatures (10.5% at 30 ºC) (Murai 2000) . The optimal temperature for onion thrips in terms of fecundity is approximately 23 ºC, which is a result of increased longevity of females that lay more eggs and have low mortality (Murai 2000). Gravid females lay eggs in the same areas that they feed and continually move toward younger leaf tissue (Theunissen and Legutowska 1991). Hot and dry weather leads to shorter generation time and rapid population increases (Rueda et al. 2007), which in addition to the higher rates of metabolism might also be due to factors such as a reduction in mortality from rain and pathogens (Gill et al. 2015).

Management. Many methods of control exist which reduce the damage caused by onion thrips. Onion cultivars which are less attractive to onion thrips include those with a yellow-green color and glossy to semi-glossy leaf surfaces; however, no highly resistant onion cultivars exist (Diaz-Montano et al. 2010, Diaz-Montano et al. 2012a, Diaz-Montano et al. 2012b, Damon et al. 2014). Field location is also important when trying to manage onion thrips populations. Crops such as alfalfa and small grains often host overwintering populations of onion thrips and should be taken into account when choosing a field location (Carter and Sorenson 2013). Intercropping

of onions with other crops such as carrots (*Daucus carota* L.) and tomato (*Solanum lycopersicum* L.) has been shown to reduce onion thrips infestations by over 50% (Uvah and Coaker 1984, Afifi and Haydar 1990). While there are known predators of onion thrips, biological control was determined not to be an effective means of control (Parrella and Lewis 1997, Diaz-Montano et al. 2011, Gill et al. 2016). Predators of onion thrips; such as, *Aelothrips* spp., green lacewing (*Chrysoperla* spp.) larvae, coccinellids (e.g. *Coleomegilla maculata*), big-eyed bug (*Geocrois* spp.) and minute pirate bug (*Orius* spp.), usually do not reach high abundances until late in the growing season, after the majority of feeding has occurred (Fok et al. 2014).

Traditionally chemical control has been used as the primary means of controlling onion thrips and dates back to first reports of naphthalene use in 1933 (Maughan 1933 and Diaz-Montano et al. 2011). However, because of their short life cycle, high fecundity and asexual mode of reproduction, onion thrips are likely to develop resistance to insecticides. Resistance to pyrethroid and organophosphate insecticides by onion thrips populations has been reported in onion growing areas around the world (Martin et al. 2003, Shelton et al. 2003, MacIntyre Allen et al. 2005, Shelton et al. 2006, Herron et al. 2008, Morishita 2008). This underscores the need to manage onion thrips insecticide resistance to maintain efficacy of currently available products. Chemical management strategies that limit the frequency of insecticide applications, rotate insecticides classes and maintain thorough coverage to prolong the effectiveness of insecticides, help mitigate resistance (Gill and Garg 2014). Applying a high spray volume of insecticides to onion improves onion thrips control and concurrently adding a penetrating surfactant critically improves the efficacy of insecticides used to control onion thrips (Nault et al. 2013).

Monitoring. It is recommended that onion growers monitor their fields regularly to determine the density of Onion thrips and time insecticide applications accordingly. Adults and larvae can be observed and counted by opening the neck of the plant (Shelton et al. 1987). The younger leaves are a preferred feeding site for onion thrips, therefore they should be the focus area of examination (Rueda and Shelton 1995). It is important that sampling is started early in the season, at the 4-5-leaf stage, and plants are checked weekly throughout the growing season, especially if weather conditions are ideal for Onion thrips development (Rueda and Shelton 1995). Other, less commonly used methods of monitoring for onion thrips include plant dissection and plant beating (Mautino et al. 2012). Plant dissection consists of collecting plants from the field and dissecting and examining them leaf by leaf to detect onion thrips. Plant beating consists of beating plants into a plastic tray and counting the onion thrips in the tray. While plant dissection and plant beating are less accurate methods of thrips sampling compared to visual sampling they still can be used in practice to adjust the number of thrips per plant recorded (Mautino et al. 2012).

Action thresholds. Under favorable conditions, onion thrips populations can rapidly increase due to their short developmental time and high fecundity (Diaz-Montano 2011) making them difficult to control. Economic action thresholds are necessary to indicate when control measures need to be implemented (Fournier et al. 1995). The determination of economic action thresholds depends on factors such as weather conditions (Pedigo et al. 1986) and insecticide efficacy (Nault and Shelton 2010), therefore no all-encompassing economic threshold exists for onion thrips on onion (Fournier et al. 1995).

In most cases economic injury thresholds are region specific, such as 30 thrips per plant in California (Kuepper 2004), 1 thrips per leaf in Texas (Edelson et al. 1989) or 5 thrips per 50 plants in Auckland, New Zealand (Jamieson et al. 2012). Action thresholds may also vary with season or weather conditions. For example, Canada has a 0.9 thrips per leaf threshold during early and severe drought conditions and a 2.2 thrips per leaf threshold under no or slight water deficit (Fournier et al. 1995). More recently, in New York, specific action thresholds were determined for different insecticides' efficacies. For example, spirotetramat (Movento), methomyl (Lannate LV) and abamectin (Agri-Mek SC) effectively manage onion thrips at a threshold level of 1 thrips per leaf. In contrast, spinetoram (Radiant SC) is effective at 3 thrips per leaf (Nault and Shelton 2010, Nault and Shelton 2012). It is important to consider geographic and environmental factors when determining an appropriate economic action threshold for onion thrips. If proper monitoring is implemented and the correct thresholds are followed onion thrips control can be optimized allowing growers to make fewer applications, save time and money, and mitigate insecticide resistance development (Gill et al. 2015).

Bacterial leaf blight of onion caused by *Pantoea agglomerans*

Pantoea agglomerans (Beijerinck 1888) comb. nov. (Gavini et al. 1989), formerly *Enterobacter agglomeran*s (Beijerinck 1888) Ewing and Fife (1972), *Erwinia herbicola* (Löhnis 1911) Dye 1964 or *Erwinia milletiae* (Kawakami and Yoshida 1920) Magrou 1937 is a gram-negative aerobic bacillus in the family *Enterobacteriaceae* which can be found in many ecological niches, most of which are associated with plants (Rezzonico 2009). However, strains of *P. agglomerans* are common symbionts in arthropods such as bees (Loncaric et al. 2009, Lozo et al. 2015), ants (Suen et al. 2010) and termites (Potrikus et al. 1977). There are also

a few reports of *P. agglomerans* causing infections in species of mammals or fishes (Dutkiewicz et al. 2016). It was identified as the cause of a hemorrhagic disease in dolphin fish (*Coryphaena hippurus*), after being isolated from their kidneys (Hansen et al. 1990) and it was also identified as the potential cause of equine abortion (Gibson et al. 1982). Lastly, strains of *P. agglomerans* have been associated with human infections which follow piercing or laceration of skin with plant material such as a thorn and subsequent inoculation of the plant-residing bacteria (Dutkiewicz et al. 2016).

Most commonly associated with plants, *P. agglomerans* is an epi- or endophytic symbiont as well as a plant pathogen. In *Eucalyptus* (*Myrtaceae*), *P. agglomerans* strain 33.1 was shown to possess beneficial characteristics such as plant growth promotion (Ferreira 2008). Certain strains of *P. agglomerans* have been identified as biocontrol agents for fire blight in pear orchards (Özaktan and Bora 2004) and bacterial wilt in bean (Costa et al. 2002 Hsieh et al. 2005). However, *P. agglomerans* has been known to cause diseases in a variety of cropping systems such as bacterial leaf blight in rice (*Oryza sativa*) in Korea (Lee et al. 2010), bacterial seed and boll rot of cotton (*Gossypium hirsutum*) (Medrano and Bell 2007), bacterial spot disease of Chinese taro (*Alocasia cucullata*) in Brazil (Romeiro et al. 2007), brown apical necrosis of walnut (*Juglans regia*) in China (Yang et al. 2011), leaf blight and vascular wilt in maize (*Zea mays*) and sorghum (*Sorghum bicolor*) in Mexico (Morales-Valenzuela et al. 2007) and bacterial leaf blight and center rot of onion (Dutta et al. 2014).

As a pathogen of onion, *P. agglomerans* was originally described as *Erwina herbicola*, and was initially identified as a pathogen affecting onions in South Africa (Hattingh and Walters 1981). The first US report of this disease came from Georgia, in 2006, and symptoms were described as water-soaked margins of the leaves and stalk, or necrotic tissue (Edens et al. 2006).

In 2011, *P. agglomerans* was reported in onion fields in six counties across Michigan (Tho et al. 2011), and is part of a disease complex, the 'center rot complex', with another gram-negative bacterium, *Pantoea ananatis*, which has been linked to bacterial bulb rot during storage. During its first reported year in Michigan, bacterial leaf blight incidence progressed throughout the season and reached an incidence of 80% or greater (Tho et al. 2011), causing concern for growers.

Bacterial center rot of onion caused by *Pantoea ananatis*

Pantoea ananatis is a gram-negative, aerobic, rod-shaped and yellow-pigmented bacteria in the family Enterobacteriaceae (Coutinho and Venter, 2009). First identified in The Philippines, *P. ananatis* was first discovered as the causal agent of fruitlet rot in pineapple (Serrano 1928). Originally it formed part of the *Erwinia herbicola–Enterobacter agglomerans* complex, but in 1989 it was assigned to the genus *Pantoea* (Gavini et al. 1989).

Similar to *P. agglomerans*, *P. ananatis* has a cosmopolitan distribution and has been found in a variety of environments across the globe (Weller-Stuart et al. 2017). As a saprophyte, *P. ananatis* has been isolated from soil, freshwater and even aviation fuel tanks (Gasser et al. 2012, Pileggi et al. 2012, Rauch et al. 2006). *Pantoea ananatis* has been associated with a plethora of insects, such as fleas and ticks (Murrel et al. 2003), brown leaf hoppers (Watanabe 1996), tobacco thrips (Wells et al. 2002, Gitiatis et al. 2003, Dutta et al. 2016) and onion thrips (Dutta et al. 2014). It was also responsible for causing bacteremic infection in a 73-year-old man (De Beare et al. 2004).

Pantoea ananatis; however, is more commonly associated with plants and has been isolated from a variety of cropping systems as an epiphyte, endophyte or plant pathogen. Many

associations of *P. ananatis* as an epiphyte of economically important plant species, including wheat heads (Legard et al. 1994), Mulberry (Takahashi et al. 1995), rice (Watanabe et al. 1996), cotton lint (Chun and Perkins 1997), poplar trees (Zeng et al. 1999), barley, buckwheat (Coplin and Kado 2001) and maize (Paccola-Meirelles et al. 2001), have been identified. It is also a known epiphyte of 25 asymptomatic weed species, including crabgrass, sicklepod and yellow nutsedge (Gitaitis et al. 2002). As an endophyte, *P. ananatis* has been isolated from coffee (Nunes and de Melo 2006), ginseng (Cho et al. 2007), pepper (Kang et al., 2007) and papaya (Thomas et al., 2007). *Pantoea ananatis* has been identified as a plant pathogen where is can cause diseases, such as brown rot of pineapple (Serrano 1928), graywall of tomato (Stall et all 1969), leaf blotch of sudangrass (Azad et al. 2000), leaf blight and bulb decay of onion (Gitaitis and Gay 1997, Schwartz and Otto 2000), bacterial blight of *Eucalyptus* species (Coutinho et al. 2002), internal fruit rot of netted melon (Kido et al. 2008) and leaf spot of maize (Pérez-y-Terrón et al. 2009).

As a pathogen of onion, *P. ananatis* was first identified in Georgia where crop losses due to bleached leaves and rotted bulbs were reported (Gitaitis and Gay 1997). The symptoms of this disease include white streaks and water-soaked margins along the length of the leaf that turn necrotic as the disease progresses. The bacterium may progress further and infect the bulb, promoting colonization by secondary microbes which results in rotting of the onion bulb (Gitaitis and Gay 1997). Yield losses due to bacterial center rot may be as high as 100% under favorable conditions (Gitaitis et al. 2002).

The primary source of inoculum for *P. ananatis* and *P. agglomerans* is unknown, however there have been a few hypotheses. In Georgia, *P. ananatis* resides as a common epiphyte on more than 20 weed species in onion fields (Gitaitis et al. 2002). Thus, it has been

suggested that weed hosts may be an inoculum source for *P. ananatis*. Another hypothesis is that both *P. ananatis* and *P. agglomerans* are transmitted via seeds (Walcott et al. 2002); vertical transfer of endophytic *P. agglomerans* from eucalyptus seeds to seedlings has already been demonstrated (Ferreira et al. 2008). In the case of onions, dissemination of these bacteria usually occurs by wind and splashing water, however, tobacco thrips (*Frankliniella fusca* (Hinds)) and onion thrips are able to obtain and transmit these pathogens (Gitaitis et al. 2003 and Dutta et al. 2014).

Onion thrips as disease vectors

Onion thrips are known vectors of a variety of plant viruses and diseases; they are the only known vectors of the tospovirus ISVY (Cortês et al. 1998, Ngata et al. 1999, Pozzer et al. 1999, Kritzman et al. 2001, Hsu et al. 2010). Their feeding injury has also been associated with the spread of purple blotch caused by the fungal pathogen *A. porri* (McKenzie et al. 1993) which uses areas of insect damage as alternative penetration sites and can cause infection on younger leaves that are injured by onion thrips (McKenzie et al. 1993). Recently onion thrips were also identified as a vector of *P. agglomerans* and *P. ananatis* (Gitaitis et al. 2003, Dutta et al. 2014).

Interactions between thrips and plant-pathogenic bacteria, specifically of the family Enterobacteriaceae, are poorly understood and until recently the ability of thrips to acquire and transmit *P. ananatis, P. agglomerans* and other related pathogens has not been studied. However, recently two published studies documenting interactions between thrips species and Enterobacteriaceae bacteria described the mechanisms of transfer, acquisition and transmission rates of *P. agglomerans* and *P. ananatis* by onion thrips and the interactions between *P. ananatis* and tobacco thrips are now understood (Dutta et al. 2014, Dutta et al. 2016).

Onion thrips are able to acquire both *P. agglomerans* and *P. ananatis* after just one hour of feeding on infected onion tissue with a mean acquisition percentage of 20% for *P. ananatis* and 12% for *P. agglomerans*. However, after 48 hours of feeding, greater than 90% of onion thrips adults acquire these pathogens (Dutta et al. 2014). Similar results were observed for tobacco thrips and *P. ananatis* (Dutta et al. 2016). After one hour of feeding on infected plants both adults (7.1%) and larvae (4.2%) were able to acquire *P. ananatis* and after 48 hours >70% of adults and larvae acquired the bacterium (Dutta et al. 2016). Results also demonstrated that onion plants which were fed on by infected onion thrips developed bacterial leaf blight and center rot symptoms. Isolations confirmed the identities of the bacteria as *P. agglomerans* and *P. ananatis* (Dutta et al. 2014). In summary, onion thrips and tobacco thrips are able to vector *P. agglomerans* and *P. ananatis* in onion under laboratory conditions (Dutta et al. 2014, Dutta et al. 2016).

To investigate the mechanism of transfer, researchers used immunolocalization to determine distribution of *P. ananatis* in onion thrips and tobacco thrips (Dutta et al. 2014, Dutta et al. 2016). When the sections of onion thrips and tobacco thrips were immunolabeled with primary and secondary antibodies and observed under a fluorescent microscope, *P. ananatis* was visible in the oesophagous, midgut and hindgut. This suggests that bacteria are only present in the digestive tract, but not in the salivary glands or other organs. Lastly, by comparing onion plants inoculated with onion thrips feces rinsates to plants inoculated with salivary secretions they were able to determine a stercorarial (via feces) method of transmission (Dutta et al. 2014).

With repeated evidence of trans-stadial persistence of enterobacteraceae bacteria in thrips it is important to determine whether thrips fitness is affected by this relationship. Dutta et al. (2016) investigated whether or not *P. ananatis* affected the fecundity, survival and

developmental time of tobacco thrips by monitoring the time to complete a generation (adult to adult) and adult development on surface sterilized peanut leaflets with and without epiphytic populations of *P. ananatis*. There were no differences between adult survival and developmental time between treatments which contained *P. ananatis* and those that did not. Results of these experiments indicated that the presence of epiphytic populations of *P. ananatis* had no effect on thrips development (Dutta et al. 2016).

My thesis research is the first investigation of the relationship between onion thrips and pathogenic *Pantoea agglomerans* in the field. Onion thrips are able to acquire and transmit *P. ananatis* and *P. agglomerans* on onions in the laboratory (Dutta et al. 2014) but, evidence of this relationship in the field was lacking. In addition, the effect onion thrips have on the spread of bacterial diseases in onion fields is unknown and concern from growers has been increasing ever since bacterial leaf blight was first reported in Michigan in 2011 (Tho et al. 2011). If a vector relationship exists in the field between onion thrips and *P. agglomerans* it may be more important to focus management on the insect instead of the bacteria. An understanding of the relationship between onion thrips and *P. agglomerans* in a commercial field setting is paramount to achieving proper control of this issue.

Thesis objectives

First, I determined the role of onion thrips as a vector of *P. agglomerans* in commercial onion fields in Michigan using real time polymerase chain reaction (qPCR) to evaluate the presence of the disease in field-collected onion thrips. Secondly, I assessed how the temporal dynamics of onion thrips and *P. agglomerans* are related in the field. I also investigated the effectiveness of combining insecticides and bactericides for managing both onion thrips and *P.*

agglomerans in the field. Lastly, I determined the role of feeding injury by onion thrips in influencing the pathogenicity of *P. ananatis*.

CHAPTER 2:

Assessing the relationship between onion thrips (*Thrips tabaci*) and bacterial leaf blight in Michigan onion fields

Introduction

Pantoea agglomerans (syn. *Erwinia herbicola;* causal agent of bacterial leaf blight) results in symptoms of leaf blight on onion, *Allium cepa* L. (Amaryllidaceae), including necrotic streaking with water-soaked margins along the length of the leaf leading to wilting in severe cases (Edens et al. 2006). Leaf blight caused by *P. agglomerans* produces symptoms similar to those by *Pantoea ananatis* (causal agent of center rot); thus *P. agglomerans* is considered part of the onion center rot 'complex' (Edens et al. 2006). Bacterial leaf blight is a cosmopolitan pest, first reported in onions in South Africa in 1981 (Hattingh and Walters 1981). In the United States, this disease was first found in the field in Georgia in 2006 (Edens et al. 2006) and, in Michigan, it was confirmed on onions in six counties in 2013 (Tho et al. 2015).

Onion thrips, *Thrips tabaci* Lindeman 1889 (Thysanoptera: Thripidae), is an important pest of onion and many other crops worldwide (Lewis 1997). The larvae and adults feed on leaf tissue, removing chlorophyll, thus impairing photosynthesis (Molenaar 1984, Parrella and Lewis 1997), ultimately reducing bulb size and causing yield losses (Fournier et al. 1995). In Michigan, as in many other places in the world, onion thrips are the main insect pest of onions and have been directly and indirectly associated with the transmission of several onion pathogens, emphasizing the need for effective management (Diaz-Montano et al. 2011). Insecticides are commonly used for onion thrips management (Diaz-Montano et al. 2011) but onion thrips have become resistant to many different chemistries (Lewis 1997). Growers are advised to rotate

insecticides classes within a growing season and apply them based on established thresholds (Byrne and Szendrei 2013).

Onion thrips are known to play an important role in the transmission of a plant pathogenic virus, Iris yellow spot virus (Hsu et al. 2010, Kritzman et al. 2001, Pozzer et al. 1999), and a pathogenic fungus that causes purple blotch (McKenzie et al. 1993). Laboratory experiments confirmed that onion thrips can acquire and transmit both *P. ananatis* and *P. agglomerans*, and that the mode of transmission of *P. ananatis* was stercorarian (Dutta et al. 2014). Tobacco thrips (*Frankliniella fusca* (Hinds)) can also transmit *P. ananatis*, and while the pathogen can persist through multiple life stages, it has no effect on tobacco thrips fitness (Dutta et al. 2016). Based on these laboratory findings, onion thrips act as a vector for *P. agglomerans*, but field research is needed to determine whether there is a link between onion thrips and leaf blight incidence in onions.

In the United States the onion industry is valued at $1 billion dollars per year (USDA/NASS, 2015). Michigan's onion industry is valued at approximately $10 million per year, grown on about 1,000 hectares, producing just over 45 million kilograms per year (USDA/NASS 2015). Most of Michigan's commercial onions are produced for dry bulbs stored through the winter, and are direct seeded. In Michigan, bacterial leaf blight incidence can reach up to 80% or more in some commercial onion fields (Tho et al. 2015). The long-term effects of *P. agglomerans* on the onion industry are unknown, but with its association to other center rot causing bacteria, there is potential for it to cause economic losses (Dutta et al. 2014). Yield losses caused by onion thrips feeding are variable but have been documented as high as 60% (Waiganjo et al. 2008).

The objectives of this study were to determine whether there is a relationship between onion thrips and bacterial leaf blight incidence in the field and to test the efficacy of insecticides and bactericides in limiting onion thrips and bacterial leaf blight.

Methods and Materials

Thrips and leaf blight incidence. Onion plants from two fields from each of four Michigan commercial onion farms located in Newaygo, Allegan, Ingham and Clinton counties were sampled in 2015 and 2016 (N=8 per year). All of the selected fields had muck soils and field sizes ranged from 3.0 - 4.5 hectares. For each of the eight fields in both seasons, ten sampling plots (7.62 m^2 each) were established per field.

Onion foliage was sampled for thrips and bacterial leaf blight symptoms weekly between 15 June and 10 August 2015 (nine sampling dates) and 6 June and 8 August 2016 (ten sampling dates). Plants were between 2 and 4 leaf stage when sampling began; five onion plants were randomly collected from each of the ten plots for a total of 400 onions collected per week across all fields. Individual plants were removed from the soil, placed singly in plastic bags in order to retain all thrips and placed into coolers for transport to the laboratory.

Each sample was assessed for thrips by counting the total number of adults and juveniles. Between 1 and 15 thrips per plant were collected to screen for *P. agglomerans*. Insects selected for DNA extraction were frozen in 90% ethanol.

Whole thrips DNA extractions were performed using the DNeasy blood and tissue kit (Qiagen, Hildon, Germany). The protocol was modified by using 25% of all extraction buffers to optimize the concentration of DNA (25.0 ± 3.0 ng / µl) in samples containing 1 to 15 thrips that were eluted with 50 µl buffer. Extracted DNA was screened for the presence of *P. agglomerans*

using quantitative PCR (qPCR) with the species specific pagRrt2 primer set and pagR2 probe (Braun-Kiewnick et al. 2012). qPCR reactions were performed in final reaction volumes of 10 µl containing 2 x 5 µl TaqMan master mix (Life Technologies, Grand Island NY), 0.4 µl ddH20, 900 nM pagRrt2 forward and reverse primers, 200 nM pagR2 TaqMan probe and 2 µl extracted DNA. PCR reactions were performed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems®, Foster City, CA). Cycling conditions were 2 min at 50 ºC, 10 min at 95 ºC, followed by 45 cycles of 15 s at 95 ºC and 2 min at 60 ºC. All PCR reactions were conducted as duplicates of each sample. Any sample with an average cycle threshold (C_T) value < 40 was considered positive (Braun-Kiewnick et al. 2012).

Plants were visually assessed for the presence or absence of bacterial leaf blight symptoms. A single symptomatic plant was randomly selected from each plot for bacterial isolation and identification. Plants were rinsed with running tap water and the foliage was allowed to dry for approximately 1 hr. Leaf tissue from the margin of the water-soaked tissue was excised (1.0 x 1.0 cm^2) using a sterile scalpel blade, dipped briefly in 95% ethanol, rinsed with sterile distilled water twice, dried for 3 min, and placed on a clean microscope slide. The disinfected leaf piece was cut into half using a sterile scalpel blade. A 50 µl drop of sterile distilled water was pipetted onto the cut area where bacterial cells were released from the tissue into the water. The suspension was streaked onto nutrient broth yeast extract (NBY) agar using a sterile bacterial loop, and incubated at 30ºC for 2 to 7 days. Each bacterial colony that had different morphological characteristics (form and color) was selected and transferred to fresh NBY agar twice, resulting in pure cultures for identification. Representative colonies were identified to species using the BIOLOG GN Microtiter Plates (BIOLOG Inc., Hayward, CA, USA) according to the manufacturer's instructions.

In 2015, selected samples were further confirmed via sequencing using the following protocol: DNA extraction was performed according to DNA extraction manual using the Wizard Genomic DNA Purification kit (Promega Corp., Leiden, The Netherlands). DNA quantity was measured using Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE). 16S rDNA was amplified with 16S primer set. A total volume of 25 µl of PCR mixture contained 1x PCR buffer, 0.2 µM dNTPs, 0.2 µM of each primer, and 0.625 U Taq polymerase (Promega, Madison, WI). The PCR reactions were conducted in a Mastercycler Pro thermocycler (eppendorf, Hauppauge, NY) with an initial denaturation at 94℃ for 5 min, followed by 3 cycles of denaturation at 94℃ for 45 sec, annealing at 52℃ for 2 min, and extension at 72℃ for 1 min, and another 30 cycles of denaturation at 94℃ for 20 sec, annealing at 52℃ for 1 min, and extension at 72℃ for 1 min. The final extension step was performed at 72℃ for 7 min. The PCR products were visualized on 1.5% agarose gel, dissolved in 1x Tris-borate-EDTA (TBE) buffer, and sequenced with a single primer by submitting to Macrogen Corp. (Macrogen USA, Rockville, MD). The nucleotide sequences were compared to the nucleotide collection in NCBI using a BLASTn search (http://blast.ncbi.nlm.nih.goc.Blast) analysis.

Pearson product-moment correlation coefficients were computed to assess the relationships between mean thrips per plant, proportion of thrips positive for *P. agglomerans* and proportion of plants with bacterial leaf blight symptoms. Differences among dates for each dependent variable were compared with an analysis of variance (ANOVA). Differences between dates were separated using a Tukeys HSD (R, version 3.2.2).

Insecticide and bactericide trial. To assess the effectiveness of different bactericides applied alone or in combination with an insecticide, a field trial was conducted during the 2015 and 2016

growing seasons. ‘Sedona’ onions were direct seeded on 29 April 2015 and 16 April 2016 in a commercial field in Stockbridge, MI. The trial was arranged in a randomized complete block design with 13 treatments replicated across four blocks. Each plot was three rows wide and 6 m long with a 60 cm buffer zone in between each plot. Three bactericides were tested: two copper hydroxide products (Kocide 3000®, DuPont™, Wilmington, DE and Nu-Cop® 50, Albough Inc., Ankeny, IA), and an antibiotic (Kasugamycin®, Hokko Chemical Industry Co. Ltd, Tokyo, Japan) (Table 2.1). A grower standard insecticide program was tested alone or in combination with the bactericides and a non-ionic surfactant (Dyne-Amic®, Helena Chemical Company, Collierville, TN) (Table 2.1). In 2015, the insecticide program consisted of two applications of spinosad (Radiant® SC, Dow™ AgroSciences, Indianapolis, IN) followed by two applications of abamectin (Agri-Mek® SC, Syngenta®, Basel, Switzerland) at weekly intervals. In 2016, the insecticide program consisted of weekly sprays consisting of the following: two applications of a spirotetramat (Movento®, Bayer CropScience LP, RTP, NC), followed by two applications of spinosad, followed by two applications of abamectin followed by one application of methomyl (Lannate® LV, DuPont™, Willmington, DE). A penetrating surfactant (Dyne-Amic, Bayer CropScience LP) was added at 0.05% v/v in some treatments (Table 2.1). These treatments were compared to an untreated control. The bactericide program was initiated on 28 May 2015 and 22 June 2016 when plants had 3 to 5 leaves. The insecticide program was based on thrips/leaf thresholds (Nault and Shelton 2010) and was initiated on 22 July 2015 and 29 June 2016. All combination treatments were tank mixed before application. All products were applied using a CO_2 backpack sprayer and a broadcast boom equipped with three XR8003 flat-fan nozzles (TeeJet® Technologies, Wheaton, IL) with the outer nozzles angled towards the center of the plot, calibrated at 50 psi and delivering 467.5 L ha^{-1}.

Prior to initiating insecticide applications, plants were scouted weekly and the number of thrips per plant was used to calculate the thrips per leaf threshold. After the insecticide program began, thrips were counted on ten randomly selected plants in each plot weekly. The total number of leaves per plant was also counted on twenty randomly selected plants in the field. The average number of thrips per leaf for each treatment was calculated.

Leaf necrosis was visually evaluated according to the Horsfall-Barratt rating system where 1 indicates no disease symptoms and 12 indicates up to 100% foliar necrosis (Horsfall Barratt 1945). Plants were evaluated one week following the final pesticide application: on 24 August in 2015 and on 17 August in 2016.

The seasonal mean number of thrips per plant was compared among the thirteen treatments using an ANOVA where treatment was the fixed factor and block by date interaction was the random factor. Mean necrotic leaf tissue was also compared among the thirteen treatments using an ANOVA where treatment was the fixed factor and block was the random factor. Tukey's HSD tests were used to determine differences among treatment means.

Results

Onion thrips and leaf blight incidence. The number of thrips per plant, thrips (%) that tested positive for *P. agglomerans*, and plants (%) with bacterial leaf blight symptoms increased during each growing season. The mean number of thrips per plant significantly differed among sampling dates (2015: $F = 35.9$, $df = 8, 62$, $p < 0.01$; 2016: $F = 2.6$, $df = 9, 61$, $p < 0.01$), reaching about four thrips per plant by the end of the growing season. Thrips (%) positive for *P. agglomerans* also differed among sampling dates (2015: $F = 9.7$, $df = 8, 62$, $p < 0.01$; 2016: $F = 4.3$, $df = 9, 61$, $p < 0.05$; Figure 2.1). Plants (%) with bacterial leaf blight significantly differed

among sampling dates (2015: F = 31.8, df = 8, 62, p < 0.01; 2016: F = 16.0, df = 9, 61, p < 0.01) reaching 100% by the end of the growing season (Figure 2.1).

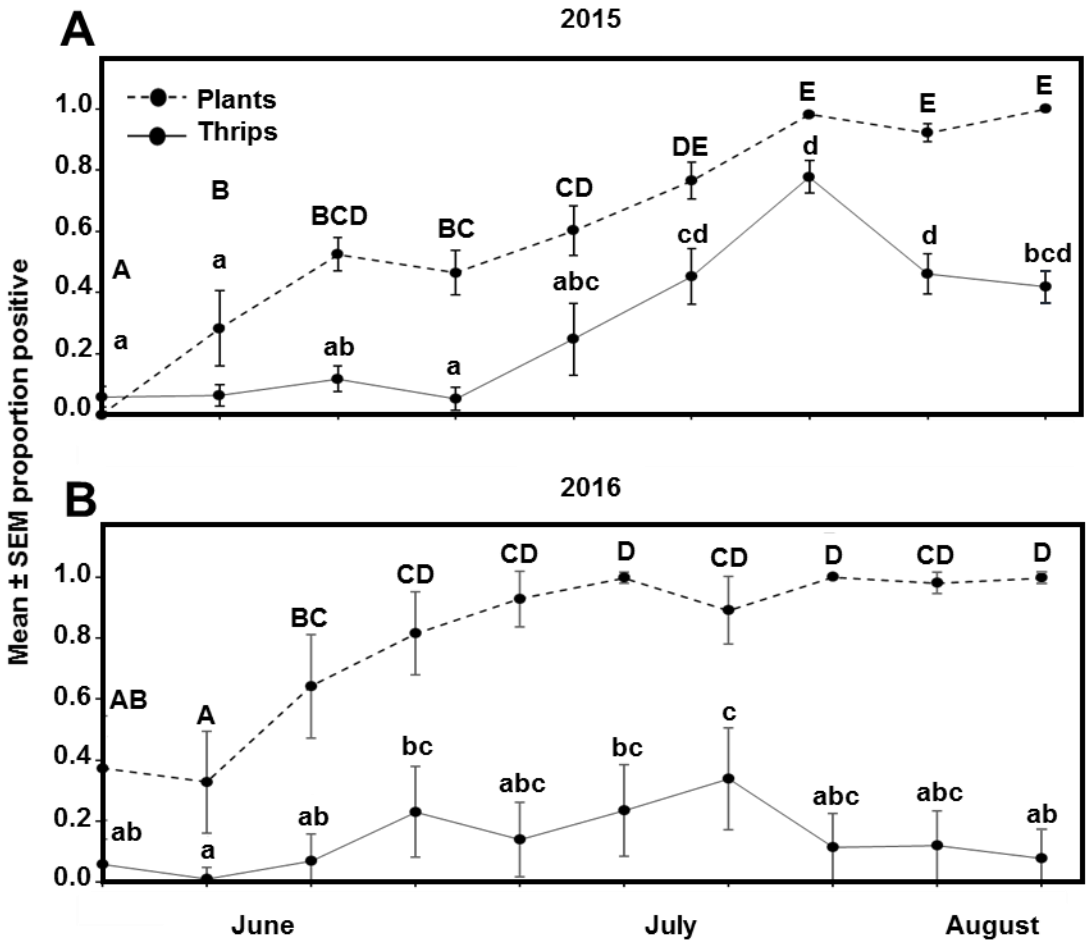

Figure 2.1. Mean (± SEM) proportion of thrips that screened positive for *P. agglomerans* using qPCR and mean (± SEM) proportion of plants with bacterial leaf blight symptoms at each sampling period during the 2015 (A) and 2016 (B) growing seasons. Each point represents the average across eight fields. Uppercase letters represent mean differences in plants with bacterial leaf blight symptoms and lowercase letters represent mean differences in thrips positive for *P. agglomerans*. Points with the same letter are not statistically significant from each other.

There was a significant positive correlation between plants (%) with bacterial leaf blight symptoms and the mean number of thrips per plant in both growing seasons (2015: $r^2 = 0.26$, $p < 0.05$; 2016: $r^2 = 0.30$, $p < 0.01$; Figure 2. A, B). There was a significant positive correlation

between plants (%) with bacterial leaf blight symptoms and thrips (%) that screened positive for *P. agglomerans* in both growing seasons (2015: $r^2 = 0.59$, $p < 0.01$; 2016: $r^2 = 0.23$, $p < 0.05$; Figure. 2 C, D).

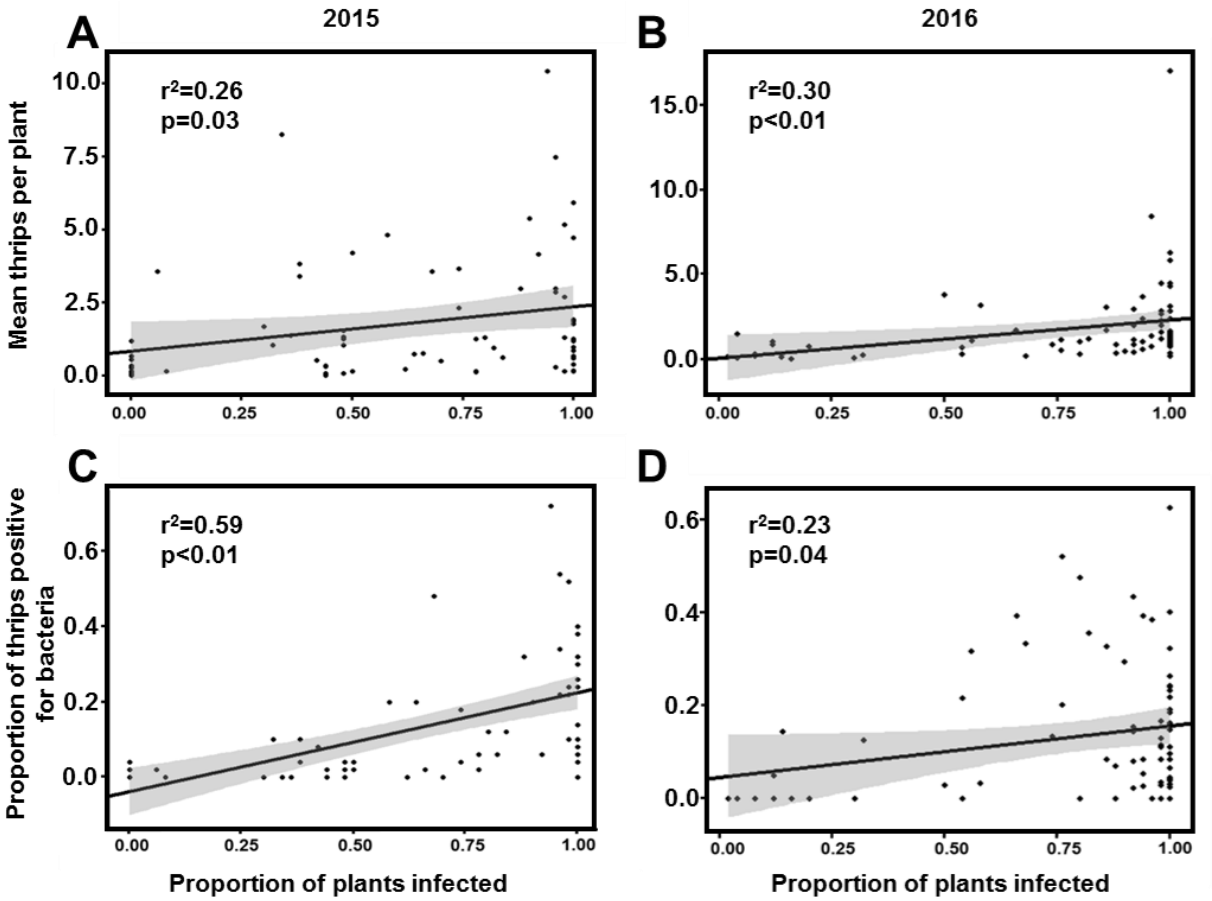

Figure 2.2. The proportion of plants with bacterial leaf blight symptoms correlated with the mean (± SEM) thrips per plant in 2015 (A) and 2016 (B). The proportion of plants with bacterial leaf blight symptoms correlated with the proportion of thrips that screened positive for *P. agglomerans* in 2015 (C) and 2016 (D). Each point represents one field for each date.

Pesticide trial. In both growing seasons, all treatments with an insecticide had significantly fewer thrips than treatments without insecticide (2015: $F = 57.5$, df = 12, 12, $p < 0.01$; 2016: $F = 71.4$, df = 12, 24, $p < 0.01$; Table 2.1). In 2015, the treatments containing Kasugamycin® and insecticide provided better control of thrips than the insecticide treatments containing the other two bactericides (Kocide®, NuCop®). The number of thrips among treatments containing

bactericides mixed with insecticide compared to insecticide alone did not differ in 2015 and 2016. Adding a surfactant did not significantly impact thrips control or leaf blight severity (Table 2.1).

There was a significant treatment effect on leaf blight severity in the pesticide trial in both growing seasons (2015: $F = 6.5$, $df = 12, 36$, $p < 0.01$; 2016: $F = 24.8$, $df = 12, 36$, $p < 0.01$; Table 2.1). In 2015, all treatments that contained an insecticide showed 26% less plant area with leaf blight symptoms than the treatments without insecticide, but this effect was not significant. In 2016, all treatments with an insecticide had 35% less plant area with bacterial leaf blight symptoms than treatments without insecticide ($t > 5.2$, $df = 12$, $p < 0.01$). Leaf blight severity was similar among the eight treatments that contained an insecticide in both years (Table 2.1).

Table 2.1. Seasonal average number of thrips per leaf and bacterial leaf blight incidence in 2015 and 2016 when treated with a bactericide and/or an insecticide program.

				2015		2016	
Product Name	Active ingredient[v]	Insecticide Program (+/-)	Surfactant (+/-)[w]	Seasonal avg. no. thrips/leaf ± SE[x,z]	Necrotic leaf tissue ± SE[y,z]	Seasonal avg. no. thrips/leaf ± SE[x,z]	Necrotic leaf tissue ± SE[y,z]
-	-	-	-	3.7 ± 0.27 a	8.0 ± 0.63 abc	8.2 ± 0.47 b	6.5 ± 0.29 a
Kocide 3000	$Cu(OH)_2$	-	-	3.8 ± 0.27 a	8.0 ± 0.63 abc	11.5 ± 0.75 a	6.0 ± 0.00 a
Kasugamycin	Kasugamycin	-	-	3.0 ± 0.21 a	8.1 ± 0.00 abc	9.9 ± 0.63 ab	6.8 ± 0.25 a
NuCop 50	$Cu(OH)_2$	-	-	3.3 ± 0.26 a	9.2 ± 0.63 a	10.4 ± 0.82 a	6.5 ± 0.29 a
Kocide 3000	$Cu(OH)_2$	+	-	1.3 ± 0.13 bcd	5.8 ± 0.25 c	4.8 ± 0.32 c	3.8 ± 0.25 b
Kasugamycin	Kasugamycin	+	-	0.8 ± 0.08 d	7.0 ± 0.85 bc	4.2 ± 0.31 c	3.8 ± 0.25 b
NuCop 50	$Cu(OH)_2$	+	-	1.4 ± 0.12 b	6.0 ± 0.41 c	4.5 ± 0.32 c	4.8 ± 0.25 b
Kocide 3000	$Cu(OH)_2$	+	+	1.4 ± 0.13 b	5.8 ± 0.48 c	3.9 ± 0.25 c	4.5 ± 0.29 b
Kasugamycin	Kasugamycin	+	+	0.9 ± 0.12 cd	6.3 ± 0.48 bc	3.2 ± 0.24 c	4.0 ± 0.00 b
NuCop 50	$Cu(OH)_2$	+	+	1.4 ± 0.12 bc	6.3 ± 0.25 bc	3.7 ± 0.24 c	4.5 ± 0.29 b
-	-	+	-	1.2 ± 0.13 bcd	6.3 ± 0.48 bc	3.7 ± 0.29 c	4.3 ± 0.25 b
-	-	+	+	1.0 ± 0.10 bcd	5.8 ± 0.48 c	3.7 ± 0.27 c	4.0 ± 0.00 b
-	-	-	+	3.1 ± 0.21 a	8.6 ± 0.50 ab	10.2 ± 0.61 a	6.5 ± 0.29 a

[v] All treatments that contain copper hydroxide ($Cu(OH)_2$) were applied at 1.68 kg ha^{-1} and all treatments that contain Kasugamycin were applied at 2.34 L ha^{-1}.

[w] Non-ionic surfactant (Dyne-Amic ®) was added at 0.05% v/v.

[x] The seasonal average number of thrips per leaf was calculated by averaging the sum of thrips per leaf and dividing by the average number of leaves per plant.

[y] Necrotic leaf tissue was measured at the end of the season using the Horsfall-Barratt rating system (1 = 0-3% of necrotic leaf tissue and 12 = 100% of leaf necrosis).

[z] Different letters indicate differences among treatments, Tukey's HSD, $\alpha < 0.0$.

Discussion

Results from this study indicate that onion thrips are likely contributing to the spread of the bacterial leaf blight pathogen in onion fields. In each season, I detected bacterial leaf blight symptoms approximately three weeks before thrips populations began to increase. Thrips populations started increasing in late July in both growing seasons, which coincided with 90 to 100% incidence of bacterial leaf blight symptoms across all fields. Thrips that were positive for *P. agglomerans* peaked at approximately 20% by the end of the sampling period, close to the time of harvesting the bulbs. Insecticide applications are often decreased near harvest time that can lead to an increase in thrips populations and this also coincides with exodus flights of thrips from the field (Smith et al. 2015). The role of overwintering thrips in introducing the bacterium to subsequent onion crops is unknown; however, the fact that 6% of the thrips samples were positive for *P. agglomerans* in the first samples taken for both years indicates that this may be a potential mechanism for the disease to survive and spread.

The field data alone cannot prove whether onion thrips are vectoring the bacterium since the incidence of plant disease could be expected to increase during the growing season if not managed effectively (Pullman and DeVay 1982). However, the field surveys together with data from the pesticide trial support thrips as vectors of *P. agglomerans* because insecticide treatments significantly reduced leaf blight symptoms. The incidence of *P. agglomerans* throughout the field can be associated with multiple methods of thrips transfer. While stercorarian mode of transmission has been verified (Dutta et al. 2014), it is likely that under field conditions thrips also carry bacteria on their integument. Furthermore, it is likely that thrips feeding provides entrance points for *P. agglomerans* inoculation. A similar relationship has been documented between onion thrips and the fungal pathogen *Alternaria porri*, causal agent of

purple blotch (McKenzie et al. 1993). When thrips injury was present, *A. porri* was able to penetrate through areas damaged by onion thrips. These methods did not differentiate whether thrips were carrying bacteria in their guts or on their integument; therefore, I am not able to distinguish the relative importance of the mode of transmission. However, *P. agglomerans* may also be carried to the plants by machinery, wind and splashing water (Wiriyajitsomboon et al. 2014), then use thrips feeding injury as entry points into the plant.

Pantoea agglomerans is part of a disease complex that includes the bacterial center rot pathogen, *P. ananatis*. It is important to prevent both *Pantoea* species from progressing into the bulb late in the growing season because they can cause rot during long-term storage (Edens et al. 2006, Vahling-Armstrong et al. 2016). Currently, *P. ananatis* is not as prevalent as *P. agglomerans* in Michigan's commercial onion industry, but the similar acquisition rates and modes of vectoring of the two bacteria indicate that onion thrips are able to spread both pathogens in a similar way (Dutta et al. 2014, Dutta et al. 2016). Effectively managing onion thrips populations could reduce the risk of losses from bulb rot during long-term storage.

A better understanding of *P. agglomerans* epidemiology is needed to further refine an effective control strategy. For instance, *Pseudomonas syringae* pv. *tomato* (Okabe) survives on the tomato plant only becoming symptomatic under appropriate environmental conditions (Smitley and McCarter 1982). Understanding the population threshold that must be exceeded for symptom development could be useful in order to time insecticide applications for thrips control to minimize pathogen transmission and spread. Susceptibility to *P. agglomerans* at different onion plant ages could also be a future avenue for research. In the case of purple blotch (*Alternaria porri*), onion leaves are more susceptible to infection as they age and emerging leaves are more susceptible in late season, closer to bulb maturity (Miller 1983).

The inoculum source for *P. agglomerans* is currently unknown but it has been previously identified from seeds (Walcott et al. 2002), various weed species (Gitaitis et al. 2002) and a variety of other crops such as Sundangrass (Azad et al. 2000), muskmelons (Bruton et al. 1986), cantaloupe (Bruton et al. 1991), *Eucalyptus* (Coutinho et al. 2002), pineapple (Serrano 1928), and honeydew melons (Wells et al. 2011). Onion thrips are known to feed and overwinter on a variety of weed species (Smith et al. 2011) before colonizing volunteer onions in the field the following spring (Larentzaki et al. 2007). If onion thrips are interacting with *P. agglomerans* on other host plants before the start of the growing season, they may be introducing it into the onion field. Screening volunteer onions and weeds early in the growing season for bacterial leaf blight symptoms as well as thrips carrying *P. agglomerans* could provide important insight as to how the pathogen is being introduced into onion fields.

This study demonstrates that increasing thrips populations are associated with increased leaf blight in commercial onion fields in Michigan. Future research should focus on revising action thresholds to optimize onion thrips and bacterial leaf blight management.

CHAPTER 3:

Onion thrips feeding promotes infection by *Pantoea ananatis* in onion

Introduction

Onion thrips, *Thrips tabaci* Lindeman (Thysanoptera: Thripidae), is a primary pest insect of onions (*Allium cepa* L.), directly affecting the United States' onion industry annually valued at $1 billion (USDA/NASS, 2015). Feeding by onion thrips results in the destruction of epidermal and parenchyma tissue (Chisholm and Lewis 1984, Hunter and Ullman 1989) and leads to chlorophyll loss that reduces photosynthetic conductivity (Diaz-Montano et al. 2011, Gill et al. 2015). In severe cases, high thrips infestation rates can result in bulb weight reductions of up to 60% (Kendall and Capinera 1987, Fournier et al. 1995, Rueda et al. 2007, Waiganjo et al. 2008, Diaz-Montano et al. 2011). In addition to directly feeding on plant tissue, onion thrips are also known vectors of several onion diseases (Gill et al. 2015). For example, they are able to acquire and transmit *Pantoea ananatis* (Serrano) Mergaert, a bacteria that causes center rot in onions (Gitaitis and Gay 1997, Lewis 1997, Dutta et al. 2014, Dutta et al. 2016). *Pantoea ananatis* and close members of the onion center-rot complex, *Pantoea agglomerans* (syn. *Erwinia herbicola*) and *Pantoea allii* (Brady et al. 2011), produce leaf-symptoms with white streaks and water-soaked margins along the length of the leaf that turn necrotic as the disease progresses; they also cause center rot during storage (Gitaitis and Gay 1997, Vahling-Armstrong et al. 2016). Yield losses associated with center rot may be as high as 100% under favorable conditions (Gitaitis et al. 2002).

Multiple species of thrips are able to acquire *P. ananatis* through feeding and transmit the bacteria through their feces, i.e., by stercorarial means (Gitaitis et al. 2003, Dutta et al. 2014,

Dutta et al. 2016). Under laboratory conditions, 20% of onion thrips acquired *P. ananatis* after 1 hour of feeding on infected onions; 48 hours of feeding resulted in 100% acquisition (Dutta et al. 2014). Center rot symptoms were also observed on 70% onion seedlings after 15 days of feeding by *P. ananatis*-infected thrips (Dutta et al. 2014). Similarly, 65% of onion seedlings inoculated with rinsates containing *P. ananatis*-infected thrips feces developed center rot symptoms indicating the *P. ananatis* could be transmitted from the thrips gut via feces following oral ingestion (Dutta et al. 2014). However, this relationship has not been tested under field conditions where more biotic and abiotic factors are at play. Recent research in Michigan determined that thrips in commercial onion fields play a role in the transmission of *Pantoea* sp., where higher abundances of onion thrips was correlated with a higher proportion of necrotic leaves in the onion plantings (Grode et al. 2017).

Bacterial infections, such as those caused by *P. ananatis*, are spread in agricultural fields during wind and rain events (Wiriyajitsomboon et al. 2014). The bacteria frequently enter plant tissue through mechanical wounds (Giataitis et al. 1978, Gitaitis et al. 2002). A recent field trial demonstrated that a season-long insecticide program led to lower thrips populations as well as reduced bacterial symptoms, regardless of whether onions were also treated with bactericides (Grode et al. 2017). These results suggest that thrips feeding may lead to an increase in infection by *Pantoea* sp. in the field. A similar relationship has been documented with onion thrips and purple blotch, a fungal disease, where thrips feeding wounds enhanced the development of purple blotch by providing alternative entry points for it (McKenzie et al. 1993). Similarly, feeding wounds caused by onion thrips may provide entry points for *P. ananatis*, leading to increased necrotic leaf tissue and center rot development.

The objectives of this study were to 1) determine the role of onion thrips feeding injury in facilitating the development of *P. ananatis*, 2) investigate the relationship between thrips abundance on plants and center rot development and, 3) quantify morphological changes in onion tissue from onion thrips feeding and *P. ananatis* infection.

Materials and Methods

Onion thrips colony. Onion thrips used in these experiments were from a laboratory colony kept in cages (50 cm by 50 cm by 50 cm; MegaView, Taichung, Taiwan) at Michigan State University (East Lansing, MI). Insects originated from wild populations of onion thrips collected from a commercial onion farm (Stockbridge, MI) in 2015. The colony was maintained on potted onion plants (cv. 'Sedona'; High Mowing Organic Seeds, Wolcott, VT) and allowed to feed *ad labitum*; new plants were added every one to two weeks allowing thrips to transfer from old to new plants. The colony was kept at room temperature (22-25 °C), with ambient humidity.

Experiment 1. 'Sedona' onions were seeded (High Mowing Organic Seeds) in 4 cm square pots (FarmTek, Dyersville, IA) and maintained in an environmental growth chamber (16:8 L:D, 28 ºC, 40% RH). Plants received water and fertilizer (20-20-20 N-P-K; Jack's Professional®, J.R. Peters, Inc., Allentown, PA) every other day. After 5 weeks, onion plants of uniform size (3-5 leaf stage) were transplanted into 12 cm square pots (The HC Companies: ITML, Middlefield, OH). An experimental unit consisted of a plant surrounded by a ventilated cylindrical cage (11 cm tall, 2.5 cm wide) made of polyester plastic (ACCO Brands, Inc.: Apollo, Lincolnshire, IL), embedded in soil (2-3 cm deep) with a top made of nylon netting attached with hot glue (160 µm mesh size, MegaView). Cages prevented the movement of thrips from plant to plant but allowed

light and air movement into the cages. Plants in the environmental chamber were arranged in a randomized complete block design with six treatments and 15 replications. The six treatments were: (1) an undamaged control, (2) thrips only, (3) *P. ananatis* only, (4) thrips 7 days prior to *P. ananatis* inoculation, (5) mechanical injury at the same time as *P. ananatis* and (6) thrips at the same time as *P. ananatis* inoculation. Plants receiving thrips were infested with one adult thrips/leaf (3-5 thrips/plant). All treatments receiving bacteria (treatments 3, 4, 5 and 6) were inoculated with a virulent strain of *P. ananatis* (~1×10^8 CFU/ml, Dutta et al. 2014), isolated from symptomatic onion plants grown in a commercial onion field (Stockbridge, MI). Inoculations were done using a 250 ml spray bottle (Meijer Inc., Grand Rapids, MI) until leaves were fully wetted, at 7 days after the initial thrips infestation. Mechanical damage was inflicted by poking 5 holes/leaf randomly using a pin to mimic thrips feeding wounds. Plants were grown for 7 days in an environmental growth chamber (16:8 L:D, 28 ºC, 40% RH), then returned to the laboratory for rating, after which they were removed from soil and placed singly in ziptop bags to retain thrips for later counting.

The number of leaves per plant and the length of each leaf was recorded prior to adding treatments and after the 14-day treatment period. Total leaf area was calculated using leaf length as an index of leaf area (Gamiely et al. 1991). Growth of each plant was calculated by subtracting the total leaf area of each plant prior to thrips infestation from the total leaf area of each plant calculated at 7 days after inoculation. The percentage of leaf area affected by *P. ananatis* and thrips feeding was visually estimated for each plant (Horsfall and Barratt 1945). Thrips damage was assessed by estimating the percentage of leaf area fed on by thrips and the total number of adults and juveniles were counted on each plant.

Five symptomatic plants were randomly selected from each treatment for bacterial isolation and identification. Leaf tissue from the water-soaked margin of the leaves was excised (1.0 x 1.0 cm^2) using a sterile scalpel blade, dipped briefly in 95% ethanol, rinsed with sterile distilled water twice, dried for 3 min and placed on a clean microscope slide. The disinfected leaf piece was macerated using a sterile scalpel with a # 10 blade (Bard-Parker®, Becton Dickinson Acute Care, Franklin Lakes, NJ). A 50 µl drop of sterile distilled water was pipetted onto the cut leaf where bacterial cells were released from the tissue into the water. The suspension was streaked onto nutrient broth yeast extract (NBY) agar using a sterile bacterial loop, and incubated at 28 °C for 2 days. Each bacterial colony that had different morphological characteristics (form and color) was selected and transferred once to fresh NBY agar resulting in pure cultures for identification.

Representative colonies were identified to species by sequencing the small subunit of the bacterial ribomosomal DNA gene. First, DNA was extracted using the DNeasy® Blood and Tissue Kit (Qiagen®, Hilden, Germany). DNA was quantified using a Qubit® 2.0 Flourometer and Qubit® dsDNA HS Assay Kit (ThermoFisher Scientific Inc., Waltham, MA). The 16S rDNA gene was amplified with a 16S primer pair (forward primer, AGTTTGATCCTGGCTCAG, reverse primer TACCTTGTTACGACTTCGTCCCA; De Baere et al. 2004). A total volume of 50 µl PCR mixture contained, 38.5 µl PCR-grade H_2O, 10x PCR buffer, 0.2 µM dNTPs, 0.2 µM of each primer, and 0.625 U Taq polymerase (Promega, Madison, WI). PCR reactions were conducted in a Mastercycler Pro thermocycler (Eppendorf, Hauppauge, NY) with an initial denaturation at 94 ºC for 5 min, followed by 3 cycles of denaturation at 94 ºC for 45 sec, annealing at 52 ºC for 2 min and extension at 72 ºC for 1 min, and another 30 cycles of denaturation at 94 ºC for 20 sec, annealing at 52 ºC for 1 min and extension at 72 ºC for 1 min.

The final extension step was performed at 72 ºC for 7 min. The PCR products were visualized on 1.5% agarose gel stained with 7.5 µl GelRed nucleic acid stain (Phenix Research Products, Candler, NC). Reactions with sufficient PCR product were purified using the QIAQuick® PCR Purification Kit (Qiagen®) and sequenced with a single primer by submitting to the Michigan State University Genomics Core Facility (East Lansing, MI). The nucleotide sequences were compared to the nucleotide collection in NCBI using a BLAST® nucleotide search analysis.

Mean plant growth, leaf area, number of leaves, bacterial leaf blight severity, thrips damage and thrips per plant were compared among treatments using an analysis of variance (ANOVA). All of the variables met the assumptions of ANOVA. Tukey's HSD tests were used to determine differences among treatment means. All statistical analysis was conducted using R software (R Core Development Team, 2015).

Experiment 2. Plants in this experiment were grown and maintained as described in *Experiment 1* except they were grown for 7 weeks (4-6 leaf stage). In order to measure the effects of varying degrees of thrips feeding damage on bacterial leaf blight severity, plants were arranged in a randomized complete block design with four treatments and 11 replications. An experimental unit consisted of a plant surrounded by a ventilated cylindrical cage as described in *Experiment 1*. The four treatments included: (1) *P. ananatis* only, (2) 4 thrips per plant with *P. ananatis*, (3) 12 thrips per plant with *P. ananatis* and (4) 20 thrips per plant with *P. ananatis*.

Eight days after thrips infestation, all treatments were inoculated with a virulent strain of *P. ananatis* (~1×10^8 CFU/ml) as described in *Experiment 1*. Onions were rated 7 days following bacterial inoculation as described in *Experiment 1*.

Mean plant growth, leaf area, number of leaves, bacterial leaf blight severity, thrips damage and thrips per plant were compared among treatments using an ANOVA. All of the variables met the assumptions of an ANOVA. Tukey's HSD tests were used to determine differences among treatment means. Pearson product-moment correlation coefficients were computed to assess the relationships between the number of thrips present on plants or thrips feeding damage (%) and leaf blight severity.

Experiment 3. Plasmid pSCH476, derived from the wide-range vector PBBR1MCS-3 in Gram-negative bacteria, contained a *gfp* gene expression cassette and a tetracycline resistance gene. It was successfully used for labelling *Delftia* sp. Cs 1-4 (Chen and Hickey 2011) and attempted in *P. ananatis*.

To make the competent cells for transformation of pSCH476, the pathogenic *P. ananatis* strain was grown for 24 hours at 28 ºC in 10 ml of Luria-Bertani (LB) liquid medium, harvested by centrifugation at 4 ºC and resuspended in 25 ml of ultrapure H_2O. After being washed three times with water, cells were resuspended in 2 ml of 10% ice cold glycerol (J.T. Baker® - Avantor™, Center Valley, PA). Aliquots (200 µl) of cells were electroporated (2.5 kV; 25 mA; 25 µF and 400 Ω) with 0.165 ng plasmid. After transformation, 1 ml LB broth was added, incubated for 1 hour at 28 ºC, plated on LB medium supplemented with tetracycline (50 µg/ml) and grown at 28 ºC for 48 hours. Colonies emitting GFP fluorescence under UV were purified and used for later study.

'Sedona' onions were grown as described in *Experiment 1.* Plants were arranged in a randomized complete block design with 4 treatments and 3 replications. The four treatments included: (1) an undamaged control, (2) thrips injury alone, (3) *P. ananatis* alone and (4) thrips

injury in combination with *P. ananatis* inoculation. Plants receiving thrips were infested with 12 adult thrips/plant.

Eight days after thrips infestation, all treatments were inoculated with the GFP strain of *P. ananatis* (~1×10^8 CFU/ml) using a 250 ml spray bottle until leaves were fully covered and started to drip. Plants were maintained as described in *Experiment 1*.

Leaf tissue from each plant was analyzed for the GFP-tagged *P. ananatis* strain in order to compare the bacterial density on leaf tissue colonized by thrips to other non-symptomatic leaf tissue. Three leaf pieces were excised from each plant (n = 36). Areas where thrips feeding injury was present were chosen in those treatments which received thrips (treatments 2 and 4), symptomatic areas were chosen in treatments inoculated with bacteria (treatments 3 and 4) and random areas were chosen in the treatment which received neither (treatment 1). Leaf tissue was excised (1.0 cm^2) and surface sterilized as described in *Experiment 1*. The resulting suspension was diluted ten-fold, twice, and 50 µl of each concentration of the dilution series was streaked onto LB medium supplemented with tetracycline (25 µg/ml) and incubated at 28 °C for 48 hours. The number of colonies with the GFP fluorescence were identified using UV light, counted on each plate containing the lowest dilution and multiplied by 100 in order to calculate total bacterial density. Mean bacterial density (colony forming units: CFU) per cm^2 of leaf tissue was compared between treatments using a t-test. The number of CFUs was log transformed to meet normality requirements of a t-test.

For microscopy, onion plants were removed from pots 8 days after inoculation and washed under running tap water. Leaf tissue (1.0 cm^2) was excised and mounted on a microscope slide. Areas where thrips feeding injury was present were chosen in those treatments which received thrips (treatments 2 and 4), symptomatic areas were chosen in treatments inoculated

with bacteria (treatments 3 and 4) and random areas were chosen in the treatment which received neither (treatment 1). Fluorescence microscopy was carried out on a Nikon Eclipse E800 epifluorescence microscope (Nikon Corporation, Tokyo, Japan) with the Nikon B – 2A fluorescence filter (Nikon Corporation) for GFP analysis. GFP-tagged bacterial cells were excited using a 490 nm filter and the images were captured with a Samsung Galaxy S6 Active camera (Samsung Group, Seoul, South Korea).

Results

Sequencing of the 16S rDNA indicated that *P. ananatis* was present in 100% of the treatments that were inoculated with the bacteria. None of the untreated or thrips only treatments were positive. The most similar GenBank sequence results for the bacterial isolates were *P. ananatis* (96 – 98% match, accession KT957000.1).

Experiment 1. The number of leaves per plant and total leaf area before manipulation were statistically similar between treatments (leaf number: $F = 0.95$, $df = 5, 70$ $p = 0.45$; leaf area: $F = 0.54$, $df = 5, 70$ $p = 0.75$). No statistical differences were found among the treatments at the end of the experiment in the number of leaves per plant, total leaf area and plant growth (leaf number: $F = 0.68$, $df = 5, 70$, $p = 0.64$; leaf area: $F = 1.14$, $df = 5, 70$, $p = 0.35$; growth: $F = 1.35$, $df = 5, 70$, $p = 0.25$; Table 3.1). All treatments inoculated with *P. ananatis* had significantly more bacterial leaf blight symptoms than treatments which did not receive bacteria ($t > 3.39$, $df = 5$, $p < 0.01$; Table 3.1). The percentage of tissue damaged by thrips was significantly higher in treatments with thrips alone (treatment 2; numbers correspond to treatment numbers in Table 3.1) and thrips with *P. ananatis* (treatment 4) compared to all other treatments ($t > 4.60$, $df = 5$ p

< 0.01; Table 3.1). Significantly more thrips were counted on all treatments which received thrips relative to those without thrips ($t > 3.87$, $df = 5$, $p < 0.01$; Table 3.1). There were approximately three times more thrips on the treatment that received *P. ananatis* with thrips simultaneously (treatment 6) compared to the other two treatments with thrips (treatments 2 and 4) ($t > 8.06$ $df = 5$, $p < 0.01$; Table 3.1).

Table 3.1. The effects of onion thrips (*Thrips tabaci*), bacterial leaf blight (*Pantoea ananatis*) and mechanical damage (five pricks using a pin) on onions. When thrips and bacteria were present together, bacteria were either added to plants 7 days after thrips or simultaneously. Thrips (3-4 adults/plant) were added to plants from a laboratory colony. Bacterial (1×10^8 CFU) solution was sprayed onto plants using a hand spray bottle. Experiments were conducted for 14 days in a growth chamber at Michigan State University, East Lansing, MI. N = 15. Numbers (1-6) in the second row correspond to treatment numbers for *experiment 1* used in the text.

Damage Parameters	Treatments[a,b] (mean ± SE)					
	-/- (1)	th/- (2)	-/pa (3)	th/pa (4)	md/pa (5)	th/pa [st] (6)
Number of leaves	6 ± 0.17a	6.06 ± 0.18a	6.13 ± 0.17a	5.93 ± 0.12a	6.13 ± 0.17a	6.33 ± 0.21a
Total leaf area, cm^2 [c]	584.67 ± 26.78a	520.62 ± 33.98a	586.56 ± 31.16a	512.45 ± 44.23a	547.62 ± 23.73a	589.38 ± 38.04a
Growth, cm^2 [d]	456.56 ± 18.95a	414.79 ± 19.40a	463.15 ± 19.4a	411.34 ± 35.77a	426.10 ± 14.20a	463.46 ± 20.50a
Estimated % leaf blight[e]	0.00 ± 0.00b	0.00 ± 0.00b	1.93 ± 0.50a	2.13 ± 0.27a	1.46 ± 0.39a	1.80 ± 0.28a
Estimated % thrips injury[f]	0.00 ± 0.00b	9.30 ± 2.41a	0.00 ± 0.00b	10.60 ± 2.75a	0.00 ± 0.00b	2.60 ± 0.69b
Thrips per plant[g]	0.00 ± 0.00c	3.86 ± 0.60b	0.00 ± 0.00c	4.26 ± 0.88b	0.00 ± 0.00c	12.60 ± 1.47a

[a] Different letters within a row indicate differences among treatments, Tukey's HSD, $\alpha < 0.05$.
[b] Thrips infestation (th), *P. ananatis* inoculation (pa), mechanical damage (md), thrips and bacteria added at the same time [st].
[c] (Gamiely et al. 1991).
[d] Growth = total leaf area prior to rating – total leaf area 7 days after inoculation.
[e] Estimated percentage of leaf damage from bacterial leaf blight cause by *P. ananatis* (Horsfall and Barratt 1945).
[f] Estimated percentage of leaf damage from thrips feeding.
[g] Number of thrips counted on each plant

Experiment 2. The number of leaves per plant and total leaf area before manipulation did not differ between treatments (number of leaves: $F = 0.95$, $df = 3, 30$ $p = 0.45$; leaf area: $F = 0.54$, $df = 3, 30$ $p = 0.75$). The number of leaves per plant, total leaf area and plant growth were similar among treatments after experimental manipulation (leaf number: $F = 1.00$, $df = 3, 30$, $p = 0.41$; leaf area: $F = 2.23$, $df = 3, 30$, $p = 0.11$; growth: $F = 2.28$, $df = 3, 30$, $p = 0.10$; Table 3.2). The treatment that received 20 thrips exhibited the most severe blight symptoms at the end of the experiment, with about 35% more leaf tissue necrosis than any of the other treatments ($t = 5.18$, $df = 3$, $p < 0.01$; Table 3.2). The percentage of leaf tissue damaged by thrips was 12% and 15% higher when initial infestation was 12 and 20 thrips per plant (treatments 3 and 4; numbers correspond to treatment numbers in Table 3.2) ($t > 5.16$, $df = 3$, $p < 0.01$; Table 3.2), respectively, compared to the treatments with lower numbers of thrips. The number of thrips per plant at the end of the experiment was seven times higher than when initial thrips infestation was 20 thrips per plant (treatment 4) ($t = 2.31$, $df = 3$, $p < 0.01$; Table 3.2) compared to when initial infestation was 0 or 4 thrips per plant (treatments 1 and 2). There was a significant positive correlation, across all treatments, between the number of thrips on each plant and the percent leaf area damaged by *P. ananatis* ($R^2 = 0.68$, $p < 0.01$; Figure 3.1A). Thrips feeding damage symptoms on leaves (%) and severity of leaf blight symptoms (%) were positively correlated ($R^2 = 0.70$, $p < 0.01$; Figure 3.1B).

Table 3.2. Effects of varying numbers of onion thrips (*Thrips tabaci*) per plant in combination with bacterial leaf blight (*Pantoea ananatis*) on onions. Thrips (0, 4, 12 or 20/plant) were added to plants from a laboratory colony. Plants were inoculated with a liquid culture of *P. ananatis* (1×10^8 CFU), sprayed onto plants. Experiments were conducted for 15 days in a growth chamber at Michigan State University, East Lansing, MI. N = 11. Numbers (1-4) in the second through fifth rows correspond to treatment numbers for *experiment 2* used in the text.

Damage Parameters	Treatments[a,b] (mean ± SE)			
	0 th/pa (1)	4 th/- (2)	12 th/pa (3)	20 th/pa (4)
Number of leaves	7.73 ± 0.20a	7.18 ± 0.26a	7.36 ± 0.20a	7.36 ± 0.28a
Total leaf area, cm^2 [c]	1006.92 ± 42.78a	821.51 ± 52.79a	877.18 ± 41.09a	956.82 ± 64.90a
Growth, cm^2 [d]	558.78 ± 33.90a	374.23 ± 52.57a	446.59 ± 42.00a	486.42 ± 56.65a
Estimated % leaf blight[e]	11.27 ± 0.54b	14.09 ± 0.80b	13.27 ± 0.96b	19.72 ± 1.59a
Estimated % thrips injury[f]	0.00 ± 0.00b	3.45 ± 0.53b	15.72 ± 1.65a	18.09 ± 2.61a
Thrips per plant[g]	0.00 ± 0.00b	1.63 ± 0.53b	6.18 ± 0.67ab	12.27 ± 3.16a

[a] Different letters within a row indicate differences among treatments, Tukey's HSD, $\alpha < 0.05$.
[b] Thrips infestation (th), *P. ananatis* inoculation (pa), mechanical damage (md), added at the same time (st).
[c] (Gamiely et al. 1991).
[d] Growth = total leaf area prior to rating – total leaf area 7 days after inoculation.
[e] Estimated percentage of leaf damage from bacterial leaf blight cause by *P. ananatis* (Horsfall and Barratt 1945).
[f] Estimated percentage of leaf damage from thrips feeding.
[g] Number of thrips counted on each plant.

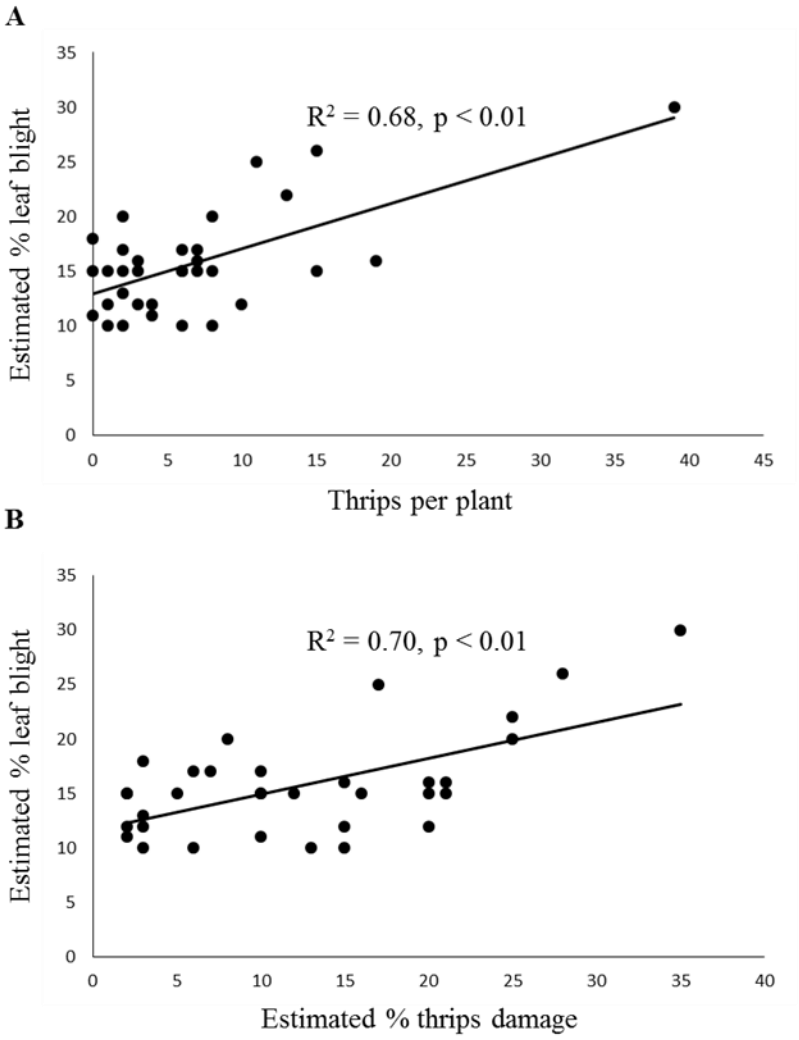

Figure 3.1. Correlation between the number of onion thrips (*Thrips tabaci*) per plant and the percent leaf area with leaf blight symptoms (A). Correlation between the percent leaf area with thrips feeding damage and the percent leaf area with leaf blight symptoms (B). Data are all treatments from *Experiment 2* which received thrips (treatments 2, 3 and 4; 4 – 20 thrips/plant). Plants were inoculated with a liquid culture of *P. ananatis* which was sprayed onto plants (1×10^8 CFU). Experiments were conducted in a growth chamber at Michigan State University, East Lansing, MI. N = 11.

<u>*Experiment 3.*</u> *Pantoea ananatis* was not recovered from any of the treatments which did not receive bacteria (treatments 1 and 2; numbers correspond to treatment numbers in Table 3.1). *Pantoea ananatis* colonies containing the GFP gene were cultured from symptomatic leaf tissue

on plants which received *P. ananatis* without thrips (treatment 3) and *P. ananatis* in combination with thrips (treatment 4) with mean bacterial densities of 5.83×10^5 and 3.01×10^5 CFU, respectively. Fluorescent colonies were also isolated from random non-symptomatic areas on plants which received *P. ananatis* alone (treatment 3) and from feeding sites on plants which received *P. ananatis* in combination with thrips (treatment 4) with mean bacterial densities of 1.44×10^3 and 1.58×10^5, respectively. The average bacterial density per plant did not differ between treatments ($t = 1.75$, $df = 34$, $p = 0.09$). Bacterial density on leaf tissue with disease symptoms from plants which received *P. ananatis* without thrips (treatment 3) compared to symptomatic leaf tissue from plants which received *P. ananatis* in combination with thrips (treatment 4) was statistically similar ($t = 0.95$, $df = 16$, $p = 0.36$). On plants which received both *P. ananatis* and thrips, bacterial density was significantly higher in leaf tissue containing thrips feeding damage (treatment 4) compared to areas without thrips feeding damage (treatment 3) ($t = 3.32$, $df = 16$, $p < 0.01$).

Pantoea ananatis was not detected in leaf tissue without bacterial application regardless of thrips damage (Figure 3.2A, B). Fluorescence microscopy confirmed the colonization of onion leaf tissue by the GFP strain of *P. ananatis*, mainly in intercellular regions of damaged cells (Figure 3.2C). Fluorescent *P. ananatis* colonized thrips feeding sites, resulting in necrotic lesions (Figure 3.2D).

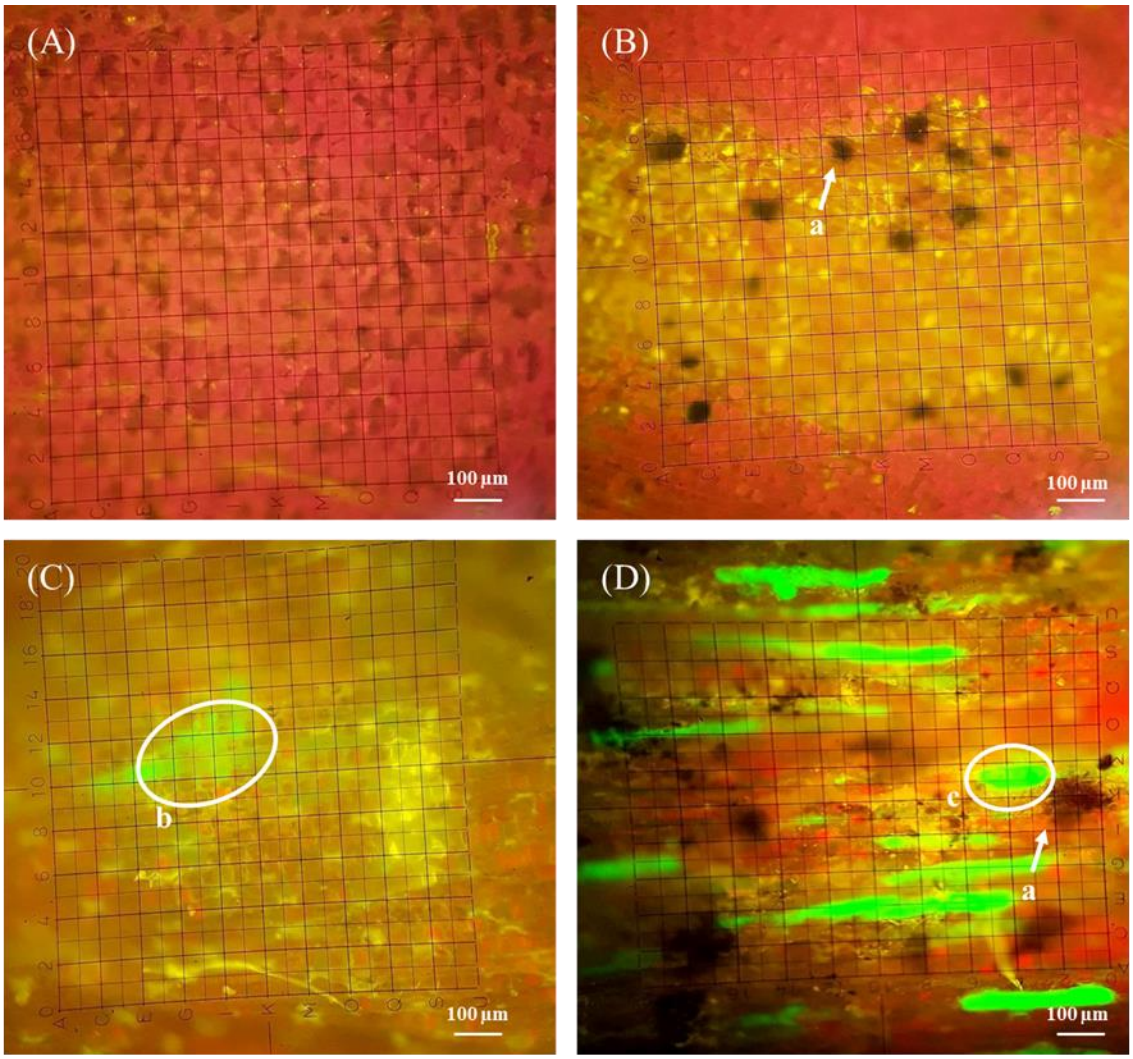

Figure 3.2. Representative samples of undamaged onion leaf tissue (A), onion tissue damaged by thrips (*Thrips tabaci*) feeding (B), necrotic leaf tissue damaged by the GFP strain of *P. ananatis* (C) and onion leaf tissue damaged by both thrips feeding injury and the GFP strain of *P. ananatis* (D). White arrows (a) indicate where thrips mandible was inserted into the leaf tissue. Yellow pigmentation is the result of chlorophyll removal by thrips feeding (B, D). Fluorescent *P. ananatis* colonizing intercellular spaces of leaf cells (C) and regions where cells were damaged by thrips feeding (D), indicated by the white circles (b and c, respectively). Plants were inoculated with a liquid culture of the GFP strain of *P. ananatis* which was sprayed onto plants (1×10^8 CFU). Experiments were conducted in a growth chamber at Michigan State University, East Lansing, MI. Photos were taken at 10x magnification and each square of the reticle = 50 µm^2. The scale bar = 100 µm.

Discussion

These results suggest that onion thrips feeding injury promotes the colonization of onion leaves by *P. ananatis* and bacterial symptoms are more abundant with increasing thrips numbers. Previous research demonstrated that thrips were able to acquire this pathogen after a few hours of feeding on an infected plant and that bacteria was able to survive transstadially without affecting the fitness of the insect host (Dutta et al., 2014, Dutta et al. 2016). Thrips are able to spread this pathogen to new plants via stercorarial means, i.e., through their feces (Dutta et al. 2014, Dutta et al. 2016), and their feeding injury provides entry points for bacterial infection. A similar relationship was recorded, where the feeding injury of thrips promoted the development of purple blotch by providing alternative penetration sites for *A. porri* (McKenzie et al. 1993). By utilizing fluorescence microscopy, I was able to visualize the GFP-tagged strain of *P. ananatis* colonized areas of the onion leaf tissue damaged by thrips, providing evidence that feeding sites act as disease entry points.

Onion thrips reproduce asexually through parthenogenesis and the rate of reproduction is dependent on environmental conditions. Under hot and dry conditions, onion thrips oviposition rates increase and generation time decreases, leading to rapid population growth (Rueda et al. 2007, Gill et al. 2015). These results demonstrate that as the number of thrips per plant and their feeding damage increases, so do the symptoms of bacterial center rot caused by *P. ananatis* (Figure 1). These results are similar to previous findings from an insecticide trial where plots of onions receiving insecticides through the growing season had significantly less necrotic leaf tissue compared to plots with unmanaged thrips populations (Grode et al. 2017). Therefore, it is important for onion growers to manage onion thrips populations, particular during hot weather, in order to minimize the development of bacterial rot in onions, and future research should focus

on understanding the application threshold for insecticides for reducing thrips populations along with bacterial symptoms.

The interaction between onion thrips and *P. ananatis* during the early growing season is poorly understood, and while the inoculum source of *P. ananatis* is currently unknown, it has been previously identified from onion seeds (Walcott et al. 2002) and a variety of other crops (Bruton et al. 1986, Wells et al. 1987, Bruton et al. 1991, Azad et al. 2000, Coutinho et al. 2002). This bacterium has also been identified as a common epiphyte of various weed species (Gitaitis et al. 2002). Onion thrips are known to overwinter on many weed species surrounding onion fields (Smith et al. 2011) before colonizing volunteer onions in the field during the spring (Larentzaki et al. 2007). Monitoring and managing weed species during early growing season for both onion thrips and *P. ananatis* may prevent the disease from spreading to the field. A better understanding of this interaction may provide insights for improving early-season management of this pest complex.

These laboratory results will need to be verified under field conditions in the future as much less is understood about *P. ananatis* and thrips relationships under field conditions. Bacterial pathogens, such as *P. ananatis,* can be disseminated throughout the field via wind, splashing water (Wiriyajitsomboon et al. 2014) and contaminated machinery (Gitaitis et al. 1978), therefore the relative contribution of thrips and different abiotic factors to pathogen spread in onion fields needs to be better understood. In addition, while field research demonstrated that onion thrips are contributing to the spread of a closely related pathogen, *P. agglomerans* (Grode et al. 2017), the acquisition rates for *P. ananatis* and *P. agglomerans* are not the same (Dutta et al. 2014), thus results with one pathogen should not be readily assigned to the other.

CHAPTER 4:

Conclusions and future directions

The purpose of this project was to determine the relationship between onion thrips and the bacterial pathogens responsible for bacterial leaf blight and bacterial center rot in onion, *Pantoea agglomerans* and *P. ananatis*. In the second chapter of this thesis, I examined the relationship between onion thrips and *P. agglomerans* in the field in 2015 and 2016 and found positive correlations between onion thrips population densities, numbers of onion thrips positive for *P. agglomerans*, and bacterial leaf blight incidence in onion fields. During the early weeks of the 2015 and 2016 growing seasons onions symptomatic with bacterial leaf blight reached > 60%; however, only a small proportion of onion thrips screened positive for *P. agglomerans* (< 10%) during this time. As the season progressed, bacterial leaf blight reached 100% incidence in the fields by late July in 2015 and early July in 2016. The number of onion thrips positive for *P. agglomerans* also increased throughout both growing seasons reaching peak positive proportions in mid-late July. Since the proportion of onion thrips carrying *P. agglomerans* did not increase until several weeks following an increase in bacterial leaf blight incidence, it is unlikely that onion thrips are introducing the bacteria into the fields. In pesticide trials conducted in 2015 and 2016, insecticides reduced both onion thrips abundance and bacterial leaf blight incidence, while bactericides showed no effect on controlling bacterial leaf blight severity.

In the third chapter, I examined the effects of injury to onion by onion thrips and its role in infection by *P. ananatis* and development of bacterial center rot symptoms under laboratory conditions. When inoculated with a liquid culture of *P. ananatis*, onions with onion thrips feeding injury had the highest percentage of necrotic leaf tissue. Also, significant positive

correlations were determined between the number of thrips per plant and the severity of leaf blight symptoms as well as the percentage of feeding damage and the severity of leaf blight symptoms. Lastly, by utilizing a GFP tagged strain of *P. ananatis*, I observed the colonization of onion thrips feeding injury by *P. ananatis*, and compared the bacterial concentration in onions with disease and no onion thrips to onions with disease and onion thrips. I found that the concentration of bacteria was higher in areas of thrips feeding injury but when the total concentration on plants was compared no significant differences were found.

Onion thrips populations fluctuate based on temperature and populations increase drastically when temperatures rise above 25C in the field (Rueda et al. 2007). Insect data collected during the 2015 and 2016 growing season's show population increases in late July, a typically hot period during Michigan summer's, which coincided with high incidences (> 90%) of bacterial leaf blight in sampled fields. Similarly, over the course of late July and early August, onion thrips positive for *P. agglomerans* reached highest proportions, suggesting that during optimal weather conditions onion thrips were more actively feeding and acquiring more bacteria. It is important to note, the time when the highest proportion of onion thrips were positive for *P. agglomerans* occurs only a few weeks before when onions senesce, a time when onion thrips are of least concern and insecticide applications are often reduced. In cases where infection by *P. agglomerans* and *P. ananatis* is severe early senesce of onions may occur (Mary Hausbeck verbal communication) which could also correspond with a period of increased onion thrips pressure. During the late part of the growing season, some onion thrips undergo exodus flights and travel long distances, often field to field (Smith et al. 2015). If a high proportion of onion thrips making exodus flights are carrying *P. agglomerans*, or other related bacteria, long-distance disease spread could occur.

The inoculum source of *P. agglomerans* as well as *P. ananatis* is currently unknown, however both bacteria are found in other cropping systems as both plant pathogens and epiphytes (Serrano 1928, Bruton et al. 1986, Wells et al. 1987, Bruton et al. 1991, Azad et al. 2000, Coutinho et al. 2002, Procópio, 2004, Medrano and Bell 2007, Morales-Valenzuela et al. 2007, Romeiro et al. 2007, Ferreira et al. 2008, Lee et al. 2010, Yang et al. 2011). *Pantoea ananatis* is also known to naturally infest onion seeds and this has been hypothesized as an inoculum source for bacterial disease in the field (Walcott et al. 2002). It may be important to screen onion seedlings for *P. ananatis* and *P. agglomerans* during the early growing season to determine if the bacteria originates from seeds. The role of onion thrips as an inoculum source for *P. agglomerans* and *P. ananatis* has not been investigated. Before colonizing volunteer onions in the field the following spring (Larentzaki et al. 2007), onion thrips feed and overwinter on an assortment of weed species. Similarly, bacteria such as *P. ananatis* are known to be a common epiphyte of various weed species (Gitaitis et al. 2002). If an interaction between onion thrips and *Pantoea* sp. is occurring in weed species before the start of the growing season, onion thrips may be introducing these diseases to the field. It is known that *P. ananatis* can persist in tobacco thrips transtadially (Dutta et al. 2016) however it has not been investigated whether these bacteria can persist in thrips during overwintering. According to the field data, 6% of the field caught onion thrips were positive for *P. agglomerans* during the first sampling period suggesting that thrips are either acquiring the disease from another source, such as weeds, or it is persisting in overwintering populations. Early season screening of onion thrips populations residing in nearby weeds may provide insight into the inoculum source of these diseases.

Results from this thesis establish a field relationship between onion thrips and *P. agglomerans* and an association between onion thrips feeding damage and *P. ananatis*

development. While these bacteria are similar and are often found together in the field, is important to investigate differences between the two and their relationship with onion thrips and other thrips species. The laboratory results which showed a correlation between onion thrips feeding damage and severity of bacterial center rot by *P. ananatis* will need to be verified under field conditions. Prior laboratory research demonstrated that onion thrips have the ability to acquire and transmit *P. ananatis* and *P. agglomerans* (Dutta et al. 2014). Future research will need to explore the differences between onion thrips direct and indirect association with *Pantoea* sp. directly as a vector and indirectly through feeding damage in onion fields. Aside from transmission by onion thrips, *P. ananatis* and *P. agglomerans* are known to be disseminated throughout onion fields via contaminated machinery (Gitaitis et al. 1978), wind or splashing water (Wiriyajitsomboon et al. 2014). Therefore, it is also important to investigate the relative contribution of onion thrips and other abiotic factors to the spread of *Pantoea* sp. throughout onion fields.

The research presented in chapter two of this thesis is the first to investigate management strategies which control both onion thrips and bacterial leaf blight. Based on the results of the pesticide trail, it is clear that onion thrips are contributing to the severity of bacterial leaf blight and the only way to reduce bacterial leaf blight incidence was to reduce the thrips populations. It is likely that the feeding damage from onion thrips is causing the increase in bacterial leaf blight severity. This would match the results from chapter three which established a correlation between the number of thrips per plant and the amount of necrotic leaf tissue on onion leaves. Current management practices utilize economic action thresholds as a way to determine when an insecticide application is necessary (Fournier et al. 1995). Future research should focus on investigating various application thresholds for insecticides for reducing thrips populations along

with bacterial leaf blight and center rot symptoms. Effectively managing onion thrips populations could reduce the risk of losses from bulb rot during long-term storage and reduce the continued spread of pathogens to other onion fields.

APPENDIX

Record of Depositions of Voucher Specimens

The specimens listed below have been deposited in the named museum as samples of those species or other taxa, which were used in this research. Voucher recognition labels bearing the voucher number have been attached or included in fluid preserved specimens.

Voucher Number: 2017-02

Author and Title of thesis:
Author: Ari Grode
Title: The Relationship between Onion Thrips (*Thrips tabaci*) and the Bacterial Pathogens *Pantoea agglomerans* and *Pantoea ananatis*

Museum(s) where deposited:
Albert J. Cook Arthropod Research Collection, Michigan State University (MSU)

Specimens:

Table S. 1. Voucher specimens deposited at the Albert J. Cook Arthropod Research Collection (Michigan State University).

Family	Genus-Species	Life Stage	Quantity	Preservation
Thripidae	*Thrips tabaci*	Adult	10	alcohol

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