ERWINIA TRACHEIPHILA PATHOSYSTEM:
VECTOR-PATHOGEN INTERACTIONS

A Thesis in
Entomology
by
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ABSTRACT

Cucurbitaceae is a diverse plant family that includes cultivated species in the genera *Cucumis, Cucurbita, Citrullus, Lagenaria* and *Luffa*. Within Cucurbitaceae, there is a tight association between the bacterial pathogen *Erwinia tracheiphila* and the required diabroticite beetle vector, *Acalymma vittatum* (the striped cucumber beetle). This thesis expands on prior knowledge of this tri-trophic system by providing insights into vector competence of diabroticite beetles that exist outside of the geographic range of *Erwinia tracheiphila*. Identification of a novel biological control agent, *Pseudomonas fluorescens*, targeting microbial gut interactions within the vector beetle to control *Erwinia tracheiphila*.

Two diabroticite beetle species were selected from regions of the United States that currently do not have the causal agent of bacterial wilt of cucurbits, *Erwinia tracheiphila*: the western striped cucumber beetle (*Acalymma trivittatum*) from California and the banded cucumber beetle (*Diabrotica balteata*) from Florida. Based on ecological and phylogenetic similarities to *Acalymma vittatum*, *Acalymma trivittatum* could serve as a competent vector that would provide *Erwinia tracheiphila* a route into the western United States. Likewise, if *Diabrotica balteata* is also a competent vector, it would provide avenues for *Erwinia tracheiphila* into the southern United States. The three diabroticite species were subjected to feeding bioassays to determine transmission capabilities. Analysis of frass through polymerase chain reaction and plate culturing demonstrated presence and viability of *Erwinia tracheiphila* after passage through the beetles. While the results indicated that *Acalymma trivittatum* was able to transmit *Erwinia tracheiphila*, there was insufficient statistical power to compare its transmission efficiency relative to *Acalymma vittatum*. *Diabrotica balteata* was also able to transmit *Erwinia tracheiphila*, but due to low beetle survival rates during the course of the experiment, the sample
size of the surviving beetles was too small to make statistically relevant conclusions about its vector status.

Current control methods for *Erwinia tracheiphila* target the vector through pesticide application, but with possible geographic expansion by other vector species, an alternative strategy may be required. The bacterial wilt of cucurbit disease cycle is maintained by *Erwinia tracheiphila* gut colonization of the vector beetle, effectively overwintering until the next growing season. By disrupting *Erwinia tracheiphila* gut colonization, the number of infected vectors could be greatly reduced. This has proven to be an effective biological control approach for *Erwinia amylovora* (the fire blight of tree fruit pathogen) using a commercially available isolate of *Pseudomonas fluorescens*.

In this study, I challenged isolates of *Erwinia tracheiphila* with an isolate of *Pseudomonas fluorescens* (Pf55) through *in vitro* plating assays and *in vivo* co-localization gut assays. *In vitro* assays confirmed that *Pseudomonas fluorescens* successfully inhibited *Erwinia tracheiphila* growth. Because *Pseudomonas fluorescens* was detected in the mid and hindguts of *Acalymma vittatum* (coinciding with the sites of *Erwinia tracheiphila* gut colonization), the *in vitro* assay results supported the hypothesis that *Pseudomonas fluorescens* could potentially inhibit *Erwinia tracheiphila* *in vivo*. I have shown that other diabroticite beetles can successfully transmit *Erwinia tracheiphila* and demonstrated the potential use of *Pseudomonas fluorescens* as a new method of biological control for *Erwinia tracheiphila*. 
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Chapter 1

Introduction

Cucurbitaceae includes three major plant genera: cucumbers and melons are in the genus *Cucumis*, squash, gourds and pumpkins are in the genus *Cucurbita*, and watermelons in the genus *Citrullus*. (Egel, 2011; Kysan & Miller, 1986). Two major problems for Pennsylvanian growers of *Cucumis* and *Cucurbita* are the insect pest *Acalymma vittatum* (Fabricus) and the plant pathogen that causes bacterial wilt, *Erwinia tracheiphila* (Smith). This tri-trophic system has important ecological and agricultural implications for growers pertaining to the Luperini leaf beetles (Coleoptera: Chrysomelidae: Galerucinae) that co-evolved with Cucurbitaceae and the plant pathogen *Erwinia tracheiphila*.

Cucurbitaceae plant family

Cucurbitaceae is an economically important plant family around the world (Kumbhalkar et al., 2013). Two genera within the within Cucurbitaceae that include domesticated species, *Cucumis* and *Cucurbita*, are particularly susceptible to bacterial wilt (Main & Walker, 1971; Stoffers, 1980; Watterso et al., 1972). Cucurbitaceae originated in Asia with several geographic dispersion events (Schaefer et al., 2009). Cucumbers (*Cucumis sativus*) and their closest relatives evolved from a common ancestor approximately ca 3 MYA in Africa. Pumpkins and squash (*Cucurbita spp*) diverged from the sister clade *Peponopsis* from South America approximately 16 MYA. North American Cucurbitaceae evolved from several expansions of South American lineages over millions of years (Schaefer et al., 2009). Europe recently had *Citrullus lanatus*, *Cucumis melo*, *Cucumis sativus* and *Cucurbita pepo* introduced most likely due to casual escapes from cultivation (Schaefer et al., 2009). Many species within Cucurbitaceae produce a secondary
plant kairomone called cucurbitacin that evolved to provide herbivory protection. However, cucurbitacin became a feeding stimulant for a group of Chrysomelidae beetles within the Luperini tribe which cause these beetles to co-evolve with Cucurbitaceae (Metcalf et al., 1980). This association of Diabrotica originated in Central and South America where Cucurbita diverged and has the highest diversity of diabroticites (Metcalf et al., 1980).

**Chrysomelidae: Diabrotica and Acalymma**

Chrysomelidae is a large diverse family in the order Coleoptera, including many pests of agricultural crops (e.g. corn, potatoes, cucurbits). Diabroticite (Chrysomelidae: Galerucinae: Luperini: Diabroticina) beetles co-evolved with host plants, dating back to 45 MYA, with beetle speciation events co-occurring with the speciation of the Cucurbitaceae (Clark et al., 2001). It is this close-knit co-evolution between diabroticites and cucurbits that *Erwinia tracheiphila* has exploited.

**Diabrotica**

*Diabrotica* is a large genus in Chrysomelidae and is composed of three species groups, *signifera*, *fucata* and *virgifera* (Eben & Espinosa de los Monteros, 2013; Krysan & Miller, 1986). There are 338 species divided among these species groups, with eleven species in *signifera*, 305 species in *fucata* and 21 species in *virgifera*. The major pest species are grouped into *fucata* and *virgifera* species groups (Branson & Krysan, 1981; Clark et al., 2001; Eben & Espinosa de los Monteros, 2013; Krysan & Smith, 1987). The *fucata* species group can withstand northern temperate climates due to the diapause capabilities allowing adult beetles to overwinter, whereas *virgifera* species group overwinter in the egg stage (Krysan & Miller, 1986; Krysan & Smith, 1987). Mitochondrial and DNA sequencing have verified the phylogeny of Diabroticites.
*Acalymma* and *Diabrotica* have an average mitochondrial distance of 16.02%, however, within the *Diabrotica* species there is an average mitochondrial distance of 13.84% (Clark et al., 2001).

**Acalymma**

*Acalymma* was recently elevated to genus from *Diabrotica* with 25 species being transferred to *Acalymma* in the 1950s and 60s by Bechnyé (Cabrera & Durante, 2001). It is currently thought that *Acalymma* diverged from *Diabrotica* 44.6 million years ago (Eben & Espinosa de los Monteros, 2013). The striped cucumber beetle, *Acalymma vittatum*, is a specialist, requiring cucurbits during the entire life cycle. Adult beetles feed on foliage and fruits of the cucurbit plants, eggs are laid in the soil where the larva propagate and feed on the root-hairs of the cucurbit plants (Cuthbert et al., 1968; Eben & Barbercheck, 1997; Howe & Zdarkova, 1971; Reed et al., 1984; Smith, 1965). Compared to the distribution of most diabroticites, the genus *Acalymma* has successfully expanded north into the continental regions with severe cold temperatures due to overwintering behavior and diapause capabilities (Smith, 1965). *Erwinia tracheiphila* exploits *Acalymma vittatum* and *Diabrotica undecimpunctata howardi*, the spotted cucumber beetle, as vectors due to the host plant coinciding with these two species and the bacterium. Fecundity and lifespan of the non-diapausing beetles were deemed unaffected by gut colonization of *Erwinia tracheiphila* (Shapiro et al., 2013).

**Taxonomy of the genus *Erwinia***

*Erwinia* is a phytopathogenic genus within Enterobacteriaceae, which also includes other animal and plant pathogenic genera (e.g. *Pantoea* and *Enterobacter*) (Garrity, 2005; Naum et al., 2008; Starr & Chatterjee, 1972). The genus *Erwinia* primarily causes wilt and blight diseases of plants, mostly within tree fruit and vegetable crops (Garrity, 2005). Prior to the early 1980s, the
genus *Erwinia* consisted of 15 species and 3 subspecies. Since then there have been several taxonomic reassignments into and out of *Erwinia*. In 1999 Hauben et al. proposed that genus *Erwinia* should be further subdivided into *Erwinia, Pantoea* and *Enterobacter* based on the phylogenetic analysis of nearly full or full-length 16S rDNA sequences available at that time (Hauben et al., 1999). Other independent research provided additional support for this subdivision (Hauben et al., 1999; Kwon et al., 1997; Waleron et al., 2002). Former species *E. herbicola, E. milletiae* (Gavini et al., 1989), *E. ananas, E. uredovora*, and *E. stewartii* (Mergaert et al., 1993) were reassigned to the genus *Pantoea*. Three species, *Erwinia dissolvens, E. nimipressuralis* (Brenner et al., 1986), and *E. cancerogena* (Dickey and Zumoff, 1988) were reclassified as *Enterobacter*. Additionally, five new species and two subspecies were added to the genus *Erwinia*: *E. alni* (Surico et al., 1996), *E. billingiae* (Mergaert et al., 1999), *E. cacticida* (Alcorn et al., 1991), *E. persicina* (Hao et al., 1990), *E. psidii* (Rodrigues-Neto et al., 1987), *E. carotovora subsp. odorifera* (Gallois et al., 1992), and *E. carotovora subsp. wasabiae* (Goto and Matsumoto, 1987). *E. amylovora, E. rhapontici, E. persicinus, E. malotivora, E. psidii*, and *E. tracheiphila* remained classified as the “true” *Erwinia* species (Brosius et al., 1981; Hauben et al., 1999; Waleron et al., 2002). *Erwinia tracheiphila* is distantly related to all the other *Erwinia* species within the true *Erwinia* species group and has an average similarity with all other *Erwinia* species of 95.5% of the 16S rDNA sequence (Hauben et al., 1999; Waleron et al., 2002).

**Bacterial Wilt of Cucurbits: *Erwinia tracheiphila***

**Biology**

*Erwinia tracheiphila* (Enterobacteriae: Enterobacteriaceae)(Smith), the etiological agent of bacterial wilt of cucurbits, was first described in 1911 at the Pennsylvania State University’s Experimental Station. The vasculature of infected cucurbit plants were described as being filled
with a “milky, stringy mass of bacteria”, which is a key sign for the pathogenicity of the bacteria (Fulton et al., 1911). The bacteria form a xylem-blocking biofilm within the plant causing the characteristic wilt symptoms: pale wilting leaves, foliar necrosis, drying of leaves and stems, and eventual death (Fulton et al., 1911; Sherf & Macnab, 1986). Many other Enterobacteriaceae have been well-studied leading to the determination of different strains. Recently, phenotypic and genetic variations have been identified in *Erwinia tracheiphila* in relation to host plant origin. Strain isolates from the *Cucumis* genera of plants have a slower disease progression in the *Cucurbita* genera of plants and vice versa (Rojas et al., 2013). *Erwinia tracheiphila* uses *Acalymma vittatum* (Fabricus), the striped cucumber beetle, to transmit the bacteria-contaminated frass between individual plants into herbivory wounds and floral nectaries (Mitchell & Hanks, 2009; Sasu et al., 2010). *Erwinia tracheiphila* infected plants have altered foliar and floral volatile profiles (Shapiro et al., 2012). Elevated attractiveness of infected plant volatiles results in more beetles preferentially feeding on symptomatic foliage, increasing the likelihood of *Erwinia tracheiphila* acquisition. Concurrently, flowers from healthy plants were more attractive than those from wilting plants, thereby increasing beetle congregation in healthy flowers to facilitate transmission of *Erwinia tracheiphila* (Sasu et al., 2010; Shapiro, 2012). To survive the winter, the bacteria colonize the gut of the striped cucumber beetles (de Mackiewicz et al., 1998; Garcia-Salazar et al., 2000). *Acalymma vittatum* foregut and hindgut share a similar cuticular epithelial morphology with spines and folds that provide a surface to which bacteria can attach (Garcia-Salazar et al., 2000). However, the effects on pathogenicity and host specificity after passage through *Acalymma vittatum* have not been explored.

**Geographic range and control**

This pathogen ranges from the Northeastern United States to the Midwest and Mid-Atlantic states and northward into southern Canada (Rojas et al., 2015). The first documented
cases of bacterial wilt in the southwest have recently been published (Sanogo et al., 2011). Beetle pressure and cultivar susceptibility play a major role in controlling the spread of *Erwinia tracheiphila* (Bach, 1980; Brust & Rane, 1995; Ferguson et al., 1983). On average crop losses from bacterial wilt of cucurbits ranges from 10-20% (Latin, 2001)(see Appendix 1-1), however crop loss up to 80% by August from *Erwinia tracheiphila* has been recorded by Cornell University (McGrath, 2001)(see Appendix 1-2). Currently, the primary method of control for this pathogen is the use of insecticidal seed treatments and foliar sprays for the striped cucumber beetle and removal of infected plants to decrease beetle acquisition (Brust et al., 1996; Courter et al., 1979; Egel, 2011; Pair, 1997; Sherf & Macnab, 1986).

**Biological control via Pseudomonas fluorescens**

**Biology of Pseudomonas fluorescens**

*Pseudomonas fluorescens* is a common opportunistic soil and plant bacterium. This species is non-pathogenic, Gram-negative, rod-shaped, motile bacteria within the Pseudomonadaceae: *Pseudomonas fluorescens* complex (Silby et al., 2009; Yamamoto et al., 2000). This group of bacteria are characterized by an ability to grow at 4 °C (psychrophilic) and can be saprophytic (Yamamoto et al., 2000). However, the optimum temperature range for growth is 20-25 °C in an aerobic environment (Breed et al., 1957). *Pseudomonas fluorescens* is capable of antibiosis and siderophore production, which allow for competitive exclusion of other bacteria (Temple et al., 2004). Several of these characteristics have been exploited for biological control of several fungal and bacterial plant pathogens that are important in wheat, legumes and tree fruit (Whipps, 2001; Yamamoto et al., 2000).
Biological control

*Pseudomonas fluorescens* has been shown to be a biological control agent for several soil-borne plant pathogens (Couillerot et al., 2009). *Pseudomonas fluorescens* was successfully used to reduce the field transmission of the bacterial agent of fire blight of apple and pear, *Erwinia amylovora* (Burrill) to flowers and immature fruits (Cabrefiga et al., 2007; Pujol et al., 2005). These studies determined that certain strains of *Pseudomonas fluorescens* competitively exclude other organisms by sequestration of finite minerals in the environment (Cabrefiga et al., 2007; Whipps, 2001). *Pseudomonas fluorescens* strains also produce antibiotics and can induce plant resistance as other modes of action against other organisms (Whipps, 2001).

Significance of this study

Within this thesis, basic biological questions will be answered providing a better understand of the full pathosystem, enabling for improved targeting for controlling the pest and pathogen.

Objectives of proposed research

In this thesis I cover the following topics:

- Chapter 2: Vector Competence of Diabroticite Beetles Associated with Cucurbit Crops Outside the Geographic Range of *Erwinia tracheiphila*
- Chapter 3: Potential Biological Control of *Erwinia tracheiphila* by Internal Gut Interactions in *Acalymma vittatum* with *Pseudomonas fluorescens*
- Chapter 4: Final Conclusions and Future Research
References: Chapter 1


Pair SD (1997) Evaluation of Systemically Treated Squash Trap Plants and Attracticidal Baits for Early-Season Control of Striped and Spotted Cucumber Beetles (Coleoptera: Chrysomelidae) and Squash Bug (Hemiptera: Coreidae) in Cucurbit Crops. Journal of Economic Entomology 90: 1307-1314.


Chapter 2

Vector Competence of Diabroticite Beetles Associated with Cucurbit Crops Outside the Geographic Range of *Erwinia tracheiphila*

Introduction

Coleoptera is one of the largest insect orders. Many of the members of the Chrysomelidae (the second most diverse family within Coleoptera) are pests of corn, tomato, pepper, eggplant, cabbage, cucurbits, sweet potatoes, asparagus, onions, spinach, lettuce, carrots, and potatoes. Diabroticites refers to the genera that are in subtribe Diabroticina. The subtribe Diabroticina includes the large genus *Diabrotica* (Eben & Espinosa de los Monteros, 2013), which is composed of three species-groups: *signifera*, *fucata* and *virgifera*, with the latter two species-groups containing the major pest species (Krysan & Miller, 1986). *Signifera* is the smallest species-group with only 11 species, mostly found in South America. The *virgifera* group, defined by their oligophagous diet and univoltinism, has twice as many species as the *signifera* group. The largest group, with over 300 species, is the *fucata* group where variable polyphagous, multivoltine species that did not fit into *virgifera* or *signifera* have been placed (Branson & Krysan, 1981; Clark et al., 2001; Eben & Espinosa de los Monteros, 2013; Krysan & Smith, 1987). Most *fucata* species can withstand the northern climates due to diapause capabilities of the adult stage. However, species in the *virgifera* group cannot survive subfreezing temperatures as adults due to a lack of diapause, and overwinter in the egg stage (Krysan & Miller, 1986; Krysan & Smith, 1987).
Figure 2-1. Phylogeny of Luperini tribe of Chrysomelidae beetles from COI, 12S and 16S mitochondrial sequences and 28S and ITS2 nuclear fragment sequences with diet changes mapped. Cerotoma and Acalymma are monophyletic whereas Diabrotica is polyphyletic due to fucata and phagy characterizations. Reproduced from Eben & Espinosa de los Monteros (2013).

In the 1940s, Barber separated Acalymma from the genus Diabrotica based on morphological dissimilarities of Acalymma barber compared to other diabroticites (Barber, 1947). Bechnyé then transferred 25 additional species from Diabrotica to Acalymma in the 1950s and 60s (Cabrera & Durante, 2001). Several species within the diabroticites are major pests of cultivated corn or cucurbit crops in the Northeastern United States, such as the western corn rootworm (Diabrotica virgifera virgifera), the twelve spotted cucumber beetle (Diabrotica undecimpunctata howardi), and the striped cucumber beetle (Acalymma vittatum) (Haynes & Jones, 1975; Radin & Drummond, 1994; Siegfried & Mullin, 1990). Cucurbit pests can damage
plants and fruits by feeding and pathogen transmission. *Acalymma vittatum* is the described vector of the causal agent of bacterial wilt of cucurbits, *Erwinia tracheiphila* (Balduf, 1922; Rand & Enlows, 1916).

I studied three closely related pest species within the subtribe Diabroticina that are found in three distinct geographic areas across the United States: the striped cucumber beetle, *Acalymma vittatum* (Fabricus), the western striped cucumber beetle, *Acalymma trivittatum* Mannerheim, and the banded cucumber beetle, *Diabrotica balteata* LeConte. The geographic range of *Erwinia tracheiphila* provided a guide to select pest species that exist outside of this range, which lead to *Acalymma trivittatum* and *Diabrotica balteata*. *Acalymma vittatum* and *Acalymma trivittatum* are closely related cucurbit specialists that can cause major crop loss and occur on opposite coasts of the United States (Balduf, 1922). *Diabrotica balteata* is a relative of *Acalymma vittatum* and is found in the Southern United States. This species is polyphagous, multi-voltine, and cold-intolerant in contrast to *Acalymma*, which is monophagous, univoltine and cold tolerant (Clark et al., 2001).

*Acalymma vittatum* (Fabricus)

The striped cucumber beetle, *Acalymma vittatum*, is one of the major pest species in the Northeastern United States and was previously in the genus *Diabrotica* (Munroe & Smith, 1980). Occurrence of the beetle ranges from southern Canada south to North Carolina, but can be seen as far south as Texas, see Figure 2-2 (Balduf, 1922).
Figure 2-2. Distribution of *Acalymma vittatum* and *Acalymma trivittatum* in North America. Reproduced from Munroe & Smith (1980).

*Acalymma vittatum* is a monophagous specialist on plants within the family Cucurbitaceae. Adult beetles feed on foliage, flowers and fruits of the cucurbit plants, and eggs are laid in the soil where the larva develop and feed on the root-hairs of the cucurbit plants (Cuthbert et al., 1968; Howe & Zdarkova, 1971; Reed et al., 1984; Smith, 1965). Adult beetles diapause during the winter in leaf litter and soil and re-emerge in mid-May. Development from egg to adult takes approximately 4 weeks at 25 °C (Ellers-Kirk & Fleischer, 2006). *Acalymma vittatum* early season field activity is speculated to be associated with average daily temperatures exceeding 12 °C, based on initial crop colonization (Radin & Drummond, 1994). Early season field activity on squash “trap” flats was modeled as Gompertz functions, with initiation of adult activity at >224.7 + 62.9 DD\(_{55F}\) (Bachmann, 2012). Temperature thresholds for mating and oviposition were determined in laboratory settings. Foliage availability did not affect mating, however temperatures ≥ 13°C was required for mating to occur (Radin & Drummond, 1994). However, oviposition activity occurred at 10°C and was affected by foliage availability (Radin &
Drummond, 1994). Life-tables were developed to determine optimum developmental temperature ranges (Ellers-Kirk & Fleischer, 2006). At the lowest temperature tested, 18°C, beetles required 72 days to develop; whereas at the highest survivable temperature, 33°C, beetles developed in 24 days (Ellers-Kirk & Fleischer, 2006). Female beetles can lay as many as 125 eggs at a rate of 0-4 eggs/female/day in their life span of approximately 80 days in laboratory settings (Ellers-Kirk & Fleischer, 2006). Electron microscopy and immunolocalization assays demonstrated that Erwinia tracheiphila colonizes in the beetle gut (Garcia-Salazar et al., 2000); however, little is known about the bacterial-beetle interactions. Fecundity and lifespan of the beetles were not significantly altered by colonization of Erwinia tracheiphila in acquisition and retention experiments (Shapiro et al., 2013).

_Acalymma trivittatum_ Mannerheim

_Acalymma trivittatum_, the western striped cucumber beetle, is closely related to _Acalymma vittatum_ as indicated by genetic analysis (Eben & Espinosa de los Monteros, 2013). The geographic range covers the majority of the west coast and as far south as Texas as of the late 1940s, Figure 2-2 (Barber, 1947; Munroe & Smith, 1980). It has been suggested, based on its relation to _Acalymma vittatum_, that the _Acalymma trivittatum_ is a specialist of the Cucurbitaceae family and diapauses in protected leaf-litter and soils (Alston & Worwood, 2012; Barber, 1947; Gonzales, 1962). The adults feed on the foliage, flowers and fruits of cucurbit plants and eggs are laid in the soil where larva feed on the root hairs of the plants. Although it has a similar ecology to _Acalymma vittatum_, to our knowledge, bacterial wilt has not been documented in the western USA, and I have not encountered any studies of the potential of _Acalymma trivittatum_ to vector Erwinia tracheiphila.
**Diabrotica balteata LeConte**

The banded cucumber beetle, *Diabrotica balteata*, is grouped within the *fucata* group. A large genetic distance in the COI region (15.6%) and ITS region (14.4%) from *Acalymma vittatum* indicates high genetic dissimilarity between *Diabrotica balteata* and *Acalymma vittatum* (Clark et al., 2001). The geographic range in the United States is restricted to the southern regions due to its inability to survive subfreezing temperatures (Clark et al., 2001; Krysan & Miller, 1986; Saba, 1970). Since 1910, *Diabrotica balteata* range has expanded from southern Texas and Arizona to include all of the southern states from the east to the west coast as shown in Figure 2-3 (Krysan & Miller, 1986; Saba, 1970; Smith, 1965).

![Figure 2-3](image)

**Figure 2-3.** The distribution of *Diabrotica balteata* in the United States reproduced from Smith (1965).

Unlike some diabroticites, the banded cucumber beetle has a broader host plant range, including plants within the families Cucurbitaceae, Rosaceae, Fabaceae, and Brassicaceae (=polyphagous) (Saba, 1970). Feeding damage from adult banded cucumber beetle activity affect several important vegetable crops including cucumber, squash, beans, sweet potato, lettuce,
and onion (Saba, 1970). Adults feed on foliage and fruits of host plants, eggs are laid in the soil and larva feed on root hairs. In contrast to *Acalymma vittatum* and *Acalymma trivittatum*, lab rearing protocols often utilize plants other than, or in addition to cucurbits (Saba, 1970). I am also unaware of any studies on the ability of *Diabrotica balteata* to transmit *Erwinia tracheiphila*.

Co-evolution of the diabroticite beetles and host plants has been dated back to 45 MYA with beetle speciation events co-occurring with the speciation of the Cucurbitaceae (Clark et al., 2001). It is this close-knit co-evolutionary relationship between the diabroticites and cucurbits that *Erwinia tracheiphila* has exploited for successful transmission and maintenance of propagules.

*Erwinia tracheiphila* (Smith)

The causal agent of bacterial wilt of cucurbits, *Erwinia tracheiphila*, is part of the bacterial family Enterobacteriaceae, which includes fecal coliforms such as *Escherichia coli* and pathogens such as *Salmonella typhimurium* and *Yersinia pestis* (Naum et al., 2008). This pathogen is found in the Northeastern United States, ranging from Mid-Atlantic states to the Midwest, and into southern Canada (Rojas et al., 2015). The bacteria form a xylem-blocking biofilm within the plant causing the characteristic wilt symptom and leading to eventual death of the plant (Fulton et al., 1911).

Many species within *Erwinia* have been well studied and designated by different strains, or variants. For example, sequence variation of *Erwinia amylovora* has been studied for identification and pathogenicity possibly caused by host and location (Momol et al., 1997). This concept has been recently demonstrated in *Erwinia tracheiphila*, where strain variants have been identified and mapped to host plant origin (Rojas et al., 2013). Isolates from the plant genus *Cucumis* have a slower disease progression in the genus *Cucurbita* and vice versa (Rojas et al.,
Acalymma vittatum can transmit *Erwinia tracheiphila* between individual plants via frass inoculations of herbivory wounds and floral nectaries (Mitchell & Hanks, 2009; Sasu et al., 2010). *Erwinia tracheiphila* infected plants have altered volatile profiles that result in differing attractiveness of foliage and flowers to beetles. Collectively, these changes to attractiveness results in 1) beetles preferentially feeding on symptomatic foliage, thereby facilitating acquisition, and 2) congregation toward healthy flowers, thereby facilitating transmission (Sasu et al., 2010; Shapiro, 2012). *Erwinia tracheiphila* colonizes the guts of the striped cucumber beetles (de Mackiewicz et al., 1998; Garcia-Salazar et al., 2000) and remain dormant over winter. Serological assays of *Acalymma vittatum* captured from emergence traps in the spring prior to feeding on any host plants demonstrated that 7 to 10% of beetles tested positive for *Erwinia tracheiphila* (Fleischer et al., 1999). *Acalymma vittatum* foregut and hindgut share similar cuticular epithelial morphology including high surface area structures such as spines and folds that provide a surface to which bacteria can attach (Garcia-Salazar et al., 2000). Currently, control strategies for this pathogen include insecticidal seed treatments and foliar sprays for the striped cucumber beetle and removal of infected plants to decrease *Erwinia tracheiphila* acquisition by beetles (Brust et al., 1996; Egel, 2011; Pair, 1997).

While the relationship of *Erwinia tracheiphila* and *Acalymma vittatum* has been well-documented, there are no studies on the potential spread of *Erwinia tracheiphila* outside of the current geographic range. I examined the vector competence, i.e., the ability of these beetles to acquire, maintain and transmit the *Erwinia tracheiphila* bacteria, and survivorship of two potential vectors: the western striped cucumber beetle (*Acalymma trivittatum*) and the banded cucumber beetle (*Diabrotica balteata*). The biological characteristics and preferred host plants shared between *Acalymma vittatum* and these two related species suggest both species will be
able to transmit *Erwinia tracheiphila* with little impact on beetle survivorship and thus could enable the geographical expansion of the causal agent of bacterial wilt of cucurbits, *Erwinia tracheiphila*.

**Methods**

**Acquisition and Establishment of Beetle Species**

We acquired APHIS permits to allow the shipment of *Acalymma trivittatum* from Dr. Larry Godfrey at University of California in Davis, California and *Diabrotica balteata* from Dr. Gregg Nuessly at University of Florida in Belle Glade, Florida. Both species were shipped in July of 2014. The beetles were reared in a quarantine greenhouse with extra lighting to maintain a 16h light:8h dark cycle all year with a temperature range of 22-28 °C (fluctuations were due to ambient environmental conditions outside of the greenhouse). Rearing protocols were previously established for *Acalymma vittatum* (Cuthbert et al., 1968; Howe & Zdarkova, 1971; Shapiro, 2012). While there were rearing protocols available for the maintenance of *Diabrotica balteata* there were none for *Acalymma trivittatum* (Cuthbert et al., 1968; Huang et al., 2002; Saba, 1970; Schalk & Peterson, 1990).

*Acalymma vittatum* beetles were collected from the Rusell E. Larson Research and Extension Center, Pennsylvania Furnace, PA to initiate a colony. Adult beetles were placed in a 30.5cm×30.5cm×30.5cm (12in×12in×12in) mesh cage (Bioquip, CA 1466AV) with a plastic sandwich container of moist Pro-mix potting soil (Premier Horticulture Reviere Du Loup, Quebec Canada) for egg deposition. Three times per week, cucumber leaves (*Cucumis sativus*: Northern Pickling) and flowers were given to the adults to feed *ad libitum*. The whole cage was then placed within a mesh bag and tied closed to provide double containment. Each week the soil was collected from the adult cages and placed into one 5L-plastic bin. Squash seedlings (*Cucurbita*
pepo: Yellow Crookneck) and extra soil were added to provide food and substrate for larval development. This “larval bin” was then placed into a mesh bag and tied closed to provide containment for adult beetles as they emerged. Bins were checked three times per week and seedlings were provided as needed. After 4-6 weeks, newly emerged adult beetles were collected and placed into a mesh cage with other Erwinia tracheiphila-free beetles from the colony. Field collected beetles were maintained in a separate cage to prevent Erwinia tracheiphila contamination.

This rearing protocol was also used in colony establishment for Acalymma trivittatum in the quarantine greenhouse. The Diabrotica balteata rearing protocol (Huang et al., 2002) was initially adapted to mimic the “larval bin” used for Acalymma. Diabrotica balteata, however, did not establish a colony; hence regular shipments of field-collected beetles were used for the following experiments.

**Erwinia tracheiphila culturing protocols**

We acquired APHIS permits for the shipment of the Erwinia tracheiphila plant pathogen from our collaborators at Iowa State University. Cultures included 3 cucumber (Cucumis) strains and 3 squash (Cucurbita) strains. However, only one Cucumis strain (TedCu10) and one Cucurbita (BuffGH) strain were selected for this experiment. The bacterial strains were initially propagated on nutrient broth media, but Erwinia tracheiphila is slow-growing and was often overgrown by other contaminants. Strains resistant to rifampicin that had been selected by Iowa State University collaborators were used to enhance recovery of Erwinia tracheiphila from frass samples and combat difficulties with contamination. Additionally, I tested growth behavior of these Erwinia tracheiphila strains on other media, including a semi-selective media for Pseudomonas spp. called King’s B medium. King’s B contains glycerol as the sole carbon source instead of glucose, and only bacteria capable of utilizing glycerol can grow on this medium.
Because plant pathogenic *Erwinia* are also able to grow on glycerol media, I cultured *Erwinia tracheiphila*+rifampicin on King’s B agar plates amended with 0.75ug/ul of Rifampicin to select pure bacterial colonies that were suspended in 750ul of 1× phosphate buffered solution (1×PBS) for each treatment. For the following experiments, TedCu10Rif (*Cucumis*) and BuffGHRif (*Cucurbita*) strains of *Erwinia tracheiphila* were used as the treatments.

**Experimental Treatment**

Colony-reared *Acalymma vittatum*, *Acalymma trivittatum* and field-collected *Diabrotica balteata* beetles were aspirated, 10 beetles per vial, and starved for 48 hours. Each *Acalymma vittatum* and *Acalymma trivittatum* was provided a 0.6mm squash cotyledon leaf disk (*Cucurbita pepo*: Yellow Crookneck) and each *Diabrotica balteata* was provided a 0.6mm lima bean leaf disk (*Phaseolus lunatus*: Fordhook 242). Each leaf disk was coated on one side with 5ul of the bacteria suspended in 1X phosphate buffered solution or only 1×PBS and placed into a 2ml micro-centrifuge tube with one beetle to feed *ad libitum* for 24 hours. Following treatment, beetles were transferred to a new 2ml micro-centrifuge tube with an untreated leaf disk for 24 hours to collect day 1 frass. Beetles were then transferred to 28ml (1oz) plastic cups, “home” containers, with lids and ample cucumber leaves (*Cucumis sativus*: Northern Pickling) for *Acalymma vittatum* and *Acalymma trivittatum* and lima bean leaves for *Diabrotica balteata* in between collections. This frass collection process was repeated again on day 5 and day 10 post-treatment, Table 2-1 and 2-3. The environmental conditions for treatment and frass collection were maintained in a growth chamber at 16h light:8h dark cycle and 25 °C. Beetle survivorship was recorded at each time point for all species and treatments.

After the frass was collected, 300ul of 1×PBS was added to each 2ml micro-centrifuge tube and vortexed. This frass suspension was tested for the presence of *Erwinia tracheiphila* using 3 methods: two involving culturing on plates (‘plate recovery’), and one involving direct
assay of the frass suspension (‘frass detection’). For the culturing methods, nutrient broth plates and King’s B plates amended with Rifampicin [0.75ug/ml] (NBA and KB) were inoculated with 5ul of the frass suspension. Any bacterial growth was collected from the media and placed in 1×PBS every day until colonies were overgrown or dead. Bacterial samples were boiled in water at 95 °C for 10 minutes to lyses cells. Then samples were pelleted and the supernatant used for direct colony polymerase chain reaction. The remaining frass suspension was transferred to a 1.7ml micro-centrifuge tube and tested directly by DNA extraction using E.Z.N.A tissue DNA kit from Omega bio-tek (Norcross, GA). The DNA was detected by polymerase chain reaction (PCR) using *Erwinia tracheiphila*-specific oligonucleotides:

ETC1 (5_GCACCAATTCCGCAGATCAAG_3)

ETC2 (5_GCGAGGATGTTACGCTTAACG_3)

These primers were designed by Mitchell and Hanks (2009) to amplify a 426 base pair region of the carbamoylphosphate synthetase gene (Mitchell & Hanks, 2009). Carbamoylphosphate synthetase is a ligase enzyme in the mitochondria that participates in the production of urea. Genomic DNA extracted from beetle frass was tested at least once using these primers following the protocol defined in Mitchell and Hanks (2009) for presence of *Erwinia tracheiphila*. The final volume was a 10μl reaction: 5.0μl 2×Taq PCR Master Mix with green loading dye (Promega), 0.2μl of each primer [100pmol] and 3.6μl molecular grade water. The protocol consisted of 94°C for 2 min, followed by 35 cycles of 1 min at 94°C (denaturation), 1 min at 53°C (annealing), and 1 min at 76°C (extension). Samples were run on a 0.5× TAE-1% agarose gel stained with Midori Green (Bulldog Scientific), and visualized using a UV transilluminator. Any DNA from beetles that tested positive in PCR, were confirmed in triplicate. Samples were confirmed as positive when both the first assay, and two of the subsequent 3 assays, were positive. A schematic of this experimental design is in Appendix 2-4.
Vector competence experiments were initiated with 84 *Acalymma trivittatum*, 29 *Diabrotica balteata*, and 88 *Acalymma vittatum*. Both *Erwinia tracheiphila* strains were initiated with 33 *Acalymma trivittatum* and 35 *Acalymma vittatum*. An additional 14 *Acalymma trivittatum* and 16 *Acalymma vittatum* were treated with 1×PBS as controls. However, *Diabrotica balteata* had a small sample size overall due to lack of survivorship during the shipment process. *Diabrotica balteata* started with 11 beetles given the *Cucumis* strain, 4 beetles given the *Cucurbita* strain and 3 beetles given 1×PBS as controls.

**Data Analysis**

Survivorship curves were plotted to illustrate any lethal effects *Erwinia tracheiphila* might have imposed on the beetles. Frass suspension, nutrient broth and King’s B plate polymerase chain reaction data was used to compare transmission of each *Erwinia tracheiphila* strain to the buffer controls within each species and collection date using 1-tail Fisher’s exact test. Collection dates and methods that were significant were then used to compare transmission rates against the known vector, *Acalymma vittatum*, using 2-tail Fisher’s exact test. This allows for determination of vector capabilities and bacterial effects on the life span of *Diabrotica balteata* and *Acalymma trivittatum* compared to the vector *Acalymma vittatum*. All analyses were performed using SAS 9.4 with an $\alpha=0.05$.

**Results**

**Beetle survivorship**

All three species survived over time regardless of bacterial treatment, as indicated in Figure 2-4. Under these conditions, adult survival of both species of *Acalymma* (vittatum and trivittatum) appeared to exceed that of *Diabrotica balteata*, however the sample size of the
Acalymma tests (35 in both the Cucumis and Cucurbita strain with Acalymma vittatum, and 33 and 37 for the Cucumis and Cucurbita strain with Acalymma trivittatum) was greater than Diabrotica balteata (19 and 6 for the Cucumis and Cucurbita strain).

Figure 2-4. Treatment sample size and survival rate across days post-acquisition for (A) Acalymma vittatum, (B) Acalymma trivittatum and (C) Diabrotica balteata for the Cucumis strain, the Cucurbita strain and the buffer control.
The following results were analyzed with Fisher’s Exact test for experimental effects for each date and were found to be not significant, allowing for pooling of the data across experimental date.

*Acalyymma vittatum* Vector Competence

*Acalymma vittatum* was examined for transmission rate differences between the *Erwinia tracheiphila* strains, and the buffer control, to verify vector competence status in frass and frass cultures. Overall, polymerase chain reaction assays detected *Erwinia tracheiphila* up to 10 days post acquisition, including detection of live cells based culturing on King’s B media, Table 2-1.

One day post-acquisition detection in the frass was high for both the *Cucumis*, 12 of 35 (34%), and the *Cucurbita*, 13 of 35 (37%), strain. The *Cucumis* strain was recovered from 13 of 35 nutrient broth cultures (37%) and 11 of 35 King’s B cultures (31%) indicating viability of the *Erwinia tracheiphila* following passage through the alimentary canal. The *Cucurbita* strain was recovered on the plates at a lower rate, 2 of 35 nutrient broth cultures (6%) and 4 of 35 King’s B cultures (11%). However, there were 4 of 16 frass samples (25%) that were positive in the buffer controls by PCR. Detection of *Erwinia tracheiphila* in frass was not significant compared to beetles given the buffer control (p=0.2). However, these same buffer controls had low rates of recovery (≤6%) on the plates. The plate recovery rates from beetles given the *Cucumis* strain was significantly higher than those given the buffer control for both the nutrient broth plates (p=0.003) and the King’s B plates (p=0.04), Table 2-1.

Significantly higher numbers of *Cucumis* and *Cucurbita* strains were detected in the frass five days post-acquisition compared to the buffer. Frass recovery rates from 16 of 34 beetles inoculated with *Erwinia tracheiphila Cucumis* strain (47%) and 13 of 31 beetles inoculated with *Erwinia tracheiphila Cucurbita* strain (42%) were significantly higher than detected from 2 of 15 buffer-inoculated beetle controls (13%) (*Cucumis*>buffer p=0.02; *Cucurbita*>buffer, p=0.04).
The plate recovery rates of the *Cucumis* (5 of 34 NBA and 7 of 34 KB) and *Cucurbita* strains (7 of 31 NBA and 1 of 31 KB) varied from 3% to 23%, but were not significantly higher than beetles given the buffer controls (1 of 15 NBA and 3 of 15 KB).

Ten days post-acquisition the proportion of beetles with *Erwinia tracheiphila* detection in the frass decreased for both the *Cucumis*, 16 of 34 to 6 of 31 (47% to 19%), and the *Cucurbita*, 13 of 31 to 10 of 31 (42% to 32%), strain. Detection in the frass of the buffer controls remained consistent with detection rates on day 5. No *Erwinia tracheiphila* was recovered using nutrient broth medium, but 3 of 31 *Cucumis* strain (10%) and 2 of 31 *Cucurbita* strain (6%) were recovered using King’s B. Although 2 of 14 the buffer controls used for each strain tested positive by PCR, no *Erwinia tracheiphila* colonies grew on either the nutrient broth or the King’s B media from these same suspensions. This suggests that the presence of *Erwinia tracheiphila* in frass may not represent viable cells and that PCR alone is insufficient to distinguish between live and dead *Erwinia tracheiphila*.

**Table 2-1.** Number of *Acalymma vittatum* with positive PCR detection in frass and plate recovery on nutrient broth and King’s B media (and total number tested) at 1, 5, and 10 days post treatment. Fisher’s Exact test compared treated beetles to buffer control. Cells designated with n/a were unable to be analyzed due to zeros.
**Acalymma trivittatum Vector Competence**

To determine if *Acalymma trivittatum* is capable of transmitting *Erwinia tracheiphila*, I compared frass detection rates and plate recovery rates of beetles treated with the *Cucumis* and *Cucurbita* strain to beetles treated with the buffer control, Table 2-2. One day post-acquisition, there was no frass detection from beetles given the *Cucumis* strain or the buffer control, but I did observe low frass detection rates from beetles given the *Cucurbita* strain, 1 of 33 (3%). No *Cucumis* strains were detected on nutrient broth or King’s B media, low levels of *Cucurbita* strain grew on nutrient broth, 4 of 33 (12%), and the King’s B media, 1 of 33 (3%). However, I also observed low rates of plate recovery from beetles given the buffer control (1 or 2 out of 14) and there was no difference in plate recovery rates.

Five days post-acquisition there was no PCR detection of *Erwinia tracheiphila* directly from the frass suspensions, but *Erwinia tracheiphila* was recovered consistently on both nutrient broth and King’s B plates. Live cells from the *Cucumis* strain were recovered from 6 of 33 (18%) of the beetles using nutrient broth, and 4 of 33 (12%) of the beetles when using King’s B. Live cells of the *Cucurbita* strain were recovered from 3 of 32 (9%) of the beetles when using either nutrient broth or King’s B. Colonies were also recovered from 1 of 14 (7%) buffer control samples on nutrient broth agar, but no *Erwinia tracheiphila* was detected from buffer control samples on King’s B, nor was *Erwinia tracheiphila* detected by PCR from frass suspensions.

Ten days post-acquisition there was no frass detection from the buffer controls. The frass detection rate was high for the *Cucumis* strain, 9 of 32 (28%) and only the King’s B cultures recovered the *Cucumis* strain, 2 of 32 (6%). The frass detection of the *Cucumis* strain compared to the buffer control was significant (p=0.05). We also observed 3 of 32 (9%) frass detection from beetles given the *Cucurbita* strain, which is biologically relevant for potentially maintaining *Erwinia tracheiphila* from season to season like *Acalymma vittatum*. Only the nutrient broth
cultures recovered the *Cucurbita* strain, 3 of 32 (9%), and none of the beetles given the buffer control resulted in plate recovery.

**Table 2-2.** Number of *Acalymma trivittatum* with positive PCR detection in frass and plate recovery on nutrient broth and King’s B media (and total number tested) at 1, 5, and 10 days post acquisition. Fisher’s Exact test compared treated beetles to buffer control. Cells designated with n/a were unable to be analyzed due to zeros.

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</tbody>
</table>

*Acalymma vittatum* was compared to *Acalymma trivittatum* only when there were significant frass detection or plate recovery rates of the *Erwinia tracheiphila* compared to the buffer control within each species, day, and method. The *Cucumis* strain on day 1 from the nutrient broth agar cultures and King’s B cultures for *Acalymma vittatum* were higher compared to *Acalymma trivittatum*, Table 2-3 (p=<0.0001). In these assays, *Acalymma vittatum* transmitted live *Erwinia tracheiphila* whereas *Acalymma trivittatum* did not. On day 5, frass detection for both strains was also higher for *Acalymma vittatum* compared to *Acalymma trivittatum* (p=<0.0001). As before, *Acalymma vittatum* excreted *Erwinia tracheiphila* whereas *Acalymma trivittatum* did not. However, frass detection on day 10, in which both beetle species showed
significant frass detection relative to the buffer controls, did not differ in transmission rates between the two species (p=0.6).

**Table 2-3.** Transmission rates of *Acalymma vittatum* compared to *Acalymma trivittatum* on those days and methods where transmission was significantly greater than buffer controls for either species (see Tables 2-1 and 2-2).

<table>
<thead>
<tr>
<th>Day</th>
<th>Method</th>
<th><em>Et Strain</em></th>
<th><em>Acalymma vittatum</em></th>
<th><em>Acalymma trivittatum</em></th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Nutrient Broth</td>
<td><em>Cucumis</em></td>
<td>13/35</td>
<td>0/33</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>5</td>
<td>King's B</td>
<td><em>Cucumis</em></td>
<td>11/35</td>
<td>0/33</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Frass Detection</td>
<td><em>Cucumis</em></td>
<td>16/34</td>
<td>0/33</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>10</td>
<td>Frass Detection</td>
<td><em>Cucumis</em></td>
<td>6/31</td>
<td>9/32</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>Frass Detection</td>
<td><em>Cucumis</em></td>
<td>6/31</td>
<td>9/32</td>
<td>0.6</td>
</tr>
</tbody>
</table>

*Diabrotica balteata* Transmission

*Diabrotica balteata* was also able to excrete *Erwinia tracheiphila* cells, although at low rates and samples sizes were small. One day post-acquisition the *Cucumis* strain was detected in 3 of 11 of the frass suspensions (27%) and recovered alive on 2 of the 11 nutrient broth plates (18%). There was no frass detection or plate recovery for *Cucurbita* strain or buffer controls, but the sample sizes involved only 3 or 4 replicates.
Five days post-acquisition the *Cucumis* strain was detected in 1 of the 9 frass suspensions (11%) and 1 of the 3 buffer controls (33%), however nothing was recovered alive on the nutrient broth or King’s B plates.

Ten days post-acquisition the detection in the frass was 33% for both the *Cucumis* (2 of 6) and the *Cucurbita* (1 of 3) strain, but there was also frass detection from the single beetle still alive as a buffer control. Nothing was recovered from the nutrient broth plates. However, *Erwinia tracheiphila* *Cucurbita* strain was recovered on 1 of the 3 King’s B plates (33%). Under these lab conditions and small sample size, *Diabrotica balteata* frass detection and plate recovery of *Erwinia tracheiphila* was not significant.

Table 2-4. Number of *Diabrotica balteata* with positive PCR detection in frass and plate recovery on nutrient broth and King’s B media (and total number tested) at 1, 5, and 10 days post-acquisition. Fisher’s Exact test compared treated beetles to buffer control. Cells designated with n/a were unable to be analyzed due to zeros.

<table>
<thead>
<tr>
<th>Method</th>
<th>Strain</th>
<th>Day 1</th>
<th></th>
<th>Day 5</th>
<th></th>
<th>Day 10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Et</td>
<td>Buffer</td>
<td></td>
<td>Et</td>
</tr>
<tr>
<td>Frass Detection</td>
<td><em>Cucumis</em></td>
<td>3/11</td>
<td>0/3</td>
<td>0.5</td>
<td>1/9</td>
<td>1/3</td>
</tr>
<tr>
<td></td>
<td><em>Cucurbita</em></td>
<td>0/4</td>
<td>0/3</td>
<td>n/a</td>
<td>0/4</td>
<td>0/3</td>
</tr>
<tr>
<td>Nutrient Broth</td>
<td><em>Cucumis</em></td>
<td>2/11</td>
<td>0/3</td>
<td>0.6</td>
<td>0/9</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td><em>Cucurbita</em></td>
<td>0/4</td>
<td>0/3</td>
<td>n/a</td>
<td>0/4</td>
<td>0/3</td>
</tr>
<tr>
<td>King's B</td>
<td><em>Cucumis</em></td>
<td>0/11</td>
<td>0/3</td>
<td>n/a</td>
<td>0/9</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td><em>Cucurbita</em></td>
<td>0/4</td>
<td>0/3</td>
<td>n/a</td>
<td>0/4</td>
<td>0/3</td>
</tr>
</tbody>
</table>
Discussion

Beetle Survivorship of *Erwinia tracheiphila* treatments

Both *Acalymma* species maintained survivorship rates of ≥ 80% during the 10-day bioassays from beetles given *Erwinia tracheiphila* and these rates were similar for beetles given buffer controls, Figure 2-4. *Acalymma vittatum* had an overall survival rate of 84%, and *Acalymma trivittatum* of 80%, suggesting that *Erwinia tracheiphila* has no immediate effect on adult survival. Age and gender of the beetles were not taken into account. Although *Erwinia tracheiphila* may impact beetle fitness, our lab assays occurred during times of good survival and any declines we cannot attribute solely to the bacterial treatment. This is consistent with beetle fitness from transmission and retention assays of *Erwinia tracheiphila* in *Acalymma vittatum* (Shapiro, 2012; Shapiro et al., 2014). *Erwinia tracheiphila* strains seem to have no impact *Acalymma* survivorship soon after acquisition.

*Diabrotica balteata* (Figure 2-4) had the lowest overall survival rate of the three species at ≤50%. The proportion of beetles given the *Cucumis* strain tended to be lower, whereas the proportion of beetles given the *Cucurbita* strain had survival rates closer to that observed from the buffer controls. For this reason, there insufficient statistical power to make definitive conclusion about the effect of *Erwinia tracheiphila* on *Diabrotica balteata* adult survivorship.

Vector competence of two species outside of the geographic range of *Erwinia tracheiphila*

Polymerase chain reaction may be more sensitive than the plating assay but has limitations. For vector capacity studies, I needed to demonstrate presence or absence of *Erwinia tracheiphila* but also successful transmission of viable propagules. Frass analysis via PCR and plating allowed for determination of successful retention and subsequent passage of *Erwinia tracheiphila* through the alimentary canal of two potential vectors that reside outside the
geographic range of the bacteria for possible expansion of cucurbits at risk. *Acalymma vittatum* was the transmission control due to the previous establishment of vector competence (Gould, 1944; Haynes & Jones, 1975; Mitchell & Hanks, 2009). Due to recent recognition of plant host variation among *Erwinia tracheiphila* strains, our assays considered retention and passage for each strain separately.

**Acalymma vittatum Vector Competence**

*Acalymma vittatum*, the striped cucumber beetle, excreted viable *Erwinia tracheiphila Cucumis* strain and *Cucurbita* strain at all time points, albeit at varying rates. On day 1, detection from frass suspensions had similarly high rates for the *Cucurbita* (37%) and the *Cucumis* (34%) strain. Low plate recovery rates from beetles given the *Cucurbita* strain (6% and 11% when using nutrient broth and King’s B, respectively) indicate that most of these *Cucurbita*-strain cells were nonviable. However, of the beetles give the *Cucumis* strain, 31%-37% passed viable cells through their guts as detected by nutrient broth and King’s B plating. This viable recovery of the *Cucumis* strain was significantly higher than beetles given the buffer control. This ingestion and live excretion of *Erwinia tracheiphila* verifies that the fecal transmission does occurs with this species in the first 1 or 2 days, which is important for within-field dissemination of the pathogen.

On day 5 both strains were excreted alive based on the recovery of *Erwinia tracheiphila* on the plates. From the 34 beetles given the *Cucumis* strain, I recovered cultures from 5 and 7 beetles using nutrient broth or King’s B, and from the 31 beetles given the *Cucurbita* strain, I recovered 7 and 1 cultures using nutrient broth or King’s B. Compared to day 1, fewer beetles (only 15-21%) fed *Erwinia tracheiphila Cucumis* strain excreted viable cells on nutrient broth or King’s B, while the beetles fed *Erwinia tracheiphila Cucurbita* strain was recovered at similar rates on the plates as on day 1 (3-22%). Although some of the frass detection buffer controls were contaminated (25%), significantly more beetles fed either *Erwinia tracheiphila* strain were
positive by PCR of frass detection than the buffer control. While frass detection PCR was similar between days 1 and 5 for both strains, there were fewer beetles that were positive by plate recovery rates for the *Cucumis* strain, suggesting that some bacterial cells are not surviving passage through the beetle alimentary canal. By day 10 both strains were excreted alive, as indicated by positive plate recovery in the treatments. Two of 14 beetles given the buffer controls were positive by frass detection PCR, but the cells were not recovered alive on the plates.

*Acalymma trivittatum* Vector Competence

*Acalymma trivittatum*, the western striped cucumber beetle, tended to have low frass detection and plate recovery of both *Erwinia tracheiphila* strains, but detection was repeatedly present, and reached 28% in one case. Due to the low frass detection rates, and the sample sizes, frass detection of the *Cucumis* strain on day 10 was the only significant point at $\alpha=0.05$, Table 2-2.

One day post-acquisition the *Cucumis* strain was not detected or recovered, however the *Cucurbita* strain was. The buffer control did not detected *Erwinia tracheiphila* in the frass but was recovered alive on the plates. As is the case with *Acalymma vittatum*, *Acalymma trivittatum* can mechanically transmit the *Cucurbita* strain of *Erwinia tracheiphila*, through ingestion and live excretion 24-48 hours later.

Five days post-acquisition the *Cucumis* strain, the *Cucurbita* strain and the buffer controls were not detected in the frass. However, *Erwinia tracheiphila* was recovered on the plates in all treatments showing that the bacterium was being excreted alive but in low concentrations. The *Cucumis* strain was recovered at the highest rate on both medias, the *Cucurbita* strain was recovered at the same rate on both media, and the buffer control was only recovered on the nutrient broth media at a low rate.
Ten days post-acquisition the *Cucumis* and the *Cucurbita* strains were detected in the frass at the highest amounts compared to day 1 and day 5. Frass detection from beetles given the *Cucumis* strain was significant compared to the buffer control, which was free of *Erwinia tracheiphila*. However, the plate recovery rate of the *Cucumis* strain was low, indicating the majority of cells were dead. Frass detection from beetles given the *Cucurbita* strain was not significant, but ~9% of beetles were excreting the bacteria based on plate recovery using nutrient broth.

The *Cucurbita* strain was not significant in detection compared to the buffer control, but was found in frass at day 1 and day 10 much like the colonization pattern of *Erwinia tracheiphila* in *Acalymma vittatum* (Shapiro, 2012; Shapiro et al., 2014). Age and gender were not taken into account and the sample sizes of the treatment were small, thus an inability to make statistically supported conclusions in some cases may be due to a lack of power. Despite these issues with this method, I showed that *Acalymma trivittatum* can successfully transmit live *Erwinia tracheiphila*, which is biologically significant because this beetle is a specialist on cucurbits much like it’s close relative, *Acalymma vittatum*.

**Problematic methods and future directions**

Polymerase chain reaction of the frass only indicates presence or absence, not viability, of the bacteria. Every group of DNA extractions was verified with an extraction of only the chemicals used to extract DNA (negative extraction) to ensure no *Erwinia tracheiphila* contamination during the protocol. Colony-reared beetles were tested using the same extraction method and primers for *Erwinia tracheiphila* and were negative for *Erwinia tracheiphila*.

Mitchell and Hanks (2009) designed primers that were *Erwinia tracheiphila* specific, which we verified with a BLAST search (National Center for Biotechnology Information, U.S. National Library of Medicine Bethesda, MD). Despite the verification of *Erwinia tracheiphila*
free conditions, we had two issues arise during the bioassays. The first was positive frass detection even in the absence of plate recovery. The BLAST search indicated that there were several other organisms that matched one of the two primers, which could account for the nonspecific binding creating alternative bands in the PCR from the frass suspensions. The false detection of *Erwinia tracheiphila* in the frass suspension also suggests that conditions external to the beetles are conducive to bacterial cell attachment.

Frass detection in data from *Acalymma vittatum* given buffer controls on day 10 illustrates this first issue. In some assays, *Erwinia tracheiphila* was detected in the frass of *Acalymma vittatum* by PCR but had lower rates of *Erwinia tracheiphila* recovery on semi-selective media. That suggests PCR detection of *Erwinia tracheiphila* in frass in absence of *Erwinia tracheiphila* recovery represents false positives. The false positive could be due to surface cross contamination of dead bacterial cells.

The second issue was lack of positive PCR detection of *Erwinia tracheiphila* from frass despite successful plate recovery. According to Mitchell and Hanks (2009), these primers produced the correct bands with “as little as 25 cells”, but they noted that frass samples contained inhibitors that altered the sensitivity of the primers. They refined their methods, but indicated that they then could not detect *Erwinia tracheiphila* in frass when there were less than 500 cells. The results suggest that this may be occurring in some of the bioassays: I may have achieved plate recovery from fewer than 500 cells, while missing detection in frass when titers were this low. As shown when using immunology to detect *Erwinia tracheiphila* (Fleischer et al., 1999), our accuracy in determining live transmission directly from frass suspensions may be low when titers are low.

Data from *Acalymma trivittatum* on day 5 illustrates this second example. In *Acalymma trivittatum*, the Cucurbita strain was recovered alive at a higher rate after plating the frass suspension on nutrient broth agar than from direct PCR assays of the frass suspension. Five days post-acquisition, direct assay of the frass suspension were negative, while *Erwinia tracheiphila*
was recovered on plates showing that the bacterium was being excreted alive. This suggests that the bacterium is being excreted, but at concentrations too low to be detected with PCR of the frass suspension. To account for possible low yield in direct frass PCR assays, I first plated frass suspensions to confirm *Erwinia tracheiphila* identities using PCR. This indicates that low levels of bacterial cells can go undetected in frass by PCR, but when grown on proper substrate, e.g. cucurbits, the *Erwinia tracheiphila* can replicate to a detectable and possible disease-causing state. This phenomenon could account for the plate recovery in the buffer controls but contamination of plate recovery is also a possibility and either cannot be rejected.

**Transmission efficiency of *Acalymma vittatum* versus *Acalymma trivittatum***

Frass detection and plate recovery rates were significantly higher for *Acalymma vittatum* when compared to *Acalymma trivittatum* except for the frass detection of the *Cucumis* strain on day 10 (p=0.6), Table 2-3. Both species excreted live *Cucumis*-strain *Erwinia tracheiphila* in frass by day 10 (as detected by PCR), which indicates that *Acalymma trivittatum* can transmit *Erwinia tracheiphila* at rates similar to *Acalymma vittatum*. While low levels of *Erwinia tracheiphila* were detected from buffer controls in both species, this could be due to cross contamination, especially considering *Acalymma vittatum* frass samples were all negative in the buffer control.

These differences in vector competence could be due to genetic, behavioral, or morphological dissimilarities between these two species. Age and gender were not taken into account in this study but could also provide explanations for the differences in detection and recovery rates. These data indicate that *Acalymma trivittatum* can acquire *Erwinia tracheiphila* by ingestion and viable bacteria can be excreted in frass, similar to *Acalymma vittatum*. Detection and recovery of *Erwinia tracheiphila* from *Acalymma trivittatum* was similar to *Acalymma vittatum*, however it did occur later in time, which could be due to biological
differences between these species. Future studies should look at differences in transmission rates over the whole life span of the beetles and how gut colonization delays could change the disease cycle.

**Diabrotica balteata Vector Competence**

*Diabrotica balteata*, the banded cucumber beetle, was able to transmit live *Erwinia tracheiphila*. One day post-acquisition the *Cucumis* strain was detected and recovered at low rates. Ten days post-acquisition the *Cucumis* strain was detected in frass but not recovered alive. These data suggest that *Diabrotica balteata* is capable of mechanical transmission, via ingestion and excretion 24-48h post-acquisition, of the *Cucumis* strain. Ten days post-acquisition the *Cucurbita* strain was detected in frass and recovered alive at low rates. These data suggest that *Diabrotica balteata* can excrete live *Erwinia tracheiphila* up to ten days after acquisition. There were difficulties associated with studying this beetle that ultimately resulted in an inconclusive vector capacity status of *Diabrotica balteata*. In addition to the small sample sizes, the feeding assay for acquisition of the *Erwinia tracheiphila* was conducted using lima bean cotyledons, which are not affected by this pathogen. The beetles readily consumed the lima beans, while the cucurbit leaves were not consistently taken. The likelihood that *Diabrotica balteata* will be a competent vector will depend on the extent of beetle contact with cucurbits under field conditions.

**Conclusion**

These data were more conclusive for *Acalymma trivittatum* as a competent vector of *Erwinia tracheiphila*. I have illustrated the potential of two diabroticite species outside the current geographic range of *Erwinia tracheiphila* to transmit the bacteria. We need a better
understanding of how the bacteria interact within the alimentary canal of all three beetle species. Contamination of media was an issue in culturing *Erwinia tracheiphila* and could have attributed to positive beetles in the buffer control and PCR detection of non-*Erwinia tracheiphila* fragments could have led to false positives. Age and gender were not taken into account and some of the sample sizes are not favorable. Despite these factors, we were able to illustrate that these beetles species would provide an avenue for the spread of *Erwinia tracheiphila* into new geographic regions and place additional cucurbits crops at risk.
References: Chapter 2


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Radin AM & Drummond FA (1994) Patterns of Initial Colonization of Cucurbits, Reproductive Activity, and Dispersion of Striped Cucumber Beetle, \textit{Acalymma vittata} (F.) \textit{(Coleoptera: Chrysomelidae)}. Journal of Agriculture Entomology 11: 115-123.


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Chapter 3
Potential Biological Control of *Erwinia tracheiphila* by Internal Gut Interactions in *Acalymma vittatum* with *Pseudomonas fluorescens*

Introduction

The plant family Cucurbitaceae contains cucumbers, melons, squash, and pumpkins. A major pest of cucurbits is the striped cucumber beetle, *Acalymma vittatum* (Fabricus). This beetle’s life cycle is dependent on cucurbits: eggs are laid in the soil, larvae feed on the roots of the plants and adults feed on foliage and fruits (Cuthbert et al., 1968; Howe & Zdarkova, 1971). In addition to herbivory damage, *Acalymma vittatum* is also a vector and overwintering host of the bacterial wilt of cucurbits, *Erwinia tracheiphila* (Smith) (Brust & Rane, 1995; Haynes & Jones, 1975; Mitchell & Hanks, 2009). Controlling this plant pathogen is primarily done by pesticide applications against the insect vector (Gould, 1944). New control methods for the pathogen and beetle could allow for a decrease in pesticide use. A method that could prevent the disease cycle by disrupting pathogen-vector interactions would provide the least evolutionary pressure. This approach has been proven in other insect-pathogen interactions with similar pathogen biology to *Erwinia tracheiphila* (Killiny et al., 2012).

*Pseudomonas fluorescens*

*Pseudomonas fluorescens* is an extremely common opportunistic soil and plant bacterial species that is physiologically and genetically diverse (Silby et al., 2009; Yamamoto et al., 2000). Three isolates of *Pseudomonas fluorescens* only shared 61% of their genes and can tolerate significant gene rearrangement (Silby et al., 2009). *Pseudomonas fluorescens* is a Gram-negative, non-pathogenic, bacillus with polar flagella for motility (Breed et al., 1957). The optimum temperature range for growth is 20-25 °C in an aerobic environment (Breed et al.,
1957). This species has several beneficial characteristics for plants, such as suppression of pathogens by competitive or allelopathic effects, induction of plant growth hormones, and direct elicitation of plant defenses (Silby et al., 2009). One key characteristic of *Pseudomonas fluorescens* is the production of pyoverdin (also known as fluorescein) in low iron environments. The pyoverdin functions as a siderophore for iron sequestration from the environment (Cabrefiga et al., 2007; Crosa, 1989; Lenhoff, 1963; Meyer & Abdallah, 1978). Pyoverdin can readily be observed under UV light when *Pseudomonas fluorescens* is cultured on a low iron medium such as King’s B (Temple et al., 2004).

**Pseudomonas fluorescens** as a biological control

Some isolates of *Pseudomonas fluorescens* have been shown to be effective biological control agents for plant pathogens (Couillerot et al., 2009). For example, successful reduction in the fire blight of tree fruit, *Erwinia amylovora* (Burrill), transmission was achieved using *Pseudomonas fluorescens* (Cabrefiga et al., 2007; Pujol et al., 2005). Initially, it was assumed that the inhibition of *Erwinia amylovora* by *Pseudomonas fluorescens* was through competitive exclusion, which is one organism out competing another and hence preventing growth of one of the competing organisms (Cabrefiga et al., 2007). However, further studies revealed an additional mechanism of antibiosis (Stockwell et al., 2002). Due to restrictions on the use of biological control agents that produce antibiotics in Europe, a new solution was needed that did not involve antibiotic production. A non-antibiotic-producing strain of *Pseudomonas fluorescens* (EPS62e isolate) was used to determine the mechanism used to suppress pathogen growth, specifically with *Erwinia amylovora*. *Pseudomonas fluorescens* EPS62e can use 51 carbon sources and of those 21 of those sources overlap with *Erwinia amylovora’s* 27 usable carbon sources (Cabrefiga et al., 2007). These studies determined that certain strains of *Pseudomonas*
*Pseudomonas fluorescens* achieve biological control through competitive sequestration of finite minerals in the environment (e.g. iron) through siderophore production (Cabrefiga et al., 2007; Whipps, 2001). However, *Pseudomonas fluorescens* EPS62e was only able to inhibit *Erwinia amylovora* growth when inoculated first for cell-to-cell interaction due to lack of antibiosis (Cabrefiga et al., 2007).

**Relationship of *Erwinia tracheiphila* and *Erwinia amylovora***

The phylogeny of *Erwinia* has undergone significant changes based on molecular evidence. The most recent taxonomic placement of *Erwinia* was determined by 16S rDNA sequencing (Hauben et al., 1999). Figure 3-1 depicts a portion of the *Enterobacteriaceae* dendrogram in which the originally described genus *Erwinia* was further divided into 4 genera: *Pantoea, Erwinia, Pectobacterium,* and *Brenneria* (Hauben et al., 1999; Waleron et al., 2002).

![Figure 3-1. A portion of the Enterobacteriaceae dendrogram depicting the 16S rDNA phylogenetic relationships of the genera Pantoea, Erwinia, Pectobacterium, and Brenneria (Brosius et al., 1981).](image)

The genus *Pectobacterium* now contains *Erwinia carotovora,* *Erwinia chrysanthemi,* *Erwinia caeticida* and *Erwinia cypripedii,* while *Erwinia alni,* *Erwinia nigrifluens,* *Erwinia paradisiaca,* *Erwinia quercina,* *Erwinia rubrifaciens* and *Erwinia salicis* were placed in a new
The last genus, *Pantoea*, contains *Erwinia ananas*, *Erwinia herbicola*, *Erwinia milletiae*, *Erwinia stewartii* and *Erwinia uredovora*. The “true” *Erwinia* species include *Erwinia amylovora*, *Erwinia rhapontici*, *Erwinia persicinus*, *Erwinia malotivora*, *Erwinia psidii*, and *Erwinia tracheiphila* (Hauben et al., 1999; Waleron et al., 2002). *Erwinia tracheiphila* is distantly related to all the other *Erwinia* species within the true *Erwinia* species group and has an average similarity with all other *Erwinia* species of 95.5% of 16S rDNA sequence. The phylogeny helps to elucidate the relationship between *Erwinia amylovora* and *Erwinia tracheiphila*, which are 94.4% similar in the 16S rDNA 1472 to 1498 section (Hauben et al., 1999).

**Erwinia tracheiphila biology**

*Erwinia tracheiphila*, the causal agent of bacterial wilt of cucurbits, is a Gram-negative rod-shaped bacterium that is within the Enterobacteriaceae family (Garrity, 2005; Naum et al., 2008). It is a xylem-limited, biofilm producing plant pathogen that blocks the xylem causing subsequent wilting, and eventual death of the infected host plant (Fulton et al., 1911). Recently, *Erwinia tracheiphila* had strain variants identified and mapped to host plant origin (Rojas et al., 2013). Isolates from the plant genus *Cucumis* have a slower disease progression in the genus *Cucurbita* and vice versa (Rojas et al., 2013). To date, *Acalymma vittatum* is the only described vector of *Erwinia tracheiphila* (Bassi, 1982; Sasu et al., 2010). However, in Chapter 2, I describe experiments demonstrating that a related diabroticite beetle, *Acalymma trivittatum*, also has biological and ecological traits required to transmit *Erwinia tracheiphila* (Roberts, 2015). *Erwinia tracheiphila* colonizes the guts of the striped cucumber beetles (de Mackiewicz et al., 1998; Garcia-Salazar et al., 2000) and remains dormant over winter. Currently, control strategies for this pathogen use insecticidal treatments for the striped cucumber beetle and removal of
infected plants to decrease *Erwinia tracheiphila* acquisition by beetles (Brust et al., 1996; Egel, 2011; Pair, 1997).

**Testing the potential application of *Pseudomonas fluorescens* as a biocontrol agent against *Erwinia tracheiphila*.**

Interbacterial antagonism can sometimes be used as an effective method of controlling plant pathogens. There has been evidence to support the efficacy of *Pseudomonas fluorescens* to inhibit *Erwinia amylovora* and fire blight progression (Cabrefiga et al., 2007; Pujol et al., 2005; Temple et al., 2004). Based on the similarity of *Erwinia tracheiphila* and *Erwinia amylovora*, and given that *Pseudomonas fluorescens* is a ubiquitously occurring soil-borne bacterial species; I hypothesize that *Pseudomonas fluorescens* should be antagonistic towards *Erwinia tracheiphila*. Here, I test the potential interaction of *Pseudomonas fluorescens* with *Erwinia tracheiphila* in *vitro* and co-localization of both bacteria in *vivo*.

**Methods**

**Bacterial strain acquisition**

APHIS permits were acquired to receive shipments of the *Erwinia tracheiphila* strains from Iowa State University. We received 6 strains, 3 *Cucumis* and 3 *Cucurbita*, and propagated colonies on nutrient broth media (Roberts 2015, Chapter 2). Contamination became an issue due to the slow-growing nature of *Erwinia tracheiphila*, which was solved by the use of the semi-selective media King’s B for the *in vitro* experiments with *Erwinia tracheiphila* strain TPINCu1. A rifampacin resistant *Erwinia tracheiphila* strain TedCu10^Rif, that Iowa State University created,
was used for the *in vivo* experiments. Nutrient broth media was amended with 0.75μg/ml rifampacin for growth of the TedCu10rif strain only.

I obtained an isolate of *Pseudomonas fluorescens* strain 55 from Dr. Tim McNellis, who isolated the strain from a Centre county Pennsylvania pear orchard. *Pseudomonas fluorescens* strain 55 was cultured on King’s B media, which is a low-iron content, semi-selective medium designed to induce fluorescein production in fluorescent *Pseudomonads* (King et al., 1954). Because *Erwinia tracheiphila* can also convert the carbon source glycerol in King’s B, this medium was ideal for studying the interactions between the two bacterial species.

**Acalymma vittatum rearing methods**

Adult beetles were placed in a 30.2cm×30.2cm×30.2cm (12in×12in×12in) pop-up mesh cage (Bioquip, Rancho Dominguez, CA 1466AV) with a plastic sandwich container of moist soil for egg deposition in a greenhouse with lighting to maintain a 16h light:8h dark cycle and a temperature range of 22-28 °C (some fluctuations occurred due to ambient environmental conditions outside of the greenhouse), following the protocols in Cuthbert et al (1968), Howe & Zdarkova (1971), and Shapiro (2012). The whole cage was then placed within a mesh bag and tied closed to provide secondary containment. Every three days cucumber leaves (*Cucumis sativus*: Northern Pickling) and flowers were given to adults for *ad libitum* feeding. Each week the soil was collected from the adult cages and placed into one 5L-plastic bin. Squash seedlings (*Cucurbita pepo*: Yellow Crookneck) and extra soil were added to provide food and substrate for larval development. This “larval bin” was then placed into a mesh bag and tied closed to provide containment for adult beetles as they emerged. Bins were checked three times a week and seedlings were provided as needed. After 4-6 weeks, newly emerged adult beetles were collected and placed into a mesh cage with other beetles from the colony. The colony was established from field-collected beetles from Rock Springs and State College, PA. Each field season the colony
was supplemented with newly collected beetles to maintain genetic diversity. To minimize *Erwinia tracheiphila* contamination within the colony, the field-collected beetles were kept separate from all other beetles.

**In vitro experiment**

The *in vitro* antagonism assays were performed on King’s B media plates with *Erwinia tracheiphila* strain TPINCu1 and *Pseudomonas fluorescens* strain 55, grown at 25 °C for a minimum of 5 days. Accepted cross-culturing methods were used to test interactions of bacteria, as described by Nguyen & Ranamukhaarachchi (2010), via a modified agar diffusion assay, which consists of a lawn plating of one bacterial with a central inoculation point of the opposing bacteria at the same time, Figure 3-2. The zone of inhibition, the area without opposing bacterial growth as illustrated in Figure 3-3, was measured from the inoculation point daily for a minimum of 4 days. Secretion of pyoverdin (=fluorescein) was assessed using UV light for visualization, Figure 3-#.

![Diagram of in vitro experimental design set up of *Erwinia tracheiphila* lawn on King’s B plate with a central disk of *Pseudomonas fluorescens*, a modified agar diffusion assay.](image)

Figure 3-2. Diagram of *in vitro* experimental design set up of *Erwinia tracheiphila* lawn on King’s B plate with a central disk of *Pseudomonas fluorescens*, a modified agar diffusion assay.
**In vivo experiment**

I used fluorescence in situ hybridization (FISH) to observe the interactions between *Erwinia tracheiphila* and *Pseudomonas fluorescens* within the *Acalymma vittatum* gut. There were several FISH probes designed to target *Pseudomonas* species, and I chose Pseudo120 probe with a green fluorophore (Saha et al., 2012). Because there were no FISH probes designed specifically for *Erwinia tracheiphila*, I designed a species-specific probe based on the published 16S RNA sequences using the following approach. I downloaded representative 16S RNA sequences for all *Erwinia* spp. published in the National Center for Biotechnology Information (NCBI) nucleotide database (as of September 2014) and aligned them to *E. coli* in MEGA6 software (Tamura et al., 2013) to determine hyper-variable regions (Chakravorty et al., 2007; Hauben et al., 1999; Kwon et al., 1997; Naum et al., 2008). I selected short nucleotide sequences (15-30 base pairs) within the hyper-variable regions, where the *Erwinia tracheiphila* base pairs differed from all other *Erwinia* spp. sequences. I confirmed each candidate probe sequence using TestProbe (http://www.arb-silva.de/search/testprobe/), an online program that utilizes the SILVA ribosomal RNA database to compare nucleotide sequences to all published sequences for matches (Klindworth et al., 2013; Quast et al., 2013; Yilmaz et al., 2014). Of the possible sequences, I identified only one probe that matched exclusively to *Erwinia tracheiphila*:

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5'_CACAGAAGCTTAGCACAG_3'
```

I used this probe labeled with a green (5’-6FAM) or red fluorophore (5’-Rhodamine or 5’-Cy5) from Integrated DNA Technologies. I used *Erwinia tracheiphila*-6FAM (green) for samples when only *Erwinia tracheiphila* was the target, and *Erwinia tracheiphila*-Rho (red) for co-localization with *Pseudomonas* using the Pseudo120-6FAM (green) probe to distinguish the two species. I verified specificity of this probe for *Erwinia tracheiphila* by performing fluorescent in situ hybridization on bacterial cells isolated from plates and on *Acalymma vittatum* alimentary
canals with *Erwinia tracheiphila* as controls. RNase-treated alimentary canals were used to assess non-specific binding of the probe.

I tested 6 treatments; each consisting of a pair of doses spaced two days apart, to examine bacterial co-localization in the alimentary canal of *Acalymma vittatum*, Table 3-1. Colony-reared *Acalymma vittatum* were starved for 24 hours before dose 1 (water, *Erwinia tracheiphila* TedCu10^rif^ strain, or *Pseudomonas fluorescens* strain 55), which was a solution containing 500ul of water with or without bacteria with 5ul of blue food dye for visualization, see Table 3-1. Treatments were orally injected (~100nl) with a glass needle under a chilled temperature (11°C) to slow the beetle’s movement. Two days post dose 1, beetles were given ~100nl of dose 2 (water, *Erwinia tracheiphila* TedCu10^rif^ strain, or *Pseudomonas fluorescens* strain 55), see Table 3-1. One day after the second dose, beetles were placed in acetone for preservation and dehydration.

Following methods outlined by Koga *et al.* 2009, I dissected out the alimentary canals from beetles in 70% ethanol and fixed them over night in 500 ul Carnoy’s solution (6:3:1 absolute ethanol:chloroform:glacial acetic acid) at room temperature (25 °C). After fixation, alimentary canals were removed from Carnoy’s and placed in 100% ethanol in -20 °C for storage. The alimentary canals were rehydrated in 1X PBSTx (Phosphate buffered saline: 0.003% Tritonx-100) and incubated in hybridization buffer (900mM of 5M NaCl, 20mM of 1M Tris/HCl ph [8.0], 30% formamide, and 0.01% of 10% SDS) for at least 15 minutes. After replacing the hybridization buffer with hybridization buffer + 100pmol/ml fluorescent probe, I incubated the samples overnight at room temperature. Samples were mounted on slides with Slow-Fade antifade solution with DAPI (ThermoFisher Scientific, Waltham, MA) and imaged (Koga *et al*., 2009). Guts were analyzed for visual co-localization of *Pseudomonas fluorescens* and *Erwinia tracheiphila*. 
Data Analysis

The *in vitro* experiment was analyzed to determine the rate at which *Pseudomonas fluorescens* inhibits growth of *Erwinia tracheiphila*.

The *in vivo* experiment was analyzed using fluorescent *in situ* hybridization to visualize location of *Pseudomonas fluorescens* and *Erwinia tracheiphila* in the alimentary canal of *Acalymma vittatum* for co-localization.

Results

*In vitro* experimental results

King’s B plates inoculated with a lawn of *Pseudomonas fluorescens* and a central disk of *Erwinia tracheiphila* did not produce zones of inhibition. Also, the *Erwinia tracheiphila* did not extend in growth from the central disk and did not inhibit the fluorescein production by *Pseudomonas fluorescens*.

In contrast, King’s B plates inoculated with a lawn of *Erwinia tracheiphila* and a central disk of *Pseudomonas fluorescens* did produce zones of inhibition. Plates with a lawn of *Erwinia tracheiphila* and a central disk of *Pseudomonas fluorescens* showed an increase in the percentage of plates that produced a zone of inhibition across observation days, Figure 3-4. The zone of inhibition increased and was maintained through the course of the observations, Figure 3-5.

The growth of the *Pseudomonas fluorescens* from the central disk also increased across time, Figure 3-6. The *Pseudomonas fluorescens* also excreted fluorescein into the media at increasing diameters from the central disk. Figure 3-7 shows the fluorescein diameter increased independent of production of a zone of inhibition against the *Erwinia tracheiphila*. 
Figure 3-3. King’s B plate with a lawn of *Erwinia tracheiphila* and a central disk of *Pseudomonas fluorescens* illustrating a zone of inhibition, the clearing of bacterial around the central disk.

Figure 3-4. Total number of King’s B plates inoculated with a lawn of *Erwinia tracheiphila* and a central disk of *Pseudomonas fluorescens* (Total Plates) and the number of those King’s B plates with zones of inhibition (ZOI).
Figure 3-5. Zone of inhibition diameter progression across observations days for King’s B plates (n=31) with a lawn of *Erwinia tracheiphila* and a central disk of *Pseudomonas fluorescens*; plates denoted by an X did not produce a zone of inhibition, plates denoted by a diamond did produce a zone of inhibition.

Figure 3-6. Diameter of *Pseudomonas fluorescens* growth from central disk on King’s B media (n=31) across observation days; plates denoted by an X did not produce a zone of inhibition, plates denoted by a diamond did produce a zone of inhibition.
Figure 3-7. King’s B (n=31) plates inoculated with a lawn of Erwinia tracheiphila and a central disk of Pseudomonas fluorescens across observation days showing the diameter of fluorescein excreted into the media. Plates denoted by an X did not produce a zone of inhibition; plates denoted by a diamond did produce a zone of inhibition.

**In vivo experimental results**

Full Acalymma vittatum alimentary canals were analyzed for presence of the bacteria.

Figure 3-8. Scanning electron microscope image of the full alimentary canal of Acalymma vittatum.
Fluorescent in situ hybridization Erwinia tracheiphila probe

Initial trials of the Erwinia tracheiphila 16S probe viability were done on slide-mounted bacterial cells from all 6 Erwinia tracheiphila strains. These tests confirmed specificity to all Erwinia tracheiphila strains and no cross-reaction with Pseudomonas fluorescens strain 55. The following images are of the same Acalymma vittatum alimentary canal after performing fluorescent in situ hybridization showing that the probe can selectively detect Erwinia tracheiphila, Figure 3-9. RNase-treated alimentary canal controls had no binding of the probe, confirming specificity for Erwinia tracheiphila, Figure 3-10.

Figure 3-9. Acalymma vittatum hindgut of the alimentary canal stained with DAPI for nuclei (top image 40X) and stained with 6FAM for Erwinia tracheiphila (bottom image 40X) same image exposed under different light for each fluorophore.
Figure 3-10. RNase-treated alimentary canal of an *Acalymma vittatum* stained with 6FAM *Erwinia tracheiphila* 16s probe (4x).

**Bacterial co-localization in the alimentary canal**

**Controls.** In samples where 16S rRNA probes to both *Erwinia tracheiphila* and *Pseudomonas fluorescens* were used, *Erwinia tracheiphila* probe was hybridized with a rhodamine-labeled (red) probe and *Pseudomonas fluorescens* was hybridized with a 6FAM-labeled (green) probe. The background presence of *Pseudomonas spp.* in the negative control (water-fed) *Acalymma vittatum* was ~18% of beetle midguts and 6% of beetle hindguts. This *Pseudomonas spp.* detected in the negative control has the possibility of being another species due to the Pseudo120 probe being *Pseudomonas* group I-specific. *Erwinia tracheiphila* was not detected in the alimentary canals of the negative controls, Figure 3-11.

Beetles dosed with *Erwinia tracheiphila* and water (‘*Erwinia tracheiphila* only’) had both *Erwinia tracheiphila* and *Pseudomonas fluorescens* in the mid and hindgut. Of the *Erwinia*
*tracheiphila* positive control beetles, 42% of the midguts and 29% of the hindguts had *Erwinia tracheiphila*, where as *Pseudomonas fluorescens* was at seen at lower rates than the negative control, of 6% of midguts and 2% of hindguts. This indicates that *Erwinia tracheiphila* can survive within the alimentary canal for a short time span, Figure 3-12.

Beetles dosed with *Pseudomonas fluorescens* and water (‘*Pseudomonas fluorescens only’”) showed *Pseudomonas fluorescens* to be in 41% of the mid guts and 32% of the hindguts. These controls illustrate that *Pseudomonas fluorescens* can survive in the alimentary canal short term. *Erwinia tracheiphila* was not found in any of the *Pseudomonas fluorescens* positive controls, Figure 3-13.

**Table 3-1.** Treatment regimen given to *Acalymma vittatum* for gut co-localization imaging, TedCu10^Rif= *Erwinia tracheiphila* strain and P. f. = *Pseudomonas fluorescens* strain 55.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sample size</th>
<th>Presence of E. tracheiphila</th>
<th>Presence of Pseudomonas spp.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dose 1</td>
<td>Dose 2</td>
<td>mid gut</td>
</tr>
<tr>
<td>water</td>
<td>water</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>TedCu10^Rif</td>
<td>water</td>
<td>48</td>
<td>20</td>
</tr>
<tr>
<td>P. f.</td>
<td>water</td>
<td>63</td>
<td>0</td>
</tr>
<tr>
<td>TedCu10^Rif and P. f.</td>
<td>water</td>
<td>29</td>
<td>5</td>
</tr>
<tr>
<td>TedCu10^Rif P. f.</td>
<td>P. f.</td>
<td>24</td>
<td>4</td>
</tr>
<tr>
<td>P. f.</td>
<td>TedCu10^Rif</td>
<td>24</td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 3-11. Negative control *Acalymma vittatum* alimentary canals stained with DAPI (blue) for nuclei, Pseudo120 6FAM (green) for *Pseudomonas spp.* and Et16s Rhodamine (red) for *Erwinia tracheiphila*. Left image is of a midgut (4X) and right image is of a hindgut (4X).

Figure 3-12. *Erwinia tracheiphila* positive control *Acalymma vittatum* alimentary canals stained with DAPI (blue) for nuclei and Et16s 6FAM (green) for *Erwinia tracheiphila*. Left image is of a midgut (4X) and right image is of a hindgut (4X).
Figure 3.13. *Pseudomonas fluorescens* positive control *Acalyymma vittatum* alimentary canals stained with DAPI (blue) for nuclei and Pseudo120 6FAM (green) for *Pseudomonas fluorescens*. Left image is of a midgut (20X) and right image is of a hindgut (4X).

**Treatments.** When *Acalyymma vittatum* was orally injected with a mix of both *Erwinia tracheiphila* and *Pseudomonas fluorescens* simultaneously followed by water there was a decrease in the number of beetles harboring either bacteria compared to the positive controls. *Erwinia tracheiphila* was found in 17% of beetle midguts and in 21% of beetle hindguts, which is a decrease compared to the *Erwinia tracheiphila* positive controls (42% of mid gut and 29% of hindgut controls). *Pseudomonas fluorescens* was found in 31% of beetles mid gut and in 24% of beetles hindgut compared to the *Pseudomonas fluorescens* positive controls, which had the bacteria in 41% of beetles midgut and 32% of beetles hindgut, Figure 3.14.

Beetles dosed with *Erwinia tracheiphila* and challenged with *Pseudomonas fluorescens* had both bacteria in the mid and hindguts. *Erwinia tracheiphila* was seen less in the mid and hindguts (17% and 8%, respectively). *Pseudomonas fluorescens* was seen at the highest rates in the midgut (71% of beetles) and in 38% of hindguts, Figure 3.15.

Beetles dosed with *Pseudomonas fluorescens* and challenged with *Erwinia tracheiphila* had only *Pseudomonas fluorescens* in the mid and hindguts (50% and 29%, respectively). *Erwinia tracheiphila* was not found in the alimentary canal of these beetles, Figure 3.16.
Figure 3-14. *Erwinia tracheiphila* with *Pseudomonas fluorescens* dosed *Acalymma vittatum* alimentary canals stained with DAPI (blue) for nuclei, Pseudo120 6FAM (green) for *Pseudomonas fluorescens* and Et16s Rhodamine (red) for *Erwinia tracheiphila*. Top image is of a mid gut (20X) and bottom image is of a hindgut (20X).
Figure 3-15. Erwinia tracheiphila challenged with Pseudomonas fluorescens Acalymma vittatum alimentary canals stained with DAPI (blue) for nuclei, Pseudo120 6FAM (green) for Pseudomonas fluorescens and Et16s Cy5 (red) for Erwinia tracheiphila. Top image is of a mid gut (20X) and bottom image is of a hindgut (4X).
Figure 3-16. *Pseudomonas fluorescens* challenged with *Erwinia tracheiphila* Acalymma vittatum alimentary canals stained with DAPI (blue) for nuclei, Pseudo120 6FAM (green) for *Pseudomonas fluorescens* and Et16s Cy5 (red) for *Erwinia tracheiphila*. Top image is of a mid gut (20X) and bottom image is of a hindgut (4X).

Discussion

*In vitro interactions of Pseudomonas fluorescens and Erwinia tracheiphila*

Based on studies of *Pseudomonas fluorescens* inhibition of *Erwinia amylovora*, and its close phylogenetic relationship with *Erwinia tracheiphila*, I hypothesized that *Pseudomonas*
fluorescens would likewise inhibit *Erwinia tracheiphila*. Contamination of *Erwinia tracheiphila* cultures, due to the slow growing nature of the bacteria, led to the need for a semi-selective media that would provide an environment less hospitable to the competitive contaminants. While I did have a rifampicin-resistant *Erwinia tracheiphila* strain, *Pseudomonas fluorescens* was not resistant. Because I needed a semi-selective medium that supported both bacterial species to study their interaction, I used King’s B medium. I confirmed that *Erwinia tracheiphila* would grow on King’s B. Additionally, the production of pyoverdin was a characteristic that allowed me to distinguish between the *Pseudomonas* and *Erwinia* on the King’s B.

Using a modified agar disk diffusion assay provided an easy test for potential interactions of *Erwinia tracheiphila* and *Pseudomonas fluorescens* in vitro. When a lawn of *Pseudomonas fluorescens* was challenged from a central disk of *Erwinia tracheiphila*; the *Erwinia tracheiphila* did not extend in growth outwards from the central disk nor did it produce a zone of inhibition against the *Pseudomonas fluorescens*. Also, there was no prevention of *Pseudomonas fluorescens* fluorescein production. This illustrates that *Erwinia tracheiphila* cannot overcome *Pseudomonas fluorescens*, which is a trait required for control.

In contrast, when a lawn of *Erwinia tracheiphila* was challenged with a central disk of *Pseudomonas fluorescens*, *Pseudomonas fluorescens* produced a zone of inhibition against the *Erwinia tracheiphila*, Figure 3-3. The number of plates that did produce a zone of inhibition against *Erwinia tracheiphila* increased over time indicating that growth of *Pseudomonas fluorescens* was necessary to inhibit *Erwinia tracheiphila* but was not a constant interaction, as illustrated in Figure 3-4. However, once a zone of inhibition was present, *Pseudomonas fluorescens* maintained the inhibition of *Erwinia tracheiphila* over 7 days and increased in diameter, Figure 3-5. This could be explained by the increase in growth of the *Pseudomonas fluorescens* from the central disk, Figure 3-6, as nutrients became less available in the immediate area of inoculation. With this data, I concluded in a restricted nutrient environment *Pseudomonas fluorescens* prevents the growth of *Erwinia tracheiphila*. I cannot state the mechanism of the
inhibition, whether it is caused by antibiosis or siderophore excretion. *Pseudomonas fluorescens* did secret fluorescein into the media in all replicate plates and the diameter of the fluorescein increased over time as depicted in Figure 3-7. Fluorescein production was not indicative a zone of inhibition against the *Erwinia tracheiphila*, as shown in Figure 3-7. There could have been some selection for or against either bacteria in regards to specific pathways being turned on or off, such as the pathway for fluorescein production in *Pseudomonas fluorescens* and biofilm production in *Erwinia tracheiphila*.

**In vivo analysis of Pseudomonas fluorescens and Erwinia tracheiphila**

I used a co-localization FISH assay for *Erwinia tracheiphila* and *Pseudomonas fluorescens* to determine whether they would 1) colonize the same region of the alimentary canal of the beetle and 2) whether *Pseudomonas fluorescens* would inhibit establishment of *Erwinia tracheiphila*, and provide evidence that *Pseudomonas fluorescens* may be a potential biological control candidate. I demonstrated that the *Erwinia tracheiphila*-specific probe hybridizes with 6 strains of *Erwinia tracheiphila* and will also work within the alimentary canal of *Acalymma vittatum*, Figure 3-9. No probe cross-reactivity was detected with any other bacteria or host DNA, as illustrated in Figure 3-9 by the use of DAPI stain for all other DNA. The probe was also tested for specificity with RNase-treated alimentary canals to determine if other DNA segments could provide a non-specific binding target, upon which there was no reaction, Figure 3-10.

*Erwinia tracheiphila* was not detected in any of the negative control (water only) *Acalymma vittatum*, Figure 3-11. A small percentage of beetles were positive for *Pseudomonas* sp. but because Pseudo120 probe is specific for Group I *Pseudomonas*, I cannot be certain what species was detected. I did not identify this naturally occurring isolate. If the unidentified *Pseudomonas* sp. was not *Pseudomonas fluorescens*, it may be another candidate for biological control since it resides in the same location of the beetle alimentary canal as *Erwinia tracheiphila*. 64
I have shown that *Erwinia tracheiphila* can survive and be retained in the alimentary canal of *Acalymma vittatum* 4 days after inoculation, Figure 3-12. This is consistent with prior vector competence and bacterial retention studies (Shapiro et al., 2014).

I confirmed that *Pseudomonas fluorescens* could be detected in both the mid and hindgut of the alimentary canal of *Acalymma vittatum*, Figure 3-13. *Pseudomonas fluorescens* was retained for 4 days, which indicates that the gut is a micro-aerobic environment and provides enough oxygen for the bacteria. This gives support for the possibility of these bacteria interacting within the gut due to presents in the same regions of the gut. However, *Pseudomonas fluorescens* survival of the alimentary canal was not determined in this experiment but could easily be performed by culturing *Acalymma vittatum* frass for live *Pseudomonas fluorescens* and confirmation by PCR. When considering the potential of both bacteria interacting within the alimentary canal, Figure 3-12 and 3-13 suggest that both bacteria will be found in the hindgut, which further supports co-localization potential.

I demonstrated that when I fed *Acalymma vittatum* a mix of both bacteria simultaneously, *Erwinia tracheiphila* and *Pseudomonas fluorescens* could be found in the same portion of the hindgut in close proximity, Figure 3-14. In a field setting, it would be possible for a beetle to ingest both bacteria simultaneously by feeding on a symptomatic plant that had been treated with *Pseudomonas fluorescens*. Although the *Pseudomonas fluorescens* did not eradicate the *Erwinia tracheiphila* in this treatment, it did limit the establishment and maintenance of *Erwinia tracheiphila* providing partial control that could slow disease spread through a crop.

When *Erwinia tracheiphila* is challenged by *Pseudomonas fluorescens* in *Acalymma vittatum* alimentary canal, *Erwinia tracheiphila* was present in fewer beetles compared to the beetles fed only *Erwinia tracheiphila*. In contrast, there was about the same number of beetles with *Pseudomonas fluorescens* as compared to the beetles fed only *Pseudomonas fluorescens*, Figure 3-15. Taken together, these data suggest that *Pseudomonas fluorescens* shows promise as a potential inhibitor of *Erwinia tracheiphila* maintenance in beetles.
When *Acalymma vittatum* was first inoculated with *Pseudomonas fluorescens* and challenged with *Erwinia tracheiphila*, I did not detect *Erwinia tracheiphila* in the alimentary canal, Figure 3-16. *Pseudomonas fluorescens* was retained approximately the same as the positive control. This indicates that *Pseudomonas fluorescens* can prevent *Erwinia tracheiphila* from colonizing the gut and potentially inhibit transmission/maintenance by the beetle vector. If these findings hold true under field conditions it may provide an effective treatment for newly emerged beetles that have not been inoculated with *Erwinia tracheiphila* and disrupt the overwintering stage of the disease cycle.

These images indicate the potential of *Pseudomonas fluorescens* acting as a biological control of *Erwinia tracheiphila*, but this data does not indicate inhibition of either bacterium within the alimentary canal. To confirm inhibition, as we saw in the *in vitro* experiment, more replicates with different concentrations of bacteria should be tested. Further, viability of each bacterium confirmed with plating assays. Transmission efficacy should also be assessed. Vector capacity of *Acalymma vittatum* is dose-dependent based on the acquisition time of the beetle (Shapiro et al., 2014). This data provides evidence of the possibility of managing the plant pathogen via interactions within *Acalymma vittatum* by decreasing vector capacity of the beetle.
References: Chapter 3


Pair SD (1997) Evaluation of Systemically Treated Squash Trap Plants and Attracticidal Baits for Early-Season Control of Striped and Spotted Cucumber Beetles (Coleoptera: Chrysomelidae) and Squash Bug (Hemiptera: Coreidae) in Cucurbit Crops. Journal of Economic Entomology 90: 1307-1314.


Chapter 4

Final Conclusions and Future Research

This thesis provides new insights into diabrotic vector species outside the geographic range of *Erwinia tracheiphila* and a biological control by *Pseudomonas fluorescens* targeting interactions inside the alimentary canal of the beetle. *Acalymma vittatum* has been verified as a vector of *Erwinia tracheiphila* as previously illustrated (Brust, 1997; Brust & Rane, 1995; Haynes & Jones, 1975; Mitchell & Hanks, 2009; Shapiro, 2012) and adult survival is not impacted by bacterial status (Shapiro et al., 2014). *Acalymma trivittatum* is a competent vector of *Erwinia tracheiphila*, whereas *Diabrotica balteata* is capable of transmitting *Erwinia tracheiphila* but vector status is less clear. Adult *Acalymma trivittatum* survival was not impacted by bacterial status, but *Diabrotica balteata* did decrease in survivorship that may or may not be due to bacterial status.

Sample sizes were not favorable for all three species, which led to statistical power issues in analyzing vector competence for *Diabrotica balteata*. However, we were still able to show significant detection of *Erwinia tracheiphila* in both *Acalymma* species. The frequency of time points were based on key bacterial concentrations for *Acalymma vittatum* (Shapiro et al., 2014) and feasibility of the three detection methods, but these time points limited the resolution for the survivorship of the beetles. The PCR method of detection had several issues in both the frass and plate recovery of *Erwinia tracheiphila*. There were false positives in the frass suspension samples and loss of accuracy for detection of bacterial cells below a threshold that is common in frass. In the future, to combat these issues, I would design new PCR primers and use another more sensitive assay to verify potential false positive such as real-time PCR.
Real-time polymerase chain reaction protocols have been established for this insect-pathogen interaction (Shapiro, 2012) but were not feasible for this vector competence study. I used the culturing methods and polymerase chain reaction to ensure the bacteria were being viably excreted, which provide field applicable information. However, these methods did not guarantee detection of Erwinia tracheiphila. Also, there have not been any studies in potential vertical or horizontal transmission of Erwinia tracheiphila between beetles, which could account for the detection in the buffer controls of Acalymma vittatum. New more sensitive methods that are affordable and less time consuming would be useful in elucidating a finer time line of Erwinia tracheiphila acquisition and excretion for these new vector species. Other possible diabroticite beetles should also be tested for vector competence to determine how economically important this pathogen could be in each region.

In vitro assays showed that Pseudomonas fluorescens inhibited the growth of Erwinia tracheiphila. I designed a fluorescent in situ hybridization probe specific to Erwinia tracheiphila for in vivo co-existence assays. Erwinia tracheiphila and Pseudomonas fluorescens are retained independently in the alimentary canal of Acalymma vittatum for at least 4 days post-inoculation. The number of Acalymma vittatum harboring Erwinia tracheiphila after being challenged with Pseudomonas fluorescens was fewer than Acalymma vittatum fed only Erwinia tracheiphila. Acalymma vittatum given Pseudomonas fluorescens before challenging with Erwinia tracheiphila had no retention of Erwinia tracheiphila.

Only one strain of Erwinia tracheiphila and Pseudomonas fluorescens were tested in vitro on the same media, hence other strains should be tested to determine if the interaction is similar. The in vivo assays had small sample sizes and a short duration that only allows visualization of the bacteria in the alimentary canal. I therefore cannot conclude anything about the viability or colonization status of either bacterium. This data does not provide causal proof
that *Pseudomonas fluorescens* is responsible for the decrease or prevention of *Erwinia tracheiphila* retention in the alimentary canal of *Acalymma vittatum*. However, we have some suggestive evidence that, when visually compared to the positive controls, presence of *Erwinia tracheiphila* is affected by presence of *Pseudomonas fluorescens*.

To further evaluate interactions between *Erwinia tracheiphila* and *Pseudomonas fluorescens*, more strains of both bacteria need to be tested. Further *in vitro* environments mimicking nutrient availability of a field setting should be explored to determine feasibility of field applications. A method for determining viability and colonization of both bacteria while in the alimentary canal is needed to determine if field applications would be sustainable. Molecular gut assays to locate where and how *Erwinia tracheiphila* colonizes and survives the alimentary canal would provide an insight into how *Pseudomonas fluorescens* could interact with *Erwinia tracheiphila* for a more specific mechanism. Some assays should focus on cellular adhesion and biofilm production of *Erwinia tracheiphila* for gut colonization as a potential target for disruption. Also nutrient availability within the alimentary canal should be examined as a possible environment that supports *Pseudomonas fluorescens* as opposed to *Erwinia tracheiphila*. *Pseudomonas fluorescens* efficacy should be examined for duration and viability of retention in the alimentary canal for long-term control. This would help create a targeted control method for *Erwinia tracheiphila* by eradicating the bacteria from the vector. Focus on disrupting the *Erwinia tracheiphila* survival and maintenance in a diapausing beetle could provide a greater chance at controlling transmission of *Erwinia tracheiphila* from season to season. Field trials should be conducted to assess interaction potential of both bacteria and feasibility of application of *Pseudomonas fluorescens* to a crop.

Currently, there is little known about how *Erwinia tracheiphila* survives within the alimentary canal of *Acalymma vittatum*. More sensitive and specific methods need to be developed that would provide quick and affordable identification of *Erwinia tracheiphila*. In
addition, there is a general lack of knowledge on the diapause biology of *Acalymma vittatum* and how *Erwinia tracheiphila* survives within overwintering adult beetles. These topics should also be addressed in other possible vector species such as *Acalymma trivittatum* and *Diabrotica balteata*. 
References: Chapter 4


Appendix A. Relevant pages from Purdue University extension website about bacterial wilt of cucurbits, accessed July 11, 2015.

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Purdue University
Cooperative Extension Service
West Lafayette, IN 47907

Diseases and Pests of Muskmelons and Watermelons

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Extension Specialist, Plant Pathology*
BACTERIAL WILT

Bacterial wilt is a common disease of muskmelons, but does not affect watermelons. The bacterial pathogen, *Erwinia tracheiphila*, multiplies within the vascular system of infected plants, causing rapid wilt and collapse of vines. The pathogen is transmitted by cucumber beetle vectors. Losses due to bacterial wilt can range from 10-20% in a disease-favorable season.

Initial symptoms of bacterial wilt include flagging or wilting of leaves on one or more vines (Figure 17). Symptom development proceeds rapidly; entire plants may collapse and die within a few days (Figure 18). Most of the loss caused by bacterial wilt occurs up to three weeks before harvest begins and coincides with springtime increases in cucumber beetle populations. In some seasons, wilt and collapse continues to occur after harvest begins. A diagnostic test for bacterial wilt can be performed in the field by cutting a wilted vine close to the main stem, rejoining the cut surfaces, then slowly drawing the sections apart. Bacterial wilt can be positively diagnosed if a thin strand of slime extends between the two sections (Figure 19).

| Disease cycle: | * polycyclic |
| Source of primary inoculum: | * adult cucumber beetles that emerge in mid-spring |
| Secondary inoculum: | * bacteria produced in infected plants |
| Spread: | * the bacteria are transmitted from infected plants to healthy plants by cucumber beetles |

**Disease Control**

- **Disease resistance:** *No muskmelon varieties are resistant to bacterial wilt. Watermelons are not affected by the disease.*
- **Cultural control:** *Cultural practices appear to have no effect on disease development.*
- **Chemical control:** *Bacterial wilt control is directly related to control of cucumber beetles. Soil-applied insecticides and repeated applications of foliar contact insecticides are necessary for adequate disease control.*
Recently there has been a dramatic increase in the occurrence of bacterial wilt, especially in pumpkin and squash. Initial symptoms of wilt are pale, wilted sections of leaves that are often associated with feeding injury (Figure 1). Symptoms of bacterial wilt progress from localized leaf symptoms to collapse of individual vines and eventually to plant death (Figure 2). The bacterium causing this disease (*Erwinia tracheiphila*) cannot be controlled directly with pesticides, therefore, management practices have targeted the insects that harbor and vector the pathogen, which are the striped and spotted cucumber beetles (Figures 3 and 4). Control is complicated because the presence of beetles...
organically is after the description of conventional practices.

1. Select less susceptible varieties.

2. Apply Admire at planting.

3. Scout weekly for cucumber beetles and wilt symptoms.

4. Foliar insecticides may be needed if beetle counts are above 1 beetle/plant, wilt is developing, and the variety is highly susceptible.

1. **Differences among cucurbit crop types and among varieties** in attractiveness to beetles and in occurrence of wilt were detected through research conducted recently on Long Island. This information will be helpful for tailoring management programs. Less susceptible varieties should be chosen when possible; else a good insecticide program should be implemented.

One important finding was that **attractiveness to beetles was not always related to wilt susceptibility**. For example, although fewer beetles per plant were observed in cucumber than in most other cucurbit crop types, and little feeding injury was observed, a higher percentage of cucumber plants developed wilt. Muskmelon had similarly low beetle densities but less wilt than cucumber. Compared to cucumber, zucchini was more attractive to beetles and less susceptible to wilt.

The **gourd** Turk's Turban (*Cucurbita maxima*) was very attractive to beetles and was severely affected by wilt, differing substantially from Pear Bicolored (*Cucurbita pepo*). This documents an important difference between these species that most likely extends to other gourds. Initial symptoms of wilt were first seen 13 and 19 days after cucumber beetles were first seen in 2000 and 1999, respectively. In the absence of insecticide treatment, all Turk's Turban plants died before producing fruit while about 25% of the Pear Bicolored plants died by late August.

Differences in wilt occurrence were also detected among **pumpkin** varieties. This was not related to attractiveness to cucumber beetles or to damage from their feeding, which suggests these varieties differ in their susceptibility to wilt. A high percentage of Merlin plants developed severe wilt (at least 50% of the plant affected) by late August in both years (89-97%). Magic Lantern was also severely affected in 2000 when insect and disease pressure was higher than in 1999 (98% in 2000 versus 22% in 1999). These varieties did not have more beetles per plant or more feeding injury than Harvest Moon and Howden. Percentage of plants of these varieties that were severely wilted was 3% and 13% in 1999 and 53% and 58% in 2000, respectively. The more susceptible varieties have powdery mildew resistance (PMR). Fortunately there does not appear to be a general correlation between wilt susceptibility and PMR as the PMR muskmelon and yellow summer squash varieties examined in this study (Eclipse, Athena, and Sunray) were not more susceptible to wilt than the other varieties examined.

Waltham Butternut (*C. moschata*) had fewer beetles and less feeding damage than other **winter squash** varieties, and it was the last to develop wilt symptoms, which were not seen until 15 Aug 2000. It was less susceptible than Golden Delicious or Blue Hubbard; Table Ace and Burgess Buttercup were intermediate. Winter squashes Golden Delicious and Blue Hubbard had higher beetle densities, more feeding injury and higher incidence of wilt than Waltham Butternut and Table Ace.
Appendix C. Number of beetles with positive frass defined by PCR and total number tested at 1, 5, and 10 days post-treatment for *Acalymma trivittatum*, *Diabrotica balteata* and *Acalymma vittatum* on each starting date and *Erwinia tracheiphila* strain used as treatment.

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Appendix D. Number of beetle frass samples collected from individual beetles 1, 5 and 10 days post-treatment that grew *Erwinia tracheiphila* cultures defined by PCR on nutrient broth and King’s B media amended with Rifampicin by treatment strain and experiment date.

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Appendix E. Polymerase chain reaction electrophoresis gels used to visualize *Erwinia tracheiphila* carbamoylphosphate synthetase gene (426 base pairs) against 5Prime 100XL bp ladder. The top image illustrates the alternative banding patterns from *Acalymma vittatum* frass and the bottom image illustrates the expected banding pattern from *Erwinia tracheiphila* pure bacterial cultures. L = 5Prime 100XL bp ladder, Et = positive control, N = negative control.
Appendix F. Schematic of experimental design for the path of a single beetle.