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John R. Caudle, Student

Dr. Mark A. Williams, Major Professor

Dr. Mark S. Coyne, Director of Graduate Studies

CONTROL OF *ERWINIA TRACHEIPHILA* IN *CUCUMIS MELO*

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy
in the College of Agriculture, Food and Environment
at the University of Kentucky

By

John Robert Caudle

Lexington, Kentucky

Director: Dr. Mark A. Williams, Associate Professor of Horticulture

Lexington, Kentucky

2013

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ABSTRACT OF DISSERTATION

CONTROL OF *ERWINIA TRACHEIPHILA* IN *CUCUMIS MELO*

Currently there is no control of bacterial wilt disease, *Erwinia tracheiphila*, in susceptible cucurbit crops, once infection of the plant occurs. Conventional and organic production systems rely on insecticide applications to kill the vectors, striped and spotted cucumber beetles, *Acalymma vittatum* and *Diabrotica undecimpunctata,* respectively, prior to transmission of the pathogen which indirectly controls the disease to some extent. Physical barriers such as row covers are used to exclude the vectors from plants prior to flowering; however, pollination requirements expose plants to potential infection. Experimental field plots were developed to test various enhanced organic production systems in an effort to increase productivity of the "Athena" variety cantaloupe melon crop, *Cucumis melo,* which is highly susceptible to bacterial wilt infection. The rotations included enhanced duration row cover applications as well as season long covering of the crop and application of bumble bee hives for pollination. The most successful enhanced production method included the removal of row covers and application of organic pesticides during flowering and recovering the crop until the end of the season. In this scenario, reduction in the cost of pesticide application and reduced risk due to less exposure to infection are the key enhancements to the system. During pollination, the melon plants are at risk of infection from bacterial wilt because organic production methods cannot include systemic insecticides. Only shorter residual contact insecticides are available, thus exposing the melon plants to vectors after the contact insecticide becomes ineffective. Application of an off-label biocontrol bacterium, *Pseudomonas fluorescens* A506, found in the organically certified product BlightBan®A506, was found to significantly increase control of *Erwinia tracheiphila* infection in plants, thereby allowing for increased productivity. Additionally, development of a Real-Time Polymerase Chain Reaction, RT-PCR, primer set and probe improve the detection of *Erwinia tracheiphila* in melon plants. This new primer set was tested against numerous related and associated pathogens to document the specificity of this particular screening test.

KEY WORDS: *Erwinia tracheiphila*, organic agriculture, *Cucumis melo*, Real-Time PCR, *Pseudomonas fluorescens* A506

John Robert Caudle

December 4, 2013

CONTROL OF *ERWINIA TRACHEIPHILA* IN *CUCUMIS MELO*

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CHAPTER 1

LITERATURE REVIEW

Conventional agriculture has successfully managed many plant pathogens through the application of synthetic chemical treatments that suppress or kill pathogens before the pathogens are detrimental to the agriculturally valuable product. However, the practice of organic agriculture is a production system that reduces outside synthetic inputs in order to improve the health of the environment (Gomiero, 2011). Specifically, synthetic fertilizers and pesticides, as well as genetically modified organisms are not used in organic systems (Mason, et al., 2006). The United States Department of Agriculture defined sustainable agriculture in in the 1990 Farm Bill in terms of "an integrated system of plant and animal production practices have a site-specific application that will, over the long term: satisfy human food and fiber needs, enhance environmental quality, efficiently use nonrenewable resources, sustain farm operations economic viability, and enhance the quality of life for farms and society (Food, Agriculture, Conservation, and Trade Act of 1990, 1990). This research explores a specific pest-plant pathogen interaction that is due to the vectoring of a bacterial pathogen by insect pests in organically produced muskmelon (*Cucumis melo L.*). Conventional muskmelon systems currently use synthetically produced pesticides to kill the vectoring pest (Brust, et al., 1996; Jasinski, et al., 2009). As a consequence of intentional non-use of synthetic pesticides organic and sustainable muskmelon production systems can experience significant damage (Brust, 1997). This research focused on developing techniques suitable for use in organically-managed muskmelon systems.

Organic Production Systems

The Organic Foods Protection Act (OFPA) of 1990 guides organic methods in the United States that directed the United States Department of Agriculture (USDA) to develop national standards for organic agricultural products. This law is cited at 7 U.S.C. 6501-6522 of the federal code. The USDA formed the National Organic Standards Board (NOSB) to advise the USDA concerning organic agriculture related regulation (USDA, 2014). Separately, the Organic Materials Review Institute (OMRI) developed a list of fertilizer, pesticides, antibiotics, food additives, irradiation, and the use of sewage sludge, that are approved for organic production systems (Organic, 2014). This list of products includes naturally obtained, as well as synthetic products, deemed essential to production agriculture. With the materials allowed for organic production, organic pest management procedures, much like Integrated Pest Management protocols for conventional crops, are applied to organic production systems. Practices include naturally sourced pesticides like chrysanthemum- based pyrethrin, soil-source *Bacillus subtilus* Cohn bacterial solutions, as well as fabric and mechanical materials used to exclude pest and pathogens by covering plants in a spun-bond fabric tunnel to exclude pests (Bierbaum, 2014). Field crop management is typically handled much like conventional systems except that organic fertilizers, soil amendments, and pesticides are used in place of synthetics (USDA, 2014). Organic post-harvest products are also available to increase post-harvest longevity (Suslow, 2000).

More specifically as relating to this research, organically produced melons are a high value crop, however, pathogen pressure can cause significant damage to production yield

(Brust, 1997). Organic melon production typically consists of the use of spun-bond fabric material row covers to exclude the vectors of pathogens (Batzer and Gleason, 2012). Wire hoops are laid down along the row at various intervals to hold the row cover material up and off the plants, as well as, allowing for air movement while excluding pests. The addition of predator beneficial insects such as lady beetles (*Hippodamia convergens* Guerin) or green lacewing larvae (*Chrysoperla sp.*) under the row covers can help manage melon aphids that commonly occur under row covers. Row covers are normally removed after flowering begins (anthesis) in order to allow for pollinator access to the melon flowers. Anthesis here is defined as the period during which the female flowers are open. A combination of certified pesticides are applied to combat pests that transmit various pathogens based on Integrated Pest Management practices (Integrated, 2014). The pesticides are applied at varying intervals based on insect pressure until harvest. In this research several products were applied in order to increase the plant protection potential from just one product. Specifically, Surround WP Crop Protectant (NovaSource, Phoenix, Arizona), which is a fine Kaolin clay powder that when added to water is sprayed over the melon plants so that when beetles feed on the melon plants, encounter the clay material which ultimately interferes with reproduction. (Dufour, 2001). Additionally is irritates and repels the insects, creates an unsuitable feeding location and causes the object sprayed to be less identifiable as feeding material (Insect, 2014). Another product applied to melon plants in this research was PyGanic Crop Protectant EC (McLaughlin, Gormley, and King Co., Minneapolis, Minn.) that is a chrysanthemum-derived powder that contains pyrethrin that is a neurotoxin to insects. It is a non-persistent, biodegradable insecticide that is applied in a water solution to the entire plant.

When insect vectors alight on the plant, they encounter this product causing incapacitation or death. A third organic certified product used in this research is neem oil that is product of the neem tree (*Azadirachta indica* Juss). The active agent is azadiractin that acts as feeding inhibitor and growth-disruptor insecticide. This biodegradable material is added to water and is sprayed over the plants so that insect vectors encounter this material when alighting to feed on the muskmelon. The insect ingests the azadiractin molecule that is similar to insect growth hormones whereby the reproductive cycle is disrupted. Various pathogens can impact the crop including fungal and bacterial pathogens such as *Podosphaera xanthii* Braun & Shishkoff (powdery mildew*),* *Pseudoperonospora cubensis* [(Berkeley & M. A. Curtis) Rostovzev] *(*downey mildew), *Colletotrichum orbiculare* (Berk. & Mont.) Arx *(*anthracnose*),* and *Erwinia tracheiphila* (Smith) Bergey et al *(*bacterial wilt*)*(Sherf, 1986).

One pathogen that has no direct control in either conventional or organic systems is *Erwinia tracheiphila*, which is primarily vectored by spotted and striped cucumber beetles (*Diabrotica undecimpunctata howardi* Barber and *Acalymma vittatum* Fabricius, respectively) (Rand and Enlows, 1916). Additionally, the western spotted cucumber beetle (*D. undecimpunctata undecimpunctata* Mannerheim), the banded cucumber beetle (*D. balteata* LeConte) are known to be vectors as well (Rand and Enlows, 1916). The striped and spotted cucumber beetle, the common names of the main insect vectors, is further described by their physical appearance as stripped or spotted. The common understanding of how *Erwinia tracheiphila* survives in temperate climates from year to year is through overwintering in the digestive track of the vectors. The vectors overwinter in the soil and debris on the forest/agricultural floor and reappears in the

spring. The pathogen enters the plant from vector mouthparts during feeding on the host plant, as well as, from insect frass that is deposited on plant leaves (Mitchell and Hanks, 2009). The bacteria can continue to live in the frass and rain or moisture can wash the bacteria into damaged leaf surfaces (Mitchell and Hanks, 2009). Once the bacteria colonizes into the plant tissues, it spreads to the tracheid vessels of the xylem (Smith, 1911). Current understanding of the progress of the disease is that the bacteria multiply to the point of clogging xylem vessels leading to wilting of the plant (Agrios, 1978). Conventional control of the pathogen mainly involves the seed treatment or soil application of synthetically derived systemic insecticides such as imidicloprid (Bayer Crop Science, Research Triangle Park, N.C.) as well as foliar application of synthetic insecticides to control the vector. Current organic pathogen control is to kill the vector with biodegradable pesticides or exclusion of the vector with row covers and then use of organic pesticides with the addition of feeding deterrent materials (Saalau-Rojas, 2011). Both conventional and organic melon production systems can suffer from incomplete control of *Erwinia tracheiphila* leading to periodic significant production losses.

Two of the research projects in this research program explore new methods of controlling *Erwinia tracheiphila*, one through modification of the standard organic production system to better exclude the vector, and the second explores the potential for biocontrol of *Erwinia tracheiphila* by application of a normally benign soil bacterial on the above ground plant surfaces to directly control the bacterial pathogen through increased plant immune system responses.

***Erwinia tracheiphila*, bacterial wilt in cucurbits**

Erwinia tracheiphila was first identified in the early 1900s (Smith, 1911). Subsequent research directly linked the bacteria with the development of bacterial wilt in cucurbits (Leach, 1964). Since that time no direct control of the bacteria has been developed. Row covers have been found to protect muskmelon from diseases (Perring et al., 1989). Also spun-bound row covers have been traditionally used in conventional production for non-disease related benefits such as season extension through temperature modification (Jenni, 1996; Wells and Loy, 1985). Row covers were found to provide delayed onset of bacterial wilt in conventional muskmelon crops (Mueller et al., 2006). Research in conventional systems systematized the application of the synthetic systemic insecticide, imidicloprid, to control *E. tracheiphila* through control of the main vectors, striped cucumber beetle and spotted cucumber beetle (Jasinski, et al., 2009). Additional research on conventional production using row covers to reduce pesticide inputs, while limiting insect feeding found that delaying the removal of the row covers past anthesis, supplying bumble bees (*Bombus impatiens* Cresson) for pollination or opening the ends of row covers at anthesis provided improved production (Gaye et al., 1991; Vassiere, 1996). Recent organic production research has focused on improving row cover efficacy by extending the duration of row covers (Saalu-Rojas, et al., 2011).

Organic production research has investigated the efficacy of companion crop planting and plastic mulches in conjunction with other organic production methods (Cline, et al., 2008). Previous research has investigated the removal of row covers from the muskmelon crop at anthesis followed by the application of conventional and organic

insecticides (Mueller et al., 2006). None of the treatments completely protected the crop from bacterial wilt incidence because organic insecticides have had limited control and short residual efficiency requiring repeated application to be effective thereby increasing the cost of production by each application. The limited efficacy of organic insecticides allows the bacterial wilt vectors opportunities to feed, wound plants, and deposit pathogens that often results in midseason infection. Once the pathogen gets into the xylem of the plant, it takes about two weeks until the plant wilts (Smith, 1911). During that time, the pathogen can move into ripening fruit making it unmarketable due to reduced sugar migration into the ripening fruit, as well as, wilting vine branches and the entire plant (Latin, 2012). The most effective way to stop the plant-to-plant infection transmission is to remove the wilted plants from the production site and reduce the level of pathogens available for vector acquisition (Latin, 2012).

Bacterial wilt Detection Via Real-Time Polymerase Chain Reaction Assay

Real-time PCR is an innovation that allows for quantitative analysis of amplified quantities of DNA. Real-time PCR entails the generation of shorter strands of DNA (<200 base pairs) in order to increase the cycling efficiency of the PCR reaction. Also an intercalating dye or photosensitive probe is introduced into the PRC reaction whereby photosensitive emissions are emitted during the PCR reaction. It is the accumulative light emissions that are quantitatively analyzed to assess the quantity of DNA in the reaction tube.

For *Erwinia tracheiphila,* numerous conventional PCR primer sets have been published (Mitchell and Hanks, 2009; Bruton, et al., 1999; Waleron, et al., 2002; Dallaire,

2009). However, there are no published primer sets specifically developed for Real-time PCR that have undergone specificity testing. This research provides these results.

The innovation that Real-time PCR allows is the testing of time sequence samples that track the multiplying bacteria in the xylem, thereby providing a tool to track the progress of the disease, particularly during the application of various materials that potentially could modify the growth cycle of the bacteria. Other diagnostic systems only provide presence/absence information with varying levels of accuracy. The Real-Time PCR assay allows for specific DNA level identification of the pathogen. DNA characterization has been conducted on bacterial wilt to the extent that a unique segment of DNA to *E. tracheiphila* can be used as the basis of the assay. Since this assay is currently using state-of-the-art diagnostic analysis, DNA characterization is not available for all pathogens; however, this will soon not be a limiting factor in expansion of this technique to many pathogen identification problems. The sensitivity of the test allows for only minute quantities of the pathogen DNA to be present for a positive reaction and quantification. By conducting cross-reactive selectivity testing with associated pathogens, the potential for false positive reactions with the assay are significantly reduced.

Biocontrol for *Erwinia tracheiphila*

Biocontrol incorporates a variety of pathogen and pest control approaches, however for this research biocontrol will focus on the use of naturally occurring bacteria to control pathogenic bacteria. This approach to pathogen control was promoted by Rachel Carson in *Silent Spring,* which started the reexamination of the Green Revolution legacy (Carson, 1963). Biocontrol uses the processes of mycoparasitism, antibiosis,

competition, hypovirulence, inhibition of enzymatic activities and induced resistance (Xu, et al., 2011). Biocontrol can be accomplished by application of specific bacterium or yeast isolates to specific pathogen host complex environments (McSpadden-Gardener, 2002). The effect of biocontrol activity can be degradation of the ability of the pathogen to act against the host or it can directly compete aggressively for the same nutrients needed by the target pathogen (McSpadden-Garderner, 2002). For example, *Bacillus mojavensis* RRC 101 is reported to be an endophyte with an influence on plant growth as well as producing antibiotics (Babu, 2011). Current research continues to investigate the action of individual and combinations of biological control agents (McSpadden Gardener, 2002). Biocontrol is a natural application to sustainable agriculture due to the emphasis on naturally sourced solutions (Singh, et al., 2009) The United States Department of Agriculture (USDA) has expended significant effort in understanding and developing biocontrol systems for plant diseases (Roberts, et al., 2003).

For the research in this study, a frequently studied soil bacterium, *Pseudomonas fluorescens* Migula, was identified as a potential biocontrol agent due to its application on the pathogen-plant complex of *Erwinia amylovora* Winslow *et al.* 1920 on apple and pear trees that causes Fire Blight (Stockwell and Stack, 2007). *Erwinia amylovora* is a close relative to *Erwinia tracheiphila* and acts in a similar fashion in woody tissue (Hauben, 1998, Stockwell and Stack, 2007). Research on *Pseudomonas fluorescens* has found that the soil-borne microorganism provides plant-growth promotion, anti-fungal metabolites, induced resistance against a wide variety of pathogens, and enhances tolerance to abiotic stress among other actions (Haas, 2003). All of these attributes are focused on interactions in the rhizosphere. Additional research on the specific strain of

Pseudomonas fluorescens A506 used in this research found the mode of action that defeated *Erwinia amylovora* was competitive out-competition (Stockwell and Stack, 2007). The biocontrol bacterium utilized available nutrients faster than the target pathogen, thereby retarding growth. (Stockwell and Stack, 2007) Specifically, research found that *Pseudomonas fluorescens* A506 is an excellent floral tissue colonizer (Wilson and Lindow, 1993). From these observations, this researcher made a connection between the similar symptoms of *Erwinia amylovora* (wilt and necrosis of tissue) with *Erwinia tracheiphila* (wilt and desiccation of tissue). Then recognizing the near relatedness of the pathogens from work on the specificity testing of the Real-time PCR screen discussed previously, it was hypothesized that *Pseudomonas fluorescens* A506 could have a similar effect of retarding the growth of *Erwinia tracheiphila* on melon as *Erwinia amylovora* with apple and pear. Prior to the recognition of this potential biocontrol agent in this research, numerous natural chemical compounds were applied on melon plants in an effort to challenge the plant defense system of *Cucumis melo* to resist Erwinia *tracheiphila* (based on conversations with Dr. J. Kuc, Kuc, 2000, Xie, 1997). Material that were screened included jasmonic acid, salicylic acid, azalaic acid, Serenade®, chitosan, hydrogen peroxide, Actigard®, Messenger®, and BlightBan A506® among others. Of all the natural compounds screened, *Pseudomonas fluorescens* A506 in the formulation of BlightBan®A506 (NuFarm Americas, Inc., Alsip, Illinois, USA) was the most effective natural chemical or biocontrol agent screened by physical observation.

Real-Time PCR Generated Genetic Evidence of Plant Defense Responses

When considering how to document a biochemical process in a plant, there have been two approaches historically pursued by plant pathologists: a biochemical approach and a genetic approach (Zipfel and Robatzek, 2010). As tools for examination of genetic materials have outpaced the ability to identify minute quantities of biochemicals diluted in a complex matrix of plant products, application of genetic tools have continued to expand the understanding of biochemical pathways (Zipfel and Robatzek, 2010). A recent query of biochemical pathways for *Arabidopsis thaliana* Heynh. on the online Plant Metabolic Network site produced 984 separate pathways of both individual products and processes (Plantcyc, 2012). Systemic acquired resistant (SAR) plant defense signaling is currently understood to occur in one biochemical pathway that includes the production of methyl salicylate (salicylic acid) (Kachroo and Robin, 2013). This signaling pathway has many metabolic building blocks that are sequentially triggered until the production of plant defense metabolites occur (Shah, 2013). Shah reviews the subject and reports that the methyl salicylate pathway involves a variety of gene responses and ongoing research is identifying new pathways that induce a resistance response. In order to quantify an increased activity in the signaling pathway leading to the production of pathogenesis-related protein 1 (PR1), which is one component of a family of proteins produced by plants to defend against pathogens (Shah, 2013), it is necessary to quantify the time-relative production of mRNA transcripts (White, 1979). Simply speaking, PR1-like proteins are involved in the activation of plant metabolites that act against invading plant pathogens to slow or stop pathogen progress in the plants that can cause plant diseases (Shah, 2003). Before production of PR1 occurs in the plant

in response to pathogen introduction, a not-fully-understood cascade of delicately-balanced biochemical processes occur from the first identification of the pathogen in a leaf or other plant structure (Shah, 2013). Recently, a gene called non-expressor of PR1 (NPR1) with its associated genes, NPR3 and NPR4, has been found to act at the beginning of the plant defense process generating "receptors" for the salicylic acid pathway which leads to systemic acquired resistance (SAR) or plant defense against pathogens (Fu, et al., 2012). Further, it was found that NPR3 acts as the specific receptor for a primed (pre-treated) plant system (Moreau, et al., 2012; Tsai, et al., 2011).

Priming is a technique where biocontrol or chemical agents are used to artificially stimulate the plant defense system, particularly when the plant does not recognize a pathogenic agent as a pathogen. The pre-application of the stimulating agent initiates the SAR defense signaling system (Conrath, 2006). Priming in my research herein is associated with a pre-treatment with BlightBan A506 (*Pseudomonas fluorescens* A506), which is non-pathogenic bacterium, to stimulate the salicylic acid pathway prior to inoculation with *Erwinia tracheiphila*. Once salicylic acid concentration is increased in the plant, it is proposed that the NPR3 gene acts as the receptor or initiator of the SAR response in the infected plant. This initiation of the process occurs by the transformation of the NPR1 through coupling with NPR3 that then binds to salicylic acid allowing Effector-Triggered Immunity (ETI) to develop and then the biochemical pathway proceeds to induce SAR (Moreau, et al., 2012; Jones, et al., 2006). This ETI process is still not fully understood.

For a primed treatment system, an indicator of its effect is to observe that increased salicylic acid production has been triggered prior to the introduction of the plant pathogen (Henry et al., 2013). Precursors to salicylic acid in the plant biochemical pathways are phenylalanine ammonia lyase (PAL) and cinnamyl-alcohol dehydrogenase (CAD) (Walters, 2011; Chen, et al., 2009; Shah, 2003). In order to determine if PAL and CAD have increased *in plantae* after priming, it is necessary to determine if quantitatively significant mRNA transcripts for these genes have increased in production after priming, thereby leading to production of the pathway components leading to salicylic acid. Quantitative analysis of mRNA via real-time PCR analysis of cDNA can provide evidence of up or down regulation of genes indicating production of plant products. By applying the Real-time PCR process to mRNA samples collected from treated and non-treated plants at 1 hour and 2 day intervals, it is possible to see the up or down regulation of the NPR3, PR1, CAD and PAL genes thereby providing evidence to support the idea that the SAR system has been activated in the plant due to the priming and pathogen inoculation of the model plant. This process is the method of determining the mode of action of the BlightBan A506 in this research.

The research conducted for this dissertation included the development of a field production system that provides greater protection from the vectoring of *Erwinia tracheiphila* relative to the current production system of providing fabric covers over the muskmelon crop until anthesis and then application of organic pesticides until harvest. Additionally, a goal of the research was to provide a new quantitative tool for researchers interested in *Erwinia tracheiphila*. This was accomplished through the development of a

Real-Time PCR assay, specific to *Erwinia tracheiphila*. Finally, additional research was conducted to provide support to the developed field production system through the evaluation of a novel biocontrol agent. Research into biocontrol agents identified a bacterium that will potentially induce plant defense resistance prior to infection with the pathogen, *Erwinia tracheiphila*. This added protection would be available when the muskmelon plant is vulnerable to vector feeding during anthesis and afterward, depending upon the production method selected.

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CHAPTER 2

DEVELOPMENT OF AN ORGANIC MUSKMELON PRODUCTION SYSTEM AGAINST BACTERIAL WILT DISEASE

Revised version:(Caudle, J.R., Coolong, T., Williams, M.A., Vincelli, P. and Bessin, R. 2013. Development of an organic muskmelon production system against bacterial wilt disease. Acta Hort. (ISHS) 1001:249-254.
http://www.actahort.org/books/1001/1001_27.htm)

INTRODUCTION

In the southeast United States, the warm, humid summer climate produces many challenges for vegetable growers, particularly those using organic practices. In particular, the cucurbit family of vegetables is plagued by bacterial wilt disease (*Erwinia tracheiphila*) vectored by stripped and spotted cucumber beetles (*Acalymma vitatta* and *Diabrotica undecimpunctata*) (Watterson, 1971; Rand, 1916). Additionally, the western spotted cucumber beetle (*D. undecimpunctata undecimpunctata* Mannerheim), the banded cucumber beetle (*D. balteata* LeConte) are known to be vectors as well (Rand and Enlows, 1916). Although first identified in the 1890s (Smith, 1911), there are no direct controls for this disease and as a result farmers often experience significant crop damage from its affects (Brust, 1997). Current control practices in conventional systems focus exclusively on eliminating the disease vectors using systemic insecticides such as imidicloprid (Jasinski, et al., 2009).

Spun-bound row covers have been traditionally used in conventional production for non-disease related benefits, such as earliness (Jenni, 1996; Wells and Loy, 1985). Row covers have also been found to protect muskmelon from insect vectored diseases (Perring, et al., 1989). Recently, row covers have been examined in convention melon

production and were found to provide delayed onset of bacterial wilt (Mueller et al., 2006) through the exclusion of insect vectors. Additional research on conventional production found that delaying the removal of the row covers past anthesis, supplying bumble bees for pollination under the row covers or opening the ends of row covers for pollination provided improved production and lower disease incidence (Gaye et al., 1991; Vassiere, 1996). Recent conventional production research has focused on improving row cover efficacy as an alternative to insecticide usage (Saalu-Rojas, et al., 2011).

Organic production research on controlling bacterial wilt has investigated the efficacy of companion crop planting and colored plastic mulches in conjunction with other organic production methods (Cline, 2008). The Kentucky State University research found that the number of trapped striped and spotted cucumber beetles was significantly reduced by the planting of companion plants known to repel vectors with the combination of radish (*Raphanus sativus*), tansy (*Tanacetum vulgare*), and nasturtium (*Tropaeolum* spp.) or to attract beneficial insects utilizing the triple planting combination of buckwheat (*Fagopyrum esculentum*), cowpeas (*Vigna unguiculata*), and sweetclover (*Melilotus officinalis*). Recent studies with row covers in organic systems have evaluated using them until anthesis, when they are typically removed for insect pollination. After removal insecticides are applied to suppress insect vectors until harvest. Although this use of row covers has provided some level of control, they have not been shown to completely protect the crop from bacterial wilt incidence (Mueller et al., 2006).

This research project expands the study of row covers in organic systems by evaluating different timing practices in relation to anthesis and pollination. The objectives of this project were to (1) assess the efficacy of extended duration row cover

application to control bacterial wilt incidence and (2) reduce the use of insecticides in an organically managed muskmelon system.

MATERIALS AND METHODS

Field trials were conducted for three years at the University of Kentucky Horticultural Research Farm in Lexington, Kentucky in 2008, 2009, and 2010. Untreated "Athena" muskmelon seeds (Seedway, LLC; Hall, NY) were planted in 72 cell trays containing organic potting mix. Seedlings were transplanted on June 23, 2008, June 9, 2009, and July 2, 2010. Transplants were transplanted into black plastic mulch raised beds with drip irrigation at a 0.3 m spacing. The raised beds had 1.8 m spacing between the beds and each subplot was 9.1 m long, consisting of three parallel beds with sampling conducted on the middle row. Each treatment was replicated four times in a randomized complete block experimental design. Fertilization was provided by an incorporated hairy vetch/rye cover crop and a granular fertilizer (NatureSafe (13-0-0) (Griffin Industries, LLC, Cold Spring, KY) during bed shaping. Fertigation with Phytamin (6-1-1) (California Organic Fertilizers, Inc., Fresno, CA) was used to bring the total fertilizer application to a recommended 125 lbs N/acre. Immediately after transplanting, treatments were applied, including covering seedlings with polypropylene spun bond row covers (Agribon, Polymer Group Inc., Charlotte, NC), which was supported with wire hoops at a spacing of .3 to 1 meter and edges secured to the ground. Various weights of spun-bound fabric were successfully applied based on what was available and what was suitable for the environmental conditions. The untreated control had no row cover applied.

Over the three growing seasons, various treatments were applied in a completely random manner to the plots to assess row cover efficacy. During the 2008 season, treatments were: (1) Control receiving no row cover or insecticides, (2) No row covers but weekly application of organic insecticide mix (Pyganic, Neem oil and Surround, used for all insecticide applications), (Peaceful Valley Organic Farm and Garden Supply, Grass Valley, CA, USA) (3) Row covers removed at anthesis, followed by weekly organic insecticide applications based upon manufacturer recommended rates until harvest. Anthesis herein is defined as the period during which the female flowers are open. (4) Row covers removed two weeks after anthesis, followed by weekly organic insecticide application until harvest with a single bumblebee hive (Koppert Biological Systems Inc., Romulus, MI) inserted under the row cover for the duration of its use, and (5) Row covers in place throughout the season with a single bumblebee hive inserted under the row cover with no organic insecticide applications. During the 2009 season, the following treatments were applied: (1) Control receiving no row cover or insecticides, (2) Row covers removed at anthesis for two weeks for pollination and then reapplied for the duration of the season; organic insecticide mix was applied while row covers were removed and ladybeetles, *Hippodamia convegens* (BioControl Network, Brentword, TN) were inserted under the covers after reapplication to control aphids as necessary, (3) Row covers applied and ends opened for two weeks at anthesis, with no organic insecticide application, with ladybeetles inserted under the covers to control aphids as necessary, and (4) Row covers maintained throughout season with one, worker bee only, bumblebee hive (*Bombus impatiens*) inserted under the row cover, no organic insecticide application and ladybeetles inserted under the covers to control aphids as

necessary. The 2010 season used the following treatments: (1) Control receiving no row cover or insecticides, (2) Row covers removed at anthesis followed by insecticide application until the end of the season, (3) Row covers removed at anthesis for one week and reapplied until harvest, organic insecticide, same pesticide mix as Treatment 2, applied during removal period, ladybeetles inserted under the covers to control aphids as necessary and, (4) Row covers removed at anthesis for two weeks and then reapplied until harvest, organic insecticides were applied during the removal period, equal quantities of ladybeetles inserted under the covers to control aphids as necessary.

Harvesting of melons was conducted on a weekly basis once marketable melons were produced. Harvesting was conducted over the entire treatment plot of three rows. Marketable melons were considered to be round to oval and had a clear net over the surface of the melon with a yellow, but not browning, skin color under the netting. Cull melons were melons with damage or were unsuitable due to size and conformation. USDA standards were not used due to determine marketable size and quality due to the greater focus on pollination efficiency of the system rather than harvest marketability.

Statistical analysis of the harvest data was conducted on JMP 9, SAS software for MAC. One-way ANOVA analysis was performed on the three seasons' data sets individually.

RESULTS

Analysis of harvest data was performed on combined cull and marketable weights in recognition that pollination effectiveness is an appropriate surrogate for harvest performance.

Irrigation and applications of organic pesticides were applied as needed. Fertilizer application was as recommended at 120 lbs/acre of N for muskmelons from the 2008 UK Extension Vegetable Production Guide for Commercial Growers (Bessin, 2008).

Year 1, tabulation of marketable produce quantities by treatment option is found in Table 1. During the first field season, an aphid infestation severely damaged Treatments 4 and 5. Application of ladybeetles to control the aphids was not effective due to the severity of the damage. Row covers were removed at anthesis where indicated and returned as described per treatment. Statistical comparisons between the treatments found that there was no significant difference between treatments 1, 2, and 3 ($P \geq .71$) and between treatments 4 and 5 ($P = 0.98$). However, there was significant difference between treatments 1,2,3 and 4, and 5 ($P \leq 0.002$). Treatments 1, 2 and 3 were valuable as baseline data for subsequent years' treatments. Figure 1 illustrates the total harvest weights with standard error bars.

The potential for bacterial wilt in the 2008 crop was gauged by collecting striped and spotted cucumber beetles on yellow sticky cards under and outside row covers. Table 2 depicts the beetle counts during this season. These numbers represent relatively low cucumber beetles in the project area considering that sites in other counties of the state experienced high beetle counts. However, bacterial wilt was experienced in the study plot during the season.

Year 2 tabulation of the harvest data can be found in Table 3. Production skills improved during this season, leading to negligible culls. Row covers were removed at

anthesis where indicated and returned as described per treatment. Statistical analysis of the harvest data found that there were significant differences between treatments 1, 4 and 2, 3 ($P \leq 0.0085$). There was no significant difference between treatments 1,4 ($P = 0.77$) and treatments 2,3 ($P = 0.59$). Figure 2 illustrates the total harvest weights. Table 3 tabulates the beetle counts during the season. Insect counts were higher in the 2009 season, which was reflected by lower productivity in the untreated control, Treatment 1.

Year 3 tabulation of the harvest data can be found in Table 5. Late planting and high temperature stress conditions taxed the plants leading to lower overall quality, however, by focusing on developed fruit, harvest data comparable to previous season data was collected. Row covers were removed at anthesis where indicated and returned as described per treatment. Statistical analysis of the harvest data found that there was no significant difference between Treatments 1, 2 and 4 ($P \geq 0.06$) and between Treatments 1 and 3 ($P=0.50$). However there was significant difference between Treatments 3 and 4 ($P=0.018$) and 2 and 3 ($P=0.025$). Table 6 tabulates the beetle counts for the season. Beetle counts were slightly higher than Year 1 and lower than Year 2, however, once again, bacterial wilt symptoms were in evidence in the study plot. Reduced productivity in the control plot illustrated the affect of the cucumber beetle presence and bacterial wilt.

DISCUSSION/CONCLUSIONS

This experiment analyzed several different combinations of row cover timing and insecticide application. After three seasons, it was found that treatments utilizing row covers until anthesis, followed by a two-week removal for pollination and then

reapplication of the covers, had productivity equal to the typical organic practice of removing the covers at anthesis and then spraying insecticides for the duration of the season.

Row covers have been previously documented to be an effective exclusion technique for the bacterial wilt vector (Mueller, 2006). Extended application of the row covers after flowering provided additional protection from late season transmission of bacterial wilt beyond what is typically employed in organic melon production. Additionally row cover exclusion produced comparable production results to the typical organic treatment. Cessation of pesticide application on the extended duration treatments during the final weeks of melon ripening reduced chemical costs by omitting 2-3 insecticide sprays. This could be a significant cost reduction depending upon the size of the planting.

Complete exclusion from the vector and applying bumble bees under the row covers for pollination was not effective over two seasons where lack of pollination significantly reduced productivity. One season of comparison of one week versus two weeks of removing the row covers for natural pollination demonstrated that two weeks produced comparable pollination with the typical organic treatment that remained uncovered for the remainder of the season.

Bacterial wilt in organic production of cucurbits susceptible to the disease has continued to experience loss of production despite the production methods applied including systemic insecticides, limited duration row covering and contact insecticides. This research demonstrated that extending the duration of row covers after anthesis to the end of the season provided additional protection from the insect vectors with the

application of the row covers as well as reduced costs associated with continued application of insecticides by the cessation of the application of insecticides compared to the typical organic production practice. Two weeks of exposure to natural pollinators with periodic application of contact insecticides provided comparable production during a relatively low pathogen pressure situation. It is anticipated that higher pest pressure would reduce typical organic production results while continuing to provide protection for the extended duration cover treatment of the cucurbit crop.

Treatment #	Average Cull Weight, kg	Average Marketable Weight, kg
1	29.7	35.9
2	22.5	43.5
3	13.4	47.4
4	8.6	2.1
5	5.1	0.54

Table 2.1 2008 Melon Production by Treatment Option

Treatment	7-25-08	8-7-08	8-20-08	9-12-08
1	9.3(0.6)	5.0(0.25)	11.75(0.0)	2.25(0.0)
2	8.0(0.33)	6.0(1.0)	11.5(0.0)	8.75(0.25)
3	0.5(0.0)	1.75(0.25)	1.5(0.0)	2.0(0.0)
4	0.0(0.0)	0.0(0.0)	4.5(0.0)	3.0(0.25
5	0.0(0.0)	0.0(0.0)	0.0(0.0)	0.25(0.0)

Table 2.2 2008 Insect Counts by Treatment Plot

The first number in the column is the average Striped Cucumber Beetle Count per Treatment and the number in parentheses is the average Spotted Cucumber Beetle Count Treatment. The action threshold for IPM control of Bacterial Wilt is 2.0 beetles per 25% of plants. Based on this criterion, the action threshold was exceeded during all reporting periods.

Treatment #	Average Total Marketable Weight, kg
1	14.2
2	54.5
3	48.5
4	11.0

Table 2.3 2009 Melon Production by Treatment Option

Treatment	6/18/09	6/26/09	7/3/09	7/10/09	7/15/09	7/17/09	7/24/09	7/31/09
1	6(1)	47.5(2.0)	19.5(2.0)	25.75(4.0)	NA	60.0(2.0)	39.0(3.25)	65.5(2.25)
2	NA	NA	NA	10.25(0.25)	15.25(0.25)	NA	NA	NA

Table 2.4 2009 Insect Counts by Treatment Plot
The first number in the column is the average Striped Cucumber Beetle Count per Treatment and the number in parentheses is the average Spotted Cucumber Beetle Count Treatment. The action threshold for IPM control of Bacterial Wilt is 2.0 beetles per 25% of plants. Based on this criterion, the action threshold was exceeded during all reporting periods. NA is data not available.

Treatment #	Average Cull Weight, kg	Average Total Marketable Weight, kg
1	17.1	19.5
2	24.2	16.0
3	18.6	12.3
4	27.8	9.2

Table 2.5 2010 Melon Production by Treatment Option

Treatment	7/9/10 Count	7/15/10 Count	7/23/10 Count	7/30/10 Count	8/6/10 Count	8/20/10 Count	8/28/10 Count
1	4.25(2.0)	50.25(2.0)	16.0(0.25)	12.5(3.25)	27.75(1.25)	45.75(4.0)	45.0(5.5)

Table 2.6 Year 3 Insect Count

The first number in the column is the average Striped Cucumber Beetle Count per Treatment and the number in parentheses is the average Spotted Cucumber Beetle Count Treatment. The action threshold for IPM control of bacterial wilt is 2.0 beetles per 25% of plants. Based on this criterion, the action threshold was exceeded during all reporting periods.

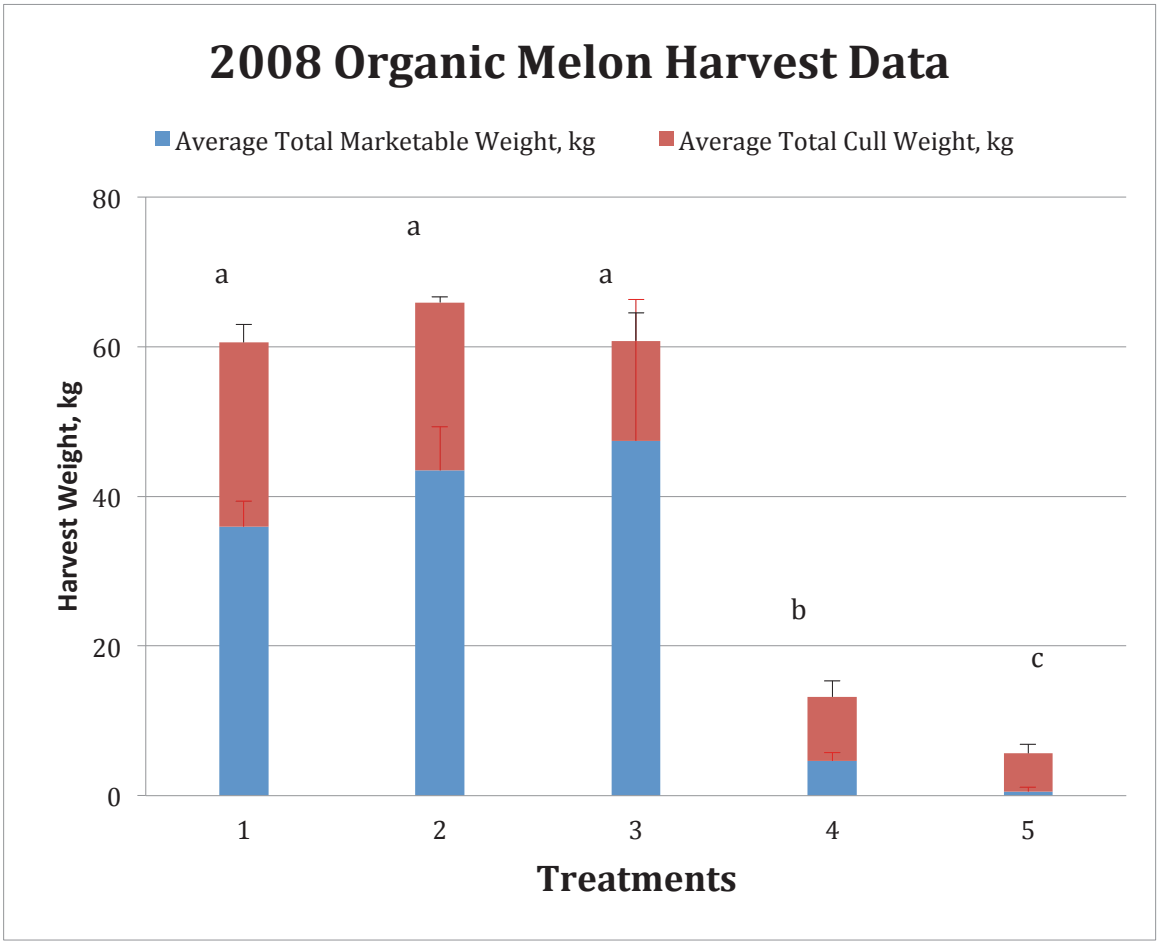

Figure 2.1 2008 Harvest Data by Treatment Option
Treatment 1– Control, no treatment
Treatment 2 – Organic insecticides only
Treatment 3 – Covers off after anthesis, insecticide sprayed after uncovering
Treatment 4 – Covers off for two weeks, insecticides sprayed while uncovered, covers replaced until harvest
Treatment 5 – Row covers throughout season, bumblebees under covers.
Statistical significance noted with letters indicating a significant difference between treatments of the same letter. Comparisons between Treatments1, 2, 3 and 4 and 1,2,3 and 5 were significant at $P \leq 0.05$.

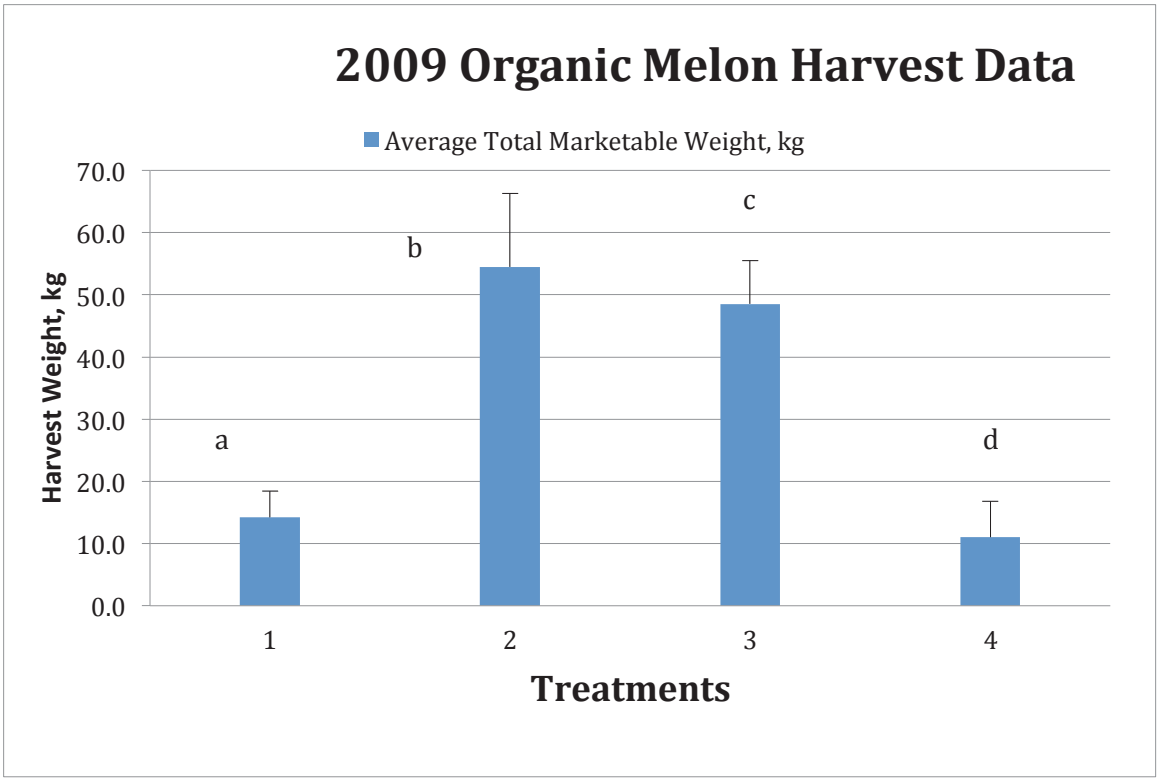

Figure 2.2 2009 Harvest Data by Treatment Option
Treatment 1 – Control, no treatment
Treatment 2 – Row covers until anthesis, covers off for two weeks, insecticides sprayed while uncovered, row covers returned until harvest.
Treatment 3 – Row covers until anthesis, end of row covers uncovered for two weeks, covers returned until harvest
Treatment 4 – Row covers for entire season, bumblebees under covers for season.
Statistical significance noted with letters indicating a significant difference between treatments of the same letter. Comparisons between Treatments1 and 4 and between 2 and 3 were significant at $P \leq 0.05$.

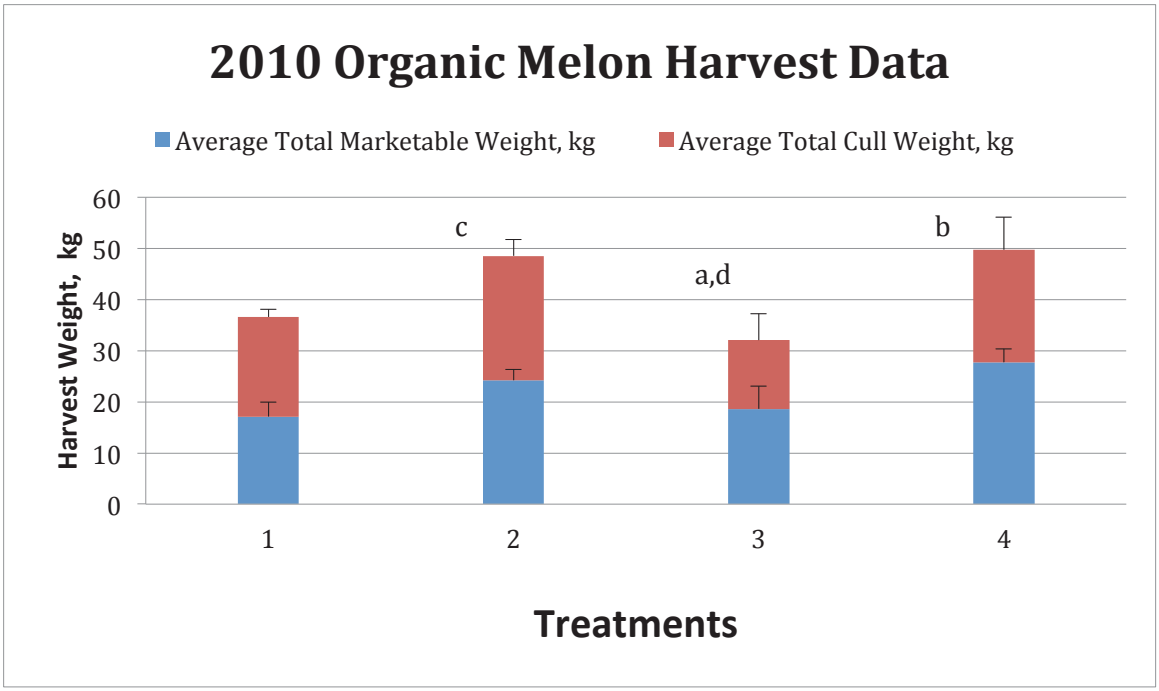

Figure 2.3 2010 Harvest Data by Treatment Option
Treatment 1 – Control, no treatment
Treatment 2 - Row covers at anthesis, covers off for rest of season, insecticides sprayed while uncovered.
Treatment 3 – Row covers until anthesis, covers off for one week, insecticides sprayed while uncovered, covers returned until harvest.
Treatment 4 - Row covers until anthesis, covers off for two weeks, insecticides sprayed while uncovered, covers returned until harvest.
Statistical significance noted with letters indicating a significant difference between treatments of the same letter. Comparisons between Treatments 3 and 4 and between 2 and 3 were significant at $P \leq 0.05$.

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CHAPTER 3

DEVELOPMENT OF A SELECTIVE REAL-TIME PCR ASSAY FOR *ERWINIA TRACHEIPHILA*

INTRODUCTION

Erwin F. Smith first named *Erwinia tracheiphila* in 1895 as *Bacillus tracheiphilus,* a bacterial pathogen that causes bacterial wilt of cucurbits (Ainsworth, 1981). The *Erwinia* nomenclature appears to be a posthumous acknowledgement of Dr. Smith, whereas tracheiphilus is a combination of tracheid and philus indicating an organism attracted to the tracheid for food or habitat. *Bacillus tracheiphilus* was subsequently revised to *Bacterium tracheiphilus,* then *Erwinia tracheiphila*, and *Erwinia amylovora var. tracheiphila*, and most recently *Erwinia tracheiphila* emend. (Smith, 1895, Bergey et al 1929, Dye 1968, Hauben et al., 1998). *E. tracheiphila* is a member of the Enterobacteriaceae family of gram-negative bacteria. (Zitter, 1997).

E. tracheiphila is a rod-shaped bacillus with peritrichous flagella. It is facultatively anaerobic, chemoorganotrophic and prefers temperatures of 27-30C (Zitter, 1997). *E. tracheiphila* infection in cucurbits is characterized by wilting of the leaves and subsequent death of the plant. The presence of vascular exudate with milky, sticky qualities is a common symptom identifier for the bacterial infection (Smith, 1911, Zitter, 1997). A field assay for the disease consists of cutting the stem, reconnecting the parts and slowly pulling the stem parts apart to reveal the characteristic sticky bacterial ooze (Prend and John, 1961; Smith 1895). *E. tracheiphila* is currently found in North America, Europe, South Africa, Japan and Korea. Additional qualities of *E. tracheiphila* can be as found in the Korean online resource (Plant Bacteria Culture Collection, 2013).

Dr. Smith first documented that *E. tracheiphila* infection causes bacterial wilt of cucurbits and that the bacteria were vectored by striped cucumber beetle, *Diabrotica vittata*, later renamed *Acalymma vittatum* (Fabricius) (Smith, 1911, Rand, 1915). It was Rand's research that first documented that *E. tracheiphila* was overwintered by a striped cucumber beetle vector where he observed infection and wilting of a cucurbit (Rand, 1915). Subsequent research identified spotted cucumber beetles, *Diabrotica undecimpunctata howardi* Barber, as an additional vector (Rand and Enlows, 1916). Research also found that common wild-type herbaceous non-cucurbit plants were not significant reservoirs of *E. tracheiphila* through the winter (de Mackiewicz, 1998). From early investigations, it was recognized that the pathogen was likely found in the mouthparts of cucumber beetles, however, recent research also identified *E. tracheiphila* in the frass of the vectors (Mitchell and Hanks, 2009, Smith, 1911). In addition to transmission via wounding on leaves and stems, recent research also found transmission of the pathogen through host plant flower parts (Sasu et al., 2010).

Various laboratory methods of introduction of the pathogen to a host plant have been documented, including syringe introduction into the vascular system, leaf rubbing, multiple prick artist airbrush, pinch and needle puncture (Reed, 1982; Watterson et al., 1971). Once the pathogen is inside the plant, progression of the symptoms occur at varying rates, however the symptoms are similar: appearance of a flaccid dull green area around the area of inoculation, with progression of wilting in the inoculated leaf, ultimately resulting in wilting of leaves further down the stem until the plant dies (Main and Walker, 1971, Watterson et al., 1971). Transpiration rate in the infected plant continues to increase for a limited number of days until it drops off to zero as the plant

dies (Main, 1970). Bacterial plugs are observed in stem and petiole xylem as the associated leaf wilts (Bacterial, 2014).

The significance of the impact of *Erwinia tracheiphila* was documented by research testing over 100 varieties of muskmelon for susceptibility to the pathogen. None of the commercially grown cultivars tested were resistant to the pathogen (Reed and Stevenson, 1985). In an organic production environment, tools available to manage the pathogen are limited to physical means and Organic Materials Review Institute (OMRI) certified pesticides. Application of OMRI pesticides can be detrimental to pollinators, thereby interfering with pollination during anthesis (Vanbergen, 2013). Antibiotic sprays have been found to provide control of *Erwinia tracheiphila*, however these sprays are not available for organic or conventional production application (Williams and Lockwood, 1956). Other research has documented the speed at which an *Erwinia tracheiphila* infection can spread within the plant. Findings indicate that initial wilting of leaves happens within two days of infection and vascular deterioration was witnessed after six days (Main and Walker, 1971). Severity of crop infection varies seasonally, however it has caused near complete crop failure (Rand and Enlows, 1920; Sherf, 1986).

Methods of detection of the pathogen include the visual field symptoms described earlier. Higher level diagnostic signs of *E. tracheiphila* infection include visual microscopic visualization with bacterial culture identifier tests. Additional tests used to identify *E. tracheiphila* include an ELISA test developed by Garcia-Salazar (Garcia-Salazar et al., 2000), conventional PCR tests (Bruton et al., 1999, Dallaire, 2009, Waleron et al., 2002), and most recently a SYBRGreen Real-Time PCR test (Sasu et al., 2010). The assays that have been prepared to date are not specific to *Erwinia*

tracheiphila because they have not been tested against associated pathogens that could be present in the plant tissue sample. False positive or false negatives can occur where specificity testing has not been thoroughly conducted (Lou, 2011). Further, the typical size of the amplicon, or template, produced by previous researchers is larger than template sizes recommended for use with quasi-quantitative SYBR Green Real-Time PCR testing. Small template size increases speed and efficiency of the Real-Time PCR thermocycling process. This research describes the development of a TaqMan Real-Time PCR screen. The TaqMan probe screen increases specificity of the PCR testing so that random and non-target DNA fragments are not quantified during the Real-Time PCR process, thereby providing a significantly more accurate quantitative assay.

METHODS AND MATERIALS

Bacterial Isolates

Fourteen *E. tracheiphila* isolates were obtained from the laboratory of Dr. Mark Gleason in the Department of Plant Pathology, Iowa State University. These *E. tracheiphila* isolates were collected from various locations in the state of Iowa as well as Kentucky. Additional pathogens, which are either commonly associated with *Cucumis melo* in field production situations or are closely phylogenetically related to *Erwinia tracheiphila*, were obtained from various laboratory sources for assay specificity testing. These pathogens included both bacterial and fungal pathogens to *Cucumis melo* and the sources are listed in Table 3.1.

Plant Material

Untreated seeds of the muskmelon (*Cucumis melo*) 'Athena' were obtained from SeedWay, LLC., Hall, New York 14463. Seedlings were raised in a commercially obtained organically certified Sunshine grow mix (SunGro Horticulture, Agawam, Mass.). The plants were grown in a laboratory setting with metal halide lamps located approximately 1 foot above the plants throughout the testing cycle. Ambient room temperature of approximately 22.2 degrees C was maintained with a 14 hours on, 10 hours off cycle per day. Plants were typically grown up to the anthesis stage to mirror field conditions when exposure to vectors occurs in a typical organic production system.

Bacterial Inoculation

Erwinia tracheiphila SCR-3 Rif was grown on sterile culture plates containing 75 ug/ml of Rifampicin. The bacteria was harvested from the plates and resuspended in 10 mM PBS solution. The Optical Density of the inoculum was diluted to an average of 0.7 OD based on NanoDrop readings at 640 nm. Inoculation of the *Cucumis melo* plants occurred at different times depending upon the test conducted, however the manner of inoculation was consistent. A 50-ul aliquot of the inoculum was placed on a leaf randomly on the surface of the leaf and then a multi-pin frog was inserted into the leaf through the inoculum drop on the leaf. Numerous holes (up to 50) were punctured into the leaf to allow for bacterial transmission into the leaf (protocol obtained from Erika Saalau-Rojas, Iowa State University, Plant Pathology Department, Ames Iowa, USA).

DNA extraction

Bacteria and fungi in this study were grown on various media as appropriate to the specific pathogen. Media recommendations were obtained from the American Type Culture Collection online records for various pathogens. The culture media for *E. tracheiphila* was recommended by Iowa State University as the following: 23 g of Nutrient Agar (Difco Laboratories Inc., Detroit, Michigan), 5 g of Bacto Agar (Difco Laboratories, Inc.), 5 g of Bacto Peptone (Becton, Dickinson and Company, Sparks, Maryland), 1 liter dd Milliq H_2O. DNA extraction, when utilized, was performed using the CTAB extraction protocol (Lopez et al., 2003). The concentration of the bacterial culture was measured with spectrophotometers (NanoDrop2000 and BioMate3, Thermo Fisher Scientific, Inc.). The optical density was recorded at 640 nm and its density adjusted for serial dilution testing with the Real-Time PCR assay.

In addition to pure bacterial cultures, which were sampled directly from the culture plate or media, a bacterial culture was collected from infected plants. Slices of infected plant tissue were collected using sterile technique. The tissue was placed in a sterile culture tube along with sterile Milliq water and the bacteria was allowed to stream out of the tissue for a limited amount of time of approximately 20 minutes. Direct aliquots of the liquid from the combined plant tissue material and ultra pure water were collected and added into the Real-Time PCR reaction tube. Also, crushing of the plant material in the mini-tube using a micro pestle was performed as an alternative to direct streaming of bacteria into the ultra-pure water. See the PCR Protocol section for more details as to the method of preparation of the sample for analysis.

Sequencing and PCR primers design

Previous research utilized an *Erwinia tracheiphila* strain ICMP 5845 carbamoylphosphatase synthetase small subunit (*carA*) gene GenBank # DQ859839, for primer development (Mitchell and Hanks,2009). From the GenBank information, the FASTA record of the gene was inserted into Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) in order to obtain an amplicon of less than 200 base pairs that would be appropriate for Real-Time PCR analysis. Several primer sets of various base pair sizes were designed and tested. An amplicon was selected to place the separation between the amplicon and the lowest region of the gel electrophoresis lane so as not to confuse primer/dimers with the amplicon on a visual observation of a gel electrophoresis. The primer sequence that met the requirements is listed in Table 3.2 and the nucleotide sequence from which the assay was developed is listed in Table 3.3.. The TaqMan probe was produced with the FAM/3 -BHQ-1 fluorescing dye label.

These primers and probe were obtained from Elim Biopharmaceuticals, Hayward, California. The calculated annealing temperature for these primers was 60°C and the anticipated length of the amplicon was 161 base pairs. A veriflex analysis was performed on the primer pair using an Applied Biosystems Veriti Thermocycler and the best annealing temperature for the primer set was 52ºC. The primer pair was tested on numerous *E. tracheiphila* strains, (See Table 3.1 for *E. tracheiphila* isolates).

PCR Protocol

Previous PCR screens developed on diverse segments of the *E. tracheiphila* genome have been 710, 700, 529, and 426 base pairs (bp) long (Bruton et al., 1999; Dallaire, 2009; Minsavage et al., 1994; Waleron et al., 2002, respectively). Using Primer3Plus, the carbamoylphosphatase synthetase gene (GenBank Accession No. DQ859839.1) was used to select a primer–probe set. To increase the efficiency of the Real-Time amplification process, a shorter amplicon was developed that was 161 bp long.

PCR and RT-PCR assay conditions in E. tracheiphila detection

The PCR reactions consisted of 1 ul of DNA of various dilutions or bacterial cell culture template quantified on the NanoDrop spectrophotometer, (Thermo Scientific), 5 ul of 10X Taq Buffer with KCL, 3 ul of 25mM $MgCl_2$, 0.5 ul 10 uM forward primer and 0.5 ul 10 uM reverse primer, 0.5 ul of 10mM dNTP, and 0.1 ul Taq polymerase (Fermentas, Glen Burnie, Maryland). When the probe was used in the reaction, 0.5 ul of 2.5 uM probe was added into the reaction. The amplification protocol for these runs was as follows: 5 minutes at 95°C denaturing, followed by 35-40 cycles of 95°, 52°, 72°C and then a final 5 minute extension of 72°C for the PCR reactions. For a SYBRGreen assay, the Fast SYBRGreen MasterMix (Applied Biosystems) was used following the protocol that accompanies the materials.

Specificity in E. tracheiphila detection

The specificity of the PCR assay was checked by amplification of 14 *E. tracheiphila* isolates and 31 pathogens related or associated with *E. tracheiphila.* All the

E. tracheiphila isolates were positively amplified and the 31 pathogens failed to amplify, with appropriate actin housekeeping genes for bacterial or fungal DNA identification co-processed for verification of DNA presence and production of sequencing amplicons. (fungal: ITS1 & 4(~400 bp), bacterial: 8F & 357R(349 bp), 16S927F & 16S1492R (565 bp). By providing the housekeeping marker test along with the *E. tracheiphila* assay, the target pathogen tested for cross-reaction is also positively identified as present in the sample and therefore a negative response to the *E. tracheiphila* will not be because the target DNA is not present. Amplicon sequencing was performed by Elim Biopharmaceuticals. Specificity-tested pathogens and *E. tracheiphila* sequences were Blast searched against the GenBank database for verification.

Sensitivity in E. tracheiphila detection

The sensitivity of the PCR assay was determined by serial dilution of *E. tracheiphila* DNA from 100 pg/ul to 1 fg/ul and bacterial cultures from 10^6 to 10^4 CFU/ml. DNA serial dilutions were prepared in Milliq H_2O. Bacterial culture was prepared from 3-day old *E. tracheiphila* isolate Rif SCR3 in serial dilutions of dd Milliq H_2O. These serial dilutions were used directly in the PCR reactions. Additional testing was conducted with *Cucumis melo* tissue infected with *E. tracheiphila* to determine if direct and rapid testing could be conducted. For this study, the PCR was performed in quadruplicate.

RESULTS

Real-Time PCR specificity assay

The specificity of this assay was determined based on positive or negative response to the *Erwinia tracheiphila* Real-Time PCR assay applied to the extracted DNA from the listed pathogens in Table 3.1. The *E. tracheiphila* primers described in this study positively identified all the *E. tracheiphila* strains with a 161 bp amplicon. The 31 related or associated (commonly found to infect host plant) bacterial and fungal DNA did not amplify. The bacterial and fungal isolates were selected from both phylogenetically related bacteria, as well as pathogens, both bacterial and fungal, that are commonly found in the environment and in the host, *Cucumis melo*. The E. tracheiphila assay product or amplicon was sequenced by Elim Biopharmaceutical, Inc. and was verified to be the target assay.

Real-Time PCR sensitivity assay

Testing was conducted on DNA, cell culture suspensions and plant tissue samples infected with *E. tracheiphila*. All testing could be conducted on extracted DNA, however, for a more rapid analysis, focus was placed on utilizing intact bacterial cells rather than adding the additional extraction step where possible. It is possible to test intact bacteria because the cell wall and various organelle membranes easily disassemble at the temperatures of the PCR reactions leaving the DNA and RNA structures free to interact with the polymerase enzymes. The primer set produced an amplicon of 161 bp shown in Figure 3.2 in the center well. In the figure, comparison is made to the Bioline Hyperladder V DNA ladder (Bioline USA Inc., Taunton, Massachusetts) with bold bands

at 100 and 200 bp on the left and with a previously published *Arabidopsis thaliana* mutant marker ciw6 at 162 bp in length on the right (Lukowitz et al., 2000). Application of the Real-Time PCR assay to bacteria extracted from the plant tissue was successful with no interference from inhibitors after one serial dilution of the original dilution solution.

Mean threshold cycle (Ct) values for each Real-Time PCR test against DNA and bacterial suspension dilution series are listed in Table 3.4. The equation of the log CFU/ml versus the Ct values obtained was $y = -4.1786x + 34.672$ with an R^2 of 0.9989. The equation for the log fg/ul versus the Ct values obtained was $y = -2.7685x + 21.439$ with an R^2 value of 0.9213. See Figure 3.1 A & B. Also, direct testing of diluted plant tissue infected with E. tracheiphila was successfully demonstrated and the ability to detect E. tracheiphila was not inhibited by the presence of plant DNA.

DISCUSSION

This study describes the development of a Real-Time PCR detection assay for *E. tracheiphila*. Whereas previous researchers have designed conventional PCR assays, this research provides a 161 bp Real-Time PCR tool that increases quantitative abilities for future researchers. Additional diagnostic specificity was obtained by testing this assay against closely related and associated pathogens.

The *E. tracheiphila* assay utilizes a small sub-unit of the carbamoylphosphatase synthetase gene that is a common precursor in the arginine and pyrimidine pathways (Piette et al., 1984; Reed, 1982). Although the gene function is found in a wide diversity

of organisms, this study found that the sequence used for this assay is unique for *E. tracheiphila* among closely related and associated pathogens (Lawson et al., 1996). In this study, evaluation for specificity of 14 strains of *E. tracheiphila* and 31 strains of closely related and associated pathogens was conducted. The results demonstrate that the assay is an effective identifier of *E. tracheiphila* while at the same time the primers do not cross-react with potential pathogens often found in association with *E. tracheiphila* in the host plant. Also, testing documented that the assay can be conducted on bacteria DNA, cells, and mixed plant tissue samples.

The Real-Time PCR assay was shown to be sensitive down to 1 fg/ul of DNA in solution. The $R^2 > 0.92$ supports the reliability of the results. Further, the cell culture suspension detected as many as 10,000 CFU/ul with an R^2 of >0.99 which strongly supports the reliability of the results. These results vary between assays and sampling conditions, however, the results are comparable to similar published Real-Time PCR assays (Lou, 2011, Cooling et al., 2008)

In this study, *E. tracheiphila* testing was conducted on *Cucumis melo* tissue infected with *E. tracheiphila* after one serial dilution without DNA extraction. This provides for a rapid assay for field identification of *E. tracheiphila*.

Table 3.1: Bacterial strains associated or phylogenetically related to *Erwinia tracheiphila* used in this study and sources.

Pathogen Identification	**Researcher, Institution**	**[1]PCR amplification**
Acidovorax avenae, 30071	Dr. Norm Schaad, USDA-ARS Fort Detrick, Maryland	-
Acidovorax avenae subsp. Citrulli, 30080	Dr. Norm Schaad, USDA-ARS Fort Detrick, Maryland	-
Alternaria alternate, EGS35-193	Dr. Peever, Washington State University	-
Alternaria cucumarina, AC1	Dr. Tony Keinath, Clemson University, CREC	-
Alternaria cucumarina, AC2	Dr. Tony Keinath, Clemson University, CREC	-
Alternaria cucumarina, AC3	Dr. Tony Keinath, Clemson University, CREC	-
Alternaria cucumarina, AC4	Dr. Tony Keinath, Clemson University, CREC	-
Colletotrichum obiculare, 24-050025	Dr. Bruton, USDA-ARS Lane, Oklahoma	-
Colletotrichum obiculare, 28-090045	Dr. Bruton, USDA-ARS, Lane, Oklahoma	-
Erwinia amylovora, CU 0273	Dr. Steven Beer, Cornell University	-

Table 3.1 (continued)

Erwinia quercina, 40280	Aaron Sechler, USDA-ARS, FDWSRU	-
Erwinia rhapondici, CU 3652	Dr. Steven Beer, Cornell University	-
Erwinia salicis, 40288	Aaron Sechler, USDA-ARS, FDWSRU	-
Erwinia salicis, 40289	Aaron Sechler, USDA-ARS, FDWSRU	-
Erwinia tracheiphila, isolates: FishCu 3-1, McM2-4, MCA1-1, GHM3-1, HCU1-4, Mcal-1, GHM2-1a, FCu1-8, FCu1-4, UnisCu1-1, ZimMusk, SCR3, Rif SCR3, KYMusk	Dr. Mark Gleason, Iowa State University	+
Fusarium oxysporum melonis, 0600002B	Dr. Bruton, USDA-ARS Lane, Oklahoma	-
Fusarium oxysporum melonis, 0600001E	Dr. Bruton, USDA-ARS Lane, Oklahoma	-
Macrophomina phaseolina, 47-090001	Dr. Bruton, USDA-ARS Lane, Oklahoma	-
Macrophomina phaseolina, 47-090008	Dr. Bruton, USDA-ARS Lane, Oklahoma	-

Table 3.1 (continued)

Pectobacterium carotovora, ECC71	Dr. Blackwell, University of Wisconsin, Chemistry Department	-
Pseudomonas syringae p.v. lachrmans, 20213	Aaron Sechler, USDA-ARS, FDWSRU Fort Detrick, MD	-
Pseudomonas viridiflava, 20131	Aaron Sechler, USDA-ARS, FDWSRU Fort Detrick, MD	-
Rhizoctonia solani, 46-070106	Dr. Bruton, USDA-ARS Lane, Oklahoma	-
Rhizoctonia solani, 27-050013E	Dr. Bruton, USDA-ARS Lane, Oklahoma	-
Serratia marcescens, 01B102-C	Dr. Bruton, USDA-ARS Lane, Oklahoma	-
Serratia marcescens, 03B336-2	Dr. Bruton, USDA-ARS Lane, Oklahoma	-
Serratia marcescens, 02B313	Dr. Bruton, USDA-ARS Lane, Oklahoma	-
Serratia marcescens, 02B327	Dr. Bruton, USDA-ARS Lane, Oklahoma	-
Xanthomonas campestris, 10434	Dr. Norm Schaad, USDA-ARS, Fort Detrick, Maryland	-

Table 3.1 (continued)

Xanthomonas cucurbitae, 10536	Aaron Sechler, USDA-ARS, FDWSRU Fort Detrick, Maryland	-
Xanthomonas vesicatoria, 11611	Dr. Norm Schaad, USDA-ARS Fort Detrick, Maryland	-

[1]The PCR amplification "-" indicates whether the bacterial or fungal pathogen cross reacted with the Real Time PCR assay developed in this study.

Table 3.2: Real Time PCR Assay nucleotide sequences for detection of *Erwinia tracheiphila* with the polymerase chain reaction.

Primer Name	Sequence (5'-3')	Length (nucleotides)	Amplicon length (bp)
Forward	GGACGGCGTATTTCTTTCAA	20	161
Reverse	TCATCTTGACCGTTTTTGCTC	21	…
Taqman Probe	CAGCTGCTGGCACTCGCCAG	20	…

Table 3.3 Basis of Nucleotide Sequence for the *Erwinia tracheiphila* assay.

GenBank Ascession: DQ859839.1 *Erwinia tracheiphila* strain ICMP 5845 carbamoylphosphate synthetase small subunit (carA) gene, partial cds
http://www.ncbi.nlm.nih.gov/nuccore/DQ859839

1 ttcacggtcg agccattggg gctttaggtt cggcagtggg ggaagtggtt ttcaacacgt
61 caatgaccgg ttatcaagaa atcctcacag acccttccta ttcccgccag attgtcaccc
121 tcacttatcc ccatatcggc aatgtcggca ccaattccgc agatcaagaa tcctctcagg
181 tccatgcaca agggctgatt attcgtgacc tgccgctgat aaccagcaac ttccgcagtg
241 aagaagggct gtctgcttat ctggaacgcc acaacatcgt tgctattgct gatattgata
301 cccgtaaact cacgcgtttg ctgcgcgaga aaggcgcaca gaacggctgc attattgccg
361 gagatgcccc gaatgcggcg cttgcactgc agcaggcaca ggcatttcct ggccttaaag
421 ggatggatct ggcaaaagaa gtgaccacca gcgaaaccta tagctggttg cagggcagct
481 ggcagctgga aggcctgcct gcccctaaaa atgaagacgg gcagtctttt catgtggtag
541 cttacgacta cggcgttaag cgtaacatcc tgcgtatgct ggtggaccgt ggctgccgac
601 tgacggttgt tcctgcgcaa accccggcag aagaagtctt caagctcaat ccggacggcg
661 tatttctttc aaacggtccg ggagacccgg aaccctgtga ttatgccatc acggctatcc
721 agaaattgtt ggaaactgac gttcccgtgt ttggtatttg tctggggcat cagctgctgg
781 cactcgccag cggagcaaaa acggtcaaga tgaagctcgg ccatcacggt ggtaatcatc
841 cggtaaaaga cctggataat aatacggtga tgatcaccgc acaaaaccac ggttttgccg
901 tcgatgaccg taatttacct gcaaatctgc gcgtgacgca tacctccttg tttgaccata
961 cggtgcaagg tatccaccgt

Assay Target Product: nucleotides 653 to 813

ccggacggcg
661 tatttctttc aaacggtccg ggagacccgg aaccctgtga ttatgccatc acggctatcc
721 agaaattgtt ggaaactgac gttcccgtgt ttggtatttg tctggggcat cagctgctgg
781 cactcgccag cggagcaaaa acggtcaaga tga

Table 3.4: *E. tracheiphila* DNA concentration, bacterial dilution series and mean threshold cycle (Ct) values generated during Real-Time polymerase chain reaction (PCR) assays.

E. tracheiphila cell and DNA concentration	Ct value[a]
10^6 CFU/ml	+(13.86 ± 0.15)
10^5 CFU/ml	+(17.80 ± 0.15)
10^4 CFU/ml	+(22.25 ± 0.15)
100 pg/ul	+(8.92 ± 0.24)
1 pg/ul	+(10.88 ± 0.43)
1 fg/ul	+(21.90 ± 0.47)
ddH_2O control	-(No Ct)

[a] Ct: PCR cycle number (in parentheses) at which fluorescence is first detected during a 40-cycle PCR; + and - = positive and negative reactions respectively.

Figure 3.1: Determination of Real-Time PCR amplification efficiency of the assay. R^2 are reported on the graph.

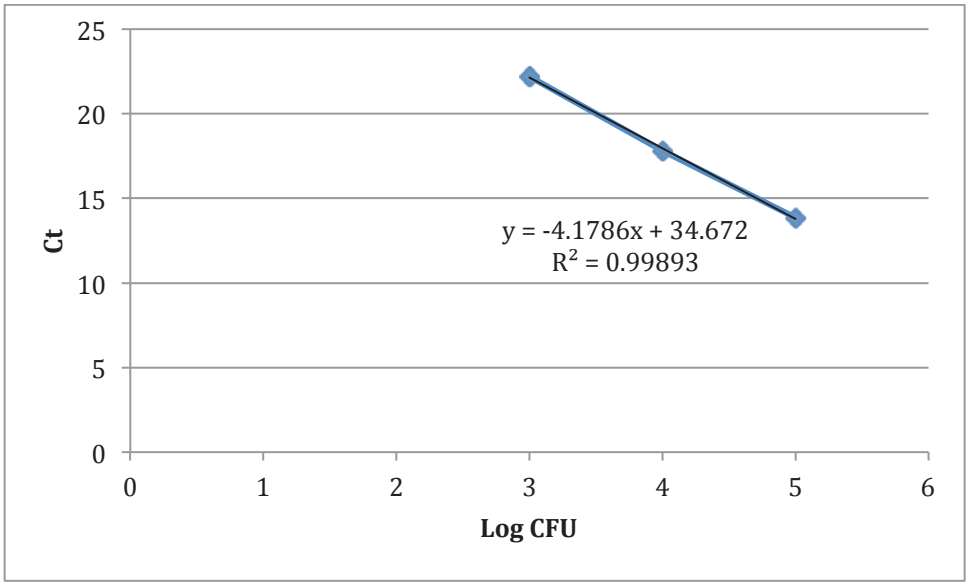

Figure 3.2 Determination of Real-Time PCR detection limits of *E. tracheiphila* DNA. R^2 are reported on the graph. Cycle threshold (Ct) is defined as the number of cycles required for the fluorescent signal to cross the threshold. For amplification efficiency testing, Ct values are the average of five replicates.

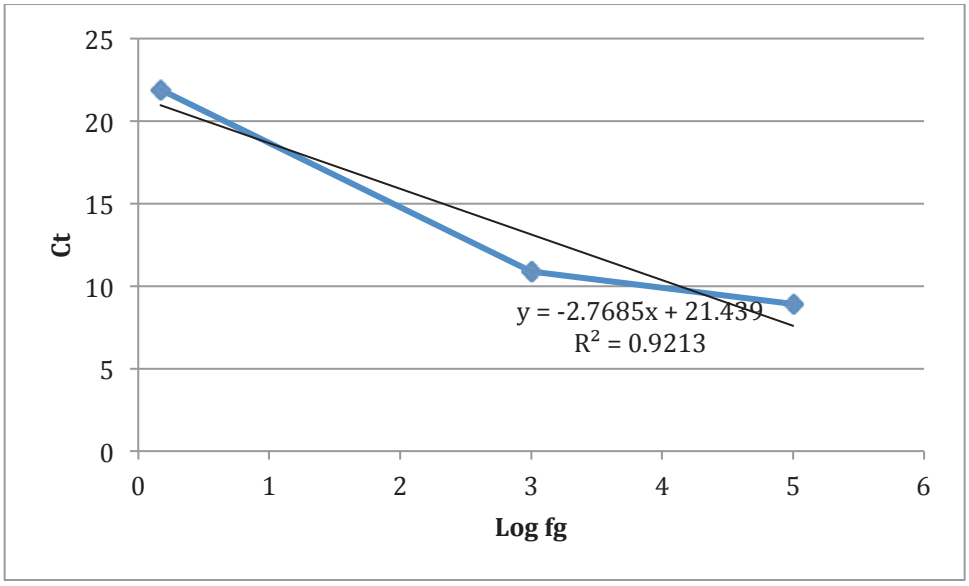

Figure 3.3: DNA amplification using the Real-Time PCR primer set. Lane 1 is Bioline HyperLadder™ V 25-bp DNA ladder, Lane 2 is *Erwinia tracheiphila* isolate Rif SCR3 amplicon at 161 bp, Lane 3 is *Arabidopsis thaliana* ciw6 marker at 162 bp for Columbia type (Lukowitz, 2000).

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CHAPTER 4

BIOCONTROL OF *ERWINIA TRACHEIPHILA* BY *PSEUDOMONAS FLUORESCENS* A506 IN MUSKMELONS

INTRODUCTION

Bacterial wilt is a significant disease of cucurbits that causes substantial production losses in the Eastern and Midwest United States, as well as various locations around the world (Brust, 1997). The disease is caused by *Erwinia tracheiphila*, which is mainly vectored by, striped and spotted cucumber beetles, *Acalymma vittatum* and *Diabrotica undecimpunctata*, respectively. Conventional plant protection recommendations for bacterial wilt rely on the use of systemic and contact insecticides to control the vectors (University, 2012; Bessin et al., 2010). Organic productions practices typically exclude the insect vectors through the use of spun-bonded polyethylene row covers until anthesis, followed by regular applications of approved insecticides. Severity of crop infection varies seasonally, however *Erwinia tracheiphila* infection in muskmelons has caused near complete crop failure, most notably in organic production systems (Rand, 1920; Sherf, 1986).

The bacterial pathogen is transmitted through vector mouthparts or frass, and enters the plant through feeding wounds. The pathogen is currently understood to migrate to the xylem vessels of the infected plant, increasing in numbers until it restricts transmission of plant fluids, thereby causing wilting and subsequent death of the plant (Mitchell and Hanks, 2009). The symptoms of the disease are typically identified in the field by the stringy exudate exposed between severed plant parts (Latin, 2012). Recent

advances have provided PCR primer sets for identification of the pathogen (Chapter 3, Mitchell and Hanks,2009).

Biocontrol of pathogens in agricultural crops has an extensive history (Cook, 2000). The soil-borne bacteria *Pseudomonas spp.* has been found to induce systemic resistance (ISR) as well as systemic acquired resistance (SAR) by a variety of modes of action (Bakker et al., 2007, Rasmussen et al., 1991). In previous research by other authors, *Pseudomonas fluorescens* A506 was found to be effective in enhancing control of *Erwinia amylovora* infections in apple and pear trees (Stockwell and Stack, 2007). The disease enters apple trees through flowers and nectaries and causes wilt and necrosis of shoots. *P. fluorescens* A506 was found to act competitively in the infection sites to out-compete *Erwinia amylovora* (Stockwell and Stack, 2007).

In order to investigate the potential of using *Pseudomonas fluorescens* A506 to control bacterial wilt two experiments were conducted. The first experiment evaluated two different concentrations of BlightBan®A506 (Nufarm, Americas, Alsip, Illinois) – the highest manufacturer recommended rate and 4 times the highest manufacturer rate - applied to *Cucumis melo* plants at the flowering stage. In the same test, the concentration treatments were applied to separate groups of plants on day one and day seven in order to document the time response of the plants to the treatments. A second, and separate, experiment was conducted to identify a potential mode-of-action of *Pseudomonas fluorescens* A506 regarding its inhibition to the spread of bacterial wilt. Four defense-related genes were assayed to evaluate whether the plant defense system was activated by the treatments. The genes fell into three groups First, nonexpressor of Pathogenesis-Related genes 3, *NPR3*, which is a paralogue of *NPR1*, which is a transcription cofactor

nonexpressor (Fu, 2012). *NPR3* functions as an adaptor of the Cullin-3 ubiquitin E3 ligase to mediate *NPR1* degradation, and thereby, is a salicylic acid receptor. In a primed (pretreatment with BlightBan® A506) treatment system such as this study, *NPR3* acts in a higher salicyclic acid concentration environment to degrade *NPR1*, thereby triggering the SAR response (Moreau, 2012). Presence of *NPR3* in this experiment could indicate that the plant has recognized a pathogen and is acting to trigger the SAR biochemical process. The second group, phenylalanine ammonia-lyase, *PAL*, and cinnamyl alcohol dehydrogenase, *CAD*, are enzymes that are intermediaries in the salicylic acid pathway and indicate whether the pathway has been activated. The third group, pathogenesis-related protein, *PR1*, is a protein that is the result of the biochemical production of the salicylic acid pathway and indicates that the plant defense system has been activated.

This study was designed to evaluate the control of *Erwinia tracheiphila* in *Cucumis melo* through the use of *Pseudomonas fluorescens* A506, and to elucidate the mode of action of the biocontrol agent through the examination of defense response-related gene expression.

MATERIAL AND METHODS

Plant Culture

Untreated muskmelon seeds (*Cucumis melo*. "Athena") were obtained from Seedway, LLC. (Hall, New York, USA). Plants were grown from seed in a Percival E41H0 growth chamber (Percival Scientific, Perry, IA) (14 hr day, 10 hr night, 25° C) during November/December, 2011 at the University of Kentucky Horticultural Research Farm in Lexington, Kentucky, USA. The plants were located approximately 12-18 inches,

depending upon growth stage, from the growth lights in the chamber with all lamps operating. The *Cucumis melo* plants were grown up to the flowering stage at which point BlightBanA506 was applied based on the treatment design and subsequent *Erwinia tracheiphila* inoculations. The plants continued to grow in the growth chamber until rankings as to the response of the treatments were collected and samples were collected for examination of progress of the *Erwinia tracheiphila* infection.

Bacterial Culture

All *Erwinia tracheiphila* strains were obtained from Iowa State University, Plant Pathology Department. *Erwinia tracheiphila* was grown on solid media plates containing 23 g Nutrient Agar, 5 g Bacto Peptone, 5 g Bacto Agar in 1 liter of Milliq water (components available from Difco Laboratories, Sparks, MD) (conversation with Erika Saalau-Rojas at Iowa State University, Ames, Iowa, USA). Also for selection purposes, the *Erwinia tracheiphila* SCR-3 rifampicin-resistant strain was used and grown on the same media with 75 ug/ml Rifampicin added to the growth culture. Additionally, *Erwinia tracheiphila* was grown in liquid culture consisting of Nutrient Broth and 75 ug/ml Rifampicin (Fisher Scientific, Pittsburgh, PA).

BlightBan*® A506 *Application

Pseudomonas fluorescens A506 has been formulated in the product BlightBan® A506 for application on fruit trees for suppression of Fire Blight and other

crops for reduction of frost and frost damage. *Cucumis melo.* plants at the flowering stage of at least one flower per plant were treated, by surface application with BlightBan® A506 using the highest recommended equivalent rate of 0.0237 g BlightBan® A506 / 30 ml dechlorinated water as well as at 4X the recommended rate. Untreated control and mock treatment with dechlorinated water were also maintained alongside the treated plants. Although the experiment was conducted multiple times the final run of the experiment was conducted with six replicate plants that were maintained for each treatment and the entire experiment was two complete sets of the experiment run.

Bacterial Inoculation

Erwinia tracheiphila SCR-3 Rif was grown up on sterile culture plates containing 75 ug/ml of Rifampicin. The bacteria was harvested from the plates and resuspended in 10 mM PBS solution. The Optical Density of the inoculum was diluted to an average of 0.7 OD based on NanoDrop readings at 640 nm. Inoculation of the *Cucumis melo* plants occurred at different times depending upon the test conducted, however the manner of inoculation was consistent. A 50-ul aliquot of the inoculum was placed on a leaf randomly around the surface of the leaf and then a multi-pin pin frog was inserted into the leaf through the inoculum drop on the leaf. Numerous holes (up to 50) were punctured into the leaf to allow for bacterial transmission into the leaf (protocol obtained from Erika Saalau-Rojas, Iowa State University, Plant Pathology Department, Ames Iowa, USA).

Symptom Progression Experimental Design

To observe the progress of the disease relative to the action of the biocontrol treatment, seven treatments were applied to *Cucumis melo* "Athena" flowering plants in the greenhouse. Six plants per treatment in a completely randomized design were maintained, and the entire experiment was replicated two times.. The treatments were: (1) untreated control plants, (2) mock treatment of BlightBan®A506 for 1 day inoculation of experimental treatments (1X & 4X), (3) mock treatment of BlightBan®A506 for 7 day inoculation of experimental treatments (1X & 4X), (4) 1x BlightBan®A506 with 1 day inoculation of experimental treatments, (5) 1x BlightBan® A506 with 7 day inoculation of experimental treatments, (6) 4x BlightBan®A506 with 1 day inoculation of experimental treatments, and (7) 4x BlightBan®A506 with 7 day inoculation of experimental treatments. 1X describes the maximum concentration of application recommended by the manufacturer (1g BlightBan®A506 /1261 ml dechlorinated water) and 4X represents four times the recommended application rate from the manufacturer. The seven-day inoculation schedule arose from previous laboratory experiments that demonstrated a higher control efficacy after a delay in inoculation after initial treatment at 1X level.

Data Analysis

Two weeks after inoculation (second and third week), plant health rankings were recorded based on the following criteria, 0 = 0% spread, 1 = 1-10% spread of pathogen

symptoms on the foliage of the plant, 2 = 11-25%, 3 = 26-50%, 4 = 51-75%, and 5 = 76-100%. Typical symptoms recorded were wilting of leaves. Rankings were collected at two weeks after inoculation. Statistical analysis was conducted on JMP9 software (SAS Institute Inc., Cary, N.C.). Duplicate test ranking data was analyzed using Likelihood-ratio test Prop>Chi square test for significance to determine whether the duplicate data could be combined for final analysis. Significance analysis for pathogen progress ranking data was conducted using the Kruskal-Wallis method of analysis since plant/pathogen outcomes were typically bi-modally ranked rather than normally distributed.

Defense Gene Expression Experiment: Pathogen Sampling

In order to observe the progression of the *Erwinia tracheiphila* infection, leaf disc samples were collected from the inoculated leaves at set intervals after inoculation:1 hour, 2 days, 4 days. Three leaf discs were collected per plant; three plants per treatment were sampled per time-period. Leaf discs were sampled using a #4 punch and the discs were placed in microtubes with 300 ul of 10 mM $MgCl_2$ solution. The discs were crushed using an autoclaved micro pestle and 700 ul of 10 mM $MgCl_2$ solution was added to the microtube. After 10 min incubation at room temperature each sample was serial diluted into four subsequent tubes. From each tube, 70 ul of diluent was added to 630 ul of 10 mM $MgCl_2$ solution for a 1:10 dilution. 50 ul of each serial diluent was placed on culture plates that contained 23 g Nutrient Agar, 5 g Bacto Peptone, 5 g Bacto Agar in 1 liter of Milliq water plus to 75 ug/ml Rifampicin. These culture plates were allowed to grow at room temperature in the dark until bacterial cultures could be identified and

counted. The plates were visually observed for bacterial growth, however they were not statistically analyzed since the RT-PCR assay effectively determines presence or absence of the bacteria in the sample.

Experimental Design

To ascertain the mode of action of the biocontrol bacteria, two treatments were applied to *Cucumis melo* "Athena" flowering plants in the growth chamber. Six plants per treatment randomly assigned to each treatment and were maintained along with a complete test duplication. The treatments were the mock treatment of unchlorinated water with inoculation after 7 days, and 1X BlightBan® A506 with inoculation after 7 days. Leaf disc samples were collected for pathogen growth plate counting and mRNA analysis. Real-time PCR analysis of the mRNA converted to cDNA of the four *Cucumis melo* plant defense-related genes (*NPR3, PAL, CAD* and *PR1*) provides evidence of up- and down- regulation of the genes in the interval shortly after *Erwinia tracheiphila* inoculation of the untreated and BlightBan® A506 treated *Cucumis melo* plants (See Figure 4.4 and Table 4.1).

RNA Sampling

In order to determine the mode of action of the observed inhibition of the progression of *Erwinia tracheiphila,* RNA sampling of the plant treatments described in the previous section was conducted. Approximately four leaf discs were collected using

a #6 punch from three plants per treatment at the 1 hr. and 2 day time periods. These leaf discs were immediately placed in liquid nitrogen for preservation. The samples were stored at -20 C until processed.

RNA Analysis

Leaf disc samples collected for RNA analysis were processed for RNA extraction using TRIzol® (Life Technologies™, Grand Island, NY). The tissues were first ground using an autoclaved micropestle, then the residue was placed in 1 ml of cold TRIzol® reagent and the TRIzol protocol for RNA extraction was followed (Portillo, 2006).

Reverse transcriptase PCR was run on the samples using Random Hexamer primers and rt-PCR reagents (Fermentas, ThermoScientific, Pittsburgh, Pa.). The 20 ul samples consisted of 1 ul of *Cucumis melo* RNA at approximately 2600 ng/ul, 1.0 ul of Random Hexamer Primer, 50 uM (Applied Biosystems, Life Technologies, Grand Island, New York), 10 ul of RNA use water, 4 ul of Buffer, Rxn 5X, 1.0 ul of SUPERase-in RNase Inhibitor (Life Technologies™), 2.0 ul of dNTP Mix (Fermentas), 10 mM, 1.0 ul of ReVert Aid Reverse Transcriptase (Fermentas). The samples were processed in an Applied Biosystems thermocycler for the following cycles: 42 °C for 60 min, 70 °C for 10 min, and 4 °C for 60 min.

For the genes in this study previously published primers for *Cucumis sativus*, *Cucumis melo* or *Arabidopsis thaliana* were used to identify DNA/RNA homologues in *Cucumis melo*. These sequences were BLAST searched against the *Cucumis melo* database at the Cucurbit Genomics Database (http://www.icugi.org/) for homologues to *PR1, PAL, CAD and nPR3* with a >70% match. The following primers were used:

- NPR3: forward, 5'-ATAAGTTCGTGGCGGATGAC-3', reverse 5'-TTGGTACCATGCTTCAACGA-3', from *Arabidopsis thaliana* (Genbank # NM_123879.2) nPR3 to *Cucumis melo nPR3* homologue
- Actin: forward, 5'- AGGCCGTTCTGTCCCTCTAT-3', reverse 5'-CAGTAAGGTCACGACCAGCA-3', for housekeeping, *Cucumis melo* (Genbank # AB640865.1)(www.ncbi.nlm.nih.gov/genbank/).
- PAL: forward, 5'- AACGGTTTGCCTTCTAATCTT-3', reverse 5'-GTTGTGTGGCTCAGCACTCT-3', *Cucumis sativus* (GenBank # DQ645596.1) to *Cucumis melo* homologue
- CAD: forward, 5'-ACCCAAGGCGGTTTCTCC-3', reverse 5'-AGCCCACTCTGTTTCAGTCC'3', *Cucumis sativus* (GenBank # DQ178938.1) to *Cucumis melo* homologue
- PR1: forward, 5'-TGCTCGACAATATGCGAACC-3', reverse 5'-CCAGCCGCACATGTATTG-3', *Cucumis sativus* (GenBank # DQ641122.1) to *Cucumis melo* homologue.

All primers were synthesized by Elim Biopharmaceuticals, Haywood, CA.

Real-Time PCR was conducted on an Applied Biosystems StepOne and StepOne Plus instrument. A reaction volume of 10 ul consisted of 5 ul of Fast SYBR® Green Master Mix (Applied Biosystems, see above), 1 ul of cDNA from the rt-PCR reaction, 3.8 ul of RNase-free water, 0.2 ul of both 10 mM forward and reverse primers. Each reaction was replicated three times.. The following amplification conditions were used: 95 °C for 20 sec – 1 cycle, 95 °C for 3 sec and 58 °C for 30 sec – 40 cycles, and Melt Curve analysis of the amplicon was run after the end of the thermo cycling.

Figure 4.1 illustrates the testing schematic and the four treatment comparisons illustrate the gene responses between treatment conditions:

- The $\Delta\Delta C_t$ analysis of the pair BA is the comparison between the ΔC_t of Treatment (B) which was sampled one day after BlightBan®A506 and 1 hour after inoculation with *Erwinia tracheiphila* and the ΔC_t of Treatment (A) which was sample one day after mock BlightBan®A506 treatment and one hour after inoculation with *Erwinia tracheiphila*.
- The $\Delta\Delta C_t$ analysis of the pair CA is the comparison between the ΔC_t of Treatment (C) which was sampled three days after mock BlightBan®A506 treatment and two days after inoculation with *Erwinia tracheiphila* with the ΔC_t of Treatment (A) which was sampled one day after mock BlightBan®A506 treatment and one hour after inoculation with *Erwinia tracheiphila*.
- The $\Delta\Delta C_t$ analysis of the pair DB is the comparison between the ΔC_t of Treatment (D) which was sampled three days after treatment with BlightBan®A506 and two days after inoculation with *Erwinia tracheiphila* and the ΔC_t of Treatment (B) which was sampled one day after treatment with BlightBan®A506 and 1 hour after inoculation with *Erwinia tracheiphila*.
- The $\Delta\Delta C_t$ analysis of the pair DC is the comparison between the ΔC_t of Treatment (D) which was sampled three days after treatment with BlightBan®A506 and two days after inoculation with *Erwinia tracheiphila* and the ΔC_t of Treatment (C) which was sampled three days after mock treatment and two days after inoculation with *Erwinia tracheiphila.*

The $\Delta\Delta C_t$ fold difference method does not incorporate statistical comparisons between treatment combinations. The results are simple arithmetic progressions/fold increase/decreases representing relative quantities of the initial sample template/amplicon relative to compared samples.

Real-Time PCR analysis provided C_t, threshold cycle values for the mRNA samples. Further analysis of the C_t values in conjunction with the housekeeping control gene, Actin and utilizing the 2^(-Delta Delta C_t) method (Livak, 2001; Applied, 2008) provided relative up- or down- gene regulation values.

RESULTS

Affect of Pseudomonas fluorescens A506 on the progression of bacterial wilt symptoms

Cucumis melo 'Athena' leaf disc samples collected after inoculation and grown on specialized culture media verified successful inoculation of *Erwinia tracheiphila* through visual examination of the plates and counting of bacterial colonies developed on the culture plates. Additional verification of pathogen infection consisted of visual progression of the symptoms of bacterial wilt at two weeks after inoculation with ranking of progress as stated below. The duplicate tests were compared for statistical significance to determine whether the data could be combined for analysis. The duplicated tests data were found to be not significantly different based on Likelihood-ratio test Prop>Chi-square of 0.46, and Pearson's chi-square test Prob>Chi-square of 0.54. Therefore the duplicate tests ranking data were combined for analysis of significance.

The distribution of the ranking data was bimodal, typically either 0, 1, or 5 after two weeks of pathogen progression. See Figure 4.1 for a chart of the average ranking

versus treatment. See Figure 4.2 for an image of the Day 1 inoculated plants and Figure 4.3 for an image of the Day 7 inoculated plants.

Analysis of significance of progress ranking against level of BlightBan®A506 applied to the plants for both 2nd week observations were found to be highly significant overall with the Likelihood-ratio test Prop>Chi-square of 0.00020, and Pearson's chi-square test Prob>Chi-square of 0.0015. However, the Treatment 5x7 and 4x6 comparisons were not significant with (P = 0.26) and (P = 0.41), respectively. The 4x7 and 5x6 comparisons were significant (P < 0.01). Visually, the observed test outcomes for application of BlightBan®A506, (0, 1X or 4X) are clearly evident in Figures 4.3 and 4.4. Analysis of significance of the inoculation day 0, 1, or 7 versus treatment overall was found to be highly significant (< 0.01). The 4x5 and 6x7 comparisons were significant (P <0.01). Further, there was a significant difference between the 1 and 7 day inoculation at 1X BlightBan®A506 application (4x5 comparison) (P<0.01) and 1 and 7 day inoculation 4X BlightBan®A506 application (6x7 comparison) (P=0.0003).

For the 3rd week observations there was a significant difference between Treatment 1 and the remainder of the Treatments 2-7 (P<0.01). Also the Treatment 7 was significant from all the other Treatments 1-6 (P<0.01).

Pseudomonas fluorescens A506 Affects on Defense –related Gene Expression

Validation of the $\Delta\Delta C_t$ fold difference calculations demonstrated that the *PR1* and *nPR3* assays met the <0.1 slope criteria, whereas the *PAL* and *CAD* assays slightly exceeded the recommended (Applied Biosystems, 2008) method criteria. In evaluating

the significance of slope exceedance, it was determined the assays were sufficiently representative of the data and further modification of the assays was unnecessary.

Up and down regulation of plant defense genes were calculated for the ΔΔCt fold difference between treatments B & A, C & A, D & B, and D & C. The *NPR3* gene BA test indicated a 3.4 fold up regulation of the NPR3 for the BlightBan®A506 treated plant versus the mock treated plant one hour after inoculation of the pathogen. The CA test indicated relatively less up regulation relative to the BA test, however, two days after introduction of the pathogen in a mock treated plant, 0.55 fold increase of *NPR3*. The DB test where two days after inoculation a BlightBan®A506 treated indicated an up-regulation of 0.21 fold over the one hour BlightBan®A506 treated plant. This would theoretically indicate a 3.5 fold up-regulation of *NPR3* over the mock-treated and inoculated A test. The DC test where the two days inoculation and BlightBan treated plants indicated a 1.38 fold increase of *NPR3* over the mock treated plant. This would theoretically indicate a 1.9 fold increase of *NPR3* over the mock treated and inoculated A test. This suggests that the *NPR3* up-regulation response is reduced after two days relative to the one-hour response in the BA test. These *NPR3* responses parallel the findings from the Fu publication (Fu et al., 2012).

The *PAL* and *CAD* fold difference results were somewhat similar and will be discussed together. The BA tests for *PAL* and *CAD* were 2.7 and 2.6 fold up-regulation, respectively. This would tend to indicate that one hour after inoculation with the pathogen in the BlightBan®A506 treated plant, the salicylic pathway was already functioning at a heighten state in the plant. The CA test result for *PAL* and *CAD* were both 1.3 fold up-regulation, potentially indicating a common reaction for both enzymes at

that point in the test, two days after inoculation. The DB test for *PAL* and *CAD* were 1.04 and 0.75 fold up-regulation, respectively. This would indicate a relatively similar response at point in the test, two days after inoculation for BlightBan®A506 treated plants. The DC test for *PAL* and *CAD* were 2.4 and 1.8 fold up-regulation, respectively. Again, this level of up-regulation indicates a common level of enzymatic activity at this point in the study, two days after inoculation.

The *PR1* fold difference results indicated a different pattern of response to the two other groups of genes examined. The BA, CA and DB tests for *PR1* were 0.60, 0.35 and 0.40 fold up-regulation. These results indicate a relatively low level of response for the BlightBan®A506 treated plant one-hour after inoculation, the mock treatment two days after inoculation and the BlightBan®A506 treated two days after inoculation relative to the BlightBan®A506 one-hour after inoculation. However the DC test result was 3.1-fold up-regulation. This indicate that at the two day point, the BlightBan®A506 treated plants had increased plant defense protein by 3.1 fold relative to the mock treated plant at the same time point. This *PR1* gene response is consistent with previously described plant defense response processes (Loake and Grant, 2007).

DISCUSSION

Registered applications of BlightBan®A506 can be applied to perennial woody plants for protection from *Erwinia amylovora* and frost injury (Elkins et al., 2005). This study experimentally applied BlightBan®A506 to *Cucumis melo* in a laboratory setting with the objective of producing suppression of *Erwinia tracheiphila*, a close phylogenetic relative to *Erwinia amylovora,* in an annual plant. Ranking tests demonstrated that 4X

the highest recommended BlightBan®A506 application rate actively suppressed *Erwinia tracheiphila* spread through the vascular system of *Cucumis melo* during the two-week test period. Localized wilting was observed at and around the inoculation site, however plant growth progressed rather than complete plant wilting and death which were the common outcome for the mock treatment and 1X BlightBan®A506 application. The 4X application of the BlightBan®A506 significantly contributed to the observed response in the plants and not the inoculation delay of 1 to 7 days.

Previous research explained BlightBan®A506 (*Pseudomonas fluorescens* A506) mode of action as a competitive nutrient utilization that robbed *Erwinia amylovora* of necessary resources to reproduce (Nuclo et al., 1998). Also in previous research, *Erwinia amylovora* was found to colonize portions of the flower structure on apple and pear plants (Johnson et al., 2009). In this study, BlightBan®A506 was sprayed externally and specifically on the flowers. The pathogen, *Erwinia tracheiphila,* was inoculated at the base of one leaf separate from the treatment location. Pathogen inoculation occurred 1 day and 7 days after BlightBan®A506 application, which anticipated die-off of epiphytic *Pseudomonas fluorescens A506* prior to inoculation. Suppression of the pathogen occurred in this new treatment environment.

The mode-of-action of stopping the spread of bacterial wilt in this study was found to potentially be the up- and down- regulation of plant defense gene production of enzymes and proteins that function and interact as the plant's innate disease defense. Plant defense up- regulation has been found in other model plant systems, however the results of this study demonstrate an addition to the understanding of the biocontrol agent, *Pseudomonas fluorescens* A506, activity in *Cucumis melo* (Veluthakkal et al., 2012,

Geisler et al., 2012, Islam et al., 2012). Analysis of genetic material by Real-time PCR, research is already confirming what was previously hypothesized where up-regulation of plant defense genes have been identified as markers for SAR (Shah, 2003). Specifically, research on an *NPR1 (non-expressor of PR genes)* mutant *Arabidopsis thaliana* found that the SAR pathway became non-functional when induced by chemical or avirulent pathogens. *NPR3*, studied in this research, is an immediate precursor with *NPR4* prior to *NPR1* in the SAR pathway (Moreau et al., 2012.). Its function is necessary for the downstream function of *NPR1*. Wild-type *Arabidopsis thaliana* did show induction of the SAR pathway when inducing agents were applied (Cao et al., 1994). Also, research has demonstrated that artificial induction of SAR in *Arabidopsis thaliana* led to the accumulation of *PR1* mRNA that led to less disease relative to an untreated control (Zimmerli et al., 2000). A recent study focusing on herbivore instigated plant damage also investigated SAR markers and found similar results as to past findings (Koo et al., 2013). Further, a recent review details the current understandings of plant defense signaling. In it SAR and signaling pathways are described demonstrating some of the same processes found in this research (Kachroo and Robin, 2013).

The $\Delta\Delta C_t$ fold difference calculations demonstrate mRNA responses to the treatments that reflect previously documented biochemical reactions to priming to a plant defense system, as well as, subsequent pathogen challenge (Islam, 2008). The experimental design is not the same, however, the relative fold-differences are similar. Combined, the ranking test that documented the delayed progression of the pathogen after application of 4X concentration of BlightBan®A506 as well as the mode-of-action test documenting the up-regulation of the salicylic acid pathway leading to systemic

acquired resistance in the tested *Cucumis melo* plants provide strong evidence for the positive effect of *Pseudomonas fluorescens* A506 in the promotion of plant protection for an annual vascular horticultural plant. Further research to define optimal rates of application for field application, as well as, elucidating additional modes-of-action will increase the understanding of the results of this study.

TABLES

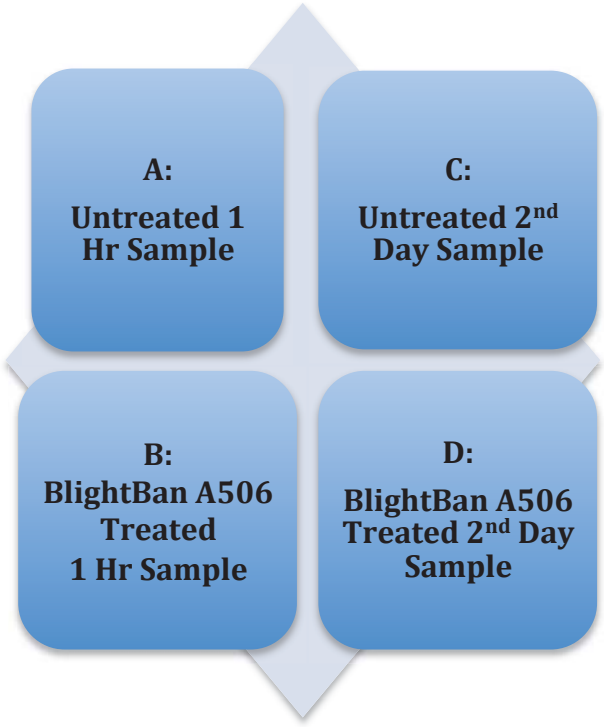
Table 4.1: *Cucumis melo* Defense Gene Response to *Pseudomonas fluorescens* A506 Application.

ΔΔCt Fold Difference Between Plant Defense Gene Up/Down Regulation and Treatments				
ΔΔCt Treatment Comparisons	Plant Defense Genes			
	NPR3	PAL	CAD	PR1
BA	3.2 (0.4-6.0)	2.7 (0.4-5.2)	2.6 (0.4-4.8)	0.6 (0.09-1.09)
CA	0.6 (0.01-1.03)	1.3 (0.1-2.4)	1.3 (0.2-2.4)	0.4 (0.02-0.7)
DB	0.2 (0.001-0.4)	1.0 (0.03-2.06)	0.8 (0.04-1.5)	0.4 (0.01-0.8)
DC	1.4 (0.8-2.7)	2.4 (0.07-4.8)	1.8 (0.1-3.4)	3.1 (0.1-6.1)

Note: $\Delta\Delta C_t$ Treatments reference Figure 4.4 Treatment comparisons, i.e., BA is a Comparison between Treatment B and A. The difference between the ΔC_t values is converted to the fold difference by the calculation of $2^{(-\Delta\Delta Ct)}$.

FIGURES

Figure 4.1: Experimental design for *Cucumis melo* Defense Gene Response to *Pseudomonas fluorescens* A506 Application*:* This figure diagrams the experimental design for the $\Delta\Delta C_t$ Fold Response testing conducted on Cucumis melo mRNA (*PAL, CAD, PR1* and *NPR3*). The treatments A-D are discussed in the research.



- BA = (B) One day after treatment with BlightBan®A506 and 1 hour after inoculation with *Erwinia tracheiphila* and compared to (A) One day after mock BlightBan®A506 treatment and one hour after inoculation with *Erwinia tracheiphila*.
- CA = (C) Three days after mock treatment and two days after inoculation with *Erwinia tracheiphila* and compared to (A) one day after mock BlightBan®A506 treatment and one hour after inoculation with *Erwinia tracheiphila*.
- DB = (D) Three days after treatment with BlightBan®A506 and two days after inoculation with *Erwinia tracheiphila* and compared to (B) One day after treatment with BlightBan®A506 and 1 hour after inoculation with *Erwinia tracheiphila*.
- DC = (D) Three days after treatment with BlightBan®A506 and two days after inoculation with *Erwinia tracheiphila* and compared to (C) three days after mock BlightBan®A506 treatment and two days after inoculation with *Erwinia tracheiphila.*

Figure 4.1 (continued)
Note: $\Delta\Delta C_t$ treatments reference above, i.e. BA is a comparison between treatment B and A. The difference between the ΔC_t values is converted to the $\Delta\Delta C_t$ fold difference by the calculation $2^{(-_{\Delta\Delta}Ct)}$.

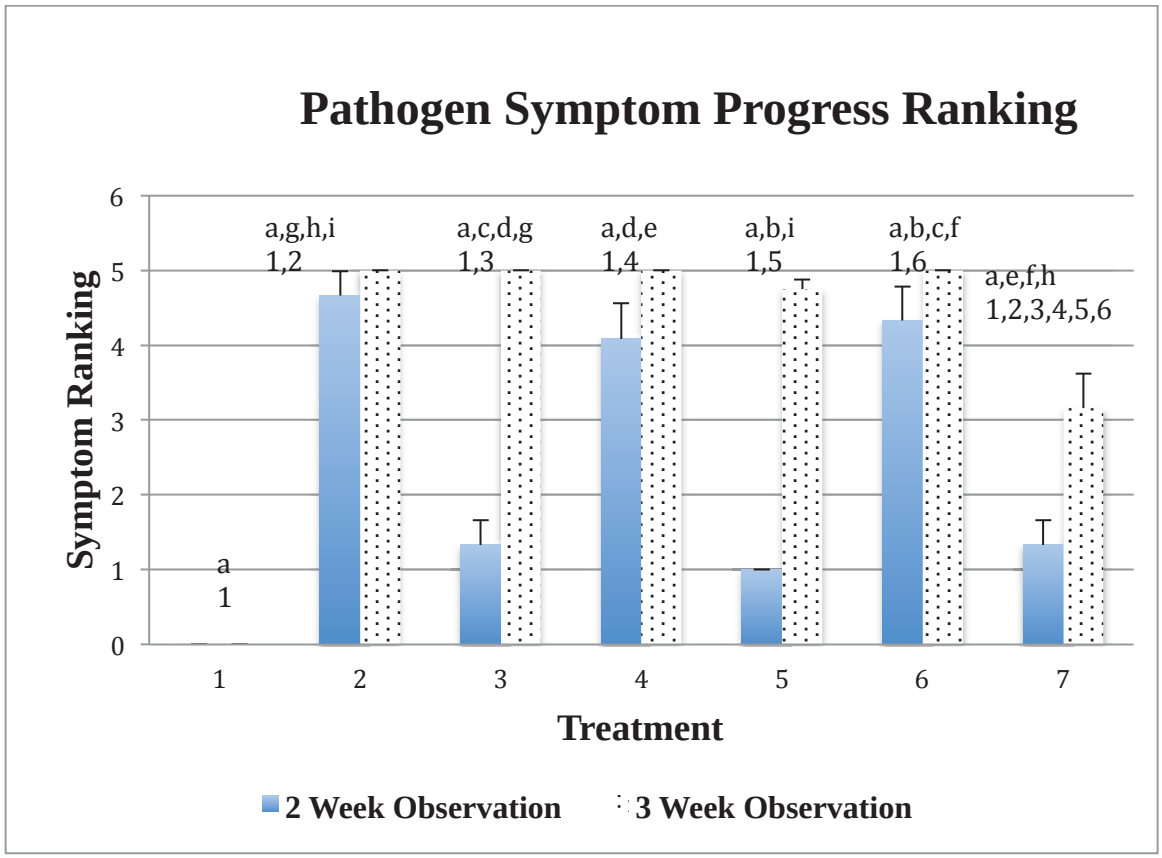


Figure 4.2: Progress of *Erwinia tracheiphila* infection by symptoms ranked by treatment.

This figure compares the treatments versus the pathogen symptom progression rankings. Treatments: 1 = Control (untreated); 2 = Mock Day1 Inoculation; 3 = Mock Day 7 Inoculation; 4 = 1X BlightBan A506, Day 1 Inoculation; 5 = 1X BlightBan A506, Day 7 Inoculation; 6 = 4X BlightBan, Day 1 Inoculation; 7 = 4X BlightBan A506, Day 7 Inoculation.

Symptom Ranking 0= 0%, 1 = 1-10%, 2 = 11-25%, 3 = 26-50%, 4 = 51-75%, 5 = 76-100% Foliage showing pathogen symptoms.

Figure 4.2 (continued)
Statistical significance noted with letters are 2 week observation and numbers are 3week observation levels of significant difference between treatments of the same letter/number. Significant comparisons were significant at $P \leq 0.05$.

Note: Treatment 1 values are 0 and 0 with 0 and 0 standard error

Figure 4.3: Pathogen Progression Ranking – Week 2:

Day 1 Inoculation Series, 2-Week Ranking

Plant 1 at far left is Treatment 1 = control (untreated);

Plant 2 is second from left is Treatment 2 = Mock Treatment, Day 1 *E. tracheiphila* inoculation to one basal leaf;

Plant 3 is second from right is Treatment 4 = 1X Blight Ban A506 treatment, Day 1 *E. tracheiphila* inoculation to one basal leaf;

Plant 4 is far right, Treatment 6 = 4X Blight Ban A506, Day 1 *E. tracheiphila* inoculation to one basal leaf.

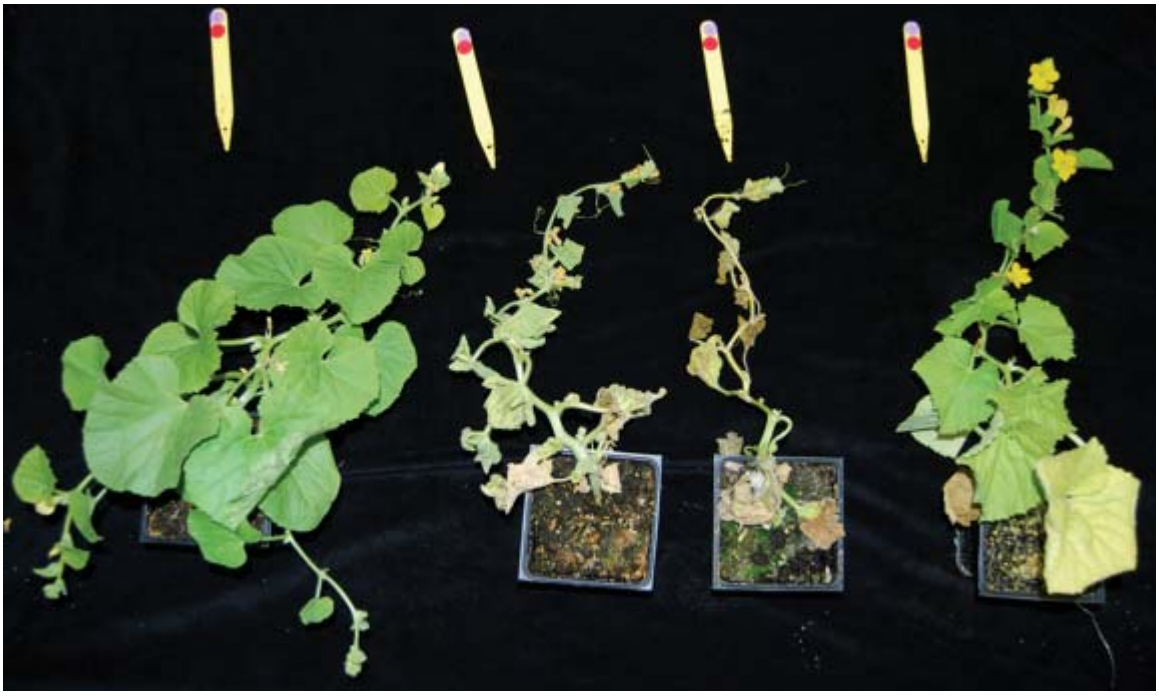


Figure 4.4: Pathogen Progress Ranking – Week 3:

Day 7 Inoculation Series, 3-Week Ranking

Plant 1 at far left is Treatment 1 = control (untreated);

Plant 2 is second from left is Treatment 3 = Mock Treatment, Day 7 *E. tracheiphila* inoculation to one basal leaf;

Plant 3 is second from right is Treatment 5 = 1X Blight Ban A506 treatment, Day 7 *E. tracheiphila* inoculation to one basal leaf;

Plant 4 is far right, Treatment 7 = 4X Blight Ban A506, Day 7 *E. tracheiphila* inoculation to one basal leaf.

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CHAPTER FIVE

ERWINIA TRACHEIPHILA AND CUCURBITS: RESEARCH SUMMARY AND FUTURE RECOMMENDATIONS

INTRODUCTION

Erwinia tracheiphila was isolated in the early part of the 20th century however at the beginning of the 21st century, understanding and control of the bacteria in cucurbit populations is limited. Physical identification of the bacteria through separation of an infected stem, reconnection and separation of the stem end pieces and witnessing the bacterial sticky/stringy fluid between the pieces has been the standard technique to verify *Erwinia tracheiphila* infection. In addition to observing wilting vines, standard progressive microbiological testing procedures that group bacteria into related groupings of bacteria into the *Erwinia tracheiphila's* physical, chemical reaction and morphological characteristics (i.e., dimensions, staining qualities and cell shape/flagella positioning) are identified and alternate bacterial identification options are eliminated. More recent molecular biological identification of the bacteria started with the development of DNA primers sequences to amplify suspected bacterial cultures through conventional polymerase chain reaction methods. This technique more quickly identifies the bacteria by DNA sequences that are uniquely associated with *Erwinia tracheiphila* DNA, thereby identifying the bacteria with the greatest current precision.

The standard understanding of the bacterial infection process is based on early observations that the bacteria prolifically reproduce in the tracheid vascular tissues (xylem) of the plant vine stem. Nourishment from the resource-rich plant fluids rising up from the cucurbit plant roots allows the multiplying bacterial cells to fill the plant xylem

to the point of restricting fluid movement and thereby causing vine extremity wilting and eventually plant death.

The infection process in the cucurbit vine commences when *Erwinia tracheiphila* is introduced into the cucurbit plant be the insect vectors, *Diabrotica undecimpunctata* and *Acalymma vittatum*, both in the family *Chrysomelidae*. The striped and spotted cucumber beetle, the common names of the main insect vectors, is further described by their physical appearance as stripped or spotted. The common understanding of how *Erwinia tracheiphila* survives in temperate climates from year to year is through overwintering in the digestive track of the vectors. The vectors overwinter in the soil and debris on the forest/agricultural floor and reappears in the spring. Some research suggests that before cucurbits are available for feeding, the vector feeds on native perennial wild vegetation that can also harbor *Erwinia tracheiphila* with no ill effect. Some larvae burrow into the soil and feed on the roots of corn and beans and are sometimes called corn rootworm. The larvae develop into adults and migrate to cucurbit crops. From direct observation, adult cucumber beetles feed on the leaves, flowers and stems of the cucurbit plants. The bitter cucurbitacin compounds produced by cucurbits, that deter other insect feeding on cucurbits, are feeding stimulants to cucumber beetles. *Erwinia tracheiphila* has been found in the feeding parts and frass of cucumber beetles (Mitchell and Hanks, 2009). Further, research has shown that infected cucumber beetles feeding on cucurbit flowers can infect the cucurbit through infection of flower parts (Sasu, et al., 2010).

At this time, no completely successful control of *Erwinia tracheiphila* has been developed for organic production except for spun-bond fabric row covers that

successfully exclude the beetles. However, flower pollination is required to produce harvestable fruit from the vines. At this point, natural pollinators are used to pollinate the flowers, which requires removal of the row covers to some extent to effectuate pollination. In both organic and conventional systems, application of contact and systemic pesticides applied for production pest control do not completely remove the risk of infection and loss of the crop prior to harvest. It is at this point that discussion of the research activities discussed in this dissertation can be summarized.

GENERAL RESEARCH FINDINGS

The first project undertaken as part of the requirements for the graduate degree was to investigate an alternative field production technique to improve the predictability of the harvest, even in high vector pressure seasons. Seven treatment options were applied to "Athena" melon plots over three seasons at the University of Kentucky Horticultural Research Farm in Lexington, Kentucky. Some generalized comments about the study include the following observations. During this study, cucumber beetle pressure varied from year-to-year due to unknown factors. Timing of the overwintering adult die-off prior to the emergence of the new season adults varied from year-to-year. These general observations are made from the insect counting conducted during the three seasons of the field study. Also in general, with the variability of the cucumber beetle season, farmers are often not prepared for the pest management practices required to insure a marketable harvest after the cucurbit crop is exposed to *Erwinia tracheiphila* vectors from anthesis to harvest, which is the common conventional and organic production method for cucurbit crops. Variability in climate also may lengthen the

duration from anthesis to harvest as happened in the second season of this project. Cool weather for approximately a month after anthesis significantly delayed the harvest, thereby extending the time the plants were exposed to infected vectors and time for *Erwinia tracheiphila* could develop and wilt/kill the plants and making unsalable the infected harvest.

The results of the project, described in Chapter 2 of this dissertation, found that a study treatment whereby the melon study plots were covered with fabric row covers for all but two weeks for pollination provided similar results to the organic standard where the melon study plots are left exposed to the infecting vectors from anthesis to harvest. The typical organic production method requires continued application of pesticides until harvest to control vector populations. Covering the plots until harvest provided exclusion of the vector that significantly reduced the risk of further infection and crop wilting/death prior to harvest. Although vector pressure was not as heavy at the study location as at other locations known to the researcher near the study site based on anecdotal evidence from other competent researchers, the treatment technique should work to higher relative efficacy when the crop is under higher pressure by significantly reducing pesticide and application costs as well as risk of infection and loss of the crop.

In order to better understand the biological processes *Erwinia tracheiphila* produces once infecting the cucurbit crop, more tools are necessary in order to quantify the reproduction and habits of the bacteria. It is with this area of research in mind that the second project was developed. Previously, conventional PCR primer sequences have been made available publically for researcher to identify *Erwinia tracheiphila* and these primer sets can be used for quantitative PCR testing based on using SYBRGreen

polymerase. SYBRGreen operates by intercalating a fluorescent dye into the middle of each polymerase chain reaction produced in the thermo-cycling process. In the exponential growth of the PCR products, non-target products are fluorescently tagged as well as the target and herein is the complication for utilizing the SYBR Green process for quantitative processes when you are working with tiny samples of bacteria and trying to differentiate progress of processes within the plant. A more sensitive process can be developed using the TAQ probe rather than using the SYBR Green method. In the TAQ process, only the target DNA product is identified with the fluorescent tag, thereby the quantification is more accurate. A TAQ probe optimized for quantitative PCR processing as well as tested against numerous associated pathogens to avoid false-positive responses was the outcome of the second project. With the TaqMan probe tool, a researcher can more carefully quantify bacterial processes.

The third project was developed in an effort to increase the potential protection of the cucurbit crop during the exposed-to-vector anthesis period inherent in the production method developed in the first project. In the first project, bumble bees were tested in a treatment option and were found to be ineffective for unknown reasons. Without an alternate pollinator that could effectively pollinate under the effective row covers, exposure to vectors is necessary during the anthesis/pollination period. A further treatment was conducted in the final season in an effort to define how long row covers need to be removed to produce at least as good as the yield of the standard organic treatment method. One week of exposure to pollinators was found to produce significantly less melons than the two-week exposure. Two weeks of exposure was enough to produce not significantly different results from the standard organic method.

However, even with this information, the crop is still exposed to the infecting vector during this two week time period with only contact pesticides in the case of organic production. Research has found that different chemicals and biological agents can artificially stimulate the plant defense system to increase defenses in the plant against insects and pathogenic agents. The third research project subjected melon plants to pretreatment with a variety of previously researched plant-based compounds as well as benign biological agents to gauge development of resistance to a subsequent infection of *Erwinia tracheiphila*. It was the beneficial bacteria *Pseudomonas fluorescens* A506 that provided the best candidate for further investigation of the melon plant defense system. In the screening tests the melon plant lived significantly longer after infection compared to the non-treated plant. From the visual observation of control of the progress of the infection, research turned to the investigation as to whether the plant defense system had actually been activated by the application of the *Pseudomonas fluorescens* A506. Messenger RNA (mRNA) is the molecular biological element that indicates whether the plant DNA processes have been activated to create proteins that act as defense within the plant. Other researchers have identified mRNA sequences that were found to directly trigger the plant defense system. Homologues of these mRNA sequences were used to quantitatively test the theory in *Cucumis melo* as to whether the defense system had been activated. As relatively little detailed information as to levels of plant defense system products are necessary to combat a particular pathogen, the research focused on assessing the intermediary mRNA relative increased production to act as an indicator was to whether the defense system had been activated. Four different mRNA sequences were tested using a SYBR Green PCR technique. Based on relatively recent research into the

biochemical processes involved in the plant defense system, the results of the mRNA PCR tests indicated that the plant defense system had been activated by the application of the Pseudomonas fluorescents A506 treatment (Moreau, 2012). With this information in hand, this researcher had found an organic compound to potentially elicit activation of the plant defense system to defend a melon from attack from *Erwinia tracheiphila*.

RESEARCH IMPLICATIONS

The research conducted during the graduate studies described in this dissertation has both applied and theoretical applications. From the applied perspective, the field research results can be recommended to growers in order to increase reliability of the production methods currently utilized.

The molecular biological test development as an applied application where the test can be used by diagnosticians to determine the presence of the bacteria with greater reliability and may be able to track the progression of the infection. Additionally, theoretical work can be pursued using the test in order to develop a better understanding of the dynamics of the progression of the disease as well as other issues related to vascular plant processes relative to *Erwinia tracheiphila*.

Finally, the third project was developed with the idea of producing a totally new means of controlling a previously uncontrollable bacterial pathogen. With a means of slowing if not controlling the bacteria, reduced applications of pesticides or other control methods may be possible. This project found that *Pseudomonas fluorescens* A506 significantly retarded the progress of infection in treated plants. Although *Pseudomonas fluorescens* A506 is a currently available product as BlightBan®A506, it is not labeled

for the application described in this research and therefore this project could potentially add another tool to the producer's toolbox of methods to produce a successful cucurbit crop where *Erwinia tracheiphila* causes significant damage to an otherwise high value crop, however, only if the manufacturer re-labels the product.

RECOMMENDATIONS FOR FUTURE RESEARCH

Based on the results from the projects undertaken in this graduate program, various additional projects could come from these activities. From the first project, the development of an improved production system, additional projects that could be undertaken could be investigated:

(1) if this new melon production method can be applied to other cucurbit crops since other cucurbits have different flowering habits,

(2) whether a combination of species or different species of natural predators could better control the aphid population under the row covers,

(3) whether a different row cover configuration application might provide a better environment for *Bombus* spp. to provide pollination services under the row cover, and

(4) natural enemies of cucumber beetles and how to lure more predator insects to the cucurbit crop.

From the second project, the development of a Real-Time PCR assay for Erwinia tracheiphila, additional research that could be undertaken could be investigated: whether there is a difference in virulence between the overwintered *Erwinia tracheiphila* bacterial infections in the cucumber beetle relative to the newly emerged adult cucumber beetles with newly acquired *Erwinia tracheiphila* infestations.

From the third project, the investigation of an organic elicitor of the plant defense system, additional research that could be undertaken could be investigating

(1) to optimize the method and rate of application of BlightBan®A506 to obtain control of *Erwinia tracheiphila* throughout the remainder of the season to allow for predictable harvests,

(2) whether *Pseudomonas fluorescens* A506 is producing an antibiotic within the plant vascular tissues that could be contributing to the control of *Erwinia tracheiphila* within the vascular system,

(3) whether *Pseudomonas fluorescens* A506 is acting from the outside of the plant or actually enters and travels inside the vascular tissues of the plant effecting the results witnessed,

(4) whether application of BlightBan ®A506 to flowers is necessary to activate the plant defense system or can the biocontrol be sprayed before flowering occurs to activate the defense system,

(5) whether BlightBan®A506 can be applied from transplanting to harvesting in the place of row covers and pesticide applications, and

(6) if BlightBan®A506 can be comingled in the application tank with various pesticide applications to further reduce labor costs.

With the reduction in research resources, future research may need to be undertaken in independent laboratory research settings.

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Publications

Caudle, J. R., et al. 2014 proposed. Real-Time PCR Assay for *Erwinia tracheiphila*. Plant Disease.

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Presentations

Organic melon production method to improve control of bacterial wilt on cucurbits. Kentucky Fruit and Vegetable Growers Conference in Lexington, KY, January, 2010

Control of bacterial wilt in Organically Grown Muskmelon, International Organic Fruit Research Symposium, Leavenworth, Washington, June 19, 2012.