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Plant improvement for insect resistance: Testing of the candidate organism Beauveria bassiana, transgenic tobacco expressing protease inhibitors, and rapid screen of insect resistance genes in an agroinfiltration transient expression system

Brian M. Leckie

To the Graduate Council:

I am submitting herewith a dissertation written by Brian M. Leckie entitled "Plant improvement for insect resistance: Testing of the candidate organism Beauveria bassiana, transgenic tobacco expressing protease inhibitors, and rapid screen of insect resistance genes in an agroinfiltration transient expression system." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Plants, Soils, and Insects.

C. Neal Stewart, Jr, Major Professor

We have read this dissertation and recommend its acceptance:

Feng Chen, Ranjan Ganguly, Bonnie Ownley

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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A Dissertation
Presented for the
Doctor of Philosphy
Degree
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Brian Michael Leckie
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Dedication

This dissertation is dedicated to my loving wife Nicola, my faithful dog Jackson, and to my parents Sara and Dave.

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Abstract

This study focused on three aspects of plant improvement for insect resistance including: testing of candidate organisms for their production of insecticidal proteins, testing of transgenic plants expressing insect resistance genes, and testing novel systems for the evaluation of insect resistance genes. In the initial part of this study, the candidate fungus *Beauveria bassiana* was tested for its production of insecticidal proteins through a series of insect bioassays containing fungal protein extracts. These extracts were shown to be orally toxic to *Plutella xylostella* (diamondback moth) and *Spodoptera frugiperda* (fall armyworm). Assays involving protease treatments significantly decreased mortality indicating the presence of a protein based oral toxin. The following research tested transgenic tobacco plants expressing proteinase inhibitors from *Brassica oleracea* (cabbage) and *Manduca sexta* (tobacco hornworm) on the insect pests *Helicoverpa zea* (corn earworm) and *Heliothis virescens* (tobacco budworm). Insects fed transgenic tobacco were able to adapt to the recombinant proteinase inhibitors to varying degrees and resulted in no major impacts on insect growth and development. The last part of this study tested a novel insect resistance gene screening system. Agroinfiltrated tobacco transiently co-expressing genes encoding GFP with either a known insecticidal protein (Bt Cry1Ac) or a candidate gene (*Brassica oleracea* proteinase inhibitor, BoPI) were fed to larval *H. zea*. Insects fed the known insecticidal protein experienced high mortality. Insects fed tobacco expressing GFP and BoPI showed significant decreases in growth compared to those fed GFP only tissue. Insects feeding on GFP only tissue showed unexpected increases

in growth and development compared to insects fed control tissue. Agroinfiltration coupled with an insect bioassay constitutes an efficient system for the evaluation of candidate insect resistance genes.

Table of Contents

List of Figures

Introduction

Impacts of Insects in Agriculture. Global agriculture has been severely impacted by insect pests for many years. In 2004, it was estimated that arthropod pests were responsible for the destruction of 18% of the world's crop production and 20% of stored grains. These losses were estimated at 100 billion US dollars annually (Nicholson 2007). The global cost of controlling arthropod pests in 2001 was estimated at 7.56 billion US dollars, with pesticides targeting lepidopteran pests constituting a significant portion (36%) of this total (Beckmann and Haack 2003). These high losses in food production combined with an estimated 40% increase in the global population by 2050 (U.S. Census Bureau, 2006) highlight the need for improved insect pest control.

The production of transgenic plants expressing *Bacillus thuringiensis* δ-endotoxins for protection against insect pests has consistently grown since first commercialization in 1996. In 2007, 114.3 million hectares of transgenic crops were grown, consisting of approximately 18% or 20.3 million hectares planted for insect resistance (not including plants expressing multiple stacked genes). The advantages of these crops are seen in both crop yield and the reduction in chemical insecticide usage which can have major environmental impact (James 2007). Global reliance on Bt crops for insect control has spurred concerns for years that insects may develop resistance to these insecticidal proteins (Stewart 1999). Recently, field populations of Bt resistant corn earworm (*Helicoverpa zea*) have developed resistance to Bt toxins in Mississippi and Arkansas (Tabashnik et al. 2008). These researchers proposed that resistant populations developed as a result of improper pest management issues, including lack of proper

refuge size and dosages that did not meet the minimum requirements. These recent events underscore the need to develop alternatives to the current biotechnology strategies for insect control.

Bacillus thuringiensis. The existing transgenic plant technologies for insect control are highly reliant on the use of proteins from *Bacillus thuringiensis*. *B. thuringiensis* (Bt) are spore-forming gram positive bacterium which are a well known pathogen of lepidopterans (Schnepf et al. 1998). In 1977, *B. thuringiensis* subsp. *israelensis* was discovered, which was shown to have specific toxicity to dipterans (Goldberg and Margalit 1977). This toxicity was due to ingested bacteria producing insecticidal proteins, called δ-endotoxins, in the insect midgut during sporulation,. Two types of δ-endotoxins, parasporal crystal toxins (Cry) and cytolitic toxins (Cyt), have been identified in Bt (Bravo et al. 2007). The cloning and expression of the first Bt δ-endotoxin gene occurred in *Escherichia coli* (Schnepf and Whiteley 1981). The potential usage of these insecticidal genes in agriculture was identified and the first transgenic tomato and tobacco plants expressing δ-endotoxins were produced (Barton et al. 1987, Fischhoff et al. 1987, Vaeck et al 1987). These plants were shown to have insect resistance to a variety of lepidopteran pests.

Cyt δ-endotoxins are mainly toxic to dipterans, while Cry δ-endotoxins have specificity to nematodes, coleopteran, dipteran, hymenopteran, and lepidopteran insects. Cry toxins are a diverse group with more than 50 subgroups (Bravo et al. 2007). The Cry toxins produced by the Bt bacterium have a protoxin form, which requires the proteolytic

digestion of one or both terminal ends of the protein for proper activation. Upon the ingestion of the bacterium by the insect host and sporulation in the insect midgut, the protoxin is activated by endogenous insect proteases, which cleave off the termini (Schnepf et al. 1998). The activated form of the toxin folds into a new confirmation that can bind to and insert into the brush border membrane of the insect midgut. Insertion into the brush border membrane causes the formation of pores in the midgut epithelium which leads to cell lysis and ion leakage. Eventually disruption of the midgut allows for movement of the bacteria into the hemolymph and the insect dies of septicemia (Bravo et al. 2007). Cry toxins in Bt transgenic plants are now truncated forms which lack the need for proteolytic activation (Peferoen 1997). Insects feeding on the Bt transgenic plants die from disruption of the midgut and the loss of ion channel control (Kumar et al. 1996).

Transgenic plants employing the expression of Bt δ-endotoxins have been effective at controlling a variety of insect pests. Commercialized Bt transgenic plants have focused on the use of Cry1Ab, Cry1Ac, and Cry9C for the control of lepidopteran pests (Shelton et al. 2002). Theses variants have been effective at controlling *Ostrinia nubilalis* (European cornborer) (Koziel et al. 1993), *Helicoverpa armigeria* (corn earworm) (Chakrabarti et al. 2000), *Helicoverpa* zea (corn earworm) (Burkness et al. 2001), *Diatraea grandiosella* (southwestern corn borer) (Reed and Halliday 2001), and *Plutella xylostella* (diamondback moth) (Stewart et al. 1996). Control of coleopterans such as *Leptinotarsa decemlineata* (Colorado potato beetle) has been achieved by the use of Cry3a (Coombs et al. 2003). Insect control through the use of these transgenic plants was effective for around a decade with only lab reared insects developing resistance

(Tabashnik 1994, Tabashnik et al. 1996, Perez and Shelton 1997, Tabashnik et al. 1997). The recent detection of field populations of corn earworms (*Helicoverpa zea*) with resistance to Bt Cry1Ac (Tabashnik et al. 2008) demonstrates the need for advances in the management of transgenic plants and for new alternatives in transgenic technologies.

Candidate Genes for Insect Resistance in Transgenic Plants. Insecticidal proteins including Bt endotoxins have been utilized in transgenic plants. These proteins represent a wide variety of classes including proteinase inhibitors, cholesterol oxidases, lectins and chitinases. Proteinase inhibitors interfere with digestive enzymes in the insect gut by competitive inhibition (Haq et al. 2004). Proteinase inhibitors have been shown to be effective in reducing herbivory in transgenic crops and usually result in developmental delays in the insect pest (Hoffman et al. 1992, Hilder et al. 1987, Sane et al. 1997, Xu et al. 1996). Recent studies have identified a variety of insects with the ability to quickly adapt to these inhibitors, which may reduce their usefulness (Bown et al. 2004, Briochi et al. 2007, Volpicella et al. 2006). Cholesterol oxidases are a group of enzymes with the ability to attack the insect midgut causing it to be lysed similar to the mode of action seen with Bt (Shen et al. 1997). Transgenic tobacco expressing a cholesterol-oxidase gene was demonstrated to be toxic to the boll weevil (*Anthonomus grandis*), a major pest of cotton (Corbin et al. 2001). Lectins are proteins found in a wide range of organisms with the ability to bind to sugars, They are also toxic to coleopterans, lepidopterans, and homopterans (Carlini and Grossi-de-Sá 2002). While the mode of action of these toxic proteins has not been elucidated, they have been transformed into oilseed rape and potato

and have exhibited deleterious effects on the pollen beetle (*Meligethes aeneus*) (Melander et al. 2003) and peach potato aphids (*Myszus persicae*) (Gatehouse et al. 1996), respectively. Chitinases are enzymes that degrade chitin, the main polymer of the insect cuticle and a major constituent in the peritrophic membrane (Kramer and Muthukrishnan 1997). In transgenic plants, these enzymes have been effective against insects including *Leptinotarsa decemlineata* (Colorado potato beetle) (Lawrence and Novak 2006) and tobacco budworm (Ding et al. 1998). Similar to proteinase inhibitors, α-amylase inhibitors inhibit the breakdown of sugars. Alpha-amylase inhibitors have shown limited success in transgenic plants as they are effective against a number of weevils (Franco et al. 2002). Other more recent candidate genes with proven insecticidal activity have come from pathogenic nematodes (ffrench-Constant et al. 2007), black widows (Rohou et al. 2006), and scorpions (Wang et al. 2005). While there are many candidate genes for insect resistance in plants, none of the genes discussed above have been shown to be as effective as Bt. It may be that the next generation of insect resistance strategies in transgenic plants will involve the stacking or pyramiding of multiple, less effective genes, with Bt δ-endotoxins (Christou et al. 2006), or new toxins with novel modes of action will need to be discovered.

***Beauveria bassiana*: a Candidate Organism for Insecticidal Gene Discovery.**

Beauveria bassiana is a ubiquitous soil-inhabiting entomopathogenic fungus and is the anamorph of the telomorph *Cordyceps bassiana* in the Ascomycota (Inglis et al. 2001). A variety of insects at all stages of development are susceptible hosts of *B. bassiana*

(McCoy et al. 1985). Due to its wide host range of almost 500 susceptible species of insects (Vilcinskas and Gotz 1999), *B. bassiana* has been tested as a microbial control agent against most of the economically important insect pests. Pests that have been successfully controlled by *B. bassiana* include: *Elasmopalpus lignosellus* (lesser stalk borer) (McDowell et al. 1990), *Ostrinia nubilalis* (Bing and Lewis 1991; Feng et al. 1988), *Phorodon humuli* (hop aphid) (Dorschner et al. 1991), *Trialeurodes vaporariorum* (greenhouse whitefly) (Poprawski et al. 2000), *Leptinotarsa decemlineata* (Jaros-Su et al. 1999) and a mosquito responsible for the transmission of malaria, *Anopheles stephensi* (Blanford et al. 2005). The efficacy of *B. bassiana* as a biological control agent against these insects demonstrates that the fungi's natural infection cycle, which includes the production of toxic compounds, is sufficient to cause significant mortality.

The infection cycle of *B. bassiana* begins with the contact of a conidium with the cuticle of a susceptible host. The conidium germinates and the fungus produces an array of enzymes that help degrade the outer integument. These enzymes include proteases, chitinases, and lipases. The fungus produces a germ tube that grows through the integument and toward the hemocoel. Once the hemocoel is entered, blastospore formation and toxin production begin (Boucias and Pendland 1988). As the fungus proliferates, the host dies and becomes mummified by hyphal growth that will later extrude from the cadaver through the intersegmental membranes (Pekrul and Grula 1979). Death usually occurs in three to seven days and is attributed to nutrient deficiency, water loss, or the action of toxins (Boucias and Pendland 1988).

Oral Toxicity of Beauveria bassiana.

Research on *B. bassiana* has demonstrated that this fungus may be orally toxic when ingested by lepidopterans. The first study to shed light on the subject observed that *B. bassiana* was able to grow endophytically in corn (Poaceae) (Lewis and Bing 1991) and confer resistance to insect herbivory. After foliar application to corn plants at the V8 stage, *B. bassiana* was recovered from the pith of plants. The percentage of plants with recovered *B. bassiana* was negatively correlated (r = -0.376) with insect damage per plant. Over the two-year study, plants treated with *B. bassiana* exhibited suppression of tunneling by larval European corn borer (*O. nubilalis*) ranging from 37.0 to 50.6% (Lewis and Bing 1991). In a later study, granular formulations of conidia of *B. bassiana*, applied to the foliage of corn at the whorl-stage, grew into and colonized up to 98.3% of plants (Bing and Lewis 1991). Once established in the plant, the fungus again decreased tunneling of *O. nubilalis*. Endophytic colonization of corn by *B. bassiana* showed no yield reduction or adverse effects on plants (Lewis et al. 1996). This reduction in insect herbivory may be attributed to increased plant defenses or the production of fungal compounds that serve as insect deterrents or orally active insect toxins.

Further investigation of the effects of ingested *B. bassiana* on insect pests involved corn earworm larvae (*Helicoverpa zea*) fed a synthetic diet containing dried mycelia of two *B. bassiana* isolates (3-00 and 11-98) (Leckie et al 2008). Delayed development and high mortality were observed in larvae fed the highest rates (1 and 5% w/v) of fungal diet. Weights of surviving larvae and pupae were also lower for larvae fed the higher concentrations of mycelia. After 10 days, larval mortality was 100% for the

5% mycelia diet treatment of one isolate (11-98), which was significantly greater than control diets and diets containing isolate 3-00. Some insects that died were observed to be stuck to the plastic cups by a translucent fluid that emanated from the anus of the insect. These observations may be similar to those noted by Ahmad et al. (1985), where house crickets, *Acheta domesticus* (Linnaeus), suffered from complete failure of the alimentary process due to feeding on perennial ryegrass infected with *Neotyphodium loliae,* an endophytic fungus responsible for tall fescue toxicosis. These deleterious effects were attributed to the toxic compounds in the mycelium. Differences in the effects of different isolates were attributed to the relative amounts of toxins produced by each isolate. Variations in production of toxic metabolites have been documented for a variety of entomopathogenic fungi and are not unusual (Strasser et al. 2000). These findings have prompted the evaluation of *B. bassiana* for insecticidal proteins to be utilized in transgenic plants.

Toxic Products of Beauveria bassiana.

The isolation of high molecular weight compounds produced by *B. bassiana* has revealed several toxic proteins. Two proteases were shown to be toxic when injected into *Galleria mellonella* (greater wax moth) (Kucera and Samainakova 1968). Another protein with a toxic effect when injected into *Galleria mellonella*, Bclp, was isolated from *B. bassiana* and shown to induce cuticular melanization (Fuget et al. 2004, Fuget and Vey 2004). Bassiacridin, a protein with similarity to a yeast chitin binding protein, was toxic at low dosages when injected into *Locusta migratoria* (Quesada-Moraga and Vey 2004).

Although toxic when injected into the hemoceol, none of these proteins were evaluated for oral toxicity. A single study has looked for orally toxic proteins in *B. bassiana*, and protein extracts had low toxicity (Quesada-Moraga et al. 2006).

While only a few high molecular weight toxic compounds have been isolated from *B. bassiana*, a variety of low molecular weight toxic compounds have been reported. These include beauvericin, bassianolide, and the red pigmented toxin oosporein. Cyclosporin is also produced as a secondary metabolite and is a known immunosuppressant produced by other fungi (Boucias and Penland 1998). Beauvericin, when injected into adult blowflies, *Calliphora erythrocephala* (Meig.), resulted in 15% mortality by day 2. When injected into larval yellow fever mosquitoes, *Aedes aegypti*, mortality reached 39% at 48 hours (Grove and Pople 1980). Suspensions of beauvericin added to water containing larval northern house mosquitoes, *Culex pipiens autogenicus*, killed 44% of the larvae by 48 hours (Zizka and Weiser 1993). Beauvericin, when applied to leaf disks and fed to Colorado potato beetles, had an LC_{50} of 633 ppm and an LC_{90} of 1196 ppm (Gupta et al. 1991). Conversely, beauvericin had no oral toxicity to silkworms at levels as high as 1000 ppm (Kanaoka et al. 1978). In this same study, bassianolide was administered orally to silkworms and was lethal at 8 ppm. Fermentation broth obtained from the production of *B. bassiana* and containing the red pigment oosporein caused 49.8% mortality in mealy bugs feeding on topically applied leaves (Eyal et al. 1994). To correctly evaluate the potential of *B. bassiana* to produce insecticidal proteins, these secondary metabolites would need to be ruled out as the origin of oral toxicity.

Techniques for the Testing of Insect Resistance Genes. The current technologies for the testing of insect resistance genes rely mainly on the generation of stably transformed plants. The production of these plants, which requires tissue culture, is time consuming and expensive (Li et al. 2007). An exception to this would be through rapid transformation of *Arabidopsis* such as floral dip (Sharma et al. 2005). While Arabidopsis has been shown to be an effective tool for the screening of some common insect pests, the range of insects that will readily feed on *Arabidopsis* greatly restricts its application (Santos et al. 1997).

For many years scientists have also relied on the recombinant expression of insecticidal proteins in microorganisms, such as *E. coli*, and the testing of insects through incorporation into artificial diet (Schnepf and Whiteley 1981). Since *E. coli* is prokaryotic, it lacks the ability to perform any required post-translational modifications to the proteins (Lawrence and Novak 2001). Additionally, insects fed artificial diets have been shown to have significant effects on their gene regulation (Caudron et al. 2006), which may make the interpretation of insect growth and developmental data inapplicable to transgenic plants. These unwanted effects of diet feeding tests suggest that the best method of evaluating an insect resistance gene may be in the insect's natural host plant.

Recent technologies that allow for the transient expression of genes in plants have opened new avenues for the testing of insect resistance genes. A transient expression system utilizing engineered plant viruses to produce insecticidal proteins *in planta* was suggested by Lawerence and Novak (2001). This system removed the problems of post-transcriptional modifications, as the plant transcriptional and translational processes are

employed for the production of the insecticidal protein. While a viral system of transient expression has obvious advantages (i.e., time and cost) to the previous technologies, an agroinfiltration system might prove to be more efficient.

Transient expression through agroinfiltration is a relatively simple procedure, which involves the injection of *Agrobacterium tumefaciens* containing the candidate transgene in a binary transformation vector for temporary transgene expression in leaves (Sparkes et al. 2006). Agroinfiltration has been demonstrated to be effective for transient expression in many plant species including tobacco (Shelduko et al. 2006), grapevine (Santos-Rosa et al. 2008), lettuce, tomato, *Arabidopsis* (Wroblewski et al. 2005), switchgrass (VanderGheynst et al. 2008), radish, pea, lupine, and flax (Van der Hoorn et al. 2000). Since there is a wide range of plant species susceptible to *A. tumefaciens* infection, the use of agroinfiltration for the evaluation of candidate insect resistance genes has great potential for a rapid screening on numerous target insects. Since the construction of a binary vector for the implementation of this system is required, any positive candidate genes can quickly be moved to a stable transformation system for further characterization.

Objectives

The objectives of this research were:

1. To test *Beauveria bassiana* isolate 11-98 for the production of insecticidal proteins.

2. To evaluate the growth and development of *Helicoverpa zea* and *Heliothis virescens* larvae that consume transgenic tobacco leaves expressing *Brassica oleracea* or *Manduca sexta* proteinase inhibitors.
3. To test agroinfiltration as a technique for rapidly assaying candidate insect resistance genes in plants.

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Beauveria bassiana extracts contain insecticidal proteins that are orally toxic to *Helicoverpa zea* and *Spodoptera frugiperda*[1]

[1] This manuscript will be submitted to Biocontrol.

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Abstract

Beauveria bassiana is an entomopathogenic fungus with a history of effective use as a biological insect pest control agent. Its value as a pest management tool is most often attributed to the organism's parasitic lifecycle. However, research has indicated that *B. bassiana* might contain numerous toxic compounds that would be useful as pesticides. While many studies have focused on low molecular weight secondary metabolic compounds, it is possible that a number of higher molecular weight proteins are also present that would be suitable candidates for insecticidal biocides or transgenic traits. This study evaluates protein extracts from one specific, highly insecticidal isolate (11-98) of *B. bassiana*. Proteinacious compounds were present in the fungal extracts of this isolate, which resulted in significant mortality when fed to *Plutella xylostella* (diamondback moth) and *Spodoptera frugiperda* (fall armyworm) larvae. These compounds also significantly reduced the development of larvae into adults. Treatment of the extracts with proteases significantly reduced their toxicity, suggesting that this isolate of *B. bassiana* is an suitable candidate for future research to identify potentially novel insecticidal proteins.

Introduction

Beauveria bassiana (Bals.) Vuill. is a soilborne entomopathogenic fungus with a wide host range (McCoy et al. 1985, Vilcinskas and Götz 1999). This organism has been studied and is used as a biological control agent in agriculture against many economically important insect pests (McDowell et al. 1990, Bing and Lewis 1991, Feng et al. 1988, Dorschner et al. 1991, Poprawski et al. 2000, Jaros-Su et al. 1999, Blanford et al. 2005). Early research into the mechanisms of pathenogenicity identified numerous low molecular weight (< 1.3 Kda) secondary metabolites that adversely affect insect growth and development. These compounds include beauvericins, bassianolide, cyclosporine, and oosporein (Grove and Pople 1980, Zizka and Weiser 1993, Gupta et al. 1991, Kanaoka et al. 1978, Eyal et al. 1994, Boucias and Penland 1998). More recent research has indicated the presence of higher molecular weight (> 6 Kda) proteins that are also notably toxic to insects (Fuget et al. 2004, Fuget and Vey 2004, Quesada-Moraga and Vey 2004). One protein, Bclp (28 Kda), induced cuticular melanization when injected into *Galleria mellonella* (Fuguet et al. 2004, Fuguet and Vey 2004). Another toxic protein isolated from *B. bassiana*, bassiacridin (60 Kda), is similar to chitin-binding proteins and is toxic when injected into *Locusta migratoria* (Quesada-Moraga and Vey 2004). Theses are of particular interest because the identification of an effective protein-based oral pesticide would be of significant value for agricultural pest management.

Previously reported research used either protein injection into the insect hemocoel (Kučera and Samšiňáková 1968, Fuget et al. 2004, Fuget and Vey 2004, Quesada-Moraga

and Vey 2004), which does not directly address oral toxicity, or reported low oral toxicity (Quesada-Moraga et al. 2006). The objective of this study was to evaluate the oral toxicity of protein extracts (> 5 kDa) from a specific *B. bassiana* isolate (11-98) on diamondback moth (*Plutella xylostella* L.), corn earworm (*Helicoverpa zea*), and fall armyworm (*Spodoptera frugiperda*) larvae. Preliminary research on this isolate has indicated a significant level of insect mortality when fed to neonate diamondback moth larvae. This study tests the hypothesis that the oral toxicity is, in part, due to the presence of insecticidal proteins.

Materials and Methods

Fungal material. *B. bassiana* isolate 11-98 was obtained as a sporulating culture grown on Sabouraud dextrose agar + 0.5% yeast extract (SDAY) from Dr. Bonnie H. Ownley. Conidia were collected by lightly brushing culture with a stencil brush and then stored on silica gel at -80˚C. *B. b*assiana 11-98 was originally collected from an Elaterid beetle in Scott County, TN, USA.

Bt production. *Bacillus thuringiensis* (Berliner) strain HD-73 was used as a positive control and was received from Dr. Juan Jurat-Fuentes. *B. thuringiensis* was grown, and purified Cry1ac protein extracted as described in Luo et al. (1999).

Toxigenic fungal material. To obtain a maximally toxic state, *B. bassiana* 11-98 was passed through a full infection cycle on *P. xylostella* as described here. Neonate *P.*

xylostella (Benzon Research Inc., Carlisle, PA) were reared to second instar on artificial diamondback moth diet (Bio-Serv Inc., Frenchtown, NJ). Insects were then washed with deionized H_2O and spores of *B. bassiana* 11-98 were applied topically to the insect with a size 10/0 camel hair paint brush. Larvae were then placed in a 60 ×15 mm Petri dish on moist filter paper and incubated at 24˚C for approximately 4 wk. A mixture of conidia and mycelial growth from the cadavers were collected with a wire loop and used to inoculate SDAY plates (Goettel and Inglis 1997). Plates were placed in a 24˚C incubator until sporulation (approximately 3 wk). Conida were then harvested and stored on silica gel at -80˚C. After passage through the first infective cycle, cultures were labeled 11-98 *Px*. Cultures passed through a second infective cycle were labeled 11-98 *Px*2. Cultures of 11-98 *Px*2 were submitted to the ARS NRRL collection (Peoria, Illinois), which is archived as stock # 30872.

Growth and Storage of Fungus. A 25 ml starter culture of SDY liquid medium was inoculated with spores from isolate 11-98, 11-98 *Px*, or 11-98 *Px2* and grown for 5 d on a rotary shaker (150 rpm) at room temperature. The starter culture was used to inoculate 12 L of SDY media divided into 12 two liter flasks. Theses cultures were grown for either 6 or 28 d. Fungal mycelia were collected by centrifugation at 6000 × g for 30 min and then snap frozen with liquid nitrogen before storage at -80˚C.

Protein Extraction. Mycelium collected from *B. bassiana* isolates 11-98, 11-98 *Px*, or 11-98 *Px2* were ground in liquid nitrogen via mortar and pestle. Proteins were extracted

using a HEPES buffer protein extraction protocol (Markham et al. 2006). The protein extracts were dialyzed for 24 h using 3500 MWCO SnakeSkin® pleated dialysis tubing (Pierce Biotechnology, Rockford, IL) to remove secondary metabolites and other low molecular weight compounds. Proteins were dialyzed in 20 L of buffer for 24 h. Buffer was changed twice (2 and 4 h) during dialysis. The resulting extracts were concentrated to 10 mg protein per ml buffer using a VivaSpin-20 centrifugal concentrator MWCO 5000 (Sartorius Corporation, Edgewood, NY). This process was repeated until a sufficient quantity of protein had been collected to allow for the insect bioassays post fractionation. Protein concentrations were determined with coomassie plus (Pierce, Rockford, IL).

Gel Electrophoresis. SDS PAGE was performed in a Mini Protean III system (Bio-Rad, Richmond, CA). A 5% stacking gel and 12% resolving gel were used to separate proteins. Two micrograms of each sample were added to equal volumes of sample loading buffer (10% Glycerol, 0.05M Tris Cl pH 6.8, 0.005% Bromophenol blue, 1% SDS) prior to loading. Pre-stained Novex sharp prestained protein ladder (Invitrogen, Carlsbad, CA) used for size estimation. Gels were run at 110 v for 1.5 h. Gels were stained with a modified plusone silver stain protocol (GE Healthcare, Buckinghamshrire, UK).

HPLC Procedure. HPLC was performed to detect the presence of the known secondary metabolic compound beauvericin (0.783 Kda) to confirm the efficacy of dialysis. Four

samples were analyzed; crude extracted protein (90 mg), dialyzed high molecular weight fraction (32 mg), buffer with beauvericin (500 μg), and buffer alone. Samples were lyophilized and dissolved in 250 ml of methanol. Precipitated proteins were removed by filtration. Methanol extracts were dried and residue was suspended in 1 ml methylene chloride. Columns were prepared as described in Leckie et al. (2008). Samples were analyzed by LC using a Waters 1525 binary pumping system with a 717 autosampler and a SPD-10AV UV-Vis detector (absorbance = 204 nm) (Shimadzu Corp., Kyoto, Japan). Mobile system was acetronitrile:water (80:20). A standard curve was generated for estimation of beauvericin quantities from the buffer + beauvericin sample.

Fungal Activation Bioassay. The effect of consecutive infective cycles on the oral toxicity of *B. bassiana* was evaluated in a bioassay with larval *P. xylostella*. Eggs received from Benzon Research, Inc. (Carlisle, PA) were placed into 160 cm^3 plastic containers coated with a layer of artificial diamondback moth diet on the bottom of the cup. The larvae were hatched and allowed to feed on standard diet for 24 h before being transferred to bioassay cups for experimentation. Protein extracts from the 28 d growth of *B. bassiana* strain 11-98, 11-98 *Px*, and 11-98 *Px*2 were prepared and each was incorporated into artificial diet at a rate of 1 mg/ml. Additionally, a diet containing only protein extraction buffer was used as a negative control. A 1 cm^3 square of diet was placed onto a moist piece of filter paper in a plastic specimen cup. Ten neonate *P. xylostella* larvae were placed in each cup and then sealed with the lid. Each of the treatments consisted of five replicate cups. Cups were arranged in a complete

randomized design and placed into an incubator (24˚C, 12:12-h light: dark). Additional treated diet was added as needed to maintain freshness. After 11 d, the experiment was terminated and larval mortality recorded.

Protease Bioassay. A protease treatment was applied to the *B. bassiana* extracts in order to determine if the observed oral toxicity was a result of protein activity. Purified Bt Cry1Ac protein and protein extracts from 6 and 28 d growth of *B. bassiana* 11-98 *Px*2 were used in these assays. The 6 day growth period was added to evaluate the rapid growth that would occur in an infected insect.

Beauveria bassiana (10 mg/ml) and Cry1Ac (0.26 mg/ml) protein extracts were divided into two 2 ml aliquots. One of the 20-mg samples was exposed to Pronase protease cocktail (Roche Applied Science, Indianapolis, IN) at 1 mg/ml for 1 h at 40˚C. The proteases were then inactivated by a heat treatment at 85˚C for 10 min. Protease digestion was confirmed by SDS-PAGE gel electrophoresis. Protein from both digested and undigested *B. bassiana* extracts were incorporated into the diet at 1 mg/ml. Cry1Ac protein diets had a rate of 0.026 mg/ml of diet. Quantities of digested protein samples added to the diet were based on protein concentrations prior to digestion. Extraction buffer samples with and without proteases were prepared as negative controls and added to diets at volumes equal to *B. bassiana* samples. The bioassays were conducted in the same manner as described above except that each treatment consisted of ten replicates of ten insects per replicate. Larval mortality was recorded on day 11.

Adult Development Assay. In addition to larval mortality, data were collected on the impact of *B. bassiana* isolate 11-98 *Px2* on adult development. The bioassay was conducted as described above except that it was allowed to run for 28 d (sufficient time for the diamondback moths to pupate). The experiment consisted of four treatments; an extraction buffer control, Cry1Ac, and two concentrations of *B. bassiana* 11-98 *Px2* protein extract (1 mg/ml and 0.1 mg/ml of diet). The Cry1Ac positive control diet had a rate of 0.07 mg/ml. The bioassay was performed in plastic containers, and each treatment consisted of 20 replicates with five insects each. Larval mortality was recorded on day 7 and the number of adult insects present on day 28.

Fall Armyworm and Corn Earworm Assay. *Beauveria bassiana* protein extract diets (1 mg/ml) and protease-treated diets were evaluated on fall armyworm (*S. frugiperda*) and corn earworm (*H. zea*) larvae. Bioassays were conducted in 16 cell plastic trays (Bio-Serv Inc., Frenchtown, NJ) with a single insect and approximately 0.2 cm^3 of diet per cell. The experiment was performed as a randomized block design, each treatment consisted of 8 trays (block) containing 16 replicates (128 insects per treatment). Diets were prepared as described above except that fall armyworm diet (Bio-Serv Inc., Frenchtown, NJ) was used. Mortality of larvae was recorded on day 7.

Statistical Analysis. Analysis of variance (ANOVA) was performed with the mixed procedure of SAS 9.13 (SAS Institute 2003) and mean separation conducted using Fisher's least significant difference. Cry1Ac data in the adult survivorship assay were

not included in statistical analysis because of unequal variances in the analysis, which violates the assumptions of ANOVA.

Results

Fungal Activity Assay. Insects fed the protein extracts from *B. bassiana* isolate 11-98 had significantly higher mortality than controls, ($F = 24.07$; $df = 3, 21$; $P < 0.0001$) (Fig. 1.1). Passage of this isolate through an infective cycle (11-98 *Px*) in *P. xylostella* produced a significantly increased level of mortality (86%). Undergoing a second infective cycle (11-98 *Px*2) did not significantly increase larval mortality of the fungal extracts (80%).

Protease Bioassay. Treatment of *B. bassiana* 11-98 *Px*2 proteins with Pronase successfully degraded the protein extract (Fig. 1.2) and significantly reduced oral toxicity to *P. xylostella* ($F = 46.58$; $df = 7, 92$; $P < 0.0001$) (Fig. 1.3). Both the 6 and 28 d growth protein extracts were orally toxic; larvae fed these diets had 72 and 80% mortality, respectively. When treated with protease, mortalities were reduced significantly to 45 and 42%, respectively. The inactivated Pronase control did not impact larval mortality. In our controls, purified Cry1Ac caused 99% mortality, while pronase-treated Cry1Ac was inactive (25% mortality, similar to buffer control).

Adult Development. Diet containing extracts from isolate 11-98 *Px2* caused a significant reduction of the number in insects reaching adult stage by day 28 ($F = 84.17$;

df = 2, 117; P < 0.0001) (Table 1.1). Only 0.5% of insects consuming the 1.0 mg/ml concentration of *B. bassiana* protein extract had developed into adults by day 28 in comparison to 54% of the buffer controls. While only 10% of the larvae died when consuming the 0.1 mg/ml concentration of the protein extract (F = 279.22; df = 3, 136; P < 0.0001), only 21% developed into adults by day 28, suggesting a delay of insect development.

Fall Armyworm and Corn Earworm Assay. Fall armyworm larvae fed diets containing *B. bassiana* isolate 11-98 *Px*2 had significantly higher mortality (48%) than controls (F = 27.27; df = 3, 28; P < 0.0001) (Fig. 1.4). Protease treatment of *B. bassiana* extracts resulted in a significant decrease in larval fall armyworm mortality (20%). Corn earworm larvae fed both *B. bassiana* isolate 11-98 *Px2* protein (2.3%) and protease-treated protein (1.6%) extracts had low mortalities and were not significantly different from the controls (data not shown).

Beauvericin Quantification. Beauvericin was detected only in the crude protein extract (Fig. 1.5) and the buffer + beauvericin control samples. Levels of this metabolite were estimated at 0.443 μg/mg of crude protein based on the standard curve. Beauvericin could not be detected in the dialyzed high molecular weight fraction, indicating the removal of this metabolite by dialysis.

Discussion

In this study, high molecular weight protein extracts from a specific isolate of *B. bassiana* were orally toxic to diamondback moth and fall armyworm larvae. While the origin of this toxicity has not been identified, the removal of all known orally toxic compounds by an effective dialysis procedure (Figs. 1.2 and 1.5) revealed a toxic compound/ compounds with previously unreported levels of toxicity. This information together with a protease treatment resulting in significant reductions in oral toxicity, suggest that a large portion of that toxicity is proteinacious in origin. These are encouraging results that warrant further investigation in order to determine and characterize the specific insecticidal compound(s). Our results are similar to those reported by Quesada-Moraga et al. (2006), where extracts of two isolates of *B. bassiana* were orally toxic to *Spodoptera littoralis* (Boisduval). These isolates resulted in 20-35% larval mortality when added to artificial diets at a rate of 1.8 mg/ml. Although tested on different organisms, *P. xylostella* and *S. frugiperda,* isolates 11-98 *Px* and 11-98 *Px*2 exhibited higher insect mortality (86 and 48%, respectively) (Figs. 1.1 and 1.4) at almost half of the concentration tested on *S. littoralis* (Quesada-Morgana et al. 2006). It is possible that passing the isolate through an infectious cycle increased the toxicity of the strain and it may have the same effect for the strains previously reported (Quesada-Moraga and Vey 2003). Alternatively, it is also possible that *B. bassiana* isolate 11-98 is more toxic than previously reported *B. bassiana* isolates. *Beauveria bassiana* isolate 11-98 was selected for this study because of its high level of toxicity in comparison to other *B. bassiana* isolates when dried mycelia were fed to *H. zea* larvae (Leckie et al. 2008). Interestingly,

H. zea larvae were unaffected by diets containing *B. bassiana* 11-98 proteins (data not shown). This may mean the secondary metabolic products located in *B. bassiana* mycelia are highly toxic to *H. zea*, whereas the proteins have no oral toxicity to these larvae. This lack of toxicity to *H. zea* indicates that the orally toxic protein(s) in *B. bassiana* 11-98 have species specificity. Similarly, bassiacridin was demonstrated to be toxic to *L. migratoria*, *Schistocerca gregaria* (Forsskål), and *Dociostaurus maroccanus* (Thunberg), while exhibiting no toxic effect on *S. littoralis* and *Tenebrio molitor* (L.) (Quesada-Moraga and Vey 2004). A study testing high molecular weight protein extracts from isolate 11-98 against several other *B. bassiana* isolate and with a number of insect species, including *S. littoralis*, would provide further insight into isolate specific insecticidal activity.

B. bassiana 11-98 is a multispore isolate collected from an Elaterid beetle in Scott County (TN, USA) that had been cultured on artificial medium prior to and after its storage in a -80˚C freezer stock. The virulence of an entomopathogenic fungus has been shown to decline as a result of repeated subculturing on artificial media (Schaerffenberg 1964, Morrow et al. 1989) and may be enhanced by *in vivo* passage (Hayden et al. 1992, Wasti and Hartmann 1974). In order to ensure that the isolate was at optimal virulence for evaluation it was passed through two successive infective cycles in *P. xylostella* larvae. The passage of the isolate through a host significantly increased oral toxicity (Fig. 1.1), which is consistent with research showing a similar phenomenon using injection into the hemocoel (Quesada-Moraga and Vey 2003).

A protease treatment of the *B. bassiana* protein extracts was effective in degrading the *B. bassiana* proteins (Fig. 1.2) and significantly reduced the oral lethality of extracts (Fig. 1.3), suggesting that proteins are, in part, responsible for the observed insecticidal activity. Quesada et al. (2006) observed similar reductions in oral toxicity of *Metarhizium anisopliae* proteins, where the efficacy of proteolytic digestion to reduce insecticidal activity varied based on the type of protease. The growing body of evidence suggests that high molecular weight proteins with insecticidal activity exist in at least some isolates of *B. bassiana* and it would be valuable to continue research into the discovery of the specific compounds responsible for the activity and elucidate their mode of action. This result is significant because an effective protein-based pesticide could have value as a potential biocide or transgenic trait for pest management.

Acknowledgements

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Appendix

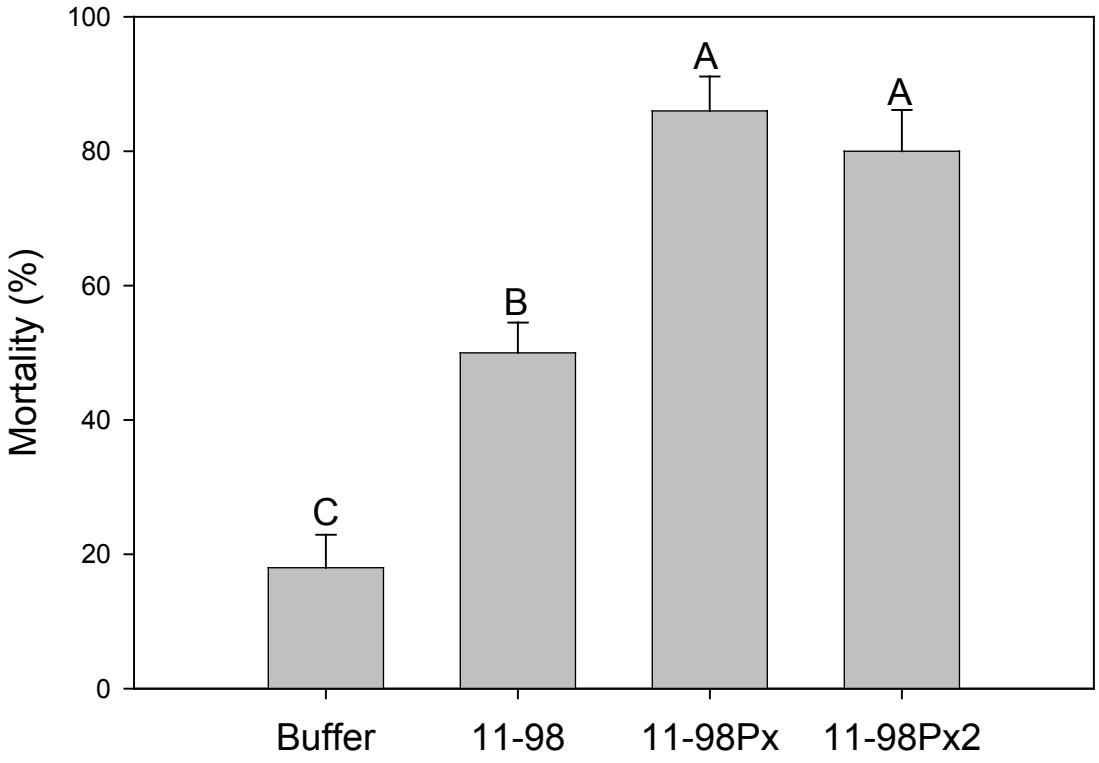

Figure 1.12. The percent mortality after 11 d of diamondback moth larvae fed synthetic diet containing protein extraction buffer, protein extracts (1 mg/ml of diet) from *B. bassiana* prior to passage through an infective cycle (isolate 11-98), after a single passage through an infective cycle (11-98 *Px*), or after passage through two infective cycles (11-98 *Px2*). Error bars indicate the standard error of the mean and bars with the same letters are not significantly different ($p < 0.05$).

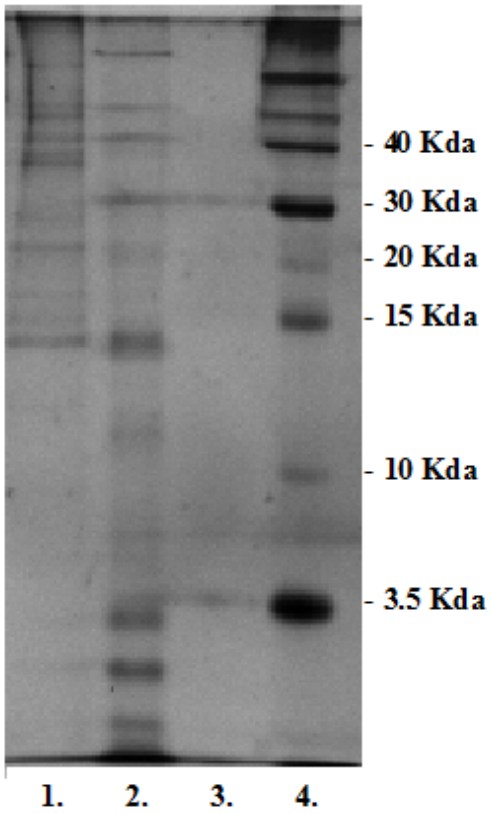

Figure 1.2~~3~~. Comparison of *B. bassiana* 11-98 Px2 1. dialyzed protein extracts, 2. crude protein extracts, 3. protease treated dialyzed protein extracts, and 4. protein ladder from on an SDS PAGE gel.

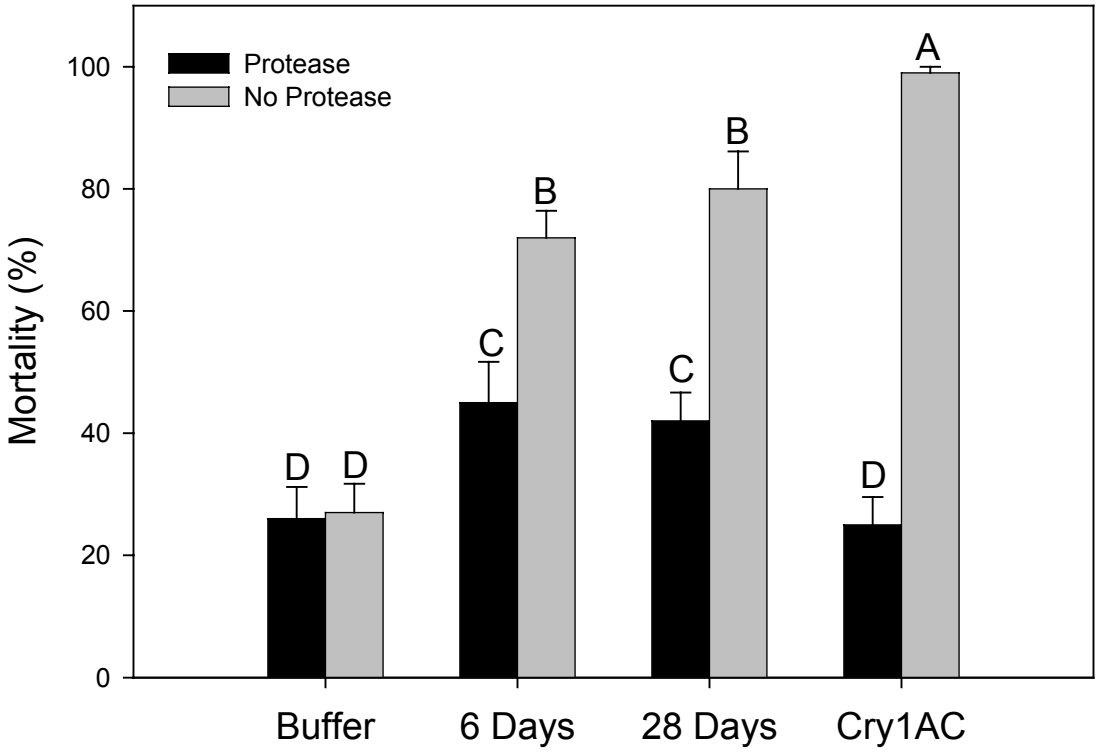

Figure 1.34. The percent mortality after 11 d of diamondback moth larvae fed synthetic diet containing protein extracts (1 mg/ml of diet) from 6 and 28 day growth of *B. bassiana* isolate 11-98 *Px2* or Cry1Ac (0.026 mg/ml of diet) with (dark bars) and without (light bars) a protease treatment. Error bars indicate standard error of the mean and bars with the same letters are not significantly different ($p < 0.05$).

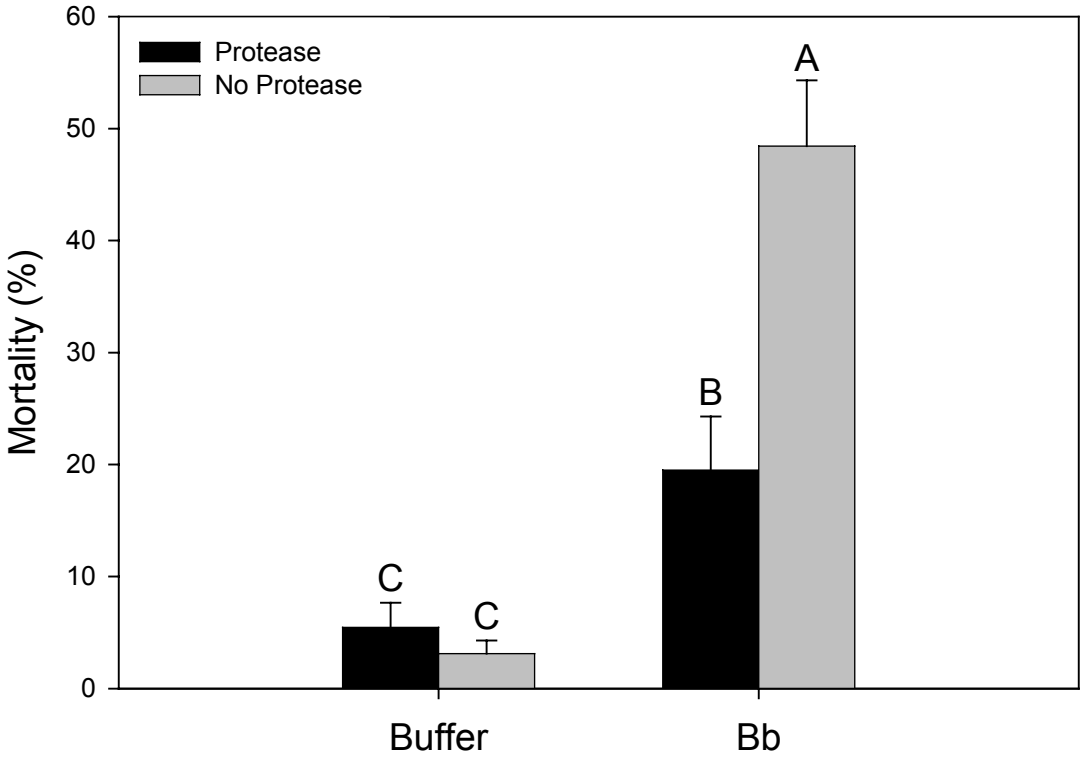

Figure 1.~~45~~. The percent mortality after 7 d of fall armyworm larvae fed synthetic diet containing protein extracts (1 mg/ml of diet) from *B. bassiana* isolate 11-98 *Px2* with and without a protease treatment. Error bars indicate standard error of the mean and bars with the same letters are not significantly different ($p < 0.05$).

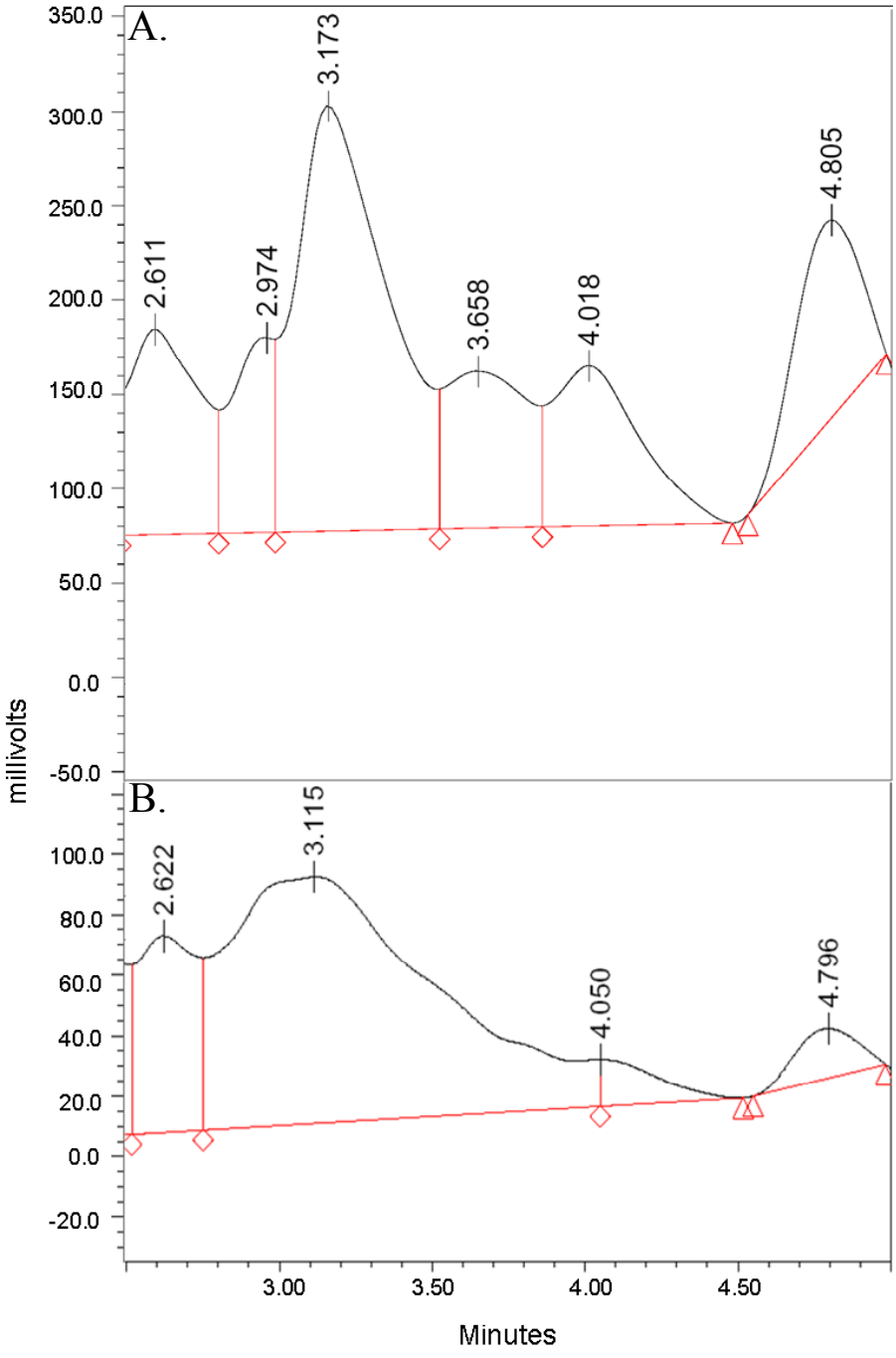

Figure 1.5~~6~~. HPLC chromatogram of *B. bassiana* A. crude protein extract and B. dialyzed high molecular weight fraction. Retention time for beauvericin is 3.658 minutes. Lyophilized extract was processed by flash chromatography as previously described for beauvericin isolation (Leckie, 2008). Mobile system was acetronitrile:water (80:20), and detection was at 204 nm.

Table 1.1. The percent mortality (day 7) and percent of developed adults (day 28) of diamondback moth larvae fed synthetic diet containing protein extraction buffer, protein extracts from *Beauveria bassiana* isolate 11-98 *Px2*, and *Bacillus thuringiensis* Cry1Ac endotoxin. Values are reported with standard error of the mean and letters denote significance ($p < 0.05$).

Diet Treatment	Protein Extract (mg/ml)	Larval Mortality (%)	Adults (%)
Buffer	0	4.5 ± 1.3^{a}	54.0 ± 3.9^{a}
Bb 11-98 *Px2*	0.1	10.0 ± 3.0^{b}	21.0 ± 3.2^{b}
Bb 11-98 *Px2*	1.0	77.5 ± 2.9^{c}	0.5 ± 0.5^{c}
Cry1Ac	0.07	99.0 ± 1.0^{d}	0

Growth and development of *Helicoverpa zea* and *Heliothis virescens* larvae that consume transgenic tobacco leaves expressing *Brassica oleracea* or *Manduca sexta* proteinase inhibitors

Abstract

Transgenic plants expressing proteinase inhibitors have been shown to be effective at controlling lepidopteran insect pests. Proteinase inhibitors from *Brassica oleracea* (BoPI) and *Manduca sexta* (VK32) were expressed in transgenic tobacco. Bioassays were performed on *Helicoverpa zea* (corn earworm) and *Heliothis virescens* (tobacco budworm) to evaluate these transgenic plants for the ability to control herbivorous insects. Larval *H. zea* and *H. virescens* were apparently able to adapt to the proteinase inhibitors since little negative effects were shown, which contrast with earlier data. While *H. virescens* developed similarly to the control insects, *H. zea* grew significantly larger on both BoPI and VK32 plants.

Introduction

Proteinase inhibitors are proteins synthesized in plant tissue that play a defensive role against herbivorous insects (Schuler et al. 1998). Their importance was first identified in tomato where they rapidly accumulated in response to mechanical or insect wounding (Green and Ryan 1972, Jongsma and Bolter 1997). These defensive compounds have since been shown to competitively inhibit the activity of proteases produced by various herbivores (Christeller et al. 1992, Hilder et al. 1987, Johnston et al. 1995).

Proteinase inhibitors act against specific groups of endoproteases that cleave internal sites of proteins and are classified based on the composition of their active sites. The common classes of proteases are aspartic, cysteine, serine, and metallo proteases (Carlini and Grossi-de-sá 2002), which contain a signature aspartate, cysteine, serine, or metal ion (Zn, Co, or Mn) in their respective active sites. In plants, serine proteases are involved in various processes including programmed cell death, tissue differentiation, and senescence (Palma et al 2002). In the lepidopteran gut, serine proteases are the predominate type of proteases accounting for up to 95% of proteolytic activity (Srinivasan et al. 2006) and commonly have trypsin-, chymotrypsin-, or elastase-like activity. A large number of serine proteinase inhibitors have been identified in plants. One of the most studied types of inhibitors is the Kunitz-type, which usually contains a single active site (Haq et al. 2004). A well characterized member of this inhibitor family is the soybean Kunitz trypsin inhibitor, which has been shown to effectively inhibit *in vitro* trypsin activity in both *H. zea* and *H. virescens* (Purcell et al. 1992). *In vitro*

activity against the proteases of these major insect pests makes them candidates as insect resistance genes for plant improvement.

Transgenic plants expressing serine proteinase inhibitors have been effective at enhancing insect resistance toward a variety of lepidopteran pests including *Manduca sexta* (Hilder et al. 1987, Johnson et al. 1989), *Chrysodexis eriosoma* (McManus et al. 1994), *Spodoptera litura* (Yeh et al. 1997), *Seramia inferens*, and *Chilo supressalis* (Xu et al. 1996). One serine proteinase inhibitor isolated from cabbage, *Brassica oleracea* (BoPI) shows 30% sequence similarity and contains a signature amino-terminal motif of the soybean Kunitz trypsin inhibitor-3 (Jofuku et al. 1989). In a previous study, this inhibitor was shown to have a high level of anti-trypsin activity and was effective at controlling two lepidopteran generalists, *H. zea* and *H. virescens* (Pulliam et al. 2001).

The present study focuses on the growth and development of *H. zea* and *H. virescens* during an extended bioassay on transgenic tobacco expressing BoPI proteinase inhibitors. In addition, a *Manduca sexta* proteinase inhibitor (VK32), which has been demonstrated to control *Bemisia tabaci* in transgenic tobacco (Thomas et al. 1995) and has anti-chymotrypsin activity (Pulliam et al. 2001) was evaluated. Transcription of BoPI in transgenic lines was confirmed by real time PCR. The hypothesis is that the transgenic lines expressing proteinase inhibitors will negatively affect the growth, development, and survivorship of larvae, when compared to insects fed wild-type (Xanthi) plant tissue. The experiments focus on two individual lines with characterized proteinase inhibitor activity (BoPI 2 and BoPI 7) and an additional uncharacterized line

(BoPI 15). We also tested the hypothesis that insects can adapt to these PIs, which would render them ineffective as insect resistance genes in transgenic plants.

Materials and Methods

Transgenic Plants. Five lines of T_1 transgenic tobacco (*Nicotiana tabacum* cv. Xanthi) expressing BoPI (*Brassica oleracea* serine proteinase inhibitor) (BoPI 2, BoPI 6, BoPI 7, BoPI 8, and BoPI 15) and a single line of VK32 (*Manduca sexta* serine proteinase inhibitor) were produced as described in Pulliam et al. 2001. Seeds from T1 plants were surface sterilized and transgenic segregants were selected on Murashige and Skoog medium (1962) (MS) containing 200 mg/L kanamycin. T_1 plants exhibiting kanamycin resistance were allowed to self-pollinate and were grown to maturity. T_2 seed was collected from T_1 plants. Homozygous lines were confirmed by screening sterilized T_2 seeds on MS medium containing 200 mg/L kanamycin, and the homozygous lines were used for further research. Wildtype tobacco (*Nicotiana tabacum* cv. Xanthi) and a high expressing homozygous line of pSAM 12 transgenic tobacco expressing both Cry1Ac and GFP5er (Bt/GFP) (Harper et al. 1999) were used as negative and positive controls, respectively.

Plants for insect bioassays and RT-PCR analysis were started from seed and grown in a growth chamber at 27˚C with 16 h light and 8 h dark photoperiod. Plants were watered and fertilized as needed. After 3 wk plants were transplanted to 4-L pots and allowed to grow for approximately 1 month before bioassays were performed.

Insects. Eggs of *H. zea* and *H. virescens* were obtained from Benzon Research (Carlisle, PA). Insect eggs were placed in a large plastic containers until they hatched. Neonate larvae were then transferred to 128-well insect trays containing synthetic fall armyworm diet (Bio-Serv Inc., Frenchtown, NJ) and held for three days before bioassays were performed.

RNA Extraction. Total RNA was extracted using an RNeasy Plant Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. Tissue samples were excised from the first true leaves of transgenic tobacco plants. Tissue was frozen in liquid nitrogen and stored in a -80˚C freezer until extraction.

Tissue for transgene expression comparisons between each of the five BoPI lines (BoPI 2, BoPI 6, BoPI 7, BoPI 8, and BoPI 15) was collected as 25 milligram samples from four plants per line and pooled for total RNA extraction and further analysis. Tissue from the first true leaf of the three BoPI plants (BoPI 2, BoPI 7, and BoPI 15) used in the second feeding assay were collected as separate 100 milligram samples.

Real Time PCR. cDNA for real time PCR analysis was generated by reverse transcription of five micrograms of total RNA using the Superscript III first-strand synthesis kit (Invitrogen Corporation, Carlsbad, CA) and oligo(dT) primers. Real time PCR was performed using an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Real time PCR reactions were carried out in 20 microliter reaction volumes consisting of gene specific primers and Power SYBR Green PCR

master mix (Applied Biosystems, Foster City, CA). The gene specific primers for BoPI were TCCCGTGAAATTCTCAAACTGG (fwd) and ACTGAGCGCAGATCGTAGGTTC (rev), and were designed with primer express software (Applied Biosystems, Foster City, CA). The reference gene used in RT-PCR analysis was ubiquitin. The ubiquitin specific primers were described in Lacomme et al. (2003). The Ct values were recorded for both the transgene and reference gene for further analysis.

RT-PCR was performed to compare transgene expression from each of the pooled samples from the original five BoPI lines (BoPI 2, BoPI 6, BoPI 7, BoPI 8, and BoPI 15). The second experiment compared transgene expression between each of the three plants per line (BoPI 2, BoPI 7, and BoPI 15) that were used in the second corn earworm feeding assay. Normalized Ct values from each of the RT-PCR experiments were analyzed in paired t-tests using the mixed procedure of SAS 9.13 (SAS Institute 2003) (Yuan et al. 2008). Ratios of expression were determined as described in Yuan et al. (2008).

Insect Bioassays. A total of three plants from each line (Xanthi, VK32, BoPI2, BoPI7, BoPI 15, and Bt/GFP) were evaluated individually in replicate corn earworm bioassays. The experimental design included 10 larvae per treatment, six treatments per replicate, three replicates with one plant per line tested per replicate. Each plant was grown as described above. Ten leaf discs (5.8 cm^2) were obtained from a single leaf from each plant. Each leaf disc was placed in a plastic cup (29.6 cm^3) (Bio-Serv Inc., Frenchtown,

NJ) containing moist filter paper. A single early second instar corn earworm larva was placed on each leaf disc. Cups were sealed with a lid and held at RT for 17 days. On the fourth day of the bioassay, leaf discs were collected to quantify insect herbivory. Larger pieces of leaf tissue from the respective plants were substituted for the removed piece. Plant tissue was frequently added to maintain a constant source of food throughout the experiment. Larval mortality and weights were recorded on days 4, 9, and 17. Larval head capsule width was recorded on day 17 and the experiment was terminated. Insect bioassays were performed also using tobacco budworm. The tobacco budworm bioassay was performed as described above, except with only two replicates. Larval weight, head capsule size, and larval mortality were analyzed with analysis of variance using the mixed procedure of SAS 9.13 (SAS Institute 2003) and mean separation conducted using Fisher's least significant difference. Tobacco budworm larval mortality was analyzed using the Glimix procedure of SAS 9.13 (SAS Institute 2003) due to its bimodal distribution.

Leaf Disc Analysis. Leaf discs collected on day 4 of each experiment were scanned with an HP Photosmart C4100 series scanner at 200 pixels per inch. Scanned images were imported into SigmaScan Pro (Systat Software, Inc., San Jose, CA), where the area of each disc was recorded and converted to cm^2.

Results

Real Time PCR. Comparisons of normalized Ct values of transgenic BoPI lines reveal no significant differences between lines, although BoPI 15 plants had the largest average ratio of expression (2.14), when compared to the internal reference gene ubiquitin (Fig. 2.1). Comparisons within lines of plants used in corn earworm bioassays showed no significant differences. Wild-type samples in all tests were negative for transgene amplification.

Corn Earworm Bioassay. Tobacco lines expressing proteinase inhibitors (BoPI and VK32) and wild-type (Xanthi) tobacco fed to larval *H. zea* resulted in significantly lower insect mortalities than insects fed transgenic tobacco expressing Cry1Ac (Bt/GFP) ($F = 5.24$; $df = 5, 12$; $P = 0.0088$) (Fig. 2.2). While all of the Bt/GFP fed insects died before day 4 (data not shown), insects fed tissue from other plant lines had statistically similar mortalities on day17. These mortalities ranged from 33.3 to 53.3% in wild-type and Line BoPI 7, respectively.

Leaf punches collected from containers with living insects on day 4 had no significant differences in the remaining total leaf punch areas ($F=1.80$; $df= 4, 98$; $P=0.1344$) (Fig. 2.3). Punches from VK32 plants (5.12 cm^2) had the smallest remaining mean leaf disc area. The largest average total leaf punch area measured was from BoPI 15 plants (5.42 cm^2).

In general, weights of *H. zea* larvae fed the plant lines expressing proteinase inhibitors were larger throughout the entire experiment (Fig. 2.4). Larval *H. zea* weights

recorded on Day 4 (F=2.64; df= 4, 98; P=0.0380) revealed that insects feeding on two transgenic plant lines, BoPI 15 (3.7 mg) and VK32 (3.6 mg), were significantly larger than those feeding on wild-type plant tissue (2.2 mg) (Fig. 2.5). *H. zea* larval weights taken on Day 9 were similar to data recorded on day 4, with insects fed tissue from BoPI 15 (32.2 mg) and VK32 (31.1 mg) plants having greater larval weights (F= 3.44; df= 4, 88; P=0.0117) than insects fed the wild-type (12.3 mg) or BoPI 2 (15.0 mg) plants (Fig. 2.6). By day 17, insects fed tissue from BoPI 15 (369.2 mg) plants were over 1.5 times the size of insects fed the wild-type (233.8 mg) plants (Fig. 2.7). *H. zea* larvae fed tissue from VK32 (326.5 mg), BoPI 7 (349.4 mg), and BoPI 15 plants were all significantly (F=2.92; df= 4, 83; P=0.0258) larger than the wild-type plants at the conclusion of the experiment.

Larval *H. zea* feeding on the transgenic and wild-type plant lines showed no significant differences in head capsule size at the conclusion of the experiment (F=0.88; df= 4, 83; P=0.4798) (Fig. 2.8). All mean head capsule sizes for insects ranged from 2.6 mm to 2.8 mm indicating that these larvae were in their 5^{th} and 6^{th} instar (Capinera 2000a).

Tobacco Budworm Bioassay. Larval *H. virescens* fed wild-type plant tissue or transgenic tobacco lines expressing proteinase inhibitors (BoPI and VK32) had significantly less mortality than insects fed transgenic plants expressing Cry 1Ac (Bt/GFP) (F=547.39; df= 5, 18; P<0.0001) (Fig. 2.9). Insects fed Bt/GFP plant tissue had complete mortality (100%), while all *H. virescens* fed on other plant lines survived.

Leaf discs fed to larval *H. virescens* and collected on day 4 of the experiment had significant differences in defoliation (F= 13.66; df= 4, 94; P<0.0001) (Fig. 2.10). Larval *H. virescens* fed wild-type and VK32 leaf punches ate significantly more leaf tissue than all other groups; remaining mean leaf areas were 3.70 and 3.93 cm^2, respectively. The largest remaining mean leaf disc areas were found for those insects fed BoPI 2 (4.99 cm^2) and BoPI 7 (4.91 cm^2), these were significantly larger than all other mean leaf disc areas.

Larval *H. virescens* fed different transgenic plant lines expressing proteinase inhibitors showed differences in weights early in the experiment but those differences had diminished by the end (Fig. 2.11). Larval *H. virescens* had significantly different mean weight by day 4 (F= 5.24; df= 4, 94; P=0.0007) (Fig. 2.12) with insects fed wild-type (22.3 mg) plant tissue having larger weights than insects fed all BoPI plant lines. Larval *H. virescens* weights on day 9 continued to have differences between treatments (F= 2.12; df= 4, 94; P= 0.001) (Fig. 2.13). Larval *H. virescens* fed wild-type (93.9 mg) plant tissues had the largest recorded weights on day 9, which were significantly larger than those larvae feeding on BoPI 2 (60.6 mg) or BoPI 15 (62.5 mg) plant tissue. By the close of the experiment, there were no significant differences in larval weights (F=1.44; df= 4, 94; P=0.2268) (Fig. 2.14).

Similar to *H. zea* larvae, *H. virescens* larvae showed no significant differences in head capsule sizes at the conclusion of the experiment (F= 1.09; df= 4, 94, P=0.3656) (Fig. 2.15). Mean larval *H. virescens* head capsule sizes ranged from 2.3 to 2.4 mm for all treatments indicating they were primarily in the 6^{th} instar of development (Capinera 2000b).

Discussion

The expected negative effects on insects feeding on transgenic tobacco expressing proteinase inhibitors were not seen in this study, with the exception of some early delayed development in those *H. virescens* feeding on transgenic BoPI lines. The majority of insects grew at rates similar to or greater than those fed wild-type tissue, which may be the result of the insects adapting to the proteinase inhibitors.

These results are extremely different than those reported in Pulliam et al. (2001), where both *H. zea* and *H. virescens* experienced high levels of mortality when fed transgenic BoPI and VK32 lines. We expected to see improvements in insect control compared to the previous study as Pulliam et al. (2001) used both hemi- and homozygous lines in their assays. A number of factors may have played into our reductions in insect mortality. First, we allowed neonates to feed on artificial diets until they reached the second instar of development, this step was added to lower the levels of background mortality observed in insects fed on wild-type tobacco tissue immediately after hatching (data not shown). While this waiting period may have improved background mortality, it may also have allow the neonate larvae to develop past crucial stage of development when proteinase inhibitor activity could have been fatal; i.e., they might be developmentally able to mount a counterdefense against proteinase inhibitors. Another factor that potentially improved our insect survivorship was the addition of only one larva per experimental unit. Both *H. zea* (Chilcutt 2006) and *H. virescens* (Gould 1986) have been shown to exhibit high levels of cannibalism. As the previous study used three insects per container there is a high likelihood that a large amount of insect mortality was

due to direct cannibalism or wounding of insects in this study. As low mortality was seen in their control treatments, there is the potential for the wild-type tobacco tissue to be a suitable enough food source to lower any aggressive behavior.

It has been demonstrated that the gut proteases of both *H. zea* and *H. virescens* can be inactivated *in vitro* by the addition of soybean Kunitz trypsin inhibitor, but in assays where the inhibitor was added to artificial diet theses insects were unaffected (Purcell et al 1992, Bayes et al. 2006). *H. zea* fed artificial diet supplemented with soybean Kunitz trypsin inhibitor have also been shown to produce a number of proteinase inhibitor insensitive proteases as a means to adapt (Volpicella et al. 2006). Recent studies on a closely related species of lepidopteran, *Helicoverpa armigeria*, have revealed that the insects immediately up regulates numerous protease genes in response to inhibitors and then selectively down regulates those proteases which are sensitive to inhibition (Bown et al. 2004). A similar "shotgun" reaction to inhibitors has been documented in *Spodoptera frugiperda*, except that the protease genes are left on without the decrease in sensitive proteases (Briochi et al. 2007). As BoPI has sequence similarity to the soybean Kunitz trypsin inhibitor, adaptations analogous to those found in *S. frugiperda* and the closely related species *H. armigeria* may be responsible for the increased weights seen in *H. zea* throughout this study. As similar weight gains were seen in those insects fed on anti-chymotrypsin VK32 plants, it may be that this "shotgun" approach is a general *H. zea* response to proteinase inhibitors regardless of their activity.

H. virescens larvae have also been documented to adapt to proteinase inhibitors. These larvae have been shown to produce inhibitor insensitive proteases when exposed to

those found endogenously in *N. tabacum* (Brito et al. 2001). Early in this study, *H. virescens* larvae fed BoPI tissues were observed to have stunted growth. It may be that they adapt more slowly than *H. zea*, but as evidenced by their equal weights and head capsule sizes they reach the same size and developmental stage as those insects fed wild-type plant material. The differing results found in this study for the two insect species, *H. zea* and *H. virescens*, are somewhat surprising as they are both generalists. While both species have been documented to colonize *B. oleracea*, it has been recognized as a poor host plant for these insects (Harding 1976). One possible explanation for these differences could be the use of lab-reared insects, which may have lost their ability to efficiently adapt to the addition of proteinase inhibitors in their diets. The use of field captured insects may help to alleviate any unintended selection brought about by lab raising of a colony.

Control of both *H. zea* and *H. virescens* has been achieved using transgenic tobacco expressing the cowpea trypsin inhibitor (Hilder et al. 1987, Hoffman et al. 1992), a Bowman-Birk type serine proteinase inhibitor with two active sites. In contrast, transgenic plants expressing Kunitz-type trypsin inhibitors have previously been demonstrated to be ineffective at controlling *H. virescens* (Gatehouse et al. 1994) and based on *H. zea* bioassay tests of artificial diets supplemented with these inhibitors (Bayes et al. 2006, Volpicella et al. 2006), our results are not surprising. While numerous insect pests have been shown to be negatively affected by Kunitz-type trypsin inhibitors there have been no examples of controlling *H. zea* or *H. virescens*, which suggests that they are poor candidates for control of these insects.

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Appendix

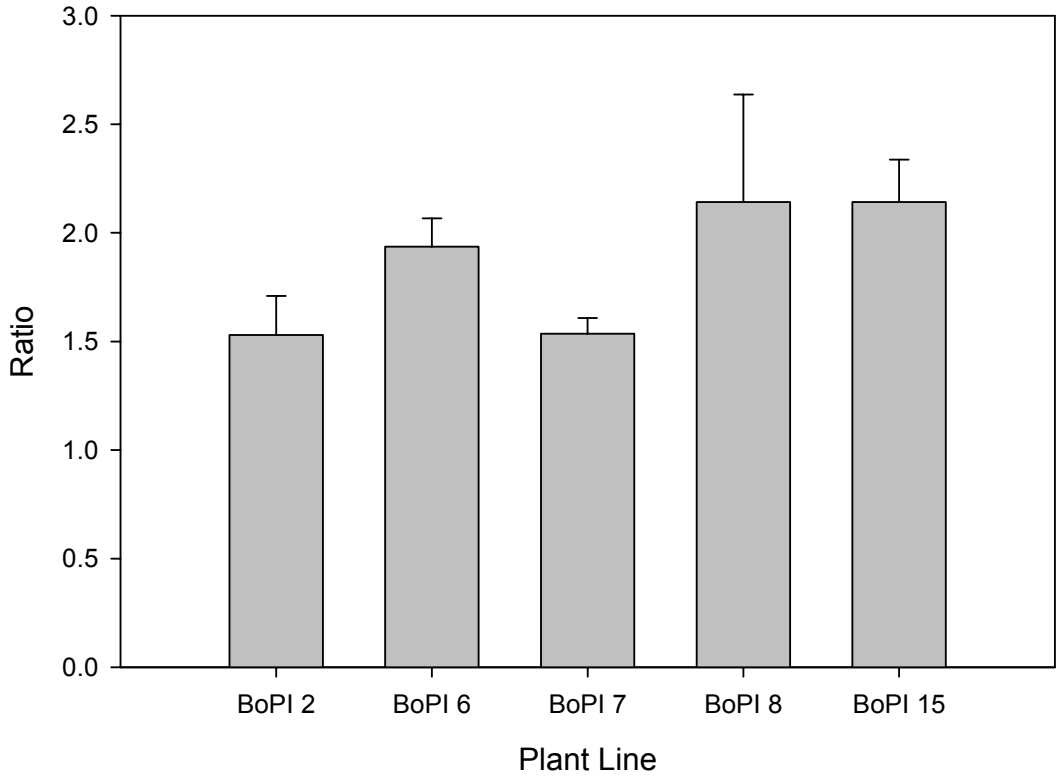

Figure 2.1. Ratio of transcription of the *Brassica oleracea* proteinase inhibitor (BoPI) transgene gene in lines (BoPI 2, 6, 7, 8, and 15) relative to the expression level of the internal reference gene ubiquitin. There was no amplification for the nontransgenic control line.

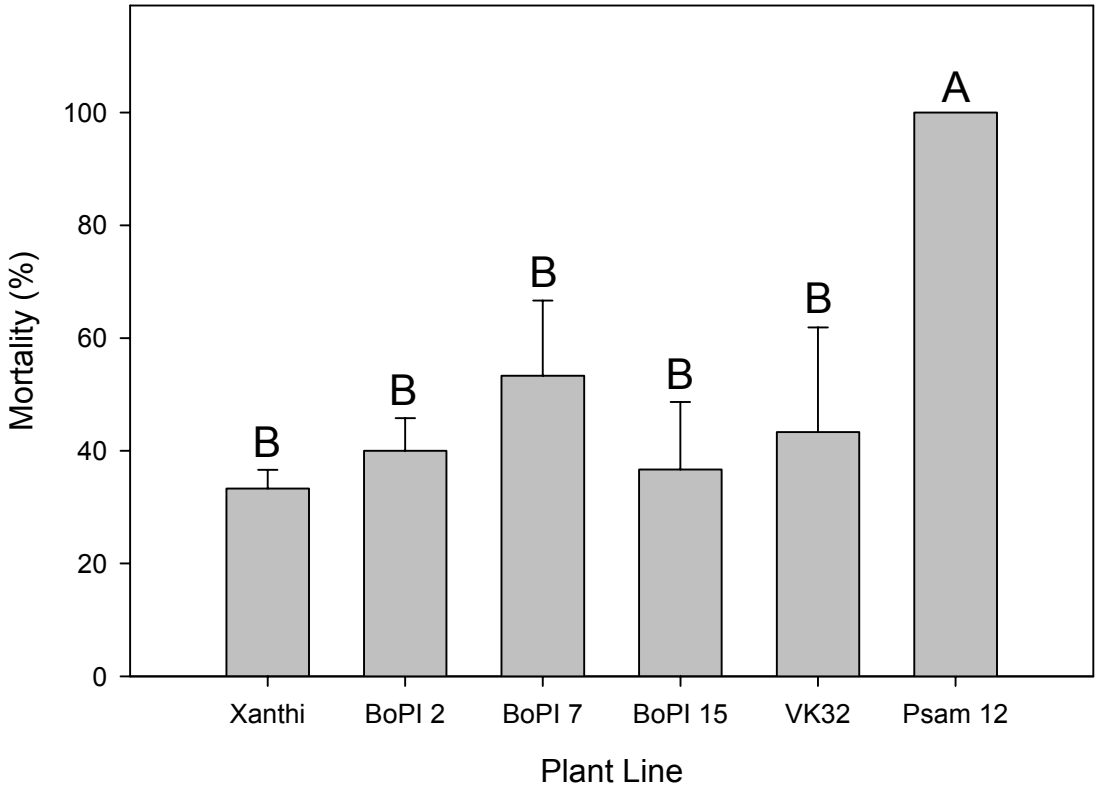

Figure 2.2. Percent mortality on day 17 of larval *Helicoverpa zea* fed wild-type (Xanthi) and transgenic (VK32 and BoPI lines 2, 7, and 15) tobacco leaf tissue. Error bars indicate standard error of the mean and bars with the same letters are not significantly different ($p < 0.05$).

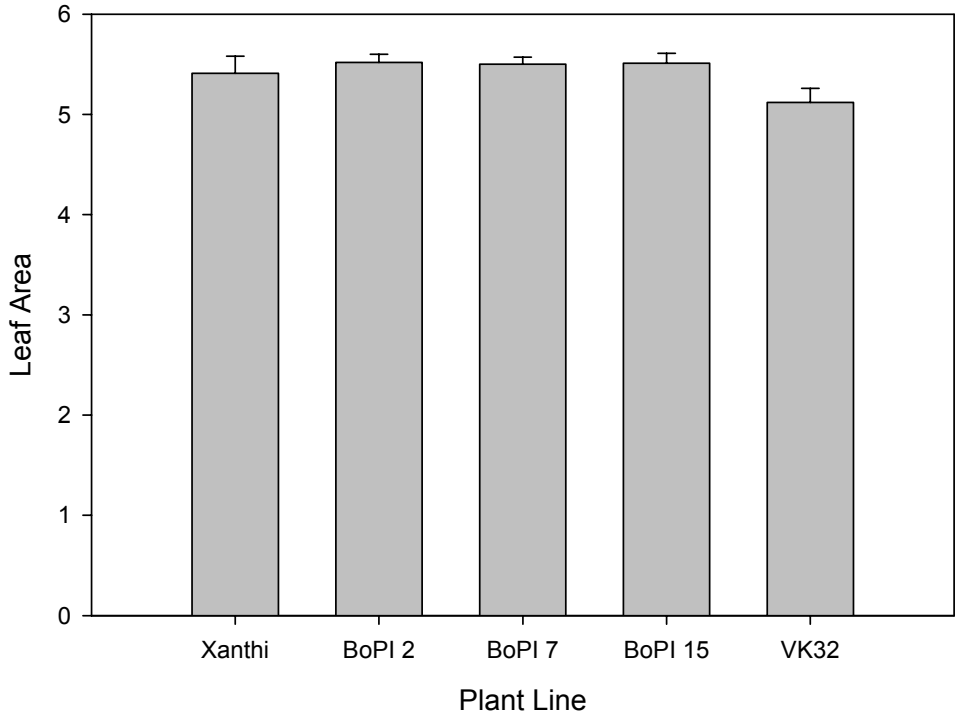

Figure 2.3. Area (cm^2) of leaf discs after four days of feeding by larval *Helicoverpa zea* fed wild-type (Xanthi) and transgenic (VK32 and BoPI lines 2, 7, and 15) tobacco leaf tissue. Leaf discs on which larvae died before day 4 were not included in analysis. Error bars represent standard errors.

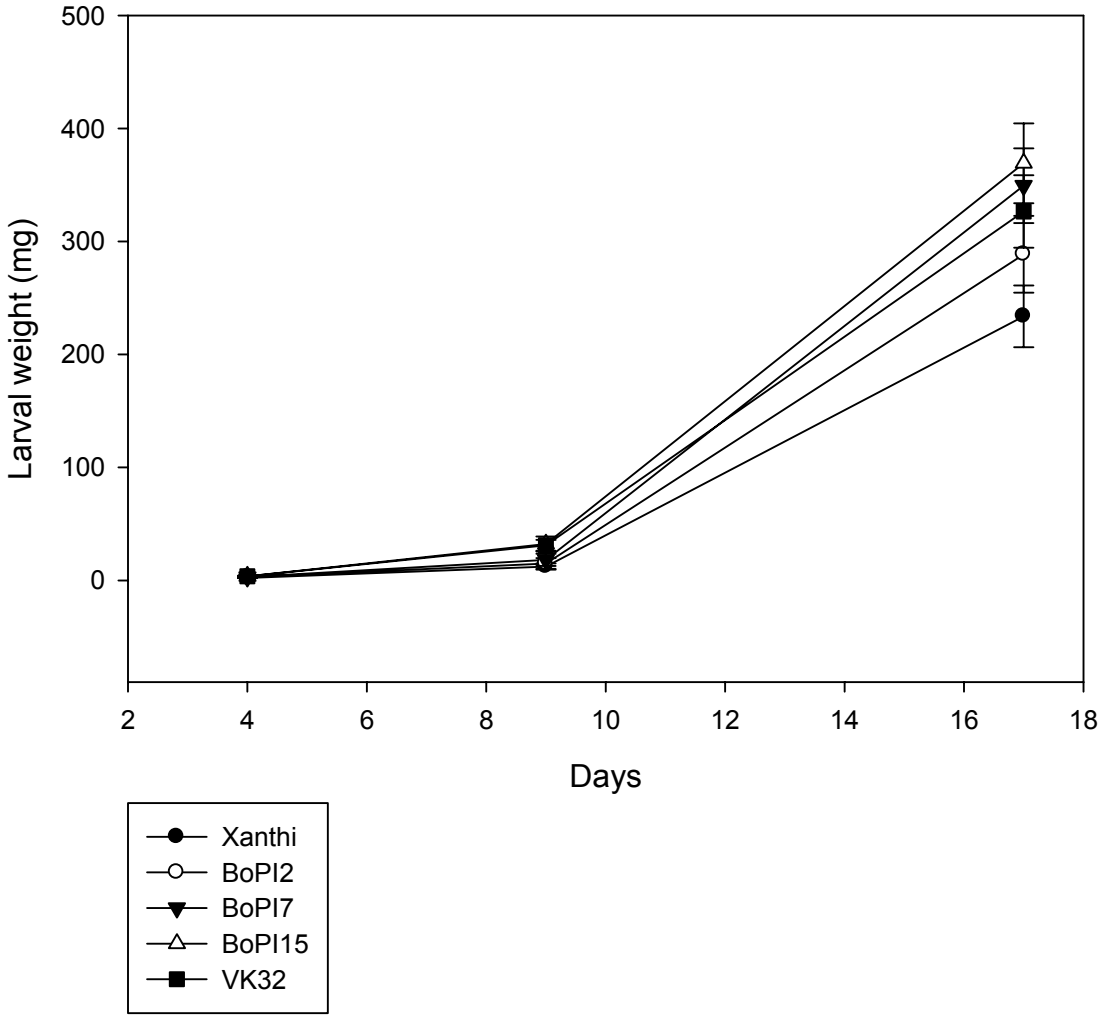

Figure 2.4. Weights of larval *Helicoverpa zea* on days 4, 9, and 17 fed wild-type (Xanthi) and transgenic (VK32 and BoPI lines 2, 7, and 15) tobacco leaf tissue. Error bars represent standard errors.

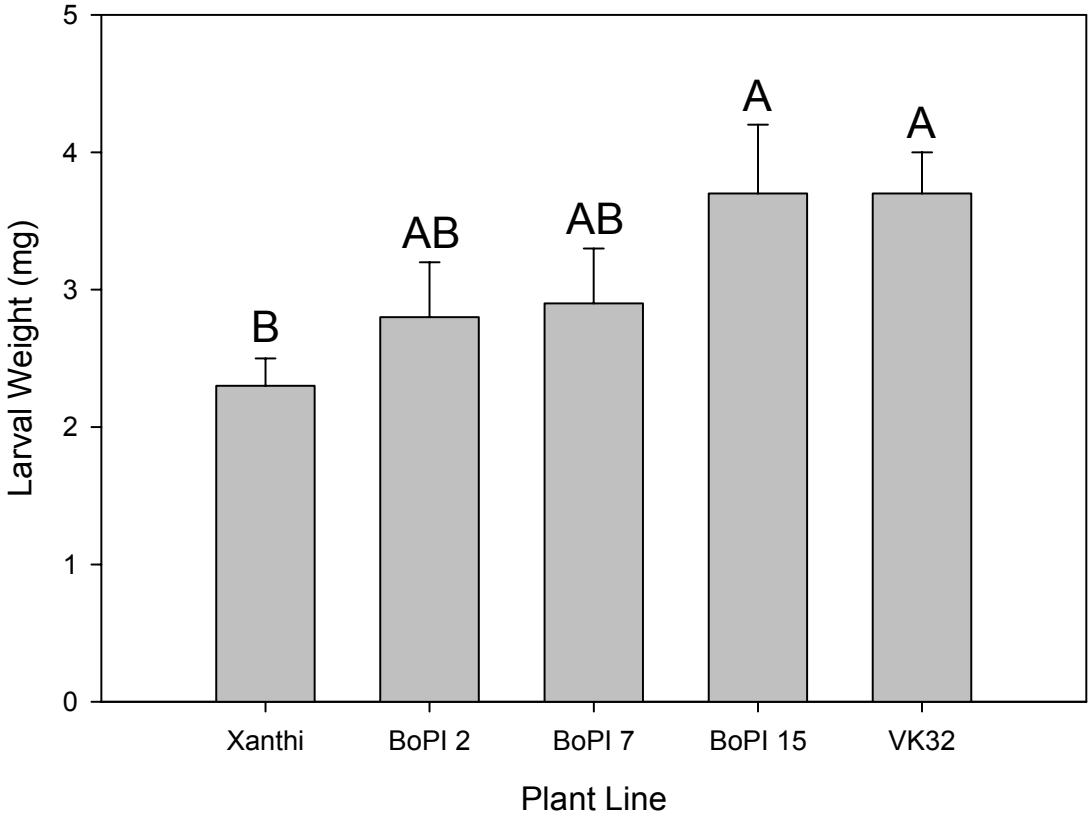

Figure 2.5. Larval weight (mg) on day 4 of larval *Helicoverpa zea* fed wild-type (Xanthi) and transgenic (VK32 and BoPI lines 2, 7, and 15) tobacco leaf tissue. Error bars indicate standard error of the mean and bars with the same letters are not significantly different ($p < 0.05$).

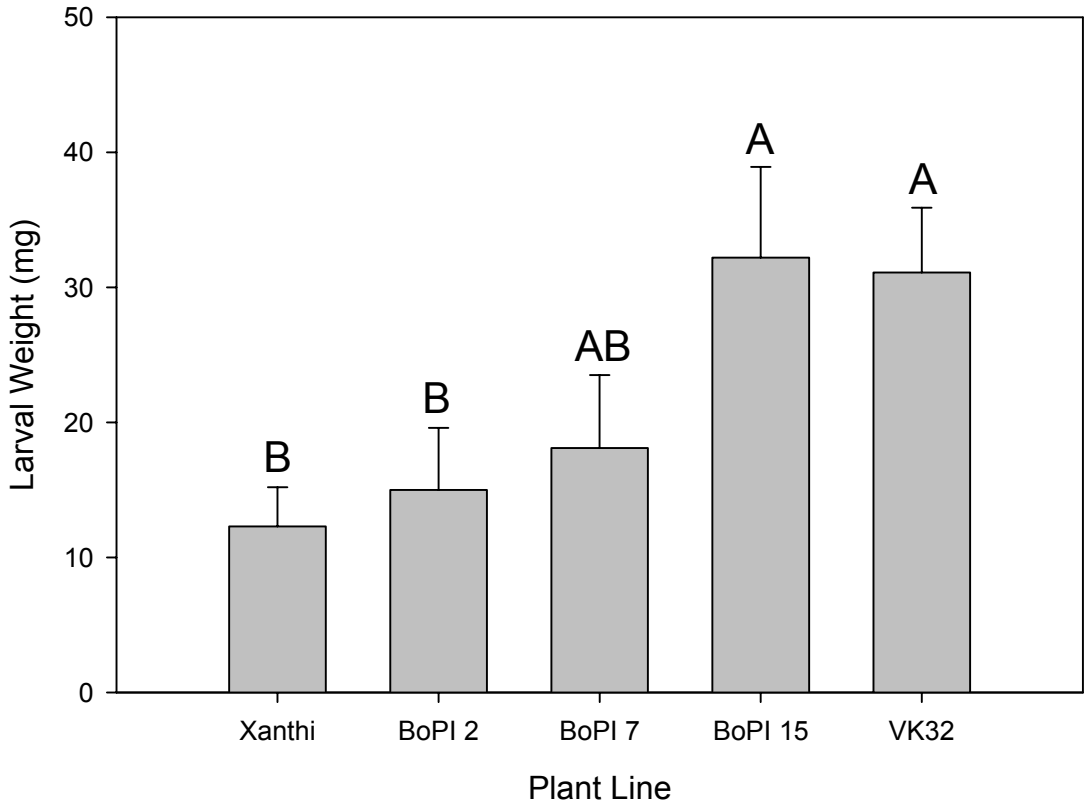

Figure 2.6. Larval weight (mg) on day 9 of larval *Helicoverpa zea* fed wild-type (Xanthi) and transgenic (VK32 and BoPI lines 2, 7, and 15) tobacco leaf tissue. Error bars indicate standard error of the mean and bars with the same letters are not significantly different ($p < 0.05$).

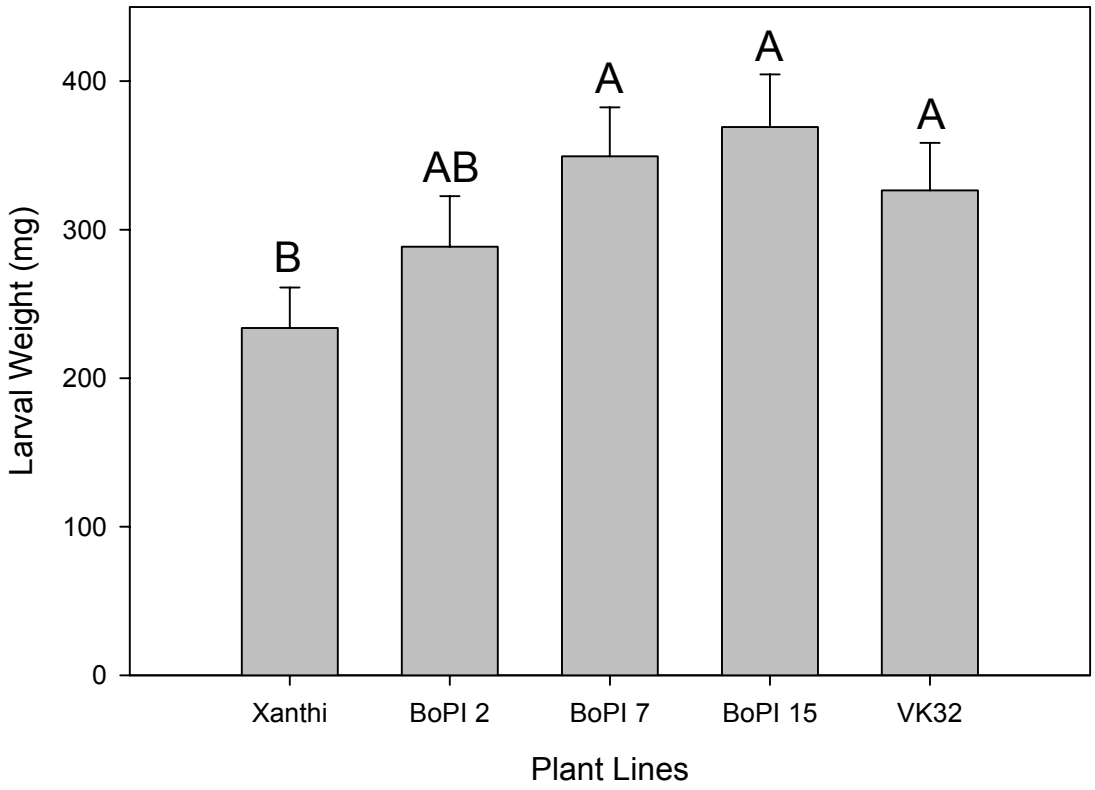

Figure 2.7. Larval weight (mg) on day 17 of larval *Helicoverpa zea* fed wild-type (Xanthi) and transgenic (VK32 and BoPI lines 2, 7, and 15) tobacco leaf tissue. Error bars indicate standard error of the mean and bars with the same letters are not significantly different ($p < 0.05$).

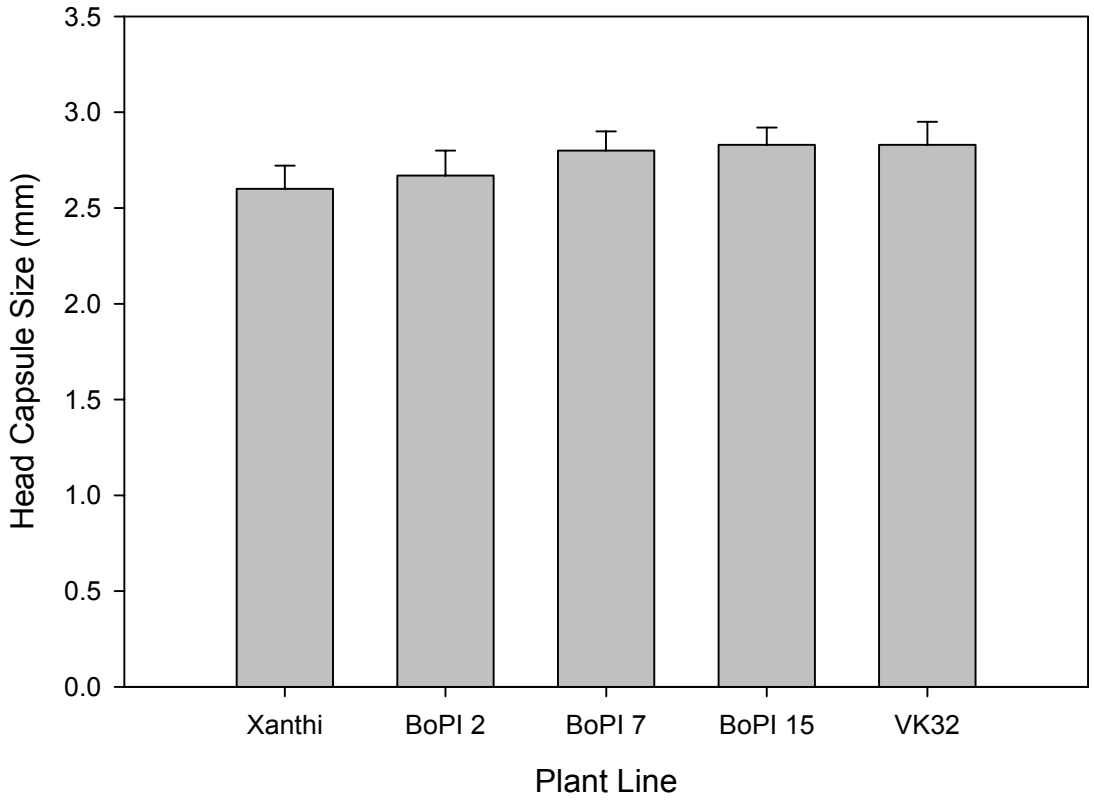

Figure 2.8. Head capsule size (mm) of larval *Helicoverpa zea* fed wild-type (Xanthi) and transgenic (VK32 and BoPI lines 2, 7, and 15) tobacco leaf tissue. Error bars represent standard errors.

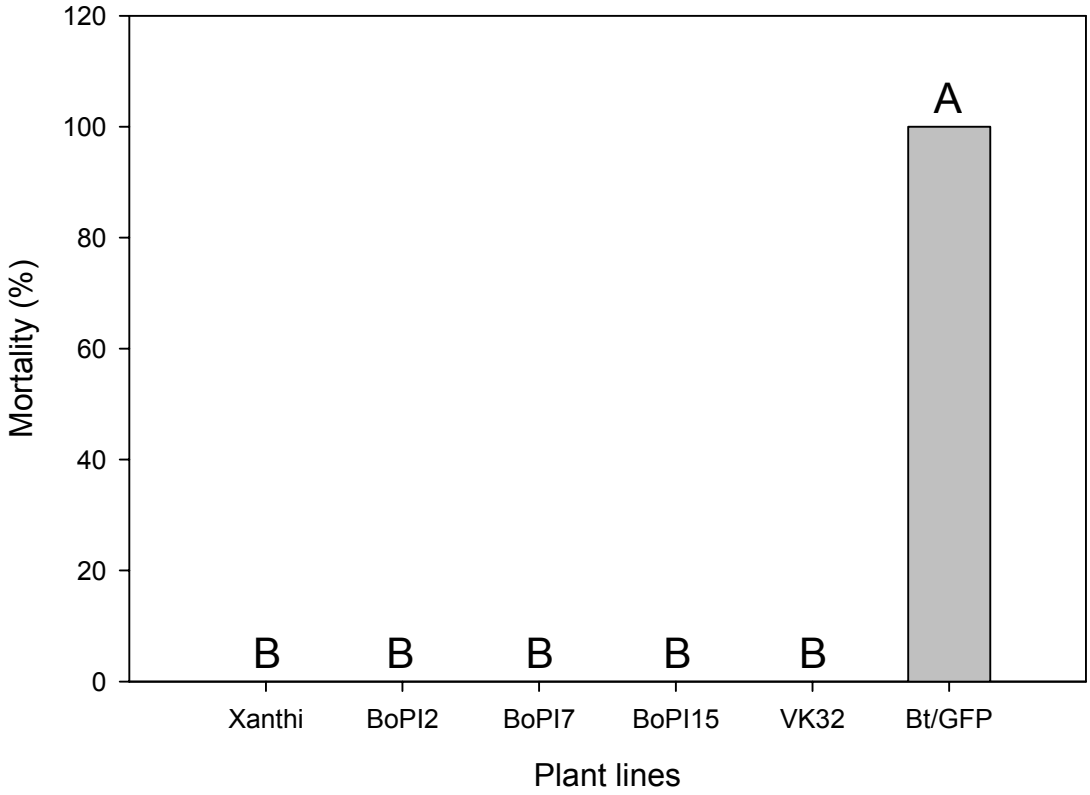

Figure 2.9. Percent mortality on day 17 of larval *Heliothis virescens* fed wild-type (Xanthi) and transgenic (VK32 and BoPI lines 2, 7, and 15) tobacco leaf tissue. Error bars represent standard errors. Error bars indicate standard error of the mean and bars with the same letters are not significantly different ($p < 0.05$).

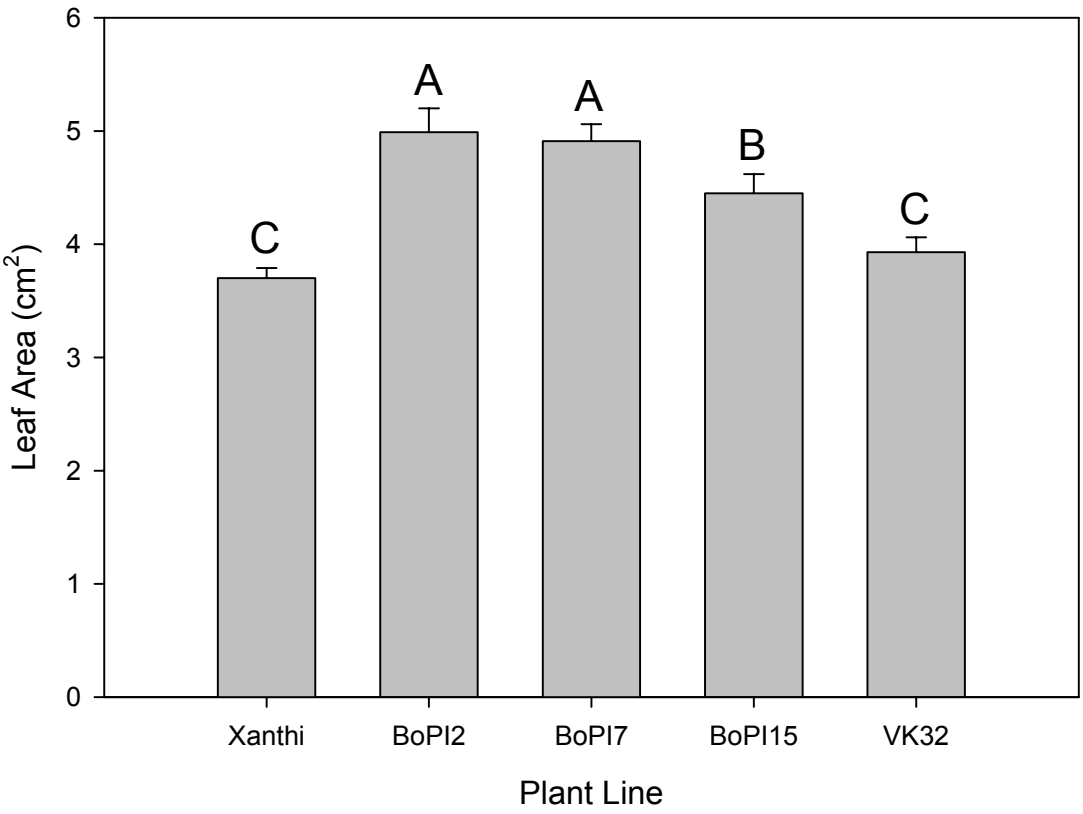

Figure 2.10. Area (cm^2) of leaf discs after four days of feeding by larval *Heliothis virescens* fed wild-type (Xanthi) and transgenic (VK32 and BoPI lines 2, 7, and 15) tobacco leaf tissue. Leaf discs on which larvae died before day 4 were not included in analysis. Error bars indicate standard error of the mean and bars with the same letters are not significantly different ($p < 0.05$).

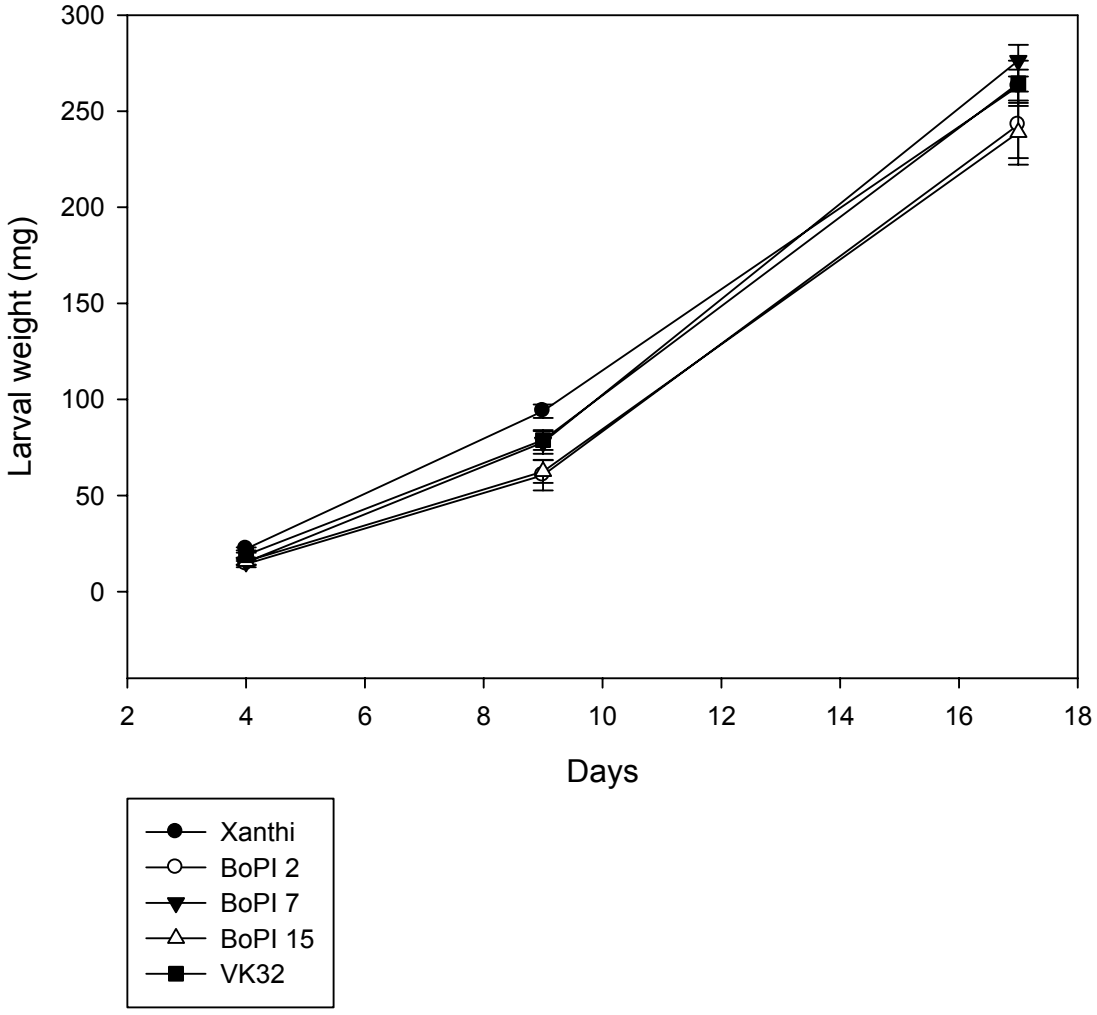

Figure 2.11. Weights of larval *Heliothis virescens* on days 4, 9, and 17 fed wild-type (Xanthi) and transgenic (VK32 and BoPI lines 2, 7, and 15) tobacco leaf tissue. Error bars represent standard errors.

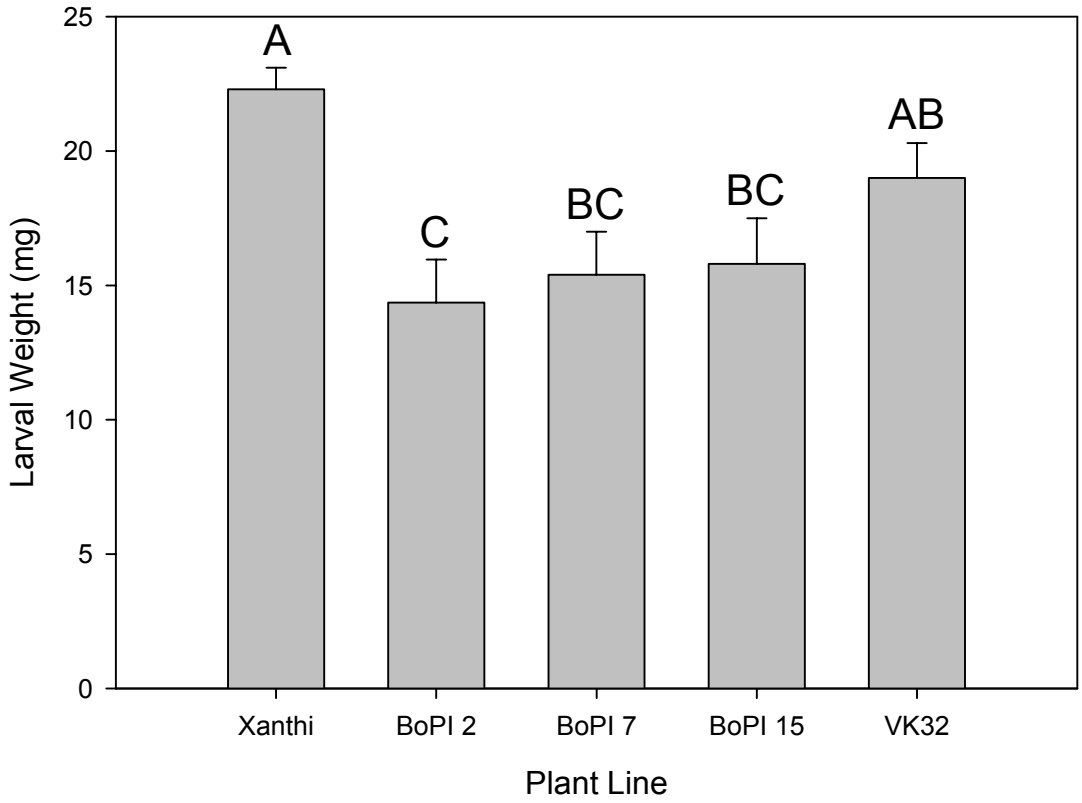

Figure 2.12. Larval weight (mg) on day 4 of larval *Heliothis virescens* fed wild-type (Xanthi) and transgenic (VK32 and BoPI lines 2, 7, and 15) tobacco leaf tissue. Error bars indicate standard error of the mean and bars with the same letters are not significantly different ($p < 0.05$).

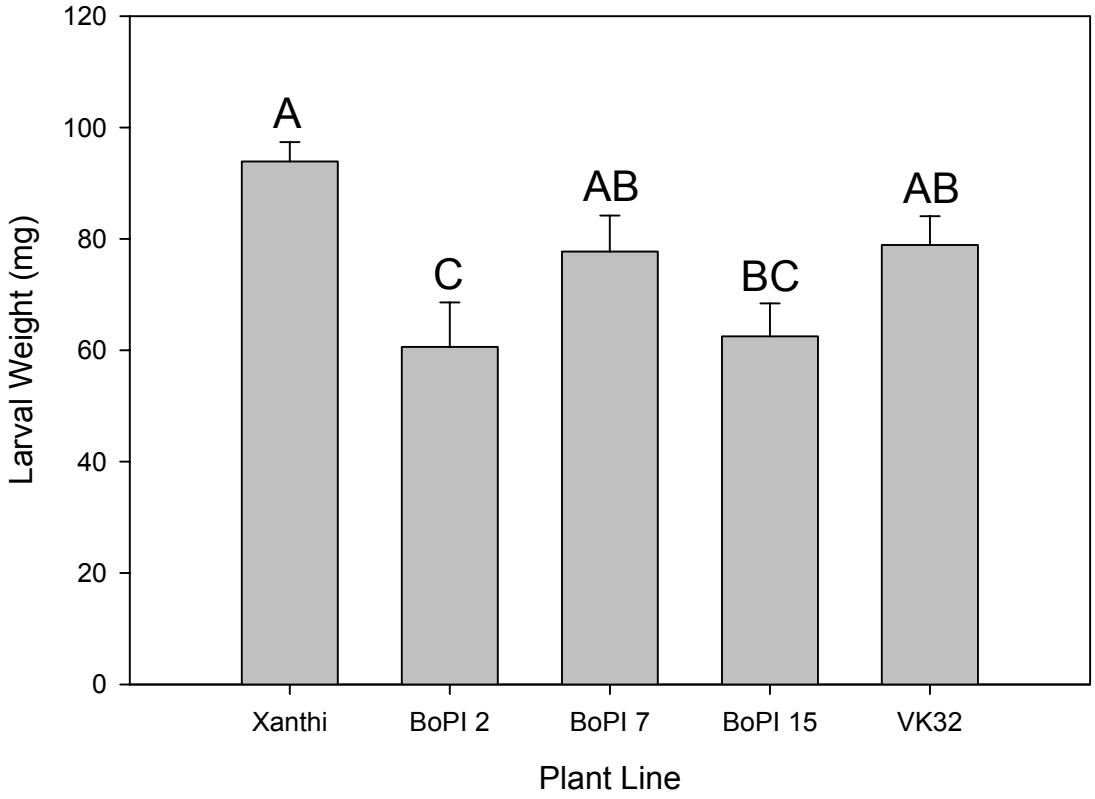

Figure 2.13. Larval weight (mg) on day 9 of larval *Heliothis virescens* fed wild-type (Xanthi) and transgenic (VK32 and BoPI lines 2, 7, and 15) tobacco leaf tissue. Error bars indicate standard error of the mean and bars with the same letters are not significantly different ($p < 0.05$).

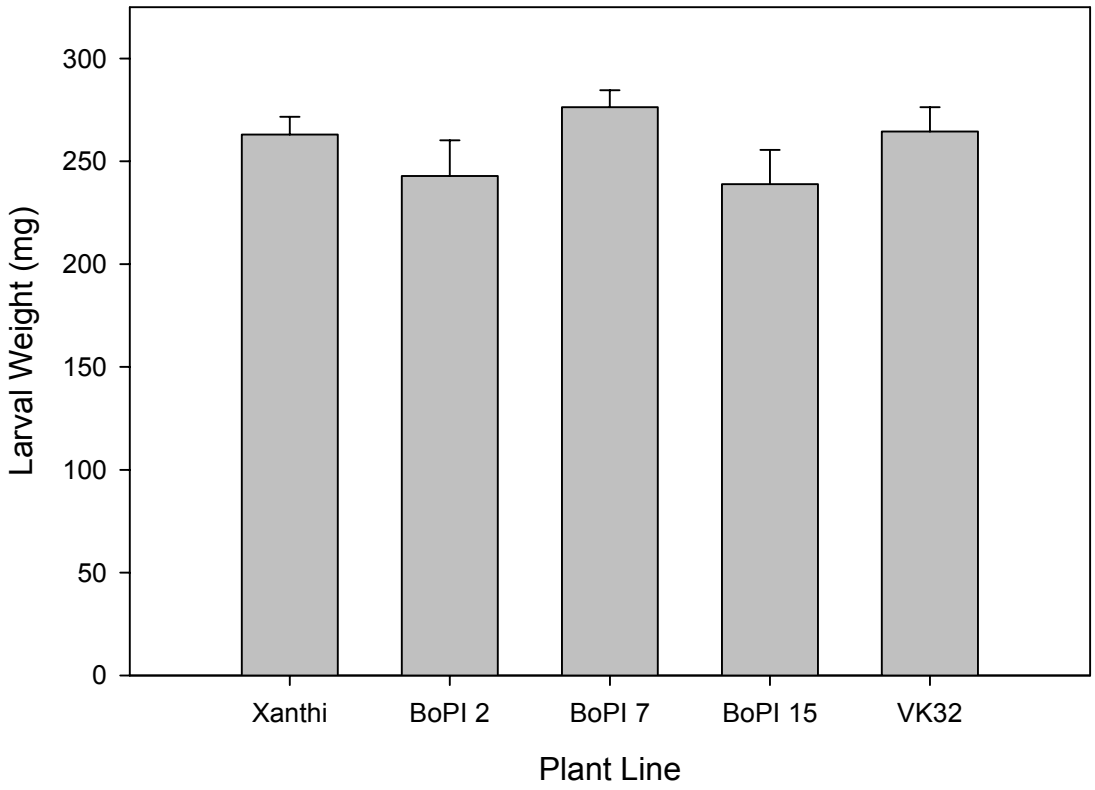

Figure 2.14. Larval weight (mg) on day 17 of larval *Heliothis virescens* fed wild-type (Xanthi) and transgenic (VK32 and BoPI lines 2, 7, and 15) tobacco leaf tissue. Error bars indicate standard error of the mean and bars with the same letters are not significantly different ($p < 0.05$).

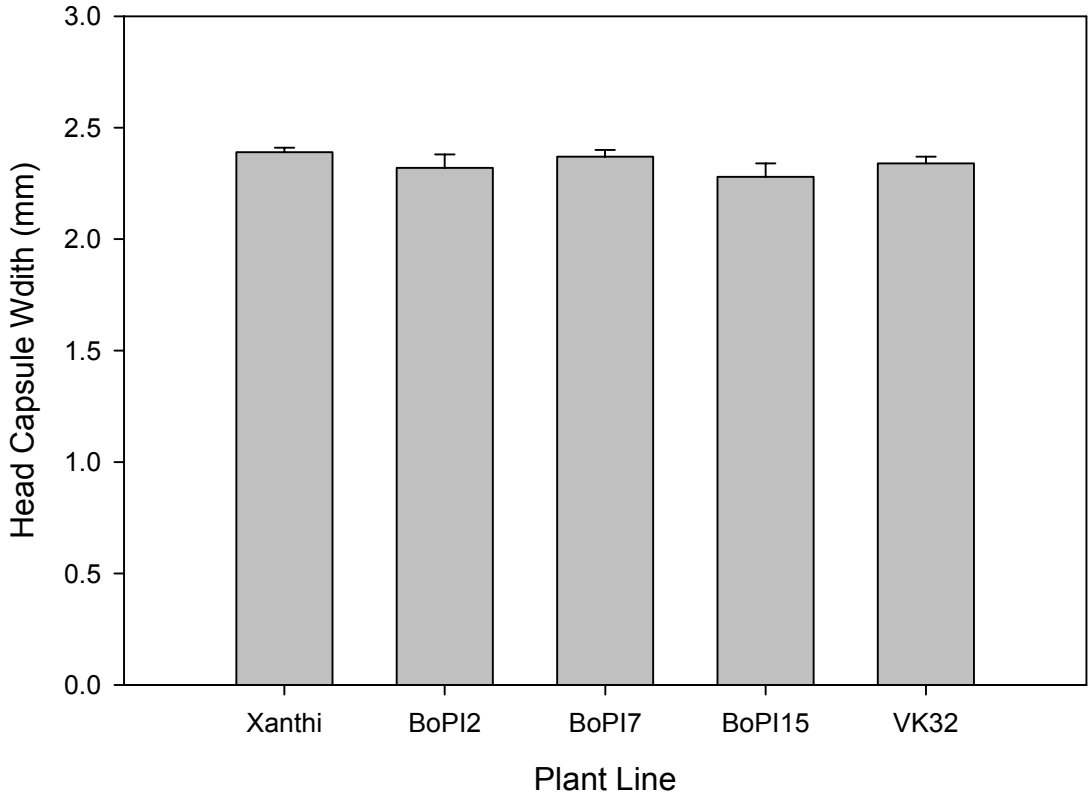

Figure 2.15. Head capsule size (mm) of larval *Helicoverpa zea* fed wild-type (Xanthi) and transgenic (VK32 and BoPI lines 2, 7, and 15) tobacco leaf tissue. Error bars represent standard errors.

Agroinfiltration as a technique for rapid assays for expressing candidate insect resistance genes in plants: exemplified by screening insect resistance genes for plant improvement[1]

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[1] This manuscript will be combined with parts of the previous paper "Growth and development of *Helicoverpa zea* and *Heliothis virescens* larvae that consume transgenic tobacco leaves expressing *Brassica oleracea* or *Manduca sexta* proteinase inhibitors" and will be submitted to the Biotechnology Journal.

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Abstract

Screening of candidate insect resistance genes is a time consuming task that may take months to years to achieve. In this study, a rapid screening technique is characterized and evaluated that combines candidate gene transient expression by means of agroinfiltration of *Nicotiana benthamiana* with a simple insect bioassay. Using this system the known insecticidal protein *Cry1Ac* is demonstrated to effectively control *Helicoverpa zea*. Insects fed tissue expressing GFP were shown to have enhanced growth and development. Additionally, a *Brassica oleracea* proteinase inhibitor (BoPI) with unproven insect resistance characteristics is demonstrated to hinder the growth and development of *H. zea*.

Introduction

The evaluation of potential insect resistance genes in transgenic plants can be arduous, given that the generation of stably transgenic plants is costly and labor intensive. Stable transformation of plants can take months-to-years to achieve and the resulting transgenic events usually vary with regards to transgene expression because of gene insertion effects and numbers of inserted genes (Wroblewski et al 2005). The combination of a robust temporary (transient) transgene expression assay combined with a reliable screening protocol could decrease the time for initial transgene evaluation, i.e., is it worthwhile to conduct in-depth screening by expression in stably transgenic plants. The use of transient expression through infiltration of *Agrobacterium tumefaciens* (agroinfiltration) harboring the transgene and promoter of interest can substantially decrease the time required to test candidate insecticidal genes and may also provide a better platform to assess the potential of these gene products. This report notes how agroinfiltration can be coupled with a bioassay procedure to determine the predictive effects of over-expressing two different candidate insect resistance genes in plants: one that is a documented insecticidal gene, and another that is much less effective in transgenic plants.

Transient expression through agroinfiltration is a relatively simple procedure after a transgene construct is produced, which involves the injection of *A. tumefaciens* containing the candidate transgene under the control of a leaf-active or constitutive promoter in a binary transformation vector (Sparkes et al. 2006). Agroinfiltration has been demonstrated to be effective for transient expression in many plant species including tobacco (Shelduko et al. 2006), grapevine (Santos-Rosa et al. 2008), lettuce,

tomato, *Arabidopsis* (Wroblewski et al. 2005), switchgrass (VanderGheynst et al. 2008), radish, pea, lupine, and flax (Van der Hoorn et al. 2000). Since there is a wide range of plant species susceptible to *A. tumefaciens* infection, the use of agroinfiltration for the evaluation of candidate insect resistance genes has great potential for rapid screening on numerous target insects.

The objective of this study is to evaluate the ability of agroinfiltration with a subsequent insect bioassay to effectively screen potential insect resistance genes for efficacy. A known insecticidal gene (Bt *Cry1Ac*) and a candidate insect resistance gene (BoPI) (*Brassica oleracea* protease inhibitor) were infiltrated into *Nicotiana benthamiana* and bioassays using larval *Helicoverpa zea* (corn earworm) were performed.

Materials and Methods

Plants. *Nicotiana benthamiana* seeds were planted and grown in a growth chamber at 25˚C with 16 h light and 8 h dark photoperiod. Plants were grown for 2 mo before bioassays were performed.

Insects. Corn earworm eggs were obtained from Benzon Research Inc. (Carlisle, PA). Eggs were hatched in a large plastic container. Neonate larvae were immediately transferred to 128 well insect trays containing synthetic fall armyworm diet (Bio-Serv Inc., Frenchtown, NJ) and held for 3 days before beginning bioassays. Second instar larvae were used in bioassays.

Bacterial Strains and Vectors. Transformed and non-transformed *Agrobacterium tumefaciens* strain GV3850 was used in all infiltrations. Binary vectors in transformed bacteria included pBin/BoPI (containing *Brassica oleracea* serine proteinase inhibitor) (Pulliam et al. 2001), pH602SBt (containing synthetic Bt *Cry1Ac*) (Stewart et al. 1996), pBin-mGFP5-ER (containing GFP5-ER only) and pSAM12 (containing both GFP5-ER and synthetic Bt *Cry1Ac*) (Harper et al. 1999). GFP was used as a visual marker of agroinfiltration and transgene expression.

Transformed *A. tumefaciens* test tube starter cultures were grown in a shaker overnight at 24˚C and 200 RPM in YEP media (1% peptone, 1% yeast extract, and 0.5% NaCl) (3 ml) containing 50 mg/L rifampicin and 50 mg/L kanamycin. Optical density at 600 nm was taken on starter cultures with a Synergy HT plate reader (BioTek Instruments, Inc, Winooski, VT). Starter cultures were used to inoculate two 25 ml cultures to an OD_{600} of 0.004 and grown overnight. Non-transformed *A. tumefaciens* were grown without kanamycin as an antibiotic selection agent.

Transient Expression Procedure. *Agrobacterium* infiltrations were performed as described in Sparkes et al. (2006). Infiltration suspensions for each vector and non-transformed *Agrobacterium* were brought to OD_{600} 0.6. Mixtures of bacterial suspensions (1:1) were made for co-infiltrations with a final OD_{600} of 0.6. Co-infiltrations suspensions included non-transformed GV3850 + pBin-mGFP5-ER (GV+GFP), non-transformed GV3850 + pSAM12 (GV+GFP/Bt), pBin-mGFP5-ER + pSG/Bt (GFP+Bt), pBin-mGFP5-ER + pBin/BoPI (GFP+BoPI), and non-transformed

GV3850 + non-transformed GV3850 (GV+GV). The addition of multiple infiltration points per infiltration spot was used to expand the area of the spot to approximately 4 cm^2. GV+GV infiltration spots were outlined with a black marker for later identification. After infiltration, plants were placed back into the growth chamber. Three days after infiltration, spots were visualized with GFP expression under a BlackRay model B100 UV light (UVP, Upland, CA) and excised with a scalpel (Fig. 3.1). Outlined spots for GV3850-only infiltrations were excised also.

Infiltration Characterization Experiment. Tobacco plants were grown and infiltrated as described above. A single plant was infiltrated in five leaves, each leaf with a different co-infiltration treatment in two spots (one on each side of the mid-vein). Three replicate plants were infiltrated for a total of six spots for each co-infiltration treatment. After three days, spots were excised and measured with a GFP meter (Opti-Sciences Inc., Hudson, NH). Tissue was snap frozen in liquid nitrogen and ground in a mortar and pestle with liquid nitrogen. Tissue was then divided into three parts: 100 mg of tissue was placed in RLT (Qiagen RNeasy mini prep) buffer for RNA extraction and stored in a -80ºC freezer; one half of the remaining tissue was added to ice cold protein extraction buffer (100 μM Tris-HCl pH 7.5, 100 μM $CaCl_2$) in a 1.5 ml microcentrifuge tube; the third portion was placed in a 2 ml cryo vial (Sarstedt Inc., Newton, NC) for potential future experiments. Both portions were stored in a -80ºC freezer.

RNA Extraction. Plant tissue samples were removed from the freezer and total RNA was extracted using an RNeasy Plant Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instructions.

Protein Extraction. Frozen samples were thawed on ice and allowed to incubate for 1 hr. Tubes were clarified by centrifugation at 13,000 x g for 10 min at 4 ºC. Supernatant was transferred to a new microcentrifuge tube and quantified using Commassie plus staining (Pierce biotechnology, Rockford, IL).

Protein ELISAs. GFP and Bt *Cry1Ac* were quantified from the infiltration characterization assay protein samples. Protein from infiltrated tissue was adjusted to 10 µg/ml for GFP quantification and 20 µg/ml for Bt quantification. Protein was quantified for GFP using a Reacti-Bind Anti-GFP ELISA plate (Pierce biotechnology, Rockford, IL) according to the manufacturer's instructions. Bt *Cry1Ac* was quantified with a Cry1Ac QuantiPlate (EnviroLogix Inc., Portland, ME) according to the manufacturer's instructions.

Real Time PCR. RT-PCR was performed to confirm expression of transgenes in all infiltrated tissues collected in the infiltration characterization experiment. cDNA for real time PCR analysis was generated by reverse transcription of two micrograms of total RNA using the Superscript III first-strand synthesis kit (Invitrogen Corporation, Carlsbad, CA) and oligo(dT) primers. Real time PCR was performed using an ABI Prism

7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Real time PCR reactions were carried out in 20 µl reaction volumes consisting of gene specific primers and Power SYBR Green PCR master mix (Applied Biosystems, Foster City, CA). The gene specific primers were BoPI (fwd) TCCCGTGAAATTCTCAAACTGG, BoPI (rev) ACTGAGCGCAGATCGTAGGTTC, Bt *Cry1Ac* (fwd) CGCTCTCTTTCCCAACTACGA, Bt *Cry1Ac* (rev) ACCGTCGAAGTTCTCGAGGACT and were designed with primer express software (Applied Biosystems, Foster City, CA). GFP gene specific primers (fwd) CAACTTCAAGACCCGCCACA and (rev) TCTGGTAAAAGGACAGGGCCA were designed and provided by Laura Abercrombie. The reference gene used in RT-PCR analysis was ubiquitin. The ubiquitin specific primers were described in Lacomme et al. (2003). Ct values were recorded for both the transgene and reference gene for further analysis. Ratios of expression compared to the reference gene were determined using ΔCt as described in Yuan et al. (2008).

Insect Bioassay. Two tobacco plants were infiltrated on five leaves for each co-infiltration as described above. Infiltrated spots were excised and GFP quantified with the GFP meter. Ten excised infiltrated plant tissue pieces from each co-infiltration and ten excised plant tissue pieces from un-infiltrated *N. benthamiana* were placed into clear plastic cups (29.6 cm^3) (Bio-Serv Inc., Frenchtown, NJ) with $1/4^{th}$ of a moist #5 Whatman filter paper (Whatman International Ltd, Kent, UK). One second instar corn earworm was place onto each infiltrated plant tissue piece. Containers were sealed with a

lid and held at 24˚C. Containers were arranged in a complete randomized design. After six days, larval mortality, larval weight and larval head capsule size were recorded. Head capsule size of larvae was measured with an eye piece micrometer in a stereoscope (Olympus SZ40, Olympus Imaging America Inc., Center Valley, PA) and larval weights were taken with a digital scale (Denver Instruments, Göttingen, Germany). This insect bioassay was replicated in space for a total of twenty insects per treatment.

GFP Meter Procedures. GFP infiltrated leaf tissue was quantified with a hand held GFP meter. Infiltrated leaf tissue was measure at four independent points and averaged according to a modified protocol described in Millwood et al. (2003).

Statistical Analysis. Larval weight and head capsule size were analyzed with analysis of variance (ANOVA) using the mixed procedure of SAS 9.13 (SAS Institute 2003). Means separation was performed with Fisher's least significant difference. Larval mortality was analyzed with the Proc Glimix procedure of SAS 9.13 (SAS Institute 2003) since the data had a bimodal distribution.

Results

Real Time PCR. Real time PCR analysis confirmed the transient expression of all transgenes in infiltrated tissue. Large variations in GFP expression were observed between the different infiltrated spots (Fig. 3.2). The highest relative GFP expression was found in a GV+GFP infiltrated spot which had 3.26 times that of the internal

reference gene ubiquitin. The lowest relative GFP expression (0.59) was in a GFP+Bt infiltrated spot. Bt *Cry1Ac* expression levels in all infiltrated spots were lower than the internal reference gene, while relative expression levels of BoPI were extremely high. Expression levels of BoPI ranged from 4.9 to 9 times the level of ubiquitin. No transgene expression was detected in GV+GV samples.

Protein Synthesis. Protein levels of GFP measured by ELISA were significantly higher in the GV+GFP (1.36 % TSP) infiltrated tissues than in all other infiltration types ($F=15.14$, $df=3,20$; $P<0.0001$) (Fig. 3.3). The lowest level of expression detected by ELISA was in GFP+Bt infiltrated tissues, which had an average GFP total soluble protein of 0.57%. No GFP was detected in GV+GV samples.

Bt *Cry1Ac* levels in infiltrated spots showed no significant differences between those co-infiltrated in different vectors (GFP+Bt) (0.022 %TSP) versus those in the same vector (GV+GFP/Bt) (0.031 %TSP) (Fig. 3.5). The range of Bt *Cry1Ac* % TSP found in individual spots ranged from 0.054 to 0.015% TSP. No significant associations were observed between Bt *Cry1Ac* %TSP and GFP % TSP for either infiltration types.

GFP Meter. GFP meter readings in the infiltration characterization experiment had a strong relationship with GFP ELISA data ($R^2=0.727$). Similar differences to those found in ELISA data were detected in average GFP meter readings ($F=22.11$; $df=4, 25$; $P=0.0001$) (Fig. 3.5). GV+GV infiltrated spots had a low level of background at 61 counts per second (CPS).

GFP meter readings in the insect bioassay had levels of GFP expression than those found in the infiltration characterization assay. Although there were significant differences found between infiltration types (F=15.51; df=4,95; P=0.0001) (Fig. 3.6) they were different than those found in the infiltration characterization assay. GV+GV infiltrated spots had a low background of 23 CPS, while all non-infiltrated tissue measured had no signal (data not shown).

Insect Bioassay. Insects fed tobacco tissue transiently expressing synthetic *Cry1Ac* had high larval mortality ($F = 772.42$; $df = 5, 18$; $P < 0.0001$) (Fig. 3.7). Both treatments GFP+Bt and GV+GFP/Bt resulted in 100% larval mortality. No insect mortality was observed in larvae feeding on all other treatments.

Larval weights were recorded for all treatments except those containing synthetic *Cry1Ac* (GFP+Bt and GFP/Bt), which had no larvae remaining on day 6. The largest average larval weight was observed in those insects feeding on the GV+GFP treatment (45 mg) ($F = 6.16$; $df = 3, 76$; $P = 0.0008$) (Fig. 3.8). Insects feeding on all other treatments had significantly lower average larval weights. The lowest average larval weight was recorded in insects fed the non-infiltrated *N. benthamiana* tissue (26.1 mg).

Average larval head capsule size was similar to average larval weight with values recorded for all treatments except those fed synthetic Cry1Ac (GFP+Bt and GFP/Bt). Insects fed the GV+GFP treatment resulted in the largest average larval head capsule size (1.59 mm) ($F = 2.65$; $df = 3, 76$; $P = 0.0551$), indicating that insects had developed to both the 4^{th} and 5^{th} instars (Caplinera 2000)(Fig. 3.9). A significantly smaller average

larval head capsule size (4th instar) was observed in those insects fed the non-infiltrated tobacco tissue (1.35 mm), when compared to the GV+GFP treatment. All other treatments were similar.

Discussion

These results demonstrate that a system combining agroinfiltration and insect bioassays can be used as a powerful tool for assessing the potential of candidate insect resistance genes. Insects feeding on tissue infiltrated with a synthetic Bt *Cry1Ac* gene suffered 100% mortality, indicating that the system is effective at evaluating genes for insecticidal activity. Levels of Bt *Cry1Ac* detected in this study were similar to levels found in transgenic canola when transformed with the same construct (Moon et al. 2007).

Interestingly, insects fed tissue infiltrated with GFP as the only transgene had enhanced levels of growth and development. This is evidenced by the increased larval weight and head capsule size, which have previously been used to determine effects on insect development (Broadway 1995, Daly 1985, De Leo et al. 1998). The increase in development is, to our knowledge, the first report of transgenic plants expressing GFP having a positive effect on an insect herbivore. This enhanced rate of growth may be explained by an increase in total soluble protein from the over-expression of GFP and potentially an increased availability of essential amino acids. Another explanation of the increased growth and development may be due to an over-production of endogenous insect proteases. From previous research, transient expression of GFP localized to the ER has been shown to up-regulate defensive genes such as protease inhibitors (Page and

Angell 2002) which may be expected to have a detrimental effect on larval lepidopterans. It has also been demonstrated that *H. zea* has the ability to adapt to plant protease inhibitors by the up-regulation of an array of protease inhibitor sensitive and insensitive proteases (Volpicella et al. 2006). This increased production of proteases may allow the insect to more efficiently gain nutrients and subsequent increased growth. Previously, *H. zea* larvae challenged with protease inhibitor rich diets have demonstrated increased weights (Baýes et al.2006, Broadway 1995); *H. zea* fed plants stably transformed with the *Brassica oleracea* proteinase inhibitor used in this study showed a significant increase in larval weight (data not shown).

When plants were co-infiltrated with both GFP and the BoPI serine protease inhibitor and fed to larval *H. zea*, a significant decrease in larval weights was detected compared to infiltrations containing GFP as the only transgene. In addition, the larval head capsule size was smaller, although not significantly, than those insects fed GFP only infiltrated tissue. These results could be due to the lower levels of GFP total soluble protein in BoPI-infiltrated lines (Fig. 3.8). This explanation is not likely due to an expected increase in growth of *H. zea* challenged with proteinase inhibitors. As mentioned previously, stably transformed BoPI plants were shown to have a positive effect on the growth and development of *H. zea* and it would be expected that BoPI in infiltrated tissue would do the same. One possible explanation could be that the levels of BoPI expression in infiltrated tissue were extremely high (ranging from ~5 to 9 times that of ubiquitin), while the levels in the stable transgenic tested were only around twice the level of ubiquitin (data not shown). This extremely high titer of BoPI may have

mitigated the efficacy of the insect's natural adaptive processes in response to both the endogenous plant proteases and BoPI. The ability to evaluate candidate insect resistance genes at levels higher than those found in typical stable transformants is a major advantage to this screening system.

The use of this system to evaluate insect resistance genes has several benefits when compared to other systems. As mentioned previously, the positional effects of transgene insertion are clouded by the transformation of individual cells and the time for preparation of transgenic tissue is drastically reduced: days instead of months (Wroblewski 2005). In addition, serious ecological risks of the escape of transgenes into the environment are drastically reduced (Li et al. 2007). When compared with previously described viral based screening systems (Lawrence and Novak, 2001), the time for expression of genes is cut in half. As viral vectors are not used to generate stable transformants, a system containing agroinfiltration can be rapidly altered for the production of stable transgenic lines. Additionally, viral vectors have size constraints in the transgenes they can produce (Gleba et al. 2007) making it a less attractive system.

As with any technology, there are also inherent pitfalls. Large variations in expression were seen within infiltrated materials in this study. There are many factors that influence transient expression from agroinfiltration. One of the major considerations is the OD of the infiltration, which has been documented to have a large effect on the expression of the transgene (Santos-Rosa et al. 2008, Sparkes et al. 2006). While this study used a standardized co-infiltration OD, it may be advantageous to adjust these ODs to suit a specific construct. In addition, species, leaf position, and age of the plant can all

have impacts on expression (Sheludko et al. 2007). Another issue with the high levels of transcription found in agroinfiltrated tissue is post transcriptional gene silencing, which by means of the plants endogenous defenses against viruses can hinder the amount and duration of transgene expression (Voinnet et al. 2003). This problem may be alleviated by the addition of a silencing suppressor, such as P19 (Voinnet et al. 2003, Wroblewski et al. 2005), which may significantly increase the level and longevity of transgene expression.

The use of agroinfiltration in tandem with insect bioassays has promise as an effective means to test insect resistance genes. This system allows for enhanced characterization of not only insecticidal genes, but genes which alter insect growth and development, which may have elusive modes of action.

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Appendix

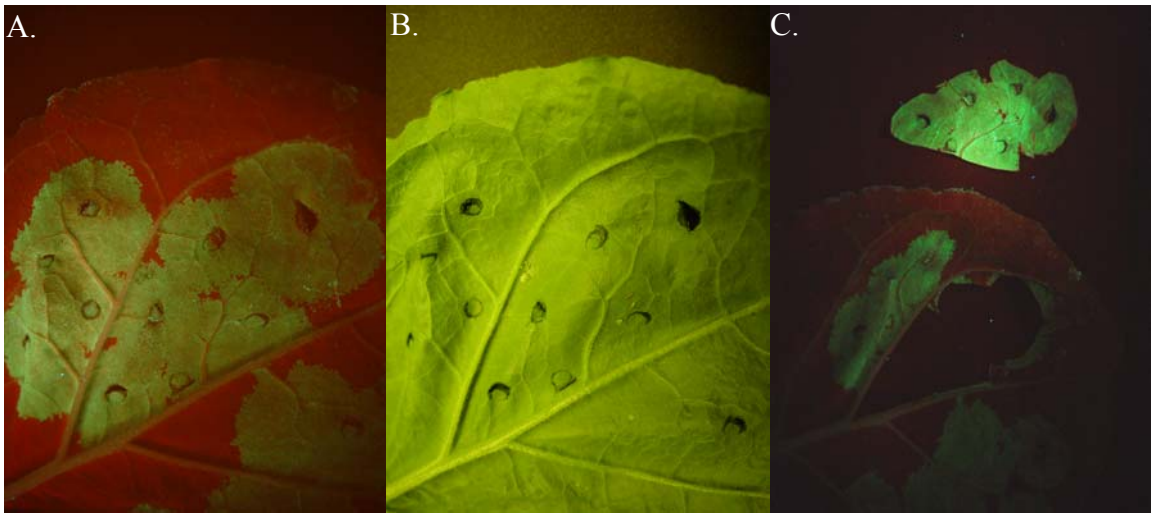

Figure 3.1. Infiltrated *N. benthamiana* tissue expressing GFP+Bt A. under ultraviolet light B. under white light, and C. excised under ultraviolet light.

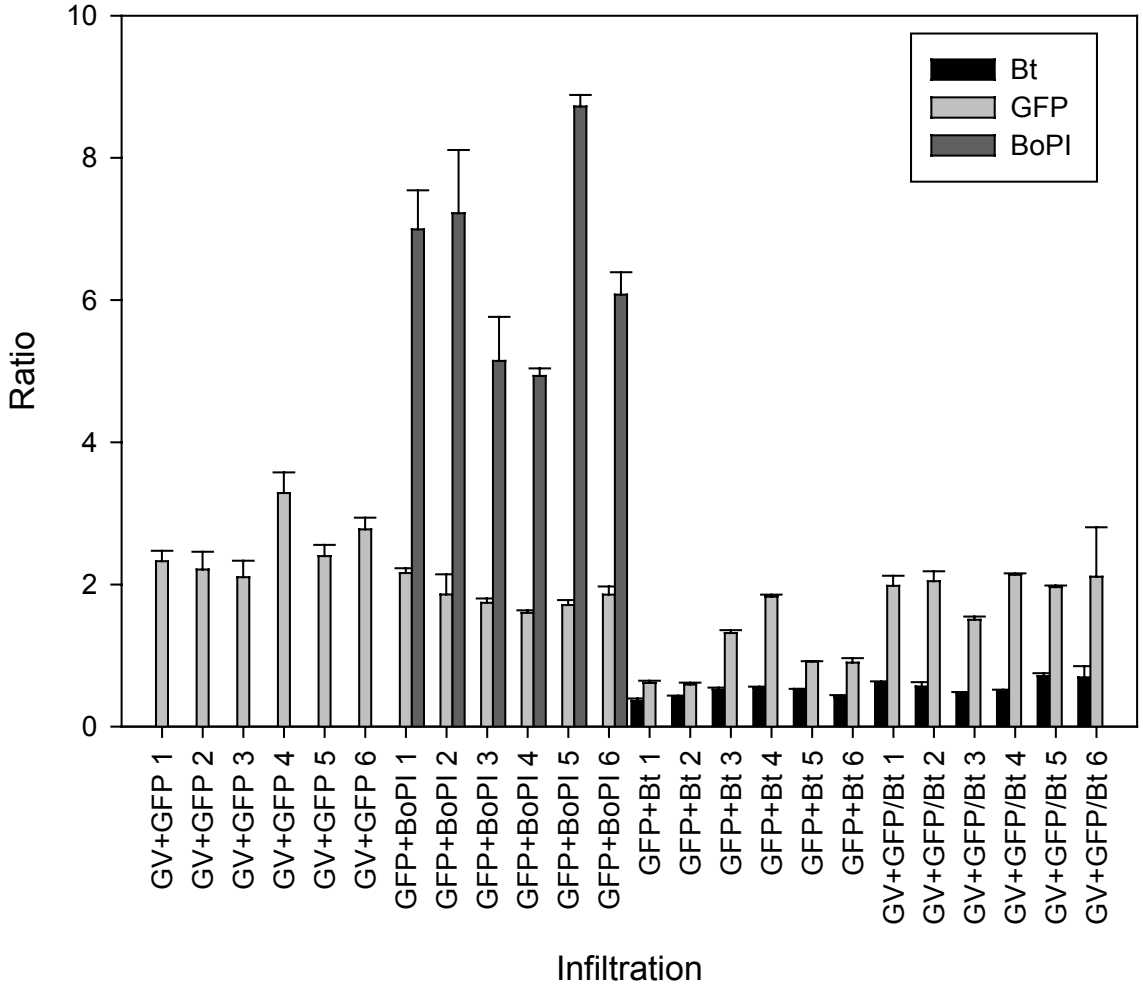

Figure 3.2. Ratio of transcription of the mGFP5-er, Bt Cry1Ac, and BoPI transgene genes relative to the expression level of ubiquitin in tobacco infiltrated with *Agrobacterium tumefaciens* containing (GV+GFP), (GFP+BoPI), (GFP+Bt), or (GV+GFP/Bt). Error bars indicate standard error of the mean.

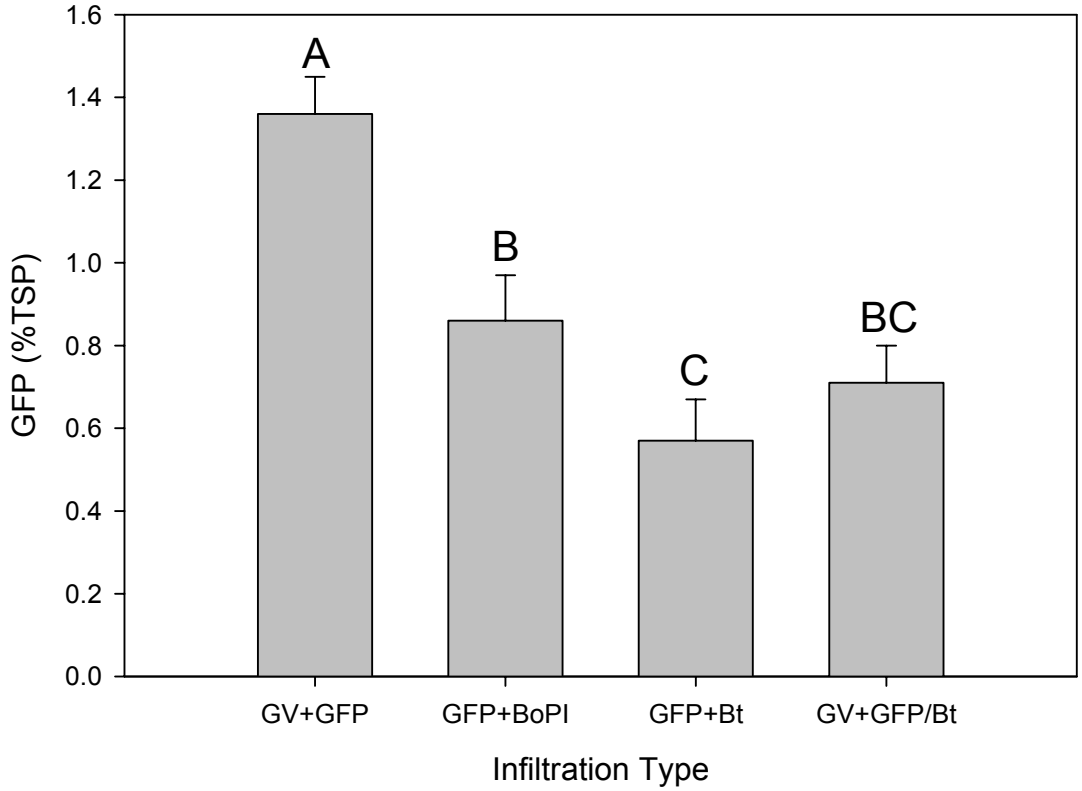

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Figure 3.3. Average GFP percent total soluble protein in tobacco tissue infiltrated with *Agrobacterium tumefaciens* containing (GV+GFP), (GFP+BoPI), (GFP+Bt), or (GV+GFP/Bt). Error bars indicate standard error of the mean and bars with the same letters are not significantly different ($p < 0.05$).

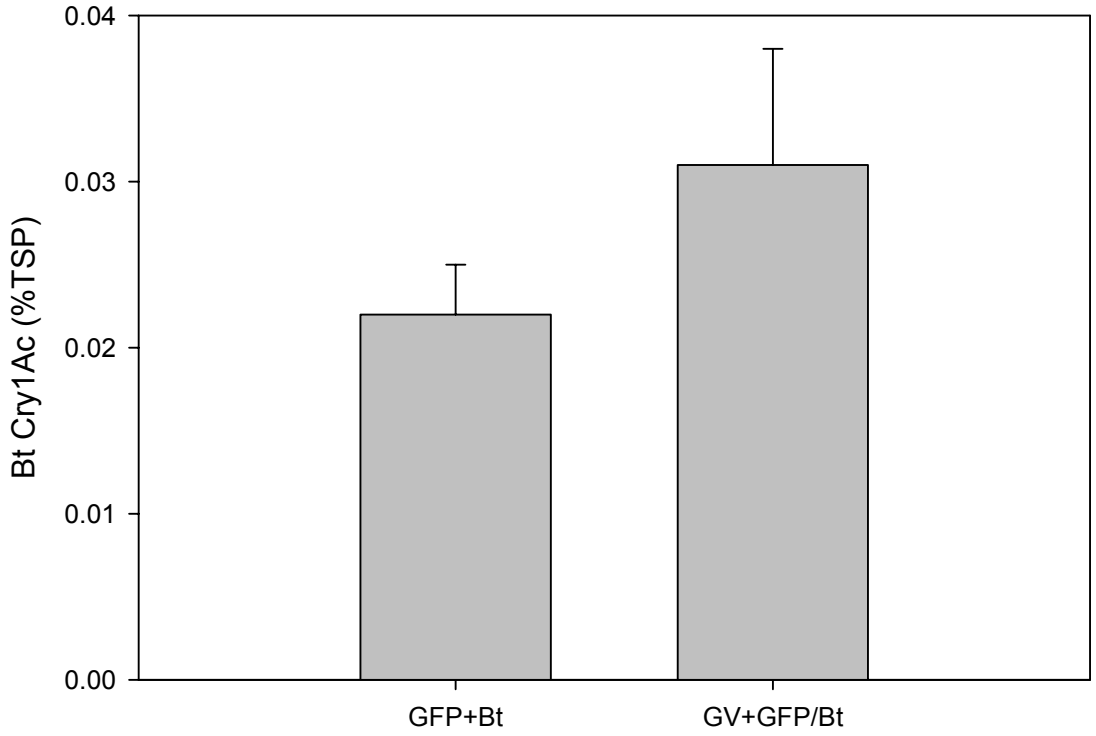

Figure 3.4. Average Bt *Cry1Ac* percent total soluble protein in tobacco tissue infiltrated with *Agrobacterium tumefaciens* containing (GFP+Bt), or (GV+GFP/Bt). Error bars indicate standard error of the mean.

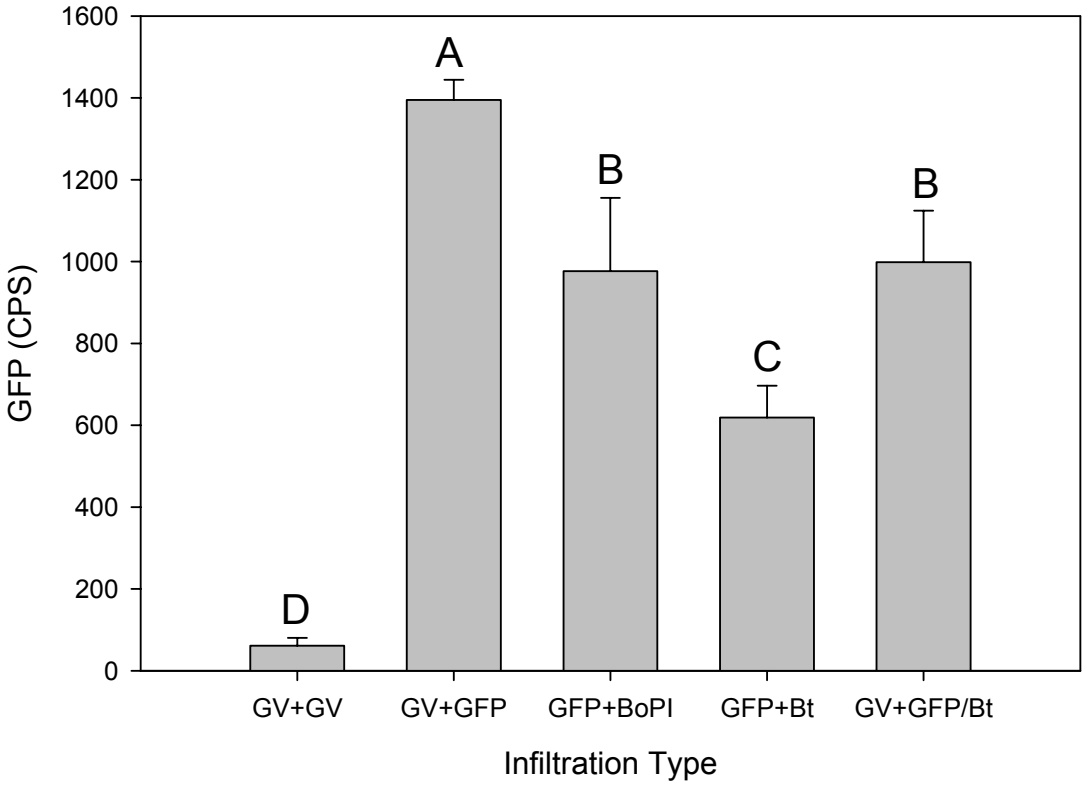

Figure 3.5. Infiltration characterization assay average GFP meter readings in counts per second of tobacco tissue infiltrated with *Agrobacterium tumefaciens* containing (GV+GV), (GV+GFP), (GFP+BoPI), (GFP+Bt), or (GV+GFP/Bt). Error bars indicate standard error of the mean and bars with the same letters are not significantly different ($p < 0.05$).

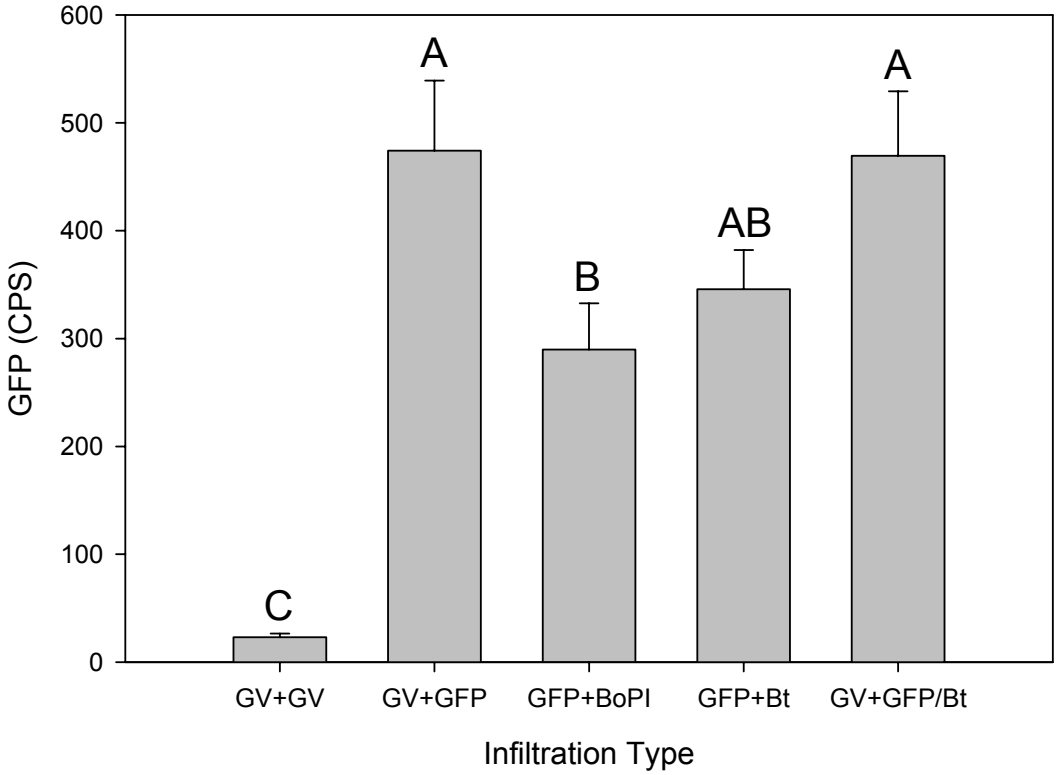

Figure 3.6. Insect bioassay average GFP meter readings in counts per second of tobacco tissue infiltrated with *Agrobacterium tumefaciens* containing (GV+GV), (GV+GFP), (GFP+BoPI), (GFP+Bt), or (GV+GFP/Bt). Error bars indicate standard error of the mean and bars with the same letters are not significantly different ($p < 0.05$).

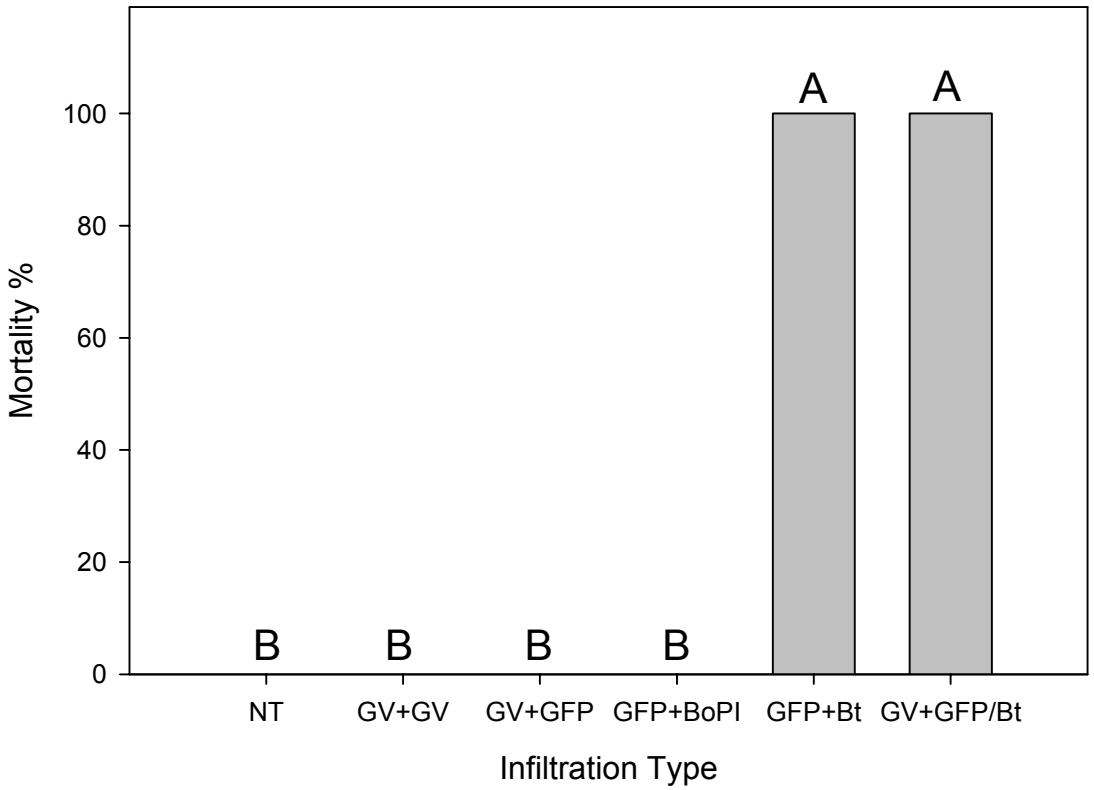

Figure 3.7. Percent mortality on day 6 of larval *Helicoverpa zea* fed *N. benthamiana* leaf tissue infiltrated with non-transformed GV3850 + pBin-mGFP5-ER (GV+GFP), non-transformed GV3850 + pSAM12 (GFP/Bt), pBin-mGFP5-ER + pH602SBt (GFP+Bt), pBin-mGFP5-ER + pBin/BoPI (GFP+BoPI), non-transformed GV3850 + non-transformed GV3850 (GV+GV), or un-infiltrated *N. benthamiana* (NT).

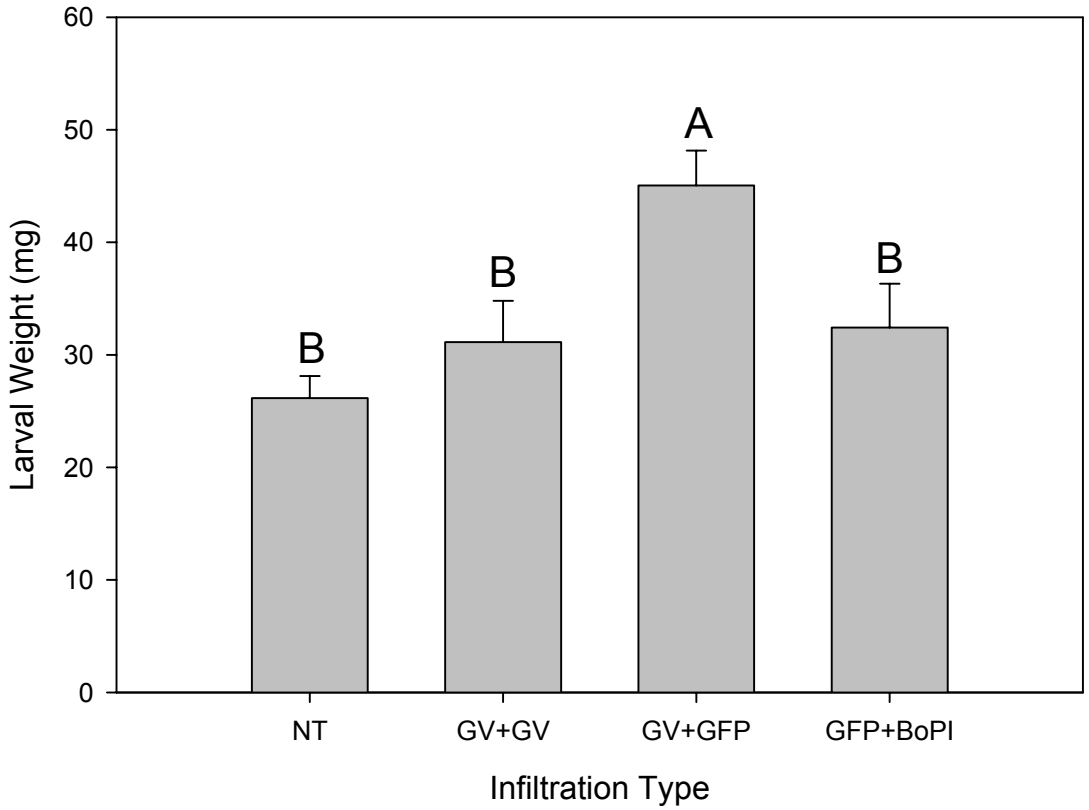

Figure 3.8. Average larval weight (mg) on day 6 of larval *Helicoverpa zea* fed *N. benthamiana* leaf tissue infiltrated with non-transformed GV3850 + pBin-mGFP5-ER (GV+GFP), pBin-mGFP5-ER + pBin/BoPI (GFP+BoPI), non-transformed GV3850 + non-transformed GV3850 (GV+GV), or un-infiltrated *N. benthamiana* (NT). Error bars indicate standard error of the mean and bars with the same letters are not significantly different ($p < 0.05$).

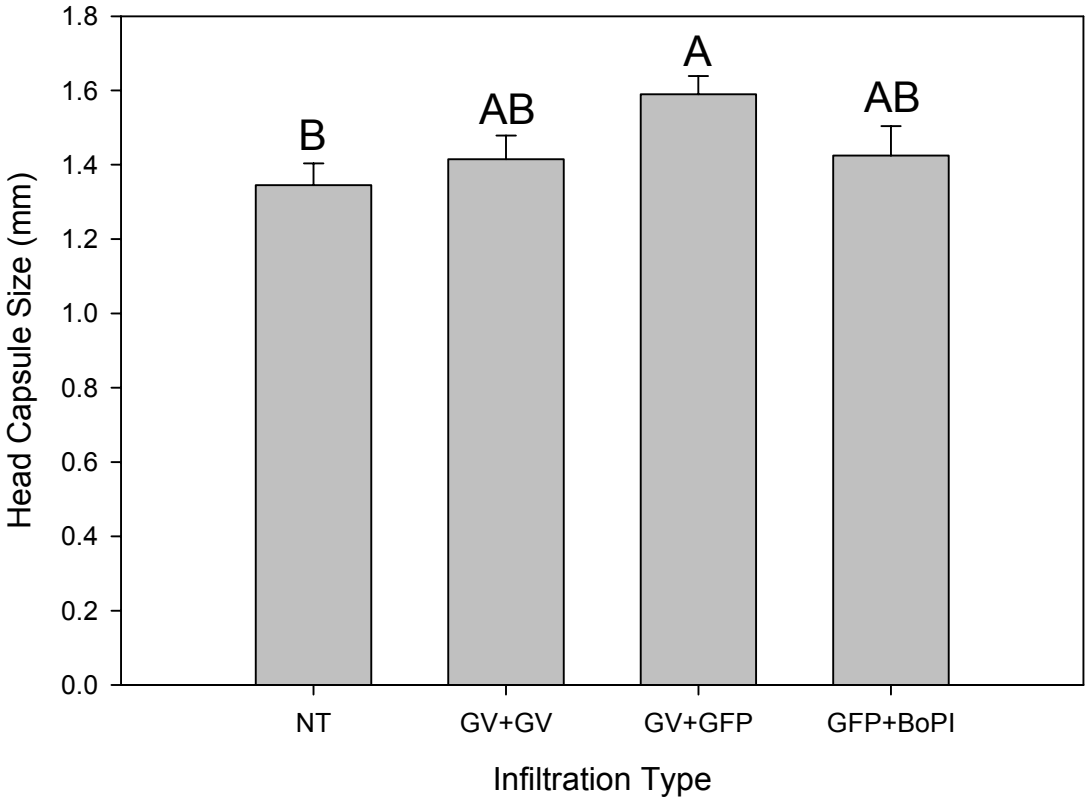

Figure 3.9. Average larval head capsule size (mm) on day 6 of larval *Helicoverpa zea* fed *N. benthamiana* leaf tissue infiltrated with non-transformed GV3850 + pBin-mGFP5-ER (GV+GFP), pBin-mGFP5-ER + pBin/BoPI (GFP+BoPI), non-transformed GV3850 + non-transformed GV3850 (GV+GV), or un-infiltrated *N. benthamiana* (NT). Error bars indicate standard error of the mean and bars with the same letters are not significantly different ($p < 0.05$).

Vita

Brian Michael Leckie was born in Knoxville, TN on June 6, 1975. Brian graduated from Bearden High School in Knoxville, Tn and later attended the University of Tennessee gaining his B.A. in Ecology and Evolutionary Biology with a minor in Chemistry in 1998, M.S. in Entomology and Plant Pathology in 2002, and Ph.D in Plants, Soils, and Insects in 2008.