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Insects Associated with *Stevia rebaudiana* Bertoni (Bertoni), and Toxicity of Compounds from *S. rebaudiana* against *Spodoptera frugiperda* (J.E. Smith) Larvae

Heather Lowery
University of Tennessee, Knoxville, hlowery@vols.utk.edu

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I am submitting herewith a thesis written by Heather Lowery entitled "Insects Associated with *Stevia rebaudiana* Bertoni (Bertoni), and Toxicity of Compounds from *S. rebaudiana* against *Spodoptera frugiperda* (J.E. Smith) Larvae." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Entomology and Plant Pathology.

Jerome F. Grant, Major Professor

We have read this thesis and recommend its acceptance:

Darrell Hensley, Juan Luis Jurat-Fuentes, Dean Kopsell

Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

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Insects Associated with *Stevia rebaudiana* Bertoni (Bertoni), and Toxicity of Compounds from *S. rebaudiana* against *Spodoptera frugiperda* (J.E. Smith) Larvae

A Thesis Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Heather Lowery
May 2017

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DEDICATION

This thesis is dedicated to my Family. For Logan and Joel, thank you for being so great about late trips to check on bioassays, “helping” with the plants, and conversations in the field because you missed me. To my wonderful spouse, Randall, I am deeply grateful for all the sacrifices you have made for this degree and all the ways you helped make it happen. This is also for Gran, who always encouraged me to keep going.

I miss your smile.

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Thank you to my wonderful spouse, who helped me every step of the way (even when I could not make a step)! Thank you, Randall, for tolerating years of insect jars all over the house!

ABSTRACT

Stevia rebaudiana (Bertoni) Bertoni (Asterales: Asteraceae: Eupatorieae), a plant native to Paraguay, produces glycosides used as zero-calorie sucrose alternatives. Despite being a relevant cash crop, little information is available on its suitability to Tennessee or on potential insect pests that may affect *Stevia* production in Tennessee. In addition, the market value for *Stevia* products could be enhanced by additional uses. One example is the potential use of *Stevia* glycosides as insecticides. To resolve these current knowledge gaps, the primary research goals of this project were to: 1) determine if differences in the number of insect visitations exist among three cultivars (‘Candy,’ ‘Sownatural,’ and ‘Stevie’) of *S. rebaudiana* in Tennessee throughout vegetative and reproductive stages; 2) identify the Orders and Families of insect visitors to *Stevia*, and assess differences among cultivars; and 3) assess insecticidal activity of *Stevia* compounds against larvae of the fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), as a relevant pest model considering its polyphagy and cases of resistance to currently available pesticides.

Significant differences in insect visitation were evident, with the highest number of visitors found on ‘Stevie’, which also had the highest average visitation per observation. The lowest average visitation per observation and the fewest total visitations were found on ‘Sownatural’. Among the visiting insects, six Orders and 30 Families were identified. Hymenoptera was the most observed Order, followed by Diptera and Hemiptera. No seriously damaging pests were observed.

Bioassays of FAW larvae indicated toxicity of *Stevia* glycosides, as well as an impact on weight gain and development. Stevioside had the lowest LC_{50} [LC50], while

whole leaf powders demonstrated the most growth reduction. Similar results were observed when performing bioassays with a colony of FAW with resistance against transgenic corn producing the Cry1Fa protein from *Bacillus thuringiensis* (Berliner) (Bt).

The outcome of this research is an insect profile of *Stevia* which may be used for the development of insect management plans for use in large-scale production. The use of *Stevia* glycosides as insecticides is also supported and should increase the value of this crop.

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CHAPTER I

Introduction and Literature Review

Chronic human diseases, including those related to weight gain, type 2 diabetes, hypertension, cardiovascular disease, and cancers, are responsible for more than 17 million global deaths each year (Singh et al. 2015). Excess calories from sugar consumption, mostly in the form of sucrose or high fructose corn syrup, have been linked with several chronic diseases including excess weight gain, and the onset of adult diabetes (Singh et al. 2015). Since 1970, sugar consumption has increased in the United States by 20%, an equivalent of 64 kg per person per year (Anton et al. 2010). This increased sugar consumption has led to numerous health concerns, including obesity. Thus, a need exists to reduce the excess calories from sugar consumption. This reduction is possible with the use of sucrose alternatives, such as artificial sweeteners, sugar alcohols, and natural sweeteners, which have fewer calories (Priebe & Kauffman 1980).

The artificial sweetener saccharin was first marketed in 1886, and the distributor claimed many health benefits. In addition to calorie reduction, saccharin was touted as a cure for gastritis (Priebe & Kauffman 1980). These claims led to a controversial period of evaluations by government, private, and industry researchers to determine any impacts on human health. Links to saccharin and bladder cancer led to a proposed prohibition in food products in 1977 (Priebe & Kauffman 1980). This proposed prohibition was not successful and currently the name brand distribution of saccharin, primarily known as Sweet'N Low®, continues. The controversy over artificial sweeteners and the need to reduce calorie consumption from sugar fueled interest in natural sweeteners, and none has captured the global market more notably than the extracts from the Paraguayan plant,

Stevia rebaudiana (Bertoni) Bertoni (Asterales: Asteraceae: Eupatorieae) (Gardner 2011).

Overview of *Stevia rebaudiana*

Description of *Stevia rebaudiana*

Stevia rebaudiana is a perennial herb or tender annual (depending on growing location), with filiform roots. Cultivated plants reach 70-80 cm in height with either a straight single stem or multiple lateral stems if harvested before reaching 10 nodes (Soejarto 2002). Stems are slender, brittle, highly pubescent with trichome hairs throughout. Leaves are highly variable in shape, but are generally simple, ovate, opposite and pubescent, with complete margins which are often toothed (Soejarto 2002) (Figure A-1) (all figures and tables are placed in Appendix).

Inflorescences of the plant are arranged in a corymbose cluster at the terminal end of a branch or stem with capitulum that consists of an involucre of five linear phyllaries arranged in a whorl (Soejarto 2002). Florets are perfect, white, tubular, and arranged in a whorl with each floret opposite a phyllary (Soejarto 2002). Stamens are inserted, while the style branches generally exceed or protrude beyond the corolla tube (Soejarto 2002) (Figure A-2). Seeds are contained within slender cylindrical achenes with persistent pappus bristles (Goettemoeller & Ching 1999). Seeds that are clear or translucent are not viable, while tan to black seeds, depending on cultivar, are viable with black seeds yielding a greater percentage of germinating seedlings (Goettemoeller & Ching 1999).

History of *Stevia rebaudiana*

Grown and consumed by natives in Paraguay, Ka'a he'e, which means "sweet herb" or "sweet leaf" (Chesterton & Yang 2016), is the only known sweet-tasting plant in this genus of more than 200 species (Chesterton & Yang 2016). The genus *Stevia* is named after Petrus Jacobus Stevus, a 16th century botanist (*Stevia*: rom niche 2013). While the newest members of this species were described in 2001 (Soejima et al. 2001), *S. rebaudiana* was first described by a Swiss naturalist, Moisés Santiago Bertoni, in 1899 (Lemus-Mondaca et al. 2012). Bertoni sent samples of this newly discovered plant to a Paraguayan chemist, Ovidio Rebaudi, who was the first to identify one of the main sweeteners within the plant (Chesterton & Yang 2016) and is presumably the person whom the rebaudioside compounds are named. In 1931, after the plant gained exposure in Europe, methods for extracting the compounds were developed by M. Bridel and R. Lavielle (referenced in Chesterton & Yang 2016).

The discovery of the plant, its sweeting qualities, and how to extract sweetening compounds would have been lost if not for sugar shortages during World Wars I and II. In Japan, sugar supplies were low and a ban was activated on all artificial sweeteners. These conditions led the Japanese government to seek natural alternatives, which were found in *Stevia* (the name used collectively for the plant and the sweet compounds from the plant). Starting with importing the plant through the 1950s, the Japanese government moved to growing its own supply, including designing research centers dedicated to the development of best growing practices. From the mid-1970s, commercial production of sweetened products has relied heavily on the plant and its extracts, which are included in

products such as: soy sauce, yogurt, soft drinks and others (reviewed in Chesterton & Yang 2016).

Not as accepting as Japan, the United States Food and Drug Administration (FDA) banned importation of *Stevia* in 1991 for human consumption due to questions of safety (Chesterton & Yang 2016). While whole-leaf sales of the plant as a sweetener are still prohibited, the FDA did not object to "highly refined *Stevia* preparations" from the plant--namely rebaudioside A and stevioside--from being considered generally regarded as safe (GRAS) (U.S. FDA 2016).

Compounds in *Stevia*

The sweetness of *S. rebaudiana* is attributed to steviol glycosides found in the leaf tissue (Kaushik et al. 2010). A steviol glycoside is a simple sugar bound to an aglycone which is steviol, an ent-kaurane diterpenoid (ent-kaurane describes the back bone of the skeleton of a compound consisting of two terpenes with the formula $C_{10}H_{16}$) (Nosov et al. 2014). The two most used glycosides in *Stevia* are stevioside and rebaudioside A (Figure A-3). Stevioside is the most abundant at 4-13% dry mass (DM) followed by rebaudioside A (2-4% DM), rebaudioside B (1-2% DM), rebaudioside C (1-2% DM), rebaudioside D (0.3% DM), rebaudioside E (0.3% DM), rebaudioside F (0.3% DM), and dulcoside (0.3% DM) (Tavarini & Angelini 2013). Stevioside has been reported to have a strong licorice aftertaste, and is less favorable than rebaudioside A, which has a sweeter taste without an aftertaste (Tavarini & Angelini 2013). In addition, rebaudioside A has a higher melting point (242-244 ºC) than stevioside (196-198 ºC) (Kinghorn 2002), which allows more uses in food production.

Market for Sugar Substitutes

The term "Stevia products" or "Stevia" can be used to refer to any commercial sweetener that uses steviol glycosides, as the United States FDA does not require the label to indicate the glycoside(s) used. Products using *Stevia* are second in total sales in the sugar substitute market with a share of 34% behind Splenda®, a sucralose based sweetener (*Stevia*: rom niche 2013). Overall the sugar substitute world market is valued at $7.4 billion (USD), and is predicted to increase 5.9% by 2019 ("Worldwide Low-Calorie Food Market," 2016). The largest increase in *Stevia* consumption is expected to be in North America, where consumption only accounted for 12% of global consumption in 2012 (*Stevia*: rom niche 2013). In the United States, two major beverage companies have both marketed and started using *Stevia* products since the GRAS status in 2008 to sweeten their beverages: Cargill manufactures Truvia® (the leading United States *Stevia* sweetener) for Coca-cola® and Merisant produces PureVia® for Pepsi® (Engber 2014).

Research on Health Benefits of *Stevia*

Both stevioside and rebaudioside A are used as sucrose alternatives for human consumption and therefore have been studied for their impact on mammalian health. Tumor growth in both male and female Wistar rats, *Rattus norvegicus* (Rodentia: Muridae), was studied over a two-year period. Tumor growth was not significantly ($p>0.05$) different between the control diets and stevioside treatments, which ranged from 0.2 to 1.2% of diet, with a maximum of 838.9 and 748.6 mg/kg/day for females and males, respectively (Xili et al. 1992). Stevioside was also used in an experiment with spontaneously hypertensive rats and confirmed to be an effective antihypertensive agent

(Lee et al. 2001). Rebaudioside A also showed no difference in organ weight or testicular lesions relative to dose of consumption with Wistar rats after 4 weeks and high ingestion rates (Curry & Roberts 2008).

Stevia products lowered calories consumed and promoted a greater sense of satiety than sucralose in a human consumption trial (Anton et al. 2010). Consumption of *Stevia* products also lowered blood glucose and insulin levels when compared to sucralose and aspartame in human trials (Anton et al. 2010). Moderate cytotoxicity by *Stevia* alcohols against human lung carcinoma cells has also been shown (Cerda-García-Rojas et al. 2009).

While the results of health studies may comfort some consumers, others are concerned with the "naturalness" of a substance which is so highly processed. Cargill agreed to a $6.1 million (USD) settlement for Truvia® in 2014, over the use of term "natural" when the end product is so highly processed (Sweet rewards 2014). In a similar class action lawsuit, Cumberland Packing Corporation agreed to a settlement for their product Stevia in the Raw® to avoid lengthy litigation over the accuracy of the label in claiming "100% natural" (Settlement 2016). This lawsuit included all of the products purchased for personal use between 9 October 2009 and 1 July 2014 (Settlement 2016). Neither company admits to wrongdoing, but both have either suspended the use of the word "natural" on their labels or have included additional information as to the processing of the product on their website (Sweet Rewards 2014).

Current Cultivation of *Stevia rebaudiana*

Demands for *Stevia* compounds have led many countries, companies and universities into researching commercial cultivation of this crop. Publications on proper growth can be found for Brazil, Canada (Carneiro 2007), China, Egypt, India, Italy (Martini et al. 2016), Mexico, New Zealand, Russia (Kostjukov et al. 2015), and Turkey. In the United States, *Stevia* has been grown in California, Georgia, and North Carolina where warm, humid climates occur. Sweet Green Fields, a *Stevia* producer, is providing farmers in Georgia with plants for production (*Stevia*: rom niche 2013). This new crop is targeted to tobacco (*Nicotiana tabacum* L.) farmers, whose crop demands have shrunk in recent years (*Stevia*: rom niche 2013).

Stevia rebaudiana is difficult to grow in the United States, especially from seed. Germination has been the main hindrance regardless of the location, as *S. rebaudiana* has sporophytic self-incompatibility (Martini et al. 2016). Greater viability can be achieved by using selected genotypes which have an increased number of blooms (Martini et al. 2016). The greater number of blooms both increases the number of available reproductive structures, which allows for more cross-pollination, and draws the attention of local pollinators, allowing for greater pollen dispersal than wind pollination alone (Martini et al. 2016). Thus, many insect-related studies with *S. rebaudiana* have focused on pollinating species and their efficacy at increasing germination, specifically when examining cross-pollination (Martini et al. 2016). Families of insects previously confirmed to carry *Stevia* pollen in Italy are Apidae (Hymenoptera), Calliphoridae (Diptera), Halictidae (Hymenoptera), Lycaenidae (Lepidoptera), and Syrphidae (Diptera) (Martini et al. 2016). A previous pollination study conducted in Italy on improved

germination rates for *Stevia*, did not give any indication of potential pest families (Martini et al. 2016). This focused on the presence of pollinators, their abundance, and the links between cultivar morphology and cross-pollination fertility, which improved viable seed percentages (Martini et al. 2016).

Difficulties with germination have led most commercial producers to take advantage of the natural tendency of *Stevia* to lodge and develop lateral growth. Plugs or stem cuttings are recommended for field plantings, especially large scale (Martini et al. 2016). A separate market for the bulk sale of these clones, as many varieties are covered under plant patents for increased durability, leaf mass, better taste profiles and compound percentages, has developed (United States patents, S&W 2014). Clone plugs can be field planted using commercial equipment (Davis & Gaskill 2015), but harvesting of the leaves is still a manual process.

Stevia in field trails in North Carolina were damaged by mammalian pests (mainly deer and rabbits), which fed on the *Stevia* (Davis & Gaskill 2015). Wildlife consumption of *Stevia* was also observed in Tennessee in 2015 (personal observation). Russian trials that focused on insect pests reported the consumption of *Stevia* by cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), and bordered straw moth, *Heliothis peltigera* (Denis & Schiffermüller) (Lepidoptera: Noctuidae), during the budding phase of *Stevia*, but both were controlled without insecticidal use by early harvesting of leaves before damage was extensive (Kostjukov et al. 2015). A study of field production of *Stevia* by the University of Kentucky indicated few pest problems (Kaiser & Ernst 2015), however, their results do not imply that plants grown in

Tennessee will be pest free, nor that the cultivars will have an equal absence of pest problems.

Complications arise for insect monitoring due to the misunderstanding of the different growth stages of *Stevia*. Two main phases, vegetative and reproductive, are used in general to describe the development of the plant, but they can further be categorized into many smaller stages (Figure A-4) (Carneiro 2007). Many reports, such as the Russian study, did not use the sub-stages when reporting insect presence (Kostjukov et al. 2015). Timing, fertility and stress can alter the percentage of compounds that the plants produce, but the budding or reproductive phase is based on the length of darkness (Carneiro 2007).

To circumvent the production difficulties with *Stevia* in the field, companies are developing ways to produce the compounds without the whole plant, such as genetically altering yeast to release compounds through fermentation (Engber 2014). In fact, Cargill has contracted with Evolva to produce some of the minor glycosides from *Stevia* that are produced within the plant in concentrations too low to be cost effective for extraction (Sweet Irony 2015). The reported glycoside choice of Cargill is rebaudioside M, which accounts for less than 1% of the plant's biomass, but is devoid of the lingering aftertaste of the other glycosides (Sweet Irony 2015).

Insecticidal Properties of *Stevia*

Stevia is in the same plant Family, Asteraceae, as *Chrysanthemum roseum* and *C. cinerariaefolium*, which are sources for a botanical insecticide (pyrethrum) (Schleier & Peterson 2011). Pyrethrins (the collective name of the pyrethrum compounds) are

lipophilic esters that have a high photo-degradation and are known for fast “knockdown” of insect populations (Schleier & Peterson 2011). Unfortunately, resistance to pyrethrins and pyrethroids (the synthetic version of pyrethrins) has developed in some insect species by overuse of the voltage-gated sodium channel binding mode of action in pesticides. Dichlorodiphenyltrichloroethane (DDT) has a similar mode of action and the broad usage of this pesticide resulted in cross-resistance to both DDT, pyrethrins, and pyrethroids (Schleier & Peterson 2011). Resistance to pesticides costs $1.4 billion (USD) per year in crop losses (Schleier & Peterson 2011).

While the insecticidal mode of action for the sugar alcohol erythritol (main sweetener used in Truvia®) is unknown, it was found to reduce longevity in direct proportion to the percent consumed by the fruit fly *Drosophila melanogaster* (Meigen) (Diptera: Drosophilidae) using an amount well below the Advisable Daily Intake (ADI) for humans (Baudier et al. 2014). Choice tests were given in this study, and the flies preferred to consume erythritol compared to sucrose food sources offered (Baudier et al. 2014). Using erythritol as an attractant or insecticide may be a safe way to manage indoor fruit fly pests, as it has demonstrated no harm to humans or mammals, including household pets. A lower development of resistance may also be expected for home use, as the genetic diversity of indoor insects is limited in a constricted environment as entire populations are eliminated without harbor areas where they can breed.

Alcohols made from *Stevia* products were also used in experiments with green peach aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae), and cotton leafworm, *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae). These compounds acted as antifeedants for both herbivores when applied to lettuce plants (Cerda-García-Rojas et al.

2009). This study also noted that the alcohols were not phytotoxic to the plants, which allows for the direct application of the compounds for use in field crop settings. *Stevia* compounds could be used as a direct spray insecticide with little concern of skin exposure, ingestion, inhalation or human mortality, and could reduce the estimated $10 billion (USD) that is associated with environmental damages related to insecticide application (Pimentel 2005). Further, the bio-pesticide market is predicted to reach $5 billion (USD) by the end of 2020 and is the largest growing section in the $54 billion (USD) pesticide market for all pesticides worldwide (Bunge 2014). However, to enter this market *Stevia* products must be researched to prove the level of mortality (if any) on economically important pests, specifically those that have shown a history of adaptation to pesticides. To test the potential of *Stevia* in the bio-pesticide market, the fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), was chosen as a model insect species for this study.

Overview of Fall Armyworm, *Spodoptera frugiperda*

Importance in Tennessee

The FAW was used to advance the insecticidal activity knowledge of *Stevia* products, as it made a suitable herbivore model. In field observations, a fourth-instar yellow-striped armyworm, *Spodoptera ornithogalli* (Guenée) (Lepidoptera: Noctuidae), was found on a *Stevia* leaf, however, the larva did not consume it (personal observation). Yellow-striped armyworm is a general feeder that damages a variety of crops, however, the presence of this species is of smaller economic concern in Tennessee than its relative, the FAW, which is more mobile than the yellow-striped armyworm (Sparks 1979). The

FAW larvae also feed on several of the state's most economically important crops: soybean, corn and cotton (Lockman 2012). Thus, FAW was selected as a model species to determine the impact of Stevia products through multiple bioassays**.**

Description of Fall Armyworm

This nocturnally active moth is native to tropical regions in the Americas, and in the United States it overwinters in Texas and Florida and then disperses throughout the Eastern and Central United States, going north into Southern Canada (Figure A-5) (Sparks 1979). The biology of this insect has been well described (Sparks 1979, Capinera 1999). Mating occurs after a feeding period that starts after dusk and is initiated by a sex pheromone the female emits through extension of her ovipositor. Eggs are deposited shortly after mating in clusters generally consisting of 100-200 eggs/cluster. Dome-shaped eggs with a point at the apex are approximately 0.4 mm in diameter with a height of 0.3 mm. After 2 to 4 days, larvae emerge, consume the egg shell, and then disperse to feed upon host material.

Young larvae are light green with a dark head during the first instar. During the second and third instars, the dorsal surface becomes brown with white lines running laterally from the head to the anus. In the fourth through sixth instars, the head is reddish brown with an inverted "Y" while the body has elevated spots along the dorsal side. Growth from the first to the last instar averages 14 days during warmer seasons and 30 days during cooler seasons. Pupation typically takes place 2 to 8 cm deep in the soil where the reddish-brown pupa develops for 9 days in warm temperatures and up to 30 days in cool temperatures.

After emergence, the adult female initiates mating in 3 to 5 days and continues to mate throughout her life, which ranges from 7 to 21 days. The adult female forewing is grayish brown to a mottling of brown and gray. Hindwings on both the male and female are iridescent silver-white with a narrow dark border along the edge. The male forewing has a distinct pattern of white triangular spots near the center and along the tip in a shaded brown and gray background.

Host Plants of Fall Armyworm

This insect has a wide host range, but prefers grasses, particularly sweet corn (*Zea mays* L. var. *rugosa*), field corn (*Zea mays* L.), sorghum (*Sorghum bicolor* L.), Bermudagrass (*Cynodon dactylon* L.), wheat (*Triticum* spp. L.) and crabgrass (*Digitaria* spp. Haller) (Capinera 1999). Other crops not in the Family Poaceae (Gramineae) (i.e., grasses) can serve as host plants with significant losses that may occur with heavy infestations. Non-grass hosts include alfalfa (*Medicago sativa* L.), barley (*Hordeum vulgare* L.), clover (*Trifolium* spp. L.), cotton (*Gossypium* spp. L.), millet (*Pennisetum glaucum* L.), peanut (*Arachis hypogaea* L.), soybean (*Glycine max* L.), sugarbeet (*Beta vulgaris* L. subsp. vulgaris), and tobacco (Capinera 1999). The damaging stage of FAW is the larval stage, and larvae can defoliate whole plants and even whole fields (Capinera 1999). In addition to defoliation, FAW larvae in corn also burrow into the growing point of the fruit, causing damage to the cob (Capinera 1999).

Field Resistance of Fall Armyworm to Transgenic *Bacillus thuringiensis* (Bt) Corn

In 1996 cotton and corn containing Bt genes became available for purchase and have dominated >80% of the hectarage of both crops in the United States (USDA 2016). By using so many Bt transgenic crops, both in the United States and around the world, farmers have consistently exposed insects to the Bt toxin, and some resistant insect populations have been documented (Tabashnik et al. 2013). In the case of FAW, field trials found that third-instar FAW had a survivorship of 25-76% on field corn expressing Bt crystal proteins Cry1Ab and Cry1F (Hardke et al. 2011). Field-evolved resistance to Cry1Fa was discovered and confirmed for populations of FAW from Puerto Rico in 2010 (Storer et al. 2010). A population named 456 was generated from insects collected in the corn fields in Puerto Rico at that time (Blanco et al. 2010) and was used for the present study. Moreover, some of these populations display cross-resistance to organophosphates, which are often used as a contact insecticide to kill larvae that are not affected by Bt toxins (Zhu et al. 2015). By exhibiting resistance to both, FAW becomes difficult to manage and a new method of control is needed.

Research Objectives

Observations of insect activity on *Stevia* are important to determine if detrimental herbivores will impede its growth in Tennessee. By observing insects, the interactions with this new crop and existing insects can be noted and used to plan cropping systems. Further, by recording the number and type of visitation that occurs on selected cultivars, the potential of *Stevia* as a crop can be further assessed. An understanding of how *Stevia* compounds impact FAW larvae, both to determine if this species will be a pest on *Stevia*,

and to determine any uses for *Stevia* as an effective insecticide for this damaging pest, is necessary. The specific goals of this research are to:

1) Determine if differences in insect visitation exist among cultivars of *S. rebaudiana* throughout vegetative and reproductive stages,
2) Identify insect visitors of *S. rebaudiana*, and assess differences among cultivars, and
3) Assess insecticidal activity of *Stevia* compounds against FAW through consumption bioassays, including tests with a strain of FAW with field-evolved resistance to the Cry1Fa protein of *Bacillus thuringiensis* (Bt) as produced by transgenic corn.

CHAPTER II

Insect Visitation of *Stevia rebaudiana* Bertoni

Introduction

The number of viable seeds from each generation of *Stevia rebaudiana* Bertoni (Bertoni) is low due to poor germination rates and obligatory cross-breeding as a result of sporophytic self-incompatibility (Martini et al. 2016). Thus, many insect-related studies with *S. rebaudiana* have focused on pollinating species and their efficacy at increasing germination, specifically by cross-pollination (Martini et al. 2016). Insect families previously confirmed to carry *Stevia* pollen in Italy are: Apidae (Order Hymenoptera), Calliphoridae (Order Diptera), Halictidae (Order Hymenoptera), Lycaenidae (Order Lepidoptera), and Syrphidae (Order Diptera) (Martini et al. 2016). This study did not indicate potential pest families, but it does illustrate the presence of pollinators, their abundance, and the links of cultivars to fertility with cross-pollination (Martini et al. 2016). A correlation in the number of flowers and attractiveness to pollinators, which is tied with the germination rate, is possible (Martini et al 2016). Relationships of cultivar morphological characteristics and herbaceous insect visitation, however, have not been explored.

Pests are more of a concern than pollinators for those in commercial production or those growing *Stevia* on a small scale, as cloned plugs are the primary source for plant material instead of seeds (Martini et al. 2016). Although no major pests were discovered in production of *Stevia* in Kentucky (Kaiser & Ernst 2015), those results do not mean that plants grown in Tennessee will be pest free, nor that the cultivars will have equal insect visitation. Insect visitation is an indication of the plant's ecological niche, and the

interactions of the compounds in *Stevia*, physiology, and bloom time on insect visitors are unknown in Tennessee. Of primary concern in this study is the overall number of insects that visit *Stevia* and how, if at all, the cultivars impact insect visitation. This study focused on the following question: does insect visitation vary among cultivars, planting dates, or growth stages? The objectives of this study are:

1) Determine if the average number of insect visitations varies among cultivars.

2) Determine if the mean total number of insect visitations varies among cultivars.

3) Determine the impact of insect visitations among the three growth stages of *Stevia*.

Materials and Methods

A study was designed to investigate the role of cultivars, planting date, and growth stages on the total number of insect visitations and the average number of insect visitations on *S. rebaudiana.* Three cultivars and two planting dates were arranged with three replications per planting date. This study was conducted on the Lowery Farm (35.87 ºN Lat. and -84.30 ºW Long.) in Loudon County, TN. The following information details the experimental design of this study.

Field Study

To determine if insect visitations differ among cultivars of *S. rebaudiana*, seeds of three cultivars, *S. rebaudiana* ‘Candy’, *S. rebaudiana* ‘Sownatural’, and *S. rebaudiana* ‘Stevie,’ were acquired on 29 October 2015 from Ritcher’s Seed Company (Goodwood, Ontario, Canada). These cultivars were chosen due to variation in germination rates, which is closely tied to the number of pollinator visitations as higher germination rates

are achieved with the greater number of flowers produced for pollinator attraction (Martini et al. 2016). Germination rates ranged from 5-70%, with ‘Candy’ at 5%, ‘Sownatural’ at 30% and ‘Stevie at 70% (Phone interview, Ritcher’s Seed Company). Seeds were stored at 1-4ºC in the original packaging after initial receipt.

On 6 April 2016, one-half of the seeds were removed from the packaging (the other one-half remained under refrigeration until 26 April 2016 for a second planting date) and immersed into 100 ml of deionized water. Seeds were soaked for 48 h in complete darkness. Seeds were separated between the black (viable) seeds and the tan (non-viable) seeds. This separation was done both by the flotation method (viable seeds will sink to the bottom) and by visual identification of dark and swollen seeds. Non-viable seeds were discarded. Viable seeds were placed manually into a saturated growing media (Sungrow Professional Growth Mixture, Agawam, MA) with the following number of seeds per unit: ‘Candy’ 20, ‘Sownatural’ 3, and ‘Stevie’ 2. Each cultivar had two trays of 50 units per tray that were placed into a dark incubation chamber at 26ºC for 4 days. Once the plants had reached the seedling stage of vegetative growth (Figure A-4c), trays were removed and placed onto a greenhouse bench in a random pattern, where they were watered as needed throughout the 60-day growing period. Relative humidity and temperature were recorded inside the greenhouse by Watchdog environmental monitoring system (Spectrum Technologies, Inc., Aurora, IL); daily averages are displayed in Table A-1 for the entire 60-day growing period. Overall, temperatures ranged from 18.62°C to 27.17ºC, with relative humidity ranging from a low of 12.76 to a high of 88.51%.

Seedling stage plants with 4-7 nodes (Figure A-4,) were transplanted on 9 June 2016 (first planting date) into tilled field sections with the following conditions: pH 5.8, P 13 mg/kg, K 46 mg/kg, Ca 784 mg/kg, and Mg 155 mg/kg, with 4.7% organic matter (Waypoint Analytical, Memphis, TN, 13 June 2016) (Figure A-6). The temperature on 9 June 2016 was 28ºC during planting, but dropped to a seasonal low of 13°C that evening. Transplants were placed individually by hand into pre-drilled holes approximately 5.08 cm in circumference by 7.62 cm deep formed from an auger bit attached to a cordless drill (Ryobi 18 volt).

The remaining seed for the second planting date were handled similarly to the first planting. Relative humidity and temperature were recorded inside the greenhouse for the second planting; daily averages are displayed in Table A-2. Overall temperatures ranged from 18.62°C to 30.09°C, with relative humidity ranging from 35.49% to 88.51%.

Once the plants for the second planting were 60-days old, seedling stage with 4-7 nodes (Figure A-4), they were transplanted into tilled field sections on 29 June 2016 (Figure A-6). The high and low temperatures were 32ºC and 19°C, respectively.

The field design for this study is shown in Figure A-6. The entire study area measured 26.21 m by 9.75 m. Each planting date was separated into nine sections (0.91 m by 1.22 m) with 12 plants spaced 0.30 m apart inside each section (total of 216 plants in the field study with 108 plants per planting date and 72 per cultivar). Each of the three cultivars were represented in three replications per planting date (Figure A-6). Each of the nine sections within the planting dates were tilled and raked prior to planting and were separated by 3 m of untilled soil. A border of 0.91 m (Figure A-6) was placed around the perimeter of each planting to allow for mowing between the field and the

fence. Due to previous difficulties with mammalian herbivores, an electric fence with six spaced rows of wire ranging from 7.62 to 91.44 cm above the ground was installed around the entire field. Watering and weeding were conducted as needed, with water applied usually every other day and weeding occurred twice per week. Neither activity took place prior to observation and sampling to prevent disruption of insect activity.

Dead plants were replaced as necessary until the supply of replacement plants were exhausted (‘Candy’ 15, ‘Sownatural’ 25, and ‘Stevie’ 11). Due to above normal temperatures and below average relative humidity, Loudon County, TN was determined to be in a drought area (D0 or abnormally dry) (Figure A-7) in August, and conditions intensified as the growing season continued. By the end of the study (15 October), the area where the field was located was classified as D3, which is extreme drought. Environmental stresses, such as excessive temperatures and low relative humidity, led to mortality of more plants in sections than could be replaced to maintain the 12-plant total per section. Once this occurred, the entire field was surveyed prior to each observation to determine the minimal number of living plants in each section (living plants counted for all of the 18 sections). Once the minimum living number of plants per section was determined, only that number of plants per section were observed and recorded in both planting dates. By observing the same number of plants per section, the impacts of the cultivar on insect visitations could be assessed independently from the fitness of the cultivar to the growing conditions.

Three growing stages were used during this study to assess the impact of plant stage on insect visitation. The three stages are: Stage 1(Immature)-plants with less than

12 nodes, Stage 2 (Mature)-plants with 13 or more nodes, and Stage 3 (Reproductive)-plants with buds and/or blooms. All three stages were present in both planting dates.

Observations

Visitation of a plant was defined as the physical contact of the plant by an insect. Each insect visitation was recorded using visual observation and included insect contact with any part of the plant. Insects that sheltered overnight on the plant were also observed and recorded as a visitation. Each section of plants (Figure A-6) was observed for a total of 8 min with an equal number of plants observed and recorded for each observation and sampling date. This method has been used for pollination studies, generally with an interval of 10 min to observe flower visitation (Kearns & Inouye 1993); however, the number of flowers observed in that study had to be altered to improve observation accuracy. Since the number of plants could not be altered by the time restrictions or the abundance of visitors as in the Kearns & Inouye (1993) method, 8 min was selected to be the optimal time per section to observe and record visitations throughout the study.

All observations were conducted twice each week between 13 June 2016 and 15 October 2016. Insect visitations on *Stevia* (first planting date) were observed throughout the study period with a total of 22 observations. Plants (first planting date) were exclusively observed from 13 June 2016 until 29 June 2016 when the second planting date was implemented. Thereafter, *Stevia* from each planting date were observed once per week. Insect visitations of *Stevia* (second planting date) were observed from 5 July 2016 until 3 October 2016 for a total of 19 observations. Before each observation, sections within a planting date were randomized for the order of observation using a ten-sided polyhedral die and general weather conditions were determined by a Weather Channel

phone application (temperature, relative humidity and UV index). The number of insect visitations were recorded on data sheets (Figure A-8) in the field and later placed into Excel for analysis. Sampling and observations occurred on selected days at different time intervals [AM (8:00AM-11:00AM), MID (12:00PM-3:00PM) and PM (5:00PM-8:00PM)] to observe the variety of visitors to the plants throughout the day as well as the growing stage. Vegetative stages (Immature and Mature) were present until 10 September 2016 when both planting dates entered the Reproductive stage.

Data Analysis

The influence of cultivar, planting date, and interactions of cultivar and planting date on the average number of insect visitations and the mean total number of insect visitations from both planting dates were analyzed in SAS 9.4 using a randomized complete block design using the GLIMMIX procedure and a Gaussian distribution (SAS Institute Inc., Cary, NC). Least Squares Means were separated with a Tukey adjustment and 0.05 significance level for mean separation Three cultivars, two planting dates and three blocks were used for data analyses. Growth Stages were analyzed using the same SAS procedures with three cultivars, three stages of growth, and three blocks.

Results and Discussion

The average number of insect visitations per observation were significantly different among cultivars, but no differences between planting date or interactions (cultivar*block or block*planting date) were detected (Table A-3). The average number of insect visitations on ‘Sownatural’ (2.12) were significantly lower than those on the other two cultivars; however, the average number of visitations per observation (8 min

per section) was not significantly different between ‘Candy’ (3.16) and ‘Stevie’ (3.20) (Figure A-9).

The mean total number of insect visitations were significantly different among cultivars but not for planting date or for the interaction of cultivar and planting date (Table A-4). The total visitation number was significantly lower (44.0) on ‘Sownatural’ than on the other two cultivars, ‘Stevie’ (66.3) or ‘Candy’ (65.8); however, the total number of visitations were not significantly different between ‘Candy’ and ‘Stevie’ (Figure A-10).

The average number of visits per observation was significantly different among growth stages, but not for the interaction of cultivar and growth stage (Table A-5). The average visitation per observation was significantly higher (5.07) during Stage 3 (Reproductive) than the other two stages of growth (Figure A-11). Average visitation was not significantly different between Stage 1 (Immature) plants (2.22) and Stage 2 (Mature) plants (2.67) (Figure A-11).

Abiotic stress on the plants and insects was high due to the environmental stresses that intensified as the season progressed. Insect visitation totals may be lower for ‘Sownatural’ due to the extreme environmental conditions experienced in 2016. As noted in ‘Materials and Methods’, the number of replacement plants used for ‘Sownatural’ was greater than those for the other two cultivars. An inability to adapt may have played a role in the reduced total number of visitations; however, ‘Sownatural’ plants bloomed at the same time as the other two cultivars. Also, the number of plants used to observe visitation was equal among the sections, regardless of the number of replaced plants.

The average number of insect visitations per observation period and total number of visitations were different among cultivars. Fluctuations in the number of insect visitations on ‘Stevie’ were the greatest, with this cultivar having visitations ranging from 0 to 12 visitations during each observation. This trend explains the high average and overall mean total number of visitation for ‘Stevie.’ The opposite incidence occurred with ‘Sownatural,’ which had the lowest average number of visitations per observation, lowest mean total insect visitations, and a range of 0 to 8. The average number of insect visitations for ‘Sownatural’ indicates a small, but steady number of insect visitations throughout the growing season.

Stage 3 reproductive growth was observed for a shorter amount of time (5 observations) than Stage 2 (27 observations) or Stage 1 (9 observations); however, this growth stage had the highest average visitation. This greater average of visitation suggests that this plant may be more attractive to insects that feed on flower products produced by the plant. Higher numbers of visitation, however, may have been a product of the plant presence over time, or been attributed to the presence of blooms when other sources of nectar and pollen were absent.

The highest average number of insect visitations, a 40% germination rate, and the least number of plants replaced (11) were associated with ‘Stevie,’ and are possible indicators of crop potential in Tennessee, which needs to be further explored. ‘Candy’ had a high number of insect visitations per observation, but also had a low (5%) germination rate, making this cultivar a possible poor choice for seed production; however, it may be a good choice for Tennessee farmers, who use plant plugs in large numbers for tissue production. This opposing trend was also observed with ‘Sownatural’

as this cultivar had the lowest average visitation per observation and mean total visitations, despite the highest germination rate (70%). Abiotic stresses could have had a larger impact on visitation than the morphology of the plant, as ‘Sownatural’ demonstrated an inability to survive the drought (25 replaced plants).

Summary

Differences in the number of insect visitations, both mean total and average number, were seen among the three cultivars and among growth stages, but not between planting dates, or blocks. While abiotic factors may have influenced numbers of insect visitors, these factors were the same across the entire field, throughout the study. Reproductive growth Stage 3 had the largest average visitation per observation as both planting dates entered the reproductive stage of growth simultaneously as the short-day photoperiod encouraged blooming.

CHAPTER III

Insect Visitors of *Stevia rebaudiana* Bertoni

Introduction

Stevia rebaudiana has great potential as a cash crop, especially as a possible replacement of tobacco (*Nicotiana tabacum* L.) for farmers (*Stevia*: rom niche 2013). Companies that process *Stevia* are now located in the United States, which provide an outlet for sales for farmers who grow *Stevia* and a demand for a product that is predicted to rise ("Worldwide Low-Calorie Food Market," 2016). FDA standards for the commercial sale of "highly refined products" (U.S. FDA 2016) have given those with a desire for products with minimal processing limited legal options other than personal plant supplies. The availability of seeds, plugs, and plants has outpaced the information on what pest problems *Stevia* may possess.

Little is known about insects that visit *Stevia*, and it is important to identify the visitors of *Stevia* to determine those species that feed on or inhabit *Stevia* in Tennessee to assess pest, predator and pollinator populations. Although some of the insect families have been confirmed to carry *Stevia* pollen as reported in Italy (Apidae [Hymenoptera], Calliphoridae [Diptera], Halictidae [Hymenoptera], Lycaenidae [Lepidoptera] and Syrphidae [Diptera]) (Martini et al. 2016), it has not been demonstrated that these families will perform the same pollinating functions in this region. It is also important to assess *Stevia* as a potential secondary host of pests of other crops, or as a plant which may potentially attract beneficial insects to a field or landscape. Of interest for residential landscape use is the possible attraction to harmful (stinging) insects, or other nuisance species. Therefore, a study was designed to identify insect visitors to *Stevia*. This study

answers the following questions: What Orders of insects are found on *Stevia*? Is there a difference in insect Orders observed among the three cultivars? What insect families are found on *Stevia*? What guilds of insects are present on *Stevia*? Are there any seriously damaging pests of *Stevia* present in this area? The objectives of this study are:

1) Determine insect Orders and Families observed on *Stevia*.
2) Asses differences among cultivars for each Order.
3) Identify guilds of insects present on *Stevia.*
4) Determine seriously damaging pests, if any, of *Stevia.*

Materials and Methods

A study was designed to determine the Orders, Families, and guilds present on *Stevia*, and if differences in average number of insects present among the cultivars for each Order were evident. Three cultivars and two planting dates were arranged with three replications per planting date. This study was conducted on the Lowery Farm (35.87 ºN Lat. and -84.30 ºW Long.) in Loudon County, TN. The following information details the experimental design of this study.

Field Study

This study was part of a larger study described in Chapter II. Thus, the same methodology described in Chapter II was utilized to complete this study.

Order Observation

During each observation for visitation data (Chapter II), the classification of Order and number of insects were recorded (Figure A-12) for each section twice each week between 13 June 2016 and 15 October 2016. Immatures of adult insects, insect

behaviors, and other arthropods found in the sections were also noted. Common names and Order classification were used until further identification could be made to Family. For some insects, such as butterflies and bees, the Family was identified and recorded at the time of the observation. Each section of plants (Figure A-6) was observed for a total of 8 min with an equal number of plants observed and recorded for each sampling/observation date.

Before observations began, sections within a planting date were randomized for the order of observation using a ten-sided polyhedral die, and general weather conditions (temperature, relative humidity, and UV index) were recorded using a Weather Channel phone application. Sampling and observations occurred on selected days at different time intervals [AM (8:00AM-11:00AM), MID (12:00PM-3:00PM) and PM (5:00PM-8:00PM)] to observe the variety of visitors to the plants throughout the day as well as the growing stage. Vegetative stages (Immature and Mature) were present until 10 September 2016 when both planting dates entered the Reproductive stage.

Collections

Insects were collected for identification after the observation period for each section was completed as to not interfere with normal insect visitation patterns. Insects were collected by hand into 5.08 cm wide-mouth sample jars (Uline, Prairie, WI) and labeled with the planting date, section number, date, and time. Samples were then identified to Family using standard keys (*Insects: Their Natural History and Diversity* [Marshall 2006], *Kaufman Field Guide to Insects of North America* [Eaton & Kaufman 2007], *The Bees in Your Backyard* [Wilson & Carril 2016], and *Garden Insects of North America* [Cranshaw 2004]). Order, Family, guild of insect (herbivore, predator or

pollinator), and numbers of insects were recorded in Excel. Insect samples were stored in vials containing alcohol for a voucher collection.

Most of the Diptera were not identifiable in the field to a Family group due to their small physical size. These were classified in a general category as “small fly” and taken to the laboratory to be identified to Family.

Data Analysis

The influence of cultivar on average number of insects observed for each Order was analyzed in SAS 9.4 using a randomized complete block design using the GLIMMIX procedure and a Gaussian distribution (SAS Institute Inc., Cary, NC). Least Squares Means were separated with a Tukey adjustment and 0.05 significance level for mean separation. One analysis was completed for each Order with three cultivars, two planting dates and three blocks.

Results and Discussion

The total number of insects identified during the observation period was 884, which was fewer than the total number of insect visitations (1,057), as described in Chapter II. Some insects moved from one plant to another, increasing the number of insect visitations while not affecting the total number of observed insects. Other arthropods, namely spiders, were also found and noted on *Stevia*. Because insects were the primary focus of this study, non-insect arthropods were recorded, but not identified or included in the totals.

Insect Orders and Families Found on Stevia

Six Orders of insects were found on *Stevia* during this field study and included: Coleoptera, Diptera, Hemiptera, Hymenoptera, Lepidoptera, and Orthoptera (Figure A-13). The greatest number of insects (n=332) were found in the Order Hymenoptera (Figure A-13), which accounted for 38% of all observed insects. Diptera was the second most commonly observed Order, with 27% (n=243) of the total (Figure A-13). Hemiptera was the third most commonly observed Order, with 18% (n=163) of all insects (Figure A-13). Coleoptera, Lepidoptera, and Orthoptera composed 11% (n=96), 4% (n=31), and 2% (n=19), respectively, of all insects observed (Figure A-13). Each Order was analyzed separately, and no significant difference in the average number of insects per Order per observation among the three cultivars were documented.

Thirty families were identified from 95 samples collected (Table A-6). Families are arranged alphabetically under each Order, which are arranged in alphabetical order. Hemiptera had the greatest number of identified Families (9) on *Stevia*, while Diptera and Hymenoptera both had six. There were five Families identified for Coleoptera and two each for Lepidoptera and Orthoptera.

The ant Family (Formicidae), accounted for 76% (n=251) of all Hymenoptera observed, as well as 28% of the overall total (n=884). The remaining 24% (n=81) of Hymenoptera included bees (Apidae and Halictidae), parasitic wasps (Chalcididae and Diapriidae) and predaceous wasps (Crabronidae) (Table A-6). Both Apidae and Halictidae were confirmed in the Italian study to carry pollen of *Stevia* (Martini et al. 2016). This Order did not have an equal presence on all cultivars, as the numbers of Hymenoptera were lower on ‘Sownatural’ than on the other two cultivars (Figure A-14).

None of the Hymenoptera showed negative impacts on growth of *Stevia.* Ants did exhibit harvesting and trophallaxis behaviors with the exudates of *Stevia*. Worker ants were observed removing exudates from the trichomes along the stem and either passing these compounds to a courier on the plant, or carrying these to the colony themselves. Several ant colonies were found inside the study area. It is unclear, however, if these groups existed prior to the planting of the *Stevia*, or were attracted to the plant and established after planting.

Among Diptera, the most notable (and easily identified) Family was Syrphidae, which accounted for 26% (n=62) of all Diptera based on field identification. Syrphidae may serve as a predator or as pollinator on *Stevia* and were present on the field during both vegetative (Stage 1-Immature and Stage 2-Mature) (June-September) and reproductive (Stage 3-Reproductive) (September-October) stages of *Stevia*. Syrphidae were also confirmed to carry pollen of *Stevia* by Martini et al. (2016). Small flies accounted for 70% (n=171) of all Diptera and 19% of all total insects observed. The small fly families were identified as: Agromyzidae, Chloropidae, Rhyparochromidae, and Sciaridae (Table A-6). It can be noted that the Agromyzidae may have been responsible for some minor leaf-mining damage. This damage did not result in the death of the leaf, nor plant mortality. The remaining flies (n=10) were blow flies (Calliphoridae), who were confirmed to carry pollen of *Stevia* in Italy (Martini et al. 2016), filth flies (Muscidae), or flesh flies (Sarcophagidae) but they were not consistently observed throughout the study period. In contrast to Hymenoptera, the number of Diptera were similar among the three cultivars (Figure A-15).

Most (94%) (n=153) Hemiptera were visually identified as “hoppers,” which included leaf- (Cicadellidae), plant- (Caliscelidae and Delphacidae), and froghoppers (Cercopidae) (Table A-6). “Hoppers” composed 17% (n=16) of all total insect samples identified on *Stevia* and were present on *Stevia* during both developmental stages of the plant. Spittlebug masses were found on the plants periodically, but their numbers were not included. In contrast to the “hopper” families, which were present in abundance, the other 6% (n=10) of Hemiptera (Anthocoridae, Miridae, Pentatomidae, Reduviidae, and Rhopalidae) (Table A-6) were only captured in late August after full bloom. A greater number of Hemiptera were found on ‘Candy’ than on the other two cultivars (Figure A-16). None of the members of this Order, however, was observed to cause serious damage.

Among Coleoptera, flea beetles (Chrysomelidae), lady beetles (Coccinellidae) and solider beetles (Cantharidae) were commonly seen on plants. Most of the Coleoptera were predaceous (Table A-6), with lady beetles and soldier beetles comprising 41% (n=39) and 39% (n=37), respectively, of all Coleoptera. Flea beetles comprised 20% (n=18) of all Coleoptera. Only one each of Dermestidae and Lampyridae were seen and captured on *Stevia*. After bloom, numerous numbers of Cantharidae could be found on all plants, presumably for the pollen. The Cantharidae was the only Family noted to actively mate on *Stevia* plants. Greater numbers of Coleoptera were present on ‘Candy’ than the other two cultivars (Figure A-17), but there was no significant difference in the average number of insects observed among the cultivars.

Lepidoptera observed in this study included all life stages, with larvae found in June and adults in September through October. This Order contained the only species found to damage the plant. Larval Lepidoptera consumed the apex stems of *Stevia.* This

damage, however, did not result in plant death, nor did it alter the plant's vertical growth. Adult Lepidoptera were identified into two families: Nymphalidae and Pyralidae (Table A-6). While the members of Pyralidae (snout moths) have species that are economically important as pests, the damage to *Stevia* did not cause serious loss of plants or leaf tissue. Greater number of Lepidoptera were found on 'Candy' than on the other two cultivars, with the lowest number of Lepidoptera found on 'Sownatural' (Figure A-18), however, the average number of insects observed did not significantly differ among the three cultivars.

Orthoptera consisted of katydids (Tettigoniidae) and grasshoppers (Acrididae) (Table A-6). Adults were the primary stage of life observed for this Order, with only one nymph noted. Tettigoniidae and Acrididae were found to shelter in *Stevia*, with no damage observed from insects of either Family. The number of Orthoptera did increase when the area surrounding the plot was mowed for hay, presumably as the insects sought shelter from the habitat disturbance. Orthoptera were found more frequently on 'Candy' than on either 'Stevie' or 'Sownatural' (Figure A-19).

Insect Guilds

Three main guilds (herbivores, predators, and pollinators) of insects were observed on *Stevia* (Table A-6). Most of the insects observed were herbivores (77%) while pollinators comprised 13% and predators 10% of all insects observed (Figure A-20). Of the 95 insects identified to Family, 73 were herbivores, which is expected over such a long vegetative stage of growth. This guild had the most variety of families, 21. Fomicidae and Miridae were the two most common families in this guild. From the first observation (4 d after transplant), herbivores were found on *Stevia.* Chewing, piercing,

sucking, and boring feeders were all seen on the plants, but only leaf mining and minor chewing damages were observed, neither of which caused plant mortality or visual growth reduction.

Four families of pollinators (Apidae, Halictidae, Nymphidae, and Syrphidae) were observed from 10 September (first bloom) until 15 October 2016 (last observation). While pollinator diversity was not as great as herbivore diversity, the number of pollinators on the plants once the plants bloomed was noteworthy. Often, all four pollinator families were present on plants at the same time. Kaleidoscopes of four to six butterflies would feed on plants together, and honey, bumble and carpenter bees would seek nectar on the same inflorescence. Number of pollinators was evenly distributed throughout both planting dates, as reproductive stages for *Stevia* are triggered by photoperiod.

Predator flies, Syrphidae, were present during the entire study. This Family was one of seven in the predator guild. Coccinellidae were also common throughout the study, although aphids, the preferred food source for lady beetles, were not found. Cantharidae were not observed until after bloom, but were present in every observation afterwards. Soldier beetles (Cantharidae) were also observed to mate on *Stevia.* It is unknown if eggs were laid on the plants, or if any larvae developed. Assassin bugs (Reduviidae) were less numerous than other predators, but were obvious on the plants. Of all the predator families, members of Reduviidae were the only ones observed to feed on other organisms, which included leafhoppers, in this study. Parasitoid wasps (Chalcididae, and Diapriidae) and predaceous wasps (Crabronidae) were found on *Stevia*, although not until after bloom, or in large numbers.

Summary

Six insect Orders were observed on *Stevia* plants during this study. Hymenoptera were the most numerous, followed by: Diptera, Hemiptera, Coleoptera, Lepidoptera, and Orthoptera. When identified in the field, ants (Formicidae) were the most numerous Family, often having colonies inside the field sections of *Stevia*. Numerous numbers of the "small fly" group (Agromyzidae, Chloropidae, Rhyparochromidae, and Sciaridae) were observed flitting between plants. Cercopidae, Cicadellidae, and Delphacidae or the "hopper" group was the first to be observed on *Stevia*, and were consistently found on the plants throughout the study. Flea beetles were also observed for the entire study. Once plants bloomed, bees (Apidae and Halictidae) visited the flowers along with syrphid flies, which were present early in the study and are known members of the predator guild.

While Lepidoptera larvae did cause minor chewing damage, and some leaf boring was present, no serious damage was observed. Insect damage was not serious relative to this small-scale production system; however, large-scale production systems may exhibit different results. Also, the growing conditions during the experiment may have impacted the insect Orders present and under different weather conditions, the Orders and Families could have changed. It can be noted that four of the five families (Apidae, Calliphoridae, Halictidae, and Syrphidae) reported in the Italian study (Martini et al. 2016) were also found in this experiment. Calliphoridae was not found in populations high enough to indicate a suitable pollinator in this study.

CHAPTER IV

Toxicity of *Stevia rebaudiana* Bertoni Compounds to *Spodoptera frugiperda* Larvae

Introduction

The fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), is known to cause economic losses in several financially important crops in Tennessee (Lockman 2012). Damages to corn (*Zea mays* L.), cotton (*Gossypium* spp.), and soybean (*Glycine max* L.) can be devastating as the larval stage of FAW can defoliate entire fields (Capinera 1999). Crops genetically engineered to produce *Bacillus thuringiensis* Berliner (Bt) toxins have reduced the amount of damage from FAW (USDA 2016). However, FAW populations in Puerto Rico have been confirmed to be Cry1F resistant (Storer et al. 2010). The emergence of resistant FAW populations increases the need for a new effective insecticide that has minimal negative impact on human health and/or the environment.

In an earlier study, leaves from *Stevia rebaudiana* (Bertoni) Bertoni (Asterales: Asteraceae: Eupatorieae) were fed to fifth-instar FAW larvae until pupation. It was noted that the raw leaf tissue had an impact on survival and pupation of FAW. These results prompted bioassays that followed using the extracted compounds of *S. rebaudiana*, rebaudioside A and stevioside, as well as the dried whole leaf powder. Food and/or pharmacy grade powders were used for further bioassays due to better distribution into the diet

This study answers the following questions: Are *Stevia* compounds toxic to FAW larvae? Are Cry1Fa-resistant strains of FAW susceptible to *Stevia* compounds? What are

the LC50 values for *Stevia* compounds? The specific objectives of this study were:and a 95% purity to meet FDA standards (U.S. FDA 2016).

1) Determine if the powdered compounds had an impact on FAW survival.
2) Asses susceptibility to *Stevia* in Cry1Fa-resistant FAW.
3) Determine the lethal concentration at 50% mortality (LC_{50}) of *Stevia* on FAW.
4) Explore any non-lethal impacts of compounds on FAW larvae.

Materials and Methods

Bioassays

These studies examined three compounds (rebaudioside A, stevioside, and whole *Stevia* leaf powders) at five different treatment levels for each compound. The basic diet (Frontier Agricultural Sciences, Newark, DE product #F9219B) for FAW rearing was used as a control, and the five concentrations of the three compounds (total of 15) were added to this basic diet in a no choice consumption test. Bioassays were conducted in an incubator, with the specific design of each study detailed below.

Amounts of powder for the treatments varied with the concentration to be tested (Table A-7). Rebaudioside A powder (56.70 g) was purchased online from Prescribed for Life (Fredericksburg, TX) at a purity level of 99%. Stevioside powder (125 g) was purchased from Wholesale Health Connection (Easton, MD) with 100% purity. Whole leaf powder (453.59 g) was purchased from Starwest Botanicals (Sacramento, CA) and was labeled as organic. Treatments were all incorporated in beet armyworm, *Spodoptera exigua* (Hübner), diet (Frontier Agricultural Sciences product #F9219B) at a rate of 161.6 g/L for the diet with 19.8 g/L of agar and 900 mL/L of ultrapure water. Each mixture of

diet and treatment was poured into 32 wells of a bioassay tray (C-D International, product #BIO-BA-128) and allowed to cool prior to neonate placement. Eggs of the Benzon FAW strain were purchased from Benzon Scientific (Carlisle, PA) and placed in an incubator (Percival Scientific) at 27°C with 46% relative humidity, until 1 day after hatching (24-36 h old). Larvae were removed from the incubator and placed individually into one well (n=32) of the bioassay tray using a small horse-hair brush and secured inside of the well with a plastic film that adhered to the sides of the well without impeding larval movement.

Determination of the concentration needed to inflict mortality on 50% of the population (LC_{50}) was performed by testing five incremental concentrations each of rebaudioside A, stevioside, and whole leaf compounds in the bioassays. Each of the 16 treatments (five concentrations of three compounds plus a control) had 32 neonates. Bioassay trays were returned to the incubator at 27 ºC with 46% relative humidity and observed daily to determine larval mortality. Insect death was recorded for 9 days or until pupation, when bioassay trays were frozen. Insects that were determined to be alive at the time of bioassay termination were examined and weighed with notations made on the numbers of pupae and larvae. These experiments were replicated twice, for a total of 64 larvae per treatment.

Strain 456, which has demonstrated resistance to Cry1Fa corn (Jakka et al. 2016), was used to test *Stevia* compound effectiveness on Bt resistant FAW larvae. Each of four treatments (control, rebaudioside A, stevioside, and whole leaf powder) contained 113 larvae. Only one concentration (18.35 g/L) of each *Stevia* compound powder was incorporated in beet armyworm diet (Scientific Services product #F9219B) for each of

the treatments. Insects that were determined to be alive after 7 days were kept on ice to be weighed and recorded to the nearest mg, with notations made on the number of pupae and/or larvae.

Larval Weight Comparisons

During the bioassays, a difference in larval weight and pupation was observed for whole Stevia leaf material. While mortality was the primary goal, weight and pupation numbers were also recorded and analyzed to better understand non-lethal impacts of treatments on larvae. To compare larval weight, frozen trays of 9-day-old larvae were removed from the freezer and weighed, comparing only the larvae that were alive at the time of bioassay termination. There were six treatments: whole leaf 40%, whole leaf 20%, whole leaf 10%, whole leaf 5%, whole leaf 2.5%, and the control.

Data Analysis

For the bioassay with Benzon larvae, mortality levels were analyzed by SAS 9.4 using a randomized complete block design and the GLIMMIX procedure and a Gaussian distribution (SAS Institute Inc., Cary, NC). Each treatment (total of 16 treatments) had 32 neonates per block. Means were separated using Least Squares Means with a Tukey adjustment and 0.05 significance level for mean separation. For the weight comparison of Benzon FAW, six total treatments were used in a randomized complete block design using SAS ANOVA Least Square Means with a 0.05 significance level for mean separation. LC_{50} data were analyzed using the Probit (Probit Software LTD Herzliya, Israel) and the chi-square test for probability on a 95% confidence limit.

Larval weight was recorded to the nearest mg, entered into Excel, and analyzed by SAS 9.4 using a randomized complete block design and the GLIMMIX procedure and

a Gaussian distribution (SAS Institute Inc., Cary, NC). Means were separated using Least Squares Means with a Tukey adjustment and 0.05 significance level for mean separation.

Comparisons of Benzon FAW larval mortality percentages to those of the 456 strain of FAW were analyzed by SAS 9.4 and the GLIMMIX procedure with a Gaussian distribution (SAS Institute Inc., Cary, NC). Means were separated using Least Squares Means with a Tukey adjustment and 0.05 significance level for mean separation.

Results and Discussion

Lethal Concentration 50% (LC_{50})

Stevioside had an LC_{50} of 9.50 g/L (± 0.42) with a 95% confidence range of 8.00 to 11.17 (Table A-8). Rebaudioside A had an LC_{50} of 15.42 g/L (± 0.44) with a 95% confidence range of 12.36 to 18.91 g/L (Table A-8). Whole leaf powder had an LC_{50} of 48.82 g/L (± 1.19) with a 95% confidence range of 38.40 to 88.66 (Table A-8). Mortality was observed in bioassays with Cry1Fa-resistant FAW larvae (456) at the tested rate of 18.35g/L for stevioside (34.5%), rebaudioside A (30.1%) and whole leaf (2.65%) treatments. There was no significant difference between the mortality of the 456 strain and the Benzon treatments (stevioside, rebaudioside and whole leaf) at 10, 15, or 20 g/L with significance at $\alpha<0.05$.

Compounds and Concentration Comparison

Mortality of FAW could be grouped statistically different among the three compounds and five concentrations tested (Figure A-21). The treatment with the highest mean mortality (100%) was Stevioside 40%, which was significantly different from all other treatments (Figure A-21). Mean mortality of FAW fed Rebaudioside A 40% and

Stevioside 15% was 90% and 73%, respectively, and was not significantly different; however, they were significantly different from other treatments (Figure A-21). Mortality of whole leaf 40% (43%) and rebaudioside A 15% (42%) were not significantly different from each other, but were significantly different from other treatments (Figure A-21). Mean mortality of all other treatments was less than 18.8%. No mortality was observed when larvae were fed whole leaf 5% (Figure A-21).

Larval Weight Comparisons

Weights of 9-day-old larvae were significantly different among treatments, as those in the control (127.65mg ± 102.61) weighed significantly more than larvae fed any other diets (Figure A-22). Larvae fed whole leaf 2.5% (72.51 mg ± 68.33) weighed significantly more than those fed the other whole leaf treatments (Figure A-22). However, weights of FAW larvae fed whole leaf 5% (32.50 mg ± 33.60), whole leaf 10% (16.67 mg ± 38.12), whole leaf 20% (2.48 mg ± 3.78), and whole leaf 40% (3.35 mg ± 10.23) were not significantly different (Figure A-22).

Stevia compounds, regardless of the concentrations, influenced viability of FAW. The mode of action of the tested compounds is unknown and needs to be further explored if *Stevia* compounds are to be used for insect control. With all the trials on mammal health undergone by *Stevia* products, it is likely that mortality is caused by a mechanism that insects possess and that the products would be safe for human consumption. Impact on mortality by stevioside compounds was larger than rebaudioside A and whole leaf. Stevioside is also found with the most abundance in plant tissues, and had the lowest LC_{50}. Compounds also demonstrated toxicity on Bt resistant larvae strain 456, which supports that *Stevia* compounds would be of great benefit where Bt and insecticide

resistant strains of FAW have been discovered. The effectiveness of stevioside is noteworthy because production of products made with stevioside for human consumption is declining due to the undesired aftertaste; however, during extraction of rebaudioside A, the stevioside could be used for the purposes of insect control instead of being discarded. The toxicity of this stevioside on FAW may allow *Stevia* production companies to develop a value-added product as the market for bio-pesticides is predicted to increase over time (Bunge 2014).

Rebaudioside A showed an impact on FAW mortality, although not as high as stevioside, even when used in the same concentrations. While this compound is found with one-third the frequency of stevioside in plant biomass, the lack of an aftertaste has driven the demand for its production over that of stevioside, even driving the market for genetically altered yeast production (Engber 2014). This new and inexpensive method of product for human sugar substitutes may make it more profitable to use this compound instead of the more abundant stevioside (Engber 2014).

Whole leaf powders, while not having the lowest LC_{50}, need further exploration for their impact into the growth regulation that was observed on larvae which consumed the compound. It is unclear whether it is a glycoside compound in the dehydrated and powdered leaves that was the active source of toxicity, or one of the other components of the leaf tissue, such as a steroid, or phenol complexes (Kinghorn 2002). Regardless, smaller larvae that never reach reproductive stages would reduce the overall numbers of insects, effectively lowering the total population. This growth inhibition is especially advantageous due to the unprocessed nature of the powder, which requires little advanced machinery, and the small concentrations needed to cause growth inhibition.

While *Stevia* compounds have shown to be toxic to FAW larvae, the percentages used for these compounds in concentrations are larger than some of the synthetic pyrethroids previously tested as dosages. Stevioside had the lowest LC_{50} (9.50 µg/ml) of the tested *Stevia* compounds, but this LC_{50} is many folds larger than the LD_{50} values reported for permethrin (0.0041µg/insect), Pounce® (0.0271 µg/insect), or diazinon (0.4907 µg/insect) when tested on third-instar larvae (Gist & Pless 1985). Further research is needed to isolate the exact mode of action of *Stevia* compounds to increase the efficiency of the compounds in insect control.

Summary

Impacts on mortality and weight gain were observed during the FAW larvae bioassays when using *Stevia* compounds. Impacts on mortality occurred regardless of Bt resistance, and with the same compound at different concentrations. In addition to insect death, growth regulation was also observed with larvae consuming the whole leaf compounds when using concentrations as low as 5 g/L.

CHAPTER V

Conclusions

Stevia rebaudiana (Bertoni) Bertoni (Asterales: Asteraceae: Eupatorieae) is a natural, zero-calorie sweetener with potential as a cash crop. Little is known regarding the impacts of *Stevia* cultivars on insect visitation, potential damaging pests of *Stevia* in Tennessee or the impacts of *Stevia* compounds on insect mortality. This study explored insect visitation to three *Stevia* cultivars, identified six Orders of insects that were found on *Stevia*, and conducted bioassays to determine mortality rates of model insects fed diets of *Stevia* compounds.

Insects were observed on all three *Stevia* cultivars during both the vegetative and reproductive stages of growth. Among the three cultivars, the average number of insect visitations, the total number of insect visitations, and average number of insect visitations by growth stage were significantly different. Abiotic conditions may have impacted the results and a replication of the entire study during a more typical growing season would better clarify the extent of impact of cultivar differences. Additional evaluations also would determine if a correlation exists between number of visitations and germination rates, which was not observed in this study.

Six Orders of insects were found on *Stevia*, the majority (38%) belonged to the Order Hymenoptera. Insects of Hymenoptera and Lepidoptera had larger numbers of insects observed on ‘Stevie’ and ‘Candy’ cultivars over ‘Sownatural.’ The preferences of these two Orders may indicate an attraction quality to the blooms of the two cultivars, as both Orders contain known pollinators. Cultivar preferences were not as distinct for the other four Orders or for any feeding damage, as minor damage was seldom and randomly

found throughout the plot. No major damage or economically important pest was observed in this study. Further studies using a large-scale production system could provide more optimal conditions to aid in determining damaging pests that may be experienced on a commercial production level.

The fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), is an economic pest of important crops in Tennessee, and populations have developed resistance to Bt corn expressing Cry1F proteins in Puerto Rico (Storer et al. 2010, Zhu et al. 2010, Blanco et al. 2010). In no-choice tests, the *Stevia* compounds had an impact on FAW viability. The LC_{50} was lowest for Stevioside, which had a 100% mortality rate at 40g/L. While rebaudioside A was not the most effective on mortality of FAW, it did exhibit a significant impact on FAW larvae mortality with concentrations of 15% and higher. Whole leaf powders of *Stevia* were not as effective at inducing larval mortality, however, these compounds did demonstrate a reduction in larval weight gain in the no-choice tests. The compounds also demonstrated an impact on viability of 456 strain FAW larvae.

Further tests using topical applications, other insects, and other methods of introduction are needed to determine the full capacity of *Stevia* compounds as insecticides. For field applications, phototoxicity, photodegradation, and dispersal ease (among many others) of *Stevia* compounds are necessary to determine the effectiveness of the compounds as a potential bio-pesticide. Compounds must also be tested on the health of birds, reptiles and fish if field applications are to be used. Additionally, determining the exact mode of action may better facilitate the reduction of the lethal concentration to be comparable to some of the other products on the market.

This study provided valuable information on the impacts of cultivars of *S. rebaudiana* to insect visitations, numbers of insect visitations, and Orders of insects found on *Stevia,* including potential damaging pests. The LC_{50} of three *Stevia* compounds were determined, and were found to be impactful on the mortality of the 456 strain, which has shown Bt Cry1F Bt resistance. The potential of *Stevia* compounds as insecticides was confirmed, but warrants further research.

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APPENDIX

Table A-1. Average Daily Values for Environmental Conditions[a] in the Greenhouse for the First Planting of *S. rebaudiana* (60 days)[b]

Date	% Relative Humidity	Temperature (°C)	Date	% Relative Humidity	Temperature (°C)
4/8/2016	63.54	22.39	5/10/2016	53.61	24.93
4/9/2016	12.76	21.46	5/11/2016	66.99	24.58
4/10/2016	19.56	21.53	5/12/2016	66.95	24.85
4/11/2016	36.65	21.86	5/13/2016	54.23	23.76
4/12/2016	41.24	21.58	5/14/2016	49.80	20.74
4/13/2016	36.52	21.89	5/15/2016	35.83	18.79
4/14/2016	43.68	22.64	5/16/2016	46.80	18.62
4/15/2016	35.23	22.30	5/17/2016	52.06	21.96
4/16/2016	31.96	22.45	5/18/2016	64.25	22.47
4/17/2016	33.57	23.17	5/19/2016	53.34	22.82
4/18/2016	36.88	23.75	5/20/2016	73.88	20.49
4/19/2016	39.37	23.67	5/21/2016	66.89	22.52
4/20/2016	50.46	23.11	5/22/2016	47.43	23.61
4/21/2016	52.36	22.02	5/23/2016	51.78	22.72
4/22/2016	77.11	20.07	5/24/2016	54.79	23.45
4/23/2016	60.89	20.27	5/25/2016	60.95	23.58
4/24/2016	51.69	21.70	5/26/2016	71.56	23.69
4/25/2016	58.38	22.23	5/27/2016	72.80	24.87
4/26/2016	52.08	25.35	5/28/2016	66.73	26.65
4/27/2016	51.73	26.73	5/29/2016	66.57	26.52
4/28/2016	58.18	25.73	5/30/2016	66.49	26.89
4/29/2016	51.06	24.50	5/31/2016	59.93	27.17
5/1/2016	66.85	24.73	6/1/2016	63.86	26.34
5/2/2016	69.12	23.29	6/2/2016	71.84	25.38
5/3/2016	58.18	22.12	6/3/2016	74.72	24.82
5/4/2016	40.72	22.08	6/4/2016	80.68	24.34
5/5/2016	35.49	20.47	6/5/2016	88.51	22.57
5/6/2016	42.39	19.93	6/6/2016	80.34	22.53
5/7/2016	43.18	22.62	6/7/2016	66.19	22.32
5/8/2016	48.09	25.07	6/8/2016	54.43	21.29
5/9/2016	58.58	23.62			

[a] Environmental conditions recorded by Watchdog environmental monitoring system
[b] Values were recorded 60 days prior to transplant in the field

Table A-2. Average Daily Values for Environmental Conditions[a] in the Greenhouse for the Second Planting of *S. rebaudiana* (60 days)[b]

Date	% Relative Humidity	Temperature (°C)	Date	% Relative Humidity	Temperature (°C)
4/28/2016	58.18	25.73	5/29/2016	66.57	26.52
4/29/2016	51.06	24.50	5/30/2016	66.49	26.89
5/1/2016	66.85	24.73	5/31/2016	59.93	27.17
5/2/2016	69.12	23.29	6/1/2016	63.86	26.34
5/3/2016	58.18	22.12	6/2/2016	71.84	25.38
5/4/2016	40.72	22.08	6/3/2016	74.72	24.82
5/5/2016	35.49	20.47	6/4/2016	80.68	24.34
5/6/2016	42.39	19.93	6/5/2016	88.51	22.57
5/7/2016	43.18	22.62	6/6/2016	80.34	22.53
5/8/2016	48.09	25.07	6/7/2016	66.19	22.32
5/9/2016	58.58	23.62	6/8/2016	54.43	21.29
5/10/2016	53.61	24.93	6/9/2016	59.92	21.14
5/11/2016	66.99	24.58	6/10/2016	64.60	22.42
5/12/2016	66.95	24.85	6/11/2016	65.65	25.02
5/13/2016	54.23	23.76	6/12/2016	65.35	26.48
5/14/2016	49.80	20.74	6/13/2016	72.38	26.08
5/15/2016	35.83	18.79	6/14/2016	75.62	24.32
5/16/2016	46.80	18.62	6/15/2016	82.88	24.85
5/17/2016	52.06	21.96	6/16/2016	66.31	27.03
5/18/2016	64.25	22.47	6/17/2016	64.10	26.12
5/19/2016	53.34	22.82	6/18/2016	57.86	25.53
5/20/2016	73.88	20.49	6/19/2016	58.06	24.19
5/21/2016	66.89	22.52	6/20/2016	61.18	24.72
5/22/2016	47.43	23.61	6/21/2016	67.36	25.66
5/23/2016	51.78	22.72	6/22/2016	66.51	28.77
5/24/2016	54.79	23.45	6/23/2016	56.09	30.09
5/25/2016	60.95	23.58	6/24/2016	64.86	27.61
5/26/2016	71.56	23.69	6/25/2016	61.40	27.87
5/27/2016	72.80	24.87	6/26/2016	63.93	28.15
5/28/2016	66.73	26.65	6/27/2016	75.09	26.89
			6/28/2016	69.43	26.27

[a] Environmental conditions recorded by Watchdog environmental monitoring system
[b] Values were recorded 60 days prior to transplant in the field

Table A-3. Influence of Cultivar and Planting Date on Average Number of Insect Visitations to *S. rebaudiana*, Loudon Co., 2016

Effect	Degrees of Freedom	F value	P > F
Block	2	2.05	0.1302
Cultivar	2	6.11	0.0025
Planting date	1	0.17	0.6819
Cultivar*Planting date	2	0.03	0.9708
Cultivar*Block	4	0.26	0.9023
Block* Planting date	2	0.41	0.6661

SAS ANOVA using GLIMMIX Least Means Squares with a Tukey adjustment for multiple comparisons at 0.05 significance.

Table A-4. Influence of Cultivar and Planting Date on Total Number of Insect Visitations to *S. rebaudiana*, Loudon Co., 2016

Effect	Degrees of Freedom	F Value	P > F
Block	2	4.29	0.3453
Cultivar	2	4.29	0.0452
Planting Date	1	1.68	0.2237
Cultivar*Planting Date	2	0.02	0.9838

SAS ANOVA using GLIMMIX Least Means Squares with a Tukey adjustment for multiple comparisons at 0.05 significance.

Table A-5. Influence of Plant Growth Stage on Average Number of Insect Visitations to *S. rebaudiana*, Loudon Co., 2016

Effect	Degrees of Freedom	F Value	P > F
Block	2	1.93	0.1472
Stage	2	19	<0.0001
Cultivar	2	9.72	<0.0001
Cultivar*Stage	4	1.72	0.1445

SAS ANOVA using GLIMMIX Least Means Squares with a Tukey adjustment for multiple comparisons at 0.05 significance.

Table A-6. Orders, Families and Guilds Found on *S. rebaudiana* in Loudon Co., TN 2016

Order	Family	Guild
Coleoptera	Cantharidae	Predator
Coleoptera	Chrysomelidae	Herbivore
Coleoptera	Coccinellidae	Predator
Coleoptera	Dermestidae	Herbivore
Coleoptera	Lampyridae	Herbivore
Diptera	Agromyzidae	Herbivore
Diptera	Calliphoridae	Herbivore
Diptera	Chloropidae	Herbivore
Diptera	Rhyparochromidae	Herbivore
Diptera	Sciaridae	Herbivore
Diptera	Syrphidae	Predator/Pollinator
Hemiptera	Anthocoridae	Herbivore
Hemiptera	Caliscelidae	Herbivore
Hemiptera	Cercopidae	Herbivore
Hemiptera	Cicadellidae	Herbivore
Hemiptera	Delphacidae	Herbivore
Hemiptera	Miridae	Herbivore
Hemiptera	Pentatomidae	Herbivore
Hemiptera	Reduviidae	Predator
Hemiptera	Rhopalidae	Herbivore
Hymenoptera	Apidae	Pollinator
Hymenoptera	Chalcididae	Predator
Hymenoptera	Crabronidae	Predator
Hymenoptera	Diapriidae	Predator
Hymenoptera	Formicidae	Herbivore
Hymenoptera	Halictidae	Pollinator
Lepidoptera	Nymphalidae	Pollinator
Lepidoptera	Pyralidae	Herbivore
Orthoptera	Acrididae	Herbivore
Orthoptera	Tettigoniidae	Herbivore

Samples collected from *Stevia* plants, identified to Family and preserved in a voucher collection

Table A-7. Treatments (compounds and concentrations) Tested against *Spodoptera frugiperda* Larvae in Bioassays

Name	Concentrations of Treatment (g/L)
Control	0
Rebaudioside A	40
Rebaudioside A	15
Rebaudioside A	5
Rebaudioside A	2.5
Rebaudioside A	0.5
Stevioside	40
Stevioside	15
Stevioside	5
Stevioside	2.5
Stevioside	0.5
Whole leaf	40
Whole leaf	20
Whole leaf	10
Whole leaf	5
Whole leaf	2.5

Table A-8. Toxicity of *Stevia* Compounds and Whole Leaf Powders to *Spodoptera frugiperda* Larvae in No-Choice Bioassays[a]

Stevia Compounds	LC_{50} (g/L± SE)[b]	95% Confidence Range	
		Lower	Upper
Rebaudioside A	15.42 ± 0.44	12.36	18.91
Stevioside	9.50 ± 0.42	8.00	11.17
Whole Leaf	48.82 ± 1.19	38.40	88.66

[a]Two replications of 32 neonates each.
[b]LC_{50} determined using Probit analysis

Figure A-1. Leaf structures of *Stevia rebaudiana* (H. Lowery)

Figure A-2. Florets of *Stevia rebaudiana* (H. Lowery)

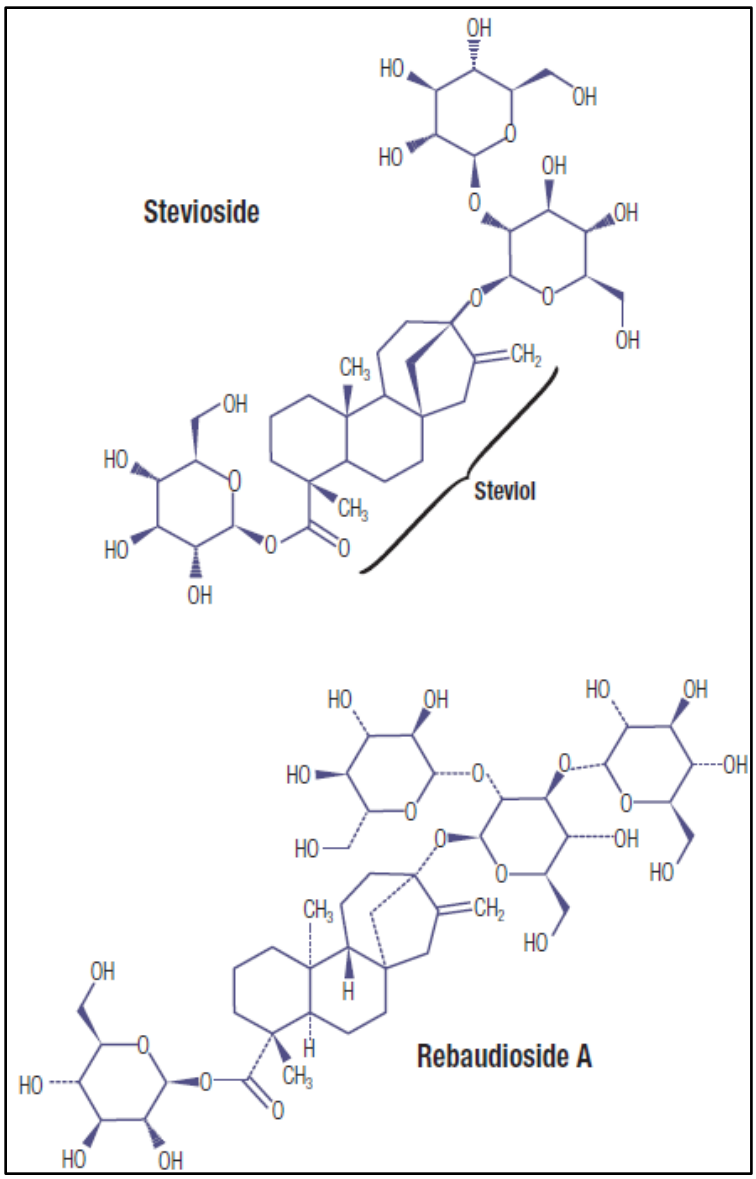

Figure A-3. Structures of stevioside and rebaudioside A (Thermo Scientific)

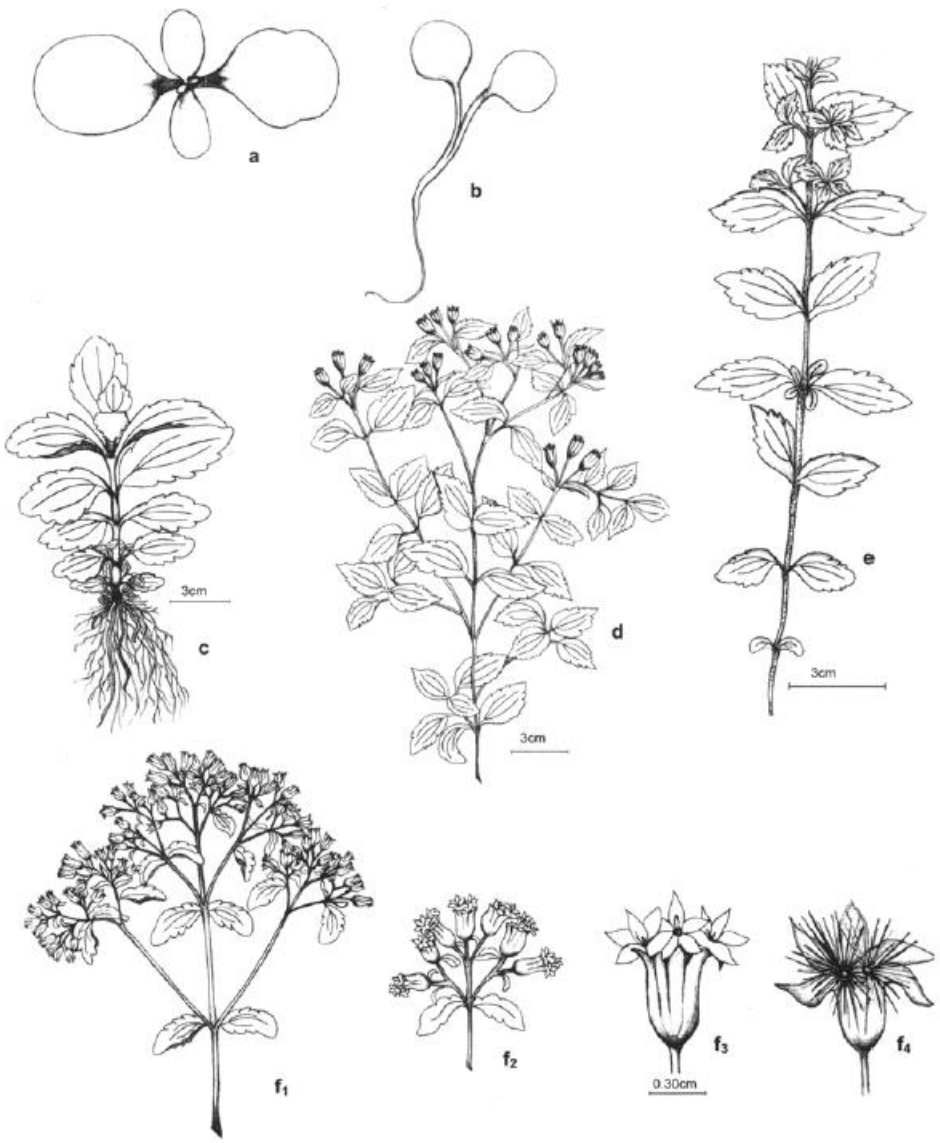

Figure A-4. Growth stages of *Stevia rebaudiana* (from Carneiro 2007)

Vegetative

(a) establishing seedling
(b) normal seedling
(c) overgrown seedling with roots
(d) plant grown under short-day photoperiod
(e) regrown stem

Reproductive

(f1) cyme of corymbs
(f2) cyme detached to count the reproductive stages,
(f3) capitulum containing five white flowers,
(f4) capitulum containing seeds ready for dispersion.

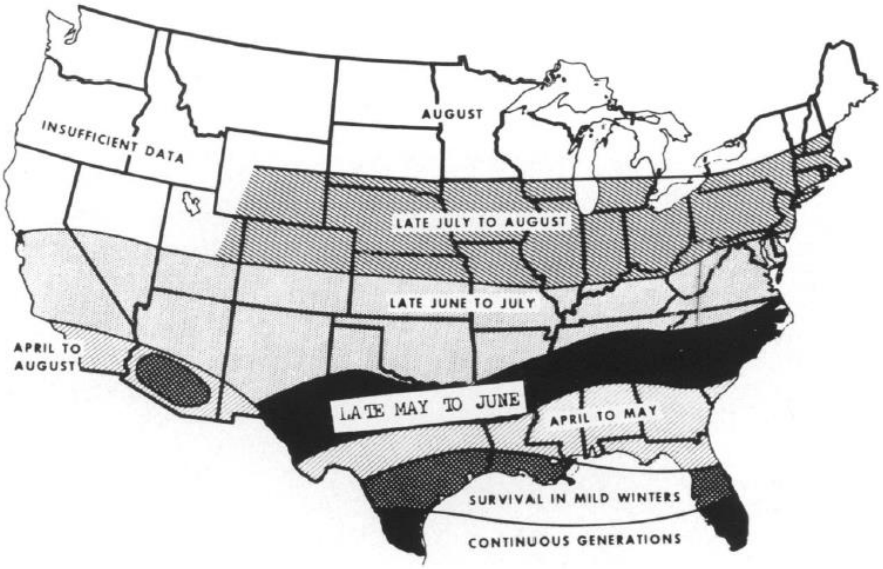

Figure A-5. Seasonal distribution of the fall armyworm, *Spodoptera frugiperda*, in the United States (from Sparks 1979)

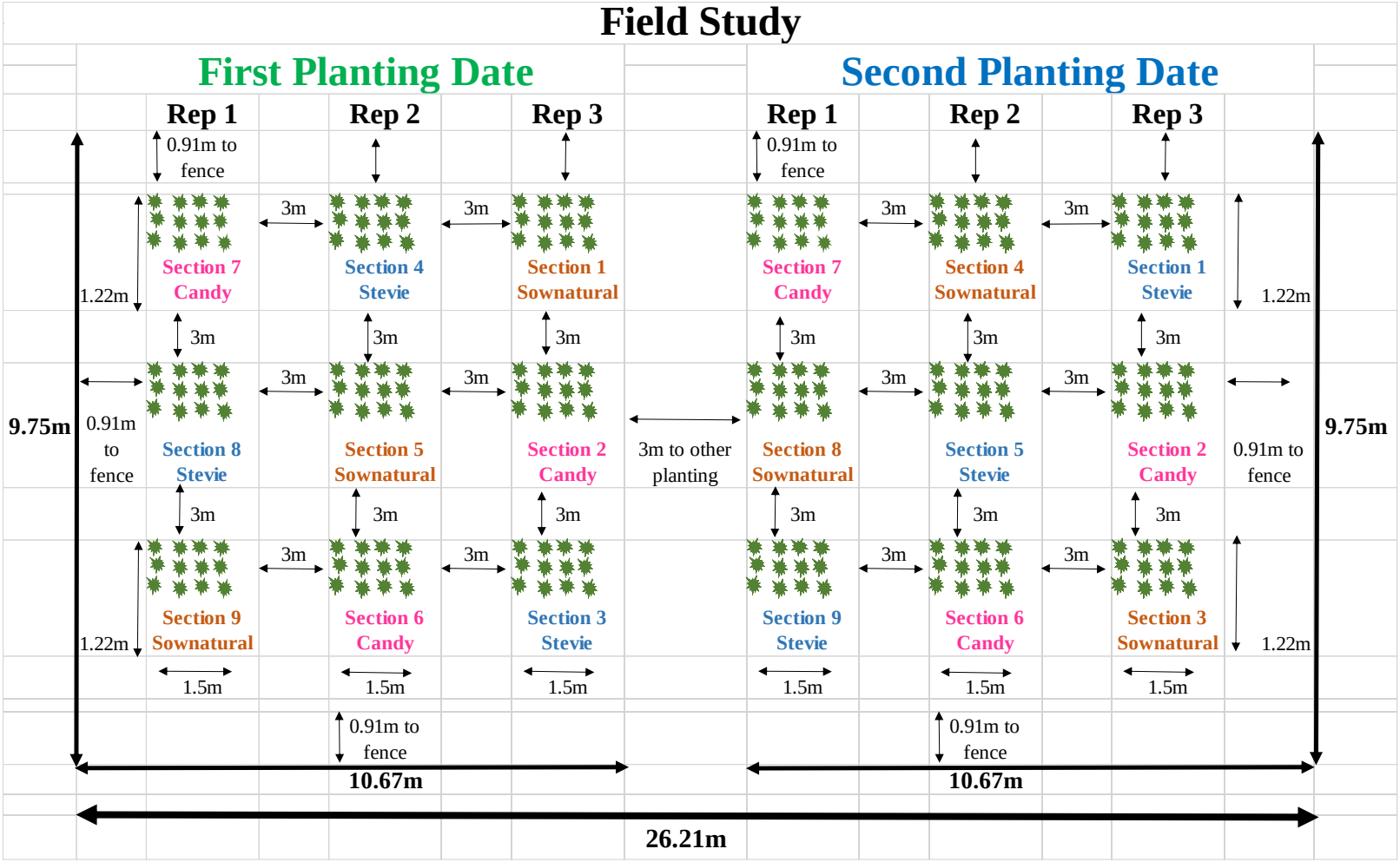

Figure A-6. Design of field study to assess impact of cultivar, planting date, and growth stage, Loudon Co., 2016

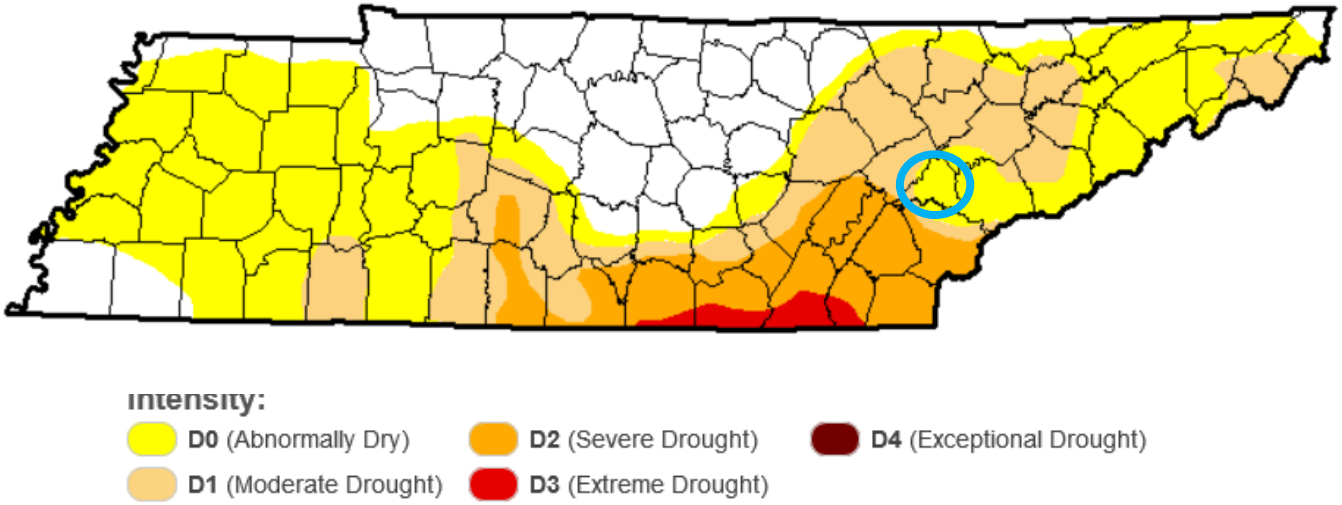

Figure A-7. Drought status for Tennessee, with emphasis on Loudon Co., August, 2016 (National Drought Mitigation Center)

Sampling Data Sheet

Date:
Time:
Plot:
General conditions:
Temperature:
UV index:
Humidity:

7	4	1
8	5	2
9	6	3

field

Section 7		
Time in		Time out
Number of visits:		
Number of Insects:		
Types of insects:		

Section 4		
Time in		Time out
Number of visits:		
Number of Insects:		
Types of insects:		

Section 1		
Time in		Time out
Number of visits:		
Number of Insects:		
Types of insects:		

Section 8		
Time in		Time out
Number of visits:		
Number of Insects:		
Types of insects:		

Section 5		
Time in		Time out
Number of visits:		
Number of Insects:		
Types of insects:		

Section 2		
Time in		Time out
Number of visits:		
Number of Insects:		
Types of insects:		

Section 9		
Time in		Time out
Number of visits:		
Number of Insects:		
Types of insects:		

Section 6		
Time in		Time out
Number of visits:		
Number of Insects:		
Types of insects:		

Section 3		
Time in		Time out
Number of visits:		
Number of Insects:		
Types of insects:		

Figure A-8. Example of data sheet used to record insect visitation to *S. rebaudiana*

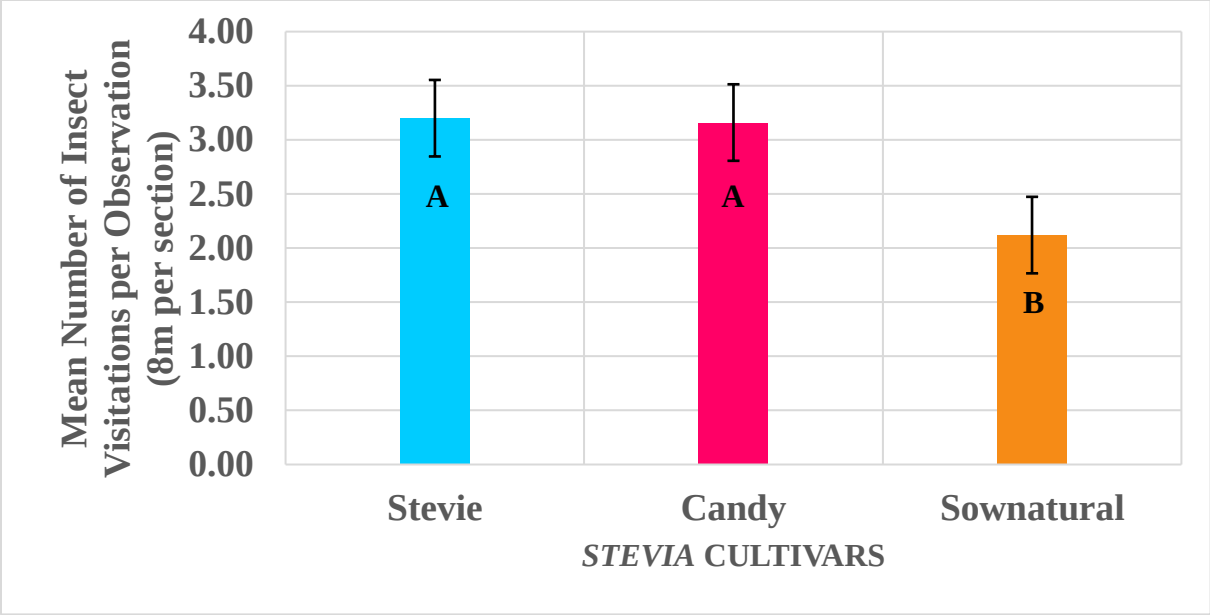

Figure A-9. Mean number of insect visitations per observation on *Stevia* cultivars, Loudon Co., 2016

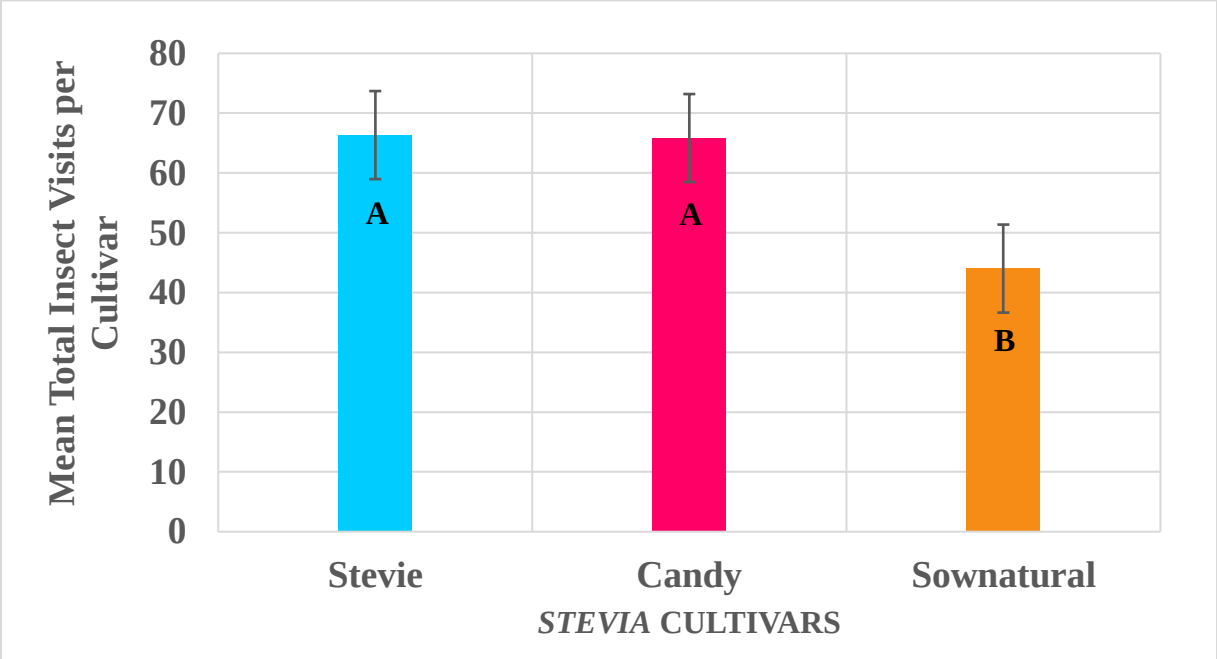

Figure A-10. Mean total number of insect visitations per *Stevia* cultivar, Loudon Co., 2016

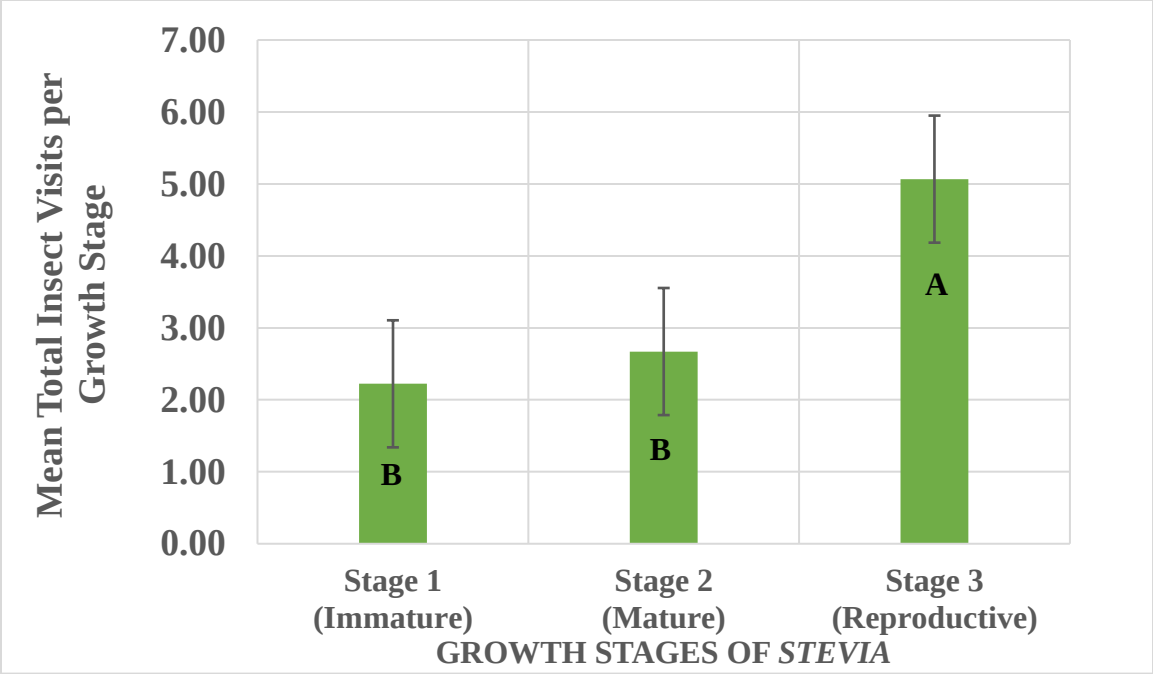

Figure A-11. Mean total number of insect visitations per *Stevia* growth stage, Loudon Co., 2016

Section 5		
Time in		Time out
Number of visitations:		10
Number of insects:		5
Types of insects:		
bee	2	Hymenoptera
leafhopper	3	Hemiptera

Figure A-12. Example of data sheet to record insect identification during field observation

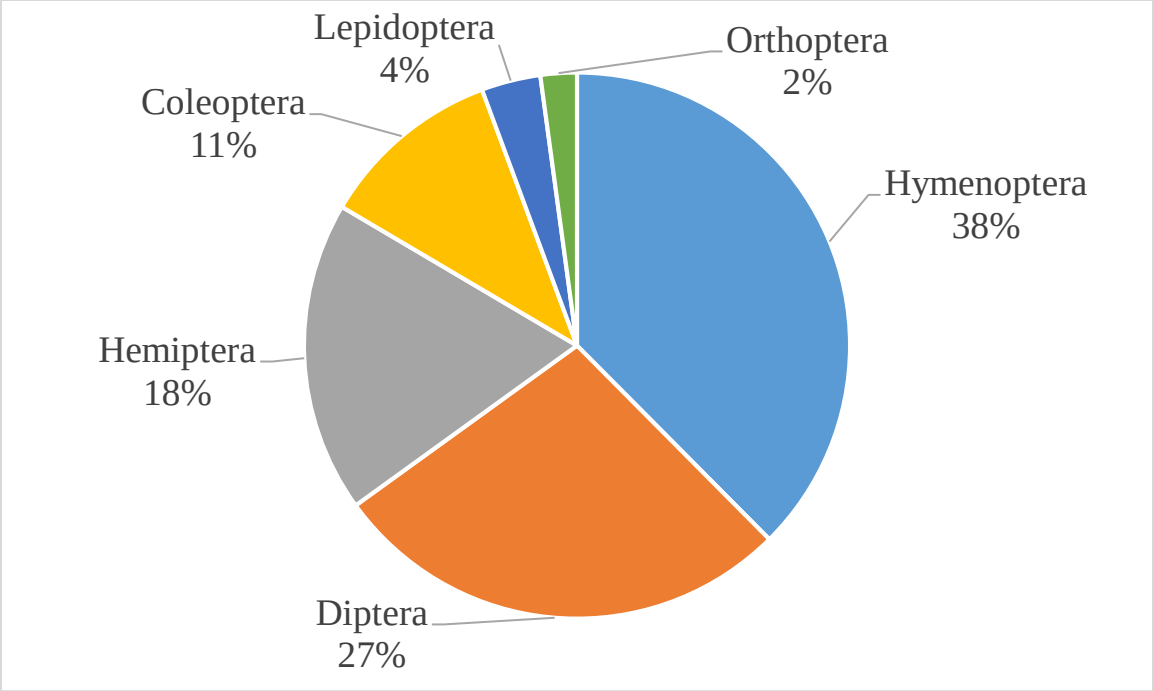

Figure A-13. Percent of insects (n=884) found on *Stevia* and categorized based on Orders, Loudon Co., 2016

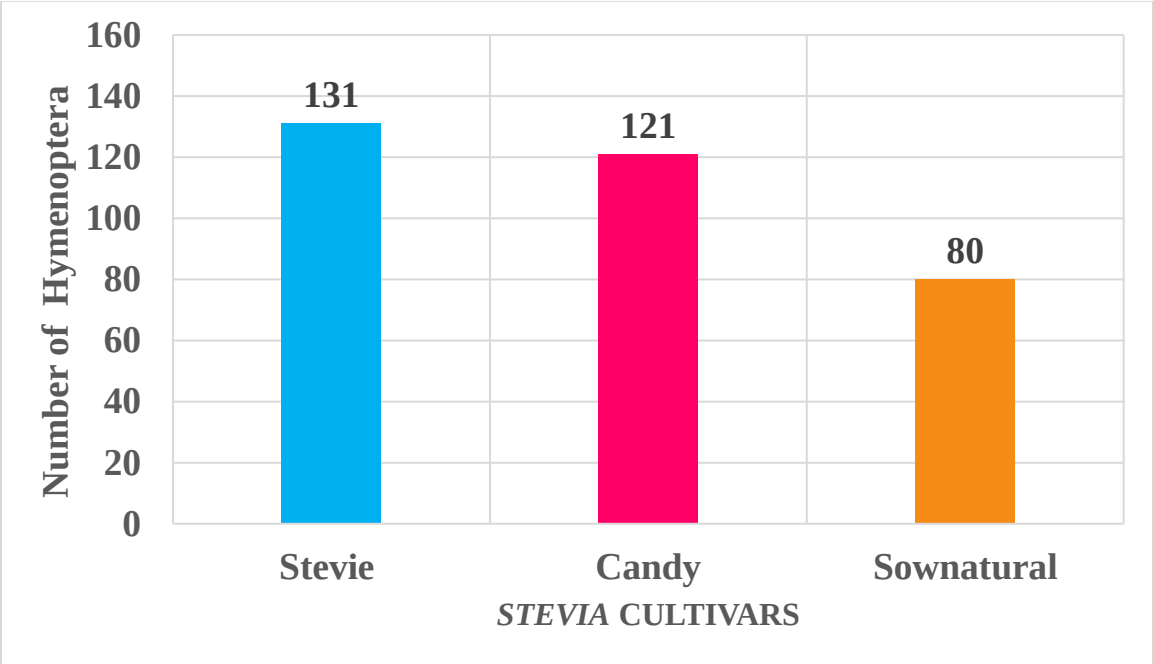

Figure A-14. Total number of Hymenoptera among three *Stevia* cultivars in Loudon Co., 2016

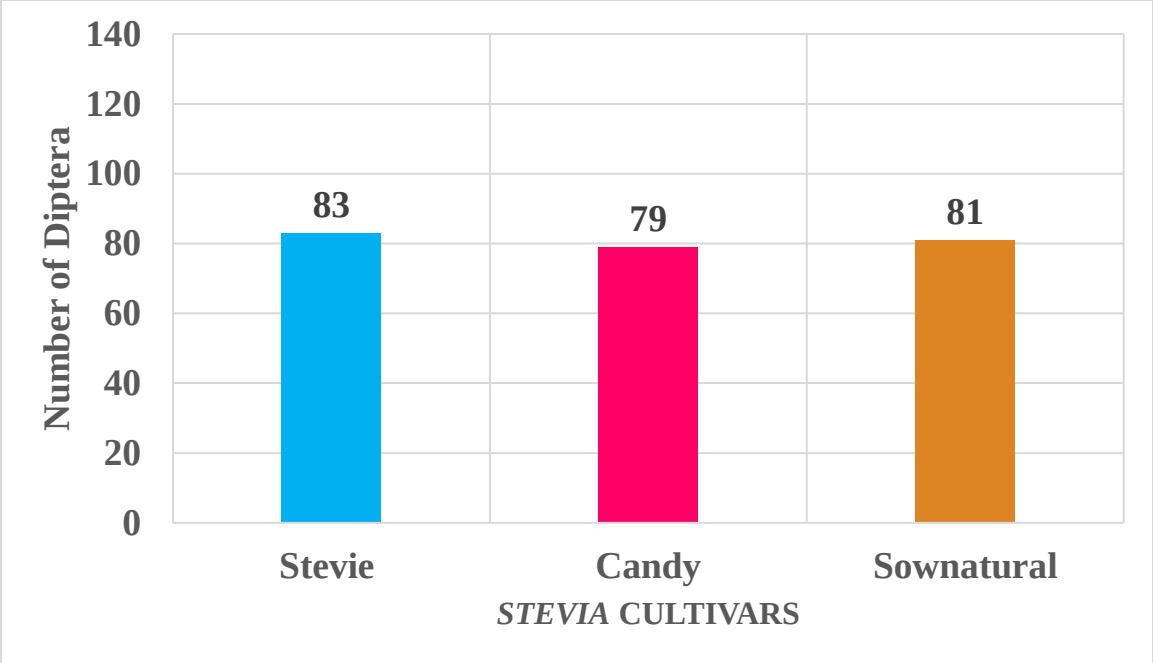

Figure A-15. Total number of Diptera among three *Stevia* cultivars in Loudon Co., 2016

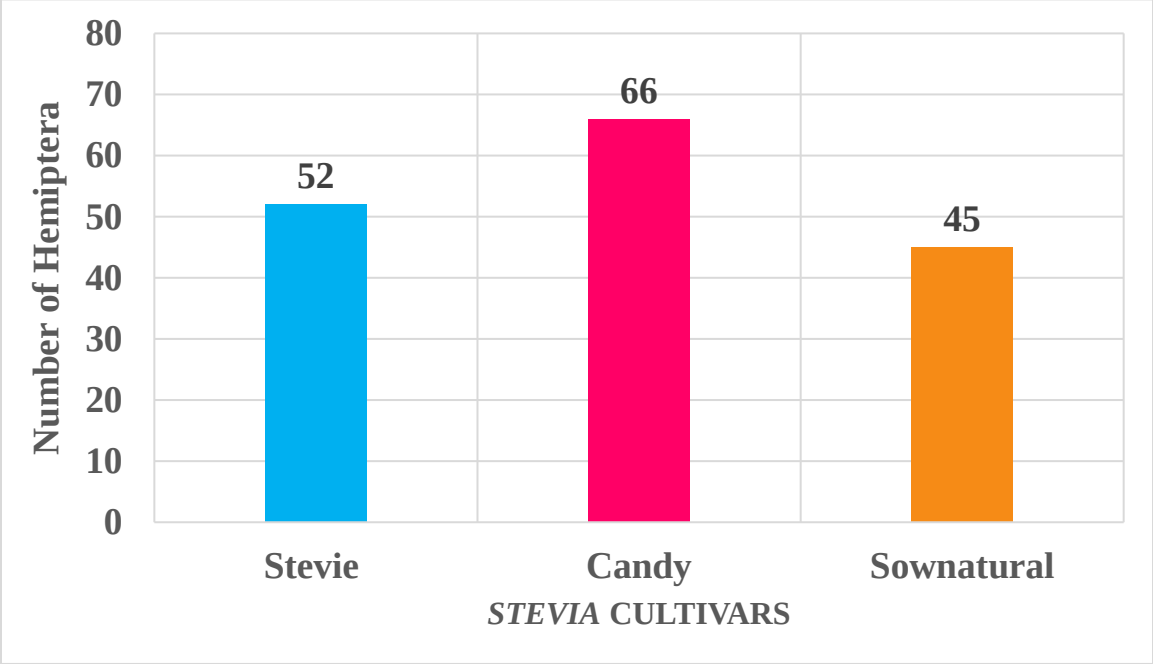

Figure A-16. Total number of Hemiptera among three *Stevia* cultivars in Loudon Co., 2016

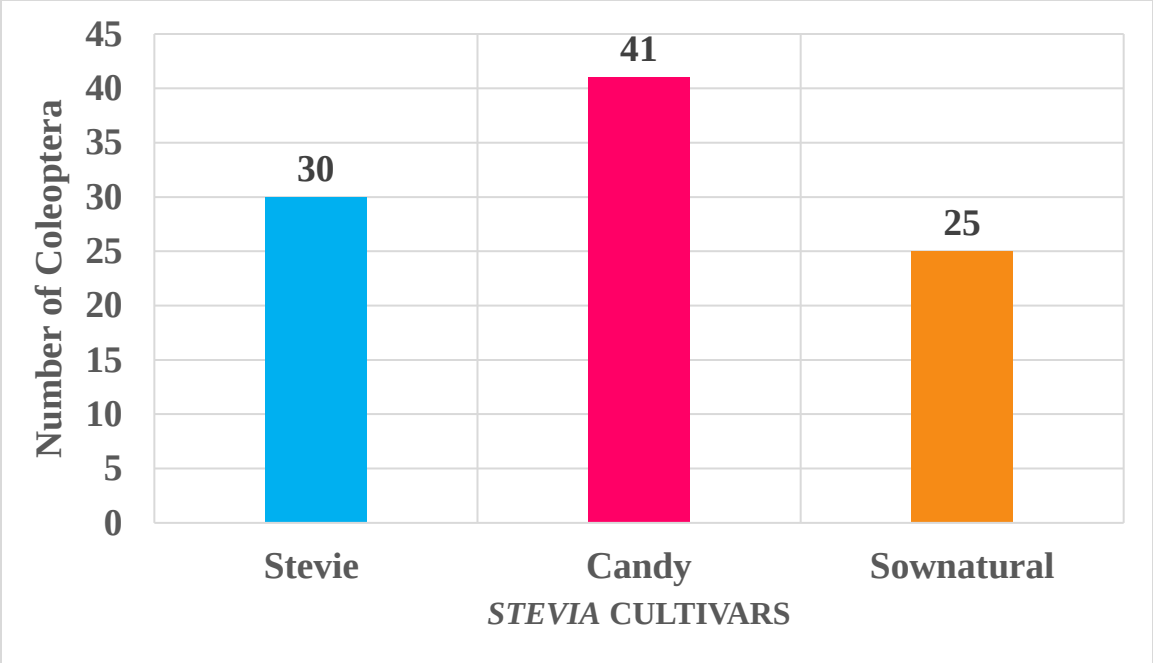

Figure A-17. Total number of Coleoptera among three *Stevia* cultivars in Loudon Co., 2016

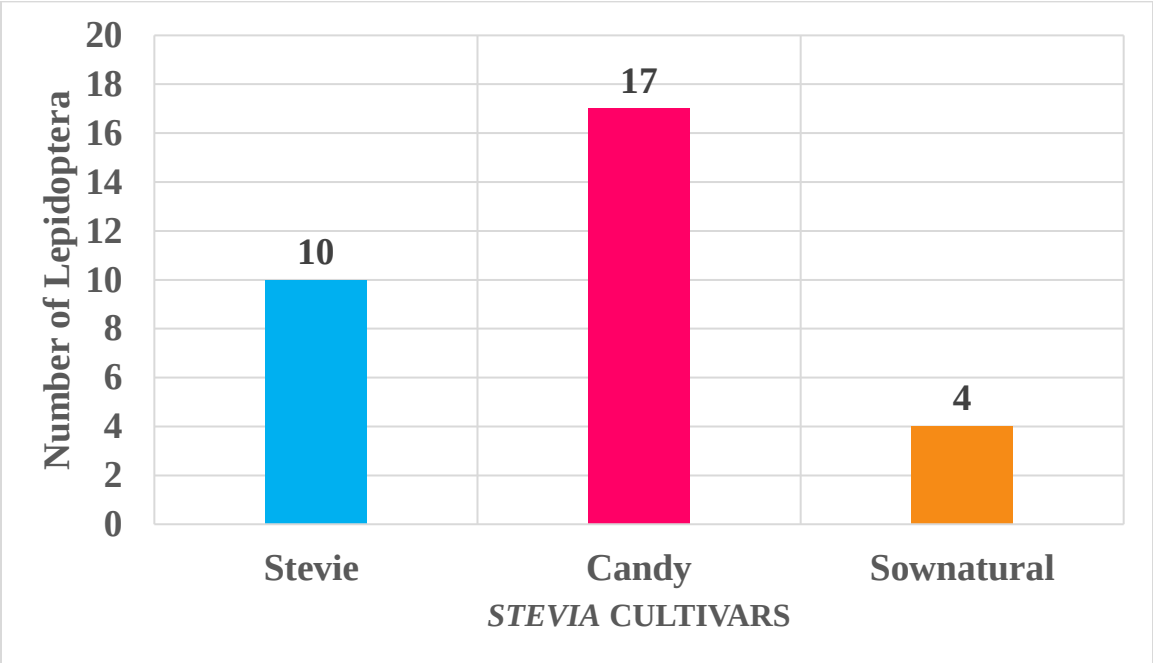

Figure A-18. Total number of Lepidoptera among three *Stevia* cultivars in Loudon Co., 2016

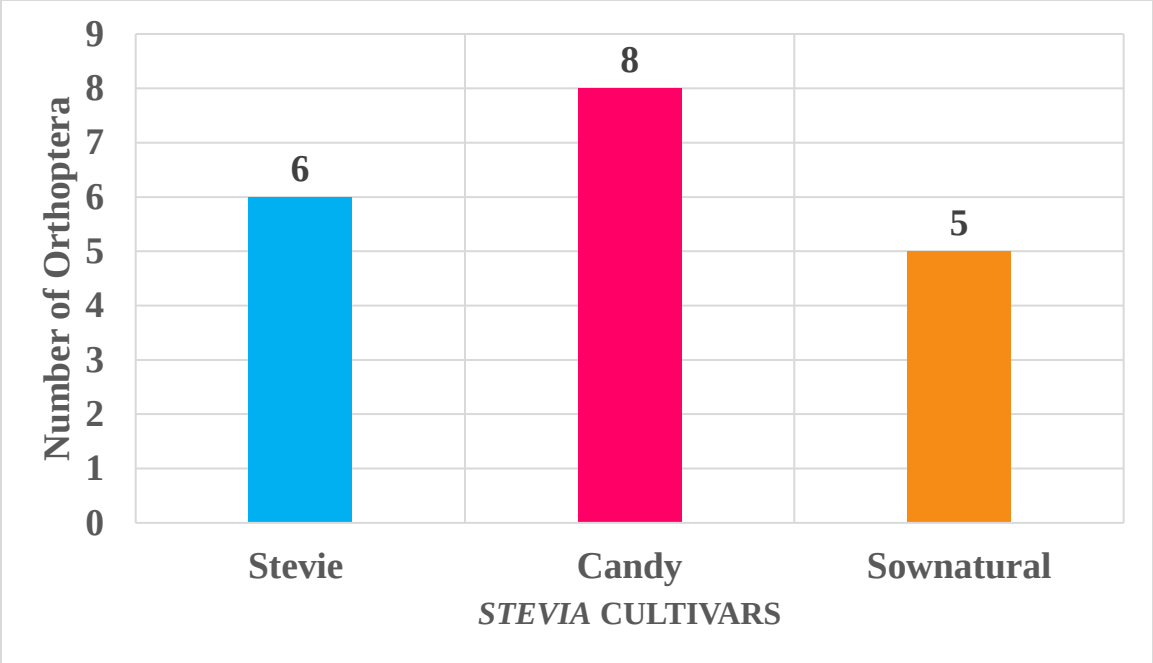

Figure A-19. Total number of Orthoptera among three *Stevia* cultivars in Loudon Co., 2016

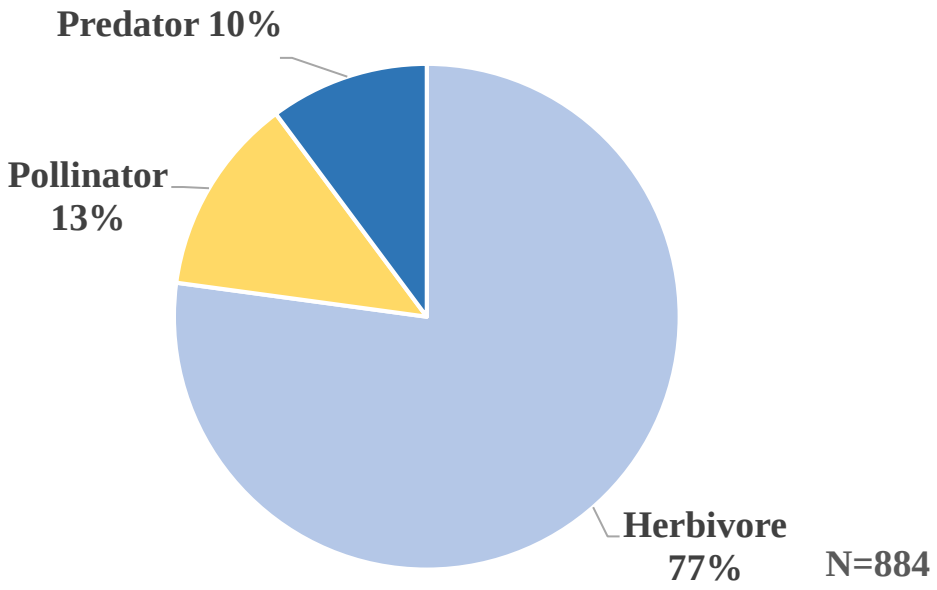

Figure A-20. Guilds of insects observed on *Stevia*, Loudon Co., 2016

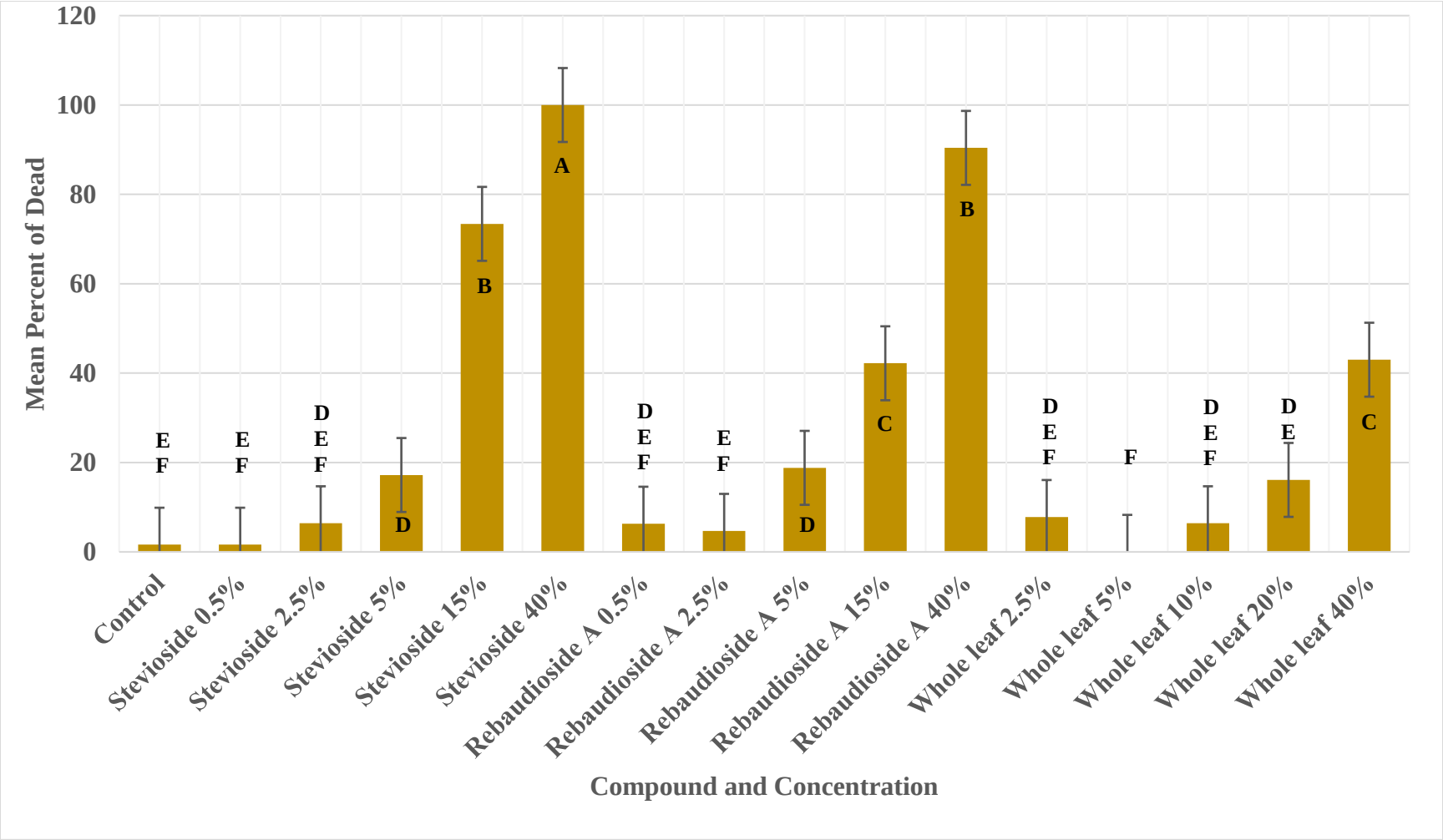

Figure A-21. Percent mortality of fall armyworm larvae by *Stevia*, compound and concentration

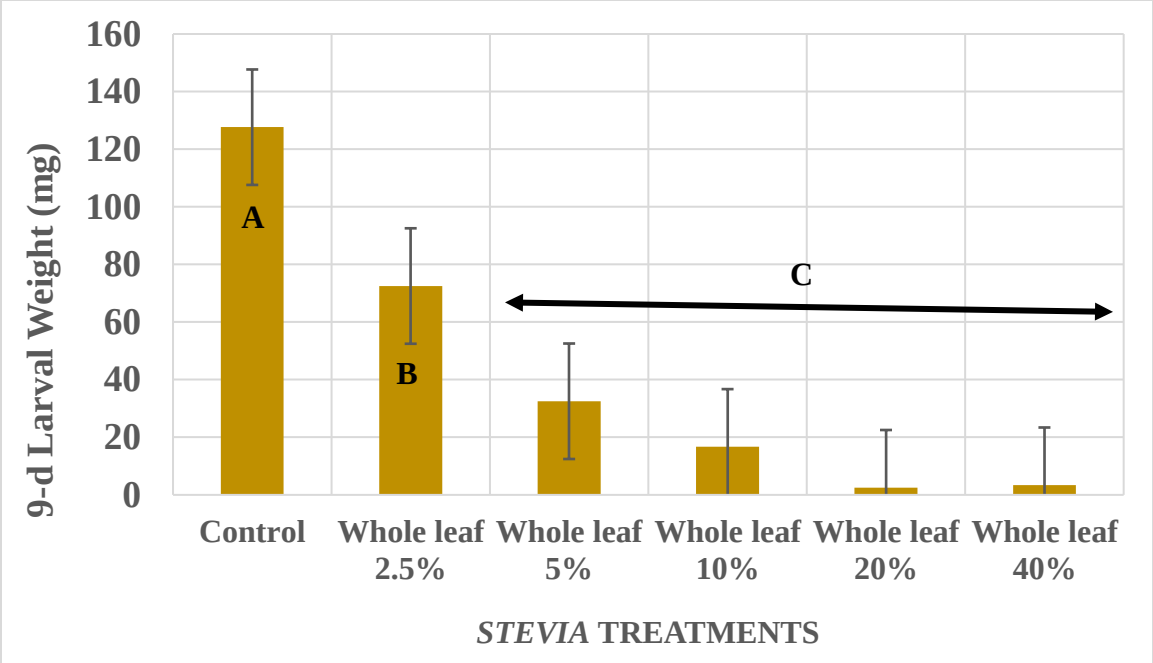

Figure A-22. Fall armyworm larval weight after 9-day exposure to *Stevia* whole leaf and control treatments

VITA

Heather Lowery is a first-generation college graduate, who was born in Knoxville, TN, but grew up in Jefferson County, TN, where she graduated from high school. After some time in the workforce, getting married and having two children, Heather returned to college. She obtained an Associate of Science degree from Pellissippi State Community College and then transferred to the University of Tennessee, Knoxville. After getting a Bachelor of Science degree in Plant Sciences with a concentration in public horticulture, she chose to pursue a Master's degree in Entomology.

Heather has a passion for outreach and education, which she has pursued during her time at UT, and wants to continue to share her knowledge of agriculture. She loves to spend time with her family hiking, camping, canoeing, and being outdoors. Beekeeping, cross-stitching, reading, and table-top war games are also some of her enjoyed activities.