COMPARISON OF ELECTRICAL PENETRATION GRAPH WAVEFORMS OF SQUASH BUG FEEDING ON WATERMELON AND ITS RELATIVES

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TABLE OF CONTENTS

Chapter Page

LIST OF TABLES

Table Page

Chapter II

Chapter III

LIST OF FIGURES

Figure Page

Chapter II

Chapter III

Figure Page

Chapter IV

Figure Page

CHAPTER I

INTRODUCTION

The squash bug, *Anasa tristis* DeGeer, is a serious pest of cucurbits in the United States (Beard 1940). It is distributed throughout North America (Elliot 1935, Fargo et al. 1988, Riley et al. 1998, Eichmann 1945, Provancher 1886, Van Duzee 1917). Squash bugs attack squash, pumpkin, watermelon, cantaloupe, and cucumber and can be found in large numbers in the commercial growing areas of southern Oklahoma (Edelson et al. 1999). The squash bug is also regarded as the primary pest of watermelon (Riley et al. 1998). It has piercing and sucking mouthparts which it uses to feed on the plant sap, primarily xylem, causing the plant to wilt and die (Beard 1940). This wilting condition is known as "Anasa Wilt" (Robinson and Richards 1931). The squash bug is also a vector of cucurbit yellow vine disease (Bruton 2003).

Cucurbit yellow vine disease (CYVD) is a destructive disease of cucurbits that causes heavy losses. Losses to CYVD can exceed 10% of watermelon, cantaloupe, and pumpkin crops in central Texas and Oklahoma. Cucurbit yellow vine disease is caused by a phloem-inhabiting bacterium, *Serratia marcescens*. The diagnostic characteristic of this disease is the honey brown discoloration of phloem in the crown and primary root (Bruton et al. 1998). Host plant resistance has been shown to be an effective strategy for controlling many pests and diseases (Clough and Hamm 1995, Liu et al. 2007,

Prischmann et al.2007). It is an ecofriendly method and can be integrated with other pest management programs. One of the most promising methods to control squash bug and CYVD in watermelon would be to develop a cultivar that is resistant to these pests.

There are watermelon relatives that have been shown to be resistant to some watermelon diseases and insects, but the level and mechanisms of resistance are unknown (Davis et al. 2007). Likewise, there are no identified watermelon cultivars resistant to the squash bug and CYVD. Electrical penetration graph (EPG) technology has been used to screen and identify plant germplasm resistant to piercing-sucking insects. However, very little work has been done using EPG in watermelon resistance studies. Using traditional host plant resistance studies and EPG technology, my goal is to identify melon germplasm resistant to the squash bug which may eventually be used to develop a commercial watermelon cultivar resistant to CYVD and the squash bug.

Therefore my research objectives are:

Objective 1: Describe major EPG waveform patterns of the squash bug probing on watermelon

Objective 2a: Screen watermelon relatives *Citrullus colocynthis*, *Praecitrullus fistulosus*, and watermelon hybrid USVL-200 (*C. lanatus* x *C. colocynthis*) for behavioral resistance to the squash bug

Objective 2b: Compare the EPG waveforms produced by the squash bug feeding on watermelon to waveforms produced on *C. colocynthis, P. fistulosus* and hybrid USVL-200.

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CHAPTER II

LITERATURE REVIEW

Squash bug biology

The squash bug, *Anasa tristis* (DeGeer) belongs to the order Hemiptera and family Coreidae. It is a serious pest of cucurbits in United States (Beard 1940). It attacks pumpkin, squash, cantaloupe, watermelon and other vine crops of the Cucurbitaceae family. Squash bugs prefer squash and pumpkin as hosts (Elliot 1935, Bonjour et al. 1990) and are a serious pest of these crops in Texas and Oklahoma. The squash bug is regarded as the primary pest of watermelon (Riley et al. 1998) even though it does not prefer it (Bonjour and Fargo 1989, Bonjour et al. 1990, Bonjour et al. 1991). Watermelon, cantaloupe and cucumber are considered to be alternate hosts for the squash bug (Hoerner 1938, Eichmann 1945).

The squash bug is native to United States (Beard 1940) and is distributed throughout North America in many states such as Connecticut (Elliot 1935), Illinois (Fielding 1990), Indiana (Cook and Neal 1999), Kansas (Wadley 1920), Oklahoma (Fargo et al. 1988), Texas (Riley et al. 1998), and Washington (Eichmann 1945). It can also be found in some parts of Canada (Provancher 1886, Van Duzee 1917) and also in Mexico and Central America (Uhler 1878, Wadley 1920). It can be found in high

numbers in commercial growing areas of southern Oklahoma as a serious pest of watermelon (Riley et al. 1998, Edelson et al. 1999).

Eggs are fairly large, about 1.48 mm in length and 1.02 mm in width, and white to yellow color when deposited but gradually darken to bronze at the time of hatching (Beard 1940). Egg masses, each containing about a dozen or more depending on the availability of food and suitable temperature, are usually deposited on the undersides of the leaves in the angles formed by the veins. It takes 10 days or more for nymphs to emerge from the eggs, depending on the temperature. First instar nymphs are brightly colored upon hatching, with red legs, antennae, head and thorax and a green abdomen, which measures about 2.5 mm in length. After a few hours, the red body turns black and the abdomen remains green. After the first molt, nymphs are about 3 mm in length. Just after molting, the body regains the reddish color (Beard 1940). After the second molt, the insect can be distinguished by the development of the wide penultimate segment of the antennae. Third instar nymphs lack wing pads and measure about 4 mm in length. The development of wing pads and the size of the nymph help to distinguish the 4^{th} and 5^{th} instar from other stages. The 4^{th} and 5^{th} instar nymphs measure about 6-7 mm and 9-10 mm in length, respectively (Beard 1940). The adults are larger, about 14-16 mm in length with well-developed wings and brownish black color. Males are smaller than females and weigh on average 27% less than females (Bonjour and Fargo 1989).

Squash bugs overwinter as adults throughout the winter season. Overwintering sites of the squash bug include buildings, tree trunks, under stones, weed and wood piles and crevices (Wadley 1920). In late spring, the overwintering squash bugs move to nearby commercial squash fields soon after seedling emergence (Fargo et al. 1988). They

start ovipositing once they leave their overwintering sites depending on the day length and availability of food (Fielding 1990).

Overwintered females produce most of the eggs during June and early July (Nechols 1987). In Connecticut and Kansas, adult squash bugs emerge between late May and mid June and continue to oviposit until about early August (Beard 1940, Nechols 1987), and in Oklahoma they appear in late spring after seedling emergence (Fargo et al. 1988). Squash and pumpkin are the preferred hosts for oviposition by the squash bug compared to watermelon, muskmelon and cucumber (Bonjour et al. 1990). In Oklahoma, the squash bug completes two to three generations per year (Fargo et al. 1988). In the northern part of the United States, the squash bug has only one generation (Elliot 1935, Davidson and Lyon 1979), but in Kansas there are two generations (Nechols 1987). The temperature and availability of food are the most dependent factors to keep this insect active.

Squash bugs overwinter in reproductive diapause, a condition in which they neither copulate nor oviposit (Chittenden 1908, Wadley 1920). Short day length induces reproductive diapause in newly molted, sexually mature and previously diapaused adult squash bugs, while long day length and high temperature prevents reproductive diapause (Fielding 1988). Squash bugs exposed to a photoperiod of 14:10 L:D or lower day length entered reproductive diapause, while those exposed to 14.5:9.5 L:D did not enter diapause (Nechols 1988). Similarly, squash bugs terminated diapause when exposed to long day length (17:7 L:D), while the diapause continued when they were exposed to short day length (12:12 L:D) (Fielding 1988). During diapause, the insect undergoes some physiological changes. The rate of respiration was lower in diapausing squash bugs

compared to non diapausing bugs (Fielding 1990). Thus, photoperiod, temperature and availability of food are responsible for stimulating the squash bug to break the diapause and leave the overwintering sites (Fargo et al. 1988, Nechols 1988, Fielding 1990). Absence of food after terminating diapause increases mortality of the squash bug (Fielding 1990).

Plant damage caused by squash bugs

Like other Heteropterans, squash bugs have piercing and sucking mouthparts and use their stylets to puncture and suck the contents from plant tissues, especially from collenchyma, xylem, and probably phloem (Bonjour et al. 1991, Neal 1993). Neal (1993) described squash bug feeding as the lacerate and flush type. When feeding on the plant vascular system, the plant wilts because of leaking of sap from the wound made by insect feeding or due to deposition of sheath saliva, blocking xylem transport of water (Neal 1993). This wilting condition is known as "*Anasa wilt*" (Robinson and Richards 1931). Plant wilting is followed by plant death, but no toxin was identified in the condition (Neal 1993). Squash bug feeding also causes loss of nutrients from the plant and interferes with the photosynthetic process, which results in slow growth and low productivity of squash (Woodson and Fargo 1991). Watermelon seedlings are vulnerable to squash bug feeding, hence, the mortality of the seedlings is high even in relatively low densities of the squash bug adults (Edelson et al. 2002).

Osmotic pump feeding by coreid bugs

Coreid bugs have a different feeding strategy compared to other Heteropterans. The feeding activities of coreids leaves water soaked lesions on the surface of the plant and straight and unbranched intracellular stylet tracks, which can be identified by the

presence of a thick, solidified salivary sheath. The salivary, or stylet, sheath is formed around the stylet bundle, forming a channel which lubricates and anchors the stylets (Miles 1987). The author also reported that coreid feeding leaves melanized lesions in the cells, beyond the feeding puncture, whose contents have been removed, suggesting that the contents of those cells had been drawn into a single locus and then removed by the insect. Miles and Taylor (1994) described coreid feeding as "osmotic pump feeding," in which the salivary enzyme sucrase is secreted into the area near sieve elements. The sucrose of the nearby cells or tissues is hydrolyzed into glucose and fructose. This causes a gradient of the solutes in the area, resulting in the movement of cell solutes into a pool outside of the sieve element. Thus, the insect can ingest hydrolyzed phloem sugars without actually penetrating the phloem. Although Miles generalized about coreid osmotic pumping, this activity has not yet been demonstrated for *A. tristis*.

Squash bug as a vector of cucurbit yellow vine disease

In addition to killing the plants by sucking sap and blocking xylem transport, the squash bug is also a vector of the phloem-colonizing bacterium, *Serratia marcescens*, the causal agent of cucurbit yellow vine disease (Bruton et al. 2003). Pair et al. (2004) showed that feral squash bugs have the ability to retain the pathogen in their body throughout the winter. In laboratory experiments, the transmission rate of *S. marcescens* was 9.2% for single inoculative insects (Bruton et al. 2003). Bextine (2001) hypothesized that *A. tristis* transmitted *S. marcescens* in a noncirculative manner but Wayadande et al. (2005) suggested, based on evidence of *S. marcescens* transmission by adults after nymphal acquisition, that the bacterium circulates throughout the body of the insect and is transmitted to its plant host via salivary secretions.

Sequential aspect of plant acceptance and feeding

Plant feeding of an insect consists of a series of sequential events. Generally, insect preference differences among its hosts is based on color, intensity of light, mechanical stimuli (physical structures and surface of the plant), and chemical stimuli (odors and tasting). Colors and light play an important role in insect attraction to the host. Before landing on plants, insects respond to color and light as reflected from the surface of the plant (Painter 1968). Once hemipterans reach the host and have explored the plant surface, they oppress the rostrum to the surface and secrete a salivary flange on the surface of the plant. The function of this salivary flange is to act as a conduit for surface compounds to contact the chemosensilla at the tip of the insect rostrum. The chemical cues detected by the chemosensilla mediated further stylet insertion by the insect. The piercing and sucking insects have precibarial chemosensilla within the food canal used for host and tissue selection (Backus and McLean 1982, Backus 1985, Backus and McLean 1985).When the insect inserts its stylets, it draws liquid through the food canal to the precibarial chemosensilla that line the epipharyngeal wall. If cues are positive, the insect will continue to insert stylet deeper into the plant. During stylet insertion they secrete saliva inside the plant tissue that solidifies as the stylets go forward, forming a stylet sheath. Once they reach a preferred tissue, sustained ingestion will ensue (Backus 1985).

Cucurbit yellow vine disease

Cucurbit yellow vine disease (CYVD) is a destructive disease of cucurbits. It was first observed in squash and pumpkin grown in the cross timbers region of Oklahoma and Texas in 1988 (Bruton et al. 1995a). In watermelon, it was observed in 1991 near Rush

Springs and Terral, Oklahoma, and Deleon and Gustine, Texas (Bruton et al. 1998). In addtition to Oklahoma and Texas, CYVD was also observed in Tennessee (Bost et al. 1999), Massachusetts (Wick et al. 2001), Kansas, Arkansas, Colorado, Nebraska (Rascoe et al. 2003), Missouri (Kabrick 2002) and Kentucky (Bessin 2003).

The most prominent symptoms of CYVD are yellowing of the lower canopy leaves and inward curving of the terminal leaves, followed by gradual or rapid decline and death of the vine, especially in early planted fields (Fig.2.1) (Bruton et al. 1998). The stunting, yellowing, and gradual decline begin about 10 to 14 days prior to harvest in older plants. In immature plants, rapid wilting occurs and plant collapse follows within a single day without the associated plant yellowing (Bruton et al. 1998). The diagnostic characteristic of this disease is the honey brown discoloration of phloem in the crown and primary root (Fig.2.2) (Bruton et al. 1998). In watermelon, the affected vines produce chlorotic, unmarketable fruit (Bruton et al. 1998).

Bruton et al. (1995b) first suggested that CYVD might be transmitted by insects. The disease symptoms were often observed in watermelon plants in small patches (Duthie et al. 1993) and weekly application of insecticides significantly reduced the incidence of the disease (Bruton et al. 1998). Squash plants fed upon by squash bugs that previously had been caged on diseased plants exhibited symptoms of phloem discoloration (Pair et al. 2000). Similarly, 20-25% of uncovered squash plants showed the presence of the yellow vine bacterium by polymerase chain reaction (PCR) but no bacteria were found in plants protected by row cover (Bextine 2001). Finally, Bruton et al. (2003) confirmed by the completion of modified Koch's postulates that the squash bug transmitted the bacterium *S. marcescens*.

Serratia marcescens

Serratia marcescens, the causal agent of CYVD, is a rod shaped, gram negative, motile, facultatively anaerobic, non spore-forming, walled bacterium belonging to the family Enterobacteriaceae (Yu 1979, Hejazi and Falkiner 1997, Rascoe et al. 2003). *S. marcescens* was found associated with phloem vessels of infected cucurbit plants (Bruton et al. 1998). There are many strains of *S. marcescens* which can be found in different plant associated niches, including the root and stems of rice (Gyaneshwar et al. 2001) and cotton (Wei et al. 1996), but these strains were not reported to be pathogenic to the host plant. Unlike these strains, some strains of *S. marcescens* are known to cause crown root of alfalfa (Lukezic et al. 1982). *S. marcescens* is also found in water, soil, food products, insects and animals (Lium 1977, Rosenzweig and Stotzky 1980, Seitz et al. 1987, Grimont and Grimont 1992, Ahrenholtz et al. 1994).

Many of the strains of *S. marcescens* produce a water insoluble red pigment, prodigiosin, also known as bloody discoloration of food (Yu 1979, Sikorowski and Lawrence 1998). Most of the human isolated strains of *S. marcescens* are non-pigmented but some from insects are red pigmented (Sikorowski and Lawrence 1998). Some strains are nonsocomial pathogens (Hejazi and Falkiner 1997). They cause infection in patients with urinary tract infections (UTI), septicemia, meningitis, endocarditis, wound infections and debilitating disorders (Okuda et al. 1984, Johnson et al. 1998). *S. marcescens* was also used as a biological marker to understand the dissemination and mechanism of infection in humans (Cumming 1920, Davis et al. 1970) and also used as markers by the US military in 1950 and 1952 (Yu 1979).

Several strains of *S. marcescens* cause disease in insects (Sikorowski et al. 2001) especially in insectary-reared insects (Steinhaus 1959, Sikorowski and Lawrence 1994), causing a lethal septicemia after invasion of the homocoel. A small number of *S. marcescens* in the digestive tract of insects is not pathogenic (Sikorowski 1985.) but when the bacteria reach the homocoel they multiply rapidly and kill the host (Sri-Arunotai et al. 1975, Tanada and Kaya 1993). In soil, some of these bacteria produce a nematotoxic volatile that kills the larvae of root knot nematode, *Meloidogyne incognita,* in tomato plants (Zavaleta and Gundy 1989a, 1989b). Research showed that insects like the German cockroach, *Blattella germanica,* and ants act as carriers of *S. marcescens* in hospitals (Flower et al. 1993, Kim et al. 1995).

In early times, *S. marcescens* was considered as a saprophytic microorganism living in soil. The bacterium plays a role in metabolizing organic iron and dissolving gold and copper (Pares 1964) and also was used as a biological control agent to suppress summer patch disease of Kentucky bluegrass (Kobayashi et al. 1995). *S. marcescens* also inhibits the growth of *Botrytis fabae,* causal agent of chocolate spot disease (Akutsu et al. 1993) and protects the leaves of *Vigna radiata* from *Xanthomonas campestris,* causal agent of bacterial leaf spot (Bora et al. 1993). It is also known as the most frequent contaminant of laboratory cultures of bacteria (Hejazi and Falkiner 1997).

Two strains of *S. marcescens*, WO1-A and ZO1-A, isolated from watermelon and zucchini squash, respectively, cause yellow vine disease in cucurbits. The sequence analysis of CYVD revealed the microbes as proteobacteria that differ significantly from other non-cucurbit strains of *S. marcescens* isolated from different environmental niches in biological functions and characteristics. These differences were demonstrated based on

the substrate utilization assays and fatty acid analysis and comparison of genomic DNA through repetitive elements-based polymerase chain reaction and DNA-DNA hybridization (Rascoe et al. 2003, Zhang et al. 2003). The CYVD associated bacteria can be detected by PCR using nonspecific primers designed from prokaryotic 16S rDNA (Francisco et al. 1998, Luo 2006). Rascoe et al. (2003) showed that the CYVD strains obtained from watermelon and zucchini produced smooth, circular, entire, convex, non pigmented colonies within 24 hrs and did grow under anaerobic conditions. They also illustrated that the *groE* sequence of these two strains were similar to each other but were significantly more distant to the strains isolated from cotton.

S. marcescens' ability to cause infectious disease in an organism is facilitated by many factors. This species of bacteria has pili, hair like appendages found on the bacterial surface that are used to adhere to the host epithelial surface (Yamamoto et al. 1985). The hydrophobic nature of these bacteria helps in distribution by air, water and oil, water interface and in the attachment to solid surfaces (Mudd and Mudd 1924, Ashkenazi et al. 1986). Some of the genes in *S. marcescens* are responsible for the synthesis of lipopolysaccharide, a biologically active constituent of endotoxin that is effective against other strains of *S. marcescens* and *E. coli* strains (Traub 1980, Guasch et al. 1995). *S. marcescens* produces several extracellular enzymes that can degrade chitin. One of the extracellular proteins produced by these bacteria is HasA (for heme acquisition system) which binds heme and acquires iron from heme and hemoglobin (Letoffe et al. 1999).

The Cucurbitaceae

Squash bugs are oligophagous on the plants of the Cucurbitaceae. This cucurbit family includes gourds, pumpkin, squash and melons. They are found in tropical and

subtropical regions. The family Cucurbitaceae consists of two subfamilies, about 118 genera, and 825 species (Jeffrey 1990). The subfamily *Zanoniodeae* includes cucurbits that have medicinal value and *Cucurbitoidae* includes the four major cucurbit crops: watermelon, cucumber, melon, and squash, along with other species.

The cucurbit crop in the United States composed of fresh cucumber (used for quick consumption in the raw state), processing cucumber (processed and preserved for future consumption), cantaloupe, honeydew, pumpkin, squash and watermelon. Total production of cucurbits in the United States is 109 million metric tons on 229,000 hectares, with a value of $1.43 billion in 2004. The major states for cucurbit production are Florida, North Carolina, Michigan, Texas, California, and Georgia (Cantliffe et al. 2007). Watermelon, cantaloupe, squash and cucumber contribute about 9.3% of the value of the total vegetable production in the United States. Watermelon, cantaloupe, squash and cucumber are ranked as 11^{th}, 12^{th}, 14^{th} and 15^{th}, respectively, in total production value (Cantliffe et al. 2007). Table.1.1 shows total harvested area, production and value of seven classes of cucurbit crops in 2004.

Watermelon and its relatives

Watermelon, *Citrullus lanatus* (Thunberg, Matsumura & Nakai), is one of the economically important crops grown in the southern region of North America. It is a leading melon crop of United States in terms of planted area (176,827 acres in 2000-2002), production, and per capita consumption (Lucier and Plummer 2003). In the United States, Florida, Texas, Georgia, Arizona, California, Indiana, North Carolina, South Carolina, and Delaware are the major watermelon growing states, producing more than 1,000,000 cwt (Dogramaci et al. 2004, Cantliffe et al. 2007, Hassell et al. 2007). Besides

these states, Oklahoma, Missouri, Maryland, Arkansas, Alabama, Mississippi, Virginia, Louisiana, and Hawaii also grow watermelon (USDA 2010a). According to 2000-02 data, the farm value of watermelon production was $282 million, which is a 19% increase from a decade earlier (Lucier and Plummer 2003). In 2009, total watermelon production in the United States was 40,122,000 cwt from 126,300 harvested acres, with a value of $460,778,000 (USDA 2010a).

Watermelon is a popular summer crop that is native to Africa (Marr and Tisserat 1998). It thrives well in tropical and sub tropical climates with sandy or sandy loam soil. It can be consumed fresh, as roasted seeds, pickled rind or watermelon juice.

Currently there are many seedless cultivars of watermelon grown in the United States. In 2009, more than 80% of the watermelon shipments in the United States are seedless (USDA 2010b). Some of the seedless cultivars adapted to grow in the US are Firecracker, Quality, Millionaire, AC 532, Crimson Trio, Genesis, King of Hearts, and Merrilee III (W1025) (Mayberry et al. 2003, Maynard 2003).

This economically important crop is attacked by many pests and diseases causing a significant loss in the production every year. The major diseases of watermelon are cucurbit yellow vine disease (CYVD), caused by the bacterium *S. marcescens* (Bruton et al. 2003), Fusarium wilt, caused by *Fusarium oxysporum* f.sp *niveum* (Ay and Erklc 2008), watermelon vine decline disease, caused by squash vein yellowing virus (Kousik et al. 2009), watermelon bud necrosis, caused by a tospovirus (Jain et al. 1998), bacterial fruit blotch, caused by *Acidovorax avenae* subsp. *citrulli* (Latin and Hopkins 1995), powdery mildew, caused by *Podosphaera xanthii* (Davis et al. 2007), bacterial leaf spot, caused by *Xanthomonas cucurbitae* (Pruvost et al. 2009), papaya ringspot, caused by

papaya ringspot virus-watermelon strain PRSV-W, watermelon mosaic, caused by watermelon mosaic virus (WMV), and zucchini yellow mosaic, caused by zucchini yellow mosaic virus (ZYMV) (Guner and Wehner 2008). Major insect pests of watermelon are the squash bug vector of CYVD, thrips vector of watermelon bud necrosis virus (Kamanna et al. 2010), whitefly vectors of Geminiviruses, (Isakeit et al. 1994), and aphid vectors of squash leaf curl disease (Dodds et al. 1984).

Some of the watermelon relatives have been reported to have resistance characteristics against some pathogens. Plant introduction (PI) accessions of *Citrullus* spp.*, Praecitrullus* spp. and watermelon cultivars have been screened for resistance to diseases and nematodes (Boyhan 1994, Thies and Levi 2003, Davis et al. 2007). *Citrullus colocynthis* PIs have been shown to be resistant to certain pathogens like watermelon mosaic virus and powdery mildew (*Podosphaera xanthii*) race 1W and moderately resistant to Fusarium wilt (*Fusarium oxysporum* f. sp. *niveum*) race 2 (Levi et al. 2001). Similarly, high to moderate resistance against *P. xanthii* race1W was seen in *Praecitrullus fistulosus* PIs (Davis et al. 2007). Similarly, these watermelon relatives are also resistant to some insect pests. *C. colocynthis* lines are resistant to arthropods, including the two spotted spider mite, *Tetranychus urticae* (Lopez et al. 2005), and whitefly, *Bemisia tabaci* (Simmons and Levi 2002). *P. fistulosus* is resistant to the white fly, *B. tabaci,* the vector of watermelon vine decline disease (Levi et al. 2005). These germplasm lines have not been tested for other disease and insect resistance traits and could be a source of resistance to other pathogens and insects.

C. colocynthis is a congener of watermelon and native of tropical Africa (*C. colocynthis* PI 525082 – origin Egypt and *C. colocynthis* PI 386015 – origin Iran). This

species is highly drought tolerant and grows on sandy soils throughout Northern Africa, southwestern Asia and the Mediterranean (Whitaker and Davis 1962, Jeffrey 1975, Whitaker and Bemis 1976). The seeds are rich in oil content and the oil is used for cooking. The seeds can also be roasted and eaten (Soliman et al. 1985).

P. fistulosus is popular in south Asia, especially in India and Pakistan. Immature fruit is used as a vegetable and seeds are also eaten. *P. fistulosus* is a distant relative of watermelon and is also known as Indian round gourd, apple gourd or Indian baby pumpkin. Earlier it was considered to be *Citrullus lanatus,* but was later placed in its own taxonomic category due in part to its difference in monoploid chromosome number (Sujatha and Seshadri 1989).

USVL-200 is a watermelon breeding line developed by crossing the watermelon cultivar "Royal Sweet" (*C. lanatus* var. *lanatus*) with *C. colocynthis* PI 386015. USVL-200 contains the nuclear genome of *C. lanatus* var. *lanatus* and the chloroplast and mitochondrial genomes of *C. colocynthis* (Levi et al. 2006). Its fruit characteristics include globular fruit, a dark green rind and yellow to pink flesh. USVL-200 can be used for enhancing watermelon germplasm, and in studies of lycopenes versus carotenoid production in watermelon.

Host plant resistance

Resistance of plants to insect or pathogen attack may be defined as the relative amount of heritable qualities possessed by the plant that influence the ultimate degree of damage done by the insect or pathogen (Painter 1968). Resistance of a crop can be determined by comparing the yield of the plant, survival of the plant, intensity of damage,

biological parameters of an insect or insect feeding duration between the susceptible and resistant cultivars.

According to Painter (1968) the three mechanisms of resistance are antibiosis, antixenosis and tolerance. Antibiosis is an association between two or more organisms that is detrimental to at least one of them. In case of host plant resistance, the plant has toxic substances that have a negative impact on the insect pest biology. Some examples of antibiosis in plant include cotton genotype PA 183, which has an antibiotic effect on the development and survival of the whitefly, *B. tabaci*, and is regarded as a resistant genotype (Jindal et al. 2008). The high amount of SiO_2 in leaf sheaths in the rice cultivars Zhendao2, Xudao3 and Xieyou136 is one of the factors responsible for resistance against the small brown planthopper, *Laodelphax striatellus* Fallen which causes high mortality, low fecundity and low hatchability (Liu et al. 2007).

Antixenosis is the plant resistance mechanism that affects the behavior of the pest. The plant has the ability to repel insects causing reduction in oviposition and/or feeding which will reduce the pest population. Antixenosis can be due to certain types of plant structures or volatiles. For example, increased flavonoid content in the wild species of genus *Vigna* was correlated with resistance to aphids (Lattanzio et al. 2000). Similarly, the presence of the *Vat* gene in the resistant melon cultivar, Margot, inhibited sustained phloem ingestion by the aphid, *Aphis gossypii* Glover (Chen et al. 1997).

According to Painter (1968) “Tolerance is a basis of resistance in which the plant shows an ability to grow and reproduce itself or to repair injury to a marked degree in spite of supporting a population approximately equal to that damaging a susceptible host”. The yield from tolerant plants does not decrease and they remain in the same vigor

as in the absence of the damaging population. Prischmann et al.(2007) explained that the synthetic maize hybrid CRW8-3 has tolerance to the root damage caused by the western corn rootworm larvae, *Diabrotica virgifera virgifera* LeConte. Similarly, hybrids OsSK 617, OsSK 602 and OsSK 596 showed tolerance to the corn rootworm *D. virgifera virgifera* Le Conte (Ivezic 2007).

Resistance of cucurbits to hemipterans and diseases has been previously documented. Collins et al. (1994) tested antixenosis and tolerance effects of muskmelon cultivars against the melon aphid and concluded that some were resistant to the melon aphid on the basis of fewer aphid numbers on the plants. Similarly, the effect of the *Vat* (monogenic, dominant) resistance gene of melon on *A. gossypii* phloem ingestion behavior using EPG showed that the expressed gene had some effect against the insect. The expression of the *Vat* gene inhibited the sustained ingestion of phloem from the melon plant, thus causing antixenosis resistance against the aphid (Chen et al. 1997). After genetically altering three yellow crookneck squash (*Cucurbita pepo*) and five cantaloupes (*Cucumis melo*), Clough and Hamm (1995) tested the resistance of these plants against zucchini yellow mosaic virus and watermelon mosaic virus. The transgenic plants showed significant reduction in disease incidence and yielded more marketable fruit than the non transgenic plants.

There is some evidence of host plant resistance to coreid bugs. Koona et al. (2002) documented antibiosis effects of four wild accessions of cowpea, *Vigna unguiculata* subsp. *dekindtiana*, to *Clavigralla tomentosicollis*. The wild accession, TVnu 151, causes high mortality (>50%) of *C. tomentosicollis* while TVnu 369, TVnu 517, and TVnu 707 extended the developmental period, causing the surviving adults to gain little

weight and oviposit few eggs. Similarly, Koona et al. (2003) tested the effect of extracts from seeds and glandular trichomes of different *Vigna* species and found that the trichome extracts from all genotypes caused significantly higher insect mortality and longer total developmental time of the insect. The bug avoided normally edible seeds treated with trichome extract from wild *Vigna,* suggesting antixenosis resistance.

Some work has been done on resistance in cucurbits to the squash bug. The development and feeding behavior of the squash bug nymphs on cucumber, *Cucumis sativus*, and pumpkin, *Cucurbita pepo*, was compared earlier by Cook and Neal (1999). Margolies et al. (1998) documented that the survival rate of the squash bug increased from 20% in the first generation to 45% in the fifth generation when it was reared continuously on resistant squash cultivars. No squash bug resistant watermelon cultivars have been identified.

Electrical penetration graph technology

Electrical penetration graph technology was first developed by D. L. McLean and M. G. Kinsey of the University of California, Davis, in the 1960s to record characteristics of aphid feeding and salivation (McLean and Kinsey 1964, 1965, 1967). Originally it was referred to as the electronic feeding monitor or electronic measuring/monitoring system but later it became known as electrical penetration graph (EPG) technology after the convention of Tjallingii (Tjallingii 1985). Two different types of EPG systems are currently used to study insect feeding behavior, the alternate current (AC) system (McLean and Kinsey 1964) and direct current (DC) system (Tjallingii 1988). EPG works by detecting small changes in an electrical current passing through a feeding insect. The EPG system consists mainly of a voltage source and an input resistor. The voltage source

and input resistor are electrically connected inside a box. Outside the box is an output receptacle, which is connected to the voltage source, and an input receptacle attached to the input resistor (Fig.2.3). The output receptacle in the other end is attached to the plant electrode (an electrode that has contact with the plant or the pot soil), while the input receptacle is attached to the insect electrode (an electrode that has contact with the insect). When the insect penetrates the plant tissue, voltage flows from the voltage source through the output receptacle to the input receptacle and input resistor and returns to the voltage source, forming a circuit (Walker 2000). The electrical signal is sent to a rectifier amplifier and computer, which records the waveform output. The change in resistance, or voltage, in this circuit is translated into trace patterns, called waveforms. In EPG technology, the plant and insect are the biological components of the circuit and they resist the current flow from the voltage source. The change in resistance is due to the different electrical conductivity of the fluids (saliva, plant sap) that pass through the insect mouthparts (Cline and Backus 2002).

Electrical penetration graph technology is used to study the feeding behavior of piercing and sucking insects by correlating the waveforms produced by the insect feeding on the plant surface with the stylet activities in the plant tissues. This technique allows the characterization of probing behavior (Hunter and Backus 1989, Bonjour et al. 1991, Walker and Perring 1994, Cline and Backus 2002, Almeida and Backus 2004, Backus et al. 2005, Joost et al. 2006), investigation of plant resistance mechanisms (Dorschner and Baird 1988, Kimmins 1989, Montllor and Tjallingii 1989), and the mode of action of pesticides (Caillaud et al. 1995, Harrewijn and Kayser 1997, Sauge et al. 1998, Annan et al. 2000, Kaufmann et al. 2004) and identification of behaviors necessary for plant

pathogen transmission (Wayadande and Nault 1993, Prado and Tjallingii 1994, Martin et al. 1997, Lett et al. 2001). Feeding behavior of many homopteran insects, including aphids (McLean and Kinsey 1964, Prado and Tjallingii 1994, Annan et al. 2000), whiteflies (Jiang et al. 2000, Johnson et al. 2002, Jiang and Walker 2003), heteropterans such as the Lygus bug (Cline and Backus 2002), squash bug (Bonjour et al. 1991, Cook and Neal 1999) and thrips (Harrewijn et al. 1996, Kindt et al. 2003, Kindt et al. 2006), has been studied using EPG.

Electrical penetration graph technology was used to understand host plant resistance mechanisms. Caillaud et al. (1995) compared the EPG waveforms produced by the cereal aphid (*Sitobion avenae*) feeding on resistant (*Triticum monococcum*) and susceptible (*Triticum aestivum*) wheat lines. They characterized the EPG waveforms produced on susceptible wheat lines and then compared them to those produced on resistant lines. Repeated stylet penetration and reduced vascular tissue ingestion were observed for insects given access to the resistant plants. The aphid also delayed sap ingestion when on the resistant cultivar as compared to the susceptible one. Total time spent by the aphid in ingesting the sieve elements was reduced by 72% on the resistant plants.

Similarly, Kimmins (1989) used the EPG technique to study the feeding behavior of the planthopper *Nilaparvata lugens* on different rice varieties showing different levels of resistance. The duration of the EPG waveform produced during phloem and xylem ingestion on resistant rice was shorter than that on the susceptible variety, but the time taken to produce these patterns from the beginning of the recording did not differ

between varieties, suggesting that the resistance factor was located in the vascular tissue and not in the epidermis or mesophyll.

Bonjour et al. (1991) described the stylet activities of first instar squash bugs on squash seedlings. The authors described six different types of waveforms produced during insect feeding, waveforms B (a non probing wave corresponding to the baseline), P (insertion of the stylets into a leaf), Wa (high amplitude peaks), Wb (medium amplitude peaks), Wc (low amplitude peaks - the ingestion pattern) and E (exit peak – removal of stylets from the leaf). Histological sections of tissues excised during production of waveform Wc contained the the termination point of a single sheath located near the vascular bundle. In most of the tissue sections, salivary sheaths terminated in or near xylem tissue, but no difference in waveform patterns was found between alleged phloem and xylem ingestion.

For insect species that have been electronically monitored, there is a noticeable difference between the phloem and xylem ingestion waveform patterns because of the differences in cibarial muscle pumping requirements and because of the different sap composition in these two tissues. Phloem ingestion patterns usually have higher amplitude than xylem ingestion patterns when recorded using low input resistance, presumably because of higher electrical conductivity due to higher sugar content in phloem (Walker 2000). Cucurbit phloem sap is dissimilar to the phloem sap of other species as it contains high protein, low total sugar, and a high proportion of sugars as monosaccharide (Richardson et al. 1982). It also has a high pH and high K^+ concentration. Xylem sap is similar among species, as it contains little protein or sugar, but has high nitrate and ammonia contents (Richardson et al. 1982). It has lower K^+ and

lower pH than phloem exudates. Since, the electrical conductivity of the sap affects the waveform pattern the difference in the ionic makeup of phloem and xylem should result in different waveform characteristics.

Relevant waveforms must be identified, correlated and compared to explain the squash bug stylet activities on different watermelon relatives and cultivars. The squash bug is considered to be a xylem drinker (Neal 1993) but the CYVD pathogen resides in the phloem. The process of transmission of *S. marcescens* by the squash bug is still under investigation. The characterization and comparison of the EPG waveforms produced by the squash bug on watermelon and its different relatives will show whether this insect probes in both xylem and phloem. This information will be used further to understand the inoculation process of *S. marcescens* into phloem tissue and also to screen melon lines to develop commercial cultivars resistant to CYVD and to the squash bug.

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Table.2.1. Total harvested area, yield and value of seven classes of cucurbit in 2004.

Crop	Harvested area (ha)	Yield (tons)	Value ($1000)
Cucumber (F)	9,213	434,340	212,734
Cucumber (P)	18,620	1,054,800	
Cantaloupe	14,755	912,150	300,578
Honeydew	3,560	229,005	89,731
Pumpkin	7,400	448,875	99,835
Squash	8,628	349,020	222,718
Watermelon	23,160	1,656,720	313,458

Source: National Agricultural Statistics Service, U.S. Department of Agriculture. Vegetable Summary 2004.

F = fresh market, P = processed.

Fig.2.1 Yellowing of cantaloupe vines by cucurbit yellow vine disease (Courtesy B.D. Bruton)

Fig.2.2 Phloem discoloration of watermelon crown caused by cucurbit yellow vine disease (Courtesy B.D. Bruton)

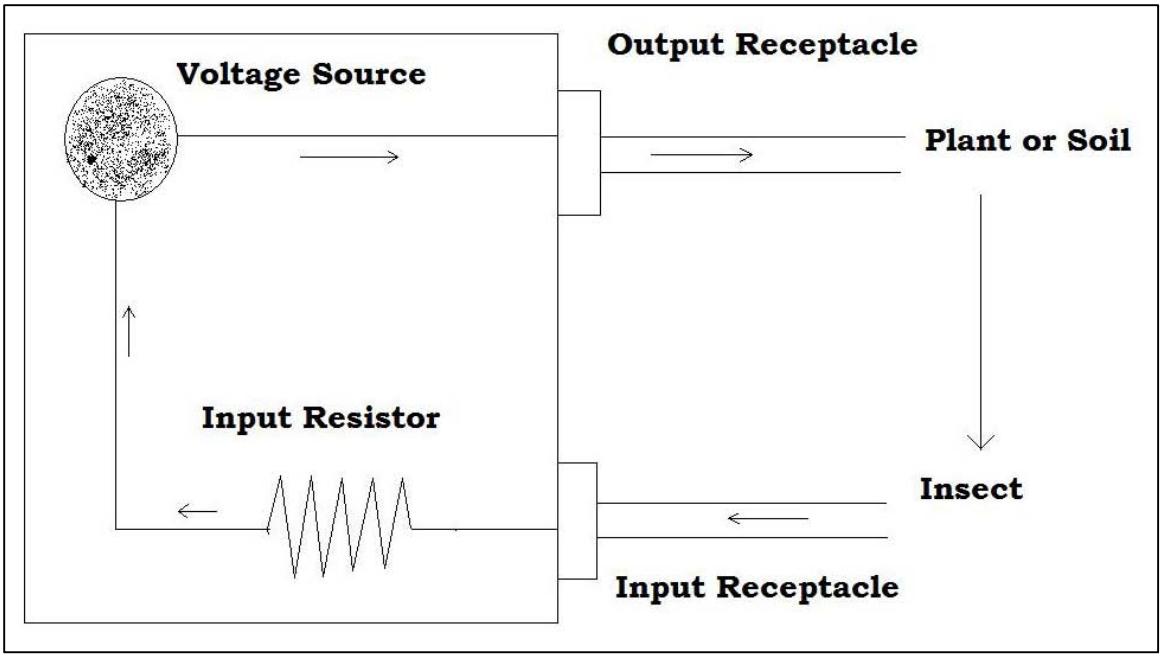

Fig.2.3. Flow of current through voltage source to input resistor and returned to the voltage source forming a circuit in an electrical penetration graph.

CHAPTER III

CHARACTERIZATION OF ELECTRICAL PENETRATION GRAPH WAVEFORMS PRODUCED BY SQUASH BUG NYMPHS FEEDING ON WATERMELON

Abstract

The squash bug, *Anasa tristis*, is a major pest of cucurbits and causes serious damage to watermelon plants by direct feeding on the plant and by transmitting the bacterium, *Serratia marcescens*, the causal agent of cucurbit yellow vine disease. Previous work on feeding behavior of this insect did not fully describe probing activities. This study was conducted to describe the major EPG waveform patterns of squash bug probing on watermelon plants. Waveforms were correlated with probing activities using the previously published interpretations and by direct observation and histology of probed tissue. Four different types of waveforms were identified and their respective voltages were measured. These waveforms and their associated behavioral activities were: 1. non probing 2. test probing 3. salivation/pathway and 4. ingestion. Voltages measured during the ingestion waveform C were relatively low compared to those measured during other waveforms. The squash bug spent more than 60% of its

time performing non probing behavior and the remainder was spent performing test probes, salivating and ingesting. Ingestion events averaged 30 min in duration, some lasting for several hours. Frequent short test probes before an ingestion probe was typical for this insect on watermelon. The function of these test probes is not fully understood but could be related to conditioning the plant tissue for ingestion or tasting fluid or overcoming plant defense responses. Histology of the probed tissue during ingestion waveform C showed the salivary sheaths of the squash bug terminated near xylem, therefore, waveform C is consistent with xylem ingestion. The characterization of these waveforms produced by the squash bug on watermelon can be used to compare the probing behavior of the squash bug when it feeds on different host and non host plants to determine host acceptability by this insect.

Keywords: EPG, Squash bug, watermelon, *Serratia marcescens*, cucurbit yellow vine disease, feeding behavior

Introduction

Cucurbit yellow vine disease (CYVD) is a destructive disease of cucurbits in the major cucurbit growing regions in the United States. CYVD was first observed in central Texas and Oklahoma in squash and pumpkin (Bruton et al. 1995). In watermelon, it was first observed in the field near Rush Springs, OK, causing total loss in some fields but only a few patches of vine yellowing in others (Bruton et al. 1998). CYVD can cause losses <5 -100%, equivalent to several hundred thousand dollars of grower income annually (Bruton et al. 2003). Infected plants show symptoms of stunting and yellowing, followed by gradual decline of the vines 7-14 days prior to harvest. The causal organism of this disease is a bacterium, *Serratia marcescens (Sm)*, which resides in the phloem of

infected plants, causing a honey brown discoloration of phloem tissue by the invasion of secondary microorganisms (Bruton et al. 2003).

S. marcescens is transmitted among cucurbits by the squash bug, *Anasa tristis* DeGeer, which is one of the more serious pests of cucurbits (Bruton et al. 2003). It attacks pumpkin, squash, watermelon, cantaloupe and cucumber and causes damage to the plant by penetrating plant tissues with its stylets, sucking the sap from xylem tissue and blocking xylem vessels with salivary sheath saliva (Neal 1993). High squash bug density is associated with reduced plant growth and plant death (Edelson et al. 2002). The mechanisms of squash bug feeding are still not clear but Miles and Taylor (1994) proposed that coreids suck the sap from phloem by a mechanism called osmotic pumping, which gives the insect indirect access to phloem sugars without piercing the sieve elements.

Electrical penetration graph (EPG) is an advanced technology used to study the probing behavior of piercing and sucking insects. It records waveforms when the insect comes in contact with the electrified plant or artificial diet chamber. Different waveforms are the result of measurable changes in resistance when fluids of varying conductivity pass through the insect mouthparts during probing (Cline and Backus 2002). EPG is used to study the stylet activities of a probing insect (Backus et al. 2005, Joost et al. 2006) and also to identify the proper time and place for pathogen acquisition and inoculation to and from the plant (Wayadande and Nault 1993, Lett et al. 2001). It has been used also to screen for plant resistance by comparing the waveforms produced by an insect probing on susceptible and resistant lines (Calderon and Backus 1992, Caillaud et al. 1995). EPG was used to record probing behavior of many Homopterans (Tjallingii 1978, Wayadande

and Backus 1989, Rapusas and Heinrichs 1990, Van Giessen and Jackson 1998) and a few Heteropterans (Bonjour et al. 1991, Joost et al. 2006, Backus et al. 2007). Bonjour (1991) recorded 1st instar squash bug feeding behavior using an old battery-operated monitor on three dual-pen strip-chart recorders, but these waveforms lacked detail. New EPG technology has the ability to expand and compress the waveforms with less background noise, greater stability and finer detail (Backus and Bennett 1992). This study uses the latest EPG technology to characterize the waveforms produced by squash bug feeding on watermelon.

Materials and Methods

Plants

Watermelon var. "Royal Sweet" (a CYVD - susceptible cultivar) was seeded in 4.5 in. diameter plastic pots (Smurfit Stone Container Corporation, Jefferson, OH) containing Sungrow Metromix 200 (Sun Grow Horticulture Products, Belleview, WA) potting soil in a greenhouse maintained at 80-90^{o}F. Plants were fertilized using Miracle-Gro ™ (Stern's Miracle-Gro Products Inc., Port Washington, NY) as directed by the manufacture every seven days. Plants were used at the 4-5 true leaf stage.

Insects

A squash bug colony was maintained in an insect rearing room at Oklahoma State University at 70-80^{o}F with a 16:8 (L:D) photoperiod. Bugs were reared on *Cucurbita pepo* var. "Lemon Drop" and supplemented with squash fruit purchased at a local grocery store. The colony was started in 2007 with feral squash bugs collected from squash plants in Perkins and Coyle, Oklahoma. The eggs were collected and transferred to separate

metal cages (8” x 12” x 12” with mesh screening). Resulting nymphs were used to supplement the colony.

Electrical penetration graph recordings

Fourth and fifth instar squash bug nymphs of undetermined sex were starved for 24 hours prior to use. The insects were tethered at the pronotum with a 0.025 inch diameter gold wire (Sigmund Cohn, Mount Vernon, NY), attached to the pronotum using silver print paint (Ladd Research Industries, Burlington, VT). Each insect was restrained to the table surface using paper tape placed across the head and the abdomen of the insect, during tethering process in order to facilitate attachment of the wire to the insect. Each tethered bug was then placed into a single head-amplifier receptacle via a brass nail glued to copper wire. Insect waveforms were recorded at 60-70^{o}F under constant light conditions, using an alternating current (AC) EPG “Missouri Monitor” (Backus and Bennett 1992) with four channels using Windaq/Pro+ software (Dataq Instruments Inc, Akron, Ohio). A 100-mV AC electrical current was applied to the test plant via an electrode inserted into the rhizosphere. A 10^{8} Ω input resistance level was used with a gain of 1 x 2000 25%. Data output was digitized using a DI-720 analog-to-digital board (DATAQ® Instruments, Inc., Akron, Ohio) with a 16-bit resolution and 100-Hz sampling rate per channel before sending signals to a Dell Latitude computer. Plants were adjusted so that the bug had easy access to the abaxial surface of the leaf. All recordings were done for 20 hours inside a Faraday cage (2’ X 2’ X 4’, constructed of an aluminum frame with a steel base and wire mesh). Once recordings were complete, the plant and insect were discarded.

Histology

To correlate the stylet tip termination points with the suspected ingestion waveform, probed plant tissues were histologically sectioned. When a specific waveform was produced, the monitor was turned off and the feeding site was marked with a fine tip permanent marker. Then the bug was forced to pull out its stylets using paint brush and the small piece of probed tissue was excised using a sharp razor blade. The tissues were transferred into an ELISA plate well, submerged in 2% glutaraldehyde in 0.1M phosphate buffer, and stored at -20^{o}C. The tissues were subjected to a standard ethanol dehydration sequence and embedded in Paraplast wax (Oxford Labware, St. Louis, MO). The embedded tissues were then sectioned at 10 µm using an A20 rotary microtome (American Optics Buffalo, NY) and heated fixed on the glass slides. The sectioned tissues were then dewaxed and stained using 0.5% safranin and 0.1% fast green and a coverslip was attached using Permount (Fisher Scientific, Pittsburgh, PA). Tissues were examined using an Olympus BX51 compound microscope at 200X and 400X magnification and digital images were captured using an Olympus DP7000 digital camera.

Measurement and Data Analysis

Waveforms were measured and analyzed using WinDaq waveform browser 2.61 software (DATAQ® Instruments, Inc., Akron, Ohio) and means of each parameter (described later) were calculated. For measurement of waveform voltages and frequency of peaks during ingestion, 24 systematically selected waveforms were measured, at least two from each insect recorded (N =12).

Waveform characterization

Primary, secondary and tertiary waveform structures were described using the convention of Cline and Backus (2002). Insect posture was also noted by direct observation of the squash bug during probing and non probing activity. The ingestion waveform produced by the squash bug was correlated with its stylet's termination point inside the plant tissue.

Waveform Parameters

Four non sequential parameters were considered for describing the waveforms.

1. Duration of waveform event: Total duration of waveform events was calculated and means were obtained by dividing the total duration by 12 insects.

2. Frequency of occurrence of waveforms: For each type of waveform, frequency was calculated by dividing the total numbers of each waveform event by 12.

3. Waveform voltage: For each type of waveform event, the voltage was calculated and the mean was derived from the data. A total of 24 waveform event was selected from the middle of the 20 hr recording period and the voltage was measured from the midpoint of each waveform event.

4. Number of peaks per unit time during ingestion: During ingestion, the number of waveform peaks produced per unit time was calculated. A total of 24 ingestion waveforms were systematically selected from the middle of the 20 hrs recording and number of peaks per second of each ingestion waveform was calculated.

Results

Overall description of probing activity on watermelon

A typical, highly compressed EPG waveform sequence produced by a squash bug is shown in Fig.3.1. This nine hour sequence includes non probing, short duration test probes and ingestion probes. At this level of compression, it is difficult to resolve individual waveform events. In general, the squash bug does some non probing activities when it comes in contact with the plant. They perform a few to several test probes at the beginning followed by the probes which include salivation and ingestion waveforms.

Insect posture as it relates to probing activity

Non probing activities of the squash bug were discernable both in the body posture and in the waveform. Non probing can be identified because of its irregular, high relative amplitude waveform structure. The rostrum was extended along the ventral side of the body during extended periods of non probing, except when the insect extended the rostrum without apparent stylet penetration during labial dabbing. Stylet insertion was associated with telescoping of the rostrum, which exposed the stylets from the labial sheath. During feeding, squash bug stylets are pressed into the plant at a right angle to the head (Fig.3.2).

EPG waveforms

Squash bug EPG waveforms were placed into four different categories (given a letter designation Z, A, B and C after the convention of Cline and Backus 2002) (Fig.3.3). The waveforms were assigned and interpreted based upon their similarity to those of *Lygus hesperus* (Cline and Backus 2002) and *A. tristis* (Bonjour et al. 1991) waveforms.

Non probing (Z)

Non probing waveforms occurred in both high and low relative amplitude, and displayed irregular shape and patterns. There was no consistent association of signal frequency with non-probing. These waveforms were produced by squash bugs during walking, standing/ resting, surface exploration with antenna, grasping with tarsal claws, labial dabbing and excretion activities. Non probing waveforms (Fig.3.4-I and II) were produced when insects were either resting or involved in the non probing activities mentioned above. The voltage of non probing waveforms ranged from 0 to the maximum voltage settings (variable). When the insects were resting, the voltage remained low, approximately equal to base line. When the insects moved, the voltage varied, but upper limits were raised to the maximum.

Test probes (A)

Test probes were usually of short duration, less than one minute, but sometimes lasted for more than two minutes. Test probes were the most prevalent type of probe performed by the squash bug. Squash bug test probes consisted of three component stereotypic subpatterns: 1) Stylet insertion peak, which has one or two high amplitude peaks; 2) waveforms of lower amplitude relative to the insertion peak; and 3) a single high amplitude stylet withdrawal peak (Fig.3.5).

Salivation or Pathway (B)

Waveform B, which occurred primarily in longer probes containing ingestion events, was characterized by a few high amplitude chevron type peaks that immediately followed an insertion peak (Fig.3.6-B). The average duration of waveform B was shorter

than that of ingestion waveform (C), and it was usually of uneven shape. Waveform B had higher average amplitude than the ingestion waveform, but the B waveform amplitude gradually declined after insertion of the stylets. Waveform B often occurred one or more times in a single probe. Table 3.1 shows the average voltage of waveform B.

Ingestion (C)

Waveform (C) was the waveform of longest duration during squash bug probing. It was always preceded by waveform B and had lower relative amplitude. Waveform C had a definite frequency associated with it (3-5 peaks per second or 3-5 Hz) (Fig.3.6-C). The formation and appearance of peaks was uniform. However, there was a high degree of variation in the number of, and amplitude, of ingestion peaks. The average duration, frequency and voltage are shown in Table 3.1. There was some variability in appearance of waveform C, but these different subpatterns were not individually measured.

Histology

Several leaf tissues excised during waveform C were sectioned and examined as described above. Only one tissue showed a clear view of the salivary sheath termination point (Fig.3.7-I and II) near the vascular bundle. The stained salivary sheath was terminated adjacent to a xylary vessel and not in phloem.

Discussion

Host plant acceptance by hemipterans is partitioned into a number of sequential events beginning with orientation to the plant via visual and chemical cues progressing through plant surface exploration and test probing, and culminating in sustained ingestion (Backus 1985). Although these activities are well described for aphids and leafhoppers

(Wayadande and Nault 1993, Lett et al, 2001, Backus et al, 2005), they are poorly understood in the Heteroptera. Prior to this study, only a few of true bugs had been studied comprehensively using electrical penetration graph technology. Bonjour et al. (1991) described the major EPG waveforms produced by first instar squash bug nymphs on squash seedlings based on data obtained using an older model electronic feeding monitor that recorded waveforms on three dual-pen strip-chart recorders at a chart speed of 0.5 cm/min. The strip chart recording provided fewer details about the waveforms produced and did not allow expansion and contraction of the highly compressed waveforms. Here we describe squash bug waveforms obtained from older nymphs using a newer version of the AC DC Missouri monitor with computer interface capability. The digitized and compressed waveforms were expanded horizontally and vertically to see more detail at different time scales, thus revealing more measurable information.

This work shows that squash bugs probed frequently, ingested primarily from xylem, and followed a stereotypical sequence of events while feeding on watermelon, confirming many of the observations made by Bonjour et al. (1991). Most of the waveforms produced by the squash bug resembled those of *Lygus hesperus* (Hemiptera: Miridae) as described by Cline and Backus (2002), except that they differentiated and described nine different feeding and non-feeding waveforms, while in this study only four of those waveforms were characterized for the squash bug. There were fewer waveform types for *A. tristis* compared to *L. hesperus* because Cline and Backus (2002) named several non probing activities as separate waveforms. Non-probing activities of the squash bug included several behaviors, including walking, moving, antennal surface

exploration, fecal discharge, pulling, grasping, and labial dabbing, all of which were considered as a single category for ease of analysis.

Non probing behavior comprised the predominant activity of this species on watermelon. The non-probing activities occurred frequently, both before and after probes. Some of these behaviors may have been due to the effect of the wire tether attached to pronotum, which restrained the insect's movement to certain portions of the leaf. The unusual restriction of movement may have influenced their behavior on the leaf. Pretreatment, such as handling, tethering and other manipulations during the period preceding the EPG access period, is another important factor to consider as it also could alter the normal insect behavior during EPG, increasing variation (Montllor and Tjallingii 1989). Spiller et al. (1990) showed that aphids starved during the period preceding recording had greater xylem ingestion compared to non-starved aphids. Experimenters usually reduce the stress encountered during handling and wiring by placing the insects on the rearing plant or in a petri dish for several hours before placing them on the test plant of the EPG experiment (Van Helden and Tjallingii 1993). In this case, squash bugs were not placed on a rearing plant for conditioning after wiring because if they fed during this time they would not feed afterwards during the EPG access period. Non-starved squash bugs do not readily feed when wired for EPG recordings and can survive for many days without food (personal observation).

All hemipterans electronically recorded to date produce a stereotypic waveform that is associated with salivation (Wayadande and Nault 1996, Walker 2000). The squash bugs in this study produced waveform "B" which was identical in appearance to homologous waveforms produced by other piercing sucking insects (Bonjour et al. 1991,

Wayadande and Nault 1993, Cook and Neal 1999, Cline and Backus 2002). Also termed "pathway" in the literature, this waveform represents deposition of the salivary sheath as the insect stylets are pushed deeper into plant tissue. In this study, waveform B occurred one or two times primarily in longer ingestion containing probes. The production of waveform B before ingestion waveform may be due to secretion of saliva by the insect to form a salivary sheath. However the production of waveform B after ingestion may also be due to secretion of saliva to facilitate easy withdrawal of stylets from the probing site.

Our work shows greater detail of the ingestion waveform C than was possible in the EPG studies by Bonjour et al. (1991) and Cook and Neal (1999). This waveform was interpreted as ingestion by these two previous studies, but the histology was inconclusive. Sheath termination points in six out of fifteen tissue samples were in vascular bundle in phloem or xylem or unclear. Bonjour et al. (1991) examined histological sections of the probed tissues taken during ingestion, and showed that the termination points of the salivary sheaths were in vascular tissue. Some of these termination sites were near phloem sieve elements but the diffuse nature of the squash bug salivary sheaths made it impossible to pinpoint the site with the same level of accuracy possible with aphid or leafhopper salivary sheaths. Neal (1993) conducted a more thorough histological study of squash bug salivary sheaths and concluded that this species feeds primarily from xylem. Our data is consistent with these previous studies. We correlated one sheath termination point with the C waveform that was adjacent to a xylary element.

We can now discern that waveform C has low amplitude peaks, at regular intervals, which are likely the result of cibarium pumping action. Xylem sap is under negative pressure, so insects feeding from xylem must suck strongly using large cibarial

muscles. During ingestion of sap from the xylem, the cibarial muscles contract and relax, forcing electrolyte fluid to enter the food canal and causing a charge difference between the two ends of the food canal and hence a change in resistance. This phenomenon of increasing and decreasing resistance during ingestion results in the formation of peaks in the waveform. The 3-6 Hz frequency of the peaks in waveform C is generally consistent with the frequency of xylem ingestion waveforms measured for other insects (Lett et al. 2001, Joost et al. 2006), but frequency alone is not diagnostic for tissue specific ingestion. In homopterans, honeydew droplet frequency and pH are used as indirect measures of ingestion and sap source. Unlike homopterans, however, squash bugs do not produce honeydew. Therefore, we were not able to use honeydew to distinguish xylem ingestion from alleged phloem ingestion during production of waveform C.

According to Miles and Taylor (1994), some coreids suck the plant sap (xylem and phloem) from a pool of liquid created by the enzymatic release of cellular contents (a phenomenon termed osmotic pumping). Coreid saliva contains the enzyme sucrase, which hydrolyzes sucrose into glucose and fructose, creating a sucrose gradient. This gradient causes an outflow of water and amino acids from the adjacent phloem. We attempted to identify a unique EPG waveform that might be associated with squash bug osmotic pumping, but were unable to do so. We were not able to correlate different squash bug ingestion waveforms because of recalcitrant nature of the bug

In this work, we have described four different types of squash bug EPG waveforms that are similar to those reported by Bonjour et al. (1991). We combined all non-probing (resting and non resting) activities of the squash bug into a single category, termed “Z”. All test probes were considered group “A” and salivation events before

ingestion group “B”. We combined all ingestion activities into a single group, “C”, though we recorded different subpatterns of the ingestion waveform. The variability in appearance of this waveform may be a characteristic unique to the squash bug. The data and interpretations are useful for further detailed study of this insect, and can be used in further studies to understand *S. marcescens* transmission behavior and EPG screening for *A. tristis* resistant germplasm.

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Table.3.1. Average duration, mean number and voltage of the EPG waveform events produced by squash bug nymphs (4^{th} and 5^{th} instar) feeding on watermelon.

Waveform Events	Average duration (sec) (±sd)	Average number (±sd)	Average voltage
Non Probing (Z)	44895±18904.09	160.75±113.99	Min-max
Test Probe (A)	5135±4057.06	148.75±110.40	3.80
Salivation (B)	664.6±740.40	15.25±13.36	3.78
Ingestion (C)	21302±18121.36	12.33±10.05	3.55

List of Figures

Figure 3.5 EPG waveform produced by squash bug nymph (4^{th} and 5^{th} instar) feeding on watermelon var. “Royal Sweet”. Different test probes (A): Insertion (1) and exit (3) peaks with salivation waveform (2) in between which is a general characteristic of a test probe. Each grid represents 1 sec per division (I) and 5 sec per division (II). All waveforms are measured from left to right.

Figure 3.6 EPG waveform produced by squash bug nymph (4^{th} and 5^{th} instar) feeding on watermelon var. “Royal Sweet”. Waveform showing salivation (B) and ingestion (C) waveform. Waveform in big box is a part of a 20h recording. Inset B: Peaks during salivation. Inset C: Peaks during ingestion. Each grid in big box represents 8 sec per division and 0.2 sec per division in small boxes. All waveforms are measured from left to right.

Figure 3.7 Cross section of “Royal Sweet” watermelon leaf showing a single stained salivary sheath left by a squash bug producing waveform C: I) single-branched salivary sheath stained red with Mc Bride’s stain II) next section showing the salivary sheath termination point in the vascular bundle, near a xylary element.

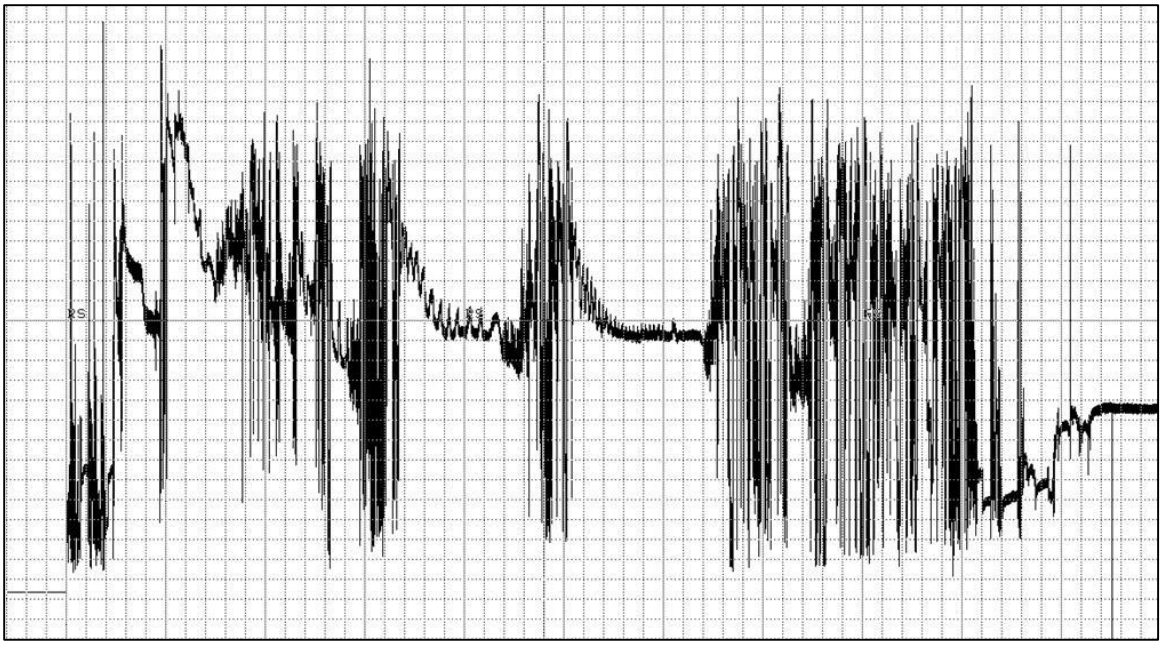

Fig.3.1

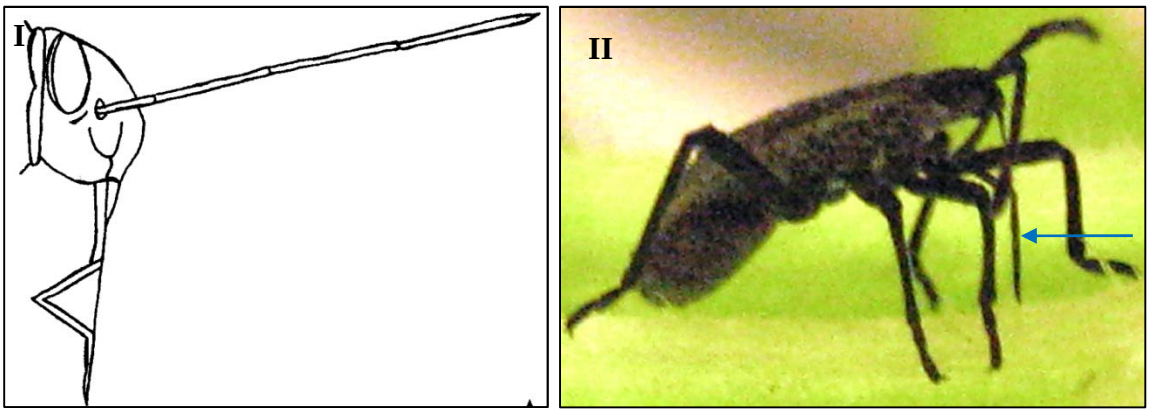

Fig.3.2

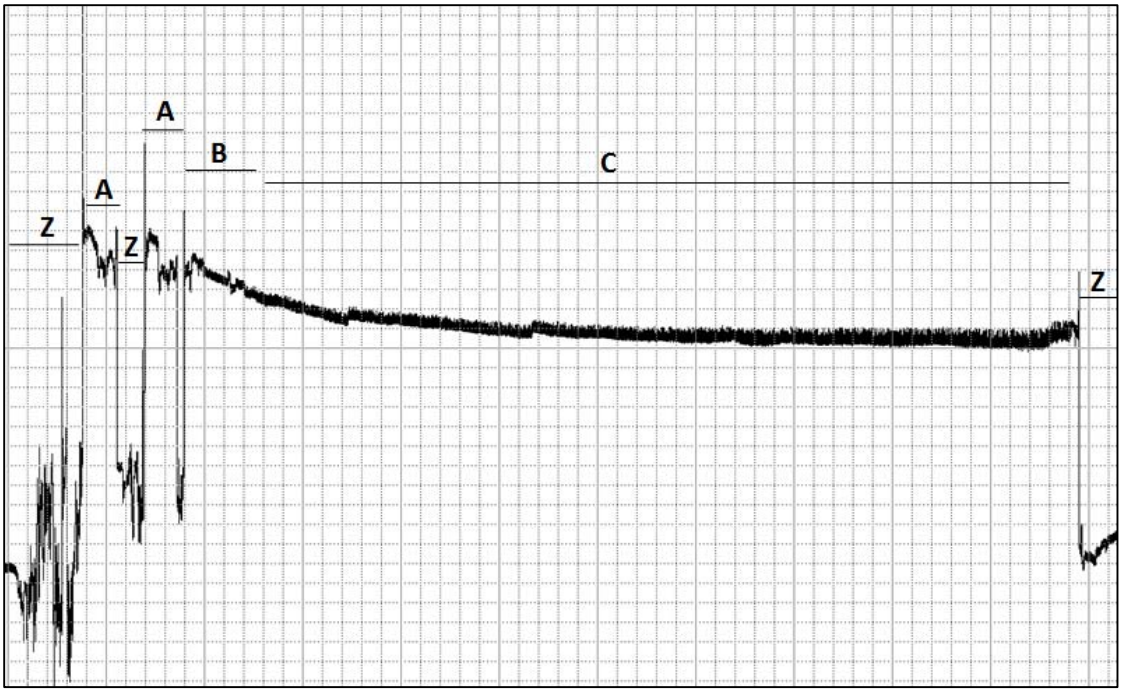

Fig.3.3

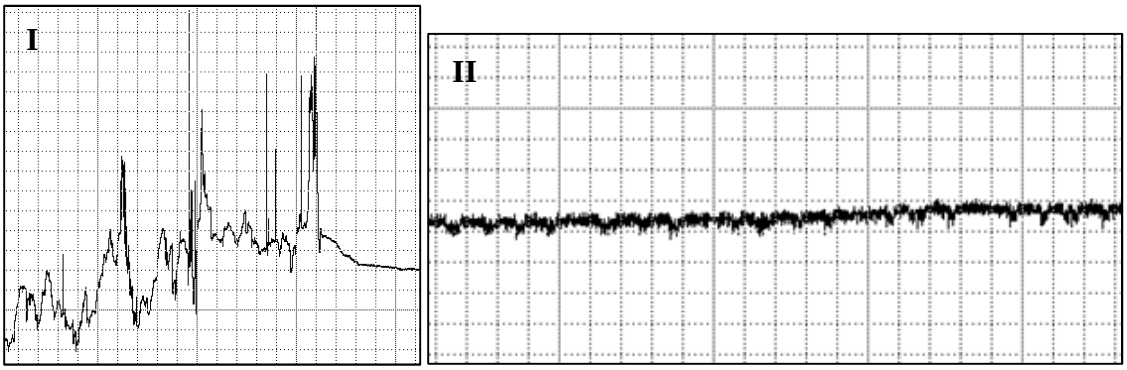

Fig.3.4

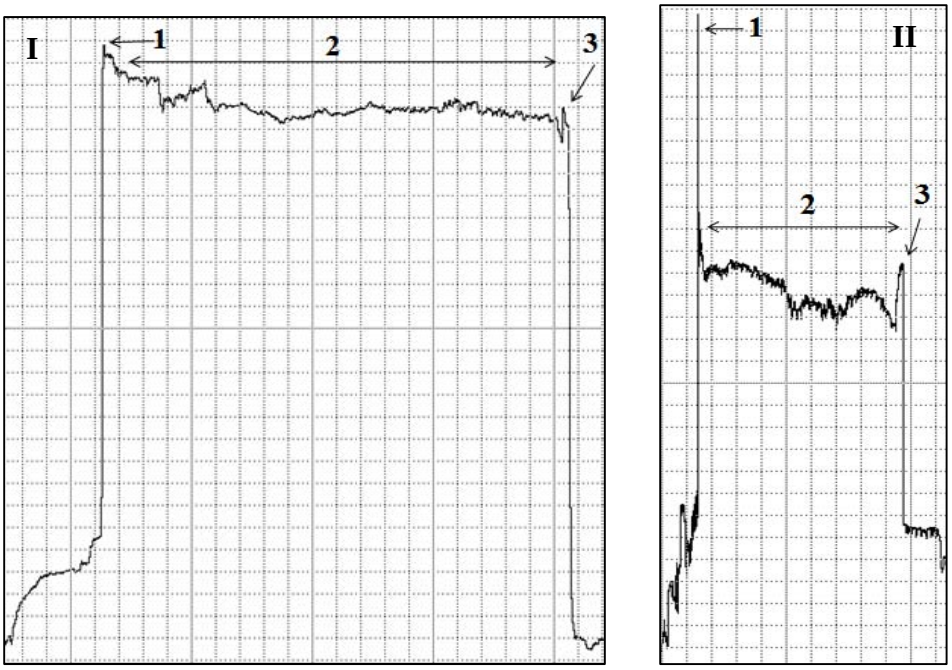

Fig.3.5

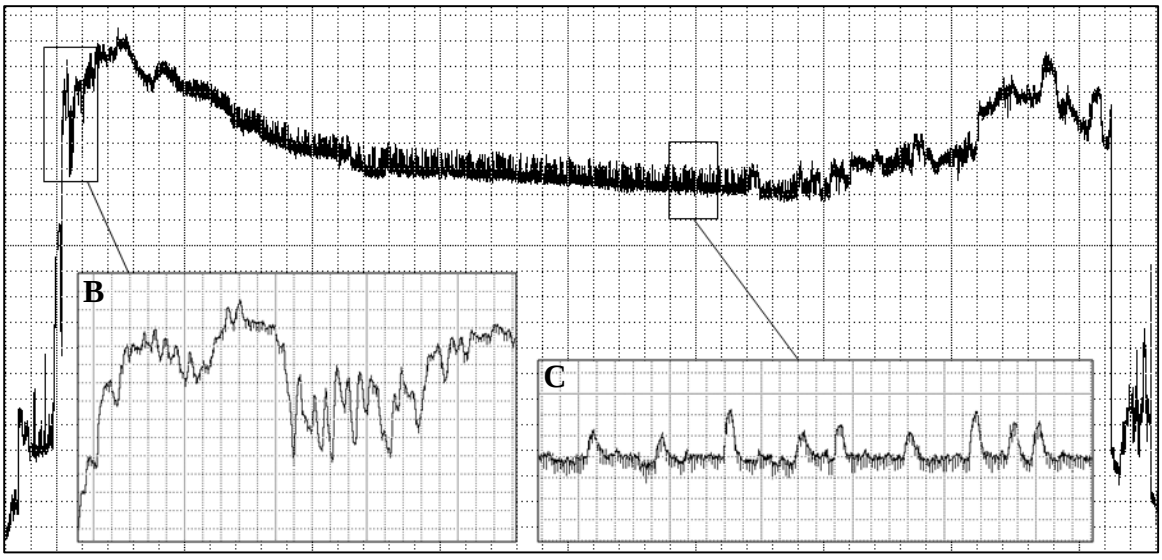

Fig.3.6

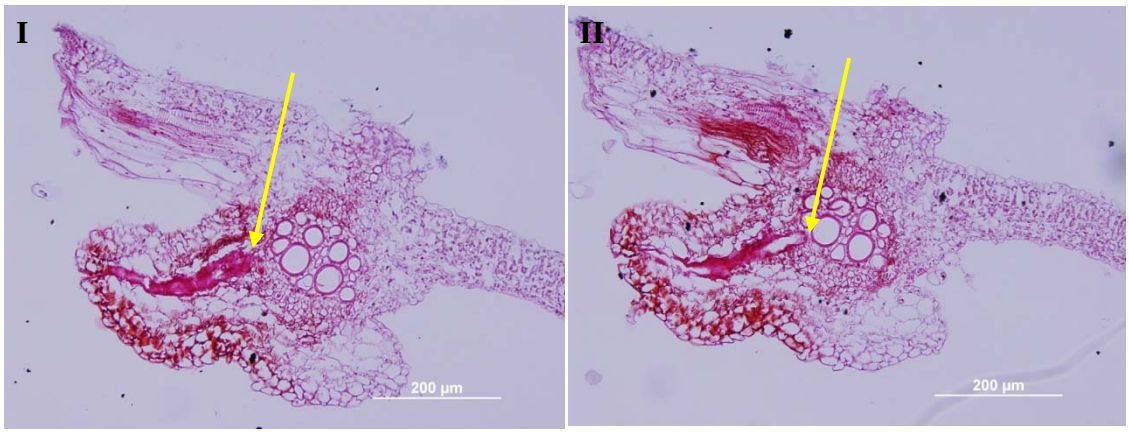

Fig.3.7

CHAPTER IV

COMPARISON OF THE EPG WAVEFORMS PRODUCED BY SQUASH BUG NYMPHS ON WATERMELON, *CITRULLUS LANATUS*, AND ITS RELATIVES, *CITRULLUS COLOCYNTHIS* AND *PRAECITRULLUS FISTULOSUS*

Abstract

Cucurbit yellow vine disease (CYVD) is a disease of cucurbits caused by the bacterium, *Serratia marcescens* and it is transmitted by the squash bug, *Anasa tristis*. The squash bug attacks most cucurbits and watermelon serves as an alternate host for this bug. Very little work has been done on host plant resistance of the squash bug on watermelon. To identify potential resistant germplasm, squash bug acceptance was compared on watermelon, *Citrullus lanatus* var. "Royal Sweet", *Citrullus colocynthis* lines, *Praecitrullus fistulosus* and a watermelon x *C. colocynthis* hybrid, USVL-200 using behavioral assays and electrical penetration graph (EPG) comparisons. Host acceptance of the squash bug was assessed by choice and no choice tests. A number of bugs chose USVL-200 followed by "Royal Sweet" but a significant number remained off of the plants. Similarly, the number of salivary sheaths on leaves during no choice test was

highest for squash var. “Lemon drop” followed by “Royal Sweet” and USVL-200. Based on EPG comparisons, there were no significant differences among the treatments in the time to first probe, first probe duration, time to first probe with ingestion or duration of first probe with ingestion probes. However, total probing duration and ingestion duration were significantly longer on “Royal Sweet” than on the other test plants. Bugs on USVL-200 and “Royal Sweet” probed more frequently than bugs on the *C. colocynthis* lines and on *P. fistulosus*. These data suggest that plant resistance factors in the latter two hosts are encountered after initiation of probing during ingestion, and are probably associated with the vascular tissue. This information can be used further for identifying the resistance factor(s) present in these plants which can be used to develop squash bug resistant watermelon lines.

Key Words: Cucurbit yellow vine disease, squash bug, watermelon, electrical penetration graph

Introduction

Cucurbits are one of the economically important crops in the United States with a total production of 109 million metric tons on 229,000 hectares, and a value of $1.43 billion in 2004 (Cantliffe et al. 2007). Watermelon, cantaloupe, squash and cucumber contribute about 9.3% of the total vegetable production value in the United States. Watermelon, cantaloupe, squash and cucumber are ranked 11^{th}, 12^{th}, 14^{th} and 15^{th}, respectively, for the total production value (Cantliffe et al. 2007). In 2009, total watermelon production in United States was 40,122,000 cwt from 126,300 harvested acres with a value of 460,778,000 US dollars (USDA 2010).

Every year economic losses in cucurbit production are attributed to diseases and pest attacks. Cucurbit yellow vine disease (CYVD) is a destructive disease causing 5 to 100% loss in production (Bruton et al. 2003). CYVD is caused by *Serratia marcescens*, a phloem colonizing bacterium transmitted by the squash bug, *Anasa tristis* (Bruton et al. 2003). The general symptoms of CYVD are stunting, yellowing, wilting and collapse of the vine and a honey brown discoloration of the phloem tissue (Bruton et al. 2003). The squash bug vector attacks most cucurbit crop species and causes damage by sucking plant sap and blocking xylem transport (Neal 1993). Seedlings are vulnerable to squash bug feeding, hence, the mortality of the seedlings is high even in relatively low densities of the squash bug adults (Edelson et al. 2002).

Bextine (2001) hypothesized that *A. tristis* transmits *S. marcescens* in a noncirculative manner but Wayadande et al. (2005) suggested, based on evidence of *S. marcescens* transmission by adults after nymphal acquisition, that the bacterium circulates throughout the body of the insect and is transmitted to its plant host via salivary secretions. In laboratory experiments, the transmission rate was 9.2% (Bruton et al. 2003). Reducing vector numbers by using insecticides resulted in a reduction in CYVD incidence (Bruton et al. 1995). Single management practices (chemical, cultural, biological, or host plant resistance) are not effective in controlling squash bugs (Zavala 1991, Olson et al. 1996) because they develop resistance to insecticides and resistant cultivars within a few generations (Margolies et al. 1998). Until now, no watermelon germplasm resistant to CYVD or the squash bug was reported, but some watermelon relatives have resistance characteristics against some disease and insects.

C. colocynthis, commonly known as Egusi (Ng 1993), bitter apple (Levi et al. 2001, Dane et al. 2007), Tumba (Sharma et al. 2004), Handal (Ageel et al. 1987), and wild gourd (Qureshi and Bhatti 2007), is a relative of watermelon and a native of tropical Africa (*C. colocynthis PI 525082* - origin Egypt and *C. colocynthis PI 386015* - origin Iran). This species is highly drought tolerant and grows on sandy soils throughout northern Africa, southwestern Asia and the Mediterranean region (Whitaker and Davis 1962, Jeffrey 1975, Whitaker and Bemis 1976). It is used as a medicinal herb due to its strong purgative impact, due to the presence of several types of alkaloids (Qureshi and Bhatti 2007). The seeds are rich in oil which is used for cooking. The seeds can also be roasted and eaten (Soliman et al. 1985). *Citrullus colocynthis* PIs are resistant to watermelon mosaic virus, watermelon vine decline disease, caused by the whitefly-transmitted squash vein yellowing virus (Kousik et al. 2009) and powdery mildew (*Podosphaera xanthii*) race 1W and moderately resistant to Fusarium wilt (*Fusarium oxysporum* f. sp. *niveum*) race 2 (Levi et al. 2001). *C. colocynthis* lines have been reported to be resistant to some arthropods, including the two spotted spider mite, *Tetranychus urticae* (Lopez et al. 2005) and the whitefly, *Bemisia tabaci* (Simmons and Levi 2002).

P. fistulosus, a distant relative of watermelon (Levi et al. 2005), is commonly known as round gourd, round melon (Dahiya et al. 2007), or tinda (Davis et al. 2007), and is popular in south Asia, especially in India and Pakistan. Its immature fruit is used as a vegetable and the seeds are also eaten. Previously named *Citrullus lanatus,* it was later placed in its own taxon due in part to its difference in monoploid chromosome number, x=12 (Sujatha and Seshadri 1989). High to moderate resistance against powdery mildew

(*P. xanthii*) race1W was reported for *Praecitrullus fistulosus* PIs (Davis et al. 2007). *P. fistulosus* also has moderate resistance to watermelon vine decline disease, caused by the whitefly-transmitted squash vein yellowing virus (Davis et al. 2007, Kousik et al. 2009). It is also resistant to the whitefly, *Bemisia tabaci* (Levi et al. 2005). These germplasm lines have not been tested for other disease and insects resistance traits and could be a source of resistance to other pests.

Plant introduction (PI) accessions of *Citrullus spp.*, *Praecitrullus spp.* and watermelon cultivars were screened for resistance to diseases and nematodes (Boyhan 1994, Thies and Levi 2003, Davis et al. 2007). Watermelon relatives can be sources of *S. marcescens* and squash bug resistance genes, which can be incorporated into commercial watermelon varieties. Electrical penetration graph (EPG) technology is a useful tool for comparing the feeding activities of piercing and sucking insects on resistant and susceptible cultivars (Calderon and Backus 1992, Annan et al. 2000). In this study squash bug responses while feeding on watermelon and several of its relatives were compared using behavioral assays and EPG recordings. These findings will help to identify resistance mechanisms and resistance genes, which can then be used to develop watermelon cultivars resistant to the squash bug.

Materials and methods

Plants

Watermelon var. “Royal Sweet”, USVL-200 (a hybrid of *C. colocynthis* PI 386015 x *C. lanatus)*, *C. colocynthis* PI 525082, *C. colocynthis* PI 386015 *P. fistulosus* PI 179660 and *Cucurbita pepo* var. “Lemon Drop” (yellow squash) were grown in 4.5 inch diameter plastic pots (Smurfit Stone Container Corporation, Jefferson, OH) containing

Sungrow Metromix 200 (Sun Grow Horticulture Products, Belleview, WA) potting soil in a greenhouse at 80-90^{o}F. Plants were fertilized using Miracle-Gro ™ (Stern's Miracle-Gro Products Inc., Port Washington, NY) every seven days. When the plants reached the 4-5 true leaf stage, they were used for EPG recordings. Seeds of *C. colocynthis* lines, USVL-200 and *P. fistulosus* were provided by Dr. Benny Bruton at USDA-ARS Lane, Oklahoma.

Insects

Insects were collected from fields near Coyle, Perkins and Terrel, Oklahoma. Feral squash bugs were reared inside aluminum cages surrounded with nylon mesh. Eggs were collected and transferred to another cages. Newly hatched nymphs were reared on squash plants (variety “Lemon drop”) and fruits (from local grocery store) at a temperature between 60-70^{o}F with a 16:8 (light: dark) photoperiod.

Behavioral Assays

These experiments were done to determine the host acceptance by the squash bug. Squash bug preference was tested by giving access to five different plants (“Royal Sweet”, *C. colocynthis* PI 525082, *P. fistulosus* PI 179660, USVL-200, and *C. colocynthis* PI 386015) in a choice test. For the no choice test, the probing behavior of the squash bug was evaluated by giving bugs access to a single plant leaf during whole experiment. In this test squash was used as a control.

Choice test

After a 24 hr starvation period, ten fourth or fifth instar squash bug nymphs of undetermined sex were released in the middle of an arena constructed of a cardboard

panel formed into a circle and surrounded by nylon mesh (Fig.4.1). After the squash bug nymphs were released, the open end of the arena was closed to prevent insect escape. Within the arena, one each of the five host plant species was placed at the edge of the circle, equidistant from the point of insect release. After 24 hours, the number of insects on each plant was counted. Insects off the plants (on the walls of the arena) were also counted. Insects not on the plant, but on the plastic pot, were counted for that plant. Each treatment was replicated eighteen times.

Data were analyzed using SAS 9.2 software. Significant differences in the mean number of bugs per plant in each treatment were determined using ANOVA, and means compared by the least significance difference test with p values at the 0.05 level.

No choice test

Individual third instar squash bugs of undetermined sex were placed onto two different leaves (2^{nd} and 3^{rd} true leaves) of four-leaf stage plants. After a 24 hr starvation period, each bug was placed inside a cylindrical cage, with a mesh window for aeration, together with the leaf for 24 hours. Bamboo sticks were used as a stand to hang the circular cages in an upright position (Fig.4.2). After 24 hr, the bugs were removed and the leaves were collected by cutting at the petiole. Excised leaves were stained for the presence of salivary sheaths using the method outlined in Backus et al. (1988). Leaves were soaked in McBride's stain (0.2% acid fuschin in 1:1 acetic acid and ethanol 95%) for 24 hours in a petri dish, submerged in a clearing solution (1:1:1 glycerin, lactic acid and distilled water) and autoclaved at 121^{o}C for 1 minute to reveal the stained salivary sheaths. After cooling, the clearing agent was removed and fresh clearing agent was

added. The leaf was examined using a Wild Heerbrugg M15 stereomicroscope (10 X - 50X) and salivary sheaths were counted.

This experiment was conducted using a completely randomized design (CRD). There were total of seven treatments including squash leaves without bugs as a negative control and squash leaves with bugs as a positive control. Each treatment was replicated 20 times (N = 40 for each treatment).

Statistical Analysis

Data were analyzed using SAS 9.2 software. Significant differences in the mean number of salivary sheaths in each treatment were determined using ANOVA and means compared using the least significance difference test with p values at the 0.05 level.

EPG recordings of squash bugs on watermelon and watermelon relatives

Squash bug 4^{th} and 5^{th} instar nymphs of undetermined sex were starved for 24 hours prior to recording. Each insect was restrained to the table surface using paper tape placed across the head and the abdomen of the insect during tethering process in order to facilitate attachment of the wire to the insect. The insects were tethered at the pronotum with a 0.025 inch diameter gold wire (2.5cm long) (Sigmund Cohn, Mount Vernon, NY), using silver print paint (Ladd Research Industries, Burlington, VT). Each tethered bug was then placed into a single head-amplifier receptacle via a brass nail glued to copper wire (2.5cm long). Insect behavioral waveforms were recorded at 60-70^{o}F under constant light condition, using an alternating current (AC) EPG "Missouri Monitor" (Backus and Bennett 1992) with four channels using Windaq/Pro+ software (Dataq Instruments Inc, Akron, Ohio). A 100-mV AC electrical current was applied to the test plant via an

electrode inserted into the rhizosphere. A 10^8 Ω input resistor level was used with a gain of 1 x 2000 25%. Data output was digitized using a DI-720 analog-to-digital board (Dataq Instruments, Akron, OH) with a 16-bit resolution and 100-Hz sampling rate per channel before sending signals to a Dell Latitude computer.

Plants of each line were selected daily using a completely randomized design. Plants were adjusted so that the bug had easy access to the abaxial surface of the second or third fully expanded leaf. All recordings for each insect were done for 20 hours inside a Faraday cage (2' X 2' X 4', constructed of an aluminum frame with a steel base and wire mesh) to reduce external noise. Once recordings were complete, the plant and insect were discarded. A total of 12 insects were recorded for each treatment.

Measurement and Data Analysis

During EPG recording, the probing and non probing behavior of the squash bug were recorded in the form of waveforms using WinDaq software. These waveforms were later measured using the WinDaq waveform browser 2.61 software (DATAQ® Instruments, Inc., Akron, Ohio). This software enabled amplification and compression of waveform for ease of identification. Measured waveforms and various EPG parameters commonly used to assess host plant resistance (Van Helden and Tjallingii 2000) were tabulated in a Microsoft Excel Spreadsheet (2007). The data were analyzed in SAS 9.2 and subjected to analysis of variance (ANOVA) and a pair wise comparison using the Least Significant Difference (LSD) Test. Differences at $p<0.05$ level were considered significant.

EPG Waveform Parameters

Waveform descriptions are the same as those described in Chapter III. Five sequential parameters and six non-sequential parameters (described below) were compared.

Sequential parameters

Means were generated by totaling all events or durations or numbers, then dividing by the number of insects (12). In cases in which the ingestion was not achieved by the end of the experiment (20 hr), the time to first probe with ingestion was considered to be the same as the total time of EPG recording. Therefore, 72,000 seconds or 20 hr was given as the time to reach the first ingestion in those replications in which the squash bug did not initiate ingestion during the recording period of 20 hours. Similarly, the duration of the first probe with ingestion is given as zero because there was no ingestion recorded during the 20 hour access period.

Time to first probe: Average time between initial access and initiation of the first probe. Sum of time required for an individual bug to initiate first probe in each treatment divided by the number of replication or number of insect (N=12) per treatment.

Duration of first probe: Average duration of first probe per insect per treatment. Sum of duration of first probe in each treatment divided by the number of bugs per treatment

Time to first probe with ingestion: Average time between initial access and initiation of first probe with ingestion. Sum of time required for an individual bug to initiate first probe with ingestion in each treatment divided by the number of insects per treatment.

Duration of first probe with ingestion: Average duration of the first probe with ingestion

per insect per treatment. Sum of duration of first probe with ingestion in each treatment divided by the number of bugs per treatment

Distribution of test probes per ingestion probe: Average number of test probes per ingestion probe per insect per treatment. Sum of average test probe (A) per each ingestion probe (C) in each replication divided by number of bugs.

Example: Average A per C is calculated by dividing total number of A per replication by total number of C in that replication. Average A per C in rep 1 is 10 and rep 2 is 8, then proportion of A per each C per treatment will be the average of 10 and 8 i.e. 9.

Nonsequential parameters

Means were generated by adding duration or number of events per insect then dividing by the number of insects (12). To avoid calculation errors during measuring mean waveform events duration and number, we included the waveforms that were artificially ended. This will result in the underestimation of the mean duration. According to Walker (2000), the level of underestimation of the mean duration by including the last artificially ended waveform is lower than the level of underestimation that would occur if the last occurrence was excluded from the calculations.

Duration of individual waveform events: Average duration of waveform events (A, Z, B or C) per insect per treatment. It is calculated by adding the total duration of each type of waveform in each replication and dividing by the number of bugs per treatment.

Example: Total duration of waveform Z (sum of all Z waveform in one rep) in rep 1 is 100 sec and rep 2 is 200 sec, then the duration of Z per treatment is average of 100 sec and 200 sec i.e. 150 sec.

Probing duration: Average probing duration (A+B+C) per insect per treatment. Duration of all the activities between stylets insertion and exit are regarded as probing duration. Probing duration is calculated by adding all the probes from each replication and dividing the sum by the number of bugs or number of replication per treatment.

Number of probes (frequency of probes): Average number of probes (A+C) per insect per treatment. We considered the sum of the test probe number (A) and the sum of the ingestion probe number (C) as the number of total probes. B is not included in the calculation because B and C occur within a single probe.

Results

Squash bug preference test

Squash bug nymphs were subjected to choice and no choice tests to determine whether they showed preference for any of the watermelon relatives and, if given no choice, whether they would feed anyway. In the choice test, the squash bugs were more attracted to USVL-200 than to "Royal Sweet", *C. colocynthis* lines and *P. fistulosus* (Fig.4.4) as indicated by higher bug numbers. Squash bug numbers on "Royal Sweet" were significantly higher than those on *C. colocynthis* PI 525082. The number of squash bugs found off of the plant was significantly higher than on "Royal Sweet", *P. fistulosus* and *C. colocynthis* lines.

Squash bug no choice test

Salivary sheaths were easily observable after staining and clearing (Fig.4.3). In the no choice test the squash bug salivary sheath number was significantly higher in squash leaves than in the other treatments (Fig.4.5). “Royal Sweet” and USVL-200 had significantly higher numbers of salivary sheaths than *C. colocynthis* lines and *P. fistulosus*. No salivary sheaths were observed on the negative control plants.

Comparison of sequential EPG parameters

The EPG parameters for all five treatments were compared and results are shown in Fig.4.6-4.13. The squash bugs took less time to initiate the first probe on “Royal Sweet” and USVL-200 than on the *C. colocynthis* lines and *P. fistulosus*, but the differences were not significant (Fig.4.6). First probes did not differ statistically in duration among the treatments, but this may have been due to the high level of variation observed in probe duration (Fig.4.7). First probes were longest on “Royal Sweet”, followed by those on *C. colocynthis* PI 525082, *P. fistulosus* PI 179660, USVL-200 and *C. colocynthis PI 386015* in that order. No significance differences were detected in the time to, and duration of, the first probe with ingestion among “Royal Sweet”, USVL-200, *P. fistulosus* and *C. colocynthis* lines (Fig.4.8-4.9).

Fig.4.10 shows the proportion of test probes (A) before ingestion probes. The mean number of test probes per ingestion probe was not significantly different among the treatments but the pattern shows that squash bugs do more short probes preceding ingestion probes on *P. fistulosus*, than when on other plants.

Comparison of non sequential EPG parameters

Fig.4.11 shows the comparison of mean duration of waveform events in the five different treatments. Squash bugs spent significantly more time feeding on "Royal Sweet" than on USVL-200, *C. colocynthis* lines and *P. fistulosus*. Test probes (A) and pathway (B) were significantly longer on "Royal Sweet" than on *C. colocynthis* lines and *P. fistulosus* but indistinguishable in length from those made on USVL-200. Squash bugs also spent more time ingesting on "Royal Sweet" than on USVL-200, *P. fistulosus* and *C. colocynthis* lines. The number of test probes and pathway events was significantly higher on "Royal Sweet" and USVL-200 than on *P. fistulosus* and the *C. colocynthis* lines. Similarly, the mean number of ingestion waveforms was significantly higher when insects fed on "Royal Sweet" and USVL-200 than other treatments. Squash bugs spent significantly more time probing on "Royal Sweet" than USVL-200, *C. colocynthis* and *P. fistulosus* (Fig.4.12). Similarly, the number of probes on "Royal Sweet" was greater than on other experimental plants (Fig.4.13).

Discussion

Squash bugs, like other true bugs, feed very differently than the better-studied aphids and leafhoppers. Most sheath-feeding homopterans make a few test probes to determine host suitability, then proceed to long term ingestion, with most successful ingestion probes lasting up to several hours when the insect is ingesting from phloem sieve elements (Kimmins 1989, Wayadande and Nault 1996). Thus, fewer probes coupled with long-term ingestion are an indication of a suitable feeding source for homopterans. Previous studies of squash bug and other heteropteran feeding behavior suggest that true bugs probe much more frequently on a preferred host plant (Bonjour et al. 1991, Cook

and Neal 1999, Cline and Backus 2002) than do the homopterans. Cline and Backus (2002) reported that frequent *Lygus hesperus* test probes executed before longer ingestion probes might function to soften the plant tissue or to overcome plant defense activities. *Lygus* ingestion probes were of much shorter duration than aphid or leafhopper ingestion probes, which typically last several hours. We observed similar probing activities for squash bug feeding on watermelon. Insects initiated several test probes at the feeding site before starting an ingestion probe and the subsequent ingestion probes were, on average, 30 minutes in duration. The sequence was repeated several times as the insect moved up or down a leaf vein, confirming the findings of Bonjour et al. (1991) and Cook and Neal (1999), that repeated bouts of short test probes followed by a longer probe with short periods of ingestion is typical for this species on an acceptable plant host. This observation was further supported by the high number of salivary sheaths observed in the no-choice test control (highly preferred) squash plants. There were exceptions to this, however. A few probes lasted several hours, demonstrating that a long-term sustained ingestion strategy, previously thought to be exclusive to the Homoptera, is also used by this coreid bug.

Choice and No choice test

Host plant acceptability was reflected in the results of the two behavioral assays. Given a choice, squash bugs showed no preference for any of the plants offered, but this finding may be an artifact of the test conditions. These insects are recalcitrant feeders and it may be that a longer period of time in the testing arena would have resulted in more insects moving to the plants. Of those that did make a choice, more bugs selected the hybrid USVL-200, which is a cross between *C. colocynthis* and “Royal Sweet”

watermelon, than “Royal Sweet”, the two *C. colocynthis* lines and *P. fistulosus*. Similarly, the significantly fewer salivary sheaths left by bugs exposed to the two *C. colocynthis* lines and *P. fistulosus* suggest lower palatability of these plants. The two *C. colocynthis* lines have leathery leaves and are much tougher in texture than watermelon, USVL-200. *P. fistulosus* leaves are more succulent than those of the two *C. colocynthis* lines, but have longer trichomes.

EPG comparisons

The EPG data also reflected lower palatability of *C. colocynthis* and *P. fistulosus* in several parameters frequently used to assess resistance to homopterans (Kimmins 1989, Calderon and Backus 1992, Diaz-Montano et al. 2007). Considered together, these data suggest that, once in contact with the plant, squash bugs probed just as readily on the watermelon relatives as they did on “Royal Sweet” or the hybrid USVL-200. However, once probing was initiated, the ingestion events within these probes were shorter, and there were fewer total probes made by each insect suggesting that *C. colocynthis* lines and *P. fistulosus* either lacked additional host acceptance cues or possessed some gustatory or mechanical cue negative to prolonged and repeated feeding.

Most hemipteran insects that have been studied using EPG were shown to ingest from several different plant tissues including phloem, xylem, and mesophyll, and the duration and frequency of probing from these tissues can be used as an indicator of acceptance of the plant host (Wayadande and Backus 1989). Sauge et al. (1998) compared the feeding behavior of *Myzus persicae* on resistant and susceptible genotypes of peach and found that the resistance of the host was linked with the ingestion behavior of the aphid. They found reduced phloem sap ingestion (E2), reduced percentage of salivary secretion (E1)

into sieve elements followed by ingestion and increased number of shifts from E1 to E2 by the aphid on the resistant genotype of peach. Similar results were reported for aphids electronically recorded on resistant and susceptible genotypes of melon and alfalfa (Klingler et al. 1998, Jiang and Walker 2003). The authors all concluded that the resistance factors were not encountered as aphid stylets moved through the epidermis and parenchyma, but were likely located in the phloem sieve elements. The presence of the *Vat* gene (virus aphid transmission factor) in melons resistant to aphids has been shown to have a negative effect on probing and phloem contact (Chen et al. 1997). The observed reduction in squash bug ingestion probes and sustained ingestion on *C. colocynthis lines* and *P. fistulosus* suggests that a similar gene or genes mediates vascular tissue chemistry in these plants. The next line of inquiry should be to identify putative compounds in the watermelon relatives and the genes that regulate expression in plants.

CYVD transmission

We found similar results of some of the parameters of EPG waveform of squash bug recorded on watermelon and its relatives and *S. marcescens* transmission by the squash bug on these test plants (described in Appendix B). A low percentage of “Royal Sweet” and USVL-200 were CYVD positive whereas *C. colocynthis* PI 386015 and *P. fistulosus* PI 179660 showed no or very little *S. marcescens* transmission. The mean number of probe data paralleled the percent transmission data. Higher number of probes by a squash bug on “Royal Sweet” and USVL-200 was associated with the higher percentage of *S. marcescens* transmission by the squash bug. Similarly, the longer duration of ingestion and total probes seen on “Royal Sweet” and USVL-200 is also associated with the higher percentage of *S. marcescens* transmission by the squash bug to

these plants. Although, there was a significant difference in the mean probing duration and ingestion duration between “Royal Sweet” and USVL-200, the trend does not seem different than that of the transmission data. The mechanical inoculation of the bacteria *S. marcescens* shows that all the test plants were infected by CYVD. Therefore, resistance in *C. colocynthis* and *P. fistulosus* is not against the pathogen, *S. marcescens* but to the vector, *A. tristis*.

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List of Figures

Figure 4.5 Mean number of squash bug salivary sheath left on test plants during 24 hr access period. The bar represents standard error of the mean and $p<0.0001$. Means followed by different letters are significantly different ($p\geq0.05$).

Figure 4.6 Mean time taken by the squash bug nymphs to initiate first probe from the beginning of the 20hr EPG recording per treatment. The bar represents standard error of the mean and $p=0.16$. Means followed by different letters are significantly different ($p\geq0.05$). 4^{th} and 5^{th} instar squash bug nymphs recorded on watermelon var. "Royal Sweet", USVL-200 (a hybrid of *Citrullus colocynthis* PI 386015 x *C. lanatus)*, *C. colocynthis* PI 525082, *C. colocynthis* PI 386015 and *P. fistulosus* PI 179660 using electrical penetration graph technology.

Figure 4.7 Mean duration of first probe produced by the squash bug nymphs during 20hr EPG recording. The bar represents standard error of the mean and $p=0.5$. Means followed by different letters are significantly different ($p\geq0.05$). 4^{th} and 5^{th} instar squash bug nymphs recorded on watermelon var. "Royal Sweet", USVL-200 (a hybrid of *Citrullus colocynthis* PI 386015 x *C. lanatus)*, *C. colocynthis* PI 525082, *C. colocynthis* PI 386015 and *P. fistulosus* PI 179660 using electrical penetration graph technology.

Figure 4.8 Mean time taken by the squash bug nymphs to initiate first probe with ingestion during 20hr EPG recording. The bar represents standard error of the

mean and p=0.35. Means followed by different letters are significantly different ($p \geq 0.05$). 4th and 5th instar squash bug nymphs recorded on watermelon var. "Royal Sweet", USVL-200 (a hybrid of *Citrullus colocynthis* PI 386015 x *C. lanatus)*, *C. colocynthis* PI 525082, *C. colocynthis* PI 386015 and *P. fistulosus* PI 179660 using electrical penetration graph technology.

Figure 4.9 Mean duration of first probe with ingestion produced by the squash bug nymphs during 20hr EPG recording. The bar represents standard error of the mean and p=0.88. Means followed by different letters are significantly different ($p \geq 0.05$). 4th and 5th instar squash bug nymphs recorded on watermelon var. "Royal Sweet", USVL-200 (a hybrid of *Citrullus colocynthis* PI 386015 x *C. lanatus)*, *C. colocynthis* PI 525082, *C. colocynthis* PI 386015 and *P. fistulosus* PI 179660 using electrical penetration graph technology.

Figure 4.10 Mean number of test probe per each ingestion probe produced by the squash bug nymphs on watermelon and its relatives during 20 hr EPG recording period. The bar represents standard error of the mean and p=0.83. Means followed by different letters are significantly different ($p \geq 0.05$). 4th and 5th instar squash bug nymphs recorded on watermelon var. "Royal Sweet", USVL-200 (a hybrid of *Citrullus colocynthis* PI 386015 x *C. lanatus)*, *C. colocynthis* PI 525082, *C. colocynthis* PI 386015 and *P. fistulosus* PI 179660 using electrical penetration graph technology.

Figure 4.11 Mean duration of waveform events produced by the squash bug nymphs on watermelon and its relatives during 20 hr EPG recording period. Means followed by different letters are significantly different ($p \geq 0.05$). 4^{th} and 5^{th} instar squash bug nymphs recorded on watermelon var. "Royal Sweet", USVL-200 (a hybrid of *Citrullus colocynthis* PI 386015 x *C. lanatus)*, *C. colocynthis* PI 525082, *C. colocynthis* PI 386015 and *P. fistulosus* PI 179660 using electrical penetration graph technology. Waveform events test probe (A) ($p=0.0044$), non-probing (Z) ($p<0.0001$), salivation or pathway (B) ($p=0.056$) and ingestion (C) ($p<0.0001$) on X-axis and the mean time of each waveform events per treatment on Y-axis.

Figure 4.12 Mean duration of probing waveform (A+B+C) produced by the squash bug nymphs on watermelon and its relatives during 20 hr EPG recording period. The bar represents standard error of the mean and $p<0.0001$. Means followed by different letters are significantly different ($p \geq 0.05$). 4^{th} and 5^{th} instar squash bug nymphs recorded on watermelon var. "Royal Sweet", USVL-200 (a hybrid of *Citrullus colocynthis* PI 386015 x *C. lanatus)*, *C. colocynthis* PI 525082, *C. colocynthis* PI 386015 and *P. fistulosus* PI 179660 using electrical penetration graph technology.

Figure 4.13 Mean number of probing waveform (A+C) events produced by the squash bug nymphs on watermelon and its relatives during 20 hr EPG recording period. The bar represents standard error of the mean and $p=0.001$. Means followed by different letters are significantly different ($p \geq 0.05$). 4^{th} and 5^{th} instar squash bug

nymphs recorded on watermelon var. “Royal Sweet”, USVL-200 (a hybrid of *Citrullus colocynthis* PI 386015 x *C. lanatus), C. colocynthis* PI 525082, *C. colocynthis* PI 386015 and *P. fistulosus* PI 179660 using electrical penetration graph technology.

Fig.4.1

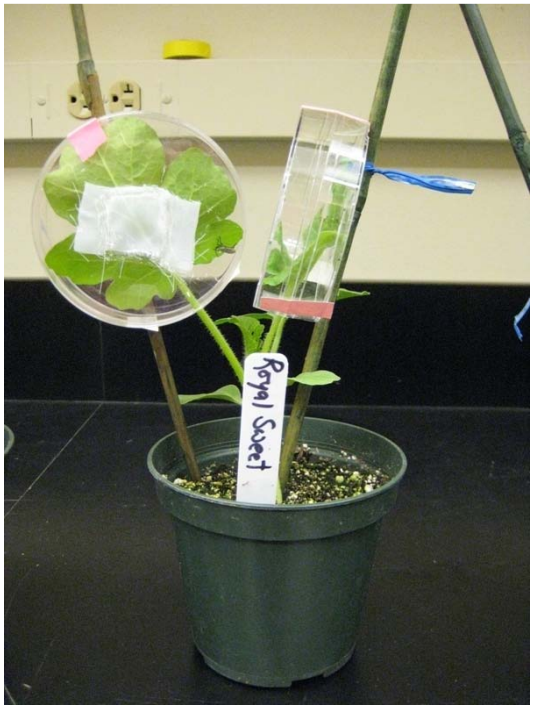

Fig.4.2

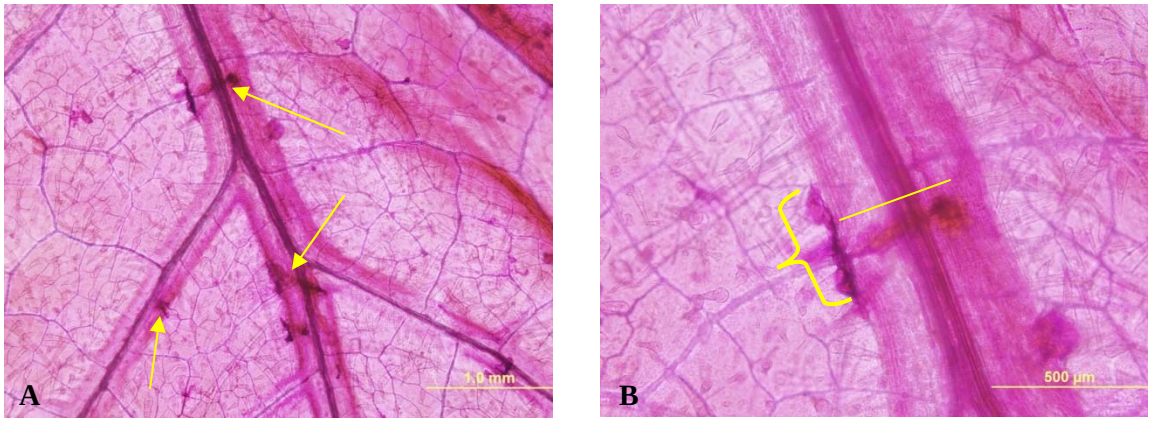

Fig.4.3

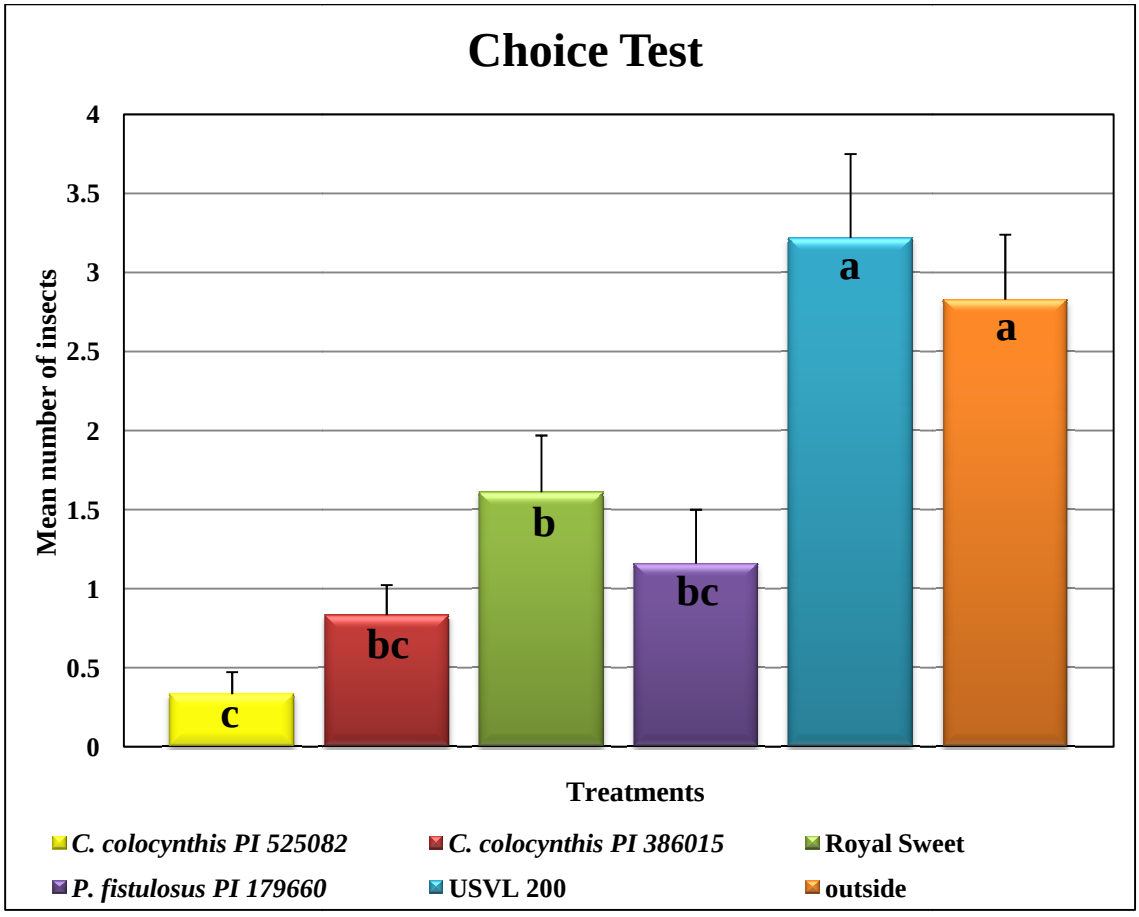
Choice Test
Mean number of insects
4
3.5
3
2.5
2
1.5
1
0.5
0
c
bc
b
bc
a
a
Treatments
C. colocynthis PI 525082
C. colocynthis PI 386015
Royal Sweet
P. fistulosus PI 179660
USVL 200
outside

Fig.4.4

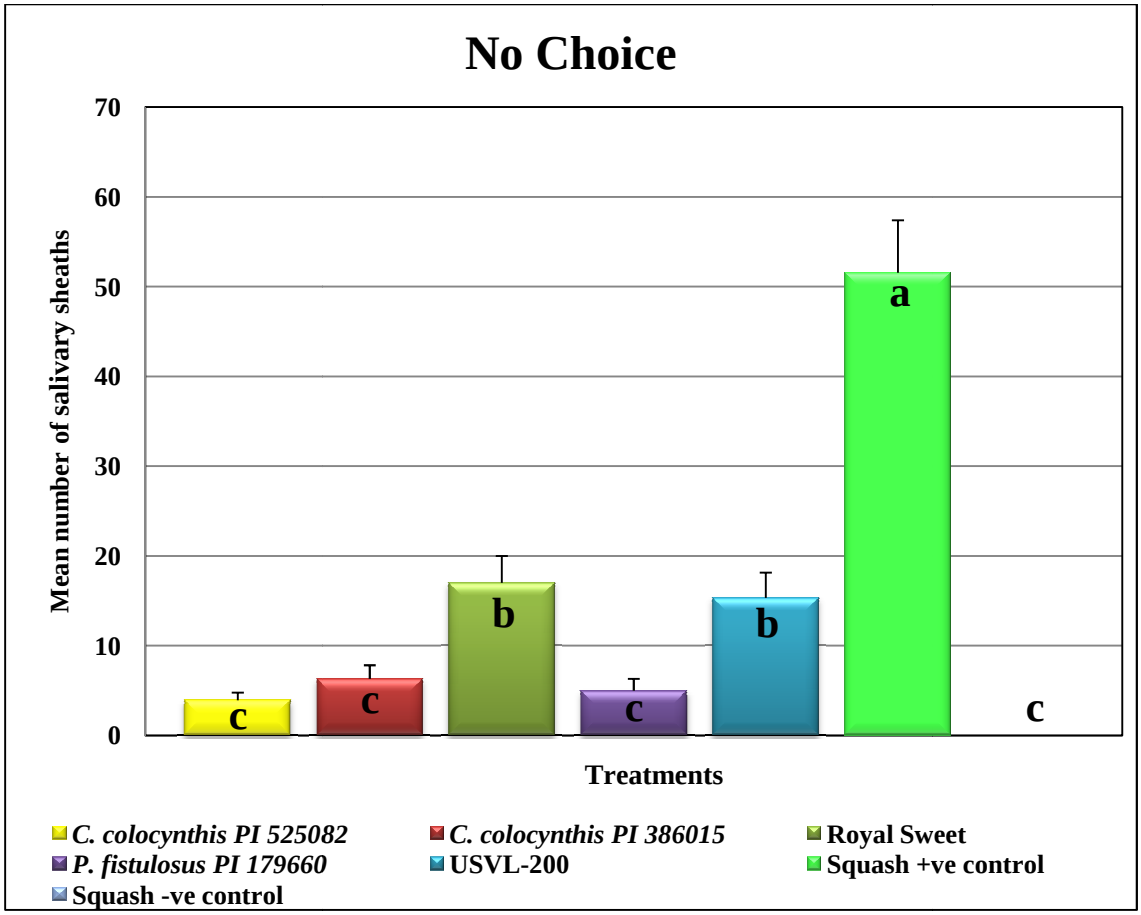
No Choice
Mean number of salivary sheaths
70
60
50
40
30
20
10
0
c
c
b
c
b
a
c
Treatments
C. colocynthis PI 525082
C. colocynthis PI 386015
Royal Sweet
P. fistulosus PI 179660
USVL-200
Squash +ve control
Squash -ve control

Fig.4.5

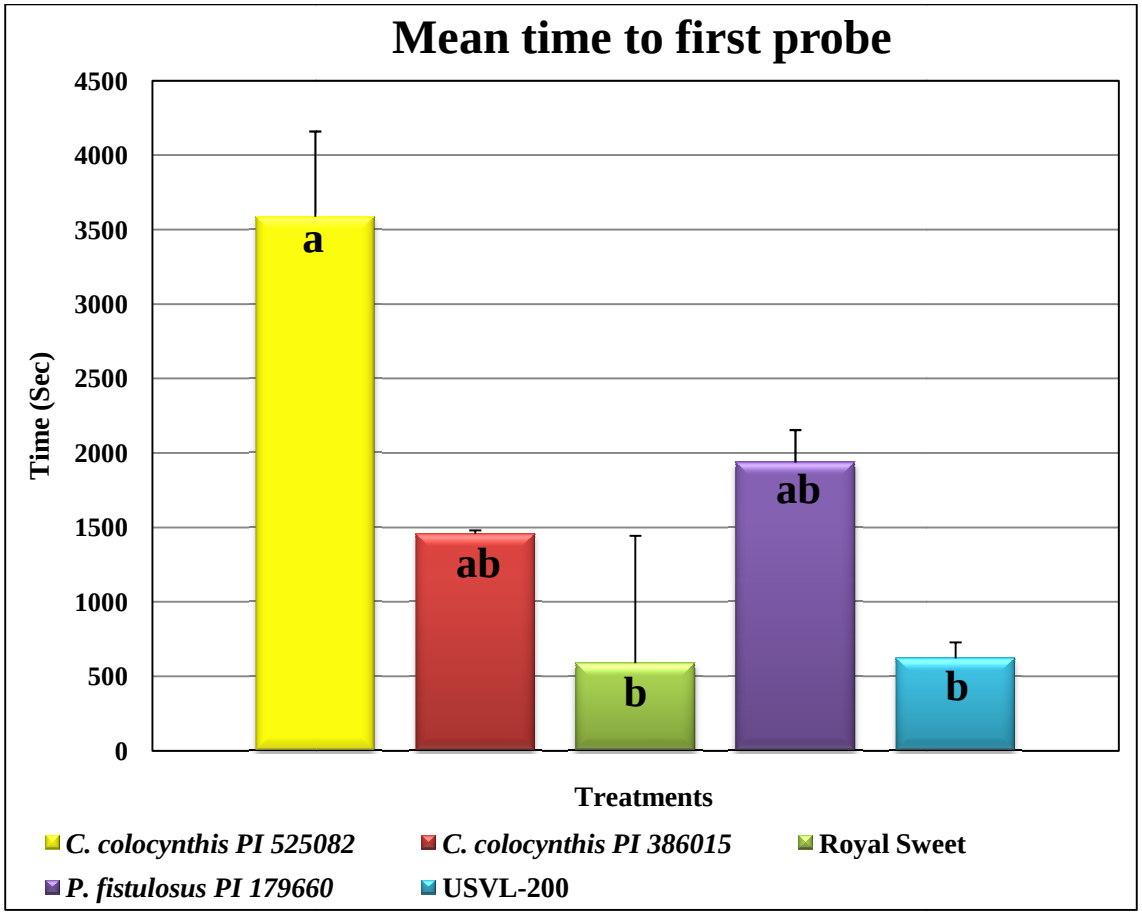
Mean time to first probe
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Time (Sec)
a
ab
b
ab
b
Treatments
C. colocynthis PI 525082
C. colocynthis PI 386015
Royal Sweet
P. fistulosus PI 179660
USVL-200

Fig.4.6

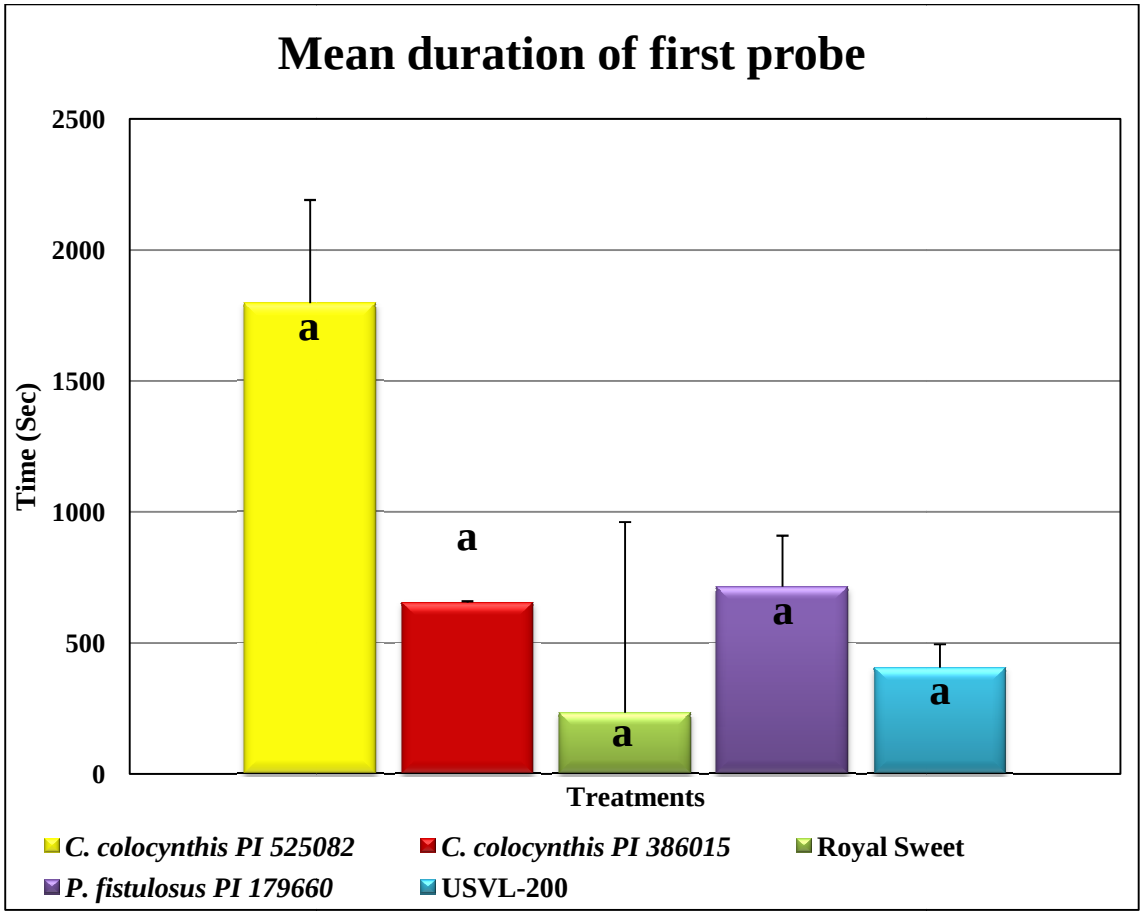
Mean duration of first probe
2500
2000
1500
1000
500
0
Time (Sec)
a
a
a
a
a
Treatments
C. colocynthis PI 525082
C. colocynthis PI 386015
Royal Sweet
P. fistulosus PI 179660
USVL-200

Fig.4.7

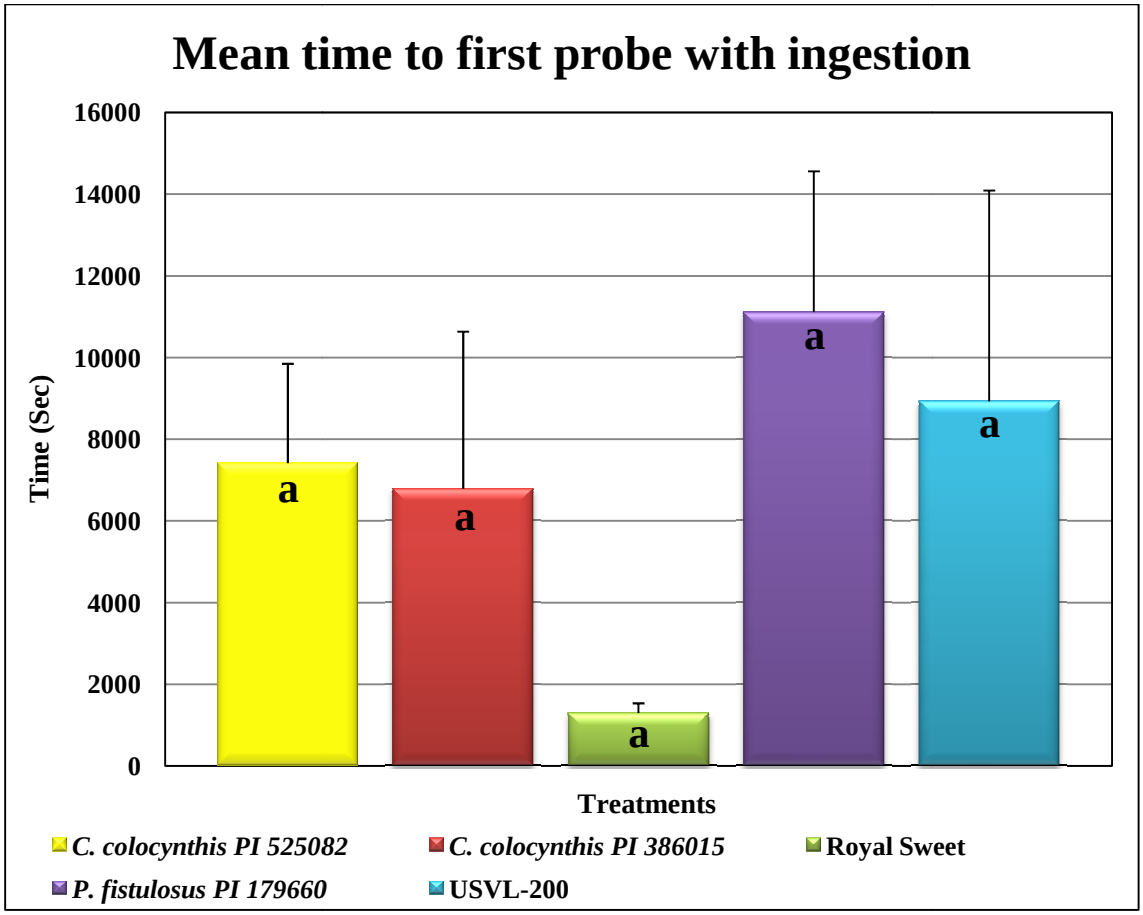
Mean time to first probe with ingestion
16000
14000
12000
10000
8000
6000
4000
2000
0
Time (Sec)
a
a
a
a
a
Treatments
C. colocynthis PI 525082
C. colocynthis PI 386015
Royal Sweet
P. fistulosus PI 179660
USVL-200

Fig.4.8

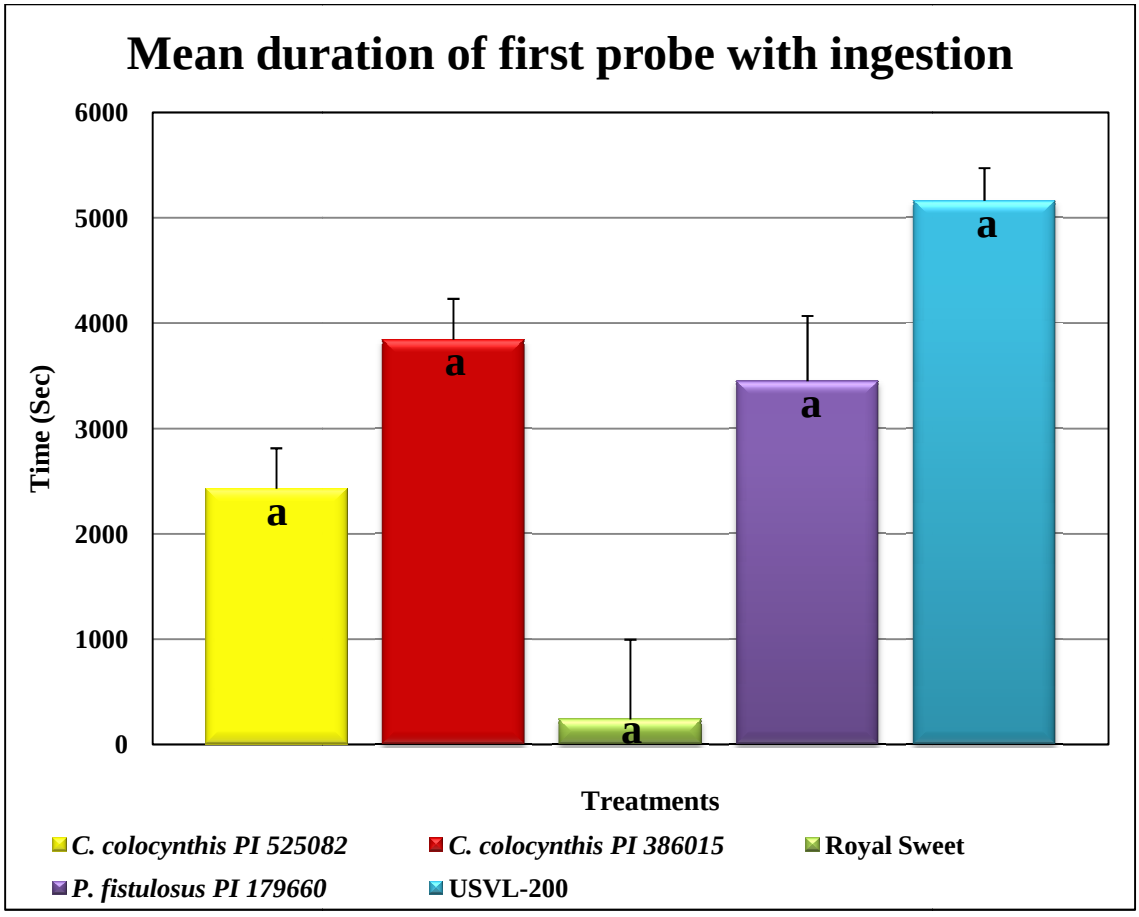
Mean duration of first probe with ingestion
6000
5000
4000
3000
2000
1000
0
Time (Sec)
a
a
a
a
a
Treatments
C. colocynthis PI 525082
C. colocynthis PI 386015
Royal Sweet
P. fistulosus PI 179660
USVL-200

Fig.4.9

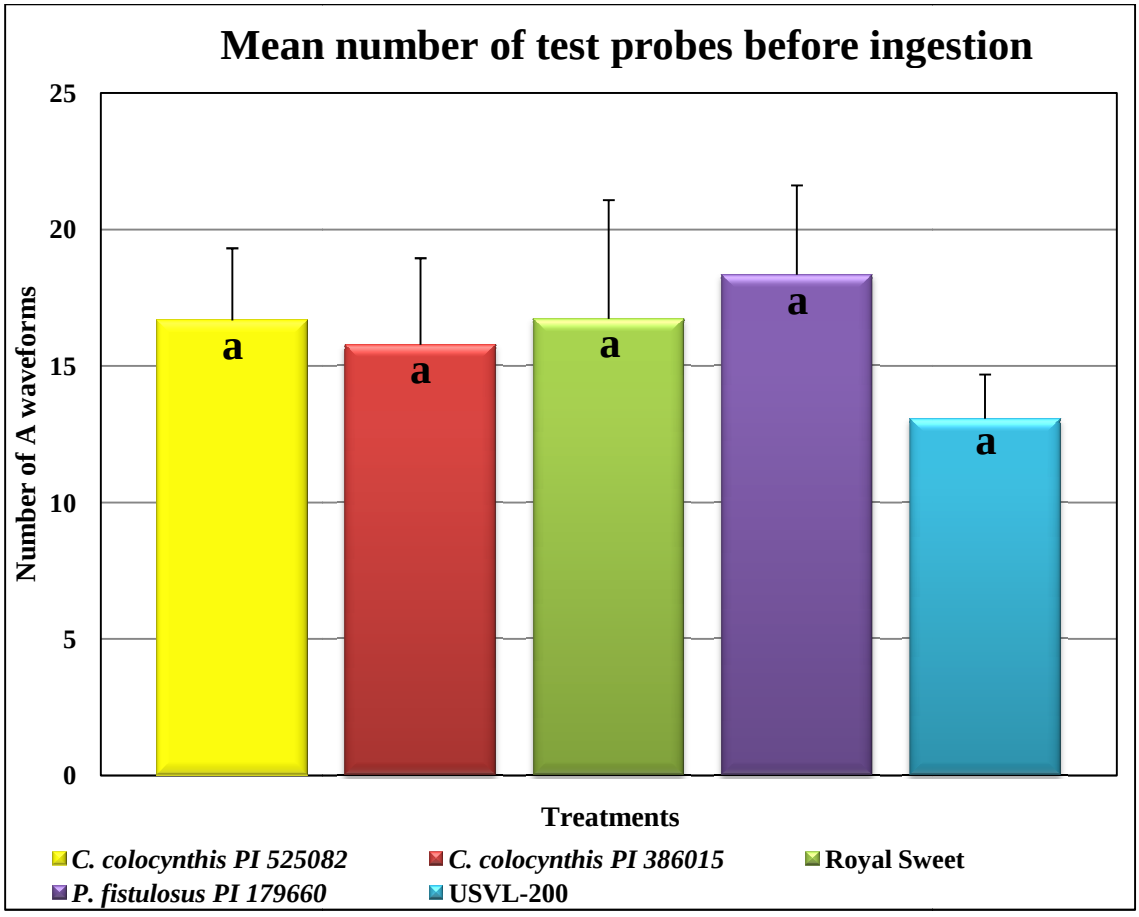
Mean number of test probes before ingestion
Number of A waveforms
25
20
15
10
5
0
a
a
a
a
a
Treatments
C. colocynthis PI 525082
C. colocynthis PI 386015
Royal Sweet
P. fistulosus PI 179660
USVL-200

Fig.4.10

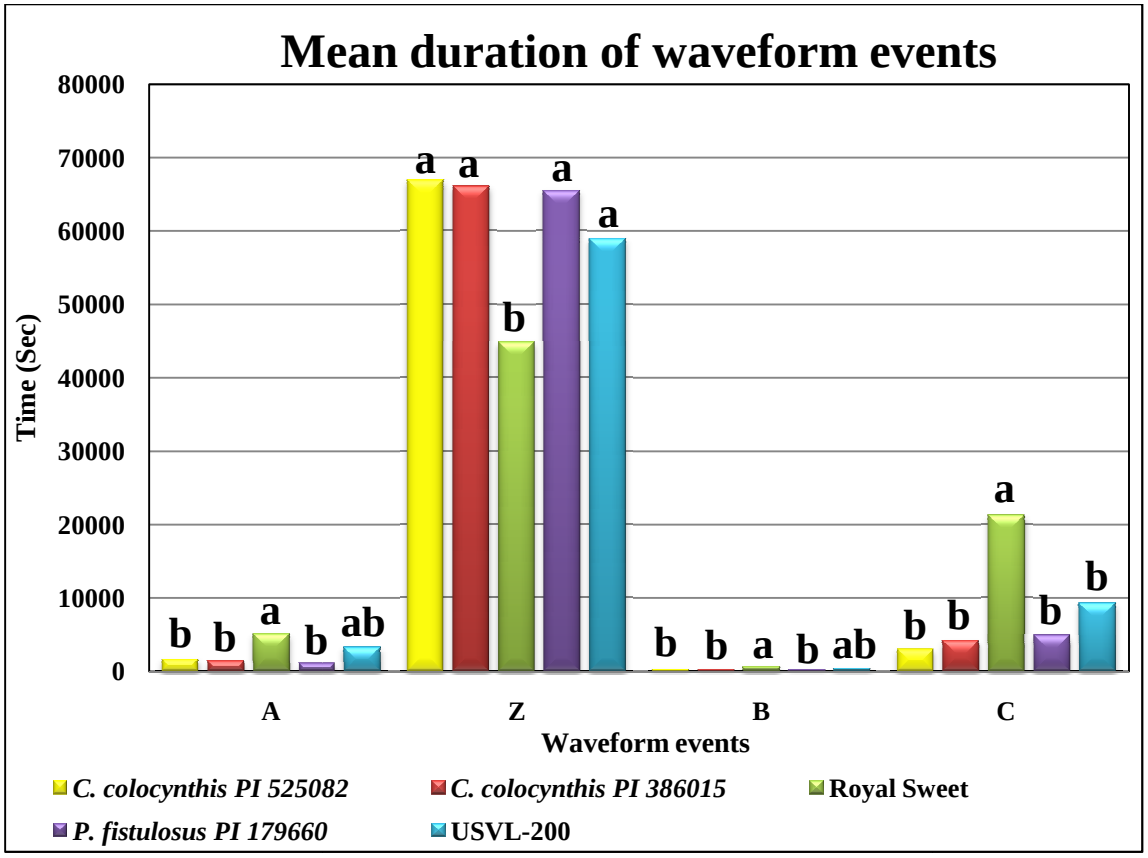
Mean duration of waveform events
Time (Sec)
80000
70000
60000
50000
40000
30000
20000
10000
0
b b a b ab
a a b a a
b b a b ab
b b a b ab
A
Z
B
C
Waveform events
C. colocynthis PI 525082
C. colocynthis PI 386015
Royal Sweet
P. fistulosus PI 179660
USVL-200

Fig.4.11

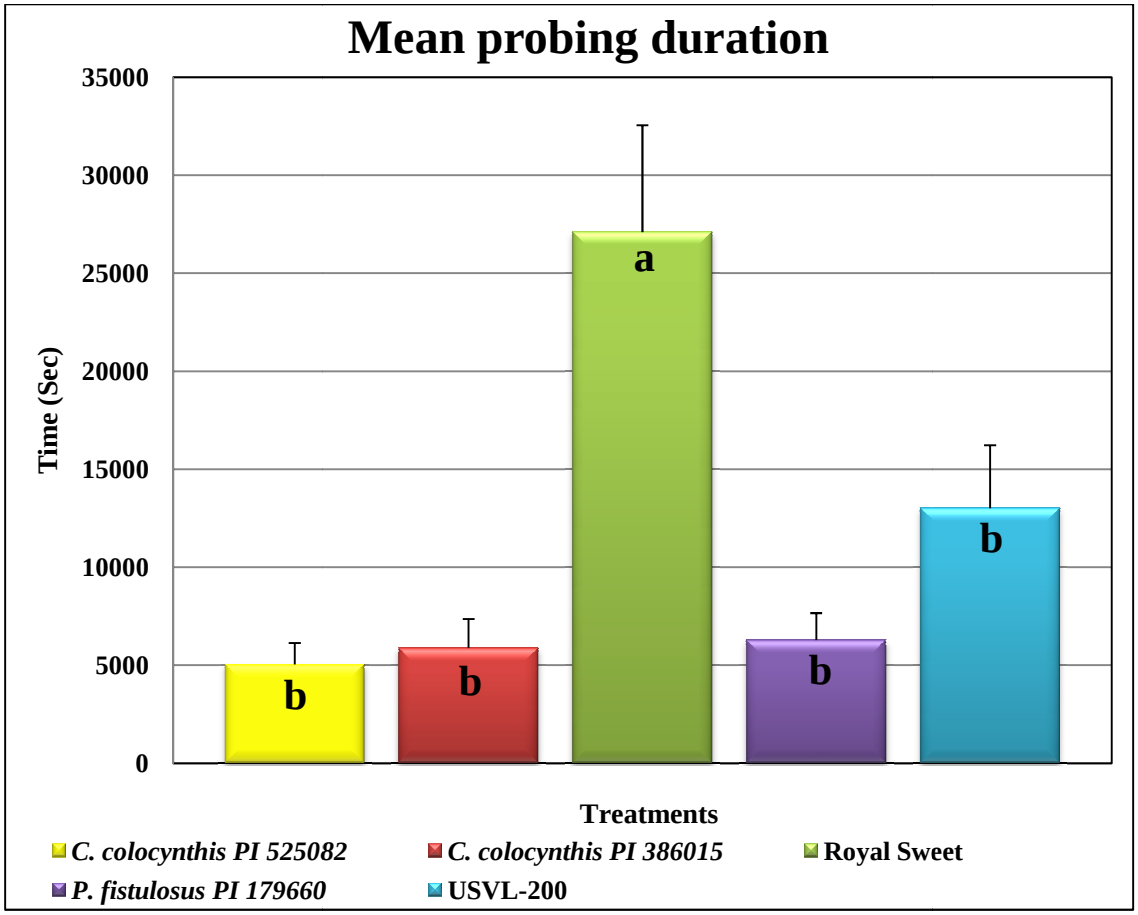
Mean probing duration
35000
30000
25000
20000
15000
10000
5000
0
Time (Sec)
a
b
b
b
b
Treatments
C. colocynthis PI 525082
C. colocynthis PI 386015
Royal Sweet
P. fistulosus PI 179660
USVL-200

Fig.4.12

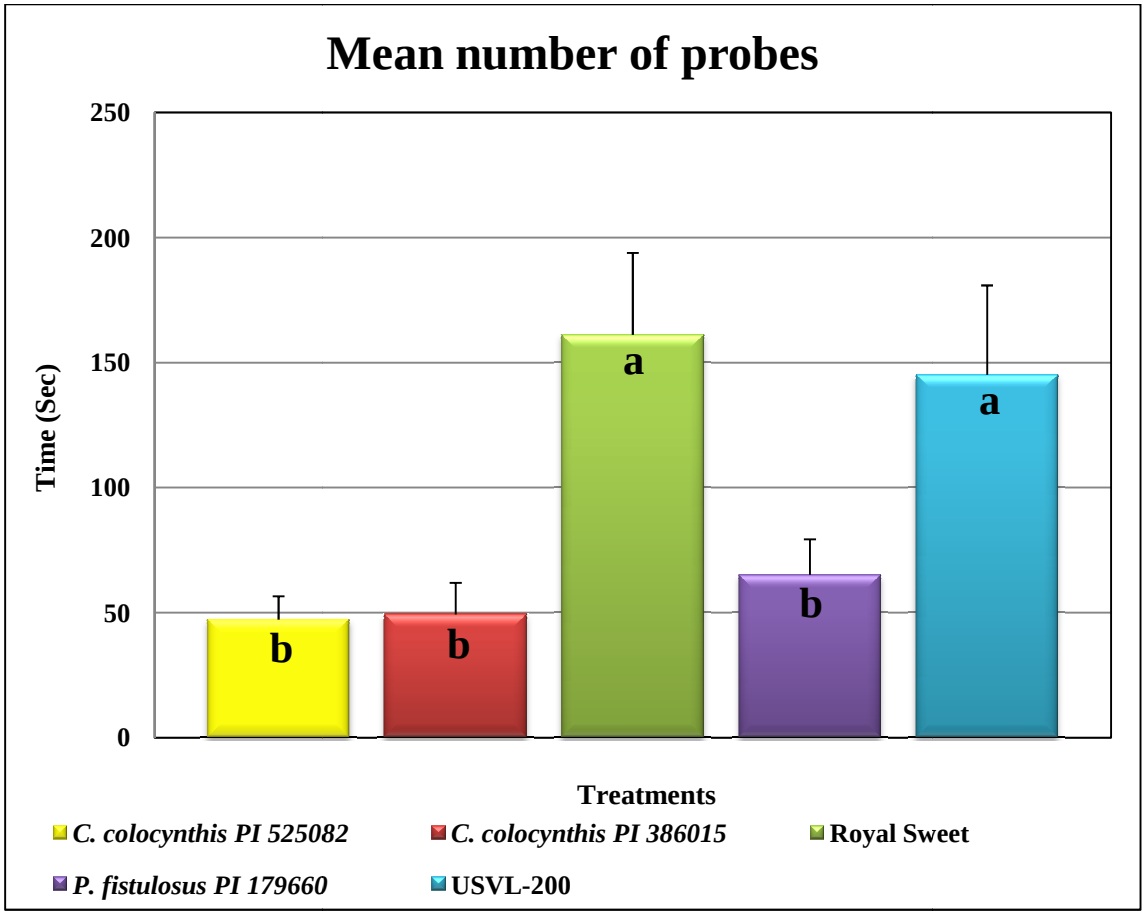
Mean number of probes
250
200
150
100
50
0
Time (Sec)
b
b
a
b
a
Treatments
C. colocynthis PI 525082
C. colocynthis PI 386015
Royal Sweet
P. fistulosus PI 179660
USVL-200

Fig.4.13

Chapter V

Epilogue

In this study, I characterized the probing and non probing behavior of fourth or fifth instar squash bug (*Anasa tristis*) nymphs on watermelon var. “Royal Sweet”, using an advanced electrical penetration graph (EPG) technology which converts unobservable stylet activities of an insect into measureable waveform patterns. I compared squash bug probing and non probing EPG waveforms on watermelon with the waveforms produced on watermelon relatives *Citrullus colocynthis*, *Praecitrullus fistulosus* and the hybrid USVL-200. The EPG waveforms produced by the squash bug nymphs on watermelon were divided into four different categories: 1. Test probes (A) 2. Non probing (Z) 3. Salivation or pathway (B) and 4. Ingestion (C). The waveforms observed during this study were similar to those of first instar squash bug nymphs obtained with a battery-operated monitor on three dual-pen strip-chart recorders (Bonjour et al. 1991 and Cook and Neal 1999). Using the convention of Cline and Backus (2002) to describe *Lygus hesperus* waveforms, we assigned the letters to each of these waveform events. I combined all the non probing activities in a single group “Z” and all ingestion probing into a single group “C” for the ease of analysis. I found that squash bugs did several test probes before an ingestion probe, and concluded that the function of these test probes

might be to soften the tissues or overcome the plant defensive response by the squash bug during feeding. Waveform “B”, which is also known as pathway, occurred during secretion of salivary sheaths before and after ingestion. I also observed that the squash bug spent more of its time on non probing activities than probing activities. This might be an effect of wiring and restraining bugs during EPG recording. I found several types of uniform EPG waveforms during ingestion “C” which had a 3-5 hz frequency of the peaks. However, I was not able to distinguish between xylem and phloem ingestion based on this frequency. Histological sections of one probed tissue taken during ingestion waveform “C” showed that the squash bug salivary sheath tip was terminated in vascular tissue, very close to the xylem.

To determine host acceptance by the squash bug, I tested older nymphs using feeding bioassays by placing them in choice and no choice situations. The choice test revealed no preference of squash bug among watermelon and its relatives which may be due to the test conditions. In the no choice test, squash was used as a control. The no choice test demonstrated that squash bugs left fewer salivary sheaths on the watermelon relatives, the *C. colocynthis* lines and *P. fistulosus*, suggesting that these plants lacked host acceptance cues. When the feeding activities of squash bug nymphs were compared between watermelon and its relatives using EPG technology, the data showed that the squash bug probed longer and more often in watermelon var. “Royal Sweet” and the ingestion duration in watermelon was longer than duration on other test plants. It demonstrated the same results of non palatability of the *C. colocynthis* lines and *P. fistulosus* as in the no choice test. The sustained ingestion phase in *C. colocynthis* lines and *P. fistulosus* was shorter than in “Royal Sweet” indicating that the plant resistance

factor(s) to the squash bug were encountered after initiation of ingestion, suggesting that the resistance factor(s) are probably present in the vascular tissue. Future work will include the identification of putative compound(s) present in the vascular sap of the watermelon relatives.

The percent transmission of *S. marcescens* by the squash bug on *C. colocynthis* and *P. fistulosus* was less than “Royal Sweet” and USVL-200. The higher number of probes, longer duration of probes and longer ingestion on “Royal Sweet” and USVL-200 was consistent with higher pathogen transmission. Mechanical inoculation of the pathogens did not show any difference in the percent transmission among the test plants, suggesting that the host resistance of *C. colocynthis* and *P. fistulosus* is not directed to the pathogen but to the vector.

APPENDIX A

Serratia marcescens transmission by the squash bug, _Anasa tristis_, at Oklahoma State University, Stillwater, Oklahoma

The purpose of this study was to compare the transmission rates of the bacterium *Serratia marcescens*, by the squash bug (*Anasa tristis)*, to watermelon var. "Royal Sweet" and its relatives *Praecitrullus fistulosus* PI-179660*, Citrullus colocynthis PI*-525082*, C. colocynthis PI*-386015 and USVL-200. Squash bug adults were exposed to *S. marcescens* vacuum infiltrated squash cubes ($5mm^3$) for 24 hours. After 24 hours, individual squash bugs were placed on a single test plant inside a cylindrical cage. After one week, the bugs were transferred into another set of new plants of same species and kept for another one week. Bugs were removed from the second set of plants after a week and the plants were kept in the green house for symptom development. After 40-45 days the crown root of the plants were excised and brought in to the lab. The section of crown root was stained with Diene's stain and examined under the microscope to see the presence of bacteria in the phloem. There were total of 10 test plants for each treatment and each treatment was replicated four times (N=40).

Table Transmission test of *Serratia marcescens* by the squash bug.

S.N.	Plants	Sample number (N)	No of positive plants for CYVD/No. exposed
1	*C. colocynthis* PI 525082	40	0/40
2	*C. colocynthis* PI 386015	40	0/40
3	*P. fistulosus* PI-179660	40	0/40
4	USVL-200	40	0/40
5	Royal Sweet (+ve control)	40	0/40
6	Royal Sweet (-ve control)	40	0/40

Fig. Transmission test set up keeping plant inside a cylindrical plastic cage.

APPENDIX B

Serratia marcescens transmission by the squash bug, _Anasa tristis_, at United States Department of Agriculture, Lane, Oklahoma

Dr. B. D. Bruton did a similar transmission test in 2009 using squash var. "Lemon drop" (preferred host), "Royal Sweet" (susceptible to squash bug), *Citrullus colocynthis* PI 386015, *P. fistulosus* PI 179660 and USVL-200 in Lane, Oklahoma. Newly hatched squash bug adults were starved for 24 hours and then placed on the vacuum imbibed squash cubes with WO1 strains of *S. marcescens* suspension (10^8 CFU/ml) for 48 hours. After acquisition period, single bugs were transferred to each test plant for seven days. Plants were assessed for honey brown discoloration of phloem tissue after five to six weeks.

Table Transmission of *S. marcescens* by the squash bug

S.N.	Plants	Sample number (N)	No of positive plants for CYVD	Percentage of total positive
1	Squash var "Lemon drop"	138	5	3.6
2	*C. colocynthis* PI 386015	136	1	0.7
3	*P. fistulosus* PI-179660	127	0	0
4	Royal Sweet	126	6	4.7
5	USVL-200	130	10	7.6

APPENDIX C

Mechanical Inoculation of *Serratia marcescens* in watermelon and its relatives

Dr. B. D. Bruton mechanically inoculated six different host plants with *S. marcescens*, a phloem colonizing bacteria. The bacteria were grown on the LB plates and were scrapped and suspended in PBS buffer for concentration of 10^{10}-10^{11} CFU/ml. The cotyledon stage plants were inoculated with the suspension using inoculation fork at the stem cotyledon junction making 10 puncture per plant. The plants were kept in green house for approximately one month and the crown root was examined for honey brown discoloration of phloem tissue.

Table Mechanical inoculation of *S. marcescens*

S.N.	Plants	Sample number (N)	No of positive plants for CYVD	Percentage total positive
1	Squash var "Lemon drop"	361	124	34.34
2	*C. colocynthis* PI 525082	306	21	6.86
3	*C. colocynthis* PI 386015	285	22	7.71
4	*P. fistulosus* PI-179660	306	30	9.8
5	Royal Sweet	373	43	11.52
6	USVL-200	341	78	22.87

S.N.	Plants	Sample number (N)	No of positive plants for CYVD	Percentage total positive
7	Pumpkin	9	2	22.22
8	Charleston Gray X *C. colocynthis* PI 386015	187	14	7.48

APPENDIX D

Definitions

Probe: An uninterrupted time period in which stylets are inserted into the plant tissue. A probe may be long or short, high or low amplitude and include different waveform events (specific waveform within a probe).

Waveform: Graphic structure formed due to the insect activity (probing and non probing) in the electrified system. It consists of different subtypes.

Event: An event is a single waveform type during a waveform. Events can occur several times within a waveform.

Probing number: Number of occurrences of a repeating event (probe) per unit time

Waveform number: Number of occurrences of a waveform per unit time

Non-sequential parameter: Parameter that does not include sequential order of the waveform in EPG

VITA

KAUSHAL MASKEY

Candidate for the Degree of

Master of Science

Thesis: COMPARISON OF ELECTRICAL PENETRATION GRAPH WAVEFORMS OF SQUASH BUG FEEDING ON WATERMELON AND ITS RELATIVES

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Education: Received Bachelor of Science in Agriculture from Institute of Agriculture and Animal Science (IAAS), Tribhuvan University, Rampur, Chitwan, Nepal in 2003.Completed the requirements for Master's of Science with a major in Entomology at Oklahoma State University, Stillwater, Oklahoma in July, 2010.

Experience: Graduate Research Assistant at Oklahoma State University, from August 2006 to December 2009.

Professional Memberships: Entomological Society of America

Name: Kaushal Maskey Date of Degree: July, 2010

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: COMPARISON OF ELECTRICAL PENETRATION GRAPH WAVEFORMS OF SQUASH BUG FEEDING ON WATERMELON AND ITS RELATIVES

Pages in Study: 125 Candidate for the Degree of Master of Science

Major Field: Entomology

Scope and Method of Study:

Cucurbit yellow vine disease is caused by the bacterium *Serratia marcescens*, which is transmitted by the squash bug, a serious pest of cucurbit. Watermelon is an economically important crop and serves as an alternate host of the squash bug. There is no any watermelon cultivar resistant of CYVD and squash bug. In this study, we characterized and compared the squash bug feeding behavior on watermelon with the feeding behavior on watermelon relatives using electrical penetration graph technology to understand squash bug host preference.

Findings and Conclusions:

Feeding behavior of squash bug on watermelon was categorized into four different waveform patterns; non probing, test probe, salivation or pathway and ingestion. Non probing waveform which includes squash bug activities other than stylet insertion was longest than test probe, pathway and ingestion waveform. Test probes were the most prevalent probing waveforms. Pathway waveform was shorter with high amplitude than ingestion, and occurs in the same probe which includes ingestion. Ingestion waveform was the longest waveform among squash bug probing waveform. Labial angle during probing was associated with the ingestion behavior.

Squash bugs were more attracted to watermelon hybrid, USVL-200 when given choice and probed more on watermelon cv. Royal Sweet and USVL-200 when given no choice showing that *C. colocynthis* lines and *P. fistulosus* are less preferred by the squash bug. There was no difference in the time taken by the insect to initiate first probe and first probe with ingestion, and duration of first probe and first probe with ingestion. It indicated that the squash bug probing was not affected by the host physical structure or plant volatiles. Longer duration of ingestion in Royal Sweet than on other test plants species indicate that squash bug ingestion behavior on watermelon relatives was altered when the stylet reached vascular bundle. There might be some difference in the sap content of these plants which reduced sustained ingestion by the squash bug. Information from this study can be used further to understand the mechanism of pathogen inoculation by the squash bug and to identify the possible gene responsible for squash bug resistance.

ADVISER'S APPROVAL: Astri Wayadande