INTERACTIONS BETWEEN *FRANKLINIELLA FUSCA* (THYSANOPTERA: THRIPIDAE) AND *TOMATO SPOTTED WILT VIRUS* IN THE PEANUT PATHOSYSTEM

by

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(Under the Direction of Rajagopalbabu Srinivasan)

ABSTRACT

This research investigated interactions between tobacco thrips, *Frankliniella fusca* (Hinds) and *Tomato spotted wilt virus* (TSWV) in the peanut pathosystem. First objective optimized a thrips-mediated transmission protocol. Incidence of TSWV infection in plants increased with thrips density. Thrips-mediated inoculation was more efficient than mechanical inoculation. Further, younger plants were more susceptible to TSWV and accumulated greater TSWV titers than older plants. Second objective investigated effects of resistant genotypes on TSWV and *F. fusca*. Resistant genotypes were equally susceptible to TSWV and accumulated substantial TSWV titers. Genotypes had minor effects on thrips biology but not on size. Third objective assessed effects of TSWV on *F. fusca*. TSWV infection in plants and in thrips did not affect their settling but influenced feeding behavior and oviposition efficiency. Further, TSWV infection in thrips as well as in plants adversely affected thrips biology. TSWV infection also increased free amino acids levels in plants.

INDEX WORDS: *Frankliniella fusca*, *Tomato spotted wilt virus,* resistant genotypes

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CHAPTER 1

INTRODUCTION

Peanut (*Arachis hypogaea* L.) is an important field crop in the United States. Georgia ranks first in peanut production in the United States (Smith and Smith 2011). In 2010, 565,000 acres of peanut was planted in Georgia (NASS 2010, Smith and Smith 2011). For several decades, peanut production in the United States has been challenged by various pests and diseases. Spotted wilt disease, caused by *Tomato spotted wilt virus* (TSWV) has been the most detrimental (Culbreath et al. 2003). TSWV is transmitted exclusively by thrips (Thysanoptera: Thripidae) in natural epidemics (Sakimura 1962, Cho et al. 1989, Wijkamp et al. 1995, Mound 1996, Moyer 1999, Chatzivassiliou et al. 2002). Several species of thrips are reported to transmit TSWV (Todd et al. 1990). Western flower thrips, *Frankliniella occidentalis* (Pergande) and tobacco thrips, *F. fusca* (Hinds) are the primary vectors of TSWV in peanut (Goldbach and Peters 1994, Ullman 1996, Mound 2001, Reitz 2009). However, in Georgia, *F. fusca* is considered the predominant vector as it readily colonizes peanut plants (Todd et al. 1995, Culbreath et al. 1990).

Spotted wilt disease was first documented in peanut in Brazil (Costa 1941). Subsequently, the disease became epidemic in peanut growing areas of South Africa, Australia, and the United States (Helms et al. 1961, Klesser 1966, Halliwell and Philey 1974). In the United States, spotted wilt disease was first documented in peanut in Texas, 1971 (Halliwell and Philey 1974). From 1985 to 1991, yield losses to spotted wilt increased, with up to 95% loss in individual fields in the southwest Texas (Mitchell et al. 1991). In Georgia, incidence of spotted

wilt increased from 1989 throughout the mid 1990's resulting in severe yield losses (Culbreath et al. 1991, 1992b). A recent review estimated average annual losses to spotted wilt to be $12.3 million from 1996 to 2006 in Georgia alone (Riley et al. 2011).

Although thrips are the only vector of TSWV in natural epidemics, management of TSWV has been very difficult mainly due to the wide host range and high reproductive capacities of thrips (Whitfield et al. 2005). Application of insecticides is one of the options for thrips management. Insecticides such as phorate has been consistently effective in thrips management for several years (Todd et al. 1995, 1996). However, no insecticide besides phorate has provided consistent suppression of spotted wilt, and application of phorate alone has not been adequate to economically reduce yield losses. Thus, several management tactics have been incorporated to suppress spotted wilt incidence in peanut. Planting of resistant genotypes along with cultural management tactics such as planting date, plant density, and tillage practices are widely adopted to manage spotted wilt in peanut (Culbreath et al. 1992a).

Use of resistant genotypes is the single most effective tool to reduce spotted wilt incidence (Culbreath et al. 1996a, 1996b). At present, several moderate to highly resistant peanut genotypes are available. Until recently, extremely susceptible peanut genotypes such as Florunner, Georgia Runner, SunOleic 97R, and GK-7 were planted in Georgia and other peanut growing areas of the southeastern United States (Culbreath et al. 2003). Continuous breeding efforts resulted in the release of first generation runner type genotypes that exhibited moderate levels of field resistance to TSWV. Such genotypes included Georgia Green, Georgia Browne, Tamrun 96, UF MDR 98, and ViruGard (Gobert et al. 1987; Culbreath et al. 1994, 1996a). Among all runner type genotypes, Georgia Green was predominantly planted in the southeastern United States (Culbreath et al. 2003). Although, Georgia Green provided moderate levels of field

resistance to TSWV, under severe conditions, it was susceptible to severe losses to spotted wilt. More recently, second generation genotypes such as Georgia-06G, Georganic, Georgia-02C, Georgia-10T, Tifguard, and Florida-07 were developed (Culbreath and Srinivasan 2011). These genotypes have great potential to reduced yield losses to spotted wilt and are expected to provide flexibility to TSWV management (Culbreath et al. 2003, Culbreath and Srinivasan 2011).

Susceptibility of genotypes and breeding lines to TSWV are generally evaluated under field conditions (Lynch 1990). However, in fields, thrips and TSWV pressure can vary profoundly from season to season and also from one location to another (Mandal et al. 2001, Culbreath et al. 2003). Thus, inconsistency in TSWV and thrips pressure can impede resistance evaluation in peanut genotypes (Mandal et al. 2001). Furthermore, screening genotypes in fields is time consuming as it is limited to one growing season and it is also labor intensive (Al-Saleh et al. 2007). Most of the peanut genotypes and breeding lines developed till today exhibit resistance and/or tolerance to TSWV. In contrast to other crops, the genes and mechanisms that impart resistance to TSWV have not been identified in peanut. In tomato (*Solanum lycopersicon* L.) and pepper (*Capsicum annuum* L.), single dominant genes, *Sw-5* and *Tsw*, respectively conferring resistance to TSWV have been identified (Black et al. 1991, Stevens et al. 1991, Boiteux and de Avila 1994). These single dominant genes are known to confer resistance by triggering hypersensitive reaction (HR) (Stevens et al. 1994, Moury et al. 1997). Several studies have demonstrated that reduced incidence of spotted wilt in peanut was not due to reduced attractiveness to thrips vector or reduced reproduction of thrips vectors (Culbreath et al. 1992b, 1994, 1996b, 2000). Based on field observations, it is believed that in peanut genotypes, resistance is mainly imparted against TSWV but not against thrips.

As TSWV infection in peanut results in severe spotted wilt disease, the presence of TSWV in thrips also may affect thrips. Among insect transmitted plant viruses, thrips and TSWV share the most complex interaction (Hull 2001). TSWV is transmitted by thrips in persistent propagative manner. Since TSWV multiplies and circulates in thrips body, several studies have investigated the effects of TSWV on thrips. Studies have demonstrated both positive and negative effects of TSWV on biology and behavior of *F. occidentalis* (Robb 1989, DeAngelis et al. 1993, Bautista et al. 1995, Maris et al. 2004). However, it is not certain if the behavioral changes observed with *F. occidentalis* are consistent across all thrips species.

In this study, three objectives were set. The first objective was to develop a thrips-mediated transmission protocol under a greenhouse conditions with an intention to provide supplemental information that would aid conventional breeding. The second objective was to investigate on the effects of newly released TSWV-resistant genotypes on thrips and TSWV to speculate on the mechanisms of TSWV resistance in peanut genotypes. The third objective was to investigate the effects of TSWV on settling, feeding, oviposition, and biology of *F. fusca*.

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CHAPTER 2

LITERATURE REVIEW

Peanut Production in the United States

Peanut (*Arachis hypogaea* L.), also known as groundnut, is a self-pollinating legume that is cultivated for its high quality protein and oil content (Venkatachalam and Sathe 2006, Fernandes et al. 2010). Peanut originated in South America and was subsequently introduced to Europe, Africa, Asia, and North America during the European expansion in the sixteen century (Kochert et al. 1991). The United States ranks third in the world peanut production after China and India (Lynch 1990). In 2010, the United States produced a total of 1.981 million tons of peanut (NASS 2010, Smith and Smith 2011). More than 70% of the peanut production acreage in the United States lies in Alabama, Florida, and Georgia (NASS 2010). Georgia ranks first in peanut production in the United States. In 2010, Georgia produced approximately 50% of the peanuts in the United States (NASS 2010, Smith and Smith 2011).

Peanut production in the southeastern United States is affected by various pests and diseases, of which spotted wilt is regarded as the most detrimental. Spotted wilt is caused by thrips-transmitted *Tomato spotted wilt virus* (TSWV). Since late 1980s, spotted wilt has become a major constraint to peanut production. (Hagan et al. 1990, Mitchell et al. 1991, Culbreath et al. 1992c, Groves et al. 2003).

History and economic importance of *Tomato spotted wilt virus* (TSWV)

Spotted wilt was first identified in tomato (*Lycopersicon esculentum* Miller) in Australia in 1915 (Brittlebank 1919). Later, thrips involvement in transmission of the casual agent of

spotted wilt disease was reported (Pittman 1927). In 1930, the agent causing spotted wilt was identified as a virus (Samuel et al. 1930). The disease was subsequently observed in several parts of the world affecting a variety of agronomic, vegetable, and ornamental crops (Helms et al. 1961, Klesser 1966, Reddy et al. 1968, Halliwell and Philey 1974, Prins and Goldbach 1998). Annual losses to spotted wilt is estimated to be over $1 billion throughout the world (Prins and Goldbach 1998).

Spotted wilt disease in peanut was first observed in Brazil (Costa 1941). Subsequently, the disease was documented in peanut in South Africa (Klesser 1966), Australia (Helms et al. 1961), and the United States (Halliwell and Philey 1974). The disease was documented in the United Stated in Texas in 1971 and soon became the most serious disease of peanut (Halliwell and Philey 1974; Black et al. 1986, 1987; Culbreath et al. 1992c). Losses to spotted wilt in peanut producing areas of southern Texas ranged from approximately 50% to 100% in some individual fields (Black et al. 1986). In the southeastern United States, the incidence of the disease increased from late 1980s through 1997 and became endemic to the region (Culbreath et al. 2003). During this time period, in Georgia alone, yield losses to spotted wilt were estimated to be approximately $40 million (Bertrand 1998). A recent study conducted to assess the losses due to spotted wilt in peanut from1996 to 2006 indicated that the average annual losses were $12.3 million (Riley et al. 2011). In recent years, the incidence of spotted wilt has decreased in peanut growing areas likely due to improved management practices. However, it still continues to pose a serious challenge to peanut growers in the southeastern United States.

Host Range and Symptoms of TSWV

TSWV has a wide host range. TSWV can infect at least 1090 different plant species in 69 dicotyledonous and 15 monocotylenous families (Parrella et al. 2003). TSWV induced symptoms

vary greatly with host plant species, genotypes, and environmental conditions (German et al. 1992, Culbreath et al. 2003). Common symptoms of TSWV on infected plants include concentric ringspots (yellow or brown rings), black streaks on petioles or stems, mottling, local lesions, tip die back, and stunting (Whitfield et al. 2005, Tsompana and Moyer 2008). TSWV also induces symptoms on the fruits of infected plants such as necrotic rings and ringspots on tomato (Maluf et al. 1991, Aramburu et al. 2000), necrotic streaks and spots on fruits such as pepper, and superficial ring symptoms and severe internal necrosis in tubers as in the case of potato (Wilson 2001).

TSWV also induces a wide range of symptoms in peanut (Culbreath et al. 2003). Some of the earliest symptoms on TSWV infected peanut include brown speckles on the abaxial leaflet surface with chlorotic spots and mottling on the adaxial leaflet surface (Hagan 1998). These symptomatic leaflets quickly wilt and fall off. Furthermore, TSWV causes terminals and leaf petioles to droop and they display yellowing and mottling appearance. In mature leaflets, TSWV induced symptoms include concentric chlorotic ring-spots, ring-spots with green centers, and chlorotic line patterns (Culbreath et al. 1992b, 1992c; Hagan 1998). Seeds produced by TSWV infected peanut are typically smaller in size and fewer in number than seeds produced by non-infected plants. Also, infected seeds have poor germination rates (Culbreath et al. 1992b). The severity of spotted wilt disease depends on the time of the season at which peanut plants get infected. Infection at an early crop stage in peanut usually results in stunting and infected plants produce fewer pods than those that are infected at a later stage (Culbreath et al. 1992b). Severe stunting during the seedling ages often may result in death. Late season TSWV infections result in yellowing of the foliage, decline in plant vigor, and reduction in yield (Hagan 1998). TSWV also is known to affect the root system of peanut. Roots of TSWV infected peanut plants often

are discolored and partially wilted (Culbreath et al. 1991). In many peanut fields in Georgia, asymptomatic infections have been observed. TSWV infections in asymptomatic peanut leaflets were found to be as high as those with symptomatic leaflets (Culbreath et al. 1992a). However, yield loss to asymptomatic infections has never been reported.

Thrips: Taxonomy, Biology, Ecology, and Vector Competence

TSWV is exclusively transmitted by thrips. Thrips are tiny hemimetabolous insects about a few millimeters in length that belong to the insect order Thysanoptera and family Thripidae (Mound 1997). There are two suborders, Terebrantia and Tubulifera. Most thrips of agricultural importance are found in Terebrantia (Ananthakrishnan 1993). Thrips have a slender, cylindrical, and elongated body with colors that vary from yellow to orange to black (Morse and Hoddle 2005). Thrips go through six stages in their life cycle: an egg, two larval stages, prepupal and pupal stages, and an adult stage (Riley et al. 2011). Adult females insert tiny eggs into young leaves or buds, which hatch within 3 to 4 days depending on the species and the temperature (Hansen et al. 2003). The first and second instar larval stages last about 5 to 6 days followed by a prepupal stage. During the prepupal stage, thrips stop feeding and often drop off from the host plant to pupate in soil or litter, which lasts 2 to 4 days (Broadbent et al. 2003). Subsequently, adults emerge and resume feeding. The life cycle of thrips lasts for approximately 20 to 30 days (Ananthakrishnan 1993). However, duration of thrips life cycle varies with host, temperature, and relative humidity (Ananthakrishnan 1993, McDonald et al. 1998, Riley et al. 2011).

Thrips are polyphagous insects that feed on wide range of plant species and plant organs. Thrips feeding causes serious damage to many crops (Morse and Hoddle 2005). With their piercing-sucking mouthparts, thrips feed on pollen, flower, and other vegetative parts of plants (Ullman et al. 1992b). Concentrated feeding by thrips results in discoloration (silvery patches)

and distortion of plant tissues which affects the cosmetic value of crops (Culbreath et al. 2003). Besides direct feeding damage, thrips also cause indirect damage by serving as a vector for many disease causing microbial pathogens such as bacteria and virus (Ananthakrishnan 1993). Thrips transmit many plant infecting viruses, among which TSWV is by far the most important economically (Sakimura 1962, 1963; Cho et al. 1989; Ullman et al. 2002).

Of an estimated 6000 species of thrips that are recorded in the world, only 9 have been implicated in TSWV transmission. All the 9 thrips species belong to the family Thripidae (Goldbach and Peters 1994, Wijkamp 1995, Mound 2001, Mound and Morris 2007, Ciuffo et al. 2008, Pappu et al. 2009, Riley et al. 2011). Despite the presence of many thrips species, there is not a clear explanation as to why one species of thrips is a vector of tospoviruses and others are not (Mound 2001). Speculations have been made that either most thrips species have lost the ability to transmit the virus or the ability to acquire the virus has occurred independently (Mound 2001). Western flower thrips *Frankliniella occidentalis* (Pergande) and Tobacco thrips, *Frankliniella fusca* (Hinds) are two confirmed vectors of TSWV in peanut in the southeastern United States. The increased incidence of spotted wilt in peanut producing area of the southeastern United States has been associated with the introduction of *F. occidentalis* (Peters et al. 1996). However, *F. fusca* is considered the most competent vector in peanut as it occurs in a large number and reproduces efficiently on peanut when compared with *F.* occidentalis (Weeks and Hagan 1991, Todd et al. 1995).

Taxonomy and Genome Organization of TSWV

TSWV is a type species of the genus *Tospovirus* that contains plant-infecting members of the family *Bunyaviridae* (Francki et al. 1991, Goldbach and Peters 1994, Murphy et al. 1995). On the contrary, the other four genera of *Bunyaviridae*, i.e. *Orthobunyavirus, Hantavirus,*

Nairovirus, and Phlebovirus are exclusively animal infecting members (Elliott 1996). Currently, *Tospovirus* contains 20 distinct species of plant infecting viruses (Ciuffo et al. 2008, Pappu et al. 2009). The recognized species of plant viruses are defined on the basis of genome structure and organization, molecular characteristics of their nucleocapsid protein genes, vector specificity, and plant host range (German et al. 1992, de Avila et al. 1993, Goldbach and Peters 1996). The morphology, genome structure, and organization of TSWV is similar to other *Bunyaviridae* members (Milne and Francki 1984, Fauquet et al. 2005). TSWV virions are enveloped spherical particles, about 80-120 nm in diameter (Mohamed et al. 1973, 1981; Tas et al. 1977). The core of TSWV virions is made up of single stranded, tri-partite RNA genomes of negative or ambisense polarity that are encapsidated in nucleocapsid protein (N) (Avila et al. 1993). These RNA genomes are aptly named as S (small), M (medium), and L (large) RNAs (Kormelink et al. 1992a). The 8.9 kb L RNA has a negative sense orientation and encodes for RNA-dependent RNA polymerase (RdRp) (331.5 kDa), which facilitates initial rounds of replication of the RNA genome (Kormelink et al. 1992a; van Poelwijk et al. 1993, 1996; Adkins 2000). Unlike other genera of *Bunyaviridae*, the M and S RNAs of TSWV and all other tospoviruses are ambisense (de Haan et al. 1990, Kormelink et al. 1992b). The 4.8 kb M RNA encodes a nonstructural protein (NSm) (33.6 kDa) in the viral sense (v) and precursor to two membrane glycoproteins: Gn (78 kDa) and Gc (58 kDa) in the viral complementary (vc) sense (Kormelink et al. 1992b). NSm is an additional gene that is found only in tospoviruses. It is considered a putative movement protein that may likely modify the size of plasmodesmata to move from one cell to another (Kormelink et al. 1994, Prins et al. 1997, Storms et al. 1998). Also, NSm is associated with tubule formation in plants. Further, the 2.9 kb S RNA encodes for NSs (52.4 kDa) in the v sense and N protein (29 kDa) in the vc sense (de Haan et al. 1990, Bucher et al. 2003). RNA

silencing is one of the plant defense mechanisms against plant viruses, which degrades RNA in sequence-specific manner (Bucher et al. 2003). The NSs of TSWV is involved in suppression of plant defense by inhibiting RNA-silencing (Bucher et al. 2003). The L, M, and S RNAs of TSWV have about 65 to70 nucleotides that are complementary at the 5' and 3'. These complementary RNA end sequences lead to the formation of pseudo-circular structure of RNAs that serve as a promoter for replication. These three RNA genomes also contain eight identical nucleotides at the 5' and 3' terminal sequences, which is strictly conserved among all tospoviruses and is associated with genome replication and transcription (de Haan et al. 1989, Tsompana and Moyer 2008).

TSWV RNA genomes are enclosed by a lipid bilayer membrane of the host origin and consist of two virus-encoded glycoproteins projecting at the surface (Kitajima et al. 1992, Goldbach and Peters 1996, Ullman et al. 2002). Because Gn and Gc are positioned on the outer surface of the lipid membrane, they are considered to be the first viral components to interact with thrips vectors. Thrips are believed to possess putative cellular receptors (possibly 50 kDa and 94 kDa) (Kikkert et al. 1998, Meideros et al. 2000) on the epithelial cells of the midgut, the first site of viral infection (Ullman et al. 1992a, Tsuda et al. 1996). TSWV glycoproteins serve as viral attachment proteins and bind to these receptors to mediate entering into thrips midgut cells most likely through receptor-mediated endocytosis (Goldstein et al. 1985, Bandla et al. 1998). The specificity associated with TSWV transmission by certain thrips species is believed to be due to the presence of these putative receptors in thrips species. Several studies have demonstrated that TSWV glycoproteins are required for thrips recognition and acquisition but not for plant infection. Repeated mechanical inoculations of TSWV lead to deletions and point mutations in the sequences encoding the glycoproteins. The inoculations generated mutants that

lacked the envelope structure and glycoproteins (Ie 1982, Resende et al. 1991). Studies have found that these mutants were able to infect host plants; however, the mutants were not thrips transmissible (Wijkamp et al. 1995). Therefore, it further confirms that thrips recognition sites occur putatively in TSWV glycoproteins.

Transmission of TSWV

Among insects transmitted plant viruses, tospoviruses involve the most complex transmission mechanism (Hull 2001). Tospoviruses such as TSWV are transmitted in a persistent propagative manner, i.e. they are able to multiply in their vectors, thrips. I will briefly attempt to explain the transmission process with emphasis to TSWV. Thrips transmission of TSWV involves three specific events: acquisition of the virus, latent period, and inoculation of the virus.

a. Acquisition

Acquisition of TSWV is a stage specific event. TSWV can be acquired by the first and early second instar larvae only if fed on infected plants (Sakimura 1962, Lewis 1973, Wijkamp and Peters 1993). In general, acquisition time for persistently transmitted virus varies from hours to days (Hull 2001). Minimum acquisition access period for TSWV is estimated to be approximately 15 minutes (Hull 2001). The competence to acquire TSWV decreases as thrips develop (Ullman et al. 1992a, van de Wetering et al. 1996). However, once acquired thrips retain the virus through molting, pupation, and adult emergence (Sakimura 1962, Kritzman et al. 2002). Adult thrips are able to transmit TSWV for the remainder of their lives. But there is no evidence of transovarial transmission (Wijkamp et al. 1996). Hence, individual thrips must acquire the virus during larval stages to transmit the virus (German et al. 1992).

b. Latent period

Latent period of TSWV is the time period between acquisition of the virus by larvae and the time it is inoculated into host plants (Hull 2001). A latent period of approximately 3 to 7 days is associated with TSWV transmission (Ullman et al. 1998, Hull 2001). This is mainly due to fact that TSWV has to migrate across several membrane barriers as it travels from initial ingestion through feeding to the salivary glands through the midgut (Ammar 1994). While feeding on infected plants, larvae ingest infected cells containing TSWV, which travel through the lumen of the foregut into the midgut (Ullman et al. 1992a, Nagata et al. 1999, Assis Filho et al. 2002). The first membrane barrier that the virus encounters is a brush border of microvilli that extends into the midgut lumen. Once the virus travels through the brush border, it replicates in the midgut epithelial cells and exits the basement membrane, which is the second barrier encountered by the virus (Ullman et al. 1993). Subsequently, the virus spreads to visceral and longitudinal muscular cells that surround the midgut epithelial cells (Ullman et al. 1993, Tsuda et al. 1996). The virus encounters third and fourth membrane barriers while escaping from these muscle cells to enter into the salivary glands of thrips (Nagata et al. 1999, Assis Filho et al. 2002). Virus entry into the salivary glands is mediated through a temporary ligament like structure that connects membranes of the visceral muscles and the salivary glands during thrips larval stage (Tsuda et al. 1996, Nagata et al. 1999, Moritz et al. 2004). As the virus reaches the salivary glands, it has to transverse the basal membrane of the salivary glands, which is the last barrier encountered by the virus. Once inside the salivary glands, the virus replicates (Ullman et al. 1992a, Tsuda et al. 1996) and eventually flows with the salivary secretions during thrips feeding (Whitfield et al. 2005). The connection between the salivary glands and the midgut epithelial cells through a thin ligament is temporary and is formed during thrips larval stages

only. It is believed that during early larval stages, thrips larvae have large cibarial muscles that lead to the displacement of the brain into the prothoracic region and establish an association between the salivary glands and the midgut cells, as larvae develop, the enlarged muscles retract back into the head and the connection between the salivary glands and the midgut epithelial cells is eventually lost (Moritz et al. 2004).

c. Inoculation

Inoculation is the process of transmitting the acquired virus into host plants. TSWV inoculation is mediated through late second instar larvae and adults while feeding. To feed, thrips first puncture the leaf epidermis with their single mandible and ingest the cytoplasm from the mesophyll cells by using a pair of maxillary stylets (Ullman et al. 1992b, Ananthakrishnan 1993). While doing this, thrips inject saliva containing TSWV into plant cells. Upon entry into plant cells, the virus initially spreads into parenchyma cells and reach the phloem (Agrios 2004, Tsompana and Moyer 2008). Although successful transmission of TSWV requires longer inoculation access period, studies have reported successful inoculation in as early as 5 to 30 minutes (Sakimura 1963).

Factors Affecting TSWV Transmission Efficiency

TSWV transmission involves several factors that determine a successful transmission event. Thrips can transmit TSWV only if acquisition occurs during larval stages. Secondly, the amount of virus ingested, the rate of virus replication in the midgut epithelium cells, and the amount of virus load on the salivary glands of thrips (Wijkamp 1995, van de Wetering et al. 1996, Nagata et al. 1999, Kritzman et al. 2002) also have impact on TSWV transmission efficiency. Furthermore, TSWV transmission efficiency was found to be different between male and female *F. occidentalis* (Rotenberg et al. 2009). The observed difference was probably

associated with differences in feeding behavior between males and females. Male thrips were found to be more mobile during probing of plant tissues, which resulted in minimal damage to plant cells. Females however, fed at a particular site for a longer period emptying plant cells, thus making plant cells unsuitable for viral replication (Rotenberg et al. 2009). Hence, TSWV transmission efficiency of thrips might be affected by the sex ratio of that particular thrips population.

Sex ratio of thrips populations depends on their reproductive strategy. Thrips are haplodiploid insects (Lewis 1973, Ananthakrishnan 1993, Mound 1996, Chatzivassiliou et al. 2002). Females are referred as diploid as they contain a complete set of chromosomes whereas males are called haploid as they contain only the half the number of chromosomes in the nucleus (Whitfield et al. 2005). The most common form of reproduction in thrips is arrhenotoky in which unfertilized eggs develop into haploid males (Kumm and Moritz 2008). Some thrips species are thelytoky in nature. In this type of reproduction, female thrips produce exclusively female offsprings. Hence, males are very rare in this type of thrips populations (Lewis 1973). Furthermore in some thrips species, reproductive strategy can fluctuate between arrhenotoky and thelytoky based on geographical areas (Lewis 1973).

Management of TSWV

In natural epidemics, transmission of TSWV in peanut is only possible through thrips. TSWV is not transmitted through physical contact of plants (Culbreath et al. 2003). TSWV has been found in pods and testae of seeds from infected plants. However, planting TSWV infected seeds has not resulted in infected plants (Culbreath et al. 2003). Managing thrips in peanut has been a challenge mainly due to wide host range and high reproductive capacities of thrips (Culbreath et al. 2003). Despite the severe impact of TSWV on peanut production, none of the

available management practices can single-handedly reduce yield losses to TSWV. To solve the problem of spotted wilt in peanut, spotted wilt eradication action team (S.W. E. A.T) was formed by group of scientists from multiple disciplines, departments and areas of expertise. The group includes members from various universities such as the University of Georgia, the University of Florida, Auburn University, and the USDA-ARS. When eradication efforts failed, an integrated management system (IMS) that incorporates multiple suppressive factors was developed and adopted widely to effectively manage TSWV in the southeastern United States. IMS combined genetic, chemical, and cultural practices to reduce yield losses to TSWV in peanut (Culbreath et al. 2003).

a. Resistant Genotypes

Selection of peanut genotypes that exhibit field resistance to TSWV is an integral component of IMS. Resistant genotypes have played a significant role in the management of spotted wilt in the southeast United States (Culbreath et al. 2003). Many peanut genotypes with moderate levels of field resistance to TSWV are commercially available and are widely planted in the southeastern U. S. (Culbreath et al. 2003). In 1980s, peanut genotypes such as Florunner, Georgia Runner, SunOleic 97R, and GK-7 were widely planted (Culbreath et al. 2003). However, these genotypes were extremely susceptible to TSWV. After an intensive screening of genotypes and breeding lines, genotypes with moderate levels of field resistance to TSWV were developed. Georgia Green (Culbreath et al. 1996), Georgia Browne (Culbreath et al. 1994), Tamrun 96 (Smith et al. 1998), UF MDR 98 (Culbreath et al. 1997) and ViruGard (Shelton 2000) were some of the first generation runner-type genotypes that exhibited moderate levels of field resistance to TSWV in comparison with susceptible genotypes. Georgia Green and Georgia Browne were developed from crosses between Sunbelt Runner and Southern Runner (Gorbert et

al. 1986). Among moderately resistant genotypes, Georgia Green was grown predominantly in the southeastern United States over ten years (Branch 1996). Use of Georgia Green combined with other cultural practices significantly reduced spotted wilt incidence and increased yield (Mckeown et al. 2001, Tillman et al. 2007). However, Georgia Green and other moderately field resistant genotypes were susceptible to under heavy TSWV pressure. This led to the development of second-generation genotypes with greater field resistance greater than Georgia Green, such as GA-06G, Georganic, Georgia-02C, Georgia-10T, Tifguard, and Florida-07 (Culbreath and Srinivasan 2011). Use of these new genotypes might provide more flexibility in management of TSWV than moderately resistant genotypes (Culbreath et al. 2010a).

Resistant genotypes are developed through conventional breeding programs (Mandal et al. 2001). Usually, several breeding lines are screened for TSWV and thrips under the field conditions (Mandal et al. 2001). Breeding lines exhibiting field resistance to TSWV were crossed to develop genotypes displaying high levels of TSWV resistance. Peanut genotypes immune to TSWV have not been developed until now. Few of the genotypes are resistant to TSWV (reduce disease incidence), whereas few of them are tolerance to TSWV (reduce severity in infected plants). The mechanism of TSWV resistance in peanut genotypes is not understood yet (Culbreath et al. 2003). However, it is believed that resistance to TSWV in peanut is against TSWV not thrips (Culbreath et al. 1996, 2000).

Genetically engineered resistance has been actively investigated in recent years to facilitate development of resistant genotypes with enhanced TSWV resistance (Li et al. 1997). Unlike conventional breeding, in which thousands of breeding lines are screened to develop a resistant genotype, in genetically engineering technology, only the gene conferring resistance is transferred to susceptible plants. This results in faster development of resistant genotypes. In

recent years, several studies have generated transgenic peanut genotypes by inserting the nucleocapsid (N) gene of TSWV (MacKenzie and Ellis 1992, Pang et al. 1992). AT-120 and MARC I are transgenic peanut lines that exhibited field resistance to TSWV compared to non-transgenic peanut lines (Ozias-Akins et al. 1993, Magbabua et al. 2000, Ozias-Akins et al. 2002). Although transgenic peanut plants can effectively suppress spotted wilt incidence, production of transgenic peanut has been limited due to the limited national and international market for transgenic peanut. There is very low public acceptance of transgenic technology mainly due to ecological and food related concerns (Bruening 2000).

b. Insecticides and Chemical Control

Application of incidences to manage thrips is one of the TSWV management practices. Over the years, several insecticides have been tested to assess their efficacy in managing thrips populations and spotted wilt incidences. Some insecticides such as chloropyrifos and carbofuran demonstrated little or no effect in reducing spotted wilt incidence (Chamberlin et al. 1993, Todd et al. 1996) while, application of the neonicotinoid insecticide, imidacloprid, increased the incidence of spotted wilt by 20-200% when compared with non-treated control treatment (Todd et al. 1994, Riley 2007). But, application of the organophosphate and organocarbamate insecticides such as phorate and aldicarb, resulted in appreciable levels of spotted wilt suppression (Todd et al. 1996). In Georgia and Florida, application of phorate in-furrow at planting significantly reduced spotted wilt incidence in 63 out of 93 tests between 1987 and 2000 (Culbreath et al. 2003). Phorate application has provided consistent suppression of TSWV and is widely adopted in the southeastern US for TSWV management. At present, several new insecticides are available which need to be tested against thrips and TSWV. In addition to insecticides, chemicals that activate plant defenses such as Acibenzolar-S-methyl (a salicylic

acid analogue) can be used to suppress spotted wilt incidence. Acibenzolar-S-methyl effectively suppressed spotted wilt incidence in tobacco (Pappu et al. 2000) but in peanut, its performance was not consistent (Wells et al. 2002). Furthermore, imazapic (a herbicide) when applied along with phorate increased TSWV suppression in peanut (McDonald et al. 1999). However, the mechanism of TSWV suppression is unknown.

Most of the insecticides have been only effective in managing thrips larval feeding. Thrips have the potential to move between various cropping systems. Viruliferous thrips from neighboring fields also can migrate and inoculate TSWV in peanut fields treated with insecticides (Chamberlin et al. 1992, 1993; Todd et al. 1996). Thus, insecticides have not been very efficient in controlling thrips that are migrating from neighboring fields. Moreover, excessive and continuous use of insecticides has resulted in the development of insecticide resistance (Zhao et al. 1995). In recent years, thrips have developed resistance to three major classes of insecticides which are organophosphates, carbamates, and pyrethroids (Herron and James 2005, Bielza 2008). Further, use of insecticides at higher doses can have adverse effects on avian and mammalian populations. Recently, US Environmental Production Agency EPA has withdrawn the use of aldicarb (Temik) as it can pose unacceptable dietary risks especially to infant and children. Hence, alternative strategy for TSWV management is highly desirable.

c. Cultural Practices

Use of moderate to highly resistant genotypes along with several cultural practices effectively suppressed incidences of spotted wilt in peanut (Culbreath et al. 2003). In the southeastern United States, selecting an appropriate planting date has become an important factor for TSWV management in peanut (Mckeown et al. 2001, Culbreath et al. 2003, Tillman et al. 2007). Fluctuations in thrips populations are seasonal. Thus, planting peanut in coincidence with

low thrips densities effectively reduced TSWV epidemics (Culbreath et al. 1999, Tillman et al. 2007). In Georgia, the population densities of *F. fusca* in peanut were greater on peanut when planted in early April or June than in May. Subsequently, incidences of spotted wilt were significantly less in peanut planted during the first two weeks of May compared to earlier plantings in April (Mckeown et al. 2001, Culbreath et al. 2010a).

Several studies have shown that manipulation of row patterns and tillage systems minimized losses to spotted wilt. Planting of peanut in twin rows spaced 18 to 24 cm apart suppressed spotted wilt incidence, improved grade, and increased yield in several studies (Tillman et al. 2006, Culbreath et al. 2008). Furthermore, peanut grown under minimum tillage conditions compared with conventional tillage system resulted in reduced thrips density, less feeding injury, and higher yield in peanut (Brandenburg et al. 1998). Increased seeding rate or plant populations stands also have been very effective in suppressing spotted wilt incidence. In Georgia peanut fields, increasing plant populations from < 6.6 to 6.6-13 to >13 plants/m of row significantly reduced spotted wilt incidence by lowering the percentage of TSWV infected plants (Brown et al. 2005). Similarly, studies conducted in Georgia, Florida, and Alabama revealed that an increment in seeding rates from 34 to 101 kg/ha along with higher plant populations substantially lowered losses to TSWV (Gobert and Shokes 1994, Wehtje et al. 1994, Branch et al. 2003). Hence, in Georgia, it is recommended to maintain uniform stands of > 13 plants/ m of row even with resistant genotypes to minimize losses to TSWV (Brown et al. 1997b, 2005; Culbreath et al. 2010b).

Conclusions

Tomato Spotted Wilt Virus (TSWV) is an economically important pathogen that has been severely affecting peanut production in the southeastern United States (Black et al. 1986, Hagan

et al. 1990). TSWV is transmitted by several thrips species in the southeast. However, in Georgia, *F. fusca* is believed to be the most important vector of TSWV in peanut (Todd et al. 1994). Management of spotted wilt in peanut has been a challenge mainly due to wide host range of TSWV and also its thrips vectors. Application of insecticides to reduce thrips populations has not been effective except for phorate (Todd et al. 1994). Further, several thrips species have developed resistance to many insecticides. Lack of a single management strategy to lower spotted wilt incidences has resulted in an adoption of integrated management system (IMS). Integration of several TSWV suppressing factors such as the use of resistant genotypes, in furrow application of phorate, manipulation of planting date, plant population, row pattern, and tillage practices has significantly reduced yield losses to spotted wilt (Culbreath et al. 2003).

Information on several spotted wilt suppressing factors has led to the development of spotted wilt risk index (Brown et al. 1997a, 1997b). Spotted wilt risk index is a great tool available for the growers to quantify the relative risk of spotted wilt associated with specific combination of production practices in order to avoid high-risks situations (Brown et al. 2000). Spotted wilt risk index was developed on the basis of the research findings in Georgia, Florida, and Alabama. The index was initiated in Georgia in 1996 to address yield losses to TSWV in peanut. Since then it has been modified and improved annually to help farmers to avoid high-risk of losses to the disease (Brown et al. 1997a, 1999).

Scope of Investigation

This investigation attempted to answer a few important questions pertaining to *F. fusca* and *Tomato spotted wilt virus* (TSWV) interactions in the peanut pathosystem. There were three research objectives. The first objective was to assess various transmission parameters. Also, thrips-mediated transmission was compared with mechanical transmission. The ultimate aim

was to utilize this information to successfully screen peanut genotypes under greenhouse conditions merely to provide supplemental information that would aid conventional breeding. The goal of the second objective was to identify the effects of newly released TSWV resistant genotypes on thrips and on TSWV, and also, to speculate on the mechanisms of TSWV resistance in peanut genotypes. Through the third objective we intended to assess the effects of TSWV on *F. fusca* with emphasis to peanut. Below is a prelude to each objective; each objective is separately addressed in a chapter.

Chapter 1

TSWV is the single most important constraint for peanut production. Planting of resistant genotypes is an important management strategy deployed against TSWV in peanut. These genotypes are typically derived through conventional breeding. Most of the screening for thrips and TSWV is conducted under field conditions. However, field screening is limited by inconsistent TSWV and thrips pressure, time, and costs. The first objective of the research focused on developing a thrips-mediated transmission protocol under a greenhouse condition in order to supplement field screening associated with conventional breeding programs. The hypothesis was that thrips density, inoculation access period, plant age, and inoculation method would influence TSWV transmission efficiency. To develop thrips-mediated transmission protocol, three parameters associated with TSWV transmission: thrips density, thrips inoculation access period, and peanut age were optimized. Thrips transmission efficiencies at different seedling ages were compared with efficiency of mechanical-inoculation at different seedling ages. Further, titer levels of TSWV in thrips-inoculated plants were compared with mechanically inoculated plants.

Chapter 2

The genetic basis of resistance to TSWV has been identified in some crops. In tomato and pepper *Sw-5* and *Tsw*, respectively have been identified as single dominant genes conferring resistance to TSWV (Black et al. 1991, Stevens et al. 1991, Boiteux and de Avila 1994). These resistant genes respond to TSWV infection by triggering a hypersensitive reaction (Black et al. 1991, Rosello et al. 1998). Unlike in tomato and pepper genotypes, mechanism of TSWV resistance in peanut genotypes has not been identified yet. In the second objective, an attempt was made to assess the effects of TSWV resistant genotypes on thrips and the virus. The hypothesis was that resistant genotypes would be differentially susceptible to TSWV and differentially influence thrips biology and size. To conduct the experiments, four resistant and susceptible genotypes were selected. These resistant genotypes were never evaluated under the greenhouse conditions and doing so would provide more insights on the interactions of these genotypes with thrips and TSWV. In this study, TSWV transmission efficiency was determined for each genotype. Also, TSWV titer levels in each genotype were assessed. It is believed that resistance in peanut genotypes is against TSWV but not against thrips. But until now, no studies on thrips reproduction and development were conducted to confirm such effects (Culbreath et al. 1992c, 1994). Effects of resistant and susceptible genotypes on thrips biology and thrips size also were investigated in this study. Furthermore, TSWV acquisition ability of thrips from resistant and susceptible genotypes also was assessed.

Chapter 3

Biological transmission of plant viruses can range from a passive transport of viruses to a complex phenomenon involving specific interactions between the vector and virus (Hull 2001). TSWV transmission by thrips belongs to the most complex category in which the virus is

transmitted in a persistent and propagative manner. TSWV has been demonstrated to have positive and negative effects on behavior and fitness of Western flower thrips, *F. occidentalis* (Robb 1989, DeAngelis et al. 1993, Bautista et al. 1995, Maris et al. 2004). However, it is not certain if the behavioral changes observed with *F. occidentalis* are consistent across all thrips species (Stumpf and Kennedy 2005). Hence, in the third objective, effects of TSWV infection on *F. fusca* were studied. The hypothesis was that TSWV infection in plants as well as in *F. fusca* would influence thrips biology and behavior. Experiments were conducted on TSWV infected and non-infected plants to determine their effects on settling, feeding, oviposition, and biology of potentially viruliferous and non-viruliferous *F. fusca*. Viruses are known to severely affect plant physiology (Shalitin and Wolf 2000). In TSWV infected peanut, physiological changes such as decrease in photosynthesis, and water-use efficiency have been observed (Rowland et al. 2005). In this study, the effect of TSWV in free amino acid contents in peanut plants was investigated. Amino acids are important building blocks of proteins. In insects, amino acids are specifically important to synthesize yolk proteins for egg production. Hence, to determine if there is any correlation between free amino acids content and thrips fecundity, an analysis was performed to compare free amino acids levels between TSWV infected and non-infected peanut leaflets.

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CHAPTER 3

THRIPS-MEDIATED AND MECHANICAL TRANSMISSION OF *TOMATO SPOTTED WILT VIRUS* TO ASSESS TSWV SUSCEPTIBILITY IN PEANUT GENOTYPE[1]

[1] Shrestha. A., R. Srinivasan, D. Riley, and A. Culbreath. 2011. To be submitted

Abstract

Planting resistant genotypes is the most effective management option for thrips-transmitted *Tomato spotted wilt virus* (TSWV) in peanut. Most of the commercially available peanut genotypes are developed by conventional breeding. As a part of the breeding process, peanut genotypes are regularly screened under field situations. Despite the numerous advantages associated with field screening, it is limited by inconsistent thrips and TSWV pressure. To supplement these efforts, we attempted to optimize a greenhouse based screening assay. Attempts were made to optimize the various parameters associated with thrips-mediated TSWV transmission including thrips density, inoculation access period, and plant age. Thrips inoculation with three, five, and ten thrips resulted in more TSWV incidence in plants than with one thrips. Irrespective of thrips densities, increase in inoculation access periods (15 min, 1 h, 1 day and 4 days) did not result in increased incidence of TSWV infection in plants. Thrips-mediated TSWV inoculation at various plant ages (one- week, two- week, and four-week old plants) was compared with that of mechanical inoculation. The infection rate with thrips-mediated inoculation was greater than with mechanical inoculation. Both thrips-mediated and mechanical inoculation resulted in greater TSWV incidence in one-week and two-week-old plants than in four-week-old plants. In plants inoculated by thrips, TSWV titer levels were greater in one- and two-week old plants when compared with four-week old plants. TSWV titer levels at different plant ages were not different in mechanically inoculated plants.

Introduction

Peanut (*Arachis hypogaea* L.) is the second most economically important legume in the United States (Chamberlin et al. 2010). More than 70% of the peanut production in the United States occurs in Georgia, Alabama, and Florida (NASS 2010). In the past several years, spotted

wilt caused by *Tomato spotted wilt virus* (TSWV) has seriously affected peanut production (Black et al. 1986, 1987). Spotted wilt was first observed in peanut in Texas in 1971 (Black et al. 1986). In Georgia, spotted wilt incidence significantly increased since 1986 (Culbreath et al. 1991). A recent review estimated an average annual crop losses to spotted wilt from 1996 to 2006 to be $12.3 million (Riley et al. 2011).

TSWV is a member of the genus *Tospovirus* within the family *Bunyaviridae* (Fauquet et al. 2005). TSWV is exclusively transmitted by a few species of thrips (Thysanoptera: Thripidae) (Mound 1996, Chatzivassiliou et al. 2002). In the southeastern United States, Tobacco thrips, *Frankliniella fusca* (Hinds), and Western flower thrips, *Frankliniella occidentalis* (Pergande), are known to transmit TSWV in peanut (Todd et al. 1995). However, in peanut, *F. fusca* is considered to be the primary vector of TSWV as it occurs in greater numbers and reproduces efficiently on peanut when compared with *F. occidentalis* (Todd et al. 1996). In natural epidemics, thrips are the only transmittal agent of TSWV. However, reducing spotted wilt incidence by managing thrips has been rare. Application of insecticides to manage spotted wilt has not been effective except for phorate (Todd et al. 1996, 1998). Growing genotypes with resistance or tolerance to TSWV has been the most important management option (Culbreath et al. 1996a). Before the spotted wilt became a major concern, genotypes extremely susceptible to TSWV such as Florunner, Georgia Runner, SunOleic 97R, and GK-7At were planted (Culbreath et al. 1992). In mid-1980s, Southern Runner was identified as the first genotype with moderate levels of field resistance to TSWV (Black 1987). Continuous breeding efforts resulted in the release of first generation runner type genotypes such as Georgia Green, Georgia Browne, Tamrun 96, UF MDR 98, and ViruGard (Culbreath et al. 1996b, 2000). These genotypes exhibited moderate levels of field resistance to TSWV (Culbreath et al. 1997a, 2003). Among all

runner type genotypes, Georgia Green was the most accepted genotype. Approximately 90% of the acreage was planted with Georgia Green during 2002 in Georgia (Culbreath et al. 2003). More recently, second generation genotypes such as Georgia-06G, Georganic, Georgia-02C, Georgia-10T, Tifguard, and Florida-07 were developed (Culbreath and Srinivasan 2011). These new genotypes display greater levels of field resistance to TSWV than the first generation genotypes. In order to assess TSWV susceptibility, breeding lines and genotypes typically are evaluated under field conditions (Lynch 1990). However, under field conditions, thrips and TSWV pressure can vary profoundly from season to season and among locations within seasons (Mandal et al. 2001, Culbreath et al. 2003). Inconsistency in TSWV and thrips pressure can impede resistance evaluation in peanut genotypes (Mandal et al. 2001). Furthermore, screening genotypes in fields is limited to one growing season per year. To conduct second season screening, breeders will have to either wait until next year or travel to warmer places (Al-Saleh et al. 2007). Therefore, we intend to develop a greenhouse based thrips transmission protocol to screen peanut genotypes. Screening peanut genotypes under controlled conditions can help in assessing consistent performance of genotypes against thrips and TSWV. The goal here is not to replace field screening, but to supplement breeding efforts.

In order to conduct transmission assays using peanut seedlings, transmission parameters need to be optimized. Previous studies have optimized such parameters using tissue-based assays such as leaf disc assays (Wijkamp and Peters 1993, Wijkamp et al. 1996; Chatzivassiliou et al. 1999). Also, a few studies revealed significant differences between leaf disk assays and whole plant assays. Wijkamp et al. 1996 demonstrated that TSWV transmission rate in lettuce (*Lactuca sativa* L.) leaf discs was 81.7% whereas, the transmission rate was only 25% on whole plants. Differences in TSWV transmission rate were thought to be due to rapid multiplication of

TSWV in leaf discs and lower susceptibility of lettuce plants to TSWV. Also, it was speculated that plant defense mechanisms could be greater in whole plants when compared with leaf discs.

Mechanical inoculation is a common technique for TSWV inoculation to plants (Culbreath et al. 1997b, Mandal et al. 2001). Screening of genotypes via mechanical inoculation can provide important information on how different genotypes react to TSWV inoculation (Culbreath et al. 1997b). Although mechanical inoculation is a convenient and effective method for screening genotypes, it completely discounts the role of thrips in TSWV transmission, whereas in nature, TSWV is almost exclusively transmitted by thrips under field conditions (Culbreath et al. 1997b). Thrips-mediated transmission assays encompass all three components of TSWV-peanut pathosystem i. e., peanut, thrips, and TSWV. Incorporation of these important components in transmision assays could best replicate TSWV associated events under field conditions. TSWV transmission efficiency can likely be affected by *F. fusca* populations, inoculation access periods, and plant ages.

In this study, we attempted to optimize three parameters: thrips density, inoculation access period, and seedling age to evaluate peanut genotypes.

We attempted to assess TSWV transmission efficiency with four thrips densities (one, three, five, and 10 thrips), four inoculation access periods (15 minutes, 1 hour, 1 day, and 4 days) and three plant ages (one-week, two-week, and four-week old plants). Also, TSWV transmission efficiency of thrips-mediated inoculation was compared with mechanical inoculation. Furthermore, TSWV titers present in thrip inoculated and mechanically inoculated plants at different ages were assessed.

Materials and Methods

Non-infected peanut plants. Peanut plants of the cultivar Georgia Green were used for all experiments. Seeds were pre-germinated in moistened paper towels and incubated in a growth chamber at 25 to 30˚C for one week. Sprouted peanut seeds were transplanted into 4-inch diameter plastic pots (Hummert International, St. Louis, MO) in commercial potting mix, Sunshine mix (LT5 Sunshine® mix, Sun Gro® Horticulture Industries, Bellevue, WA). Peanut plants were maintained in thrips-proof cages (47.5 cm^3) (Megaview Science Co., Taichung, Taiwan) in a greenhouse at 25 to 30˚ C and at 80 to 90% RH with a 14 h photoperiod.

TSWV infected peanut plants. TSWV infected peanut plants of the cultivar, Georgia Green were initially collected from the Belflower Farm, Coastal Plain Experimental Station, Tifton, GA in 2009. Plants were maintained in a greenhouse under the conditions described previously. Leaflets from infected plants were placed in a Munger cage (11.43 X 8.89 X 1.77 cm) (Munger 1942) and non-infected *F. fusca* were released. After the next generation potentially viruliferous adults emerged, they were transferred to one-week-old plants with a paint brush (fine camel hair #2 with aluminum ferrules, Charles Leonard Inc., Hauppauge, NY). Plants were maintained in thrips-proof cages in the greenhouse as described previously. Three weeks post thrips inoculation, TSWV infection in thrips inoculated plants was assessed by double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) (Clark and Adams 1977). Leaf tissue (approximately 0.1 g) from one-third above ground portion of the plant was obtained from each plant and DAS- ELISA was performed in a 96 well microtiter plate (Maxisorp, Nunc, Rochester, NY). Along with samples, a positive control (TSWV infected peanut leaf tissue) and a negative control (non-infected peanut leaf tissue) were included in each plate. Primary antibody (anti-TSWV IgG) was used at a dilution ratio of 1:200 and the secondary

antibody (anti-TSWV IgG conjugated with alkaline phosphatase) also was used at a 1:200 dilution ratio (Agdia®, Elkhart, IN). Incubation and washing steps were followed as per the manufacturer's instructions. Final absorbance values were measured at 405 nm in a photometer 1 h after substrate addition (Model Elx 800, Bio-Tek®, Kocherwaldstr, Germany). An average absorbance value of negative control samples plus four standard deviations was considered positive. TSWV infected plants were subsequently generated by thrips-mediated inoculation.

Maintenance of non-viruliferous *F. fusca*. A *F. fusca* colony was established in 2009 on non-infected Georgia Green leaflets with thrips collected from peanut blooms at the from the Belflower Farm, Coastal Plain Experimental Station, Tifton, GA. Since then, thrips were maintained on non-infected leaflets in Munger cages in a growth chamber (Thermo scientific, Dubuque, IA) at 25 to 30˚C and a 14h photoperiod.

Maintenance of potentially viruliferous *F. fusca*. Similar to non-viruliferous thrips, a colony of potentially viruliferous thrips was maintained on TSWV infected peanut leaflets in Munger cages in a separate growth chamber as described previously. In both colonies, a majority of thrips over 95% were females. Thus, for all experiments, predominantly females, up to 2 days old were used. Infection status of thrips in colonies was assessed with antigen coated plate enzyme linked immunosorbent assay (ACP-ELISA) (Bandla et al. 1994). Adult thrips were ground individually, and ACP-ELISA was performed in a 96 well microtiter plate (Maxisorp, Nunc, Rochester, NY). Primary antibody (TSWV IgG, polyclonal nonstructural protein (NSs) (Agdia Inc., Elkhart, IN) was used at 1:500. Subsequently, secondary antibody (Anti-Mouse TSWV IgG conjugated with Alkaline Phosphatase) (SIGMA, St. Louis, MO) was used at 1:15,000. Incubation and washing steps were followed as per the manufacturer's instructions. Final absorbance values were measured at 405 nm in a photometer 1 h after substrate addition

(Model Elx 800, Bio-Tek®, Kocherwaldstr, Germany). An average absorbance value of negative control samples plus four standard deviations was considered positive.

***F. fusca*-mediated TSWV inoculation.** Four densities, one, three, five and 10 of potentially viruliferous adult *F. fusca* were used for TSWV inoculation. Ten plants were used for each thrips density and the experiment was repeated twice (N = 30 for each thrips density). Respective densities of potentially viruliferous adult thrips were transferred with a paintbrush to a 1.5 ml microcentrifuge tube. Subsequently, thrips were released on one-week old plants. After releasing thrips, approximately 0.05 g of pine (*Pinus taeda* L.) pollen grains was dusted to facilitate thrips feeding. Each plant was enclosed in a Mylar® film (Grafix®, Cleveland, PA) cage with a copper mesh top (mesh pore size-170 microns) (TWP®, Berkeley, CA). After three weeks of thrips inoculation, plants were tested by DAS-ELISA as described above.

Statistical analysis was performed to compare TSWV inoculation efficiency at different thrips densities. Treatments (thrips densities) were arranged using a completely randomized design (CRD). Treatments were considered fixed effects, and replications were considered random effects. TSWV infection was treated as a binomial response (positive or negative), and difference among treatments was evaluated using PROC GENMOD in SAS (SAS Enterprise 4.2, SAS Institute, Cary, NC). The statistical significance of differences between treatments were estimated using pairwise contrasts at $P = 0.05$.

Thrips density and inoculation access periods. Inoculation efficiencies of four densities of potentially viruliferous adult *F. fusca* (one, three, five, and 10) were evaluated at four different inoculation access periods (IAP) (15 minutes, 1 hour, 1 day, and 4 days). For each thrips density and inoculation access period, 10 plants were inoculated. The experiment was repeated once (N = 20 for each IAP and thrips density). Each density of potentially viruliferous

adult thrips was confined in a 1.5 ml microcentrifuge tube and released on one-week-old plants for each IAP. After the release of thrips, each plant was dusted with pine pollen and enclosed in a Mylar® film cage. Immediately after the IAP, plants were sprayed with an insecticide, Radiant (Dow AgroSciences, Indianapolis, IN). Three weeks post inoculation, leaflets from inoculated plants were tested with DAS-ELISA as described previously.

TSWV inoculation efficiency of each thrips density at various inoculation access periods was evaluated by using PROC GENMOD in SAS as described previously.

Plant age and TSWV susceptibility. Peanut plants of different ages were subjected to thrips-mediated inoculation to determine TSWV susceptibility. One-week, two-week, and four-week old plants were selected for the experiment. Ten potentially viruliferous adult thrips were placed in a 1.5 ml microcentrifuge tube and released on ten plants individually. The experiment was repeated twice (N = 30 for each plant age). Following thrips release, plants were dusted with pine pollen and enclosed in a Mylar® film cage as previously described. Three weeks post inoculation, TSWV infection status in inoculated plants was tested by DAS-ELISA as described previously.

TSWV infection rates among different plant ages were evaluated by using PROC GENMOD in SAS as describe previously.

Plants that tested positive for TSWV by DAS-ELISA were selected to estimate TSWV titers using reverse transcriptase real time polymerase chain reaction (RT-RT-PCR). Thirteen, twelve, and seven plants from one-week, two-week, and four-week old plants, respectively were selected. Approximately 0.1g of leaf tissue from each plant was collected from top one-third above ground portion of the plant for RNA extraction. Total RNA was extracted by using RNeasy plant mini kit (Qiagen®, Valencia, CA) as per manufacturer's instructions. The

extracted RNA was subsequently used for cDNA synthesis. Complementary DNA synthesis was performed using the GoScript™ reverse transcription system (Promega corporation, Madison, WI) following the manufacturer's instructions. Oligo (dT) (5 µM) was used as a primer for cDNA synthesis. Three microliters of RNA, 1 µl of Oligo (dT), and 1 µl of nuclease-free water were mixed and placed in a dry bath heat block (Fisher Scientific, Dubuque, IA) preheated to 70˚C for 5 min. Subsequently, 4 µl of Go script reaction buffer, 2 µl of $Mgcl_2$, 1 µl of deoxynucleotide triphosphates (dNTP), 0.5 µl of RNase inhibitor, 1 µl of reverse transcriptase, and 6.5 µl of nuclease-free water were added to the reaction mix to a total volume of 20 µl. The reaction mix was placed in a thermocycler (Bio-Rad, Hercules, CA) at 25˚C for 5 min, 42˚C for 1 h, and 70˚C for 15 min. Obtained cDNA was used as a template for RT-RT-PCR.

RT-RT-PCR was conducted using N gene specific forward and reverse primers, 5′ GCTTCCCACCCTTTGATTC3′ and 5′ ATAGCCAAGACAACACTGATC3', respectively (Rotenberg et al. 2009). The reaction mix for RT-PCR consisted of 1 µl of synthesized cDNA, 12.5 µl of GoTaq qPCR MasterMix (Promega Corporation, Madison, WI), 0.5 µl of each of the forward and reverse primers, and the final volume of reaction mix was brought to 25 µl by adding nuclease-free water. The reaction was run at 95˚ C for 2 min, followed by 40 cycles at 95˚C for 15 s, 55˚C for 60 s, and 72˚C for 20 min in a Realplex Mastercycler (Eppendorf, Hamburg, Germany). Subsequently, melting curve analysis was done after the final PCR cycle by incubating the reaction at 95˚C for 15 s, 60˚C for 15 s and then increasing the temperature by 0.5˚C per min for 20 min. Each sample was duplicated per PCR run. Also, negative (non-infected peanut tissue) and positive (TSWV infected peanut leaf tissue) controls were included in each PCR run.

Linearized plasmids and N-gene inserts were used as external standards. Plasmids with N-gene inserts were obtained by TOPA cloning following manufacturer's recommendations (Invitrogen, Carlsbad, CA). Plasmids with cloned inserts were digested with a restriction enzyme for linearization. Linearized plasmids were purified using Qiaquick PCR purification kit (Qiagen®, Valencia, CA). Following purification, they were quantified using a Nanodrop (Thermo Scientific, Wilmington, DE) and copy numbers were estimated (URI Genomics & Sequencing Center, 'http://www.uri.edu/research/gsc/resources/cndna.html'). Following which, the plasmids were serially diluted and used as external standards. The number of N-gene copies in standards ranged from 6.4 X 10^9 copies to 6.4 copies. For each standard, threshold cycle (C_t) was calculated and a standard curve was generated. TSWV titer levels in leaf tissue samples were estimated by using the standard curve.

Statistical analysis was done to compare TSWV titers in different plant ages. Treatments (plant ages) were considered as fixed effects and replications were considered as random effects. The statistical significance of differences in TSWV titers in different ages were estimated using least squares means. The analysis was performed in PROC GLIMMIX in SAS (SAS Enterprise 4.2, SAS Institute, Cary, NC)

Mechanical inoculation at different plant ages. Peanut seedlings of varying ages, one-week, two-week, and four-week old were subjected to mechanical inoculation. Mechanical inoculation was conducted using one-week-old peanut plants as described by Mandal et al. (Mandal et al. 2008). TSWV (GA isolate) infected tobacco leaves were used as a source of inoculum. For every plant, 0.1 g of infected tobacco tissue was ground in 1 ml of 0.1 M phosphate buffer (pH 7.0, containing 1.7% potassium phosphate dibasic, 1.4% potassium phosphate monobasic, 0.2% sodium sulfite and 0.01 M mercatoethanol). To this, 1% Celite 545

(Across Organics, Geel, Belgium) and 1% of Carborundum (320 grit, Fisher Scientific, Fair Lawn, NJ) were added @ 0.01g/ml of buffer. Tissues were ground until they reached soupy consistency. Before applying inoculum mix, adaxial leaf surfaces of plants were dusted with Carborundum. Subsequently, a piece of cheesecloth (American Fiber & Finishing, Inc., Burlington, MA) was soaked in the inoculum mix and applied on fully expanded leaflets with moderate pressure. After 15 min, plants were washed with water and placed in thrips-proof cages and maintained in the greenhouse as described previously. TSWV infection status in inoculated plants was assessed by DAS-ELISA three weeks post inoculation as described previously.

Ten plants were inoculated for each plant age and the experiment was repeated twice (N = 30 plants). Inoculated plants were kept in thrips-proof cages until further testing. Three weeks post inoculation, TSWV infection status in inoculated plants was tested by DAS-ELISA as described previously. Thirteen, seven, and six plants from one-week, two-week, and four-week old plants, respectively, that was DAS-ELISA positive were selected for RT-RT-PCR to estimate TSWV titer levels.

Differences in TSWV infection rates in mechanically inoculated plants at various ages were determined by using PROC GENMOD in SAS. Differences in TSWV titers in mechanically inoculated peanut plants at various plant ages were assessed using PROC GLIMMIX in SAS.

Results

***F. fusca*-mediated TSWV inoculation**: TSWV infection rate at different densities did not vary with the repeats of the experiment ($\chi 2 = 0.62$; df = 2, 27; $P > \chi 2 = 0.7334$). Hence data from all the repeats of the experiment were pooled for statistical analysis. Thrips inoculations at all densities resulted in TSWV infection in peanut plants. The incidence of TSWV infection

ranged from 46.6 ± 8.8% to 80 ± 11.5% (mean ± standard error) (Fig 3.1). The incidence of TSWV infection in plants inoculated with a single potentially viruliferous thrips was less than the infection incidence in plants inoculated with three ($\chi2 = 8.71$; df = 1, 58; $P > \chi2 = 0.0032$), and five thrips ($\chi2 = 8.71$; df = 1, 58; $P > \chi2 = 0.0032$), respectively. TSWV infection rates in peanut plants were not different when three or five potentially viruliferous thrips per plant were used for inoculation purposes (Fig. 3.1) ($\chi2 = 0$; df = 1, 58; $P > \chi2 = 1.0$). Similarly, the infection rates in peanut plants when inoculated with 10 potentially viruliferous thrips were not different than the infection rates obtained in plants inoculated with one thrips ($\chi2 = 2.95$; df = 1, 58; $P > \chi2 = 0.0859$), three thrips ($\chi2 = 1.61$; df = 1, 58; $P > \chi2 = 0.2045$), and five thrips ($\chi2 = 1.61$; df = 1, 58; $P > \chi2 = 0.2045$), respectively.

Thrips density and inoculation access periods: As in the previous case, attempted inoculations with all thrips densities resulted in TSWV infection in peanut plants. When individual thrips were used to inoculate peanut plants, TSWV infection incidence of 5 ± 5 % (mean ± standard error) was noticed on plants when provided with an IAP of 1 and 4 days (Fig 3.2). With three thrips, an IAP of 1 h and 1 day resulted in TSWV infection of up to 30 ± 30% (mean ± standard error) (Fig 3.2). Furthermore, when five potentially viruliferous thrips were used to inoculate peanut plants, an IAP of 15 min and 1 h resulted in up to 40 ± 40% (mean ± standard error) TSWV infection (Fig 3.2). With ten potentially viruliferous thrips, all IAPs resulted in TSWV infection up to 35 ± 35% (mean ± standard error) infection (Fig. 3.2). The incidence of TSWV infection with ten potentially viruliferous thrips was less when thrips were provided with IAP of 1 h compared to an IAP of 15 min ($\chi2 = 7.08$; df = 1, 38; $P > \chi2 = 0.0078$) and 1 day ($\chi2 = 5.33$; df = 1, 38; $P > \chi2 = 0.0210$) (Fig 2.2d). However, the incidence of TWSV infection was not different when thrips were provided with an IAP of 1h and 4 days ($\chi2 = 2.39$;

df = 1, 38; $P > \chi2 = 0.1221$). Also, no difference in TSWV infection incidence was observed in plants when provided with an IAP of 4 days and 15 min ($\chi2 = 1.40$; df = 1, 38; $P > \chi2 = 0.2367$) and 4 days and 1 day ($\chi2 = 0.65$; df = 1, 38; $P > \chi2 = 0.4201$). These results indicated that the TSWV infection increases with thrips density and with an IAP up to 1 day. Results reiterate that under favorable conditions, even single thrips with an IAP of 15 min can successfully transmit TSWV to susceptible peanut plants.

Plant age and TSWV susceptibility. TSWV infections at different seedling ages varied with the repeats of the experiments ($\chi2 = 10.68$; df = 2, 27; $P > \chi2 = 0.0048$). Hence, statistical analysis was performed individually for each experiment (Table 3.1). In all the repeats of the experiment, the incidences of TSWV infection incidences were different among plant ages (Table 3.1). The incidence of TSWV infection was greater in one-week old plants than in two-week and four-week old plants in the first repeat of the experiment (Table 3.1). In the second and third repeats of the experiment, the incidence of TSWV infection was greater in one-week and two week old plants than in four-week old plants (Table 3.1). TSWV N gene copies varied from $7.3 \text{ X } 10^5 \pm 356258.31$ to $13.3 \text{ X } 10^5 \pm 328432.26$ (mean ± standard error) (Fig. 3.3). The number of N gene copies in samples from four-week-old plants was less than in one-week ($F = 12.32$; df = 1, 18; $P > F = 0.0025$) and two-week old plants ($F = 9.20$; df = 1, 17; $P > F = 0.0075$). The number of N gene copies in samples from one-week and two-week old plants was not different ($F = 0.39$; df = 1, 23; $P > F = 0.5384$).

Mechanical inoculation at different plant ages. The incidence of TSWV infection in mechanically inoculated plants varied with the repeats of the experiment ($\chi2 = 10.94$; df = 2, 27; $P > \chi2 = 0.0042$). Hence, the data were analyzed separately for each repeat of the experiment. In the first repeat of the experiment, the incidence of TSWV infection in plants was different among

plant ages (Table 3.2). The incidence of TSWV in one-week old plants was greater than in four-week old plants. However, the incidence of TSWV in two-week old plants was similar to one-week old and four-week old plants. In the second and third repeats, the incidence of TSWV infection in plants at all ages was similar (Table 3.2).

The number of TSWV N gene copies in mechanically inoculated plants ranged from 11.8 X 10^5 ± 340426.63 to 18.3 X 10^5 ± 708753.73 (mean ± standard error) (Fig. 3.4). TSWV N gene copies did not vary among plant ages. TSWV titers did not vary between one-week and two-week old plants (F = 3.26; df = 1, 18; $P > F = 0.0877$), one-week and four-week old plants (F = 0.70; df = 1, 17; $P > F = 0.4144$), and two-week and four-week old plants were (F = 0.75; df = 1, 11; $P > F = 0.405$).

Thrips-mediated inoculations resulted in an incidence of TSWV infection of up to 85 ± 15% (mean ± standard error) whereas, mechanical inoculations resulted in up to 43.3 ± 12.01 % (mean ± standard error) infection. In both thrips-mediated inoculations and mechanical inoculations, the incidence of TSWV infection in one-week old plants was greater than in two-weeks and four-week-old plants. Differences in the number of TSWV N gene copies were observed in thrips inoculated plants of various ages. However, in mechanically inoculated plants of various ages, TSWV N gene copies were not different.

Discussion

Thrips density, IAP, and susceptibility of host plant stage can play an important role in *Tospovirus* transmission. All experiments were performed with the peanut genotype, Georgia Green. Georgia Green though considered as moderately resistant to TSWV, is one of the most susceptible genotypes available today and is used as a susceptible check in all genotype evaluation studies (Yang et al. 1993, Culbreath et al. 2003, Culbreath and Srinivasan 2011). In

this study, we found that thrips densities influenced TSWV infection rate, the incidence of TSWV infection was greater on plants inoculated with multiple thrips than with individual thrips. TSWV inoculation by individual thrips indicates that viral titers or viral copies released by individual thrips during feeding is sufficient to systemically infect peanut plants. Thus, lower inoculation efficiency of thrips does not necessarily indicate a titer (in thrips)-dependent response. It rather suggests that the lower incidence of TSWV infection in plants inoculated with single thrips could be due to the TSWV infection rate in individual thrips. ACP-ELISAs and western blotting targeting the non-structural protein, NSs indicated the infection rates in thrips ranged from 20 to 45% in our potentially viruliferous *F. fusca* colony. The incidence of TSWV infection in plants with 10 thrips was lower than plants inoculated with three or five thrips, though this result was not statistically significant. The slight reduction could have been due to feeding associated with various thrips densities. Feeding by greater numbers of thrips could have induced a greater degree of plant cell damage than feeding by fewer thrips, thus making them unsuitable for virus replication. Also, it has been demonstrated that female thrips feed more frequently and for longer intervals (van de Wetering et al. 1998, 1999). Unlike male thrips, females move less frequently between each probe resulting in empty cells or severely damaged cells. Predominantly female thrips were used in this study. However, increasing the number of thrips also increases the probability of viruliferous thrips (transmitters).

Even though, interactions between thrips density and IAPs were noticed, no consistent pattern of TSWV infection was observed across IAPs. An increase in IAP did not result in increased TSWV inoculation efficiency. This inconsistency could have been caused by the variability of TSWV infection rates in thrips, the distribution and the amount of titer in individual leaflets on which the potentially viruliferous thrips were reared could have influenced

this outcome. Nevertheless, results reiterate that at least 5 individual thrips can successfully inoculate a peanut plant in as few as 15 min and 1 thrips requires 1 day of IAP to inoculate TSWV in plants. Transmission assays with plants of various age groups indicated that the younger plants were more susceptible to TSWV than the older plants. Mandal et al. 2001 also observed similar results in their study. They observed an 87% TSWV infection rate when plants were mechanically inoculated at 5 days after planting. The increased susceptibility of younger plants could be due to thrips feeding preference to younger plants with less leaf thickness and high nutrient concentrations such as Nitrogen (Taiz and Zeiger 2010). Physiological changes in older plants could be attributed to the mature plant phenomenon such as leaf thickness, reduced nutrients contents, and elevated concentrations of secondary metabolites. Studies with mechanical inoculation indicated that the inoculation efficiency was less when compared with thrips-mediated inoculation and the results often were inconsistent. Several other studies have found inconsistent results with mechanical inoculation of TSWV (Halliwell and Philey 1974, Clemente et al. 1990, Pereira 1993, Hoffmann et al. 1998, Mandal et al. 2001). The precise reasons for these differences are unknown, but we speculated that the differences could be due to the tissue damage associated with mechanical inoculation. Factors associated with mechanical inoculation such as tissue damage induced by abrasives can render the cells unsuitable for TSWV replication whereas, thrips feeding often causes minimal tissue damage as opposed to mechanical inoculation. Plant response to tissue damage typically results in cell and or tissue death. Such a response could limit virus infection and systemic spread.

An estimation of TSWV N-gene copy numbers indicated that TSWV accumulation was greater in younger plants (one-week and two-week-old plants) than in older plants. This difference could be once again attributed to mature plant resistance. However, such a difference

was not documented in the case of mechanically inoculated plants. Unlike thrips-mediated inoculations, it is possible to deliver higher titers of TSWV through mechanical inoculation when compared with thrips-mediated inoculations. That being said the validity of mechanical inoculation for genotype evaluations is often questionable, as under natural conditions, TSWV is exclusively transmitted by thrips. The role of thrips in TSWV transmission is completely overlooked during mechanical inoculation (Culbreath et al. 1997b). TSWV is transmitted by thrips in a persistent and propagative manner and there are numerous intricate interactions between thrips and TSWV (German et al. 1992, Ullman et al. 1998), these details often are discounted during mechanical inoculation. That being said, mechanical inoculations can be beneficial particularly in a greenhouse setting where thrips contamination could become a serious problem.

In this study, we optimized various parameters associated with thrips-mediated TSWV transmission to develop a greenhouse based screening protocol. The goal of optimizing screening protocol is to supplement breeding programs in screening peanut genotypes. The protocol could provide an opportunity to screen promising genotypes throughout the year, which would hasten breeding efforts. Through the protocol, peanut genotypes also can be screened for different TSWV isolates and different *F. fusca* biotypes which might not be always possible in fields due to inconsistent thrips and TSWV pressure. Further, screening of genotypes under the controlled conditions can assess environmental impact and also provide an opportunity to analyze various other parameters such as virus titers (virus-plant interactions) and TSWV infection rates in thrips (thrips-virus interactions). The screening protocol can further be very useful to evaluate peanut genotypes when sufficient amounts of seeds are not available for field screening. Thus, thrips-mediated transmission protocol can be useful in several ages of the breeding process. Generally

in conventional plant breeding, genotypes are selected based on morphological traits to develop improved cultivars. Recently, marker-assisted selection (MAS) has been introduced in breeding programs to improve selection strategies by using biochemical and molecular markers (Ibitoye and Akin 2010). The greenhouse based screening protocol can be useful in screening breeding lines and genotypes derived through MAS, thus resulting in faster development of resistant genotypes.

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Figures

Fig 3.1. Incidence of *Tomato spotted wilt virus* (TSWV) infection in plants inoculated with potentially viruliferous adult at four different densities (1, 3, 5, and 10). Thirty plants were tested for each thrips density. After three weeks of thrips inoculation, infection status of plants was assessed with double antibody coated enzyme-linked immunosorbent assay (DAS-ELISA) using antibodies specific to the nucleocapsid protein (N).

Fig. 3.2. Thrips-mediated inoculation of one-week old plants with four potentially viruliferous thrips densities (1, 3, 5, and 10 thrips) at four inoculations access periods (15 min, 1 h, 1 day, and 4 days). Twenty plants were tested for each thrips density and each inoculation access period. Following inoculation, plants were given three weeks for symptoms development. *Tomato spotted wilt virus* (TSWV) infection in plants was then assessed with double antibody coated enzyme-linked immunosorbent assay (DAS-ELISA) using antibodies specific to the nucleocapsid protein (N).

Fig. 3.3. *Tomato spotted wilt virus* (TSWV) titer levels in thrips inoculated plant ages assessed with reverse transcriptase real time reverse transcriptase polymerase chain reaction (RT-RT-PCR). Plant ages were inoculated with 10 potentially viruliferous thrips. Thirteen, twelve, and seven plants from one-week, two-week, and four-week old plants, respectively, that were double antibody coated enzyme-linked immunosorbent assay (DAS-ELISA) positive were selected for RT-RT-PCR. DAS-ELISA was performed by using antibodies specific to the nucleocapsid protein (N). Linearized plasmids with N-gene inserts were purified and subsequently quantified to determine the copy number. TSWV titer levels in leaf tissue samples were estimated by using the standard curve. Average threshold cycle, C_t values of one-week, two-week and four-week old plants were 15.95, 16.57, and 17. 16, respectively. Average C_t value for healthy peanut tissue

(negative control) was 32.02 and TSWV infected peanut tissue (positive control) was 15.30.

Fig. 3.4. *Tomato spotted wilt virus* (TSWV) titer levels in different plant ages that were mechanically inoculated were assessed with reverse transcriptase real time polymerase chain reaction (RT-RT-PCR). Mechanical inoculation was performed using TSWV infected tobacco (*Nicotiana tabacum* L.) leaf tissue. Thirteen, seven, and six plants from one-week, two-week, and four-week old plants, respectively, that were double antibody coated enzyme-linked immunosorbent assay (DAS-ELISA) positive were selected for RT-RT-PCR. DAS-ELISA was performed by using antibodies specific to the nucleocapsid protein (N). Linearized plasmids with N-gene inserts were purified and subsequently quantified to determine the copy number. TSWV titer levels in leaf tissue samples were estimated by using the standard curve. Average threshold cycle, C_t values for one-week, two-week, and four-week old plants were 18.59, 18.78, and 16.97, respectively. Average C_t value for healthy peanut tissue (negative control) was 32.50 and TSWV infected peanut tissue (positive control) was 16.16.

Fig 3.1.

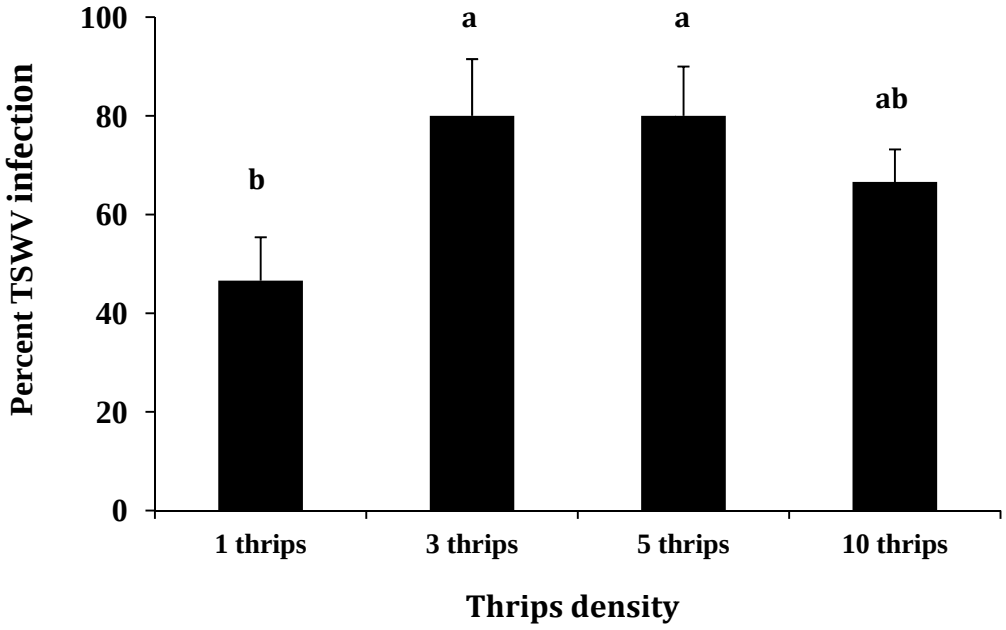
100
80
60
40
20
0
Percent TSWV infection
a
a
ab
b
1 thrips
3 thrips
5 thrips
10 thrips
Thrips density

Fig. 3.2

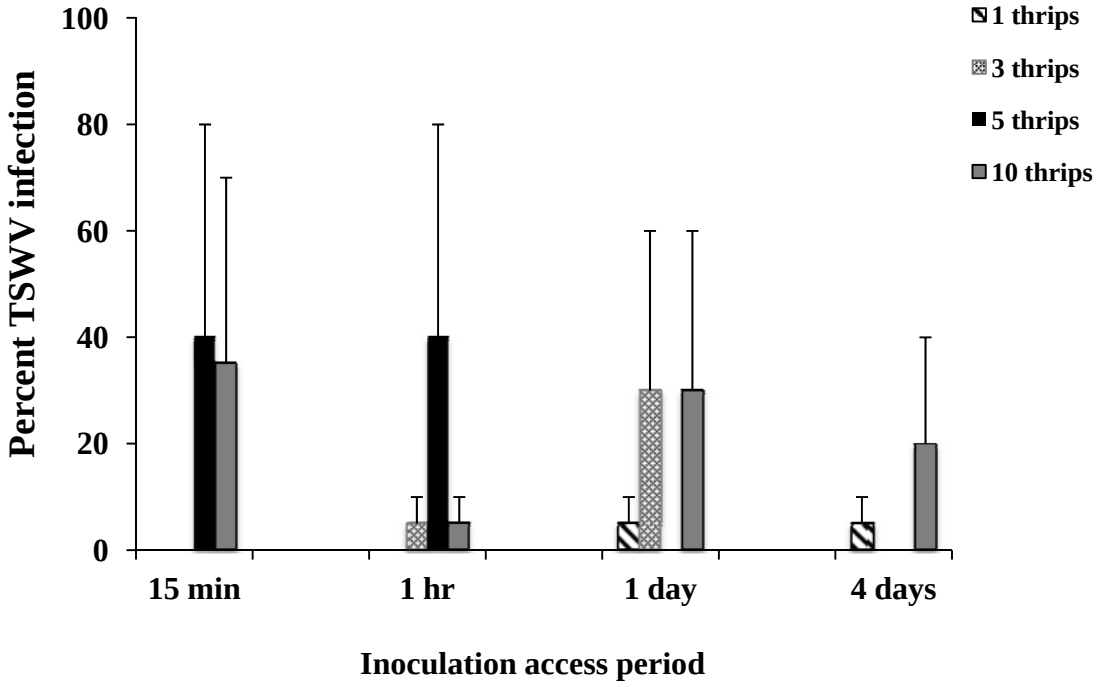
100
80
60
40
20
0
Percent TSWV infection
1 thrips
3 thrips
5 thrips
10 thrips
15 min
1 hr
1 day
4 days
Inoculation access period

Fig. 3.3

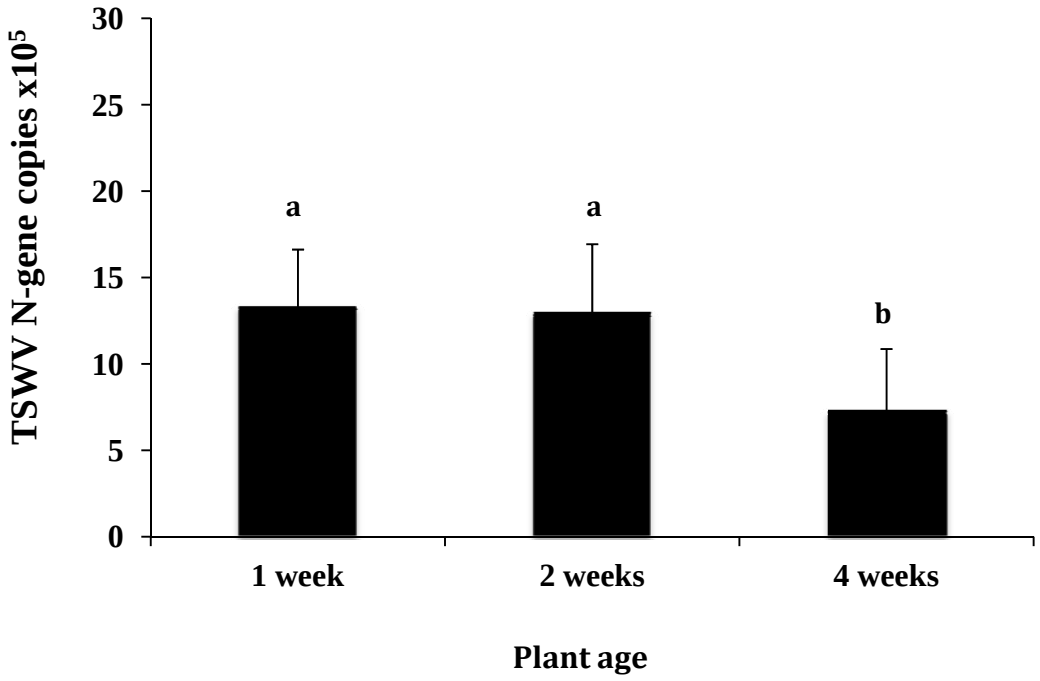
TSWV N-gene copies x10^5
30
25
20
15
10
5
0
a
a
b
1 week
2 weeks
4 weeks
Plant age

Fig. 3.4.

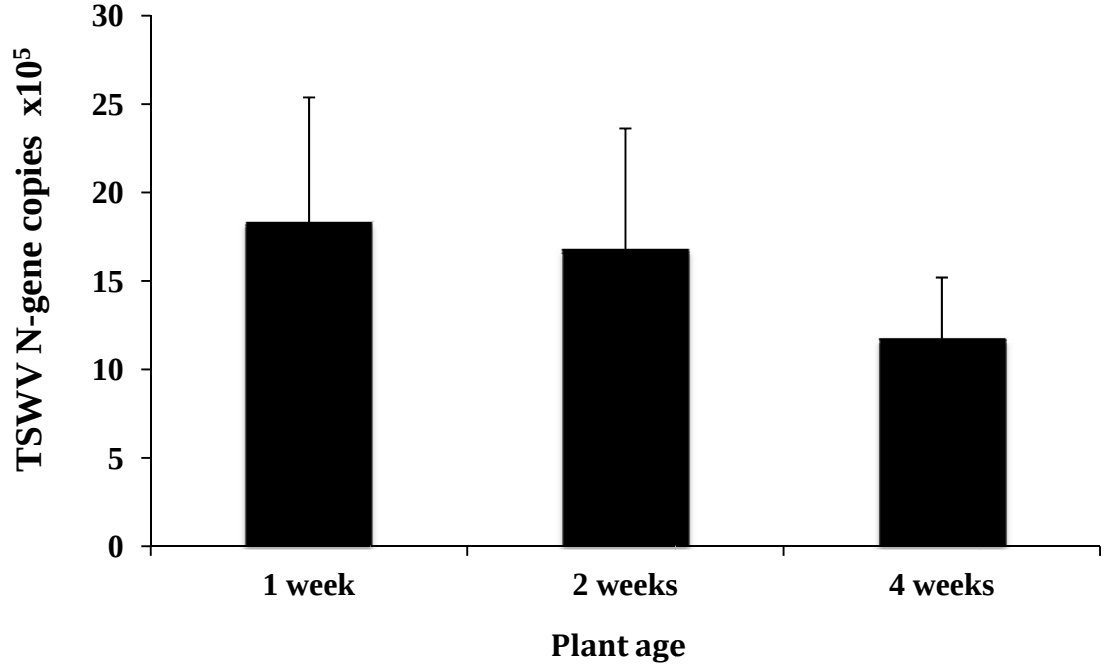
TSWV N-gene copies x10^5
30
25
20
15
10
5
0
1 week
2 weeks
4 weeks
Plant age

Table 3.1. Incidence of *Tomato spotted wilt virus* (TSWV) infection in different plant ages through thrips-mediated inoculation

	Number of plants infected/inoculated [y]			
	Repeats of the experiment [z]			
Plant ages [x]	**I**	**II**	**III**	**Incidence (%) of infected plants (Mean ± standard error)**
One week old	10/10 (A)	10/10 (A)	4/10 (A)	80 ± 20
Two weeks old	6/10 (B)	10/10 (A)	6/10 (A)	73.3 ± 13.33
Four weeks old	6/10 (B)	6/10 (B)	0/10 (B)	40 ± 20
Proc GENMOD type III analysis (df 2, 27)				
$\chi 2$	7.95	10.16	10.16	
$P > \chi 2$	0.0188	0.0062	0.0062	

[x] Plants of varying ages were subjected to thrips inoculation. Ten plants from each age were selected and 10 potentially viruliferous adults thrips (each plant) were released. The experiment was repeated twice (N = 30 plants for each plant stage). $\chi 2$ and $P >$ chi-square values are presented to indicate differences in the incidence of TSWV infection in thrips inoculated different plant ages, calculated using PROC GENMOD procedure in SAS.

[y] Thrips inoculated plants were assessed with double antibody coated enzyme-linked immunosorbent assay (DAS-ELISA) using antibodies specific to the nucleocapsid protein (N).

[z] Roman numerals I to III indicate numbers of repeats of the same experiment.

Table 3.2. Incidence of *Tomato spotted wit virus* (TSWV) infection in mechanically inoculated plants at three plant ages

	Number of plants infected/inoculated [y]			
	Repeats of the experiment [z]			
Plant ages [x]	**I**	**II**	**III**	**Incidence (%) of infected plants (Mean ± standard error)**
One week old	6/10 (A)	5/10 (A)	2/10 (A)	43.4 ± 12.01
Two weeks old	5/10 (A, B)	1/10 (A)	1/10 (A)	23.3 ± 13.33
Four weeks old	4/10 (B)	2/10 (A)	0/10 (A)	20.0 ± 11.54
Proc GENMOD type III analysis (df 4, 46)				
$\chi 2$	10.16	4.15	3.07	
$P > \chi 2$	0.0062	0.1256	0.2155	

[x] Plants of varying ages were subjected to mechanical inoculation. Ten plants from each stage were mechanically inoculated with TSWV infected tobacco (*Nicotiana tabacum* L.) leaf tissues and the experiment was repeated twice (N = 30 plants for each plant stage). $\chi 2$ and $P >$ chi-square values are presented to indicate differences in the incidence of TSWV infection in mechanically inoculated plants of various ages, calculated using PROC GENMOD procedure in SAS.

[y] Mechanically inoculated plants were assessed with double antibody coated enzyme-linked immunosorbent assay (DAS-ELISA) using antibodies specific to the nucleocapsid protein (N) there weeks post inoculation.

[z] Roman numerals I to III indicate numbers of repeats of the same experiment.

CHAPTER 4

EVALUATION OF *TOMATO SPOTTED WILT VIRUS* (TSWV)-RESISTANT AND SUSCEPTIBLE PEANUT GENOTYPES AGAINST THRIPS-MEDIATED TSWV TRANSMISSION[1]

[1] Shrestha. A., R. Srinivasan, A. Culbreath, and D. Riley. 2011. To be submitted

Abstracts

Spotted wilt disease caused by thrips transmitted *Tomato spotted wilt virus* (TSWV) is a major threat to peanut growers in the southeastern United States. Reducing yield losses to TSWV has largely relied on the use of resistant genotypes. Several genotypes and breeding lines exhibit resistance and/or tolerance to TSWV. Mechanisms responsible for resistance to TSWV in peanut genotypes have not been determined. In this study, we investigated effects of resistant genotypes (Georganic, Georgia-06G, Tifguard, NC94022) and a moderately resistant genotype (Georgia Green) on thrips and TSWV. Thrips-mediated inoculation resulted in TSWV infection in all the genotypes. Estimation of TSWV titers indicated that some resistant genotypes accumulated less TSWV titers than susceptible genotypes. Western blotting demonstrated that thrips can acquire TSWV from both resistant and susceptible genotypes. Further, thrips biology studies on different genotypes revealed no differences in the number of adults produced among genotypes except for Georgia Green. The developmental time required to complete one generation (adult to adult) also was greater on Georgia Green than on other genotypes. Further, head capsule measurements of thrips reared on different genotypes showed no differences in head capsule length and width. These results indicate that resistance in peanut genotypes may be governed by a multitude of minor genes resulting in a phenomenon similar to tolerance. Results also indicate that peanut genotypes can have a minor impact on thrips biology.

Introduction

Tomato spotted wilt virus (TSWV) is an economically important pathogen that causes spotted wilt disease in peanut. Since the first report of spotted wilt in peanut in Texas in 1971, yield reduction of up to 95% has been observed in several counties in Texas (Halliwell and Philey 1974, Black 1987). In Georgia, spotted wilt incidence became increasingly common after

1986 (Culbreath et al. 1990, 1991). Average annual losses to spotted wilt from 1996 to 2006 have been estimated to be $12.3 million in Georgia alone (Riley et al. 2011). TSWV is transmitted persistently by several species of thrips (Thysanoptera, Thripidae) (German et al. 1992, Pappu et al. 2009, Riley et al. 2011). Tobacco thrips, *Franklinella fusca* (Hinds) and western flower thrips, *Frankliniella occidentalis* (Pergande) are the two known vectors of TSWV in peanut (Culbreath et al. 1997b). However, *F. fusca* is considered most important in the southeastern United States as it readily colonizes peanut plants (Todd et al. 1995, 1996).

Soon after occurrence of spotted wilt in the southeastern United States, application of insecticides such as aldicarb and phorate were evaluated for utility in spotted wilt management. However, only phorate provided any consistent suppression of spotted wilt. Due to the severity of spotted wilt epidemics, application of phorate was not adequate to substantially reduce yield losses on susceptible genotypes (Culbreath et al. 2003). Subsequently, planting of TSWV resistant peanut genotypes in combination with altered cultural practices such as planting date, plant density, and tillage options significantly reduced yield losses (Culbreath et al. 2003). Currently, planting resistant genotypes is considered to be the most important option for spotted wilt management. In the recent years, numerous genotypes exhibiting moderate to high levels of field resistance to TSWV have been developed by breeding programs in Georgia and Florida (Culbreath et al. 1999a, 1999b; Mandal et al. 2006). For most of the genotypes developed to date, PI 203396 is the presumed source of resistance to TSWV (Isleib et al. 2001). Recently, a new source of resistance, PI 576638 also has been used to develop several breeding lines (Barrientos-Priego et al. 2002).

Until recently, in the southeastern United States, first-generation genotypes with moderate levels of field resistance to TSWV were grown widely. Several runner type genotypes

such as Southern Runner, Georgia Browne, Georgia Green, Tamrun 96, UF MDR 98, and ViruGard were released from 1984 to 1997 (Gobert et al. 1987; Culbreath et al. 1994, 1996; Smith et al. 1998; Gobert and Shokes 2002; Branch et al. 2003). These genotypes appreciably suppressed spotted wilt when compared with susceptible standards that were grown before TSWV epidemics became severe such as Florunner, Georgia Runner, SunOleic 97R, and GK-7 (Culbreath et al. 2003). Among the runner type genotypes, Georgia Green became the predominant cultivar grown in production areas of Georgia, Alabama, and Florida after 1998 (Culbreath et al. 2000). Use of Georgia Green combined with other cultural practices provided consistent suppression of spotted wilt and also increased yield (Culbreath et al. 2003). Despite being moderately resistant to TSWV, severe epidemics of spotted wilt were common in Georgia Green in high-risk situation such as when early planting dates were used and high plant populations were not established. Research efforts have led to the development of second generation TSWV resistant genotypes such as Georganic, Georgia-06G, and Tifguard (Culbreath and Srinivasan 2011). These genotypes exhibit more resistance to TSWV than Georgia Green and allow more flexibility in managing spotted wilt. Breeding lines with high levels of field resistance to TSWV also have been identified such as F NC 94002 and F NC 94022 (Baldessari 2008, Culbreath et al. 2005). All the resistant genotypes that have been developed so far impart varying levels of partial resistance to TSWV. None of the genotypes developed to date are immune to TSWV (Culbreath et al. 1997b). In contrast to other crops, the genes and mechanisms that impart resistance to TSWV have not been identified in peanut. Genes conferring resistance to TSWV have been identified in tomato (*Solanum lycopersicon* L.) and pepper (*Capsicum annuum* L.). Dominant genes, *Sw-5* and *Tsw* induce resistance to TSWV in tomato and pepper, respectively (Stevens et al. 1991, Boiteux and de Avila 1994, Stevens et al. 1994, Black et al.

1996, Rosello et al. 1998). These single dominant genes confer resistance by triggering hypersensitive reaction (HR) (Stevens et al. 1994, Moury et al. 1997). Several studies have demonstrated that reduced incidence of spotted wilt in peanut was not due to reduced attractiveness to thrips vector or reduced reproduction of thrips vectors (Culbreath et al. 1992, 1994, 1996, 2000). Based on these observations, it is believed that in peanut genotypes, resistance is mainly imparted against TSWV.

The objective of this study was to examine the effects of TSWV resistant genotypes on thrips and TSWV. We evaluated second-generation newly released peanut genotypes by thrips-mediated transmission and speculated on the mechanisms of resistance. Some of the resistant genotypes selected for this study have never been evaluated in a greenhouse or laboratory conditions and doing so would provide more insights on the interactions of these genotypes with thrips and TSWV. Along with new resistant genotypes, Georgia Green was included as a standard in this study. Although Georgia Green has moderate levels of field resistance to TSWV, when compared with recently released second-generation genotypes, it is much more susceptible to TSWV. Hence, at present, Georgia Green is used as a susceptible check for all field evaluations. We evaluated TSWV transmission efficiency, TSWV associated symptoms, and TSWV titers in resistant and susceptible genotypes. We further determined TSWV acquisition ability of *F. fusca* from resistant and susceptible genotypes. Effects of TSWV resistant and susceptible genotypes on thrips biology also were examined.

Materials and Methods

Non-infected peanut plants. Peanut plants of the genotypes Georgia Green, Georganic, Georgia-06G, Tifguard, and NC94022 were used for all experiments. Georgia Green was released in 1995 (Branch 1996), Georganic in 2006 (Holbrook and Culbreath 2008), Georgia-

06G in 2006 (Branch 2007), and Tifguard in 2007 (Holbrook et al. 2008). NC94022 was developed from a cross-made by T. G. Isleib in North Carolina between N91026E, an early maturing virginia-type line, and a selection from a hirsuta-type line, PI 576638 from Mexico (Culbreath et al. 2005, Sanchez-Dominguez and William 1993). Seeds were pre-germinated in moistened paper towels and incubated in a growth chamber at 25 to 30˚C for one week. Sprouted peanut seeds were transplanted into 4-inch diameter plastic pots (Hummert International, St. Louis, MO) in commercial potting mix, Sunshine mix (LT5 Sunshine® mix, Sun Gro® Horticulture Industries, Bellevue, WA). Peanut plants were maintained in thrips-proof cages (47.5 cm^3) (Megaview Science Co., Taichung, Taiwan) in a greenhouse at 25 to 30˚ C and 80 to 90% RH with a 14 h photoperiod. For all experiments, one-week old plants were used.

TSWV infected peanut plants. TSWV infected peanut plants of the genotype Georgia Green were initially collected from the Belflower Farm, Coastal Plain Experimental Station, Tifton, GA in 2009. Plants were maintained in a greenhouse under conditions previously described. Leaflets from infected plants were placed in a Munger cage (11.43 X 8.89 X 1.77 cm) (Munger 1942) and non-infected *F. fusca* were released. After the next generation of potentially viruliferous adults emerged, they were transferred to one-week old plants with a paint brush (fine camel hair #2 with aluminum ferrules, Charles Leonard Inc., Hauppauge, NY). Plants were maintained in thrips- proof cages in the greenhouse as described previously. Three weeks post thrips inoculation, TSWV infection in thrips inoculated plants was assessed by double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) (Clark and Adams 1977). Leaf tissue (approximately 0.1 g), was obtained from each plant and DAS-ELISA was performed in a 96 well microtiter plate (Maxisorp, Nunc, Rochester, NY). Along with samples, a positive control (TSWV infected peanut leaf tissue) and a negative control (non-infected peanut leaf

tissue) were included in each plate. Primary antibody (anti-TSWV IgG) was used at a dilution ratio of 1:200 and the secondary antibody (anti-TSWV IgG conjugated with alkaline phosphatase) also was used at a 1:200 dilution ratio (Agdia®, Elkhart, IN). Incubation and washing steps were followed as per the manufacturer's instructions. Final absorbance values were measured at 405 nm in a photometer 1 h after substrate addition (Model Elx 800, Bio-Tek®, Kocherwaldstr, Germany). An average absorbance value of negative control samples plus four standard deviations was considered positive. TSWV infected plants were subsequently generated by thrips-mediated inoculation. Similar to Georgia Green, TSWV infected plants of the genotypes Georganic, Georgia-06G, Tifguard, and NC94022 were obtained through thrips-inoculation.

Maintenance of non-viruliferous *F. fusca*. A colony of *F. fusca* was established in 2009 on Georgia Green non-infected leaflets with thrips collected from peanut blooms from the Belflower Farm, Coastal Plain Experimental Station, Tifton, GA. Since then, thrips were maintained in Munger cages on non-infected Georgia Green leaflets. Munger cages were maintained in a growth chamber (Thermo scientific, Dubuque, IA) at 25 to 30˚C and a 14h photoperiod.

M**aintenance of potentially viruliferous *F. fusca***. Similar to non-viruliferous thrips, a colony of potentially viruliferous thrips was maintained on TSWV infected Georgia Green leaflets in Munger cages in a growth chamber as described previously. In both colonies, over 95% of the thrips were females. Thus, for all experiments, predominantly females, up to 2 days old were used. Infection status of thrips colonies was regularly assessed with antigen coated plate enzyme linked immunosorbent assay (ACP-ELISA) (Bandla et al. 1994). Adult thrips were ground individually and ACP-ELISA was performed in a 96 well microtiter plate (Maxisorp,

Nunc, Rochester, NY). Primary antibody (TSWV IgG, polyclonal nonstructural protein (NSs) (Agdia Inc., Elkhart, IN) was used at 1:500. Subsequently, secondary antibody (Anti-Mouse TSWV IgG conjugated with Alkaline Phosphatase) (SIGMA, St. Louis, MO) was used at 1:15,000. Incubation and washing steps were followed as per the manufacturer's instructions. Final absorbance values were measured at 405 nm in a photometer 1 h after substrate addition (Model Elx 800, Bio-Tek®, Kocherwaldstr, Germany). An average absorbance value of negative control samples plus four standard deviations was considered positive. ACP-ELISA indicated that the infection rate in the potentially viruliferous thrips was from 20 to 45%.

TSWV transmission to resistant and susceptible genotypes. Four resistant genotypes (Georganic, Georgia-06G, Tifguard, and NC94022) and a susceptible genotype (Georgia Green) were evaluated for TSWV susceptibility. Ten plants of each genotype were used in the experiment, and the experiment was repeated twice (N = 30 plants for each genotype). Ten potentially viruliferous *F. fusca* developed on TSWV infected leaflets were transferred to a 1.5 ml microcentrifuge tube (Fisher Scientific, Pittsburgh, PA) by a paint brush (fine camel hair #2 with aluminum ferrules, Charles Leonard Inc., Hauppauge, NY). Thrips were subsequently released on peanut plants (ten potentially viruliferous thrips per plant) that have been dusted with approximately 0.05 g of pine (*Pinus taeda* L.) pollen grains. Each plant was enclosed in a Mylar® film (Grafix®, Cleveland, PA) cage with a copper mesh top (mesh pore size-170 microns) (TWP®, Berkeley, CA). Plants were maintained in the greenhouse for three weeks, following which TSWV infection status was assessed with DAS-ELISA as described before.

A completely randomized design (CRD) was used. Treatments (genotypes) were considered as fixed effects and replications were considered random effects. TSWV inoculation efficiency was compared among the different genotypes. TSWV infection was treated as a

binomial response (positive or negative), and differences among treatments were estimated by using PROC GENMOD in SAS (SAS Enterprise 4.2, SAS Institute, Cary, NC). The statistical significance of differences between treatment pairs were estimated using pairwise contrasts at $P = 0.05$.

TSWV titer levels in TSWV resistant and susceptible peanut genotypes. Titer levels were estimated using reverse transcriptase real time polymerase chain reaction (RT-RT-PCR). Leaf samples from plants that tested positive by DAS-ELISA were used for RT-RT-PCR. Numbers of plants selected from Georgia Green, Georganic, Georgia-06G, Tifguard, and NC94022 for RT-RT-PCR were thirteen, eight, ten, eight, and eight respectively. Symptomatic leaflet tissues (approximately 0.1g) were collected from top one-third above ground section of the plant for RNA extraction. Total RNA was extracted by using RNeasy plant mini kit (Qiagen®, Valencia, CA) as per manufacturer's instructions. The extracted RNA was subsequently used for complementary DNA (cDNA) synthesis. cDNA synthesis was performed using the Go-Script™ reverse transcription system (Promega corporation, Madison, WI) following the manufacturer's instructions. Oligo (dT) (5 µM) was used as a primer for cDNA synthesis. Three microliter (µl) of RNA, 1 µl of Oligo (dT), and 1 µl of nuclease-free water were mixed and placed in a dry bath heat block (Fisher Scientific, Dubuque, IA) preheated to 70˚C for 5 min. Subsequently, 4 µl of Go script reaction buffer, 2 µl of $Mgcl_2$, 1 µl of deoxynucleotide triphosphates (dNTP), 0.5 µl of RNase inhibitor, 1 µl of reverse transcriptase, and 6.5 µl of nuclease-free water, were added to the reaction mix to a total volume of 20 µl. The reaction mix was placed in a thermocycler (Bio-Rad, Hercules, CA) at 25˚C for 5 min, 42˚C for 1 h, and 70˚C for 15 min. Obtained cDNA was used as a template for RT-RT-PCR.

RT-RT-PCR was conducted using N gene specific primers. The forward and reverse primers, 5′ GCTTCCCACCCTTTGATTC3′ and 5′ ATAGCCAAGACAACACTGATC3', respectively were used (Rotenberg et al. 2009). The reaction mix for RT-PCR consisted of 1 µl of synthesized cDNA, 12.5 µl of GoTaq qPCR MasterMix (Promega Corporation, Madison, WI), 0.5 µl of each of the forward and reverse primers, and the final volume of reaction mix was brought to 25 µl by adding nuclease-free water. The reaction was run at 95˚ C for 2 min, followed by 40 cycles at 95˚C for 15 s, 55˚C for 60 s, and 72˚C for 20 min in a Realplex Mastercycler (Eppendorf, Hamburg, Germany). Subsequently, melting curve analysis was done after the final PCR cycle by incubating the reaction at 95˚C for 15 s, 60˚C for 15 s and then increasing the temperature by 0.5˚C per min for 20 min. Each sample was duplicated per PCR run. Also, negative (non-infected peanut tissue) and positive (TSWV infected peanut leaf tissue) controls were included in each PCR run.

Linearized plasmids and N-gene inserts were used as external standards. Plasmids with N-gene inserts were obtained by TOPA cloning following manufacturer's recommendations (Invitrogen, Carlsbad, CA). Plasmids with cloned inserts were digested with a restriction enzyme for linearization. Linearized plasmids were purified using Qiaquick PCR purification kit (Qiagen®, Valencia, CA). Following purification, they were quantified using Nanodrop (Thermo Scientific, Wilmington, DE) and copy numbers were estimated (URI Genomics & Sequencing Center 'http://www.uri.edu/research/gsc/resources/cndna.html'). Following which plasmids were serially diluted and used as external standards. The number of N-gene copies typically ranged from 6.4 X 10^9 copies to 6.4 copies. For each standard, threshold cycle (C_t) was calculated and a standard curve was generated. TSWV titer levels in leaf tissue samples were estimated by using the standard curve.

Treatments (genotypes) were considered as fixed effects and replications were considered as random effects. TSWV titers were compared among the genotypes. The analysis was performed by using PROC GLIMMIX in SAS (SAS Enterprise 4.2, SAS Institute, Cary, NC). The statistical significance of differences between TSWV titers in different genotypes were estimated by using least squares means.

TSWV acquisition by *F. fusca* from TSWV infected resistant and susceptible genotypes. Non-viruliferous adult thrips were transferred to Munger cages with TSWV infected leaflets of resistant (Georganic, Georgia-06G, Tifguard, and NC94022) and susceptible genotypes (Georgia Green). Thrips were maintained on TSWV infected leaflets belonging to each genotype independently. The next generation potentially viruliferous adults from each colony were subjected to western blotting to detect their ability to transmit TSWV using antibodies for the non-structural protein (NSs) of TSWV. The presence of NSs is an indication of TSWV replication inside its thrips vector and its TSWV transmission status (Ullman et al. 1993). A total of three runs were conducted. In the first run, one thrips from each genotype was tested for the presence of TSWV-NSs. Similarly, three and five thrips from each genotype also were tested by western blotting.

Western blotting was conducted as described by Laemmli 1970. The gel cast (BIO-RAD, Hercules, CA) was assembled with glass plates. Resolving gel (12%) was prepared with 3.75 ml of 1.5 M Tris-HCL, pH 8.8, 6 ml of 30% acrylamide, 4.9 ml of sterile water, 150 µl of 10% SDS, 150 µl of 10% ammonium persulphate (APS), 5 µl of Tetramethylethylenediamine (TEMED). This solution (7 ml) was poured between glass plates and covered with sterile water and left to set for 30 min. After which, water was poured off and 4% stacking gel was prepared. Stacking gel included 1.5 ml of 0.5 M Tris-HCL, pH 6.8, 800 µl of 30% Acrylamide, 3.575 ml of sterile

water, 60 µl of 10% SDS, 60 µl of 10% APS, 5 µl of TEMED. Stacking gel was poured on the top of already set resolving gel and a gel comb was inserted. This was allowed to set for 30 min the comb was removed.

The gel was removed from casting apparatus and placed in a gel tank (BIO-RAD, Hercules, CA). The gel tank was filled with running buffer containing 3 g of 25 mM Tris base, 14.4 g of 250 mM Glycine, and 1% SDS in 1000 ml sterile water. To extract total proteins, adult thrips were ground in 50 µl PBS (0.01 M sodium-potassium phosphate buffer pH 7.4, containing 0.02% sodium azide (w/v), 0.8% sodium chloride (w/v), 2% PVP mol wt. 40,000 (w/v)). Subsequently, 50 µl of the mixture (2.5 ml of 0.5 M Tris-HCL, pH 6.8, 2ml glycerol, 2ml of 10% SDS, 500 µl beta-mercaptoethanol, 0.001 g of bromophenol blue) was added to samples and boiled in a water bath (Thermo Scientific, Marietta, OH) at 95˚F for 5 min. Samples (20 µl each) were then loaded into wells. Each gel included a pre-stained protein marker (10 to 170KDa) (Fisher Scientific, Pittsburg, PA). A positive (TSWV infected peanut leaf tissue) and a negative control (Non-infected peanut leaf tissue) also were loaded in each gel. The gel was run at 75 V with a BioRad PowerPac (BIO-RAD, Hercules, CA) until the maker started to separate and then it was increased to 100 V.

Contents of the gel were transferred to nitrocellulose membrane (Whatman, Schleicher & Schuell, Dassel, Germany). The gel was removed from the glass plates and carefully sandwiched in a transfer cassette in the order of: black cassette, sponge, blotting paper (BIO-RAD, Hercules, CA), gel, nitrocellulose membrane, blotting paper, sponge, and white side of cassette. The transfer cassette was loaded into the gel tank and transfer buffer (3.03 g of 25mM Tris base, 14.4 g of 250 mM, Glycine, 200 ml of methanol, 1 L of sterile water, pH 8.3) was poured. Electrophoresis was performed at 75 V for 3 h.

Following transfer of proteins, the membrane was blocked with 5% non-fat dry milk (LabScientific, Inc., Pleasant Avenue Livington, NJ) in PBS at 4˚C overnight. The membrane was washed 3 times for 10 min with PBS/Tween (0.05%). Subsequently it was incubated with the primary antibody (TSWV IgG, polyclonal nonstructural protein (NSs) (Agdia Inc., Elkhart, IN) for 2 h at a 1:500 dilution ratio in PBST and 5% non-fat dry milk. Following 3 washes, the membrane was incubated with the secondary antibody (Anti-Mouse TSWV IgG conjugated with Alkaline Phosphatase) (SIGMA, St. Louis, MO) same as above at 1:15,000 for 2 h at the room temperature. The membrane was again washed 3 times and incubated in 15 ml sterile water with a Sigma FAST^{TM} (5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium) (BCIP/ NBT) tablet (SIGMA-ALDRICH, St. Louis, MO) for 5 min. Samples that tested positive for TSWV-NSs produced a band (approximately 55kDa).

Effects of TSWV resistant genotypes on thrips reproduction and development. Leaflets from four resistant genotypes (Georganic**,** Georgia-06G, Tifguard, NC94022) and a susceptible genotype (Georgia Green) were used for this experiment**.** Ten Munger cages were set up for each genotype with two non-infected leaflets of respective genotypes. The experiment was repeated once (N = 20 cages for each genotype). Ten non-viruliferous female adults (up to 2 days old) were released in each Munger cage. The released adult thrips were removed from the cage five days later. The cages were monitored daily under a compound microscope (MEIJI TECHNO, Santa Clara, CA) and the number of newly hatched larvae were recorded. The number of adults emerging from each cage was recorded at 24 h intervals and the adults were removed. The cages were monitored until there were no more larvae or adults in each cage. Differences in the number of adults produced and the developmental time of adults reared on different genotypes were determined by using PROC GLIMMIX in SAS as described previously.

Effects of TSWV resistant genotypes on thrips size. Head capsule length and width of thrips reared on TSWV resistant (Georganic, Georgia-06G, Tifguard, NC94022) and susceptible (Georgia Green) peanut genotypes were measured. Ten female adult thrips (up to 2 days old) from each genotype were used and the experiment was repeated once (N = 20 thrips from each genotypes). Head capsule was measured using an ocular micrometer fitted in a dissecting microscope (MEIJI TECHNO, Santa Clara, CA). Number of ticks in the ocular scale of the microscope was calibrated with a stage micrometer (LEICA, Buffalo Grove, IL) at 75X magnification. Comparison of head capsule length and width of thrips reared on different genotypes were made by using PROC GLIMMIX in SAS as described previously.

Results

TSWV transmission to resistant and susceptible genotypes. Incidence of TSWV infection varied with the repeats of the experiment ($\chi 2 = 12.19$; df = 2, 27; $P > \chi 2 = 0.0068$). In the first repeat of the experiment there were no differences in the incidence of TSWV infection among genotypes (Table 4.1). But, in the second repeat of the experiment, the incidence of TSWV infection varied among genotypes (Table 4.1). TSWV infection rate in Georgia Green was greater than the infection rates in all other genotypes. In the third repeat of the experiment again there was no difference in TSWV infection rate among genotypes (Table 4.1).

Thrips-mediated inoculation lead to TSWV symptoms development in all the genotypes with no variations (Fig. 4.1). All the genotypes expressed typical TSWV symptoms. Symptoms appeared as early as 7 to 9 days in some of the genotypes such as Georgia Green and Georgia-06G (Fig. 4.1a and 4.1b, respectively). TSWV induced symptoms were first observed in terminals. Terminals of infected genotypes were typically yellow in color and had droopy appearance. In immature leaflets, TSWV initially induced chlorotic spots on adaxial leaflet

surfaces. As the disease progressed, leaflets were found with several necrotic spots and often appeared mottled. These leaflets quickly fell off. Stunting of the plants was also observed in all the infected genotypes. In mature leaflets, chlorotic spots, ring spots with necrotic centers and chlorotic line pattern were observed. These symptoms were prominent in Georgia Green than rest of the genotypes (Fig. 4.1a). Also, TSWV induced symptoms were expressed in more number of leaflets of the genotypes, Georgia Green (Fig. 4.1a), Georgia-06G (Fig 4.1b), and Tifguard (Fig 4.1d) than in Georganic (Fig. 4.1c) and NC94022 (Fig. 4.1e).

TSWV titer levels in TSWV resistant and susceptible peanut genotypes. TSWV titers were estimated as equivalents of N-gene copy numbers. TSWV titers varied with genotypes ($F = 4.64$; df = 4, 42; $P > F = 0.0034$). The numbers of N-gene copies in Georgia Green and NC94022 were greater than all other genotypes (Fig 4.2). The numbers of N-gene copies were not different between Georgia Green and NC94022 ($F = 0.13$; df = 1, 20; $P > F = 0.7222$). Also, there were no differences in TSWV N-gene copy numbers among GA-06G, Tifguard, and Georganic (Fig. 4.2).

TSWV acquisition by *F. fusca* from TSWV infected resistant and susceptible genotypes. Western blotting demonstrated the presence of NSs in thrips that developed on all genotypes tested except for Georgia-06G. A 55 KDa band in the blots is an indication of the presence of TSWV-NSs. The incidence of TSWV infection in thrips reared on different genotypes varied with the number of thrips tested. When individual thrips from each genotype was tested, only thrips reared on Georgia Green tested positive for TSWV-NSs (Fig 4.3a). When three and five thrips were tested, thrips that developed on TSWV infected Georgia Green, Georganic, Tifguard, and NC94022 were positive for TSWV-NSs (Figs. 4.3b and Fig 4.3c). The

presence of NSs in thrips indicated that the virus replicates in thrips and that thrips have successfully acquired TSWV and can potentially transmit TSWV.

Effects of TSWV resistant genotypes on thrips reproduction and developmental time. The number of adults produced on leaflets of different genotypes did not vary with the repeats of the experiment (F = 0.63; df = 1, 18 *P* > F = 0.4312). Hence data from both repeats of the experiment were pooled for statistical analysis. The number of adults produced by each female released on different genotypes ranged from 1.12 ± 0.25 to 2.02 ± 0.36 (mean ± standard error) (Fig. 4.4). The number of adults produced on Georgia Green leaflets was more than the number of adults produced on Tifguard leaflets (F = 5.32; df = 1, 38; *P* >F = 0.0266). No differences in adult emergence rates were noticed among other genotypes (Fig. 4.4).

The developmental time required to complete one generation (adult to adult) on leaflets of different genotypes did not vary with the repeats of the experiment (F = 1.79; df = 1, 18; *P*> F = 0.197). Hence, data from both repeats were pooled for statistical analysis. The developmental time ranged from 11.5 ± 0.37 to 14.55 ± 0.37 days (mean ± standard error) (Fig. 4.5). Thrips reared on Tifguard leaflets developed faster than thrips that were reared on leaflets of Georganic (F = 13.48; df = 1, 38; *P* >F = 0.0007), Geogia-06G (F = 23.97; df = 1, 38; *P* >F = 0.0001), NC94022 (F = 25.07; df = 1, 38; *P* >F = 0.0001), and Georgia Green (F = 46.07; df = 1, 38; *P* >F = 0.0001). Also, thrips reared on Georganic leaflets developed faster than thrips reared on leaflets of Georgia Green (F = 9.71; df = 1, 38; *P* >F = 0.0035).

Effects of TSWV resistant genotypes on thrips size. Thrips head capsule length and width did not vary with the repeats of the experiment (F = 0.17; df = 1,18; *P* >F = 0.6850) and (F = 3.32; df = 1,18; *P* >F=0.0851), respectively. Hence, data from both repeats were pooled for statistical analysis. Head capsule length of adult thrips that were reared on different genotypes

did not vary with genotypes (F = 0.53; df = 4, 95; *P* >F = 0.7140) (Fig 4.6a). Also, head capsule width of adult thrips that were reared on different genotypes did not vary with genotypes (F = 1.55; df = 4, 95; *P* >F = 0.1941) (Fig 4.6b). These results indicate that the genotypes did not have any effect on thrips size.

Discussion

Thrips-mediated inoculations resulted in TSWV infection in both resistant genotypes and Georgia Green. However, TSWV infection rates were lower in resistant genotypes when compared with Georgia Green. Culbreath et al. 2005 also documented reduced spotted wilt incidence on Georganic and NC 94022 when compared with Georgia Green during field screening. Further, typical TSWV symptoms such as yellowing of the terminals with droopy appearance, concentric ringspots, necrotic spots, chlorotic line patterns and stunting were noticed on all TSWV infected plants of all genotypes. But, observations revealed that fewer symptomatic leaflets were found on Georganic and NC 94022, these responses also were documented by Culbreath et al. (2005) during field screening. Despite the variation in the severity, TSWV associated symptoms often were observed throughout the plant, even on leaflets with no evidence of thrips feeding injury. This indicated that the virus movement was not restricted to the inoculation sites alone. Studies that conducted mechanical inoculation on resistant and susceptible genotypes indicated that the TSWV infection in genotypes was systemic (Mandal et al. 2002, 2006). These responses were very different from what was observed with other crop hosts that are resistant to TSWV, such as tomato and pepper. HR is often associated with *Tospovirus* inoculations in tomato and pepper that are resistant to TSWV (Stevens et al. 1994, Moury et al. 1997). Resistance genes are known to induce HR, during which, cells at or around virus entry sites are rapidly terminated (Flor 1942). This type of response helps in

confining viral infections to lesions and their adjacent cells thus, preventing systemic movement of the virus. HR induced TSWV resistance in tomato and pepper is believed to be mediated by a single dominant gene (Stevens et al. 1994, Black et al. 1996, Moury et al. 1997). The mechanisms of TSWV resistance in peanut are not known and genes that are associated with TSWV resistance have not been identified and/or characterized so far. However, in this study no evidence of the presence of major genes or dominant genes that confer resistance to TSWV was found.

Attempts to quantify TSWV titers by estimating TSWV N gene copies in resistant and susceptible genotypes revealed significant differences among genotypes. TSWV titers in Georgia Green and NC94022 were greater than in Georganic, Georgia-06G, and Tifguard. Despite the reduced TSWV infection rates, TSWV titer levels in NC94022 were similar to Georgia Green. This revealed that TSWV infection rates in genotypes do not necessary correlate with their TSWV titer levels. Further, this study indicated that resistant genotypes also can accumulate high TSWV titers. Mandal et al. 2002, estimated TSWV titer levels in mechanically inoculated Georgia Green, Georgia Runner, and C-99R. TSWV titer levels in these genotypes differ significantly. Also, their results indicated that TSWV infection rates varied with temperature.

Western blotting using antibodies against the NSs protein indicated that *F. fusca* that had fed on leaflets of TSWV infected genotypes tested positive for the presence of NSs. This indicated that *F. fusca* larvae acquired TSWV from infected leaflets of peanut genotypes and could potentially transmit TSWV. When individual thrips reared on TSWV infected genotypes were tested, only *F. fusca* reared on TSWV infected Georgia Green leaflets tested positive for TSWV-NSs. However, when three and five *F. fusca* adults reared on leaflets of all TSWV

infected peanut genotypes tested positive for TSWV-NSs, except Georgia-06G. It was not clear as to why *F. fusca* reared on leaflets of TSWV infected Georgia-06G tested negative for TSWV-NSs. As shown in this study and in other studies, Georgia-06G can get infected with TSWV and accumulate substantial TSWV titer. Also, it was possible to rear thrips on TSWV infected Georgia-06G leaflets for a whole generation. The inability to detect TSWV-NSs in thrips reared on leaflets of infected Georgia-06G result may not be associated with the variation in TSWV titer levels as thrips developed on genotypes with TSWV titer levels similar to Georgia-06G tested positive for TSWV-NSs.

Numerous field studies have attempted to assess the effect of peanut genotypes on *F. fusca* or *F. occidentalis* (Culbreath et al. 1996, 1997a, 1997b, 2000). However, these studies only focused on field counts and on observed foliar damage. In this study, we attempted to evaluate the impact on newly released TSWV resistant genotypes on thrips biological parameters and size. In this study, the number of adults emerged per each adult released was greater on Georgia Green leaflets than on other TSWV resistant peanut genotypes. It may not be unreasonable to assume that some of the resistant genotypes tested also may differentially interact with *F. fusca*. Physiological differences in secondary metabolites and nutrient contents along with variations in morphological traits such as leaflet thickness and wax contents may be responsible for the observed differences in thrips biology. On the contrary, the observed differences could merely be a cultivar/genotype effect. Prior to initiating this experiment all thrips were reared for numerous generations on Georgia Green. Further, no differences in developmental time (time for adult emergence) were noticed among all genotypes, except for Tifguard. The next generation adults emerged on Tifguard leaflets sooner than on leaflets of all other genotypes. When compared to other TSWV resistant genotypes tested in this study,

Tifguard has a different pedigree and also possesses nematode resistance (Holbrook et al. 2008). These traits also may inadvertently affect thrips and Tifguard interactions. The short developmental period may just be a strategy employed by thrips to overcome unfavorable traits of Tifguard, and remains to be investigated in detail. Head capsule measurements of thrips reared on various genotypes did not reveal any differences reiterating that the genotypes had no effect on thrips size. In this study, effects of genotypes on biology and size of thrips that developed on detached leaflets were investigated. The observed results could vary when thrips are developed on leaflets attached to plants.

Thrips-mediated inoculations resulted in slightly reduced TSWV infection in all resistant genotypes. The infected genotypes displayed typical TSWV associated symptoms, although the symptoms were somewhat less severe in some resistant genotypes. Similar observations were made by Culbreath et al. 2005. The incidence of spotted wilt often was less severe in some resistant genotypes in peanut fields (Culbreath et al. 2005). This could be due to absence of major gene/s that can confer resistance to TSWV in peanut. The systemic infection and accumulation of TSWV titers seem to indicate that existence of a phenomenon similar to tolerance. The environmental factors also may likely influence these interactions, more detailed studies need to be undertaken to decipher such intricate interactions. Resistant genotypes, if at all, only seem to have a minor impact on thrips development and growth, reiterating that TSWV resistance is mainly imparted against the virus and not against the vector. The absence of a HR based resistance may actually be of benefit to peanut production, as there may not be enough selection pressure induced by TSWV as well as thrips to overcome the currently operating resistance and/or tolerance mechanisms and likely will not jeopardize the usefulness of these genotypes in the near future.

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Figures

Fig. 4.1. *Tomato spotted wilt virus* (TSWV) induced symptoms on resistant and susceptible genotypes. One week old plants from susceptible and resistant genotypes: Georgia Green (a), Georganic (b), Georgia-06G (c), Tifguard (d), and NC94022 (e), respectively were inoculated with 10 potentially viruliferous adult *Frankliniella fusca*. Plants were maintained in the greenhouse for TSWV symptoms development.

Fig. 4.2. *Tomato spotted wilt virus* (TSWV) titers in leaflet samples of TSWV infected resistant and susceptible genotypes inoculated with 10 potentially viruliferous thrips (N = 30 plants for each genotype) were estimated by using reverse transcriptase real time polymerase chain reaction (RT-RT-PCR). The number of plants assessed with RT-RT-PCR for Georgia Green, Georganic, Georgia-06G, Tifguard, and NC94022 were thirteen, eight, ten, eight, and eight, respectively. Threshold cycle (C_t) for each sample was calculated and a standard curve was generated using linearized plasmids with N-gene inserts. TSWV titers in leaf tissue samples were estimated by using the standard curve. Average C_t values for Georgia Green, Georganic, Georgia-06G, Tifguard, NC94022 were 15.95, 19.12, 19.64, 18.49, and 16.67 respectively. Average C_t value for healthy peanut tissue (negative control) was 33.02 and TSWV infected peanut tissue (positive control) was 15.66.

Fig. 4.3. Western blotting of *Frankliniella fusca* adults that developed on *Tomato spotted wilt virus* (TSWV) infected resistant (Georgia-06G, Georganic, Tifguard, and NC94022) and susceptible (Georgia Green) genotypes using antibodies specific to the nonstructural protein (NSs) of TSWV. Western blotting was individually performed with one (a), three (b), and five

(c) thrips from each genotype. In each blot, the samples were loaded in the following order: a marker, Georgia Green, Georganic, Tifguard, NC94022, Georgia-06G, non-viruliferous *F. fusca*, non-infected Georgia Green, and TSWV-infected Georgia Green. Thrips that tested positive for TSWV-NSs produced a band, approximately 55 kDa (indicated by an arrow in blots).

Fig. 4. 4. Adults produced per female released on leaflets of resistant (Georganic, Georgia-06G, Tifguard, and NC94022) and susceptible (Georgia Green) genotypes. Ten Munger cages were set up for each genotype and the experiment was repeated once (N = 200 thrips released for each genotype). Ten non-viruliferous female adults were released in each Munger cage. The number of adults emerging from each cage was recorded at 24 h intervals.

Fig. 4.5. Developmental time required to complete one-generation (adult to adult) on resistant (Georganic, Georgia-06G, Tifguard, and NC94022) and susceptible (Georgia Green) genotypes. Ten Munger cages were set up for each genotype and the experiment was repeated once (N = 20 cages for each genotype). Ten non-viruliferous female adults were released in each Munger cage. Cages were monitored at 24 h intervals to record newly emerged adults. Mean developmental time of adults produced in each cage was calculated for each genotype.

Fig. 4.6. Measurement of head capsule length (a) and width (b) of thrips developed in resistant (Georganic, Georgia-06G, Tifguard, and NC94022) and susceptible (Georgia Green) genotypes using dissecting microscope equipped with ocular micrometer (N = 20 thrips for each genotype). Number of ticks in the ocular scale of the microscope was calibrated with a stage micrometer at 75X magnification.

Fig. 4.1

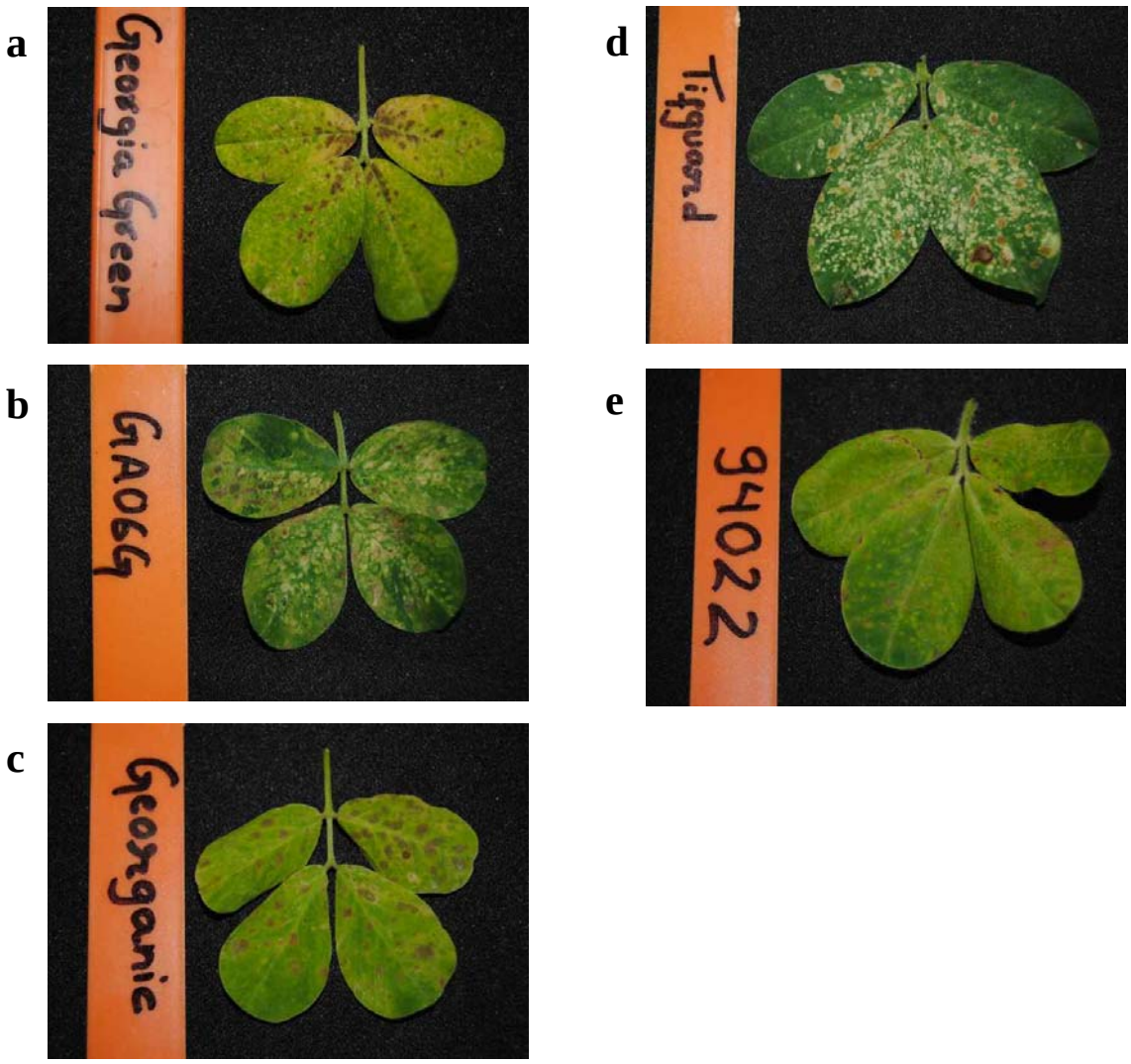

Fig. 4.2

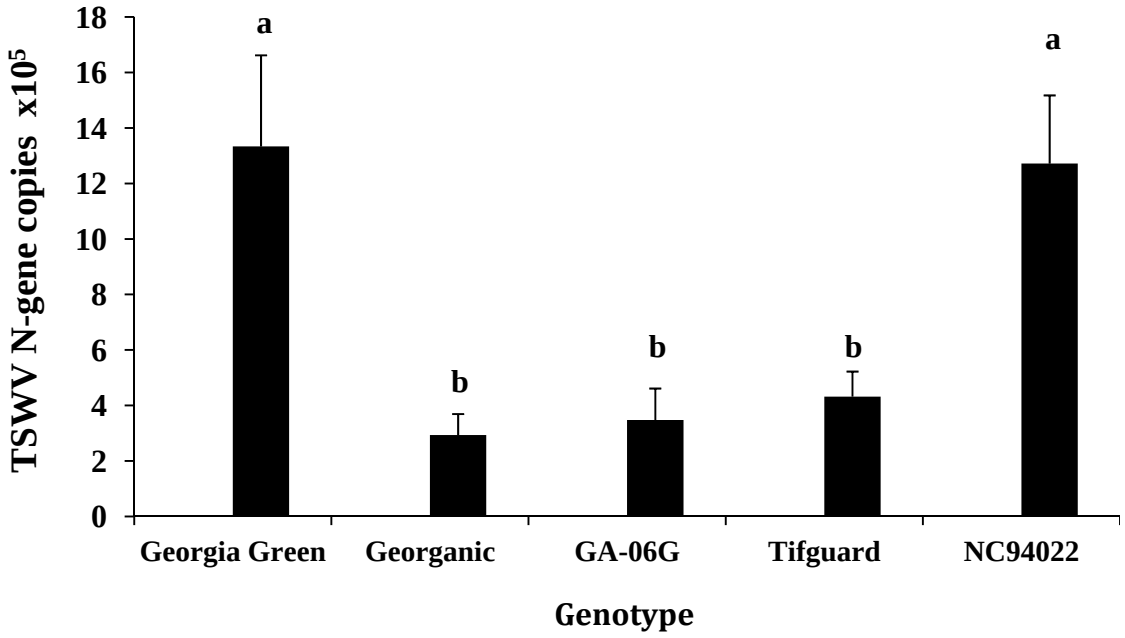
TSWV N-gene copies x10^5
18
16
14
12
10
8
6
4
2
0
a
b
b
b
a
Georgia Green
Georganic
GA-06G
Tifguard
NC94022
Genotype

Fig. 4.3

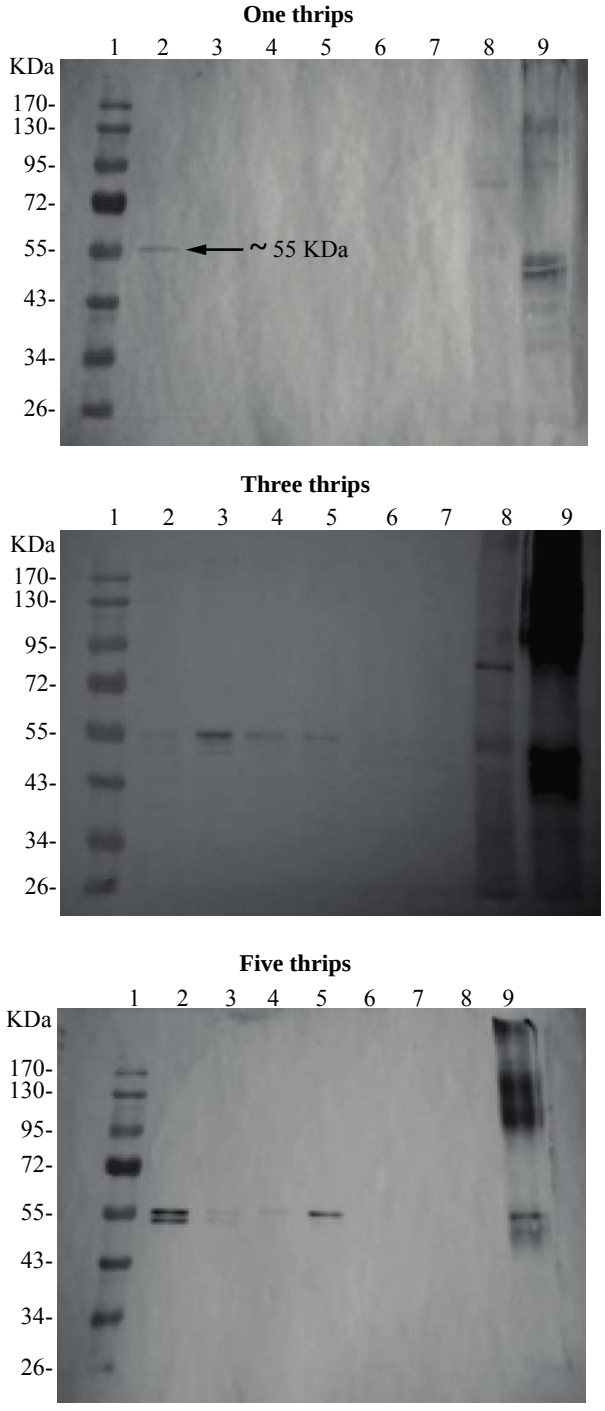
One thrips
1 2 3 4 5 6 7 8 9
KDa
170-
130-
95-
72-
55-
43-
34-
26-
~ 55 KDa
Three thrips
1 2 3 4 5 6 7 8 9
KDa
170-
130-
95-
72-
55-
43-
34-
26-
Five thrips
1 2 3 4 5 6 7 8 9
KDa
170-
130-
95-
72-
55-
43-
34-
26-

Fig. 4.4

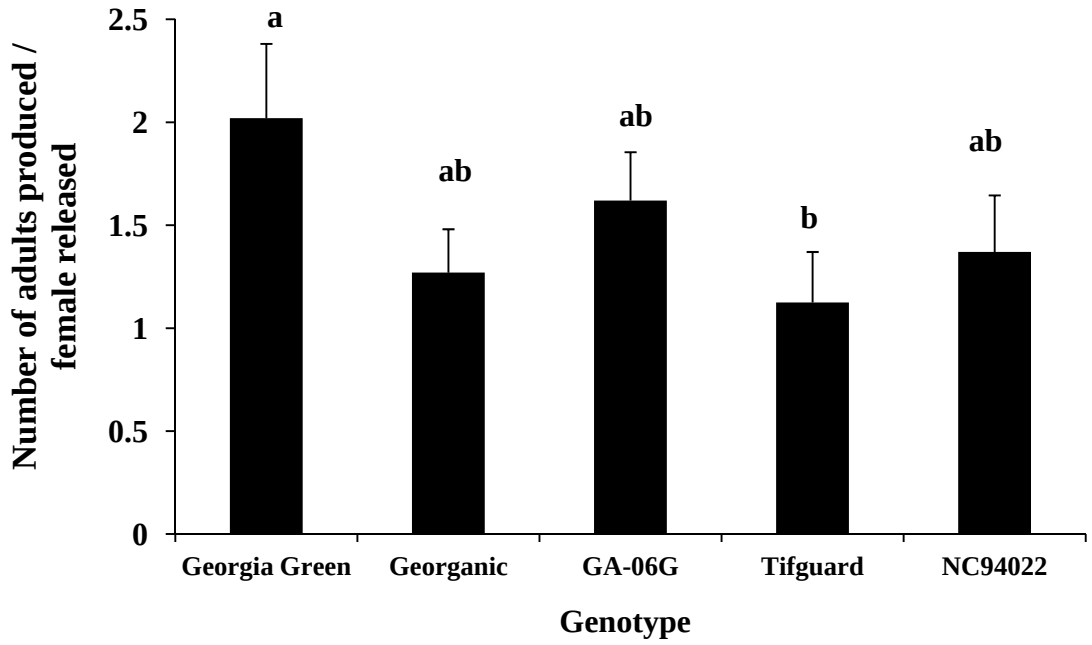
Number of adults produced / female released
2.5
2
1.5
1
0.5
0
a
ab
ab
b
ab
Georgia Green
Georganic
GA-06G
Tifguard
NC94022
Genotype

Fig. 4.5

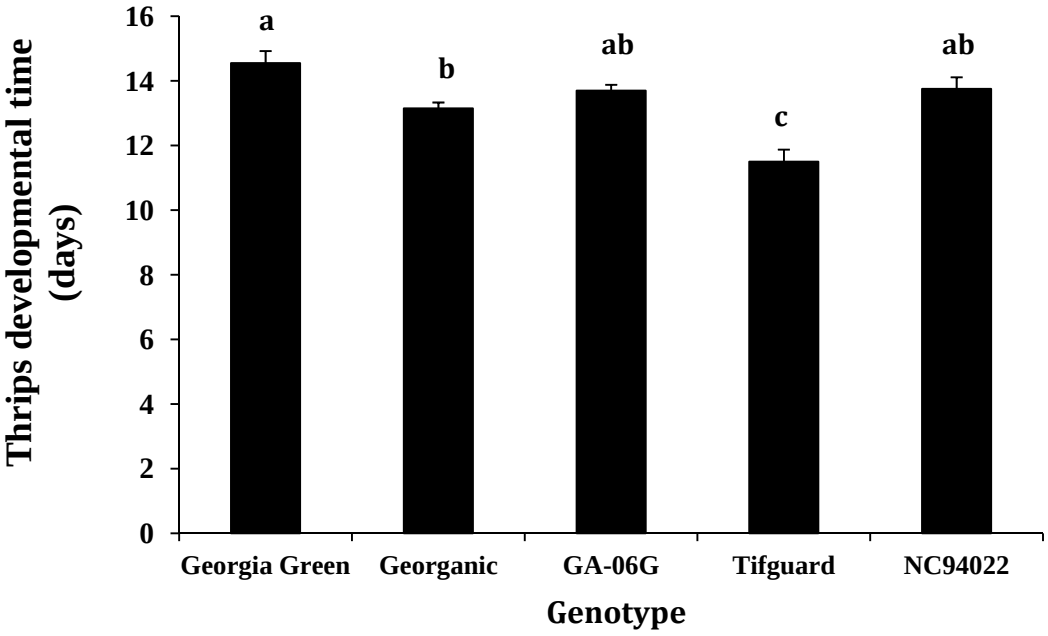
Thrips developmental time (days)
16
14
12
10
8
6
4
2
0
a
b
ab
c
ab
Georgia Green
Georganic
GA-06G
Tifguard
NC94022
Genotype

Fig. 4.6a

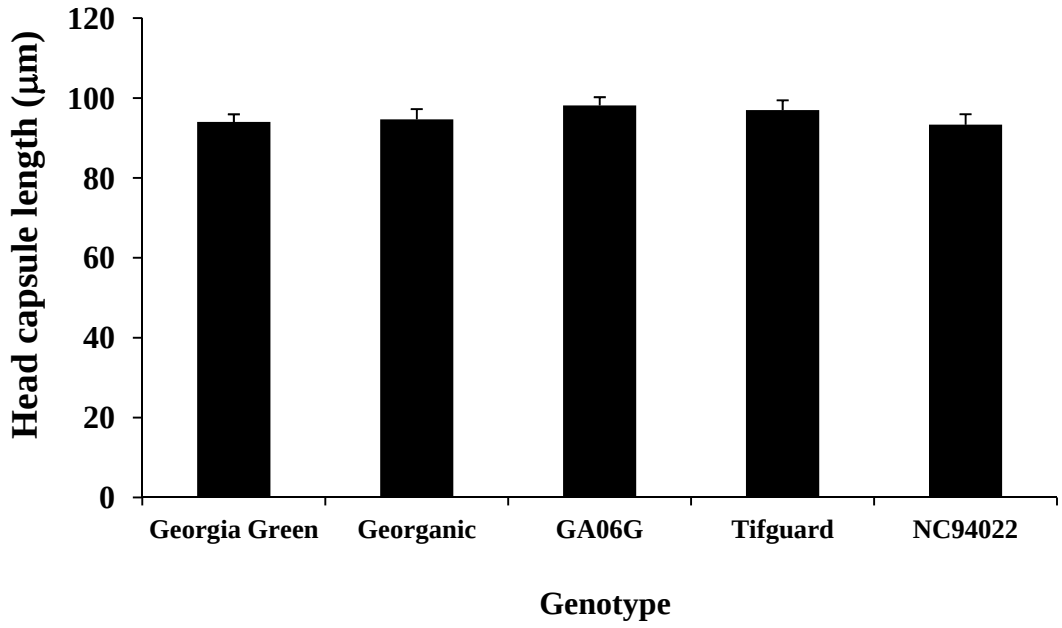

Fig. 4.6b

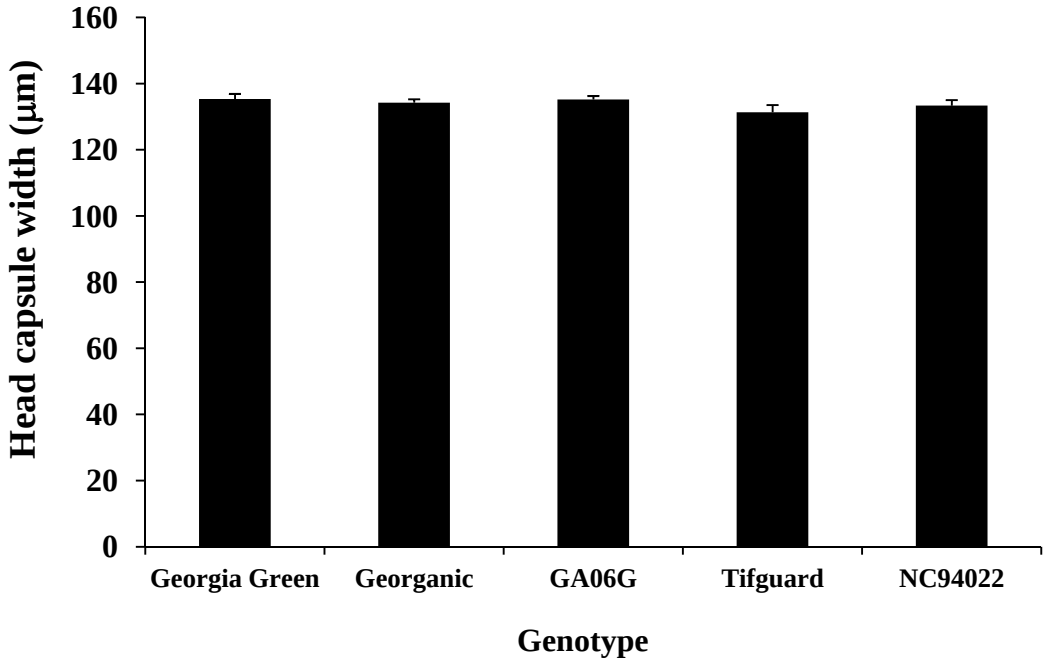

Table 4.1. Incidence of *Tomato spotted wilt virus* (TSWV) infection in susceptible (Georgia Green) and resistant (Georganic, Georgia-06G, Tifguard, NC94022) genotypes.

	Number of plants infected/inoculated [y]			
	Repeats of the experiment [z]			
Peanut genotypes [x]	**I**	**II**	**III**	**Incidence (%) of infected plants (Mean ± standard error)**
Georgia Green	8/10	9/10	7/10	80 ± 5.77
Georganic	7/10	2/10	4/10	43.3 ± 14.53
Georgia-06G	8/10	2/10	4/10	46.7 ± 17.64
Tifguard	9/10	5/10	3/10	56.7 ± 17.64
NC94022	6/10	3/10	4/10	43.3 ± 8.82
Proc GENMOD type III analysis (df 4, 46)				
$\chi 2$	3.65	15.6	3.92	
$P > \chi 2$	0.4554	0.0036	0.4169	

[x] Peanut genotypes inoculated with 10 potentially viruliferous *Frankliniella fusca* (per plant). For each genotype, 10 plants were tested and the experiment was repeated two twice (N = 30 plants for each genotype). $\chi 2$ and $P >$ chi-square values are presented to indicate differences in the incidence of TSWV infection in different peanut genotypes, calculated using the PROC GENMOD in SAS.

[y] Infection status of plants was assessed with double antibody coated enzyme-linked immunosorbent assay (DAS-ELISA) using antibodies specific to the nucleocapsid protein (N).

[z] Roman numerals I to III indicate numbers of repeats of the same experiment.

CHAPTER 5

EFFECTS OF *TOMATO SPOTTED WILT VIRUS* (TSWV) INFECTION ON SETTLING, FEEDING, OVIPOSITION, AND BIOLOGY OF *FRANKLINIELLA FUSCA*[1]

[1] Shrestha. A., R. Srinivasan, D. Riley, and A. Culbreath. 2011. To be submitted

Abstract

Phytovirus infections can affect the fitness and behavior of their arthropod vectors positively and negatively. Mixed outcomes have been documented with tospoviruses and thrips. In this study, we investigated the effects of *Tomato spotted wilt virus* (*Tospovirus*) infection on *Frankliniella fusca*'s feeding ability and other fitness parameters using peanut as a model host. Results indicated that neither TSWV infection in thrips nor TSWV infection in peanut influenced thrips preference to TSWV infected or non-infected plants. Feeding assays revealed that TSWV infection in *F. fusca* can impair its feeding ability. TSWV infection in *F. fusca* increased its egg production capacity. However, TSWV infection in peanut reduced egg production. These contrasting results led to investigations on the availability of vital nutrients such as free amino acids in TSWV infected and non-infected plants. Leaflets from TSWV infected plants had elevated levels of free amino acids than leaflets of non-infected plants. Greater amino acid titers thus could have influenced the differential oviposition capabilities. Despite the spike in free amino acid titers, TSWV infection in peanut, overall, negatively affected the reproductive fitness and development of *F. fusca*. This suggested that free amino acids and possibly others may play a role in deceptive arrestment of thrips on TSWV infected plants. Such an arrestment may be beneficial for a *Tospovirus* whose acquisition and transmission is specific to vector stages. This reiterates that *Tospovirus* infection of thrips can directly impact thrips development; however, through host mediated deceptive arrestment they can manipulate thrips to aid its own proliferation.

Introduction

Arthropod-borne viruses are obligate parasites, and their interactions with their arthropod vectors and hosts often have favored their proliferation (Hurd 2003). Virus induced host

physiological changes, vector responses to such changes, and subsequently viral epidemics have been extensively studied in botanical pathosystems (Mann et al. 2008). Virus induced host cues have affected biology of vectors in numerous ways. Increase in fitness of arthropod vectors upon feeding on infected plants has been demonstrated in the case of persistently transmitted viruses (Belliure et al. 2005, Stout et al. 2006, Jiu et al. 2007). On the contrary, decrease in vector fitness has also been documented in arthropod vectors (Robb 1989, Costa et al. 1993, DeAngelis et al. 1993). In other studies, no effects of virus induced host changes were documented on vector fitness (Wijkamp et al. 1996, Roca et al. 1997). It is not clear if these changes are influenced by the intricacy of interactions between the arthropods and the viruses. Positive benefits to arthropods seem to be predominantly associated with the persistently transmitted viruses compared to non-persistently transmitted viruses (Eigenbrode et al. 2002, Jimenez-Martinez 2004, Srinivasan et al. 2006, Mauck et al. 2010). However, virus induced adverse effects seem to be common in arthropods transmitting viruses in a persistent and propagative fashion (Robb 1989, DeAngelis et al. 1993, Garica et al. 2000, Stumpf and Kennedy 2005). One explanation for this might be that persistent and propagatively transmitted viruses often are enveloped and are closely related to animal infecting viruses (Fauquet et al. 2005).

Besides influencing vector biology arthropod-borne viruses have been implicated in modifying vector behavior. Viruses can alter host plant phenotypic traits and subsequently vector responses (Mann et al. 2008). Variation in phenotypic traits such as virus induced yellowing in infected host plants attracted more vectors than non-infected plants (Baker 1960, Macias and Mink 1969, Montllor and Gildow 1986, Smiths et al. 2000, McLeod 2005). Similarly, virus induced volatile cues (volatile organic compounds (VOCs)) also are known to influence arthropod behavior. Viruses induced VOCs are known to attract and arrest arthropod vectors.

Examples include potato and wheat plants infected with luteoviruses, *Potato leafroll virus* and *Barley yellow dwarf virus*, respectively attracted more aphids than their non-infected counterparts (Eigenbrode et al. 2002, Jimenez-Martinez 2004).

In general, manipulations in vector biology and/or vector behavior associated with virus induced physiological changes in host plants seem to favor pathogen spread (Hurd 2003). However, whether the altered behaviors in vectors are the result of true manipulation is still debatable (Hurd 2003). Also, it is not clear if these interactions are dictated by the intricacy of interactions between the pathogen and the vector or if they are predominantly influenced by their physiological manipulations of their host plants. For instance, conflicting reports have been associated with thrips-transmitted *Tomato spotted wilt virus* (TSWV), a plant-infecting member of the *Bunyavirus* genus (*Tospovirus*) (Francki et al. 1991, Goldbach and Peters 1994, Murphy et al. 1995, Elliott 1996). TSWV is transmitted by several thrips species in a persistent and propagative manner (German et al. 1992, Ullman et al. 1992, Whitfield et al. 2005). Thrips also exhibit stage-specific acquisition and transmission characteristics (Ullman et al. 1992, Kritzman et al. 2002). TSWV infection positively influenced the fitness of western flower thrips, *Frankliniella occidentalis* (Pergande) (Bautista et al. 1995, Maris et al. 2004, Stafford et al. 2011), whereas, in some cases, deleterious or no effects were observed (DeAngelis et al. 1993, Wijkamp et al. 1996, Roca et al. 1997).

Most of the studies pertaining to TSWV were conducted with experimental or indicator host plants and with western flower thrips. In this study, we investigated the effects of TSWV on Tobacco thrips, *Frankilniella fusca* (Hinds) using a natural crop host, peanut (*Arachis hypogaea* L.). *F. fusca* also were reared on peanut foliage. *F. fusca* is the most common competent vector of TSWV in peanut in the southeastern United States (Culbreath et al. 2003).

We attempted to characterize the effect of TSWV on *F. fusca* settling, feeding, oviposition and thrips biological parameters with and without TSWV infection. Also, we investigated the effect of TSWV on nutrient concentrations in peanut plants.

Materials and Methods

Non-infected peanut plants. Peanut plants of the genotype Georgia Green were used for all experiments. Seeds were pre-germinated in moistened paper towels and incubated in a growth chamber kept at 25 to 30˚C for one week. Sprouted peanut seeds were transplanted into 4-inch diameter plastic pots (Hummert International, St. Louis, MO) in commercial potting mix, Sunshine mix (LT5 Sunshine® mix, Sun Gro® Horticulture Industries, Bellevue, WA). Peanut plants were maintained in thrips-proof cages (47.5 cm^3) (Megaview Science Co., Taichung, Taiwan) in a greenhouse at 25 to 30˚ C and 80 to 90% RH with a 14 h photoperiod. For all the experiments, one-week-old plants were used.

TSWV infected peanut plants. TSWV infected plants of the genotype, Georgia Green were initially collected from Belflower Farm, Coastal Plain Experimental Station, Tifton, GA in 2009. Plants were maintained in a greenhouse under conditions described previously. Leaflets from TSWV infected plants were placed in a Munger cage (11.43 X 8.89 X 1.77 cm) (Munger 1942) and non-infected *F. fusca* were released. After the next generation adults emerged, potentially viruliferous adults were transferred to one-week-old plants with a paint brush (fine camel hair #2 with aluminum ferrules, Charles Leonard Inc., Hauppauge, NY). Plants were maintained in thrips- proof cages in the greenhouse as described previously. Three weeks post thrips inoculation, TSWV infection in thrips inoculated plants was assessed by double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) (Clark and Adams 1977). Leaf tissue (approximately 0.1 g) from one-third above ground portion of the plant was obtained from

each plant and DAS- ELISA was performed in a 96 well microtiter plate (Maxisorp, Nunc, Rochester, NY). Along with samples, a TSWV infected peanut leaf tissue and non-infected peanut leaf tissue were included as positive and negative controls, respectively, in each plate. Primary antibody (anti-TSWV IgG) was used at a dilution ratio of 1:200 and the secondary antibody (anti-TSWV IgG conjugated with alkaline phosphatase) also was used at a 1:200 dilution ratio (Agdia®, Elkhart, IN). Incubation and washing steps were followed as per the manufacturer's instructions. Final absorbance values were measured at 405 nm in a photometer 1 h after substrate addition (Model Elx 800, Bio-Tek®, Kocherwaldstr, Germany). An average absorbance value of negative control samples plus four standard deviations was considered positive. TSWV infected plants were subsequently generated by thrips-mediated inoculations.

Maintenance of non-viruliferous *F. fusca*. A *F. fusca* colony was established in 2009 on Georgia Green non-infected leaflets with thrips collected from peanut blooms from the Belflower Farm, Coastal Plain Experimental Station, Tifton, GA. Since then, thrips were maintained in Munger cages on non-infected foliage of the peanut genotypes Georgia Green. Munger cages were maintained in a growth chamber (Thermo scientific, Dubuque, IA) at 25 to 30˚C and a 14h photoperiod.

Maintenance of potentially viruliferous *F. fusca*. Similar to non-viruliferous thrips, a colony of potentially viruliferous thrips was maintained on TSWV infected leaflets of the peanut cultivar in Munger cages in a separate growth chamber as mentioned above. In both colonies, a majority of thrips over 95% were females. Thus, for all experiments, predominantly females, up to 2 days old were used. TSWV infection in individual thrips was tested using an antigen coated plate enzyme linked immunosorbent assay (ACP- ELISA) (Bandla et al. 1994). Adult thrips were ground individually and ACP-ELISA was performed in a 96 well microtiter plate (Maxisorp,

Nunc, Rochester, NY). Primary antibody (TSWV IgG, polyclonal nonstructural protein (NSs) (Agdia Inc., Elkhart, IN) was used at 1:500. Subsequently, secondary antibody (Anti-Mouse TSWV IgG conjugated with Alkaline Phosphatase) (SIGMA, St. Louis, MO) was used at 1:15,000. Incubation and washing steps were followed as per the manufacturer's instructions. Final absorbance values were measured at 405 nm in a photometer 1 h after substrate addition (Model Elx 800, Bio-Tek®, Kocherwaldstr, Germany). An average absorbance value of negative control samples plus four standard deviations was considered positive. ACP-ELISA indicated that the infection rate in the potentially viruliferous thrips was from 20 to 45%.

Effects of TSWV infection on *F. fusca* settling. A Y-shaped glass tube olfactometer (Analytical Research systems, Gainesville, FL) was used to assess settling of potentially viruliferous and non-viruliferous *F. fusca* (up to 2 days old) on TSWV infected and non-infected peanut leaflets. A tripod (Fisher Scientific, Pittsburgh, PA) was set up directly below lights in the laboratory at the room temperature and the Y-tube was held in horizontal position (90 degree between Y-tube and tripod) (Kogel et al. 1999). A symptomatic leaflet attached to a TSWV infected plant was inserted into one arm and a leaflet attached tp from a non-infected plant was inserted into the other arm of the tube. Both leaflets were held in position using parafilm (American National Can Company, Greenwich, CT). Settling patterns of twenty-five potentially viruliferous or non-viruliferous female thrips (up to 2 days old) were tested separately for each experiment and the experiment was repeated 24 times. Thrips were released at the base of the Y-tube by using a paintbrush (finecamel hair #2 with aluminum ferrules, Charles Leonard Inc., Hauppauge, NY). Thrips were given 24 h to settle on either leaflet, following which, the number of thrips settled on each leaflet was recorded. To avoid positional and directional effects, after

each set of experiment, the Y-tube was rotated 90 degrees and the position of the plants was exchanged. Also, for each experiment new plants were used.

Statistical analysis was done to compare settling of potentially viruliferous and non-viruliferous thrips on TSWV infected and non-infected leaflets. Treatments were considered as fixed effects and replications were considered as random effects. The analysis was performed by using PROC GLIMMIX in SAS (SAS Enterprise 4.2, SAS Institute, Cary, NC). The statistical significance of differences between treatments was estimated using least squares means.

Assessment of *F. fusca* feeding damage on non-infected and TSWV infected peanut plants. Feeding damage on TSWV infected and non-infected peanut plants by potentially viruliferous and non-viruliferous thrips were assessed separately using no-choice tests. TSWV infected plants were obtained through mechanical inoculation. Mechanical inoculation was conducted on one-week-old peanut plants as described by Mandal et al. (Mandal et al. 2008). TSWV (GA isolate) infected tobacco (*Nicotiana tobacum* L.) leaves were used as a source of inoculum. For every plant, 0.1 g of infected tobacco tissue was ground in 1 ml of 0.1 M phosphate buffer (pH 7.0, containing 1.7% potassium phosphate dibasic, 1.4% potassium phosphate monobasic, 0.2% sodium sulfite and 0.01 M mercatoethanol). To this, 1% Celite 545 (Across Organics, Geel, Belgium) and 1% of Carborundum (320 grit, Fisher Scientific, Fair Lawn, NJ) were added @ 0.01g/ml of buffer. Tissues were ground until they reached a soupy consistency. Before applying inoculum mix, adaxial leaf surfaces of plants were dusted with an abrasive Caroborundum. Subsequently, a piece of cheesecloth (American Fiber & Finishing, Inc., Burlington, MA) was soaked in the inoculum mix and applied on fully expanded leaflets with moderate pressure. After 15 min, plants were washed with water and placed in thrips-proof cages and maintained in the greenhouse as described previously. TSWV infection status in

inoculated plants was assessed using DAS-ELISA three weeks post inoculation as described previously.

Five TSWV infected plants and five non-infected plants approximately 3 weeks old were selected and placed separately in a thrips proof cage randomly (one plant type per cage). Plants were dusted with approximately 0.05 g of pine (*Pinus taeda* L.) pollen grains and 10 potentially viruliferous female adults (up to 2 days old) were released on each plant. Each experiment was repeated three times (N = 200 potentially viruliferous thrips on TSWV infected and non-infected plants respectively). Similarly, the experiment was conducted with non-viruliferous thrips on TSWV infected and non-viruliferous peanut plants separately. After releasing thrips, each plant was inspected visually for the presence of feeding scars at 48 h intervals for 2 weeks. Feeding damage index (FDI) was calculated based on a formula proposed by Maris et al. 2003 with slight modifications (Maris et al. 2003).

$$\text{FDI} = \frac{\text{No. of leaflets with feeding damage}}{\text{Total no. of leaflets in a plant}} \times \text{intensity of feeding scar}$$

Intensity of feeding scars was based on an arbitrary scale (1= 0-20%, 2 = 20-40%, 3 = 40-60%, 4 = 60-80%, and 5 = 80-100%).

To compare statistical differences in feeding damage produced by potentially viruliferous and non-viruliferous thrips on TSWV infected and non-infected leaflets, feeding at three time intervals, 4, 10, and 14 days after thrips release were selected. Treatments were considered as fixed effects and replications were considered as random effects. The analysis was performed by using PROC GLM in SAS (SAS Enterprise 4.2, SAS Institute, Cary, NC). The same thrips population was observed throughout the sampling period and hence the observations were treated as repeated measures. The statistical significance of differences between treatments was estimated using least squares means.

Effects of TSWV infection on *F. fusca* oviposition efficiency. Leaflets from mechanically inoculated plants and non-infected plants were used for this study. Mechanical inoculation was conducted on one-week-old plants as described previously.

A TSWV symptomatic leaflet was placed in a Munger cage with a non-infected leaflet. Ten Munger cages were set up and in each cage 10 potentially viruliferous or non-viruliferous thrips (up to 2 days old) were released. The experiment was repeated twice (N = 300 viruliferous and non-viruliferous thrips respectively). Leaflets used for the experiment were of same age and same size. Thrips were allowed to oviposit for 72 h following which they were removed from the cages. Subsequently, leaflets were stained with acid-fuschin to count the number of eggs on each leaflet (Simonet and Pienkowski 1977). Leaflets were first boiled for 20 to 40 min in a 1:1:2 (by volume) solution of glacial acetic acid, 10% lactic acid, and 95% ethanol at 60˚C until leaflets turned white. Fixed leaflets were then boiled in a lacto phenol acid solution consisting of a 1:2:1:1:1 solution of 10% lactic acid, 50% glycerin solution, distilled water, saturated phenol buffered at pH 4.3 and 1 g/l of acid fuschin high purity biological stain (Acros Organics, Morris Plains, NJ). Upon staining for 2 to 4 min, leaflets were allowed to cool. Subsequently, excess stain on leaflets was removed with warm water. Stained eggs on individual leaflets were observed under a dissecting microscope (MEIJI TECHNO, Santa Clara, CA).

To compare the number of eggs oviposited by potentially viruliferous and non-viruliferous thrips on TSWV infected and non-infected leaflets statistical analysis was performed by using PROC GLIMMIX in SAS as previously described.

Effects of TSWV infection on free amino acid levels in peanut plants. Five TSWV infected plants and five non-infected plants of same age (approximately 2 and half weeks old) were selected. TSWV infected plants were obtained through thrips-mediated inoculation as

described previously. Leaf tissue (approximately 0.15 g) collected from top one-third portion of each plant were free amino acids analysis (Hacham et al. 2002). Samples were frozen in liquid nitrogen (Airgas south, Tifton, GA) and ground with 600 µl of water:chloroform:methanol (3:5:12 v/v). Ground samples were transferred to 1.5 ml microcentrifuge tubes (Fisher Scientific, Pittsburgh, PA) and centrifuged (Eppendorf, Hamburg, Germany) at full speed (14,000 rpm) for 2 min. Supernatants were then collected and placed in 2 ml tubes (Fisher Scientific, Pittsburgh, PA). Residues in the tubes were reextracted with another 600 µl of water:chloroform:methanol extraction buffer and centrifuged at full speed for 2 min. Supernatants were again collected and combined with the previously obtained in 2 ml tubes. Subsequently, 300 µl of chloroform and 450 µl of water were added to the combined supernatants and centrifuged at full speed for 2 min. The upper water:methanol phase of the tubes were transferred to new 2 ml tubes following which tubes were placed in a speed vac (Thermo Fisher Scientific, Asheville, NC) at 45˚C for 1 h. The samples were then kept at -20˚C until they were sent to Proteomics Core Facility, UC Davis, CA for free amino acids analysis. The facility used L-8900 Hitachi, amino acid analyzer to quantify free amino acids present in samples. The L-8900 Hitachi analyzer quantified free amino acids by utilizing a lithium buffer system. Prior to analysis, samples were acidified with sulfosalicyclic acid to remove any intact proteins. Subsequently, the analyzer used ion-exchange chromatography to separate amino acids followed by a “post-column” ninhydrin reaction detection system. The Na system quantified individual amino acids down to the pmole level (approximately 100 pm).

To determine the differences in free amino acids levels in TSWV infected and non-infected leaflets, statistical analysis was performed by using PROC GLIMMIX in SAS as described previously.

Effects of TSWV infection on thrips reproduction and development. Leaflets from mechanically inoculated plants and non-infected plants were used for this experiment. Mechanical inoculation was performed as described previously. In a Munger cage, two TSWV symptomatic leaflets and two non-infected leaflets were placed separately. In each cage, ten potentially viruliferous thrips (up to 2 days old) were released. Ten cages were set up and the experiment was repeated once (N = 200 potentially viruliferous thrips on TSWV infected and non-infected leaflets respectively). Similarly, the experiment was repeated with non-viruliferous thips on TSWV infected leaflets and non-infected leaflets separately. Munger cages were kept in the growth chamber maintained as described previously. The released adult thrips were removed from the cage five days later, and the cages were monitored daily under a compound microscope (MEIJI TECHNO, Santa Clara, CA) and newly hatched larvae were recorded. The number of adults emerging from each cage was recorded at 24 h intervals and removed. The experiments were continued until there were no more larvae or adults in each cage.

Differences in the number of adults produced by each adult/10 adults released and the time required to complete one generation (adult-adult) also was estimated. The time required for the first adult to develop in each Munger cage was estimated for each potentially viruliferous and non-viruliferous thrips on TSWV infected and non-infected leaflets. Analysis of variance was conducted using PROC GLIMMIX in SAS as described previously.

Results

Effects of TSWV infection on *F. fusca* settling. Regardless of the infection status of thrips no differences in settling were observed between the treatments ($F = 2.10$; df = 3, 96; $P > F = 0.1053$) (Fig 5.1). This indicates that in *F. fusca* settling is not affected by TSWV infection status in thrips as well as in peanut plants.

Assessment of *F. fusca* feeding damage on non-infected and TSWV infected peanut plants. The leaf damage induced by feeding of non-viruliferous thrips and potentially viruliferous thrips on non-infected and TSWV infected peanut plants is illustrated in Fig 5.2. Feeding damage induced by thrips feeding varied with the experiments conducted and hence data from each experiment were analyzed individually. The same thrips population was observed at 2-day time intervals; in essence the same population was sampled at each time interval. Hence, three representative intervals, 4, 10, and 14 days after thrips release were chosen for statistical analysis.

Non-viruliferous *F. fusca* fed plants had more thrips feeding scars than plants that were fed by potentially viruliferous and consequently the damage index was greater on non-viruliferous *F. fusca* fed plants than potentially viruliferous *F. fusca* fed plants (Fig. 5. 2). This difference was consistent across all time intervals and across all experiments (Table 5.1). This suggested that non-viruliferous thrips fed more rapidly on TSWV infected or non-infected peanut plants rapidly than potentially viruliferous thrips.

Feeding damage index varied with TSWV infection and this response differed with time intervals and experimental repeats. Feeding damage index was greater on non-infected plants than on TSWV infected plants in the second repeats of the experiment at four days post thrips release (Table 5.1). No differences were observed in other experiments (Table 5.1). At 10 days post release, feeding damage index was greater on non-infected plants than on TSWV infected plants in first, second, and fourth repeats of the experiment (Table 5.1). No such differences were observed in the third repeat of the experiment (Table 5.1). At 10 days post release this difference was found only in the fourth repeat of the experiment (Table 5.1).

These results clearly indicated that feeding damage induced by non-viruliferous thrips was more than the feeding damage induced by potentially viruliferous thrips. Also, feeding damage on TSWV infected plants were greater than the damage on non-infected plants. However, this difference was not consistent.

Effects of TSWV infection on *F. fusca* oviposition efficiency. Oviposition efficiency of potentially viruliferous thrips and non-viruliferous thrips varied with the repeats of the experiment (Table 5.2). Regardless, of TSWV infection in peanut leaflets, viruliferous thrips produced more eggs than non-viruliferous thrips (Table 5.2). This difference was consistent across all the repeats of the experiment (Table 2). Regardless of TSWV infection in *F. fusca*, *F. fusca* produced more eggs on non-infected leaflets than on TSWV infected leaflets (Table 5.2). However, this trend was observed in two of the three repeats only (Table 5.2). In the third repeat, no differences were observed between TSWV infected and non-infected leaflets (Table 5.2).

Effects of TSWV infection on free amino acid levels in peanut plants. The titers of ten essential free amino acids (Isoleucine, Leucine, Lysine, Methionine, Phenylalanine, Threonine, Tryptophan, Valine, Histidine, and Tyrosine) were greater on TSWV infected leaf tissue samples than on non-infected leaf tissue samples (Fig. 5.3a) (Table 5.3a). The titers of nine non-essential amino acids (Alanine, Arginine, Aspartic acid, Glutamic acid, Glycine, Proline, Serine, Asparagine, Glutamine) also were greater on TSWV infected leaf tissue samples than on non-infected leaf tissue samples (Fig. 5.3b) (Table 5.3b).

Effects of TSWV infection on thrips reproduction and development. TSWV infection in thrips and in peanut leaflets affected thrips reproduction and development. Also, these effects varied with the repeats of the experiment. Regardless of TSWV infection in peanut leaflets, non-

viruliferous thrips produced more adults than potentially viruliferous thrips (Table 5.4). This effect was consistent across all repeats of the experiment (Table 5.4). Regardless of TSWV infection in thrips, more adults were recovered from non-infected leaflets than on TSWV infected leaflets. However, this difference was observed in experiment one only. No differences between TSWV infected and non-infected leaflets were observed (Table 5.4). These results indicate TSWV infection in thrips can reduce thrips reproductive potential and that the reproductive potential of thrips is greater on non-infected host plants than on TSWV infected host plants. Treatment differences did not vary with the repeats of the experiment; hence the data were pooled for statistical analysis ($F = 0.59$; df = 1, 18; $P > F = 0.4524$). The developmental time or the time required to complete one generation (adult to adult) varied with TSWV infection of thrips. Development of thrips was delayed when they were infected with TSWV (Table 5.5). Also, thrips development was not influenced by TSWV infection status of host plants (Table 5.5). Results suggest that thrips development can lead to delayed development.

Discussion

TSWV infection in thrips and in host plants did not influence *F. fusca* settling behavior. This is in contrast to earlier studies, which documented preferential settling of *F. occidentalis* and onion thrips, *Thrips tabaci* (Lindeman) on TSWV infected host plants when compared with non-infected host plants (Carter 1939, Yudin et al. 1998, Maris et al. 2004). Yudin et al. (1998) speculated that visual cues induced by chlorosis following viral infection could have attracted thrips to infected plants. A number of other studies also have emphasized the importance of visual cues associated with host plant selection of *F. occidentalis* (Heinz et al. 1992, Matteson and Terry 1992, Teulon et al. 1999, Smiths et al. 2000). Smiths et al. (2000) demonstrated that other cues besides visual cues could be involved in host selection of *F. occidentalis*. On the

contrary, there seems to be no involvement of either visual or other cues that influencing settling in the case of *F. fusca*. The observed difference could be due to the fact that *F. fusca* is not primary a flower colonizer and also TSWV induces a multitude of symptoms ranging from chlorosis to ring spots on peanut (Kirk 1985, Cho et al. 2000).

Feeding damage index estimations indicated that fewer feeding scars were found on plants that were fed by potentially viruliferous thrips than non-viruliferous thrips. This suggested that reduced feeding rates were associated with viruliferous thrips. More probing of male *F. occidentalis* was observed on TSWV infected plants than on non-infected plants. However, this difference was not observed with female thrips (Stafford et al. 2011). Our thrips colony was thelytokus in nature and only adult females were used in our experiments. Our results indicate a direct effect of TSWV infection on thrips feeding behavior. Also, regardless of TSWV infection in thrips, a greater amount of thrips feeding was observed on TSWV infected plants over non-infected plants, but the difference was not consistent.

When compared with feeding, contrary results were obtained with thrips oviposition. Viruliferous thrips produced more eggs than non-viruliferous thrips regardless of the infection status of their host plants. It is not clear how the reduced feeding effects could translate into increased oviposition efficiency. Feeding damage was only a result of adult feeding whereas oviposition could be influenced by thrips development in a diet rich in vital nutrients. Virus infection can affect the nutrient composition in infected plants and accumulated nutrient pools such as free amino acids, which can lead to enhanced vector fitness. Plant nutrients such as free amino acids are the main ingredients for vitellogenesis (Klowden 2007). In several hematophagous female insects such as mosquitoes and other flies, amino acids acquired from erythrocyte are utilized to synthesize yolk proteins for egg production (Cohen 1989, Hurd et al.

1995, Wheeler 1996, Davey 1997). Thus, increased oviposition of viruliferous thrips may be due to the availability of increased free amino acid concentrations in TSWV infected plants. Perhaps, this also may explain increased fitness of thrips when reared on a pollen rich diet; pollen grains are rich in free amino acids (Angelella and Riley 2010). Viruliferous thrips also produced more eggs on non-infected host plants. Thus, increased oviposition by potentially viruliferous thrips on non-infected plants in association with feeding may favor virus spread.

The development of thrips also was affected by virus infection in thrips as well as in host plants. The reproductive fitness of viruliferous thrips was lower than non-viruliferous thrips. These results correspond with feeding results, reiterating a direct effect on TSWV on thrips. Thrips development also was affected by TSWV infection status of host plants, adult emergence was reduced in TSWV infected leaflets when compared with non-infected leaflets. Robb 1989 also reported reduced survival and reproductive potential of *F. occidentalis* maintained on TSWV infected plants versus non-infected plants. Another study conducted using closely related *tospovirus*, *Impatiens necrotic spot virus* (INSV) also led to adverse effects on *F. occidentalis* biology. Also, thrips developmental time (adult-adult) was longer in potentially viruliferous thrips than on non-viruliferous thrips. Such an effect may be attributed to a direct effect of the virus on thrips. Stumpf et al. (2005) also demonstrated direct effects of TSWV on developmental time of female thrips. Their experiments also revealed that TSWV infection reduced the head capsule width of viruliferous *F. fusca*.

Even though, greater concentrations of free amino acids were found on TSWV infected plants their overall nutrient quality may be compromised (Shalitin and Wolf 2000). Rowlanda et al. 2005 reported reduced photosynthesis and water use efficiency and increased transpiration rate in TSWV infected peanut plants when compared with non-infected plants (Rowland et al.

2005). Several studies have linked the events of increased free amino acids with water deficiency in plants (Jones et al. 1980, Stewart and Larher 1980, Zagdanska 1984, Navari-Izzo et al. 1990). One of the effects of TSWV on peanut plants is reduced water storing capacity (Rowland et al. 2005). It has been speculated that increase in free amino acids have been documented to influence the osmotic potential of virus infected host plants (Navari-Izzo et al. 1990).

Despite the availability of higher concentrations of free amino acids, the overall quality of the TSWV infected peanut plants and/or leaflets may be inferior. Thus, greater amino acid concentrations on infected leaflets may serve as an incentive for thrips feeding and development until or shortly after adult emergence. This deceptive arrestment of thrips vectors may be vital for virus acquisition and transmission. Stage-specific acquisition and transmission of tospoviruses by thrips may benefit from this deceptive arrestment. Wherein, only early instar larvae of thrips can acquire the virus and late larval instars and adults can transmit the virus efficiently (van de Wetering et al. 1996). Therefore, it is essential for thrips to feed on *Tospovirus* infected plants until adult emergence. Deceptive signals in the form of increased amino acids may encourage the thrips to stay on *Tospovirus*-infected host plants besides the overall poor nutrient quality in TSWV infected plants. On the contrary, thrips feeding on TSWV infected plants did not affect the fitness of thrips or at times increased their fitness (Wijkamp et al. 1995; 1996; Marris et al. 2004). This indicates that there could be strong host effects involved, particularly the interactions between host plants and the viruses, tospoviruses are known to produce a multitude of symptoms within a single host due to their interactions with the environment, vectors, virus isolates, and plant defense mechanisms (Culbreath and Srinivasan

2011). These differences in host plant appearance and quality also may differentially influence vector fitness.

These results clearly demonstrated negative fitness benefits associated with TSWV infection on thrips. Negative effects of TSWV infection in host plants on thrips fitness also were observed, but not consistently. This study suggested that the possibility of deceptive arrestment in the form of increased amino acid concentrations and possibly other nutrients may manipulate vector biology, behavior, and fitness. However, this brings us back to the question, whether the alterations in vector traits are a result of true manipulation by the pathogen to benefit its own proliferation? Despite the evidence of deceptive arrestment and/or signaling severe *Tospovirus* epidemics are often observed only on a few hosts, even though some tospoviruses have a very broad host range (TSWV has > 1000 recorded hosts). This suggests that the manipulations are random and are not always dictated by the intricacy of vector-pathogen interactions.

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Figures

Fig. 5.1. Settling of non-viruliferous thrips (gray bar) and potentially viruliferous thrips (black bar) on *Tomato spotted wilt virus* (TSWV) infected and non-infected leaflets conducted with Y-shaped glass tube olfactometer. A TSWV symptomatic leaflet was inserted into one arm of the tube and a non-infected leaflet was inserted into the other arm of the tube. Twenty-five potentially viruliferous and non-viruliferous thrips were released separately at the base of the tube and the experiment was repeated 24 more times (N = 625 viruliferous and non-viruliferous thrips respectively). After 24 h interval, the number of thrips settled on each leaflet was recorded.

Fig. 5.2. Feeding damage on Non-infected (Non-inf.) and TSWV infected (TSWV inf.) produced by non-viruliferous (Non-vir.) and potentially viruliferous thrips (Vir.). Five TSWV infected plants (mechanically inoculated) and five non-infected plants were placed separately in a thrips proof cage randomly and ten potentially viruliferous female adults were released on each plant. Each set of experiment was repeated three times (N = 200 viruliferous thrips on TSWV infected and non-infected plants respectively). Similar to potentially viruliferous thrips, the experiment was conducted with non-viruliferous thrips also. After releasing thrips, feeding scars produced on each plant were recorded at 48 h interval for 2 weeks. Feeding damage on each plant was estimated by multiplying the number of leaflets with thrips feeding by intensity of feeding scars divided by total number of leaflets in the plant. Intensity of feeding scars was based on an arbitrary scale (1 = 0-20%, 2 = 20-40%, 3 = 40-60%, 4 = 60-80%, and 5 = 80-100%).

Fig. 5. 3. Differences in the levels of essential (a) and non-essential amino acids (b) between TSWV infected and non-infected leaflets (N = 5 leaflets from TSWV infected and non-infected

leaflets respectively). From each plant, 0.15 g of leaf tissue was taken from top one-third portion of the plant to extract samples for free amino acids analysis.

Fig. 5.1

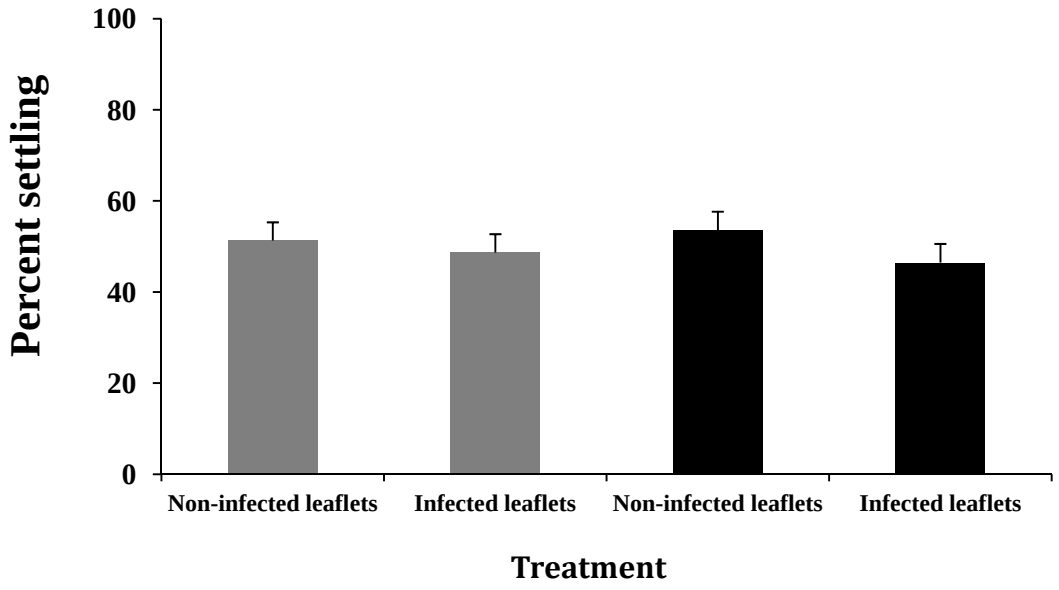
Percent settling
100
80
60
40
20
0
Non-infected leaflets
Infected leaflets
Non-infected leaflets
Infected leaflets
Treatment

Fig. 5.2.

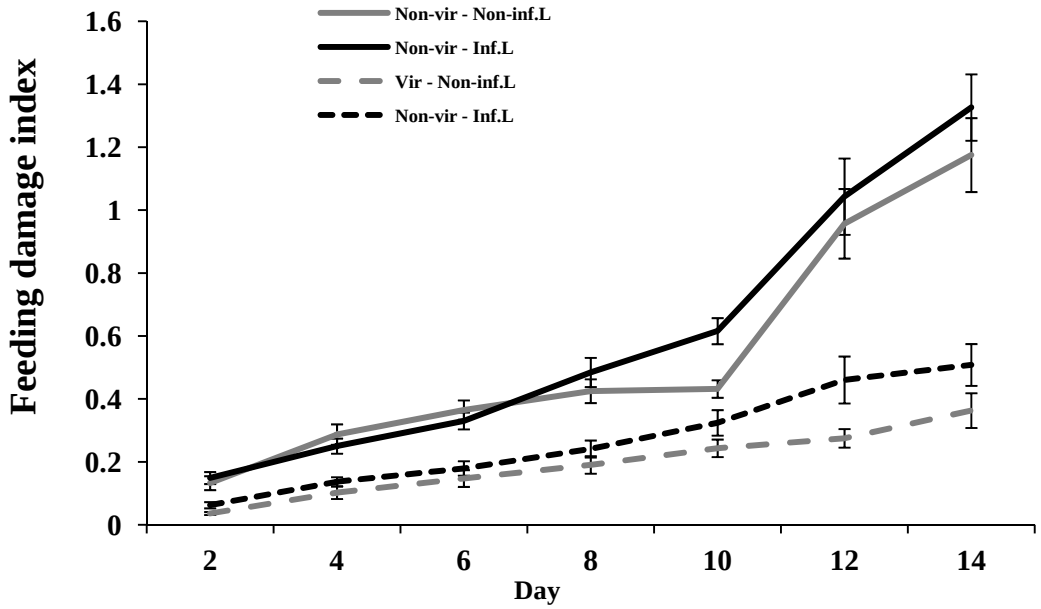
Non-vir - Non-inf.L
Non-vir - Inf.L
Vir - Non-inf.L
Non-vir - Inf.L
Feeding damage index
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
2
4
6
8
10
12
14
Day

Fig. 5. 3 a

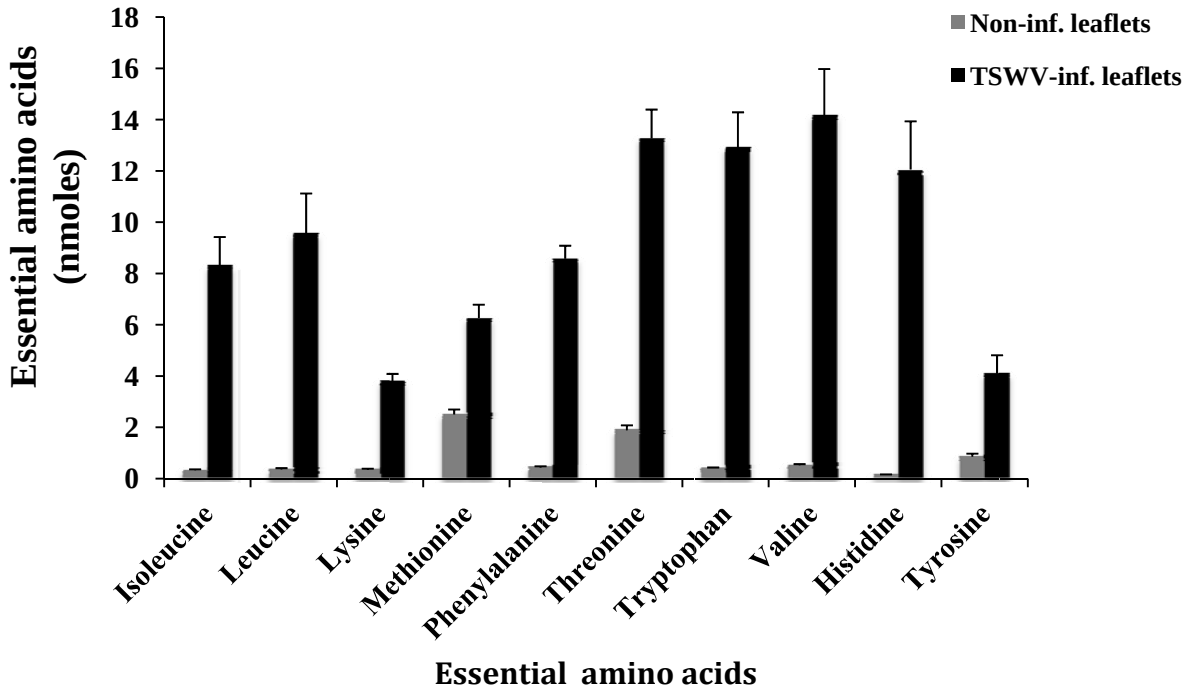

Fig. 5.3b.

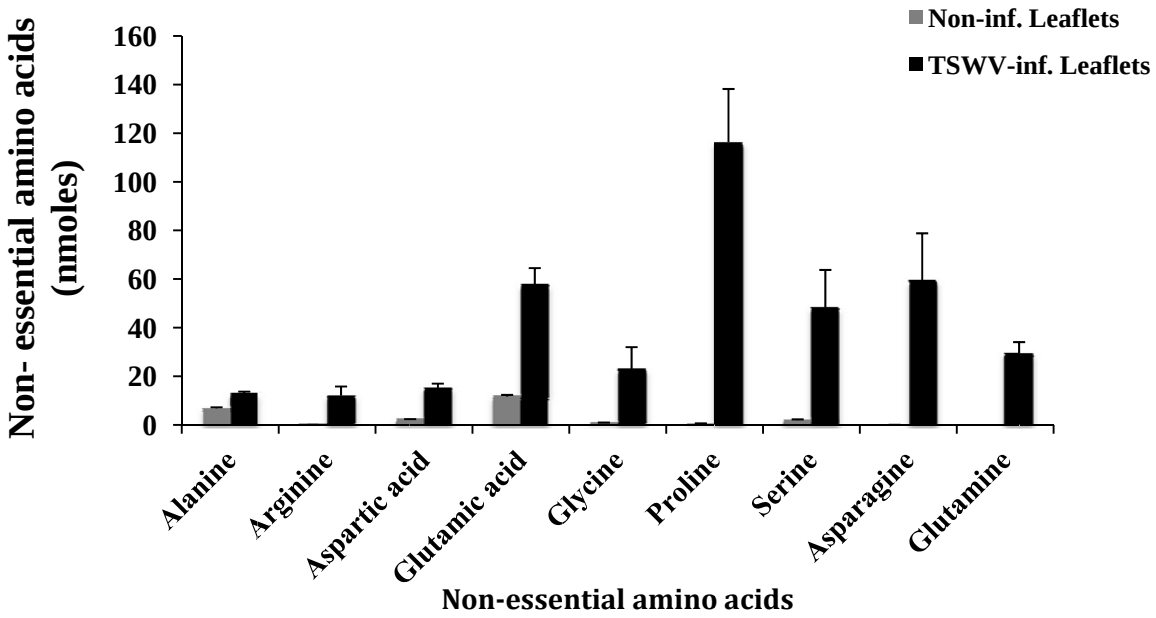

Table 5.1. Feeding damage index on *Tomato spotted wilt virus* (TSWV) infected and non-infected leaflets produced by potentially viruliferous thrips and non-viruliferous thrips.

	Feeding damage index (Mean ± Standard) [y]				
	Repeats of the experiment [z]				
Treatments [x]	**I**	**II**	**III**	**IV**	**Total feeding damage (Mean ± standard error)**
			Time 4		
Non-vir. thrips on Non-inf. leaflets	0.23 ± 0.05	0.38 ± 0.05	0.26 ±0.09	0.27 ± 0.052	0.29 ± 0.03
Non-vir. thrips on TSWV inf. leaflets	0.26 ±0.04	0.22 ± 0.03	0.29 ± 0.57	0.23 ± 0.07	0.25 ± 0.02
Vir. thrips on Non-inf. leaflets	0.05 ±0.005	0.12 ± 0.06	0.14 ± 0.04	0.09 ± 0.04	0.10 ± 0.02
Vir. thrips on TSWV inf. infects	0.13 ± 0.02	0.10 ± 0.03	0.14 ±0.03	0.17 ± 0.03	0.13 ± 0.01
Type III analysis (3, 16)					
F value	6.88	9.61	1.41	2.50	
P > F	0.0035	0.0007	0.2764	0.0965	
Pairwise contrast of Non-vir. thrips with Vir. thrips (df 3, 36)					
F value	18.16	21.21	4.16	6.10	
P > F	0.0001	0.0001	0.0234	0.0057	

Pairwise contrast of Non-inf. leaflets with TSWV inf. leaflets (df 3, 36)					
F value	1.98	4.60	0.05	0.17	
P > F	0.1576	0.0166	0.9847	0.9151	
Time 10					
Non-vir. thrips on Non-inf. leaflets	0.35 ± 0.02	0.46 ±0.02	0.52 ± 0.07	0.39 ± 0.07	0.43 ± 0.03
Non-vir. thrips on TSWV inf. leaflets	0.59 ± 0.13	0.70 ± 0.03	0.56 ± 0.09	0.61 ± 0.05	0.62 ± 0.04
Vir. thrips on Non-inf. leaflets	0.12 ± 0.06	0.20 ± 0.06	0.33 ±0.05	0.31 ± 0.02	0.24 ± 0.03
Vir. thrips on TSWV inf. infects	0.41 ± 0.12	0.21 ± 0.05	0.29 ± 0.07	0.37 ± 0.04	0.32 ± 0.04
Type III analysis (3, 16)					
F value	5.28	23.41	2.87	7.69	
P > F	0.0101	0.0001	0.0690	0.0018	
Pairwise contrast of Non-vir. thrips with Vir. thrips (df 3, 36)					
F value	6.09	58.49	8.41	11.47	
P > F	0.0058	0.0001	0.0014	0.0003	
Pairwise contrast of Non-inf. leaflets with TSWV inf. leaflets (df 3, 36)					
F value	9.69	6.15	0.02	8.64	
P > F	0.0007	0.0055	0.9960	0.0012	
Time 14					
Non-vir. thrips on Non-inf. leaflets	1.39 ± 0.19	1.55 ±0.15	1.19 ± 0.17	0.55 ± 0.17	1.17 ± 0.12

Non-vir. thrips on TSWV inf. leaflets	1.20 ± 0.25	1.30 ±0.23	1.55 ± 0.12	1.23 ± 0.25	1.32 ± 011
Vir. thrips on Non-inf. leaflets	0.19 ± 0.02	0.28 ±0.09	0.59 ± 0.16	0.39 ±0.02	0.36 ± 0.05
Vir. thrips on TSWV inf. infects	0.60 ± 0.25	0.44 ± 0.15	0.56 ± 0.17	0.44 ±0.06	0.51 ± 0.08
Type III analysis (3, 16)					
F value	13.75	12.95	7.52	7.12	
P > F	0.0001	0.0002	0.0023	0.0030	
Pairwise contrast of Non-vir. thrips with Vir. thrips (df 3, 36)					
F value	36.64	37.39	20.48	10.58	
P > F	0.0001	0.0001	0.0001	0.0004	
Pairwise contrast of Non-inf. leaflets with TSWV inf. leaflets (df 3, 36)					
F value	0.51	0.08	0.83	6.17	
P > F	0.6810	0.9699	0.4966	0.0055	

[x] Non-viruliferous and potentially viruliferous thrips released on TSWV infected and non-infected plants respectively to investigate feeding damage index. Five TSWV infected (TSWV inf.) plants (mechanically inoculated) and five non-infected (Non-inf.) plants were placed separately in a thrips proof cage and ten potentially viruliferous (Vir.) or non-viruliferous (Non-vir.) female adults were released on each plant. Each set of experiment was repeated three times (N = 200 viruliferous thrips or non-viruliferous thrips on TSWV infected and non-infected plants respectively). F and *P* > F values are presented to indicate differences in the thrips feeding damage calculated using PROC GLM procedure in SAS.

[y] Mean feeding damage index of each treatment at three time interval, 4, 10, and 14 day post thrips release. Feeding damage was

estimated by multiplying the number of leaflets with thrips feeding by intensity of feeding scars divided by total number of leaflets in the plant. Intensity of feeding scar was based on arbitrary scale ranged from 1 to 5 (1 = 0-20%, 2 = 20-40%, 3 = 40-60%, 4 = 60-80%, and 5 = 80-100%).

[z] Roman numerals I to IV indicate numbers of repeats of the same experiment.

Table 5.2. Oviposition efficiency of potentially viruliferous and non-viruliferous thrips on TSWV infected and non-infected leaflets

	Number of eggs (Mean ± Standard error) [y]			
	Repeats of the experiment [z]			
Treatments [x]	**I**	**II**	**III**	**Total number of eggs (Mean ± standard error)**
Non-vir. thrips on Non-inf. leaflets	37.1 ± 5.7	14.2 ± 9.4	7.8 ± 5.28	19.7 ± 4.57
Non-vir. thrips on TSWV inf. leaflets	2 ± 1.2	1.2 ± 0.4	1.1 ± 0.6	1.4 ± 0.45
Vir. thrips on Non-inf. leaflets	82.7 ± 11.4	85.9 ± 8.6	56.6 ± 10.5	74.8 ± 6.18
Vir. thrips on TSWV inf. infects	17.9 ± 6.26	5.1 ± 2.3	50.1 ± 14.8	24.4 ± 6.29
Proc GENMOD type III analysis (df 3, 36)				
F value	30.15	35.62	7.2	
P > F	0.0001	0.0001	0.0008	
Pairwise contrast of Non-vir. thrips with Vir. thrips (df 3, 36)				
F value	24.36	31.96	21.22	
P > F	0.0001	0.0001	0.0001	
Pairwise contrast of Non-inf. leaflets with TSWV inf. leaflets (df 3, 36)				
F value	65.25	49.2	0.39	
P > F	0.0001	0.0001	0.7609	

[x] Non-viruliferous (Non-vir.) or potentially viruliferous (Vir.) thrips released on Munger cages containing a non-infected (Non-inf.) and TSWV infected (TSWV inf.) leaflets to investigate ovipositional preference. Ten Munger cages were set up with a non-infected

leaflet and a TSWV infected leaflet in each cage. Ten non-viruliferous thrips or ten potentially viruliferous thrips were released on each cage and the experiment was repeated twice (N = 300 viruliferous and non-viruliferous thrips separately). Thrips were allowed to oviposit for 72 h following, which leaflets were dyed with acid fuschin to count numbers of eggs on each leaflet. F and *P* > F values are presented to indicate differences in the number of eggs in various treatments, calculated using PROC GLIMMIX procedure in SAS.

[y] Number of eggs oviposited by non-viruliferous thrips and potentially viruliferous thrips on non-infected and TSWV-infected leaflets were counted by using dissecting microscope.

[z] Roman numerals I to III indicate numbers of repeats of the same experiment.

Table 5.3a Statistical differences in essential amino acids levels between TSWV infected and non-infected leaflets

Essential amino acids	F value	Degree of freedom (d. f)	*P* > F [x]
Isoleucine	49.47	1, 8	0.0001
Leucine	34.60	1, 8	0.0004
Lysine	147.30	1, 8	0.0001
Methionine	52.51	1, 8	0.0001
Phenylalanine	256.66	1, 8	0.0001
Threonine	126.65	1, 8	0.0001
Tryptophan	534.0	1, 8	0.0001
Valine	55.85	1, 8	0.0001
Histidine	39.77	1, 8	0.0002
Tyrosine	22.18	1, 8	0.0015

Table 5.3b. Statistical differences in non-essential amino acids between TSWV infected and non-infected leaflets

Non-essential amino acids	F value	Degree of freedom (d. f)	*P* > F [x]
Alanine	25.04	1, 8	0.0010
Arginine	10.01	1, 8	0.0133
Glycine	6.02	1, 8	0.0397
Proline	9.51	1, 8	0.0150
Serine	9.05	1, 8	0.0169
Asparagine	9.09	1, 8	0.0167
Glutamine	40.36	1, 8	0.0002
Glutamic acid	42.06	1, 8	0.0002
Aspartic acid	60.70	1, 8	0.0001

[x] Differences in the levels of essential (a) and non-essential amino acids (b) between TSWV infected and non-infected leaflets calculated using PROC GLIMMIX procedure in SAS. Five TSWV infected and non-infected plants were tested for free amino acids analysis. From each plant, 0.15 g of leaf tissue was collected from the top one-third portion of the plant to extract samples for

free amino acids analysis.

Table 5.4. Number of adults produced by potentially viruliferous and non-viruliferous thrips on *Tomato spotted wilt virus* (TSWV) infected and non-infected leaflets.

	Number of adults (Mean ± standard error) [y]		
	Repeats of the experiment [z]		
Treatments [x]	**I**	**II**	**Total number of adults (Mean ± standard error)**
Non-vir. thrips on Non-inf. leaflets	19.3 ± 3.59	14.5 ± 4.20	16.9 ± 2.75
Non-vir. thrips on TSWV inf. leaflets	7.14 ± 1.59	16.2 ± 4.25	12.5 ± 2.57
Vir. thrips on Non-inf. leaflets	3 ± 0.89	5.9 ± 1.44	4.5 ± 0.89
Vir. thrips on TSWV inf. leaflets	2.4 ± 1.02	0.4 ± 0.22	1.4 ± 0.55
Proc GENMOD type III analysis (df 3, 36)			
F value	12.73	6.29	
P > F	0.0001	0.0015	
Pairwise contrast of Non-vir. thrips with Vir. thrips (df 3, 36)			
F value	18.50	16.98	
P > F	0.0001	0.0001	
Pairwise contrast of Non-inf. leaflets with TSWV inf. leaflets (df 3. 36)			
F value	8.23	0.41	
P > F	0.0003	0.7468	

[x] Non-viruliferous (Non-vir.) and potentially viruliferous (Vir.) female thrips released on TSWV infected (TSWV inf.) and non-infected (Non-inf.) leaflets respectively to investigate adult productions. Twenty Munger cages were set up with TSWV infected leaflets and non-infected leaflets separately. Ten non-viruliferous female adults or potentially viruliferous female adult thrips were released separately in each cage (N = 200 viruliferous and non-viruliferous thrips on TSWV infected and non-infected leaflets respectively). F and *P* > F values are presented to indicate differences in the number of adults in various treatments, calculated using the PROC GLIMMIX procedure in SAS.

[y] Newly emerged adults on non-infected (Non-inf.) leaflets and TSWV infected (TSWV inf.) leaflets by non-viruliferous (Non-vir.) thrips and potentially viruliferous (Vir.) thrips were recorded at 24 h interval.

[z] Roman numerals I to II indicate numbers of repeats of the same experiment.

Table 5.5. Developmental time (adult to adult) of non-viruliferous and potentially viruliferous thrips on non-infected and *Tomato spotted wilt virus* (TSWV) infected thrips

	Developmental time (days) (Mean ± standard error) [y]		
	Repeats of the experiment [z]		
Treatments [x]	**I**	**II**	**Total number of days (Mean ± standard error)**
Non-vir. thrips on Non-inf. leaflets	14.7 ± 0.49	14.1 ± 0.62	14.4 ± 0.39
Non-vir. thrips on TSWV inf. leaflets	16 ± 0.91	14.3 ± 0.48	15 ± 0.51
Vir. thrips on Non-inf. leaflets	17.75 ± 0.55	15.6 ± 0.54	16.6 ± 0.45
Vir. thrips on TSWV inf. infects	18.4 ± 0.72	16.5 ± 0.67	17.9 ± 0.51
Proc GENMOD type III analysis (df 3, 36)			
F value	3.36	2.41	
P > F	0.0292	0.0829	
Pairwise contrast of Non-vir. thrips with Vir. thrips (df 3, 36)			
F value	9.03	5.32	
P > F	0.0001	0.0039	
Pairwise contrast of Non-inf. leaflets with TSWV inf. leaflets (df 3. 36)			
F value	1.63	0.18	
P > F	0.1995	0.9093	

[x] Non-viruliferous (Non-vir.) and potentially viruliferous (Vir.) female thrips released on TSWV infected (TSWV inf.) and non-infected (Non-inf.) leaflets respectively to investigate developmental time. Twenty Munger cages were set up with TSWV infected leaflets and non-infected leaflets separately. Ten non-viruliferous female adults or potentially viruliferous female adult thrips were released separately in each cage (N = 200 viruliferous and non-viruliferous thrips respectively on TSWV infected and non-infected leaflets respectively). F and *P* > F values are presented to indicate differences in the number of adults in various treatments, calculated using the PROC GLIMMIX procedure in SAS.

[y] Mean developmental time required to complete one generation (adult to adult) of non-viruliferous and potentially viruliferous thrips reared on TSWV infected and non-infected leaflets separately. Munger cages were monitored at 24 h interval to record newly emerged adults.

[z] Roman numerals I to II indicate numbers of repeats of the same experiment.

CHAPTER 6

SUMMARY

The goal of this research was to investigate interactions between tobacco thrips, *Frankliniella fusca* (Hinds) and *Tomato spotted wilt virus* (TSWV) in the peanut pathosystem. Three objectives were set for the research. The first objective was to develop a greenhouse based thrips-mediated transmission protocol. The intention of developing a screening protocol was to provide supplemental information that would aid conventional breeding. The second objective was to investigate the effects of newly released TSWV resistant genotypes on thrips and on TSWV in order to speculate on the mechanisms of TSWV resistance in peanut genotypes. The third objective was to assess the effects of TSWV on biology and behavior of *F. fusca*.

For the first objective, various parameters associated with thrips-mediated TSWV transmission including thrips density, inoculation access period (IAP), and plant age were optimized. Among four thrips densities (one, three, five, and 10), three, five, and ten thrips resulted in greater incidence of TSWV infection in plants than with one thrips. With each thrips density, an increase in IAP (15 min, 1 h, 1 day, and 4 days) did not result in an increased TSWV infection in plants. However, the study demonstrated that an individual thrips requires 1 day of IAP to successfully inoculate TSWV in peanut plants and five thrips can inoculate plants in as few as 15 min. Thrips-mediated inoculation resulted in greater incidence of TSWV infection in plants than with mechanical inoculation at different plant ages (one week, two week, and four week-old plants). Irrespective of inoculation methods, one week and two-week old plants were more susceptible to TSWV than four-week old plants. Thrips-mediated and mechanical

inoculation both resulted in greater TSWV titer levels at different plant ages. Unlike in thrips-mediated inoculation, TSWV titer levels were not different among plant ages with mechanical inoculation.

For the second objective, effects of resistant (Georganic, Georgia-06G, Tifguard, NC94022) and susceptible genotype (Georgia Green) on thrips and TSWV were investigated. Thrips-mediated inoculation resulted in greater TSWV infection incidence in Georgia Green compared to other resistant genotypes. All the genotypes exhibited typical TSWV symptoms. Estimation of TSWV titers indicated that Georgia Green and NC94022 accumulated greater levels of TSWV titers than the other genotypes. Thrips developed on all genotypes were able to acquire TSWV from both resistant and susceptible genotypes. Thrips biology such as adult productions and developmental time required to complete one generation (adult to adult) did not vary significantly among resistant genotypes. Further, both resistant and susceptible genotypes did not affect thrips head capsule length and width. In this study, evidence for a single dominant gene conferring resistance to TSWV was not found.

For the third objective of the research, effects of TSWV infection on settling, feeding, oviposition, and biology of *F. fusca* were studied. Subsequently, the effects of TSWV in free amino acids levels in peanut plants was investigated. TSWV infection in plants as well as in thrips did not influence thrips settling behavior. However, non-viruliferous thrips fed more than potentially viruliferous thrips demonstrating the direct effects of TSWV on thrips feeding. Both potentially viruliferous and non-viruliferous thrips produced more eggs on non-infected leaflets than on TSWV infected leaflets. Also, potentially viruliferous thrips produced more eggs than non-viruliferous thrips. Free amino acids analysis revealed that both essential and non-essential amino acids levels were significantly more in TSWV infected leaflets than non-infected leaflets.

Greater amino acid titers thus could have influenced the differential oviposition capabilities in thrips. TSWV infection in leaflets as well as in thrips had negative impact on thrips biology. More numbers of adults were produced on non-infected leaflets than on TSWV infected leaflets. Further, non-viruliferous thrips produced more adults than potentially viruliferous thrips. Developmental time of adults was not affected by TSWV infection in plants. However, development of thrips was delayed when they were infected with TSWV. Despite the spike in free amino acid titers, TSWV infection in peanut, overall, negatively affected the reproductive fitness and development of *F. fusca*. This suggested that free amino acids and possibly others may play a role in deceptive arrestment of thrips on TSWV infected plants. Such an arrestment may be beneficial for a *Tospovirus* whose acquisition and transmission is specific to vector stages.