MONITORING OF THRIPS-TRANSMITTED ORTHOTOSPOVIRUS AND WILD SPECIES-MEDIATED RESISTANCE IN THE PEANUT PATHOSYSTEM

by

YI-JU CHEN

(Under the Direction of Rajagopalbabu Srinivasan)

ABSTRACT

Tobacco thrips, *Frankliniella fusca* (Hinds), is a major vector of *tomato spotted wilt orthotospovirus* (TSWV) in peanut of the southeastern United States. TSWV infection in peanut causes the yield-limiting spotted wilt disease. In this study, temporal changes in TSWV accumulation in peanut and TSWV acquisition and inoculation by *F. fusca* were assessed and detection of TSWV-transmitting thrips on sticky cards was optimized. Results indicated that TSWV transmission varied temporally, and these temporal changes were caused by the percentage of viruliferous thrips and the field inoculum available throughout the cropping season. Resistant cultivars are widely used for TSWV management, and new cultivars with improved resistance can increase the sustainability of TSWV management. However, sources of genetic resistance in cultivated peanut are limited. Related/wild *Arachis* species are important sources of resistance against TSWV and *F. fusca*. Experiments were conducted to examine resistance/tolerance against thrips and/or TSWV in 16 wild peanut genotypes. Plausible TSWV resistance in diploid species and tetraploid hybrids was characterized by reduced TSWV infection, virus accumulation and/or thrips damage. Lastly, to explore resistance mechanisms in wild peanuts, transcriptional responses in two diploid species and their hybrid in response to TSWV were compared by gene expression. The changes in gene expression in response to TSWV infection seems to be more pronounced in *A. valida* accession GK30011 (BB genome) than in *A. stenosperma* V10309 (AA genome).

INDEX WORDS: *Frankliniella fusca*, *Tomato spotted wilt orthotospovirus*, *Arachis*, host plant resistance, wild peanut, transcriptome, resistant mechanisms, transmission, inoculum, thrips resistance

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by

YI-JU CHEN

BS, National Chung Hsing University, Taiwan, 2008

MS, National Taiwan University, Taiwan, 2010

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YI-JU CHEN

Major Professor:	Rajagopalbabu Srinivasan
Committee:	Mark R Abney
	Soraya Leal-Bertioli
	Sudeep Bag

Electronic Version Approved:

Ron Walcott
Vice Provost for Graduate Education and Dean of the Graduate School
The University of Georgia
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DEDICATION

For Ting-Ying, the love of my life. Thanks for supporting me thoroughly and being unselfish all because of love. For my parents, whose endless love let me become myself. For my brother and sister, who always stand behind me. For my family-in-law, who filled me up with more love than I had. For my son, I love you and hope you would understand my absence from your childhood.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Introduction

Thrips-transmitted viruses cause severe diseases in many crops around the world. Tomato spotted wilt orthotospovirus (TSWV) infection, which causes spotted wilt disease, is a serious problem in peanut production in the southeastern United States. The use of field-resistant peanut cultivars with cultural practices has reduced crop losses due to the disease. Tritrophic interactions between thrips, plants, and viruses are intricate; understanding these interactions between the major thrips vector, *Frankliniella fusca,* and TSWV in cultivated and wild peanut is essential for sustainable management of spotted wilt disease.

Peanut

The origin and distribution of peanut. The peanut (*Arachis hypogaea* L.) is an allotetraploid (2n=4x=40, AABB-type genome) containing four sets of chromosomes (Husted, 1936) from the hybridization of two wild diploid species (2n=2x=20) (Bertioli et al., 2016). The *Arachis* genus in the family Fabaceae is native to South America and contains 83 described species; all of which are leguminous (Santana & Valls, 2015; Seijo et al., 2021; Valls & Simpson, 2005, 2017; Valls et al., 2013). New genomic data of wild and cultivated *Arachis* species have helped clarify their origin and evolution (Stalker & Wilson, 2015); the parental species are believed to be *A. ipaensis* (BB-type genome) and *A. duranensis (*AA-type genome)

(Bertioli et al., 2016). Plant habit and branching patterns are used to identify *A. hypogaea* into subspecies, varieties, or market types. The major market types in the US are Runner, Virginia, Spanish, and Valencia (Tillman & Stalker, 2009).

The economic importance of the peanut. The peanut seed has been cultivated for thousands of years and is an attractive food and oil source for humans (Bertioli et al., 2016).. China (36%), India (14%), Nigeria (9%), and the US (5%) are the major producers (FAOSTAT, 2021). In the US, peanut production was valued at 1.3 billion dollars in 2020 at 3.1 million tons (USDA NASS 2020). Most of the US peanut crop was planted in the Southeast region, with 50% in Georgia. Georgia produced 1.6 million tons of peanuts on 800,000 acres, and total annual production was valued at over US$656 million (USDA NASS 2020).

Thrips as peanut pests. Before harvest, fungal diseases are the major yield reducers. Early leaf spot, late leaf spot, and rust are widespread, and soil-fungal disease, nematodes, and spotted wilt are important diseases in the US (Tillman & Stalker, 2009). Arthropod pests are categorized by the feeding site: foliar arthropods (thrips, leafhoppers, caterpillars, and mites) and soil insects (rootworms and burrow bug) (Brandenburg, 2020; Lynch and Douce, 1992). The pest species and their populations often vary greatly among production regions and production years (Stalker, 1997). Four different market types are distinguished by pod length, the number and size of kernels, and primary retail market (Anco & Croft, 2019; Koppelman et al., 2016; Puppala et al., 2018). In Georgia, most peanut pests are in the four orders which are Thysanoptera (family: Thripidae), Lepidoptera (families: Pyralidae and Erebidae), Hemiptera (families: Cydnidae, Membrancidae, and Cicadellidae) and Coleoptera (family*Chrysomelidae* in the class Insecta, and one order Trombidiformes (family: *Tetranychidae*) in the class Arachnida (Abney, 2020). Thrips serve as vectors of plant viruses. Six genera, including *Frankliniella, Scirtothrips, Thrips,*

Megalurothrips, Caliothrips, and *Enneothrips* are known to feed peanut foliage. Among them, *F. fusca* is very efficient in transmitting TSWV in Georgia (Srinivasan et al., 2018).

Thrips

Thrips vectors of orthotospovirus and related viruses. TSWV is a species of *Orthotospovirus*, a genus that includes 26 International Committee on Taxonomy of Virus (ICTV)-approved species (2021. Jan. 16). Previous studies showed that less than 0.3 % (17 of ~6,300) thrips species transmit orthotospoviruses (Riley et al.2011, Chen et al., 2019, ThripsWiki, 2021). But thrips vectors are taxonomically distantly related (Mound 2002). Thrips vectors are placed in seven genera including *Ceratothripoides*, *Franklniella*, *Thrips*, *Scirtothrips*, *Dictyothrips*, *Neohydatothrips*, and *Megalurothrips* (Oliver and Whitefield, 2016; Chen et al., 2019). *Megalurothrips abdominals* and *Frankliniella tritici* have been added to the existing 15 species (Chen et al., 2019; Mound et al., 2022; Mound & Cavalleri, 2021; Riley et al., 2011). Thrips vectors are taxonomically distantly related (Mound, 2002). There are 11 potential thrips vector species of Orthotospovirus in seven genera, including *Frankliniella schultzei*, *F. occidentalis*, *F. fusca*, *F. gemina*, *F. intonsa* , *F. bispinosa*, *F. cephalica*, *Scirtothrips dorsalis*, *Thrips palmi*, *T. setosus*, *T. tabaci, and Megalurothrips abdominals* (Chen et al., 2019; Culbreath et al., 2003; Rotenberg et al., 2015; Sharman et al., 2020, Mound et al., 2022; Oliver & Whitfield, 2016). Seven orthotospoviruses infect peanuts: capsicum chlorosis orthotospovirus (CaCV) (Chen et al., 2007), groundnut bud necrosis virus (GBNV) (Reddy et al., 1992), groundnut chlorotic fan-spot virus (GCFSV) (Chu et al., 2001), groundnut ringspot virus (GRSV) (de Avila et al., 1993), groundnut yellow spot virus (GYSV)(Satyanarayana et al., 1998), impatiens necrosis spot virus (INSV)(Pappu et al., 1999), tomato spotted wilt virus

(TSWV) (Halliwell & Philley, 1974), watermelon bud necrosis virus (WBNV) (Culbreath et al., 2003; Rotenberg et al., 2015), and tomato chlorotic spot virus (TCSV) (Adegbola et al., 2016).

The morphology, biology, and ecology of tobacco thrips.

(1) Geographic distribution: The tobacco thrips, *Frankliniella fusca* (Hinds) (Thysanoptera: Thripidae), is a pest on several major crops (Watson, 1922). Tobacco thrips is widespread in North America (Canada, Cuba, Martinique, Mexico, Puerto Rico, and USA) and common in the Southeastern US (LaTora et al., 2022). Tobacco thrips also been reported in Europe (Netherlands) and Asia (Nakahara & Minoura, 2015; Nakao et al. 2011; Vierbergen, 1995).

(2) Morphology and taxonomy: Thrips are tiny insects, with body lengths of 1.0-1.8 mm. Tobacco thrips have sexual and wing dimorphism. Both sexes have long-winged (macropterous) and short-winged (brachypterous) morphs (Newsom et al., 1953). Females (1.2-1.8 mm) have a medium to dark brown color; males (1.0-1.3 mm) are smaller with pale yellow color (Nakao et al., 2011). These features can be used to distinguish *F. fusca* from other *Frankliniella* species: 1) eight-segmented antennae; 2) ocular setae III are more than three times longer than postocular setae; and 3) a comb is absent on the posterior margin of tergite VIII (Nakahara, 1997).

(3) Life stages and reproduction: The life cycle of *F. fusca* includes six stages: egg, two larval stages (first instar and second instar), two pupal stages (propupa and pupa), and adult. Most individuals complete the cycle in two to three weeksdepending on host type and abiotic factors (Ananthakrishnan, 1993b). Thrips show temperature-dependent growth. The development time from egg to adult on peanut for tobacco thrips was 11.5 and 23.9 days at 20 and 35°C, respectively (Lowry et al., 1992). Thrips exhibit haplodiploid reproduction and can reproduce

asexually (arrhenotokous parthenogenesis) and sexually. The males develop from unfertilized eggs with half the number of chromosomes of a female (Mound, 2009). The development time and reproductive rate of thrips can be affected by virus infection status and insecticide resistance (Huseth et al., 2016; Shrestha et al., 2012). Potentially viruliferous (TSWV) thrips have longer developmental times and lay more eggs than nonviruliferous thrips. Previous reports support that tobacco thrips overwinter as female adults near or in the roots of grasses (Newsom et al., 1953). However, the ecology of overwintering adult *F. fusca* is not completely known.

(4) Feeding injury, pathogen transmission, and potential pollinator status: Thrips are polyphagous, piercing-sucking cell-feeders, and adults and larvae have similar feeding behaviors (Chisholm & Lewis, 1984; Ullman et al., 1992). The mouthparts are uneven, and only the left mandible stylet pierces plant epidermal cells. Once thrips make a hole, cell components such as whole chloroplasts are sucked through one pair of maxillary styles, which form a single food canal (Chisholm & Lewis, 1984). After feeding, a silvered patch develops on the leaves because the concentrated punctures result in complete internal cell disruption and empties mesophyll cells. The effect of leaf feeding can be more severe especially in hot and dry weather in the case of onion thrips to onion plants (Lewis, 1973).

The role of thrips as pollinators is unclear, but species within the family Melanthripidae have transported pollen grains probably since 110-105 million years ago (Ananthakrishnan, 1993a; Peñalver et al., 2012). One study showed that sprinkling pine pollen on host plants can increase oviposition several fold, implying that pollen could be a main food source (Angelella & Riley, 2010).

The economic importance of tobacco thrips in the US. Tobacco thrips is best known by a pest of tobacco and commonly found in peanut, tomato, cotton, and onion crops in the

southeast (Watson, 1922; Riley et al., 2011). They are also found on minor crops, such as strawberries, oats, turnips, dewberries, mustard, and plantains (Watson, 1922). In addition to causing feeding injury on foliage, tobacco thrips transmit TSWV, which caused about US$12.3 million annual average losses in peanut between 1996 and 2006 in Georgia (Riley et al., 2011). The economic damage of tobacco thrips in peanuts caused by direct feeding injury is not clear. Over three decades, reports have shown that seed yield were increased up to 32% (Funderburk et al., 1998) by thrips control and in-furrow chemical control reduced thrips injury and TSWV incidence (Herbert et al., 2007). However, thrips control is uneconomical in some cases because using chemical insecticides suppresses thrips populations that did not increase yield (Lynch et al., 1984; Tappan & Gorbet, 1979; Whalen et al., 2015). Funderburk et al. (1998) mentioned that thrips injury may be economically important when combined with other factors for certain cultivars that are sensitive to early-season stress. It's not easy to rule out other factors. Overall, thrips injury is most likely cause to yield loss (LaTora et al, 2021). For example, the economic threshold in peanut for thrips injury is 25%, growers need to control thrips to reduce yield loss (Jordan et al., 2017).

Thrips populations and seasonal dynamics in peanut. Tobacco thrips occur seasonally on crops and non-crop plants (Chellemi et al., 1994; Groves et al., 2003; Newsom et al., 1953; Sweeden & McLeod, 1993). Thrips population is typically high early in the season when peanuts are young (Tappan & Gorbet, 1979; Todd et al., 1995). Tobacco thrips are found in the crop once the seedlings break out though the soil (Newsom 1953; Garcia et al., 2000). The peak of thrips adult on peanut is around 10-31 days after planting (Todd et la., 1995; Tappan & Gorbet, 1979) and populations begin to decline after two to four months later (Tappan & Gorbet, 1979; Tappan, 1986; Todd et al., 1995; Wells et al., 2001). The adults and larvae are dominant in flowers and

terminals, respectively (Tappan & Gorbet, 1979). Since few larval thrips were observed on flowers, and studies showed that thrips did not move from the feeding site to flowers, Tappan (1986) implied that the thrips had immigrated to flowers. Cage experiments indicated that soil planted with crops are not the main source of thrips attacking newly planted crops (Barbour & Brandenburg, 1994).

The timing of thrips occurrences can be predicted by weather parameters (Chappell et al., 2020). Also, temperature and precipitation are important factors for thrips flight (Garcia & Brandenburg, 1995). Populations of thrips declined to low levels during the late summer in Georgia's peanut during 1987-1992 (Todd et al. 1995). Natural enemies of tobacco thrips such as parasitic nematode (*Thripinema fuscum*) would be one of the reasons for thrips decline (Funderburk et al., 2002). The seasonal decline in populations may be caused by the decaying of winter hosts that harbor immigrant thrips (Morsello et al., 2008). The reasons for thrips population declines in summer are not well characterized.

Thrips-Transmitted Tomato Spotted Wilt Orthotospovirus (TSWV)

TSWV: History, host range, and symptoms. Spotted wilt disease was first reported on tomato in Australia in 1915 (Brittlebank, 1919), and was later found to be transmitted by thrips (Pittman, 1927) and caused by TSWV (Samuel et al., 1930). By the late 1980s, TSWV was widespread in peanut-producing areas (Goldbach & Peters, 1994). TSWV has a broad host range, infecting thousands of species in 69 families of monocots and dicots, including many crops, weeds, landscape plants, and wild plants (Parrella et al., 2003). TSWV symptoms vary with host plant, time of year, and environmental conditions (German et al., 1992). Infected peanuts show variable and systemic symptoms, ranging from mild ringspots and/or chlorosis on

a leaflet to severe stunting of the entire plant. Symptoms appear on leaves, stems, and seeds and include: concentric ringspots, chlorosis, yellow spot, and/or ragged leaflets, wilting and stunting of partial plants, and reddish color and cracking of seeds (Culbreath et al., 2003; Sundaraj et al., 2014). Mixed infections of TSWV, peanut mottle virus (PMotV), and peanut stripe virus (PStV) are common in the field (Gillaspie et al., 2000; Kuhn et al., 1984). TSWV can be detected in 95% of symptomatic plants with uneven virus distribution (Kresta et al., 1995). However, the early symptoms of TSWV are similar to PMotV. Therefore, identification of TSWV should not solely rely on symptom-based diagnosis, particularly in the early stage of the disease process (Hoffmann et al., 1998).

Taxonomy and genome organization. TSWV belongs to the order *Bunyavirales*, which consists of 13 families of tri-segmented, negative-sense RNA viruses, infecting a diversity of plants, animals, and insects (Leventhal et al., 2021). The family *Tospoviridae* contains only one genus, *Orthotospovirus,* which infects both animals and plants. The name of the species tomato spotted wilt virus was updated to tomato spotted wilt orthotospovirus by the International Committee on the Taxonomy of Viruses (ICTV) in 2019 (Walker et al., 2020). TSWV was the only member in the genus until the early 1990s and became the type species after more species were recognized by serological relationships (Murphy et al., 2012; Oliver & Whitfield, 2016). So far, 26 distinctive *Orthotospovirus* species are known (Table1.1). The TSWV virion is a pleiomorphic particle of about 80-110 nm in diameter and contains 5% RNA, 70% proteins, 5% carbohydrates, and 20% lipids (Adkins, 2000). The virion particle is enclosed by ribonucleoproteins (RNP), with a host-derived and double-layered membrane in which the two glycoproteins are embedded. The core of the virion contains RNP composed of the ssRNA

covered by the N protein and an RNA-dependent RNA polymerase (RdRP) (Leventhal et al., 2021) (Figure 1.1).

The virus also codes for structural proteins and non-structural proteins. The typical TSWV genome consists of three linear RNAs: small (S; 2.9 kb), medium (M; 4.8 kb), and large (L; 8.9 kb) (German et al., 1992). The RNA S is an ambisense segment with two open reading frames that code for nucleocapsid (NP) proteins in viral complementary (vc) sense and NSs in viral (v) sense orientation (De Haan et al., 1990). N protein is the most abundant, and it regulates the switch from transcription to replication and encapsulations of the genome (Qiu & Moyer, 1999). NSs is a silencing suppressor, a crucial defense suppressor in plants and insects (Csorba et al., 2015; Takeda et al., 2002). The M RNA is an ambisense segment with two open reading frames (ORF). Non-structural homologue, NSm, is encoded in v sense, and glycoprotein precursor proteins (Gn and Gc) are encoded in vc sense. NSm is associated with host range determination, cell to cell movement, and symptoms in plants (Kormelink et al., 1994; Silva et al., 2001). Glycoproteins are associated with the recognition of thrips but not important for TSWV infection (Sin et al., 2005). The L RNA is a negative sense segment with one ORF in the vc sense encoding RdRp, which is one of the replicases and catalyzes the initiation and elongation of RNA synthesis. The replication occurs in the cytoplasm.

The economic importance of TSWV. TSWV crop loss was estimated at over US$1 billion dollars in 1994, it was the second most economically important plant virus due to scientific/economic importance (Scholthof et al., 2011). From 1996 to 2006, estimated losses in the US were US$1.4 billion and US$326 million in Georgia (Riley et al., 2011). TSWV is still a major concern in peanut production in the southeastern US (Culbreath & Srinivasan, 2011).

TSWV transmission efficacy. Multiple factors influence TSWV transmission efficiency. The thrips development stage, sex, isoline, and individual genetics within a species are the factors affecting thrips' acquisition and inoculation of TSWV (Ogada et al., 2016; Linak et al., 2020; Ogada & Poehling, 2015; Van De Wetering et al., 1999). Virus isolates and virus accumulation (virus load) affect transmission. A number of vectors and virus load in individual thrips showed positive effects on virus spread (Rotenberg et al., 2009; Shrestha et al., 2015). The virus load in the leaf is also associated with the efficiency of acquisition by the vector (Okazaki et al., 2011). However, the quantitative relationship is not always straightforward. The interaction between thrips and TSWV is specific and complex and supports local adaption (Linak et al., 2020).

Plant age, nutrient content, and genotype can affect TSWV transmission. Thrips acquire the virus from plants and transmit the virus to another plant. The nutrient content of the leaf may influence vector biology and preference, thereby affecting later transmission efficiency. For example, thrips prefer TSWV-infected leaflets that contain more amino acids compared with non-infected ones and facilitate TSWV acquisition and transmission (Shrestha et al., 2012; Shalileh et al., 2016). Young plants are sensitive to being infected (Shrestha et al., 2015). In addition, environmental factors such as water stress affect transmission efficiency. TSWV systemic infection in tomato is slower in water stressed than in well-watered plants(Córdoba et al., 1991). Therefore, the irrigation status may affect the virus spread in peanut.

Interactions Within Thrips-Orthotospovirus Pathosystems

Tospovirus-thrips interactions. Understanding the interactions between thrips and viruses is crucial to reducing virus transmission. A vector is a species that can successfully

transmit the virus (Wijkamp et al., 1995). Vector competence is based on the efficiency with which a thrips vector can transmit TSWV (Sakurai et al., 1998). 'Viruliferous thrips' is an umbrella term that includes virus carriers (TSWV-infected) and transmitters (Ogada et al., 2016).

Viruliferous thrips. Although the transmission efficiencies of the two wing type of *F. fusca* have not been shown to be different, macropterous (long-winged) thrips are a greater concern for the primary spread of TSWV (Arthurs et al., 2018; Wells et al., 2002). Macropterous populations often have higher proportions of viruliferous thrips, compared with brachypterous populations (Barbour & Brandenburg, 1994; Camann et al., 1995; Wells et al., 2002). Even though brachypterous thrips can transmit TSWV, their ability to move long distances is limited. Secondary spread of TSWV within the field could be aided by brachypterous thrips.

Monitoring thrips abundances do not reflect the proportion of carriers and/or transmitters within populations (Boonham et al., 2002). Therefore, early detection of TSWV in thrips might be useful for long-term, sustainable management (Wan et al., 2020). In peanut fields, the proportion of viruliferous thrips with TSWV were found to be 0-10 % as determined by N-gene based ELISA (Chamberlin et al., 1993) and 1.5-2.3 % by NSs ELISA (McPherson et al., 2005). Less is known about the relationship between virus load in thrips and their transmission efficiency than the relationship between the plant's virus load and vector acquisition (Bag et al., 2014). The interactions between viruses and thrips and the factors affecting thrips competence are still poorly explored.

Thrips vector competence and virus transmission. Determining which thrips species is the most important vector in the local cropping system is critical for thrips-borne virus management. The mechanism of vector competence of thrips species in relation to TSWV is not well determined (de Assis Filho et al., 2005). In India, *Frankliniella schultzei* and *Scirtothrips*

dorsalis are the vectors of TSWV in peanut, *S. dorsalis* is a less efficient vector (Amin, 1981). Similarly, in the US peanut system, there are more than one thrips vector of TSWV, *F. fusca* is a more efficient vector than *F. occidentalis* (Arthurs et al., 2018). The thrips competence, which is the ability of a thrips to transmit the vrius varies on plant hosts. For example, *F. occidentalis* (77%) is a better transmitter than *F. fusca* (38%) on *Datura stramonium* (Kritzman et al., 2002). Estimate of TSWV accumulation in thrips is a method to compare the vector competence, and it showed a positive correlation between virus loads and frequency of successful transmission on leaf disk assay using *Datura stramonium* (Rotenberg et al., 2009). An estimate of virus accumulation in salivary glands is more convincing to determine the status of thrips competence rather than virus accumulation in individuals (Han et al., 2019). Intraspecies differences such as gender and developmental stage also affect transmission efficiency (Kritzman et al., 2002; Rotenberg et al., 2009). Male thrips of *F. occidentalis* are better transmitters due to virus accumulation, feeding behavior, and mating behavior than female thrips (Ogada & Poehling, 2015; Rotenberg et al., 2009; Wan et al., 2020). But TSWV transmission via *F. fusca* was not affected by sex (male and female) and wing type (macropterous and brachypterous wings) (Arthurs et al., 2018). Thrips transmit the virus in a circulative-persistent and propagative method, and only thrips that acquired the virus through feeding as larvae are able to transmit it (Ullman, 1993). Circulative-persistent transmission means viruses circulate within the vectors, and vectors remain transmitters for their life. Since the virus propagates, or replicates, within vectors, vectors do not need to acquire the virus multiple times. Ingestion of the virus by adult thrips does not lead to transmission (Nagata & Peters, 2001). For instance, *Thrips palmi*, *F. occidentalis,* and *F. fusca* that acquire TSWV as adults could not transmit the virus, even if virus

replication was observed (de Assis Filho et al., 2005; Bandla et al., 1994; Seepiban et al., 2015). The barriers to virus transmission by thrips have been discussed in Whitfield et al. (2005).

TSWV transmission in thrips. The interaction between thrips and TSWV is specific to species. The virus must overcome at least two barriers after the virus is acquired by thrips. The first barrier is the binding of glycoproteins on virus particles to specific receptors on the thrips' midgut epithelial cells (Badillo-Vargas et al., 2019). The movement of the virus from midgut cells to salivary glands and the resulting replication that occurs is the second barrier (Hogenhout et al., 2008). It is unknown how the virus moves from the midgut to the salivary glands. Three possible routes have been proposed by Whitfield et al. (2005). The virus does not spread to the salivary glands in the adult stage (Montero-Astua et al., 2016). It is believed that the virus reaches the salivary glands before pupation (Kritzman et al., 2002; Nagata et al., 2002).

Techniques of virus detection in thrips. Many techniques have been developed for TSWV detection in thrips, including observation of virus particles by protein-based assay (Amin, 1981; Cho et al., 1991; Bandla et al., 1994; Aramburu et al., 1996; Arthurs et al., 2018), electron microscope (EM) (Ullman et all, 1992), and nucleic acid-based assays (Tsuda et al., 1994; Boonham et al., 2002; Roberts et al., 2000). In 1980s, protein-based hemagglutination was used to detect viral antigens in groups of thrips (Amin, 1981). In 1990, virus accumulation was detected in individual thrips using enzyme-linked immunosorbent assays (ELISA) targeting N protein (Cho et al., 1991) and immunological squash blot (Aramburu et al., 1996). The ELISA methods are not used widely due to sample size limitations and concerns of false positives. Arthurs et al. (2018) pointed out that ELISA failed to detect the virus in a few thrips samples, (Arthurs et al., 2018; Bandla et al., 1994). A false positive may occur when adults obtain the virus after feeding on infected plants, which can be detected by ELISA.

RT-PCR was used to detect TSWV RNA in individual thrips in the early 1990s (Tsuda et al., 1994). Later, a sensitive and robust Taq-man probe RT-PCR method was developed for a specific target gene, and it also worked for samples taken from sticky cards in a greenhouse (Boonham et al., 2002). The methodology showed the potential of field survey, and it has been used in other tospovirus studies, such as for IYSV (Smith et al., 2015). Quantitative RT-PCR detection has many benefits over other methods: (1) automated reaction and analysis, (2) closed tubes reduce cross-contamination risks, (3) no post-PCR manipulation is required, (4) up to 96 samples can be tested at once, and (5) probe enhances sensitivity and specificity (Roberts et al., 2000). However, no field study has yet applied this method, as it could be cost-prohibitive.

TSWV transmission. The steps of TSWV transmission by thrips are as follows: (1) passive or active movement to a new host; (2) vector feeding and delivery of the virus into plant cells; (3) vector acquires virus by feeding and ingesting contagious fluid; (4) virus retention and replication within the vector; and (5) virus inoculation to a new plant host (Dáder et al., 2017). Details of the initial recognition of the virus in the vector, the role of salivary glands for inoculation, and the initial onset of infection in a new plant are poorly documented (Blanc et al., 2011). Once TSWV is delivered into the cytoplasm of the plant cells, the subsequent steps are uncoating or disassembling virus particles, replicating RNAs, assembling components, and movement of virions between cells (Chen et al., 2019).

Mechanisms driving virus spread. TSWV spread relies on horizontal transmission (Nagata & Peters, 2001). There are two factors that drive horizontal transmission (1) increased vector population growth between hosts or (2) increasing the probability of contact between hosts and vectors with different infection status movements and the vector's preference. Both are

important, but the vector growth population may have the greatest influence on the rate of viral spread (Shaw et al., 2017).

Disrupting vector transmission. The first step in disrupting transmission is to reduce the thrips vector populations, particularly the number of viruliferous thrips. The virus load is critical for thrips competence (transmission ability), and salivary gland infection is required for the efficiency (likelihood of transmitting) of the thrips-borne virus (Okazaki et al., 2011; Rotenberg et al., 2009). Thrips that carry a low virus load will not be able to successfully transmit TSWV (Margaria et al., 2014). However, the tool for differentiation between carrier and transmitter thrips is beneficial to evaluate the virus spread. Secondly, we should focus on condition-dependent factor. In general, the virus manipulates the vector in direct and indirect (plant-mediated) ways. For instance, the virus can modify vector feeding behavior, which directly affects transmission efficiency (Gutiérrez et al., 2013; Stafford et al., 2011). Both direct and indirect vector manipulation occurs in the TSWV-*F.fusca* pathosystem (Maris et al., 2004; Stafford et al., 2011). Potentially viruliferous *F. fusca* laid more eggs and had a reduced survival rate (Shrestha et al. 2012).

Studies on transcriptomes and proteomes have helped to identify more potential factors involving interactions of plant virus and insect vector (Dietzgen et al., 2016). The differential gene expression pattern via next-generation sequencing (NGS) showed that the gut tissue of larvae changes over time following TSWV infection, particularly in the midgut epithelium (Han & Rotenberg, 2021).

Host resistance to TSWV. There are two types of host-plant resistance against viruses: non-host resistance and host-response resistance (de Ronde et al., 2014). In the case of TSWV and plants, we focus on host-response because plant viruses replicate and cause infection only in

specific host plants. Identifying resistant (R) genes in plants has received much attention from breeders (de Ronde et al., 2014). Vertical host resistance is controlled by one or two dominant genes resulting in high-level resistance (Parlevliet & Zadoks, 1977). Vertical host resistance is common because more than 80% of viral resistance is controlled by single genes, half of which are dominantly inherited (Kang et al., 2005). However, the resistance mechanism could be easily broken under high vector selection pressure (Fabre et al., 2012; Kobayashi et al., 2014), particularly in a single dominant R gene. For instance, the *Tsw* gene and *Sw5* gene confer resistance to TSWV in pepper and tomato, respectively. The *Sw5* gene in tomato suppresses cell-to-cell movement and causes a hypersensitive response (HR) on the inoculated leaf. Related *Avr* gene is NSm gene on M RNA of TSWV (Hoffmann et al., 2001). Studies have shown that resistance conferred by the *Sw5* gene can be overcome by mutation, virus re-assortment, and synergistic interactions following mixed infection (López et al., 2011; Webster et al., 2011). The resistant mechanism of the *Tsw* gene is also related to HR, but the related *Avr* gene is either N gene or NSs gene (Lovato et al., 2008; Margaria et al., 2007). The *Tsw* gene can also be overcome by a single mutation (Margaria et al., 2007; Moury et al., 1997).

General horizontal host resistance is often controlled by several genes with minor effects. In the case of TSWV and peanuts, field-resistant peanuts have shown stable horizontal resistance (Branch & Culbreath, 2015). The conferred resistance is not well understood but offers long-term stability and is hard to break (Branch & Culbreath, 2018). The TSWV resistance mechanism in peanuts is not clear and could be conferred by multiple genes. TSWV resistance on non-peanut hosts is regulated by a single resistance gene with a HR, which prevented virus dissemination (Hoffmann et al., 2001; Lovato et al., 2008; Margaria et al., 2007). However, the peanut was infected by TSWV systemically and did not show local lesions (Lai, 2015).

Interactions Between TSWV and *F. fusca* Within the Peanut Pathosystem

Application of resistant peanut for TSWV management. The use of resistant cultivars is the major tactic for TSWV management in peanut cropping systems. Using peanut cultivars with moderate field resistance combined with other cultural practices. Details are included into the peanut mitigation risk index (peanut Rx), which was made for growers to assess the risk for TSWV (Culbreath & Srinivasan, 2011; Srinivasan et al., 2017). Even though resistant cultivars cannot be a stand-alone tactic and new sources of resistance to TSWV are needed for effective long-term management (Culbreath et al., 2005; Srinivasan et al., 2017).

The susceptibility of peanut cultivars to thrips and TSWV. Knowledge about the mechanisms of thrips resistance/tolerance is limited, and antixenosis (non-preference) and antibiosis (biological interaction) could contribute to resistance (Srinivasan et al., 2018). No indirect selection for TSWV resistance via plant traits were found in Anderson et al. (1996). A thrips-mediated inoculation protocol was developed for evaluating TSWV resistance (Nascimento et al., 2006; Shrestha et al., 2013). Few studies have examined the resistance to thrips in peanuts and demonstrated that some TSWV resistant genotypes had lower thrips feeding and survival than TSWV susceptible genotypes (Shrestha et al., 2013; Sundaraj et al., 2014). The interaction between resistance to thrips and TSWV is not very clear.

Exploring mechanisms of TSWV resistance in peanut. The cultivated peanut has a genome size of approximately 2.7 Gb, which is composed of subgenomes from two wild, diploid ancestors: A (37,059 genes) and B (46,650 genes) (Bertioli et al., 2016). The B subgenome is nearly identical to the genome of *A. ipaensis*, and *A. duranensis* is the ancestor of A subgenome (Bertioli et al., 2016). Homologous recombination has created diversity, as evidenced by

phenotypic changes such as color in the new polyploid hybrid (Bertioli et al., 2019). Markers associated with resistance are useful for the identification of candidate genome regions in *Arachis* species (Moretzsohn et al., 2009). The release of the peanut genome benefits the transcriptome analysis against diseases (Zhuang et al., 2019; Catto et al., 2021).

Transcriptome analysis, gene expression, and resistance mechanisms in peanuts. Transcriptome analysis is beneficial for understanding the mechanisms conferring resistance against pathogens in peanuts, such as late leaf spot disease and TSWV. For late leaf spot disease, RPP-13 like (Aradu.P20JR) and NBS-LRR (Aradu.Z87JB) on the peanut A subgenome have been identified as R genes due to upregulation in resistant cultivars (Gangurde et al., 2021). Defense-related genes have been found upregulated in TSWV-resistant cultivars compared with the TSWV-susceptible cultivars (Catto et al., 2021). However, gene expression may be caused by epigenetics, such as DNA methylation, rather than base pair changes in the genome (Gibney & Nolan, 2010). Temperature stress could influence gene expression through cytosine-5 DNA methyltransferase and demethylase (Wang et al., 2016); both proteins are used to determine the levels of methylation (Cao et al., 2014). Bhat et al. (2020) concluded that the A subgenome may be less susceptible to environmental changes because it possesses fewer DNA methylation sites at 21 days post sowing compared with the B subgenome. However, this conclusion has limitations, as the number of DNA methylation sites can vary at different plant developmental stage. Understanding patterns of DNA methylation may explain the independent variation of gene annotation (Bertioli et al., 2016). Transcriptomics have been used to understand the pathway for TSWV pathogenicity via comparing the biogenesis of unique viral small interfering RNA (vsiRNA) profiles of TSWV-infected thrips and plants (Fletcher et al., 2016).

Utilization of *Arachis* Species for Peanut Improvement

Using wild *Arachis* species as a resistance source. The cultivated peanut is a self-pollinating allotetraploid crop with very low genetic variability (Stalker, 2017; Stalker et al, 2013). The benefits of utilization of wild species for a peanut breeding system include: (1) wild species harbor more alleles with abundant variability, (2) most pathogen resistance is vertical resistance controlled by one or few genes and relatively simple for introgression (Leal-Bertioli et al., 2015). The sexual incompatibility of differences in ploidy level have been challenging for peanut crossing. The development of a synthetic allotetraploid of *Arachis* species could overcome the situation and induced tetraploid genotypes could cross with the highly productive peanut cultivars (Leal-Bertioli et al., 2015).

Late leaf spot and rust are major foliar diseases in peanut, and mechanisms of resistance to the causal pathogens is complex and polygenic in nature (Dwivedi et al., 2002). However, disease-resistant traits have been successfully introgressed to peanuts by artificial hybridization (Leal-Bertioli et al., 2015; Simpson, 1991; Stalker, 2017). More potential allotetraploids are expected in breeding programs for cultivated peanut.

TSWV and/or thrips resistance in wild diploid species. The mechanisms conferring resistance to TSWV in peanuts is unknown, but field studies support that it could be horizontal resistance controlled by multiple genes (Branch & Culbreath, 2015). Many diploid *Arachis* species, such as *A. cardenasii*, *A. correntina*, *A.diogoi*, *A. stenosperma*, and *A. villosa* have shown resistance to TSWV (Lyerly et al., 2002). The diploid species *A. diogoi* is likely immune to TSWV, and five related resistance markers were found in the hybrid *A. kuhlmannii* x *A. diogoi* (Milla et al., 2005).

Resistance to thrips in peanut has often been evaluated by measuring rating damage or insect abundances on leaves during field screening, rather than by quantifying thrips preference (Campbell & Waynne, 1980; Janini et al., 2010; Michelotto et al., 2017; Stalker & Campbell, 1983; Yang et al., 1993). Thrips preference has evaluated by measuring cuticular lipids of foliage from five wild species (*A. glandulifera*, *A. batizocoi*, *A. ipaensis*, *A. chacoense* [=A. *diogoi*], and *A. paraguariensis*) (Yang et al., 1993). Resistance against *F. fusca*, *F. scultzei*, *S. dorsalis*, and *Enneothrips flavens* has been evaluated in wild peanut species. Many diploid species, such *as A. batizocoi*, *A. williamsi, A. duranensis*, *A. magna*, *A.batizocoi*, *A. diogoi*, *A. correntina*, A. *duranensis*, *A. monticola*, *A. stenosperma*, *A. villosa*, *A. villosa-correntina* were considered potential sources of resistance against *F. fusca* (Campbell & Waynne, 1980; Lyerly et al., 2002; Stalker & Campbell, 1983). Additionally, diploids have shown resistance of *F. schultzei* and *S. dorsalis*, which are important pests and virus vectors in Asia, and to the non-vector thrips, *E. flavens,* in Brazil (Amin, 1981; Amin & Mohammad, 1980; Michelotto et al., 2017). However, varying degrees of resistance have been reported among accessions in the same species (Stalker, 2017).

Resistance induced by synthetic allotetraploids against TSWV and/or thrips. A study evaluating resistance against thrips among induced allotetraploids observed that *A. duranensis* x *A. kempff-mercadoi*, *A. gregoryi* x *A. stenosperma*, and *A. magna* x *A. cardenasii* have substantial levels of resistance to *Enneothrips enigmaticus,* which was misidentified as *Enneothrips flavens* (Lima et al., 2022; Michelotto et al., 2017). Additionally, a study of a hybrid genotype (*A. hypogaea* cv. Gregory x *A. diogoi*) implied that wild resistance genes can be transferred to cultivated peanuts (Stalker, 2017). Studies have also observed negative effects on fecundity and development time of *F. fusca* following feeding on *A. diogoi* and its hybrid (*A.*

hypogaea X *A. diogoi*), suggesting antibiosis as a resistance mechanism (Lai, 2015; Srinivasan et al., 2018).

Current Strategies for Thrips and TSWV Management

Management strategies for vector-borne pathogens vary with the pathogen's mode of transmission (Eigenbrode et al., 2018). These are tritrophic interactions (e.g., peanut-thrips-TSWV), but management usually relies on peanut-TSWV, TSWV-thrips, or thrips-peanut interactions. For example, current strategies include the intervention of virus vectors using insecticides (thrips-peanut), resistant cultivars, (peanut-TSWV) or through interference of vector transmission (TSWV-thrips) (Bragard et al., 2013). The peanut season in Georgia is from April to October. In general, viruliferous thrips inoculate peanuts after planting, and the TSWV incidence increases after 50–60 days of planting (Culbreath & Srinivasan, 2011). Reducing TSWV inoculum may benefit its management; however, it is not possible to remove all overwintering host plants of viruses and/or thrips. Avoiding and controlling thrips is possible using a combination of cultural and chemical tactics. In summary, the use of moderately resistant cultivars plays an important role in management, but highly resistant cultivars and non-chemical control are more desirable due to increased flexibility of cultural practices and chemical use and the long-term sustainability of peanut production (Culbreath & Srinivasan, 2011; Jordan et al., 2020).

Research Objectives

Knowledge of the epidemiology of TSWV in peanut enriches the management of thrips-transmitted TSWV. Although field-resistant cultivars have reduced losses due to TSWV since

the 1990s, TSWV is still a major threat to Georgia peanut production. We still need a better understanding of how TSWV spreads during and after the peanut season and tobacco thrips and TSWV interacts in the peanut pathosystem. We must also develop improved resistance in peanut cultivars to improve the sustainability of TSWV management in the future. Knowing the proportion of thrips transmitting TSWV in the field could help predict TSWV spread.

The first objective was to assess temporal patterns of TSWV accumulation in peanut and TSWV acquisition and inoculation by *F. fusca* and to optimize methods of TSWV detection and/or quantitation in individual *F. fusca* transmitters. The second objective was to evaluate the resistance/tolerance against thrips and/or TSWV in several wild peanut species and their hybrids in order to expand the pool of wild resources that can be used to improve TSWV resistance in peanut cultivars. The third objective was to explore the underlying plant response to TSWV infection based on expression analyses using the transcriptome of three wild genotypes with TSWV-resistance traits. This information will fill current knowledge gaps and prepare for future challenges.

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Table 1.1. Taxonomy of genus *Orthotospovirus*. Taxonomical classification of the 26 species according to the ICTV taxonomy update

#	List of orthotospovirus species	Acronym
1	*Alstroemeria necrotic streak orthotospovirus*	ANSV
2	*Alstroemeria yellow spot orthotospovirus*	AYSV
3	*Bean necrotic mosaic orthotospovirus*	BeNWV
4	*Calla lily chlorotic spot orthotospovirus*	CCSV
5	*Capsicum chlorosis orthotospovirus*	CaCV
6	*Chrysanthemum stem necrosis orthotospovirus*	CSNV
7	*Groundnut bud necrosis orthotospovirus*	GBNV
8	*Groundnut chlorotic fan spot orthotospovirus*	GCFSV
9	*Groundnut ringspot orthotospovirus*	GRSV
10	*Groundnut yellow spot orthotospovirus*	GYSV
11	*Hippeastrum chlorotic ringspot orthotospovirus*	HCRV
12	*Impatiens necrotic spot orthotospovirus*	INSV
13	*Iris yellow spot orthotospovirus*	IYSV*
14	*Melon severe mosaic orthotospovirus*	MSMV
15	*Melon yellow spot orthotospovirus*	MYSV
16	*Mulberry vein banding associated orthotospovirus*	MVBaV
17	*Pepper chlorotic spot orthotospovirus*	PCSV
18	*Polygonum ringspot orthotospovirus*	PolRSV
19	*Soybean vein necrosis orthotospovirus*	SVNV*
20	*Tomato chlorotic spot orthotospovirus*	TCSV
21	*Tomato spotted wilt orthotospovirus*	TSWV*
22	*Tomato yellow ring orthotospovirus*	TYRV
23	*Tomato zonate spot orthotospovirus*	TZSV
24	*Watermelon bud necrosis orthotospovirus*	WBNV
25	*Watermelon silver mottle orthotospovirus*	WSMoV
26	*Zucchini lethal chlorosis orthotospovirus*	ZLCV

*viruses transmitted by *Frankliniella fusca*

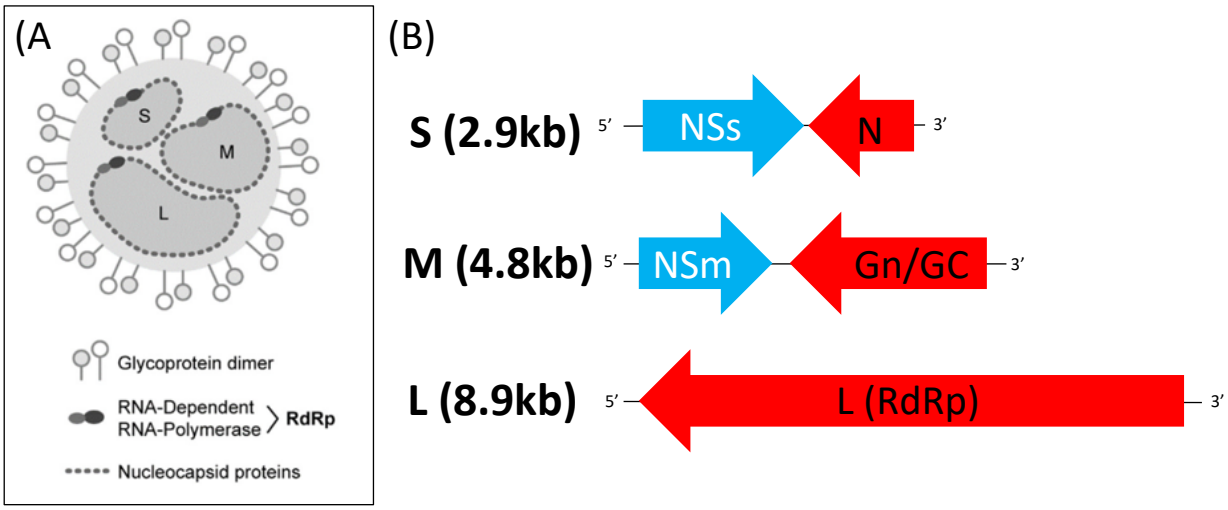

Figure 1.1. Bunyavirus generalized virion and tri-segmented genome. (Leventhal et al., 2021. (B) Open reading frames (ORFs) of N gene, glycoproteins (Gn/Gc), and L gene are on the vc sense (red arrow) of S, M, L segments; and ORFs of NSs and NSm are on the v sense (blue arrow) of S segment and M segment of tri-genome.

Table 1.2. List of thrips species capable of transmitting *Orthotospoviruses*. (Y.Chen et al., 2019; Mound et al., 2022; Oliver and Whitfield, 2016; Riley et al., 2017; Sharman et al., 2020)

	Species	Common name	
1	*C. claratris*	Oriental tomato thrips	CaCV
2	*F. bispinosa*	Florida flower thrips	TSWV
3	*F. cephalica*	Florida flower thrips	TSWV
4	*F. fusca*	tobacco thrips	INSV, IYSV, SVNV, TSWV
5	*F. gemina*	-	GRSV, TSWV
6	*F. intonsa*	Eurasian flower thrips	CSNV, INSV, TCSV, TSWV, GRSV
7	*F. occidentalis*	Western flower thrips	ANSV, CSNV, GRSV, INSV, TCSV, TSWV, TZSV
8	*F. schultzei*	Tomato thrips	CaCV, CSNV, GBNV, GRSV, GYSV, TCSV, TSWV
9	*F. tritici*	Eastern flower thrips	SVNV
10	*F. zucchini*	-	ZLCV
11	*T. palmi*	Melon thrips	CCSV, CaCV, GBNV, MYSV, WBNV, WSMoV, TZ
12	*T. setosus*	Japanese flower thrips	TSWV
13	*T. tabaci*	Onion thrips	AYSV, IYSV, TSWV, TYRV
14	*S. dorsalis*	Chilli thrips	GBNV, GCFSV, GYSV
15	*D. betae*	-	PolRSV
16	*N. variabilis*	Soybean thrips	SVNV
17	*M. abdominalis*	Composite thrips	TYRV CaCV

CHAPTER 2

TEMPORAL VARIATION OF TOMATO SPOTTED WILT ORTHOTOSPOVIRUS (TSWV) ACCUMULATION IN THE INOCULUM SOURCE UNDER FIELD CONDITIONS MODULATES THRIPS ACQUISITION AND INOCULATION

[1] Y.-J. Chen, M.R. Abney, S. Bag, S. Leal-Bertioli, and R. Srinivasan. 2022. To be submitted to *Peanut Science*.

Abstract

Tobacco thrips, *Frankliniella fusca* (Hinds), is a major vector of tomato spotted wilt orthotospovirus (TSWV) in peanut in the Southeastern United States. TSWV causes spotted wilt disease in peanut, which can be severely yield-limiting. Prior studies have indicated that TSWV secondary spread is continuous throughout the production season. *Frankliniella fusca* should acquire TSWV from in-field (crop) inoculum sources and inoculate peanut (*Arachis hypogaea* L.) plants to facilitate the secondary spread. Virus interactions with peanut plants throughout the peanut growing season could determine the extent of secondary spread. In this study, TSWV accumulation in peanut and TSWV acquisition and inoculation by thrips thereafter were assessed temporally. Results indicated that TSWV accumulation in peanut foliage declined temporally, and the decline was correlated to TSWV acquisition by thrips and inoculation subsequently. In this process, TSWV detection and/or quantitation in individual transmitting thrips was optimized by using an RT-qPCR assay. Assessing the proportion of TSWV transmitting thrips under field conditions would beneficial to evaluating TSWV spread within the production season (to assess secondary spread) and during the non-crop season (to assess primary spread). To accomplish the same, detection of TSWV-transmitting thrips on sticky cards was optimized. Laboratory-reared TSWV-transmitting thrips was used as the positive control for optimization. Results indicated that TSWV-transmitting thrips could be identified on yellow sticky cards effectively.

Introduction

Tobacco thrips, *Frankliniella fusca* (Hinds) (family *Thripidae*, Order *Thysanoptera*) is known to infest plants belonging to 11 diverse families including peanuts, *Arachis hypogaea* L. (family *Fabaceae,* Order *Fabales*) (Latora et al., 2022; Poos, 1945; Watson, 1922). Although thrips infestation causes economic damage to peanut crops, the most significant losses result from the transmission of the tomato spotted wilt orthotospovirus (TSWV), which causes the spotted wilt disease. TSWV is a member of the genus *Orthotospovirus* and family *Tospoviridae*. TSWV is a negative-stranded/ambi-sense RNA virus with three-segmented RNA genome, namely small-(S), medium-(M), and large-(L) RNA enclosed within a host-derived and double-layered membrane embedded with two glycoproteins (German et al., 1992). TSWV is exclusively transmitted by thrips in a persistent and propagative mode (Whitfield et al., 2005). The virus is also transmitted in a stage-dependent manner. Thrips can inoculate the virus successfully only when it is acquired by early instar larvae (Ullman, 1993), and these viruliferous thrips are referred to as transmitters. In contrast, when thrips acquire the virus for the first time as adults, they cannot inoculate the virus and are referred to as non-transmitters (Bandla et al., 1994; de Assis Filho et al., 2005; Nagata & Peters, 2001; Seepiban et al., 2015). Previous studies have indicated that TSWV primary spread is initiated by immigrating viruliferous transmitters soon after planting (Camann et al., 1995). This primary mode of spread was presumed to be the sole contributor to TSWV epidemics in any given season (Camann et al.,1995). Weeds are believed to be inoculum sources for primary infection of TSWV in peanut (Barbour & Brandenburg, 1994; Groves et al., 2002). Several weeds within and surrounding fields were identified in Southeastern USA as potential inoculum sources of TSWV (Groves et al., 2001; Russell L. Groves et al., 2002; Srinivasan et al., 2014). Nevertheless, a recent study indicated

that besides primary spread aided by non-field inoculum sources, a substantial amount of secondary spread occurs aided by inoculum sources within the field and peanut colonizing thrips populations (Lai, 2021). This secondary spread continued throughout the season right until harvest. The impact of late season infections on yield is limited, but evidence exists for continuous secondary spread of TSWV.

TSWV-peanut host interactions and the resulting TSWV accumulation could be affected by peanut age due to matured plant resistance among other factors. It is likely that TSWV accumulation decreases in peanut plants temporally. Consequently, these temporal changes on virus accumulation levels could affect thrips acquisition and impact thrips inoculation efficiency. To assess TSWV acquisition and inoculation efficiency, it is vital to distinguish TSWV transmitting thrips from non-transmitting thrips. Serological and molecular detection and/or quantitation exist to make the distinction (Amin, 1981; Aramburu et al., 1996; Arthurs et al., 2018; Bandla et al., 1994; Boonham et al., 2002; Chamberlin, Todd, et al., 1993; Cho et al., 1991; McPherson et al., 2005; Roberts et al., 2000; Rotenberg et al., 2009; Tsuda et al., 1994). However, they are not reliably optimized for individual *F. fusca* adults on peanut plants. Furthermore, assessing TSWV transmitter status in thrips caught within the peanut fields would be benefical for determining the extent of secondary spread of TSWV. The same assessment could be used to assess the extent of the primary spread of the virus.

The objective of this study was to assess temporal variation in TSWV accumulation in peanut leaves and its impact on TSWV acquisition by thrips and the subsequent inoculation of non-infected peanut seedlings. Reverse transcription-quantitative PCR (RT-qPCR) was used to assess variation in TSWV levels in peanut tissues (Boonham et al., 2002; Rotenberg et al., 2009). The technique to distinguish TSWV transmitting thrips from non-transmitting thrips was

optimized under laboratory conditions using lab-reared viruliferous thrips that had acquired TSWV from field-collected peanut leaflets. Following which, a series of acquisition and inoculation assays were conducted using peanut seedlings. In addition, a method to detect TSWV transmitting thrips as an indirect measure for determining secondary spread using yellow sticky cards also was optimized. Nonetheless, the usefulness of this method remains to be validated under field conditions.

Material and Methods

Peanut plants and TSWV inoculum source. All experiments were performed using peanut (*Arachis hypogaea* L.) cv. Georgia Green. Seedlings were maintained in thrips-proof mesh cages (47.5 cm^3) (Megaview Science, Taichung, Taiwan) at 25-30°C and ~80% humidity. TSWV-infected leaflets were collected from Jones Farm (Tifton, GA, USA) at two-week interval and stored in zipper bags at 2-4°C. The leaflets were then surface-sterilized using a 1% bleach-water solution, rinsed with tap water twice, and dried with paper towels before using them for TSWV acquisition by thrips.

Maintenance of non-viruliferous thrips colony. Non-viruliferous thrips were maintained on leaflets of non-infected plants (*A. hypogaea* cv. Georgia Green) within Petri dishes with a wet cotton round (Lai et al., 2021). Colonies were maintained by successive releases of ten adult female thrips, allowed to oviposit for 48h on a peanut leaflet dusted with a trace of pine pollen as a supplement and placed in growth chambers (Thermo Scientific, Waltham, MA, USA) at 28-30°C and a photoperiod of 14h:10h (L:D). Fresh leaflets and water were added to the Petri dishes three times a week until the emergence of the next generation.

Maintenance of viruliferous thrips colony. Viruliferous thrips colony was maintained on TSWV-infected leaflets collected from the field in a separate growth chamber. Newly hatched first instar larvae were maintained on TSWV-infected foliage in Munger cages until adult emergence at 28-30°C and 14h:10h (L:D) as described in Shrestha et al. (2013). Female adults (less than three days old) were used for RT-qPCR-based detection.

Standardization of TSWV detection and quantitation in thrips. Total RNA from five thrips was individually extracted using one of three RNA methods: a) RNeasy Micro Kit (Qiagen, Valencia, CA), b) Chelex 100 (Bio-Rad, Hercules, CA, USA), and c) InstaGene Matrix (Bio-Rad, Hercules, CA, USA). One non-infected thrips was included in each experiment as a negative control. Each extraction method was repeated three times (n=15). For the RNeasy Micro Kit, individual thrips samples were homogenized in 50 ul of grinding buffer, and the extraction protocol was followed as per the manufacturer's instructions. RNA was extracted from thrips using 50% Chelex and InstaGene Matrix as described in Boonham et. al. (2002) and Srinivasan et al. (2012), respectively. The extracted RNA samples (4ul for the RNA Micro Kit and 10.5ul for Chelex/InstaGene Matrix) were used to synthesize first-strand cDNA followed by qPCR using two TSWV gene-specific primers (Supplementary Table 2.1). To estimate the percentage of TSWV-infected thrips within viruliferous colonies, five thrips were collected from a potentially viruliferous colony and replicated three times. Each thrips was used for total RNA extraction using a RNeasy Micro Kit, followed by RT-qPCR detection as described in Shrestha et al. (2013).

Accumulation of TSWV RNA in thrips was quantitated using RT-qPCR. TSWV quantitation was done separately using the N gene- and NSs gene-specific primers with 1 µl of cDNA as a template with the cycling conditions of 95°C for 2 min, followed by 40 cycles at

95°C for 15s, 58°C for 60s, and 72°C for 20s. The reaction was extended with a melting curve analysis in a QuantStudio 3 System (applied biosystems by Thermo Fisher Scientific, Waltham, MA, USA) to rule out non-specific binding. Both specific primer sets (N gene and NSs gene) were used for determining TSWV RNA accumulation in individual thrips. Each sample was duplicated, and the absolute number of TSWV-N gene copies and TSWV-NSs gene in the samples were quantified using the standard curve protocol described by Shrestha et al. (2013). The N gene (777bp) and NSs gene (148bp) inserts were cloned into pJET1.2 vector (GenScript, Piscataway, NJ, USA) and their serial dilutions were used to generate the respective standard curves.

TSWV accumulation in the inoculum source under field conditions, thrips acquisition, and inoculation.

(1) TSWV detection and quantitation in plant tissues from field. Symptomatic leaflets used for TSWV acquisition were collected from an insecticide-free field at the USDA-ARS Jones Farm, Tifton, GA, USA, at two-week intervals in 2021. Symptomatic leaflet tissues (ca 0.03 g) from infected samples were used for RNA extraction. Total RNA was extracted by RNeasy Plant Mini Kit (Qiagen, Valencia, CA) and complementary DNA (cDNA) was synthesized by GoScript reverse transcription system (Promega Corporation, Madison, WI) following the manufacturer's instructions. Synthesized cDNA was used as a template for RT-qPCR. TSWV-N gene accumulation in twelve leaflets from each sampling date was measured via RT-qPCR following the protocol by Shrestha et al. (2013) with modifications.

(2) **Thrips acquisition and thrips-mediated transmission assay.** Ten viruliferous thrips from each experiment were sub-sampled for total RNA extraction before transmission in 2020 and 2021. Total RNA from thrips samples in 2020 was individually extracted using Chelex 100, and

thrips samples in 2021 using RNeasy Micro Kit. The TSWV detection and TSWV-N gene accumulation in thrips were estimated following the aforementioned protocol. The whole-plant TSWV transmission assay was conducted via *F. fusca*-mediated inoculation (Shrestha et al., 2015). Inoculated plants were maintained in thrips-proof cages in the greenhouse at 25-30°C, ~80% humidity, and at 14h:10h (L:D) photoperiod.

(3) Evaluation of Transmission efficiency and quantitation in infected plants. The percentages of infected plants in 2020 and 2021were estimated at three weeks post-inoculation via double antibody enzyme-linked immune sorbent assay (DAS-ELISA) and RT-qPCR, respectively. To determine TSWV infection status, plant tissue (ca 0.03 g) was collected from the first fully expanded leaf below the terminal for TSWV detection. In 2020, the DAS-ELISA was conducted following the manufacturer's protocol (Adgia, Elkhart, IN, USA). In 2021, the RNA extraction, and cDNA synthesis were conducted following the aforementioned protocol for plants. TSWV detection and TSWV-N gene accumulation were conducted via RT-qPCR (Shrestha et al., 2013).

TSWV detection and quantitation in thrips from sticky cards. Ten viruliferous thrips were collected and placed on sticky traps (Dual-sided yellow sticky traps, Gemplers Inc., Janesville, WI, USA) in a Munger cage (11.43 x 8.89 x 1.77cm^3) (Munger, 1942) at 29°C. Thrips were removed with insect pins after 24 hours. The pins with thrips were soaked in Histo-clear reagent (Electron Microscopy Sciences, Hatfield, PA, USA) for 20 minutes in a microcentrifuge tube to unglue thrips. Detached and intact thrips were moved to filter paper using a brush pen for drying and then stored in 95% ethanol (supplementary Fig. 2.1). Ten thrips from the same colony cohort were collected and stored in a 1.5ml centrifuge tube and were used as a control treatment. Each thrips was used for total RNA extraction using a RNeasy Micro Kit, followed by RT-qPCR

detection as described in Shrestha et al. 2013. Each treatment was replicated five times (N=50 for each treatment).

Statistical Analyses. The infection status data (present/non-present) in thrips were analyzed using a generalized linear mixed-effects model (GLMM) with a binomial distribution by using the "glmer" in R software (Core R Team, 2019). The random effect was the experimental repeat, and the fixed effect was the treatment in the analysis. The virus copies' data in thrips and plants were analyzed using linear mixed model (LMM) with normal distributions by using the "glm" function in R software. The experimental repeats were considered as random effects, and the treatments were considered as fixed effects. To determine the difference between treatments, least-square means (LS-mean) were used for multiple comparisons at the 95% significance level ($\alpha = 0.05$) with Tukey adjustment. The percentage infection and TSWV loads data determined with two primer sets were compared with a paired t-test using "t.test" in R.

A correlation matrix with significance levels of $\alpha = 0.05$ was developed to assess the correlation between the following parameters for each transmission experiment viz., percentage of infected leaf, virus loads in samples from field-collected plants, percentage of viruliferous thrips, virus loads in thrips, transmission percentage, and virus loads in inoculated plants (N=16). The data were analyzed using "rcorr" function in software R and "corrplot" was used for visualizing the correlation.

Results

Standardization of TSWV detection and quantitation in thrips. The acquired RNA concentrations from individual *F. fusca* using Chelex 100, InstaGene, and the RNeasy Micro Kit were lower than 16 ng/ul (Table 2. 1). The RNA purity ratio (A_{260}/A_{280}) of individual thrips

averaged 1.96-2.38. RNA extraction via all three methods were usable for RT-qPCR, but the highest proportion of infected thrips was observed using the RNeasy Micro Kit, with TSWV being detected in > 70% of thrips. The proportion of infected thrips was higher when RNeasy Micro Kit was used than when InstaGene Matrix was used ($F_{2,42}$=3.6921, p=0.02492; Fig. 2. 2).

TSWV N gene and NSs genes located on the S RNA were used as target genes for TSWV detection from the potential viruliferous colony. Both primers for TSWV were able to amplify the virus, and the percentage of infected thrips was higher when the N-gene than NSs was amplified (t=4.3225, df=71, p<0.0001; Fig. 2. 3A). TSWV N-gene accumulation was greater than the NSs gene (t=13.692, df=19, p<0.0001; Fig. 2. 3B).

TSWV detection and quantitation in plant tissues and thrips-mediated transmission assay. In 2021, symptomatic peanut leaves were collected in the field during the growing season and used as TSWV inoculum sources for acquisition by *F. fusca*. TSWV accumulation in peanut leaves varied by sampling dates (DAP, days after planting). TSWV was detected in all field-collected symptomatic leaves using RT-qPCR between June 2nd (28 DAP) and July 29th (70 DAP); however, some leaves collected after August 12th (84 DAP) tested negative via RT-qPCR (Fig. 2. 4A). In addition, TSWV loads in field peanut leaves were significantly higher from June to July (from 28 to 70 DAP) than from August to September (from 84 to 126 DAP) ($F_{7,76}$=97.35, p<0.0001; Fig. 2. 4B).

The percentage of TSWV-infected thrips ranged from 0% to 57% and 0% to 50% in 2020 and 2021, respectively (Fig 2. 5A&B). A reduced percentage of thrips acquired TSWV from the inoculum that was collected later in the cropping season than earlier in the season. Thrips did not acquire TSWV successfully from TSWV-infected leaves in September (132 DAP in 2020 and 126 DAP in 2021; Fig. 2. 5A&B). Virus accumulation in thrips in 2020 was higher on

August 26th (118 DAP) than on June 17th (49 DAP) and June 1st (63 DAP) with subtle differences ($F_{4,11}$=5.573, p=0.0135; Fig. 2. 5C). Virus accumulation in thrips did not vary substantially over the season in 2021 ($F_{6,22}$=2.155, p=0.087; Fig 2. 5D).

The viruliferous thrips that acquired TSWV from field-collected leaves were used for thrips-mediated inoculation. The TSWV infection percentage of thrips-inoculated plants at three weeks post-inoculation ranged from 0% to 80% and 0% to 70% in 2020 and 2021, respectively (Fig 2. 6A&B). Plants were not infected via thrips-mediated TSWV transmission when the leaflets from peanut plants collected later in September were used as inoculum (Fig. 2. 6A &B).

Virus accumulation in TSWV-infected plants in 2020 varied with the sampling dates in which the inoculum were collected ($F_{4,44}$=3.555, p=0.01345; Fig. 2. 6 C). However, TSWV accumulation in TSWV-infected plants did not vary statistically with the sampling dates in which the inoculum were collected in 2021 ($F_{6,48}$=2.177, p=0.06158; Fig. 2. 6D). Overall, the temporal variation of TSWV transmission in 2020 and 2021 was similar as determined by DAS-ELISA and RT-qPCR, respectively (Fig. 2. 6A&B).

Relationship between virus accumulation in field plants, thrips acquisition, and TSWV secondary spread. The percentage of field-collected TSWV-infected leaves, virus copies in TSWV-infected leaves, the percentage of TSWV-infected thrips, virus copies in TSWV-infected thrips, transmission percentage after thrips inoculation, and virus loads in TSWV-infected plants in 2021 were correlated. The greatest correlation was observed between the percentage of TSWV-infected thrips and the transmission percentage (correlation=0.793, p=0.0002; label C&E; Fig. 2. 7). Also, the percentage of TSWV-infected leaves was positively correlated with the virus copies in TSWV-infected leaves in the field (correlation=0.689, p=0.0045; label A&B; Fig. 2. 7). Lastly, the virus copies of infected leaves in the field were

positively correlated with the percentage of viruliferous thrips (correlation=0.611, p=0.0156; label B&C; Fig. 2. 7) and TSWV transmission efficiency (correlation=0.679, p=0.0054; label B&E; Fig. 2. 7).

TSWV detection and quantitation in thrips from sticky cards. The percentage of TSWV-infected thrips was numerically lower on the sticky cards (33%) compared with the control (48%; Fig. 2. 8A), but they were not significantly different (t=0.82377, df=83, p=0.4124; Fig. 2. 8A). However, TSWV loads in thrips collected from sticky traps were lower than in the control, albeit not by much (t=2.3525, df=42, p=0.0234; Fig. 2. 8B).

Discussion

This study explored how virus-host (peanut) interactions could influence the secondary spread of TSWV through a series of laboratory experiments. TSWV detection and quantitation through RT-qPCR were optimized for thrips samples. Results showed that TSWV loads in the inoculum sources declined temporally throughout the peanut cropping season, and this decline was correlated to TSWV acquisition by thrips and thrips-mediated TSWV inoculation thereafter. Furthermore, this study optimized TSWV detection and quantitation in thrips trapped on the sticky card. TSWV detection and/or quantitation under field situations would provide an indirect measure of TSWV inoculum present in the field and/or the landscape.

TSWV detection in thrips and assessing if they are transmitters can be critical to understanding both the primary and secondary spread of the virus. Several detection techniques are currently in place to detect viruses from vector samples. In terms of TSWV detection in thrips, a large sample size (20 to 30 thrips) produced more accurate results compared with one thrips via DAS-ELISA (Arthurs et al., 2018). In another study, the nucleotide-based detection

method, RT-PCR, was designed for individual thrips (Tsuda et al., 1994). However, most of the techniques did not facilitate a distinction between transmitters and non-transmitters. Thrips that acquire the virus as adults cannot transmit the virus and are called non-transmitters. Chamberlin et al. (1993) used an N-gene based DAS-ELISA to assess the proportions of TSWV-infected thrips and estimated that 0 to 10 % of thrips were viruliferous. This technique used a structural protein-based ELISA and did not distinguish transmitters from non-transmitters. McPherson et al. (2005) used the non-structural gene (NSs) antibody-based antigen-coated ELISA (ACP-ELISA) and assessed that 1.5 to 2.3% of thrips were transmitters from April to May sampling. The presumption behind using the NSs-based ACP-ELISA was that non-structural proteins are indicative of virus replication, and virus replication is a prerequisite for TSWV transmission (Bandla et al., 1994). Nevertheless, even NSs-based ACP ELISA cannot rule out virus replication in the non-transmitters completely. A quantitative assay (RT-qPCR) based on absolute quantitation was then developed based on N-gene specific primers to identify transmitters in the case of western flower thrips, *Frankliniella occidentalis* (Pergande) (Boonham et al., 2002; Rotenberg et al., 2009). This technique has since been used for *F. fusca*, but it required further optimization. The current study attempted to address the issue by optimizing the extraction technique and evaluating two genes (N and NSs genes). Results indicated that using the RNeasy Microkit produced the best quality of RNA from individual thrips and resulted in the highest detection percentage. The N-gene-based assay resulted in increased TSWV detection and/or accumulation in thrips compared to the NSs-based RT-qPCR assay.

Thrips transmitters in the beginning of the season have been considered important due to their contribution in primary spread (Camann et al., 1995). Primary spread is believed to be the main driver of TSWV epidemics. However, recent research has indicated that TSWV

transmission via secondary spread continues to occur throughout the season (Lai, 2021). The TSWV-host interactions could have a pivotal part in influencing secondary spread. This study examined TSWV infection percentages and TSWV accumulation in field-planted peanut over time; the results indicated that TSWV infection percentage and TSWV accumulation decreased over time. Consequently, thrips feeding on the foliage also showed a similar trend (Lai, 2021). Though peanut is indeterminate in its growth, as peanut plants age, matured plant resistance by enhancing plant defenses could be limiting the virus accumulation (Garcia et al., 2003; Kresta et al., 1995; Rowland et al., 2005). However, despite feeding on leaflets with reduced virus loads, the TSWV accumulation levels in thrips did not vary as much in relation to the inoculum source. Similar results were found in the case of *T. tabaci*, wherein TSWV accumulation in *T. tabaci* was unrelated to virus accumulation in the leaf tissue from which they acquired the virus, and the factor that was more relevant to determining transmission was local adaptation (Linak et al., 2020). This could be due to the fact that TSWV is a propagative virus and virus replication in the salivary glands is the critical determinant of virus accumulation (Okazaki et al., 2011; Rotenberg et al., 2009).

The results of this study suggest that TSWV-peanut interactions lead to the substantial accumulation of the virus that would favor thrips acquisition and virus inoculation to non-infected plants thereby facilitating secondary spread. These results reiterate the findings by Lai (2021) indicating TSWV secondary spread well past the 77 days after planting. However, that study also showed that the increase of TSWV infection presumably due to secondary spread decreased temporally. The secondary spread simulation via the transmission assay adopted in this study indicated that TSWV infection ranged from >60% to about 20% later in the season. This magnitude of secondary spread might be an overestimation of the actual secondary spread

of TSWV at the same time points under field conditions. The overestimation could be due to the two-week-old seedlings used in the greenhouse transmission assay, and the older plants in the field might be less susceptible to TSWV in the field than the seedlings used in the transmission assay.

These results of this study could have tangential implications for greenhouse based TSWV screening assays evaluating TSWV resistance in germplasm materials. Estimation of TSWV infection and accumulation in inoculum materials (used for thrips acquisition) could help gauge the results accordingly, Also, using a susceptible cultivar and an infection threshold in that cultivar could help under or over-estimating TSWV incidences in germplasm materials.

The virus detection in thrips used in this study to assess TSWV secondary spread cannot be readily adapted to assess forecast TSWV incidences in the field. Establishing a reliable proxy would be useful. In addressing the need, this study attempted to use sticky cards to assess prevalent TSWV infection in thrips and their transmitting status, which in turn would provide an estimate or forecast TSWV incidence in subsequent weeks. Sticky cards should be easily adoptable as they are already used by scouts in row cropping systems such as peanut and cotton. The results in this study indicate sampling TSWV in thrips on sticky cards within 24h was slightly less efficient than estimating TSWV incidences in laboratory-reared thrips. The sticky cards could also be used earlier in the season even prior to planting to assess prevalent TSWV infection in thrips and their transmission ability. A practical application would be to delay planting slightly to evade TSWV transmitting thrips peak densities.

Conclusions

This study evaluated the secondary spread contributing factors such as temporal TSWV infection status and TSWV accumulation in peanut plants under field settings, and TSWV acquisition and inoculation abilities of thrips. This study provides evidence of secondary spread and its temporal decline that would occur naturally in the field. In the process, the current study optimized TSWV detection and quantitation in individual thrips including RNA extraction and selecting the appropriate TSWV gene. In addition, this study optimized TSWV detection and quantitation in thrips on sticky cards. This tool could have substantial implications in forecasting subsequent epidemics. However, field validation remains to be conducted, and the relevance of such a tool in terms of management remains to be deciphered.

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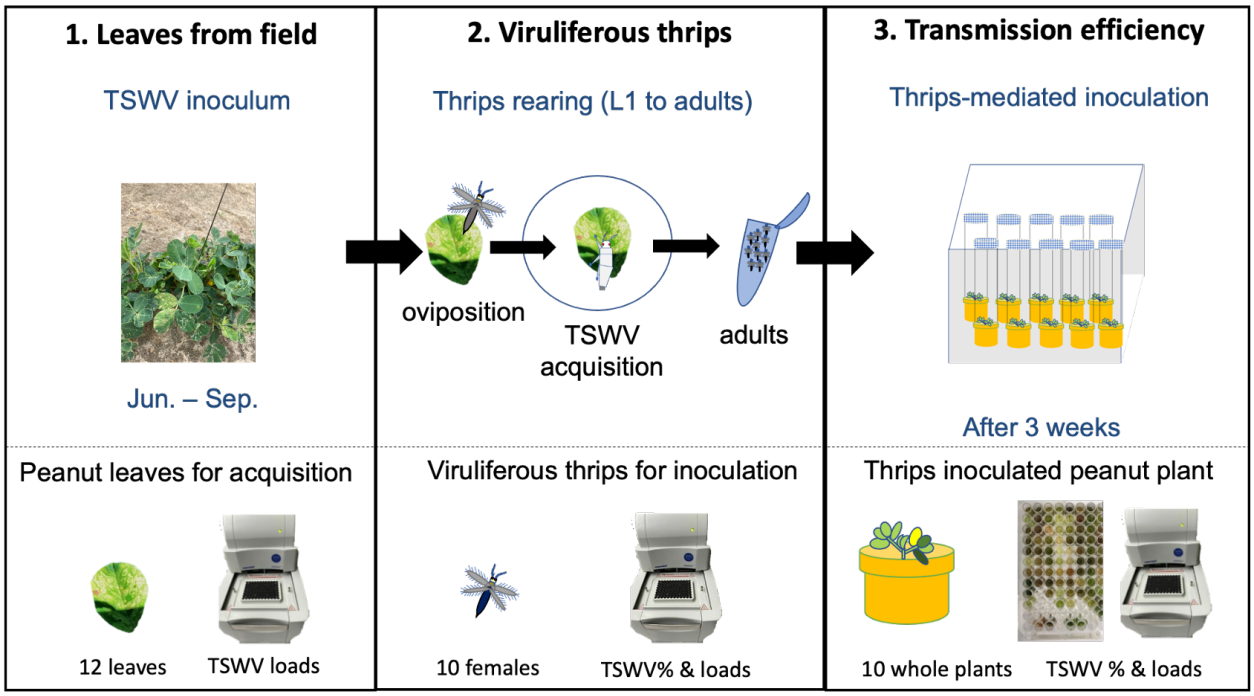
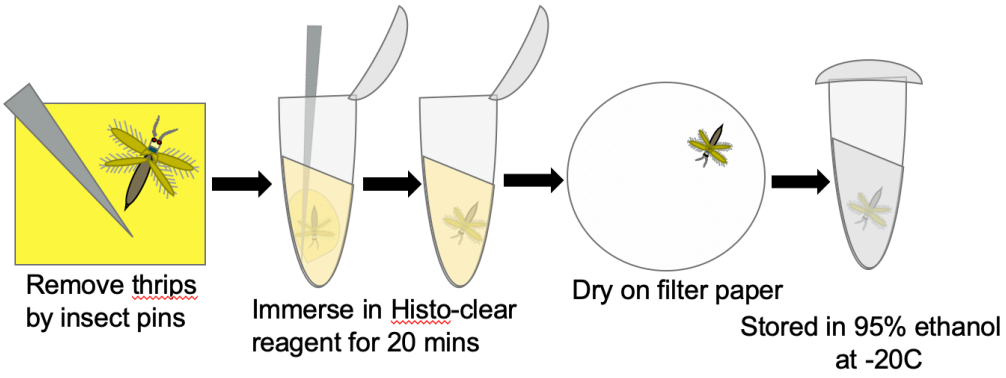
Supplementary Table 2. 1. Primers used for detection and quantitation of TSWV

Sequence (5'-3')	Target gene	Product size
GCTTCCCACCCTTTGATTC ATAGCCAAGACAACACTGATC (Rotenberg et al., 2009)	N gene	139 bp
CAGCATCCAAATCCCTTCATGG GCATACGCTTCCTTAACCTT	NSs gene	148 bp



Supplementary Figure 2.1. Diagram of experimental design



Supplementary Figure 2.2 Removal thrips from sticky traps

Table 2.1. Quantification of RNA from *Frankliniella fusca* samples determined by absorbance using Nanodrop and cost of RNA extraction per thrips.

RNA extraction method	Total RNA concentration (ng/ul)	RNA purity ratio (A_{260}/A_{280})
Chelex 100	15.4 ± 0.69	1.96 ± 0.03
InstaGene	6.95 ± 0.46	2.38 ± 0.14
RNeasy Micro Kit	9.76 ± 1.59	1.95 ± 0.16

Figure 2. 1. Adults of tobacco thrips *Frankliniella fusca* (Hinds) on peanut leaves.

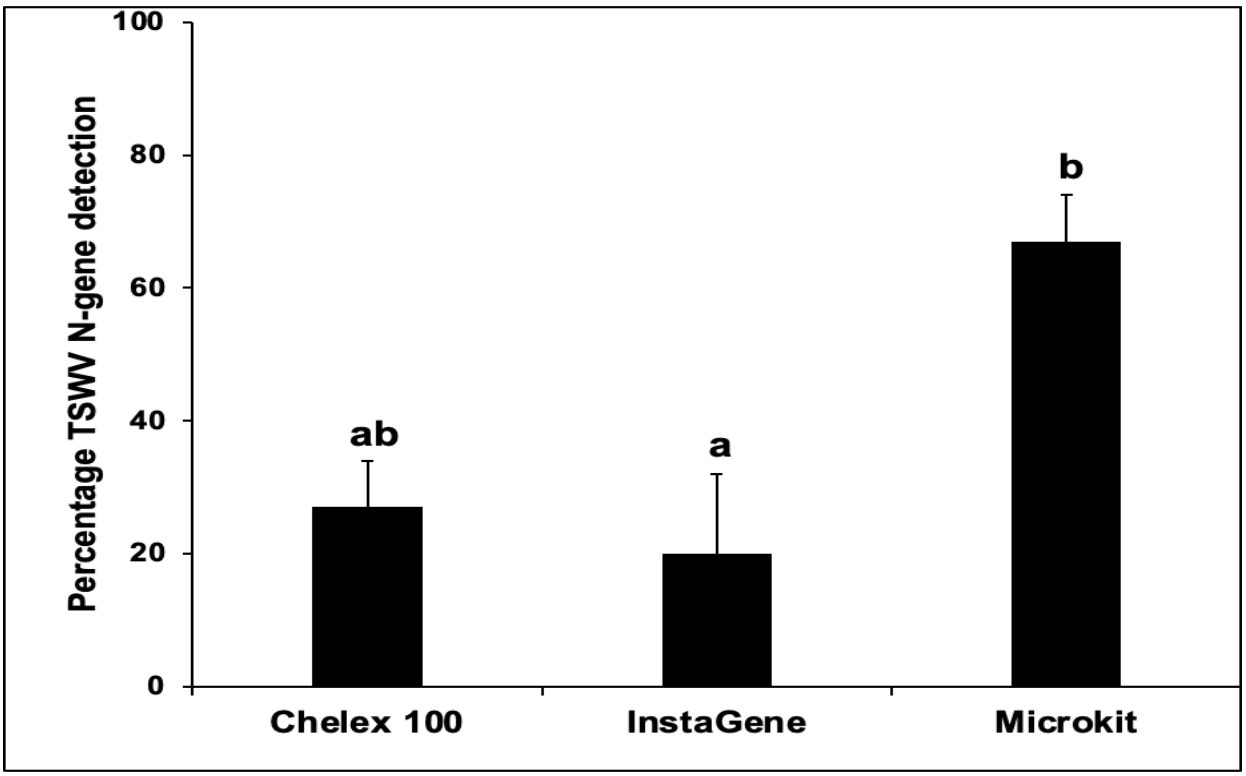


Figure 2. 2. Percent infection of tomato spotted wilt virus (TSWV) in thrips using three RNA extraction methods. Five potentially viruliferous tobacco thrips were tested for each extraction method, and the experiment was repeated two times (N=15). The non-viruliferous thrips was used as the control for each experiment. The infection status of thrips was determined by RT-qPCR by targeting the N gene of TSWV. Different letters on standard errors of means indicate significant differences between means separated by LSD with Turkey method at α=0.05.

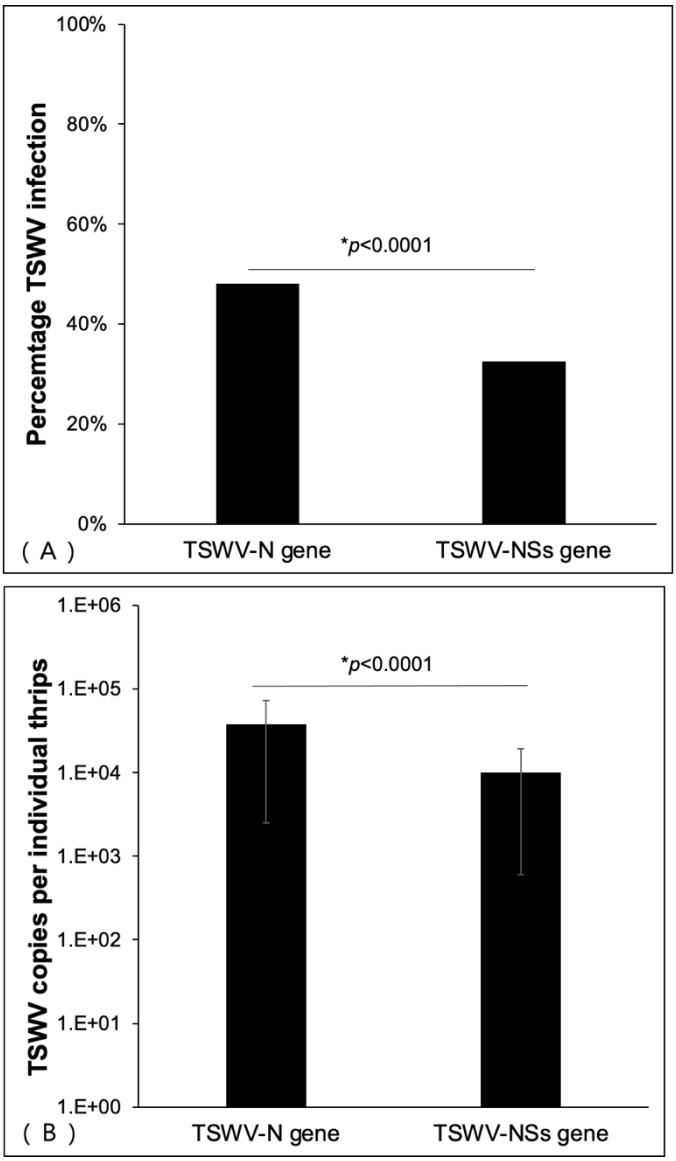


Figure 2. 3. Percentage infection of tomato spotted wilt virus (TSWV) and TSWV accumulation in viruliferous tobacco thrips (*Frankliniella fusca*). The viruliferous thrips acquired the virus from field-collected leaflets from TSWV-infected plants from June to July 2021 (A). Different letters on standard errors of means indicate significant differences between means separated by LSD with Turkey method at α=0.05. The accumulation of TSWV in viruliferous thrips (B) was measured by obtaining TSWV-N gene copies and TSWV-NSs gene copies via RT-qPCR.

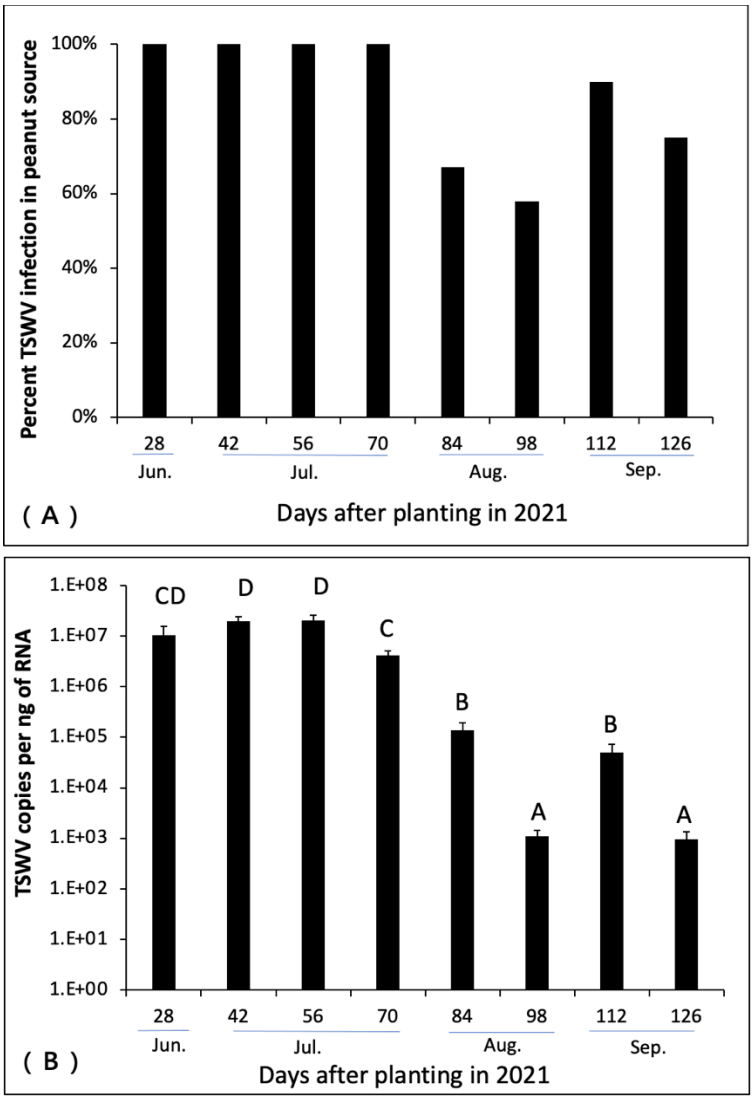


Figure 2. 4. Percentage detection of tomato spotted wilt virus (TSWV) in symptomatic leaflets (*A. hypogaea* cv. Georgia Green) and TSWV accumulation under field conditions over the cropping season. Twelve leaves collected from the field at the two-week interval were estimated by RT-qPCR. TSWV loads from infected leaflet samples were estimated via absolute quantitation using plasmids containing TSWV N-gene inserts as standards. Different letters on standard error of means (SE) indicate significant differences between means separated by LSD with Tukey method at $\alpha = 0.05$.

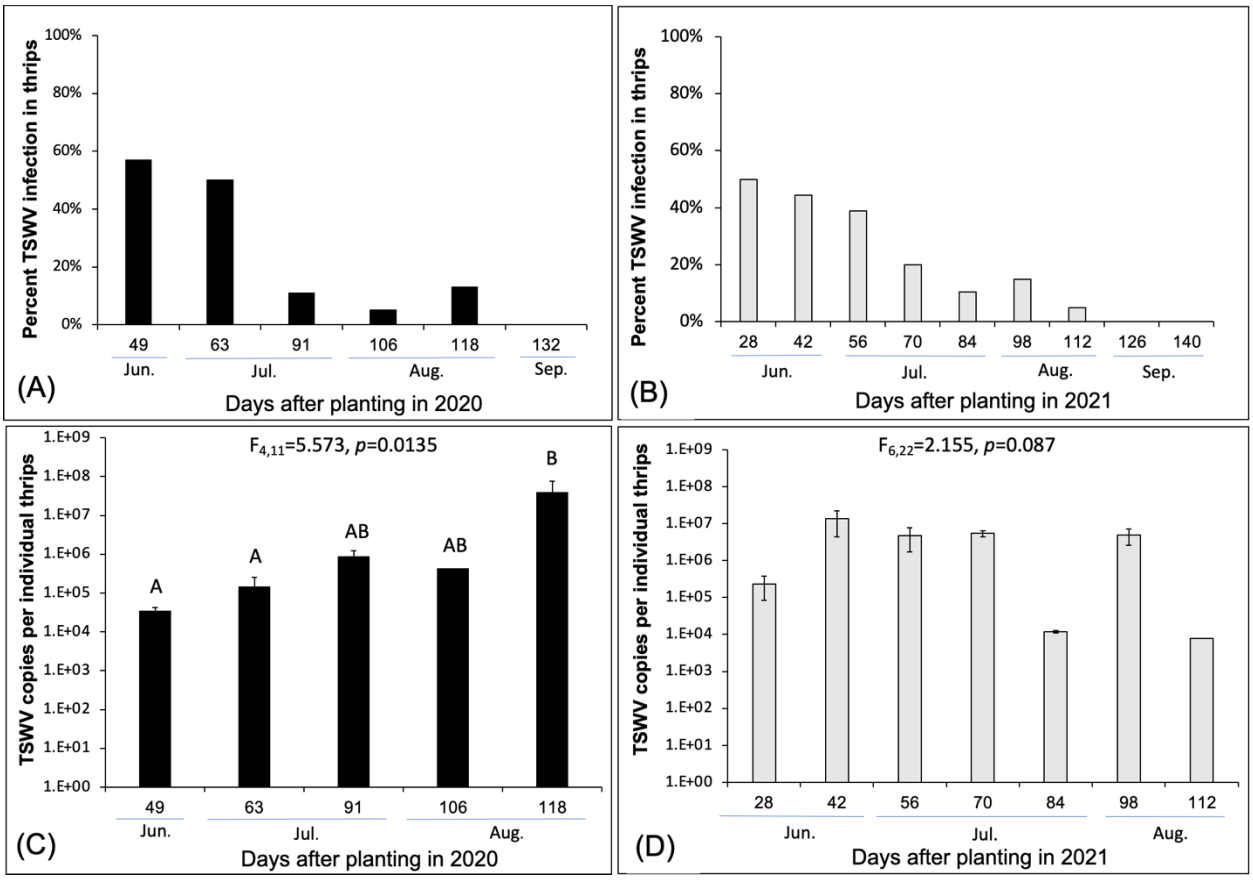


Figure 2. 5. Percent infection of tomato spotted wilt virus (TSWV) and TSWV accumulation in viruliferous thrips (*Frankliniella fusca*) following acquisition from TSWV-infected field-collected leaflets in 2020 and 2021. Potentially viruliferous tobacco thrips were used for thrips-mediated transmission and ten subsamples were used for TSWV detection. The infection status of thrips was determined by RT-qPCR and the accumulation of TSWV-infected thrips was measured by estimating TSWV-N gene copies.

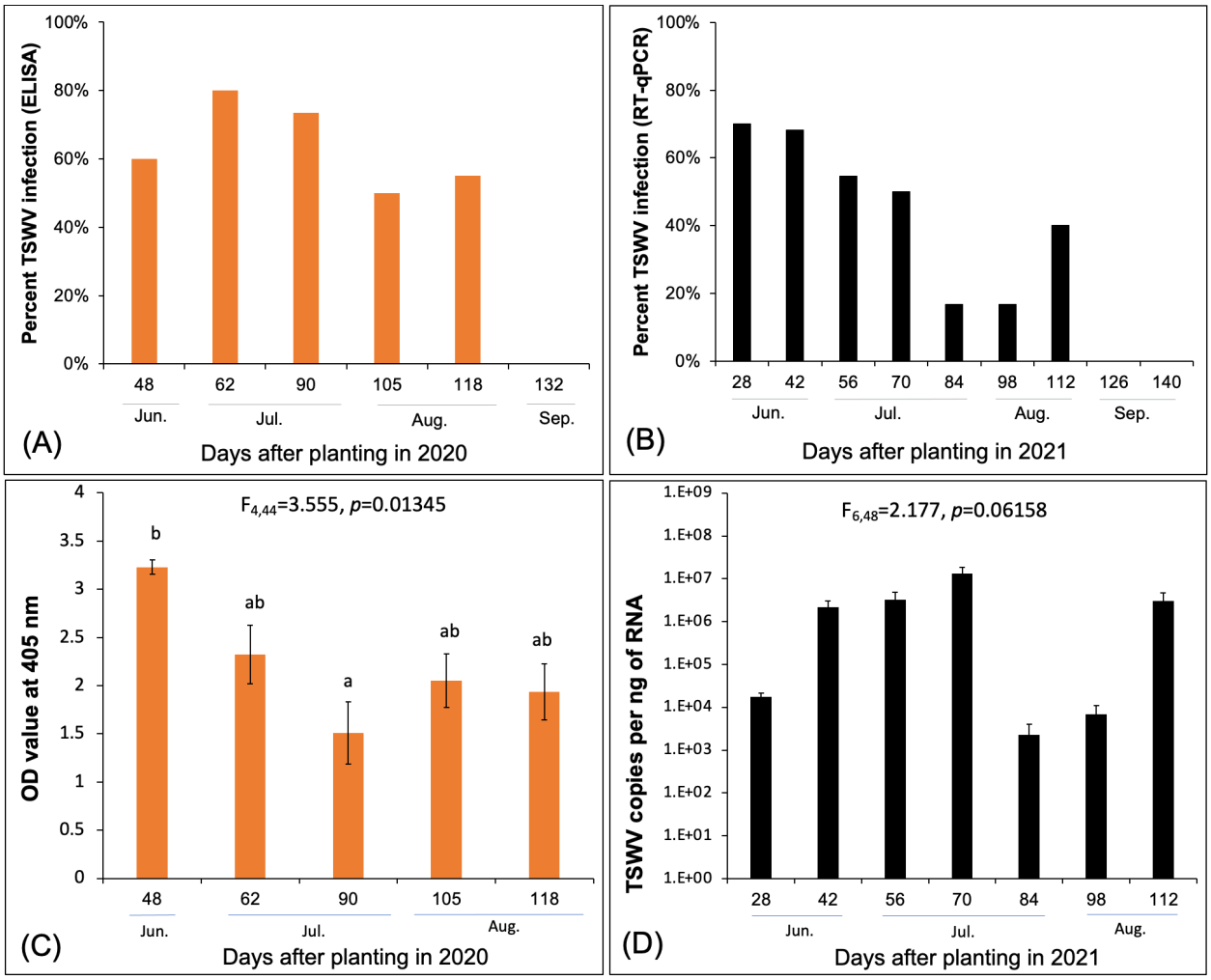


Figure 2. 6. Transmission of tomato spotted wilt virus (TSWV) following thrips acquisition on TSWV-infected field-collected peanut leaflets (*A. hypogaea* cv. Georgia Green). TSWV infection (A) and accumulation (C) following transmission in 2020 by DAS-ELISA and TSWV infection (B) and accumulation (D) following transmission in 2021 by RT-qPCR.

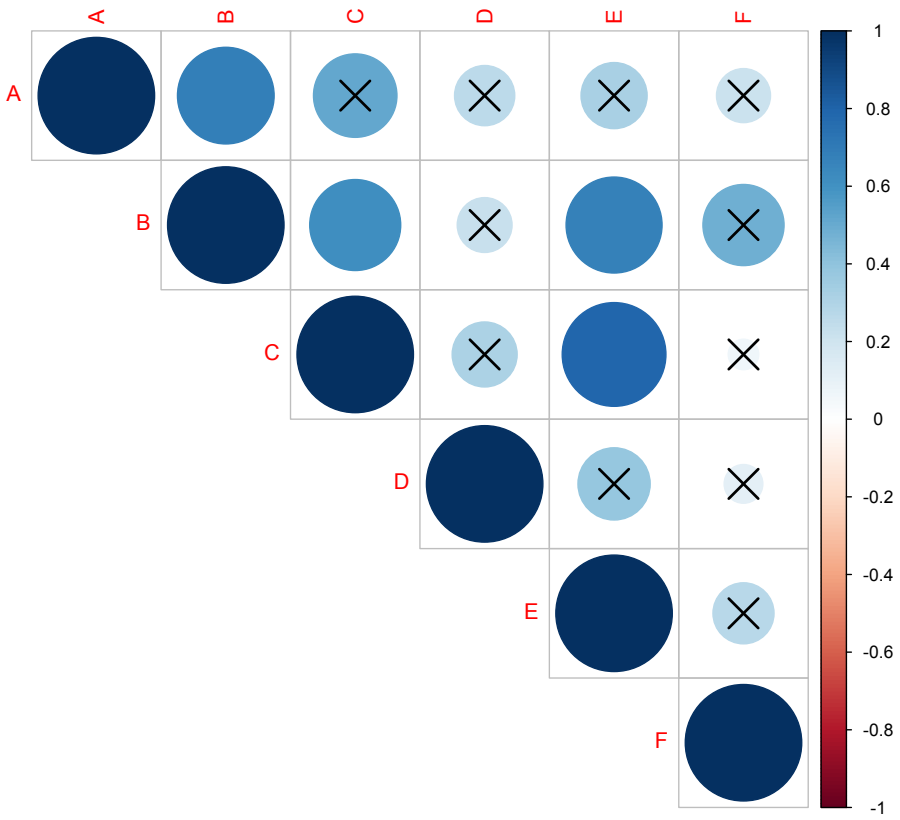


Figure 2. 7. Relationship between TSWV accumulation, thrips acquisition, and transmission in 2021. Six parameters in 16 experiments were used in the correlation matrix, the parameters include A (percentage of infected leaf), B (virus load in field-collected plants), C (percentage of viruliferous thrips), D (virus loads in thrips), E (transmission percentage), and F (virus loads in inoculated plants). "X" in circle means there is no significant difference at 0.05 level. Y-axis represents the strength of correlation.

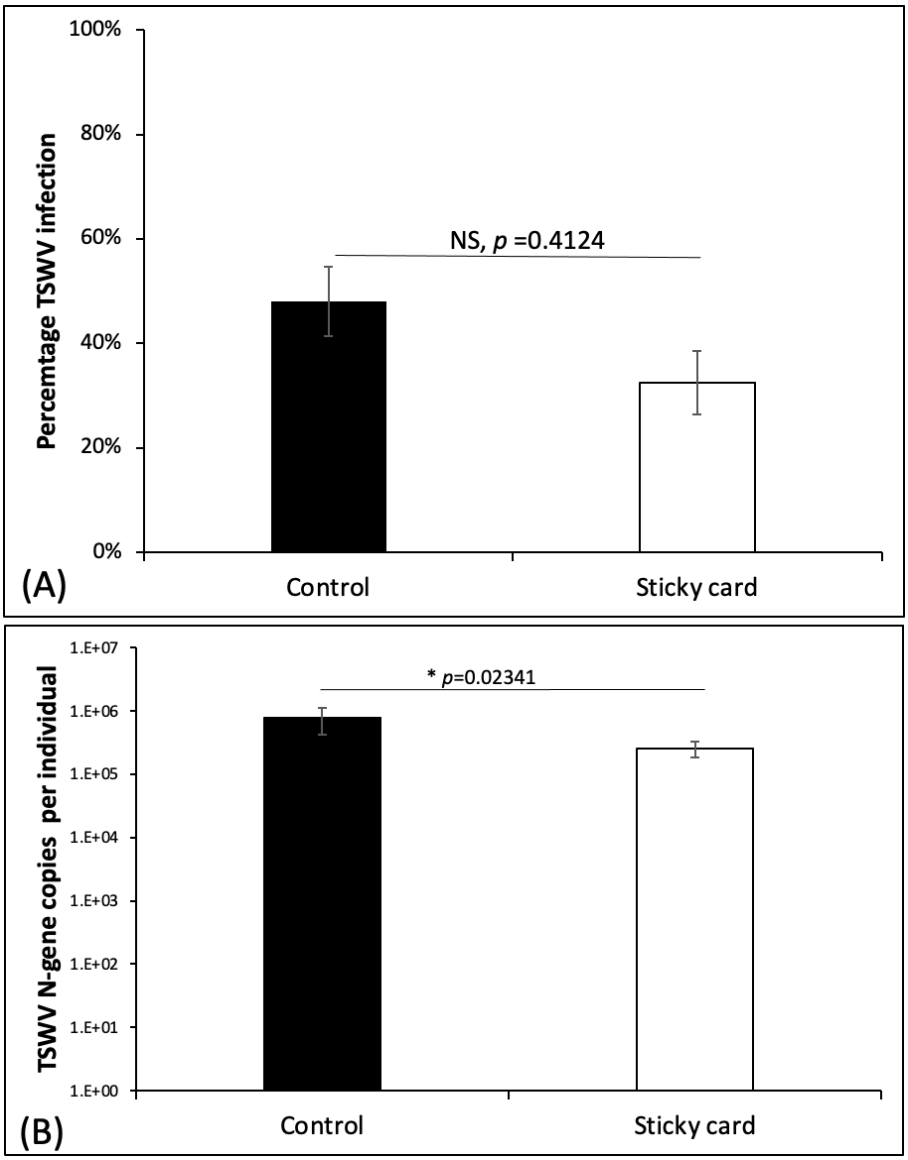


Figure 2. 8. Percent infection of tomato spotted wilt virus (TSWV) in *Frankliniella fusca* collected from sticky traps and TSWV accumulation in detected thrips. Ten potentially viruliferous tobacco thrips were tested for each treatment, and each experiment was repeated four times (N=50). The infection status of thrips (A) was determined by RT-qPCR and the accumulation of TSWV-infected thrips (B) was measured by obtaining TSWV-N gene copies.

CHAPTER 3

EVALUATION OF WILD PEANUT GENOTYPES FOR RESISTANCE AGAINST THRIPS AND THRIPS-TRANSMITTED TOMATO SPOTTED WILT ORTHOTOSPOVIRUS (TSWV)

[1] Y.-J. Chen, S. Leal-Bertioli, M.R. Abney, S. Bag, and R. Srinivasan. 2022. To be submitted to *Phytopathology*.

Abstract

Tomato spotted wilt orthotospovirus (TSWV) is a serious plant pathogen transmitted by thrips in persistent and propagative manner. TSWV infection in peanut causes the yield limiting spotted wilt disease (SWD). Peanut breeding programs over the past three decades have been engaged in developing resistant cultivars against thrips and/or TSWV. However, the genetic resistance available in cultivated peanut is limited/narrow. Wild peanut genotypes are important sources of resistance against pests and pathogens. With recent technological advances it is possible to introgress genes conferring resistance against pest and/or pathogens from wild diploid *Arachis* species (AA, BB, and KK sub-genome) into cultivated allotetraploid peanut, *Arachis hypogaea* L. (AABB genome). This study examined resistance/tolerance against thrips and/or TSWV in several wild peanut species and induced allotetraploids. In this study, 10 wild *Arachis* and six induced allotetraploid genotypes were screened in the greenhouse. Thrips' feeding assays and thrips-mediated transmission assays were used for screening against thrips and TSWV. Three parameters were evaluated: percent TSWV infection, virus accumulation, and temporal severity of thrips feeding injury. Results indicated that the diploid *Arachis stenosperma* accession V10309 and induced allotetraploid ValSten1 had the lowest TSWV infection incidences among the evaluated genotypes. Allotetraploid BatDur1 had the lowest thrips-inflicted damage, while diploid *A. batizocoi* accession K9484 and *A. duranensis* accession V14167 had lower feeding damage in three weeks than the others. Plausible TSWV resistance in diploid species and tetraploid hybrid was characterized by reduced percent TSWV infection

and/or virus accumulation. Further, a few diploids and tetraploid hybrids displayed antibiosis against thrips. Results together document evidence for resistance against thrips and TSWV in wild diploid *Arachis* species and peanut-compatible induced allotetraploids.

Introduction

Tomato spotted wilt orthotospovirus (TSWV) is the causal agent of the spotted wilt disease in peanut in the southeastern United States. *Tomato spotted wilt orthotospovirus* is a type species in the genus *Orthotospovirus* and family *Tospoviridae*. The genome of TSWV is comprised of a tri-segmented negative or ambi-sense RNA molecules designated as S (small, 2.9kb), M (medium, 4.8 kb), and L (large, 8.9 kb) enclosed within host-derived and double-layered membrane embedded with two glycoproteins (German et al., 1992). TSWV is transmitted by several species of thrips in a persistent and propagative manner. The tobacco thrips, *Frankliniella fusca* (Hinds) (Thripidae: Thysanoptera) is the major vector of TSWV in peanut in southeastern United States (Chaisuekul et al., 2003; Groves et al., 2001; Riley et al., 2012; Todd et al., 1995). The other well-known TSWV vector, *Frankliniella occidentalis* (Pergande), also is present on peanuts (Cultbreath et al, 2003). Besides transmitting TSWV, direct feeding injury of thrips may also contribute to economic losses in peanut production under certain circumstances (LaTora et al., 2022).

TSWV was first documented in the United States in 1971 in Texas (Halliwell & Philley, 1974). Since then, it has spread eastward and has constrained peanut production with yield loss peaking during 1997 at an estimated $40 million (Bertrand, 1998; Clevenger et al., 2017). Georgia accounts for over 50% of the peanut acreage (800,000 acres). From 1996 to 2006, an annual average loss of $12.3 million due to TSWV was assessed in peanut production in Georgia

(Riley et al., 2011). Virus and/or vector-resistant cultivars were not available at the time of TSWV introduction into the United States, and management relied heavily on chemical and cultural tactics predominantly targeting the vector (Srinivasan et al., 2017). Simultaneously, a heavy emphasis was placed on breeding for resistance against TSWV and/or thrips.

TSWV resistance in peanut has been built incrementally over time since the 1990s. The first generation of field resistant (against TSWV) cultivars were released in the 1990s. The most popular first generation TSWV-resistant cultivar was 'Georgia Green'. The cultivars released in the last twenty years (second- and third-generation cultivars) are significantly more field resistant to TSWV than Georgia Green (Srinivasan et al., 2017). TSWV field resistance in peanut seems to be different than the typical hypersensitive response (HR) defined by local lesions induced by cell death at the site of virus inoculation (López et al., 2011; Margaria et al., 2007; Webster et al., 2011). The hypersensitive responses on tomato and pepper are conferred by single genes, whereas evidence suggests that TSWV resistance resides in multiple chromosomes and that multiple genes could be involved in conferring field resistance. TSWV field resistant cultivars are not immune to the virus and exhibit mild symptoms upon infection (Shrestha et al., 2015; Sundaraj et al., 2014).

TSWV accumulation in some field-resistant cultivars seem to be lower than that of TSWV susceptible cultivars (Shrestha et al., 2015; Srinivasan et al., 2017; Sundaraj et al., 2014). The susceptibility of field-resistant cultivars also seems to vary with thrips and/or inoculum pressure, thereby indicating that the resistant cultivars cannot serve as 'stand-alone' management options. Chemical and cultural tactics are still used in conjunction with field resistant cultivars to offset yield losses (Culbreath et al., 2003; Culbreath & Srinivasan, 2011; Srinivasan et al., 2017).

Given the status of field resistant cultivars, peanut production would benefit by enhancing resistance. However, the available sources of resistance within the cultivated peanut seems to be narrow. The USDA accession, PI203396, collected from Brazil in 1952 is the sole source of resistance for almost all TSWV-resistant cultivars released in the United States, however this resistance is not very strong. Moreover, cultivated peanut is self-pollinated and has limited diversity and low genetic variability, and therefore not an effective source for resistance against pests and pathogens such as thrips and TSWV (Stalker, 2017).

In contrast, wild *Arachis* species (diploid) have high genetic diversity resistance and could be good sources of resistance against thrips and/or TSWV (Leal-Bertioli et al., 2015; Stalker et al., 2013; Stalker, 2017). But sexual incompatibility associated with ploidy level differences and other fertility barriers present considerable difficulties in crossing with cultivated (tetraploid) peanut (Lyerly et al., 2002; Stalker et al., 2013; Stalker, 2017). Induced tetraploid genotypes of *Arachis* spp. could help overcome these barriers and be amenable to breeding for resistance in cultivated peanut (Leal-Bertioli et al., 2015). Thus far, host plant resistance derived from wild peanut genotype against root-knot nematode (RKN, *Meloidogyne arenaria* (Neal)), late leaf spot (LLS, *Nothopassalora personata*), and early leaf spot have been effectively introgressed into cultivated peanut (Ballén-Taborda et al., 2019; Chu et al., 2021; Holbrook et al., 2017; Isleib et al., 2006; Simpson et al., 2003)

The goal of this study was to evaluate ten diploid *Arachis* species and six induced tetraploid hybrids as potential resistant sources against thrips and/or TSWV. An optimized thrips-mediated TSWV transmission assay was used as a high throughput screening platform to evaluate the above-stated genotypes. Thrips feeding assays and fitness studies were conducted to assess the diploids and tetraploid hybrids as resistance sources against thrips.

Materials and Methods

Maintenance of thrips colonies. Non-viruliferous thrips and viruliferous thrips were maintained on detached peanut leaflets. Non-viruliferous thrips were collected in Georgia and established in 2009 at the University of Georgia and maintained on leaflets of non-infected plants (cv. Georgia Green) in Petri dishes with a wet cotton round. Colonies were maintained by successive releases of ten adult female thrips, allowed to oviposit for 48h on a peanut leaflet dusted with a trace of pine pollen as a supplement (Angelella & Riley, 2010), and placed in growth chambers at 28-30°C and a photoperiod of 14:10 (L:D). Fresh leaflets and water were added to the Petri plates three times a week until emergence of the F1 generation. Viruliferous thrips (TSWV) colony was maintained similarly on TSWV-infected leaflets collected from the field in a separate growth camber as described in Shrestha et al. (2013). During the off-season, viruliferous thrips were maintained on TSWV-infected leaflets generated by mechanical inoculation as described by Marasigan (2014) in the greenhouse.

Non-infected peanut genotypes. Ten accessions of nine diploid *Arachis* species (*A. stenosperma* V10309, *A. duranensis* V14167, *A. cardenasii* GKP10017, *A. ipaensi* K30076, *A. valida* GK30011 (PI 468154), *A. diogoi* V10602, *A. villosa* V12912, *A. batizocoi* K9484, *A. magna* 30092, and *A. magna* 30097; Table 3.2) and six induced allotetraploid hybrid genotypes of *Arachis* species (BatDur1, BatSten1, IpaVillo1, ValSten1, MagDur1, and MagSten1; Table 3.2) from University of Georgia at Athens, GA, USA were used for thrips-mediated inoculation in the greenhouse in 2019, 2020, and 2021. The common tetraploid cultivar "Georgia Green" was used as a susceptible reference check in all inoculation assays. Seeds of diploids and tetraploid genotypes were treated with 2-3 ml of a 0.5% solution of Florel® Growth Regulator (Monterey

Lawn and Garden, Fresno, California, USA) and incubated in a Petri dish at 28°C for 18-24h to break seed dormancy. Seeds were sown in individual 4" pots with potting mix that included 4 parts sand, 2 parts field soil, 2 parts ProMix (Premier Horticulture Inc, Quakertown, PA, USA), 1 part Perlite (Therm-O-Rock East Inc, New Eagle, PA). Seeds of Cultivar "Georgia Green" were pre-geminated in moisten paper towel and incubated in a growth camber kept at 28°C for 2-3 days. Ten seedlings (ca. one-to-two-week-old with one-to-two nodes and up to 16 leaflets) of each genotype were used for TSWV transmission.

Thrips-mediated TSWV transmission. TSWV transmission was conducted via *F. fusca*-mediated inoculation (Shrestha et al., 2015). For each genotype, ten seedlings were placed in a thrips-proof cage (47.5 cm^3) (Megaview Science, Taichung, Taiwan).Ten thrips (female adults up to three days old) from viruliferous thrips colony were released on each seedlings dusted with pollen as a supplement and enclosed with in a plastic film cage ($\pi r^2 h$=3.14 x 16 x 39 cm^3). Each plant was evaluated for thrips injury at one-week interval and used for TSWV detection at three weeks after thrips release

(1) Transmission efficiency. The percentage of infected plants was estimated three weeks post inoculation. To determine TSWV infection status, plant tissue (ca 0.03 g) was collected from the first fully expanded leaf below the terminal and subjected to a double antibody enzyme-linked immune sorbent assay (DAS-ELISA). DAS-ELISA (Adgia, Elkhart, IN, USA) was used to detect TSWV in peanut tissue in the laboratory as per the protocol outlined earlier (Lai et al., 2021).

Statistical analysis was performed to compare TSWV infection between wild diploid species and their tetraploid hybrids with Georgia Green. The data from two experimental repeats were pooled (n=20 for each genotype) and subjected to generalized linear mixed model analysis using the "glmer" function in software R assuming a binomial distribution using the logit link

function (Core R team., 2014). Genotypes (treatments) were considered as the fixed effect and replications and experimental repeats were considered as random effects. Least square means (LS-mean) was used for multiple comparisons at a significance level of $\alpha = 0.05$ with Tukey adjustment to determine the difference between genotypes using the "lsmeans" function in R.

(2) Quantitation of virus loads in infected samples. The plants testing positive for TSWV by DAS-ELISA were selected for quantitation of TSWV via a two-step reverse transcription-quantitative polymerase chain reaction (RT-qPCR) using TSWV-N gene-specific primers (Rotenberg et al., 2009; Shrestha et al., 2012). Symptomatic leaflet tissues (ca 0.03 g) from infected samples were used for RNA extraction. Total RNA was extracted by RNeasy Plant Mini Kit (Qiagen, Valencia, CA) and complementary DNA (cDNA) was synthesized by Go-Script reverse transcription system (Promega Corporation, Madison, WI) following the manufacturer's instructions. Synthesized cDNA was used as a template for qPCR.

The reaction mix for qPCR included 12.5 µl 2x GoTaq qPCR Master Mix (Promega, Madison, WI), forward and reverse primers (final concentration of 0.2 µM), 1 µl cDNA, and nuclease-free water for final reaction volume of 25 µl. The reaction started at 95°C for 2 min, followed by 40 cycles at 95°C for 15s, 58°C for 60s, and 72°C for 20s. The reaction was extended with a melting curve analysis in a QuantStudio 3 System (applied biosystems by Thermo Fisher Scientific, Waltham, MA, USA) to rule out non-specific binding. Each sample was tested in duplicate, and absolute number of TSWV-N gene copies in the samples was quantitated using the standard curve protocol described by Shrestha et al., (2013).

Statistical analysis was performed to compare TSWV-N gene copies between diploid species and their tetraploid hybrids with Georgia Green using the "glmer" function (the generalized linear mixed model analysis, GLMM) in software R (Core R team., 2014) with Gaussian

distribution. Genotypes (treatments) were considered as fixed effects and replications and experimental repeats were considered as random effects. Least square means (LS-mean) were used for multiple comparison at a significance level of $\alpha = 0.05$ with Tukey adjustment to determine the difference between genotypes using the "lsmeans" function in R.

(3) Thrips feeding damage index. Feeding injuries were cataloged by the feeding damage index (Maris et al., 2003; Sundaraj et al., 2014). Feeding intensity (FI) was rated from 0 to 3. Foliage without feeding scars was rated as 0; foliage with less than 25% of feeding scars was rated as 1; foliage with 25 to 50% of feeding scars was rated as 2, and foliage with higher than 50% of feeding scar was rated as 3 (Supplementary Figure 3.1). Feeding damage (FD) indicates the proportion of leaflets with feeding scars. Thrips feeding injuries were evaluated as feeding damage index (FDI, formula = FD X FI) on one-, two-, and three weeks post-inoculation (Maris et al., 2003). Each genotype had at least ten plants. The experiment was conducted two times (n=20 for each genotype). Data from all the repeats were pooled, genotypes (treatments) were considered as fixed effect and experimental repeats and replications were considered as random effects. The observation over time were analyzed separately for each time point. Thrips feeding injury data was subjected to GLMM analysis using "glmer" function in software R assuming a normal distribution (Core R Team, 2019). Least square means (LS-mean) were used for multiple comparison at a significance level of $\alpha = 0.05$ with Tukey adjustment to determine the difference between genotypes using the 'lsmeans' function in R.

(4) Thrips fitness. The effects of wild peanut genotypes on thrips fitness were evaluated by assessing thrips development time and the number of adults produced. Ten diploid and six induced tetraploid genotypes were used for evaluating TSWV susceptibility. Five Munger cages (Munger, 1942) were set up for monitoring the fitness parameters. Two leaflets (similar leaf size)

of each genotype were placed in one Munger cage. Ten non-viruliferous female thrips (up to 3 days old) were transferred to leaflets using a fine brush for oviposition and removed after 72h. Cages were kept in growth camber at 29±1°C with 14h:10h (L:D) photoperiod. The dates of newly hatched larvae were recorded and newly emerged adults were counted and removed daily. The experiment was repeated once (N=10 Munger cages for each genotype). The median developmental time required for the adults to develop was evaluated on each genotype and the statistical significance of the difference between genotypes was estimated by Kruskal-Wallis test using "kruskal.test" function in R. Thrips fitness data (number of adults produced) was subjected to GLMM analysis using "glmer" function in software R assuming a Poisson distribution. Least square means (LS-mean) was used for multiple comparisons at a significance level of α =0.05 with Tukey adjustment to determine the difference between genotypes using the "lsmeans" function in R.

(5) The correlation between final TSWV infection and thrips feeding injury. To assess the correlation between feeding injury on one-, two-, and three- weeks post-inoculation and TSWV infection (infection percentage on three weeks post-inoculation) data from all the experiments on 17 genotypes were pooled. The comparisons were made by Pearson's product-moment correlation 'cor.test' at 95% confidence interval in software R.

Results

1.TSWV symptoms on wild peanuts. All wild diploid and tetraploid genotypes showed visible TSWV systemic symptoms in infected plants (Fig 3.1, and Fig. 3.2).The common symptoms associated with TSWV infection in evaluated diploid and tetraploid genotypes

included ringspots, chlorotic spots, yellowing, necrosis, wilting, stunting, and death of terminal buds. Diverse symptoms were observed on each genotype, and TSWV symptoms varied with disease progression. Most wild peanut genotypes showed concentric ringspot on leaflets and continued to grow as in the case of ValSten1. However, the plants with dry terninal buds resulted in the death of plants such as *A. duranensis* V14167 and *A. ipaensis* K30076. Overall, the severity of TWSV symptoms on the most evaluated genotypes was somewhat more subdued than in the susceptible cultivar, Georgia Green.

2. Transmission efficiency. TSWV infection percentage varied within wild genotypes of *Arachis* species ($F_{16,697}$=4.6332; p<0.001; Fig. 3.3). Diploids, *A. stenosperma* V10309 (Sten, 22.0 ± 7.3%) and *A. cardenasii* GKP10017 (Car, 26.7 ± 8.8%) were infected at a reduced percentage than the cultivated genotype *A. hypogea* cv. Georgia Green, which was used as the control genotype in each experiment. The infection percentages in *A. villosa* V12912 (Villo, 26.1 ± 17%), *A. diogoi* V10602 (Dio, 29.4 ± 12.0%), *A. ipaensis* K30076 (Ipa, 36.0 ± 3.4%), *A. valida* GK30011 (Val, 37.6 ± 14.7%),and *A. batizicoi* K9484 (Bat, 44.3 ± 9.1%) were lower but not different from *A. hypogea*. TSWV infection percentages in *A. duranensis* V14167, *A. magna* 30097, and *A. magna* 30092 were higher than that of *A. stenosperma* V10309. In allotetraploids, TSWV infection percentage in ValSten1 (14.1 ± 4.1%) was significantly lower than the control genotype (Georgia Green, 65.5 ± 3.1%).

3. **Quantitation of virus loads in infected samples**. Virus loads in terms of TSWV-N gene copies were compared between diploids and tetraploids along with Georgia Green. The results showed differences between genotypes examined among diploids ($F_{10,66}$=1.91, p=0.06; Fig. 3.4A) and tetraploids ($F_{6,54}$=0.64, p=0.70; Fig.3.4B). However, TSWV loads in diploids

and/or tetraploids were not significantly different to those of the control genotype (Fig. 3.4). In diploids, TSWV loads in *A. villosa* V12912, *A. batizocoi* K9484 and *A. magna* 30092 were significantly higher than in *A. cardenasii* GKP10017, A. *valida* GK30011, and *A. duranensis* V14167 (Fig. 3.4A). In induced tetraploid genotypes, TSWV loads in MagSten1, BatSten1, and BatDur1 were significantly higher than in MagDur1 via RT-qPCR (Fig. 3.4B).

4. Thrips feeding damage index. In diploid genotypes, feeding injury differences were observed at one week ($F_{10,397}$=16.94; p<0.0001; Fig. 3.5A), two weeks ($F_{10,423}$=16.01; p<0.0001; Fig. 3.5B), and three weeks ($F_{10,419}$=23.57; p<0.0001; Fig. 3.5C) post thrips release. More feeding injuries were recorded in *A. magna* 30097 alone than the cultivated genotype (Georgia Green) one week after thrips release, and *A. batizocoi* K9484 and *A. duranensis* V14167 had significantly fewer feeding scars than the control genotype (Fig. 3.5A). Also, every diploid, except *A. diogoi* V10602, at three weeks post thrips release showed fewer thrips injuries than *A. magna* 30097 (Fig. 3.5A-C).

In tetraploid genotypes, feeding injury differences were observed at one week ($F_{6,270}$=14.39, p<0.0001; Fig. 3.5D), two weeks ($F_{6,293}$=7.51, p<0.0001; Fig. 3.5E), and three weeks ($F_{6,272}$=15.36, p<0.0001, Fig. 3.5F) after thrips release. One week after thrips release, thrips feeding injuries were lower in BatDur1 and higher in both MagSten1 and IpaVillo1 than the control genotype, Georgia Green (Fig. 3.5D). Two weeks after thrips release, only BatDur1 had fewer thrips feeding scars in comparison with the control genotype (Fig. 3.5E). At three weeks after thrips release, ValSten1, BatDur1, and BatSten1 had less thrips injury than the control genotype (Fig. 3.5F).

Based on the accumulation of feeding injury, the thrips injury increased by time except genotype BatSten1 (Fig. 3.6). BatSten1 showed obvious defoliation after thrips injury. Therefore, there were fewer feeding scars observed on BatSten1 at three week than two week after feeding. Also, the defoliation of lower leaves was observed on Sten, Val, and MagSten1.

5. Thrips fitness. The survival of thrips was estimated by the number of adults emerging from each cage (ten females releasing per cage). The number of adults varied between genotypes in diploids ($F_{10, 105}$=134.35, p<0.0001; Fig. 3.7A) and tetraploids ($F_{6, 53}$=79.005, p<0.0001; Fig. 3.7B). The survival of thrips feeding on diploids was five times lower than in the control, Georgia Green. Similar to the diploids, the number of adults emerged on tetraploids was lower than in the control. Within the tetraploid genotypes, thrips survival was the highest in MagSten1 and lowest in BatDur1.

The median developmental time of *F. fusca* ranged from 13 to 16 d on 17 *Arachis* accessions at 29°C. Developmental time varied with diploids (x^2=43.2, df=10, p<0.0001; Fig. 3.8A) and with tetraploids (x^2=41.6, df=6, p<0.0001; Fig. 3.8B). The median developmental time was shorter on the cultivated genotype 'Georgia Green' (12.8 d) than on diploids and tetraploids. The median developmental time was the shortest and longest time on *A. villosa* V12812 (14.3 d ± 0.26) and *A. ipaensis* K30076 (15.9 d ± 0.27), respectively. In tetraploids, the median developmental time was the shortest and longest on BatSten1 (13.7 d ± 0.40) and MagDur1 (16.0 d ± 0.20), respectively.

6. Correlation between final TSWV infection and thrips feeding injury. Seven days post thrips-mediated inoculation, no correlation between thrips feeding and percentage of TSWV infection in diploids (y=0.1428x+0.3061, R^2=0.047, Correlation=0.216, p=0.2345; Fig. 3.9A) and tetraploids (y=-0.03692x+0.60439, R^2=0.004, Correlation=-0.06, p=0.7245; Fig. 3.9B) was

identified. While thrips feeding injury recorded after two weeks showed a positive correlation in the case of diploid genotypes (y=0.1736x+0.1813, R^2=0.167, Correlation=0.408, *p*=0.01832; Fig. 3.9C) but the positive correlation was not observed in tetraploids (y=0.09238x+0.40721, R^2=0.097, Correlation=0.312, *p*=0.094; Fig. 3.9D) After three weeks feeding, a positive correlation between thrips feeding injury and TSWV transmission was identified on both diploids (y=0.221x+0.02921, R^2=0.366, Correlation=0.605, *p*=0.00011; Fig. 3.9E) and tetraploids (y=0.1840x+0.1240, R^2=0.268, Correlation=0.518, *p*=0.002; Fig. 3.9F).

Discussion

Thrips-mediated inoculation of TSWV indicated that some diploids such as *A. stenosperma* V10309 (AA genome) were infected at a lower level than the other diploids and the cultivar, Georgia Green. *Arachis stenosperma* V10309 has been shown in earlier studies to possess resistance against fungal pathogens and nematodes (RKN) (Leal-Bertioli et al., 2010; Leal-Bertioli et al., 2016; Proite et al., 2008). Among the induced allotetraploids evaluated, ValSten1 had a reduced TSWV infection percentage (~30%) in comparison with others. An earlier field study also documented ValSten1 to be tolerant to TSWV compared with the susceptible cultivars 'Gregory' and 'Florunner' (Chu et al., 2021). Also, ValSten1 was documented to exhibit resistance to early and late leaf spot and rust pathogens (Gao et al., 2021). These additional traits besides TSWV resistance make this genotype promising for peanut research and breeding, and it is vigorous and highly fertile (Gao et al., 2021). Some of the results observed in this study were in contrast with earlier findings. *A. diogoi* V10602 did not display higher levels of TSWV resistance and exhibited TSWV symptoms upon inoculation. Three

accessions of *A. diogo*i V10602 previously showed no symptoms following TSWV inoculation (Lyerly et al., 2002). While the current study used thrips-mediated inoculation, the Lyerly et al (2002) study used mechanical inoculation. Thrips-mediated TSWV transmission has shown to be more effective than mechanical inoculation (Shrestha et al., 2015). The current study and Lai et al (2015) used thrips-mediated inoculation of *A. diogoi* V10602, both studies indicated that *A. diogoi* V10602 accession can get infected with TSWV. However, TSWV symptoms and virus accumulation were slightly higher in the current study than in Lai et al. study.

Despite the reduced TSWV infection percentages in some diploids and tetraploids in comparison with others evaluated in this study as well as with Georgia Green, TSWV accumulation levels were not congruent to the infection percentages. For instance, the genotypes, *A. cardenasii* GKP10017, *A. valida* GK30011, and *A. duranensis*V14167 were infected at higher percentages but accumulated reduced virus loads when compared with other diploids and tetraploids. In none of the genotypes evaluated in this study there was evidence for hypersensitive response against TSWV. Instead, all diploid and tetraploid genotypes displayed systemic TSWV symptoms. Most wild species developed the same characteristic TSWV symptoms, such as ringspots, yellowing, and stunting as in the case of peanut cultivars, but ringspots were not observed in the case of a few genotypes such as *A. villosa* V12812. Overall, the severity of TWSV symptoms on the evaluated genotypes was somewhat milder than in the susceptible cultivar, Georgia Green. However, dry terminal bud was a severe TSWV symptom in a few wild peanut genotypes. Given that seedlings of wild peanut genotypes often have one or very few terminal bud(s) compared with cultivated peanut, drying of terminal buds could be lethal. These results suggest that the mechanism of TSWV resistance in *Arachis* diploids and tetraploids is similar to what has been observed in the case of tetraploid cultivars obtained via conventional plant breeding

(Shrestha et al., 2015; Sundaraj et al., 2014) The field resistance to TSWV in peanuts is identified in numerous quantitative trait loci (Agarwal et al., 2019; Khera et al., 2016; Pandey et al., 2017) and suggests that the resistance against TSWV in peanut is different from the single gene governed resistance observed in the case of solanaceous crops such as tomato and pepper (Margaria et al., 2007; Moury et al., 1997). The results from this study with diploids and induced tetraploids together also supported the possibility of involvement of multiple genes associated with TSWV resistance in peanut (Branch & Culbreath, 2015, 2018; Catto et al., 2021).

Previous studies have indicated that besides reduced TSWV infection in some of the field-resistant cultivars, there was also enhanced resistance and/or tolerance against tobacco thrips, *F. fusca* (Srinivasan et al., 2017). Thrips feeding damage was evaluated in this study. A number of diploids such as *A. batizocoi* K9484 and *A. duranensis*V14167 as well as tetraploids such as BatDur1 had reduced thrips feeding induced damage when compared with the susceptible genotype, Georgia Green, two and three weeks after thrips infestation. A previous study also provided evidence for reduced thrips susceptibility in *A. batizocoi* K9484 (=PI298639) and PI468329 (Stalker & Campbell, 1983; Yang et al., 1993). Overall, less feeding injury was recorded in the diploids than in the induced tetraploids. Similarly, Michelotto et al., (2017) observed that certain allotetraploids showed higher susceptibility to another thrips species (*Enneothrips flavens=E. enigmaticus*) than its parental diploids. Reduced thrips feeding injuries in some diploids and a few tetraploids could be due to antixenosis (non-preference).

Besides antixenosis, antibiosis (negative effects on thripts fitness) is another factor that could be responsible for resistance against thrips. The fitness experiments conducted under controlled conditions showed that the diploid genotypes of wild *Arachis* species were unsuitable hosts for thrips as many individuals were not able to complete their lifecycle.

Leaf traits can be the first physical barrier against thrips, the types and density of trichomes contributed to thrips resistance in strawberries and wild tomato (Mouden & Leiss, 2021). Since *Frankliniella fusca* showed less infestation in diploid peanuts and wild diploid genotypes have higher trichome density than induced allotetraploids (Leal-Bertiolo et al., 2017). It is possible that the thrips resistance may be correlated to the density of the trichome or its associated chemicals/metabolites but it needs further investigation (Kortbeek et al., 2021).

Thrips survival percentage was consistently lower in diploid genotypes than others evaluated in this study. In tetraploid genotypes, the pattern of adult emergence was consistent with that of diploids. But in some instances, there was reduced heritability of resistance/tolerant traits in tetraploids when compared with parental diploids. For example, MagDur1 was more susceptible to thrips than both parental genotypes. Thrips resistance trait of ValSten1 was likely inherited from the AA genome progenitor (Sten1). Overall, thrips on the diploids and tetraploids took a longer time to complete one generation. This delayed developmental time is also another indication of antibiosis induced effects on thrips development.

Several diploids and induced tetraploids were infected at a reduced percentage versus others and several of them also exhibited resistance/tolerance against thrips. However, the correlation between TSWV and thrips resistance was not substantial. This indicated that TSWV and thrips resistance are not governed by the same set of genes and that lack of correlation along with lack of reduction of TSWV accumulation in many diploids and tetraploids presents a significant challenge to plant breeders. The current genetic base in peanuts for TSWV resistance is extremely limited. While there are appreciable levels of resistance in diploid peanut species, the challenge has been to overcome the ploidy level differences. With the ability to generate synthetic allotetraploids, the issues that arise due to ploidy level differences could be avoided.

Furthermore, availability of the synthetic allotetraploids increases the genetic base for thrips and/or TSWV resistance.

Conclusion

TSWV remains one of the most serious pathogens that infects peanuts. The development of TSWV-resistant cultivars has been a vast improvement prior to their existence. Despite the usage of these resistant cultivars, losses continue to occur, and they are often proportional to the thrips and TSWV inoculum pressure. Almost all currently available peanut cultivars have been generated from a single accession with TSWV resistance. With such a narrow genetic base, there is room for evolution of resistance breaking variants' development, as has been shown in other crops (López et al., 2011; Margaria et al., 2015; Moury et al., 1997). There is no evidence of high levels of host resistance-induced virus selection pressure against TSWV at this juncture (Lai et al., 2021); however, the intense cultivation of peanuts in over a million acres annually might facilitate the development of resistance in the future. To avoid such a scenario and to broaden the genetic base of TSWV resistance, the synthetic tetraploids present a viable option. By combining resistance from two diploid species, the tetraploids, when bred with commercial cultivars, could impart higher levels of TSWV resistance than in the current scenario. Diversifying the TSWV resistance sources might also delay/avoid the development of resistance-breaking TSWV variants. Nevertheless, the challenge remains to develop TSWV resistant tetraploids given that TSWV resistance in diploids is not all that high.

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Scale (0-3)	0	1	2	3
Feeding scar intensity of individual leaflet	None 	<25% 	25-50% 	>50% 

Supplementary Figure 3.1. Thrips feeding scar intensity scale 0-3.

Table 3.1. Wild diploid *Arachis* genotypes used for thrips-mediated inoculation.

Wild diploid	Accession no. (USDA no.)	Genome type	Collection site
A. batizocoi	K9484 (PI 298639)	KK	Parape, Bolivia
A. cardenasii	GKP10017	AA	Robore, Bolivia
A. diogoi	V10602 (PI 276235)	AA	Paraguay
A. duranensis	V14167	AA	Salta, Argentina
A. ipaensis	K30076	BB	Gran Chao, Bolivia
A. magna	30092 (PI 468337)	BB	Bolivia
A. magna	30097	BB	Santa Cruz, Bolivia
A. stenosperma	V10309 (PI 666100)	AA	Mato Grosso, Brazil
A. valida	GK30011 (PI 468154)	BB	Mato Grosso, Brazil
A. villosa	V12812	AA	Bella Union, Uruguay

Table 3.2. Wild induced allotetraploid *Arachis* genotypes used for thrips-mediated inoculation.

Induced allotetraploids	Plant ID	Genome type	Collection/ registration
A. batizocoi K9484 x *A. duranensis* V14167	BatDur1	AAKK	Leal-Bertioli et al., 2015
A. batizocoi K9484 x *A. stenosperma* V10309	BatSten1	AAKK	Leal-Bertioli et al., 2015
A. ipaensis K30076 x *A. villosa* V12812	IpaVillo1	AABB	Leal-Bertioli et al., 2017
A. magna 30097 x *A. duranensis* V14167	MagDur1	AABB	Unpublished
A. magna 30097 x *A. stenosperma* V10309	MagSten1	AABB	Bertioli et al., 2021
A. valida GK30011x *A. stenosperma* V10309	ValSten1	AABB	Gao et al., 2021

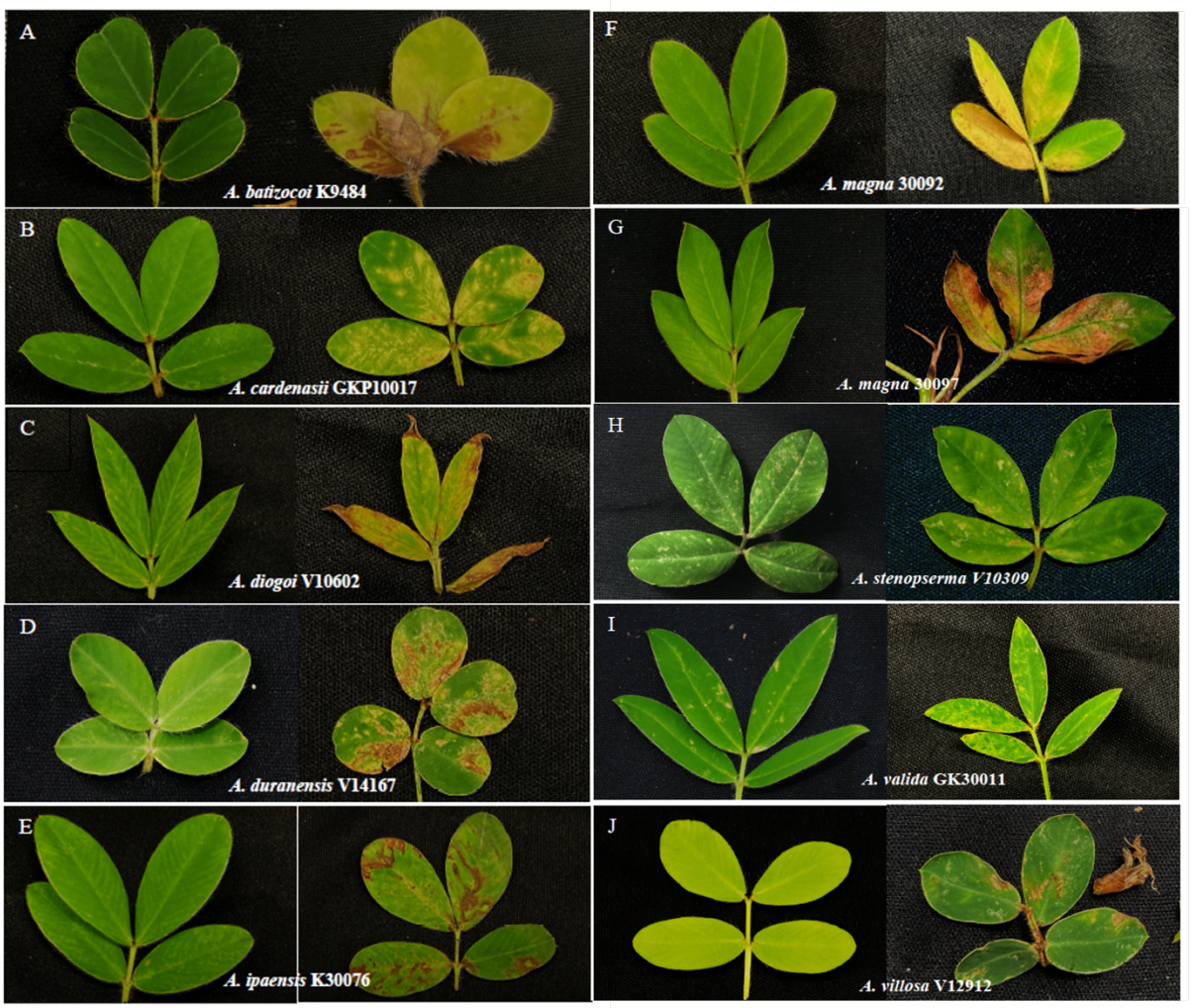

Figure 3.1. Symptoms of tomato spotted wilt virus (TSWV) infection on leaflets of diploid *Arachis* genotypes. TSWV non-infected leaf (left) and TSWV-infected leaf (right).

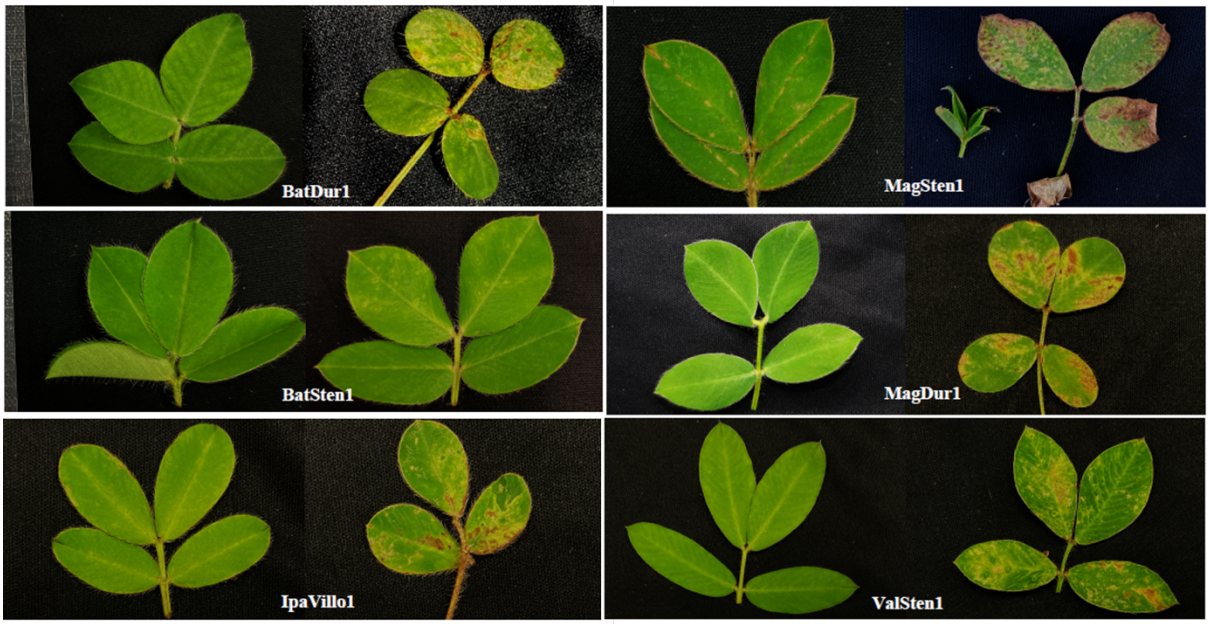

Figure 3.2. Symptoms of TSWV infection on leaflets of tetraploid *Arachis* genotypes. TSWV-non-infected leaf (left) and TSWV-induced symptoms (right) on *Arachis* induced allotetraploids .

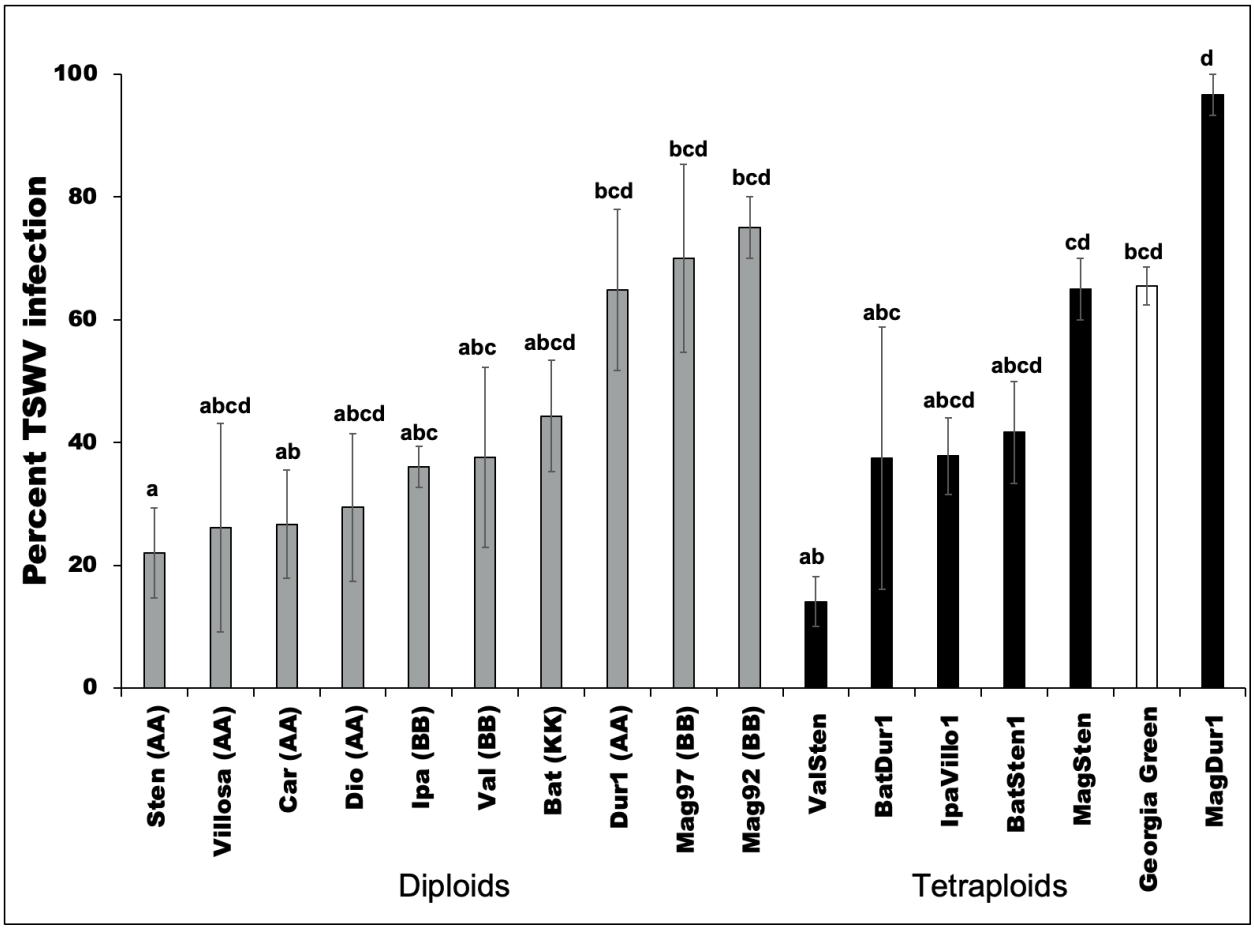

Figure 3.3. Percent infection of tomato spotted wilt virus (TSWV) in wild species of *Arachis* genotypes with AA, BB, or KK genomes and their induced allotetraploids genotypes three weeks post-inoculation. Ten potential viruliferous tobacco thrips were used to inoculate a single plant. Ten plants were tested for each genotype and the experiment was repeated twice (N=20). Cultivated tetraploid (*A. hypoaea* cv. Georgia Green) was used as the control for each experiment. The infection status of inoculated plants was determined by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) targeting the nucleocapsid protein (N) of TSWV. Different letters on standard error of means (SE) indicate significant differences between means separated by LSD with Tukey method at $\alpha = 0.05$.

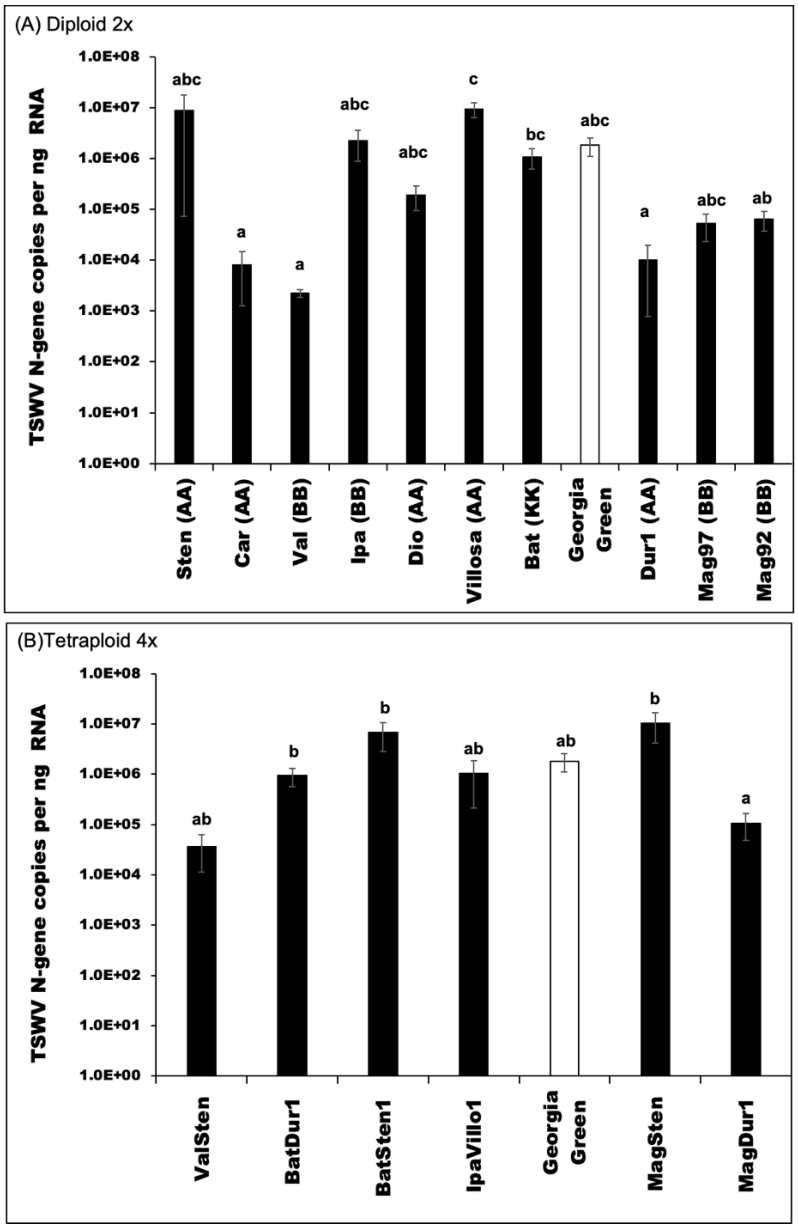

Figure 3.4. Tomato spotted wilt virus (TSWV) accumulation in *Arachis* genotypes three weeks post-inoculation. Virus loads of TSWV from infected leaflet samples in wild diploids, induced allotetraploids, and cultivated peanut (*A. hypoaea* cv. Georgia Green) were estimated by reverse transcription quantitative polymerase chain reaction followed by absolute quantitation using plasmids containing TSWV N-gene inserts as standards. Different letters on standard error of means (SE) indicate significant differences between means separated by LSD with Tukey method at $\alpha = 0.05$.

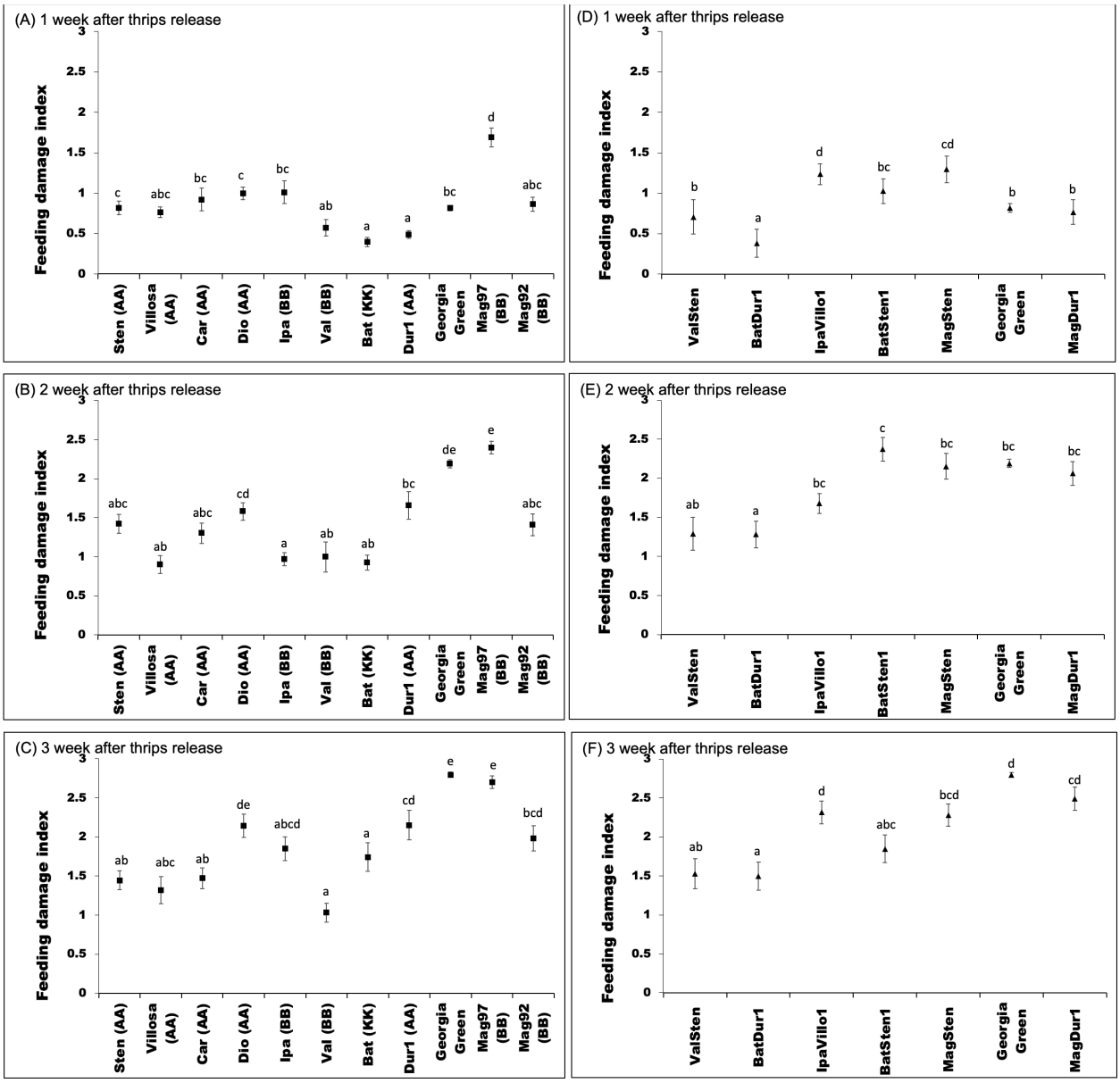

Figure 3.5. Thrips feeding injury on *Arachis* genotypes. Feeding damage indices (FDI) were evaluated at one week, two weeks, and three weeks post thrips release on wild diploids (A, B, C) and induced allotetraploids (D, E, F). Cultivated tetraploid (*A. hypoaea* cv. Georgia Green) was used as the control at each experiment. Mean feeding damage indices are presented in one-week intervals. Different letters on standard error of means (SE) indicate significant differences between means separated by LSD with Tukey method at $\alpha = 0.05$.

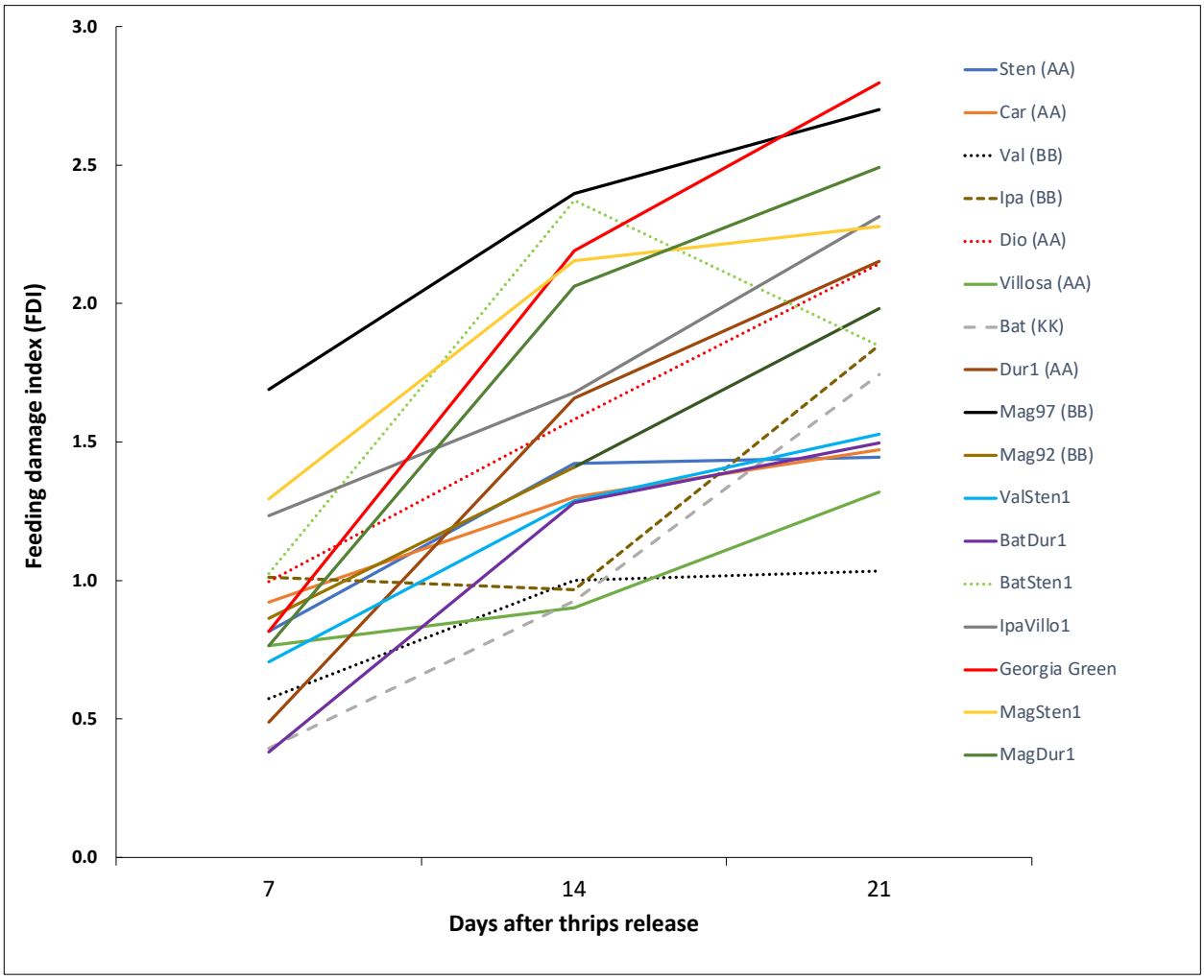

Figure 3.6. Accumulation of thrips feeding injury on *Arachis* genotypes over time. Feeding damage indices (FDI) were evaluated at one week, two weeks, and three weeks post thrips release on wild peanut genotypes. Cultivated tetraploid (*A. hypoaea* cv. Georgia Green) were used as the control at each experiment.

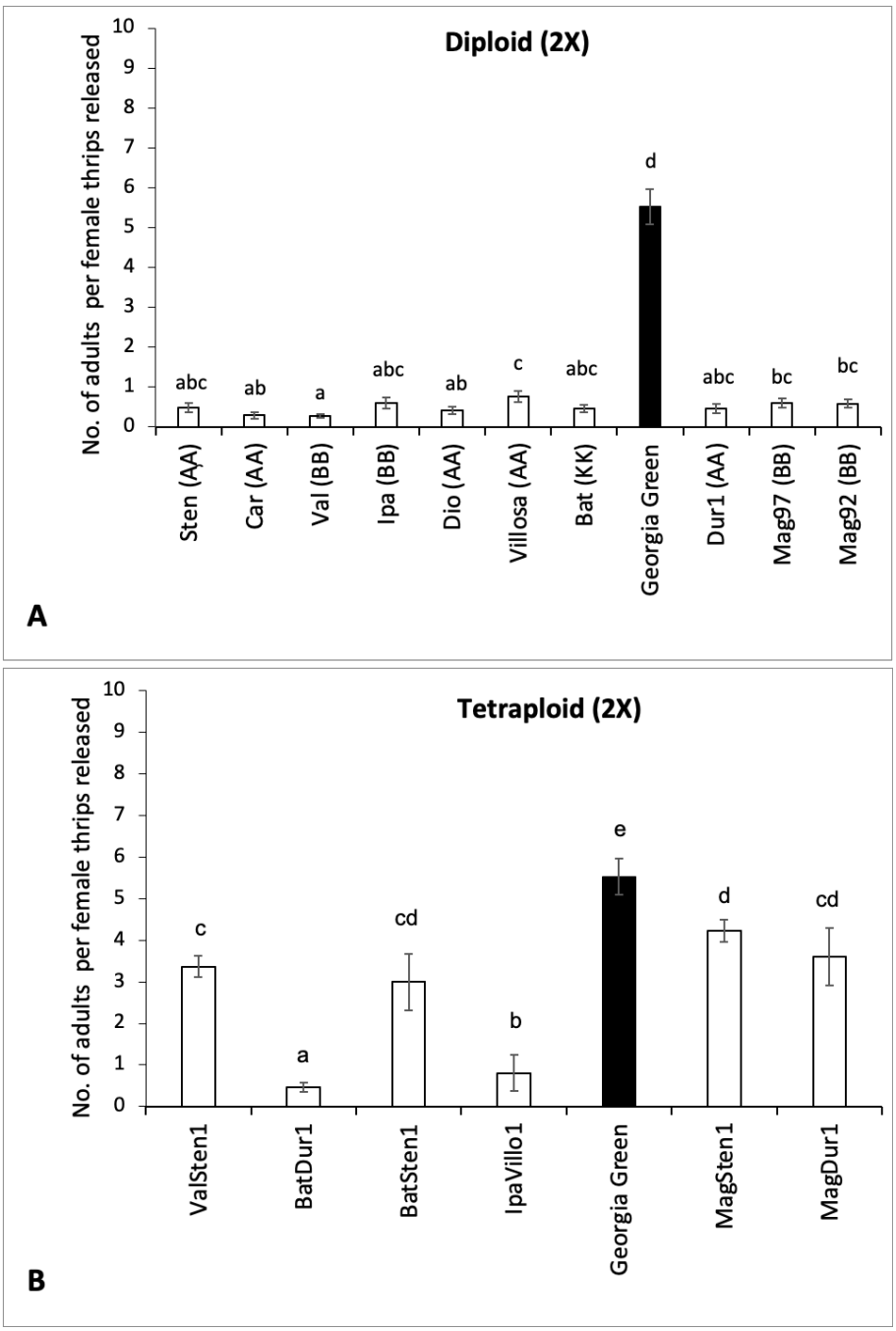

Figure 3.7. Mean number of adults emerging per *Franklinella fusca* female thrips released on the leaflets of non-infected *Arachis* genotypes. Ten non-viruliferous female thrips were released on non-infected leaflets on each Munger cage. Approximately ten Munger cages were set up for each genotype. The number of adults emerged from each cage were recorded at 24h intervals.

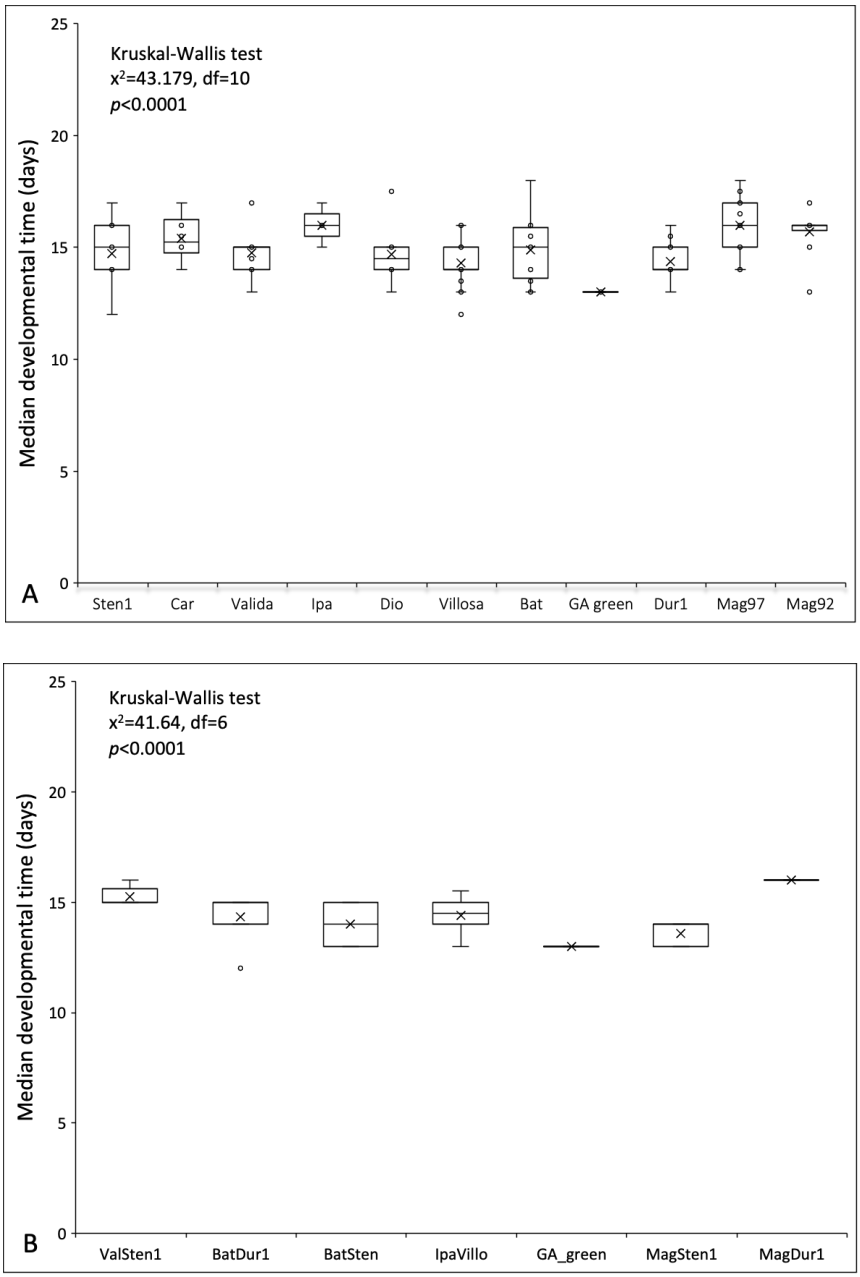

Figure 3.8. Median developmental time of *Franklinella fusca* on plants. The time required for thrips to complete one generation on leaflets of non-infected *Arachis* genotypes was recorded daily intervals. The 'horizontal line' and 'X' in the box are median and mean of median developmental time.

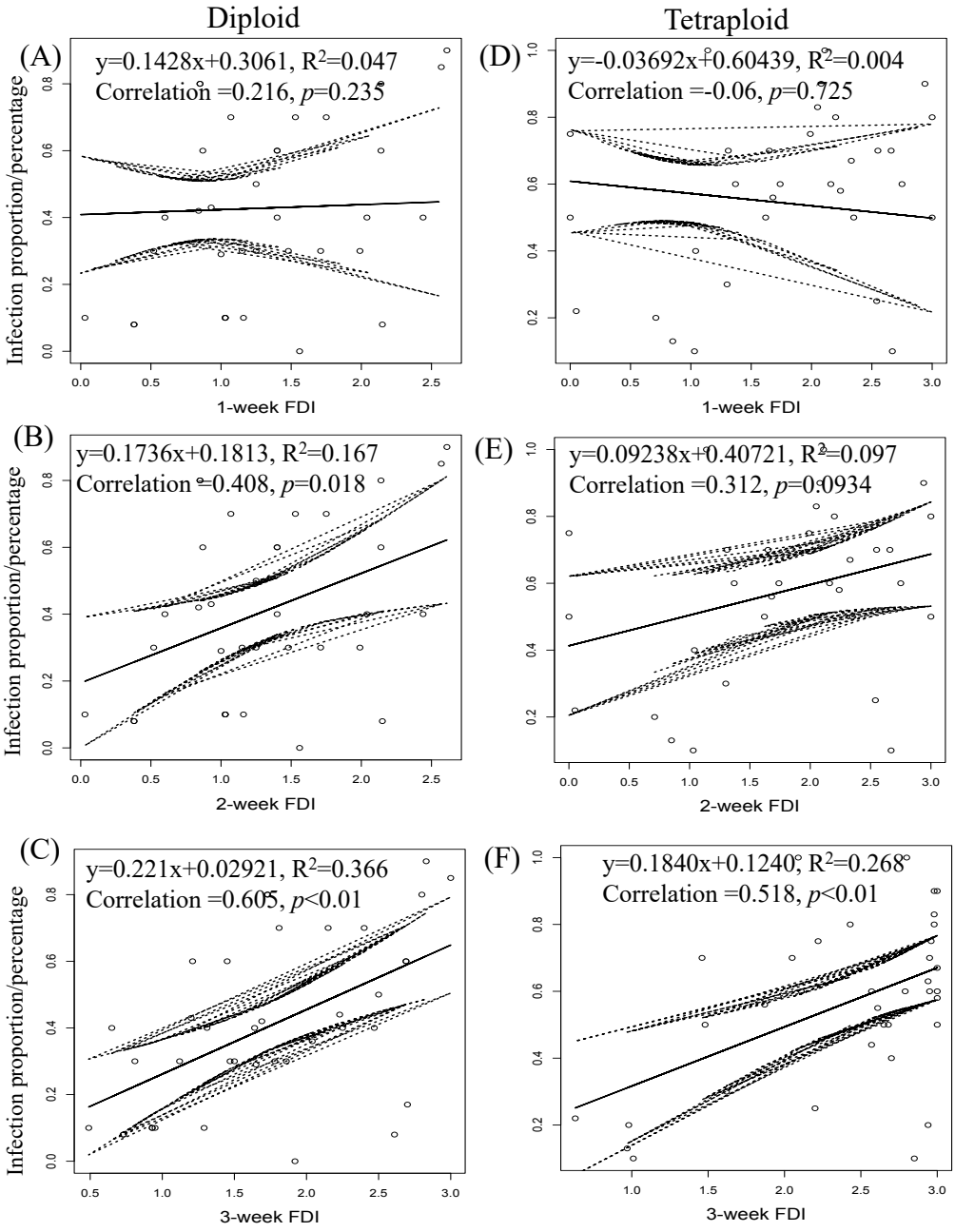

Figure 3.9. Correlation of final percent infection of tomato spotted wilt virus and thrips feeding injury by time. Feeding scars produced on each plant were recorded three times at one-week interval during the thrip-mediated inoculation. Pooled data from all experiment on wild diploids and tetraploid genotypes are presented. Equation of linear regression and R square are shown.

CHAPTER 4

TRANSCRIPTIONAL RESPONSES IN TWO DIPLOID SPECIES AND THEIR HYBRID IN RESPONSE TO TSWV INFECTION

[1] Y.-J. Chen, M.R. Abney, S. Bag, S. Leal-Bertioli, and R. Srinivasan. 2022. To be submitted to *Peanut Science*.

Abstract

Tomato spotted wilt virus (TSWV) transmitted by thrips causes significant yield loss in peanut (*Arachis hypogaea L.*) production. Use of peanut cultivars with moderate field resistance has been critical for TSWV management. However, current TSWV resistance is often not adequate, and the resistance pedigree is extremely narrow. Wild-species related allotetraploids obtained by crossing diploid species could help broaden the TSWV resistance base in peanut. Thrips-mediated TSWV screening identified two diploids and their allotetraploid possessing the AA, BB, and AABB genomes *A. stenosperma* V10309 (Sten), *A. valida* KG30011 (Val), and ValSten1 that conferred TSWV resistance. These genotypes had reduced TSWV infection and accumulation in comparison with others evaluated. Transcriptomes from TSWV-infected and non-infected samples from *A. stenosperma* V10309 (Sten), *A. valida* KG30011 (Val), and ValSten1 were assembled and differentially expressed genes (DEGs) following TSWV infection were assessed. There were 608, 10,396, and 1,475 significant DEGs in *A. stenosperma*, *A. valida*, and ValSten1, respectively. Higher proportion of genes were downregulated for *A. stenosperma* and ValSten1, whereas they were upregulated in *A. valida*. Higher numbers of defense-related genes in relation to abiotic/ biotic stress were differentially expressed in *A. valida* followed by ValSten1 and *A. stenosperma*. Overall, fewer defense-related gene categories were upregulated in two diploid species and their hybrid than in two cultivated genotypes. Plant phytohormone and photosynthesis genes also were more differentially expressed in *A. valida* followed by ValSten1 and *A. stenosperma*, with a slant towards downregulation. Taken together, response to TSWV infection seems to be more pronounced in the case of *A. valida* with the BB

genome than in the case of *A. stenosperma* with the AA genome. Response to TSWV infection in the hybrid ValSten1 with the AABB genome seems somewhat subdued in comparison with *A. valida*.

Introduction

Tomato spotted wilt orthotospovirus (TSWV) is transmitted by thrips in a persistent propagative manner (Ullman, 1993). TSWV infection in peanut causes the spotted wilt disease (SWD). SWD has been the major concern in peanut production in the Southeast United States for the past three decades (Culbreath & Srinivasan, 2011; Srinivasan et al., 2017). Successful breeding efforts have led to the release of numerous peanut cultivars with moderate field resistance to TSWV(Culbreath & Srinivasan, 2011; Janila et al., 2016). Peanut cultivars with moderate field resistance combined with other cultural practices have been instrumental in managing the SWD (Culbreath & Srinivasan, 2011; Srinivasan et al., 2017).

Field resistant peanut cultivars are not immune to the virus. They can be systemically infected with the virus and display TSWV characteristic symptoms upon infection (Srinivasan et al., 2017). The mechanism of field resistance to TSWV seems to be different in peanut than in other crops such as tomato and pepper, wherein resistance is governed by single dominant genes such as *Sw5* and *Tsw* (Hoffmann et al., 2001; Moury et al., 1997; Stevens et al., 1991). In contrast, in peanut, multiple quantitative trait loci (QTL) particularly in chromosome A01 have been associated with TSWV resistance (Agarwal et al., 2019; Tseng et al., 2016; Zhao et al., 2018). Unlike tomato and pepper wherein the selection pressure induced by TSWV has led to resistance breaking strains, no such resistance-breaking strains have been documented in peanut thus far (Lai et al., 2021; Sundaraj et al., 2014). Therefore, it is likely that TSWV resistance in

peanut is governed by multiple genes. TSWV incidence in these moderately field resistant cultivars is not robust and often dependent upon external factors such as vector and virus pressure. Peanut cultivars developed thus far with TSWV resistance are mostly from one peanut accession PI 203396 (Clevenger et al., 2018). Therefore, there is a critical need to breed for robust TSWV resistance from other durable sources.

Arachis genus is native to South America and contains 83 described species (Santana & Valls, 2015; Seijo et al., 2021; Valls & Simpson, 2005, 2017; Valls et al., 2013). Many diploid *Arachis* accessions from *A. cardenasii*, *A. correntina*, *A.diogoi*, *A. stenosperma*, and *A. villosa* have exhibited resistance to TSWV (Lyerly et al., 2002). For instance, *A. diogoi* GKP 10602 was identified as resistant to TSWV among 46 wild *Arachis* accessions (Lai, 2015; Milla et al., 2005; Stalker, 2017). Several QTLs linked to TSWV resistance have been mapped in wild diploid genotypes. Moretzsohn et al. (2013) found five markers for TSWV resistance from two A-genome wild species, *A. kuhlmannii* (VRGeSv 7639) and *A. diogoi* GKP 10602. In addition to TSWV, wild species also have been documented to confer resistance to its vector- thrips. Twelve diploid species were considered as potential sources to *F. fusca* resistance (Campbell & Waynne, 1980; Lyerly et al., 2002; Stalker & Campbell, 1983), and antibiosis-based resistance to thrips was also found in *A. diogoi* and its hybrid (*A. hypogaea* Gregory X *A. diogoi*) (Lai, 2015; Srinivasan et al., 2018).

The cultivated allotetraploid peanut was generated from the natural hybridization of two wild diploid species (2n=2x=20), and the subgenomes of *A. ipaensis* (BB-type genome) and *A. duranensis* (AA-type genome) can be used for identifying disease-resistant genes (Bertioli et al., 2016). Cultivated peanut is a self-pollinating crop with very low genetic variability (Moretzsohn et al., 2013) and contains four sets of chromosomes (2n=4x=40, AABB-type genome) (Husted,

1936). Consequently, resistance to TSWV and other pathogens is limited. On the contrary, several diploid wild species possess more resistance to TSWV and many other pathogens than cultivated peanut. However, transferring TSWV resistance across ploidy levels has been limiting due to hybrid incompatibility. Recent advancements have overcome such issues and have developed tetraploids from diploids by artificial hybridization (Leal-Bertioli et al., 2015; Simpson, 1991; Stalker, 2017). These allotetraploids are expanding the utilization of wild species in peanut breeding (Chu et al., 2021; Stalker, 2017).

In induced tetraploid genotypes, TSWV QTLs were located on A03 and B08 on ValSten1, B05, B10 on IpaCor, and A02, A05, A06 on IpaCor (Levinson, 2021). More wild related materials have been registered as TSWV resistant genotypes, such as ValSten1-GA-NC, IpaCor2-GA-NC, and IpaDur3-GA-NC (Chu et al., 2021). Despite the conferred resistance, it is not clear how the virus-host interactions would differ from the cultivated peanut. Next-generation sequencing (NGS) and transcriptome analysis have provided insights on virus-host interactions in TSWV susceptible and resistant cultivars (Catto et al., 2021). Defense responses in general were upregulated following TSWV infection, and more so in the case of TSWV-resistant cultivar than in the susceptible cultivar (Catto et al., 2021). It is not clear if the TSWV-induced responses in cultivated peanuts would differ from that of diploid species and/or their hybrids. Screening of diploids and their hybrids in the greenhouse led to the identification of some genotypes with TSWV and/or thrips resistance (chapter 3 in this dissertation). Two diploid species viz., *A. stenosperma, A. valida*, and their hybrid displayed TSWV and/or thrips resistance. The goal of this study was to develop transcriptomes using a NGS platform, examine differential gene expression following TSWV inoculation, and quantitatively validate DEGs.

Materials and Methods

Maintenance of A*rachis species* plants. Two diploid species and their allotetraploid hybrid, namely *Arachis valida* GK 30011 (PI468154; Val), *A. stenosperma* V10309 (PI666100; Sten), and ValSten1 (*A. valida* KG30011x *A. stenosperma* V10309 (PI695393) were used in this study (Chu et al., 2021; Gao et al., 2021). *Arachis valida* is a diploid species with the A-genome; *A. stenosperma* is a diploid species with the B-genome; and induced allotetraploid ValSten1 was a cross between *A. valid*a x *A. stenosperma* with AB-genome. Seeds of these genotypes were treated with two to three ml of a 0.5% solution of Florel® Growth Regulator (Monterey Lawn and Garden, Fresno, California, USA) and incubated in a petri dish at 28°C for 18-24h to break seed dormancy. Seeds were sown in individual 4" pots with commercial potting mix Promix (Premier Horticulture Inc, Quakertown, PA, USA). The plants were kept in thrips-proof cages (47.5 cm^3) (Megaview Science, Taichung, Taiwan) at 25-30°C, 80-90% RH, and a photoperiod of L14: D10 in the greenhouse. Seeds of the cultivar "Georgia Green" were pre-geminated in moistened paper towel and incubated in a growth chamber kept at 28°C for two to three days and used for thrips maintenance. One-to-two-week-old seedlings with one-to-two nodes and up to 16 leaflets of each genotype were used for TSWV transmission.

Thrips maintenance. Non-viruliferous thrips and viruliferous thrips were maintained. Non-viruliferous thrips were maintained on leaflets of non-infected plants (cv. Georgia Green) within Petri dishes stuffed with a wet cotton round. Colonies were maintained by successive releases of ten adult female thrips, allowed to oviposit for 48h on a peanut leaflet dusted with a trace of pine pollen, and placed in growth chambers at 28-30°C and a photoperiod of L14: D10. Fresh leaflets and water were added to the Petri plates three times a week until emergence of the

F1 generation. Viruliferous thrips (TSWV) colony was maintained similarly on TSWV-infected leaflets collected from the field in a separate growth camber as described in Shrestha et al. (2013). During the off-season, viruliferous thrips were maintained on TSWV-infected leaflets generated by mechanical inoculation in the greenhouse (Marasigan 2014; Shrestha et al., 2015).

Thrips-mediated inoculation of diploids and their hybrid. *Frankliniella fusca*-mediated inoculation was conducted as per the established protocol (Shrestha et al., 2015). The experiment included two treatments: mock inoculation via non-viruliferous *F. fusca* thrips, and TSWV inoculation via viruliferous thrips. Inoculated plants were maintained in thrips-proof cages (47.5 cm^3) in the growth chamber at 27°C and ~80% humidity (Conviron, Pembina, ND, USA). After two weeks, the first fully expanded leaf of inoculated peanuts (ca 0.03 g) was tested by RT-qPCR followed as per the methods described by Shrestha et al. (2015) to assess TSWV-infection status or lack thereof.

Sample preparation, total RNA extraction, and quality control. Samples from plants two-to-three weeks post-inoculation were used. Five replications for each genotype were used. Leaflets were collected from the first fully expanded leaf below the terminal of each plant for RNA extraction. Total RNA of plants was extracted by RNeasy plant mini kit following the manufacturer's protocol (Qiagen, Valencia, CA, UGA). For each replicate, a leaf sample was from an individual plant. Thus, a total of 30 RNA samples were prepared for sequencing (three genotypes X two infection status X five replications) and were stored at -80°C before shipping. Prior to library preparation, each sample integrity (RNA integrity number, RIN) was measured by using Aglient 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) for RNA quality control (QC). Two samples failed the QC test; therefore 28 samples were used for library preparation and sequencing.

Library preparation and sequencing. The complementary DNA (cDNA) synthesis, cDNA libraries (messenger RNA library), and sequencing were undertaken by Novogene Corporation Inc. (Sacramento, CA, USA). Illumina sequencing libraries were constructed using TruSeq RNA sample preparation kits. Briefly, mRNA was selected, fragmented, and first-strand cDNA was synthesized using random primers and reverse transcriptase. Subsequently, Polymerase I and RNase H were used to make the second-strand cDNA. An Illumina TruSeqLT adapter was ligated to the DNA fragments, and PCR amplification was performed for a minimal number of cycles with standard Illumina primers to produce the final cDNA libraries. Twenty-eight libraries were constructed using two lanes in the Illumina NovaSeq 6000 platform (pair-end 150 cycle sequencing setting, > 6G raw data per sample).

(1) Raw read processing for transcript abundance. In advance of the *A. valida*, *A. stenosperma*, and ValSten1 transcriptome assemblies, FastQC v0.11.9 and multiQC v1.11 were used to check the quality of raw reads before and after trimming (Andrew et al., 2010; Magnusson et al., 2016). Trimmomatic v0.39 software was used with the default setting to remove adapters (Bolger et al., 2014). Also, Sortmerna v4.3.3 software was used with the SILVA database to remove rRNA contamination (Kopylova et al., 2012). The rRNA decontaminated trimmed reads were converted from interleaved to paired files using BBMap v38.93 software for configuring files and for transcriptome assembly (Fu et al., 2012; Slater & Birney, 2005; Wu et al., 2016).

(2) Transcriptome assembly. The r Ribosomal RNA (rRNA) decontaminated trimmed reads from *A. valida*, *A. stenosperma*, and ValSten1(*A. valida* x *A. stenosperma*) were used to generate respective *de novo* assemblies using Trinity v2.10.0 software with the default parameters (Grabherr et al., 2011). The sra2genes v4 software was used to clean up the

assemblies using prior evidence from closely related species to address the possibility of over assembly of the transcriptome (Figure 4.2). Sra2genes is a complete pipeline to reconstruct genes from RNA data sources, and it includes several tools such as CD-HIT v4.8.1, Exonerate v2.4.0, Blast+ 2.10.1, and GMAP-GSNAP (Fu et al., 2012; Slater & Birney, 2005; Wu et al., 2016). CD-HIT v4.8.1 was used for the removal of potentially chimeric or misassembled transcripts from the input reads. Exonerate v2.4.0 was involved in the removal of all duplicated sequences. Blast+ 2.10.1 was used to separate the transcripts as various isoforms. GMAP-GSNAP was used to align the reads to the assemblies. BUSCO v4.0.6 was used to determine assembly completeness before and after cleaning of the *de novo* assemblies against the Insecta odb10 lineage (Simão et al., 2015).

(3) Mapping of reads and differential expression profile. Trimmed reads were mapped to the respective transcriptome assemblies using Bowtie2 v2.4.1 with default mapping parameters (Langmead & Salzberg, 2012). Gene count estimates were derived from the mapped read files using RSEM v1.3.3 for each of the transcriptomes (Li & Dewey, 2011). Custom R script was used to determine the FPKM across all samples on R v4.1.0 using the following R libraries: dplyr, tidyverse, and stringr. DESeq2 was used to measure differentially expressed genes by comparing the gene counts from non-infected samples to virus-infected samples where genes that had a false discovery rate (FDR) ≤ 0.05 and a $\log_2$ fold change $\geq |\pm 4|$ were classified as being significantly differentially expressed (Love et al., 2014).

(4) Functional Annotation. The *de novo* assemblies for *A. valida*, *A. stenosperma*, and ValSten1 were compared against a subset of the NCBI databases for non-redundant proteins (NR) and RefSeq genes were filtered for *Arachis* using the default of e^{-3} in BLASTx using OmicsBox (Camacho et al., 2009; Omicbox, 2019). The OmicsBox tool also performed

Blast2GO and GO mapping to assign functional annotations to the genes within each assembly (Conesa et al., 2005; Götz et al., 2008; Mi et al., 2019). Additional annotations were performed using InterProScan and the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Jones et al., 2014; Kanehisa & Goto, 2000). The GO term distributions were visualized using the WEGO 2.0 web tool (Ye et al., 2018).

Validation of RNA sequence using qPCR. Quantitative reverse transcription-polymerase chain reaction (RT-qPCR) was utilized to validate *Arachis* species transcripts following TSWV infection. Three sequences from each genotype with the log2fold change value of > 4 and p-value of < 0.05 were randomly selected. The sequences were extracted with the tool "seqtk". RT-qPCR was performed on plant samples obtained from four biological repeats from the remaining samples. Primers for targeted DEGs were designed by NCBI primer design (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer sequences are listed in Table 4.1.

The cDNA was synthesized by a Go-Script reverse transcription system (Promega Corporation, Madison, WI) following the manufacturer's protocol and then diluted 20-fold for quantitative polymerase chain reaction (qPCR). The reaction mix for qPCR included 2x GoTaq qPCR Master Mix, 1 µl of sequence-specific primers (final concentration of 250 mM), 2 µl cDNA of sample, and nuclease-free water for a final reaction volume of 20 µl. The reaction was run at 95°C for 2 min, followed by 40 cycles at 95°C for 15s, 58°C for 20s, and 72°C for 30s. The reaction was extended with a melting curve in a QuantStudio 3 System (applied biosystems by Thermo Fisher Scientific) to rule out non-specific binding. Two repeats for targeted transcripts and the reference gene (alcohol dehydrogenase class III), and water control were included in each RT-qPCR run. The log2fold change of each target transcript in infected plants against mock-inoculated plants was calculated after normalization to the reference gene, which

belonged to alcohol dehydrogenase class III. The log2 transformed (ratio of infected samples/ratio of non-infected samples) expression of target genes (transcripts) was correlated with Pearson's correlation using the function "cor" in software R.

Results

(1) Illumina sequencing and reads assembly. Total raw reads obtained from infected plants and non-infected plants of *A. valida* (GK 30011), *A. stenosperma* V10309 (PI666100), and ValSten1 (*A. valida* KG30011x *A. stenosperma* V10309) were assembled *de novo* using Trinity platform. Total raw reads generated from the three genotypes were 222, 234, and 253 million pair reads, respectively, which after trimming amounted to 218, 231, and 250 million pair reads, respectively. The percentage of reads mapped to the *de novo* assembled transcriptome for *A. stenosperma*, *A. valida*, and ValSten1 genotypes were 86%, 87%, and 80%, respectively (Table 4.1). These reads were assembled into 141,144 (*A. valida*), 106,374 (*A. stenosperma*), and 137,039 (ValSten1) contigs. The complete assembly obtained from assembled contigs of *A. stenosperma* was 4,571, which included 2,571 (48%) single-copy and 2000 (37%) duplicated orthologs. Similarly, for *A. valida* 4,724 complete assemblies were generated, which included 2,545 (47%) single-copy and 2,179 (41%) duplicated orthologs. For ValSten1, 4,670 complete assemblies were generated, which included 2,209 (41%) single-copy and 2,461 (46%) duplicated orthologs. The assembly parameters for the three genotypes are summarized in Table 4.1. One infected sample of *A. stenosperma* showed low RIN (RNA integrity number) and one non-infected sample of *A. valida* that showed unsmooth baseline at QC were not processed for libraries. The reads were from 28 samples (libraries).

(2) Quantitation of differential expression analysis profile. The reads obtained from infected and non-infected samples from the three genotypes were normalized and clustered using fragment per kilobase of transcript per million mapped reads (FPKM) and principal component analysis (PCA) for comparison. The PCA clustered TSWV infected samples of the three genotypes separately from the non-infected ones (Fig. 4.3). However, one sample (asten_paired_V3B) in *A. stenosperma* was removed due to the unexpected clustering in PCA, although it did not have a reduced FPKM value. Differentially expressed genes (DEGs) observed for *A. stenosperma*, *A. valida*, and ValSten1 in response to TSWV were 608 (184 upregulated and 424 downregulated; Fig. 4.4), 10,396 (7,319 upregulated and 3,077 downregulated; Fig. 4.5), and 1,475 (756 upregulated and 719 downregulated; Fig. 4.6), respectively. TSWV-infected samples of *A. valida* had higher DEGs (10,396) compared to ValSten1 (1,475) or *A. stenosperma* (608). Higher percentage of DEGs for *A. stenosperma* were downregulated, whereas more upregulated genes were expressed in *A. valida*. Similar numbers of down-regulated genes and up-regulated genes were expressed in ValSten1.

(3) Functional annotation of genes. DEGs observed in the wild species in response to TSWV infection were functionally annotated. There were 107,043 genes , 149,877 genes, and 138,389 genes (non-significant and significant genes) included in the analysis to check the DEGs against the background for significant enrichment of *A. valida*, *A. stenosperma,* and ValSten1, respectively.

Gene ontology (GO) provides context for the functionality of genes and is broken into three major classes: biological process, cellular component, and molecular function. Within each category, the levels 2 and 3 were chosen as the focus as these levels provide broader context for gene functionality. Significantly enriched GO terms were determined by the WEGO tool (Ye et

al., 2018) by comparing the GO terms distribution from DEGs to that of the background genes of the entire transcriptome. GO terms within the biological processes category provide biological relevance by attributing biological objectives to gene products. Within the GO term distribution of *A. stenosperma*, DEGs were found to have significant ($p<0.05$) enrichment against the background for the biosynthetic process category of biological processes (Fig .4.8).

In *A. valida*, DEGs were involved in 22 categories including (1) macromolecule localization, (2) cellular localization, (3) biological regulation, (4) regulation of biological process,(5) regulation of molecular function,(6) metabolic process, (7) methylation, (8) regulation of metabolic process, (9) biosynthesis process, (10) small molecular metabolic process,(11) oxidation-reduction process, (12) regulation of the cellular process, (13) cell cycle, (14) cellular component organization, (15) cell wall organization, (16) response to stimulus, (17) response to chemical, (18) response to stress, (19) response to endogenous stimulus, (20) development process, (21) anatomical structure development, and (22) cellular component organization or biogenesis (Fig .4.9).

In ValSten1, DEGs were involved in 20 categories including (1) biological regulation, (2) regulation of biological quality, (3) regulation of the biological process, (4) regulation of molecular function, (5) metabolic process, (6) organic substance metabolic process,(7) primary metabolic process, (8) cellular metabolic process, (9) methylation, (10) regulation of the metabolic process, (11) biosynthesis process, (12) small molecule metabolic process, (13) cellular process, (14) regulation of cellular process, (15) cell communication, (16) protein folding, (17) cell wall organization or biogenesis, (18) response to stimulus, (19) response to chemical, (20) response to endogenous stimulus (Fig .4.10).

(5) Comparison of DEGs between genotypes. To determine the transcriptional changes in each genotype related to TSWV infection, the number, and the list of DEG between *A. stenosperma*, *A. valida*, and ValSten1 were compared. A core set of 126 DEGs was shared by three genotypes, 701 DEGs were commonly shared by any two genotypes such as *A. stenosperma* vs. *A. valida* (158), *A. valida* vs. ValSten1 (519), and ValSten1 vs. Sten (24); remaining 1,095 DEGs were specific to *A. stenosperma* (13), *A. valida* (1,057), and ValSten1 (25) (Fig .4.11).

The DEGs from *A. stenosperma* (113), *A. valida* (1,074), and ValSten1 (243) were grouped into three major categories viz., defense, phytohormone, and photosynthetic pathways in response to TSWV infection, respectively (Table 4.2). The categories were chosen based on the study with resistant and susceptible cultivated peanuts (Catto et al., 2021). Upregulation of defense-related DEGs of the 20 examined categories was higher in *A. valida.* The percentage (No. of upregulated DEGs) in *A. stenosperma, A. valida,* and ValSten1 were 34% (25), 64% (490), and 55% (69). Less than 15% of 20 examined categories (2/20) in *A. stenosperma* were upregulated genes that belong to heat shock protein and leucine zipper (Table 4.3). In addition, examples of DEGs downregulated in *A. stenosperma* and ValSten1 include the P450, WRKY transcription factor, leucine-rich repeats (LRR), serine/threonine-protein kinase, and TMV resistance protein N.

A similar pattern was observed in the case of phytohormone-related DEGs. Upregulation of phytohormone-related DEGs of the 8 examined categories was higher in *A. valida*. The percentages (No. of upregulated DEGs) in *A. stenosperma, A. valida,* and ValSten1 were 9% (1), 51% (100), and 36% (16), respectively (Table 4.2). Downregulated phytohormone-related genes were higher in *A. stenosperma* in 50% (4/8) (Table 4.3). Three categories (3/8) in ValSten1 and

A. valida have more downregulated DEGs. Examples of DEGs related to auxin were downregulated in wild *Arachis*.

Regarding photosynthesis-related DEGs, upregulation of phytosynthesis-related DEGs of the four examined categories were higher in *A. valida*. The percentages (No. of upregulated DEGs) in *A. stenosperma, A. valida,* and ValSten1 were 10% (3), 46% (249), and 38% (28). DEGs pertaining to NADP-dependent malic enzymes were upregulated in *A. valida* and ValSten1 (Table 4.2).

(6) Validation of RNA-sequencing. Three DEGs from each genotype were randomly selected and their expression were validated using RT-qPCR (Fig. 4.12). The log2fold change of expression in RNA-seq and qRT-PCR indicated good correlation (cor= 0.87, t=4.6, df=7, p=0.002.). expand this section

Discussion

Peanut diploid genotypes *A. stenosperma* and *A. valida* and their hybrid had reduced TSWV infection and accumulation than other diploids and tetraploids evaluated in a study following thrips-mediated inoculation (Chapter 3). For gainig insights on host-virus interactions in TSWV-resistant genotypes at the transcript level, the molecular response of wild related peanut after systemic infection at two-to-three weeks post-inoculation were examined. TSWV infection symptoms in these wild genotypes did not vary from other genotypes and that of the cultivated peanut. However, the molecular responses of wild genotypes to TSWV infection showed interspecific interactions compared with peanut cultivars, and the results identified the candiate genes/contigs associtated with TSWV infection. In this study overall gene expression was substantially higher in *A. valida* than in the case of *A. stenosperma* and their tetraploid

hybrid. Numerous genes pertaining to defense against biotic stress including pathogens were upregulated in *A. valida* with the B-genome following TSWV infection. In contrast, previous studies showed that A-genome was diverse and harbored more loci on QTLs than the B-genome (Bertioli et al., 2016; Pandey et al., 2017). In addition to defense related genes, genes associated with plant physiology such as phytohormones and photosynthesis were examined. Based on de *de novo* assemblies, differential expression pattern was not similar in all three genotypes evaluated following TSVW infection. Also, the DEGs in the wild species and their inudced hybrid did not mirror the pattern observed with TSWV-resistant cultivar in an earlier study (Catto et al., 2021). In that study, most defensed related genes were up-regulated than susceptible tetraploid cultivated peanut varieties (Catto et al. 2021).

Gene ontology (GO) terms obtained following enrichment analysis for functional annotation were divided into three categories, which were cellular components, molecular functions, and biological processes. More GO terms with DEGs were found in tetraploid (ValSten1) than in diploids. However, a higher number of DEGs was found in the B-genome (*A. valida*) than ValSten1 and the A-genome (*A. stenosperma*). Defense category genes were further examined in greater detail. A greater proportion of contigs associated with defense such as heat shock protein, lectins, and leucine zipper were upregulated in *A. valida* followed by ValSten1 and *A. stenosperma*. Heat shock protein was associated with virus infection in *Arabidopsis thaliana* (Roux & Bergelson, 2016). Lectins are known to upregulate plant defenses by facilitating recognition of plant pathogens (Fliegmann et al., 2004). Other proteins involved in plant immunity such as leucine rich repeats (LRR) are known to provide defense against a range of pathogens including viruses (Mishra et al., 2019; Noman et al., 2017). In fact, LRRs genes were upregulated in the TSWV resistant and susceptible cultivars following TSWV infection

(Catto et al. 2021). LRR genes and a greater proportion of them were upregulated in *A. valida* than in *A. stenosperma* and in their hybrid in this study. These results provide some mechanistic reasons for the observed response against TSWV in *A. valida*. It was surprising that *A. valida* had more defense-related DEGs than *A. stenosperma,* which thus far has been the most resistant diploid. It implied that the resistance to TSWV in wild related peanut may have interspecific differences and unkown resistance factors were excluded in the examined catagories. The pathogen resistant genes in wild peanut can be diverse and uinique (Yin at al., 2020; Stalker, 2017). Also, the time for severe TSWV symtom expression was not recorded in the study, but *A. stenosperma* showed TSWV-specific symptoms later than *A. valida* and ValSten during the test. The time-series DEGs profile will be benefial for understanging changing pattern of gene expression to TSWV.

Several other defense proteins also were differentially expressed. Hypersensitive response inducing genes such as nucleocapsid N gene from tobacco, *Nicotiana glutinosa* L., that imparts resistance to several tobamoviruses including the tobacco mosaic virus (TMV) as well as the disease resistance (R) proteins were downregulated in three resistant wild genotypes in this study, but they were upregulated in two cultivated genotypes in a previous study (Catto et al. 2021). In tomato, the disease- resistant R gene *Mi* conferred resistance against nematodes and potato aphids (Rossi et al., 1998). Within the three wild genotypes evaluated in this study, a greater proportion of hypersensitive response (HR) associated genes were upregulated in *A. valida* than in the others. However, HR can be upcoupled with resistance and may vary depending on species in some cases (Balint-Kurti, 2019). Generally, R protein in plants recognizes the effectors in pathogens and are known to trigger a defense response . WRKY transcription factors also are involved in triggering immunity against a range of pathogens

including viruses by recognizing pathogen associated molecular patterns (PAMPs) (Pandey & Somssich, 2009). WRKY was upregulated in tomato genotype with resistance to TSWV (Catoni et al., 2009). Similarly, WRKY contigs were substantially upregulated in *A. valida* and slightly in the hybrid. More upregulation of pathogen-associated immunity/hypersensitive response genes in *A. valida* again reiterates a robust defense system in *A. valida* than the others.

The expression of a suite of other defense genes such as calcium-modulated calmodulin, stilbene synthase, and serine carboxypeptidases were upregulated substantially in the case of *A. valida* followed by the hybrid, and *A. stenosperma*. These genes have been documented to mediate resistance against a wide array of pathogens including viruses (Catto et al., 2021; Fraser et al., 2005; Hong et al., 2017; Takabatake et al., 2007; Yu et al., 2005). The DEGs pattern seems to be consistent, wherein defense genes upregulation in *A. valida* was almost always higher than in the hybrid and least in the other diploid, *A. stenosperma*. In addition, induced response related genes such as those associated with RNA interference and salicylic acid were upregulated in a similar pattern in *A. valida* followed by the hybrid and *A. stenosperma*. Despite this reoccurring pattern of upregulation of defense related genes in *A. valida* and its hybrid, overall, comparison of functional annotation in defense-related DEGs between cultivated peanuts (AB) and wild peanuts (A-, B-, and AB-genome) showed that genes in many categories were downregulated in wild species than in the case of cultivated peanuts (Table 4.3). The host phenotype alteration in the wild species and their hybrid in comparison with the tetraploid cultivars following TSWV infection was not as severe. This could have resulted in less physiological perturbances in the wild diploid species and their hybrid than the cultivated tetraploids.

In addition to differential expression of defense related genes, other genes such as phytohormones and photosynthesis related genes also were differentially expressed. Altogether,

more than half-a-dozen phytohormones were differentially expressed, trending towards downregulation in *A. stenosperma* and the hybrid ValSten1. Phytohormones were slightly upregulated in the case of *A. valida* . Phytohormones can induce systemic resistance and inhibit infection of viruses such as TSWV (Zhao et al., 2020). In another study, auxin-related pathway in pepper induced hypersensitive resistance against thrips-borne virus (Zhao et al., 2022). Results in this study showed that genes related to abscisic acid (ABA) and auxin were downregulated in wild peanuts. Although ABA plays a role against bacterial and fungal diseases (Alazem & Lin, 2017), the virus infection did not upregulate ABA in some incompatible interactions (Baetz & Martinoia, 2014; Kovač et al., 2009). For example, infection by potato virus Y (PVY) of the resistant potato cultivar did not induce ABA (Kazan & Manners, 2009). PVY, like TSWV, is also non-tissue specific. Results in this study are in contrast with the tetraploid cultivars examined in another study, wherein phytohormone related genes were upregulated (Catto et al. 2021). The upregulation was more prominent in the TSWV-resistant cultivar, Tifguard than in the susceptible cultivar (Catto et al. 2021).

Chloroplast and photosynthesis related genes also were downregulated overall in both diploids and their hybrid, with the downregulation being more prominent in *A. stenosperma* followed by the hybrid and ValSten1. These results were consistent with the other study, in which photosynthesis related genes were downregulated in both TSWV and susceptible genotypes, with the downregulation being substantial in the case of the TSWV-susceptible cultivar, SunOleic 97R (Catto et al. 2021). Similarly, in the current study, the downregulation of photosynthesis related genes was less substantial in the case of the *A. valida* followed by the hybrid and *A. stenosperma*. Overall, this study reveals gene expression dynamics between *A. valida, A. stenosperma,* and ValSten1 in response to TSWV. Most of them were down-regulated

in *A. stenosperma* and up-regulated in *A. valida* after TSWV infection. 126 DEGs were shared and further functional studies of those genes may provide the clues on the mechanism of TSWV resistance.

Conclusion

TSWV resistance base in cultivated peanut is narrow. TSWV resistance in wild diploid species and their hybrids may play a pivotal role in broadening the resistance base. Transcriptomes developed in this study with two wild diploid species and their hybrid provide insights into virus-host interactions. The differential gene expression analyses indicated that defense related genes were upregulated in the diploid species with the B genome as opposed to the species with the A genome. Results in this study suggest the resistance in certain wild species could be tapped to enhance TSWV resistance. The challenge remains in choosing appropriate diploid species and retaining higher levels of TSWV resistance in their tetraploid hybrids.

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Figure 4.1. TSWV-resistant *Arachis* species: (A) *A. stenosperma* V10309 (B) *A. valida* KG30011, and (C) the allotetraploid hybrids ValSten1. Left photograph represents a non-infected leaf, middle photograph represents a TSWV-infected leaf, and right represents the whole plant after two weeks of thrips-mediated inoculation including infected and non-infected plants.

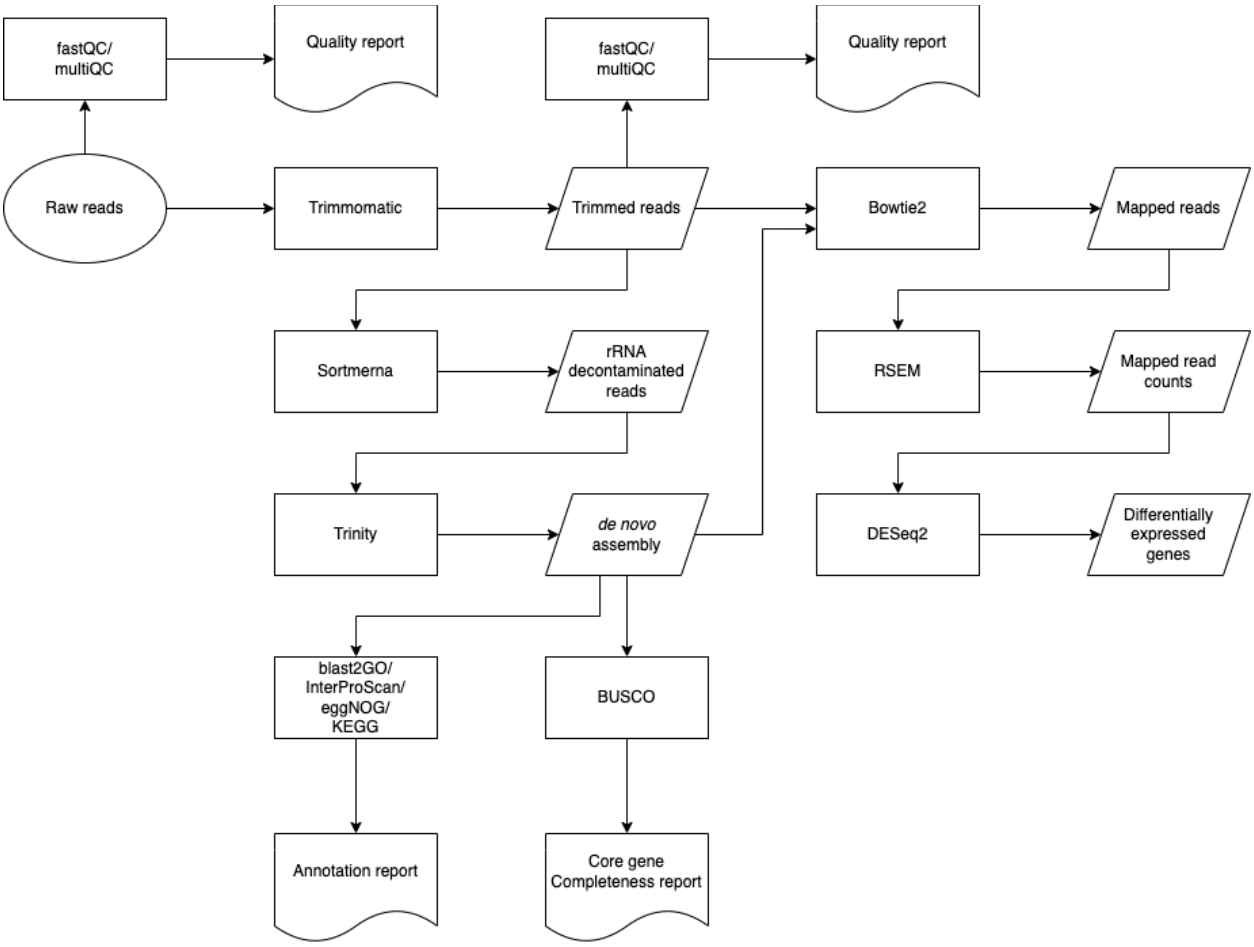
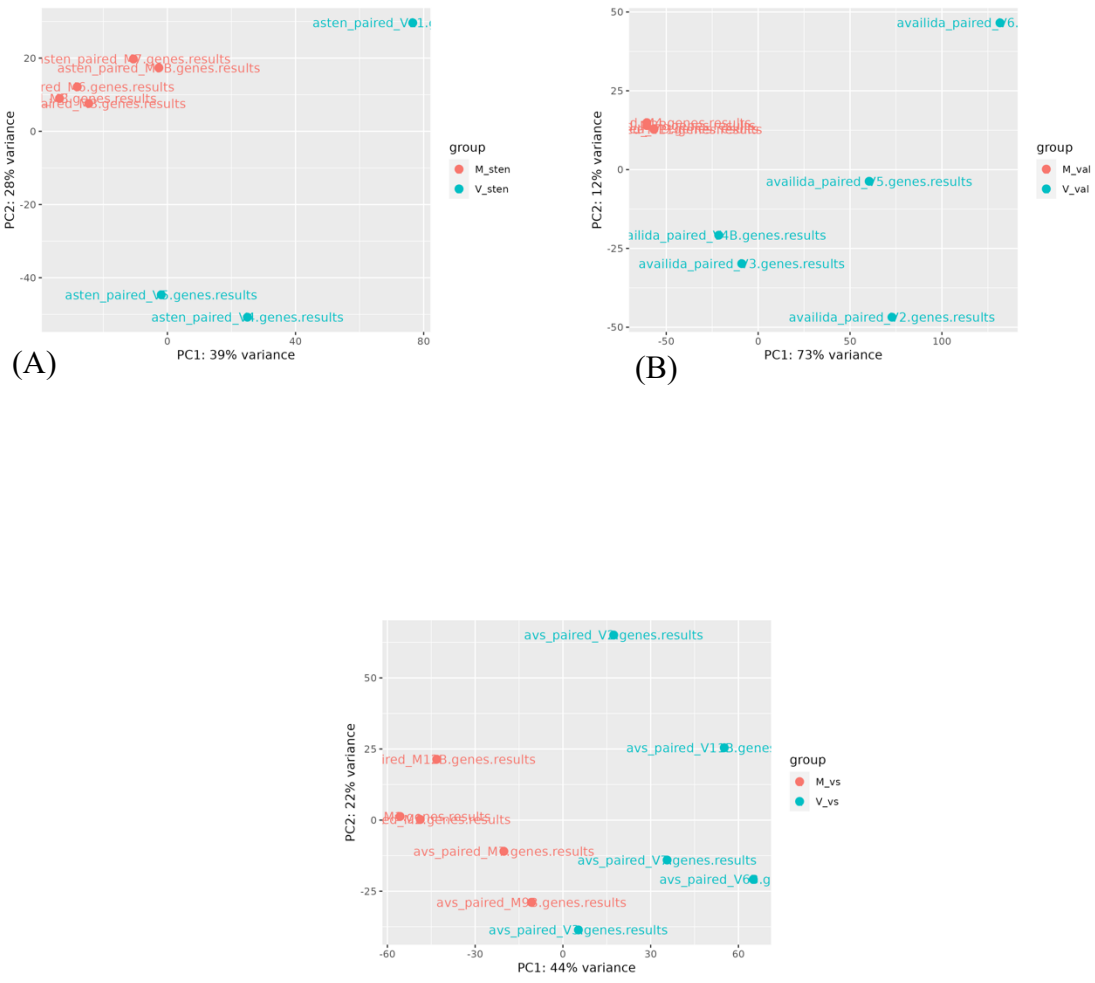


Figure 4.2. Pipeline for raw read processing, transcriptome assembly, mapping, and differential expression. Tools within the pipeline were applied to each of the species of interest: *A. valida* KG30011, *A. stenosperma* V10309, and ValSten1 (*A. valida X A. stenosperma*).

Table 4. 1. Summary of the RNA-seq dataset generated from *Arachis stenosperma*, *Arachis valida*, and ValSten mock-inoculated and TSWV-infected TSWV plants

Sample ID	Assmbly ID	Number of Input Reads	Number of Uniquely Mapped Reads	Number of Multiply Mapped Reads	Total Percentage Mapped to Assembly
Sten _Mock_rep1	asten_paired_M3	29,368,534	9,988,009	15,697,451	87%
Sten _Mock_rep2	asten_paired_M6	19,140,614	6,529,068	9,439,229	83%
Sten _Mock_rep3	asten_paired_M7	20,035,941	6,908,722	9,848,170	84%
Sten _Mock_rep4	asten_paired_M8	19,636,769	6,572,595	10,378,289	86%
Sten _Mock_rep5	asten_paired_M9B	13,343,007	4,358,975	6,828,647	84%
Sten _TSWV_rep1	asten_paired_V11	20,645,348	6,879,846	11,002,955	87%
Sten _TSWV_rep2	asten_paired_V3B	25,261,650	9,031,296	13,139,371	88%
Sten _TSWV_rep3	asten_paired_V4	21,704,792	7,995,981	10,894,302	87%
Sten _TSWV_rep4	asten_paired_V5	26,158,639	9,325,634	13,595,665	88%
Valida _Mock_rep1	availida_paired_M11	25,039,985	8,035,875	13,637,503	87%
Valida _Mock_rep2	availida_paired_M3B	23,990,954	8,010,782	13,041,759	88%
Valida _Mock_rep3	availida_paired_M4	28,464,507	8,893,077	16,095,196	88%
Valida _Mock_rep4	availida_paired_M9	24,543,724	7,970,176	13,614,611	88%
Valida _TSWV_rep1	availida_paired_V2	22,322,834	8,431,916	10,642,835	85%
Valida _TSWV_rep2	availida_paired_V3	22,577,536	7,675,313	12,046,347	87%
Valida _TSWV_rep3	availida_paired_V4B	21,781,422	7,255,017	11,934,059	88%
Valida _TSWV_rep4	availida_paired_V5	26,397,622	9,369,474	14,042,895	89%
Valida _TSWV_rep5	availida_paired_V6	26,567,169	9,513,309	13,498,361	87%
ValSten_Mock_rep1	avs_paired_M1	34,029,958	9,465,683	18,113,337	81%
ValSten_Mock_rep2	avs_paired_M12B	23,513,720	6,846,594	12,241,433	81%
ValSten_Mock_rep3	avs_paired_M2	30,896,630	8,387,837	16,486,540	81%
ValSten_Mock_rep4	avs_paired_M7	22,442,379	6,438,906	11,587,985	80%
ValSten_Mock_rep5	avs_paired_M9B	19,626,010	5,594,556	10,396,012	81%
ValSten_TSWV_rep1	avs_paired_V11B	21,492,612	5,948,362	10,671,202	77%
ValSten_TSWV_rep2	avs_paired_V2	24,191,325	7,259,928	12,448,410	81%
ValSten_TSWV_rep3	avs_paired_V3	19,856,626	5,364,146	10,729,067	81%
ValSten_TSWV_rep4	avs_paired_V6B	20,691,308	6,476,137	10,001,928	80%
ValSten_TSWV_rep4	avs_paired_V7	21,536,150	6,578,758	10,786,593	81%



(A) (B)

(C)

Figure 4. 3. Principal component analysis of three genotypes. (A) *A. stenosperma* V10309, (B) *A. valida* KG30011, and (C) ValSten1 clustered together according to being either Mock-inoculated (M, in red color) or TSWV-infected (V, in blue color).

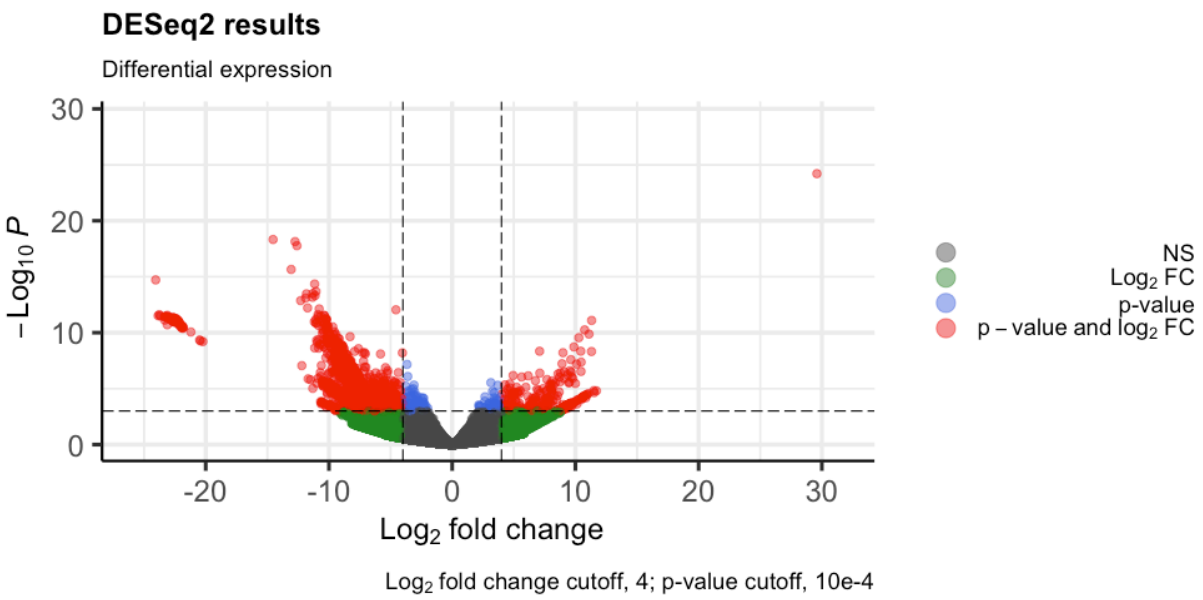


Figure 4.4. Volcano plot detailing the differential expression profiles of TSWV-infected versus non-infected samples of *Arachis stenosperma* V10309 (PI666100). All transcripts with a p-value $\geq 10 \times 10^{-4}$ and $\log_2$ fold-change ≥ 4 are highlighted in red (608 genes).

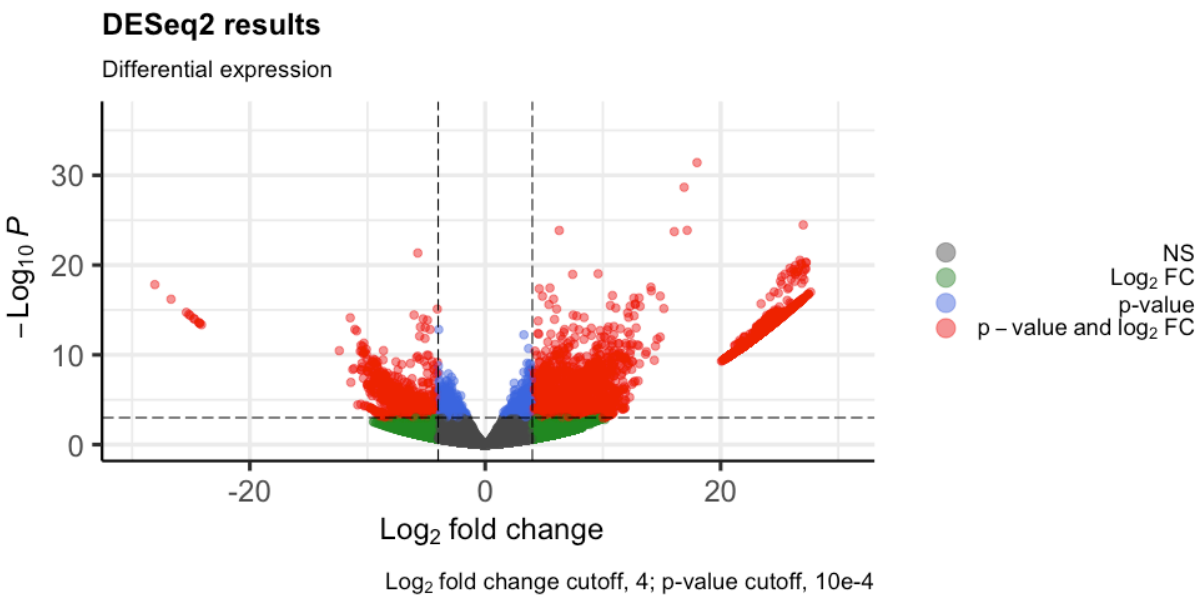


Figure 4.5. Volcano plot detailing the differential expression profiles of TSWV-infected versus non-infected samples of *Arachis valida* GK 30011. All transcripts with a p-value ≥ of 10×10^{-4} and $\log_2$ fold-change ≥ 4 are highlighted in red (10,396 genes).

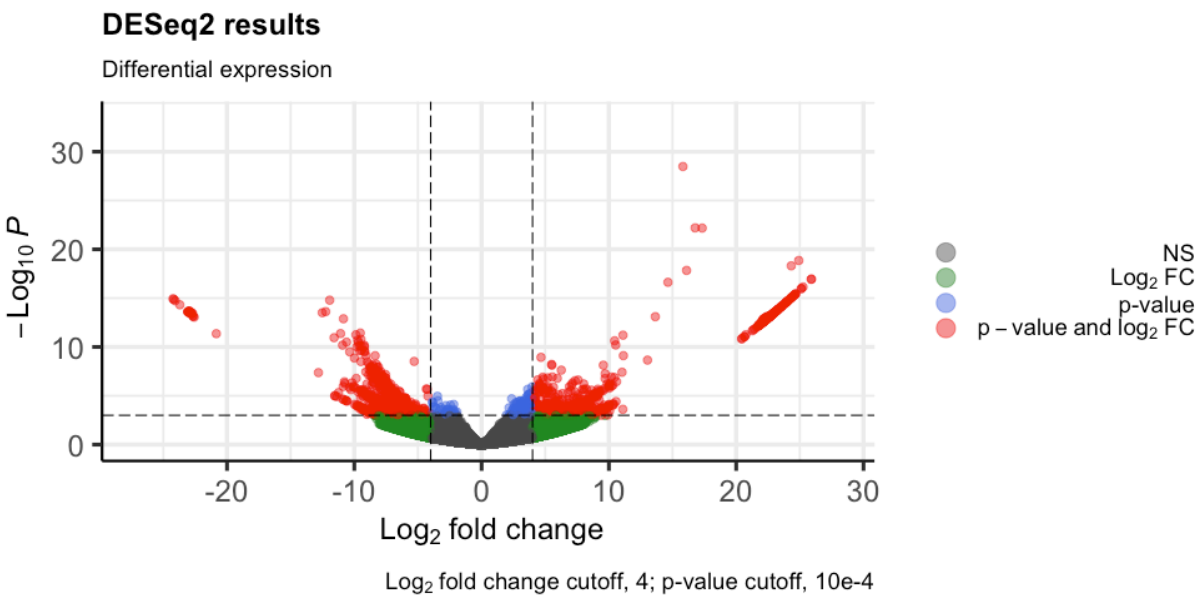


Figure 4.6. Volcano plot detailing the differential expression profiles of TSWV-infected versus non-infected samples of the *Arachis* genotype, ValSten1 **(**PI695393**).** All transcripts with a p-value of $\geq 10 \times 10^{-4}$ and $\log_2$ fold-change ≥ 4 are highlighted in red (1475 genes).

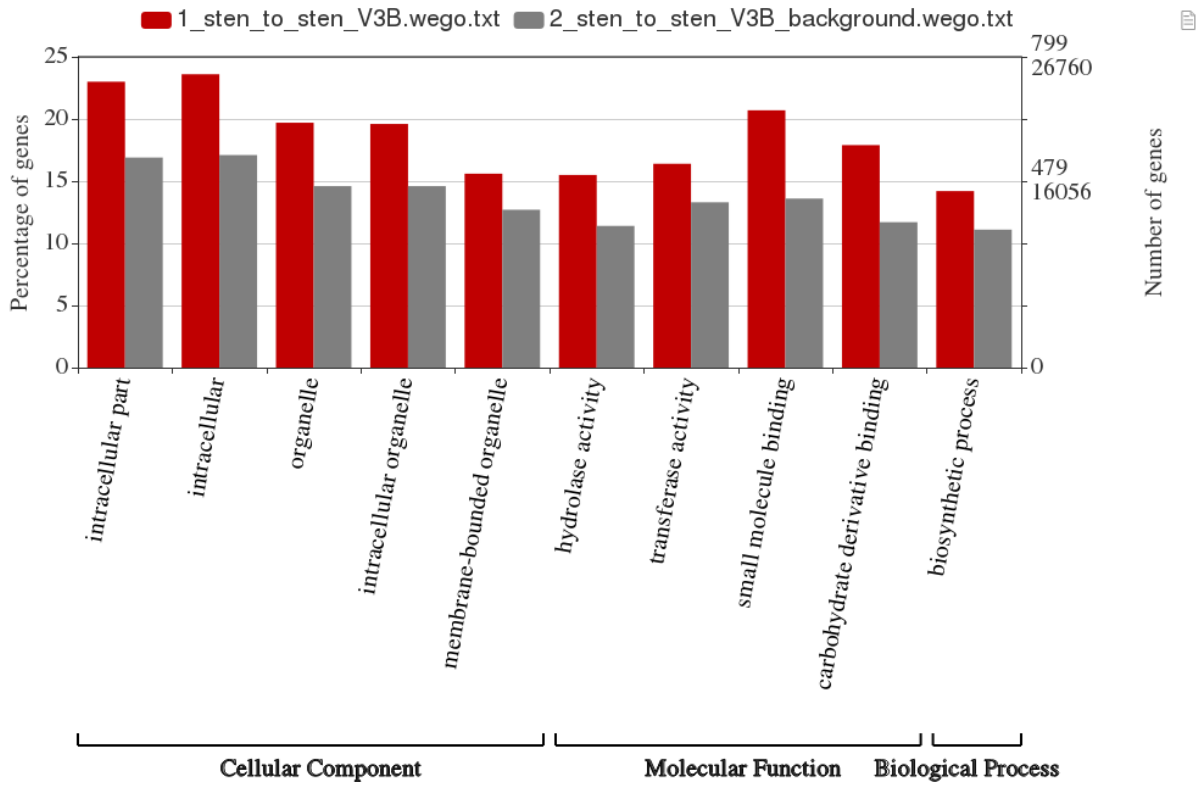


Fig. 4.7. *Arachis stenosperma* V10309.Gene Ontology (GO) terms. Distribution of all significant level 2 and 3 GO terms assigned to differentially expressed genes (DEGs) in *A. stenosperma* V10309. The DEG total is included for comparison. The x-axis shows the GO terms selected from the GO trees. The left y-axis shows the percentages of selected GO terms and the right y-axis shows the gene numbers (WEGOID67108154156388).

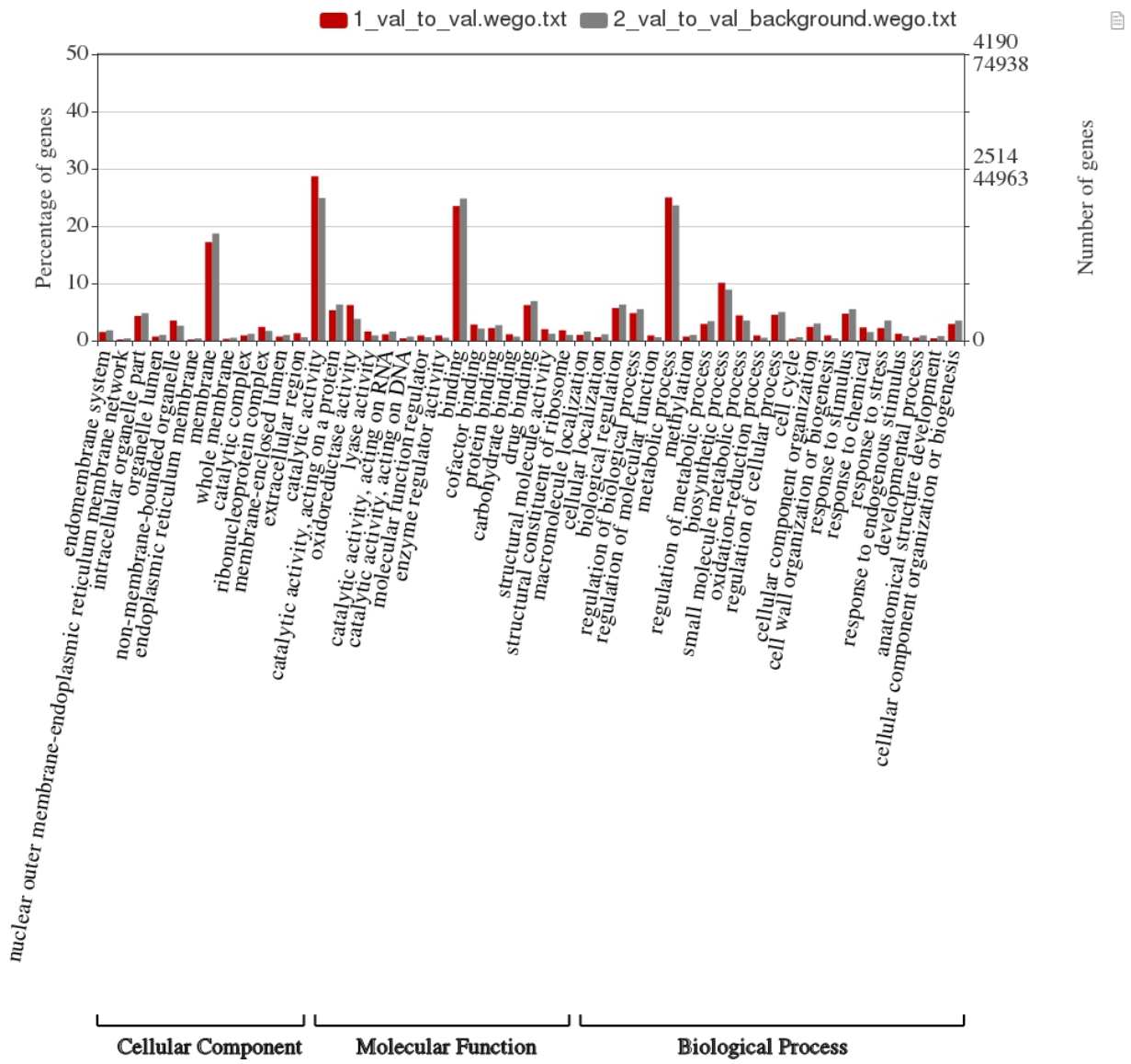


Fig. 4.8. *Arachis valida* KG30011Gene Ontology (GO) terms. Distribution of all significant level2 and 3 GO terms assigned to differentially expressed genes (DEGs) in *Arachis valida.* The The DEG total is included for comparison. The left y-axis shows the percentages of selected GO terms and the right y-axis shows the gene numbers (WEGOID34193536156389).

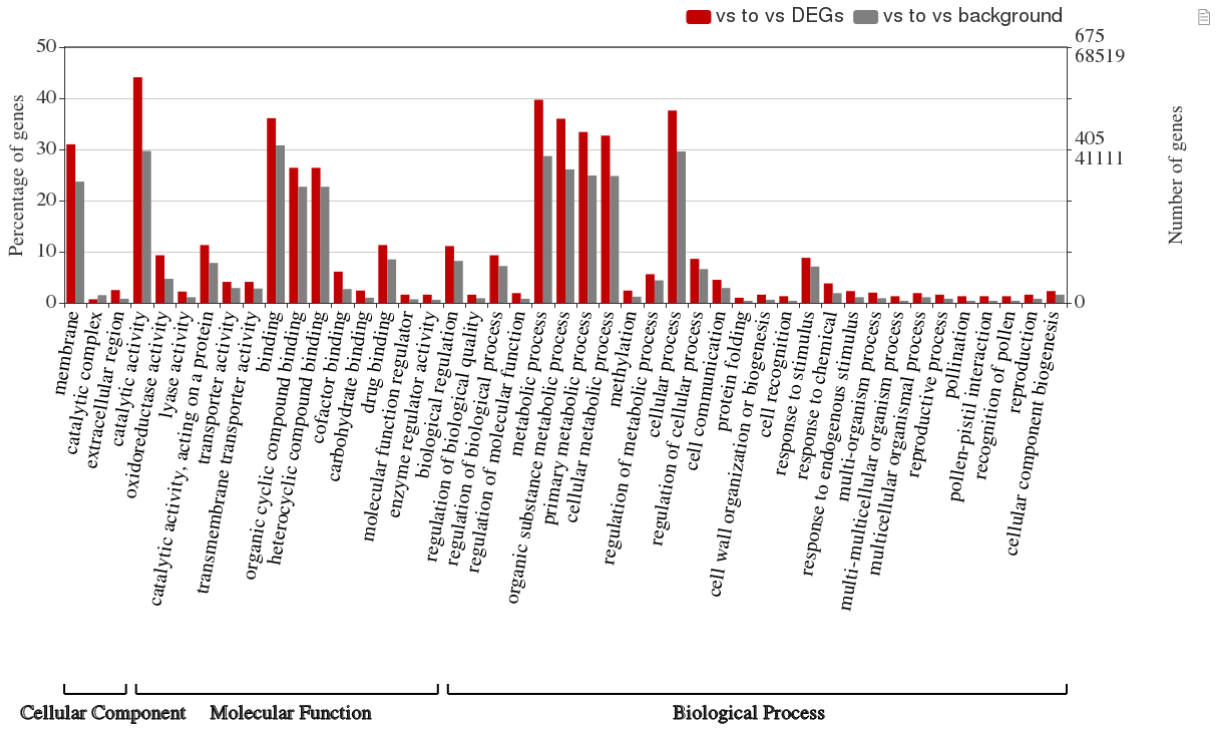


Fig. 4.9. ValSten1 Gene Ontology (GO) terms. Distribution of all significant level2 and 3 GO terms assigned to differentially expressed genes (DEGs) in ValSten1. The DEG total is included for comparison. The left y-axis shows the percentages of selected GO terms and the right y-axis shows the gene numbers (WEGOID06471673156390).

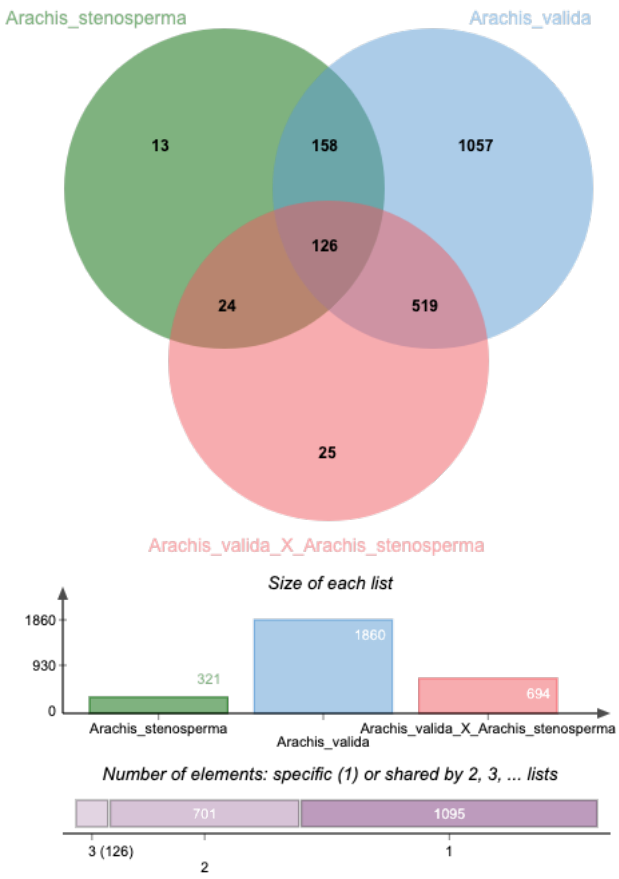


Figure 4.10. Summary of shared and differentially expressed genes (DEGs) between wild peanut (*Arachis spp*.) with different chromosomes in response to TSWV infection. Venn diagram represents the expressed common, unique, and core set of DEGs between *Arachis stenosperma*, *Arachis valida,* and ValSten1.

Table 4.2. Counts of defense-, phytohormone-, and photosynthesis-related significant differentially expressed genes above the $|\log_2|FC > 4$ cutoff in wild *Arachis* species in response to TSWV infection.

Gene description	*A. stenosperma* ($Sten^{2x}$)		*A. valida* (Val^{2x})		$ValSten^{4x}$	
	Upregulated	Downregulated	Upregulated	Downregulated	Upregulated	Downregulated
Argonaute	0	0	1	6	0	0
MATH domain	0	0	1	1	0	0
Dicer	0	1	3	1	0	0
Heat shock protein	11	0	49	1	15	0
Lectin	1	2	47	12	12	2
Leucine zipper	1	0	10	4	3	1
Mitogen-activated protein kinase	0	1	13	4	0	0
MYB	1	2	23	13	2	2
P450	1	4	51	18	6	8
PAMP	0	0	1	0	0	0
Disease resistance (R) protein	1	12	11	49	4	13
WRKY transcription factor	0	1	22	3	2	0
LRR	1	4	24	25	1	2
Serine/threonine	7	11	129	89	14	23
Salicylic acid	0	0	3	1	0	0
Calmodulin	0	1	25	12	3	0
TMV resistance protein N	1	8	25	20	2	5
Stilbene synthase	0	0	33	0	0	0
Serine Carboxypeptidase	0	1	19	11	5	0
Alpha-Dioxygenase	0	0	0	3	0	1
(Total of genes related to defense)	(25)	(48)	(490)	(273)	(69)	(57)
Auxin	0	1	7	37	3	14
Gibberellin	0	0	5	8	1	3
Cytokinin	0	0	13	4	2	2
Abscisic acid	0	1	5	8	0	0
Ethylene	0	1	29	9	5	1
Brassinosteroid	0	0	0	0	1	0
Salicylic acid	0	0	0	0	1	0
ABC transporter	1	7	41	32	3	8
(Total of genes related to phytohormones)	(1)	(10)	(100)	(98)	(16)	(28)
Chloroplastic	3	23	243	256	26	44
Protochlorophyllide	0	0	1	3	0	0
Photosystem	0	1	0	26	0	1
NADP-dependent malic enzyme	0	2	5	2	2	0
(Total of genes related to phytosynthesis)	(3)	(26)	(249)	(287)	(28)	(45)

Table 4.3. The regulated direction of defense-, phytohormone-, and photosynthesis-related significant differentially expressed genes above the $log_2FC > 4$ cutoff in *Arachis* species in response to TSWV infection.

	Gene description	Wild peanut			Cultivated peanut*	
		Sten^{2x}	Val^{2x}	ValSten^{4x}	Tifgurard^{4x}	Sunoleic^{4x}
1	Argonaute	-	↓	-	↑	↑
2	MATH domain	-	⇅	-	↑	↑
3	Dicer	↓	↑	-	↑	↑
4	Heat shock protein	↑	↑	↑	↑	↑
5	Lectin	↓	↑	↑	↑	↑
6	Leucine zipper	↑	↑	↑	↑	↑
7	Mitogen-activated protein kinase	↓	↑	-	↑	⇅
8	MYB	↓	↑	⇅	↑	↑
9	P450	↓	↑	↓	↑	↑
10	PAMP	-	↑	-	↑	↑
11	Disease resistance (R) protein	↓	↓	↓	↑	↑
12	WRKY transcription factor	↓	↑	↑	↑	↑
13	LRR	↓	↓	↓	↑	↑
14	Serine/threonine	↓	↑	↓	↑	↑
15	Salicylic acid	-	↑	-	-	↑
16	Calmodulin	↓	↑	↑	↑	↑
17	TMV resistance protein N	↓	↑	↓	↑	↑
18	Stilbene synthase	-	↑	-	↑	↑
19	Serine Carboxypeptidase	↓	↑	↑	↑	↑
20	Alpha-Dioxygenase	-	↓	↓	↑	↓
	No. of upregulated categories to defense	2	15	6	19	18
1	Auxin	↓	↓	↓	⇅	↑
2	Gibberellin	-	↓	↓	⇅	↓
3	Cytokinin	-	↑	⇅	↑	↑
4	Abscisic acid	↓	↓	-	↑	↑
5	Ethylene	↓	↑	↑	↑	↑
6	Brassinosteroid	-	-	↑	↑	-
7	Salicylic acid	-	-	↑	-	↑
8	ABC transporter	↓	↑	↓	↑	↑
	No. of upregulated categories to phytohormone	0	3	3	5	6
1	Chloroplastic	↓	↓	↓	↑	↓
2	Protochlorophyllide	-	↓	-	↓	↓
3	Photosystem	↓	↓	↓	↓	↓
4	NADP-dependent malic enzyme	↓	↑	↑	↑	⇅
	No. of upregulated categories to phytosynthesis	0	1	1	2	0

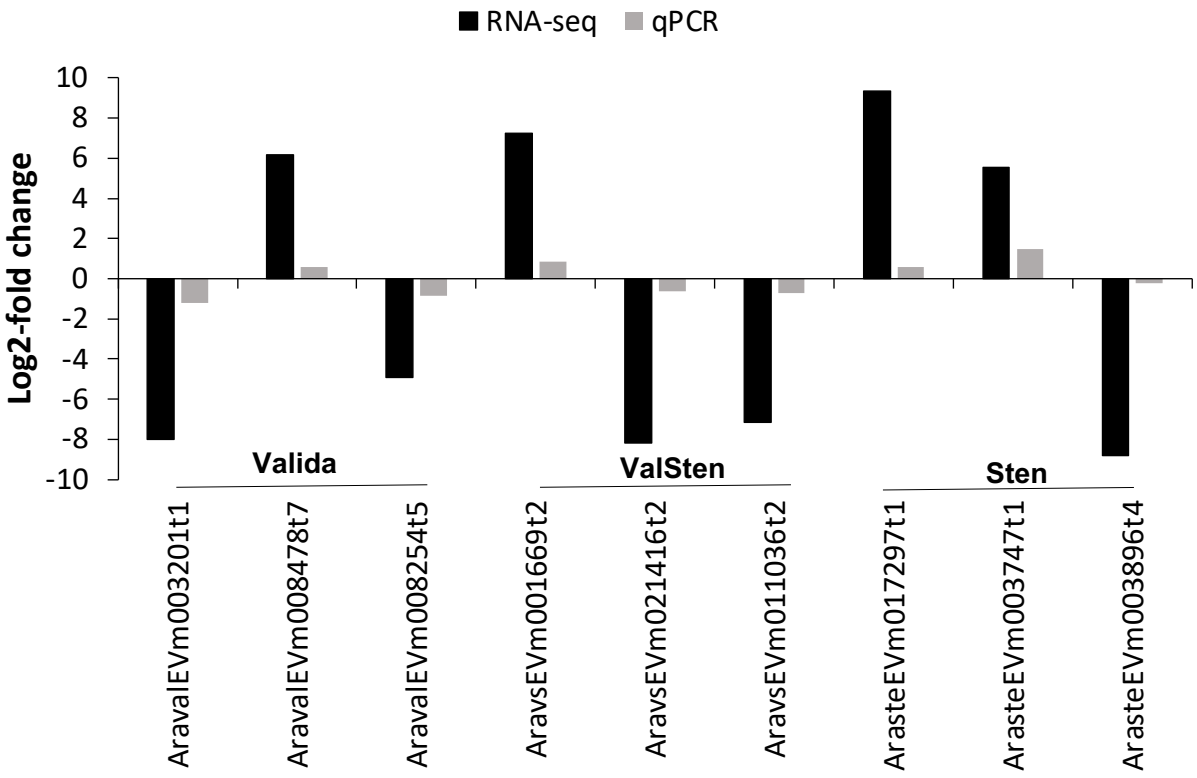


Figure 4.11. Quantitative PCR validation of the RNA-seq analysis using selected differentially expressed Genes (DEGs) in three *Arachis* genotypes in response to TSWV infection.

Supplementary Table 4.1. Primer pairs designed for normalized quantitation of differentially-expressed genes (DEGs) in response to tomato spotted wilt orthotospovirus (TSWV) infection in *Arachis* genotypes.

Genotype	Transcript ID	Reverse Sequence (5'->3')
A. valida	AravalEVm003201t1	TATCTTTGGGCGCCACTTGT
		TCGGTCCTGCAATGCTTCAT
A. valida	AravalEVm008478t7	TGGTGTGGTCTGGAATGGTT
		TGCACAATGTGGCTCAACAG
A. valida	AravalEVm008254t5	CCGTGGCTACACACTTACCC
		TATGGCCGAAGCAAGCATGA
ValSten1	AravsEVm001669t2	TTGCTAGGACATTTGCCGGA
		GCCAAAGGCGAACACATCAG
ValSten1	AravsEVm021416t2	CTCCAGATGCGACACCTGTT
		GCGCCAGTTGCTTAATTCGT
ValSten1	AravsEVm011036t2	TGACGGTTAGAACGGCAACA
		TTGAGGGCAGCCACTGTAAT
A. stenosperma	ArasteEVm017297t1	CCACGAGGGTGAGTTGTTGT
		GCAATAAACGGGCGAACACC
A. stenosperma	ArasteEVm003747t1	GGATGCAGGGGCAATTTCGG
		ATCTCCGGCAGTGGCTTTCA
A. stenosperma	ArasteEVm003896t4	CTGGGCGGGTGAGTATGGAG
		TTGGAAGGGCAGAAGTCGCA
A. hypogaea	(Internal reference)	GACGCTTGGCGAGATCAACA
		AACCGGACAACCACCACATG