THE ROLE OF *BEMISIA TABACI* IN THE TRANSMISSION OF VEGETABLE VIRUSES IN THE FARMSCAPE OF GEORGIA

by

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ABSTRACT

Bemisia tabaci Gennadius- transmitted viruses limit vegetable production in the Southeastern United States. Two whitefly-transmitted begomoviruses (Cucurbit leaf crumple virus and Tomato yellow leaf curl virus) and one *Crinivirus*, *Cucurbit yellow stunting disorder virus*, are very important in Georgia. Cucurbit yellow stunting disorder virus (CSYDV), and cucurbit leaf crumple virus (CuLCrV) occur as mixed infections in squash. Experiments were conducted to study the effects of CYSDV and/or CuLCrV infections on whitefly fitness and preference. Whiteflies preferred non-infected over infected plants, but no effects were observed on whitefly fitness. In Georgia, multiple crops host for CuLCrV are planted next to each other. Transmission studies were conducted to evaluate whitefly-mediated transmission of CuLCrV from different hosts into squash. It was concluded that CuLCrV epidemics in squash largely depend on the primary source of infection. During screening for CuLCrV, another *Begomovirus*, *Sida golden mosaic virus* was detected for the first time in snap bean in Georgia. Host range studies revealed that sida golden mosaic virus (SiGMV) can infect plants in three families: hollyhock, marshmallow, okra, country-mallow, prickly sida, tobacco and snap bean.

Phylogenetic analysis revealed that SiGMV identified in this study is related to other sida viruses reported from the New World. *B. tabaci* has been in the U.S. for three decades, but its genetic structure at the farmscape level is poorly understood. In order to better understand the population structure of *B. tabaci* at the farmscape level, we collected 36 different populations of whiteflies from different farmscapes across Georgia. Five different types of whiteflies were recorded: the banded-wing whitefly, the greenhouse whitefly, the citrus whitefly, and two sweetpotato whitefly cryptic species: MEAM1 and MED. Population genetics analysis revealed minor difference between *B. tabaci* populations colonizing different host plants, but for the first time, MED was found in field-grown snap bean in the Oconee, County of Georgia. To assess the impact of introduction of MED in Georgia's farmscape, we compared the transmission of tomato yellow leaf curl virus (TYLCV) and CuLCrV by MEAM1 and MED biotypes. TYLCV was transmitted by both MED and MEAM1. However, CuLCrV was transmitted only by MEAM1.

INDEX WORDS: Tomato yellow leaf curl virus, cucurbit leaf crumple virus, sida golden mosaic virus, cucurbit yellow stunting disorder virus. *Bemisia tabaci*, Phylogenetics, Vector, Mixed infections, Population genetics

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INTRODUCTION

Introduction

Whitefly-transmitted viruses cause severe diseases in multiple crops around the world. Outbreaks of the MEAM1 biotype of *Bemisia tabaci* in the Southeastern United States have led to the emergence of multiple plant pathogenic viruses, particularly in the genera *Begomovirus* and *Crinivirus.* Whitefly-virus pathosystems consist of whitefly, plant, and virus.

Whitefly

There are around 1500 species of whiteflies (Homoptera: Aleyrodidae) (Inbar and Gerling. 2008). Because of their significant pest status in multiple commercial crops, whiteflies have been studied extensively. Among all identified species, most studied whitefly species are the spiraling whitefly (*Aleurodicus disperses,* Russell), the greenhouse whitefly (*Trialeurodes vaporariorum* Westwood)*,* and the sweet potato whitefly (*Bemisia tabaci,* Gennadius). Middle East-Asia Minor 1 (MEAM1, formerly biotype B) and Mediterranean (MED, formerly biotype Q) are the most invasive and damaging members of the *B. tabaci* cryptic species complex (De Barro et al. 2011).Biotype A of *B. tabaci* is known to be present in the United States of America (USA) since the late 1800s, although until 1986, it didn't pose a serious economic threat. After

introduction of MEAM1 in 1986, *B. tabaci* started attacking crops that it had not infested earlier, such as poinsettia, and quickly became resistant to earlier effective insecticides. Introduction of MEAM1 in Florida caused serious economic losses, first in poinsettia, followed by high infestations in field-grown tomatoes (*Solanum lycopersicum* L.) (Hamon and Salguero 1987, Schuster et al. 1989, Hoelmer et al. 1991). MEAM1 moved rapidly to Texas, Arizona, and California, where it caused severe economic losses in various crops, such as melons, cotton, and vegetable crops (Perring et al 1991, Perring et al. 1992, Perring et al. 1993, Gonzalez et al. 1992).

MED was first reported in Spain in 1997 (Guirao et al. 1997). In the USA, it was first reported in Arizona in an ornamental retail store in December 2004 on poinsettias (Dennehy et al. 2005). Concurrently with confirmation about the presence of MED biotypes in the USA, there were reports of whitefly control failure from various commercial nurseries. Recently, MED has been reported from open field environments in Florida (McKenzie et al 2017). MED is morphologically indistinguishable from MEAM1. However, MED has a high propensity to develop to resistance to neonicotinoid insecticides (Elbert and Nauen 2000, Horowitz et al. 2004), the cornerstone of current whitefly management programs in cotton, vegetables, and ornamentals.

Physical characteristics of whiteflies

Whiteflies have opisthognathous piercing-and-sucking types of mouthparts. Both sexes have two pairs of membranous wings and follow incomplete metamorphosis. The whitefly anus opens

within the vasiform orifice located on the dorsum of the ninth abdominal segment in males and eighth abdominal segment in females (Gupta, 1972). The anus is covered by a lingua that helps nymphs to catapult the honeydew away from their bodies (Byrne and Bellows, 1991). Both nymph and adult secrete waxes that cover their bodies. In nymphs, waxes can be secreted as gelatinous masses, plumes, columns or setae. In *B. tabaci* adults, waxy threads of 1-micron diameter thickness are secreted; the hind legs and forelegs help to distribute the wax over the wings and other body parts. Wax of *B. tabaci* is mainly composed of triacylglycerols (65-75%), and has minute amounts of esters, free fatty acids, alcohols, and hydrocarbons (Byrne and Hadley 1988).

Biology of *B. tabaci*

There are six stages in the whitefly life cycle: egg, four nymphal stages, and winged adults. A female can lay about 300 eggs in her lifetime (Liburd et al. 2008). *B. tabaci* follows arrhenotokous parthenogenetic reproduction. All unfertilized eggs develop into males and fertilized eggs, which are diploid, develop into females. Mating occurs immediately after emergence once the wings have hardened, usually within one hour (Byrne and Bellows, 1991). Eggs are oval-shaped; the apex is acute, and the basal part is broadly attached to the leaf surface with a pedicel or stalk. Immediately after hatching, first instar nymphs (crawlers) move actively to find a suitable feeding site. Crawlers can move anywhere from a few centimeters to another leaf in search of feeding sites. Once they settle themselves on the underside of the leaf, they start feeding on plant phloem. The remaining three instars are immobile and complete their life cycle on the same site. Two pairs of mycetomes, located on the dorsum of nymphs, have symbiotic

bacteria that helps in nutrition. Pharate adults develop within the cuticle of the fourth instar prior to emergence. Duration of development depends on the temperature and host plant species. Typically, *Bemesia* spp. take around three weeks to complete their life cycle at 80°F. Chemoreceptors present on the mouthparts help whiteflies to decide the suitability of plant hosts. If the host is suitable, whiteflies insert their stylets deep into the phloem for feeding. Females lay eggs while feeding.

Damage

Whiteflies can damage plants in several ways. Direct damage includes feeding on the plant phloem and phytotoxicity caused by saliva secreted during feeding. Whiteflies can also cause indirect damage by secreting sugary honeydew, which provides a suitable growth medium for sooty mold, which blocks sunlight and reduces photosynthesis (Chen et al. 2004, Gerling 1990, Henneberry 2000, Yee et al. 1996). Feeding by *B. tabaci* can cause uneven fruit ripening in tomato, and silver leaf disorder in squash. In addition to these damages, whiteflies also transmit plant viruses (Brown and Czosnek 2002).

Plant responses to whiteflies

Plants respond to insect attacks by accumulating harmful secondary metabolites in their damaged parts, or by releasing secondary metabolites, which attracts natural enemies. Accumulated metabolites can operate in several ways: by influencing feeding behavior, increasing mortality, decreasing reproductive potential, or increasing developmental time. Whitefly feeding does not do

lots of direct damage to the plants; therefore, plant responses to whiteflies resemble their responses to plant pathogens via salicylic acid (SA) and jasmonic acid (JA) /ethylene–dependent pathways (Walling 2000). Tomato leaves attacked by whiteflies produce pathogen-related proteins β-1,3-glucanase, chitinase, peroxidase (Inbar et al 1999, Mayer et al 1996). In squash (*Cucurbita pepo* L.), expression levels of two genes, *SLW1* and *SLW3,* are increased in response to whitefly nymph feeding (Walling 2000). Levels of expression of these genes vary depending upon whitefly biotype. Whitefly nymph feeding produces silverleaf (SSL) disorder in squash characterized by silvering of the veins resulting in reduced photosynthesis, stunting and lower yields (McAuslane et al. 2004).

Whitefly-transmitted viruses

Insects are the most common vectors of plant pathogenic viruses, approximately 70% of plant infectious viruses are transmitted by the arthropod vectors. Global distribution of *B. tabaci* has led to the widespread emergence of whitefly-transmitted viruses (Navas-Castillo et al. 2011). There are five known genera of viruses transmitted by *B. tabaci*: *Begomovirus (Geminiviridae), Ipomovirus (Potyviridae), Crinivirus (Closteroviridae), Carlavirus (Betaflexiviridae),* and *Torradovirus (Secoviridae)*. Among these genera, begomviruses and criniviruses are the two most important plant pathogens in the Southeastern United States.

Based on the mode of transmission, *B. tabaci*-transmitted viruses can be classified into semipersistent (crinivirus) and persistent (begomovirus) types. Whiteflies acquire virus particles with thier stylets while feeding on infected phloem. Virus retention sites vary depending upon the

mode of transmission. Semipersistent virus particles move from stylet to esophagus and are retained in the foregut (Chen et al. 2011). Virus-encoded coat protein (CP) is reported to play a major role in virus transmission by whiteflies. For lettuce infectious yellows, the crinivirus capsid is composed of major and minor capsid proteins (CP and CPm, respectively). CPm is reported to interact with the receptors in the foregut of *B. tabaci* (Ng and Falk, 2006). Persistently transmitted viruses reach the midgut after crossing the esophagus; they cross the filter chamber and midgut into the hemolymph through receptor-mediated endocytosis. In hemolymph, an endosymbiont-encoded chaperone protein called GroEL helps in the translocation of virus to primary salivary glands (PSG). At the PSG, virions move into the PSG lumen through receptor-mediated endocytosis. From the lumen, virus particles are egested with saliva into plant phloem (Czosnek et al. 2017). *B. tabaci* can acquire semipersistent viruses within minutes or hours of feeding and retain them in the foregut for hours or days. In contrast, persistently-transmitted viruses are acquired after hours of feeding and can stay with *B. tabaci* for either weeks or up to the duration of life. During the whole process of persistent transmission, coat protein is the only virus-encoded protein reported to interact with the receptors at the midgut and PSG.

Begomovirus

Viruses within the genus *Begomovirus,* depending upon their genome organizations, are either monopartite (DNA A~2.7 kb) or bipartite (DNA-A and -B, each approx. ~2.6 kb) (Brown 2001). Begomoviruses are transmitted exclusively by *B. tabaci* in a persistent, non-propagative manner. Members of the *B. tabaci* species complex are known to transmit begomoviruses with different efficiencies. For examples, whiteflies of the Asia II-1 clade transmit the tomato yellow leaf curl

virus (TYLCV) at half the efficiency of MEAM1 and MED biotypes (Li et al. 2010). Begomovirus transmission is also influenced by *B. tabaci* feeding behavior, preference, amount of virus, and virus distribution within the plant (Azzam et al. 1994). Endosymbionts have been reported to influence begomovirus transmission by whiteflies (Gottlieb et al. 2010).

Tomato Yellow Leaf Curl Virus

YLCV causes tomato yellow leaf curl disease (TYLCD) in tomatoes (Moriones and Navas-Castillo 2000). TYLCV is a monpartite virus with a single circular DNA-A. DNA-A consist of six open reading frames (ORF). Two ORFs are present on the viral sense strand (V1 and V2) and four are present on the complementary strand (C1, C2, C3 and C4). V1 encodes for viral capsid proteins; V2 helps to suppress the defense gene expression in infected tomato plants. ORFs present on the C strand encode for proteins involved in replication, pathogenicity, and movement. In addition, there is an intergenic region (IR) on the TYLCV genome, which encodes for promoter and regulatory genes involved in viral replication. TYLCV is transmitted exclusively by *B. tabaci*. However, a single study has shown that castor whitefly, *Trialeurodes ricini* Misra*,* can act as a vector for TYLCV (Idriss et al. 1997), although these finding have never been tested by any other group independently.

Acquisition, retention, circulation, and transmission of TYLCV by B. tabaci

Whiteflies acquire TYLCV from the phloem of infected plants while feeding on phloem. Virus acquisition access period (AAP) can vary between 10-60 min (54-59). AAP in whiteflies varies

from individual to individual. Using the PCR detection method, 20% of whiteflies tested positive for viral DNA following a 5-min AAP and 100 % after 10 min (Navot et al. 1992, Atzmon et al. 1998, Ghanim et al. 2001). Following acquisition, TYLCV is retained in the whitefly for life (Czosnek and Ghanim). Virus transmission frequency is dependent on the AAP. *B. tabaci* can acquire about 0.5 X10^{9} virions (Polston et al. 1990), or 6 X 10^{8} virus genomes (Zeidan and Czosnek 1991). Rate of virus transmission decreases with whitefly age, and females transmit TYLCV better than males. Once ingested, the virus must move from the mouth to the midgut, and from midgut to salivary glands before it can be transmitted to the next plant through saliva. The latent period for TYLCV is about 8h (Ghanim et al. 2001). TYLCV can be detected in the saliva after a 7-h AAP; however, whiteflies are only able to successfully transmit it after 8 hours. These observations suggest that the minimum number of virions must be present in the salivary glands before successful transmission to the next plant. Studies have reported that most whitefly species can acquire the majority of begomoviruses (Polston et al. 2014). However, they differ significantly in their ability to transmit these viruses. Furthermore, transmission ability also differs between different populations of the same whitefly species (Kollenberg et al. 2014). Differential transmission ability may be attributed to different feeding habits, life histories, endosymbionts, and different genetic makeups (Rosen et al. 2015). Movement of virus from midgut to salivary glands via hemolymph is mediated by multiple receptors, although such receptors have not been discovered yet. Using fluorescence in-situ hybridization (FISH), different studies have localized the virus in the midgut and salivary glands (Czosnek et al. 2002).

Cucurbit leaf crumple virus

Cucurbit leaf crumple virus (CuLCrV) is a bipartite begomovirus with DNA-A and DNA-B components, each about 2.6 kb in size. CuLCrV can infect many plant species in the family *Cucurbitaceae,* tobacco, and certain cultivars of common bean (*Phaseolus vulgaris*) (Brown, et al. 2002, Hagen et al 2008, Hernandez et al. 2001). CuLCrV was first discovered in the Imperial Valley of California in 1988 (Guzman et al. 2000). It was first detected in the Southeastern US in 2006, and in 2010, it was found in snap beans in Georgia (Larsen and Kmiecik 2010). Recently, CuLCrV has been reported from cucurbits in South Carolina, demonstrating its ever-expanding geographical range (Keinath et al. 2018). In the early stages of CuLCrV infection, plants show stunting. Subsequent symptoms vary among known host plants. In yellow summer squash and snap bean, disease symptoms are severe: stunted growth, curled and crumpled young leaves. Fruits of zucchini squash show no obvious symptoms. However, fruits of yellow summer squash develop green streaks (Webb et al. 2007). In cantaloupe and watermelon, disease symptoms are mild, and economic damage is relatively low. Infected plants mostly remain green, with little or no yellowing on younger leaves.

Sida golden mosaic virus

Sida golden mosaic virus (SiGMV) is a bipartite begomovirus with DNA-A and DNA-B components, each about 2.6 kb in size (Fiallo-Olive et al. 2010). SiGMV was first reported in 2006 in Alachua County, Florida (Durham et al 2010). In 2010, a new strain, sida golden mosaic Florida virus-*Malvastrum* (SiGMFV-Ma), was reported in Cuba infecting mallow weed,

Malvastrum coromandelianum (L.) (family Malvaceae) (Fiallo-Olive et al. 2010). Several *Sida* spp. have also been reported as hosts for begomoviruses (hereafter referred to as sida viruses) (Fiallo-Olive et al. 2010; Stewart et al. 2014; Wyant et al. 2011). Sida viruses have been reported from both the Old World (OW) (Duan et al. 2019; Ha et al. 2008) and New World (NW) (; Echemendia et al. 2004; Fontenele et al. 2018; Tavares et al. 2012). *Sida* spp. are ubiquitous in the Southeast US. Around ten *Sida* spp. have been reported from Florida alone (http://www.plantatlas.usf.edu). *Sida* spp. are frequently found in proximity to important food and fiber crops, which makes *Sida* spp. potential natural reservoirs for sida viruses that could also infect important food and fiber crops. SiGMV infection in susceptible host plants in characterized by development of golden mosaic on younger leaves.

Crinivirus

Genomes of viruses included in the genus *Crinivirus* are composed of linear, positive-stranded ssRNA of approximately 15.3–17.7 kb in size (Wisler et al 1988**)**. Except for potato yellow vein virus, which has three, genomes of criniviruses are composed of two independently encapsulated RNA molecules. RNA1 codes proteins involved in replications, and RNA2 codes for coat protein, movement protein, and proteins involved in vector transmission (Karasev 2000, Livieratos et al. 2004, Martelli et al. 2002). Criniviruses are phloem-limited viruses, transmitted exclusively by whiteflies in two genera, *Trialeurodes (T. vaporariorum* and *T. abutiloneus)* and *Bemisia (B. tabaci),* in a semi-persistent manner.

Cucurbit yellow stunting disorder virus

Cucurbit yellow stunting disorder virus (CYSDV), (genus *Crinivirus,* family *Closteroviridae*) was first reported in the Middle East from the United Arab Emirates in 1982 (Hassan and Duffus 1991). In the Southeastern US, CYSDV was first reported from Florida in 2007 (Polston et al. 2008) and then later in 2017, it was found in squash in Georgia (Gadhave et al. 2018). CYSDV can infect members of the *Cucurbitaceae,* alfalfa (*Medicago sativa*), lettuce (*Lactuca sativa)*, certain cultivars of snap beans (*P. vulgaris*), and many wild weed species (Wintermantel et al. 2009). CYSDV infection in squash is characterized by severe interveinal chlorosis, especially in the older leaves. CYSDV infection can result in significant decreases in sugar production in melons, resulting in poor, unmarketable yields. CYSDV has become a serious production problem for cucurbit farms in the Southern United States, Mexico, and Central America. Once whiteflies acquire CYSDV from infected plants, they can remain viruliferous for up to 9 days (Celix et al. 1996, Wisler et al. 1988).

Interactions within whitefly-transmitted viral (TYLCV, CuLCrV, SiGMV, CYSDV) pathosystems

Plant viruses are ubiquitous and cause economically important diseases. Many plant pathogenic viruses depend on arthropod vectors such aphids, thrips and whiteflies for transmission (Andret-Link and Fuchs 2005). Successful transmission of vector borne viruses largely depend on the nature of the interactions between the host and vector (McElhany et al. 1995). This dependency

of plant viruses on arthropod vectors has led to the evolution of virus induced traits in host plants and vectors that positively influence the vector mediated transmission. Indirect interactions between the virus and vector can occur via the host plant, an example could be increased plant nutritive quality (Chen et al. 2013) or altered plant mediated visual and olfactory cues for the vector (Chen et al. 2013, Fereres et al. 2016, Hodge and Powell 2008, Srinivasan et al. 2008). Direct interactions can occur when virus particles are assimilated by the vector and can led to changes in behavior that enhances virus transmission (Liu et al. 2013; Stafford et al. 2011). Therefore, the broader goal of the current project was to understand the interactions within the whitefly-transmitted pathosystems and their implications in virus epidemics. First, I examined the effects of single and mixed infections of CuLCrV and CYSDV on virus accumulation in host plants, and how single and mixed infections of CuLCrV and CYSDV affect the fitness, preference and virus accumulation in MEAM1. Second, I examined effects of single and mixed infections of CuLCrV and CYSDV on CuLCrV transmission by MEAM1. I also studied the host range, symptoms, and SiGMV accumulation in different host plants, and SiGMV accumulation in MEAM1 feeding on different host plants. Furthermore, I examined the phylogenetic relationships between SiGMV and other sida viruses. In addition, I also compared the transmission efficiency of TYLCV and CuLCrV by MEAM1 and MED biotypes. For many insect's species genetic structure is constantly shaped by local/region conditions. Framscape ecology, cropping patterns and agricultural practices can have significant effects on local whitefly populations and currently no information is available on genetic structure of whiteflies in Georgia. Therefore, I collected whitefly samples from major agricultural production regions of Georgia and studied the population genetic structures of *B. tabaci* at the Georgia farmscape level.

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CHAPTER 2

THE EFFECTS OF MIXED-VIRAL INFECTIONS IN HOST PLANTS AND IN THE VECTOR (WHITEFLY) ON VECTOR PREFERENCE AND FITNESS AND IMPLICATIONS FOR EPIDEMICS

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Abstract

A plethora of studies have examined the effects of single-plant virus infections on their vectors; however, very few have assessed the impacts of mixed-virus infections on their vector/s. The primary reason being that mixed-infections can tremendously increase the complexity of the pathosystem. Our earlier studies clearly demonstrated that mixed-infections in host plants can differentially alter the plant phenotype, influence virus acquisition and transmission, and vector fitness, than single-virus infections. Our current whitefly-virus pathosystem in the southern United States is incredibly complex. This pathosystem has two facets: 1. Mixed-infection in a host plant due to multiple viruses transmitted by the same vector, and 2. Mixed- infection in the vector (whitefly) due to acquisition of multiple viruses from multiple host plants in the farmscape. For the first facet, we examined the effects of cucurbit leaf crumple virus, (CuLCrV, a begomovirus) and cucurbit yellow stunting disorder virus (CYSDV, a crinivirus) infecting squash on whitefly (*Bemisia tabaci* Gennadius MEAM1) preference and fitness. Mixed-infection of CuLCrV and CYSDV in squash drastically altered its phenotype and affected whitefly settling, wherein whiteflies seem to prefer non-infected plants, and the magnitude of such preference varied between viruliferous and non-viruliferous whiteflies. Mixed-infected plants despite their altered phenotype (increased symptom severity), had fewer viral copies of at least one of the component viruses than singly-infected plants, and this difference affected virus acquisition by whiteflies. For the second facet, we evaluated the combined acquisition (mixed-infection) of tomato-infecting tomato yellow leaf curl virus (TYLCV) and squash-infecting

CuLCrV by whiteflies. Mixed infection of CuLCrV and TYLCV in whiteflies enhanced settling towards non-infected tomato and squash plants. In one instance, CuLCrV accumulation was significantly lower in mixed-infected than singly-infected whiteflies. The fitness study involving whiteflies infected with CuLCrV and/or TYLCV was conducted on a virus non-host (cotton), and results revealed that the mere presence of the viruses (either alone or together) in the vector alone did not affect its fitness. Taken together, the results indicate that mixed-infections of viruses in host plants and within the vector could have implications for virus accumulation, virus acquisition, vector preference and epidemics that sometimes are different from single-virus infections.

Introduction

Many plant pathogenic viruses rely on arthropod vectors for transmission (Andret-Link and Fuchs 2005; Hogenhout et al., 2008). Super vectors such as the sweetpotato whitefly (*Bemisia tabaci* Gennadius) can transmit multiple viruses simultaneously leading to mixed infections in host plants (Gilbertson et al., 2015). The nature of interactions between multiple viruses co-infecting host plants and vectors is often complex, dynamic, and could have variable consequences for epidemics of each of the co-infecting viruses. A variety of these virus–virus interactions within host or within vector are either synergistic or antagonistic in nature (Syller, 2011). In a synergistic interaction, at least one, ideally both, of the co-infecting viruses facilitate the replication and/or transmission of their virus partner/s in a plant host (García-Cano et al., 2006; Rentería-Canett et al., 2011; Untiveros et al., 2007). On the contrary, in an antagonistic

interaction, only one of the virus partners benefit, if at all, while lowering the replication and/or transmission of the co-infecting virus/es in a plant host (Calap et al., 2019; Chen et al., 2018; Crespo et al., 2019; Wintermantel et al., 2008). Unlike plants, mixed-infection in vectors and their effects on virus transmission has not been exclusively studied.

Virus-virus interactions within plant hosts and possibly in vectors could then also modulate interactions between host plants and vectors. Such interactions, as in the case of single-infections, albeit differently, could lead evolution of virus-induced traits in host plants (Chen et al., 2013; Fereres et al., 2016**;** Hodge and Powell, 2008; Srinivasan et al., 2008) and in vectors (Liu et al., 2013; Stafford et al., 2011**)**, and successful transmission of vector-borne viruses largely depend on these virus-induced traits (McElhany et al., 1995). A few earlier studies have shown that mixed-infections in plants alter the acquisition, retention, and inoculation of plant viruses by their vectors **(**Chen et al., 2018; Li et al., 2014; Tatineni et al., 2010**;** Wintermantel et al., 2008**).** This involves virus-driven modulation of the preference and fitness of vectors in multiple ways (Chen et al., 2018; Fereres et al., 2016; Jiu et al., 2007; Srinivasan and Alvarez, 2007; Srinivasan et al., 2012).

In the southeastern United States**,** the sweetpotato whitefly, *Bemisia tabaci* Gennadius cryptic species Middle East-Asia Minor 1 (MEAM1, formerly biotype B), is a major pest of many agricultural crops mainly because of its ability to transmit multiple plant viruses (Brown et al., 2002; Navas-Castillo et al., 2011). The outbreaks of whiteflies have resulted in the emergence of a wide array of viruses, most notably begomoviruses and criniviruses in cucurbits and tomato farmscapes (Adkins et al., 2011). Begomoviruses are transmitted by whiteflies in a persistent

circulative manner, whereas criniviruses are transmitted in a semi-persistent manner and are retained only in the foregut of whiteflies. Majority of plant-whitefly-virus interactions studies have explored single virus pathosystems, while a few have examined whitefly-virus interactions following multiple virus infections (Dalmon et al., 2009; Wintermantel et al., 2008). Whiteflies are increasingly transmitting multiple plant viruses that co-infect an array of single to multiple host plants (Abrahamian et al., 2015; Gadhave et al., 2018; Gil- Salas et al., 2011; 2012, Kuo et al., 2007**;** Turechek et al., 2010), it is critical to understand whether and how mixed infections of whitefly-transmitted viruses affects the vector preference and fitness, virus accumulation and their implications for virus epidemics.

Cucurbit leaf crumple virus is a species in the genus Begomovirus and family Geminiviridae, and *Cucurbit yellow stunting disorder virus* is a species in the genus Crinivirus and family Closteroviridae. Both cucurbit leaf crumple virus (CuLCrV) and cucurbit yellow stunting disorder virus (CYSDV) often are detected as mixed-infections in squash in the southeastern United States (Gadhave et al., 2018; Kuo et al., 2007). CuLCrV is single-stranded bipartite DNA virus with two circular components DNA-A and-B (Hagen et al., *2008)*. On the contrary, CYSDV is a positive-sense single-stranded RNA virus with two linear RNA molecules RNA 1 and 2 (Karasev, 2000). Both CuLCrV and CYSDV are phloem-limited viruses, transmitted by the common vector, the whitefly. Squash, whitefly, CuLCrV, and CYSDV pathosystem was used to examine mixed infection in host plants and impacts on the vector in the current study.

The whitefly-transmitted tomato yellow leaf curl virus, also a begomovirus, is an economically important disease of tomato (*Solanum lycopersicum* L.) in the southeastern United States

(Polston et al., 1999). Squash and tomato are two of predominant summer crops in the region, with squash sometimes planted as a trap crop in the vicinity of tomato plots to attract whiteflies (Schuster, 2004). Since whiteflies transmit economically important begomoviruses in each of these crops, we studied the effects of sequential and combined acquisition squash-infecting CuLCrV and tomato-infecting TYLCV) on whitefly preference and fitness.

The key objectives of the present study were to examine (i) whether plant viruses (CuLCrV and CYSDV in squash (facet 1), and CuLCrV and TYLCV in whiteflies (facet 2) accumulate differentially in single and mixed infections and interact differently in mixed infections in squash and in whiteflies. (ii) whether and how single and mixed infections of plant viruses (CuLCrV and CYSDV) in squash, and CuLCrV and TYLCV acquired by whiteflies influence the vector preference and fitness. Our hypotheses were: (1) mixed-infected plants would accumulate more amounts of one or two component viruses, and have a severely-altered phenotype that is more attractive to the non-viruliferous vector, consequently enhance the acquisition and inoculation of one or more component viruses than singly-infected plants (Fig. 2. 1); (2) mixed-viral infection in whiteflies could lead to increased attraction to non-infected plants and enhanced fitness.

Materials and Methods

Plants and insects

Two seeds of squash cv. Goldstar (Johnny's Selected Seeds, ME, USA) and tomato cv. Florida 47 (Seminis Vegetable Seeds, MO, USA) were sown independently in a 10 cm diameter x 8 cm

tall pots (Hummert International, Earth City, MO) using Sunshine LP5 Plug Mix (SunGro Horticulture Industries, Bellevue, WA, USA). Water-soluble Miracle-Gro (Scotts Miracle-Gro products, Inc., OH, USA) fertilizer was applied at weekly intervals. The potted plants were placed in whitefly-proof cages (Megaview Science Co., Taichung, Taiwan) [47.5(*l*)×47.5(*w*)×93(*h*) cm] in the greenhouse and maintained at 25°C, 60% RH, and 16L:8D photoperiod. Plants were thinned one week after sowing to retain one seedling per pot. The whiteflies (*B. tabaci* cryptic species MEAM1) used in the present study were first collected in Tifton, Georgia, and have been reared on cotton plants since then in 10 cm diameter x 8 cm tall pots in whitefly-proof cages in the greenhouse at above-stated conditions. The purity of the colony was periodically confirmed (once every few months) by partially sequencing the mitochondrial cytochrome oxidase I (COI) gene (Frohlich et al., 1999).

Virus source and maintenance

Fifteen whitefly-infested squash plants showing symptoms such as crumpling and yellowing were originally collected from a research plot in Tifton, GA served as initial inoclum sources. The presence of CuLCrV was tested using primer 3FAC3 (5′-TTTATATCATGATTTTCGAGTACA-3′) and 5RAC1 (5′-AAAATGAAAGCCTAAGAGAGTGGA-3′) targeting the 525 bp amplicon of AC3, AC2 and AC1 genes of CuLCrV DNA-A component. Total genomic DNA was extracted with the GeneJET Plant Genomic Purification Kit (ThermoFisher Scientific, Waltham, MA) following the manufacturer's protocol, and PCR was performed with 2X GoTaq Green Master Mix (Promega, Madison, WI) using Eppendorf Mastercycler Pro thermocycler (Eppendorf, Hauppauge, NY).

The 10 µl PCR mixture contained 5 µl of Master Mix, 0.5µM of forward and reverse primers, 20 ng DNA, and nuclease-free water. The PCR conditions were: 5 min of initial denaturation at 94°C followed by 40 cycles of 94°C for 1 min, 54°C for 30 sec, 72°C for 1 min, and a final extension of 72°C for 10 min. PCR products were analyzed by 1% agarose gel electrophoresis. PCR products were cloned using pJET1.2 cloning vector (Thermo Fisher Scientific, Waltham, MA) following the manufacturers guidelines. The integrity of inserts was confirmed by sequencing of purified plasmids (Eurofins Genomics, Louisville, KY). The obtained sequences (MN543080-81) showed >99% identity to deposited CuLCrV sequences in the NCBI database.

For CYSDV, total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA) following the manufacturers guidelines, and subjected to cDNA synthesis using the *GoScript Reverse Transcription System (*Promega, Madison, WI). The cDNA was amplified using CYSCPf (5′-ATGGCGAGTTCGAGTGAGAATAA-3′) and CYSCPr (5′ - ATTACCACAGCCACCTGGTGCTA-3′) primers, which target a 755 bp of the coat protein gene (Rubio et al. 2001). PCR mixture was prepared using 2X GoTaq Green Master Mix as described above. PCR cycling conditions were: 5 min of denaturation at 94°C followed by 40 cycles at 94 °C for 30 sec, 50 °C for 45 sec, 72 °C for 2 min, and a final extension at 72°C for 5 min. PCR products were analyzed by 1% agarose gel electrophoresis. The samples were cloned, sequenced and percent homology was determined as described above. The obtained sequences (MN557851-52) showed >99% identity to deposited CYSDV sequences in the NCBI database. The presence of another probable whitefly-transmitted virus was ruled out by testing for squash vein yellowing virus (SqVYV) by RT-PCR (Adkins et al., 2007).

After a transmission passage via tobacco (*Nicotiana tabacum* L.), a host for CuLCrV but not for CYSDV, CuLCrV was separated from the squash infected with CuLCrV and CYSDV (hereafter referred as "mixed-infected squash"). Viruliferous whiteflies were obtained by allowing whiteflies to feed on mixed-infected squash for an acquisition access period (AAP) of 48h. Using clip cages, whiteflies (100 adults/plant) were attached to the first true leaf of the four-week old tobacco, and provided with an inoculation access period (IAP) of 48h. After four weeks, total DNA/RNA from 100 mg of young leaf tissue was extracted and subjected to PCR analysis for CuLCrV and CYSDV as described above. As expected, tobacco was infected only with CuLCrV not with CYSDV. From CuLCrV-infected tobacco, CuLCrV-infected squash plants were generated and maintained through repeated inoculations using viruliferous whiteflies in squash.

Some of the field collected plants were only infected with CYSDV. CuLCrV infection or absence thereof was repeatedly confirmed by PCR in those plants. These plants served as inoculum sources for generating CYSDV-infected squash plants via whitefly-mediated transmission following an AAP and IAP of 48h each. CYSDV was since maintained in squash through repeated inoculations using viruliferous whiteflies.

TYLCV isolate used in the present study was first collected in 2010 from a TYLCV-infected commercial tomato field in Montezuma (Macon County, GA, USA) (Srinivasan et al. 2012). Since then, TYLCV has been maintained in a susceptible tomato cultivar Florida 47 through repeated inoculations of 4–6 weeks old plants with viruliferous whiteflies (Legarrea et al. 2015).

Virus accumulation

CuLCrV and/or *CYSDV in squash and whiteflies:*

Total DNA/RNA from 100 mg of leaves was extracted from non-infected, CuLCrV-infected, CYSDV-infected, and mixed CuLCrV-CYSDV-infected squash using the protocol described above. Each treatment was replicated ten times, and the experiment was repeated twice (n=30). Whiteflies were provided with an AAP of 48h on non-infected and virus-infected (CuLCrV and/or CYSDV) squash. After 48h, whiteflies were transferred to cotton using clip cages for another 48h. Following which, for CuLCrV assessment, total DNA was extracted from individual whiteflies using a specially formulated Chelex resin, InstaGene Matrix (Bio-Rad, Hercules, USA). For CYSDV assessment, total RNA from individual whiteflies was extracted using the RNeasy Micro Kit (Qiagen, Valencia, CA) and subjected to cDNA synthesis. Twenty individual whiteflies representing each treatment was used, and the experiment was repeated twice (n=60). CuLCrV and CYSDV copy numbers in plant samples and individual whiteflies were estimated using quantitative PCR protocols described below.

CuLCrV:

Quantitative PCR to determine CuLCrV-DNA accumulation in plants and whiteflies was carried out using 2X GoTaq qPCR Master Mix (Promega, Madison, WI) in a Mastercycler ep realplex (Eppendorf, Hauppauge, NY). Primers CuLCrV-QF (5'- CCTCAAAGGTTTCCCGCTCT-3') and CuLCrV-QR (5'-CCGATAGATCCTGGGCTTCC-3') amplifying a 110 bp region of the

coat protein gene of CuLCrV were used. GoTaq qPCR Master Mix was combined with forward and reverse primers (final concentration of 0.5 μM), 10 ng DNA, and nuclease-free water for a final reaction volume of 25 μl. Cycling parameters were as follows: 95°C for 2 min; 40 cycles of 95°C for 1 min, 63°C for 15 sec, and 72°C for 20 sec. Upon completion of the run, melting curve analysis was performed to confirm the specificity of the primer pairs. Each sample was tested in duplicate, and absolute number of copies in the samples were quantified using the standard curve protocol described by Legarrea et al., (2015).

CYSDV:

CYSDV accumulation in plants and whiteflies was quantified using the primers and cycle conditions described by Gil-Salas et al. (2007) with some modifications. CYSDV-For (5'-GCTTAATGTGGGAGAAGTTCTCCTA-3') and CYSDV-Rev (TCTGGATATAACCTTCAG ACACTC CTT) were combined with the GoTaq qPCR master mix, 10 ng DNA, and nuclease-free water for a final reaction volume of 25 μl. Upon completion of the run, melting curve analysis was performed, and copy numbers were quantified as described by Legarrea et al., (2015).

CuLCrV and/or TYLCV in whiteflies:

Total DNA from 100 mg of leaves was extracted from non-infected, CuLCrV-infected squash, and TYLCV-infected tomato plants, and virus accumulations were determined. Whiteflies were provided with an 48h AAP on both infected squash and tomato plants separately and sequentially

on both plants one after the other (CuLRV first and TYLCV next and vice versa). Following which, all whiteflies were transferred to cotton plants in clip cages for 48h. Total DNA was extracted from whiteflies using Chelex resin (Srinivasan et al. 2012). CuLCrV copy numbers were estimated using the protocol stated above, and TYLCV accumulation in infected tomato plants was estimated four weeks-post-inoculation using the protocol described by Legarrea et al. (2015). Twenty whiteflies were processed for each treatment. The experiment was repeated twice (n=60).

Whitefly settling

CuLCrV and/or CYSDV in squash and whiteflies:

The settling of non-viruliferous or viruliferous whiteflies on non-infected, CuLCrV-infected, CYSDV-infected, and mixed CuLCrV-CYSDV-infected squash was studied using a dual choice settling arena. The schematic representation of the setup of arena is described in Lagarrea et al., (2015). In brief, the arena consisted of a clear plastic (Mylar® film) cylinder (150 mm diameter x 310 mm height) closed with a 15-cm diameter petri plate at the top. Two narrow slits each 5 mm wide x 70 mm long were made exactly opposite to each other 9 cm from the top. These two slits were lined with a rubber foam strip (1.9 mm wide x 11.1 mm thick), and were used to position the infected leaf on one side and the same sized non-infected leaf on the other side. Each leaf was intact to their respective plants. For each plant, two choices were offered to non-viruliferous and viruliferous whiteflies: non-infected versus CuLCrV- or CYSDV-infected squash plants, and non-infected versus mixed (CuLCrV-CYSDV)-infected squash plants. Four types of adult

whiteflies used in the experiments were (1) non- viruliferous whiteflies emerged within 48 h on cotton plants, whiteflies independently viruliferous for (2) CuLCrV and (3) CYSDV, with 48 h AAP on CuLCrV or CYSDV- infected squash respectively, and (4) whiteflies viruliferous for both CuLCrV and CYSDV with 48 h AAP on mixed-infected squash. The infection status of a batch of these whiteflies for all treatments was confirmed via PCR as described above. Depending on the whitefly treatment, one hundred adults were collected into a 10-ml glass vial (VWR, Radnor, PA) using aspirator and released at the bottom of the arena. The number of whiteflies settling on each leaf was recorded after 24 h. The experiment was conducted with 10 arenas (replications) set up with previously unused virus-infected and non-infected replicate plants under laboratory conditions (25°C; 12h L:12 h D), and the experiment was repeated twice (n=30).

CuLCrV and/or TYLCV-infection in whiteflies:

Whitefly settling on non-infected versus virus-infected squash and tomato plants was studied using a dual choice settling arena described above. Two choices were offered to non-viruliferous and viruliferous whiteflies: non-infected versus CuLCrV-infected squash plants, and non-infected versus TYLCV-infected tomato plants. Five types of adult whiteflies used in the experiment were (1) non-viruliferous whiteflies emerged within 48 h on cotton plants, whiteflies viruliferous for (2) CuLCrV and (3) TYLCV, with 48 h AAP on squash and tomato plants, respectively (4) whiteflies that acquired virus from CuLCrV-infected squash with 48 h AAP followed by a 48 h AAP on TYLCV-infected tomato plants, and (5) whiteflies that acquired virus from TYLCV-infected tomato with 48 h AAP followed by a 48 h AAP on CuLCrV-

infected squash plants. The remainder of the experimental set up was same as described above. Each choice test included ten replications, and the experiment was repeated once (n=20 for each choice test).

Whitefly fitness

CuLCrV and/or CYSDV infection in squash:

Whitefly fitness was studied on non-infected, CuLCrV-infected, CYSDV-infected, and mixed CuLCrV-CYSDV-infected squash using clip cages. A pair of non-viruliferous whitefly adults (male and female) were attached to the lower leaf surface of squash plants. After 48 hr, adults were removed, and total number of eggs laid were recorded. Plants with the clip cages were transferred into whitefly-proof cages and maintained in the greenhouse under the conditions described above. Clip cages were observed every morning, and time required to develop from egg to adult was recorded. Each treatment had 10 replications, and the experiment was repeated twice (n=30). Emerged adults were collected for fecundity studies. Pair of whitefly adults (male and female) emerged within 24 h from non-infected, CuLCrV-infected, CYSDV-infected, and mixed CuLCrV-CYSDV-infected squash were clip caged on cotton. For next three weeks, every week, whitefly adults were transferred to new clip cages attached to cotton leaves. Egg bearing leaves were excised from the plants, and number of eggs laid were recorded under the dissecting microscope (10X). Each treatment had 10 replications, and the experiment was conducted for a total of three times (n=30).

CuLCrV and/or TYLCV infection in whiteflies:

The fecundity of four types of whiteflies i.e. non-viruliferous, viruliferous for CuLCrV, TYLCV, and for both CuLCrV-TYLCV was measured on cotton plants. A pair of non-viruliferous whitefly adults (male and female) were clip caged to the lower leaf surface of respective hosts, and were provided with a 48h AAP and transferred to cotton. For next three weeks, every week, whitefly adults were transferred to new clip cages attached to cotton leaves, and fecundity was estimated as described above for squash. Each treatment had seven replications and the experiment was repeated once (n=14).

Statistical Analyses

Data analyses were performed in R version 3.4.2 (The R Foundation for Statistical Computing). Differences in CuLCrV and CYSDV accumulation in squash and whiteflies and TYLCV accumulation in tomato and whiteflies, and whitefly fitness parameters *(*Whitefly settling & fecundity) were analyzed using one-way ANOVA and means were separated with Tukey's HSD posthoc test ('aov' and 'TukeyHSD' functions in R). Whitefly developmental time (egg to adult) was analyzed using a non-parametric test (Kruskal-Wallis) in R.

Results

Virus accumulation

CuLCrV and/or CYSDV infection in squash and whiteflies:

CuLCrV- and CYSDV-infected plants exhibited typical symptoms associated with their infection and were in general stunted and smaller than non-infected plants (Fig. 2. 2). Mixed-infected plants (CuLCrV-CYSDV) had more severe symptoms than CuLCrV/CYSDV infected plants (Fig 2. 2). CuLCrV accumulation between CuLCrV-infected versus mixed-infected (CuLCrV-CYSDV) squash plants was not significantly different (F= 1.939; df=1,58; *P*=0.16) (Fig. 2. 3A). On the contrary, CYSDV accumulation in mixed-infected (CuLCrV-CYSDV) squash plants was substantially lower than in CYSDV-infected plants (*F* = 11.85; df=1,58; *P*< 0.0001) (Fig. 2. 3B). Consistent with the virus accumulation in squash plants, both CuLCrV and CYSDV accumulation in whiteflies showed a similar trend. CuLCrV accumulation in whiteflies that fed on individual CuLCrV-infected versus mixed infected (CuLCrV-CYSDV) squash plants was not significantly different (F= 2.465; df=1,118; *P*=0.120) (Fig. 2. 3C). However, whiteflies feeding on mixed-infected (CuLCrV-CYSDV) squash acquired significantly fewer copies of CYSDV than whiteflies feeding on CYSDV-infected squash plants (*F* = 26.85; df= 1,118; *P*< 0.0001) (Fig. 2. 3D).

CuLCrV and/or TYLCV in whiteflies:

CuLCrV accumulation in squash plants was significantly lower than TYLCV accumulation in tomato plants (F= 17.940; df=1,38; *P*=0.0004) (Fig. 2. 4A).

CuLCrV accumulation in singly-infected whiteflies following a 48 h AAP on CuLCrV-infected squash was higher than in mixed-infected (CuLCrV-TYLCV) whiteflies (F= 15.291; df=2,78; *P*<0.0001) (Fig. 2. 4b). In the mixed-infected whiteflies, the sequence of virus acquisition by whiteflies significantly altered CuLCrV accumulation in whiteflies, in that whiteflies acquiring CuLCrV after TYLCV had lower accumulation of CuLCrV than the whiteflies acquiring CuLCrV before TYLCV.

On the contrary, TYLCV accumulation in singly-infected whiteflies following a 48 h AAP on TYLCV-infected tomato was not different from TYLCV-infection in mixed-infected whiteflies, irrespective of the order of TYLCV acquisition (F= 0.342; df=2,84; *P*= 0.70) (Fig. 2. 4c).

Whitefly settling

CuLCrV and/or CYSDV infection in squash and whiteflies:

Significant differences in settling of non-viruliferous whiteflies were observed between non-infected squash versus mixed-infected (CuLCrV-CYSDV) (F= 83.8; df=1,58; *P*<0.0001), non-

infected squash versus CuLCrV-infected (F= 400; df=1,58; *P*<0.0001), and non-infected squash versus CYSDV-infected squash (F= 10.5; df=1,58; *P*<0.0001) was significantly different (Figs. 2. 5A-C)

viruliferous whiteflies also showed similar trends, in that settling differences were observed between non-infected versus mixed-infected (CuLCrV-CYSDV) squash (F= 3234.02; df=1,58; *P*<0.0001), non-infected versus CuLCrV-infected squash (F= 68.10; df=1,58; *P*<0.0001), and non-infected versus CYSDV-infected squash (F= 1850.32; df=1,58; P<0.0001) (Figs. 2. 5D-F). For both non-viruliferous and viruliferous (CuLCrV and/or CYSDV) whiteflies, the percent settling was significantly higher on non-infected versus virus-infected squash regardless of virus type.

CuLCrV and/or TYLCV in whiteflies:

Significant differences in percent settling of non-viruliferous versus viruliferous whiteflies were observed between non-infected and virus-infected squash and tomato plants. On squash, settling of all four whitefly types i.e. non-viruliferous (F= 70.2; df=1,38; *P*<0.001), viruliferous for CuLCrV (F= 29.3; df=1,38; *P*<0.0001), TYLCV (F= 6.9; df=1,38; *P*=0.015) and a combination of CuLCrV and TYLCV (F= 33.4; df=1,38; P<0.001) was substantially higher on non-infected plants when compared with infected plants (Figs. 2. 6A-D). On the contrary, non-viruliferous whiteflies preferred TYLCV-infected tomato plants over the non-infected plants (F= 5.03; df=1,38; *P*=0.035) (Fig. 2. 6E). However, the viruliferous whitefly settling on tomato showed a similar trend as observed on squash, in that whiteflies that acquired TYLCV (F= 7.19; df=1,38;

P=0.013), CuLCrV (F= 9.31; df=1,38; *P*=0.02), and both CuLCrV and TYLCV (F= 17.7; df=1,38; *P*<0.0001) (Figs. 2. 6F-H) significantly preferred to settle on non-infected tomato plants over virus-infected plants.

B. tabaci **MEAM1 fitness**

CuLCrV and/or CYSDV infection in squash:

B. tabaci was able to complete its life cycle on non-infected, and singly- as well as mixed-infected (CuLCrV-CYSDV) squash with. Median development time from egg to adults did not differ between whiteflies developing on any of the squash plants regardless of infection status (X^2 = 0.3848; df=3,636; *P*> X^2=0.9434). Similarly, no significant differences were observed in the fecundity of whiteflies developed on any of the squash plants regardless of the plant infections status (F= 0.827; df= 3,116; *P*=0.481) (Table 2. 1A).

CuLCrV and/or TYLCV in whiteflies:

Fecundity of all 4 whitefly types i.e. non-viruliferous, viruliferous for CuLCrV, TYLCV and a mixture of CuLCrV and TYLCV on cotton did not significantly differ between non-viruliferous or viruliferous whiteflies (F= 1.43; df=3,46; *P*= 0.240) (Table 2. 1B).

Discussion

We examined two facets of mixed virus infections in this study: 1. Mixed virus infections in plants and in vectors following acquisition of viruses from the same host, and 2. Mixed virus infections in the vector (whitefly) following acquisition of viruses from multiple hosts. These studies were conducted using two important whitefly-virus vegetable pathosystems representative of the southeastern United States. For the first facet, we studied the mixed-infection of multiple viruses in squash and consequently in the vector (whitefly). For the second facet, we studied mixed-infection in the vector (whitefly) that acquired virus from multiple host plants (tomato and squash). Overall, our plant virus accumulation results in squash show harmony with previous studies documenting neutral and antagonistic interactions between plant viruses in mixed infections **(**Syller, 2011**).** Our results also indicate that mixed-infections of viruses in host plants and within the vector could differentially influence vector preference, and virus accumulation and epidemics in a context specific manner.

In squash, both CuLCrV and CYSDV are phloem restricted viruses, and their interactions in mixed-infected (CuLCrV-CYSDV) squash appeared to be neutral and antagonistic. CuLCrV accumulation was not significantly different in singly- versus mixed-infected squash plants. On the contrary, CYSDV accumulation was substantially reduced in the mixed-infected than singly-infected plants. Numerous studies have examined virus accumulation in mixed-infected plants involving whiteflies and have observed results across the interaction spectrum (facilitative-neutral-antagonistic) (Calap et al., 2019; Chen et al., 2018; Crespo et al., 2019; Wintermantel et al., 2008). A recent study by Calap et al., (2019) showed partly contrasting results than ours in a

similar mixed virus pathosystem: CYSDV and watermelon mosaic virus (WMV) transmitted by whiteflies and aphids, respectively in melon. This study showed reduced levels of WMV and higher levels of CYSDV in CYSDV-WMV infected melon plants compared with single infections. The key difference in ours versus Calap et al., (2019) study was that CYSDV accumulated in substantially lower levels in CuLCrV-CYSDV mixed infected squash plants, whereas it accumulated in higher levels in CYSDV-WMV infected melon plants. This suggests context-specificity in interactions in the mixed virus pathosystems, and such interactions could have variable consequences for the transmission and spread of each virus. With whiteflies being phloem feeders, their ability to acquire viruses seem to follow a density-dependent pattern, whiteflies that fed on mixed-infected squash plants in this study acquired similar levels of CuLCrV and reduced levels of CYSDV in comparison with singly-infected plants. Our results are in harmony with an earlier study conducted by Wintermantel et al., (2008), which showed that virus transmission efficiency of tomato chlorosis virus (ToCV) and tomato infectious chlorosis virus (TICV) by various whitefly species corresponded with virus concentration in the *Physalis wrightii* (Miers) Sandw. and *Nicotiana benthamiana* Domin in both single and mixed infections.

In the second facet of this study, interactions between two begomoviruses acquired by whiteflies from two different hosts typically present in proximity in a farmscape was examined. When CuLCrV-TYLCV mixed-infected whiteflies acquired CuLCrV either before or after TYLCV with 48 hr AAP for each virus, CuLCrV accumulation was significantly lowered when compared with whiteflies that acquired CuLCrV alone. Furthermore, the sequence of CuLCrV acquisition in the mixed infected whiteflies appear to be crucial for the overall CuLCrV accumulation in

whiteflies. For instance, mixed-infected whiteflies acquiring CuLCrV first had significantly greater number of CuLCrV copies than whiteflies acquiring CuLCrV second. On the contrary, TYLCV accumulation remained unaffected in the mixed infected whiteflies versus singly-infected whiteflies, and the sequence of acquisition of CuLCrV in mixed-infected whiteflies did not alter TYLCV accumulation levels. This could partly be due to significantly higher accumulation of TYLCV in tomato plants than CuLCrV in squash plants. It is also possible that TYLCV—being a predominant virus— outcompeted CuLCrV accumulation in the vector.

Mixed infections of plant viruses, especially phloem-limited viruses, are known to more drastically alter the plant phenotype than single infections. Such alterations could be realized in the form of changes in plant biochemistry (increased soluble sugars & free amino acids), increased defense suppression, and enhanced visual apparency to vectors in mixed-infected than singly-infected plants (Gil-Salas et al., 2011, 2012; Srinivasan and Alvarez 2007, Wintermantel et al., 2008). Consequently, mixed-infected plants could be more attracted to vectors, and provide more fitness benefits to vectors than singly-infected plants (Srinivasan and Alvarez, 2007). In the current squash study system, mixed-infected plants were more symptom expressive when compared with either CuLCrV- or CYSDV-infected plants. Despite the enhanced phenotype (visual) apparency to whiteflies in mixed-infected squash plants, non-viruliferous whiteflies preferred to settle on non-infected whiteflies. In an ideal epidemic-inducing scenario, increased attraction of non-viruliferous whiteflies to virus-infected hosts and vice versa would be the norm. Such epidemic-conducive relationships have already been observed in one of our earlier studies in a whitefly-begomovirus system (Legarrea et al., 2015). On the contrary, in the current squash viral pathosystem, both non-viruliferous and viruliferous

(CuLCrV and/or CYSDV) preferred to settle on non-infected plants. It is not clear how such a preference towards non-infected plants would be beneficial for utilizing inoculum sources for virus acquisition and initiating epidemics. Perhaps, such preferences can become irrelevant when overwhelming vector densities (population explosions) are present in a farmscape. In the Southeastern United States CuLCrV and CYSDV epidemics more often coincide with heavy whitefly pressure when compared with TYLCV epidemics. TYLCV epidemics seem to be more consistent and often occur even under moderate whitefly pressure. Settling patterns following mixed infection (CuLCrV-TYLCV) in whiteflies following acquisition from multiple hosts were not different than singly-infected whiteflies, and remained consistent with results observed with an earlier study on TYLCV (Leggarrea et al. 2015) as well as in facet 1 of this study. In another study, Fereres et al., (2016) reported partly harmonious results with our CuLCrV study, in that viruliferous whiteflies carrying the persistent circulative begomovirus tomato severe rugose virus (ToSRV) clearly preferred non-infected tomato plants.

Besides settling, mixed infections of viruses in host plants have been known to offer additional fitness benefits than single infections (Jiu et al., 2007, Srinivasan and Alvarez, 2007). No significant differences in the life history of whiteflies on either non-infected or any of the virus infected squash plants were observed. We speculate that no changes in whiteflies fitness in the present study were possibly due to the lack of CuLCrV or CYSDV-mediated modulation of the visual, olfactory and gustatory (sugars and amino acids) cues in singly- or mixed-infected squash plants. Since whiteflies naturally preferred non-infected squash plants, volatile manipulation may not be required for CuLCrV spread. Our findings show disparity with the earlier work showing that the mixed viral infections affect the fitness of virus vectors (Chen et al., 2018; Fereres et al.,

2016; Jiu et al., 2007; Srinivasan and Alvarez, 2007). In particular, a similar study by Jiu et al., (2007) showed that the mixed infections of two begomoviruses tobacco curly shoot virus (TbCSV) and tomato yellow leaf curl China virus (TYLCCNV) in tobacco provide fitness benefits to their insect vector, *B. tabaci* MEAM1. However, the fitness benefits of whiteflies infected with two begomoviruses (CuLCrV& TYLCV) in this study did not differ from that of singly-infected whiteflies. A number of previous studies have shown contrasting results, in that begomovirus-infected whiteflies tend to show greater fitness on begomovirus-infected plants when compared with non-infected plants (Liu et al., 2010; Moreno-DeLafuente et al., 2013; Maluta et al., 2014; Shi et al., 2014; Wang et al., 2012). This fitness study involving single and mixed-infections of (CuLCrV and/or TYLCV) was evaluated a non-host of both viruses, and results suggest that at least in this begomovirus system a lot of the fitness benefits could be driven by suitable host plants rather than by the virus infection itself.

In mixed infections, the ability of each virus to adapt to the host (infectivity, movement etc.), to interact with their counterparts— synergistically and/or antagonistically and with their vectors predominantly determine their fitness, transmission and spread (Elena et al., 2014; Martin and Elena, 2009). Plant viruses that adapt well to most, if not all of these conditions, thrive well in mixed infection in both host and in vectors (Syller and Grupa, 2014). More specifically, a few predominant factors that determine virus success in mixed infection include (i) rapid multiplication in host, (ii) better exploitation of host resources, (iii) efficient movement from cell-to-cell and plant-to-plant (vector-mediated), (iv) efficient silencing suppression capability, and (v) successful evasion of plant defenses. A number of these factors often lead to one of the viruses in mixed infection pathosystems being predominant and thus antagonizing their

counterpart in varying magnitudes. For instance, in present study, CuLCrV appeared to antagonize CYSDV accumulation in mixed infected squash plants and TYLCV appeared to antagonize CuLCrV accumulation in whiteflies. It is not clear if each of these interactions is likely to have variable consequences on the epidemics of interacting viruses.

Concluding remarks

Plant-virus-vector interactions in a pathosystem are dynamic and complex. Multiple viruses co-infecting host plants and vectors add another layer of complexity to these highly specific interactions, and often differentially impact the transmission and spread of each of the partnering viruses (Mauck et al., 2012; Syller, 2014; Syller and Grupa, 2014). This study examined two facets of mixed-virus infections in hosts plants and in the whitefly vector. The first facet of mixed-virus infections in plants has already been explored in numerous pathosystems, and their interactions range from facilitative, neutral, and antagonistic. Synergistic interactions in several instances seem to have enhanced the fitness of vectors. On the contrary, mixed infection (CuLCrV-CYSDV) examined in squash seems to be antagonistic to one of the viruses (CYSDV) and did not yield any fitness benefits to whiteflies in this study. The fact that squash is already a preferred host for whiteflies, and that mixed (CuLCrV-CYSDV) infections might be extremely altering the plant chemical and visual phenotype in a fashion that might be limiting to whitefly feeding and fitness, further studies on host plant biochemical profiles (free amino acids, soluble sugars, and volatile organic compounds) could help comprehend this phenomenon better. The Southeast United States vegetable production farmscapes are diverse, and often presents a scenario in which multiple virus hosts are present along with multiple virus infections in a host.

Consequently, mixed infections within vectors either acquiring multiple viruses from the same host as well as from different hosts are a possibility. In both instances, no fitness benefits were identified between whiteflies infected with either one or two viruses. In terms of preference, all the squash-virus laden and non-viruliferous whiteflies preferred non-infected plants, whereas non-virulifeorus whiteflies preferred TYLCV-infected tomato, and viruliferous whiteflies preferred non-infected tomato. The ideal epidemic-inducing settling preference scenario seems to favor TYLCV and not squash viruses. This indicates appearance to vectors is very different between hosts within the same farmscape, whereas in one scenario (TYLCV-tomato) a directed settling could favor epidemics, whereas in the squash scenario, a more random dispersal could aid in virus spread. These findings reiterate that effects of mixed-infections in hosts and in vectors could vary with each pathosystem and have variable ecological and epidemiological implications.

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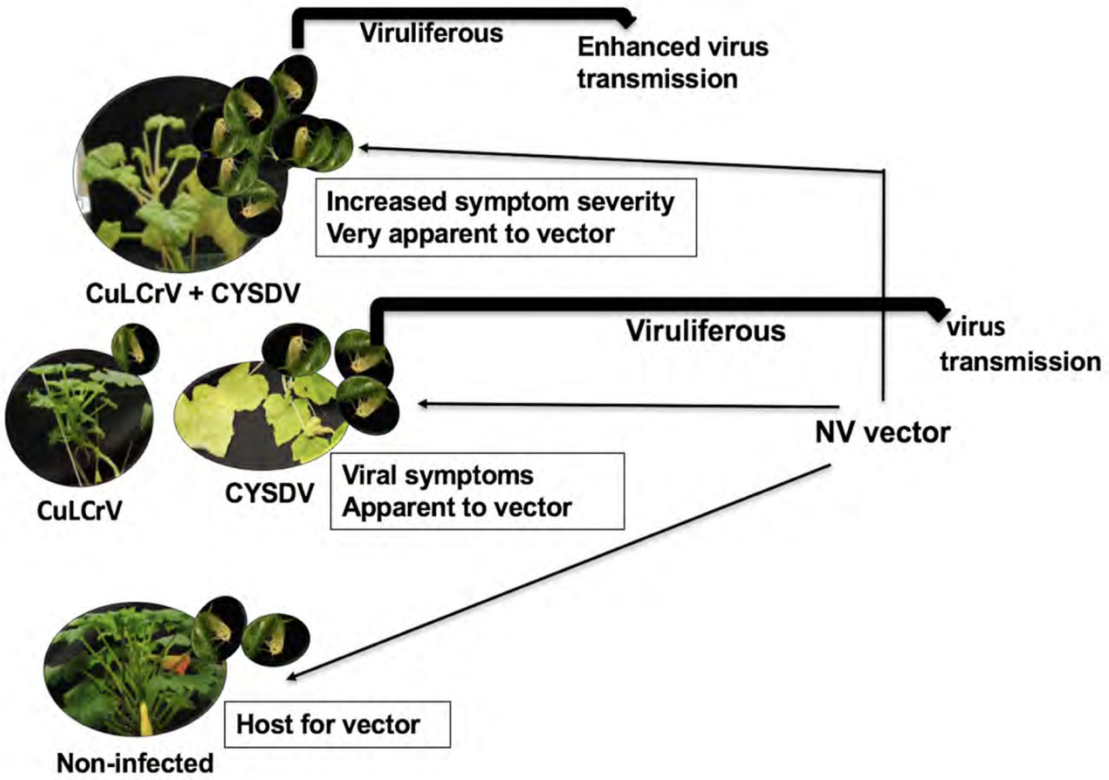

Figure 2. 1. Model for virus acquisition between single and mixed infections in squash.

Model explaining the hypothesis that mixed-infected plants would accumulate more amounts of one or two component viruses, have a severely-altered phenotype, consequently, are more attractive to the vector, and enhance the acquisition and inoculation of one or more component viruses than singly-infected plants

Fig. 2

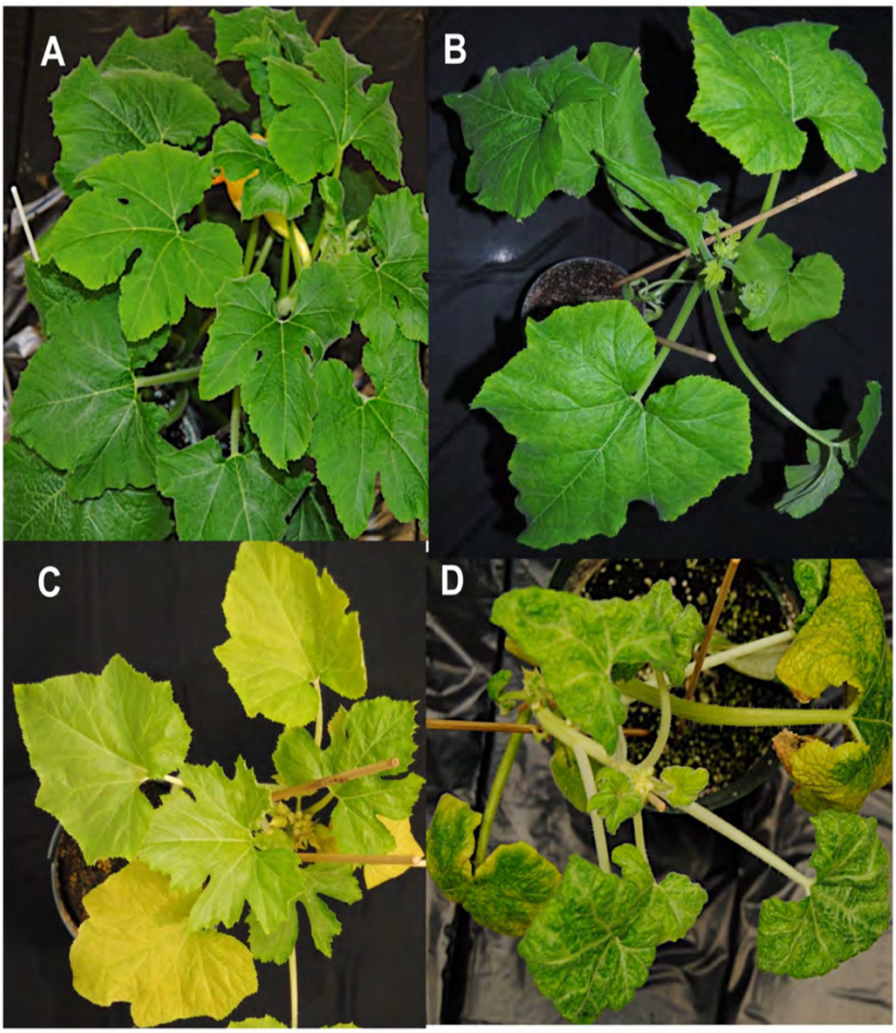

Figure 2. 2. Photographs of (A) non-infected, (B) CuLCrV-infected, (C) CYSDV-infected, and (D) mixed-infected (CuLCrV and CYSDV) squash plants. Virus-infected squash plants were inoculated with ~100 viruliferous whitefly adults following a 48h acquisition access period on inoculum sources. Photographs were taken approximately four weeks post inoculation.

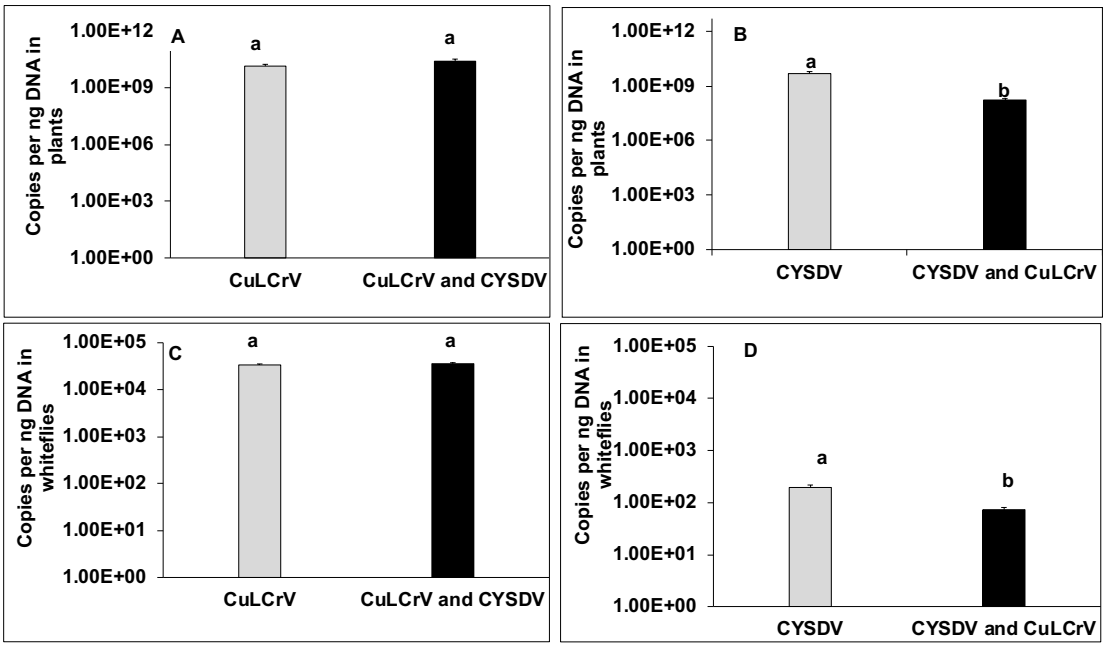

Figure 2. 3. CuLCrV and CYSDV accumulation in singly-infected (CuLCrV or CYSDV) versus mixed (CuLCrV&CYSDV)-infected squash and whiteflies

Bars with standard errors represent average number of CuLCrV and CYSDV copies per ng DNA in squash or whiteflies: (A) CuLCrV accumulation in CuLCrV-infected versus mixed-infected (CuLCrV-CYSDV) squash (B) CYSDV accumulation in CYSDV-infected versus mixed-infected (CuLCrV-CYSDV) squash (C) CuLCrV accumulation in CuLCrV-infected versus mixed-infected (CuLCrV-CYSDV) whiteflies (D) CYSDV accumulation in CYSDV-infected versus mixed-infected (CuLCrV-CYSDV) whiteflies. CuLCrV and CYSDV copy numbers were estimated by qPCR followed by absolute quantification using plasmids containing CuLCrV AV1 gene or 5′ non-coding region of CYSDV RNA 2 inserts as standards. Different letters on bars indicate significant differences between means separated with Tukey's HSD posthoc test at $\alpha = 0.05$. Y-axis represents a logarithmic scale.

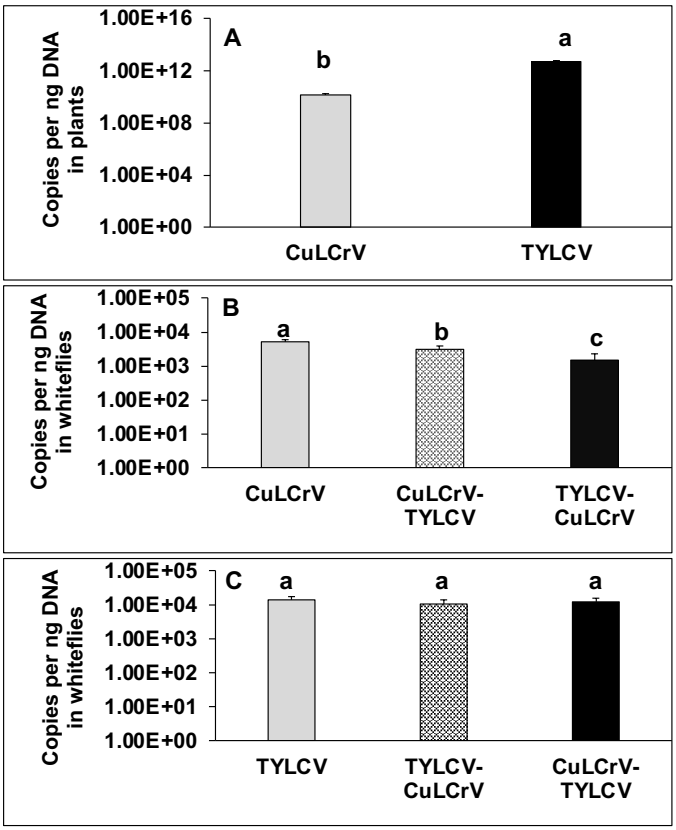

Figure 2. 4. CuLCrV and TYLCV accumulation in individually infected plants, and singly- and mixed-infected whiteflies

Bars with standard errors represent average number of CuLCrV and TYLCV copies per ng DNA: (A) CuLCrV or TYLCV copies accumulated in CuLCrV-infected squash and TYLCV-infected tomato (B) CuLCrV accumulation in whiteflies acquiring virus from CuLCrV-infected squash plants alone (48 h AAP) versus CuLCrV accumulation in mixed-infected whiteflies acquiring CuLCrV and TYLCV sequentially (48 h AAP each) (C) TYLCV accumulation in whiteflies acquiring virus from TYLCV-infected tomato plants (48 h AAP) alone versus TYLCV accumulation in mixed-infected whiteflies acquiring CuLCrV and TYLCV sequentially (48 h AAP each). Copy numbers for CuLCrV and TYLCV were estimated by qPCR followed by absolute quantification using plasmids containing CuLCrV AV1 gene or TYLCV C2 gene inserts as standards. Different letters on bars indicate significant differences between means separated with Tukey's HSD posthoc test at $\alpha = 0.05$. Y-axis represents a logarithmic scale.

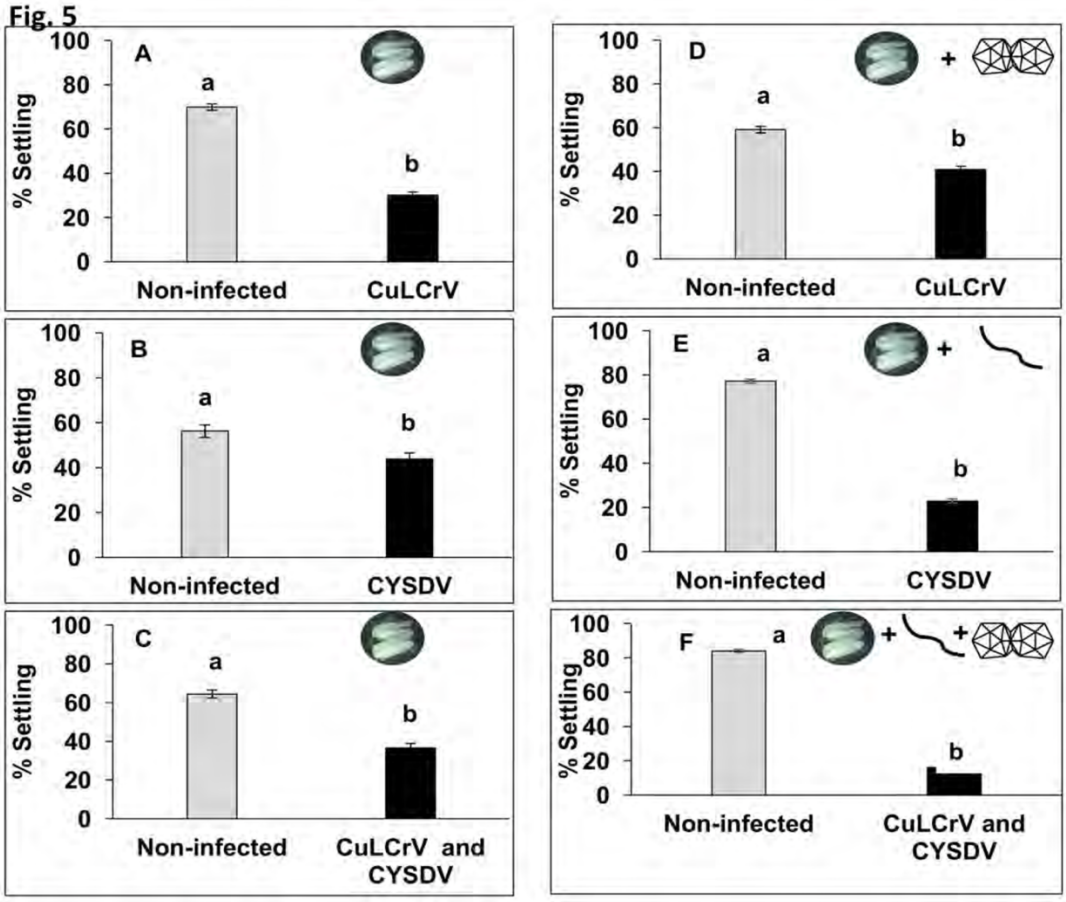

Figure 2. 5. Settling of non-viruliferous and viruliferous whiteflies on non-infected and virus-infected squash plants

Bars with standard errors indicate percent settling of non-viruliferous (A-C) and viruliferous (D-F) whiteflies 24h after release on non-infected versus singly- as well as mixed- (CuLCrV and CYSDV) infected squash leaves. Different letters on bars indicate significant differences between means separated with Tukey's HSD posthoc test at $\alpha = 0.05$.

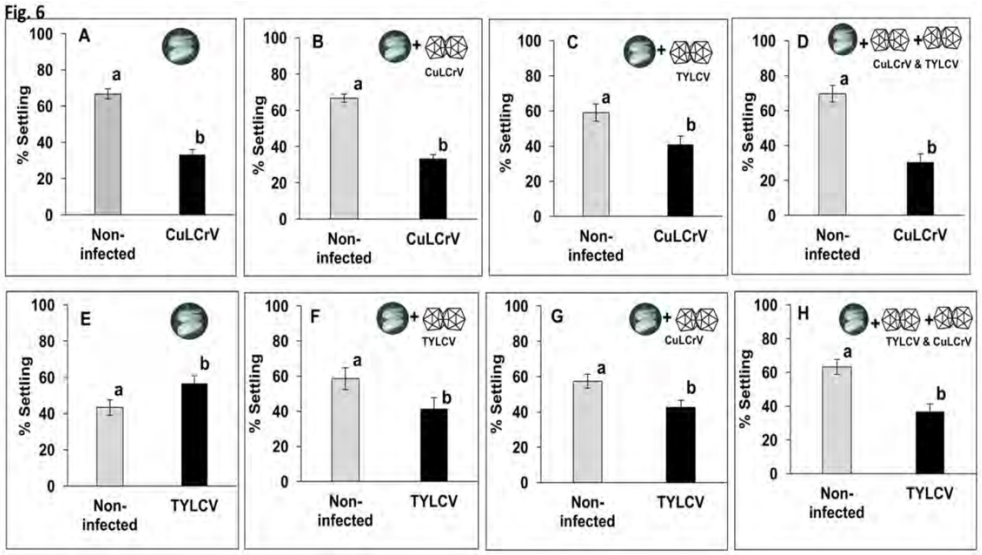

Figure 2. 6. Settling of non-viruliferous and viruliferous whiteflies on multiple virus-infected and non-infected hosts.

Bars with standard errors indicate percent settling of four types of adult whiteflies: 1) non-viruliferous (NV); whiteflies independently viruliferous for 2) CuLCrV; and 3) TYLCV; and 4) for both TYLCV and CuLCrV. Percent settling of four types whiteflies 24h after release on (A-D) non-infected versus CuLCrV -infected squash leaves, and (E-H) Percent settling of four types whiteflies 24h after release on (A-D) non-infected versus TYLCV -infected tomato leaves. Different letters on bars indicate significant differences between means separated with Tukey's HSD posthoc test at $\alpha = 0.05$.

Table 1. Developmental time of whiteflies on non-infected and virus-infected (CuLCrV and/or CYSDV) squash

Treatments	**N[x]**	**Egg-adult[Y]**
Non-infected	147	23 (19-32)
CuLCrV	165	22 (18-30)
CYSDV	172	22 (18-33)
CuLCrV & CYSDV	156	23 (20-31)
$X^2 = 0.3848$		
Df = 3, 636		
P> X^2 = 0.9434		

[x] = number of eggs monitored to adulthood.
[y]= median development time from egg to adult with range in parentheses.

Table 2. A) Fecundity (mean ±SE) of whiteflies that developed on non-infected and virus-infected (CuLCrV and/or CYSDV) squash, and B) Fecundity (mean ±SE) of non- viruliferous, and viruliferous (CuLCrV and/or TYLCV) whiteflies on cotton.

(A) CuLCrV and/or CYSDV infection in whiteflies

Treatments	**Fecundity** [x]
Non-infected	58.4 ±15.2 a
CuLCrV	47.4 ±12.1 a
CYSDV	54.4± 7.4 a
CuLCrV and CYSDV	51.4 ± 4.1 a
F = 0.827	
Df = 3, 116	
P = 0.481	

((B) CuLCrV and/or TYLCV infection in whiteflies

Treatments	**Fecundity** [y]
Non-viruliferous	29.7 ± 8.1 a
CuLCrV	26.2 ± 4.1 a
TYLCV	26.7 ± 4.7 a
CuLCrV and TYLCV	31.6 ± 5.2 a
F = 1.43	
Df = 3, 46	
P = 0.24	

x Fecundity of non-viruliferous and viruliferous (CuLCrV and/or CYSDV) whiteflies that developed on respective squash plants and clip caged on cotton for three weeks.

y Fecundity of non-viruliferous and viruliferous (CuLCrV and/or TYLCV) whiteflies that acquired CuLCrV and TYLCV from squash and tomato, respectively on cotton for three weeks.

CHAPTER 3

EFFECTS OF HOSTS AND THEIR INFECTION STATUS ON ACQUISITION AND TRANSMISSION OF CULCRV BY WHITEFLIES, *BEMISIA TABACI* MIDDLE EAST-ASIA MINOR 1

Gautam, S., Gadhave, K. R., Buck, J. W., Dutta, B., Coolong, T., Adkins, S., Srinivasan, R. To be submitted to Plant Disease

Abstract

Bemisia tabaci Gennadius (MEAM1)-transmitted cucurbit leaf crumple virus (CuLCrV) is a serious production problem for squash growers in the Southeastern United States. CuLCrV is often found with another whitefly-transmitted virus, cucurbit yellow stunting disorder virus (CYSDV). CuLCrV also infects snap bean under field conditions and tobacco under experimental conditions. No information is available on how these various hosts and their infection status influence the acquisition and transmission of CuLCrV by whiteflies. In this study, we compared the whitefly-mediated CuLCrV transmission from mixed (CuLCrV and CYSDV; squash) vs singly-infected (CuLCrV; snap bean and tobacco) plants to squash. Whiteflies feeding on mixed-infected squash and singlytobacco accumulated significantly higher CuLCrV-DNA than whiteflies feeding on snap bean. Squash infected from whiteflies feeding on mixed-infected squash and singly-infected tobacco had severe disease phenotype and accumulated significantly higher CuLCrV-DNA than squash infected from whiteflies feeding on CuLCrV-infected snap bean. Furthermore, using squash with different levels of CuLCrV accumulations and infection status (mixed or single) as inoculum sources, transmission assays were carried out to evaluate squash-to-squash transmission of CuLCrV. Irrespective of infection status of squash (mixed or single), CuLCrV-DNA accumulation in whiteflies was dependent on the virus accumulation in squash. Differential CuLCrV-DNA accumulations in whiteflies resulted in differential transmission and CuLCrV-DNA accumulation in squash following inoculation access. Overall, results demonstrate CuLCrV accumulation in whiteflies depends on the CuLCrV accumulation in host plants, and differential CuLCrV accumulation in whiteflies can significantly affect the CuLCrV transmission and subsequent virus spread.

Introduction

Successful survival of plant viruses in the natural ecosystem depends on the availability of susceptible hosts (Cunniffe and Gilligan 2010). Generally, most emerging and damaging pathogens have broad host ranges that ensure maintenance in the ecosystem (Haydon et al. 2002). Further, most emerging pathogens are vector-transmitted (Woolhouse et al. 2001). Vectors can transmit multiple viruses simultaneously, leading to mixed infections in host plants (Gilbertson et al., 2015). Co-infections can alter the disease phenotype, plant biochemistry, and can influence the vector-plant interactions and subsequent transmissions (Peñaflor et al. 2016; Pinto et al. 2008; Wintermantel, 2005). Therefore, interactions between vector, host plants, and co-infected viruses can influence the reservoir potential of host plants.

Mixed infections are ubiquitous in nature (Mascia and Gallitelli 2016; Roossinck et al. 2015; DaPalma et al. 2010) and many co-infecting viruses interact synergistically (Syller, 2012). Several agriculturally important plant viruses produce enhanced disease phenotypes in mixed infection status (Rentería-Canett et al. 201, Li et al. 2015, Wintermantel et al., 2008, Gil-Salas et al., 2011, 2012; Srinivasan and Alvarez 2007). Insects are the most important vectors of plant-infecting viruses (Eigenbrode et al. 2018; Fereres and Moreno, 2009). Polyphagous vectors such as whitefly, *Bemisia tabaci* Middle East-Asia Minor 1 (MEAM1) (Gennadius) (Hemiptera: Aleyrodidae) moving through a habitat may encounter multiple host plants (reservoir hosts) and acquire viruses during the process, resulting in single- or mixed-infections in subsequently encountered susceptible host plants. In the Southeastern United States, MEAM1 transmits a wide range of viruses (Adkins et al. 2011).

Recently, a whitefly-transmitted virus, cucurbit leaf crumple virus (CuLCrV) (genus, *Begomovirus)* (Larsen and Kmiecik 2010) is increasingly becoming important in the region, especially in squash production. CuLCrV is transmitted by MEAM1 in a persistent and circular manner. Once acquired by the whitefly, CuLCrV can remain associated with it for rest of the life span. CuLCrV can infect many plant species in the family *Cucurbitaceae,* and certain cultivars of common bean (*Phaseolus vulgaris* L.) (Brown, et al. 2002, Hagen et al. 2008, Hernandez et al. 2001). Furthermore, tobacco has been reported as an experimental host (greenhouse) for CuLCrV (Brown et al. 2002). CuLCrV was first reported in the US in the Imperial Valley of California in 1988 (Guzman et al. 2000). It was first detected in the Southeastern US in 2006 in Florida (Akad et al. 2008); and in 2010 was detected in snap beans in Georgia (Larsen and Kmiecik 2010). Recently, CuLCrV has been detected in cucurbits in South Carolina, demonstrating its expanding geographical range (Keinath et al. 2018). CuLCrV is often found with another whitefly-transmitted virus, cucurbit yellow stunting disorder virus (CYSDV) (genus *Crinivirus* and family *Closteroviridae*) (Kuo et al. 2007). CYSDV is a semipersistent virus, and once acquired by whiteflies feeding on infected plants, whiteflies can remain viruliferous for up to 9 days (Celix et al. 1996, Wisler et al. 1988). CYSDV can infect members of the *Cucurbitaceae,* lettuce (*Lactuca sativa* L.*)*, alfalfa (*Medicago sativa* L.), and snap bean (*P. vulgaris* L.), and many wild weed species (Wintermantel et al. 2009).

Squash and snap bean are two commercially important hosts of CuLCrV in Southeastern United States. Snap bean shares a growing season with squash in Georgia. Both in squash and in certain cultivars of snap beans, CuLCrV is found to be mixed infected with CYSDV (Kuo et al. 2007; Larsen and Kmiecik 2010; Wintermantel et al 2009). The frequency with which viruses co-infect

the same host depends on the affinity for co-existence (Ludwig and Reynolds 1988). Therefore, frequent co-infections of CuLCrV with CYSDV raise two important epidemiological questions. First, how does the infectious status (single/mixed infection) affect the ability of whiteflies to acquire and transmit CuLCrV? Second, how much does inoculum source infection status (single/mixed infection) affect the CuLCrV disease epidemic?

In order to answer the above questions, we used squash infected with CuLCrV and CYSDV (hereafter referred to as "mixed-infected squash") as an inoculum source for CuLCrV and conducted a series of MEAM1-mediated transmissions bioassays (Fig. 1). (i) CuLCrV transmission from mixed-infected squash to tobacco, snap bean, and squash
(ii) CuLCrV transmission from mixed- (squash) vs singly-infected (tobacco and snap bean) plants to squash (iii), CuLCrV transmission from mixed- vs singly-infected squash to squash.

Materials and methods

Plants and insects

Three different plant species were used. Yellow summer squash (*Cucurbita pepo* cv. "Gold Star, F1 hybrid") and snap bean (*P. vulgaris* cv. "Provider") were procured from Johnny's Selected Seeds (Winslow, ME, USA). Tobacco (*Nicotiana tabacum* cv. "L326") seeds were obtained from UGA extension services. Throughout these studies, two-week old squash, four-week-old tobacco, and four-week-old snap bean seedlings were used for transmission experiments. Plants were

maintained in a greenhouse (25–30°C with a 14h L:10h D photoperiod) in insect-proof cages (Megaview Science Co., Taichung, Taiwan), @ 5 plants per cage. The whiteflies (*B. tabaci* cryptic species MEAM1) used in the present study were first collected in Tifton, Georgia, and have been reared on cotton plants in whitefly-proof cages in the greenhouse at above-stated conditions (Legarrea et al. 2015). The purity of the colony was periodically confirmed (once every few months) by partially sequencing the mitochondrial cytochrome oxidase I (COI) gene (Frohlich et al., 1999).

Virus maintenance

Inoculum source

In September 2017, fifteen whitefly-infested squash plants showing symptoms of begomovirus and crinivirus infections (crumpling and yellowing) were collected from a research plot in Tifton, GA, which served as initial inoculum sources. Symptomatic leaves were excised and surface sterilized using a two-step surface sterilization. Leaves were first washed in autoclaved distilled water, followed by 1 min rinsing in 1% bleach, followed by a 1 min wash in 70% ethanol, and finally three rinses with sterile distilled water to remove the sterilizing agents. 100 mg of surface-sterilized leaf tissues were used for the DNA/RNA extractions. Total genomic DNA was extracted with the GeneJET Plant Genomic Purification Kit (ThermoFisher Scientific, MA, USA) following the manufacturer's protocol. The presence of CuLCrV was tested using primers 3FAC3 (5′-TTTATATCATGATTTTCGAGTACA-3′) and 5RAC1

(5′-AAAATGAAAGCCTAAGAGAGTGGA-3′) targeting the 525 bp amplicon of AC3, AC2 and AC1 genes of the CuLCrV DNA-A component. PCR was performed with 2X GoTaq Green Master Mix (Promega, Madison, WI) using an Eppendorf Mastercycler Pro thermocycler (Eppendorf, Hauppauge, NY). The 10 µl PCR mixture contained 5 µl of Master Mix, 0.5 µM forward and reverse primers, 20 ng DNA, and nuclease-free water. The PCR conditions were: 5 min of initial denaturation at 94°C, followed by 40 cycles of 94°C for 1 min, 54°C for 30 sec, 72°C for 1 min, and a final extension of 72°C for 10 min. PCR products were analyzed by 1% agarose gel electrophoresis. PCR products were cloned using pJET1.2 cloning vector (Thermo Fisher Scientific, Waltham, MA) following the manufacturer's guidelines. The integrity of inserts was confirmed by sequencing of purified plasmids (Eurofins Genomics, Louisville, KY). The obtained sequences (MN543080-81) showed >99% identity to deposited CuLCrV sequences in the NCBI database.

For CYSDV, total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA) following the manufacturer's guidelines, and subjected to cDNA synthesis using the GoScript Reverse Transcription System *(*Promega, Madison, WI*).* The cDNA was amplified using CYSCPf (5′-ATGGCGAGTTCGAGTGAGAATAA-3′) and CYSCPr

(5′ -ATTACCACAGCCACCTGGTGCTA-3′) primers, which target a 755 bp of the coat protein gene (Rubio et al. 2001). The PCR mixture was prepared using 2X GoTaq Green Master Mix as described above. PCR cycling conditions were: 5 min of denaturation at 94°C followed by 40 cycles at 94°C for 30 sec, 50°C for 45 sec, 72°C for 2 min, and a final extension at 72°C for 5 min. PCR products were analyzed by 1% agarose gel electrophoresis. The samples were cloned, sequenced and percent homology was determined as described above. The obtained sequences

(MN557851-52) showed >99% identity to deposited CYSDV sequences in the NCBI database. The presence of another probable whitefly-transmitted virus was ruled out by testing for squash vein yellowing virus (SqVYV) by RT-PCR (Adkins et al., 2007).

Single- and mixed-infection sources

Field-collected, mixed-infected (CuLCrV and CYSDV) squash plants were used as an inoculum source to obtain the singly-infected (CuLCrV) plants. Using viruliferous whiteflies, CuLCrV was transferred from mixed-infected squash to tobacco and snap bean via whiteflies. Both tobacco and snap bean got infected only with CuLCrV, not with CYSDV. From CuLCrV-infected tobacco and snap bean, CuLCrV-infected squash plants were generated and maintained through repeated inoculations using viruliferous whiteflies. Mixed-infected (CuLCrV and CYSDV) squash plants were generated and maintained through repeated inoculations using viruliferous whiteflies.

CuLCrV transmission from mixed-infected (squash) to tobacco, snap bean, and squash

Mixed-infected plants were used as a CuLCrV inoculum source. Viruliferous whiteflies were obtained by allowing whiteflies to feed on mixed-infected squash for an acquisition access period (AAP) of 72h. Using clip cages, whiteflies (100 adults/plant) were attached to the first true leaf of the four-week-old tobacco and snap bean, or two-week-old squash, for an inoculation access period (IAP) of 5 days. After four weeks, total DNA/RNA from 100 mg of young leaf tissue was extracted and subjected to PCR analysis for CuLCrV and CYSDV as described above. Each treatment had 10 replications and the experiment was replicated thrice (n=30).

CuLCrV acquisition and transmission from mixed- (squash) vs singly-infected (tobacco and snap bean) plants to squash

Mixed-infected squash and singly-infected snap bean and tobacco were used as sources of inoculum for CuLCrV. Infection status of inoculum sources was confirmed with PCR analysis as described above. Viruliferous or non-viruliferous whiteflies were obtained by allowing whiteflies to feed on infected plants (single or mixed infection) or non-infected for a 72-hr acquisition access period (AAP). Following AAP, whiteflies were attached to cotton plants using clip cages. After 48 h on cotton, whiteflies were collected, and surface sterilized to get rid of honeydew residues using the protocol described above. For CuLCrV, total DNA was extracted from single individual whiteflies using a specially-formulated Chelex resin, InstaGene Matrix (Bio-Rad, Hercules, USA), and subjected to PCR analysis as described above. For CYSDV, total RNA from individual whiteflies was extracted using the RNeasy Micro Kit (QIAGEN, MD, USA) and subjected to cDNA synthesis and, subsequently, PCR analysis. Percent infection of whiteflies was estimated using endpoint PCR as described above. CuLCrV copy numbers in infected whiteflies were estimated using the Quantitative PCR. Quantitative PCR to determine CuLCrV-DNA accumulation in plants and whiteflies was carried out using 2X GoTaq qPCR Master Mix (Promega, Madison, WI) in a Mastercycler ep realplex (Eppendorf, Hauppauge, NY). Primers CuLCrV-QF (5'- CCTCAAAGGTTTCCCGCTCT-3') and CuLCrV-QR (5'- CCGATAGATCCTGGGCTTCC-3') amplifying a 110 bp region of the coat protein gene of CuLCrV were used. GoTaq qPCR Master Mix was combined with forward and reverse primers (final concentration of 0.5 μM), 10 ng DNA, and nuclease-free water for a final reaction volume

of 25 µl. Cycling parameters were as follows: 95°C for 2 min; 40 cycles of 95°C for 1 min, 63°C for 15 sec, and 72°C for 20 sec. Upon completion of the run, melting curve analysis was performed to confirm the specificity of the primer pairs. Each sample was tested in duplicate, and absolute number of copies in the samples were quantified using the standard curve protocol described by Legarrea et al., (2015). Each treatment had 20 replications, and experiments were replicated thrice (n=60).

Using clip cages, viruliferous or non-viruliferous whiteflies (100 adults/plant) were attached to the first true leaf of two-week-old squash and given an IAP of 5 days. Viruliferous or non-viruliferous whiteflies were obtained by allowing whiteflies to feed on infected plants (single or mixed infection) or non-infected for a 72-hr acquisition access period (AAP). Four weeks after the 5-day IAP, total DNA/RNA from 100 mg surface-sterilized young leaf tissue was extracted and subjected to PCR analysis as described above. Percent CuLCrV and/or CYSDV infection in squash was measured using endpoint PCR as described above. CuLCrV copy numbers in infected squash were estimated using the protocol stated below. Each treatment had 10 replications and the experiment was replicates thrice (n=30).

CuLCrV transmission from mixed- vs singly-infected squash to squash

Mixed-infected squash and singly-infected squash (obtained from snap bean and tobacco) were used as sources of inoculum for CuLCrV. CuLCrV transmission from single/mixed- infected squash was carried out using the protocol described above. Percent infection and CuLCrV accumulation in plants and whiteflies was estimated using the protocol described above.

Statistical analysis

Whitefly and plant percentage infection data was evaluated assuming a binomial response (infected vs. noninfected) using logit model in R and CuLCrV accumulation data in plants were analyzed using one-way ANOVA after log transformation. Data was pooled for replications and during analysis replications were considered as random effect and treatments were considered as fixed effect. Means separation was performed using Tukey's HSD posthoc test. CuLCrV accumulation in individual whiteflies was analyzed using the Kruskal-Wallis test with the mean separation analyzed using the Dunn test. Data analyses were performed in R version 3.4.2 (The R Foundation for Statistical Computing).

Results

CuLCrV transmission from mixed-infected (squash) to tobacco, snap bean, and squash

Significantly higher percent plant infection rates were observed in squash comparative to tobacco and snap bean the tested plant species ($\chi 2$ = 86.04*;* df = 2, 80 P <0.0001) (Fig. 3. 2A). Highest infection was observed in squash (93%), followed by tobacco (68%), and the lowest percent infection was observed in snap bean (62%). CuLCrV accumulation differed significantly between the plant species **(**F = 10.43; df = 2, 68; P < 0.0001**).** Highest CuLCrV accumulation was observed in the mixed-infected squash and the lowest accumulation was observed in snap bean (Fig. 3. 2 B).

CuLCrV transmission from mixed- (squash) vs singly-infected (tobacco and snap bean) plants to squash

Percentage infection in whiteflies differed significantly depending upon the host plant species ($\chi 2$ =18.41, df = 2, 177; P< 0.0001). Highest percent infection was observed for whiteflies feeding on mixed-infected squash (95%), followed by tobacco (83%) and snap bean (64%) (Fig. 3. 3 A). CuLCrV accumulation in whiteflies differed significantly depending upon the host plant species (X^2 = 62.304, df = 2, 144, P <0.0001). CuLCrV accumulation in whiteflies feeding on mixed-infected squash was significantly higher than that of whiteflies feeding on tobacco and snap bean (Fig. 3. 3 B). Within single infections, whiteflies feeding on tobacco acquired significantly higher virus than whiteflies feeding on snap bean (Fig. 3. 3 B).

Percent infection and virus accumulation differed in infected squash depending on the CuLCrV source. Highest percent infection was observed in squash infected from mixed-infected squash (84%) and lowest percent infection was observed in squash infected from snap bean (40%) ($\chi 2$ =12.7; df = 2, 81, P <0.0001) (Fig. 3. 3 C). CuLCrV accumulation in infected squash was dependent on the CuLCrV accumulation in the source plants. There was no significant difference in the CuLCrV accumulation in squash infected from mixed-infected squash and tobacco. Squash infected from snap bean accumulated significantly lower amounts of CuLCrV than squash infected from mixed-infected squash or tobacco (F = 628.6; df = 2, 72; P < 0.0001) (Fig. 3. 3 D). Symptom development in squash varied depending upon CuLCrV inoculum source (Fig. 3. 4). Mild symptoms (thickened leaves) developed on squash inoculated from snap bean (Fig 3. 4 E). Squash inoculated from tobacco had characteristic CuLCrV symptoms (stunted growth, curled and

crumpled young leaves) (Fig. 3. 4 F). Mixed-infected squash had symptoms of both CuLCrV and CYSDV. The younger leaves had CuLCrV symptoms (stunted growth, curled and crumpled young leaves), and older leaves had CYSDV symptoms (severe interveinal chlorosis) (Fig. 4. 4 A).

CuLCrV transmission from mixed- vs singly infected squash

CuLCrV percent infection in whiteflies feeding on squash was dependent on the CuLCrV accumulation in squash ($\chi 2$ =10.22, df = 2, 177; P< 0.0001). Whiteflies feeding on mixed- infected squash had the highest percent infection (95%). Within single infections, whiteflies feeding on squash infected from tobacco had a higher percent infection (77%) than squash infected from snap bean (40%) (Fig. 3. 5 A). CuLCrV-DNA accumulation in whiteflies feeding on the mixed-infected squash and squash infected from tobacco was significantly higher than that of whiteflies feeding on squash infected from snap bean (X^2= 73.8; df = 2, 124; P <0.0001) (Fig. 3. 5 B).

Very mild symptoms (slower growth) developed on squash inoculated from squash infected from snap bean. Squash inoculated from squash infected from tobacco had characteristic CuLCrV symptoms (stunted growth, curled and crumpled young leaves). Mixed-infected squash had symptoms of both CuLCrV and CYSDV. The younger leaves had CuLCrV symptoms (stunted growth, curled and crumpled young leaves), and older leaves had CYSDV symptoms (severe interveinal chlorosis). Percent infection and virus accumulation differed in infected squash depending on the CuLCrV source. Highest percent infection was observed in squash infected from mixed-infected squash (85%) and within single infection sources, percent infection in squash infected from squash that was originally infected from tobacco was higher (80%) than squash

infected from squash originally infected from snap bean (20%) ($\chi 2$ =20.11, df = 2, 82; P< 0.0001) (Fig. 3. 5 C). CuLCrV accumulation in squash infected from the mixed-infected squash and squash infected from squash originally infected from tobacco was significantly higher than in squash infected from squash originally infected from snap bean (F = 478.4; df = 2, 57; P <0.0001) (Fig. 3. 5 D).

Discussion

We examined the transmission of CuLCrV by MEAM1 feeding on CuLCrV-infected plants with differential infection status and varying levels of CuLCrV accumulation. We found that the percent infection and CuLCrV accumulation in whiteflies was proportional to the CuLCrV accumulation in host plants irrespective of mixed or single infection status. Disease phenotype development and percent infection in squash were dependent on both the infection status (mixed or single) and the amount of CuLCrV transmitted by whiteflies. CuLCrV accumulation in squash was directly proportional to the amount of CuLCrV in the inoculum source plants. Overall, our results provide experimental evidence that MEAM1-mediated CuLCrV disease epidemics in squash largely depend on the CuLCrV accumulation in the source host.

MEAM1 whiteflies were able to acquire CuLCrV from both mixed- (squash) and single-infected plants (tobacco and snap bean), confirming that CuLCrV accumulation in plants was above the threshold that would limit its acquisition. However, CuLCrV accumulation in whiteflies was proportional to the virus accumulation in the host plants. Similar patterns of

begomovirus accumulations in whiteflies that are dependent on the host plants have been reported earlier for tomato yellow leaf curl virus (TYLCV) (Lapidot et al. 2001). Studies with solutions of purified virions of TYLCV have also proved that TYLCV accumulation in whiteflies is proportional to the virus load in the source (Kollenberg et al 2014). In squash, both CuLCrV and CYSDV are phloem-restricted viruses, and their mixed infections in squash resulted in severe disease symptoms and higher CuLCrV accumulation. Previous studies on mixed infection with CuLCrV and cucumber mosaic virus (CMV) found that CMV aids CuLCrV replication in zucchini by suppressing the host's immune system (Hagen et al. 2006) resulting in an increase in CuLCrV accumulation. CYSDV might be playing a similar role in mixed-infected squash. CYSDV infection in snap bean is reported to be cultivar dependent. Wintermantel et al. (2009) reported that during greenhouse transmission studies, two cultivars of snap bean (Shade and Top Crop) were infected by CYSDV, while a third cultivar (Shara) remained uninfected, following feeding by viruliferous whiteflies. Therefore, the snap bean cultivar used in the present study (Provider) might not be a host for CYSDV.

Within single infections, squash infected from snap bean had the least CuLCrV-DNA accumulation and less severe symptoms compared with squash infected from tobacco. In fact, symptoms and CuLCrV-DNA from squash infected from snap bean disappeared after eight weeks of infection. Recovery phenotypes has been observed in certain begomovirus-infected plants, coupled with a reduction in viral accumulation and symptom severity, is the result of host defense responses (Hagen et al. 2008, Raja et al. 2010, Rodriguez-Negrete et al. 2009). Begomoviruses are reported to trigger the host's plant defense systems, which can lead to recovery from the infection (Carrillo-Tripp et al. 2007, Chellappan et al. 2004, Chellappan et

al. 2005, Pooggin et al. 2003), but it can also suppress the host's plant defense systems system (Bisaro 2006, Moissiard and Voinnet 2004). Therefore, infection depends on the ability of the begomovirus to suppress the host's immune system. For aphid-transmitted potyviruses, there are studies that had measured the inoculum threshold of virus particles required to induce infection (Moury et al 2007, Pirone and Thornbury 1988). However, studies quantifying inoculum thresholds for begomovirus particles to induce infection are lacking. Results from the current study and studies done on potyviruses imply that in single infections, whitefly feeding on snap bean did not inject enough CuLCrV particles into squash for the virus to overcome the plant's immune system completely. However, whiteflies feeding on tobacco were able to acquire and transmit virus particles above the threshold required to start irreversible infection.

Depending on the host's suitability, MEAM1 is reported to feed differentially on different host plants (Liu et al. 2012). Differential feeding can affect the virus transmission by whiteflies (Ning et al. 2015). Therefore, results obtained in the back-transmission studies (CuLCrV transmission from mixed- (squash) vs singly-infected (tobacco and snap bean) plants) to squash assays might be the result of differential feeding of MEAM1 on squash, tobacco, and snap bean along with differential CuLCrV accumulations in the three hosts. Through squash-to-squash transmission assays (CuLCrV transmission from mixed- vs singly-infected squash), we removed the potential impact of the host plant. Results from squash-to-squash transmission agreed with results obtained from back-transmission studies. CuLCrV-DNA accumulation in the whiteflies was dependent on the CuLCrV accumulation in the inoculum source plant. This in turn affected the disease phenotype and CuLCrV accumulation in squash subjected to

viruliferous whiteflies. Previous studies have obtained similar results with TYLCV (Lapidot et al. 2001; Legarrea et al., 2015; Srinivasan et al. 2012)

Vector-borne pathogens are known to influence host phenotype in ways that stimulate their transmission by the vector (Mauck et al. 2012; Lefévre and Thomas, 2008). Results from our earlier studies with TYLCV-infected tomato demonstrated that non-viruliferous whiteflies were attracted to susceptible genotypes with higher TYLCV-accumulation, and they accumulated higher TYLCV compared to whiteflies feeding on resistant hosts with lower TYLCV accumulation (Legarrea et al. 2015). Therefore, results from the current study and previous findings prove that apparent symptomatic phenotypes with enhanced virus accumulation could drive the virus spread/epidemics faster than hosts with less severe disease phenotype and reduced virus accumulation.

CuLCrV was first reported in the Imperial Valley of California in 1988 (Guzman et al. 2000) and didn't cause many problems until it moved to the Southeastern United States around 2006 (Akad et al. 2008). Processes of viral disease emergence are complex and predicting what will trigger the next epidemic is difficult. Results from the current study show that CuLCrV epidemics largely depend on the number of virus particles acquired and transmitted by the whiteflies. Therefore, there could be wild host plants that accumulate high levels of CuLCrV and serve as long-term reservoirs and efficient inoculum sources for CuLCrV. However, it has been proposed that viruses generally don't cause apparent disease in wild plants because they cannot afford to kill their reservoir host (Roossinck, 2005, Roossinck et al. 2015). Therefore, high vector pressure observed in summer and fall in the region might be driving the current

CuLCrV epidemic. Primary infection might be initiated by large numbers of viruliferous whiteflies injecting enough virus particles in at least some plants to kickstart the infections and subsequent secondary spread from infected susceptible host plants, resulting in widespread epidemics. Since squash accumulate higher amount of CuLCrV, it can serve as an efficient inoculum source for snap bean than vice versa. But given the population explosions of whiteflies it might be possible for snap bean to serve as an inoculum source as well.

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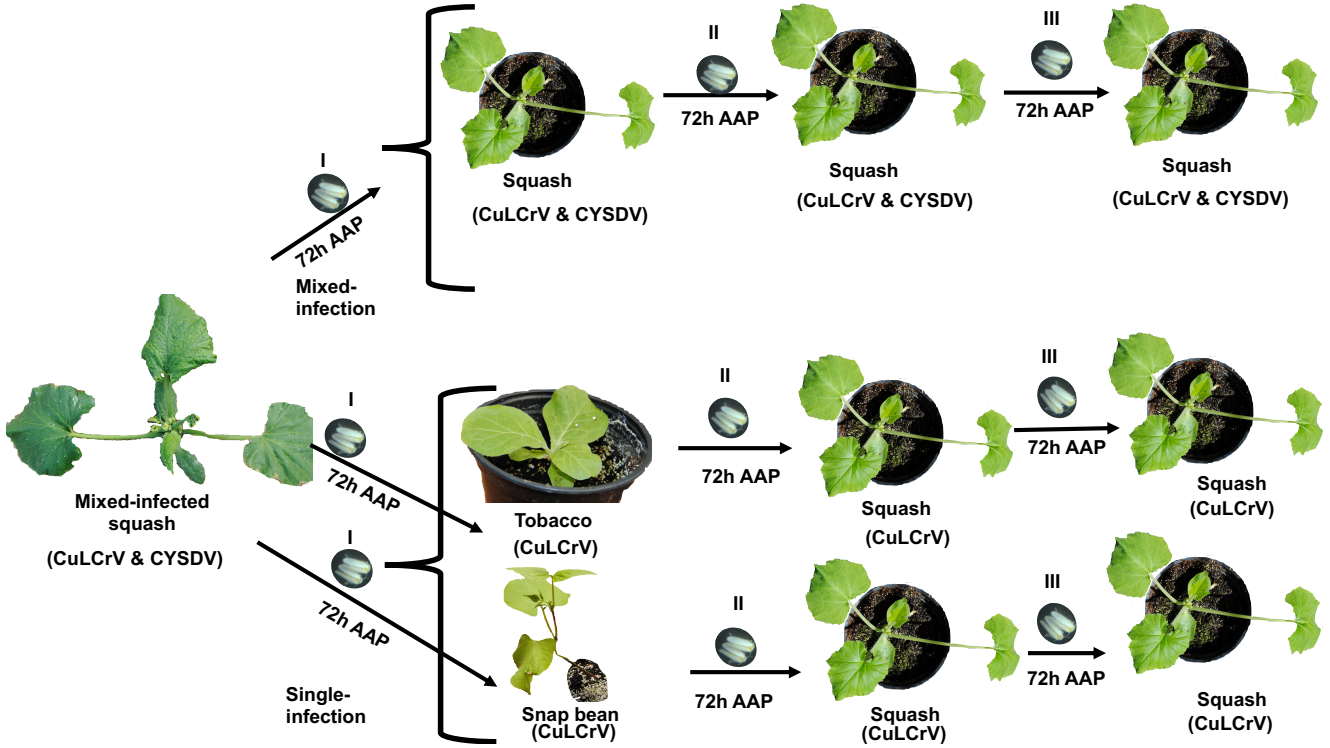

Figure 3. 1. Schematic representation of whitefly-mediated transmission and back-transmission bioassays.

I, Whitefly mediated CuLCrV transmission from mixed-infected squash (CuLCrV and CYSDV) to tobacco, snap bean, and squash. II, Whitefly mediated CuLCrV transmission from mixed- infected squash (CuLCrV and CYSDV) vs singly-infected tobacco and snap bean (CuLCrV) plants. III, Whitefly mediated CuLCrV transmission from mixed (CuLCrV and CYSDV)- vs singly (CuLCrV)-infected squash

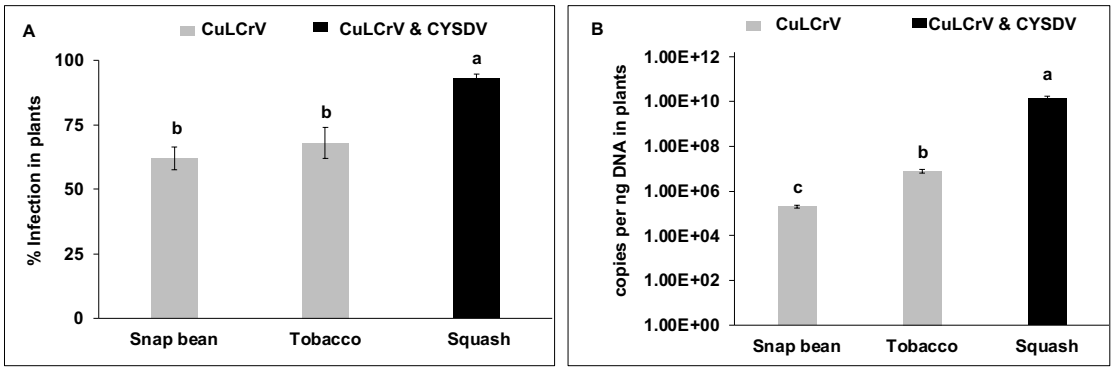

Figure 3. 2. CuLCrV transmission from mixed-infected squash to squash, tobacco, and snap bean.

A, Percent CuLCrV infection in snap bean, tobacco, and squash infected from mixed-infected (CuLCrV and CYSDV)- squash. B, CuLCrV accumulation in snap bean, tobacco, and mixed-infected squash. Values are means ± SE. Means with different letters are significantly different (HSD test at $P < 0.05$).

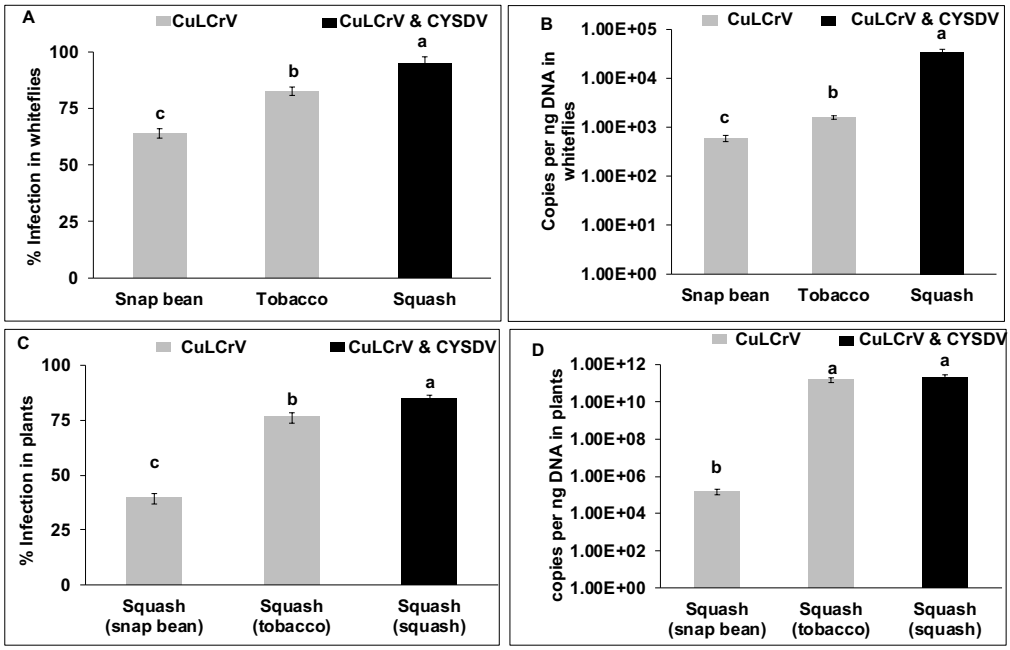

Figure 3. 3. CuLCrV transmission from mixed- (squash) vs single-infected (tobacco and snap bean) plants to squash.

A, Percent infection in whiteflies feeding on single (CuLCrV)- (tobacco and snap bean) or mixed-infected (CuLCrV and CYSDV) (squash) plants. B, CuLCrV accumulation in whiteflies feeding on single (CuLCrV)- (tobacco and snap bean) or mixed-infected (CuLCrV and CYSDV) (squash) plants. C, Percent infection in squash infected from single (CuLCrV)- (tobacco and snap bean) or mixed-infected (CuLCrV and CYSDV) (squash) plants. D, CuLCrV accumulation in squash infected from single (CuLCrV)- (tobacco and snap bean) or mixed-infected (CuLCrV and CYSDV) (squash) plants. Values are means ± SE. Means with different letters are significantly different ($P < 0.05$)

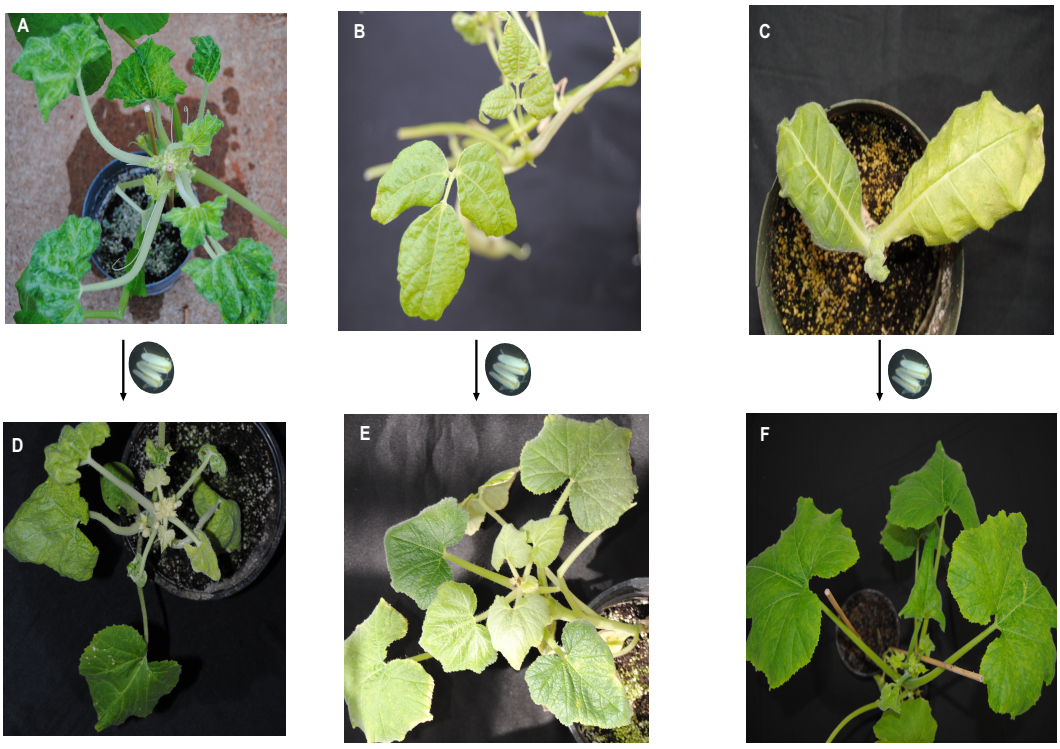

Fig. 3. 4. Symptoms

Symptom development in plants infected with CuLCrV or CuLCrV and CYSDV. Symptoms are shown during the symptomatic phase of infection as they appear on systemically infected leaves. A, mixed-infected squash with CuLCrV and CYSDV; B, snap bean infected with CuLCrV; C, tobacco infected with CuLCrV; D, mixed-infected squash with CuLCrV and CYSDV infected from mixed infected squash; E, squash infected with CuLCrV from snap beans; D, squash infected with CuLCrV from tobacco.

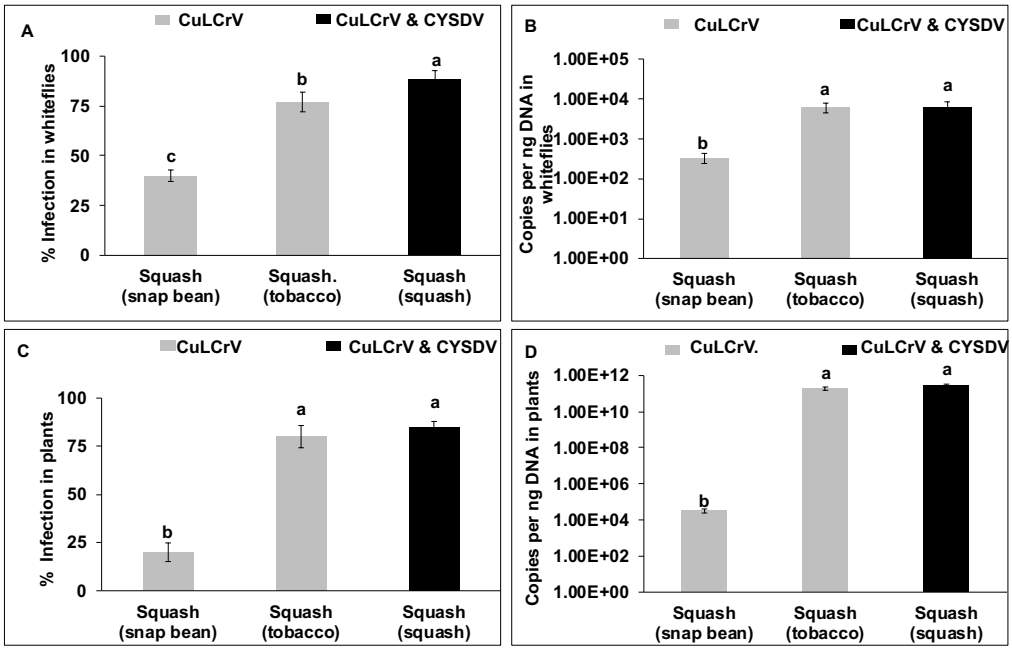

Figure 3. 5. CuLCrV transmission from mixed- vs single-infected squash to squash

A, Percent CuLCrV infection in whiteflies feeding on single (CuLCrV) - (squash infected from bean or tobacco) or mixed-infected (CuLCrV and CYSDV) squash (squash infected from mixed-infected squash). B, CuLCrV accumulation in whiteflies feeding on single- (CuLCrV) (squash infected from snap bean or tobacco) or mixed-infected (CuLCrV and CYSDV) squash (squash infected from mixed-infected squash). C, Percent CuLCrV infection in squash infected from single- (squash infected from bean or tobacco) or mixed-infected (CuLCrV and CYSDV) squash (squash infected from mixed-infected squash). D, CuLCrV accumulation in squash infected from single (CuLCrV) - (squash infected from bean or tobacco) or mixed-infected (CuLCrV and CYSDV) squash (squash infected from mixed-infected squash). Means with different letters are significantly different ($P < 0.05$).

CHAPTER 4

SYMPTOMS, EXPERIMENTAL HOST RANGE, AND PHYLOGENETIC ANALYSIS OF SIDA GOLDEN MOSAIC VIRUS INFECTING SNAP BEAN (*PHASEOLUS VULGARIS* L.) IN GEORGIA

Gautam, S., Gadhave, K. R., Buck, J. W., Dutta, B., Coolong, T., Adkins, S., Srinivasan, R. To be submitted to Plant Disease

Abstract

Whitefly-transmitted begomoviruses are economically important viruses affecting a wide range of crop and weed plants. Timely *Begomovirus* species identification, along with understanding host range and relationships with closely related viruses, is crucial for development of effective management strategies. *Sida golden mosaic virus* is a member of the genus *Begomovirus* in the family *Geminiviridae*. Sida golden mosaic virus (SiGMV) was isolated from common bean (*Phaseolus vulgaris* L.) and prickly sida (*Sida spinosa* L.) plants for the first time in Georgia, USA. The virus has been documented from Florida earlier in the United States. To examine the experimental host range of SiGMV, eight plant species belonging to three different families; Malvaceae (hollyhock (*Alcea rosea* L.), march-mallow *(Althaea officinalis* L.), okra (*Abelmoschus esculentus* (L.) Moench*),* cotton *(Gossypium hirsutum* L.), Country Mallow (*Sida cordifolia* L.) and prickly sida (*S. spinosa*), Solanaceae (tobacco (*Nicotiana tabacum* L.), and Fabaceae (snap bean (*P. vulgaris*) were inoculated using viruliferous whiteflies (*Bemisia tabaci* Gennadius). All tested plant species except *G. hirsutum* became infected with SiGMV, and infection percentages and virus accumulation levels varied among host species evaluated. SiGMV accumulation and percent infection was higher in *S. spinosa*, *A. rosea*, and *P. vulgaris* than *N. tabacum, A. esculentus A. officinalis*, and *S, cordifolia*. SiGMV percent infection in whiteflies and accumulation levels following a 72h AAP on infected plants followed similar pattern observed among different hosts. Complete sequences of DNA-A from the total DNA extracts from SiGMV-infected field-collected prickly sida plants shared 97% identity with SiGMV sequences reported from Florida. To the best of our knowledge, this is the first report of SiGMV in Georgia. Phylogenetic analysis revealed that SiGMV identified in this study is more closely related to the sida viruses reported from the New World, as opposed to those from the Old World. The

relevance of SiGMV in the vegetable production landscape is currently being examined in detail.

Introduction

Begomovirus is the largest genus within the family *Geminiviridae* and causes devastating diseases in multiple crops around the world (Brown 1994). Viruses within the genus *Begomovirus,* depending upon their genome organization, are either monopartite (DNA A~2.7 kb) or bipartite (DNA-A and -B, each approx. ~2.6 kb) (Brown 2001). Begomoviruses also show a distinct phylogeographical distribution, with most bipartite viruses being reported from the New World (NW), and most monopartite viruses being reported from the Old World (OW) (Briddon et al. 2010; Rojas et al. 2005). Begomoviruses are transmitted exclusively by a whitefly cryptic species complex commonly known as *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) in a persistent-circulative manner (Rosen et al. 2015). Once acquired by *B. tabaci*, begomoviruses can remain associated with them for the duration of their lifespan. *B. tabaci* causes significant damage to plants, directly by feeding on the foliage leading to silvering, and indirectly by transmitting numerous plant pathogenic viruses including begomoviruses (Jones 2003). Outbreaks of *B. tabaci* have facilitated widespread epidemics of whitefly-transmitted viruses in several crops worldwide leading to substantial losses (Morales and Anderson 2001; Polston and Anderson 1997).

Tomato yellow leaf curl virus (TYLCV) was the first reported monopartite begomovirus to occur in the continental United States, and it has established itself as a major tomato pathogen (Polston et al. 2004). Cucurbit leaf crumple virus (CuLCrV), a bipartite begomovirus, is increasingly becoming important in commercial production of cucurbits and

beans in the Southern United States. CuLCrV was first reported in Florida in 2006 in squash (Akad et al. 2008) and in Georgia in snap bean (*Phaseolus vulgaris* L.) in 2009 (Larsen and Kmiecik 2010). In August 2018, snap beans with characteristic begomovirus infection symptoms (crumpled, curled, and thickened leaves) were found in Tifton, Georgia, and these snap bean plants were very heavily infested with whiteflies. The plants were initially presumed to be infected with CuLCrV. When representative samples from these plants were tested for CuLCrV confirmation via PCR, all were negative. Subsequent PCR with degenerate begomovirus primers, cloning, and sequencing revealed the presence of a previously unreported begomovirus, sida golden mosaic virus (SiGMV).

SiGMV is a bipartite *Begomovirus* with DNA-A and DNA-B components, and each about 2.6 kb in size (Fiallo-Olive et al. 2010). SiGMV was first reported in snap bean in 2006 in Alachua County, Florida (Durham et al 2010). In 2010, a new strain, sida golden mosaic Florida virus-*Malvastrum* (SiGMFV-Ma), was reported in Cuba infecting mallow weed, *Malvastrum coromandelianum* L. (family Malvaceae) (Fiallo-Olive et al. 2010). *Sida* spp. are ubiquitous in the Southeast US and around ten *Sida* spp. have been reported from Florida alone (http://www.plantatlas.usf.edu). Several *Sida* spp. have also been reported as hosts for begomoviruses (hereafter referred to as sida viruses) (Fiallo-Olive et al. 2010; Stewart et al. 2014; Wyant et al. 2011). Sida viruses have been reported from both the Old World (OW) (Duan et al. 2019; Ha et al. 2008) and New World (NW) (Echemendia et al. 2004; Fontenele et al. 2018; Tavares et al. 2012). *Sida* spp. are frequently found in proximity to important food and fiber crops, which makes *Sida* spp. potential natural reservoirs for sida viruses that could also infect important food and fiber crops. Thus far, there has been no reported epidemics of SiGMV in the Southeast; however, this virus is largely understudied, and given

the distribution of *Sida* spp., in the cropping landscape of southern United States, there is certainly potential for SiGMV to become an economically important pathogen.

Although SiGMV has been present in the Southern United States for more than a decade, not much information is available about its host range and interactions with its vector. The objectives of the present study were: 1) to investigate the experimental host range of SiGMV by inoculating selected host species using viruliferous whiteflies; 2) to characterize virus-induced symptoms, assess the virus accumulation levels in different host plants, and subsequently affects virus accumulation in whiteflies feeding on them; and 3) to study the phylogenetic relationships of SiGMV and closely related sida viruses.

Materials and Methods:

Virus source and maintenance

Twenty snap bean plants exhibiting begomovirus-associated symptoms were collected from the field in August 2018. Total DNA was extracted from 100 mg of leaf tissues of the symptomatic plant using the GeneJET Plant Genomic Purification Kit (ThermoFisher Scientific, USA, K0791) following guidelines supplied by the manufacturer. Initial testing for the suspected CuLCrV turned out to be negative. Presence of another begomovirus was examined through PCR using a degenerate primer pair PAR1c496 and PAL1v1978 (5'GCCCACATYGTCTTYCCNGT-3' and 5'-GGCTTYCTRTACATRGG-3'), and to amplify a 1.159 kb region of DNA-A (Rojas et al. 1993). PCR products were sequenced, and subsequent BLAST analysis confirmed the presence of a previously unreported begomovirus from Georgia. The sequences shared >95% similarity with the DNA-A of sida golden mosaic

Florida virus (SiGMFV) reported from Florida (Durham et al. 2010). SiGMV-specific primers were designed using Primer3 software (Untergasser et al. 2012) (Table 3). SiGMVF and SiGMVR that amplify 574 bp region of DNA-A were used for end-point PCR. For PCR, GoTaq Green Mastermix buffer (2x) (Promega, Madison, WI) was combined with forward and reverse primers (SiGMVF and SiGMVR, Table 3) (final concentration of 0.5 μM), 20 ng of DNA, and sterile nuclease-free water for a final volume of 10 μl. PCR was performed using a T-100 thermocycler (Bio-Rad, Hercules, CA). An initial denaturation step (2 min at 95°C) was followed by 40 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min; ending with a final extension of 72°C for 10 minutes.

In September 2018, prickly sida weeds exhibiting putative golden mosaic symptoms were collected from just outside a *P. vulgaris* field in Tifton, GA (USA) where SiGMV-infected plants were discovered in August 2018. Approximately twenty plants were dug up with a shovel to a depth of 25 cm and placed individually in plastic pots (2.37 L). The field-collected prickly sida plants were maintained in a greenhouse (25–30°C, 14h L:10h D) in whitefly-proof cages (45Lx45Wx90H) (Megaview Science Co., Taichung, Taiwan), four plants per cage. Total DNA was extracted from single leaf of the symptomatic plant using the GeneJET Plant Genomic Purification Kit and subjected to PCR analysis using the primer pairs (SiGMVF and SiGMVR) listed in Table 3, following the procedure described above. PCR products were cloned using the CloneJET PCR Cloning Kit (ThermoFisher Scientific, USA, K1231) and sequenced to confirm the presence of SiGMV. Positive plants were used as a virus inoculum source. Throughout the study, SiGMV was maintained in seed-germinated prickly sida through repeated inoculations with viruliferous whiteflies (Muniz and Nombela 2001; Srinivasan et al. 2012). Prickly sida was used as an inoculum source for SiGMV instead of snap beans, as it was easier to maintain SiGMV in these plants.

Plants and insects

Eight plants species representing three different families commonly present in the Southern United States were selected. Seeds of prickly sida were collected from mature flowers of plants that tested negative for SiGMV. Mallow seeds were procured from different suppliers listed on Amazon.com: Country Mallow (*Sida cordifolia* L.) from Asklepios Seeds, Germany; Marsh-Mallow (*Malva parviflora* L,) from Outsidepride, OR, USA; and hollyhock (*Alcea rosea* L.) from Seedusa, USA. Cotton (*Gossypium hirsutum* L. cv. 'ST 6182 GLT'), okra (*Abelmoschus esculentus* (L.) Moench*)*, tobacco (*Nicotiana tabacum* L. cv. 'L326) seeds were obtained from UGA extension services. Snap bean (*P. vulgaris* L. cv. 'Provider') seeds were obtained from Johnny's selected seeds (Winslow, ME, USA). Plants were grown in Sunshine propagation mix (SunGro Horticulture Industries, Bellevue, WA, USA) in 10-cm plastic pots (depth 8 cm). Two seeds per pot were sown and maintained in a greenhouse under the conditions described above. One week after germination, seedlings were thinned to one per pot. Plants at the 4-5 leaf stage (~4 weeks) were used for experiments. The whiteflies (*B. tabaci* cryptic species MEAM1) used in the present study were first collected in Tifton, Georgia, and have been reared on cotton plants since then in whitefly-proof cages in the greenhouse at above-stated conditions. The purity of the colony was confirmed every few months by partially sequencing the mitochondrial cytochrome oxidase I (COI) gene (Frohlich et al.1999).

Host range, symptoms, and SiGMV accumulation in host plants

Viruliferous and non-viruliferous whiteflies were obtained by allowing the whiteflies to feed on the infected or non-infected prickly sida for 72h. After the 72h AAP, 20 viruliferous or non-viruliferous whiteflies were tested for the presence of SiGMV via PCR. Total DNA was extracted from individual whiteflies using a specially formulated Chelex resin, InstaGene matrix (Bio-Rad, USA) following manufacturer instructions, and subjected to PCR analysis. Using a clip cage, 100 viruliferous or non-viruliferous whiteflies were attached to the first true leaf of each test plant for a week. Symptom development of the plants was recorded for the next eight weeks. After 8 weeks of inoculation, 100 mg of tissue from the youngest leaf of the test plants was collected and surface sterilized by washing with 1% bleach, then rinsing with autoclaved distilled water, then by a single wash with 75% ethanol, and finally three rinses with autoclaved distilled water. Total DNA from surface-sterilized tissues was extracted and subjected to PCR analysis as described above. Transmission percentage of SiGMV was measured as the number of plants infected divided by the total number of plants exposed to viruliferous whitefly feeding. Plants infested with non-viruliferous whiteflies were used as a control. For each plant species, ten replicates were used, and the experiment was conducted three times (n=30).

Virus accumulation in the infected plants was estimated through qPCR. SiGMV-QF and SiGMV-QR that amplify 114 bp region of DNA-A of SiGMV were used for quantitative PCR (qPCR). Quantitative PCR was performed using GoTaq qPCR Master Mix (Promega, Madison, WI). GoTaq qPCR Master Mix (2X) was combined with forward and reverse primers (SiGMV-QF and SiGMV-QR, Table 3) (final concentration of 0.25 μM), 20 ng of DNA, and nuclease-free distilled water for a final volume of 12.5 μl. Quantitative PCR was

performed using a Mastercycler ep realplex[4] (Eppendorf, Hauppauge, NY). An initial denaturation step (2 min at 95°C) was followed by 40 cycles of 95°C for 15 sec, 63°C for 10 sec, and 72°C for 20 sec. Melting curve analysis was performed to test the specificity of fluorescence signals. Absolute viral copy numbers were estimated using the procedure described by Legarrea et al. (2015).

Virus accumulation in whiteflies

Whiteflies were collected using an aspirator and attached to the infected or non-infected plants using clip cages (100/cage). After a 72h AAP, whiteflies were removed and re-attached to 4-week-old cotton plants maintained in the greenhouse under the conditions described above. Forty-eight hours later, whiteflies were collected from the cotton plants and placed in vials with 70% ethanol. Whiteflies were then stored at -80°*C* until DNA extraction. In order to remove any residual honey dew from whiteflies, prior to DNA extraction, whiteflies were surface-sterilized using the protocol described above for plant tissue surface sterilization. Total DNA from individual whiteflies was extracted using a specially formulated Chelex resin, InstaGene matrix and subjected to PCR as described above. Infection percentages of whiteflies infected with SiGMV for each acquisition host (prickly sida, hollyhock, marshmallow, country mallow, okra, snap bean and tobacco). Virus accumulation in infected whiteflies was estimated through qPCR as described above. Twenty insects were used for every host plant species (single plant/experiment). The experiment was conducted three times (n=60).

Phylogenetic analysis

Total DNA obtained from infected prickly sida was used to obtain the DNA-A sequence of SiGMV. SiGMVf 5'-CCTAAGCGCGATTTGCCAT-3' and SiGMVr 5'-TACAGGGAGCTAAATCCAGCT-3' primers specific to SiGMV were used to amplify the 1.5 kb region of the DNA-A (Durham et al. 2010). The remaining portion of DNA-A was amplified through the degenerate primer pairs PAR1c496 and PAL1v1978 (Rojas et al. 1993). Amplicons obtained from specific and degenerate primer pairs were cloned and sequenced. Obtained sequences were compiled to generate the complete sequence of DNA-A (2645 bp, GenBank Accession No. MK387701). Sequenced DNA-A shared 97% identity with SiGMFV (GenBank Accession No. AF049336) isolated from *P. vulgaris* from Florida (Durham et al. 2010). Sequenced DNA-A was used for phylogenetic analysis. DNA-A sequences of other sida viruses were downloaded from GenBank (Table 4). Phylogenetic analysis was performed with the R statistical program, version 3.6 (R Core Team 2014). All sequences were aligned with the MSA package (Bodenhofer et al. 2015). For phylogenetic analysis, the function modelTest was used to compare different nucleotide substitution models (Schliep, 2011). The best fitting model was selected based on the Akaike Information Criterion (AIC). Phylogenetic tree was construction using the Maximum-likelihood (ML) method using optim.pml command in Phangorn package (Schliep, 2011). Using bootstrap.pml command in Phangorn bootstrap support values were assigned to the nodes.

Statistical analyses

Differences in the percent infection in whiteflies and plants was evaluated assuming a binomial response (infected vs. noninfected) using logit model in R and virus accumulation in whiteflies and plants were analyzed using one-way ANOVA after log transformation. Data was pooled for replications and during analysis replications were considered as random effect and treatments were considered as fixed effect. Means for virus accumulation and percent infection in both plants and insects were compared with Tukey's HSD posthoc test ('TukeyHSD' functions in R). All statistical calculations were performed with the R statistical program, Agricolae version 3.6 (De Mendiburu 2015).

Results

Host range, symptoms, and SiGMV accumulation in host plants

SiGMV infection in test plant species was significantly different ($\chi 2$ = 23.41; df = 7, 232; $P<$ 0.00001). Among the eight plant species tested, all but cotton were infected with SiGMV (Table 5). The highest percent infection was found in hollyhock and pricky sida, followed by country mallow, snap bean, marsh-mallow, tobacco, and okra had the least percent infection (Fig. 4. 1 A). Typical SiGMV infection symptoms were observed after ~4-6 weeks of inoculation (Fig. 4. 2). Characteristic golden mosaic SiGMV symptoms were observed in hollyhock, bean, marsh-mallow, country mallow, and prickly sida (Fig. 4. 1). SiGMV accumulation differed significantly between plant species (F = 14.58; df = 6, 163; $P<$ 0.00001). Accumulation of SiGMV was significantly higher in the leaf tissues of bean, hollyhock, and prickly sida than all other plant species (Fig. 1 B). SiGMV accumulation was

lowest in country mallow. SiGMV accumulation was did not differ among tobacco, okra, and marsh-mallow (Fig. 4. 1 B). None of the whitefly feeding on non-infected plants was found positive for the SiGMV.

Percent infection and virus accumulation in whiteflies

SiGMV DNA-A was detected in *B. tabaci* adults that were subjected to a 72h AAP on different host plants, verifying that whiteflies were able to successfully acquire virus from all infected plant species. Variation in SiGMV infection in whiteflies was dependent upon the host plants ($\chi 2$ = 83.39; df = 6, 413; P< 0.00001). The highest percent infection was observed in whiteflies feeding on prickly sida and hollyhock followed by bean and tobacco (Fig. 4. 3 A). Similar infection percentages were observed in whiteflies feeding on okra, marsh-mallow, and country mallow (Fig. 4. 3 A). Trends of SiGMV accumulation in whiteflies were similar to virus accumulation in host plants (Fig 4. 4). Whiteflies feeding on prickly sida accumulated significantly higher amounts of SiGMV than all other whiteflies (F = 8.9; df = 6, 206; P< 0.00001). No differences were observed in the virus accumulation in whiteflies feeding on tobacco, okra, and marsh-mallow (Fig. 4. 3 B).

Nucleotide similarity and phylogenetic analysis

BLASTn analysis of DNA-A revealed 97% nucleotide sequence identity with SiGMFV (GenBank Accession No. MK387701) reported from Florida (Durham et al. 2010). Phylogenetic analysis revealed that sida viruses form five clusters (Fig. 4. 4). However, sida golden yellow spot virus (SiGYSV) and sida chlorotic mottle virus (SiCMoV) were not associated with any cluster. Viruses that originated in the New World and Old World were

grouped under two different clades. Within the New World clade, *sida golden mosaic virus* (SiGMV), *sida golden mosaic Florida virus* (SiGMFV), and *sida yellow mottle virus* (SiYMoV) constituted a monophyletic group. The majority of sida viruses reported from Brazil clustered together**.**

Discussion

For the first time, our study reports previously unreported begomovirus, SiGMV from Georgia along with its host range and transmission efficiency by MEAM1 whiteflies. Since the first report of SiGMV in 2007, in Florida, the virus has been described in snap beans and in a weed, *M. coromandelianum* (Durham et al. 2010; Fiallo-Olive et al. 2010). The infection of prickly sida, a widely-distributed weed, can contribute to the spread of SiGMV in the landscape. Prickly sida can also overwinter in Georgia; thereby can assist with the establishment of the pathogen continuously in the landscape.

Our experimental host range study shows that SiGMV can infect plants belonging to three different families, including commercially important crops (okra, bean, and tobacco). Since we used vector-mediated transmission in place of plasmid mediated transmission, the results here depict realistic scenarios observed under field conditions. We observed a direct relationship between severity of symptom development and the level of SiGMV accumulation in susceptible host plants (hollyhock, prickly sida, snap bean, marsh-mallow, country mallow, tobacco, and okra). Of the susceptible plants tested here, hollyhock, snap bean, and prickly sida exhibited symptoms typical of SIGMV infection, golden mosaic (Fig. 1). Sever golden mosaic was observed in hollyhock. Symptoms in bean, marsh-mallow and prickly sida were stunted growth accompanied by chlorotic spots, leaf crumpling and mild

foliar golden mosaic. Country mallow exhibited stunting along with severe interveinal chlorosis. Young leaves of okra developed chlorotic spots on infection with SiGMV. Symptoms in tobacco were thickened and leathery young leaves.

Sida spp. belong to the mallow family, Malvaceae. Mallow plants are widely distributed throughout the Americas (Hill 2012), and as reported here, SiGMV can infect multiple mallow species. Prickly sida has been reported as weed in six or more important crops in Georgia (Webster and MacDonald, 2001). Prickly sida is a reservoir for SiGMV, and also a suitable host for whiteflies, which are able to complete their life cycle on this host (greenhouse observations). Whiteflies were able to transmit SiGMV after 72 hours spent feeding on infected prickly sida. Earlier studies have reported that once a *Begomovirus* becomes established in commercially-cultivated crops, it quickly adapts itself and becomes distinct from parental populations (Brown 2001). So far, there has been no reported widespread epidemic of SiGMV. However, considering the host range of SiGMV, begomovirus adaptability, and ability of the whiteflies to transmit it efficiently from prickly sida, it could pose a serious risk to beans, okra, or other Malvaceous crops in the future.

We observed a direct relationship between SiGMV accumulation in whiteflies to SiGMV accumulation in susceptible host plants, except for prickly sida. Although prickly sida, hollyhook, and bean accumulate the same amount of SiGMV DNA-A, whiteflies feeding on prickly sida acquired more viral DNA. Previous studies with electrical penetration graphs have shown that whiteflies spending more time feeding acquire more virus than whiteflies with interrupted feeding (Ning et al. 2015). One possible explanation is that whiteflies on prickly sida might be spending more time feeding, resulting in increasing opportunities for virus acquisition. Earlier studies have also reported a direct correlation between tomato

yellow leaf curl virus (TYLCV) accumulation in whiteflies following feeding on source plants with varying levels of TYLCV (Lapidot et al. 2001; Legarrea et al. 2015). Results from the present study and earlier studies show that the higher the begomovirus level in the host plant, the higher the begomovirus in the whitefly. Although it may be premature to assume such a direct relationship just based on the work on two begomoviruses, this is clearly a possibility. Further studies are needed to confirm how differential SiGMV accumulation by whiteflies affects virus transmission. Both the highest and lowest SiGMV accumulations were observed in mallow plants. The highest SiGMV accumulation was observed in hollyhock and the lowest was observed in country mallow, although it seems to be above the threshold that would limit SiGMV acquisition by whiteflies.

Phylogenetic relationships of SiGMV with other related begomoviruses have been previously studied (Fiallo-Olive et al. 2010). Here, we have reported phylogenetic relationships between sida viruses identified in different parts of the world. Begomoviruses are considered separate species based on less than 91% pairwise similarity in the DNA-A of different viruses, and the cutoff for strain demarcation has been set at 94% (Brown et al. 2015). Based on nucleotide sequence analysis, the SiGMV reported here is identical to SiGMFV reported previously from Florida in 2007. Olive et al. (2010) reported that a strain of SiGMV from *M. coromandelianum,* which belongs to a group of begomoviruses that infect malvaceous weeds in the Caribbean (Fiallo-Olive et al. 2010). Our results also indicate that SiGMV is more closely related to sida viruses reported from Central and South America.

Epidemics of plant infectious viruses depend on the availability of reservoir hosts to survive, susceptible hosts to increase in populations, and efficient vectors for rapid transmissions (Hily et al. 2014). As reported here, SiGMV can infect multiple weeds/crop plants, multiply

efficiently in some of them and whiteflies are able to acquire SiGMV after brief feeding on infected plants. Furthermore, weeds belonging plant families - Fabaceae, Malvaceae, and Solanaceae are widespread in Georgia (Webster and MacDonald, 2001). Therefore, chances that SiGMV has established itself in southeastern US/Georgia are very high. Processes of viral disease emergence are complex and predicting what will trigger the next epidemic is difficult. Recently, SiGMV has been found in mixed infections with CuLCrV and TYLCV in snap bean and tomatoes, respectively (unpublished data). Mixed infections are ubiquitous in nature (Mascia and Gallitelli 2016; Roossinck et al. 2015; DaPalma et al. 2010) and many co-infecting viruses interact synergistically (Syller, 2012). Several agriculturally important plant viruses produce enhanced disease phenotype in mixed infection status (Rentería-Canett et al. 201, Li et al. 2017, Gil-Salas et al., 2011, 2012; Srinivasan and Alvarez 2007). Therefore, co-infections of SiGMV with other begomoviruses could trigger SiGMV disease epidemics.

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Table 3. Primers used for amplification of a segment of DNA-A of SiGMV.

Name	Sequence (5' – 3')	Product size (bp)
SiGMV-QF	CTCAAAGGTTAGCCGCAACG	114
SiGMV-QR	CGGTAGATCCTGGGCTTCCT	
SiGMVF	TTCTCCTCGTGCAGGTAGTG	574
SiGMVR	ACTTGCCAGCCTCTTGATGA	

Table 4. List of sida viruses used for phylogenetic analysis

Sr. No.	Viruses	Acronym	GenBank (Access No.)	Location
1	Sida angular mosaic virus	SiAMV	KX691407	BR (NW)
2	Sida bright yellow mosaic virus	SiBYMV	NC_038991	BR (NW)
3	Sida chlorotic mottle virus	SiCMoV	NC_038990	BR (NW)
4	Sida chlorotic vein virus	SiCVV	KX691402	BR (NW)
5	Sida ciliaris golden mosaic virus	SicGMV	NC_038456	VE (NW)
6	Sida common mosaic virus	SiCMV	NC_038457	BR (NW)
7	Sida golden mosaic Braco virus	SiGMBcV	NC_038458	JM (NW)
8	Sida golden mosaic Buckup virus	SiGMBuV	HQ008338	JM (NW)
9	Sida golden mosiac Costa Rica virus	SiGMCRV	X99550	CR (NW)
10	Sida golden mosaic Florida virus-Malvastrum strain	SiGMFlV	HM003779	USA (NW)
11	Sida golden mosaic Honduras virus	SiGMHV	Y11097	HN (NW)
12	Sida golden mosaic Lara virus strain Venezuela	SiGMLaV	NC_038459	VE (NW)
13	Sida golden mosaic virus - Florida	SiGMV	GQ357649	USA (NW)
14	Sida golden mottle virus	SiGMoV	GU997691	USA (NW)
15	Sida golden yellow spot virus	SiGYSV	NC_038992	BR (NW)
16	Sida leaf curl virus	SiLCuV	AM050730	CN (OW)
17	Sida micrantha mosaic virus	SiMMV	AJ557451	BR (NW)
18	Sida mosaic Alagoas virus	SiMAV	JF694471	BR (NW)
19	Sida mosaic Bolivia virus 1	SiMBoV1	HM585441	BO (NW)
20	Sida mosaic Bolivia virus 2	SiMBoV2	HM585443	BO (NW)
21	Sida mosaic Sinaloa virus	SiMSinV	DQ520944	MX(NW)
22	Sida mottle Alagoas virus	SiMoAV	JX871385	BR (NW)
23	Sida mottle virus	SiMoV	AY090555	BR (NW)
24	Sida yellow blotch virus	SiYBV	JX871380	BR (NW)
25	Sida yellow leaf curl virus	SiYLCV	NC_038461	BR (NW)
26	Sida yellow mosaic Alagoas virus	SiYMAV	JX871383	BR (NW)

27	Sida yellow mosaic China virus	SiYMCNV	AM048837	CN (OW)
28	Sida yellow mosaic Yucatan virus	SiYMYuV	DQ875872	MX (NW)
29	Sida yellow mosaic virus	SiYMV	AY090558	BR (NW)
30	Sida yellow mottle virus	SiYMoV	NC_016082	CU (NW)
31	Sida yellow net virus	SiYNV	JX871376	BR (NW)
32	Sida yellow vein Madurai virus	SiYVMV	AM259382	IN (OW)
33	Sida yellow vein Vietnam virus	SiYVVV	DQ641696	VN (OW)
34	Sida yellow vein virus	SiYVV	Y11099	HN (NW)
35	Sida golden mosaic virus[a]	SiGMV	MK387701	USA (NW)

[a]SIGMV sequence obtained in this study

[b] Locations: NW (New World), OW (Old World), HN (Honduras), VN (Vietnam), IN (India), BR (Brazil), CU (Cuba), MX (Mexico), CN (China), VE (Venezuela), CR (Costa Rica), JM (Jamaica)

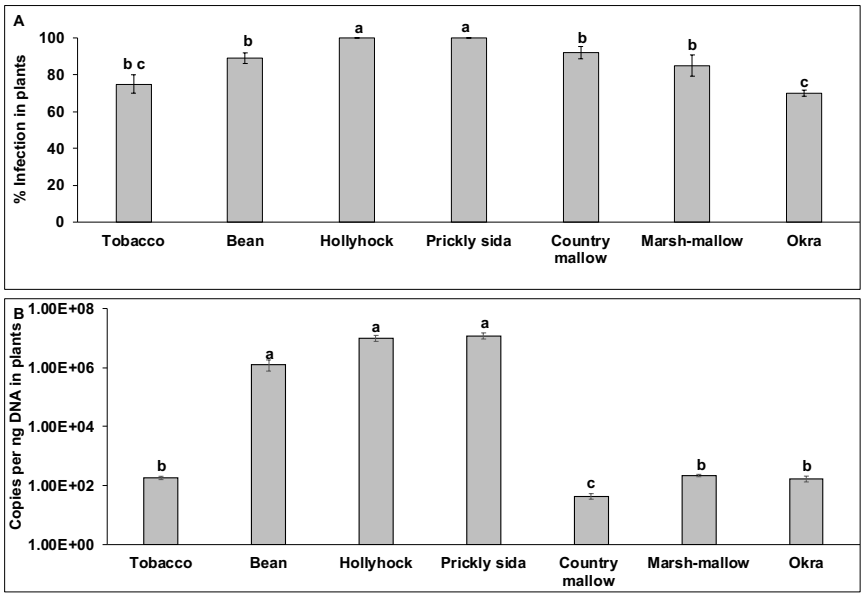

Figure 4. 1. SiGMV percent infection and accumulation in different host plants.
A, Bar with standard errors represent the average percent infection in susceptible plant species. B, Bar with standard errors represent the average number of SiGMV copies accumulated in the leaf tissues of SiGMV susceptible plants species. For copy numbers Y-axis is on logarithmic scale. Means with different letters are significantly different (Tukey-HSD, $P < 0.05$).

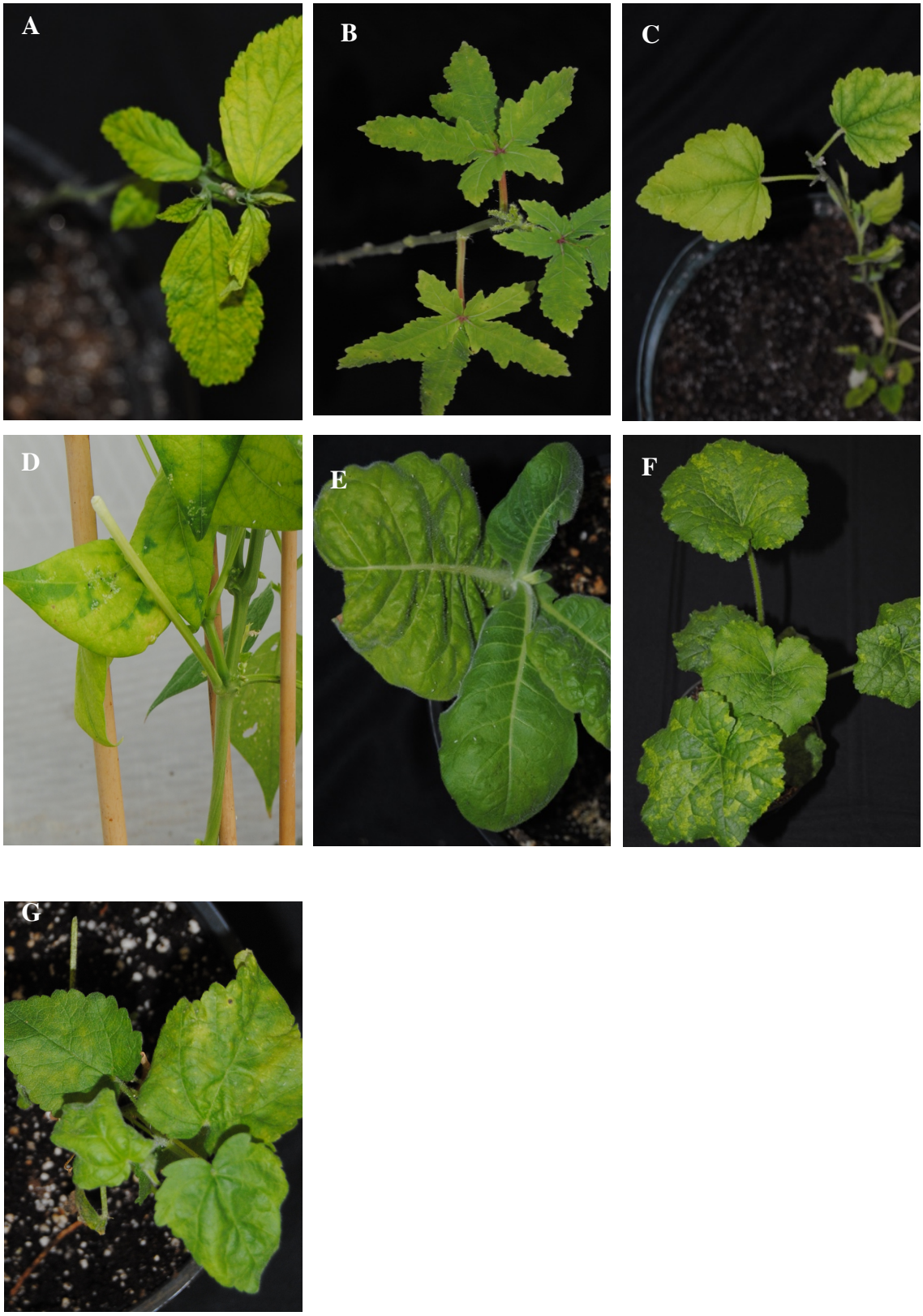

Fig. 4. 2. Symptomatic plants plant infected with SiGMV. **A**, prickly sida; **B**, okra; **C**, country mallow; **D** bean; **E**, tobacco; **F**, hollyhock; **G**, marsh-mallow.

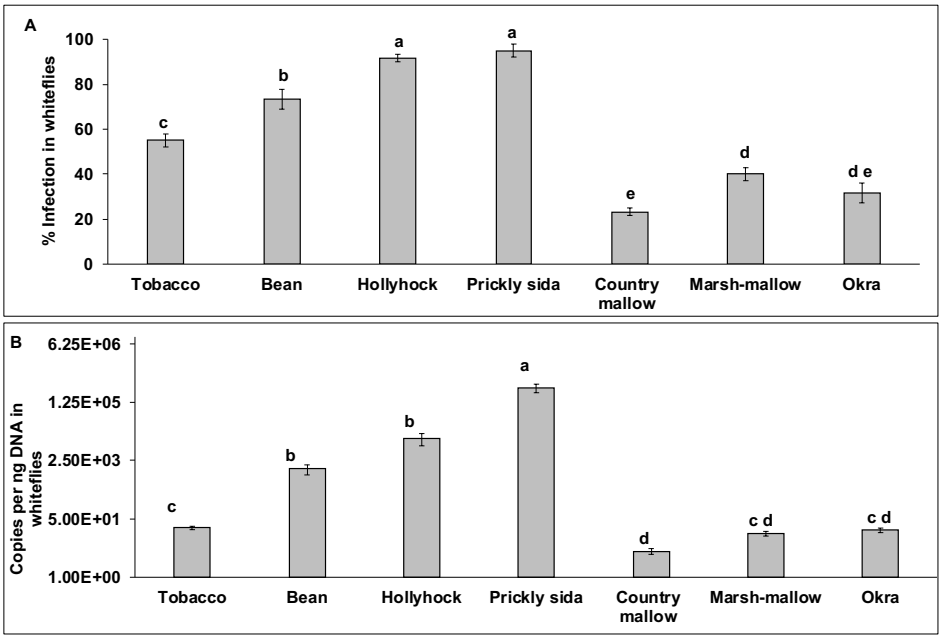

Figure 4. 3. Percent infection and SiGMV accumulation in whiteflies feeding on infected plants

A. Bar with standard errors represent the average percent infection in whiteflies feeding on SiGMV-susceptible plant species. B, Bar with standard errors represent the average number of SiGMV copies accumulated in whiteflies feeding on SiGMV-susceptible plant species. For copy numbers Y- axis is on logarithmic scale. Means with different letters are significantly different (Tukey-HSD, $P < 0.05$).

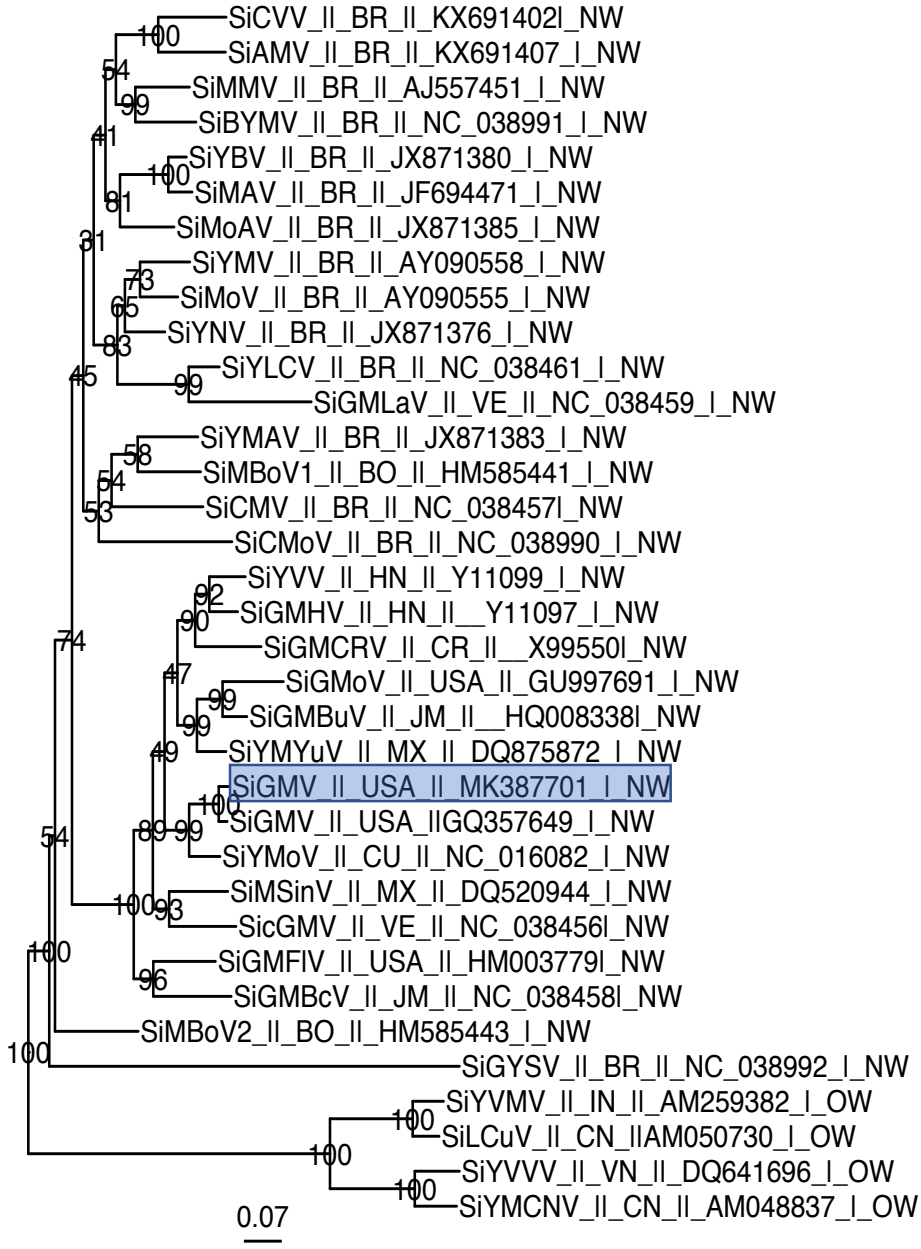

Figure 4. 4. Phylogenetic relationships among the DNA-A of SiGMV sequence obtained in this work and previously described DNA-A of sida viruses. Relationships were inferred by Maximum Likelihood analysis of the sequences. Support for nodes in a bootstrap analysis with 1000 replicates is shown. Tree branch lengths are drawn to scale and bar indicates number of nucleotide differences between branch nodes. Node tip shows virus abbreviation according to the International Committee on Taxonomy of Viruses, country of isolation, accession number and location (OW or NW). Highlighted sequence obtained in this study.

CHAPTER 5

SINGLE *BEMISIA TABACI* POPULATION DOMINATES MAJOR CROPS WITHIN A FARMSCAPE

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Abstract

The sweet potato whitefly, *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae), is one of the most important pests of agricultural crops worldwide. *B. tabaci* Middle East- Asia Minor 1 (MEAM1) cryptic species was introduced to the US three decades ago and continues to cause serious economic damage in multiple crops across the country directly by feeding on the plant phloem and indirectly by transmitting numerous plant pathogenic viruses including begomoviruses. To the best of our knowledge, no information is available on the genetic diversity of *B. tabaci* colonizing multiple crops at a farmscale level. Therefore, using a set of six microsatellite markers, we studied the population genetic structure of whiteflies in important agricultural regions of Georgia. Whitefly populations were collected from thirty-six locations from the Coastal Plain and Piedmont regions of Georgia. Whitefly samples from each location were identified to species/cryptic species level using mitochondrial cytochrome oxidase I (mtCOI) gene analysis. For the first time in Georgia, we found Mediterranean (MED, also known as Q biotype) in field-grown snap bean in the Clarke county. Analysis of molecular variance (AMOVA) and clustering analysis revealed no genetic differences between the populations colonizing different host plants at a farmscape level. Our results are in agreement with previous reports that, within a geographical area, a single whitefly population dominates the agro-ecosystem.

Introduction

In the southeastern USA, the landscape of the state of Georgia is very diverse, with multiple ecological zones and diverse agricultural practices (Rudd et al. 2018). The Georgia landscape can be divided into five distinct physiographic regions based on climatic conditions: the Appalachian Plateau, the Valley and Ridge, the Blue Ridge, the Piedmont, and the Coastal

Plain. Agricultural activities are primarily centered in the Coastal Plain (Rudd et al. 2018), with some crops (mainly wheat, soybean, corn, and cotton) planted in the Piedmont. The Coastal Plain agriculture is very diverse with multiple vegetables, cotton, soybean, pecan, peanuts, and onions planted in the region. Population structure of an organism depends on its dispersal ability, ecological differences and agricultural practices (Sun et al. 2012). Therefore, Georgia agroclimatic conditions and agricultural practices provide ideal conditions to test the effects of spatial separations on the population genetics of a polyphagous pest, *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae).

B. tabaci is a serious pest of open-field crop production systems throughout the world. *B. tabaci* causes direct damage to plants by feeding on the phloem (Brown et al. 1995, Schuster et al. 1996), and indirect damage by acting as a vector for multiple plant-pathogenic viruses (Castillo et al. 2011). *B. tabaci* cryptic species complex composed of at least 42 species, based on mitochondrial cytochrome oxidase subunit I (mtCOI) gene analysis (Boykin et al. 2007; Boykin and De Barro, 2011; Firdaus et al. 2013; Hu et al. 2018; Marubayashi et al. 2013; Roopa et al. 2015). Middle East-Asia Minor 1 (MEAM1, formerly biotype B) and Mediterranean (MED, formerly biotype Q) are the most invasive and damaging members of the *B. tabaci* cryptic species complex (De Barro et al. 2011). Biotype A of *B. tabaci* is known to be present in the United States of America (USA) since the late 1800s, although until 1986, it didn't pose a serious economic threat. After introduction of MEAM1 in 1986, *B. tabaci* started attacking crops that it had not infested earlier, such as poinsettia, followed by serious economic losses in field-grown tomatoes, *Solanum lycopersicum* L. (Hamon and Salguero 1987, Schuster et al. 1989, Hoelmer et al. 1991). Since then, *B. tabaci* has become a serious pest on almost all field-grown vegetable crops in the Southeastern United States. *B.*

tabaci readily colonize cucurbits, tomato, snap bean, soybean, cotton, and other vegetable crops, while transmitting a wide range of viruses in vegetables (Adkins et al. 2011).

Understanding the genetic structure of whiteflies would be helpful in predicting population dynamics and spread of resistance genes, if and/or when they arise. Owing to their ease of use, high polymorphism, co-dominant inheritance microsatellite markers are frequently used in population genetics studies (Schlötterer, 2001). Many microsatellites have been developed for whitefly population genetic analysis (Dalmon et al. 2008, De Barro et al. 2003, Delatte et al. 2006, Gauthier et al. 2008, Hadjistylli et al. 2014, Tsagkarakou and Roditakis, 2003, Tsagkarakou et al. 2007), providing valuable insights into host association (Saleh et al. 2012), detecting hybridization (Delatte et al. 2006), detecting insecticide resistance levels (Gauthier et al. 2014), and population genetics at a country or global level (Hadjistylliet al. 2016). However, to the best of our knowledge, microsatellite markers have not been tested to study whitefly population genetic structure at farmscape level.

In the current study, we collected thirty-six whitefly populations from different crops grown in two major agricultural growing regions of Georgia (Piedmont and Coastal Plain). We examined the population genetics and structure of *B. tabaci* using six polymorphic nuclear microsatellite loci. Our main focus was to determine whether 1) whitefly populations colonizing different host plants at the farmscape level show host-associated genetic differentiation, and 2) if whitefly populations collected from different regions show spatial genetic differentiation.

Materials and Methods

In July-Sept 2019, whiteflies were collected from different crops from major agricultural regions of Georgia. A total of 36 different populations of whiteflies were collected from four areas of two different regions [Piedmont (northeast and northwest); Coastal Plain (Southeast and Southwest] (Table 1). ("Population" here refers to whiteflies collected from a single site.) Populations were collected from plants that have been reported as hosts for whiteflies. Using an aspirator, whitefly adults were collected from the underside of the leaves. Collected whitifies were transferred immediately into a vial with 95% ethanol. Minimum 25 whiteflies were collected from every site. In order to limit the migration effects, the sampling was done in early fall, when whitefly populations are low. If not used immediately, samples were stored at -80 °C. Total DNA was extracted from individual whiteflies using a specially-formulated Chelex resin, InstaGene Matrix (Bio-Rad, Hercules, USA). For every population, twelve females were genotyped at six loci using the primers: Bem6, Bem11, Bem15, Bem23, Bem25, and Bem31 by De Barro et al (2003) (Table 2). PCR amplification for microsatellite primers was conducted in 12.5-µl reactions composed of 6.25 µl of microsatellite PCR master mix **(**Qiagen, Valencia, USA), 1 µl of sterile water, 1.25 µl of forward and reverse primer mix (10 pmol), and 4 µl of DNA template. The forward primers were labeled with the fluorescein derivative 5-carboxyßuorescein (FAM) (Eurofin Genomics, USA) for microsatellite scoring, and the PCR cycling parameters were 94°C for 7 min, followed by 35 cycles of 1 min at 94°C (denaturation), 1 min at 47°C. (annealing), 1 min at 73°C (extension), and a final extension of 72°C for 1 h (McKenzie et al. 2012). One µl of PCR product was submitted to UGA Genomics (Athens, GA, USA) for genotyping and amplicon size analysis. Briefly, 1 µl of PCR product was combined with 8.5 µl of 8.5 µl of Hi-Di formamide (Applied Biosystems Inc., CA, USA) and 0.5 µl size standard (Genescan

ROX-500, Applied Biosystems Inc.). One µl of diluted PCR products was run on were run on an ABI 3730xl DNA analyser. Allele size were determined by comparing the mobility of the PCR products to that of Genescan ROX-500 size standards in R using Poppr package. Duplicate genotypes of Middle East Asia Minor (MEAM1) (20%) and Mediterranean (MED) (100%) were generated to measure the error in genotyping. Alleles were treated as missing data in cases of amplification failure, three or more peaks in the electropherogram, or a peak of insufficient height.

Whitefly species detection

Species diversity in the collected populations was determined based on morphological characteristics under a dissecting microscope at 10X magnification, followed by mitochondrial cytochrome c oxidase subunit I – COI gene analysis (Folmer et al. 1994). Three *B. tabaci* females/site were identified to the cryptic species level by amplification of 840 bp mitochondrial-CO1 gene fragments using the primers and conditions described by Mugerwa et al. (2018). Amplified PCR products were cleaned using the Gene JET PCR Purification Kit (Thermo Scientific, Waltham, USA). The cleaned PCR products were sequenced (Eurofins Genomics, Louisville, USA). Whitefly biotypes were determined based on direct sequence comparisons using the web-based NCBI BLAST sequence comparison application (http://blast.ncbi.nlm.nih. gov/Blast.cgi).

Genetic diversity of *B. tabaci*

Within *B. tabaci* cryptic species, the allelic and genotypic frequencies per locus were calculated in R statistical program version 2.93 (R core team 2013). Number of alleles, diversity, expected

heterozygosity, and evenness in allele diversity for polymorphic loci were calculated using the R statistical package POPPR. Whiteflies from each site were assessed for the presence of null alleles and departure from Hardy–Weinberg equilibrium (HWE) using the R statistical program POPPR. While accounting for null alleles, observed and expected heterozygosities were calculated using GENEPOP 3.1d (Raymond and Rousset, 1995). Whiteflies from each location were tested for linkage disequilibrium for each locus using the R statistical program POPPR (Table 3).

Determining population structure and number of populations

Analysis of molecular variance (AMOVA) was performed between different populations to identify the significance of host association and geographical partitioning (South East, South West, North East, and North West) using the R package POPPR. Population structure was analyzed using discriminant principal components analysis (DPAC) implemented in GenAlex 6.41 (Peakall and Smouse 2006) using the POPPR R package.

Results

Whitefly species detection

The banded-wing whitefly (*Trialeurodes abutilonea* Haldeman) was present in 100% of the collection sites. The greenhouse whitefly (GHWF), *Trialeurodes vaporariorum* (Westwood), was detected from field-grown squash in Griffin. The citrus whitefly, *Dialeurodes citri* (Ashmead), was detected from horseweed growing with cotton in Mitchell and Sumter counties. MED was detected from field-grown snap bean and eggplant in Clarke and

Spalding counties, respectively. MEAM1 was present at every site (100%) and was far more abundant than other species/biotypes.

Genetic diversity of *B. tabaci*

A total of 47 alleles were scored across all loci in the studied populations. All loci had good expected heterozygosity (0.19-.79) and evenness (0.43- 0.84) in allele distribution, confirming effectiveness of these microsatellite markers for detecting variability (Table 3). The average frequency of null alleles ranged between 0.02 to 0.12. The number of alleles per locus ranged from 9–5 (Table 3). The observed heterozygosity (Ho) ranged from 0.05 (BEM 23) to 0.63 (BEM25) and expected heterozygosity (He) ranged from 0.13 (BEM23) to 0.72 (BEM15) (Table 3). The fixation index (F_{ST}) measures the genetic differentiations between the populations. Its range is from 0–1; F_{ST} close to zero means no difference, and F_{ST} close to one means high genetic variation between populations. In the current study, F_{ST} ranged from 0.001 to 0.42 (Table 4). The lowest F_{ST} was between populations collected from cotton in worth county and Toombs county. The hiighest F_{ST} was observed with MED populations collected from Spalding county and MEAM1 collected from tomato in Tifton.

Determining population structure and number of populations

AMOVA revealed that the highest genetic variation was found within samples (66.1%), followed by genetic variation between samples within a population (23.4%) (Table 3). In DAPC analysis, populations were clustered together with high overlap between them. MED was clustered separately with very low overlap with other populations (Fig. 2A). NW and NE

populations were clustered with medium overlap with other populations, whereas populations from SE and SW had very high overlap with each other (Fig. 2B).

Discussion

Host specialization by pest populations can have significant implications for pest management (Kennedy, 1992). Host specialization drives survival of individuals with certain genotypes over others. Host specialization can result in genetic clustering in different crops planted at a farmscape level. Here, we studied the possibility of genetic clustering of *B. tabaci* populations collected from different crops planted at a farmscape level. We did not observe genetic differences between populations collected from different crops planted within the same region or different regions. Earlier studies have shown that whiteflies collected from the same geographical areas tend to cluster together in population genetic analysis (Dinsdale et al. 2012). Therefore, earlier results and those of this study show that a single genetic cluster of whiteflies dominates all crops planted within a geographical area.

Based on Mt COI sequence, banded-wing whiteflies and *B. tabaci* (MEAM1) were present in all locations, although MEAM1 was far more abundant. For the first time, we found MED in field-grown snap bean and egg plant in North Georgia. However, at both locations, MED individuals were present with MEAM1 and were limited in numbers (less than 10%). MED has replaced MEAM1 as the dominant whitefly in certain regions of China (Chu et al. 2010, Wang et al. 2010b, Yuan et al. 2012). MED has been in the US for more than 15 years, and has been restricted mostly to greenhouses on ornamentals (McKenzie et al. 2017). But recently, MED has been detected from residential landscapes in Florida, and now we found it in field-grown snap bean in Athens, GA. Predicting what will trigger MED outbreaks in the

U.S., is difficult. However, field reports of MED have increased the risk of MED spreading to important food and fiber crops in the Southeastern US. We didn't find any native American *B. tabaci* species (NW, Biotype A), confirming the indigenous biotype-A has been replaced by MEAM1 in Georgia. In this study, the greenhouse whitefly, *T. vaporariorum,* was found outside of the greenhouse on squash, reinforcing the belief that *T. vaporariorum* is not limited just to greenhouses.

Genetic variation in aphid populations spread over a landscape are reportedly dependent on geographical isolation between collection sites. Furthermore, aphids are reported to develop host-adapted genetic groups, which can be clustered separately using microsatellite markers (Ferrari et al. 2006; Peccoud et al. 2009; Eigenbrode et al. 2016). This is understandable, given that aphids are weak fliers (Dixon & Howard, 1986; Taylor, Woiwod, & Taylor, 1979). Also, correlation between host plant and *B. tabaci* cryptic species have been reported from Africa and Asia (Abdullahi et al. 2003, Dinsdale et al. 2010, Delatte et al. 2011). However, we didn't observe the host plant co-relation within the collected populations. The estimated low genetic diversity suggests that during the last three decades, *B. tabaci* has suffered multiple genetic bottlenecks, limiting the amount of genetic diversity compared to what is observed in its native ranges in Africa/Asia (Wosula et al. 2017). Furthermore, continuous gene flow between populations resulting from whitefly clouds generated every year during cotton defoliation ensures a genetically uniform *B. tabaci* populations in the region.

B. tabaci population genetic analysis carried out over small geographical areas tend to show no or minimum genetic differences (Delatte et al., 2006; Dalmon et al., 2008; Tsagkarakou et al., 2012; Tahiri et al., 2013). However, studies carried out over large geographical areas have reported population structures (Dıaz et al. 2015; Simon et al. 2007). Therefore, *B. tabaci*

populations tend to structure between regions more so than within regions. Our results partly agree with these reports; *B. tabaci* populations collected from the Coastal Plain region (SE and SW) were clustered together with high overlap compared to populations collected from Piedmont (NE and NW). However, we didn't observe high levels of genetic diversity between these regions, probably due to lack of geographical barriers between them. Single MED populations analyzed in the current study cluster separately in DAPC, confirming microsatellite markers used in the current study can differentiate between MEAM1 and MED. Some individuals from Spalding and Oconee counties overlapped with MED clusters showing the presence of MED individuals in these populations, and confirming the results obtained though mt-*COI* gene analysis.

Anthropogenic factors such as transport of whitefly-infested plants can significantly impact whitefly genetics in the region. For instance, in two provinces of South Korea, Jeju and Pyeongtaek, whitefly genetic clustering evolved very differently over the same period of time. Over a period of three years, Jeju whiteflies had a single genetic cluster. However, Pyeongtaek whitefly genetic clustering changed every year. Farmers in Jeju produce their own tomato seedlings, thus decreasing the chances of introduction of new genotypes. On the other hand, farmers at Pyeongtaek procure tomato seedling from different nurseries, possible allowing the introduction of different populations of whiteflies into the region (Park et al. 2019). Although we didn't gather data on actual sources of seedlings in the case of vegetables, some of our populations were taken from row crops (cotton and soybean), which are seed-planted, and we didn't find genetic differences between whiteflies collected from vegetables or row crops. Therefore, an anthropogenic factor in whitefly population genetics may not be playing a big role in our case.

Whitefly population genetics at a geographical level with respect, to introduction route and establishment, have been studied extensively (Abdelkrim et al. 2017; McKenzie et al. 2012, Li et al. 2017). Here for the first time, we have reported whitefly population genetics at a farmscape level. Homogeneity of populations shows continuous gene flow between them. Therefore, any new trait arising anywhere in the population could be distributed throughout the population in a short period of time. Furthermore, introduced *B. tabaci* biotype MED, with its favorable traits such as insecticide resistance, will find it easy to replace the single MEAM1 population quickly. Therefore, identification of "hot spot" areas with higher introduction potential or development for insecticide resistance traits in local whitefly populations is extremely important for development of long-term whitefly management programs.

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Table 5. *B. tabaci* **regions, county and source plants surveyed in Georgia farmscape levels**

Sr. No	Region [a]	County	Source plants	Codes used in analysis
1	NE	Clarke	Snap bean	NE_CL_SN
2	NE	Oconee	Lantana	NE_OC_LN
3	NW	Spalding	Okra	NW_SP_OK
4	NW	Spalding	Dandelion	NW_SP_DND
5	NW	Spalding	Eggplant	NW_SP_EG
6	NW	Spalding	Lantana	NW_SP_LN
7	NW	North Georgia	Poinsettia	NE_NG_PO
8	SW	Sumter	Cotton	SW_SM_CT
9	SW	Colquitt	Cotton	SW_CQ_CT
10	SW	Tift	Snap bean	SW_TF_SN
11	SW	Tift	Squash	SW_TF_SQ
12	SW	Tift	Tomato	SW_TF_TM
13	SW	Mitchell	Cotton	SW_MT_CT
14	SW	Mitchell	Horseweed	SW_MT_HW
15	SW	Tift	Soybean	SW_TF_SY
16	SW	Tift	Tobacco	SW_TF_TB
17	SW	Worth	Cotton	SW_WR_CT
18	SW	Worth	Redroot pigweed	SW_WR_PG
19	SW	Colquitt	Squash	SW_CQ_SQ
20	SW	Colquitt	Eggplant	SW_CQ_EG
21	SW	Decatur	Cotton	SW_DC_CT
22	SW	Tift	Cotton	SW_TF_CT
23	SE	Burke	Cotton	SE_BR_CT
24	SE	Toombs	Soybean	SE_TM_SY
25	SE	Toombs	Cotton	SE_TM_CT
26	SW	Echols	Cotton	SW_EH_CT
27	SW	Echols	Squash	SW_EH_SQ
28	SW	Echols	Eggplant	SW_EH_EG
29	SW	Echols	Lantana	SW_EH_LN
30	SE	Candler	Cotton	SE_CH_CT
31	SE	Candler	Snap bean	SE_CH_SN
32	SE	Candler	Squash	SE_CH_SQ
33	SE	Candler	Redroot pigweed	SE_CH_PG
34	SE	Candler	Morning glory	SE_CH_MG
35	SE	Wheeler	Cotton	SE_WH_CT
36	SE	Montgomery	Cotton	SE_MT_CT

[a] NE: Northeast; NW: Northwest; SE: Southeast; SW: Southwest

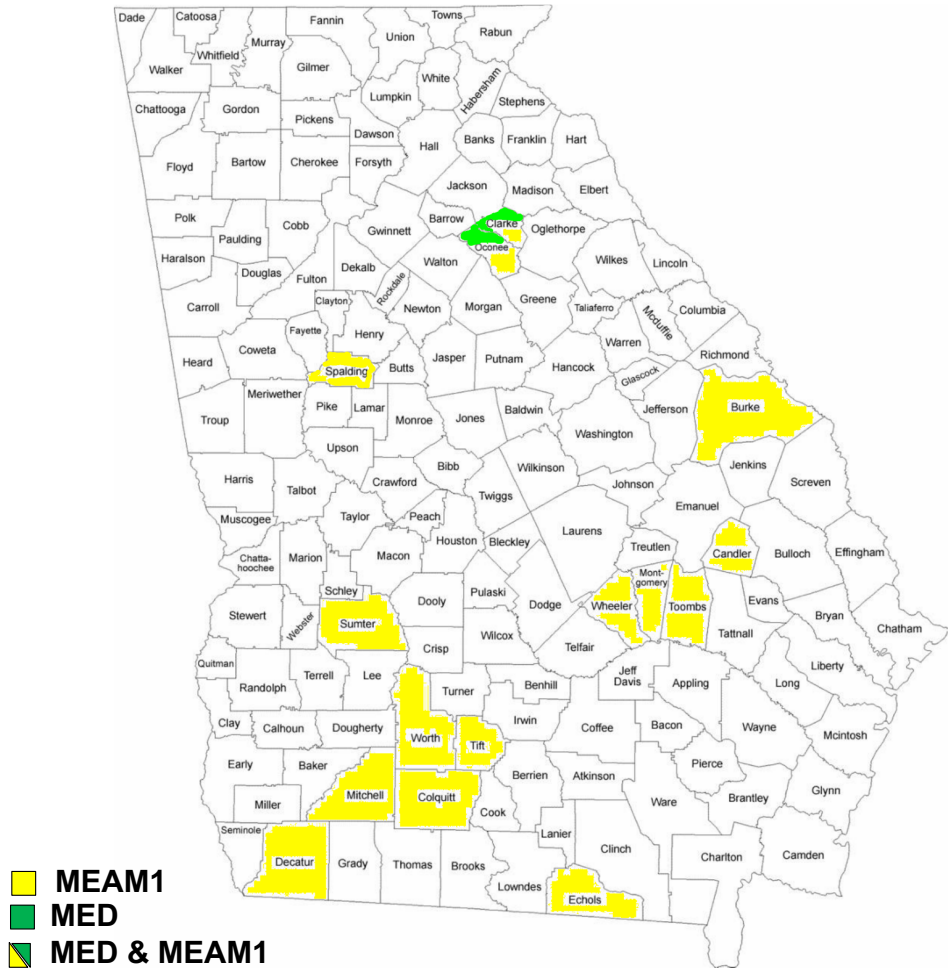

Figure 5.1. Collection sites of *B. tabaci* populations. Color denotes presence of *B. tabaci* cryptic species, MEAM1 (yellow), MED (green), and both, MEAM1 and MED (yellow and green matrix).

Table 6. Primer sequences

Loci	Primers (5′–3′ direction)	Repeat motif	Size range	No. of Alleles	PCR T_a(°C)	Source of microsatellite	H_O	H_E	GenBank no.
BEM6	F: TTACACTTAACACCAGAACT R: GATGGCTTATGTTATAATACTA	$(CA)_8$imp	133–413	25	50	Australasia/ Oceania	0.01	0.904	AY145452
BEM11	F: TTCAATGATGCTTTCCTGAC R: CAAATAAATACACCATTTACA	$(CT)_4(AG)_5$ $(GA)_{11}$imp	130–216	18	50	B biotype	0.078	0.808	AY145453
BEM15	F: AGCAGCATCAACAGGCTC R: CTAGATTCTGCTTGAAAGG	$(CAA)_6(CAG)_4$ $(CAA)_4$	142–262	21	50	Asia 2	0.243	0.852	AY145456
BEM23	F: CGGAGCTTGCGCCTTAGTC R: CGGCTTTATCATAGCTCTCGT	$(GAA)_{31}$imp	153–387	34	55	B biotype	0.075	0.931	AY145461
BEM25	F: AAGTATCAACAAATTAATCGTG R: TGAAGAATAAGAATAAAGAAGG	$(CTT)_{10}$	95–188	19	50	B biotype	0.063	0.844	AY145462
BEM31	F: AAGAACTAGCCAGGGACAAAC R: GTCATTTCTGGATTCTCAGCA	$(TGG)_4(TTG)_1($ $TGG)_3(TTG)_2($ $TGG)_2$	98–149	15	50	Asia 1	0.065	0.820	AY145463

Table 7. Genetic diversity of loci.

Loci	Allele[a]	Hexp [b]	Evenness [c]	Ho [d]	Fis [e]	Fst [f]
BEM6	9.0	0.19	0.43	0.06	0.39	0.41
BEM11	7.0	0.60	0.74	0.63	-0.12	0.06
BEM15	7.0	0.78	0.83	0.58	0.18	0.08
BEM23	5.0	0.18	0.44	0.05	0.59	0.27
BEM25	8.0	0.69	0.72	0.63	-0.01	0.09
BEM31	5.0	0.22	0.43	0.09	0.29	0.31
mean	6.8	0.44	0.60	0.34	0.09	0.13

[a] Allele: Number of allele observed at each loci; [b] Hexp: heterozygosity within Loci; [c] Evenness: evenness in the distribution of hetetrozygosity [d] Ho: observed heterozygosity within Loci; [e] Fis: inbreeding coefficient per overall loci; [f] Fst: fixation index per overall loci;

Table 8. Analysis of molecular variance (AMOVA) for the 36 *B. tabaci* populations collected from major agricultural regions of Georgia, based on six microsatellite markers

Source of variation	Degrees of freedom	Sums of squares	Mean sums of squares	% variation	*P* -value
Between regions	4	27.620	9.200	2.841	0.008
Between counties within the region	11	36.510	3.310	-1.000	0.320
Between populations within the counties	19	70.990	3.731	8.712	> 0.001
Between samples within the population	418	522.070	1.252	23.341	> 0.001
Within the samples	452	330.850	0.731	66.102	< 0.001

Significance at $P < 0.01$ based on 999 permutation

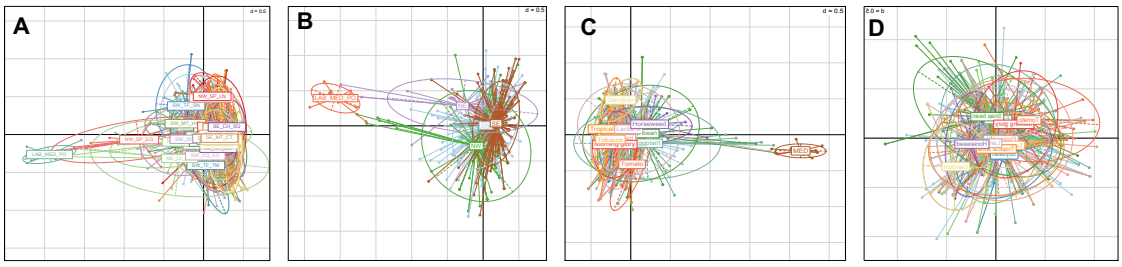

Figure 5. 2. Discriminant analysis of principal components (DAPC) of 36 *B. tabaci* populations collected from major agricultural regions of Georgia.

A, Scatter plot depicting clustering of *B. tabaci* populations collected from different locations. B, Scatter plot depicting clustering by region. C, Scatter plot depicting clustering by host. D, Scatter plot depicting clustering by host without outgroup. LAB_MED_PO (MED lab population); NE (populations from northeast); NW (populations from northwest); SW (populations from southwest); SE (population from southeast).

CHAPTER 6

DIFFERENTIAL TRANSMISSION OF A NEW WORLD AND AN OLD WORLD BEGOMOVIRUSES BY MEAM1 AND MED, MEMBERS OF THE *BEMISIA TABACI* CRYPTIC SPECIES COMPLEX

Gautam, S., Gadhave, K. R., Buck, J. W., Dutta, B., Coolong, T., Adkins, S., Srinivasan, R. To be submitted to PLOS Pathogens

Abstract

Bemisia tabaci Gennadius is a serious pest of vegetable crops worldwide. Middle East Asian Minor (MEAM1) and Mediterranean (MED) are the two most invasive and damaging members of the *B. tabaci* cryptic whitefly-species complex. Both MEAM1 and MED are excellent vectors of begomoviruses. In 2016, MED was reported for the first time outside of the greenhouse in Florida, USA, increasing the risk that field populations of MED would alter the whitefly-disease complex in important vegetable crops in the Southeastern United States. Whitefly-transmitted, squash-infecting cucurbit leaf crumple virus, (CuLCrV, bipartite New World begomovirus) and tomato-infecting tomato yellow leaf curl virus (TYLCV, monopartite Old World begomovirus) are two important whitefly-transmitted begomoviruses in the Southeastern US. Therefore, to better understand the implications of the introduction of a new vector on the epidemiology of these viruses, we compared the transmission of TYLCV and CuLCrV between already-established MEAM1 and newly introduced MED whiteflies. MEAM1 was able to efficiently transmit both TYLCV (100%) and CuLCrV (86.7%). However, MED was only able to transmit TYLCV (100%). In order to ascertain the reason for non-transmission of CuLCrV by MED, we compared the percent infection, acquisition, retention, and localization of TYLCV and CuLCrV in MED and MEAM1. TYLCV acquisition and retention were significantly higher in MED than MEAM1; whereas CuLCrV percent infection, acquisition, and retention were significantly higher for MEAM1 than MED. Fluorescence *in-situ* hybridization of whitefly organs (salivary glands and midgut) revealed that CuLCrV failed to accumulate in the primary salivary glands of MED, resulting in non-transmission of CuLCrV by MED. Our results are in agreement with previous reports of MED being a more efficient vector for TYLCV than MEAM1. However, for

the New World begomovirus CuLCrV, MEAM1 seems to be a better vector than MED. This study is the first to compare the transmission of a New and an Old World begomovirus by MEAM1 and MED, and provides valuable insights into the differential transmission characteristics of MEAM1 and MED.

Introduction

Inadvertent introduction of new species into new geographical areas can cause large-scale economic and ecological consequences (Aukema et al. 2011; Liebhold et al. 2012). Insects form the biggest part of all non-native invasive animal species on the planet (Seebens et al. 2017). Many insect species are vectors of important plant pathogens, particularly viruses (Eigenbrode et al. 2018). Introduction of efficient vectors can activate the dormant native/introduced pathogens (Anderson et al. 2004; Bar-Joseph 1989; McClean 1975,). For instance, citrus tristeza virus (CTV), an introduced pathogen in South America, didn't cause any problem until the introduction of an efficient aphid vector, *Toxoptera citricidus* (Bar-Joseph 1989; McClean 1975). Introduced insect vectors interact with and can competitively displace closely related indigenous/introduced species (Liu et al. 2007; Zang et al. 2005), and significantly alter the local pathogen dynamics (Pan et al. 2012).

In 2004, *Bemisia tabaci* Gennadius Mediterranean (MED, also known as Q biotype) whitefly was first detected in Arizona, USA (Dennehy et al. 2005). MED is now present in greenhouses in 23 states in the United States (Dennehy et al. 2005). For the first time in 2016, MED was detected outside of the greenhouse in Florida (Mckenzie and Osborne, 2017). This has increased

the risk of MED moving into open production systems. MED is a member of the *B. tabaci* cryptic species complex composed of at least 42 species, based on mitochondrial cytochrome oxidase subunit I (mtCOI) gene analysis (Boykin et al. 2007; Boykin and De Barro, 2011; Firdaus et al. 2013; Hu et al. 2018; Marubayashi et al. 2013; Roopa et al. 2015). In many parts of the world, invasion by members of *B. tabaci* cryptic species have resulted in outbreaks of whitefly-transmitted viruses (Brown, 2007; Brown, 1994; Pan et al. 2011; Pan et al. 2012; Seal et al. 2006; Varma and Malathi, 2003). For example, introduction and establishment of *B. tabaci,* Middle East Asia Minor1 (MEAM1, formerly known as B biotype) whitefly in Brazil resulted in emergence of previously unreported whitefly-transmitted begomoviruses (Rojas et al. 2005), presumably because polyphagous MEAM1 feeds on multiple native host plants and facilitates the transmission of begomoviruses found in common non-cultivated plant species into cultivated plants (Bedford et al. 1994).

B. tabaci-transmitted plant viruses, such as ss (DNA) begomoviruses, are increasingly becoming problematic worldwide (Islam et al. 2018; Rojas et al. 2008; Saeed and Samad, 2017; Varma and Malathi 2003). Viruses within the genus *Begomovirus show phylogenetic geographical distribution based on genome organization. B*egomoviruses reported from the Old World (OW [Europe, Africa, Asia, and Australia]) are mostly monopartite, whereas those reported from the New World (NW [the Americas]) are bipartite (Briddon et al. 2010; Nawaz-ul-Rehman and Fauquet 2009; Rojas et al. 2005). Both NW and OW begomoviruses supposedly evolved from common ancestors and were separated via continental drift (Lefeuvre et al. 2011). Phylogenetic analysis of begomoviruses based on coat protein sequences grouped them into five major clades based on geographical distribution (Padidam et al. 1995; Rybicki 1994;). Whitefly phylogenetics

based on mitochondrial DNA markers reveal the coinciding geographical distribution (Brown, 2001; Frohlich et al., 1999), and also suggesting vector-virus co-evolution in geographical isolation.

Previous studies on begomovirus acquisition, retention, and inoculation by MEAM1 and MED, have reported MED as a better vector than MEAM 1for tomato yellow leaf curl virus (TYLCV; (Pan et al. 2012, Liu et al. 2013, Ning et al. 2015). *Tomato yellow leaf curl virus* is a species in the genus *Begomovirus* and Family, *Geminiviridae* MED is believed to have evolved in the OW (De-Barro et al. 2011). TYLCV also supposedly evolved in the OW (Lefeuvre et al. 2010). Therefore, it is reasonable to assume that coevolution of MED with TYLCV might have made it an excellent vector for TYLCV. Introduction of MED in the NW has raised questions about how MED will affect the agriculturally important OW and NW begomovirus disease epidemics.

Squash and tomato are two important summer crops in the Southeastern United States, a NW bipartite begomovirus, Cucurbit leaf crumple virus (CuLCrV) is increasingly becoming important in squash production systems. TYLCV, a monopartite OW begomovirus, is a serious threat to tomato production worldwide (Moriones and Navas-Castillo, 2000; Navas-Castillo et al. 2011). *Cucurbit leaf crumple virus* is a species in the genus *Begomovirus* and Family, *Geminiviridae*. Currently, under field conditions, both TYLCV and CuLCrV are transmitted by MEAM1. Therefore, in order to evaluate the possible impact of MED on CuLCrV and TYLCV disease epidemics, we compared the transmission of CuLCrV and TYLCV by MED and MEAM1, and we compared the acquisition and retention of CuLCrV and TYLCV by MED and

MEAM1. Lastly, we analyzed the localization of CuLCrV and TYLCV in MED and MEAM1 salivary glands and midgut.

Materials and Methods

Plants, virus inoculums, and whiteflies

Yellow summer squash (*Cucurbit pepo* cv Goldstar, Jonhny's Selected Seeds, ME, USA) and tomato (*Lycopersicon esculentus* cv. Florida 47, Harris Moran Seeds Company, CA, USA) seeds were planted in 1 L plastic pots using LP5 Plug Mix (SunGro Horticulture Industries, WA, USA). Plants were maintained in a greenhouse (25–30°C with a 14 L:10 D photoperiod) in insect-proof cages (Megaview Science Co., Taichung, Taiwan), with 5 plants per cage. Plants were fertilized every week with water-soluble Miracle-Agro fertilizer (Scotts Miracle-Gro products, Inc., OH, USA) as per label recommendations. The CuLCrV isolate used in this study was collected from infected squash plants from Tifton, GA in 2016, and has been maintained on susceptible squash (Goldstar F1 hybrid) through repeated inoculations using viruliferous MEAM1. The TYLCV isolate used in this study was collected from commercial tomato farms located in Montezuma (Macon County, GA, USA) in 2009, and has been maintained on TYLCV-susceptible tomato variety Florida 47 through repeated inoculations using viruliferous MEAM1. For all experiments, four-week-old plants were infected with the viruses.

MEAM1 and MED colonies were maintained on cotton in whitefly-proof cages kept in two separate greenhouses 500 meters away from each other. Every month, individuals were tested for

the purity of the colony by partially sequencing the *mtCOI* gene using universal COI primers C1-J-2195 and L2-N-3014 (Frohlich et al. 1999). The whiteflies (*B. tabaci* cryptic species MEAM1) used in the present study were first collected in Tifton, Georgia, and have since been reared on cotton plants in 10 cm diameter x 8 cm tall pots in whitefly-proof cages in the greenhouse at above-stated conditions. MED colonies were obtained from Dr. Ronald D. Oetting, Dept of Entomology, UGA. Originally, MED individuals were collected from poinsettia plants from a nursery located in North Georgia, USA. In order to obtain insect free CuLCrV and TYLCV inoculum sources for transmission studies, viruliferous MEAM1 were obtained by releasing whiteflies for a 72h acquisition access period (AAP) on CuLCrV-infected squash or TYLCV-infected tomato. Following the AAP, 20 whiteflies were tested via PCR to confirm virus acquisition and viruliferous MEAM1 (~100/plant) were attached to the first true leaves of susceptible squash or tomato using clip cages (36.5 x 25.4 x 9.5 mm). Individual plants with clip cages were placed in insect-proof cages under the conditions described above. One week later, leaves with clip cages were excised, and plants were carefully examined for the presence of whitefly adults, nymphs and eggs using a hand lens. Four weeks post-inoculation, infection was confirmed through PCR (described below). Viruliferous whiteflies were obtained by releasing MEAM1/MED on insect-free CuLCrV-infected squash or TYLCV-infected tomato for 72h (AAP).

DNA extraction from plant and insect samples

DNA from single whiteflies was extracted using a specially formulated Chelex resin, InstaGene Matrix (Bio-Rad, Hercules, USA) (Srinivasan et al., 2012). Individual whiteflies were macerated

in 1000 µl of autoclaved distilled water in 1.5 ml centrifuge tubes, followed by centrifugation at 12000 rpm for 1 min. Supernatant was discarded and 100 µl of InstaGene matrix was added to the pellet, which was then incubated at 56 °C for 20 min, followed by vortexing at full speed for 10 sec. Tubes were then placed in a 100°C heating block for 8 min, followed by vortexing at full speed for 10 sec. Finally, tubes were centrifuged at 12000 rpm for 3 min and stored at −20°C until used.

For plants, the topmost leaf of the non-infected or CuLCrV-infected squash, or TYLCV-infected tomato, was removed 4 weeks after inoculation. Excised leaves were surface-sterilized using a six-step surface sterilization protocol. Each leaf was washed in running autoclaved distilled water, followed by 1 min rinsing in 1% bleach, followed by a 1-min wash in 70% ethanol, and finally three rinses with sterile distilled water to remove sterilizing agents. One hundred mg of surface-sterilized leaf tissue were used for DNA extraction. Total genomic DNA was extracted with the GeneJET Plant Genomic Purification Kit (ThermoFisher Scientific, MA, USA) following the manufacturer's protocol.

TYLCV or CuLCrV infection and accumulation in plants or whiteflies

TYLCV infection status and accumulation in whiteflies and plants were determined using the primers and conditions described earlier by Legarrea et al. (2015). CuLCrV infection in samples was determined through end-point PCR. PCR primers 3FAC3 (5′-TTTATATCATGATTTTCGAGTACA-3′) and 5RAC1 (5′-AAAATGAAAGCCTAAGAGAGTGGA-3′) targeting the 525 bp amplicon of AC3, AC2, and

AC1 genes of CuLCrV at a final 0.5 µM concentration were combined with 5 µl of GoTaq Green Master Mix (Promega,WI, USA), 2 µl distilled water, and 20 ng of DNA to make the final volume of 10 µl. PCR cycling conditions were as follows: 95 °C for 5 min, followed by 40 cycles of 94°C for 30 sec, 54°C for 30 sec, and 72°C for 1 min and a final extension at 72°C for 5 min. CuLCrV accumulation in samples was estimated through quantitative PCR. Quantitative PCR to determine CuLCrV accumulation in plants and whiteflies was carried out using 2X GoTaq qPCR Master Mix (Promega, WI, USA) in a Mastercycler ep realplex (Eppendorf, Hauppauge, NY). Primers CuLCrV-QF (5'- CCTCAAAGGTTTCCCGCTCT-3') and CuLCrV-QR (5'-CCGATAGATCCTGGGCTTCC-3') amplifying a 110 bp region of the coat protein gene of CuLCrV were used. GoTaq qPCR Master Mix was combined with forward and reverse primers (final concentration of 0.5 µM), 10 ng DNA, and nuclease-free water for a final reaction volume of 25 µl. Cycling parameters were as follows: 95°C for 2 min; 40 cycles of 95°C for 1 min, 63°C for 15 sec, and 72°C for 20 sec. Upon completion of the run, melting curve analysis was performed to confirm the specificity of the primer pairs. Each sample was tested in duplicate, and absolute number of copies in the samples were quantified using the standard curve protocol described by Legarrea et al. (2015).

Transmission of CuLCrV and TYLCV by MEAM1 and MED

Successful transmission of CuLCrV or TYLCV by MEAM1 and MED was assayed three times. Each time, 1500 non-viruliferous whiteflies (MEAM1 or MED) were transferred onto to a single insect-free CuLCrV-infected squash or TYLCV-infected tomato for virus acquisition. Whiteflies were allowed to feed on infected plants for 72h, after which twenty individual whiteflies from

infected squash or tomato were tested for the presence of virus. Using a clip cage, potentially viruliferous whiteflies (100 adults/plant) were attached to the leaf of a non-infected squash or tomato. Plants were then held in the greenhouse conditions described above. After a 5-day inoculation access period (IAP), plants were sprayed with Admire pro (Bayer CropScience LP, NC, USA) to kill the whiteflies. Four weeks later, total genomic DNA was extracted from100 mg of tissue from the youngest leaves and subjected to endpoint PCR analysis to determine each plant's infection status. Each treatment had 10 replications, and the experiment was conducted three times (n=30).

Acquisition and retention of CuLCrV and TYLCV by MEAM1 and MED

For retention studies, MEAM1 and MED adults were allowed to feed on insect-free non-infected or CuLCrV-infected squash or TYLCV-infected tomato for 72h. Whiteflies were then transferred to four-week-old non-host cotton plants. Twenty whitefly adults were randomly collected from the cotton plants at five separate time intervals (72h, 7d, 14d, 21d, and 28d). Total genomic DNA from individual whiteflies was extracted using the protocol described above and used for both endpoint and qPCR. CuLCrV or TYLCV infection and accumulation at each interval was estimated using endpoint PCR and qPCR, respectively as described above. Percent infection was measured as whiteflies found positive for the virus over total whiteflies tested into 100. Virus accumulation was estimated in the positive whiteflies using qPCR. Each treatment had 20 whiteflies. The experiment was conducted three times (n=60).

Localization of CuLCrV and TYLCV in MEAM1 and MED

MEAM1 and MED were transferred onto insect-free CuLCrV-infected squash and TYLCV-infected tomato for virus acquisition. Whiteflies were allowed to feed on infected plants for 72h, after which twenty individual whiteflies from infected squash or tomato were tested for the presence of the virus. Viruliferous whiteflies were transferred to four-week-old cotton plants. After 72h, using an aspirator, whitefly adults were collected in 10 ml plastic vials and chilled on ice. Twenty minutes later, individual adult whiteflies were collected using fine-tip forceps, Dumont Tweezer, Style 5 (Electron Microscopy Science, PA, USA) and dissected on a glass slide for midgut and primary salivary glands (PSG) in a drop of saline buffer. The fluorescence in-situ hybridization (FISH) procedure followed the methods and protocols of Kliot et al. (2014). Briefly, dissected organs were fixed using Carnoy's fixative (VWR, PA, USA). Following fixation, organs were washed with hybridization buffer (20 mM Tris-HCl, pH 8.0, 0.9 M NaCl, 0.01% [wt/vol] sodium dodecyl sulfate, 30% [vol/vol] formamide). After washing, specimens were hybridized overnight at 4°C in 500 µl hybridization buffer supplemented with 10 pmol of Cy3-labeled fluorescent oligonucleotide probes. After 12h, using a single-eyelash brush, organs were transferred to a new slide containing 30 µl of new hybridization buffer containing DAPI (0.1 mg/ml in 1x PBS, Gene TEX, CA, USA). After 5 min, organs were gently covered with a glass cover slip, sealed with Permount (Fisher Scientific, MA, USA), and observed under an Olympus BX 60 confocal microscope. At least twenty samples were observed per treatment. The DNA probe (CuLCrV-Probe: [Cy3]5’-GCCGAAGCGCGATGCCCCAT-3’) specific to CuLCrV DNA-A was designed using primer3 software (Rozen and Skaletsky, 2000). TYLCV was localized using the TYLCV-probe ([Cy3]5'-GGAACATCAGGGCTTCGATA-3) reported earlier

by Kliot et al. (2014). FISH analysis was also performed on the organs of whiteflies feeding on non-infected plants.

Statistical analyses

Data analyses were performed in R version 3.4.2 (The R Foundation for Statistical Computing). For analysis data from experimental repeats was pooled. Percent infection in plants was evaluated assuming a binomial response (infected vs. noninfected) using Logit Model in R. Repeated-measures ANOVAs were used to compare percent infection and virus retention at different time intervals in MEAM1 or MED following feeding on CuLCrV-infected squash or TYLCV-infected tomato. Mixed effects model was used for repeated-measures ANOVAs using the lme function in the nlme package of R. Replications were treated as random variable and treatments were considered as fixed effect. Means were compared by highest significant difference (HSD) tests at $P < 0.05$.

Results

Transmission of CuLCrV and TYLCV by MEAM1 and MED

MEAM1 and MED transmitted TYLCV at the same rate ($X^2 = 0$; df= 1, 58; $P = 1$) (Fig.1). However, CuLCrV transmission rates differed significantly between MEAM1 and MED ($X^2 = 24.04$; df= 1, 51; $P < 0.0001$). More than 80% of CuLCrV infection was observed in the squash

subjected to CuLCrV-infected MEAM1 feeding. However, none of the squash subjected to CuLCrV-infected MED feeding were found to be infected with CuLCrV (Fig.1).

Percent infection and retention of CuLCrV and TYLCV by MEAM1 and MED

As indicated by endpoint PCR, percent infection in MEAM1 for CuLCrV or TYLCV decreased gradually over time, when viruliferous whitifies were transferred to non-host cotton (Fig. 2 A, B). At the end of the 4th week, approximately 73% of MEAM1 had TYLCV and 33 % MEAM1 had CuLCrV (Fig. 2 A, B). On the other hand, percent infection of TYLCV in MED didn't show gradual decline with time; at the end of the 4th week, about 90 % of MED had TYLCV (Fig.2 A). CuLCrV percent infection in MED dropped to 7 % by the end of the 4th week (Fig.2 B).

Data from qPCR revealed that amounts of TYLCV in MEAM1 and MED feeding on TYLCV-infected tomato decreased gradually with time, when viruliferous whiteflies were transferred to non-host cotton (Fig. 3 A, B). The amount of CuLCrV declined gradually in MEAM1. However, in MED, there was a sharp decline in CuLCrV after seven days. MED retained more TYLCV than MEAM1 at every time interval (Fig. 3 A). MEAM1 feeding on CuLCrV-infected squash retained significantly higher CuLCrV than MED at intervals (Fig. 3 B).

Localization of CuLCrV and TYLCV in MEAM1 and MED

Probes for TYLCV and CuLCrV provided hybridizing signals from infected organs. FISH revealed that both TYLCV and CuLCrV in MEAM1 and MED accumulated in higher amounts

in the midgut (Fig.4). Most viruses were localized within the filter chamber (Fig. 1). There was some localization in descending and ascending midgut. CuLCrV localization in dissected midguts was relative low comparative to TYLCV (Fig. 4). FISH signals for TYLCV were detected in MEAM1 and MED PSG (Fig. 5 A, B). However, for CuLCrV, FISH signals were observed in PSG of MEAM1 but not from MED (Fig.5 C, D). Both TYLCV and CuLCrV primarily localized in the secretory section of the central region of PSG. No signals were detected in MEAM1 or MED feeding on non-infected tomato or squash.

Discussion

Here, we provide evidence that although MED is a better vector of TYLCV than MEAM1, this may not be true for NW bipartite begomoviruses such as CuLCrV. MED was able to acquire and retain both TYLCV and CuLCrV after feeding on infected plants. However, it was only able to transmit TYLCV. On the other hand, MEAM1 was able to successfully acquire, retain, and inoculate non-infected tomato and squash with TYLCV or CuLCrV after feeding on TYLCV-infected tomato or CuLCrV-infected squash. Results from FISH suggest that CuLCrV accumulated in the midgut of MED, but failed to accumulate in the PSG, resulting in the non-transmission of CuLCrV by MED.

It has been documented multiple times that transmission of begomoviruses differs between whitefly cryptic species (Bedford et al. 1994; McGrath et al. 1995). For instance, MEAM1 and MED differ in their ability to acquire and transmit TYLCV (Ning et al. 2015). Biotype A (New World 1) of *B. tabaci* is a better vector of chino del tomate virus than MEAM1 (Idris et al. 2001).

Some of these transmission differences between whitefly cryptic species may be due to the feeding habit of whiteflies. For example, MED does more prolonged and continuous feeding on TYLCV-infected plants than MEAM1, resulting in higher accumulation and better transmission of TYLCV by MED (Ning et al. 2015). In the current study, MED accumulated and retained significantly higher TYLCV than MEAM1, although this didn't translate into differential transmission. Similar transmission rates appear to be associated with the ability of whiteflies to acquire the virus particles above the threshold that would limit virus transmission. Upon transfer to non-host cotton, TYLCV declined gradually in MED (5-9%/day) and MEAM1 (8-14%/day). Our results are consistent with previous findings on gradual decline in TYLCV in viruliferous whiteflies once transferred to a non-host (Ning et al. 2015). The rate of TYLCV decline observed in the current study is much higher than the 1–2% per day reported earlier (Caciagli and Bosco, 1997, Su et al. 2013). Su et al (2013) reported the TYLCV rate of decline in viruliferous whiteflies on non-host cotton was associated with presence or absence of a symbiont, *Hamiltonella,* in the whiteflies. Higher decline rates observed in the current study might be the result of differences in symbionts harbored by the whiteflies, experimental conditions, virus isolates, or whitefly genetics.

CuLCrV accumulation in MEAM1 and MED was less than TYLCV, but this might be because of differences in virus accumulations in the host plants. TYLCV accumulation in tomato, in terms of copies per ng DNA, is higher than CuLCrV accumulation in squash (unpublished data). This correlation between begomovirus accumulation in vectors and hosts has been reported in the previous study from our lab (Lagarrea et al. 2015). Furthermore, a similar correlation between virus accumulation in host and vector has been reported for watermelon chlorotic stunt

virus and tomato yellow leaf curl virus in populations of *B*. *tabaci* (Kollenberg et al. 2014). Taken together, these results provide clear evidence that begomovirus accumulation in whiteflies is a function of begomovirus accumulation in the host plants. MED accumulated significantly less CuLCrV than MEAM1 and failed to transmit it. However, MEAM1 readily transmitted CuLCrV. After transfer to non-host cotton, within a week, a sharp decline (90%) in CuLCrV accumulation was observed for MED, whereas in MEAM1, CuLCrV declined gradually (6-10%/day). Czosnek et al. (2002), while comparing the retention of TYLCV between vector (*B. tabaci*) and non-vector (*Trialeurodes vaporariorum*) whiteflies, reported that after a 4 d AAP on TYLCV-infected tomato, *B. tabaci* was able to retain TYLCV for 15 days, whereas, *T. vaporariorum* lost TYLCV within a few hours after transfer to non-host plants. In the current study, MED acquired CuLCrV from CuLCrV-infected squash after a brief feeding of 72 h and retained it much longer that the non-vector might. In fact, some MED individuals had PCR-detectable CuLCrV-DNA in them after 28 days following transfer to cotton. Why MED retained CuLCrV for so long without transmitting it is very intriguing.

Begomoviruses are transmitted by whiteflies in a persistent-circulative fashion (Brown and Czosnek, 2002; Czosnek et al. 2017). Circular translocation of both bipartite and monopartite begomoviruses within whiteflies is broadly accepted. However, the mechanism behind circulative transmission is not well understood. Begomovirus particles acquired during feeding from infected plants move to the midgut and trafficked across midgut epithelium in vesicles (Uchibori et al. 2013, Xia et al. 2018) into the hemolymph. In hemolymph, the chaperone protein GroEL encoded by bacterial endosymbionts guides begomoviruses to PSG (Kliot and Ghanim, 2013), from which they are discharged into plant phloem with saliva during

feeding (Czosnek et al. 2017). During this process, begomoviruses cross multiple barriers, including midgut epithelium and the basal lamina of PSG (Hogenhout et al. 2008, Brown and Czosnek 2002). Since coat protein (CP) is the only begomovirus protein reported to influence acquisition and transmission by whiteflies (Harrison et al. 2002), CP interactions with putative receptors (clathrin-mediated endocytosis or peptidoglycan recognition proteins (PGRPs)) at midgut and PSG basal lamina determine non-transmissibility or transmissibility of begomoviruses by whiteflies (Pan et al. 2017; Wang et al. 2016). For instance, Pan et al. (2018) reported differential transmission of cotton leaf curl Multan virus (CLCuMuV) by four members of the *B. tabaci* species complex (MED, Asia 1, Asia II, and MEAM1) depends on the efficiency of CLCuMuV to cross the midgut; differential ability to cross midgut barriers was implicated with the interactions with CP and putative receptors at the midgut epithelium. Furthermore, transmission of TYLCV and tomato yellow leaf curl China virus (TYLCCNV) by MEAM1 and MED depends on the interactions between CP of TYLCV or TYLCCNV with putative receptors on the PSG (Wei et al. 2014). MEAM1 transmits both TYLCV and TYLCCNV, while MED is only able to transmit TYLCV. TYLCCNV is able to reach the PSG of MED, but fails to enter into the PSG lumen, resulting in non-transmission of TYLCCNV by MED. GroEL protein supposedly protects translocating viruses in the hemolymph from proteolysis by the insect immune system (Morin et al., 2000, Gottlieb et al., 2010). Therefore, incompatible interactions of GroEL with CP or non-availability of GroEL protein can also result in non-transmission of begomoviruses by whitefies.

In the current study, CuLCrV accumulated only in the midgut of MED; it failed to accumulate in the PSG of MED, resulting in non-transmission of CuLCrV by MED. Therefore, there are a

couple of scenarios that might explain non-accumulation of CuLCrV in MED PSG. First, either CuLCrV failed to cross the midgut barrier into the hemolymph, or there might be non-compatible interactions between CuLCrV-CP and begomovirus receptors on the PSG. A third scenario of non-compatible interactions between CP of CuLCrV and GroEL is also possible. Experiments are currently underway in our lab to measure the accumulation and retention of CuLCrV and TYLCV in the midgut, PSG, and hemolymph of MED and MEAM1. Furthermore, we are also studying the diversity of endosymbionts in our MED and MEAM1 populations.

In conclusion, we report the transmission or non-transmission and localization of NW and OW begomoviruses by two of the most invasive and destructive members of the *B. tabaci* species complex. Since the first report of MED in the US in 2004, it has spread to almost every major agricultural region in the country. The recent report on field populations of MED in Florida (McKenzie and Osborne, 2017) has raised questions about possible impacts of MED on local agriculture. MED has been shown to have a higher propensity to develop resistance to commonly used insecticides in whitefly management programs in horticultural and row crops (Ellsworth and Martinez-Carrillo JL. 2001; McKenzie and Osborne 2017; Palumbo et al. 2001). Under higher insecticide pressure, MED can outcompete MEAM1 (Elbert and Nauen 2000; Yao et al. 2005,). In China, Japan, and South Korea, MED has started to displace MEAM1 as the predominant whitefly biotype (Horowitz et al., 2003; Pan et al., 2015; Pascual and Callejas, 2004; Shatters et al., 2006). In China, the current epidemic of TYLCV has been associated with field outbreaks of MED (Liu et al. 2013). Previous experiences with MED outbreaks and our study strongly suggest that the establishment of MED in the US can have severe impacts on whitefly-begomovirus epidemics, particularly in tomatoes. However, similar trends may not be

observed for NW begomoviruses such as CuLCrV. Further studies are required on the interactions of MED with other important whitefly-transmitted begomoviruses and criniviruses to fully understand the impact of MED on local agriculture.

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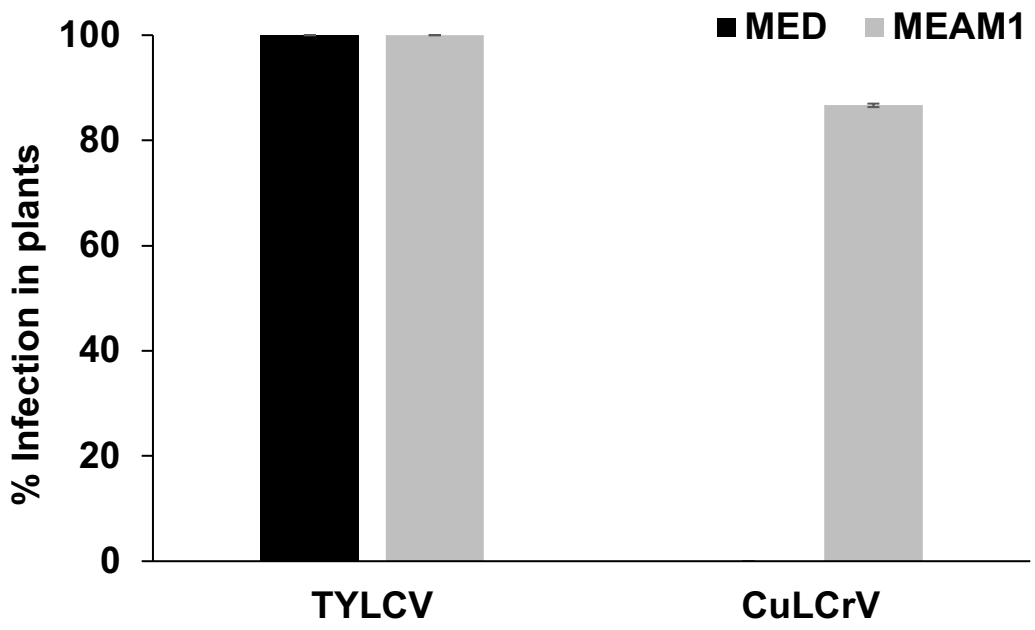

Figure 6. 1. Percent infection in plants.

Bars with standard errors indicate percent CuLCrV or TYLCV infection in tomato and squash subjected to viruliferous MEAM1 and MED feeding. Viruliferous MEAM1 and MED were obtained by 72 h AAP on TYLCV-infected tomato or CuLCrV-infected squash. Viruliferous MEAM1 and MED were given 5-day inoculation access period (IAP) on non-infected four-week-old tomato or squash plants. Four weeks later, infection status was confirmed via endpoint PCR.

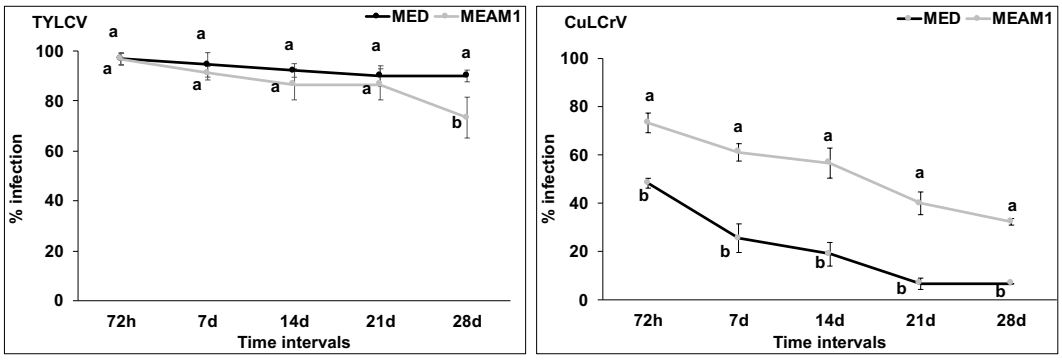

Figure 6. 2. Percent infection in *B. tabaci* adults.

Values are means ± SE. Means with different letters are significantly different (HSD test at P<0.05). A, Percent infection in *B. tabaci* adults (MEAM1 and MED) randomly collected from cotton plants at five-time intervals (72h, 7d, 14d, 21d, and 28d) after a 72h AAP on TYLCV-infected tomato plants. B, Percent infection of *B. tabaci* adults (MEAM1 and MED) randomly collected from cotton plants at five-time intervals (72h, 7d, 14d, 21d, and 28d) after a 72h AAP on CuLCrV-infected squash plants.

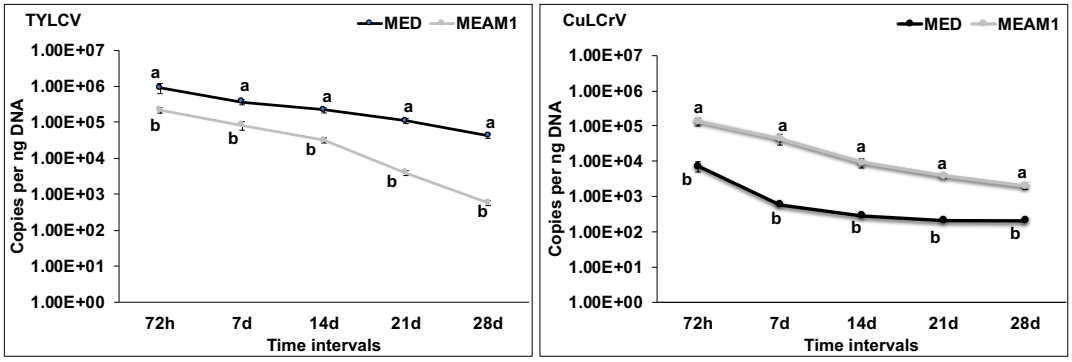

Figure 6. 3. Virus accumulation in *B. tabaci* adults.

Values are means ± SE. Means with different letters are significantly different (HSD test at P<0.05). A, TYLCV accumulation in *B. tabaci* adults (MEAM1 and MED) randomly collected from cotton plants at five-time intervals (72h, 7d, 14d, 21d, and 28d) after a 72h AAP on TYLCV-infected tomato plants. B, CuLCrV accumulation in *B. tabaci* adults (MEAM1 and MED) randomly collected from cotton plants at five-time intervals (72h, 7d, 14d, 21d, and 28d) after a 72h AAP on CuLCrV-infected squash plants. Y-axis is shown in a logarithmic scale.

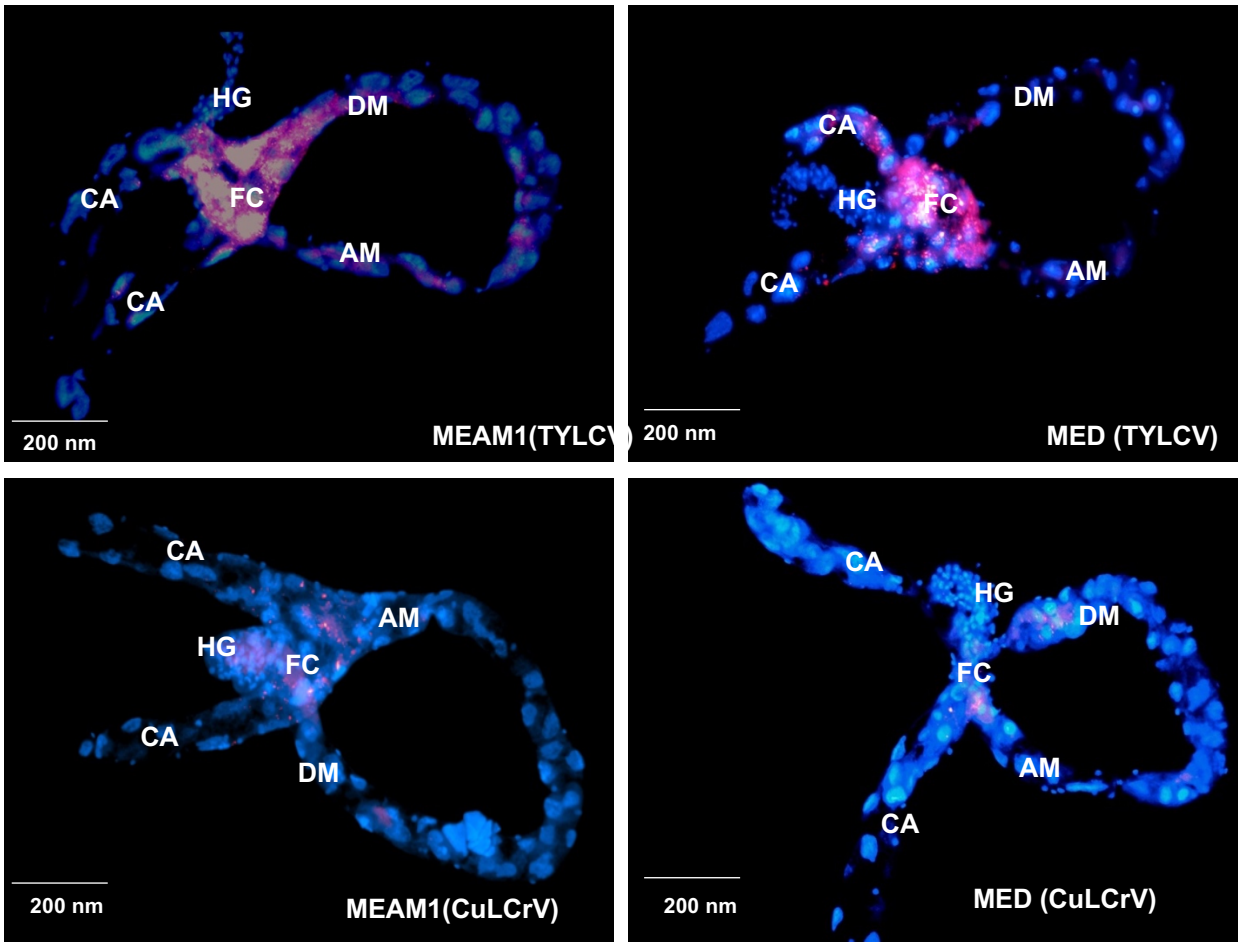

Figure 6. 4. TYLCV and CuLCrV localization in the midguts dissected from *B. tabaci* adults (MEAM1 and MED) that fed on TYLCV-infected tomato or CuLCrV-infected squash for 72h.

FISH signal(red) and DAPI stained nuclei (blue) as seen from confocal microscope. Annotations under the corresponding picture represent whitefly species-virus combinations. FC: filter chamber; DM: descending midgut; AM: ascending midgut; CA: caeca; HG: hindgut. A, Midgut of MEAM1 infected with TYLCV. B, Midgut of MED infected with TYLCV. C, Midgut of MEAM1 infected with CuLCrV. D, Midgut of MED infected with CuLCrV.

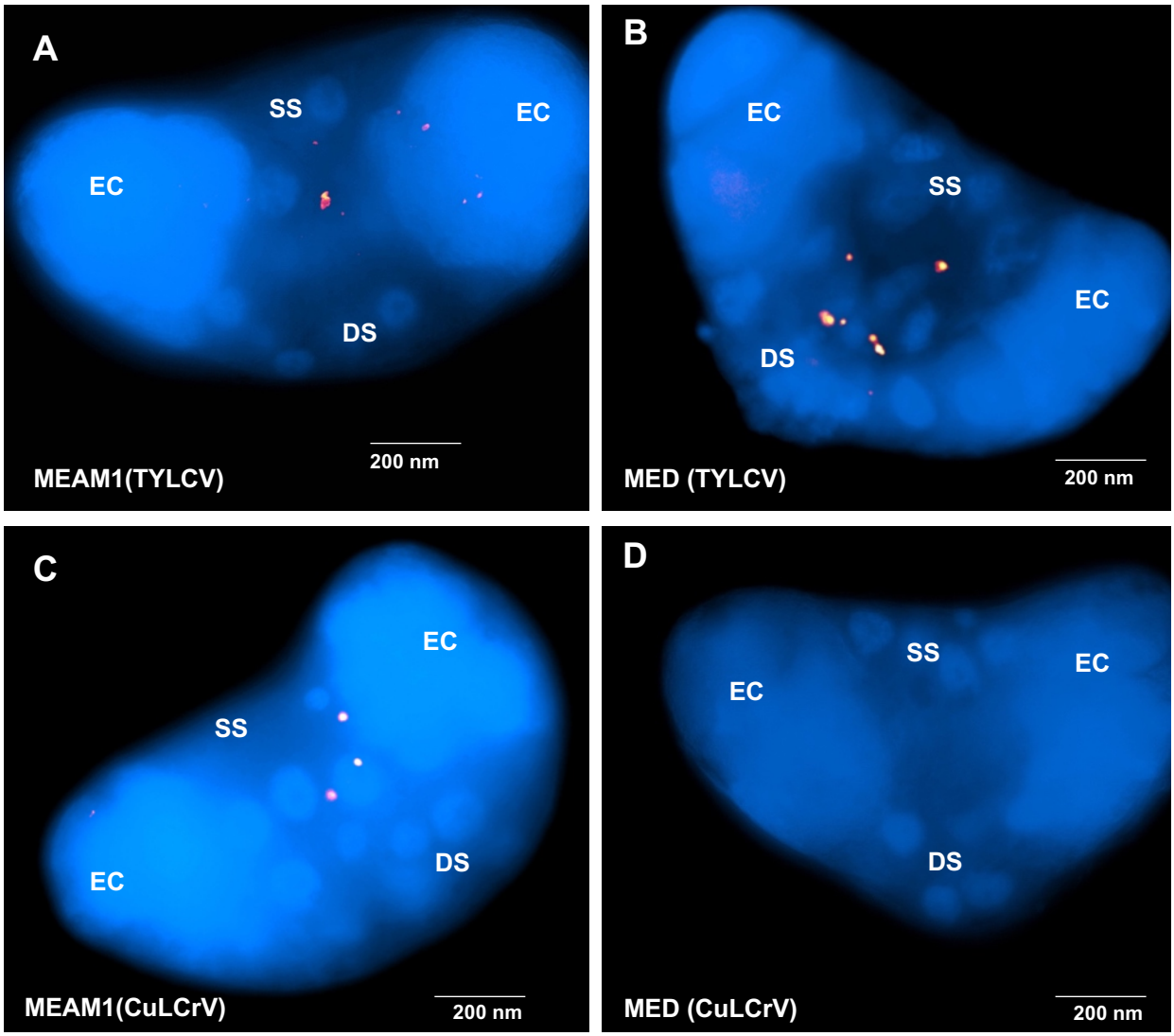

Figure 6. 5. TYLCV and CuLCrV localization in the primary salivary glands (PSG) dissected from *B. tabaci* adults (MEAM1 and MED) that fed on TYLCV-infected tomato or CuLCrV-infected squash for 72h.

FISH signal(red) and DAPI stained nuclei (blue) as seen from confocal microscope. Annotations under the corresponding picture represent whitefly species-virus combinations. DS, ductal section of the central region; EC, end cap; SS, secretory section of the central region. A, PSG of MEAM1 infected with TYLCV. B, PSG of MED infected with TYLCV. C, PSG of MEAM1 infected with CuLCrV. D, PSG of MED infected with CuLCrV.