Investigating Vector-Virus-Host Plant Interactions That Influence the Acquisition and Transmission of Mixed Infections

By

Autumn Alyce McLaughlin

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Approved by

Alana Jacobson, Chair, Professor of Entomology and Plant Pathology
Arthur Appel, Professor of Entomology and Plant Pathology
Kira Bowen, Professor of Entomology and Plant Pathology
Kathleen Martin, Assistant Professor of Entomology and Plant Pathology

Abstract

Tomato yellow leaf curl virus (TYLCV) and *Tomato mottle virus* (ToMoV) (*Geminiviridae, Begomovirus*) are two economically important, tomato-infecting viruses that occur in single and mixed infections in the United States. Mixed infections of plant viruses can be important factors in influencing disease severity, genetic diversity of the co-infecting viruses, transmission rates, and virus evolution. The role of the vector in propagating these mixed infections has been largely ignored. In this study, the role of vector transmission on the propagation of single and mixed infections of TYLCV and ToMoV by the whitefly vector, *Bemisia tabaci* (Gennadius), in tomato was investigated. A transmission experiment was designed to investigate how the acquisition of these viruses by *B. tabaci*, either sequentially from a single infection or mixed infections scenarios, influences the probability of transmission.

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TYLCV	Tomato yellow leaf curl virus
TYLCD	Tomato yellow leaf curl disease
ToMoV	Tomato mottle virus
CMG	Cassava mosaic geminiviruses
WF	Whitefly
SS	Sucrose Sachet
GWFSS	Grouped Whitefly Sucrose Sachet
MEAM1	Middle East-Asia Minor 1
MED	Meditarinean
DPI	Days post-inoculation
AAP	Acquisition access period
IAP	Inoculation access period
PSG	Primary salivary glands
ssDNA	Single-stranded DNA
dsDNA	Double-stranded DNA
MtCO1	Mitochondrial cytochrome oxidase I
%	Percent
ºC	Degrees in Celsius
s	Second
min	Minute
hrs	Hours
g	Gram

mm	Millimeter
cm	Centimeter
m	Meter
ml	Milliliter
µl	Microliter
pH	Potential of hydrogen
×g	Times gravity
LB	Lysogeny broth
DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
Ct	Threshold cycle

Chapter One

Literature Review

The vector – Whitefly (*Bemisia tabaci*)

The majority of plant viruses depend on insect vectors to move from one plant to another to ensure their survival. Although several organisms are vectors of plant viruses, insects in the suborder Sternorrhyncha, a group of phloem-feeding insects that includes aphids, scale, and whiteflies, are the most common and transmit fifty-five percent of plant viruses (Fereres and Moreno 2009; Navas-Castillo et al. 2011; Chandi et al. 2018). Sternorrhynchans are very well-suited to transmit plant viruses because their piercing-sucking mouthparts allow the insect to feed without killing the plant cell, allowing virus infection of the cell to occur. Whiteflies (Hemiptera;Sternorrhyncha:Aleyrodidae) are considered the second most important group of insect vectors because they transmit the second highest number of plant viruses. The family of the whiteflies, Aleyrodidae, is comprised of more than 1,500 species in 126 genera, but only five species are known to transmit plant viruses (Basu 1995; Jones 2003).

The most economically important whitefly vector, *Bemisia tabaci* (Gennadius), was first described in 1889 as a pest of tobacco in Greece, resulting in the common name, tobacco whitefly (Georgia and 1889 n.d.). Accurate description of this species was debated by scientists until 1957 when it was finally synonymized into a single taxon, yet several other common names exist (Russell, 1957). The amount of diversity within the species complex of *Bemisia tabaci* has been studied extensively, and biological characteristics such as plant host preference, fecundity, vector competency, insecticide resistance, and dispersal had been previously used to determine biotypes (Brown et al. 1995, De Barro et al. 2011). More recently, molecular characteristics such as mitochondrial cytochrome oxidase 1 (mtCO1) has shown genetic evidence of at least 36 putative cryptic species. The most globaly important pest species are Middle East-Asia Minor 1 (MEAM1, formally know as biotype B) and Mediterranean (MED, formally known as biotype

Q) (De Barro et al. 2011, Liu et al. 2012, Chen et al. 2016). In the mid-1980, invasive sub-species *B. tabaci* (MEAM1) was introduced into the Eastern hemisphere by the movement of infested ornamental plants, and quickly became a threat to cultivated crops (Perring et al. 1993, McKenzie et al. 2012). This newly introduced sub-species of whitefly quickly displaced native Biotype A in the southwestern US by 1991 (J K Brown et al. 1995; Polston and Anderson 1997). *B. tabaci* MEAM1 populations have caused significant harm to crops by phloem-feeding, introducing phytotoxic disorders, excretion of honeydew and, most significantly, transmission of plant viruses (Li et al. 2010). *B. tabaci* MEAM1 has been reported to transmit plant viruses in the genera *Begomovirus (*family *Geminiviridae)*, *Ipomovirus (*family *Potyviridae)*, *Crinivirus* (family *Closeroviridae)*, *Carlavirus* (family *Betaflexiviridae*), and *Torradovirus (*family *Secoviridae)* (Navas-Castillo et al. 2011).

The Host- Tomato

Tomato, *Solanum lycopersicum* L.*,* (*Solanaceae*) is an herbaceous plant with hundreds of varieties, which differ in size, taste, and color. The tomato plants cultivated today originated from wild plants in the Andes and were later domesticated in Mexico. Interestingly, earlier cultivation of the plant was for aesthetic needs and not for consumption because of its resemblance to the very poisonous wolf peach. It wasn't until the 20th century that its importance as an edible fruit emerged (Aggie Horticulture n.d., *Tomato Health Management* 2017, McLaurin 2018).

Today, tomatoes are the largest crop in terms of production and the second most valued crop in the U.S.Tomatoes are cultivated in fields, greenhouses, screen houses, and homes in almost every country in the world. ("FOA Stat" 2019, Agricultural Statistics Service 2020).

Globally, the tomato business has two markets of production; one deals with producing fresh tomatoes and the other deals with producing processing tomatoes. In 2017, approximately 1.42 million tons of fresh-marketed tomatoes and 14.7 million tons of processed tomatoes were harvested from 311,500 acres in the U.S. with a total value of $1.6 billion ("Tomatoes | Agricultural Marketing Resource Center"). Nevertheless, due to its constant large-scale production, it is vulnerable to a variety of pathogens. In addition to certain bacterial and fungal pathogens that cause serious tomato infections, many viruses are also very detrimental (*Tomato Health Management* 2017).

Bemisia tabaci feeds on tomato, and indirectly transmits more than 60 distinct begomoviruses to this one host (King et al. 2011). The tomato variety, 'Florida Lanai', is a fast-growing dwarf tomato variety developed by the University of Florida that reaches 30-45 cm high with regular leaf and determinate growing habit (Augustine et al. 1981). The use of 'Florida Lanai' as a susceptible host of begomoviruses in laboratory has been studied and found to be a great model for experiments (Momotaz et al. 2007; McKenzie 2002; Nava et al. 2013; Rajabu et al. 2018).

The viruses - Whitefly transmitted Begomoviruses

The *Geminiviridae* family are a group of plant viruses characterized by twin icosahedral "geminate" capsids and circular single-stranded DNA (ssDNA) genomes that replicate in infected cells through double-stranded DNA (dsDNA) intermediates (Hanley-Bowdoin et al. 2000). Geminiviridae is currently comprised of nine genera, *Begomovirus*, *Mastrevirus, Curtovirus*, *Topocuvirus, Grablovirus, Eragrovovirus, Turncurtovirus, Becurtovirus, and*

Capulavirus based on genome organization, host range, and insect vectors (Zhou 2013, Zerbini et al. 2017). Begomoviruses have become the most destructive group of plant viruses in tropical and subtropical regions of the world because of the high number of viruses in this genus, the ability to infect multiple plant species, and their potential to evolve quickly through recombination and mutation (Seal et al. 2006, Vinutha et al. 2014). Phylogenetic studies have divided begomoviruses into two distinct origin groups based on the genome, described as the old world and new world (Rybicki 1994; Stanley et al. 2005). Old world begomoviruses (Eastern hemisphere, Europe, Africa, Asia, Australia) have both monopartite and bipartite genomes, whereas,new world (western hemisphere, North and South America) begomoviruses have bipartite genomes (Fauquet et al. 2003). Members of the genus *Begomovirus* may have either two (bipartite, DNA-A and DNA-B) or one (monopartite, DNA-A-like) circular ssDNA genomic molecules of approximately 2800 nucleotides each. Each is encapsulated separately in a 22 × 38 nm geminate particle made up of 110 copies of a single protein type, the coat protein (CP). Monopartite begomovirus DNA-A codes for virus replication, movement, and plant defense neutralization. Bipartite begomoviruses possess two genome components, a DNA-A and DNA-B, and both components are required to get a systemic infection (Hanley-Bowdoin et al. 2000; Czosnek et al. 2017).

The majority of viruses transmitted by whiteflies are begomoviruses, and *B. tabaci* is reported to transmit ~320 *begomovirus* species (ICTV 2020). Begomoviruses are reported to be transmitted by whiteflies in a persistent circulative manner, but evidence of replication in whiteflies have been described for one begomovirus species (He et al. 2020b). With its stylet, the whitefly ingests viral particles, which pass through the food canal and reach the esophagus and filter chamber.The virions then cross the filter chamber and the midgut into the hemolymph,

where they move to the primary salivary glands (PSG) then translocate into the salivary ducts. Once in the salivary ducts, virions can be injected with the whitefly saliva into plant phloem (Gray et al. 2014, Czosnek et al. 2017).

Tomato yellow leaf curl virus (TYLCV)

Tomato yellow leaf curl virus (TYLCV) is a complex of begomovirus species causing Tomato yellow leaf curl disease (TYLCD) which is one of the most devastating diseases in the tomato producing industry throughout tropical and sub-tropical regions of the world (Moriones and Navas-Castillo 2000; Abhary et al. 2007; ICTV 2017). Although TYLCV was first reported in Israel in 1939, it was not until the 1960s that its causal agent was identified (Cohen and Nitzany 1960; Cohen and Harpaz 1964). This disease has since spread to most European countries and parts of sub-Saharan Africa, Asia, Australia, North America, and South America (Accotto et al. 2000; Khan et al. 2013). To date, seven species of TYLCV hace been characterized; TYLCV-Is (TYLCV- Israel), TYLCV-Ch (TYLCV- China), TYLCV-Sar (TYLCV- Sardinia virus), TYLCV-Tz (TYLCV- Tanzania), TYLCV-Th (TYLCV- Thailand), TYLCV-Ng (TYLCV-Nigeria), and TYLCV-SSA (TYLCV- Southern Saudi Arabia) (Moriones and Navas-Castillo 2000; Brown et al. 2015). TYLCV strains are identified with nucleotide sequence homology and share at least ninety one percent pairwise sequence identity for the full-length genome or DNA-A component, whereas TYLCV species are demarked by sharing less than ninety-one percent pairwise sequence identity (Brown et al. 2015). Most TYLCV species have distinct geographic ranges, such as TYLCV-China and TYLCV-Thailand that are only found within those countries (Moriones and Navas-Castillo 2000). However, multiple distinct TYLCV species can also exist in the same area, with examples of this occurring in Spain and

Morocco (Sánchez-Campos et al. 1999, Belabess et al. 2015). TYLCV-Is and TYLCV-Sar were the first begomoviruses characterized as having a monopartite genomic DNA structure comprised of 2.7-2.8 nucleotides, and most TYLCV species have been characterized as having monopartite genomes that are typical for old world begomovirus species (Navot et al. 1991; Hanley-Bowdoin et al. 2000; Gronenborn 2007). However, the genome of TYLCV-Th has been characterized as bipartite with A and B components (Rochester et al. 1994, Czosnek et al. 2017).

In the early 1990s, TYLCV-Is entered the Western Hemisphere via asymptomatic TYLCV-infected tomato transplants (McGlashan 1994, Zubiaur et al. 1996, Momol et al. 1999). In July 1997, symptoms characteristic of TYLCV-Is were first seen on a tomato plant in a field in southwest Florida. At almost the same time, four tomato plants with symptoms typical of TYLCV-Is were found at retail garden centers in West Central Florida (Polston et al. 1999). Subsequently, the virus spread across most of the southern United States including: Georgia, Alabama, North Carolina, South Carolina, Texas, Mississippi, Arizona, and California (Momol et al. 1999; Ingram and Henn 2001; Polston et al. 2002; Ling et al. 2006; Akad et al. 2007; Isakeit et al. 2007; Rojas et al. 2007; de Sá et al. 2008). Estimations of TYLCV-induced losses vary with country, so no official estimate of losses is shown in tomato, though it is assumed to be in tens of millions of dollars (Glick et al. 2009). A study conducted in Georgia, USA during 2017, demonstrated that TYLCV can cause 50%-100% total loss if no management actions were implemented (Fonsah et al. 2018)

TYLCV has a very wide host range of dicotyledonous plants of both cultivated crops and weed species. Other than tomato, TYLCV is known to infect 15 families with over 30 plant species, this includes crops such as common bean, pepper, tobacco, eustoma, tomatillo, soybean, and some cucurbit species (Cohen et al. 1995, Navas-Castillo et al. 1999, Reina et al. 1999, Ying

and Davis 2000, Font et al. 2005, Salati et al. 2010, Papayiannis et al. 2011, Kil et al. 2017). TYLCV infection may produce symptoms such as prominent upward curling of leaflet margins, reduction and distortion of leaflet area, yellowing (marginal chlorosis) of young leaves, stunting, and flower abortion (Moriones and Navas-Castillo 2000; Lapidot et al. 2001). Disease severity can vary depending on the time of infection, growing conditions, and host. Asymptomatic infections also occur in some crops, such as sweet pepper, that can serve as virus reservoirs in the landscape (Kil et al. 2014). In laboratory studies TYLCV can be transmitted artificially via grafting, DNA-coated particle bombardment, or agroinoculation using *Agrobacterium tumefaciens* (Scholthof et al. 2011). Seed transmission of TYLCV-Is has been reported in tomato and soybean (Kil et al. 2016, 2017).

TYLCV is a phloem-limited virus that is transmitted exclusively by the vector, *B. tabaci* (Gronenborn 2007). The acquisition, latent period, and transmission parameters of TYLCV required for transmission by *B. tabaci* have been well studied (Atzmon et al. 1998, Li et al. 2010, Polston and Capobianco 2013, Rosen et al. 2015, Czosnek et al. 2017). The acquisition access period (AAP) is the feeding time required for an insect to aquire the virus, while the inoculation access period (IAP) is the feeding time required for the insect to transmit the virus. The latent period is the time required between acquisition and transmission for the virus to circulate in the vector before it reaches the salivary ducts. TYLCV has been reported to be acquired and detected in the *B. tabaci* MEAM1 head after 5-10 minutes of feeding, and in the midgut after 40 minutes, it takes 30 more minutes to cross the midgut and to be detected in the hemolymph. After first being detected in the hemolymph, it takes 5.5 hours to be detected in the salivary glands. One hour after being detected in the salivary glands, the virus can be inoculated into another host(Ghanim et al. 2001a, Czosnek and Ghanim 2012). The latent period of TYLCV

is reported to be between 6-8 hours (Ghanim et al. 2001b, Rosen et al. 2015). Following acquisition, TYLCV is reported to be retained in *B. tabaci* for its lifespan (Czosnek and Ghanim 2012), however, the amount of TYLCV accumulating in individual adult *B. tabaci* during continuous feeding on infected tomato plants reaches a saturation point after 12–48 hrs (Czosnek et al. 2017).

Over the past two decades,replication of TYLCV in the vector has been debated, with some studies reporting no replication (Becker et al. 2015, Sánchez-Campos et al. 2016) while others detecting replication (Sinisterra et al. 2005, Pakkianathan et al. 2015). Recent studies have shown that replication can occur (He et al. 2020a) but it depends on the biotype-virus isolate interactions and not all TYLCV isolates are able to replicate in all whitefly biotypes. Horizontal transmission of TYLCV has been shown to occur between mating pairs of *B. tabaci,* though it has been not been proven, it is hypothesized through physical contamination (Ghanim and Czosnek 2000). Vertical transmission has been shown to occur in progeny of viruliferous whiteflies, and TYLCV DNA has been found in the eggs, nymphal and adult stages (Ghanim et al. 1998, Pan et al. 2012, Wei et al. 2017, Fonsah et al. 2018, Guo et al. 2019).

***Tomato mottle virus* (ToMoV)**

Tomato mottle virus (ToMoV) was first documented in Florida in 1989 and is believed to originate from Florida, USA (Simone et al. 1990; Abouzid et al. 1992; Polston 1993). ToMoV has been reported in Tennessee, South Carolina, Virginia (Polston 1995), Alabama (Akad et al. 2007), Puerto Rico (Brown et al. 1995), and Mexico (Garrido-Ramirez and Gilbertson 1998). Epidemics of ToMoV have been sporadic, and virus incidence may vary by site and year (McGovern et al. 1995), however, severe outbreaks of ToMoV have been observed with yield

loss up to 50% reported (Abouzid et al. 1992). ToMoV symptoms include mottling, cupping and curling of leaves with yellow mosaic distortion and plant stunting (Polston 1993), though less severe than TYLCV. ToMoV is an new world virus documented in fewer host plants including tomato, common bean, tobacco, tomatillo, and a few weed species (Polston 1993, R.J. McGovern et al. 1995). ToMoV has a bipartite genomic DNA structure that includes an A component comprised of 2601 nucleotides and B component comprised of 2541 nucleotides (Abouzid et al. 1992). ToMoV is exclusively transmitted by *B. tabaci* MEAM1 (Schuster et al. 1989, Polston 1993). In the laboratory, ToMoV can be inoculated artificially using *A. tumefaciens* (Rajabu et al. 2018). *Tomato yellow leaf curl* and *Tomato mottle virus* have been documented in Alabama, and may co-infect plants (Akad, et al. 2007).

Mixed Viral Infections

Viral infections in plants have been long studied with primary focus on single infection models in field crops and ornamentals due to economic losses viruses cause (Méndez-Lozano et al. 2003; Blanc et al. 2011; Tollenaere et al. 2016;Moreno and López-Moya 2020). Mixed infections are assumed to occur frequently in nature because viruses are found infecting weed and crop hosts in the landscape (Borer et al. 2010; Syller 2012; Alexander et al. 2014). The extent of viruses co-infecting plants is still a wide area of study, as there are many complexities to still be explored. Mixed infections occur through co-infection or superinfection which are distinguished by time of infection. In co-infection, virus inoculation occurs simultaneously or within a short time, while super-infection occurs when viruses are inoculated at two different time points (Kumar et al. 2018, Moreno and López-Moya 2020). In the disease triangle, outcomes of plant disease severity and occurrence are typically expressed by a three-way

interaction between host, pathogen, and the environment. Mixed infections can also be added to the simplistic disease triangle, but complex interactions may occur between viruses in the vector and the host plant that affect transmission, fitness, and disease severity (Syller and Grupa 2016, Islam et al. 2020). What is known about these interactions will be described in the sections below by interaction-type.

Virus-Virus Interactions

Virus-virus interactions can lead to synergism, antagonism, or neutralism in the infected plant host (Syller 2012, Moreno and López-Moya 2020), however, these terms are ambiguous when talking about the outcomes of two viruses. During a mixed viral infection, the viruses involved may interact between themselves in a range of outcomes from synergism to antagonism. These interactions can impact the fitness of one or both of the viruses in a mixed infection and are always compared to the how a virus will act in a single infection. A focus of these virus-virus outcomes in studies is a measure of viral fitness, that is defined as virus replication and titers that accumulate in hosts (Alcaide et al. 2020). In the neutralism outcome, two viruses in a host have no effect on fitness of either virus. If one virus has no effect, while the other's viral fitness increases or decreases, the outcome is defined as neutral synergism and neutral antagonism, respectively. In the double syngergism outcome, viruses interacting will both have increased fitness, while in double antagonism both viruses will have a decrease in fitness. The final outcome, is the inverse outcome, where one virus will have an increase in fitness, while the other's fitness will decrease. Theses virus-virus outcomes are variable and can be influenced by plant host species, virus species/strain, mode of transmission/vector, and environment.

Virus-virus interactions can have impacts on a host organism by the disease that may develop (Syller and Grupa 2016, Islam et al. 2020). Virus-virus interactions impact disease severity and can cause a more than additive increase in symptoms expressed in a host plant that affects growth parameters such as plant height, weight, and seed production (Murphy and Bowen 2006, Syller 2012, Moreno and López-Moya 2020). Virus-virus interactions inside the host can have effects on virus movement and localization into new plant tissues called tissue trophism (Morra and Petty 2000, Wege et al. 2001, Amaku et al. 2010, Caracuel et al. 2012), and can indirectly cause changes to transmission efficiency. In one case, the genus *Umbravirus* are only able to be transmitted by an aphid vector in the presence of a virus in the family *Luteoviridae* (Syller 2003). With respect to disease management, multiple viruses can be a major threat to food security in affected regions. One well documented case would be the epidemic caused by Cassava mosaic disease in Africa (Pita et al. 2001, Tiendrébéogo et al. 2012, Jacobson et al. 2018). In contrast, virus-virus interactions can also be beneficial for management if the virus interactions are antagonistic. A phenomenon called cross-protection is defined when one strain prevents an infection of another closely related strain. A successful case reported using mild isolates of *Citrus tristeza virus* (Folimonova 2013), however this is still a highly controversial topic and it takes many years to develop and test a local mild strain (Moreno and López-Moya 2020).

Vector-Virus Interactions

Viruses are obligate parasites, and most require an insect vector for movement. Early research into the biology of specific relationships between viruses and their insect vector began with the mode of transmission (Dietzgen et al. 2016). Four types of transmission are described

based on acquisition period, inoculation thresholds, and latent period within the vector (Gray et al. 2014; Whitfield et al. 2015). Non-persistent transmission is characterized by short acquistion access periods (AAP) requiring seconds of insect feeding on virus-infected leaf tissue, and inoculation access periods (IAP) occurring within minutes, and the virus is not retained long in the vector as an infectious form. Semi-persistent transmission occurs when the virus localizes in insect foreguts, which increases AAP and IAP times to minutes or hours. Non-persistent and semi-persistent have been categorized as non-circulative modes of transmission because virions attach to binding sites on the stylet or anterior chitin lining of the digestive system, respectively. Non-propagative,persistent transmission takes longer (hours to days) to complete a circulative cycle through the gut cells into the hemolymph to the salivary glands, without replicating, which distinguishes it from propagative,persistent transmission in which virus replication occurs in the vector (Ng and Falk 2006; Blanc et al. 2011; Dáder et al. 2017). Virus-vector interactions underlying acquisition, circulation, replication and transmission are beginning to be characterized, but huge knowledge gaps of the complex interactions that occur within the vector remain (Dietzgen et al. 2016).

Indirect and direct effects of plant viruses on their insect vectors have been identified and reviewed (Bosque-Pérez and Eigenbrode 2011; Dietzgen et al. 2016;Dáder et al. 2017). Virus-host interactions indirectly cause chemical and physical modifications in the plant host, such as changes in volatile emissions, coloration, and nutritional composition. These modifications play a role in effective transmission benefitting the virus (Fereres and Moreno 2009; Ingwell et al. 2012; Fereres et al. 2016). Direct effects on vector fitness are also reported to alter growth rate, reproduction, and longevity (Gutiérrez et al. 2013; Wang et al. 2014; Maluta et al. 2019). In a recent study, TYLCV-infected whiteflies preferred healthy tomato plants over virus infected

plants. It was determined that TYLCV induced apoptotic neurodegeneration in the brain of the *B. tabaci* MED vector which was hypothesized to impair host selection, which increases the likelihood TYLCV will to be transmitted to the non-infected host (Wang et al. 2020). Alterations to vector behavior also likely affect the virus epidemiology but these complex interactions are not well understood (Madden et al. 2000; Jeger et al. 2009).

Multivirus-vector-host interactions and their influence on evolution

Interactions between co-infecting viruses, vectors, and a susceptible host can be detrimental to an agro-ecosystem (Elena et al. 2011, Alexander et al. 2014). Mixed infections have been identified as an important driver of virus evolution responsible for epidemics and emergence of viruses threatening food security (Méndez-Lozano et al. 2003). Many mixed viral infections including *Maize lethal necrosis disease*, *Sweet potato virus disease, Cassava mosaic disease,* and *Potato virus Y disease* have triggered catastrophic disease outbreaks (Syller 2012; Untiveros et al. 2007; Scheets 1998; García-Andrés et al. 2007; Tiendrébéogo et al. 2012). Many of these outbreaks have been by heterologous viruses, although homologous viruses such as the genus *Begomovirus* have been reported as mixed infections that speed up evolution through mutation, recombination and pseudorecombination events (Martín and Elena 2009, Sanfaçon 2017). Recombination occurs by producing novel combinations of pre-existing nucleotide polymorphisms. However in certain cases, the replication protein encoded with the DNA-A will recognize the DNA-B of another virus which is known as pseudorecombination or reassortment (Martin et al. 2011). Several cassava mosaic geminiviruses (CMG) (of both monopartite and bipartite genomes) that cause *Cassava mosaic disease* are a result of intra- and inter- species

recombination as a case in point, leading to severe crop loss of cassava in Africa (Tiendrébéogo et al. 2012).

Conclusion

Virus- vector- host interactions are complex and have been focused on one virus affecting a plant pathosystem. Studies on vector-virus interactions have been predominantly studied single virus and vector species, and there is a paucity of studies on mixed infections include vector transmission. We are unaware of any single study that examines viral fitness of two viruses during vector acquisition, circulation in the vector, transmission by the vector, and fitness in inoculated plants. This research was conducted to address this knowledge gap by examining the role of the vector in propogating mixed infections, and examining virus-virus-vector interactions during acquisition, transmission and subsequent infection in hosts using the *B. tabaci* MEAM-TYLCV-ToMoV tomato pathosystem.

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Chapter Two

"Investigating Vector, Virus, and Host Plant Interactions That Influence the Acquisition and Transmission of Mixed Infections by *Bemisia tabaci*"

Introduction

Studies on plant viruses largely focus on single infection models in plant pathosystems of field and ornamental crops (Méndez-Lozano et al. 2003; Blanc et al. 2011; Tollenaere et al. 2016), but mixed infections of more than one virus species are a common occurrence in nature, and are becoming detected more frequently in agricultural crops (Alexander et al. 2014, Sanfaçon 2017). Mixed infections have been identified as an important driver of virus evolution responsible for epidemics and emergence of viruses threatening food security (Clark et al. 2012, Tiendrébéogo et al. 2012, Naidu et al. 2014). Most plant viruses rely on vector-mediated transmission for dispersal and infection of new hosts (Andret-Link and Fuchs 2005, Hogenhout et al. 2008, Heck 2018), and the majority of plant virus vectors are peripatetic arthropods, moving to plant to another throughout vast landscapes (Syller 2014). Research on the numerous and complex virus-vector-host interactions required for vector acquisition, vector transmission and host infection is still in its infancy, and knowledge on more complex mixed virus models is limited (Allen et al. 2019, Alcaide et al. 2020). The majority of studies on mixed infections examine the within plant-host dynamics of two viruses, and their outcomes on disease severity and viral fitness (Martín and Elena 2009, Elena et al. 2014, Syller and Grupa 2016, Moreno and López-Moya 2020). A paucity of these studies use insect vector-transmission or examine vector-related effects (Gildow 1980, Wintermantel et al. 2008, Ng and Chen 2011, Carroll et al. 2016, Mondal et al. 2017). In this study we investigate how the acquisition of two plant viruses influences the fitness of each to be acquired and transmitted by their vector, and how co-inoculation by their vector influences fitness after transmission.

There is an expanding area of research on how viruses interact directly and indirectly with their vectors and hosts to increase their fitness by increasing spread and replication. Virus

fitness can be altered when their vectors exhibit altered growth rates, reproduction, and longevity either directly due to virus-vector interactions occurring within the vector, or indirectly by mediating changes in host plant quality (Bosque-Pérez and Eigenbrode 2011, Gutiérrez et al. 2013, Mauck et al. 2019). Virus-host interactions that cause chemical and physical modifications in the plant host may alter settling and feeding behaviors responsible for virus acquisition and/or transmission, and are hypothesized to be adaptations that have evolved to promote virus spread (Colvin et al. 2006, Ingwell et al. 2012, Dáder et al. 2017b, Moeini et al. 2020). In mixed infection pathosystems, the presence of one virus may alter the distribution of the viruses within the plant, which may affect acquisition of one or both viruses from plant tissues the vector feeds on (Wintermantel et al. 2008, Amaku et al. 2010, Caracuel et al. 2012, Gutiérrez et al. 2013, Singhal et al. 2020). Viral titers in plant tissues can also influence the amount of virus ingested by the vector (Wintermantel et al. 2008, Martinière et al. 2013).

The set of optimal within-vector and within-host interactions that maximize viral fitness during acquisition, transmission, and systemic infection of hosts will depend largely on the mode of transmission of the virus (Gray and Banerjee 1999, Blanc 2007, Dietzgen et al. 2016, Tollenaere et al. 2016). Persistent,circulative viruses must be ingested during feeding events, move through the vector and later be egested with the saliva in order to infect another host (Brown and Czosnek 2002, Blanc et al. 2011). Virus- virus interactions within the vector remain an understudied area for persistently transmitted viruses. It has been hypothesized that non-persistently transmitted viruses may compete for receptor binding sites in the stylet (Mondal et al. 2017), and it is possible that this occurs for persistently transmitted viruses as they move through midgut and salivary gland membrane barriers along the circulation route through the vector (Gray et al. 2014). Other within-vector processes that may influence virus-virus

interactions include competition for circulation-facilitating proteins in the hemolymph (Kanakala and Ghanim 2016), and the vector's immune response to presence of a virus (Wang et al. 2016).

Mixed infections have been described by two terms that define the timing of infection. In co-infection, virus inoculation occurs simultaneously, whereas super-infection occurs when viruses are inoculated at two different time points (Kumar et al. 2018, Moreno and López-Moya 2020). Once two viruses enter a plant and a mixed infection is established, the viruses may interact in a range of outcomes from synergism to antagonism (Syller 2012). These interactions can impact the fitness of one or both of the viruses in a mixed infection when compared to how the viruses act in single infections. A focus of studies examining virus-virus outcomes is measuring viral fitness, which is defined as virus replication and titers that accumulate in hosts (Alcaide et al. 2020) (Figure 14). Virus-virus interactions impact disease severity and can cause more severe symptoms expressed in a host plant including growth parameters such as plant height, weight, and seed production (Murphy and Bowen 2006, Syller 2012, Moreno and López-Moya 2020). Virus-virus interactions inside the host can have effects on virus movement and localization into new plant tissues called tissue trophism (Morra and Petty 2000, Wege et al. 2001, Amaku et al. 2010, Caracuel et al. 2012), and can indirectly cause changes to transmission efficiency of one or both viruses.

The objective of this study was to investigate the role of vector transmission on the fitness of two persistently transmitted plant viruses, *Tomato yellow leaf curl virus* (TYLCV) and *Tomato mottle virus* (ToMoV) (Family: Geminiviridae, Genus: *Begomovirus*). These viruses are transmitted by the whitefly, *Bemisia tabaci* (Gennadius), and occur in single and mixed infections in tomato production systems in the southeastern U.S.A. A transmission experiment

was designed to investigate the influence of vector acquisition from single or mixed infection source plants on the probability of acquisition, transmission, and host-infection of both viruses. Virus titer data were used to compare virus-virus interactions and viral fitness of *Tomato yellow leaf curl virus* (TYLCV) and *Tomato mottle virus* (ToMoV) after acquisition, transmission and host-infection.

Materials and Methods

Whitefly Colony

In 2016, a whitefly colony was established from a greenhouse infestation at the Auburn University Plant Science Research Center,Auburn, AL. Whiteflies were identified to be *B. tabaci* MEAM1 by sequencing a partial mitochondrial COI gene (data not shown). Whiteflies were kept in in the same conditions as described by Gong (2018). *Solanum melongena* L., Pinstripe Hybrid(Park Seed, Greenwood, SC), a dwarf variety that is not a host for ToMoV or TYLCV, was used to rear the whiteflies. Eggplants were sown in six-pack trays (The HC Companies Inc., Middlefield, OH) and transplanted at the two-true-leaf stage into six-inch pots (The HC Companies Inc., Middlefield, OH). Plants used for insect rearing were grown in a growth chamber (Percival Scientific Inc., Perry, IA) with a photoperiod of 16:8 hr light/dark cycle, at 25ºC with a relative humidity of 80±5% to keep them free of insects and pathogens before use in the greenhouse. The two-true-leaf stage eggplants were grown in insect-proof cages in the greenhouse until the 6-8 true leaf stage before using them to rear the whiteflies. Healthy plants were placed in the adult cages for 1-2 weeks, then adults were removed from the plants by gentle aspiration using a 5 ¾” glass pipet (Thermo Fisher Scientific Inc, Waltham, MA, USA), and

moved to a separate cage and reared to adulthood. 4-7 days after the whiteflies had reached adulthood, females were selected and used for these experiments.

Susceptible Host Plant

Solanum lycopersicum L., variety 'Florida Lanai', is a fast-growing dwarf tomato variety that is asusceptible host for TYLCV and ToMoV, and easily fits in incubators for experiments (Rajabu et al. 2018). Lanai tomatoes were sown and grown as seedlings using the same methods described for eggplant. They were transplanted into 11.5 cm pots when at the two-true leaf growth stage, and remained in incubators from seed until the end of the experiments described below (The HC Companies Inc., Middlefield, OH). Tomatoes that had reached the four-true leaf growth stage were used in agroinoculations to generate source plants for the virus during whitefly transmission experiments, and two-true leaf stage tomatoes were used as recipient plants during whitefly transmission experiments.

Agrobacterium Mediated Inoculation

For this experiment, agro-inoculated clones were used to inoculate TYLCV, ToMoV, and a mixed infection of the two viruses in source plants used for virus acquisition in the whitefly transmission experiments. Agro-inoculated clones allow us to start this experiment with a characterized virus population. *Agrobacterium tumefaciens* strain ABI was transformed with plasmids pNSB1906-ToMoVA, pNSB1877-ToMoVB, pNSB1736-TYLCV, or pMON721 (empty vector) as described by Reyes (2013). Sterile 50 ml polypropylene tubes (BD Falcon Brand, Corning Company, Corning, NY, USA) were used to grow the *Agrobacterium* clones of

TYLCV, ToMoV-A, ToMoV-B, and pMON721 separately in autoclaved Luria-Bertani (LB) Broth (Sigma Cat. No. L9234) with 25µl Spectinomycin (Tokyo Chemical Industry Co., Ltd., Japan). The cultures were then incubated in a Classic C25 shaker (New Brunswick Scientific Co., Inc., Edison, NJ, USA) at 30 ºC shaken constantly at 140 RPM for 48 hrs. Agroinoculations were performed by loading *Agrobacterium* clones in 1ml syringes (BD Luer-LokTM, Franklin Lakes, NJ) with 0.26×13mm TSK STERiJECT hypodermic needles (TSK Laboratory, Japan). Cultures of ToMoV-A and ToMoV-B components were mixed at a 1:1 ratio before inoculation, and a 1:1:1 ratio of TYLCV and ToMoV components were mixed together before inoculation. *Agrobacterium* cultures were inoculated separately into Lanai tomatoes by piercing the area below the apical meristem and the first nodes approximately 5 times on each side of the plant (10 total punctures), while slowly letting 200µl seep into the puncture wounds. Agro-inoculations were performed on 20 plants for each infection status: TYCLV, ToMoV and mixed infections. Five plants were mock inoculated with the empty vector to be used as controls in this experiment. All experiments were conducted in growth chambers (Percival Scientific Inc., Perry, IA) with a photoperiod of 16:8 light: dark cycle at a temperature of 25±3 ºC and relative humidity of 80±5%. Two strips of plant Grow LED lights (Litever®, Guangdong, China) were placed above each shelf in the incubators to increase lighting for optimized plant growth and enhanced symptom expression. Infections were confirmed using PCR methods described below. Three plants per treatments were selected as source plants by infection status (TYLCV, ToMoV, MIXED) for the whitefly transmission experiments.

Whitefly-Acquisition and Transmission

Acquisition scenarios were designed to mimic how whiteflies could acquire and transmit a single virus, or both viruses potentially leading to mixed infections (Table 1). Each treatment was comprised of three replications; each replicate of treatments 1-4 used one infected source plant and each replicate of treatments 5-7 used one ToMoV and one TYLCV-infected plant. All replications for each experiment were initiated the same day. Source plants were placed individually into a 32.5cm×32.5cm×32.5cm cage (MegaView Science Co., Ltd., Taiwan Product: BugDorm-4S3030D) with 500 non-viruliferous whiteflies for a total of 48h for all treatments.

Cohorts of six whiteflies were removed from agro-inoculated source plants and used to transmit to ten recipient plants and five sucrose sachets of each replicate. Individuals were aspirated together into a 1.5 mL tube that was connected to a clip cage (Figure 18) to release whiteflies onto a leaf of a healthy two-true-leaf tomato seedling, or a sucrose sachet (Figure 17). Sucrose sachets are artificial diet packets used to collect virions transmitted in the whiteflies' saliva during feeding. Sucrose sachets were made with a 20% sucrose solution. The lids of a 1.7 mL tubes were colored with a green Sharpie® (Sanford, L.P., Oak Brook, IL, USA) to artificially simulate the color of plants to encourage whitefly feeding. After the lids were colored, parafilm (PARAFILM® M, Neenah, WI) was stretched over the lid opening to form a small bowl. A volume of 20µl of sucrose solution was pipetted into the shallow bowl and another layer of parafilm was stretched very thin over the top to create a feeding membrane.

Whitefly cohorts from plants and sucrose sachets were allowed to feed for 48 hours, then collected and stored in -80ºC Revco UxF freezer (Thermo Fisher Scientific Inc) for later analysis to examine virus acquisition. Recipient tomato plants were maintained for 28 days post-inoculation (dpi) at a temperature of 25.0℃ with a photoperiod of 16:8 light/dark cycle and

relative humidity of 80±5% in a growth chamber (Percival Scientific Inc., Perry, IA). At 14 dpi, a soil drench using imidacloprid (Admire® Pro, Bayer CropScience, St. Louis MO) was applied to kill whitefly nymphs.

Tissue sampling and DNA extraction

Leaf tissue samples were taken from the tomatoes 28 dpi. A sterilized 1-Hole Punch (6mm in diameter) (Staples®, Birmingham, AL) was used to take samples from fully expanded terminal leaves. Two leaf discs were taken from the first fully expanded leaf, 1 leaf disc was taken from 2nd fully expanded leaf, and 1 leaf disc was taken from third fully expanded leaf, then all four leaf disc were moved with an autoclaved toothpick into 1.5 ml microcentrifuge tube (VWR Cat. No. 10025-726) with three 4 mm glass beads (Millipore Sigma Darmstadt, Germany). Liquid nitrogen was used to flash freeze plant tissue samples until stored at -80℃ in a Revco UxF freezer (Thermo Fisher Scientific Inc, Waltham, MA, USA).

For DNA extraction, frozen plant tissue samples were transported in liquid nitrogen to and from a Mini-BeadBeater™ (BioSpec Products, Inc., Bartlesville, OK) where the samples were ground into a fine powder for the DNA extraction. The DNeasy® Plant Mini Kit (QIAGEN, Hilden, Germany) was used to following manufactures instructions with minor revisions,as described in Gong (2018) to extract DNA. DNA samples were stored in -20℃ freezer (VWR). The DNA concentration was quantified using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA). If DNA concentrations were below 20ng/µl, a Qubit 3.0 Fluorometer (Thermo Fisher Scientific Inc., Waltham, MA) was used following the manufacture instruction.

PCR Virus Confirmation

Virus confirmation was conducted by PCR. Amplification of TYLCV-DNA was achieved using primers TYLCV convFor and TYLCV convRev while ToMoV-DNA A was amplified using primers TomoV-convFor and TomoV-convRev (Gong 2018). Only TYLCV DNA-A and ToMoV DNA-A were used for virus detection in recipient plants by PCR. To visualize the amplified product, 1µl of gel Red and 1ml gel loading dye (6x) no SDS (New England Biolabs Inc., Ipswich, MA, USA) were mixed within the gel loading dye tube. After gel red + purple loading dye was mixed, 2 µl of the mixture was added to 10µl PCR product along with 100 KB DNA ladder. Samples were then run on a 2% agarose gel in 1× TAE buffer and visualized using UV Transilluminator (BT Lab Systems, St. Louis, MO, USA) to confirm infection status.

Whitefly DNA Extraction

DNA was extracted from cohorts of six whiteflies that fed on sucrose sachets using Qiagen® DNA and Tissue Kit (QIAGEN, Hilden, Germany) by following the manufacture's protocol. Whitefly DNA was then stored in -20ºC freezer until further analysis by qPCR.

Quantitative PCR (qPCR) amplification of viral DNA

Titers of each virus component, TYLCV-A, ToMoV-A and ToMoV-B, were quantified from virus source plants, whitefly cohorts that fed on sucrose sachets, sucrose sachets, and

recipient plants. Aforementioned primers were used for quantification of TYLCV and ToMoV-A. ToMoV-B was quantified using primers ToMoV-B-Fw and ToMoV-B-Rv mentioned in Gong (2018). Recipient plant DNA was standardized to 25ng of total DNA per qPCR reaction, and total DNA of whitefly cohorts was standardized to 1ng per reaction. Sucrose sachet samples were prepared by adding 5µl of sucrose solution to each reaction. Amplification was performed with C1000 Touch TM thermocycler (Bio-Rad Laboratories, Inc., Hercules, CA) with CFX96TM Real-Time System. All samples were run in triplicate and qPCR was performed at the same conditions used by Gong (2018). An absolute quantification of titers were performed using a standard curve of known copy numbers created by Gong (2018). Sucrose sachet and whitefly samples were determined to be positive by the lowest point on the standard curve average plus three standard deviations.

Virus titers were quantified from five whitefly cohorts and the sucrose sachets they fed on. Co-inoculation of TYLCV and ToMoV in the mixed infection scenario treatments resulted in both single and mixed infection outcomes in recipient plants. Single infected plants were selected to examine whether or not co-inoculation of viruses altered replication in-planta to produce a synergistic or antagonistic effect on the resulting virus. One infected plant was targeted from each replicate of each acquisition scenario treatment, for a total of six plants tested per treatment. A fewer number of plants were tested for treatments where transmission of one virus outcome was low and resulted in fewer than six infected plants in these experiments. No single infections of ToMoV were observed in treatment 4 (agro-inoculated mixed infection),and only 3 mixed infections were observed in treatments 4 (agro-inoculated mixed infection) and treatment 5 (Sequential acquistion TYLCV first), and all eight mixed infected plants from treatment 7

(Mixed whitefly cohort) were tested because the number of mixed infections was very low in the other mixed acquistion scenarios.

Statistical analysis

All statistical analyses were performed using SAS PROC GLIMMIX version 9.4 (SAS Institute, Cary, NC). Tukey's tests were used to perform LS means comparisons using a significance value of $P < 0.05$ for all analyses. The proportion of samples testing positive for virus were compared among acquisition scenario treatments using a binary distribution. Data collected from the PCR reactions used to confirm virus infection in recipient plants were used to analyze the proportion of plants with systemic infection of TYLCV, ToMoV or mixed infections. Sucrose sachet and whitefly cohort data was collected from qPCR reactions to analyze the proportion of these samples that TYLCV, ToMoV or both viruses. Titer data were also used to examine the interactions of the two viruses in whitefly cohorts after acquisition, sucrose sachets after transmission, and recipient plants that were infected with TYLCV, ToMoV, and both viruses. The average virus titers of TYLCV-A, ToMoV-A and ToMoV-B DNA components were analyzed separately for whitefly cohorts, sucrose sachets and recipient plants using the main effects of acquisition scenario treatment (Table 1), viral DNA component, and their interaction term as main effects. Titer data were $\log^{10}$ transformed before analysis. Co-inoculation of TYLCV and ToMoV in the mixed infection scenario treatments resulted in both single and mixed infections of these viruses in recipient plants, and the titers from these three infection outcomes were analyzed separately.

Results

Transmission Efficiency of TYLCV

TYLCV acquisition, transmission and systemic infection were examined by quantifying the proportion of whiteflies that acquired TYLCV, the proportion of sucrose sachets that TYLCV was transmitted into, and the proportion of recipient plants that were systemically infected by TYLCV, respectively. There were no significant differences in the probability of TYLCV being acquired by whiteflies among the acquisition scenario treatments ($F_{4,175}$ =0.32, P= 0.8620), but there were significant differences in the probability of TYLCV transmission to sucrose sachets ($F_{5, 216}$ = 4.51, P = 0.0006), and systemic infection in recipient plants ($_{4, 214}$ = 4.51, P= 0.0122) (Table 2). Transmission of TYLCV to sucrose sachets was highest in the sequential acquisition scenario where ToMoV was acquired first, or in the single infection of TYLCV acquisition scenario, and was lowest in the mixed cohort scenario treatment where it was acquired and transmitted separately from ToMoV. In the recipient plants, significantly higher infection of TYLCV was observed in the single infection acquisition of TYLCV compared to all other scenarios, and there were no differences in systemic infection of TYLCV in any of the mixed acquisition scenario treatments.

Transmission Efficiency of ToMoV

ToMoV transmission efficiency was examined by quantifying the proportion of whiteflies that acquired ToMoV, the proportion of sucrose sachets that ToMoV was transmitted to, and the proportion of recipient plants that were systemically infected by ToMoV. Similar to TYLCV, there were no significant differences among the acquisition scenario treatments in the probability of ToMoV acquisition by whiteflies ($F_{4, 180}$ = 0.35, P= 0.8448), but there were differences in the probability of transmission to sucrose sachets ($F_{3, 202}$ = 3.90, P= 0.0045) and systemic infections

of recipient plants ($F_{4, 225} = 4.16$, $P = 0.0029$) (Table 3). The highest transmission of ToMoV with sucrose sachets was observed for the single infection acquisition scenario, but this was only significantly higher than both of the sequential acquisition scenarios. In the recipient plant analysis, the highest infection was observed when ToMoV was acquired first during sequential acquisition, as a single infection, or separately from TYLCV in the mixed whitefly cohort scenario.

Transmission Efficiency of Both Viruses

Acquisition and transmission of mixed infections (TYLCV-A, ToMoV-A, and ToMoV-B) was examined by quantifying the proportion of whiteflies that acquired both viruses, the proportion of sucrose sachets both viruses were transmitted into, and the proportion of recipient plants that were systemically infected by both viruses. Acquisition of mixed infections was high while transmission and systemic infection among all treatments were low. There were no significant differences among virus acquisition scenarios in analyses of whiteflies ($F_{3, 161} = 0.46$, $P = 0.7071$), sucrose sachets ($F_{3, 173} = 0.46$, $P=0.7294$), or the recipient plants ($F_{3, 174} = 0.46$, $P=0.1978$) (Table 4).

Viral Titers in the Whiteflies

In the comparison of average viral titer in the whiteflies, the main effects of acquisition scenario treatments ($F_{5, 192} = 2.81$, $P = 0.0180$) (Figure 1), DNA component ($F_{2, 192} =16.18$, $P<0.0001$) (Figure 2) and their interaction ($F_{14, 185} =4.63$, $P<0.0001$) (Figure 3), were all significant. A comparison of the average titer showed the virus titers were higher in all

acquisition scenarios where viruses were acquired individually (whiteflies only acquired one virus), and were generally lower in acquisition scenarios where the viruses were acquired together. The titer of TYLCV-A in whiteflies is significantly higher than either ToMoV components in the acquisition scenarios where the viruses are acquired together, but not in the virus acquisition scenario with mixed whitefly cohorts.

Viral Titers in the Sucrose Sachets

The average viral titers transmitted into sucrose sachets were not significantly different among virus acquisition scenario treatments ($F_{5,194}$=1.14, P=0.3423) (Figure 4), but both ToMoV components were significantly higher than the TYLCV-A component when averaged across treatments ($F_{2,194}$ =8.99, P=0.0002) (Figure 5), and the interaction between treatment and DNA component was also significant ($F_{14,187}$ =2.44, P=0.0036) (Figure 6). In contrast to the results observed in whiteflies, the titers of both ToMoV components were either not significantly different than TYLCV-A, or were higher than TYLCV-A in sucrose sachets.

Viral Titers in Recipient Plants – Comparing Mixed Infections to Single Infections

Average titers were significant for the main effects of treatment ($F_{5, 65}$ =7.83, P<0.0001) (Figure 7), components ($F_{2, 65}$ =10.28, P=0.0001) (Figure 8), and their interaction ($F_{14, 58}$ =5.45, P<0.0001) (Figure 9). The average titer of all three components in the single infection of ToMoV, agro-inoculated mixed infection and the sequential acquisition of TYLCV first were significantly higher than the other three treatments. On average, TYLCV is significantly higher than the ToMoV components, but this varies among acquisition scenario treatments. A

comparison of the titers of each viral component by treatment shows that co-acquisition and co-inoculation of these viruses together produced an antagonistic effect in specific acquisition scenario treatments. To determine whether there was no effect, a positive effect or a negative effect of virus – virus interactions, the titer of each virus component of mixed acquisition scenario treatments was compared to titers in the single acquisition treatments. There were no differences in titers for the agro-inoculated mixed infection and the sequential acquisition of TYLCV first treatments, however, it should be noted that sample size is low because only three mixed infections were observed in both of these treatments over the course of these experiments. In the sequential acquisition of ToMoV first and the mixed whitefly cohort treatments a significant reduction in titers of both ToMoV components was observed.

Viral Titers in Recipient Plants – Co-Acquisition and Co-inoculation that Resulted in a Single Infection Outcome – TYLCV

In the analysis conducted to investigate whether or not there is an effect of co-inoculation on the virus titers observed in single infection outcomes, the overall ANOVA model was not significant ($F_{4, 24} = 1.79$, P=0.1636). However, pairwise LS means comparisons between the single and mixed infection acquisition scenarios showed a significant increase in titers when TYLCV was acquired first ($t_{24} = -2.44$, P=0.0223) and when ToMoV was acquired first ($t_{24} = -2.16$, P=0.0406) (Figure 10). Titers were numerically higher in all mixed acquisition scenario treatments.

Viral Titers in Recipient Plants – Co-Acquisition and Co-inoculation that Resulted in a Single Infection Outcome – ToMoV

In the analysis of ToMoV component titers in co-inoculated plants the main effect of treatment was significant ($F_{3, 29} = 9.97$, P=0.0001) (Figure 11) , but the main effect of virus DNA component and the interaction term was not significant ($F_{1, 29} = 0.00$, P=0.9774) (Figure 12) ($F_{3, 29} = 0.19$, P=0.9046) (Figure 13). Generally, lower titers of both components were observed in the mixed acquisition scenario treatments. Titers in the sequential acquisition of ToMoV first and the mixed whitefly cohort treatments were significantly lower than the single acquisition of ToMoV treatment. Titers were not significantly lower in the sequential acquisition of TYLCV first scenario, but sample size was low because only two ToMoV infected plants resulted during these experiments. No ToMoV infections were observed in the agro-inoculated mixed infection treatment. Overall, these results suggest that co-inoculation with TYLCV has an antagonistic effect on ToMoV fitness.

Discussion

The aims of this study were to understand the how the whitefly vector may propagate a mixed infection between two different *Begomovirus* species, and examine the influences of transmission on virus- virus interactions through time (acquisition, transmission, and systemic infection). We have demonstrated that the order of acquisition does influence the transmission efficiency of two persistent, circulate viruses. The order of acquisition had an influence on viral fitness of the mixed infections propagated, and we also observed an effect of fitness in single virus outcomes resulting from mixed infection acquisitions. Our results suggest virus – virus interactions occurring at both within-vector and at the host level influence transmission and plant infection.

Vector acquisition and transmission

Past studies examining vector transmission of two viruses were conducted on semi-persistently and non-persistently transmitted viruses, and focus on the effect of acquisition scenarios on transmission efficiency of two viruses. One study examined sequential acquisition of two semi-persistently *Crinivirus* species and reported that *Lettuce infectious yellows virus* (LIYV) was transmitted more efficiently and always interfered with the transmission of *Lettuce chlorosis virus* (LCV) regardless of order of acquisition, suggesting there may be interference due to these viruses sharing a similar transmission pathway in the vector (Ng and Chen 2011). A study conducted on two strains of non-persistently transmitted Potato virus Y (PVY) examined sequential acquisition treatments and a mixed cohort scenario on the probability of transmission of both strains (Carroll et al. 2016). Regardless of acquisition or transmission scenario, the PVY^{NTN} strain was transmitted more efficiently, suggesting that the order of acquisition overall did not affect the transmission efficiency of either strain.

This study used persistently transmitted viruses that must successfully circulate in the vector and localize in salivary ducts for transmission to occur. Our data suggest that acquisition of more than one virus by the vector population alters the transmission efficiency of one or both viruses depending on the acquisition scenario (Tables 1-3). Detection of virus in sucrose sachets provides a measure of what is transmitted by the whiteflies that is not obscured by replication of viruses in host plant tissues after transmission. TYLCV transmission efficiency to sucrose sachets was the highest when acquired from single infection of TYLCV or when ToMoV was acquired first. ToMoV transmission efficiency was also highest in the single acquisition ToMoV scenario, or when it was acquired from a mixed infection or the mixed cohort of whiteflies. Overall, transmission efficiency of both viruses was reduced in all mixed infection acquisition

scenarios, and transmission of mixed infections was very low. The titers of all three virus components transmitted, however, was very similar (Figure 6), but in some virus acquisition scenarios there were higher titers of ToMoV than TYLCV.

Differences in transmission were not due to differences in the accumulation of virus in the whiteflies during acquisition because higher titers of TYLCV were observed in whitefly cohorts from all acquisition scenarios (Figure 3). In this experiment, we examined the viral load within whole whiteflies, but only virions successfully localizing in salivary ducts of individual whiteflies will be transmitted (Wei et al. 2014). ToMoV is a persistent virus that circulates through the whitefly and has not been reported to replicate within the vector (Polston 1993), whereas TYLCV-Is has been reported to replicate within the *B. tabaci* MEAM1, indicating that the mode of transmission is persistent,propagative (Sinisterra et al. 2005, Pakkianathan et al. 2015, He et al. 2020a). If TYLCV is replicating in the vector this may explain why TYLCV titers were higher in the whitefly than both the ToMoV components. Our observations that titers of TYLCV and ToMoV were generally similar after transmission suggesting there is not strong competition of these two viruses along their circulation pathways in the vector. Mechanisms underlying virus-vector interactions required for circulation and transmission remain poorly understood and future research is needed in this area to understand how viruses interact within the vector.

Virus – Virus Interactions in Mixed Infection Recipient Plants

During a mixed viral infection, virus-virus interactions may lead to a range of outcomes from synergism to antagonism, or neutral effects. These interactions can impact the fitness of one or both viruses compared to what would be observed in a single infection (Syller 2012), and this discussion, we will use the definitions of virus-virus interactions and viral fitness provided by

Alcaide. (2020) (Figure 14). Transmission efficiency and viral titers were the two measures of viral fitness measured in this study. Single infections were propagated by most mixed acquisition scenarios with the exception being the agroinoculated mixed infection treatment, and mixed infections of the viruses were propagated by whitefly cohorts in all mixed acquisition scenarios, but. the frequency of co-infection is very low regardless of how the viruses are acquired and co-inoculated. Although mixed infections occur frequently in nature, our results are consistent with those from other studies that report a low frequency of mixed infections in laboratory experiments (Ng and Falk 2006, Wintermantel et al. 2008, Carroll et al. 2016). Our results also suggest that when both viruses are acquired by the vector there may be an antagonistic effect on transmission. Transmission of TYLCV was highest in the single-infected acquisition scenario compared to all the mixed acquisition scenarios, suggesting the acquisition of ToMoV by the vector reduces transmission efficiency of TYLCV (Table 2). The transmission of ToMoV was not consistently reduced when TYLCV was acquired by the vector; this only occurred when TYLCV was acquired first or together from a mixed infection source (Table 3). It is not clear how these viruses are interacting, and a better understanding of circulation pathways and vector-virus interactions are required to speculate about the underlying mechanisms of virus-virus interactions in the vector. These results also suggest that a vector-mediated effect influences virus replication after transmission because the patterns of transmission efficiency in plants 28 dpi did not always correspond with transmission rates observed in sucrose sachets. The vector could influence temporal dynamics of virus transmission if circulation and localization in salivary glands occurs at different rates based on the acquisition scenarios. Virus-vector interactions may also be influenced by a behavioral, physiological or biochemical aspects of vector feeding required for transmission.

Virus titer is a measure of virus replication and fitness in host tissues. For this pathosystem, titers of each virus component are compared because both virus components of bipartite begomoviruses must be present for a systemic infection to occur (Hanley-Bowdoin et al. 2000). Our data suggest that order of acquisition influences the relative fitness when quantifying titers of viral components in this specific virus- virus- vector- host system. Component titers in our mixed acquisition scenarios compared to the titers in the single infection scenarios (Figure 3C) showed there was a neutral virus-virus effect observed in the agro-inoculated mixed infection and the sequential acquisition of TYLCV first scenarios (Figure 14). The results for the sequential acquisition of ToMoV and the mixed whitefly cohort treatments show that the TYLCV component was not significantly different from the single infection TYLCV, but the ToMoV components were significantly lower than the single infection of ToMoV, defining this virus-virus outcome as neutral antagonism (Figure 14). When the titers of the three virus components are compared by treatment for each mixed virus acquisition scenarios (Figures 3C), the titers are generally not significantly different from each other if the whiteflies acquired both viruses, but they are significantly different when the viruses were co-inoculated by whiteflies that only acquired one of the viruses in our mixed cohort scenario. This suggests there is an important spatial aspect of co-inoculation that influences infection and virus-virus outcomes. The temporal aspect of inoculation has been studied by examining superinfection, which refers to inoculation of two strains of a virus, or two closely related, viruses at different points in time. There has been less attention paid to the spatial aspect of mixed infections; speculation has been made about the influence of viruses infecting the same or neighboring cells (Mascia and Gallitelli 2016, Allen et al. 2019), but we were not able to find mention of a study that investigated co-inoculation by different vectors on the same or different plant tissue. In this

experiment, both viruses were co-inoculated into the plant by whiteflies that had acquired both viruses in three of the four mixed acquisition scenarios. When a single whitefly harbors both viruses it is possible that the viruses are inoculated into the same cell or neighboring cells, but it cannot be determined from this study which scenario occurred. Although we did not observe competition between these two viruses based on titer data from mixed-infected plants it is possible that competition early in the virus replication cycles is occurring, and this is a potential explanation for the overall low number of mixed infections observed in these experiments. This is supported by our observation that the highest number of mixed infections occurred in the mixed whitefly cohort scenario, where each viruses were co-inoculated, but by different whiteflies.

Co-inoculation and the effects virus- virus interactions on single infection outcomes

Three different plant infection outcomes are reported to occur after a vector acquires two viruses; a single infection of either virus or a mixed infection with both viruses (Wintermantel et al. 2008, Carroll et al. 2016, Syller and Grupa 2016). Studies examining virus-virus outcomes in the plant have measured titers in mixed infected plants to examine competition and fitness of the replicating viruses. Replication and competition are measured at specific time points during the infection cycle, but they are not static or consistent interactions (Mascia et al. 2010, Chávez-Calvillo et al. 2016). It is possible that early interactions between co-inoculated viruses may lead to single-infection outcomes, and that these early interactions may influence subsequent replication of the remaining virus, but we are unaware of any studies quantifying the titers of single infections after co-inoculation with another virus. Data from the sucrose sachets demonstrates that both viruses are being transmitted at the same relative titers in our co-

inoculation scenarios, therefore, we investigated whether or not there was an effect of co-inoculation on the fitness of viruses detected as a single infection (Figure 19).

Differences in titers of recipient plants were observed between single and mixed-infection acquisition scenario treatments. Co-inoculation of ToMoV increased the titers of TYLCV-infected recipient plants in all mixed acquisition scenario treatments (Figure 10), and titers were significantly higher in both sequential acquisition scenarios compared to the single infection treatment. These results indicate that co-inoculation in these two treatments had a synergistic effect on TYLCV replication. In contrast, co-inoculation of TYLCV reduced replication of ToMoV (Figure 13). No single-infections of ToMoV resulted from the mixed-infected source plant scenario, and an antagonism was observed in sequential acquisition ToMoV first treatment and the mixed cohort scenarios. There was a neutral effect observed in the sequential acquisition of TYLCV first treatment, however, there were only two ToMoV infected plants observed in this treatment. Due to the low transmission and significant reduction in titers there was an overall antagonistic effect of TYLCV co-inoculation on ToMoV replication.

Conclusion

This research was conducted to generate knowledge about the role of the vector in propagating mixed infections by examining virus-virus-vector interactions during acquisition, transmission and subsequent infection in hosts using the *B. tabaci* MEAM1-TYLCV-ToMoV tomato pathosystem. Our results demonstrate that a mixed infection may be propagated by all mixed acquisition scenarios mentioned in our study, but the frequency of a mixed infection

outcome was low, and likely due to virus-virus interactions that occur in the vector and host plants. In contrast to other studies examining transmission of mixed infections (Ng and Falk 2006, Carroll et al. 2016), the order of acquisition did influence transmission efficiency of the viruses. Viral titer results clearly demonstrate that co-inoculation is influencing virus-virus interactions in the host, and that co-inoculation influenced the replication of viruses in both single and mixed-infections that propagated after vector transmission. Further studies need to be conducted on virus-virus interactions in vectors and host plants including, mechanisms underlying circulation in vectors, spatial aspects of co inoculation of host plants, and cellular cross talk during virus replication in vectors and plants.

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Table 1- A list of single and mixed infection acquisition scenario treatments used in this study.

TREATMENT	VIRUS ACQUISITION SCENARIOS
1 Healthy Control	Whiteflies feed on a healthy, non-infected source plant.
2 TYLCV Single Infection	Whiteflies feed on a *Tomato yellow leaf curl virus* (TYLCV) infected source plant only.
3 ToMoV Single Infection	Whiteflies feed on *Tomato mottle virus* (ToMoV) infected source plant only.
4 Agro-inoculated Mixed Infection	Whiteflies co-acquire TYLCV and ToMoV together from a mixed-infected source plant.
5 Sequential Acquisition TYLCV First	Whiteflies feed on TYLCV infected source plant for 24 hours, followed by ToMoV infected source plant for 24 hours.
6 Sequential Acquisition ToMoV First	Whiteflies feed on ToMoV infected source plant for 24 hours, followed by TYLCV infected source plant for 24 hours.
7 Mixed Cohorts of Virulferous Whiteflies	Whiteflies feed on a TYLCV or ToMoV infected source plant (whiteflies acquired one virus), and were then placed together on the recipient plant.

Table 2 –The average proportion of whiteflies, sucrose sachets, and recipient plants that *Tomato yellow leaf curl virus* (TYLCV) was detected in. Results not shown for Treatment 1 (Control) because acquisition from this treatment did not result in any samples testing positive for TYLCV. LS means comparisons were conducted separately for whitefly cohorts, sucrose sachets and recipient plants using Tukey's method at P =0.05 level

Treatment	Acquisition Order[1]	Whitefly Cohorts		Sucrose Sachets		Recipient Plants	
		Mean (SE)	# Positive /# Tested	Mean (SE)	# Positive /# Tested	Mean (SE)	# Positive/ # Tested
2	TYLCV	1.0(0.00) a	15/15	0.60(0.13) ba	9/15	0.73(0.06) a	37/51
4	MIX	0.89(.05) a	13/15	0.42(0.08) bc	6/15	0.48(0.08) b	21/43
5	TYLCV » ToMoV	0.96(0.03) a	14/15	0.33(0.07) bc	5/15	0.53(0.07) b	26/49
6	ToMoV » TYLCV	1.0(0.00) a	15/15	0.73(0.07) a	11/15	0.36(0.07) b	16/46
7	TYLCV+ToMoV	1.0(0.00) a	15/15	0.27(0.07) c	4/15	0.48(0.08) b	20/41
	Statistics[2]	F (4,175)=0.32, P=0.8620		F (5,216)=4.51, P=0.0006***		F(4, 214) = 3.28, P = .0122**	

[1] Acquisition Order: Single Infection of TYLCV (Treatment 2), Agro-Inoculated Mixed Infection (Treatment 4), Sequential Acquistion of TYLCV First (Treatment 5), Sequential Acquisition of *Tomato mottle virus* (ToMoV) First (Treatment 6), Mixed Whitefly Cohort (Treatment 7)

[2] Num DF, Den DF, F Value, Pr>F. Not significant (P>0.05) or significant at P<0.05(*), 0.01(**) or 0.001(***).

Table 3 – The average proportion of whiteflies, sucrose sachets, and recipient plants that *Tomato mottle virus* (ToMoV) was detected in. Results not shown for Treatment 1 (Control) because acquisition from this treatment did not result in any samples testing positive for ToMoV. LS means comparisons were conducted separately for whitefly cohorts, sucrose sachets and recipient plants using Tukey's method at P =0.05 level.

Treatment	Acquisition Order[1]	Whitefly Cohorts		Sucrose Sachets		Recipient Plants	
		Mean (SE)	# Positive /# Tested	Mean (SE)	# Positive /# Tested	Mean (SE)	# Positive/ # Tested
3	ToMoV	1.0(0.00)a	10/10	0.47(0.09)a	7/15	0.30(0.06)a	17/51
4	MIX	0.89(0.05)a	9/10	0.36(0.07)ba	5/15	0.07(0.04)b	3/43
5	TYLCV » ToMoV	0.93(0.03)a	14/15	0.07(0.04)c	1/15	0.10(0.03)b	5/49
6	ToMoV » TYLCV	0.1.0(0.00)a	15/15	0.2(0.06)bc	3/15	0.38(0.07)a	17/46
7	TYLCV+ToMoV	0.93(0.07)a	14/15	0.27(0.07)ba	4/15	0.32(0.07)a	13/41
Statistics[2]		F (4,180)=0.35, P=0.8448		F (4,202)=3.90, P=0.0045***		F(4, 225) = 4.16, P= .0029***	

[1] Acquisition Order: Single Infection of ToMoV (Treatment 3), Agro-Inoculated Mixed Infection (Treatment 4), Sequential Acquistion of *Tomato yellow leaf curl virus* (TYLCV) First (Treatment 5), Sequential Acquisition of ToMoV First (Treatment 6), Mixed Whitefly Cohort (Treatment 7)
[2] Num DF, Den DF, F Value, Pr>F. Not significant (P>0.05) or significant at P<0.05(*), 0.01(**) or 0.001(***).

Table 4 – The average proportion of whiteflies, sucrose sachets, and recipient plants that *Tomato mottle virus* (ToMoV) and *Tomato yellow leaf curl virus* (TYLCV) were detected in. Results not shown for Treatment 1 (Control) because acquisition from this treatment did not result in any samples testing positive for either virus. LS means comparisons were conducted separately for whitefly cohorts, sucrose sachets and recipient plants using Tukey's method at P =0.05 level.

Treatment	Acquisition Order [1]	Whitefly Cohorts		Sucrose Sachets		Recipient Plants	
		Mean (SE)	# Positive /# Tested	Mean (SE)	# Positive /# Tested	Mean (SE)	# Positive/ # Tested
4	MIX	0.88(0.05)a	9/10	0.00(0.00)a	0/15	0.07(0.04) a	3/43
5	TYLCV » ToMoV	0.96(0.03)a	14/15	0.07(0.03)a	1/15	0.06(0.03) a	3/49
6	ToMoV » TYLCV	1.0(0.00)a	15/15	0.13(0.05)a	2/15	0.13(0.05) a	6/46
7	TYLCV+ToMoV	0.93(0.03)a	14/15	0.13(0.05)a	2/15	0.20(0.06) a	8/41
Statistics [2]		F (4,180)=0.35, P=0.8448		F (4,202)=3.90, P=0.0045***		F(4, 225) = 4.16, P= .0029***	

[1] Acquisition Order: Agro-Inoculated Mixed Infection (Treatment 4), Sequential Acquistion of TYLCV First (Treatment 5), Sequential Acquisition of ToMoV First (Treatment 6), Mixed Whitefly Cohort (Treatment 7)

[2] Num DF, Den DF, F Value, Pr>F. Not significant (P>0.05) or significant at P<0.05(*), 0.01(**) or 0.001(***).

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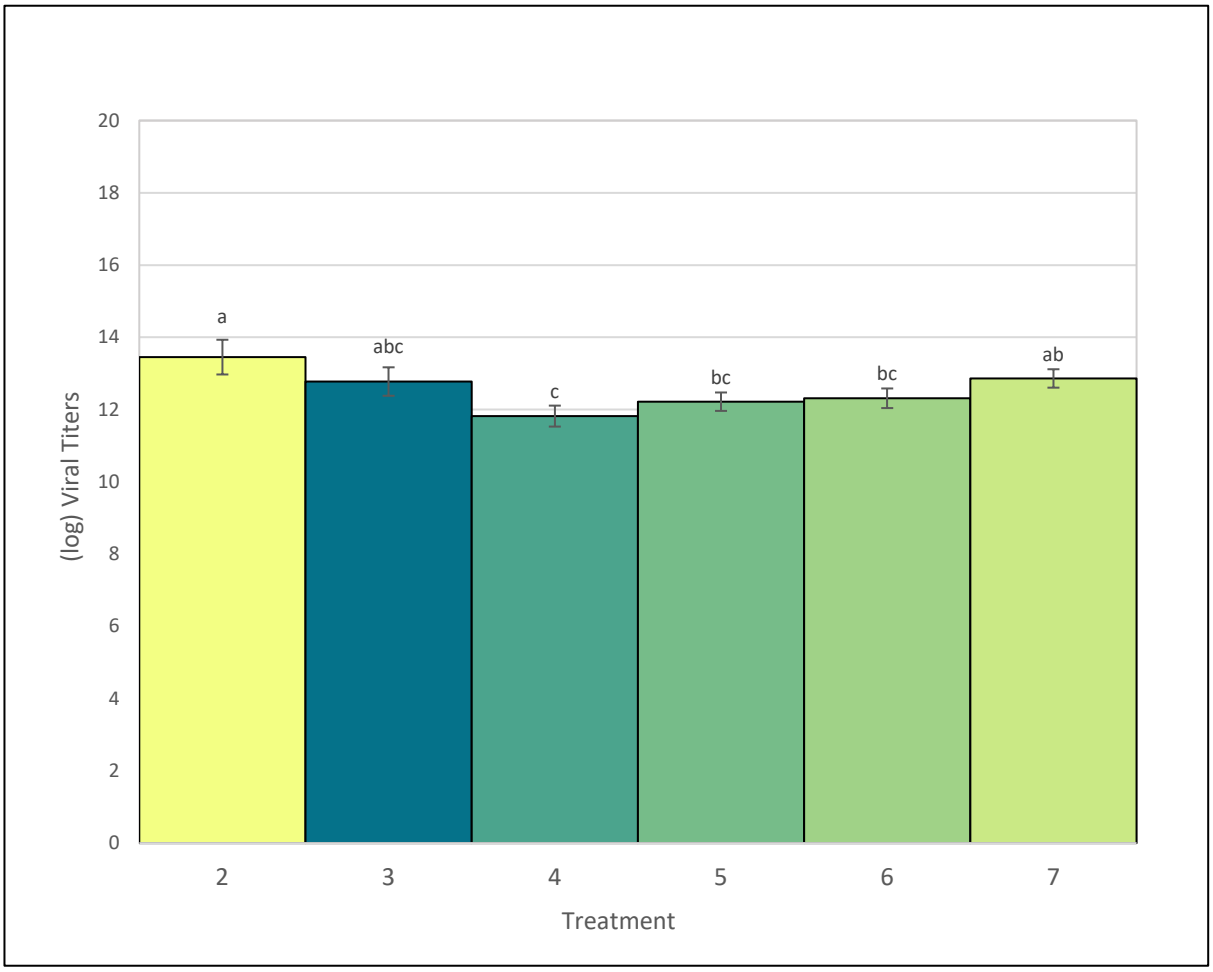

Figure 1: The average titers of *Tomato yellow leaf curl* (TYLCV) and/or *Tomato mottle virus* (ToMoV) DNA quantified by treatment in whiteflies. Treatment 1 (Control, no positives, data not shown), Treatment 2 (Single infection TYLCV), Treatment 3 (Single infection ToMoV), Treatment 4 (Agro-inoculated mixed infection), Treatment 5 (Sequential acquisition of TYLCV first), Treatment 6 (Sequential acquisition of ToMoV first), Treatment 7 (Mixed whitefly cohort). LS means comparisons were conducted using Tukey's method at P=0.05.

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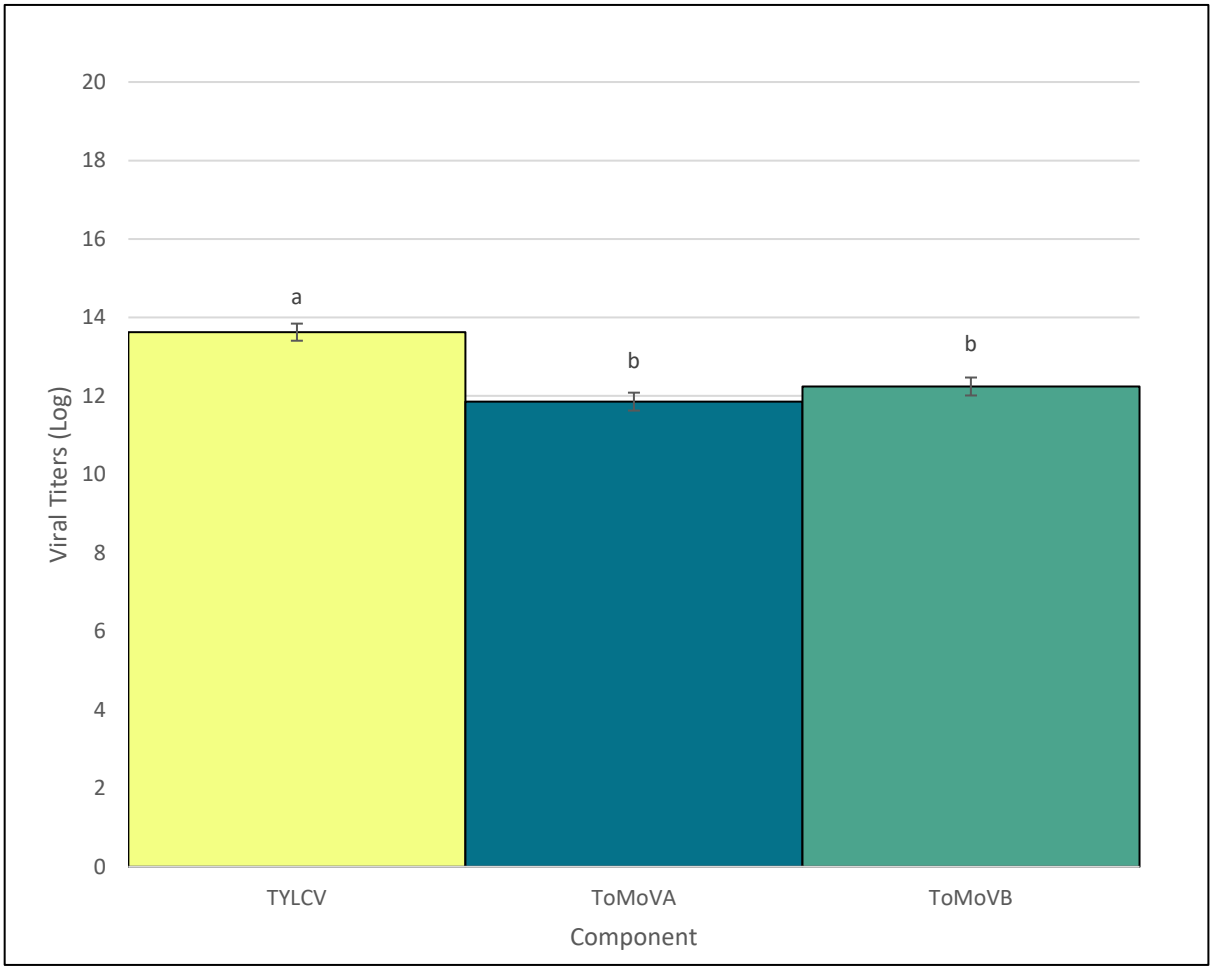

Figure 2: The average titer of *Tomato yellow leaf curl* and *Tomato mottle virus* by DNA components in whitefly cohorts across all treatments. Yellow (TYLCV DNA-A), Blue (ToMoV DNA-A), Green (ToMoV DNA-B). LS means comparisons were conducted using Tukey's method at P =0.05 level.

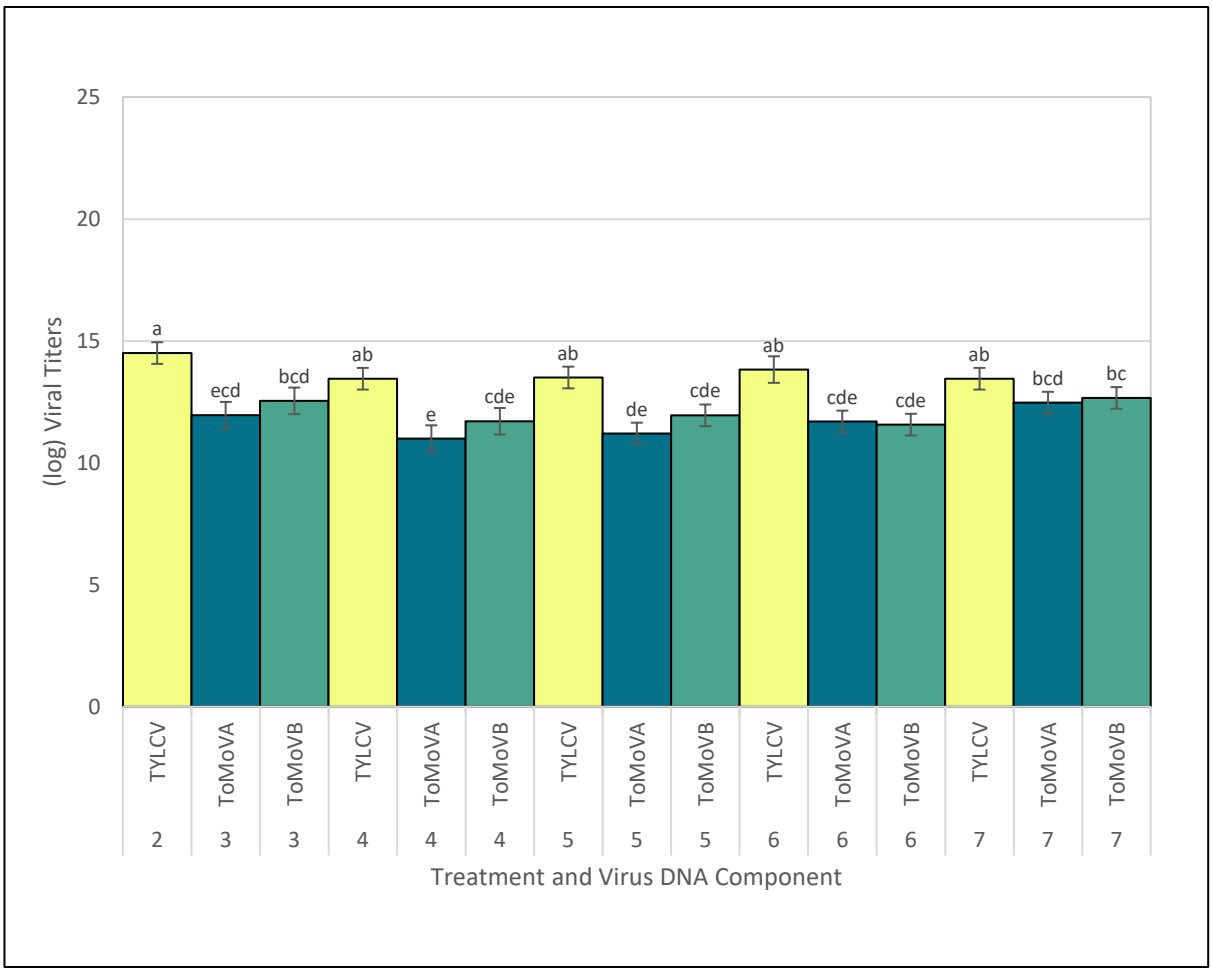

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Figure 3: The average titer of *Tomato yellow leaf curl* (TYLCV, DNA-A) and *Tomato mottle virus* (ToMoV, DNA-A and DNA-B) by component and treatment in whitefly cohorts that acquired virus in acquisition scenario treatments.Treatment 1 (Control, no positives, data not shown), Treatment 2 (Single infection TYLCV), Treatment 3 (Single infection ToMoV), Treatment 4 (Agro-inoculated mixed infection), Treatment 5 (Sequential acquisition of TYLCV first), Treatment 6 (Sequential acquisition of ToMoV first), Treatment 7 (Mixed whitefly cohort). LS means comparisons were conducted using Tukey's method at P=0.05.

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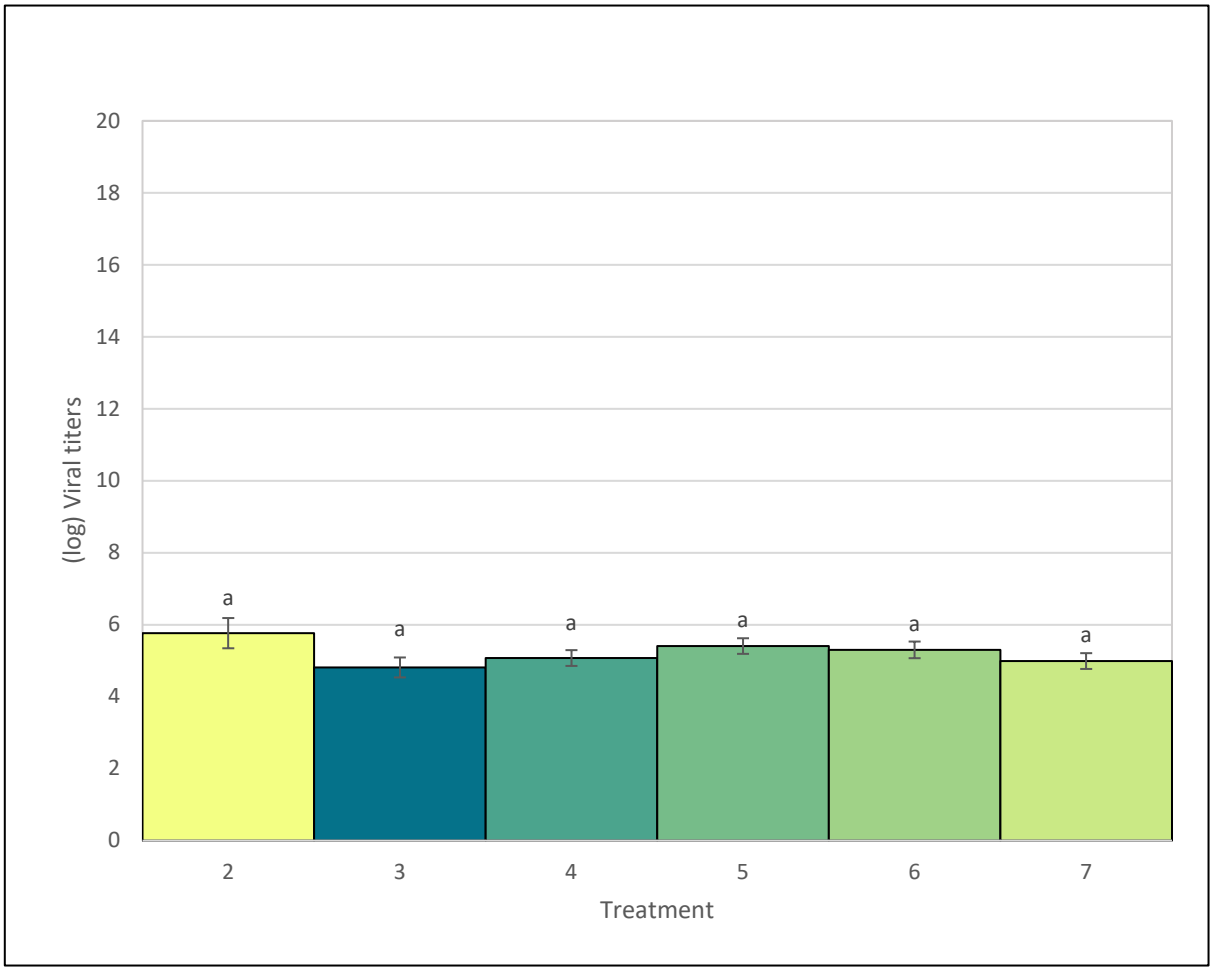

Figure 4: The average titers of *Tomato yellow leaf curl* (TYLCV) and/or *Tomato mottle virus* (ToMoV) DNA quantified by treatment in sucrose sachets. Treatment 1 (Control, no positives, data not shown), Treatment 2 (Single infection TYLCV), Treatment 3 (Single infection ToMoV), Treatment 4 (Agro-inoculated mixed infection), Treatment 5 (Sequential acquisition of TYLCV first), Treatment 6 (Sequential acquisition of ToMoV first), Treatment 7 (Mixed whitefly cohort). LS means comparisons were conducted using Tukey's method at P=0.05.

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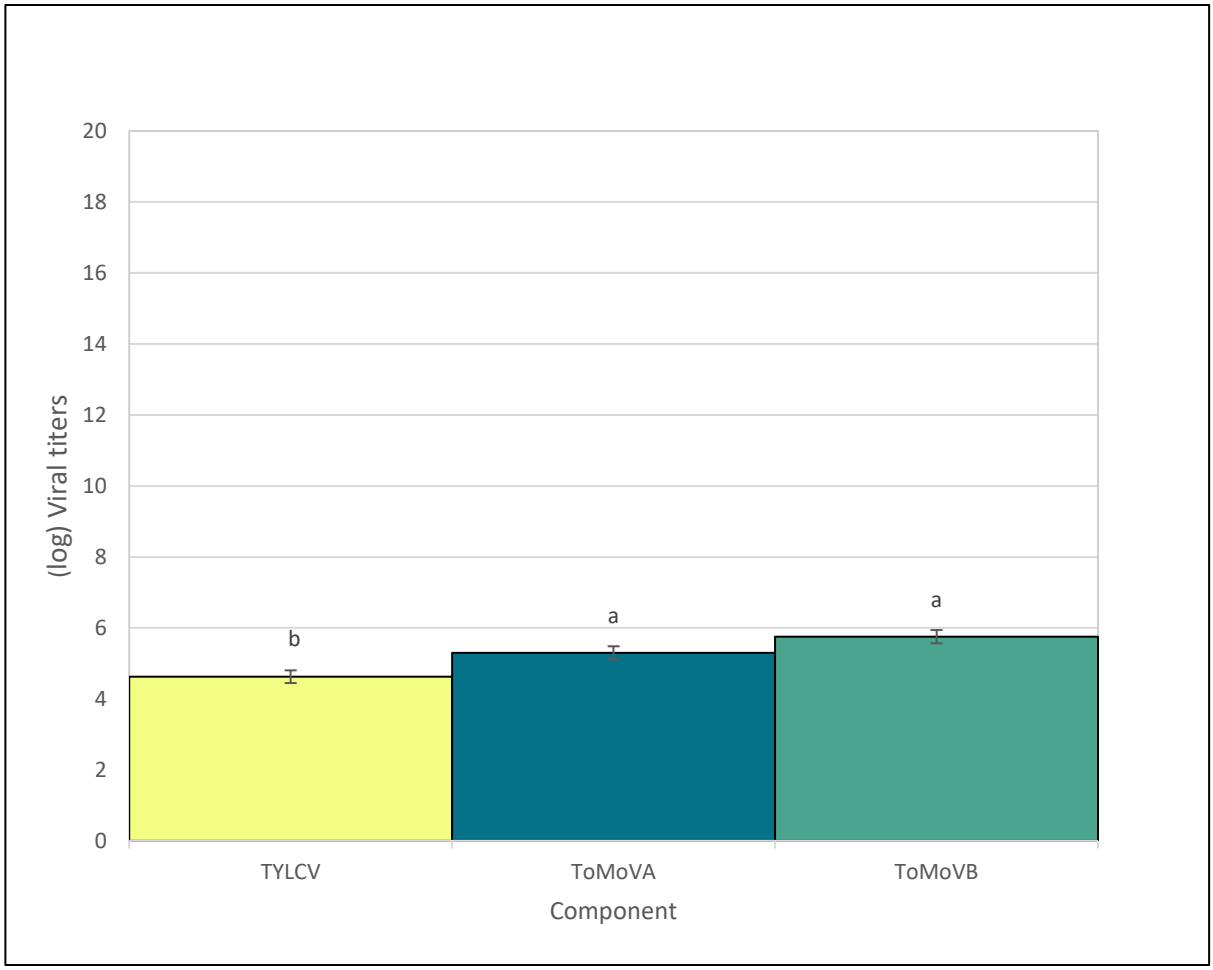

Figure 5: The average titer of *Tomato yellow leaf curl* and *Tomato mottle virus by* DNA components in sucrose sachets across all treatments. Yellow (TYLCV DNA-A), Blue (ToMoV DNA-A), Green (ToMoV DNA-B). LS means comparisons were conducted using Tukey's method at P =0.05 level.

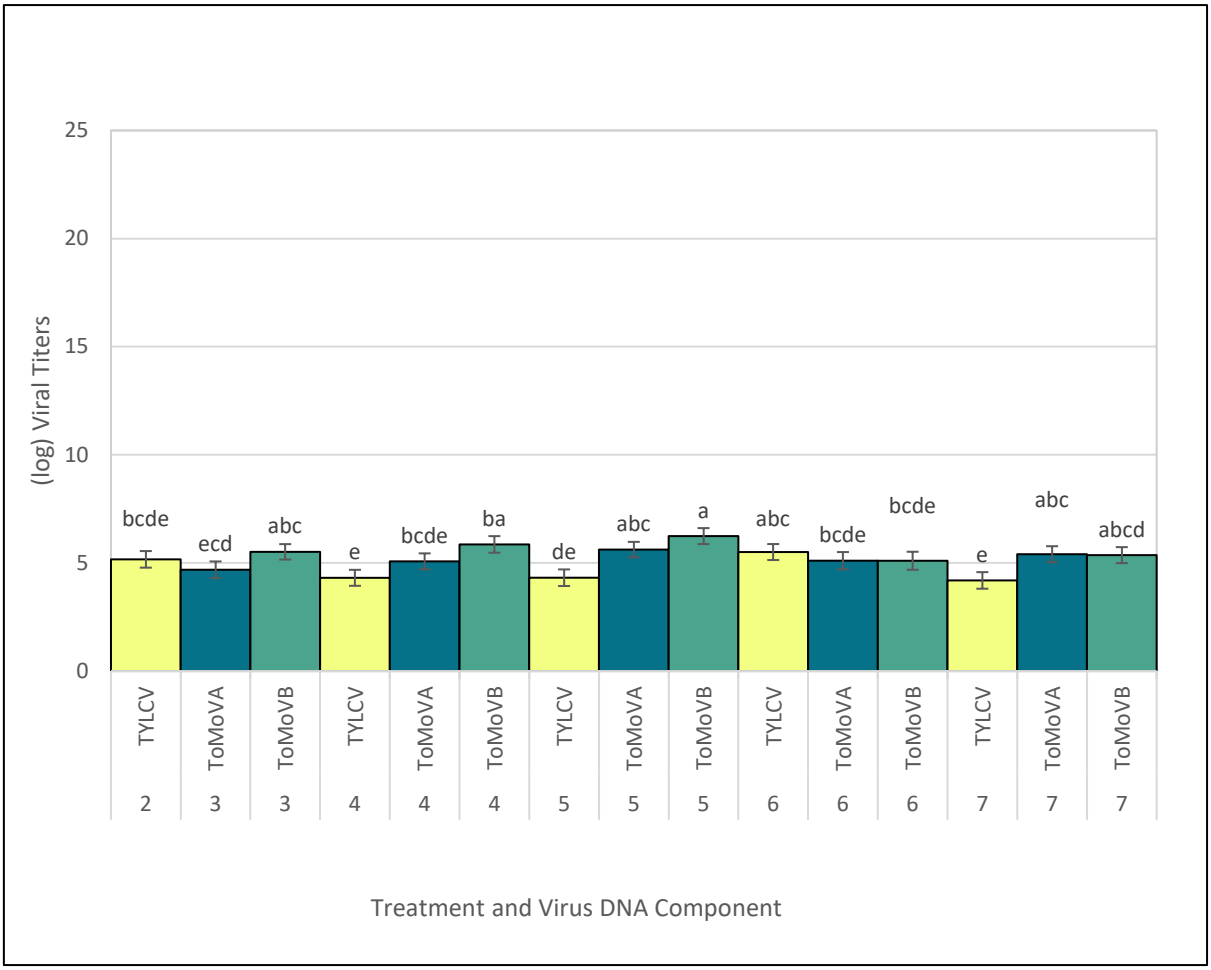

Figure 6: The average titer of *Tomato yellow leaf curl* (TYLCV, DNA-A) and *Tomato mottle virus* (ToMoV, DNA-A and DNA-B) by component and treatment in sucrose sachets that were fed on by whitefly cohorts after acquisition scenario treatments. Treatment 1 (Control, no positives, data not shown), Treatment 2 (Single infection TYLCV), Treatment 3 (Single infection ToMoV), Treatment 4 (Agro-inoculated mixed infection), Treatment 5 (Sequential acquisition of TYLCV first), Treatment 6 (Sequential acquisition of ToMoV first), Treatment 7 (Mixed whitefly cohort). LS means comparisons were conducted using Tukey's method at P=0.05.

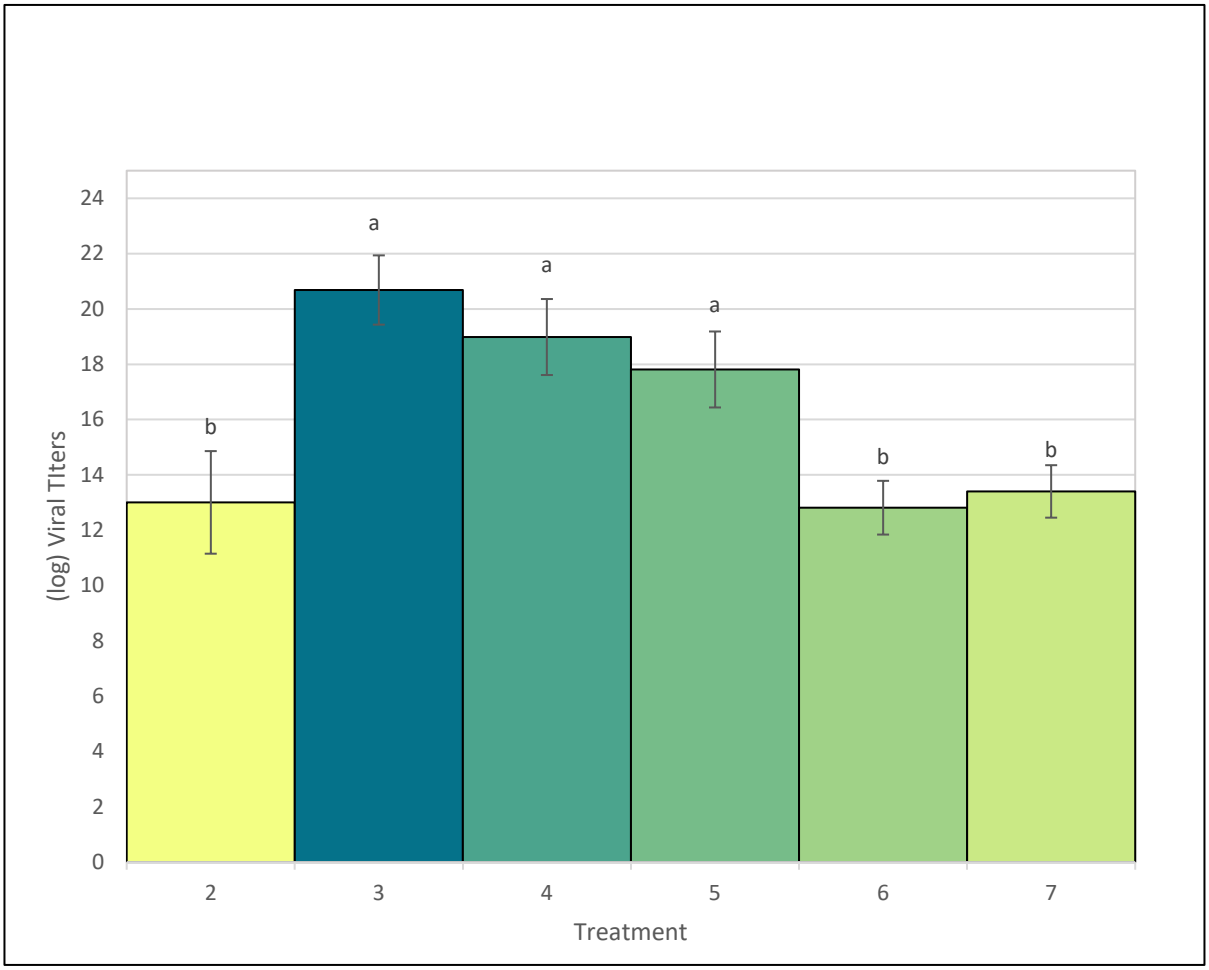

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Figure 7: The average titers of *Tomato yellow leaf curl* (TYLCV) and/or *Tomato mottle virus* (ToMoV) DNA quantified by treatment in recipient plants 28 days post inoculation. Treatment 1 (Control, no positives, data not shown), Treatment 2 (Single infection TYLCV), Treatment 3 (Single infection ToMoV), Treatment 4 (Agro-inoculated mixed infection), Treatment 5 (Sequential acquisition of TYLCV first), Treatment 6 (Sequential acquisition of ToMoV first), Treatment 7 (Mixed whitefly cohort). LS means comparisons were conducted using Tukey's method at P=0.05.

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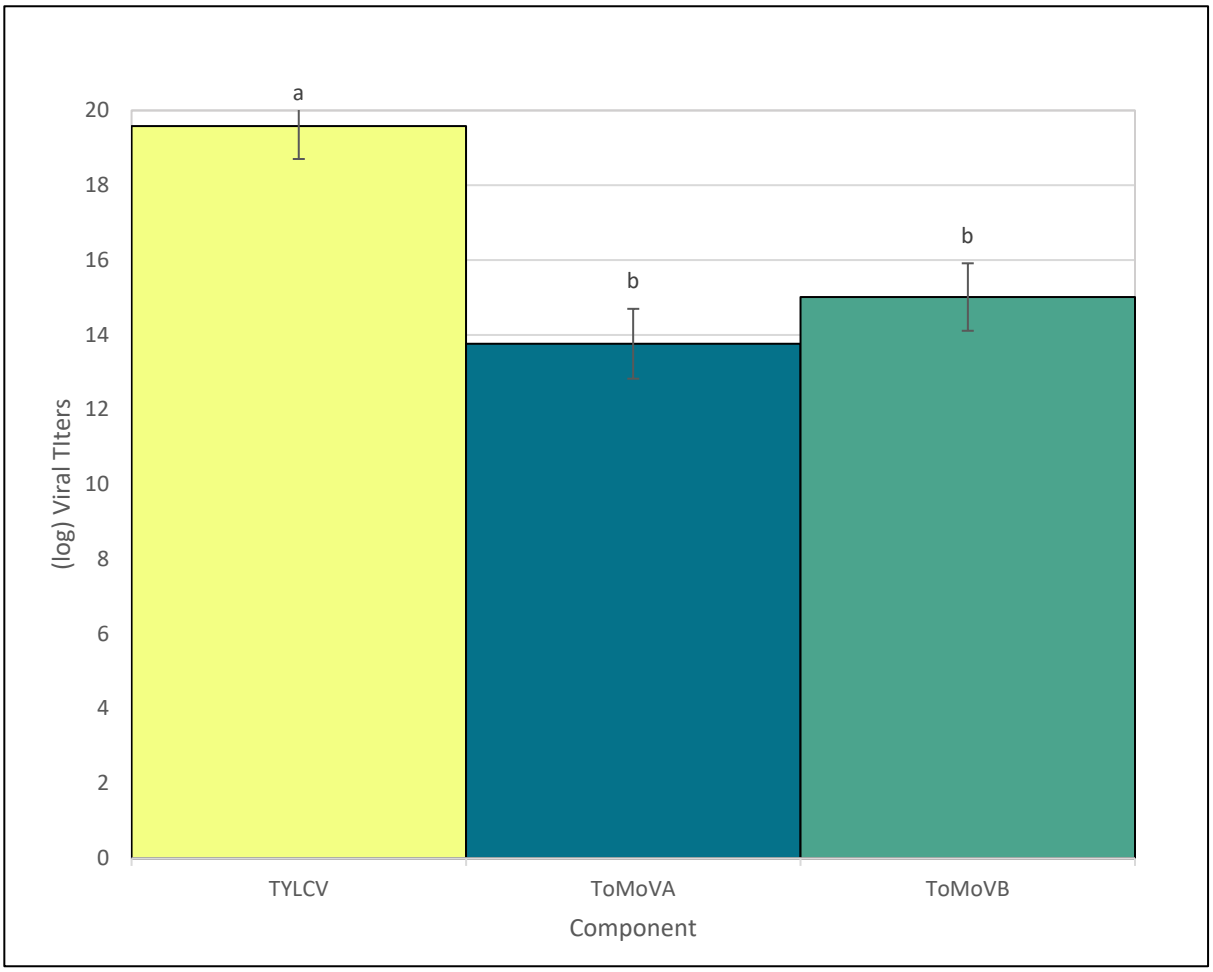

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Figure 8: The average titer of *Tomato yellow leaf curl* and *Tomato mottle virus* by DNA components in recipient plants across all treatments. Yellow (TYLCV DNA-A), Blue (ToMoV DNA-A), Green (ToMoV DNA-B). LS means comparisons were conducted using Tukey's method at P =0.05 level.

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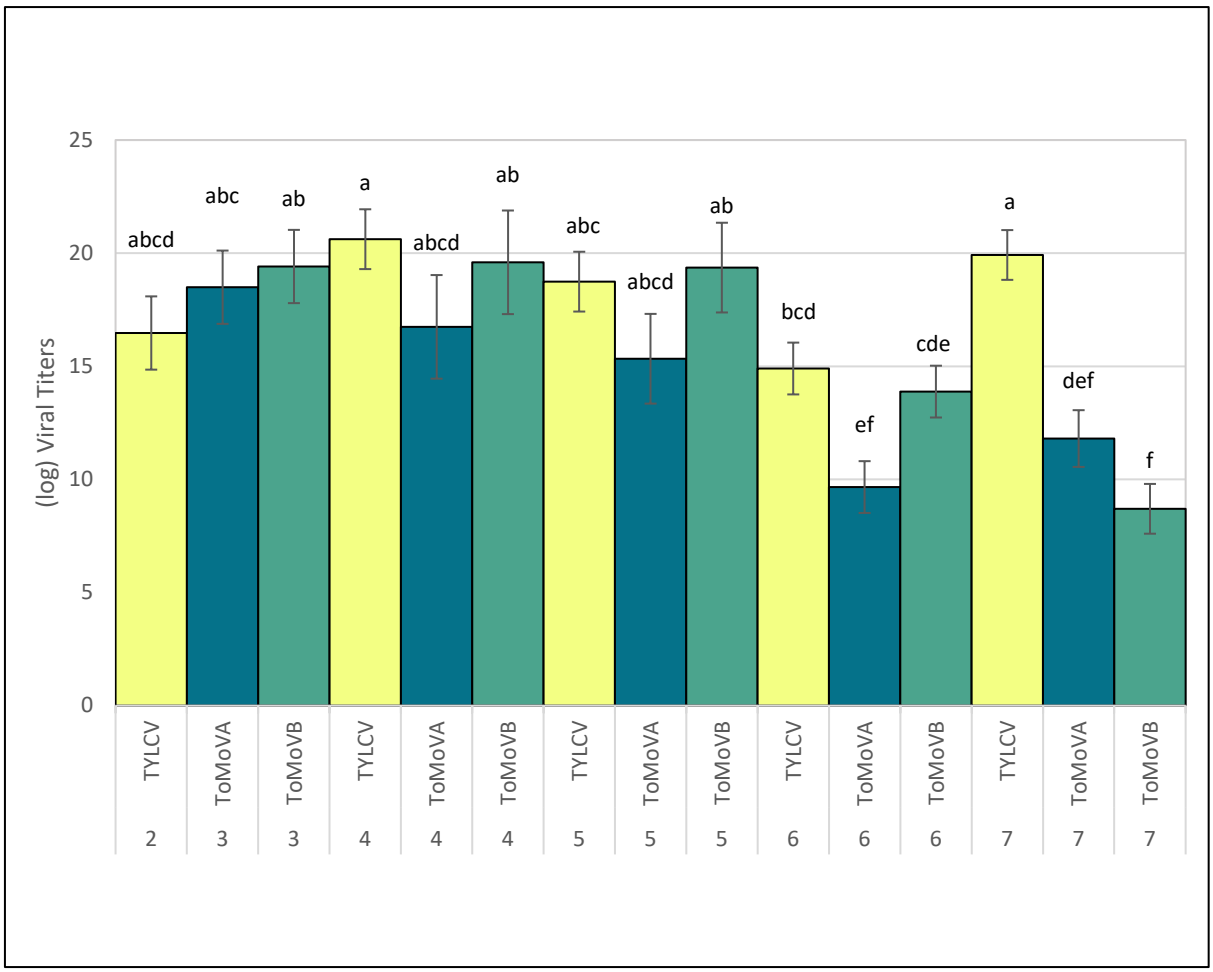

Figure 9: The average titer of *Tomato yellow leaf curl* (TYLCV, DNA-A) and *Tomato mottle virus* (ToMoV, DNA-A and DNA-B) by component and treatment in recipient plants 28 days post inoculation.Treatment 1 (Control, no positives, data not shown), Treatment 2 (Single infection TYLCV), Treatment 3 (Single infection ToMoV), Treatment 4 (Agro-inoculated mixed infection), Treatment 5 (Sequential acquisition of TYLCV first), Treatment 6 (Sequential acquisition of ToMoV first), Treatment 7 (Mixed whitefly cohort). LS means comparisons were conducted using Tukey's method at P=0.05.

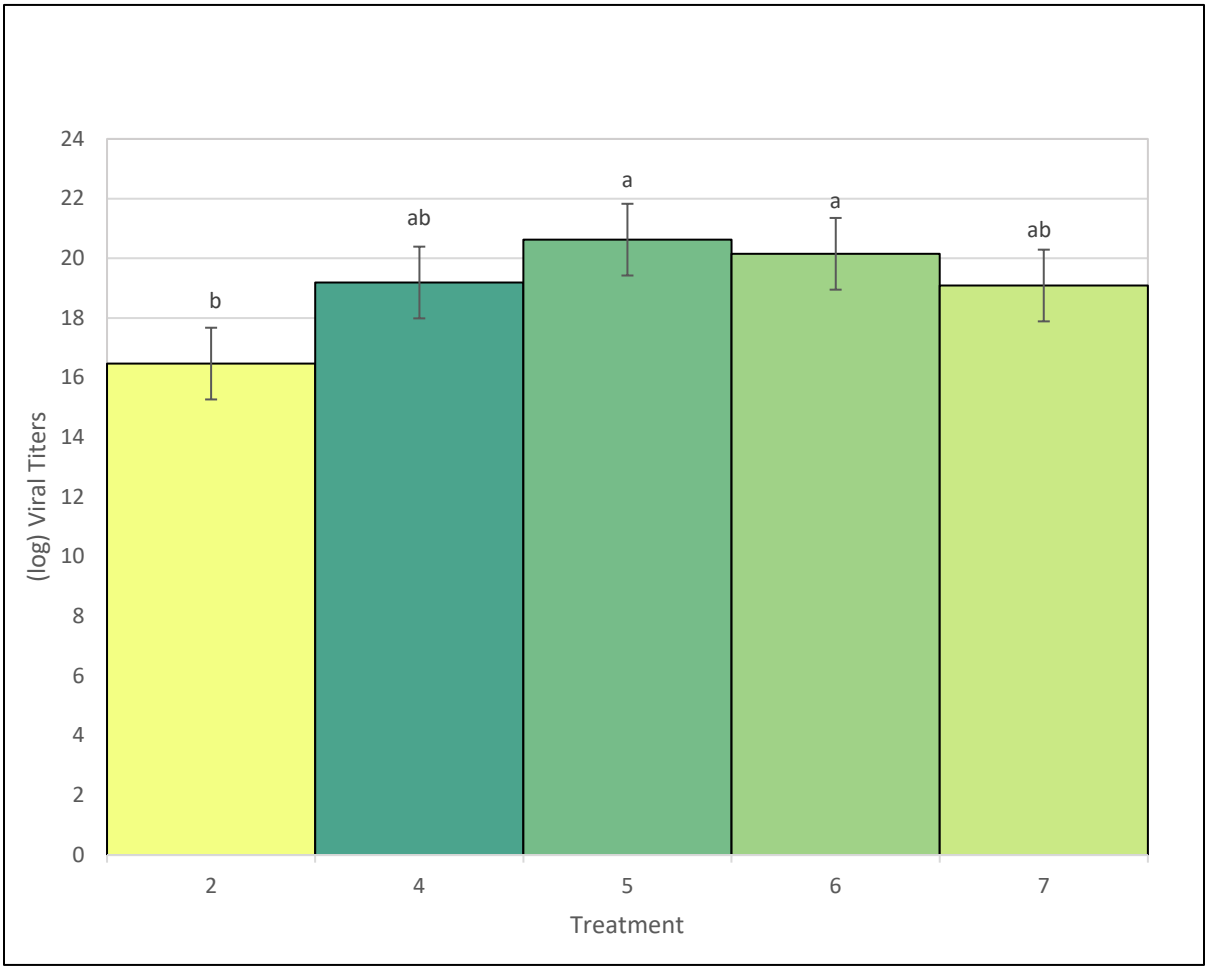

Figure 10: The average titers of *Tomato yellow leaf curl* (TYLCV) DNA quantified by treatment in recipient plants that only test positive for TYLCV 28 days post inoculation. Treatment 1 (Control, no positives, data not shown), Treatment 2 (Single infection TYLCV), Treatment 3 (Single infection ToMoV), Treatment 4 (Agro-inoculated mixed infection), Treatment 5 (Sequential acquisition of TYLCV first), Treatment 6 (Sequential acquisition of ToMoV first), Treatment 7 (Mixed whitefly cohort). LS means comparisons were conducted using Tukey's method at P=0.05.

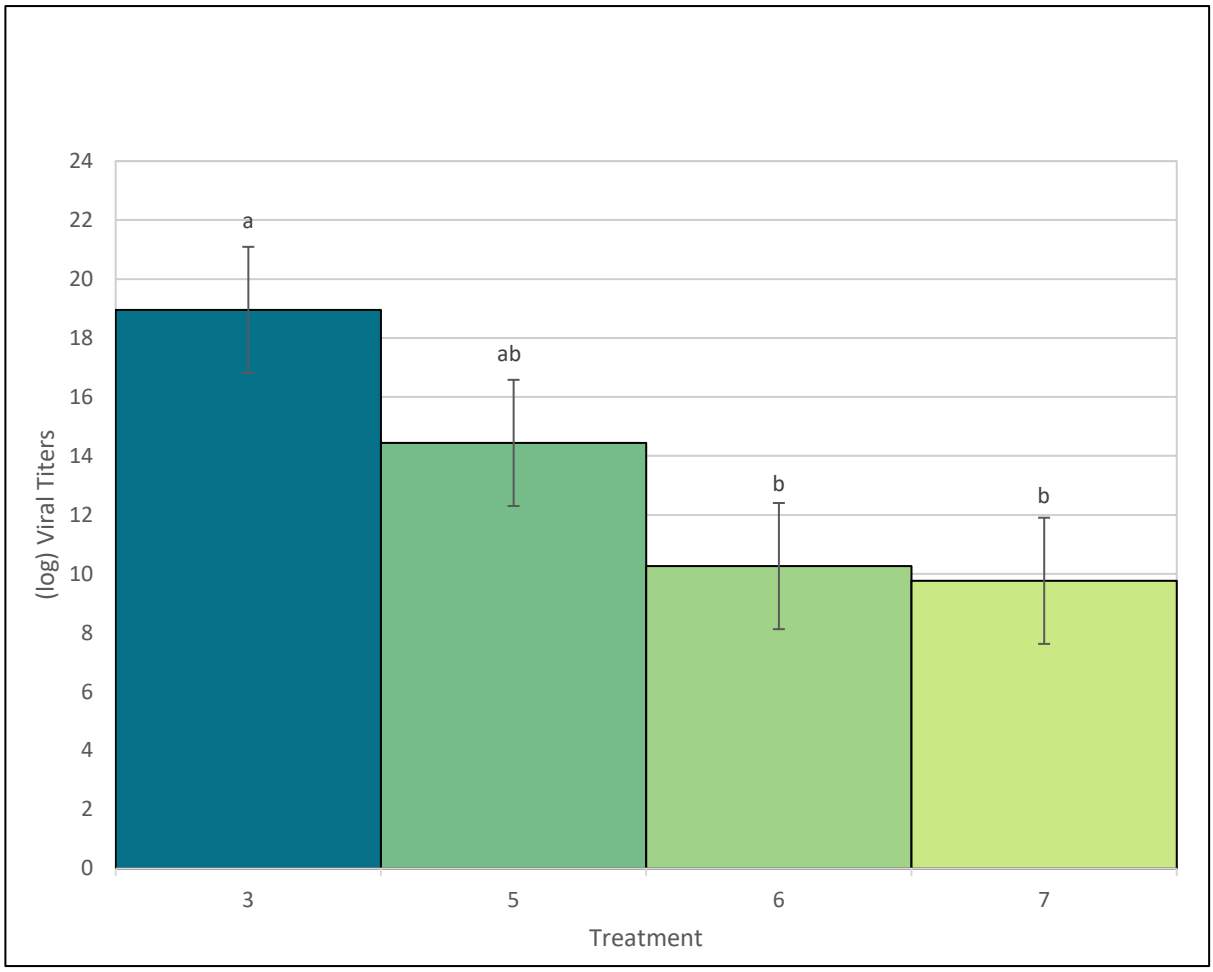

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Figure 11: The average titers of *Tomato mottle virus* (ToMoV) DNA quantified by treatments in recipient plants that only test positive for ToMoV 28 days post inoculation. Treatment 1 (Control, no positives, data not shown), Treatment 2 (Single infection TYLCV), Treatment 3 (Single infection ToMoV), Treatment 4 (Agro-inoculated mixed infection), Treatment 5 (Sequential acquisition of TYLCV first), Treatment 6 (Sequential acquisition of ToMoV first), Treatment 7 (Mixed whitefly cohort). LS means comparisons were conducted using Tukey's method at P=0.05.

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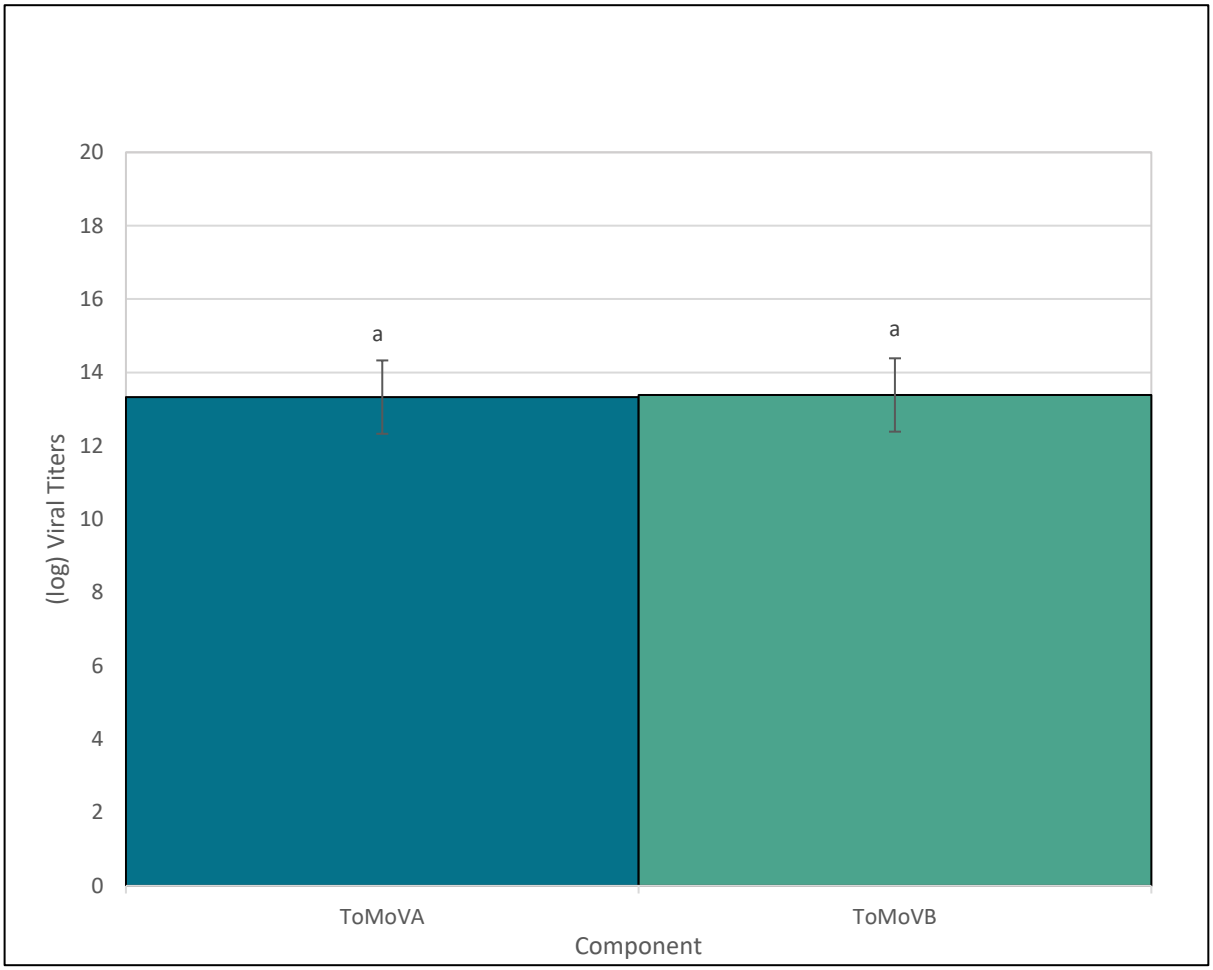

Figure 12: The average titer of *Tomato mottle virus* by DNA components in single infection recipient plants across all treatments. Blue (ToMoV DNA-A), Green (ToMoV DNA-B). LS means comparisons were conducted using Tukey's method at *P* =0.05 level.

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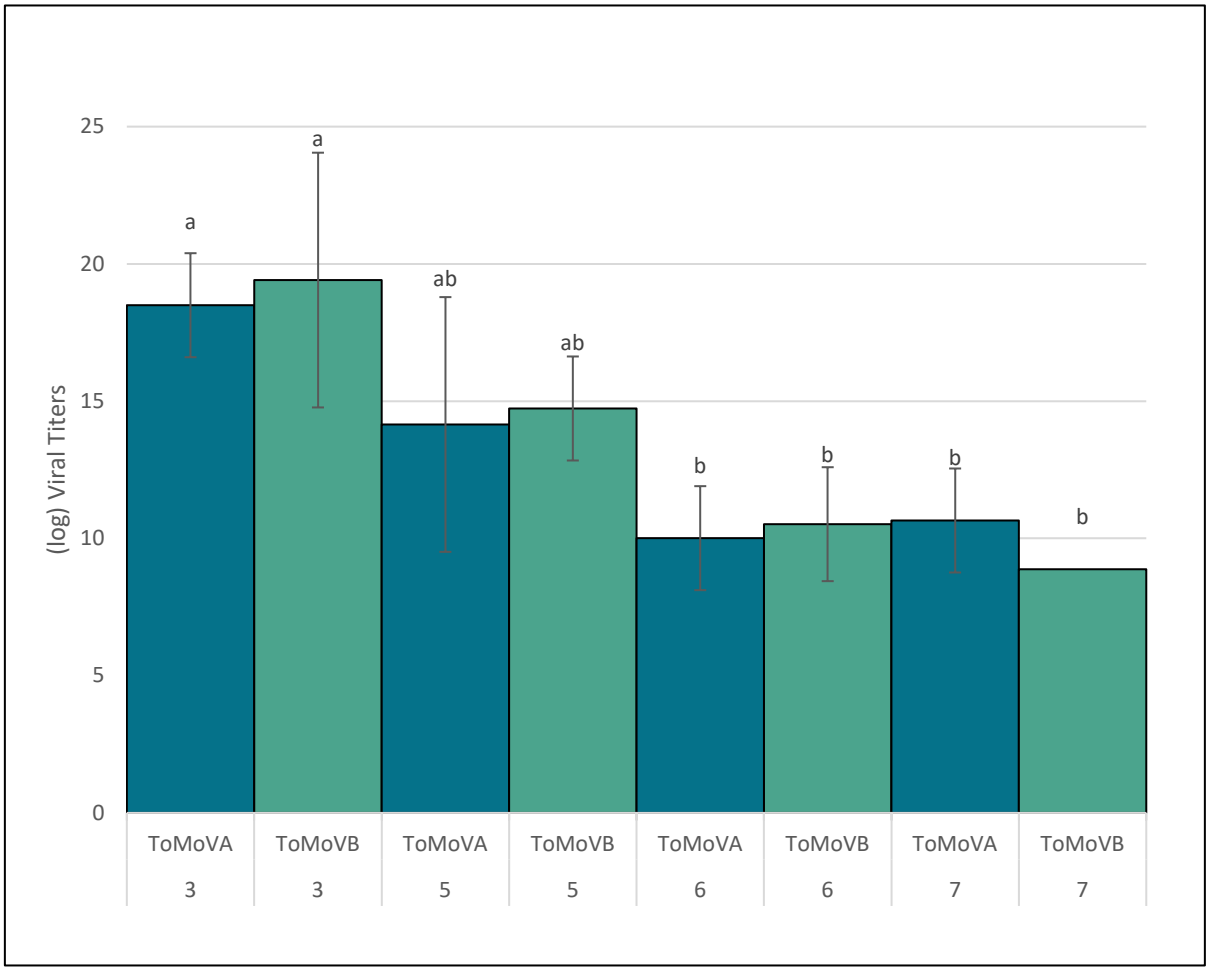

Figure 13: The average titer of *Tomato mottle virus* (ToMoV, DNA-A and DNA-B) by component and treatment in single infection recipient plants 28 days post inoculation. Treatment 1 (Control, no positives, data not shown), Treatment 2 (Single infection TYLCV), Treatment 3 (Single infection ToMoV), Treatment 4 (Agro-inoculated mixed infection), Treatment 5 (Sequential acquisition of TYLCV first), Treatment 6 (Sequential acquisition of ToMoV first), Treatment 7 (Mixed whitefly cohort). LS means comparisons were conducted using Tukey's method at P=0.05.

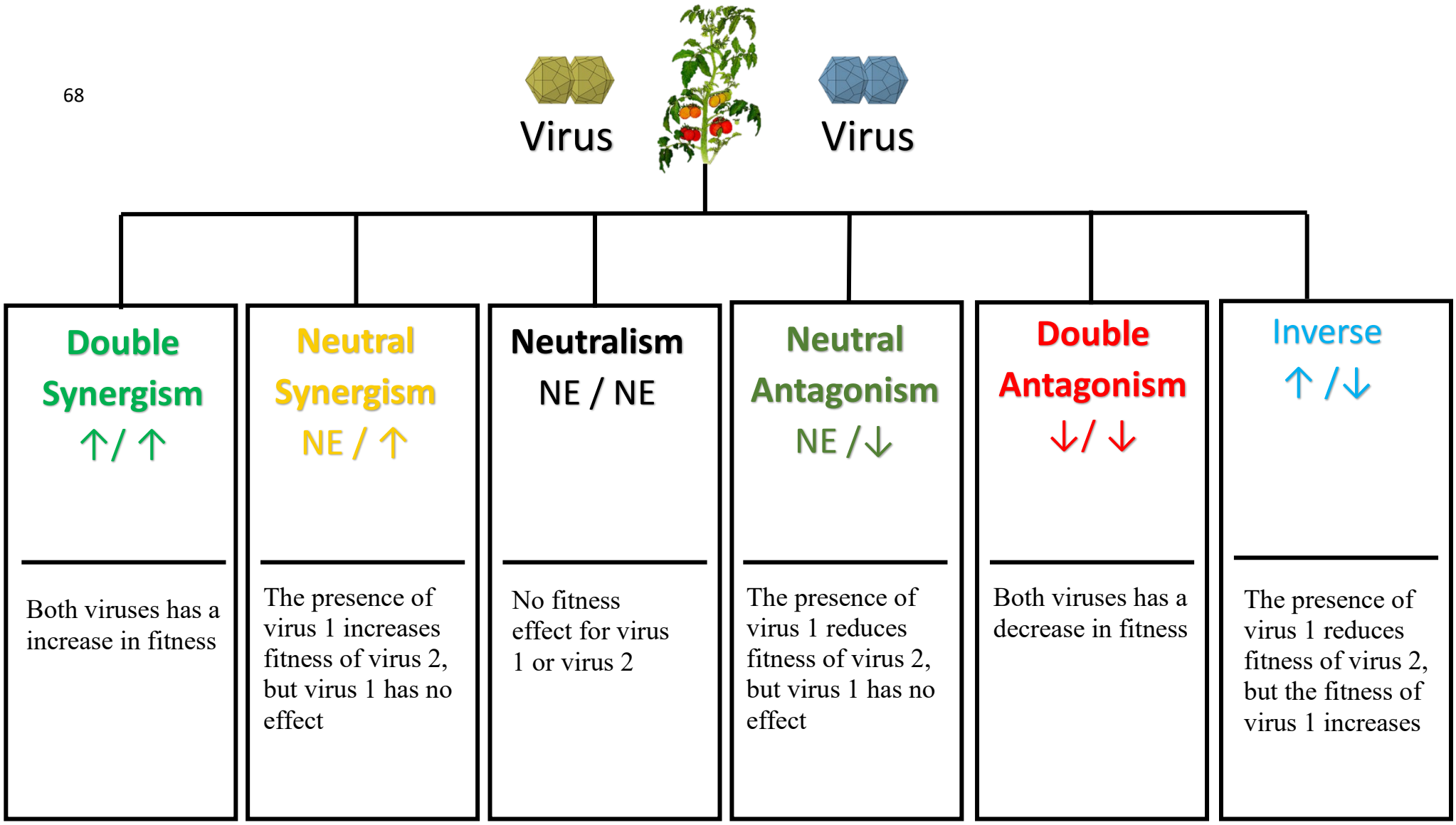

Figure 14: Possible neutral, synergistic, or antagonistic virus-virus interaction outcomes (modified from Alcaide et al, 2020): ↑ = Increase in fitness by increasing titer, ↓ = Decrease in fitness by decreasing titer, NE = No effect or no change in virus titers.

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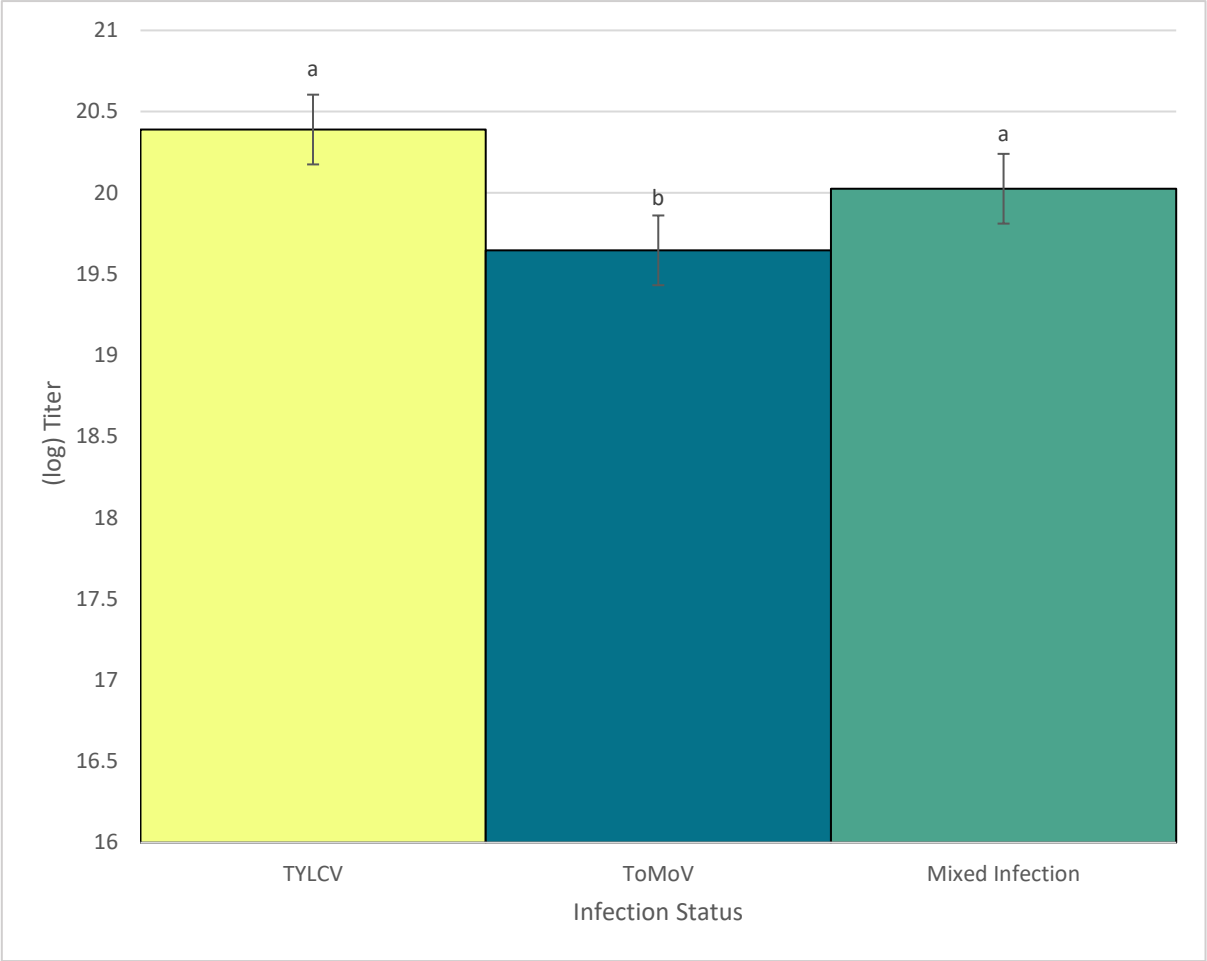

Figure 15: Average Titer of Source Plants (Supplemental Data) ($F_{2, 66} = 10.05$, P=0.0002).

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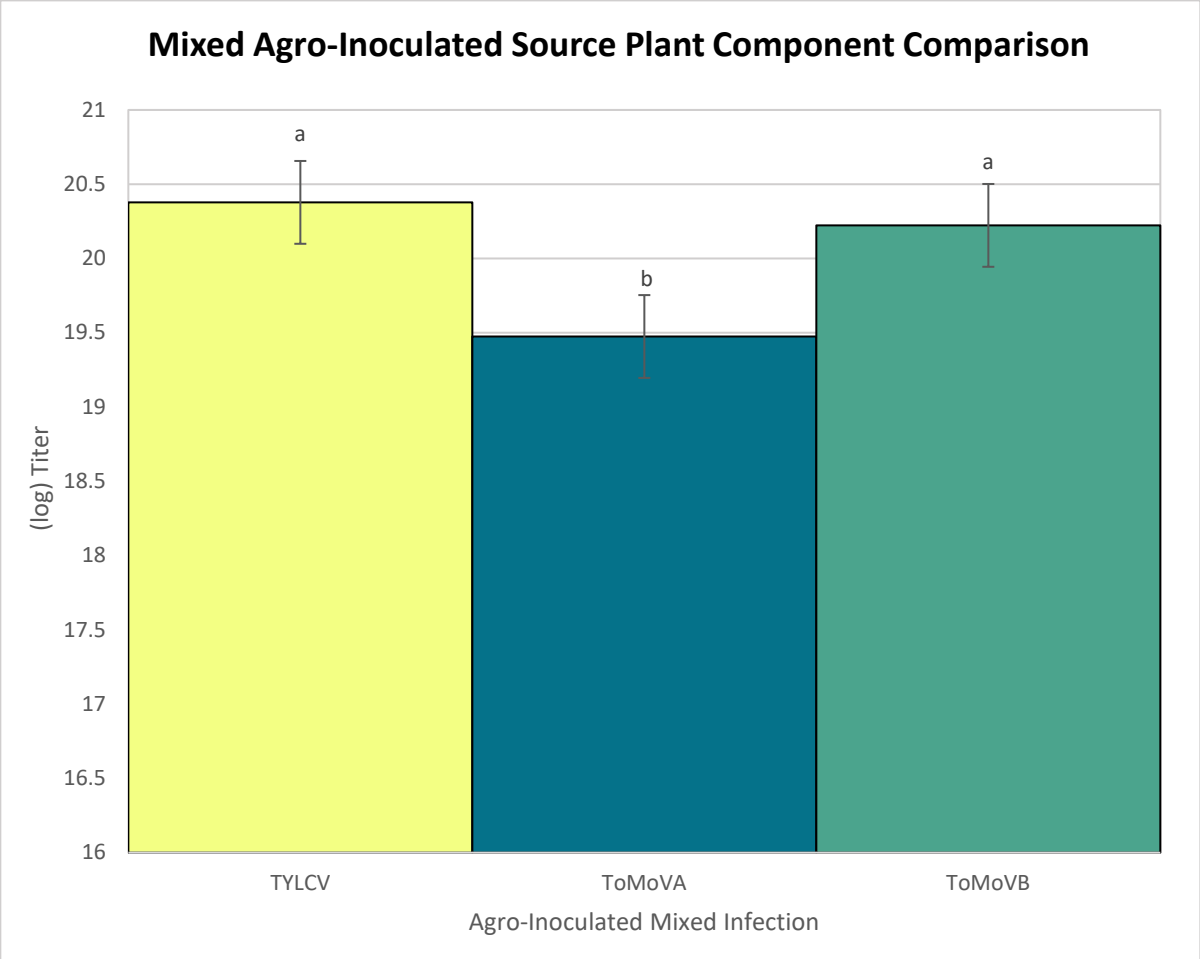

Figure 16: Average Titer of DNA Component Agro-Inoculated Source Plant (Supplemental Data) ($F_{2, 14} = 8.45$, P=0.0039).

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Figure 17: Sucrose Sachet – Artificial diet packet used to collect whitefly salvia

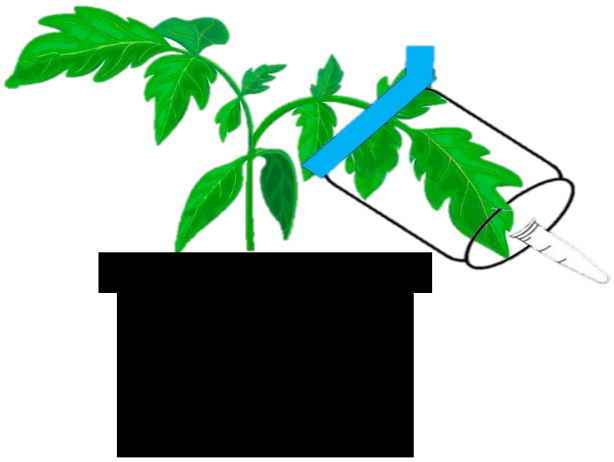

Figure 18: Clipcage used to confine whiteflies to a single leaf

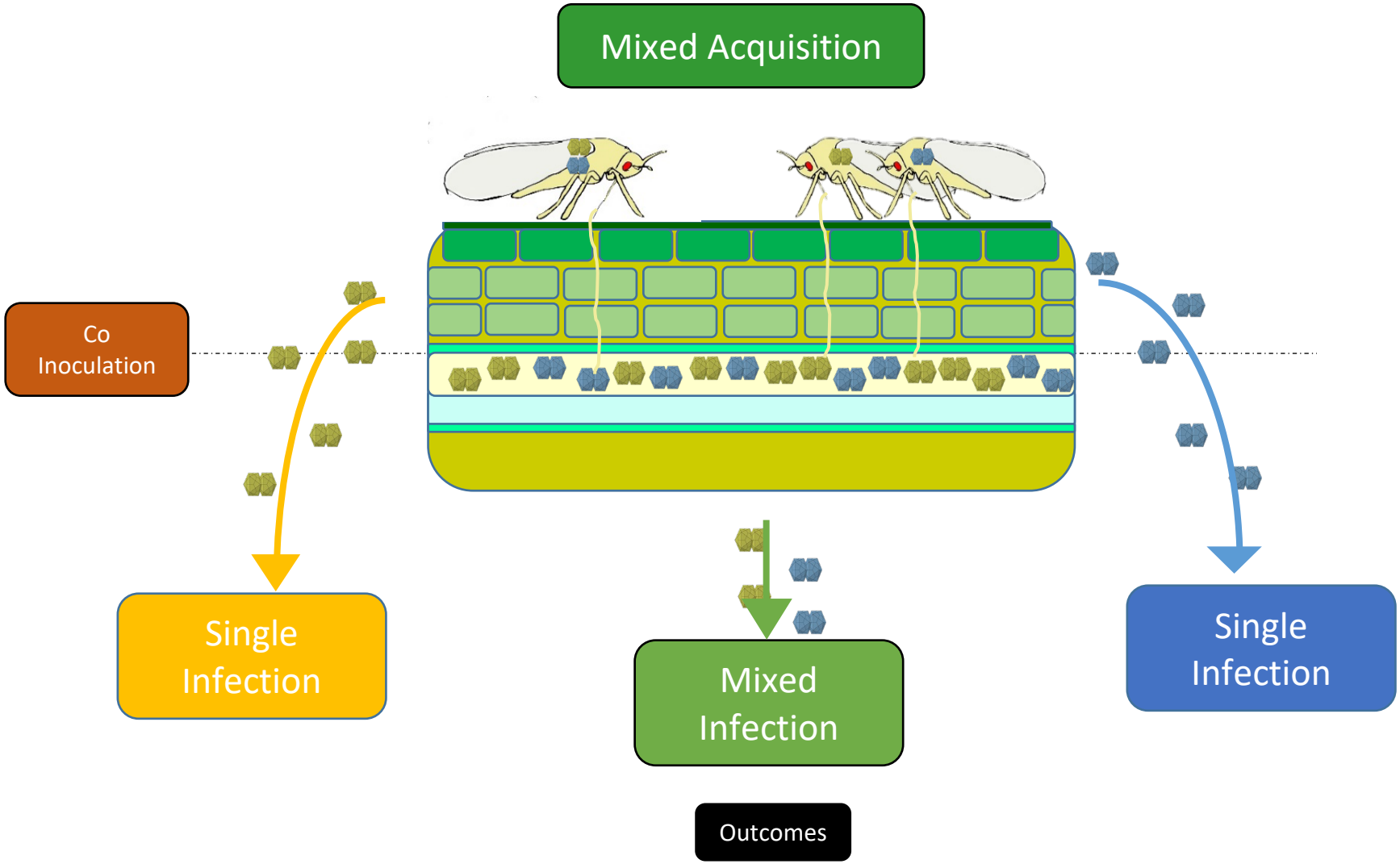

Figure 19: Acquisition of two viruses by a vector does not always lead to transmission of mixed infections; both single and mixed infection outcomes occur.