OCCURRENCE AND DISTRIBUTION OF VIRUSES ON WATERMELON AND CANTALOUPE AND HOST-RESPONSE TO WHITEFLY-TRANSMITTED VIRUSES ON SQUASH

by

ISMAILA ADEYEMI ADELEKE

(Under the Direction of SUDEEP BAG)

ABSTRACT

Viruses transmitted by whiteflies affect many agricultural crops and cause a global decline in food production. In fall production in the United States, viruses that infect cucurbits include the cucurbit chlorotic yellows virus (CCYV), cucurbit yellows stunting disorder virus (CYSDV), cucurbit leaf crumple virus (CuLCrV), and watermelon crinkle leaf associated virus 1 (WCLaV-1). Management of these viruses depends on early detection and understanding of the spread and biology of the virus. A survey was conducted in cantaloupe and watermelon-producing counties in Georgia and high throughput sequencing was used to study the prevalence of the viruses in spring crops. To develop a better management strategy, plant introduction lines were accessed for resistance to single infection of CCYV and CuLCrV under greenhouse conditions.

INDEX WORDS: Cucurbits, Viruses, Resistance, Watermelon, Cantaloupe, Squash, CCYV, CYSDV, CuLCrV, WCLaV-1, HTS, RT-PCR, Real-time PCR.

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DEDICATION

To my beloved wife (Dr. T.R. Adeleke, PharmD)

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CHAPTER 1

INTRODUCTION

Cucurbits are important vegetable crops cultivated worldwide. They are commonly grown either in a garden, greenhouse, or open field for human consumption and they serve as a good source of income for the growers and economic return for the state. However, several biotic and abiotic factors continuously challenge the production of cucurbits. Biotic factors include pathogenic organisms fungi, bacteria, nematodes, and viruses. In Georgia, most of the production is central to the southern part of the state and includes cantaloupe (*Cucumis melo* var. *cantalupensis* Naudin), cucumber (*Cucumis sativus* L.), honeydew (*Cucumis melo* L. (Inodorus Group) ‘Honey Dew’), muskmelon (*Cucumis melo*), pumpkin, yellow squash (*Cucurbita pepo* L.), watermelon (*Citrullus lanatus*), and zucchini (*Cucurbita pepo* L.) (Adeleke et al., 2022b).

In recent years, the production of cucurbits has experienced a drastic decline due to the high incidence of whitefly (*Bemisia tabaci*) (Hemiptera: Aleyrodidae) and the viruses transmitted by them. Whiteflies are important insect pests of crops responsible for global food losses through direct feedings on the crop and, most significantly, their ability to serve as a vector for numerous plant viruses, including genus begomovirus, crinivirus, and ipomovirus. (Brown et al., 1995, Henneberry and Castle 2001, Kavalappara et al., 2021b, Lagarrea et al., 2015, Legg et al., 2010, Schuster et al., 1995, Srinivasan et al., 2012). Whiteflies have a piercing and sucking mouth part for feeding on the plant (Bethke, 2016). During feeding, whitefly excretes sticky honeydew that accumulates on plants. This silvery accumulation can block sunlight thereby reducing the photosynthetic ability of the plant (Sparks et el., 2018).

Sweetpotato whiteflies have a wide crop and non-crop host range which enables them to survive year-round. In Georgia, their population is usually low on leafy vegetables during winter. During spring, cucurbits such as cantaloupe, squash, and watermelon are host crops to whiteflies. Although these crops are grown extensively, in most years, whiteflies populations might be mild and require less insecticide management. Most whitefly damages occur on fall-grown cucurbits in Georgia when there is usually a high population. In most years, this population emerged from cotton grown during summer. Cotton is known to support a high population of whiteflies which may require management with insecticide (Sparks et el., 2018).

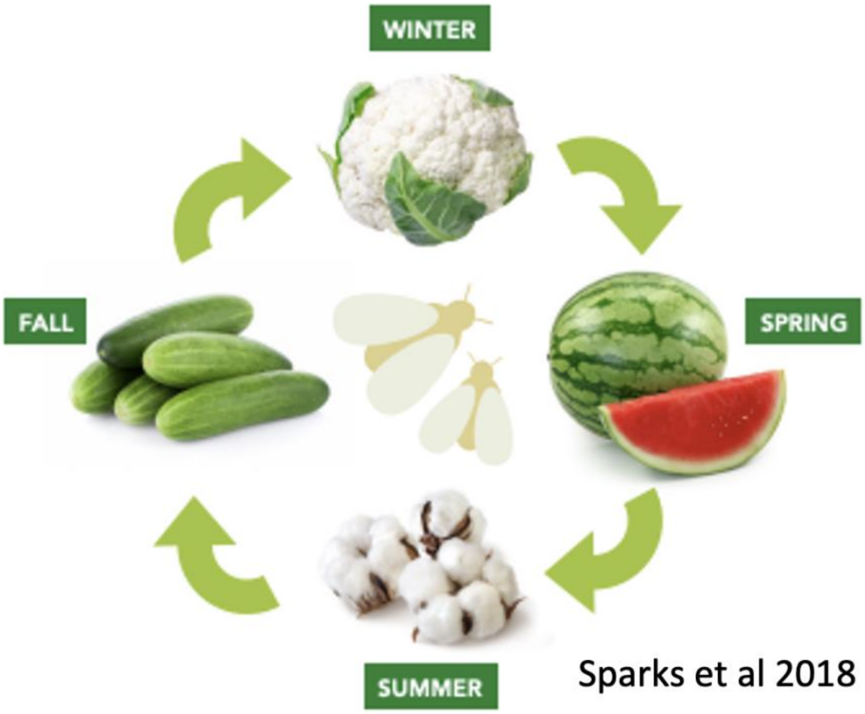

Figure 1.1: Silver leaf whitefly (SLWF) circle and survival in Georgia cropping systems all year round. They move from winter leafy vegetables to spring cucurbits, to cotton during summer, to fall cucurbits, and back to winter leafy vegetables (Sparks et al., 2018).

The spring-grown cucurbits contribute to the presence of whiteflies on the field year-round. Although crops like squash support lower populations, they are very sensitive to feeding which can result in foliar silver symptoms. These crops are also susceptible to many viruses transmitted by whiteflies (Kavalappara et al., 2021b, Sparks et al., 2018).

This phytophagous insect pest of crops is known to transmit more than 300 known plant pathogenic viruses (Navas-Castillo et al., 2011). They are particularly known as an agricultural notorious pest because of their ability to infest and cause tremendous damage to more than 1000 known species of plants across the world (Abd-Rabou et al., 2010).

The most prominent whitefly-transmitted viruses (WTVs) of cucurbits in Georgia include cucurbit chlorotic yellows virus (CCYV) (Kavalappara et al., 2021a), cucurbit yellow stunting disorder virus (CYSDV) (Gadhave et al., 2018), and cucurbit leaf crumple virus (CuLCrV) (Larsen, 2010). CCYV and CYSDV are single-stranded RNA viruses of the genus *Crinivirus* of the family *Cloisteroviridae* (Navas-Castillo et al., 2011). Members of this group are considered emerging threats to cucurbit production regions. CuLCrV belongs to the genus *Begomovirus* of the *Geminiviridae* family and is usually found as a mixed infection with criniviruses on the field thereby contributing to significant threats (Kavalappara et al., 2021b Adeleke et al., 2022b).

Another potential WTVs of importance is the squash vein yellowing virus (SqVYV) due to its presence in the neighboring state of Florida. The incidence of this virus was only reported once in 2011 by Webster and Adkins (2012). However, SqVYV has not been consistently detected in GA since then. *Criniviruses* are considered emerging viruses of cucurbits on a global scale while the genus *Begomovirus* has been known for decades to cause significant yield losses in dicotyledonous agricultural crops (Ndunguru et al., 2005, Varma and Malathi, 2003, Salaudeen et al., 2019).

Other than the whitefly-transmitted viruses of cucurbits, another virus of potential concern in Georgia is the newly identified watermelon crinkle leaf-associated virus 1 (WCLaV-1) in watermelon (Adeleke et al., 2022a). This virus belongs to the genus coguvirus (Zhang et al., 2021). It was first detected in watermelon in China (Xin et al., 2017) and later in 2021 in the United States (Hernandez et al., 2021). It has not been detected on any other cucurbit crops and the insect vector of this virus is still not known yet.

Furthermore, persistent viruses, such as cucumis melo endornavirus (CmEV), cucumis melo amalgavirus (CmAV-1), and cucumis melo cryptic virus (CmCV) infection are also common in cucurbits (Adeleke et al., 2022b). Although these viruses do not show any obvious disease symptoms on their host plant, their roles in other symptomatic viruses on cucurbit still need to be investigated. Many of these viruses have been detected on watermelon and cantaloupe within the United States with the aid of high throughput sequencing (HTS) (Adeleke et al., 2022b, Sabanadzovic et al., 2016, Villamor et al., 2019).

In addition to the phenotypic expressions of plant infected by viruses, different diagnostic assays are employed for the detection, identification, and characterization of known and novel viruses. These include electron microscopy, hybridization, enzyme-linked immunosorbent assay (ELISA), and the polymerase chain reaction (PCR). However, in recent years, with the advancement of molecular and sequencing technologies, the identification of plant pathogenic viruses has improved tremendously, and many novel viruses have been identified using high throughput sequencing (HTS) (Adams et al., 2012, Adeleke et al., 2022b, Charon et al., 2020, Hernandez et al., 2021, Li et., 2012, Sabanadzovic et al., 2016, Tomasechova et al., 2019, Villamor et al., 2019) thereby enhancing the decision for the strategic management.

Insect pests and pathogens pose major threats and constraints to cucurbit production and this menace requires attention to new and adaptive approaches to mitigate the economic impact in the southern part of the United States. Based on this, it is essential to understand the prevalence and distribution of these viruses on the field and develop a long-lasting strategic approach such as resistant varieties against viruses and vectors. To have better knowledge, my research objectives were designed to include surveying cantaloupe and watermelon fields as well as screening yellow squash plant introductions (PI Lines) for resistance to prevailing WTVs cucurbits in Georgia.

These objectives were to:

- Determine if spring-grown cantaloupe and watermelon are reservoirs of WTVs that cause damage in the fall, determine the diversity of viruses infecting spring-grown cucurbits, and assess their prevalence in Georgia through a survey of major cantaloupe and watermelon-producing counties.
- Evaluation of plant introduction lines of yellow squash *(Cucurbita pepo)* for resistance against cucurbit chlorotic yellows virus (CCYV) and cucurbit leaf crumple virus (CuLCrV).

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CHAPTER 2

LITERATURE SURVEY

This chapter discussed previous research works and their importance to the current and ongoing research on whitefly-transmitted viruses (WTVs) of cucurbits.

Production and Economic Importance of Cucurbits

The family Cucurbitaceae is one of the largest vegetable groups of valuable horticultural crops cultivated globally (FAOSTAT, 2021). Although a high number of minor cucurbits are largely produced in many parts of the world for basic food and sustenance, most cucurbits are found in the genera of watermelon (*Citrullus lannatus*), cucumber, and melons (*Cucumis sativus* and *Cucumis melo*); and zucchini, pumpkin, squash, and gourd (*Cucurbita* spp) (Rolnik and Ola, 2020). In the US, the estimated field production of all economically important cucurbits with other vegetables averaged 679 metric tons on 2.3 million hectares, contributing to an economic value of USD 12.8 billion (USDA NASS, 2021).

Among the cucurbit-producing states in the United States, Georgia ranked 4th based on production values (Table 2.1) with an economic contribution of about USD 322 million annually (Georgia Farm Gate Report 2021). Most of these economically important species are concentrated in southern Georgia (Adeleke et al., 2022b; Kavalappara et al., 2021b) with watermelon being the largest produced followed by cucumber and yellow squash (Fig 2.1 and Fig. 2.2).

Table 2.1. Top 10 Vegetable producing states in the United States (USDA's National Agriculture Statistics Service 2020).

State	Rank	Total vegetable production ($)
California	1	7,744,144,000
Arizona	2	1,628,351,000
Florida	3	1,222,850,000
Georgia	4	515,530,000
North Carolina	5	499,681,000
Washington	6	362,740,000
New York	7	226,905,000
Michigan	8	217,138,000
Oregon	9	194,586,000
Texas	10	184,896,000
United States		12,796,821,000

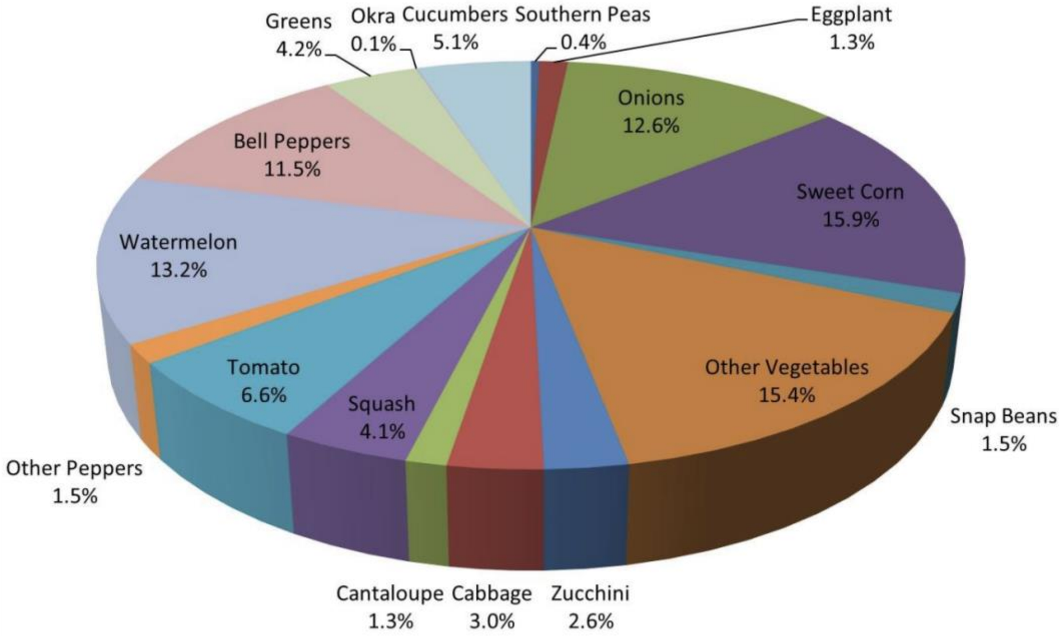

Figure 2.1. Georgia vegetable production value by crops (Georgia farm gate report 2021)

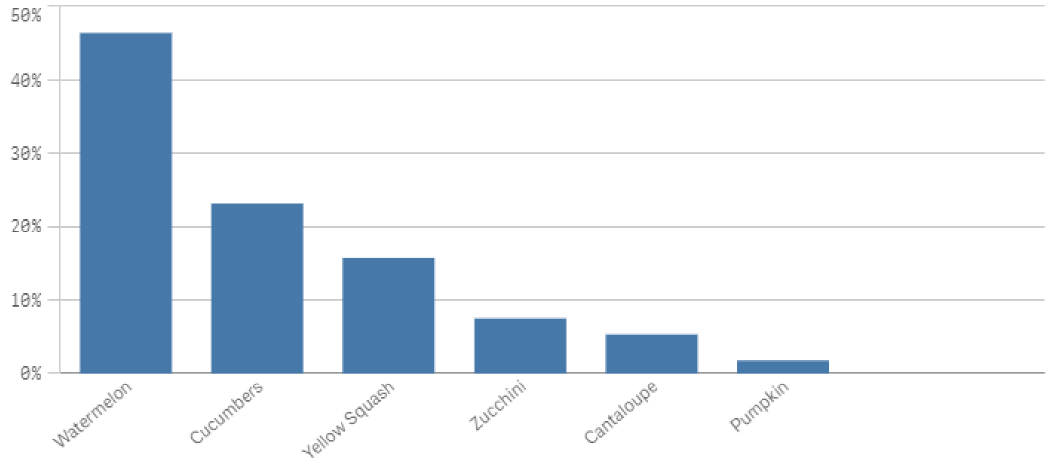

Figure 2.2. Percentage of cucurbit production by crops in Georgia (Georgia Farm gate report 2021).

Production Constraints and Economic Loss

In general, infections and damages caused by plant viruses are responsible for more than USD 30 billion in annual crop loss (Sastry and Zitter, 2014). Over the years, the production of cucurbits has been very challenging due to several abiotic and biotic factors. These biotic factors include fungi, bacteria, nematodes, and numerous plant viruses. In cucurbits, the majority of these viruses are transmitted by silverleaf whiteflies (SLWF) (Sparks et al., 2018). These viruses are usually a mixed infection of more than one virus of the same or different genera at the same time (Kavalappara et al., 2021b). Important whitefly-transmitted viruses (WTVs) of cucurbits include the cucurbit chlorotic yellows virus (CCYV), cucurbit yellow stunting disorder virus (CYSDV), cucurbit leaf crumple virus (CuLCrV), squash vein yellowing virus (SqVYV) among others (Adeleke et al., 2022b; Kavalappara et al., 2021b; Sparks et al., 2018). They are found in different plant virus groups such as the begomovirus, crinivirus, and ipomovirus.

Owing to the rapid geographical expansion and distribution of whitefly-transmitted viruses (WTVs) is the increase in whitefly population and availability of non-host crops for these viruses (Abrahamian and Abou-Jawdah 2014; Kavalappara et al., 2022b; Wintermantel et al.,2009).

Epidemiology of the Whitefly-Transmitted Viruses

These viruses are known to be introduced into new geographical areas through the movement and exchange of infected plant materials where the viruses were not previously found. However, plant-to-plant spread within the field and local spreads between fields can only occur upon the presence and the population of whiteflies when viruses are present. Adult viruliferous whitefly of the genus *Bemisia* and *Trialeurodes* can transmit these viruses in a persistent and semipersistent mode of virus transmission during feedings. The emergence of WTVs is considered one of the major threats to global cucurbits production, owing to the spontaneous establishment of their insect vectors leading to billions of dollars in losses annually. The majority of these viruses are often found together on the field as mixed infections, thereby contributing to more significant losses in vegetables, reduced sugar content production, and unmarketable cucurbit fruits (Tzanetakis et al., 2013). Many of these viruses are found in both the *Crinivirus* and *Begomovirus* genera.

The Genus Crinivirus

Criniviruses are semi-persistently transmitted plant viruses in the family closteroviridae by whiteflies (Navas-Castillo et al., 2011; Abrahamian and Abou-Jawdah, 2014). Members of this genus constitute one of the three members of the family with filamentous nonenveloped with either monopartite, bipartite, or tripartite positive-sense single-stranded RNA (ssRNA) viruses. The genome of bipartite such as the CCYV and CYSDV consist of RNA-1 and RNA-2 encoding replication gene and transmission gene as well as encapsidation and movement protein respectively (Navas-Castillo et al., 2011).

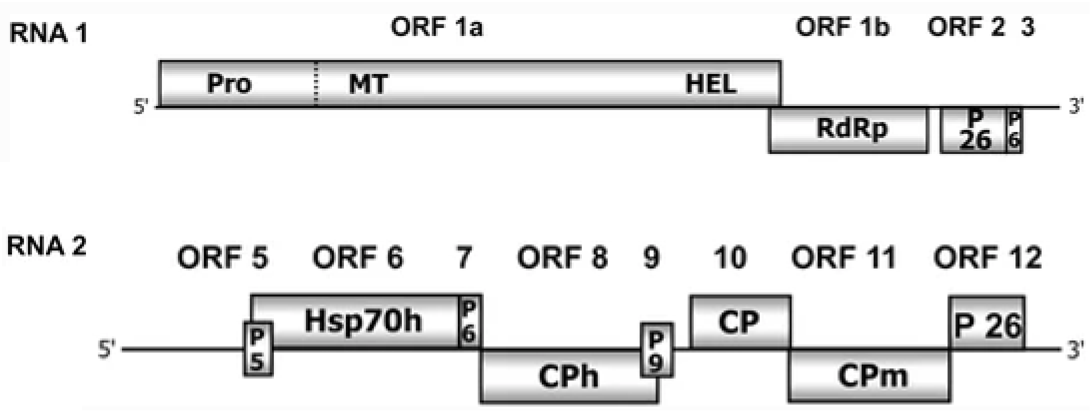

Figure 2.3. Genome organization of crinivirus (Martín et al., 2008)

They are considered newly emerging plant viruses posing a serious threat to cucurbit production in tropical and subtropical regions of the world due to the upsurge of the whiteflies population in production areas (Martelli et al., 2011; Navas-Castillo *et al.*, 2011). These viruses can also infect non-host crops, which makes them survive during the off-season of economically important crops (Abrahamian and Abou-Jawdah, 2014; Kavalappara et al., 2022b). The first member of this group to be identified was the beet pseudo-yellows virus (BPYV) identified in the 1960s (Duffus, 1965) on cucurbits.

Among the crinivirus, the cucurbit yellow stunting disorder virus (CYSDV) is one of the most widely distributed WTVs in cucurbit production regions worldwide. This virus was first described in the United Arab Emirates (Hassan and Duffus, 1991). Together with cucurbit chlorotic yellows virus (CCYV), they are considered a prevalent cucurbit disease throughout many tropical and subtropical production areas, including the Mediterranean basin and the Middle East, as well as China and North and Central America (Abou-Jawdah et al., 2000; Bellows et al., 1994; Gyoutoku et al., 2009; Kuo et al., 2007; Polston et al., 2008; Wisler et al., 1998).

Effective transmission and spread of the virus may be related to the upsurge increase in the population and distribution of whiteflies as well the continuous diversities in resistance to pesticides by invasive whiteflies. Favorable environmental conditions such as the temperature and abundance of susceptible host plants during the fall cropping season, non-crop hosts can also support the prevalence of the whiteflies and the viruses on the field (Sparks et al., 2018). Other members of this family include the lettuce chlorosis virus (LChV) (Duffus et al., 1996b), tomato chlorosis virus (ToCV) (Wisler et al., 1998b), and lettuce infectious yellows virus (LIYV) (Duffus and Flock, 1982). The LIYV is the most studied member of the genus crinivirus (Tzanetakis et al., 2013). The genome of crinivirus encodes specific proteins which enhance the members to efficiently establish infection, propagate within the host plant and cause disease.

Following the first report of CYSDV in the United Arab Emirates (Hassan and Duffus 19991), the epidemiology of the virus has been consistently reported throughout the Middle East (Abou-Jawdah et al., 2000) and Mediterranean Basin. In the United States, this virus was first reported in the New World by Kao et al 2000 in Texas in 1999 as well as in the Imperial Valley of California and adjacent Yuma on melons (*Cucumis melo*) (Kuo et al., 2007). It was also detected in watermelon (Citrullus lanatus) in Central Arizona and Sonora Mexico (Brown et al., 2007).

Furthermore, in the Southern United States, CYSDV was first reported on squash and watermelon in Florida and Georgia (Polston et al., 2008; Gadhave et al., 2018).

Cucurbit chlorotic yellows virus (CCYV) is another emerging WTVs of the genus crinivirus (Okuda et al., 2010). In 2009, CCYV was first reported on melon in association with cucurbit yellows disease in Japan where the name cucurbit chlorotic yellows virus was then proposed (Okuda et al., 2010). This was reported in Taiwan in 2010 by Huang et al when a large population of whiteflies was observed with diseased cucurbit crops. More so, several viruses have been detected in cucurbits from where cucurbits are being grown worldwide. These plants usually exhibit several symptoms and considerable crop loss associated with yield loss and declining economic returns. CCYV is exclusively transmitted in a semipersistent mode (Li et al., 2016) by Mediterranean (MED) and Middle East-Asia Minor 1 (MEAM 1) cryptic species of whitefly complex (Gyoutoku et al., 2009; Okuda et al., 2010).

The epidemiology of the virus through geographical expansion and several reports in verse majority of important cucurbit crops worldwide makes the virus an economically important pathogen and one of the major threats to cucurbit production. It was also reported on cucumber (Cucumis sativus), melon (Cucumis melo), and watermelon (Citrullus lanatus) from China with initial disease symptoms of chlorosis starting from the older and middle leaves followed by the development of chlorotic spots on the leaf lamina (Gu et al., 2011). The geographical expansion was followed by the report on cucumber in Lebanon (Abrahamian et al., 2012), muskmelon (Cucumis melo) and cucumber in Sudan (Hamed et al., 2011), and Zucchini (Cucurbita pepo) in Greece (Orfanidou et al., 2014).

The first report of CCYV in the United States was on melons from the new world (Wintermantel et al., 2019), followed by Georgia on yellow squash (Kavalappara et al., 2021a), Texas, Alabama, and the central valley of California (Hernandez et al., 2021; Mondal et al., 2021a; Mondal et al., 2021b; Jailani et al., 2021) and also recently in watermelon in Georgia (Adeleke et al., 2022b).

The Genus Begomovirus

Begomoviruses are the most important and largest group of viruses of the family *Geminiviridae* with genome sizes ranging from 2.6kb to 2.8kb depending on the species (Ho et al., 2014; Vivek et al., 2021). Members of this genus have circular single-stranded DNA (ssDNA) with either monopartite or bipartite genome organization (Fiallo-Olivé et al., 2021).

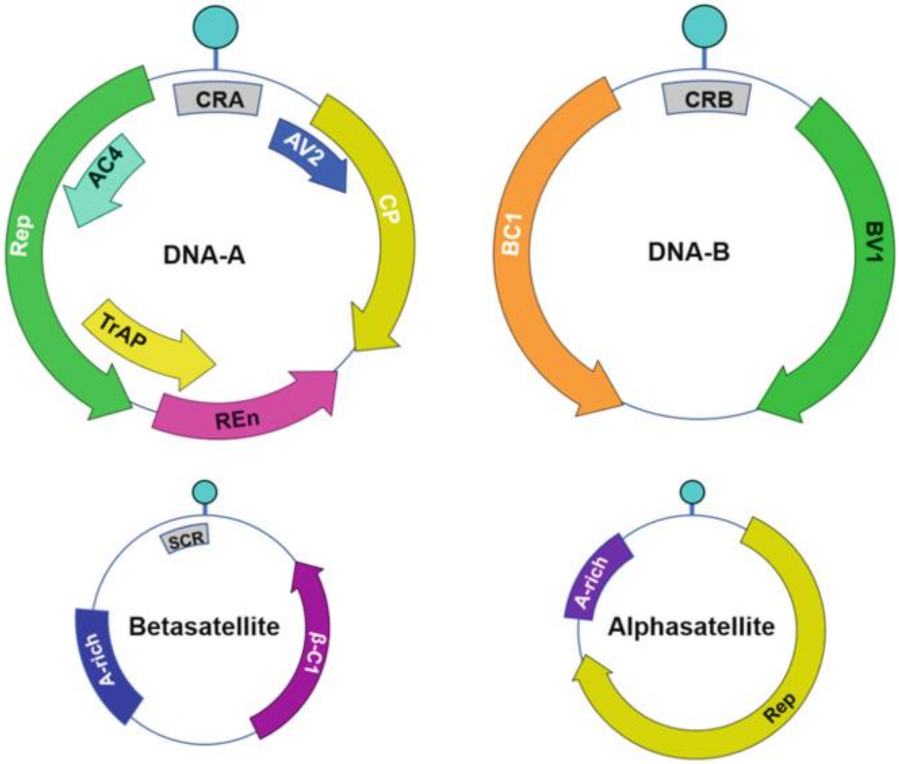

Figure 2.4. Genome organization of bipartite Begomovirus and its associated satellites like alphasatellite and betasatellite (Kumar and Chakraborty 2021).

These viruses have emerged as one of the major WTVs of cucurbits and many other agricultural crops causing a considerable economic setback to monocot and dicot plants in tropical and subtropical regions of the world (Brown, 1994; Brown et al., 2000; Seal et al., 2006). Symptoms of plants infected with members of this viruses include deformed and stunted growth, yellowing, curling, and deformed leaves, a mosaic pattern on the leaves, interveinal chlorosis, and yellow spots (Dasgupta et al., 2003; Legg et al., 2010; Kavalappara et el., 2021; Rishi, 2009). They are responsible for significant yield loss in cassava production in African countries (Hillock et al., 2002). The infection of the tomato yellow leaf curl virus (TYLCV) on tomato production in the United States of America is also significant and causes tremendous yield loss (Morales and Anderson, 2001). Tomato leaf curls New Delhi virus (ToLCNDV) is another important begomovirus infecting several *Solanaceae* and other vegetable crops in the Asia continent (Briddon, 2002; Moriones et al., 2017).

Begomovirus consists of the diverse and largest genus of the family *Gemiriviridae* (Fauquet et al., 2008; Zerbibi et al., 2017;) as a result of the worldwide cultivation of susceptible cultivars, rapid increase, and geographical expansion of polyphagous biotypes of whiteflies (Briddon, et al., 2010). More than 300 known species of this group have been identified to infect economically important crops (Brown, et al., 2015). Most begomoviruses that originated from the New World have a bipartite genome with DNA-A and DNA-B components, whereas those from the Old World have either bipartite or monopartite genomes (Brown, et al., 2002). This Old World begomovirus is usually associated with satellite viruses (Alphasatellites, Betasatellites, and Deltasatellites), usually for their replication and ability to cause symptoms in an infected host plant (Gnanasekaran, et al., 2019; Fiallo-Olivé, et al., 2021; Li et al., 2018; Zhou et al., 2013). This can also be distinguished based on phylogenetics (Polston and Anderson, 1997). The genome of DNA-A found in both monopartite and bipartite are similar both in genomic structures and organization of virus-encoded proteins (Harrison and Robinson, 1999; Zhao et al., 2022).

In the United States, the first reported whitefly-transmitted begomovirus squash leaf curl virus (SLCV) of cucurbit was in the early 1980s from the Imperial Valley of California. This was found on a squash (Cucurbita pepo) plant exhibiting curling of leaves accompanied by stunted growth (Cohen et al., 1983; Flock and Meyhew, 1981). Following the geographical expansion of the virus, it was found infecting watermelon in Grand Valley Texas (Isakeit et al., 1994)

In 1998, chlorotic symptoms, leaf curl, and crumpling on the leaves were observed on watermelon volunteers from the Imperial Valley of California, Polymerase Chain Reaction (PCR) and subsequent phylogenetic analysis revealed the presence of cucurbit leaf crumple virus (CuLCrV) (Guzman et al., 2000). Following its initial identification, it has been detected in many cucurbit-producing areas within the United States (Adkins et al 2011; Keinath et al., 2018) CuLCrV is a bipartite ssDNA virus transmitted in a persistent manner by whiteflies. Its first report in the southeastern US was from a commercial watermelon field in Florida (Polston et al., 2008). Recently, it was detected in a mixed infection with CCYV and CYSDV (Adeleke et al., 2022b; Kavalappara et al., 2021b) in cucurbits.

Symptom Expression and Susceptible Plants

Symptoms identification of WTVs of cucurbit is often difficult if not impossible due to several factors such as the mixed infection of two or more viruses on the field, and mimics of nutrient deficiency: a typical yellowing appearance of the plant infected with either single or mixed infection of CCYV and CYSDV. Often time, mixed infections of these viruses are common in the field and this phenomenon constitutes and subjects the plant to more severe stress and damage than a single virus infection. Usually, the criniviruses are often associated with chlorotic spots at the early stage of infection which will later progress to complete yellowing of the leaves (Fig 2.5).

Single infection of CuLCrV symptoms is often identified with curling as well as yellowing of the leaves (Fig 2.5). However, the identification of viruses on infected plants based on foliar symptoms is not enough to draw a conclusion about the infecting virus. Symptom expression of CCYV differs from plant to plant and stages of infection among the cucurbits. On an infected cucumber crop, the older leaves appeared brittle with interveinal chlorotic spots, while younger leaves are also chlorotic with yellowing between the veins (Salem et al., 2020).

A plant infected with both CCYV and CYSDV shows initial characteristics of chlorotic spot symptoms from the lower leaves. As the symptom of CYSDV progresses, the plant becomes stunted, and the leaves turn completely yellow. Depending on the crop, the leaves of the plant mimic water stress and show a discolored patch between veins. This patch may later turn into bright yellow interveinal chlorosis. Small green spots may develop on the leaves of certain cucurbit types. Expressed symptoms differ from one virus to another and from plant to plant and the age of the plant. Time of infection also plays a significant role in symptom prevalence in the field (Adkins et al., 2011; Ghadhave et al., 2012; Kavalappara et al., 2021b; Polston et al., 2008)

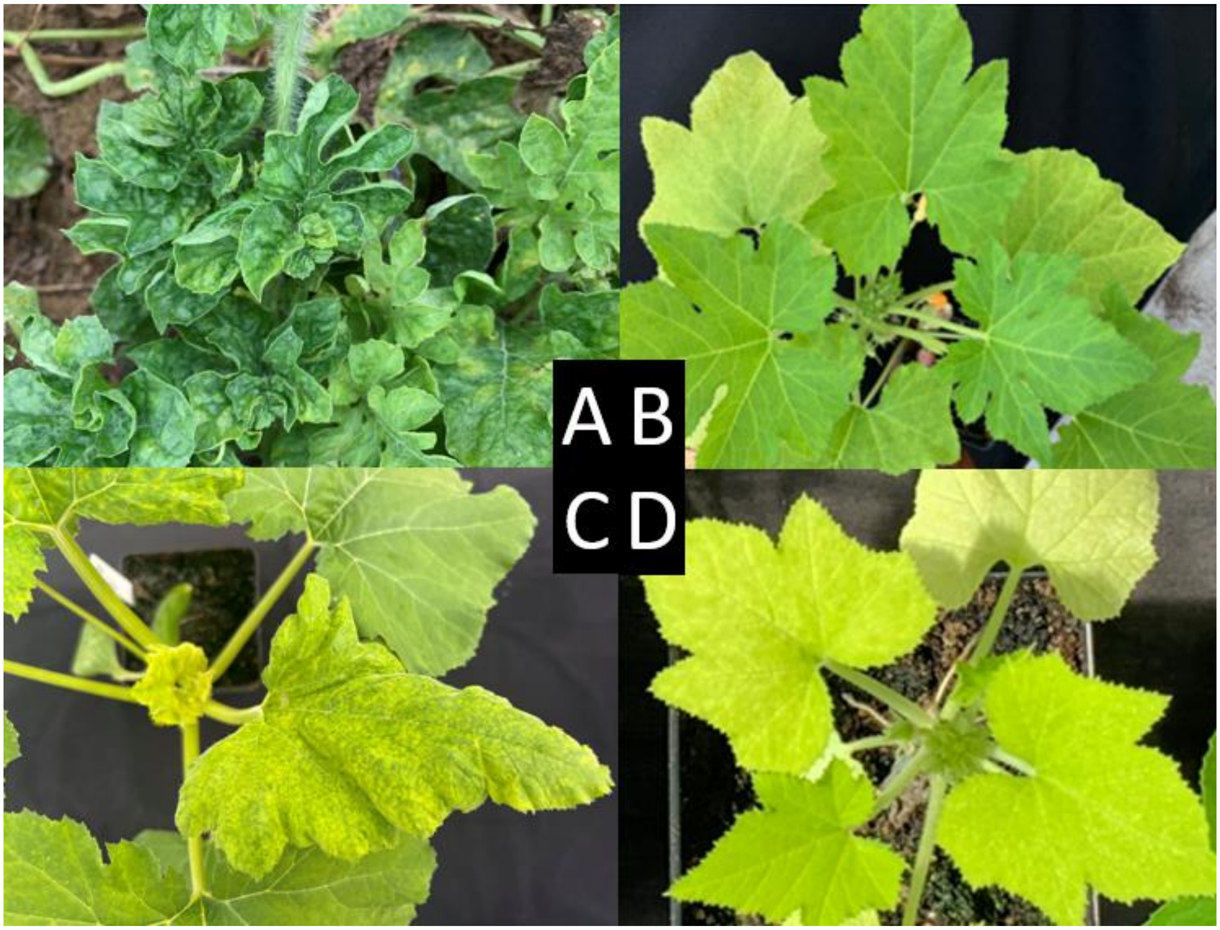

Figure 2.5. Symptoms expression of cucurbit viruses. A) watermelon crinkle leaf associated virus 1 (WCLaV-1) on watermelon, B) cucurbit yellow stunting disorder virus (CYSDV), C) cucurbit leaf crumple virus (CuLCrV), D) cucurbit chlorotic yellows virus (CCYV) on yellow squash.

Leaf crumpling and curling of younger leaves are prominent symptoms associated with the cucurbit leaf crumple virus (CuLCrV). A plant infected by this virus often shows symptoms of small irregular patches on the leaf, in the early stages of infection, the plant exhibits interveinal chlorosis, which is progressively followed by the downward curling and crumpling of the leaf. Symptom of CuLCrV starts from the younger leaves and progresses toward the older leaves. The leaf shows irregular patches of yellow and green discoloration, which can subsequently turn chlorotic and eventually died (Fig 2.5 C). Prominently, the leaves become crumpled, which is a sign of CuLCrV infection (Gilbertson et al., 2017). As the symptoms progress with time, infected leaves show characteristic symptoms, including twisted, thick, crumpled, and yellowed leaves (Rodríguez-Negrete et al., 2021).

In the family Cucurbitaceae, squashes and pumpkins are more susceptible to leaf crumple infection than other members. Plants show distorted and thickened leaves with light green to yellowing coloration. Yellow squash (*Cucurbita pepo*) leaves are curled and crumpled with rounded edges appearance (Gilbertson et al., 2017). Although zucchini also shows a similar appearance with exception of a rounded leaf margin (Gilbertson et al., 2017). Muskmelons (*Cucumis melo*) exhibit crumpled leaves and the appearance of downward curling of the leaves. Watermelon (*Citrullus lanatus*) infected plant develops initial characteristics of stunted symptoms with crumpled and yellowing leaves. In general, CuLCrV-infected plant shows deformed leaves, rugosity, and mild mottling (Webb et al., 2007).

Transmission of Whitefly-Transmitted Viruses

Most plant-infecting viruses need a vector for effective transmission due to the immobility of the plants and their strong cell wall layers. The most common group of this insect vector is the hemipterans among which whiteflies are classified due to their piercing and sucking mouthparts nature (Nault, 1997; Mitchell, 2004). The most predominant and damaging worldwide species among whiteflies are the biotype cryptic species (B cryptic species) (Brown et al., 1995, Brown, 2007). These viruses are transmitted in different ways depending on the interaction between the host plant, vector, and the virus itself. The transmission can either be semi-persistent (days) or persistent (lifetime of the insect) mode of virus transmission. This mode of virus transmission is categorized based on the period the virus remains within the vector (Hohn, 2007; Hongenhout et al., 2008; Ng and Falk, 2006; Li et al., 2016). Therefore, understanding the transmission mechanisms of these viruses is important for the development of effective and adaptive strategic management approaches.

CCYV and CYSDV belong to the genus crinivirus (Okuda et al., 2010), viruses of this genus are said to be transmitted exclusively by whitefly complex in a semipersistent mode of plant virus transmission (Celix et al., 1996). They are retained in the foregut of the insect, unlike the nonpersistent viruses which are retained in the stylet (Chen et al., 2011; Ng and Falk, 2006; Ng, 2013). However, the transmission and incidence impact of these viruses depends on several interactive factors such as the host range of the virus, specie of the whitefly, crops on the adjacent fields of infected field, non-crop hosts, climatic conditions as well the virus itself (Celix et al., 1996; Kavalappara et al., 2022b). The effective acquisition and transmission of crinivirus are different on the type of virus and the specie of the insect vector. (Li et al., 2016; Wintermantel et al., 2017).

CuLCrV is a member of the genus begomovirus. The genus comprises emerging and economically important insect-transmitted viruses that are transmitted in a persistent mode by whitefly (King et al., 2012; Ran et al., 2015). During feeding on an infected plant, the insect acquires the virus from the plant phloem using its stylet for the acquisition period which may last an hour depending on the virus (Bird et al., 1970; Brown, 1994; Mehta et al., 1994). However, the frequency of begomovirus transmission by the vector increases when the feeding period increases (Brown and Czosnek, 2002).

Following the acquisition, translocation within the insect occurs through the midgut to the hemolymph and then to the salivary gland (Ghanim et al., 2001).

Diagnosis of Plant Viruses

The success of virus disease management depends upon diagnostics methods and early detection of the virus. The traditional method of virus diagnosis involved the use of hybridization, electron microscopy, enzyme-linked immunosorbent assay (ELISA), and polymerase chain reaction (PCR) using virus-specific primers/probes, reagents, and antibodies.

PCR is one of the widely used and key elements of nucleic acid-based diagnostic technique in plant virology, this tool employs the use of multiple and stepwise temperatures with polymerase enzyme to target and amplify a strand of DNA or cDNA transcribed from RNA (Bustin, 2000; Mullis et al., 1986). The use of PCR and reverse transcription-PCR are both generally used for the detection of DNA and RNA viruses which are then run on agarose gel electrophoresis for visualization (Burkhalter and Savage, 2017; Liu et al., 2018; Lin et al., 2020; Wadhwa et al., 2017). The gel electrophoresis works on the principle of electricity to separate the DNA bands base on the fragment sizes (Motohashi, 2019). This might give a semi-result due to the use of virus-specific reagents or the assay might fail if the samples contain a very low concentration of the targeted virus (Lin et al., 2020; Motohashi, 2019).

Another nucleic acid-based assay is the real-time quantitative polymerase chain reaction (qPCR). This uses specific DNA dyes such as the SYBR Green that binds to all the double-stranded DNA across the reaction to facilitate virus detection (Navarro et al., 2015). The use of real-time qPCR has high specificity compared with the conventional PCR, but this is more expensive due to the initial cost of the machine, sample preparation, which is usually done with commercially available isolation kits as well as the cost of reagents (Clark et al., 2016). This is rapid and efficient for testing multiple samples at a time (Eigner et al., 2019; Ou et al., 2020). Among others, loop-mediated isothermal amplification (LAMP), recombinant polymerase amplification (RPA), and rolling circle amplification (RCA) are also used for diagnosing plant viruses (Notomi et al., 2000; Mohsen and Kool, 2016; Piepenburg et al., 2006). The method of RCA is effective for detecting circular DNA viruses such as the CuLCrV in a sample (Kavalappara et al., 2021b).

Rapid and portable diagnosis usually on the field is also possible with the use of an immunoassay-based viral diagnostics assay that primarily uses antibodies for the detection (Ma and O'Kennedy, 2015). Unlike the traditional techniques described, the use of high throughput sequencing is very promising in the field of virology due to the fact that multiple viruses can be detected more rapidly with no prior knowledge of the viruses. This stand-alone diagnostics assay can simultaneously detect both the DNA and RNA viruses in each sample and differentiate detected viruses from the host plant genome (Adams et al., 2009). It is effective in the diagnosis of novel and emerging viruses and very effective for the surveillance of virus infections (Adeleke et al., 2022b; Coleman and Sigler, 2020; Duarte et al., 2020; Eiras et al., 2018; Rott et al., 2018).

In HTS application for virus detection, several RNA templates such as the small RNA (sRNA), double-stranded RNA (dsRNA), and total plant RNA can be used. However, among all these, the use of sRNAs has been proven to be the most promising for identification and detection due to the ability to identify both the DNA and RNA viruses (Hagen et al., 2011; Kreuze et al., 2009; Nanarro et al., 2009). Furthermore, these sRNAs are produced abundantly within the host plant which also plays crucial roles in gene expressions (Li et al., 2012).

Management of Whitefly and the Whitefly-transmitted Viruses

About 1500 species of whiteflies are found in approximately 126 genera (Martin et al., 2004). Out of these, only species from about 5 genera which include the *Bemisia* (Priesner &

Hosny), *Bemisia tabaci* complex, *Parabemisia myricae* Kuwana (bayberry whitefly), *Trialeurodes abutilonea* Haldeman (they are the banded winged type of whiteflies), and *Trialeurodes vaporariorum* Westwood (greenhouse whitefly) (Gamarra et al., 2010, Hogenhout et al., 2008, Navas-Castillo et al., 2011, Ng and Falk, 2006) are known to transmit important plant viruses. Plant viruses are prevalent pathogenic organism that depends on the host for their replication mechanisms. The majority of these viruses are transmitted by insects such as whiteflies, therefore knowledge of the vector is very important and crucial to the management of the disease caused by them. Since there are no post-infection chemicals for the control of viruses, the management focused more on controlling the insect with insecticide, proper quarantine methods, and screening for disease-resistant varieties where available.

Although often time insect management may not be the best due to invasive species and resistance against insecticide by the insect vectors. Research on plant viruses focused more on disease management of the crop aspect. However, comprehensive information and basic knowledge are required for the virus's natural ecosystem to better understand the ecology, virus-virus, vector-virus interaction, and virus-host interaction (Kamitani et al., 2016). The management of whiteflies and the viruses transmitted by them is very challenging due to the insect's broad host range and relatively short generation time (Soumia et al., 2021). Currently, in Georgia, there is no single management approach effective for the management of whiteflies and the viruses transmitted by them. However, current management focused on the integrated management approach which includes the existing cultural and chemical methods (LaTora et al., 2022).

Although whiteflies are usually managed by the multiple application of insecticides of a broad spectrum, consistent use may lead to increased resistance. However, their applications play significant roles in reducing whitefly numbers and the spread of viruses on the field where resistant crop varieties are not available (Lapidot et al., 2014). Their application should be monitored with proper timing for the whiteflies population through the field scout and insecticides with different modes of action should be rotated to prevent whiteflies from developing resistance against them. Neonicotinoids are an important soil-drenched and sprayed systemic insecticide class. They are effective in managing whitefly-transmitted viruses due to readily available to feeding whiteflies on the field through systemic mobility within the plant after the application (Lapidot et al., 2014, Mineau and Palmer, 2013).

Whiteflies and their largely transmitted viruses can also be managed by cultural management approaches that have to do with the planting and the manner in which the crop is managed throughout the crop life cycle on the field. These with other control strategies form an efficient integrated management system (Stansly and Natwick, 2010). In Georgia, the use of plastic (Polyethylene) mulch to cover the soil has been proven effective for the natural production of cucurbits on the field. This approach is either used to attract the whitefly to the mulch instead of the crop or reflect lights and discourage whiteflies from landing on the crop based on the plastic mulch color. The use of yellow mulch attracts whiteflies to itself instead of the host plant while the white or aluminum reflective mulch discourages the whiteflies from landing on the crop (Cohen and Lapidot, 2007; Simmons et al., 2010; Polston and Lapidot, 2007). This also added advantages to the field in form of weed control strategies in place of herbicide application (Ngouajio and Ernest, 2004). The use of reflective mulch has been successfully used to reduce the whitefly population and the spread of the viruses transmitted by them within the field. However, this must be done at the early stage of planting.

Whiteflies transmitted viruses can also be managed with the use of virus-free vegetative plant parts, planting the new crop in an isolated or virus-free area from the older and infected host plant, (Clark et al., 2012; Ellsworth & Martinez-Carrillo, 2001). A significant reduction in the abundance of whiteflies can also be achieved by trap crops depending on the crop of interest. In the southeastern US, squash plants are more attracted to whiteflies than tomato plants, therefore, planting squash close to a tomato field might reduce the number of whiteflies and the spread of whitefly-transmitted viruses in tomato (Schuster, 2004). In subsistence agriculture, intercropping cucurbits with Corn, Okra, Cowpea, and Sunhemp can help lower the prevalence of whiteflies and the viruses transmitted by them (Adb-Robou and Simmons, 2012; Manandhar et al., 2009).

Cultural methods such as the proper photosanitation may be effective for controlling the initial inoculum of the virus on the field. Some of these viruses can infect noncrop hosts during the off-season of economically important crops and serve as a source of initial inoculum during the early stages of the season (Kavalappara et al., 2022b; Wintermantel, et al., 2009). WTVs such as the CYSDV and CuLCrV can also infect lettuce (Lactuca sativa) and snap bean (*P. vulgaris*) (Wintermantel et al., 2014).

Overall, the knowledge and understanding of the reservoir hosts and the whitefly life cycle as well as these viruses are very significant toward reducing sources of viruses in the field and preventing the spread of the viruses. The management of insect vector and viruses are more effective when an approach is combined with other practices for integrated pest and disease management.

Resistance Against Whitefly and Whitefly-Transmitted Viruses of Cucurbits

Among all, the use of genetically viral-resistant crop varieties for the management of whiteflies and the whitefly-transmitted viruses of cucurbits are considered the most effective management approach where available. This approach unlike the use of chemicals is safe from polluting the environment. Resistant crop varieties may be stable and long-lasting, and it addresses the issue of resistant development to insecticides by the whiteflies. However, the breeding and screening for the resistant varieties of crops are usually difficult due to the breeding timelines and identification of resistance loci which are usually found in the wild relatives (Lapidot et al., 2014).

Similar to the use of resistant crop varieties for managing the whitefly-transmitted viruses, the whitefly itself may also be potentially managed by the use of crops that are resistant to whitefly feedings. This can be achieved using plant secondary metabolites to prevent insects from settling and feeding on the host plant. Some of these metabolites may also prevent the insects from laying eggs on the host plant thereby preventing and reducing the available insect population for the virus's transmission and spread on the field (Bleeker et al., 2011; Mutschler and Wintermantel, 2006; Nombela and Muniz, 2010).

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CHAPTER 3

PERSISTENT AND ASYMPTOMATIC VIRAL INFECTIONS AND WHITEFLY-TRANSMITTED VIRUSES IMPACTING CANTALOUPE AND WATERMELON IN GEORGIA, USA

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Adeleke, I.A.; Kavalappara, S.R.; McGregor, C.; Srinivasan, R.; Bag, S. (2022). Persistent, and Asymptomatic Viral Infections and Whitefly-Transmitted Viruses Impacting Cantaloupe and Watermelon in Georgia, USA. *Viruses*, *14*, 1310; (reprinted here with the permission of the publisher).

Adeleke, I.A.; Kavalappara, S.R.; Torrance, T.; Bennett, J.E.; McGregor, C.; Srinivasan, R.; Bag, S. (2022). First report of watermelon crinkle leaf-associated virus 1 naturally infecting watermelon (*Citrullus lanatus*) in Georgia, USA. *Plant Dis.* **(**reprinted here with the permission of the publisher).

Abstract:

Cucurbits in the Southeastern USA have experienced a drastic decline in production over the years because of economically important viruses, mainly those transmitted by the sweet potato whitefly (*Bemisia tabaci* Gennadius). In cucurbits, these viruses can be found as a single or mixed infection, thereby causing significant yield loss. During the spring of 2021, surveys were conducted to evaluate the incidence and distribution of viruses infecting cantaloupe (*n* = 80) and watermelon (*n* = 245) in Georgia. Symptomatic foliar tissues were collected from six counties and sRNA libraries were constructed from seven symptomatic samples. High throughput sequencing (HTS) analysis revealed the presence of three different new RNA viruses in Georgia: cucumis melo endornavirus (CmEV), cucumis melo amalgavirus (CmAV1), and cucumis melo cryptic virus (CmCV). Reverse transcription-polymerase chain reaction (RT-PCR) analysis revealed the presence of CmEV and CmAV1 in 25% and 43% of the total samples tested, respectively. CmCV was not detected using RT-PCR. Watermelon crinkle leaf-associated virus 1 (WCLaV-1), recently reported in GA, was detected in 28% of the samples tested. Furthermore, RT-PCR and PCR analysis of 43 symptomatic leaf tissues collected from the fall-grown watermelon in 2019 revealed the presence of cucurbit chlorotic yellows virus (CCYV), cucurbit yellow stunting disorder virus (CYSDV), and cucurbit leaf crumple virus (CuLCrV) at 73%, 2%, and 81%, respectively. This finding broadens our knowledge of the prevalence of viruses in melons in the fall and spring, as well as the geographical expansion of the WCLaV-1 in GA, USA.

INTRODUCTION AND LITERATURE REVIEW

Cantaloupe and watermelon are economically important crops in Georgia; they are cultivated on an estimated average of 2400 acres, contributing 193 million USD to the state economy in 2019 (Georgia Farmgate Value 2019). They are grown extensively during the spring and summer seasons on open fields. Cantaloupe (*Cucumis melo* var. cantalupensis Naudin) and watermelon (*Citrullus lanatus*) are commercially produced and are concentrated in the southern part of the state. Other cucurbits produced in the state include cucumber (*Cucumis sativus* L.), honeydew (*Cucumis melo* L. (Inodorus Group) 'Honey Dew'), muskmelon (*Cucumis melo*), pumpkin, yellow squash, and zucchini (*Cucurbita pepo* L.).

In recent years, cucurbit production in Georgia has incurred severe annual losses due to increased whitefly (*Bemisia tabaci* Gennadius) populations and the incidence of viruses transmitted by them. Three whitefly-transmitted viruses (WTVs), the cucurbit leaf crumple virus (CuLCrV) (Genus *Begomovirus*, family Geminiviridae) (Larsen, 2010), cucurbit yellow stunting disorder virus (CYSDV) (Gadhave et al., 2018), and cucurbit chlorotic yellows virus (CCYV) (Genus *Crinivirus*, family Closteroviridae) (Kavalappara et al., 2021a) have been reported on major cucurbits, including cantaloupe, cucumber, squash, watermelon, and zucchini in Georgia [Gadhave et al., 2018, Kavalappara et al., 2021a, Kavalappara et al., 2021b). Recently, the presence of watermelon crinkle leaf-associated virus 1 (WCLaV-1) was reported in watermelons in Georgia (Adeleke et al., 2022). Furthermore, whitefly-transmitted squash vein yellowing virus (SqVYV) (Baker et al., 2008) and watermelon crinkle leaf-associated virus 2 (WCLaV-2) (Hernandez et al., 2021, Hendricks et al., 2021) are a concern to watermelon production in the state due to their recent reports in the neighboring state of Florida.

Although much of the damage due to whitefly-transmitted viruses (WTVs) is inflicted on fall-grown cucurbits, their incidence is assumed to build up slowly over spring and summer, with an increasing whitefly population on fall-grown cucurbits. Furthermore, the majority of the WTVs of cucurbits previously identified in Georgia, USA were on fall-grown vegetables (Kavalappara et al 2021b).

The objectives of this study were:

1) To determine if spring-grown cantaloupe and watermelon are a reservoir of WTVs that cause damage in the fall.
2) To determine the diversity of viruses infecting spring-grown cucurbits and assess their prevalence in Georgia.

Viruses present in the watermelon and cantaloupe samples were identified by high throughput sequencing (HTS) of small (s) RNAs which enable simultaneous screening and detection of known and novel viruses (Kavalappara et al., 2021b). This technique employs the use of RNA interference (RNAi), a natural antivirus defense mechanism presents in the host cell, to generate small RNAs that are 21–24 nt long for the analysis of the viral population present in a tested plant sample (Mlotshwa et al., 2008). The technique has become an important tool and has been used widely in the diagnosis, identification, and characterization of viruses in recent years (Kavalappara et al., 2021b, Boonham et al., 2014, Bag et al., 2015, Roossinck et al., 2015, Pooggin 2018, Massart et al., 2019, Fontenele et al., 2020, Fowkes et al., 2021, Kwibuka et al., 2021, Pecman et al., 2017).

MATERIALS AND METHODS

Survey Route and Sample Collection

In spring 2021, an extensive survey was conducted on open fields in Colquitt, Crisp, Tift, Turner, Wilcox, and Worth counties (Figure 1), which represent the major cantaloupe and watermelon-producing areas of the state. On symptomatic watermelons, 60 foliar tissues each were collected from both Crisp and Wilcox counties, 40 samples each were collected from both Turner and Tift counties, while 25 and 20 samples were collected from Colquitt and Worth counties, respectively. Forty symptomatic cantaloupe samples were collected from each of Turner and Tift counties. A total of 245 watermelons and 80 cantaloupes were collected to be processed by PCR and reverse transcription-polymerase chain reaction (RT-PCR). In addition, seven samples representing the symptoms observed on the field were also collected, surface sterilized three times with distilled water, and frozen on dry ice for HTS. Samples were transported on ice to the Crop Virology Laboratory, University of Georgia, Tifton Campus, and stored at -80 ◦C for further laboratory assays. In addition to this, 43 symptomatic leaf tissues of the watermelon collected in the fall of 2019 in Colquitt County were also tested for the presence of viruses detected by HTS.

RNA Extraction and HTS of Small RNA

Leaf tissues from one cantaloupe and six watermelon samples representing the distinct symptoms (Figure 2) observed in the field were macerated in liquid nitrogen. Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions. RNA integrity (RIN) was analyzed using a Qubit 4.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and samples with RIN values above 8.0 were sent to Beijing Genomic Institute (BGI, San Jose, CA, USA) on dry ice for HTS of sRNA. A DNA Nanoball (DNB) small RNA sequencing platform was used for the construction of small RNA libraries with a single end read of 1 × 50 bp (BGI, Hong Kong, China).

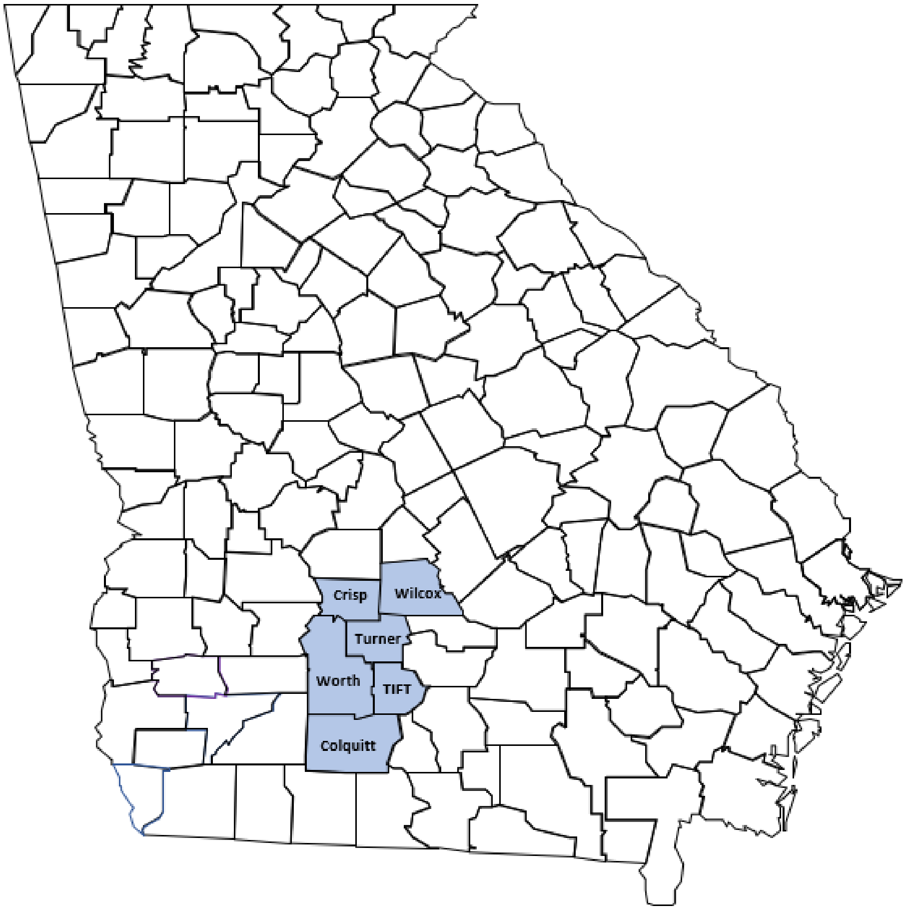

Figure 3.1: Map of Georgia showing counties from where samples were collected during the spring 2021 survey.

Analysis of HTS Data

Analysis of small RNA to identify the virome community in the samples were performed with CLC Genomics Workbench 21 (Qiagen, Redwood City, CA, USA). Sequences with low-quality adapters and those having more than two ambiguous nucleotides were removed. Reads with 18–30 nucleotides in length were filtered and analyzed. de novo assembly of the contigs (minimum 50 nt) was carried out using published parameters (mapping mode-create simple contig sequences(fast), mismatch cost-2, insertion cost-3, deletion cost-3, alignment mode-local, minimum contig length-50) (Pecman et al., 2017)

A local virus database was created from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/genome/viruses) (downloaded on 6 January 2021) using the Create Database feature of CLC Genomics Workbench 21. Contigs were compared against all sequences in the database for possible similarities using BLASTn (Altschul et al., 1990) with default settings in CLC Genomics Workbench 21. Contigs that mapped to non-plant virus sequences were not considered for further analysis.

Total Nucleic Acid Extraction and Virus Detection by PCR and RT-PCR

Total nucleic acid (TNA) isolation was done using 100 mg of symptomatic leaf tissue with magnetic bead mill technology which allowed simultaneous processing and preparation of a large number of samples for the detection of viruses. Samples were grinded using 4 M guanidine thiocyanate (GTC) buffer (pH 5.0) with Bead Mill 24 Homogenizer (Thermo Fisher Scientific, Waltham, MA, USA). TNA was isolated from homogenized samples with MagMAX 96 viral RNA kit using the KingFisher Flex Purification System (Thermo Fisher Scientific, Waltham, MA, USA) with the manufacturer’s instructions without the DNase treatment. More specific details of the RNA isolation protocol have been described earlier (Kavalappara et al., 2021b).

The quantity and purity of isolated TNA were assessed using a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Samples were stored at 80 °C for further laboratory analysis, pending the results from HTS. Complementary DNA (cDNA) was synthesized using 1 µL of 10 µM random primers denatured with 10 µL TNA at 70 °C for 5 min. This was chilled at 4 °C. Subsequently, the reverse transcription master mix was prepared using 8 µL of reverse transcriptase buffer, 2 µL of 100 mM DTT, 4 µL of 10 mM dNTPs mix, 2 µL Superscript III (200 U/µL) (Invitrogen, Carlsbad, CA, USA), 1 µL RNase out (50 U/µL), and 3 µL RNase free water with a final volume of 20 µL.

PCR reaction was done using 5 µL of 5X GoTaq green buffer with $MgCl_2$, 0.5 µL dNTPs (10 mM), 0.5 µL each of forward and reverse primers (10 µM), 16.25 µL of RNase-free water, 0.25 µL GoTaq polymerase (5 U/µL) (Promega, Madison, WI, USA), and 2 µL cDNA or TNA, amounting to a final volume of 25 µL. PCR was performed with an initial denaturation at 95 ◦C for 2 min, followed by 35 cycles each of 95 ◦C for 30 s, annealing for 30 s, 72 ◦C for 1 min/kb, with the final extension of 72 ◦C for 5 min. All primers and annealing temperatures used for the detection of each virus are given in Table 1.

cDNA preparations and PCR reactions were performed in a T100 thermal cycler (Bio-Rad, Hercules, CA, USA). Plasmids with the fragments of the virus being amplified by the primers used for testing were used as positive controls for CCYV, CYSDV, and CuLCrV. While the subset of samples used for HTS and in which CmAV1, CmCV, CmEV, and WCLaV-1 were detected was used as controls for these viruses. Leaf tissue of yellow squash plants grown in an insect-proof cage in the greenhouse was used as negative controls. Water was also used as a no template control. The final PCR products were analyzed on 1% agarose gel horizontal electrophoresis with 1X TAE buffer containing Gel Red (Biotium, Fremont, CA, USA). The PCR products were gel purified and Sanger sequenced for the confirmation of the target amplification. The sequences were annotated and submitted to NCBI GenBank.

Table 3.1. Primers used for PCR and RT-PCR for confirmation of the viruses.

Primer Name	Sequences 5'-3'	Tm (°C)	Amplicon Size	References
CmAV-2459F	AACCTCCCACATTCTGGA	55	740	[21]
CmAV-3189R	TCCAGTCAGCATAGGTCTCC			
CmEV-1F	ACCCCACTATTAGATATGCTAAGGTC	55	680	[23]
CmEV-1R	CTCCAGGAGTAAGATATAATGTAACCG			
CmCV-109F	ACTGAAGGATGAGTTCGCA	55	640	[21]
CmCV-751R	CCATCGGCATTCAGAACT			
CCYV_RDRP_1515	CTCCGAGTAGATCATCCCAAATC	62	953	[4]
CCYV_RDRP_1515	TCACCAGAAACTCCACAATCTC			
CuLCrV CP 259 F	TCAAAGGTTTCCCGCTCTGC	58	588	[5]
CuLCrV CP 846 R	TCCTGCTTCCTGGTGGTTGTAG			
CYSDV_RDRP_1542	TTTCGGCTCCCAGAGTTAATG	58	492	[22]
CYSDV_RDRP_1542	CGATCTCCGTGGTGTGATAAG			
WCLaV-1F	GGTGAGTTAGTGTGTCTGAAGG	55	881	[8]
WCLaV-1R	GAGGTTGCCTGAGGTGATAAG			

Abbreviations used for viruses: Watermelon crinkle leaf-associated virus 1 (WCLaV-1), cucumis melo amalgavirus (CmAV1), cucumis melo endornavirus (CmEV), cucumis melo cryptic virus (CmCV), cucurbit chlorotic yellows virus (CCYV), cucurbit yellow stunting disorder virus (CYSDV) and cucurbit leaf crumple virus (CuLCrV).

Construction of Consensus Viral Genome Sequences

Reference-based mapping was used to assemble the consensus sequences using CLC Genomics Workbench 21. Small RNA reads were aligned with the reference sequences of viruses potentially present in the samples. Consensus sequences were assembled from the mappings with the following parameters: mismatch cost = 2, insertion cost = 3, and deletion cost = 3. For graphical visualization of coverage in each region of the assembled genome, read tracks showing maximum, minimum, and average coverage values were created. The alignment of consensus sequences with the reference genome was inspected for discrepancies with CLC Genomics Workbench 21.

RESULTS

Symptoms

Symptoms resembling those caused by viruses were observed on watermelons in all fields of six counties surveyed in spring 2021. The main symptoms observed on spring-grown watermelons were thickened, wrinkled leaves with a severe bunchy top and upward curling, accompanied by a mild yellowing appearance (Figure 2A) in Wilcox County. Similar foliar symptoms were also observed on Tift County watermelon fields (Figure 2B) but with less crinkling and a bunchy appearance of the leaves. Watermelon samples collected in the fall of 2019 from Colquitt County displayed symptoms of severe interveinal chlorosis, mild crumpling, and yellowing (Figure 2C). Cantaloupe plants from the two counties surveyed in spring 2021 displayed yellow mottling with interveinal chlorosis (Figure 2D). These symptoms were observed in both Tift and Turner counties. Symptoms observed on cantaloupe were more restricted to the crown, while on watermelon, they were evenly distributed except for the bunchy and wrinkling appearance restricted to the young leaves.

Viruses Detected by HTS and Their Characteristics

sRNA libraries were constructed from one cantaloupe and six watermelon samples. After quality control and removal of adapters, a total of 17 to 28 million reads were retained (Table 2). All samples had a similar length distribution, with 21–24 nt sRNAs being the most abundant.

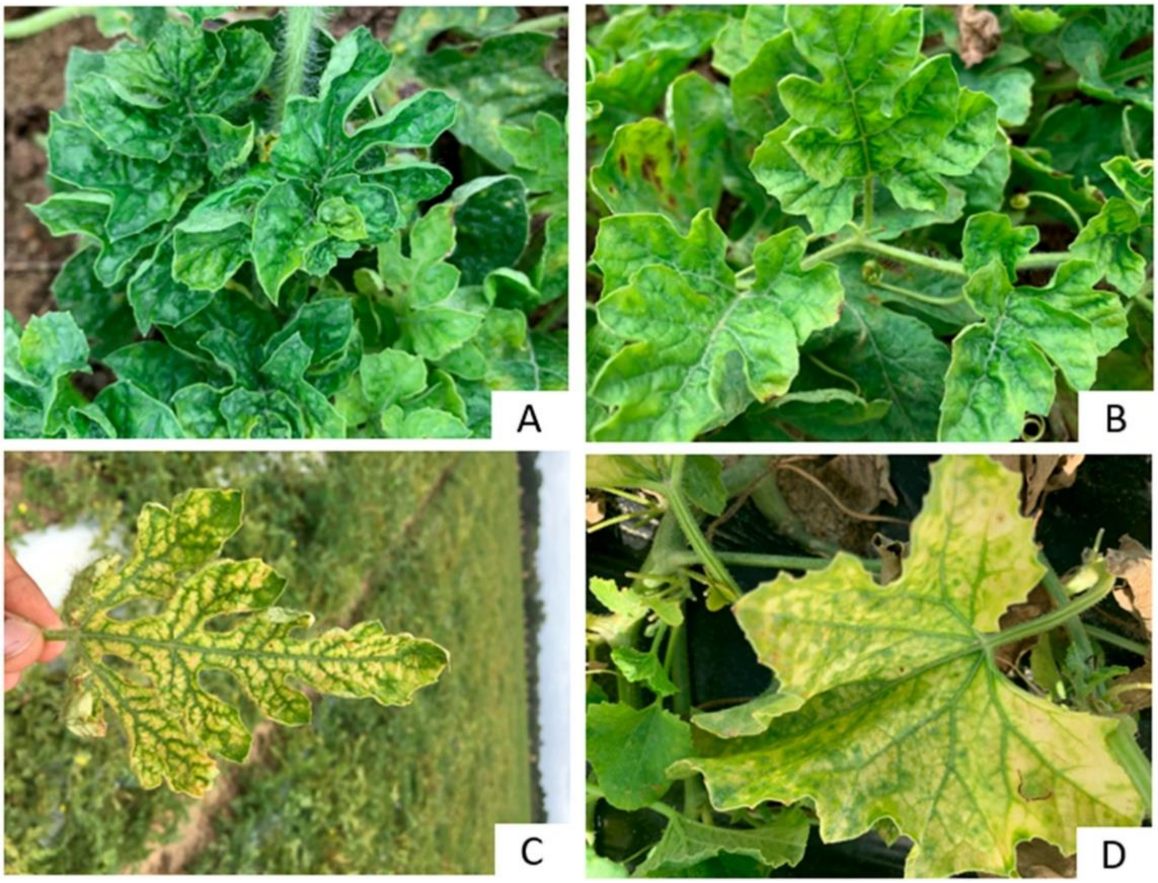

Figure 3.2. Symptoms observed on the field during the survey include: on watermelon (**A**–**C**), leaf crinkling and bunchy top with upward curling appearance of young leaves (**A**), crinkling of young leaves with mild yellowing (**B**), severe interveinal chlorosis of the leaf (**C**). On cantaloupe, yellowing and interveinal chlorosis on the older leaf (**D**). Watermelon (**A**,**B**) and cantaloupe (**D**) was collected in spring 2021 and watermelon (**C**) was collected in fall 2019.

Contigs with a minimum length of 75 bp were assembled and compared with virus sequences on the NCBI database for identification using BLASTn. Only viruses infecting plants were further analyzed. Four different plant RNA viruses were identified after *de novo* assembly and BLASTn searches against the NCBI database. One of them was the recently reported watermelon crinkle leaf-associated virus 1 (WCLaV-1) (Adeleke et al., 2022) and the other three were persistent viruses: cucumis melo amalgavirus 1 (CmAV1), cucumis melo cryptic virus (CmCV), and cucumis melo endonavirus (CmEV).

The large number of sRNA reads aligning to the genome (Table 2) and covering the entire length of their genome (Figure 3) added confidence to their identification. The percentage of viral sRNA sequences varied from sample to sample (Table 2). CmAV1 was detected in one cantaloupe and four out of six watermelon samples. CmCV and CmEV were detected only in watermelon samples, while WCLaV-1 was detected in one sample each of watermelon and cantaloupe.

In addition, reads from each sample were also aligned to the reference sequences of WTVs, CCYV, CuLCrV, and CYSDV (CuLCrV: DNA A-NC_002984, DNA B-NC_002985; CCYV: RNA1-NC_018173.1, RNA2-NC_018174.1; CYSDV: RNA1-NC_004809.1, RNA2-NC_004810.1), however these viruses were not detected in any samples collected in the spring, either on watermelon or cantaloupe. Near complete genomes of CmAV1, CmCV, CmEV, and WCLaV-1 were assembled from the sRNA sequences. The nucleotide sequence identity of CmAV1 isolates from watermelon (OM751927) ranged from 99.18 to 99.85% to that of CmAV1 reported in China (MH479774).

The genome of CmCV from watermelon in Georgia was 99.69% identical with RNA1 (MH479772) and 99.94% identical with RNA 2 (MH479773) with the isolate from China. CmEV sequences on watermelon matched 96.68% with that of CmEV reported from Mississippi (KT727022).

The watermelon (RNA1-OM751928 and RNA2-OM751930) and cantaloupe isolate of WCLaV-1 Georgia shared 99.71% and 99.73% identity with RNA 1 and 99.30% with RNA 2 of WCLaV-1 isolates reported in Brazil in BLASTn analysis (Table 2)

Prevalence and Distribution of the Viruses

Foliar tissues of watermelon collected in the spring of 2021 (*n* = 245) and the fall of

2019 (*n* = 43) and cantaloupe collected in spring 2021 (*n* = 80) were tested for the presence of viruses detected by HTS in this study (Table 2), as well as the WTVs previously reported to infect cucurbits in the region (Table 3). The presence of viruses, detected via HTS, were validated using RT-PCR on the portion of seven symptomatic samples sent for HTS.

Table 3.2. Viruses identified in sRNA reads from watermelon and cantaloupe samples collected in spring from Georgia and their characteristics

Virus Detected	**Sample ID & Location**	**Genome size of Refseq (nt)**	**Total sRNA reads**	**Reads Matching to Virus**	**Coverage (%)**	**Nucleotide Identity (%)**
CmAV1	WM3 Wilcox	3424	17,110,156	54, 623 (0.31)	3395 (99.1)	99.18
	WM4 Wilcox	3424	19,040,070	1,89,757 (0.99)	3397 (99.2)	99.24
	CA Turner	3424	18,645,801	27819 (0.14)	3419 (99.8)	99.85
	WM2 Wilcox	3424	28,380,401	16477 (0.05)	3398 (99.2)	99.24
	WM5 Wilcox	3424	21,552,412	30070 (0.13)	3398 (99.2)	99.27
CmCV	WM2 Wilcox	1592 RNA 1 1715 RNA 2	28,380,401	1,44,683 (0.5)	1714 RNA 1 (99.9) 1587 RNA 2 (99.6)	99.94 RNA 1 99.60 RNA 2
CmEV	WM 1 Tift	15078	18,645,801	2,73,320 (1.46)	14577 (96.6)	96.68
WCLaV-1	WM2 Wilcox	6645 RNA 1 2682 RNA 2	28,380,401	5,57,592 (1.96)	6599 RNA 1 (99.3) 2678 RNA 2 (99.8)	99.71(RNA 1) 99.30 (RNA 2)
	CA Turner	6645 RNA 1 2682 RNA 2	18,645,801	2,39,859 (1.28)	6601 RNA 1 (99.3) 2678 RNA 2 (99.8)	99.73 (RNA 1) 99.30 (RNA 2)

All samples except WM3 and WM4 were leaves. WM3 was fruit skin and WM4 was peduncle from watermelon. Viruses detected: watermelon crinkle leaf-associated virus 1 (WCLaV-1), cucumis melo amalgavirus (CmAV1), cucumis melo endornavirus (CmEV), cucumis melo cryptic virus (CmCV). Complete genome: Genome length of virus sequence with the highest identity in BLAST. CmEV (KT727022, Mississippi); WCLaV-1 (RNA 1, LC636068.1; RNA 2 LC636069, Brazil); CmAV1 (MH479774, China); CmCV (MH479773, China). Reads matching the virus: values in parenthesis represent the percentage of total reads aligning with the virus genome. Coverage: Percentage of the GenBank genome covered by contigs assembled from the sample. Percentage nucleotide identity: Identity to the GenBank sequence with the highest nucleotide identity in BLAST of all contigs aligned to the sequence.

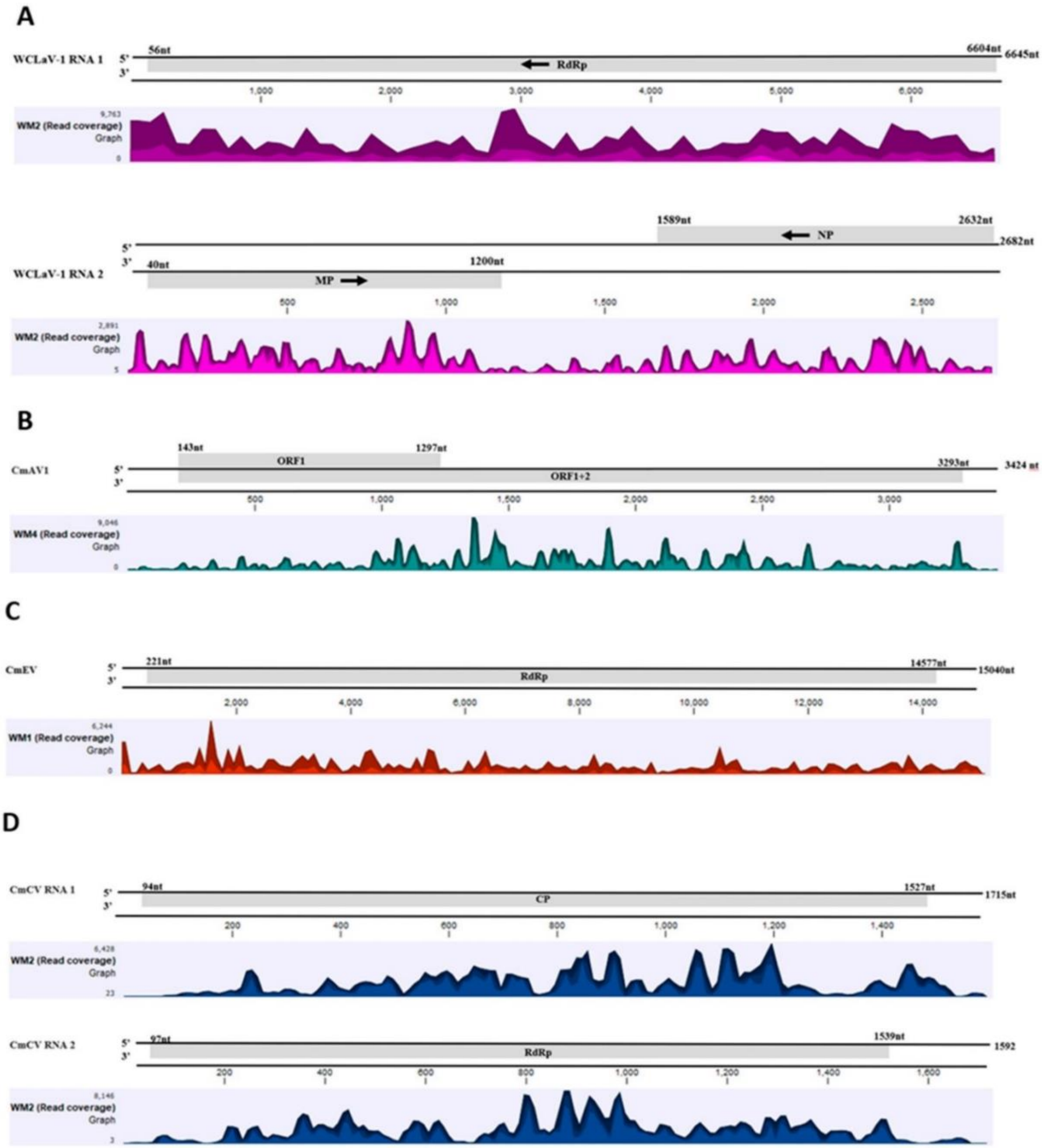

Figure 3.3. Read coverage maps of the virus genomes detected by HTS of small RNAs of symptomatic watermelon and cantaloupe from Georgia. Watermelon crinkle leaf-associated virus 1 (WCLaV-1) (**A**), cucumis melo amalgavirus 1 (CmAV1) (**B**), cucumis melo endornavirus (CmEV) (**C**), and cucumis melo cryptic virus (CmCV) (**D**). Genome positions of the virus are presented to scale above the histograms and the coverage in number of reads is represented on the *Y*-axis. Within the specified aggregation bucket, the colors mean: the maximum, average, and the minimum coverage values (read counts), from top to bottom.

Table 3.3. Incidence and prevalence of viruses from fall 2019 and spring 2021 cantaloupe and water- melon samples in Georgia as detected by PCR and RT-PCR.

Virus	2021								2019	Detected
	Watermelon						Cantaloupe		Watermelon	
	Colquitt	Crisp	Worth	Wilcox	Turner	Tift	Turner	Tift	Colquitt	
WCLaV-1	2(8)	-	1(5)	17(28)	33(83)	**39(98)**	-	-	-	92
CmAV1	-	**47(78)**	1(5)	37(62)	27(68)	20(50)	1(3)	6(15)	-	139
CmEV		-	-	-	8(20)	-	**40(100)**	**40(100)**	-	88
CCYV	-	-	-	-	-	-	-	-	**34(79)**	34
CYSDV	-	-	-	-	-	-	-	-	**1(2)**	1
CuLCrV	-	-	-	-	-	-	-	-	**35(81)**	35
CmCV	-	-	-	-	-	-	-	-	-	-
Total	25	60	20	60	40	40	40	40	43	

Virus acronyms used: Watermelon crinkle leaf-associated virus 1 (WCLaV-1), cucumis melo amalgavirus (CmAV1), cucumis melo endornavirus (CmEV), cucurbit chlorotic yellows virus (CCYV), cucurbit yellow stunting disorder virus (CYSDV), and cucurbit leaf crumple virus (CuLCrV). All samples collected in this study were also tested for squash vein yellowing virus (SqVYV), watermelon crinkle leaf-associated virus 2 (WCLaV-2), but none were detected. The number of samples in which a virus was detected, and their percentages (in parenthesis) are presented. The numbers for the virus detected at the highest frequency on a crop in a particular year are shown in bold.

In the samples collected in spring 2021, CmAV1 was detected by RT-PCR on both watermelon and cantaloupe samples in different counties. CmAV1 was detected in a high percentage of watermelon samples in Crisp (78%), Tift (50%), Turner (68%), and Wilcox (62%) counties, with only 5% from Worth County. On cantaloupe, CmAV1 was detected in 15% and 3% of samples from Tift and Turner counties, respectively. CmEV was detected in 100% of the cantaloupe samples from Tift and Turner counties and 20% of the total watermelon samples tested from Turner County.

WCLaV-1 was also detected on watermelons in all counties surveyed, except for Crisp County. The percentage of samples in which WCLaV-1 was detected varied in each county. It was detected in 8% of the total samples tested from Colquitt, 5% of samples from Worth, 28% of samples from Wilcox, 83% of samples from Turner and 98% of samples from Tift County (Table 3). The presence of WCLaV-1 was only detected on watermelon and not in cantaloupe using RT-PCR, even though WCLaV-1 was detected from a cantaloupe sample by HTS. However, CmCV was not detected from any samples tested using RT-PCR. Mixed infection of more than one virus was also identified (Supplementary Material Table S1). Surprisingly, none of the WTVs, including CCYV, CYSDV, CuLCrV, previously identified in the cucurbits in Georgia, were detected in any of the samples tested.

RT-PCR and PCR analysis of the symptomatic foliar tissues of the watermelon collected in the fall of 2019 revealed the presence of CCYV in 79%, CYSDV in 2%, and CuLCrV in 81% of the total samples tested (Table 3). Dual infection of CCYV and CuLCrV was detected in 67% of the total samples, while the mixed infection of the three viruses was found in 2% of the total samples tested (Supplementary Material Table S1). However, viruses detected in spring 2021, CmAV1, CmCV, CmEV, and WCLaV-1, were not detected in any of the samples collected in the fall of 2019. The partial sequence of the CCYV coat protein (MW915456; MW915457) and heat shock protein (MW915459; MW915460), the CmAV1 RNA-dependent RNA polymerase (ON364007), and the CmEV polyprotein (ON364008) from this study were submitted to the NCBI GenBank.

DISCUSSION

The application of HTS has become an important tool for detecting and identifying known and novel viruses in plant samples. In addition, many viruses infecting important food crops have been characterized (Zhan et al., 2019, Sabanadzovic et al., 2016, Mondal et al., 2021, Tomasechova et al., 2019, Villamor et al., 2019, Charon et al., 2020, Adams et al., 2009, Li et al., 2012, Xin et al., 2017). Unlike traditional assays like hybridization, ELISA, and PCR, metagenomic diagnostic techniques are unbiased and can detect pathogens without prior knowledge of their existence (Adams et al., 2009, Xin et al., 2017). In many cases, the virome of a host plant has been used as a basis for field diagnosis (Kavalappara et al., 2021b, Charon et al., 2020). siRNA sequencing has the advantage of detecting all types of viral and viroid genomes (Charon et al., 2020, Xin et al., 2017). In this study, surveys were conducted in the major cantaloupe and watermelon growing counties in Georgia and HTS was employed to explore the diversity of viruses infecting cantaloupe and watermelon.

A large number of samples collected during the survey were tested by RT-PCR to determine the distribution and prevalence of viruses identified by HTS. sRNA sequences from one cantaloupe and six watermelon samples were generated by HTS. Four different RNA viruses (CmAV1, CmCV, CmEV, and WCLaV-1) were identified from the sRNA sequences. Among the four viruses identified, only WCLaV-1 is reported to cause symptoms on watermelon, while CmAV1, CmCV, and CmEV express asymptomatic infections. WCLaV-1 was detected in one of the six watermelon samples analyzed by HTS. This virus was recently identified in Georgia on watermelon (Adeleke et al., 2022) and the samples from which it was detected showed symptoms of mosaic, crinkling, and bunching (Figure 2A), as described previously (Hernandez et al., 2021, Zhang et al., 2021, Maeda et al., 2022). Based on the molecular features and phylogenetic re-constructions, WCLaV-1 has been provisionally assigned to the genus Coguvirus (family *Phenuiviridae*) (Zhang et al., 2021). Since its discovery in China (Zhang et al., 2021) in 2017, the virus has been found in Brazil (Maeda et al., 2022) and the USA (Hernandez et al., 2021). The isolate of WCLaV-1 from Georgia (RNA1-OM751928 and RNA2-OM751930) was identical to those from Brazil (100% for RNA 1-LC636070 and 99% for RNA 2-LC636069;) and China (99% for RNA 2-MW751424.1) based on the nucleotide sequence of the near-complete genome sequence of RNA 1 and RNA 2. Even though WCLaV-1 was identified in the USA only recently, the virus appears to be widely distributed in the state and was detected from most of the counties surveyed in spring 2021.

WCLaV-1 has been found to be consistently associated with WCLaV-2 wherever it was detected, including Brazil (Maeda et al., 2022), China (Zhang et al., 2021), and the USA (Hernandez et al., 2021, Hendricks et al., 2021). However, in this study, WCLaV-2 was not detected in any of the samples tested either by HTS or RT-PCR. WCLaV-1 and WCLaV-2 are rather recently discovered viruses and much has yet to be learnt about both, including vector relations.

CmAV1, CmCV, and CmEV are classified as persistent viruses based on their lifestyle. They infect economically important crops, although they do not cause any obvious symptoms and remain in the host for a long time (Roossinck 2010). So far, most members of this plant virus group identified have a double-stranded RNA genome. They generally do not code for movement proteins or move from cell to cell. These viruses are almost 100% vertically transmitted by seed in hosts and not horizontally by insects or grafting.

Plant persistent viruses are not well-studied. Their genomes code for RNA-dependent RNA polymerase (RdRp) and a coat protein. Endornaviruses are an exception since they do not have domains that code for a coat protein and instead, only code for a polyprotein that functions as RdRp (Roossinck et al., 2015). Cryptic viruses, which are currently classified in the family *Partitiviridae* (Fauquet and Stanley 2005), were the earliest members identified in this group in the 1960s and 1970s (Boccardo et al., 1987). Other viruses with similar characteristics were discovered and described later (Fukuhara et al., 2019, Fukuhara and Moriyama 2008, Fukuhara et al., 2006, Valverde et al., 2019). Earlier methods of the discovery of persistent viruses from dsRNA preparations were not efficient. Particularly in cases like endornaviruses, in which the members do not form classic virions ("capsid-less viruses") (Mondal et al., 2021, Roossinck 2010) their naked RNA can go unnoticed while contaminating DNA in dsRNA analysis (Roossinck 2010).

Metagenomic studies have accelerated the discovery of new viruses in this category (Roossinck et al., 2015, Fukuhara et al., 2006, Valverde et al., 2019, Bernado et al., 2018, Park et al., 2018, Osaki and Sasaki 2018, Susi et al., 2019, Bejerman et al., 2020, Ma et al., 2020, Galipienso et al., 2021, Nabeshima and Abe 2021). In addition, the genomes and sequences generated from these studies will improve our understanding of these viruses, including phylogeny, host relation, and transmission.

CmEV belongs to the genus *Endornavirus* and family *Endornavirudae* (Sabanadzovic et al., 2016), and CmAV1 belongs to the genus *Amalgaviruses* and family *Amalgaviridae* (Zhan et al., 2019). Endornaviruses are persistent within the host and infect every tissue of the plant (Boccardo et al., 1987, Fukuhara 2019, Fukuhara and Moriyama 2008). Pepper endornaviruses were found in all cultivars of bell pepper and could be transmitted to other cultivars by crosses (Valverde and Gutierrez 2007). In contrast, CmEV was identified in plants belonging to three different genera in the family Cucurbitaceae (*Cucumis*, *Luffa* and *Praecitrullus*), which are not cross- compatible (Sabanadzovic et al., 2016). In this study, CmEV was identified from watermelon (*C. lanatus*), which is not cross-compatible with either of the three genera from which CmEV was reported earlier. These findings suggest the long association of endornaviruses with their hosts and their co-divergence with hosts since they are not transmitted horizontally. It is also the first time CmEV and CmAV1 have been identified on watermelon (Table 3).

All virus isolates identified in this study had very high genetic similarity with isolates of corresponding viruses described earlier. The phylogenetic relations and detailed genome characteristics of CmAV1 (Zhan et al., 2019), CmCV (Nibert et al., 2014, Vainio et al., 2018), CmEV (Sabanadzovic et al., 2016), and WCLaV-1 (Zhang et al., 2021) have been described earlier and are not repeated here.

Although sequences of CmCV and WCLaV-1 were detected on watermelon and cantaloupe in HTS (Table 2), their presence could not be verified in any of the samples by RT-PCR. This could have resulted from cross-contamination during RNA preparation, which is not uncommon in HTS (Lusk 2014, Merchant et al., 2014, Tosar et al., 2014, Ballenghien et al., 2017). This suggests the importance of not relying on HTS alone and the reliability of the use of alternate methods to confirm presence of a virus identified by HTS. Similarly, no virus was detected in some samples that displayed virus-like symptoms. This could be due to other factors, including environmental conditions, nutrient imbalances, chemicals, or non-viral pathogen infections.

Overall, this study did not find any evidence of a buildup of previously reported WTVs viz CuLCrV, CYSDV, and CCYV affecting cucurbits on spring-grown cantaloupe and watermelon in Georgia. This indicates non-crop hosts are likely sources of inoculum for the buildup of WTVs in the fall crops. After the fall season harvest of cucurbits, the whiteflies migrate to winter brassica crops and weeds, likely carrying the viruses with them (Kavalappara 2022a). However, the spring population of whiteflies is much lower than what is seen in the fall (Barman et al., 2021) and may not be enough to spread the viruses from alternate crop or weed hosts, such as the wild radish (Kavalappara 2022b), to infect the spring cucurbits.

Nevertheless, spring cantaloupe and watermelon in Georgia support a diverse array of viruses, including the recently discovered WCLaV-1 and three persistent viruses. WCLaV-1 appears to have spread in Georgia since it was detected on watermelon in five different counties. Being a rather recently discovered virus, biological properties, including vector relations, are unknown. Watermelon is an economically important crop in Georgia and worldwide. Further studies are required to better understand WCLaV-1 and its impact on watermelon production. The mode of spread/transmission, including vectors, if any, crops and weed hosts of the virus, and most importantly, the economic impact of the virus on its crop hosts are important questions to be answered.

CONCLUSIONS

Applications of HTS and surveillance reveal the presence of new persistent plant viruses in cantaloupe and watermelon in GA. CmAV1 and CmEV are widespread and prevalent in the region, along with the newly identified WCLaV-1 in GA. The WTVs (CCYV, CYSDV, CuLCrV, and SqVYV) were not detected in the spring cantaloupe and watermelon, suggesting these viruses may overwinter in another crop and non-crop host. These results present the first report of CCYV, CmAV1, and CmEV on watermelon in Georgia, USA. The impact of these viruses on the agroecosystem in the state is yet to be investigated.

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Supplementary Table 1: Prevalence and distribution of mixed infection of the v*iruses* detected during 2019 and 2021 cantaloupe and watermelon survey.

Virus	**2021**								**2019**
	Watermelon						**Cantaloupe**		**Watermelon**
	Colquitt	**Crisp**	**Worth**	**Wilcox**	**Turner**	**Tift**	**Turner**	**Tift**	**Colquitt**
CCYV + CuLCrV	-	-	-	-	-	-	-	-	29(67)
CmAV + CmEV	-	-	-	-	-	-	3(1)	15(6)	-
CmAV + WCLaV-1	-	-	-	-	50(20)	50(20)	-	-	-
CmEV + WCLaV-1	-	-	-	-	10(4)	-	-	-	-
CuLCrV + CCYV + CYSDV	-	-	-	-	-	-		-	1(2)
CmAV + CmEV + WCLaV-1	-	-	-	-	10(4)	-	-	-	-
Total number of samples tested	25	60	20	60	40	40	40	40	43

Virus acronyms used: cucurbit chlorotic yellows virus (CCYV), cucurbit leaf crumple virus (CuLCrV), cucumis melo amalgavirus (CmAV), cucumis melo endornavirus (CmEV). cucurbit yellow stunting disorder virus (CYSDV) and watermelon crinkle leaf-associated virus 1 (WCLaV-1).

First report of watermelon crinkle leaf-associated virus 1 naturally infecting watermelon (*citrullus lanatus*) in georgia, united states

Watermelon (*Citrullus lanatus*) is one of the major vegetable crops grown in Georgia during the spring and summer seasons, contributing $180 million of farmgate value to the state's economy (Economic). During the summer of 2021, watermelon plants with foliar symptoms such as yellow mottling, chlorosis, and wrinkling with thickened, bunchy, and upward curling were observed on commercial fields in Georgia, United States. Infection incidence of 15 to 20% in 56 ac. in Tift County and 10 to 15% in 60 ac. in Wilcox County was observed.

The symptoms observed were similar to those described for watermelon crinkle leaf-associated viruses (WCLaV-1 and WCLaV-2) from Florida (Hendricks et al. 2021) and Texas (Hernandez et al. 2021). Symptomatic leaves from Tift (*n* = 40) and Wilcox (*n* = 20) Counties were collected, surface sterilized with 0.1% bleach, and used for total nucleic acid extractions using the Mag-MAX 96 Viral RNA isolation kit (ThermoFisher Scientific, Waltham, MA, U.S.A.) following the manufacturer's instructions without DNase treatment.

The potential introduction of WCLaV-1 and WCLaV-2 into Georgia was tested by a reverse transcription (RT)-PCR assay using specific primers targeting RNA-dependent-RNA polymerase (RdRp) and movement protein (MP) genes of both viruses (Hernandez et al. 2021). The expected amplicon sizes for RdRp (900 nt) and MP (500 nt) genes of WCLaV-1 located on RNA 1 and RNA 2 segments, respectively, were observed in 39 of 40 (97.5%) samples from Tift and 7 of 20 (35%) samples from Wilcox. How- ever, WCLaV-2 was not detected in any of the tested samples. All 60 samples also tested negative for the whitefly-transmitted viruses prevalent in the region, including cucurbit chlorotic yellows virus, cucurbit yellow stunting disorder virus, and cucurbit leaf crumple virus using virus-specific primers (Kavalappara et al 2021)

A subset of the samples analyzed by RT-PCR was also tested by SYBR green-based real-time RT-PCR assay targeting the MP gene of WCLaV-1 using primers WCLaV-1FP (59TCCACAAGCTTGATGGAGGG39) and WCLaV-1RP (59TCCCGAGTGAGGAAGCTAGT39). The virus was detected in samples from both counties, and the results matched those obtained by the conventional RT-PCR assays. The presence of WCLaV-1 was further con-firmed by sequencing (Genewiz, South Plainfield, NJ, U.S.A.) coupled with BLASTn analysis of amplicons that resulted from the conventional RT-PCR from three randomly selected samples. The partial RdRp sequences (OL469153 to OL469155) were 99.3 and 99.9% identical to the corresponding sequences of WCLaV-1 isolates from China (KY781184) and Texas (MW559074), respectively. The partial MP sequences (OL469150 to OL469152) were 100% identical to those from China (KY781185) and Texas (MW559077). WCLaV-1 and WCLaV-2 were first discovered in Asia (Xin et al. 2017).

Both viruses were subsequently reported from North and South America (Hendricks et al. 2021; Hernandez et al. 2021; Maeda et al. 2021), indicating their geographical expansion. Biological information, including vector relations, is unknown for both viruses and other members of the genus *Coguvirus* (family *Phenuiviridae*), to which they are provisionally assigned (Zhang et al. 2021). Further studies are also required to understand the biology and impact of both viruses on watermelon pro- duction and other crops if any.

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CHAPTER 4

EVALUATION OF PLANT INTRODUCTION LINES OF YELLOW SQUASH *(CUCURBITA PEPO)* FOR RESISTANCE AGAINST SINGLE INFECTION OF CUCURBIT CHLOROTIC YELLOWS VIRUS (CCYV) AND CUCURBIT LEAF CRUMPLE VIRUS (CuLCrV)

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INTRODUCTION AND LITERATURE REVIEW

Infections and damage caused by whitefly-transmitted viruses (WTVs) have emerged as a major threat to global food production over the past several decades. Affected include crops in cucurbitaceae and solanaceae (Polston and Anderson 1997; Polston et al., 1999; Guzman et al., 2000; Adkins et al., 2009; Navas-Castillo et al., 2011). Commercially produced cucurbits such as squash (*Cucurbita pepo*) are centered in South Georgia and in neighboring Florida, where a high incidence of WTVs of cucurbits has been reported (Adeleke et al., 2022). Squash production in Georgia accounts for about 3% of total vegetable production with a total farmgate value of USD 38 million (Georgia Farm Gate Value 2021).

However, squash production under field conditions has been continuously threatened and drastically reduced due to whiteflies and the complex of viruses transmitted by them (LaTora et al., 2022; Moodley et al., 2019; Kavalappara et al., 2021a). The severity of these viruses on the crop depends on the time of infection, plant vigor, and form of infection, which could be a single or mixed infection with multiple viruses simultaneously (De Barro et al., 2011; Lapidot et al., 2014). Cucurbit chlorotic yellows virus (CCYV) (Kavalappara et al., 2021b), cucurbit yellow stunting disorder virus (CYSDV) (Gadhave et al., 2018), and cucurbit leaf crumple virus (CuLCrV) (Larsen, 2010) are major WTVs on cucurbit crops reported in the region. Management of whiteflies and the complex of viruses transmitted by them is very challenging due to the broad host range, the short generation of whiteflies, as well as resistance to insecticides (Hidayat et al., 2018). Current management of WTVs relies heavily on using insecticides and resistant varieties when available. But unlike in other crops such as melons, where host-resistance to WTVs has been reported, host-resistance to WTVs is not available in commercial squash cultivars (Vidavski et al., 2008; Jennings, 1994; Codod et al., 2022). In the absence of host-resistance in squash, several plant introductions (PI) were screened for resistance under field conditions over two consecutive years in Florida and Georgia (Luckew et al., 2022). Mixed infection of WTVs is a natural phenomenon in open field conditions, which makes it challenging to assess the impact and severity of individual viruses and plant response. Based on the phenotypic response to the mixed infection of the WTVs and reduced virus accumulation, two PI lines of *C. pepo* were selected for further evaluation under greenhouse conditions for 1) resistance against single infection of CCYV and CuLCrV and 2) quantitation of the virus load in individual plants at different sampling points.

MATERIALS AND METHODS

4.1 Whitefly Colony and Maintenance of Virus Isolates

A whitefly colony was established and maintained on cotton (*Gossypium spp*) (a non-host for viruses) in an insect-proof cage (Mega View, Taiwan) at 25 ± 2 °C inside a greenhouse. The abundance of whiteflies in the cages was maintained by replacing cotton seedlings every four weeks and allowing the insect to migrate onto new plants. A pure culture of CCYV was maintained in a separate insect-proof cage on the squash variety Gold Star (Seedway Hall, NY) and used as the inoculum source. CuLCrV was obtained from Dr. Rajagopalbabu Srinivasan (UGA, Griffin). The isolate was initially maintained on snap bean (*Phaseolus vulgaris*) and transferred to Gold Star.

4.2 Plant Materials

Selfed seeds from two *C. pepo* lines, PI 171625 (UGA26) and PI 171627 (UGA28) (here after being referred to as UGA26 and UGA28 respectively) were obtained from Dr. Cecilia McGregor (UGA, Athens), as well as one susceptible cultivar Gentry (Seedway Hall, NY).

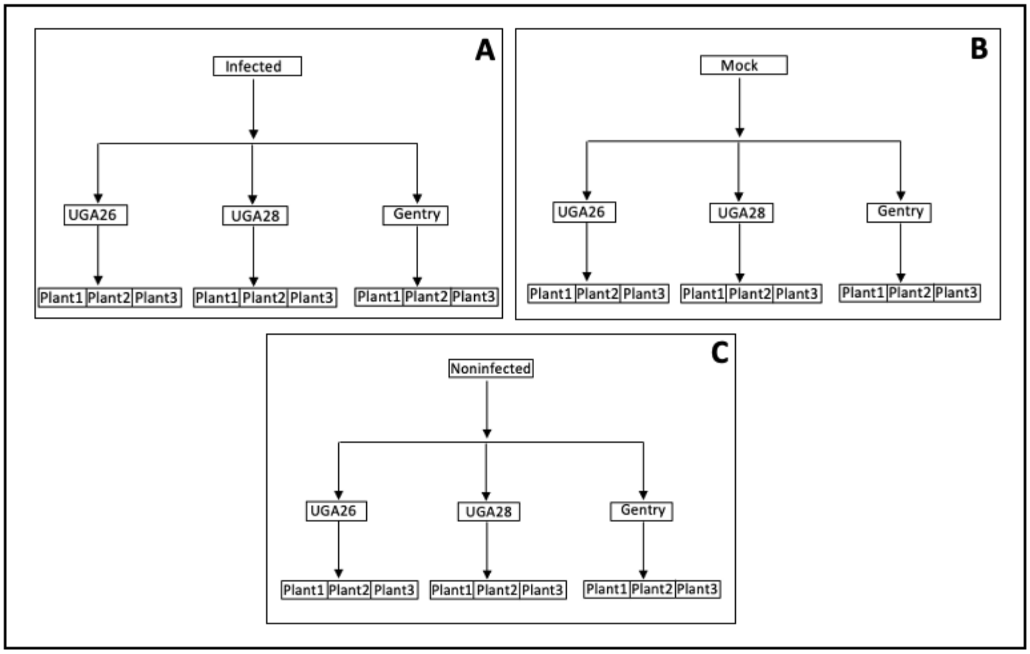

Fig. 4.1: Schematic diagram of the experimental layout. **A)** infected plants treated with viruliferous whiteflies carrying infection of CCYV or CuLCrV. **B)** Mock plants treated with non-viruliferous whiteflies. **B)** Non-infected plants with no whiteflies or virus treatment. UGA26 and UGA28 are plant introduction lines. Gentry is the susceptible line. Plants 1, 2, and 3 are the replicates of each line. The three plant replicates from each line were maintained within the same cage.

Seeds (UGA26 n=3, UGA28 n=3, and Gentry n=3) were germinated and three biological replicate plants of each line were maintained within the same cage (Fig. 4.1A). This layout was also used for both mock (non-viruliferous whitefly) and non-infected control (Fig. 4.1B and C). In each trial, a total of 9 insect-proof cages were maintained and monitored in the greenhouse. This layout was used for both CCYV and CuLCrV trials and each trial was repeated once more. Trials for CCYV were conducted in the spring of 2022, while CuLCrV was conducted in the fall of 2022.

4.3 Virus Transmission and Maintenance of Plant

Non-viruliferous whiteflies were released onto the virus source plant (Gold Star) and allowed an acquisition access period (AAP) of 48 hours in an insect-proof cage. To achieve a high transmission efficiency, approximately 50 adult viruliferous whiteflies per clip cage were transferred onto squash seedlings at the 2-3 true leaf stage, two weeks after planting for an inoculation access period (IAP) of 48 hours. Prior to inoculation, a single infection of CCYV or CuLCrV was confirmed on the source plants using SYBR Green-based qPCR (Kavalappara et al., 2022). After an IAP, plants were treated with the insecticide ASSAIL (UPL, New Zealand) (0.92g/L) to kill the whiteflies.

Plants were also fertilized (Miracle-Gro, Marysville, OH) (15g/L) every two weeks, and leaf sampling was done at two weeks and four weeks post inoculation (wpi)

4.4 Plant Evaluation and Sampling

Plant response to virus infection was monitored and rated weekly for the severity of symptoms starting from the first wpi. This was conducted for four consecutive weeks at 7, 14, 21, and 28 days after inoculation (DAI). In both CCYV and CuLCrV experiments, an overall score was assigned to individual plants using the symptoms severity scale ratings as follows: 0 = no symptoms; 1 = 1% to 20%; 2 = 21% to 40%; 3 = 41% to 60%; 4 = 61% to 80%; and 5 = 81% to 100%. This scale was used for both CCYV and CuLCrV based on the typical symptoms expressed.

In CCYV trials, approximately 0.1g of leaf tissue was collected from the lower 2-3 leaves from each plant at 2 and 4 wpi, while the upper 2-3 leaves from CuLCrV-infected plants were sampled. Samples were frozen immediately in liquid nitrogen, and kept at -80℃ pending further laboratory analysis.

4.5 Nucleic Acid Isolation and Quantitative Analysis

Nucleic acid was isolated from approximately 0.1g of leaf tissue of CCYV and CuLCrV single-infected plants and controls using the Trizol RNA extraction protocol (Thermofisher Scientific, Waltham, MA) and DNeasy plant mini kit (Qiagen, Germantown, MD), respectively following manufacturers instructions. The concentration of extracted nucleic acid was determined using a Nanodrop Spectrophotometer (Thermofisher Scientific) and normalized to 100 ng/µl for each sample.

Complementary DNA (cDNA) was synthesized from CCYV-infected samples following the previously described protocol (Adeleke et al., 2022). SYBR Green qPCR was developed and standardized to determine the virus accumulation at two different time points. To calculate the copy number of the virus in each sample, each sample's cycle threshold (Ct) value was compared with the standard cycle threshold (Ct values). A fragment size of 87-bp of the conserved region of Mitochondrion cytochrome oxidase subunit I (CyOXID) was amplified with the primer set (CyOXIDF and CyOXIDR) according to (Papayiannis et al., 2011) for the housekeeping gene. This number of copies was estimated based on the formula described by (Kavalappara et al., 2022, Rotenberg et al., 2009, and Tamang et al., 2021).

The qPCR assay was carried out with 10 µL of SSOAdvanced Universal SYBR Green Supermix (BioRad, Hercules CA, USA), 1 µL reverse primer (10 µM), 1 µL forward primer (10 µM), 12 µL RNAse free water, and 2 µL cDNA or DNA to a final volume of 25 µL. Details of all primer sets used in this analysis for the detection of virus accumulation are given in Table 1. Three technical replicates for each biological sample were included. A plasmid carrying the targeted gene of each virus was used as the positive control, and nuclease-free water was included as no template control (NTC). A separate healthy squash plant maintained in an insect-proof cage was used for the negative control. The assay was carried out using CFX96 Touch Deep Well Real-Time PCR System® (Bio-Rad, Hercules, CA) with the run-time profile of an initial denaturation step of 3 min at 95 °C followed by 35 cycles of denaturation for 30 s at 95 °C and a combined step of annealing and extension for 30 s.

Table 4.1. List of primers used for the real-time qPCR analysis of criniviruses and begomovirus

Primer Name	Sequence 5'-3'	Gene	Amplicon Size	Reference
qPCR-CCVY-F	GGTTTACACACCCGGTGAGTTT	RdRp	91	Orfanidou et al., 2021
qPCR-CCYV-R	TGAAATTAGGGCTTGCTTCCA			
CuLCrV-F	CCTCAAAGGTTTCCCGCTCT	CP	110	Agarwal et al., 2021
CuLCrV-R	CCGATAGATCCTGGGCTTCC			
CyOXID-F	TGGTAATTGGTCTGTTCCGATT	CyOXI D sub1	87	Papayiannis et al., 2011
CyOXID-R	TGGAGGCAACAACCAGAATG			

Abbreviations used for viruses: cucurbit chlorotic yellows virus (CCYV), cucurbit leaf crumple virus (CuLCrV)

4.6 Statistical Analysis

The mean of the three technical replicates for all individual biological replicates of plant sample data obtained from the qPCR was subjected to a Two-way analysis of variance (ANOVA) using R version 4.0.3. (R Core Team, 2020). For each line, virus accumulation was analyzed using the two sampling points, followed by multiple mean comparisons using Tukey's significant difference ($P < 0.05$) to compare virus severity on each line. One-way analysis of variance (ANOVA) was used to analyze AUDPC and mean separation was done with Tukey's HSD mean separation.

RESULTS

4.7 Detection of CCYV and CYSDV from the Source Plants

Prior to the inoculation of the experimental plants, nucleic acid was isolated from both CCYV and CuLCrV source plants and tested to ensure the presence of CCYV or CuLCrV and the absence of contamination of other whitefly-transmitted viruses (WTVs). The results show a single infection of each virus on the individual plant with no contamination with other WTVs.

4.8 Disease Phenotyping and Plant Response to Virus Infection

Two independent trials were carried out for each experiment. In both experimental trials for an individual virus, the plant's response to virus infection was observed throughout the stages of the experiment (Fig. 4.2A and B).

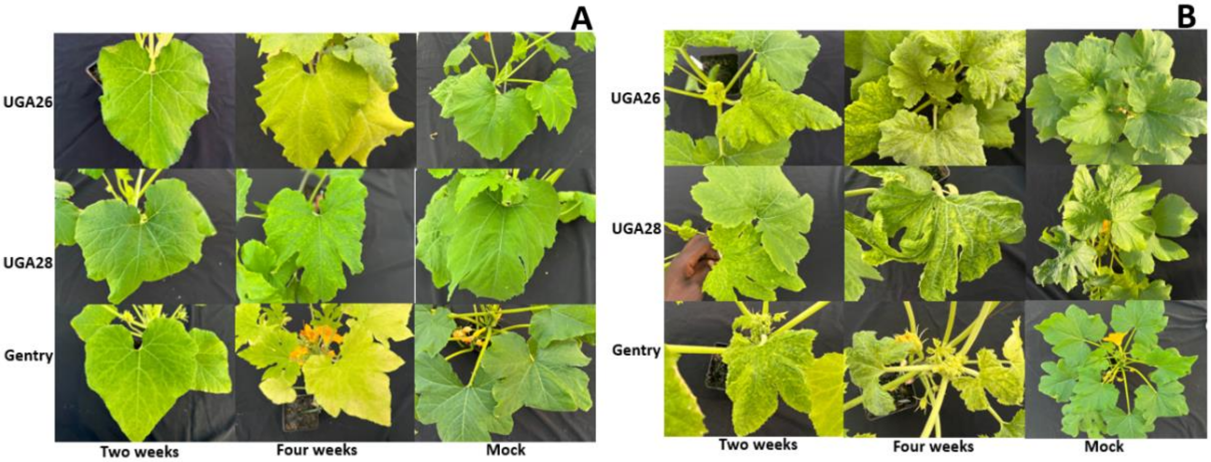

Fig. 4.2: Phenotypic response of experimental plants to virus infection. **A)** cucurbit chlorotic yellows virus (CCYV). **B)** cucurbit leaf crumple virus. Nonviruliferous whitefly treated plants (Mock) UGA26 and UGA28 are plant introduction test lines while Gentry is the susceptible line.

Plants infected with CCYV expressed chlorotic spots on the lower leaves, the symptom progressed to interveinal chlorosis and later to a complete yellowing as the plants aged (Fig. 4.2A). In CuLCrV-infected plants, the symptoms started on the younger leaves and progressed toward the lower leaves. At the initial stage, the younger leaves expressed a minimal light-green and yellow mottling, which progressed to thickened and distorted leaves (Fig. 4.2B). Leaves of infected plants show characteristic curling of the CuLCrV symptoms.

4.8.1 Cucurbit chlorotic yellows virus (CCYV)

In the CCYV experiments, at two wpi, the initial symptom of chlorotic spots was observed on the lower leaves of both the UGA26 and Gentry (Fig. 4.2A). This symptom was not initially exhibited by UGA28 at two wpi. At four wpi, the symptoms progressed extensively with the entire lower leaves of both UGA26 and Gentry turning completely yellow with mild interveinal chlorosis on the lower leaves of UGA28 (Fig. 4.2A). Overall, the symptoms were more severe on the susceptible line Gentry compared to the PI lines.

4.8.2 Cucurbit leaf crumple virus (CuLCrV)

In the CuLCrV experiments, typical symptoms of CuLCrV infection were observed on both PI lines as well as the susceptible line at two wpi. These symptoms started with mild interveinal chlorosis on young leaves which later extended to crumpling of leaves, and downward curling of only the young leaves as the plant ages. At four wpi, the crumpling of the younger leaves was more pronounced on the susceptible line with yellowing compared to both the PI lines (Fig. 4.2B). The symptoms observed on CuLCrV-infected plants were only restricted to the younger leaves. The leaves of Gentry were more brittle with severe crumpling and yellowing with interveinal chlorosis. In both experiments and the repeated trials, no symptoms were observed in both mock or non-infected controls (Fig. 4.2A and B).

4.9 Disease Severity and Area Under Disease Progress Curve

Symptoms severity on individual plants was recorded for four weeks post-inoculation (wpi) to estimate area under the disease progress curve (AUDPC) and analyzed by genotype (line). In CCYV experimental trials, UGA28 shows less disease severity progression (Fig. 4.3A and B). There was a significant difference among all the cultivars AUDPC in the first trial ($F=211.8_{\text{DFn=2, DFd=6}}$) ($P< 0.05$) as well as the second trial ($F=402.4_{\text{DFn=2, DFd=6}}$) ($P< 0.05$). The effect of the trials was also compared for the AUDPC, there was no significant difference between the two trials. However, in both trials, Gentry showed more disease severity and high AUDPC (Fig. 4.3 and 4.4).

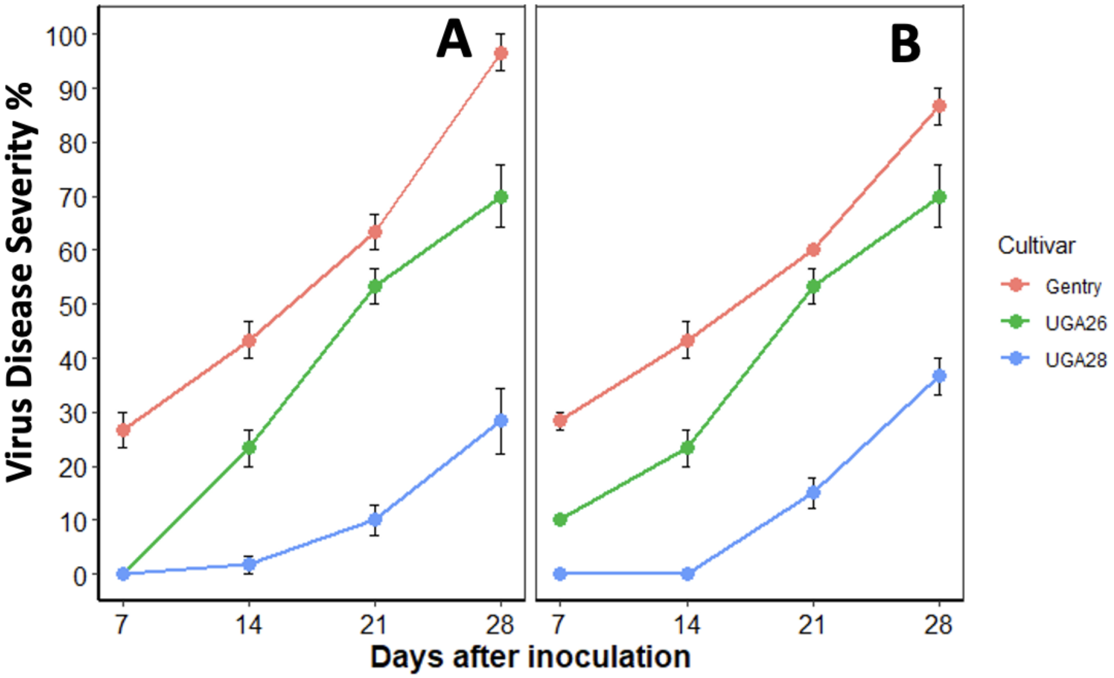

Fig. 4.3: Mean disease progression over time of cucurbit chlorotic yellows virus (CCYV): **A)** CCYV first trial. **B)** CCYV second trial. Y-axis represents the severity of infection at a time point. The x-axis represents the day after inoculation at which disease incidence was recorded. UGA26 and UGA28 are plant introduction lines. Gentry is the susceptible line. The error bar presented here is the standard error.

In CuLCrV experimental trials, all three cultivars show an increase in disease severity over time, with a significant difference between the AUDPC of UGA26 and Gentry. There was no difference between UGA28 and either UGA26 and Gentry (Fig. 4.5 and 4.6) ($F=10.95_{\text{DFn}=2, \text{DFd}=6}$)) ($P< 0.001$)) and the second trial ($F=20.78_{\text{DFn}=2, \text{DFd}=6}$) ($P< 0.001$)). The effect of trials was also compared and there was no significant difference between the trials ($F=0.379_{\text{DFn}=1, \text{DFd}=16}$) ($P> 0.05$).

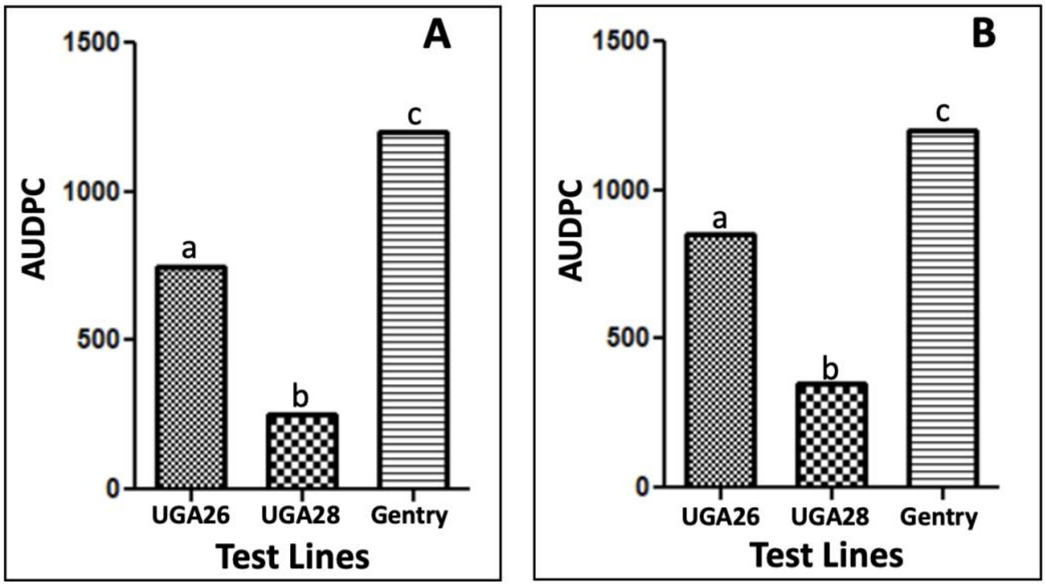

Fig. 4.4: Area under disease progress curve (AUDPC): **A)** CCYV first trial. **B)** CCYV second trial. Y-axis represents the AUDPC. The x-axis represents the lines used. UGA26 and UGA28 are plant introduction lines. Gentry is the susceptible line. The statistical difference is represented with a, b, and c.

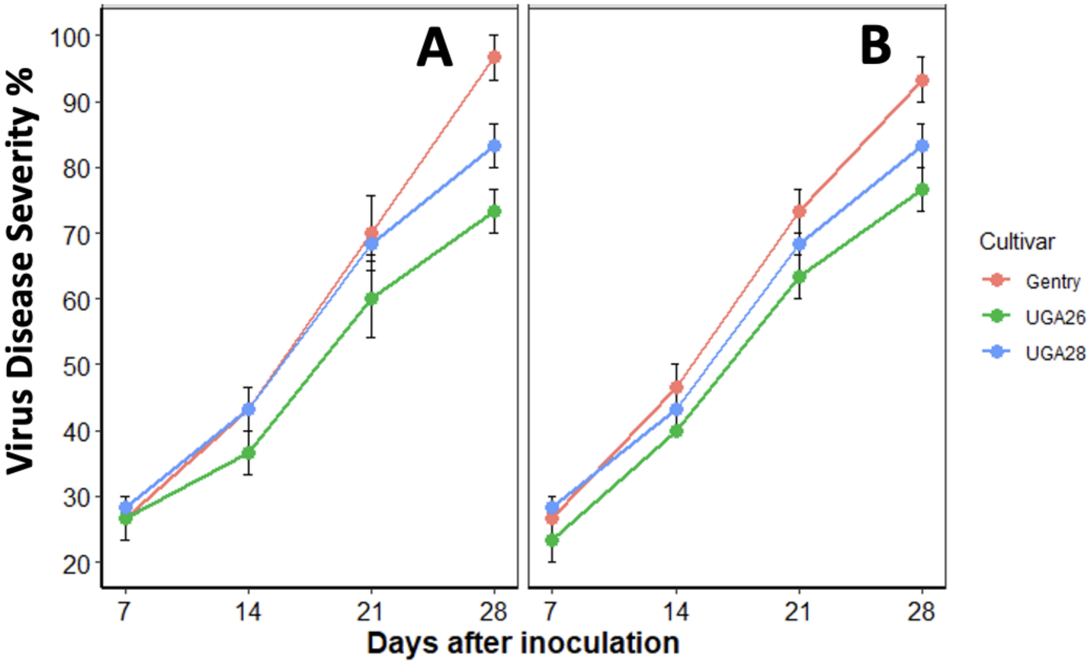

Fig. 4.5: Mean disease progression over time cucurbit leaf crumple virus (CuLCrV) disease progression over time: **A)** CuLCrV first trial. **B)** CuLCrV second trial. Y-axis represents the severity of infection at a time point. The x-axis represents the day after inoculation at which disease incidence was recorded. UGA26 and UGA28 are plant introduction lines. Gentry is the susceptible line. The error bar presented here is the standard error.

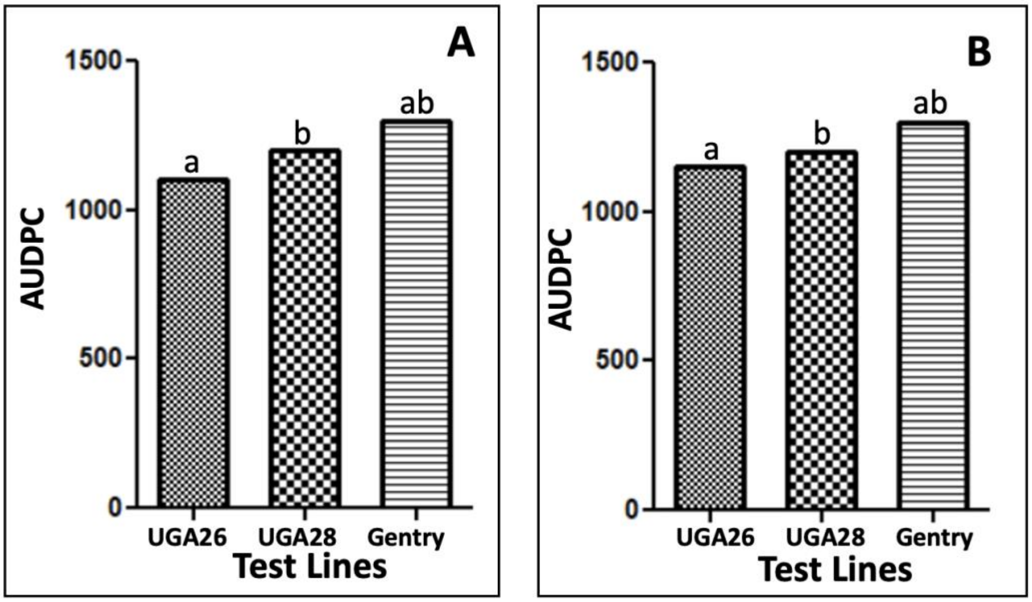

Fig. 4.6: Area under disease progress curve (AUDPC): **A)** CCYV first trial. **B)** CCYV second trial. Y-axis represents the AUDPC. The x-axis represents the lines used. UGA26 and UGA28 are plant introduction lines. Gentry is the susceptible line. The statistical difference is represented with a and b.

4.10 Standard Curve

The standard curve was developed by 10-fold serial dilutions using plasmid carrying the RdRp and CP fragments of CCYV and CuLCrV, respectively. The amplification efficiency was 92.3 % for CCYV (Fig. 4.7A) and 90.1% for CuLCrV (Fig. 4.7B). The linearity (R^2) of the standard curve assay for both viruses was >0.99, validating the reliability of the assay for the quantification.

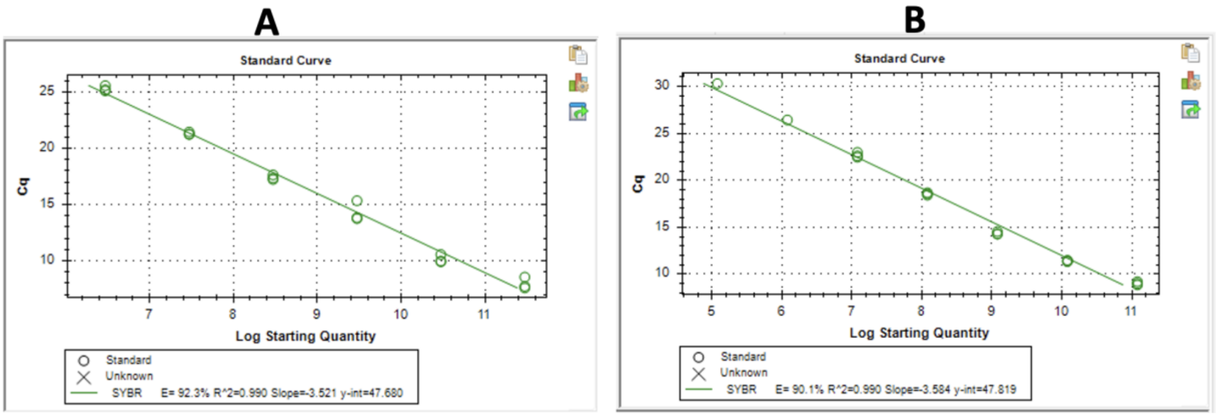

Fig. 4.7: SYBR Green quantitative polymerase chain reaction standard curve showing the assay's application efficiency and linearity. **A)** cucurbit chlorotic yellows virus (CCYV). **B)** cucurbit leaf crumple virus (CuLCrV).

4.11 Accumulation of Viruses in Tested Sample

In all repeated experiment trials, CCYV and CuLCrV single infection virus loads were calculated on both PI lines and Gentry (Fig.8).

4.11.1 Cucurbit chlorotic yellows virus

In CCYV first trial, the virus load in Gentry was higher than both PI lines at two- and four weeks post-inoculation. Although CCYV was detected in all three lines at two wpi, the virus load was lower (higher Ct value) in UGA28 (no symptoms). However, there was no significant difference between UGA26 and UGA28 [($F=5.56_{\text{DFn=6, DFd=6}}$) $P< 0.05$] at two wpi. At four wpi, there was a significant difference among all the lines ($F=86.57_{\text{DFn=2, DFd=6}}$) ($P \leq 0.001$) with Gentry having the highest virus accumulation (Fig. 4.8A) In the second trial, there was no significant difference between the PI lines at two and four wpi. However, there was a significant difference between the PI lines and Gentry at four wpi ($F=119.76_{\text{DFn=2, DFd=6}}$) $P< 0.001$) (Fig. 4.8B). The effect of trials was compared and there was a significant difference between the two repeated trials for the CCYV experiment ($F=0.579_{\text{DFn=1, DFd=16}}$) ($P<0.05$)

4.11.2 Cucurbit leaf crumple virus

In both repeated trials of CuLCrV experiments, there was no significant difference among all the lines at two wpi or at four wpi and between the two repeated trials (Fig.4.8C and 4.8D).

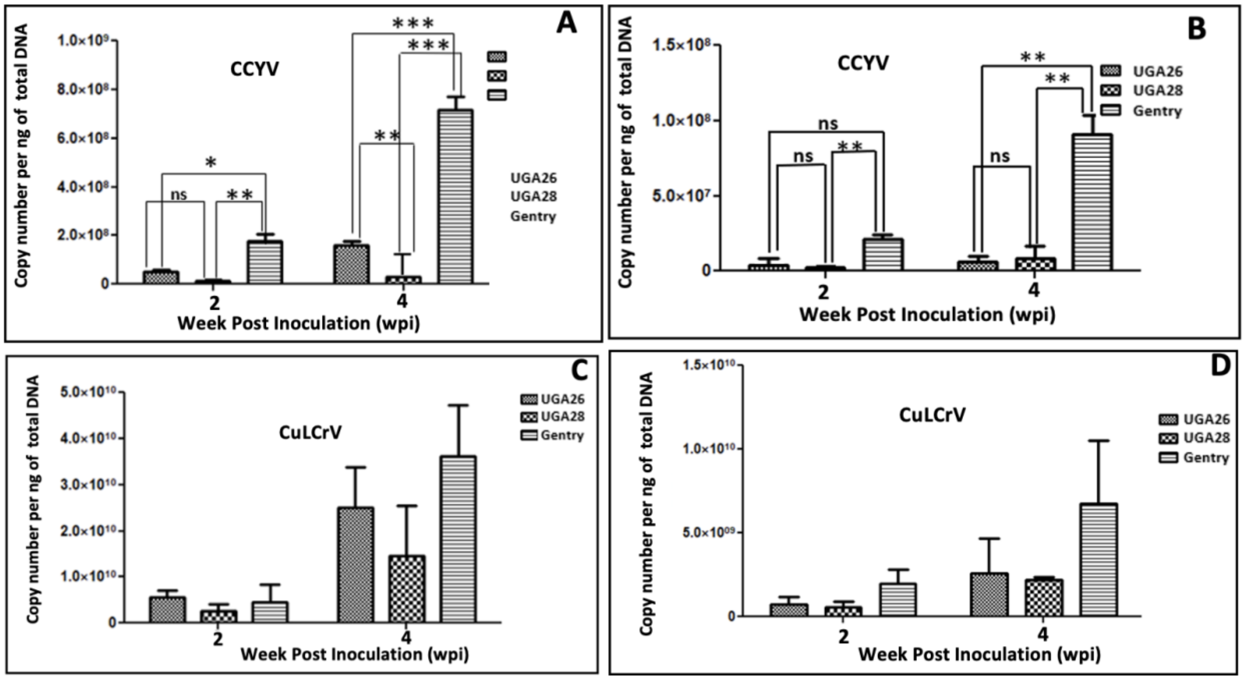

Fig. 4.8: Quantification and the virus accumulation in the inoculated plants. **A)** cucurbit chlorotic yellows virus (CCYV) first trial. Cucurbit chlorotic yellows virus (CCYV) second trial. **C)** cucurbit leaf crumple virus (CuLCrV) first trial **D).** cucurbit leaf crumple virus (CuLCrV) second trial. Each bar with standard errors represents an average of virus copies number per nanogram of DNA. Y-axis represents a logarithmic scale from the virus accumulation titer value. Significant differences between means were separated with Tukey's HSD test at $\alpha = 0.05$. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. UGA26 and UGA28 are plant introduction test lines while Gentry is the susceptible line.

DISCUSSION

Cucurbita pepo genotypes were evaluated against single infection of CCYV and CuLCrV in the greenhouse. In most cases, these two viruses together with CYSDV are usually in a mixed infection in the field, thereby making it nearly impossible to access the severity of individual viruses under natural production conditions. There is currently no resistant yellow squash cultivar to these viruses (Codod et al., 2022).

CCYV is an ssRNA bipartite virus member of the genus crinivirus (Okuda et al., 2010). CCYV is transmitted in the semipersistent mode by whiteflies (Celix et al., 1996). CuLCrV on the other hand is a ssDNA bipartite virus of the genus begomovirus transmitted in a persistent mode by whiteflies. Two yellow squash PI lines' response and virus accumulation were evaluated against CCYV and CuLCrV. Virus accumulation was recorded and compared at two-time points (2 wpi and 4 wpi).

Our results indicated that plants inoculated with CCYV showed chlorotic spots, interveinal chlorosis, and yellowing symptom of CCYV. UGA28 performed better than the susceptible Gentry and UGA26 as the onset of symptoms was delayed and the virus accumulation within the plant tissue was reduced.

With CuLCrV single infection, there was no significant difference in virus accumulation between both the PI lines and the Gentry throughout the experiment. UGA28 has a reduced disease severity and AUDPC to CCYV (Fig. 4.3 and 4.4) compared with UGA26 and Gentry. Furthermore, in CuLCrV, there was a significant difference between UGA26 and Gentry (Fig. 4.5 and 4.6). In field experiments, UGA26 was among the top 10 lines that performed better to CuLCrV load and symptom expression in mixed infection with CYSDV (Luckew et al., 2022). The reduction in virus load and symptom expression in UGA26 to CuLCrV under natural conditions might be due to the interaction of mixed infection with other viruses. Although in contrast to field evaluation by Luckew et al (2022), Gautam et al (2020) found that there was no reduction in CuLCrV virus load in both single and when co-infected with CYSDV in the greenhouse.

UGA26 (PI 171625) was also previously identified to be resistant to cucumber mosaic virus (CMV) (Lebeda and Kristkova, 1996). However, when compared with Gentry in the greenhouse, the line shows reduced CuLCrV symptom severity (Fig.4.5) but no reduced virus load (Fig.4.8C and D). In CCYV experiment, there was no reduction both in virus severity and virus load in UGA26. The severity of CCYV tends to reduce when co-infected with CYSDV (Orfanidou et al., 2021) but the transmission of one does not antagonize the other one, however, infected plants show reduced virus load compared with a single infection (Orfanidou et al., 2021). This phenomenon might be the reason why a high CCYV virus load was recorded in the plant tissue in single infections. To further confirm the tolerance level of these lines, a different combination of WTVs mixed infection will be needed to evaluate and confirm the plant responses.

Resistance to CYSDV has been previously identified in melon PI 313970 and TGR-1551 (López-Sesé and Gómez-Guillamón 2000; McCreight and Wintermantel, 2008; McCreight and Wintermantel, 2011). However, PI 313970 is not resistant to CuLCrV in either field and greenhouse (McCreight et al., 2008) and shows a typical yellowing phenotypic response when CYSDV co-infected with CCYV (Tamang et al., 2021). In addition, in melons, McCreight et al (2008) found a potential source of resistance to CuLCrV to greenhouse infection in eight different PI lines, but the plant response in natural field conditions is currently unknown. Complete resistance to CuLCrV was also found in MR-1 melon to both greenhouse and field conditions (McCreight et al., 2008).

A resistant variety must be able to tolerate a combination of different WTVs infections in both natural growing conditions and the greenhouse. This is challenging in a breeding program due to the whitefly population upsurge, and the source of resistant genes from the exotic germplasm (Tamang et al., 2021).

UGA26 and UGA28 have been previously screened under natural conditions for resistance to WTVs and were found to perform better based on phenotypic expression and reduced CuLCrV accumulation in mixed infection with CYSDV (Luckew et al., 2022). However, mixed infection of WTVs makes it nearly impossible to assess the phenotypic severity of individual viruses in a pathosystem. Furthermore, the response of these germplasm materials to CCYV infection was previously not assessed. Although mixed infection of these viruses can reduce the virus accumulation of both viruses in plant tissue, their transmission efficiency by *B. tabaci* was not affected (Orfanidou et al., 2021; Gautam et al., 2020).

Plant resistance to a virus prevents virus infection or limits the virus multiplication within the plant. On the other hand, tolerance to viruses enables an infected plant to recover from virus infection or to withstand the virus attack with less significant yield loss or symptom expression. Several mechanisms such as modulation of gene expression, regulation of metabolic pathways, and activation of defense responses contribute to tolerance to viruses in a plant (Mitter et al., 2013).

CONCLUSIONS

The use of host-resistant crops is the most efficient and effective management strategy for managing the whitefly-transmitted viruses of cucurbits. This study further elucidated the WTVs resistance in yellow squash to single infection of CCYV and CuLCrV in the greenhouse. The result showed that UGA28 is more tolerant as compared to UGA26 against single infection of CCYV infection.

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