Transmission and Host Plant Resistance of *Cotton leafroll dwarf virus* (CLRDV) in the Southeastern United States

By

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Abstract

Cotton leafroll dwarf virus (CLRDV) is an aphid transmitted *Polerovirus* responsible for severe yield losses in Brazil and is the first virus reported to infect cotton in the Southeastern United States. As a new strain, transmission and host plant resistance to CLRDV-AL are unknown and require investigation. Seed, mechanical, and grafting transmission were evaluated, an aphid-based screening assay protocol developed, and transmission by the aphid vector, *Aphis gossypii*, was characterized in this study. CLRDV-AL was not transmitted by seed or mechanical inoculation but was transmissible by grafting and *A. gossypii*. Aphids were shown to acquire the virus in 30 minutes, retain it for 23 days, and could inoculate in 15 minutes. The leaf disc assay proved that all lines tested could be inoculated with CLRDV-AL. Next, these screening protocols need to be utilized on a diverse range of germplasm to screen for resistance.

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List of Abbreviations

°C	Degrees Celsius
AAP	Acquisition access period
ACBD	Atypical Cotton Blue Disease
ASG	Accessory salivary gland
bp	Base pair
BYDV	Barley yellow dwarf virus
CBD	Cotton Blue Disease
CBTV	Cotton bunchy top virus
cDNA	Complementary DNA
CLCrV	Cotton leaf crumple virus
CLRDV	Cotton leafroll dwarf virus
cm	Centimeter
CtRLV	Carrot red leaf virus
ddH_2O	Double distilled water
EPG	Electrical penetration graphs
g	Gram
ha	Hectare
hr	Hour
IAP	Inoculation access period
kg	Kilogram
L	Liter
m	Meter

mg	Milligram
mil	Million
min	Minute
ml	Milliliter
mm	Millimeter
mM	Millimolar
mRNA	Messenger RNA
nM	Nanometer
ORF	Open reading frame
oz	Ounce
PCR	Polymerase chain reaction
PEMV	Pea enation mosaic virus
PLRV	Potato leaf roll virus
ppm	Parts per million
RH	Relative Humidty
RNAseq	RNA sequencing
RT-PCR	Reverse-transcriptase polymerase chain reaction
s	Second
SbDV	Soybean dwarf virus
spp.	Species
µl	Microliter
µM	Micromole

Chapter One

Literature Review

Cotton (*Gossypium* spp.) is a perennial plant that has been cultivated for 8,000 years (NCCA) due to its versatility in producing fiber, feed, and fuel (Innes, 1992). Worldwide, the United States is the largest exporter and third largest producer of cotton fiber. In 2019, the US produced 19.9 million bales of cotton valued at $6 billion, representing a direct portion of the $21 billion industry (NASS 2020; USDA-ERS 2020). Upland Cotton (*Gossypium hirsutum* L.) makes up 95% of the United States cotton production and is grown from California to Virginia (USDA-ERS 2019). *Gossypium barbadense*, also known as “Pima” or “Egyptian” cotton accounts for the remaining 5% of cultivated cotton in the United States. Although it produces longer and stronger fibers, it has lower yield compared to Upland cotton (Wendel et al. 1992). Both are tetraploid species although diploid species of cotton also exist.

Viruses in Cotton

Cotton, like many crops, is affected by a multitude of pathogens. There are over 20 viruses that are known to infect cotton, 16 of which are transmitted by hemipteran vectors including aphids and whiteflies (Brown 2001). Currently, the only confirmed viruses to exist in the United States are *Cotton leaf crumple virus* (CLCrV) and *Cotton leafroll dwarf virus* (CLRDV) that are transmitted by *Bemisia tabaci* and *Aphis gossypii,* respectively (Olsen and Silvertooth, 2001; Avelar et al. 2019; Heilsnis et al. 2020). CLCrV, a geminivirus, was identified in 1924 in the Southwestern United States but did not become economically significant until 1954 when ratooning cotton for perennial cultivation became widespread, causing yield losses of 16-85% (Brown 1992). CLCrV is controlled by a combination of resistant varieties and the

elimination of stub cotton as a growing practice. In 1991, a cross between Cedix and Deltapine 90 demonstrated resistance to CLCrV (Wilson and Brown, 1991). CLCrV has not been detected east of Texas so far (Idris and Brown, 2004).

***Cotton leafroll dwarf virus* (CLRDV)**

CLRDV is a member of the *Luteoviridae* family (Genus *Polerovirus*), which are monopartite, single stranded, positive sense RNA viruses (Harrison 1999). Other genera in the family include *Luteoviruses* and *Enamoviruses* (Dormier 2012). Viruses of the *Luteoviridae* family are 5.7-5.9 kb in length and are encapsidated in a 25-30 nm isometric icosahedral particle (Hull 2002b). They are difficult to transmit due to low titer, the restriction of particles to the phloem, and high vector specificity (Rochow and Duffus, 1981; Katis et al. 2007). Luteoviruses are phloem-limited and exclusively transmitted by aphids in a persistent, circulative fashion. Virus particles are introduced directly into sieve elements by aphids and are restricted to the phloem but can replicate in tissues outside of the phloem when other viruses capable of systemic infection are present (Goncalves et al. 2020; Taliansky and Barker, 1999; Hull 2002c). Transmission of luteoviruses are reliant entirely on the aphid vector in the field, as they are not reported to be mechanically or seed transmitted (Katis et al. 2007). General transmission characteristics of luteoviruses are that they take hours to days to acquire, are retained for weeks, and have a latent period of hours to days. They do not replicate in the vector and are not transmitted in a transovarial manner. Grafting has proven an effective means of luteovirus transmission for *Cotton bunchy top virus* (*Polerovirus*), *Potato leafroll virus* (*Polerovirus*), and *Peach-associated luteovirus* (*Luteovirus*) (Reddall et al. 2004; Derrick and Barker, 1997; Wu et

al. 2017). Grafting is a successful method for luteovirus transmission due to the direct phloem contact of the scion and rootstock.

Common symptoms of luteoviruses include yellowing or reddening of leaves, stunted growth, leaf rolling, and leaf brittleness, although symptoms vary depending on the age at which the plant was infected (Harrison 1999; Rochow and Duffus, 1981). Plants infected at a younger age typically have more severe symptoms and can result in death (Brown 2019; Cauquil and Vaissayre, 1971). Overall, symptoms of luteoviruses are often mild and can be mistaken as herbicide damage or nutritional deficiencies, leading to late diagnosis.

Luteoviruses are known to interact with other viruses in the host plant. Umbraviruses, which do not encode their own coat protein, are transcapsidated into helper luteovirus coat proteins for transmission by aphids (Hull 2002a; Falk and Tian, 1999). An aphid feeding on a mixed-infected plant can acquire and transmit both to a new host, resulting in a virus complex. However, if the receiving plant is a non-host for one of the viruses, only a single infection will occur. In addition, Luteoviruses will transcapsidate luteovirus-associated RNA's, satellite RNA's, and viroids, resulting in virus complexes. Carrot motley dwarf virus complex is an example of a triple-infection in which the luteovirus *Carrot red leaf virus* (CtRLV) encapsidates both an umbravirus (*Carrot mottle virus)* and a luteovirus-associated RNA, enabling aphid transmission of all three (Falk and Tian, 1999).

The first paper published on CLRDV was in the Central Republic of Africa in 1971 by Cauquil and Vaissayre who indicated the disease, "La maladie bleue" (Cotton Blue Disease, "CBD"), had been in the country since 1949 (Cauquil 1977). Other countries reporting CBD include Timor Leste, India, Thailand, Argentina, and Brazil (Ray et al. 2016; Mukherjee et al. 2012; Sharman et al. 2015; Distefano et al. 2010; Silva et al. 2008). CBD may have existed in

Brazil since 1937, however, PCR did not yet exist to confirm with molecular diagnosis (Costa and Forster, 1938). The Brazilian disease of 1938, "mosaico das nervuras" was described as having similar symptoms and sharing a vector, *A. gossypii,* with current day CBD. It is suspected to be either an early strain of CLRDV or closely related (Miranda et al. 2008). In 1962, a disease referred to as "vein mosaic var. Ribeirão Bonito" was observed in Brazil and Argentina and was believed to be a more virulent strain of the previously reported CBD (Costa and Carvalho, 1962; Miranda and Suassuna 2004). In 2005, Correa named CLRDV as the causal agent of CBD in Brazil using molecular characterization (Correa et al. 2005). From the nine clones isolated and amplified, Correa et al. (2005) determined that CLRDV was a result of a single Polerovirus.

Symptomatology and transmission have remained consistent across countries and years, with the primary symptoms being dark blue-green leaves that roll under, shortened internodes, epinasty, red petioles and veins, and yield loss (Brown 2001; Galbieri et al. 2017). Symptom severity decreases with increased plant age at the time of infection, as plants infected 50 days post-emergence had no yield and plants infected at 100 days post-emergence had yield losses of 15-20% (Brown 2001). Brazil has recorded yield losses of up to 80% in fields planted with susceptible cultivars (Silva et al. 2008). This yield loss is due to a combination of fewer flowers forming and increased boll shed. Infected plants produce poorer quality lint, particularly reducing both length and resistance (Cauquil and Follin, 1983). Plant mortality is fairly low, with fields averaging 3.4-4.5% death attributed to CBD (Cauquil 1977). Symptom remission has been documented in plants, with 1.2-6% of plants observed recovering from CBD. Remission is dependent on the age of the plant at infection and the amount of virus particles inoculated into the plant. All documents consistently report *A. gossypii* as the vector and vector competency work has ruled out *Bemisia tabaci* Genn., Hemitarsonemus spp., and Empoasca spp. as vectors

of CLRDV (Cauquil and Follin, 1983). A recent study sequencing viral genomes extracted from soybean aphids collected in China discovered CLRDV to be present in the aphid (Feng et al. 2017). However, transmission experiments involving *Aphis glycines* have yet to be reported.

Management strategies suggested by both Miranda et al. (2008, Brazil) and Cauquil (1977, Africa) include planting early, removing symptomatic plants from the field mid-season, clearing fields of all plants after harvest, removing weed hosts, and utilizing resistant cultivars. For control of colonizing aphids, rotating chemical control is suggested. Cauquil (1977) notes that *G. arboreum* is resistant to CBD, where the highly cultivated *G. hirsutum* and *G. barbadense* are not. Argentina's primary form of control comes from resistant cultivars and 99% of the varieties grown are resistant to typical CBD (Agrofolio et al. 2017).

Atypical Cotton Blue Disease

In 2006, a new strain of CLRDV was observed in Brazil that infected CBD-resistant cotton varieties (Silva et al. 2008). The disease caused by the new strain was termed atypical cotton blue disease (ACBD) or "de mosaic das nervuras atípico" (atypical vein mosaic disease) (Galbieri et al. 2010). Symptoms of the atypical CBD include mild typical CBD symptoms along with withered, reddish leaves and accentuated verticality or "whip-top" (Silva et al 2008; da Silva et al. 2015). The majority of varieties (90%) grown in Brazil have proven susceptible to ACBD and in 2017, ACBD was determined to be predominate over CBD in major growing areas (Chitarra and Galbieri, 2015; Galbieri et al. 2017). During the 2009/2010 growing season, ACBD was detected in northern Argentina, with the viral isolate having 95% amino acid similarity to the Brazilian isolate characterized by da Silva et al. (2015) (Agrofolio et al. 2017).

A third strain of CLRDV was discovered in the U.S. in 2017 and the completed genome sequence published in 2020 (Avelar et al. 2020). Characterization efforts involving CLRDV

isolates have determined two strains to exist in South America (CLRDV 'typical' and CLRDV 'atypical') and one in North America (CLRDV-US). The demarcation for a new strain in the family *Luteoviridae* is >10% divergence in amino acid sequence identity of any gene product (Dormier 2012). In the case of CLRDV, that product is the P0 gene located in ORF0 (Agrofolio et al. 2019; da Silva et al 2015; Avelar et al. 2020). In Brazilian and Argentinian isolates, the P0 genes of CLRDV-atypical were 86.1% and 87-88.2% similar to CLRDV-typical, respectively (da Silva et al. 2015; Agrofolio et al. 2017). The P0 gene of Alabama isolate CLRDV-AL is 82.4-88.5% similar to the isolates from South America (Avelar et al. 2020). Across all seven genotyped CLRDV isolates, ORF1-ORF5 have maintained 91.9% and greater sequence identity. Studies on *Poleroviruses* confirm that the P0 gene is the least conserved across the genus (Distefano et al. 2010). It is therefore unsurprising that the P0 gene is the source of resistance breaking in atypical CLRDV (Agrofolio et al. 2019).

Plants have a natural anti-viral defense mechanism referred to as the RNA silencing pathway in which viruses are neutralized via enzymes that digest the viral RNA into smaller fragments (Cascardo et al. 2015). Luteoviruses are capable of suppressing the RNA silencing pathway in the host plant via the P0 gene. Resistance to this suppression was discovered in *G. hirsutum* lines containing African source material (Fang et at. 2010). The gene conferring resistance to CLRDV-typical is a single dominant gene, *Cbd*, and is located on chromosome 10 of *G. hirsutum* (Fang et al. 2010; Agrofolio et al. 2019). Single dominant resistance genes are easy to breed with but are also more likely to be overcome by resistance-breaking viral isolates than either polygenic or recessive genes (Khetarpal, et al. 1998). *Cbd* may possibly confer resistance to CBTV, which shares 90% RdRp amino acid identity located in ORF2 (Ellis et al. 2013; Ellis et al. 2016; Agrofolio et al. 2019). Genetic mapping done by Ellis et al. (2016),

indicates that the *Cbt* gene is likely adjacent to the *Cbd* gene, however, this was based on two separate mapping populations. Flanking markers NC0204310 and NG0211495 were in a different order between the two populations, indicating a potential chromosome rearrangement event (Fang et al. 2010; Ellis et al. 2016). It is proposed that CBTV is the result of a recombination event between CLRDV and an unknown Polerovirus. CLRDV has also been shown to infect chickpeas in Uzbekistan, therefore presenting a danger to regions growing both chickpeas and cotton (Kumari et al. 2020).

Several cultivars resistant to CLRDV-typical, such as 'Delta Opal' and BRS '286' (Ellis et al. 2013; Galbieri et al. 2010), have tested positive for CLRDV-atypical and shown mild to no symptoms without significant yield loss (Silva et al. 2009; Galbieri et al. 2010; da Silva et al. 2015).

CLRDV in the United States

CLRDV is the first virus reported to infect cotton in the Southeastern United States and was first recognized in 2017 in Alabama (Avelar et al. 2019; ACES 2019a). The virus has since been detected in 13 cotton producing states from Virginia to Texas (Bag et al. 2020). Due to high whitefly pressure coinciding with disease symptoms in 2017, the pathogen was originally believed to be a begomovirus (Avelar et al. 2019). However, after shotgun sequencing both DNA and RNA, the causal agent was found to be *Cotton leafroll dwarf virus*. Further vector competency work confirmed *A. gossypii* as the vector (Heilsnis et al. 2020). Analysis of isolates collected from Barbour, Elmore, and Macon counties have 99% amino acid identity, strongly indicating that CLRDV-AL is the result of a single, and recent, introduction into the country (Avelar et al. 2020). Symptoms produced by CLRDV-AL vary by location and cotton cultivar but can typically be described as downward curling of leaves, reddening of the petioles, rugosity,

shortened internodes, accentuated verticality, and reduced boll set. These symptoms are similar to those caused by ACBD in Brazil. Yield loss in Alabama was estimated at 560 kg/ha, resulting in a total loss of $19 mil in 2017 (Avelar et al. 2019).

CLRDV has been shown to overwinter in common weeds such as white clover, henbit, evening primrose, and carpet weed (Kassie Conner, Personal Communication; Nichols 2019). Winter weeds and cotton regrowth act as virus reservoirs, enabling virus spread in the spring. Current recommendations in the U.S. include earlier planting dates, controlling weeds around cotton fields, and using resistant cultivars (Brown et al. 2019). Cultivar screening in the U.S. has shown that CLRDV-AL is capable of infecting CLRDV-typical and CLRDV-atypical resistant varieties from Brazil. As of 2020, there is no known commercial cultivar resistance to CLRDV-AL.

Aphis gossypii

The cotton or melon aphid, *Aphis gossypii*, is a major agricultural pest found worldwide in over 170 countries (CABI 2019). *A. gossypii* has the greatest host diversity and geographical range of all aphid species (Blackman and Eastop, 2007). Cotton and cucurbit crops are especially attractive to and affected by large *A. gossypii* populations, giving rise to the common names "cotton aphid" and "melon aphid". Approximately 1.5mm long, *A. gossypii* present in a range of colors from pale yellow to green and to black. Pale yellow is associated with poor host conditions and decline of the colony. Populations can double every 40 hours at 25°C on cucumber (Zamani et al. 2006). Aphids give live birth and lay an average of 3.1 nymphs per day and a 28.3 nymphs total at 25°C on cotton (Xia et al. 1999). Aphids are parthenogenic and reproduce asexually spring-fall on herbaceous hosts. In the southeastern U.S., *A. gossypii*

displays heteroecious life cycle behavior, alternating hosts between the winter and summer months (Williams and Dixon, 2007). Eggs laid in the fall hatch after winter to produce the viviparous fundratrix which give rise to the fundatrigenia, both of which are apterous (wingless) morphs. The fundatrigenia produce alates (winged) morphs that leave the primary winter host plant in search of herbaceous summer crops. This spring migration is also known as "first flight" and is used by epidemiologists and growers alike. For growers, an early first flight means that the crops are young and may not recover from a sustained infestation of aphids (Irwin et al. 2007). For epidemiologists, the first flight indicates the time and pattern an epidemic will take that year. The alates land on spring crops and birth apterous morphs which colonize the field. Flights of viruliferous alates landing in a field are considered the primary source of viral infection. Secondary spread occurs within a field when colonizing populations grow and spread to new plants and can be caused by alate and apterous morphs (Williams and Dixon, 2007). Parthenogenesis is the primary form of reproduction throughout the summer. Apterous morphs will produce alatoids (alate nymphs) in response to deteriorating host plant conditions, crowding, and unfavorable temperatures (Williams and Dixon, 2007). The alates will disperse and seek out new host plants to colonize. When the temperatures drop and day length decreases, alates are produced that fly back to the primary winter host. There, they birth the sexual morphs that will mate and lay eggs to overwinter.

Management Strategies

Common management strategies for aphids are primarily chemical based in the Southeastern U.S. At-plant insecticide seed treatments, or in-furrow applications of insecticides primarily used for thrips management are also reported to suppress early-colonizing aphid populations. Normally, aphid populations on cotton in the Southeast U.S. crash due to a fungus,

Neozygites fresenii, in July, negating the need for further chemical intervention (ACES 2019b; Greene 2015). Additional foliar sprays may be applied to manage populations mid-late season and are typically used when heavy aphid populations occur on young plants, or to prevent reduced lint quality caused by excessive feeding and the excretion of honeydew on open bolls.

Modes of Transmission

Aphids are one of the most important insect vectors for plant viruses with 190 aphid species reported to transmit approximately half of all insect-borne plant viruses (Katis et al. 2007; Nault 1997). Insect transmitted viruses are characterized by the time it takes to acquire, retain, and inoculate said viruses as well as the period of latency. In chronological order:

1. Acquisition access period (AAP): time it takes for the vector to ingest or acquire the virus.
2. Latent period: time it takes from acquisition of the virus until the vector is able to inoculate a susceptible host.
3. Inoculation access period (IAP): time it takes for a viruliferous host to inoculate a susceptible host.
4. Retention period: length of time after acquisition that a vector is capable of transmitting the virus.

Differences in these characteristics impact the design and implementation of vector-based disease management strategies and define the viruses' mode of transmission.

Virus transmission by insects is organized into three distinct modes: non-persistent, semi-persistent, and persistent. Non-persistent viruses make up 75% of the aphid transmitted viruses (Nault 1997). They are acquired and transmitted in under one minute and are viruliferous for minutes to hours. The speed of transmission is possible due to the virions adhering to the stylet

and foregut during feeding, and the expulsion of virions when the aphid excretes saliva into a punctured cell (Katis et al. 2007). Due to their location, virions are lost during molting. Non-persistent viruses invade several cell types, including epidermal, and are therefore readily available to probing aphids.

Semi-persistent viruses share several characteristics with non-persistent viruses including the ability to be acquired in minutes, the shed of virions with molting, and the inability to cross into the haemocoel (Katis et al., 2007; Harris 1977). What all five genera of semi-persistent viruses share that differentiates them from non-persistent is the increase of transmissibility with increased feeding periods (Harris 1977). Some of the genera also employ the use of helper components (Hull 2002a). These components aid in uptake, transmission, and movement within the plant, but are not exclusive to semi-persistent viruses.

Persistent viruses are cross out of the mid- or hind guts and into the vector haemocoel via receptor mediated endocytosis (Katis et al., 2007). There are two subcategories of persistent viruses: propagative and non-propagative (Hull 2002a). For propagative viruses, *Rhabdoviridae* is the only virus family that can replicate within the aphid vector (Sylvester 1980). Non-propagative, or circulative, viruses do not replicate in their insect vector. The number of virions present are in direct correlation with the length of time an aphid has fed. The majority of persistent circulative viruses belong to the *Luteoviridae* family, which are phloem limited. Virions are acquired with sustained feeding wherein the stylet reaches the phloem. For *Luteoviruses*, the fastest AAP time recorded was 15 minutes for *Rhopalosiphum padi* to transmit *Barley yellow dwarf virus* (Family *Luteoviridae*, Genus *Luteovirus*) (Gray et al. 1991). It is more common for AAP's to take several hours.

The persistent, circulative pathway inside the aphid vector is species specific, but generally follows the same trend. As an aphid feeds on sap, the virions are carried up the food canal, through the foregut, and accumulate in the mid- or hindgut (Gray and Gildow 2003). Which gut the viruses pass out of depends on the virus-vector relationship. Non-compatible viruses are excreted with the honeydew. Compatible virions are actively transported across the epithelial tissue via receptor mediated endocytosis and are released into the haemocoel (Hull 2002a; Gray and Gildow 2003). Viruses can survive several weeks in the hemolymph, although not much is known about what occurs during this time. After release into the haemocoel, virions are passively transported up to the accessory salivary gland. Within the ASG there are two barriers: the extracellular basal plasmalemma and the basal plasmalemma. If the vector-virus relationship is compatible, the virions will cross both membranes and mix with saliva to be expelled at the next feeding. The time it takes for the virions to cross the epithelial membrane, travel up to the ASG, and cross the plasmalemma barriers is referred to as the latent period. As a consequence of the gut and ASG barriers, there is a higher degree of vector-specificity with persistent viruses than with either non- or semi-persistent viruses. In the case of *Soybean dwarf virus*, SbDV-D virions can cross the gut membrane of both *Aulacorthum solani* (glasshouse-potato aphid) and *Myzus persicae* (green peach aphid), but not the basal plasmalemma of *M. persicae* (Gildow et al. 2000). Therefore transmission of SbDV-D occurs only in *A. solani* (Damsteegt et al. 2011).

Feeding Behavior

After landing on a plant, an aphid will probe to determine if the plant is a suitable host before committing to a sustained feeding. These test probes last 5-25 seconds (Nault and Gyrisco, 1966). During a probe, the aphid pierces the epidermis with its stylet and searches intercellularly for the phloem (Pollard, 1977). As the aphid maneuvers its stylet through the

epidermal layer, it samples and analyzes sap from the cells to orient itself (Will et al. 2013). Upon reaching the phloem, the stylet punctures the sieve tube, excretes saliva, and begins to ingest sap. Aphids are passive phloem feeders, relying on the turgor pressure of the host plant to ingest sap (Pettersson et al. 2007; Tjallingii 1997).

Electrical penetration graphs (EPG's) are an accurate method to determine aphid stylet activity while in the plant. To create an EPG, a host plant and aphid are connected by gold wire that runs through an EPG monitor (Walter 2000). When the aphid inserts its stylet into the plant, it completes the electrical circuit and the current activity can be monitored. The changes in waveforms on the graph are respective to aphid stylet activity inside the plant. Stylet activity is broken down into three phases: the pathway phase wherein the stylet enters the epidermis and probes, the sieve element or phloem phase where the stylet breaches the phloem and commits to a sustained feeding, and the final phase from sustained ingestion to stylet withdrawal (Tjallingii 1990; Xue et al. 2009). Literature analyzing EPG's has indicated that aphids do not always reach the phloem during probing and when they do, can spend days feeding from one sieve element. In terms of the first phase, *Rhopalosiphum padi* (Bird cherry-oat aphid) has been shown to reach the phloem of wheat plants in 18-24 minutes while Aphis fabae reached the phloem of broad bean plants in 45-72 minutes (Prado and Tjallingii, 1997). For *Aphis gossypii* feeding on *Cucumis melo* L. cv. Siglo, the aphids were able to reach the phloem in 7.72-105.17 minutes (Gonzalez-Mas et al. 2019).

The EPG results are in contrast with Michelotto and Busoli (2007) who recorded that a single *A. gossypii* alate was able to transmit CLRDV in 40 seconds. In that study, plants were grown out 60 days after inoculation and then scored for symptoms. Virus presence was not confirmed via molecular methods. A review of EPG experiments with *R. padi* demonstrated that

transmission of BYDV-PAV could occur without sustained feeding on phloem elements, leading Katis et al. (2007) to hypothesize that infection occurred from virions expelled via salivation during the brief cell punctures observed.

Aphid Transmission of Persistently Transmitted Luteoviruses

Experiments involving aphid transmission of *Luteoviridae* viruses include *Cotton bunchy top virus* (CBTV), CLRDV – 'la maladie bleue', CLRDV – 'mosaico da nervuras', and BYDV. In the case of CBTV and BYDV, several aphid species were tested and found that virus isolates were transmissible by one aphid species and not another (Ali et al. 2007; Gray et al. 1991). CBTV has been partially sequenced with results indicating that it belongs in the same genus as CLRDV, *Polerovirus*. The two viruses share 90% amino acid identity at the RNA-dependent-RNA polymerase and are the only known *Poleroviruses* to infect cotton (Ellis et al. 2013). Reddall et al. (2004) was able to achieve 100% transmission of CBTV by placing 100 viruliferous *A. gossypii* onto 20 twelve-node 'Sicot 70' cotton plants for 10 days. Plants were monitored for symptoms over 5 weeks. Ali et al. (2007) investigated further by examining the transmission rate of CBTV by 1, 3, 5, 10, or 20 *A. gossypii* per plant, allowing aphids an AAP and IAP of 2 days. A single aphid was able infect 5% (1/20) of plants with CBT, whereas groups of 3 or 5 aphids infected 50%. Groups of 20 aphids had the highest transmission rate that resulted in 80% of plants infected. These results indicate that CBTV is potentially a low-titer virus. AAP and IAP were also examined using groups of 20 aphids and it was found that the virus could be acquired in 5 minutes but took a minimum IAP of 1 hour to inoculate. 100% infection was achieved only after an IAP of 48 hours. Infection was determined by the presence of symptoms 5 weeks after treatment, not by PCR.

Studies on aphid transmission of CLRDV have been conducted in both Africa and Brazil. The earliest research was conducted in the 1970's in Africa on "La maladie bleue", believed to be caused by CLRDV (Cauquil and Vaissayre, 1971). Aphids were collected from symptomatic plants in the field and then confined on seedling cotton plants in groups of 15 using clip cages for an IAP of 24 hours. Transmission of "La maladie bleue" occurred in 91% of cotyledon stage cotton and 85% of 2-true leaf cotton, based on symptomatology.

In Brazil, studies were conducted using field collected cotton plants displaying archetypal symptoms of blue disease. Takimoto (2003) was unable to achieve transmission or acquisition in less than 60 minutes by groups of either 1, 3, 9, or 27 aphids. He was able to attain 50% transmission on a highly susceptible cultivar by placing 10 viruliferous aphids on each plant to feed for 3 days. In that experiment, it was demonstrated that more susceptible cultivars required fewer viruliferous aphids to develop symptoms while more resistant cultivars required a minimum of 10 aphids to develop symptoms. Michelotto and Busoli (2007) examined IAP and virus persistence in the aphid vector using single aphids. They determined that apterous aphids were able to transmit CLRDV for up to 12 days. Alates were more efficient at inoculating the virus, transmitting as early as 40 seconds whereas apterous did not transmit until 1.5 hours. Transmission increased as inoculation time increased with alates transmitting 60% at an IAP of 48 hrs. Overall, their study indicated that alates transmitted CLRDV more efficiently at shorter inoculation times (<1.5 hours) and apterous aphids transmitted more efficiently with longer inoculation times (>1.5 hours).

One of the most studied luteoviruses is *Barley yellow dwarf virus*. It is a group of luteoviruses that are transmitted by several aphid species (Gray et al. 1991). The results of an investigation between acquisition, transmission, and virus titer of three BYDV isolates and two

of its aphid vectors, *R. padi* and *Sitobion avenae*, indicated that BYDV could be acquired in 15 minutes, however, depending on aphid species and isolate combination, the AAP required to obtain transmission in 50% of the aphids varied from 1-12 hrs. Transmission efficiency was dependent on the virus isolate-aphid combination as well as the titer levels of the source leaves. *S. avenae's* ability to transmit its respective isolates was significantly reduced when the virus was acquired from source leaves containing low titer levels. Power et al. (1991), using the same isolates and aphid species as Gray et al. (1991) above, demonstrated that increased inoculation times increased transmission and that *R. padi* transmitted BYDV more efficiently that *S. avenae*.

There are many internal and external factors that influence virus transmission by an insect vector. The aphid itself has an impact on transmission efficiency, as not all aphids within a species transmit equally (Gray 1999). Large viral transmission discrepancies have been reported between clones of the same aphid species for BYDV, PEMV, and SbDV. In the case of BYDV, four biotypes of *R. maidis* (corn leaf aphid) were examined for transmission efficiency and found to transmit at 87%, 46%, 44%, and 28% (Saksena et al. 1964). Rochow (1960), demonstrated that Wisconsin collected clones of *S. graminium* transmitted the BYDV-SGV isolate efficiently whereas Florida collected clones transmitted inefficiently and the South Carolina clones not at all. Additionally, there are discrepancies between morphs and instars. For CLRDV, Michelotto and Busoli (2007) recorded that alates transmitted more often at shorter IAPs while apterous aphids transmitted more often at IAPs longer than 1.5 hours. Ali et al. (2007) reported that CBT virus transmission increased as instar stage increased, with adults transmitting 100%.

Virus titers vary in concentration within a host plant's tissues, most notably between old and new tissue. In their work with BYDV, Gray et al. (1991), found that older leaves of oat seedlings had significantly lower titer values than young leaves, which did and did not affect

transmission depending on the aphid species and virus strain combination tested. In only one of the four combinations (*R. padi* transmitting BYDV-RPV) did the difference in titer level not have an effect on transmission efficiency. While CLRDV titer concentrations within a host plant have not yet been quantified, the virus is more reliably detected from roots, stems, and petioles than from new growth (Kassie Conner, Personal Communication).

Abiotic factors, such as temperature, can also impact the transmission of luteoviruses. Damsteegt and Hewings (1987), demonstrated that *Aulacorthum solani* (glasshouse-potato aphid) exhibited higher transmission of SbDV between 20-22°C than at temperatures above 29°C and below 10°C. For BYDV-PAV, a 5°C increase in temperature halved the latent period for *R. padi* from 62 h at 15°C to 32 h at 20°C (van der Broek and Gill, 1980). An additional BYDV study found that transmission of BYDV-RPV by *R. Padi* and *M. avenae* increased as temperatures increased from 15°C to 30°C (Rochow et al. 1969). *M. avenae* did not transmit when the acquisition and inoculation temperatures were below 20°C and 25°C, respectively. In contrast, that same study found that temperature had no effect on the ability of *R. maidis* and *S. graminum* to transmit BYDV-RPV. Temperature has been shown to affect both aphids and the infected host plant. Syller (1987) noted increased transmission of *Potato leaf roll virus* (PLRV) from infected plants kept at 12°C prior to the acquisition access period, but higher transmission when acquisition and inoculation occurred at 26°C. Additionally, the acquisition period of PLRV was more sensitive to temperature changes than the inoculation period.

Disease Management Strategies

Control of luteoviruses is based on preventative measures. Cultural practices such as planting date and density can alter incidence of aphid transmitted viruses by affecting timing of infection and landing behavior (Katis et al. 2007). Reducing weeds around fields and removing

the previous year's plants after harvest can also decrease virus reservoirs. Chemical control is difficult, as aphids can transmit viruses in minutes after landing. Systemic pesticides are capable of slowing down the spread of a virus in a field but cannot completely prevent it (Hull 2002d). The most effective and long-term solution to insect-transmitted viruses is growing resistant plant genotypes (Rochow and Duffus, 1981). There are three types of resistance: immunity, cultivar resistance, and acquired resistance (Hull 2002c). Immunity is defined as the inability of an introduced virus to replicate in the hosts' cells. If a whole species is immune, then they are considered non-hosts. Cultivar resistance is when some cultivars in a species are resistant and some are not. Lastly, acquired resistance occurs when a susceptible individual is inoculated with a virus and gains resistance. Acquired resistance is non-heritable (Fraser 1998). Cross protection is a form of acquired resistance where a mild strain of a virus is used to confer protection against more severe strains (Hull 2002d). This is not a common practice as identifying a mild strain that provides systemic protection, is genetically stable, and is not easily distributed to neighboring crops is challenging. Additional problems may arise if mixed infections occur that result in reduced protection by the mild strain or induce a synergistic response.

The primary focus for plant breeders is cultivar resistance. Most virus resistance genes are monogenic (78%) and dominant (51%), and resistance-breaking isolates occur more frequently in this group than in recessive or polygenic resistance (Fraser 1998; Khetarpal et al. 1998). These genes confer resistance through mechanisms such as vector resistance, resistance to virus movement, resistance to virus multiplication, and hypersensitivity (intolerance) (Barker and Waterhouse, 1999). Dominant genes have a strong correlation with resistance mechanisms that prevent virus movement from the point of inoculation (Fraser 1998). In gene-dose dependent resistance, virus replication is reduced, especially in homozygous plants. The mechanisms can be

classed as either constitutive or induced. Constitutive resistance inhibits essential viral functions whereas induced resistance is a defense reaction by the host plant stimulated by the presence of the virus (Khetarpal et al. 1998). A hypersensitive local response occurs when the host plant initiates programmed cell death and kills healthy cells surrounding the infected cells to quarantine the virus (Hull 2002c). This is seen as necrotic lesions on the surface of the tissue. Tolerance to a virus is resistance to symptom development as the virus infects and replicates in the host plant (Hull 2002d) and is also referred to as 'field resistance' by plant breeders. Tolerant cultivars are asymptomatic and act as a virus reservoir. Opinions on breeding for tolerance differ. One school of thought disregards tolerance as an acceptable breeding objective given that it allows for fields to become virus reservoirs and those same fields provide increased opportunity for resistance-breaking mutations (Hull 2002d). The competing school of thought believes tolerance to be an acceptable method, as it is less likely to be overcome than monogenic dominant resistance and less resource-intense to achieve (Salomon 1999; Hull 2002d).

Host Plant Resistance to Aphids

Host plant resistance to aphids can take the form of anatomical, physiological, or biochemical resistance and are categorized into two main types: antixenosis and antibiosis (van Emden 2007). Antixenosis is the resistance to colonization and is determined by the proportion of alates that take off after landing. Mechanisms include off putting color, palatability, waxiness, physical resistance to feeding, local necrosis, and trichomes that prevent alates from landing. These are primarily physical obstructions to aphids as compared to antibiosis, whose mechanisms are primarily chemical. Antibiosis resistance principally consists of toxins that reduce aphid survival on the host plant. In cotton, that chemical is the polyphenol gossypol. Gossypol is a secondary metabolite stored in cotton glands that is produced as a defensive

measure and has recorded toxicity to bollworms, nematodes, fungi, and is poisonous to monogastric animals (Bell 1986). Aphids feeding on cotton plants with high gossypol content had reduced lifespans and lower fecundity than aphids feeding on low-gossypol plants (Du et al. 2004). Cottonseed is considered a high protein feed source for livestock and recent breeding efforts have concentrated on producing low-gossypol cultivars (Cherry et al. 1986; Cai 2010). However, this has resulted in glandless cotton plants being infested with insect species that were not previously recorded to feed on glanded cotton and plants accrued more damage from pests, birds, and rodents (Bell 1986).

Grafting

Grafting is an ancient plant propagation technique that joins two or more plant tissues together. Common uses of grafting include asexual propagation, disease management, virus indexing, and size management (Mudge et al. 2009; Ahktar and Haq, 2003; Lee and Oda, 2003). Tissues used in grafting vary based on the desired result. Roots, buds, stems, and terminal tips are typical tissues used in grafting. A graft consists of a scion and rootstock, and in rare cases an interstock. Interstocks are inserted between a scion and rootstock and are primarily used to overcome graft incompatibilities. The rootstock is the recipient tissue containing the roots in a graft. Conversely, the scion is the tissue being introduced that will grow to become the top portion. Once the scion has been cut, it is necessary to place the scions in bags and coolers to retain moisture and reduce transpiration until the scions are ready to be grafted. Regardless of the graft type used, the vascular cambium of the rootstock and scion need to be aligned for the graft to take. If the graft is rejected due to misalignment of the cambiums or incompatibility, the scion will die.

Callus tissue grows to bridge the gap between the scion and rootstock. Cambial cells will differentiate from the callus to form new xylem and phloem, connecting the scion and rootstock (Janick 1986). Once the union is established, water, photosynthates, mRNAs, and virions can cross from scion to rootstock (Lee and Oda, 2003; Harada 2010).

Graft type is dependent on the relative size of the rootstock and scion. Grafts using the same size scion as rootstock include whip-and-tongue, wedge, saddle, and approach grafts. Grafts using dissimilar sized rootstock and scions include side-veneer, t-graft, chip budding, and cleft. Chip budding is a common practice for virus indexing of agricultural trees. Virus indexing is the use of indicator plants that are sensitive to the pathogen being screened and will exhibit characteristic symptoms of the disease after inoculation (Laimer 2003). In virus indexing, the potentially diseased bud tissue is always grafted onto the indicator plant. Performing the reverse is discouraged due to a lack of clear, defined symptoms produced in the growing bud (Dijkstra and de Jager, 1998).

The type of graft used depends on the tissue type used and the desired end goal. In fruit tree grafting, it is a common practice to use chip buds in a t-graft in order to extend a small supply of scions over a large quantity of rootstocks. In tomato and watermelon grafting, the entire top portion of a one-two week old seedling is replaced with a desirable cultivar of similar size using pin-hole grafting (Lee and Oda, 2003).

As a perennial, cotton has two stages of growth. Herbaceous tissue is produced first, which is easier to graft, followed by lignification of that same tissue, turning it woody. It is possible to graft woody cotton plant tissue; however, young herbaceous tissue is preferred due to its fast growth, bark slippage, and higher percentage of graft take.

Grafts can die quickly if suitable healing conditions are not observed. In the case of herbaceous grafts, high humidity and low light conditions are essential. Humidity is necessary because the scion has no source of water until the callus forms. The scion can dehydrate and die before the graft has healed. Use of fully turgid scions is recommended for both ease of grafting and for graft success. Humidity is raised by firstly wrapping the graft union in parafilm or buddy tape, ensuring overlapping coverage above and below the graft as well. Second, the graft is bagged, placed in an incubator, or placed in a mist chamber to keep the humidity above 90%. For woody grafts performed during the dormant season, buddy tape is sufficient. Once graft union is established, humidity is slowly decreased as plants are reacclimated to standard growing conditions. Lighting is reduced to decrease transpiration and reduce the stress load photosynthesis places on the scion tissues.

Virus Transmission by Grafting

In the case of phloem-limited viruses such as *Poleroviruses* wherein mechanical transmission fails, viruses can be transmitted by grafting. Union of the cambial tissues is not necessary for viral transmittance and neither is the survival of the diseased scion (Dijkstra and de Jager, 1998). Contact between diseased and healthy phloems is all that is needed for the virus to transfer. Prolonged contact increases the chances of infection. Due to this, interfamilial grafting for virus transmission is possible. Traditionally, interfamilial grafts are incompatible; callus will not form and the scion will die. By using an approach graft in which both the diseased donor plant and the healthy recipient retain their own roots and stems, turgidity and subsequent phloem movement can be maintained, giving the virus time to cross into the recipient plant.

Grafting with the intent of virus transmission circumvents the need for the vector. This is useful for when the vector is difficult to acquire, maintain, or rear. The disadvantage to grafting

viruses is that all pathogens present in the diseased donor plant will be transferred to the recipient plant. Akhtar and Haq (2003) developed a grafting screening protocol for *Mungbean yellow mosaic virus* in Mungbean due to difficulties and inconsistencies in using the whitefly vector to screen. Using an approach graft, the researchers were able to introduce infected scions to rootstock for screening. Koeda et al. (2017) used a combination of agroinoculations and grafting to transmit *Pepper yellow leaf curl Indonesia virus*, a whitefly-transmitted bipartite begomovirus, to pepper plants. Initial agroinoculations resulted in 55-75% infection and subsequent cleft grafting, using the confirmed infected agroinoculated plants as scions, resulted in 100% infection. Infection was confirmed using PCR. Grafting viruses in cotton has also proven to be successful. Akhtar et al. (2002) used a modified approach graft, "bottle-shoot graft", to transmit *Cotton leaf curl virus* to six-week cotton plants with 100% success rate. Reddall et al. (2004) and Ali et al. (2007) both included grafting in their work on *Cotton bunchy top disease*. Reddall used a wedge graft on seedlings, approximately 4 weeks old, to transmit CBT with 100% infection. Ali grafted CBT infected scions to rootstocks for the purpose of examining the effect of the virus on the root system. All CBT grafted plants demonstrated a drastic root aberration compared to the healthy grafts.

Objectives

In conclusion, there is limited information on transmission of CLRDV. Therefore, the focus of this study is to characterize the mode of transmission of CLRDV-AL and to develop screening methods for the virus. The objectives are:

1) Determine if the virus can be seed transmitted and/or mechanically inoculated
2) Characterize the mode of transmission in *A. gossypii*

3) Develop an aphid-based leaf disc screening assay

4) Investigate various grafting methods for virus transmission

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Chapter 2

Seed and Mechanical Transmission of *Cotton leafroll dwarf virus* in *Gossypium hirsutum*

Introduction

Cotton leafroll dwarf virus (CLRDV) is an emerging threat to the Southeastern U.S. cotton industry. It has been studied in Brazil, where up to 80% yield loss has been observed in cotton growing regions (Silva et al. 2008). Molecular characterization in 2005 and 2015 identified two separate strains existing in South America with the second strain, 'CLRDV-atypical', capable of infecting CLRDV-typical resistant varieties (Correa et al. 2005; da Silva et al. 2015). The strain in the US is classified as a distinct third strain and has been detected in both CLRDV-typical and CLRDV-atypical field resistant varieties (Avelar et al. 2020; Brown et al. 2019). First noticed in Southern Alabama in 2017, CLRDV-AL was initially identified by RNAseq analysis in 2018 (Avelar et al. 2019). Work is currently being performed across the cotton belt at both private and public institutions to characterize and understand this emerging virus.

CLRDV is in the genus *Polerovirus* (family Luteovirus) which are phloem-limited viruses transmitted by aphids in a persistent, circulative fashion (Katis et al. 2007). Aphid transmission by *Aphis gossypii* has been confirmed in all three reported strains of CLRDV (Cauquil and Vaissayre, 1971; Miranda et al. 2008; Heilsnis et al. 2020). In early 2019, CLRDV-AL was detected in the seed coat of cottonseeds harvested from infected plants in Baldwin Co., AL, and raised concern of possible seed transmission. If seed transmission of an infectious agent was to be discovered, the cotton industry would be quarantined until phytosanitary controls could be established and could be potentially devastating.

Luteoviruses are not reported to be seed transmitted (Katis et al. 2007). However, seed transmission has been reported in one-seventh of known plant viruses (Hull 2002) and when co-infection with compatible *umbraviruses* occur, such as with *Pea Enation Mosaic Virus* (PEMV-2), luteoviruses have been detected in the embryo (Timmerman-Vaughan et al. 2009). Although seedborne, seed transmission was not detected in the case of PEMV. Rates of seed transmission in viruses are strain-specific and have been documented below 1% (Hull 2002; Njeru et al. 1997). CLRDV is a low-titer virus and could be replicating at extremely low levels.

There are two general types of seed transmission: infection of the seedling by mechanical damage from the seed coat, and more commonly, infection of the embryo. Seed transmitted viruses can persist for years inside a seed, and with commercial distribution, can be spread long-distances. Therefore, the objective of this study is to investigate both seed and mechanical transmission for CLRDV-AL in order to understand the mode of transmission.

Materials and Methods

In 2018, a commercial field growing the cultivar, NextGen 5711, in Baldwin County, AL, exhibited severe symptoms of CLRDV including: accentuated verticality, excessive vegetation, and absence of bolls in the upper half of the canopy. The upper most open boll was collected from ~50 plants. The number of seeds per boll and size were reduced in infected bolls. In addition to their small size, some of the collected seed showed evidence of malformation including sunken sections in the seed coat and splitting of the seed coat along the seam, exposing the embryo (Fig. 1). Incidence of CLRDV in this field was documented to be 100% which was confirmed through PCR. Seed coat experiments and one seed transmission utilized this seed source.

A large research trial was conducted in Elmore county in 2019. Three varieties that tested positive for CLRDV were selected, *G. hirsutum* 'Delta Opal', *G. hirsutum* 'Macha WR 2', *G. barbadense* 'Tidewater (Seabrooks)', to be grown out for seed transmission studies. In addition, a commercial bag of 'DeltaPine 1646' was used to test for presence in a commercially available source.

Seed Coat Detection

Cotton fiber was separated from the seed by hand and seed was delinted with 93% sulfuric acid. The seeds were soaked in 10% bleach for 2 min and rinsed with ddH_2O. The seed coat from 25 seeds were excised from the internal embryo and RNA was extracted from each seed coat and embryo separately using RNeasy Plant Mini Kit (Qiagen, Germantown, MD) following manufacturer's recommendations. Modifications to the manufacturer's protocol are as follows: Prior to step 1, plant tissue was prepared by finely chopping 100mg petiole tissue using razor blades then transferred into a 2ml bead beating tube (Biospec XXtuff Reinforced Microvials) containing two 5mm borosilicate glass beads (KIMAX®). The tubes were then flash frozen in liquid nitrogen for five minutes before being loaded into a bead beater (BioSpec Mini-Beadbeater™, Bartlesville, OK) and disrupted for 15 seconds. Tubes were removed and the ends sharply hit against each other to dislodge the compressed, powdered tissue. Tubes were refrozen in liquid nitrogen for an additional two minutes before being disrupted a second time in the bead beater. Tubes are removed and the ends sharply hit against each other to dislodge the compressed, powdered tissue from the ends of the tube. Extractions continued using the RNeasy® Plant Mini Kit following the manufacturer's instructions from step 2 onwards.

cDNA was synthesized using SuperScript IV first strand synthesis system (ThermoFisher Scientific, Waltham, MA) performed in a 20 µl reaction containing: 2 µM Pol3982R, 10 µM

dNTP mix, 6 µl ddH_2O, and 5 µl RNA in the annealing step and 4 µl 5xSSIV buffer, 1 µl 100mM DTT, 1 µl ribonuclease inhibitor, and 1 µl SuperScript® IV Reverse Transcriptase for the RT step under conditions described by the manufacturer. The cDNA was then amplified with CLRDV-specific primers CLRDV3675F/Pol3982R targeting a 307 bp section of the coat protein gene (Table 1). Positive (plants that had previously tested positive) and negative (plants that had been grown in a controlled environment in the absence of aphids) controls were included. PCR was performed in 25 µl volumes containing: 200 nM of each primer, 200 mM dNTPs, 1.75 mM MgCl2, 2.5 µl cDNA template, 17.585 µl ddH2O, 2.5 µl 10X PCR buffer, and 1 unit Platinum Taq polymerase (Invitrogen). PCR cycling conditions were comprised of an initial denaturation of 95°C for 60 s, then 35 cycles of: 95°C for 15 s, 62°C for 20 s, 56°C for 10 s and 72°C for 40 s; followed by a final annealing of 72°C for 3 min, carried out using a Bio-Rad thermal cycler (Bio-Rad T-100™, Hercules, California). PCR products were examined by gel electrophoresis.

Seed Transmission

NextGen 5711 seeds collected in January 2019 from a PCR-confirmed infected field in Baldwin Co., AL were sown in 4 inch pots (The HC Companies Inc., Middlefield, OH) filled with peatlite (PRO-MIX 'BX', Quakertown, PA) and grown out for 28 days in a growth chamber (Conviron, Winnipeg, Canada) at 25°C, 12/12 light cycle, and 50% RH. Fertigation with 250ppm 20-10-20 (Everris NA Inc., Dublin, OH) began 14 days after emergence. Plants were visually observed every two days for reported symptoms including red stems and petioles, leaf curling, and blue tinting. A total of 157 plants were tested.

Seed harvested in October 2019 from PCR-confirmed infected field plants was sown in 300s pots (Nursery Supplies®, Chambersburg, PA), placed in constructed insect-free cages made of 100 micron screen (Econet 1515, AB Ludvig Svensson, Charlotte, NC) in a full sun

greenhouse, and grown out for 60 days in July 2020 under induced stress conditions. To create stress conditions, fertilizer was withheld completely and plants were maintained in a drought condition without killing them. 100 plants from each line were selected and compositely tested in groups of five.

The second most expanded leaf with its attached petiole was harvested from each plant for total RNA extraction as described above. Nested PCR was used for the seed transmission grow out test as CLRDV is a low-titer virus and a nested protocol increases the sensitivity of detection. cDNA was synthesized as described above, with the substitution of Pol4021R in place of Pol3982R. First round primers Pol4021R and Pol3628F amplified a 393 bp fragment (Table 1). The first round PCR product was diluted in ddH_2O (1:4) and amplified with the CLRDV-specific primers listed previously, CLRDV3675F/Pol3982R. Both PCR reactions were performed as described above.

Mechanical Transmission

CLRDV-infected leaves were collected from infected plants transplanted from the field and maintained at the Plant Science Research Center greenhouses in Auburn, AL. Inoculum buffer was prepared by grinding the infected leaves with 50 mM potassium phosphate and 10 mM sodium sulfite in an ice-cold mortar and pestle until a homogenized slurry was formed. The mortar containing the inoculum was kept on ice while the recipient seedlings were prepared.

Twenty 2-3 true leaf, healthy cotton seedlings were sown in 6-packs (The HC Companies Inc., Middlefield, OH), grown in an incubator (Percival Scientific Inc., Perry, IA) and tested for mechanical inoculation. A single true leaf per plant was selected and lightly dusted with carborundum powder. Cheesecloth was then soaked in the inoculum and gently wiped 2-3 times

onto each selected leaf. Seedlings were returned to the incubator for 30 days. Total RNA was extracted and single round PCR performed as described above.

Results and Discussion

Seed Coat Detection

CLRDV was detected in all 25 seed coat samples. It was not detected in the embryo samples. This finding is consistent with phloem tissue being present in the seed coat but not in the embryo, supporting the phloem-limited nature of *luteoviruse*s (van Dongen et al. 2003; Katis et al. 2007).

Mechanical Inoculation

Cottonseed hulls have been known to damage cotyledons during emergence, and with CLRDV present in the seed coat, mechanical inoculation from seed coat to embryo by tissue damage is possible. However in this study, CLRDV was not detected in any of the mechanically inoculated samples, consistent with transmission characteristics of the Luteoviridae family and recent studies (Katis et al. 2007; Reddall et al. 2004). This suggests that transmission of CLRDV from the seed coat to the embryo during germination is unlikely to occur. However, mechanical transmission of luteoviruses has been shown to occur when present in a mixed infection with an *umbravirus* (Ryabov et al. 2001; Mayo et al, 2000; Doumayrou et al 2016). Therefore, further work on multiple virus interactions may be warranted to determine seed transmissibility of CLRDV.

Seed Transmission

CLRDV is a low-titer virus and stress conditions are known to weaken the plant's defenses (Hatmi et al. 2018), potentially allowing for increased viral incidence. Therefore, the

growing protocol was altered between the first and second years to prevent false negatives. Regardless, none of the 557 plants grown out in either year tested positive for CLRDV, confirming that it is unlikely that CLRDV-AL is seed transmitted (Table 2).

U.S. cottonseed production occurs primarily in Arizona and CLRDV has not yet been reported west of Texas (Alabi et al. 2020). However, it is unknown if the virus has been spread by commercial production. The results from the DeltaPine 1646 commercial seed test indicate that U.S. grown cottonseed available to growers is not a source of CLRDV infection, however more testing is needed to confirm. This result is consistent with the observation that luteoviruses are known to be phloem restricted. The appearance of the NextGen 5711 seed, (small size and malformation, Figure 1) and low germination rate (30%) indicate a severe issue for commercial seed growers if their crop becomes infected. Other potential issues for commercial seed production include the reduced yield of infected plants due to boll drop and the reduced number of seeds per boll (Brown 2001; Silva et al. 2008).

Conclusion

In conclusion, the negative results obtained in seed and mechanical inoculation transmission studies are consistent for luteoviruses. While it is highly unlikely the CLRDV-AL is seed transmitted, seed transmission rates may be below 0.5% (Njeru et al. 1997). This is reassuring, as the cotton industry would be severely impacted if cottonseed production were restricted. Although seed transmission is unlikely, future work should focus on testing larger quantities of seed from infected plants and multiple virus interactions to provide a more definitive answer.

Table 1. CLRDV-specific primers for Nested PCR

Primer Name	Sequence (5' to 3')	Expected product size (bp)	Target Region	Reference
Nested PCR Round 1				
Pol3628F	TAATGAATACGGYCGYGGSTAG	393	CP	Sharman et al. 2015
Pol4021R	GGRTCMAVYTCRTAAGMGATSGA			This Study
Nested PCR Round 2/Single Round Primers				
CLRDV3675F	CCACGTAGRCGCAACAGGCGT	307	CP	Sharman et al. 2015
Pol3982R	CGAGGCCTCGGAGATGAACT			Sharman et al. 2015

Table 2. Results of seed transmission for *Cotton leafroll dwarf virus*

Year Tested	Variety	Seed Source	Infected source[a]	Species	Positive Plants/Total Plants Tested[a]
2019	NextGen 5711	Baldwin Co	Yes	*G. hirsutum*	0/157
2020	Delta Opal	Elmore Co	Yes	*G. hirsutum*	0/100
2020	Macha WR 2	Elmore Co	Yes	*G. hirsutum*	0/100
2020	Tidewater (Seabrooks)	Elmore Co	Yes	*G. barbadense*	0/100
2020	DeltaPine 1646	Commercial	No	*G. hirsutum*	0/100

[a]Confirmed by using PCR

Figure 1. Damaged seeds from heavily infected CLRDV plants

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Chapter 3

Characterizing the Mode of Transmission for *Cotton leafroll dwarf virus* by *Aphis gossypii* in the U.S.

Introduction

Cotton leafroll dwarf virus (CLRDV) is an aphid-transmitted *Polerovirus* (family Luteoviridae) that infects upland cotton in the American and African continents (Avelar et al. 2019; Bag et al. 2020; Distefano et al. 2010; Silva et al. 2008; Miranda et al. 2008; Cauquil and Vaissayre, 1971). In North America, it was first observed in coastal counties of Alabama in 2017 and has since been identified in 13 cotton producing states across the South (Avelar et al. 2019; Bag et al. 2020). Symptoms include downward curling of leaves, reddening of the petioles, rugosity, shortened internodes, accentuated verticality, and reduced boll set (Avelar et al. 2020; Bag et al. 2020). CLRDV has been most extensively studied in Brazil, where symptoms were first documented in 1938 (Miranda et al. 2008). In 2006, a resistance-breaking isolate of CLRDV was observed infecting CLRDV-resistant fields in Mato Grosso, Brazil (Silva et al. 2008). Completed genomes in 2015 identified the resistance-breaking isolates as a new strain of CLRDV, referred to as CLRDV-atypical (da Silva et al. 2015). CLRDV-atypical is reported to cause yield losses up to 80% (Silva et al. 2008). The strain in the U.S. is classified as a third strain and has been detected in both CLRDV-typical and CLRDV-atypical field resistant cultivars (Avelar et al. 2020; Brown et al. 2019). For example, the variety 'BRS 286' has shown field resistance to CLRDV-typical and CLRDV-atypical in Brazil (Galbieri et al. 2017), however this variety appears to be susceptible to CLRDV-AL. In other parts of the world *Aphis gossypii* Glover is reported as the primary vector responsible for spreading CLRDV in the landscape (Ray et al. 2016; Mukherjee et al. 2012; Sharman et al. 2015; Distefano et al. 2010; Silva et al. 2008;

Heilsnis et al. 2020; Cauquil and Follin, 1983) and this cosmopolitan species is distributed throughout the U.S. cotton belt. Knowledge on how quickly *Aphis gossypii* acquires and transmits CLRDV-AL is needed to understand spread and devise management strategies for disease caused by this virus.

The mode of transmission of an insect-transmitted virus characterizes how long it takes for a virus to be acquired (acquisition access period, AAP), the time it takes for a virus to circulate in the vector before it can be transmitted (latent period), how long it takes for a vector to feed in order to transmit the virus (inoculation access period, IAP), and how long the virus can be retained and transmitted by the vector (retention time). An understanding of these four parameters is critical for devising disease management strategies. Disease management strategies typically include a combination of host resistance, cultural, biological, and chemical controls tailored for individual pathosystems (Jones, 2004), and a strategy needs to be developed for CLRDV-AL in the U.S. Historically, luteoviruses are controlled using preventative measures including altering planting date and density, reducing weed hosts, and growing resistant cultivars (Katis et al. 2007). Chemical control is difficult as aphids can transmit viruses quickly after landing, and often before an insecticide causes a cessation of feeding or mortality of the vector. Knowledge on the speed at which aphids can acquire and inoculate viruses is key to developing disease management strategies.

Aphis gossypii adult apterous and alates are capable of transmitting the virus (Michelotto and Busoli, 2007; Heilsnis et al. 2020), and previous studies from Brazil characterized inoculation access periods and retention time for CLRDV (Michelotto and Busoli, 2007). CLRDV-typical can be retained for up to 12 days by the apterous aphid. Alates are reported to transmit CLRDV-typical in 40 seconds and apterous are reported to transmit in 1.5 hours.

Transmission rates increased with increased feeding times in both morphs. Michelotto and Busoli (2007) also reported CLRDV to be transmitted in under 40 sec of feeding by alate aphids. Previously, the fastest inoculation time recorded for a luteovirus was 15 min (Watson and Mulligan, 1960). A closely related cotton infecting polerovirus, *Cotton bunchy top*, was reported to be acquired by *A. gossypii* in 5 minutes (Ali et al. 2007). The time required to acquire CLRDV-typical by *A. gossypii* was not characterized by Michelotto and Busoli (2007).

The objectives of this study were to characterize the speed at which alates and apterous are able to acquire and inoculate CLRDV-AL, as well as how long virions are retained in the aphid body over the course of the aphid's lifetime.

Materials and Methods

Rearing and Maintenance of Healthy Cotton Plants

Healthy cotton plants were grown in a greenhouse in 1.6m x 0.7m x 1.6m cages covered with 100-micron insect-proof screen (AB Ludvig Svensson, Charlotte, NC). Seed was sown in six-packs (The HC Companies Inc., Middlefield, OH) filled with peat-lite (PRO-MIX 'BX', Quakertown, PA), watered as needed and fertilized weekly with 20-10-20 (Everris NA Inc., Dublin, OH). Seedlings were maintained in this manner for use as leaf disc material.

Rearing and Maintenance of CLRDV Infected Cotton Plants

CLRDV infected cotton plants were obtained from cotton plots in the fall of 2018. Plants were transplanted into 3 gallon containers (Nursery Supplies®, Chambersburg, Pennsylvania), and maintained in a greenhouse in the same manner as the healthy cotton until use in this study.

Rearing of Insect Colonies

A non-viruliferous *Aphis gossypii* colony is maintained in a greenhouse at the Pesticide Research Building, Auburn, AL in BugDorm insect rearing tents (MegaView Science Co., Taiwan). Colonies were sustained on 606 flats of 1-3 true leaf cotton seedlings by transferring 2 adult apterous aphids to individual healthy seedlings once a week by moving them gently with a fine paintbrush. Aphids were originally collected from a cotton field in Tallassee, Alabama in 2019.

Cutting and Maintaining Leaf Discs in Transmission Experiments

A leaf disc assay was used to characterize the mode of transmission (Hughes and Woolcock, 1965; Moreno et al. 2011; Czosnek et al. 1993; Kalleshwaraswamy and Kumar, 2008; Di Mattia et al. 2020). Experimental arenas were constructed from 1.25 oz plastic food containers (Dart, Mason, MI) that were modified to contain insects and leaf discs. Holes (2 cm diameter) were cut from lids using a soldering iron, and aphid-proof screen (Equinox, Williamsport, PA) was secured over the hole using hot glue. Plant agar (RPI, Mt. Prospect, IL) was mixed with distilled water at 5.5g/L and heated until dissolved. When the agar was warm to the touch, but had not started to solidify, a 60mL syringe was used to deliver 5mL of plant agar to each 1.25 oz cup. Agar was allowed to cool and solidify in the cups, and was either used immediately for experiments or were covered with cellophane and stored at 5℃ until used in the experiments. Expanded, mature leaves were collected from healthy cotton plants, placed upside down on a piece of cardboard, and cut into with a 2.5cm disc using a cork borer, ensuring to include a vein in the resulting disc. Using a 5mL syringe, a single drop of water was added to each agar cup prior to placing leaf discs upside-down on the agar to help hold the leaf disc flat on the agar (aphids feed on the undersides of leaves). Discs were lightly pressed into the agar to ensure adhesion to the plant agar and prevent curling or desiccation. Leaf discs were cut the

same day the experiments were performed. For all experiments performed below, after the leaf discs were exposed to viruliferous aphids they were held for a total of 5 days in the growth chamber to allow for virus replication to occur before testing for CLRDV using molecular diagnostics.

Acquisition Access Period Protocol

To determine the AAP for alates and adult apterous, 400 adult aphids were collected from the healthy colony, placed in 50x9mm petri dishes with tight-fitting lids (Becton Dickinson, Franklin Lakes, NJ) modified with aphid-proof screen and starved overnight. Aphids were then moved in groups of 50 to each of five leaves on a CLRDV-infected plant and confined to the leaves using two weigh boats sealed with parafilm (Fig. 2). Acquisition occurred at room temperature (approximately 23°C) on an infected plant housed inside a BugDorm that was covered with a blanket to block out the light to stimulate the alates to settle on the plant. Aphids were allowed to feed for the following eight AAP's: 0.5, 1, 2, 4, 6, 12, 24, or 48 hrs. As each of the AAP's ended, the weigh boats were unsealed, 10 aphids removed from each leaf, and the weigh boats resealed with parafilm. Groups of five aphids were then placed on each of 10 leaf discs such that each leaf disc had one aphid from each of the five CLRDV infected leaves to account for varying titer levels in the host plant. Leaf discs with aphids for all experiments described were kept in a growth chamber (Percival Scientific Inc., Perry, IA) at 25°C, 12:12 light:dark cycle, and 50% RH for 5 days before the aphids were manually removed and the leaf discs frozen in a -80°C freezer.

Inoculation Access Period Protocol

IAP treatment times were based on Michelotto and Busoli (2007), with an additional ninth IAP of 15 minutes due to a report that aphids can reach the phloem in 15-30 minutes (Gray

et al. 1991). Aphids were collected from the healthy colony and moved to a CLRDV-infected plant and allowed to colonize and feed for a minimum of 4 days.

To determine the IAP of CLRDV-AL, 450 viruliferous adults were collected from the infected plant, placed in petri dishes with modified lids, and starved overnight. Aphids were then placed in groups of five on leaf discs, and a total of 10 leaf disc replicates were included in each IAP treatment. Treatment times investigated were: 40 sec, 15 min, 45 min, 1.5, 3, 6, 12, 24, and 48 hrs. Leaf discs were placed in a growth chamber for the duration of each IAP. At the end of each IAP, aphids were manually removed and the leaf discs returned to the chamber for the remainder of their 5 days. For the 40 sec IAP treatment, aphids were observed under a microscope and timed on a stopwatch for 40 seconds once the stylet was inserted.

Retention Time

Viruliferous aphids were collected from CLRDV plants infested for IAP experiments. Virus persistence in the aphid was determined by placing 20 viruliferous adult apterous aphids and 20 viruliferous alates individually onto healthy leaf discs and transferring the aphids every 24 hours to new leaf discs. This continued until aphid death. Leaf discs were held for a total of 5 days before being frozen in a -80°C and tested for presence of the virus.

Confirmation of CLRDV

Virus presence in leaf discs was confirmed using the extraction and nested PCR protocols described in Chapter 2.

Results & Discussion

Acquisition Access Period

Apterous aphids were able to acquire CLRDV with as little as 30 minutes of feeding time (Table 3). Alates were not able to acquire the virus at any time point less than 24 hrs. For both alates and apterous, acquisition was highest at the longest feeding periods of 24 and 48 hrs, respectively.

The IAP and AAP attempts documented in this study were conducted using 5 aphids per leaf disc because transmission using one aphid was unreliable and infrequent. CLRDV transmission was not observed in alate cohorts that fed for less than 24 hrs on an infected plant, but apterous aphid cohorts were able to acquire within 30 minutes of feeding. This may be explained by differences in biological and feeding behavior described for apterous and alate morphs (Boquel et al. 2011). Apterous aphids are the primary colonizers and are therefore more likely to engage in prolonged undisturbed feeding periods (Williams and Dixon, 2007; Boquel et al. 2011). Alates are created as a result of overcrowding and poor host quality and will disperse to seek out new hosts. Several possibilities for the alates being unable to acquire CLRDV with less than 24 hrs access exist. Alates may not have settled on the infected plant to feed, due to crowding within the weigh boat clamshell, non-preference of the CLRDV leaf, or because they did not have a long enough flight period (Johnson 1958). The effect of a short flight can be overcome by placing the alates in darkness, which encourages them to settle. A second possible explanation for the lack of transmission is that the alates may not have acquired enough virions to produce a positive test due to their probing and feeding behavior.

Inoculation Access Period

Apterous aphids were not observed to transmit CLRDV with less than a 24 hr feeding period (Table 4). Due to difficulty performing transmissions, only one replicate of the IAP experiments was recorded for apterous aphids. This study is not in accordance with Michelotto

and Busoli (2007), who recorded apterous transmitting CLRDV-typical as quickly as 1.5 hrs, with the highest rates of transmission (40-75%) occurring between 3 and 48 hours. More data would be needed to verify the minimum IAP time for apterous aphids transmitting CLRDV-AL. Transmission by alates was detected for all inoculation times 15 min and longer. This correlates with Michelotto and Busoli (2007) who indicated that alates could transmit at all IAP's. However, no transmission occurred at 40 sec in this study. There was a general trend of increasing transmission with increased feeding period. From a disease management standpoint, with the current tools available, it is not possible to prevent CLRDV from entering cotton fields with alates able to transmit in 15 minutes.

Retention

Of the 20 alates in the study, 11 transmitted on at least one day (Table 5). Most transmission occurred between days 5 and 8 although one alate transmitted as early as day 2. Two alates were able to transmit 23 days after feeding on an infected plant. These two aphids were observed to transmit on more days during their lifetime; CLRDV transmission was detected for 7 days each, and they both lived 27 days. The longest time between positive infections by the same aphid was 9 days. Most positive infections were intermittent across the aphid's lifespans.

Of the 20 apterous in the study, 14 transmitted on at least one day (Table 6). Most transmission occurred between day 1 and 10 with over 50% of aphids transmitting on days 1,2, and 5. A single apterous was able to transmit at day 15. This aphid also had the highest number of transmitting days at 8 days. The longest time until first positive infection was 9 days. Apterous aphids had the highest transmission in their first 10 days with peaks of over 50% of aphids transmitting on days 1,2, and 5.

Both morphs of *A. gossypii* are capable of retaining CLRDV-AL for longer than has been reported in Brazil. A single apterous was able to transmit 15 days after acquisition, whereas in Brazil, a single apterous was only able to transmit up to 12 days. Two alates in this study retained CLRDV-AL for 23 days, twice as long as was previously reported in Brazil by apterous aphids. This is especially significant in the alates, as they can be carried by wind currents hundreds of kilometers during long-distance migrations (CABI 2019). With the ability to spread CLRDV across the country, increased testing of cotton and host weed species and monitoring for CLRDV is needed.

The time between positive infections by the same aphid can be explained by the persistent, circulative nature of *luteoviruses.* Virions travel passively in the hemocoel to reach the accessory salivary gland (ASG) and therefore reach the ASG at different times (Katis et al. 2007). It is also possible that there were more positive infections, but the titer could have been low enough to be undetectable by nested PCR. This was proven by an early IAP attempt where a cotton plant that had previously tested positive for CLRDV had extremely low transmission rates by single apterous aphids (3/90, data not shown). The plant was retested a week after the study was conducted and tested negative in samples collected from both old and new petiole tissues.

Conclusion

The results of transmission of CLRDV-AL by US populations of *Aphis gossypii* indicate that *A. gossypii* has the capacity to spread CLRDV long distances as alates can be carried by wind currents hundreds of kilometers (CABI 2019). With the ability to transmit in 15 minutes, chemical control of CLRDV-AL is not practical. The next step in understanding CLRDV is to characterize the titer levels that occur within an infected plant and that result from seasonal

fluctuations. The seasonal information, paired with data indicating timing of aphid-flights, will be instrumental in developing cultural control of CLRDV.

Table 3. Acquisition access period (AAP) of CLRDV-AL assessed in two morphs using groups of 5 *Aphis gossypii* in a leaf disc assay.

AAP (time)	Apterous	Alate	Totals
30 min	1/20[a]	0/20	1/40
1 hr	2/20	0/20	2/40
2 hrs	1/20	0/20	1/40
4 hrs	0/20	0/20	0/40
6 hrs	0/20	0/20	0/40
12 hrs	1/20	0/20	1/40
24 hrs	1/20	10/20	11/40
48 hrs	5/15	-	5/15

[a] Number of leaf discs infected/total tested

Table 4. Transmission of CLRDV-AL assessed in two morphs of *Aphis gossypii* using groups of 5 aphids in a leaf disc assay.

IAP (time)	Apterous	Alate	Totals
40 sec	0/9[a]	0/30	0/40
15 min	0/9	1/30	1/40
45 min	0/9	2/30	2/40
1.5 hrs	0/9	1/30	1/40
3 hrs	0/9	4/30	4/40
6 hrs	0/9	6/30	6/40
12 hrs	0/9	4/30	4/40
24 hrs	3/9	6/30	9/40
48 hrs	5/9	11/30	16/40

[a] Number of leaf discs infected/total tested

Table 5. Transmission of *Cotton leafroll dwarf virus* (CLRDV-AL) by individual alate *Aphis gossypii* during successive 24 hr inoculation access periods using a leaf disc assay

Alates	Day																											
(reps)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	Total
1	-	+	-	-	-	+	-	+	-	-	+	-	+	-	+	-	-	-	-	-	-	-	+	-	-	-	-	7/27
2	-	-	-	-	-	-	+	+	-	-	-	-	-															2/13
3	-	-	+	-	+	-	-	-																				2/8
4	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-								1/20
5	-	-	+	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-						3/22
6	-	-	-	-	-	+	-	-	-	+	-																	2/11
7	-	-	-	-	-	+	+	-	-	-	-																	2/11
8	-	-	-	-	+	-	-	+	-	-	-	-	-	-														2/14
9	-	-	-	-	-	+	-	+	-	-	+	-	-	-	-	-	+	+	-	-	-	+	+	-	-	-	-	7/27
10	-	-	-	+	+	-	-	-	+	-	-	-	-	-	-	-	-											3/17
11	-	-	-	-	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-				4/24
Percent of aphids transmitting	0	9	18	9	45	55	27	36	20	10	20	0	13	0	33	0	17	20	0	0	0	33	100	0	0	0	0	

[a] Total number of days an aphid transmitted CLRDV-AL over the course of its lifespan

[b] Result of transmission, either positive (+) or negative (-) by a single aphid after a 24 hr feeding period.

Table 6. Transmission of *Cotton leafroll dwarf virus* (CLRDV-AL) by individual apterous *Aphis gossypii* during successive 24 hr inoculation access periods using a leaf disc assay

Apterous	Day																										
(reps)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	Total[a]
1	-[b]	-	-	-	+	+	-	-	-	-																		2/10
2	+	+		+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-						5/22
3	+	+	+	+	+	-	-	+	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-		8/26
4	-	+	+	-	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-									5/19
5	+	+	+	+	+	-	-	-	-	-	-																	5/11
6	-	+	+	+	-	+	-	+	-	+	-	-	-	-														6/14
7	+	+	+	-	-	-	+	-	-	-	-	-	-	-	-													4/15
8	-	+	-	-	-	-	+	-	-	-	-	-																2/12
9	-	+	-	+	+	+	-	+	+	-	-	-																6/12
10	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3/27
11	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-													2/15
12	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-											3/17
13	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-									1/19
14	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-							1/21
Percent of aphids transmitting	50	71	36	43	57	36	21	23	14	21	0	0	0	0	11	0	0	0	0	0	0	0	0	0	0	0	0	

[a] Total number of days an aphid transmitted CLRDV-AL over the course of its lifespan

[b] Result of transmission, either positive (+) or negative (-) by a single aphid after a 24 hr feeding period.

Figure 2. Apterous aphids on a leaf inside a weigh boat clam-shell structure designed to confine them to on one leaf.

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Chapter 4

Screening Protocol 1: Developing an Aphid-Based Rapid Screening Assay

Introduction

Cotton leafroll dwarf virus (CLRDV) is an aphid transmitted *Polerovirus* reported as the causal agent of cotton blue disease (CBD) of cotton in Africa, Asia and South America (Cauquil and Vaissayre, 1971; Ray et al. 2016; Mukherjee et al. 2012; Sharman et al. 2015; Distefano et al. 2010; Correa et al. 2005). In 2017, a new strain, CLRDV-AL, was identified in the southeastern United States (Avelar et al. 2020), and it has been detected in all commercially grown cotton cultivars (Brown et al. 2019). Breeding and deploying cotton varieties that are field resistant to CLRDV has been used as an effective management strategy in Africa (Cauquil 1977) and South America (Miranda et al. 2008). Developing resistant crop varieties is the most effective disease management strategy against viral pathogens, and is a current focus of cotton breeding research efforts in the U.S. In this report, we describe a rapid screening method that can improve the speed and efficiency of identifying sources of resistance to CLRDV-AL in cotton germplasm collections.

There are two plant defense mechanisms that can be identified and selected for in crop breeding programs: resistance and tolerance. Host plant resistance to prevent infection by viruses can be achieved through mechanisms such as resistance to virus movement, resistance to virus multiplication, and hypersensitivity (Barker and Waterhouse, 1999). In contrast, tolerance is characterized by viral replication in asymptomatic plants (Hull 2002), meaning that the plants become infected with the virus but are able to compensate and do not exhibit reduced yields. Tolerance is considered to be a less durable management approach if resistance can be identified,

but does not always exist in germplasm collections. Methods of screening germplasm for resistance to virus include field screening in areas where the pathogen naturally occurs, and under controlled conditions using vector transmission or artificial inoculation methods. Both methods require growing and monitoring infections and symptom expression, and some require months or an entire growing season to complete. Field screening for CLRDV-AL is underway, however, it requires the full growing season to determine susceptibility of the cultivar because it is unknown if or when plants were infected during the season. Previous work on determining CLRDV resistance in the greenhouse involved placing viruliferous aphids on two-true leaf cotton seedlings and evaluating their symptoms 9-28 days (Africa) or 60 days (Brazil) later (Cauquil and Vaissayre, 1971; Michelotto and Busoli, 2007). Both methods allow tolerance and resistance mechanisms to be evaluated when symptom expression is easy to identify and monitor.

An additional complication of screening for resistance to CLRDV-AL is the high degree of variation in symptom expression that occurs throughout the cotton belt. Many plants are asymptomatic, even in areas where PCR-testing shows up to 100% of plants are infected with the virus (data not shown). Screening for resistance based on virus symptom expression will not be effective until the specific genotype x environment interactions are understood and can be replicated. The reduced virulence of CLRDV-AL will complicate efforts to identify either tolerance or resistance mechanisms in breeding trials of any kind. Therefore, the initial breeding strategy for developing a CLRDV-AL resistant cultivar is to screen existing elite and landrace germplasm for resistance.

Leaf disc laboratory assays are commonly used to study virus transmission by virologists and vector-biologists. They are easily adapted as a high-throughput and quick screening method and may aide in rapid testing of germplasm collections. These assays have not been examined in

this context; however, they have been used reliably to test for virus replication after vector transmission of plant viruses (Wijkamp and Peters, 1993; Inoue et al. 2004; Jacobson and Kennedy, 2013; Nagata et al. 2007; Mason et al. 2003; Ohnishi et al. 2006). Leaf discs have been shown to support aphid survival and replication of persistently transmitted, phloem restricted viruses (Hughes and Woolcock, 1965; Moreno et al. 2011; Czosnek et al. 1993; Kalleshwaraswamy and Kumar, 2008; Di Mattia et al. 2020). The objective of this study was to develop a leaf disc assay to screen cotton germplasm for resistance to CLRDV infection. Results obtained from a field screening trial were used to identify candidates for this screening and included 'Delta Opal', a variety highly tolerant to CLRDV-typical and -atypical (Fang et al. 2010; da Silva et al. 2015). These selected lines were grown in a growth chamber to generate healthy tissue that was then used in a leaf-disc aphid transmission assay to determine whether or not these lines were resistant to infection by CLRDV.

Materials and Methods

CLRDV Trial

In 2019, a large-scale field-screening trial was implemented encompassing 821 cotton varieties at the EV Smith Research Center in Tallassee, AL. Plots consisted of single 10 foot long rows with 2 replications. The trial was arranged in a randomized complete block and was planted at 3 seeds per foot in a Decatur silt loam soil. Plots were irrigated as needed and fungicides were applied to prevent foliar pathogens. Okra lines were planted randomly throughout the field to attract aphids. Brazilian cultivars, 'BRS 293' and 'BRS 286', were included because they are resistant to CLRDV-typical. BRS 286 is also resistant to CLRDV-atypical. At the end of the growing season, all lines were tested for CLRDV using molecular

diagnostics. If a negative result (indicating no virus) was obtained from the first replication, samples were collected and tested from the second replication. All varieties that tested negative in both replications were advanced to leaf disc assay studies.

Leaf Disc Assay Using Aphid Transmission of CLRDV

Forty-one cotton varieties were planted in 606 inserts (The HC Companies Inc., Middlefield, OH) in peatlite (PRO-MIX 'BX', Quakertown, PA) (Table 7). Seedlings and experiments were maintained in a growth chamber (Conviron, Winnipeg, Canada) at 25°C with a 12:12 light:dark cycle and 50% RH until the 3rd true leaf stage. Five plants from each line were chosen at random for the assay. A single leaf disc was cut per plant from the 2nd most expanded leaf. The five leaf discs per line were combined for a composite sample during RNA extractions. Transmission assays with leaf discs were conducted following the leaf disc protocol listed in Heilsnis (2020), Chapter 3. Viruliferous aphids (created using the methods stated in Heilsnis (2020), Chapter 3), were used for transmission at 5-10 aphids per leaf-disc, depending on the number of aphids that reproduced and were available for use in this experiment. If a line receiving 5 aphids per leaf disc tested negative, the line was tested again using 10 aphids. Aphids were allowed to feed on the healthy leaf discs for 5 days in the growth chamber before being manually removed, the leaf discs placed in tubes and frozen in the -80°C until extraction. Virus presence was confirmed by molecular methods.

Results and Discussion

This leaf disc assay was successful at transmitting CLRDV-AL to all lines tested, including the control 'Delta Opal' (Table 7). Therefore, all lines tested in the field for CLRDV-AL were able to be infected by the virus. This result was not unexpected as there has only been

one monogenic dominant resistance gene (*Cbd*) identified for CLRDV-typical derived from Delta Opal (Fang et al. 2010). Monogenetic dominant resistance genes are easy to breed with but are also more likely to be overcome by resistance-breaking viral isolates than either polygenic or recessive genes (Khetarpal et al. 1998).

CLRDV-AL and CLRDV-atypical are resistance breaking as they are able to infect cotton varieties containing the *Cbd* gene (da Silva et al. 2015). *Cbd* is believed to be inherited from *G. arboreum* African germplasm and all lines in the leaf disc assay were *G. hirsutum* (Fang et al. 2010). *Gossypium hirsutum*, or 'Upland cotton' constitutes 95% of U.S. production with *G. barbadense* making up the remaining 5% (USDA-ERS, 2019).

The leaf disc assay is a test for resistance, it does not distinguish tolerance. Tolerance can only be ascertained in a grow out trial where symptoms are monitored, and tissue samples taken to confirm systemic infection. The lines selected from the 2019 Auburn CLRDV screening trial may be tolerant, but more years of data would be needed to confirm. It is possible that they failed to test positive in the field due to limited feeding by viruliferous aphids, the host plant recovering, or titer levels were too low to be detected by PCR. Host plant recovery has been documented with CLRDV, most often associated with single-infection events or older age of the plants at the time of infection (Cauquil 1977; Follin and Campagnac, 1981).

Transmission rates generally increase when plants are exposed to a higher number of viruliferous insects (Kalleshwaraswamy and Kumar, 2008; Ali et al. 2007). A few lines were able to be infected with as few as 5 aphids, whereas some required 10. This is likely due to variation of virus titers within the CLRDV source plant the aphids fed on to acquire the virus. There is currently no reported qPCR method to assist with virus quantification, but we have observed wide variation in transmission rates among CLRDV-infected source plants, or even the

same source plants used during different times of the year (personal observation). This is not unexpected for an attenuated, low-titer virus, but due to this variation it is important to include a known susceptible in all screening trials and increase the number of aphids to at least ten to achieve desired transmission rates. The benefit of this leaf disc assay is quick results. The assay can be completed in 4 weeks, from sowing to final PCR result. Leaf disc assays can also be used with artificial vectors. When new research tools are available for artificial inoculation, the efficiency of this method may be further simplified by eliminating the expertise for insect colony rearing and maintenance.

Conclusion

A leaf disc rapid screening assay for resistance to CLRDV-AL is possible when using a minimum of 10 aphids per leaf disc to overcome titer issues. This assay is used to confirm that a variety can be infected with the virus but will not indicate any level of tolerance. One of the benefits of this assay is speed; results can be obtained in 4 weeks. Lastly, this study confirmed that all 821 varieties in the field screening trial can be infected with CLRDV-AL

Table 7. Cotton varieties tested in the CLRDV aphid assay, using either 5 or 10 A. *gossypii*

Variety	Source		Result
1115-36	Arkansas	10	Positive
Acala 1064 New Mexico	GWAS	10	Positive
ALLEN 33	GWAS	10	Positive
Arkot 0012	Arkansas	10	Positive
Arkot 0016	Arkansas	10	Positive
Arkot 0114	Arkansas	10	Positive
BRONCO 360	GWAS	10	Positive
CA30	GWAS	10	Positive
CASCOT C 13	GWAS	10	Positive
Cedix	USDA	10	Positive
Delta Opal	Monsanto	10	Positive
DUNN 1047	GWAS	10	Positive
DUNN 325	GWAS	10	Positive
GSC 30	GWAS	10	Positive
H1220	USDA	10	Positive
SOUTHLAND 400	GWAS	10	Positive
TAMCOT SP 37	GWAS	10	Positive
128089	Alabama	5	Positive
Barbren 713	USDA	5	Positive
Chaco 510 INTA	USDA	5	Positive
SATU 65	USDA	5	Positive
REBA B 50	USDA	5	Positive
ALA 70-1	USDA	5	Positive
UKA J2 (72) 017	USDA	5/10[a]	Negative//Positive
0908-52	Arkansas	5	Positive
1102-55	Arkansas	5	Positive
Miscot 7813	Arkansas	5	Positive
Arkot 8710	Arkansas	5/10	Negative/Positive
Arkot 9108	Arkansas	5/10	Negative/Positive
Arkot 9304a	Arkansas	5	Positive
Arkot 9706	Arkansas	5	Positive
UA212ne	Arkansas	5	Positive
0822-48	Arkansas	5/10	Negative/Positive
LA11309005	Lousiana	5	Positive
Guazuncho	USDA	5	Positive
Cedix	USDA	5	Positive
Cedix[b]	USDA	5	Positive
CD3HHARCIH 1 88	GWAS	5	Positive
GP 1005	GWAS	5	Positive

SA 2328	GWAS	5	Positive
UK 64	USDA	10	Positive

[a] These lines tested positive when retested using 10 aphids per leaf disc

[b] Open pollinated source

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Chapter 5

Screening Protocol 2: Graft Transmission of *Cotton leafroll dwarf virus* in Cotton (*G. hirsutum*)

Introduction

Studies on CLRDV in Brazil have reported yield losses of up to 80% in major cotton growing areas (Silva et al. 2008). There are two reported strains, -typical and –atypical, in South America (Correa et al. 2005; Silva et al. 2008). In Brazil and Argentina, CLRDV-typical is controlled by growing resistant varieties of cotton (Miranda and Suassuna, 2004). However, with the advent of CLRDV-atypical in 2006, 90% of varieties grown in Brazil have proven susceptible. In 2018, *Cotton leafroll dwarf virus* (CLRDV) was the first virus reported to infect cotton in the Southeastern United States (Avelar et al. 2019) and is transmitted by *Aphis gossypii*. The virus in the U.S., CLRDV-AL, is classified as a third strain and has been shown to infect cotton varieties resistant to CLRDV-typical and CLRDV-atypical (Avelar et al. 2020; Brown et al. 2019; Heilsnis, Chapter 4).

As an emerging threat to the U.S. cotton industry, efforts are focused on finding host plant resistance to CLRDV-AL in current cotton populations. Breeding efforts cannot begin until resistance has been identified. As this is a recently identified virus, the only cotton germplasm that has been phenotyped are current commercial cultivars. Therefore large quantities of diverse breeding material need to be screened. This can be done by field screening, vector transmission, mechanical inoculation, grafting, or through the use of an infectious clone.

Field screening is a simple method that relies on viruliferous insects in the landscape. However, it requires a full growing season and is not time effective. There is also the possibility that the vector may not be present or transmitting the virus, which is problematic for screening.

Vector transmission assays ensure specificity of the virus of interest but requires maintaining robust insect colonies. This type of screening can be completed in several months. Mechanical inoculation is a fast screening method but is ineffective for phloem-limited viruses, such as *luteoviruses* (Katis et al. 2007). An infectious clone is the optimal method for resistance screening of germplasm; however, it is costly and clones can be difficult to produce, especially when producing a cDNA clone (Beckett and Miller, 2007). In the absence of an infectious clone, and with limited time and the uncertainty of insect availability; graft transmission is a suitable replacement.

A graft is the union of two plant tissues referred to as the scion and the rootstock. The scion is the bud or stem being introduced that will grow to form the top portion of the plant and is grafted onto the rootstock, which will form the root system for the new composite plant (Hartmann et al. 2014a). Graft types can be divided into two groups based on the relative size of the scion to the rootstock. Grafts using the same size scion as rootstock include whip-and-tongue, wedge, saddle, and approach grafts. Grafts using dissimilar sized rootstock and scions include side-veneer, t-graft, chip budding, and cleft.

Grafting is primarily used in horticulture and therefore there is little information on grafting in agronomic crops (Hartmann et al. 2014a). In cotton, grafting has been reported for virus transmission and is typically performed on 4-8 week old rootstock (Ahktar et al. 2002; Ahktar et al. 2013; Reddall et al. 2004; Rahman et al. 2005). As a perennial, cotton has two stages of growth. Herbaceous tissue is produced first, followed by lignification of that same tissue. It is possible to graft woody cotton plant tissue; however, young herbaceous tissue is preferred due to its fast growth, bark slippage, and higher percentage of graft take. Grafts used

successfully in cotton include wedge, modified bottle shoot, and T-graft. (Karaca et a. 2020; Reddall et al. 2004; Ahktar et al. 2002; Sharman, Personal Communication).

The primary objective of this paper is to develop a screening protocol for CLRDV-AL by investigating different graft types and necessary healing conditions in cotton.

Materials and Methods

Grafting Protocol Development

Preliminary: T-graft and Bottle-shoot

Thirty Stoneville '6182' cotton seeds were sown in 3 gallon pots (Nursery Supplies®, Chambersburg, Pennsylvania), 3 plants per pot, in peatlite (PRO-MIX 'BX', Quakertown, PA) and placed on a greenhouse bench in full sun until the 4th true leaf stage, approximately 12 weeks. At this time, self-grafts were performed on 15 of the plants, 10 T-grafts and 5 bottle-shoot. All grafts were made by cutting the terminal shoot off 0.5cm below the 3rd node. The terminal was trimmed to 15cm, all leaves below the two topmost expanded leaves were removed, and the terminal placed in a jar of water while the rootstock was prepared.

For the T-grafts, two cuts were made in the shape of a "T" on the stem of the rootstock above the 2nd node, lightly cutting through to the cambial layer (Table 8; Fig. 3). Using the bark lifting edge on the back of the grafting knife (Felco 3.90 20, Felco SA, Switzerland), the epidermal layer was lifted up and water dripped into the cut (Fig. 4). The terminal shoot was then prepared by trimming one side of the basal end into a wedge shape (Fig. 5). The basal end was placed inside the T-cut, cambial layers touching, and bound together with parafilm for stability and moisture retention (Fig. 6).

The bottle-shoot graft is a modified approach graft (Fig. 7) in which the basal end of the scion is placed in a test tube of water that is bound to the rootstock (Fig. 8). The graft was performed by making a single long shaving cut to the rootstock that removed the epidermal layer, making an identical cut to the scion, then aligning the cuts together and binding them with parafilm (Table 8). The cut on the scion was made 10-15cm above the bottom of the stem. A test tube was then fitted over the basal end of the scion and taped to the rootstock for stability. The test tube of water was removed from the bottle-shoot grafts after the scions had regained turgidity (approximately seven days).

Five of the T-graft plants had a clear one-gallon plastic bag placed over them and taped shut below the union using grafting tape. The remaining five T-grafts were left uncovered. Bottle shoot grafts were not bagged since their water source is taped to the rootstock.

Types of grafts

The wedge, saddle-graft, whip-and-tongue, bottle-shoot, and T-graft were performed on the remaining 15 cotton plants with each graft type designated to a pot of 3 plants. Wedge grafts were performed by preparing the terminal as stated above, except for both sides of the terminal were cut into a wedge shape (Fig. 5). The rootstock was cut by centering the grafting blade on top of the cut stem and carefully rocking the blade to split the stem downward (Hartmann et al. 2014b). The scion was inserted into the split, ensuring cambial alignment between scion and rootstock along at least one side, then wrapped in parafilm (Table 8; Fig. 9).

A saddle-graft is an inverse wedge: the rootstock was cut into the pointed wedge shape and the scion was split in half. The scion was then split over the apex of the wedge, cambiums aligned, and wrapped in parafilm (Fig. 10).

For the whip-and-tongue, identical, angled cuts are made to both the scion and the rootstock. A second angled cut is made under the first, creating the tongue. The tongues are inserted behind each other as the rootstock and scion are brought together, locking the graft together (Fig. 11). The graft was then wrapped in parafilm.

T- and bottle-shoot grafts were performed as above, with modification to the bottle-shoot graft. Bottle-shoot grafts were modified from the preliminary experiment by increasing the length of the scion to 20cm.

All grafts were bagged to prevent desiccation of the scion (Fig. 12). After grafts had regained turgidity (approximately 5 days), the bags were clipped to allow airflow and humidity acclimation. Three days later, the bags were fully removed.

Graft Screening – CLRDV Transmission

Twenty ‘DeltaPine 1646’ plants were sown as described above and grown for 8 weeks for use in graft transmission of CLRDV-AL. The first 10 grafts were performed using scions from CLRDV-infected cotton plants that had been transplanted from a field in Elmore County, AL (source A) onto a greenhouse bench at the end of the 2018 growing season. The second set of 10 grafts were performed using scions from greenhouse grown cotton plants (source B) that were placed in a field to acquire the virus under natural conditions before being returned to the greenhouse.

Plants were prepared for grafting by cutting the terminal shoot off 0.5cm below the 3rd node. Scions were collected and kept basal end down in a jar of distilled water to prevent loss of turgidity while rootstocks were prepared. T-grafts were performed on the side of the stem using CLRDV infected scions prepared as above. Scions from the first source were 10cm long due to a zig-zag growth pattern and short internodes, while scions from the second source were 20cm,

and straighter. As cuts were made to the rootstock, water was dripped into the wounds to prevent them from drying out. Due to the use of diseased tissue, grafts were placed under a bench top shade structure (Agribon®, Berry Global, Evansville, IN) to reduce light stress and transpiration until fully healed (Fig. 13). To test for presence of the virus, three leaves were collected from the first node below the graft for a composite sample. Virus presence was confirmed via PCR (Heilsnis 2020, Chapter 2).

Results and Discussion

Grafting Protocol Development

Graft success

A successful graft take is observed when xylem and phloem tissue are produced that connect the scion and rootstock. Graft take is dependent on a myriad of factors, the most important to this study being cambial alignment and healing conditions. Out of the 50 grafts performed in these experiments, 14 were unsuccessful. The preliminary experiment proved that cotton grafts need the humidity because the scion desiccated and died before the graft had healed (Fig. 14). This has been reported in herbaceous grafts, as high humidity and reduced light conditions are essential (Vu et al. 2013).

The scion and rootstock must be aligned so that the vascular systems can grow together. The first step to this successful union is callus formation, which fills the gaps between the tissues, thereby holding the scion and rootstock together (Hartmann et al. 2014b). All bagged t-grafts survived because callus was able to form before the scion desiccated. In the unbagged T-grafts, transpiration drew water away from the graft union and out of the leaves before callus could form (Fig. 15). Scions wilted in less than two hours and were dead in 3 days. By protecting

the scions with plastic bags some transpiration occurred, but that transpiration created a high humidity environment that sustained the scion long enough for the callus to form (Vu et al. 2013). Therefore all further experiments were bagged.

Types of grafts

T-graft

The T-graft proved to be the best graft overall due to the high amount of cambial contact, required little time to perform, and was the easiest to bind with parafilm as the epidermal layer of the rootstock held the scion in place. The high amount of cambial contact was contributed by the rootstock as the entire cambium was exposed to the scion when the scion was inserted under the epidermal layer. High amount of cambial contact greatly increases the chances of both graft take and virus transfer (Mudge 2008). This graft is extremely easy to perform on young cotton tissue as the epidermal layer slips off the cambium.

Bottle shoot

Bottle-shoot grafts are not recommended for cotton grafting due to the difficulty in maintaining cambial alignment during binding, the need for 20 cm long, straight scions, and the added involvement of maintaining water levels in the test tubes. In the methods described by Akhtar et al. (2002) and (2013), he removed the test tube from grafted cotton plants after 5 days and 7 days, respectfully, and was successful in both disease transmission and graft take. However, in this study, once the test tube of water was removed after the scions regained turgidity (approximately 7 days), the scions subsequently died within the next seven days. This was unexpected as the grafts had regained turgidity and there was mild callus formed within this time period to sustain the scion. It is possible that there was not enough vascular tissue formed between the scion and rootstock in this experiment to sustain the scion without an external water

source. This is a time-intensive set-up to assemble and maintain compared to the wedge and T-grafts. A symptom of CLRDV is zig-zag stems and these stems would be problematic to fit inside the test tube. If a bottle or other large water source were to be used, support structures would have to be added to take the weight of the bottle making it impractical.

Wedge

Wedge grafts were effective and easy to cut, but difficult to maintain cambial alignment during binding due to the sap flow making conditions slippery. Care had to be taken with the cut downward into the stem as it was easy to injure oneself or break the stem if too much pressure was applied. This graft became more difficult when performed on practice plants older than 12 weeks but that had not yet lignified. Cotton forms pith in the center of the stem that is spongy and resists being cut (Janick 1986). The downward cut into the stem was hindered and the structural integrity of the scion wedge weakened by pith. This graft is best performed on young cotton or old cotton that is fully lignified.

Saddle

This graft was successful but had similar issues to the wedge graft regarding the pith but was less safe to perform. Cutting the wedge was more dangerous as the grafter now had to make long upward cuts on the rootstock in a less controlled fashion. When cutting a wedge on a scion, the scion is placed between the thumb and blade and pulled through without moving the thumb or blade. This maneuver is not possible on a potted rootstock and therefore the cuts made were more erratic. Rea (1931) used the saddle graft in cotton with a success rate of 46.2-71.3%. His reduced success rate may be attributed to his use of mature wood as opposed to the faster growing herbaceous tissues.

Whip-and-Tongue

Whip-and-tongue grafts were easy to perform on true herbaceous tissue, but not when pith was present. The benefit of the interlocking tongues are structural integrity and increased cambial contact (Hartmann et al. 2014b), both of which would aid novice grafters. While not reported in cotton, whip-and-tongue was included as it is useful for small diameter material (<1cm) and increased cambial contact, which is necessary for virus transmission.

CLRDV Graft Transmission

Six of ten grafts using scion source A transmitted CLRDV, two of which were dead grafts. All of the grafts performed with scion source B survived and eight of ten transmitted. The results prove that union and scion survival are not necessary for transmission of CLRDV. The grafts that died most likely did so because the scions were weakened by disease and once cut, they were unable to survive long enough for callus to form. This is especially likely as the plant that scions were sourced from (A) was stressed from both disease and having been transplanted. The more robust state of scion source B likely contributed to the 100% survival rate.

Akhtar et al (2002) and Reddall (2004) both indicated 100% transmission of *Cotton leaf curl virus* (bottle-shoot) and *Cotton bunchy top virus* (wedge), respectively, in their studies. Transmission in this study was likely affected by fluctuating viral titers within the plant that the scions were harvested from. CLRDV is a low-titer virus and can be present in a plant and undetectable by PCR (Heilsnis 2020, Chapter 3). Current molecular diagnostics do not exist that could characterize titer in the host plant. CLRDV is therefore graft transmissible and an effective screening method for resistant lines. The longer an infectious scion has living contact with a rootstock, the higher the probability of infection.

Conclusion

CLRDV-AL can be transmitted via both living and dead scions, indicating that grafting is a viable method for resistance screening. The overall recommended graft with the highest cambial contact is the T-graft. All grafts investigated worked in cotton under greenhouse conditions, except for bottle-grafts. These studies have demonstrated the need to protect herbaceous cotton grafts from desiccation by providing humidity control. The next step with grafting is to set up a screening trial and examine cotton varieties for resistance. Based on the percent success rate in this study, it is recommended to use five plants per line to determine a result. It is also recommended to use the most robust of the available diseased tissues to increase the chances of both graft take and virus transmission.

Table 8. Grafts types examined for cotton grafting in the Southeastern U.S.

Type of Graft	Description	Virus	Reference
T-graft	https://grapes.extension.org/grafting-grape-vines/	*Cotton leafroll dwarf virus*	*This study*
Wedge	Scion Stock https://galveston.agrilife.org/event/wedge-grafting-a-hands-on-demonstration/	*Cotton bunchy top virus*	Reddall et al. 2004

Saddle	https://etc.usf.edu/clipart/51200/51209/51209_saddle_graft.htm	n/a	Rea 1931
Approach graft	Spliced Approach Graft https://irrecenvhort.ifas.ufl.edu/plant-prop-glossary/06-grafting/02-graftingtypes/01-grafting-approach.html	n/a	n/a
Bottle-shoot (modified approach graft)	From: Fuller,A.S.,1897,The propagation of plants, Orange Judd Co., NY Fig. 92. BOTTLE GRAFTING, MODIFIED.	*Cotton leaf curl virus*	Akhtar et al. 2002

Whip-and-tongue	Prepared scion Prepared stock Graft in position with stock and scion matched *Figure 2: 'Whip-tongue Graft'.* http://rfcarchives.org.au/Next/FruitImages/MangoGraftA11-84.png	n/a	n/a

Figure 3. T-graft. T-shaped cut on rootstock, prior to scion insertion.

Figure 4. Epidermal layer of a 12-week old cotton plant lifted using the bark lifting edge of a grafting knife.

Figure 5. Basic wedge shapes of a scions used in wedge, saddle, whip-and-tongue, and T-grafts.

Figure 6. A T-graft prior to wrapping with parafilm

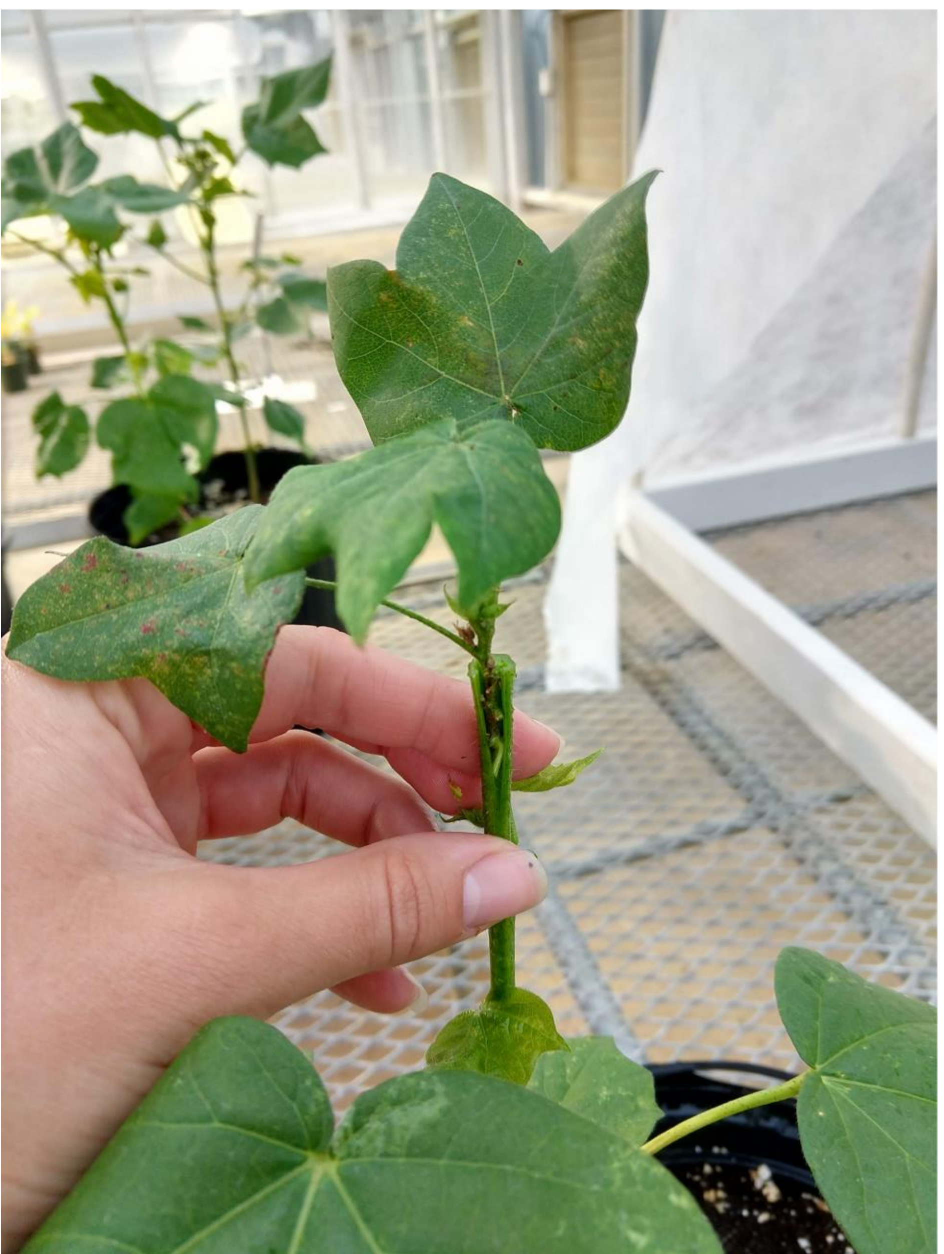

Figure 7. Healed approach graft. This is the type of graft used in a bottle-shoot graft

Figure 8. Bottle-shoot graft. A standard approach graft fitted with a test-tube for water supply.

Figure 9. Healed wedge graft.

Figure 10. Healed saddle graft

Figure 11. Whip-and-tongue graft, cuts outlined in blacker marker, prior to wrapping with parafilm

Figure 12. Bagged grafts in the acclimation phase of healing. Bags are clipped to allow humidity acclimation.

Figure 13. Benchtop shade structure for healing grafts.

Figure 14. Dead T-graft in cotton. No humidity control applied.

Figure 15. Dead T-graft. Scion pulled away from the rootstock to demonstrate lack of callus.

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