The *Journal of Cotton Science 26:23–30 (2022)*
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ARTHROPOD MANAGEMENT AND APPLIED ECOLOGY

Vector Competency of *Aphis gossypii* and *Bemisia tabaci* to Transmit Cotton Leafroll Dwarf Virus

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ABSTRACT

A new variant of cotton leafroll dwarf virus (CLRDV) (genus: *Polerovirus*, family: Solemoviridae) was discovered in cotton (*Gossypium hirsutum* L.) fields that were reported to be infested with aphids and whiteflies in southern Alabama in 2017. Prior to the confirmation of CLRDV, speculation focused on whiteflies as a potential vector of the then-unknown virus. Although the only vector reported to transmit CLRDV to cotton is the cotton aphid, *Aphis gossypii* (Glover), two recombinant poleroviruses have been reported recently to be transmitted by the whitefly, *Bemisia tabaci* (Genn.). Due to the emergence of a new CLRDV variant in the U.S., and the recent studies on recombinant poleroviruses, conflicting messages that whiteflies and/or aphids could be transmitting CLRDV have been relayed to growers and stakeholders in the Cotton Belt. The objective of this study was to determine if *A. gossypii* or *B. tabaci* (B Mitotype) transmit CLRDV to cotton. The results demonstrated that the CLRDV-AL variant was transmissible by alate and apterous morphs of *A. gossypii,* but not by *B. tabaci*. These findings emphasize the importance of screening insect vectors for the transmission of novel plant virus variants to correctly identify the vector(s) and provide growers and stakeholders with appropriate information to make informed management decisions.

Cotton leafroll dwarf virus (CLRDV) (family: Solemoviridae, genus: *Polerovirus*) is a phloem-limited virus transmitted by the cotton aphid, *Aphis gossypii* (Glover) (Hemiptera: Aphididae). The mode of transmission of CLRDV is persistent and non-propagative; alate *A. gossypii* are reported to transmit in under a minute and up to 12 d after acquisition (Cauquil and Vaissayre, 1971; Michelotto and Busoli, 2003, 2007). CLRDV contains a monopartite, single-stranded, positive-sense RNA approximately 5.7 kb in length. The viral genome encodes multiple proteins involved in insect-vector mediated transmission and infection of the host (Avelar et al., 2020; Tabassum et al., 2021). CLRDV is reported to infect cotton, *Gossypium hirsutum* L., in Africa, Asia, and the Americas (Cauquil and Vaissayre, 1971; Distéfano et al., 2010; Mukherjee et al., 2012; Ray et al., 2016; Sharman et al., 2015; Silva et al., 2008;). The first observation of disease and yield loss caused by CLRDV in the U.S. occurred in cotton plant samples collected late-season from Alabama in 2017 (Avelar et al., 2019; Brown et al., 2019; Lawrence et al., 2019). CLRDV has been reported since in 10 additional states within the eastern Cotton Belt (Aboughanem-Sabanadzovic et al., 2019; Alabi et al., 2020; Ali and Mokhtari, 2020; Avelar et al., 2019; Faske et al., 2020; Iriarte et al., 2020; Price et al., 2020; Tabassum et al., 2019; Thiessen et al., 2020; Wang et al., 2020). In 2017, cotton plants later determined to be infected with CLRDV showed virus-like symptoms including foliar distortions, bluish-green discoloration, vein clearing, internodal shortening, and reduced boll set late-season following heavy infestations of the sweetpotato whitefly, *Bemisia tabaci* (Genn.) (B Mitotype) (Avelar et al., 2019), initially leading to the hypothesis that a whitefly-transmitted virus could be the causal agent. Infestations of *A. gossypii* also occurred mid-season, ultimately corroborating the preliminary identification of the polerovirus CLRDV (Avelar et al., 2019; Bag et al., 2021). Although most viruses in the family Solemoviridae are transmitted by aphids, recent reports of two recombinant poleroviruses transmitted by whiteflies have caused uncertainty

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regarding the vector status of novel virus variants and prompted this investigation to determine whether *B. tabaci* or *A. gossypii* transmits CLRDV in the U.S.

Characteristically, plant viruses are transmitted by insect vectors that belong to the same taxonomic family [e.g., by Aphididae (aphids), Aleyrodidae (whiteflies), or Thysanoptera (thrips)], and most often by the same genus (Nault, 1997) due to the species-specific nature of vector-virus interactions required for successful virus acquisition, retention/circulation within the vector, and transmission (Andret-Link and Fuchs, 2005; Gallet et al., 2018; Gray et al., 2014). Poleroviruses have been recognized historically to be transmitted exclusively by aphids, but unexpectedly, recent studies from Israel and Brazil demonstrated whitefly-transmission of recombinant poleroviruses (Costa et al., 2020; Ghosh et al., 2019). Although the vector of these recombinant viruses changed, this research supports the species-specific nature of compatible vector-virus interactions because the poleroviruses tested were transmitted by either aphids or whiteflies, but not both. The questions raised by this research, however, have resulted in mixed messages regarding the vector status of these insects to transmit CLRDV, and stakeholders from multiple U.S. states have been told that both insects could be transmitting CLRDV.

Determining the vector of CLRDV in the U.S. has important implications for guiding future research on epidemiology, management, insecticide use, and virus spread because the distribution, timing, and pest status of *A. gossypii* and *B. tabaci* infestations vary by production region (Leclant and Deguine, 1994). *Aphis gossypii* annually infests cotton across the U.S. Cotton Belt (Abney et al., 2008; Rosenheim et al., 1997), whereas *B. tabaci* is considered a primary pest only in parts of the Southeast, Texas, and the Southwest (Barman et al., 2019; Ellsworth and Martinez-Carrillo, 2001; Goolsby et al., 1998). Unwarranted increases in insecticide use will have negative consequences on profit margins, beneficial insect populations, selection for insecticide resistance, and could flare secondary pests that will require additional management interventions.

The objective of this study was to determine if the CLRDV-AL variant is transmitted by *A. gossypii* or *B. tabaci* in the U.S., due to the practical implications for continuing research, extension, and integrated pest management. Based on the genetic similarity of U.S. CLRDV variants and aphid-transmitted CLRDV variants in South America (Aboughanem-Sabanadzovic et al., 2019; Avelar et al., 2020; Distéfano et al., 2010; Michelotto and Busoli, 2003; Tabassum et al., 2020, 2021), we expected CLRDV-AL to be aphid-transmitted. In this study we also present methodology for conducting leaf disc and whole-plant transmission assays with vectors of CLRDV.

MATERIALS AND METHODS

All insect colonies were contained in BugDorms (MegaView Science Co., Taiwan) or cages made with insect-proof screen (Econet 1515, AB Ludvig Svensson, Charlotte, NC). A colony of whiteflies collected from Auburn, AL in 2017 and identified by sequencing a partial mtCOI as *B. tabaci* (B Mitotype) (Moya et al., 2019; Shatters et al., 2009) was maintained in a greenhouse on eggplant, *Solanum melongena* L. 'Pinstripe Hybrid' (Park Seed, Greenwood, SC), a non-host of CLRDV. A colony of *A. gossypii*, collected from a cotton field in Tallassee, AL in 2019, was maintained on healthy two to three true leaf cotton seedlings. All cotton used in these experiments were variety 'Deltapine 1646' (Deltapine®, Dekalb Genetics Corporation, Dekalb, IL). Eggplant and cotton seeds were sown in 606 cell packs in with peatlite (PRO-MIX 'BX', Quakertown, PA) and grown indoors in 100-**μ** insect-proof screen cages (Econet 1515, AB Ludvig Svensson, Charlotte, NC) on greenhouse benches. After the four true leaf stage, eggplant and cotton were transplanted into 2.4 L plastic pots (model C300S, Nursery Supplies®, Chambersburg, PA). Plants were watered daily. Beginning 14 d after emergence, plants were fertilized weekly using a solution consisting of 9 g dry fertilizer (20-10-20, Everris NA Inc., Dublin, OH) dissolved in 1.5 L of water.

Multiple plants confirmed to be infected with CLRDV-AL by polymerase chain reaction (PCR) were transplanted from fields in Tallassee, AL in 2018; sequences of the P0 gene of CLRDV-AL variants collected in 2018 were 98 to 100% similar (Brown et al., 2019). Infected plants were maintained insect-free in greenhouses until used in this study. CLRDV infection was confirmed using PCR-

based methods. Total RNA was extracted using Qiagen RNeasy Plant Mini kits (Qiagen, Germantown, MD), cDNA was synthesized using a SuperScript IV first-strand synthesis system (ThermoFisher Scientific, Waltham, MA), and cDNA was amplified with CLRDV-specific primers CLRDV3675F/Pol3982R (Sharman et al., 2015) targeting a 307-bp segment of the coat protein gene.

Experimental Approach. A two-step approach was used to confirm transmission of CLRDV. First, transmission of CLRDV was confirmed using a leaf disc assay. If transmission was detected in leaf disc assays, then follow-up experiments were performed using whole plants to confirm systemic infection after transmission. Leaf disc assays are a sensitive, reliable, and rapid method for detecting transmission of plant viruses in host plant tissue (Jacobson and Kennedy, 2013; Wijkamp, 1993), including phloem-infecting viruses persistently transmitted by whiteflies and aphids (Czosnek et al., 1993; Di Mattia et al., 2020; Moreno et al., 2011) and low frequency transmission events (Jacobson and Kennedy, 2013). Leaf disc assays have been used to rear aphids (Hughes and Woolcock, 1965) and can support insect feeding over several days. Leaf disc experiments can be completed in 1 wk and require little space in an environmental chamber. In comparison, cotton plants grow large during the 30 d required for reliable CLRDV detection (Galbieri et al., 2010) and thus require a larger chamber. A cork borer was used to cut 2.5 cm diameter leaf discs from fully expanded leaves of healthy five to eight true leaf cotton, and a mid-vein was included in each disc. The leaf discs were placed abaxial side up on 5 ml plant agar (5.5g/L, RPI, Mt. Prospect, IL) solidified in 37 ml plastic food containers. Lids of containers were modified to have a 2 cm diameter insect-proof screen for ventilation.

Virus acquisition for *A. gossypii* and *B. tabaci* were conducted in Percival growth chambers (Percival Scientific Inc., Perry, IA) at 25 °C and 12:12 h (L:D) day-length cycle with 30 to 50% relative humidity. The same CLRDV infected plants were used for virus acquisition by *A. gossypii* and *B. tabaci*, but because both insects do not successfully establish together on the same plant, *B. tabaci* acquired CLRDV from excised leaves and *A. gossypii* were allowed to feed on the CLRDV infected plants (see below). To optimize transmission parameters and increase the probability of transmission, there were slight differences in *A. gossypii* and *B. tabaci*, protocols. Modifications were based on conditions that yielded successful transmission of poleroviruses with aphids or whiteflies in previous studies. These differences should not have significantly impacted the outcomes of these experiments because conditions included the maximum ranges required for successful acquisition and transmission by the respective insect vectors (Costa et al., 2020; Ghosh et al., 2019; Michelotto and Busoli, 2003; Polston and Capobianco, 2013).

Virus-free *A. gossypii* and *B. tabaci* on healthy (CLRDV-free) cotton plants served as a negative control for any contamination in the colonies or source material. CLRDV infection was confirmed in leaf discs using nested PCR to amplify a fragment of the coat protein. Primers Pol3628F/Pol4021R were used to amplify a 395-bp fragment in the first round PCR reaction, and then first round PCR product was diluted in ddH_2O (1:10) and amplified with primers CLRDV3675F/Pol3982R targeting a 307-bp fragment as described in Mahas et al. (2022) and Sharman et al. (2015). Results were recorded as positive or negative infection, and the average proportion (±standard error) of leaf discs or plants infected with CLRDV are reported.

Transmission Experiments with Whiteflies. *Bemisia tabaci* fed freely and exhibited negligible mortality on excised cotton leaves. Therefore, acquisition access periods (AAP) for *B. tabaci* were conducted over 96 h on excised, fully expanded leaves from CLRDV-infected plants. This AAP is 48 h longer than those reported for *B. tabaci*-transmitted poleroviruses in Israel and Brazil (Costa et al., 2020; Ghosh et al., 2019), one of which occurred on excised leaves (Costa et al., 2020). Petioles of excised leaves were placed in 22 ml glass test tubes containing water, secured with parafilm, and placed in insect-proof cages with adult *B. tabaci*. Inoculation access periods (IAP) were performed by allowing the putatively viruliferous adults from the AAPs to feed on leaf discs. Groups of 10 adult female *B. tabaci* were confined on each leaf disc for a 48 h IAP. Afterwards, insects were removed and leaf discs were incubated for another 96 h before CLRDV testing. Leaf discs infested with *B. tabaci* were examined under a microscope for eggs, which were removed with a straight tip dissection needle before RNA extraction for CLRDV testing. The

IAP was the same length of time as those used for transmission of other poleroviruses (Costa et al., 2020; Ghosh et al., 2019) and is a time reported to optimize transmission of whitefly-transmitted viruses more generally (Polston and Capobianco, 2013). The IAP was limited to 48 h for *B. tabaci* to reduce the number of eggs laid and time required to remove them so the leaf discs could be processed the same day. Ten leaf discs were tested during each replicate, and these experiments were replicated twice at different times and using different source plants for virus acquisition. A total of 20 leaf discs and 200 individuals were tested for transmission.

Transmission Experiments with Aphids. *Aphis gossypii* did not establish well on mature excised cotton leaves from CLRDV-infected plants, therefore, adult females were allowed to colonize a CLRDV-infected cotton plant and their adult offspring were used in transmission experiments, consistent with the methods of Michelotto and Busoli (2003, 2007). Different *A. gossypii* morphs were tested for their ability to transmit at 23, 25, and 27 °C, and a 12:12 L:D cycle with 30 to 50% relative humidity. One alate, one apterous, or a group of five alate adults were confined on individual leaf discs for a 120 h IAP before being removed manually and the leaf discs tested for presence of the virus. This IAP is 24 h longer than that reported by Michelotto and Busoli (2003) and 72 h longer than reported in Michelotto and Busoli (2007) for successful transmission of CLRDV to plants in Brazil. Ten leaf discs were tested during each replicate. Because transmission was observed in the first replicate, we proceeded with whole-plant experiments without performing a second replicate. A total of 10 leaf discs and 100 individuals were tested at each temperature.

Transmission of CLRDV to a total of 60, two true leaf cotton plants was used to confirm CLRDV transmission observed in the aphid leaf disc assays. Seedlings were planted into 0.95 L plastic containers modified with drainage holes. Another set of containers were modified by gluing a 2 cm diameter insect-proof screen to the bottom, and these were used as lids by inverting them over the seedling and securing it with parafilm to confine insects. Experiments were conducted at 23, 25, and 27 °C, and a 12:12 L:D cycle. Ten putatively viruliferous *A. gossypii* (7:3, apterous: alate) were transferred by paintbrush to the plants for a 72 h AAP. After 72 h, the plants were sprayed with flupyradifurone (Sivanto Prime®, Bayer Crop Sciences, St. Louis, MO) mixed at 11 ml of concentrate per liter of water to kill the aphids. Lids were removed completely 96 h after the spray to allow the cotton to grow, and plants were tested using PCR 28 d after infestation. The third, upper-most expanded leaf was collected to confirm systemic infection.

RESULTS AND DISCUSSION

The results of this study corroborate evidence that *A. gossypii* is the vector of CLRDV in the U.S. and provide direct evidence that *B. tabaci* does not transmit the CLRDV-AL variant to cotton (Table 1). CLRDV was not detected in leaf discs fed upon by 20 groups of 10 putatively viruliferous *B. tabaci* in two replicated experiments (200 individuals total), which provides support that *B. tabaci* does not transmit the CLRDV-AL variant. Results show that *A. gossypii* transmitted CLRDV-AL to leaf discs fed upon by alate (6/30), apterous (4/30), and both life stages (18/30) of *A. gossypii* (Table 1). Similar proportions of infected leaf discs were observed when individual apterous and alate aphids transmitted CLRDV-AL to leaf discs, and 3.5- to 6-fold higher transmission rates were observed when groups of five individuals were used. Transmission of CLRDV-AL by *A. gossypii* resulting in systemic infection of cotton seedlings was also confirmed (27/60) (Table 1). Virus-plant interactions and host plant defenses can inhibit systemic infections from inoculation points represented in the leaf disc assay. Therefore, it is important to demonstrate systemic infection of the host in whole plants if transmission is detected in leaf discs. CLRDV-AL was detected in approximately half of the plants tested when groups of 10 aphids were used to transmit the virus. Temperatures within the range of 23 to 27 °C did not influence transmission in these experiments ($p < 0.05$, data not shown), but additional experiments with larger sample sizes are required to investigate the influence of temperature. No symptoms were observed on these plants in the greenhouse during the 30 to 60 d they were observed (data not shown), and symptoms of CLRDV in the U.S. are typically not observed in infected cotton plants maintained in the greenhouse (Jacobson and Conner, personal observation).

Table 1. The proportion (±standard error) of samples testing positive for cotton leafroll dwarf virus (sample size) in transmission experiments testing vector competence of *Aphis gossypii* and *Bemisia tabaci*

Sample Type:	Cotton Leaf Discs				Cotton Plant
Temperature	10[z] Adult *B. tabaci*	1 Alate *A. gossypii*	1 Apterous *A. gossypii*	5 Apterous *A. gossypii*	10 mixed[y] *A. gossypii*
23°C	—	0.20(±0.1) (10)	0.10(±0.1) (10)	0.50(±0.2) (10)	0.45(±0.1) (20)
25°C	0.00 (20)	0.20(±0.1) (10)	0.30(±0.2) (10)	0.70(±0.2) (10)	0.50(±0.1) (20)
27°C	—	0.20(±0.1) (10)	0.10(±0.1) (10)	0.60(±0.2) (10)	0.40(±0.1) (20)
Total	0.00 (20)	0.20(±0.1) (30)	0.16(±0.1) (30)	0.60(±0.1) (30)	0.45(±0.1) (60)

[z] **Number of insects per plant or leaf disc.**

[y] **7 Apterous + 3 Alate aphids were used to transmit CLRDV to cotton seedlings.**

Our results confirming vector competence of *A. gossypii* is supported by knowledge to date about CLRDV sequence similarity in the Americas, and sequences of recombinant poleroviruses that are transmitted by whiteflies and not aphids (Costa et al., 2020; Ghosh et al., 2019). Sequence analyses of whitefly-transmitted poleroviruses detected mutation and recombination events in genes responsible for vector transmission but did not identify specific mechanisms responsible for the change in vector transmissibility. A common finding in the sequence analyses was the whitefly-transmitted isolates originated from an interspecific recombination event between two polerovirus species that resulted in the alteration of the P3/P5 region of the genome. Polerovirus P3/P5 is a multifunctional protein required for aphid transmission and movement within the vector (Brault et al., 1995). How recombination altered vector competence is not clear because the P3 and P5 regions of the two whitefly-transmitted poleroviruses exhibited low genetic similarity at 49.8 and 17.4%, respectively (Costa et al., 2020). Recent studies in the U.S. show that CLRDV variants from Georgia, Alabama, and Texas are 95 to 98% identical to each other (Tabassum et al., 2020). The full genome sequence of the CLRDV-AL shares 94.8 to 95.2% nucleotide identity with CLRDV variants from Argentina and Brazil (Avelar et al., 2020). The P3 region of CLRDV-AL shares 97.0 to 98.0% nt (98.5 to 99.5% aa identity) and the P3-P5 protein shared 95.3 to 96.0% nt (96 to 97.3% aa identity) with South American variants. P3 sequences from Mississippi and Georgia share 98.4 to 100% nt and 98.8 to 100% aa identity with South American variants (Avelar et al., 2020; Tabassum et al., 2020). No interspecies recombination events were detected in analyses of the CLRDV-AL genome (Avelar et al., 2020). Taken together, the low genetic diversity reported in studies suggests a recent introduction and common origin of CLRDV in the U.S., and that the CLRDV-AL variant used in this study is not produced from recombination events that have been identified for the whitefly-transmitted poleroviruses. Based on this knowledge, further experimentation examining whitefly transmission of CLRDV to whole plants was not conducted because the time, space, and high cost of CLRDV diagnostics was difficult to justify.

Experiments with aphids also demonstrated that leaf disc assays are useful for studying transmission of CLRDV with individuals or groups of aphids. This method has been used reliably for other aphid- and whitefly-transmitted viruses (Czosnek et al., 1993; Di Mattia et al., 2020; Moreno et al. 2011) and could be valuable in future transmission studies with CLRDV due to the large amount of space required to conduct experiments with cotton plants. Maintaining experimental plants in greenhouses is risky because infestations of both whiteflies and aphids can compromise experiments if they occur before virus testing is complete, and environmental chambers are only suitable for short-term experiments. Leaf discs can be collected and tested five d after they are initially exposed to viruliferous aphids, whereas CLRDV detection in plants requires an incubation period of at least 30 d (Galbieri et al., 2010), but is most accurate 60 d after inoculation with CLRDV-AL (Michelotto and Busoli, 2007).

Future studies should continue to monitor the genetic diversity and evolution of CLRDV, especially in areas where other poleroviruses occur. Additional information is also needed to complete our knowledge on the mode of transmission of CLRDV by *A. gossypii*, and whether additional aphid species are vectors of CLRDV in the U.S.

ACKNOWLEDGMENTS

Funding for this project was provided by the Foundation for Food and Agricultural Research ROAR-0000000020, Cotton Incorporated, Alabama Cotton Commission, and the Alabama Agricultural Experiment Station.

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