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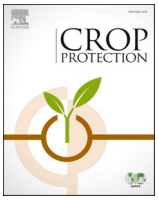

Investigating the effects of planting date and *Aphis gossypii* management on reducing the final incidence of cotton leafroll dwarf virus

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ABSTRACT

This is the first study to research management strategies for cotton leafroll dwarf virus (CLRDV) in the southeastern U.S. The efficacy of aphid vector management to reduce final CLRDV incidence was investigated concurrent with efforts to monitor aphid population dynamics and timing of CLRDV spread. Adjusting the planting date and insecticide applications did not reduce the final incidence of CLRDV, which was confirmed in 60–100% of plants per plot using RT-PCR. Aphid population density was reduced, but not eliminated with foliar insecticide applications. *Aphis gossypii* was the only species observed on cotton and was the dominant species collected in pan traps. Three distinct periods of virus spread were detected with sentinel plants including early, mid-and late-season. Most virus spread occurred during large aphid dispersal events.

1. Introduction

The cotton aphid, *Aphis gossypii* Glover, has been reported to transmit over thirty viruses to crops worldwide (Ebert and Cartwright, 1997), and is the only vector reported to transmit cotton leafroll dwarf virus (CLRDV, genus: *Polerovirus*, family: *Solemoviridae*) to cotton, *Gossypium hirsutum* L, in a persistent-circulative and non-propagative manner (Cauquil J and Vaissayre M, 1971; Heilsnis et al., 2020; McLaughlin et al., 2020; Michelotto and Busoli, 2007, 2003). This virus has been reported from Africa, Asia, and South America with losses up to 1500 kg ha^{-1} in South America (Cauquil J and Vaissayre M, 1971; Corrêa et al., 2005; Distéfano et al., 2010; Galbieri et al., 2017; Mukherjee et al., 2016; Ray et al., 2016; Sharman et al., 2015; Silva et al., 2008). Management of disease caused by CLRDV in Brazil, and of a related cotton-infecting polerovirus from Australia, is achieved using resistant varieties and aphid management (Ellis et al., 2016; Galbieri et al., 2017; Reddall et al., 2004). CLRDV is the first virus reported to infect cotton in the southeastern United States (U.S.)(Avelar et al., 2019) and the virus isolates detected in the U.S. are distinct from those previously reported from South America (Avelar et al., 2020; Tabassum et al., 2020). CLRDV was first observed from Alabama (AL) in 2017, and is currently distributed in cotton growing states from North Carolina to west Texas (Aboughanem-Sabanadzovic et al., 2019; Alabi et al., 2020; Ali and Mokhtari, 2020; Avelar et al., 2019; Iriarte et al., 2020; Price et al., 2020; Tabassum et al., 2019; Thiessen et al., 2020; Wang et al., 2020). Compared to symptoms in South America, disease caused by CLRDV in the U.S. is highly variable among locations and asymptomatic infections are common (Brown et al., 2020). Virus incidence based on symptomatology ranges from 2 to 100% (Aboughanem-Sabanadzovic et al., 2019; Alabi et al., 2020; Ali and Mokhtari, 2020; Avelar et al., 2019; Brown et al., 2020; Tabassum et al., 2019). Reported yield losses are variable (Avelar et al., 2019; Brown et al., 2020). Extreme yield loss has occurred at some locations, but many commercial fields where CLRDV was detected in the past two years in AL and Georgia (GA) met production

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goals (Roberts, Bag, Toews, Conner, Jacobson, personal observation). It is unknown whether variation in disease and yield loss among locations is due to varietal, environmental, crop age, or vector related factors. CLRDV has been detected in all commercial cotton varieties tested in the U.S., and management tactics for reducing CLRDV have not been investigated in the U.S. (Brown et al., 2020).

Cultural and chemical practices may reduce virus incidence in some pathosystems, but their efficacy depends on the seasonal dynamics of reservoir hosts and vectors in the landscape, and how quickly the vector acquires and transmits the plant virus. Mitigation programs aim to disrupt either primary spread events, in which vectors spread the virus from reservoir hosts to crop host plants, or secondary spread events caused by colonizing vector populations that spread virus throughout the crop field as populations increase (Momol et al., 2004; Reitz and Funderburk, 2012; Swenson, 1968). Adjusting the planting date may decrease virus incidence by temporally isolating young crops from vectors because older plants are generally more tolerant of virus infection (Beaudoin et al., 2009; Kone et al., 2017; McMechan and Hein, 2016; Srinivasan et al., 2017). Insecticides are only reported to reduce primary spread if they have antifeedant properties, and it takes longer periods of feeding for virus transmission to occur (Chappell and Kennedy, 2018; Groves et al., 2001; Jacobson and Kennedy, 2011; Li et al., 2019; Pappu et al., 2000). Secondary spread can be managed with insecticides by reducing vector populations in the field (Momol et al., 2004; Reitz and Funderburk, 2012; Swenson, 1968). Studies on CLRDV from Brazil reported a 200–300 kg ha^{-1} increase in cotton yield, and a 1.5–2 point decrease in disease severity rating (1–5 scale), on susceptible cotton varieties when insecticides were applied at a threshold of 5, 20, 40, or 60% of plants infested with colonies of 5–10 aphids (Galbieri et al., 2017). CLRDV is reported to be transmitted by alate aphids in less than 1 min of feeding (Michelotto and Busoli, 2007) which suggests insecticide applications reduce the secondary spread of CLRDV.

The efficacy of vector population management for reducing virus incidence also depends upon the magnitude of primary spread into a crop, and the relative contribution of primary versus secondary spread to final virus incidence. Initial analyses of virus incidence in AL and GA have shown that up to 80–100% of plants test positive for CLRDV using PCR (Brown et al., 2020), strongly suggesting that the majority of plants in a given field experienced aphid infestation during the growing season. Previous studies examining *A. gossypii* in the U.S. cotton belt quantified population size, but not the proportion of plants infested, and knowledge of the timing and magnitude of season-long aphid dispersal into cotton is limited (Abney et al., 2008; Weathersbee and Hardee, 1994).

The high incidence of CLRDV could also be caused by the presence of multiple vectors. The primary vector in Africa, India, and South America is *A. gossypii* (Cauquil J and Vaissayre M, 1971; Michelotto and Busoli, 2007, 2003; Mukherjee et al., 2016), but the cowpea aphid, *Aphis craccivora* Koch, and the green peach aphid, *Myzus persicae* Sulzer, have also been reported to transmit CLRDV to chickpea crops in India (Mukherjee et al., 2016; Reddy and Kumar, 2004). A study from China reported detecting CLRDV in *Aphis glycines* Matsumura collected from soybean, however, this species is not reported to feed on cotton and vector competence has not been confirmed (Feng et al., 2017). *Aphis gossypii* is the only known vector of CLRDV in the U.S. (Heilsnis et al., 2020; McLaughlin et al., 2020), but seven other aphid species are reported to colonize cotton in the U.S. (Stoetzel et al., 1996) including: *A. craccivora* (Blackman and Eastop, 2000); bean aphid, *Aphis fabae* Scopoli (Blackman and Eastop, 2000); potato aphid, *Macrosiphum euphorbiae* Thomas (Blackman and Eastop, 2000); *M. persicae* (Blackman and Eastop, 2000; Kennedy et al., 1962); corn root aphid, *Protaphis middletonii* Thomas (Blackman and Eastop, 2000); rice root aphid, *Rhopalosiphum rufiabdominale* Sasaki (Blackman and Eastop, 2000); and the bean root aphid, *Smynthurodes betae* Westwood (Blackman and Eastop, 2000). All of these species or at least one of their junior synonyms (e.g., in *P. middletonii* as *Aphis armoraciae* Cowen (Chan et al., 1991) lists five viruses associated with *A. armoraciae*) are known to transmit at least one plant virus. The status of these aphid species as vectors of CLRDV is not referenced in (Chan et al., 1991) and is currently unknown in the U.S.

The primary objective of this study was to determine whether aphid management practices reduce CLRDV transmission to cotton under field conditions. A secondary objective was to monitor aphid population dynamics and the timing of virus spread around the field plots using insect traps and sentinel plants, respectively, to identify timing of aphid dispersal into the crop, and which aphid species are present when virus spread occurs. Our first hypothesis was that insecticide sprays for aphids would not reduce primary virus spread due to the quick transmission times reported for alates (Michelotto and Busoli, 2007), but may reduce secondary spread. Our second hypothesis was that planting date adjustments would reduce final CLRDV incidence if crop susceptibility changes due to mature plant resistance reported for other crops (Beaudoin et al., 2009; Kone et al., 2017; McMechan and Hein, 2016; Srinivasan et al., 2017). Two site-years of data are presented from replicated small plot field trials in south AL and south GA where a high incidence of CLRDV was observed in the preceding year.

2. Methods and materials

2.1. Small plot experiment

Two field trials were performed in 2019, one in Brewton, Escambia County, AL (31.141700, −87.050000) and one in Tifton, Tift County, GA (31.489738, −83.519721). Each plot was 4-rows wide (0.91 m centers) and 9.14 m long. Plots were separated by a skip row on each side and a 2.13 m alley on each end to minimize aphid spread between plots. A split-plot design with four replications was used for these experiments; planting date was the main plot effect and insecticide treatments used for aphid management was the subplot effect. There were two planting date treatments and four insecticide treatments; see Table 1 for descriptions and rationale. Natural infestations of *A. gossypii* were managed with acetamiprid (Assail 70 WP United Phosphorus, Inc., King of Prussia, PA) at a rate of 182 ml ha^{-1} (Table 1). This active ingredient was used because whitefly, *Bemisia tabaci*, infestations also occur in the southeast, and this active ingredient manages both pests. Applications were made with tractor mounted sprayers using TeeJet 8002 nozzles, 0.46 m spacing, 18 L ha^{-1}, and 35 psi.

Variety DP1646B2XF (DeltaPine®, Dekalb Genetics Corporation, Dekalb, IL) was used for these trials. The seed contained an imidacloprid seed treatment (0.375 mg a.i./seed) (Gaucho, Bayer Crop Science, Research Triangle Park, NC) for thrips management. When required, two-spotted spidermites, *Tetranychus urticase* Koch, were managed with abamectin (Agri-Mek, Syngenta, Pensacola, FL) at a rate of 402 ml ha^{-1} and stink bugs (Hemiptera: Pentatomidae) were managed using dicrotophos (Bidren8, AMVAC, Axis, AL) at a rate of 585 ml ha^{-1} (Table 1). Weeds, pathogens and fertility were managed based on standard local practices (Alabama Cooperative Extension System, 2019; Whitaker et al., 2019).

The proportion of plants infested with aphids, the number of aphids per plant, the final incidence of CLRDV, and yield were recorded from the middle two rows of each plot. To determine the proportion of plants infested with aphids, presence/absence was recorded from ten consecutive plants in each of the middle two rows (20 plants per plot) in AL; in GA, ten plants were randomly selected from rows two and three (ten plants per plot). Aphid population size was recorded as the total number of live aphids on the fourth fully expanded leaf below the terminal for ten random plants per plot (Hardee et al., 1993; Weathersbee et al., 1994). This corresponds to a leaf position where consistently high numbers of aphids are observed (Table S1). Final virus incidence was confirmed by testing ten plants per plot for the presence of CLRDV using RT-PCR (see below); plots were sampled 12 August in AL and 31 July in GA. Plots were machine harvested from the middle two rows of each plot to examine yield (Table 1).

Table 1
Dates of management activities for small plot trials.

Location	Planting Date	Treatment[a]	Date Initiated[b]	Spidermite[c]	Stink Bug[d]	Harvest[f]
Alabama	May 2	First True Leaf	5/24/2019	6 June, 13 August	16 July	25 September
		First Colonization	6/21/2019			
		Early July	7/2/2019			
		Control				
	June 4	First True Leaf	6/19/2019			11 October
		First Colonization	6/21/2019			
		Early July	7/2/2019			
		Control				
	All[e]	All	7/16/2019			
Georgia	May 2	First True Leaf	5/10/2019	27 July	12 & 31 July	24 September
		First Colonization	6/14/2019			
		Early July	7/3/2019			
		Control				
	June 3	First True Leaf	6/14/2019			4 November
		First Colonization	6/21/2019			
		Early July	7/3/2019			
		Control				
	All	All	7/12/2019			

[a] Weekly insecticide applications of acetamiprid targeting *Aphis gossypii* were initiated at the "First true leaf" growth stage in an attempt to deter colonization; at "First Colonization" to suppress population buildup; or "Early July" to eliminate populations after colonization and population increase, or no application was made as a "Control".
[b] Date weekly foliar sprays were initiated.
[c] Dates all plots were sprayed with miticides to manage spidermite infestations.
[d] Dates all plots were sprayed with insecticide to manage stink bug infestations.
[e] All plots were oversprayed after *Aphis gossypii* populations crashed due to fungal epizootics.
[f] Dates plots from each planting date were harvested.

2.2. Monitoring aphid dispersal and CLRDV spread

Pan traps, 21 cm diameter x 7.5 cm height, and painted with Krylon® "Gloss Sunbeam" yellow spray paint (Sherwin-Williams, Cleveland, OH), were used to monitor aphids following previously described methods (Heathcote et al., 1969; Kring, 1972; Nielson and Wolfenbarger, 1970). Four yellow pan traps were placed around the perimeter of the small plot trials, with one pan trap located in the middle of each field edge, and surrounded by bare soil season-long to increase alightment (Döring et al., 2004; Kennedy et al., 1961). Each trap was filled with 50% propylene glycol, and a drop of liquid dish soap to reduce the surface tension. Every seven days, trap contents were collected and stored individually in 70% ethanol. In the laboratory, adult alate aphids were counted and identified to species using existing identification keys (Stoetzel et al., 1996; Stoetzel and Miller, 2001).

Morphological characters of individuals were examined in ethanol using an Olympus SZX12 microscope with an Olympus DR PLAPO 1X PF objective (Olympus Corporation of the Americas, Center Valley, PA). The eight aphids reported from cotton in the U.S. were targeted for identification (Stoetzel et al., 1996). Other aphid species were counted and listed as "other" with the exception of the rusty plum aphid, *Hysteroneura setariae* Thomas, which was one of the predominant species collected and a first report in AL. Voucher specimens of each species were slide-mounted using the protocol of the Systematic Entomology Laboratory – USDA ARS (USDA) and deposited at Auburn University Museum of Natural History (AUMNH), accession numbers 215557–215583.

Sentinel plants were used to monitor the timing of virus spread. Healthy cotton (DP 1646B2XF) that did not have a field rate of seed-applied insecticide was planted in 3601 standard plant tray inserts (BWI, Nash, Texas) using ProMix MX General Purpose (Premier Horticulture Inc., Quebec Canada) soil and grown in virus and insect-free incubators. Three-four true-leaf plants were transplanted individually to 15.24 cm pots (Blow-Molded Classic Line, part C600, Nursery Supplies Inc., Chambersburg, PA), fertilized with 20–10–20 Peat-Lite Special, Base Formulation, M-77 Chelating Formula (Peter Professional, Summerville, SC), and covered with a 60.5 (height) by 34 (diameter) cm sleeve cage made out of 100-μm thrips-proof screen (Ludvig Svensson, Sweden). Four sentinel plants remained in the greenhouse as control plants in future virus testing, and to monitor for unintended virus spread in the greenhouse. Eight sentinel plants were held for one to two days in the greenhouse and then transported to the field. Two plants were placed on each field edge, uncovered so aphids could access them, and were surrounded by bare soil to increase alightment (Döring et al., 2004; Kennedy et al., 1961). Seven days after being placed in the field, the cohort of eight sentinel plants was replaced with a new cohort. After collection, sentinel plants were transported back to Auburn University greenhouses, sprayed with Flupyradifurone (Sivanto™ Prime, Bayer Crop Science, Research Triangle Park, NC) at a rate of 1 L ha^{-1} to remove aphids, and were grown insect-free in a greenhouse for six to eight weeks (Galbieri et al., 2010) before being tested for CLRDV infection by RT-PCR as described below. Sentinel plant monitoring was conducted concurrently with aphid trapping in AL only, because the current cost of diagnostics was cost prohibitive.

2.3. PCR confirmation of CLRDV

CLRDV infections in field plots and sentinel plants were confirmed using nested RT-PCR. The nested-PCR assay targeting the CLRDV partial coat protein gene is often the best approach for increased sensitivity and reduced non-specific binding in the second round PCR. The coat protein gene is encoded on a sub-genomic RNA and is at a higher copy number relative to most of the virus genome making it a good target for a low titer virus such as CLRDV. Two petioles were collected from each plant, one from old growth and one from new growth, and combined into one sample. RNA was extracted from the petiole tissue of each sample using Qiagen RNeasy Plant Mini kits (Qiagen, Germantown, MD) following the manufacturer's recommendations. cDNA was synthesized using SuperScript IV first-strand synthesis system (ThermoFisher Scientific, Waltham, MA) and first round PCR done using polerovirus PCR primers Pol3628F and Pol4021R (Table 2) targeting a 395 nt genome segment of the partial coat protein gene.

First round PCR product (Pol3628F/Pol4021R) was diluted 1:10 and amplified in a second round nested PCR using CLRDV-specific primer

Table 2
Primer sequences used for detection of cotton leafroll dwarf virus in cotton plants.

Primer name	Primer direction	Sequence (5′ – 3′)	Round[a]	Product size	Reference
Pol3628F	Forward	TAATGAATACGGYCGYGGSTAG	1	395 bp	Sharman et al., (2015)
Pol4021R	Reverse	GGRTCMAVYTCRTAAGMGATSGA			
CLRDV3675F	Forward	CCACGTAGRCGCAACAGGCGT	2	310 bp	Sharman et al., (2015)
Pol3982R	Reverse	CGAGGCCTCGGAGATGAACT			Sharman et al., (2015)

[a] Designates which primer pair is used for the first (1) and second (2) amplification for the nested PCR.

CLRDV3675F and polerovirus primer Pol3982R (Table 2; (Sharman et al., 2015)) targeting a 310 nt section of the coat protein gene located within the first round PCR target. Both rounds of PCR contained 25 μl reaction volumes with 1 unit Platinum *Taq* polymerase (Invitrogen, Carlsbad, CA), 1.75 mM $MgCl_2$, 200 mM dNTPs, 200 nM of each primer and 2 μl of cDNA template for the first round of PCR or 1 μl of diluted PCR product in the second round PCR. Temperature cycling parameters for both rounds of PCR consisted 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 denaturation of 72 °C for 3 min. Positive controls (plants that had previously tested positive) and negative (plants that had been grown in a controlled environment in the absence of aphids) were included in each run, and PCR products were examined by gel electrophoresis.

2.4. Statistical analyses

Data from this split-plot experiment were analyzed using PROC GLIMMIX in SAS (Version 9.4; SAS Institute, Inc., Cary, NC, USA). Data were analyzed to compare values among treatments separately for each data collection date. Aphid incidence (presence/absence), aphid counts, and CLRDV incidence (presence/absence) were analyzed separately for each location using planting date, aphid management regime and their interaction term as fixed effects, and main plot and subplot as random effects. Count data were modeled using a negative binomial distribution. Incidence data were modeled using a binomial distribution. Yield data from AL and GA were analyzed together using a Gaussian distribution with planting date, insecticide treatment, and their interaction term as fixed effects, and location and main plot as random effects; data were pooled because there was no significant difference between locations in preliminary analyses. If the interaction between the fixed effects was not significant this term was removed from the final model. When interaction terms were significant between the fixed effects, the SLICE option was used to examine the simple effects while controlling for the other factor.

3. Results

3.1. Aphids infestations on cotton

The proportion of plants infested with aphids in the small plot experiments was determined by inspecting plants for the presence of aphids. These in-field assessments began on 17 June for both planting dates in AL, and on 30 May and 20 June for May and June plant dates, respectively, in GA when aphids were first detected. Only two aphids were found 30 May in GA, so no statistical analyses were performed for this date. In GA, no aphid counts were conducted for the June-planted cotton from 6/6/2019-6/13/2019 because the first true leaf was not present; there were no planting date comparisons made for these dates.

In AL, infestations were higher in May-planted cotton on 17 June ($F_{1,608} = 48.77$, $P < 0.0001$), 24 June ($F_{1,608} = 7.66$, $P < 0.0001$), but weren't different in GA on 20 June ($F_{1,288} = 0.00$, $P = 0.9600$). At both locations, 100% of the plants were infested with aphids (Fig. 1) during the next two evaluation weeks regardless of plant date or insecticide treatment. After this time, the proportion of plants infested were significantly higher in the June-planted cotton in AL on 15 July ($F_{1,608} = 27.5$, $P < 0.0001$) and 22 July ($F_{1,608} = 9.07$, $P = 0.0027$), and GA on 11 July ($F_{2,228} = 15.82$, $P < 0.0001$) (Fig. 1A and B). The only date a significant reduction in the proportion of plants infested due to insecticide treatment was observed in AL on 24 June ($F_{3,608} = 1.8$, $P < 0.0001$). No significant differences among insecticide treatments were observed in AL on 17 June ($F_{3,608} = 1.8$, $P = 0.1500$), 15 July ($F_{3,608} = 0.49$, $P =$

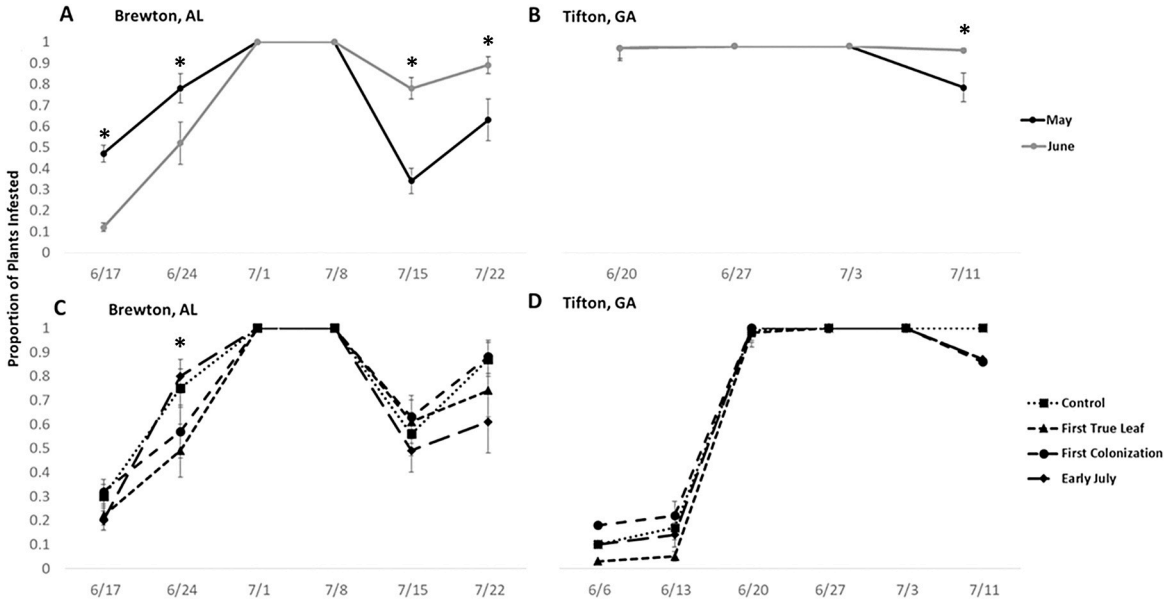

Fig. 1. Means (±standard errors) of the proportion of plants infested with aphids among planting dates (main plot effect, A & B) and insecticide treatments (sub-plot effects, C & D). Asterisks denote significant differences by date using Tukey's method at $P \leq 0.05$.

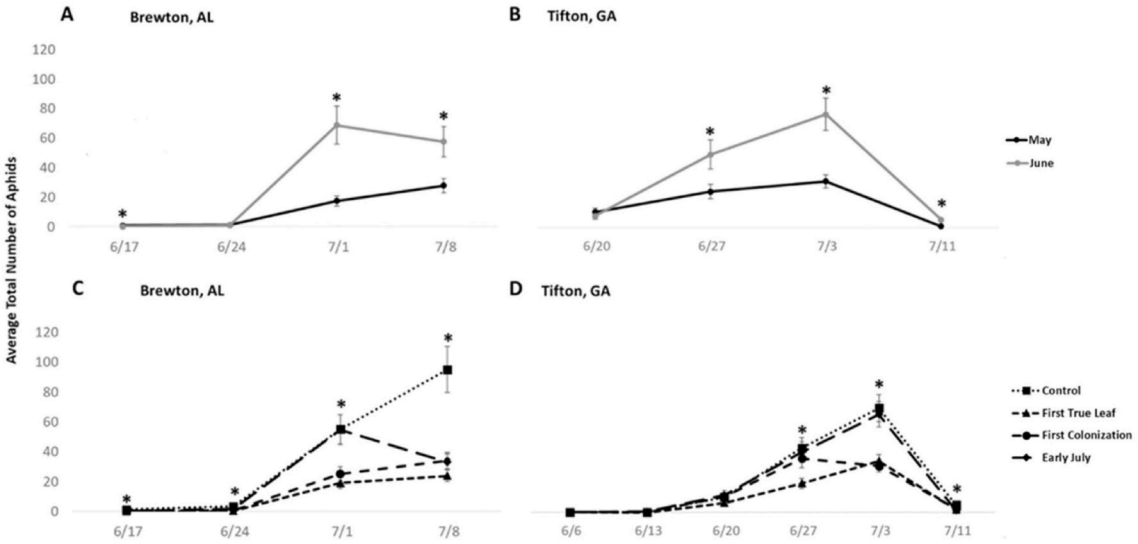

Fig. 2. Means (±standard errors) of the total number of aphids compared among planting dates (main plot effect, A & B) and insecticide treatments (sub-plot effects, C & D). Asterisks denote significant differences by date using Tukey's method at $P \leq 0.05$.

0.6897), 22 July ($F_{3,608} = 0.16$, $P = 0.1590$), or in GA on 6 June ($F_{3,144} = 1.38$, $P = 0.2523$), 13 June ($F_{3,144} = 1.44$, $P = 0.2344$), 20 June ($F_{3,288} = 0.79$, $P = 0.5031$), 11 July ($F_{3,288} = 0.02$, $P = 0.9947$) (Fig. 1C and D). The only significant interaction between insecticide treatment and planting date for aphid incidence occurred in AL, on 16 June, ($F_{3,608} = 5.52$, $P = 0.0010$), where aphid incidence was greater across insecticide treatments in the May-planted cotton compared to the June-planted cotton.

Aphid counts were performed to monitor population size. The only date May-planted cotton had a significantly higher number of aphids was on 17 June in AL ($F_{1,288} = 10.02$, $P = 0.0035$). There were no differences among planting dates on 24 June in AL ($F_{1,288} = 0.09$, $P = 0.7677$) or 20 June in GA ($F_{1,288} = 0.29$, $P = 0.5900$). Means were higher for June-planted cotton in AL (Fig. 2A and B) on 1 July ($F_{1,288} = 25.96$, $P < 0.0001$), 8 July ($F_{1,288} = 8.39$, $P = 0.0041$), and in GA on 27 June ($F_{1,288} = 6.40$, $P = 0.0120$), 3 July ($F_{1,288} = 19.72$, $P < 0.0001$), and 11 July ($F_{1,288} = 22.57$, $P < 0.0001$). Differences in aphid numbers were not observed among insecticide treatments in GA on 6 June ($F_{3,144} = 0.74$, $P = 0.5275$), 13 June ($F_{3,144} = 0.99$, $P = 0.3994$), 20 June ($F_{3,288} = 1.22$, $P = 0.3014$), but they were always numerically highest in the non-sprayed plots. Weekly insecticide sprays significantly reduced the number of aphids in insecticide-treated, compared to the non-treated plots, in AL on 17 June ($F_{3,288} = 4.63$, $P = 0.0035$), 24 June ($F_{3,288} = 6.4$, $P = 0.0003$), 1 July ($F_{3,288} = 14.96$, $P < 0.0001$), 8 July ($F_{3,288} = 26.12$, $P < 0.0001$), and in GA on 27 June ($F_{3,288} = 10.01$, $P < 0.0001$), 3 July ($F_{3,288} = 18.90$, $P < 0.0001$), 11 July ($F_{3,288} = 27.13$, $P < 0.0001$) (Fig. 2C and D). Significant interactions between insecticide treatment and planting date occurred in AL on 8 July ($F_{3,288} = 6.64$, $P = 0.0002$) and in GA on 3 July ($F_{3,288} = 5.50$, $P = 0.0011$) and 11 July ($F_{3,288} = 21.26$, $P < 0.0001$).

3.2. Proportion of plants infected with CLRDV and yield

There were no reductions in final virus incidence among plots due to plant date ($F_{1,288} = 0.26$, $P = 0.6116$) or insecticide treatment ($F_{3,288} = 1.78$, $P = 0.1513$) in AL (Table 3) where CLRDV was confirmed in 60–100% of the plants tested in each plot. In GA incidence ranged from 90 to 100% of all plots. No statistical analyses could be performed on data from GA because only 6/320 plants tested negative for CLRDV, and most plots of each treatment x plant date combination had 100% incidence.

No yield differences were observed among insecticide treatments. Yield differences were observed between plant dates but this was likely due to differences in season-long growing conditions experienced by

Table 3
Mean (±standard error) cotton leafroll dwarf virus (CLRDV) incidence and yield and in small plot field trials conducted in Alabama and Georgia. Means comparisons analyzed the main plot effect of planting date and sub-plot effect of insecticide treatment using Tukey's method at $P = 0.05$.

	Final CLRDV Incidence		
	Alabama	Georgia	Lint Yield (kg ha-1)
Planting Date			
May	0.79 (0.05)	0.98	1642.16 (113.68) a¶
June	0.83 (0.05)	0.99	1356.17 (113.68) b
Insecticide Treatment[a]			
Control	0.82 (0.05)	0.99	1565.07 (112.39) a
First True Leaf	0.84 (0.05)	0.95	1467.27 (112.39) a
First Colonization	0.70 (0.07)	0.99	1496.85 (112.39) a
Early July	0.85 (0.05)	1	1467.46 (112.39) a
Significance of Main Effects			
PD	$F_{1,288} = 0.26$, P=0.6116	-[b]	F1,44 = 21.29, P < 0.0001
IT	$F_{3,288} = 1.78$, P=0.1513	–	F3,44 = 2.38, P = 0.0819

¶Means followed by different letters are significantly different from each other.
†2 May (AL and GA), 4 June (AL) and 3 June (GA).
[a] Indicates when weekly foliar sprays were initiated.
[b] No statistical analyses were performed on Georgia because incidence was 100% in most plots.

Table 4
Weekly number of aphids collected in pan traps, and detection of cotton leafroll dwarf virus (CLRDV) spread in cohorts of sentinel plants.

Trapping Start Date	*Aphis gossypii*[a]	*Myzus persicae*[a]	*Aphis craccivora*[a]	*Protaphis middletonii*[a]	*Rhopalosiphum rufiabdominale*[a]	*Macrosiphum euphorbiae*[a]	*Aphis fabae*[a]	*Hysteroneura setariae*[a,b]	Other[a]	Total[c]	CLRDV[d]
Brewton, AL											
5/13/2019	6.5 (1.3)	0.8 (0.8)	0.5 (0.3)	51.3 (1.5)	0.3 (0.3)	0	0	0	4.0 (0.4)	253	2/8
5/20/2019	5.0 (1.1)	0	0.3 (0.3)	33.0 (5.4)	0.5 (0.5)	0	0	0	4.0 (1.4)	171	1/8
5/27/2019	3.8 (1.6)	0	0	17.3 (2.8)	0.5 (0.3)	0	0	0.3 (0.3)	6.5 (1.2)	113	0/8
6/3/2019	4.8 (1.7)	0	0	7.3 (2.5)	2.3 (1.4)	0	0	0.5 (0.3)	6.8 (1.4)	86	0/8
6/10/2019	7.0 (1.6)	0	0.8 (0.8)	12.5 (0.9)	0.8 (0.3)	0	0	0.8 (0.3)	6.3 (1.4)	112	0/8
6/17/2019	6.0 (1.7)	0	0	4.8 (1.8)	0.5 (0.5)	0	0	2.5 (0.7)	4.5 (1.0)	73	1/8
6/24/2019	142.8 (51.7)	0	0.3 (0.3)	14.3 (1.7)	2.0 (1.1)	0	0	3.0 (1.2)	23.5 (4.3)	748	8/8
7/1/2019	238.8 (40.1)	0	0	22.8 (2.0)	0.5 (0.3)	0	0	11.3 (4.0)	8.8 (1.1)	1128	5/8
7/8/2019	117.8 (19.5)	0	0	8.0 (1.7)	0.3 (0.3)	0	0	13.3 (1.3)	15.3 (5.6)	618	4/8
7/15/2019	20.3 (4.0)	0	0	2.0 (0.8)	0.3 (0.3)	0.3 (0.3)	0	10.8 (4.2)	4.5 (1.2)	152	0/8
7/22/2019	11.8 (2.8)	0	0	9.5 (2.5)	4.5 (1.9)	1.0 (0.7)	0	11.5 (4.7)	0.8 (0.5)	156	0/8
7/29/2019	2.0 (0.7)	0	0.5 (0.3)	7.3 (1.7)	0	0.8 (0.5)	0	4.3 (1.5)	4.3 (1.1)	76	0/8
8/6/2019	0.8 (0.5)	0	1.0 (0.4)	8.5 (2.4)	0	0.3 (0.3)	0	1.0 (0.7)	6.0 (2.0)	70	1/8
8/12/2019	1.8 (0.9)	0	0.8 (0.5)	2.3 (0.9)	0	0	0	0.8 (0.8)	4.0 (0.4)	38	2/8
8/19/2019	1.0 (0.7)	0	1.0 (0.6)	1.3 (0.5)	0	0	0	1.0 (0.4)	2.5 (1.3)	27	2/8
8/26/2019	7.5 (4.0)	0	1.3 (0.5)	2.5 (1.0)	0	0	0	0.3 (0.3)	5.8 (1.5)	69	4/8
Total[c]	2309	3	25	817	49	9	0	244	434	3890	–
Tifton, GA											
5/13/2019	2.5 (1.6)	4.0 (2.4)	0	7.5 (2.9)	0.5 (0.3)	0	0	0	0.3 (0.3)	59	–
5/20/2019	0.5 (0.3)	0.3 (0.3)	0	8.5 (2.6)	0.5 (0.3)	0	0	0	1.8 (0.5)	46	–
5/27/2019	0.8 (0.3)	0	0.3 (0.3)	5.3 (1.0)	0	0	0	0	1.25 (0.6)	30	–
6/3/2019	0.3 (0.3)	0	0	1.0 (0.4)	0	0	0	0	0.5 (0.3)	7	–
6/10/2019	1.5 (1.5)	0	0	0	0.3 (0.3)	0	0	0.3 (0.3)	0.5 (0.5)	10	–
6/17/2019	21.5 (6.6)	0.3 (0.3)	1.8 (0.8)	0.8 (0.8)	0	0	0	0.3 (0.3)	6.5 (2.1)	124	–
6/24/2019	349.3 (29.8)	0	2.0 (0.7)	1.3 (0.8)	0	0	0	0.8 (0.5)	16.5 (2.6)	1479	–
7/1/2019	336.8 (131.7)	0	0.8 (0.3)	1.3 (1.0)	0	0	0	0.3 (0.3)	42.0 (9.9)	1524	–
7/8/2019	12.8 (5.1)	0	0.5 (0.3)	0.8 (0.5)	0	0	0	0.3 (0.3)	4.8 (1.5)	76	–
7/15/2019	1.3 (0.6)	0	0	0	0	0	0	0	2.5 (0.6)	15	–
7/22/2019	0.3 (0.3)	0	0	0	0	0	0	0	0.5 (0.5)	3	–
7/29/2019	0.8 (0.5)	0	0	0.3 (0.3)	0	0	0	0	1.3 (0.6)	9	–
8/6/2019	1.0 (1.0)	0	0	0	0	0	0	0	3.0 (2.0)	16	–
8/12/2019	0.3 (0.3)	0	0.3 (0.3)	0	0	0	0	0	0.5 (0.3)	4	–
8/19/2019	0.5 (0.5)	0	0	0	0	0	0	0	0.3 (0.3)	3	–
8/26/2019	0.3 (0.3)	0	0	0	0	0	0	0	0	1	–
Total[c]	2920	18	22	106	5	0	0	7	328	3406	–

[a] Mean (standard error) of alates in four traps per location.
[b] Not reported to infest cotton or transmit CLRDV. First record of species in AL.
[c] Total number of alates in four traps per location (all species).
[d] Proportion of weekly sentinel plants testing positive for CLRDV; to detect virus spread in landscape.

plots after each planting date (Table 3). Conclusions about the relationships between CLRDV infection and yield cannot be made because experimental treatments did not significantly reduce virus incidence. Lint quality analyses were also conducted and are provided in Table S2.

3.3. Monitoring seasonal aphid dynamics

A total of 7296 aphids were captured in AL and GA pan traps, of which 6434 were the eight species reported to infest cotton (Table 4). *Aphis gossypii* was the most abundant and accounted for 60% and 86% of individuals collected in AL and GA, respectively. *Aphis gossypii* were captured each week of trapping, and a large increase in numbers was observed late-June and early-July. *Myzus persicae* and *A. craccivora*, were observed in low numbers, with *M. persicae* primarily present in May, while *A. craccivora* was captured throughout the collection period. *Protaphis middletonii* were observed every week in AL, and in higher numbers than at GA, where this species was captured May–July, but not in August. One or fewer *M. euphorbiae* individuals were collected per trap at AL during July and August, but were not captured in GA. *Rhopalosiphum rufiabdominale* were present in low numbers May–July at AL, and only sporadically May–June in GA. *Aphis fabae* and *S. betae* were not collected at either location. *Hysteroneura setariae* was the third most abundant aphid species in AL and represents a new state record. *Hysteroneura setariae* host alternates between *Prunus* spp. and Poaceae species, (Blackman and Eastop, 2000; Nasruddin, 2013; Stoetzel and Miller, 2001) and is a pest of corn, rice, sugarcane, wheat, (Blackman and Eastop, 2000; Stoetzel and Miller, 2001) and soybeans, *Glycine max* L.(Jahn et al., 2005). This species is known to transmit numerous plant viruses (Blackman and Eastop, 2000; Chan et al., 1991; Masumi et al., 2011; Saleh et al., 1989), but none that infect cotton. It has not been recorded from cotton, and we did not observe species other than *A. gossypii* on cotton plants.

3.4. Timing of CLRDV spread using sentinel plants

CLRDV was detected in sentinel plant cohorts the first two weeks of monitoring when *P. middletonii* was the most abundant species trapped, three weeks later during peak flights of *A. gossypii*, and at the end of August when captures of all species were low (Table 4). None of the control plants that remained in the greenhouse throughout the course of this study tested positive for CLRDV, indicating that virus spread did not occur in the greenhouse.

4. Discussion

The overarching goal of this study was to determine whether aphid management practices reduce CLRDV transmission under natural field conditions. Although adjusting the plant date and making weekly insecticide applications did not reduce the final incidence of CLRDV, these negative results provide valuable information about management for CLRDV. The aphid insecticide management treatments (Table 1) reduced aphid population size (Fig. 2), but did not prevent infestations, or reduce the proportion of plants that became infested with *A. gossypii* (Fig. 1). At both locations, 100% of the plants were infested with aphids during two evaluation weeks (Fig. 1) which indicates the potential for primary spread is high. Current management recommendations for *A. gossypii* suggest avoiding insecticide sprays because yield reductions due to aphid feeding are not generally observed (Abney et al., 2008; Johnson et al., 2002; Layton et al., 1999; Marti and Olson, 2006; O G Marti and Olson, 2007; Sanchez-Peña, 1993; Weathersbee and Hardee, 1994). Using aphid management as a component of a CLRDV disease mitigation approach would increase the season-long economic and environmental costs of cotton pest management by requiring additional insecticide applications for both aphids and secondary pests that are commonly flared by insecticide use, and is not warranted at this time.

We present RT-PCR results to report the CLRDV incidence. This is the most reliable method to report virus incidence in our plots because symptomatology for CLRDV in the U.S. is not formally defined, and many asymptomatic infections occur (Bag, Conner personal communication). Symptomatology was monitored (data not shown), but no obvious disease or boll loss was observed. No yield differences were observed among treatments and we cannot make conclusions regarding the effects of CLRDV infection on yield due to the high virus incidence in all plots. Future studies using aphid-proof cages to manipulate virus spread are needed to compare yield between infected and healthy plants. Caged plants were not included here because our objective was to examine the efficacy of stakeholder management practices to reduce CLRDV incidence under natural conditions.

Aphid monitoring during this study identified seven of the eight species of aphids reported to infest cotton at both locations. *Aphis gossypii*, was the most abundant species, captured every week in traps, and the only species that colonized cotton at both locations. Our results suggest that *A. gossypii* was responsible for a significant amount of virus spread to the crop based on the timing and magnitude of flights, and the colonization that occurred when virus spread was detected during four consecutive weeks June–July. The role of this vector in spreading CLRDV early and late-season is less clear. Virus spread was detected in sentinel plants beginning the first two weeks after the May plant-date, indicating that adjusting the plant date may not have prevented virus spread to seedling cotton. At the time, *P. middletonii* populations were highest at both locations (Table 4). At Brewton *P. middletonii* comprised 81% and 71% of the total aphids collected during these two weeks and were up to 9-fold higher than *A. gossypii*. The captures of all other species were also low during this time. Colonizing aphids were not observed in the field plots during this time, however, the neonicotinoid seed treatment used for thrips management may have suppressed colonization. The absence of aphids does not equal the absence of transmission because there may be transient vectors that feed on but do not colonize crops. Transient vectors are reported to contribute significantly to virus spread in other pathosystems (Halbert et al., 1981; Kalleshwaraswamy et al., 2007). CLRDV was also detected in the sentinel plants during the last four collection dates, however, numbers of all species were low during this time, including those not identified. Cotton plots were not monitored for aphid populations late-season, but it is possible that the virus spread occurring in August was due to the secondary spread of the virus from the cotton plots. Future research is needed to better understand the magnitude of virus spread that occurs throughout the growing season, and vector species responsible for spread.

5. Conclusion

The results of this study suggest that increasing insecticide use is not an effective management strategy for reducing CLRDV spread. These experiments were conducted where high populations of aphids resulted in 100% infestation of our plots and up to 100% incidence of CLRDV. Virus spread is determined by the amount of inoculum in the environment, transmission efficiency of the vectors, distance between inoculum and crop, number of vector species, seasonal population dynamics, vector dispersal behavior, and susceptibility of the crop to the virus (Jacobson, 2019; Jeger et al., 2004). Future research is needed to examine yield effects of CLRDV under controlled conditions, vector population sizes, virus spread by the vector, and how the susceptibility of cotton changes during different phenological stages. More information is needed to better understand the biotic and abiotic interactions underlying risk of virus spread.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cropro.2022.106005.

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