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Natural host range, incidence on overwintering cotton and diversity of cotton leafroll dwarf virus in Georgia USA

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ABSTRACT

In 2018–19, cotton leafroll dwarf virus (CLRDV) was reported from several cotton-producing states in the southern United States. An extensive survey was conducted in the spring, summer, and fall of 2019 to identify weeds and overwintering cotton as sources for primary inoculum for the next season crop. Foliage samples of 57 different weed species were collected from fields formerly planted to cotton and analyzed using RT-PCR with CLRDV specific primers. CLRDV was detected from 23 weed species belonging to 16 different botanical families. Overwintering cotton stalks (48%) and regrowth leaves (75%) both harbored CLRDV. A phylogenetic analysis conducted using nucleotide and amino acid sequences of complete ORF 0, ORF 3, and ORF 4 determined that the CLRDV from all weeds and overwintered cotton stalks from Georgia clustered with CLRDV isolates collected in the USA but differed from isolates reported from South America and Asia. Pairwise nucleotide and amino acid identity showed 91–100% sequence similarity for complete ORF3. For ORF4, pairwise identity among the nucleotide ranged from 92-100%, while amino acids ranged from 90.2-100% with isolates reported from the USA and South America, with the exception of three Asian CLRDV isolates and two weed isolates from Georgia. Similarly, CLRDV isolates from Georgia weeds shared 91.6–93% nucleotide and 88–90.8% amino acid for silencing suppressor compared to most of the isolates from North America and South America. The role of alternative hosts on disease incidence and spread has not been studied in cotton-producing countries where the disease is prevalent. This is the first comprehensive study that identifies weeds and overwintering cotton as a potential green bridge for the year-round survival of CLRDV.

1. Introduction

Cotton (*Gossypium hirsutum* L.) is the most common natural fiber used as a raw material for textile manufacturing. Multiple biotic and abiotic stresses challenge cotton production. Cotton leafroll dwarf virus (CLRDV) that has the potential to cause severe damage to cotton was recently identified from Alabama (Avelar et al., 2019) and subsequently from all major cotton-growing regions in the southern United States (Aboughanem-Sabanadzovic et al., 2019; Alabi et al., 2020; Ali and Mokhtari, 2020; Avelar et al., 2019; Faske et al., 2020; Huseth et al., 2019; Iriarte et al., 2020; Price et al., 2020; Tabassum et al., 2019; Thiessen et al., 2020; Wang et al., 2020). Plants infected with cotton leafroll dwarf disease (CLRDD) show symptoms that include reddening, curling, and drooping of leaves, internodal shortening, intense dark green foliage, and moderate to severe stunting (Agrofoglio et al., 2016; Distéfano et al., 2010; Sharman et al., 2015; Tabassum et al., 2020). CLRDV was also detected from plants showing reduced fruiting and whip-like cotton plants (Tabassum et al., 2019, 2020). Losses up to 50–80% have been reported from Argentina (Agrofoglio et al., 2016; Distéfano et al., 2010) and up to 1500 kg/ha seed cotton from Brazil (Corrêa et al., 2005). CLRDV is a phloem limited positive-sense single-stranded RNA virus of the genus Polerovirus; family *Luteoviridae*

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(Distéfano et al., 2010). It is transmitted by cotton aphids (*Aphis gossypii*) in a persistent-circulative and non-propagative manner (Cauquil and Vaissayre., 1971; Galbieri et al., 2017; Pupim Junior et al., 2008; Silva et al., 2008; Takimoto, 2003).

There is no host-resistance against CLRDV established, reported, or deployed in the USA; however, cotton blue disease (CBD) resistance has been reported in a cotton variety (Delta Opal) with a single dominant gene (*cbd*) (Fang et al., 2010). Preventive management by destroying alternative hosts that harbor CLRDV is an option to manage the disease. Weeds act as a reservoir of viruses and their vectors and play an essential role in the ecology and epidemiology of plant viruses (Alyokhin et al., 2012; Duffus et al., 1971; Kazinczi et al., 2004; Norris and Kogan, 2005; Srinivasan et al., 2008). Many *Poleroviruses* have been reported to infect weeds. Beet western yellow virus (BWYV) was detected in common weeds including *Citrullus lanatus* (Afgan or wild melon), *Conyza* spp. (fleabane), *Navarretia Squarossa* (stinkweed), *Solanum nigrum* (black-berry nightshade), and in *Brassica napus* (volunteer canola) (Coutts et al., 2006). Beet mild yellowing virus has been reported from 93 different weeds (Kozłowska-Makulska et al., 2007). A ubiquitous weed, hairy nightshade (*Solanum sarrachoides*), is a shared host for the potato leafroll virus, green peach aphid (*Myzus persicae*), and potato aphids (*Macrosiphum euphorbiae*) in the potato agroecosystem of southeastern Idaho (Srinivasan et al., 2008, 2012; Thomas, 2002). This weed has a significant impact on the transmission of PLRV in potato fields.

Volunteer crops that emerge after the harvest harbor viruses from one season to the next and serve as a source of inoculum (Hsu et al., 2011; Hull, 2014). Field sanitation is an integral part of the management of iris yellow spot virus on onions (Gent et al., 2004), beet western yellows virus on canola (Coutts et al., 2006) and wheat streak mosaic virus on wheat (Coutts et al., 2008) to minimize the primary inoculum from volunteer crops. In Georgia, cotton is harvested in late fall, and the stalks are often left in the field. Unless destroyed, overwintered cotton stalks can survive mild winters along the lower Coastal Plain in Georgia. Infected cotton that successfully overwinters may be a source of virus inoculum to pass to the next cropping season.

The objective of this study was to identify weeds that harbor CLRDV and investigate if CLRDV can be detected in weeds and overwintering cotton. Extensive surveys were conducted in cotton-growing counties in Georgia to detect the presence of CLRDV on common weeds as well as on overwintered cotton stalk. Both weeds and overwintered cotton stalk may act as a green-bridge and primary inoculum source for the onset of disease in the next growing season.

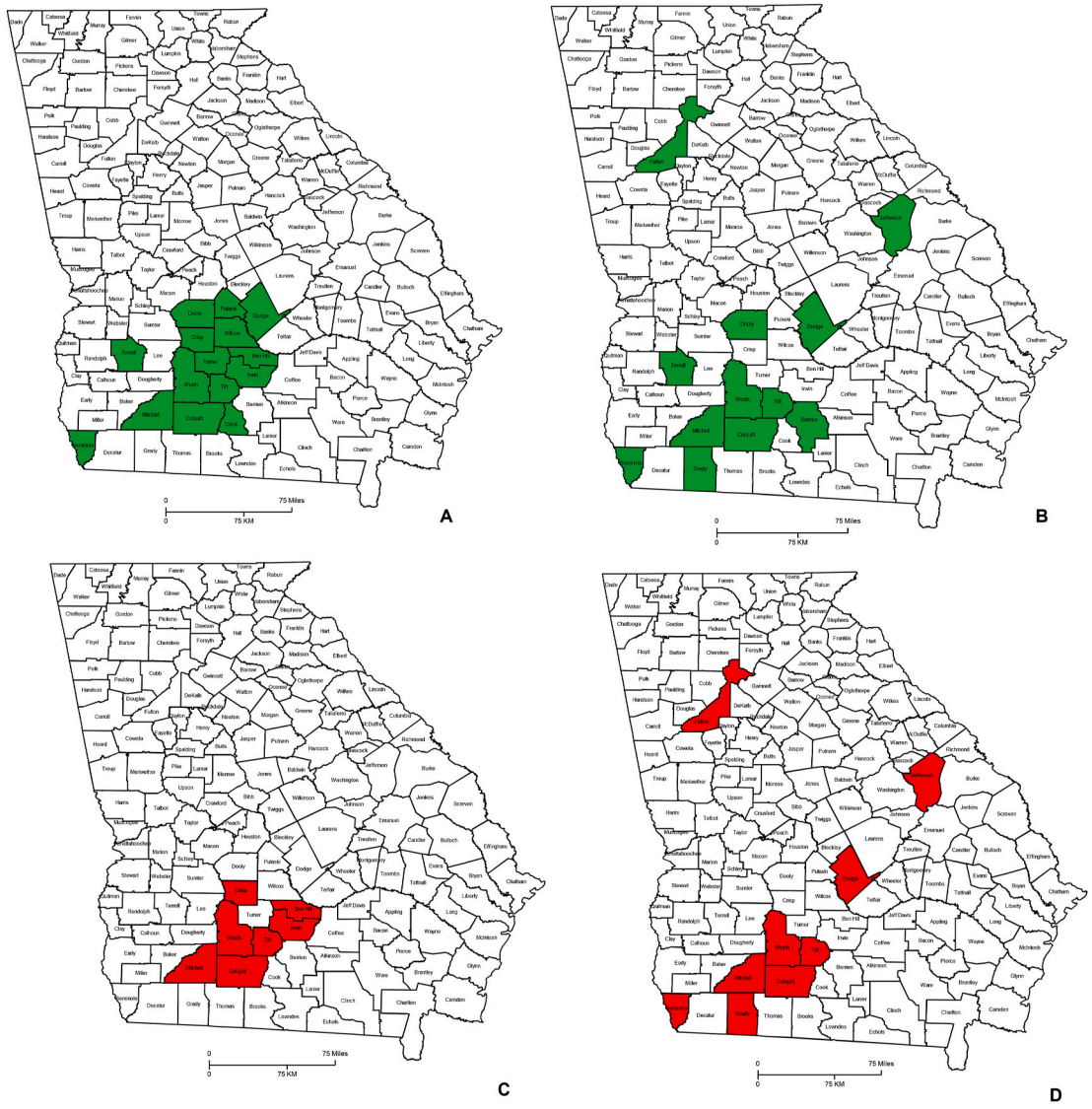

Fig. 1. Counties from where weed (A) and overwintering cotton stalks (B) were surveyed and collected. Counties where the cotton leafroll dwarf virus was detected on weeds (C) and overwintering cotton stalks (D).

2. Materials and methods

2.1. Survey and sample collection of weeds

Common weeds growing in commercial cotton fields of Georgia were collected irrespective of the presence or absence of any visible symptoms to provide an unbiased estimation of the frequency of CLRDV occurrence. In the spring of 2019, weeds were collected from 15 different counties. In the summer and fall of 2019, fields in two counties were surveyed (Fig. 1A). In each county, five fields were surveyed. In each field, five areas of 10 m^2 were arbitrarily selected, and five plants were collected from each weed species growing within that area. Samples were enclosed in plastic bags and stored in an ice cooler to prevent viral RNA degradation while transporting from the field to the laboratory for further analysis. A total of 2,055 weed samples from 57 different species belonging to 24 families were collected. Weeds of the same species from one location, *i. e,* one 10 m^2 sampling unit, were pooled in the laboratory. The weeds collected from the fields were photographed and identified to species level based upon the identification keys (Weakley, 2015). In the Spring of 2021, an individual weed screening was carried out in five ubiquitious weed species (*Cerastium glomeratum, Gamochaeta pensylvanica*, *Lamium amplexicaule*, *Oenothera laciniata*, and *Raphanus raphanistrum*) which had been screened for CLRDV earlier in 2019. The screening was done in a minimum of 15 samples/weed. Samples were collected from 10 different fields in Colquitt county and tested individually.

2.2. Overwintered cotton stalks and regrowth sample collection

Overwintered cotton stalk samples were collected from 12 counties during the spring and early summer of 2019 and 2020. Bark tissues from overwintered cotton stalks (n = 114) submitted by county advisors were tested as a pool consisting of 2–8 stalks per pool. Fifty-two overwintering cotton stalks were also tested on an individual basis. During April–June 2020, new regrowth (foliage that grew from overwintered cotton stalks) leaf tissues (100 mg) from each overwintering cotton stalks (n = 150) were collected from a commercial cotton fields in Ben Hill county and UGA research farms in Tift county. The leaf tissues were tested individually for the presence of CLRDV.

2.3. RNA isolation from weeds and overwintered cotton stalks

Leaves and petioles of weed samples and bark tissues of overwintering cotton stalks were sterilized with 1% bleach and cleaned by washing thrice with distilled water before processing to remove any contaminants. Approximately 100 mg of the leaf or bark tissue in liquid nitrogen was ground to a fine powder in an autoclaved mortar and pestle. Samples were processed with 2% CTAB buffer (2% CTAB, 4M NaCl, 0.5 M EDTA, 1M Tris HCl, 2% PVP-40) containing 0.2% β-mercaptoethanol for total RNA extraction (Murray and Thompson, 1980). The final pellet was washed in 70% ethanol and centrifuged at 13,000 rpm for 5 min, air-dried, and suspended in 150 μL of nuclease-free ultra-pure water (Invitrogen, Carlsbad, CA USA). Total RNA from asymptomatic/healthy cotton grown in an insect-free controlled growth chamber and symptomatic CLRDV-infected cotton plant from the 2018 cropping season was included as a negative and positive control respectively. The quality and quantity of RNA were checked using NanoDrop One (Thermo Scientific, Waltham, MA USA) before complementary DNA (cDNA) synthesis.

2.4. CLRDV detection by RT-PCR

To confirm the presence of CLRDV, OFR0 (RNA silencing suppressor), ORF3 (coat protein-CP), ORF4 (movement protein-MP), and partial ORF5 (aphid transmission protein) were targeted by reverse transcription PCR (RT-PCR) using gene specific primers (Table 1). Since ORF 4 is embedded within ORF 3, a single set of primers was used to amplify both ORFs. cDNA was synthesized using 2.5 μg of the total RNA, gene-specific reverse primer targeting complete ORF0, ORF3, or partial ORF 5 (1 μL of 10 mM), dNTP mix (1 μL of 10 mM), 5X First-Strand buffer (4 μL), DTT (1 μL of 0.1 M), RNaseOut (40 u) and Reverse Transcriptase (200u SuperScript III, Invitrogen, USA) following manufacturer's recommendation. The temperature cycling parameters for the cDNA synthesis consisted of 5 min at 25 °C, 60 min at 55 °C, followed by 15 min of enzyme deactivation at 70 °C. The PCR assays for CLRDV detection were performed with cDNA (1 μL), Platinum Taq DNA Polymerase high fidelity (0.1 μL of 5u/μL), gene-specific forward and reverse primers (0.5 μL of each 10 mM stock), 10x high fidelity PCR buffer (2.5 μL of 10X), dNTP mix (0.5 μL of 10 mM), $MgSO_4$ (1 μL of 50 mM) and DNAse and RNAse free ultra-pure water for making final reaction volume of 25 μL per reaction. PCR reaction was carried out with initial denaturation at 94 °C for 3 min followed by 35 cycles of 30 s at 94 °C, 45 s at a temperature of annealing (T_m) for primers as indicated in Table 1 and 2 min at 72 °C, and a final extension at 72 °C for 10 min. PCR products were analyzed in 0.8% agarose gel by electrophoresis, and the amplicons were visualized in the UVsolo touch gel documentation system (Analytik Jena, Upland, CA USA).

2.5. Cloning, sequencing, and analysis of the amplified fragments

Amplicons of complete ORF0 encoding putative silencing suppressor was purified with PCR purification kit (Qiagen, Valencia, CA USA) whereas ORF3 encoding CP genes were gel purified using the QIAquick Gel extraction kit (Qiagen, USA) and cloned into pCR 2.1-TOPO vector (Invitrogen, USA). Recombinant clones were screened by colony PCR and restriction digestion using *Eco*RI. Three recombinant plasmids from independent colonies were sequenced (GeneScript, Piscataway, NJ USA) to confirm the presence of virus genes. The gene sequence were annotated and analyzed using NCBI- BLAST (Altschul et al., 1990), and MEGA X with inbuilt MUSCLE alignment tool (Edgar, 2004). Phylogenetic distance trees were constructed using MEGA X software (Kumar et al., 2018) using the maximum-likelihood method and sequence demarcation tool (SDT) version 1.2 was used to create the sequence identity matrix.

Table 1
Primer details used for detection of cotton leafroll dwarf virus.

S. No	Primers	Sequences (5′-3′)	Position on CLRDV genome (bp)	Target	Expected amplicon size (bp)	Annealing temperature (Tm °C)	References
1	Primer 17	GCTGCACGCGCAGTGGAAGTG	4729–4749	ORF 5 (Partial)	1066	68	Distéfano et al. (2010)
	Primer 18	TGCCTATCCTTTCGGAGTCGTTCC	5794–5771				
2	SB11F	AGGTTTTCTGGTAGCAGTACCAATATCAACGTTA	3544–3569	ORF 3 and ORF 4	803	60	Tabassum et al. (2019)
	SB11R	TATCTTGCATTGTGGATTTCCCTCATAA	4346–4319				
3	SB28F	CACTTGAGACATAACTCGCTT	29–49	ORF 0	964	59	This study
	SB28R	GCGGTGAGGAGACCATACTCA	972–992				

Table 2
Weed species on which cotton leafroll dwarf virus was detected in different cotton growing regions of Georgia in 2019. Weeds (n = 5) of the same species from one location, *i.e* one 10 m^2 sampling unit, were pooled and analyzed using RT-PCR.

S. N	Family	Scientific name	Common name	Location (County)	Number of pooled samples tested positive/total number of tests				CLRDV ORFs identified (NCBI Accession numbers)	
					Spring (Jan–May)	Summer (Jun–Jul)	Fall (Aug–Dec)	Total	ORF 3	ORF 4
	Annuals									
1	Amaranthaceae	*Amaranthus palmeri*	Pigweed	Colquitt	0/0	0/0	1/7	1/7	MT750288[a]	MT750288[a]
2	Asteraceae	*Gamochaeta pensylvanica*	Cudweed	Irwin	1/30	0/0	0/1	1/31	MT750286[a]	MT750286[a]
3	Asteraceae	*Hypochaeris radicata*	Flatweed	Crisp	1/7	0/0	0/0	1/7	MT559373	MT559392
4	Asteraceae	*Erigeron annuus*	Daisy fleabane	Colquitt	0/0	1/1	1/1	1/1	MT559371	MT559390
5	Brassicaceae	*Lepidium coronopus*	Swinecress	Colquitt	0/0	1/1	0/0	1/1	MT559376	MT559395
6	Brassicaceae	*Lepidium virginicum*	Virginia pepperweed	Colquitt	0/0	1/1	0/1	1/2	NA	NA
7	Brassicaceae	*Raphanus raphanistrum*	Wild radish	Colquitt	0/16	2/2	0/0	2/18	MT559383	MT559402
8	Campanulaceae	*Wahlenbergia marginata*	Southern rock bell	Colquitt	0/0	1/1	0/0	1/1	MT559388	MT559407
9	Caryophyllaceae	*Cerastium glomeratum*	Mouse-ear chickweed	Colquitt	1/8	0/0	0/0	1/8	MT559370	MT559389
10	Convolvulaceae	*Jacquemonita tamnifolia*	SF morning glory	Colquitt	0/0	3/5	0/5	3/10	MT559374	MT559393
11	Fabaceae	*Arachis glabrata*	Perennial peanut	Tift	1/1	0/0	0/0	1/1	MK656891	MK656891
12	Fabaceae	*Trifolium campestre*	Low hop clover	Crisp	1/4	0/0	0/0	1/4	MT559386	MT559405
13	Fabaceae	*Medicago polymorpha*	Burr clover	Colquitt	0/0	1/1	0/0	1/1	MT559377	MT559396
14	Geraniaceae	*Geranium carolinianum*	Geranium	Mitchell	2/4	0/0	0/0	2/34	MT559372	MT559391
15	Lamiaceae	*Lamium amplexicaule*	Henbit deadnettle	Ben Hill, Irwin, Tift	3/57	1/1	0/0	4/58	MT559375	MT559394
16	Molluginaceae	*Mollugo verticillata*	Green carpet weed	Colquitt	0/0	6/7	2/8	8/15	MT559378, MT559379	MT559397, MT559398
17	Portulacaceae	*Portulaca pilosa*	Pink purslane	Colquitt	0/0	7/10	0/8	7/18	MT559382	MT559401
18	Verbenaceae	*Glandularia pulchella*	Mock vervain	Colquitt	0/8	1/4	0/2	1/14	MT559387	MT559406
	Biennials									
19	Onagraceae	*Oenothera laciniata*	Cut-leaf	Colquitt, Mitchell, Worth	3/28	4/8	0/1	7/37	MT559380, MT559381	MT559399, MT559400
20	Rubiaceae	*Richardia scabra*	Florida parsley	Colquitt	0/0	3/5	1/5	4/10	MT559384	MT559403
	Perennials									
21	Asteraceae	*Solidago altissima*	Goldenrods	Colquitt	0/0	1/1	0/2	1/3	MT559385	MT559404
22	Malvaceae	*Sida rhombifolia*	Arrow leaf sida	Colquitt	0/0	1/1	0/0	1/1	NA	NA
23	Solanaceae	*Physalis minima*	Ground cherry	Colquitt	0/0	1/1	0/1	1/2	MT750287[a]	MT750287[a]

NA Target gene was amplified but the PCR product was not cloned and sequenced.
[a] Indicates amplification of partial ORF3 and ORF4.

3. Results

3.1. Detection of CLRDV on weeds

In 2019, an extensive survey of weed species was conducted in 15 different cotton-growing counties in Georgia to identify potential weed hosts for the virus. Pooled samples from 57 different species belonging to 24 families were tested in this study. Total RNA from samples were analyzed by RT-PCR directed at multiple targets on the viral genome for detection. A sample was considered positive when fragments of 964 bp, 803 bp or 1066 bp were amplified using primers for ORF0, ORF3, ORF4 and partial ORF 5, respectively.

CLRDV was detected from 18 annuals, two biennials, and three perennial weeds (Table 2). The number of weed species harboring CLRDV was higher in summer than in fall and spring. In the spring of 2019, CLRDV was detected in eight different weed species of 20 tested: *Arachis glabrata, Cerastium glomeratum, Geranium carolinianum, Gamochaeta pensylvanica*, *Hypochaeris radicata*, *Lamium amplexicaule*, *Oenothera laciniata,* and *Trifolium campestre.* In the summer, CLRDV was detected from 15 of 35 weed species tested including *Erigeron annuus, Glandularia pulchella, Jacquemontina tamnifolia, Lamium amplexicaule, Lepidium coronopus, Lepidium virginicum, Mollugo verticillata, Oenothera laciniata, Physalis minima, Portulaca pilosa, Raphanus raphanistrum, Richardia scabra, Sida rhombifolia, Solidago altissima,* and *Wahlenbergia marginata*. In the fall, CLRDV was detected from only three weed species of 16 tested, *Amaranthus palmeri, Mollugo verticillata,* and *Richardia scabra*. In this study, 57 weed species were tested, and CLRDV was detected in 23 species belonging to 16 families (Table 2; Fig. 2). We were able to amplify and sequence complete ORF3, ORF4, and partial ORF5 from representative samples of all these 23 species suggesting the presence of the virus. Similarly, complete ORF0 was sequenced from the eight prevalent weed species, including *Amaranthus palmeri*, *Arachis*

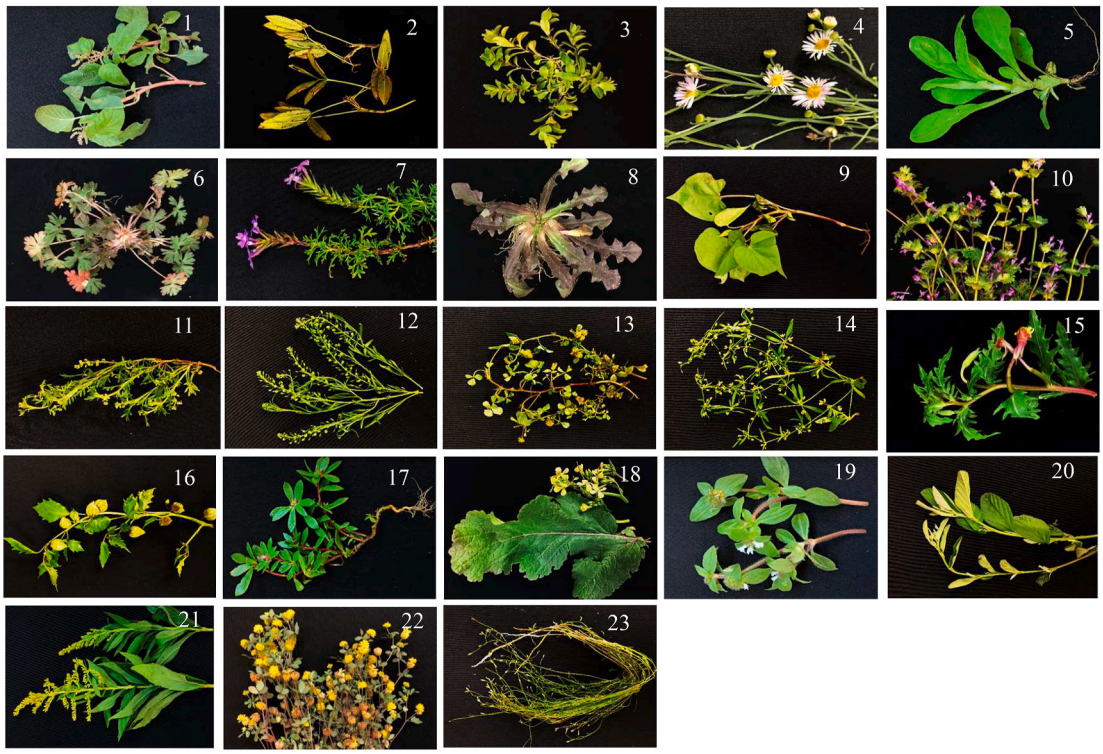

Fig. 2. Weeds on which the cotton leafroll dwarf virus was detected in Georgia. (1) *Amaranthus palmeri* (2) *Arachis glabrata*, (3) *Cerastium glomeratum*, (4) *Erigeron annuus*, (5) *Gamochaeta pensylvanica,* (6) *Geranium carolinianum*, (7) *Glandularia pulchella,* (8) *Hypochaeris radicata*, (9) *Jacquemonita tamnifolia* (10) *Lamium amplexicaule* (11) *Lepidium coronopus*, (12) *Lepidium virginicum*, (13) *Medicago polymorpha,* (14) *Mollugo verticillata*, (15) *Oenothera laciniata*, (16) *Physalis minima,* (17) *Portulaca Pilosa*, (18) *Raphanus raphanistrum*, (19) *Richardia scabra*, (20) *Sida rhombifolia,* (21) *Solidago altissima*, (22) *Trifolium campestre*, (23) *Wahlenbergia marginata.*

Fig. 3. Overwintering cotton stalks (A) Tift County (Jun 01, 2020), (B) Colquitt County (Feb 22, 2019), and cotton regrowth (C) Ben Hill County (May 23, 2020) and (D) Tift County (Jun 17, 2020) which survived the winter were collected in spring. Volunteer cotton regrowth was collected after the emergence of the new crops and tested for the presence of the cotton leafroll dwarf virus.

glabrata, *Lamium amplexicaule*, *Mollugo verticillata*, *Oenothera laciniata, Portulaca Pilosa, Physalis minima*, and *Richardia scabra*. Thirty-four other weed species belonging to 16 different families have also been screened, but the presence of virus has not been detected (Supplementary Table 1; Supplementry Fig. 1). In spring 2021, CLRDV detection was higher in *Lamium amplexicaule* (45%), *Oenothera laciniata* (40%), and *Raphanus raphanistrum* (30%) and comparatively low in *Gamochaeta pensylvanica* (13.3%) and *Geranium carolinianum* (6.67%).

3.2. Seasonal overlap of weeds found to harbor CLRDV

Most of the weeds that were tested in the survey were annuals, although some were biennials or perennials (Table 2). *Glandularia pulchella, Oenothera laciniate,* and *Solidago altissima* were found throughout most of the year in South Georgia in and around commercial cotton and vegetable fields. Spring weeds such as *Cerastium glomeratum, Gamochaeta pensylvanica, Geranium carolinianum, Hypochaeris radicata, Lamium amplexicaule, Trifolium campestre, Verbena hastata* were present in the field starting from January until June. Summer annuals such as *Amaranthus palmeri, Jacquemonita tamnifolia, Lepidium virginicum,*

Mollugo verticillata, *Physalis minima*, *Portulaca pilosa*, and *Richardia scabra* were present in fields throughout the summer and fall (June - November).

3.3. Detection of CLRDV on overwintered cotton stalks and regrowth leaves

CLRDV was detected on overwintered cotton stalks from 9 out of 12 counties tested (Fig. 1C and D) during February–April before the sowing of the next crop (Table 3). Bark tissues of 114 overwinter cotton stalks were tested as 22 pooled samples, and CLRDV was detected from six pooled samples (Table 3). Overwintered cotton stalks (n = 52) (Fig. 3A and B) were tested individually, and CLRDV was detected in 25 stalks (48%) samples in 2019–2020.

CLRDV was also detected from cotton regrowth leaves collected during April–May 2020 in Tift county before sowing and in May–June 2020, immediately after the emergence of the new cotton seedlings. Cotton regrowth leaves (n = 150) from the overwintering cotton were tested, and CLRDV was detected from 113 (75%) samples (Table 3, Fig. 3C and D). These results confirm the presence of CLRDV in overwintered cotton stalks and regrowth that survive the winter and into the next crop, potentially act as the primary inoculum source.

3.4. Sequence characteristics of CLRDV isolated from weeds and overwintered cotton stalks in Georgia

To confirm the presence of CLRDV, PCR amplicon of CP, MP and silencing suppressor gene were sequenced and analyzed. The silencing suppressor gene amplicon was 786 nt long encoding 261 amino acids of approximately 29.7 kDa protein. The CP gene was 606 nt long and coded for 201 amino acids (~22.43 kDa) and the MP was 525 nt long and coded for 174 amino acids (~19.88 kDa). The nucleotide sequences of ORF0 (91.6–93%), ORF3 (91–100%), and the ORF 4 were 92–100% identical with the other CLRDV isolates reported from the US and other cotton-growing regions of the world. In the phylogenetic analysis, all CLRDV isolates from Georgia, including those from weed and cotton, were clustered together and formed a clade separated from South American and Asian isolates (Fig. 4A and B). This indicates that the CLRDV population from Georgia is a single strain that has spread across the state. The phylogenetic trees based on nucleotide and amino acid identities were also congruent. The sequences generated from this study were submitted to NCBI GenBank with accession numbers ORF 3 and ORF 4 (MT559370-MT559407) and ORF 0 (MW629382-MW629391).

Table 3
Overwintered cotton stalks and cotton regrowth screened for the presence of cotton leafroll dwarf virus in Georgia collected during 2019–2020.

S. N	Collection Time	Location (County)	Total number of samples	Number of stalks per pool	Number of positive/ Number of test
	Overwintered cotton stalks (Pooled)				
1	March 2019	Berrien	16	8	0/2
2	March 2019	Seminole	63	7	3/9
3	March 2019	Tift	4	2	1/2
4	March 2019	Worth	15	5	1/3
5	April 2019	Dodge	8	4	1/2
6	April 2019	Terrell	8	4	0/2
	TOTAL		**114**		**6/22**
	Overwintered cotton stalks (Individual)				
7	February 2019	Colquitt	10	_	5/10
8	March 2019	Dooley	1	_	0/1
9	March 2019	Mitchell	2	_	0/2
10	June 2019	Tift	30	_	13/30
11	July 2019	Grady	1	_	1/1
12	March 2020	Jefferson	3	_	2/3
13	March 2020	Wilcox	5	_	4/5
	TOTAL		**52**		**25/52**
	Regrowth leaves (Individual)				
14	April 2020	Tift	40	_	31/40
15	May 2020	Ben Hill	39	_	39/39
16	May 2020	Tift	51	_	27/51
17	June 2020	Tift	20	_	16/20
	TOTAL		**175**		**113/150**

3.5. Population structure and sequence characteristics of CLRDV isolates based on the complete ORF0, ORF3, and ORF4 sequences

The phylogenetic relationships among the CLRDV isolates from different weed species were studied on the basis of ORF0, ORF3, and ORF4 that encode the RNA silencing suppressor, structural (CP), and non-structural (MP) proteins, respectively. In the analysis, the complete ORF3 and ORF4 sequences of CLRDV isolates were resolved into three major clades consisting of North American, South American, and Asian isolates. Phylogenetic analysis of ORF3 and ORF4 sequences of the Alabama (MN071395), Georgia (MK290759, MK290760), and Texas (MN872302) isolates (from cotton) clustered together with Georgia CLRDV isolates (from weeds) (MT559370-MT559407) and was separated from the South American (KF359947, KF359964, NC014545, GU167940, KF906260, GQ379224, HQ827780, GQ401151, EU871551, EU871550) and Asian viruses (KX588248, KP176644, KP176643). Among the US isolates, the ORF3 and ORF4 *nt* sequences were resolved further into three major clades. Sequences from cotton isolate from Alabama (MN071395), Early county-Georgia (MK290760), Tift county-Georgia (MK290759), Texas (MN872302), and weed sequences from twelve different species, namely *Cerastium glomeratum* (ORF3-MT559370, ORF4-MT559389), *Erigeron annuus* (ORF3-MT559371, ORF4-MT559390), *Glandularia pulchella* (ORF3-MT559387, ORF4-MT559406), *Jacquemonita tamnifolia* (ORF3-MT559374, ORF4-MT559393), *Lamium amplexicaule* (ORF3-MT559375, ORF4-MT559394), *Lepidium coronopus* (ORF3-MT559376, ORF4-MT559395), *Medicago polymorpha* (ORF3-MT559377, ORF4-MT559396), *Mollugo verticillata* (ORF3-MT559378, ORF4-MT559397), *Portulaca pilosa* (ORF3-MT559382, ORF-4MT559401), *Raphanus raphanistrum* (ORF3-MT559383, ORF4-MT559402), *Solidago altissima* (ORF3-MT559385, ORF4-MT559404), and *Wahlenbergia marginata* (ORF3-MT559388, ORF4-MT559407) belonging to Colquitt, Ben Hill, and Tift counties of GA were grouped in one clade. However, sequences from *Oenothera laciniata* (ORF3-MT559381, ORF4-MT559400), *Mollugo verticillata* (ORF3-MT559379, ORF4-MT559398), and *Richardia scabra* (ORF3-MT559384, ORF4-MT559403) Colquitt County, GA grouped into a separate clade. Similarly, sequences from *Arachis glabrata* (MK656891, Tift County), *Geranium carolinianum* (ORF3-MT559372, ORF4-MT559391 Mitchell County), *Hypochaeris radicata* (ORF3-MT559373, ORF4-MT559392 Crisp County), *Oenothera laciniata* (ORF3-MT559380, ORF4-MT559399 Mitchell County), and *Trifolium campestre* (ORF3-MT559386, ORF4-MT559405) were separated into third separate clade. However, bootstrap values for the major clades within the phylogeny were relatively low (<60%), and the short branches within the phylogenetic tree indicate the close genetic relationship between the isolates.

Similarly, the phylogenetic analysis of the ORF0 sequences were resolved into four major clades. ORF0 sequences from *Amaranthus palmeri*, *Arachis glabrata*, *Lamium amplexicaule*, *Mollugo verticillata*, *Oenothera laciniata*, *Physalis minima*, *Portulaca pilosa*, and *Richardia scabra* were sequenced and submitted to GenBank (MW629382-MW629391). The uppermost clade consisted of the CLRDV isolates reported from the US (North American isolates) and separated out of the South American cluster (Brazil and Argentina) and one Asian (KX588248) isolate. Phylogenetic analysis of ORF0 sequences reported from the Texas

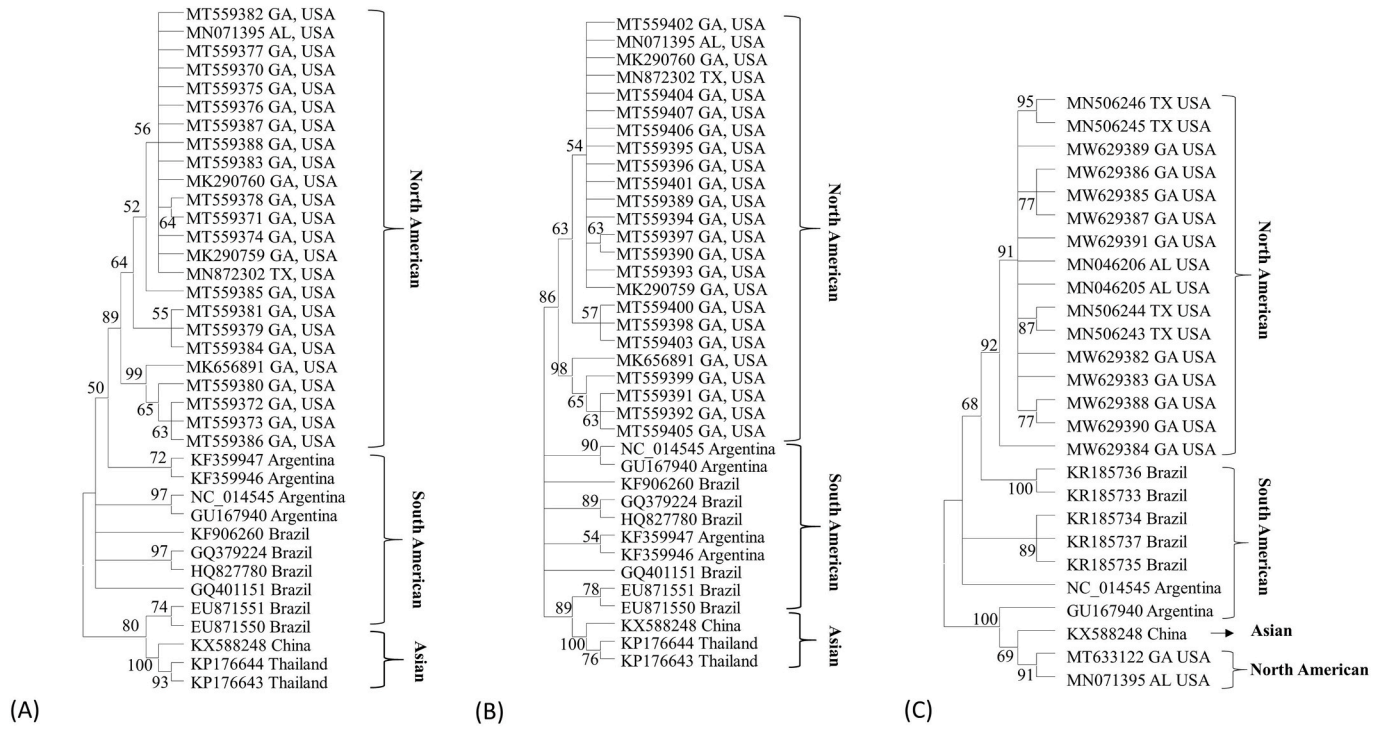

Fig. 4. Phylogenetic relations and sequence identities of cotton leafroll dwarf virus (CLRDV) isolated from weeds and cotton collected in Georgia with other CLRDV isolates from the US and the rest of the world. Phylogenetic tree based on the alignment nucleotide sequences of (A) ORF 3 encoding P3 (Coat protein), (B) ORF 4 encoding P4 (movement protein) and (C) ORF 0 encoding P0 (RNA silencing supressor). The phylogenetic tree was inferred using the Maximum-likelihood method. Bootstrap values (1000 iterations) are shown at the nodes.

(MN506243-MN506246) and Alabama (MN046205, MN046206) isolated from cotton clustered together with Georgia (MW629382-MW629391) CLRDV isolates (from weeds) which indicates the possibility of sharing the common ancestry among the viruses. Similarly, Brazilian isolates (KR185733-KR185737) clustered with Argentinian (GU167940, NC_014545) isolates and clearly separated out of the North American cluster. However, two US isolates, Alabama (MN071395) and another from Georgia (MT633122), were genetically close to the Argentinian (GU167949, NC_014545) and Chinese (KX588248) isolate (Fig. 4C). In contrast, two Brazilian isolates (KR185733 and KR185737) clearly separated out of the other Brazilian viruses (KR185734, KR185737) because these divergent virus isolates were reported to be obtained from the formerly resistant cotton varieties in Brazil and proposed as the atypical strain of the cotton leafroll dwarf virus (Cascardo et al., 2015). CLRDV isolates from weeds shared 97.7–100% identity for ORF0 both at nucleotide and amino acid levels with cotton CLRDV isolates reported from other US states (AL, and TX), while 91.6–93% nucleotide and 88–90.8% amino acid identity compared to rest of CLRDV isolates from North America and South America except two Brazilian atypical strain of CLRDV (84.2–85.8%). CLRDV isolates from weeds revealed a lower pairwise identity for ORF0 amino acids with three CLRDV isolates; MN071395 AL (68–69%), MT633122 GA (70.2–70.5%) and KX588248 (70.7–71%).

4. Discussion

There are several viral diseases reported on cotton worldwide, that could pose a threat to the sustainability of the cotton industry (Nelson, 1998). Among them, CLRDV is likely to cause serious economic damage to cotton in South America and parts of Asia (Agrofoglio et al., 2016; Avelar et al., 2019; Costa and Carvalho, 1962; Costa and Forster, 1938; Corrêa et al., 2005; da Silva et al., 2015; Nelson, 1998). This virus has recently been identified in most cotton-growing regions of the United States (Aboughanem-Sabanadzovic et al., 2019; Alabi et al., 2020; Ali and Mokhtari, 2020; Avelar et al., 2019; Faske et al., 2020; Huseth et al., 2019; Iriarte et al., 2020; Price et al., 2020; Tabassum et al., 2019; Thiessen et al., 2020; Wang et al., 2020) and poses an imminent threat to cotton production there.

CLRDV is a single-stranded RNA virus that belongs to the genus *Polerovirus* and is transmitted by cotton aphids. Insecticide applications for control of aphids are not generally made in Georgia since a fungus, *Neozygites fresenii*, kills the majority of cotton aphids. This fungi reduce the aphid population below the levels where they can cause economic damage due to feeding. However, in early summer, aphid populations tend to build rapidly within a two weeks period before the fungal attack (Abney et al., 2000), giving them enough time to infect cotton. Insecticide programs directed to control the aphids are reportedly not helpful to reduce the spread of the virus (Unpublished: Personal Communication Phillip Roberts). In the absence of an insecticide program to control the spread of CLRDV and no known CLRDV resistant varieties suitable for the region, greater emphasis needs to be placed on minimizing the initial inoculum sources in and around cotton fields for disease management. Viruses depend on alternative hosts to ensure their survival, as they are obligate intracellular parasites. However, limited information is available on the non-crop host of CLRDV. Weeds (Duffus et al., 1971) and volunteer crops (Hsu et al., 2011; Hull, 2014) are known to play an important role in the epidemiology of plant viruses. In an attempt to identify possible green bridges for the virus, weeds and overwintered cotton stalks and regrowth were tested for the presence of CLRDV and found to contribute as a potential primary inoculum source for the next season's crop.

Fifty-seven different weed species were tested and CLRDV was detected from 23 species belonging to 16 families. These includes *Lamium amplexicaule*, *Cicer arietinum, Sida acuta,* and *Hibiscus sabdariffa* reported earlier from Alabama (Hagan et al., 2019). CLRDV was detected on weeds throughout the year in Georgia. In addition to the detection of the virus in weeds during the cotton growing season, we also detected the virus during non-cropping seasons. Virus was detected

in the weeds primarily in the summer (15 of 35 tested) compared to spring (8 of 20 tested) or fall (3 of 16 tested). CLRDV was also detected from summer and winter annuals that have overlapping growth periods. Detection of CLRDV from common weeds, such as cut-leaf evening primrose, mock vervain, and goldenrod present over multiple seasons throughout the year, could potentially act as a green-bridge allowing the virus to persist in the field and native flora between two consecutive cropping seasons for disease transmission.

Weeds can influence the spread of viruses by acting as propagative hosts for aphids and allowing the vector to acquire the virus from infected plants. CLRDV was detected on spring weeds such as henbit deadnettle, geranium, and annual as cut-leaf evening primrose which are also reported to be a host for the vector *A. gossypii* (Young and Garrison, 1949).

In this research, several weed species have been identified as potential green-bridges for CLRDV. Adaptation of field sanitation is a primary preventive technique that could help reduce the primary virus inoculum in the field. Modern herbicides are often effective, in controlling weed populations. However, large scale use of Roundup Ready crop (ex. corn, cotton, and soybean) and glyphosate application as a principal herbicide resulted in the emergence of glyphosate-resistant weeds (Culpepper et al., 2006; Gilbert, 2013; Koetz and Asaduzzaman, 2020; Koger et al., 2004; Sosnoskie et al., 2011; Spaunhorst et al., 2019; VanGessel, 2001; Ward et al., 2013). Some of the weeds from which CLRDV was detected as "*Amaranthus palmeri*" and "*Conyza canadensis*" have developed resistance against commonly used herbicides (Culpepper et al., 2006; Gilbert, 2013; Koger et al., 2004; Sosnoskie et al., 2011; VanGessel, 2001) may need to be managed with alternative strategies. Also, the presence of CLRDV on overwintered cotton and annual and biennial weeds favor the persistence of the virus and the vector.

The role of volunteer plants in virus disease epidemiology has been well studied in several crops, including potato (Thomas, 2002), onion (Gent et al., 2004; Hsu et al., 2011), sugarbeet, groundnut (Hull, 2014), and wheat (Coutts et al., 2008). In this study, we found that CLRDV survives in overwintered cotton stalks in Georgia and this helps to preserve the virus inoculum into subsequent cropping season. The virus was detected in 48% of individual overwintered cotton stalks and six out of 20 pooled samples tested. It was also detected in 75% of cotton regrowths from overwintered cotton stalks in spring, 2020 before planting and the cotton emergence. The presence of CLRDV in a high percentage of overwintered cotton stalks and regrowth suggests that these could be a potential primary source of inoculum for the next season's crop in Georgia, where farmers typically do not destroy them after harvest.

Sequence characteristics of CLRDV isolates from weeds in Georgia displayed the clustering of the nucleotide (*nt*) and amino acid (*aa*) sequences reported from the United States in the same clade with Georgia cotton isolates and separated out of the South American and Asian cluster. Pairwise *nt* and *aa* identity showed 91–100% sequence similarity for complete ORF3. For ORF4, pairwise identity among the *nt* ranged from 92 to 100%, whereas *aa* ranged from 90 to 100% for all isolates except for the Asian isolates (KX588248, KP176644, KP176643) and two weed isolates (*Gamochaeta pensylvanica* and *Jacquemonita tamnifolia*). This result illustrates that *nt* and *aa* sequences for the CLRDV isolates were similar to each other irrespective of the natural host and its geographic origin but there could be exceptions that need to be investigated. Weed isolates shared 97.7–100% identity at nucleotide and amino acid levels for ORF0 with cotton CLRDV isolates reported from the other US states justifies a close genetic relationship among the CLRDV isolates within the United States. In contrast, CLRDV isolates from weeds shared 91.6–93% nucleotide and 85.4–90.8% amino acid identity compared to the rest of the CLRDV isolates from North America and South America. Further efforts are needed to understand the role of weeds and overwintered cotton stalks and regrowth in the epidemiology of cotton leafroll dwarf disease. However, the findings of this study will help to formulate initial management practices to minimize losses from this recently identified threat to the sustainable cotton production in Georgia and other cotton-growing regions.

5. Conclusion

The recent identification of CLRDV from major cotton-growing regions of the USA is a reason for concern since the virus has the potential to cause significant losses. There is no insecticide program or resistant variety identified to manage the disease in the USA. Today, the adoption of field sanitation measures, to include weed roguing, crop rotation, and destruction of cotton stalks could be helpful to reduce the virus inoculum and manage the disease. We identified the association of CLRDV with 23 different weed species belonging to 16 families. These weeds have the potential to complicate the viral pathosystem by acting as a reservoir of either vectors or viruses or both. CLRDV isolates from weeds in Georgia clustered together with isolates from different US states namely; Alabama, Georgia, and Texas and were separated from the South American and Asian cluster in phylogenetic analysis. To our knowledge this is the first report of the natural infection of CLRDV on *Amaranthus palmeri*, *Arachis glabrata*, *Cerastium glomeratum*, *Erigeron annuus*, *Gamochaeta pensylvanica*, *Geranium carolinianum*, *Glandularia pulchella*, *Hypochaeris radicata*, *Jacquemonita tamnifolia*, *Lamium amplexicaule*, *Lepidium coronopus*, *Lepidium virginicum*, *Medicago polymorpha*, *Mollugo verticillata*, *Oenothera laciniata*, *Physalis minima*, *Portulaca Pilosa*, *Raphanus raphanistrum*, *Richardia scabra*, *Sida rhombifolia*, *Solidago altissima*, *Trifolium campestre*, *Wahlenbergia marginata* in the United States.

The presence of CLRDV-positive overwintered cotton stalks in the field after the cropping season shows the residual cotton stand to be a suitable reservoir and overwintering host for the virus. Adoption of proper chemical rotation and integration of the cultural practices such as the use of cover crops, trap crops, crop rotation, and elimination of weed escapes could help manage weeds as well as reduce the reservoirs of the virus.

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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

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