

## Recovery Plan

**Cotton leaf curl disease caused by a leaf curl virus complex (*Begomovirus*, *Geminiviridae*):  
A group of whitefly- transmitted ssDNA viruses with ssDNA satellites, causing diseases of  
cotton, vegetables, and ornamentals**

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This recovery plan is one of several disease-specific documents produced as part of the National Plant Disease Recovery System (NPDRS) called for in Homeland Security Presidential Directive Number 9 (HSPD-9). The purpose of the NPDRS is to ensure that the tools, infrastructure, communication networks, and capacity required to mitigate the impact of high consequence plant disease outbreaks are such that a reasonable level of crop production is maintained.

Each disease-specific plan is intended to provide a brief primer on the disease, assess the status of critical recovery components, and identify disease management research, extension and education needs. This document is not intended to be stand-alone documents that address all possible diverse and inter-related aspects of a plant disease outbreak, or the subsequent actions that may be required to initiate recovery from an introduction or establishment. It is, however, a document that will help USDA guide further efforts directed toward understanding the pathogen-host-vector components necessary to inform plant disease response and recovery action plans.

### **Executive Summary**

Cultivated cotton, *Gossypium* species (L.), has been a major source of food, feed, and fiber, worldwide, for at least 7,000 years. Globally, about 32.6 million hectares are devoted to cotton cultivation, with production estimated at ~27 million tons (<http://www.ers.usda.gov/topics/crops>). Cotton is the leading cash crop in the United States, with annual business revenue stimulated by cotton exceeding 120 billion dollars to the economy [www.cottoncounts.net](http://www.cottoncounts.net). This accounts for

approximately 35 percent of the total world's fiber used, half of which is exported <http://www.ers.usda.gov/amber-waves/2013-june/crop>. In the U.S. alone the industry generates about 200,000 jobs and accounts for more than 25 billion in products and services in China, India, the United States, and Pakistan accounting for more than 70 percent of global cotton production in 2013-14, while other important cotton producing countries are Australia, the African Franc Zone, Brazil, China, India, and Pakistan, countries lead global cotton mill use and account for a combined 65 percent of world consumption during 2013-14 (Meyer et al., 2013).

Cotton leaf curl disease (CLCuD) is caused by a complex of whitefly-transmitted plant viruses in the genus, *Begomovirus* (family, *Geminiviridae*). Infected cotton and other cultivated host species, such as vegetable and tropical fruit crops, and some ornamentals develop symptoms of leaf curling, and/or leaf-like enations on the underside or surface of leaves, overall stunting, and reduced yield and quality. The cotton leaf curl disease is most damaging when cotton plants become infected during the early growth stages, with losses occurring due to mid- or late- season infection are usually minimal. In all locations where the disease occurs early season infection of cotton has during some years resulted in a total loss of the crop.

In the Eastern Hemisphere, leaf curl disease of cotton occurs in the Sahel region of Africa, and in India and Pakistan where cotton is grown, whereas, in the Americas leaf curl (crumple) disease occurs in cotton-growing regions of the US, Mexico, and Central America. The disease has been managed to some extent in Africa and the Americas by persistent whitefly control and the use of tolerant varieties when available. However, in Asia, during the past four decades, Pakistan and India have experienced two major outbreaks that have led to economically crippling epidemics. The most recent one, began in 2004-present, resulted from the emergence of a resistance-breaking strain of a recombinant virus endemic to the Punjab region of Pakistan that has overcome host-plant resistance in varieties developed to combat the first outbreak caused by the *Cotton leaf curl Multan virus* (CLCuMV) (Gutierrez, 2008; Rahman et al., 2005), which emerged small-scale during the 1960-1980's (Hussain and Ali, 1975) and from 1990-2004, was widespread in cotton, erupting into an epidemic (Zafar and Brown, 2011). There is great concern that CLCuD could spread from its current endemic geographical range in Pakistan and India, to other cotton growing areas of the world where, although the disease is not present, the whitefly vector is prevalent and the environmental conditions are suitable for disease establishment. Recently, this fear has proven well-founded, given that CLCuD has been reported in ornamental species (malvaceous), and in cotton and okra, representing at least three locations in China (Cai et al., 2010; He et al., 2010; Mao et al., 2008; Tang et al., 2014), and soon thereafter, in the Philippines (Dolores et al. 2014), to where it was also probably transported on infected plants and/or by viruliferous whiteflies, albeit, by an unknown route(s).

Currently no resistance to the Asian leaf curl virus complex is available in cotton or other cultivated species that are hosts, including vegetables or ornamentals. Even so, the reliance on genetic resistance for disease management in cotton has been the primary means under consideration for disease management in Pakistan and India. The ability of this highly differentiated virus complex and its satellites to break the resistance within 3-4 years in a recently introduced variety that became widely grown by 2001 (Ahuja et al., 2007; Arshad et al., 2009; Mahmood et al., 2003; Zafar et. al., 2003, 2007) underscores the concern that the virus complex, which has a broad host range, may easily spread to additional currently uninfected areas through the incidental movement of unknowingly, virus-infect ornamentals and/or other plants transported through commercial trade. Efforts are underway in India and Pakistan to identify genes in cotton germplasm sources that could yield viable resistance or at least tolerance to early season infection.

In addition, genetic engineering of cotton plants is being explored as a means of devising effective virus-derived resistance (Wang et al. 2016) or of killing the whitefly vector, using RNA interference (RNAi) (Malik et al., 2016; Vyas et al., 2017).

As is so for other well-studied members of the geminiviruses, the viruses of the CLCuD complex are not seed transmitted. The virus complex is transmitted from plant to plant through by the whitefly *Bemisia tabaci* (Genn.) [Hemiptera: s.o. Homoptera, Aleyrodidae) sibling species group in a circulative and persistent manner, and so once acquired, can be harbored, and therefore, transmitted for the life of the vector. Begomoviruses are not transovarially (passed through the egg) or sexually transmitted, nor are they propagative (replicative) in the vector (albeit, one exception has been reported for *Tomato yellow leaf curl virus*-Israel). Mechanical transmission has not been demonstrated for any members of the CLCuD complex. These viruses can, however, be experimentally transmitted by grafting, and by inoculation of plants with infectious viral clones using biolistic inoculation, particle bombardment, or Agro-inoculation. The tobacco species, *Nicotiana benthamiana* (Domin), is a useful bioassay host when infectious viral clones are available, owing to its general susceptibility to most plant viruses; however, most haplotypes of the whitefly *B. tabaci* vector do not readily feed on this species, and so it is unreliable as a test plant when whitefly-mediated inoculation is desired.

Collectively, the severity of the disease, the ease with which the members of the leaf curl complex are transmitted several predominant biotypes (sibling species) of the whitefly vector, and propensity of begomoviruses to undergo genetic/genomic changes in response to corresponding changes in the host plant genetics (documented, in particular for cotton), together with the broad host range of the viruses are reasons for predicting the likely expansion of the geographic range of members of the CLCuD complex. Such expansions would pose considerable risks to a variety of high-cash value agronomic and horticultural crops outside the locales currently affected by these viruses.

The leaf curl disease of cotton crops is primarily managed by the use of pesticide treatments; often frequent, to kill the whitefly vector to reduce virus transmission by the vector. The lack of alternative control options has led to the profuse, and often overuse, of pesticides to reduce vector populations. This overuse of pesticides leads to the development of insecticide resistance in the whitefly vector, which subsequently undermines abatement of virus transmission to reduce damage and losses.

The current lack of accurate, rapid virus detection tests for individual species and/or groups within the complex underscores the present need to develop molecular and genomic pathology diagnostic methods to facilitate early- detection of existing strains and species, as well as potentially emerging species and strains. The capability for molecular surveillance of the leaf curl complex in plants and the whitefly vector would greatly inform epidemiological studies, and aid in the prevention of further spread locally where the virus occurs, and to international destinations where it has not yet been reported, as well as provide badly needed support for breeding and research programs involved in developing disease resistant germplasm.

Because viruliferous whiteflies can and do infest non-host plants, a non-leaf curl host of the virus can serve as a potentially important vehicle for the accidental transport of these viruses. Such a scenario can be envisioned when viral host and non-host plant species are produced in the same greenhouse facility. In addition, virus-infected, symptomless plants harboring adults of the whitefly vector or the immature instars that develop to adulthood later, likewise can serve as vehicles that

unknowingly introduce virus and its whitefly vector, simultaneously. Therefore, ornamentals or vegetable seedlings transported from high-risk areas provide the most likely mechanisms of unexpected routes of entry for the members of the leaf curl complex.

Finally, international ornamentals industry transports and distributes large numbers of plants to and from a wide range of geographical locales, often spanning continents. Movement of asymptomatic begomovirus-infected plants and/or those infested with viruliferous whiteflies (virus hosts or non-hosts) into temperate or humid-subtropical regions that are not high risk regions for endemics because whiteflies do not naturally over-season there except in greenhouses, such as much of Europe, and therefore pose little perceived threat, are potential pathways for these introductions. Other pathways that could be envisioned are *via* whitefly-infested ornamentals or vegetable seedlings transported from Europe to Canada to the US, or directly from Europe to cotton-growing areas in the US, Central America-Caribbean, and South America. Also, Asian-Pacific routes of human and goods transport continues to represent a direct line for introductions into the Pacific Islands, North America, and the Caribbean region. In Central America and the Caribbean region cuttings or other propagated materials are received and grown to maturity from Africa, Asia, Europe, and the Middle East, and shipped to a variety of end-users in many locations, worldwide. Most recently, plant trade has expanded between Asia and West and East African countries, and from Asia to the Arabian Peninsula/Middle East where cultivated and ornamental plants are now transported, at times with minimal attention to quarantine concerns, and recent reports have already pointed to virus introductions in both directions, involving the leaf curl complex.

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## ***Cotton leaf curl virus-satellite complex (Begomovirus, Geminiviridae): A group of whitefly-transmitted viruses causing leaf curl diseases of cotton, vegetable crops, and ornamentals***

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### **I. Introduction**

Cultivated cotton, *Gossypium* species (L.), and its seed have been a major source of fiber, food (oil), and animal feed for at least 7,000 years. Globally about 32.6 million hectares, on average, are devoted to cotton cultivation, with recent production estimated at 25-27 million tons (Anonymous, 1997; Meyer et al., 2013; Sattar et al., 2013). Cotton is the leading cash crop in the United States, with annual business revenue stimulated by cotton exceeding 120 billion dollars to the U.S. economy alone [www.cottoncounts.net](http://www.cottoncounts.net). The crop accounts for approximately 35 percent of the total world's fiber used, and half of the U.S. crop is exported <http://www.ers.usda.gov/amber-waves/2013-june/crop>. In the U.S. alone the industry generates about 200,000 jobs and accounts for more than 25 billion in products and services (<http://www.ers.usda.gov/topics/crops>). China, India, the United States, and Pakistan accounting for more than 70 percent of global cotton production in 2013-14 (Mayer, et al., 2013), and China, India, and Pakistan are expected to lead global cotton mill use and account for a combined 65 percent of world consumption in 2013-14, while other important cotton producing countries are Australia, Brazil, the Africa Franc Zone, and Central Asia (Mayer, McDonald, and Kiawu, 2013).

Both abiotic (drought, flooding, heat or cold stress) and biotic stresses are responsible for yield losses in cotton production, with insects, plant fungal and viral pathogens, and weeds contributing to reduced production. This report specifically addresses plant viral pathogens, given the rising importance of whitefly-transmitted viruses that threaten the sustainability of cotton production, and evidence of their impending spread from a major center of origin in the Indian Subcontinent, elsewhere where cotton and cotton-vegetable production systems prevail. A number of plant virus-like symptoms associated with decreased yield and quality, even loss of the entire crop has been described in cotton from different parts of the world. Over 20 virus-like diseases of cotton have been described (Brown, 1990, 1992; Kirkpatrick and Rothrock, 2001), but only a few have been confirmed to be of viral etiology, including a luteovirus belonging to the genus, *Polerovirus* (Corrêa et al., 2005; Distéfano et al., 2010; Silva et al., 2008), and the whitefly-transmitted geminiviruses, or begomoviruses. These are represented by a 'core' group of six major species of virus currently recognized as causal agents of leaf curl symptoms. They are the *Cotton leaf crumple virus* (CLCrV) endemic to the Americas-Caribbean region (Brown and Nelson, 1984, 1987; Idris and Brown, 2004), at least four species belonging to the leaf curl complex endemic to India and Pakistan (Briddon et al., 2000; 2001; Mansoor et al., and the *Cotton leaf curl Gezira virus* (CLCuGeV), endemic to the African Sahel region (Idris et al., 2000). Currently, these species are the most economically important cotton-infecting begomoviruses, and for which an etiological agent has been established. All pose a serious threat to cotton production either locally and potentially, globally (Briddon and Markham, 2000; Brown, 1992; 2002; Mansoor et al., 2006, Idris and Brown, 2002; 2004).

Geminiviruses are circular, single-stranded, circular DNA viruses with a small genome that is encapsidated in a twinned-icosahedral or 'geminata' particle. Geminiviruses infect either

monocotyledonous or dicotyledonous plants, and are taxonomically classified into seven genera based on insect vector, genome organization, and viral host range. Taxonomically, begomoviruses are grouped into two main taxonomic clades: those originating either from the Western Hemisphere (WH) or Eastern Hemisphere (EH). The WH begomoviruses have a genome consisting consists of two components of approximately 2,600 nucleotides (nt) in size, referred to as the DNA-A and DNA-B component, respectively. Each component is encapsidated in a separate icosahedral particle such that each virus particle contains either a DNA-A or DNA-B component, and both are required for systemic infection of the plant host. This type of virus occurs in both the hemispheres.

Monopartite begomoviruses have a single genomic component, referred to as DNA-A (<http://link.springer.com/article/10.1007%2Fs00705-015-2398-y>; Brown et al., 2015). Monopartite viruses, thus far are known to occur only in the EH, leading to the hypothesis that ancestral geminiviruses have originated first in the EH (Briddon et al., 2010; Nawaz-ul-Rehman and Fauquet, 2009). Within the *Geminiviridae*, the genus, *Begomovirus* contains the greatest number of taxa, and is economically most important because its members have recently emerged in cultivated crop species as agriculture has expanded over the last two centuries. They occur naturally and are widespread in diverse wild (endemic or introduced) eudicot hosts throughout in tropical, subtropical, and mild climate zones of the world, from where they have been introduced into cultivated plants, in many instances by a phenomenon known as ‘host-shifting’. They are transmitted by the whitefly *Bemisia tabaci* (Gennadius) sibling species group (Aleyrodidae; S.o. Homoptera; O. Hemiptera) (Brown, 2010) in a circulative, persistent manner (Harrison et al., 1997).

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## Satellites associated with 'helper' begomoviruses

Satellites are defined as viruses or nucleic acids (DNA or RNA) that are dependent on a helper virus for replication, while lacking extensive nucleotide sequence homology with the 'helper virus', but are dispensable for helper virus proliferation. Until recently, the majority of plant viral-associated satellites were RNA satellites associated with RNA viral genomes, often contributing discernably to symptom phenotype in the infected plant. Most recently, monopartite begomoviruses have been found associated with one or more small, non-viral (~1350 nt) circular ssDNA molecules, referred to as betasatellites or alphasatellites. Still poorly understood, begomoviral helper viruses vary in the degree to which they require one or more satellites to systemically infect the host and cause wild type disease symptoms.

Betasatellites are approximately half the size of their helper virus genome and have a highly conserved structure, despite their sequences sharing as little as 45% nt sequence identity (Bridson et al., 2003; 2008). They encode a single gene, beta C1 ( $\beta$ C1), in the complimentary sense, are rich in adenine, and contain an 80-100 nt fragment that is highly conserved among all betasatellites. The conserved region is referred to as the satellite conserved region (SCR) (Bridson et al., 2001, 2003, 2008). The role of betasatellites in the infection cycle has been attributed to the product of the single gene they encode,  $\beta$ C1. It has been shown to be a pathogenicity (symptom) determinant and has various effects on symptom development (Iqbal et al., 2012; Paul et al., 2008, 2011; Saeed et al., 2005, 2007, 2010; Saunders et al., 2000, 2004), and functions as a suppressor of post-transcriptional gene silencing that in part facilitates systemic infection of the plant host (Amin et al., 2011; Cui et al., 2005; Idris et al., 2011; Saeed et al., 2007). Betasatellites have been found to readily diversify (evolve), and to be highly promiscuous and therefore dynamic, in that they are capable of interacting with many different begomoviral helper viruses cotton, as well as in other host plant species (Aktar et al., 2014; Bridson et al., 2015; Das et al., 2008; Hameed et al., 2014; Hussain et al., 2009; Iqbal et al., 2012; Mubin et al., 2009; Paul et al., 2008b; Saeed, 2010; Sartaj et al., 2014; Shahid et al., 2007; Ur-Rehman et al., 2013; Xie et al., 2010;). The second type, referred to as alphasatellites, was first identified as a class of molecules called DNA-1 (Mansoor et al., 1999; Saunders et al., 2000). The alphasatellites comprise a group of closely related ssDNA molecules that encode a single protein, a rolling-circle replication initiator protein, or 'replication-associated protein' (Rep) that is capable of autonomous replication. The alphasatellite Rep protein shares an evolutionary relatedness to the Rep of the nanoviruses (*Nanovirus; Nanoviridae*) ([http://ictvonline.org/taxonomyHistory.asp?taxnode\\_id=20153162&taxa\\_name=Nanoviridae](http://ictvonline.org/taxonomyHistory.asp?taxnode_id=20153162&taxa_name=Nanoviridae)), a group of aphid-transmitted, multi-segmented (6-11), single-stranded circular DNA viruses, with each segment encoding one protein. For all other functions, alphasatellites depend on the helper begomovirus, including movement within the plant, and whitefly-mediated transmission. Alphasatellites share no significant levels of sequence identity to their helper begomoviruses, except for a predicted hairpin structure within the loop, referred to as the nonanucleotide sequence, the cleavage site for rolling circle replication (Saunders et al., 2000; Saunders and Stanley, 1999). They rely on the helper virus for encapsidation, vector transmission, and perhaps indirectly for other critical functions in the infection cycle of the virus. The precise role(s) in the disease complexes that infect cotton has not yet been clarified (reviewed in Sattar et al. 2013). They are not thought to be essential for systemic infection of the helper virus, however, the contributions of alphasatellites to begomovirus host plant infection are beginning to be clarified (Hameed et al., 2014; Idris and Brown, 2011; Nawaz-ul-Rehman et al., 2010; Xie et al., 2010).

Because disease symptoms can be experimentally produced in certain host plants inoculated with different combinations of helper viruses and betasatellites (Saunders et al., 2004), the assumption

has been that alphasatellites are of little etiological consequence. However, recent evidence suggests that the alphasatellites can contribute importantly to disease severity by modulating helper virus virulence, an observation that is based on the reduction of symptom severity and decreased levels of betasatellite DNA accumulation when an alphasatellite was co-inoculated with the helper-betasatellite complex (Idris et al., 2011). In addition, expression of an alphasatellite Rep gene has been shown to suppress host-plant induced gene silencing in the youngest leaves, a phenomenon mediated by alphasatellite encoded Rep (protein), in the presence of begomoviral Rep and C4 protein expression (Nawaz-ul-Rehman et al., 2010).

The three most widespread begomoviruses of cotton are those comprising the cotton leaf curl disease 'core' complex (CLCuD) (Bridson et al., 2001; Das et al., 2008; Iqbal et al., 2012; Kumar et al., 2010; Mansoor et al. 1993; 1999; 2006; 2003b; Mubin et al. 2010; Saleem et al., 2016; Sartaj et al., 2014; Zaffalon et al, 2011), four that are endemic to India and Pakistan in Asia, the *Cotton leaf curl Gezira virus* (CLCuGeV), endemic to the African Sahel region (Idris and Brown, 2002a,b; Idris et al., 2005), and the *Cotton leaf crumple virus* (CLCrV) (Brown and Nelson 1984, 1987; Brown et al., 1987; Butler et al., 1986; Dickson et al., 1954; Idris and Brown, 2002; Idris and Brown, 2004; Wilson et al. 1991), which is endemic to and currently restricted to North and Central America.

In Asia, CLCuD is caused by one or more of four begomoviral species that are endemic to India and Pakistan. Studies have shown that they have unexpectedly broad host ranges, including vegetable crops, ornamentals, and numerous wild, uncultivated species. Viral-betasatellite complexes can occur in mixtures in the same host plant. Thus, the Asian originating 'core' leaf curl begomoviral species and/or strains are *Cotton leaf curl Alabad virus* (CLCuAIV), *Cotton leaf curl Bangalore virus* (CLCuBaV), *Cotton leaf curl Kochran virus* (CLCuKoV), *Cotton leaf curl Multan virus* (CLCuMuV) (**Fig. 1**).

The predominant species occurring in India and Pakistan are CLCuKoV (the CLCuKoV-Bur strain causing the second leaf curl disease outbreak) and CLCuMuV (first leaf curl disease outbreak), however, additional strains of both of these major species have been reported (Amrao et al., 2010; Bridson et al., 2001; Mansoor et al.; 2003b; Mubin et al. 2010; Nawaz-ur-Rehman et al., 2012; Sartaj et al., 2014; Sattar et al., 2013; Saleem et al. 2016; Zaffalon et al, 2011). Of them, the most widespread CLCuKV-Bur is recognized as the resistance-breaking strain (Mansoor et al., 2003a), a recombinant composed of about half of the genome each of CLCuKoV and CLCuMuV, referred to as 'the Burewala strain' (AM421522). The CLCuAIV and CLCuBaV are detectable in leaf curl affected cotton and/or vegetable crops species, but they have not been linked to an epidemic, thus far. In addition, several 'non-core' cotton leaf curl geminiviruses have been reported to occur in mixed infections with 'core leaf curl' species, including the *Chickpea chlorotic dwarf virus* (CpCDV) (also identified in tomato) (Ur-Rehman et al., 2015), *Okra enation leaf curl virus* (OEnLCV), *Papaya leaf curl virus* (PaLCV), *Tomato leaf curl Bangalore virus* (ToLCBaV), and *Tomato leaf curl New Delhi virus* (ToLCNDV) (Saeed, 2010; Zaidi et al., 2016). The role of these secondary species and of other strains of the 'core' leaf curl viruses in disease outbreaks or severity are not well-established. Among all of the species and strains identified in cotton, Koch's postulates have been established for only CLCuMuV, CLCuKoV and PaLCuV (Sattar et al. 2013). All of these begomoviral species/strains occur in and are known to be native to India and Pakistan.

#### *History of leaf curl disease in the Indian Sub-Continent*

Cotton leaf curl disease in Pakistan was first reported during 1967 near Multan (Hussain and Ali, 1975). This disease is characterized by an upward curling of leaves, thickening of veins and

laminar outgrowth on underside of the leaves referred to as enations (Mahmood, 1999; Khalid et al., 1999; Akhtar et al., 2002a). Attention was drawn to the disease in 1973 when leaf curl symptoms became prominent in several important cotton varieties, including 149-F and B-557. Symptoms were observed late in the season and only on the flush growth. By 1987, the incidence increased to as high as 80% in some fields, damaging 60 hectares of the crop in the Multan District. During 1991, leaf curl disease affected 14,000 hectares in Multan, Khanewal, and Vehari Districts, and by 1992, 48,500 hectares were infected. During the 1993 season the disease spread to the entire cotton belt of the Punjab damaging 889,000 hectares. The increased incidence in leaf curl disease during the mid-1970's and early 1980's has been attributed to the decline in popularity of the smooth leaf cotton varieties, because they became highly susceptible to jassid infestation. This caused production varieties to shift to hairy or hirsute leaf types, which were not susceptible to jassid (R. Chaudhry, ICAC; personal communication). The hirsute varieties were then found to stimulate cotton infestation by the cotton whitefly *B. tabaci*, which preferred the hirsute over the smooth-leaf varieties that were previously the predominant type of cotton grown there. The widespread use of insecticides to control the upsurging whitefly populations resulted in the development of insecticide resistance, and whitefly vector populations spiraled out of control, leading to rapid spread of leaf curl disease (then of unknown etiology), first near Multan and then other cotton growing areas of Pakistan (Hussain and Ali, 1975), and finally into India. The pathogen responsible for the leaf curl epidemic was identified as a new, previously undescribed whitefly-transmitted begomovirus, *Cotton leaf curl Multan virus* (CLCuMV) (Bridson et al., 2001; Mansoor et al. 1993, 1999).

The effects of the leaf curl disease on production, during 1991-1999 proved disastrous. Pakistan reported that in the first year that the epidemic reached full scale, yields declined by one million bales from a record production of 12.82 million bales. By 1994-1995, the yields were further reduced, to 7.9 million bales (Anonymous, 1997). After 1995 leaf curl disease outbreaks occurred annually, and the industry was debilitated. Breeding efforts were launched by the Pakistan government (national and provincial) to develop resistance varieties to combat the disease, and for a short period of time, cotton production was returned to pre-epidemic levels. However, during 2001-02 another outbreak occurred, beginning in the Burewala territory of the Punjab Province where affected the cotton varieties were shown to those developed to combat CLCuMV infection, the causal agent of the 1994-95 epidemic (Mahmood et al., 2003; Mansoor et al., 2003b). Studies to determine the causal agent of the 'Burewala outbreak', led to the identification of a new, emergent begomovirus strain, initially referred to as Cotton leaf curl Burewala virus, and later correctly named *Cotton leaf curl Kochran virus-Burewala* (Brown et al., 2015).

Unexpectedly, the CLCuKoV-Bur was found to be a recombinant virus with the genome consisting of two previously recognized species, CLCuKoV and CLCuMV, the latter having been the causal species of the 1990's pandemic. Although CLCuKoV was known to be associated with leaf curl symptoms in cotton in certain locations, how widespread it was, was not known until the latter discovery spurred additional investigations (Amin et al., 2006; Amrao et al., 2010a).

The recombinant CLCuKoV-Bur, apparently originating in Burewala where evidence of resistance-breaking was first observed in cotton, was found to be more virulent than CLCuMV in cotton, particularly when infection occurred in the early growth stages (Arshad et al., 2006). Taken together with the ability of at least several endemic whitefly vector haplotypes to transmit the recombinant virus (Ahmed et al., 2011; Ashfaq et al., 2014), has led to rapid virus spread into the once, most productive cotton-growing areas in central Punjab (Khanewal, Multan, Lodhran, Vehari, Bahawalnagar, Bahawalpur) (Aktar et al., 2014). All varieties harboring resistance to

CLCuV-Multan are susceptible to CLCuKoV-Bur infection (Mahmood et al., 2003; Saleem et al., 2016; Tahir et al., 2004).

Due to the geographical proximity of the Punjab regions of Pakistan and India, and the direction of the prevailing winds, it is hypothesized that the Multan and Burewala CLCuD epidemics first caused by CLCuMV and then by CLCuKV-Bur, likely spread eastward from Pakistan into northwestern India from where it then moved further into the other northwestern state (based on the distribution of the disease). Further evidence taken from the more recently discovered species, CLCuMuV, CLCuKoV and CLCuBuV first identified in Pakistan, also are now present in cotton in northwestern India (Rajagopalan et al., 2012; Zaffalon et al., 2011), whereas, several begomoviruses identified in cotton in India have not been detected in Pakistani cotton. For example, a lesser known species, CLCuRaV, occurs widely in cotton in India (Kumar et al., 2010), and has been detected in Pakistan, in at least one cotton species maintained in the outdoor 'living herbarium' in Multan (Nawaz-ul-Rehman et al., 2010) and in tomato (Shahid et al., 2007), indicating it is endemic throughout the region, and could pose a threat to cotton crops in the future. Another species, ToLCBaV has been identified in cotton in India, but is not widely prevalent there. In addition, the bipartite *Tomato leaf curl New Delhi virus* (ToLCNDV) DNA-A and DNA-B components has been reported in cotton from India (EF063145), together with a betasatellite (Jyothsna et al., 2013), an unusual affiliation among bipartite begomoviruses. However, until recently, it had not been considered an important viral pathogen of cotton (Zaidi et al., 2016). Indeed, cotton plants co-infected by ToLCNDV and CLCuKoV developed more severe symptoms than plants inoculated with CLCuKoV alone, suggesting that together, they exhibit 'enhanced virulence' (Jyothsna et al., 2013), by increasing the accumulation of CLCuMB, which encodes a pathogenicity (symptom) determinant (Zaidi et al., 2016). Further, a ToLCNDV-associated betasatellite (ToLCND $\beta$ ) has been shown to serve as the possible source of a fragment also present in a recombinant CLCuMuV-like satellite (Amin et al., 2006), frequently associated with CLCuKoV-Bur. These lines of evidence indicate that the bipartite ToLCNDV has intermingled with at least some monopartite virus-betasatellite complexes found in cotton in Pakistan prior to the outbreak caused by the CLCuKoV-Bur resistance-breaking strain.

Research undertaken to determine the mechanism of resistance-breaking, showed that the recombinant CLCuKoV-Bur produced a truncated C2 protein, despite encoding a complete C2 open reading frame (ORF) (Akbar et al., 2012; Amrao et al., 2010b). In begomoviruses, the C2 protein is involved in suppression of gene silencing by the plant host (Amin et al., 2011), the latter being a host immune response designed to counter begomoviral infection. This observation strongly suggested that the resistance-breaking phenomenon was directly or indirectly associated with the expression of the shorter-than wild type gene product, which feasibly, was selected for by the as yet poorly understood mechanism of genetic resistance to virus infection (Khan et al., 2007; Mahmood et al., 2003; Rahman et al., 2005; Rajagopalan et al., 2012). Following its spread throughout much of Pakistan, CLCuKoV-Bur was identified infecting cotton in the neighboring Indian Punjab, where it infected CLCuMV resistant varieties as well, resulting in extreme crop losses in both countries during 2009-2010. Since that time, CLCuKoV-Bur has become the predominant begomovirus in cotton and certain vegetable crops in northern India (Rajagopalan et al., 2012; Zaffalon et al., 2011).

Studies of begomovirus diversity in cotton in India since the emergence of CLCuKV-Bur indicate that CLCuBuV and CLCuRaV are the predominant species in cotton there (Rajagopalan *et al.*, 2012; Zaffalon et al., 2011). This differs from the current situation in Pakistan where only CLCuBuV is widespread (Amrao et al., 2010b). Also, in India, CLCuMV has been detected in

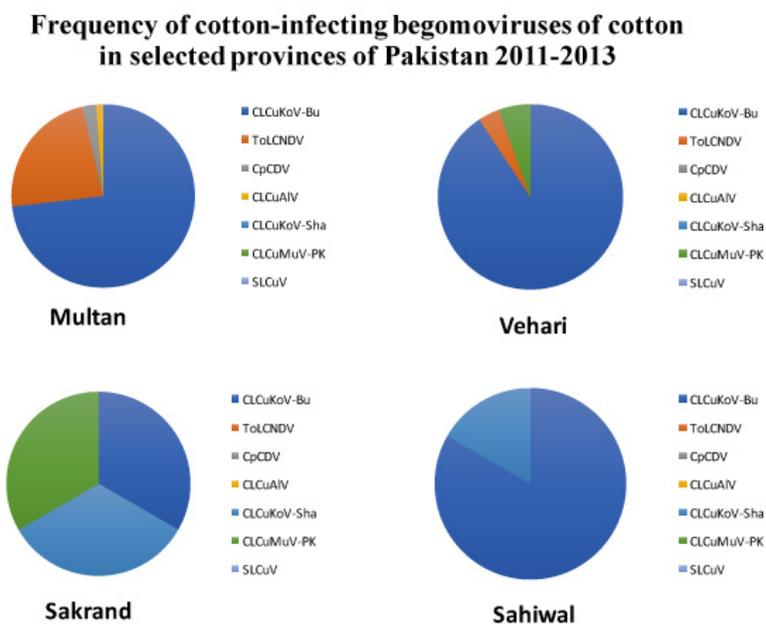
malvaceous species other than cotton, including species of *Hibiscus* grown as ornamentals (Srivastava et al., 201), and fiber crops such as *Hibiscus cannabinus* and *Hibiscus sabdariffa* (Das et al., 2008; Paul et al., 2008; Roy et al., 2009).

Previous studies have shown that okra is a host of CLCuMV (Zhou et al., 1998), implicating non-cotton cultivated plant species as hosts of this ‘core’ leaf curl virus. Recently, in Pakistan *Okra leaf curl virus* has been shown to infect cotton together with the associated Cotton leaf curl Multan beta (CLuMB) and alpha-satellites (Hameed et al., 2014) and chilli peppers (Hussain et al., 2003), whereas, CLCuKoV-Bur was detected with CLCuMB and *Gossypium darwinii* symptomless alphasatellite in symptomatic *Luffa cylindrical* plants (Ur-Rehman et al, 2013). These and other reports confirm that this virus is not restricted to cultivated plants, and can be harbored by numerous wild hosts (Mubin et al., 2009; Mubin et al., 2010; Srivastava et al., 2016). Thus, in both India and Pakistan, cotton-infecting species have been detected in wild species and in an extensive array of vegetable and ornamental hosts, suggesting that these viruses may infect species and plant families beyond the currently recognized range of hosts.

Owing to this and the ease with which they are transmitted by the whitefly vector, they could feasibly spread from the Indian subcontinent and establish in distant cotton-vegetable producing areas in Africa, Australia, Latin America, and the United States. Indeed, China and the Philippines have already reported the introduction of highly homologous isolate of CLCuMV *via* malvaceous ornamental hosts (Cai et al., 2010; Mao et al., 2008; Tang et al., 2010; Du et al., 2015; Delores et al., 2014). Further, CLCGeV has been introduced from Africa into the Arabian Peninsula (Al-Saleh et al., 2015; Idris et al., 2014), and from Africa to Pakistan (Tahir et al., 2011). Indeed, in analogous thread, *Squash leaf curl virus* (SLCuV), endemic to North and Central America was identified in squash plants in Egypt, Israel, Jordan, and other locales in the Middle East (Idris et al., 2006) from where it has spread into cotton in southern Pakistan (**Fig.1**)

### Africa

Leaf curl in Africa was first reported in Nigeria in 1912 (Farquharson, 1912), followed by Sudan (Golding, 1930) and Tanzania (Kirkpatrick, 1931). When leaf curl symptoms were observed in



**Figure 1.** Results of a virus survey of selected cotton-growing locales in Pakistan during 2011-2013, indicating uneven distribution of viruses in different provinces in Pakistan (courtesy, Ur-Rehman, Hameed, Haider, Avelar, Chingandu, Ilyas, and Brown (in preparation).

Pakistan during the 1970's (Hussain and Ali, 1975) the name 'cotton leaf curl' was erroneously adopted, because it was not yet recognized that the viruses in Africa were quite different from those species endemic to Pakistan and India.

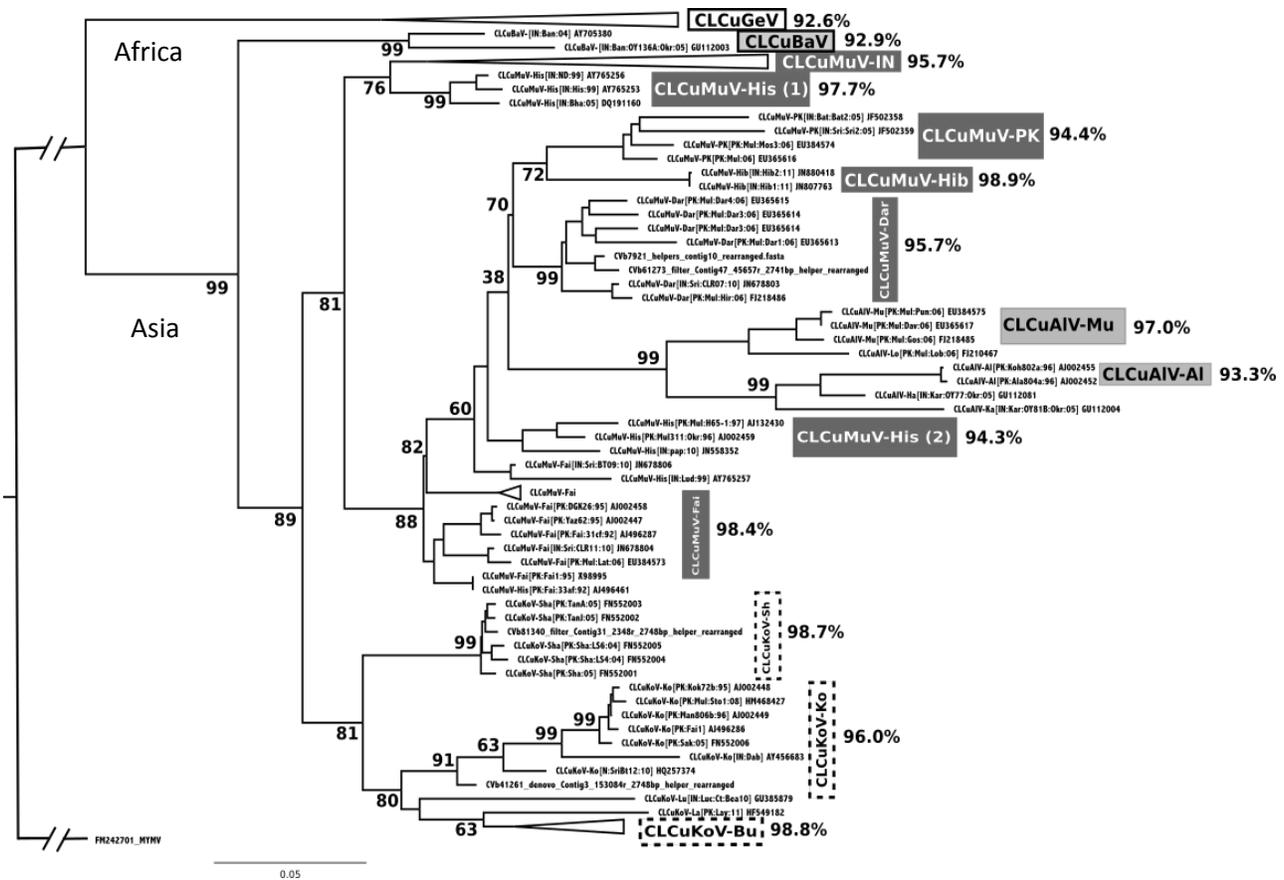
The begomoviral species that predominates in cotton crops in Africa, *Cotton leaf curl virus-Gezira* virus (CLCuGeV) (Idris and Brown, 2002) and its betasatellite, represent a fifth species of the 'core leaf curl complex. It has been found widespread throughout the cotton belt (in cotton) in sub-Saharan Africa, mainly in the Sahel region. The first isolate of this viral species to be characterized at the molecular level was the Gezira isolate from *Sida* spp. and okra plants in Sudan (Idris and Brown, 2002). As with the Asian leaf curl viruses, CLCuGeV is associated with a betasatellite, *Cotton leaf curl Gezira betasatellite* (CLCuGB). The virus and associated satellites are genetically and phylogenetically distinct from those occurring on the Indian subcontinent (Idris and Brown, 2002; Idris et al., 2005). Recent studies have demonstrated that CLCuGeV is present not only in Egypt, Sudan, and other African countries where cotton is grown, but also in the Arabian Peninsula (UA and, elsewhere) and Jordan. Collectively, CLCuGeV is known to infect cotton, hollyhock, okra, *Sida* spp. and perhaps other malvaceous species (Tahir *et al.*, 2011; Idris et al. 2002a, b; 2013; 2014).

Although begomoviruses have been described in cultivated malvaceous hosts such as hollyhock and okra in sub-Saharan Africa, only CLCuGeV has been detected infecting cotton in the region (Idris and Brown, 2002; Tahir et al., 2011). Other begomoviruses causing leaf curl and other diseases in okra and tomato are widespread in West Africa (Leke et al., 2013; 2015; Tiendrébéogo et al., 2010) and could possibly infect cotton and ornamental species. So far, only a few other begomoviruses associated with cotton have been characterized from sub-Saharan Africa. These latter viruses endemic to the region may therefore become of potential importance to cotton production in Africa (Leke et al., 2015; Sattar et al. 2013).

### *Americas*

The sixth begomoviral species infecting cotton is *Cotton leaf crumple virus* (CLCrV), a bipartite virus of New World origin. It is represented as a group of closely related variants. Symptoms of the disease are foliar discoloration, leaf crumpling, shortening of internodes, and stunting in both cotton, and in experimentally and naturally infected common bean [*Phaseolus vulgaris* (L.)]. Disease severity is dependent upon plant age at time of infection. Damaging outbreaks of cotton leaf crumple disease may be exacerbated by rationing, a practice in which cotton is pruned and allowed to re-grow the following year. In most years, infection occurs after cotton plants have developed to the 10-14-leaf stage, so CLCrV has not been considered an economically important disease of cotton. In years when whitefly populations build to high levels early in the season, plants become infected during the early growth stages and damage can be extensive. However, management is usually confined to controlling the whitefly vector to reduce virus transmission and secondary spread. Several virus-tolerant lines have been selected from efforts to develop disease resistant cotton using 'Cedix variety' as the source of genes for introgression (Wilson et al., 1991).

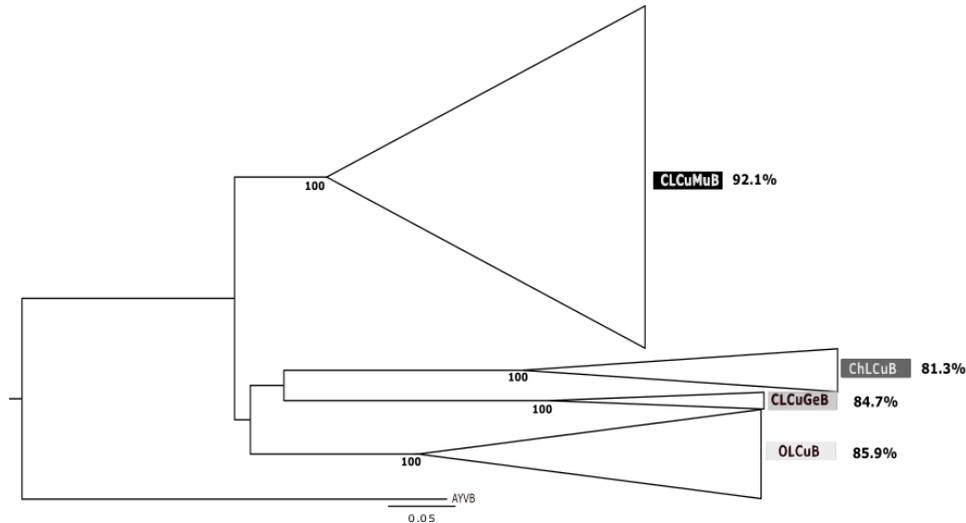
### *Genetic diversity among cotton-infecting begomoviruses*



**Figure 2.** Phylogenetic tree (NJ, MEGA7) showing relationships between cotton-infecting begomoviruses in the Old World, and illustrating the extents of genomic variability among leaf curl viruses originating from the Eastern Hemisphere, with the most extensive genomic variability in Asia and substantially less among viruses of African origin. The tree was reconstructed using the complete genome for 233 sequences (GenBank), and 219 field isolates collected in Pakistan during 2011-2013, respectively (Brown et al., unpubl. data). Colors indicate five of the six ‘core’ cotton leaf curl virus species, at  $\geq 91\%$  (per Brown et al., 2015) (Courtesy, M. Ilyas and J.K. Brown, Virus Res. (in prep)).

In the EH, two major groups or divergent clades of cotton-infecting begomoviruses are recognized, one centered on the Asian Continent and another in Africa. The two major clades are overall highly divergent from the CLCrV group that is species endemic to the Americas. This suggests that the ancestors of all three groups of viruses evolved independently and adapted and diversified with cotton post-domestication. The African cotton-infecting begomovirus isolates known thus far are only distantly related those of Asian origin, as is illustrated by the complete genome sequences (Fig. 2). In Sudan, where the leaf curl disease affected cotton production during the early part of the 20th century, one predominant begomovirus species CLCuGeV has been associated with the disease there (Idris and Brown, 2002). In studies using infectious clones constructed from viral DNA isolated from *S. alba*, the inoculated cotton plants did not become infected even though *S. alba* plants were systemically infected, suggesting that *S. alba* is either a less recalcitrant host to experimental inoculation than cotton, or that slight differences in genome sequences differences account for host range differences. Comparisons of CLCuGeV and associated alpha- and beta-satellites from okra in Burkina Faso and malvaceous and solanaceous-infecting viruses in other West African countries, with other begomoviruses of hollyhock, okra, and tomato, indicate that the range of host species of these viruses is similar to the host range(s) observed in Pakistan and India,

(Leke et al., 2015; Tiendrébéogo et al., 2010), thereby also providing a fertile ground for diversification through mixed infections, recombination, and reassortment. In addition, CLCuGeV occurs in the Arabian Peninsula (Idris et al., 2014), but how widespread it is distributed there is not known. The genome sequences of the isolates from Arabia share 88-93% nt identity to previously reported strains of CLCuGeV from Africa. In addition, the CLCuGeV betasatellites share ~ 60% nt identity with several associated *Cotton leaf curl Gezira* betasatellites reported from the Nile Basin and sub-Saharan Africa (**Fig. 3**). However, nt sequence comparisons revealed that one alphasatellite shared 88% nt identity with Cotton leaf curl Gezira alphasatellite (DNA-1 type), while the second satellite shared the highest nt identity (64%) with Ageratum yellow vein Singapore alphasatellite (DNA-2 type) from Oman and Singapore (data not shown). The combined



**Figure 3.** Phylogenetic tree (NJ, MEGA7; 1000 bootstrap iterations) showing the four betasatellite groups associated with the four monopartite ‘core’ cotton leaf curl begomoviruses from African and Asia. The four clades comprise 506 sequences downloaded from the GenBank database (minus 100% identical sequences). The cutoff for group demarcation is  $\geq 78\%$  shared nt identity. The extensive within and between-clade variability is illustrated. Courtesy, M. Ilyas and J.K. Brown, *Virus Res.* (in prep).

high variability and relatively low nt identities of these molecules with previously reported satellites suggest they are endemic, and that they are not the result of recent introductions (Idris et al., 2013). The CLCuGeV also has been found in southern Pakistan, and the genome sequence for the Pakistani isolate is so similar to those from Sudan that it appears to have been recently introduced there (Tahir et al., 2011). Some time ago, in

Egypt, a distinct begomovirus has been identified in the malvaceous host, hollyhock, *Hollyhock leaf crumple virus* (Idris et al, 2002). This virus infects hollyhock and other malvaceous species, suggesting that it may be able to cause disease in cotton but to date it has been not identified infecting cotton, and whitefly-transmitted virus-like symptoms in Egyptian cotton have been relatively rare (Aly Abdel-Salam, J.K. Brown, A.M. Idris, pers. observation).

In the Americas (e.g. WH), a single species consisting of several strains or variants (mostly owing to recombination) prevails, spanning the southwestern U.S. (AZ, CA, TX), northwestern and southwestern Mexico, and Guatemala. In Puerto Rico and the Dominican Republic, virus-like symptoms have been observed and confirmed to be begomoviruses, but they represent different species whose closest relatives are found in endemic, uncultivated malvaceous species. Although minor variants occur in the USA (AZ, CA, and TX), Mexico, and Guatemala they comprise a single species, at 0-4% nt divergence across the genome (Brown and Nelson, 1984; Idris et al., 2004). A comparison of the CLCrV ORFs with those of closely related begomoviruses indicated that the CLCrV AC3 ORF shares a maximum nt identity with *Potato yellow leaf mosaic virus* (PYMV), at 87%. Also, the CLCrV AV1 shares 83 % nt identity with its close relative, *Sida yellow*

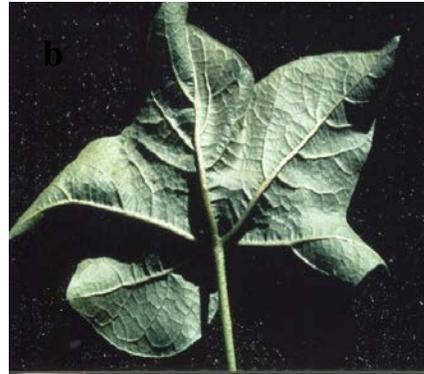
vein virus (SiYVV), comparisons at the amino acid level indicate that the AV1 for the two viruses is 93% identical. Similarly, CLCrV AC2 and AC3 ORFs shares high nt identity with SiYVV, at 82-83%, and a correspondingly high amino acid sequence identity (Idris and Brown, 2004).

## II. Symptoms

Cotton leaf curl symptoms were first reported in Nigeria in *G. barbadense* cotton (Farquharson, 1912). In 1924, similar symptoms were reported to be widespread in the Sudan cotton crop (Golding, 1930), and subsequently, in 1926 the disease broke out in Tanzania (Kirkpatrick, 1931). When leaf curl symptoms were observed in Pakistan cotton crops during 1967 the 'leaf curl disease' name was incorrectly adopted based on the 'leaf curl' symptom phenotype, irrespective of the particular agent(s), later shown to be highly divergent species. Leaf curl symptoms in Sudan and Pakistan are characterized as curling of the leaf margins, either upward or downward, and a crinkled appearance of the leaves, now referred to as 'enations', consisting of leafy tissue developing directly from the leaf veins. The veins of the affected leaves become thickened and more pronounced on the underside (**Fig. 4a-c**). In Africa, two types of vein thickening are reported for CLCuGeV, small vein thickening and main vein thickening. Small-vein thickening is the most common phenotype, and is characterized by small green bead-like thickening on the young leaves (**Fig. 5a-d**). The irregular thickening gradually extends and coalesces to form a continuous reticulation of the small veins. Main vein thickening first appears near the leaf margin, and extends inward to form a network of dark green thickened main vein. In extreme cases, leaves form cup-shaped, and leaf-like outgrowths appear on the underside of the leaves. Tarr (1951) reported spirally twisted petioles, fruiting branches and tall stems and elongated internodes in *G. barbadense*. Most varieties in Africa infected by CLCuGeV exhibit dwarfing, overall stunting, and reduced boll number and boll weight.

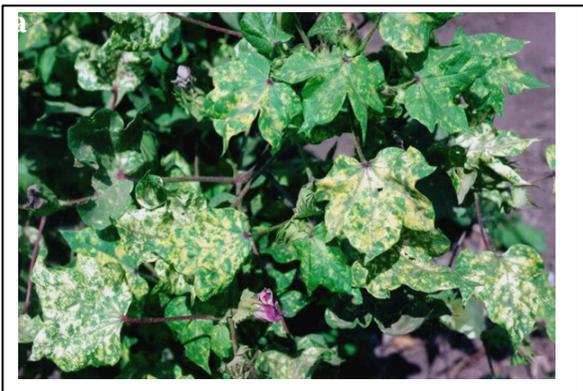


**4a-c.** (a) Leaf curl disease symptoms in cotton, (b) compound foliar enation on the underside of a leaf, and (c) cup-shaped veinal-enation on the leaf underside, all from Pakistan (photos, courtesy R. Briddon and S. Mansoor).



**Figure 5 a-d.** Symptoms of CLCuGeV from Sudan in (a) cotton plants, (b) underside of cotton leaf, (c) hollyhock plant, lower leaf surface, and (d) hollyhock plant, upper surface.

Symptoms caused by CLCrV in the Americas are fairly similar from one location to another but differences are noted with certain varieties. Cotton (**Fig. 6a-b**) and kenaf (**Fig. 6d**) plants infected in the seedling stage develop severe leaf curling, blistering, and crumpling on the newest growth. Symptoms persist in the leaves throughout the season (**Fig. 6a**). Some varieties also develop yellow-green mosaic symptoms (**Fig. 6b**). In plants infected at later growth stages, leaf symptoms are mild or absent, unless flush growth is stimulated, and then all of the latter leaves will develop typical leaf crumple symptoms. Flower petals (**Fig. 6c**) and bolls also develop symptoms, particularly in plants inoculated prior to the 8-10 leaf stage. Yield and fiber quality are reduced substantially (Brown et al., 1987) (photos, courtesy A.M. Idris and J.K. Brown).





**Figure 6 a-d.** Symptoms of *Cotton leaf crumple virus* infection in (a) cotton plants in Arizona, USA (b) cotton plants in Caborca, Mexico, (c) flower petals, and (d) in kenaf plants, Texas, USA (photos, courtesy J. K. Brown).

### III. Spread and Risk

Members of the complex are not seed transmitted, a property consistent with other well-studied geminiviruses. The leaf curl virus complex is spread by the whitefly *B. tabaci* sibling species group in a circulative, persistent manner. Begomoviruses are not transovarially or sexually transmitted (with one possible exception, *Tomato yellow leaf curl virus*, from Israel), nor has mechanical transmission been demonstrated for the leaf curl complex viruses.

The *B. tabaci* sibling species comprises a cryptic (morphologically indistinguishable) group of *Bemisia* sibling species that may exhibit restricted gene flow (or not), indicating that speciation has occurred or is impending within this whitefly. Members of sibling species group exhibits different biological characteristics, harbors distinct suites of endosymbionts, have a variety of phenotypes, and are adapted to different environments (Brown, 2010). The group as a whole colonizes over 500 plant species, however the host range of most haplotypes is probably not overridingly broad, in general, because it seems likely that environment-related fitness and constraints would co-govern adaptation and host preference to certain locally-available suites of hosts. Genetically distinct *B. tabaci* populations that have been characterized with respect to biological characteristics, are referred to as ‘biotypes’ whereas, those for which only a molecular marker sequence is available, are referred to as ‘haplotypes’, however, the group as a whole is considered a sibling species group, and so can be considered ‘separate species’, however, they can only be distinguished by molecular analysis, and the criteria for species-cutoffs are unresolved (see references in: Brown, 2010).

Virus-satellite complexes can be experimentally transmitted by grafting (Akhtar et al., 2013), and by inoculation of plants with infectious viral clones using biolistic inoculation, particle bombardment, or agroinoculation (Idris and Brown, 2000; 2004; numerous others). The latter types of inoculation methods do not necessarily result in the development of wild type disease symptoms in cotton. The tobacco species, *Nicotiana benthamiana* (Domin), is a useful bioassay host when infectious viral clones are available, owing to its susceptibility to most plant viruses.

Pathways for entry into the United States include cuttings and propagative host materials. These methods are regulated by USDA-APHIS under the authority of the Plant Protection Act with

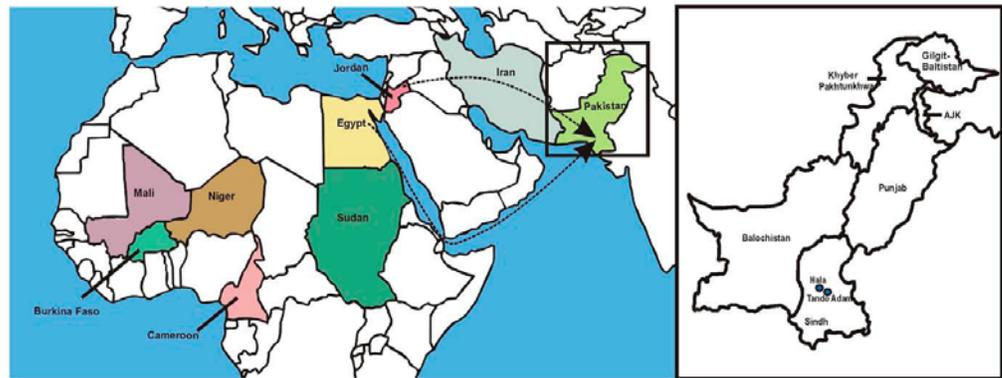
regulations in 7 CFR Part 319, which prohibit or restrict entry of certain plants and plant products to prevent introduction of plant pests and pathogens into the United States. All cotton (*Gossypium* sp.), okra (*Abelmoschus esculentus*), and tomato (*Solanum lycopersicum*) plants are prohibited, except for the seed. Hibiscus (*Hibiscus* sp., and *Hibiscus cannabinus*) is prohibited from Africa, Brazil and India, and also must meet Federal Order effective May 11, 2011 (specifically for the Importation of host material of *Anoplophora chinensis* (Forster), the Citrus Longhorned Beetle and *Anoplophora glabripennis*, Asian Longhorned Beetle). These materials are subject to size restrictions and must undergo 2-year post-entry quarantine.

Natural spread of the leaf curl disease is mainly by the whitefly vector (*B. tabaci*). It can complete the transmission cycle from the acquisition of the virus to infection of a new host plant, within 6.5 hours (citation). *B. tabaci* is capable of establishing high population levels, particularly in crops grown under irrigated, arid conditions in both field and greenhouse environments. In addition, this whitefly has the potential to colonize a wide range of dicotyledonous species, including vegetable and fiber species of great importance to worldwide agricultural production. Studies have shown that there are numerous variants of *B. tabaci*, referred to as biological types (biotypes) that can differ with respect to fecundity, feeding damage, insecticide resistance, and virus transmission efficiency (competency) (Bedford et al., 1994; Brown and Bird, 1992; Brown et al., 1995; Brown, 2010, and references therein; see refs in Brown, 2010; Maruthi et al., 2002).

Recent introductions:

Based on historical knowledge and the extent of nucleotide divergence in viral genomes and associated beta- and alpha-satellites present different locations, it is possible to ascertain whether a virus is endemic or recently introduced from a zone of endemism. The introduction of exotic, genetically divergent begomovirus-satellite complexes to cotton-growing areas where the viruses are not endemic has

great potential to cause outbreaks because tolerance or resistance to the exotic viruses is not likely to exist in local germplasm. This is because resistance has been selected in the presence of the endemic virus and whitefly vector populations. The demonstrated high likelihood for

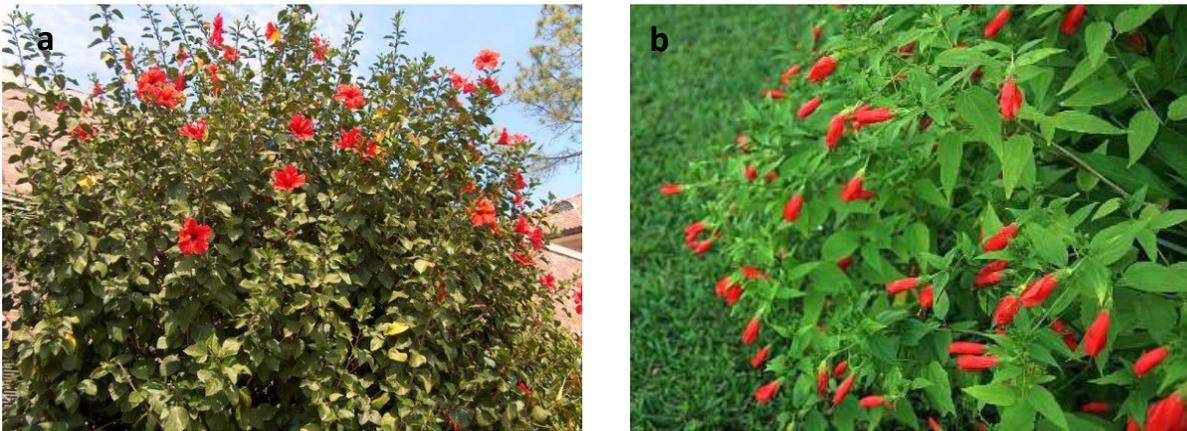


**Figure 7.** Proximity of cotton growing regions in Africa and Indian-subcontinent, illustrating the ease with which plant viruses can be moved by human activity involving exportation/importation of plants between the continents (from Tahir et al., 2011).

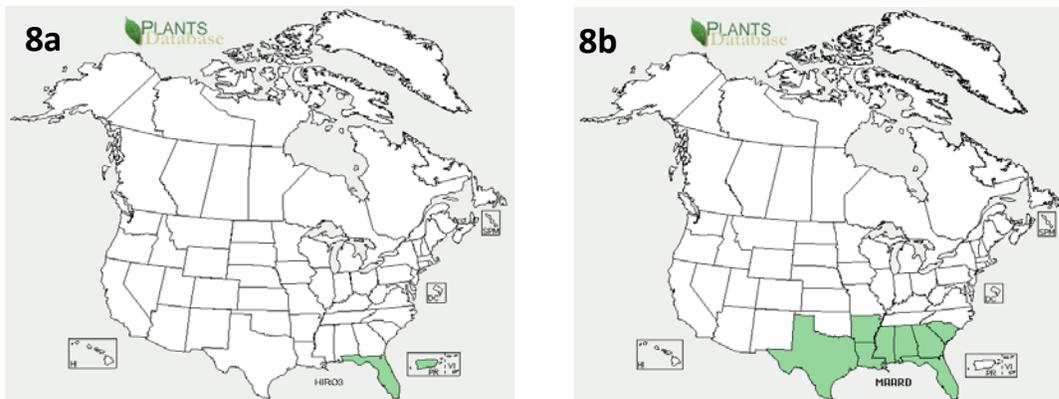
recombination between helper virus genomes and beta- and alpha-satellites, as well as reassortment of satellite-helper complexes, makes possible the emergence of new variants. Presently, there is evidence that cotton-infecting viruses have spread from their endemic to previously uninhabited locales.

An isolate of the CLCuGeV (endemic to Africa) was detected infecting cotton plants in Southern Pakistan for the first time (Tahir et al., 2011), and there have been no further reports of additional

discoveries or further spread in the country. The percentage nucleotide (nt) sequence identities between the Sudan and Burkina Faso isolates and the Pakistan isolates are greater than 95%, suggesting that the Pakistan isolate is a recent introduction from Africa. So far it has been found south of the Punjab Province, suggesting it was transported there from Sub-Saharan Africa by human activity (*via* major ports such as in Karachi) (**Fig. 7**). In 2008, an isolate of CLCuMV and its associated CLCuMB satellite, the main virus associated with the first epidemic in the Punjab region of Pakistan e.g. ~1990-2004 was detected in two Chinese provinces, Guangdong and Guangxi. The first report occurred in Guangzhou (Ghangdong Province) from infected the ornamental *Hibiscus rosa-sinensis* plants (rose mallow) (Mao et al., 2008). Then in 2009, CLCuMV and CLCuMB satellite were identified in cotton in the Guangxi Province (Cai et al., 2010). Within the next few years the virus appeared to have spread from Asia to China, based on extremely high shared CLCuMV-betasatellite nt identities, at >98.5%, features consistent among isolates from symptomatic okra (*Hibiscus esculentus*) and *Hibiscus rosa-sinensis* (He et al., 2010), and *Malvaiscus arboreus* (Turks cap) (Tang et al., 2014), and as would be expected from a single introduction event (Du et al., 2015). The latter CLCuMV isolates were reported to have a defective betasatellite, a feature also reported for CLCuGeV from Saudi Arabia (Idris et al., 2013). Further, CLCuMV has been identified in naturally-infected *Hibiscus rosa-sinensis* plants in India and Pakistan (Akhtar et al., 2014; Srivastava et al., 2016). All of these cultivated species are grown or found in the United States (**Figs. 8, 9**) cotton belt. The significance of these defective betasatellites is not yet known, and additional information is required to understand the etiology and possibly additional complexity of the leaf curl isolates now circulating in China. However, it is thought that the CLCuMV-betasatellite complex was transported from Pakistan to the Gulf States on *H. rosa-sinensis* plants from where it was apparently later imported into China on infected cuttings or plants. These observations suggest that the exportation of the latter virus likely occurred before the emergence of CLCuKoV-Bur in Pakistan, and therefore had been in China for a longer period than previously documented.



**Figure 8a-b.** Photos showing *Hibiscus rosa-sinensis* (L.) plants (rose mallow) (left), and *Malvaiscus* (also, *Malvaviscus*) *arboreus* var. *drummondii* (Torr. & Gray) Schery (Turks cap) (right) plants, hosts of *Cotton leaf curl Multan virus* originating in Pakistan and recently introduced to China and the Philippines (photos courtesy, contributions to the internet).



**Figure 9a-b.** Maps illustrating the distribution of hibiscus (*H. rosa-sinensis*) (left) and Turks Cap (*M. arboreus*) (right) in the southern United States and/or Puerto Rico, however, *H. rosa-sinensis* is not restricted to Florida, as is shown in this map, but is grown in many locations in the U.S. owing to its wide distribution through the nursery trade. These ornamental species are two of many ornamental species that are known or suspected hosts of CLCuD complex (taken from <http://plants.usda.gov/>; [http://www.wildflower.org/plants/result.php?id\\_plant=MAARD](http://www.wildflower.org/plants/result.php?id_plant=MAARD)).

#### IV. Detection and Identification

Knowledge of when and where mutations occur and/or are fixed in the viral- satellite populations by host (or whitefly vector) selection will alert cotton producers and breeders to the potential emergence of a potentially damaging new threat to currently cultivated varieties and cotton germplasm under consideration in genetic improvement programs. The ability to detect subtle changes in the viral genome and population structure in near real time will warn of the impending spread of the leaf curl-satellite complexes from their regions of endemism to exotic ones where extensive damage would likely occur in unprotected cotton cultivars (those not bred for resistance or tolerance to exotic viral pathogens). Employing this multifaceted, proactive approach to viral population structure analysis will provide the most proactive means of initiating proper actions and subsequently, recovery from the effects of disastrous outbreaks, and identify potential resistant germplasm.

Currently, most begomoviruses and satellite complexes may be detected by polymerase chain reaction using virus specific or degenerate primers (Briddon et al., 2002; Brown et al. 2001; Bull et al., 2003; Wyatt and Brown, 1996). To amplify an informative region of the begomoviral coat protein of many begomoviruses, degenerate primers are available (Wyatt and Brown, 1996). These primers were updated in 2006 (Idris and Brown, unpublished). The former primers facilitate amplification and sequencing of an informative fragment of the coat protein gene in one forward and reverse reaction. If not confounded by recombination, the reactions permit tentative identification of begomoviral species without the requirement to sequence the entire genome. A non-sequence specific amplification and cloning of genome-length units, referred to as rolling circle amplification (RCA) is used to circumvent the lack of virus-specific primers to facilitate amplification of all known helper viral genomes, and the multitude of recombinants (Haible, et al., 2004; 2006; Inoue-Nagata et al., 2004). PCR primers that amplify the majority of, but not all, begomovirus-associated satellites are available (Amrao et al., 2010; Briddon et al., 2002; Bull et al., 2003; Idris et al., 2002; 2005; 2011). Polymerase chain reaction and RCA diagnostics should be implemented concurrently because RCA does not always detect satellite molecules that may be present. Most recently genomic pathology approaches are being applied to begomovirus detection

(Idris et al., 2014) that does not rely on the *a priori* knowledge of the plant virome, as do primer-dependent or serological methods. In the future, it is expected that the increased use of high-throughput sequencing platforms for virus detection in plants and the insect vector, as well as improved approaches that rely on specific primers and amplification, such as isothermal methods that can be carried out at room temperature, including real-time, under field conditions. Also, with increased knowledge of viral helper and satellite variability at the genome/component levels, it will become possible to vastly improve primer design.

Because betasatellites (and perhaps alphasatellites too) contribute to begomoviral helper virulence, symptom severity, and resistance breaking, it is essential to accurately detect and identify the entire complex of helper virus and the associated satellites. Once achieved, the next important step is to identify the source of the introduction, and to understand the key biological features of the complex, in particular, the host range. Some leaf curl disease hosts may not show symptoms until late in infection or at all, particularly ornamentals or some wild hosts. Further, symptoms can be confused with other well-known endemic viruses and an introduction can be easily overlooked until the virus complex has spread and become established over large areas, in ornamental, vegetable, and fiber crops (Bridson et al., 2014; Saleem et al., 2016; Sattar et al., 2013).

## **V. USDA Pathogen Permits and Regulations**

USDA-APHIS-PPQ permit and registration requirements for plant diseases and laboratories fall under two authorities, the Plant Protection Act (7 CFR Part 330) and the Agricultural Bioterrorism Protection Act of 2002 (7 CFR Part 331). Laboratories receiving suspect infected plant material or cultures are required to have PPQ permits. Laboratories possessing, using, or transferring Select Agents are required to be registered; however, diagnostic screening laboratories that identify select agents from a suspect sample are exempt from this requirement as long as an APHIS/CDC Form 4 is completed, and the culture(s) are destroyed within 7 calendar days (Floyd, 2007).

The Plant Protection Act permit requirements apply to all plant pests and infected plant material, including diagnostic samples, regardless of their quarantine status, that when shipped interstate require the receiving laboratory to have a permit. For further guidance on permitting of plant pest material, consult the PPQ permit website at <http://www.aphis.usda.gov/ppq/permits/> [accessed August 11, 2009] or contact PPQ Permit Services at 301-734-0841.

The Agricultural Bioterrorism Protection Act of 2002 (7 CFR Part 331) specifies the requirements for possession, use, and transfer of organisms listed as Select Agents such as R3b2. Once an unregistered diagnostic laboratory identifies or suspects a Select Agent, they must immediately notify the APHIS Select Agent Program (within 24 hours of confirmation), complete an APHIS/CDC Form 4 and either destroy or transfer the agent to a registered laboratory within 7 days. In compliance with this Act, if a diagnostic laboratory held back part of a screened sample or culture for voucher purposes and that sample forwarded to the USDA Beltsville Laboratory came back as positive for a Select Agent, the diagnostic laboratory is required to notify the APHIS Select Agent Program immediately. This must take place within 7 calendar days of results notification and a PPQ Officer must be provided with the opportunity to witness the destruction of the sample or culture within that time period. Clarification of this and other information related to adherence to the Select Agent regulations is available on the following APHIS website: [http://www.aphis.usda.gov/programs/ag\\_selectagent/index.shtml](http://www.aphis.usda.gov/programs/ag_selectagent/index.shtml) or contact the APHIS Select Agent Program 301-734-5960.

Researchers wishing to work with foreign plant pathogens in the U.S. should review the websites listed above and contact the PPQ permit unit to understand how best to comply with the permitting requirements.

## **VI. Response**

The response to all plant health emergencies is under USDA-APHIS-Plant Protection under The Plant Protection Act of 2000 (7 CFR Part 330) and the Agricultural Bioterrorism Protection Act of 2002 (7CFR Part 331).

The planned immediate response to a begomoviral-like suspect would be to determine the identity of the causal agent(s), including the helper virus and associated satellites (alpha or beta complex). Although there are very few RNA viruses known to infect cotton, their presence should not be ruled out until additional information, including the prospective insect vector(s) on site (or observed consistently with the symptoms) are explored. Subsequent to ruling out other potential causes that present similar symptoms in the plants, immediate action should be taken to detect and determine the identity of the suspect viral complex associated with symptomatic plants, followed by completion of Koch's Postulates, in so far as this is possible (predominant viral helper and beta satellites, at the least) given the need to construct full-length infectious clones (see *Detection and Identification*, section IV).

After a confirmed detection by the USDA-APHIS-PPQ recognized authority, APHIS, in cooperation with the Department of Agriculture is in control of the response. The response is an immediate assessment consisting of investigation and delimitation of the site of initial detection to prevent pathogen spread and to establish extent of the affected area. The team will also assess whether the introduction was intentional or accidental. As a plant pathogen on the select agent list, CLCuV is covered under the Agricultural Bioterrorism Protection Act of 2002; federal and local law enforcement may be involved to determine if a bioterrorism event has occurred.

APHIS imposes quarantines and regulatory requirements to control and prevent the interstate movement of quarantine-significant pathogens or regulated articles and works in conjunction with states to impose these actions parallel to state regulatory actions to restrict intrastate movement.

## **VII. Economic Impact and Compensation**

Cotton and textile industries are central to the economic well-being of developed and lesser-developed countries. Cotton production contributes heavily to food security in Africa, Asia, and Latin America. It is grown in 100 countries and occupies about 2% of the world's arable land. It is among the most significant crops after field grains and soybeans. Over the last three decades, the leading cotton-producing countries have been China, India, the United States, and Pakistan accounted for about 75% of the world's production in 2010. Cotton production activities involve over 250 million individuals, and millions more in related industries, worldwide. Cotton is widely traded and over 150 countries are involved in import and/or export activities related to cotton. The industry produced 22 million tons of cotton worth approximately 37 billion U.S. dollars in 2010-2011. On average, the cost to produce cotton is about sixty cents (U.S.) per pound minus the cost of land rent and seed after ginning, excluded from the cost of (U.S. \$1.22/kg lint) (Cost of Production of Raw Cotton: Technical Information Section, International Cotton Advisory Committee, 2010; personal communication, R. Chaudhry, ICAC).

Yields of cotton have risen steadily over time from 230 kilograms per hectare (kg/ha) in the 1950's to 600 kg/ha in 1991-92. Since then yields have stagnated due to insect and disease problems, which have increased with increased intensity of production and the introduction of varieties having high yields and superior quality, while lacking pest and disease resistance. Varietal improvement through breeding programs and biotechnology made possible yields of 795 kg/ha in 2007-2008. The average rate of increase between 1950 and the present has been 9kg/ha per year.

The widespread production of cotton in Asia and Africa since the initial outbreak and spread of, and the recent trade in ornamentals and vegetable seedlings between CLCuMV and CLCuKoV-Bur infected and uninfected areas is cause for concern. The introduction and establishment of extant, as well as potential emergent, variants of the 'core' cotton leaf curl virus complex endemic to Asia and Africa, outside their zone(s) of endemism could result in serious outbreaks. Because haplotypes of the whitefly vector are present in all cotton growing regions of the world, a single introduction followed by spread could rapidly overtake production areas where the virus is not endemic. Because all local varieties would likely be susceptible, extremely rapid spread, not only to cotton crops but also to certain susceptible vegetable crops and to ornamentals grown in landscapes in the rural- urban interface would be expected. The rapid spread into the U.S. cotton and vegetable crops alone would result in huge economic losses similar to those experienced with the Asian complexes in Pakistan and India. This is because our ornamental, vegetable, and cotton production areas in the United States and elsewhere in Latin America are tightly interconnected geographically. As a result, an introduction into one commodity can readily affect another particularly when it concerns the highly polyphagous whitefly vector and begomovirus complexes they transmit, both of which have broad and overlapping host ranges. Further, many ornamental hosts may harbor both the virus and the vector making the situation even more precarious because the whitefly vector can transmit the virus for life, once acquired, and so do not necessarily require transport on a virus-infected plant to pose a threat, when coming into contact with a virus-susceptible host. Not ornamentals or vegetable seedlings that are infected exhibit easily recognizable symptoms, particularly if they are shipped shortly after inoculation, and before full-blown symptom become apparent. A number of ornamental host species apparently are somewhat tolerant to the virus and so can serve as symptomless carriers. Finally, the host range of the collective leaf curl virus complexes are poorly studied, and the most recent research underway in Pakistan and India is revealing that the host range of these viruses span far more genera and families of plant than previously known (Kirthi et al., 2004; Nawaz-ul-Rehman et al., 2012; Rajagopalan et al., 2012; Paul et al., 2008a, 2008b; Roy et al., 2009).

No information was found regarding compensation.

## **VIII. Mitigation and Disease Management**

### *Whitefly control*

The recommendations for managing population size and density apply to thresholds established for management of the whitefly *B. tabaci* as a pest, not as a vector. The population threshold size per plant for *B. tabaci* as a vector is one, because a single viruliferous whitefly is capable of transmitting the virus, despite the overall reduction in whitefly population sizes in general (due to overuse of pesticides that leads to resistance). At different times of the year the frequency of viruliferous whiteflies in a population is expected to vary, and to increase as the season progresses, owing to secondary virus infection rates in cotton and alternate hosts. Thus, whitefly control alone, particularly in cotton- vegetable cropping systems, or when planting dates overlap, not allowing a

sufficient host free period, will not result in quarantine significant control of the vector populations. This is particularly true during early growth stages of the crop when the primary virus inoculum levels are high. This latter situation occurs frequently in the crop in Pakistan owing to the prevalence of diverse viruses having broad host range.

Cultural control of whiteflies is possible only if the dates of planting and harvesting of all whitefly-susceptible crops can be synchronized over a broad area, and crop-free periods are established and adhered to. Because most of the *B. tabaci* variants (biotypes or sibling species) associated with agricultural systems have a broad host range, and generally disperse moderate to long distances, cultural control of the whitefly has not proven very effective.

#### Insecticide control of whitefly to reduce virus spread

Insecticide programs in place vary depending on the cotton growing area of the United States. Specific references can be sought out for those areas through the Cooperative Extension Service and the Western Region Integrated Pest Management Center (<http://www.wrpmc.ucdavis.edu>). Selective insecticides, i.e. whitefly-specific versus broad spectrum, have the greatest success because they do not kill natural enemies, need fewer treatments per season, and tend to develop resistance more slowly. It is important not to mix broad spectrum and selective insecticides unless a mixture is required to manage a complex of insect problems in the crop. The choice of materials depends on the risks of economic loss, the potential for unmarketable lint due to honeydew contamination, and the risk of developing resistance to valuable non-chemical and chemical tactics that promote survival of predators and parasitoids, as well as the other natural fauna.

Insecticide resistance management is accomplished by limiting the number of treatments, using a diverse class of compounds and modes of action, and partitioning and sharing chemistries across crops. Refer to the three-stage management plan for controlling *B. tabaci* at pest thresholds, as outlined in Ellsworth et al., (2006).

#### Protective measures

Reflective mulches and floating row covers deter and protect plants from whitefly infestations, respectively. This is relevant to cotton-vegetable mixed cropping systems because only certain kinds of vegetables in certain production settings are economically feasible. Row covers are effective only if plants are protected during early- and mid-growth stages because once plants become infected with begomoviruses, growth is arrested and fruit is harvestable primarily from the portion of the plant in production at the time of removal. Row covers are expensive and not practical for cotton production and large-scale vegetable production. Greenhouses and screen houses (e.g., for ornamentals or controlled environment vegetable production such as tomatoes and peppers) can be protected by outfitting the structures with fine mesh screens to protect against whitefly infestation and therefore reduced inoculation that results in transmission of virus to plants by dispersing, viruliferous whiteflies. This is not practical for field crops.

#### Resistance to Cotton leaf curl complex and other begomoviruses of cotton

Disease resistance is the only effective way of managing leaf curl disease, particularly when infection occurs early and routinely in the production season. The variability in the natural incidence of disease depends upon the genetic makeup of the cultivar, concentration of inoculum of the disease and cultural management at different sites. Further, the pressure of whitefly with

concurrent presence of inoculum in the area influences disease incidence (Baluch, 2007; Tahir and Mahmood, 2005).

Conventional selection and breeding approaches that involved transferring resistance genes from wild species to Upland cotton eventually yielded varieties with excellent resistance to CLCuMV even though early screening and selection trials revealed a wide range in degrees of resistance across the offspring (Ahuja et al. 2007; Baluch, 2007; Naveed and Anjum, 2007; Tahir and Mahmood, 2005; Tahir et al., 2005). The widespread cultivation of the resistant varieties throughout Pakistan (except in Sindh) subsequently resulted in highly effective management of CLCuD. A genetic study of the offspring of crosses and selections from the parental donors of the resistance genes, LRA5166, CP-15/2, and Cedix, indicated that three genes conferred resistance. Two of the genes that contributed to virus resistance and a further gene imparted suppression of the symptom development (Tahir and Mahmood, 2005).

Currently, sources of genetic resistance are lacking to manage the recently emergent, recombinant CLCuKV-Bur that predominates in Pakistan and northwest India. Breeding efforts are again underway in the region to screen a wide array of germplasm to identify sources of resistance genes. It has been reported that the genetic stock maintained by different research institutions in Pakistan has a narrow genetic base, and that all are highly susceptible to (Mahmood, 1999; Mahmood et al., 2002; Tahir and Mahmood, 2005). Therefore, the introduction of exotic materials could possibly aid in widening the genetic base for inclusion of resistance in Upland cottons. The variability in reaction of the different cotton stock is dependent upon its genetic makeup and environmental conditions, including the extent of whitefly vector and therefore virus pressure.

Diploid cotton (*G. arboreum* and *G. herbaceum*) grown across Asia and Africa prior to the introduction of tetraploid cottons (*G. hirsutum* and *G. barbadense*) is immune to CLCuD (Mahmood, 1999). A recent study on cotton species grown in the 'living herbarium' maintained at CCRI Multan has identified other sources of resistance in wild species of cotton (Azhar et al., 2010). The major obstacles are ploidy barriers that require several steps to introduce characters from diploid to tetraploid cotton. This is problematic due to the lack of understanding of resistance mechanisms in diploid Asiatic species, and the absence of DNA markers linked to disease resistance. Other strategies have involved the incorporation of traits from *G. hirsutum* into *G. arboreum* ('hirsutization' of *G. arboreum*), and cloning genes from *G. arboreum* for transgenic introgression. Even so, additional efforts are required to determine the durability of the varieties produced using either of these strategies.

Several promising lines have been identified with tolerance or resistance to CLCrV in the United States (Wilson and Brown, 1991). Resistance has not been incorporated into varieties grown there because infection occurs mid to late season in most years. In years when whitefly population levels increase earlier than usual, inoculation of cotton by viruliferous whiteflies occurs when cotton is in the early growth stages and susceptible to significant damage (Brown et al., 1997; Butler et al., 1986; van Schaik et al., 1962).

#### Transgenic resistance to begomovirus infection

A number of research efforts have been initiated and/or are underway to explore the use of virus-derived resistance (Asad et al., 2003; Beachy, 1997) to manage the leaf curl complex in cotton and/or in alternate hosts such as tobacco or tomato (Brown et al., unpublished; in progress). Sense, anti-sense, and more recently small RNA-interference approaches have been investigated. Various

viral-encoded genes as well as the beta-satellite C1 and C2 ORFs have been considered as targets, as well as combinations of multiple targets yielded some promising results when tested in easy to transform species such as tobacco, tomato, and ultimately, cotton (Asad et al., 2003; Hashmi et al., 2011; Ilyas et al., 2011; Mubin et al., 2007; Zafar and Brown, 2011; Zafar et al., 2003).

The extensive genetic diversity in the extant viral species in Pakistan and India and the ability of begomoviruses and beta satellites to occur in mixed infections and undergo recombination warrants careful scrutiny to select regions of high homology across the entire complex to avoid selection of a highly virulent recombinant. The use of small RNAi strategies (Hamilton and Baulcombe, 1999), that although highly sequence dependent, require only short targets to be effective. Another important constraint has been the difficulty surrounding the ability to reproduce authentic leaf curl symptoms in cotton when inoculated with the cloned viral-betasatellite components of CLCuMV helper virus-betasatellite complexes (Briddon et al., 2000), although in surrogate tobacco plants, the clones are shown to be infectious, producing leaf curl symptoms following inoculation of the plants (Briddon et al., 2001). The subsequent recovery of the viral helper genome and betasatellite DNAs from symptomatic plants has demonstrated that at least in tobacco the clones have activity that are expected to be transferable to cotton likewise inoculated with them.

## **IX. Current Infrastructure, Needs and Experts**

Little infrastructure is in place in the United States other than in a few laboratories specializing in begomovirus diseases of cotton. There are several U.S. virology labs specializing in geminiviruses that could provide assistance with emergency detection. There are no certified protocols or action plans in place for doing so at this time. Several molecular and genomic pathology diagnostic tools are under development and are expected to become available, once validated.

### **Scientific experts:**

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#### **Whitefly management and insecticide resistance in cotton/field and vegetable crops:**

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## **X. Research, Education and Extension: relating to whitefly management**

<http://cals.arizona.edu/crops/cotton/cotton.html>

<http://www.ipm.ucdavis.edu/PMG/r114300311.html>

<http://ag.arizona.edu/crop/cotton/insects/wf/ipm6.html>

[http://www.icac.org/projects/commonfund/ipmc/proj\\_02\\_final.pdf](http://www.icac.org/projects/commonfund/ipmc/proj_02_final.pdf)  
<http://cals.arizona.edu/pubs/insects/az1402.pdf>  
[http://www.fsca-dpi.org/Homoptera\\_Hemiptera/Whitefly/whitefly\\_catalog.htm](http://www.fsca-dpi.org/Homoptera_Hemiptera/Whitefly/whitefly_catalog.htm)

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