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HISTORICAL WORK TO COMBAT WITH COTTON LEAF CURL DISEASE (CLCUD) IN PAKISTAN

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ABSTRACT

Cotton leaf curl disease (CLCuD) was first reported in Nigeria in 1912 which then appeared in other cotton growing countries of the world like Sudan, Tanzania and Pakistan in pandemic form and is endemic to India and China. The disease is caused by a monopartite begomovirus (family *Geminiviridae*) and transmitted by whitefly (*Bemisia tabaci* Gennadius) as vector. Pakistan has faced two epidemic outbreaks of the disease during past three decades.The higher mutation and recombination rate in viruses leads to multiple strains infection, which makes its control difficult. Availability of alternate host plants, the farming practices, and favorable environmental conditions have further embellished the situation. Successful efforts were made to develop resistant varieties by conventional breeding techniques during late 1990s. However, during 2001-2002, outbreak of Cotton Leaf Curl Burewala (CLCuBuV) mutant strain broke down the resistance and the varieties, which were released as resistant fell prey to the new virus. At the moment, no single immune or completely resistant variety is available however, a reasonable number of varieties are found with varying degree of tolerance. The combination of traditional and molecular breeding techniques may lead to develop genetically resistant genotypes. Development of Transgenic cotton plants resistant to CLCuV by employing pathogen and non-pathogen derived resistance is under progress. Furthermore, screening of resistant plants, utilization of molecular markers for efficient insertion of targeted genes, and CRISPR/Cas based genome editing techniques would be helpful for the development of resistant genotype with optimum yield and improved fiber quality.

KEYWORDS: *Gossipium hirsutum;* CLCuV; strategies; geminiviruses; causal agent; small vein thickening; Pakistan

INTRODUCTION

Cotton is one of the leading natural fiber crop, which is under cultivation since ancient human civilization. Evidences of cotton cultivation about 7000 years ago have been documented from different areas in Mexico. Desi cotton (*G. arboreum*) cultivation was traced in Mohenjo-daro (Sindh, Pakistan) 6000 BC (Moulherat *et al.*, 2002). Gradually, cotton was not only introduced worldwide but also substituted the diploid species with tetraploid species due to its higher yield and fiber quality traits. It is remarkable to note that American cotton introduction occurred ~200 years earlier while its commercial cultivation started in 1930s, parallel to the textile industry revolution (Rahmat *et al.*, 2014). Currently, cotton is cultivated in more than 80 countries on 32.6 million hectares with a produce of 24.1 million tons per year. The major cotton producing counties are China, India, USA, Brazil and Pakistan.

In various parts of the world, cotton yield is stagnant or lagging behind the world average due to climate change pattern, insects pest pressure and disease emergence. Among these, CLCuD has been observed as major menace for Cotton crop (Rahman *et al.*, 2017). In Pakistan, CLCuD is a major yield fluctuating factor as shown in the Fig. 1.

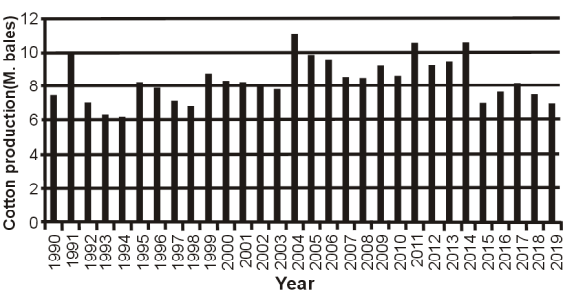

Fig. 1. Year wise cotton production in Pakistan from 1990-2019 (Source: United States Department of agriculture)

The first evidence of CLCuD was reported in 1912 from Nigeria on *G. barbadense* (Kirkpatrick, 1931). In

1924, it was recorded in Nigeria and Oyo with typical symptoms: curling of leaves, change in color and texture of leaf. In 1924, it was also observed in Sudan and Bailey and in 1934 again reported in Sudan. It has been one of the major issues in these countries and may destroy the cotton crop. In Pakistan, 'Stenosis' mentioned by Ali (1992) exhibiting crumpling of leaves was reported, but was of physiological nature. According to Idris (1990), cotton leaf curl virus (CLCV) was first recorded in Gezira during1923 and in Delta, Shambat, Takar in 1929. It was observed that virus transmission is carried out via *B. tabaci* and grafting. SVT (small vein thickening) was more prevailing in Gezira while MVT (main vein thickening) in Shambat.

After about four decades, CLCuD was first observed near Multan in the year 1967 on a few individual plants and has been noted consistently since then (Hussain and Ali, 1975). In the early days, the scientists did not take serious notice due to its informal happening and less economic importance. The disease became noticeable with its appearance in heaviest patch on a few genotypes e.g. B-557 and 149-F. CLCuV attack appeared in the late season on the upper portion of the plants. The disease occurred in the same manner until1986. Hussain and Mahmood (1988) reported that in 1987, the disease covered 80% area of certain fields. In 1988, it destroyed 150 acres cotton cultivated in Multan. Later on, its prevalence intensified every year. In 1989 and 1990, it damaged 500 and 2000 acres, respectively. In 1991, the disease was recorded in epidemic form and > 35000 acres in district Vehari, Khanewal and Multan were affected. In 1992, the disease covered more than 120000 acres leading to monetary losses. In 1993, the CLCuV attack extended to the whole cotton zone of the Punjab with varied intensity and affected the cotton crop on an area of 2.2 million acres. During 1996-97, the disease was also recorded from D.G. Khan and Sindh.

During 1991-1992, cotton production was the ever-highest (12.8 million bales) in Pakistan (GoP, 2001). In1992-1993, CLCuV attack was observed in epidemic form, which reduced the production to 9.05 million bales and decreased further to 8.04 million bales during 1993-94. During 1990s, a loss of 7.1 million bales was recorded. The estimated loss occurred to Pakistan economy during 1992-1997 was approximately 5 billion US$ (Idris, 1990). Cotton yield in million bales from 1990 to 2019 is shown in Fig. 2. The disease then also bellowed-out across the border towards Indian cotton grown zone at the boundary of Pakistani Punjab and after that diffused toward northern India (Reddy *et al.*, 2005). According to new classification, nine different variants of Begomovirus are responsible for the CLCuD complex: cotton leaf curl Bangalore (CLCuBaV), cotton leaf curl Gezira virus (CLCuGeV), cotton leaf curl Alabad virus (CLCuAlV), cotton leaf curl Kokharan virus (CLCuKoV), cotton leaf curl Multan virus (CLCuMuV), Tomato leaf curl Karnataka virus, cotton leaf curl Rajasthan virus (CLCuRV), Papaya leaf curl virus (PaLCuV) and cotton Leaf curl New Dehli virus ((Mansoor *et al.,* 1999; Muhire *et al.*, 2014). Cai *et al.,* 2010, reported CLCuMuV in China and Philippines. The virus complex is still under variation, which may evolve into new complex strains. The Burewala strain, also called Cotton leaf curl Kokhran virus-Burewala strain (CLCuKoV-Bur), was the result of recombination between CLCuKoV and CLCuMuV and resulted into second epidemic attack in Pakistan. The historical landmark with reference to cotton leaf curl disease attack is presented in the Fig. 2.

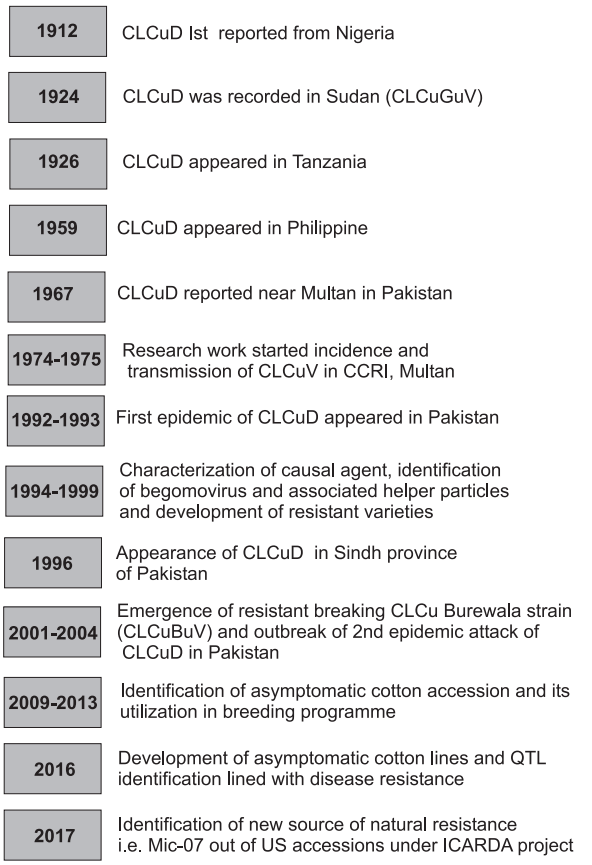

Fig. 2. Landmarks in the history of cotton leaf curl virus attack

Cotton cultivation in China, Pakistan and India provides more than 60% of World's total yield. Sustainable cotton production is under a constant threat in these countries. Presently no resistant variety is available against CLCuD. However, efforts are ongoing for the

development of CLCuD resistant through conventional breeding as well as transgenic cotton having resistance against CLCuD (Vyas *et al.*, 2017). The objective of this review is to explore the historical attack of CLCuV, particularly in Pakistan and the research work conducted for the management of this notorious disease.

Symptoms of CLCuD attack

Cotton leaf curl virus infected plant exhibit particular features e.g. MVT (main vein thickening), and SMT (small vein thickening), upward or downward curling of leaves (Fig 3). Diseased leaves look dark green, thick and brittle than healthy ones. In server disease condition, a small outgrowth emerges on the abaxial side of the leaves known as enations and results into restricted plant growth due to less intermodal distance and leads to yield loss of 15-70% (Brown, 2002; Farooq *et al.*, 2014). Kirkpatrick (1931) first time named the disease as leaf curl and observed that the disease symptoms develop according to the cultivar. However, in all cases peculiar curling of the leaf margins and enation may be produced. Necrosis may also be observed. The possible reason of curling is increased veinal tissues on the lower side of the leaf (Moustafa, 1961). Thickening first develops adjacent to leaf margins, later spreads towards main vein and convert it to thick dark green (Briddon *et al.*, 2000). The typical symptoms of CLCuV attack develop within 14-21 days of inoculation via whitefly followed by leaf margins curling in downward or upward direction, darkened and swelled veins and leaf enation.

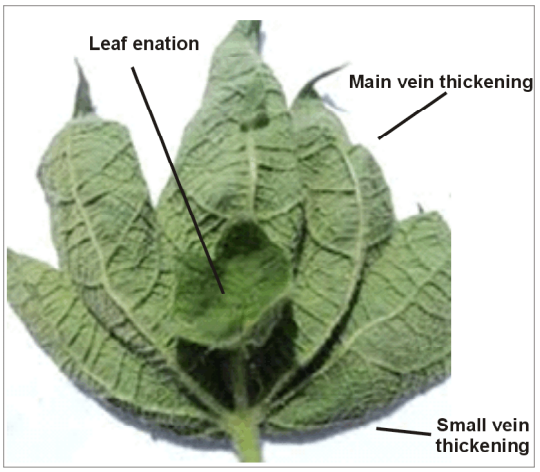

Fig. 3. Symtoms of cotton leaf curl virus attack

Leaf enation is also seen on leaves with mild vein thickening (Muhammad *et al.*, 2008). Vein thickening is of two types; small vein and large vein thickening and it is more pronounced on underside of the leaf. These irregular thickening join to form a reticulation of small veins (Briddon *et al.*, 2000; Yassin and Nur, 1970).

Losses caused by CLCuD

Cotton leaf curl disease (CLCuD), caused by gemini virus and transmitted by whitefly (*Bemisia tabaci* Gennadius) is a serious problem in Pakistan and Northern India, affecting the productivity to a great extent. Depending upon the severity of infection in susceptible varieties, the diseasecan cause up to 90.0% yield losses. Besides this, it also causes deterioration in fiber quality.

Early stage of cotton is more sensitive to virus attack as seedling infection leads to little omo lint production. It was recorded that the degree of damage relied on plant age at the time of infection. If young, the result is reduced growth and ultimately lessharvest (Hussain *et al.,* 2012). Ahmad *et al.* (2002) reported 70.3% yield reduction due to CLCuV. Singh *et al.* (2013) reported 52.7% and 54.2% reduction in the number of bolls and boll weight, respectively. The disease also affected the quality of cotton fibre, reducedlength by 9.9%, strength by 7.5% and micronaire valueby 3.8%. According to Idris (1990), the disease reducedyield by 20% in 1930-31 in Gezira although the quality of fiber was not affected.

In Pakistan, CLCuV causes about 30% yield loss every year (Ashraf *et al.,* 2013). Yield loss due to CLCuD relies on time of infection, cultivar, and type of infection (single or mixed), whitefly population and environmental conditions. The pronounced damage of CLCuD is at initial growth stages; however, at later stages minor infections have been observed (Akhtar *et al.*, 2003). CLCuD damage differs on different plant parts and lead to yield reduction. Khan and Shah (2001)demonstrated that in a spatial and temporal experiment diverse cotton cultivars responded differently with reference to CLCuD severity and yield loss. Cultivars SLS-1, BH-95 and FH-682 were found tolerant with less disease attack followed by NIAB-Karishma, FH- 672, S-12, MNH-329, NIAB-78 and B-622 were found more sensitive. The disease also adversely affected the yieldand fiber quality. Maximum reduction in seed cotton and fiber length was 83.7% and 7.1%, respectively in CIM-70. In S-12, disease imparted pronounced effectson GOT percentage (18.2%) and fineness (23.8%) (Ahmad *et al.*, 2002). Akhtar *et al.* (2009) stated that CLCuD imparts significant negative effect on GOT%, uniformity index, fiber length, fiber bundle strength, short fiber index, maturity ratio and yellowness. It was also reported that CLCuD effects major fiber constituents e.g. cellulose, protein, pectin and wax.

Cotton leaf curl disease (CLCuD) management inother countries

For long-term management, development of resistant varieties is the economical solution. Until early sixties, CLCuD was one of the major yield limiting factors of long fiber cotton (*G. barbadense*) in the Sudan. The consistent efforts of breeders and pathologists led to the evolution of practically immune varieties, viz. Barkat (B-16) and VS (B-65) varieties of Lambert and Sakel cotton. Cleaning up of primary inoculum reduced disease incidence of small vein thickening (SVT). The disease spread along the direction of the wind and was controlled by the population of the whitefly. Plant stage, plenty of the inoculum sources and environmental conditions influenced the incidence of disease. The period of highest infection rate correlates with plant growth activity and population density.

In Sudan, resistant varieties had solved the problem and since the release of barkat (BGL), leaf curl is no longer a constraint in cotton growing in Sudan Idris (1990). Typical SVT symptoms were observed in extra-long staple lines of EB series. It was possible that different strains of CLCuV existed in Sudan. American tested cultivars showed great resistance to SVT type, while main vein thickening (MVT) was found to infect okra and cotton. It was suggested that SVT and MVT symptoms were probably caused by different viruses.

Strategies adopted in Pakistan to combat the CLCuD threat

Viral diseases caused severe yield loss in Pakistan. Among these, begomoviruses are a major risk for economically important crops including cash crop (cotton), vegetables (chilies, cucurbits, tomato, and radish),leguminous plants (mungbean) and ornamental plants (honeysuckle) (Mansoor *et al.,* 1999).

Review of work and identification of causal agent

In Pakistan, the epidemic of CLCuV disease is one of the best precedence of the drastic shift in significance of a previously minor disease. The unprecedented drop in yield of this vital crop necessitated immediate steps to recover its productivity. Review of literature was undertaken focusing on the African experience to gain information on regarding nature of the disease, alternate hosts, transmitting agents, control measures etc. A delegation of Pakistaniscientists visited Sudan to get firsthand information for the control of the disease.

According to Farquharson (1912), the causal agent of cotton leaf curl was *Gossypium* virus-1. Massey and Andrews (1932) discovered that infected plants carry comparatively higher N_2 content in their tissues. Leaf curl mosaic virus complex in the African cotton belt was associated with at least four diseases; leaf curling, vein thickening and mosaic. Cauquil and Follin (1983) have discussed very comprehensively about the symptoms, causes, mode of transmission, damage caused and control measures for African leaf curl, American leaf crumple, mosaic, blue disease and other leaf roll diseases. Nelson (1991) stated that *Gemini* and *Carla* viruses caused CLCuD in Pakistan.

Research work on the incidence and transmission of CLCuV in Pakistan was first undertaken at the Central Cotton Research Institute (CCRI), Multan during 1974-75 (Ali, 1992). The symptoms of the disease were observed on a number of plants of different cotton varieties *viz.,* 149-F, Deltapine, Dunn, Acala and Carolina Queen in Multan area (Mahmood, 1999). Symptoms similar to CLCuD were also noted on *Althea rosa, Abutilon sp., Hibiscus rosa-sinensis, Hibiscus esculentus* and *Ponsitia spp.* Successful transmission of the disease from cotton to cotton was achieved through grafting. Insect transmission using whitefly from cotton to cotton and okra to cotton was quite successful. Efforts to transmit the disease through seed, soil or mechanical contact gave negative results (Ali, 1992).

Mohsin *et al.* (1992) isolated virus from CLCuV affected leaves, after examination under the electron microscope, they concluded that the virus belonged to the *Gemini* group, which has whitefly as the vector. Mirza (1992) attempted serological identification of the virus by employing monoclonal antibodies prepared from *Indian cassava mosaic virus* (ICMV) through triple antibody sandwich enzyme linked immunosorbant assay (TAS-ELISA). The findings of the serological tests confirmed the existence of a geminivirus in infected cotton. Hashmi (1993) confirmed that CLCuV was a Gemini virus and proved whitefly to be the vector of CLCuV. They also claimed successful purification and identification of CLCuV as *Gemini virus* through electron microscopy.

Mansoor *et al.* (1993) proved the relation of a Gemini virus with the CLCuD by Polymerase Chain Reaction (PCR) amplification and sequence analysis. This was the first report published on virus characterization. However, full-length infectious clones of CLCuV could not incite the typical symptoms of CLCuD in cotton. Thus, other factors associated with CLCuD were examined.

Khalid (1994) reported that CLCuV particles were observed by immunosorbant electron microscopy and also detected the virus by PCR in plant tissue and individual viruliferous whitefly (*B. tabaci* Genn). While studying the molecular aspects of *cotton leaf crumple virus* (from North America) and cotton leaf curl virus (from Pakistan), Nadeem *et al.* (1997) found that

these viruses were only distantly related. Harrison *et al.* (1997) described techniques for virus detection by ELISA with monoclonal antibodies and by PCR. They also used these methods for differentiation of CLCuV- PK from five other geminiviruses found in different crops and weeds in Pakistan. Pakistani crops are attacked by a range of distinguished but comparatively closely associated whitefly transmitted geminiviruses, certain of which resemble those found in India. Zhou *et al.* (1998) compiled the sequence of DNA-A of 9 Pakistani geminiviruses taken from CLCuV infected plants. Isolates from cotton showed four types sequences; isolates fromleaf curl infected okra showed sequence similarity withthat of cotton isolate sequence. Isolates from yellow vein mosaic infected okra were of two types (OYVMV types 301 and 201) both of these were different from each other but closely associated with the cotton leaf curl virus. It was concluded that CLCuD epidemic in Pakistan was associated with various distinct variants with recombination events comprising *okra yellow vein mosaic virus* (OYVMV) and other Gemini viruses., possibly have participated in their evolution.

Contrary to monopartite begomoviruses, contagious clones of these viruses caused infections but symptoms severity was very little when inoculated into the original hosts, proposing presence of supplementary new component in the viruses. Continuous studies resulted in the discovery of minor component named as DNA 1 accompanying with both (AYVV and CLCuV) of these diseases (Mansoor *et al.*, 1999).

DNA 1 has the capability of self-replication in plant cells but needs helper Begomovirus for its spread in plants and for transfer in insects. However, it was observed that DNA 1 did not participate in disease symptoms induction. Later on, an extra small (*c.* 1350 nucleotides) ssDNA molecule named as DNA β was isolated from CLCuD affected cotton plants. DNA β needs CLCuV for multiplication and encapsidation, and when co-transmitted with CLCuV to cotton plants, typical symptoms of CLCuD were developed. Thus, the begomovirus (CLCuV) DNA β complex shows the infectious unit responsible of CLCuD (Briddon *et al.*, 2001). Ahmed *et al.* (2011) explored genetic diversity among prevailing species of whitefly in Sindh and Punjab provinces and reported three putative species viz: Middle East-Asia Minor 1, Asia 1 and Asia II 1. Asia 1 was reported from both Punjab and Sindh while Asia II 1 and Middle East-Asia Minor 1were found in Punjab and Sindh, respectively. Asia II 1 is responsible for higher occurrence of CLCuD. MiddleEast-Asia Minor 1 is associated with lower incidenceof CLCuD. This may be one of possible explanation ofless CLCuD attack in Sindh province.

Possible vectors/transmission of the disease

The results of six experiments conducted in cages demonstrated that the symptoms of CLCuD were observed within 11-24 days by using whitefly as a vector (Golding, 1930). The vector (whitefly) is shown in Fig**. 5**. He also reported that Jassid was not a vector of this disease in Southern Nigeria. Hopkins (1936) demonstrated successful transmission of the leaf curl disease found on wild species of *Sida* to cotton by budding and grafting. Yassin and El Nur in 1970 successfully transmitted the disease by a single whitefly. Watkins (1981) observed that CLCuV was transmitted by whitefly feeding which can complete its whole life cycle inside the vector within 6.5 hours. The Plant Pathology Division of CCRI, Multan, in 1974-75 reported that the virus could be transmitted from infected to healthy plants through grafting and whitefly. Further, it was also found that CLCuV was not transferred via mechanical source and not carried out by seeds or soil. In some experiments conducted at Faisalabad, whitefly was not able to transfer the disease from diseased to healthy plants. However, transmission through grafting proved to be successful (Mirza, 1992). The most efficient “improved bottle shoot grafting technique” described by Akhtar *et al*. (2002) is presented in Fig. 4.

Fig. 4. Improved bottle shoot grafting technique

The reported whitefly vectors of begomoviruses are sweet potato, cotton or tobacco whitefly, B biotype of whitefly and *B. argentifolli* (Kedar *at el.,* 2014). Whitefly has the ability to colonize various dicotyledonous plants including vegetables and fibre plant species.

It was reported that there were various *B. tabaci* populations that behaved differently to some extent in their capability to form dense population and cause damage through direct feeding, in the degree of their host ranges, and in the effectiveness with which they can transfer viruses (Barboza *et al.,* 2019).

Jiskani (2005) concluded that CLCuD was transferred by whitefly feeding in 6.5 hours. A single viruliferous female has the capability to transmit virus in many plants. The virus could not be transferred via soil, sap or seed. He further stated that it can live on 50 species and responsible of causing 23 crop diseases in the region. However, throughout the world it could infest 600 plant species (De-Moraes *et al.*, 2018). Globally, whitefly is an important pest in subtropical and tropical regions along with greenhouses. It could adopt easily in new geographical region and host plant species throughout the globe except Antarctica (Martin *et al.*, 2000; Kanakala and Ghanim, 2019).

Bemisia tabaci belongs to order *Hemiptera* and family *Aleyrodidae* with 506 host plant species in the world (Lee *et al.,* 2013). In Pakistan, during 1996-1999, it was reported on 160 host species including 113 genera and 42 families e.g. fruits, ornamental plants and weeds (Attique *et al.*, 2003). It completes 12 generations yearly and remains active during whole year. Among all host species, it has best survival on pumpkin. CLCuVD enhances the whitefly egg's viability percentage. Whitefly deposits comparatively less eggs on diseased plant than healthy one. Furthermore, the developmental period is also less on infected plant with short nymphal and pupal stages. However, both male and female whitefly have shorter life duration on virus-infected plant in comparison to healthy plant (Guo *et al.*, 2010).

Begomoviruses are transmitted by whitefly in a circulative, persistent manner and all begomoviruses are transferred by only one whitefly species; below the species in whitefly there is found vector specificity (Fig. 5). As Bedford *et al.* (1994) exhibited that though the biotype B of whitefly can transfer all tested fifteen begomoviruses, other biotypes may not transfer some begomoviruses and some viruses are transferred efficiently by certain biotypes. Thus, it was confirmed that some of begomoviruses have been evolved to be transferred by particular biotypes. This may describe why during the first CLCuD epidemic in Pakistan, Sindh province remained disease free (Panhwar *et al.*, 2001). In Central and southern Sindh, incidence of CLCuV attack has been observed since 1996. However, the range of CLCuD causing begomoviruses is more in Sindh than Punjab (Amrao *et al.*, 2010a) which may be because of earlier absence of this disease in the province and the cotton growers did not pay attention for cultivation of resistant genotypes.

Fig. 5. The vector of cotton leaf curl virus

However, the most probable reason for this difference in Sindh and Punjab is not clear yet. Ahmad *et al.* (2002) collected whitefly samples from Punjab and Sindh reported two different biotypes (categorized as species) prevailing in Pakistan. Both species were found in Punjab while in Sindh only one species was recorded. However, it is still unclear whether the biotype found in Sindh is unable to transfer the CLCuD or the other biotype is prevailing in the Sindh. Entomologists have categorized B. *tabaci* as a species complex and subdivided it into 24 indistinguishable species by using their sequences data (De-Barro *et al.*, 2011).

Optimum condition for whitefly population and CLCuD

Incidence of CLCuD is rather complex as it depends upon type of variety, type of begomovirus strain, availability of inoculum, prevailing weather condition and whitefly population etc. The severity of the disease depends on rainfall, temperature and wind. The optimum conditions are listed in Table 1. Maximum incidence was observed on 1.5 months old seedlings, later it decreased with the increase in the plant age. As cotton is an annual crop, alternate weeds and other host species actas virus reservoirs. Primary sites of infection are developed in cotton fields. Primary site acts as a source for secondary outbreak (Rajendran *et al.,* 2018). In cotton the rate of oviposition was found maximum (10 eggs/ day) from 25°C to 30°C and reduced at 20°C (Kutuk and Yigit, 2007). The mean development time is also reduced to 20 days between 25-30°C; however, at 20°C it takes 37 days (Khanzada *et al.*, 2016). For the development of whitefly eggs and nymphs, temperature from 25°C to

30°C is favorable (Darwish *et al.*, 2000). Maximum temperature positively correlates with the whitefly population. Whitefly respiration enhances with the increase in temperature from 25 to 46°C (Kumawat *et al.*, 2000).

Table 1. Optimum conditions to promote whitefly growth

Parameter	Optimum condition	Reference
Temperature	Maximum: :34-35.5°C	(Kutuk and Yigit, 2007)
	Minimum: 25.75-26.25 °C	
Relative humidity	64.14 to 66 %	(Saeed *et al.*, 2018)
Rainfall	1-2mm	

Identification of alternate hosts

Alternate host plants play a vital role in the dissemination of the virus to cotton crop. They provide inoculum to the vector for transmission the same to cotton. A number of plants have been recognized as alternate hosts of CLCuV. Hussain (1932) informed with reference to Mathur (1932) that the leaf curl virus was present on garden *Zinnia* in Northern India and it was identical to that recorded on cotton in Sudan. This disease was readily transmissible to cotton from *H. esculentus* and *H. cannabinus.* Tarr (1957) recorded it on Lobia (*Dolicus lablab*), *Phaseolus trilobus* and green gram (*P. mungo*) and further stated that *H. esculentus, H. sabdoriffa* and *H. cannabinus* serve as the primary inoculum of this disease to cotton.

Hussain and Mahmood (1988), based on their review of literature and visual observations, reported that in Pakistan the alternate hosts of CLCuV were more than 40, although the exact identification of this virus on these plant species was not done at that time. In collaboration with virology section of Ayub Agricultural Research Institute (AARI), Faisalabad. Malik *et al.* (1999) by using ELISA test confirmed that CLCuV attacks a wide variety of alternate hosts belonging to several families of dicots as well as monocots. Out of 68tested plant species, 61 gave positive results by using a very specific, sensitive and reliable test than ELISA. For the identification of alternate hosts based on Polymerase Chain Reaction (PCR) and DNA probe technology, proved that *H. esculentus* (okra), *H. rosasinensis* (China rose), *H. cannabinus* (sunkukra), *H. tiliaceus* (saklai), *Ageratum* and Sida are alternate hosts of CLCuV, while chillies, tomato, tobacco and zinnia are not alternate hosts of CLCuV. These plants showed leaf curl symptoms and were infected with a geminivirus but it proved to be different from CLCuV. There were no reports of CLCuD affecting chili pepper until 2003 but, Hussain *et al.* (2003) by southern- blotting confirmed the existence of CLCuD DNA β in chili pepper plants showing symptoms such as leaf curling and vein thickening.

Serological tests

Serological identification of the virus was attempted by Mirza (1992). As reported by him, leaf samples obtained from cotton fields from different areas were tested by using several monoclonal bodies in the first hatch and antisera of two monoclonal bodies in the second hatch. These results were reported to be similar to the leaf samples of CLCuV from Northern India. Later on another batch of 23 leaf samples was tested but the results illustrated no indication for the degree of varietal resistance to CLCuV. The above serological tests however showed that the causal viruswas CLCuV belonging to *Gemini* group.

Development of resistant varieties

On being trapped in the CLCuV complex, essentially the first step was to find out some of the existing cotton (*G. hirsutum* L.) cultivars, which could withstand the attack of the disease and save the cotton culture of Pakistan from total massacre. Trials on the under cultivation of cultivated genotypes was carried out to discover some resistant sources, but not a single genotype showed complete resistance. Instead different genotypes varied in their tolerance level. The disease incidence was more on S-12 and CIM-70. In Sindh province, the genotype “Rehmani” was also found susceptible (Ali, 1992). In addition to testing of the available varieties, efforts were also focused to evolve new resistant strains. Initially the genetic base of the hybrids was explored and discovered that Indian accessions CP- 15/2 and LRA-5166 were proved resistant against CLCuV. Attention was given to sort out the hybrids by using these two varieties as parents. From a cross of 492/87 × CP-15/2, 1098 and 1100 emerged as resistant source against CLCuV. Resultantly, crosses were made at CCRI, Multan which resulted in the development of resistant varieties i.e. CIM-1100 and CIM-448 and were released in 1996. During 1997 and 1998, approximately 20.1% and 28.4% area was covered with these two varieties, respectively. In 1998, two more new varieties namely CIM-443 and CIM-446 were released. Similarly, two virus resistant cultivars *viz*., FH-634 and FVH-53 were also developed at AARI, Faisalabad suited to Faisalabad conditions. With the passage of time some more resistant varieties like CIM-473, NIAB Karishma, FH-900, FH-901 FH-1000, NIAB-98 etc. were approved and released for general cultivation in the Punjab.

Breakdown of resistance

In Pakistan, molecular genetics and molecular virology was first time employed in 1990s during the first epidemic of CLCuD. Although, begomovirus association was already suspected due to existence of *B. tabaci* on diseased plants. Until 1992, experimental disease transfer via insects was not attained (Briddon and Markham, 2000). The involvement of begomovirus in causing CLCuD was first confirmed by PCR amplification (Mansoor *et al.*, 1993).

Zhou *et al.* (1998) first time did the complete sequencing of begomoviruses (CLCuAV, CLCuKoV and CLCuMuV). Later on, more begomovirus species were elucidated: PaLCuV, CLCuRaV and ToLCBaV (Mansoor *et al.*, 1999). In Asia, the etiology of CLCuD was depicted overwhelmingly with Koch's postulates for monopartite begomovirus with betasatellite, which is currently termed as CLCuMuβ (Briddon *et al.*, 2001). Moreover, all field collected samples showed association of an alphasatellite with the begomovirus (Briddon *et al.*, 2004). Hence, during 1990s, the epidemic attack of CLCuD in Pakistan was associated with numerous monopartite begomoviruses, with several plants carrying more than one species of begomovirus, single betastaellite species and various alphasatellites. During the cropping season 2001-02, all the newly developed resistant cotton genotypes became susceptible because of the emergence of a resistance breaking strain known as Cotton Leaf Curl Burewala (CLCuBuV) strain which changed the virus complex (Mansoor *et al.*, 1999). This lead to initiation of second epidemic attack, which covered all the cotton-growing areas in Pakistan except Sindh and into northwestern India. The betasatellite was characterized rapidly which was linked with new epidemic while begomovirus took a little longer. For the most part, the betasatellite was shown to be CLCuMuβ that was linked with first epidemic but was recombinant, with approximately 100 nucleotide sequences reported in tomato. This betasatellite was named as "Burewala strain" of CLCuMuB (CLCuMuβ^{Bur}), with the original betasatellite was termed as the Multan strain (CLCuMuβ^{Mul}). Amin *et al.* (2006) reported that the recombinant fragment might increase interaction with the helper begomovirus. However, the relation of helper begomovirus-encoded *Rep* with betasatellites remained unresolved. CLCuBuV species was a recombinant of CLCuKoV and CLCuMuV (Amrao *et al*., 2010b). However, CLCuBuV did not carry *C2* gene. Although, this gene was not necessary for infectivity but studies showed that mutant viruses were infectious (Baliji *et al.*, 2007). Cloned virus's sequence analysis has depicted that CLCuBuV is the causal organism of CLCuD and this finding is the affirmation of previous results that, unlike first epidemic of CLCuD when a group of begomoviruses were damaging the cotton, there is a single virus CLCuBuV attacking the cotton (Hina *et al.*, 2012). The emergence of Burewala strain of CLCuBV in Burewala District Vehari, during 2001-02 resulted in the breakdown of resistance and all the varieties which were initially developed as resistant to the disease became susceptible to the disease. The breakdown of resistance which started in Burewala area, rapidly amplified into the whole cotton belt; and from 2003 onward, there is not even a single genotype of *G. hirsutum* available which could be claimed to have complete resistance against the disease. However, the today`s cotton varieties developed by gigantic efforts of our local breeders only show different levels of tolerance.

The phenomenon of recombination lead to the evolution of CLCuBuV, CLCuMuV and CLCuShV. Cotton leaf curl Shahdadpur virus (CLCuShV) is a recombinant derived from begomovirus species responsible of epidemic attack in Punjab during 1990s (Amrao *et al.*, 2010a; Monga *et al.*, 2011). Resistance durability is reliant if no resistance breakdown has occurred or it works for 25 or more years (García-Arenal and McDonald, 2003). However, in CLCuD resistance, durability is less and has not been maintained for more than 3 or 4 years as the parents CP-15/2 and LRA-5166 employed in development of resistant cultivars in Pakistan carried a narrow genetic base along with the evolution of new virus strains (Rahman *et al*., 2005).

Management strategies of CLCuV

Balanced fertilizer application assists cotton in reducing the attack of CLCuD and also helps in recovering the damage. Nitrogen and potassium ratio should be kept adequate as N lowers the resistance against CLCuD while K improves it (Zafar and Athar, 2013). It was observed that the application of Potassium @ of 250 Kg/ha lowered the disease incidence up to 12-38% (Panhwar *et al*., 2001). Potassium interferes with disease by effecting the metabolic activity of plant and alter the host-parasite relationship (Kafkafi *et al.*, 2001). In Pakistan, to trickle-down the effect of CLCuD, it is suggested to control the population of whitefly on cotton plants from emergence until 70 to 90 days. For this purpose, the farmers are advised to treat the seed with some suitable insecticide, which protects the crop from whitefly up to 45 DAS (Singh *et al.*, 2002). Later on, different chemicals are sprayed alternatively to keep the whitefly population

under control. This practice works effectively if the population is kept control up to 70- 90 days after sowing. After it, the plant is able enough for disease escape. Bio-pesticides are also getting attention however; their efficacy is yet to be realized (Sarwar and Sattar, 2016). The availability of alternate hosts favors the increased recombination events between different viruses, which results into emergence of new strains. Therefore, ban on the sowing of alternate hosts during off-season in cotton cultivated areas may assists in breaking whitefly life cycle (Muhammad *et al.*, 2008).

Iqbal *et al.* (2007) concluded that the genotypes, which are more prone to CLCuD attack, could be tackled to withstand the damage via increased plant population and N application for getting optimum yield. Iqbal and Khan (2010) documented that planting time is effective in controlling CLCuD and suggested that early planting with more plant-to-plant distance and late planting with less distance is effective for its management and optimum yield. Disease intensity and incidence increased with late sowing. The long term and economical management of CLCuD is to develop resistant varieties. Currently, no cotton variety is available which showed complete absenceof CLCuD. However, almost all the currently available cotton varieties in the Punjab show varied degree of effective tolerance against this deadly disease.. Besides conventional approaches, advance biotechnological tools are also being used for the development of resistance. V2 (pre-coat protein) and CP (coat protein) proteins play role in encapsidation, transmission, virus movement in the plant and host defense suppression (Rojas *et al.*, 2001). RNAi technique was used successfully for the silencing of V2 gene of CLCuKoV-Bur in two cotton varieties VH-289 and MNH-786. The gene was transformed on 6th and 16th chromosome number of cotton. Under controlled conditions, the stable transgenic lines exhibited less titter of virus in the presence of whitefly It was concluded that V2 RNAi amplicon has the potential to control CLCuV (Yasmeen *et al.*, 2016). Under prevalent situation of failure in managing CLCuD, more precise controlling system is required. CRISPR/Cas9 system offers considerable advantage for controlling viruses and success has been achieved in controlling various geminiviruses. It could be a future tool for controlling CLCuD (Iqbal *et al.*, 2016; Gollardo, 1999; Lewin, 1927; Moustafa, 1961; Singh *et al.,* 1999).

G. arboretum has a robust defense mechanism againstall types of *Begomoviruses* linked with CLCuD (Khan *et al.*, 2016). However, the exact mechanism impartingresistance against CLCuD is still unrevealed. Mushtaq *et al.* (2018) identified and isolated a set of genes conferring resistance against CLCuD in *G. arboreum*. They made successful incorporation of CLCuD resistant genes from *G. arboreum to G. hirsutum* through backcross breeding. F_1 showed complete resistance against CLCuD. While in BC1, BC2 and BC3 the disease incidences were 1.7-2%, 1.8-4.0% and 4.2-7.0%, respectively. Comparatively disease attack was much lower on these backcrossed progenies than check variety (Nazeer *et al.*, 2014).

Future outlook

The globalization of agriculture has enhanced the dispersal of viral diseases. The disaster caused by the geminivirues in different countries is a typical example of it. Particularly in Pakistan, cotton-growing areas have conducive environment for the proliferation of CLCuV and its transmission agent (*B. tabaci*). Breeders have worked a lot on conventional breeding of cotton to control this havoc disease, still resistant material against newly emerged strains has not been developed. Now with the discovery of new biotechnological tools, RNAi technique has been used successfully for the silencing of V2 gene of CLCuKoV-Bur depicting its potential use in controlling the CLCuD. CRISPR/ Cas9 system has offered considerable advantage for controlling viruses and success has been gained in controlling various geminiviruses. Therefore, besides conventional breeding, employment of biotechnological tools could be the future strategy to curb the menace of deadly CLCuVD.

CONCLUSION

CLCuD caused immense damage to cotton crop in Pakistan. Despite the development of resistant varieties, evolution of the new virus complex breakdown the resistance. The diploid species of cotton, *G. arboretum* is a good source of resistance against cotton leaf curl disease. Under prevalent situation of failure in managing CLCuD, more precise controlling system is required. In addition to sowing the resistant material, early sowing faces less incidence of cotton than late sowing. Balanced use of fertilizer could also be effective for controlling the disease. As long as the CLCuV resistant varieties have not been developed, tolerant genotypes should be cultivated to minimize the losses caused by this disease. Furthermore, in late sowing, plant population and inputs should be increased to get economical yields.

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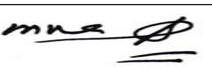
Sr. No.	Author's name	Contribution	Signature
1.	Ghulam Sarwar	Conceived the idea and prepared the initial draft of manuscript	
2.	Iram Sharif	Proof read the manuscript	
3.	Abia Younas	Helped in manuscript write-up	
4.	Amna Nazir	Collected the literature	
5.	Jehanzeb Farooq	Finalized contents of the manuscript