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Present status of cotton leaf curl virus disease (CLCUVD): a major threat to cotton production

[1] Irum Hasan, [1] Sumaira Rasul, [2]Tassawar Hussain Malik, [3]Muhammad Kamran Qureshi, [1]Kashif Aslam, [1]Ghulam Shabir, [4]Habib-ur-Rehman Athar and [1]Hamid Manzoor*

[1]Institute of Molecular Biology and Biotechnology, Bahauddin Zakariya University, Multan, Pakistan,
[2]Director Agricultural Research Pakistan Central Cotton Committee,
[3]Department of Plant Breeding and Genetics, Bahauddin Zakariya University, Multan, Pakistan,
[4]Institute of Pure and Applied Biology, Bahauddin Zakariya University, 60800 Multan, Pakistan.

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Cotton is ranked 1st fiber crop that is cultivated in more than 60 countries of the world with an approximate production of 120 million bales annually. From sowing till harvesting, cotton is exposed to more than 75 destructive diseases that are responsible for about 50% yield losses annually with a worth of $50 billion in the world. Among these diseases, *Cotton leaf curl virus disease (CLCuVD)* is one of the significant factors responsible for yield reduction worldwide. This disease is caused by cotton leaf curl virus which is mainly acquired and transmitted by the vector Whitefly (*Bemisia tabaci)*. This disease causes economic losses estimated millions of US$ per year throughout the world. It is difficult to control this disease due to occurrence of virulent viral strains or related species and higher recombination rate of cotton leaf curl virus complex. The problem is more complicated due to availability of alternate host plants like okra, tomato, tobacco etc., and mixed type farming practices which could be the source of evolution of new viral strains and vectors. Resistance developed by using host plant resistance using two gene based-resistances remained successful until the evolution of new resistance breaking strain named Cotton leaf Curl *Burewala virus*. To date, management *(CLCuVD)* is the only way to overcome the damaging effects. However, efforts are underway to develop new strategies in order to protect cotton from *CLCuVD.* In this regard, biological control and development of transgenic cotton varieties by using both non-pathogen and pathogen derived approaches are in progress. Modern molecular techniques like RNAi and CRISPR-Cas technology can be potentially used to develop resistance against *CLCuVD* and its viral causal agents. In this review, we have tried to summarize the existing knowledge regarding *CLCuVD* and the possible solutions to overcome this devastating disease.

Key word: Cotton; geminiviridae; begomovirus; whitefly; bipartite; biological control; cotton leaf curl virus disease.

INTRODUCTION

Cotton is an important crop cultivated throughout the world for fiber, feed, fuel and food (Chakravarthy *et al.*, 2014). Cotton seeds are also enriched in high quality protein and oil contents of approximately 23% and 21% respectively (Sunilkumar *et al.*, 2006). It is ranked 1st for fiber and 2nd among oil seed crops in the world (Gul *et al.*, 2014). Cotton belongs to the genus *Gossipier* and family *Malvaceae* (Khan *et al.*, 2016). This genus contains 52 species in which *Gossypium barbadense* and *Gossypium hirsutum* are allotetraploid while *Gossypium herbaceum* and *Gossypium arboreum* consist of diploid genomes (Bakhsh *et al.*, 2015). Among them, the most widely cultivated specie is *Gossypium hirsutum* cultivated in more than 80 countries (Shakeel *et al.*, 2011). Moreover, *Gossypium hirsutum* shares 90% thus, ranked 1st in the total worldwide production of cotton regarding yield and quality. However, *G. barbadense* shares 8% thus, ranked 2nd whereas *G. herbaceum* and *G. arboreum* share 2% of the world's cotton production (Khan *et al.*, 2015).

In the ever-changing environmental conditions, cotton is exposed to a number of (a) biotic factors that are responsible to decrease the cotton production. Cotton is attacked by various important diseases like *Fusarium* wilt, bacterial blight and *(CLCuVD)*. Among all the biotic stresses, *Cotton leaf curl virus disease (CLCuVD)* is considered as the most devastating. This disease causes an estimated loss of millions of U.S. dollars annually, throughout the world (Leke *et al.*, 2015). The history of *CLCuVD* dates back to 1912 in Nigeria where it was first time noticed on *Gossypium vitifolia* and *Gossypium peruvianum.* This disease is caused by *Cotton leaf curl virus (CLCuV)* which is mainly acquired and transmitted by the vector whitefly (*Bemisia tabaci*) (Nogia *et al.*, 2014). Once *(CLCuV)* is acquired by the Whitefly (*B. tabaci)*, it is retained by it throughout the life. *CLCuV* replicates in the nucleus of infected cells. Plants affected by *CLCuV* develop symptoms including downward or upward leaf curling, veins thickening, vein swelling, yellowing, enations (outgrowth) on the underside of the leaves and in severe cases stunted growth (Farooq *et al.*, 2014).

Begomoviruses consists of small circular, single-stranded DNA (ssDNA) genomes that are either monopartite or bipartite (Ha *et al.*, 2008). Bipartite *begomoviruses* consists of DNA-A and DNA-B components of each about 2.8kb (Rosario *et al.*, 2016). These are native to both the Old World (eastern hemisphere including Australia, Asia, Europe, and Africa) and New World (western hemisphere such as the Americas and Caribbean), and are less dominant as compare to monopartite *begomoviruses*. Both these components are essential for the severity of disease (Ha *et al.*, 2008). On the other hand, monopartite *begomoviruses* are native to the Old World and primarily consists of satellite like DNA complexes. These components are responsible for the severity of disease in these cotton growing countries (Briddon and Stanley, 2006; Zhou, 2013). In Pakistan, Resistant cotton

cultivars were developed by crossing the local susceptible cultivars with the resistant sources (CP15/2, LRA 5166 and Cedix). In 2001, new resistance breaking strain named *cotton leaf curl Burewala virus (CLCuBuV)* was identified in Vehari District of Punjab. This strain affected all the resistant cultivars of cotton. Later, this strain also identified from cotton field in India and replaced the old strains (*CLCuKoV* and *CLCuRV)* (Rajagopalan *et al.*, 2012). Thus, Pakistan, India and China together contributes more than 60% of the world cotton production are at potential risk to *CLCuD* (Vyas et al., 2017). This highlights the need for undertaking control measures to overcome the disease severity in all the cotton growing countries in the world. Therefore, the physiological, biochemical and molecular approaches are reviewed in the following sections.

History: The history of infection with *Geminiviruses* dates back to 752 AD (Saunders *et al.*, 2003). However, recently phylogenic studies demonstrated that most of the *Geminiviruses* are classified based on their geographical origins (Sattar *et al.*, 2013). In 1912, *Cotton leaf curl disease* was first time reported scientifically from Nigeria on *Gossypium vitifolia* and *Gossypium peruvianum* (Farquharson, 1912). Later on, it was also reported from Tanzania and Sudan in 1926 and 1934 (Bailey, 1934). Later, it was observed from Philippine in 1959 (Hussain and Ali, 1975). However, this disease has been constantly being reported during the past few years from several countries in Africa and South Asia, and more specifically in China, Pakistan and North-Western regions of India. However, In Pakistan, it was first time noticed in 1967 near Multan on few plants with mild effects (Hussain and Ali, 1975). This disease was not considered as a serious threat up to 1987 but later it appeared as epidemic form in 1992-93 and damaged 1.3 million bales of cotton over an area of 24.28 hectares. In 1994, this disease caused a decrease in cotton production (7.9 million bales). In 1992-97, it caused an estimated economic losses ~5 billion US$ to the nation (Briddon *et al.*, 2001). In 1997, disease was observed in Sindh Province of Pakistan which was previously free from *Cotton leaf curl disease* (Mansoor *et al.*, 1998). Later, this disease spread throughout the country and its severity increased that causes the colossal economic losses (Mansoor *et al.*, 1998). In India, it was first noticed in New Dehli in the experimental fields of Indian Agricultural Research Institute (1989) on a few isolated plants of *G. barbadense* (Kirthi *et al.*, 2004).

Components of cotton leaf curl virus: *Geminiviruses* consists of circular single-stranded DNA genomes of approximately 2.8kb and are encapsidated in a twinned icosahedral pattern. Among them, *begomoviruses* replicate via rolling circle replication (Haible *et al.*, 2006), in which ssDNA is transcribed into double stranded DNA (dsDNA) as an intermediate, and then this dsDNA is used as a template for the formation of mature ssDNA genomes (Farooq et al., 2011). *Begomoviruses* are composed of monopartite (DNA-A and satellite molecules) and bipartite (DNA-A and DNA-B) genomic organization (Sahu *et al.*, 2014). These components are discussed here in the following section

Single stranded DNA- A and DNA-B: Bipartite *begomoviruses* consists of DNA-A and DNA-B of approximately equal size 2.8 kb. These viruses are native to New World while few are also present in the Old World (Ali, 2010). Both these components are required for symptom development, and share a common region containing nanomeric sequence and the repeat sequence called "iterons" (Argüello-Astorga and Ruiz-Medrano, 2001). This common region 5'-TAATATTAC-3' is essential for the maintenance of DNA-B. The DNA-A encodes six proteins essential for the control of transmission and replication of virus (Saunders *et al.*, 2004). The DNA-A component of both monopartite and bipartite Begomoviruses encode two genes in the virion sense strand and four genes in the complementary sense strand (Figure 1a) Among them, coat protein *(CP)* is encoded by the virion sense gene and involved in vector mediated transmission, encapsidation and movement in host plants. The *(A)V2* protein is also encoded by the virion sense gene and might be a pathogenicity determinant, and can be involved in movement of virus in infected plants (Rojas *et al.*, 2018). The viral proteins encoded by the complementary sense genes are replication-associated protein *(Rep)* involved in initiating the rolling circle replication of viral DNA, replication enhancer (*REn)* which in combination with *Rep* provides a suitable cellular environment for replication of virus, *(A)C4* protein necessary for symptom determination and host range, and might be involved in viral movement and *C2/TrAP* which in case of bipartite *begomoviruses* involved in up-regulating late, host genes and virion sense genes (Rahman *et al.*, 2017).

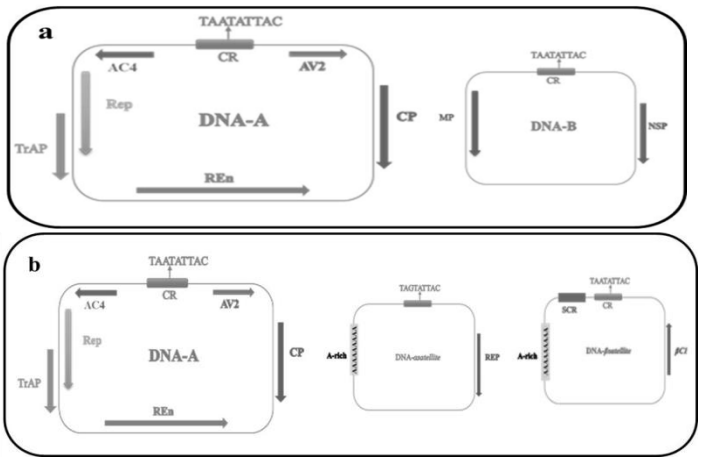

Figure 1: Components of *Begomoviruses*.

Components (a) present in NW consist of small, circular single stranded DNA-A and DNA-B, DNA-A encodes six genes and DNA-B encodes only two genes either on the sense and complementary sense strand while common region in stem loop structure is present in all the components of NW and OW *begomoviruses*. However, *AV2* gene is absent in bipartite *begomoviruses* that are present in Old World. (b) *Begomoviruses* native to OW are also composed of small, single stranded DNA-A and also satellite like molecules (DNA- αsatellite and DNA-β satellite), both these components encode only one gene either on the sense or complementary sense strand while adenine rich region is present in these components. Satellite common region is present in all the β-satellites which spans the ori.

The DNA-B component encodes two proteins; nuclear shuttle protein *(NSP or BV1)* in the virion sense and movement protein (*MP*) in the complementary sense (Gilbertson *et al.*, 2003). These proteins are essential for the viral movement in the host plants either from nucleus to the cytoplasm or from cell to cell via plasmodesmata, respectively (Farag *et al.*, 2005).

DNA α-satellite and β-satellite: DNA-α and β-satellites both

require the helper *begomovirus* (DNA-A) for encapsidation, whitefly mediated transmission, cell to cell and systematic movement within the infected plants. However, exact function is still unknown (Briddon and Stanley, 2006; Zhou, 2013). These satellite molecules share only conserved stem loop structure with DNA-A molecule. The genome of these satellites is approximately 1.4kb (Briddon and Stanley, 2006; Zhou, 2013). Both these satellites display conserved structures that share some broad features. In addition, these molecules contain a putative origin of replication (ori) distinct by a canonical nonanucleotide motif (NANTATTAC) present at the apex of a hairpin structure (Rosario *et al.*, 2012). However, betasatellites consists of same nonanucleotide motif (TAATATTAC) seen in vast majority of *begomoviruses*, while α-satellites consists of nonanucleotide motif (TAGTATTAC) observed in family *Nanoviridae***.** Intriguingly, DNA α-satellite is described as satellite like molecule because it can autonomously replicate itself. Both satellite molecules contain an adenine-rich region that might function as a "stuffer" to increase their size and allow systemic movement and efficient encapsidation by the helper begomovirus (Zhou, 2013). Both these satellite molecules encode single protein. Alphasatellites encode a replication-associated protein *(Rep)* involved in inducing the rolling circle replication for the replication of virus within the host cells. Conversely, helper begomovirus is necessary for the replication of betasatellite molecules. Betasatellites also encodes a multifunctional protein *(βC1)* essential for determination of pathogenicity (Figure 1b) (Zhou, 2013). Moreover, betasatellite molecules share approximately 120nt long common region called satellite common region (SCR) (Briddon, 2003).

Relationship of CLCuV with whitefly: Whitefly is the main source of viral transmission. There are 24 cryptic species of *B. tabaci* complex that are identified using biological and molecular tools (De Barro *et al.*, 2011). A wide variety of vegetables, ornamental plants and major crops like tobacco, cotton are significantly affected by whiteflies of this species complex via direct feeding and transmission of over 200 plant viruses predominantly *begomoviruses* (Inoue-Nagata *et al.*, 2016). It was first time reported as one of the most devastating insect pest in Northern India in the late 1920s and is now distributed all over the world, except in Antarctica (Chaubey *et al.*, 2015). However, genetic complexity of *B. tabaci* was first time identified in the late 1950's (Bird, 1957). Recently, in several laboratories the sequencing of genomic organization of *B. tabaci* has been completed using Next-generation sequencing (NGS) which would help in determining the phylogenies of the *B. tabaci* complex and studying their relationship with their host (Zhu *et al.*, 2016).

Whitefly (*Bemisia tabaci)* transmitted *CLCuV* to the host plant in a circulative, persistent manner (Ruiz *et al.*, 2006). These viruses do not replicate in the vector while it has been reported that genes of *CLCuV* may be transcribed in *B. tabaci* (Sinisterra *et al.*, 2005). However, the exact mechanism is not yet clear. After the acquisition of virus, *B.tabaci* transmits it to the haemolymph over the epithelial cells of midgut (Ohnishi *et al.*, 2009). During the next feeding cycle, these viral particles circulate in haemolymph and then transferred to the salivary glands for transmission to the healthy ones (Czosnek *et al.*, 2002). Moreover, the intimate relationship suggests active cellular and molecular interactions between proteins and genes of that virus and *B.tabaci*. These interactions might trigger innate immunity as well as stress-responsive genes of whitefly (Liu *et al.*, 2013). Currently, only few proteins related with endosymbionts has been reported that are involved in *B. tabaci* mediated transmission of *begomoviruses*. For example, production of Arsenophonus of *B. tabaci and* bacterial endosymbionts *Hamiltonella* derived 63 kDa *of GroEL* protein are involved in transmission of *CLCuV* and *TYLCV*, respectively (Gottlieb *et al.*, 2010).

Disease etiology and symptom development: Cotton leaf curl disease is caused by *CLCuV* that is mainly transmitted by Whitefly *(B.tabaci)*. Once this virus is acquired by its vector Whitefly it is retained in the body throughout its life. Whitefly acquires the virus from infected ones and requires 30 minutes of acquisition feeding period (AFP) on infected plants and a latent period of 24 hours and then 30 minutes of inoculation feeding period (IFP) for the transmission of virus to the healthy plants (Czosnek, 2007). However, transmission efficiency depends on abiotic factors; vector behavior, host plant and virus strain (Mann, 2011). It causes unremarkable changes at the early stage to significant variations at later stages in growth patterns of cotton plant development. In mature plants, Whitefly *(B. tabaci)* may reduce the vigor and yield. It can also cause death of seedlings (Czosnek, 2007).

The plants affected with CLCuVD primarily appear greener as compared to non-infected plants because of proliferation of chloroplast-containing tissues (Shahid *et al.*, 2015). Symptoms of CLCuVD depend on the severity of the infection. Symptoms include yellowing, swelling and thickening of veins, outgrowth development at underneath of the leaf known as 'enations' and decrease in inter nodal distance that leads to the stunted plant growth (Qazi *et al.*, 2007). Furthermore, symptoms development depends on the cotton variety as well as the plant age at the time of infection. Late season infection primarily caused minor changes and slight yield reduction. Conversely, cotton plants infected soon after germination leads to tightly rolled leaves, stunted growth and produce no harvestable lint (Sunilkumar *et al.*, 2006).

Detection/identification methods for CLCuVD: Before establishing the management program, it is essential to confirm that the given disease is caused by the cotton leaf curl virus. Following detection methods that are discussed here under:

Sick plot technique: It's very economical and easy method practiced frequently by cotton pathologists at several Cotton research stations for phenotypic evaluation of the target cultivars. In this method, the most susceptible and popular genotype (S-12) is used as spreader in rows among the genotypes under investigation for the natural spreading of disease (Rashida *et al.*, 2005).

Grafting method: Grafting is the most convenient method used for the transmission of whiteflies because grafted plants develop symptoms within 14-30 days based on the type of variety to be tested. In this method, "root stock" is used as a "resistant" and "scion" as a "susceptible" source for the transmission of disease. Then, the viral presence was confirmed by ELISA test (Farooq et al., 2011). Three grafted methods are commonly used by the researchers including top cleft, wedge graft and bottle graft.

Late sowing: This method is economically most convenient used for the screening of segregating population, germplasm

and candidate genotypes resistant to *CLCuVD*. New resistant cotton genotypes are screened by choosing an appropriate sowing time (early or late sowing) along with the disease nursery (Iqbal and Khan, 2011). It has been reported that *CLCuVD* attack reached maximum when cotton sown in late season (first week of July). The incidence of *CLCuVD* occurs after sowing within 100 days. Thus, segregating material or candidate genotypes tested for disease tolerance should be sown late in July (Iqbal *et al.*, 2014).

Viral detection by PCR: The causal agents of *CLCuD* are primarily amplified using specific or degenerate primers using PCR. Moreover, primers for the detection of satellites like molecules are also available (Idris *et al.*, 2011). Rolling circle amplification (RCA) method has also been used for the amplification of various helper viruses and their recombinants (Haible *et al.*, 2006). However, user friendly identification assays are still unknown. In future, with the development of genome sequencing tools new assays can be designed for the detection and identification of whole virus and vector complex in the field and monitoring of spreading to various other crop species (Saleem *et al.*, 2016).

Pollen irradiation technique: In this technique tolerant material against *CLCuD* is developed to produce genetic variability in the cotton germplasm (Aslam and Elahi, 2000). Different crosses for the creation of more genetic variability after applying irradiation doses of gamma rays ranging about 5-10 Gy (Farooq *et al.*, 2014).

Viruliferous whiteflies: It is another method used for the screening of cotton germplasm against *CLCuVD* via the inoculation of Viruliferous whiteflies in the net house either by open choice or via the release of counted Viruliferous whiteflies on the tested genotypes in polyhouse under plastic jar for fixed intervals (Monga *et al.*, 2011).

Plant self defense mechanisms: Plants have effective self-defense mechanism against pathogens such as viruses. Defense response is generated when plants detect the conserved pathogen derived molecules. However, pathogens secrete effector proteins to suppress the host defense and to control the metabolism for their nutrition (Klein *et al.*, 2015). Conversely, plants contain resistance R genes that play significant role in developing resistance against pathogens. After viral attack R gene become active against that particular virus and kill the infected cells by triggering localized cell death that seem as large spots which in turns prevent the spreading of infection (Khan *et al.*, 2015). Furthermore, plants produce natural disinfectants including salicylic acid, Reactive oxygen molecules and Nitric oxide that destroy the pathogens (Sattar *et al.*, 2013). RNA silencing is considered as a major line of defense against pathogens such as viruses. Plants also have the capability to produce siRNA in response to dsRNA (Rishishwar and Dasgupta, 2019). However, most of the viruses encode proteins against this plant defense response. In case of injury plants may also reduce transport through plasmodesmata (Su *et al.*, 2015).

Biological control: Biological control is considered as one of the novel approaches for the protection of plant species against pathogen invasion (Bal *et al.*, 2014). Diverse species of entomopathogenic fungi are used as natural enemies against several pests (Eilenberg *et al.*, 2001). These fungi are capable to kill the insect pests at all stages from egg to the adult stage (Hajek and Delalibera, 2010; Mensah *et al.*, 2012). More than 20 different species of entomopathogenic fungi have been known to infect whitefly *(B. tabaci)* but *B. bassiana*, *Lecanicillium* and *Paecilomyces* are most widely used and studied against pests (Cuthbertson *et al.*, 2012). These fungi are applied in the field as an alternative to insecticides as they are eco-friendly and persistent for a longer period of time. *Trichoderma* releases various compounds that trigger the localized or systemic resistance responses, showing their lack of pathogenicity to plants (Leelavathi *et al.*, 2014). *Trichoderma longibrachiatum* is present mainly in warm climates on decaying plant material throughout the world. However, its ecological significance considered from purely saprotrophs to parasite of other saprotrophic fungi (Samuels *et al.*, 2012). In another study, beneficial effect of this specie has been reported on canola *(Brassica napus* L.*)* against mustard aphid *(Lipaphis erysimi)* (Ujjan and Shahzad, 2012). In addition, recent study showed the maximum mortality rate (73%) after conidial spore suspension of *T. longibrachiatum* was sprayed against *B. tabaci*. However, *T. longibrachiatum* activity was severe on nymphal as compare to adult stage of *B. tabaci* (Anwar *et al.*, 2016). Recently, in another study maximum inhibition of CLCuV has been observed when mixture of bacterial isolates including *Bacillus spp.* isolates, JS3HR2 and JS2HR4, *Burkholderia sp.* S1HL4 and *P. aeruginosa* S1HL3 was used under greenhouse conditions (Ramzan *et al.*, 2016).

Management of CLCuVD: Various factors affect the occurrence, incidence, severity and economic losses caused by cotton leaf curl disease. In "Integrated pest management" approach, these factors are considered in order to select the control measure that are used to overcome the disease severity and economic losses. Some general strategies for the management of CLCuV are discussed in the following section:

Physiological adaptations of plants against viruses: Insects usually attach themselves to the plant surfaces and cause infection. Most of the plants develop some physiological adaptation to prevent the viral interaction with the plant surfaces (Khan *et al.*, 2015). Some of the physiological adaptions against viruses particularly CLCuV is discussed in the following section:

Hindering insect movement: Wild cotton *G. arboreum* can hinder the movement of insect by its specialized structures referred as 'trichomes'. It confers difficulty to the viruses in movement, piercing and sucking (Whitney and Federle, 2013). It depends on the length and density of the trichomes as it is vital for insect behavior. Intriguingly, sometimes trichomes act as an indicator of insect attack while, defense chemicals are released by plants when/or after trichomes are broken (Alcorn *et al.*, 2012).

Deprivation of food for insects: Inorganic salts and calcium oxalate are present on the outer surfaces of the terrestrial plants such as, cotton. These chemical compounds damage the gut and mouthparts of insects when they try to eat plants (Lucas *et al.*, 2000; Khan *et al.*, 2015).

Epicuticular waxes: The outer film of cuticle is referred as "cuticular wax" that physically exposed from surface of fruits, leaves and stem by means of aqueous glue (Buschhaus and Jetter, 2011). Chemically these are composed of very long chain aliphatics (VLC; C20-C34) that lack functionality (alkanes), functional group of single terminal oxygen such as aldehydes, primary alcohols and fatty acids, or in chain group: ketones,

alkyl esters and secondary alcohols (Jetter *et al.*, 2008). However, in most of the plant species alicylic compounds such as tocopherols and triterpenoids are found at comparatively high concentrations along with VLC (Joubès and Domergue, 2018). Moreover, the composition and amount of cuticular wax markedly varies among plant species even among organs of the same plant. Moreover, cuticle chemistry can also alter during organ development and is affected by environmental conditions. The cuticular wax layer play central role to defend the plants against a(biotic) stresses and also function like a waterproof barrier (Ahmad *et al.*, 2015). The cuticular waxes play pivotal role in eliminating the affection as well as growth of insects, enhance resistance of plants in response to phytopathogens such as bacteria and fungi, viruses and hence, weaken the host-pathogen interactions (Carver and Gurr, 2008). For example, the aphid spends more time in wax deficient pea mutant plants (Chang *et al.*, 2004). Except for their glossy appearance most of the epicuticular wax mutants show morphological similarities when compared with the wild type plants (Barozai and Husnain, 2014). Epicuticular wax mutants were first time identified (1979) in Arabidopsis namely, eceriferum (cer) which in Latin is 'without wax'. Later, epicuticular wax mutants have been identified in multiple plant species such as *Brassica napus* barley *(Hordeum vulgare)* and maize (*Zea mays* L.) (Rostás *et al.*, 2008). In another study, Asiatic *G. arboreum* wax mutants *(GaWM3)* with 50% less wax were produced. It seems that mutant plants were more susceptible to CLCuV when compared with wild type (Khan *et al.*, 2011). However, quantity and quality of wax is important in feeding of whiteflies (Khan *et al.*, 2016).

Biochemical modifications: Incidence and occurrence of Cotton leaf curl virus disease can be controlled by using following biochemical modifications

Identification of plant host enzymes: Viruses generally target the host proteins after successful interaction. DNA-β satellites of CLCuMuV encodes a pathogenicity determinant protein ($\beta C1$) that physically binds with the host Ubiquitin conjugating (E2) enzyme *S1UBC3* (Eini *et al.*, 2009). In this interaction, transgenic plants overexpressing $\beta C1$ inhibited the high level of polyubiquitinated proteins. It has been reported that this interaction is associated with severity of disease (Bachmair *et al.*, 1990). However, more research is needed to explore such interactions in cotton plants for limiting the risk of viral invasion.

Identification of plant host hormones: Phytohormones play indispensable role in initiating signal cascades against a wide-variety of pathogens (Bari and Jones, 2009). Among them, jasmonic acid hormone is involved to develop resistance against herbivores, insects as well as necrotrophic pathogens. In recent study, this hormone induces resistance in young Arabidopsis plants against insects, and is regulated by miR156-targeted-SPL9 (negatively associated with JA expression) (Mao *et al.*, 2017). However, more research is needed to exploit such novel pathways in cotton plants against whitefly and other insect pests.

Plant extracts against CLCuVD: Synthetic pesticides are most commonly used to control the population of insect pests. However, efforts are underway to develop alternative strategies against multiple insect pests as they developed resistance against these products. Use of essential oils is an alternative approach that helps the plants against several household as well as field insect pests (Sarwar *et al.*, 2013). Moreover, petroleum oil and several oils obtained from plants act as repellents against various types of insect pests including whiteflies (Du *et al.*, 2016). It has been reported that about 2000 plant species contain insecticidal activities. The essential oils are well known complex, volatile, and natural compounds formed in plants that produce distinctive odors due to secondary metabolites. Such compounds display an important role to provide protection against bacteria, fungi, viruses, herbivores as well as insect pests (Sarwar and Salman, 2015). These oils contain poisoning effect when interact with the fatty acids of the insects and affect the metabolism. The air hole is restricted through which the insects breathe which causes asphyxiation, and hence kill the insect population (Sarwar and Salman, 2015). In a recent study, four most common and effective repellents including cinnamon, aframomum, savory and geranium were seemed to be irritant and toxic plant extracts against *B. tabaci.* However, among them, the most effective repellent extracts used against *B. tabaci* were aframomum and lemongrass, although savory geranium and cinnamon were also found to be effective repellents at higher doses (Emilie *et al.*, 2015). Another study suggested the use of *P. hystophorous* and *D. alba* extracts that confer resistance in cotton against whitefly population (Ali *et al.*, 2015). It has also been reported that exogenous application of 2 or 3% concentration of mustard oil is also effective in decreasing population of whiteflies (*Bimisia tabaci*) (Arif *et al.*, 2009). In another study, *A. indica* (plant extract) and the specific concentration of salicylic acid were seemed to be most effective against *B. tabaci*. Other treatment of plant extracts such as *E. globules* and *A. sativum, Datura stramonium* L.*, Aloe barbadensis Mill. Calotropics procera* were also evaluated but found to be less effective in comparison with *A. indica* (Plant extract) against whitefly *Bimisia tabaci* and *CLCuV*. It has been showed that *A.indica* (Neem) and 3% Salicylic acid significantly decreased the numbers of whiteflies, ability of hatching eggs and adult emergence (Ali *et al.*, 2010).

Netting: Netting is seems to be barrier for the sucking insect pests (Kumar and Poehling, 2006). Different types of insect proof nets are used to control the whiteflies. However, the only use of nets against whiteflies is not effective in tropical areas because of humidity and temperature while, $< 50 \text{mesh}/cm^2$ reduces the ventilation and increases the number of pathogens. Therefore, it has been suggested that netting is effective in combination with repellents against whiteflies. Taken as a whole, netting along with pyrethroid, a-cypermethrin is proved to be significantly (100%) reduces the number of whiteflies and aphids (Ali *et al.*, 2010).

Conventional breeding for viral tolerance and quality characters: Breeders are interested to develop new varieties that are adapted well to the environment for high yield, better quality, high response to the fertilizer and to increase tolerance against pathogenic attack (Hussain, 2015). Furthermore, as cotton yield is affected due to environmental and genetic factors resulting in a difficulty for breeders to select the high yielding plants (Farooq *et al.*, 2013). Globally, Breeders are trying to improve the yield and quality of cotton plants via conventional methods. However, the progress is very slow and sometimes ineffective as it consists of visual screening (Liang *et*

al., 2014). Other major disadvantages include sudden environmental changes, unavailability of genetic resources and the effect of minor genes that are not easy to measure in the presence of major genes. Selection of resistant varieties against leaf curl virus is based on a small number of visually scorable traits like plant growth. Furthermore, generally yield is the dominant factor in selection. The viral damage can be reduced after breeding of broad diversity resistant variety with the virus using either genetic engineering or classical breeding (Ashokkumar *et al.*, 2014). Simultaneous selection is used to produce viral resistant genotype with higher yield and quality (Rahman *et al.*, 2005). It has been shown that biparental mating maintains the broad genetic base thus, desirable genotypes of viral resistance can be produced. It requires adequate criteria of selection and screening techniques. Molecular techniques are used to solve the problems. The development of molecular techniques and molecular markers give a new dimension in to traditional era of plant breeding (Shang *et al.*, 2015).

Molecular approaches: Cotton leaf curl virus disease can be controlled by using following molecular techniques

Identification of DNA markers in Molecular Breeding: Conventional breeding is used for the improvement of valuable traits but the progress is very slow. Moreover, these are quantitative traits thus it is difficult to conduct the phenotypic selection (Zhang *et al.*, 2015). Breeders use DNA markers to select the valuable traits on molecular basis to avoid the phenotypic screening. Major traits such as quality, yield and disease resistance are under the control of multiple genes called "quantitative trait" (Bolek *et al.*, 2016). Identification of markers tightly linked to the quantitative trait loci allows the breeders to easily identify the target trait in early growth stages of plants (Boopathi *et al.*, 2015). The identification of molecular markers linked to *CLCuVD* resistance has the potential to improve both the efficacy and efficiency of selection in cotton breeding programs. Breeding material can be easily identified by marker-assisted selection (MAS) (Liang *et al.*, 2014). Efforts are underway to identify the QTLs tightly linked to disease resistance by using inter and intra-specific crosses. Random amplified polymorphic DNA (RAPD) assay was used to identify the markers linked to the genes associated with disease resistance. A bi-parental F2 mapping population was derived by using highly resistant cultivar *G. hirsutum* LRA-5166 and most susceptible variety S-12 (Rahman *et al.*, 2005). Bulked sergeant analysis (BSA) was used by combining equal quantity of genomic DNA of susceptible and resistant plants of F2 in two different pools. A total of 520 decamer random primers were assessed on these bulks but, polymorphic RAPD primers were not recognized. Afterwards, these primers were evaluated on the parental genotypes. In total, 13% of the amplicons were polymorphic. Recombination frequency approximately (14%) was identified in RAPD marker during *trans* phase. Whereas, OPY-21080, OPQ-14325 and OPO-19460 were identified with recombination frequency (0-5%) in coupling phase. These primers were found associated with disease resistance (Rahman *et al.*, 2002). In another study, genetic diversity in 18 cotton varieties were evaluated using Random Amplified Polymorphic DNA (RAPD). Three RAPD primers: OPO-2, OPQ-14, and OPY-19 were linked with the resistance. Marker assisted screening showed two cotton cultivars CIM-443 and CIM-240 were resistant to *CLCuV* (Mumtaz *et al.*, 2010). Recently, a total of 10 cotton cultivars (four highly susceptible, five highly tolerant, and one immune) of diverse origin were selected. In total, 322 SSRs derived from bacterial artificial chromosome (BAC) end sequences of *Gossypium raimondii* were screened. Out of these, only 65 primer pairs were found polymorphic. These cotton genotypes were then grouped into susceptible and tolerant genotypes, respectively. Out of the polymorphic markers, two SSR markers: CM-43 and PR-91 were amplified only in tolerant genotypes displayed significant association with *CLCuVD* (Abbas *et al.*, 2015).

CRISPR-Cas: The clustered regularly interspaced palindromic repeat (CRISPR)-CRISPR-associated protein system is considered as a genome editing tool used by bacteria against mobile genetic elements, viruses and archaea (Sander and Joung, 2014). This technique derived the attention of the scientists from all the disciplines particularly plant biologists due to higher level of specificity (Iqbal *et al.*, 2016). It provides an adaptive immunity against the nucleic acids of these invading pathogens. Now, in eukaryotes, it has been adapted as a genome editing tool (Sander and Joung, 2014). In this technique, CRISPR spacers chopped the nucleic acids of the invading viruses and archaea. The resultant molecules are about 20nt long that are similar to the size of sequences produced by RNAi (Marraffini and Sontheimer, 2010). These are found in about 40-90% of the bacterial and archaea genomes, respectively (Grissa *et al.*, 2007). This technique can be used against various complex organisms by inserting the *Cas9* protein and guide RNAs into the host cell (Rojas *et al.*, 2018). It can also be used against Geminiviruses due to ease of engineering, adaptability and robustness (Iqbal *et al.*, 2016). It has been used to edit the genomes of *Bean yellow dwarf virus (BeYDV)*, *Merremia mosaic virus*, *Cotton leaf curl Kokhran virus (CLCuKoV)*, *TYLCV* and *beet severe curly top virus (BCTV)* (Ali *et al.*, 2015; Baltes *et al.*, 2015). In these studies, coding and non-coding regions of viral genomes are targeted by constructing the sgRNAs (Baltes *et al.*, 2015). This approach can be used against *CLCuVD*. However, there are no reports of *Cas9* or an edited host factor that inhibits the transcription or replication and hence confers resistance against geminiviruses. Thus, more research is needed to demonstrate the potential of CRISPR-Cas against *CLCuVD* and its viral causal agents.

Pathogen derived resistance (PDR): Resistance produced using genetic engineering is classified into Pathogen derived or non-pathogen derived resistance (NPDR). In PDR, a complete or part of viral gene is introduced into the host plant that later blocks the replication process of that particular virus. Conversely, PDR is not found to be effective against these viruses because of induction of gene silencing by viruses (Yousaf et al., 2015). It is divided into two categories with or without protein expression. Replication associated protein *(Rep)* is essential and play vital role to replicate the virus inside the host plant (Su *et al.*, 2015). This is also involved in host cell replication and in the synthesis of viral components by bringing the cell into S phase. This protein is also used for the induction of resistance to overcome viral infection. siRNA strategy is used to interfere the structure of protein (Khan *et al.*, 2015). If Rep protein structure is suppressed, then viral components cannot be made. However, not any commercial variety is developed up till now by using this technology (Yousaf *et al.*, 2013). Currently, intergenic region of *Ageratum conyzoides* are transformed

against *CLCuV* but transgenic are still under trial (Nagata *et al.*, 2015).

RNA interference: RNAi technology is considered as a powerful tool based on transcriptional gene silencing (TGS) and post transcriptional gene silencing (PTGS) used to improve the expression of valuable traits (Meena *et al.*, 2017). This technology is widely used to study the function of Agronomically, biologically and physiological valuable genes that are involved to develop resistance against (a)biotic stresses and also for early maturity, embryogenesis fertility, improve fiber quality, increases yield potential and improve the oil quality of the seeds (Abdurakhmonov *et al.*, 2016). Recently, biotech cotton has been developed by using this technology. In eukaryotes, RNA interference is first time identified by Andrew Fire and Craig C. Mello in *Caenorhabditis elegans* and later it is used to induce resistance in a majority of host plants (Tian *et al.*, 2015). The main steps of RNAi include the formation of small RNAs (sRNA) about 21-25 nt by the action of enzymes called Dicers and then incorporation of one of the strand (loading strand) into the RNA-induced silencing complexes (RISCs) containing Argonaute *(Ago)* proteins that directly lead to gene silencing at the post transcriptional or transcriptional levels as shown in Figure 2 (Brodersen *et al.*, 2008). There are two main classes of small RNAs: small interfering RNAs (siRNAs) and microRNAs that are involved in gene silencing. SiRNAs are formed when endonuclease dicer enzymes act on long dsRNA and cleave them which later bind with their complementary sequence for transcriptional or post transcriptional gene silencing. Contrarily, microRNAs are transcribed by their own genes or from fold back structures with double stranded regions from introns (Rishishwar and Dasgupta, 2019). In plants, these primary microRNAs are processed in the nucleus by the dicer enzymes into pre or precursor miRNA which later form duplexes before being exported to the RISCs (Sattar *et al.*, 2013).

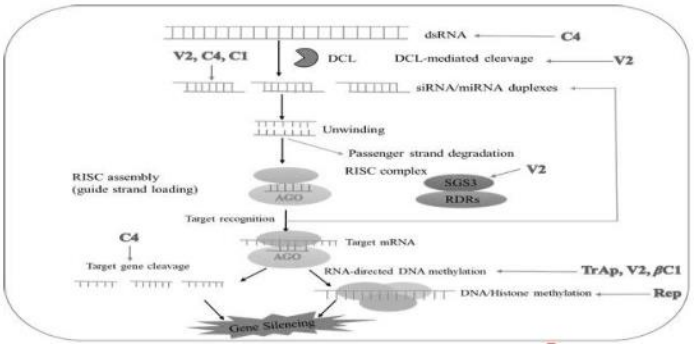

Figure 2: The main steps in the gene silencing pathway where Geminiviral derived suppressor proteins have been displayed to act. The RNA duplexes of about 21-30nt (siRNA/miRNA) are formed after the cleavage of long dsRNA. One of the strand; guide stranded is incorporated into the RISC complex which have AGO protein while the other strand called passenger strand is degraded. The siRNA guides this protein to the target mRNA sequences that leads to gene silencing by several mechanisms. The green arrows show the inhibition of steps of silencing pathway by suppressor proteins.

This technology has also been used against viruses such as, *African cassava mosaic virus (ACMV), mung bean yellow mosaic virus (MYMV)* and various others. Recently, it has been reported that *G.hirsutum ghr-miR166b* targeted the *ATP synthase* gene of *B. tabaci*, and its overexpression has the potential to function as biopesticides for decreasing *B. tabaci* population and whitefly mediated viral transmission (Wamiq and Khan, 2018). However, viruses encode suppressor proteins as a counter defense to avoid silencing (Amin *et al.*, 2011). It has been reported that all viruses encode one or more suppressors against silencing to defend themselves. It has also been reported that *CLCuV* encode 4-5 suppressors particularly *V2, C2, C4* and *βC1*. The *AC4/C4* and *βC1* have been shown to attach and separate or remove the siRNA thus, avoid their integration into RNA induced silencing process (Figure 2) (Sattar *et al.*, 2013). Moreover, *V2* shows the higher suppressor activity, protein did not form when siRNA bind to the specific mRNA. The *V2* of Cotton leaf curl Multan virus (CLCuMV) has been reported to sequester the long dsRNA and inhibits its dicer mediated cleavage (Amin *et al.*, 2011). The *AC2/C2* protein has been reported to function as both TGS and PTGS suppressor (Van Wezel *et al.*, 2002). This protein has been reported to act as PTGS suppressor in Vigna *Mungbean yellow mosaic virus* (MYMV-Vig), *East African cassava mosaic Cameroon virus*, *CLCuMV* and *Tomato yellow leaf curl virus-China* (Van Wezel *et al.*, 2002; Vanitharani *et al.*, 2004; Trinks *et al.*, 2005). Furthermore, *C2* has been shown to inhibit the components of methylation machinery including adenosine kinase, *SUCROSE NONFERMENTING1 (SNF1)* Kinase, and certain histone methyltransferases (Buchmann et al. 2009). Therefore, it function as TGS suppressor (Zhang *et al.*, 2011).

Anti-sense RNA: Anti-sense RNA is complementary to the mRNA that forms duplex with the target mRNA and prevented it from being translated (Amudha *et al.*, 2011). It is most widely used and effective technique against several viruses such as, *Tomato Yellow Leaf Curl virus* and *Cotton Leaf Curl Kokhran virus (CLCuKoV)* (Khan *et al.*, 2015). In transgenic cotton, *rep* protein was targeted by using this technology which inhibited the replication of attacking virus (Amudha *et al.*, 2010). Another study was also conducted for targeting the viral *AV1* which in turns suppressed the viral movement, encapsidation and replication in transgenic cotton plants (Amudha *et al.*, 2011). Recently, transgenic cotton *(G. hirsutum cv. Coker-310)* was introduced after inserting viral gene *βC1* in antisense orientation under 35S promoter. In cotton genome, successful insertion of this gene was confirmed using southern blot hybridization. However, No symptoms were observed after insertion of this gene in transgenic cotton (Sohrab *et al.*, 2016).

Non-pathogen derived resistance: Introduction of genes from host or non-host plants are deployed to induce resistance against the various diseases. For instance, introduction of genes responsible for providing antiviral antibodies, coat binding proteins, DNA binding proteins, etc., in plants to develop resistance against CLCuV (Castellano *et al.*, 1999). The first report of development of transgenic resistance against plant virus involved the expression of *Tobacco mosaic virus (TMV)* derived viral coat protein *(CP)* gene and this strategy was later used against *Geminiviruses* (Khan *et al.*, 2015). Recently, a mechanism has been explored in mosses and ferns that confer resistance against phytophagous insects. For instance, a protein *Tma12* was identified in fern that provides resistance against whitefly. This protein encoding gene was introduced in cotton cv. Coker-312. Only one cotton line has displayed increased resistance (>99%) against whitefly (Shukla *et al.*, 2016). Hence,

this protein can be used in future to induce resistance against whitefly in many other plant species.

Cell death induction: In transgenic plants, this approach has been used to inhibit the replication of geminiviruses. It was developed by using the *Bacillus amyloliquefaciens* derived combined action of barstar and barnase proteins. Barstar acts and suppresses the barnase activity which is a ribonuclease (RNase). These two transgenes should express at the same levels when there is no geminivirus infection for limiting the RNase production. This approach was successfully used against *tomato leaf curl New Delhi virus (ToLCNDV)* and the viral transmission to the plant tissues was arrested (Vanderschuren *et al.*, 2007). Recently in transgenic tobacco plants, restriction of whitefly population expressing the insecticidal genes has been reported under the phloem promoter (Javaid *et al.*, 2016). In future, this approach can be used to protect the cotton plants against geminiviruses.

Recent advances using biotechnological tools: Conventional breeding methods have some limitations such as sudden climatic changes and availability of limited resources. However, by exploiting plant biotechnology now it is easy to control various diseases such as CLCuV via cloning certain viruses and development of defense strategies which in turns improve the plant yield (Farooq et al., 2011). Today, Biotech cotton is most widely accepted, marketed and cultivated on more than 24 million hectares throughout the world. First GM crop was commercialized in 1996 and now 70% biotech cotton is adapted globally (Bakhsh *et al.*, 2015). Now it is possible via biotechnology or genetic engineering to incorporate resistant gene into the commercially valuable crops against diseases. *G. arboreum* is resistant against CLCuV and several other bacterial and fungal diseases. *G. arboreum* has been used for incorporation and isolation of resistant genes into susceptible cultivars by using genetic engineering. However, genetic variation is insufficient in *G. arboreum* (Niu *et al.*, 2008). Pathogen derived resistance (PDR) approach is deployed to protect the plants against various viruses lacking natural disease resistance. By exploiting transcriptional control two truncated forms of replicase *(tACI)* gene expressing C-Terminal 783bp (3'ACI) and N-terminal 669bp (5'ACI) nt was introduced via cloning into *G.hirsutum*. This gene inhibits the replication of DNA- β satellites and viral genomes (Hashmi *et al.*, 2011). In transgenic cotton, a strain LBA 4404 *Agrobacterium tumefaciens* was used via interference technology to combat *CLCuV* (Briddon and Markham, 2000). However, when these transformed plants are compared with non-transformed control plants the over expression of nucleotides develop resistance by inhibiting β satellites and viral genomic DNA components. Northern blot hybridization showed high transgene expression in late and early growth stages (Farooq *et al.*, 2014).

Control Measures with management Practices: The plant's ability to recover from the damages caused by *CLCuVD* depends upon the balanced uptake and utilization of nutrients. Application of nutrients and management of planting time are essential to control the disease severity (Farooq et al., 2011). It has been reported that resistance in host plants against diseases can be improved by adequate supply of potassium due to maintenance of osmoregulation, energy gradient and production of molecular compounds. Potassium (K) causes significant effects on various diseases via regulation of metabolic function that alters the host-parasite compatible interaction which suggests its potential effectiveness against *CLCuVD* (Kafkafi *et al.*, 2001). It has been reported that low potassium level significantly decreased the chances of disease severity in cotton susceptible variety S-12 (Zafar and Athar, 2013). It has also been reported that application of potassium upto 250kg per ha significantly reduced the spreading of disease approximately 12-38% and, increases the grain yield up to 37% when or/after compared with zero-K. Thus, nitrogen and potassium concentration (N:K) must be maintained, as nitrogen decreases disease resistance while potassium improves it (Farooq *et al.*, 2014). Cotton plants are susceptible to cotton leaf curl virus because of too early or late sowing as well as environmental changes. Environmental factors including humidity, rainfall (prior to seedling), wind temperature (33-45°C or 25-30°C) are also responsible for the occurrence of *CLCuVD* (Mahmood *et al.*, 2015). Choosing an appropriate sowing time depending upon particular variety and region is difficult as too late or early sowing may cause diseases. It has been reported that early sowing significantly decreases disease severity. Appropriate planting time preferably mid-April to mid-May can reduce the disease incidence as infection occurs in late season thus, losses can be avoided by too early or late sowing (Iqbal *et al.*, 2014). It has also been reported that increased spacing between plants in case of early sowing and decreased spacing in case of late sowing is effective against *CLCuVD*. In addition, 15cm plant spacing has been suggested in order to control the disease incidence in case of sowing later than 15th of June (Farooq *et al.*, 2014).

CONCLUSION: It is concluded that all above mentioned measures can be implemented for the control of cotton leaf curl disease depending upon the conditions. Only way to check the disease is production and commercial cultivation of the *CLCuVD* resistant varieties. However, losses can be minimized by the cultivation of highly tolerant and tolerant varieties which are available for general cultivation. In this context, currently the CRISPR/Cas technology can be potentially used to develop resistance against *CLCuVD* and its viral causal agents.

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