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Molecular Diagnosis and Transmission Studies of *Chilli Leaf Curl Virus* by Asia-I Cryptic Species of Whitefly (*Bemisia tabaci* Genn.)

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ABSTRACT

Chilli (*Capsicum annuum* L.)is major vegetable cum spice crop known to be infected with several begomoviruses resulting in severe yield losses. Chilli samples showing typical begomovirus symptoms were collected from farmer fields and assessed for detection using ChiLCV coat protein gene specific primers. The genome analysis showed the virus had 98.3 per cent nucleotide identity with *Chilli leaf curl virus* (ChiLCV). The whitefly samples collected from farmer's fields were identified as Asia-I cryptic species based on *mtCO1* gene analysis. The point mutations in coat protein were identified and subjected to *in silico* analysis with bioinformatic tools to determine the effect of amino acid substitutions as deleterious or neutral as it influences the transmission characteristics of whitefly species. The interaction studies of ChiLCV with Asia-I cryptic species revealed that twenty whiteflies with 24 h of acquisition access period (AAP) and inoculation access period (IAP) each successfully transmitted the virus with 100.0 per cent transmission. Asia-I cryptic species females found to be more efficient transmitters (86.6%) of ChiLCV than males (53.0%). The susceptibility of chilli seedlings to leaf curl virus is inversely proportional with age. This study established the tripartite interaction relationship between ChiLCV, Asia-I cryptic species of whitefly and chilli host.

Keywords: Begomovirus, Virus-vector relationship, *mtCO1*, Coat protein, Asia-I cryptic species

CHILLI (*Capsicum annuum* L.) is an important vegetable crop that has been cultivated both for industrial (oleoresin and capsaicin) purpose and spice (Saxena *et al*., 2016). *Chilli leaf curl virus* (ChiLCV) is an important begomovirus vectored by whitefly (*Bemisia tabaci* Genn.) in persistent and circulative manner and it is a major constraint in chilli cultivation both in tropical and subtropical regions of India (Thakur *et al*., 2018). The transmission route of the virus within whitefly begins with acquisition by stylets, then virus moves through oesophagus into midgut and reaches the haemolymph. Then, virus enters into salivary glands and from where it is inoculated into healthy plants (Czosnek *et al*., 2017). Typical ChiLCV disease symptoms include upward curling, puckering, thickening and swelling of veins, reduction in leaf size and stunting of entire plant. Severely affected chilli plants produce fewer, smaller and deformed fruits (Thakur *et al*., 2018).

In recent decades, the plant viruses specifically begomoviruses are responsible for frequent epidemics in agricultural ecosystems as they evolve quickly and adopts to the global climatic changes due to selection pressure (Lefeuvre *et al*., 2019). Mutations and recombinations induce genetic variations in the begomoviruses which indirectly affect the virus and insect vector interactions as virus coat protein (CP) play a crucial role in transmission of begomoviruses by whitefly (*Bemisia tabaci* Genn.) cryptic species (Pan *et al*., 2020). Whitefly cryptic species complex consists of 34 morphologically indistinguishable species but genetically differentiated by molecular analysis of mitochondrial cytochrome oxidase-1 (*mtCO1*) gene sequences with nucleotide divergence of 3.5 per cent. There is a strong disparity in the transmission efficiency of same begomovirus was reported among whitefly cryptic species complex (Wei *et al*., 2014) and efficiency disparity also noted in transmission of different begomoviruses by same cryptic species (Fiallo-olive *et al*., 2020). The differential transmission efficiencies may be due to genetic variations in the CP region of the virus. In begomoviruses, CP region between conserved amino acids (GCEGPCKVQS and

LYMACTHASN) found to be responsible for distinct transmission efficiency of whitefly cryptic species (Wei *et al*., 2014; Guo *et al*., 2018 and Pan *et al*., 2018).

Plant viruses undergo genetic variations through mutations and recombinations over time resulted in formation of new strains of begomoviruses and transmission efficiency of these viruses varies with cryptic species of whitefly vector in particular geographical regions (Pan *et al*., 2020). Therefore, it is essential to standardize the transmission of begomovirus with specific species of whiteflies. The present study was aimed to determine the genetic variations in the CP of ChiLCV, identification of cryptic species of *B, tabaci* and to establish the triangular interaction between virus, insect vector and host which helps in determining the ability of specific whitefly cryptic species involved in transmitting ChiLCV.

Material and Methods

Maintenance of ChiLCV Inoculum and Molecular Detection

During summer 2019, chilli plants showing prominent leaf curl symptoms were collected from farmer fields and maintained separately under insect proof cages as source of inoculum. In order to avoid mixed infections, whitefly transmission of leaf curl virus was done repeatedly thrice to healthy chilli plants and allowed for symptom expression under insect proof cages. Total genomic DNA was isolated from both symptomatic and asymptomatic chilli plants by cetyltrimethyl ammonium bromide method (CTAB) method (Dellaporta *et al*., 1983). Further, these samples were amplified through polymerase chain reaction (PCR) with universal deng primers (Deng *et al*., 1994) and ChiLCV CP gene specific primers (ChiLCVF- CATATGCTCCAGACACTCTG and ChiLCVR- CTAACCTTCCGAATCTGGACG). PCR amplicons were analysed on one per cent agarose gel and amplified samples were sequenced in both orientations. Phylogenetic tree was constructed by neighbourhood joining method with 1000 bootstrap replications in Mega-X software (Tamura *et al*., 2011). Single nucleotide polymorphisms (SNPs) in sequence data were subjected for analysis with different bio-informatic tools *viz*., sorting tolerant from intolerant (SIFT), protein variation effect analyzer (PROVEAN) and polymorphism phenotyping v2 (Polyphen-2) which predicts the effect of amino acid substitutions on the protein function. The variants with SIFT, PROVEAN and Polyphen-2 score of <0.05, <2.5 and 1 respectively are deleterious in nature.

Whitefly Maintenance and Identification of its Cryptic Species

Virus free whitefly (*Bemisia tabaci*) stock culture was maintained on cotton in glasshouse insectary at MRS, Hebbal, Bengaluru. Adult whiteflies were collected in 70 per cent alcohol and stored at -20 ^{0}C to characterise the whitefly cryptic species. Total genomic DNA was isolated from individual whitefly by Chelex 100 resin method (Walsh *et al*., 1991) and samples were amplified in PCR with mitochondrial cytochrome oxidase gene subunit 1 (*mtCO1*) universal primers (C1-J-2195-TTGATTTTTTGGTCATCCAGAAGT) and reverse primer (L2-N-3014-TCCAATGCACTAATCTGCCATATTA) (Jiu *et al*., 2017). A negative control was maintained without DNA to avoid false positive results. PCR amplicons were analysed on one per cent agarose gel and amplified samples were sequenced. Phylogenetic tree was constructed by neighbourhood joining method with 1000 bootstrap replications in Mega-X software (Tamura *et al*., 2011) to study the per cent homology.

Virus-Vector Relationship Studies

Acquisition Access Period (AAP) : To determine the effect of different acquisition feeding periods on rate of ChiLCV transmission, healthy whiteflies were fed separately for 15 min, 30 min and 1, 2, 4, 8, 10, 12 and 24 h of acquisition on ChiLCV infected source. After acquisition access, viruliferous *B. tabaci* adults were allowed to feed on healthy chilli seedlings for 24 h at the rate of 20 insects per plant and kept in insect proof glasshouse for symptom expression. After 24 h of IAP, whiteflies were killed by spraying imidacloprid 17.8 per cent SL@0.05 per cent. The experiment was repeated thrice to find out the accuracy of transmission. Ten chilli plants were inoculated and observations on per cent transmission was recorded.

Inoculation Access Period (IAP) : The effect of different IAP on the rate of ChiLCV transmission was determined by transmitting healthy *B. tabaci* adults on ChiLCV infected source for 24 h of AAP and viruliferous whiteflies were transmitted to healthy chilli seedlings with an IAP of 15 min, 30 min and 1, 2, 4, 8, 10, 12 and 24 h at the rate of 20 insects per plant. After inoculation access, whiteflies were killed by spraying 0.05 per cent imidacloprid and kept under insect proof glasshouse for development of symptoms and the experiment was repeated thrice. Ten chilli seedlings were included in each treatment and per cent disease transmission was recorded.

Minimum Number of B. tabaci Adults required for Transmission of Leaf Curl Virus Infecting Chilli : To determine minimum number of viruliferous whiteflies required for the transmission of ChiLCV, whiteflies were collected from stock culture and allowed for acquisition access of 24 h on virus source. Then viruliferous whiteflies were released on healthy chilli seedlings at two leaf stage in batches of 1, 3, 5, 7, 10, 15 and 20 per seedling separately and 10 plants were inoculated in each treatment. After inoculation access of 24 h, whiteflies were killed by spraying imidacloprid 17.8 per cent SL@0.05 per cent and inoculated plants were kept in insect proof glasshouse for symptom expression. The same experiment was repeated thrice and per cent disease transmission was recorded.

Efficiency of Whitefly Gender on Transmission of ChiLCV : To compare the efficiency of whitefly gender on the rate of transmission of ChiLCV, male and female *B. tabaci* adults were distinguished and collected into separate collection bottles. Later, these flies were allowed for AAP of 24 h separately on virus source. After prescribed AAP, male and female adult whiteflies were allowed separately to feed on healthy chilli seedlings. After 24 h IAP, whiteflies were killed as mentioned above and kept in insect proof glasshouse for symptom expression. In each treatment, 10 chilli seedlings were inoculated and observed for symptom expression.

Efficiency of Virus Transmission on Age of Seedlings : To assess the rate of virus transmission on plant age, twenty viruliferous whiteflies were inoculated at different leaf stages *viz*., 10, 15, 20 and 30 days old seedlings. Twenty whiteflies were given 24 h of AAP and IAP and whiteflies were killed with imidacloprid 17.8 per cent SL@0.05 per cent after 24 hrs of inoculation access. Ten chilli plants at different leaf stages were inoculated in each treatment and experiment was repeated for three times. The inoculated plants were kept under insect proof glasshouse and observations recorded on per cent disease transmission.

Results and Discussion

PCR Amplification and Sequence Analysis of Begomovirus

PCR amplication of chilli genomic DNA with Deng primers resulted in expected amplicon size of 550 bp (Plate 1a) indicated the presence of begomovirus infection. Further, genomic DNA characterised using ChiLCV CP gene specific primers which yielded an amplicon size of 1 kb (Plate 1b). Full length CP gene of 770 bp was obtained from sequence data. Sequence analysis revealed that CP had highest nucleotide identity of 98.3 per cent with C*hilli leaf curl virus*. Sequence data under study was aligned with reported sequences available at NCBI database using clustal-W algorithm implemented in Mega-X software. In phylogenetic tree, isolate under study was closely clustered with CP gene of *Chilli leaf curl virus* isolates (Fig. 1). Point mutation was noticed in the CP amino acid sequence at 86th position where valine replaced the isoleucine and this mutation is categorized as neutral substitution based on SIFT, PROVEAN and

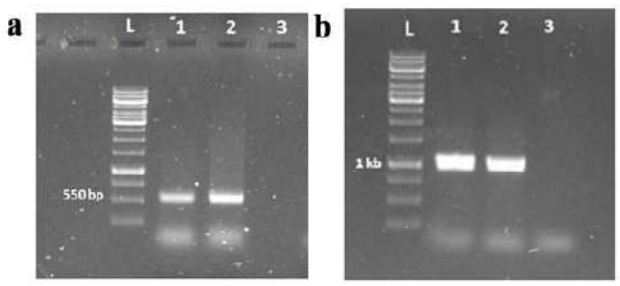

Plate 1: PCR based detection of begomovirus under study with Deng and *Chilli leaf curl virus* (ChiLCV) specific primers. Lane L:1 kb ladder; Lane 1-2: Chilli DNA sample, Lane 3: Negative control

Polyphen-2 analysis (Table 1). The results clearly indicate that SNPs in CP gene under study do not have any deleterious effect in transmission by whiteflies. Hohnle *et al*. (2001) reported that three amino acid substitutions *viz*., Q124K, H149Q and L174M in CP region results in transmission of *Abutilon mosaic virus* by the whitefly. Noris *et al*. (1998) reported that whitefly lost its ability to transmit *Tomato leaf curl sardinia virus* due to amino acid substitutions *viz*., Q129P and Q134H in CP gene.

TABLE 1

Analysis of Single nucleotide polymorphism (SNPs) in the coat protein gene of *Chilli leaf curl virus* (ChiLCV) by different bioinformatics tools

Bio-informatic tools	Amino acid change	Score	Prediction
SIFT	V86I	1.00	Neutral
PROVEAN	V86I	-0.10	Neutral
PolyPhen-2	V86I	0.00	Neutral

*SIFT (Sorting tolerant from intolerant) score - Deleterious (<0.05), Neutral (e"0.05); PROVEAN (protein variation effect analyser) score - Deleterious (<-2.50), Neutral (>-2.50); Polyphen-2 (polymorphism phenotyping v2) score - Deleterious (>0.5), Neutral (<0.5)

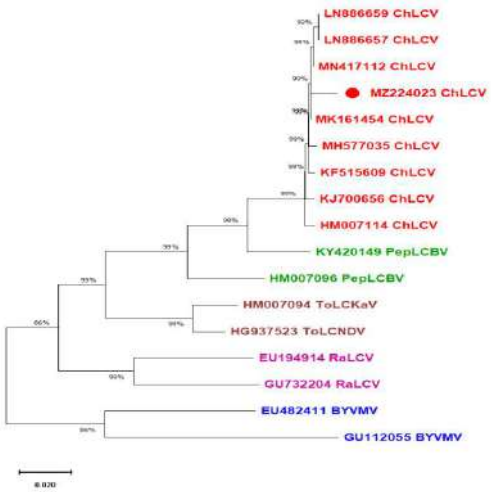

Fig. 1: Phylogenetic tree constructed from nucleotide sequences of ChiLCV coat protein amplicon sequence with other selectedbegomovirusesretrived from NCBI database using the Neighbor-joining algorithm in MEGA-X. Acronyms: *Tomato leaf curl New Delhi virus* (ToLCNDV), *Tomato leaf curl Karnataka virus* (ToLCKaV), *Pepper leaf curl Bangladesh virus* (PepLCBV), *Chilli leaf curl virus* (ChiLCV), *Radish leaf curl virus* (RaLCV) and *Bhendi yellow vein mosaic virus* (BYVMV)

Molecular Detection of Cryptic Species of Whitefly

Total genomic DNA was isolated from the individual adult whitefly and PCR amplification with *mtCO1* primers yielded an expected amplicon with a size of 0.8 kb (Plate 2). Amplified PCR samples were sequenced in both orientations. Sequence analysis revealed that *mtCOI* sequence under study shared highest nucleotide identity of 99.5 per cent with Asia-I genetic species of whitefly. Multiple sequence alignment with reported sequences available at NCBI database were done using clustal-W algorithm

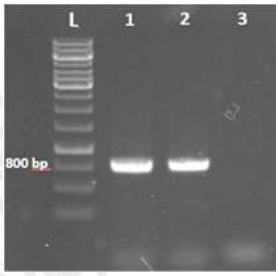

Plate 2: PCR based detection of whitefly (*B. tabaci*) cryptic species using *mtCOI* primers. Lane L:1 kb ladder; Lane 1-2: Whitefly DNA sample, Lane 3: Negative control

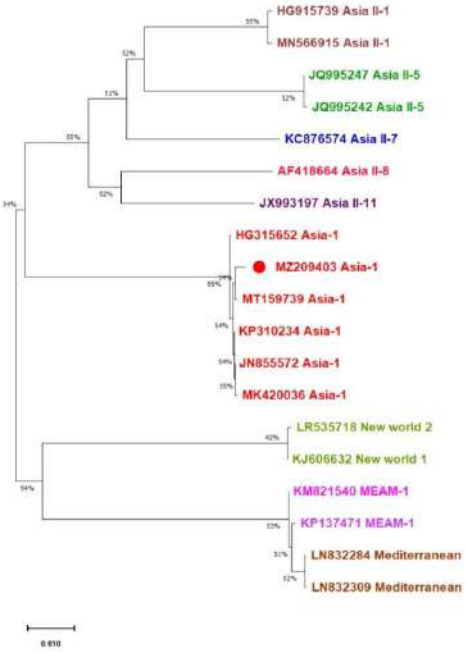

Fig. 2: Phylogenetic tree constructed from nucleotide sequence of whitefly Asia-I with selected sequences of other cryptic species of whitefly retrived from NCBI database using the Neighbor-joining algorithm in MEGA-X

implemented in Mega-X software. In phylogenetic analysis, sequence data under study formed a close cluster with Asia-I cryptic species isolates (Fig. 2). Chowda Reddy *et al*. (2012) identified the presence of five genetic species *viz*., Asia-I, Asia II-5 Asia II-7, Asia II-8 and MEAM-1 based on differences in *mtCOI* sequences. Three whitefly cryptic species *viz*., Asia I, Asia II-1 and Asia II-7 were detected based on *mtCOI* sequences in major soybean growing states of India (Prasanna *et al*., 2015).

Acquisition Access Assay

The minimum acquisition access period of 60 min was required for successful transmission of ChiLCV by Asia-I adults and rate of transmission was increased with increase in duration of acquisition access. However, 100.0 per cent transmission of virus to the assay plants was achieved with 24 AAP (Table 2). These findings are in conformity with Senanayake *et al*. (2012) who reported that a minimum of 3h AAP is required for successful transmission of ChiLCV while Anokhe *et al.,* (2018) reported that 6h AAP with both Asia-I and Asia II-1 cryptic species resulted in 100 per cent transmission of *Mungbean yellow mosaic virus* (MYMV). Jyothi and Nagaraju (2013) reported that a minimum of 1h AAP showed 40 per cent transmission of *Pole bean yellow mosaic virus* and 100 per cent transmission was achieved at 12 h AAP.

TABLE 2

Per cent transmission of *Chilli leaf curl virus* (ChiLCV) by whitefly (*B. tabaci*) Asia-I cryptic species at different acquisition access period

AAP	Asia-I cryptic species of whitefly		
	No. of chilli seedlings infected(R)/ inoculated (N) [a, b]	Transmission rate (R/N)	Transmission (%)
15 min	0/10	0.00	0.00
30 min	0/10	0.00	0.00
1 h	1/10	0.10	10.00
2 h	3/10	0.30	30.00
4 h	4/10	0.40	40.00
8 h	6/10	0.60	60.00
10 h	7/10	0.70	70.00
12 h	8/10	0.80	80.00
24 h	10/10	1.00	100

[a]20 *B. tabaci* adults were used;
[b]24 h IAP (Inoculation access period)

Inoculation Access Assay

ChiLCV was transmitted to healthy chilli plants with minimum IAP of 60 min by Asia-I whitefly adults and transmission rate increased with increase in inoculation access. Further, 100.0 per cent transmission of inoculated plants was achieved with 24 h IAP (Table 3). Vindhyashree *et al*. (2018) reported that 6 h IAP resulted in 100.0 per cent transmission of *Sunflower leaf curl virus*. The presence of virus in the inoculated plants was tested in PCR from 1st day of inoculation till the appearance of symptoms as virus requires an incubation period of 10-12 days for symptom expression. The results from PCR assays showed the presence of virus from the 5th day onwards as virus titre may be low in inoculated plants and may require latent period to replicate in plants after inoculation (Plate 3). *Bhendhi yellow vein mosaic virus*was detected immediately *i.e*., 2nd day after

TABLE 3

Per cent transmission of Chilli leaf curl virus (ChiLCV) by whitefly (*B. tabaci*) Asia-I cryptic species at different inoculation access period

IAP	Asia-I cryptic species of whitefly		
	No. of chilli seedlings infected(R)/ inoculated (N) [a, b]	Transmission rate (R/N)	Transmission (%)
15 min	0/10	0.00	0.00
30 min	0/10	0.00	0.00
1 h	1/10	0.10	10.00
2 h	2/10	0.20	20.00
4 h	4/10	0.40	40.00
8 h	5/10	0.50	50.00
10 h	7/10	0.70	70.00
12 h	8/10	0.80	80.00
24 h	10/10	1.00	100.00

[a]20 B. tabaci adults were used, IAP-Inoculation access period
[b]24 h AAP (Inoculation access period)

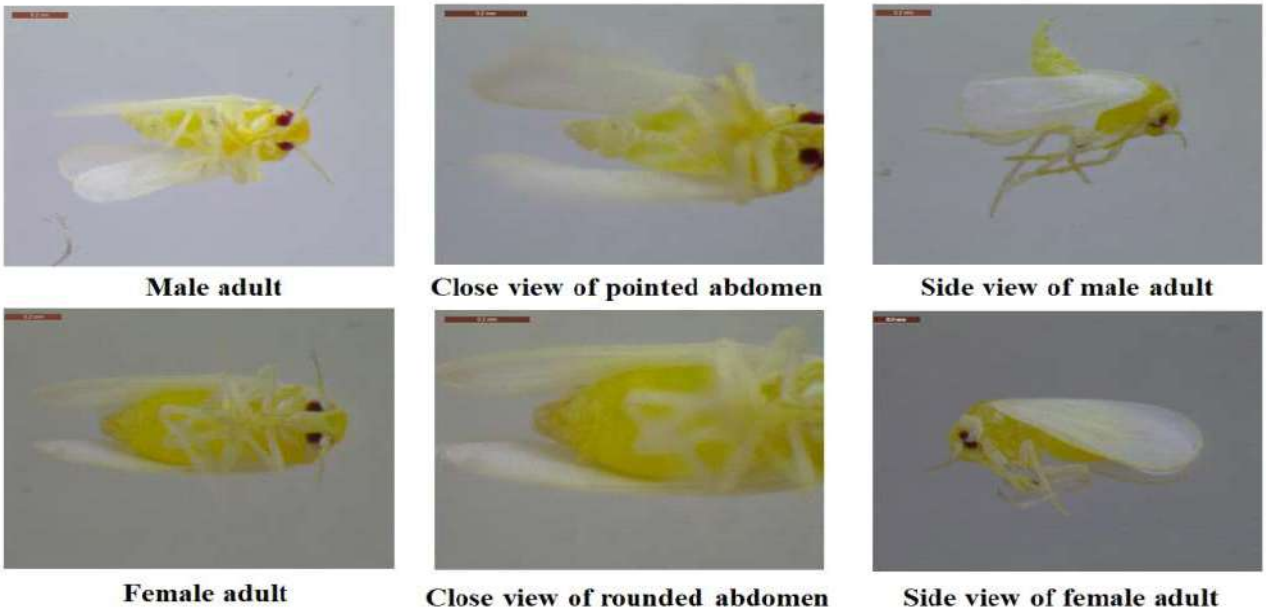

Plate 3: Images for illustrations of male and female whitefly (*B. tabaci*)

inoculation in okra and symptom was expressed at 8-10 days after inoculation (Venkataravanappa *et al*., 2017).

Number of Whitefly Adults and Transmission Efficiency

Asia-I cryptic species of whitefly successfully transmitted the ChiLCV with 24 h of acquisition and inoculation access. The rate of transmission influenced by number of whitefly vectors used in the inoculation experiment. Individual adult whitefly transmitted ChiLCV with an efficiency of 10.0 per cent and transmission efficiency was inflated with increase in viruliferous whitefly population. However, 100.0 per cent transmission was achieved with groups of 20 whitefly adults (Table 4). This clearly indicate that single Asia-I whitefly adult was enough for successful transmission of ChiLCV in chilli and transmission rate was positively correlated with whitefly population used for inoculation. Similar findings were observed inSenanayake *et al*. (2012) who reported that single whitefly able to transmit ChiLCV to 66.6 per cent of inoculated plants and 100.0 per cent transmission achieved with eight whiteflies. *Alternanthera yellow vein mosaic virus* (Chetan *et al*., 2012) was successfully transmitted with single whitefly and recorded 100.00 per cent transmission with five whiteflies. MEAM1 and Asia II-7 transmits the *Papaya leaf curl China virus* with higher efficiency than MED and Asia-I cryptic species (Pan *et al*., 2018).

TABLE 4

Per cent transmission of Chilli leaf curl virus (ChiLCV) with varying whitefly (*B. tabaci*) number

No. of white flies	Asia-I cryptic species of whitefly		
	No. of chilli seedlings infected (R)/ inoculated (N) [a, b]	Transmission rate (R/N)	Transmission (%)
0	0/10	0.00	0.00
1	1/10	0.10	10.00
2	1/10	0.10	10.00
3	2/10	0.20	20.00
5	4/10	0.40	40.00
7	5/10	0.50	50.00
10	6/10	0.60	60.00
15	8/10	0.80	80.00
20	10/10	1.00	100.00

[a]Ten days old chilli seedlings were inoculated;
[b]24 h Acquisition access period (AAP);
24 h Inoculation access period (IAP)

Transmission Efficiency of Asia-1 Cryptic Species of Whitefly Gender on ChiLCV Transmission

Male and female *B. tabaci* adults were distinguished based on size and shape of the abdominal tip (Plate 4). The difference in transmission efficiency

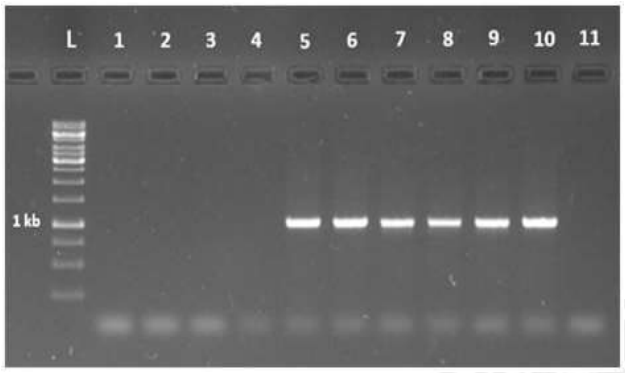

Plate 4: Detection of leaf curl virus in chilli plants at different intervals using ChLCV coat protein gene primers after inoculation access of 24 h. Lane L: 1 kb ladder; Lane 1-10: 1 to 10 days after inoculation of ChLCV to chilli; Lane 11: Negative control.

between male and female cryptic species revealed that females (86.6%) of Asia-I are highly efficient in transmission of ChiLCV to inoculated plants than males (53.0%) (Table 5). Ning *et al*. (2015) reported that females of B and Q biotypes acquire and transmit in greater quantity of virus than males based on the number of probes and duration of probing measured through electrical penetration graphs. Similarly, Xie *et al*. (2012) reported that female whiteflies transmit *Tomato yellow leaf curl virus* with higher efficiency than males as endosymbionts produce GroEL protein in large quantity in females which protects the begomovirus from degradation in the haemolymph.

Effect of Age of Chilli Seedlings on Susceptibility to ChiLCV

The susceptibility studies related to transmission rate of ChiLCV on different aged seedlings revealed that eight days old chilli seedlings are highly susceptible and all seedlings expressed initial leaf curl symptoms within 10 days after inoculation. Further, 80.00 per cent and 40.0 per cent transmission was achieved, respectively with 15 and 30 days old seedlings indicating that disease incidence was inversely proportional to age of the seedlings (Table 6). Asia-I cryptic species successfully transmitted *Bhendi yellow vein mosaic virus* to one week old seedlings with 100.00 per cent efficiency and subsequently decreased with increase in age (Venkataravanappa *et al*., 2017).

TABLE 6

Transmission of Chilli leaf curl virus (ChiLCV) to different aged seedlings of chilli through whitefly (*B. tabaci*) vector

Age of chilli seedlings (Days)	Asia-I cryptic species of whitefly		
	No. of chilli seedlings infected(R)/ inoculated (N) [a, b]	Transmission rate (R/N)	Transmission (%)
10	10/10	1.00	100
15	8/10	0.80	80.00
20	6/10	0.60	60.00
30	4/10	0.40	40.00

[a]20 B. tabaci adults were used;
[b]24 h Acquisition access period (AAP);
24 h Inoculation access period (IAP)

TABLE 5

Transmission efficiency of whitefly (*B. tabaci*) gender of Asia-1 cryptic species on *Chilli leaf curl virus* (ChiLCV) transmission

Sex of *B. tabaci* adults	No. of whiteflies/plant	No. of chilli seedlings infected (R) / inoculated (N) [a, b]	Transmission rate (R/N)	Transmission (%)
Female	20	13/15	0.86	86.66
Male	20	8/15	0.53	53.00

[a]24 h Acquisition access period (AAP); 24 h Inoculation access period (IAP)

Virus-vector interaction studies clearly indicated that a single Asia-I whitefly could able to transmit ChiLCV and transmission efficiency inflated with increase in whitefly number, AAP and IAP. Female whitefly transmits ChiLCV with higher efficiency than males and susceptibility of chilli seedlings to ChiLCV decreased with age. The differential transmission of ChiLCV with whitefly species and virus-vector interaction studies would help greatly to assess the disease risk and which provide a basis for further research on cryptic species with specific virus interaction.

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