Pharmacogn. Mag.
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ORIGINAL ARTICLE

Molecular Cloning and Quantitative Real-time PCR Analysis to Study the Expression of Tryptophan Decarboxylase Gene from Chillies (*Capsicum annuum* L.) against Whitefly

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Submitted: 04-Aug-2021 Revised: 14-Oct-2021 Accepted: 29-Dec-2021 Published: 07-Jul-2022

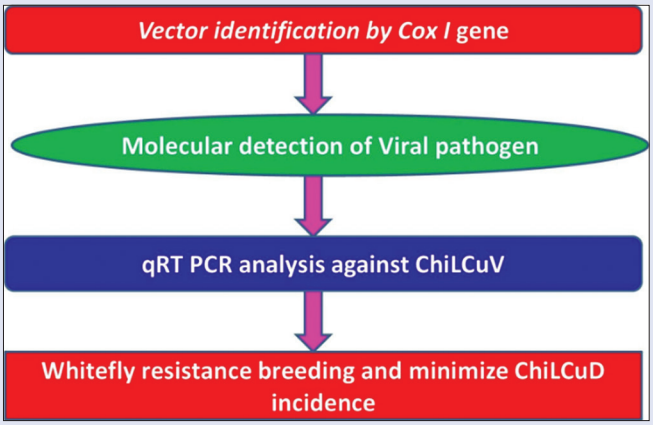
ABSTRACT

Background: One of the amazing and economical spices is Chilli (*Capsicum annum* L.) and also called "wonder spice," and it gives great significance to traditional household use and export as well as such a remarkable cash crop in India. However, the important constraints in chilli production are chilli leaf curl disease which has been caused by whitefly (*Bemisia tabaci*) transmitted by begomoviruses and leads to a major decline in yield. **Aim:** To explore the stability of the insect resistance gene (tryptophan decarboxylase [TDC]) in resistant genotypes after being challenged with a known number of whiteflies. Since, biotic stress defense allied mechanism regulated during tryptophan biosynthesis. **Materials and Methods:** The presence of endogenous genes was verified using the polymerase chain reaction (PCR), cloning (T/A vector), and gene expression level (quantitative real-time PCR). **Results:** Amplification of mitochondrial *coxI* gene fragment using the primer (C1-J-2195 and L2-N-3014) produced *B. tabaci* specific ~ 800 bp band. Besides, the whitefly sequence was aligned with the NCBI blast where 92% of identity was observed. TDC gene expression was greater in P2 accession leaves at 48 h post-infestation with 20 number of *B. tabaci* after feeding and downregulation in less TDC gene expression level at moderately resistant accession ACC 20. Further, all the amplified gene sequences were aligned using NCBI BLAST. The expression of TDC genes in chillies could demoralize the *B. tabaci* fitness causes mortality. **Conclusion:** The satisfactory control of viral disease may be achieved with the application of molecular biology, DNA, and RNA-based technologies include the cloning of insect resistance genes.

Key words: *Bemisia tabaci,* ChiLCuV, molecular cloning, phylogenetic analysis, quantitative real-time polymerase chain reaction, tryptophan decarboxylase gene

SUMMARY

- Amplification of mitochondrial cytochrome oxidase cox I gene fragment using the primers (C1-J-2195 and L2-N-3014) produced *B. tabaci*-specific 800 bp band. Amplification of leaf curl virus using AC1 (replication initiation protein gene) (nt1521-2606) specific primers produced 1086 bp band and further confirmation of chilli leaf curl virus achieved by using the coat protein (CP) gene. The amplification of leaf curl virus using CP gene produced 750 bp band. Amplification of TDC using gene-specific primers produced 1509 bp band on different chillies accessions.
- Amplified full-length TDC gene (1509 bp) was cloned into T/A cloning vector (pTZ57R/T). The transformed colonies were selected by LB medium with ampicillin and X-gal based on blue/white colony selection. The transformed white colonies and non transformed blue colonies were further confirmed by the colony polymerase chain reaction (PCR).
- In quantitative real-time PCR analysis, there was a significant difference in TDC gene expression levels either between or within accessions. Although TDC gene expression was greater in P2 accession and downregulated in less TDC gene expression level at moderately resistant accession ACC 20. At the same time, relatively TDC gene expression was also detected in ACC 01 and ACC 16. Within each accession also, there were significant differences in TDC gene expression levels when compared against respective control of the accession. The low level of expression was observed on within the accessions of ACC 16 and ACC 20.



Abbreviation used: ChiLCuD: Chilli leaf curl complex disease; ChiLCuV: Chilli leaf curl virus; TDC: Tryptophan decarboxylase; PCR: Polymerase chain reaction; qRT-PCR: quantitative real-time PCR; coxI: mitochondrial DNA coxI; NCBI: National Center for Biotechnology Information.

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DOI: 10.4103/pm.pm_362_21

Access this article online
Website: www.phcog.com
Quick Response Code:

Cite this article as: Jeevanandham N, Marimuthu M, Natesan S, Gandhi K, Prabakar N, Elango P, *et al*. Molecular cloning and quantitative real-time PCR analysis to study the expression of tryptophan decarboxylase gene from chillies (*Capsicum annuum* L.) against whitefly. Phcog Mag 2022;18:476-93.

INTRODUCTION

Chilli is regarded as a profit-making pungency crop and also named as a wonder spice. It is cultivated for several purposes, namely vegetables, pickles, and condiments. The pericarp and placenta of fruits were confined with an alkaloid called "capsaicin" which elicits pungency. Capsaicin is widely used in industries such as pharmaceuticals, cosmetics, and beverage industries. *Capsicum annuum* L. (red pepper) comes under the genus capsicum, *Solanaceae* family.[1] The maximum existence of diversity for different quality parameters among chillies has paved the way for the improvement of chillies in India. India is the leading exporter of chillies around the world.[2] Nevertheless, the crop is infested by more than 21 insects and noninsect pests[3] Particularly, the production of chillies is seriously limited by many viral diseases mainly the occurrence of chilli leaf curl disease (ChiLCuD) transmitted by *Bemisia tabaci* is a grave threat to chillies[4] *B. tabaci* (G) (Hemiptera: Aleyrodidae) is significant agricultural and horticultural pest in India as well as the world since it has more adaptability, i.e., wide host range and capability to act as a vector could transmit more than 110 plant pathogenic viruses. Of them, huge begomoviruses were transmitted rigorously by phloem-feeding hemipteran such as *B. tabaci*. The *Begomovirus* genus includes at least 200 species[5] which are primarily transmitted by whiteflies.[6] The leaf curl disease of chilli is caused by chilli leaf curl virus transmitted by viruliferous whiteflies which have come under the begomoviruses group. The ChiLCuV is the most important pathogen associated with chillies and causes severe yield loss which has been transmitted by the vector in a persistent circulative manner. The genus, namely, *Carlavirus*, *Potyvirus*, *Begomovirus*, *Closterovirus*, *Nepovirus*, and *Geminivirus* were transmitted by *B. tabaci*. However, *Begomovirus* is a major issue in the vegetable cultivation. As a result, these investigations were carried out. One of the interesting facts is that, several years ago, it was found that, TDC (EC 4.1.1.28) catalyzes tryptophan into tryptamine which has ultimately been involved in the defense against herbivores.[7]

Whiteflies show outstanding skewing in host preference and are largely recognized to the secondary plant metabolites accumulated in the host plants principally, terpenoid indole alkaloids (TIAs), biosynthesis regulated by enzyme tryptophan decarboxylase (TDC) and discovered that it influenced the settling behavior of whitefly for feeding and oviposition. Any intrusion in the multiplication of vectors will retard the dispersal of diseases. TDC interferes with whitefly survival and offered a single gene-based defense.[8] Thus, cultivated and wild species of *Capsicum* line were noticed for the presence of TIAs and TDC enzyme. Accessibility and availability of DNA markers for the TDC gene may help in the selection of the resistance genotypes in a breeding program without the disease. Further cloning of the TDC gene was useful for molecular breeding programs. It was isolated and amplified from chilli accessions using a polymerase chain reaction (PCR) approach and perform cloning purposes to understand the biological influence on whiteflies. Thus, the study envisages the use of molecular approaches to select resistant lines was useful for whitefly resistance breeding and minimize ChiLCuD incidence in chillies.

MATERIALS AND METHODS

Polymerase chain reaction-mediated amplification

Genomic DNA was isolated from chillies accessions using the CTAB method. TDC gene was isolated and amplified using TDC the gene-specific primers were designed based on the sequences from NCBI using the Primer Premier 6.0 software (Premier Biosoft International, Palo Alto, CA, USA) [Figure 1]. A guideline for designing gene-specific primers was explained by Nirali and Nutan.[9] The list of gene-specific primers used in this study is listed in Table 1. The primer sequences were synthesized from Eurofins Genomics India, Bangalore, India, were used for PCR reaction. The PCR was conducted in a Fast PCR (Medline, U. K). The thermocycler was set for 34 cycles of denaturation at 94°C 30 s, annealing at 65.3°C for 30 s, and extension at 72°C for 45 s. Runs included an initial denaturation at 94°C for 5 min and a final extension step at 72°C for 7 min. PCR products were electrophoresed on 1.2% agarose gels stained with ethidium bromide (10 mg/ml) and were viewed in a gel documentation system (Bio-rad, USA) [Plate 1]. The amplified DNA was purified using a Genei pure gel extraction kit (Bangalore, India).

Cloning and sequencing of DNA fragments

Elution and purification of the polymerase chain reaction product

Amplified PCR products were electrophoresed in 1.5% (w/v) agarose gel stained with ethidium bromide [Plate 1]. PCR fragments of the individual amplicon were excised under a UV transilluminator and purified using Gel Elution Kit (GeNei™ Bangalore, India), by essentially following the manufacturer's instructions.

Technique

- A PCR fragment of the individual amplicon was excised under UV transilluminator (Fotodyne, USA) to a preweighed fresh 1.5 ml vial, and the gel weight was noted down
- The Gelsolubliser (about 300 µL) was added to the vial
- Then, the sample was incubated at 55°C until the contents were dissolved and 400 µL of isopropanol was added to the contents
- The GeneiPure™ Column was placed in a 2 ml collection tube and the sample was loaded. The contents were centrifuged at 11,000 rpm for 1 min
- The flow was discarded and the GeneiPure™ Column was placed in a 2 ml collection tube and 150 µL of wash buffer was diluted with 600 µL of ethanol
- The diluted wash buffer (about 700 µL) was added to the column and once again centrifuged at 11,000 rpm for 1 min
- The flow was discarded and the GeneiPure™ Column was placed in a 2 ml collection tube. The collection tube with a column was centrifuged for 12,000 rpm for 2 min to remove the traces of the wash buffer
- The GeneiPure™ Column was placed in a sterile 1.5 ml collection tube and 40 µL of Elution buffer was added. The contents were centrifuged at 11,000 rpm for 1 min
- The eluted DNA was stored at −20°C.

Cloning into T/A vector

The purified DNA product was cloned into a T/A cloning vector (InsTAclone™ PCR Cloning Kit Cat. no. #1213, Thermo Scientific) according to the manufacturer's protocol.

Ligation

The ligation reaction was carried out with a linearized T/A vector and PCR amplified DNA fragment using T4 DNA ligase as described in the manufacturer's protocol. The reaction cocktail was as follows: Nuclease free water 16 µL, PCR amplified and purified DNA fragment4 µL, 5X Ligation Buffer6 µLVector pTZ57R/T (0.17 pmol) T4 DNA ligase 1 µL, Total volume is 30 µL. Vortexed briefly and centrifuged for 3–5 s, after that incubated the ligation mixture at the room temperature (22°C) for 1 h. If the maximal number of transformants is required, incubated overnight at 4°C. 5 µl of the ligation mixture was used directly for bacterial transformation.

Figure 1: The mRNA tryptophan decarboxylase gene sequence of *Capsicum annuum* from NCBI with the primer information used for the amplification of the gene

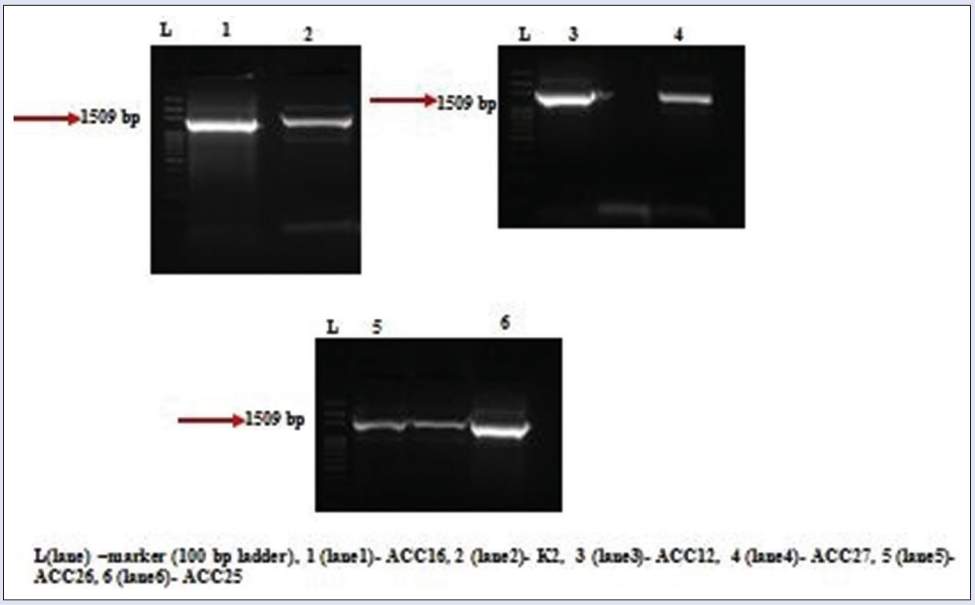

Plate 1: Polymerase chain reaction amplification of tryptophan decarboxylase gene using gene specific primers on chiilies accessions for cloning

Table 1: List of gene-specific primers used for amplification of tryptophan decarboxylase gene from chillies

Primer name	Forward primer	Reverse primer	Length (bp)
TDC 1	GCTCGTTGTCTATGGTTCT	CTCAGGATTCGTGCTCAAT	463
TDC1V2	GTACTTACCTGAGTCATTGGA	GTATGGATCGGACCTAACG.	1036
TDCV3NEW	CCCCAAATAACATGGGTAGCCT	GTAGCAAAGCATCGGCACAG	1509

TDC: Tryptophan decarboxylase

Transformation

- The overnight bacterial culture (150 µl) was added to 1.5 ml of prewarmed C-medium
- Incubated 20 min at 37°C in a shaker
- It was then transferred to 1.5 ml mini centrifuge tubes and the cell suspension was pelleted by centrifugation at 3000 rpm for 3 min, at 4°C and the supernatant was discarded
- An equal volume (250 µl) of each T-Solution (A) and T-Solution (B)

was pipetted out in an Eppendorf tube kept in ice
- From this, 300 µl was transferred to the pelleted cells. Pellet was dissolved and kept in ice for 5 min
- Resuspended the cells in 300 µl of T solution and incubated on ice for 5 min. The cell suspension was again pelleted by centrifugation at 3,000 rpm for 3 min, at 4°C and the supernatant was discarded
- Resuspended the pelleted cells in 120 µl of T solution and incubated for 5 min on ice
- For 50 µl of the above prepared competent cells, 5 µl of the ligated mixture was added and kept on ice for 5 min.

Selection

- Appropriate quantity of cells (50–60 µl) was inoculated on LB agar containing 100 µL ampicillin for every 100 ml LB medium (50 mg/ml) and X-Gal (40 µl/plate) and incubated at 37°C for 24 h
- After incubation, blue (transformed but self-ligated plasmids) and white (transformed with a gene of interest) colonies have appeared on the plate
- The white colonies were picked up and grow on another LB plate with ampicillin for 24 h.
- The transformed colonies were further confirmed by colony PCR which produced the specific 1509 bp product
- The genuine colonies were picked up and streaked on another LB plate with 20 µL ampicillin (50 µg/ml) for 12 h [Plate 2].

Experimental setup to collect tissue to study the tryptophan decarboxylase gene expression

- The procedure suggested by Murugan and Smith[10] with modification was followed. Experiments were conducted in the greenhouse of Insectary, Agricultural College and Research Institute, Madurai
- Plants were maintained at 30°C–35°C temperature and 70%–80% of relative humidity
- Chillies plants (ACC 01, ACC20, ACC27, P2, and ACC16) were grown in 6.5 cm diameter mud pots with coco pith and soil potting mix
- Ten days after sowing, four-leaf stage chillies were selected with similar growth conditions by size and appearance
- Plants were organized in a completely randomized design with three replicates
- Each of the infested and noninfested plants was enclosed in a glass chimney (6.5 cm dia × 15 cm height) in an inverted position with a mouth to the bottom and base at the top that was lined with a 100 µ mesh cloth to prevent the escape of adult whiteflies
- Three levels (0, 10, and 20/plant) of whitefly infestation were followed and plant tissue harvest was done 48 h post-infestation
- A 30 min buffer period was allowed for introduced whiteflies to settle
- Three biological replicates of one plant per replicate were used to collect the required plant tissues.

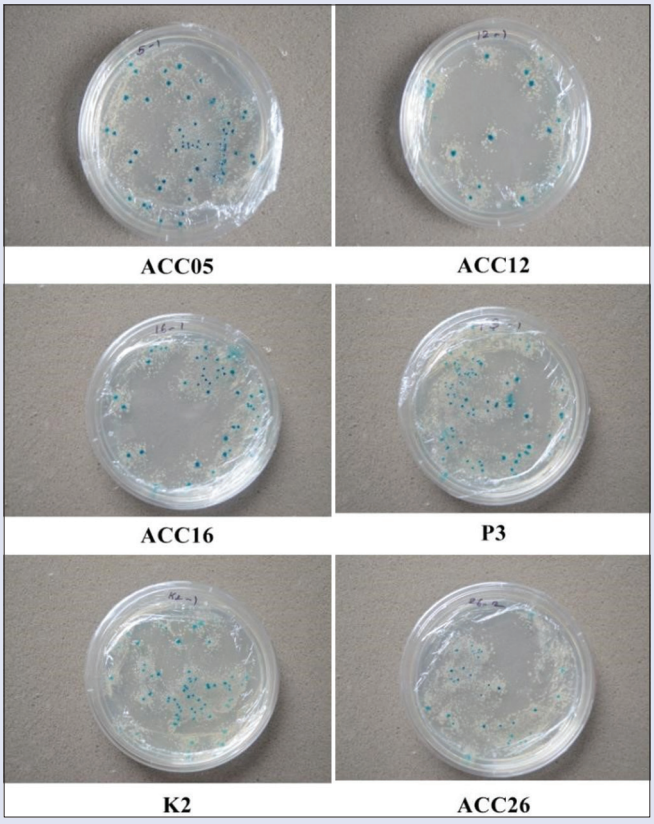

Plate 2: Cloning of tryptophan decarboxylase gene from chillies with vector pTZ57R/T

Expression of tryptophan decarboxylase gene under whitefly infestation

Isolation of total RNA

Total RNA was extracted from frozen whole plant tissue of the following chillies accession *viz.*, ACC 01, ACC20, ACC27, P2, and ACC16 by Trizol® method [Plate 3]. The working table, micropipettes were cleaned with 75% ethanol to avoid RNase contamination from the floor and body.

Method

- Tissue samples of 1 g were ground to a fine powder using liquid nitrogen
- About 1 ml of TRI reagent (Sigma, USA) was added into this powdered tissue, and the tubes were vigorously shaken for homogenous mixing of TRI reagent (Sigma) with the sample
- The samples were allowed to complete dissociation of nucleoprotein complexes and release of RNA by incubating at the room temperature for 5 min
- The tubes were centrifuged at 12,000 rpm for 10 min at 4°C. All the insoluble materials such as cellular membranes, high molecular weight DNA, and polysaccharides precipitated at the bottom of the tubes
- As the RNA is small in molecular weight and they will be separated in the supernatant, along with low-molecular weight DNA and proteins
- The supernatant containing the RNA was transferred to a fresh tube and to that 200 µl of the chloroform was added

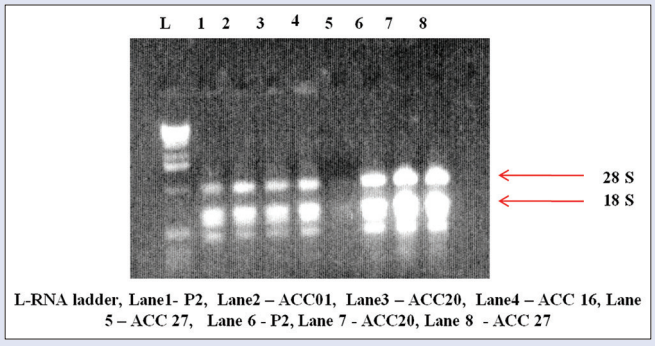

Plate 3: Total RNA on different chillies accessions challenged with whitefly *Bemisia tabaci*

- The tubes were shaken vigorously for 15 s and again incubated at the room temperature for 5–10 min and later centrifuged at 12,000 rpm for 15 min at 4°C
- After centrifugation, the upper aqueous phase was transferred to a fresh centrifuge tube
- With the available supernatant, 250 µl of isopropanol and 250 µl of 2M NaCl was added, mixed by inversion, and incubated at the room temperature for 30 min and the solution was centrifuged at 12,000 rpm for 10 min at 4°C
- The supernatant was drained from the Eppendorf tube and the RNA was pelleted at the bottom of the Eppendorf tube and was washed with 1 ml of 75% ethanol and centrifuged at 12000 rpm for 5 min at 4°C
- The supernatant was discarded and the pellet was air-dried for 10 min and dissolved with 50 µl of 1% DEPC treated water and stored at −80°C.

RNA quality check by agarose gel electrophoresis

The quality of the RNA was checked in 1% agarose gel by visualizing the intactness of RNA bands by performing agarose gel electrophoresis. Two microliter of RNA sample dissolved in DEPC water was pipette out and mixed with 2 µl of 6x loading dye and loaded in 1% gel cast the using 1 × TBE buffer and DEPC water. The gel was to run at 120 volts for 1 h 30 min and bands were visualized and documented in the gel documentation system. The viewed picture was photographed and saved for further scrutiny.

Protocol

The open ends of the Pyrex gel casting plate were sealed with cello tape and placed on a perfectly horizontal platform. Agarose (1%) was added to 1 × TBE, boiled until the agarose dissolved and cooled to lukewarm temperature completely. It was then poured into the gel mold and the comb was placed properly and allowed solidifying. After solidification of the agarose, the comb and the cello tape were removed carefully. The cast gel was placed in the electrophoresis unit with wells toward the cathode and submerged with 1 × TBE to a depth of about 1 cm.

Loading the products

Three microliters of RNA were loaded with 2 µl of loading dye. The voltage was maintained at 120 volts for 1 h 30 min. The bands were visualized and documented in the gel documentation system. The viewed picture was photographed and saved for further scrutiny.

RNA quantification

The quantity of extracted RNA was checked using Eppendorf Biospectrometre® Kinetic Germany.

Protocol

The instrument was initialized with 1.0 µL of Milli Q® water. 1.0 µL of Milli Q® water was used as blank and 1.0 µL of unknown RNA was added to the instrument and the quantity measured. To ensure accuracy, the quantification of each unknown sample is repeated twice.

cDNA synthesis

The total RNA was reverse transcribed to complementary DNA (cDNA) using iScript™ cDNA Synthesis Kit (Bio-rad, USA) following the manufacturers' protocol and was used as a template for qRT-PCR analysis. Reverse transcription reaction consisted of 5 X iScript reaction mix (4 µl), iScript Reverse Transcriptase, 20 U/µl (1 µl) and nuclease-free water (14 µl), 1 µg/µl of total RNA as template in a final reaction volume of 20 µl. The PCR was conducted in a Fast PCR (Medline, U. K) programmed as, Priming −25°C for 5 min; Reverse transcription at 46°C for 20 min; RT inactivation done using 95°C for 1 min, Hold the sample at 4°C.

Primer design

Specific primers were designed for selected gene-specific sequences using Beacon Designer ® software (Premier Biosoft International) under SYBR Green Design, BLAST searched for cross-homology to complete sequences in the barley genome and tested for template structure to avoid the formation of primer secondary structures. The primer sequences were synthesized from Eurofins Genomics India (Bangalore, India) was used for PCR reaction. The *C. annuum* actin gene[11] was used as an internal reference gene and primers were designed using the same method.

Quantitative real-time–polymerase chain reaction

- Quantitative real-time PCR (qRT-PCR) reactions were performed using a Roche Light Cycler 480 (Roche Diagnostics, India)
- Each qRT-PCR reaction consisted of 10 µl 2x iQ SYBR Green Supermix (Bio-Rad), 0.8 µl each of forward and reverse primers (10 mM), and 2 µl of cDNA as the template in a final reaction volume of 20 µl
- Each PCR plate included noninfested susceptible controls for making comparisons and calculations of different genes at different time points
- Each plate carried three technical replicates of each cDNA biological replicate and the actin internal control gene
- The qRT-PCR amplification protocol was 95°C for 15 min and 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 40 s each
- The threshold was determined as the first cycle above the background fluorescence in which all samples were in the exponential amplification phase
- After the amplification step, the machine continued to proceed for dissociation (melt) curve analysis to determine if only one product was amplified during annealing and amplification
- A single peak in the dissociation curve implied that only the gene of interest was amplified. For the dissociation curve, PCR conditions were 95°C for 1 min, 55°C for 1 min, and an increase in set point temperature after cycle 2 by 0.5°C for each cycle at every 10 s.

Quantitative real-time–polymerase chain reaction calculation

Efficiency ranged from 97.6% to 101.1% for all primers. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method[12] with BioRad Gene Expression Macro Version 1.1, where ΔΔCt = ([Ct for sample cDNA-Ct for control cDNA] $_{TDC}$)−([Ct for sample cDNA-Ct for control cDNA] $_{ACTIN}$). TDC was the gene of interest and ACTIN was the internal control gene. For TDC gene, fold change was calculated as, $2^{-\Delta\Delta Ct}$ where ΔΔCt = ([Ct for sample cDNA-Ct for control cDNA] $_{TDC}$)−([Ct for sample cDNA-Ct for control cDNA] $_{ACTIN}$). Here, the susceptible uninfested plant is treated as control. Within each accession, the fold change was calculated as, $2^{-\Delta\Delta Ct}$ where ΔΔCt = ([Ct for sample cDNA-Ct for control cDNA] $_{TDC}$)-([Ct for sample cDNA-Ct for control cDNA] $_{ACTIN}$). Here, the respective uninfested plant is treated as control [Plate 7,8,9,10,11,12 and 13]. When relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method, the time 0 uninfested susceptible control (ACC 27) gave a fold change value of 1.

Comparison of tryptophan decarboxylase genes amplified from susceptible and resistant chillies accessions

DNA from one resistant ACC 16 accession, ACC 03 moderately resistant accession, and susceptible accessions ACC 27 and ACC 10 were

Table 2: List of primers set used for comparison of tryptophan decarboxylase genes amplified from susceptible and resistant chillies accessions

PCR product (30 µl each)	Sequenced			
	Forward sequencing		Reverse sequencing	
	I	II	I	II
16Replication1	TDCV3F + TDC1R	TDC1F + TDCV3R	TDCV3F + TDC1R	TDC1F + TDCV3R
16Replication2	TDCV3F + TDC1R	TDC1F + TDCV3R	TDCV3F + TDC1R	TDC1F + TDCV3R
27Replication1	TDCV3F + TDC1R	TDC1F + TDCV3R	TDCV3F + TDC1R	TDC1F + TDCV3R
27Replication1	TDCV3F+ TDC1R	TDC1F + TDCV3R	TDCV3F + TDC1R	TDC1F + TDCV3R

TDC: Tryptophan decarboxylase; PCR: Polymerase chain reaction

amplified with the 1509 bp primer set, and the amplified products were sequenced [Plate 4, 5, 6] after purification with multiple replications as mentioned in Table 2.

Sequencing

The eluted PCR fragments were sent for sequencing. The Sanger sequencing was done with M13 forward and reverse primers at the Agri genome, Kochi, Kerala, India. Sanger sequencing, also known as the chain termination method, is a technique for DNA sequencing based upon the selective incorporation of chain-terminating dideoxynucleotides (ddNTPs) by DNA polymerase during *in vitro* DNA replication.[13] With the automated procedures, the reactions were performed in a single tube containing all four ddNTP's., each labeled with a different color dye. Since the four dyes fluoresce at different wavelengths, a laser then reads the gel to determine the identity of each band according to the wavelengths at which it fluoresces. The form of the chromatogram, which was a diagram of colored peaks that correspond to the nucleotide in that location in the sequence.[14] The color represents the four bases; blue was C, green was A, black was G, red was T.

Polymerase chain reaction product sequencing

The amplified products of the target gene of interest were purified using Hi Pure A™ PCR product purification kit/Gel Elution Kit (GeNei™ Bangalore, India), by essentially following the manufacturer's instructions and sent for sequencing.

Method

- In a 2.0 ml capped collection tube, 5 volumes of PCR Binding Solution (SPB) to 1 volume of the PCR sample were added and mixed well by pipetting
- Then transferred the mixer to the HiElute miniprep spin column (capped) and centrifuged for 1 min at 12,000 RCF. Discarded the flow-through and replace the column in the same collection tube
- After that, 700 µl diluted wash solution (HPE) was added to the column and centrifuged for 1 min at 12,000 RCF in a tabletop microcentrifuge
- Discarded the flow-through and replaced the column in the same collection tube. Centrifuged for 1 min at 12,000 RCF to remove excess ethanol
- Transferred the column to a clean 2.0 ml uncapped collection tube, pipetted 50 µl of elution buffer (ET) to the center of the column and incubate at the room temperature (15°C–25°C) for 1 min. Centrifuged for 1 min at 12,000 RCF in a tabletop microcentrifuge
- Transferred the eluted product to a fresh capped 2 ml collection tube for longer DNA storage. The purified PCR amplification product present in the eluate is ready for immediate use
- For long-term storage, −20°C or lower temperature (−80°C) was recommended. For using the above method, full-length sequences of TDC genes, *coxI* sequences of *B. tabaci*, and replication initiation protein gene of chillies leaf curl virus were processed and sent to further studies and sequencing.

BLAST analysis

Sequences of the gene were well-surveyed in the database (available in GenBank). The BLAST program was linked to the gene of interest database and GenBank, enabling the user to input the target gene sequence of an "unknown' isolate and received a diagnostic report that provides target gene identification, or the closest match as an "output." BLAST searches of the target gene database could be carried out at the site and yield an output that contains a list of the highest scores and percent sequence identities. The closest match to a taxon in the database with the highest sequence homology was also considered.

Analysis of sequence

Homology search was carried out using Blast (http://www.ncbi.nlm.nlh.gov) and the difference in the sequences was determined using the sequence alignment editor Bio Edit version (7.1) and compared against the consensus sequences of National Center for Biotechnology Information (NCBI). The alignment was further analyzed using the MEGA 5.0 program, using the Neighbor-joining method with a "bootstrap" value of 1000.

Phylogenetic analysis for *Bemisia tabaci*, ChiLCuV using AC1-specific primer, ChiLCuV using coat protein gene and tryptophan decarboxylase genes

Fifteen sequences covering the mitochondrial DNA *coxI* sequences of *B. tabaci* were used in the study and aligned using clustalW. Two sequences covering the replication initiation protein gene of pepper leaf curl virus were used in the study and aligned using clustalW. Twelve sequences covering the coat protein (CP) gene of chilli leaf curl virus were used in the study and aligned using clustalW. Thirteen sequences covering the TDC gene sequences were used in the study and aligned using clustalW. Multiple sequence alignments were produced using the clustalW available in MEGA v. 5 and subsequently trimmed using the MEGA v. 5[15] Phylogenetic relationships were inferred using the neighbor-joining method[16] with bootstrapping.[17] Evolutionary distances were computed using the P-distance method[18] using MEGA v. 5. Bootstra *P* values (1000 iterations) were calculated based on the >70% majority rule and confidence limits were placed at the major nodes of the tree.

Statistical analysis

Data from the resistance category experiments were analyzed using a one-way analysis of variance.[19] The data on percentages were transformed with arcsine √ percent values and the population numbers $\sqrt{x+0.5}$ before the statistical analysis. The data obtained from laboratory experiments were analyzed in a completely randomized design, while the same from field trials were analyzed in RBD.[20]

RESULTS

Polymerase chain reaction-mediated amplification of tryptophan decarboxylase gene

Amplification of TDC using gene-specific primers produced a 1509 bp band [Plate 1]. Chillies genome sequence was larger in size. However, to get accurate result at the maximum of 1.5 kb was isolated and amplified. TDC gene was determined using the sequence alignment editor Bio Edit version (7.1) and compared against the consensus sequences (GenBank: FJ710789.1 *Capsicum annuum* TDC1 mRNA, complete cds) of NCBI [Figure 2]. Furthermore, the TDC gene sequence was aligned using NCBI BLAST and 98% of homology was observed with the TDC gene sequence (GenBank: FJ710789.1) deposited in NCBI [Figure 2].

Cloning and sequencing of polymerase chain reaction fragments of tryptophan decarboxylase gene

The TDC gene expressed chillies accessions were found, eluted [Plate 1]. The purified DNA product was cloned into a T/A cloning vector [Figure 3]. The transformed colonies were selected by LB medium with ampicillin and X-gal based on blue/white colony selection [Plate 2].

Expression of tryptophan decarboxylase gene under whitefly infestation using quantitative real-time polymerase chain reaction analysis

In qRT-PCR analysis, there were significant differences in TDC gene expression levels either between or within accessions

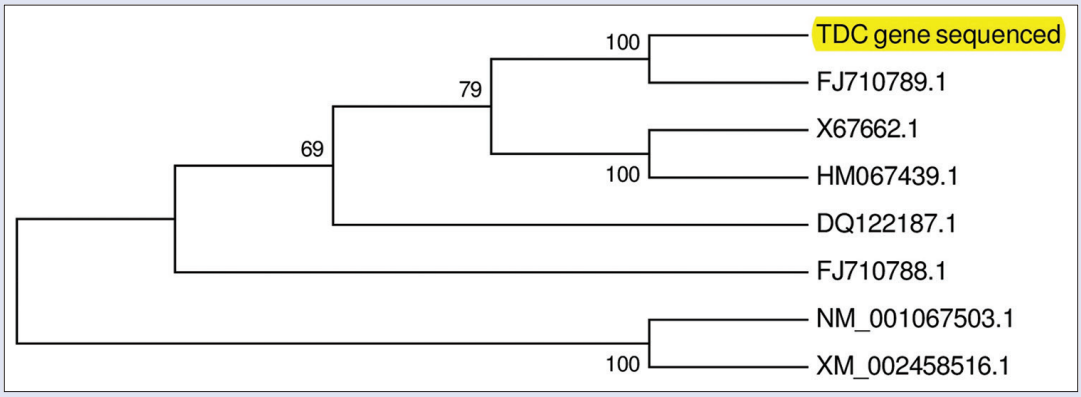

Figure 2: Cladogram of tryptophan decarboxylase genes of different plant species. . NM_001067503.1| *Oryza sativa* Japonica Group mRNA, complete cds; DQ122187.1| *Gossypium hirsutum* tryptophan decarboxylase mRNA, partial cds; FJ710788.1| *Capsicum annuum* tryptophan decarboxylase (TDC2) mRNA, complete cds; FJ710789.1| *Capsicum annuum* tryptophan decarboxylase (TDC1) mRNA, complete cds; XM_002458516.1| Sorghum bicolor hypothetical protein, mRNA; X67662.1| *C. roseus* tdc gene for tryptophan decarboxylase; HM067439.1| *Rauvolfia verticillata* tryptophan decarboxylase (TDC) mRNA, complete cds

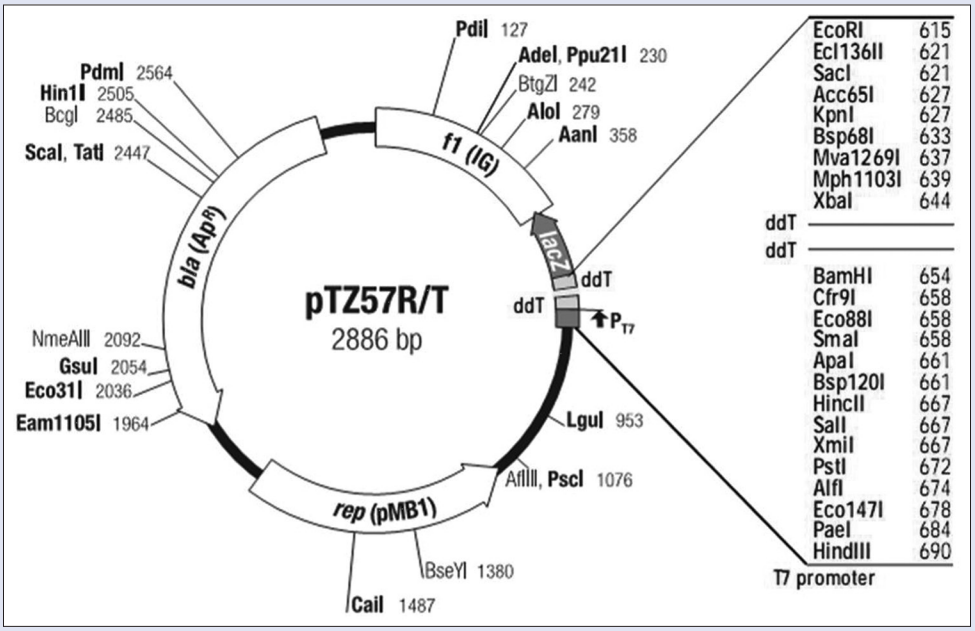

Figure 3: Map of the P TZ57R/T cloning vector

Table 3: Upregulation of tryptophan decarboxylase gene on different chillies accessions after feeding by *Bemisia tabaci* adults for 48 h infestation

Chillies accessions	48 h post-infestation	Fold change (TDC gene) $2^{-\Delta\Delta Ct}$
ACC 27 (S)	0	Susceptible control
	10	
	20	
P2 (R)	0	4.22
	10	8.76
	20	10.38
ACC01 (R)	0	2.07
	10	7.77
	20	5.74
ACC20 (MR)	0	5.23
	10	7.46
	20	6.29
ACC16 (R)	0	8.89
	10	6.87
	20	7.69

TDC: Tryptophan decarboxylase

Table 4: Upregulation of tryptophan decarboxylase genes within the chillies accession with with varying challenged numbers of *Bemisia tabaci* adults for 48 h infestation

Chillies accessions	48 h post-infestation	Fold change (TDC gene) $2^{-\Delta\Delta Ct}$
ACC 27 (S)	0	Control
	10	1.54
	20	1.83
P2 (R)	0	Control
	10	2.37
	20	1.62
ACC01 (R)	0	Control
	10	4.03
	20	2.91
ACC20 (MR)	0	Control
	10	1.32
	20	1.17
ACC16 (R)	0	Control
	10	0.99
	20	0.91

TDC: Tryptophan decarboxylase

[Tables 3 and 4] [Plate 9-13] Although TDC gene expression was greater in P2 accession leaves at 48 h postinfestation with 20 numbers of *B. tabaci* after feeding and downregulation in less TDC gene expression level at moderately resistant accession ACC 20. At the same time, relatively TDC gene expression was also detected in ACC 01 and ACC 16. Within each accession also, there were significant differences in TDC gene expression levels when compared against respective control of the accession [Table 3]. A low level of expression was observed within the accessions of ACC 16 and ACC 20. A significant difference was detected on susceptible accession ACC 27 at 0, 10, and 20 numbers of adult feeding after 48 h of postinfestation. There were significant differences evident between resistant and susceptible accessions [Table 4].

Comparison of tryptophan decarboxylase genes amplified from susceptible and resistant chillies accessions

The TDC gene sequences comparison was studied on the accessions ACC 16 (resistant), ACC03 (moderately resistant), ACC10 (susceptible), and ACC 27 (susceptible) with *C. annuum* TDC1 mRNA [Plate 4]. The conserved regions (1134 bp) were furnished in Plate 5. The results revealed that at 17 bp T was present in ACC03 and ACC 10 while it was absent at 21 bp. At 27 bp and 31 bp A was present in ACC03 and ACC 10 whereas C was present at 31 bp. At 32 bp and 50 bp T was present in ACC03 and ACC 10 whereas G was present at 50 bp. However, 53 bp and 54 bp A were present in ACC03 and ACC 10 whereas T was present at 54 bp. Nucleotide T was absent at 63 bp and 64 bp in ACC 27. The nucleotide T was absent in ACC 03, ACC 10 at 75 bp. In ACC, 27 G was present at 92 bp. The nucleotide C was absent in ACC 03, ACC 10 at 101 bp. The nucleotide C was absent in ACC 16, *C. annuum* TDC1 mRNA, and ACC 27 at 142 bp. In *C. annuum* TDC1 mRNA nucleotide T was present at 271 bp. There were no G, T nucleotides at 347 bp, and 426 bp in ACC 16, respectively. In *C. annuum* TDC1 mRNA nucleotide T was present at 490 bp. ACC 27 was observed with the absence of T at 505 bp. There was no G nucleotide at 534 bp in ACC 16. The accession ACC 27 was observed with the presence of C and G at 597 bp and 598 bp, respectively. The nucleotide G was absent in ACC 27 at 615 bp. At 619 bp and 683 bp, the nucleotide T was absent in ACC 16. The nucleotide A was present in ACC 27 and *C. annuum* TDC1 mRNA at 854 bp while it was G in ACC 03 and ACC 10, and also, it was absent in ACC 16. At 982 bp, there was no nucleotide in ACC 16 and ACC 27, whereas it was G on ACC 03, ACC 10. *C. annuum* TDC1 mRNA consists of T at 982 bp [Plate 6].

Phylogenetic analysis of *Bemisia tabaci* using coxI fragments

Results of the present study revealed that the amplification of mitochondrial *coxI* gene fragment using the primer (C1-J-2195 and L2-N-3014) produced *B. tabaci* specific ~800 bp band in the sample of whitefly. Thirteen sequences covering the mitochondrial DNA *coxI* sequences of *B. tabaci* were used in the study and aligned using clustalW [Figure 2]. Our results of the whitefly sequence are grouped into *B. tabaci* isolate Asia _II_7 cytochrome oxidase subunit I gene, partial cds; mitochondrial since 99% of homology was observed [Figure 2 and Table 5]. Analysis of the sequence using BLASTN program the virus showed highest identity (100%) [Table 5] with *Bemisia tabaci* from India cytochrome oxidase subunit I (COI) gene.

Phylogenetic analysis for ChiLCuV using AC1 gene fragment

Amplification of leaf curl virus using AC1 (replication initiation protein gene) (nt1521-2606) specific primers produced a 1086 bp band. Thirteen sequences covering the replication initiation protein gene sequences of leaf curl virus were used in the study and aligned using clustalW [Figure 4]. Our results of replication initiation protein gene sequence grouped into Pepper leaf curl virus since 95% of homology was observed [Figure 4 and Table 6]. Analysis of the sequence using BLASTN program the virus showed highest identity (95%) [Table 6] with Pepper leaf curl virus isolate ANGC_CREP replication initiation protein gene.

Phylogenetic analysis for ChiLCuV using coat protein gene fragment

Amplification of leaf curl virus using the CP gene produced a 750 bp band. The sequenced ChiLCuD information was aligned using NCBI BLAST and 99% of homology was observed with the CP gene sequences deposited in NCBI. Sixteen sequences covering the CP gene sequences of leaf curl virus were used in the study and aligned using clustalW [Figure 5]. Our results of CP gene sequence grouped into pepper leaf curl virus CP since 97% of homology were observed [Figure 5 and Table 7]. On blasting, the sequence using BLASTN

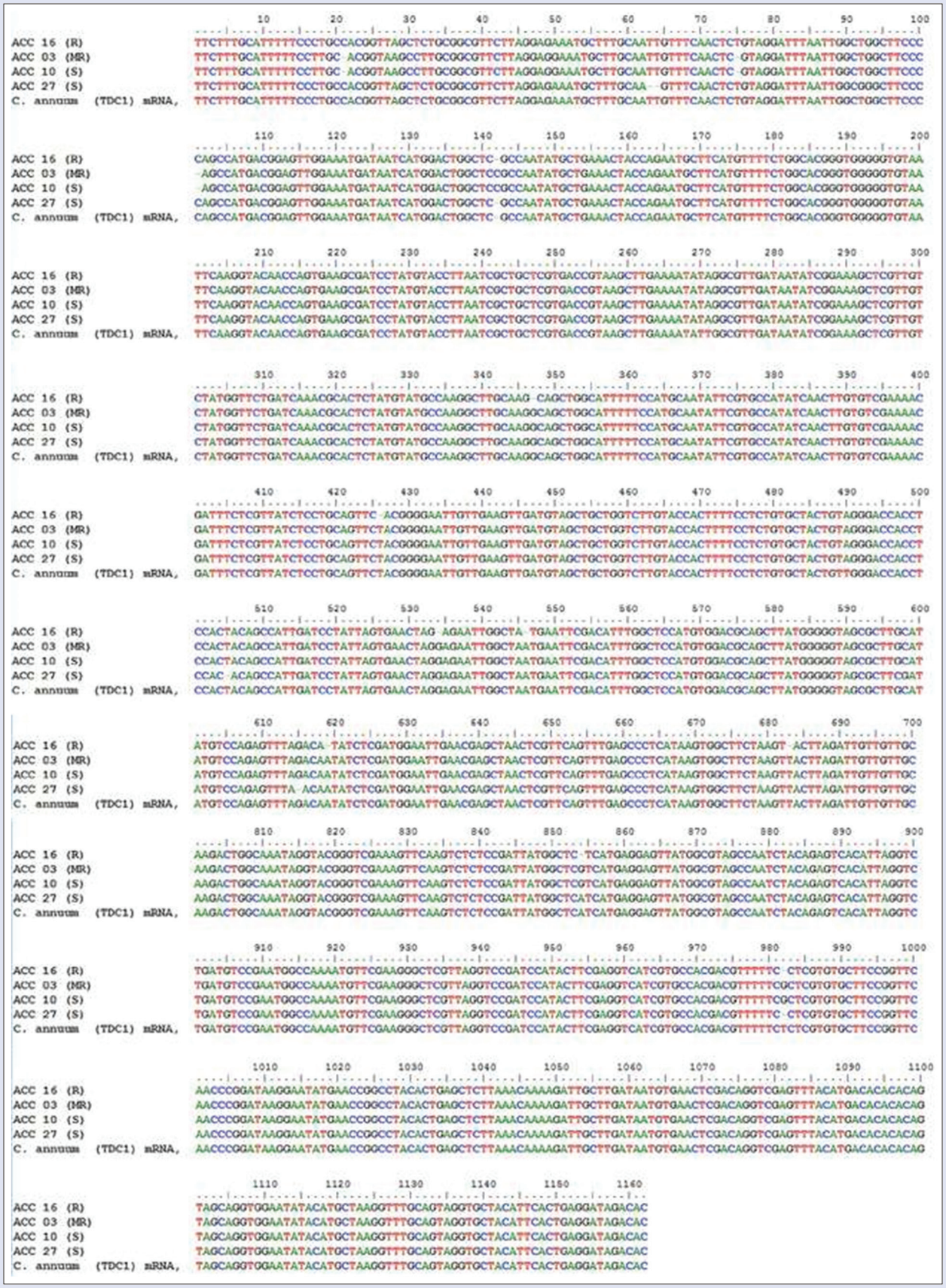

Plate 4: Comparison of tryptophan decarboxylase genes amplified from susceptible and resistant chillies accessions

Table 5: The coxI regions of *Bemisia tabaci* with their accession numbers from National center for biotechnology information, GenBank database used for sequence analysis and phylogenetic comparison of the *Bemisia tabaci* coxI gene amplified

Description	Accession number	Homology of nucleotide sequence (%)
B. tabaci from India COI gene, partial cds; mitochondrial	GQ281715.1	100
B. tabaci isolate Asia_II_7 gene, partial cds; mitochondrial	KM821541.1	99
B. tabaci mitochondrial partial COI gene for cytochrome oxidase I, isolate Coimbatore 3	AM408899.1	99
B. tabaci from India COI gene, partial cds; mitochondrial	GQ281720.1	99
B. tabaci mitochondrial partial COI gene for cytochrome oxidase I subunit, specific host parthenium hysterophorus, from India, Karnataka, Ranibennur, Sulibele	AJ748372.1	99
B. tabaci isolate KL05-M COI gene, partial cds; mitochondrial	JQ995246.1	99
B. tabaci mitochondrial partial COI gene for cytochrome oxidase I subunit, specific host watermelon, from India, Karnataka, Kolar, Chintamani, Talagavara	AJ748378.1	99
B. tabaci mitochondrial genes for, tRNA-Leu, cytochrome c oxidase subunit II, partial cds and complete sequence, isolate: AsianII_7-42	LC159296.1	98
B. tabaci mitochondrial genes for, tRNA-Leu, cytochrome c oxidase subunit II, partial cds and complete sequence, isolate: AsianII_7-1	LC159295.1	98

COI: Cytochrome oxidase subunit I; *B. tabaci: Bemisia tabaci*

Table 6: Chilli leaf curl disease with their accession numbers from National center for biotechnology information, GenBank database used for sequence analysis and phylogenetic comparison of the chilli leaf curl disease AC1 gene amplified (replication initiation protein gene)

Description	Accession number	Homology of nucleotide sequence (%)
Pepper leaf curl virus isolate ANGC_CREP replication initiation protein gene, partial cds	JN887127.1	95
Pepper leaf curl virus isolate CAK226 replication initiation protein gene, partial cds	JN887125.1	94
Pepper leaf curl virus isolate CESC_CREP replication initiation protein gene, partial cds	JN887128.1	94

Table 7: Chilli leaf curl disease with their accession numbers from National center for biotechnology information, GenBank database used for sequence analysis and phylogenetic comparison of the chilli leaf curl disease coat protein gene amplified

Description	Accession number	Homology of nucleotide sequence (%)
Pepper leaf curl Lahore virus complete genome, clone P1	AM691745.1	97
Pepper leaf curl Varanasi virus isolate Jorehat segment DNA A, complete sequence	JN663861.1	97
Pepper leaf curl Lahore virus (Pakistan:Lahore2:2004) segment A, complete sequence, clone PeAL2	AM491589.1	96
Pepper leaf curl Lahore virus isolate Nagpur segment DNAA, complete sequence	JN663864.1	96
Chilli leaf curl virus isolate India: Amritsar:Papaya:2009 segment DNA A, complete sequence	GU136803.1	97
Chilli leaf curl Multan virus av2 gene for precoat protein, segment DNAA, isolate UN	HG932561.1	96
Chilli leaf curl Multan virus coat protein (AV1) gene, complete cds	KT835649.1	96
Pepper leaf curl Lahore Virus (Pakistan:Lahore1:2004) segment A, complete sequence, clone BpAL 4 (3)	AM404179.1	96
Pepper leaf curl virus isolate Palampur segment DNAA, complete sequence	JN663870.1	96
Chilli leaf curl virus India coat protein (AV1) gene, complete cds	KT835648.1	95

Table 8: Tryptophan decarboxylase gene with their accession numbers from National center for biotechnology information, GenBank database used for sequence analysis and phylogenetic comparison of tryptophan decarboxylase sequenced from chillies accession

Description	Accession number	Homology of nucleotide sequence (%)
C. annuum tryptophan decarboxylase (TDC1) mRNA, complete cds	FJ710789.1	98
C. annuum aromatic-L-amino-acid decarboxylase-like (LOC107877290), mRNA	NM_001325087.1	98

TDC1: Tryptophan decarboxylase; *C. annuum*: *Capsicum annuum*

program the virus showed the highest identity (97%) [Table 7] with Pepper leaf curl Lahore virus complete genome.

Phylogenetic analysis of chillies tryptophan decarboxylase genes

Amplification of TDC using gene-specific primers produced a 1509 bp band [Plate 1]. Eight sequences covering the TDC were used in the study and aligned using clustalW [Figure 6]. On blasting the sequence using the BLASTN program, the virus showed the highest identity (98%) [Table 8] with *Capsicum annuum* TDC1 mRNA.

DISCUSSION

Conventionally, the morphological character of the fourth instar nymph was used to identify *B. tabaci* populations[21] The invasion of a *B. tabaci* variant who behaved differently from the native population at that time only the concept of biotype in *B. tabaci* came into distinction[6] in the Southern United States. One of the modern methods to identify the species is depends on DNA markers such as mitochondrial cytochrome c oxidase subunit I (coxI). Using this technique, 33 extant biotypes of *B. tabaci* have been reported. Succeeding research suggested that *B. tabaci* contained 11 well-defined genetic groups[6,22] Nevertheless, because *B. tabaci* contains at least 24 morphologically indistinguishable species that scarcely interbreed and form different phylogenetic clades, it is regarded as a cryptic species complex[6,22] The molecular identification of insect species is a valuable resource to identify and understand the genetic difference between two or more species. Furthermore, it's less time-consuming and the results were readily available in a self-explanatory way. Underlying this viewpoint the present study

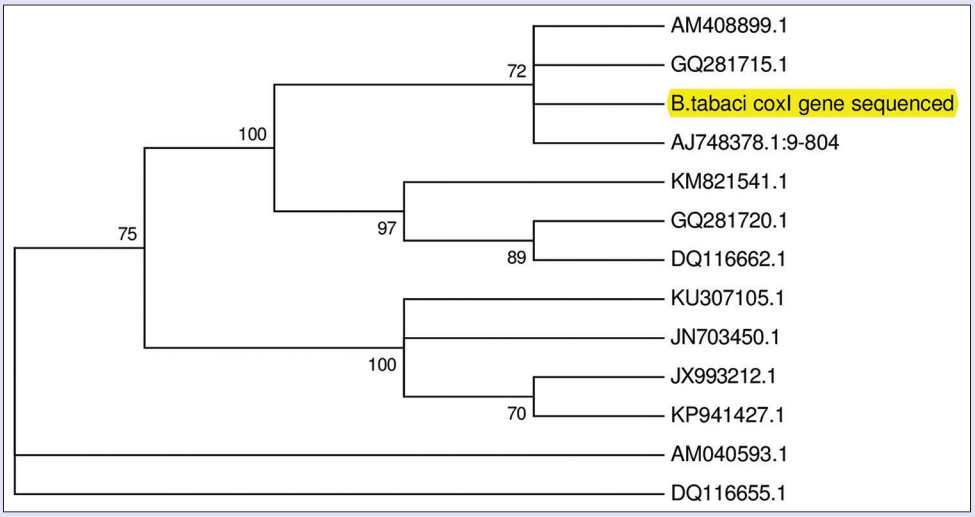

Figure 4: Phylogenetic analysis for *Bemisia tabaci* using coxI gene fragment. KM821541.1 *Bemisia tabaci* isolate Asia_II_7 cytochrome oxidase subunit I gene, partial cds; mitochondrial; AM408899.1 *B. tabaci* mitochondrial partial COI gene for cytochrome oxidase I, isolate Coimbatore 3; GQ281715.1 *B.tabaci* from India cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial; AJ748378.1:9-804 *B. tabaci* mitochondrial partial coi gene for cytochrome oxidase I subunit, specific host watermelon, from India, Karnataka, Kolar, Chintamani, Talagavara ; GQ281720.1 *B. tabaci* from India cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial DQ116662.1 *Bemisia tabaci* isolation-source sunflower cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial; KU307105.1 *Bemisia tabaci* isolate C15 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial;; JN703450.1 *Bemisia tabaci* voucher HS124 cytochrome oxidase subunit I gene, partial cds; mitochondrial; JX993212.1 *Bemisia tabaci* isolate Ludhiana II cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial; >KP941427.1 *Bemisia tabaci* isolate Asia_1 cytochrome oxidase subunit I gene, partial cds; mitochondrial; AM040593.1 *Bemisia tabaci* mitochondrial COI gene for cytochrome oxidase subunit I, isolate Kolar; DQ116655.1 *Bemisia tabaci* isolate 3 isolation-source sunflower cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial

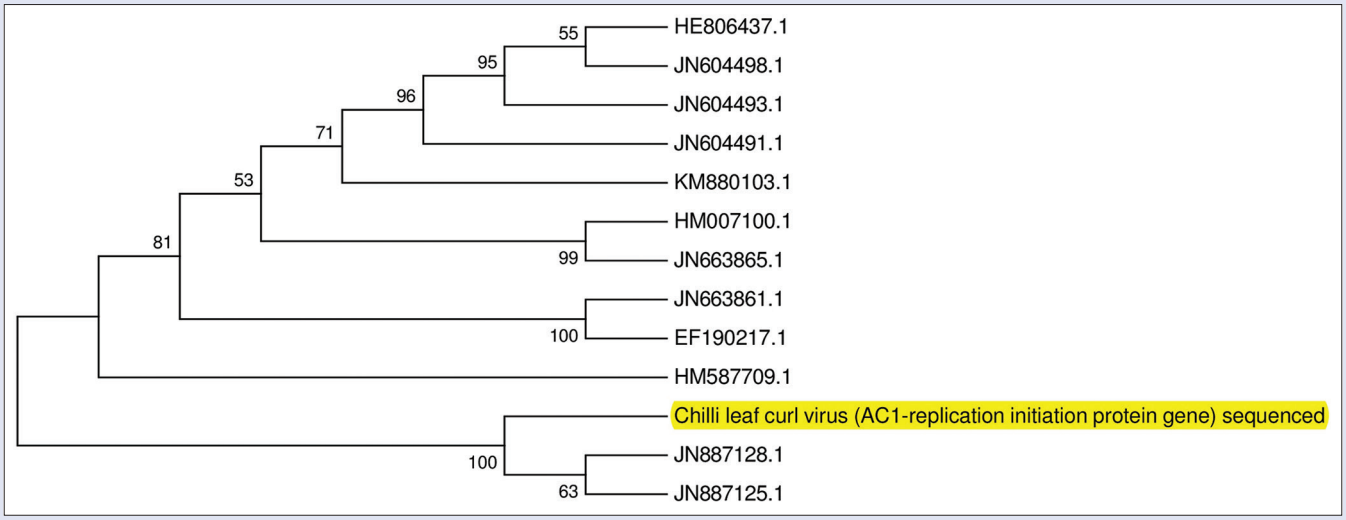

Figure 5: Cladogram of chilli leaf curl virus using replication initiation protein (AC1) specific primer. HE806437.1 Chilli leaf curl virus complete genome, isolate T-37; JN604498.1 Chilli leaf curl virus isolate Th3, complete genome; JN604493.1 Chilli leaf curl virus isolate Taq2, complete genome; JN604491.1 Chilli leaf curl virus isolate Sh1, complete genome; KM880103.1 Chilli leaf curl Ahmedabad virus-India [India/Ahmedabad/2014] complete genome; HM007100.1 Chilli leaf curl Multan virus-India [India/Guntur/2009] clone pChGuB16 segment DNA-A, complete sequence; JN663865.1 Chilli leaf curl Multan virus isolate Nagpur segment DNA-A, complete sequence; JN663861.1 Pepper leaf curl Varanasi virus isolate Jorehat segment DNA-A, complete sequence; EF190217.1 Pepper leaf curl virus isolate Varanasi, complete genome; HM587709.1 Chili leaf curl Pakistan virus isolate YN1045, complete sequence; JN887128.1 Pepper leaf curl virus isolate CESC_CREP replication initiation protein gene, partial cds; JN887125.1 Pepper leaf curl virus isolate CAK226 replication initiation protein gene, *partial cds*

reveals that, amplification of mitochondrial *coxI* gene fragment using the primer (C1-J-2195 and L2-N-3014) produced *B. tabaci* specific ~ 800 bp band. Besides, the whitefly sequence was aligned with the NCBI blast where 92% of identity was observed [Figure 2]. Another recent study suggested that the diversity of *B. tabaci* complex in soybean-growing states of India including Tamil Nadu lined with Asia I, Asia II 1, and Asia II 7 as well, denoted as major groups[23] There is no universal strategy for managing *B. tabaci*. However, phylogenetic as well as population genetics, as offered in this study, may enable pest control practitioners to carry out different pest control strategies at the state level. Based on the

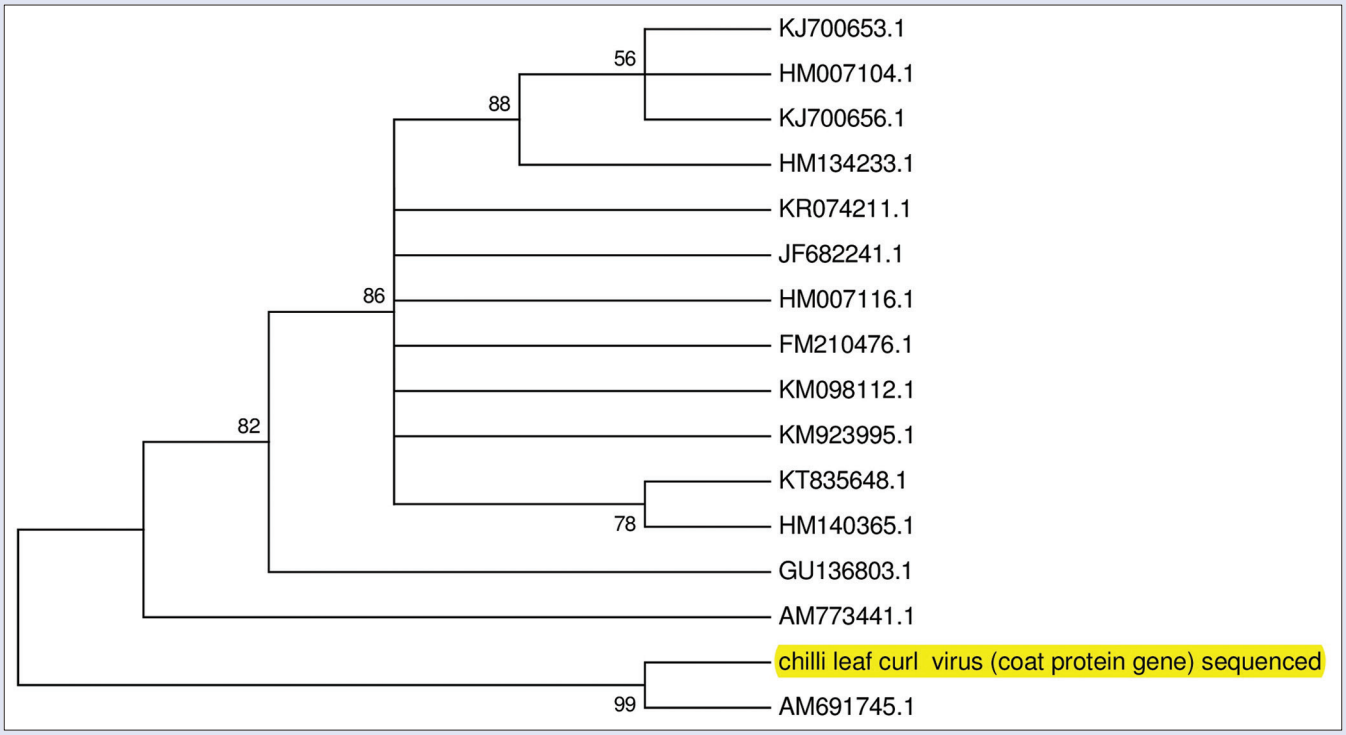

Figure 6: Phylogenetic analysis for chilli leaf curl virus using the coat protein gene. KJ700653.1 Chilli leaf curl virus isolate RK01, complete genome; HM007104.1 Chilli leaf curl virus-India [India/Jodhpur/2009] clone pChJodB26 segment DNA-A, complete sequence; KJ700656.1 Chilli leaf curl virus isolate RK02, complete genome; HM134233.1 Chilli leaf curl virus-DU [India:New Delhi:Papaya:2009] coat protein gene, complete cds; KR074211.1 Chilli leaf curl virus isolate Ludhiana; JF682241.1 Chilli leaf curl virus isolate Lucknow coat protein (AV1) gene, complete cds; HM007116.1 Chilli leaf curl virus-India [India/Pataudi/2007] clone pChPataB9 segment DNA-A, complete sequence; FM210476.1 Chilli leaf curl virus partial cp gene; KM098112.1 Chilli leaf curl virus coat protein gene; KM923995.1 Chilli leaf curl virus isolate LC coat protein gene; KT835648.1 Chilli leaf curl virus-India coat protein (AV1) gene, complete cds; HM140365.1 Chilli leaf curl virus-HD [India:New Delhi:Papaya:2007], complete genome; GU136803.1 Chilli leaf curl virus isolate India:Amritsar:Papaya:2009 segment DNA A, complete sequence; AM773441.1 Chilli leaf curl virus partial cp gene; AM691745.1 Pepper leaf curl Lahore virus complete genome, clone P1

information generated from phylogeography, the effective management strategies can be developed to target-specific populations. To control the spread of *B. tabaci*, new cryptic species should be monitored and their origins assessed. We need to examine diversity and the pattern of its spread in a particular region to develop strategies to control *B. tabaci*. The development of suitable *B. tabaci* control measures requires a thorough understanding of the phylogeography of the target pest species because the population structure and dynamics usually vary from region to region and effective control strategies, such as the development of host plant resistance.

The classification and phylogenetic relationships of begomoviruses based on three components, namely, complete monopartite viral genomes or the component A of bipartite viruses, CP, and/or replication association protein gene (Rep) trees.[24-26] Gene-specific marker is an incredible molecular tool to identify the diseased plant, healthy plant, and also a type of vector. The ChiLCV genome encodes six proteins namely, two from the virion sense strand (CP and precoat protein) and four from the complementary sense strand (replication initiator protein, transcription activator protein, replication enhancer protein, and pathogenicity determinant protein).[27] Therefore, amplification of leaf curl virus using AC1 (replication initiation protein gene) (nt1521-2606) specific primers produced a 1086 bp band. The results were in line with Nirbhay *et al.* (2015) detected ChiLCuV using AC1 (nt1521-2606) specific primers. The sequenced ChiLCuD information was aligned using NCBI BLAST and 85% of homology was observed with the replication initiation protein gene sequences deposited in NCBI [Figure 4].

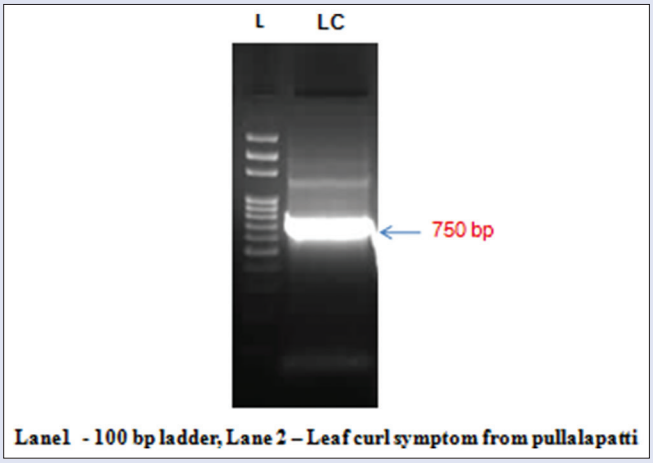

Figure 7: Ploymerase chain reaction a amplification of coat protein gene in chillies

The ChiLCuV detection and confirmation were also done by using CP gene primers [Figure 7]. The CP gene is the most highly conserved in the family *Geminiviridae*.[28] Therefore, through this gene amplification, we could detect the disease. The utility of the CP sequence for this rationale is likely promising and feasible because the CP sequence is most advantageous since conserved regions to

Plate 5: Conserved regions of tryptophan decarboxylase genes amplified from susceptible and resistant chillies accessions

TTCTTTGCATTTTTCCTGCACGGTAGCTGCGGCGTTCTTAGGAGAATTGCAAGTTTCAACTAGTAGGATTTAATTGGCGGCTTCCCAGCCATGACGGAGTTGGAAATGAT
AATCATGGACTGGCTCGCCCAATATGCTGAAACTACCAGAATGCTTCATGTTTTCTGGCACGGGTGGGGGTGTAATTCAAGGTACAACCAGTGAAGCGATCCTATGTACCTTAATCGCTGCTCGT
GACCGTAAGCTTGAAAATATGGCGTTGATAATATCGGAAAGCT
CGTTGTCTATGGTTCTGATCAAACGCACTCTATGTATGCCAAGGCTTGCAAGCAGCTGGCATTTTTCCATGCAATATTCGTGC
CATATCAACTTGTGTCGAAAACGATTTCTCGTTATCTCCTGCAGTTCACGGGGAATTGTTGAAGTTG
ATGTAGCTGCTGGTCTTGTACCACTTTTCCTCTGTGCTACTGTGGGACCACCTCCACTACAGCCATTGATCCTATTAGTGAACTAGAGAA
TTGGCTATGAATTCGACATTTGGCTCCATGTGGACGCAGCTTATGGGGGTAGCGCTTATA
TGTCCAGAGTTTAGACATATCTCGATGGAATTGAACGAGCTAACTCGTTCAGTTTGAGCCCTCATAAGTGGCTTCTAAGTAC
TTAGATTGTTGTTGCATGTGGGTGAAAGAACCAAGCGTGCTAGTCAAGGCATTGAGCACGAATCCT
GAGTATCTGAGGAATAAACGTTCCGAACATGGCTCAGTTGTGGATTACAAAGACTGGCAAATAGGTACGGGTCGAAAGTTCA
AGTCTCTCCGATTATGGCTCTCATGAGGAGTTATGGCGTAGCCAATCTACAGAGTCACATTAGGTCTGATGTCCGAATGGCCAAAATGTTCGAAGGCTCGTTAGGTCCGATCCA
TACTTCGAGGTCATCGTGCCACGACGTTTTTCCTCGTGTGCTTCCGGTTCAACCCGGATAAGGAATATGAACCGGCCTACACTGAGCTCTTAAACAAAAGATTGCTTGATAATGTGAACTCGACA
GGTCGAGTTTACATGACACACACAGTAGCAGGTGGAATATACATGCTAAGGTTTGCAGTAGGTGCTACATTCACTGAGGATAGACAC

predict are mostly close to accuracy and more in line with the extent of sequence variation and conservation across the entire genome.

Therefore, in the present study also, the CP gene primers were used for further identification.

The highly variable 5' end of the CP gene sequence (200 nt) is proposed as a reliable molecular marker for predicting *Begomovirus* identity.[25]

The CP region is sufficient to provide a simple, rapid, and reliable method for *Begomovirus* identification and classification. Here, we demonstrated the utility of the CP region for predicting the identities of the *Begomovirus* relatives. Amplification of leaf curl virus using CP gene produced a 750 bp band. The results were in line with Sinha[29] who found that PCR-based detection conditions were optimized for chilli leaf curl virus using CP primers 5'-AGAATTATGTCCAAGCGACCA-3' and 5'-AAGCGTTGGGGATACACAAA-3 and revealed that the 750 bp amplicon of CP gene was amplified and sequenced. The sequenced ChiLCuD information was aligned using NCBI BLAST and 99% of homology was observed with the CP gene sequences deposited in NCBI. Padidam *et al.*[25] referred that, genetic distances of all CP gene fragments were nearly equally useful for making accurate predictions. Our results of CP gene sequence grouped into pepper leaf curl virus CP since 99% of homology were observed [Figure 5]. Results are akin to the observation of Sinha[29] reported that phylogenetic reconstructions of CP sequence with other *Begomovirus* sequences retrieved from GenBank reveal a close relationship among Indian and other Asiatic isolates.

The indole-alkaloid derivatives of tryptamine may affect whitefly feeding[30] and reproduction[8] Tryptamine accumulation may influence the whiteflies' development and growth as a single gene-based plant protection strategy. Only a few recent studies have investigated plant responses to phloem-feeding whiteflies. The endogenous gene was present in both the resistant and susceptible accessions of chillies [Plate 4-6]. The accessions ACC16 registered as resistant, ACC 20 and ACC 03 recorded as moderately resistant and received with a lower number of adults in-field screening (0.32 adults/3leaves), (0.45 adults/3leaves), (0.48 adults/3leaves), and 25%, 45%, and 35% of ChiLCuD damage were recorded, respectively. In laboratory screening also, these accessions were recorded as less preferred for adult settlement, lower number of eggs, and reduced percentage of nymphal and adult emergence also registered. We hypothesized that feeding by *B. tabaci* on resistant accessions activates the defense gene when compared with susceptible plants. The results were akin to Gill[36] who reported that in transgenic poplar and tobacco plants ectopically expressing TDC1 accumulated elevated levels of tryptamine leads to adverse effects on feeding behavior and physiology of *M. disstria* and *Manduca sexta* L.(tobacco hornworm, THW).

Further, in an earlier era, it was reported that weight reductions of 60 and ≥90% have been found for *S. frugiperda* larvae fed diets made with corn silks containing ≥0.2% and 0.7% fresh weights of maysin, respectively[31] Similarly, Klingler *et al.*[32] registered that a single dominant gene, AKR (*Acyrthosiphon kondoi* resistance) showed antixenosis effect on migratory aphids (*Acyrthosiphon kondoi)* where the aphids deterred from settling on resistant plants within 6 h of release, preferring to settle on susceptible plants. Cloning of resistance-related endogenous genes (TDC gene) in chillies is a novel approach to develop transgenic plants resistant to whitefly, *B. tabaci,* and ChiLCuD. The results of this study provide further evidence that insect-resistant traits associated with endogenous genes could provide effective control of primary insect pests in agricultural and horticultural ecosystems. Currently, insect resistance has entered the commercialization stage and has a larger crop-growing region.

Plate 6: Variations of tryptophan decarboxylase genes amplified from susceptible and resistant chillies accessions

Accessions	17 bp	21 bp	27 bp	31 bp	32 bp	50 bp	53 bp	54 bp	55 bp	56 bp
ACC 16 (R)	C	C	T	T	C	A	T	G	C	T
ACC 03 (MR)	T	-	A	C	T	G	A	T	G	C
ACC 10 (S)	T	-	A	C	T	G	A	T	G	C
ACC 27 (S)	C	C	T	T	C	A	T	G	C	T
C. annum TDC1 (mRNA)	C	C	T	T	C	A	T	G	C	T
Accessions	**63 bp**	**64 bp**	**75 bp**	**92 bp**	**101 bp**	**142 bp**	**271 bp**	**347 bp**	**426 bp**	**490 bp**
ACC 16 (R)	T	T	T	T	C	-	A	-	-	A
ACC 03 (MR)	T	T	-	T	-	C	A	G	T	A
ACC 10 (S)	T	T	-	T	-	C	A	G	T	A
ACC 27 (S)	-	-	T	G	C	-	A	G	T	A
C. annum TDC1 (mRNA)	T	T	T	T	C	-	T	G	T	T
Accessions	**505 bp**	**534 bp**	**546 bp**	**597 bp**	**598 bp**	**615 bp**	**619 bp**	**683 bp**	**854 bp**	**982 bp**
ACC 16 (R)	T	-	-	G	C	G	-	-	-	-
ACC 03 (MR)	T	G	A	G	C	G	T	T	G	G
ACC 10 (S)	T	G	A	G	C	G	T	T	G	G
ACC 27 (S)	-	G	A	C	G	-	T	T	A	-
C. annum TDC1 (mRNA)	T	G	A	G	C	G	T	T	A	T

TDC1: Tryptophan decarboxylase; *C. annuum: Capsicum annuum*

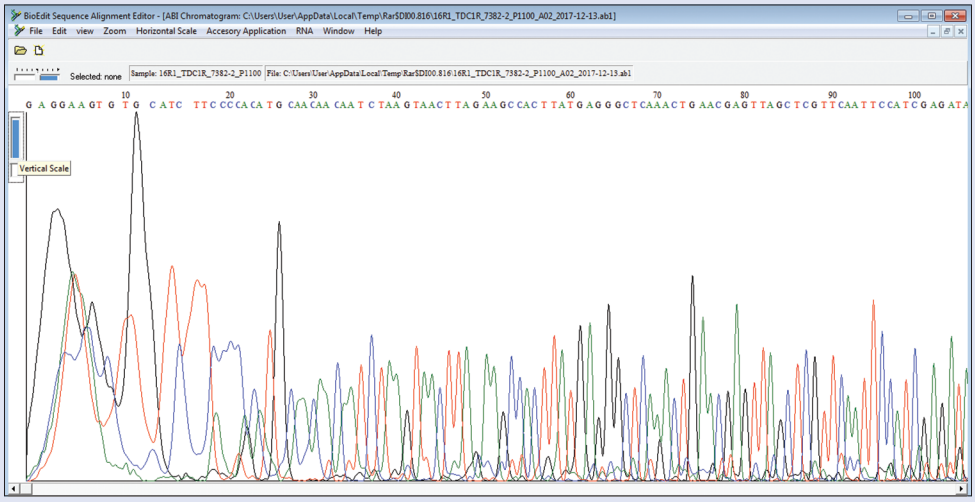

Plate 7: Chromatogram of sequenced resistant accession ACC 16

Plate 8: Chromatogram of sequenced susceptible accession ACC 27

Numerous plant biochemical pathways regulating plant defense, development, and metabolism are simultaneously or sequentially involved in these interactions. Plants produce a rich diversity of secondary metabolites. Amines and their derivatives are known to influence insect behavior involved in feeding and reproduction. Plant-derived amine-generating transgene, explants were transformed with TDC (TDC, EC 4.1.1.28) on *Poplar* spp[33] express the defense strategy. TDC catalyzes the conversion of Trp to tryptamine[34] providing a common backbone for many secondary metabolites that have potentially divergent biological activities regulated by developmental and environmental factors. Some of these have prominent functions in defending plants from abiotic stresses such as pathogen infection[35] and insect attack.[36] In some crops, antibiotic metabolites or precursors derived from tryptamine accumulate after pathogen infection[37] and rice leaves treated with an inhibitor of TDC resulted in reduction in severe damage by *Bipolaris oryzae*.[39] These findings suggested important roles for TDC in plant resistance to pathogens. Tryptamine and tryptamine-derived alkaloids, when

expressed in plants, may act as anti-oviposition and antifeedant agents and or inhibitors of larva1 and pupal development, providing an example of a single-gene-based approach to further our understanding of plant-insect interactions.[8]

In the present study, results revealed that TDC gene expression was greater in P2 accession leaves at 48 h postinfestation with 20 numbers of *B. tabaci* after feeding [Table 3]. The results revealed that gene expression was induced and higher expression was observed by *B. tabaci* feeding.

TDC gene expression was downregulated in moderately resistant accession ACC 20. The results are in line with Gelvin[40] who reported that the level of gene expression varied among the different transgenic lines. In our results showed that a low level of expression was observed within the accessions ACC 16 (resistant) Plate 7 and ACC 20 (moderately resistant) [Table 4]. Similar results were proposed by Gelvin[40] who observed that in an extreme case, there can be an apparent absence of gene expression. The TDC transgenic plants showed that the tryptamine accumulation was consistently associated with adverse effects on feeding potential and physiology

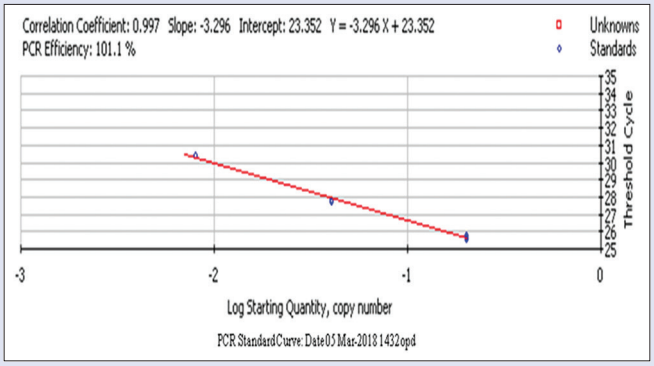

Plate 9: Primer efficiency of tryptophan decarboxylase gene and housekeeping (ACTIN) gene

of *Malacosoma disstria* (forest tent caterpillar) on *Populus* spp[33].The TDC gene was isolated and amplified using gene-specific primers and produced 1509 bp. Similarly, Thomas[8] and Ueno[38] observed that when the TDC gene (isolated from *Cantharanthus roseus* (periwinkle) when expressed in transgenic tobacco, the 55-kD TDC enzyme and tryptamine accumulated had caused a 97% reduction in *B. tabaci* reproduction. These results are in agreement with several previous reports on the overexpression of TDC in herbaceous crops, which demonstrated an increase in the accumulation of tryptamine in transgenic lines[7] Similarly, a 30-fold higher percentage of tryptamine was found in the "basic" fraction of transgenic line extracts, in comparison to the "basic" fraction of wild-type plant extracts of *Populus* spp[33] against *M. disstria*. Within each accession also, there were significant differences in TDC gene expression levels when compared against respective control of the accession [Table 4]. A significant difference was detected on susceptible accession ACC 27 at 0, 10, 20 numbers of adult feeding after 48 h of post infestation [Plate 8]. The results were in line with Murugan and Smith[10] registered that the TDC and T5M (tryptophan 5-monooxygenase) IAA (Indole acetic acid) genes were upregulated to extraordinarily high levels in Russian wheat aphid susceptible Otis plants, and of these, only TDC was belatedly expressed in tolerant barley variety Stoneham to *Diuraphis noxia* feeding of barley. In the present study, there were significant differences evident between the resistant and susceptible accessions gene expression.

Similar results were also obtained by Li *et al.*[41] who registered that the expression of two *Ae*VTDCs imparts resistance to cereal cyst nematode and root-knot nematode. The *Ae*VTDCs gene expression levels were detected in all samples at different levels (roots, stems, leaves of 20-day-old seedling and roots, stems, leaves, flower buds, flowers of mature plants at 3 days after flowering). Besides, transgenic rice plants that overexpressed TDC accumulated higher levels of serotonin than the wild type and showed a protective role against reactive oxygen species, pathogen infection, and leading to delayed senescence of rice leaves. Thus, it is persistent that the TDC gene and its downstream products have a significant role in reducing the whitefly *B. tabaci* population.

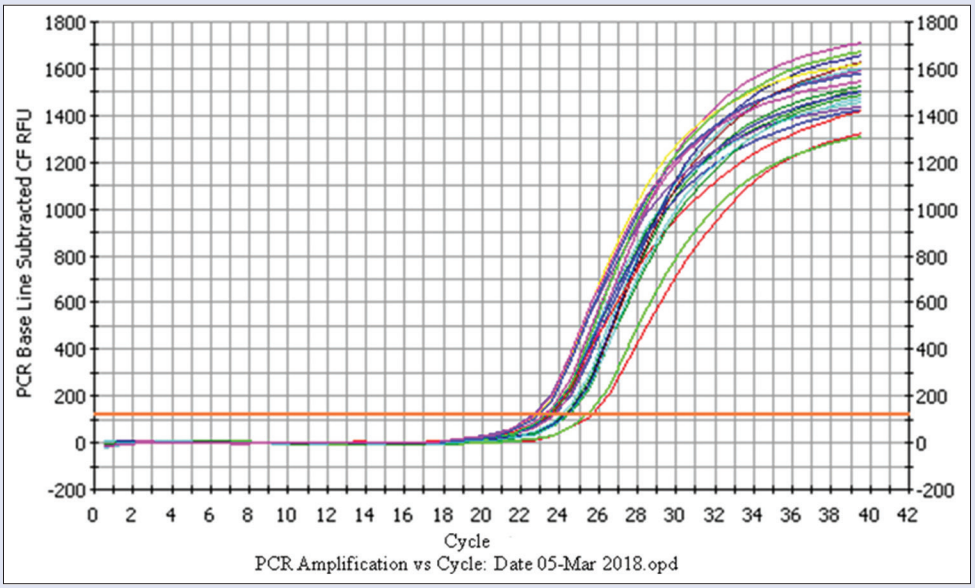

Plate 10: Upregulation of action gene different chillies accessions (ACC 01, ACC20, ACC27, P2 and ACC16) after feeding by *Bemisia tabaci* adults for 48 h infestation

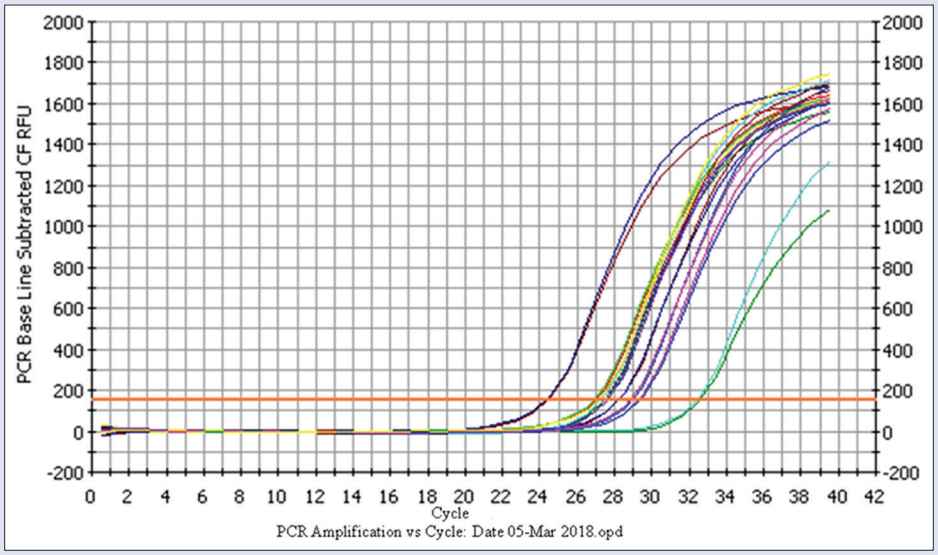

Plate 11: Upregulation of tryptophan decarboxylase gene on different chillies accessions (ACC 01, ACC20, ACC27, P2 and ACC16) after feeding by *Bemisia tabaci* adults for 48 h infestation

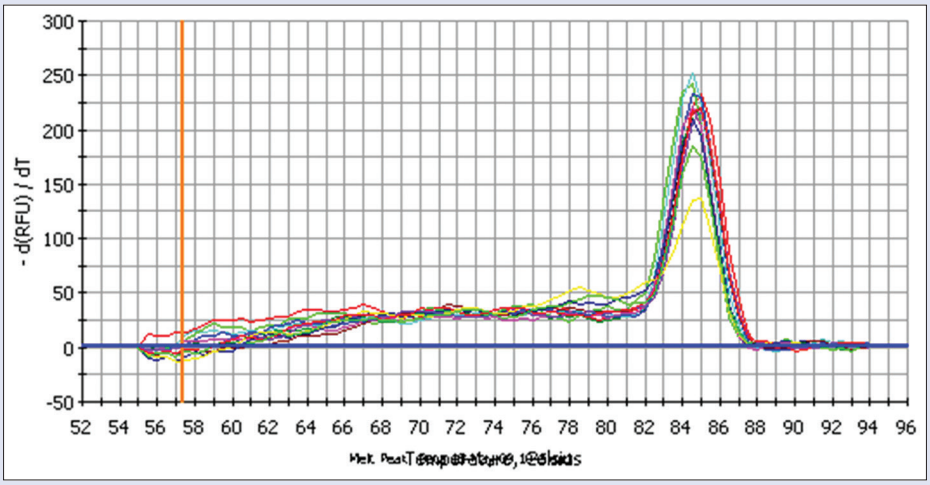

Plate 12: Dissociation curves of tryptophan decarboxylase on different chillies accessions (ACC 01, ACC20, ACC27, P2 and ACC16)

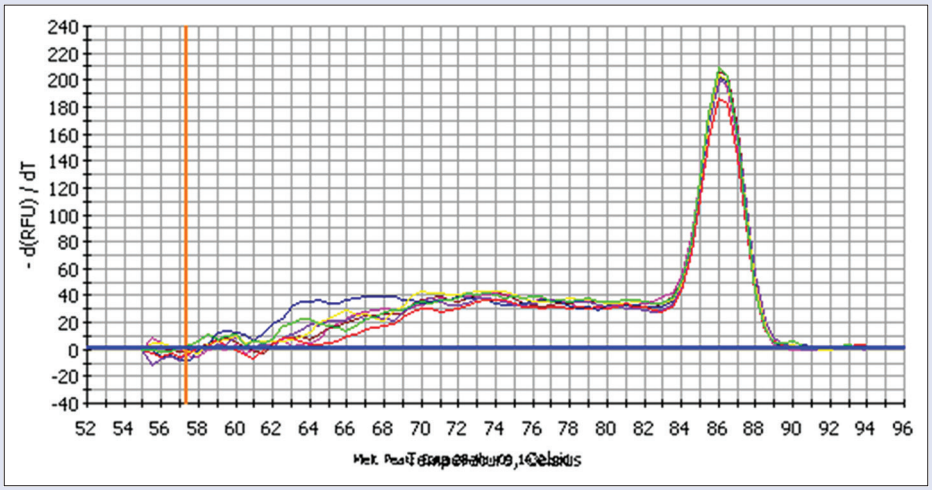

Plate 13: Dissociation curves of housekeeping gene (ACTIN) on different chillies accessions (ACC 01, ACC20, ACC27, P2 and ACC16)

CONCLUSION

The satisfactory control of viral disease may be achieved with the application of molecular biology, DNA, and RNA-based technologies include the cloning of insect resistance genes, their expression studies showed great potentiality in promoting insect resistance germplasm and making biotechnology more and more important, to modern agriculture can enable the achievement of food security for present and future generations. To better understand the application of novel technologies in agriculture and achieve the goals to promote their utilities in current agriculture to enhance productivity without the disease. Insect and disease-resistant crops are going to be an essential part of our life and the enormous potential of biotechnology must be exploited to the benefit of humankind. Thus, the study envisages the use of molecular approaches to select resistant lines was useful for whitefly resistance breeding and minimize ChiLCuD incidence in chillies.

Acknowledgements

The authors are also grateful to Dr. K. Prabakaran Department of Agricultural Economics, Agricultural College and Research Institute, Madurai, Tamil Nadu, India, for his guidance to perform the statistical analysis of the data. I owe a great debt to the honorable one of the members of the advisory committee as well as Professor and Head of Center of Innovation, Biotechnology, Agricultural College and Research Institute, Madurai, Dr. N. Senthil.

Financial support and sponsorship

The financial support is given by the Department of Biotechnology, Government of India under project No. DR/P7/S2/DBT Scheme/2015 dt: 25.05.15 is kindly acknowledged.

Conflicts of interest

There are no conflicts of interest.

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