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Differential expression of *Citrus tristeza* virus across various age groups of Assam lemon (*Citrus limon* Burm.)

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Assam lemon (*Citrus limon* Burm.), locally known as Kaji Nemu, is a widely cultivated and economically important fruit crop in the Rutaceae family. *Citrus tristeza* virus (CTV) is a global destructive pathogen that severely impacts citrus production. In this study, leaf samples from Assam lemon plants of ages 3, 5, 10, 15, 20, and 25 years were analysed for CTV infection using serological (DAS-ELISA) and molecular (RT-PCR and qPCR) techniques. Results showed that all samples tested positive for CTV. qPCR study revealed a progressive increase in CTV coat protein (CP) gene expression from 3 to 15 year-old plants, followed by a decline in older plants. The highest CTV titer in 15 year-old plants showed a weak positive correlation with reduced fruit yield, indicating a negative impact on productivity. Phylogenetic analysis showed close genetic associations between CTV isolates from Assam lemon and those from Northeast India and neighbouring regions.

Keywords: ELISA, RT-PCR, qPCR, *Citrus tristeza* virus, Assam lemon, differential expression

Citrus (*Citrus* spp.), one of the major fruits of global economic importance belonging to the family Rutaceae is widely cultivated across tropical and subtropical regions[1]. However, its production is severely threatened by *Citrus tristeza* virus (CTV), a devastating pathogen responsible for the death of over 100 million trees worldwide[2]. Considered to be the largest plant RNA virus[3], the pathogen now poses a serious threat to citrus cultivation in Northeastern India. In Assam, Citrus is cultivated over an area of 33.01 thousand hectares, yielding a total production of 369.56 thousand metric tonnes[4]. The CTV disease incidence in Assam lemon (*Citrus limon*) can be as high as 56.0% in NE India[5]. CTV induces three distinct syndromes, which vary depending on the strain, graft, and rootstock combination: quick decline, stem pitting, and seedling yellows[6]. The virus is efficiently transmitted in a semipersistent manner by aphid vectors (*Toxoptera* spp.)[7], which are abundant in the Northeast region of India[8,9]. CTV shortens the lifespan of Assam lemon trees, with quick decline typically affecting older trees[10]. However, the impact of the virus on different age groups of Assam lemon remains underexplored, highlighting a key knowledge gap. To address this, a study was conducted to examine the incidence. and the viral load of CTV across six different age groups of Assam lemon. Current study aimed to identify the age group most affected by the virus and assess its impact on the yield.

Materials and Methods

Survey, sampling and assessment

A survey was conducted in July 2022 to assess the incidence and severity of *Citrus tristeza* virus infection across *Citrus* growing regions within the Lower Brahmaputra Valley Zone (LBVZ) of Assam. Six age groups of Assam lemon plantations in three locations, with trees aged 3, 5, 10, 15, 20, and 25 years, were sampled for CTV-associated symptoms and vector incidence. Symptomatic and symptomless leaf samples were collected from twenty randomly selected trees from each location and age group.

Serological and molecular detection

Serological detection of CTV was performed by assaying the samples using a commercial Double Antibody Sandwich- Enzyme Linked Immunosorbent Assay (DAS-ELISA) kit (Bioreba, AG, Switzerland) according to the protocol.

Molecular detection was carried out with the following steps: (i) total genomic RNA was isolated from 100 mg leaf tissue samples using both the CTAB[11] and TRIzol[12] (Invitrogen) methods. The quality and quantity of the extracted RNA were

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assessed using a Nanodrop Bio-Spectrophotometer (Eppendorf). (ii) PrimeScript 1st strand cDNA synthesis kit (Takara Bio) was used to synthesise the cDNA following the manufacturer's protocol. (iii) A 10 uL reaction was setup to perform polymerase chain reaction using EmeraldAmp GT PCR Master Mix (Takara Bio) with 1 uL of cDNA template. The experimental setup included a positive control (known infected CTV sample), a negative control (CTV-free citrus sample), and a no-template control (NTC). Primer pair p20F and p20R of CTV were used to amplify the target CP gene (422 bp)[13]. Additionally, the presence of mild and severe strains was checked in two representative samples from individual age groups using the primers CN 119F and CN 219R for mild strain 400 bp and CN 218F and CN 120R primers for severe strain 300 bp[14]. The PCR conditions were set similar to an earlier study[10]. Post-PCR, the amplified products were subjected to electrophoresis on a 1.5% agarose gel in 1× TBE buffer containing 0.5 µg/mL of Ethidium bromide. A 5 µL aliquot of a 100 bp DNA ladder was included to determine the molecular weight of the PCR products.

Gene expression studies using quantitative Real-time PCR (qPCR)

Comparative quantification using the SYBR Green method was employed to assess viral titre across different age groups of Assam lemon. CTV-positive samples from each age group were selected for real-time PCR analysis.

cDNA synthesised from each sample was diluted to 100 ng/µL and used as a template for the Real-time PCR assay, performed with the TB Green Premix Ex Taq kit (Takara Bio). The actin gene served as an endogenous control[15] for which the primer pair Actin Forward and Actin Reverse were used. Similarly, the primer pair JRT51F and JRT51R was used to amplify CP gene (114 bp)[16]. qPCR was performed in a 10 µL reaction volume using the Applied Biosystems 7500/7500 Fast Real-time PCR system, following the manufacturer's protocol. Thermal cycling conditions included an initial denaturation at 94°C for 2 min, followed by 40 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 45 s, with a melt curve step: 95°C for 15 s, 60°C for 1 min, 95°C for 30 s, and 60°C for 15 s. Each sample was run in triplicate.

Collection of yield data and correlation with virus titer of CTV

Yield was recorded from individual plants within each age group, and the average yield for each group was calculated and expressed in tonnes per hectare (t/ha). This average yield was then correlated with the corresponding virus titer measured for each age group and statistical analysis was performed.

Molecular characterisation of CTV isolates through CP gene sequencing

Six CTV isolates, one from each age group, were selected for molecular characterisation. PCR products were sequenced by Bioserve Biotechnologies India Pvt. Ltd. and the assembled sequences were submitted to National Center for Biotechnology Information (NCBI) GenBank. These were compared with closely related isolates, and phylogenetic trees were constructed based on nucleotide similarity.

Results

Symptomatology and aphid survey

Differential symptoms and vectors were recorded in different age groups of Assam lemon. The most typical symptoms exhibited by the plants were chlorosis of the lamina and midrib, vein clearing, vein banding, vein thickening, and interveinal chlorosis (Fig. 1).

DAS-ELISA

CTV was detected serologically using DAS-ELISA. The absorbance values at 405 nm (OD_{405}) for the negative control, positive control and cutoff were 0.089, 2.774 and 0.267, respectively. All tested

Fig. 1 — Common symptoms of CTV infection of Assam lemon. Yellowing of midrib (A), Chlorosis of lamina (B), Vein banding (C), Vein thickening (D), Interveinal chlorosis (E).

samples showed OD_{405} values ranging from 0.270 to 2.575, indicating positivity for CTV infection. Based on OD_{405} readings, samples within each age group were classified into three ranges of CTV infection: low, medium, and high (Table 1).

Reverse transcriptase Polymerase chain reaction (RT-PCR)

RT-PCR analysis confirmed the presence of CTV in all the samples of each age group which was indicated by the amplicon size of 422 bp (Fig. 2).

Table 1 — ELISA values (OD_{405}) for CTV under different age groups of Assam lemon

	Number of ELISA positive samples			
	Range of ELISA value (OD_{405})			
Age groups	0.270-1.000 Low range	1.001-1.800 Medium range	1.801-2.575 High range	Total
3-year	14	4	2	20
5-year	11	6	3	20
10-year	11	4	5	20
15-year	10	4	6	20
20-year	9	7	4	20
25-year	8	8	4	20

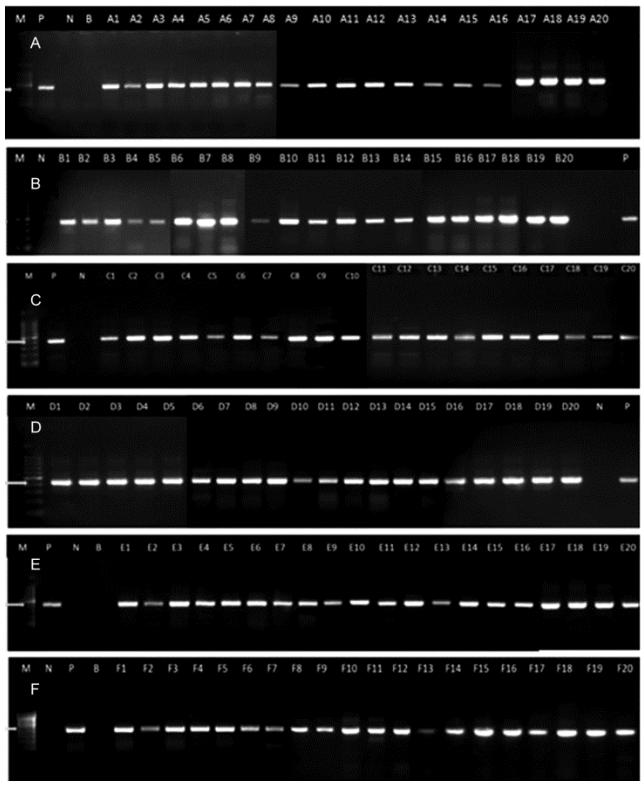

Fig. 2 — Amplified RT-PCR product of CP gene of CTV 422bp (A) 3-year age group plants A1-A20 = samples; (B) 5-year age group plants B1-B20 = samples; (C) 10-year age group plants C1-C20 = samples; (D) 15-year age group plants D1-D20 = samples; (E) 20-year age group plants E1-E20 = samples; (F) 25-year age group plants F1-F20 = samples. [M= 100 bp ladder, P= positive control, N=negative control B= blank]

Quantitative Polymerase chain reaction (qPCR)

The qPCR analysis indicated that the 3-year age group showed the highest Ct value, indicating lower gene expression, which corresponded to the lowest relative quantification (RQ) value. On the other hand, the 15-year age group showed the lowest Ct value, indicating higher gene expression, and correspondingly the highest RQ value (Fig. 3, Table 2).

Correlation between virus titer and yield in Assam lemon across different age groups

The average yield (t/ha) of Assam lemon plants was compared with the Relative Quantification (RQ) values of *Citrus tristeza* virus (CTV) across different age groups, revealing a trend between viral titer and yield with increasing plant age (Fig. 4).

There was a strong positive correlation between the viral titer and age (years) of the plants (0.83) showing that viral load increase with the age of plant. Similarly, a positive yet non-significant correlation (0.062) was

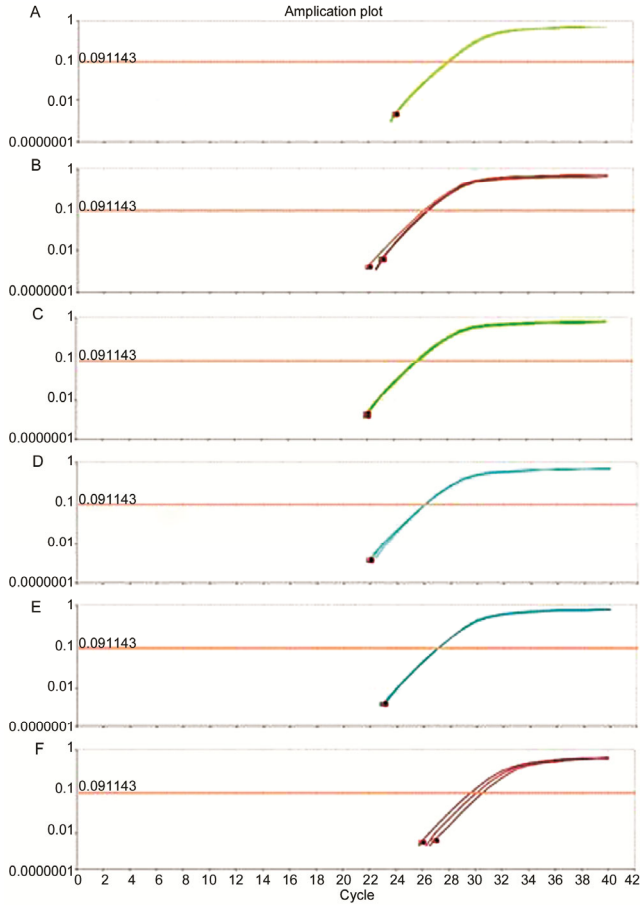

Fig. 3 — Amplification plot of comparative Ct quantification of Real-time PCR assay carried out for CTV-infected samples (A) 3-year (B) 5-year (C) 10-year (D) 15-year (E) 20-year (F) 25-year age group of Assam lemon.

Table 2 — Relative quantification of CTV infected samples of different age groups of Assam lemon

Samples	Mean Cycle threshold (C_t)	Relative Quantification
3-year	30.01	8.62
5-year	28.06	15.08
10-year	26.41	28.87
15-year	25.73	47.05
20-year	26.06	42.84
25-year	27.02	38.11
Healthy	31.29	1.00

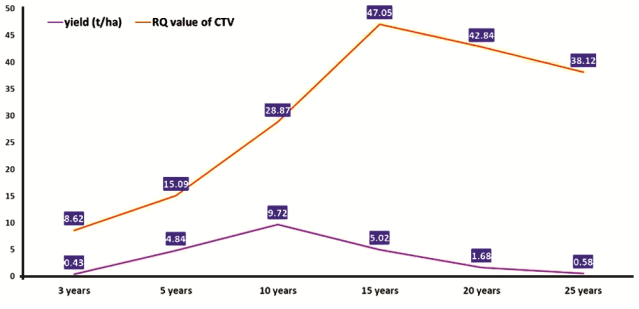

Fig. 4 — Yield trends and CTV Relative Quantification in Assam lemon across different age groups.

recorded between viral titer and yield (t/ha) of the plants. However, a negative correlation (-0.28) was observed between yield (t/ha) and age (years) of the plants revealing that with increase in the age of the plant, there was decrease in the yield.

CTV strain reconfirmation by sequencing

National Center for Biotechnology Information Basic Local Alignment Search Tool (NCBI BLAST) indicated that the consensus sequences showed maximum nucleotide similarity to CTV. These sequences were submitted to NCBI and their accession numbers were obtained. These are OP293340 (3-year), OP312166 (5-year), OP278676 (10-year), OP278675 (15-year), OP278672 (20-year) and OP278673 (25-year).

Phylogenetic analysis of the CTV isolates of each age group

Phylogenetic analysis was conducted to determine the degree of relatedness between the CTV isolates of the Assam lemon from each age group. Sequence analysis showed that isolates from 3-year and 5-year age group shared 100 and 98.1% similarities with previously reported Tinsukia isolate CTV AR1 (KC590488.1)[9]. CTV isolates from 10-year and 15-year age groups showed maximum similarity percentages of 99.46 and 98.20, respectively, with another Indian isolate K7 reported from Darjeeling (GQ475543.1).[17] CTV isolate from 20-year age group shared maximum nucleotide similarity with K20 isolate reported from Darjeeling (GQ475550.1). Similarly, 25-year age group isolate also shared maximum similarity of 100 % with TK1 isolate from Tinsukia, Assam (KC986386.1). Phylogenetic analysis of the six CTV isolates from each age group revealed that the isolates from the three, five, ten, and fifteen year age groups formed two clusters in one group, whereas isolates from the twenty and twenty-five year age groups formed separate groups (Fig. 5).

Discussion

Numerous CTV epidemics have been reported in countries such as Brazil, Argentina, California, Spain, Cuba, Mexico, Israel, and Florida, as well as in many other regions worldwide[18].Given its large genome, limited host range within the slow-growing Rutaceae family, confinement to vascular tissues, and high genetic complexity, CTV presents significant research challenges[19]. For the past few decades, CTV has been extensively studied to find effective means of control in order to eradicate this notorious disease which causes significant losses in fruit production[20]. In India, research has been carried out to report the incidence of CTV in various Citrus species[21-24]. CTV incidence across India ranges from moderate to high, with the highest levels observed in the Northeast[25,26]. Assam lemon showed the greatest susceptibility, followed by Khasi mandarin and Assam rough lemon[27]. However, no prior attempt has been made to study the trend of viral load in various age groups of Citrus species through a molecular approach. Hence, this current investigation is the first of its kind, carried out in Assam lemon of different age groups, to study the behaviour of the virus.

The study was carried out on six Assam lemon age groups (3, 5, 10, 15, 20 & 25 years) as the sampling units, with twenty plants randomly selected from each plantation for sample collection and yield data recording. The symptoms observed included midrib yellowing, lamina chlorosis, vein clearing, vein thickening, and interveinal chlorosis. However, diagnosing CTV is challenging because its symptoms are similar to some bacterial infections, such as citrus canker, phytoplasma disease, or nutrient issues[20,22].

CTV infection was confirmed by ELISA and RT-PCR, using coat protein gene-specific primers. ELISA OD_{405} values for all samples across six age groups of Assam lemon ranged from 0.270 to 2.575, indicating a potentially high incidence of CTV. A significant population of *Toxoptera* species, the primary

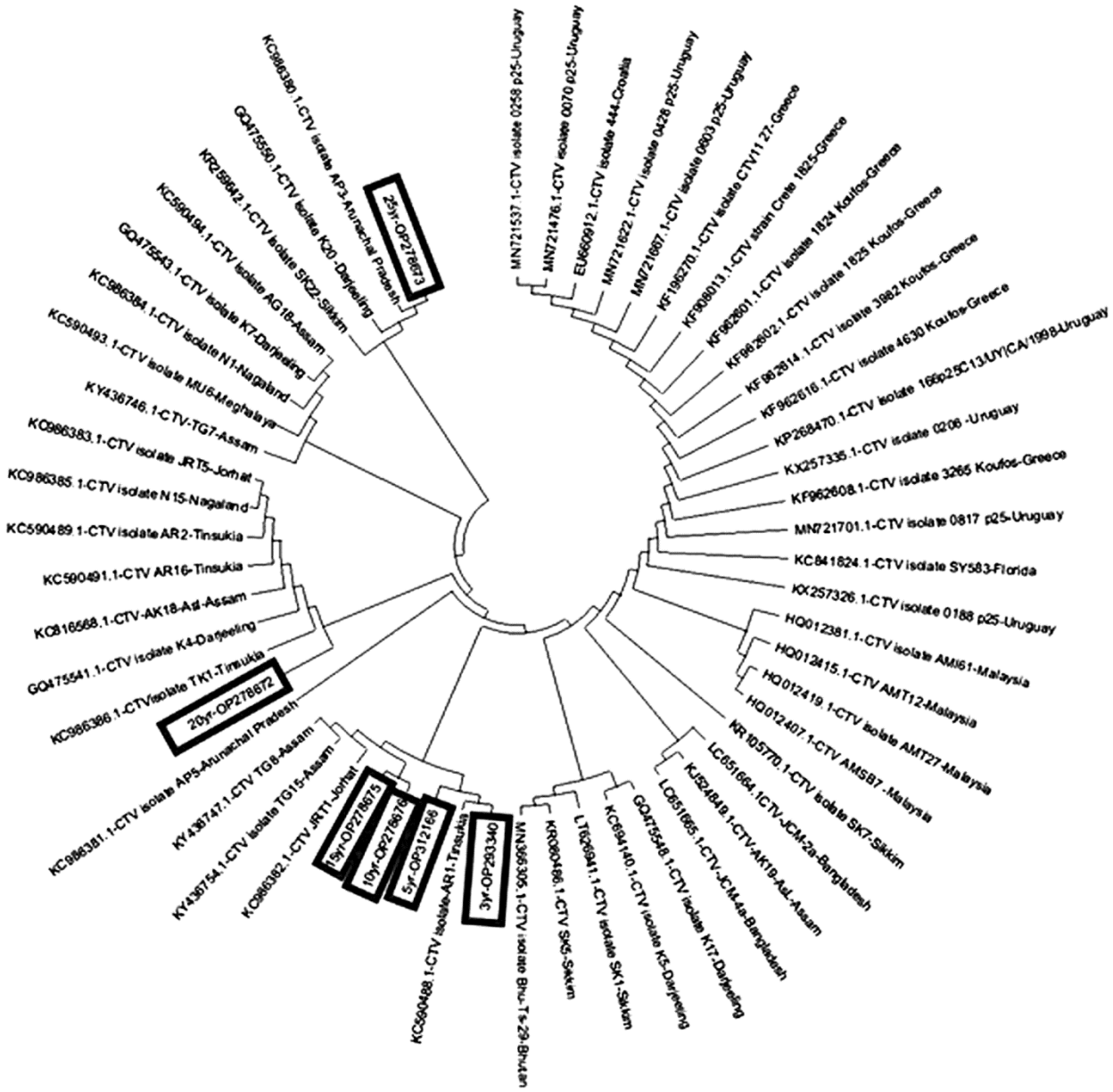

Fig. 5 — Phylogenetic relationships of CTV isolates from each age group with other Indian and international CTV isolates based on CP gene.

vector, was also recorded, varying from moderate to high levels. Previous studies have reported both high vector prevalence and efficient CTV transmission through grafting[28,29]. Additionally, host plant preference can influence the transmission efficiency of aphid vectors[30]. The results were further confirmed by RT-PCR, with agarose gel electrophoresis depicting the expected 422bp amplicons in all samples.

Real-time PCR was conducted to compare viral titer across different age groups of Assam lemon using the Relative Quantification method, with the *Actin* gene as an endogenous control. Ct values for the test samples ranged from 25.73 to 30.01, while the healthy sample had a Ct value of 31.29. The 3-year age group showed the smallest RQ value (8.62), with an increase to a peak RQ of 47.05 in the 15-year age group. Beyond this point, RQ values decreased to 42.84 in the 20-year and 38.11 in the 25-year age groups, suggesting a decline in viral load with advanced age, likely due to natural senescence reducing viral replication. However, specific host factors may also have a role in the gene expression of

the virus[31]. Also, abiotic factors, such as temperature fluctuations, along with co-infections by other viruses or pathogens, can significantly influence the manifestation of symptoms in host plants[32].

Yield data from Assam lemon plants showed that productivity increased up to 10 years and then declined until 25 years. Correlating yield with Relative Quantification values revealed a critical age when the virus titer peaks, significantly reducing productivity. CTV plays a key role in reducing citrus yield, contributing to Citrus decline[33]. However, other factors, such as ageing, physiological changes, and crop management practices, may also influence the decreased output[34].

CTV isolates from all age groups of Assam lemon showed the highest similarity with Indian isolates from Northeastern states and nearby regions, such as Darjeeling, Bhutan, and Bangladesh. International similarity was observed with isolates from Uruguay, Greece, and Florida. Phylogenetic analysis revealed that isolates from the 3, 5, 10, and 15-year age groups clustered together, indicating close relatedness, while isolates from the 20 and 25-year age groups formed separate clusters. The 25-year old isolate was the most genetically distant. This nucleotide variation may be due to the error-prone nature of RNA polymerases, selection pressures, superinfection, genetic drift, or recombination in CTV isolates[35].

Efforts were made to detect mild and severe strains of CTV in Assam lemon across different age groups. Mild strains of CTV often cause no visible symptoms in most Citrus species despite replicating to high titres[36] while severe strains can cause decline and stem pitting[37]. Two plants from each age group were randomly selected, and RT-PCR followed by agarose gel electrophoresis revealed the presence of the severe strain in all age groups. The mild strain was scattered in the 3, 5, 10, 15, and 20-year groups but was absent in the 25-year group.

Conclusion

This study highlights the significance of a global pathogen *Citrus tristeza* virus (CTV) and its impact on Assam lemon (*Citrus limon*), posing a substantial threat to fruit yield. Notably, plants in the 15-year age group exhibited the greatest decline in productivity, indicating a possible age-related vulnerability. Molecular diagnostics, particularly qPCR, have proven effective in unravelling disease trends and highlighting the importance of early detection for effective management.

However, several limitations of this research must be acknowledged. While a low Ct value determining high viral load, may often be related with more severe symptoms and increased mortality, this relationship may not always be true and can be influenced by multiple confounding factors such as host immune responses, physiological conditions, and varietal resistance[38]. Environmental variables, such as temperature fluctuations and drought stress, further complicate disease progression[32]. Additionally, the virulence of specific CTV strains plays a crucial role, as certain strains can induce severe symptoms even at lower viral titres[39].

Further, comprehensive studies are needed to isolate, characterize, and document the CTV strains prevalent in Assam lemon grown under different agro-ecological conditions across the state. Pathogen derived resistance has been developed in transgenic plants against CTV but could not be maintained[40]. Advanced tools like CRISPR-based genome editing has been used to identify new isolates[41] and can offer promising avenues for enhancing citrus resilience against CTV[42].

Conflict of interest

The authors declare no conflict of interests.

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