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RESEARCH ARTICLE

Age-dependent epidemiological insights into *Citrus tristeza virus* in Assam lemon cultivation

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Abstract

Assam lemon (*Citrus limon* Burm.), a valuable indigenous horticultural crop from Northeast India, is facing a serious threat from *Citrus tristeza virus* (CTV), a global pathogen responsible for citrus decline. A study conducted in the Lower Brahmaputra Valley Zone (LBVZ) of Assam aimed to investigate the dynamics of CTV in Assam lemon across six plant age groups (2, 5, 10, 15, 20 and 25 years). Serological [double antibody sandwich- enzyme linked immunosorbent assay (DAS-ELISA)] and molecular [reverse transcriptase-polymerase chain reaction (RT-PCR)] tests confirmed 100 % CTV incidence in the surveyed area. The study examined the influence of plant age and climatic factors on vector incidence, disease severity and yield. Analysis revealed that minimum temperature, rainfall and evening relative humidity had strong positive correlations with vector presence and disease severity. Regression analysis showed that older plants experienced significantly greater disease severity, with a one-year increase in age leading to a 5.71 % rise in disease severity. Additionally, disease severity negatively impacted fruit yield, with a 1 unit increase in disease severity causing yield reductions ranging from 21.5 % to 42.9 %, depending on the age group. Disease severity increased with plant age, while fruit yield peaked at younger stages and declined in older plants. These findings underscore the critical need for early detection and targeted disease management to mitigate losses and maintain the long-term productivity of Assam lemon orchards. Early intervention strategies are essential to protect this economically important crop from the devastating effects of CTV.

Keywords: correlation; DAS-ELISA; epidemiology; regression; RT-PCR

Introduction

Assam lemon (*Citrus limon* Burm.), locally known as Kazi Nemu, is an indigenous and economically important fruit crop of Assam in northeastern India (1). The fruit is highly valued for its distinctive aroma and characteristic flavour traits that have contributed to its recognition with a geographical indication (GI) tag in 2019 (2, 3). Cultivation of Assam lemon not only supports the agricultural economy but also plays a crucial role in the livelihoods of small and marginal farmers in the region (2). However, in recent years, the sustainability of Assam lemon cultivation has been increasingly threatened by the rapid spread of *Citrus tristeza virus* (CTV), a globally recognized pathogen responsible for citrus decline (4, 5). CTV leads to a range of physiological and morphological symptoms, including chlorosis, vein banding, vein thickening, stunted growth and a significant reduction in fruit yield (6). Infected trees often exhibit dieback and decline over time, severely affecting orchard productivity and farmer livelihoods. According to reports, the prevalence of CTV disease is 26.3 % in central India, 47.1-56.0 % in northeastern India, 36-50 % in south India and 16-60 % in north-northwest India (7). Also, it has been reported that 76.47 % of Assam lemons had CTV disease, followed by 61.18 % of Khasi mandarins and 52.03 % of Assam rough lemons (6).

CTV is transmitted mechanically by cleft grafting (8, 9). It is also transmitted in a semi-persistent manner by aphid vectors, particularly species of the genus *Toxoptera*, which are highly efficient in spreading the virus across citrus orchards (10). The widespread presence of these aphids in Assam's humid subtropical climate further exacerbates the risk of CTV transmission (11). While numerous studies have addressed different aspects of CTV epidemiology, the specific influence of weather variables and plant age on disease severity and yield trends remains unexplored in Assam lemon (7, 10). To bridge this knowledge gap, the present study was undertaken to: (i) assess disease incidence and examine the influence of key weather parameters on aphid population dynamics and disease severity; (ii) assess the relationship between plant age and disease severity and (iii) evaluate the impact of disease severity on yield across different age groups of Assam lemon. Understanding these relationships is crucial for developing effective disease management strategies and ensuring the long-term sustainability of Assam lemon cultivation.

Materials and Methods

Survey and sampling

An investigation was carried out during 2021-22 where Assam lemon plantations of six age groups 2, 5, 10, 15, 20 and 25 years were surveyed from a Citrus orchard in Horticultural Research Station, Kahikuchi located in the Lower Brahmaputra Valley Zone (LBVZ) of Assam for disease incidence, severity and vector count. Recommended horticultural practices were followed and no insecticides were sprayed for natural epiphytotics to occur. Both symptomatic and asymptomatic leaf samples were collected from randomly selected twenty plants of each age group.

Serological (DAS-ELISA), molecular (RT-PCR) detection of CTV and calculation of percent disease incidence

Leaf samples from individual plants across all age groups were tested for CTV using a commercial DAS-ELISA kit (Bioreba AG, Switzerland) following the manufacturer's protocol. Reverse transcriptase-polymerase chain reaction was performed to confirm DAS-ELISA results. Total RNA was extracted using both CTAB and TRIzol reagent (12, 13). cDNA was synthesized using the PrimeScript 1st strand cDNA synthesis kit (Takara Bio) following the manufacturer's instructions. PCR amplification targeting the coat protein gene (422 bp) was performed using p20F (5'GATGTGCGTCAGTTGGGTAC3') and p20R (5'CCAGCTCCGGTCAAGAAATC3') under thermo cycling conditions (14, 15). The amplified products were resolved on a 1.5 % agarose gel. Disease incidence was calculated as per the standard method (7).

Record of weather parameters, disease severity, vector incidence and fruit yield

Weather data, including morning relative humidity (RH1), evening relative humidity (RH2), maximum temperature (T_{max}), minimum temperature (T_{min}), rainfall and bright sunshine hours (BSH), were collected from the Regional Agricultural Research Station, Gossaigaon, for the period from 1st February to 15th August (2021-22). All weather parameters were expressed as weekly averages, except for rainfall, which was recorded as the total amount per week.

Disease severity was assessed for each age group of Assam lemon plants using a standardized 0-3 rating scale, where 0 = symptomless, 1 = mild symptoms, 2 = moderate symptoms and 3 = severe symptoms (16). For each plant, ten leaves were randomly selected and scored (0-3) and the mean severity score was calculated. Aphid incidence was recorded as the average number of aphids per twig, based on observations from ten twigs per plant. Both disease severity and aphid incidence were monitored weekly from February through the second week of August. Fruit yield was recorded in the second week of August for each individual plant and was expressed in kg.

Statistical analysis

Correlation (Pearson) analysis was performed to examine the relationships among weekly weather parameters, aphid population levels and disease severity over the study period. Simple linear regression was conducted to evaluate the effect of plant age on disease severity. Additionally, regression analysis was used to assess the impact of disease severity on fruit yield across the fruit-bearing age groups (5 to 25 years). All statistical analyses were carried out using R software and Microsoft Excel.

Results

Symptomatology

Distinct CTV-associated symptoms such as chlorosis, interveinal chlorosis, vein banding and vein thickening were recorded across all age groups. Symptom expression ranged from asymptomatic to mild in 2-year-old plants, while 10- and 15-year-old plants predominantly exhibited mild to moderate symptoms. In 20- and 25-year-old plants, symptoms were more pronounced, ranging from moderate to severe. Advanced infections in older plants were further characterized by dieback, indicating progressive disease infection (Fig. 1).

Confirmation of CTV and disease incidence

DAS-ELISA results revealed the presence of CTV in all assayed samples across the different age groups. The recorded OD_{405} values ranged from 0.270 to 2.575, exceeding the threshold cutoff value of 0.267 (positive control: 2.774; negative control: 0.089). RT-PCR further validated the ELISA results, with all representative samples yielding the expected 422 bp amplicon, confirming the presence of CTV across all age groups (Fig. 2). These findings indicated a 100 % incidence of CTV among the sampled plants in the surveyed region.

Influence of weather parameters on aphid population and disease severity

The Pearson's correlation analysis revealed a strong and statistically significant positive association of minimum temperature (T_{min}), evening relative humidity (RH2) and rainfall with aphid population and disease severity (Fig. 3 & 4). However, morning relative humidity (RH1) showed a strong positive correlation with disease severity and a weak positive correlation with vector abundance. Maximum temperature (T_{max}) and bright sunshine hr (BSH) showed positive and negative correlations respectively, with both disease severity and aphid population but none of these relationships were statistically significant (Fig. 3 & 4). Overall, the study demonstrates a clear association among weather parameters, vector incidence and disease severity.

Fig. 1. *Citrus tristeza virus* symptoms (A) chlorosis, (B) vein banding and (C) dieback.

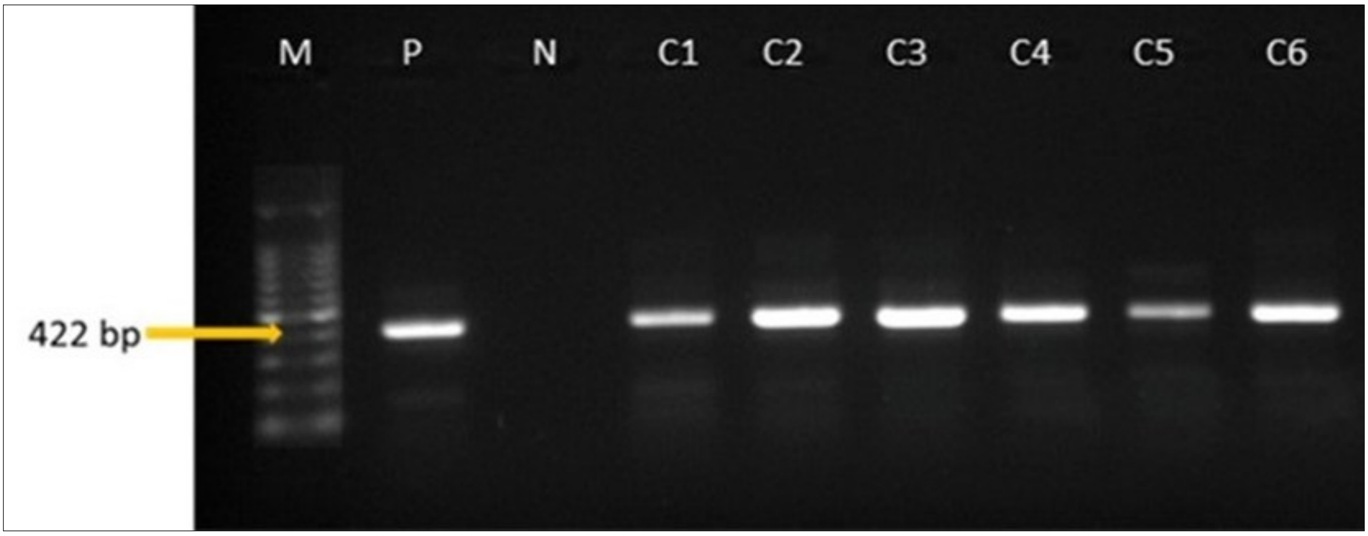

Fig. 2. Agarose gel electrophoresis showing amplified RT-PCR product of CP gene of representative *Citrus tristeza virus* infected samples of different age groups; M = 100 bp ladder, P = positive control, N = negative control, C1= 2-years age group plant sample, C2= 5-years age group plant sample, C3= 10-years age group plant sample, C4= 15-years age group plant sample, C5= 20-years age group plant sample, C6= 25-years age group plant sample.

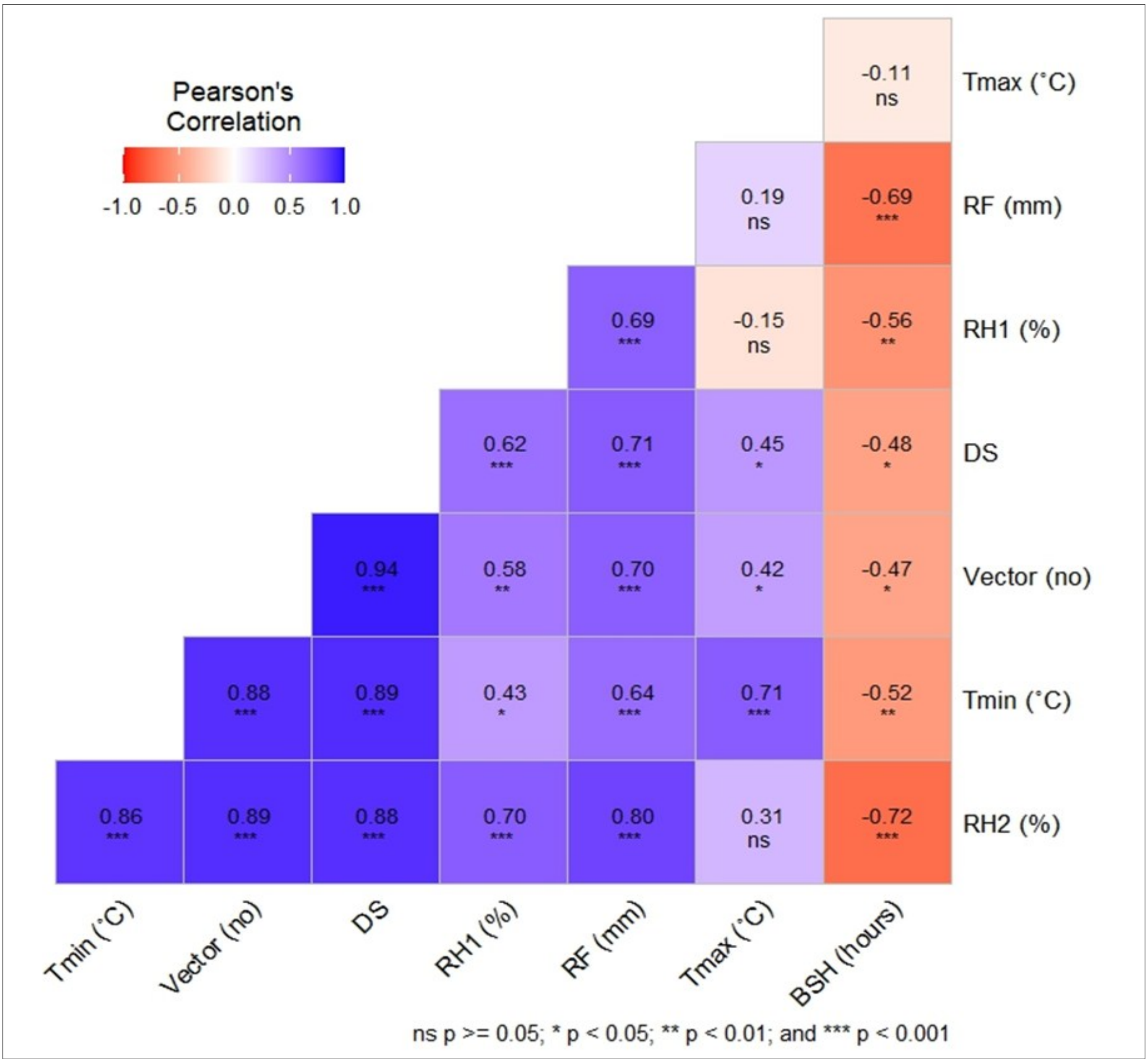

Fig. 3. Pearson correlation among weather parameters (T_{max}, T_{min}, RH1, RH2, BSH and rainfall), vector population, disease severity with correlation coefficients.

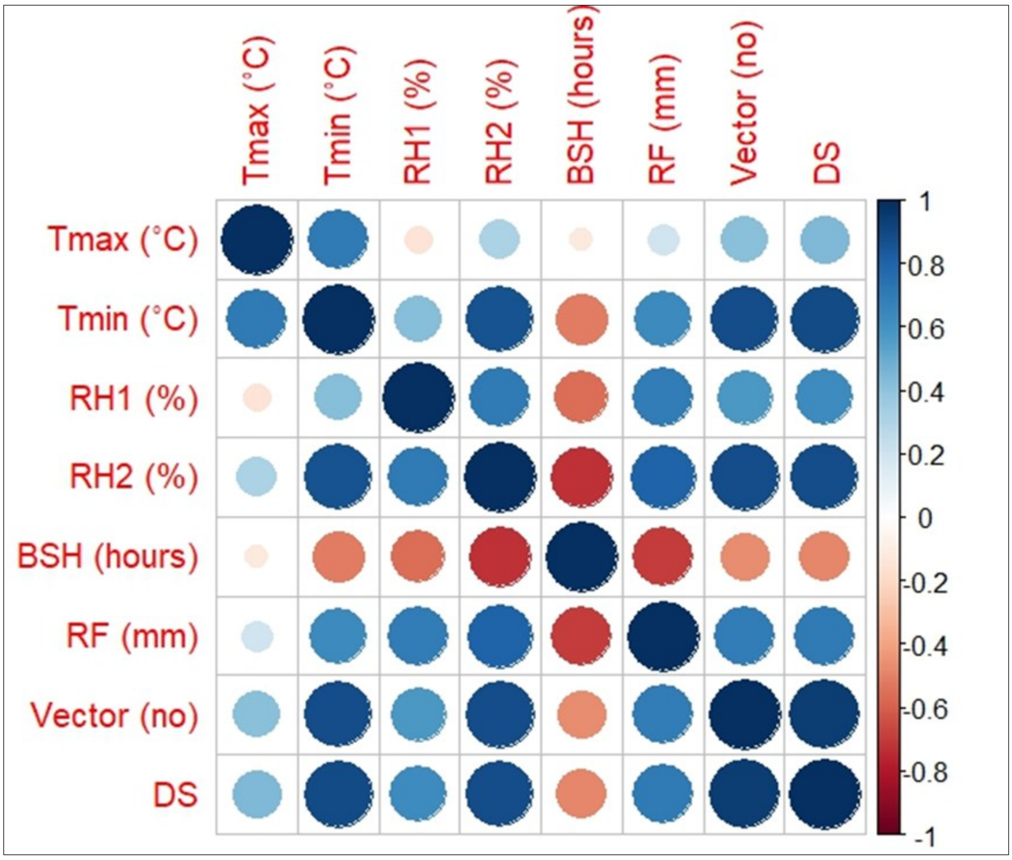

Fig. 4. Corrplot (R studio) showing correlation strengths among weather parameters (T_{max}, T_{min}, RH1, RH2, BSH, rainfall, vector population and disease severity.

Regression analysis

Simple linear regression analysis between plant age (independent variable) and disease severity (dependent variable) indicated that a one-year increase in plant age corresponded to a 5.71 % increase in disease severity (Table 1, Fig. 5). Regression analysis across all age groups revealed that a 1 unit increase in disease severity corresponded to a 21.5-42.9 % reduction in yield (Table 1, Fig. 6). A clear increasing trend in disease severity was observed with advancing plant age, while yield showed a consistent decline, particularly between the 10- and 25-year age groups (Fig. 7). Most of these relationships were statistically significant at $p = 0.05$. However, the regression between disease severity and yield was not statistically significant for the 5-year age group (Table 1).

Discussion

CTV has long been a devastating threat to citrus cultivation, having caused the death of over 100 million trees worldwide (17). In India, considerable research has been conducted on CTV across various citrus species. Despite these efforts, the virus continues to pose a significant challenge to orchard health and fruit productivity due to its rapid spread and complex interactions with host and environmental factors.

In this study, six age groups of Assam lemon (2, 5, 10, 15, 20 and 25 years) were surveyed in LBVZ of Assam. To assess the disease incidence as well as impact of weather variables on vector incidence and disease severity and their combined impact on yield. Serological as well as molecular (RT-PCR) detection confirmed 100

Table 1. Regression analysis of plant age effects on *Citrus tristeza virus* disease severity and yield across 5-25 years age groups

	Variables	Coefficients	Percent gain in disease severity / percent loss in yield	Standard errors	*p*-values
Plant age and disease severity (DS)	Intercept	0.317		0.123	0.015
	X (plant age)	0.1095	5.71	0.008	1.056e-13
DS and yield (5-year age group)	Intercept	10.318		2.626	0.004
	X (DS)	-4.32	41.9	2.006	0.063
DS and yield (10-years age group)	Intercept	15.845		2.003	4.74e-05
	X (DS)	-3.41	21.52	1.19	0.020
DS and yield (15-years age group)	Intercept	13.71		1.34	7.44e-06
	X (DS)	-3.78	27.57	0.57	0.0001
DS and yield (20-years age group)	Intercept	4.57		1.08	0.002
	X (DS)	-1.43	31.29	0.41	0.008
DS and yield (25-years age group)	Intercept	3.66		1.07	0.009
	X (DS)	-1.12	30.60	0.37	0.016

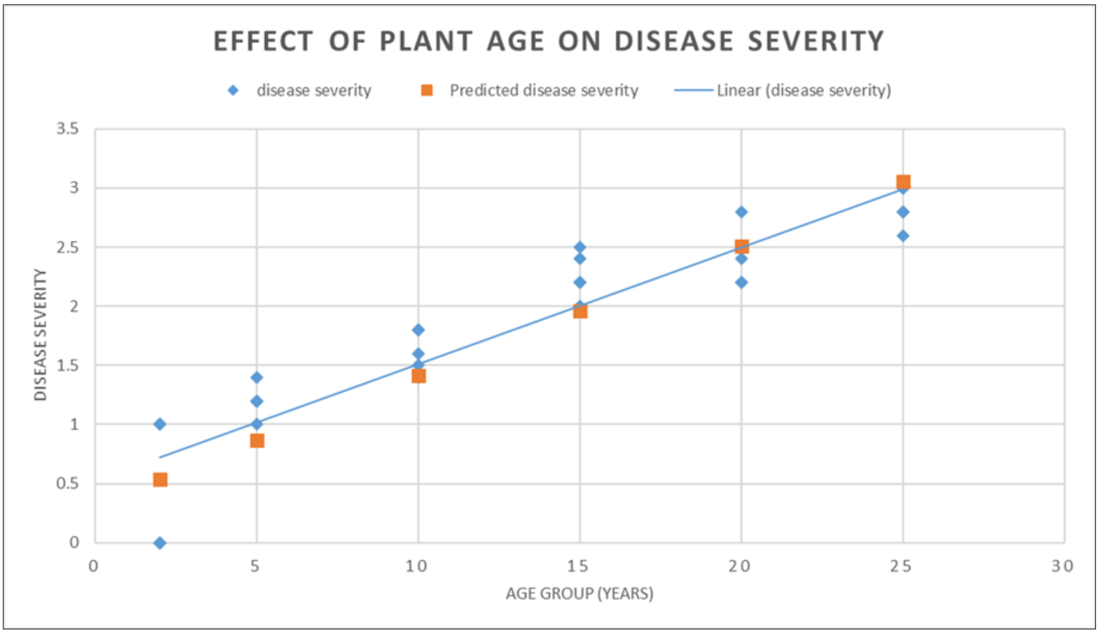

Fig. 5. Regression analysis showing influence of plant age on disease severity across six different age groups in Assam lemon.

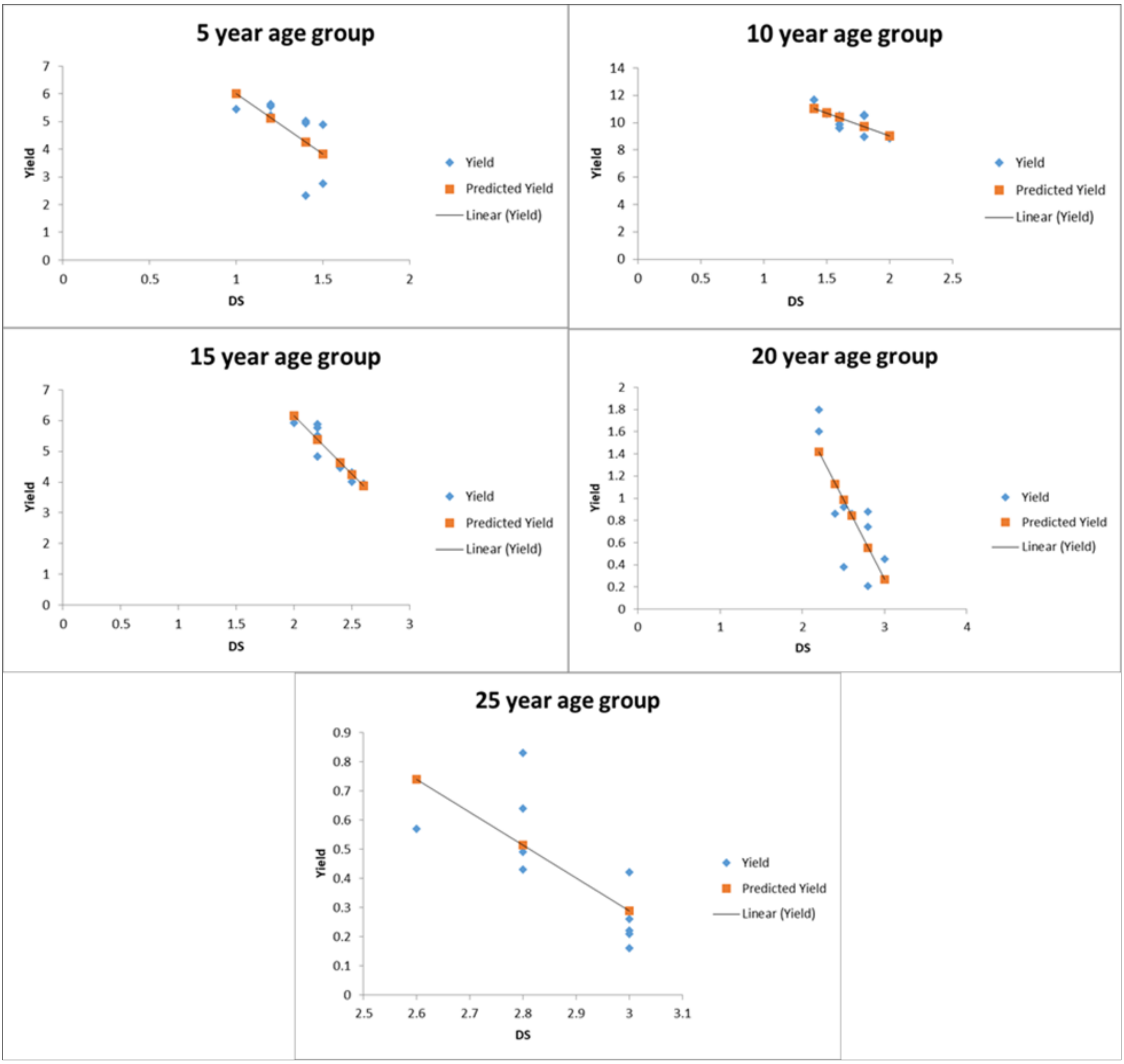

Fig. 6. Scattered plot charts showing relationship between disease severity and yield in 5, 10, 15, 20 and 25 years age groups.

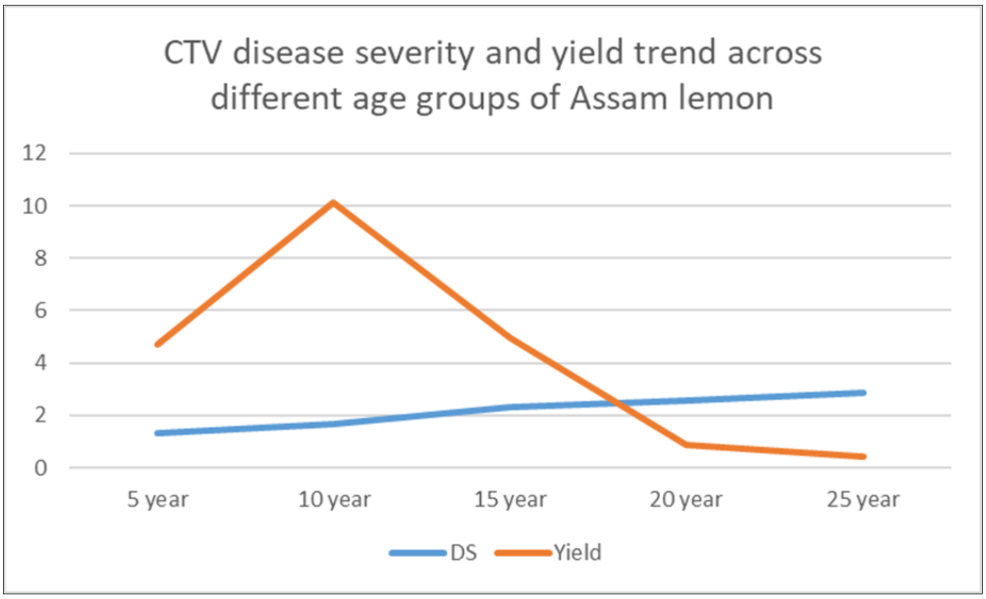

Fig. 7. *Citrus tristeza virus* disease severity vs. yield trend in Assam lemon plants of varying age groups.

% CTV incidence in the surveyed Assam lemon plants, confirming the widespread presence of the virus. Detection of the virus in symptomless trees indicates the existence of a mild strain, which may not cause visible disease symptoms but can still contribute to the persistence and spread of the virus in the orchard. Previous studies have also identified Assam lemon as the most susceptible citrus species, with higher incidence rates than Khasi Mandarin and rough lemon (15). CTV incidence as high as 63.5 % across various citrus species in Assam, with Assam lemon showing the highest at 76.47 % (6). These findings highlight the extensive distribution of the virus and its potential threat to the citrus industry in Assam and the Northeast region.

Correlation analysis indicated a strong positive association among aphid population, disease severity and weather parameters such as minimum temperature (T_{min}), evening relative humidity (RH2) and rainfall. Maximum temperature (T_{max}) also had a strong positive association with disease severity but a weak positive relationship with aphid population. Weather conditions play a critical role in influencing the production of new flushes, aphid population dynamics and the replication of CTV. These interconnected factors collectively impact the temporal patterns of virus incidence and facilitate its spread both within and across citrus orchards (18). Mean temperature and precipitation were also seen to have a positive relationship with aphid population in clementine citrus (19).

Plant age was also seen to have a direct influence on the progression of CTV. Regression analysis revealed that with one year increase in plant age, disease severity is predicted to increase by 5.71 %. Similar regression analyses across other age groups revealed that with 1 unit increase in disease severity, yield is expected to decrease by 21.5-42.9 % (20). Due to the small sample size and data collection limited to a specific orchard from a single agroclimatic zone, multivariate regression could not be applied in the present study. However, this statistical approach holds promise for future research, as it could offer deeper insights into the complex relationships between weather parameters, plant age, vector populations, disease severity and yield. Further studies across multiple locations and replications would be valuable to confirm the broader applicability of the results.

However, decrease in yield cannot be solely contributed to CTV. Biological aspects such as plant vigour and natural senescence may also influence fruit yield (21). Also, factors affecting yield, such as soil fertility, nutrient status or tree pruning history should also be considered as these could confound disease-yield relationships. In the present study, disease severity showed a consistent increasing trend with plant age, whereas yield initially increased and then declined in older age groups. These observations suggest a potential cumulative impact of disease and age-related physiological decline on productivity. Similar findings were reported that older orchards tend to harbor higher levels of disease inoculum compared to younger ones (15).

Conclusion

The study underscores the widespread prevalence and significant impact of CTV on Assam lemon across all age groups in the LBVZ of Assam. Symptom expression and disease severity increased progressively with plant age, with older plants exhibiting more pronounced symptoms and greater yield loss. Serological and molecular diagnostics confirmed 100 % CTV incidence, highlighting its pervasive threat. Correlation and regression analyses further revealed that weather parameters such as minimum temperature, evening humidity and rainfall significantly influence aphid populations and disease severity. The findings demonstrate that both plant age and environmental conditions play critical roles in CTV progression and associated yield decline, emphasizing the urgent need for integrated disease management strategies in citrus orchards of the region.

Effective management of CTV requires an integrated approach combining cultural, genetic and chemical strategies. Early and regular scouting helps in timely detection of symptoms and vector populations. Removal of old, severely infected trees helps reduce inoculum sources and limits disease spread. The use of resistant rootstocks, such as *Poncirus trifoliata* and its hybrids can minimize yield losses and maintain orchard longevity. Vector control should be timed with weather patterns that favour vector activity, during periods of moderate temperatures and new flush growth to enhance insecticide effectiveness and reduce virus transmission. Mild strain cross protection, pathogen derived resistance and RNA silencing can also help in combatting the viral disease.

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Authors' contributions

DS planned and conducted all the lab experiments and drafted this manuscript. KSDSRK provided valuable suggestions in conducting the experiment in the laboratory. SS, VU and SD helped in the field data collections. PDN conceived the idea and guided throughout the study. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

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