



Research Article

ELISA Based Monitoring of Citrus Tristeza Virus in Declining Orchards of Sargodha, Pakistan

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Abstract | This study assesses the current status of citrus tristeza virus (CTV) in three tehsils of the Sargodha district: Kot Momin, Sargodha, and Bhalwal. The incidence and severity of CTV were evaluated using three citrus varieties: Musambi, Feutrel's Early, and Kinnow. Disease incidence was calculated using the ELISA test, while disease severity was determined by counting the number of pits per square inch. The results revealed significant variation in CTV incidence and severity across the three citrus varieties. Musambi exhibited the highest incidence rate at 4.5, followed by Feutrel's Early at 2.89 and Kinnow at 1.71. Musambi also had the highest severity score at 53.5 pits per square inch, followed by Feutrel's Early at 27.5 and Kinnow at 11.5. Geographical analysis of CTV incidence indicated that Kot Momin had the highest incidence rate at 4.73, followed by Bhalwal at 2.89 and Sargodha at 1.9. Correspondingly, Kot Momin also showed the highest severity score at 33.5 pits per square inch, with Bhalwal and Sargodha recording 25.5 and 12.5, respectively. The research provides deep insights and understanding for the identification of high-risk areas in Punjab and susceptible varieties, which are crucial for developing effective CTV mitigation strategies. This research contributes to the existing body of knowledge by presenting novel insights into the geographical and varietal differences in CTV impact, paving the way for future studies and interventions.

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Introduction

Pakistan is the world's sixth-largest producer of Kinnow. Among the nations that produce citrus fruit, Pakistan ranks low in production (Mubeen *et al.*, 2024). Citrus trees suffer devastating losses due to various diseases, such as bacteria, fungi, viruses, viroids, nematodes and phytoplasma (Iftikhar *et al.*, 2024; Mubeen *et al.*, 2015a, b). Citrus tristeza virus (CTV) is the deadliest virus. CTV is a virus that belongs to the family *Closteroviridae* and the genus *Closterovirus*. It is spread by vectors such as mealybugs, aphids, and whiteflies (Karasev *et al.*, 1997). *Aphis gossypii* is the principal vector that effectively transfers different CTV strains from plant to plant and from orchard to orchard (Roistacher and Moreno, 1991). The rootstock and the desired citrus cultivar affect the severity of disease in citrus trees. Two prominent symptoms are stem pitting, which causes aberrant development of vascular structures, and decline, ultimately killing the tree. Graft-transmissible viruses and viral diseases significantly constrain citrus production globally (Roistacher, 1991; Satpute and Fadli, 2022). Among these, Citrus Tristeza Closterovirus (CTV) is one of the most devastating and widespread diseases affecting citrus-growing regions worldwide. Once a citrus tree is infected with the virus, it remains infected for life, and there is no known method of eliminating the virus from the plant. Citrus Tristeza Virus degenerates the cambium layers, causing the infected trees to progressively decline. When a certain strain of CTV attacks a tree, the infected tree may develop symptoms (Vives *et al.*, 1999). Approximately 20 kilobytes in length, with some untranslated regions at each end, CTV comprises positive-sense single-stranded RNA, as (Karasev *et al.*, 1997) reported. It is formatted with 12 ORFs for easy reading. CTV is a single-stranded RNA Closterovirus characterized by flexuous, rod-shaped particles measuring approximately 12 x 2,000 nm (Gaur *et al.*, 2023). The virus has numerous complex strains that vary significantly in their effects, ranging from mild to severe stem-pitting isolates (Roberts *et al.*, 2001; Sharma, 2023; Su, 1998). CTV affects all citrus species, cultivars, and hybrids, irrespective of the rootstock, leading to a spectrum of symptoms from mild or undetectable to severe (Coletta-Filho *et al.*, 2020). Sweet oranges and other varieties grafted onto sour orange rootstock are especially prone to severe infections. Additionally, the virus causes disease in grapefruit, lemon, lime, and calamondin (Satpute and

Fadli, 2022). Since viruses must consume their hosts to reproduce, they are universally seen as parasites in the natural world. Infection requires establishing a favorable interaction between the host and the Virus (Muller and Costa, 1987). Plants use several immune responses to combat virus infection (Voinnet, 2005). When genetic diversity occurs in the wild, it may impact symptom phenology. Stem pitting in the main trunk, large and tiny lateral branches, and twigs are symptoms of CTV virulence. Huge losses might be caused, however, by very aggressive CTV strains or isolates. Fruit quantity and quality are diminished when the citrus plant's growth ability is inhibited. In good circumstances and severe disease, plants dry up and die (Niblett *et al.*, 2000). CTV is prevalent in all citrus-growing regions worldwide and has led to the death of millions of trees in countries such as Brazil, Spain, and Argentina (Ayana, 2021; Cambra *et al.*, 2000; Mooney and Harty, 1992). By 1981, the global loss due to CTV was estimated at 50 million trees, however, this figure had escalated to 100 million trees in Argentina, Brazil, Spain, California, Venezuela, and other regions due to the rapid decline caused by the virus in 1991 (Bar-Joseph *et al.*, 1982; Jyothi and Shilpa, 2020; Mooney *et al.*, 1994). In Pakistan, the presence of CTV was confirmed in citrus species in Punjab and NWFP through ELISA and electron microscopy (Anwar and Mirza, 1992; Catara *et al.*, 1988; Iftikhar *et al.*, 2009). The virus poses a serious threat to citrus production in major citrus-growing areas of Punjab. CTV is primarily spread by the citrus brown aphid (*Toxoptera citricidus* Kirk.), Khan *et al.* (2022) which, fortunately, has not been reported in Pakistan. However, other significant aphid species, such as *Toxoptera aurantii*, *Myzus persicae*, and *Aphis gossypii*, may potentially serve as vectors, although no disease transmission has been documented so far (Iftikhar *et al.*, 2009, 2012). However, previous studies also indicated that Citrus Tristeza Virus (CTV) incidence and severity are significantly higher in the Musambi Iftikhar *et al.* (2009), variety and in the Kot Momin tehsil compared to other citrus varieties and tehsils within the Sargodha district, suggesting that both varietal susceptibility and geographic location play critical roles in the spread and impact of CTV. Given the potential threat, extensive surveys of citrus orchards in Pakistan were deemed necessary to collect data on the distribution of CTV in citrus cultivars, assess the percentage of disease incidence, Severity and confirm CTV infection through ELISA.

Materials and Methods

Survey and CTV-infected sample collection

Citrus plantations in three Tehsils-Sargodha, Bhalwal, and Kot Momin underwent extensive surveys across selected villages (Figure 1). Each Tehsil included five carefully chosen villages for sampling. In Kot Momin, the selected villages were Mateela, Moazzamabad, Chak 19, Chak 26, and Uppi. Similarly, Bhalwal chosen villages were Chak 7 NB, Chak 4 SB, Chak 13 NB, Chak 2 NB, and Chak 10. Sargodha's villages included Chak 89 SB, Chak 90 SB, Chak 94 SB, Chak 86 SB, and Chak 81 SB. The study focused on three citrus fruit varieties, Musambi, Feutral's early, and Kinnow. Morphological, like dying off, pitting stems, and yellowing leaves, identified diseased samples. Collected samples showing symptoms were immediately placed in polythene bags and stored in an ice bucket. These samples were then sent to the Citrus Research Institute in Sargodha to determine the presence of CTV.

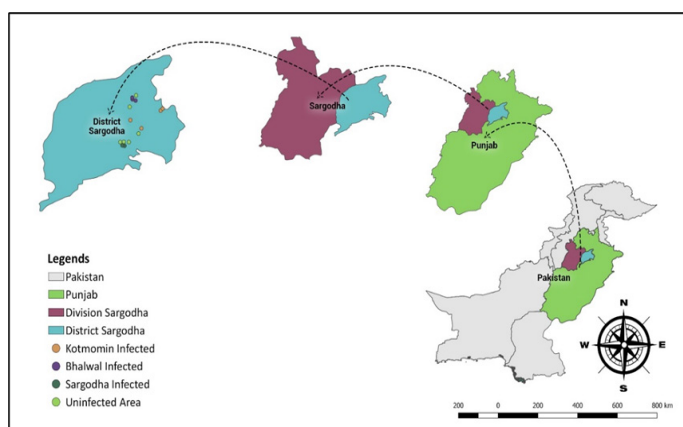


Figure 1: Spatial distribution of CTV in citrus orchards of Sargodha.

ELISA test

The enzyme-linked immunosorbent assay (ELISA) technique was used to determine the presence of the virus. According to Clark and Adams (1977), infected material was taken from the field and tested using a double antibody sandwich (DAS-ELISA) to determine whether or not CTV was present. The ELISA test used polyclonal antibodies from Bioreba (www.bioreba.com). Several buffers were utilized in the ELISA assay, all of which were utilized prior to the tests beginning.

ELISA procedure

In the ELISA process, Microtiter plates were coated with CTV-specific antibodies (1000-fold dilution

in coating buffer), incubated at 30°C for 4 hours, and washed thrice. Antigen (1:10 w/v) in extraction buffer (100µl/well) was loaded, followed by overnight incubation at °C and additional washing. Reference controls were included as Positive and Negative controls. CTV conjugated antibody (1000-fold dilution in conjugate) was added (100µl/well) and incubated at 30°C for 5 hours, followed by washing. Substrate (p-nitrophenyl-phosphate) at 1mg/ml (100 µl/well) was added, and the incubation occurred at room temperature for at least an hour. The procedure was repeated multiple times for precision in CTV detection.

Colour development

The appearance of a yellowish hue in the wells was the most telling sign of CTV infection, with the intensity of the color correlating to the level of viral activity in the plant. Examining how yellow was perceived allowed us to distinguish between the positive and negative samples. The reaction was halted by adding 50 L of a 1N sodium hydroxide solution, and the plates were imaged afterwards.

Severity of CTV

The Musambi orchards were discovered to have a severe CTV infestation. The initial step was to classify Musambi orchards into five distinct age categories based on their estimated age at the time of planting up to 12 years, up to 14 years, up to 16 years, up to 18 years, and up to 20 years. To acquire an accurate picture of the scope of the issue, we sampled two fields from each cluster. A total of 10 plants were then selected at random from each plot. The research used these plants. It was determined how many pits were, thereby cutting a square inch of bark from the area where the rootstock and scion stock union met. After determining the percentage of the 1 square inch region that was pitted, we used the chart to evaluate the severity of the pitting (Table 1). To calculate this, divide the area covered by pits by the area of one square inch.

Table 1: Number of pits per square inch of bark (%).

Rating	Severity level	No. of pits per Sq. inch
1	Least severe	0-15
2	Slightly severe	15-30
3	Moderately severe	31-45
4	Severe	46-60
5	Highly severe	> 60

Statistical analysis

The research involved implementing a two-way analysis of variance (ANOVA) and subsequent application of the least significant difference (LSD) test in Software named as SPSS and Statistix 8.1 to determine the spatial distribution of the Citrus tristeza virus in the orchard of Sargodha (Steel and Torrie, 1997).

Results and Discussion

Spatial distribution of CTV in Sargodha citrus orchards

During survey of 3 Tehsils of Sargodha, including Kot Momin, Bhalwal, and Sargodha it was observed that 4 villages in Kot Momin showed a higher vulnerability to CTV. These villages, namely Mateela, Moazzamabad, Chak 19, and Uppi were highlighted on maps using the color red, however, old plantation in Kot Momin, this tehsil appeared to be high in susceptibility to CTV. Likewise, three villages in Bhalwal exhibited susceptibility to CTV, while to a lesser degree compared to Kot Momin. These villages, namely Chak 13 NB, Chak 7 NB, and Chak 4 SB, were represented in blue on the maps. In contrast, the villages in Sargodha demonstrated minimal susceptibility to CTV in comparison to the other Tehsils. Only two villages, Chak 94 SB and Chak 90 SB, displayed low levels of susceptibility, indicated by the color green. The areas marked in yellow on the map represented the non-infectious status of citrus.

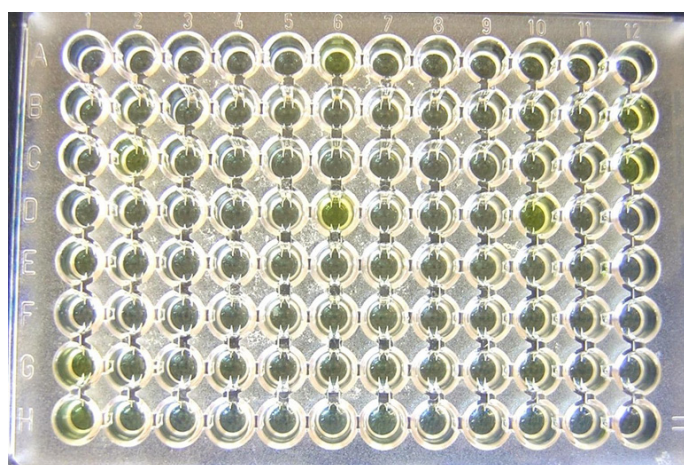


Figure 2: The intensity of the yellow hue indicated a higher concentration of the virus in the wells.

Confirmation of virus

The wells' more brilliant color indicated a larger viral load than the others (Figure 2). The presence of the virus was inferred from the findings of the ELISA test, which revealed that the wells had taken

on a yellow hue as a consequence. The Incidence of disease is mainly dependent upon the number of positive ELISA values.

Disease incidence for varieties

This study explores disease prevalence across three cultivars using ELISA. Deeper yellow hues in ELISA wells correspond to higher CTV quantities. Diseased Musambi had OD values ranging from 3.8 to 5.5 (4.5), while healthy samples were below 3.7. For Feutal's Early, diseased OD values ranged from 2.5 to 3.5 (2.89), with healthy samples below 2.5. Kinnow exhibited OD values from 1.5 to 2.5 (1.71), and healthy samples were consistently below 1.5. Musambi showed higher susceptibility to CTV than Feutal's Early, while Kinnow exhibited the least susceptibility. Graphs visually represent these findings, with Diseased Musambi displaying higher OD values, indicating increased CTV presence, and Feutal's Early and Kinnow showing lower OD values, suggesting reduced CTV presence (Figure 3).

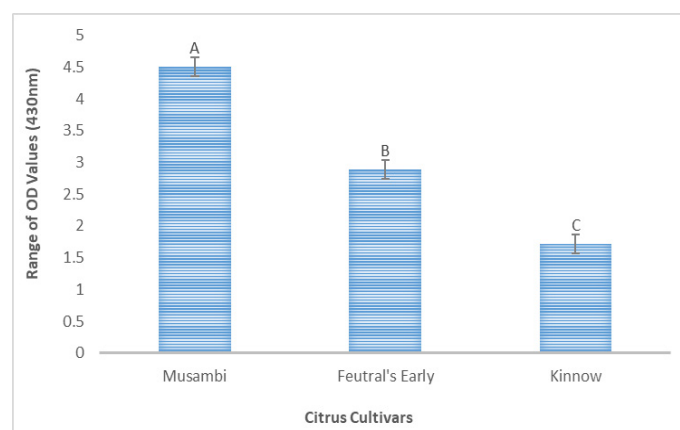


Figure 3: Different disease incidence for varieties.

Disease incidence for three tehsils of Sargodha

In the Kot Momin region, OD values ranged from 3.2 to 4.2, with results showing 4.73 for diseased samples and values below 3.0 for healthy citrus samples. Bhalwal exhibited OD values ranging from 2.6 to 3.2, with results at 2.89 for diseased samples and values below 2.2 for healthy citrus. Sargodha displayed OD values from 1.8 to 2.8, with results at 1.9 for diseased samples and values below 1.8 for healthy citrus. Comparative analysis revealed Kot Momin's higher susceptibility to CTV infection compared to Bhalwal, while Sargodha showed the least susceptibility. Graphs in Figure 6 visually depict these findings, confirming higher ELISA values for Kot Momin, lower values for Bhalwal, and the least susceptibility in Sargodha (Figure 4).

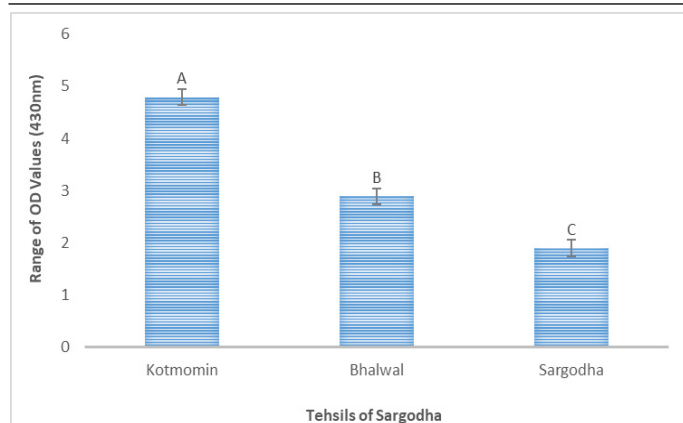


Figure 4: Different disease incidence for Sargodha district.

Disease incidence of CTV in three tehsils on 3 citrus cultivars

CTV infection levels were markedly lower in Tehsil Bhalwal and Sargodha compared to Tehsil Kot Momin across all three citrus species, with Musambi exhibiting the highest disease burden, followed by Feutal's Early and Kinnow. In Tehsil Kot Momin, OD values for diseased Musambi samples ranged from 3.8 to 5.5, with results at 4.68, while in Bhalwal, the range was the same (3.8 to 5.5), resulting in 4.69. In Sargodha, the range was 3.5 to 4.5, with results at 3.69. Feutal's Early showed a lower OD range of 2.5-3.5 in Kot Momin, with results at 3.14, while in Bhalwal, the range and results were 2.5-3.5 and 2.88, respectively, lower than Kot Momin. Sargodha exhibited the lowest values (2.5-3.5). Kinnow had the lowest OD values of 1.8 to 2.5 in Kot Momin, with results at 2.0. In Bhalwal, the range was 1.5-2.5, with results at 1.68. Sargodha showed the lowest values (1.2 to 2.2), with results at 1.4. Overall, the three citrus cultivars had the highest incidence in Kot Momin, lower incidence in Bhalwal, and the least incidence in Sargodha (Table 2; Figure 5).

Table 2: OD Values of CTV in three tehsils on 3 citrus cultivars (430 nm).

Varieties/ tehsils	Kot Momin	Bhalwal	Sargodha
Kinnow	2 (D)	1.68 (DE)	1.48 (E)
Musambi	4.8 (A)	4.69 (A)	3.93 (B)
Feutal's early	3.14 (C)	2.88 (C)	2.65 (C)

Disease severity for cultivars of citrus

The results of the experiments showed the disease severity for three varieties, which were counted by visual observation, and the rating for the disease severity (Table 3). In diseased Musambi, the number of pits per square inch varied from 55.2 to 55.5;

in case of Feutal's Early, the no. of pits per square inch lies anywhere from 25.5 to 29.5 Same is the case with Kinnow, the no. of pits per square inch was varied from 10.5 to 12.5 By comparing three different cultivars of Musambi we came to know that Musambi is more susceptible to the attack of CTV, while Feutal's Early is slightly severe to CTV, as compared to Musambi, while Kinnow is least severe to CTV (Figure 6).

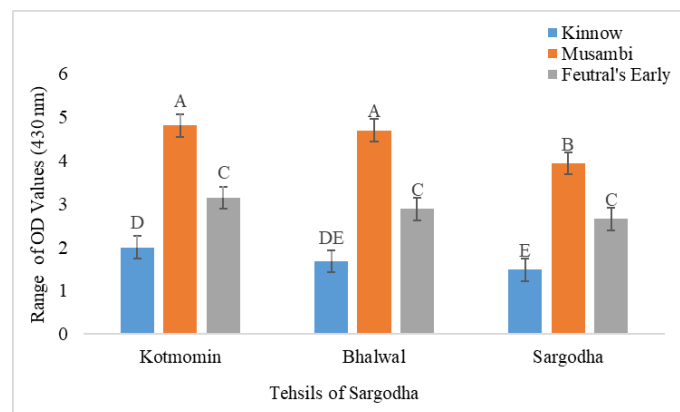


Figure 5: Disease incidence of CTV in three tehsils on 3 citrus cultivars.

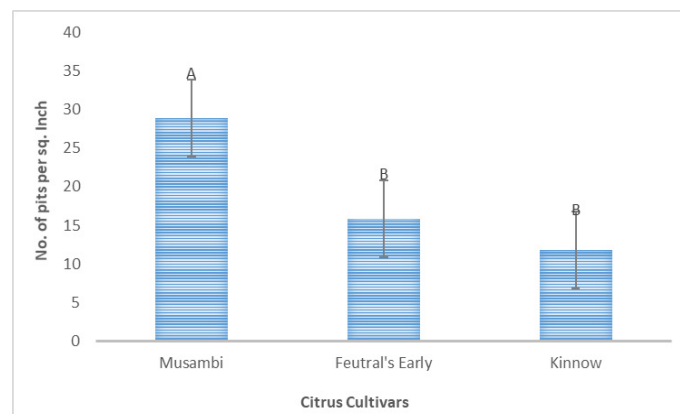


Figure 6: Different disease severity for varieties.

Table 3: Disease Severity of CTV in three tehsils on 3 citrus cultivars.

Varieties/Tehsils	Kot Momin	Bhalwal	Sargodha
Kinnow	17.1 (BC)	9.5 (C)	8.8 (C)
Musambi	39.3 (A)	30.8 (AB)	16.5 (BC)
Feutal's Early	23.4(ABC)	13.5 (C)	10.6 (C)

Disease severity for tehsils of Sargodha

The results of experiments show the incidence of disease for three tehsils of Sargodha. In Kot Momin, the citrus shows the most severity of the disease because in this area, they have a very dense and old plantation, the no. of pits per square inch in this region varies from 34.5 to 36.5. In the case

of Bhalwal, the values of no. of pits per square inch lie anywhere from 24.5 to 26.5. The same is the case with Kinnow; the no. of pits per square inch varies from 13.5 to 16.5. By comparing these results with the rating scale, we came to know that Kot Momin is moderately severe to CTV, Bhalwal is slightly severe, and Sargodha is least severe to Citrus Tristeza Virus (Figure 7).

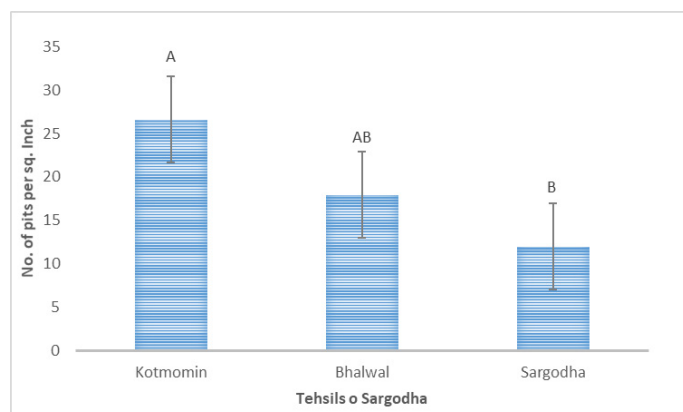


Figure 7: Different disease severity of Sargodha district.

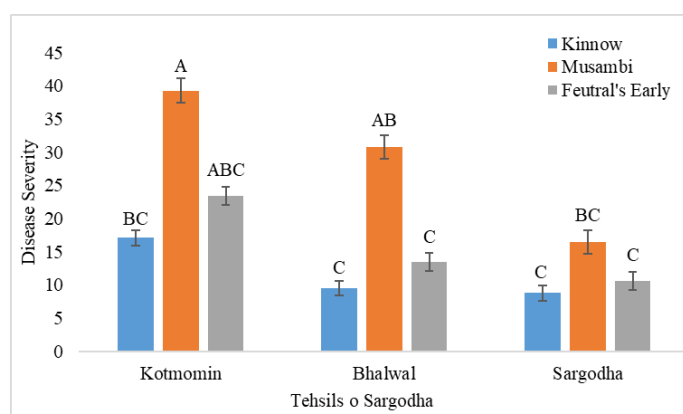


Figure 8: Disease severity of CTV in three tehsils on three citrus cultivars.

Comparison of disease severity of CTV in three tehsils on three citrus cultivars

CTV infection levels were significantly lower in Tehsil Bhalwal and Sargodha than Tehsil Kot Momin for all three citrus species. Musambi exhibited the highest disease caseload, followed by Feutal's Early and Kinnow. The number of pits per square inch in diseased Musambi samples ranged from 52.5 to 57.5 in Tehsil Kot Momin, 48.5 to 52.5 in Bhalwal, and 43.5 to 48.5 in Sargodha. Feutal's Early had fewer pits per square inch, ranging from 32.5 to 34.5 in Bhalwal, lower than Kot Momin. Sargodha showed the lowest number of pits per square inch (29.4 to 33.6). Kinnow had pit values ranging from 23.4 to 27.4 in Kot Momin, 22.5 to 24.7 in Bhalwal (lower than Kot Momin), and 18.4 to 21.8 in Sargodha (the

lowest). These results indicate that the severity of CTV was highest in Kot Momin, lower in Bhalwal, and least severe in Sargodha among the three cultivars (Figure 8).

Citrus tristeza has spread fast throughout the nation without anybody noticing. The researchers wanted to discover how widespread CTV disease is, how dangerous it is, and what environmental variables contribute to its spread. So, effective management methods might be established to forestall future losses of this kind. Five villages were chosen randomly from each of the three tehsils that make up the Sargodha District. The presence of CTV in the collected samples needed to be confirmed using ELISA. Once the confirmation procedure was completed, the survey was completed to ascertain how often the virus was discovered in Musambi, Kinnow, and Feutal's early. Musambi had the greatest incidence, followed by Feutal's early and Kinnow. Environmental conditions showed that somewhat warm and humid conditions are ideal for the vector to spread disease. According to (Iftikhar *et al.*, 2009), the CTV ELISA test yielded good findings. They used ELISA to confirm CTV, finding that the average OD at 405 nm was 0.60 for Sweet Orange, 0.42 for Kinnow, and 0.31 for Grapefruit. After conducting analyses, these results were judged to reflect CTV. Using an ELISA test similar to the previously described one, Yasin *et al.* (2020) successfully detected CTV. They found that the stronger the yellow colour, the more it matched the pathogen in the plate wells. Using the ELISA test, Catara *et al.* (1988), confirmed the presence of CTV in Pakistan the same year. CTV infected citrus trees despite the scion/rootstock hybrid's genetic makeup; in 2007, CTV incidence was recorded at 48% in the Bhalwal region of Punjab and at 40% in the Mardan Region of KPK (Iftikhar *et al.*, 2009); in 2013, CTV incidence was reported at 67% in Sargodha and 39% in Mardan (Naseem *et al.*, 2016). Our findings, supported by several investigations, demonstrate that the prevalence of CTV in citrus groves is increasing. Several drugs' efficacy against the aphid that transmits citrus tristeza virus was studied (Gottwald *et al.*, 2002). Moreno *et al.* (2008) suggest that citrus tristeza virus (CTV) disease may be controlled by using tristeza-resistant rootstock, budwood certification initiatives, and other types of quarantine. It is also possible to prevent the development of tristeza by using rootstock that is resistant to the disease. They

reasoned that reducing the disease's severity would require an intensive investigation of the relationship between hosts, vectors, and the viral protein. The citrus tristeza virus may be carried by various kinds of aphids. Therefore, the vector population significantly impacts the disease's prevalence and severity. One approach to controlling citrus tristeza is to keep the virus responsible for the disease under control. Effective chemical insecticides and other plant extracts are mostly responsible for controlling CTV. [Mordue and Blackwell \(1993\)](#), claim that neem plant extract is the most effective of all plant extracts. This is because neem plant extract reduces the number of aphids in citrus trees by a greater margin than any other treatment. You may use any of these things alone or in addition to mineral oils. The most used insecticide for mealy bugs is Chlorpyrifos ([Peleg, 1995](#); [Urbaneja et al., 2020](#)). According to [Arif et al. \(2005\)](#), between 2002 and 2010, the infection rate in Pakistan's northwest varied between 24 and 44 percent, as reported by. This happened somewhere between 2002 and 2010. The prevalence of CTV was also found to be higher in regions where sweet orange was planted as a rootstock. The citrus tristeza virus (CTV) is a global danger to the citrus industry due to the destruction it causes in citrus groves ([Bar-Joseph et al., 1979](#); [Catara et al., 1988](#)). [Catara et al. \(1988\)](#), discovered softer financing in a few areas of Pakistan's northwest. According to the exhaustive study and survey results, the prevalence of infection rose from 24% to 44% after the outbreak. The average prevalence of CTV was 27%, as reported by ([Arif et al., 2005](#)). According to research by [Sharma et al. \(2012\)](#), as much as 39% of delicious oranges were infected. Both *C. reticulata* and *C. lemon* were shown to be immune to CTV. CTV may exist on plants in several ways, including the twigs, leaves, and bark. Studies have shown that these three factors have changed significantly during the last two years. Petioles and midribs are the best tissues for identifying CTV using DAS-ELISA ([Anfoka et al., 2005](#)). The research by leaf section was an efficient method, as a result. However, there are a few limitations, including unequal viral dispersal and varying virus quantities in different plant parts ([Anfoka et al., 2005](#)). Regarding plant life, there are two distinct types of viral transmission: localized transmission and long-distance dispersal. The number of viruses in the stem is negligible compared to the rest of the body. Due to the virus's phloem-restricted distribution, the age of the host plant and

the specific virus species or strain must be considered when deciding on a diagnostic strategy. [Ghosh et al. \(2014\)](#), found that the viral concentration was higher in traditional Mandarin. The aphid population and the plant's advanced age probably affected this heightened concentration. The ELISA test is the most precise approach for identifying the types of viruses present in plant cells. It is a common agricultural practice in citrus-growing regions due to its low cost ([Hilf and Garnsey, 2002](#)). This method uses the high specificity of antibodies isolated from animals to identify the viral coat protein. According to [Webster et al. \(2006\)](#), this method facilitates the rapid and accurate identification of CTV and the processing of many samples in a single experiment.

Conclusions and Recommendations

This study aimed to investigate the impact and frequency of the Citrus Tristeza Virus (CTV) disease on three citrus varieties in Sargodha, Pakistan. Samples were collected from 8 fields across three Tehsils and analysed through symptomatology and serological tests. The results showed that Musambi cultivars were the most susceptible to CTV, with Feutral's early and Kinnow following suit. Additionally, older orchards were more susceptible to the disease than younger ones. This research is expected to aid farmers in predicting and managing the onset and severity of CTV disease through the use of environmentally friendly insecticides and the establishment of a citrus disease-free nursery. As for future contributions, researchers could explore more effective control measures and management techniques for CTV disease, such as developing resistant citrus cultivars or utilising biological control strategies. Further studies could also investigate the economic impact of CTV disease on the citrus industry in Pakistan and other citrus-producing countries.

Novelty Statement

Sero-Diagnostic assay for the detection of Citrus tristeza virus in relation to declining orchards of Sargodha, Pakistan will help in continuous monitoring of virus for timely detection.

Author's Contribution

Talha Shafique: Conducted the research trial.

Mustansar Mubeen: Statistical analysis, software and writing original draft.

Yasir Iftikhar: Conceptualization and supervised the trial.

Qaiser Shakeel, Haseeb Ahmed Lalika and Muhammad Irfan Zafar: Analyzed the data and helped in editing.

Muhammad Ahmad Zeshan and Malik Abd-ur-Rehman: Co-supervised the trial.

Atta ur Rehman and Farwa Seemab: Helped in field work and technical assistance for lab analysis.

Conflict of interest

The authors have declared no conflict of interest.

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