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Research Article

Current status of CTV in new plantation of citrus in Layyah and Bhakkar, Pakistan

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ABSTRACT

Citrus fruit is widely regarded as a valuable commodity on a global scale, especially within the country of Pakistan, due to its great flavor, nutritional advantages, and therapeutic attributes. The Citrus Tristeza Virus (CTV) is a significant pathogen that poses a substantial threat to the citrus industry's stability and productivity. The tree that has been impacted by the disease displays symptoms of deterioration, including leaf chlorosis, twigs dieback, and stem pitting. The objective of this study was to document the occurrence and severity of CTV disease using symptomatology and serological studies. A survey was conducted in two districts of Punjab named as District Bhakkar and Layyah. This research carried out in each district included identification of seven sample agricultural fields and the evaluation of three types of citrus fruits including Musambi, Feutral's Early and Kinnow. Serological test known as ELISA was used for an analysis of the samples that were suspected to contain CTV. The study was observed that the extent of the condition was present in all the regions. The study also shows that among the sites, identified the Musambi was the most affected by the climatic change, trailed by Feutral and Kinnow respectively. Similarly District Layyah presented higher disease severity as compared to the District Bhakkar. The study shows that the level of disease incidence in orchards increases with the age of CTV in a given orchard. Future research should explore efficient strategies for controlling CTV disease, including cultivar resistance through gene silencing and examine the economic impact on citrus businesses in Pakistan and other countries.

Keywords: Citrus Tristeza Virus, ELISA, new plantation, Layyah and Bhakkar.

INTRODUCTION

The agricultural industry in Pakistan includes a vast citrus portion that occupies around 2500 hectares of citruses (Cheema and Jamali, 2020; Dogar et al., 2017). These groves help to yield 412300 metric tons (Mt) of citrus fruits every year (Siddique and Garnevska, 2018). Pakistan especially Punjab in particular is well known for practicing intensive fruit farming particularly for the citrus fruits at a large

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extent (Asad et al., 2019; Cheema and Jamali, 2020). It results to a high average production of 12,782 Mt of fruit per hectare planted to Citrus tree (Jabbar et al., 2022; Shah et al., 2020). Pakistan proudly ranks as the sixth largest producer of kinnow, a delicious fruit belonging to the citrus family which is loaded with nutrients (Kousar et al., 2019). As per the information by the Pakistan Bureau of Statistics for the year 2018-2019, Pakistan's area under the kinnow was a vast area of about 8.5453 million hectares (Nazeer et al., 2019). This large area under cultivation led to a significant production of 123.69 million Mt of kinnow (Siddique et al., 2023). The leading producers of kinnow in Pakistan comprises of Sargodha, Toba Tek Singh, Sahiwal, Bhakkar and Layyah districts all located in central Punjab (Umar et al., 2022).

Due to the large quantities of citrus, the system needs to be very organized, complex and careful design (Mubeen et al., 2015a,b; Ahmad et al., 2018; Ghafoor et al., 2010). It is predicted that the citrus sector in Pakistan particularly kinnow would have a rate of increase in its profits coming from better practice where handling, processing and transport of the kinnow fruit both nationally and internationally are concerned (Ghafoor et al., 2010). The cultivation of cirtus crop is carried out in a total of 64 countries in the world (Hamid et al., 2024). Pakistan was successful in producing 123.69 Mt tons of Kinnow during 2018-2019 (Anwaar et al., 2020). The region of Central Punjab consisting of Sargodha, Toba Tek Singh, Sahiwal, Bhakhhar and Layyah is now contributing to 95% production of the globally produced kinnow (Umar et al., 2022). However, Citrus trees are known to experience significant and detrimental impacts as a result of various diseases (Iftikhar et al., 2024; Mubeen et al., 2024; Jaouad et al., 2020). Citrus Tristeza Virus (CTV), is widely recognized as one of the most destructive viruses identified in the scientific community. Citrus Tristeza Virus is classified as a member of the family *Closteroviridae* and the genus Closterovirus (Folimonova and Sun, 2022; Hajeri and Yokomi, 2023). The transmission of the aforementioned disease is facilitated by various vectors, including mealybugs, aphids, and whiteflies, as studied by Agranovsky (2021), and Karasev et al. (1997). Aphis gossypii, commonly known as the cotton aphid, serves as the primary vector responsible for the efficient transmission of various strains of CTV between individual plants as well as across different orchards (Huang et al., 2024; Roistacher and Moreno, 1991).

The significant economic impact of CTV underscores its paramount importance. The severity of the disease in young citrus trees varies depending on the type of rootstock and cultivar (Forner-Giner et al., 2020). Two distinct signs can be seen on trees affected by the disease. The first sign is called stem pitting, which relates to the alterations in the structures of the vessels in the tree (Sharma, 2023). This condition results to formation of pits on the stem causes hindering of nutrients and water flow in the plant stem (Van Wyk, 2024). The second symptom, decline, is severe and leads to the death of the tree and is the ultimate stage of the disease (Singh et al., 2022). Such symptoms, when observed, can be deemed as noticeable sign of any problem that may be causings concern to the tree's growth and production of health (Satpute and Fadli, 2022). Vives et al. (1999) have noted that depending on the strain of CTV that infects any tree, then the infected tree may manifest different symptoms (Moreno et al., 2022). CTV genetic material includes positive-sense single-stranded RNA. Concerning the genomic organization of CTV, the size estimate of about 20 kilobybase with untranslated region sequences at both termini of the virus. This has been designed and implemented within the document by the accommodation of 12 Open Reading Frames (ORFs) (Folimonova, 2020; Ghorbani et al., 2023; Karasev et al., 1997; Moreno et al., 2022). Infection process requires the creation of a symbiotic relationship between the host organism and the invader (viruses) (Li et al., 2022; Muller and Costa, 1987). The affected plant utilizes different immune responses to combat the viral diseases as part of the immune system (Han, 2019; Voinnet, 2005). The presence of genetic diversity in ecologically relevant environments could affect the phenology of symptoms. Stem pitting occurrence in both the primary trunk and other larger and smaller lateral branches and twigs signifies the high virulence of CTV (Gautom et al., 2021). The rootstock utilized for commercial applications, such as the sour orange, exhibits immunity against decline caused by mild isolates of CTV (Sharma, 2023). Significant losses can potentially arise due to the presence of highly aggressive CTV strains or isolates (Folimonova et al., 2020). According to Niblett et al. (2000), under favorable conditions as well as in the presence of severe disease, plants undergo desiccation and ultimately perish (Upadhyay, 2020). The Mealybug, a diverse group of insects, encompasses more than 60 distinct species. However, only a limited number of these species are considered significant pests, capable of causing considerable damage and leading to substantial economic losses within the citrus industry (Mwanauta et al., 2021). Specifically, it impacts the overall development and productivity of plants, as well as the desirable attributes of fruits, including color, weight, and juice content. Sooty mould is a type of fungal growth that specifically emerges on particular species of the primary mealy bug (Mathulwe et al., 2022). This fungus is known to generate a concealed substance called honeydew, which serves as an enticing attractant for a multitude of fruit-boring

moths. These moths, in turn, are responsible for the transmission of various diseases. (Silva and Mexia, 1999). The brown citrus aphid, a vector for CTV, is handled by aphid management programs. The disease's impact is proportional to the density of its vector population (Raza and Younas, 2023). The citrus aphid, known as Brown, is a severe danger to the citrus sector, although it may be managed using effective pesticides (Sharma et al., 2019). The extract of some higher plants may be used as a pesticide against a wide variety of insects, including every known species of aphid. When it comes to reducing disease in citrus groves by reducing the number of aphids, neem extract is at the top of the list (Mordue and Blackwell, 1993). According to Bushong and Timmer (2000), the chemical approach provides a quick response after infection. Most farmers choose chemical treatment because it is effective, readily accessible, and eradicates diseases more rapidly than other methods. The CTV is a significant causative agent responsible for substantial agricultural losses in citrus crops across diverse geographical regions worldwide. Citrus Tristeza Virus, has emerged as a newly recognized disease in Pakistan, exerting detrimental effects on both the quality and quantity of fruit production within the region. The global trade of fruits is currently being influenced by a variety of factors that have significant implications for the industry. There is an urgent need for the development of effective treatment strategies for the disease, as well as the identification of risk factors that contribute to its spread. Additionally, it is imperative to conduct thorough investigations into the potential of CTV-resistant plant species as a means of mitigating the impact of the disease. The main objective of current study was to assess the Prevalence and Incidence of CTV in Bhakkar and Layyah districts. Moreover, this study also provide to identify the potential factors that influence the spread and severity of CTV in Bhakkar and Layyah, Pakistan.

MATERIALS AND METHODS

CTV-infected sample collection and survey

The citrus plantations in district Layyah and Bhakkar were investigated through extensive surveys conducted across various villages in these districts. Total three villages were carefully selected from each district for sampling purposes. In District Layyah, the chosen villages included Layyah (Latitude: 30.9693, Longitude: 70.9428), Chobara (Longitude: 71.4774291, Latitude: 30.9057881), Karor Lal Esan (Latitude: 31.2272, Longitude: 70.9517), Kot Sultan (Longitude: 70.9368, Latitude: 30.7750), Fatehpur (Latitude: 31.178949. Longitude: 71.209016) and similarly in District Bhakkar three villages were included due to less citrus plantation at Bhakkar, the names of the villages of Bhakkar includes Kalur Kot (Latitude: 32.1575, Longitude: 71.2696), and Darya Khan (Latitude: 31.7895, Longitude: 71.1077) Three varieties of citrus fruits, Musambi, Feutral's Early, and Kinnow, were examined for this study. Dying off, pitting the stems, and yellowing the leaves were some morphological markers used to identify diseased samples. After collecting the samples in polythene bags as indicated by the symptoms, they were immediately added to the ice bucket. The samples were then sent to the Citrus Research Institute in Sargodha to determine whether or not they contained CTV.

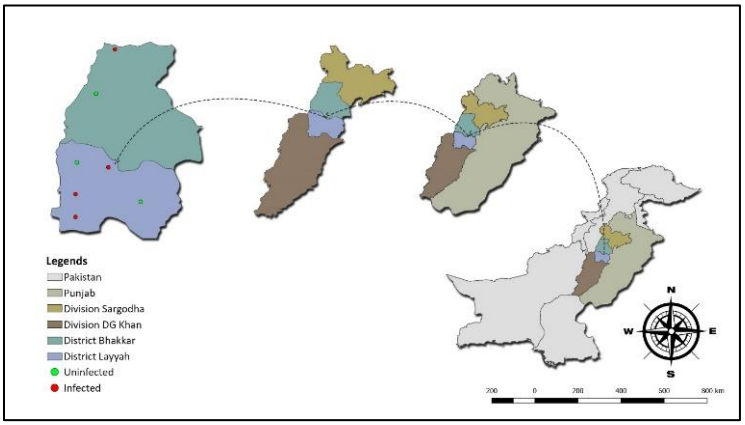

Figure 1. Current distribution of CTV in Layyah and Bhakkar District

Confirmation of Virus

To confirm the presence of the virus the Enzyme Linked Immunosorbent Assay (ELISA) was used. Following the analysis of the findings of the work done by Clark and Adams (1977), a sample of infected material was obtained from the field and tested through a double antibody sandwich enzyme-linked immunosorbent assay technique. The ELISA test utilized polyclonal antibodies obtained from Bioreba a company that can be visited at www.bioreba.com. Multiple buffers were used in the ELISA that were prepared before the start of the experiment was conducted.

Buffer formulations

A. Carbonate coating buffer/Liter:

- Sodium carbonate (anhydrous) (Na_2CO_2) 1.59g
- Sodium bicarbonate ($NaHCO_2$) 2.93g
- Sodium azide (NaN_3) 0.20g

pH 9.6 was maintained, and the buffer was stored at 4°C.

B. PBST buffer (Wash buffer)/Liter:

- Sodium chloride (NaCl) 8.00g
- Sodium phosphate (dibasic) (Na_2HPO_4) 1.15g
- Potassium phosphate (monobasic) (KH_2PO_4) 0.20g
- Potassium chloride (KCl) 0.20g
- Tween-20 20.0ml

pH 7.4 was maintained, and the buffer was stored at 4°C

C. Extraction buffer/Liter:

Extraction buffer was prepared by dissolving the following chemicals in PBST.

- Sodium sulfite (anhydrous) (Na_2SO_3) 1.30 g
- Polyvinylpyrorolidone (PVP) (C_6H_9NO) 20.0g
- Sodium azide (NaN_3) 2.00g
- Egg (chicken) albumin 2.00g
- Tween-20 20.0g

pH 7.4 and stored at 4°C

D. Conjugate Buffer

Alkaline phosphatase labelled antibodies were added to the extraction buffer at a dilution of 1:1000

- Bovine serum albumin (BSA) ($C_{123}H_{193}N_{35}O_{37}$) 2.00 g
- Polyvinylpyrorolidone (PVP) (C_6H_9NO) 20.0g
- Sodium azide (NaN_3) 0.20g

pH 7.4 and stored at 4°C

E. PNP or Substrate Buffer

- Magnesium chloride hexahydrate ($MgCl_2$. $6H_2O$) 0.10g
- Sodium azide (NaN_3) 0.20g
- Diphenylamine($C_{12}H_{11}N$) 97.0ml
- Distilled water (H_2O) 800 ml

The volume was adjusted to one liter, and the pH was adjusted to 9.8 with HCl. The buffer was prepared 5 minutes before use, and p-nitrophenyl phosphate was dissolved at 1mg/1 ml.

DAS-ELISA Procedure

The preparation of the aforementioned buffers, the ELISA procedure was subsequently carried out. The microtiter plates were coated with antibodies specific to CTV, which were diluted 1000-fold in a coating buffer. The ELISA plates were subjected to incubation at a temperature of 30°C for a duration of 4 hours. The ELISA plates underwent three washes using washing buffer. Freshly manufactured antigen at a concentration of 1:10 weight/volume in extraction buffer was dispensed into the wells of the plates, with a volume of 100µl per well. The plates were then incubated overnight at a temperature of 4°C, after which they were subjected to a washing step. In addition, reference samples were added to serve as blank, negative, and positive controls. The CTV conjugated antibody was found to be 1000 fold diluted in the conjugate solution. 100µl of this diluted antibody was added in each well and the plate put at a temperature of 30°C for 5hrs. After that, washing of the plate was done. In each well) the substrate, p-nitrophenyl-phosphate was added at 100µl and 1mg/ml concentration. This mix was left at laboratory temperature to react fully for not less than one hour before analysis. The above technique was performed severally in order to prove the efficiency of CTV detection.

Statistical Analysis

Two way analysis of variance (ANOVA) was carried out and followed by least significant difference (LSD) test to map out the viral disease CTV in the orchard of district Layyah and Bhakkar.

Color Development

The appearance of yellow color in the wells was the most diagnostic sign of CTV infection, where intensity of the color is proportional to the level of activity within the plant. By utilizing this methodology, the study were able to segregated the positive and negative samples using perception to the colour yellow. The reaction was stopped by quenching with 50 liter of 1N sodium hydroxide solutions. Afterwards, the plates were taken through image processing.

Severity of CTV

A significant infestation of Citrus Tristeza Virus (CTV) was detected in the Musambi orchards. The first step involved the classification of Musambi orchards into five discrete age categories, determined by their anticipated age at the time of planting. These categories include orchards up to 12 years old, up to 14 years old, up to 16 years old, up to 18 years old, and up to 20 years old. In order to obtain a precise understanding of the extent of the matter, we used a sampling procedure including two fields from each cluster. Subsequently, a random selection of 10 plants was made from each plot, resulting in a total of 10 plants. The study employed these botanical specimens. The number of pits was ascertained by extracting a square inch of bark from the region where the rootstock and scion stock were joined. Upon calculating the proportion of the pitted area within the 1 square inch zone, we proceeded to employ the subsequent chart for the purpose of assessing the extent of pitting severity. In order to perform the calculation, the area encompassed by the pits was divided by the area equivalent to 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

RESULTS

Current Status and Distribution of CTV in District Layyah and Bhakkar

A series of extensive surveys were carried out in various villages within the District Layyah and Bhakkar. By observing the visible symptoms associated with Citrus Tristeza Virus (CTV), it was observed that Layyah have more citrus plantation as compared to the District Bhakkar. Among the District Layyah five villages were surveyed and three of them have a higher vulnerability to CTV. These villages, named as Layyah, Kot Sultan and Fatehpur. Likewise, in the villages of District Bhakkar, only one area named as Kalur Kot shows the symptoms of CTV. The area having high disease incidence of CTV were denoted by red color while area having no disease incidence were denoted by yellow color.

Elisa Test Results

A more brilliant color in the wells indicated a larger viral load than the others. 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 depend upon the no. of positive values of ELISA.

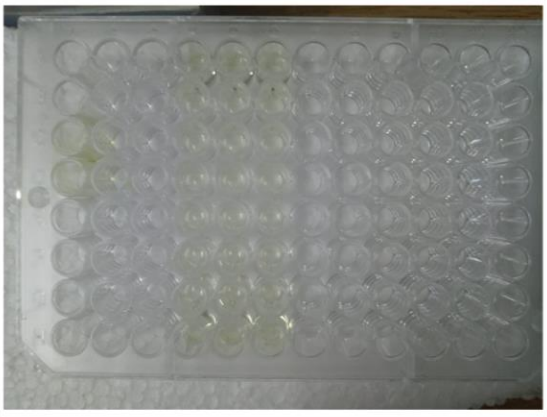

Figure 2. The intensity of the yellow hue indicated a higher concentration of the virus in the wells

Disease Incidence for Varieties

This study presents experimental findings regarding the prevalence of disease across three distinct citrus cultivars. Disease incidence was assessed using the ELISA methodology. The analysis of ELISA values revealed a notable association: wells with deeper yellow hues exhibited higher levels of CTV compared to those with lighter yellow hues. Specifically, for Diseased Musambi, optical density (OD) values ranged from 3.8 to 5.5, with diseased samples averaging 4.5, while healthy Musambi samples showed significantly lower values (<3.7). Similarly, for Feutral's Early, diseased OD values ranged from 2.5 to 3.5, with an average of 2.89, whereas healthy Feutral's early samples consistently displayed values below 2.5. A similar pattern was observed for Kinnow, where OD values ranged from 1.5 to 2.5, with an average of 1.71, while healthy Kinnow samples consistently exhibited values below 1.5. Comparative analysis among the three Citrus cultivars revealed that Musambi exhibited higher susceptibility to CTV infection compared to Feutral's Early, which displayed relatively lower susceptibility. Kinnow exhibited the least susceptibility to CTV infection among the cultivars under investigation. Furthermore, graphical representations were generated to visually depict the results. Figure 5 presents a bar graph illustrating the OD values of Diseased Musambi, healthy Musambi, Feutral's Early, and Kinnow. The graph clearly illustrates higher OD values in Diseased Musambi, indicating a greater presence of CTV in diseased samples, while Feutral's Early and Kinnow both exhibited relatively lower OD values, suggesting a reduced presence of CTV in both diseased and healthy samples of these cultivars.

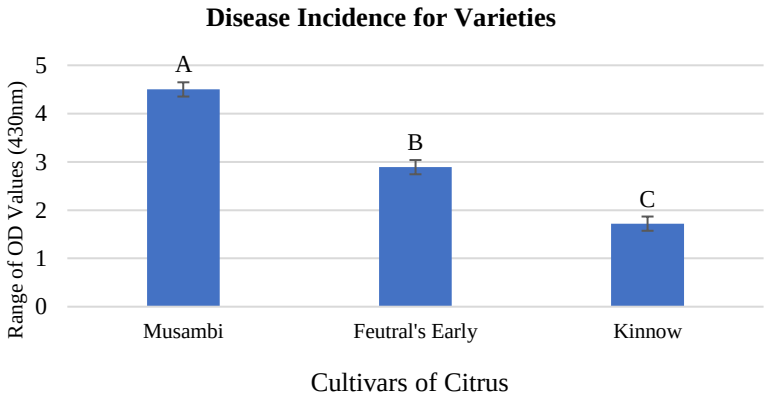

Figure 3. Comparisons Test of Disease Incidence for Varieties

Disease Incidence for district Layyah and Bhakkar

The study evaluated disease incidence across two district of Punjab named as District Layyah and District Bhakkar, using the ELISA test. In the district Layyah, three regions of district Layyah exhibited high incidence the OD values ranged from 3.2 to 4.2, with an average of 4.73 for diseased citrus samples, while healthy citrus samples from the same region consistently showed significantly lower values (<3.0). Likewise, in District Bhakkar, OD values ranged from 2.6 to 3.2, with an average of 2.89 for diseased samples, whereas healthy citrus samples from the same region displayed markedly lower values (<2.2). Comparative analysis among the two district revealed that the Layyah Region exhibited a higher susceptibility to CTV infection compared to Bhakkar, which displayed relatively lower susceptibility. To visually represent these findings, graphical representations were generated, as presented in Figure 6. The graph visually confirms the higher ELISA values in the district Layyah region, indicating an increased presence of CTV in diseased samples. In contrast, district Bhakkar exhibits a lower range of ELISA values, suggesting reduced CTV infection in diseased samples.

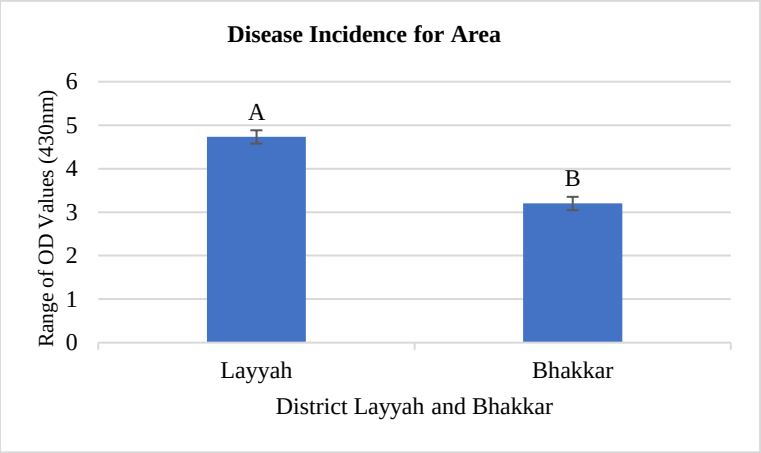

Figure 4: Comparisons Test of Disease Incidence for Area

Disease Incidence of CTV in Two District of Punjab (Bhakkar and Layyah)

CTV infection levels were notably lower in District Bhakkar when compared to District Layyah across all three citrus varieties. Mosambi exhibited the highest disease prevalence, followed by Feutral's Early and Kinnow. In District Layyah, OD values for diseased Mosambi samples ranged from 3.8 to 5.5, with an average of 4.68. In District Bhakkar, the range was similar (3.8 to 5.5), with an average of 4.69. For Feutral's Early, the range of OD values in District Layyah was 2.5-3.5, with an average of 3.14. In District Bhakkar, the range was the same (2.5-3.5), with an average of 2.88, lower than in Layyah. Kinnow exhibited the lowest OD values, ranging from 1.8 to 2.5 in District Layyah, with an average of 2.0. In District Bhakkar, the range was 1.5 to 2.5, with an average of 1.68. In summary, the mentioned citrus cultivars displayed the highest CTV incidence in District Layyah, while District Bhakkar had lower incidence rates than Layyah, and exhibited the lowest CTV incidence. Graphical representations of these results are provided below.

Table 2. Disease Incidence of CTV in Two District on 3 Citrus Cultivars

Varieties/District	Layyah	Bhakkar
Kinnow	2 (D)	1.48 (E)
Musambi	4.8 (A)	3.93 (B)
Feutral's Early	3.14 (C)	2.65 (C)

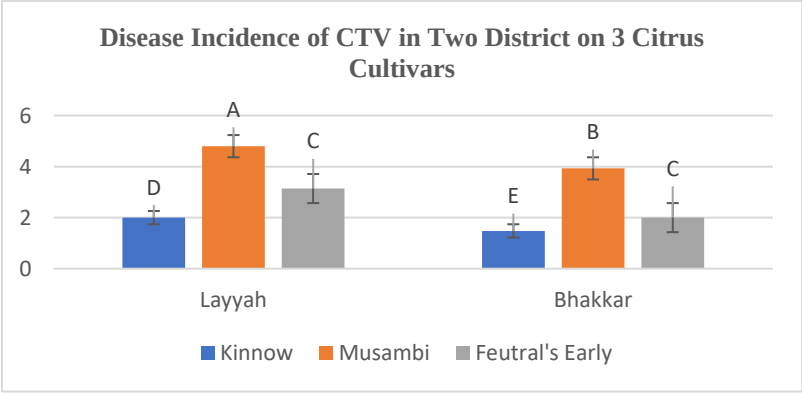

Figure 5. Comparison of Disease Incidence of CTV in two districts on 3 citrus cultivars

Disease Severity for District Bhakkar and Layyah:

The study focuses on assessing disease severity across the two citrus growing district of Punjab named as District Layyah and District Bhakkar. In particular, District Layyah stands out as having the highest disease severity among these districts. This heightened severity can be due to the extensive and well-established citrus plantations. In Layyah, the number of citrus pits per square inch is notably high, ranging from 34.5 to 36.5, which indicates a dense and mature citrus crop. Continuing to district Bhakkar, it possesses comparatively lower disease severities as compared to Layyah. There are 24.5 to 26.5 citrus pits per square inch in Bhakkar making the plantation a little sparse was observed in

Layyah. Likewise, in these districts, less number of pits per square inch has been obtained which varies from 13.5 to 16.5 of Kinnow. This implies a rather younger and less compact citrus growing region in these areas. To obtain better results in the extent of the diseases, compare these results with a rating scale. Based on this scale, Layyah is in the group of moderate-severe CTV infection because of high pit count and disease prevalence. Bhakkar is categorized as slightly severe, reflecting its lower disease severity compared to Layyah but still notable. For a visual representation of these results, graphical representations are provided below to offer a clearer understanding of the disease severity across these districts.

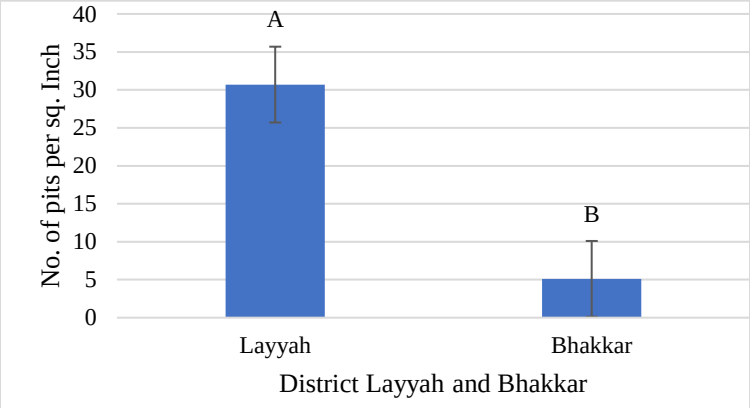

Figure 6. Comparisons Test of Disease Severity for Area

Comparison of Disease Severity of CTV in District Punjab (Layyah and Bhakkar)

CTV infection levels were significantly lower in District Bhakkar as compared to District Layyah for all three citrus species. The number of pits per square inch of diseased samples of Musambi ranged from 52.5 to 57.5 in district Layyah, while diseased samples of Musambi in District Bhakkar ranged from 48.5 to 52.5. Feutral's early had the lower number of pits per square inch, varying from 32.5 to 34.5 in District Bhakkar, which is lower as compared to district Layyah having 43.5 to 48.5. Kinnow had the values of pits per square inch, which varied from 23.4 to 27.4 in district Layyah, while District Bhakkar showed fewer pits, ranging from 22.5 to 24.7, which is lower as compared to district Layyah, while this cultivar of Citrus, i.e., Kinnow, in District Layyah, showed the lowest pits per square inch compared to District Bhakkar, which were 18.4 to 21.8. Based on these results, we came to know that the above-mentioned three cultivars of Citrus showed the highest severity in district Layyah, while District Bhakkar has lower severity as compared to district Layyah. The graphical representation of these results is given below.

Table 3. Disease Severity of CTV in two district on 3 citrus cultivars

Varieties/District	Layyah	Bhakkar
Kinnow	17.1 (BC)	8.8 (C)
Musambi	39.3 (A)	16.5 (BC)
Feutral's Early	23.4(ABC)	10.6 (C)

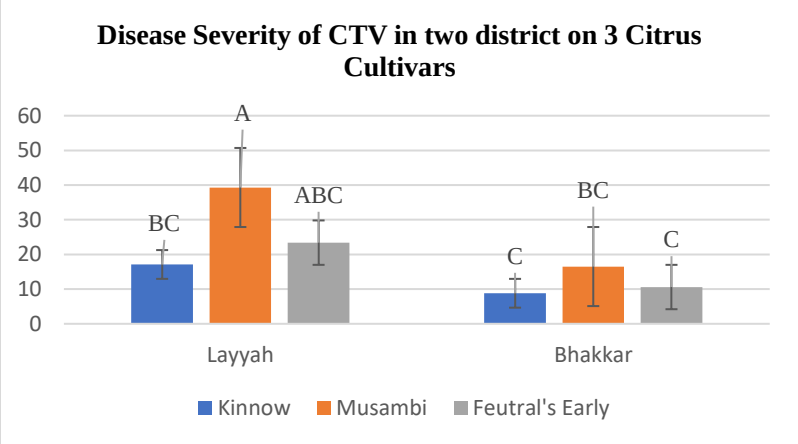

Figure 7. Comparison of Disease Severity of CTV in two district on 3 citrus cultivars

CTV Disease Severity in Mosambi Orchards

Since CTV disproportionately negatively impacts Mosambi orchards, the severity of the disease is magnified in these trees. More pits were left behind following CTV assaults on older orchards, indicating that the severity of the attacks increased with orchard age. When the collected data was evaluated, it was shown that the amount of damage produced by CTV was proportional to the age of the plant. The CTV disease may get so bad in older orchards that all of the trees die.

Figure 8. Honey-comb formation beneath the Surface of stem pitting at scion and root stock part

Figure 9. Visual symptoms of CTV, a dieback symptoms

DISCUSSION

Citrus tristeza virus (CTV), a highly destructive disease affecting citrus crops, had been largely overlooked until recently, despite its rapid spread across the nation going unnoticed. Researchers aimed to assess the extent of CTV disease, its severity, and the environmental factors contributing to its spread. Their goal was to establish effective management strategies to prevent future losses in citrus production. To achieve this, five villages were randomly selected from each of the two district of Punjab named as District Layyah and District Bhakkar. In the present study, CTV in collected samples was confirmed using the ELISA method. Early detection of virus in citrus varieties like Musambi, Kinnow, and Feutral was found to be more effective due to their susceptibility to the disease's vector in a slightly warm and humid climate. Iftikhar et al. (2009) confirmed the presence of CTV in Sweet Orange, Kinnow, and Grapefruit by observing high OD at 405 nm, and Yasin et al. (2020) identifying samples with yellow intensity correspond to pathogen intensity. Catara et al. (1988) identified the same virus in Pakistan in the same year, but through ELISA test only. Using the records of other papers, it can be concluded that the cases of CTV presence in citrus orchards have been on the increase in the recent past (Sajid et al., 2022). Other related researches conducted by Gottwald et al. (2002) were focused more on the efficiency of employing various chemical on the eradication of the aphid vector of CTV. Imidacloprid and acephate treatments results indicated the disease progress was monitored over time. Most significant of all, the CTV can only spread from infected to non-infected plants that are separated by an appreciable physical distance. The effectiveness of Imidacloprid and acephate in preventing CTV transmission was found to be limited. Interestingly, acephate not only prolonged the life of citrus trees but also reduced damage caused by Diaprepes to the plant roots, although it made them more susceptible to CTV due to increased aphid attraction.

Aphids play a pivotal role in CTV transmission, with migratory aphids like *Aphis gossypii* and *Toxoptera citricida* contributing to disease spread. However, efforts to reduce aphid populations using pesticides did not yield statistically

significant benefits. Cross-protection of plants did not significantly lower CTV infection compared to non-protected plants in the same citrus plots. Moreno et al. (2008) proposed control measures for CTV, including the use of tristeza-resistant rootstock, budwood certification programs, and quarantine procedures. Rootstock resistant to the disease can also prevent tristeza development.

Managing the disease effectively requires an in-depth understanding of the host-vector-virus relationship. Various aphid species can carry CTV, making the vector population a crucial factor in disease prevalence and severity. Effective chemical insecticides and plant extracts, such as neem plant extract, have been used to control CTV. Common insecticides include Dimethoate, Malathion, Pyrimifos methyl, and Chlorpyrifos, which can be used alone or in combination with mineral oils. Chlorpyrifos is often used for mealy bugs. Between 2002 and 2010, infection rates in Pakistan's northwest ranged from 24% to 44%, with higher prevalence in regions using sweet orange as rootstock. CTV poses a global threat to the citrus industry (Bar-Joseph et al., 1979; Catara et al., 1988), and softer financing was observed in some areas of Pakistan's northwest following the outbreak. According to Sharma et al. (2012), up to 39% of delicious oranges were infected with CTV, while *C. reticulate* and C. lemon were found to be immune. CTV can be found in various parts of citrus plants, including twigs, leaves, and bark, with variations noted in these factors over the past two years. Petioles and midribs have proven to be efficient tissues for CTV identification using DAS-ELISA. However, limitations exist, such as uneven viral distribution and varying virus quantities in different plant parts (Anfoka et al., 2005; Cambra et al., 2019).

Regarding viral transmission in plant life, two distinct types exist: Localized transmission and long-distance dispersal. The virus concentration in the stem is minimal compared to the rest of the plant, and the host plant's age and specific virus strain must be considered when selecting a diagnostic approach. Ghosh et al. (2014) reported higher levels of virus in traditional Mandarins which they attributed to increased aphid activity as well as plant maturity. PCR is a technique that is precise in determining the viral types in plant cells, but it is costly due to its complex implementation, the ELISA tests are therefore mostly used in the citrus growing regions due to their cost (Hilf and Garnsey, 2002). This method employed the ability of antibodies from animals to bind specifically to coat proteins of viruses, making it easy to identify CTV in different samples at the same time. The best measures must be taken to avoid any possibility of CTV getting to the citrus industry as this will destabilize the production and supply chain. Physical distance should be ensured to avoid virus transmission, and nurseries should offer farmers only healthy, certified clean stocks. Farmers should also be enlightened through seminars as well as workshops organized by extension workers against the use of sour oranges as rootstock. Such information needs to be provided to farmers in time so that the necessary preventive measures can be put into practice immediately.

CONCLUSION

This study aimed to investigate the presence and consequences of CTV disease on three citrus varieties in the Bhakkar and Layyah districts of Punjab, Pakistan. Samples were collected from seven fields across two districts, and a detailed analysis was conducted using symptom-based observations and serological testing. The study revealed that Musambi cultivars were the most susceptible types to CTV, while Feutral’s Early and Kinnowes exhibited almost equal susceptibility. Interestingly, orchards which were proven positive for CTV were more common in the older varieties. The finding involves important consequences, Farm producers can take benefit out of it for the prediction and management of CTV disease. Further research could focus on effective ways of containing and suppressing CTV disease for example through breeding of virus resistant citrus cultivars; or making use of the biological control techniques. Moreover, future studies can look at the impact of CTV disease on the citrus industry in Pakistan and other countries that rely on citrus production.

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AUTHOR CONTRIBUTIONS

Talha Shafique and Javaid Khan: Conducted the research trial and writing original draft. **Yasir Iftikhar:** Conceptualization and supervised the trial. **Qaiser Shakeel and Sara Anum**: Finalization, writing– review and editing. **Muhammad Ahmad Zeshan**: Co-supervised the trial. **Muhammad Irfan Zafar, Talha Riaz and Imran Ali**: Software, writing– review and editing. **Muhammad Hamza Saeed and Rabia Safdar:** Helped in field work and technical assistance for lab analysis.

COMPETING OF INTEREST

The authors declare that the research was carried without any commercial or financial relationships that could be construed as a potential conflict of interest.

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