



Research Article

Current Status of Citrus Tristeza Virus in Major Citrus Growing Areas of Sargodha, Pakistan

Asim Ali Khan¹, Muhammad Ahmad Zeshan¹, Yasir Iftikhar^{1*}, Mustansar Mubeen¹, Maham Sohail¹, Sonum Bashir¹, Malik Abdul Rehman² and Muhammad Usman Ghani³

¹ Department of Plant Pathology, College of Agriculture, University of Sargodha, Sargodha, Pakistan; ² Citrus Research Institute, Sargodha, 40100, Pakistan; ³ Institute of Soil and Environmental Sciences, University of Agriculture Faisalabad, Pakistan.

Abstract | Tristeza disease is caused by citrus tristeza virus (CTV) expresses salient symptoms like stem pitting, yellowing of leaves, stunted growth of large, small lateral branches and twigs. The study was carried out to quantify the spatial and temporal distribution of CTV disease in major citrus growing areas of Sargodha district (Sargodha, Kot Momin, and Bhalwal). The samples from the declined orchards were collected based on symptomology and confirmed through ELISA. CTV was detected in 3 varieties viz., Kinnow (*Citrus reticulata*), Mosambi (*Citrus sinensis*) and Feutrell's early (*Citrus reticulata* cv feutrell's early). The maximum disease incidence was recorded in Mosambi (90%), followed by Feutrell's early (74%) and Kinnow (57%). Mosambi was the most susceptible within the range of OD values (0.82-2.35) followed by Feutrell's early (0.45-0.64) and Kinnow (0.25-0.56). The disease severity was recorded only on Mosambi as it was more affected than Feutrell's early and Kinnow. The rating scale to observe the severity of tristeza disease was based on percentage of pits in the 1 sq. inch piece of bark at bud union of infected tree. This rating scale has been developed for the first time to record the tristeza disease severity. The maximum disease severity was recorded in Kot Momin (62%) followed by Bhalwal (56%) and Sargodha (50%). The study concluded that CTV is one of the most promising factors playing role in the citrus decline.

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***Correspondence** | Yasir Iftikhar, University of Sargodha, Sargodha, Pakistan; **Email:** yasir.iftikhar@uos.edu.pk

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Keywords | CTV, Disease severity, citrus decline, Disease mapping, Sargodha



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Introduction

Citrus is cultivated in more than 64 countries of the world and its worldwide production is 140 million tons annually (Hussain *et al.*, 2021; Sajid *et al.*, 2021). Citrus tristeza virus (CTV) is an economically important pathogen because it attacks the citrus orchards throughout the world and left millions of

trees unproductive or completely destroyed. Since the emergence of CTV in the 20th century, the epidemic form of CTV had destroyed more than 60 million citrus trees (Iftikhar *et al.*, 2021). CTV belongs to the genus *Closterovirus*; family *Closteroviridae* and is transmitted very efficiently by different species of aphids (Lin *et al.*, 2006). CTV has different strains from mild to severe and the symptoms of CTV are

strain dependents (Iftikhar *et al.*, 2021). Mild isolates of CTV cannot cause decline whereas virulent strains cause huge losses. The main symptoms are stem pitting in twigs, honeycombing, vein clearing in lime, and gummosis on small lateral branches and twigs (Iftikhar *et al.*, 2013). CTV was first documented in Pakistan in the late 80s during a survey of citrus plantations in KPK and Punjab. It was confirmed through ELISA and electron microscopy (Lin *et al.*, 2006). The CTV was also documented in different areas of Punjab and KPK during extensive surveys in 2006-2007 (Abbas *et al.*, 2008; Iftikhar *et al.*, 2009). The highest incidence of CTV (48%) was recorded in Bhalwal (Punjab) and 40% incidence was found in Mardan (KPK) (Iftikhar *et al.*, 2009). Again in 2013, 67% incidence in Sargodha and 39% in Mardan was reported (Naseem *et al.*, 2016). Presently, very rare studies were conducted regarding spatio-temporal dynamics of CTV disease in Sargodha which is also known as California of Pakistan. Keeping in view the above-mentioned facts about CTV, the current study was aimed to quantify the spatial and temporal distribution of CTV disease in 3 Tehsils of Sargodha because Bhalwal, Kot Momin and Sargodha are major citrus growing regions in the district. The assessment of CTV disease occurrence in 3 citrus cultivars i.e., Kinnow, Mosambi and Feutrel's early which are readily available in the mentioned areas would provide a base to map out its role in the citrus decline and to devise sustainable management options.

Materials and Methods

Survey and collection of infested samples

A survey of citrus fields was conducted in different fields of 3 major citrus growing Tehsils of Sargodha District viz., Sargodha, Bhalwal and Kot Momin where 18 orchards were surveyed from each Tehsil. This survey included 3 varieties of citrus viz., Mosambi (*Citrus sinensis*) Feutrel's early (*Citrus reticulata* cv Feutrell's early) and Kinnow (*Citrus nobilis* x *Citrus deliciosa*). Salient features of CTV such as; dieback, stem pitting, honeycombing in bark and leaf yellowing were used as morphological markers to identify the diseased samples at initial step. The samples based on symptoms were collected in polythene bags and immediately shifted to ice bucket. The samples were collected in such a way that 6 orchards of each variety i.e. Kinnow, Mosambi and Feutrell's early were selected in every Tehsil and 3 samples were collected from each orchard; thus 54 samples/ Tehsil were

brought to the lab for further processing to confirm the virus. The orchards were selected on the basis of 3 age groups i.e., up to 5 years, 6-10 years and 11-15 years.

Extraction of virus sap

The collected samples were washed with tap water to remove the dust particles followed by 3 washings with distilled water and drying on blotter paper. The samples were crushed in a pre-chilled pestle and mortar by using extraction buffer. The extract was sieved through muslin cloth and collected in Eppendorf separately for each sample (Byamukama *et al.*, 2013).

Confirmation of virus

The presence of Virus was confirmed through enzyme linked immunosorbent assay (ELISA). The suspected samples were subjected to double antibody sandwich (DAS) ELISA as described by Iftikhar *et al.* (2009) as follows.

Microtiter plates were coated with CTV-specific antibody diluted 1000-folds in coating buffer followed by incubation at 30°C for 4 hours. After the incubation, ELISA plates were washed thrice with washing buffer to remove the excessive antibodies. Freshly prepared antigens (1:10 w/v) in extraction buffer were charged (100µl/well) and the plates were again incubated overnight at 4°C followed by repeating the washing. Reference blank, negative and positive controls were also included in the ELISA plate. CTV enzyme-conjugated antibodies were diluted 1000-fold in the conjugate and ELISA plate wells were coated by adding 100µl/well and incubated at 30°C for 5 hours followed by washing. Substrate (p-nitrophenyl-phosphate) was added @ 1mg/ml and incubated at room temperature for an hour at least. The reaction strength was rated visually as: = no reaction; + = weak reaction; ++ = definite reaction; +++ = strong reaction; ++++ = very strong reaction.

Disease incidence data recording

Disease incidence was recorded, after the confirmation of CTV through ELISA, in the samples collected from the surveyed orchards, the disease incidence data were calculated by using the following formula:

$$\text{Disease incidence (\%)} = \frac{\text{Number of ELISA positive samples}}{\text{Total number of samples}} \times 100$$

Severity of CTV

The severity of CTV was calculated in Mosambi orchards as characteristics symptoms were more pronounced in Mosambi trees affected with CTV. Based on apparent symptomology, the plants were selected from each field in a randomized pattern and 1 square inch piece of barks were taken out from bud union and the number of pits were observed. The area covered by pits was calculated out of a total 1sq inch area and determined the severity by the percentage of the pits (Table 1). The pits are salient symptoms of CTV disease while leaf yellowing and mottling etc. also resemble with other diseases, that's why pits were selected to calculate disease severity. Among all the citrus varieties, Mosambi was the most affected citrus variety therefore, disease severity was recorded only in Mosambi.

Table 1: Percentage of pits per square inch of bark.

Severity level	Pits (%)
Least severe	0-20%
Slightly severe	21-40%
Moderately severe	41-60%
Severe	61-80%
Highly severe	81-100%

Results and Discussion

The random survey was carried out including diseased and healthy citrus orchards of Sargodha, Bhalwal and Kot Momin, where 18 orchards were surveyed from each Tehsil. Out of 18 orchards at each Tehsil 6 orchards were marked per cultivar i.e., Kinnow, Mosambi and Feutrell's early that was further divided into three age groups where 2 diseased and 1 healthy

sample was collected (Table 2).

ELISA showed the presence of the virus by developing yellow colour in the wells having positive samples (Figure 1). The optical density (OD) of the colour showed the intensity regarding the presence of virus in the wells. OD values of infected samples were compared to those of healthy and control plants (Table 2). The OD values indicated that wells with dark yellow color have more concentration of CTV as compared to light yellow colour. In Tehsil Kot Momin, the OD values for infected samples of Mosambi were ranged from 0.89 to 2.35 while healthy samples of the same area showed very low value (0.17). The OD values for infected samples of Feutrell's early in Tehsil Kot Momin was less than Mosambi which ranged from 0.45 to 0.61 while in case of healthy samples it was 0.12. The infected samples of Kinnow showed the least OD values as compared to Mosambi and Feutrell's early ranging from 0.28 to 0.53 which was only 0.11 in case of healthy Kinnow samples.



Figure 1: Density of the colour showed the increased presence of virus in the wells.

Table 2: The survey of citrus orchards.

Cultivar	Tehsils								
	Bhalwal			Kot Momin			Sargodha		
	Kinnow	Mosambi	FE*	Kinnow	Mosambi	FE	Kinnow	Mosambi	FE
AG 1**	2	2	2	2	2	2	2	2	2
AG 2	2	2	2	2	2	2	2	2	2
AG 3	2	2	2	2	2	2	2	2	2
Total	6	6	6	6	6	6	6	6	6
S/O***	3	3	3	3	3	3	3	3	3
T/C^	18	18	18	18	18	18	18	18	18
GT	18			18			18		

*FE= Feutrell's early; **AG= Age group; AG1= up to 5 years; AG2= 6-10 years; AG3= 11-15 years; ***S/O = Number of samples per orchard; ^T/C = Number of samples per cultivar (S/O × Total).

Table 3: Optical density (OD) values in ELISA of diseased and healthy plants.

Varieties	Sargodha		Kot Momin		Bhalwal	
	Diseased	Healthy	Diseased	Healthy	Diseased	Healthy
Citrus limetta	0.82-2.22	0.18	0.89-2.35	0.17	0.85-2.32	0.13
<i>C. reticulata</i> cv Feutrel's early	0.46-0.64	0.15	0.45-0.61	0.12	0.47-0.64	0.15
<i>C. reticulata</i> cv Kinnow	0.25-0.56	0.13	0.28-0.53	0.11	0.26-0.52	0.12

The intensity of CTV infection was less in Tehsil Bhalwal and Sargodha as compared to Kot Momin among all 3 citrus cultivars. However, the infection was more in Mosambi followed by Feutrell's early and Kinnow, respectively in both Tehsils. The range of OD values for infected samples of Mosambi in Tehsil Bhalwal was 0.85-2.32 while it was 0.82-2.22 in Sargodha. The healthy samples of Mosambi showed mean OD values of 0.13 and 0.18 values in Bhalwal and Sargodha, respectively. Feutrell's early gave least OD values as compared to Mosambi in both Tehsils i.e., Bhalwal and Sargodha which range from 0.47-0.64 and 0.46-0.64, respectively. The healthy samples of Feutrell's early gave 0.15 OD value in both Tehsils.

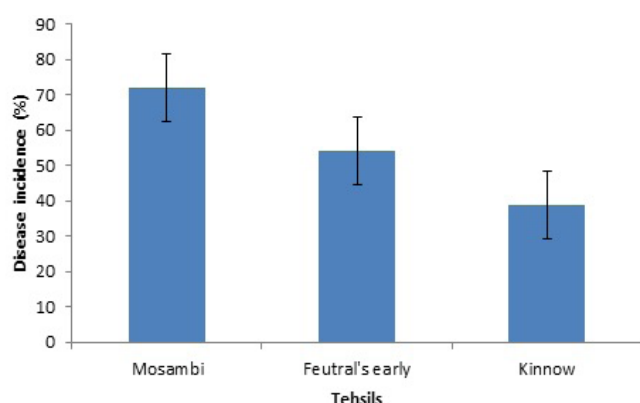


Figure 2: The incidence of CTV in three Tehsils.

Disease incidence in three Tehsils of Sargodha

The orchards of (Mosambi, Kinnow and Feutrell's early) were surveyed based on symptomology in Sargodha, Bhalwal and Kotmomin to record disease incidence. The disease incidence was calculated based upon the salient CTV disease symptoms i.e., stem pitting and the abnormal development of root and scion stock union. Mosambi orchards were examined thoroughly for the detection of CTV based on symptomology. CTV showed the highest incidence rate of 91% in Kot Momin followed by 85% in Bhalwal and 64% in Sargodha (Figure 2). Similarly, symptomology-based detection was performed for 3 cultivars separately, among which Mosambi showed maximum disease incidence in all Tehsils followed by Feutrell's early and Kinnow (Figure 3). The disease incidence of 3 citrus cultivars was recorded in following order i.e.,

Kot Momin, Bhalwal and Sargodha, respectively. The overall disease incidence and severity increased with every observation in all cultivars and Tehsils.

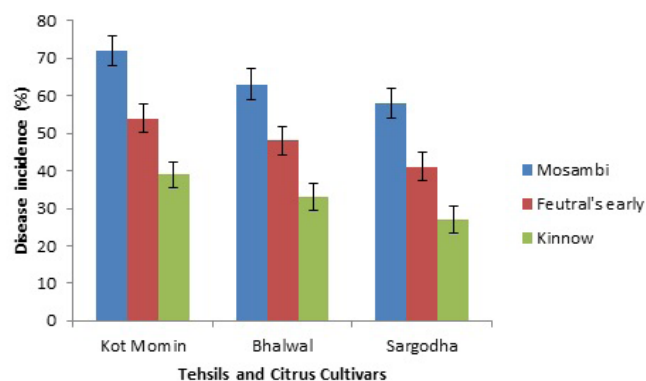


Figure 3: The incidence of CTV in three Tehsils on 3 citrus cultivars.

CTV disease severity in Mosambi orchards

Mosambi is one of the most affected citrus variety by the CTV therefore, the severity level of CTV was much higher in Mosambi plants. The increasing age of orchards showed more severe attacks of CTV by having the large number of pits in infected plants. It was estimated that the greater the age of the plant, the greater will be the severity of the virus. The maximum disease severity was recorded in age group 3 (11-15) years followed by age group 2 (6-10 years) and age group (up to 5 years), respectively (Figure 4).

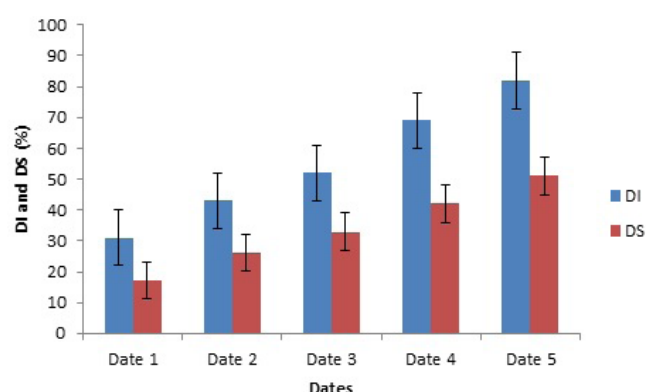


Figure 4: Overall temporal distribution of CTV disease incidence and disease severity.

Citrus tristeza virus (CTV) is one of the most important diseases, but it has not been given much importance in the past. It has spread fast in the country without being noticed. CTV is a huge threat

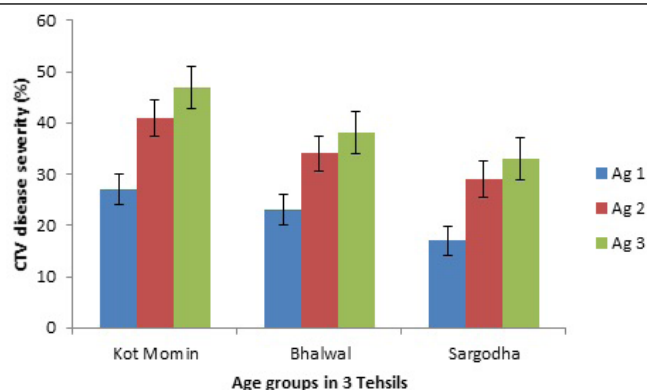


Figure 5: The severity of CTV on Mosambi orchards in 3 age groups.

to the citrus industry all over the world as it is destroying the citrus orchard (Lin *et al.*, 2006). CTV resulted in many disease epidemics worldwide and caused significant losses (Korkmaz *et al.*, 2008). The citrus fields surveyed for sample collection and CTV was confirmed through ELISA. ELISA is the most efficient method of detecting the virus in plant cells amongst all methods of detecting virus. It is the cost effective, so it is commonly used in citrus growing countries (Hilf *et al.*, 2005). The technique uses the capability of antibodies (which were extracted from animals) to recognize proteins, especially the viral coat protein. It is a quick and efficient method of detecting CTV and at a time large quantity of samples can be run (Folimonova, 2020). CTV was first reported in Pakistan in 1988 on a few citrus plants in KPK and Punjab and confirmed through ELISA (Bester *et al.*, 2021). The suspected CTV samples were subjected to DAS-ELISA for the confirmation of virus. The intensity of yellow colour favours the intensity of the pathogen in the wells of the plate (Iftikhar *et al.*, 2021). The wells of the plate with denser colour showed high OD values when placed in ELISA reader. A similar study for the detection of CTV was conducted by Iftikhar *et al.* (2009) and found the OD values at 405nm 0.60, 0.42 and 0.31 for sweet orange, Kinnow and grapefruit, respectively. The petioles and midribs were best tissues to detect CTV through DAS-ELISA (Anfoka *et al.*, 2005). Wu *et al.* (2015) studied biological and serological characters of CTV for the better understanding of its dynamics. The darker colour of positive reaction is the indication of more virus titre as compared to light colours in the diseased samples of less affected plants. This study showed that maximum CTV disease incidence was recorded in Mosambi followed by Feutrel's early and Kinnow. Mosambi orchards were selected for the calculation of CTV disease severity being the more affected among others. *Citrus limetta* (Mosambi) orchards showed increased disease severity in older

orchards than younger ones. Previously, it was found by Sharma *et al.* (2011) that *Citrus reticulata* and *Citrus limon* were resistant against CTV disease. It is evident from all the studies that there was more disease incidence in sweet oranges. CTV disease incidence also varied on Kinnow with respect to different areas and it has serious role in citrus decline. In Florida, declined orchards were sampled randomly and subjected to high throughput sequencing (HTS) for the detection of exact cause and CTV was found (Britt *et al.*, 2022). CTV disease was more severe in Sargodha than Bhalwal and Kot Momin. Naseem *et al.* (2016) recorded maximum CTV disease incidence in Sargodha (67%) followed by Bhalwal (48%) and Mardan (39%) collectively on all citrus cultivars. The variation in the level of incidence and severity may be the result of varied growing conditions and soil factors. The incidence of CTV disease depends upon the prevalence of aphid population (Harper *et al.*, 2018). Arif *et al.* (2005) conducted extensive survey of citrus orchards and reported that percentage of infection was high in North western area of Pakistan which was 24-44%. CTV is a phloem limited virus where it replicates in sieve elements and interrupts the normal physiological functions of the citrus plants and also obstructs the translocation of prepared food (Britt *et al.*, 2020). Moreno *et al.* (2008) revealed that (CTV) disease can be managed by using tristeza resistant rootstock, certifications programs of bud woods as well as through different quarantine measures. They observed that a complete study of the interaction between hosts its vectors as well as viral protein will provide a significant role for minimizing disease severity. The disease incidence and severity were increased with the passage of time, it is evident both from different dates of disease observation and varied age groups of the citrus cultivars in current study. Ippolito *et al.* (2020) used ordinary differential equations (ODEs) to describe the possible CTV disease outbreak with reference to time. He specified the joint likelihood function as poisson binomial distribution. Vidal *et al.* (2012) described that CTV incidence and severity increased in older plantations than in younger ones. The spatio-temporal distribution of CTV disease depends upon susceptibility of citrus cultivars, infectivity of virus isolates and population dynamics of vector (Moreno *et al.*, 2008). The CTV disease severity was more in higher age groups which gradually decreased in lower age groups (Singh *et al.*, 2017). In current study, CTV disease severity was more in Mosambi followed by Feutrell's early and Kinnow, respectively. The research of Singh *et al.*

(2017) endorsed the present study as they compared CTV disease incidence and severity in various citrus cultivars and found more in sweet oranges. In various countries, the negative impacts of CTV disease were countered by incorporating a cross-protection technique. Plants were inoculated with mild-strain of CTV to reduce the devastating effects of subsequent infections that can be introduced by aphids (Shilts *et al.*, 2020).

Conclusions and Recommendations

The incidence and severity of CTV was high in sweet orange. CTV can be detected in three main parts of plants like twig, leaves and barks. This study provided a new quantitative information regarding spatial and temporal dynamics of CTV disease. Such data enabled the method of sampling designs were needed to quantify and compare the impact of climate, new management tactics and integrated disease management. The incidence and severity of CTV was more in Kot momin followed by Bhalwal and Sargodha. The old plantation is more in all these citrus growing areas. Therefore, it is concluded that CTV is well established in old plantations and the source of spread in new citrus plantations. Diagnosis of CTV in citrus nurseries and insect vector in Pakistan is the need of time.

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Novelty Statement

The spatial and temporal distribution of CTV disease based upon serological confirmation was studied for the first time in Sargodha, Pakistan.

Author's Contribution

Muhammad Ahmad Zeshan and Yasir Iftikhar: Conceived the idea and supervised the research.

Asim Ali Khan and Maham Sohail: Conducted the experimentations.

Malik Abdul Rehman and Sonum Bashir: Helped in identification and collection of samples.

Muhammad Usman Ghani: Analyzed the data statistically.

Mustansar Mubeen: Refined and finalized the first draft.

Conflict of interest

The authors have declared no conflict of interest.

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