Pak. J. Phytopathol., Vol. 32 (02) 2020. 197-202 DOI: 10.33866/phytopathol.030.02.0591

Official publication of Pakistan Phytopathological Society
Pakistan Journal of Phytopathology
ISSN: 1019-763X (Print), 2305-0284 (Online)
http://www.pakps.com

EVALUATION OF CITRUS CULTIVARS AGAINST TRISTEZA CLOSTERO VIRUS

Muhammad U. Yasin*, Saba Saeed, Azhar Mustafa, Muhammad Iqbal

Plant Virology Section, Plant Pathology Research Institute, AARI, Faisalabad, Pakistan.

A B S T R A C T

Citrus tristeza disease is the emerging devastating threat of citrus plants worldwide particularly in Punjab and KPK provinces of Pakistan. To evaluate the resistant cultivars, fourteen hundred leaf samples of 14 citrus cultivars were collected from Citrus Research Institute Sargodha, Pakistan. For the detection of tristeza disease, leaf sap was tested through a Double Antibody Sandwich Enzyme-linked Immunosorbent Assay technique. Reaction observed by yellow color development. The intensity of color development is directly proportional to the amount of pathogen enzyme trapped. Among all the cultivars highest was infection recorded in the Gil-Gil *Citrus maxima* i.e., 24% and lowest in seedless kinnow *C. reticulata* i.e., 3%. Seedless kinnow has a maximum level of resistance against citrus tristeza Clostero virus disease.

Keywords: CTV Resistance LISA, incidence, Citrus nursery screening.

INTRODUCTION

Citrus fruits represent approximately 40% of all fruit crops grown in Pakistan. Pakistan holds a good position among top citrus-producing countries by exporting 318.93 thousand tons of citrus (Govt. of Pakistan, 2019. It is cultivated in four provinces but mainly in Punjab and Khaber Pakhtun Khawa (KPK) (Catara, 1987; Catara *et al*., 1988). The major citrus growing areas in Punjab are Sargodha, Jhang, Mianwali, Sahiwal, TobaTek Singh, Multan, Layyah, Rahim Yar-Khan (GOP, 2009) and in KPK are Haripur, Peshawar, Charsadda, Swabi, Malakand, Nowshera, Mardan, Swat, Dir, D. I. Khan (Arif *et al*., 2015).*Citrus reticulata* is a major cultivar in Punjab, whereas *C. sinensish* is mainly grown in KPK (Arif *et al*., 2005).However, per hectare production is very low as compared to other countries due to insect pests and diseases.

Virus and linked diseases considered major production decline problems (Satir *et al*., 2016; Naqvi *et al*., 2017; Fateh *et al*., 2017). In the 1970s, many cases of citrus decline have been report from Punjab and KPK and consider that the tristeza clostero Virus plays key role in declining of citrus plants (Bove, 1995).

In 1988, Pakistan Agricultural Research Council (PARC) conducted a survey with the collaboration of Ministry of Foreign Affairs and a group of Italian and Pakistani experts jointly detected the presence of CTV in major citrus growing areas of Punjab and KPK by enzyme-linked immune assay (ELISA) and electron microscopy techniques (Catara *et al*., 1988). The detection of CTV from selected orchards of two provinces Punjab and KPK also reported as a potential threat (Iftikhar *et al*., 2009). The increasing trend of citrus decline by CTV year after year could be horrific in Pakistan (Arif *et al.*, 2015). The epidemic of CTV destroyed the citrus industry in many citrus growing countries in the past.

CTV was firstly originated from Orient where it became epidemic in citrus growing areas worldwide through infected bud-wood and plants (Bar-Joseph *et aI*., **l**979; Roistacher *et aI*., l991; Roistacher and Moreno, **l**991; Rocha-Pena *et aI*., l995). During the 1930s, over 30 million citrus trees have been killed in Argentina and Brazil (Bar-Joseph *et aI*., l989). In 1960 to 1980, almost 10 million trees in Spain and 6.6 million trees in

Submitted: September 08, 2020
Revised: December 15, 2020
Accepted for Publication: December 16, 2020
* Corresponding Author:
Email: aropp2017@gmail.com

Venezuela were killed due to this disease (Cambra *et al*., 2000; Rocha-Pena *et al*., 1995).

Size of viral particles varies up-to 2000-11nm and these are of round flexure rod in shape (GonsaIves *et al*., 1978; Bar-Joseph and Iee, 1989). Majority of the other viruses these have also single strand RNA and the size of its RNA is about 20kb which is covered by a protein known as coat protein (Gonsalves *et al*., 1978; Bar-Joseph *et al*., 1985; Bar-Joseph and lee, 1989; Sekiya *et al*., 1991). Moreover, the characterization of Citrus Tristeza Virus on molecular basis has also been performed (Biswas, 2010; AI-Sadi *et al*., 2012; Davino *et al*., 2013).

The basic source of disease spreading is the use of uncertified infected scion in the new plantation. Various aphid species are also involved in disease transmission but their efficiency varied, *Toxopetra citricida* found the most efficient vector (Roistacher and Bar-Joseph, 1987; Rocha-Pena *et al*., 1995). *Aphis gossypii*is also an effective vector for transmission of severing strains in all citrus-growing areas around the world (Roistacher and Bar-Joseph, 1987; Yokomi and Garnsey, 1987). *Aphis spiraecola, A. aurantii* and *A. craccivora* are some important species of the insect vactor which are responsible for disease transmission (Yokomi and Garnsey, 1987; Norman and Grant, 1958; Roistacher and Bar-Joseph, 1987; Bar-Joseph and Iee, 1989). While in Pakistan, two species of aphid including *A. spiraecola* and *A, gossypii* are responsible for disease transmission (Catra, 1987).

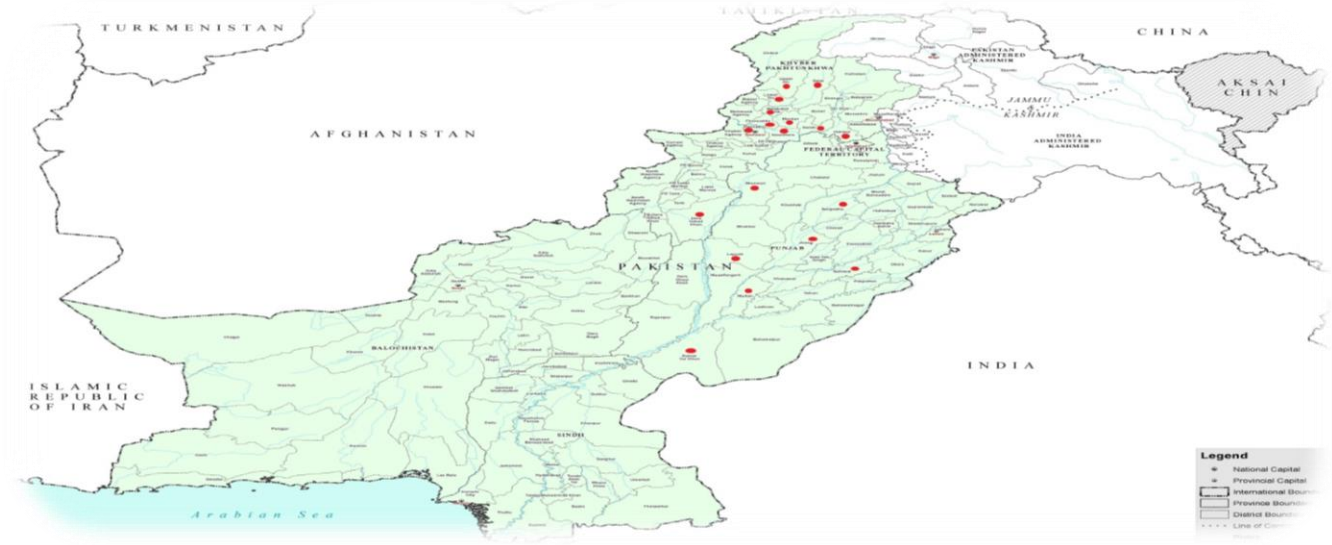

Figure 1. Major citrus-growing areas in Punjab and KPK

The present study carried out to detect the prevalence of CTV in different citrus cultivars in Sargodha and Faisalabad Districts of Punjab, Pakistan. By using its results, we will be able to measure the level of resistance in different cultivars against CTV. Therefore, we can produce fruits of high quality and quantity in a sustainable way by using resistant cultivars.

MATERIALS AND METHODS

Collection of samples: Fourteen hundred leaf samples of 14 citrus cultivars (100 of each cultivar) were collected from Citrus Research Institute Sargodha and brought in to serological laboratory, Plant Virology Section, PPRI, AARI Faisalabad (31.24N, 73.3E) for the detection of citrus tristeza clostero virus .

Detection of CTV through DAS-ELISA

For the detection of CTV, leaf samples tested through a Double Sandwich Antibody Immunosorbent Assay technique (DAS-ELISA).ELISA test conducted by following four steps.

1st step: Coating antibody diluted in coating buffer as recommended on the bottle label and added 100 µl to the required number of wells for the test. ELISA plate was placed in a plastic bag with some damp paper towels and sealed tightly. The plate was incubated at 4°C overnight Figure 2 (a).

2nd step: Washing buffer (Phosphate buffered saline + Tween 20 (0.5%) PBST) and extraction buffer were prepared according to the instructions given in ELISA-kit. Washing buffer was used to wash the wells of ELISA plate and extraction buffer used for extraction of sap from citrus samples. The sap extracted by grinding 1g of plant tissue in 10 ml of extraction buffer. Before injecting the antigen (sap), the wells of the ELISA plate were washed (Three times) and 100 µl positive and negative control were

injected into four wells diluted in distilled water. The sample sap (antigen) 100 µl was injected into each well of ELISA-plate with single micro-titer, placed in a plastic bag and incubated at 4°C for overnight Figure 2 (b).

3rd step: ELISA plate was washed with washing buffer 8thtimes and the antibody-enzyme conjugate diluted in the conjugate buffer as recommended on the label. Then 100-µl conjugate buffer was injected into each test well and pack the plate in a plastic bag for incubation at 4°Cfor overnight Figure 2(c).

4th step: The ELISA plate was washed 8th times with a washing buffer to ensure the removal of all unbounded antibody-enzyme conjugates from the wells. The substrate buffer was prepared just before the use and p-Nitro phenyl Phosphate (PNP) was added at 1mg/ml to substrate buffer. Substrate buffer was added into the wells and then the plate was placed in a plastic bag and incubated in dark at room temperature for half to 1 hour Figure 2 (d).

Observations of reactions: The reaction was observed after half-hour yellow color development noted visually. The intensity of yellow color development is directly proportional to the amount of pathogen enzyme trapped. By inserting ELISA plate into ELISA reader, accurate results were taken.

STATISTICAL ANALYSIS

To analyze data whether all the cultivars having any relevancy regarding disease spread it is assumed that all cultivars having a similar trend of resistance against the CTV disease and strong relevancy of resistance occurred among all the cultivars. Therefore, to determine the association of various citrus cultivars regarding resistance against CTV infection spread, the chi-square test used (Microsoft Excel 2013) SS.

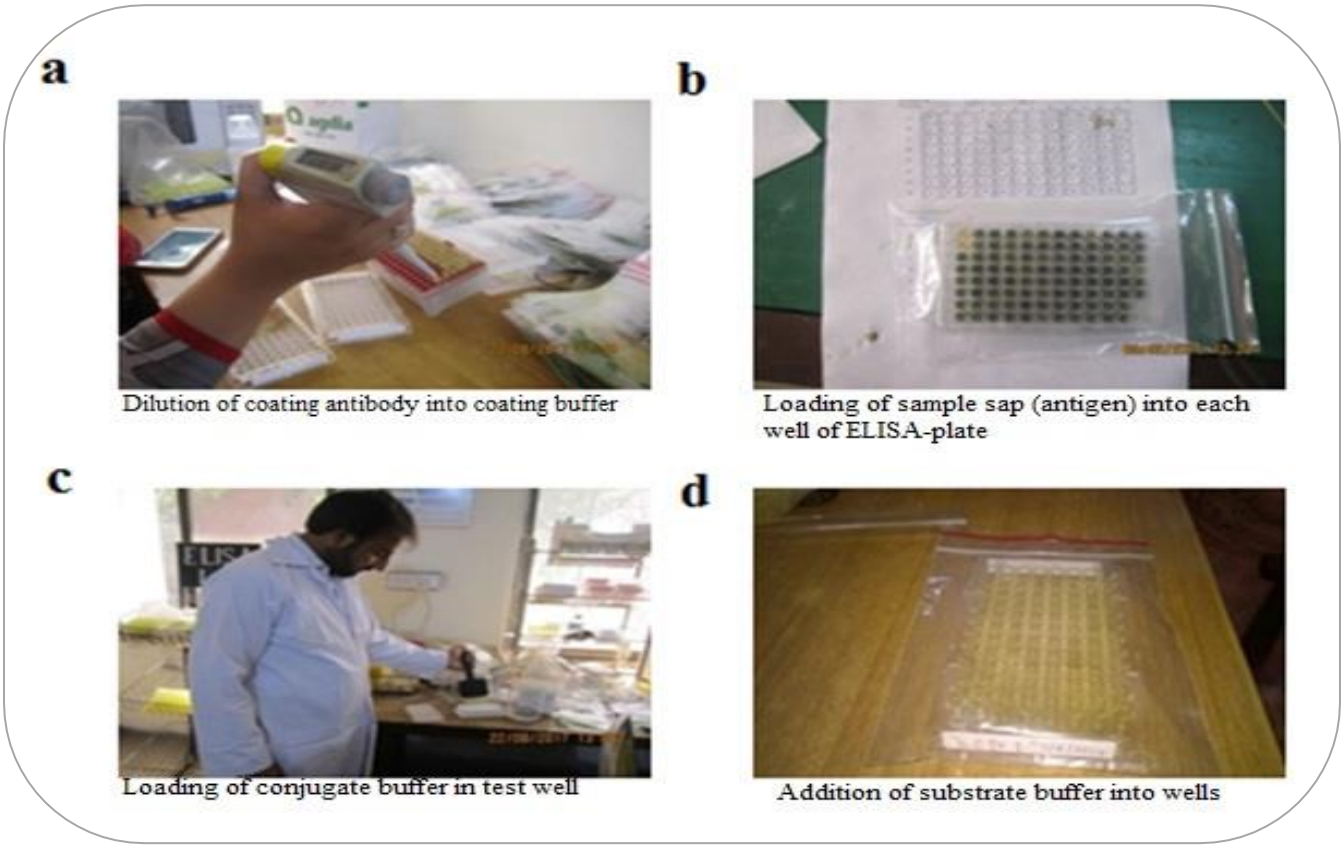

Figure 2. Steps of DAS-ELISA

RESULTS AND DISCUSSION

When the data analyzed through the Chi-square test, the results confirmed the incidence of CTV in all major citrus growing cultivars. All cultivars have great variations of CTV infection.

Total Samples observed	1400
Overall Chi-Square	45.62
P-value	0.0000
Degree of Freedom	13

Overall Chi-Square value (45.62) is greater than p-value (0.00) which depicted that results are highly significant and each cultivar has a different response to the

infection of CTV. Some cultivars have shown more resistance and less infection while others are vice versa. The average percentage of CTV in major citrus cultivars of Pakistan ranged from 3-24%. Highest infection was found in *C. maxima* i.e., 24%and lowest in *C. reticulata* i.e., 3%. Other cultivars Shember, Mussambi, Salustiana, Kinnow, FSD-07, Metha, Kala blood, Taracco, Euriko, Moro blood, Pashwarimussambiand Gara grand found 7%, 8%, 8%, 9%, 9%, 10%, 11%, 11%, 18%, 18%, 20% and 20% respectively Figure 3.

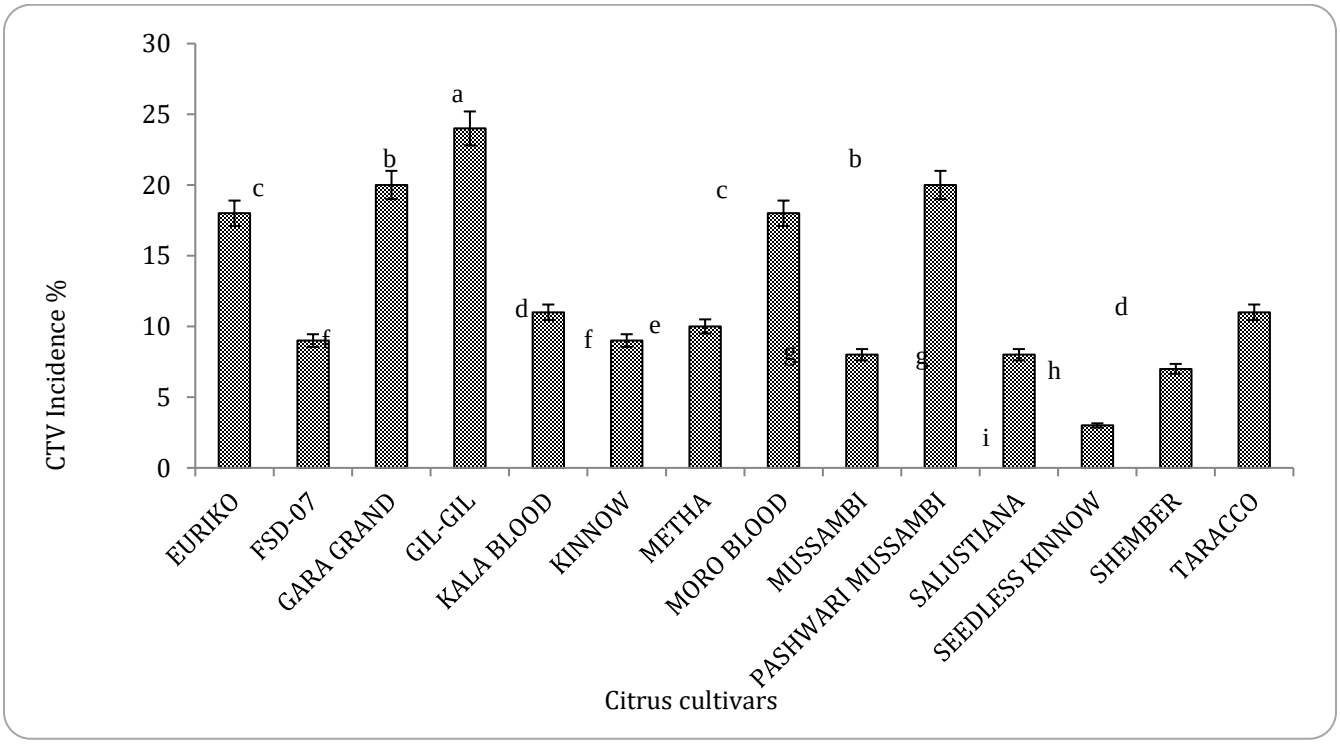

Figure 3. Evaluation of Citrus cultvars against citrus tristiza virus

Results indicated that *C. reticulata* is the highly resistant but *C. maxima* most susceptible cultivars among all others. Findings are in arrangement with Arif *et al*., (1962) and Akhtar and Ahmad (1999) founded infection in various citrus cultivars are observed as 25-40% in *C. sinensis*, 22% in *C. reticulata*, 15% in *C. paradisi*, 10% in *C. aurantifolia*, and 2% *C. limon*.

Table 1. Association of various citrus cultivars regarding resistance against CTV infection by applying chi-square test through Microsoft Excel 2013).

Infected cultivar	Observed			Expected		Cell Chi-Sq	
EURIKO	82	18	100	87.43	12.57	0.34	2.34
FSD-07	91	9	100	87.43	12.57	0.15	1.01
GARA GRAND	80	20	100	87.43	12.57	0.63	4.39
GIL-GIL	76	24	100	87.43	12.57	1.49	10.39
KALA BLOOD	89	11	100	87.43	12.57	0.03	0.20
KINNOW	91	9	100	87.43	12.57	0.15	1.01
METHA	90	10	100	87.43	12.57	0.08	0.53
MORO BLOOD	82	18	100	87.43	12.57	0.34	2.34
MUSSAMBI	92	8	100	87.43	12.57	0.24	1.66
PASHWARI M	80	20	100	87.43	12.57	0.63	4.39
SALUSTIANA	92	8	100	87.43	12.57	0.24	1.66
SEEDLESS KINOW	97	3	100	87.43	12.57	1.05	7.29
SHEMBER	93	7	100	87.43	12.57	0.36	2.47
TARACCO	89	11	100	87.43	12.57	0.03	0.20

Most of the CTV infected plants do not show any foliar symptom, symptom development largely depends upon citrus cultivars, environmental conditions and virus strain (Bar-Josph and Dawson 2008; Biswas 2008). Visual

diagnosis of the disease is not possible, so advance tools like ELISA have been used in the Indian Sub-continent (Chakraborty *et al*., 1992; Biswas, 2008).

Arif *et al*., (2015) founded that average infection percent increased from 24-44% during 2002-10 in the Northwest province of Pakistan. Moreover, the severity of CTV was found high in areas where sour orange used as root stock for sweet orange.

CTV is a viroid which is becoming a potential threat to citrus growing areas throughout the world including Pakistan (Catara *et aI*., **l**988; Bar-Joseph *et aI*., **l**989; Iee *et aI*., l994; Rocha-Pena *et aI*., l995). Catara *et al*., (1987, 1988) reported CTV in low intensity at the few localities of Northwest of Pakistan. After the broad-spectrum survey and comprehensive examination, it was revealed that CTV incidence has been increased from 24-44 %., (Coehran, **l**976; Catara *et aI*., l988) Arif *et aI*., 2005 founded a 27% average incidence of CTV.

Sharma *et al*., 2012 found that sweet orange *C. sinensis* was more susceptible to CTV up to 39% incidence. *C. lemon* and *C. reticulata* did not show any CTV infection.

Jaywant *et al*., (2013) detected the CTV disease infection 16.6-20.5 in mussambi *C. aurantifolia* through the direct antigen-coated ELISA and reverse transcriptase-polymerase chain reaction (RT-PCR).

Serious and consistent efforts are required to minimize the CTV infection for running the citrus industry in a smooth way. To avoid the introduction of a virus into new geographical area, quarantine rules imposed by government should be strictly followed. Government and public nurseries should arrange the only certified un-infected scion and rootstock available for the farmers. Extension experts should arrange the workshops in which guide the farmers that do not use sour orange as a rootstock.

CONCLUSION

It is concluded from this study that seedless kinnow *C. reticulata* have highest resistance level against the citrus tristeza clostero virus among all other cultivars. In other words, we can produce high quality and quantity of fruits by using this cultivar to manage the disease.

REFERENCES

Akhtar, M. and I. Ahmad. 1999. Incidence of citrus greening disease in Pakistan. Pakistan Journal of Phytopathology, 11: 1-5.

Al-Sadi, A., S. Al-Hilali, R. Al-Yahyai, F. Al-Said, M. Deadman, I. Al-Mahmooli and G. Nolasco. 2012. Molecular characterization and potential sources of Citrus tristeza virus in Oman. Plant Pathology, 61: 632-640.

And Condiments Statistics Of Pakistan.

Arif, A. G., M. Akhtar and M. Ibrahim. 1962. Citrus diseases and their control. Punjab Fruit Journal, 26: 369-370.

Arif, M., A. Ahmad, M. Ibrahim and S. Hassan. 2005. Occurrence and distribution of virus and virus-like diseases of citrus in north-west frontier province of Pakistan. Pakistan Journal of Botany, 37: 407-421.

Arif, M., W. Khan, M. Ibrahim and M. Fahim. 2015. Citrus tristeza virus: An increasing trend in the virus occurrence and distribution in citrus fruits of Northwest, Pakistan. African Journal of Biotechnology, 14: 2352-2360.

Bar-Joseph M. and W.O. Dawson.2008. Citrus tristeza virus. Encyclopedia of Virology pp 520–525.

Bar-Joseph, M. and R. Lee. 1989. Citrus tristeza virus. AAB Descriptions of plant viruses, pp. 353.

Bar-Joseph, M., D. Gumpf, J. Dodds, A. Rosner and I. Ginzberg. 1985. A simple purification method for citrus tristeza virus and estimation of its genome size. Phytopathology, 75: 195-198.

Bar-Joseph, M., R. Marcus and R. F. Lee. 1989. The continuous challenge of citrus tristeza virus control. Annual review of phytopathology, 27: 291-316.

Bar-Joseph, M., S. Garnsey and D. Gonsalves. 1979. The clostero viruses: a distinct group of elongated plant viruses, Advances in virus research. Elsevier, pp. 93-168.

Biswas, K. 2008. Molecular diagnosis of Citrus tristeza virus in mandarin (*Citrus reticulata*) orchards of Darjeeling hills of West Bengal. Indian Journal of Virology, 19: 26-31.

Biswas, K. 2010. Molecular characterization of Citrus tristeza virus isolates from the Northeastern Himalayan region of India. Archives of virology, 155: 959-963.

Bové, J. M. 1995. Virus and virus-like diseases of citrus in the Near East region.

Cambra, M., M. T. Gorris, C. Marroquın, M. P. Román, A. Olmos, M. C. Martınez, A. H. de Mendoza, A. Lopez and L. Navarro. 2000. Incidence and epidemiology of Citrus tristeza virus in the Valencian Community of Spain. Virus Research, 71: 85-95.

Catara, A. 1987. Compendium of citrus diseases and disorders in Pakistan. Results of a preliminary

survey. Agrotec SPA, Rome project for research and development in the cultivation of fruit, vegetables and olives. PARC, Islamabad, Pakistan: 14-15.

Catara, A., A. Azzaro, S. Mughal and D. Khan. 1988. Virus, viroid and prokaryotic diseases of citrus in Pakistan. Proceedings of 6th Conference of the International Organization of Citrus Virologist. pp. 957-962.

Chakraborty, N., Y. Ahlawat, A. Varma, K. J. Chandra, S. Ramapandu and S. Kapur. 1993. Serological reactivity in Citrus tristeza virus strains in India. International Organization of Citrus Virologists Conference Proceedings (1957-2010).

Cochran, L. 1976. The occurrence of greening disease in Pakistan. International Organization of Citrus Virologists Conference Proceedings, pp. 108-112.

Davino, S., A. Willemsen, S. Panno, M. Davino, A. Catara, S. F. Elena and L. Rubio. 2013. Emergence and phylodynamics of Citrus tristeza virus in Sicily, Italy. PLoS One, 8: e66700.

Fateh, F. S., T. Mukhtar, M. R. Kazmi, N. A. Abbassi and A. M. Arif. 2017. Prevalence of citrus decline in district Sargodha. Pakistan Journal of Agriculture Sciences, 54: 9-13.

Gonsalves, D., D. Purcifull and S. Garnsey. 1978. Purification and serology of citrus tristeza virus. Phytopathology, 68: 553-559.

Govt. of Pakistan. 2009. Pakistan statistical yearbook. Federal bureau of statistics, Statistics division, Islamabad.

Govt. of Pakistan. 2018-19. .Ministry of National Food Security and Research. Fruit, Vegetables

Iftikhar, Y., M. A. Khan, A. Rashid, S. Mughal, Z. Iqbal, A. Batool, M. Abbas, M. Khan, S. Muhammad and M. Jaskani. 2009. Occurrence and distribution of Citrus Tristeza Closterovirus in the Punjab and NWFP, Pakistan. Pakistan Journal of Botany, 41: 373-380.

Lee, R. F. and P. Baker. 1994. The Citrus tristeza virus (CTV): An introduction to current priorities, with special reference to the worsening situation in Central America and the Caribbean. 634.3.

Naqvi, S. A. H., S. Atta, H. Liu, A.-u. Rehman and A. A. Khan. 2017. Serological and molecular based detection of graft-transmissible pathogens associated with citrus from non-core areas of Pakistan. Pakistan Journal of Agricultural Sciences, 54.

Rocha-Peña, M. A., R. F. Lee, R. Lastra, C. Niblett, F. M. Ochoa-Corona, S. M. Garnsey and R. K. Yokomi. 1995. Citrus tristeza virus and its aphid vector Toxoptera citricida: Threats to citrus production in the Caribbean and Central and North America. Plant Disease, 79: 437-445.

Roistacher, C. and P. Moreno. 1991. The worldwide threat from destructive isolates of citrus tristeza virus-a review. International Organization of Citrus Virologists Conference Proceedings, 1: 7-19.

Roistacher, C., D. Gumpf, J. Dodds and R. Lee. 1991. The threat of" The Killer. Calif. Citrogr, 76: 8-12.

Roistacher, M. 1987. Aphid transmission of citrus tristeza virus: a review. Phytophylactica, 19: 163-168.

Satir, N. Y., I. Ortas and O. Satir. 2016. The influence of mycorrhizal species on sour orange (*Citrus aurantium* L.) growth under saline soil conditions. Pakistan Journal of Agricultural Sciences, 53.

Sekiya, M. E., S. D. Lawrence, M. McCaffery and K. Cline. 1991. Molecular cloning and nucleotide sequencing of the coat protein gene of citrus tristeza virus. Journal of General Virology, 72: 1013-1020.

Sharma, S. K., A. Tarafdar, D. Khatun, K. Sumita and K. K. Biswas. 2012. Intra-farm diversity and evidence of genetic recombination of Citrus tristeza virus in Delhi region of India. Journal of plant biochemistry and biotechnology, 21: 38-43.

Singh, J. K., A. Tarafdar, S. K. Sharma and K. K. Biswas. 2013. Evidence of Recombinant Citrus tristeza virus Isolate Occurring in Acid Lime cv. Pant Lemon Orchard in Uttarakhand Terai Region of Northern Himalaya in India. Indian Journal of Virology, 24: 35-41.

Yokomi, S. 1987. Transmission of citrus tristeza virus by *Aphis gossypii* and *Aphis cirticola* in Horida. Phytophylactica, 19: 169-172.

Contribution of Authors:

Muhammad U. Yasin	:	Conceptualized the study. Formal analysis, methodology and writing of original manuscript
Saba Saeed	:	Supervised, reviewed and edited the manuscript. Provided technical guidelines during the study
Azhar Mustafa	:	Designing of survey format and compilation and interpretation of data
Muhammad Iqbal	:	Field visits for collection of data and Figures and Graphs in Microsoft excel