Quantitative and Qualitative Differences of Inclusion Bodies Induced by *Citrus tristeza virus*

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ABSTRACT

The inclusion body characteristics, virus titer and foliar symptoms in Mexican lime [*Citrus aurantifolia* (Christm.) Swing.] and sour orange (*C. aurantium* L.) plants inoculated with a mild isolate (T-TX8) or a severe isolate (CTV-2) of *Citrus tristeza virus* (CTV) were compared over a period of 11 weeks. In Mexican lime, the formation time of inclusion bodies was three weeks after inoculation with CTV-2 and four weeks with T-TX8. In sour orange, we detected inclusion bodies at the seventh week with both isolates. Comparison of the color intensity of inclusion bodies revealed that sections from Mexican lime yielded darker purple inclusion bodies with CTV-2 than with T-TX8 infection. Out of 349 sections examined from CTV-2 infected Mexican lime plants, 251 showed inclusion bodies, while only 43 out of 376 sections from the plants inoculated with T-TX8 showed inclusion bodies. The most average inclusion bodies per section were 32.0 for CTV-2 infection and 3.2 for T-TX8 infection in Mexican lime. Statistical analysis of inclusion body numbers and ELISA OD_{405} values showed that there were significant differences between Mexican lime and sour orange with CTV-2 infection ($p \leq 0.05$), and between Mexican lime infected with CTV-2 vs. T-TX8 ($p \leq 0.05$). The inclusion body characteristics were positively correlated with the severity of the virus isolate, virus titer, and the symptoms in Mexican lime plants but not in sour orange.

RESUMEN

Se compararon las características de las inclusiones virales, la concentración del virus y los síntomas foliares en plantas de lima mexicana [*Citrus aurantifolia* (Christm.) Swing.] y de naranjo agrio (*C. aurantium* L.) inoculadas con un aislamiento moderado (T-TX8) o un aislamiento severo (CTV-2) del virus de la tristeza de los cítricos (VTC) durante un período de 11 semanas. En las plantas de lima mexicana, las inclusiones virales se formaron 3 semanas después de la inoculación con CTV-2 y 4 semanas después de la inoculación con T-TX8. En naranjo agrio, se detectaron inclusiones virales en la séptima semana en ambos aislamientos. Al comparar la intensidad del color de las inclusiones virales se encontró que las inclusiones virales en los cortes de lima mexicana fueron de un color púrpura mas intenso en las plantas inoculadas con CTV-2 que con las infectadas con T-TX8. En las plantas de lima mexicana infectada con CTV-2, 251 de los 349 cortes examinados mostraron inclusiones virales, mientras que solo 43 de los 376 cortes revisados de las plantas inoculadas con T-TX8 mostraron inclusiones virales. El número promedio de inclusiones virales observadas por corte fue de 32 en el caso de la infección con CTV-2 y de 3.2 con la infección con T-TX8. El análisis estadístico del número de inclusiones virales y de los valores DO_{405} reveló que hubo diferencias significativas entre la lima mexicana y el naranjo agrio infectados con CTV-2 ($p \leq 0.05$) y entre la lima mexicana infectada con CTV-2 vs. T-TX8. Las características de las inclusiones virales se correlacionaron positivamente con la severidad del aislamiento del virus, la concentración del virus y los síntomas en las plantas de lima mexicana pero no en las plantas de naranjo agrio.

Additional index words: Citrus tristeza virus (CTV)*; Mexican lime; sour orange; mild isolate; severe isolate.*

Citrus tristeza virus (CTV) causes one of the most serious disease problems of citrus worldwide. Its presence has been already detected in Texas (Skaria et al., 1995, Solis-Gracia et al., 2001). CTV has different isolates that produce variable symptoms ranging from severe stem-pitting, tree decline, and seedling yellows to no visible damage in commercial *Citrus* spp. From a disease management perspective, it is important to distinguish between severe and mild isolates as well as to select mild isolates for cross protection against severe isolates of CTV (van Vuuren et al., 1993). Biological characterization is the most reliable procedure to accurately document the severity of an isolate of CTV by indexing on to a standard host range (Garnsey et al., 1987). A monoclonal antibody, CTV- MCA13, reacts to decline-inducing, seedling yellows, and stem-pitting isolates of CTV from Florida, California, and Spain. It has the potential for rapidly identifying and screening the severe

Table 1. The color intensity of virus inclusion bodies, foliar symptoms, and virus titer compared over 11 weeks after inoculation.

CTV isolate	Host	Test	1st wk	2nd wk	3rd wk	4th wk	5th wk	6th wk	7th wk	9th* wk	10th wk	11th wk
CTV-2	Mexican	Inclusions	0[x]	0	4	4 a[z]	5 c	5 c	4 c	5 c	4 b	4 b
	lime	OD_{405}	0.38-[y]	0.60 -	1.52 +	1.72 +	1.84 +	1.58 +	1.86 +	1.89 +	1.83 +	1.82 +
	Sour	Inclusions	0	0	0	0	0	0	2	2	1	0
	orange	OD_{405}	0.35 -	0.39 -	0.33 -	0.39 -	0.40 -	0.43 -	0.42 -	1.11 +	0.64 -	0.33 -
T-TX8	Mexican	Inclusions	0	0	0	1	3	2	3 a	2 a	0 a	1 a
	lime	OD_{405}	0.32 -	0.37 -	0.62 -	1.42 +	1.30 +	0.94 +	0.75 +	0.83 +	0.36 -	0.80 +
	Sour	Inclusions	0	0	0	0	0	0	1	1	0	1
	orange	OD_{405}	0.33 -	0.33 -	0.33 -	0.33 -	0.4 -	0.37 -	1.28 +	0.36 -	0.33 -	0.34 -
Healthy	Mexican	Inclusions	0	0	0	NA**	NA	NA	NA	NA	NA	NA
	lime	OD_{405}	0.30 -	0.36 -	0.36 -	NA	NA	NA	NA	NA	NA	NA
	sour	Inclusions	0	0	0	0	0	0	0	NA	NA	NA
	orange	OD_{405}	0.31 -	0.33 -	0.33 -	0.32 -	0.34 -	0.35 -	0.34 -	NA	NA	NA

[x]The color intensity of inclusion bodies: 0 = no inclusion bodies, 1 = very light purple, 2 = light purple, 3 = purple, 4 = dark purple, 5 = very dark purple.

[y]ELISA OD_{405} values and results: + = positive, - = negative.

[z]Symptom severity: a = very light vein clearing and leaf cupping, b = moderate vein clearing and leaf cupping, c = severe vein clearing and leaf cupping.

*There were no suitable leaves for the test at the 8th week after inoculation.

**NA = Not analyzed.

isolates of CTV elsewhere (Permar et al., 1990).

CTV has been known to produce virus inclusion bodies in large aggregates which were found in phloem and other tissues of the host plants (Brlansky, 1987, 1991; Brlansky and Lee, 1990; Christie and Edwardson, 1977; Garnsey et al., 1980; Kitajima and Costa, 1968). These structures are excellent tools for tristeza disease diagnosis (Schneider, 1957; Schneider and Sasaki, 1972). Although Kitajima and Costa (1968) observed more inclusion bodies in susceptible hosts infected with a severe CTV isolate, they did not quantify them. Bar-Joseph et al. (1976) reported differences in numbers of inclusion bodies with different isolates of CTV in Israel. Brlansky and Lee (1990) showed that the inclusion bodies and the virus titer produced by mild and severe CTV isolates in seven hosts varied according to the severity of the isolates. Broadbent et al. (1996) showed that the numbers of inclusion bodies in West Indian lime in Australia were significantly higher for severe isolates and subisolates than for the mild ones.

Since inclusion bodies result from viral infection, a better understanding of their quantity and quality could reveal valuable information about the biological severity of a virus isolate in a known host. In this paper, we report the quantitative and qualitative differences of the inclusion bodies produced by a severe and a mild CTV isolate infections over a period of 11 weeks.

MATERIALS AND METHODS

Virus isolates and plant materials. CTV-2, an isolate that causes severe vein clearing and leaf cupping of Mexican lime [*Citrus aurantifolia* (Christm.) Swing.] indicator plants, and a positive reaction to monoclonal antibody 13 (CTV- MCA13) in double antibody sandwich enzyme-linked immunosorbent assay (ELISA), was considered a severe isolate. Another isolate, T-TX8 that causes only light vein clearing and light leaf cupping of Mexican lime and a negative reaction to CTV-MCA13, was regarded as a mild isolate. Two-year-old seedlings of Mexican lime and sour orange (*C. aurantium* L.) were used as test plants throughout this study.

Virus inoculation. Test plants were inoculated using bud grafting (Roistacher, 1991). Three buds from an infected Mexican lime source tree of CTV-2 or T-TX8 were grafted onto each of six Mexican lime and four sour orange seedlings. All plants, including the same numbers of un-inoculated controls, were trimmed after grafting to induce uniform new growth and maintained in a greenhouse at 27.5ºC to 32.8ºC. The temperature was recorded with a hygro-thermograph (Model 594, The Bendix Corp., Baltimore, MD).

Sampling. Each week after inoculation, the foliar symptoms of plants were recorded and classified into one of three types as severe, moderate or mild reactions. Two fully expanded leaves as described by Brlansky and Lee (1990) from two different flushes were collected weekly from each plant. Four leaves chosen at random were used as one sample from each treatment for inclusion body and ELISA assays.

Azure A staining and quantification of inclusion bodies. Free-hand cross sections were prepared weekly using a razor blade from the abscission zone of the four fresh leaf petioles. Among the prepared sections, 16 to 55 thin sections were chosen and stained in 0.1% azure A solution as described by Christie and Edwardson (1977). A compound light microscope under transmitted light was used to examine the sections. The inclusion bodies were counted at 400X magnification by focusing at the center of the section which included the xylem, phloem areas and parenchyma cells outside the phloem tissue. The color intensity of inclusion bodies was rated on a 0 to 5 scale, using 0 for no inclusion bodies, 1 for very light purple color, 2 for light purple, 3 for purple, 4 for dark purple and 5 for very dark purple. The color intensity differences were recorded photographically. The sections from healthy controls

Table 2. Number of inclusion bodies produced by CTV-2 and T-TX8 of CTV in two citrus hosts.

CTV isolate	Host	1st wk	2nd wk	3rd wk	4th wk	5th wk	6th wk	7th wk[y]	9th wk	10th wk	11th wk
CTV-2	Mexican lime	0.0	0.0	3.3	5.1	18.2	18.1	24.0	32.0	7.6	9.3
	Sour orange	0.0	0.0	0.0	0.0	0.0	0.0	0.3	2.0	0.3	0.0
T-TX8	Mexican lime	0.0	0.0	0.0	0.4	3.2	1.2	1.2	2.8	0.0	0.1
	Sour orange	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.04	0.0	0.2
Healthy	Mexican lime	0.0	0.0	0.0	NA[z]	NA	NA	NA	NA	NA	NA
	Sour orange	0.0	0.0	0.0	0.0	0.0	0.0	0.0	NA	NA	NA

[y]There were no suitable leaves for the test at the 8th week after inoculation.
[z] NA = Not analyzed.

were stained and checked until the inoculated plants showed inclusion bodies.

ELISA for monitoring CTV titer. The leftover tissues from the four petioles and the four midveins after cutting the sections as one sample were saved and kept in a freezer (-20ºC) each week. An ELISA test, as described by Bar-Joseph, et al. (1979, 1980), was done at the end of the experiment to test the virus titer of all the samples at one time. Samples were considered positive when the OD_{405} values were twice as much as the OD405 of the healthy control.

Statistical analysis. The inclusion body numbers and the ELISA values of each treatment from the 1st to the 11th weeks were tested using Duncan's multiple range test for analysis of variance between the hosts and the virus isolates.

RESULTS

Location and structure of inclusion bodies. Inclusion bodies were observed in phloem, xylem and parenchyma cells (Fig. 1, 2). At higher magnification, needle-like or fibrous paracrystal inclusion bodies were similar to that described for CTV by Christie and Edwardson, 1977 and by Garnsey et al., 1980 (Fig. 3).

The purple color intensity. CTV inclusion bodies showed a purple color with 0.1% azure A staining. The color intensity of inclusion bodies in Mexican lime produced by CTV-2 and T-TX8 ranged from 4 to 5 and 1 to 3, respectively (Table 1). The very dark inclusion bodies were seen with CTV-2 infection (Fig. 1) but not with T-TX8 infection (Fig. 2). For a given growth stage, darker purple colored inclusion bodies were seen in the plants infected with CTV-2 than with T-TX8 (Table 1).

The formation time of inclusion bodies. In Mexican lime, the inclusion bodies were seen as early as three weeks after inoculation for CTV-2 and four weeks for T-TX8 (Table 2). In sour orange, we found inclusion bodies at the seventh week after inoculation with both isolates. No apparent differences on inclusion body formation time (Table 2) were seen in sour orange with the two isolates. Inclusion bodies were not found in any healthy control plants.

The number of inclusion bodies. Out of 349 sections examined from CTV-2 infected Mexican lime plants, 251 sections had inclusion bodies. From T-TX8 infected Mexican lime plants, only 43 out of 376 sections showed inclusion bodies. The most average inclusion bodies per section were 32.0 for CTV-2 infection and 3.2 for T-TX8 infection in Mexican lime (Table 2). Statistical analysis of inclusion bodies developed weekly from the 1st to 11th weeks after inoculation showed that the number of inclusion bodies in Mexican lime was significantly higher with CTV-2 than with T-TX8 infection (AB, $p \leq 0.05$, Table 3). The inclusion bodies formed more

Table 3. Comparison of inclusion body numbers between two hosts and two isolates.

	No. of Inclusion Bodies		OD_{405}	
Host	CTV-2	T-TX8	CTV-2	T-TX8
Mexican lime	11.760[x] a[y] A[z]	0.890 a B	1.518[x] a[y] A[z]	0.776 a B
Sour orange	0.260 b A	0.054 b A	0.493 b A	0.452 a A

[x]Each mean was average number of inclusion bodies of each treatment from the 1st week to the 11th week. Means with the same letters are not significantly different at the 0.05 level according to Duncan's multiple range test.
[y] a or b: comparison between Mexican lime and sour orange with the same CTV isolate.
[z] A or B: comparison between CTV-2 and T-TX8 with the same CTV host.

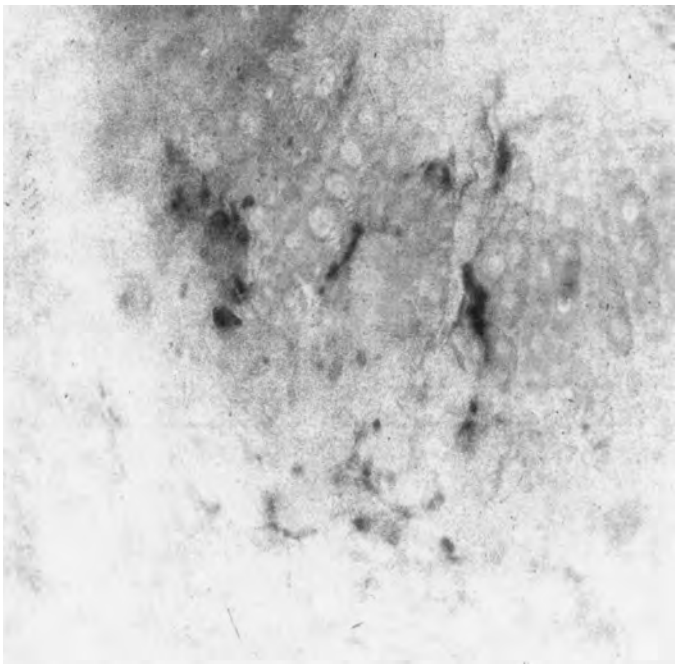

Fig. 1. The inclusion bodies in a petiole cross section from the Mexican lime plant infected by CTV-2 (approximately 100X).

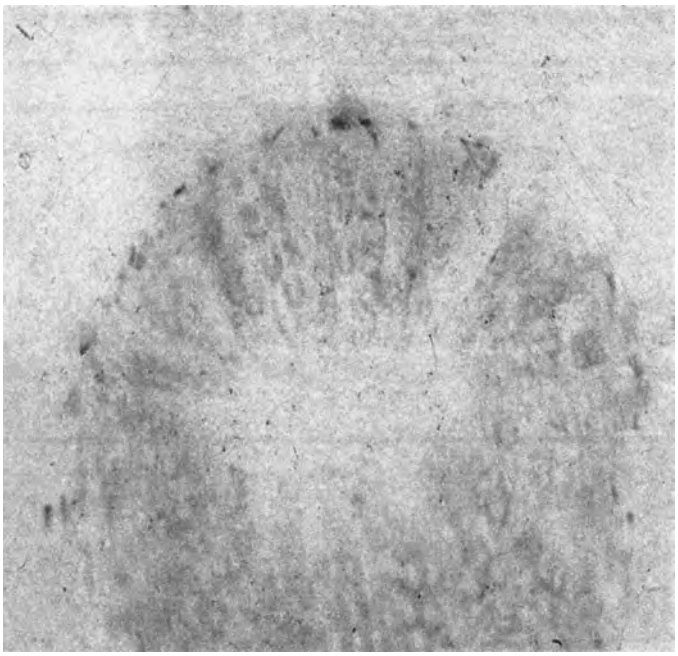

Fig. 2. Inclusion bodies in a petiole cross section from the Mexican lime plant infected by T-TX8 (approximately 33X).

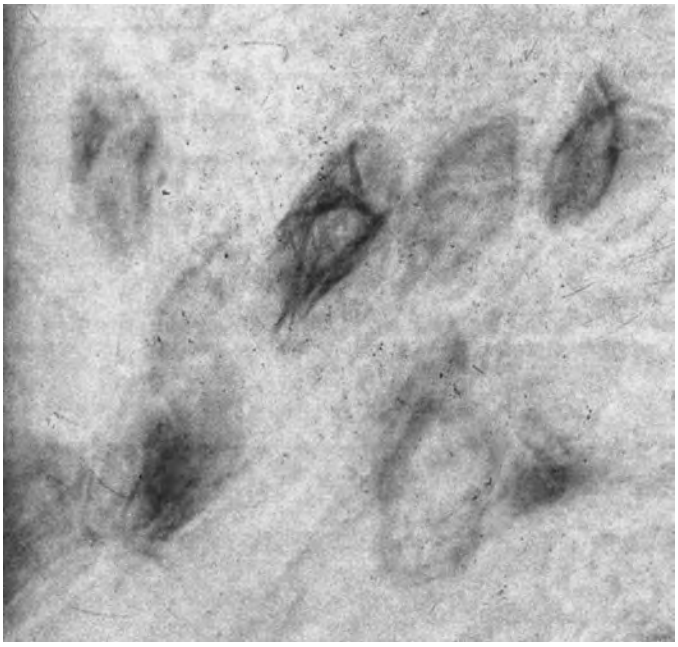

Fig. 3. Fibrous inclusion bodies in a petiole cross section from the Mexican lime plant infected by CTV-2 (approximately 250X).

consistently in Mexican lime than in sour orange for CTV-2 infection (ab, $p \leq 0.05$, Table 3). In sour orange, there was no statistical difference between the numbers of inclusion bodies caused by the two isolates (AA, $p \geq 0.05$, Table 3).

The virus titer. Statistical analysis of ELISA OD_{405} values showed that the virus titer was significantly higher in Mexican lime than in sour orange for CTV-2 infection (ab, $p \leq 0.05$, Table 3), and in Mexican lime infected with CTV-2 than T-TX8 (AB, $p \leq 0.05$, Table 3). There were no significant differences in virus titer in sour orange between the two isolates (AA, $p \geq 0.05$, Table 3) or between Mexican lime and sour orange inoculated with T-TX8 (aa, $p \geq 0.05$, Table 3).

Comparison of azure A staining procedure, ELISA and biological detection of CTV. For CTV-2 infection in Mexican lime, positive results were achieved uniformly from all three comparison procedures. For T-TX8 infection in Mexican lime, the inclusion bodies and the positive ELISA results were achieved three weeks prior to the symptom appearance. In sour orange plants infected with CTV-2 or T-TX8, there were no visible symptoms during the experimental time (Table 1). In Mexican lime, for both isolates at the first time that the inclusion bodies were found, the ELISA tests were positive. However, the purple color intensity and the number of inclusion bodies were different between CTV-2 and T-TX8 (Table 1) .

DISCUSSION

A study of the quantitative and qualitative differences of inclusion bodies in differential hosts over a period of time, performed with a light microscope, has several advantages it is rapid, reliable, simple, inexpensive, and it can be performed in any laboratory that has a microscope.

Selection of an indexing procedure should be based on specific needs and the availability of appropriate materials, equipments, and skills. Serological detection of CTV using ELISA is simple, fast, inexpensive, and effective, especially for a large scale survey (Bar-Joseph, et al., 1979; Solis-Gracia et al., 2001). However no serology procedure provides information on the physical conditions of the virus particle or its distribution, aggregation in the hosts, and visible biological reactions. Staining with azure A clearly shows the virus inclusion bodies in aggregation. Therefore, we suggest carrying out inclusion body assay after a large-scale survey of CTV using ELISA to further understand the information on the biology of CTV isolates.

The results from our experiment clearly showed the difference of the inclusion body characteristics, which not only confirmed the previous reports on inclusion body number and virus titer (Brlansky and Lee, 1990), but also revealed some new findings on the purple color intensity and the formation time of inclusion bodies. All these features were correlated with the isolate severity and host type.

The inclusion body features described here were based on the results with only two isolates. It would be more desirable to test more isolates from different CTV sources. It would also be very useful and practical if the severity of a CTV isolate could be distinguished from the field samples by comparing inclusion body features. Garnsey et al. (1980) reported results of inclusion body assays from field samples of Valencia orange, Marsh and Duncan grapefruits.

In our experiment, inclusion bodies and positive ELISA results were not consistent in sour orange infected with CTV-2 or T-TX8; however the replication experiment carried out in an insectary showed consistent results. The temperature in the greenhouse ranged from an average of 27.5ºC to 32.8ºC; in the

insectary it ranged from 24.5ºC to 28.7ºC. This suggests that when collecting samples from the field for CTV surveys, especially for the detection of mild isolates from commercial cultivars, the cool season may be more appropriate. In our experiment, mild isolate infection seemed more sensitive to unfavorable conditions.

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LITERATURE CITED

Bar-Joseph, M., G. Loebenstein, and J. Cohen. 1976. Comparison of particle characteristics and cytopathology of *Citrus tristeza virus* with other morphologically similar viruses. p. 39-46. *In*: Proc. Conf. Int. Organ. Citrus Virol. 7th. E. C. Calavan, ed. IOCV, Riverside.

Bar-Joseph, M., S.M. Garnsey, D. Gonsalves, M. Moscovitz, D.E. Purcifull, M.F. Clark, and G. Loebenstein. 1979. The use of enzyme-linked immunosorbent assay for detection of *Citrus tristeza virus*. Phytopathology 69:190-194.

Bar-Joseph, M., S.M. Garnsey, D. Gonsalves, and D.E. Purcifull. 1980. Detection of *Citrus tristeza virus*. I. Enzyme-linked immunosorbent assay (ELISA) and SDS-immunodiffusion methods. p. 1-8. *In*: Proc. Conf. Int. Organ. Citrus Virol. 8th. E. C. Calavan, S. M. Garnsey, and L.W. Timmer, eds. University of California Press, Riverside.

Brlansky, R.H. 1987. Inclusion bodies produced in *Citrus* spp. by *Citrus tristeza virus*. Phytophylactica 19:211-213.

Brlansky, R.H., and R.F. Lee. 1990. Numbers of inclusion bodies produced by mild and severe strains of *Citrus tristeza virus* in seven citrus hosts. Plant Dis.74:297-299.

Brlansky, R.H. 1991. Detection of *Citrus tristeza virus* inclusion bodies using azure A staining and *in situ* immunofluorescence. p. 261-264. *In*: Graft-transmissible diseases of citrus. Handbook for detection and diagnosis. C. N. Roistacher, ed. Food and Agriculture Organization of the United Nations, Rome.

Broadbent, P., R.H. Brlansky and J. Indsto. 1996. Biological characterization of Australian isolates of *Citrus tristeza virus* and separation of subisolates by single aphid transmissions. Plant Dis. 80:329-333.

Christie, R.G., and J.R. Edwardson. 1977. Light and electron microscopy of plant virus inclusions. Fla. Agric. Exp. Stn. Monogr. Ser. 1-10, p. 134-135.

Garnsey, S.M., R.G. Christie, K.S. Derrick, and M. Bar-Joseph. 1980. Detection of *Citrus tristeza virus*. II. Light and electron microscopy of inclusions and viral particles. p. 9-16. *In*: Proc. Conf. Int. Organ. Citrus Virol. 8th. E. C. Calavan, S. M. Garnsey, and L. W. Timmer, eds. University of California Press, Riverside.

Garnsey, S.M., D.J. Gumpf, C.N. Roistacher, F.L. Civerolo, R.F. Lee, and M. Bar-Joseph. 1987. Toward a standardized evaluation of the biological properties of *Citrus tristeza virus*. Phytophylactica 19:145-149.

Kitajima, E.W., and A.S. Costa. 1968. Electron microscopy of the tristeza virus in citrus leaf tissues. p. 59-64. *In*: Proc. Conf. Int. Organ. Citrus Virol. 4th. J. F. L. ed. University of Florida Press, Gainesville.

Permar, T.A., S.M. Garnsey, D.J. Gumpf, and R.F. Lee. 1990. A monoclonal antibody that discriminates strains of citrus tristeza virus. Phytopathology 80:224-228.

Roistacher, C.N. 1991. Graft-transmissible diseases of citrus. Handbook for detection and diagnosis. 169-173, 187p. Food and Agriculture Organization of the United Nations, Rome.

Schneider, H. 1957. The anatomy of tristeza-virus-infected citrus. p. 73-84. In: Proc. of the Conf. on Citrus Virus Diseases. J. M. Wallace, ed. University of California Press, Berkeley.

Schneider, H. and P.J. Sasaki. 1972. Ultrastructural studies of chromatic cells in tristeza-diseased lime. p. 222-228. *In*: Proc. Conf. Int. Organ. Citrus Virol. 5th. W. C. Price, ed. University of Florida Press, Gainesville.

Skaria, M., H. Miao, and N. Solis-Gracia. 1995. Use of virus inclusion bodies for preliminary detection of severe types of *Citrus tristeza virus*. Phytopathology 85: 383. (Abstract)

Solis-Gracia, N., C.J. Kahlke, C.M. Herron, J.V. da Graca, K.L. Essau, H.Q. Miao, and M. Skaria. 2001. Survey for *Citrus tristeza virus* in Texas 1991-2000. Subtropical Plant Sci. 53:4-8.

van Vuuren, S.P., R.P. Collins, and J.V. da Graca. 1993. Evaluation of *Citrus tristeza virus* isolates for cross protection of grapefruit in South Africa. Plant Dis. 77:24-28.