Distribution of Citrus Tristeza Virus in Grapefruit and Sweet Orange in Florida and South Africa*, **

R. F. Lee, S. M. Garnsey, L. J. Marais, J. N. Moll, and C. O. Youtsey

ABSTRACT. The distribution of citrus tristeza virus (CTV) in field trees was determined by enzyme-linked immunosorbent assay on individual flushes collected from citrus trees in Florida and South Africa. In Florida, most CTV isolates were unevenly distributed in grapefruit trees, especially in late summer. However, one mild isolate with some cross-protecting ability (T26) was consistently distributed throughout the trees regardless of season. Citrus tristeza virus was more evenly distributed in sweet orange trees in Florida, but occasionally very young flush tissue was found CTV-free. In South Africa, CTV was found evenly distributed throughout both grapefruit and sweet orange trees. South African isolates were highly invasive in recently inoculated plants. The even distribution of CTV within trees is a trait which may be important for effective cross protection by mild CTV strains.

Index words. ELISA, cross protection, stem pitting.

Citrus tristeza virus (CTV), a member of the closterovirus group, is the most economically important citrus virus worldwide (1). There are many strains of CTV which differ in their biological activity (1, 3). Quick decline of trees on sour orange rootstock induced by CTV has killed millions of trees worldwide, but this aspect of tristeza can be controlled by the use of CTV-tolerant rootstocks. Strains of CTV which cause severe stem pitting on scions cause losses even in trees on CTV-tolerant rootstocks. For these severe stem pitting strains of CTV, the use of mild strains for cross protection is probably the most effective control strategy (5, 7). With mild strain cross protection, a plant previously infected with a mild virus strain will show interference or delay in the expression of severe symptoms after being challenge-inoculated with a severe strain of the same virus. Severe stem-pitting strains of CTV have been controlled successfully by use of mild strain cross protection in Brazil (5) and in South Africa (7).

While mild CTV strains are common, few of these strains are capable of effective cross protection. Selection of cross-protecting CTV strains has thus far been an empirical process. The distribution of the mild strain of virus within the plant host may play an important role in its ability to cross protect the host against subsequent challenge by severe isolates (9).

We used the enzyme-linked immunosorbent assay (ELISA) to study the distribution of CTV within grapefruit and sweet orange trees in Florida where severe stem-pitting strains of CTV are not yet present and in trees in South Africa where severe stem pitting strains are endemic. Results are presented in this paper and discussed in relation to cross protection success.

METHODS AND MATERIALS

Virus isolates. For some experiments, Marsh grapefruit trees on sour orange rootstock were inoculated with the previously characterized CTV isolates T3, T4, or T26 (11). These trees were in an experimental field plot in central Florida. Isolate T3 causes decline of sweet orange on sour orange rootstock and causes seedling yellows (SY). Isolate

*Florida Agricultural Experiment Station Journal Series No. 7952.

**Mention of a trademark, warranty, proprietary product, or vendor does not constitute a guarantee by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

T4 causes strong stunting, stem pitting and vein clearing in Mexican lime, but no decline of sweet orange on sour orange and no SY. Isolates T26, T32, and T55 produce mild symptoms and little stunting on Mexican lime, and cause no decline of sweet orange on sour orange, or SY. Isolate Ti33 is an uncharacterized CTV field strain which produces moderate vein clearing and stunting on Mexican lime.

The Nartia mild, Bolton severe, and Nkwalini mild CTV isolates from South African have been previously described (6, 10). The CSFRI CTV strains from South Africa were naturally-occurring field selections which did not cause decline of sweet orange on sour orange rootstock. Isolate GFSS-1 is a severe strain of CTV which severely stunts grapefruit, and GFMS-10 produces moderate stem pitting and mild stunting on grapefruit (12).

Sample collection and enzyme linked immunosorbent assay (ELISA). Samples of young flush which had the basal leaves at or near full expansion were collected from field trees and stored in plastic bags at 4 C until processed for ELISA, usually within 1-7 days. A 0.25-gm aliquot of chopped bark tissue was homogenized in 5.0 ml of 0.05 M Tris-HCl buffer, pH 8.0. The double antibody sandwich ELISA procedure (2) was used with antisera prepared against unfixed CTV (4). These antisera have reacted to all CTV isolates tested. Healthy tissue gave OD_{405} readings of 0.02-0.03, and values twice this were considered as the threshold for a CTV-positive reaction. Most OD values were 0.4 or greater; only rarely were OD values less than 0.4 in the samples declared CTV positive.

Tissue sources—Florida. Thirty individual branches were labeled on Marsh grapefruit trees systemically infected with CTV isolates T3, T4, T26, and Ti33. Samples of new flush tissue were collected from the same individual branches in July, September, and February. Similar repetitive assays were made from four sweet orange trees. Some trees infected with naturally-occurring CTV strains were sampled only one time by collecting the indicated number of flushes from throughout the tree canopy. Root sprouts were forced by exposing and cutting some of the roots in an 80-year-old grove of Marsh grapefruit on rough lemon rootstock located in central Florida. About 8 weeks later, new flush tissue was collected from the forced root sprouts and also from new flushes on the grapefruit scions.

Tissue sources—South Africa. Twenty-five flushes were collected from individual trees from 11-year-old Nartia grapefruit in the Nkwalini Valley, Natal Province, and from 10-year-old Rose grapefruit propagated from the Bolton budwood source. Samples were also collected from 5-year-old seedling Marsh grapefruit trees on an experimental plot near Malelane, Transvaal Province. Twenty-five samples were collected from each of five trees infected with three different CTV isolates in the latter location.

A greenhouse experiment was set up in South Africa to determine the uniformity of CTV infection within the inoculated plants 4 weeks after graft inoculation. Two large Marsh grapefruit seedlings and two Mexican lime plants were inoculated using leaf pieces infected with Florida isolates T55 and T32 and South African isolates Nartia, GFMS-10 and GFSS-1. After 4 weeks, the young growth from four different branches of each plant was collected for assay.

RESULTS

Distribution of CTV within grapefruit trees in Florida. In a preliminary test, 30 flushes were collected from each of two 12-year-old Marsh grapefruit trees on rough lemon rootstock during the spring and were assayed by ELISA. Only eight of the flushes from one tree and 12

from the other reacted positively for CTV. In a test of the scions and the rootsprouts from an 80-year-old grove of Marsh grapefruit on rough lemon rootstock, CTV was present in both the scion and rootsprouts of 21 trees, in only the scion of seven trees, and in only the rootsprout of six trees. It was not detected in either scion or rootstock of nine trees.

The apparent erratic distribution of CTV within grapefruit trees was examined more closely by individually labeling 30 separate branches on four 6-year-old Marsh grapefruit trees, each inoculated with a different CTV strain. Young flush tissue was collected for ELISA from each tagged branch in summer (July), late summer (September), and during spring flush (February) (table 1). Isolate T26 was well distributed throughout the tree with 90% of the branches positive on all three sampling dates. Isolate Ti33 was very unevenly distributed throughout the tree. Only 53% of the branches indexed CTV positive in July, while 37% were positive in September, and 83% were positive in February. Only 13% of the tagged branches were CTV positive on all three sampling dates, whereas 10% of the branches were CTV negative on all three sampling dates. Isolates T4 and T3 were intermediate in their distribution.

Distribution of CTV in sweet orange trees in Florida. Samples were collected from 30 separate new flushes on four naturally CTV-infected Hamlin sweet orange budwood source trees on rough lemon rootstock. Branches were tagged and samples collected in late spring (April), in summer (July), and fall (September) (table 2). Citrus tristeza virus was well distributed within these trees on all sampling dates. From 90 to 100% of the individual branches assayed positively for CTV infection on all three sampling dates. In a separate test, 30 flushes from two 12-year-old Valencia trees on rough lemon rootstock were sampled in June. All 30 flushes from both trees tested positively for CTV infection.

In another study, 22 Pineapple sweet orange trees on sour orange rootstock were sampled at three intervals. The first collection was January 1985, after a severe freeze had defoliated the trees and the new flush was about 2-4 cm long. Only eight of the 22 trees were found CTV positive using a sampling of 25 flushes from each tree. Subsequent collections of five flushes from each tree were made in April 1985 and August 1986, and all flushes from all trees tested positively for CTV infection.

Distribution of CTV in grapefruit and sweet orange in South Africa. Several grapefruit and sweet orange trees were tested for the presence of CTV by collecting 25 flushes from around the canopy of each tree. A list of the trees tested and the severity of the CTV symptoms is given in table 3. All individual flushes from all trees tested in South Africa assayed positively for CTV.

The movement of Florida and South African CTV isolates into freshly inoculated plants was determined by testing the new flush tissue from plants 4 weeks after graft inoculation. All South African isolates were detected in all flushes from grapefruit and Mexican lime seedlings. The Florida isolates T55 and T32 were present in all Mexican lime flushes 4 weeks after inoculation. However, isolate T55 was detected in only three of four flushes from each grapefruit plant, and T32 was detected in two of four and one of four flushes, respectively, from each of two grapefruit plants tested.

DISCUSSION

The distribution of the mild strain within the plant is important when considering mild strain cross protection as a control strategy for CTV (9). The virus must distribute itself in all parts of the plant and have the ability to rapidly invade new growth flushes. Any part of the plant which is virus-free, even temporarily, provides an opportunity for an aphid to infect the plant with a severe CTV strain which,

TABLE 1
DISTRIBUTION OF DIFFERENT CITRUS TRISTEZA VIRUS (CTV) ISOLATES IN GRAPEFRUIT TREES DURING DIFFERENT TIMES OF THE YEAR IN FLORIDA

CTV isolate	Flushes infected with CTV			Percent of branches[y]	
	July	Sep.	Feb.	Always positive	Always negative
Ti33	16/30[z]	11/30	25/30	13	10
T3	20/30	18/30	27/30	43	3
T4	23/30	25/30	30/30	60	0
T26	30/30	27/30	30/30	90	0

[z]Number of flushes infected with CTV/total number of flushes samples. Presence of CTV in tissue was determined by ELISA.
[y]New flush was collected at each period from the same labeled branch.

given the advantage of starting in virus-free tissue, could ultimately result in the breakdown of cross protection.

Most CTV strains tested from Florida were unevenly distributed within grapefruit trees (table 1), but well distributed within sweet orange trees (table 2). While most Florida CTV strains were unevenly distributed within grapefruit, isolate T26 was present in 90% of the same branches on three different sampling dates which included summer and winter conditions in Florida (table 1). Isolate T26 appears to have cross-protecting potential in Mexican lime plants in field tests in Hawaii, where severe stem-pitting strains of CTV and the efficient CTV aphid vector, *Toxoptera citricida* Kirk are present (Garnsey & Yokomi, unpublished data).

TABLE 2
DISTRIBUTION OF CITRUS TRISTEZA VIRUS (CTV) WITHIN SWEET ORANGE TREES IN FLORIDA DURING DIFFERENT TIMES OF THE YEAR

Tree code	Flushes infected with CTV		
	April	July	Sep.
BA7	30/30[z]	30/30	29/30
BA9	29/30	30/30	29/30
BA12	30/30	30/30	30/30
BA25	30/30	29/30	27/30

[z]Number of flushes infected with CTV/total number of flushes sampled. Presence of CTV in tissue was determined by ELISA. Flushes were collected from the same labeled branches at each period.

The severity and titer of CTV is influenced by temperature. More severe symptoms are produced in cooler temperatures, and extremely hot growing conditions reduces CTV titer and can result in temporary thermotherapy (8, Lee, unpublished data). This is consistent with our finding that the distribution of CTV within grapefruit trees was poorest in September (table 1). At this sampling date, the mean daily temperature had been 30 C for 3-4 months and the overnight low has not been below 21 C for the same period.

Finding flush from 14 of 22 Pineapple sweet orange trees in Florida CTV negative just after defoliation by a freeze was unexpected. Although these trees had not been sampled prior to the freeze, all were presumed CTV infected and this was confirmed by positive tests for all trees in later sampling. While severe freezes have been rare in Florida, there may be a correlation between defoliation of field trees and periodic epidemics of CTV-induced decline 1-2 years later. More research needs to be done on virus distribution within recently defoliated trees.

The erratic distribution of CTV within grapefruit trees in Florida is in contrast to the uniform distribution of CTV in grapefruit observed in South Africa. This uniform distribution of South African CTV isolates was also apparent in the greenhouse tests where Mexican lime and grapefruit seedlings, inoculated with differ-

TABLE 3 DISTRIBUTION OF CITRUS TRISTEZA VIRUS (CTV) WITHIN GRAPEFRUIT AND SWEET ORANGE IN SOUTH AFRICA

Tree code		Isolate[y] severity	CTV infection
Nartia grapefruit			
Bedlane	M8	Mild	25/25[z]
	M1	Mild	25/25
	S3	Severe	25/25
Rose grapefruit			
Bolton	R4T7	Severe	25/25
	R6T9	Severe	25/25
	R7T6	Mild	25/25
Marsh grapefruit sdlgs.			
Nartia	1	Mild	25/25
	2	Mild	25/25
	3	Mild	25/25
	4	Mild	25/25
	5	Mild	25/25
Nkwalini	1	Mild	25/25
	2	Mild	25/25
	3	Mild	25/25
	4	Mild	25/25
	5	Mild	25/25
Bolton	1	Severe	25/25
	2	Severe	25/25
	3	Severe	25/25
	6	Severe	25/25
	7	Severe	25/25
Valencia/sour orange			
CSFRI	R8T14	Mild	30/30
	R7T12	Mild	30/30
	R4T8	Mild	30/30

[z]Number of flushes infected with CTV/total number of flushes tested. Presence of CTV was determined by ELISA.
[y]Isolate severity as determined by stem pitting induced on grapefruit.

ent CTV isolates, assayed uniformly for the presence of CTV in flushes 4 weeks after inoculation. These results also indicated that differences in field assays between Florida and South Africa were not solely due to environment. This inherent rapid and uniform distribution of South African CTV isolates may reflect natural selection over many years for highly invasive isolates. In this area, severe CTV isolates are endemic, efficient aphid vectors are present, and trees are naturally challenged repeatedly by different sources. The erratic distribution of CTV isolates from Florida, particularly in grapefruit trees, suggests that problems may be encountered, if at some future date, the citrus industry in Florida has to rely on these existing mild strains for protection against severe stem-pitting strains of CTV such as those present in South Africa. Further selection of protecting strains for Florida conditions is indicated.

All of the characteristics of CTV isolates associated with a good cross protecting ability are still not known. A mild selection from an invasive isolate or from a mild isolate obtained from a mutagenized invasive isolate may be good cross protecting candidates. While thorough distribution within the protected plant seems important, it may not be the only consideration. For example, CTV is well distributed in sweet orange in Florida, yet decline apparently occurs readily when plants infected with many mild isolates are challenged by severe decline inducing isolates.

ACKNOWLEDGMENTS

The technical assistance of H. Lasater and M. Washington is gratefully acknowledged. This research was supported in part by Grant No. US-874-84 from United States-Israel Binational Agricultural Research and Development Fund.

LITERATURE CITED

1. Bar-Joseph, M., S. M. Garnsey, and D. Gonsalves
1979. The closteroviruses: a distinct group of elongated plant viruses. Adv. Virus Res. 25: 93-168.
2. Bar-Joseph, M., S. M. Garnsey, D. Gonsalves, M. Moscovitz, D. E. Purcifull, M. F. Clark and G. Loebenstein
1979.The use of eyzyme-linked immunosorbent assay for detection of citrus tristeza virus. Phytopathology 69: 190-194.

3. Bar-Joseph, M., C. N. Roistacher, S. M. Garnsey, and D. J. Gumpf
1981. A review on tristeza, an ongoing threat to citriculture. 1981 Proc. Int. Soc. Citriculture 1: 419-423.
4. Brlansky, R. H., S. M. Garnsey, R. F. Lee, and D. E. Purcifull
1984. Application of citrus tristeza virus antisera in labeled antibody, immuno-electron microscopical, and sodium dodecyl sulfate-immunodiffusion tests. Pages 337-342. *In* Proc. 9th Conf. IOCV. IOCV, Riverside, CA.
5. Costa, A. S., and G. W. Muller
1980. Tristeza control by cross protection: a U.S.-Brazil cooperative success.Plant Dis. 64: 538-541.
6. da Graca, J. V., L. J. Marais, and L. A. von Broembsen
1984. Severe tristeza stem pitting decline of young grapefruit in South Africa. Pages 62-64. *In* Proc. 9th Conf. IOCV. IOCV, Riverside, CA.
7. de Lange, J. H., S. P. van Vuuren, and G. S. Bredell
1981. Groei-punt enting suiver sitrusklone vir die superplantskema van virusse. Subtropica 2(5): 11-16.
8. 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. Pages 9-16. *In* Proc. 8th Conf. IOCV. IOCV, Riverside, CA.
9. Lee, R. F., R. H. Brlansky, S. M. Garnsey, and R. K. Yokomi
1987. Traits of citrus tristeza virus important for mild strain cross protection of citrus: the Florida approach. Phytophylactica 19: 215-218.
10. Marais, L. J., R. F. Lee, J. V. da Graca, and J. M. Kotze
1988. Environmental effect on Marsh grapefruit (*Citrus paradisi* Macf.) infected with different isolates of citrus tristeza virus. Phytophylactica 19: in press.
11. Rosner, A., R. F. Lee, and M. Bar-Joseph
1986. Differential hybridization with cloned cDNA sequences for detecting a specific isolate of citrus tristeza virus. Phytopathology 76: 820-824.
12. van Vuuren, S. P., and J. N. Moll
1987. Glasshouse evaluation of citrus tristeza virus isolates. Phytophylactica 19: 219-221.