Surveys for *Citrus tristeza virus* in Texas 1991-2000

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ABSTRACT

Surveys for *Citrus tristeza virus* (CTV) were conducted throughout the citrus growing areas of Texas over the period of 1991-2000, during which over 11,000 trees were sampled. Midvein and petiole samples were collected from commercial orchards, nurseries, mother trees, backyard citrus, and TAMUK-Citrus Center orchards in the Lower Rio Grande Valley (LRGV), and from trees across south Texas from Carrizo Springs to Corpus Christi, Dallas, and the East Texas counties of Harris, Orange, Hardin, Newton, and Jefferson. Our survey showed a 1.1% incidence of CTV in the LRGV where infection was detected mostly in commercial orchard and nursery trees. No economic losses due to CTV have been reported and no CTV-induced symptoms have been observed in the field during the survey period. An 18% CTV incidence was found in East Texas. Four isolates from East Texas, as well as three other isolates collected from Meyer lemon in the LGRV in the 1970s, reacted with monoclonal antibody 13, an antibody raised against a severe decline isolate in Florida.

RESUMEN

Se realizaron muestreos para detectar al virus de la tristeza de los cítricos (VTC) en áreas productoras de este árbol frutal en Texas durante el período de 1991-2000. Alrededor de 11,000 árboles fueron muestreados durante el estudio. Muestras consistentes en pecíolos y nervaduras centrales fueron colectadas de árboles en huertas, viveros, bloques de plantas donadoras de yemas, patios particulares y en las huertas del Centro de Cítricos-Universidad Texas A&M-Kingsville localizados en el Bajo Valle del Río Grande asi como de árboles en otras áreas del estado como Carrizo Springs, Corpus Christi, Dallas y los condados de Harris, Orange, Hardin, Newton, y Jefferson en la región este de Texas. Se encontró una incidencia de 1.1% de VTC en el Bajo Valle del Río Grande encontrándose los mayores niveles de infección en árboles de huertas y viveros comerciales. No se observaron síntomas de la enfermedad o pérdidas económicas causadas por la infección con el VTC en árboles en campo durante el tiempo que duró el muestreo. Se encontró una incidencia de VTC de 18% en el área del este de Texas. Cuatro aislamientos colectados en el este de Texas, asi como otros 3 aislamientos colectados durante los años 70's en limón Meyer en el Bajo Valle del Río Grande reaccionaron con el anticuerpo monoclonal 13, un anticuerpo producido en contra de un aislamiento severo colectado en Florida que causa declinamiento.

Commercial citrus production in Texas is confined mainly to the Lower Rio Grande Valley (LRGV) where mostly grapefruit (*C. paradisi* Macf.) and sweet orange [*C. sinensis* (L) Osbeck] on sour orange (*C. aurantium* L.) rootstock are grown. Cold tolerant varieties on trifoliate rootstocks are grown in small orchards and backyards in other areas of the state, mostly along the Texas Gulf Coast. Tristeza, caused by *Citrus tristeza virus* (CTV), is one of the most devastating citrus diseases, characterized by quick decline of trees on sour orange, and stem pitting of several species such as grapefruit, Mexican lime [*Citrus aurantifolia* (Christm.)Swing.] and sweet orange (Bar-Joseph et al., 1989; Rocha-Peña et al., 1995). CTV is very efficiently transmitted by the brown citrus aphid (BrCA), *Toxoptera citricida* Kirkaldy. BrCA has not yet been reported in Texas, but has been moving northward from South America and is now in Florida (Hardy, 1995), and Mexico (Michaud and Alvarez, 2000). In view of the expected arrival of the BrCA, the Texas citrus industry is at risk of experiencing a tristeza epidemic due to the almost exclusive use of sour orange rootstock.

The presence of CTV in Texas was first reported in 1954 by Olson and Sleeth who observed typical CTV leaf flecking symptoms in Mexican lime seedlings bud inoculated with Meyer lemon (*C. limon* hybrid) tissue from trees growing in the LRGV and near Carrizo Springs. A number of CTV-infected satsumas [*C. unshiu* (Macf.) Marc.] was also found in Winter Haven (Olson and McDonald, 1954). In further studies, 14 infected trees of eight varieties introduced in the 1940's mainly from Japan, India, Australia, and New Zealand were found growing in a variety collection in Weslaco, TX. Similarly, in the LRGV, two Valencia sweet orange trees were found to be infected out of 250 Valencias and grapefruit tested; one of these trees had thin foliage, die back and honeycombing of the sour orange bark below the bud union. In addition, an infected calamondin (*C. mitis* Blanco), and a Mexican lime tree adjacent to infected Meyer lemon trees were found (Olson, 1955). A severe strain of CTV was found at Carrizo Springs in a Sueoka satsuma tree

which originated from Japan (Olson, 1956). CTV was also found in Meyer lemon, satsuma and grapefruit in the upper Gulf Coast area (Malouf, 1959). Davis et al. (1984) were unable to detect CTV in commercial citrus using ELISA, but did find infected dooryard Meyer lemon and satsuma, and three varieties (navel, tangelo and calamondin) in the collection in Weslaco, as well as nine out of 101 satsuma nursery samples from Port Neches.

CTV presence has therefore been well documented in Texas for many decades. This study was conducted to determine the current extent of CTV infections throughout the LRGV and other citrus growing areas in the state. In this paper we present the results of several surveys conducted over the period 1991-2000, during which over 11,000 trees were sampled. Some preliminary results of the survey have been reported earlier (Skaria 1993, 1996; Skaria et al. 1993, 1996, 1997).

MATERIALS AND METHODS

Sample collection. Samples were collected from commercial orchards, nurseries, mother trees, dooryard citrus, and from TAMUK-Citrus Center orchards in the LRGV. Sampling was also done across south Texas from Carrizo Springs and Catarina in the west, through San Antonio, to Victoria and Corpus Christi in the east, Dallas, and the East Texas counties of Harris, Orange, Hardin, Newton, and Jefferson. Samples were collected at various times of the year since a study at the Citrus Center showed that CTV was detectable in leaf samples collected monthly over a 12-mo period (Solís-Gracia and Skaria, unpublished data). Individual samples consisted of 4 - 6 young twigs with leaves, or in a few cases fruit peduncles, collected from different quadrants of the trees. One leaf was selected from each twig, and the tissue used for testing consisted of the midvein and the petiole which were cut from the rest of the leaf. Samples were either processed immediately or stored at -20º C until testing.

ELISA. CTV detection was performed by double antibody sandwich (DAS) or indirect double antibody sandwich (DAS-I) ELISA (Bar-Joseph et al. 1979; Clark and Adams, 1977; Garnsey and Cambra, 1991). The antisera were provided by Dr. R. F. Lee (University of Florida, Citrus Research and Education Center, Lake Alfred, FL) and Dr. S.M. Garnsey (USDA, Agricultural Research Service, Orlando, FL). The samples were ground using mortar and pestle, a roller sap extractor (Maxi-Torq, Dayton Electric Co., Chicago, IL), leaf roller grinder (Ceres 2000 Inc., Winterhaven, FL) or a tissue pulverizer (Model 8200, KLECO, Visalia, CA). The tissue pulverizer was the most efficient method of extraction and consequently most of the samples were extracted using this method. Four to six midveins or peduncles (one from each twig) were ground adding 5 ml of PBS-Tween buffer (pH 7.4) +2% polyvinylpyrrolidone (PVP-40, Sigma Chemical Co., St. Louis, MO). Routinely, polystyrene flat bottom 96 well Immulon 4 microtiter plates (DYNEX Technologies, Inc. Chantilly, VA) were coated with 110 µl/well of 1 µg/ml purified IgG in carbonate buffer (pH 9.6) and incubated at either 37ºC for 4 h or overnight at 4ºC. One hundred microliters of sap were added per well in duplicate. Plates with antigen were incubated either overnight at 4ºC or 4 h at 37ºC. After incubation, 100 µl/well of enzyme conjugate antibody (DAS-ELISA) or secondary antibody (DAS-I ELISA) dissolved in conjugate buffer (PBS-Tween +2% PVP + 0.2 % ovalbumin) were added and plates were incubated as previously described. An additional step of adding 100 µl/well of enzyme conjugate anti-goat antibody (Sigma Chemical Co., St. Louis, MO) at a 1:30,000 dilution was included when performing DAS-I ELISA and plates were incubated as described above. The reaction with 1 mg/ml of p-nitrophenyl phosphate (Sigma) in 10% triethanolamine (pH 9.8) was measured at 405 nm (OD_{405}) using a microplate reader (EIA Reader Model EL307, BIO-TEK Instruments, Inc. Winooski, VT 05404). Three washings with PBS-Tween were performed after incubation between all the ELISA steps. An OD reading of twice the negative control was recorded as positive. For controls, leaf samples from a healthy (negative) and an infected (positive) field tree were included in each assay.

Several Texas CTV isolates were tested for reaction against monoclonal antibody 13 (MCA-13) which is known to react against severe CTV isolates (Permar et al., 1990). The MCA-13 reaction was tested by a direct tissue blot immunoassay (Garnsey et al. 1993). Both ends of known CTV positive twigs were cut with a sharp razor blade and the freshly cut surfaces were then blotted onto nitrocellulose membranes. Membranes were then mailed to Nokomis Corp. (Altamonte Springs, FL) for analysis.

RESULTS

Citrus Center Variety Block and Orchards. Only eight out of the 1163 trees surveyed at the Citrus Center tested positive by ELISA during the period 1991- 2000 (Table 1). Six of the CTVpositive samples were from the variety block and included a Cytrangeuma-hybrid seedling, a nucellar Pineapple sweet orange, two Thornton tangelos, and two Bell tangerines (*C. reticulata* Blanco). The other positive samples were collected in the nursery and were two young Bearss lime (*C. lattfolia* Tan.) trees produced using budwood from a local nursery and intended to be part of the variety collection at the Citrus Center. No samples from the grapefruit or orange orchards tested positive.

LRGV commercial orchards and nurseries. From 1991 to 1993, 1,912 samples of commercial varieties were collected from orange and grapefruit orchards, mother trees, and nurseries

Table 1. Results of surveys of citrus for *Citrus tristeza virus* (CTV) by ELISA in the Lower Grande Valley of Texas 1991-2000.

Sample Site	Years	Total tested	CTV positives
Citrus Center	1991-1997	1,163	8
Commercial citrus	1991-1993	1,912	75
orchards and	1994-1996	1936	15
nurseries	1999-2000	641	0
Dooryard citrus	1994-1996	3,417	8
	2000	583	0
Commercial and			
Dooryard combined	1997	624	11
TOTAL	1991-2000	10,276	117

in the LRGV (Table 1). Forty-seven samples from commercial orchards and 28 samples from nurseries and mother trees reacted positively for CTV by ELISA (Table 1). A total of 1,936 trees from LRGV commercial sites were tested from 1994 to 1996 of which only 15 were CTV-positive. None of the positive samples came from commercial orchards; twelve samples were collected from five different nurseries which included three variegated lemon [*C. limon* (L.)Burn.f.] trees, one Cara Cara navel orange, five unidentified sweet orange trees, one calamondin, and two Bell tangerine trees. The other three CTV-positive trees were Valencia sweet orange (Olinda, Frost, and Campbell) from a private variety collection block. In 1997, seven positive trees were found in two nurseries, ie six Cara Cara and one Bell tangerine. None of the 641 samples collected in 1999 and 2000 tested positive for CTV.

LRGV backyard sampling. A total of 3,417 dooryard citrus trees were surveyed from 1994 to 1996 (Table 1). Eight trees (four sweet oranges and one each of lime, lemon, tangerine, and variegated lemon) from McAllen, Edinburg, Harlingen, Mission, and Weslaco were CTV positive. Four dooryard trees, two sour orange in Mission, and a satsuma and an Ortanique tangor (*C. reticulata* X *C. sinensis*) in La Feria, out of the 624 samples collected from dooryard and commercial sites in 1997, were positive for CTV. Both the satsuma and the tangor had been previously purchased from a local nursery. No positive samples were found out of the 583 samples tested from Harlingen, Bayview, Rio Hondo, San Benito, and Brownsville during the 2000 survey.

Carizzo Springs, Catarina, San Antonio, Dallas, Victoria and Corpus Christi. Ninety-nine samples were collected for CTV testing in Corpus Christi in 1993 and 1996, all of which were negative (Table 2). However, seven out of the 69 samples collected in 1997 gave a borderline positive reaction. One Arizona-propagated Kinnow mandarin tree in a San Antonio backyard gave a positive reaction, as did a sample from an unidentified tree originating from Louisiana collected in a nursery in Dallas. Two of three unidentified samples from Victoria were CTV positive. However, none of the 193 samples collected in Carrizo Springs and Catarina was CTV positive.

Table 2. Results of surveys of citrus for *Citrus tristeza virus* (CTV) by ELISA in various Texas locations outside the Lower Grande Valley of Texas 1991-2000.

Sample Site	Years	Total tested	CTV positives
Carrizo Springs	1991-1992	65	0
	1996	80	0
Catarina	1996	48	0
Corpus Christi	1993	3	0
	1996	96	0
	1997	69	7
San Antonio	1997	2	1
Dallas	1997	2	1
Victoria	1999	3	2
East Texas	1994-1995	92	24
	1996	178	32
	1997	99	15
	1999	231	39
TOTAL	1991-2000	968	121

East Texas. A total of 369 samples were collected from 36 locations in East Texas from 1994 to 1997. Seventy-one samples from 17 different locations in Orange, Deweyville, El Campo, Houston, and Port Neches, were CTV positive. An additional 231 samples were collected in 35 locations in the East Texas counties in 1999, of which 39 samples from 16 of the locations were infected. The infected trees found in East Texas included several satsuma mandarins (Armstrong early, Owari, Neopolitan, and Okitsu), tangerines (Honeybell, Sunburst, Changsha, Fallglo, Empress, Obawase, and Clementine X Uzu), sweet oranges (Hamlin and Navel), lemons (Meyer, Variegated, Eureka, Ponderosa, and Spanish pink), Murcott tangor, sour orange, bloodsweet grapefruit, pummelo, Nippon orangequat, Thornless Mexican lime, Sulcatta, and Sunquat.

Some citrus trees from the LRGV that were growing in two nurseries in East Texas (Owari satsuma, Eureka Lemon, Meyer lemon, Ponderosa lemon, Algerian tangerine, Orlando tangelo, Calamondin, and Kumquat) were all negative for CTV.

Reaction with MCA-13. Twenty-six Texas CTV isolates were tested by monoclonal antibody MCA-13 in 1992 and 1997. Seven of them gave a positive CTV reaction. Three were isolates collected from Meyer lemon in 1972 (L.W.Timmer, unpublished) and maintained in a greenhouse at the Citrus Center. The other four isolates were from Nippon orangequat, Armstrong Early Satsuma, Owari Satsuma, and Meyer lemon, all collected in East Texas.

DISCUSSION

Our survey conducted from 1991 to 2000 showed an overall low CTV incidence (1.1%) in the LRGV. Most of the positive samples were found in orchards and nurseries. No economic losses due to CTV have been reported and no symptoms of the disease have been observed in the field during the time this survey was conducted. The low CTV incidence may be due to the fact that the LRGV citrus industry has suffered several severe freezes, the last in 1989. The budwood used to establish new trees was probably cut from CTV-free trees. Budwood sources were biologically indexed in the voluntary budwood program of the 1950s (Sleeth, 1959). CTV-infected samples would also have been eliminated if they did not grow well on sour orange. For example the Meyer lemon which was introduced into the LRGV before 1923 was soon reported to grow slowly on sour orange rootstock, indicating that the original plants were infected. In 1930, a sour orange-tolerant variety, Rickett's Meyer lemon, was found (Friend; 1954) which was shown to be CTV-free (Olson and Sleeth, 1954). None of the Meyer lemon trees surveyed in the LRGV tested positive for CTV; they likely are propagations of this Rickett variety.

Herron and Sabal (1997) found in Belize that samples from infected citrus trees taken in the hot summer months did not react with either polyclonal or MCA-13 antibodies. Dodds et al. (1987) have shown that antigen titer of CTV does drop in summer, but the optical density values were still significantly higher than the negative control. CTV-infected leaf samples collected in the summer and tested by ELISA in Texas (Solís-

Gracía and Skaria, unpublished data) and in Cuba (Peña et al., 2000) all gave positive results. During the surveys reported in this paper, positive field controls were always included. However, in view of the evidence of low titer, and the non-detection in Belize, it is possible that some CTV-infected samples collected in Texas in summer were missed.

The presence of infected nursery plants and mother trees constitutes a serious risk for the propagation of the virus. A low incidence such as the one found in this study might lead one to suggest an eradication program; however, such action elsewhere has been found to be unpopular and rejected by growers (Bar-Joseph et al., 1989). The importation of citrus trees or budwood into Texas is illegal and the Texas Department of Agriculture is committed to enforcing these regulations. Another essential step is to promote the use of virus-free budwood. Currently, Texas has a virus-free budwood program which is aimed at producing enough budwood for commercial and ornamental purposes in the near future, at which point it will become mandatory (Skaria et al., 1997). CTV presence in dooryard citrus also represents a risk since these trees could be a virus source for aphid transmission, especially when the brown citrus aphid arrives in the area.

The low incidence of CTV, despite the fact that the virus has been present in the state for many years, indicates that there has been very little natural spread. Transmission of Texas CTV isolates by local populations of the spirea aphid, *Aphis spiraecola* Patch, has been demonstrated (Smith and Farrald, 1988; Cutrer 1998). On the other hand, transmission by native or Florida populations of the cotton aphid, *Aphis gossypii* Glover, has not been observed in Texas (Dean and Olson, 1956; Smith and Farrald, 1988). When the BrCA arrives, the situation will change since this is a much more efficient aphid vector of the virus.

Although there is no commercial citrus close to the LRGV in Mexico, citrus trees are common as dooryard trees in the border region of Tamaulipas state. There is no information on the status of CTV in these trees, however, studies in other areas in Mexico where commercial production occurs shows a situation similar to that found in Texas in which CTV is present in low incidence, but no disease symptoms have been found (Silva-Vara et al., 2001; Villarreal et al., 1996), despite the use of sour orange rootstock. In neighboring Belize, where sour orange is also widely used, a survey has shown some 3% of trees to be infected (Herron and Sabal, 1997).

Tristeza symptoms are not apparent in the LRGV possibly because of the low CTV incidence, but also because the prevalent CTV strains are mild and the efficient aphid vector is not present. Results of MCA-13 analysis with some of the Texas CTV isolates detected in our survey in the LRGV showed negative reactions. However, three isolates previously collected in LRGV, gave positive reactions. Severe isolates in mixed infections have previously been shown to be masked by milder ones, but can be separated out when transmitted by the BrCA (van Vuuren et al., 2000).

The low incidence of CTV in the LRGV contrasts with the much higher incidence in East Texas (18.3%). In neighboring Louisiana, an initial survey reported three positive samples out of 34 tested in one area in 1996 (Valverde et al., 1996). In a 1997 survey, 38 out of 196 samples (19.4%) were found to be CTV positive (Skaria, unpublished), with a similar figure (138/820; 16.8%) being found more recently (Valverde, pers. comm., 2001). Since trees in East Texas and Louisiana are grown on trifoliate rootstock for cold tolerance, CTV infection is asymptomatic. The possibility of movement of plant material from these areas to the commercial citrus growing area in the LRGV, represents a serious risk of the introduction of severe CTV strains. In our study, we showed that some of the isolates from East Texas reacted with MCA-13 which may indicate they are severe, thereby posing a risk to the LRGV citrus industry if infected trees are moved there and *T. citricida* enters the area and transmits CTV to the commercial trees on sour orange.

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