Effectiveness of Different Citrus Species as Donor Hosts for Graft Transmission of Citrus Tristeza Virus*

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ABSTRACT. Citrus tristeza virus (CTV) was transmitted by graft inoculation from *Citrus excelsa*, Mexican lime, and Madam Vinous sweet orange (donors), to Madam Vinous, Mexican lime, and grapefruit plants (receptors), by using either leaf or bark pieces as inoculum. There were significant differences in the rate of transmission in each donor/receptor and host/virus isolate combination. Transmission rates were 89.2% and 75.6% when leaf and bark pieces, respectively, were used as inoculum. The overall rate of transmission for all donor/receptor and host/virus isolate combinations was 72.5%, 85.2%, and 90.6% from *C. excelsa*, Mexican lime, and Madam Vinous sweet orange, respectively. Virus titer in *C. excelsa* and Madam Vinous sweet orange donor hosts varied in the different tissues assayed, with bark having the highest virus concentration. The suitability of different citrus species as donor hosts for graft transmission of CTV is discussed.

Index words: DAS-ELISA, virus isolates, virus titer.

Citrus tristeza virus (CTV) is an aphid-transmitted, phloem-limited closterovirus of about 2,000 x 12 nm in size (1), that causes one of the most economically important diseases of citrus (4,22).

Extensive research work on CTV is conducted every year throughout the world to characterize virus particles (2,11,12,23,24) identify and characterize CTV isolates (13,17,30), disease detection (3,8,19,26-30) and disease control (4,6-8,10,18,21,28,29). CTV has long been experimentally transmitted by budding and other grafting procedures (1,4,22,31), because of the lack of an efficient method of mechanical inoculation (14-16). Several grafting procedures involving the use of leaf pieces as inoculum with different degrees of efficiency in the transmission have been reported (5,9,11,32,34). Some of them (5,15) have been used in numerous tests and routine work for many years with CTV and some other citrus viruses (5,7), and in the characterization of the biological properties of diverse worldwide collection of CTV isolates (13,17,30). In general, leaf piece grafts are especially advantageous when large numbers of plants are to be inoculated with limited sources of inoculum (14).

*Florida Agricultural Experiment Station Journal Series No. R-01443.

During several experiments with CTV in Florida (28-30) large numbers of plants were inoculated by leaf piece grafts with several CTV isolates that were propagated in different citrus hosts. There were notable differences in the efficiency of transmission of some CTV isolates from different donor hosts, and in some cases no transmission was achieved even after repeated inoculations. The objectives of this research were to evaluate the effect of different citrus hosts on the efficiency of graft transmission of CTV, and to determine the relative distribution of the virus in different host tissues.

MATERIAL AND METHODS

Virus isolates and donor hosts. Three previously described isolates of CTV, T26, T30, and T66a (17,20,36), were used in this study. The isolates were propagated in *Citrus excelsa*, Mexican lime and Madam Vinous sweet orange plants, herein referred to as donor hosts, and maintained in a greenhouse with mean night and day temperatures of 21 to 38 C. All donor hosts were indexed serologically by DAS-ELISA (see below) to confirm the presence of CTV, before being used for graft inoculation.

Grafting procedures and receptor hosts. Rectangular leaf and bark pieces of about 3 X 15 mm were cut from donor hosts with a sharp knife

and inserted under corresponding bark flaps cut on the stem of one-year-old Madam Vinous sweet orange, Mexican lime, and grapefruit seedlings plants, herein referred to as receptor hosts. A portion of the grafted tissue (2-3 mm) was left exposed at the top of bark flaps to monitor tissue survival at 21 days post inoculation. A minimum of five seedlings of each receptor host were inoculated with four pieces of either leaf or bark tissue for every donor host/virus isolate combination tested. Serological indexing by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (see below) was done on receptor hosts at three and five months post-inoculation.

Inoculated receptor plants were grown in a commercial potting mixture (Pro-Mix BX) in three liter plastic containers, fertilized with a mixture of NPK (20-10-20) every other week, and given normal pest and disease management.

Virus distribution and antigen concentration in host tissues. Individual Madam Vinous sweet orange and *C. excelsa* plants infected with CTV isolates T26 or T66a were used to study the relative distribution and antigen concentration of the virus in different tissues of the host plant. Bark, petioles, midribs, and leaf blades of four individual branches of each test plant, were assayed individually by DAS-ELISA. At least four plants were assayed for every host/virus isolate combination tested.

Purification of CTV. Citrus tristeza virus was purified from tender new tissue of *C. excelsa* greenhouse grown plants infected with CTV isolate T26, by the Driselase method (23). The final virus preparations were read in a spectrophotometer at 260 nm (OD_{260}) and adjusted with 0.05 M Tris buffer to optical density values of 0.4 and stored in one ml aliquots at -18 C.

Serological tests. The double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (3) was conducted with polyclonal antiserum No. 1053 prepared against whole, unfixed CTV isolate T26 (R. F. Lee, unpublished) using polystyrene Immulon II microtiter plates (Dynatech Laboratories). Unless otherwise stated, 200 µl were used per well of microtiter plate. Three washings with phosphate buffered saline (PBS)-Tween [PBS = 8 mM Na_2HPO_4, 14 mM KH_2PO_4, 15 mM NaCl, pH 7.4, (+ 0.1 % Tween 20)] were performed between steps. Host tissue (bark, petioles, midribs, etc.) was finely chopped with a razor blade and ground in a Tekmar Tissumizer in extraction buffer (PBS-Tween + 2% polyvinyl pyrrolidone (PVP-40 Sigma) at a 1:20 (w/v) dilution. Microtiter plates were coated with 2.0 µg/ml of purified CTV specific IgG in carbonate buffer (0.015 M $NaHCO_3$, 0.03 M $NaCO_3$, pH 9.6) and incubated for 6 hr at 37 C. Antigen samples were added to the wells and incubated for 18 hr at 5 C. CTV specific IgG conjugated to alkaline phosphatase was used at a dilution of 1:1,000 in conjugate buffer (PBS-Tween + 2% PVP + 0.2% bovine serum albumin) and incubated for 4 hr at 37 C. The reaction with one mg/ml of p-nitrophenyl phosphate (Sigma) in 10% triethanolamine, pH 9.8, was measured after 120 min at 405 nm (OD_{405}) with a Bio-Tek EL-307 ELISA plate spectrophotometer. For the graft transmission experiment, samples were considered positive when OD_{405} values were higher than 0.1 or three times the mean of healthy controls, whichever was greater. There were two replications per sample in each microtiter plate. To estimate the relative antigen concentration of CTV in test samples, a standard curve prepared by diluting purified CTV T26 to OD_{260} values of 0.02, 0.01, 0.005, 0.0025 and 0.00125 in a PBS-Tween + 2% PVP buffered extract of healthy *Citrus excelsa*, was included as a positive control in every test. Negative controls included PBS-Tween + 2% PVP, conjugate buffer, and extract from healthy *C. excelsa*, Madam Vinous sweet orange, Mexican lime and grapefruit plants.

RESULTS

Graft transmission of citrus tristeza virus isolates. At 21 days post-inoculation the survival rate of grafted tissue in the whole experiment (270 plants) was 83 and 66 % for leaf and bark pieces, respectively. Overall there was at least one surviving or successful graft per plant in 92 and 90 % where leaf or bark tissues, respectively, had been used (Table 1). In calculating the percent of virus transmission for each donor/receptor and host/virus isolate combination, only those plants with at least one (of four) surviving inoculum piece were considered. Thus, overall there was a greater efficiency of transmission with leaf pieces (89.2%) than with bark pieces (75.6%) for the whole experiment (Table 1). There were three plants of 270 in the entire experiment, one Mexican lime and two grapefruit that became infected even though no successful graft was scored three weeks post inoculation.

The overall rate of transmission of CTV by graft inoculation (leaf and bark combined) for each donor/receptor host combination is shown in Table 2. With *C. excelsa*as the donor host there was 72.4%, 86.9%, and 60.7% transmission to Madam Vinous, Mexican lime and grapefruit, respectively. With Mexican lime as the donor host, there was 93.1%, 76.9% and 89.3% transmission to Madam Vinous, Mexican lime and grapefruit, respectively. With Madam Vinous as the donor host there was 86.7%, 100%, and 84.6% transmission to Madam Vinous, Mexican lime and grapefruit, respectively (Table 2). There was an overall efficiency of transmission of 72.5% from *C. excelsa*, 85.2% from Mexican lime, and 90.6% from Madam Vinous. Statistical analysis showed a significant differences ($P \leq 0.05\%$) for all donor-receptor host combinations. Likewise, according to Duncan's multiple range comparison test, there were statistical differences between some of the hosts tested (Table 2).

The rate of transmission for the three different CTV isolates tested with each donor host is shown in Table 3. The T26 isolate was transmitted at a rate of 69.0% to 92.8% and the transmission for T30 and T66a isolates ranged from 65.2% to 100% and from 71.4% to 96.7%, respectively, from all hosts tested. While there were statistical differences in the rates of transmission for the virus isolate/donor host combination, the overall average of transmissions showed no statistical differences (Table 3).

The overall statistical analysis for percent transmission of the interaction among the different donor/receptor/virus isolate/inoculum piece combina-

TABLE 1
TRANSMISSION OF CITRUS TRISTEZA VIRUS BY GRAFT INOCULATION BETWEEN SELECTED CITRUS HOSTS: I. EFFICIENCY OF LEAF AND BARK PIECES AS INOCULUM

Inoculum tissue	Inoculum survival[z] (%)	Plants with at least one successful graft (%)	Transmission[y] (%)
Leaf	83.0[x] a[w]	92.0	89.2 a
Bark	66.0 b	90.0	75.6 b

[z]Measured at 21 days post-inoculation.
[y]Percent transmission to plants with at least one inoculum piece (of four) alive, measured serologically by DAS-ELISA at 3 and 5 months post-inoculation. Number indicates overall transmission for all donor/receptor/virus isolate combinations.
[x]A total of 270 plants (135 each) were inoculated with four pieces of either leaf or bark tissue. Number indicates overall survival for all donor/receptor/virus isolate combinations.
[w]Numbers in the same column followed by different letters are statistically different by Duncan's multiple range test ($P \leq 0.05$).

TABLE 2
TRANSMISSION OF CITRUS TRISTEZA VIRUS BY GRAFT INOCULATION BETWEEN SELECTED CITRUS HOSTS: II. OVERALL RATE OF TRANSMISSION

Donor host	Receptor host			
	Madam Vinous	Mexican lime	Grapefruit	Average
Citrus excelsa	72.4[z]b[y]	86.9ab	60.7b	72.5[x]b
Mexican lime	93.1 ab	76.9b	89.3a	85.2 ab
Madam Vinous	86.7 ab	100.0a	84.6a	90.6 a

[z]Percent transmission to plants with at least one inoculum piece (of four) alive 21 days post-inoculation. Number indicates all of transmission for all virus isolates combinations. Each value represents a minimum of 27 plants.
[y]Numbers in the same column followed by different letters are statistically different by Duncan's multiple range test ($P \leq 0.05$).
[x]Number indicates the overall of transmission for all receptor/virus isolate combinations.

tions, indicated no significant differences for receptor and virus isolates alone, and for the combinations of donor/receptor virus isolate. However, significant differences ($P \leq 0.05$) were found for donor and inoculum pieces alone, and for the interactions between donor/receptor, donor/virus isolate, and virus isolate/inoculum pieces.

Virus distribution and antigen concentration in host tissues. The relative antigen titer of CTV as measured by DAS-ELISA in each host tissue/virus isolate combination is illustrated in Table 4. The optical density values at 405 nm (OD_{405}) for bark tissue ranged from 0.221 to 0.349 for the T26 isolate and from 0.266 to 0.336 for the T66a isolate in Madam Vinous and *C. excelsa*, respectively. The OD_{405} values found in the other tissues assayed in both hosts for T26 and T66a isolates were in the range of 0.137-0.188 and 0.099-0.238 for petioles, 0.086-0.120 and 0.040-0.133 for midribs, and 0.044-0.065 and 0.030 for leaf blades, respectively. There were significant statistical differences between *C. excelsa* and Madam Vinous for bark tissue with T26 isolate, and for both petioles and midribs with T66a isolate. The overall analysis showed that the highest OD_{405} values in both hosts for both T26 and T66a isolates, were found in bark, followed by petioles and midribs. Leaf blade gave the lowest OD_{405} values of all tissues assayed in both hosts and isolates tested. Some differences in the OD_{405} values were found between different parts of the same plant, and from one plant to another in some virus isolate/host combinations. However, the statistical analysis did not show signif-

TABLE 3
TRANSMISSION OF CITRUS TRISTEZA VIRUS BY GRAFT INOCULATION BETWEEN SELECTED CITRUS HOSTS: III. EFFECT OF VIRUS ISOLATES

Virus isolate	Donor host			
	Citrus excelsa	Mexican Lime	Madam Vinous sweet orange	Average
T26	69.0[z]a[y]	89.3a	92.8a	83.5[x]a
T30	76.7 a	65.7a	100.0a	80.7 a
T66a	71.4 a	96.7a	77.3b	83.5 a

[z]Percent transmission to plants with at least one inoculum piece alive 21 days post-inoculation. Number indicates overall of transmission for all donor/receptor host combinations. Each value represents a minimum of 27 plants.
[y]Numbers in the same column followed by different letters are statistically different by Dundan's multiple range test ($P \leq 0.05$).
[x]Number indicates overall of transmission for all donor/receptor host combinations.

TABLE 4
RELATIVE ANTIGEN TITER OF CITRUS TRISTEZA VIRUS IN DIFFERENT TISSUES OF *CITRUS EXCELSA* AND MADAM VINOUS SWEET ORANGE HOST PLANTS, AS MEASURED BY ENZYME-LINKED IMMUNOSORBENT ASSAY

Virus isolate	Donor host	Host tissue			
		Bark	Petioles	Midribs	Leaf blade
T26	*Citrus excelsa*	0.349[z] a[y]	0.137 a	0.086 a	0.044 a
	Madam Vinous	0.221 b	0.118 a	0.120 a	0.065 a
T66a	*Citrus excelsa*	0.336 a	0.238 a	0.133 a	0.030 a
	Madam Vinous	0.266 a	0.099 b	0.040 b	0.029 a

[z]Mean of optical density (OD_{405}) per 10 mg plant tissue after 120 min of substrate reaction. There were four replicates per plant and four plants per host/isolate combination. Control reaction with the same tissue from healthy plants averaged OD_{405} = 0.001-025. This has not been subtracted from the values above.
[y]Numbers in the same column per host/isolate combination followed by different letters are statistically different by Duncan's multiple range test ($P \leq 0.05$).

icant differences among them (data not shown). From the standard curve prepared with purified T26 isolate (Fig. 1), it was estimated that an OD_{405} value of 0.465 was approximately equivalent 20 μg/ml of CTV, assuming an extinction coefficient of 2.0 (16). Therefore, the CTV antigen concentration in the test samples (10 mg of tissue/200 μl) ranged from an average of 0.2-0.5 μg in leaf blades to 1.9-2.0 μg in bark tissue.

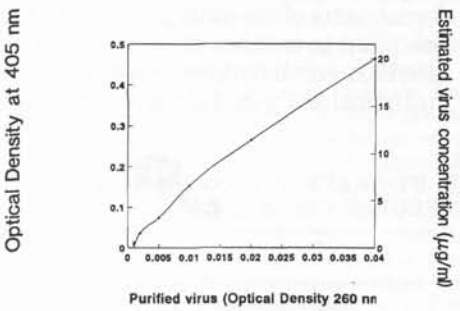

Fig. 1. Plot of purified citrus tristeza virus (CTV) against optical density. Bark of healthy *Citrus excelsa* (0.25g) tissue was ground in 5.0 ml of phosphate buffered saline, pH 7.6, + 0.05% Tween + 2% polyvinyl pyrrolidone and mixed with purified CTV T26 isolate to give the desired optical density at 260 nm (OD_{260}). DAS-ELISA was performed as described in Materials and Methods. An extinction coefficient of 2.0 was assumed to estimate the relative virus concentration.

DISCUSSION

The results obtained in this work showed significant differences in the efficiency of the three donor hosts tested to transmit CTV. Likewise, differences were found in the rate of transmission for each donor/receptor host combination. There were a number of instances, i.e. 22 of 80, and 12 of 81, respectively, when *C. excelsa* and Mexican lime were used as donor hosts, where no transmission was achieved even on those receptor plants where at least one grafted tissue piece was still alive 3 weeks post-inoculation. Similar results were obtained, but to a lesser degree (7 of 75) when Madam Vinous sweet orange was the donor host. Furthermore, some of the receptor plants where no transmission was scored had all four grafted pieces still alive even three and five months post-inoculation (data not shown).

C. excelsa showed a low 72.4% and 60.7% of transmission to Madam Vinous and grapefruit, respectively; whereas, 86.7% was obtained to Mexican lime plants. With Mexican lime as donor host, there was a transmission rate of 89.3% and 93.1% to grapefruit and Madam Vinous, respectively, and a 76.9% rate to Mexican lime. Transmission from Madam Vinous sweet orange ranged from 84.6 to 100% in all

receptors tested. It was surprising that transmission rates between the same species were only 86.7% for Madam Vinous, and 76.9% for Mexican lime (Table 2).

Madam Vinous sweet orange was the most efficient donor host with the three receptors tested (90.6%), followed by Mexican lime (85.2%) in efficiency. *C. excelsa* was a poor donor host (72.5%), being relatively efficient only when inoculated to Mexican lime (Table 2). These results provide further evidence of the suitability of Madam Vinous sweet orange as a propagation host for CTV and perhaps other citrus viruses as reported previously (31).

Previous studies on the transmission of CTV by grafting procedures have shown that a period of at least ten days of contact between grafted tissues is needed to obtain transmission of the virus to the receptor host (33,35). In this study, the survival of grafted tissue was scored 21 days post-inoculation, but the inoculated tissue was left in the receptor plants for up to five months. This should have been ample time for contact between the inoculum and receptor cambium to establish a tissue union with a subsequent transmission of CTV. Furthermore, when leaf pieces were used as inoculum, a small portion of the midrib was included in every piece to increase the success of the grafting. The overall rate of successful grafts were about 83% and 66% for leaf and bark pieces, respectively (Table 1).

The reason why a low percentage of graft transmission of the virus was found from some donor hosts, the absence of an expected 100% when the donor-receptor combination was of the same species, as well as why *Citrus excelsa*, considered as an excellent propagation host for purification purposes of CTV (23,24), was a poor donor host for graft transmission, are unknown. A possible explanation could be differences in the virus distribution and/or concentration in the donor tissues used as inoculum. Even though the statistical analysis did not show significant differences in antigen titer in the different parts of the same plant or from one plant to another (data not shown). There were some instances where OD_{405} values were as low as the healthy controls, indicating a possible absence of virus in those tissues. This raises the possibility that occasionally the tissue used for graft transmission may be virus-free, with a subsequent failure in the transmission. Uneven distribution of CTV in host tissues is known to occur in grapefruit and in a lesser degree in sweet orange (25). Other possibilities could be an occasional absence of phloem connections between the donor and receptor tissues with a subsequent absence of movement of the virus across the junction, or the requirement of a minimum of virus particles present in the tissue used as inoculum in order to accomplish transmission.

Citrus tristeza virus is phloem-limited (1,22), and is normally found at higher concentrations in young phloem-rich tissues (1,3,4). However, the virus titer frequently decreases as the tissues reach maturity or when the plants are exposed to warm environments (11,25). The OD_{405} values obtained in this research were low if compared with those found when DAS-ELISA is used routinely for CTV diagnosis (3,26,27). This part of the work was addressed to determine the virus titer in the tissues suitable for graft transmission, and young tender tissue normally is not a good source of inoculum for leaf and bark piece grafts (personal observations). In this regard, bark tissue contained the highest titer with OD_{405} values in the range of 0.221 and 0.349 in both *C. excelsa* and Madam Vinous with both CTV isolates tested (Table 4). These values were, in some instances, more than double those found in petioles and midribs, and at least triple those found in the leaf blade. Further studies are needed to determine why bark tissue, even though it showed the highest virus concentration, was less efficient than leaf pieces to transmit the virus.

The overall analysis of the results obtained indicates that the efficiency of the graft transmission of CTV is conditioned primarily by the donor/receptor host combination, and secondly by the virus isolate involved, but apparently not by the interaction of the three. For example, *C. excelsa* showed an overall rate of transmission in the range of 72.5% with all receptor hosts tested (Table 2), and a similar low pattern between 69% and 76.7% (≈ 72.8%) was obtained for the three isolates tested (Table 3). Likewise, when Madam Vinous was used as donor host, there was an overall rate of transmission of 90.6% (Table 2). A relatively similar example of 77.3-100% (≈ 88.6%) occurred from this host with the three isolates tested (Table 3). A similar result was also scored when Mexican lime was the donor host (Table 2 and 3). The statistical significance found for the interaction of donor/receptor and donor/virus isolate, and no significance for the interaction of donor/receptor/virus isolate supports this conclusion.

The use of leaf and/or bark pieces for graft transmission of CTV, may be advantageous when large numbers of plants are to be inoculated with limited sources of inoculum (11,17). However, in the light of the results of this research, in order to achieve a high success in transmission, the particular efficiency of any donor host and the donor/receptor host combination should be considered.

ACKNOWLEDGMENTS

This work was supported in part by USDA Specific Cooperative Agreement 58-43YK-0-0008. Support by Consejo Nacional de Ciencia y Tecnología, Mexico, for first author is also acknowledged. The authors thank Dr. Ben Lye for advice on the statistical analysis of the data, and to N. E. Berger, T. Nguyen and S. Jackson for technical assistance.

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