Susceptibility of Citrus Varieties, Species, Citrus Relatives, and Non-Rutaceous Plants to Slash-Cut Mechanical Inoculation with Citrus Tristeza Virus (CTV)[1]

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ABSTRACT. The susceptibility of 47 citrus species, cultivars, hybrids and relatives to citrus tristeza virus (CTV) infection was tested by stem-slash inoculation of greenhouse-grown plants with concentrated, partially purified preparations of CTV. Infection in 26 of 31 citrus selections inoculated was detected by symptoms and/or by ELISA (enzyme-linked immunosorbent assay). The ELISA procedure was valuable for detecting symptomless infections. CTV-induced vein clearing was observed in several normally latent hosts such as Rangpur lime at the onset of systemic infection. High rates of infection were observed in Etrog citron, Rangpur lime, calamondin, Mexican lime, sweet orange and *Citrus hystrix*. Grapefruit and Cuban Shaddock were difficult to infect. Six of 16 citrus relatives mechanically inoculated were infected: *Aegle marmelos* (L.) Corr., *Aeglopsis chevalieri* Swing., *Afraegle paniculata* (Schum.) Engl., *Citropsis gilletiana* Swing. & M. Kell., *Microcitrus australis* (Planch.) Swing., and *Pamburus missionis* (Wt.) Swing. No CTV infections were detected by ELISA in the 55 different woody and herbaceous nonrutaceous plants inoculated. Tristeza was retransmitted mechanically from *A. marmelos* to Etrog citron.

Since the 1940's a large number of citrus types and a few citrus relatives have been tested for susceptibility to the citrus tristeza virus (CTV), at the Instituto Agronômico, Campinas, Brazil (3, 5, 10, 11). Inoculations have been made by grafting, by aphid vectors, and with several species of dodder (1, 3). Graft transmission of CTV has been limited to hosts graft-compatible with citrus (13), but CTV has been transmitted to several citrus relatives, such as *Aeglopsis chevalieri* by aphids. Repeated attempts also have been made to extend the host range of CTV outside the Rutaceae (6, 12), but this has been successful only with *Passiflora gracilis* Jacq. inoculated by aphids (1, 14).

The recently developed procedure to mechanically transmit CTV to citrus (7) and the adaptation of the ELISA test to quickly detect CTV infections (2) offered further possibilities to test citrus, citrus relatives and nonrutaceous plants as hosts of CTV. We hoped that herbaceous hosts could be discovered which would be better assay and/or increase plants for CTV.

In this paper, we report the results of inoculating and indexing numerous citrus, citrus relatives, and nonrutaceous plants to determine their susceptibility to CTV infection by mechanical inoculation.

MATERIALS AND METHODS

Plant materials and growing conditions. Tests were conducted in an air-cooled, partly shaded glasshouse in Orlando, Florida. Temperatures ranged from 22 to 27 C in winter and spring and 22 to 35 C in summer. Plants were grown in a sterilized potting mix, fertilized and sprayed as needed to maintain healthy, vigorous growth. Plants were grown from seeds or from rooted cuttings of clonal, virus-free sources.

A variety of nonrutaceous plants were chosen for testing.

[1]Use of a company or product name by the U.S. Department of Agriculture does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

Some were woody, some were hosts for other viruses, viroids, and prokaryotic pathogens (15- 16), some were weeds found in citrus orchards, and one has been described as a nonrutaceous host of *T. citricida* (17). Etrog citron plants which are susceptible to CTV infection by mechanical inoculation (7) were included in every experiment to verify infectivity of the inoculum.

Inoculation preparation. Young stem bark from greenhouse-grown Etrog citron or sweet lime plants infected with CTV isolate T-3 (a seedling yellows (SY) isolate) or CTV isolate T-4 (a non-SY isolate), was peeled and wrapped in 5 g bundles with "Parafilm®." The bundles were sectioned on a Hooker® microtome and finely diced with a razor blade at a 1:3 (w/v) ratio in extraction buffer (0.05 M Tris-HCl buffer [Tris (hydroxymethyl) aminomethane hydrochloride], pH 7.8 which contained 100 mg/l sucrose and 2 μl/ml of 2-mercaptoethanol). The extract was squeezed through cheesecloth by hand press and reextracted with a small volume of buffer. All extraction steps were done at 0 to 4 C. Extracts were clarified by centrifugation for 15 minutes at 12,000 RPM in a Sorvall® SS-34 rotor and layered in 2.5 x 7.5 or 2.5 x 8.75 cm centrifuge tubes containing a freshly prepared sucrose step gradient (5 ml of 250 mg/ml and 5 ml of 600 mg/ml sucrose in Tris buffer) (8). Tubes were centrifuged overnight at 21,500 RPM in a Beckman® SW25.1 rotor or at 19,000 RPM in a SW28 rotor. Half ml fractions were collected in premarked test tubes by puncturing the bottom of the gradient tube. Gradient fractions were tested for CTV content by ELISA (bead procedure) and the leading (lowest) 2-3 CTV-containing fractions were combined and diluted 1:1 with 0.05 M Tris to use as inoculum. Approximately 6 to 9 ml of inoculum was recovered from 25 g of bark tissue.

Inoculation. Most inoculations were done from January through April and during September and October 1980, but some were also done in the spring of 1981, 1982 and 1983. Inoculations were done by the stem-slash method (7) with 30 cuts at each of 3 sites on the plant stem. Most stems were 0.4 to 0.8 cm in diam at the point of inoculation. Usually a minimum of 10 plants of each type were inoculated. Fresh, partially purified preparations were used for each inoculation test and Etrog citron plants were used to verify infectivity of each batch.

Initially, cuts on citrus and citrus relatives were wrapped with Stericrepe® (Beacon & Janis Ltd., London, W.C.I., England). Later, however, this was found unnecessary. Some succulent nonrutaceous plants were covered with plastic bags and on others the inoculation cuts were wrapped with "Parafilm" and the plants were not covered. Leaf midribs were also slashed on some herbaceous plants. After inoculation, the herbaceous plants were put in the shade for 48 hours before the plastic bags were removed.

Citrus and most woody plants were cut back immediately to force new growth and the herbaceous plants were cut back 2 weeks after inoculation. Rapidly growing plants were cut back several times before testing for CTV infection. Three to 5 uninoculated control plants were kept for each species tested.

Assay for infection. All citrus cultivars, species and relatives were examined periodically for vein clearing and the noncitrus plants were examined for vein clearing plus any other abnormalities.

After the Etrog citron control plants showed vein-clearing symptoms, succulent stem bark tissue was collected from inoculated citrus

and citrus relatives. Whole young stem was collected from succulent nonrutaceous hosts. Chopped tissue (0.5 g) was placed in a 2.5 cm-diameter glass tube with 5 ml of PBST-2 PVP buffer (phosphate-buffered saline with 0.05% Tween® 20 and 2% polyvinylpyrrolidone, m.w. 40,000) (2, 4) and homogenized about 15 seconds with a SDT-Tissumizer® (Tekmar Co., Cincinnati, Ohio 45222). The extracts were filtered through glass wool and the ELISA tests were conducted by the double antibody sandwich method (4). The CTV antiserum used was prepared to unfixed, whole virus of the T-4 isolate (9). IgG was purified and conjugates to alkaline phosphatase prepared as described previously (2, 4). We used polystyrene Micro ELISA® plates (Dynatech Labs, Inc., Alexandria, Virginia 22314) with round-bottom wells. Healthy extracts, CTV-infected extracts, and buffer controls were included in each plate for reference. Plates were scored visually and results recorded by photography. In some cases, the absorbance at 405 nm was also determined spectrophotometrically on diluted samples to verify assessment. Samples were not considered CTV-positive unless the $O.D._{405}$ was 2X that of the healthy controls.

RESULTS

Inoculation of citrus varieties, species and relatives: Over 600 plants comprising 47 citrus varieties, species and relatives were mechanically inoculated with CTV (table 1). Calamondin, Mexican lime, Rangpur lime and Etrog citron showed the highest incidence of CTV infection. Some Mexican lime plants showed CTV vein clearing within 30 days after inoculation, and Etrog citron plants often showed vein-clearing symptoms 35 to 45 days after inoculation (in the second flush of growth). Clear-cut, vein-clearing symptoms not previously reported were observed in Rangpur lime (fig. 1A) 40-50 days after inoculation. Faint vein clearing was observed in Rusk citrange and in some sweet orange plants. The initial vein clearing was more pronounced than that in subsequent flushes, especially in plants which are normally asymptomatic. The other citrus cultivars and types showed varying rates of infection. Cleopatra mandarin, Duncan grapefruit, Cuban Shaddock, and Gomri and Soh jhalia rough lemon selections were not infected (table 1).

Six of 16 citrus relatives tested, *Aeglopsis chevalieri, Aegle marmelos, Afraegle paniculata, Citropsis gilletiana, Microcitrus australis* and *Pamburus missionis*, were infected. Rates of infection were lower than in most citrus selections (table 1); however, the infected plants of *A. chevalieri, A. marmelos* and *A. paniculata* which became infected showed very clear, persistent vein-clearing symptoms (fig. 1B).

Tristeza was partially purified from *A. marmelos* and was retransmitted mechanically to Etrog citron.

Inoculation of nonrutaceous plants. Plants of 55 species belonging to 26 families outside the Rutaceae were inoculated with CTV. They are listed alphabetically by family as follows: AMARANTHACEAE—*Gomphrena globosa* L.; ANACARDIACEAE — Brazilian pepper tree (*Schinus terebinthiofolius* Raddi); APOCYNACEAE—*Cantharanthus roseus* L.; ARACEAE—*Dieffenbachia* sp.; BUXACEAE—*Buxus sempervirens* L. cv. Wintergreen; CAPRIFOLIACEAE—*Viburnum dentatum* L., and *Viburnum odoratissimum* Ker; CHENOPADIACEAE — *Chenopodium amaranticolor* Costa & Reyn, *Chenopodium quinoa* Wild, and *Beta vulgaris* L. cvs. Early Wonder and Swiss Chard; COLEA-

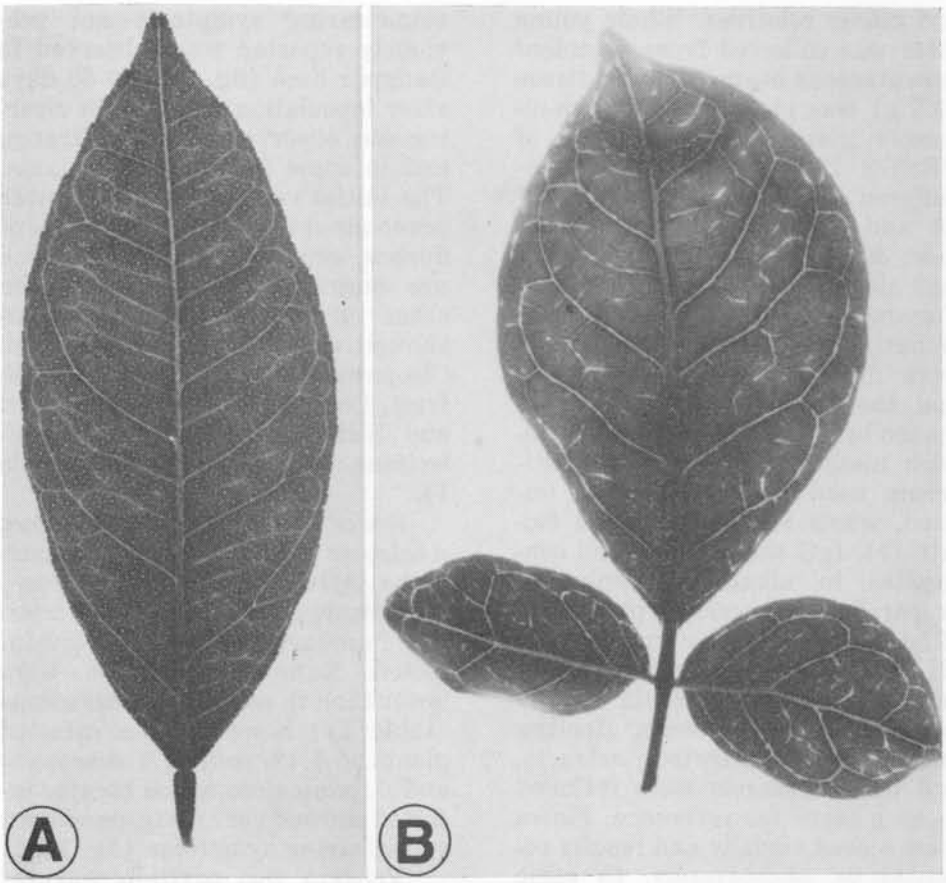

Fig. 1. Citrus tristeza virus-induced vein clearing in mechanically inoculated Rangpur lime (A) and *Aegle marmelos* (B).

CEAE — *Ligustrum japonicum* Thunb. and *Ligustrum sinense* Lour.; COMPOSITAE — *Bidens pilosa* L., *Chrysanthemum morifolium* Ramat, *Gynura aurantiaca* DC, *Gynura sarmentosa* DC, and *Zinnia elegans* Jacq. cv. Peppermint, *Wedelia trilobata* Hitchc.; CUCURBITACEAE — *Momordica balsamina* L.; *ELAEGNACEAE*— *Eleagnus commutata* Bernh.; ERICACEAE — *Rhododendron indicum* (L.) cv. Formosa, and *R. obtusum* (Lindl.) cv. Christmas Cheer; EUPHORBIACEAE — *Acalypha wilkesiana* Muell. Arg.; LABIATEAE — *Coleus blumei* Benth.; LEGUMINOSE — *Phaseolus vulgaris* L., *Crotalaria spectabilis* Roth and *Cassia* sp.; MALVACEAE — *Hibiscus rosa sinensis* L. and *Hibiscus esculentus* L. cv. Clemson spineless; MORACEAE Osage orange (*Maclura pomifera* (Raf.) Schneid); MYRSINACEAE — *Ardisia crispa* (Lam.) A. DC; PASSIFLORACEAE — *Passiflora gracilis* Jacq., and *Passiflora* sp.; PEDALIACEAE—White sesame (*Sesamum indicum* L.); PITTOSPORACEAE — *Pittosporum tobira* Ait *tobira* cv. variegated; PODOCARPACEAE — *Podocarpus* sp.; ROSACEAE — *Malus sylvestris* Mill cv. York; RUBIACEAE — *Ixora* spp., *Pentas* spp., and *Gardenia veitchii* Hort.; SOLANACEAE — *Nicotiana clevelandii* Gray, *Nicotiana edwardsonii* Christie & C. W. Hall, *Nicotiana glauca* Graham, *Nicotiana glutinosa* L., *Nicotiana megalo-*

TABLE 1
TRANSMISSION OF CITRUS TRISTEZA VIRUS (CTV) BY MECHANICAL INOCULATION TO CITRUS CULTIVARS AND RELATIVES

Citrus variety	No. plants inoculated	% infection	Citrus relative	No. plants inoculated	% infection
Calamondin	10	100	*Citropsis gilletiana*	10	30
Mexican lime	15	87	*Afraegle paniculata*	35	20
Etrog citron (S-1)	40	80	*Microcitrus australis*	20	15
Rangpur lime	20	75	*Aegle marmelos*	26	8
Palestine sweet lime	10	70	*Aeglopsis chevalieri*	36	8
C. webberi	10	70	*Pamburus missionis*	20	5
Etrog citron (RMA 861)	36	64	*Atalantia monophylla*	10	0
Iran lemon	10	60	*Balsamocitrus dawei*	5	0
C. hystrix	10	60	*Clausena excavata*	5	0
Valencia orange	10	50	*Clausena lansium*	1	0
Gou Tou sour orange	10	50	*Glycomis pentaphylla*	10	0
Florida rough lemon	10	50	*Hesperethusa crenulata*	5	0
Rusk citrange	10	50	*Murraya koenigii*	14	0
Alemow	10	50	*Murraya paniculata*	10	0
C. karna	9	44	*Swinglea glutinosa*	10	0
Navel orange	17	35	*Triphasia trifoliata*	10	0
Coorg lime	10	30			
Sour orange	40	30			
C. excelsa	10	30			
Temple tangor	10	30			
Milam lemon	15	27			
C. myrtifolia	10	20			
Soh Jhalia (rough lemon)	10	10			
Eureka lemon	20	10			
Volkamer lemon	10	10			
Orlando tangelo	10	10			
Marsh grapefruit	21	5			
Cleopatra mandarin	10	0			
Duncan grapefruit	20	0			
Gomri rough lemon	10	0			
Cuban Shaddock	10	0			

siphon Heurck & Muel, *Nicotiana tabacum* L., *Solanum nigrum* L., *Lycopersicon esculentum* Mill. cv. Patio, *Capsicum annuum* L. cv. California Wonder, *Datura metel* L., *Datura stramonium* L., and *Solanum seaforthianum* Andr.; SCROPHULARIACEAE — *Scopolia sinensis* Hensl., and *Antirrhinum majus* L.

No symptoms which would indicate CTV infection were observed, except for some vein clearing in *H. rosa sinensis,* which was also present in noninoculated controls. All nonrutaceous plants (many of which were assayed twice) tested negative for CTV infection by ELISA.

DISCUSSION

Although high rates of infection are sometimes obtained, the mechanical inoculation procedures used in these studies are not highly efficient for transmitting CTV to most citrus hosts. Many different species of citrus can become infected with CTV by extensive mechanical inoculation with concentrated *in vitro* sources of inoculum; however, accidental transmission of CTV as a contaminant on cutting tools in the field is less likely with unconcentrated inoculum and fewer wounds.

Partially purified tristeza isolates can be inoculated directly to

plants of interest without need for an easily infected, intermediate host such as Etrog citron. This may be of special interest for introducing mild CTV isolates from one country to another in purified form for cross-protection studies or other experimental uses.

The mechanical transmission of CTV between citrus and citrus relatives which are not graft compatible will also permit use of these alternate hosts for virus increase purposes should they prove especially suitable host plants. Because mechanical inoculations to normally receptive hosts, such as Etrog citron, often give somewhat erratic results, the relative rates of infection reported are significant only in a general sense. However, it appears that some citrus cultivars such as grapefruit are more difficult to infect than others such as Etrog citron or Mexican lime. Negative results for individual selections where only a single test was made should not be regarded as definitive at this point; however, in all cases reported, the same inoculum caused extensive infection in known susceptible hosts. No striking differences were noted between the 2 isolates tested. Variations between preparations of the same isolate were as great as variations between isolates. Accurate estimates of the concentration of intact, infectious particles are difficult to obtain and generally were not made on the inocula used here.

A. chevalieri, A. paniculata and *P. missionis* have all been previously reported as reactive hosts of CTV (12, 13), and CTV has been transmitted from the former 2 species to citrus by aphids (12). *Aegle marmelos* was reported nonreactive to aphid inoculation with CTV by Knorr (12), but was infected in our tests. Positive serological reactions in ELISA with CTV specific antibodies confirm the diagnosis of CTV infection in these citrus relatives made previously by symptoms, and also confirm the utility of ELISA for indexing for CTV infection in plants which are not graft-compatible with citrus indicators.

In most cases, we did not attempt to infect the citrus relatives which were nonsusceptible to mechanical inoculation by other means. However, we did find that CTV could not be detected in graft-inoculated *Swinglea glutinosa* plants by ELISA.

Although we had only negative results with nonrutaceous plants, further tests may yet show that some of these are hosts of CTV. Additional genera of the Rutaceae, such as the genus *Fagara* which has a number of species in the Amazon area of Brazil, could be tested. Plants in other families related to the Rutaceae are other possible candidates.

The stem-slash inoculation procedure may not be highly suitable for some of the nonrutaceous plants tested. We did not infect *Passiflora gracilis* even though more than 50 plants were inoculated in several tests. Since it is a known host of CTV which is infectable by aphids and the inoculum used here was infectious, it appears that the slash-cut procedure was not appropriate for this host. Other sap inoculation procedures, including leaf-abrasion inoculation have either not been successful or were less successful than the stem-slash method for inoculating citrus (Garnsey and Müller, unpublished), and we did not have sufficient inoculum to test numerous different procedures on different hosts.

The use of ELISA was a great advantage for indexing for CTV infection in symptomless plants, especially nonrutaceous ones. We believe that if any of the nonrutaceous plants tested had been highly susceptible to CTV infection and

able to support CTV multiplication, we should have been able to detect this. Although ELISA may not detect very low levels of infection, we detected CTV in citrus extracts at dilutions 100 to 200X those used for the assays here.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the expert technical assistance of C. Henderson and R. Whidden. Also acknowledged are Dr. Robert Knight of the Miami Plant Introduction Station; Dr. M. Cohen, University of Florida, Fort Pierce, Florida; and Dr. J. B. Carpenter, USDA, Indio, California, for furnishing plant material; and Dr. Condorcet Aranha, Department of Economic Botany, Instituto Agronômico, Campinas, Brazil, for assistance in classifying the nonrutaceous plants.

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