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Title

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Journal

International Organization of Citrus Virologists Conference Proceedings (1957-2010), 13(13)

ISSN

2313-5123

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Publication Date

1996

DOI

10.5070/C507j7q11w

Peer reviewed

Differential Susceptibility of Pummelo and Swingle Citrumelo to Isolates of Citrus Tristeza Virus

S. M. Garnsey, H. J. Su, and M. C. Tsai

ABSTRACT. Differential susceptibility of pummelo and Swingle citrumelo to different isolates of citrus tristeza virus (CTV) have been observed in Taiwan, Hawaii, and the United States. Glasshouse experiments were conducted to determine the extent and nature of this susceptibility/resistance to different isolates of CTV. Pummelo selections tested were: (a) Peiyu (Taiwan); (b) a pink pummelo from Hawaii (HA Pink) which has resisted natural infection by severe challenge of CTV for many years; (c) Thong Dee (Florida) CTV; and (d) three seedling clones of HA-Pink. These were graft-inoculated with four different isolates: B3, a Florida decline isolate; B31, a Hassaku dwarf (CTV-HD) isolate from Japan; B51, a Florida mild isolate; and B280, a pummelo dwarf-inducing (CTV-D) CTV isolate from Taiwan. Test plants were evaluated for symptoms and tested for virus replication by ELISA. All pummelos were infected by B31 and B280 and all except the HA-Pink showed strong stem pitting or stunting symptoms. B3 infected all but the HA-Pink selection and B51 infected only Peiyu and Thong Dee. Seventy-nine seedlings of HA-Pink inoculated with B31 showed mild to severe reactions. Seedling and clonal propagations of Swingle citrumelo on rough lemon were graft-inoculated with five different CTV isolates. Swingle citrumelos were infected by B3 and B28 (a severe isolate from Ellendale mandarin), but were not infected by B2, B51, or B52 (three Florida mild isolates). These results confirmed the differential susceptibility of pummelos and Swingle citrumelo to different CTV isolates. The isolate-specific resistance to CTV isolates in pummelo and Swingle are apparently different, and both are distinct from the inheritable CTV resistance factor in trifoliolate orange. These differential hosts may be useful for characterization of isolates within the CTV group.

Key words. Tristeza stem pitting, virus resistance, ELISA, Hassaku dwarf, pummelo dwarfing.

Most citrus species and cultivars are considered susceptible to infection by CTV, although infections may be symptomless. However, some observations have indicated that susceptibility to CTV in some pummelos is isolate specific. Pummelo was grown for many years in Taiwan without apparent injury from citrus tristeza virus (CTV). Pummelo trees were often affected with Huanglongbing (greening or likubin) but did not show stem pitting symptoms or other evidence of CTV infection. Infections did not persist in pummelos inoculated with seedling yellows (SY) isolates commonly found in Ponkan and Tankan trees (11). In 1978, pummelo trees were observed which were stunted and exhibited extensive stem pitting (11). An isolate of CTV was recovered from these trees which readily infected healthy pummelos and caused stem pitting, but caused only mild symptoms in Mexican lime indicators and

little or no SY (9, 11). This isolate was designated CTV dwarfing strain (CTV-D). CTV-D continued to spread in Taiwan and has become a serious disease problem in pummelo plantings. Stem pitting problems have also been observed in pummelo in China (13). Some similarities between CTV-D and an isolate of CTV which causes severe stem pitting and dwarfing in Hassaku (a cultivar similar to pummelo) in Japan have been noted (10, 11).

Surveys for CTV were conducted in Hawaii from 1978 to 1980 to locate sites favorable for establishment of mild strain cross-protection tests (6). In the course of the survey, a vigorous pink pummelo (shaddock) tree was observed in a citrus variety planting. This tree, designated here as HA-Pink, was surrounded by grapefruit and sweet orange trees with obvious symptoms of stem pitting and was heavily infested with the brown citrus aphid, *Toxoptera*

citricida (Kirkaldy). No stem pitting was observed and the tree tested negatively for CTV by ELISA in repeated assays (6). Bud propagations of HA-Pink were made and graft-inoculated with several isolates of CTV from the CTV isolate collection at Beltsville, MD (5). HA-Pink plants inoculated with the Has-saku dwarf isolate (CTV-HD) showed mild stem pitting and tested positively when assayed by ELISA, but plants inoculated with other isolates did not show symptoms and tested negatively by ELISA (2).

Swingle citrumelo, a trifoliolate orange hybrid, has become a popular rootstock for citrus in Florida and some other citrus areas (1). Trees propagated on Swingle rootstock are tolerant to tristeza-induced decline and apparently are tolerant to citrus blight. Trifoliolate orange is highly resistant or immune to infection by nearly all isolates of CTV (3). This resistance is associated with a dominant genetic factor that is heterozygous in most sources of trifoliolate orange tested. Many trifoliolate hybrids, such as Carrizo citrange, inherit this resistance or immunity factor from trifoliolate orange. Initially, it appeared that Swingle citrumelo had also inherited CTV resistance from trifoliolate orange, but subsequently, infection by several isolates was observed (S. Garnsey, unpublished data).

Serological assays, such as ELISA, have provided a means to determine the relative replication of CTV in citrus plants and are particularly valuable for distinguishing between a tolerant response where the virus replicates without causing symptoms, and an immune or resistance response where the virus fails to replicate (3). Differences in replication rates of different isolates in different hosts have also been shown (7).

This paper reports results from a series of tests that were conducted to compare the reaction of different

pummelos to infection with CTV-D, CTV-HD, and two Florida isolates and experiments conducted to further evaluate the CTV susceptibility of Swingle citrumelo. Selective susceptibility to infection by different CTV isolates was confirmed in both pummelo and Swingle citrumelo. Implications for the evaluation of citrus germplasm for CTV resistance and international exchange of citrus germplasm are discussed.

METHODS AND MATERIALS

Propagation of test plants. Six selections of pummelo were used in these tests. Peiyu pummelo plants were bud-propagated from a virus-free source obtained by shoot-tip grafting in Taiwan. Plants of Thong Dee were also bud-propagated from an old line CTV-free Florida source. Plants of HA-Pink were propagated as budlings from a glasshouse-grown subpropagation made earlier from the original tree. This plant indexed free of CTV infection by ELISA and by graft-inoculation to Mexican lime indicators. Three seedling clones of HA-Pink, which were coded M802, M806, and M813, were propagated as budlings on rough lemon seedlings. Individual seedlings of HA-Pink were used in some tests. Swingle citrumelo plants were either nucellar seedlings or bud propagations from a single nucellar seedling on rough lemon. Bud propagations of virus-free Valencia sweet orange, Marsh grapefruit, and Cleopatra mandarin on rough lemon were used for comparison in some citrumelo tests.

Greenhouse conditions. Tests on pummelo were conducted in the quarantine facility for citrus pathogens, Beltsville Agricultural Research Center, USDA, ARS, Beltsville, MD (5). This house was lightly shaded and cooled by evaporative coolers in summer. From fall through spring night temperatures averaged 19° to 23°C, and day tem-

peratures were 25° to 32°C. Day maxima were higher in summer, and sometimes exceeded 37°C for several hours at midday. Tests on Swingle were conducted at the U.S. Horticultural Research laboratory, Orlando, FL, in glasshouses equipped with evaporative coolers. Conditions were generally similar, but maximum temperatures in the summer months were somewhat lower in Orlando. Plants were grown in Metro 500 (Grace-Sierra, Milpitas, CA) or Vertigro Bark Mix A (Verlite, Tampa, FL) potting mixes and fertilized by fertigation. Pesticides were applied as needed to control insect and mite pests. Growth of healthy plants was vigorous at both locations.

Virus isolates and inoculation procedures. Four isolates of CTV were used in the tests of pummelos. These were: B3 (Florida decline isolate T36 (8)); B51 (Florida mild isolate T4, an isolate used previously for testing host resistance to CTV infection (3)); B280 (CTV-D isolate WTdw-F-2-3, an isolate from a severely dwarfed Wentan pummelo near Hualein, Taiwan); and B31, an isolate of CTV-HD from Japan. These isolates were all maintained

in the CTV isolate collection at Beltsville (5).

The B51 and B3 isolates were also used for tests of Swingle. Additional isolates tested on Swingle were Florida mild isolates B2 (T30) and B52 (T55-1)(12), and B28 (T68), a more severe isolate recovered from Ellendale mandarin trees propagated from budwood imported without authorization into Florida (retained only for experimental use). Symptoms expressed by B28, and the other isolates in standard indicators (5) are summarized in Table 1. none of these isolates replicate in trifoliolate orange or Carrizo citrange at levels detectable by ELISA (data not shown).

Test plants were graft-inoculated using several blind buds or leaf pieces per plant. The plants were topped to force new growth approximately 3 wk after inoculation. New growth was confined to a single stem.

Sample collection and ELISA.

Unless noted otherwise, tissue samples were collected from new growth flushes. The sample consisted of 0.5 g of either the petiole and leaf midrib tissue of leaves approaching full leaf expansion, or bark tissue from a

TABLE 1
SYMPTOM EXPRESSION OF CITRUS TRISTEZA VIRUS ISOLATES IN STANDARD HOSTS

B Code ^a	Isolate ^b	Lime Reaction ^c	Decline Reaction ^c	SY ^c	GSP ^c	OSP ^c
B51	T4	2	0	0	0	0
B2	T30	0.5	0	0	0	0
B3	T36	2	2	1.5	0?	0
B52	T55-1	0.5	0	0	0	0
B28	T68	2.5	3	2.5	2.0	1.5
B280	CTV-D	1	1.5	0	2	0
B31	CTV-HD	2	0?	0	3	1.5

^aCode for isolate in citrus tristeza virus collection at Beltsville, MD.

^bOriginal identification of isolate, see text for more details.

^cRatings on a 0 to 3.0 scale, where 1 = mild, 2 = moderate and 3 = severe. Lime reaction is the composite score for vein clearing, stunting, and stem pitting in Mexican lime clonally propagated on Alemow. Decline reaction is stunting and chlorosis in grafted combinations of sweet orange on sour orange rootstock, SY is the seedling yellows reaction on sour orange and grapefruit seedlings, GSP is the rating for stem pitting in seedlings of Duncan grapefruit, and OSP is the rating for stem pitting in seedlings of Madam Vinous sweet orange. Data is from tests conducted at Beltsville, MD.

stem section collected at a point where leaves were nearly expanded. In the experiments where CTV titer was compared in different hosts, samples were collected as the flush reached optimum maturity on each individual plant and were stored frozen at -20°C or stored dry over Silica gel at 4°C until all plants were sampled. In citrusmelo tests, samples were collected for ELISA beginning with the second flush after inoculation. Two subsequent assays on new growth flushes were made. Two assays were made at 6-mo. intervals for the pummelo test.

Samples were assayed for presence of CTV by double antibody indirect (DAS-I) ELISA (4). Samples were extracted in 5 ml PBST with a dispersion homogenizer (Polytron, Brinkman Instruments, Inc.) or a Kleco tissue pulverizer (Kinetic Laboratory Equipment Co., Visalia, CA 93292). A ratio of tissue to buffer of 1 to 20 was used for most tests, but a 1 to 50 ratio was used in tests for isolate susceptibility in Swingle to better detect titer differences. The polyclonal antibody for coating was IgG purified from polyclonal antisera to Florida isolates T36 (1052) or T3 (908), and it was used at 1 µg/ml. Costar High Binding EIA plates (Costar, Cambridge, MA 02140) were used in most tests. The secondary antibody was a mixture of the monoclonal antibodies (MABS) 3E10 and 11B1 which were prepared to an isolate of CTV-D (9). These MABS individually react to most isolates of CTV, and in combination have detected all isolates tested to date (S. Garnsey, unpublished data). A commercial source (Boehringer Mannheim, Indianapolis, IN 46250) of goat anti-mouse IgG conjugated to alkaline phosphatase was used to detect bound CTV-specific MABS. Each sample was tested in two wells and each plate included PBST, extracts of healthy citrus, and extracts from citrus infected with B2 and B3 for reference.

Evaluation of symptoms.

Growth of the pummelo plants after inoculation was measured and plants were evaluated periodically for symptom expression. Ratings were made on a 0 to 3 scale for general visual symptoms which included vein clearing, chlorosis and leaf epinasty (Fig 1c). Stem pitting (Fig. 1d) was evaluated at the termination of the experiment by peeling the bark from the entire stem of inoculated plants. No visual symptoms were observed in Swingle and no ratings were made.

RESULTS

Effects of different CTV isolates in Pummelo. Effects of CTV infection in propagations of six selections of pummelo inoculated with four isolates are shown in Table 2. B51 caused mild symptoms in Thong Dee, and none in the other selections. B3 and B280 caused more wide spread visual symptoms and stunting (Table 2), and isolate B31 severely affected all selections except HA-Pink. Severity of foliar symptoms closely correspond to the growth ratings. Response of Peiyu and HA-Pink selections to the four isolates is shown in Fig. 1a and 1b. B31 caused stem pitting in all cultivars and B280 caused stem pitting in all except HA-Pink. Symptoms were generally more severe in plants inoculated with B31. HA-Pink was the least affected, but plants infected with B31 showed mild pitting (Table 2).

Based on ELISA results, all four isolates replicated in Peiyu and Thong Dee. B3 replicated in all pummelos except HA-Pink. Titer was lower in M806 than the other selections. Only B31 and B280 replicated well in HA-Pink (Table 2).

Susceptibility of HA-Pink seedlings. In the first test, graft propagations of 54 seedlings of HA-Pink inoculated with isolate B51 showed variable susceptibility with

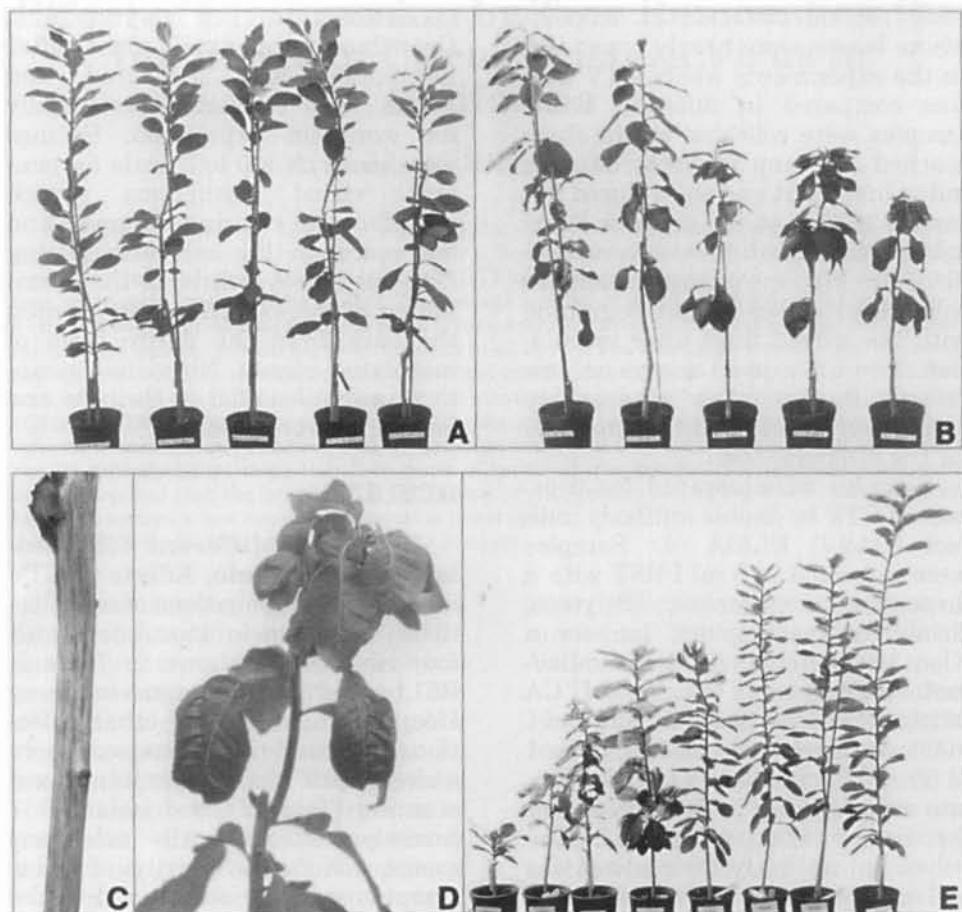


Fig. 1. Reaction of pummelo selections and seedlings to infection with citrus tristeza virus. A) Bud propagations of Hawaiian Pink Pummelo (HA-Pink) on rough lemon rootstock from left, uninoculated, inoculated with CTV isolates B51 (T4 from Florida), B3 (T36 from Florida), B280 (CTV-D from Taiwan) and B31 (CTV-HD from Japan). B) Bud propagations of Peiyu pummelo from Taiwan inoculated as in A. C) Stem pitting in Peiyu induced by CTV-D. D) Close up of leaf epinasty, leaf distortion and stunting in Peiyu infected with CTV isolate B31. E) Reaction of individual seedlings of HA-Pink 6 months after inoculation with CTV isolate B31.

generally poor CTV replication. Propagations of three seedlings (M802, M806, and M813) were tested at Beltsville, and all were susceptible to B31 and B280 (Table 2). A second group of 79 vigorous seedlings of HA-Pink pummelo were graft-inoculated with B31 at Beltsville in an attempt to find a tolerant or resistant seedling. All but six of the seedlings showed obvious effects of inoculation. Sixty-three seedlings showed vein clearing or leaf epinasty symptoms and 73 showed at least

mild stunting. All seedlings showed stem pitting and it was moderately strong to severe in 65 plants. The range of response of HA-Pink seedlings to B31 is shown in Fig. 1e.

Susceptibility of Swingle Citrumelo. CTV isolates B3 and B28 replicated well in Swingle while B51, B2, and B52 did not (Table 3, Test 1). B3 and B28 were detected at a 1/500 dilution from Swingle (OD_{405} of 0.32 and 0.29 respectively) indicating that replication of these isolates was more than 10-fold than

TABLE 2
RELATIVE GROWTH, STEM PITTING AND REPLICATION OF CITRUS TRISTEZA VIRUS AS MEASURED BY ELISA IN SIX PUMMELOS INOCULATED WITH FOUR CTV ISOLATES

Isolate	Cultivar					
	PEIYU	Thong DEE	M802	M813	M806	HA-Pink
Relative growth^a						
None	100	100	100	100	100	100
B51	118	103	101	106	95	103
B3	46	91	60	94	98	100
B280	41	72	80	88	104	100
B31	42	68	72	30	48	97
Stem pitting^b						
None	0	0	0	0	0	0
B51	0	1.0	0	0	0	0
B3	1.0	?	?	0.2	0	0
B280	1.5	2.0	1.0	1.0	1.0	0
B31	1.0	2.5	2.2	3.0	1.2	0.5
Relative OD₄₀₅^c						
None	1	1	1	1	1	1
B51	3	3	1	1	1	1
B3	4	5	3	3	1.5	1
B280	6	4	3	5.5	5	3
B31	6	5	5	4	5.5	3
Growth (cm)	147	114	158	139	216	161
OD ₄₀₅ -H	0.22	0.22	0.21	0.17	0.25	0.21

^aRelative growth as % of growth of healthy control for each cultivar (shown in lower box), mean of four replications.

^bRatings on 0 to 3.0 scale where 1.0 is mild, 2.0 is moderate, and 3.0 is severe.

^cOD₄₀₅ expressed as a multiple of the reading for extracts of the healthy control (mean for two assays, four replications). OD₄₀₅ values for healthy extracts (OD₄₀₅-H) of each cultivar are shown in lower box.

that for B51, B2, and B52, since the latter were not detectable even at a 1/50 dilution. In contrast, all isolates replicated well in Valencia and Marsh grapefruit, and assays of extracts diluted to 1/500 were still 10- to 30-fold greater than those of the healthy control. Results for Valencia sweet orange and Marsh grapefruit were essentially the same and only data for Valencia is shown Table 3. ELISA values for extracts from Cleopatra mandarin were consistently lower than those observed in Valencia orange, especially for B3 (Table 3). However, in contrast to Swingle, B51, B2, and B52 were all

detectable, even at a 1/500 dilution in Cleopatra.

A second test was conducted with Swingle and the same isolates. In this test we inoculated clonal propagations, as in test one, and seedling plants. The results for seedlings and bud propagations were the same and only results for the grafted propagations are shown in Table 3 (Test 2). To preclude the possibility that negative assay results with B51, B2, and B52 were due to inoculation failure, we assayed a bark patch removed from the rough lemon rootstock of each plant. All isolates were readily detected by ELISA in the

TABLE 3
DETECTION OF FIVE ISOLATES OF CITRUS TRISTEZA VIRUS IN SWINGLE CITRUMELO
AND THREE OTHER CITRUS CULTIVARS BY ELISA

Isolate	Test 1 ^a			Test 2 ^b	
	Valencia	Cleo	Swingle	Swingle Scion	R. Lemon Rootstock
None	0.02	0.02	0.02	0.04	0.06
B51	1.61	0.84	0.01	0.05	1.20
B2	1.38	0.50	0.01	0.07	0.85
B52	1.02	0.57	0.04	0.06	1.25
B3	1.33	0.10	0.86	0.51	1.49
B28	1.51	0.91	0.98	1.03	1.60

^aOD₄₀₅ values are mean of three separate assays for each of four replications of each cultivar grafted on rough lemon rootstock.

^bOD₄₀₅ readings are mean values for assays from three replications. Assays made from young bark tissue of Swingle scion and from bark patch taken from rough lemon rootstock. All extracts tested at a 1 to 50 ratio of tissue to extraction buffer.

rough lemon rootstock indicating that infection had occurred.

DISCUSSION

These results clearly demonstrate that infection and replication of CTV in pummelo vary according to the cultivar and CTV isolate involved. CTV-D and CTV-HD infected all pummelos tested. B3 infected all but HA-Pink while B51 infected only two selections. The susceptibility of Peiyu to B3 in these tests was somewhat surprising because Peiyu has not been affected by CTV isolates other than CTV-D in Taiwan. The HA-Pink selection was relatively tolerant in these short term glasshouse tests to CTV-D and CTV-HD, but the long term effects of these isolates on fruit quality and production in the field are unknown. Our results may help explain the divergent observations made on susceptibility of pummelo to CTV stem pitting in different locations.

We failed to find seedling selections of HA-Pink with resistance to CTV-HD, and most seedlings were apparently less tolerant than the parent. Screening larger populations and other pummelo selections may yet yield tolerant seedlings. Since the gene for CTV resistance in trifo-

liate orange is apparently effective against CTV-D and CTV-HD, hybridization of pummelo with a parent carrying the trifoliolate orange gene for CTV resistance may be the best approach to obtain selections with resistance to all isolates of CTV. Accurate evaluation of CTV resistance in new pummelo selections may be more complex than previously recognized, and it will require use of several isolates, including a source of CTV-D or CTV-HD. Selections tested only against local isolates in one location may perform differently when exposed to isolates originating from other locations.

The response of the pummelo selections tested was similar to CTV-D and CTV-HD, although symptoms were consistently more severe in plants infected by CTV-HD. Variations in severity among isolates of CTV-D and CTV-HD have been observed so comparisons based on a single isolate may not be representative. The origin of CTV-D and CTV-HD is unknown, but both probably arose via natural spread from another citrus cultivar. Hassaku is a Japanese cultivar similar to pummelo which apparently arose as a chance seedling in Japan and has been extensively exposed to natural infection. Spread of CTV-D into

pummelo in Taiwan was also apparently by vector from another cultivar. It is possible that a cultivar that is common to both Taiwan and Japan is the original source for both isolates. Many similarities have been observed between CTV-D and CTV-HD, but there are also some differences in biological properties, and a more detailed comparison at the molecular level is needed to establish the actual degree of relationship.

Our results also confirm that susceptibility of Swingle citrumelo to CTV infection is isolate dependent. The differences in susceptibility seem to be qualitative rather than quantitative since those isolates which do infect Swingle apparently replicate well. The lack of replication of Florida mild isolates in Swingle cannot be due to failure of inoculation or variation in host plants since variation in isolate replication occurred in clonal propagations, and these isolates replicated well in rootstocks of the same plant from which the Swingle scion tested negatively for CTV infection.

A negative ELISA test does not preclude the possibility that virus replication below the threshold of detection occurred, or that replication occurred sporadically under certain conditions. Application of more sensitive techniques, such as RT-PCR or graft-inoculation back to a permissive host, may clarify that issue; however, ELISA results from diluted extracts from plants infected with B3 and B28 effectively show that significant differences exist.

An extensive comparison of HA-Pink pummelo and Swingle for susceptibility to different isolates of CTV has not been done; however, results obtained with B3 suggest that the factors involved may be different because Swingle is susceptible to infection with B3 and HA-Pink is not. The selective resistance in both Swingle and pummelo is differ-

ent from the general CTV resistance factor in trifoliolate orange and none of the isolates used in these tests will replicate in trifoliolate orange at levels that are serologically detectable (S. Garnsey, unpublished data). Evaluation of hybrids for CTV resistance where strain-specific resistance factors and the more general trifoliolate orange resistance factor are involved requires use of several isolates of CTV to assess the nature of resistance present.

While ELISA values were lower overall for all isolates in Cleopatra than in sweet orange, they were especially low for B3. A similar variability in replication rates among CTV isolates in satsuma mandarin has also been observed (7).

The standard set of indicators commonly used to evaluate biological characteristics of CTV isolates (5) was chosen to measure reactions that are economically important in sweet orange and grapefruit. Use of cultivars such as HA-Pink pummelo and Swingle citrumelo may also be useful for further biological grouping of different CTV isolates.

Recognition that resistance to CTV in some cultivars may be selective has practical implications for movement of citrus germplasm. Introduction of infected cultivars, even into areas where CTV is already endemic, can introduce a new hazard. Similarly a cultivar considered resistant based on exposure to isolates in one location may behave quite differently when propagated at another site and exposed to another source of infection. For example, introduction of HA-Pink to areas where CTV-HD is endemic would result in infections that would be unexpected based on observations made only in Hawaii.

ACKNOWLEDGMENTS

The technical support of C. Gouin Behe, C. Halliday, and C. T. Henderson is gratefully acknowledged.

Acknowledgment is also made to Dr. M. Koizumi for making available the isolate of CTV-HD used in these studies. This work was supported in part by funding provided under the joint U.S.-Taiwan project TWARS 20 administered by RSED, FAS, USDA,

and the Council of Agriculture of Taiwan. It was also made possible by access to CTV isolates and facilities at the USDA quarantine greenhouse, Beltsville Agricultural Research Center, USDA, ARS, Beltsville, MD.

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