Numbers of Inclusion Bodies Produced by Mild and Severe Strains of Citrus Tristeza Virus in Seven Citrus Hosts

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ABSTRACT

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Inclusion bodies produced by citrus tristeza virus (CTV) in seven citrus hosts (*Citrus aurantifolia, C. aurantium, C. excelsa, C. hystrix, C. limon, C. paradisi*, and *C. sinensis*) infected with five biologically different isolates were stained with azure A and enumerated. Over all citrus hosts, the number of the inclusion bodies was positively related to strain severity and to virus titer as determined by ELISA. This trend was most evident in the more susceptible *C. aurantifolia, C. excelsa, C. hystrix*, and *C. sinensis* and least apparent in the less susceptible *C. aurantium, C. limon*, and *C. paradisi*. No differences were noted in the types of inclusions produced in the various hosts.

Plant closteroviruses are characterized by the occurrence of inclusion bodies that are confined mostly to the phloem and associated tissues and appear as large aggregates in arrays that are often cross-banded. Citrus tristeza virus (CTV), a closterovirus, has been shown to produce these inclusions, which are useful in diagnosis (2,6). Schneider (17) in 1959 first reported inclusions in infected citrus tissues as dark staining masses of stranded or needlelike objects in the cytoplasm of chromatic cells and suggested that these masses were aggregates of virus because of their staining characteristics.

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The presence of large aggregates of the flexuous CTV particles in the phloem cells of CTV-infected plants has been demonstrated by means of electron microscopy (4,11,13,18). Christie and Edwardson (5) and Garnsey et al (7) showed the presence of magenta-staining structures in azure A-stained sections of the phloem of CTV-infected citrus. Kitajima and Costa (11), in an electron microscopic study of CTV, reported that the most striking feature was the presence of fibrous inclusions that varied in size and appeared to be composed of elongated particles as well as other unidentified components. They observed that more inclusions occurred with severe CTV isolates in the more susceptible hosts but did not attempt to quantify them. Bar-Joseph et al (1) compared the cytopathology of three CTV strains from Israel and reported differences in the numbers of particles in seedling yellows strains vs. an ordinary strain but found no unique particles or structures. Sasaki et al (16) separated mild and severe strains of CTV using an immunofluorescent technique to label virus in sections of infected tissues but did not identify any of the structures as inclusions. Recently, Brlansky et al (3) showed that the inclusions that stain magenta using the azure A procedure are the same structures that fluoresce when immunofluorescent techniques are used.

In this paper, we report differences in the number of inclusions in various citrus hosts and relate the number to strain severity and to virus concentration in tissue.

MATERIALS AND METHODS

Virus isolates and plant materials. Five isolates of CTV were used throughout this study. The T-3 isolate, originally described by Grant and Higgins (10), causes severe decline of sweet orange (*Citrus sinensis* (L.) Osbeck) grafted on sour orange (*C. aurantium* L.), a seedling yellows (SY) reaction on Eureka lemon (*C. limon* (L.) Burm. f.), and severe veinclearing and stem pitting on Mexican lime (*C. aurantifolia* (L.) Swingle) and is rated as a severe isolate using the standardized host range (SHR) of Garnsey et al (8). Isolates T-36, T-4, T-30, and T-26 were described in detail by Rosner et al (14). The T-36 isolate is rated severe on the SHR (8); it causes decline of sweet orange on sour orange rootstock and severe symptoms on Mexican lime

but a mild SY reaction. The T-4 isolate, rated moderate on SHR, causes strong veinclearing, stunting, and stem pitting on Mexican lime but no visible decline of sweet orange on sour orange rootstock and no SY reaction. Isolates T-30 and T-26 are rated as mild isolates on SHR and produce mild symptoms on Mexican lime but no decline of sweet orange on sour orange and no SY reaction.

Each CTV isolate was graft-inoculated into five plants each of *C. hystrix* DC., Mexican lime, sweet orange, sour orange, *C. excelsa* L., Duncan grapefruit (*C. paradisi* Macf.), and Eureka lemon. These plants and healthy, noninoculated control plants were maintained in an air-cooled greenhouse at 21–30 C and arranged in a randomized complete block design on a greenhouse bench. Plants were trimmed to induce uniform new growth, and CTV infection was verified using double antibody sandwich enzyme-linked immunosorbent assay (ELISA) as previously described for CTV (1).

Sampling. Leaves were marked and measured for length and width daily in order to harvest plant tissues at a uniform stage of development. When the measurement was the same on two consecutive days, a leaf was considered fully expanded and was harvested. Three leaves from three growth flushes were harvested from each inoculated and control plant. Petiole samples approximately 1 cm long were excised from the leaves at the abscission zone, dried over silica gel, and stored as described previously (3). Samples were rehydrated in phosphate-buffered saline (PBS), pH 7.4, and 30- to 40-μm transverse sections were prepared using a Harris WRC cryostat-microtome (Harris Manufacturing, Inc., North Billerica, MA). The sections were stained with azure A according to the method described for CTV (7). Three randomly selected leaf petioles were sectioned from each plant, the sections were mounted, and the total number of inclusions were determined in 10 randomly selected sections.

Virus titer. The virus titer of each sample was determined from the remaining leaf petiole and 1 cm^2 of bark below the petiole using double sandwich ELISA (1). Antisera specific to whole unfixed CTV isolate T-26 was used for coating and conjugate.

Statistical analysis. The means for inclusion body and ELISA values were calculated for each of the plant-CTV isolate combinations. Data were subjected to analysis of variance, orthogonal contrasts, and Duncan's multiple range test, and correlations were tested using SAS (15).

Table 1. Number of inclusions and virus titer of five citrus tristeza virus isolates in seven citrus hosts

Host	Virus isolate	Number of inclusions/petiole section[y]	ELISA value (OD 405)
Citrus aurantifolia (Mexican lime)	T-3	61.82 a[z]	0.64 a
	T-36	49.96 b	0.70 a
	T-4	30.32 c	0.58 b
	T-30	12.78 d	0.50 c
	T-26	10.60 d	0.52 bc
C. hystrix	T-3	39.92 a	0.78 a
	T-36	43.86 a	0.80 a
	T-4	30.46 a	0.78 a
	T-30	10.60 b	0.70 b
	T-26	5.58 b	0.62 c
C. sinensis (sweet orange)	T-3	32.38 a	0.54 a
	T-36	17.98 b	0.55 a
	T-4	7.80 c	0.56 a
	T-30	7.46 c	0.46 b
	T-26	8.88 c	0.51 ab
C. excelsa	T-3	30.40 b	0.62 b
	T-36	56.14 a	0.72 a
	T-4	11.72 c	0.52 c
	T-30	9.18 c	0.46 d
	T-26	6.88 c	0.55 c
C. aurantium (sour orange)	T-3	4.64 b	0.37 b
	T-36	8.50 ab	0.46 ab
	T-4	13.76 a	0.54 a
	T-30	5.52 b	0.47 ab
	T-26	9.66 ab	0.43 b
C. limon (Eureka lemon)	T-3	11.56 b	0.35 b
	T-36	7.60 b	0.42 b
	T-4	27.90 a	0.59 a
	T-30	4.18 b	0.37 b
	T-26	6.50 b	0.42 b
C. paradisi (Duncan grapefruit)	T-3	20.92 a	0.38 a
	T-36	15.98 a	0.53 a
	T-4	24.28 a	0.56 a
	T-30	9.86 a	0.45 a
	T-26	15.92 a	0.49 a

[y] Average number of inclusions calculated from total number of inclusions in 10 sections randomly selected from three petioles from each of three plant virus combinations.
[z] Mean separation within host by Duncan's multiple range test, $P \leq 0.05$.

RESULTS

By use of the azure A staining procedure, inclusions were observed in all the citrus hosts infected with each of the five CTV isolates. Inclusions were not found in any healthy control plant. In Mexican lime and *C. hystrix*, the numbers of inclusions induced by the two severe isolates, T-3 and T-36, were significantly higher than those formed by the mild isolates, T-30 and T-26 (Table 1). In Mexican lime, the severe isolates had more inclusions than the moderate isolate and the moderate isolate had more than the two mild isolates. In *C. hystrix*, the moderate isolate had as many inclusions as the two severe isolates. The severe isolates also were easily separated from the moderate and mild isolates in sweet orange and *C. excelsa*. In the other three hosts—sour orange, Eureka lemon, and Duncan grapefruit—there was no relationship between the severity of the isolate and the number of inclusions. The largest number of inclusions was found with isolate T-3 in Mexican lime and with T-36 in *C. excelsa*.

When the isolates were compared using orthogonal contrasts, the number of inclusions, considered across all hosts, was significantly higher ($P \leq 0.001$) for severe isolates than for the moderate isolate, for the moderate isolate than for the mild isolates, and for the severe isolates than for the mild isolates. When similarly compared, there also was a significant relationship between isolate severity and ELISA values ($P \leq 0.05$). There was a significant positive correlation ($P \leq 0.0001$) between the number of inclusions and the ELISA values ($r = +0.65$). When viewed separately, there was no significant relationship in the numbers of inclusions in the severe isolates vs. the mild isolates in sour orange, Eureka lemon, and Duncan grapefruit. Only in *C. excelsa, C. hystrix*, and Mexican lime was a significant relationship ($P \leq 0.0001$) found in the ELISA values of severe vs. mild isolates.

DISCUSSION

At present, severe isolates of CTV can be differentiated from mild and moderate isolates only by their biological activity on a variety of citrus indicator plants. This method requires high-quality indicator plants, good plant growth facilities, and several months to complete. The differentiation of severe CTV isolates from other isolates is important for certification of plants in budwood registration programs as well as for selection and use of mild isolates for cross-protection against severe isolates. In this study, four citrus hosts were

identified as ones in which severe CTV isolates could be separated from mild isolates on the basis of the number of inclusion bodies formed and three citrus hosts were identified in which strains could be separated by ELISA titer values. In *C. hystrix*, separation of the severe isolates and the moderate isolate from mild isolates was done on the basis of ELISA titer. Across all hosts, there was a significant correlation between the number of inclusions produced and the ELISA titer of the virus in the tissue. There was a significant interaction for the numbers of inclusions between cultivar and isolates, indicating that all isolates do not behave similarly in all these hosts. This was probably because the moderate isolate (T-4) produced more inclusions than the severe isolates in hosts more resistant to CTV, i.e., Eureka lemon, sour orange, and Duncan grapefruit.

Muller and Garnsey (12) listed Mexican lime, *C. hystrix*, and sweet orange as some of the easier hosts to mechanically infect with CTV. Sour orange, Eureka lemon, and Duncan grapefruit were found to be more difficult to infect. Our results with ELISA titer value and number of inclusions agrees with the findings of both studies. Garnsey et al (9) studied the multiplication of CTV in various hosts using ELISA and found *C. hystrix* was the best host overall for all five CTV isolates tested. Mexican lime and *C. excelsa* also were found to be good hosts, whereas Eureka lemon and sour orange were found to be poor hosts. The results of our study were similar, showing that Mexican lime, *C. hystrix, C. excelsa*, and sweet orange were good hosts for formation of inclusions as well as for virus titer.

The differences in the number of inclusions formed by various isolates in the different plant species might reflect the biological activity of a particular virus isolate at the cellular level that is later expressed at the tissue and the whole plant levels. No apparent differences were noted in the types of inclusions formed by the various isolates, although different types of inclusions have been described.

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