Rapid Diagnosis of Citrus Tristeza Virus Infections by Sodium Dodecyl Sulfate-Immunodiffusion Procedures

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ABSTRACT

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An antiserum to sodium dodecyl sulfate (SDS)-degraded citrus tristeza virus (CTV) coat protein was efficiently produced by toe-pad injection of a rabbit. This serum reacted in SDS agar gel double-diffusion tests to extracts of citrus hosts infected with biologically different CTV isolates. The serum did not react to extracts of healthy citrus or extracts of citrus infected with other viruses. Purified CTV could be detected at concentrations as low as 1–2 μg/ml. Detectable amounts of CTV were found in most phloem-containing vegetative tissues, but the highest titer was consistently found in young shoot bark. Virus titer was highest in young tissues and declined, often quite rapidly under warm conditions, as tissues matured. Virus could be concentrated from aqueous extracts by precipitation with 6% polyethylene glycol 6000 and centrifugation. Tissue could be stored frozen, lyophilized, or air dried for testing. Results obtained from SDS-immunodiffusion tests of 120 field trees correlated well with indexing results obtained from graft inoculation of *Citrus aurantifolia* 'Mexican' lime indicators. The SDS-immunodiffusion procedure provides a simple, rapid approach to CTV identification applicable to both research and practical needs.

Many thousands of citrus trees are indexed annually to eliminate infected trees and to suppress further natural spread of citrus tristeza virus (CTV) in areas such as Israel (16) and parts of California (17), where extensive commercial plantings on the CTV-susceptible sour orange (*Citrus aurantium* L.) rootstock remain. Large numbers of trees also are indexed in certification and research programs. Although mechanical transmission of CTV was demonstrated recently (8), no herbaceous hosts have been found, and most indexing is done by graft inoculation of the indicator plant, Mexican lime (21). This procedure requires from 1 to 6 mo plus propagation of the indicator plants. Rapid determination of CTV infection can be made by electron microscopic examination of negatively stained extracts (3,10), but availability of electron microscopes and the limited number of samples that can be processed restrict use of this technique. Attempts to develop antisera to CTV have been reported (6,13,20); however, the production of a clearly specific CTV serum was first reported by Gonsalves et al (11). They purified a single isolate of CTV and produced antisera to formaldehyde-fixed and unfixed whole virus. The serum to unfixed virus reacted to sodium dodecyl sulfate (SDS)-degraded virus coat protein (15) and was used to identify CTV in crude extracts of citrus tissue by the SDS-immunodiffusion procedure (10,11). Although these results were highly promising, problems were noted with nonspecific reactions and with antibodies to healthy antigens. Application of the SDS-immunodiffusion system to practical diagnosis was suggested but not critically evaluated. Diagnosis of CTV by other serological methods has been suggested (6,20) but not thoroughly demonstrated.

We describe an efficient procedure for producing a specific CTV antiserum and define the factors essential for field detection of CTV by an SDS-immunodiffusion procedure.

MATERIALS AND METHODS

Virus isolates.—Three biologically distinct isolates of CTV (T-4, T-3, and T-26) were used extensively in this work. The T-4 isolate, to which our antisera were prepared, causes strong vein-clearing and stem-pitting symptoms in Mexican lime (8). It does not cause either visible decline of sweet orange (*C. sinensis* [L.] Osbeck) trees grafted on sour orange rootstocks or seedling yellows symptoms (14,21). The T-3 isolate was originally described by Grant and Higgins (12) and causes severe symptoms in Mexican lime, a decline in sweet orange trees on sour orange rootstock, and seedling yellows in Eureka lemon (*C. limon* [L.] Burm. f.) and sour orange seedlings. The T-26 isolate produces mild symptoms in Mexican lime but does not cause decline or seedling yellows symptoms. All of the above isolates are free of other detectable citrus viruses. Other CTV isolates are described as pertinent.

Virus purification.—Powdered frozen tissue was ground in a mortar and pestle in the presence of 0.1 M Tris (tris[hydroxymethyl]-aminomethane) buffer, and the extracted virus was concentrated and partially purified by two cycles of polyethylene glycol (PEG-6000) precipitation. It was further purified on Cs_2SO_4 or CsCl gradients. Virus centrifuged on CsCl gradients was fixed with formaldehyde prior to centrifugation. Purified virus

preparations were stored frozen at −20 C in 0.05 M Tris buffer, pH 8.0, which contained 5% sucrose, or were stored lyophilized in the same medium. These procedures have been described in detail (11).

Gel electrophoresis of citrus tristeza virus coat protein.—About 2 mg (assuming A_{260} = 2.0 for 0.1% concentration) of partially purified virus (after a single cycle of Cs_2SO_4 centrifugation) was centrifuged for 1.5 hr at 45,000 rpm in a Beckman Ti-60 rotor (Beckman Instruments, Inc., Palo Alto, CA 94304). The virus pellet was resuspended in 2–3 ml of 0.01 M sodium phosphate buffer, pH 7.0, which contained 1% SDS and 0.1% 2-mercaptoethanol. The mixture was heated for 1–2 min at 100 C and made 10% (w/v) with sucrose. Electrophoresis was done in 7.5% (w/v) polyacrylamide (24) with a Bio-Rad Model 301 (Bio Rad Labs., Richmond, CA 94804) fitted with one 13-mm or two 8-mm OD glass tubes. Current was applied at 8 mA per each 20-mm^2 cross-sectional area of gel (100 mm length). Gels were run for 6 hr at approximately 15 C and incubated at 4 C for 3 hr. The whiter protein-containing zone was visible without staining (18,23) and was excised with a razor blade and cut into 1-2 mm thick disks. Protein was eluted from the disks by gentle shaking in a small volume of distilled H_2O at 21 C for 8 hr. The elution process was repeated twice, and the eluates were checked by immunodiffusion. Most of the coat protein eluted in the first two washes. All eluates were combined, centrifuged 10 min at 6000 *g* to remove bits of gel, and concentrated by lyophilization.

Immunization of rabbits.—An aliquot of lyophilized, purified coat protein was dissolved in H_2O and emulsified with Freund's adjuvant (Difco, Detroit, MI 48232). A 0.15-ml aliquot of the emulsion that contained approximately 80–100 μg of protein was injected into the foot pad of a white rabbit (13). The antigen was emulsified in complete adjuvant for the initial injection and in incomplete adjuvant for the three subsequent injections given at 2, 8, and 21 wk. The rabbit was bled at approximate 1-wk intervals beginning 1 wk after the second injection. Antiserum was processed and stored as previously described (15). The antiserum to unfixed whole CTV (UFW-CTV) was as described previously (11).

Plant materials and growing conditions.—Tissues were collected from greenhouse, screenhouse, and field trees from March through December 1977. The greenhouse was air-cooled, partially shaded, and provided mild temperature conditions (21–27 C) in spring and fall and warm conditions (30–32 C) in summer. The temperatures in the field and screenhouse were lower than in the greenhouse at night in spring and fall and somewhat higher during the day in summer, with the daily maximum often reaching 35–37 C.

Most field material was collected from trees 3–5 yr old at experimental plots near Dundee and Orlando, Florida.

Tissue storage.—Unless noted otherwise, tissues were stored after harvest in plastic bags at 2–4 C. Samples were placed in stoppered glass vials or in sealed plastic bags for long-term storage at −20 C or −50 C.

Preparation of extracts.—Unless noted otherwise, tissues were triturated with a mortar and pestle in 0.2–0.5% SDS or in H_2O. Expression of undiluted sap from citrus is difficult, and extracts were normally prepared in two to three volumes (w/v) of extraction fluid. Extracts used for dilution tests were filtered through a small pad of glass wool in a 2–10-ml disposable syringe to remove debris.

Preparation of plates.—The medium used in most tests contained 0.8% Noble agar (Difco), 0.5% SDS (Sigma Chemical Co., St. Louis, MO 63178), and 1.0% NaN_3 (J. T. Baker Chemical Co., Phillipsburgh, NJ 08865). The SDS and NaN_3 were added after melting the agar. In some instances, to avoid nonspecific reactions, the SDS and NaN_3 had to be dissolved separately in distilled water before they were added to the melted agar. Normally, 12 ml of media was poured in each 15 × 100-mm disposable plastic petri dish. Wells, 7 mm in diameter and spaced 5 mm edge to edge, were cut with an Auto-gel punch (Grafar Corp., Detroit, MI 48238) in a standard pattern of six peripheral wells around a center well.

Reactants were loaded into the plate wells by pipet. An adjustable pipet with disposable, tapered plastic tips (Rainin Pipetman P-200D, Rainin Instruments Co., Brighton, MS 02135) was convenient, especially for dilution studies. Tips were trimmed to a wider orifice to pipet unfiltered extracts.

Recording of results.—Starting after 12–16 hr incubation, formation of precipitin zones was recorded periodically on data sheets that contained a diagram of the plate and a list of reactants. Plates were photographed 24–48 hr after loading under dark field illumination provided by a light box constructed with a black truncated cone over a circular fluorescent tube and an elevated, flat, black center area. Polaroid 665 film (Polaroid, Cambridge, MA 02139) provided a convenient combination of print and negative for our purposes. Norit A charcoal (Matheson, Coleman & Bell, Norwood, OH 45212) 15% w/v in H_2O was added to the wells prior to photography.

RESULTS

Reactivity of CTV-CP antiserum.—The antiserum prepared to the gel-electrophoresed, SDS-degraded CTV coat protein (CTV-CP) was free of detectable antibodies to healthy antigens (crude extracts in H_2O or 0.5% SDS and extracts concentrated approximately six-fold by high-speed centrifugation).

The CTV-CP antiserum reacted visibly to purified CTV (two cycles of centrifugation on Cs_2SO_4 gradients) at concentrations of 1–2 μg/ml in 0.5% SDS. Addition of healthy extracts (1:3, w/v in 0.5% SDS) to the purified CTV did not affect the sensitivity.

All bleedings contained detectable antibody to CTV, although the titer of the CTV-CP antiserum as measured by SDS immunodiffusion, was not very high. The dilution end point (DEP) for bleedings collected after the first two injections was 1/4 to 1/8. It increased to 1/16 after each subsequent booster injection but gradually declined to the initial level. In microprecipitin tests with purified virus (10 μg/ml), the DEP was 1/64 or greater. Undiluted antiserum was best for SDS-immunodiffusion tests; however, antiserum with a titer of 1/16 could be used diluted 1:1 or 1:2 with 5% bovine serum albumin in Tris-buffered normal saline (15) or normal serum. The γ-globulin fraction precipitated with $(NH_4)_2SO_4$ from CTV-CP antiserum and resuspended in normal saline did not react in SDS tests, whereas the same γ-globulin fraction resuspended in normal serum was reactive.

The precipitin lines in SDS plates were normally visible after 12-hr incubation and were quite distinct after 24 hr. Some additional strengthening of the reaction occurred in the next 24–36 hr, and weak reactions were sometimes detected only after 36–48 hr incubation. Generally, no improvement occurred after 48-hr incubation, and the precipitin lines gradually faded and often disappeared after 4–7 days. Addition of activated charcoal to the wells after 24-hr incubation (15) stabilized the precipitin lines for a number of days. However, addition of activated charcoal at 24 hr inhibited subsequent development of weak reactions.

Effects of agar composition and extraction media.—An agar medium that contained 0.8% Noble agar, 0.5% SDS, and 1.0% NaN_3 was effective with the

CTV antigen-antibody system (Fig. 1-a). A nonspecific reaction to the SDS in the extraction media occurred if SDS was omitted from the agar medium (Fig. 1-b). The nonspecific reaction disappeared in 5–8 days when charcoal was added to the wells after 24-hr incubation. No reaction occurred if NaN_3 was omitted from the agar (Fig. 1-c). An agar medium similar to that described by Tolin and Roane (19), which contained 0.8% Noble agar, 0.2% SDS, 0.7% NaCl, and 0.1% NaN_3, also worked well with the CTV system, although precipitin zones were broader and weak lines were more difficult to read. Pyrrolidine-degraded CTV extracts reacted to CTV-CP serum in agar containing 0.85% NaCl, 0.05M Tris pH 7.2, 0.03% NaN_3, and 0.7% Ionagar No. 2 (Difco); however, the precipitin zones were broad and sensitivity was no greater than in the SDS system.

Extracts of CTV-infected tissue, which were prepared in water, 0.2% SDS, and 0.5% SDS, all reacted with CTV-CP antiserum in plates containing SDS. Precipitin lines were sharpest with 0.5% SDS, however, and sensitivity was approximately 50% less when SDS was omitted.

No sap-related nonspecific reactions were observed with extracts from the citrus hosts tested.

Sample preparation and storage of samples.—Extracts for testing were effectively prepared by grinding tissue in the extraction medium (usually 0.5% SDS) with a mortar and pestle or with a homogenizer (SDT-Tissumizer, Tekmar Co., Cincinnati, OH 45222). The SDS was added after grinding when the homogenizer was used. The simplest procedure for routine purposes was to mash approximately 50 mg of tissue in a disposable polystyrene 5-ml beaker (Fisher Scientific Co., Pittsburgh, PA 15219) with a short stainless-steel rod, add 0.2 ml of 0.5% SDS, and incubate the contents for 30 min before loading the immunodiffusion plates.

In comparative tests, CTV titer was highest in extracts from tissues that were most completely disrupted , but detectable CTV was present even when tissue was only coarsely diced. Extracts prepared in SDS reacted about equally well at 30–120 min, and 24 hr (stored at 4 C) after preparation. Precipitin lines were slightly broader with extracts incubated for 24 hr.

Extracts in 0.5% SDS stored frozen at −20 C reacted as well as fresh extracts.

For large-scale indexing, collection of tissue during the spring growth flush and storage of samples for later testing is advantageous. To compare effects of storage procedures on virus titer, a composite batch of diced, sweet orange young bark from infected, greenhouse-grown plants was divided into uniform aliquots and stored as follows: fresh at 4 C, frozen at −20 C, dried in a desiccator at room temperature, and lyophilized at −20 C. No appreciable difference was observed among fresh tissue, tissue stored 16 days at 4 C, and tissue stored frozen, dried or lyophilized for 59 days. The titer in repeated tests was 1/64 to 1/128 for all samples. Dilution end points for field-collected tissue stored at −20 C for 6 to 9 mo were similar to those from fresh tissue.

Tissue selection and virus distribution and host effects.—Several experiments were conducted to determine which host tissues contained the highest CTV titer. When greenhouse-grown plants were sampled while producing a new growth flush under mild growing conditions (<30 C), CTV was detected in all parts sampled (Table 1, Fig. 4), with the highest titer occurring in the bark of the young flush. We have also observed that young flush bark has a higher titer than other vegetative tissues in numerous tests of greenhouse-grown and field-grown trees.

Bar-Joseph et al (1) (*unpublished*) observed that the bark from the fruit pedicel contains numerous CTV particles, and we found that CTV titer is higher in pedicel bark than in bark of comparable age from nonfruiting branches in field collections made in June and July; the CTV titer also is sometimes higher in pedicel bark than in bark from young growth of field trees. Albedo of immature fruit had a lower titer than pedicel bark of the same fruit, and CTV was barely detectable by SDS-immunodiffusion tests of albedo of mature Valencia oranges. We detected CTV in young feeder roots collected from field trees on sour orange and rough lemon rootstocks in July, August, and September.

To test for the distribution of CTV in a single plant, young bark from 13 shoots of field-grown Marsh grapefruit, collected April 21, was assayed separately. Twelve of the 13 shoots tested positively, and some differences in reaction strength were noted. In a subsequent test, 12 uniform shoots of greenhouse-grown sweet orange infected with T-4 were assayed separately. Although bark from all shoots assayed positively at a 1/8 dilution in 0.5% SDS, differences in reaction strength were apparent. Serial dilutions from the strongest and weakest samples yielded DEP of 1/128 and 1/16, respectively. Extracts from a single leaf midrib (including petiole) from the midregion of each of the 12 shoots gave comparable but weaker reactions. All seven leaves from one shoot tested positively. Extracts from the lower leaves reacted more strongly than extracts from the leaves near the tip. These results, coupled with observations from other tests, show that CTV is well distributed throughout infected plants but that variations in titer occur.

The titer of CTV in extracts from comparable young flushes of greenhouse-grown plants of Eureka lemon, *C. excelsa* Wester, sweet lime (*C. limettioides* Tan.), and sweet orange was similar. In all hosts, young bark extracts contained two to four times the titer of leaf midrib extracts. We have detected CTV in a wide variety of other hosts, including Etrog citron (*C. medica* L.), Hamlin,

TABLE 1. Titer of citrus tristeza virus in extracts[a] of different tissues from single greenhouse-grown plants[b] as determined by dilution end point in SDS immunodiffusion tests

	Reciprocal of dilution end point			
Tissue	*Citrus excelsa*	Eureka lemon	Sweet orange	Marsh grapefruit
Bark				
Young flush	64–128	64	128	128+[c]
Mature flush	64	32	32	8
Trunk	16	16	16	4/8
Leaf				
Midrib, young flush	64	16	32	64
Lamina, young flush	16	8	4	8
Young feeder roots	16	32	16/32	32

[a]Extracts prepared and diluted in 0.5% SDS.
[b]Plants infected with T-4 isolate to which CTV-CP antiserum was prepared.
[c]No dilutions greater than 1/128 were tested, but reaction strength at 1/128 indicated that further dilution was possible.

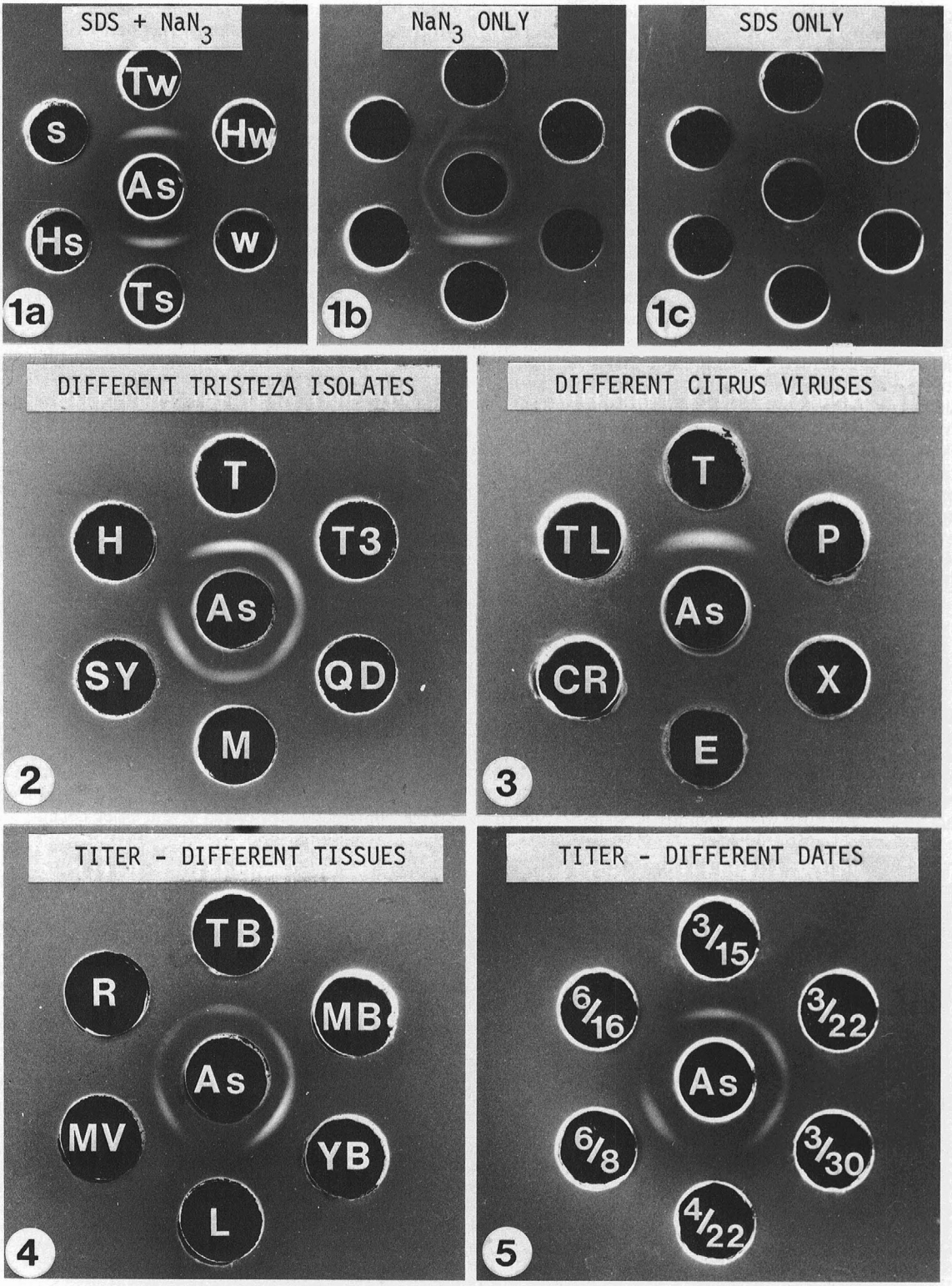

Fig. 1-5. Factors affecting serodiagnosis of citrus tristeza virus (CTV) in sodium dodecyl sulfate (SDS)-immunodiffusion tests. All tests involved media containing 0.8% Noble agar, 0.5% SDS, and 1.0% NaN_3, except as indicated in Fig. 1 a,b,c. Photos taken after 24–32 hr incubation. **1)** Effect of media composition on reaction with extracts from CTV-infected (T) and healthy (H) sweet orange prepared in H_2O (w) and 0.5% SDS (s) plus (w) and (s) controls; antiserum (As). **1a)** Medium contains 0.8% Noble agar, 0.5% SDS and 1.0% NaN_3. **1b)** SDS omitted and **1c)** NaN_3 omitted. **2)** Reaction of CTV coat protein antiserum to extracts of young greenhouse-grown sweet orange bark healthy (H) and infected with the homologous CTV T-4 isolate (T), the severe seedling yellows isolates T-3 and SY (from Meyer lemon), a Florida quick decline isolate (QD), and a Florida mild isolate (M). **3)** Reaction of the same serum to extracts of comparable young bark tissue from plants infected with tristeza (T), psorosis (P), xyloporosis (X), exocortis (E), citrus ringspot (CR), and tatter leaf (TL). **4)** Reaction with extracts from different tissues of an individual greenhouse-grown sweet orange plant at 1/8 dilution (w/v) in 0.5% SDS. Sources are trunk bark (TB), bark of mature green branch (MB), bark of young shoot (YB), leaf blade tissue (L), leaf and petiole midvein (MV), and young white feeder and pioneer roots (R). **5)** Reaction at 1/8 dilution of extracts from shoot bark collected from single Valencia orange tree at dates indicated. March and April samples from spring flush as it matured and June samples from newly formed and maturing summer flush.

Jaffa, Madam vinous, navel, Parson Brown, Pineapple, Pope Summer, Shamouti, and Valencia oranges, Murcott orange (*C. reticulata* Blanco hybrid), Temple orange (*C. sinensis* hybrid), sour orange, Marsh, Duncan, and Ruby Red grapefruits (*C. paradisi* Macf.), rough lemon (*C. jambhiri* Lush.), Orlando and Minneola tangelos (*C. reticulata* × *C. paradisi*), Mexican lime, and *C. macrophylla* Wester.

Trifoliate orange (*Poncirus trifoliata* [L.] Raf.) and some of its hybrids such as Troyer and Carrizo citrange (*P. trifoliata* × *C. sinensis*) are considered highly resistant or immune to CTV infection. Comparable young bark extracts were tested from a grafted combination plant of Carrizo citrange and sweet lime infected with the T-3 isolate of CTV. The extract from Carrizo was negative, whereas the extract of sweet lime was strongly positive.

Effect of sampling time.—To determine favorable times for collecting field samples and to determine changes in titer as new growth matured, three shoots were collected periodically during maturation of the spring and summer flushes from selected field-grown trees of Valencia orange and Marsh grapefruit at Dundee, Florida, and from seedling sweet orange and Duncan grapefruit trees near Orlando. The trees at Dundee were naturally infected, and the trees near Orlando were previously graft-inoculated with seven tristeza isolates, including T-4, T-3, and T-26. An initial test of most collections was made on extracts of 50 mg of fresh bark tissue mashed in 0.2 ml of 0.5% SDS. Additional tissue was stored frozen at −20 C to compare samples collected on different dates.

As shown in Fig. 5 and Table 2, the CTV titer was high in young spring-flush tissue of most host-isolate combinations tested and declined slowly over a period of several weeks as the flush matured. After 4–6 wk, the flush was mature and virus titer had dropped markedly. A new flush of growth (summer flush) appeared early in June under warm conditions (daily maximum, 33–37 C). Virus titers in this new flush were generally high and exceeded the titer in the spring flush at a comparable stage of development in some cases (Table 2). This flush matured rapidly, and the virus titer dropped more rapidly than during the spring flush. The overall pattern of cyclic fluctuation in virus titer was consistent for all isolate host combinations. Some variability noted may be isolate effects but also may reflect variation in tissue age, despite efforts to select tissue of comparable maturity at each collection date.

Samples also were collected sporadically from new flushes later in the summer. These were generally positive, but virus titer was not as high as that in earlier flushes.

When necessary, CTV can be concentrated by a single cycle of PEG-6000 precipitation. We diluted an aqueous extract of T-4 infected young bark to a concentration (1/32) in which the virus was barely detectable and added 6% PEG-6000 (w/v) and NaCl to 0.15 M. After 60-min incubation (room temperature), the mixture was centrifuged for 10 min at 10,000 *g* and the pellet resuspended in 1 ml of 0.5% SDS. The resuspended "concentrate" reacted very strongly, and redilution showed recovery of most of the original antigen.

We also mixed 1 g of infected tissue with 9 g of healthy tissue and prepared an extract in 40 ml of H_2O. The extract was filtered through glass wool, concentrated by PEG-6000 precipitation as above, and resuspended in 5 ml of 0.5% SDS. The initial extract (1/50 dilution of the virus sample) reacted faintly, but the resuspended pellet (ten-fold concentration of the extract) yielded a very strong precipitin zone in immunodiffusion tests. We could not precipitate the degraded CTV coat protein from extracts prepared in 0.5% SDS by the same PEG-6000 treatment.

Reaction to different citrus tristeza virus isolates and to other citrus viruses.—A key question for application of serology to indexing is the reactivity of an antiserum to different isolates. Limited tests (10,11) indicated that the antiserum prepared to unfixed, whole T-4 would react to other isolates that vary considerably in biological properties.

To confirm this observation, additional tests were conducted with an extensive collection of Florida CTV isolates. These included isolates from field trees on sour orange rootstock that showed severe quick decline, slow decline symptoms, and no obvious symptoms. The isolates also varied widely in their effects on Mexican lime. Several isolates that induce seedling yellows (SY) were tested, as well as one SY recovery isolate (22). In total, more than 109 sources were tested and all reacted

TABLE 2. Effect of sampling time on titer, as measured by SDS-immunodiffusion, of citrus tristeza virus in field-collected young bark tissue

Host	Tristeza isolate	Reciprocal of dilution end point[a]						
		March		April	May	June		
		21	31	14	16	8	21	29
Location A—Hiawassee								
Sweet orange	T-4	16	16	4	0	64	4	8
Duncan grapefruit	T-4	128	64	32	8	128	16	...[b]
Sweet orange	T-1	16	16	8	0	16	4	4
Duncan grapefruit	T-1	16	8	8	0	16	4	0
Sweet orange	T-3	64	64	32	16	32	64	...
Duncan grapefruit	T-3	16	16	8	4	32	16	...
Sweet orange	T-26	32	16	8	0	8	4	4
Duncan grapefruit	T-26	32	32	8	16	128	8	...
Sweet orange	Healthy	0	0	0	0	0	0	0
Duncan grapefruit	Healthy	0	0	0	0	0	0	0

Location B—Dundee		March			April		June			
		15	22	30	6	22	8	16	23	30
Valencia orange	Ti-1	32	32	16	8	4	16	4	4	8
Valencia orange	Healthy	0	0	0	0	...	...	...	...	...
Marsh grapefruit	Ti-9	32	64	64	16	16	128	128	64	32
Marsh grapefruit	Healthy	0	0	0	0	...	...	...	...	...

[a]Collections from March, April, and May were from spring flush as it matured. Collections in June were from new summer flush as it matured.

[b]... = not tested.

positively. Most reactions were similar in intensity to the homologous T-4 reactions, and no spur formation in precipitin lines was observed. Weaker reactions were observed with some isolates (Fig. 2), but relative virus titer was not determined. There were no observable differences in heterologous sensitivity between the UFW-CTV and CTV-CP antisera in SDS-immunodiffusion tests.

Neither antiserum reacted to bark extracts from young flushes of systemically infected, greenhouse-grown citrus plants infected with the following viruses: citrus leaf rugose, citrus variegation, psorosis, xyloporosis, exocortis, tatterleaf-citrange stunt, citrus ringspot (two Florida isolates), and Algerian navel orange viruses (Fig. 3).

Field indexing.—To determine the applicability of the SDS-immunodiffusion test for indexing field trees, 120 field trees were sampled during the spring flush of 1977. These included known infected trees (based on previous indexing on Mexican lime) and recently planted healthy trees free of CTV unless naturally infected. Nine shoots of new growth were harvested from each tree, divided into three equal samples, and coded. These samples were indexed independently by the authors for the presence of CTV. The virus content of all samples was unknown to two of us, and the content of 80 samples was unknown to all when the assays were made. Extracts were prepared by: (i) grinding 0.5 g young bark tissue in 1 ml of 1% SDS with a mortar and pestle, (ii) grinding tissue in water (1:4, w/v) with a mortar and pestle, and (iii) mashing about 50 mg diced tissue in a disposable beaker and adding 0.2 ml 0.5% SDS. Antiserum to unfixed, whole virus (UFW-CTV) (10,11) was used because the CTV-CP antiserum was not yet available.

Only one discrepancy occurred among our results. In repeated tests, two of us found one Marsh grapefruit tree negative, and one found it positive. Because indexing by graft inoculation from this source also has been inconsistent, erratic distribution of the virus among the original nine shoots from the tree was indicated. The comparison of indexing results by grafting with results by serology are summarized in Table 3. All of the infections previously determined by indexing on Mexican lime (G. D. Bridges, C. O. Youtsey, and M. Cohen, *unpublished*) in sweet orange, tangelo or mandarin scions were detected serologically. In addition, we discovered four new infections that had occurred since the previous index. These new infections were confirmed by electron microscopy (10).

With grapefruit scions, only 22 of 36 previously determined infections were detected serologically. The "positive" trees were assumed positive because they had been bud-propagated from an infected parent tree or found positive by prior indexing. Comparable young-flush tissues from 10 of these presumed-positive grapefruit trees, which tested negatively by serology, were collected again 1 week after the original test and indexed by graft inoculation to three Mexican lime seedlings. All indexed negatively on Mexican lime, indicating that the disparity in results was not attributable to the serological test. Erratic indexing results from grapefruit have been obtained previously by graft inoculation (M. Cohen and E. C. Calavan, *unpublished*).

DISCUSSION

Our results show that SDS-immunodiffusion procedures are practical for indexing for CTV. The essential requirements for this test are a specific antiserum, proper selection of tissue, and the proper media.

Immunodiffusion methods require an adequate supply of a CTV-specific antiserum. The production of sufficient quantities of highly purified CTV remains a challenge despite significant improvements in the purification process and means to identify source tissues with high CTV titer. However, the use of gel electrophoresis to purify CTV-CP simplifies preparation of high-quality inject antigens. This, coupled with use of the toe-pad immunization technique, markedly improved our efficiency in producing an antiserum. We estimate that a liter or more of antiserum could be produced from 1 mg of purified coat protein, and that 100–200 samples can be

TABLE 3. Application of serological indexing for detecting field infections of citrus tristeza virus in field trees of several cultivars

Host cultivar	Total trees (no.)	Trees presumed infected[a] (no.)	Trees found positive by SDS-immuno-diffusion tests[b] (no.)
Oranges			
Valencia	21	12	12
Sweet seedling	8	7	7
Hamlin	6	4	4
Navel	6	3	4[c]
Pineapple	7	3	5[c]
Parson Brown	3	1	1
Jaffa	2	1	1
Grapefruit			
Marsh	27	16[d]	11
Duncan	19	13[d]	9
Rudy Red	14	7[d]	2
Miscellaneous			
Orlando tangelo	2	1	1
Minneola tangelo	2	1	2[c]
Murcott "orange"	1	1	1
Temple "orange"	1	1	1
Robinson tangerine	1	0	0

[a]Status determined by prior indexing on Mexican lime or in a few cases by field symptoms.
[b]Coded samples of all trees tested independently by three people, CTV content unknown at time of test.
[c]New infections that occurred since previous index on Mexican lime (infection confirmed by electron microscopy).
[d]Ten of the 14 trees that were presumed positive but were negative by serological test were reindexed on Mexican lime. All 10 were negative on the retest also indicating that the serological results were accurate.

tested per milliliter of undiluted antiserum with the test procedures described.

Fortunately, for indexing purposes, the antisera produced to the T-4 isolate reacted to all CTV isolates tested. We do not know if it will react with all isolates from other areas or whether antisera prepared to other CTV isolates will show a similar broad reactivity; however, antiserum to the HD strain of CTV recently prepared by Tsuchizaki et al (20) reacted to other CTV isolates. Antisera to the T-3 and T-26 isolates are being prepared to further evaluate serological relationships among CTV isolates.

The key factor for accurate indexing by SDS-immunodiffusion procedures is proper selection of test samples. Our results demonstrate that CTV titer is cyclic in the citrus host and that it can vary dramatically. Similar results also were reported for citrus leaf rugose (citrus crinkly leaf-type virus) (9). Young tissue formed under moderate temperature conditions is the most desirable for testing. Because tissues can be stored for extended periods without loss of titer, samples should be collected when new growth is available and tested as convenient.

Young shoot bark is the preferred vegetative tissue to test, but young feeder roots and fruit pedicel bark can be used when young bark tissue is not available, especially in summer. Young roots of susceptible hosts contain a greater quantity of CTV than indicated previously by other methods (2); however, tissue from resistant rootstocks such as trifoliate orange and Carrizo and Troyer citranges would not be useful.

The problems encountered in detecting CTV in some grapefruit trees by serological methods or by grafting will require further study.

Limited tests indicated a higher CTV titer in fruit pedicel bark than in vegetative shoot bark of the same age, and it should be a useful source. Further data on the use of pedicel bark tissue will be published elsewhere (1).

Fruit albedo was described recently as a good source of CTV (20). Our limited results with albedo of sweet orange indicate that other tissues such as young bark and pedicel bark contain a higher titer of CTV. Multiple crops of succulent young citrus shoots with a relatively high titer of CTV can be produced year around under proper greenhouse conditions. In contrast, only a single fruit crop per year is produced and trees usually must be grown outdoors to obtain fruit in quantity.

Our SDS-immunodiffusion results with CTV are consistent with those reported for other groups of viruses, and the guidelines developed by Purcifull and Batchelor (15) for SDS-immunodiffusion tests are applicable. The agar medium used by Purcifull and Batchelor (15) or the modified medium of Tolin and Roane (19) will give satisfactory results. As shown in Fig. 1, inclusion of SDS and either NaN_3 or NaCl (19) in the agar is essential. An extraction media of 0.5% SDS works well, but the SDS concentration is not critical. Where weak reactions are expected, activated charcoal should not be added until after 36–48 hr incubation. Different well patterns and dimensions can be used, and smaller wells conserve antiserum, but spacing can be critical (15). With experience the basic procedures outlined can be modified as needed.

If antisera are diluted, they should be diluted with either normal serum or a serum protein solution such as bovine serum albumin (15). The relatively low DEP of our CTV-CP antiserum in SDS tests is consistent with SDS results for antisera to other plant viruses (15).

Additional development of rapid sample preparation procedures may be desirable for large-scale use. Only modest mechanical disruption of the plant tissue is necessary, since SDS solubilizes virus protein bound in inclusions or virus aggregates and cell membranes. The sensitivity of the present SDS-immunodiffusion test is certainly adequate for many indexing applications, and it can be further increased by concentrating extracts. This may be especially useful for indexing composite samples from several trees (3).

The application of enzyme-linked immunosorbent assay (ELISA) techniques (5) for indexing CTV is also being investigated (1). Enzyme conjugates have been prepared with antiserum to fixed, whole CTV and with antiserum to CTV-CP. This serological procedure offers greater sensitivity (1,5) and more efficient use of antiserum. ELISA tests are more complicated to set up than SDS tests, however, and ELISA is not as well adapted for quick tests of small numbers of samples.

No single procedure, serological or otherwise, solves all indexing problems. We believe that the SDS procedure described herein offers some significant advantages over previous methods of indexing for CTV and will complement others, such as ELISA, serologically specific electron microscopy (7), and detection of inclusions (4), that may be developed. It offers a method for testing large numbers of field trees with minimal facilities and equipment and requires only limited technical experience and skill. It is also an extremely useful research tool that provides rapid quantitative information needed for studies on purification, transmission, host tissue relationships, etc., of CTV.

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