Table 2. Effect of water ring herbicide treatments on tree growth (expressed as percent of untreated control).

Herbicide	Rate lb./acre	Tree height	Trunk diameter	Canopy volume
Trifluralin	6.3	100	138	141
Trifluralin	12.5	109	152	157
Trifluralin	25.0	94	92	94
Trifluralin	50.0	82	74	82
Napropamide	8.0	93	93	95
Napropamide	16.0	93	91	90
EPTC	8.0	88	85	83
EPTC	16.0	94	84	88
Oxadiazon	8.0	87	71	73
Oxadiazon	16.0	99	87	83
Oryzalin	8.0	111	144	149
Oryzalin	16.0	100	113	123
Norflurazon	4.0	98	86	88
Norflurazon	8.0	103	159	166
Oxyfluorfen	4.0	94	129	131
Oxyfluorfen	8.0	95	122	128
Pendimethalin	8.0	91	110	113
Pendimethalin	16.0	102	162	178
Untreated control	—	100	100	100
L.S.D. (5%)		9	13	14

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Proc. Fla. State Hort. Soc. 97: 53-56. 1984.

USE OF DOUBLE STRANDED RNAs TO DIAGNOSE CITRUS TRISTEZA VIRUS STRAINS[1,2]

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Abstract. **Double stranded ribonucleic acids (dsRNAs) were examined as a possible way to diagnose citrus tristeza virus (CTV) strains. The dsRNAs were purified from CTV-infected bark tissue by the use of CF-11 cellulose followed by polyacrylamide gel electrophoresis. The sizes of dsRNAs purified from 5 CTV isolates which differed widely in their biological activity were compared. Each CTV isolate had a 13 X 10^6 dalton dsRNA corresponding to the reported full-length replicative form as well as several shorter (subgenomic) dsRNAs. The number and sizes of the subgenomic dsRNAs for each CTV isolate were unique from each host but seemed to be affected by the citrus cultivar used as the host.**

Citrus tristeza virus is the most economically important citrus virus (2). Millions of trees on sour orange rootstock have been killed worldwide, and the situation is becoming worse as more severe CTV strains appear in new areas and spread by aphids and budwood increases. In Florida most CTV-tolerant or resistant rootstocks are susceptible to citrus blight, a serious disease of unknown etiology, which has become a major production problem in recent years.

Several serological methods have been developed to diagnose the presence of CTV in infected tissue (8). These serological methods provide a rapid, reliable means to detect the presence of CTV, however, they do not distinguish between mild and severe strains (8, 12).

A 1979 survey conducted by the serologically based enzyme linked immunosorbent assay (ELISA) of registered budwood sources which were being commercially propagated on sour orange (*Citrus aurantium* L.) rootstocks indicated that about 89% of the sweet orange [*C. sinensis* (L.) Osb.] budwood was infected with CTV (12). At the time the survey was conducted, there was no obvious CTV-induced decline problems associated with plants being propagated on sour orange. However, recently in Florida, there have been increasing instances where very dwarfed, stunted trees have resulted due to the presence of severe CTV strains in the budwood source trees.

The only means presently available to determine the severity of CTV strains is by evaluating the reaction produced on a number of indicator plants which takes 3 to 18 months. The recent occurrences of stunting of young trees underscores the inadequacy of serology assays to differentiate CTV strains and the problems with the long period of time needed to obtain information on severity by the use of index plants.

Most plant viruses contain a positive sense, single-stranded RNA genome. When a virus infects a host cell, the virus will begin to replicate or increase in number. To replicate, the virus must make more copies of its RNA genome. It does this by a process called transcription. Transcription results in the production of a double strand-

[1]Florida Agricultural Experiment Stations Journal Series No. 6109. This research was supported by Grant No. US-336-80 from BARD—The United States-Israel Binational Agricultural Research and Development Fund.

[2]I gratefully acknowledge the expert technical assistance of Laura L. Achhiredly and the use of plant tissue provided by Dr. S. M. Garnsey.

ed RNA (dsRNA) called a replicative form which contains both a positive sense and a negative sense, single-stranded RNA. Several positive sense, single-stranded RNAs are then transcribed from the negative sense copy, thus providing more copies of the viral genome. The full length replicative form of a virus is twice the molecular weight of the single stranded, genomic RNA. Plants not infected with RNA viruses or virus-like agents do not usually contain detectable amounts of dsRNAs or replicative forms (7). The dsRNAs associated with viral infections have been recently reviewed by Dodds et al. (7) and earlier by Ralph (14).

The replicative form or dsRNAs from virus-infected plant tissues can be extracted, purified, and analyzed for size by polyacrylamide gel electrophoresis (7). The recent refinement of techniques to purify and detect dsRNA has led to the discovery of smaller subgenomic dsRNAs in virus-infected plants (4, 5), including plants infected with citrus tristeza virus (CTV) and other viruses in the closterovirus group (6).

Some recent reports (6, 7) have indicated that smaller than full length dsRNAs could be templates for subgenomic single stranded RNAs and could therefore be a "fingerprint" of the entire genome. Analysis of such subgenomic dsRNAs may be useful for differentiation of CTV strains. The purpose of this work was to see if analysis of the dsRNA patterns on polyacrylamide gels could be used to diagnose Florida CTV strains.

Materials and Methods

CTV isolates and plant material. Five isolates of CTV which vary in their biological activity were used. The T4 isolate causes strong vein-clearing, stunting, and stem-pitting in Mexican lime (*C. aurantifolia* Swingle), no visible decline on sweet orange on sour orange rootstocks and no seedling yellows (SY) symptoms on sour orange or Eureka lemon [*C. lemon* (L.) Burm. f.] (9). Isolate T3 causes severe symptoms on Mexican lime, severe decline of sweet orange on sour orange rootstock, and SY symptoms on Eureka lemon and sour orange seedlings (10). The T36 isolate produces severe symptoms on Mexican lime, mild SY symptoms on Eureka lemon and sour orange seedlings, and quick decline of sweet orange trees on sour orange rootstock (11). The T26 and T30 isolates produce very mild symptoms and little to no stunting on Mexican lime, no SY symptoms on Eureka lemon and sour orange seedlings, and no noticeable decline of trees on sour orange rootstock.

Each of the 5 CTV isolates were established in *C. excelsa* L., Mexican lime, *C. hystrix* L., and Palestine sweet lime (*C. limettioides* Tan.). The plants were grown in a partially shaded, air-cooled glasshouse with temperatures ranging from 21 to 35°C.

Extraction of dsRNAs. The dsRNAs were extracted and purified from infected citrus tissue by modifying the procedure used to detect citrus exocortis viroid (1). Four g of tender bark tissue were pulverized in liquid nitrogen, then transferred to a 50 ml centrifuge tube containing 20 ml of extraction buffer (0.2 M glycine, 0.1 M Na_2HPO_2, 0.6 M NaCl at pH 9.6), 20 μl of 10% sodium lauryl sulfate (w/v), 20 μl of 2-mercaptoethanol, 50 mg of bentonite, and 5 ml of a mixture of liquified phenol/chloroform/pentanol (25:24:1 v/v/v). The tube was stoppered, shaken vigorously several times for the next 15 to 20 min, then centrifuged for 15 min at 10,000 g at 4°C. The aqueous phase was transferred to a sterile tube and the volume adjusted to 20 ml by addition of extraction buffer. The homogenate was made to 16.5% ethanol (v/v) by the addition of 4.2 ml of cold 95% ethanol. After mixing well, 1.0 g CF-11 Whatman® cellulose powder was added, the suspension was then shaken for 10 min at 4°C, then centrifuged at 10,000 g for 10 min at 4°C. The cellulose precipitate was washed 3 times with 25 ml of STE buffer [0.05 M Tris (Hydroxymethyl) aminomethane (Tris), 0.001 M Na_2ethylenediaminetetraacetic acid (EDTA), 0.1 M NaCl, pH 6.9] containing 15% ethanol. After the final wash in the centrifuge tube, the cellulose precipitate was resuspended in about 10 ml STE buffer with 15% ethanol and poured into polypropylene columns with plastic filter discs (Code: QS-Q, Isolab Incorp., Akron, OH 44321) fitted with 25 ml extension funnels (Code: QS-S, Isolab Incorp.) and washed well with about 50 ml STE buffer containing 15% ethanol.

Materials bound to the cellulose were eluted by adding 10 ml STE buffer, collected into autoclaved tubes, precipitated by adding 2 volumes cold 95% ethanol and 3 drops of 3.0 M sodium acetate and stored overnight at −20°C. The precipitate was collected by centrifugation at 10,000 g for 20 min at 4°C. The pellet was air dried, resuspended in 800 μl sterile water, then divided into two 400 μl aliquots in sterile 1.5 ml microcentrifuge tubes. Two volumes of cold 95% ethanol were added and 40 μl of 3.0 M sodium acetate and the tube was stored at −20°C.

Electrophoresis and visualization of dsRNAs. Immediately before electrophoresis, the ethanol-precipitated nucleic acids in the microcentrifuge tubes were collected by centrifugation for 10 min in a microcentrifuge at room temperature. The pellet was dried by placing over silica gel desiccant in a vacuum chamber for 5 to 10 min and resuspended in 40 μl sterile water. Then 10 μl of a solution containing 0.1% (w/v) bromophenol blue and 20% (w/v) sucrose was added and electrophoresis was performed using 5% polyacrylamide gels as described by Morris and Smith (13) on a 16 cm slab cell using 1.5 mm spacers. Electrophoresis was for 11 to 15 hr at 90 to 100 V. Double stranded RNAs with molecular weights of 2.18, 1.99, and 1.89×16^6 purified from *Pennicillium chrysogenum* Thom infected with a mycovirus were used as molecular weight standards (3). After electrophoresis, gels were stained for 15 min in 2.5 μg ethidium bromide per ml of 0.1 mM EDTA, then destained for 4 hr in 0.1 mM EDTA. Gels were viewed on a shortwave UV light (Model C-63, Ultraviolet Products Inc., San Gabriel, CA 91778) and were photographed using Kodak Contrast Process Pan film with UV and orange filters, or alternatively, using Kodak Kodachrome 64 slide film with UV filters.

Treatment with nucleases. Some resuspended dsRNA preparations were treated with 10 μg DNase prior to loading on the gel or, alternatively, the gels were treated after electrophoresis with DNase or RNase in high (0.3 M NaCl) and low ionic (0.01 M NaCl) strength 0.01 M sodium phosphate buffer, pH 7.0, as described by Castanho et al. (3).

Results

All Florida CTV isolates examined contained a large dsRNA of about 13.0×10^6 molecular weight as well as numerous shorter, "subgenomic," dsRNAs (Fig. 1). The large dsRNA corresponds to the expected full length replicative form (RF). A line sketch of a typical example of the dsRNA banding patterns for the 5 CTV isolates after extraction of dsRNAs from CTV infected *C. excelsa* bark tissue and electrophoresis on 5% polyacrylamide gels is shown in Fig. 1A.

The intense band between the top of the gel and the RF was identified as DNA because it was susceptible to DNase digestion and resistant to RNase digestion. The other bands remained after DNase digestion. In Fig. 1B,

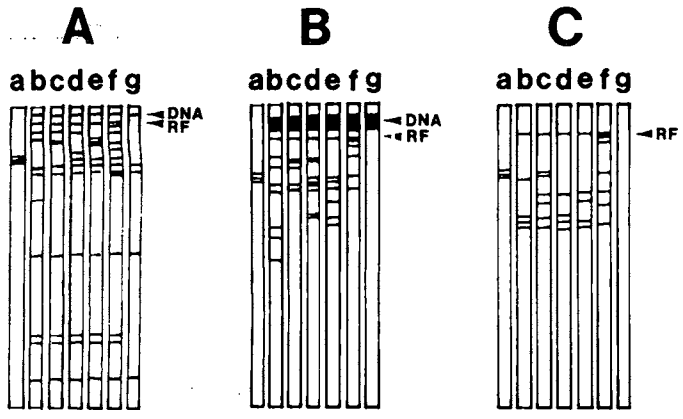

Fig. 1. Examples of typical dsRNA banding patterns on 5% polyacrylamide gels after electrophoresis. Lane a contains dsRNAs purified from mycovirus-affected *Penicillium chrysogenum* with molecular weights of 2.18, 1.99, and 1.89 x 10^6 daltons from top to bottom. Lanes b, c, d, e, and f are dsRNA extractions from bark tissue infected with CTV isolates T3, T4, T26, T30, and T36, respectively. Lane g is the extraction from healthy tissue. Extract from 2.0 g fresh weight of bark tissue was added to each lane. Band locations of DNA and full length replicative form (RF) are indicated. A) Extracts from virus-infected and healthy *Citrus excelsa*, electrophoresis was for 8 hr at 100 V. B) Extracts from virus-infected and healthy *C. hystrix*, electrophoresis was for 11 hr at 80 V. C) Extracts from virus-infected and healthy sweet lime (*C. limettioides*), electrophoresis was for 15 hr at 80 V. Samples were DNase digested prior to electrophoresis.

the DNA band is more diffuse and spread out, reflecting the longer electrophoresis time (15 hr) used for these gels as compared to the gel in Fig. 1A which was electrophoresed for 8 hr. The absence of the DNA band in Fig. 1C is because the samples were digested with DNase prior to electrophoresis.

In addition to the large dsRNA corresponding to the full length RF, each isolate also contained several smaller (subgenomic) dsRNAs (Fig. 1). These subgenomic dsRNAs, as well as the dsRNAs corresponding to full length RF, were demonstrated to be dsRNAs by their resistance to RNase 1A in high ionic strength (0.3 M NaCl) and their susceptibility to RNase 1A in low ionic strength (0.01 M NaCl).

Extractions made for dsRNAs from different plants of the same citrus cultivar infected with the same CTV isolate yielded a consistent number of dsRNAs which had the same banding patterns. At least 6 extractions have been made from each host for each CTV isolate. Typical dsRNA banding patterns for extractions from *C. excelsa, C. hystrix,* and sweet lime plants are illustrated in Fig. 1A, B, and C, respectively. However, when the dsRNA banding patterns of a particular CTV isolate was compared from the different hosts, it became apparent that the number and size of the subgenomic dsRNAs varied. For example, isolate T30 (Fig. 1A, B, C, lane e) contained a prominent dsRNA band at about 4.0 x 10^6 MW when extracted from *C. excelsa,* but not from *C. hystrix* or sweet lime.

There are some trends in the dsRNA banding patterns which seemed to be consistent for a given CTV isolate regardless of the citrus host. For example, isolate T36 consistently has a subgenomic dsRNA very close in size to the full length RF, giving the impression in the gel that the full length RF is a doublet (Fig. 1A, B, C, lane f). Isolate T4 tends to have a doublet of dsRNAs between the area where the *P. chrysogenum* dsRNAs and the full length RF migrate (Fig. 1A, B, C, lane c), but isolate T26 has a similar doublet when extracted from *C. hystrix* (Fig. 1B, lane d). The prominent dsRNA of about 4.0 x 10^6 MW is diagnostic of isolate T30 when using *C. excelsa* (Fig. 1A, lane e) or Mexican lime (data not shown) as the host.

Healthy plants used in this study did not contain any distinct dsRNA in the molecular weight range of 1.0 to 13.0 x 10^6. Occasionally, some banding patterns of low molecular weight RNA were seen in extracts from healthy tissue (Fig. 1A, B). Because of this, a dsRNA extraction from a comparable healthy control plant must always be run with each gel so that these nonviral RNAs will not be confused with viral dsRNAs.

Discussion

All the Florida CTV isolates examined contained a large dsRNA corresponding to the full length RF as well as numerous smaller subgenomic dsRNAs. The occurrence of the subgenomic dsRNAs has been previously reported (6), but only a limited comparison of 2 CTV isolates from a common host was reported previously (7).

The procedure for extracting and purifying dsRNA, while not suited to running large numbers of samples, permits one person to process and electrophorese up to 75 samples in a week. The use of slab gels permits better differentiation of small differences in molecular weight of dsRNA bands than does tube gel electrophoresis (1).

The function of the subgenomic dsRNAs is unknown although the occurrence of similar subgenomic dsRNAs has been reported for other virus groups (5, 7). It has been suggested that the subgenomic dsRNAs could be templates for transcription of viral messenger subgenomic RNAs and, as such, could be a "fingerprint" of the entire viral genome (6). This study with 5 CTV isolates which vary in biological activity suggests that the "fingerprint" of subgenomic dsRNA for each CTV isolate is indeed unique, but that the "fingerprint" may be influenced by the virus host cultivar. The variability of dsRNA patterns on gels between isolates makes it unlikely any single trait will be correlated with a specific biological activity such as decline on sour orange rootstock. The unique "fingerprints" for each CTV isolate from a given host cultivar, however, could be useful in future tests at a molecular level on mild strain cross protection. Further study is needed to determine the purpose of subgenomic dsRNAs and if they can be used to obtain probes specific for a CTV strain.

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Proc. Fla. State Hort. Soc. 97: 56-59. 1984.

RELIABILITY OF SPRAY TREATMENTS FOR REDUCING GREASY SPOT-INDUCED DEFOLIATION ON GRAPEFRUIT TREES[1]

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Abstract. **The reliability of different spray materials for controlling citrus greasy spot, caused by** Mycosphaerella *citri* **Whiteside, under heavy disease pressure was appraised. Results were compiled of 10 spraying experiments conducted from 1976 to 1983, in which the treatments were applied only once and in which heavy greasy spot-induced defoliation occurred by late winter. Basic copper sulfate, applied at 0.75 lb. product/100 gal (dilute), was the most reliable treatment for greasy spot control. Spray treatments with 1% (dilute) 435 oil or captafol at 0.5 lb. a.i./100 gal (dilute) sometimes equalled the copper treatment in effectiveness, but in some tests they failed to reduce disease severity significantly. Spray oil with 412 specifications was less effective than the standard 435 oil. Chlorothalonil reduced disease severity in some tests, but was never as effective as a copper treatment. Zineb, captan and folpet provided no control. Mancozeb significantly increased greasy spot in the one year in which it was tested.**

Greasy spot, caused by *Mycosphaerella citri,* is important mainly because it induces premature leaf drop. If excessive defoliation of citrus trees occurs before the end of the winter, the development of the spring growth flush is impaired and fruit yields can be reduced (10).

Despite the fact that greasy spot-induced defoliation has a greater potential impact on tree performance than the mere loss of photosynthetic area caused by the spots themselves, defoliation data have been used to quantify greasy spot severity only during the past 10 yr. Previously, disease severity was expressed only as the percentage of diseased leaves or leaf area with symptoms (9). This meant that disease assessments had to be made before substantial leaf drop began on nontreated check trees, often long before the end of the winter. When evaluated in that manner, some candidate materials showed more promise than was justified by later experience.

Earlier, defoliation data were not used to appraise greasy spot severity (9) because of considerations that other factors might cause premature defoliation. For example, citrus rust mite [*Phyllocoptruta oleivora* (Ashmead)] and citrus purple mite [*Panonychus citri* (McGregor)] were reported to cause leaf drop (4). After several years of monitoring of leaf drop from labelled grapefruit shoots, including some that were injured by these mites, I observed that greasy spot was the only biotic factor that could cause substantial abscission of leaves before they were 1 yr old. However, during the time of heavy seasonal leaf drop that occurs during March through May, many leaves, including healthy ones that were little more than 12 months old, did drop.

Greasy spot severity varies greatly from year to year, even in those groves that have a history of severe greasy spot. Disease severity depends on prevailing temperatures in the fall and winter (15) as well as on the amount of infection that occurs the previous summer.

Therefore, to appraise the reliability of different materials for controlling greasy spot more thoroughly, I have in this paper summarized the results of only those fungicide evaluation experiments conducted at the Citrus Research and Education Center, Lake Alfred from 1976 to 1983 in which substantial greasy spot-induced defoliation occurred by late winter. More comprehensive reports of some of these experiments have been published elsewhere (11, 13, 14, 16, 17).

Materials and Methods

The spray materials reported on in this summary of results were basic copper sulfate (53% copper), spray oils Sunspray 6E and 7E with FC412 and FC435 specifications (8), respectively; captafol (Difolatan) 4F and 80W Sprills; benomyl (Benlate) 50W; chlorothalonil (Bravo) 6F and 500; zineb (Dithane Z-78) 75W; mancozeb (Dithane M-45) 80W; captan (Orthocide) 50 W and folpet (Phaltan) 50W. No adjuvants were applied to any spray mixes. The rates of material applied are shown in Table 1.

The tests were conducted in groves of 'Marsh' and 'Ruby Red' grapefruit (*Citrus paradisi* Macf.) and sprays were applied with single-nozzled handguns. Some of the tests were made on 10- to 12-ft-high trees, with the whole canopy being sprayed. Other tests were made on 18- to 22-ft-high trees with only the southern half of each canopy treated, the other half being left as a buffer against spray drift. The volume of spray applied was equivalent to 8 to 10 gal for a 10-ft-high tree and 15 to 20 gal for a 20-ft-high tree. The only additional spray treatments that the trees received during the year of a test were with ethion, which has no effect on greasy spot and which was applied separately from the test treatments, up to 3 times per year, to control rust mites. The test treatments were applied to 6 to 9 (mostly 8) single-tree or half-canopy-tree plots arranged in a randomized block design.

In May or June, prior to spraying, shoots of the current year's spring growth flush were tagged. When the whole canopy was sprayed, 10 randomly selected shoots were labelled on the same four equidistantly spaced compass points on each tree. When only the southern half of each tree canopy was sprayed, 2 groups of 20 shoots each were labelled on the treated side of the tree. The total number of leaves on each shoot was counted before greasy spot-induced defoliation began and again in the winter, usually just as the spring growth flush began to emerge.

[1]Florida Agricultural Experiment Stations Journal Series No. 5822.