An Evaluation of Types of Citrus Tristeza Virus in Selected Sweet Orange Groves in Southern California

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ABSTRACT. Trees from thirty sweet orange groves were selected for detection and comparison of citrus tristeza virus (CTV). Most groves were expected to be potential sources of more severe forms of CTV than are commonly encountered in southern California. The initial evaluation was based on grafting pooled samples from several trees from a grove to grapefruit seedlings, which were subsequently evaluated for CTV symptoms. Ten trees from each grove were analyzed individually for CTV specific dsRNAs, and bark samples from the same trees were grafted to sweet orange seedlings which were subsequently analyzed for CTV specific dsRNAs. The dsRNA profiles tended to be fairly consistent for the trees in a given grove and varied from grove to grove. When variability was in evidence within a grove, the trees with non-conforming dsRNA profiles were invariably younger replacement trees. Specific bands that could be used to type CTV isolates were detected in the parental tree and in the sweet orange seedling used to propagate the CTV isolate in the greenhouse. A marker dsRNA (Mr 0.5 x 10^6) that is normally undetectable in typical California isolates of CTV was detected in some groves and an association was established between the detection of this dsRNA and the ranking for virulence established by the initial survey that was used as a basis for grove selection.

California has experienced severe losses to citrus tristeza virus (CTV) induced quick decline (9). This has resulted in the re-location of a major part of the citrus industry to the Central Valley where the use of sour orange and the accompanying susceptibility to quick decline was avoided in favor of rootstocks such as Troyer citrange. Plantings in the Central Valley are free of CTV for the most part (9). In southern California the citrus industry is not as fortunate and CTV is endemic. The strains normally encountered are not known to cause CTV-induced stem-pitting, and trees on tolerant rootstocks show no obvious CTV-related diseases.

Strains transmissible by *Aphis gossypii* Glover and causing stem-pitting have been detected in plantings at the University of California, Riverside. Plants harboring such strains were eradicated (1, 10). The knowledge that strains causing diseases related to scion stem pitting, chlorosis and decline, have caused severe and epidemic diseases in other countries such as Brazil (8) and Peru (11) has stimulated a new study in California. The objective of this study was to gather information on the range of virulence of CTV isolates which are currently established in the industry, so as to provide background information against which the introduction of exotic strains can be quickly recognized. This report presents a summary of a preliminary study that surveyed approximately 62,800 trees in 406 groves by biological indexing to identify potential sites where severe strains might be found. This was completed in 1986 by D. Cordas, California Department of Food and Agriculture (CDFA), and subsequently included analysis of dsRNAs of CTV isolates in field trees in 30 selected groves and in greenhouse indicator seedlings used to maintain isolates from such trees.

Previous work in our laboratory has established that characteristic dsRNAs accumulate in trees infected with CTV (2, 5, 7), and that specific dsRNAs can be used as markers for some strains of CTV (3, 4). One of the most interesting markers is a dsRNA with a Mr of 0.5 x 10^6. The ready detection of this dsRNA is associated with strains of greater virulence than is common for CTV isolates from California (6). A major portion of this study was to determine if this dsRNA could be detected in field trees and in greenhouse sweet orange subcultures

TABLE 1
ANALYSIS OF 30 SOUTHERN CALIFORNIA CITRUS GROVES FOR CTV

				CDFA survey[w]			Current survey[v]			
Grove ID[z]	Type	Age[y]	Num.[x]	DI	SPI	ELISA	dsRNA	0.5	Rating	Other
Latimer	Navel	Rp	3	na	na	na	2	1	2	0
Latimer	Navel	St	4	na	na	na	4	4	1	0
RV-0118-04-00	Valencia	St	10	1.78	0.07	+	10	9	3	2
RV-0156-01-07	Navel	St	7	1.20	0.10	+	7	7	3	3
RV-0156-01-07	Navel	Rp	3	na	na	na	3	1	3	0
RV-0209-01-01	Lemon	St	4	na	na	na	0	0	0	0
RV-0209-01-01	Navel	St	3	0.20	0.00	+	3	0	0	0
RV-0209-01-01	Valencia	Rp	3	na	na	na	1	0	0	0
RV-0221-01-00	Valencia	St	10	0.90	0.20	+	10	0	0	0
RV-0222-04-05	Valencia	St	3	2.29	0.00	+	3	3	3	1
RV-0222-04-05	Valencia	Rp	7	na	na	na	7	5	3	0
RV-0250-28-00	Lemon	St	10	0.00	0.00	+	0	na	na	na
RV-0255-07-00	Valencia	St	1	0.23	0.00	+	1	0	0	0
RV-0255-07-00	Gfruit	St	9	0.23	0.00	+	0	na	na	na
RV-0296-02-00	Valencia	St	1	0.71	0.07	+	1	0	0	0
RV-0296-02-00	Valencia	Rp	9	na	na	na	6	0	0	0
RV-0369-01-02	Valencia	St	10	1.84	1.31	−	10	1	1	0
RV-0510-01-01	Navel	St	8	0.99	0.44	+	8	8	2	0
RV-0510-01-01	Navel	Rp	2	na	na	na	2	0	0	0
RV-0614-07-00	Navel	St	9	1.15	0.40	+	9	3	2	1
RV-0614-07-00	Navel	Rp	1	na	na	na	0	na	na	na
RV-0628-01-08	Navel	St	8	1.50	0.80	+	8	8	3	0
RV-0628-01-08	Navel	Rp	2	na	na	na	1	0	0	0
RV-0636-01-08	Navel	St	10	1.10	0.50	+	10	10	1	1
RV-0641-06-01	Navel	St	2	1.60	1.00	+	2	2	3	0
RV-0641-06-01	Navel	Rp	8	na	na	na	7	7	3	0
RV-0786-06-01	Navel	St	10	0.00	0.00	+	10	3	2	0
RV-1080-01-02	Navel	St	5	1.85	0.33	+	5	5	3	5
RV-1080-01-02	Navel	Rp	5	na	na	na	5	5	2	2
RV-1138-01-00	Navel	St	3	1.15	0.05	+	3	0	0	0
RV-1138-01-00	Gfruit	Rp	7	na	na	na	0	na	na	na
RV-1185-01-00	Navel	St	10	2.20	0.00	+	10	10	3	8
RV-3497-01-01	Lemon	St	6	0.29	0.00	−	0	na	na	na
RV-3497-01-01	Lemon	Rp	4	na	na	na	0	na	na	na
SB-0036-03-00	Navel	St	10	0.00	0.00	+	10	10	4	2
SB-0090-02-00	Valencia	St	1	1.00	0.62	+	1	1	3	0
SB-0090-02-00	Valencia	Rp	7	na	na	na	7	7	3	0
SB-0090-02-00	Navel	Rp	2	na	na	na	2	2	1	0
SB-0167-01-05	nd	St	9	nd	nd	nd	9	9	3	7
SB-0168-01-00	Navel	St	6	2.60	1.00	+	6	6	3	1
SB-0168-01-00	Navel	Rp	4	na	na	na	1	1	3	0
SB-0378-01-03	Navel	St	8	0.58	0.17	+	8	8	3	7
SB-0378-01-03	Navel	Rp	2	na	na	na	1	1	3	0
SB-0412-04-00	Navel	St	9	1.45	0.35	+	9	9	2	6
SB-0412-04-00	Navel	Rp	1	na	na	na	0	na	na	na
SB-0421-02-00	Navel	St	10	0.90	0.20	+	10	2	2	0
SB-0424-01-02	Valencia	St	3	1.10	0.40	+	3	2	1	0
SB-0424-01-02	Valencia	Rp	7	na	na	na	5	4	1	0
SB-0631-05-00	Navel	St	10	0.65	0.07	+	10	0	0	2
SB-0910-02-AJ	Navel	St	10	1.20	0.50	+	10	2	2	2
SB-0910-02-00	Navel	St	5	1.20	0.50	+	5	0	0	0
SB-0910-02-00	Navel	Rp	5	na	na	na	4	1	2	0

[z]Identification system used by D. Cordas, CDFA. Data are sorted on this column.
[y]St = original, Rp = replant tree. Note that grove IDs are replicated in column 1 if the 10 sampled trees from a grove were a mixture of types.
[x]Number of trees of a given type that were sampled.
[w]Rating system used by D. Cordas, CDFA. DI = cumulative disease index (scale of 0 to 3.0, mild to severe) for the grove. The index has been assigned to the row for original trees wherever possible and is probably not applicable (na) for replants; SPI = stem pitting index which is included in the

of CTV from these individual trees. CTV-infected trees from groves with this property were detected.

MATERIALS AND METHODS

Inoculation of CTV to sweet orange. Samples were collected in the spring of 1987 from 10 trees from each of 30 groves in San Bernadino and Riverside counties, California. Groves were identified by D. Cordas, and were selected based on evaluation of the results of a CDFA survey of citrus groves in southern California. The T-bud or chip-bud technique was used to inoculate sweet orange seedlings which were maintained in a greenhouse. Inoculation consisted of four buds from budwood collected from individual field trees.

Double-stranded RNA analysis. Samples of green bark were collected in the spring of 1987 from field trees and four and 11 months later from CTV-inoculated greenhouse grown seedlings. Tissue was frozen, then ground to a powder in liquid nitrogen. Tissue powder was extracted in buffer followed by phenol, nucleic acids were purified by adjustment to 16.5% ethanol, and passed through a cellulose column to bind dsRNAs (3). Bound dsRNAs were eluted with ethanol-free buffer, concentrated by ethanol precipitation, and analyzed by 6.0% polyacrylamide gel electrophoresis (3). Three qualities were scored: the presence of the major RF dsRNA of CTV (Mr 13.3 x 10^6); detection and rating on a scale of 0 to 4 of a 0.5 x 10^6 CTV specific dsRNA (6, referred to as "0.5" in the tables); and detection of dsRNAs intermediate in size between these two extremes that were prominent in the gel lane for a specific sample, and were not one of the generally accepted additional dsRNAs commonly detected for California isolates (referred to as "other" in the tables).

RESULTS AND DISCUSSION

DsRNA in field trees from groves selected from the CDFA survey. We have analyzed 10 samples from field trees of each of the 30 groves under study. In addition, sweet orange seedlings inoculated with isolates from the 10 sampled trees from four groves have also been analyzed at two dates after inoculation. The total number of dsRNA samples analyzed is therefore approximately 340. Each result was recorded as a photographic positive of a lane of a polyacrylamide gel. Most of the data are summarized in table 1 and an additional summary of part of the data is presented in table 2. Selected gels have been used for figures.

Some observations from individual groves are as follows: Good results were obtained from 2 g of green bark tissue from CTV-infected field and greenhouse grown sweet orange trees. Navel and Valencia sweet orange gave similarly good dsRNA results from field samples and are summarized together. Grapefruit and lemon were generally negative for dsRNA, and so were not included in summary data for figure 3.

When a grove was composed of a mixture of citrus types, dsRNA data were normally positive for the sweet orange but not for the grapefruit or lemon in that grove. This may have a

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DI; ELISA = grove was positive for CTV by enzyme linked immunosorbent assay (ELISA); nd = not determined.

[v]DsRNA = number of trees which tested positive for CTV by dsRNA analysis; 0.5 = number of trees positive for the Mr 0.5 x 10^6 dsRNA marker; Rating = relative ease of detection of the Mr 0.5 x 10^6 dsRNA marker on a scale of 0 to 4 (hard to easy). Ratings of 3 and 4 have been associated with more severe forms of CTV in a previous study (6); Other = additional dsRNAs with higher molecular mass (Mr) than the Mr 0.5 x 10^6, none of which are normally detected for typical California CTV strains.

TABLE 2
COMPARISON OF dsRNAs IN FIELD TREES AND SWEET ORANGE SEEDLINGS

Grove[z] ID	Tree[y]			Field[x]				Greenhouse—1st harvest[x]				Greenhouse—2nd harvest[x]			
	Site														
	S	E	Age	dsRNA	0.5	Rating	Other[w]	dsRNA	0.5	Rating	Other[w]	dsRNA	0.5	Rating	Other[w]
SB-0910-02-00	20	4	Rp	+	+	2	−	−	na	na	na	+	+	1	−
SB-0910-02-00	5	27	St	+	−	na[v]	na	+	+	1	−	+	−	0	−
SB-0910-02-00	5	5	St	+	−	na	na	+	+	1	−	+	−	0	−
SB-0910-02-00	13	15	St	+	−	na	na	+	+	1	−	+	−	0	−
SB-0910-02-00	5	5	Rp	+	−	na	na	+	+	2	−	+	−	0	−
SB-0910-02-00	5	26	Rp	+	−	na	na	nd[v]	nd	nd	nd	+	−	0	−
SB-0910-02-00	20	12	St	+	−	na	na	+	+	1	−	+	−	0	−
SB-0910-02-00	20	11	St	+	−	na	+	+	+	1	−	+	−	0	−
SB-0910-02-00	13	15	Rp	+	−	na	na	+	+	1	−	+	−	0	−
SB-0910-02-00	20	5	Rp	−	−	na	na	−	na	na	na	−	na	na	na
SB-0424-01-02	5	5	St	+	−	na	na	−	na	na	na	+	−	0	−
SB-0424-01-02	16	5	Rp	+	+	1	−	+	−	0	−	+	−	0	−
SB-0424-01-02	16	4	Rp	+	+	1	−	+	+	1	−	+	−	0	−
SB-0424-01-02	29	4	Rp	+	+	1	−	−	na	na	na	+	−	0	−
SB-0424-01-02	29	4	St	+	+	1	−	+	−	0	−	+	−	0	−
SB-0424-01-02	7	5	St	+	+	1	+	+	−	0	+	+	−	0	+
SB-0424-01-02	27	4	Rp	+	+	1	−	+	+	2	−	+	+	1	−
SB-0424-01-02	5	5	Rp	+	−	na	na	+	−	0	−	+	−	0	−
SB-0424-01-02	7	4	Rp	−	−	na	na	−	na	na	na	−	na	na	na
SB-0424-01-02	27	4	Rp	−	−	na	na	−	na	na	na	nd	nd	nd	nd
RV-1185-01-00	20	59	St	+	+	3	+	nd	nd	nd	nd	+	+	3	+
RV-1185-01-00	5	6	St	+	+	3	+	+	+	3	+	+	+	3	+same
RV-1185-01-00	20	5	St	+	+	3	+	+	+	3	+same	+	+	3	+same
RV-1185-01-00	5	59	St	+	+	3	+	nd	nd	nd	nd	−	na	na	na
RV-1185-01-00	13	31	St	+	+	3	+	+	+	3	+same	+	+	3	+same
RV-1185-01-00	20	6	St	+	+	3	+	+	+	3	+same	+	+	3	+same
RV-1185-01-00	5	60	St	+	+	3	−	nd	nd	nd	nd	+	+	3	+same
RV-1185-01-00	5	5	St	+	+	3	+	+	+	3	+same	+	+	3	+same
RV-1185-01-00	13	32	St	+	+	3	+	+	+	3	+	+	+	3	+
RV-1185-01-00	20	60	St	+	+	2	−	nd	nd	nd	nd	+	+	3	+
SB-0090-02-00	14	16	Rp	+	+	3	−	+	+	2	−	+	+	3	−

SB-0090-02-00	25	5	Rp	+	+	3	−	+	+	2	−	+	+	3	−
SB-0090-02-00	25	27	Rp	+	+	3	−	+	+	2	−	+	+	2	−
SB-0090-02-00	25	27	Rp	+	+	3	−	+	+	3	+	+	+	3	+
SB-0090-02-00	5	27	Rp	+	+	3	−	+	+	3	−	+	+	3	−
SB-0090-02-00	5	28	Rp	+	+	3	−	−	na	na	na	+	+	3	−
SB-0090-02-00	14	16	St	+	+	3	−	+	+	3	−	+	+	3	−
SB-0090-02-00	5	5	Rp	+	+	2	−	nd	nd	nd	nd	+	+	2	−
SB-0090-02-00	5	6	Rp	+	+	2	−		nd	nd	nd	−	na	na	na
SB-0090-02-00	25	5	Rp	+	+	1	−		nd	nd	nd	+	+	1	−

[z]Identification system used by D. Cordas, CDFA.
[y]South (S) and east (E) row co-ordinates for tree location in the grove. St = original tree, Rp = replant tree.
[x]DsRNA result for samples collected from field trees, or from sweet orange seedlings graft inoculated with budwood from the corresponding individual field trees. Sweet orange seedlings were sampled four (first harvest) and 11 (second harvest) months after inoculation.
[w]The additional markers were the same (+same) or very similar (+) to those observed in the field tree used as a source of the isolate.
[v]na = not applicable; nd = not determined.

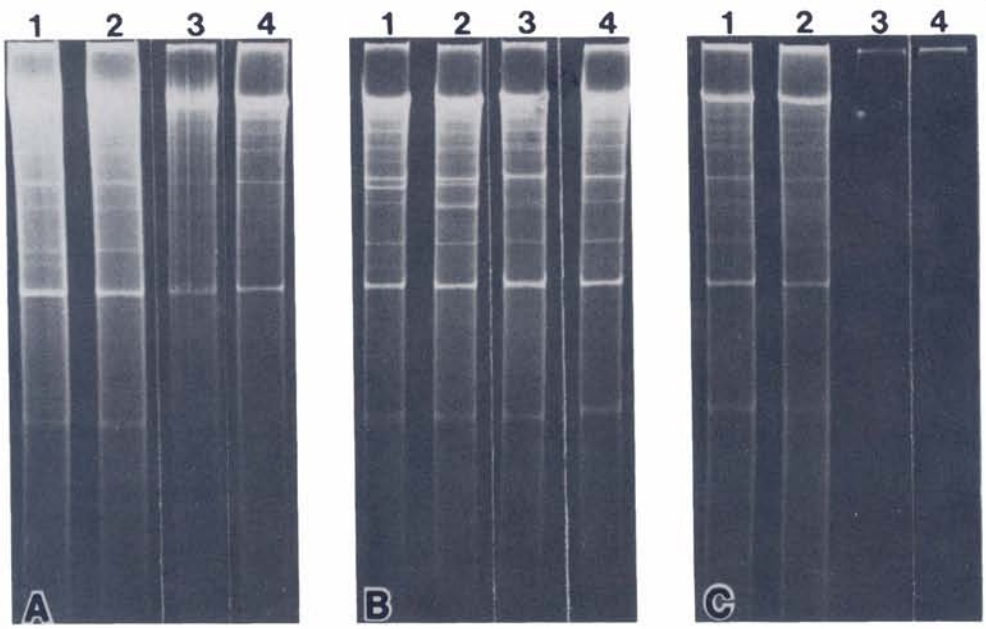

Fig. 1. Comparison of CTV dsRNAs in field trees from three sweet orange groves (A = RV-0510-01-01, B = RV-1080-01-02, C = SB-0168-01-00) in which the dsRNA result from original trees (lanes 1 and 2) differs from the results in younger replanted trees (lanes 3 and 4). In one case (grove C) the replanted trees have not yet become infected. DsRNAs were analyzed by electrophoresis in 6% polyacrylamide gels and were stained with ethidium bromide. Migration is from top (high Mr dsRNA) to bottom (low Mr dsRNA).

bearing on the data summaries of the CDFA survey, which most likely were from the grapefruits in a grove such as RV-1138-01-00. Grove RV-0209-01-01 is an interesting site which shows that spread from original navel to sweet orange replants has probably occurred without any equivalent spread to original lemons in the same grove.

Several observations lead to the conclusion that dsRNA analysis can be used to distinguish differences between CTV isolates in original and younger replanted trees in the same grove. Trees sampled from grove RV-0510-01-01 were eight older navel trees and two replants. The two replants were notable for the lack of any Mr 0.5 x 10^6 dsRNA, in comparison with the eight older trees which were all positive for this marker. Grove RV-1080-01-02 is an example of complexity in dsRNAs other than the Mr 0.5 x 10^6 dsRNA seen in the older original trees but absent from the replants. All trees in this case have readily detectable Mr 0.5 x 10^6 dsRNA. RV-0628-01-08 is another case where the two replants differ from the eight original trees, since one was not infected and the other had no detectable Mr 0.5 x 10^6 dsRNA. Figure 1 illustrates how the CTV dsRNA result of original trees may differ from the results in younger replanted trees. In contrast, grove RV-0641-06-01 has the Mr 0.5 x 10^6 dsRNA marker in replants and original trees, indicating that the grower may be propagating his own trees. These results from individual groves indicate that care should be taken in determining which trees to use for sampling, and points out a reason why new biological data are needed for individual trees to supplement the data from the CDFA survey. It also points out the value of dsRNA analysis for making potential strain distinctions. DsRNA results of CTV-infected replants are not always the same as those in the established trees in the same grove. Much more work is

needed to explain the basis for these differences.

Data for dsRNAs detected in 10 individual trees from each of four of the tested groves are summarized in figure 2. The dsRNA profiles for trees in a single grove are generally similar, and differences have been noted from grove to grove. The most notable conclusion is that the Mr 0.5 x 10^6 dsRNA that we believe to be an indicator of severity is not readily detected in some groves (fig. 2, groves A and B), but is quite obvious in other groves (fig. 2, groves C and D). A second observation is that the overall dsRNA patterns are noticeably more complex in samples from grove C and to a lesser extent from grove D than from groves A and B.

The CDFA survey was done in such a way that its value as a predictor of the type of virulence to be found in typical isolates from any grove is problematic. This is because several samples collected from one part of a grove were co-inoculated to a single Duncan grapefruit indicator seedling, and so the final reaction in the indicator, which was used to "score" a set of trees in a grove is not indicative of any single isolate. The final disease index for any grove was expressed as a number which ranged from 0 to 3.0 with 0 being healthy and 3.0 most severe. Despite these problems, we have attempted to look at the relationship between dsRNA analysis and the CDFA survey disease rating. Several criteria were used to narrow the comparison. The only groves used were those for which dsRNA data for five or more original sweet orange trees (not younger replanted trees) were available. This narrowed the comparison to 19 groves, and the data

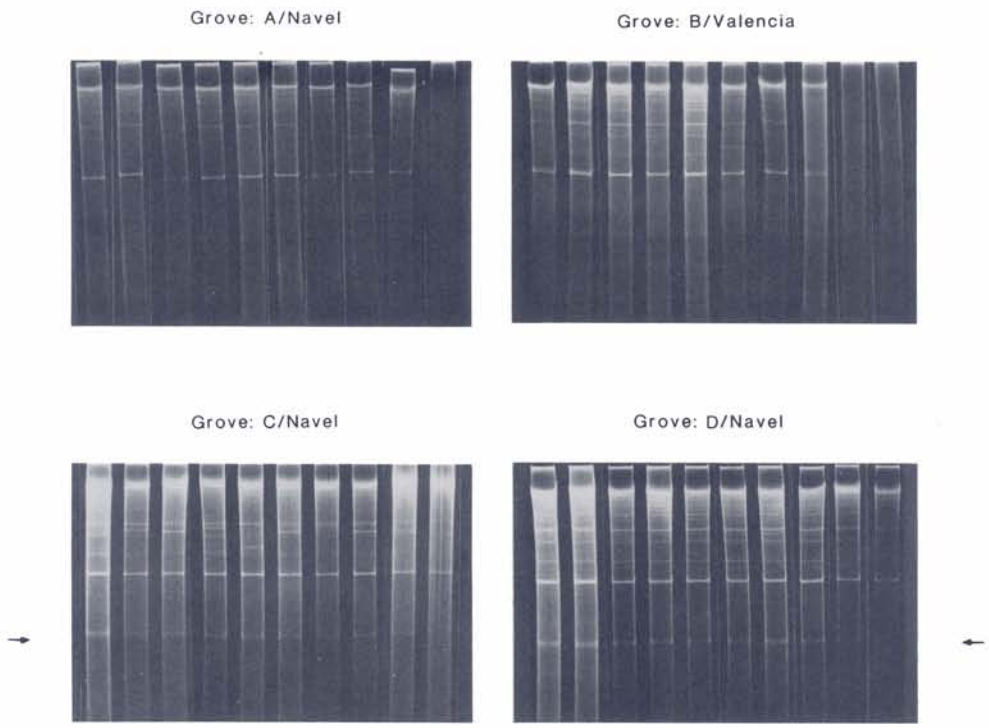

Fig. 2. DsRNA results for trees from four (A = SB-0910-02-00, B = SB-0424-01-02, C = RV-1185-01-00, D = SB-0036-03-00) of 30 groves selected by D. Cordas for this study based on a CDFA survey of CTV in California. Note the fastest migrating dsRNA component (arrowed, Mr 0.5 x 10^6) in panels C and D, which is generally absent or weakly expressed in panels A and B. DsRNAs were analyzed by electrophoresis in 6% polyacrylamide gels and were stained with ethidium bromide. Migration is from top (high Mr dsRNA) to bottom (low Mr dsRNA).

for these groves are summarized and presented graphically in figure 3.

The most general conclusion that can be drawn is that the groves with trees that show the Mr 0.5 x 10^6 dsRNA in an obvious way (ratings of 3 or 4) tended to have a high CDFA survey disease index (values of 1 to 2.4). This result is in agreement with our general conclusion that expression of the Mr 0.5 x 10^6 dsRNA is associated with virulence of CTV (6). In addition, there was a high frequency of detection of additional dsRNAs with molecular weights greater than Mr 0.5 x 10^6 in groves that were given ratings of 3 or 4 for the Mr 0.5 x 10^6 ds RNA. It is not known for certain whether these represent CTV dsRNAs or the dsRNAs of other viral agents that could be infecting these plants. On the other hand, trees from grove SB-0036-03-00 have all the characteristics of a severe isolate being present in that it is the only one with a dsRNA score of 4 for the Mr 0.5 x10^6 dsRNA, yet it had the lowest possible index value (zero) in the CDFA survey. Trees we tested that have been identified as potential sources of severe isolates based on dsRNA analysis are being biologically indexed (1, 10) (work in progress).

DsRNA analysis of selected CTV isolates in sweet orange seedlings. The results of dsRNA analysis of sweet orange seedlings used to maintain isolates from individual trees of four selected groves are summarized in table 2. Comparisons were made four and 11 months after inoculation. More seedlings were positive at the later date, and rating for the Mr 0.5 x 10^6 dsRNA was easier to read.

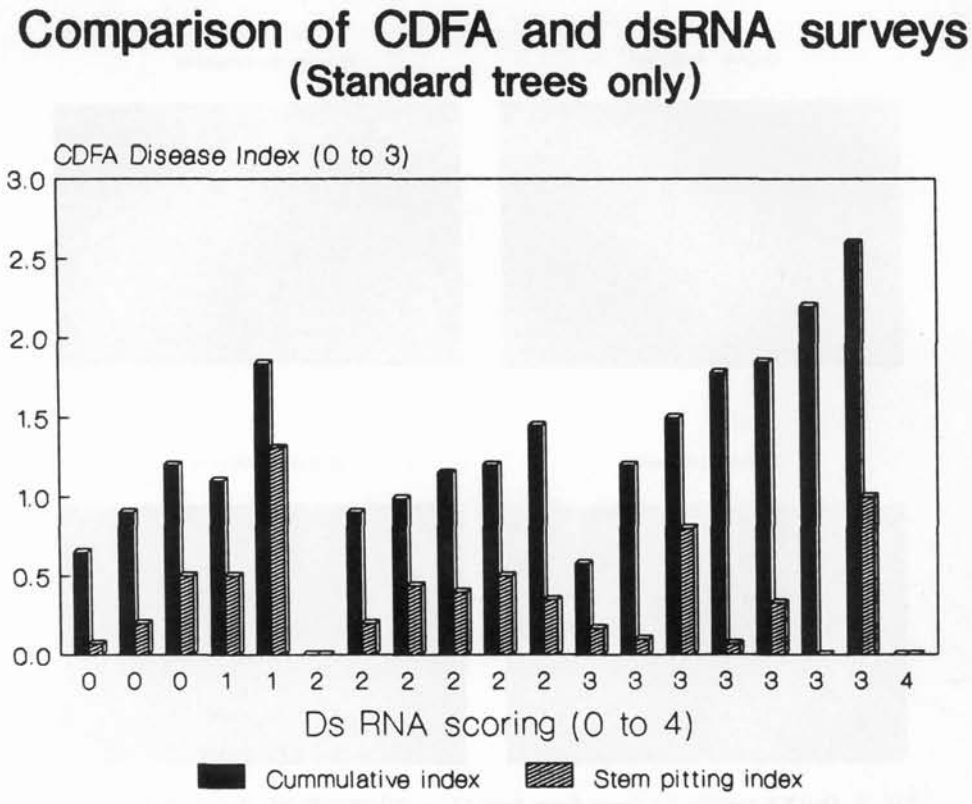

Fig. 3. Comparison of disease indexes and stem pitting indexes with ease of detection of a CTV marker dsRNA (Mr 0.5 x 10^6) in 19 groves. Each vertical group of bars represents data from one grove. In each grove five or more of the trees sampled for dsRNA analysis were original trees (not replanted trees), and these were the only trees used to establish the dsRNA score. See legend for table 1 for explanation of ratings for CDFA disease index and dsRNA scoring.

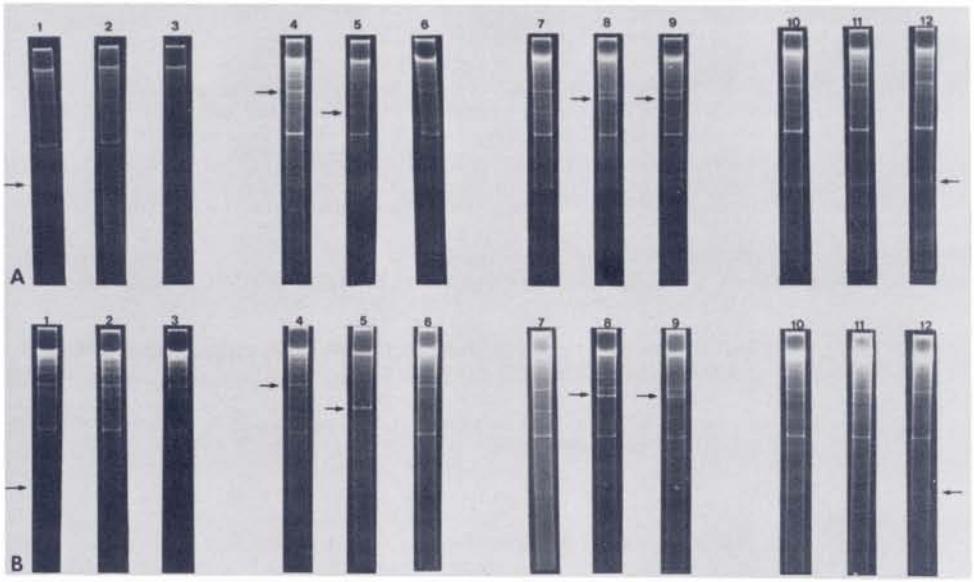

Fig. 4. Comparison of CTV dsRNAs in field trees (A) and sweet orange seedlings (B) used to maintain isolates obtained from individual trees. Results are for three trees from each of four groves (lanes 1-3 = SB-0910-02-00, lanes 4-6 = SB-0424-01-01, lanes 7-9 = RV-1185-01-00, lanes 10-12 = SB-0090-02-00). Expected migration of a marker dsRNA (Mr 0.5 x 10^6) is indicated with arrows placed on either side of the panel of results. Other higher molecular weight marker dsRNAs unique to specific isolates are indicated with additional arrows within the panel of results. DsRNAs were analyzed by electrophoresis in 6% polyacrylamide gels, and stained with ethidium bromide. Migration is from top (high Mr dsRNA) to bottom (low Mr dsRNA).

Trees from groves SB-0910-02-00 and SB-0424-01-02 were examples of the type which did not strongly express the Mr 0.5 x 10^6 dsRNA (ratings of 0 or 1). This was also true for the isolates established in sweet orange seedlings. By contrast the Mr 0.5 x 10^6 dsRNA was readily detected in samples from groves RV-1185-01-00 and SB-0090-02-00, and also in the sweet orange seedlings inoculated with these isolates.

Additional specific markers were noted in some trees, especially in grove RV-1185-01-00, and these were usually evident in corresponding isolates maintained in sweet orange seedlings. These results confirm and extend the conclusion that CTV dsRNA profiles can differ from strain to strain (6) and the differences can be quite stable, especially in sweet orange. Typical examples of dsRNAs from three trees from each of four groves are given in figure 4. Trees from groves A and B generally lack the Mr 0.5 x 10^6 dsRNA, whereas trees from groves C and D express this marker. Specific additional dsRNAs associated with individual trees from groves B and C are marked with arrows within the figure.

It appears that CTV may vary from grove to grove, and that dsRNA analysis is a good approach to monitor this variability. We believe that a continued effort to describe the variability of CTV in California is important if the detection of exotic, severe strains is to be timely, and management of such introductions is to be effective.

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