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Title

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Journal

International Organization of Citrus Virologists Conference Proceedings
(1957-2010), 15(15)

ISSN

2313-5123

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Publication Date

2002

DOI

10.5070/C58bd3p4vn

Peer reviewed

An Economic Sampling Protocol for Locating *Citrus tristeza virus* Reservoirs in a Large Area

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ABSTRACT. In citrus-growing areas where the incidence of *Citrus tristeza virus* (CTV) is low and CTV spread is suspected, a sampling strategy that maximizes limited resources and allows coverage of large areas would be useful to locate CTV reservoirs. We developed a strategy to estimate CTV incidence over a large area by uniformly sub-sampling portions of orchards. Our sampling method was tested in 79 blocks of citrus ranging in size from 2 to 16 ha in Central California. Some knowledge of the CTV incidence in each field existed from surveys conducted previously. Each orchard was considered as a separate plot. A subplot of 20 rows by 20 trees (400 trees total) or equivalent was selected in the center of each block regardless of the orchard size or planting density. Each subplot was surveyed using the hierarchical sampling (HS) method and the results compared to previous HS or singles surveys conducted over the entire orchard conducted by the Central California Citrus Tristeza Virus Eradication Agency. The subplot HS procedure was effective in finding CTV reservoirs in three small test plots where singles survey indicated virus incidence was 5.3, 5.0 and 0.5%. When tested in the 79 plots, the subplot HS method was successful and reliable at finding CTV only when incidence was >1%. Therefore, caution should be used if this abbreviated HS system is adopted where incidence is extremely low. The best strategy for its use is to survey a large area by the subplot HS method to locate areas of CTV incidence >1%. Once located, these areas can be prioritized for more thorough surveys or other actions.

Index words. Surveys, epidemiology, vector transmission, dissemination.

Citrus tristeza virus (CTV) is a regulated disease in California (2). In Central California where over 90,000 ha of commercial citrus is grown, approximately 47,000 ha are subject to mandatory eradication of CTV (3). Spread of CTV in California occurs principally by the cotton aphid, *Aphis gossypii* Glover (6). However, a CTV isolate from southern California shown to be efficiently vectored by the cotton aphid (14), has been eradicated, and CTV spread has been sporadic and slow in most areas of Central California. Surveys conducted during 1998-2001 by the Central California Tristeza Eradication Agency (CCTEA) Tulare, CA, indicate that the overall CTV incidence in the eradication area (= suppressive area which includes the Pest Control Districts of Central Valley (Fresno), Southern Tulare, and Kern Counties) is 0.137% (4). In contrast, the last survey conducted in 1996 in the Tulare County Pest Control District (non-eradication district) indicated an overall CTV incidence of 1.65%. We have con-

ducted vector tests with various isolates of CTV from Fresno to Kern Counties and have found isolates ranging in transmission efficiency by the cotton aphid from low (<1%) to over 40% with 5 to 10 aphids per plant (Yokomi, unpublished data). Significant natural spread has occurred recently in the non-eradication districts and now poses a serious risk to adjacent CTV suppressive districts.

Currently, CTV isolates in Central California do not appear to induce stem pitting at levels that reduce citrus yields. However, as in other areas, molecular evidence indicates that most CTV isolates here are composed of a population of genetically related variants (13). Moreover, aphid vectors have been shown to transmit isolates which can differ significantly from the parent isolate in molecular, serological and virulence characteristics (1, 12, 13, 15, 16). Therefore, in suppressive districts where the incidence of CTV is low or CTV is suspected, a sampling strategy that is economi-

cally efficient (e.g., maximizes limited resources and allows coverage of large areas) is useful to locate areas of higher CTV incidence or reservoirs. Once such an area is found, additional surveys can be conducted to delimit the area and appropriate eradication measures implemented.

A subplot sub-sampling system was developed and tested to locate CTV reservoirs and is reported herein.

MATERIALS AND METHODS

Plot locations and sampling methods. The test area was located in a 1,036 ha area of commercial citrus totaling 621 ha in Kern Co., CA which is in a suppressive district with an overall CTV incidence estimated to be 0.111% in 1999-2000 (4). The sample area contained 79 citrus orchards ranging in size from 2 to 16.2 ha and was mostly Navel or Valencia oranges and four blocks of Minneola tangelos. These plots were located in four adjacent sections each 259 ha in size in the same township and range. Data collected in each section were kept separate and served as four replicates for the analysis. There were 22, 26, 21, and 10 observations (individual plots) in each replicate, respectively. Two survey methods were used in this report: 1) singles sample where a total of eight leaves were taken from all quadrants of a tree and every tree was tested; and 2) hierarchical sample method (HS) as described by Hughes and Gottwald (11). The CTV status of all orchards in the study area were known from estimates made by the CCTEA during surveys conducted from 1998-99 to spring 2001 using either HS or singles survey from entire fields. Each citrus orchard was considered as one plot. A subplot of 20 rows by 20 trees (400 trees total) or equivalent was selected in the center of each block regardless of orchard size or planting density. Within these subplots,

HS sampling was conducted by the senior author's (RKY) research team. Twenty-five four-tree quadrats per subplot were systematically selected. Four leaves per tree were sampled from each tree in a quadrat; thus a composite sample consisted of tissue from 16 leaves taken from four trees.

Both HS and singles survey were conducted in three 400-tree subplots to verify that subplot HS was correctly estimating incidence in cases where CTV incidence was low. Overall evaluation of the subplot HS method was conducted by comparing the 2001 sub-plot data with the complete HS survey and singles survey provided by the CCTEA.

HS of subplot collection and ELISA. The subplots were sampled from April 10-26, 2001 when CTV titer was near seasonal highs. Tissue collection followed the procedures described by Garnsey et al. (8, 9). DAS-I ELISA was conducted using CTV polyclonal antibodies provided by D. Gumpf, UC Riverside. Goat anti-CTV, purified by DEAE cellulose (DEAE-Sephacel, Pharmacia Biotech, Uppsala, Sweden), was used for coating plates. Rabbit anti-CTV-CP, purified by Protein A agarose (KPL, Gaithersburg, MD), was used as the detecting antibody and commercial anti-rabbit IgG conjugated with alkaline phosphatase (Sigma, St. Louis, MO) was used as the conjugate. ELISA procedures followed those used by the CCTEA (5). ELISA plates were Immulon 4HBX Flat Bottom plates (Dynex, Chantilly, VA). The reactions were read at OD₄₀₅ with a plate reader (MRX II, Dynex, Chantilly, VA). The ELISA was performed by the RKY lab.

Statistical analysis. The data on estimated CTV incidence was expressed as our subplot HS result versus the CCTEA result based on whole field HS or singles survey of the same fields averaged over the four sections and was categorized as follows: 1) + vs +; 2) - vs ±; 3) + vs ±;

4) + vs -; 5) - vs +; 6) - vs -; where + = CTV incidence $\geq 1\%$; \pm = CTV incidence $< 1\%$; and - = no CTV detection. This data was then analyzed using SAS GENMOD procedure (7). The counts from the category \times field contingency table were modeled as a Poisson-distributed variable using a log link function. An adjustment for overdispersion was made using the Pearson scale factor.

RESULTS

The subplot HS procedure was first tested in three small citrus blocks (Table 1) and compared to singles surveys in the 400-tree block. The subplot HS estimated CTV incidence to be 5.4, 6.0, and 1.0% in plots 13A, 12A, and 7B, respectively. These estimates compared well with the actual incidence of 5.3, 5.0, and 0.5%, respectively.

The subplot HS procedure was then followed in 45 blocks which covered an overall area of 350.4 ha and 86,316 trees (Table 2). Of this area, 20.3% (71.1 ha) was in the subplot HS sampling grid and 5.1% (4,402 trees) of the trees were actually sampled. Overall, the subplot HS procedure estimated that CTV incidence was 0.259% which compared well with the 0.232% infection level estimated by the HS survey of the entire orchard. In comparisons of our data in 34 fields where CCTEA's singles surveys were avail-

able, the subplot HS scheme estimated CTV incidence at 1.146% compared to 2.261% indicated by the singles survey (Table 2). This difference could be explained, in part, by time differences when the samples were taken. Our samples were taken after those of the CCTEA and, in some cases, up to 2 yr later. Whenever infection is found by CCTEA's singles survey, those infected trees are removed with the cooperation of the grower; we could not have found these infections unless our samples were taken before removal activities.

When our data was collated and categorized according to the levels of infection (0, $< 1\%$, $> 1\%$) based on whole field samples, the percentages of cases in the 79 fields observed from category one to six of the subplot HS vs. CCTEA comparisons were 22%, 33%, 5%, 6%, 10%, and 24%, respectively (Table 3). LR statistics for type 3 analysis indicated significant differences in categories ($P = 0.0005$) and no differences between replicate locations (Chi-Square = 5.71, $df = 15$, $P = 0.1269$) (Table 3). Pairwise comparison of our subplot HS data to that of the CCTEA's whole field HS or singles survey indicated that categories 1, 2, and 6 were significantly different from categories 3, 4, and 5 ($P < 0.001$) (Table 3). This meant that when CTV incidence was $> 1\%$ or was zero, there was a statistically

TABLE 1
VALIDATION OF *CITRUS TRISTEZA VIRUS* (CTV) SURVEY DATA COLLECTED BY SUBPLOT HIERARCHICAL SAMPLING (HS) VERSUS SINGLES SAMPLING WITHIN 400-TREE BLOCKS

Subplot ^a	Variety	Incidence (%) by singles ^b	Estimated incidence (%) by subplot HS ^c
13A	Navel	5.3	5.4
12A	Fukumoto	5.0	6.0
7B	Navel	0.5	1.0

^aEach validation subplot was selected to represent a low ($\leq 1\%$ CTV) and moderate ($\approx 5\%$) incidence of CTV infection.

^bSingles samples from 13A were collected on June 14, 2001; 12A were collected on May 1, 2001; and 7B were collected on June 19, 2001.

^cSubplot HS samples were taken from 25 quadrats from 100 trees in a 400-tree grid in the center of the block from April 12 to 26, 2001.

TABLE 2
SUMMARY OF SUBPLOT HIERARCHICAL SAMPLING (HS) VERSUS COMPLETE HS OR SINGLES SAMPLING TO ESTIMATE *CITRUS TRISTEZA VIRUS* INCIDENCE (%) IN ORCHARDS IN A 621 HA AREA

Sampling method ^a	No. subplots	Total area sampled (ha)	No. trees	Estimated incidence ^b	Estimated incidence by subplot HS ^c
Complete HS	45	350.4	86,316	0.232	0.259
Singles	34	270.7	75,304	2.261	1.146
Totals	79	621.1	161,620		

^aVarieties were Navels and Valencias with four blocks of Minneola tangelos.

^bCCTEA took samples from the entire orchard and conducted the assays in this column. Most of the complete HS data was collected in the 1998-99 season. The singles surveys were taken from the spring of 2000 and 2001.

^cThe RKY team took subplot samples from a block of 400 trees (20 rows by 20 trees) or equivalent in the center of each block on April 12 to 26, 2001 and conducted assays in this column to determine virus infection levels.

significant correlation between our subplot HS sampling scheme and a complete field HS or singles survey. Category 2, which contained 33% of the cases, was in the same statistical group, however, and indicated that when field incidence was <1%, the subplot HS method was not reliable in finding CTV. In 5% of the cases (Category 3), however, our subplot HS survey did detect CTV when field incidence was <1%.

Observations in Category 5 where the subplot HS failed to detect CTV in 10% of the cases is, in part, attributed to our sampling after infected trees detected by the CCTEA were removed (Table 3). Infections we detected in Category 4

(Table 3) where the CCTEA tests had indicated no infections likely indicated new natural spread.

In summary, our subplot HS was successful in finding CTV reservoirs when CTV incidence was >1% but not when incidence was less.

DISCUSSION

Spatial and temporal patterns of CTV spread where the cotton aphid is the principal vector have generally shown an absence of infection in trees immediately adjacent to infected trees. Virus spread was more frequently found some distance away from source trees (e.g., 8 to 20 trees) (10). This results in lit-

TABLE 3
COMPARISON OF THE SUBPLOT HIERARCHICAL SAMPLING (HS) VERSUS KNOWN ESTIMATES OF *CITRUS TRISTEZA VIRUS* (CTV) INCIDENCE IN THE FIELD

Category	Known CTV incidence in field ^a	Subplot HS	No. obs.	% obs.
1	>1%	Detected	17	22
2	<1%	Not detected	26	33
3	<1%	Detected	4	5
4	0%	Detected	5	4
5	>1%	Not detected	8	10
6	0%	Not detected	19	24

^aData provided by the CCTEA.

Data analysis indicated that the overall effect of category (Chi-square = 21.95, $df = 15$) is significant ($P = 0.0005$). Categories 1,2,6 versus 3,4, 5 was also significant (Chi square = 19.18, $df = 15$, $P < 0.0001$).

the aggregation of CTV when incidence is low and was the assumption in developing the subplot HS system described in this paper.

CTV eradication programs require a rapid and efficient disease detection system. Over a large area, however, it is often not feasible to survey all fields with the frequency necessary to maintain zero-level infection. Therefore, the subplot HS procedure was developed to allow survey of more area per person with the purpose of reducing survey costs. This method was tested in plots in Kern Co. where CTV incidence was low. It was known, however, that some fields in this area had higher CTV incidence and aggregates of infected trees resulting from secondary spread in fields where removals of infected trees were delayed.

The subplot HS system was very successful in identifying orchards

where CTV incidence was >1% but was not reliable in identifying fields where CTV incidence was <1%. It should be mentioned, however, that in specific plots where a significant level of infection was missed, the subplot HS did find CTV in adjacent orchards. We conclude that the subplot HS system found areas where CTV incidence was high and that this method could be used to find areas of high CTV reservoirs over a large acreage. Once located, these areas should be prioritized for more extensive surveys. The subplot HS, hence, is a method to supplement, not replace, existing sampling strategies.

ACKNOWLEDGMENTS

The authors thank Robert DeBorde and AnnMarie Cornett (USDA, ARS, SJVASC, Parlier, CA) for technical assistance; Bruce MacKey (USDA, ARS, PWA, Albany CA) for help in statistical analysis.

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