Probability Model of *Citrus tristeza virus* Detection in the Tree Canopy and Reliability and Efficiency of Direct Immunoprinting-ELISA

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ABSTRACT. The serological technique, direct immunoprinting-ELISA, was evaluated with respect to DAS-ELISA with the objective to compare the reliability and efficiency of the detection of *Citrus tristeza virus* (CTV) in order to justify its application in official programs of monitoring and eradication in Mexico. In January and March of 2004, 7,430 trees of Valencia sweet orange grafted on sour orange were sampled. A total of 89,160 immunoprinting and 186 ELISA tests were performed in 11 commercial orchards in five counties in Tamaulipas State. Two groups of 63 and 68 known-positive trees were tested by immunoprinting-ELISA and DAS-ELISA methods. Immunoprinting-ELISA detected 98% and 100% of the known-positive trees, respectively, whereas DAS-ELISA detected 96.8% and 98.5%, respectively. Both techniques detected 98.5% of all trees. All positive trees (62) in one experiment were successfully detected by biological indexing, while DAS-ELISA detected 91.7% of the positive trees that were also detected by immunoprinting-ELISA. A cost reduction of 29.4% resulted when the immunoprinting method was used. The optimal imprint pattern on the nitrocellulose membrane varies with the age of the petiole. The observed frequency of positive shoots out of 2,232 tested (186 positive trees) was fitted to the beta binomial model. Using this model CTV can be detected with four petioles with 97% confidence and 33.3% cost saving. Depending on host, shoot age, and printing pattern the immunoprinting cost varied from US$0.69 to US$1.02 per tree.

The application of DAS-ELISA in eradication programs of *Citrus tristeza virus* (CTV) in Mexico is feasible by the combined use of the monoclonal antibodies 3DF1 and 3CA5, which allow the detection of a broad range of CTV isolates (2, 3). However there are several limitations. This technique requires infrastructure such as a laboratory, specialized equipment and specially trained personnel, as well as great amount of manual labor and time for processing the plant tissue. Further, when ELISA readings are lower than a threshold value, but higher than a normal healthy sample, the trees must be resampled and an additional ELISA test must be done. Thus, such a program can be costly in a time when funds are limited.

Experiments conducted in Mexico using direct immunoprinting ELISA yielded inconsistent results (8, 9, 10, 12). Loeza (8) showed immunoprinting-ELISA detected 14% (9/64) of known-positive trees in a commercial orchard of 1,200 trees of Valencia/sour orange. In another study (10), 98.8% (88/89) known-positive trees of Valencia/sour orange were detected by immunoprinting-ELISA whereas, DAS-ELISA detected 77.5% (69/89) of the known-positive trees. In Spain, the sensitivity of DAS-ELISA, immunoprinting-ELISA, and immunocapture-PCR were compared. This study showed that immunoprinting-ELISA is a reliable, sensitive and cost effective method for surveying nursery trees but it is not as reliable

for surveying mature trees in commercial groves (4).

Currently in Mexico, CTV is managed by the eradication of virus-infected trees. In order for an eradication program to be effective, it is necessary to have a reliable and cost effective method to rapidly detect CTV in mature trees. Previous studies comparing various detection methods did not address this need (4, 8, 10). Therefore, it was necessary to compare both techniques under field conditions using the established protocols of the eradication program in Mexico. The objectives of the present work were to determine the reliability and efficiency of the immunoprinting-ELISA technique, whether it was more cost effective than the currently accepted DAS-ELISA protocol, and whether it was sufficiently accurate to be used for extensive epidemiological studies.

MATERIALS AND METHODS

Area of study. In January and March of 2004, a total of 7,430 trees of sweet orange grafted on sour orange were sampled in 11 commercial orchards in five counties of Tamaulipas, Mexico (Table 1). Eight shoots with two leaves were collected uniformly around the canopy from each tree. The sampled shoots and leaves were tagged, wrapped separately in a plastic bag, and stored at 4°C until tested. Trees were classified in two categories, positive or negative, based on the DAS-ELISA results of two previous surveys. To reduce experimental error, the survey and all serological tests were performed by the same researchers.

Serological analyses. The membrane printing was performed with leaf petioles of collected shoots. A clean cut of leaf petioles was made with sharp scissors. The freshly-cut sections were pressed carefully against the nitrocellulose membrane (3, 4). The membrane prints were made with three patterns: 12 impressions in single line, 12 impressions in double line with upper line-lower line replicates and filling the membrane in continuous rows, and the same pattern but filling the membrane in columns (Fig. 1). The immunoprinting-ELISA technique was made with a commercial kit produced by Plant Prints Diagnostic (Plant Prints Diagnostic, SL. Valencia, Spain). The membranes were read using dissecting scope at 10× magnification. The presence of a purple precipitate in the vascular region of leaf petiole was considered CTV positive (4). An extract of the

TABLE 1
CHARACTERISTICS OF 11 COMMERCIAL CITRUS ORCHARDS SAMPLED TO DETERMINE THE EFFICIENCY OF IMMUNOPRINTING-ELISA IN THE DETECTION OF *CITRUS TRISTEZA VIRUS*

Counties	Orchard /ha	Variety/age (year)	Previously positive	Latest detection	Shoot age (mo)	Samples/ orchard
Güemez	G1/130	Valencia/20	9	19-02-02	6	392
	G2/27	Valencia/12	9	27-01-03	6	414
	G3/85	Mandarina/10	29	03-04-02	1	390
Llera	L1/190	Valencia/40	58	20-04-03	6	384
	L2/24	Val. Temp./15	2	23-04-02	1	427
	L3/50	Nav. Temp./14	4	21-02-02	1	433
Mante	M1/90	Valencia/20	62	28-02-03	6	2024
Padilla	P1/180	Val. Temp./13	40	26-11-02	6	1700
	P2/14	Mars/12	6	04-02-02	1	441
	P3/100	Valencia/20	4	22-11-02	1	385
Victoria	V1/8	Valencia/16	2	17-01-02	6	440

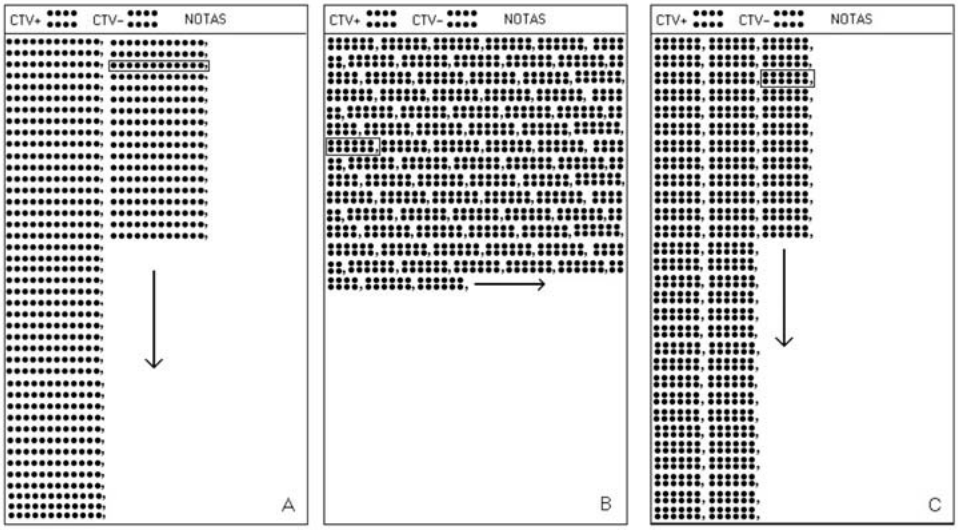

Fig. 1. Impression pattern on nitrocellulose membranes using 6 petioles in double replicates per tree: A) 12 impressions in single line; B) 12 impressions in double line with replicates upper line-lower line and filling the membrane in continuous rows; C) same as pattern (B), but filling in columns.

same plant tissue was (six shoots were used per sample and one sample per tree) also analyzed by enzyme-linked immuno-adsorption (ELISA) in the form of indirect double sandwich antibody (DAS-I) as described in Garnsey et al. (6), using a commercial Agdia kit (Agdia, Inc., Indiana, USA). The polyclonal anti-CTV antibody (catalog number CAB 78,900) was used as a coating antibody. A mixture of the 3DF1 and 3CA5 monoclonal antibodies was used as intermediate antibodies. IgG conjugated to alkaline-phosphate was used to detect CTV. Substrate (p-nitrophenol phosphate) was added to the ELISA plate and incubated for 20 minutes at room temperature. Tissue extracts from known-positive (Valencia sweet orange grafted on sour orange) trees were used as positive control. The optical density was read at 405 nm after 40 min of final reaction. The threshold for scoring positives trees was three times the average of two negative controls.

Reliability of direct immunoprinting-ELISA. Two independent experiments were conducted with known-positive trees. In the first experiment; 63 trees were tested using immunoprinting-ELISA at three intervals: in the field during tissue collection, at 24 and at 48 hr after the samples were collected. The positive trees in this experiment were biologically indexed in Valencia sweet orange grafted on Carrizo citrange. In a second experiment, 68 known-positive trees were tested using immunoprinting-ELISA made one day after the tissue was collected. In both experiments, an extract of the same plant tissue was also analyzed by standard DAS-ELISA 5 days after the sampled was collected. The performance of both techniques was assessed for reliability, which is defined as the proportion of positive samples obtained with DAS-ELISA or immunoprinting with respect to the total number of known positive trees (5).

DAS-ELISA versus immunoprinting-ELISA. The reliability of immunoprinting-ELISA for CTV detection was evaluated in 6,928 trees in 11 commercial orchards (Table 1). Two situations were considered: orchards in which the disease incidence was not known and orchards

where the trees were negative in a previous census with DAS-ELISA (orchard M1, L1, and P2). The tree samples consisted of eight shoots with two leaves collected as described previously. The procedure for the immunoprinting was made as described before. Only positive trees detected with direct immunoprinting-ELISA were also assayed using DAS-ELISA.

Effect of the pattern of membrane impression in efficiency and cost. In order to measure the cost efficiency of the immunoprinting-ELISA technique, the average number of samples (12 blots using 6 petioles by tree) that could be printed with three filling patterns in membranes (Fig. 1) were compared. The average number of samples per membrane was compared using the Student t test for independent samples. The cost per filling pattern was estimated based upon the price of the commercial kit (US$1,300).

Probability of CTV detection with immunoprinting-ELISA. Reports in Israel indicated that the distribution of CTV-positive shoots around the tree is heterogeneous (1). Thus, the probability of randomly collecting a positive shoot depends on its position in the canopy. Because each shoot can be only one of the two following conditions: positive or negative relative to CTV, the probability distribution of the number of positive shoots by tree (x) corresponds to a Binomial with parameter $n = 6$ and p unknown. The supposition of heterogeneous distribution of the positive shoots implies that p varied from one tree to another, which indicates a probability distribution, Beta, with parameters a and b, and that x has Beta-Binomial distribution:

$$f(x) = \binom{n}{x} \frac{\prod_{r+0}^{x-1}[\pi + r\theta]\prod_{r=0}^{n-x-1}[1-\pi+r\theta]}{\prod_{r+0}^{n-1}[1+r\theta]}, \quad x = 0,1,\ldots,n \qquad (1)$$

where $\pi = a/(a + b)$ and $\theta = 1/(a + b)$ (13). The parameter π is the expected value of p and represents the average probability of CTV detection using immunoprinting-ELISA and θ is the variability of p. If $\theta = 0$, the Beta-Binomial model is reduced to the Binomial model implying a random disposition of positive shoots around the canopy. For both probability distributions, the parameters were estimated using the algorithm AS R93 (13). The goodness of fit test was made with the chi-square. The reduction in information obtained in the sample due to the effects of heterogeneity was estimating using

$$n_{deff} = \frac{\sum_{i=0}^{n-1} \ln \frac{1-\hat{p}+i\hat{\theta}}{1+i\hat{\theta}}}{\ln(1-\hat{p})}$$

where $\hat{p}$ is the observed mean incidence of CTV positives shoots per tree, and $\hat{\theta}$ is the aggregation parameter of the beta binomial distribution estimated in equation 1 (7). A graphic estimation of effective sample size can be obtained by plotting n_{deff} versus a broad range of incidence.

RESULTS AND DISCUSSION

Reliability of direct immunoprinting-ELISA. In both experiments, immunoprinting-ELISA was as reliable as DAS-ELISA for detecting CTV. In the first experiment, immunoprinting-ELISA detected 62 of 63 (98.4%) of known-positive trees, whereas, 61 of 63 (96.8%) were confirmed as positive by DAS-ELISA. All positive trees started at time 0. The 62 positive trees were confirmed two mo later with immunoprinting-ELISA in the set of grafted plants. In the second experiment with 68 known-positive trees, 100% were positive by immunoprinting-ELISA whereas, 67 (98.5%) were positive by DAS-ELISA. The reliability of immunoprinting-ELISA in these studies agrees with that previously reported (10). In addition, the

agreement between both techniques was greater in this work (98.5%) than the 78.65% reported in prior studies (10). The detection of CTV by immunoprinting-ELISA was not affected by how the samples were handled in the field, even with the high temperatures, or by the duration of sample storage.

DAS-ELISA versus immunoprinting-ELISA. The results of detecting CTV using DAS-ELISA and immunoprinting-ELISA are shown in Table 2. In experiment 1 consisting of 4,146 trees with unknown CTV incidence, DAS-ELISA detected 41 positive trees whereas immunoprinting-ELISA detected 45. In experiment 2 with 2,782 known negative trees, 73 positives were detected by immunoprinting-ELISA, while 67 (91.7%) of these were positive by DAS-ELISA. These results indicate that immunoprinting-ELISA detected a greater number of positive trees than DAS-ELISA. Further, immunoprinting-ELISA is more reliable due to the visual presence of a purple precipitate rather than the optical density values given by the DAS-ELISA method which can be marginally positive or negative with an elevated reading (3, 6). This was evident in the present study with 89,160 impressions. Eleven trees were declared positive by immunoprinting-ELISA with a reaction in only one of six petioles (Fig. 2), whereas the DAS-ELISA results for these same trees were marginally under the detection threshold (Fig. 2B, C). Therefore, trees with one positive shoots could not be properly detected with DAS-ELISA and may require repeated inspection. This is time consuming and costly for eradication purposes (11).

Effect of membrane pattern of impression on efficiency and cost. The factors that determined the number of samples that could be printed on one membrane were: the petiole thickness used in the impression and the impression pattern of petioles on the membrane (Table 3). The petiole thickness was determined by the shoot age and the citrus variety. Twelve continuous impressions made in a line by tree and filling the membrane in columns (Fig. 1A) was the most efficient method for young orange petioles. However, this pattern was not optimum for petioles of greater thickness. With old petioles it was not possible to make three columns on the membrane, thereby reducing considerably the efficiency and increasing the cost (Table 3). For petioles greater than 6 mo of age, the optimum number of samples per membrane was obtained by placing the 12 impressions by tree in a double line fashion with six prints per line and filling the membrane in columns (Fig. 1C). The time to fill and space limitations of membranes was affected by the impression pattern. The pattern illustrated in Fig. 1C was the easiest to make, easiest to read and required the least amount of time to prepare.

Probability of CTV detection with immunoprinting-ELISA.

TABLE 2
EFFECT OF INFECTION TIME AND PETIOLE AGE ON THE DETECTION OF CTV BY DAS-ELISA AND DIRECT IMMUNOPRINTING-ELISA USING SAMPLES OF COMMON ORIGIN

Scene[1]	Petiole age (mo)	Trees analyzed by immunoprinting	Positives by immunoprinting	DAS-ELISA	
				Positive	Negative
1	1	1634	34	33	1
	6	2512	11	8	3
2	1	441	6	6	0
	6	2341	67	61	6

[1]Length of time tree was infected: 1) Unknown; 2) Positive for less than 1 yr.

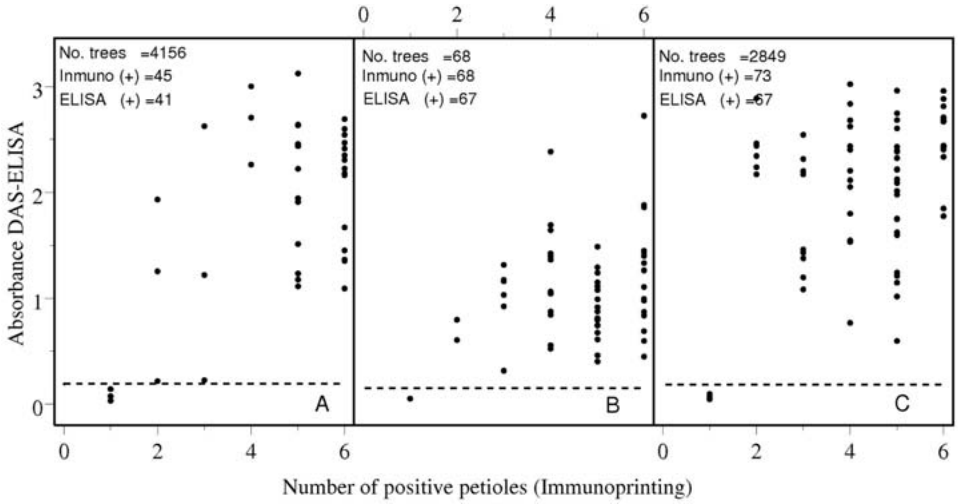

Fig. 2. CTV-positive petioles detected by immunoprinting-ELISA by tree and its respective ELISA value for three scenarios of infection: A) Unknown; B) Known-positives that had been infected for more than a year; C) Known positives that had been infected for less than a year (---s, indicate the threshold of determination between positives and negatives trees using DAS-ELISA).

The probability distribution for CTV detection in the canopy in two field sites (Figs. 2A and 2B) corresponded to the beta binomial model (Table 4). This result implies that the distribution of positive shoots around the tree canopy is heterogeneous and can be associated with a probabilistic function of detection. Therefore it is possible to estimate the probability of detecting CTV depending on the number of shoots collected per tree (Fig. 3B). Also, these results statistically verify previous empirical results (1). In the case where trees had been infected for at least 12 mo, the detectability function can be described by the binomial or beta binomial model. This allowed adequate time for the virus to invade the entire plant, thus the distribution of the positive shoots around

TABLE 3
EFFICIENCY AND COST OF IMMUNOPRINTING MEMBRANES USE OF THREE PATTERNS OF IMPRESSION FOR TWO PETIOLE AGES AND OF TWO SPECIES OF CITRUS

Citrus	Petiole age (mo)	Design[1]	Number of membranes	Trees	Samples/ Membrane[2]	Processing cost/US$	
						Sample	Campaign[3]
Mandarin	1	C	2	377	188.5 ± 4.5	0.69	4828
Orange	1	A	6	1088	181.3 ± 2.6[a]	0.72	5019
		B	7	938	134.0 ± 1.3[c]	0.97	6791
		C	7	1110	158.6 ± 1.8[b]	0.82	5738
	6	A	13	1654	127.2 ± 1.4[b]	1.02	7154
		B	12	1547	128.9 ± 1.1[b]	1.01	7059
		C	5	693	138.6 ± 1.9[a]	0.94	6566

[1]Impression pattern of membranes: A) Single line prints, B) Double line prints in rows; C) Double line prints in columns (Fig. 1).
[2]Averages with the same letter are not statistically different (comparisons within each group of petioles age in Valencia orange).
[3]Estimated cost for 7000 samples processed during the campaign in 2003-2004 against CTV in Tamaulipas. It does not include the operative costs of field work.

TABLE 4
TEST OF GOODNESS OF FIT OF THE NUMBER OF POSITIVE PETIOLES PER TREE DETECTED BY IMMUNOPRINTING-ELISA

Scenario[1]	Model	Chi Square (*p value*)	Parameters
1	Binomial	0.012	$\hat{\pi} = 0.765, \pm 0.028$
	Beta-Binomial	0.569	$\hat{\theta} = 0.151, \pm 0.068$
2	Binomial	0.000	$\hat{\pi} = 0.702, \pm 0.027$
	Beta-Binomial	0.243	$\hat{\theta} = 0.167, \pm 0.58$

[1]Status of infection: 1) Unknown; 2) Trees with less of a year has been detected positives.

the tree canopy can vary from heterogeneous to homogenous. In contrast, when infection is recent there has not been sufficient time for all the branches to become infected and therefore the distribution of the positive shoots is heterogeneous (Fig. 3A). This explanation can be applied to tree age. Young trees are readily infected and virus distribution can be homogeneous. This explains why 10 petioles were sufficient to detect CTV (3, 4). With 50% of petioles with CTV, the probability of detecting a positive tree (with almost one positive shoots) is greater than 0.95 (Fig. 3A). The effective sampling size to reach that probability is shown in Fig. 3B. The effective sampling size to detect a known positives tree are five shoots.

The estimated cost in this work with one sample per tree analyzed with immunoprinting-ELISA was US$0.98 and US$1.72 for DAS-ELISA. Immunoprinting-ELISA implied a saving of 42.35% in the cost of analysis of the samples. Another report in Mexico showed a saving of 66% in adult trees using immunoprinting-ELISA (9). With effective sampling size of five shoots per tree, the cost savings that can be obtained with petioles one month

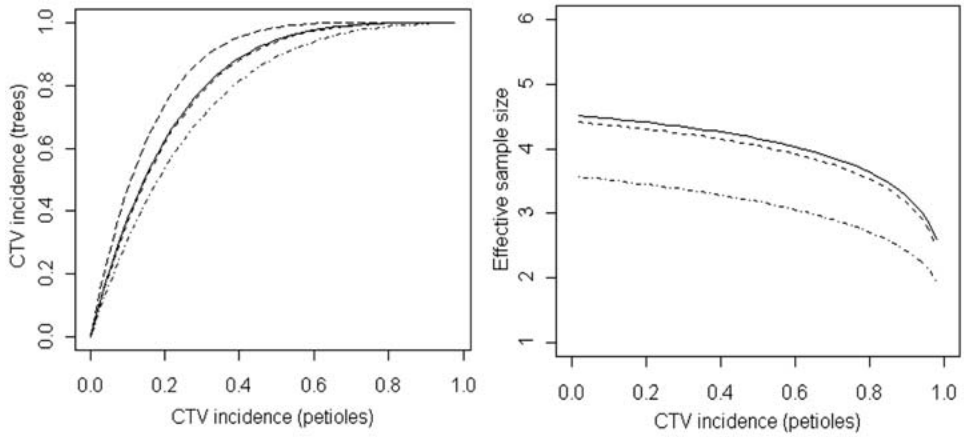

Fig. 3. A. The relationship between incidence of CTV in trees and its incidence on petioles for three scenarios of infections: i) (-- -- --) binomial and (-----) beta binomial for known-positives that had been infected for more than a year; ii) (- - -) beta binomial for positives that had been infected for less than a year; and iii) (-.-.-) positives with unknown time of infection. B. Effective sampling size to detect a positive tree with almost one shoot CTV-positive: i) (---) known-positive that had been infected for more than a year; ii) (- - -) positives that had been infected for less than a year; and iii) (-.-.-) positives with unknown time of infection.

old are 58.1% and 45.4% using petioles of six months of age. On the other hand, because only 93.2% of the positive shoots were positive in both repetitions, it is recommended that a single print for each petiole be made (3) and to use 10 petioles per tree. Ten petioles per tree increases the probability of CTV detection by examining more canopy sections. This can be important for recent CTV infections, where there is a heterogeneous distribution of positive shoots in the tree canopy (1).

CONCLUSIONS

Immunoprinting-ELISA was as reliable as DAS-ELISA for CTV detection in commercial orchards of Valencia orange grafted on sour, detecting 98.4% and 100% of known positives, whereas DAS-ELISA detected 96.8% and 98.5%. When testing trees of unknown infection, DAS-ELISA only confirmed 91.1% and 91.7% of the positive trees that were detected by immunoprinting-ELISA. With petioles one month old, it was possible to analyze 181 trees making imprints in a continuous line. When using testing 6-mo-old petioles, 139 were imprinted using a double line. In both cases the best membrane pattern was in columns. The immunoprinting-ELISA is recommended because it resulted in 97% confidence of CTV detection, and by printing five petioles with two replicates per plant it gave a cost saving of 42.4%. In trees with recent infections where virus distribution is limited, this probability can be smaller, thus it is recommended to use ten petioles per tree collected uniformly from the canopy with a single impression per petiole instead of five petioles with two replicates per each one.

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