Improvement of Viroid Diagnosis and Determination of Viroid Presence in Cuban Citrus Areas

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ABSTRACT. Presently, sour orange is being replaced by rootstocks giving tristeza tolerant scion/rootstock combinations. Since some promising rootstocks are sensitive to viroids, the plants selected as budwood sources must be viroid-free. Therefore, sensitive and reliable indexing methods are needed to certify the sanitary status of propagation materials. In the present work, nine citrus cultivars were analyzed by sequential polyacrylamide gel electrophoresis (sPAGE), imprint nucleic acid hybridization (NASH) and dot blot NASH. Statistical analyses were performed to establish the efficacy and confidence intervals of viroid detection using as gold standards results obtained by NASH from Etrog citron Arizona 861 S1. Dot blot NASH gave the highest detection efficacy, over 80% in some citrus cultivars, whereas in others the results were erratic. In all instances, detection efficacy was below the level obtained for citron, thus confirming the need to inoculate Etrog citron for reliable indexing. The chemiluminescent NASH was validated for the simultaneous detection of all citrus viroids identified from Etrog citron. With NASH, reproducibility of the results was over 97%. A survey conducted in citrus growing areas and of propagation materials using this technique showed that the number of infected plants ranged from 10-37%, with *Citrus exocortis viroid*, *Hop stunt viroid* and *Citrus viroid III* found to be the most widespread. The Germplasm Bank and propagation nurseries were found to be viroid-free.

Viroids are widespread in most citrus growing areas (1). Since the main rootstocks used currently are sensitive to these pathogens, reliable and fast detection methods are needed for their control. Some authors have reported that viroid detection from commercial cultivars is possible (5), but this had not been tested in a systematic way.

In the present work the efficacy and confidence intervals of viroid detection in 10 citrus cultivars were determined. Two plants of each cultivar were graft inoculated with an isolate containing five viroid species, *Citrus exocortis viroid* (CEVd), *Citrus bent leaf viroid* (CBLVd), *Hop stunt viroid* (HSVd), *Citrus viroid III* (CVd-III) and *Citrus viroid IV* (CVd-IV) and were then maintained in a screenhouse. Detection assays were carried out monthly over a one year period using sPAGE, imprint and dot blot NASH. The efficacy of viroid detection using the different assays was determined using viroid detection by dot blot NASH in citron as a gold standard for comparison to detection in other citrus cultivars. The data were analyzed by point estimation and confidence intervals using R Development Core Team Program (2004). The efficacy of positive detection of dot blot NASH in citron (EPD) was calculated as EPD = (PDC/PDCi) $\times$ 100, where PDC is the percentage of viroids detected in citrus cultivars and PDCi is the percentage of viroids detected in citron by dot blot NASH. Percentages are defined as the number of evaluations with positive viroid detection relative to the total number of evaluations.

The results indicated that dot blot NASH gave the highest efficacy and imprint NASH the lowest one (Table 1). In most cultivars no viroids were detected by imprint NASH. Using this method, the best

TABLE 1
CUMULATIVE EFFICACY OF VIROID DETECTION BY DIFFERENT METHODS IN CITRUS CULTIVARS

	Efficacy (%)*														
	Imprint NASH					sPAGE					Dot blot NASH				
Cultivar	CEVd	CBLVd	HSVd	CVd-III	CVd-IV	CEVd	CBLVd	HSVd	CVd-III	CVd-IV	CEVd	CBLVd	HSVd	CVd-III	CVd-IV
Washington navel sweet orange	0	0	0	42	0	75	58	25	58	58	83	75	92	92	100
Delta sweet orange	0	0	0	42	0	58	58	58	58	58	92	100	67	100	92
Troyer citrange	8	50	0	58	0	83	83	67	58	58	83	100	75	75	83
Persian lime	33	0	0	25	0	100	50	50	17	25	92	75	75	92	67
Mexican lime	42	0	0	33	0	83	25	25	17	25	75	67	58	92	83
Volkamer lemon	42	8	0	58	0	75	75	58	58	58	92	92	75	92	58
Alemow	67	83	0	50	0	92	67	58	67	58	100	92	92	83	83
Henderson grapefruit	0	0	0	0	0	33	17	0	25	8	92	92	67	75	33
Cuban shaddock	83	67	0	67	0	100	92	75	75	75	100	100	100	100	92
Etrog citron Arizona	92	92	67	100	67	100	100	100	100	100	**	**	**	**	**

*Refers to the range of percentage detection of the different viroid species.
**Technique chosen as gold standard because of 100% detection of all viroids in Etrog citron Arizona.

results were achieved in citron, with erratic results for detection of HSVd and CVd-IV. These results are in agreement with previous studies, which determined that citron is the only citrus host amenable to viroid detection by this method (4). All viroid species were detected with 100% efficiency in citron by sPAGE analysis. In general, low percentages of detection were achieved in other species, and some were occasionally zero (Table 1). The efficacy of the detection of viroids in different cultivars ranged from 0 to 92%, with CEVd detected at 100% efficiency in Persian lime and Cuban shaddock.

On all assay dates, all the viroid species were detected by dot blot NASH in citron and Alemow. But in some further evaluations made in Alemow, only CEVd was consistently detected, with positive detection ranging from 83 to 92%. CVd-IV was detected with difficulty in most cultivars. The erratic detection of this viroid in Henderson grapefruit was more noticeable, suggesting that CVd-IV is present at low concentration and is unevenly distributed in certain citrus hosts as was already pointed out by Nakahara et al. (3).

The systematic analysis of the nine cultivars indicated that detection of viroids from commercial cultivars is erratic. This confirms that, in order to guarantee reliable detection in the Budwood Multiplication Programs, inoculation on Etrog citron Arizona 861 S1 with subsequent molecular analysis provides the most sensitive assay. Considering the results obtained by dot blot NASH in Etrog citron Arizona 861 S1, this method was further validated using a mixture of five DNA probes (4). This assay was performed using 135 negative controls including non-inoculated citrons as well as plants infected with non-viroid citrus pathogens (*Citrus psorosis virus*, *Citrus tristeza virus* and concave gum) as well as RNA extracts of *Avocado sunblotch viroid*, *Chrysanthemum stunt viroid* and *Potato spindle tuber viroid*.

Viroid containing samples (200) included 189 citron plants grafted on Volkamer lemon or Troyer citrange rootstocks, with 126 of them inoculated with viroid isolates of known composition and the rest with samples of unknown viroid composition collected from infected field trees. In addition, 11 plasmids containing full-length cloned viroid sequences were included as positive controls. The efficacy, diagnostic sensitivity, specificity and predictive values of positive and negative test results were above 97% (data not presented). The detection limit was a 1:100 dilution of the extract and the reproducibility assays showed little variability with coefficients of variation that ranged within the limits established by Jacobson (2) (data not presented).

Using this viroid detection technique, the plants in the Germplasm Bank and the propagation nurseries were determined to be viroid-free, confirming the quality of the certified budwood production system. However, viroids were detected in citrus production areas, with percentages of infected plants estimated to range from 10 to 37%. CEVd, HSVd and CVd-III were widespread in Cuban citrus areas and accounted for 5.1, 8.3 and 6.0% of infections of the sources tested, respectively. CBLVd was detected for the first time in Cuban citrus areas. Low incidences of CVd-IV (2.5%) and CBLVd (0.5%) were also found. These viroids have been detected in most citrus growing areas in other countries (1, 6), because of the extensive exchange of citrus material.

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