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Survey of Citrus Viroids in Venezuela

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ABSTRACT. A survey of citrus viroids (CVd) was carried out in the main citrus areas of Venezuela to detect and characterize these pathogens. Samples of Valencia sweet orange grafted on Volkamer lemon, Cleopatra mandarin, Troyer citrange or citrumelo, and Tahiti lime grafted on Volkamer lemon were collected and analyzed. Nucleic acids extracted from bark tissue of field trees were analyzed by sequential polyacrylamide gel electrophoresis (sPAGE), by molecular hybridization with citrus exocortis viroid (CEVd) and hop stunt viroid (HSVd) specific probes and by reverse transcription polymerase chain reaction (RT-PCR). Results obtained show that six viroids, belonging to CEVd, CVd-II and CVd-III group are present in Venezuelan citrus trees.

Citrus cachexia and citrus exocortis (CEVd) diseases in Venezuela were first reported on Willowleaf mandarin by Malaguti (4) and on Tahiti lime by Estrada and Malaguti (2), respectively, but before the 1980s, these diseases had not caused important losses in this country as most citrus orchards were grafted onto sour orange rootstock.

Since 1980, citrus growers in Venezuela began experiencing large-scale destruction of citrus on sour orange rootstock caused by spread of citrus tristeza virus (CTV) decline by *Toxoptera citricida* (Kirkaldy). Therefore, the use of CTV-tolerant rootstocks became imperative and resulted in a need to distribute certified budwood to citrus growers.

With new fundamental knowledge of citrus viroids and an agreement between Venezuela and Italy, a survey was carried out in the main citrus areas of Venezuela over three years (1992 to 1994) to detect and characterize viroid incidence by several laboratory techniques.

Plant material. Plants tested in this study were local old line or supposed virus-free lines introduced illegally by local citrus growers from sources in California, Florida, Spain and Italy. The scion/rootstock combinations selected and the citrus areas surveyed are in Table 1. Young shoots were collected either from viroid symptomatic or asymptomatic trees. The following field symptoms were observed: Tahiti lime/Volkamer

lemon rootstock had leaf vein discoloration, bark striations and cracking, both on the shoots and on the old branches of the scion; Valencia sweet orange/Volkamer lemon rootstock manifested strong growth reduction, bark pegs and deep pits in the wood of the rootstock; Valencia sweet orange/Cleopatra mandarin rootstock showed decline and bumps on the inner bark which fitted into depressions in the outer wood of the rootstock; one tree of this scion/rootstock combination showed horizontal line striations; Valencia sweet orange/citrumelo rootstock had canopy chlorosis, leaf zinc-deficiency and growth reduction.

Molecular analyses. Nucleic acids from green bark tissue (~5-10 g/sample) were phenol extracted and purified by CF-11 cellulose chromatography (3). The extracts were analyzed by sequential polyacrylamide gel electrophoresis (sPAGE) at a discontinuous pH (7) and the viroid bands detected by silver staining. After sPAGE, the nucleic acids of some samples were transferred to nylon membranes and hybridized with CEVd and hop stunt viroid (HSVd) probes as previously described (1). Samples characterized by a different sPAGE pattern (CEVd, CVd-II, CVd-III) were reanalyzed by reverse transcription and polymerase chain reaction (RT-PCR) using the conditions and primers described elsewhere (9). Electrophoretic analysis showed the pres-

TABLE 1
SURVEY RESULTS OF VIROIDS IN CITRUS IN VENEZUELA

Scion/rootstock combination	State surveyed	No. trees infected/ no. tested
Valencia sweet orange/Cleopatra mandarin	Yaracuy	7/11
Valencia sweet orange/citrumelo	Carabobo	5/7
Valencia sweet orange/Volkamer lemon	Yaracuy	17/26
Valencia sweet orange/Troyer citrange	Yaracuy, Carabobo	4/5
Tahiti lime/Volkamer lemon	Aragua, Yaracuy	3/11
TOTAL		36/60

ence of RNA-viroids in 36 out of 60 tested plants distributed in all Venezuelan citrus areas examined (Table 1).

On the basis of the molecular size estimated by comparison with known markers in sPAGE, viroids detected were tentatively fitted on the consensus catalog (8) as follow: CEVd (~371 n), CVd-IIa (~309 n), CVd-IIa (~305 n), CVd-IIb (CCaVd, ~299 n), CVd-IIIb (~293 n) and CVd-IIIc (~287 n). The CVd-IIa (~309 n) is a new viroid we found and provisionally added to the citrus viroid catalog. CEVd and CCaVd were detected in 30 out of 36 infected trees and the presence of these viroids was confirmed by bioassays on indicator plants. Molecular hybridization analysis showed a positive reaction only with samples containing CEVd and CVd-II viroids, homologous to CEVd and HSVd probes, respectively. Healthy samples did not react with either probe. RT-PCR analysis gave amplification products from samples infected with CEVd, CVd-II or CVd-III when the correspondent specific primers were used, confirming the viroids group affiliation obtained by sPAGE and hybridization.

In some of the tested trees, field symptoms were related to the results obtained by molecular characterization: e.g., all trees of Tahiti lime/Volkamer rootstock with bark cracking contained CEVd, beside other CVds; all dwarfed Valencia orange on Cleopatra mandarin trees with severe

depressions in the rootstock wood were affected by CCaVd. Moreover, in the tree with horizontal line striations, CVd-IIa and CVd-IIIb were detected. In three out of five Valencia orange/citrumelo trees showing only canopy chlorosis and growth reduction, CVd-IIIb was found.

The results of this study confirm that CEVd and CCaVd are widespread in Venezuela as previously reported (5, 6) and show for the first time the presence of two additional viroids, CVd-II and CVd-III. Moreover, the present work shows that some problems of viroid identification by biological indexing can be overcome by using molecular techniques. Some viroids cannot be accurately identified by symptoms on Etrog citron when CEVd is also present, as their specific symptoms are masked by CEVd symptoms. Contrarily, the analyses used in this work readily detected all viroids even when their symptoms were not observed.

These results also confirm that sPAGE and RT-PCR allow detection of citrus viroids in young green bark sampled directly from field plants, although in temperate climates, reliable detection might be restricted to the warm season (9). Tropical conditions are extremely advantageous for viroid detection by molecular techniques as constant warm temperatures allow their replication and plant growth all the year around making tests from field samples more reliable.

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