Identification of Viroids in Citrus Orchards in Tunisia

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ABSTRACT. The cachexia disease caused by specific variants of Citrus viroid II (CVd-II) has been identified in commercial orchards of Cassar Clementine and common mandarin in Tunisia. Since commercial cultivars are usually grown on sour orange rootstock in Tunisia, exocortis symptoms have not been observed. Within the framework of the National Program for the selection of varieties free of virus and virus-like agents, indexing for citrus viroids allowed the identification of additional viroids in tolerant scion/rootstock combinations. In addition to CVd-II and *Citrus exocortis viroid*, we identified Citrus viroid I, *Citrus viroid III* and *Citrus viroid IV*.

Viroids are the smallest phytopathogenic agents known on plants. Two viroids have shown to cause diseases in citrus, exocortis and cachexia which are caused by *Citrus exocortis viroid* (CEVd) and specific variants of Citrus viroid II (CVd-II), respectively. In citrus, infection may occur as a single viroid infection, but generally viroids are found as mixtures. In Tunisia, cachexia symptoms have been mainly observed in commercial orchards of Cassar Clementine and common mandarin varieties (3). However, since commercial cultivars are usually grown on sour orange rootstock, exocortis symptoms have never been observed. Within the framework of the National Program for the selection of varieties free of virus and virus-like agents, viroid indexing was conducted with the aim of identifying those that may be present in tolerant scion/rootstock combinations.

The plant materials tested came from the INRAT collection of mother plants (Washington Navel, Maltaise demi-sanguine and Maltaise sanguine sweet oranges, Cassar Clementine, Arbi common mandarin, and Eureka lemon) which had been previously selected for their authenticity. Some plants from private orchards located in Cap-Bon, the main citrus growing region in Tunisia, were also tested.

Biological indexing was performed using Arizona 861-S1 Etrog citron grafted on rough lemon rootstock as indicator. For each cultivar tested, two indicator plants were inoculated by grafting two bark patches in each (7). The inoculated plants were maintained in a greenhouse at 28-32°C and symptoms were observed 2-6 mo after inoculation.

All the inoculated citrons developed symptoms with intensities ranging from severe to mild (Table 1). Citron plants with severe symptoms were very stunted and presented severe leaf epinasty and vein necrosis. Moderate symptoms were characterized by mild stunting, mild epinasty and petiole necrosis which sometimes were also observed along the mid-vein. A few plants presented very mild leaf epinasty affecting only a few leaves. All these symptoms were considered as evidence of viroid infection.

In order to determine which viroids were actually present, nucleic acid preparations from the inoculated citrons were subjected to sPAGE analysis. Samples of young leaf and stem tissue (5 g) were homogenised in 5ml of extraction medium (0.4 M Tris-HCl, pH 8.9; 1% (w/v) SDS; 5 mM EDTA, pH 7.0; 4% (v/v) mercaptoethanol) and 15 ml of water saturated phenol. The total nucleic acids were partitioned in 2 M LiCl and the soluble fraction was concentrated by ethanol precipitation and resuspended in TKM buffer (10 mM Tris-HCl; 10 mM

TABLE 1
BIOLOGICAL INDEXING AND sPAGE ANALYSIS OF CITRUS CULTIVARS GROWN IN TUNISIA

Cultivar	Samples	Symptoms on citron	sPAGE analysis				
			CEVd	CVd-I	CVd-II	CVd-III	CVd-IV
Washington Navel	3	Moderate	—	—	+	+	—
	1	Severe	+	+	—	+	+
Maltaise demi-Sanguine	3	Severe	+	—	—	+	—
	1	Severe	+	+	+	+	+
	2	Moderate	—	—	+	+	—
Maltaise Sanguine	2	Severe	+	—	+	+	—
	1	Severe	+	—	—	—	—
Cassar Clementine	3	Severe	+	—	—	+	—
	2	Moderate	—	+	+	+	—
	1	Severe	+	—	+	—	—
Arbi common Mandarin	2	Severe	+	—	+	+	—
	1	Mild	—	—	+	—	+
Eureka lemon	1	Severe	+	—	+	+	—
	1	Moderate	—	—	+	+	—
Total	24		15	4	16	21	3
Frequency (%)			62.5	16.7	66.7	87.5	12.5

KCl; 0.1 mM MgCl2; pH 7.4) (9). Aliquots of these preparations (300 µl) were subjected to non denaturing PAGE at constant 60 mA for 2.5 h and a segment of the gel defined by the spot of xylen cyanol was cut and placed on the top of the second gel containing 8 M urea and polymerised with pH 6.5 buffer. Electrophoresis was run at constant 18mA for an additional 4 h (6, 8) and the circular forms of the viroids were viewed after silver staining (2).

After sPAGE analysis the viroids were identified by their characteristic mobility compared with standards run in the same gel. In some instances the identity of viroid bands with closed electrophoretic mobility was confirmed by tissue imprint hybridization following the protocol of Palacio et al. (4). The results are summarized in Table 1.

The five viroid species described by Duran-Vila et al. (1) were identified in citrus grown in Tunisia. All the samples that gave a severe reaction on citron contained CEVd, whereas those showing moderate and mild symptoms contained different combinations of Citrus viroid I (CVd-I), CVd-II, *Citrus viroid III* (CVd-III) and *Citrus viroid IV* (CVd-IV). Infection with CVd-III, a viroid associated with dwarfing of trees grafted on trifoliate orange (10), was found in 21 out the 24 samples tested. CVd-II and CEVd also appear to be widespread, whereas CVd-I and CVd-IV were only found in a few samples.

The results of this study show that citrus viroids infect the main citrus cultivars grown in Tunisa. Although no attempts have been made to discriminate between CVd-II sources that induce cachexia from those that, like CVd-IIa which are non-pathogenic (5), the high frequency of CVd-II can be considered as confirmation of previous observations made by Jamoussi (3) who reported cachexia symptoms on Clementine and common mandarin. Since all the Tunisian citrus are grown on sour orange rootstocks the trees are not affected by CEVd and other citrus viroids. However, the identification of citrus viroids in the main cultivars must be taken into consideration when the citrus industry foresees in the future the utilization of new rootstocks as a strategy to control the tristeza disease.

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