Comparison of Different Diagnostic Methods for Detection of *Hop stunt viroid* and *Citrus exocortis viroid* in Citrus

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ABSTRACT. Viroids such as *Hop stunt viroid* (HSVd) and *Citrus exocortis viroid* (CEVd) are important pathogens in citrus plants. A comparative assay was performed to evaluate the viroid diagnostic techniques currently available at the Citrus Sanitation Center in Argentina. We compared the results obtained in the diagnosis of HSVd and CEVd in citrus using two molecular techniques; s-PAGE and RT-PCR and biological indexing. Biological indexing was performed in a greenhouse under controlled temperature using Etrog citron Arizona 861-S1 grafted on rough lemon rootstock as the indicator plant. Tissue samples for s-PAGE were obtained from inoculated citrons used in the biological indexing tests and DNA extraction was performed following published procedures. HSVd and CEVd specific primers were used for RT-PCR and samples were taken directly from field plants and inoculated citrons. Two nucleic acid extraction methods were compared. A total of 12 viroid isolates collected from orange, citron, grapefruit, citrumelo and lime trees, mostly from North Western Argentina were used in these assays. The three diagnostic methods compared gave similar results in 11 of 12 samples assayed. Several viroids were detected by s-PAGE and most of them were found as mixtures. HSVd and CEVd were identified by RT-PCR and no differences were found between the two extraction methods assayed. Sample R-407 was positive for exocortis with biological diagnosis and s-PAGE, but it was negative with RT-PCR. This was probably due to inefficient cDNA synthesis or to the presence of inhibitors that could interfere with the reaction. On the basis of the results obtained, it can be concluded that the three techniques assayed are complementary and useful tools for diagnosis and identification of HSVd and CEVd in citrus.

Citrus exocortis viroid (CEVd) and *Hop stunt viroid* (HSVd) cause two important citrus diseases, exocortis and cachexia respectively. These diseases produce considerable economic losses and limits the use of susceptible rootstocks. Moreover, in the field, symptoms induced by viroids are delayed or do not become evident in tolerant cultivars, and therefore can be spread infected trees to ones on susceptible rootstocks. The aim of this work was to compare HSVd and CEVd diagnosis in citrus using biological indexing and two molecular techniques: s-PAGE (sequential polyacrylamide gel electrophoresis) and RT-PCR (reverse transcriptase-polymerase chain reaction).

Twelve viroid isolates from orange, citron, grapefruit, citrumelo and lime trees, mostly collected in North Western Argentina were used. Biological indexing was performed in a greenhouse under controlled temperature using Etrog citron Arizona 861-S1 (*Citrus medica* L.) grafted on Rough lemon (*C. jambhiri* Lush) as the indicator plant. Temperatures were kept within a range from 27º to 38ºC.

Tissue samples for s-PAGE were obtained from inoculated citrons used in the biological indexing tests. DNA extraction was performed following Semancik et al. (3).

HSVd and CEVd specific primers (4) were used for RT-PCR (1) and samples were taken directly from field plants and inoculated citrons. A nucleic acid extraction method proposed by Semancik et al. (3) for s-PAGE was compared with that of Pallás et al. (2).

The three diagnostic methods compared gave similar results in 11 of 12 samples assayed (Table 1).

TABLE 1
COMPARISON OF THREE DIFFERENT VIROID DIAGNOSTIC METHODS

Isolate	Province	Cultivar	Symptoms on inoculated citron	Molecular technique			
				s-PAGE		RT-PCR	
				CEVd	Other/s	CEVd	HSVd
R-011	Tucumán	Ruby Blood Orange	+	+	+	+	-
R-069	Tucumán	Tahiti Lime	+	+	++*	+	-
R-096	Salta	Jaffa Orange	+	-	+	-	+
R-144	Entre Ríos	Etrog Citron	+	-	+	-	+
R-146	Entre Ríos	Etrog Citron	+	+	-	+	-
R-407	Tucumán	Marsh Grapefruit	+	+	+	-	+
Positive control CEVd (R-408)	Tucumán	Marsh Grapefruit	+	+	+++*	+	+
Positive control CEVd (R-410)	Tucumán	Valencia Orange	+	+	+++*	+	-
Positive control CVd-II (R-009)	Tucumán	Ruby Blood Orange	+	+	+	+	+
Positive control CVd-II (R-074)	Tucumán	Cape Nartge Orange	+	-	+	-	+
Uninoculated citron	Tucumán	Etrog Citron	-	-	-	-	-
Negative control STG mother plant	Tucumán	75 AB Citrumelo	-	-	-	-	-

*number of bands observed by PAGE

Fig 1. Etrog citron grafted on rough lemon – Left: negative control (non inoculated) and Right: positive control with exocortis symptoms.

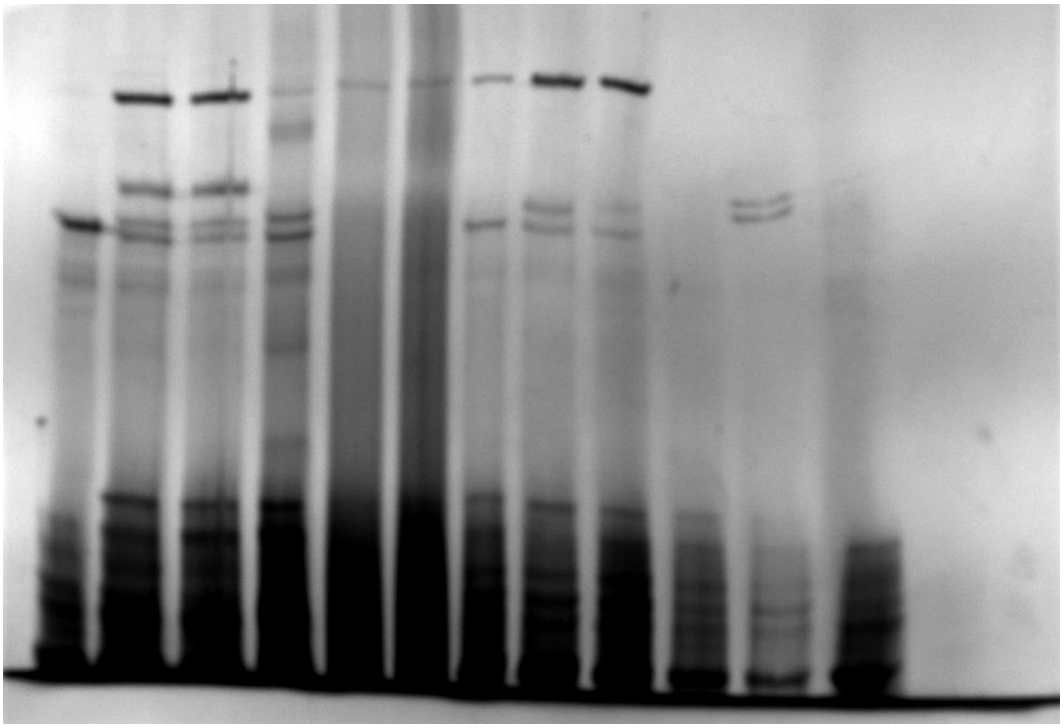

Fig 2. Silver staining of the second denaturing gel of s-PAGE showing the presence of mixedcitrus viroid infections.

Biological indexing of inoculated citron showed typical viroids symptoms as epinasty, stunting, vein and petiole necrosis, and petiole wrinkle (Fig.1). Several viroids were detected in the isolates by s-PAGE. In most cases, viroids were found as mixtures (Fig. 2).

Results obtained showed that RT-PCR could detect CEVd and HSVd in different citrus species using specific primers for each viroid.

HSVd and CEVd were identified by RT-PCR and no differences were found between the two extraction methods assayed.

The sample R-407 was positive for exocortis with biological diagnosis and s-PAGE, but it was negative for RT-PCR. This was probably due to a cDNA inefficient synthesis or to the presence of inhibitors that could interfere with the reaction.

Conclusions. On the basis of the results obtained, it can be concluded that the three techniques assayed are complementary and useful tools for diagnosis and identification of HSVd and CEVd in citrus. The two nucleic acid extraction methods were both suitable for RT-PCR. RT-PCR should not be used alone as a diagnostic technique for citrus viroids, as it failed to detect CEVd in the R-407 sample.

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