Exclusion and/or Uneven Distribution of Viroids in Four Citrus Hosts

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ABSTRACT. Four citrus cultivars: Verna lemon, Nules clementine, Frost satsuma and Navelina sweet orange were inoculated with a complex isolate of citrus viroids containing CEV, CV-Ia, CV-IIa and CV-IIId. The inoculated plants were biologically indexed on Etrog citron indicators, and were also analyzed by sequential polyacrylamide gel electrophoresis (sPAGE) of nucleic acid extracts. Citrons inoculated with infected Verna lemon and Nules clementine showed the severe reaction characteristic of the original isolate whereas Frost satsuma and Navelina sweet orange induced a milder reaction. Viroid analysis showed that Verna lemon and Nules clementine contained the same viroids that were present in the original isolate, whereas Navelina sweet orange excluded CEV and Frost satsuma showed segregation of CEV and CV-IIa.
Index words. citrus exocortis, citrus viroids, indexing.

In the routine biological indexing for exocortis, it was consistently observed that certain commercial varieties did not develop severe reactions on Arizona 861-S1 Etrog citron. The results of Etrog citron indexing of 86 cultivars done during the development of the Citrus Variety Improvement Program of Spain (6), showed that lemons and clementines always induced severe reactions, whereas satsumas and Navelina sweet orange induced mild symptoms (table 1). Since these cultivars had been equally exposed to viroid infection, an uneven distribution or exclusion of citrus viroids in certain citrus cultivars was considered the possibility for the lack of symptoms.

MATERIALS AND METHODS

Viroid source. An isolate called E-117 (2) which was originally obtained by mechanical transmission from a stunted Troyer citrange seedling which had been graft inoculated with Nules clementine buds. The above plant when indexed with Arizona 861-S1 Etrog citron gave a positive reaction with severe stunting and leaf epinasty, and necrosis of stems, petioles and veins. Nucleic acid extraction and sPAGE of the citrons showed that E-117 comprised CEV, CV-Ia, CV-IIa and CV-IIId (2). This isolate has been maintained on Pineapple sweet orange in the Citrus Virus Collection of the Instituto Valenciano de Investigaciones Agrarias (IVIA) as E-117.

Four citrus cultivars, Verna lemon, Nules clementine, Frost satsuma and Navelina sweet orange grafted on Carrizo citrange rootstock were graft inoculated with E-117 isolate. The inoculated plants were maintained in two separate greenhouses maintained at 18-25 C and 27-32 C for 9 months.

Biological indexing. Indexing was accomplished by grafting bark patches onto Arizona 861-S1 Etrog citron (8). Two separate grafts were made per inoculation and two indicator plants were used per cultivar. The symptoms were recorded 3 and 6 months after inoculation. The isolate E-117 maintained on Pineapple sweet orange was used as a positive control.

Viroid identification. Three months after inoculation the citron indicators were analyzed for viroids. The second and third flushes of citron tissue were also analyzed as they became available, 5 and 7 months after inoculation. The same plants were also analyzed over the following 6 months. Viroid identification was accomplished by nucleic acid extraction and sPAGE of inoculated citrons. Young leaf and stem tissue (5g) was homogenized in extraction medium containing water saturated phenol, and the nucleic acids were partitioned in 2M LiC1 (3). Following the first PAGE under standard conditions, a segment of the gel defined by the spot of the xylene cyanol dye was cut and placed on the top of a second gel containing 8M urea and

TABLE 1
INDEXING ON ETROG CITRON OF 86 CULTIVARS INCLUDED IN THE CITRUS VARIETY IMPROVEMENT PROGRAM OF SPAIN

Group of cultivars	Number of trees indexed	Number of trees[z]			
		Intensity of citron reaction[y]			
		+	+ +	+ + +	+ + + +
Lemons	19	0	0	6(32)	13(68)
Clementines	35	0	1(3)	28(80)	6(17)
Satsumas	7	1(14)	6(86)	0	0
Navelinas	4	2(50)	2(50)	0	0
Other Navel	21	1(5)	5(24)	14(66)	1(5)

[z]Figures in brackets are percent (%) over the number of trees indexed.
[y]Symptom intensity on citron had been rated as: severe (+ + + +); moderate (+ + +); mild (+ +); very mild (+); and no symptoms (–) six months after inoculation.

polymerized with pH 6.5 buffer (7,9). The circular forms of the viroids were viewed after silver staining (5).

RESULTS

Biological indexing onto Etrog citron, using bark patches of Verna lemon and Nules clementine that contained the isolate E-117 induced severe stunting, epinasty and tissue necrosis characteristic of the E-117 isolate. Indexing using bark patches from Frost satsuma and Navelina sweet orange induced mild wrinkle petiole, necrosis and mild leaf epinasty on Etrog citron (Fig. 1).

Three months after inoculation, the first flush of tissue of the citron indicators was analyzed for the presence of viroids. Citrons inoculated with Verna lemon and Nules clementine contained similar viroids as in Pineapple sweet orange inoculated with isolate E-117 used as control (Fig. 2). None of the citrons inoculated with Frost satsuma and Navelina sweet orange showed CEV and CV-IIa was not present in one of the citrons inoculated with Frost satsuma (Table 2).

The second and third flushes of tissue of the citrons inoculated with Frost satsuma and Navelina sweet orange also showed milder symptoms than the controls. When the second flush of tissue was analyzed for viroids five months after inoculation, the results were the same as before. However when the third flush of tissue was analyzed seven months post inoculation, both citrons inoculated with Frost satsuma showed CV-IIa. CEV was not detected then, nor in later tests performed over the following six months (Table 2).

In order to address the question of whether CEV was actually present in the inoculated Frost satsuma and Navelina sweet orange, these trees were budded with two healthy citron-buds each. The citron buds developed poorly on Navelina sweet orange and

Fig. 1. Reaction on Etrog citron: plant in the left was inoculated with bark patches from Frost satsuma, and plant in the left was inoculated with bark patches from Pineapple sweet orange which contained the E-117 isolate.

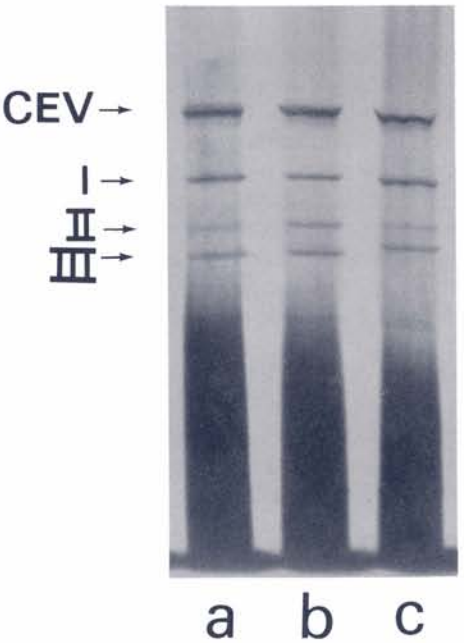

Fig. 2. Sequential polyacrylamide gel electrophoresis (sPAGE) of nucleic acid extracts of citron inoculated with the isolate from: a) Verna lemon; b) Nules clementine; and c) Pineapple sweet orange.

Frost satsuma, but after three months, enough tissue (2-3 g) was collected for viroid analysis. The buds on Verna lemon and Nules clementine did not grow at all (Fig. 3). CEV was detected from citron growing on Frost satsuma, but not from citron growing on Navelina sweet orange (Fig. 4). No differences were found between plants grown at 18-25 C or at 27-32 C.

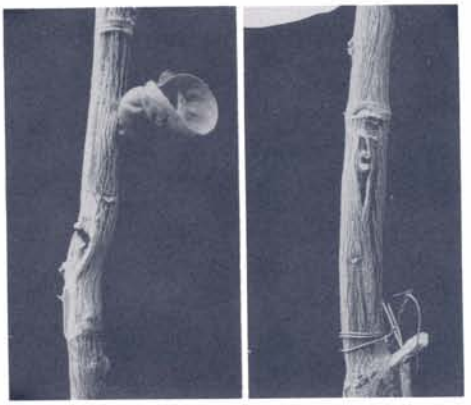

Fig. 3. Development of citron buds grafted onto Frost satsuma (left) and Verna lemon (right) infected with the E-117 isolate.

DISCUSSION

The results show that either Naveline sweeet orange was not infected with CEV or it replicated so poorly that it made detection impossible even with the most sensitive selec-

TABLE 2
DETECTION OF CITRUS VIROIDS FROM FROST SATSUMA AND NAVELINA SWEET ORANGE

	Detection of citrus viroids on citron inoculated with bark patches from Frost satsuma and Navelina sweet orange[z]							
	CEV		CV-Ia		CV-IIa		CV-IIId	
	1	2	1	2	1	2	1	2
Frost satsuma	–	–	+	+	+	+	+	+
	–	–	+	+	–	+	+	+
Navelina sweet orange	–	–	+	+	+	+	+	+
	–	–	+	+	+	+	+	+

	Detection of citrus viroids on citron budded onto Frost satsuma and Navelina sweet orange			
	CEV	CV-Ia	CV-IIa	CV-IIId
Frost satsuma	+	+	+	+
	+	+	+	+
Navelina sweet orange	–	+	+	+
	–	+	+	+

[z]Detection of citrus viroids: (1) 3-5 months after inoculation; (2) 7-21 months after inoculation.

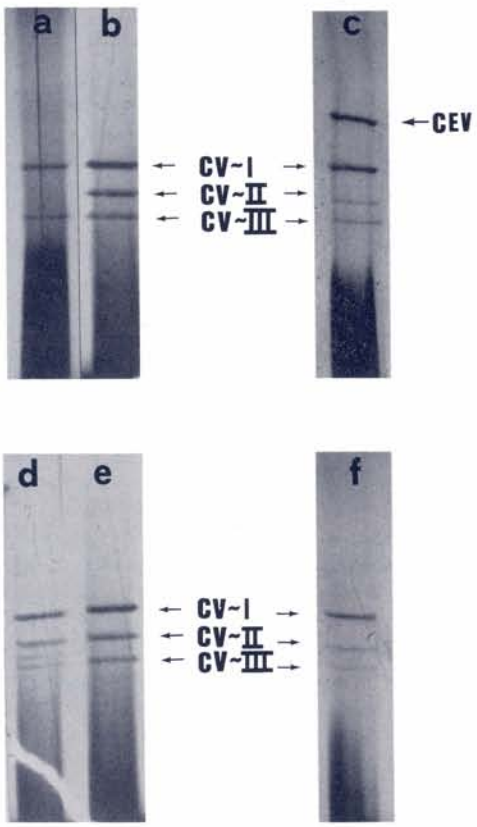

Fig. 4. Sequential polyacrylamide gel electrophoresis (sPAGE) of nucleic acid extracts of citrons inoculated with Navelina sweet orange (a,b), and Frost satsuma (d,e). Sequential polyacrylamide gel electrophoresis (sPAGE) of nucleic acid extracts of citrons budded onto Navelina sweet orange (c) and Frost satsuma (f).

tion of Etrog citron (861-S1). This citron selection has been shown to be an excellent host for all the citrus viroids described. Among the viroids inoculated, CEV replicated the best (3). High titers of CEV were also detected in citrons infected with the same isolate E-117 from other host (Fig. 2) and tissue from citron on Frost satsuma (Fig. 4). The apparent exclusion of CEV seems to be peculiar of Navelina sweet orange since CEV has been readily detected in other sweet orange varieties including other navel cultivars (table 1).

Frost satsuma was able to harbor the four viroid components of the isolate E-117. One of the viroids CV-IIa, was detected in a citron indicator three months after inoculation, whereas, it took seven months before it could be detected in the other. This result suggests that CV-IIa distribution was uneven or it replicated poorly in Frost satsuma. Poor viroid titers and/or uneven distribution might explain the difficulty encountered in detecting CEV in the citron indicators.

Our results show that the citron bioassay may fail to detect CEV in Frost satsuma even when the citron indicators were further analyzed by nucleic acid extraction and sPAGE. The poor distribution of viroids within Frost satsuma tissue may result in erroneous results in detection assays. Although it has been suggested that the use of one or two citron indicators per test coupled with nucleic acid extraction and sPAGE would provide the most sensitive test for viroid detection (4), in the case of satsuma caution must be exerted and larger number of indicators should be used (1,8).

According to the results presented here, budding Arizona Etrog citron 861-S1 onto the satsuma plants to be indexed, followed by nucleic acid extraction and sPAGE of citron tissues would provide the most sensitive technique. Whether the reported results are unique to Frost satsuma or a characteristic of all the satsuma cultivars should be further studied. These studies should be also expanded to other citrus species in order to develop better assays.

LITERATURE CITED

1. Calavan, E. C., E. F. Frolich, J. B. Carpenter, C. N. Roistacher and D. W. Christiansen 1964. Rapid indexing for exocortis of citrus. Phytopathology 54: 1359-1362.
2. Duran-Vila, N., J. A. Pina, J. F. Ballester, J. Juarez, C. N. Roistacher, R. Rivera-Bustamante, and J. S. Semanick.
1988. The citrus exocortis disease: A complex of viroid-RNAs, p. 152-164. *In* Proc. 10th Conf. IOCV. IOCV, Riverside.

3. Duran-Vila, N., C. N. Roistacher, R. Rivera-Bustamante, and J. S. Semanmcik.
1988. A definition of citrus viroid groups and their relationship to the exocortis disease. J. Gen. Virol. 69: 3069-3080.
4. Duran-Vila, N., J. A. Pina, M. I. Molins and L. Navarro
1991. A new indexing method for cachexia, p. 224-229. *In* Proc. 11th Conf. IOCV. IOCV, Riverside.
5. Igloi, G. L.
1983. A silver stain for the detection of nanogram amounts of tRNA following two-dimensional electrophoresis. Anal. Biochem. 134: 184-188.
6. Navarro, L., J. Juárez, J. A. Pina, J. F. Ballester, and J. M. Arregui
1988. The citrus variety improvement program of Spain after eleven years. p.400-406. *In* Proc. 10th Conf. IOCV. IOCV, Riverside.
7. Rivera-Bustamante, R., R. Gin, and J.S. Semancik
1986. Enhanced resolution of circular and linear forms of viroid and viroid-like RNA by electrophoresis in a discontinuous-pH system. Anal. Biochem. 156: 91-95.
8. Roistacher, C. N., E. C. Calavan, R. L. Blue, L. Navarro, and R. Gonzalez
1977. A new more sensitive citron indicator for detection of mild isolates of citrus exocortis viroid (CEV). Plant Dis. Rep. 53: 333-336.
9. Semancik, J. S., and K. L. Harper
1984. Optimal conditions for cell-free synthesis of citrus exocortis viroid and the question of specificity of RNA polymerase activity. Proc. Natl. Acad. Sci. USA, 81: 4429.4433.