Viroid Diseases of Citrus in India

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ABSTRACT. Exocortis disease of citrus in India has been known to occur for more than two decades but studies on its causal viroid have not been accomplished. The results of analysis of samples showing exocortis-like symptoms in R-PAGE revealed presence of two viroid species, one belonging to Indian Tomato bunchy top viroid (ITBVd) and the other to citrus B (CVd-IIa) group of viroids. This is for the first time in India that viroids have been found infecting citrus plantings.

Citrus exocortis disease was first described by Fawcett and Klotz (4). The involvement of a viroid with this disease was established in 1972 (10). In India, exocortis was reported in some trees in Delhi and Punjab which were grafted on Rangpur lime (5,8). These reports were based on visual symptoms and transmission to Rangpur lime of limited samples. We therefore attempted to investigate if any viroid species is present in a disease showing exocortis-like symptoms in citrus plantings in India. The results are presented in this paper.

Budwood were collected from citrus plantings in Pune (Maharashtra), Bangalore (Karnataka) and Delhi (Table 1) and graft-inoculated on rooted cuttings of Etrog citron and Rangpur lime seedlings. Grafted plants were maintained in a greenhouse (max. 25-35 C and min. 20-25 C). The leaves from symptomatic plants 6 months after inoculation were used as viroid source. Young leaf samples obtained from trees suspected to be affected with exocortis disease from Ludhiana were also analysed (Table 1). Cucumber cv. Suyo seedlings (cotyledons) were used for bioassay of the nucleic acid preparation obtained from grafted plants and samples from Ludhiana.

Six months after inoculation, conspicuous stunting was observed in inoculated Rangpur lime plants and epinasty symptoms in Etrog citron from the samples collected from Pune and Bangalore but not from Delhi source. However cucumber plants showed typical symptoms of viroid infection from Delhi samples.

TABLE 1
SOURCE OF PLANTING MATERIAL FOR VIROID DETECTION

Place of collection	Rootstock (RS)	Scion Var. (SV)	No. of trees sampled	Symptoms (RS)	Symptoms (SV)
Ludhiana (Punjab)	Rangpur lime	Kinnow mandarin	1	BS[z]	Ly
		Malta Swo[z]	1	Bs	Db
		Etrog citron	1	Bs	St
Pune (Maharashtra)	Trifoliate orange	Mosambi Swo	1	Bsp	Db
Bangalore (Karnataka)	Rangpur lime	Chini Swo	1	Bs	Db
Delhi	Not known	Trifoliate	6	No visible symptoms	
	Rough lemon	Mosambi Swo	3		

[z]Swo = sweet orange, Bs = bark scaling, Bsp = bark splitting, Db die back, St = stunting.

Young leaves of inoculated Etrog citron plants were used for nucleic acid (NA) extraction. Total NA were prepared by grinding 1 g of leaf tissue in 3 ml of extraction buffer (0.53 M NH_4OH, 0.013 M (EDTA) with Tris, 4 M LiCl, and 1% purified bentonite) and 4 ml 0.05 M Tris saturated phenol (containing 0.1 g of 8-hydroxy quinoline/ 100ml). Samples and extraction buffer were maintained at 4-5 C throughout the extraction procedure. The samples were homogenized in liquid nitrogen and centrifuged (15 min, 8,000 *g* at 4 C, and nucleic acids from upper aqueous layer were precipitated (-20 C, 30 min) by adding 0.5 volume of 7.5 M ammonium acetate solution and 2.5 volume of ethanol. The precipitate was collected by centrifugation as above, dried with a current of air, and dissolved in sterilized double glass distilled water (100 µl/g of tissue for gel electrophoresis).

Return Polyacrylamide Gel Electrophoresis (R-PAGE) was used (7,9) with some modification. Aliquots of 10 µl were mixed with 6 µl of a solution containing 1% xylene cyanol and 1% bromophenol blue prepared in 40% sucrose. First electrophoretic separation was at constant current 46 mA for 2.5 hr at 20 C on 7.5% acrylamide nondenaturing slab gel in high salt buffer (89 mM Tris, 89 mM boric acid , 2.5 mM EDTA, pH 8.3). During the second run the buffer in both the reservoirs was replaced with a low salt buffer (1:8 dilution of the high salt buffer) at boiling point (95 C) and the gel left as such without passing current for 5 min. These conditions denature the viroid. Later the run was resumed at constant current 46 mA for 2 hr at 70 C with reversed polarity. Gels were stained using silver nitrate (6). After fixing the gels in a mixture of acetic acid and ethanol for 1 to 2 hr they were kept in a solution of 0.2% silver nitrate for 1 hr. After two washes in sterile distilled water the gels were developed in a fresh solution of 375 mM KOH, 2.3 mM sodium borate, 0.5% HCHO (37% w/v) and further fixed in a solution of 70 mM sodium carbonate. Silver stained bands on electrophoretograms were evaluated based on their electrophoretic mobility in comparison to reference samples. In the present study, purified low molecular weight RNA from Indian tomato bunchy top viroid (ITBVd) and an isolate of hop stunt viroid (HSVd) from grapevine isolated by us were used as reference samples.

The results of R-PAGE revealed differences in electrophoretic mobility in bands observed in different samples. Bands observed in citron samples from Ludhiana, Mosambi sweet orange from Pune and Chini sweet ornage from Bangalore corresponded in electrophoretic mobility to ITBVd while that in trifoliate and Mosambi from Delhi to HSVd type. No bands were detected in samples of Kinnow mandarin and Malta sweet orange from Ludhiana and healthy controls.

Viroids have been detected from two type of trees - one showing typical exocortis symptoms and transmissible to its indicator hosts, whereas the other ones were without any visible symptoms. Bioassay of symptomless trees on cucumber, however, showed the presence of viroid infection. R-PAGE analysis of the test samples clearly showed the presence of viroid species belonging to ITBVd (7) and HSVd (2) in citrus plantings in the country. The band on the HSVd pattern and its infection to cucumber cv. Suyo was reported as Citrus B viroid (CBVd) and was considered as a strain of HSVd (2). CBVd is now known to be identical to CVd-IIa (13). It is therefore concluded that citrus plantings in India carried viroid infection apparently of CVd-IIa and ITBVd types.

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