PCR Diagnosis of Citrus Viroids in Field Samples

M. Tessitori, R. La Rosa, G. Albanese, and A. Catara

ABSTRACT. Several procedures for rapid and sensitive detection of different citrus viroid-RNAs in field samples were evaluated. Bark tissue of different citrus species with single or mixed infections of CEVd, CVd-II and CVd-III were collected at different seasons. Nucleic acids were phenol extracted and purified by CF-11 cellulose chromatography or extracted with SDS-potassium acetate and analyzed by sequential polyacrylamide gel electrophoresis (sPAGE), reverse transcription (RT)- or multiplex reverse transcription (MRT)- polymerase chain reaction (PCR). Both PCR procedures were more sensitive than sPAGE, partially overcoming the difficulties derived from low viroid concentrations in winter and spring. However, in the presence of the three citrus viroid groups, these procedures didn't allow a full profile of viroid content. MRT-PCR, using two sets of primers, showed about the same sensitivity as RT-PCR, giving a simultaneous diagnosis of two viroid groups in mixed infections but the amplified DNA yield was usually lower. Greenhouse-grown plants gave better DNA yield than field plants. In our operating conditions, PCR diagnosis of field samples resulted in reliable all-season detection of CEVd, from spring to autumn for CVd-II, and in summer and autumn for CVd-III; whereas CVds detection by sPAGE was restricted to June to November.

Index words: exocortis, cachexia, detection, sPAGE, PCR.

Viroids represent an important class of pathogens responsible for two serious, well-defined diseases, exocortis and cachexia, as well as distinct symptoms in some citrus species (15). Viroid detection in quarantine and certification systems is routinely accomplished by bioassay on indicator plants, which is time consuming, expensive, and not suitable for individual viroid identification when multiple infections occur.

In 1984, CEVd was detected in citrus leaves from field trees either by non-denaturing polyacrylamide gel electrophoresis (PAGE) (2) or by denaturing (d) PAGE (3). Further research showed reliability of dPAGE for detecting viroids in field citrus plants, by analyzing young bark collected during the warm season (4, 6, 10). Sequential PAGE (sPAGE) provides a better separation of viroid RNAs (14), and it has also been successfully used for diagnosis in field samples (1, 4). Additional techniques that have been applied to diagnose and to characterize citrus viroids from field samples include molecular hybridization (1, 6, 9), reverse transcription and amplification by the polymerase chain reaction (RT-PCR) (11, 17) and multiplex reverse transcription PCR (MRT-PCR) analyses (8, 12, 16).

In this paper we have compared RT-PCR or MRT-PCR analyses with routine sPAGE, using two procedures for nucleic acid extraction for diagnosis of viroids in field samples.

MATERIALS AND METHODS

Viroid sources. Field citrus plants of several cultivars, containing single viroids or mixtures of two or three of them, were used for the analyses (Table 1). Donor plants were from citrus orchards located in Calabria and Sicily, both areas characterized by temperate climate. All viroid sources and a purified CVd-IIIa source were inoculated into Arizona 861-S1 Etrog citron grown in a warm greenhouse. Healthy citrus varieties from the field were included as control.

Nucleic acids extraction and sPAGE analysis. Viroids were phenol-extracted from 5 g of young bark collected at different seasons, purified by CF-11 cellulose column chro-

TABLE 1
sPAGE VIROID PROFILE OF SOURCE PLANTS, NUMBER OF SAMPLES PROCESSED BY
PCR PER SOURCE PLANT AND NUMBER OF PCR TESTS PER VIROID

Source plant	sPAGE viroid profile				No. samples per source plant
	CEVd	CVIIa	CVIIb	CVIIIb	
Star ruby grapefruit	+	-	-	-	2
Orlando tangelo	-	-	+	-	2
Moro sweet orange	-	-	-	+	2
Howell grapefruit	-	-	+	-	2
Clementine "SRA-63"	+	+	-	+	3
Clementine	+	-	-	+	3
Femminello Fior d'Arancio lemon	+	+	+	-	14
Femminello Continella lemon	+	-	+	+	14
PCR test/viroid	36	17	32	22	

matography (7), concentrated and finally resuspended in 10 μl sterile distilled H_2O per g of tissue. Alternatively, 0.2 to 0.5 g of young bark were extracted by the SDS-potassium acetate procedure, as described by Garnsey et al. (8) and the final preparation dissolved in 10 μl H_2O per 0.1 g of tissue. Aliquots of each sample (~20 μl) were analyzed by sPAGE and visualized by silver staining (14).

RT- or MRT-PCR amplification. Viroid RT- and MRT-PCR amplification were performed using primers for CEVd, HSVd (hop stunt viroid, CVd-II homologous) and CVd-III viroids. CEVd and HSVd primers were the same as those previously selected by Yang et al. (17). CVd-III primers (C-2 and H-2) were the same as those devised by Rakowski et al. (13) on CVd-IIIb sequence.

Viroid cDNA synthesis was performed at 42°C for 1 h with M-MLV reverse transcriptase (Promega) using 0.02 to 0.2 g of total nucleic acid as template. Cycling parameters for PCR were 94°C for 1 min, 57°C for 2 min and 72°C for 1 min (30 cycles) with final extension at 72°C for 7 min in a DNA thermal cycler (12). The annealing temperature was the same for RT and MRT-PCR. RT-PCR was performed by using CEVd, HSVd or CVd-III primers to detect each viroid group separately, and MRT-PCR using CEVd+HSVd, CEVd+CVd-III or HSVd+CVd-III primers pairs, to show multiple infections in a single reaction.

RT-PCR and MRT-PCR products were analyzed by electrophoresis on 2% agarose gel (2 h, 65 V) or on 6% polyacrylamide gel (2 h, 160 V). After electrophoresis the gels were silver or ethidium bromide stained.

RESULTS

The phenol extraction and purification by CF-11 cellulose column chromatography, followed by sPAGE analysis, allowed detection of CEVd, CVd-II and CVd-III during the warm season (June to November), but only CEVd was detectable out of these months (Fig. 1). In preliminary tests, the SDS-potassium acetate extraction procedure yielded a low concentration of viroid nucleic acid that was not detectable by sPAGE. The molecular size of detected viroids, estimated by comparing their electrophoretic migration with known markers, was approximately: CEVd (~371), CVd-IIa (305), CVd-IIb (~299), CVd-IIIa (~297), CVd-IIIb (~294) nucleotides.

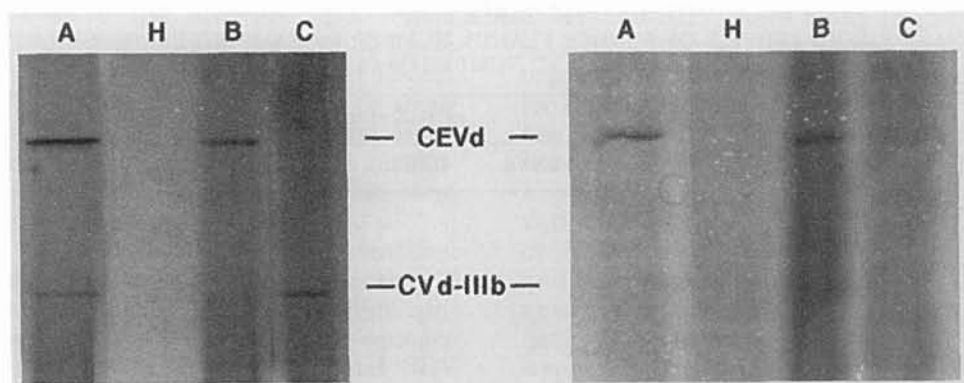RT-PCR of nucleic acids, either partially purified by CF-11 or extracted by SDS-potassium acetate

Fig. 1. Sequential polyacrylamide gel electrophoresis (sPAGE) of nucleic acid extracts from: A) clementine infected with CEVd and CVd-IIIb; H) healthy lemon; B) Star ruby grapefruit infected with CEVd; C) Moro sweet orange infected with CVd-IIIb. Samples were collected in August (on the left) or in February (on the right), respectively.

method, allowed amplification of cDNAs from CEVd in all seasons and from CVd-II group in spring, summer and autumn. In our conditions, detection of CVd-III group from field samples was only reliable during the warm season (Table 2). HSVd-primers amplified CVd-IIa and IIb cDNAs. Similarly, CVd-III-specific DNA primers detected CVd-IIIa and IIIb (Fig. 2). No PCR products were obtained when healthy extracts were analyzed with any set of primers.

MRT-PCR allowed simultaneous detection of two viroids in mixed infections in a single reaction. Thus, by using two sets of primers, the presence of CEVd, CVd-IIa and CVd-IIb (Fig. 3) or CEVd and CVd-III, or CVd-II and Cvd-III, could be detected. Samples simultaneously analyzed by RT and by MRT-PCR gave similar results, but yield obtained with the latter procedure was lower. A sample of lemon infected with CEVd, CVd-IIa and CVd-IIb, collected in January and tested by MRT-PCR showed only two bands corresponding to CEVd and CVd-IIa; whereas CVd-IIb was not detectable at that time. No amplification products were observed in healthy samples by MRT. As expected, greenhouse infected controls, processed either by RT-PCR or by MRT-PCR, always yielded higher concentration of PCR products.

TABLE 2

COMPARISON BETWEEN sPAGE AND PCR ANALYSES FOR DETECTION OF CITRUS VIROIDS (CEVd, CVd-II AND CVd-III GROUPS) IN FIELD SAMPLES COLLECTED AT DIFFERENT SEASONS

	Winter		Spring		Summer		Autumn	
Viroid	sPAGE	PCR	sPAGE	PCR	sPAGE	PCR	sPAGE	PCR
CEVd	2/4[z]	4/4	3/6	6/6	15/15	15/15	11/11	11/11
CVd-IIa	0/2	1/2	0/3	3/3	7/7	7/7	5/5	5/5
CVd-IIb	0/4	0/4	0/4	4/4	14/14	14/14	10/10	10/10
CVd-III	0/2	0/2	0/4	1/4	9/9	9/9	7/7	7/7

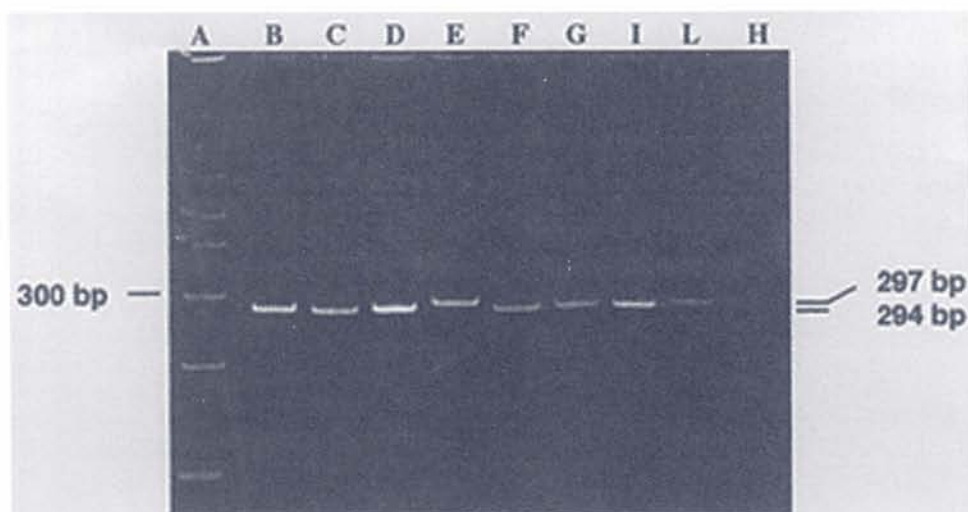[z]Number of positive viroids detection out of total tests per season.

Fig. 2. RT-PCR products synthesized with primers from CVd-III and extracts from citrus infected with: B) CVd-IIIb; C) CEVd and CVd-IIIb; D) CEVd, CVd-IIa and CVd-IIIb; E) CVd-IIIa marker; F to L) same samples as in B to E, tenfold diluted; A) DNA molecular size marker (low marker, Bioventures); H) healthy control. DNA was separated in a 2% agarose gel and stained with ethidium bromide.

DISCUSSION

Viroid replication is affected by temperature and, thus, their concentration in infected tissues is variable around the year in temperate climates with a maximum during the summer to autumn period (4, 9). In these seasons, sPAGE analysis of nucleic acids extracted from field samples and partially purified by CF-11 cellulose chromatography is fast, sensitive and reliable for viroid detection. In winter and spring, viroid concentration is very low and sPAGE analysis unsuitable for diagnosis.

PCR amplification allowed detection of CEVd and groups CVd-II and CVd-III, either alone or in mixture, for a wider period of time, although some seasonal limitations also exist due to low concentration of CVd-II and CVd-III viroids (Table 2).

MRT-PCR was faster for diagnosis of several viroids in mixed infections but, in some cases, less sensitive than RT-PCR. This was probably due to interference between two sets of primers template, that could result in advantage for one of the sets. Under our experimental conditions, a reliable MRT-amplification was obtained for the combination CEVd and CVd-II, allowing simultaneous diagnosis of exocortis and cachexia, as well as for CEVd and CVd-III viroids. Amplification of the mixture CVd-II and CVd-III yielded a faint band of CVd-II DNA compared to that of CVd-III, making it difficult to diagnose. By amplification methods, viroid detection may be achieved from a minimum amount of tissue (0.2 to 0.5 g) particularly after SDS-potassium acetate extraction as the sensitivity is increased to the level of picograms of viroids.

Electrophoretic analysis of nucleic acids extracted from field samples is a well established method for citrus viroid diagnosis. This procedure which is complementary to the bioassay on citron, has been used routinely in our laboratory since 1988 (10), and since 1993, has been adopted for indexing plants in the voluntary certification program recently approved in Italy. The method is also used in other countries after replication of viroids in
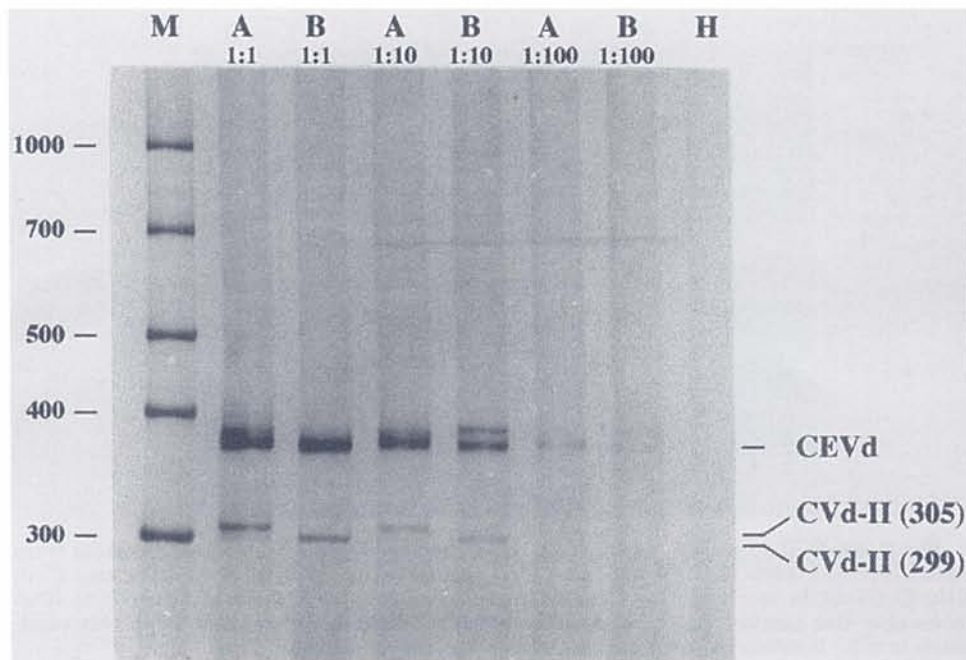
Fig. 3. Polyacrylamide gel electrophoresis (6%) of MRT-PCR products amplified from citrus samples by using primers for CEVd and CVd-II groups. Samples were: A) Femminello Fior d'Arancio lemon infected with CEVd, CVd-IIa and CVd-IIb and B) Femminello Continella lemon infected with CEVd and CVd-IIb, at different dilutions. M) DNA molecular size marker (low marker, Bioventures); H) healthy control. The DNA bands were silver stained.

citron (5). PCR-based detection methods have already been suggested by different authors to diagnose CEVd and CVd-II viroid-RNAs from greenhouse and field-grown sweet orange trees (8, 12). Our results indicate applicability of the method to additional citrus species and varieties, i.e. lemon, clementine, grapefruit, tangelo. The CVd-III group of viroids was also detected for the first time by PCR methods in field samples.

In conclusion, during the warm season, a reliable and fast diagnosis of CEVd, CVd-II and CVd-III from field samples may be achieved either using sPAGE or PCR-based methods. PCR methods allow a wider sampling period, particularly for CVd-II viroids. The choice will depend on the purpose of the diagnostic, the sampling time and the facilities available.

ACKNOWLEDGMENTS

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