

SHORT COMMUNICATION

**OCCURRENCE OF PEACH LATENT MOSAIC VIROID IN CHINA AND DEVELOPMENT OF AN IMPROVED DETECTION METHOD**

C. Turturo<sup>1</sup>, A. Minafra<sup>1</sup>, H. Ni<sup>2</sup>, G. Wang<sup>2</sup>, B. Di Terlizzi<sup>3</sup> and V. Savino<sup>1</sup>

<sup>1</sup>Dipartimento di Protezione delle Piante, Università degli Studi and Centro di Studio sui Virus e le Virosi delle Colture Mediterranee, Via G. Amendola 165/A, I-70126 Bari, Italy

<sup>2</sup>Department of Plant Protection, Research Institute of Pomology, Xingcheng, People's Republic of China

<sup>3</sup>Istituto Agronomico Mediterraneo, Via Ceglie 9, I-70010 Valenzano (Bari), Italy

**SUMMARY**

Peach latent mosaic viroid (PLMVd) was detected by indexing on peach 'GF-305' seedlings and by molecular hybridization with a digoxigenin-labelled riboprobe, in 9 out of 14 peach samples from the Liaoning province of the People's Republic of China. For molecular assays four different tissue processing methods were compared, two involving total nucleic acid (TNA) extraction with or without organic solvents followed by formaldehyde or alkaline denaturation, and two based on direct denaturation of crude leaf sap. All extracts, regardless of the method used, gave positive hybridization signals, but alkaline denaturation increased the sensitivity of detection in both TNA and crude sap preparations. Because of its simplicity and minimal manipulation, alkaline denaturation of crude sap appears to be the best method for routine detection of PLMVd.

**RIASSUNTO**

**PRESENZA DEL VIROIDE DEL MOSAICO LATENTE DEL PESCO IN CINA E MESSA A PUNTO DI UN EFFICACE METODO DI IBRIDAZIONE PER LA SUA DIAGNOSI.** Il viroide del mosaico latente del pesco è stato identificato in 9 su 14 piante di pesco della provincia cinese di Liaoning sia con indexaggio sull'indicatore "GF-305", che con ibridazione molecolare con una ribosonda marcata con digossigenina. Per i saggi molecolari sono stati provati quattro metodi di preparazione del campione, due dei quali prevedevano l'estrazione dell'RNA totale con solventi organici o no, e due erano basati sulla denaturazione diretta di succo fogliare con formaldeide o con idrossido di sodio. Tutti i tipi di estrazione, indipendentemente dal metodo adoperato, hanno dato risultati positivi alla ibridazione. La denaturazione alcalina, tuttavia, ha aumentato la sensibilità delle reazioni, per cui, a causa della semplicità e della scarsa manipolazione che richiede,

il trattamento con soda di estratti grezzi di succo fogliare appare come il metodo più indicato per la diagnosi di routine.

*Key words:* PLMVd detection, hybridization, alkaline denaturation.

Peach latent mosaic disease, first recorded from France some 20 years ago (Desvignes, 1976, 1980), is caused by peach latent mosaic viroid (PLMVd), but is generally latent in peach trees for 5-7 years before symptoms appear (Flores and Llacer, 1988; Flores *et al.*, 1990). PLMVd occurs in several countries (Skrzeczowski *et al.*, 1996; Hadidi *et al.*, 1997), including Italy (Albanese *et al.*, 1992; Shamloul *et al.*, 1996). Desvignes (1986) remarked on its presence in Chinese peach cultivars.

Detection and identification of PLMVd is currently based on biological and molecular methods. Indexing on peach 'GF-305' seedlings by cross-protection tests (Desvignes, 1976, 1980) is time consuming, expensive, and unsuitable for large scale screening. More sensitive and rapid results are obtained by nucleic acid hybridization (Ambros *et al.*, 1995; Loreti *et al.*, 1995), but these tests require extensive sample manipulation to remove inhibiting compounds. Here we describe the development of a fast, reliable and sensitive protocol for PLMVd detection by molecular hybridization, and its application for investigating the presence of the viroid in peach trees from China.

Green cuttings were collected in September 1996 in peach orchards of cv. 'Okubo', a variety originally introduced from Japan, in the Liaoning province (North-Eastern China) and brought to Bari, where buds were grafted onto healthy 'GF-305' seedlings grown in a screen-house. The seedlings were not challenge inoculated with a marker PLMVd strain. Grafts from 14 accessions survived and, during spring 1997 were used for symptom reading and molecular assays. Glasshouse-grown peach trees of cvs 'Federica', 'Independence' and 'Fairline' infected with a local isolate of PLMVd were used as positive controls, and PLMVd-free 'GF-305' seedlings were used as negative controls.

Corresponding author: A. Minafra  
Fax: +39.080. 5442911  
E-mail: csvvam05@area.ba.cnr.it

Samples of ca 300 mg of leaf tissue were processed in parallel immediately after collection or storage at 4°C overnight, using two methods involving total nucleic acids (TNA) extraction, and two applied directly to crude sap:

(i) liquid nitrogen-powdered tissues were extracted with 1.5 ml of 1x GES (0.1M glycine-NaOH, pH 9, 50 mM NaCl, 1 mM EDTA) containing 1%  $\beta$ -mercaptoethanol and 2% SDS, essentially according to White and Kaper (1989). The aqueous phase, after a phenol-chloroform extraction, was enriched for viroid RNA by chromatography through non-ionic cellulose CF11 (Whatman) columns as described by Flores *et al.* (1985);

(ii) the method was based on a slightly modified protocol for detection of hop stunt viroid in fruit trees (Astruc *et al.*, 1996). After an extraction in 15 vol. of TNE buffer (100 mM Tris-HCl pH 8.3, 500 mM NaCl, 500 mM EDTA, 10%  $\beta$ -mercapethanol), 100  $\mu$ l of 10% SDS were added to 750  $\mu$ l of extract. The samples were incubated at 70°C for 15 min, then with 200  $\mu$ l of 5 M potassium acetate on ice for 15 min. The extracts were centrifuged for 20 min and 500  $\mu$ l of the aqueous phase containing nucleic acids were ethanol-precipitated. TNA preparations resulting from (i) and (ii) were resuspended in 100  $\mu$ l of sterile water;

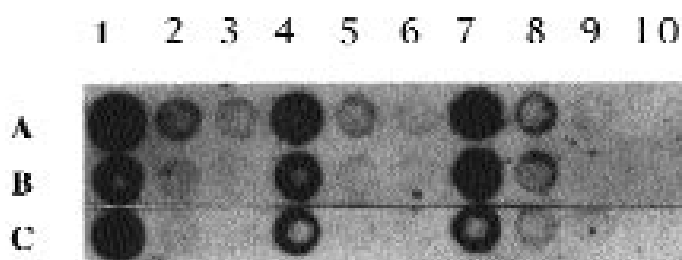
(iii) liquid nitrogen-powdered tissues were homogenized with Na-citrate buffer, containing 20 mM DIECA (Wetzel *et al.*, 1992), for 2 min, and the extract was clarified by a single low-speed centrifugation;

(iv) leaf tissues were ground in a plastic bag with a roll press in the presence of 3-6 vol of extraction solution (50 mM NaOH, 2.5 mM EDTA). The sap was centrifuged at 3000 rpm for 5 min and the supernatant was undiluted or serially diluted in water (Saldarelli *et al.*, 1996).

TNA extracts and Na-citrate extracts were denatured either with formaldehyde buffer (6x SSC-12.5% formaldehyde) (White and Bancroft, 1982) or by adding an equal volume of 100 mM NaOH containing 5 mM EDTA (alkaline denaturation). Five-fold dilutions of TNA (starting from 1  $\mu$ g) and crude sap were made in water before spotting.

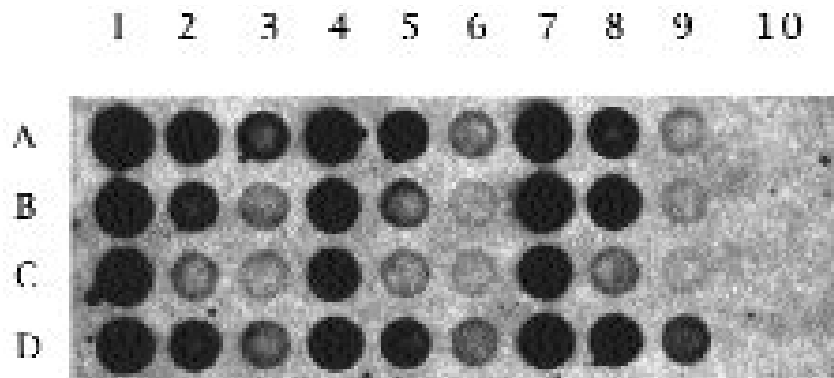
The samples were spotted onto Hybond-N+ nylon membranes (Amersham, UK) and the nucleic acids were cross-linked to the membrane by exposure to UV light (320 nm) for 5 min. The membranes were pre-hybridized for 2 h at 55°C in a hybridization mix (150  $\mu$ l cm<sup>-2</sup>) containing 5x SSC, 50% deionized formamide, 0.02% SDS, 0.1% sodium lauryl sarcosine, and 2% blocking reagent (Boehringer Mannheim). Overnight hybridization followed with 100 ng ml<sup>-1</sup> of SP6 RNA polymerase-generated full-length cRNA probe, synthesized on an Italian PLMVd isolate (Shamloul *et al.*, 1995), in a mini hybridization oven. The membranes were washed twice at room temperature with 2x SSC 0.1% SDS for 10 min and twice at 68°C with 0.1x SSC 0.1% SDS for 20 min, then incubated at room temperature in 2x SSC containing 1 mg ml<sup>-1</sup> RNase A to remove non-specifically bound ribo-probe. The membranes were processed as described previously (Finetti *et al.*, 1996) except for the chemiluminescent step in which CPD-Star (Boehringer, Mannheim) was used. Moist membranes, sealed in transparent polyester sheets, were exposed to X-ray films for 15-30 min.

Figures 1 and 2 show the outcome of dot-blot hybridization tests with digoxigenin-labelled probe carried out on TNA (Fig. 1, rows A and C; Fig. 2, rows B and D) and crude sap extracted in citrate (Fig. 1, row B; Fig. 2, row C) and alkaline solution (Fig. 2, row A) respectively, of the three PLMVd-infected peach varieties ('Federica', 'Independence', and 'Fairline'). TNA samples (up to the final dilution of 40 ng), as well as citrate spots, denatured either with formaldehyde (Fig. 1) or alkaline solution (Fig. 2), successfully hybridized. No hybridization signals were observed in the healthy controls (Figs 1 and 2, lane 10) regardless of the extraction or denaturation procedures used, indicating that, with the procedures described, spurious hybridizations due to carry-over of interfering substances were avoided. Furthermore, both crude sap extraction methods as well as the SDS-K acetate procedure were just as effective in obtaining undegraded nucleic acids, suitable for PLMVd detection, as was the standard CF 11 cellulose procedure.



**Fig. 1.** Dot-blot hybridization of a PLMVd riboprobe to extracts from three PLMVd-infected peach samples: cv 'Federica' (lane 1-3), 'Independence' (lane 4-6), 'Fairline' (lane 7-9) and healthy 'GF-305' (lane 10). Extract aliquots equivalent to 25 mg of fresh weight tissue and their serial five-fold dilutions were spotted on the membrane. Row A: TNA (1  $\mu$ g) extracted with SDS-K acetate Row B: Na citrate extracts. Row C: TNA purified on CF 11.

**Fig. 2.** Dot-blot hybridization of three PLMVd infected peach cultivars: 'Federica' (lane 1-3), 'Independence' (lane 4-6), 'Fair-line' (lane 7-9) and healthy 'GF-305' (lane 10). Samples were denatured with alkaline solution. Row **A**: direct alkaline extracts. Row **B**: TNA extracted with SDS-K acetate. Row **C**: Na citrate extracts. Row **D**: TNA purified on CF 11.



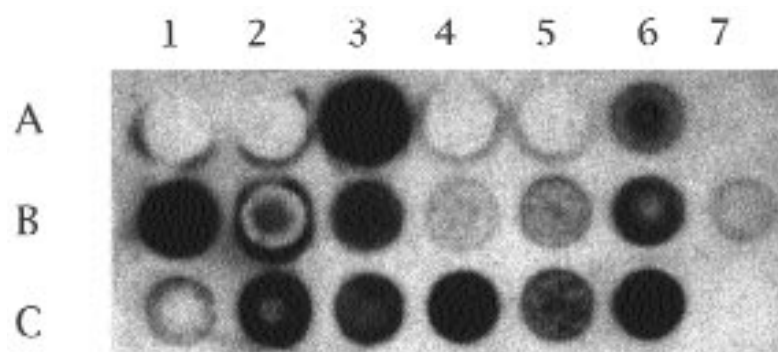
Regardless of the extraction method used, alkaline denaturation of TNA (Fig. 2) gave consistently stronger hybridization signals than formaldehyde denaturation (Fig. 1). Considering the intensity of the signal obtained with the highest TNA dilutions, it appeared that the limit of detection (Figs 1 and 2) was at least 40 ng, corresponding to 1.2 mg of fresh weight tissue. Since the dot-spots from citrate extraction gave a hybridization pattern comparable with that of TNA (Fig. 2, row C vs. B and D), with the undoubted convenience of avoiding nucleic acid extraction, a further simplification of the direct alkaline extraction was tried.

Alkaline extraction (Fig. 2, row A) gave clear-cut hybridization results with better sensitivity than TNA extracts. The method was also faster and required minimal manipulation compared to the long and cumbersome procedures of TNA extraction.

Based on the above results, the alkaline extraction procedure was used for the detection of PLMVd in Chinese peach samples. The hybridization pattern of 14 such samples is shown in Fig. 3, and Table 1 reports the comparative results of biological and molecular testing. Six of the Chinese accessions induced typical PLMVd responses in 'GF-305' seedlings, *i.e.* yellow calico-like blotching of the leaves. This reaction was sometimes accompanied by symptoms like those induced by apple chlorotic leaf spot virus (ACLSV), *i.e.* chlorotic leaf

ringspot, mottling and dwarfing. Ten clear-cut positive hybridization signals, indicating PLMVd infection, were observed (Fig. 3, A6, B1-B3, B5, B6, C2-C5), five of which were from accessions that induced yellow-calico symptoms on leaves of 'GF-305'. However, since a marker PLMVd strain was not used for challenge inoculating 'GF-305' seedlings that had been grafted with Chinese budwood, mild viroid isolates may have escaped detection. This could explain why some samples (for example B1, in Fig. 3), which did not give PLMVd symptoms in 'GF-305', showed a strong hybridization signal. Signal intensity of the positive controls was consistently high (Fig. 3, A3 and C6), whereas the signal of some tested samples was variable, sometimes below that of positive controls. For instance, Fig. 3 shows a doubtful hybridization signal (Fig. 3, C1) which corresponds to a positive indexing reaction. Whether this was due to the quality of the extraction (*i.e.* residual presence of interfering compounds) or a low viroid titre was not ascertained.

Different procedures for extracting peach tissues and denaturing sap or TNA to improve molecular hybridization assays for PLMVd detection were investigated. All the extraction methods were effective but alkaline denaturation increased detection sensitivity in both TNA and crude sap preparations as compared to formaldehyde denaturation, with no apparent loss of specificity.



**Fig. 3.** Dot-blot hybridization of PLMVd riboprobe to 14 Chinese peach samples. Positive controls: cv. 'Elberta' (A 3), 'Hale' (C 6). Negative controls: cv. 'Sweet Lady' (A1), 'Dixired' (A2), 'Sanguine' (A4), 'Titan' (A5), and 'GF-305' (C 7). Spotted sap corresponds to 30 mg of fresh tissue.

**Table 1.** Results for fourteen Chinese peach samples of dot-blot hybridization and indexing on 'GF-305'.

Sample	Dot-blot hybridization	Indexing
1	+	(A 6) PLMVd symptoms
2	-	(A 7) no symptoms
3	+	(B 1) ACLSV-like symptoms
4	?	(B 2) PLMVd symptoms, dwarfing
5	+	(B 3) PLMVd symptoms
6	?	(B 4) ACLSV-like symptoms
7	+	(B 5) dwarfing
8	+	(B 6) no symptoms
9	?	(B 7) no symptoms
10	?	(C 1) dwarfing
11	+	(C 2) dwarfing
12	+	(C 3) ACLSV-like and PLMVd symptoms
13	+	(C 4) PLMVd symptoms
14	+	(C 5) ACLSV-like and PLMVd symptoms

? : uncertain reaction. In parenthesis are the coordinates of hybridization spots in Fig. 3.

Proper denaturation is a critical step for ensuring RNA binding to membranes and availability of hybridization sites. Alkaline treatment inducing an extensive breakage of hydrogen bonds in extracted RNA and full denaturation of the molecules is thus desirable in view of the high degree of self complementarity of viroid molecules (Diener, 1979). In accordance with previous results showing that good denaturation increases the sensitivity of detection of other viroids (Macquaire *et al.*, 1984; Flores 1986; Astruc *et al.*, 1996), we successfully tried the alkaline extraction of crude peach sap without any further manipulation, obtaining consistent detection of PLMVd RNA by digoxigenin-labelled cRNA probes. This lays the basis for further developments in large scale detection of PLMVd as required by certification schemes.

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