Asian Journal of Plant Pathology

ISSN 1819-1541

Academic Journals Inc.

www.academicjournals.com

OPEN ACCESS Asian Journal of Plant Pathology

ISSN 1819-1541
DOI: 10.3923/ajppaj.2017.71.80

Research Article
Diagnostic Parameters of Northern Blot Hybridization Technique for Detection of Citrus Viroids in Field-grown Plants

[1]R. Umaña-Castro, [2]C. Pritsch, [3]R. Molina-Bravo and [2]G. Pagliano

[1]Laboratorio de Análisis Genómico, Escuela de Ciencias Biológicas, Universidad Nacional de Costa Rica, Apartado Postal 86-3000, Campus Omar Dengo, Heredia 40101, Costa Rica
[2]Laboratorio de Biotecnología, Dpto. Biología Vegetal, Facultad de Agronomía, UdelaR, Montevideo, Uruguay
[3]Laboratorio de Biología Molecular, Programa BIOVERFI, Escuela de Ciencias Agrarias, Universidad Nacional de Costa Rica, Apartado Postal 86-3000, Heredia 40101, Costa Rica

Abstract

Background and Objective: The identification and diagnosis of citrus viroids can be achieved by molecular techniques that directly detect the presence of viroid genomes within infected plant RNA pools. Several studies have reported numerous diagnostic parameters for detection of citrus viroids in field-grown plants. Herein, we report an improved molecular technique based on PAGE-Northern blotting with cDNA DIG-specific (non-radioactive) probes that is rapid, reliable and requires low amounts of tissue from field-grown samples. **Materials and Methods:** Total RNA was extracted from viroid-infected and negative control bark tissue sampled from field-grown citrus cultivars and subjected to DIG-labeled Northern blot molecular procedures. **Results:** Based on multi-probe viroid RNA hybridization sensitivity analysis, we were able to detect specific viroids with as little as 8-12 mg of fresh tissue. All cDNA DIG-specific probes yielded strong positive signal only when the specific viroid was present. Signal repeatability observed in 25 membranes was robust, with a total of 84 field samples analyzed. **Conclusion:** Overall, this molecular diagnostic technique, based on PAGE-Northern blot using cDNA-DIG probes, offered rapid, reliable and robust results, allowing for the direct detection of viroids from minute amounts of bark tissue sampled from field-grown citrus plants.

Key words: PAGE-Northern, citrus viroids, molecular diagnosis, cDNA-DIG probes, certification program

Received: September 27, 2016 Accepted: January 30, 2017 Published: March 15, 2017

Citation: R. Umaña-Castro, C. Pritsch, R. Molina-Bravo and G. Pagliano, 2017. Diagnostic parameters of Northern blot hybridization technique for detection of citrus viroids in field-grown plants. Asian J. Plant Pathol., 11: 71-80.

Corresponding Author: R. Umaña-Castro, Laboratorio de Análisis Genómico, Escuela de Ciencias Biológicas, Universidad Nacional de Costa Rica, Apartado Postal 86-3000, Campus Omar Dengo, Heredia 40101, Costa Rica

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Viroids are subviral pathogens consisting of a single-stranded, covalently closed, non-coding RNA molecule, with strong intramolecular base pairing. Citrus viroids belong to the family Pospiviroidae, which is composed of four genera *Pospiviroid*, *Hostuviroid*, *Apscaviroid* and *Cocadviroid* and has a genome size between 284-375 nucleotides[1]. Citrus viroids are transported through the phloem[2]. Accumulation of the viroids can vary depending on the citrus host-species, where intensity signals from sPAGE analysis range from high to undetectable[2]. The methods for diagnosis of diseases caused by these subviral pathogens are important worldwide. The available information on citrus viroids has increased and studies show that viroids have complex symptoms and disease aggressiveness due to synergistic interactions, host range and the possibility of recombination events[3]. The detection of virus-like infections in cultivars using diagnostic methods is important because of the potential economic impact in which viroid-infected commercial cultivars may present[4,5].

Molecular diagnostic methods have been recently developed for viroid detection based on the knowledge of the complete sequence of the pathogen[6-8]. These methods seek to avoid biological assays while achieving better sensitivity, specificity, diagnostic efficiency, safety and reduced handling time[9-11]. In Uruguay, citrus exocortis viroid (CEVd), citrus bent leaf viroid (CBLVd), hop stunt viroid (HSVd) and citrus dwarfing viroid (CDVd) have been detected in field-grown citrus plants using biological and molecular methods[12]. In contrast, citrus bark cracking viroid (CBCVd) and citrus viroid (CVd)-VI have not been detected[13], while CVd-V has not been surveyed.

Conventional diagnosis of citrus viroids is performed by biological methods based on the use of the indicator plant Etrog citron 861 (*Citrus medica*), which is the most sensitive woody-plant viroid indicator[14]. Depending on the citrus viroid species, inoculated Etrog citron plants show specific responses, however, symptoms are often non-specific due to complex interactions among viroids in multiple infections[15,16]. The long incubation periods needed for symptom expression result in biological methods being slow and expensive. Duran-Vila *et al.*[17] reported an approach that combines bioassays with the use of molecular methods, such as sPAGE followed by Northern blot with satisfactory results. Murcia *et al.*[11] developed a molecular approach based on a methodology that allows diagnostic results with high sensitivity and specificity, while at the same time handle large numbers of samples. The sensitivity, specificity and efficiency of molecular techniques for detection of viroids in citrus are important parameters for developing molecular tools for routine detection in commercial cultivars. Several authors have reported that molecular hybridization approaches without using radioactive probes can be used for the diagnosis of viruses and viroids[18-20]. The sensitivity of molecular techniques can be estimated by measuring the detection limits about titers of the viroid genomic RNA and the cut-off points that set the signal threshold to determine the presence or absence of the pathogen[21,22].

A molecular tool that discriminates among viroid species in infected plants for its use in routine assays using mixed probes is important for a reliable diagnosis. The robust specificity of a technique avoids false positives due to cross-reactions between the probe and host ribonucleoprotein complex[23,24]. A method with high specificity can be used to analyze the occurrence and the distribution of these pathogens in citrus-growing regions. Reducing the time needed for diagnosis of citrus viroids is essential when implementing an alternative technique in the production of certified planting material and in sanitation programs as a way of restricting the entry of infected material. Additionally, diagnostic protocols are particularly convenient when infected samples can be detected with small amounts of sampled tissue.

Although, testing for viroid presence is not a requirement in citrus certification programs in Uruguay, regulatory guidelines to prevent viroid contamination in propagation nurseries should be mandatory. Therefore, a standardized and reliable diagnostic method for numerous viroid evaluations is a priority[25]. The aim of the present study was to adjust and improve the methodological combination PAGE-Northern blotting with DIG-cDNA specific probes for several citrus viroids that affect nursery- and field-grown citrus plants. Additionally, we have established new sample size and parameter thresholds that support the reliability of this molecular tool.

MATERIALS AND METHODS

Plant material: Positive (20) and negative (4) controls consisted of citron plants inoculated with field-grown citrus plant species containing each viroid, grafted on rough lemon (*Citrus jambhiri* Lush.), trifoliate orange, sweet orange (W. Navel), mandarin (Common and Satsuma), Lisbon lemon and grapefruit (Star Ruby) and kept under controlled conditions[12,13] (Table 1). Fifty centimeters long by 1 cm diameter branches from orange, mandarin, lemon, grapefruit, volkamer lemon, trifoliate orange (*Poncirus trifoliata*) and kumquat trees were sampled from citrus-growing areas. Bark was collected from 42 trees in each area and infected citron samples were used as screening

Table 1: Positive and negative control citron plants and field-grown citrus plant species infected or not infected with viroid pathogens

Citrus species	Variety and code sample	Viroids infection	Reference
Citrus medica	Citron 102	None CVds infection*,#,$	Pagliano *et al.*[13]
Citrus medica	Citron 006	HSVd*,#,$	Pagliano *et al.*[13]
Citrus medica	Citron 16	CEVd*,#,$, HSVd*,#,$	Pagliano *et al.*[13]
Citrus medica	Citron 24	CBLVd*,#,$	Pagliano *et al.*[13]
Citrus medica	Citron 804Ca	CBCVd*,#,$	Pagliano *et al.*[13]
Citrus limon	Lemon Lisbon FB410	HSVd#	Pagliano *et al.*[13]
Citrus limon	Lemon Lisbon FB411	HSVd#	Pagliano *et al.*[13]
Citrus limon	Lemon Lisbon FB380	HSVd#	Pagliano *et al.*[13]
Citrus limon	Lemon Lisbon K390	CBLVd#, HSVd#, CDVd#	Pagliano *et al.*[13]
Citrus limon	Lemon Lisbon K393	CEVd*,#, HSVd#, CDVd#	Umaña *et al.*[12]
Citrus limon	Lemon Lisbon CDL386	HSVd#, CDVd#	Pagliano *et al.*[13]
Citrus limon	Lemon Lisbon K395	CEVd*,#, CBLVd#, HSVd#, CDVd#	Umaña *et al.*[12]
Citrus sinensis	Valencia late PqL175	HSVd#, CDVd#	Pagliano *et al.*[13]
Citrus sinensis	Orange Washington Navel KWN05	None CVds infection#	Pagliano *et al.*[13]
Citrus sinensis	Orange Washington Navel PEWN01	None CVds infection#	Pagliano *et al.*[13]
Citrus sinensis	Orange Washington Navel PEWN02	CEVd#, CBLVd#, HSVd#	Pagliano *et al.*[13]
Citrus sinensis	Orange Washington Navel MolWN36	HSVd#	Pagliano *et al.*[13]
Citrus sinensis	Orange Washington Navel MolWN66	CEVd#, HSVd#	Pagliano *et al.*[13]
Citrus unshiu	Mandarin Satsuma VMS434	CBLVd#	Pagliano *et al.*[13]
Citrus deliciosa	Mandarin common GS430	HSVd#, CDVd#	Pagliano *et al.*[13]
Citrus deliciosa	Mandarin common FAgS437	HSVd#, CDVd#	Pagliano *et al.*[13]
Citrus paradisi	Grapefruit Star Ruby CDL070	None CVds infection#	Pagliano *et al.*[13]
Citrus paradisi	Grapefruit Star Ruby G031	CEVd*,#, CBLVd#, HSVd#, CDVd#	Umaña *et al.*[12]
Citrus paradisi	Grapefruit Star Ruby G054	CEVd#, HSVd#, CDVd#	Pagliano *et al.*[13]

*Diagnostic screening with RT-PCR approach, #Diagnostic screening with Northern blot approach, $Biological diagnostic (indexing in citron plants)

controls. Samples were collected from 30-40 year-old trees chosen at random with or without symptoms.

cDNA-DIG probe: For the synthesis of the probes, viroid cDNA was PCR-amplified from plasmids containing the complete sequence of each viroid species (CEVd, CBLVd, HSVd, CDVd, CVd-IV and CVd-VI) using specific primer pairs corresponding to the central conserved region and were labeled with digoxigenin[12,13]. The sequences of the primer pairs used to synthesize the full viroid genome probes were obtained from Semancik *et al.*[26] (CBLVd), Sano *et al.*[27] (HSVd), Gross *et al.*[28] (CEVd), Rakowski *et al.*[29] (CDVd), Puchta *et al.*[30] (CVd-IV) and Ito *et al.*[31] (CVd-VI).

***In vitro* transcription of CBCVd and CVd-VI full genome Northern blot positive controls:** The cDNAs were cloned onto TS4.7 and S10S ElLabb plasmids[6,13]. Monomeric sense-strand transcripts were synthesized starting at the T7 RNA polymerase promoter of the pCR2.1 vector (Invitrogen®), which contained the CBCVd and the CVd-VI viroid genomes. The recombinant plasmids were purified using the high pure plasmid isolation kit (Roche Molecular Biochemicals) according to the manufacturer's instructions. One microgram of pDNA was linearized using 10 U of Hind III (Fermentas) in R buffer at 37°C for 1 h. Linearized plasmids were recovered using the Wizard® PCR Preps DNA purification systems kit (Promega) and viroid cDNA fragments were transcribed using T7 RNA polymerase (Promega) followed by DNase treatment according to the manufacturer's instructions. Viroid RNA presence was confirmed on 2% agarose gels. For Northern blots, monomeric synthesized transcripts for CBCVd and CVd-VI were used as positive controls, where assays contained a mixture of synthetic viroid cRNA and RNA from an uninfected plant to simulate a naturally-infected control (spike control/sample)[32].

Extraction of nucleic acids: Five grams of bark tissue from tree branches (total phloem RNA), leaves from field-grown trees and viroid-infected leaves from Etrog citron trees were ground in liquid nitrogen. Homogenization was carried out in extraction buffer (0.4 M tris-HCl, pH 8.9, 1% (w/v) SDS, 5 mM EDTA, pH 7.0, 2% (v/v) 2-mercaptoethanol) containing 15 mL of phenol saturated in water at neutral pH. The soluble fraction was precipitated in ethanol and resuspended in 300 µL of 1X TKM buffer (10 mM tris-HCl, pH 7.4, 10 mM KCl, 0.1 mM $MgCl_2$)[12].

PAGE electrophoresis and electroblotting: Aliquots (20 µL equivalent to 333 mg fresh weight of tissue) of the samples and positive and negative controls were analyzed by 5% PAGE in 1X TAE (40 mM tris, 20 mM sodium acetate, 1 mM EDTA, pH 7.2) under non-denaturing conditions for 3 h at 60 mA. The gel segment, including the 7S rRNA were cut and electrotransferred (400 mA, 1X TBE for 1.5 h) to positively

charged nylon membranes (Roche Applied Science)[3]. To verify that all the RNAs were electro-transferred, the resulting gel stripe was subjected to silver nitrate staining.

Northern blot and diagnostic sensitivity: The membranes containing the electro-transferred samples were exposed to 70,000 μJ cm^{-2} on a UV cross-linking oven (Hoefer-Uvc500, Amersham Biosciences Corp.) for nucleic acid fixation. Prehybridization was performed at 42°C for 2 h. Hybridization of the membranes with specific probes containing the immobilized RNAs was carried out as described by Murcia *et al.*[11] and membranes were revealed with anti-DIG-alkaline phosphatase and CSPD (Roche). The sensitivity and saturation point of the Northern blot method for detection of CEVd, CBLVd, HSVd and CDVd was measured using infected lemon trees[12]. For the molecular hybridization, a mixture of DIG-labeled cDNA probes for the 4 viroids was used. The efficiency of this method was evaluated by means of presence/absence of viroid infections. Cut-off values and detection limits for the diagnosis of HSVd and CEVd in the plant samples of 4 citrus species orange, tangerine, lemon and grapefruit were determined for samples collected from either commercial farms or experimental stations. Hybridization signals produced in the films were quantified by densitometric analyses with the 1Dscan EX 3.1.0 Eval software (Scanalytics Inc.). Signal intensities were reported in Relative Units (RU) of signal intensity.

RESULTS AND DISCUSSION

Northern blot and diagnostic sensitivity: Sensitivity analyses using PAGE-Northern blotting for detection of the four viroids (CEVd, CBLVd, HSVd and CDVd) for diagnosis of viroids in field lemon plants (*Citrus limon*) are shown in Fig. 1. The results showed that different levels of saturation of the hybridization signals obtained from plant tissue ranged from 333-8 mg (in 20-0.5 μL of suspended total phloem RNA, Fig. 1a). The signals produced by the vd-RNA-cDNA-DIG hybridization showed that the detection limit (the lowest concentration of target RNA that was visually detectable) was 1,513 ng (0.75 μL) to 1,009 ng (0.5 μL) of the total RNA extracted. Under these conditions, the threshold was equivalent to 8-12 mg of processed tissue, which showed positive signals in all infected tissues. The non-infected Washington Navel orange used as a negative control showed no signal (equivalent to 57,425 ng of total RNA in a 25 μL volume). The graphical representation of the saturation levels of the signals generated by autoradiography of the different concentrations of RNA is shown in Fig. 1b.

The Relative Units (RU) as measured using the background correction parameter (negative control test is 0 RU), showed that the signals above zero were detectable (2-83 RU, corresponding to 8-333 mg of fresh tissue), following a positive trend according to the logarithmic model and showed a strong correlation index (R^2 = 0.9781). This demonstrated that the signal intensity values were within the range of quantity of RNA analyzed (or processed tissue) and followed a logarithmic model where the amount of target RNA in the sample did not reach a saturation level. Results generated by the mixture of CEVd-, CBLVd-, HSVd- and CDVd-specific probes showed that the Northern analysis is reliable for diagnostics because the detection limit herein is well below the limit reported by Murcia *et al.*[11]. These results are consistent with the scatter plots and regression analysis that define the detection limit as the minimum volume that can be used to obtain a signal due to the presence of the viroid in the sample, as compared with the volume and signal intensity obtained with the non-infected control (Fig. 1). These results are comparable to those reported by Murcia *et al.*[11] and Mohamed *et al.*[33] because they show detectable hybridization signals associated with high viroid titers. The RNA preparations of 333 mg of tissue were subjected to PAGE and the gel segment corresponding to the area of viroid mobility (between the 2 white lines in Fig. 2) was cut, electrotransferred and hybridized with DIG-labeled CDVd and HSVd probes (Fig. 2). The CDVd was detected in all samples hybridized with the DIG-labeled CDVd probe except for tissue samples that were collected from Washington Navel orange (PEWN04), Lisbon lemon (FB380) and HSVd-infected Etrog citron (citron 006). In contrast, HSVd (Fig. 2b) was detected in all samples except for the uninfected field sample (PEWN01, lane 18). Considering the densitometry analysis of the autoradiograms, the negative controls used showed a signal intensity value of 10-30 RU, whereas those considered as positive signals (presence of viroid) by visual observation of the autoradiography showed variations in the quantification of the signal ranging from 62-160 RU (Fig. 2, 3). The cut-off for the absence/presence of infection (as estimated parameters for the image background) in this experiment was approximately 60 RU, based on the criteria view that an infected sample is twice the highest value of intensity of the negative control. Thus, it was concluded that all samples with visually identifiable signals in the autoradiographic plate fell within the acceptable range, indicating a positive infection. In the case of samples PEWN02 and FB380 (Fig. 2a, lanes 2 and 8, respectively), their infection status could not be established because they produced signal intensities below the defined cut-off (~60 RU). This was reinforced when we compared the

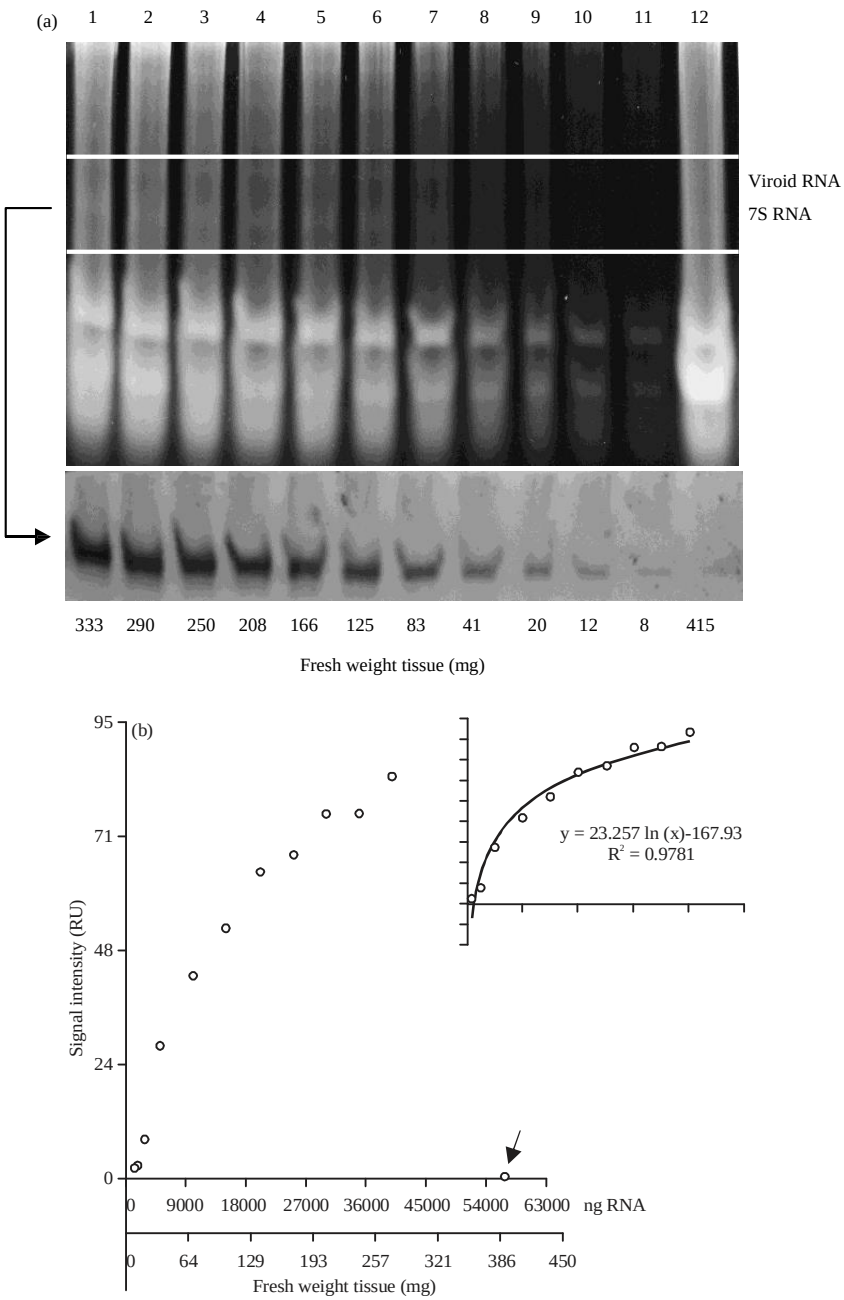

Fig. 1(a-b): Northern blot sensitivity analysis for the detection of plant viroids in field-grown lemon trees (*Citrus limon*) using a cDNA probe mixture CEVd, CBLVd, HSVd and CDVd, (a) PAGE electrophoresis, lane 1-11, Lisbon lemon sample K395, Lane 12: Negative control sample KWN05 and (b) Scatterplot and regression analysis of signal intensity values obtained from the Northern blot. Item 0 RU indicated with an arrow is the negative control test (Table 1)

amounts of tissue processed (recommended maximum 333 mg) for samples that showed a signal and the same amount of tissue processed for samples with signals below 60 RU. This is in agreement with visual observations of the signals in the x-ray film and the quantitation of signal intensities. A similar study conducted by Pagliano *et al.*[13] also supported the efficiency of this type of hybridization for diagnostic purposes on field collected samples in Uruguay.

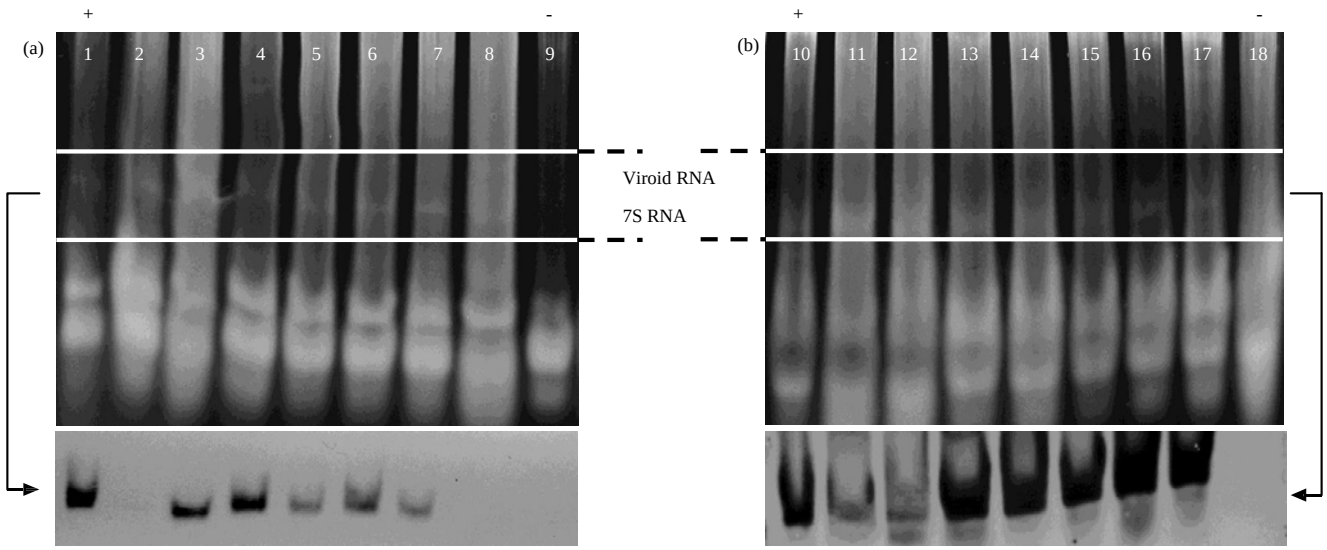

Fig. 2(a-b): Effectiveness of a Northern blot analysis using field-grown *Citrus* samples (a) PAGE-Northern blots were hybridized to CDVd and (b) HSVd viroid probes. (a) Lane 1: Sample K395, Lane 2: PEWN02, Lane 3 and 4: Samples code K390 and K393, Lane 5: GS430, Lane 6: PqL175, Lane 7: FAgS437, Lane 8: FB380, Lane 9: Citron 006, (b) Lane 10: Citron 006, Lane 11 and 12: Samples G031 and G054, Lane 13 and 14: Samples FB410 and FB411, Lane 15: GS430, Lane 16: K390, Lane 17: MolWN36, Lane 18: Negative control PEWN01 (Table 1)

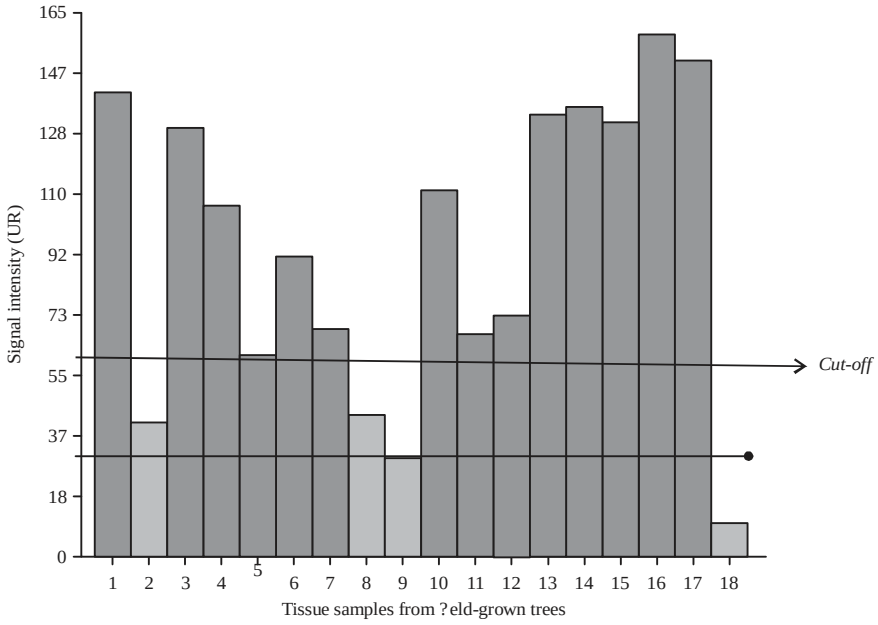

Fig. 3: Graph of signal intensity values (RU) obtained by Northern blotting of CDVd and HSVd viroid probes. Lanes 1-18: Materials tested showing the quantification of the signals obtained by autoradiography. The upper line indicates signal cut-off points, which was ratified as positive (sample 5), where the accepted intensity for an infected sample represents an intensity of at least twice the highest value of the intensity of the negative control sample (sample 9, lower line)

Viroids were found in higher concentrations in infected bark obtained from branches or infected citron leaves. Previous reports showed several difficulties in detecting viroids directly from citrus field-grown leaves due to their mobilization in the

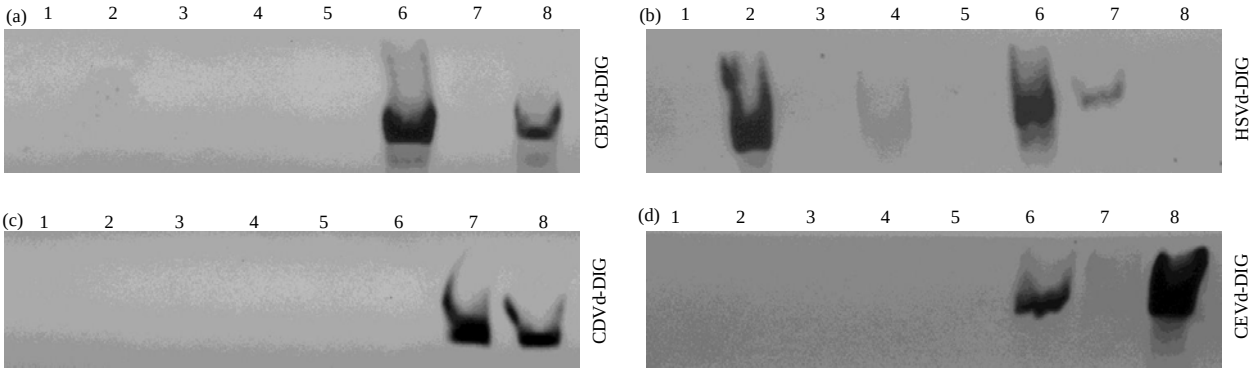

Fig. 4(a-d): Northern blot analysis with specific probes for (a) CBLVd, (b) HSVd, (c) CDVd and (d) CEVd, (a) Lane 1 and 2: Negatives controls Etrog citron 102 and KWN05 respectively, Lane 3: CDL386, Lane 4: K393, Lane 5: MolWN66, Lane 6: VMS434, Lane 7: PEWN02, Lane 8: Citron 24 positive control, (b) Lane 1 and 8: Negatives controls Etrog citron 102 and KWN05 respectively, Lane 2: GS430, Lane 3: Citron 804, Lane 4: VMS434, Lane 5: Citron 24, Lane 6 and 7: K390 and K393, (c) Lane 1 and 2: Negatives controls Etrog citron 102 and KWN01, respectively, Lane 3 and 4: Citron 24 and 006, respectively, Lane 5: MolWN66, Lane 6: Citron 16, Lane 7: G054, Lane 8: CDL386 and (d) Lane 1 and 2: Negatives controls Etrog citron 102 and KWN01, respectively, Lane 3: VMS434, Lane 4: FB410, Lane 5: CDL386, Lane 6: G031, Lane 7: FB411, Lane 8: Positive control citron 16 (Table 1)

phloem, however, using bark tissue detection approaches, we were able to detect viroid titers with high sensitivity and specificity from citron infected plants[2,34].

Specificity and repeatability: The specificity for the routine detection of viroids (CEVd, CBLVd, HSVd and CDVd) was tested and revealed that separate membranes subjected to specific probes were able to discriminate among viroids and had minimal cross-hybridization of the probe to non-specific RNA targets with specific cDNA-DIG probes. Hybridization with specific probes was highly specific for the four viroids tested. The probes were separately exposed to different viroid combinations and did not show non-specific hybridization (Fig. 4). The autoradiography revealed that the samples of plants infected with CEVd, HSVd or CDVd (Fig. 4, lanes 1, 2, 3, 4, 5 and 7) did not show positive signals when probed with CBLVd. However, a positive control from a citron infected with CBLVd (lane 8) or co-infected with CEVd, CBLVd, HSVd and CDVd (lane 6) showed a strong and compact hybridization signal (Fig. 4a). This same scenario occurred for all other probes used in the study, where strong fluorescent signal was detected only when the specific viroid was present, either by itself or co-infected with other viroids, except when the HSVd probe was tested using HSVd-positive samples co-infected with CBLVd and CDVd (Fig. 4b, lane 7). However, for the HSVd probe, DIG signal was not visible when total RNA extracted from CBLVd-infected citron trees was probed, therefore we could not conclude any viroid presence (Fig. 4b, lane 5). These analysis confirm that DIG-labeled viroid probes hybridize specifically to the viroid RNAs and demonstrate the high diagnostic specificity and efficiency of each probe without producing non-specific hybridization. This diagnostic tool has the ability to segregate viroid genomes or target sequences that partially match the specific probe.

The results from the application of CBCVd- and CVd-VI-positive controls using a mix of artificially transcribed RNA and RNA from non-infected tissue suggests high probe specificity when contrasted with tested samples from field-grown plants infected with several citrus viroids (Fig. 5). This technique simulated the possible interactions between the transcribed viroid RNAs and the host RNAs, thus synthetic vdRNAs functioned as positive controls in the absence of confirmed infected material. The use of synthetic viroids as controls was proposed by Hataya[35], where non-infectious (synthesized) PSTVd-positive controls were used for RT-PCR assays. This is particularly useful when the pathogen of interest is unavailable or is under quarantine. The CBCVd and CVd-IV Northern blot analyses reveal that each specific probe is able to discern between the 2 citrus viroids. The repeatability was confirmed by the hybridization of 25 positively charged nylon membranes that were processed in different days. This implies a robust specificity for diagnostic purposes. In all instances, positive and negative controls produced comparable hybridization signals. This technique is now used as a molecular alternative to conventional biological indexing methods for the detection of citrus viroids.

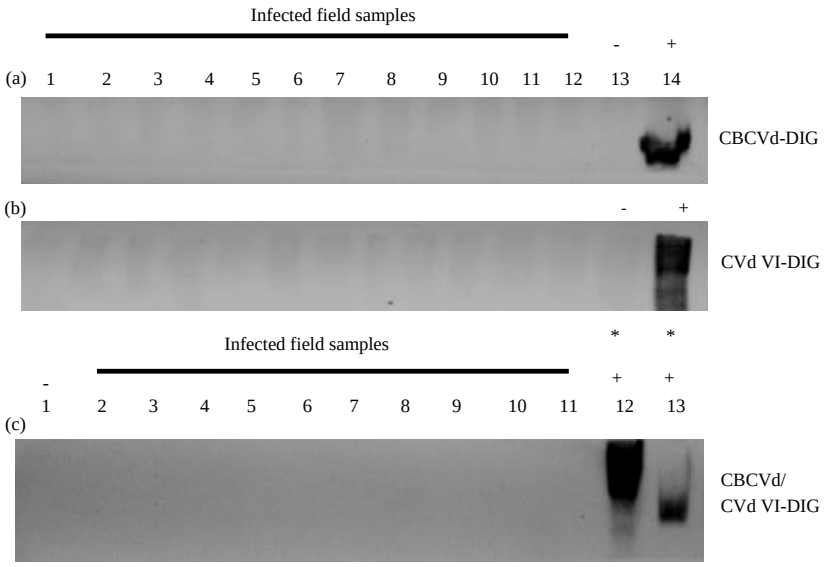

Fig. 5(a-c): Specificty analysis of DIG-labaled Northern blot probes for (a) CBCVd-DIG, (b) CVd-VI citrus viroids and (c) Routine screening for field-grown samples. (a) Lane 1-12: Total RNA extracted from the bark of field-grown citrus trees infected with one or more citrus viroids (CEVd, CBLVd, HSVd, CDVd), Lane 13: RNA extracted from the bark of healthy field-grown citrus tree (CDL070), Lane 14: Total RNA extracted from the bark of positive control citron 804, (b) Identical to (a) except lane 14 was a monomeric CVd-VI transcipt spike control and (c) Routine Northern blot hybridization analysis of a probe cocktail specific for CBCVd y CVd-VI citrus viroids using total RNA extracted from field-grown citrus trees. Lane 1: Negative control Etrog citron 102, Lanes 2-11: Total RNA extracted from the bark of field-grown citrus trees infected with one or more citrus viroids (CEVd, CBLVd, HSVd and CDVd), Lane 12: Monomeric CVd VI transcipt spike control, Lane 13: Positive control citron 804 infected with CBCVd. *Significant signal intensities of the tested probes when compared to all negative control signal of all 84 samples tested ($p<0.001$)

CONCLUSION

Molecular hybridization techniques are robust, consistent, specific and sensitive for detecting viroids in field plants and are useful for either screening or diagnostic purposes. These techniques can reduce the time needed to obtain diagnostic results and therefore are particularly helpful for certification and sanitation programs and support the quarantine system. We have demonstrated a reliable, quick technique that can be implemented in these types of programs in particular when sampled material is limited.

ACKNOWLEDGMENTS

The authors acknowledge the funding of the project BID-CONICYT PDT 74/19. We would like to thank Vicerrectoría de Investigatíon of Universidad Nacional de Costa Rica for their financial support. To Nuria Duran-Vila (IVIA, Spain) for her great support throughout the activities of this study. To Jacques Borde (DGSSAA) for giving us access to their citron controls and for his support through out the project. To Ana Bertalmío also for offering us samples of the control plants (INIA-Salto Grande). To Takao Ito (NIFTS, Japan) for providing TS4.7 and S10S recombinant plasmids.

REFERENCES

1. Flores, R., C. Hernandez, A.E.M. de Alba, J.A. Daros and F. di Serio, 2005. Viroids and viroid-host interactions. Annu. Rev. Phytopathol., 43: 117-139.
2. Barbosa, C.J., J.A. Pina, L. Navarro and N. Duran-Vila, 2002. Replication/accumulation and symptom expression of citrus viroids on some species of citrus and related genera. Proceedings of the 15th International Conference of the Organization of Citrus Virologists, (OCV'02), Riverside, CA., pp: 264-277.
3. Serra, P., C.J. Barbosa, J.A. Daros, R. Flores and N. Duran-Vila, 2008. Citrus viroid V: Molecular characterization and synergistic interactions with other members of the genus *Apscaviroid*. Virology, 370: 102-112.

4. Hashemian, S.M.B., P. Serra, C.J. Barbosa, J. Juarez and P. Aleza *et al*., 2009. Effect of a field-source mixture of citrus viroids on the performance of 'Nules' clementine and 'Navelina' sweet orange trees grafted on carrizo citrange. Plant Dis., 93: 699-707.
5. Kyriakou, A., M. Ioannou, A. Hadjinicolis, R. Hoffman and E. Antoniou *et al*., 2005. Citrus exocortis and cachexia viroids affect growth, yield and fruit quality of lapithou lemon on sour orange rootstock in cyprus. Proceedings of the 16th Conference of the International Organization of Citrus Virologists, (OCV'05), Riverside, CA., pp: 257-263.
6. Ito, T., H. Ieki, K. Ozaki, T. Iwanami and K. Nakahara *et al*., 2002. Multiple citrus viroids in citrus from Japan and their ability to produce exocortis-like symptoms in citron. Phytopathology, 92: 542-547.
7. Wang, X., C. Zhou, K. Tang, Y. Zhou and Z. Li, 2009. A rapid one-step multiplex RT-PCR assay for the simultaneous detection of five citrus viroids in China. Eur. J. Plant Pathol., 124: 175-180.
8. Wang, J., I.N. Boubourakas, A.E. Voloudakis, T. Agorastou and G. Magripis *et al*., 2013. Identification and characterization of known and novel viroid variants in the Greek national citrus germplasm collection: Threats to the industry. Eur. J. Plant Pathol., 137: 17-27.
9. Cohen, O., O. Batuman, G. Stanbekova, T. Sano, W. Mawassi and M. Bar-Joseph, 2006. Construction of a multiprobe for the simultaneous detection of viroids infecting citrus trees. Virus Genes, 33: 287-292.
10. Bernad, L. and N. Duran-Vila, 2006. A novel RT-PCR approach for detection and characterization of citrus viroids. Mol. Cell. Probes, 20: 105-113.
11. Murcia, N., P. Serra, A. Olmos and N. Duran-Vila, 2009. A novel hybridization approach for detection of citrus viroids. Mol. Cell. Probes, 23: 95-102.
12. Umana, R., C. Pritsch, J.R. Arbiza, F. Rivas and G. Pagliano, 2013. Evaluation of four viroid RNA extraction methods for the molecular diagnosis of CEVd in Citrus lemon using RT-PCR, dot blot and Northern blot. Biotecnol. Apl., 30: 131-136.
13. Pagliano, G., R. Umana, C. Pritsch, F. Rivas and N. Duran-Vila, 2013. Occurrence, prevalence and distribution of citrus viroids in Uruguay. J. Plant Pathol., 95: 631-635.
14. 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., 61: 135-139.
15. Duran-Vila, N. and J.S. Semancik, 1990. Variations in the 'cross protection' effect between two strains of citrus exocortis viroid. Ann. Applied Biol., 17: 367-377.
16. Duran-Vila, N., J.A. Pina and L. Navarro, 1991. Exclusion and/or uneven distribution of viroids in four citrus hosts. Proceedings of the 11th Conference of the International Organization of Citrus Virologists, November 6-10, 1989, Orlando, Florida, pp: 219-223.
17. Duran-Vila, N., J.A. Pina and L. Navarro, 1993. Improved indexing of citrus viroids. Proceedings of the 12th Conference of the International Organization of Citrus Virologists, (OCV'93), Riverside, CA., pp: 202-211.
18. Li, S.F., S. Onodera, T. Sano, K. Yoshida, G.P. Wang and E. Shikata, 1995. Gene diagnosis of viroids: Comparison of return-PAGE and hybridization using DIG-labeled DNA and RNA probes for practical diagnosis of hop stunt, citrus exocortis and apple scar skin viroids in their natural host plants. Jap. J. Phytopathol., 61: 381-390.
19. Nakahara, K., T. Hataya, I. Uyeda and H. Ieki, 1998. An improved procedure for extracting nucleic acids from citrus tissues for diagnosis of citrus viroids. Ann. Phytopathol. Soc. Jpn., 64: 532-538.
20. Nakahara, K., T. Hataya and I. Uyeda, 1999. A simple, rapid method of nucleic acid extraction without tissue homogenization for detecting viroids by hybridization and RT-PCR. J. Virol. Methods, 77: 47-58.
21. Rodriguez, R., P.L. Ramos-Gonzalez, V. Dorestes, K. Velasquez, R. Peral, A. Fuentes and M. Pujol, 2003. Establishment of a non-radioactive nucleic acid hybridization technique for begomovirus detection. Biotecnologia Aplicada, 20: 164-169.
22. Du, Z., B. Jin, W. Liu, L. Chen, J. Chen and M. Xu, 2007. Highly sensitive fluorescent-labeled probes and glass slide hybridization for the detection of plant RNA viruses and a viroid. Acta Biochimica Biophysica Sinica, 39: 326-334.
23. Gomez, G. and V. Pallas, 2004. A long-distance translocatable phloem protein from cucumber forms a ribonucleoprotein complex *in vivo* with *Hop stunt viroid* RNA. J. Virol., 78: 10104-10110.
24. Xu, W.X., H. Ni, Q.T. Jin, A.B.U. Farooq and Z.Q. Wang *et al*., 2009. Probe binding to host proteins: A cause for false positive signals in viroid detection by tissue hybridization. Virus Res., 145: 26-30.
25. Barbosa, C.J., J.A. Pina, J. Perez-Panades, L. Bernad, P. Serra, L. Navarro and N. Duran-Vila, 2005. Mechanical transmission of citrus viroids. Plant Dis., 89: 749-754.
26. Semancik, J.S., A.G. Rakowski, J.A. Bash and D.J. Gumpf, 1997. Application of selected viroids for dwarfing and enhancement of production of 'Valencia' orange. J. Horticult. Sci., 72: 563-570.
27. Sano, T., T. Hataya, Y. Terai and E. Shikata, 1989. Hop stunt viroid strains from dapple fruit disease of plum and peach in Japan. J. Gen. Virol., 70: 1311-1319.
28. Gross, H.J., G. Krupp, H. Domdey, M. Raba and P. Jank *et al*., 1982. Nucleotide sequence and secondary structure of citrus exocortis and chrysanthemum stunt viroid. Eur. J. Biochem., 121: 249-257.
29. Rakowski, A.G., J.A. Szychowski, Z.S. Avena and J.S. Semancik, 1994. Nucleotide sequence and structural features of the group III citrus viroids. J. Gen. Virol., 75: 3581-3584.

30. Puchta, H., K. Ramm, R. Luckinger, R. Hadas, M. Bar-Joseph and H.L. Sanger, 1991. Primary and secondary structure of Citrus Viroid IV (CVd IV), a new chimeric viroid present in dwarfed grapefrut in Israel. Nucleic Acids Res., 19: 6640-6640.
31. Ito, T., H. Ieki, K. Ozaki and T. Ito, 2001. Characterization of a new citrus viroid species tentatively termed citrus viroid OS. Arch. Virol., 146: 975-982.
32. Badiee, A., H.G. Eiken, V.M. Steen and R. Lovlie, 2003. Evaluation of five different cDNA labeling methods for microarrays using spike controls. BMC Biotechnol., Vol. 3. 10.1186/1472-6750-3-23.
33. Mohamed, M.E., S.M. Bani Hashemian, G. Dafalla, J.M. Bove and N. Duran-Vila, 2009. Occurrence and identification of citrus viroids from Sudan. J. Plant Pathol., 91: 185-190.
34. Palacio-Bielsa, A., X. Foissac and N. Duran-Vila, 1999. Indexing of citrus viroids by imprint hybridisation. Eur. J. Plant Pathol., 105: 897-903.
35. Hataya, T., 2009. Duplex reverse transcription-polymerase chain reaction system to detect *Potato spindle tuber viroid* using an internal control mRNA and a non-infectious positive control RNA. J. Gen. Plant Pathol., 75: 167-172.