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THREE VIROIDS FREQUENCY NATURALLY INFECTING GRAPEVINE IN EGYPT

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ABSTRACT

Three viroids: *Citrus exocortis viroid* (CEVd), *Hop stunt viroid* (HSVd), and *Potato spindle tuber viroid* (PSTVd) can be detected in naturally infected grapevine by biological indexing and molecular methods. In the present study, direct tissue-printing and dot-blot on the membranes, procedures has been applied on a large scale for an initial screening of HSVd, CEVd and PSTVd in Egypt. The results showed that the tissue print assays allowed clear discrimination between healthy and viroid-infected grapevine plants than dot-blot hybridization, the number of HSVd-infected grapevine plants were 10 plants, PSTVd were 10 plants. And the CEVd was detected with high incidence level in grapevine, where 12 out of 100 grapevine trees analyzed were infected. The frequency of HSVd, CEVd and PSTVd naturally infected grapevine trees recorded different percentages as in single (8.33, 8.33 and 11.11%) respectively, double (11.11, 11.11 and 19.44%) for CEVd+HSVd, CEVd+PSTVd and HSVd+PSTVd respectively and mixed infection (19.44%) with different disease symptoms. The pervious results illustrated that, tissue printing hybridization was more reliable than dot-blot hybridization in viroid detection. HSVd was isolated on *Cucumis sativus* L. cv. Alpha plants which showed specific symptoms severe mosaic, vein clearing, rugosity and yellowing spots. CEVd was isolated on *Gynura aurantiaca* plants which showed specific symptoms mild mosaic and mottling and *Lycopersicon esculantum* L. cv. Castle rock reacted with PSTVd producing leaf curl and epinasty.

Keywords: Grapevine viroids, *Hop stunt viroid* (HSVd), *Citrus exocortis viroid* (CEVd), *Potato spindle tuber viroid* (PSTVd), Nucleic acid hybridization.

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INTRODUCTION

Viroids are small, single-stranded, circular RNA molecules of about 246 to 400 nucleotides (nt) which infect higher plants and cause significant agricultural losses and are the smallest known nucleic acid-based pathogens. Sequence comparisons of naturally occurring variants of the same viroid are important for defining the conserved and variable features of the viroid genome and may indicate regions that have a role in replication or symptom expression mechanisms.

Grapevine is the oldest kind of fruit crops cultivated in Egypt, Ancient Egyptian hieroglyphics show the cultivation of grapes. The grapevine cultivated areas are about 14% from the total cultivated area with different fruit crops, El-Minia, Al-Behaira and El-Dakahlia Governorates are the main centers for grapevine production. Recently, grapevine cultivated areas spreads in a new reclamation lands in a different governorates especially in west and east regions of the Nile Delta (Min. of Agriculture, Agricultural Statistics, 2009).

Grapevines are usually affected by numerous diseases caused by bacteria, fungi, nematodes, viruses, and viroids. Viroids are widespread throughout all grapevine-growing areas of the world. According to sequence analysis, 5 distinct viroids have been recognized on grapevine (**Szychowski, *et al.*, 1988**). *Hop stunt viroid* (HSVd), *Citrus exocortis viroid* (CEVd), *Grapevine yellow speckle viroid 1* and *2* (GYSVd-1 and GYSVd-2) and *Australian grapevine viroid* (AGVd). Grapevine viroids are subdivided into three groups based on their homology within the central domain of the viroid molecule. Expression of yellow speckle is ephemeral and mostly evident at the end of summer, indicating that symptoms are strongly influenced by climatic conditions. Experiments have shown that Vein-Banding disease results from a synergistic reaction between grapevine viroids and *Grapevine fanleaf virus* (GFLV) (**Szychowski, *et al.*, 1988**). Since most measures for the control of virus and viroidal diseases are based on prevention rather than cure, it is essential to have reliable and sensitive methods for pathogen detection. In order to develop rapid and specific detection techniques for viroids infecting grapevine cultures, we will compare between

biological and molecular protocols. Ideally, these procedures should allow the rapid screening of a large number of samples, and some of them should allow the detection of viroids maintained at low levels in the host plant.

CEVd and HSVd are distributed worldwide and infect a large number of hosts **(Singh *et al.*, 2003)**. HSVd was the first viroid described in grapevines, in Japan **(Shikata *et al.*, 1984; Sano *et al.*, 1985).** After its description, other viroid species were reported, including CEVd **(Flores *et al.*, 1985; García-Arenal *et al.*, 1987)** and three members of the genus *Apscaviroid* that occur exclusively in grapevine: *Grapevine yellow speckle viroid 1* (GYSVd-1), *Grapevine yellow speckle viroid 2* (GYSVd-2) and *Australian grapevine viroid* (AGVd) **(Rezaian *et al.*, 1992; Little & Rezaian, 2003).** Despite the stunting and yellowing symptoms that HSVd induces in cucumber, no disease symptoms are observed in grapevines infected by this viroid. CEVd was also isolated from symptomless grapevines in Spain, Australia and California **(García-Arenal *et al.*, 1987; Rezaian *et al.*, 1988; Semancik and Szychowski, 1992).** CEVd and HSVd doubly infecting grapevines in Brazil (**Eiras *et al.*, 2006).** Cross-protection occurs between mild and severe isolates of several viroids. It has not been used as a control measure for viroids, but has been used to identify mild strains of PSTVd in biological indexing programs. The best means for control of viroid diseases is grower vigilance, the use of stringent hygiene procedures, and monitoring the crop for unusual symptoms.

A limited survey was carried out in Egypt to check for the presence of grapevine viroids.

MATERIALS & METHODS

1. Field inspection and collection of doubtful viroids symptoms samples :

One hundred grapevine (*Vitis vinifera*) cv. Balady/banaty trees were investigated, samples of grapevine leaves presumed to grapevine viroids-infected were collected on the basis of their symptoms. The leaves of grapevine plants showing distinctive viroid and virus like symptoms involved "yellow speckle, yellowing spots,

vein banding, vein clearing and wavy leaflets margin" were collected from symptomatic and a symptomatic grape trees during summer season 2009/2010 from grapevine farm in Fac. of Agric. Ain Shams Univ.

2. Detection of grapevine viroids:

Molecular hybridization:

i. Tissue printing:

Fresh-cut sections of (leaf petiole) were immediately printed twice onto nylon membrane to obtain duplicates spots of each sample (**Amari *et al.*, 2001**). The membrane was air-dried and irradiated with UV cross linker and kept at room temperature until hybridized. Membranes were prehybridized for blocking with blocking stock solution (blocking reagent vial (6) dissolved in 0.1 M maleic acid and 0.15 M NaCl, pH= 7.5 to final concentration of 10% (w/v) by shaking and heating either on a heating block or in a microwave oven. This stock solution is autoclaved and stored at 4C or -20°C). Blocking stock solution was applied in hybridization tube at 68°C for at least 1-3 hrs. the membrane was hybridized with 20 ml of hybridized solution containing 5-25 ng of freshly heat DIG labeled cDNA probe was denatured (Boiling water bath) per 100 cm^2 membrane. The membrane was incubated for at least 6 hrs to overnight at 68°C. The membrane was washed 2-5 min. at room temperature with at least 50 ml of washing buffer A (2xSSC, 0.1% SDS (w/v)) per 100 cm^2 membrane and 2-15 min. at 68°C with buffer B (0.1xSSC, 0.1% SDS (w/v)). Membrane was equilibrated in Genius buffer (I) (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) for 1 min. with at least 20 ml of pre-hybridization solution per 100 cm^2 of membrane. The buffer was discarded, then 100 ml of Genius buffer (II) (2% Blocking reagent dissolved in Genus buffer 1 and diluted 1:10)) was added and membranes were incubated for 30 min. at minimum. Antidigoxigenin alkaline phosphatase was diluted 1:5000 in Genius buffer (II), and incubated for 30 min. The membranes were equilibrated in Genius buffer (III) (100 mM Tris-HCl, 100 mM NaCl, 50 mM MgCl2, pH 9.5) for 2 min. then immunologically detected. The membranes were incu-bated for 3 h in 10 ml of freshly prepared color solution (45 µl Nitro blue tetrazolume and 35 µl X-Phosphate

solution were added to a 10 ml of Genius buffer (III)) in box in the dark when the spot intensities were achieved, the reaction was stopped by washing the membranes for 5 min. with 50 ml of water. The results obtained allowed clear discrimination between infected and uninfected samples. The results were documented by photography.

ii. Dot blot-hybridization

Sap extraction were prepared from grapevine leaves by grinding 100 mg of fresh plant tissue in the presence of 100 µl of AMES buffer according to **Podleckis *et al.*, 1993.** the homogenate were incubated 5 min at 37 °C before extraction with an equal volume of chloroform. The aqueous phase was collected and reserved in a fresh microcentrifuge tubes. Five µl aliquots were spotted onto a nitrocellulose membranes. The membranes were air dried and irradiated with UV cross linker and kept at room temperature until hybridized. Membranes were prehybridized in hybridization tube at 68 °C for at least 1-3 hrs. the membranes were hybridized with 20 ml per 100 cm^2 membrane of hybridized solution. The membranes were incubated for at least 6 hrs to over night at 68°C. the membranes were washed with washing buffer. Membranes were equilibrated in Genius buffer (I) for 1 min then buffer was discarded. 100 ml of Genius buffer (II) and adding antidigoxigenin alkaline phosphatase diluted and incubated. The membranes were equilibrated in Genius buffer (III). The membranes were incubated for 5 min to 6 hrs in 10 ml of freshly prepared color solution in box in the dark. When the spot intensities were achieved, the reaction was stopped by washing the membrane for 5 min with 50 ml of water. The results were documented by photography.

Viroids indexing

The infectious sap (inoculum) was prepared by grinding the infected grapevine leaves with a sterilized mortar with addition of an equal amount (w/v) of 0.02 M phosphate buffer pH 7.2 containing 0.3% 2-mercaptorhanol **(EL-Dougdoug, 1988).** Mechanical inoculation process was used several times. Healthy seedling of host plants (2-3 leaf stage) were rubbed with a cotton swab. The inoculated plants were kept under a greenhouse conditions (16h day light and 30+-2) for development of indicated

symptoms. After 15, 20 and 30 days post-inoculation of plants , ,the symptoms were recorded. All symptomatic leaves were used as a source of viroids isolates for the following identification experiments.

Indicator plants:
Viroids were isolated from infected grapevine plants on indicator plants (*Cucumis sativus* L. cv. alpha , *Lycopersicon esculantum* L. cv. Castle rock, and *Gynura auratiaca*), by mechanical inoculation **(Yang and Deng 1991)** and kept in a greenhouse for development of indicated symptoms. The symptoms were recorded 2-4 weeks after the mechanical inoculation. All the experiments were repeated at least twice.

RESULTS

1. Natural incidence of grapevine viroids:
Grapevine (*Vitis vinifera*) cv. Balady/banaty samples presumed to be viroids infected (GYSVd, HSVd, CEVd and PSTVd on the basis of their symptoms "yellow speckle, yellowing spots, vein banding, vein clearing and wavy leaflets margin" **Figure(1)** were examined by tissue printing and dot-blot hybridization assays. Data showed that tested 18 trees out of 100 were recorded grapevine viroids-infected on the basis of dot-blot hybridization, where as the infected grapevine trees were showing grapevine viroids symptoms in which chlorotic pattern, mosaic vein-banding and yellow batches **Figure (2).**

2. Detection of grapevine viroids:

a. Tissue printing hybridization:
One hundred leaf petioles of grapevine plants were printed directly on three separated nitrocellulose membranes and hybridized with HSVd dig-labeled DNA-DNA-probe, PSTVd dig-labeled DNA-DNA-probe and CEVd dig-labeled DNA-DNA-probe separately. As shown in **Figures (3,4,5)** the dig-labeled DNA successfully hybridized with naturally infected tissues of grapevine. Magnified grape tissue prints indicated that viroids are phloem restricted as shown from vascular tissue colored with purple hybridization signals. The infected grapevine leaves were strong reacted with HSVd dig-labeled

DNA-DNA-probe **Figure (3).** The reactivity of PSTVd dig-labeled DNA-DNA-probe with grape plants in tissue print hybridization assay indicating different severity reactions as shown in **Figure (4).** Natural infected grapevine plants gave positive results against specific CEVd dig-labeled DNA-DNA-probe which they were different in their reaction according to hybridization signals **Figure (5).** The reactivity of HSVd dig-labeled probe with eighteen naturally infected grape plants using tissue print hybridization assay indicating different reaction and the percentage of infected plants gave positive results were 10/100. The reactivity of PSTVd dig-labeled probe with eighteen naturally infected grape plants using tissue print hybridization assay indicating different reaction and the percentage of infected plants gave positive results were 9/100.The reactivity CEVd dig-labeled-probe with eighteen naturally infected grape plants using tissue print hybridization assay indicating different reaction and the percentage of infected plants gave positive results were 11/100, **Table (1).**

b. Dot-blot hybridization:

One hundred leaf samples were grinding in AMES buffer separately according to disease symptoms (100 mg of fresh plant tissue / 100 ul of AMES buffer) and five ul aliquots were spotted onto a nitrocellulose membranes (one membrane for each viroid) described under material and methods. The colored spots were appeared in the three membranes according to the severity of reaction. Incase of membrane no. (1) HSVd gave 11 positive results from 18 **Figure (6),** membrane no. (2) PSTVd gave 12 positive results from 18 **Figure (7)** and membrane no. (3) CEVd gave 7 positive results against 11 negative **Figure (8) , Table (1).**

Tissue print and dot-blot hybridization assays clearly demonstrate that CEVd gave single infection in 3 trees with external symptoms yellow speckle, yellowing, HSVd gave single infection in 3 trees also with external symptoms severe mosaic and yellowing and 4 trees infected with PSTVd and gave external symptoms yellow speckle, mild mosaic. CEVd and HSVd doubly infecting 4 grapevine trees, CEVd

and PSTVd doubly infecting 4 grapevine trees also. But HSVd and PSTVd were detected in 7 grapevine trees and gave chlorotic spots, vein clearing and yellow speckle. CEVd, HSVd and PSTVd mixed infections were appeared in 7 trees and gave mild mosaic, vein clearing and no symptoms this is due to viroids interference **Table (2).** On other hand yellow speckle, severe mosaic and yellow batches symptoms gave negative results with three viroids DNA-probes because of these symptoms may be for GYSVd. **Table (1).**

3. Indicator plants:

The reactions of indicator plants mechanically inoculated under the greenhouse condition indicate that these hosts are susceptible to infection with viroids isolates. They show the characteristic viroid symptoms 10-15 days post inoculation. HSVd, PSTVd and CEVd gave mosaic with chlorotic spots, epinasty and mild mosaic with *Cucumis sativus* L. cv. alpha, *Lycopersicon esculantum* L. cv. Castle rock, and *Gynura aurantiaca*), respectively, **Figures (9, 10 and 11).**

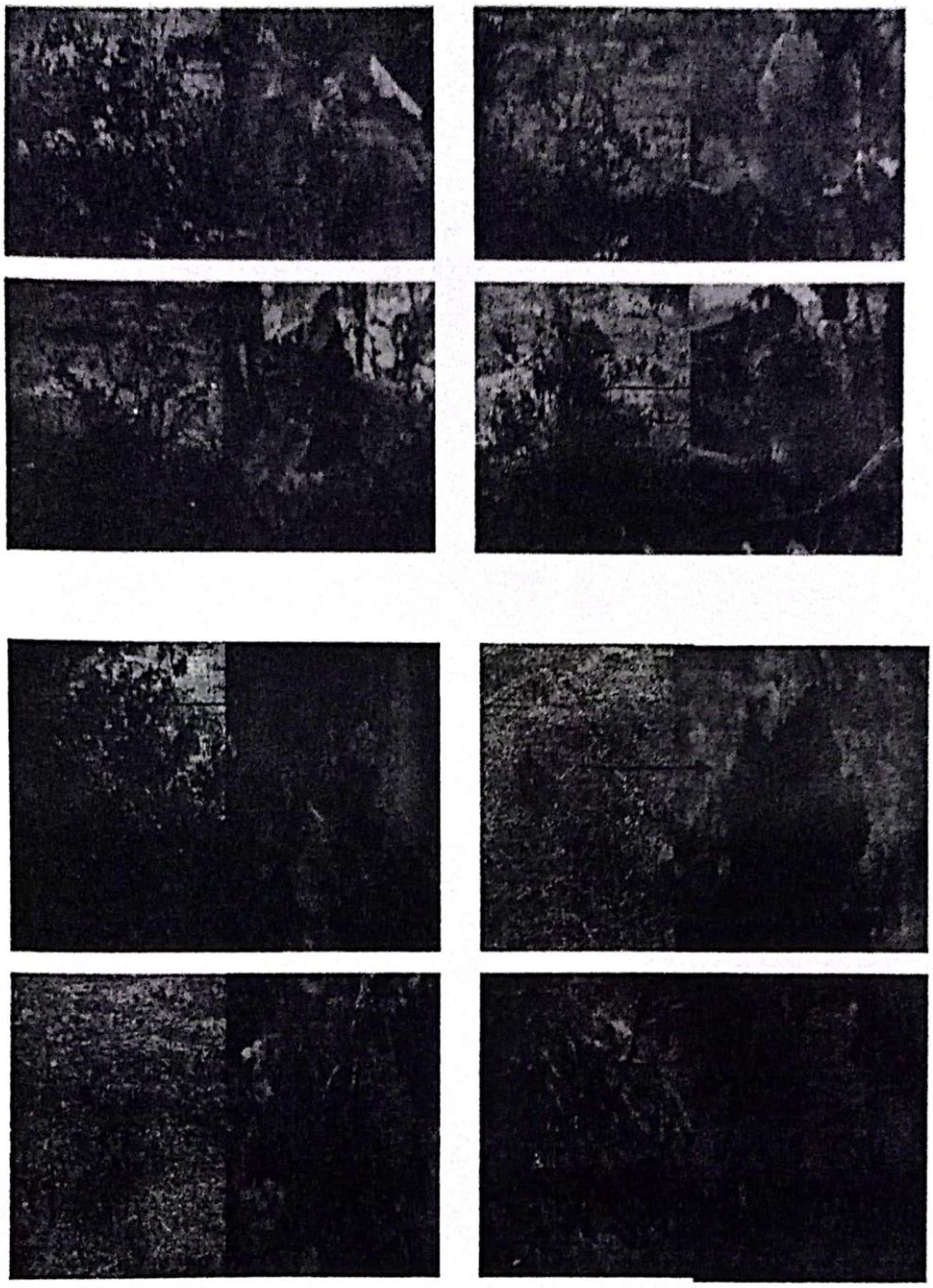

Figure 1. Grapevine trees showing viroid and virus like symptoms (yellowing, mosaic, chlorotic spots, yellow speckle and leaf deformation) in summer season at Grapevine farm Fac. of Agric. Ain Shams Univ.

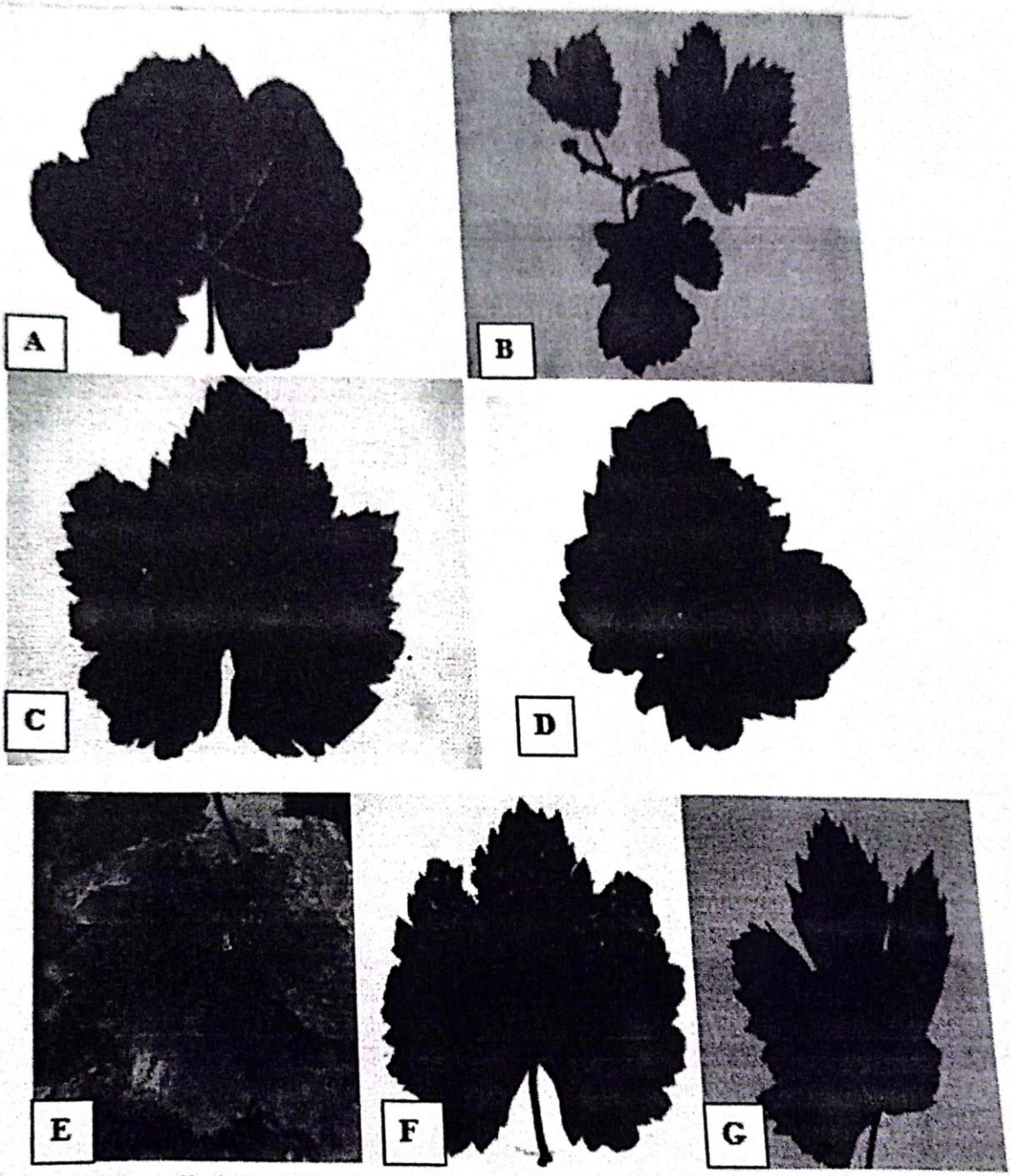

Figure 2. Naturally infected grapevine leaves with grapevine viroids showing: (A) mosaic, (B) vein banding, (C) mosaic with chlorotic spots, (D) leaf deformation and mosaic, (E) yellowing batches, (F) yellow speckle and (G) mosaic with vein banding.

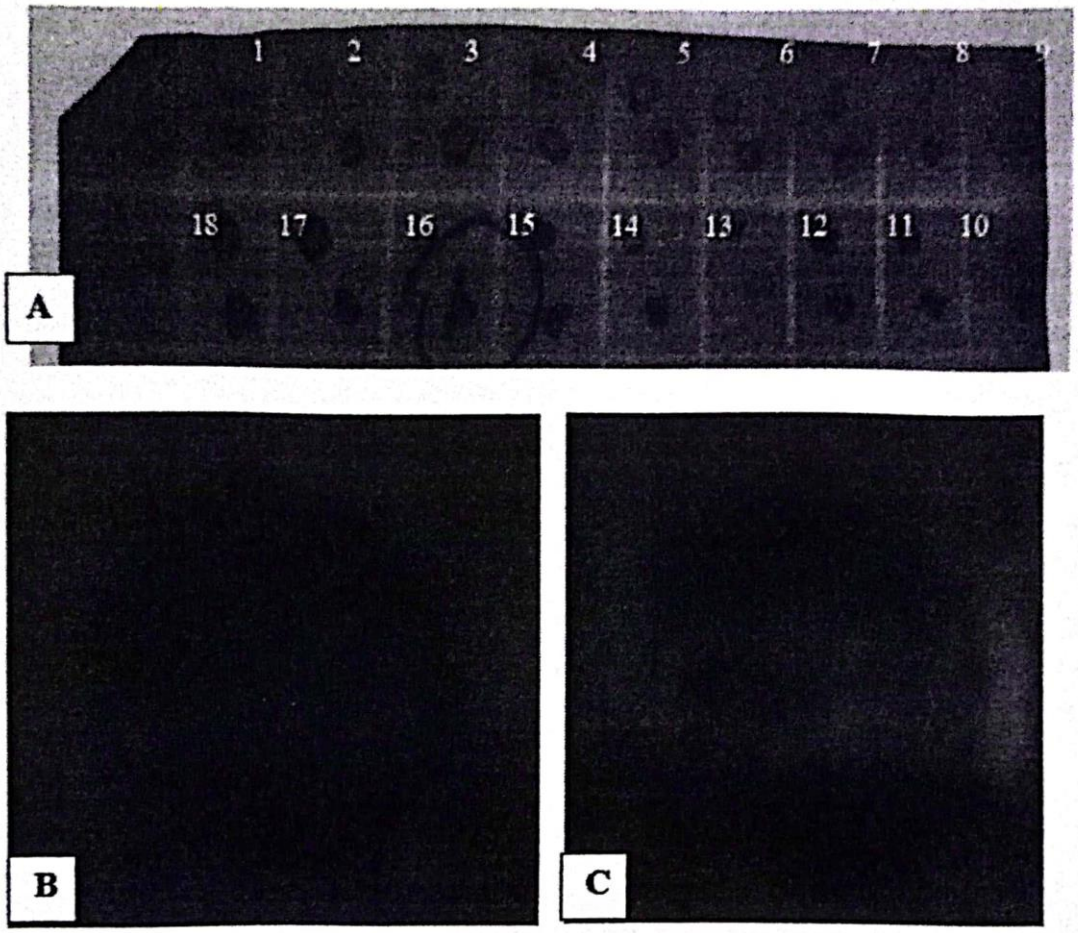

Figure 3. A. Tissue print hybridization of eighteen naturally infected and uninfected grapevine plants on nitrocellulose membrane using HSVd dig-labeled DNA-DNA-probe showing hybridization signals.

B. Magnified transverse section of grapevine petiole printed on nitrocellulose membrane showing the positive hybridization signals concentrated on phloem tissue.

C. Magnified transverse section of grapevine petiole printed on nitrocellulose membrane showing negative hybridization signals

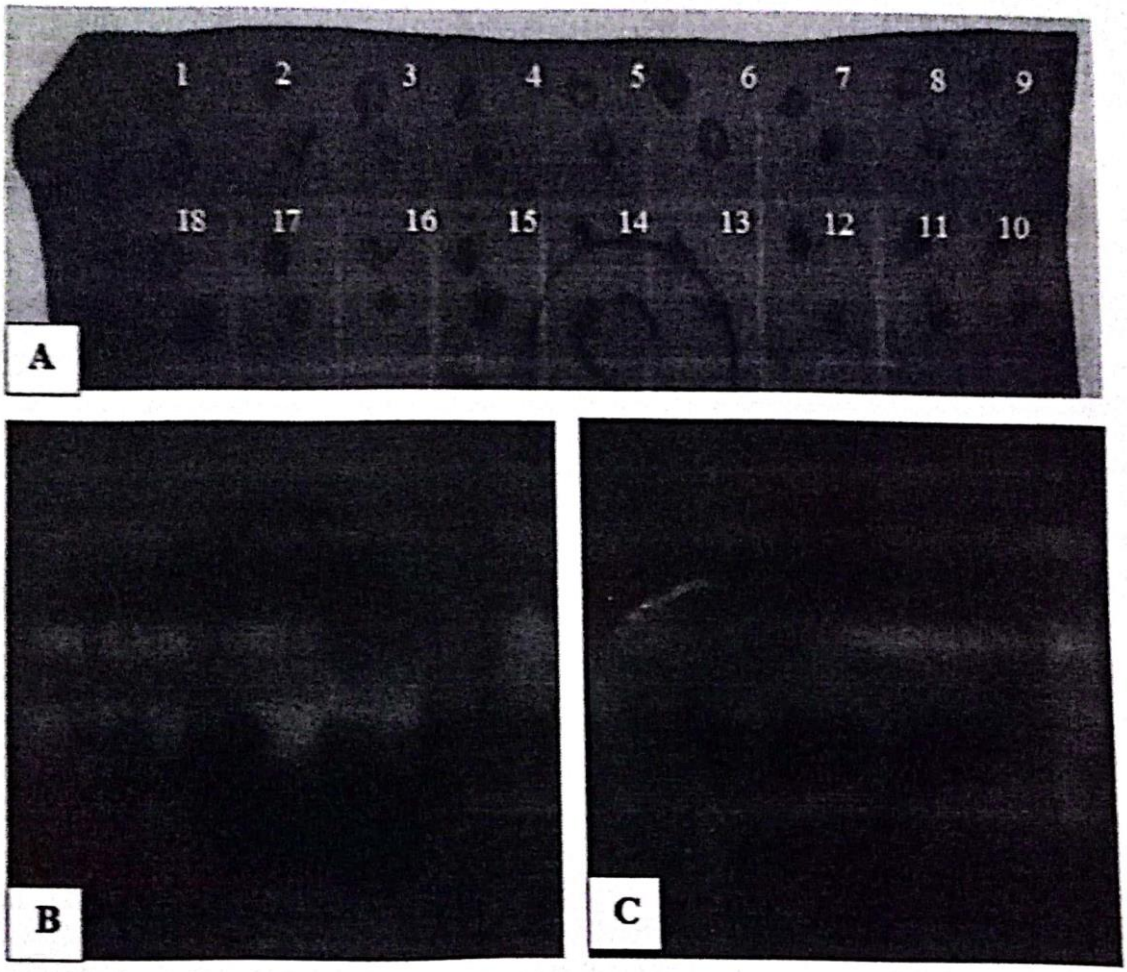

Figure 4. A. Tissue print hybridization of eighteen naturally infected and uninfected grapevine plants on nitrocellulose membrane using PSTVd dig-labeled DNA-DNA-probe showing hybridization signals.

B. Magnified transverse section of grapevine petiole printed on nitrocellulose membrane showing the positive hybridization signals concentrated on phloem tissue.

C. Magnified transverse section of grapevine petiole printed on nitrocellulose membrane showing negative hybridization signals

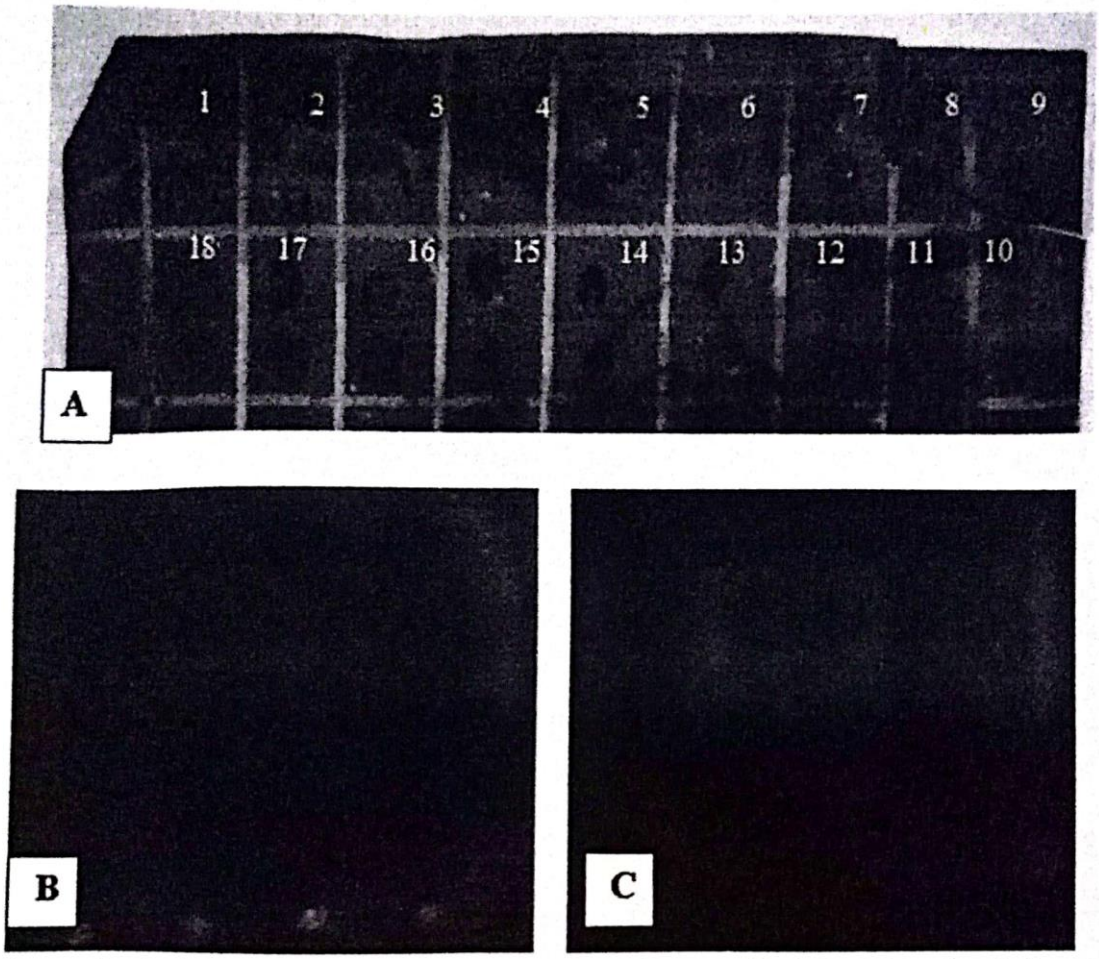

Figure 5. A. Tissue print hybridization of eighteen naturally infected and uninfected grapevine plants on nitrocellulose membrane using CEVd dig-labeled DNA-DNA-probe showing hybridization signals.

B. Magnified transverse section of grapevine petiole printed on nitrocellulose membrane showing the positive hybridization signals concentrated on phloem tissue.

C. Magnified transverse section of grapevine petiole printed on nitrocellulose membrane showing negative hybridization signals

Table 1. Tissue print and dot-blot hybridization results of the samples suspectful infected by HSVd, PSTVd and CEVd.

No. of tree s	External symptoms	Tissue print hybridization			Dot-blot hybridization		
		CEV d	HSV d	PSTV d	CEV d	HSV d	PSTV d
1	Mosaic	+	+	-	-	-	-
2	Severe mosaic	+	+	+	-	+	+
3	Chlorotic spots	-	+	+	-	-	+
4	Severe mosaic	+	-	+	-	-	-
5	Mild mosaic	+	+	+	-	+	+
6	Severe mosaic and yellowing	+	+	-	+	+	+
7	yellow speckle	+	-	-	-	+	+
8	Severe mosaic and yellowing	-	+	-	-	+	-
9	yellow speckle	-	-	-	+	-	+
10	No symptoms	+	-	-	+	-	+
11	yellow speckle	-	-	+	+	+	-
12	Mild mosaic	+	+	-	-	-	+
13	yellow speckle and yellowing	+	-	-	+	-	-
14	Yellowing spots	+	-	+	-	+	+
15	Vein clearing and yellow speckle	-	+	+	+	+	+
16	Mild mosaic	-	-	+	-	+	-
17	No symptoms	+	+	+	-	+	+
18	Vein banding	+	+	+	+	+	+
(*)		12/100	10/100	10/100	7/100	11/100	12/100

(*) 18 out of 100 grapevine trees (+) positive and (-) negative

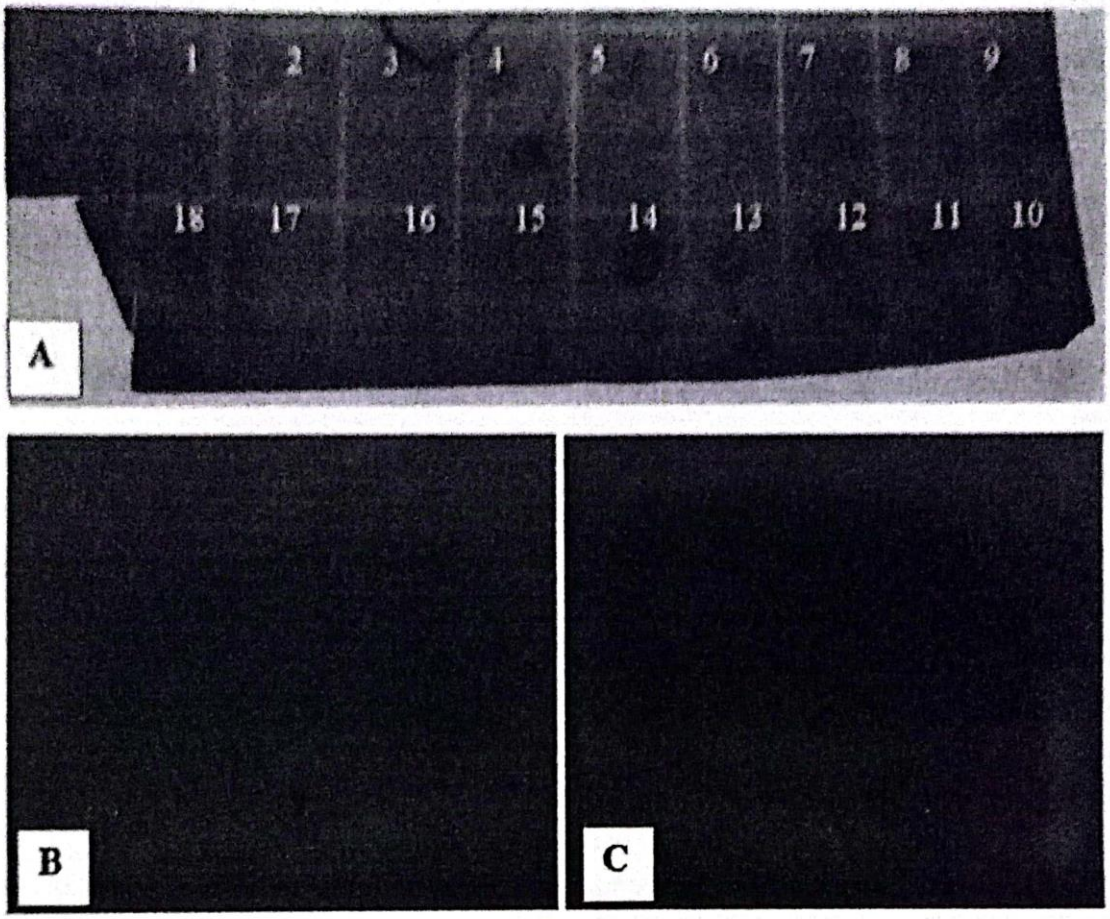

Figure 6. A. Dot blot hybridization assay showing colored blots of hybridized nucleic acid of HSVd infected grapevine plants

B. Magnified colored blots on nitrocellulose membrane showing the positive hybridized nucleic acid.

C. Magnified colored blots on nitrocellulose membrane showing the negative result.

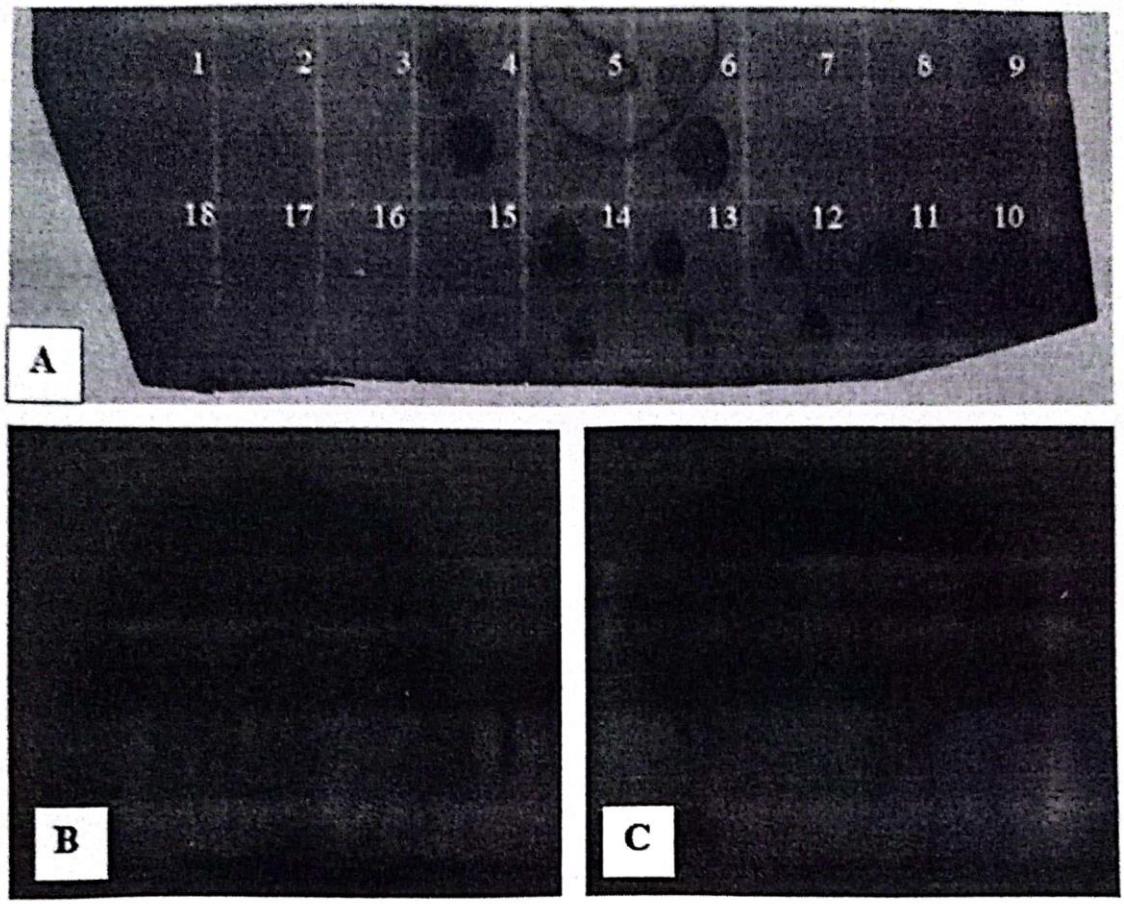

Figure 7. A. Dot blot hybridization assay showing colored blots of hybridized nucleic acid of PSTVd infected grapevine plants

B. Magnified colored blots on nitrocellulose membrane showing the positive hybridized nucleic acid.

C. Magnified colored blots on nitrocellulose membrane showing the negative result.

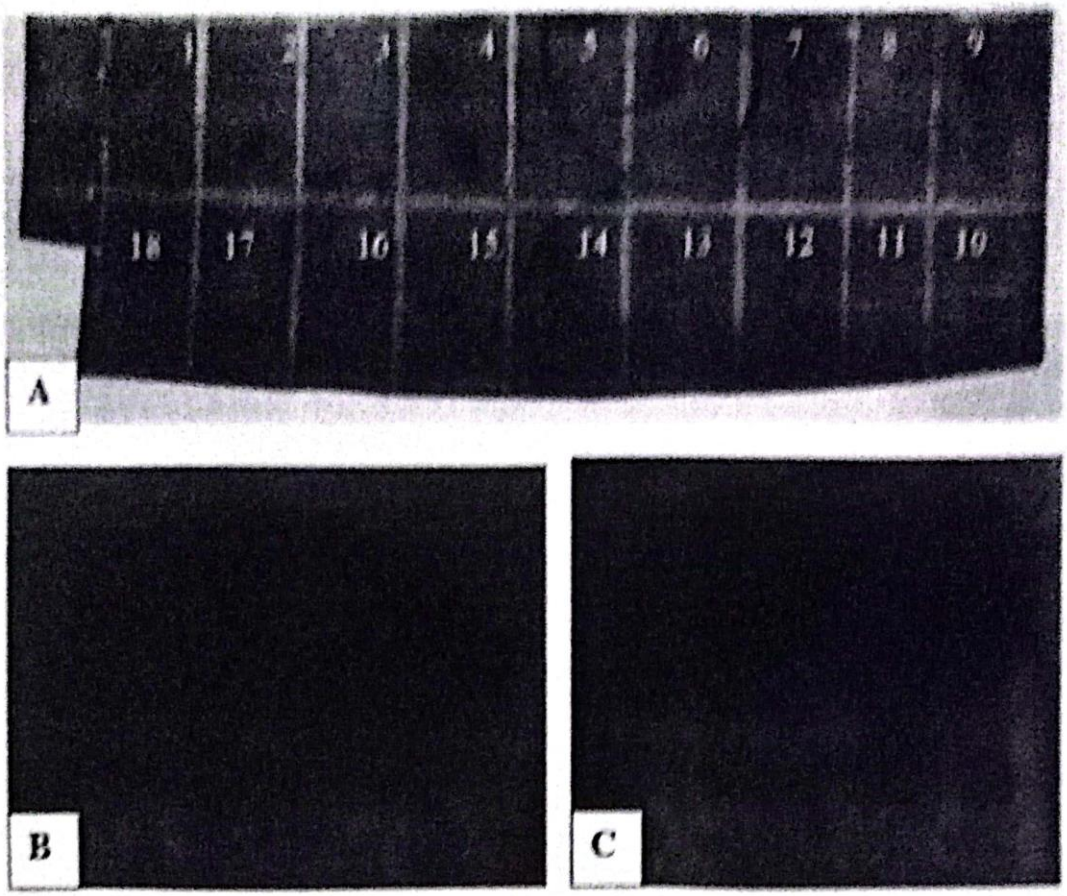

Figure 8. A. Dot blot hybridization assay showing colored blots of hybridized nucleic acid of CEVd infected grapevine plants

B. Magnified colored blots on nitrocellulose membrane showing the positive hybridized nucleic acid.

C. Magnified colored blots on nitrocellulose membrane showing the negative result.

Table 2. Frequency of HSVd, CEVd and PSTVd naturally infected grapevine trees.

Viroid	External symptoms	Tissue print hybridization (TPH)		Dot blot hybridization (DBH)		TPH + DBH
		No. of infected trees	(%) Frequency	No. of infected trees	(%) Frequency	Mean frequency (%)
CEVd	Yellow speckle, yellowing, no symptoms	3	16.66	0	0	8.33
HSVd	Severe mosaic and yellowing	1	5.55	2	11.11	8.33
PSTVd	Yellow speckle, mild mosaic	2	11.11	2	11.11	11.11
CEVd+HSVd	Mosaic, severe mosaic and yellowing, mild mosaic	3	16.66	1	5.55	11.11
CEVd+ PSTVd	Yellowing spots	2	11.11	2	11.11	11.11
HSVd+ PSTVd	Chlorotic spots, vein clearing and yellow speckle	2	11.11	5	27.77	19.44
CEVd+HSVd+ PSTVd	Mild mosaic, no symptoms, Vein banding	4	22.22	3	16.66	19.44
Total infected grapevine		**17/18**	**94.44**	**15/18**	**83.33**	**88.88**

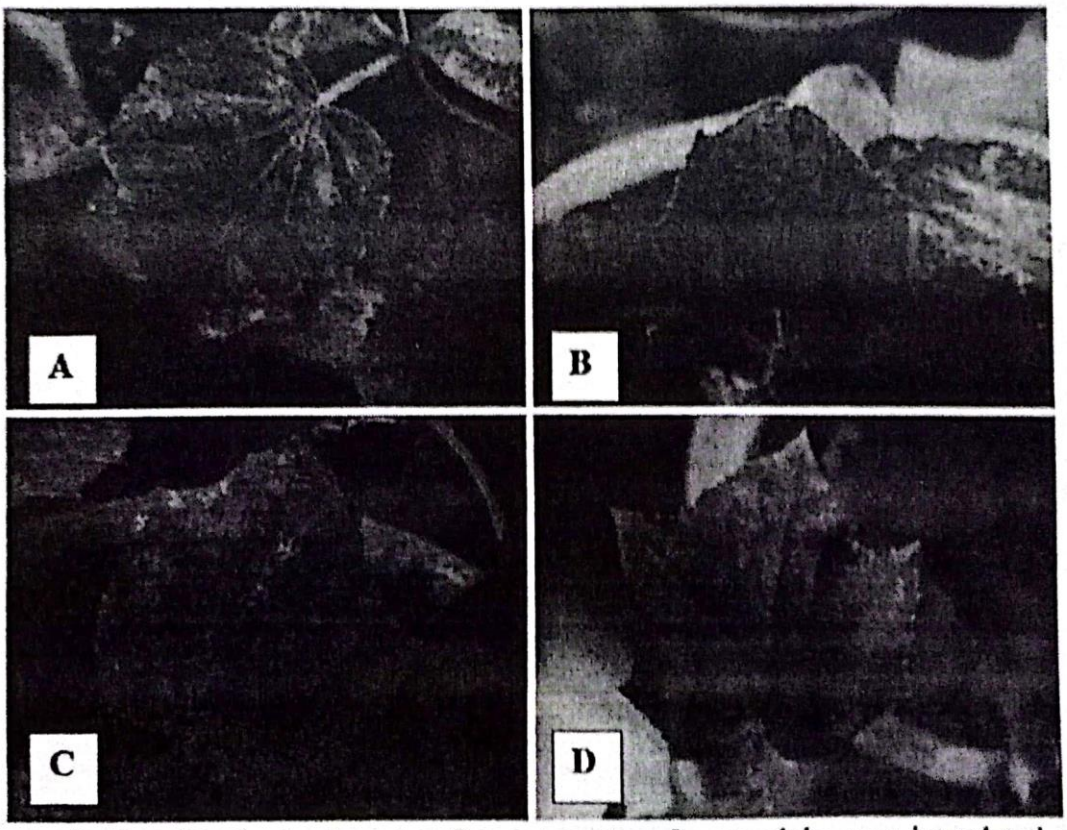

Figure 9. Developed symptoms on *Cucumis sativus* L. cv. alpha was inoculated with HSVd showing: (a) severe mosaic, (b) vein clearing and rugosity, (c) yellowing spots and (d)Top yellowing.

Figure 10. Developed symptoms on *Lycopersicon esculantum* L. cv. Castle rock was inoculated with PSTVd showing: (a) severe mosaic, leaf deformation, epinasity (b) leaf deformation, mosaic, small leaflet.

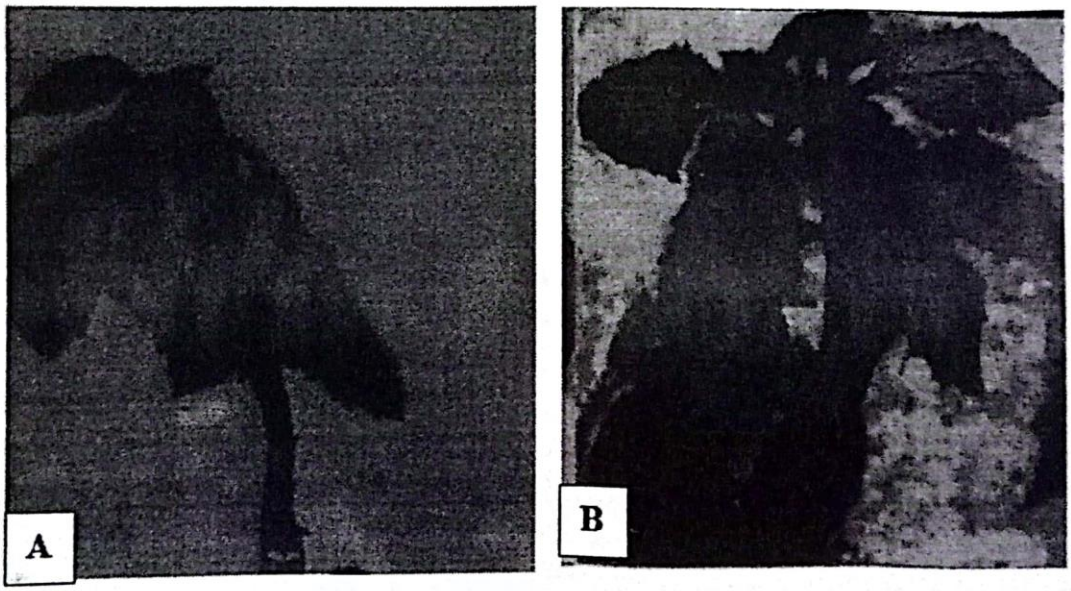

Figure 11. Developed symptoms on *Gynura aurantiaca* was inoculated with CEVd showing: (a) mosaic, leaf curl (b) mosaic .

DISCUSSION

The application of recombinant DNA technology has permitted the use of molecular hybridization for viroids detection. These techniques, combined with the application of an easy procedure for the extraction of total nucleic acids, the existence of non-radioactive HSVd, CEVd and PSTVd specific probes, are now used for routine diagnosis of viroids. More recently, tissue-imprinting hybridization, a technique that avoids sample extraction and only requires the direct transfer of the plant material has been applied successfully to the detection of these viroids in grapevine.

The results obtained in the tissue print and dot-blot hybridization assays allowed clear discrimination between infected and un infected grapevine trees, the unclear signals occasionally observed were not considered as positive. The tissue printing described rapid method for viroid detection from grapevine trees in the field and was effective and reliable. The efficiency of tissue printing method was similar to that of the Polymerase Chain Reaction (PCR). Molecular hybridization assays gave excellent results and more sensitive than S-PAGE technique. Any way, tissue print hybridization is more advisable because of its possibility to test simultaneously a large number of samples. No reaction occurred with healthy control indicating that this tissue printing preparation is able to avoid cross reaction with plant nucleic acids and the carry over deriving from the interference of substances with PCR. In addition this assay have many advantages such as the small amount of starting plant tissue, the rapidity and the possibility to print the petiole on nitrocellulose membranes in the field. All characteristics make this method useful for detection and diagnosis of viroids on grapevine trees in particular for preliminary screening of material candidate for sanitary certification programs. Tissue printing is a very convenient method for analysis of a large number of samples, faciliting the evaluation of the sanitary status of the grapevine industry in different countries. This is particularly relevant for countries where no appreciate facilities for viroid diagnosis exist.

In addition, all analysis conducted in a single facility enhancing the test reproducibility and ensuring that the same

evaluation criteria are applied to samples from all region. Interestingly, cutting originating from Egypt for viroids were collected in summer season with temperature oscillating between 35-45°C, indicating that viroid concentration in the trees is sufficient for detection under these extreme conditions.

Viroids can be detected by biological, biochemical or molecular methods the three approaches are time consuming and expensive, with the limiting step for molecular techniques being the sample preparation process.

The results obtained for CEVd are consistent with those previously reported in Egypt (**EL-Dougdoug, *et al.* 1993)** and other countries of the Mediterranean region **(Hadidi *et al.* 2003 a, b)** from grapevine.

The results showed that the tissue print assays allowed clear discrimination between healthy and viroid-infected grapevine plants than dot-blot hybridization, the number of HSVd-infected grapevine plants were 10 plants, PSTVd were 10 plants. And the CEVd was detected with high incidence level in grapevine, where 12 out of 100 grapevine trees analyzed were infected.

In Egypt, during the last decade, grape production have been developed through increased cultivated areas of field. Heavy losses caused by viral and viroidal infections are observed mainly in field crops. The severity of losses also appears to be directly related to the lack of certified cultivars and to cultivation methods, revealed a worrying situation for the future of grape cultivation in this country, if appropriate control measures are not taken.

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