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Detection of *Grapevine Fanleaf Virus* Capsid Protein Gene by RT–PCR and DNA Hybridization

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Grapevine fanleaf virus (GFLV) was isolated from symptomatic grapevine (*Vitis vinefera* L.) leaf samples obtained from El Kalubia Governorate. Three out of 25 samples were positive for GFLV by DAS-ELISA using specific polyclonal antibodies raised against purified GFLV preparation and five samples were positive by RT-PCR. RT-PCR detected GFLV in both fresh and dried tissues. A fragment (321 bp) of the coat protein gene of GFLV was amplified by reverse transcription-polymerase chain reaction (RT-PCR) using two primers specific for the coat protein gene of GFLV. Nucleotide sequences of the RT-PCR products confirmed that these sequences were amplified from the GFLV coat protein gene. A specific GFLV Dig labeled DNA probe was prepared by PCR and detected the GFLV virus in fresh leaves up to 10^{-5} dilution in dot blot hybridization assay.It was suggested that the inhibitory compounds released during the extraction of RNA constitute a limiting factor for the detection of GFLV in infected vines. Both immunological and molecular detection methods provide tools assisting in the understanding of the epidemiology and diversity of nepoviruses as well as to facilitate resistance breeding, cultivar selection, and development of strategies for control.

INTRODUCTION

Grapevine fanleaf Nepovirus (GVFL) is the most important and widespread virus of the grapevine viruses. It stunts the growth of the vine, and can significantly reduce yield, although it rarely kills the plant. Symptoms include deformation of the leaf, shortening of internodes and canes, yellow patches or yellow veins on the leaf, stunted growth and very poor fruit set.

In the early 1960s the virus received considerable attention, and several strains were partially characterized.The virus was detected in many countries of the world, i.e. Turkey, Jordan, Israel, Tunis, Taiwan, New Zealand, Germany, Switzerland, Italy, Bulgarian, Yougoslavis, Canada and Japan (Kolber and Lehoczky, 1983 and Kearns and Mossop, 1984; Tanne, 1985; Ouertani *et al.*, 1992; Tzeng *et al.*, 1994; Al Tamini *et al.*, 1998 and Koklu *et al.*, 1998).

Grapevine fanleaf virus (GFLV) is a bipartite, isometric particle, 30 nm in diameter, protein size 54 kDa (Quacquarelli *et al.*, 1976). The GFLV genome consists of 2 plus-senses single-stranded RNAs, RNA1 and RNA2 (Serghini *et al.*, 1990; and Ritzenthaler *et al.*, 1991). GFLV genomes may consist of a genetically diverse collection of mutants, the dominant members of which may vary during shifts among successive host varieties, in the manner of a quasispecies (Roossinck, 1997; Schneider and Roossinck, 2000). Extensive variability exists in the sequences of GFLV genomes (Serghini *et al.*, 1990; Sanchez *et al.*, 1991;

Esmenjaud *et al.*, 1994 and Brandt *et al.*, 1995). Nucleotide sequence differences were found to range from 8% to over 10%, with amino acid sequence differences in the range of 2 to 4%. This suggests that variability in GFLV symptomatology, from 'fanleaf' to yellow mosaic or vein banding symptoms (Krake *et al.*, 1999), may have a genetic component.

Control strategies of GFLV remain preventive. They are based on the identification and destruction of infected material to reduce disease incidence and minimize economic damage (Raski *et al.*, 1983; Walter and Demangeat, 1995). Therefore, detection of infected plants is important. Diagnosis can be accomplished by biological indexing with indicator plants, immunological tests such as ELISA (Huss *et al.*, 1986; Walter and Etienne, 1987; Walter, 1994), and molecular techniques such as hybridization (Fuchs *et al.*, 1991.; Fortass *et al.*, 1996; Harald *et al.*, 1996) and reverse transcription-polymerase chain reaction (RT-PCR) (Minafra and Hadidi, 1994).

Many reports have shown that immobilized viruses (Rowhani *et al.*, 1995; Olmos *et al.*, 1996; La Notte *et al.*, 1997) or nucleic acid captured by magnetic beads (Regan and Margolin, 1997) increase the efficiency of RT-PCR and permit the use of large amounts of crude extracts. Immunocapture IC-RT-PCR has been shown to be more sensitive than ELISA and direct RT-PCR for detecting viral RNA in infected plants (Wetzel *et al.*, 1992; Acheche *et al.*, 1999). Nolasco *et al.* (1993) applied this technique to detect GFLV in infected grapevines. However, in some cases, the sensitivity of this technique was unsatisfactory for virus detection in plant extracts. Minafra and Hadidi (1994) suggested that the recovery of RNA from bound particles is less than 100%. Specific nucleic acid probe capture methods have been described and successfully assayed for detecting the animal poliovirus RNA in groundwater (Regan and Margolin, 1997) and hepatitis A virus in stool and shellfish samples (Arnal *et al.*,1999).

The objective of this work is to develop simple and sensitive molecular diagnostic method for the detection of GFLV in Egyptian grapevines. Because standard extraction protocols were not effective for grape tissues, a successful protocol is reported using high pure RNA tissue kit. Asuccessful RT-PCR amplification for detecting GFLV was also reported. The sensitivity of the DNA probe was determined in dot-blot hybridization assay.

MATERIAS AND METHODS

Source of the virus

Grapevine leaves showing GFLV symptoms were collected from the farm of the Faculty of agriculture, Ain Shams University, El Kalubia, Egypt, and examined serologically by enzyme linked immunosorbent assay (ELISA) using ELISA kite provided by sanofi., Sante Animal, Pairs, France Five-hundred mg of grapevine shoot tips and leaves were ground in 5 ml of 50 mM phosphate buffer, pH 6·5, containing 2 % nicotine. *C. quinoa* leaves dusted with carborundum were inoculated mechanically with this infectious sap. inoculated plants were kept under green house conditions (25-30°c) and observed for symptoms development. Single local lesion technique was used for biological purification and *Ch. amaranticolor* was used as a local lesion host.

Enzyme linked immunosorbent assay (ELISA)

The double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) was used for virus detection as mentioned by Clark and Adams (1977). ELISA plates were coated with 200 µl of 1µg/ml of IgG/well in coating buffer. The plates were incubated at 4 °C overnight and then washed four times 3 minutes each with washing buffer. The tested samples were prepared by grinding 1.0 gm of infected leaves in 10 ml extraction buffer and then 200 µl were added per well followed by incubation overnight at 4 °C. Plates were washed four times and 200 µl of second antibody alkaline phosphatase conjugated IgG was added at dilution 1:1000 in conjugating buffer and then incubated at 37 °C for 3 hours. Plates were washed four times and 200 µl of pNPP substrate was used at final concentration 0.75 mg/ml. The plates were incubated at room temperature until the positive yellow colour appeared. The reaction was stopped by adding 50 µl of 3 M NaOH to each well. The ELISA values were determined at a wavelength 405 nm by ELISA reader Reading greater than twice that obtained of healthy controls was considered positive.

Preparation of total RNA

Total RNAs were prepared from young grapevine leaves according to the instruction manual of High Pure RNA tissue kit (Version 1, 2000) from Roche diagnostics GmbH, Germany. 25 mg leaf tissues were homogenized in 400-µl lysis/Binding buffer (4.5M guanidine-HCl, 100 mM sodium phosphate, pH 6.6). The lysate was centrifuged for 2 min at maximum speed in a microcentrifuge and 200 µl of absolute ethanol was added to the lysate supernatant. The high pure filter tube and the collection tube were combined and the sample was pipetted in the upper reservoir and centrifuged for 30 s at maximal speed (13,000 xg). The flowthrough was discarded and 500 µl of wash buffer I (5 M guanidine-HCl, 20 mM Tris-HCl, pH 6.6, in ethanol) was added to the upper reservoir, and centrifuged for 15 s at 10,000 rpm. The flowthrough was discarded again and 500 µl wash buffer II (20 mM NaCl, 2 mM Tris-HCl, pH 7.5 in ethanol) was added to the upper reservoir of the filter tube and centrifuged 15 sec at 8000 xg. After the flowthrough was discarded, 100 µl of elution buffer (nuclease–free, sterile, double dist. Water) was added to the upper reservoir of the filter tube and centrifuged at 8000 xg for 30 sec. The eluted RNA was stored at -80 °C for later analysis.

RT-PCR amplification

A modification of the method of Rowhani *et al.* (1995) was used for synthesizing complementary DNA strand by adding 7 µl of total nucleic ac ds primed with 50 pmol of Ist strand primer (oligo dT) in a total volume of 20 µl and placed in a water bath at 70 °C for 5 min. The reaction contained 4 µl of 5x RT buffer (Biotools, Biotechnological & Medical Laboratories, S.A. Madrid, Spain), 1 µl of 10 mM dNTPs, 1 µl of enhancing buffer, and 1.5 µl of Retrotools Reverse Transcriptase (Biotools, Biotechnological & Medical Laboratories, S.A. Madrid, Spain). The reaction was performed at 70 °C for 45-60 min. For PCR, 50 pmole of each amplification primer (forward primer) (V1) corresponding to nucleotides 762-781 (5'-ACCGGATTGACGTGGGTGAT - 3') and (reverse primer) (C1) was complementary to nucleotides 1,064-1,083 (5' CCAAAGTTGGTTTCCCAAGA-3')

(Rowhani *et al.*, 1993) were selected from the GFLV coat protein gene region, located at the 3' end of RNA2 (Sanchez *et al.*, 1991), 5 µl of each cDNA reaction, and 5 U/µl of High Expand Fidelity DNA polymerase (Roche) were used in a 5x Standard DNA buffer containing 20 mM Tris HCl, pH 8.2, 10 mM KCl, 6 mM $(NH_4)_2$ SO_4, 2 mM $MnCl_2$, 0.1% Triton X-100 and 10 µg/ml of nuclease-free BSA. The amplification reaction was carried out in a total volume of 50 µl using the UNOII system from Biometra and using 0.2 ml micro Amp PCR tubes. Hard denaturation of the DNA was performed at 95 °C for 2 min followed by 35 cycles of amplification with denaturation at 94 °C for 30 sec, annealing at 45 °C for 45 sec, and extension at 72 °C for 1 min. A single tailing cycle of long extension at 72 °C for 7 min was carried out in order to ensure flush ends on the DNA molecules. The amplified DNA was electrophoresed on 1% agarose gel and the obtained fragments were used for southern blotting step.

Southern blot hybridization and probe preparation

Southern blotting technique was performed on the RT-PCR products amplified from extracted total RNA separated on a 1.0 % agarose gel in a 1x TAE buffer, transferred to nitrocellulose membrane by capillary force (Southern, 1975) and cross linked under UV crosslinker between 2,500 and 10,000 µJoules/cm^2. Digoxigenin -11- dUTP–labeled cDNA probes, corresponding to GFLV were prepared by using 10X DNA labeling nucleotide mix (Roche, Boehringer Mannheim, Indianapolis). Digogxigenin-11-dUTP nucleotide mix was incorporated into the PCR cocktail instead of the normal nucleotide mix using the protocol described under the technical bulletin (Roche, Boehringer Mannheim, Indianapolis). The PCR reaction was performed in 50 µl total volume reaction as described above. Prehybridization and hybridizations of the membranes were done according to the manufacturer's instruction, and the color detection system (Roche, Boehringer Mannheim, Indianapolis) was used. Hybridizations were done at 65°C overnight in a hybridization oven. The membrane was incubated for 30 min at room temperature with anti-dig-alkaline phosphatase conjugate diluted 1-2500 in buffer II (100 ml buffer I and 1 gm blocking reagent) . The unbound antibodies were removed and the membrane was equilibrated by washing 2 times for 15 min with 100 ml of buffer I (0.1M maleic acid, 0.15M NaCl, pH 7.5), then washed for 2 min with 20 ml of buffer 3 containing 100 mM Tris-HCl, 100 mM NaCl, and 50 mM $MgCl_2$ pH 9.5) at room temperature. The membrane was introduced for colour detection system in 10 ml colour solution using 35 µl of 5-bromo-4-chloro-3-indolylphosphate (BCIP) and 45 µl of Nitro blue tetrazolium (NBT) to visualize the fragments. The membrane was incubated for 15 min in a suitable clean box in the dark. The reaction was stopped when desired signals were obtained using Genius buffer IV (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) for 5 min. The membrane was air dried and stored at room temperature.

Dot blot hybridization

Total clarified sap preparations were diluted ten-fold with 6XSSC buffer, heated to 95°C for 10 min and chilled on ice. 5 µl of each dilution was directly applied to nitrocellulose membrane (Roche). Membranes were air-dried and the nucleic acids were cross-linked to the membrane by exposure to UV irradiation from a

transilluminator for 3 min between 2,500 and 10,000 μJoules/cm^2. Prehybridization and hybridization with single digoxigenin labeled probe were done. Colour detection with NBT and BCIP reagents as substrate was performed as recommended by the manufacturer (Roche).

RESULTS AND DISCUSSION

A preliminary survey was conducted to assess the presence and incidence of *Grapevine fanleaf virus* among the cultivations of grapevines in Egypt including the commercial vineyards and mother plants– depending upon field inspections for symptom observations. 25 samples out of 945 grapevine showing symptoms were serologically positive by DAS-ELISA. Golino *et al.* (1992) indicated that the serodiagnostic test ELISA could not be reliably used to detect GFLV in infected field – grown vines during the summer season while detection of GFLV in infected grapevines in the field by PCR was possible during the growing season (Rowhani *et al.* 1993) and this method could be adopted to complement or substitute for ELISA. One of the positive plant cultivated in the farm of faculty of agriculture, Ain Shams University, exhibits vein banding / yellow mosaic, abnormal branching, chlorosis and fanleaf shape (Fig. 1 A) was grafted on healthy grapevine seedling (Fig. 1 B) and after 62 days, the external symptoms were observed. Similar results were recorded by AL-Tamini *et al* (1998) Their data indicated that the tested vines were infected by *Grapevine Virus* (GAV; GBV), *Leaf roll associated virus* (GLRAV), *Grapevine fanleaf Nepovirus* (GFLV) and *Grapevine fleck* virus (GFKV). Kearns and Mossop (1984) detected also the *Grapevine fanleaf Nepovirus* in 990 samples from grapevine cultivated in New Zealand. The virus was also successfully detected at all dilutions (1:10, 1:50, and 1:100) in extracts from infected grapevine (kolber and lehoczky, 1983)

Standard sample extraction procedures for PCR failed to detect GFLV in grapes, because grape vine tissues contain substantial amounts of phenolic compounds and polysaccharides that may interfere with nucleic acid preparations. This result is in agreement with that reported by Demeke and Adams (1992) The extraction method used successfully eliminated this problem and described in this paper. The results obtained by this method were consistent and reproducible, therefore, minimizes the loss of viral RNA

GFLV-RNA2 was detected by RT-PCR using primers C1 and V1 as reported by (Rowhani *et al.* 1993) Amplification of RNA2 occurred with infected samples but not from healthy one susing the same procedure The sensitivity of the PCR method reported in this paper open up a new horizon for GFLV research. It is more rapid and may offer an alternative and specific procedure to detect the virus in infected samples The retrotools cDNA/DNA polymerase kit (Biotools, Biotechnological & Medical Laboratories, S. A. Madrid., Spain) used in this paper improved our results of RT-PCR amplification due to the presence of "Enhancing buffer" that used in the RT- mixture. Incubation of the reaction mix at 60 °C for 15 to 60 minutes with oligo (dT) primer in cDNA synthesis increased the efficiency of transcription and the yields of amplification Similar results were obtained by Izadpanah *et al*

(2003) when random and oligo (dT) primers were used on viral RNA templates to synthesize cDNA for cloning and sequencing. Specificity of the PCR amplification was confirmed by southern blot analysis of the 321 bp amplified fragment of RNA2 (Fig 2 A & B). The presence of phenolic compounds liable to interfere with the enzyme system used for RT and PCR reactions was omitted by using the RNA extraction method reported in this paper, therefore increasing the efficiency of the direct RT-PCR. The use of diluted extracts to avoid inhibition of RT-PCR will reduce viral RNA concentration; therefore, GFLV can not be detectable in some infected grapevines, especially in the early diseased samples (Acheche *et al.*, 1999).

In order to increase the performance of RT-PCR, we developed DNA probe for the detection of GFLV in infected tissues. The results showed that the DNA hybridization technique is as specific as the RT-PCR (Figure 3, Lane 1). A single band of the predicted size (321 bp) was amplified using RNA2 specific primers, whereas no amplification was observed with the healthy sample (Fig 3, lane 2). Also, the DNA probe detected GFLV-RNA2 at a 10-fold lower dilution of crude extract in dot-blot assay (Figure 4). Because the DNA probe developed by this method proved to be very sensitive in GFLV detection, it may possibly be applied for the detection of viruses in which no specific antibodies are available and also in certification and clean stock programs.

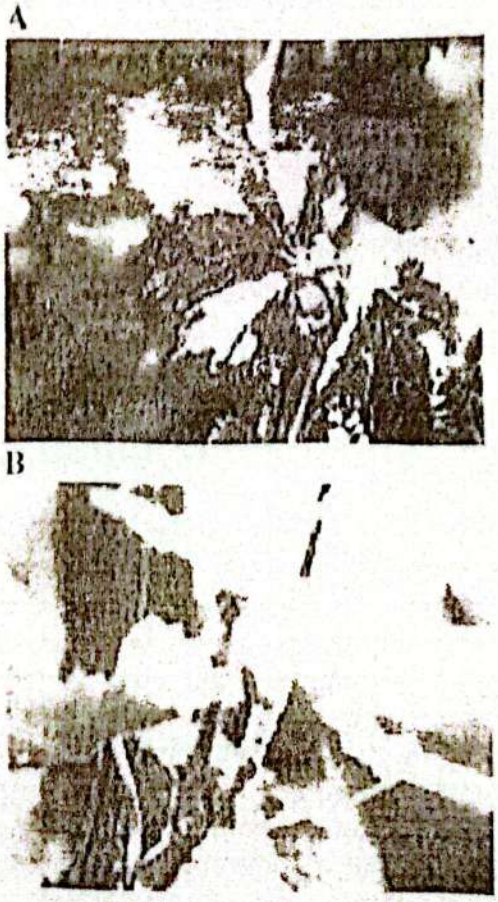

Fig. (1): (A) GFLV – infected grape vine plant showing yellowing and fanleaf shape. (B) Transmission of GFLV by grafting on healthy grapevine.

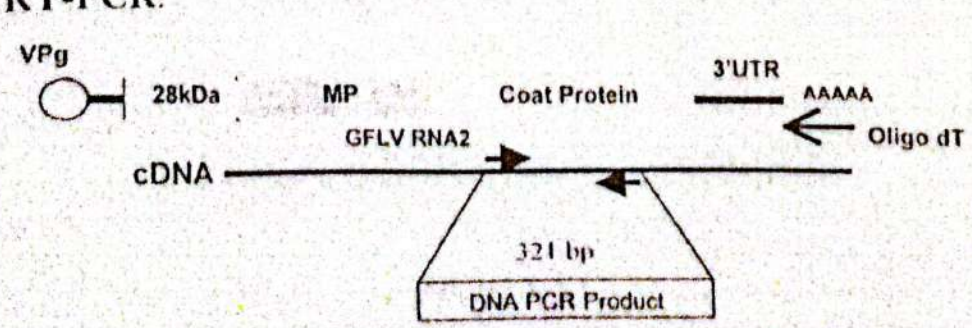

Fig.(2): Schematic diagram of RT-PCR procedure. The CP gene is 3' proximal in GFLV RNA2, adjacent to the (putative) movement protein gene. cDNA was synthesized from total RNA followed by PCR. The Oligo dT primer was used to make first strand cDNA, then the M and P primers were used for production of a 321 bp PCR product. The PCR product contains sequences from the CP region.

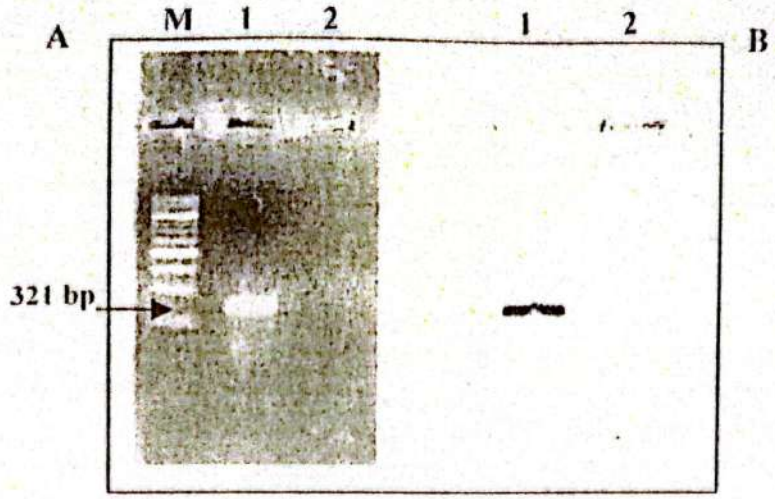

Fig. (3A): 1% agarose gel electrophoresis showing the RT-PCR product using (V1) and (C1) primers on RNA2 extracted from Vitis leaf tissues infected with GVFL. Lane 1: showing the amplified RT-PCR product of the correct size (321bp) from Vitis tissues infected with GFLV. Lane M: DNA Molecular weight marker (Roche, Applied bioscience). Lane 2. Healthy plant showing no PCR product). (B): Southern hybridization of GFLV amplified PCR fragment using a specific DNA probe labeled with dig-11-dUTP. The hybridization signal in lane (1) was RT-PCR product of correct size (321 bp).

DNA dot blot Hybridization:

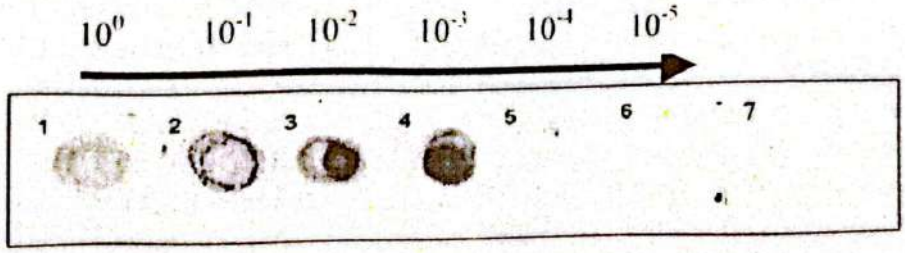

Fig (4): Dot blot hybridization of GFLV RNA extracted from mechanically infected grapevine tissue. 10 µl of total nucleic acid extracted from grapevine tissue infected with GFLV by using dot-Blot buffer were spotted on the nitrocellulose membrane in serial dilution (10^{0}-10^{-5}). The hybridization signals were very clear even in the dilution 10^{-5} of nucleic acid extract.The colour was developed after 15 minutes using BCIP- NBTsubstrate.Sample No.7(Negative)Healthy Vitis plant no signal.

REFERENCES

Acheche, H.; Fattouch, S.; M'hirsi, S.; Marzouki, N. and Marrakchi, M. (1999). Use of optimized PCR methods for the detection of GLRaV3: a closterovirus associated with grapevine leafroll in Tunisian grapevine plants. Plant Mol Biol Rep. 17: 31- 42.

Al-Tamini, N.; Digiaro, M. and Savino, V. (1998). Viruses of grapevine in Jordan. Phytopathologia. Mediterranea 37: 3. 122-126.

Arnal, C., Ferré-Aubineau, V.; Besse, B.; Mignotte, B.; Schwartzbrod, L. and Billaudel, S. (1999). Comparison of seven extraction methods on stool and shellfish samples prior to hepatitis A virus amplification. J Virol Methods 77: 17-26.

Brandt, S.; Ibl, M. and Himmler, G. (1995). Capsid protein gene sequence of an Austrian isolate

of GFLV. Archives of Virology 140, 157-164

Clark, M. F., and Adams, A. N., (1977). Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Genl. Virol .34: 475-483.

Demeke, T., and Adams, R. P. (1992). The effects of plant polysaccharide and buffer additives of PCR. Biotechniques 12:332-334

Esmenjaud, D.; Abad, P.; Pinck, L. and Walter, B. (1994). Detection of a region of the capsid protein gene of Grapevine fanleaf virus by RT–PCR in the nematodevector Xiphinema index. Plant Disease 78, 1087-1090.

Fortass, M.; Van Der Wilk, F.; Goldbach, R.W.; Bos, L. and Van der Heuvel, J. (1996). Diversity of luteoviruses infecting faba bean (Vicia faba L) in Morocco and their detection by polymerase chain reaction. Agronomie 16: 61-68.

Fuchs. M.; Pinck, M.; Etienne, L.; Pinck, L. and Walter, B. (1991). Characterization and detection of grapevine fanleaf virus by using cDNA probes. Phytopathology 81(5): 559-565.

Golino, D. A.; Verdegaal, P.; Rowhani, A. and Walker, M. A. (1992). Sampling procedures to find nepoviruses in grapevines need improvement. Calif. Agric. 46: 11-13

Harald, P.; Uwe, S. and Hans, J. H. (1996). Variation of viroid profiles in individual grapevine plants: novel *Grapevine yellow speckle viroid* 1 mutants show alterations of hairpin I. J. Gen .Virol .77: 155-161.

Huss, B.; Walter, B.; Etienne, L. and Van Regenmortel, M.H.V. (1986). *Grapevine fanleaf virus* detection in various organs using polyclonal and monoclonal antibodies. Vitis 25: 178-188.

Izadpanah, K.; Zaki-Aghl, M.; Zhang, Y. P.; Daubert, S. D. and Rowhani, A. (2003). Bermuda Grass as a potential reservoir host for Grapevine fanleaf virus. Plant Dis. 87: 1179- 1182

Kearns, C. G. and Mossop, D. W. (1984). Detection of nepoviruses of Vitis Vinefera in NewZealand using enzyme – linked immunosorbent assay (ELISA). New- Zealand Journal of agricultural research. 27:3, 431-435

Koklu, G.; Digiaro, M. and Savino, V. (1998). A survey of grapevine viruses in Turkish thrace. Phytopathological Mediterranea. 37: 3, 140-142

Kolber, M. and Lehoczky, J. (1983). detection of *Grapevine fanleaf virus* in different tissues at dormant and forced stages of grapevine by application of the ELISA technique. Kertgazdasag. 15: 2, 47-51.

Krake, L. R.; Steele Scott, N.; Rezaian, M. A. and Tylor, R. H. (1999). Graft-Transmitted Diseases of Grapevines. Collingwood, Victoria: CSIR Publishing.

La Notte, P.; Minafra, A. and Saldarelli, P. (1997). A spot-PCR technique for the detection of phloem-limited grapevine viruses. J Virol Methods 66: 103-108.

Minafra, A. and Hadidi, A. (1994). Sensitive detection of *Grapevine virus A, B,* or *Leafroll associated III* from viruliferous mealybugs and infected tissue by cDNA amplification. J Virol Methods 47: 175-188.

Nolasco, G.; De Blas, C.; Torres, V. and Ponz, F. (1993). A method combining Immunocapture and PCR amplification in a microtiter plate for the detection of plant

viruses and subviral pathogens. J Virol Methods 45: 201-213.

Olmos, A.; Dasi, M. A.; Candresse, T. and Cambra, M. (1996). Print-capture PCR: a simple and high sensitive method for the detection of *Plum pox virus* (PPV) in plant tissues. Nucleic Acids Res 24 (11): 2192-2193.

Ouertani, R.; Savino, V.; Minafra, A.; Boscia, D.; Castellano, M. A.; Martelli, G. P. and Greco, N. (1992). Properties of a previousely undescribed graevine, Nepovirus from Tunesia. Archives of Virology. 126: 107-117

Raski. D. J.; Goheen, A. C.; Lider, L. A. and Meredith, C. P. (1983). Strategies against *Grapevine fanleaf virus* and its nematode vector. Plant Disease: 335-337.

Regan, P.M. and Margolin, A. B. (1997). Development of nucleic acid capture probe with reverse transcriptase-polymerase chain reaction to detect poliovirus in groundwater. J Virol Methods 64: 65-72.

Ritzenhaler, C.; Viry, M.; Pinck, M.; Margis, R.; Fuchs, M. and Pinck, L. (1991). J. Gen. Virol. 172: 2357.

Roossinck, M. J. (1997). Mechanism of plant virus evolution.Annual Review of Phytopathology 35, 191-209.

Rowhani, A.; Chay, C.; Golino, D. A. and Falk, B.W. (1993). Development of a polymerase chain reaction technique for the detection of *Grapevine fanleaf virus* in grapevine tissue. The American Phytopatholgical Society 83: 7, 749-753

Rowhani, A; Maningas, M. A.; Lile, L. S.: Daubert, S. D. and Golino, D. A. (1995). Development of a detection system for viruses of woody plants based on PCR analysis of immobilized virions. Phytopathology 85: 347-352.

Sanchez, F.; Chay, C.; Borja, M. J.; Rowhani, A.; Romero, J.; Bruening, G. and Ponz, F. (1991). cDNA sequence of the capsid protein gene and 3′-untranslated region of a fanleaf isolate of *Grapevine fanleaf virus*. Nucleic Acids Res. 19, 5440.

Schneider, W. L. and Roossinck, M. J. (2000). Evolutionarily related Sindbis-like plant viruses maintain different levels of population diversity in a common host. Journal of Virology 74, 3130-3134

Serghini M.A.; Fuchs, M.; Pinck, M.; Reinbolt, J.; Walter, B. and Pinck, L. (1990). RNA2 of grapevine fanleaf virus: sequence analysis and coat protein cistron location. J Gen. Virol. 71: 1433-1441.

Southern, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 503-517.

Tanne, E. (1985). Appearence and spread of *Grapevine yellow mosaic* in Israël. Phytopathologia Mediterranea. 24: 15-16

Tzeng, H. L. C.; Chen, M. J. and Tzeng, D. D. S. (1994). The occurence of grapevine leafroll disease among the main grapevine cultivars and breeding stocks in Taiwan. Plant Pathology Bulletin 3: 156-167

Walter, B. (1994). Le court-noué de la vigne. (I) Avantages et limites de la détection par Elisa. Le Progrès Agricole et Viticole 13-14: 320-328.

Walter, B. and Demangeat, G. (1995). Les virus du court-noué de la vigne. (I) Les voies de la contamination. Progrès Agricoles et Viticoles 112 (13-14): 295-303.

Walter, B. and Etienne, L. (1987). Detection of *Grapevine fanleaf virus* away from the period of vegetation. Journal of Phytopathology 120: 335-364.

Wetzel, T; Candresse, T.; Macquaire G.; Ravelonandro, M. and Dunez, J. (1992). A highly sensitive immunoçapture polymerase chain reaction method for *Plum pox potyvirus* detection. J. Virol. Methods. 39: 27-37.