Yüzüncü Yıl Üniversitesi, Ziraat Fakültesi,
Tarım Bilimleri Dergisi (J. Agric. Sci.),
2005, 15(2): 153-158

Geliş Tarihi: 27.01.2005

# Comparison of DAS-ELISA and RT-PCR Methods for the Detection of *Prunus Necrotic Ringspot Virus* (PNRSV)[1]

Mustafa USTA[2] Hikmet Murat SİPAHİOĞLU[2] Bülent POLAT[2]

**Abstract:** Two methods (DAS-ELISA and RT-PCR) were compared for the detection of *Prunus necrotic ringspot virus* (PNRSV) in woody host *Prunus mahleb* isolated from Malatya. Total RNA extractions were made from serially diluted fresh leaf tissue, root, bark and one year old green bark tissue using silica-based method. Purified total RNA extracts were used as template for cDNA synthesis by reverse transcription. PCR products, about 616 base pair in size, was analyzed by gel electrophoresis and visualized by ethidium bromide staining. Comparing ELISA test, RT-PCR was found more sensitive in detecting local PNRSV isolate. Several plant tissues were used for the evaluation of best tissue in the detection of PNRSV by RT-PCR. Leaf tissue of infected *P. mahaleb* was proved to be more suitable tissue in detection of virus by RT-PCR respect to root, one-year-old green bark tissue and bark tissue.

**Key words**: PNRSV, DAS-ELISA, RT-PCR, detection, comparison

## *Prunus Necrotic Ringspot Virüs*'ünün (PNRSV) Teşhisinde Kullanılan DAS-ELISA ve RT-PCR Metodlarının Karşılaştırılması

**Özet:** İki farklı teşhis yönteminin (DAS ELISA ve RT-PCR) odunsu konukçu *P. mahaleb*'e transfer edilen Malatya izolatı *Prunus necrotic ringspot virüs*'ünü (PNRSV) teşhisindeki başarısının karşılaştırmalı analizi yapılmıştır. Total RNA ekstraksiyonu silica temelli metoda göre taze yaprak, kök, kabuk ve yeşil sürgün kabuğu dokusundan gerçekleştirilmiştir. Saflaştırılan RNA revers transkripsiyonda cDNA sentezi için kalıp olarak kullanılmıştır. 616 bp uzunluğunda çoğaltılan PCR ürünleri agaroz jel'de koşulmuş ve ethidium bromide ile boyanarak gözlemlenmiştir. ELISA testi ile kıyaslandığında lokal PNRSV izolatını teşhiste RT-PCR'ın daha hassas olduğu tespit edilmiştir. PNRSV izolatını RT-PCR ile teşhiste kullanılacak en iyi dokuyu belirlemek üzere infekteli *P. mahaleb* bitkisinin farklı doku ekstrakları hazırlanmıştır. Kök, kabuk ve yeşil sürgün kabuğu dokularına nazaran yaprak dokusunun PNRSV'yi saptamada en iyi bitkisel doku olduğu tespit edilmiştir.

**Anahtar kelimeler**: PNRSV, DAS-ELISA, RT-PCR, teşhis, karşılaştırma

## Introduction

*Prunus necrotic ringspot virus* (PNRSV) is the most common virus of cultivated species of *Prunus* and some other rosaceous plants. It occurs in many strains and forms, some of which incite serious diseases in fruit trees (Nyland *et al*., 1976). This virus is known to be transmissible through grafting and also by pollen and seeds (Gilmer and Way,1961; Marenaud and Llacer, 1976; Gella, 1980). Since the virus has many strains, in some species, the effect may depend on virus strain involved (Mink, 1992).

PNRSV exist in many strains and variants, causing diseases in different cultivated *Prunus* species. The virus in most hosts induces shock symptoms after the infection, provided that they have not been infected earlier by latent strains. In some hosts the shock symptoms, return from time to time in the years following infection (Nemeth, 1986). Spread of PNRSV from tree to tree in *Prunus* orchards has been shown to be closely associated with pollen. PNRSV is also transmitted through seeds of some *Prunus* species (Francki, 1985).

Reliable detection of PNRSV is required in certification schemes aimed to provide virus-tested propagation material to the fruit growing industry and to prevent entry of imported PNRSV infected plant materials (Stain et al., 1987). PCR is a well-established, highly sensitive diagnostic tool, shown to be superior for certain plant viruses over the commonly used serological methods (Vunsh et al., 1990). Despite such assays, detection is still severely hampered by the uneven virus distribution in infected trees (Nemeth, 1986).

[1] The study was supported by The Research Fund of Yüzüncü Yıl University–YYUBAP (ZF 2002-046) and The Scientific and Technical Research Council of Turkey-TUBITAK
[2] University of Yüzüncü Yıl, Faculty of Agriculture, Department of Plant Protection 65080 Van - TURKEY

Serological procedures have been used more than others for the detection of PNRSV (Mink and Aichele, 1984). However erratic ELISA results were reported for shoot and leaf collections made by early in May when day temperatures exceeds 38°C over a 12 day period Uyemoto et al. (1989). In addition, Scott et al., 1992 reported that, when ELISA used, PNRSV was detectable in peach tissues only until the cessation of stem elongation.

In this study a comparative analysis of DAS-ELISA and RT-PCR was carried out for the detection of PNRSV-Malatya isolate in woody host *P. mahaleb* and several plant organs were extracted and used in RT-PCR for the determination of most suitable tissue in PCR amplification.

## Materials and Methods

### Plant and virus source

A cherry isolate of PNRSV detected in the field survey of Malatya was used in this study. The isolate was transmitted to a healthy *P. mahaleb* supplied by Mediterranean Agronomic Institute of Bari (Italy). The inoculated plant served as a virus source during the trials. Leaf, root, bark and green bark tissue of one-year old shoots of systemically infected *P. mahaleb* were used in RT-PCR and ELISA tests.

### Plant total RNA extraction with silica

Total RNA was recovered according to study made by Foissac et al. (2000) with slight modifications. 100mg of leaf, root, bark and green bark tissue was homogenized in 1 ml of grinding buffer (Annex 1) in a sterile mortar. Tissue extracts were prepared from leaf, root, bark and green bark tissues by serially diluting (1:10, 1:40, 1:80: 1:160 1:320, 1:640, 1:1280, 1:2560 and 1:5120). Aliquots of 500 µl of diluted extracts were then mixed with 100 µl of 10% sodium lauryl sarcosyl solution in a new set of sterile eppendorf. Tubes were incubated at 70°C with intermittent shaking for 10 min and incubated in ice for 5 min. After centrifugation at 14000 rpm for 10 min, 300 µl of the supernatant was transferred to a new eppendorf set containing 150 µl of ethanol, 25 µl of resuspended silica (Annex 2) and 300 µl of 6 M sodium iodide. The mixture was then incubated at room temperature for 10 min with intermittent shaking. After centrifugation at 6000 rpm for 1 min, supernatant discarded and the pellet washed twice with washing buffer (Annex 1). The pellet then was resuspanded with 150 µl of RNase-free water and incubated for 4 min at 70°C followed by a centrifugation at 14000 rpm for 3 min. Finally the supernatant was transferred to a new eppendorf set and stored at –20°C.

### Synthesis of complementary DNA (cDNA)

Purified RNA from diluted extracts was used as template to generate single stranded cDNA. For this purpose SuperScript™ II Rnase H⁻ Reverse Transcriptase kit was used (Invitrogen, Cat. No:18064-022). To a nuclease free microcentrifuge tube 2µl of template RNA, 1µl (100pmol/µl) of each specific primer and 1µl of 10mM dNTP (10mM each dATP, dGTP, dCTP and dTTP) were added and completed to 12 µl with sterile distilled water. The mixture than heated to 65°C for 5 min and quickly chilled on ice. Than 4µl of 5X first strand buffer (supplied with the kit), 2µl of 0.1 M DDT (supplied with the kit) and 1µl of RNaseOUT (40 units/µl) were added and mixed gently and spined briefly. Mix contents of the tube incubated at 42°C for 2 min and 1µl of SuperScript™ II Rnase H⁻ Reverse Transcriptase was added and incubated at 42°C for 50 min. For the inactivation of reverse transcriptase (RT) the mixture was than heated to 70°C for 15 min. To remove RNA complementary to the cDNA 1µl (2 units) of *E. coli* RNaseH (Invitrogen Cat. no: 18021-014) was added and incubated 37°C for 20 min. Synthesized cDNA conserved at –20°C and used as a template for PCR amplification.

### Primers

Oligonucleotide primer sequences reported by Spiegel et al., 1999 were used to detect PNRSV. Primer I: 5'-TCACTCTAGATCTCAAGCAG-3', Primer II: 5'-GAGCTCTGGTCCCACTCAGG-3'. The amplified fragment was in length of 616 bp. The sequence of reverse complement primer (I) was used for viral cDNA synthesis. Another primer set reported by Rosner et al. (1998) was used only for confirmation of viral infection in infected *P. mahaleb*. Primer a: 5'-TCACTCTAGATCTCAAGCAG-3' Primer b: 5'-CGTTTTTCTTTCTTTCTTCC-3' amplifying 785 bp fragment. Both primer set were synthesized by Termo Hybaid (Thermo BioScience GmbH, Germany).

### PCR amplification of cDNA

PCR amplifications were performed as described by Spiegel et al. (1999). One microliter of cDNA was mixed with 24µl of the amplification mixture containing 2.5 µl of the 10X reaction buffer (200mM Tris-HCL pH:8.4, 500mM KCl) 1.5µl of $MgCl_2$ (25 mM), 0.5µl dNTPs (10mM), 0.5µl of each primer (100 pmol/µl), 0.2µl of *Taq* DNA polymerase (Promega Corporation, Madison USA, Cat. no: M1661) and 17.8µl of RNase free sterile water. Initial denaturation was at 94°C for 3 min and followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, 72°C for 1 min and a final extension at 72°C for 5 min. Ten micro liter of amplified PCR product was analyzed by agarose gel electrophoresis and ethidium bromide staining.

### ELISA test

DAS-ELISA was carried out as described originally by Clark and Adams (1977). The diagnostic reagents were purchased from AGDIA and used as indicated by the manufacturer. Polystyrene plate was coated with IgGs

diluted in coating buffer (pH: 9.6) and incubated at 37 °C for 4h. The plate then washed three times with washing buffer for 3 min intervals. Dilution series were prepared from 1:10 to 1:1280. Samples were loaded duplicate wells of polystyrene microtitre plate. After loading the diluted extracts of 100 µl quantity the plate was incubated overnight at 4 °C. Following washing, 100 µl of conjugated antibodies were added to each well and the plate was incubated at 37 °C for 4h. After 3 additional washing freshly prepared p-nitrophenylphosphate in substrate buffer (1mg/ml) were loaded to each well. The plate was incubated at room temperature and photometric measurement was done at 405 nm after 2h. Samples were considered as positive if their absorbance values were more than 2.5 times of negative control. ELISA test was carried out with four repetition including positive and negative controls.

## Results

### Detection of PNRSV by DAS-ELISA

The sensitivity of ELISA for the detection of PNRSV was assayed using serially diluted infected and healthy leaf samples of *P. mahaleb*. The absorbance values are presented as a graph in Figure 1.

As seen in graph the most significant positive was obtained at the dilution of 1/10. At the dilutions following 1/10, the positive results were in decreasing trend. The last positive response was obtained at the dilution point of 1/80 after two hours reading and determined as detection limit of DAS-ELISA kit. In the dilution of 1/160 the absorbance value was near about the positive sample threshold, which is 2.5 times of negative control value and considered as suspected. In the dilution of 1/320 extremely low signal was recorded and considered as unreliable.

### Total RNA extraction and PCR amplification

The protocol utilized to extract RNA from systemically infected *P. mahaleb* enabled to extraction of 36µg/ml, 23µg/ml, 18µg/ml and 34µg/ml of ssRNA from infected leaf, green bark, bark and root tissues respectively. The presence of the virus was confirmed by using two sets of primers designed for the detection of PNRSV (Fig. 2). Only one set of primer amplifying 616bp fragments reported by Spiegel et al. (1999) was used in comparative studies.

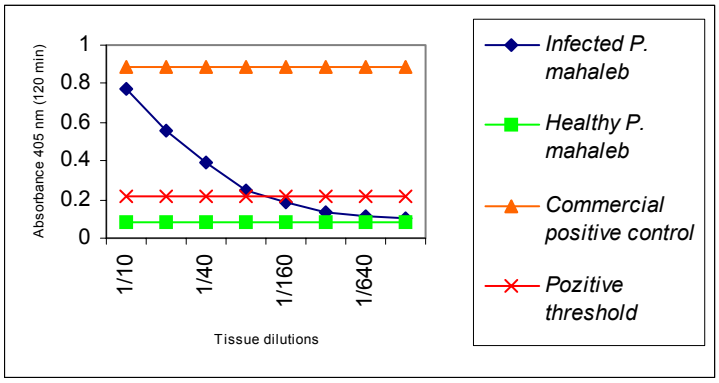


Figure 1. The graph shows the absorbans values of extracted leaf samples tested by ELISA. Each point indicates the mean of four repetitions. As seen above it was possible to have positive signal up to dilution limit of 1/80 after 2h reading. The dilutions following 1/80 gave suspected and unreliable results after 2h reading by ELISA

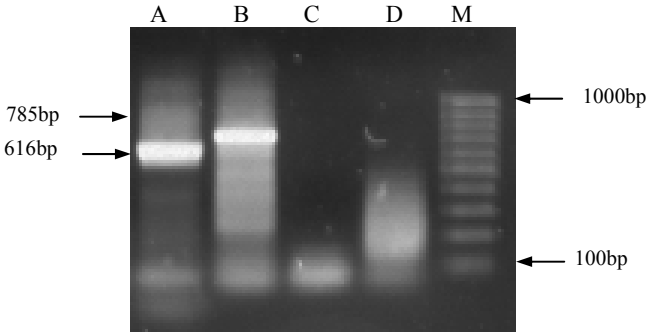


Figure 2. The presence of PNRSV isolate was confirmed by using two sets of primer. Lane A shows the DNA band of expected size of a 616 bp amplified with the first set of primer reported by Spiegel et al., 1999, Lane B shows another DNA band of expected size of 785 bp obtained by using the second set of primer reported by Rosner et al., 1998, Lane C is water control, Lane D is healthy control and Lane M is 100bp DNA ladder

RT-PCR amplification of RNA extracted from leaf, root, bark and green bark tissue of infected *P. mahleb*, using PNRSV specific primers, yielded a specific DNA band of about 616 base pairs.

Practically it was possible to detect virus from all tissues. PCR amplification of tested tissues gave a unique amplified DNA fragment of expected size. In general, RT-PCR of leaf sample yielded very high amounts of the anticipated product (Figure 3a), whereas root, bark and green bark tissue of green shoot gave lover amounts of amplified product (Figure 3b, Figure 4a and b).

The detection limit of techniques was recorded as the dilution of 1/2560 by RT-PCR and 1/80 by ELISA after 2 hours reading.

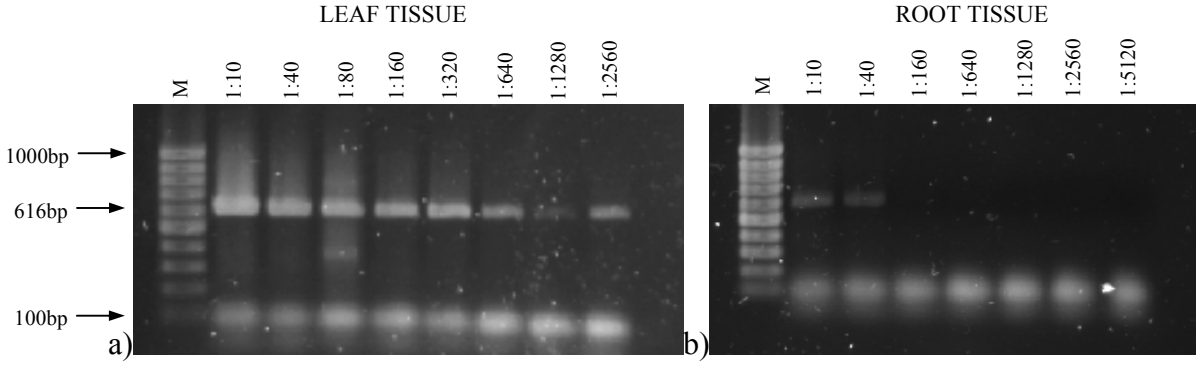


Figure 3. Ethidium bromide stained agarose gel of PNRSV specific PCR products. Homogenized leaf and root tissues were serially diluted before total nucleic acid extraction a) The picture shows the PCR amplification limit of leaf tissue of systemically infected *P. mahaleb*, b) PCR amplification limit of root tissue, M is 100bp ladder

The leaf tissue was found to be best tissue in detecting PNRSV from different tissues of infected plant. By using leaf extract it was possible to detect PNRSV up to dilution of 1/2560 (Figure 3a). When using green bark tissue, the detection limit was determined as 1/640. Root and bark tissues were gave equal amplified yield in RT-PCR test (Figure 3b and 4b). The detection limit was determined as 1/40 for both tissues.

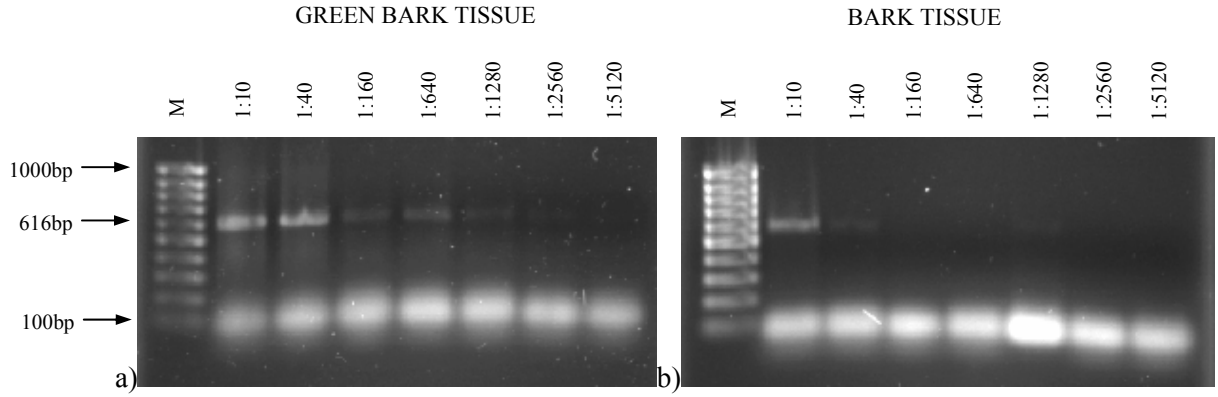


Figure 4. Homogenized bark, and green bark tissue of young shoots were serially diluted before total nucleic acid extraction and then submitted to RT-PCR reaction a) The picture shows the PCR amplification limit of green bark tissue of systemically infected *P. mahaleb*, b) PCR amplification limit of bark tissue of trunk, M is 100bp ladder

**Discussion**

Diagnostic methods used to detect plant pathogens should be reliable, and possess both high specificity and sensitivity. This is a special significance when mother plants are marked as "virus tested" in commercial nurseries (Mekuria et al., 2003), which will serve as source for subsequent vegetative propagation.

The detection limit of ELISA test by using AGDIA kit was determined as 1/80 after two hours reading. The result suggests when fresh green material is used, the technique is quite reliable in detecting PNRSV. Heleguera et al., 2001 reported that samples, collected in summer from systemically infected plants, showed a positive result for PNRSV with IC-RT-Nested–PCR and RT-PCR but produced negative result with ELISA.

When using the leaf tissue the detection limit of RT-PCR was recorded as 1/2560, which indicates more diluted samples respect to ELISA test, can be safely detected. PCR method was approved to be more effective and sensitive than ELISA test in detecting PNRSV from leaf extracts. According to the result obtained when high sensitivity and specificity is needed RT-PCR technique can serve more reliable results. This result is in agreement with previous works. Helegurea et al. (2001) obtained similar results. They compared PCR with DAS-ELISA in detecting PNRSV and Prune dwarf virus (PDV). According to study when systemically leaf samples were used PNRSV was detectable up to 1:390.000 for leaf and 1:10.000 for buds by IC-RT-Nested-PCR. They also reported that the same values were consistently reproducible when repeating the tests. This result is also in accordance with work conducted by Navarro et al. (1998). They reported that when short and simple sample processing method is used detection limit of RT-PCR was quite higher than DAS-ELISA.

The leaf tissue was found to be best tissue in detecting PNRSV from infected plant. By using leaf extract it was possible to detect PNRSV up to dilution of 1/2560. When using green bark tissue, the detection limit was determined as 1/640, which is less reliable comparing leaf extracts. Root and bark tissues gave equal amplified yield in RT-PCR test (Fig. 3b and 4b). The detection limit was determined as 1/40 for both tissues. Root and bark tissues found to be less reliable tissues in detecting virus respect to leaf and green bark tissues. These results suggest that the virus is distributed throughout actively growing woody host but more concentrated in green tissues.

**Acknowledgement**

The study was supported by a grant from The Research Fund of Yüzüncü Yil University–YYUBAP (Project no: ZF-2002-46) and The Scientific and Technical Research Council of Turkey-TUBITAK (Project number: TOGTAG-2990). The authors acknowledge YYUBAP and TUBITAK for their financial supports.

**References**

Boom, R., Sol, C.J.A., Salimans, M.M.M., Jansen, C.L., Wertheim-Van Dillen, P.M.E., Van Der Nordaa. J., 1990. Rapid and simple method for purification of nucleic acids. ***J. Clin. Microbiol.*** 28:495-503.

Clark, M.F., Adams, A.N., 1977. Characteristics of the microplate method of enzyme linked immunosorbent assay for the detection of plant viruses. ***Journal of General Virology,*** 34, 475-483.

Foissac X., Svanella-Dumas, L., Gentit, P., Dulucq, M.J., Candresse, T., 2000. Polyvalent detection of fruit tree Tricho, Capilo and Foveaviruses by nested RT-PCR using degenerated and inosine containing primers (DOP RT-PCR). ***Acta Horticulture,*** 550: 37-43.

Francki, R.I.B., 1985. ***The Plant Viruses. Polyhedral Virions with Tripartite Genomes.*** No. 1, Plenum Press, New York,. 309 pp.

Gella, R.F., 1980. Research note on the diffusion on Ilarvirus in a collection of varieties of *Prunus domestica* L. Acta Phytopathologica, Acad. Scient. ***Hung.,*** 11.351-354.

Gilmer, R., Way, R., 1961. Pollen transmission of Necrotic ringspot and Prune dwarf viruses in sour cherry. Proc. 4th Symp. Virus diseases and Fruit Trees in Europe. ***Tidsski. Planteueaul,*** 65, 111-117.

Heleguera P.R., Taborda, R., Dacampo, D.M., Ducasse, D.A. 2001. Immunocapture reverse transcription-polymerase chain reaction combined with nested PCR greatly increases the detection of Prunus necrotic ringspot virus in peach. ***Journal of Virological Methods,*** 95:93-100.

Marenaud, C., Llacer. G., 1976. Etude de la diffusion de virus de type ILAR (taches annularies necrotiques) dans un verger de ceriser (*Prunus avium*). ***Ann. Amèlior. Plantes,*** 26 (2), 357-363.

Mekuria, G., Rhames, S.A., Albersts, E., Bertozzi, T., Wirthensohn, M., Collins, G., Sedgley, M., 2003. Comparison of ELISA and RT-PCR for detection of Prunus necrotic ringspot virus and Prune dwarf virus in almond (*Prunus dulcis*). ***Journal of Virological Methods***, 114: 65-69.

Mink, G.I., 1992. ***Prunus Necrotic Ringspot Virus***. Plant Diseases of International Importance, Volume III, Disease of Fruit Tree Crops, pp. 335-356.

Mink, G.I., Aichele, M.D., 1984. Detection of Prunus necrotic ringspot virus and Prune dwarf virus in Prunus seed and seedlings by enzyme-linked immunosorbent assay, ***Plant Dis.,*** 68,378.

Navarro, S.J.A., Aporicio, F., Rowhani, A., Pallás, V., 1998. Comparative analysis of ELISA, non radioactive moleculer hybridizasyon and PCR for the detection of Prunus necrotic ringspot virus in herbaceous and Prunus hosts. ***Plant Pathology,*** 47:780-786.

Nemeth, M., 1986. ***Virus Mycoplasma and Rickettsia Diseases of Fruit Trees.*** Akademiai Kiado. Budapest, Hungary, 840 pp.

Nyland, G., Gilmer, R.M., Moore, J.D., 1976. Fruit disease and noninfectious disorders of stone fruits in North America. U.S.D.A. Agric. ***Handbk.,*** 437:104-132. Governement Printing Office, Washington, DC.

Rosner, A., Shiboleth, Y., Spiegel, S., Krisbai, L., Kölber, M., 1998. Evaluating The Use of İmmunocapture and Sap-Dilution PCR for The Detection of Prunus necrotic ringspot virus. ***Acta Horticulture,*** 472 .17 th Int.Symp.on Fruit Virus Diseases.

Scott, S.W., Bowman- Vance, V., Bachman, E.J., 1992. The use of nucleic acid probes for the detection of

prunus necrotic ringspot virus and prune dwarf virus. ***Acta Hortic*****,** 309:79-83

Spiegel, S., Tam,Y., Maslenin, L., Kolber, M., Nemeth, M., Rosner, A., 1999. Typing prunus necrotic ringspot virus isolates by serology and restriction endonuclease analysis of PCR products. ***Annals of Applied Biology***, 135 (1): 395-400.

Stain, A., Leavy, S., Lobenstein, G., 1987. Detection of prunus necrotic ringspot virus in several rosaceous hosts by enzyme-linked immunosorbent assay. ***Plant Pathology,*** 36:1-4.

Uyemoto, J.K., Luhn, C.F., Asai, W.K., Beede, K, Beutel, J.A., Fenton, R., 1989. Incidence of Ilarviruses in young peach trees in California. ***Plant Disease.,*** 73: 217-220.

Vunsh, R., Rosner, A., Stain, A., 1990. The use of polymerase chain reaction (PCR) for the detection of bean yellow mosaic virus in Gladiolus. ***Ann App. Biol.,*** 117:561-569.

**Annex 1.** Buffers used in total RNA extraction (Boom et al., 1990; Foissac et al., 2000)

| Grinding buffer | Concentration | Quantity for 10-50 ml |
|---|---|---|
| Guanidine thiocyanate | 4.0 M | 23.64 g / 50 ml |
| NaOAc | 0.2 M | 1.36 g / 50 ml |
| EDTA | 25 mM | 0.465 g / 50 ml |
| KOAc | 1.0 M | 4.9 g / 50 ml |
| PVP-40 | 2.5% wt / vol | 1.25 g / 50 ml |
| 2-ME (add just before use) | 1% vol / vol | |
| or can be replaced by | | |
| sodium metabisulfite | 1% wt /vol | |
| Washing buffer (4°C) | | |
| Tris-HCl, pH 7.5 (1 M) | 10.0 mM | 100 µl / 10 ml |
| EDTA (5 M) | 0.5 mM | 100 µl / 10 ml |
| NaCl (0.5 M) | 50.0 mM | 10 µl / 10 ml |
| ETOH | 50% | 5 ml / 10 ml |
| NaI solution (6 M) | | |
| $Na_2CO_3$ | | 0.75 g / 50 ml |
| NaI | | 36 g/ 50 ml |
| Stir until completely dissolved and store in dark bottle at 4°C. | | |

**Annex 2.** Preparation of silica suspension

In a measuring cylinder, add 60 g silica particles (Sigma 12% S5631) to 500 ml $H_2O$. Mix and let settle for 24 hours. Discard the upper 470 ml supernatant (90% of the supernatant) and add $H_2O$ to 500 ml and mix well. Let settle 5 hours and discard 440 ml (85% of the supernatant). Adjust the remaining 60 ml slurry to a pH of 2.0 with HCl. Autoclave and store in dark bottle at room temperature.