

## SEROLOGICAL, BIOLOGICAL AND MOLECULAR DETECTION OF *Prunus necrotic ringspot virus* ON *Rosa damascena* Mill. IN TURKEY

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### ABSTRACT

This study was carried out during 2012 in oil rose (*Rosa damascena* Mill.) production areas in Burdur province of Turkey. A total of 102 oil rose leaf samples showing virus-like symptoms were collected from 8 different locations. All oil rose leaf samples were tested for *Prunus necrotic ringspot virus* (PNRSV) using commercially available DAS-ELISA kit with negative and positive control. 16 samples of 102 were found to be infected with PNRSV (16%). DAS-ELISA positive samples were later inoculated onto *Cucumis sativus* L. cv. Cemre F1, *Chenopodium quinoa* Wild. and *Catharanthus roseus* L.G. Don. Inoculation with extracts from PNRSV-positive plants produced chlorotic local lesions on *Chenopodium quinoa* Wild. The symptoms was not observed on *Cucumis sativus* L. cv. Cemre F1 and *Catharanthus roseus* L.G. Don. Total RNA was extracted from leaves of oil rose samples which were positive in the DAS-ELISA and PNRSV- inoculated *Chenopodium quinoa* Wild. plants. Total RNA was isolated from EZ-10 spin column plant total RNA minipreps kit (Bio Basic, Canada Inc). Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was performed using PNRSV specific primers as described by Rosner (15). The expected fragment size of 785 bp was observed after electrophoresis of PCR products in 1% agarose gel. RNA isolated from healthy *Chenopodium quinoa* Wild. was used as a negative control. No PCR products were amplified from this sample.

**Key words:** *Rosa damascena* Mill., PNRSV, DAS-ELISA, RT-PCR

### INTRODUCTION

The *Rosa* genus belongs to the family *Rosaceae* and has over 130 species [Cairns 2001]. In the world many types of rose are available, but very few of them can be evaluated in perfume and cosmetics industry. The most important type of rose which has been cultivated in order to extract oil and which has an economical value is the *Rosa damascena* Mill. which is also known as such as 'Isparta rose', 'Kazanlik rose', 'Damascus rose', 'Pink oil rose' or only with 'Oil rose' [Timor 2011].

Turkey is the biggest rose oil producer in the world. Current demand for rose oil in the world is supplied by countries such Turkey (50 %), Bulgaria (40 %), and the remaining 10% is supplied by Iran, India, Morocco, Egypt, France, China and India and Afghanistan [Örmeci-Kart et al. 2012; Demirözer et al. 2009]. Kashan, Shiraz, Fars, Meshed and Azerbaijan are the major cultivation areas in Iran; Isparta, Burdur, Denizli, Afyonkarahisar in Turkey and the Kazanlak valley in

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Bulgaria [Ginova et al. 2012]. Burdur and Afyon provide approximately 20% of the total oil rose and 15% of the total rose oil production in Turkey. Recently, rose production is done in 4.096 decares in Burdur, particularly in central and Ağlasun districts [Timor 2011].

Viral diseases are the main problems in oil rose growing areas and limit the production of rose oil. All species and varieties of rose are susceptible to one or more viral diseases. Many viral or suspected viral diseases can affect roses [Yardimci and Çulal 2009]. Viruses that infect rose belong mainly to the genera *Ilarvirus* and *Nepovirus*. Among the *Ilarvirus*-es, *Prunus necrotic ringspot virus* (PNRSV), *Apple mosaic virus* (ApMV), and *Tobacco streak virus* (TSV); and among the *Nepovirus*-es, *Arabidopsis mosaic virus* (ArMV), *Strawberry latent ringspot virus* (SLRSV), *Tobacco ringspot virus* (TRSV), and *Tomato ringspot virus* (TomRSV) have been isolated in many rose growing regions worldwide [Moury et al. 2001]. The most common is rose mosaic caused by either ApMV (also called *Rose mosaic virus*) and/or PNRSV [Manners 1997].

*Prunus necrotic ringspot virus* a member of the genus *Ilarvirus* in the family *Bromoviridae*, occurs worldwide and is a serious pathogen of many plant species, including rose, *Prunus* spp. [Cambrá et al. 1982, Sertkaya 2010]. PNRSV transmission by seed, pollen, on cutting implements and by root grafting from infected plants to healthy plants has been reported and the results showed that root grafting is involved in the natural spread of the virus in roses [Golino et al. 2005]. The symptoms of disease include color breaking, necrotic ringspots on leaves, bud failure, and streaked petals [Moran et al. 1988].

Oil-rose viruses were detected in Isparta and Samsun provinces of the country [Erdiller et al. 1995, Yardimci and Çulal 2009] however this is the first time that it was determined of PNRSV on oil rose growing areas in Burdur provinces, Turkey. This paper describes, the identification of PNRSV on oil rose plants by symptomatology, mechanical inoculation, serology, and molecular techniques.

## MATERIAL AND METHODS

### Surveys and sample collection

A total of 102 oil rose leaf samples showing virus-like symptoms were collected from 8 different locations (tab. 1). During surveys, photographs of typical virus and virus-like symptoms were taken (figs 1, 2). Leaf samples, from each plant put into plastic bags and labeled. The samples were brought to the laboratory by placing on ice and stored at -20°C until tested.

### Double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA)

DAS-ELISA tests were carried out as described by Clark and Adams [1977] and standard procedure of the antibody's manufacturer (Bioreba, Switzerland). Polystyrene plates (Nunc Maxisorp) were first coated with virus-specific polyclonal antibody diluted in carbonate buffer (pH: 9.6) and incubated at 4°C overnight. After washing the plates with PBST buffer, samples ground in sample extraction buffer (1 g tissue/10 mL buffer) were added to wells in duplicate and incubated at 4°C overnight. Alkaline phosphatase conjugated antibody diluted in conjugate buffer was coated after washing the plates and incubated for 4 h at 30°C. Finally, p-nitrophenyl phosphate in diethanolamine substrate buffer; (pH: 9.8) was added and 405 nm values were measured at a microplate reader VersaMax microplate reader (Molecular Devices, Sunnyvale, CA) after 60–120 min. Samples with absorbance values greater than twice the mean absorbance reading of healthy controls obtained from (Bioreba, Switzerland) were considered positive for PNRSV. Positive and negative controls provided with the kits were used.

### Mechanical inoculations

Inoculum from PNRSV-infected oil rose leaves were prepared in a phosphate buffer (0.01 M, pH 7.2, 1 ml per 1 g of leaf material) and were applied to the indicator plants. *C. sativus* L. cv. Cemre F1, *Ch. quinoa* Wild. and *C. roseus* L.G. Don. were used as the indicator plants. The inoculated plants were grown in a greenhouse at 22°C–28°C. The occur-

rence and type of symptoms were observed on inoculated leaves after inoculation (fig. 3). Inoculated plants were tested by RT-PCR.

#### Total RNA extraction

Total RNA was extracted from fresh leaves of oil rose samples which were positive in the DAS-ELISA and PNRSV-inoculated *Ch. quinoa* Wild. plants. RNA isolated from healthy *Ch. quinoa* Wild. was used as a negative control. EZ-10 spin column plant total RNA minipreps kit (Bio Basic, Canada Inc) was used to extract the total RNA from infected plants according to the kit procedure.

The extraction was done following the procedure described by the manufacturer. Oligonucleotide primer sequences reported by Rosner et al. [1998] were used to detect PNRSV. Primer I: 5'-TCACTCTAGATCTCAAGCAG-3' Primer II: 5'-CGTTTTTCTTTCTTTCTTCC-3'. The amplified fragment was in length of 785 bp. The primer set were synthesized by Bio Basic, Canada Inc.

**Table 1.** Numbers of fields surveyed and samples collected in each district/village

District, villages	Total Samples	No. of samples PNRSV positive	No. of samples PNRSV negative
Ağlasun	24	3	21
Çeltikçi	14	0	14
Yazır	10	2	8
Dereköy	6	1	5
Çebişköy	18	2	16
Kibrit	12	1	11
Çamlidere	10	0	10
Yeşilbaşköy	8	7	1
Total	102	16	86

#### RT-PCR

The reverse transcription and polymerase chain reaction amplification was performed using a OneStep RT-PCR Kit (Bio Basic, Canada Inc). Reverse transcription was performed in a 50 µl reaction mixture containing, 21 µl H<sub>2</sub>O, 25 µl 2 × 1 PrimeScript One Step RT-PCR buffer (containing dNTP mixture, One step Enhancer solution), 2 µl

Prime Script 1 step enzyme mix, 1 µl 20 Mm primers. Thermocycling was carried out as follows: 50°C for 30 min, 94°C for 2 min, then 30 cycles of 94°C for 30 second, 55°C for 30 second and 72°C for 1 min., followed by 72°C for 3 min RT-PCR products were analyzed in a 1% agarose gel by electrophoresis and stained in a 0.5 µg/ml ethidium bromide solution. An image was captured after exposing the ethidium-bromide-stained gel on a transilluminator with a digital camera (UVP-Doc-It). DNA markers (100 bp DNA ladder, Fermentas) were used in each electrophoretic run.

#### RESULTS

A total of 102 oil rose leaf samples showing virus-like symptoms were collected from 8 different locations. During surveys, photographs of typical virus and virus-like symptoms were taken. Some of these symptoms photographed during field surveys are presented in (figs 1, 2).



**Fig. 1.** Chlorotic patterns on oil rose

All oil rose leaf samples were tested for PNRSV using commercially available DAS-ELISA kit with negative and positive control. 16 samples of 102 were found to be infected with PNRSV and 86 samples of them gave negative reaction to ELISA (tab. 1). Samples with DAS-ELISA values at least twice those of the healthy control were considered to be positive.

DAS-ELISA positive samples were later inoculated onto *C. sativus* L. cv. Cemre F1, *Ch. quinoa* Wild. and *C. roseus* L.G. Don. Inoculation with extracts from PNRSV-positive plants produced chlorotic local lesions on *Ch. quinoa* Wild (fig. 3) Local lesions were recorded 4 or 5 days after the inoculation. The symptoms was not observed on *C. sativus* L. cv. Cemre F1 and *C. roseus* L.G. Don.

RNA isolated from healthy *Ch. quinoa* Wild. was used as a negative control. A portion of RNA of PNRSV was amplified by RT-PCR with virus specific primers, by using total RNA extracts from symptomatic plants to confirm the presence of PNRSV infection in DAS-ELISA positive samples and the detection of PNRSV in inoculated test plants was confirmed by RT-PCR.



Fig. 2. Mottle symptoms on oil rose

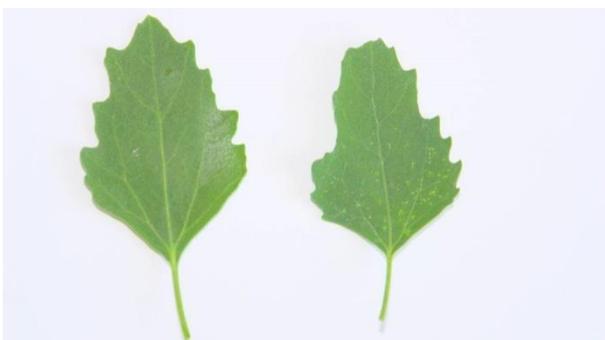


Fig. 3. Right: Chlorotic local lesion on the leaves of *Ch. quinoa* Wild inoculated with PNRSV; Left: Healthy *Ch. quinoa* Wild.

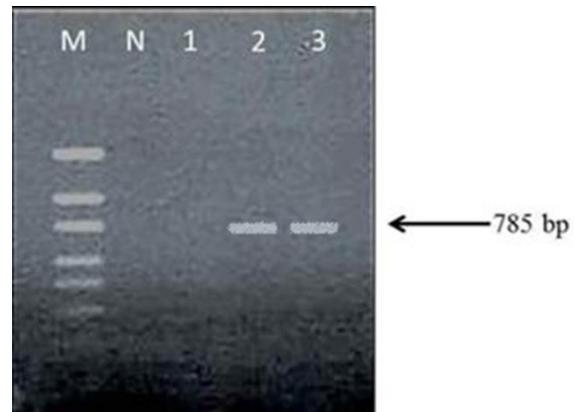


Fig. 4. Detection of PNRSV infection in oil rose plants by RT-PCR. M: Marker: 100 bp DNA ladder (Fermentas); N: Healthy *Ch. quinoa* Wild; 1. H<sub>2</sub>O control; 2. *Ch. quinoa* Wild inoculated with PNRSV; 3. Oil rose

The expected fragment size of 785 bp was observed after electrophoresis of PCR products in 1% agarose gel (fig. 4). The results of RT-PCR were in complete agreement with DAS-ELISA results. A sample of non-infected *Ch. quinoa* Wild. did not yield visible specific DNA band. Amplification was not observed in sample with H<sub>2</sub>O control, too.

## DISCUSSION

Burdur is the major cultivation area in total rose oil production in Turkey. PNRSV is the most common agent of oil rose and stone fruit tree pathogen. The data presented in this study showed that oil roses were infected with PNRSV in Burdur Province. The rate of incidence of PNRSV in oil rose was found as 15. 68% by DAS-ELISA. PNRSV inoculated on selected test plants (*C. sativus* L. cv. Cemre F1, *Ch. quinoa* Wild. and *C. roseus* L.G. Don.) are reported here. Chlorotic local lesions was observed on *Ch. quinoa* Wild. These symptoms were similar to those that were described previously for PNRSV [Sipahioğlu et al. 2001, Rakhshandehroo et al. 2006, Sertkaya 2010, Paduch-Cichal and Sala-Rejczak 2011].

Rakhshandehroo et al. [2006] found that mixed infections of PNRSV and ArMV were in all rose

samples tested by sap inoculations and ELISA in Iran. However, PNRSV was reported to be mostly distributed through the red rose varieties and ArMV was within the white varieties by serological tests.

Sertkaya [2010] found that according to the results of the Bioassay by sap inoculation and ELISA on symptomatic rose plants, the causal agent of rose mosaic disease (RMD) is PNRSV. The viruses affecting rose plants spread through cuttings from a diseased plant because new plants are generally produced by the rooting of cuttings in home gardens in Hatay province.

A survey for viruses in rose propagated in Europe resulted in the detection of only PNRSV among seven viruses screened by Moury et al. [2001].

In the survey conducted in Isparta Province of Turkey for viruses infecting oil rose, ArMV, PNRSV and ApMV were detected by ELISA. In Isparta, ArMV was found in the largest number of plants (51.8%), followed by PNRSV (35.7%), ApMV (17.8%) [Yardimci and Çulal 2009].

Methods based on the RT-PCR technique using specific primers allowed to obtain amplicons of 785 bp for PNRSV. Similar results were obtained by other authors using RT-PCR for the detection of PNRSV [Rosner et al. 1998, Verma et al. 2002, Usta et al. 2005].

In this study, the presence of PNRSV on oil rose in Burdur-Turkey were confirmed by symptomatology, mechanical inoculation on test plants, DAS-ELISA and RT-PCR methods. The present study has indicated for the first time, the presence of PNRSV in oil rose plants in Burdur province where the major cultivation area in Turkey.

## CONCLUSIONS

PNRSV is transmitted by infected buds, scions and by root stocks. The use of virus-free oil-rose plants is important to improve productivity in oil rose areas. Molecular techniques such as RT-PCR using specific primers were proven to be useful for the diagnosis and control of the disease and will also be beneficial for resistance breeding, epidemiological investigations and plant virus collections. The samples testing positive by this method may be further

examined by other confirmatory technique and phylogenetic relatedness between the different isolates of PNRSV.

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