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Seasonal variation of *Prunus necrotic ringspot virus* concentration in almond, peach, and plum cultivars

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Summary. Levels of *Prunus necrotic ringspot virus* (PNRSV) infection in almond, peach, and plum cultivars over the course of an entire year were determined by testing different plant parts of naturally infected trees, using the double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA). The data showed that spring was the best time of year for PNRSV detection in flowers, active growing buds, and young leaves. PNRSV detection was less reliable during the summer months. Young leaves of all cultivars were the most reliable source for distinguishing between healthy and infected plants, while flowers and buds yielded high values in some cultivars but not in others. Seasonal fluctuations in virus concentration did not follow the same pattern in all cultivars. It is therefore impossible to distinguish between infected and healthy trees on the basis of one single sampling time for all cultivars.

Key words: infection level, PNRSV, ELISA.

Introduction

Prunus necrotic ringspot virus (PNRSV) is a member of the genus *Ilarvirus* in the family *Bromoviridae* with a tripartite genome (Van Regenmortel *et al*., 2000). It is the most widespread and economically important virus affecting *Prunus* species (Nemeth, 1986). It has number of different strains that cause diverse symptoms on many hosts (Mink and Aichele, 1984a, 1984b). All species and cultivars of *Prunus* appear to be susceptible to one or more strains of this virus (Fulton, 1970; Nemeth, 1986). The virus causes serious diseases in nurseries and orchards resulting in reduced tree growth and yield (Pusey and Yadava, 1991). PNRSV is transmitted through seeds and pollen and is also distributed in propagating material (Fulton, 1970).

In many countries PNRSV infections are not permitted in nursery propagating material and the detection and identification of this virus is of prime importance, particularly in regard to the testing of nursery material, propagation stock, stone fruit registration and certification, and also in importation programs. In addition to indexing performed by inoculation on woody indicators, serological assays and molecular diagnostic techniques have also been employed for the detection of PNRSV (Torrance and Dolby, 1984; Stein *et al*., 1987; Rowhani *et al*., 1995; Spiegel *et al*., 1996; Rosner *et al*., 1997), leading to significant improvements in detection.

Enzyme-linked immunosorbent assay (ELISA) is now the method of choice in the routine detection of PNRSV (Barbara *et al*., 1978; Mink, 1980; McMorran and Cameron, 1983; Mink and Aichele, 1984a, 1984b; Torrance and Dolby, 1984; Luhn and

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Uyemoto, 1988). In order to use ELISA effectively, the virus titer in different plant tissues during the growing and dormant seasons must be known. The purpose of this study was to determine fluctuations in PNRSV in the course of an entire year by repeated assaying of different host tissues with the ELISA technique, and to ascertain what type of tissue gives the most reliable detection of PNRSV.

Materials and methods

Virus sources and sampling methodology

Virus titer changes for PNRSV were studied in the following *Prunus* species and cultivars: almond *(Prunus dulcis)* cv. Doma, Frinces, Ne Plus Ultra, S.F.121, Texas, and Tuono; peach *(P. persica)* cv. Babygold, Elberta, and Red Haven; and plum *(P. salicina)* cv. Angeleno. All trees grew in an open field in the Al-Hassan Station (Tafila, Jordan) and were inspected for virus symptoms on the leaves and bark.

Five trees per cultivar of the same age, which had tested positive for PNRSV by DAS-ELISA, were selected and labeled. Two virus-free trees per cultivar were selected as the negative controls. To determine virus titer fluctuations in different tree organs and at different times of year, samples were collected from designated trees on a monthly basis starting in April 2001 and continuing until March 2002. Samples were collected from all four quadrants of each tree depending on the avaibility of the tree organ at the collection date. Samples collected in the spring were from flowers, new young leaves, and roots. Those collected in the summer from mature leaves, fruits, and roots; and those in fall and winter from dormant buds and roots. Samples from the four quadrants of the same organ were mixed together and used as a single sample.

DAS-ELISA

Samples were tested for PNRSV by DAS-ELISA using the ELISA diagnostic kit supplied by Bioreba (Bioreba AG, Reinach, Switzerland) and following the manufacturer's instructions.

Samples were prepared by grinding tissue in a mortar and pestle at 1:10 (w:v) in extraction buffer (Tris-buffer pH 7.4, containing 0.8% [w:v] NaCl, 0.02% [w:v] KCl, 0.05% [v:v] Tween-20, and 2% polyvinylpyrrolidone [PVP] MW 20,000 [Sigma Chemical Co., St. Louis, MO, USA, P-8136]).

Wells of polystyrene microtiter plates (Immulon, Dynatech, Chantilly, VA, USA) were coated with purified immunoglobulin G (IgG) diluted (1:1000) in coating buffer (0.05 M carbonate buffer, pH 9.6) and incubated for 4 h at 30ºC. Wells were washed three times with washing buffer PBS-T (0.01 M phosphate buffer saline pH 7.4, containing 0.8% [w:v] NaCl, 0.02% [w:v] KC1, and 0.05% [v:v] Tween-20). The sample extracts were then placed in each well and stored overnight at 4ºC. Two wells were prepared each time from each sample. Wells were washed again as described above, and a solution of alkaline phosphatase-conjugated IgG diluted (1:1000) in conjugate buffer (Tris-buffer pH 7.4, containing 0.8% [w:v] NaCl, 0.02% [w:v] $MgCl_2$, 0.02% [w:v] KCl, 0.05% [v:v] Tween-20, 2% [w:v] PVP, and 0.2% [w:v] eggalbumin [Sigma Chemical, A-5235]) was placed in each well and incubated at 30ºC for 5 h. Lastly, *p*-nitrophenyl phosphate substrate (Sigma Chemical, N-2765) was added to plates at a concentration of 1 mg ml^{-1} in 10% diethanolamine, pH 9.8. Plates were incubated for 60–90 min at room temperature. Absorbance values at 405 nm (A_{405nm}) were recorded using a Denley WellScan ELISA reader (Denley Instruments Limited, West Sussex, UK).

All plant extracts, antibody solutions, and substrate were used at 100 µl per well. All samples were tested in two replicate wells. Absorbance readings from replicate wells were averaged. Standard negative and positive controls (Bioreba AG, Reinach, Switzerland) were included in each plate to verify the assay performance. ELISA reactions with an absorbance reading equal to or greater than two times that of the healthy controls of the same cultivar and with a visually detectable yellow color were rated positive.

Results

During the test period, PNRSV was detected in the leaves, fruits, buds and flowers, and from the roots of almond and peach trees. In plum trees the virus was detected from all tree parts except from the fruits (Table 1).

Leaves from almond, peach, and plum trees from March through April were the most reliable organ for PNRSV detection. The virus titer declined in the leaves, reaching a non-detectable level by the end of the growing season. The highest A_{405nm}

Table 1. Average absorbance values (A_{405nm}) of plant tissues (leaves, fruits, roots, buds or flowers) from PNRSV-infected almond, peach, and plum trees tested in Tafila (Jordan) during 2001–2002.

Sampling time		Average absorbance values		
Year	Month	Almond	Peach	Plum
2001	April	2.36±0.411 (leaves)[a] 0.12±0.006 [b]	2.47±0.044 (leaves) 0.15±0.014	1.27±0.062 (leaves) 0.09±0.019
	May	0.44±0.170 (fruits) 0.09±0.007	0.74±0.136 (fruits) 0.120±0.007	1.78±0.078 (leaves) 0.07±0.006
	June	0.82±0.326 (fruits) 0.06±0.010	1.98±0.226 (fruits) 0.10±0.006	0.40±0.086 (leaves) 0.06±0.004
	July	0.53±0.199 (fruits) 0.072±0.007	1.77±0.236 (fruits) 0.05±0.006	0.35±0.022 (roots) 0.03±0.003
	August	0.15±0.070 (roots) 0.01±0.004	0.53±0.355 (fruits) 0.04±0.004	0.13±0.030 (roots) 0.03±0.002
	September	0.11±0.007 (roots) 0.03±0.004	0.15±0.029 (roots) 0.04±0.005	0.17±0.023 (roots) 0.05±0.016
	October	0.22±0.114 (roots) 0.03±0.003	0.13±0.012 (roots) 0.02±0.003	0.16±0.049 (roots) 0.04±0.017
	November	0.14±0.010 (roots) 0.05±0.007	0.14±0.003 (roots) 0.06±0.004	0.14±0.031 (roots) 0.03±0.001
	December	0.25±0.077 (buds) 0.06±0.005	0.21±0.019 (buds) 0.05±0.012	0.12±0.020 (buds) 0.04±0.001
2002	January	0.53±0.114 (buds) 0.06±0.008	0.72±0.299 (buds) 0.08±0.008	0.12±0.016 (buds) 0.02±0.018
	February	0.62±0.201 (flowers) 0.07±0.011	0.88±0.068 (flowers) 0.09±0.011	0.98±0.091 (flowers) 0.08±0.006
	March	0.28±0.112 (leaves) 0.06±0.006	0.55±0.149 (leaves) 0.08±0.005	0.28±0.012 (leaves) 0.064±0.008

[a] Upper line, average absorbance (A_{405nm}) and standard error of ELISA for PNRSV-infected plants.
[b] Lower line, average absorbance (A_{405nm}) and standard error of ELISA for healthy controls.

was obtained in leaf extracts from almond and peach at the beginning of April. The titer dropped rapidly and by the end of May was very low. This drop late in the season may be due to higher field temperatures at that time (Fig. 1). In the fruits of almond and peach the absorbance value of PNRSV decreased as fruits ripened. PNRSV was not detected in plum fruits (Table 1).

The average A_{405nm} in almond indicated that the virus titer was high in all tree organs early in the growing season (from budbreak until the beginning of May). The ELISA value in the leaves dropped rapidly in May (A_{405nm}=0.21). In the summer months the titer dropped further (A_{405nm}=0.113). The highest ELISA value in the fruits was recorded in June, then it dropped rapidly in July. In the roots the highest A_{405nm} value occurred in October, then it dropped in November. In the buds the vi-

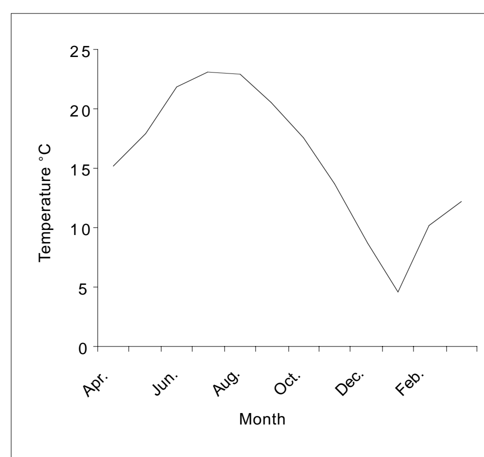

Fig. 1. Average air temperature (°C) during 2001–2002 in the Tafila area.

rus titer began to increase in December when the buds were still dormants and reached its highest value in January. In the flowers, the highest ELISA value was recorded in February. At the beginning of March the virus moved to the newly developed leaves (Table 1).

In peach trees, the average absorbance values indicated that the virus titer was high in leaf tissue during April, followed by a sharp drop in May. Virus titer increased in peach fruits from May through June, then decreased rapidly in August, but it remained constant in the roots in September, October, and November. The highest ELISA value in peach was in January in the dormant buds, and in February in the flowers (Table 1). A sharp increase in the virus titer of leaves of the plum cv. Angeleno was observed in May followed by a rapid decrease in June. The virus titer remained constant from August to January, then increased in February in the flowers (Table 1).

In general, differences between almond, peach and plum in monthly 405 nm absorbance values were slight (Table 1). Leaf samples generally had higher absorbance values than did other organs. In April 2001, ELISA values of positive samples were 2.36, 2.47, and 1.27 for almond, peach, and plum respectively, and 0.12, 0.15, and 0.07 for the healthy controls. The highest absorbance value in plum leaves was recorded in May, whereas in peach and almond it was in April.

ELISA values of the almond cultivars Doma, Frinces, Ne Plus Ultra, S.F.121, Texas, and Tuono showed three patterns. The first pattern was found in the cv. Doma, Frinces, and Tuono, where there was a gradual increase in detectable virus from May to July and from December to January, but with a considerable decrease from August to November (Fig. 2a).

The second pattern was represented by cv. S.F.121 and Texas, which showed an increase in detectable virus titer from May to June, followed by a gradual decrease in July and an increase in February (Fig. 2b).

The third pattern occurred in the cv. Ne Plus Ultra and showed a comparatively high titer throughout the year with increases in July, October, and February (Fig. 2c).

In peach cultivars, the absorbance profiles showed two patterns. The first was that of the cv. Baby Gold and Elberta, with a sharp increase from

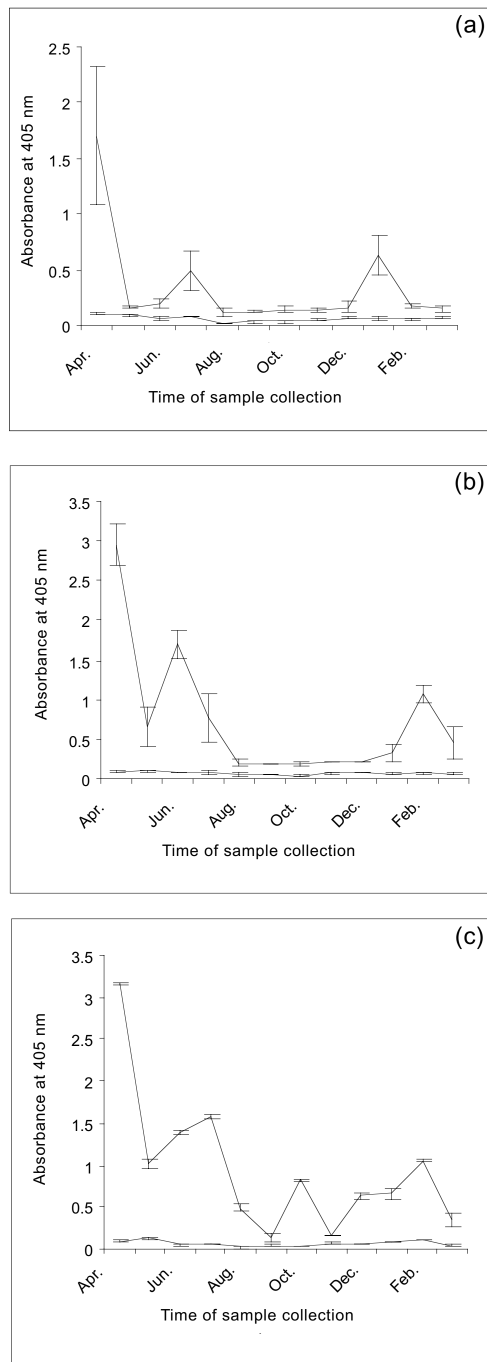

Fig. 2. Average monthly absorbance (A_{405nm}) and standard error values of sap extracted from tissues of almond cultivars: (a) Doma, Frinces, and Tuono; (b) S.F.121 and Texas; and (c) Ne Plus Ultra, infected with *Prunus necrotic ringspot virus* in 2001–2002, in the Tafila area. Baselines are the average monthly A_{405nm} of the healthy controls.

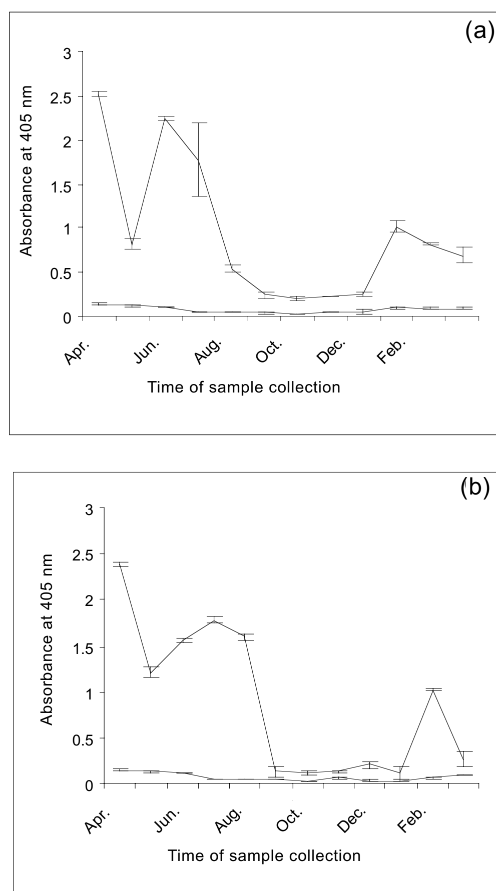

Fig. 3. Average monthly absorbance (A_{405nm}) and standard error values of sap extracted from tissues of peach cultivars: (a) Baby Gold and Elberta; and (b) Red Haven, infected with *Prunus necrotic ringspot virus* in 2001–2002 in the Tafila area. Baselines are the average monthly A_{405nm} of the healthy controls.

May to June and from December to January (Fig. 3a). The second comprised the cv. Red Haven and showed a gradual increase in July, followed by a decrease from August to December, then a sharp increase in February (Fig. 3b).

Discussion

Detection of PNRSV by DAS-ELISA in *Prunus* species was possible throughout the year in extracts of one or more of the following tree organs: leaves, fruits, roots, buds, or flowers. However, the PNRSV titer varied between organs and between recording date, so that is important to choose the particular organ that will give the optimal ELISA reading at a given time of year. Variations in the virus titer of a particular organ in the course of a season was likely the result of organ age and/or environmental conditions. Young leaves from PNRSV-infected plants gave the highest ELISA values from bud break until June. Once the shoot tips stopped growing, the PNRSV titer dropped.

PNRSV concentrations in the leaves of almond, peach, and plum trees were similar to those obtained by previous workers (Clark *et al*., 1976; Barbara, 1978). Therefore, the occurrence of sufficient PNRSV in the leaves for reliable detection seems to depend not only on ambient temperature, as has previously been suggested, but also on the physiological condition of the leaves. Detection was most reliable in young, rapidly growing leaves. Actively growing leaves produced higher ELISA readings, probably because they provided a better environment for PNRSV replication. In addition, actively growing leaves were more succulent and more easily processed in the homogenizer. Dormant or less active leaves did not produce high ELISA values. In large-scale surveys for PNRSV it is therefore recommended to use young leaves in the spring.

Reduced PNRSV detectability after April may be attributed to the rise in temperature during summer which reduced replication of the virus rendering its detection by ELISA more difficult. Similar results were reported by other investigators (Uyemoto *et al*., 1989; Scott *et al*., 1992; Varveri *et al*., 1997). PNRSV was detected in extracts of the pulp of peach fruits and almond at higher levels than in the leaf extracts tested during June. This agreed with Sanchez-Navarro *et al*. (1998) who found that PNRSV concentration was up to 125 times higher in mature peach fruits than in leaves. This implies that whenever possible the fruits of peach and almond trees should be analyzed to guarantee the most accurate detection of the virus.

PNRSV was detected in the flowers during February, therefore this tissue is recommended if testing is necessary in this month. Root samples were not as an appropriate tissue for DAS-ELISA because of the low virus titer. Moreover, root sampling from mature orchard trees is too difficult and time-consuming for routine surveys.

DAS-ELISA of the dormant buds detected PNRSV in all three *Prunus* spp.; this agreed with Opgenorth and Smith (1989) and Dal Zotto *et al*. (1999).

Seasonal variations in PNRSV concentration did not follow an identical pattern in all cultivars of almond and peach. This was probably due to differences in the phenology of the cultivars. As a result it was impossible to distinguish between infected and healthy trees on the basis of one single sampling time for all cultivars. The selection of PNRSV-free plants must therefore be based on material obtained from at least two different phenological stages.

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