Factors affecting the reliability of PDV and PNRSV detection in peach by DAS-ELISA

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Beeinflussende Faktoren für die Verlässlichkeit der Bestimmung von PDV- und PNRSV-Virosen bei Pfirsich mittels DAS-ELISA

1 Introduction

Prunus necrotic ringspot ilarvirus (PNRSV) and *Prune dwarf ilarvirus* (PDV) infect all cultivated *Prunus* species including almond, apricot, sweet and sour cherry, peach and plum (NÉMETH, 1986; DESVIGNES, 1999; RAQUEL et al., 2002). Both viruses are transmitted by mechanical inoculation, by grafting, by seed, by pollen to the seed and by pollen to the pollinated plant (BRUNT et al., 1996). These transmission modes cause rapid spread of both viruses, which are therefore distributed throughout the world (AGRIOS, 1990). PNRSV, alone or together with PDV, can cause severe losses on susceptible varieties of all its hosts (AGRIOS, 1990). Data about damages due to PNRSV infections in nurseries or orchards vary considerably (NÉMETH, 1986). The expression of symptoms depends on climate, virus isolate, host species and cultivar, nutrient supply and the age of the plant at the time of infection (NÉMETH, 1986; VAŠKOVA et al., 2000). For example, PNRSV strain causing Stecklenberg disease reduced the yield of sour cherry up to 91–98 %, whereas yield reductions observed in plum trees infected with PNRSV were negligible (NÉMETH, 1986). DESVIGNES (1999) considers most of the infections with PNRSV to be latent, but he also states that a climatic change can trigger an acute and harmful crisis. PDV causes severe damages in sour cherries, whereas sweet cherries are only slightly affected. Peach is very susceptible to PDV. Less severe strains propagate rapidly and can cause crop losses of 30 to

Zusammenfassung

Um verlässliche serologische Untersuchungen an Steinobstarten in Slowenien zu gewährleisten, wurden verschiedene beeinflussende Faktoren bei der Bestimmung von PDV und PNRSV mittels DAS-ELISA untersucht. An befallenen Bäumen der Sorte cv. Veteran wurden 2003 und 2004 verschiedene Gewebeproben gesammelt und mittels DAS-ELISA auf PDV- und PNRSV Virusbefall untersucht. In Abhängigkeit vom Zeitpunkt der Analyse, den untersuchten Gewebeproben-Arten und dem Virusbefall differierten die mittleren Absorptionswerte und die Ergebnisse auch innerhalb der Einzelanalysen. Beide Virusarten konnten jedoch verlässlich mittels DAS-ELISA in den Winter- und Herbstknospen, in den Blüten oder den jungen Blättern im Frühjahr bestimmt werden. Ein störender Einfluss von hohen Temperaturen auf die Konzentration von PNRSV und PDV in den Blättern konnte beobachtet werden.

Schlagworte: PDV, PNRSV, DAS-ELISA, hohe Temperatur, Pfirsich.

Summary

To ensure reliable serological testing of stone fruits in Slovene growing conditions, several factors influencing the detection of PDV and PNRSV by DAS-ELISA were studied. Various tissues were sampled at different dates in 2003 and 2004 on PDV and PNRSV infected peach trees of cv. Veteran and analysed by DAS-ELISA. Mean absorbance values as well as the variability of results within analysis differed depending on the date of analysis, analysed tissue and virus. Both viruses could be reliably detected by DAS-ELISA in winter and autumn buds and in flowers or young leaves in spring. A deteriorating effect of high temperatures on concentrations of PNRSV and PDV in leaves has been observed.

Key words: PDV, PNRSV, DAS-ELISA, high temperature, peach.

60 %. Severe strains are self-limiting since affected trees usually decline (DESVIGNES, 1999).

Due to their potential harmfulness and rapid spread, yearly testing of propagation stock for the presence of PDV and PNRSV by enzyme-linked-immunosorbent-assay (ELISA) is recommended by the EPPO certification scheme for stone fruits (EPPO, 2001a; EPPO, 2001b). ELISA is the most useful technique for routine diagnosis. It has been used routinely for detection of PNRSV, although this test is most reliable on plant tissues collected in spring (BERTOZZI et al., 2002). The virus appears to be unevenly distributed through the plant and this factor combined with seasonal fluctuations of viral concentrations can cause chronically infected trees to appear healthy when tested by ELISA (MEKURIA et al., 2003). Uneven distribution and fluctuation in viral titre can sometimes also make PDV infections undetectable by ELISA (UYEMOTTO, 1989). Diagnostic techniques based on reverse transcription polymerase chain reaction (RT-PCR) provide detection systems with sensitivity to overcome the above-mentioned difficulties (SAADE et al., 2000).

To provide quick, simple and reliable testing of stone fruits in Slovene growing conditions, especially for the purposes of certification, several factors influencing the detection of PDV and PNRSV by DAS-ELISA were studied. The results are presented in the paper.

2 Material and Methods

2.1 Plant material

To determine the optimal sampling time and the best analyse tissue source for a reliable detection of PDV and PNRSV by DAS-ELISA flowers, leaves, buds, bark and fruits were collected at various dates (Table 1) from the infected peach trees of the variety Veteran (planted in spring 1999).

At least, one sample for each tree was prepared. 10 flowers were collected randomly around the canopy of each tree. One leaf sample was prepared from the middle parts of 10 leaves collected around the tree crown. 4 one-year old shoots were cut from different crown parts of each tree and 2 buds per shoot, t.i. 8 buds were used for extraction. Bark tissue was dissected from each of the four one-year old shoots and cut in pieces. Fruit skin with some fruit flesh was dissected from the ripe fruits. One to two fruits were collected from trees. Each fruit was analysed separately.

Tabelle 1: Analysierte Geweberproben, Datumsangaben der Stichprobennahme und der durchgeführten DAS-ELISA-Tests in dieser Studie

Table 1: Analysed tissues, dates of sampling and dates of completion of DAS-ELISA tests for the analyses performed in the study

Analysis number	Analyse tissue	Date of sampling	Date of DAS-ELISA completion
1	Flowers	Apr. 16th, 2003	Apr. 18th, 2003
2	Young leaves from tips of one-year old shoots	May 26th, 2003	May 30th, 2003
3	Old leaves from the base of one-year old shoots	May 26th, 2003	May 30th, 2003
4	Fully developed leaves from the middle part of one-year old shoots	May 26th, 2003	May 30th, 2003
5	Fully developed leaves from the middle part of one-year old shoots	Aug. 12th, 2003	Aug. 14th 2003
6	Fully developed leaves from the middle part of one-year old shoots	Sep. 30th, 2003	Oct. 3rd , 2003
7	Fully developed leaves from the middle part of one-year old shoots	Oct. 22nd, 2003	Oct. 25th, 2003
8	Fully developed leaves from the middle part of one-year old shoots	Aug. 30th, 2004	Aug. 31st, 2004
9	Dormant buds	Oct. 22nd, 2003	Oct. 25th, 2003
10	Extracts of buds from October 2003, kept on -20°C	Oct. 22nd, 2003	Jan. 10th, 2004
11	Dormant buds	Jan. 8th, 2004	Jan. 10th, 2004
12	Dormant buds	Aug. 30th, 2004	Aug. 31st, 2004
13	Bark tissue from one-year old shoots	Jan. 8th, 2004	Jan. 10th, 2004
14	Fruits	Aug. 12th, 2003	Aug. 14th, 2003

Trees were selected on the basis of the results of the flower sample analyses for PDV and PNRSV that was performed in April 2003. Trees with high absorbance values, indicating a high concentration of both viruses, were selected for further analyses. 7 to 19 PNRSV positive trees were samples from various dates (Table 2). The number of PDV analysed trees varied from 10 to 29 (Table 2).

Tabelle 2: Ergebbusse des DAS-ELISA-Tests
Table 2: Results of DAS-ELISA testing

Analysis number*	PDV			PNRSV		
	No. of analysed samples	No. of positive samples	Thres-hold (2xNC**)	No. of analysed samples	No. of positive samples	Thres-hold (2xNC)*
1	29	29	0,271	19	19	0,092
2	11	11	0,267	7	7	0,096
3	11	11	0,267	7	7	0,096
4	18	18	0,267	14	14	0,096
5	12	12	0,152	8	0	0,051
6	29	29	0,117	19	0	0,068
7	10	10	0,212	7	0	0,114
8	18	18	0,295	14	13	0,080
9	10	10	0,212	10	10	0,114
10	10	10	0,109	10	10	0,063
11	10	10	0,109	10	10	0,063
12	18	18	0,295	14	14	0,080
13	10	10	0,109	10	10	0,063
14	20	20	0,152	10	8	0,051

* Classification see in Table 1
** Twice mean absorbance value for health control of fruit trees

2.2 DAS-ELISA

DAS-ELISA using Bioreba AG reagents for detection of PDV and PNRSV was performed essentially as recommended by the producer, except for 100 µl reactions were used. Samples were homogenised in 20 volumes (w/v) of extraction buffer. Each sample was analysed in duplicate. Absorbance was read at 405 nm in a Sunrise Remote Control Reader (TECAN Austria GmbH). Samples were considered positive when the mean absorbance value of a sample after three hours exceeded the threshold. The threshold was set as at least twice mean absorbance value for health control of fruit trees (Bioreba AG).

3 Results and Discussion

The results of the analyses from the selected positive trees are presented in Table 2, Figure 1 and 2. In Figure 1 and 2 the absorbance values (after 3 hours) of each samples are plotted as squares. The mean absorbance values within the analysis are presented as points with 95 % confidence intervals for each mean absorbance value which is a vertical lines.

Considerable fluctuations of DAS-ELISA readings were observed among the analyses. Mean absorbance values as well as the variability of the results within the analysis differed depending on the date of analysis, the analysed tissue and the virus.

Considerably higher absorbance values were observed for negative controls in analyses for PDV when compared to PNRSV (Table 2).

3.1 PDV detection

The results of the PDV detection are presented in Table 2 and Figure 1. Leaves, analysed for the presence of PDV at the end of May (Analyses 2, 3 and 4), gave much higher absorbance values than flowers collected in April (Analysis 1). The position of the leaf on one-year old shoots (Analyse 2, 3 and 4) did not have any effect on the result of the analysis. A considerable decrease of mean absorbance value was observed in leaves in summer and autumn 2003 (Analyses 5, 6 and 7). At the same time the variability of results among samples greatly increased. In contrast the analysis of leaves collected at the end of August 2004 gave very high and uniform absorbance values (Analysis 8).

Differences between the results obtained in summer 2003 and summer 2004 can be explained by notable differences in temperatures between the two years. In 2003, maximum temperatures reached 29, 5 °C already in the first decade of May and the weather remained extremely hot through whole summer. In contrast, temperatures in summer 2004 were moderate and very close to the long-term temperature mean (Table 3).

Buds proved to be a good source for PDV detection. The

Tabelle 3: Monatliche Temperaturdaten (2 Meter über dem Boden in Celsius) von Mai bis August 2003 und 2004, gemessen in 5 km Entfernung vom Obstgarten der Sorte cv. Veteran
Table 3: Monthly temperature data (2 m above the ground; in °C) from May to August 2003 and 2004, recorded 5 km away from the orchard of cv. Veteran.

	May		June		July		August	
	2003	2004	2003	2004	2003	2004	2003	2004
Mean maximum air temp.	26,4	20,6	31,6	26,0	31,3	28,5	33,9	29,2
Maximum temp.	30,6	26,0	37,0	31,0	37,5	34,0	39,0	33,0
Deviation from long-term temp.	+2,8	-1,4	+5,1	+0,7	+2,6	+0,4	+4,7	+0,9
No. of days with temp. over 25 °C	22	4	29	19	31	27	31	29

highest mean absorbance value was detected in buds collected in August 2004 (Analysis 12). Although lower, the concentration of PDV in buds in October 2003 (Analysis 9) and January 2004 (Analysis 11) still allowed a highly reliable detection of the virus. Absorbance values of the extracts kept at -20 °C for nearly 3 months before analysis (Analysis 10) were only some slightly lower than the absorbance values of fresh extracts. Bark samples, analysed in January (Analysis 13), also proved to be suitable for PDV detection.

Fruits, analysed in August 2003 for the presence of PDV (Analysis 14), gave very variable results. Although some of the fruit samples had very low absorbance values, all of them proved to be positive (Table 2). Similarly GRÜNTZIG and FUCHS (1988) detected PDV in all tested plum fruits whereas the reliability of PNRSV detection depended on the cultivar.

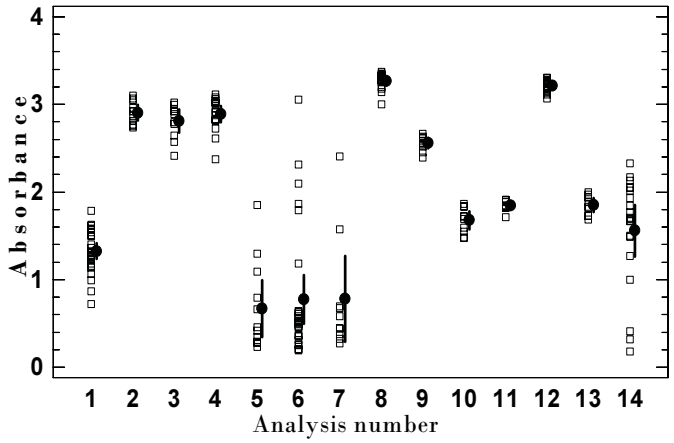

Figure 1: Absorbance values (after three hours) of individual samples (represented as squares) collected from PDV positive trees, mean absorbance values (represented as points) and 95 % confidence intervals (represented as vertical lines) for the individual analyses described in Table 1.

Abbildung 1: Absportionswerte (nach 3 Stunden) der individuellen Proben (dargestellt in Quadraten) von PDV-positiven Bäumen, Absorbtionsmittelwerte (dargestellt in Punktform) und 95 % Konfidenzintervalle (in vertikalen Strichen) für die Einzeldaten aus Tabelle 1.

3.2 PNRSV detection

Detection of PNRSV showed to be much more prone to deleterious effect of high temperatures than PDV. The results are presented in Table 2 and Figure 2. One of the leaf samples collected in summer 2004 (Analysis 8) proved to be completely negative and two had absorbance values very close to the threshold. The absorbance values of the rest of the samples were also low. In summer and autumn 2003 the results of all leaf samples (Analyses 5, 6 and 7) were completely negative. Similarly, DAL ZOTTO and NOME (1999) found absorbance values in mature peach leaves of 6 varieties tested for PNRSV in January (Córdoba, Argentina) to be similar to those of noninfected plants. In contrast, FUCHS et al. (1988) could detect PNRSV in leaves collected in peach orchards in Germany from flowering until mid-September. UYEMOTTO et al. (1989) found erratic ELISA results when testing peach shoots and leaves for the presence of PNRSV in May, when temperatures exceeded 38 °C over a 12-day period. In our study the concentration of PNRSV in leaves collected in summer was too low to allow a reliable detection by DAS-ELISA even in 2004 when the temperatures did not exceed 34 °C (Table 1). Differences among the results from different countries could be explained by their different climatic conditions. In addition, tested varieties differed among studies which could also have contributed to observed differences among studies. DAL ZOTTO and NOME (1999) reported considerable differences in average absorbance profiles among studied peach cultivars. Apart from different climatic conditions and studied host varieties, PNRSV has numerous strains with distinct biochemical and pathogenic properties (CROSSLIN and MINK, 1992), thus differences in the population structure of PNRSV in studied orchards could also have contributed to observed variability among the results of the different authors. Differences in sensitivity for high temperatures have been reported for different isolates of *Plum pox potyvirus* (GLASA et al., 2003).

In contrast to PDV, flowers (Analysis 1) gave considerably higher absorbance values for PNRSV than leaves collected at the end of May 2003 (Analyses 2, 3 and 4). Substantial differences were observed among leaf samples collected from different parts of a one-year old shoot. Although the presence of PNRSV was confirmed in all samples, young leaves from shoot tips (Analysis 2) gave much higher and more reliable results than leaves from the middle part (Analysis 4) and from the base of a one-year old shoot (Analysis 3).

Similarly to PDV, autumn (Analysis 9) and winter buds (Analysis 11) proved to be a very good source for detection of PNRSV. As with PDV, keeping the bud extract at -20 °C for nearly 3 months did not significantly affect the efficiency of detection (Analysis 10). Considerably lower results were observed in bud samples collected in August (Analysis 12) and in bark samples collected in January (Analysis 13). Since the preparation of bud samples is easier and quicker

than preparation of bark samples, buds are more suitable for DAS-ELISA analysis than the bark.

PNRSV could not be detected in 2 out of 10 tested fruit samples (Analysis 14). Additionally, three fruit samples had absorbance values which were very close to the threshold. When testing plum fruits, GRÜNTZIG and FUCHS (1988) could not detect PNRSV in all analysed samples either. The reliability of PNRSV detection differed among the studied plum varieties. It was 100 % in varieties Hauszwetsche and Frigga, 75 % in variety Kirkes Pflaume and 65 % in variety Stanley. The authors considered that 10 fruits per tree should be taken to ensure reliable diagnosis.

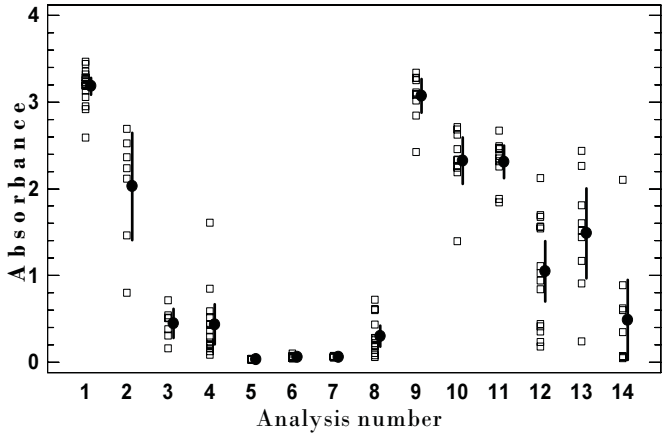

Figure 2: Absorbance values (after three hours) of individual samples (represented as squares) collected from PNRSV positive trees, mean absorbance values (represented as points) and 95 % confidence intervals (represented as vertical lines) for the individual analyses described in Table 1.

Abbildung 2: Absorptionswerte (nach 3 Stunden) der individuellen Proben (dargestellt in Quadraten) von PNRSV-positiven Bäumen, Absorbtionsmittelwerte (dargestellt in Punktform) und 95 % Konfidenzintervalle (in vertikalen Strichen) für die Einzeldaten aus Tabelle 1.

4 Conclusions

We can conclude that flowers are the best source for detection of PNRSV. Similar absorbance values were found only in buds sampled at the end of October. Nevertheless, dormant buds collected in autumn and winter also proved to be a reliable source of PNRSV for DAS-ELISA testing. High summer temperatures have a deteriorating effect on concentrations of PNRSV and PDV in leaves. Detection of PNRSV was either not possible or very unreliable in leaves in summer and autumn, whereas the efficiency of PDV detection in summer or autumn leaves depended on summer temperatures. Detection of PDV was most reliable in dormant buds in autumn or winter, in bark in winter and in leaves in May. PDV concentrations were lower and more variable in flowers, but nevertheless allowed reliable detection.

The concentration of PDV and PNRSV in extracts kept at -20 °C for nearly 3 months showed only a small decrease and allowed a reliable detection of both viruses.

PDV and PNRSV can thus be simultaneously and reliably analysed with DAS-ELISA in winter and autumn buds and in flowers or young leaves in spring. However, more efficient PCR techniques (SAADE et al., 2000) need to be used for summer testing.

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