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Direct sample preparation methods for the detection of *Plum pox virus* by real-time RT-PCR

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Summary. Direct systems to process plant materials allowed high-throughput testing of *Plum pox virus* (PPV) by real-time reverse transcription (RT)-PCR without nucleic acids purification. Crude plant extracts were diluted in buffer or spotted on membranes to be used as templates. Alternatively, immobilized PPV targets were amplified from fresh sections of plant tissues printed or squashed onto the same supports, without extract preparation. Spot real-time RT-PCR was validated as a PPV diagnostic method in samples collected during the dormancy period and showed high sensitivity (93.6%), specificity (98.0%), and post-test probability (97.9%) towards sharka disease. In an analysis of 2919 *Prunus* samples by spot real-time RT-PCR and DASI-ELISA 90.8% of the results coincided, demonstrating high agreement ($k = 0.77 \pm 0.01$) between the two techniques. These results validate the use of immobilized PPV targets and spot real-time RT-PCR as screening method for large-scale analyses. [**Int Microbiol** 2009; 12(1):1-6].

Key words: *Plum pox virus* (PPV) · sharka disease · spot real-time RT-PCR · winter or dormand diagnostic methods · post-test probability

Introduction

Sharka disease, caused by *Plum pox virus* (PPV), is considered the most devastating viral disease of stone-fruit trees, affecting mainly apricots, peaches, and plums. Since its initial detection in Bulgaria in 1917, PPV has spread to most *Prunus* growing areas [7]. Legal and illegal transport of PPV-infected plant material for vegetative propagation is the main cause of the long-distance spread of PPV. Nursery plants and plant materials are generally commercialized in winter, during the dormant period, when the virus titer is low. Since incorrect PPV analyses are among the causes of introduction of PPV in new areas, the need for accurate and validated methods for PPV diagnosis is critical.

Serological methods, such as double antibody sandwich (DAS) and double antibody sandwich indirect (DASI)-ELISA, using polyclonal or specific monoclonal antibodies, are widely used for PPV detection and characterization [6]. With the development of molecular techniques, especially PCR-based methods, tools for the sensitive detection and identification of plant viruses have become available [9]. PCR-based techniques require purified total RNA as templates in amplification reactions. The RNA purification step can be circumvented by the implementation of an immunocapture step prior to the reverse transcriptase-PCR (IC-RT-PCR) that simultaneously decreases the concentration of inhibitors [14]. Alternatively, plant crude extract spotted onto filter paper can be used for PPV detection [12], but the preparation of these extracts is time-consuming and enhances the risk of contamination. Squashes and tissue prints on membranes have been used to immobilize viral targets and obviate the need for tedious grinding steps [2].

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The main drawback of using immobilized plant samples is the small amount of sample that is loaded onto the support. This limitation could be avoided by coupling these easy sample preparation methods with highly sensitive techniques such as real-time RT-PCR. Here we describe the use of four direct sample preparation methods (dilution, spot, squash, and tissue-print) that, coupled with real-time RT-PCR, give reliable results for the routine diagnosis of PPV. Spot real-time RT-PCR has been validated for the analysis of samples collected during the winter, when plant tissues are dormant. This is a requirement for including the method in international diagnostic protocols. A preliminary report of this work has been published in conference proceedings [3].

Materials and methods

Viral isolates and plant samples. The following PPV isolates from the IVIA collection were employed: 3.3RB/GF (PPV-D), PS (PPV-M), SwC and SoC (PPV-C), and EA-B (PPV-EA). The PPV-W and PPV-Rec isolates were kindly provided by Dr. D. James, Canada, and Dr. I. Zagrai, Romania, respectively. A total of 405 *Prunus* adult trees [183 Japanese plums (*Prunus salicina* Lindl.), 113 apricots (*P. armeniaca* L.), 86 peaches (*P. persicae* (L.) Batsch), 15 almonds (*P. dulcis* (Mill.) D.A. Webb), and eight GF676 peach × almond hybrids] were analyzed to validate spot real-time RT-PCR for the detection of PPV in samples collected during the plants' dormancy period. A total of 2919 *Prunus* samples (cultivars and/or rootstocks of peach, plum, apricot, almond and peach × almond hybrid trees) were analyzed to compare DASI-ELISA and spot real-time RT-PCR in a large-scale analysis of PPV.

Sampling and preparation of crude extracts. Samples from *Prunus* adult trees were collected in winter (5–8 spurs or dards with dormant or swelling buds/tree) and in the following spring (5–8 spurs or shoots with fully expanded leaves/tree) from each branch at the internal structure of the tree. Winter samples were ground in plastic bags containing a heavy net (Plant Print Diagnostics, Valencia) with the help of a hammer, in the presence of 1:20 (w:v) PBS buffer, pH 7.2 supplemented with 2% (w:v) polyvinyl pyrrolidone and 0.2% (w:v) sodium diethyl dithiocarbamate. Spring samples were processed in the same buffer into plastic bags containing a soft net (Bioreba), using the Homex-6 machine (Bioreba). The same crude extracts were used for DASI-ELISA, IC-RT-PCR, spot real-time RT-PCR, and total RNA extraction. Serial dilutions (1:10 v:v to $1:10^{11}$ v:v) of an extract from a PPV-infected GF305 peach seedling (3.3RB/GF isolate) were prepared in an extract from a healthy GF305. PPV detection was performed by DASI-ELISA, co-operational-PCR (Co-PCR), and dilution, spot, and conventional real-time RT-PCR approaches to compare the sensitivities of these methods. Sections of leaf petioles and sectors of expanded leaves from the same infected GF305 peach seedling were used to prepare imprints and squashes on membranes.

Conventional real-time RT-PCR and dilution, spot, tissue-print, and squash versions. For conventional real-time RT-PCR, total RNA was extracted from 200 µl of crude extracts using the RNeasy plant mini kit (Qiagen) or Ultraclean Plant RNA isolation kit (MoBio). For real-time RT-PCR, 5 µl of total RNA was used as template with the plant crude extract, 5 µl was diluted in 100 µl of the conventional extraction buffer, and 5 µl of the diluted sample was used for real-time RT-PCR. The spot procedure was carried out loading 5 µl of plant crude extract onto 0.45-µm positively charged nylon membrane (Roche) or on Whatman 3MM paper filter. Tissue-printing was prepared with fresh sections of tender shoots or leaves that were pressed onto the membrane to make one or several prints. Leaf petiole imprints from a PPV-infected GF305 were combined with overlapping petiole imprints of a healthy GF305 in the same site of the membrane in ratios (infected:healthy) of 0:8, 1:7, 2:6, 3:5, 4:4, 5:3, 6:2, 7:1, 8:0. Fresh tissues were squashed on the membrane with the bottom part of an Eppendorf tube. Sections of the membranes harboring the immobilized samples (spotted, tissue-printed, or squashed plant material) were inserted into Eppendorf tubes or placed inside ELISA plate wells for large-scale assays. RNA targets were extracted with 0.5% Triton X-100 [12] or glycine buffer (0.1 M glycine, 0.05 M NaCl, 1 mM EDTA, pH 8.0) [13].

The reaction cocktail for SYBR Green chemistry contained 1× Power SYBR Green PCR Master Mix (Applied Biosystems), 6.25 units of MultiScribe reverse transcriptase (Applied Biosystems), 10 units of RNase inhibitor (Applied Biosystems), 0.3 µM primer P1, and 0.05 µM primer P2 [14]. RT-PCR variables were as follows: 48°C for 30 min, 95°C for 10 min, and 45 cycles of amplification (95°C for 15 s, 60°C for 1 min). A melting curve was obtained for temperatures between 60 and 95°C in all cases to verify the specificity of the reactions. The reaction cocktail for TaqMan chemistry was prepared as previously described [11]. Data acquisition and analysis were conducted with an ABI Prism 7000 and StepOnePlus software packages (Applied Biosystems).

Other PPV detection methods. DASI-ELISA (Durviz reinforced kit, AMR Lab), IC-RT-PCR, and Co-PCR were carried out according to the European and Mediterranean Plant Protection Organization (EPPO) protocol [6].

Variable calculation. Sensitivity, specificity, positive and negative likelihood ratios, and post-test probability of sharka disease were calculated for DASI-ELISA, IC-RT-PCR, spot real-time RT-PCR, and conventional real-time RT-PCR techniques. Sensitivity was defined as the frequency of true positives correctly identified by the method [1], with "true positive" samples being those that tested positive by two methods (serological and at least one molecular) and exhibiting symptoms of sharka disease in spring. Specificity was defined as the frequency of true negatives correctly identified by the method [1], with "true negative" samples being those that tested negative by two methods (serological and molecular) and not exhibiting symptoms of sharka disease in spring. Positive likelihood ratio was the frequency of true positives correctly identified by the technique (sensitivity) divided by the frequency of the false positive results obtained with that method (1-specificity). The negative likelihood ratio was the frequency of false negatives given by the method (1-sensitivity) divided by the frequency of true negatives correctly identified by the technique (specificity) [5]. Prevalence as defined as the incidence of the disease in a given moment at a given area, and was calculated as the number of diseased plants divided by the number of plants analyzed. Likelihood ratios and prevalence were used to calculate the probability of sharka disease for any individual plant [10]. The agreement between techniques was measured by calculating Cohen's kappa (k) index [4].

Results and Discussion

Specificity and sensitivity analysis. The four sample preparation methods assayed, when coupled to real-time RT-PCR, detected all described PPV strains using either TaqMan or SYBR Green chemistries. Comparisons of the sensitivities for PPV detection by dilution, spot, and conventional real-time RT-PCR (TaqMan) with the EPPO recommended techniques are shown in Table 1. The dilution and spot methods had the same limit of detection but were 10 and

Table 1. Comparison of DASI-ELISA, co-operational (Co)-PCR, and dilution, spot, and conventional real-time RT-PCR (TaqMan) sensitivities for the detection of *Plum pox virus* in serial dilutions prepared from an infected GF305 peach seedling extract and a healthy GF305 extract

			Real-time RT-PCR (Ct)		
Dilution	DASI-ELISA (OD_{405})	Co-PCR[a]	Spot	Dilution	RNA extraction
1:10	4.523	+	23.1	–	14.0
1:20	5.412	+	23.6	–	14.0
1:100	5.341	+	24.7	26.0	15.5
1:500	3.534	+	27.2	28.5	19.2
1:1000	2.621	+	28.2	29.6	19.5
1:5000	1.023	+	30.5	32.0	22.3
$1:10^4$	678	+	31.3	32.7	22.6
$1:5 \times 10^4$	338	+	33.9	34.6	24.6
$1:10^5$	277	+	35.3	36.8	25.3
$1:5 \times 10^5$	254	–	37.1	37.9	27.0
$1:10^6$	207	–	38.2	39.3	27.2
$1:5 \times 10^6$	213	–	–	–	29.8
$1:10^7$	203	–	–	–	32.3
$1:5 \times 10^7$	198	–	–	–	34.5
$1:10^8$	201	–	–	–	35.1
$1:10^9$	184	–	–	–	38.9
$1:10^{10}$	215	–	–	–	–
$1:10^{11}$	178	–	–	–	–
Healthy control	180	–	–	–	–

[a] + Positive amplification. – Negative detection or undetermined.

100 times more sensitive than Co-PCR and DASI-ELISA, respectively. Dilution failed to amplify the PPV targets at low dilutions (1:10 and 1:20, v:v) probably due to the presence in the reaction of high amounts of PCR inhibitors of plant origin. The most sensitive method was conventional real-time RT-PCR, which detected PPV at a dilution as high as $1:10^9$ (Ct value 38.9) (Table 1). Nevertheless, the purification of RNA from plant samples is time-consuming, costly, and not suitable for large-scale analyses. Healthy GF305 seedlings and cocktail controls tested negative by all assayed techniques.

The dilution and spot real-time RT-PCR methods were slightly more sensitive than the tissue-print and squash methods (Fig. 1). Because of the uneven distribution of PPV in infected woody hosts, the use of plant extracts is more reliable than a single tissue-print or squash. This drawback can be overcome by using multiple overlapping imprints or squashes from the same sample at the same site of the membrane. Analysis of one to eight leaf petiole imprints from a PPV-infected GF305 overlapping with leaf petiole imprints from a healthy GF305 (from 8 to 0) gave similar Ct values in all assayed combinations (average 32.8 ± 2.0), supporting the convenience of using multiple overlapping imprints per sample. Similar results were reported for viruses with a more regular distribution in the tree canopy, such as *Citrus tristeza virus* (CTV) [2].

The same efficacy was achieved using two different supports for sample immobilization (nylon and Whatman membranes). Both supports had previously shown to adequately immobilize other plant pathogens, such as viruses, bacteria, and phytoplasma [2,13]. Triton X-100 and glycine buffers had the same efficiency in releasing PPV immobilized targets. This characteristic is not shared for all pathogens, e.g., CTV targets can be much more efficiently released in glycine buffer than in Triton X-100 [2].

Validation of spot real-time RT-PCR for the detection of PPV in plants during the dormant period and in large-scale analyses. Spot real-time RT-PCR was validated as a reliable technique for the detection of PPV in plants during the dormant period, when erroneous diagnoses are more frequent due to low virus titers in the tree canopy. Figure 2 shows the number of trees found to be true positive, true negative, false positive, and false negative, following the analysis of 405 adult *Prunus* trees using different techniques with samples collected in the winter and

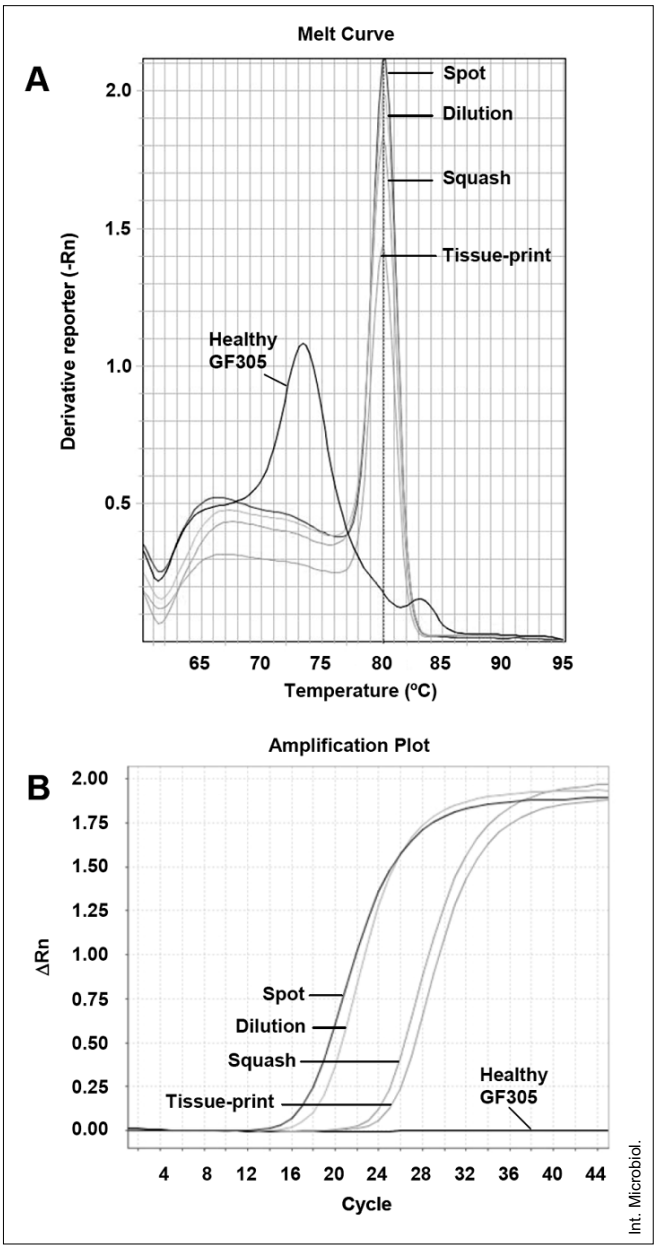

Fig. 1. Detection of *Plum pox virus* (PPV-D type) by real-time RT-PCR with SYBR Green (**A**) and TaqMan (**B**) chemistries, using four direct sample preparation methods: dilution, spot, tissue-print, and squash. Healthy GF305 peach seedlings were used as negative control.

the following spring. Of these 405 trees, 201 showed symptoms of sharka disease in the following spring, indicating a prevalence of 49.6%. This prevalence close to 50% is very adequate for validation purposes. Note that diagnostic reagents and protocols are very seldom validated by estimating practice variables.

The sensitivity, specificity, positive and negative likelihood ratios, and post-test probability of sharka disease as detected by each assayed method are shown in Table 2. The highest sensitivity for PPV detection in winter was obtained by conventional real-time RT-PCR, which detected PPV in 97.5% of the trees that developed sharka symptoms in the following spring (196 of 201). With this technique, the frequency of false negatives (5 out of 405) was extremely low, confirming the high reliability of negative results (Fig. 2). Spot real-time RT-PCR also had a high sensitivity (93.6%), followed by IC-RT-PCR (91.5%) and DASI-ELISA (86.6%). DASI-ELISA was the most specific method, correctly identifying 99.0% (202 out of 204) of the healthy trees and detecting the lowest percentage of false positives (2 out of 405) (Fig. 2), confirming the high reliability of positive results. The specificity of spot real-time RT-PCR is shown in Table 2. Based on likelihood results and prevalence data, the post-test probabilities of sharka disease were estimated. DASI-ELISA had the highest post-test probability for a positive result (98.8%), indicating that for a sample found to be positive by DASI-ELISA in winter, there is a 98.8% probability that the tree will develop sharka symptoms in the following spring. Positive post-test probabilities were 97.9, 95.8, and 89.9% for spot real-time RT-PCR, IC-RT-PCR, and conventional real-time RT-PCR, respectively. Conventional real-time PCR had the highest frequency (22 out of 405 samples) of false positives. Conversely, for a sample found to be negative by DASI-ELISA in winter, the probability of disease appearance in the following spring is 11.8%. This probability is higher than that obtained by spot (6.1%) and conventional real-time RT-PCR (2.7%). There are several possible explanations for the false positives reported by molecular methods, especially conventional real-time RT-PCR. First, certain PPV infections are latent, such that the virus may be subclinically present in the plant but is only detectable by the high sensitivity of real-time RT-PCR, although the infected tree does not later develop sharka symptoms. Second, some trees may have been recently inoculated by PPV/viruliferous aphids; therefore, the virus titer is still extremely low and localized to a restricted area near the inoculation site. In certain cases these infections may be overcome by plant defense mechanisms. Third is the presence of contaminants inherent to sampling, sample manipulation, and/or the PCR. False negatives can be attributed to insufficient sensitivity or to uneven distribution of the virus in the tree, especially, as noted above, in the case of recent inoculations by PPV/viruliferous aphid vectors.

Spot real-time RT-PCR was compared with the validated serological method DASI-ELISA in a large-scale analysis of 2919 *Prunus* samples. The same plant extracts were used in both tests. DASI-ELISA values (OD_{405} intervals) and the corresponding Ct values (threshold cycle of the real-time RT-PCR reaction) are shown in Table 3. Samples with high OD_{405}

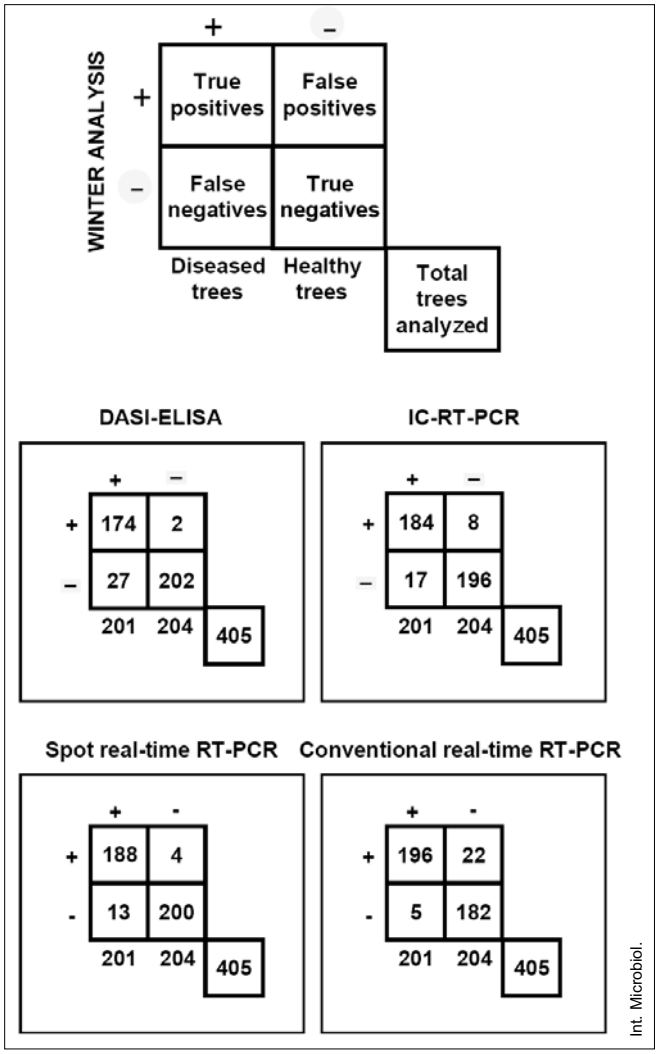

Fig. 2. Comparison of DASI-ELISA, immunocapture (IC)-RT-PCR, spot real-time RT-PCR (using crude plant extracts), and conventional real-time RT-PCR (using purified RNA) for the detection of *Plum pox virus* in 405 adult *Prunus* trees analyzed in winter and in the following spring.

values had low Ct values, and vice versa. A high agreement [8] (kappa index 0.77 ± 0.01) was observed between the two diagnostic methods and a high percentage (90.78%) of coincidental results was obtained. Among the discrepancies, 6.6% of the samples tested positive by spot real-time RT-PCR but not by DASI-ELISA, accounting for the high sensitivity of spot real-time RT-PCR (100 times more sensitive). Conversely, 2.6% of the samples tested positive only by DASI-ELISA, probably due to the inhibition of some spot real-time RT-PCR reactions. The OEPP protocol for PPV diagnosis [6] recommends the use of two different methods (biological, serological, and/or molecular) to consider a sample as healthy or infected. The combined use of DASI-ELISA and real-time RT-PCR based methods is thus recommended for accurate PPV diagnosis for quarantine purposes. Spot real-time RT-PCR could be the molecular alternative to serological detection methods during the dormancy period and in large-scale analyses.

The main advantage of these sample preparation methods is that the immobilized plant samples can be stored before processing at room temperature or at –20ºC for up to 2 years without a loss of sensitivity (data not shown). This allows the mailing of materials to be tested in other laboratories or for diagnosis confirmation, while keeping positive controls in sample collections or for the management of quarantine pathogens without risks. In addition, these methods might be easily adapted for the diagnosis of other pathogens.

The availability and use of reliable diagnostic methods are essential to controlling the introduction and spread of a given pathogen. Portable real-time PCR machines are emerging as diagnostic tools for in situ detection under field conditions. For that purpose, direct methods, especially tissue-print and squash, would be very appropriate. The results of this work demonstrate that simple sample preparation methods coupled with real-time RT-PCR constitute a reliable and

Table 2. Practice variables or parameters of serological (DASI-ELISA) and molecular [immunocapture (IC)-RT-PCR, spot and conventional real-time RT-PCR] diagnostic methods for *Plum pox virus*

Detection method	Sensitivity ± SE[a]	Specificity ± SE	Likelihood ratio		Post-test probability (%)
DASI-ELISA	0.866 ± 0.017	0.990 ± 0.005	LR+[b]	88.3	98.8
			LR–[c]	0.13	11.8
IC-RT-PCR	0.915 ± 0.014	0.961 ± 0.01	LR+	23.3	95.8
			LR–	0.09	7.9
Spot real-time RT-PCR	0.936 ± 0.012	0.980 ± 0.01	LR+	47.7	97.9
			LR–	0.07	6.1
Conventional real-time RT-PCR	0.975 ± 0.010	0.892 ± 0.01	LR+	9.0	89.9
			LR–	0.03	2.7

[a]Standard error.
[b]Likelihood ratio for a positive result by the technique.
[c]Likelihood ratio for a negative result by the technique.

Table 3. Comparison of $OD_{405\ nm}$ and Ct (threshold cycle) values in the large-scale analysis of 2919 *Prunus* samples for the diagnosis of *Plum pox virus* by DASI-ELISA and spot real-time RT-PCR

	OD DASI-ELISA	No. of plants							
Positive	4001–6000	6	30	69	138				
	2001–4000		3	32	77	26			
	1001–2000			3	48	14	12		
	601–1000				29	25	12		
	405–600				5	19	17	107	75
Negat.	203–404					37	42	12	1289
	0–202					13	72	18	689
	Ct Spot real- time RT-PCR	13–15	16–20	21–25	26–30	31–35	36–40	41–45	> 45
		Positive							Negative

Coincidental results (positive or negative by both techniques) (Total 2650; 90.8%.)

Samples positive only by DASI-ELISA. (Total 75; 2.6%.)

Samples positive only by spot real-time RT-PCR. (Total 194; 6.6%.)

validated approach for PPV diagnosis. The application of this approach for the analysis of large numbers of samples can contribute to controlling the sanitary status of commercial *Prunus* trees and to preventing the spread of sharka disease.

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