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Research Article

Optimization of Plant Tissue Selection and Sampling Time for Reliable RT-PCR Detection of *Pelamoviroid latenspruni*, and the Influence of Climatic Conditions on Detection Efficiency

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Abstract: Viroids are the smallest pathogens with no protein-coding properties, made up of a circular single-stranded RNA molecule, 246-401 nt length causing diseases in plants. PLMVd is the type member of the Pelamoviroid genus of the Avsunviroidae family. The aim of this thesis study was to find out the optimal sampling time and tissue in plants infected with PLMVd., The study was conducted on two persimmon plants number TH7 and TH9 with NCBI accession numbers MZ289071 and MZ289070, respectively, which had previously been shown to be infected with PLMVd. Between March 2021 and February 2022, sampling was performed monthly, and all available plant tissues were extracted and tested for TRNA using RT-PCR. The study concluded that the most reliable testing organs and times when there is a seasonal limitation are the flower bud, bark, and leaf tissue in April, flower, leaf, bark, and fruit tissue in May, and the bark, leaf, and fruit tissue in September. It has been concluded that bark, leaf, and fruit tissue samples provide consistent results regardless of season. The independent sample t test was used to estimate the likelihood that the PLMVd concentration in climatic data would yield positive or negative findings, as well as its significance. According to the study results, sampling is recommended between 14.40-20.90 °C, humidity rate 41.40-49.30%, mean soil temperature 7.35-11.875 °C, sunshine duration 223.10-345.00 hours, and sunshine intensity 223.56-313.33 cal÷cm². It was determined that sampling should be conducted in accordance with the viroid's biology, the host's phenological phase, and regional meteorological circumstances.

Keywords: PLMVd, Persimmon, Suitable sampling time, Suitable tissue, Climatic conditions.

1. Introduction

The persimmon (*Diospyros kaki* Thunb.) is a fruit species that mainly grows in the subtropical climate zone of the world and originated in China (Tuzcu and Yıldırım, 2000). According to data from 2022, yearly persimmon production of Türkiye is 97,560 tonnes, whereas Malatya province produces 279 tonnes. In 2021, there are 1,663,793 persimmon trees in Türkiye, with 12,824 persimmon trees in Malatya province accounting for around 1.1% of this total (TÜİK, 2023). Many fungi, bacteria, viruses, viroids, and phytoplasmas infect persimmons. Among the important fungal pathogens are *Cladosporium cladosporioides* (Kwon and Park, 2003; Palou et al., 2015). *Colletotrichum hori* (Xie et al., 2010),

Cercospora leaf spot (Ding et al., 2013), *Lasiodiplodia theobromae*, *Neofusicoccum* spp. *Pestalotiopsis clavispora*, *Colletotrichum gloeosporioides*, *Penicillium expansum*, *Alternaria alternata*, *Botrytis cinerea* (Palou et al., 2015) and *Adisciso kaki* (Yamamoto et al., 2012). *Pseudomonas syringae* pv. *syringae* (Scortichini et al., 1998) is the only bacterial pathogen infecting persimmon, while *Canditatus* phytoplasma ziziphi (Wang et al., 2015) is the only phytoplasma reported. Viruses and virus-like pathogens infecting persimmon include *Persimmon cryptic virus* (PeCV) and *Persimmon virus A* (PeVA) (Cho et al., 2016), *Persimmon viroid* and *Apple fruit crinkle viroid* (AFCVd) (Nakaune and Nakano, 2008). *Peach latent mosaic viroid* (PLMVd), which is the subject of this study, was first detected as a causal agent of infection in persimmon in a study conducted by the Virology Laboratory of the Department of Plant Protection, Faculty of Agriculture, Turgut Özal University, Malatya (Oksal et al., 2021).

Viroids are the smallest pathogens that can infect plants and consist of a circular, single-stranded RNA molecule 246-401 nt in length that does not encode proteins (Flores et al., 2004). PLMVd is a type member of the genus Pelamoviroid in the family Avsunviroidae. The scientific name was changed to *Pelamoviroid latenspruni* in 2024 by the International Committee on Taxonomy of Viruses (ICTV). The pathogen was first described by Desvignes (1976) as peach mosaic disease in France. PLMVd occurs in Argentina, Brazil, Canada, Chile, Uruguay and Mexico in Amerca in Morocco, Algeria, Tunisia and Egyp in Africa in Turkey, Spain, France, Greece, Austria, Serbia, Montenegro and Italy in Europe; in Iran, China, Japan, Jordan, Korea, Lebanon and Syria in Asia and in New Zealand and Australia in Oceania (Albanese et al, 1992; Jawhar et al. 1997; Turturo et al. 1998; Garland and Cree 1999; Osaki et al. 1999; Di Serio et al, 1999; Ismaeil et al. 2001; Choueiri et al. 2001; Paduch-Cichal and Skrzeczkowski 2001; Fiore 2003; Torres et al. 2004; Susuri et al. 2006; Škoric et al. 2008; Nieto et al. 2008; Al Rwahnih et al. 2008; Jevremovic and Paunovic 2008; Gazel et al, 2008; Hassan et al. 2009; Boubourakas et al. 2009, Yazarlou et al. 2011; Mavrič Pleško et al. 2012; De La Torre-Almaráz et al. 2015; Veerakone et al. 2015; Jo et al. 2016; Oksal et al. 2021).Thanks to the sensitive analytical methods that have been widely used in recent years, it has been detected in peach, nectarine, grapevine, ornamental plum, almond, apple, plum, mango, Japanese plum, apricot, cherry, walnut, pear, quince, persimmon, johnsongrass, a weed and pistachio (Skrzeczkowski et al, 1996; Hadidi et al, 1997; Faggioli et al. 1997; Osaki et al. 1999; Kyriakopoulou et al. 2001; Hassen et al. 2005; El-Dougdoug et al. 2012; Kyriakopoulou et al. 2017; Tuncel et al. 2020; Oksal et al. 2021; Çiftçi et al. 2021; Yılmaz, 2024). *Pelamoviroid latenspruni* is particularly latent in peach. Some viroid isolates show cream-coloured mosaic or chlorotic spots on peach leaves, albino or calico-like symptoms on some or all shoots and fruit leaves. In areas where seedlings are infected, disease symptoms appear about 2 years after planting and there is a delay of 4-6 days in foliage, flowering and ripening. The fruits of the plant show deformations and discolorations, crack-like scars as well as round and flat formations in the core. It also causes a pink coloration with dashed stripes on the petals of the flower. It causes symptoms such as bud necrosis, stem hollowing, slowing of growth and rapid ageing of the tree (Flores et al., 2003; Flores et al., 2011). *Pelamoviroid latenspruni* is easily transmitted by grafting material (eye and pin) (Desvignes, 1986), but not by seeds (Howell et al., 1997; Barba et al., 2007). *Pelamoviroid latenspruni* has been reported experimentally not to be transmitted by the aphid species *Aphis gossypi* and *Aphis spiraecola*, while it can be transmitted in low levels by *Myzus persicae* (Flores et al., 1992). Agricultural equipment and machinery (grafting knives, pruning shears, etc.) play an important role in the spread of *Pelamoviroid latenspruni* in orchards (Hadidi et al., 1997; Flores et al., 1990). Depending on the peach cultivar, *Pelamoviroid latenspruni* has been found to be transported to varying degrees by pollen but not by the roots of peach trees (Barba et al., 2007). The aim of this study was to determine the appropriate time and plant tissue for a reliable diagnosis of peach mosaic virus (*Pelamoviroid latenspruni*) in persimmon using RT-PCR.

2. Materials and Methods

2.1. Collecting plant material and total RNA extraction

The main material of the study consisted of two persimmon plants, TH7 (accession number: MZ289071) and TH9 (accession number: MZ289070), which were found to be infected with *Pelamoviroid latenspruni* by Oksal et al. (2021) in Malatya province (Figure 1). Samples were collected from the tree's vegetative tissues (flower bud, leaf bud, leaf, bark, flower, and fruit) every month between March 2021 and February 2022 to determine the most appropriate diagnostic tissue and sampling time for trees known to be infected with *Pelamoviroid latenspruni*. The collected samples were labelled properly and transported to the laboratory in polyethylene bags in ice boxes under cold chain conditions.

Between March 2021 and February 2022, samples were taken every month from bark tissue, in March, April and May from leaf bud, flower bud and flower tissue, in the seven months from April to October from leaf tissue and in the six months from May to October from fruit tissue and RT-PCR analysis was performed to determine the presence of *Pelamoviroid latenspruni*. For RNA extraction, the method of Foissac et al. (2001) was used, and total RNA was stored in a freezer at -20°C until cDNA synthesis.

Plant samples were collected from availeble plant tissues monthly from March 2021 to February 2022. In other words, bark tissue samples were collected throughout all 12 months; leaf bud, flower bud, and flower samples were collected in March, April, and May; leaf tissue samples were obtained over the seven months from April to October; and fruit tissue samples were collected during the six months from May to October. *Pelamoviroid latenspruni* was detected using RT-PCR analysis. Foissac et al. (2001) RNA extraction procedure was utilized, and total RNA was frozen at -20°C until cDNA synthesis.

Figure 1. Plant tissues infected with *Pelamoviroid latenspruni* (A) Leaf and bark samples collected in April 2021 (B) Leaf, flower and bark samples collected in May 2021 (C) Leaf, bark and immature fruit samples collected in June 2021 (D) Leaf, bark and ripe fruit samples collected in October 2021.

2.2. Complementer DNA (cDNA) synthesis and amplification

For cDNA synthesis, pathogen-specific primers (PLMVd-F-5'-AAC TGC AGT GCT GCT CCG AAT AGG GCA C-3' PLMVd-R-5'-CCC GAT AGA AAG GCT AAG CAC CTC G-3') with 25 bases of 339 bp constructed by Loreti et al. (1999) were used. A mixture containing 1 µl (2 pmol/µl) of specific primer, 3 µl of total RNA and 1 µl of 10 mM dNTP (Promega, U1515, USA) was prepared and the total volume was brought up to 12 µl with RNase and DNase-free purified water. The mixture was homogenised by vortexing and collected at the bottom of the tube by brief centrifugation (4000-5000 rpm). The mixture was then incubated in a dry heating block at 65 °C for 5 minutes and placed directly on ice to cool rapidly. To the cooled mixture, 4 µl of 5X RT buffer (Promega, M1701, USA) and 2 µl of 0.1 M DL-dithiothreitol (DTT) (Sigma, 43816, Germany) were added on ice. After vortexing, the tubes were incubated at 42°C for 2 minutes. Then, 1 µl of reverse transcriptase enzyme

(Promega, M1701, USA) was added to each tube and incubated at 42°C for 50 minutes to complete cDNA synthesis. To inactivate the enzyme, the tubes were placed in a thermal block (Thermo Fisher, 5020, Finland) at 70 °C for 15 minutes after incubation. The resulting cDNAs were stored at -20°C until RT-PCR. A total volume of 25 µl (RNAse and DNAse free water 12.3 µl, 5X PCR buffer (Promega M7805 USA) 5 µl, $MgCl_2$ (Promega M7805 USA) 1.5 µl, dNTP (10 mM) (Promega U1515 USA) 1 µl, specific primer R 1 µl, specific primer F 1 µl, Taq DNA (Promega M7805 USA) 0. 2 µl and c DNA 3 µl) and amplification was performed with specific primers designed by Loreti et al. (1999). The amplification process consisted of an initial denaturation at 95 °C for 45 seconds, followed by 40 cycles of denaturation at 95 °C for 45 seconds per cycle, annealing of the primers to the target sequence at 58 °C for 45 seconds and an extension step at 72 °C for 1 minute and a final extension at 72 °C for 7 minutes at the end of the reaction. The amplified PCR products were electrophoresed in a 2% agarose gel and visualised with a gel imaging system. For this purpose, 2 g of agarose (Biomax Prona Agarose) was added to 100 ml of 1X TAE (Tris-Acetic acid-EDTA) solution and the solidified gel was placed in the electrophoresis tank (Fisher Scientific CS-300V USA). DNA markers (100 bp) (Jena Bioscience Mid-Range M-203S Germany), PCR amplification products, positive control and negative controls were added to the gel wells and run for 40 minutes at 80 volts and 400 mA electric current.

2.3. Statistical analyses

The influence of the climatic variables on the viroid titration of bark, fruit and leaf parts of two persimmon trees known to be infected was measured during March 2021 - February 2022.

The impact of environmental variables on the viroid titration of bark, fruit, and leaf sections of two infected persimmon trees were studied from March 2021 to February 2022. Humidity, temperature, rainfall, mean soil temperature, sunshine intensity, and sunshine duration were the factors assessed. The mean and standard deviation are examples of descriptive statistics. Deviation by month was utilized to determine the difference between positive and negative sample findings, and the independent samples t-test was used to assess the hypotheses that will contribute to the study's goals.

3. Results

The flower bud, leaf bud, leaf, bark, flower and fruit tissues from persimmon trees infected with *Pelamoviroid latenspruni* were subjected to tRNA extraction and analysed according to the results of the RT-PCR procedures subsequently performed.The gel images were excluded from the study because the RT-PCR test results for the tissues obtained in July, August, October (bark, leaf, and fruit), November, December (bark), and January 2022 (bark) of 2021 were all negative. In March, the available tissues from persimmon trees, leaf bud and bark tissues, were tested. According to the test results, the leaf bud of tree number 9 (9B) gave negative results and the bark tissue (9C) gave positive results, while the leaf bud of tree number 7 (7B) gave positive results and the bark tissue (7C) gave negative results (Figure 2). Persimmon plants sampled in April were tested and the results showed that the bark, leaf and fruit tissue samples from tree 9 and 7 gave positive results (Figure 3). The persimmon plants collected in May were tested and the results showed that the bark, leaf, flower and fruit tissue samples from trees number 9 and 7 were both positive (Figure 4). The bark, leaf and fruit tissues of the persimmon plants collected in June were tested for *Pelamoviroid latenspruni* by RT-PCR. According to the test results, the samples of bark (9C), leaf (9D) and fruit tissue (9F) of tree number 9 were positive and negative, while the samples of bark (7C), leaf (7D) and fruit tissue (7F) of tree number 7 were positive (Figure 5). The persimmon plants collected in September were tested and according to the results, the bark, leaf and fruit tissue samples of tree 9 and 7 were positive (Figure 6).

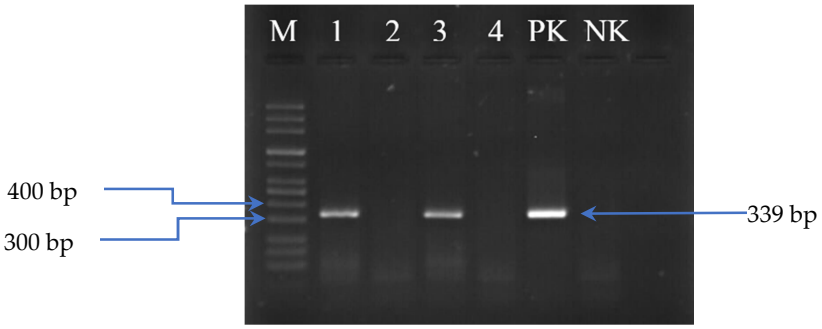

Figure 2. Gel image of *Pelamoviroid latenspruni* samples of March.

1:9C, 2:9B, 3:7B, 4:7C, M: marker, PK: positive control NK: negative control.

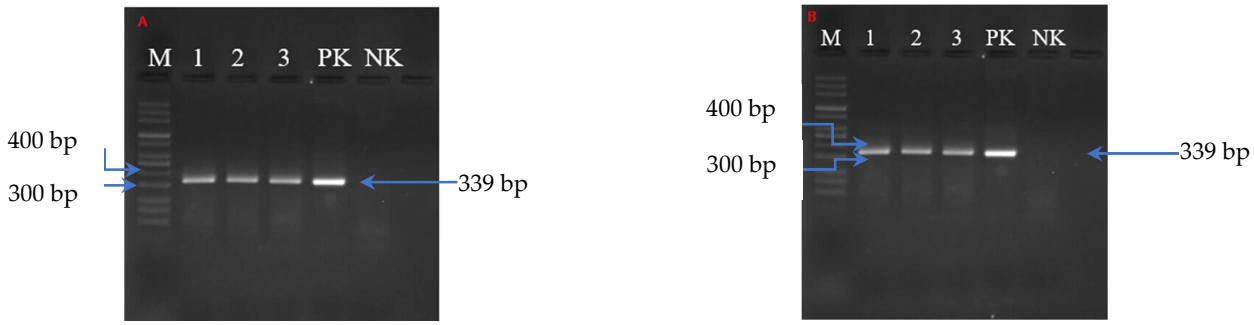

Figure 3. Gel image of *Pelamoviroid latenspruni* samples of April.

(A) 1:9A, 2:9C, 3:9D; (B) 1:7A, 2:7C, 3:7D, M: marker, PK: positive control, NK: negative control.

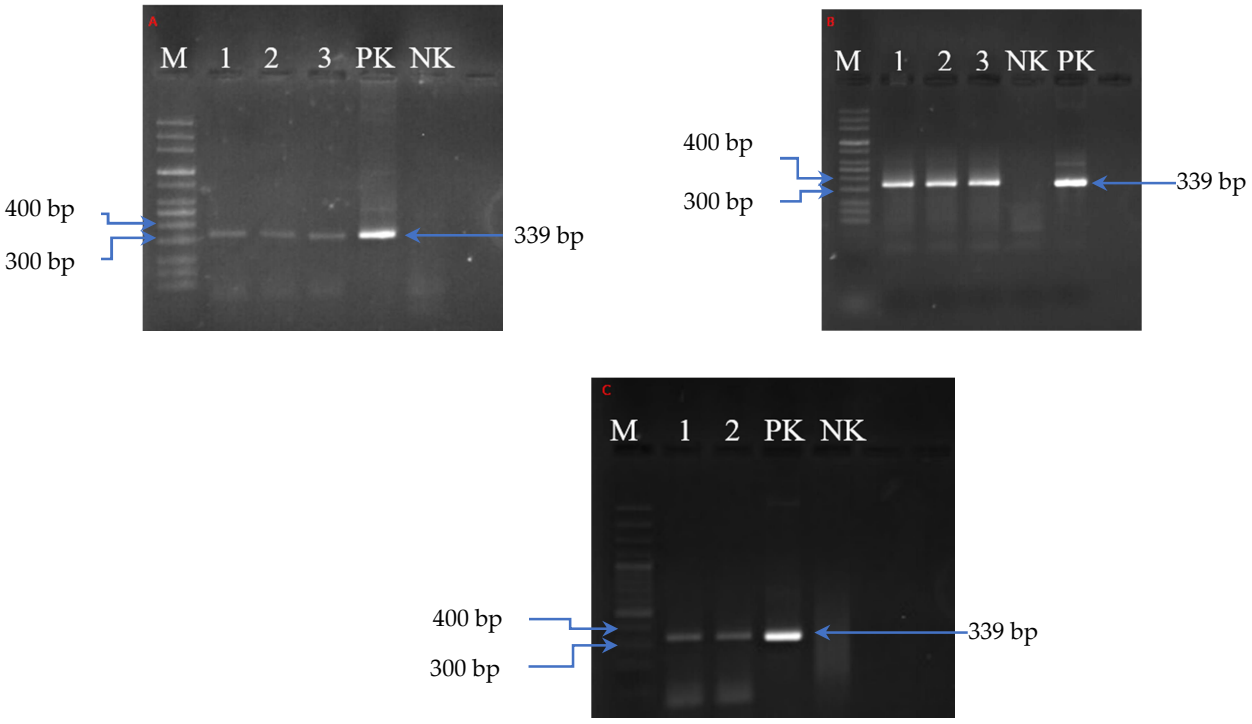

Figure 4. Gel image of *Pelamoviroid latenspruni* samples of May.

(A) 1:9C, 2:9D, 3:9F; (B) 1:7C, 2:7D, 3:7F, (C) 1:9E, 2:7E M: marker, PK: positive control, NK: negative control.

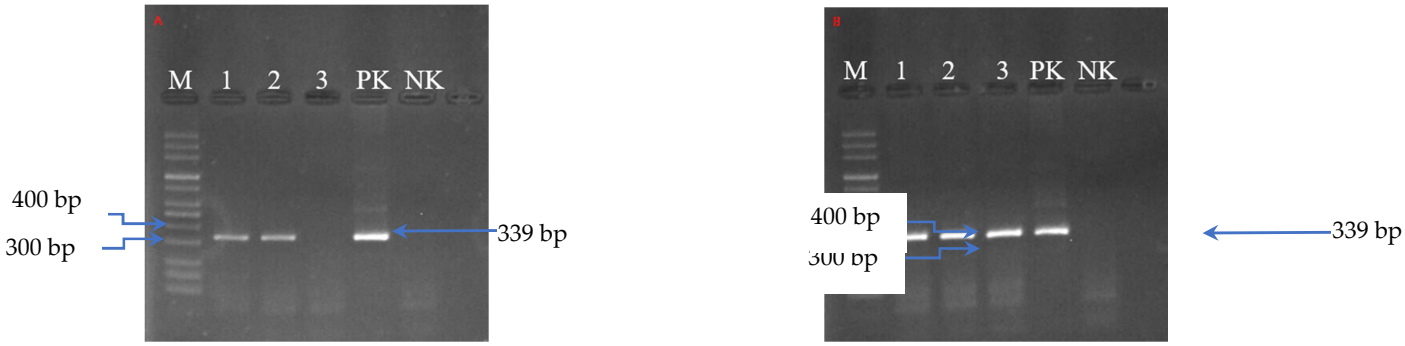

Figure 5. Gel image of *Pelamoviroid latenspruni* samples of June.

(A) 1:9C, 2:9D, 3:9F; (B) 1:7C, 2:7D, 3:7F, M: Marker, PK: positive control, NK: negative control.

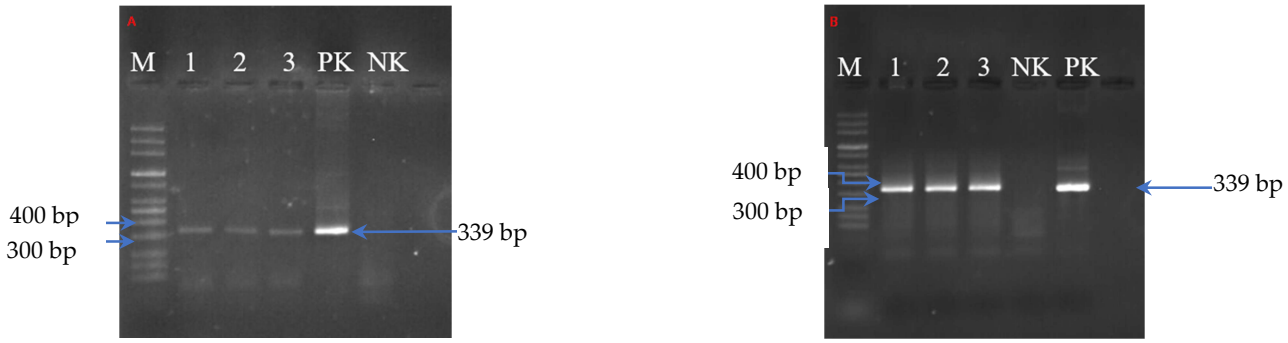

Figure 6. Gel image of *Pelamoviroid latenspruni* samples of September.

(A) 1:C, 2:9D, 3:9F; (B) 1:7C, 2:7D, 3:7F, M: Marker, PK: positive control, NK: negative control.

During sampling in March, leaf bud and bark tissues were taken from both trees and analysed. The results showed that only one tissue was positive for *Pelamoviroid latenspruni* on both trees and the infection rate was 50%. Flower bud, bark and leaf tissues were analysed during the April sampling. The results were positive in all organs of both trees and the infection rate was reported as 100%. During the May sampling, flower, bark, leaf and fruit tissues were analyzed. All tissues were positive in both trees and the infection rate for this period was determined to be 100%. According to the June results, all tissues except the fruit (bark and leaf) were positive in both trees, while in one tree only the fruit tissue was positive, so the infection rate in the fruit was determined to be 50%. In the tests carried out in July and August, bark, leaf and fruit tissues were sampled from both trees, but all tissues were negative for *Pelamoviroid latenspruni*. When the same tissues were tested in September, all organs from both trees were positive and the infection rate increased again to 100%. In October, all samples (bark, leaves and fruit) were negative. In November, December, January and February, only bark tissue was sampled and all samples were negative during this period (Figure 7).

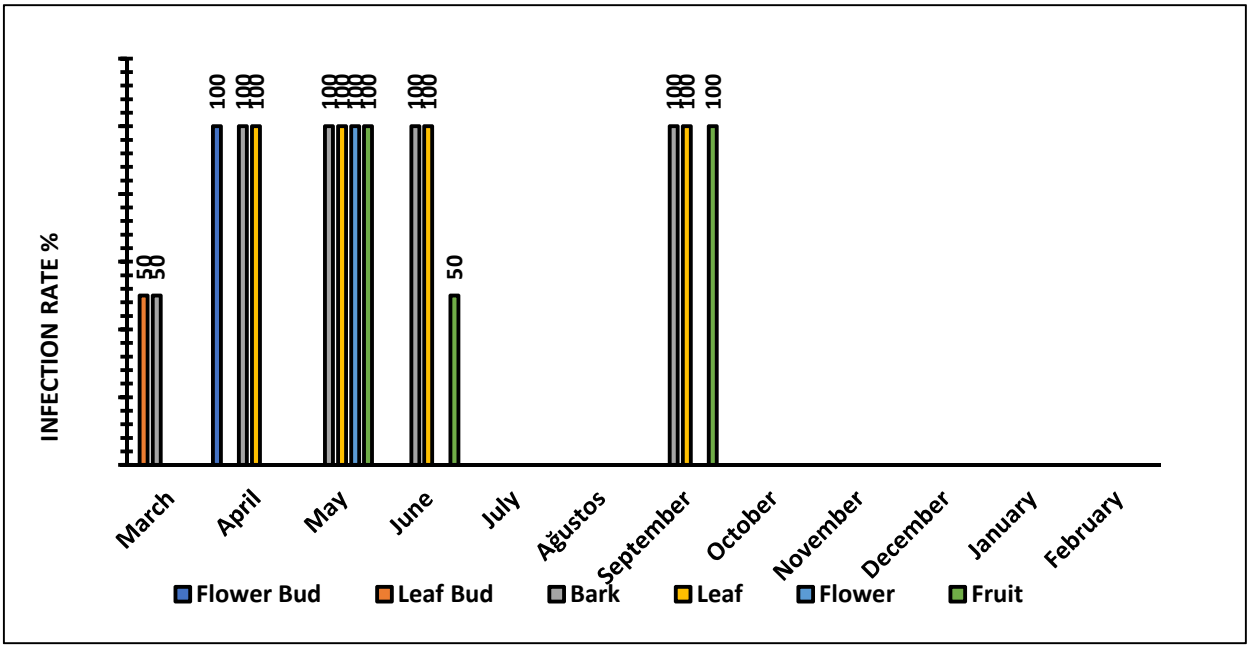

Figure 7. *Pelamoviroid latenspruni* infection rates in persimmon plant tissues by months.

In this study, independent samples t-test analyses were performed using SPSS statistical software to evaluate the possible effects of environmental factors such as humidity, temperature, rainfall, mean soil temperature, sunshine intensity and duration on *Pelamoviroid latenspruni* infection. As a result of the analyses examining the relationships between *Pelamoviroid latenspruni* positivity in bark tissue samples and humidity, temperature, sunshine intensity and duration, statistically significant differences were found between positive and negative samples. On the other hand, no significant differences were observed between *Pelamoviroid latenspruni* negativity/positivity and rainfall and mean soil temperature (Table 1).

Table 1. Group statistics of humidity, temperature, rainfall, mean soil temperature, sunshine intensity and sunshine duration of bark tissue.

Group Statistics					
BARK					
	Results	N	Mean	Std. Deviation	Std. Error Mean
Humidity	Negative	15	59,433	17,426	4,499
	Positive	9	43,478	7,676	2,559
Temperature	Negative	15	11,427	10,772	2,781
	Positive	9	18,267	5,514	1,838
Rainfall	Negative	15	11,940	14,938	3,857
	Positive	9	7,122	3,656	1,219
Mean soil temperature	Negative	15	8,347	5,497	1,419
	Positive	9	11,200	3,760	1,253
Sunshine intensity	Negative	19	178,609	94,127	21,594
	Positive	5	290,488	47,532	21,257
Sunshine duration	Negative	19	163,647	134,502	30,857
	Positive	5	325,580	27,658	12,369

Statistical analyses of fruit samples showed statistically significant differences between *Pelamoviroid latenspruni* infection and humidity, temperature, sunshine intensity, mean soil temperature, and sunshine duration. However, there was no significant difference between rainfall and *Pelamoviroid latenspruni* negativity/positivity (Table 2).

Table 2. Group statistics of humidity, temperature, rainfall, mean soil temperature, sunshine intensity and sunshine duration of fruit tissue.

Group Statistics					
FRUİT					
	Result	N	Mean	Std. Deviation	Std. Error Mean
Humidity	Negative	19	57,216	16,405	3,764
	Positive	5	39,140	2,065	0,923
Temperature	Negative	19	12,095	9,993	2,293
	Positive	5	21,200	1,136	0,508
Rainfall	Negative	19	10,874	13,523	3,102
	Positive	5	7,320	2,219	0,992
Mean soil temperature	Negative	19	8,371	5,135	1,178
	Positive	5	13,390	1,383	0,619
Sunshine intensity	Negative	19	178,609	94,127	21,594
	Positive	5	290,488	47,532	21,257
Sunshine duration	Negative	19	163,647	134,502	30,857
	Positive	5	325,580	27,658	12,369

Statistical analysis of leaf samples showed statistically significant differences between *Pelamoviroid latenspruni* infection and humidity, temperature, sunshine intensity, mean soil temperature, and sunshine duration. On the other hand, there was no significant difference between rainfall and *Pelamoviroid latenspruni* negativity/positivity (Table 3).

Table 3. Group statistics of humidity, temperature, rainfall, mean soil temperature, sunshine intensity and sunshine duration of leaf tissue.

Group Statistics					
LEAF					
	Result	N	Mean	Std. Deviation	Std. Error Mean
Humidity	Negative	19	57,216	16,405	3,764
	Positive	5	39,140	2,065	0,923
Temperature	Negative	19	12,095	9,993	2,293
	Positive	5	21,200	1,136	0,508
Rainfall	Negative	19	10,874	13,523	3,102
	Positive	5	7,320	2,219	0,992
Mean soil temperature	Negative	19	8,371	5,135	1,178
	Positive	5	13,390	1,383	0,619
Sunshine intensity	Negative	19	178,609	94,127	21,594
	Positive	5	290,488	47,532	21,257
Sunshine duration	Negative	19	163,647	134,502	30,857
	Positive	5	325,580	27,658	12,369

As the number of negative and positive tissue samples was not equal, statistical analyses were performed under the assumption of unequal variance. The results of the analyses showed that increases in temperature, sunshine duration and sunshine intensity decreased the probability of detecting the fungus in the bark. On the other hand, an increase in humidity significantly (P<0.005) decreased the probability of detection of *Pelamoviroid latenspruni* in bark tissue. On the other hand, rainfall and mean soil temperature had no statistically significant (P>0.005) effect on the detection of the viroid (Table 4).

Table 4. Independent sample test for humidity, temperature, rainfall, mean soil temperature, sunshine intensity and sunshine duration of bark tissuei.

Independent Sample Test BARK										
Environmental conditions		Levene's Test for Equality of Variances		t-test for equality of means						
		F	P	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Mean Difference	95% Confidence Interval of the Difference	
									Lower	Upper
Humidity	Equal variances assumed	14,76	0,00	2,58	22,00	0,02	15,96	6,18	3,14	28,77
	Equal variances not assumed			3,08	20,73	,006 ***	15,96	5,18	5,18	26,73
Temperature	Equal variances assumed	5,95	0,02	-1,76	22,00	0,09	-6,84	3,88	-14,90	1,22
	Equal variances not assumed			-2,05	21,67	,052 *	-6,84	3,33	-13,76	0,08
Rainfall	Equal variances assumed	4,99	0,04	0,94	22,00	0,36	4,82	5,11	-5,78	15,41
	Equal variances not assumed			1,19	16,65	0,25	4,82	4,04	-3,73	13,37
Mean soil temperature	Equal variances assumed	3,19	0,09	-1,37	22,00	0,18	-2,85	2,08	-7,17	1,46
	Equal variances not assumed			-1,51	21,48	0,15	-2,85	1,89	-6,79	1,08
Sunshine intensity	Equal variances assumed	3,21	0,09	-2,54	22,00	0,02	-111,88	43,99	-3,11	-20,65
	Equal variances not assumed			-3,69	13,36	,003 ***	-111,88	30,30	-77,17	-46,59
Sunshine duration	Equal variances assumed	8,82	0,01	-2,64	22,00	0,02	-161,93	61,44	-89,34	-34,52
	Equal variances not assumed			-4,87	21,73	,000 ***	-161,93	33,24	-30,93	-92,94

Due to the unequal number of negative and positive fruit samples, the statistical analyses were based on the assumption of unequal variance. As a result of the analyses, it was found that an increase in temperature, mean soil temperature, sunshine duration and sunshine intensity reduced the probability *of Pelamoviroid latenspruni* detection in fruit tissue. On the other hand, an increase in humidity had a significant effect (P<0.005) decreased the probability of detection. However, rainfallhad no statistically significant (P>0.005) on *Pelamoviroid latenspruni* detection (Table 5).

Table 5. Independent sample test for humidity, temperature, rainfall, mean soil temperature, sunshine intensity and sunshine duration of fruit tissue.

Independent Sample Test FRUIT										
		Levene's Test for Equality of Variances		t-test for Equality of Means						
Environmental conditions		F	P	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Mean Difference	95% Confidence Interval of the Difference	
									Lower	Upper
Humidity	Equal variances assumed	18,49	0,00	2,42	22,00	0,02	18,08	7,47	2,58	33,57
	Equal variances not assumed			4,66	19,91	,000 ***	18,08	3,88	9,99	26,16
Temperature	Equal variances assumed	10,90	0,00	-2,00	22,00	0,06	-9,11	4,55	-18,54	0,33
	Equal variances not assumed			-3,88	19,60	,001 ***	-9,11	2,35	-14,01	-4,20
Rainfall	Equal variances assumed	2,95	0,10	0,58	22,00	0,57	3,55	6,17	-9,24	16,34
	Equal variances not assumed			1,09	20,89	0,29	3,55	3,26	-3,22	10,33
Mean soil temperature	Equal variances assumed	8,18	0,01	-2,13	22,00	0,04	-5,02	2,35	-9,90	-0,14
	Equal variances not assumed			-3,77	21,83	,001 ***	-5,02	1,33	-7,78	-2,26
Sunshine intensity	Equal variances assumed	3,21	0,09	-2,54	22,00	0,02	-111,88	43,99	-3,11	-20,65
	Equal variances not assumed			-3,69	13,36	,003 ***	-111,88	30,30	-77,17	-46,59
Sunshine duration	Equal variances assumed	8,82	0,01	-2,64	22,00	0,02	-161,93	61,44	-89,34	-34,52
	Equal variances not assumed			-4,87	21,73	,000 ***	-161,93	33,24	-30,93	-92,94

As there was an imbalance in the number of leaf tissue samples with negative and positive results, an unequal variance assumption was used in the analyses. As a result of the statistical analyses, it was found that the increase in temperature, mean soil temperature, sunshine duration and sunshine intensity decreased the probability of detection of *Pelamoviroid latenspruni* in leaf tissue, while the probability of detection decreased significantly (P<0.005) with the increase in humidity. Rainfall did not have a statistically significant (P>0.005) effect on *Pelamoviroid latenspruni* detection (Table 6).

Table 6. Independent sample test for leaf tissue moisture, temperature, rainfall, mean soil temperature, sunshine intensity and sunshine duration.

Independent Sample Test LEAF										
		Levene's Test for Equality of Variances		t-test for Equality of Means						
Environmental conditions		F	P	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Mean Difference	95% Confidence Interval of the Difference	
									Lower	Upper
Humidity	Equal variances assumed	18,49	0,00	2,42	22,00	0,02	18,08	7,47	2,58	33,57
	Equal variances not assumed			4,66	19,91	,000 ***	18,08	3,88	9,99	26,16
Temperature	Equal variances assumed	10,90	0,00	-2,00	22,00	0,06	-9,11	4,55	-18,54	0,33
	Equal variances not assumed			-3,88	19,60	,001 ***	-9,11	2,35	-14,01	-4,20
Rainfall	Equal variances assumed	2,95	0,10	0,58	22,00	0,57	3,55	6,17	-9,24	16,34
	Equal variances not assumed			1,09	20,89	0,29	3,55	3,26	-3,22	10,33
Mean soil temperature	Equal variances assumed	8,18	0,01	-2,13	22,00	0,04	-5,02	2,35	-9,90	-0,14
	Equal variances not assumed			-3,77	21,83	,001 ***	-5,02	1,33	-7,78	-0,26
Sunshine intensity	Equal variances assumed	3,21	0,09	-2,54	22,00	0,02	-111,88	43,99	-3,11	-20,65
	Equal variances not assumed			-3,69	13,36	,003 ***	-111,88	30,30	-77,17	-46,59
Sunshine duration	Equal variances assumed	8,82	0,01	-2,64	22,00	0,02	-161,93	61,44	-89,34	-34,52
	Equal variances not assumed			-4,87	21,73	,000 ***	-161,93	33,24	-30,93	-92,94

4. Discussion

A literature search revealed only one study on the impact of viruses and virus-like diseases on the time and organization required to correctly diagnose *Pelamoviroid latenspruni* in fruit trees. In this study, Loreti et al. (1999) compared two different diagnostic methods to identify sensitive, reliable and less time-consuming techniques, particularly suitable for use in international certification schemes. Biological indexing and molecular techniques (dot blot, tissue blot hybridisation and RT-PCR) were tested using two different nucleic acid isolation methods with samples from different plant organs (leaf, buds and bark). Based on the results obtained, it was concluded that the most reliable testing method that can be used to obtain *Pelamoviroid latenspruni*-free peach production material for quarantine and certification programs is testing with a digoxigenin-labelled probe from bud tissue. In our study it was concluded that the most reliable test organs and times were flower bud, bark and leaf in April, flower, leaf, bark and fruit in May and bark, leaf and fruit tissues in September.

In other studies on the appropriate time and tissue for diagnosis of viruses and virus-like agents, Gazel et al. (2020) reported that when there is no seasonal limitation for diagnosis of 'Candidatus Phytoplasma pyri', the most appropriate tissues are fruit and flower, and the most appropriate tissues are root, phloem and shoot cambium tissue and leaves regardless of season. In this study it was concluded that the most reliable test organs when there is a seasonal limitation are flower bud, bark and leaf tissue in April, flower, leaf, bark and fruit tissue in May and bark, leaf and fruit tissue in September. Regardless of the season, bark and leaf tissue samples gave reliable results. EPPO (2023) Plum Pox Potyvirus Diagnostic Standard PM 7/32 (2) states that proper sampling is critical for diagnosis, that sampling should take into account the biology of the virus, and that regional climatic conditions, especially weather conditions during the growing season, are important. It has been reported that sampling should not be carried out at temperatures above 25°C, when the virus titre decreases, and that sampling should be carried out during the dormant period in winter, when the virus titre is low and the tests have the highest analytical sensitivity. In our study, a similar conclusion was reached for *Pelamoviroid latenspruni* and it was concluded that it is appropriate to sample when the *Pelamoviroid latenspruni* density decreases to the point of giving false negative results during the dormant season and the temperature is around 14.4-20.9°C. According to Tomato ringspot nepovirus diagnostic standard PM7/49(1) (EPPO 2005), accurate sampling is essential for diagnosis, and it should consider the virus's biology and regional climatic factors, particularly the weather conditions throughout the growing season. It has been reported that the best time to test samples is between November and April or when the temperature is lower, and when flowering or young leaves are tested, the spring months give the best results for woody plants. In our study, similar results were obtained and it was found that the concentration of *Pelamoviroid latenspruni* was low during the dormant period between November and February, sampling could be done between March and June with the increase in air temperature, and also flower bud, bark and leaf in April, flower, leaf, bark and fruit in May and bark, leaf and fruit tissues in September. When collecting samples for the detection of *Ca.* Phytoplasma mali, '*Ca*. P. pyri,' and *Ca*. P. prunorum, it is essential to consider the biology ohe phytoplasma and the regional climatic circumstances, particularly the weather during the growing season. PM 7/62 (3) of EPPO (2020), it was noted that root samples should be taken from at least 3 different parts of trees suspected of being infected with phytoplasma and that root tissue collected in early winter is more reliable for in the case of *Ca.* P. prunorum on apricot trees, root tissue collected in early winter is more reliable for diagnosis. Care should be taken not to test roots of Pyrus species trees grafted on *Cydonia oblonga*, as this species is not resistant to *Ca*. P. pyri' should not be tested as this species has a different susceptibility to 'Ca. When testing for 'Ca. P. pyri', it was recommended to take leaf samples. In our study it was observed that the tests gave negative results in the months when the plant was dormant, while the leaf tissue gave positive results in April, May, June and September. According to diagnostic standard PM 7/67 (1) of EPPO (2006), in the standard diagnostic method for American plum line pattern virus (APLPV), in spring, leaves are a better source of virus than flowers and bark tissue, and in summer, ripe fruits give better results than leaves. Dormant buds are a reliable source of tissue for winter testing. In our study, flower bud, bark and leaf in April, flower, leaf, bark and fruit in May, flower, leaf, bark and fruit in autumn and bark, leaf and fruit in September were found to give the best results. In Western blot experiments carried out by Martínez-García et al. (2004), the presence of Cucumber vein yellowing virus was detected in all organs and tissues (root, stem, leaf, petiole, shoot, flower and fruit) of cucumber plants inoculated with Cucumber vein yellowing virus. In our study, flower bud, bark and leaf in April in spring, flower, leaf, bark and fruit in May and bark, leaf and fruit in September in autumn gave the best results. According to EPPO (2013) diagnostic standard PM7/118(1), Tomato infectious chlorosis virus samples should be taken from fully developed leaves with slight yellowing between the veins. Virus levels are likely to be low or

undetectable in older symptomatic leaves (bright yellowing, bronzing or necrosis) and young asymptomatic leaves. This pathogen can also be detected in fruit. In contrast, in our study *Pelamoviroid latenspruni* was successfully detected in both young and old leaves, depending on the climatic conditions. Furthermore, in parallel with this study, fruit samples are also a suitable tissue for the diagnosis of *Pelamoviroid latenspruni* depending on the climatic conditions in our study. In general, the upper and fully developed young leaves, i.e. growing tissue, are best for testing. As viroid concentrations can vary significantly and depend on environmental conditions (temperature and photoperiod), the size of the sample to be taken varies depending on the type of test (Verhoeven et al., 2008; 2016). In our study, similar results were obtained, depending on the climatic conditions, bark tissue is a suitable tissue for *Pelamoviroid latenspruni*. Moreover, in summer, only in June, other tissues (bark and leaves) than fruit tissue are suitable for sampling. Temperature (14.4-20.9 °C) and photoperiod (223.1-345 h) were also discovered to be particularly relevant for sampling in our study. In the case of limited infection with certain tospovirus-host combinations (INSV Impatiens necrotic ring spot virus), samples are preferably taken from symptomatic material (Roenhorst and Verhoeven, 1997). *Pelamoviroid latenspruni* was latent and exhibited no symptoms according to our observations. Therefore, samples were taken from asymptomatic plant parts. *Ca. P. phoenicium* can be found on petals, petioles or midveins, but the highest concentration is in the phloem tissue of stems and roots. The concentration of *Ca. P. phoenicium* remains high in the phloem tissue of stems and roots in all seasons except autumn (Jawhari et al., 2015). In contrast to this study, our study found that bark, leaf and fruit tissues gave the best results in the autumn season (especially in September). According to the diagnostic standard PM7/152 (1) of EPPO (2022), the virus concentration depends on the begomovirus race or species, the host plant species, the developmental stage of the plant and the weather conditions. In addition, virus concentration can vary considerably in different parts of the plant; therefore, it is recommended to test symptomatic plants as much as possible for the diagnosis of begomoviruses. For most begomoviruses, the virus is found in higher concentrations in freshly opened young leaves at the top of the plant than in older parts of the plant. Asymptomatic infections can occur on some host plants and it is important to sample different parts of the plant if begomovirus infection is suspected. Similar results were obtained in our study, weather conditions were found to have an effect on *Pelamoviroid latenspruni* concentration and as *Pelamoviroid latenspruni* is latent, sampling was carried out from different parts of the plant. According to the survey instruction of the Ministry of Agriculture and Forestry, the survey period in orchards starts with the formation of flowers in April and May and sampling can be carried out until the end of summer (*Pelamoviroid latenspruni* Survey Instruction 2021). In our study, partly similar results were obtained. In spring, flower bud, bark and leaf in April, flower, leaf, bark and fruit in May, flower, leaf, bark and fruit in autumn, bark, leaf and fruit tissues in September were found to be the best time for sampling. However, in summer only in June, except for fruit tissues, other tissues (bark and leaf) are suitable for sampling.

5. Conclusions

Persimmon is a new host for *Pelamoviroid latenspruni*. Therefore, there is no study on the periods in which this new host can be reliably identified. Our study will serve as a guide for other researchers working on this issue. Two persimmon trees TH7 (accession no: MZ289071) - TH9 (accession no: MZ289070), which were found to be infected with *Pelamoviroid latenspruni* by Oksal et al. (2021). According to the RT-PCR test results, the best sampling time and plant tissues for the detection of *Pelamoviroid latenspruni* were flower bud, bark and leaf in April, bark, leaf, flower and fruit in May and bark, leaf, flower and fruit tissues in September. It is recommended that researchers carrying out studies on this issue should collect samples in the specified months in order to obtain accurate results. Our study was conducted in Malatya province and the study material belongs to this region, and the researchers who will conduct studies should take into account the climatic and ecological conditions in their own regions and the phenological period of the plant.

Climate data obtained from the General Directorate of Meteorology were analysed using the independent samples t-test in the SPSS statistical programme to see if there was a statistical effect on *Pelamoviroid latenspruni* concentration and detectability. According to the results of the statistical analyses of bark, fruit and leaf tissues, it was determined that high temperatures were not suitable for PLMVd testing and the most suitable sampling temperature was 14.40-20.90 °C. It was also concluded that autumn (except September) and winter months, when the plant is dormant, are not suitable for sampling. It was concluded that the appropriate moisture content for PLMVd testing was 41.40-49.30%. The mean soil temperature suitable for sampling was 7.35-14.40 °C. Sunshine duration was 223.10-345.00 hours, which was found to be more

suitable for sampling. It is more suitable for sampling when the sunshine intensity is 223.56-313.33 cal÷cm². The climate data used in our study belong to Malatya province and our results are based on these data. It is recommended that researchers who are going to carry out studies should take these conditions into consideration.

To prevent economic losses and contamination by PLMVd, it is important that the materials used in production are free of viruses and viroids. Preference should be given to certified and healthy seedlings. In addition, regular control of aphids, destruction of plants found to be infected or showing signs of disease, and regular disinfection of tools and equipment used in mechanical or cultural processes with 5-20% sodium hypochlorite (bleach) solution are effective preventive measures to limit the spread of the pathogen.

Conflicts of Interests

Authors declare that there is no conflict of interests

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Statement contribution of the authors

This study's experimentation, analysis and writing, etc. all steps were made equally by the authors.

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