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Phyto-virologic status of sour cherry in Bulgaria

Фитовирусологичен статус на вишната в България

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ABSTRACT

This study investigated the prevalence and distribution of major viruses (PNRSV, PDV, ApMV, ACLSV, CLRV, RpRSV, and PPV) in sour cherry trees across nine regions in Bulgaria. A total of 746 sour cherry trees were sampled, comprising 534 from commercial orchards and 212 from individual trees. Samples were tested using DAS-ELISA for the presence of these viruses; ELISA for ACLSV was performed by Cocktail-ELISA. Serologically identified PNRSV and PDV were further analyzed using RT-PCR after RNA isolation from fresh leaves using a modified mini CTAB method. The overall infection rate was 14.2%, with PNRSV being the most prevalent virus (10.9%), followed by PDV (2.1%) and mixed infections (1.2%). Notably, approximately 70% of infected trees displayed no visible symptoms. No ACLSV, ApMV, PPV, CLRV, or RpRSV infection was detected in the surveyed orchards. Molecular RT-PCR analysis confirmed the presence of PNRSV and PDV at the molecular level in ELISA-positive samples. These findings underscore the importance of continuous monitoring and control measures to manage viral diseases in sour cherry orchards in Bulgaria.

Keywords: *Prunus cerasus*, viruses, detection, ELISA, RT-PCR

РЕЗЮМЕ

Изследването разглежда разпространението и честотата на основните вируси (PNRSV, PDV, ApMV, ACLSV, CLRV, RpRSV и PPV) при вишната в девет региона на България. Взети са проби за анализ от 746 дървета, от тях 534 са от производствени овощни градини и 212 от единични дървета. Пробите бяха тестирани чрез DAS-ELISA за наличие на горепосочените вируси; ELISA за ACLSV бе проведен чрез Cocktail-ELISA. Серологично идентифицираните вируси PNRSV и PDV бяха допълнително анализирани чрез RT-PCR след изолация на РНК от свежи листа с помощта на модифициран мини CTAB метод. Общата степен на инфекция бе 14.2%, като PNRSV бе най-разпространен вирус (10.9%), последван от PDV (2.1%) и смесени инфекции (1.2%). Приблизително при 70% от заразените дървета не се наблюдаваха видими симптоми на вирусна инфекция. Не бе установена инфекция с ACLSV, ApMV, PPV, CLRV или RpRSV в изследваните овощни градини. Анализът чрез RT-PCR потвърди наличието на PNRSV и PDV на молекулярно ниво. Тези резултати подчертават значението на непрекъснатото наблюдение и контролни мерки за управление на вирусните заболявания в овощните градини в България.

Ключови думи: *Prunus cerasus*, вируси, идентифициране, ELISA, RT-PCR

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INTRODUCTION

The natural and climatic conditions, established traditions, and existing orchards provide a good basis for developing fruit growing in Bulgaria. Among stone fruit species, sour cherry (*Prunus cerasus* L.) occupies relatively small areas (1,453 ha in 2023). It has limited production (3,274 t in 2023) in Bulgaria (Ministry of Agriculture and Food, 2024), despite the significant economic potential of this species, particularly in the processing industry. Monitoring the phytosanitary status of the orchards is essential to achieve good production.

Diseases caused by viruses can lead to significant economic losses due to reduced yields and diminished fruit quality. In 2017, 20 viruses and 2 viroids were reported to infect sour cherry (Rubio et al., 2017). Since this last review, additional sour cherry infections have been reported, resulting in a list of 28 sour cherry-infecting viruses and 2 viroids (Desiderio et al., 2024). Among these, the most prevalent and widespread viruses belong to the genus Ilarvirus within the family *Bromoviridae* - prunus necrotic ringspot virus (PNRSV) and prune dwarf virus (PDV). These viruses represent a considerable risk to cherry production, leading to notable reductions in both yield and fruit quality, inducing leaf and fruit deformations, and delaying fruit maturation (Pallas et al., 2012). They are transmitted through infected propagating material, seeds, and pollen, contributing to their widespread distribution (Kelley and Cameron, 1986; Barba et al., 2015).

Another widespread virus affecting sour cherry trees is the apple chlorotic leaf spot virus (ACLSV) (Pérez-Sánchez et al., 2017), belonging to the genus Trichovirus. Most sweet and sour cherries are latently infected with ACLSV. However, certain virus strains are known to cause necrosis on the fruits of susceptible cultivars (Desvignes and Boye, 1989; Desvignes, 1999). The severity of necrosis on cherry fruits is exacerbated when there is a mixed infection of ACLSV and PNRSV (Nemeth, 1986; Verderevskaja and Marinescu, 1985).

The plum pox virus (PPV) is the most dangerous pathogen affecting *Prunus* species, belonging to the genus Potyvirus. This virus causes sharka disease, one of the most devastating viral infections in stone fruit crops. Ten PPV strains (D, M, Rec, EA, W, T, An, C, CR, and CV) have been identified based on full-length genome analysis. Among these, only PPV-Cherry (C), PPV-Cherry Russian (CR), and PPV-CV (Cherry Volga) strains can infect sweet and sour cherries, making them the cherry-adapted strains (Kalashyan et al., 1994; Crescenzi et al., 1995; Maxim et al., 2002; Chirkov et al., 2017; James et al., 2013; García et al., 2014).

The cherry leaf roll virus (CLRV) and raspberry ringspot virus (RpRSV), both belonging to the genus Nepovirus, are also occasionally found in sour cherries, though not as commonly (Mandic et al., 2007; Pavliuk et al., 2021).

A survey conducted in 2018 to assess the prevalence of ilarviruses in sweet and sour cherries in Bulgaria included a limited number of sour cherry samples, primarily from the collection orchard of the Institute of Agriculture in Kyustendil (Kamenova et al., 2020). This study did not provide a comprehensive understanding of the distribution of viruses in sour cherries in Bulgaria. The prevalence of numerous virus infections in commercial sour cherry plantations across the country remains unclear.

This study aimed to investigate the presence, distribution, and symptoms of major viruses (PNRSV, PDV, ApMV, ACLSV, CLRV, RpRSV, and PPV) in sour cherry trees in commercial orchards in different regions in Bulgaria. The results of this investigation should provide current information on the prevalence of sour cherry viruses in Bulgaria and offer additional data on their frequency and associated symptoms.

MATERIALS AND METHODS

Plant material

Field observations and samples were collected during the springs of 2019–2024 from nine regions of Bulgaria (Kyustendil, Pernik, Kostinbrod, Berkovitsa, Botevgrad, Plovdiv, Kazanlak, Pazardzhik, and Strelcha). A total of 746 sour cherry trees were sampled, including 534 from eight commercial sour cherry orchards and 212 from in-

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dividual trees. Four of the commercial orchards had an area of over 15 hectares, and the primary cultivar was 'Oblačinska'. The cultivar represented in the 20-year-old commercial orchard at the Institute of Agriculture in Kyustendil was 'Erdi Bőtermő'. The surveyed orchards ranged in age from 7 to 25 years. For each tree, 15-20 leaves were collected from the four quadrants. Samples were taken randomly from trees exhibiting or not exhibiting virus-like symptoms.

Serological assays

All collected samples were tested using the Double Antibody Sandwich-ELISA (DAS-ELISA) method for the presence of PNRSV, PDV, ApMV, CLRV, RpRSV, and PPV (Clark and Adams, 1977). ELISA for ACLSV followed the modification proposed by Flegg and Clark (1979), known as Cocktail-ELISA. Commercial diagnostic kits produced by Loewe Phytodiagnostica GmbH were used, following the manufacturer's recommended protocol. Samples with an optical density (OD) three times higher than the average of negative controls were considered positive.

RT-PCR analyses

Serologically identified PNRSV and PDV from a limited number of sour cherry samples, primarily from the Kyustendil region, were analyzed using the RT-PCR method. RNA was isolated from fresh leaves using a modified mini CTAB method (Li et al., 2008).

The coat proteins (CP) of PNRSV and PDV were amplified using a two-step RT-PCR method. First, cDNA was synthesised using the smART Reverse Transcriptase kit (EURx, Ltd.). For this, 2 µl of template RNA, 1 µl (10 mM) of the respective specific antisense primer (listed below), and 2.5 µl (2.5 mM) dNTPs were added and brought up to 12.5 µl with sterile distilled water. The mixture was heated to 65 °C for 5 min and quickly chilled on ice. Then, 4 µl of 5X first-strand buffer, 2 µl of 0.1 M DTT (both supplied with the kit), 0.5 µl of RNase OUT (40 units/µl), and 1 µl smART (200 U/µl) were added, mixed gently, and incubated at 50 °C for 30 minutes. The reaction was terminated by heating at 85 °C for 5 min.

Secondly, PCR amplifications were performed by mixing 2 µl of cDNA with 23 µl of the amplification mixture containing 2.5 µl of 10X Buffer, 1.5 µl of $MgCl_2$ (25 mM), 2.5 µl dNTPs (2.5 mM), 0.5 µl of each primer (10 mM), 0.25 µl OptiTaq DNA polymerase (5 U/µl; EURx, Ltd.), and 15.25 µl RNase-free sterile water.

Oligonucleotide primer sequences 5'-AGTGTGCTTATCTCACTCTAG-3', and 5'-ATGGTTTGCCGAATTTGCAATCAT-3' (Paduch-Cichal and Sala-Rejczak, 2011) were used to detect PNRSV. This primer pair amplifies a 700 bp fragment of the RNA3 CP coding region of the PNRSV.

The PDV-specific primers, 5'GTGTAGAAAGAAGAGAAGTCCGACAAG-3' and 5' ATCTAGAAGCAGCATTTCCAACTACGA-3' (Boulila, 2009) that amplify a 613 bp fragment, were used for RT-PCR for PDV detection.

Amplified products (5 µl each) were stained with SimplySafe™ (EURx, LtD.) and electrophoresed at 100–120 V for 45–60 min in a 1.2% agarose gel in 1x TBE buffer, pH 8.0

RESULTS AND DISCUSSION

Field Observations

The sour cherry trees in each orchard and the single ones were initially monitored for virus-like symptoms through visual observation. The most severe symptoms were observed in trees of the 'Oblachinska' cultivar from the 15-year-old orchard in Botevgrad, manifesting as necrosis, spots, and ruptures on the leaves (Figure 1A). In these trees, PNRSV infection was confirmed through serological testing. Yellow chlorotic spots on the leaves of individually grown trees were noted in Kyustendil (Figure 1B). These symptoms were found in trees infected with PDV. In the commercial orchard at the Institute of Agriculture – Kyustendil, chlorotic rings, spots, and chlorosis on the leaves of the 'Erdi Bötermö' cultivar infected with PNRSV were observed during the spring of the study period (Figure 1C, D). As the vegetation progressed, the symptoms diminished, and the trees appeared visually healthy.

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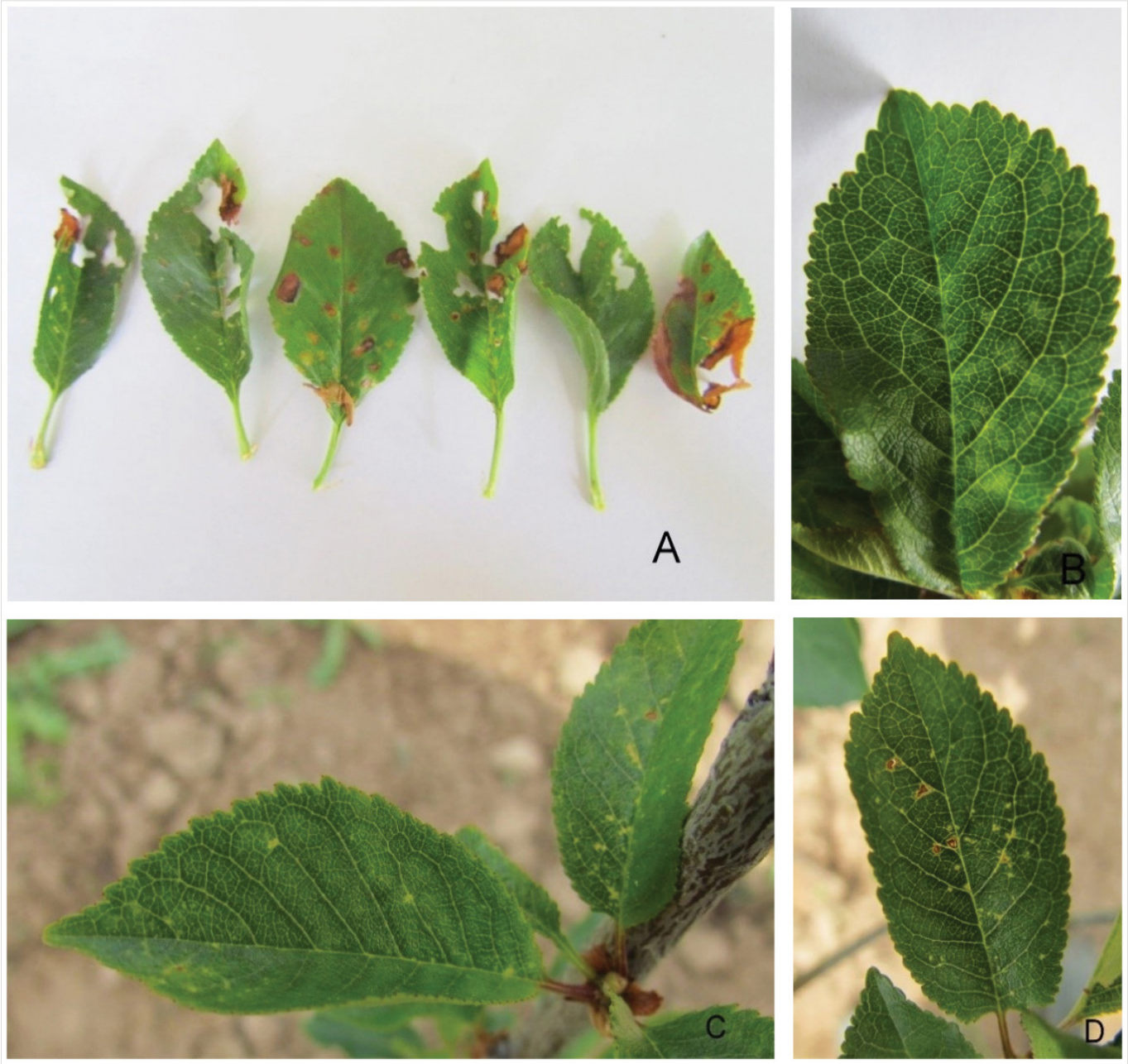

Figure 1. PNRSV and PDV symptoms on sour cherry leaves: **A**: severe necrosis, spots, and rupture on PNRSV-infected cultivar 'Oblacinska'; **B**: yellow chlorotic spots on the leaves infected with PDV; **C**: chlorotic rings, spots, and chlorosis on cultivar "Erdi Bötermö" infected with PNRSV; **D**: yellow and necrotic rings on leaves infected with PNRSV

Approximately 70% of the infected trees exhibited no obvious symptoms during data collection. The absence of visible symptoms in a significant proportion of the studied trees necessitates conducting serological and molecular tests to confirm the presence of viral infections.

Serological evaluation

A total of 746 sour cherries were screened (534 from commercial orchards and 212 single-grown trees) using enzyme-linked immunosorbent assay (ELISA) for seven common stone fruit viruses, and the results are presented in Table 1.

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Table 1. Virus infection detected by ELISA in sour cherries in different regions of Bulgaria

Region / District	Type and location of the orchard	Number tested/ number infected (%)	Positive for			
			PNRSV	PDV	PNRSV+ PDV	ACLSV, ApMV, PPV, CLRV, RpRSV
Western / Kyustendil	1 commercial orchard, IA-Kyustendil	28/15 (53.6%)	11 (73.3%)	0	4 (26.7%)	0
Western / Kyustendil	3 commercial orchards (v. Konayvo; v.Vaksevo; v. Resilovo)	112/17 (15.2%)	6 (35.3%)	10 (58.8%)	1 (5.9%)	0
Western / Kyustendil	Single trees	38/13 (34.2%)	8 (61.5%)	2 (15.4%)	3 (23.1%)	0
Western / Pernik	1 commercial orchard (v. Kondofrej)	90/1 (1.1%)	1 (1.1%)	0	0	-
Western / Kostinbrod	Single trees	46/4 (8.7%)	2 (50.0%)	2 (50.0%)	0	-
Northwest / Berkovitsa	1 commercial orchard (v.Bokilovtsi)	89/0	0	0	0	0
Northwest / Botevgrad	1 commercial orchard (v. Skravena)	127/45 (35.4%)	43 (95.6%)	1 (2.2%)	1 (2.2%)	-
South Central / Plovdiv	Single trees (v. Stroevo)	44/2 (4.5%)	2 (100%)	0	0	0
South Central / Kazanlak	Single trees	45/5 (11.1%)	4 (80.0%)	1 (20.0%)	0	0
South Central / Pazardzhik	1 commercial orchard (v. Slavovica)	88/0	0	0	0	0
South Central / Strelcha	Single trees	39/4 (10.2%)	4 (100%)	0	0	-
Total		746/106 (14.2%)	81 (76.4%)	16 (15.1%)	9 (8.5%)	0

The total virus infection rate among the studied sour cherry trees from nine regions of Bulgaria was 14.2%. The percentage of virus infection varied across different orchards, ranging from 0% in a 5-year-old garden in Pazardzhik, Southern Bulgaria, to 53.6% in a commercial orchard of the Institute of Agriculture in Kyustendil. A relatively high infection rate (35.4%) was found in the monoculture orchard in Botevgrad, Northwestern Bulgaria.

The most prevalent virus was PNRSV, found in 76.4% of infected trees, followed by PDV in 15.1% of infected trees. In nine of them (8.5%), a mixed infection of PNRSV + PDV was found. Our results confirm the higher susceptibility of sour cherry to PNRSV compared to PDV, as found in previous study of Kamenova et al. (2000), and reported by Paprstein et al. (1995) and Rouag et al. (2008). Other researchers have also detected PNRSV more frequently in sour cherry samples than in sweet cherries (Ulubaş and Ertunç, 2004; Oliver et al., 2009; Suchá et al., 2010; Çevik et al., 2011).

It should be noted that despite the relatively extensive list of tested viruses, only PNRSV and PDV were detected. Five of the studied viruses (ACLSV, ApMV, PPV, CLRV, and RpRSV) were not serologically identified in any of the

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analyzed sour cherry trees in our work. A study carried out in 2014-2015 in the Czech Republic found that out of 19 studied viruses, PNRSV and PDV were the most abundant in sweet and sour cherry orchards and wild trees (Přibylová et al., 2020).

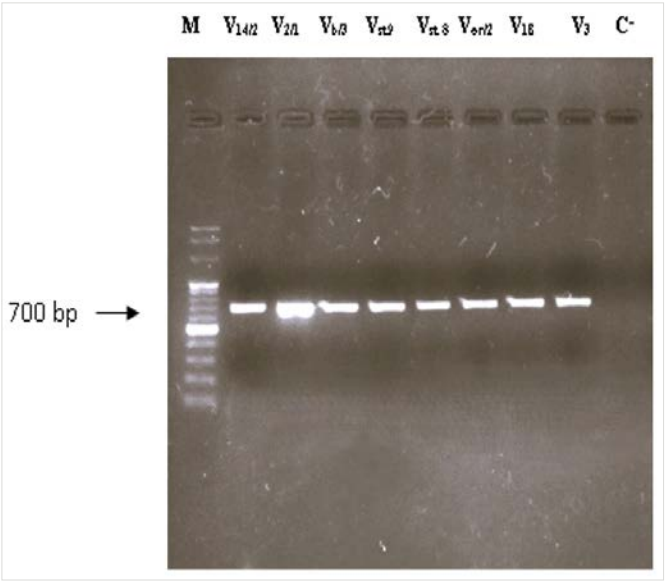
No infection of sour cherry trees by ACLSV has been reported in previous studies conducted in Bulgaria (Borisova, 2005) or in neighboring countries such as Turkey and Serbia (Sipahioğlu and Baloğlu, 2006; Mandić et al., 2007). ACLSV has a very limited distribution in sour and duke cherry-growing areas in Southwest Europe (Pérez-Sánchez et al., 2017). A survey of propagation stock orchards of sour cherry, sweet cherry, and their clonal rootstocks carried out in 2018 in Ukraine did not detect the presence of CLRV, ACLSV, ApMV, and PPV (Pavliuk et al., 2019). In subsequent research, Pavliuk et al. (2021) found TBRV and ACLSV in sour cherry orchards, and the occurrence of previously undetected sour cherry viruses once again confirms the need for testing of propagating plant materials. PNRSV was found in mixed infections with CLRV and ACLSV in declining sour cherry trees of the cultivar Marasca and has been identified as one of the main reasons for the death of cherry trees of this cultivar (Šarić et al., 1986).

The presence of a relatively high rate of PNRSV infection in this study, up to 35.4-53.6% in some orchards, and its spreading through pollen indicates that the phytosanitary situation in sour cherry plantations should not be underestimated.

RT-PCR detection

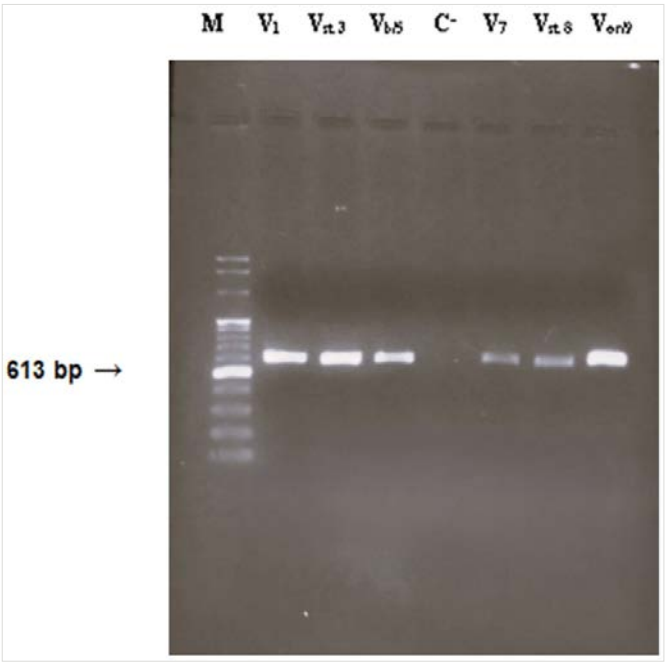
A total RNA was extracted from eight PNRSV isolates previously serologically proved by DAS-ELISA and from one PNRSV-free sour cherry tree, used as a negative control. The expected fragment of 700 bp was obtained from all analyzed PNRSV isolates (Figure 2).

RT-PCR successfully amplified the targeted genome portion of the six PDV isolates collected from ELISA-positive PDV sour cherry trees. Amplicons of 613 bp were obtained with PDV primers targeting the coat protein gene (Figure 3).



M: ladder, C -: negative control; letters and numbers at the top of starters indicate isolate names

Figure 2. RT-PCR detection of PNRSV in sour cherry trees



M: ladder, C -: negative control; letters and numbers at the top of starters indicate the names of isolates

Figure 3. RT-PCR detection of PDV in sour cherry trees

CONCLUSIONS

This study provides insights into the viral status of commercial sour cherry orchards in Bulgaria. The overall average infection rate was determined to be 14.2%. The most prevalent virus among the tested sour cherry trees was PNRSV, affecting 10.9% of the trees. Infections with PDV were observed in 2.1% of the trees, while mixed infections were found in 1.2% of the samples. Significantly, approximately 70% of the infected trees showed no visible symptoms of viral infection.

No ACLSV, ApMV, PPV, CLRV, and RpRSV infection was found in the surveyed sour cherry orchards.

The presence of PNRSV and PDV in the sour cherry trees was conclusively confirmed at the molecular level using RT-PCR analysis.

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