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Research Article

Assessment of the virus infections occurrence in new established plum and sweet cherry orchards in Transylvania, Romania

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Abstract

Sixteen plum and eleven sweet cherry newly established commercial orchards in Transylvania, Romania, were the subject of the survey during the vegetative periods of 2020-2021 in order to assess the occurrence of different virus infections. Two blocks of 200 trees from each orchard were monitored by visual observation for virus-like symptoms, especially for *Plum pox virus* (PPV) in plum orchards. Twenty trees of each plum orchard were then sampled and tested for serological detection of PPV, *Prune dwarf virus* (PDV), *Prunus necrotic ringspot virus* (PNRSV), *Apple chlorotic leaf spot virus* (ACLSV), *Apple mosaic virus* (ApMV) and *Myrobalan latent ringspot virus* (MLRSV). Similarly, ten trees of each sweet cherry orchard were sampled and tested for the presence of PDV, PNRSV, ApMV, ACLSV, PPV, *Arabis mosaic virus* (ArMV), *Cherry leaf roll virus* (CLRV), *Raspberry ringspot virus* (RpRSV), *Strawberry latent ringspot virus* (SLRSV) and *Tomato black ring virus* (TBRV) by serologic assays. Additionally, a few sweet cherry trees suspected to be infected by *Little cherry virus*-1 (LChV-1) were tested by molecular assay. Unexpectedly, no plum orchards were found to be free of PPV. The average level of PPV infection was 32%. PNRSV occurred in 8.1% and PDV in 1.2% of sampled plum trees. Four out of ten viruses were detected in sweet cherry sampled: PDV (3.6%), ACLSV (0.9%), RpRSV (0.9%) and TBRV (0.9%). No infection with ACLSV, ApMV and MLRSV in plum and PPV, PNRSV, ApMV, ArMV, CLRV, SLRSV and LChV-1 in sweet cherry was detected. Mixed infections occurred at 4.8% in plum, and at 0.9% in sweet cherry trees sampled. The average occurrence of viruses in plum and sweet cherry orchards surveyed in Transylvania was determined at 41.3%, and 6.3% respectively. Overall results revealed a critical situation especially in regards to PPV infections making the success of fruit production in the most surveyed plum orchards quite problematic. Virus infections level in the younger sweet cherry orchards were significantly lower.

Keywords: stone fruits; survey; virus incidence; young orchards

Introduction

Plum (*Prunus domestica* L.) has traditionally been the dominant fruit species in Romania followed by apple and sweet cherry. Romania ranks the first place in European plum production, with 692,670 tonnes and

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ranks second in world production behind China. In terms of plum production area, Romania is in the second place in Europe with 65,580 ha, after Serbia, and in the third place in the world behind China and Serbia. At a yield of 10.56 t/ha Romania ranks 12th place in Europe and 24th in the world (FAOSTAT, 2020). This situation raises some questions about the technologies applied, the control of diseases and pests, and also on viral status of the trees in orchards. Obviously, some of these losses could be attributed to viruses and therefore, a strict control of planting material and other prevention measures are required.

To limit the quantitative and qualitative losses caused by viral pathogens, the strategy for their control represents a priority in integrated management of orchards. Viral diseases are very difficult to control in fields because there is no curative treatment for virus infected crops (Nicaise, 2014). Therefore, applying prophylactic measures remain crucial in reducing the economic losses caused by viruses' infections. The use of virus-free plant propagating material produced according to certification schemes (EPPO 2001a, 2001b; Roy, 2011), the placement of the new orchards as far away as possible from sources of infection, using virus resistant cultivars, if any (Scorza *et al.*, 2013; Ventura *et al.*, 2019), limit the population of vector-organisms by chemical treatments, removal and burning of infected plants, control on weeds and other host plants in the vicinity of fields (Nicaise, 2014) are useful tools to prevent viral infection. Also, preventing viruses from entering a new area is essential because eradication by any methods other than tree removal is not possible once these pathogens infect an area where trees are growing (Reed and Foster, 2011).

Sometimes viruses can escape in newly established orchards due to an inadequate implementation of phytosanitary control measures. Also, free movement of propagating material within the European Union increases the risk of the spread of viruses and novel viral strains into new areas. In young orchards, occurrence of a new virus or viral strain can create serious issues and even can compromise the grower's investment if it has a high economic impact, as *Plum pox virus* (PPV) has on plum. In Romania and other plum growing countries, the plum is highly affected by PPV which causes Sharka, the most devastating viral disease of stone fruits (Barba *et al.*, 2011). The estimated costs associated with Sharka management worldwide exceed 10.000 million euros (Cambra *et al.*, 2006). Sharka disease strongly affects quality of fruits and often causes their premature dropping, and can seriously compromise most of the production of sensitive cultivars (Dunez and Sutic, 1988; Nemeth, 1994). From infected plants PPV is easily transmitted by grafting, and naturally by aphid species in a non-persistent manner (Labonne *et al.*, 1995).

For more than a century following its first description from Bulgaria in around 1917 (Atanasoff, 1932), Sharka has been spreading around the Mediterranean basin, Middle East, and from there to Western Europe, Asia, Africa (Egypt, Tunisia), North (Canada) and South (Chile, Argentina) America (Roy and Smith, 1994; Barba *et al.,* 2011) becoming a global concern. In some countries, such as United States of America, Sweden, Finland, and Estonia, PPV has been eradicated. In Lebanon and New Zealand, PPV has not been detected by surveys (EPPO, 2021). In Molecular Plant Pathology, PPV is highlighted as one of the top ten plant viruses with worldwide distribution (Scholthof *et al.*, 2011).

PPV was determined to have a high infection incidence (average 68.5%) in the main plum-growing areas from Romania, and of twenty-seven orchards surveyed none were found to be PPV-free (Zagrai *et al.*, 2010a).

Ten distinct strains of PPV are known: D (Dideron), M (Marcus), EA (El Amar), C (Cherry), Rec (Recombinant), T (Turkey), W (Winona), An (Ancestor Marcus), CR (Cherry Russian), and CV (Cherry Volga). The most common strains are D, M and Rec (Kerlan and Dunez, 1979; Candresse *et al.*, 1998; Glasa *et al.*, 2002). A large-scale survey performed in Romania revealed that only D and Rec strains are present in plum orchards (Zagrai *et al.*, 2010b). PPV-C was reported in Romania in sweet cherry trees from an orchard in Bistrița, in 2001 (Maxim *et al.,* 2002). At that time, the entire orchard was destroyed and a new survey done ten years later did not detect the presence of PPV-C in the area and in other orchards (Zagrai *et al.*, 2011).

In addition to PPV, there are many other viruses which can cause direct or indirect damage of stone fruits negatively affecting crop production (Hadidi and Barba, 2011). *Prune dwarf virus* (PDV) and *Prunus necrotic ring spot virus* (PNRSV) both belonging to genus *Ilarvirus*, transmitted by pollen and seeds, have an

economic importance in stone fruit crops as they can cause crop losses depending of the *Prunus* species, cultivar and viral strain (Nemeth, 1986; Caglayan *et al.*, 2011; Hammond, 2011). PDV and PNRSV can affect stone fruits in singular infections, but also frequently in mixed infections expressing a synergic effect that often lead to a progressive decline of stone fruit trees (Uyemote and Scott, 1992). PDV, in single and mixed infections with PNRSV, negatively influences the chemical composition of sweet cherry fruits (Maxim and Papp, 2000). *Apple mosaic ilarvirus* (ApMV) causes reduction of tree growth and decrease of fruit production (Nemeth, 1986). Occurring in mixed infections with other viruses from the ILAR group, ApMV might have significant economic impact due to the synergistic action of the viruses involved (Paunovic *et al.,* 2011), even if is not transmitted through pollen (Digiaro *et al.*, 1992) and seed (Barba *et al.*, 1986). *Apple chlorotic leaf spot trichovirus* (ACLSV) is practically present in all areas of stone fruit crops and has detrimental effects in some infected fruit species (Myrta *et al.*, 2011). Fruit quality and graft compatibility could be affected by some viral isolates of ACLSV (Delbos and Dunez, 1988; Desvignes and Boye, 1988).

Tree growth, orchard longevity, production and quality of the fruits might be affected by viruses belonging to genus *Nepovirus* that can infect different species of stone fruits in singular or mixed infections. Certification schemes of stone fruits require testing of plants for the following nematode-borne viruses: *Myrobalan latent ringspot virus* (MLRSV), *Arabis mosaic virus* (ArMV), *Cherry leaf roll virus* (CLRV), *Raspberry ringspot virus* (RpRSV), *Strawberry latent ringspot virus* (SLRSV) and *Tomato black ring virus* (TBRV) (Martelli and Uyemoto, 2011).

A viral disease with economic impact for sweet and sour cherry is Little Cherry Disease (LChD) caused by *Little cherry virus-*1 and -2 (Eastwell, 1997; Rott and Jelkmann, 2001, 2005). In severe infections, this disease produces important crop losses (Yorston *et al.*, 1981; Uyemoto and Scott, 1992).

Surveys regarding the incidence or occurrence of viruses in different types of pome or stone fruit orchards (commercial, germplasm collections) were performed in many areas where these species are growing. Some examples of viruses' occurrences determined by serological or molecular techniques were done in Albania (Digiaro *et al.*, 1994), Serbia (Mandic *et al.*, 2007), Turkey (Ulubas, 2008; Ulubas and Ertunc, 2008), Bosnia and Herzegovina (Matic *et al.*, 2008), China (Ni *et al.*, 2012), Latvia (Gospodaryk *et al.*, 2013), Lebanon (Nassar *et al.,* 2012).

Once a virus infection accidentally occurs in a young orchard, removing the infected trees remains the main measure for limiting virus spread. Therefore, early identification of infections in new orchards can sometimes be life-saving for the orchard. Thus, monitoring of viruses in the new orchards, followed by suitable measures for limiting virus spread, may reduce the damage caused by viruses.

New plum and sweet cherry commercial orchards were established in the last years by using planting material produced both in Romania and in different European countries. This was possible mainly due to EU funding within a National Rural Development Program.

The aim of this study was to assess the occurrence of viruses in some new established plum and sweet cherry orchards from Transylvania in order to obtain information concerning the initial viral status of planting material and also to provide recommendations to farmers depending on their specific situations.

Materials and Methods

Biological materials and plantations investigated

Sixteen plum and eleven sweet cherry young commercial orchards in Transylvania were the subject of the survey during the vegetative periods of 2020 (plum) and 2021 (cherry). The age of the selected plum and sweet cherry orchards for virus monitoring was between 1 and 6 years old. All planting material used to set up the orchards was provided as "Certified" category.

Field monitoring of plum orchards and sampling

Five out of sixteen plum surveyed orchards were established with planting material propagated in Hungary, one in Germany, and the other ten with material produced in Romania. Preliminary control involved an initial overall inspection in each orchard selected for survey, followed by the delineation of two representative blocks in the diagonal of the orchard, covering the entire range of cultivars, comprising a total of 200 trees (100 trees per block). Also, GPS positioning was performed in the middle of each block so that the location of the orchard could be digitally verified. All trees within the blocks were individually monitored by visual observation of virus-like symptom development. Since Sharka disease is a long and permanent issue in Romania, the field surveys were mainly focused on typical PPV symptoms on leaves that allowed a preliminary assessment of PPV occurrence based on the visual observations. Ten trees from each block were then sampled for virus detection by serological assays, as follows: when PPV-like symptoms were determined to be lower than 10% per block, one symptomatic and nine asymptomatic trees were randomly sampled. When the PPV occurrence based on visual observation was noted to be between 10 and 20% per block, two symptomatic and eight asymptomatic trees were sampled, and so on, so that when PPV-like symptoms occurred between 90-100% per block, ten symptomatic trees were sampled. If no symptomatic trees observed within the two blocks, ten symptomless trees were randomly sampled from each block. Since PPV-M, known as the most epidemic strain of PPV, was not reported so far in Romania (Zagrai *et al.*, 2010b), additional samples with typical PPV symptoms were collected from young orchards established with planting material from abroad for further molecular discrimination in order to detect the potential for cross border spread (data not shown). For virus diagnosis by serological assays, a minimum of ten leaves per tree were randomly collected throughout the canopy. Only symptomatic leaves were collected when trees showed PPV typical symptoms on leaves, such as yellow or pale rings, diffuse spots or leaf mottling. When symptoms were limited to particular branches, leaves were only sampled from symptomatic branches.

Field monitoring of sweet cherry orchards and sampling

Four out of eleven sweet cherry surveyed orchards were established by using propagated material from Romania, and seven with plant material produced in Hungary, Italy, Spain, Germany, Netherlands and Belgium. Similar to the sampling methodology for plum, two blocks with a total of 200 sweet cherry trees of each orchard were first monitored by visual observation of virus-like symptom development. Within the blocks, in depth visual checking was performed, each tree being verified for the presence of potential symptoms which could suggest possible viral infections, and then the samples were taken. Five trees of each block were randomly sampled in line for further laboratory analysis. A minimum of ten leaves per tree were collected throughout the canopy. GPS positioning was taken in the middle of each block. Seven trees from two orchards, that apparently developed early leaf reddening on leaves, were prior sampled to check for possible infection with *Little cherry virus* -1.

Inoculum sources nearby

In some cases, old orchards or old isolated trees were located within 1-200 m from the newly established plum and sweet cherry orchards under study. In these cases, symptoms of virus infection in some older trees were evaluated both visually and through serological assays.

Serological procedure and viruses tested

A total of 420 trees (310 of plum and 110 of sweet cherry) were sampled for serological virus diagnosis performed by Double Antibody Sandwich - Enzyme Linked Immunosorbent Assay (DAS-ELISA) (Clark and Adams, 1977). Polyclonal antibodies reagents against PPV, PDV, PNRSV, ACLSV, ApMV (Bioreba, Switzerland), and MLRSV (Sediag, France) were used to detect viral infections in the new established plum orchards. Similarly, complete kits (Bioreba, Switzerland) for PDV, PNRSV, ApMV, ACLSV, ArMV, PPV,

CLRV, RpRSV, SLRSV and TBRV detection were used for sweet cherry, according to the producer's recommendations. Samples consisting in fresh leaves were ground in extraction buffer 1:20 (w/v) using a HOMEX 6 homogenizer. Absorbance values were measured at 405 nm optic density after 1 h substrate hydrolysis using Magellan universal reader control and data analysis software from TECAN. Samples were considered positive if their absorbance values were more than twice those of the negative control. Positive and negative controls provided in kits were used in serological assay.

Virus occurrence

PPV infections in the plum orchards were determined by correlating the preliminary assessment of PPV based on visual observations with the results obtained by serological diagnosis. When PPV infections based on visual monitoring were confirmed by serological diagnosis, PPV infection was established based on visual symptoms. When correlations between visual and serological data were only partially correlated an adjustment was made according to serological results.

The occurrences of the other viruses, other than PPV, both in plum and sweet cherry, were determined based on laboratory tests.

Molecular diagnosis

Molecular assay RT-PCR (Reverse Transcriptase-Polymerase Chain Reaction) was used to check if *Little Cherry virus*-1 (LChV-1) was present in a few suspected trees. Seven samples from two sweet cherry orchards, which developed early leaf reddening, were tested by RT-PCR. Leaf samples were ground in liquid nitrogen to a fine powder and use for total RNA extraction using the Spectrum Total Plant RNA kit (Sigma-Aldrich, USA). cDNA was prepared from tenfold diluted total RNA using the iScript cDNA Synthesis Kit (Bio-Rad, Belgium), and for amplification FastStart Taq DNA Polymerase kit (Roche, Switzerland) was used according to the manufacturer's instructions. RT-PCR detection of LChV-1 was carried out using previously described specific primers: LCUW7090/LCUWc7389 (Bajet *et al.*, 2008), amplifying a 300-bp fragment spanning the ORF1b encoding the RNA dependent RNA-polymerase (RdRp) gene.

Results

Monitoring of virus spread in plum orchards

Symptomological observation

Typical PPV symptoms on leaves, such as yellow or pale rings, diffuse spots or leaf mottling were observed in all sixteen new established plum orchards surveyed from six counties in Transylvania (Figure 1).

Figure 1. Symptoms of PPV on plum leaves

DAS-ELISA test

All surveyed orchards were found to be infected by at least one virus (Table 1). The most widespread virus in plum orchards was PPV followed by PNRSV and then by PDV. As expected, PPV infections were confirmed in all symptomatic samples. There were only two cases of discrepancy between visual observations and serological results (orchards no. 8 and 11), where one asymptomatic sample per orchard proved to be infected with PPV. The highest PPV infection (77.5%) was noted in orchard no. 6, and the lowest (1%) in orchard no. 16.

A large range of PPV detection between orchards was found, with 1-10% in orchards 12, 14 and 16; 11-20% in orchards 1, 2, 3, 4 and 10; 21-30% in orchards 7 and 15; 31-40% in orchard 13; 51-60% in orchard 11; 61-70% in orchards 5 and 9; and 71-80% in orchards 6 and 8. Infections with PDV were found in three orchards at a rate of 5-10% (no. 7, 8 and 14), while PNRSV infections were detected in seven orchards in a rate of 5-50% (no. 7, 9, 11, 13, 14, 15 and 16). No infection with ACLSV, ApMV and MLRSV was identified by DAS-ELISA test.

Table 1. The occurrence of viruses in some new plum orchards in Transylvania (2020)

Orchard code	Location/ County	Age of trees	The origin of plant material	Cultivars in surveyed orchards	PPV inoculum sources* nearby surveyed orchards	PPV infection** (%)		Occurrence of viruses** (%) based on DAS-ELISA test				
						based on typical symptoms on leaves	corroborated with DAS-ELISA test	PDV	PNRSV	ACLSV	ApMV	MLRSV
1	Buduslau/ BH	3	Hungary	Topend plus, Jofela	-	16	16	0	0	0	0	N/A***
2	Dumitra/ BN	1	Romania	Stanley, President, C. Lepotica	+	19	19	0	0	0	0	0
3	Jelna/ BN	1	Hungary	Stanley, Topend plus	+	14	14	0	0	0	0	0
4	Ciceu-Mihaiești/ BN	4	Romania	Stanley, Topend plus	+	16.5	16.5	0	0	0	0	N/A
5	Cluj-Napoca/ CJ	6	Hungary	Topend	-	61.5	61.5	0	0	0	0	0
6	Cluj-Napoca/ CJ	6	Hungary	Topend	-	77.5	77.5	0	0	0	0	0
7	Tritenii de Sus/ CJ	4	Romania	Stanley, D'Agen	+	24	24	5	30	0	0	0
8	Turdaș/ HD	3	Romania	Anna Spath, Stanley	+	68.5	73	10	0	0	0	N/A
9	Brad/ HD	3	Romania	Tuleu gras, Stanley	+	61	61	0	50	0	0	N/A
10	Ribița/ HD	1-3	Romania	Tuleu gras, Stanley, Anna Spath	+	15.5	15.5	0	0	0	0	N/A
11	Reghin /MS	5	Hungary	Haganta, President, Blue free	+	50	55	0	10	0	0	N/A
12	Reghin/ MS	5	Germany	Tophit, Cacak	+	5	5	0	0	0	0	N/A
13	Cehal/ SM	1	Romania	Stanley, C. Lepotica, Blue free, Centenar	+	39	39	0	10	0	0	N/A
14	Cehal/ SM	3	Romania	Stanley, C. Lepotica	+	9.5	9.5	5	10	0	0	N/A
15	Săcășeni/ SM	2	Romania	Stanley	+	24	24	0	5	0	0	N/A
16	Săcășeni/ SM	2	Romania	Stanley	+	1	1	0	15	0	0	N/A

*Symptomological observation of PPV inoculum sources nearby the new plum orchards ('+' the presence of PPV by typical symptoms on leaves; '-' the absence of virus-like symptoms); ** Single-viral infection ; *** N/A – not applicable

Occurrence of mixed infections in plum

Single-virus infections were found in most of the serologically tested samples (95.2%). However, there were a few cases where mixed infections were identified (Table 2). The most common mixed infections involved two viruses, PPV+PNRSV (3.9%), followed by PPV+PDV (0.6%) and PDV+PNRSV (0.3%). Mixed infections of three or more viruses in tested samples were not detected. Mixed infections in new plum orchards surveyed in Transylvania was determined at 4.8%.

Table 2. The occurrence of mixed infections in new established plum orchards from Transylvania (2020)

Orchard code	No. of total plum trees tested	Mixed infections			
		PPV+PNRSV	PPV+PDV	PDV+PNRSV	Total mixed infections
1	20	0	0	0	0
2	20	0	0	0	0
3	20	0	0	0	0
4	20	0	0	0	0
5	20	0	0	0	0
6	20	0	0	0	0
7	20	**2**	0	**1**	**3**
8	20	0	**2**	0	**2**
9	10	**5**	0	0	**5**
10	20	0	0	0	0
11	20	**2**	0	0	**2**
12	20	0	0	0	0
13	20	**2**	0	0	**2**
14	20	0	0	0	0
15	20	0	0	0	0
16	20	**1**	0	0	**1**
Total	**310**	**12**	**2**	**1**	**15**

PPV inoculum sources nearby

The presence of PPV external inoculum sources nearby (less than 200 m) in different hosts (wild *Prunus*, ornamental, old plum orchards) have been noted in thirteen out of sixteen plum orchards by typical symptoms on leaves and virus presence was confirmed by serological diagnosis (data not shown).

Monitoring of virus spread in sweet cherry orchards

Symptomological observation

Most of the surveyed sweet cherry orchards showed no symptoms that could suggest viral infections. However, there were a few cases when typical symptoms of PDV (Figure 2), such as chlorotic diffuse rings or spots, chlorosis of veins, mottling, were observed on leaves sporadically in some sweet cherry surveyed orchards.

Figure 2. Symptoms of PDV on sweet cherry leaves

DAS-ELISA test

Four out of ten viruses checked in the eleven young sweet cherry orchards in Transylvania were detected by using DAS-ELISA test (Table 3). Four orchards (no. 20, 21, 22 and 23) revealed viral infections with one to three viruses. Serological results showed a prevalence of PDV with an incidence of 10% in each of four orchards infected. Three other viruses (ACLSV, RpRSV and TBRV) were identified in two orchards (no. 20 and 21) with a rate of 10% for each of them. No infection with PPV, PNRSV, ApMV, ArMV, CLRV and SLRSV was identified through DAS-ELISA tests.

Table 3. The occurrence of viruses in some new sweet cherry orchards from Transylvania (2021)

Orchard code	Age of trees	Location/ County	The origin of plant material	Occurrence of viruses* (%) based on										
				DAS-ELISA										RT-PCR
				PPV	PDV	PNRSV	ACLSV	ApMV	ArMV	CLRV	RpRSV	SLRSV	TBRV	LChV-1
17	3	Pinticu/BN	Belgium	0	0	0	0	0	0	0	0	0	0	N/A**
18	5	Ghinda/BN	Netherlands	0	0	0	0	0	0	0	0	0	0	N/A
19	4	Dumitra/BN	Belgium	0	0	0	0	0	0	0	0	0	0	N/A
20	2	Corpadea/CJ	Romania	0	**10**	0	0	0	0	0	**10**	0	**10**	0
21	2	Săcășeni/SM	Romania	0	**10**	0	**10**	0	0	0	0	0	0	N/A
22	2	Săcășeni/SM	Romania	0	**10**	0	0	0	0	0	0	0	0	0
23	5	Aușeu/BH	Hungary	0	**10**	0	0	0	0	0	0	0	0	N/A
24	1	Valea Mare de Cris/BH	Italy	0	0	0	0	0	0	0	0	0	0	N/A
25	1	Noșlac/AB	Romania	0	0	0	0	0	0	0	0	0	0	N/A
26	1-6	Stăuini/AB	Germany/ Netherlands/ Spain/ Italy	0	0	0	0	0	0	0	0	0	0	N/A
27	2	Cincu/BV	Italy	0	0	0	0	0	0	0	0	0	0	N/A

* Single-viral infection

** N/A - not applicable

Occurrence of mixed infections in sweet cherry

Single-viral infections generally occurred in infected sweet cherry trees, except in orchard no. 20 where in two trees three viruses were detected, one tree with mixed infection of PDV+TBRV, and the other tree with a single-viral infection with RpRSV. No other mixed infection was detected in sweet cherry trees analysed. The occurrence of mixed infections in sweet cherry orchards surveyed in Transylvania was calculated at 0.9%.

Molecular assays

RT-PCR results revealed that all seven samples (three samples from orchard no. 20 and four samples from orchard no. 22) were not infected with LChV-1 since no signal of amplification was detected (Figure 3).

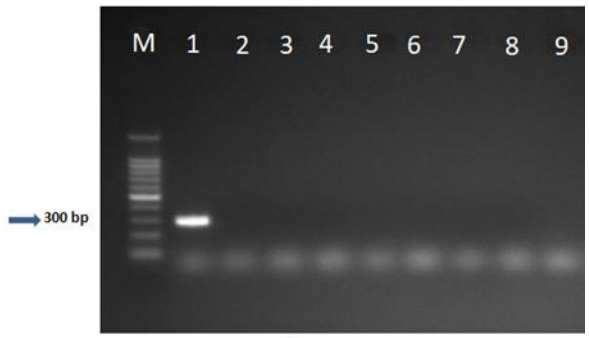

Figure 3. Gel electrophoresis (1.5% agarose) of RT-PCR amplified products using specific primer set for LChV-1

M - marker, 1 - Positive Control (+); 2-4 - samples from orchard no. 20; 5-8 - samples from orchard no. 22; 9 - Negative Control (-).

The occurrence of viruses in plum and sweet cherry orchards surveyed in Transylvania was determined at 41.3%, and 6.3% respectively (Figure 4).

In the new plum orchards from Transylvania the PPV infection rate was 32%, while PNRSV was 8.1% and PDV 1.2% of tested plum samples. No infection with ACLSV, ApMV or MLRSV was identified within the surveyed plum orchards by serological tests.

In the sweet cherry orchards from Transylvania the occurrence of PDV was 3.6%, and ACLSV, RpRSV and TBRV were 0.9% each. No infection with PPV, PNRSV, ApMV, ArMV, CLRV or SLRSV was detected in the surveyed sweet cherry orchards by serological tests.

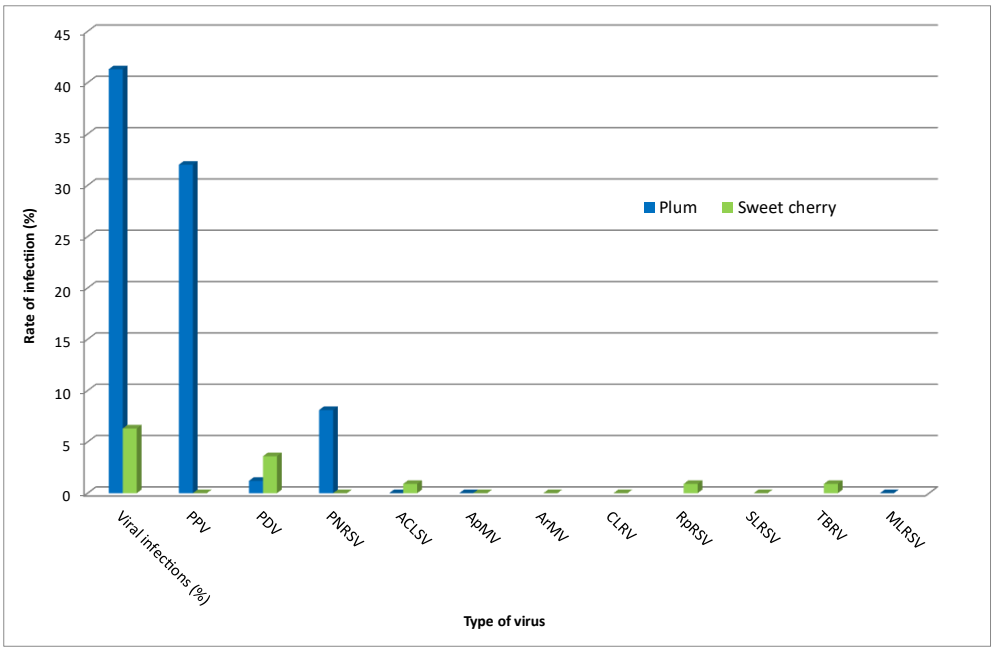

Figure 4. The occurrence of viruses in some new plum and sweet cherry orchards in Transylvania

Discussion

Overall results on the occurrence of viruses in the newly established plum orchards revealed a worrying situation in regards to PPV infections in Transylvania since all surveyed orchards confirmed the presence of the virus. It was ascertained that one third of the young plum surveyed orchards (no. 5, 6, 8, 9 and 11) recorded a very high rate of PPV infection (55-77.5%). This critical situation makes the potential success of fruit production in most of these orchards to be quite problematic. Obviously, the current situation happened as a result of an inadequate implementation of preventive measures and of recommendations regarding the establishment of new plum orchards.

On one hand, this critical situation raises question about the 'real' initial viral status of the 'Certified' planting material since the surveyed plum orchards were very young. Although there were some inoculum sources near most of the surveyed orchards, this cannot justify the high rate of PPV infection in some newly established orchards. This not necessarily means that all newly planted trees were provided in an infected state. However, high infection with PPV correlated with the young age of the trees, which in most cases developed PPV typical symptoms throughout the canopy support the hypothesis that at least as part of the reason for the early infection of the newly planted trees was a consequence of deficiencies in the chain of producing 'Certified' plum material in some nurseries.

In addition, it appeared that the recommendation to avoid setting up the new plum orchard close to potential PPV inoculum sources of infection was neglected in most cases.

Since PPV is easily transmitted in natural way from infected plants by aphid species in non-persistent manner (Labonne *et al.*, 1995), any inoculum sources near the newly established orchards would play an important role in rapid virus spread. Unexpectedly, most of the orchards (thirteen out of sixteen) were established in the proximity of PPV inoculum sources, favouring the potential for virus spread to the new orchards. The density of the orchard, its surface area, the distance from the orchard to the nearest source of infection and the distance to the first symptomatic tree previously detected influences the risk of a tree becoming infected over time (Dallot *et al.*, 2004). With the exception of three orchards (no. 1, 5 and 6) all plum orchards were located near a potential source of PPV infection. Indeed, the new plum orchards were established in a PPV endemic country, where it is difficult to find even small PPV-free areas. However, growing non-host PPV fruit trees species (i.e. apple, pear) as buffer zone around the new plum orchards could be a solution to contain PPV spread. Unfortunately, this kind of recommendation was not well understood both by farmers and consultants.

Long distance spread of PPV is possible mainly because of the introduction of infected propagative plant material resulting from inadequately controlled exchanges of propagative materials (Cambra *et al.*, 2006). This scenario is at least partly applicable to our case.

Comparing to another region from Romania, the average of PPV infection in new plum orchards in Transylvania (32%) was higher than in Moldova (19.4%) (Zagrai *et al.*, 2021).

Generally, the symptoms of PPV were very well expressed on the leaves, which mean that a visual assessment of plum orchards could represent an effective method of signalling possible outbreaks of infection and taking timely limitation measures. This statement is supported by the serologic confirmation of all symptomatic samples and only a few cases of PPV detection by DAS-ELISA in asymptomatic samples.

In most of the surveyed plum orchards 'Stanley' is the predominant cultivar. It is a cultivar that, when infected, shows few or no PPV symptoms on fruit. From this point of view, although problematic, these orchards should be economically viable if plum-specific cultivation technology is applied, such as soil maintenance, pruning, removal of suckers, chemical treatments against PPV vectors, application of fertilizers, etc. In the cases of PPV infection with low incidence it is recommended to remove trees with typical symptoms of PPV and to replace them with virus-free planting material, followed by orchard monitoring in the following years in order to reduce the spread of infection to other trees in the orchard.

PNRSV was detected in seven plum orchards and PDV in three out of sixteen surveyed in Transylvania. These viruses are transmitted by pollen and seeds (Cameron *et al.*, 1973; Amari *et al.*, 2009; Caglayan *et al.*, 2011) with implication in spreading into stone fruit trees (Mink, 1992). This raises another question concerning the health of planting material since these orchards are very young. Compared to the Moldova region, where occurrence of PNRSV in plum orchards was 4.1% (Zagrai *et al.*, 2021), in Transylvania this was almost twice as high (8.1%). The rate of PDV infection was similar in Transylvania (1.2%) and Moldova (1.8%). The absence of ACLSV, ApMV and MLRSV in surveyed plum orchards in Transylvania, and also in Moldova (Zagrai *et al.*, 2021) revealed that the most widespread viruses in the new plum orchards from Romania are PPV, followed by PNRSV and PDV. In contrast, in Latvia the most widespread viruses in plum commercial orchards were PNRSV and PDV (Gospodarek *et al.*, 2013). Studies performed in young plum orchards in California showed 20% infections with PNRSV and PDV (Uyemoto *et al.*, 1989).

Mixed infections were often reported in stone fruits orchards (Mandic *et al.*, 2007; Gospodaryk *et al.*, 2013; Pavliuk *et al.*, 2019; Borisova *et al.*, 2021). In our survey, mixed infections with two viruses (PPV+PNRSV; PPV+PDV; PDV+PNRSV) were detected on fifteen plum trees out of 310. The most prevalent combination was PPV+PNRSV in twelve trees, five of them in one single orchard (no. 9). Mixed infections with PDV and PNRSV can induce peach stunt disease (PSD) as a synergic reaction (Uyemoto and Scott, 1992). Therefore, the occurrence of mixed infections in plum trees surveyed in this study, at a level of 4.8%, together with the presence of single-virus infection with PPV, PNRSV and PDV could represent a real concern about the profitability of newly established plum orchard in Transylvania. Compared with other studies (Gospodarek *et al.*, 2013) the occurrence of mixed infections in commercial plum orchards from Latvia was higher (13.8%) than in our study, the prevalent combination of viruses was PNRSV+PDV. However, it should be mentioned that our studies were focused on young plum commercial orchards (1-6 years old).

The situation of virus infection status of sweet cherry surveyed orchards revealed a quite good situation in Transylvania. However, there were recorded infections with PDV of 3.6%, and of 0.9% for ACLSV, RpRSV and TBRV. All infected sweet cherry orchards were established with planting material propagated in Romania and Hungary. Since there were no old sweet cherry orchards or solitary trees that could have acted as virus hosts that were identified near these orchards, it is plausible to attribute the presence of these viruses to nurseries. The most eloquent example is orchard no. 20 where three viruses were detected, one of these as mixed infections and there were no potential inoculum sources in a large area surrounding this newly planted orchard.

A recent study performed in Bulgaria regarding the incidence of *Ilarviruses* (PDV, PNRSV, ApMV) in young sweet cherry orchards (Borisova *et al.*, 2021) reported a relatively high incidence of PDV (7.6%) and PNRSV (4%) compared with our results. Another survey done in Bulgaria (Kamenova *et al.*, 2019) revealed an average rate of PDV infection in sweet and sour cherry at about 14.4%, with 7.6% as mixed infection with PNRSV. Infections with PDV and PNRSV were also reported in young sweet cherry orchards from California, at about 4% (Uyemoto *et al.*, 1989). Similar to our results, in Serbia the most prevalent virus in sweet cherry was PDV (Mandic *et al.*, 2007), but with a higher incidence (20%) than in our study (0.9%).

No LChV-1 infection was detected by molecular analyses in sweet cherry samples collected from symptomatic trees, therefore the apparent symptoms do not seem to be related to the virus, but could be side effects of excess soil moisture recorded in the orchards where these symptoms were observed or other possible causes due to soil nutrient levels including deficiencies or excesses of macro- /microelements or other possible viral pathogens. In contrast, a survey on sweet cherry trees from Japan reported an occurrence of LChV-1 of 14% (Isogai *et al.*, 2004), and from Germany, 22% (Schroder and Petruschke, 2010).

A large study in stone fruits (24,000 trees serologically tested from 14 countries) led by the Mediterranean Agronomic Institute of Bari, during 1992 to 2007 (Myrta *et al.*, 2003; Pallas *et al.*, 2012), revealed high incidences of PNRSV (46.4%) and PDV (40.7%), and also of mixed infections (9.6%), most of them PNRSV and PDV. Of the stone fruits species tested, cherry was the most infected, the average of viral infections was 45.6%. A study performed in western Mediterranean region of Turkey revealed that the most

widespread virus in stone fruits was PDV (26.3%), followed by PNRSV (9.1%), and mixed infections on sweet cherry also occurred with high incidence (9.6%) (Yardimci and Culal-Kilic, 2011).

In contrast to the high infection rates from other countries, our results are interesting considering that seven out of eleven sweet cherry orchards were established by using planting material propagated in Belgium, Netherlands, Hungary, Germany, Spain and Italy, and only four of them with plant material produced in Romania. The differences noted may be due to the young age of orchards (1-6 years) that were the focus of our studies. Our findings raise some questions about the planting material produced in our country and in the neighbouring country, Hungary, which seems to have some shortcomings in the production chain of virus-free planting material in some nurseries.

The high level of PPV occurred in the new established plum orchards might reveals a dysfunction in the verification of the propagating material before being validated as belonging to the 'Certified' category. Although virus levels in the new planted sweet cherry orchards were relatively low, their presence indicate that improved virus-free verification practices are needed.

The results of this study corroborated with those obtained in Moldova (Zagrai *et al.*, 2021) and Muntenia (Plopa and Butac, 2020; Plopa *et al.*, 2021) have created the opportunity to develop a verified image about the virus infection status of some newly orchards of plum and sweet cherry in Romania, that can be used to provide to farmers recommendations for better management of viral diseases.

Conclusions

The assessment on the occurrence of viruses in the new established plum orchards in Transylvania revealed a critical situation especially in regards to PPV infections making the future fruit production of the most orchards quite problematic. Contrary, a significantly better situation was assessed on new established sweet cherry orchards which create the premises for real success.

Authors' Contributions

I.Z. and L.A.Z. conceived and designed the analysis; L.A.Z., S.D.R.M., G.M.G. and C.M. collecting the samples from plum and sweet cherry orchards; C.M. prepared samples for serological tests; L.A.Z. and G.M.G. performed the serological analyses; L.A.Z. and I.Z. performed molecular analyses, data interpretation and wrote the paper. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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