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Study of the Earliest Symptoms of Plum Pox in the Sofia Valley and Vratsa Region

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Abstract

Since ancient times, the fruits of the plum have occupied an important place on the table of the Bulgarians. To obtain high and stable yields an important condition is the proper organization and conduct of plant protection activities and the fast, the correct and accurate diagnosis of PPV - Stoneware. In our climate, Plum pattern manifests symptoms first on the first spring leaves. The described symptoms of flowers in different stone species are few and scarce, so we conducted the present study in two following years 2019 and 2020 to describe, differentiate and compare visual symptoms of flowering with the results of DAS-ELISA tests on the leaves of the studied stone species. From the surveyed PPV host species in two orchards 12 mather trees with color symptoms were selected and described in two regions of Bulgaria - the Sofia field (Vrajdebna (1,2 ha) and the Vratsa region (Roman Orchard Nursery (0,4 ha). In a total of 9 samples, PPV infection was confirmed. The established deviations were found only in fruit crops that bloom with white flowers. Based on the results and conclusions, the present study will contribute to the timely detection of the causative agent of pox virus in the earliest symptoms in nurseries and gardens and will provide the right and quick direction to overcome the damage through proper plant protection activities.

Key words: PPV; earliest symptoms; early diagnostics; blooms; Bulgaria

INTRODUCTION

In botany, the plum is classified as belonging to the *Prunus* genus of the rose family (*Rosaceae*), plum subfamily (*Prunoidea*). The basis of cultivated plum varieties is formed by the domestic plum (*Prunus domestica*), the thorn plum (*Prunus insititia*) and the cherry plum (*Prunus cerasifera*). The plum is and will be one of the main fruit crops in Bulgaria. To obtain high and stable yields from existing plum plantations, it is important to organize and implement appropriate plant protection measures and, last but not least, to make a correct diagnosis of the most important diseases. A variety of methods are used to study phytopathogenic viruses. Some of them are preferred over others for one reason or another, most often identified as simplicity or accuracy. Notwithstanding those considerations, it is good to use different methods in the study of viruses so as to avoid errors caused by side effects (Stoev, 2007).

Historical Overview of the Disease

The symptoms of this disease were first observed in Bulgaria around 1916-1917, which means it began to be referred to as "pox", or "Sharka" in Bulgarian, at the end of the First World War, although some reports show symptoms were observed in Macedonia as early as 1910. Nevertheless, the first book describing the disease's viral nature came out in 1932, where Atanasoff (1935) described it as "PLUM POX". Hristov later (1937) observed and described pox that affected apricots in Bulgaria during 1933. In Bulgaria's climate, the earliest symptoms of plum

pox appear on the first spring leaves. Pale green broad annular spots scattered throughout the leaf blade are a typical feature. As the leaves age, the symptoms are masked, becoming difficult to notice in the second half of summer and in autumn. Symptoms on the fruit vary the most: dots to large spots, dimples, single or numerous furrows and rings, grouped or scattered across the fruit, sunk more or less into the pericarp like the symptoms on the viruses descripted by Bos (1964). In yellow fruit varieties, the fruits are pale red, while in olive and dark green ones, they are green, with their color blue-violet before ripening. Under the visible symptoms, the fruit flesh is necrotic, with its color changed to pale red or red-brown and more or less resinous. In many cases, the damaged flesh is firmly attached to the stone. Fruits with symptoms of the disease have a bad taste and, very often, lower sugar content. In a number of varieties, ring- and arc-shaped symptoms appear on the stones, corresponding to the shapes on the damaged fruit flesh. The symptoms appear on the stalks of fruits and shoots. In moderately sensitive varieties, affected fruits fall off during ripening depended of the strain in Bulgaria (Kamenova & Borisova, 2019) as a reported from Serbia (Jevremović & Paunović, 2014). In highly sensitive varieties, 80-100% of the fruits fall off prematurely, making them unsuitable even for processing in the alcohol industry (Atanasoff, 1932). The virus can be transmitted by stones, although that is difficult. It was found by ELISA that the cotyledons of plum, peach and apricot seeds contain 21% to 93%. In the germ of those seeds, the infection is 0% to 11%. When using stones with a viral infection of 81%, seed infestation of 0% to 14% was found (Németh, 1963; Németh & Kolber, 1983).

The causative agent is the plum pox potyvirus (PPV). Its virions are filamentous, with a size of 764 x 20 nm, thermal inactivation point (TIP) of 50-55°C, dilution limit of 10^{-4} and stability at room temperature of up to 48 hours. The incubation period lasts 9 to 13 months. Like other plant viruses, plum pox consists of several strains based on biology, serological reactions and molecular and biological data. To date, four PPV strains or serogroups have been identified, namely M, D, Al and C (Wetzel et al., 1991; Kalashyan et al., 1994). All isolates within each strain are different and have been serogrouped. PPV-D is a Dideron strain originally isolated from apricot in south-eastern France and the most common variant of the virus in Western Europe and Bulgaria (EPPO, 2023; Kamenova et al., 2003; Jevremović & Paunović, 2014; Kamenova & Borisova, 2019). This strain is not transmitted by seeds, it can be transmitted by vectors, but that is unlikely in the real world (Sheveleva et al., 2018). The Marcus strain is known as PPV-M and was originally isolated from a peach in northern Greece and it is the main natural host of this strain in Central Europe (Németh & Kolber, 1983). It is mentioned that an isolate of PPV-M found in France is very aggressive and causes peach necrosis, resulting in rapid leaf fall and tree death (Kegler & Schade, 1971; Rancovic, 1975). **PPV-EA** is an El Amar strain originally isolated from an apricot in Egypt. So far, PPV-EA is only found in North Africa (Myrta et al., 2006). There is a **PPV-C strain** found in cherries that was originally isolated from sweet cherry hybrid and cherry in Moldova (Kalashyan et al., 1994). The disease has an endemic incidence in Bulgaria (Kamenova et al., 2017). Based on serological analysis of more than 3000 leaf samples of *Prunus* spp., the highest level of PPV infection was detected in plum (82%), followed by peach (40%) and apricot (32%) (Kamenova & Borisova, 2019). A high infection rate in plum cultivars grown in our country are reported by, Kamenova & Milusheva (2005), Dragoyski et al. (2009, 2010), Petrov (2014) and Borisova & Sotirov (2021), but by ELISA tests on leaves, not on flowers. There are no known treatments of the disease that can be used once the tree becomes infected. Infected trees have to be destroyed. Once the disease has been identified, measures to control and prevent plum pox include conducting field examinations, using certified nurseries, controlling aphids and eliminating infected trees in nurseries and orchards (Németh, 1994; Kamenova et al., 2003; Borisova & Sotirov, 2021).

Using indicator plants to diagnose PPV is the easiest and most common method. It essentially consists of applying sap from the test material on indicator (test) plants, so the virus can penetrate into their cells. When leaves are mechanically infected with sap from a diseased plant, some plant species' reaction is local necrotic damage. The number of local lesions on each leaf depends on the viral concentration in the inoculum (Ericsson, 1929). The susceptibility to mechanical infection varies widely in individual plants. Serological methods are currently considered routine diagnostic methods for

PPV. One of the most common modifications of the serological method is ELISA (enzyme-linked immunosorbent assay), which is based on the ability to establish an antigen-antibody relationship on the basis of an immunological reaction observable as an enzyme-induced change in color. ELISA has numerous variants. In this method, the intensity of staining of each well in the plate is proportional to the amount of virus in the sample. Having methods to detect plum pox is very important in making a rapid and reliable diagnosis during production of a fruit seedlings. This study will therefore contribute to detecting the causative agent of plum pox as soon as the earliest symptoms appear in bloom period nurseries and orchards and will provide appropriate and quick instructions for overcoming the damages by properly conducting plant protection activities.

GOALS AND TASKS

This study aims to: detect and identify the virus causing plum powdery mildew (PPV) in the field of experimental crops in Vrazhdebna and the nursery of the Roman city of flowers; correlate and prove the first visual symptoms during flowering with the laboratory results; and to accept them as a reliable indicator of the presence of the virus in these two regions of Bulgaria. To achieve our goals, we set the following tasks: to find and identify the cause of plum pox (Plum pox virus) in the Hostile Experimental Field and the city nursery Orchard (of Roman town); analyze the different types of symptoms in plums, cherries and peaches to demonstrate transmission and existing reservoirs of new infections; identify and differentiate viral, phytoplasma and fungal pathogens by visual signs. The final task involves summarizing the survey data and making recommendations to producers on how to prevent and combat the disease and reduce the risk to volume and quality of production.

MATERIALS AND METHODS

The study covers plant materials from orchards in 2019, 2020 in the Vrazhdebna Test Crop Field and from stock trees in the Orchard Nursery from Roman region. Plant samples. A total of 12 plant parts were subjected to laboratory analysis to identify the viral causative agent of diseases in fruit crops and use ELISA to detect already-identified pathogens (Table 1).

Preparation of the plant part samples to be tested. Flowers. They all had symptoms characteristic of PPV infection. Under laboratory conditions, those samples were reduced to weigh no more than 1 g. The samples, in a ratio of 1:10, were ground in a DAS-ELISA extraction buffer (Clark & Adams, 1977; Adams, 1978) in accordance with the manufacturer's requirements for the ELISA kit (Sediag SAS, 2018). Serological tests were performed at the Central Laboratory for Plant Quarantine (CLPQ) in Sofia, using isolates, benchmarks, buffers and reagents for serological analyses and controls made by SEDIAG, France. All laboratory analyses were performed at the Sofia CPQL, while the samples were prepared and itemized at Laboratory 19 of the University of Forestry, Sofia.

The methods we used were aimed at detecting and identifying the first symptoms of plum pox in the Vrazhdebna Test Crop Field and the Orchard Nursery in Roman town. *Morphological diagnostics.* Fruit crops, such as plum, cherry, peach and nectarine, were examined during the visual diagnostics, because there is literature evidence that they are hosts of the plum pox virus, the causative agent of pox in fruit stone crops. We compared the signs observed with the literature sources. Enzyme-linked immunosorbent assay (ELISA). PPV in plant

Table 1. Description of samples (flowers and leaves) for PPV identification by ELISA

No	Culture	Variety	Origin
1.	Cherry	Van	CLPQ
2.	Plum	Chachanska lepotitsa	Roman
3.	Cherry	Van	Vrazhdebna
4.	Peach	Red Haven	Vrazhdebna
5.	Peach	Somerset	Vrazhdebna
6.	Peach	Juneta	Vrazhdebna
7.	Plum	Black Beauty	Roman
8.	Plum	Black Star	Roman
9.	Plum	Outman	Roman
10.	Peach	Gold Haven	Vrazhdebna
11.	Plum	Stanley	Roman
12.	Nectarine	New Jersey	Roman

extract from mechanically infected indicator plants and from purified virus was identified by DAS-ELISA (PM 7/32 OEPP/EPPO, 2004). Direct DAS-ELISA was performed with reagents made by Sediag SAS France. We used a polyclonal antiserum (detected race M, D and C). We accepted as positive the samples whose optical density (OD) was more than twice the extension value of the negative control, known as "cut off". There is a third version of the results, which we interpreted as indeterminate. It is dependent on the optical density (OD) values between the negative and positive control limits (Clark & Adams, 1977).

RESULTS

Identification and differentiation by visual signs during flowering. The symptoms observed in the trees we examined during the flowering phase varied depending on the species and varietal identity of the different fruit crops. After the examinations, we focused mainly on the trees that showed signs close to that of a viral infection. As a result, we found that the flowers of some trees had significant deviations from the normal shape and color, as follows:

Flowers: the most obvious malformations and decolourisation were observed in species with pink petals, such as peaches and nectarines. In comparison with the visibly healthy flowers provisionally marked as infected, we observed a reduction in flower size and a change in flower shape. Infected petals were wrinkled and incomplete. In many cases, there were deviations from the normal color in certain parts or entire petals. Decolourisation was not observed in species with white flowers, where only the shape was affected – incomplete and wrinkled. In contrast to the visibly healthy flowers, those with changes in size and color remained on the branches for a significantly shorter time, falling off earlier than the others (Fig. 1).

Leaves: wide rings and spots appear, the shape of which is influenced by the veins of the leaves. The symptoms are identical to those described in

Figure 1. Flowers with signs of PPV: A) peach; C) Plum; C) Nectarine

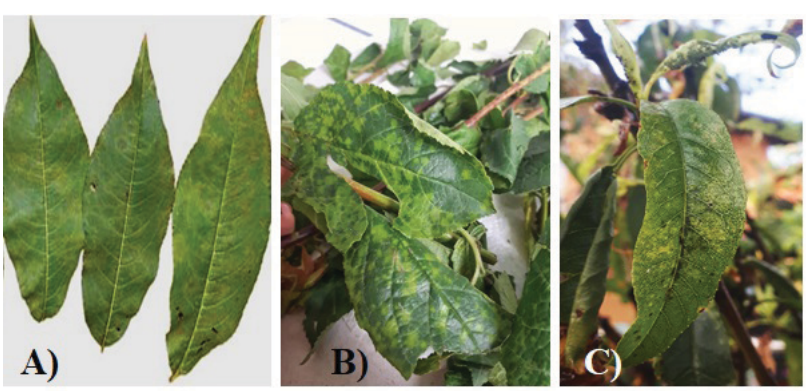

Figure 2. Leaves with simptoms of PPV infection: A) peach C) plum C) nectarine

literature (Atanasoff, 1932, 1960; Kamenova & Milusheva, 2005). The spots are pale green. As the plants transition to later phenophases, those spots become masked (Fig. 2).

The examinations performed during flowering revealed symptoms of early brown rot on the cherry in the Vrazhdebna Test Crop Field and on the peach in the Roman Orchard Nursery similar as the symptoms described from Bobev et al., 2019 (Fig. 3).

No other bacterial and phytoplasma diseases were detected. Only after some of the marked plum trees had flowered, we found visual symptoms of pocket plum gall on the fruits the Vrazhdebna Test Crop Field. The disease's causative agent is *Taphrina pruni* with the same symptoms as described in the literature (Redfern & Shirley, 2002; Ljubojevic, 2021) (Fig. 4).

Figure 3. Simptoms of early brown rot: A) cherry C) peach.

Figure 4. Simptoms of *Tafrina pruni* on Plum

The results of ELISA and the measurement of optical density (OD), performed according to the manufacturer's requirements in the last stage of ELISA for PPV, are shown in Table 2 and Table 3.

DISCUSSION OF RESULTS

Out of the total 12 stone fruit samples we analyzed, 9 samples came out positive for PPV, or one more than the visual examination. In all samples of stone fruit species with pink coloration, the visual characteristics identified on the flowers were confirmed as existing PPV infection, or 100%. The visual and serological methods did not detect one plum sample, whose flowers were white, or about 33% chance of discrepancy between existing but unproven visual identification of the infection.

The results of the serological method performed on the leaves of stone fruit species largely confirmed the already-established PPV infection. Our visual examinations showed signs of infection in 9 samples, or one more than the flower examinations. The laboratory tests performed by serological methods on 12 leaf samples of stone fruit species came out positive for PPV in 7 samples, with two samples showing indeterminate results (uncertainty of ++ / -). The difference between the two tests is probably due to substances accumulating as the vegetation of the fruit crops progresses. Those substances can act as inhibitors in ELISA. As a confirmation, we can point to the difference between the extension values of OD measured in flowers and leaves. For both samples showing an indeterminate result, additional analysis is required to prove the existing infection.

CONCLUSIONS

1. We detected the plum pox virus by the DAS-ELISA method in peach and nectarine trees in the Vrazhdebna Test Crop Field and in plum trees in the Orchard Nursery in Roman region.
2. We found that the visual characteristics of the flowers in all samples of pink stone fruit species were fully confirmed as an existing PPV infection.
3. No cherry PPV infection was detected in either test crop field.

4. There may be deviations from the visual and serological methods in examinations of fruit crops whose flowers are white, such as plum and cherry.

5. We found differences in visual and serological diagnosis of PPV in leaf samples, expressed as an indeterminate result, probably due to inhibitor substances that had accumulated as the vegetation of fruit crops progressed.

6. For samples showing an indeterminate result, additional analysis is required to prove an existing infection.

Table 2. Results of ELISA test for PPV by flowers

	Volume OD / 60min	Announcement of a positive result
Blank	0.010	Any sample showing OD values above 0.083 is positive and 0.062 to 0.083 are undetermined.. *Negative*: OD < [K(-)] : [2xOD(k+)] – 20% *Positive*: OD > 2x[OD(k-)] + 20%; *Undetermoned*: [K(-)] > OD < [K(+)].
Positive control (k+)	0.083	
Negative control (k-)	0.031	
Substrate	0.048	

№	Region	Variety	With simptoms	Results from ELISA tests		
				PPV		Results
				Volume	OD	+ / -
1	Cherry	Van	No	0,048	0,051	-
	CLPQ			0,055		
2	Plum	Chachanska lepotitsa	Yes	0,150	0,157	+
	Roman			0,165		
3	Cherry	Van	No	0,046	0,0465	-
	Vrazhdebna			0,047		
4	Peach	Red Haven	Yes	0,094	0,105	+
	Vrazhdebna			0,116		
5	Peach	Somerset	Yes	0,149	0,149	+
	Vrazhdebna			0,149		
6	Peach	Juneta	Yes	0,085	0,090	+
	Vrazhdebna			0,095		
7	Plum	Black Beauty	No	0,036	0,045	-
	Roman			0,054		
8	Plum	Black Star	Yes	0,382	0,359	+
	Roman			0,336		
9	Plum	Outman	No	0,142	0,141	+
	Roman			0,141		
10.	Peach	Gold Haven	Yes	0,158	0,154	+
	Vrazhdebna			0,150		
11.	Plum	Stanley	Yes	0,259	0,251	+
	Roman			0,244		
12.	Nectarine	New Jersey	Yes	0,157	0,165	+
	Roman			0,174		

7. We found that it is much more sensible to start laboratory identification during flowering, so the results of visual examinations can be juxtaposed with laboratory ones.

The results show this study should be continued in the following years and can be recommended to those looking to improve the phytosanitary diagnostics currently used with a view to increasing the

Table 3. Results of ELISA test for PPV in leaf samples

	Volume OD / 60min	Announcement of a positive result
Blank	0.013	Any sample showing OD values above 0.128 is positive and 0.084 to 0.128 are undetermined.. *Negative*: OD < [K(-)] : [2xOD(k+)] – 20% *Positive*: OD > 2x[OD(k-)] + 20%; *Undetermoned*: [K(-)] > OD < [K(+)].
Positive control (k+)	0.128	
Negative control (k-)	0.042	
Substrate	0.052	

№	Region	Variety	With simptoms	Results from ELISA tests		
				PPV		Results
				Volume	OD	+ / -
1	Cherry	Van	No	0,068	0,066	-
	CLPQ			0,065		
2	Plum	Chachanska lepotitsa	Yes	0,140	0,147	+
	Roman			0,155		
3	Cherry	Van	No	0,075	0,081	-
	Vrazhdebna			0,087		
4	Peach	Red Haven	Yes	0,109	0,116	+/-
	Vrazhdebna			0,121		
5	Peach	Somerset	Yes	0,190	0,201	+
	Vrazhdebna			0,213		
6	Peach	Juneta	Yes	0,149	0,149	+
	Vrazhdebna			0,149		
7	Plum	Black Beauty	No	0,076	0,065	-
	Roman			0,054		
8	Plum	Black Star	Yes	0,204	0,221	+
	Roman			0,238		
9	Plum	Outman	Yes	0,242	0,241	+
	Roman			0,241		
10.	Peach	Gold Haven	Yes	0,128	0,129	++/-
	Vrazhdebna			0,130		
11.	Plum	Stanley	Yes	0,139	0,170	+
	Roman			0,201		
12.	Nectarine	New Jersey	Yes	0,231	0,219	+
	Roman			0,208		

purity of the raw materials used to produce stone fruit seedlings.

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