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PLUM POX VIRUS SURVEY OF SWEET AND SOUR CHERRY IN BULGARIA

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Abstract

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Plum pox virus (PPV), the causal agent of sharka disease affecting the major stone fruit species is endemic in Bulgaria. To investigate PPV incidence level and distribution on sweet cherry (*Prunus avium*) and sour cherry (*P. cerasus*) 28 commercial and abandoned orchards were surveyed in 2009 and 2010, as well as some residential and wild cherries. A total of 1141 samples from individual tree were collected and tested using a commercial DAS-ELISA detection system. Some samples were subjected to additional testing by RT-PCR using PPV-specific primers. A commercial sweet cherry sample showing PPV-like symptoms, e.g. chlorotic rings and spots on the leaves and fruits was bud-grafted on GF305 hybrid peach woody indicator seedlings and tested by RT-PCR. PPV was not detected in any samples using serological and/or molecular based tests. Nevertheless, periodic systemic surveys should be conducted to evaluate PPV disease status of sweet and sour cherries in Bulgaria.

Key words: PPV, tracking, virus strain, diagnosis

Introduction

Plum pox virus (PPV), the causal agent of sharka, is the most economically damaging disease of stone fruit trees, significantly reducing fruit quality and yield. Prof. Atanasoff first reported it in Bulgaria around 1915 (Atanasoff, 1932). Since then the disease has spread rapidly throughout Europe to now be found on almost every continent except for Australia. PPV isolates vary significantly and to date have been classified into seven strains designated as PPV-M, PPV-D, PPV-Rec, PPV-EA, PPV-C, PPV-W and PPV-T (Kerlan and Dunez 1979; Glasa et al., 2004; Wetzel at al., 1991a; Kalashyan et al., 1994; James et al., 2003; Serçe et al., 2009). These strains differ in their geographical distribution, biological properties, such as transmissibility by aphids and symptomatology, host specificity and in their serological and molecular properties that impact detection and diagnosis. Three of the strains, PPV-D, PPV-M and PPV-Rec, are considered the most common strains in Europe, including Bulgaria (Kamenova et al., 2011), while the other strains, PPV-EA, PPV-C, PPV-W and PPV-T, have limited geographical distribution.

Cherry (*Prunus avium*) has historically been considered resistant to PPV (Dosba et al., 1987; Nemeth, 1986). In the last two decades, however, PPV was detected in naturally infected trees of sour cherry (*P. cerasus*) in Moldova (Kalashyan et al., 1994; Nemchinov et al., 1995), in sweet cherry in Southern Italy (Crescenzi et al., 1995), in sweet and sour cherry in Bulgaria (Topchiiska, 1996), in Hungary (Kőlber et al., 1998) and in Romania (Maxim et al., 2002). Despite multiple observations since the early 1990's, PPV in cherry is not wide spread and it is often difficult to observe and detect. PPV isolates derived from sour cherry (PPV-SoC) and sweet cherry (PPV-SwC) are closely related and have been proposed as members of the strain named PPV-Cherry (PPV-C) (Nemchinov and Hadidi, 1996; Nemchinov et al., 1998; Nemchinov and Hadidi, 1998). Based on the molecular organiza-

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tion and serological reactivity, PPV-C is defined as a unique strain, which differs from the other strains of PPV. Recently Glasa et al. (2012) have described unusual PPV isolates detected in old sour cherry trees in Russia, which have shown about 30% nucleotide sequence divergence in the NIb-CP genome region with the most common PPV-M, PPV-D and PPV-Rec isolates. Phylogenetic analyses have assigned these new cherry isolates to a distinct cluster, most closely related to PPV-C and PPV-W strains.

Sharka disease is widely distributed and endemic in Bulgaria affecting most of the *Prunus* species such as plum (*P. domestica*), peach (*P. persica*), apricot (*P. armeniaca*) and myrobalan (*P. cerasifera*) (Kamenova et al., 2011). PPV infection in cherry and sour cherry trees, however, has been found only rarely (Topchiiska, 1996). The aim of present study was, in the frame of research agreement between AgroBioInstitute and USDA-APHIS-PPQ-CPHST, to conduct large-scale analyses of PPV infection of cherry and sour cherry in Bulgaria.

Materials and Methods

Survey and samples collection

Surveys were carried out in commercial sweet cherry *(Prunus avium*) and sour cherry (*P. cerasus*) orchards of different age (from 5-6 years to 40-45 years old) located in the West, Southwest, South Central, Northeast, Southeast and North Central regions of Bulgaria in the spring of 2009 and 2010. Additionally, single cherry trees including *P. serrulata* (Japanese flowering cherry) were sampled in several city parks in Sofia. In total 1141 samples (one sample/one tree) from 28 orchards were collected. Some PPV-infected plum samples were also collected during the survey and processed together with the cherry samples. Blossoms and young leaves (not less than 20-25) were collected from scaffold branches around the canopy of each individual tree. Samples were placed in plastic bags and into an icebox and transported to the laboratory where they were immediately analyzed or frozen at -70°C.

DAS-ELISA

All samples were serologically analyzed by DAS-ELISA according the manufacturer's protocol (SRA 31505, Agdia Inc. Elkhart, IN, USA). Absorbance at 405 nm was measured with a microtiter plate reader (HUMAN GmbH, Wiesbaden, Germany) at 60 to 90 min after the addition of the substrate (p-nitro phenyl phosphate). A threshold value for positive samples was set at three times the value of the uninfected plum control.

RT-PCR

Samples that tested negative in ELISA but showing PPV-like symptoms were concentrated by immunocapture (IC) in microfuge tubes and then subjected to reverse transcription-polymerase chain reaction (RT-PCR). Samples for PCR were prepared using IC with polyclonal anti-PPV IgG #150512 from Bioreba (Reinach, Switzerland) according to Wetzel et al. (1992) or by total plant RNA extracted using the RNeasy Plant Mini kit (Qiagen, Hilden, Germany). One tube, one-step RT-PCR was performed with the SuperScript III One-Step RT-PCR System with Platinum *Taq* (Invitrogen, Carlsbad, CA, USA). The following sets of PPV specific universal primers were used: (i) 3'NTR hPPV-cPPV targeting the PPV 3'-non-coding region, (Levy and Hadidi, 1994) and (ii) P1-P2 primers amplifying C-end of the coat protein (CP) gene (Wetzel et al., 1991b). Additional primer sets were used for strain differentiation: (i) P1-PM or P1-PD for PPV-M or PPV-D strain detection, respectively (Olmos et al., 1997) and (ii) SoC-2-R-SoC/2-R (Nemchinov and Hadidi, 1998) for specific detection of PPV-C strain. One-step real-time IC-RT-PCR TaqMan assays using the same 3'NTR universal primers mentioned above in real-time format (Mavrodieva and Levy, 2004) were performed on some of the samples showing PPV-like symptoms.

Grafting and mechanical inoculation

Cherry bud-wood of cv. Van commercial cherries showing PPV-like symptoms were bud-grafted to GF305 seedlings and sap inoculated to *Nicotiana benthamiana* seedlings. Inoculated plants were maintained under greenhouse conditions to promote virus replication and symptom expression. Leaf samples were tested by one-step real-time IC-RT-PCR TaqMan assays to verify the virus presence or absence.

Results and Discussion

In this study, 28 sweet and sour cherry orchards located in four geographical regions of the country were surveyed for PPV infection. The orchards differed by age from young (approximately 5-6 years old) to very old (approximately 40-45 years old) and are represented in Table 1. Some abandoned orchards were also included in the survey. It should be noted that in many cases the surveyed cherry orchards were in close proximity to plum (West, South Central and North Central region) or peach trees (Southwest region) heavily infected with PPV. Serological and molecular analyses of most of infected plum and peach trees showed the presence of PPV-Rec and PPV-M strains. Therefore, cherry orchards surveyed were under high PPV inoculum's pressure. A total of 1141 samples from sweet cherry, sour cherry and single trees of *P. serrulata* were serologically tested by DAS-ELISA for PPV infection. All samples tested negative for PPV (results not shown). DAS-ELISA using polyclonal antibodies has relatively low sensitivity in comparison to the molecular methods and could be insufficient for

detecting PPV-C strain in cherry and sour cherry trees. Based on this knowledge, leaf samples showing PPV-like symptoms were subjected to additional molecular testing despite negative DAS-ELISA results. Several sweet cherry trees from a very old orchard located in West region and from the orchard of Fruit Growing Institute, Plovdiv (South Central region) were tested using a more sensitive IC-RT-PCR test with two sets of universal primers (P1-P2 and 3'NTR) and a set of PPV-C strain-specific primers targeting different regions of PPV genome. No PPV infection was detected (results not shown).

In the second survey year several trees of sweet cherry cv. Van from a private commercial orchard located in Banja (South Central region) showed diffuse chlorotic rings and spots on both leave and fruits. Bud-wood material of four trees was collected and shipped under permit to the CPHST Beltsville Laboratory (Maryland, USA) for additional testing. Infected plum material from nearby plum orchards was also shipped as positive control materials. Upon arrival at the USDA APHIS PPQ Plant Inspections Station in Beltsville, the plant material had to be fumigated due to heavy aphid infestation according to the USA quarantine regulation. These cherry leaf and fruit samples (and plum leaf samples) were subjected to IC-RT-PCR using two highly sensitive real-time RT-PCR (TaqMan) assays. PPV was not detected in any of the cherry samples. In comparison, PPV was easily detected in the accompanying samples of the plum trees (Table 2). Bud-wood of one cherry tree and several plum trees was bud-grafted onto GF305 woody indicator seedlings in CPHST Beltsville lab and mechanically inoculated onto *Nicotiana benthamiana* plants. PPV-like symptoms were not observed on any of the plants inoculated with cherry samples, nor were virus detected using IC-RT-PCR. In comparison, typical PPV-like symptoms were observed on seedling grafted with all 4 plum isolates and PPV was detected by IC-RT-PCR (Table 2, isolate plum 7-8 presented only).

To date the cherry strains of PPV remain rare and elusive and only a few PPV isolates have been found in cherries in Italy, Moldova, Hungary and Romania that have been determined to belong to PPV-C strain. The results of this two-year survey demonstrated that sweet cherry, sour cherry and ornamental cherry trees in Bulgaria were free of PPV. Sharka-like symptoms on leaves and fruits of sweet cherry along with typical PPV symptoms on peach and apricot leaves were first described in Bulgaria in 1934 (Atanasoff, 1934). Several years later Christoff (1944) reported no PPV transmission when bud-wood from PPV-infected plums and myrobalans (*P. ceracifera*) was grafted onto sweet and sour cherry, and Mahaleb cherry (*P. mahaleb*). Topchiiska (1992) reported ELISA detection of PPV by the use of commercial detection kit in different tissue of sweet and sour cherry later. Our survey could not verify PPV presence in large number of orchards in the major cherry producing areas in Bulgaria. Moreover, one of the sweet cherry isolates described by Topchiiska et al. (2002) and named SwC8-34 (*P. avium* cv. 'Compact Stela', South Central region) has been recently reported to belong to PPV-M strain (Milusheva et al., 2005). On the other hand,

Table 1
Cherry orchards surveyed in 2009 and 2010

Region/Orchard Location	Specie	N orchards/Average age	Samples
North Central/Pelishat	*P. avium*	1/>10	9
North Central/Troyan	*P. avium*	3/ >30 abandined	44
Southeast /Pomorie	*P. avium*	1/>10	9
Northeast/Silistra	*P. avium*	1>8	7
South Central/Banja	*P. avium*	2/>6	131
South Central/Banja	*P. cerasus*	1/>6	15
South Central/Parvenez	*P. avium*	3/>8	115
South Central/Plovdiv	*P. avium*	3/5 and 14	265
Southwest/Petrich	*P. avium*	3/4-6, 10-12 and >40-45 abandoned	118
West/Dupniza	*P. avium*	7/ >35-40	289
West/Kyustendil	*P. avium*	2/16 and 26	81
West/Kyustendil	*P. cerasus*	1/21	14
West/Sofia	*P. cerasus*	single trees/ >20	16
West/Sofia	*P. avium*	single trees/ >20	14
West/Sofia	*P. serrulata*	single trees/ >10	14
Total			1141

experimental transmission of PPV-Rec strain (apricot isolate) and PPV-M strain to Mahaleb cherry was recently reported (Milusheva, 2008). Some of the preliminary reports of PPV-M strain infection of cherry trees in Croatia (Mikec et al., 2008), and in the Czech Republic (Navratil et al., 2008) additionally complicate the knowledge of sweet and sour cherry cultivars susceptibility to different strains of PPV.

Conclusion

Presently no PPV infection of cherry and sour cherry in Bulgaria has been found. The soil, climatic and topographical conditions are very favorable for sweet cherry growing in Bulgaria. In the last several years, some expansion of areas occupied with cherry orchards is observed that together with the increase of the global trade and human migration could lead to introduction and spread of PPV-C strain or other PPV strains into cherry in Bulgaria. To prevent some eventual, future PPV infection of cherry and sour cherry into the country periodically visual surveys accompanied by serological and molecular analyses should be continued. Increased knowledge of the biology of newly emerging PPV strains, better antibodies and advanced molecular diagnostics informed by expanding sequence databases will support the effort to detect PPV in Bulgaria as early as possible thus decreasing economic damage.

Table 2
Results of real-time IC-RT-PCR analyses of cherry samples with PPV-like symptoms and plum samples infected with PPV

Location	Sample	Sub sample	Sample type	PPV-like symptoms[1]	IC-rtRT-PCR Ct 3'NCR / CP1
Banja	Cherry 10-3	1	Leaves	Yes	0.00/0.00
		2	Leaves	No	0.00/0.00
		3	Leaves	Yes	0.00/0.00
		4	Fruit	No	0.00/0.00
	Cherry 12-3	1	Leaves	Yes	0.00/0.00
		2	Leaves	No	0.00/0.00
		3	Leaves	No	0.00/0.00
		4	Twig	No	0.00/0.00
	Cherry 13-3	1	Leaves	No	0.00/0.00
		2	Leaves	No	0.00/0.00
		3	Leaves	Yes	0.00/0.00
		4	Leaves	Yes	0.00/39.71[2]
		5	Fruit	No	0.00/0.00
	Cherry 15-4	1	Leaves	Yes	0.00/39.56[2]
		2	Leaves	Yes	0.00/0.00
		3	Fruit	No	0.00/0.00
Alexandrovo	Plum 7-8	1	Leaves	Yes	n/t[3]/ 18.75
		2	Leaves	Yes	n/t/ 18.62
	P. cerasifera BG-03	1	Leaves	Yes	n/t/ 20.09
		2	Leaves	Yes	n/t/ 20.12
	IC buffer control				0.00/0.00
	IC healthy control				0.00/0.00
	IC PPV POS				19.74/19.06
	NTC				0.00/0.00

Legend:
[1] PPV-like symptoms observed on the original bud-wood
[2] rtRT-PCR Ct values above 36 are generally considered unleliable
[3] not tested
IC buffer: IC sample of the extraction buffer
IC healthy control: healthy GF305 leaves
IC PPV POS: PPV-D infected GF305 leaves
NTC: not-template control = molecular grade H_20

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