

## ***PRUNUS CERASIFERA* AS A HOST OF *PLUM POX VIRUS* IN BULGARIA**

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### **SUMMARY**

Four hundred twenty-five trees of myrobalan (*Prunus cerasifera* Ehrh.) growing along the roads and small areas within residential spaces, private gardens, and parks in Sofia and around the town were visually inspected for virus symptoms and tested for the presence of PPV by DAS-ELISA. DASI-ELISA for strain differentiation was carried out for the identification of PPV isolates, using PPV-M and PPV-D specific monoclonal antibodies. A small number PPV isolates was tested with M/D specific primers targeting two different regions: cylindrical inclusion protein (CIP) and the coat protein (CP) amino terminal. The primers targeting the 3'-terminal region of the CP gene allowed the detection of a few unknown PPV isolates. While the inspected trees showed highly variable leaf symptoms, only a small number of them showed symptoms on the fruits. The overall detection of PPV was 49%, PPV-M was found in the majority (57%) of the samples, followed by PPV-D (29%). Mixed infections occurred in 2% of the samples, and the diseased trees affected by the unknown PPV strain were 12%. PPV-Rec strain was identified in one infected tree. For the first time in Bulgaria two ornamental species, *P. cerasifera* var. *rubrum* and *P. cerasifera* cv. *Pissardii* were found to be natural hosts of PPV. Strains M and D alone or in mixture were identified in the infected trees of these species. IC-RT-PCR results showed the presence of mix infection of PPV-M and PPV-Rec strains in one tree of *Armeniaca desicarpa* (*P. cerasifera* x *P. armeniaca*). The presence of PPV in cultivated and uncultivated, edible and ornamental *P. cerasifera* species provides strong evidence that they are natural PPV reservoirs in Bulgaria.

*Key words:* *Prunus cerasifera*, survey, PPV strains, ELISA, IC-RT-PCR.

### **INTRODUCTION**

*Plum pox virus* (PPV) the causal agent of Sharka, initially described in Bulgaria more than 70 years ago (Atanassov, 1932) has spread worldwide (Roy and Smith, 1994; Cambra *et al.*, 2006) so that Sharka is currently the most important disease of *Prunus* due to its agronomic and economic impact.

PPV infects both edible and ornamental *Prunus* species. For many years it remained the most destructive and serious virus disease in Bulgaria, causing yield losses to the plum industry. PPV-M, PPV-D and PPV-Rec strains alone and in mixed infection were identified in commercial *Prunus* species, including the European plum (*P. domestica*), peach (*P. persica*) and apricot (*P. armeniaca*) (Kamenova *et al.*, 2003; Kamenova *et al.*, 2006).

*P. cerasifera* Ehrh. (myrobalan) is widespread in Bulgaria, representing the second major *Prunus* species after plum. Myrobalan was reported as a natural host of PPV as early as 1934 (Atanassov, 1934; Christoff, 1938). However, since little is known on the incidence of Sharka and specific PPV strains in wild and ornamental *P. cerasifera* in Bulgaria these aspects were investigated with the present study.

### **MATERIALS AND METHODS**

**Field surveys.** *P. cerasifera* trees grown along the roads and small areas within residential spaces, private gardens and parks in Sofia and around the town were analyzed for the presence of PPV. Myrobalan trees from an experimental orchard of the Institute of Agriculture, Kyustendil were also tested. Trees from two ornamental species, *P. cerasifera* var. *rubrum* and *P. cerasifera* cv. *Pissardii* grown around public buildings and parks were also inspected for virus symptoms. Field surveys for PPV symptoms in the leaves were done in May 2007, while the surveys for fruit symptoms were carried out in July-August.

**Serological assays.** Flowers and leaves from inspected trees were sampled for PPV detection by DAS-ELISA (Clark and Adams, 1977) with polyclonal anti-

bodies raised against a Bulgarian virus isolate (Kamenova and Peters, 1999) and the monoclonal antibody 5B (Cambra *et al.*, 1994). For PPV strain determination, the same extracts were also analyzed by DAS-ELISA (Cambra *et al.*, 1994) using specific monoclonal antibodies (MAbs), respectively raised against PPV-M (Agritest, Italy) and PPV-D (Durviz, Spain). Polystyrene microtiter plates were used for ELISA, and controls in all assays included sample buffer only, uninfected (*Nicotiana benthamiana*) and virus-infected plant tissues (*N. benthamiana* plants infected with PPV-M, -D and -Rec strains). Absorbance at 405 nm was measured with a Human reader about 60 to 90 min after the addition of the substrate (*p*-nitrophenyl phosphate). A threshold value for positive samples was set at three times the value of the uninfected control.

**Molecular assays.** Immunocapture-reverse transcription polymerase chain reaction (IC-RT-PCR), targeting two genomic regions: the coat protein (CP) amino terminal and cylindrical inclusion protein (CIP) with specific M/D PCR primers (Candresse *et al.*, 1998; Glasa *et al.*, 2002) of twenty five samples was carried out as described (Dallot *et al.*, 2006; Kamenova *et al.*, 2006). Samples that did not react with MAbs were tested with primers targeting the 3'-terminal region of the CP gene according to Wetzel *et al.* (1992). RT-PCR assays were performed using MiniCycler™ Thermal Cycler (MJ Research, USA). Positive (*N. benthamiana* tissues infected with PPV-M, -D and -Rec strains), negative (healthy *N. benthamiana*) tissue and water controls were included in each IC-RT-PCR reaction. Products were analyzed by electrophoresis on native 1.5% agarose gels and detected by ethidium bromide staining.

## RESULTS

**Field symptoms.** Different kind of symptoms, ranging from typical ringspots, lines along the veins, and diffused light green spots could be seen in early spring (Fig. 1A, B, C). The intensity of symptoms on the leaves was highly variable, from very mild to moderate or severe. PPV was latent in several symptomless trees because the virus infection was confirmed by serological test. Only a small number of infected trees showed symptoms on the skin of the fruits, consisting of rings and spots and these symptoms were more pronounced before the full ripening of the fruits.

**Serological and molecular assays.** Seventy samples (41.4%) of 169 tested from cultivated *P. cerasifera* trees grown in the experimental orchard of the Institute of Agriculture, Kyustendil were PPV-positive with PABs and MAb 5B. In 66 infected trees, only PPV-M strain (94.3%) was identified. Four PPV isolates did not react

with MAbs raised to PPV-M (Boscia *et al.*, 1997) and PPV-D (Cambra *et al.*, 1994). Therefore their strain status was not determined.

Seventy five (57.7%) of 130 tested myrobalans, naturally growing in several parks of Sofia were found to be natural sources of PPV. Forty one isolates (55%) were classified as PPV-M and 24 isolates (32%) as PPV-D. Overall, a mixed infection of both strains was found in a single tree and nine isolates were not identified at the strain level. Strikingly, one isolate was identified as strain M by DAS-ELISA and PCR test with CP PPV-M specific primers (product size of 467 bp, not shown). Using PCR detection with CIP PPV-D specific primers, this sample showed a band with the expected length (962 bp, not shown) thus indicating that this isolate belonged to the PPV-Rec strain (Glasa *et al.*, 2002).

PPV was detected in 64 (50.8%) of 126 tested myrobalans from private gardens around Sofia. The most prevalent strain was PPV-D (56.3%), followed by PPV-M (18.8%). Mixed infections (M+D) were 6.3% and 18.6% of the isolates were not identified at the strain level. The status of these PPV isolates that did not react with the used MAbs was ascertained using primers targeting the 3'-terminal region of CP in IC-RT-PCR assays (Wetzel *et al.*, 1992). The expected band of 243 bp was amplified (not shown).

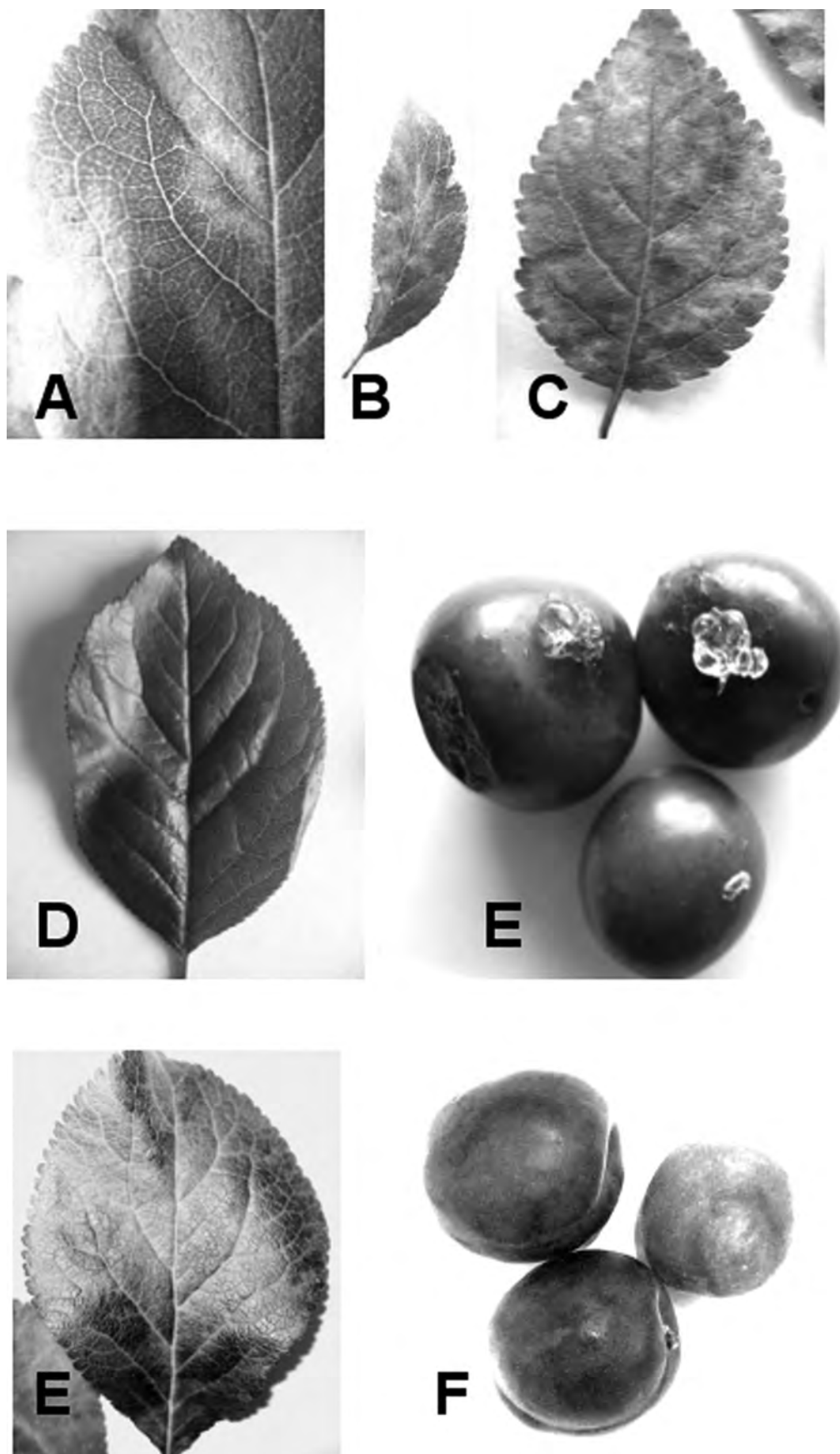
Altogether 209 (49%) myrobalans of 425 tested were affected by Sharka. PPV-M strain was the most frequent (57%), followed by PPV-D (29%). In 2% of the samples mixed infection of both strains was recorded and 12% of the samples were not identified at the strain level.

DAS-ELISA tests allowed detection of seven PPV-M, two PPV-D and one mixed infection with M and D in leaf samples from *P. cerasifera* var. *rubrum* trees. Infected leaves showed pale-red stripes and spots (Fig. 1D), while the fruits of two of the trees infected with PPV-M strain exhibited rings and spots on the surface together with oozing of resin (Fig. 1E). No symptoms were observed on the stones.

Both strains, PPV-M and PPV-D were identified by DAS-ELISA and confirmed by IC-RT-PCR in *P. cerasifera* *Pissardii*. Infected trees grown in one of the biggest parks of Sofia showed discoloration of the purple leaves, consisting of green stripes and spots along the veins (Fig. 1E). IC-RT-PCR results showed the presence of mix infection of PPV-M and PPV-Rec in one tree of *Armeniaca desicarpa* (*P. cerasifera* x *P. armeniaca*). The fruits of this tree showed rings on their surface (Fig. 1F).

## DISCUSSION

To our knowledge this is the first extensive survey for Sharka infection of *P. cerasifera* in Bulgaria addressing both the virus incidence and the distribution of specific PPV strains in *P. cerasifera*. The high rate of PPV infec-



**Fig. 1.** A-C. Sharka symptoms on the leaves of *P. cerasifera*. rings (A), chlorosis along the veins (B), mottling (C). D-E. Symptoms on a leaf (D) and fruits (E) of *P. cerasifera* var. *rubrum*. F-G. Symptoms induced by PPV-M on a leaf of *P. cerasifera* var. *Pissardii* (F) and on the fruits of *Armeniaca desicarpa* (*P. cerasifera* X *P. armeniaca*) (G).

tion found in *P. cerasifera* in Bulgaria is similar to that observed in the Czech Republic (about 50%) (Polak, 2006).

In our previous investigation on PPV strains in *Prunus* species in Bulgaria (Kamenova *et al.*, 2003) we reported that PPV-M was the only strain found in infected peach and apricot trees, and the prevalent strain in plums, with an incidence of 88.3% followed by PPV-D (5.2%). The results obtained from DAS-ELISA tests of *P. cerasifera*-infected trees showed that the incidence of PPV-M was double when compared with that of PPV-D. These results confirmed the dominant and endemic spread of PPV-M in Bulgaria. For the first time in Bulgaria we found also *P. cerasifera* tree infected only with PPV-Rec and a mixed infection of PPV-M + PPV-Rec in one *P. cerasifera* hybrid. Presence of PPV-Rec in myrobalans had been already reported by Glasa *et al.* (2003).

Natural infection of ornamental stone fruit species by PPV is important from an epidemiological point of view since, being woody perennials, they serve as virus reservoir. Similarly to the observations made in Turkey (Elibuyuk, 2006), we found that two ornamental species, *P. cerasifera* *Pissardii* and *P. cerasifera* var. *rubrum* commonly grown in the parks and around public buildings were naturally infected. However, under experimental conditions in France, Labonne *et al.* (2004) have found that *P. cerasifera* *Pissardii* is not a host of PPV.

There was no clear association between the symptoms observed on the leaves of infected *P. cerasifera* species and their intensity and the particular strain identified, since severe symptoms could be observed in trees infected with PPV-M or/and PPV-D strain.

Even though such survey was limited mainly to one region of the country, these results show that cultivated and uncultivated, edible and ornamental *P. cerasifera* species are natural reservoirs for PPV in Bulgaria.

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