Final Report

Project title (Acronym)
Determine different *Plum pox virus* strains in wild hosts and in stone fruit cultivars with different susceptibility as a part of improved control and surveillance strategies (Epi-PPV)

Project duration:

Start date:	2016-07-01
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Contents

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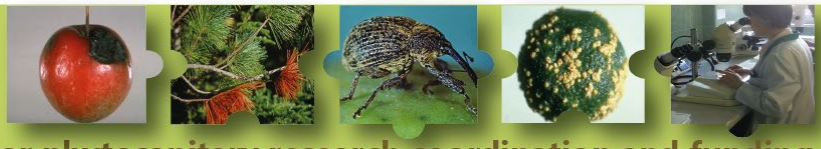

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2. Short project report

2.1 Short executive summary

Plum pox virus (PPV) is the most important and devastating virus disease causing the highest economic impact in stone fruits worldwide (Cambra *et al*., 1994). The virus, classified as a quarantine pest (EPPO A2 list, EU 2000/29 EEC, annex II), was first detected in Bulgaria in 1900, and is now widespread in most European countries. The hosts of PPV are the fruit - producing species and wild and ornamental species of *Prunus*. Wild hosts can act as a natural reservoir for PPV. Eight PPV strains have been identified based on their biological, serological and molecular properties. One example for the biological relevance of this interspecific diversity is the PPV-M strain causing more severe symptoms in peaches than the PPV-D strain. For an effective control and surveillance strategy it is important to use PPV-free plant material, tolerant/resistant cultivars and eradicate infected plants. To reduce the spread of this disease, a reliable and sensitive detection method (to be used e.g. on propagation material) is necessary. CTAB and RNeasy nucleic acid extraction coupled with highly sensitive TaqMan real-time RT-PCR was demonstrated not reliable for stone fruit propagation material routine testing. Spot TaqMan real-time RT-PCR is recommended for that purpose.
Researchers have previously observed that PPV infection produces modifications in some chlorophyll fluorescence parameters. The effect of the treatments with mandelonitrile (MD) or phenylalanine (Phe) on PPV symptomatology and in the chlorophyll fluorescence in peach GF-305 and the effect of PPV infection on cyanogenic glycoside turnover and salicylic acid (SA) biosynthesis were analysed. The results suggest that under stress conditions MD treatment, and hence the new SA biosynthetic pathway described, affect the SA-mediated H_2O_2 content. This finding supports the hypothesis that the SA biosynthesis from MD also occurred under stress conditions.

2.2 Project aims

From an epidemiological point of view, it is necessary to include also wild host in surveillance activities and to determine the strains of PPV, because of the different damages they cause. The project had the goal to collect empirical data on PPV in wild hosts and in stone fruit cultivars with different susceptibility in fruit producing and adjacent areas. The strain differentiation of the PPV isolates will give a deeper insight into the epidemiology of this pathogen. The new findings can help to develop a more effective surveillance strategy and adapt control measures to viral populations.
The apricot cultivation in Greece is under reconstruction during the last years, mainly because of the high prevalence of PPV-M in the country. Many new apricot varieties resistant or tolerant to PPV and of different origin (Greece, Italy, USA) are being tested by growers in new orchards and were ranked on the incidence of PPV symptoms developed in the field. In the nurseries they normally did not show any symptoms and were negative to the laboratory virus tests. Spanish germplasm, tested for PPV-M resistance for the first time, could provide a good alternative for the reconstruction of apricot industry in Greece.
The project also aimed at developing environmentally friendly strategies to increase plant resistance against Sharka disease, including the use of transformed plum plants.
Transformed plum line harbouring 4 copies of cytapx, encoding for cytosolic ascorbate peroxidase (APX) that proved to be tolerant to NaCl stress under *in vitro* conditions, as well as tolerant to water stress under *ex vitro* conditions. Due that long-term PPV-infection produced an oxidative stress at subcellular level in susceptible peach, apricot and pea cultivars, it was proposed the use of this plum line, with increased antioxidative defences, in order to test if they have some degree of tolerance against PPV infection.

Aims of the project:

- Collect empirical data of PPV in tolerant/resistant *Prunus* hosts;
- Collect empirical data of PPV in wild hosts;
- Identify viruses which cause the same symptoms as PPV;
- Distinguish PPV-strains with PCR techniques;
- Evaluate the PPV presence in the stone fruit propagation material in Hungary;
- Investigate different PPV strain incidence in stone fruit propagation material in Hungary;
- Compare different nucleic acid preparation techniques in real-time RT-PCR;
- Evaluate the correlation between caused symptoms and testing results;
- Study the effect of ectopic expression of cytosolic ascorbate peroxidase;
- Evaluate PPV resistance;

Finally, the project also aimed to develop strategies to increase the tolerance of *Prunus* spp. against environmental stresses in Europe and PPV infection.

2.3 Description of the main activities

Activities conducted in Greece

Surveys were carried out in apricot nurseries in Peloponnese, the most important apricot production area in Greece. Apricot propagation material was visually inspected and PPV symptoms were only observed in the old, PPV sensitive, local apricot varieties (i.e. Bebecou, Early of Tyrinth, Diamantopoulou) and only scarcely in the tolerant one (Nostos; Karayiannis *et al*., 2008). To check PPV incidence, samples from symptomatic plants were randomly collected and tested by ELISA. The virus was detected only in samples of local varieties showing symptoms. Monitoring was also undertaken in orchards in the same area, where orchards of tolerant varieties are in proximity of sensitive varieties; it is not uncommon that growers graft tolerant varieties on infected rootstocks. Trees were visually inspected in the fields and sampled, if suspicious symptoms were observed. The ELISA test used to estimate virus concentration revealed that if the resistance is overcome, the virus titres are generally high. Depending on the PPV incidence observed in the fields, tolerant apricot varieties can be listed as follows, beginning with the most tolerant one: Harcot, Tom Cot, Orange Red, Bora, Nostos, Niriis, Neraida, Tirvi, Aurora, Ninfa. In cooperation with CEBAS-CSIC, Spain (Dr. D. Ruiz) an experiment was set up to evaluate the resistance of Spanish apricot germplasms (10 varieties) to PPV-M using variety grafts on PPV-M infected rootstocks. Preliminary ELISA results showed that six out of ten varieties were not infected by PPV and one exhibited very low PPV titre. PPV strain identification of ELISA positive samples was performed by strain specific RT-PCR using P1/PD, P1/PM and mD5/mM3 primers according to Olmos et al. (1997), Šubr, Pittnerova and Glasa (2004) respectively in 2016.

Activities conducted in Hungary

The testing of stone fruit propagation material was performed in the frame of the official activities carried out by the Plant Health and Molecular Biology Laboratory, Budapest, Hungary during spring and early summer.

DASI ELISA and real-time RT-PCR positive samples were subjected to strain-specific RT-PCR in order to determine the occurrence of different PPV strains in stone fruit propagation material. PPV-D and PPV-M specific primers from EPPO standard (PM7/032(1)) were used. Different nucleic acid extraction techniques were compared:

The cheap CTAB based total nucleic acid isolation method (Li *et al*. 2008) and the expensive RNeasy Plant Mini Kit (Qiagen).

The laboratory also evaluated the correlation between caused symptoms and testing results: PPV like symptoms were reported in a stone fruit propagation material nursery by plant health inspectors. The results could not be confirmed by laboratory tests (ELISA and RT-PCR). These samples were subjected to further specific laboratory tests for other viruses.

Activities conducted in Austria
A survey of wild host plant, especially *Prunus* species, was conducted in the province Lower Austria. Symptomless plant material was randomly collected. RNA extraction was carried out with a RNeasy Plant Mini Kit (Qiagen) and a specific PCR method (Wetzel, 1991) was used for the detection of PPV. The strain characterisation was done following the EPPO standard PM7/032(1).

Activities conducted in Spain
Researchers had previously observed that PPV infection produces modifications in some chlorophyll fluorescence parameters. In the current study, PPV infection produced a non-statistically significant decrease in Y(II) and an increase in the rest of the analysed parameters in control seedlings (no chemical treatment). In PPV-infected seedlings, none of the analysed parameters were altered by the MD treatment, whereas a decrease in chlorophyll fluorescence quenching qP was observed in Phe-treated seedlings when compared to infected control seedlings. When compared with MD-treated, non-infected seedlings, MD-treated seedlings submitted to PPV infection showed a decrease in the quantum yield of photochemical energy conversion in photosystem II (Y(II)) but a strong increase in non-photochemical quenching parameters.
The effect of the treatments with mandelonitrile (MD) or phenylalanine (Phe) in the symptomatology and in the chlorophyll fluorescence in peach GF-305 and the effect of PPV infection on cyanogenic glycoside turnover and SA biosynthesis was analysed.

2.4 Main results (knowledge, tools, etc.)

Both PPV-D and PPV-M strains were detected in certified stocks. The occurrence of PPV-M was a little higher (56%) than PPV-D (43%) considering the total infected samples. Few (1%) PPV-Rec was identified. No other PPV strains were detected. Universal primers EPPO standard PM7/032(1) 3'NCR (Levy and Hadidi 1994) and P1/P2 (Wetzel et al. 1991) proved to be more sensitive than either of primer pair used for strain identification. The 3'NCR universal primers showed the highest sensitivity in the conventional RT-PCR. PPV positive trees were eradicated.
A CTAB-based total nucleic acid isolation protocol was adapted and compared with RNeasy Plant Mini Kit (Qiagen) and spot sample preparation using plant leaves extracted in PBS, 2% PVP, 0.2% DIECA (Olmos *et al*. 1996, Capote *et al*. 2009) in real-time RT-PCR (Olmos *et al*. 2005). Leaf samples from a peach orchard collected in May 2015 and May 2016 were used in comparison experiments where symptoms were inspected for several years. CTAB extraction yielded the highest nucleic acid quantity (containing both DNA and RNA). The quality of the nucleic acid extracted by CTAB method was also very good exceeding 2.00 of 260/280, 230/280 ratio values measured by NanoDrop. The highest sensitivity was achieved by CTAB and RNeasy Plant Mini Kit extraction as expected. Both extraction methods proved to be about 1000 x more sensitive than spot extraction in real-time RT-PCR. Only the samples processed with RNeasy and giving real-time RT-PCR Ct value lower than about 30 could be confirmed positive with 3'NCR primers (samples with a real-time RT-PCR Ct value < 28 were confirmed positive by P1/P2 primers). Samples processed using CTAB and RNeasy extraction methods with Ct values higher than 30 collected and tested in 2015 gave negative results in 2016. No symptoms were developed in these trees until recently. Extraction of high quantity nucleic acid (with CTAB or RNeasy) and testing with highly sensitive real-time RT-PCR led to unreliable results as previously described (Capote *et al*. 2009). Spot extraction combined to real-time RT-PCR was very reliable giving the same results in all samples in both years. Therefore, the Spot TaqMan real-time RT-PCR method was introduced in the ISO 17025 accreditation system of the Greek laboratory for screening stone fruit propagation material. CTAB extraction is a cheap alternative method compared to the very expensive RNeasy Mini Kit, but it can only be used

with the conventional RT-PCR and not with the very sensitive real-time RT-PCR assay for PPV detection.
PPV like symptoms (rings, spots, mosaics) were reported in plum (*Prunus domestica*) trees in a certified stock stone fruit propagation material by plant health inspectors in Hungary in 2014 which could not be confirmed by DASI-ELISA and RT-PCR using 3'NCR and P1/P2 universal primers. The symptoms may be associated to *Apple mosaic virus* (ApMV) and *American plum line pattern virus* (APLPV) but RT-PCR failed to confirm that. Cherry leaf roll virus (CLRV) was detected in selected plum samples using RW1/RW2 CLRV universal primers designed to 3'NCR region by RT-PCR (Buchhop *et al*. 2009). Both of PPV-D and PPV-M strains were detected in certified stocks. The occurrence of PPV-M was a little higher (56%) than PPV-D (43%) considering the total infected samples. Few (1%) PPV-Rec was identified. No other PPV strains were detected. CLRV was detected also in asymptomatic plums and some apricot trees from the same place without any symptoms. Very likely the CLRV infection cannot be associated with the symptoms reported in plum trees. *Prunus cerasiera* (myrobalan) is a natural host of CLRV-E. Plums and apricots are grafted to myrobalan rootstock, so *Prunus cerasifera* samples collected from another rootstock-seedling orchard were tested. CLRV was detected from these samples so very likely CLRV detected in plums and apricots originated from *Prunus cerasifera* rootstocks. The economic impact of CLRV in plum and apricot is not known. Also, PPV strain D and M could be detected in symptomless wild hosts (*Prunus* spp.)
MD treatment reduced the symptomatology associated with PPV infection.
Taking into account the fact that infection decreases the number of leaves per plant, the number of branches per plant was also considered. In control seedlings, PPV infection decreased the number of branches, whereas no differences were observed in MD- and Phe-treated seedlings. Interestingly, although both MD and Phe treatments did not affect other growth parameters such as plant height, in the absence of PPV infection, a decrease in the number of branches on chemically-treated plants compared to control seedlings was recorded.
Also the effect of PPV infection, in the absence or in the presence of the treatments (MD or Phe) on the photochemical [Y(II) and qP] and non-photochemical [Y(NPQ) and qN] quenching fluorescence-related parameters was analysed. The ANOVA statistical analysis showed that Y(II), Y(NPQ) and qN were affected by the PPV infection, while the interaction between both factors also had an effect on qP and qN. In non-infected seedlings, MD increased Y(II) and qP, whereas no changes were observed in Phe-treated plants. Regarding the non-photochemical quenching parameters, a decrease in Y(NPQ) due to the MD treatment and a non-significant increase in qN due to the Phe treatment were observed).
Researchers have previously observed that PPV infection produces modifications in some chlorophyll fluorescence parameters. In the current study, PPV infection produced a non-statistically significant decrease in Y(II) and an increase in the rest of the analysed parameters in control seedlings (no chemical treatment). In PPV-infected seedlings, none of the analysed parameters were altered by the MD treatment, whereas a decrease in qP was observed in Phe-treated seedlings when compared to infected control seedlings. When compared with MD-treated, non-infected seedlings, MD-treated seedlings submitted to PPV infection showed a decrease in Y(II) but a strong increase in non-photochemical quenching parameters.

Effect of PPV infection on cyanogenic glycoside turnover and SA biosynthesis
PPV-infected GF305 shoots were fed with [13C]Phe or with [13C]MD. Based on previous results, the CNglcs pathway is fully functional under our experimental conditions.
In PPV-infected shoots, there was a significant increase in amygdalin as well as a significant decrease in MD. BA levels significantly increased in non-treated and Phe-treated shoots. Accordingly, the SA levels rose significantly in these plants, while MD-treated plants maintained their SA levels, similar to salt-stressed shoots. The SA concentration was significantly higher in MD- than in Phe-treated shoots, however, as occurred in healthy plants.

Peach seedlings grown in a greenhouse were fed with either MD or Phe, under PPV infection conditions. SA biosynthetic pathway from MD is also functional under biotic (Plum pox virus infection) stress conditions, although the contribution of this pathway to the total SA pool does not seem to be important under such conditions.
In PPV-infected micropropagated shoots the chemical treatments affected the APX, POX and CAT activities, whereas PPV infection only had a significant effect on CAT. The interaction between both factors (chemical treatment vs. PPV) had a significant effect on all the antioxidants activities measured. In PPV-infected micropropagated shoots, the MD treatment did not statistically affect the activity of the any of the analyzed enzymes, whereas the Phe-treatment reduced POX and SOD activities when compared to non-treated PPV-infected shoots).
Regarding the PPV assay in peach seedlings, it is important to remember that the control plants used in this experiment were different from those used in the NaCl stress experiment. In peach seedling both factors as well as its interaction (chemical treatment vs. PPV) had a significant effect on all the antioxidants activities measured). In non-infected plants, both treatments reduced SOD activity in a similar manner, whereas Phe treated plants also showed a strong decrease in CAT activity. In control seedlings, PPV infection produced a decrease in APX and CAT activity and an increase in POX activity. In MD-treated plants, PPV infection reduced APX, POX, CAT and SOD activities. In contrast, Phe-treated plants showed a 2-fold increase in POX and CAT activities, as well as a dramatic increase in SOD activity, and these changes paralleled a significant decrease in APX activity (4.3-fold).

2.5 Conclusions and recommendations to policy makers

The study and promotion of apricot varieties resistant to PPV is the best way to control the virus and to revive apricot cultivation in the country.
Both PPV-D and PPV-M are present in Hungary and Austria. This to strains are also present in wild host without any symptoms. So it is very important to check also the incidence of PPV in wild host with molecular biological (PCR) or serological (ELISA) methods. These results should be considered for planting out a new orchard with potential PPV host plants.
CTAB and RNeasy nucleic acid extraction coupled with highly sensitive TaqMan Real-time RT-PCR is not reliable for stone fruit propagation material routine testing. Spot TaqMan Real-time RT-PCR is recommended for that purpose.
PPV-like symptoms were observed that could influence the reliability of visual inspection. We recommend laboratory tests for all cases when suspicious symptoms can be seen. CLRV was detected in both plum and apricot orchards and *Prunus cerasifera* seedlings. No economic impact of CLRV in plum and apricot is known. Very likely the CLRV infection cannot be associated with the symptoms reported.
MD treatment can be used to reduce the symptomatology associated with PPV infection. In addition, MD treatment improves some photochemical-quenching parameters.

2.6 Benefits from trans-national cooperation

Virus management is possible only through the cooperation of scientists, growers and policy makers at trans-national level. PPV has spread almost all over the world mainly through the movement of infected propagation material and the adoption of appropriate strategies and policies by all cooperating parties is a prerequisite for the continuation of *Prunus* cultivation.

The results of this project can be extended to other plant species and other pathologies in all the affected areas.

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3. Publications

3.1. Article(s) for publication in the EPPO Bulletin

None.

3.2. Article for publication in the EPPO Reporting Service

None.

3.3. Article(s) for publication in other journals

None.

4. Open Euphresco data

None.