

SharCo

Containment of Sharka virus in view of EU-expansion

Small Collaborative project of the 7th Framework Programme

Theme 2

Food, Agriculture, Biotechnologies

ML05

Upgraded PPV detection methods

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Milestone report structure

1. GENERAL PRESENTATION.....	3
1.1. OBJECTIVES OF THE WORKPACKAGE	3
1.2. CONDITIONED ISSUE OF THIS MILESTONE	3
2. DETAILED DESCRIPTION	4
3. MEANS OF VERIFICATION AND ACTUAL ACHIEVEMENTS	6
4. CONTINGENCY PLAN.....	7
5. REFERENCES:.....	7

1. General Presentation

1.1. Objectives of the workpackage

The main goal of the SharCo research programme is to provide new protocols, methods and tools for the containment of sharka disease in stone fruit trees. Different specific research activities are ongoing on a number of topics in order to achieve this purpose, both in nurseries and in orchards. One of the goals of the SharCo WPE3 workpackage is to improve detection protocols in nurseries to prevent virus spread in the nursery as well as to limit the trade of PPV infected material for planting. This goal will be attained by identifying the most accurate sampling and detection methods for large numbers of samples. The final objective is then the increase in the commercialization of *Plum pox virus* (PPV)-free plant materials by European nurseries. This strategy will thus help significantly in the containment of sharka disease.

1.2. Conditioned issue of this milestone

The term “detection” refers to the presence of a particular target organism in plant tissues, vectors, plant products or environmental samples, with emphasis on symptomless plants (López et al., 2008). Illegal traffic and exchanges of symptomless PPV infected propagative plant material are the main means of PPV spreading over long distances. Then, once the virus has become established in an orchard, aphids present in the area will transmit the virus locally, from tree to tree, in a non-persistent manner. For decades, there has been no awareness nor reliable detection methods and reagents suitable for large scale surveys of the disease. Consequently, PPV has easily escaped visual inspections and other inefficient control methods employed, spreading worldwide (Cambra et al., 2006). Despite very significant progress over the years in the efficiency of PPV detection and typing methods (EPPO 2004; García and Cambra, 2007; IPPC, 2009; Barba et al., 2010), there is still today a significant risk of dissemination of PPV infected plant material displaying "subclinical" infection. This material, whereas initially stated as PPV-free, will develop the disease once planted in another location, in another nursery or in orchards, and after a variable period of latency. Therefore, improved sanitary control of propagation material and of nursery blocks through the use of appropriate and validated protocols is needed. It is also the main object of this milestone.

Ultimately, those upgraded protocols of sampling and PPV detection are expected to facilitate the labour of Plant protection Services (PPS) inspectors and to value both the production of certified plants and the delivery of the EU sanitary passport leading to a better

prevention of PPV spread. Therefore, the conditioned issue of this milestone is the generalisation and dissemination all over Europe of accurate and easy-to-use protocols of control of the sanitary status in nurseries.

2. Detailed description

Three aspects are determining the accuracy of a detection test in woody plants:

1) Sampling (period, type of tissues collected, individual, and hierarchical or multiple collections of samples).

2) Sample preparation (extraction and RNA purification versus direct methods of sample preparation).

3) Type of detection test (serological and/or molecular techniques and reagents used).

In the first year of the project, the SharCo partners involved in WPE3 initiated or intensified activities related to the three above described items. Particular attention was devoted to the validation, including the calculation of the different parameters (see part 3 below). The objective has been accomplished and upgraded PPV detection methods, based on data presented in the DE3.2 deliverable, are described below.

2.1 Sampling:

Period: preferably in spring for improved accuracy with both serological and molecular PPV detection techniques, then autumn and winter samples. Preliminary reports related to this aspect were published by Capote et al. (2009) starting from adult trees and by Vidal et al. (2009), with nursery juvenile plants.

Plant tissues: During the vegetative period, sampling of 3 to 4 fully expanded leaves per nursery, juvenile plant or 10 leaves per adult tree (orchards, mother plants) is recommended. Leaves should be preferably selected from the internal structure of the nursery plant or collected around the canopy of each individual adult tree from the middle of each scaffold branch. During the dormancy period, in winter, 3 to 4 dormant buds per nursery plant and 15 to 20 per adult tree have to be sampled. They are indifferently originating from the apical, medium or basal part of the shoot.

PPV is detected both in leaves or buds of infected plants at any period assayed. More accurate data are expected from spring leaves than from dormant buds if using the serological (ELISA) method of PPV detection. Nevertheless, similar accuracy in PPV detection is provided if

testing by real-time RT-PCR either Spring leaves or dormant buds. This information is important if PPS inspectors and nursery men are willing to split the labour load of sampling and PPV detection over longer periods.

Individual or combined sampling: Three leaves or 3 dormant buds per nursery plant to be tested have to be collected. If detecting PPV by ELISA, one can combine up to 4 nursery plants together in one sample, leading to 3x4 (12) leaves pooled for further analysis. In winter, the analysis of nursery plants using dormant buds has to be performed individually.

If using real-time RT-PCR for PPV detection, whatever is the growing period and whatever is the type of samples (leaves or dormant buds), one can pool up to 10 plants at once (3x10 =30 leaves or buds).

Preliminary data are showing also that the one-by-one analysis of the plants in a nursery block provided a more accurate estimation of the real PPV incidence than the hierarchical method (Hughes et.al. 2002). The method is now being evaluated in different nursery blocks and the results will be compared with those of the one-by-one analysis. Preliminary results showed an over-estimation of the PPV incidence when the real prevalence of PPV in the area was less than 6%.

2.2 Sample preparation: Direct processing of the plant material through to the molecular detection of PPV should be preferred. It avoids nucleic acid purification, decreases time and labour cost while processing large amounts of tree samples. It is also providing equivalent to better accuracy compared to nucleic acid extracts issued from the same samples.

Crude extracts from PPV infected plants can either be diluted in buffer or spotted on nylon membranes before to be used as templates. Alternatively, immobilised PPV targets can be amplified from fresh sections of plant tissues printed or squashed onto a nylon membrane (Capote et al., 2009). The four sample preparation methods (dilution of extracts, spot, tissue-print and squash) assayed, coupled to real-time RT-PCR were able to detect any PPV strain using either TaqMan or SYBR Green chemistries. Comparison of sensitivity for PPV detection between dilution, spot and conventional real-time RT-PCR (TaqMan) and the EPPO (2004) recommended techniques showed that dilution and spot methods have i) the same limit of detection, ii) resulted into a 10 to 100 times more sensitive method than Co-PCR (Olmos et al., 2002) and DAS-ELISA, respectively. While using the dilution method, be aware that PPV amplification might be impaired at low dilution rates in extraction buffer (1:10 and 1:20, v:v); this might be due to the side effect of PCR inhibitors present in the plant extract. Anyway, the most sensitive method appears to conventional real-time RT-PCR in which PPV is detected at up to 1:10⁹ dilution.

In summary, simple sample preparation methods, coupled with real-time RT-PCR, are reliable and validated methods for PPV diagnosis (Capote et al., 2009). Its application for the analysis of large numbers of samples should be preferred in the upgraded PPV detection method and will contribute to a better control of the phytosanitary status of commercial *Prunus* trees.

2.3 Type of detection:

EPPO (2004) recommended PPV detection by ELISA based on the 5B-IVIA monoclonal antibody and by immunocapture (IC) RT-PCR using the P1 and P2 primers (Wetzel et al., 1992). More recently, the International Plant Protection Convention (IPPC) hosted by FAO, suggested real-time RT-PCR as the most convenient molecular method for PPV detection coupled with 5B-IVIA monoclonal ELISA (IPPC, 2009). Whatsoever, based on data obtained in the first period of SharCo, we also recommend the use of two different methods (biological, serological and/or molecular) to consider a sample as healthy or infected. The biological indexing has the main disadvantage of being time and space consuming. In consequence, the use of ELISA and real-time RT-PCR based methods are recommended for accurate PPV diagnosis. ELISA using the 5B-IVIA monoclonal antibody is the recommended method of PPV detection for routine analyses during the vegetative period due to its high specificity (high confidence in positive reactions). Spot real-time RT-PCR is an alternative, molecular method that should be preferred if one is testing dormant tissues (buds) because of its high sensitivity (high confidence in negative reactions).

3. Means of verification and actual achievements

The accuracy in PPV detection by using the validated serological (ELISA 5B-IVIA) and molecular (spot real-time RT-PCR) methods was described by Capote et al. (2009) in samples originating from adult trees and by Vidal et al. (2009) for juvenile plants. The validation of protocols for routine analyses of large numbers of nursery plant samples using diagnostic parameters (sensitivity, specificity, likelihood ratios, predictive positive and negative values, accuracy, and post-test probability) is currently being calculated. The initial data shows a high agreement (kappa index 0.88 ± 0.01) (Cohen, 1960), between the two assayed detection methods, after analysis of more than 5,000 nursery *Prunus* plants.

4. Contingency plan

No contingency plan is proposed since this ML5 was successfully achieved. The EPPO and IPPC recommended detection methods have been upgraded and validated in the course of SharCo, extending the study to the sampling methods.

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