SharCo

Containment of Sharka virus in view of EU-expansion

Small Collaborative project of the 7th Framework Programme

Theme 2

Food, Agriculture, Biotechnologies

DE.3.2

Procedures for sampling in nursery blocks and validated tools and protocols for reliable and accurate PPV detection

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Deliverable report structure

1. General Presentation

The main goal of the third workpackage (WPE3) in the SharCo project is *Plum pox virus* (PPV) containment and reduction of the impact of the virus in nurseries. For this purpose, the development of accurate PPV detection methods and protocols is essential in order to detect as soon as possible PPV outbreaks and limit the trade of PPV infected trees for planting.

PPV is a member of the *Potyvirus* genus in the *Potiviridae* family, in which, in recent years, there have been significant advances in the knowledge of the genome organization and expression, in the deciphering of function variability of the different viral genome products and in the identification of pathogenicity and host range determinants (Candresse and Cambra., 2006; Decroocq et al., 2006; James and Glasa., 2006; Salvador et al., 2006; García and Cambra, 2007; Barba et al., 2010).

PPV is efficiently transmitted in the field by different aphid species in a non-persistent manner (Ng and Falk, 2006). For decades, there has been no awareness, nor reliable detection methods and reagents suitable for a large scale application. In consequence, PPV has easily escaped visual inspections and other inefficient control methods employed up to now. The illegal traffic and/or the exchange of symptomless propagative plant material have probably been the main mean of PPV spreading over long distances (Cambra et al., 2006). In fact, sharka disease has been reported, worldwide, in most countries producing *Prunus* crop species for industrial purposes and fruit marketing (Capote et al., 2006; Barba et al., 2010). However, the impact of the disease is also non-negligeable in countries where the stone fruit production is socio-economically and locally important, such as in Eastern European and former Yugoslavian states.

Despite very significant progress in recent years in the accuracy of PPV detection methods (EPPO, 2004; IPPC, 2009), there is still to-date a significant risk of dissemination of nursery plant material with “subclinical” PPV infections, *i.e* plant material in which PPV has not been detected in nursery blocks but in which sharka will develop later on, while planted in new locations, after a variable period of latency up to three to four years. Consequently, an improved detection protocol for the control of nursery plants is needed to facilitate the labour of plant protection services (PPS) inspectors. Improved detection protocols will increase the guaranties of sorting out PPV-infected from PPV-free materials and thus value both the

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production of certified plants and the delivery of a reliable sanitary EU passport in order to prevent PPV spread.

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). Three aspects are conditioning the accuracy of a detection test in *Prunus* plants maintained in nursery blocks:

1) Sampling (period of sampling in nursery blocks, the type of tissues collected, sampling pressure, number of passages of the inspectors/owner in the nursery blocks, individual or multiple sample(s) collected at once and pooled, sample storage before analysis).

2) Sample preparation and processing (preparation of sample extracts, RNA purification, direct methods of sample preparation without nucleic acid purification).

3) Quality of the detection test (use of validated techniques and reagents).

2. Detailed description

In order to define a reliable protocol of sampling and virus detection, we conducted various experimental procedures in nursery blocks established in Valencia (Spain) and in Bari (Italy) as well as in laboratories. We thus assessed different parameters related to the three aspects described above in order to identify the best conditions for the delivery of an accurate and reliable protocol. Results are presented below.

2.1 Sampling:

Period: Traditionally, the recommended period for PPV detection was limited to spring (active vegetative period) and was frequently based on visual inspections targeting PPV symptoms. Nowadays, the better quality of current serological and molecular detection assays, due to increased specificity, sensitivity and accuracy, has lead to the definition of an extended sampling period, up to all four seasons, the dormancy period included.

While estimating accuracy by the number of true positives plus the number of true negatives divided by the total number of analysed samples, the most accurate detection assays are still those performed during the vegetative period, at the beginning of spring and at the end of fall.

Nevertheless, in order to split the labour load required for sampling and PPV detection over longer periods, we confirmed that samples can also be collected and analysed during the dormant period (winter), at the time when the nursery plants are commercialized (Capote et al., 2009).

Plant tissues: Appropriate selection of plant tissues is critical for serological or molecular detection of PPV in nursery plants. Flowers, leaves or fruits showing PPV symptoms are excellent material for analysis. Nevertheless, symptomless plants are more frequent in nursery blocks and they do represent the most critical material for further spread over long distances through their commercialisation. We thus recommend sampling from symptomless and/or juvenile plants, as follows:

- during the vegetative period, 3 to 4 fully expanded leaves per nursery plant or 10 per adult tree. Leaves should preferably be 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. The basal part of the leaves, including the peduncle, is the most appropriate leaf area for PPV detection, in symptom-free material.

- during the dormant period in winter, 3 to 4 dormant buds per nursery plant or 15 to 20 per adult tree (corresponding most of the time to mother plants), from apical, medium or basal part of shoots.

In summary, PPV can be detected from infected, symptomless plants by collecting either leaves or buds at any period of the year. However, by serological ELISA (5B-IVIA monoclonal based), the most accurate results are obtained rather in spring from fully expanded leaves than in winter with dormant buds. Nevertheless, similar accuracy in PPV detection was attained from leaves collected in spring or from dormant buds collected in winter when the analysis was performed by real-time RT-PCR based methods.

Sampling pressure (number of samples to be selected per nursery block): The analysis of 25% of the nursery plants in a single nursery block using the hierarchical method (Hughes et al., 2002) obviously lead to an accurate estimation of the PPV incidence, but this procedure is not normally achievable due to the high number of samples that need to be processed. A good

estimation of the PPV presence in a nursery block located in open fields is obtained by analyzing at least 10% of the plants by ELISA (5B-IVIA based) pooled in samples representing 4 plants all together when the expected PPV prevalence is equal or less than 0.3%. The same estimation can be done analyzing 10% of the plants by Spot real-time RT-PCR pooled in samples representing 10 plants all together when the expected PPV prevalence is equal or less than 0.3%

Nevertheless, we recommend that all mother plants in nurseries not established under screenhouses or insect-proof nets or covers must be individually tested, every year.

To increase the possibilities of PPV detection in a nursery, the most PPV susceptible rootstocks have to be preferentially selected for grafting.

Number of passages in a nursery block: The best accuracy in PPV detection is achieved when several passages for sampling and analysis are performed in the same nursery block, each year. An initial screening of the nursery block has to be done during the vegative period by ELISA and then in dormant period, before the trade of the propagative plants, by real-time RT-PCR. Mother plants or imported budsticks for grafting have to be analyzed individually by real-time RT-PCR at any time of the year, before the collection of buds for grafting.

Individual or pooled sampling: Composite (pool of) samples can be prepared but the number of samples pooled will depend on accuracy and sensitivity of the analytical (serological or molecular) method chosen:

When detecting PPV by ELISA (5B-IVIA), in spring time, similar results and accuracy were obtained by testing individual samples or by combining up to four nursery plants (3 leaves per plant and 4 plants pooled, equal to 12 leaves tested at once). However, in winter, the analysis of nursery plants starting from dormant buds has to be performed individually.

When testing by real-time RT-PCR, whatever is the growing period or whatever is the plant tissue sampled (leaves *vs* dormant buds), the same accuracy was attained from one-by-one tree samples or from composite samples obtained by pooling 10 plants (3 leaves per plant and 10 plants sampled = 30 leaves or buds tested together).

Sample storage: Store leaves at 4ºC for not more than seven days. For longer periods, leaves have to be placed in a -80°C freezer and then analyzed by real-time RT-PCR. Dormant buds can be stored for longer periods, without detaching them from the collected shoot. In part 2.2 below, other options (immobilization of PPV targets by tissue prints or squashes or extracts) are described. They correspond to a handy, alternative way of disposing of samples until PPV detection.

2.2 Sample preparation: Sample preparation has to be performed according to the EPPO (2004) or IPPC (2009) protocols. Among all the available procedures, direct detection methods that avoid nucleic acid purification allow rapid and high-throughput detection of PPV by real-time RT-PCR (Olmos et al., 1996; Bertolini et al., 2008). In those procedures, crude extracts are diluted in buffer or spotted on nylon membranes to be used later on. Alternatively, immobilised PPV targets can be amplified from sections of plant tissues freshly printed or squashed onto nylon membranes, without plant extract and/or nucleic acid preparation (Capote et al., 2009). The four above-mentionned sample preparation methods (dilution of extracts, spot, tissue-print and squash) coupled to real-time RT-PCR are efficient for successful PPV detection.

2.3 Quality of the detection: Conventional diagnosis of viral agents present in woody plants is based on biological indexing followed by visual inspections and/or serological assays. However, molecular methods are increasingly preferred for detection and characterization of plant viruses because they have the advantage of targeting the viral genome, leading to further isolate and strain identification by subsequent sequencing of the viral fragment (López et al., 2008). In general, molecular techniques also provide a supplementary method of PPV detection, usually applied after serological assays and/or biological indexing. Since molecular techniques are more sensitive, they are particularly adapted to the analysis of suspicious samples for which previous data are not conclusive or for rapid screening of critical samples maintained in the quarantine area. It is also used for PPV typing when the first PPV outbreak is detected in a given country.

EPPO's protocol (2004) suggested ELISA based on the 5B-IVIA monoclonal antibody and inmunocapture (IC) RT-PCR using P1 and P2 primers (Wetzel et al., 1992) as the most appropriate methods for PPV detection. Nevertheless, 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-based ELISA (IPPC, 2009). In the course of the SharCo WPE.3, spot real-time RT-PCR has been compared with the serological method over a large-scale analysis. The same plant extracts were used in both tests. In general, samples with high 405nm OD values showed low Ct values and *vice versa*. Interestingly, a high percentage of coincidental results between spot real-time RT-PCR and ELISA (5B-IVIA based) procedures was obtained, reaching 96.16%.of coincidental results (Table1). The Cohen's kappa index (Cohen, 1960) was 0.88 ± 0.01, indicating an excellent agreement between both detection methods (Table 1).

Table 1: Comparison of ELISA (5B-IVIA) and Spot real-time RT-PCR results for PPV detection in 5,047 nursery plants analyzed using the same plant extract.

		Total analyzed plants: 5,047	
		ELISA (5B-IVIA)	
Spot		**+**	**-**
real time	**+**	967	184
RT-PCR	**-**	10	3,886
Cohen's kappa index			5,047

The OEPP protocol for PPV diagnosis recommends the use of two distinct PPV detection methods (biological, serological and/or molecular) to consider a sample as healthy or infected. We further recommend the use of 5B-IVIA based ELISA and real-time RT-PCR methods for accurate and non-ambigous PPV diagnosis. In such situation, ELISA (using the 5B-IVIA antibody) would be the recommended technique for routine analyses during the vegetative period due to its high specificity (high confidence in positive reactions) while spot real-time RT-PCR is the detection method of choice during the dormancy period, due to its high sensitivity (high confidence in negative reactions).

3. Original specifications and actual achievements

The following specifications, as mentioned and detailed above, can be considered as significant and reliable for PPV detection:

1) PPV testing on nursery plants at any period of the year, including dormant period.
2) Use of the basal part of symptomless, fully expanded, leaves for PPV detection in nursery plants from spring to fall.
3) Buds collected during dormant period are fairly appropriate for PPV detection in winter time.
4) Accurate PPV detection can be achieved from combined sampling (pools of up to 4 nursery plants all together).
5) Direct methods of sample preparation, prior to real-time RT-PCR analyses, can be successfully used for large scale analysis of samples in a routinely manner.
6) Use of EPPO or IPPC recommended techniques of PPV detection, reagents (antibodies, primers and probes) and protocols for a more accurate and reliable detection of PPV.
7) When choosing a diagnostic test, one has to remember that there is no perfect method that would never give false positive or false negative results. It also means that it is necessary to know the capacity of each technique available and to define the one the most appropriate in each condition. This capacity is measured by the estimation of sensitivity, specificity, predictive positive and negative values according to the prevalence, hit rate or accuracy and likelihood ratios (López et al., 2008; Olmos et al., 2008). Sensitivity and specificity are not the only criteria indispensable to select a diagnostic test. The probability that a diagnostic method will result in an accurate diagnosis must be determined by calculating other predictive values (Altman and Bland, 1994). A positive predictive value represents the proportion of positive samples correctly diagnosed. A negative predictive value is the proportion of samples with negative results which are correctly diagnosed. However, the predictive values also depend on the prevalence (viral incidence) of infection in the samples tested and do not apply universally (Olmos et al., 2008; Massart et al., 2008). Prevalence can be interpreted as the probability that the sample is harboring the pathogen before the test is performed. If the prevalence of the infection is very low, the positive predictive value will not be close to 1 even if both sensitivity and specificity are high. In screening tests, it is expected that many samples issued from nursery plants and giving positive results by one specific method, are false positives. Positive and negative

predictive values can be calculated for any prevalence (López et al., 2008; Olmos et al., 2008).

8) The highest hit rate (accuracy) in routine PPV detection procedures is usually obtained by ELISA using the 5B-IVIA specific monoclonal antibody followed by RT-PCR based molecular methods. However, one has to be aware that the high sensitivity of real-time RT-PCR implies the risk of false positives. Those two techniques and reagents are providing a higher specificity, being recommended for the analysis of large numbers of samples during the vegetative period.
9) The highest sensitivity in PPV detection at any period of the year is afforded by real-time RT-PCR based methods. This technique using primers and probes as described by Olmos et al. (2005) provides a higher confidence in the negative results being very appropriate and will thus allow sorting out true PPV-free material.
10) The use of at least two validated methods (serological and molecular, in combiantion for the same samples) is recommended by EPPO and IPPC-FAO protocols for a higher accuracy in PPV detection. We confirm this recommendation.

4. Use and dissemination of the results

The protocols and recommendations related to PPV detection developed in the course of the SharCo first period or previously validated in international forums were already transferred to members of the SharCo consortium (see protocols online on the SharCo plateform or website) and are being disseminated to non-SharCo members through the SharCo website, the technical training workshops as it happened already in Poland and in Turkey and through scientific and technical publications. International policy and plant protection agencies such as EPPO and IPPC-FAO will clearly benefit from those improved protocols for PPV detection. They will be proposed for larger dissemination through the elaboration and publication of cultivation guidelines and PPV early warning system in workpackage WPA1. Finally, the direct beneficiaries will be the Plant Protection Services of the different EU Member States and associated countries (Serbia, Turkey already aware of such initiative), PPS inspectors included, since it might conditionned the delivery of the European phytosanitary passport. The application of the above recommended protocols for PPV

detection will help to contain PPV spread and to increase the guarantee of international trade of PPV-free plants.

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