SharCo

Containment of Sharka virus in view of EU-expansion

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Deliverable DE3.1

Improved methods to detect the presence of PPV-viruliferous aphids in nurseries and ranking of the susceptibility to natural PPV infection of different Prunus rootstocks

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Deliverable report structure

1. GENERAL PRESENTATION **3**
1.1. CONTEXT 3
1.2. RATIONAL 4
1.2.1. Detection and quantitation of viral targets in insect vectors *4*
1.2.2. Susceptibility of Prunus rootstocks to natural infection by Plum pox virus *6*
2. DETAILED DESCRIPTION **7**
2.1. APHID MONITORING 7
2.2. SAMPLE PREPARATION (VIRAL NUCLEIC ACID EXTRACTION FROM APHIDS AND PLANT MATERIAL) 8
2.3. REAL-TIME RT-PCR FOR *PLUM POX VIRUS* DETECTION AND QUANTITATION 9
3. ORIGINAL SPECIFICATIONS AND ACTUAL ACHIEVEMENTS **9**
4. USE AND DISSEMINATION OF THE RESULTS **10**
5. REFERENCES **11**
6. ANNEX **20**
IVIA / SHARCO EU PROJECT PROTOCOL **20**
PREPARED BY E. VIDAL, A. MORENO AND M. CAMBRA **20**
PROTOCOL FOR “STICKY SHOOT METHOD” **20**
REFERENCES: **22**

1. General Presentation

1.1. Context

The main goal of the workpackge number 3 (WPE3) of the project KBBE 204429-CE entitled "Containment of sharka virus in view of EU-expansion" is the evaluation of strategies to reduce *Plum pox virus* (PPV) incidence in nursery blocks. For this general purpose it is necessary to provide information about epidemiological data to tray to interfere with the natural spread of PPV by a number of aphid species present in each ecological area.

PPV is a member of the genus *Potyvirus* in the family *Potiviridae*, from which, in recent years there have been notable advances in the knowledge of the genome organization and expression, functions of genome products, and 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). PPV causes sharka, one of the most serious diseases of *Prunus* species (Cambra et al., 2006c). The causal agent of sharka disease is transmitted in the field by aphids in a non-persistent manner (Kunze and Krczal, 1971; Ng and Falk, 2006). In early studies, the attention was focused on *Prunus* colonizing species. *Myzus persicae*, *Brachycaudus helychrisi, Phorodon humuli,* and *Hyalopterous pruni* were reported as PPV vectors (Jordović, 1963; Kassanis and Šutíć, 1965; Minoiu, 1973). Then, it was demonstrated that more than twenty species belonging either to *Prunus* colonizing or non-colonizing species were able to transmit PPV (Labonne et al., 1995). Currently, *M. persicae* and *Aphis spiraecola* are considered the main and most efficient PPV vectors (Glasa et al.,2004; Cambra et al., 2006b; Labonne and Dallot, 2006). Nevertheless, the Illegal traffic and insufficiently controlled exchanges of infected yet symptomless propagative plant material, has probably been the main pathway of spread of PPV over long distances (Cambra et al., 2006c). In fact, sharka disease has been reported in all the most important *Prunus* industries worldwide except

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Australia, New Zealand, South Africa and California (USA) (Capote et al., 2006; García and Cambra, 2007). Despite very significant progress in recent years in accuracy of PPV detection methods (OEPP/EPPO, 2004), there is still today a significant risk of dissemination of nursery plants with "subclinical" PPV infections, which will ultimately shown symptoms in the new plantations after a variable period of latency. Consequently, improved sensitive control of nursery plants is needed, as well as the use of less sensitive or resistant rootstocks and cultivars to natural PPV infection.

1.2. Rational

1.2.1. Detection and quantitation of viral targets in insect vectors

A number of features contribute to the success of aphids as vectors of plant viruses (Ng and Perry, 2004; Cambra et al., 2006a). These include: a) their polyphagous nature (migratory behaviour) that allows them to visit and feed on a wide range of plant species, facilitating the spread of viruses that infect a large number of plant hosts, b) the ability to undergo parthenogenetic reproduction (rapid generation of large quantities of individuals), and c) the possession of a highly specialized stylet capable of piercing plant cell walls and delivering viruses into a host cell. In addition, basically three factors determine the viral incidence in field: a) the number of immigrant winged aphids and the timing of colonisation, b) their viruliferous state, and c) the dynamics of the secondary epidemic spread (Plumb, 1990). Thus, the assessment of infectivity of aphids appears to be one of the most important factors to evaluate the spread and epidemics of aphid-borne viruses and for development of any decision making-system to improve viral control strategies (by interfering in the success of the transmission event (Fabre et al., 2003; Marroquín et al., 2004; Cambra et al., 2006a).

The viruliferous state (presence of virions or viral PCR-amplifiable targets) of aphid species was, at first, indirectly assessed by observation of symptoms on plants and by experimental assays of transmission. The development of the serological ELISA technique (Clark and Adams, 1977) revolutionised diagnostics in plant

virology and, from the beginning, was successfully applied to detect circulative (propagative and nonpropagative) and noncirculative (semipersistent and nonpersistent) aphid-transmitted plant viruses (Gera et al., 1978; Denèchère et al., 1979; Clarke et al., 1980; Du Plessis and Von Wechmar, 1981; Cambra et al., 1981 and 2006a). However, when not propagative, the low concentration of viruses in single aphids was close to the normal limit of reliable detection by ELISA assays. In the last decade, due to its high sensitivity, conventional PCR and reverse transcription coupled to PCR in a single step (RT-PCR), are molecular methods frequently used for the detection of plant viruses. Different PCR or RT-PCR variants including Immunocapture (with plant extracts) (Wetzel et al., 1992; Nolasco et al., 1993) or print/squash-capture PCR (with immobilised targets on paper) (Olmos et al., 1996 and 1997), enable the detection of minute quantities of DNA or RNA targets in plant or insect tissues. Nevertheless, when targets are at very low concentration and/or the samples (plant material or insects) contain PCR-inhibitors, a more sensitive detection can be achieved by heminested or nested-PCR. These two techniques enhance sensitivity but greatly favour the risk of self-contamination when two different tubes are used in the subsequent reactions. To avoid this problem, nested-PCR in a single closed tube has been proposed using a compartmentalised Eppendorf tube (Olmos et al., 1999). Usually the PCR products are detected by gel electrophoresis but colorimetric detection of PCR products has been successfully employed increasing sensitivity and facilitating the interpretation of results (McManus and Jones, 1995; Bertolini et al., 2001).

The detection of nonpersistently transmitted viruses is very problematic due to the very low titre or viral charge of these particular viruses (formerly named stylet-borne viruses) in aphid vectors. Probably for this reason there are few reports of detection of nonpersistently transmitted viruses in individual aphid vectors using PCR-based methods (Singh et al., 1996, 2004; Olmos et al, 1997; Singh, 1998; Nie and Singh, 2001; Cambra et al., 2004). In addition, the information provided was only qualitative.

Today the use and application of real-time RT-PCR is gaining acceptance as a highly sensitive method for nucleic acids detection and quantitation (López et al., 2006). Real-time PCR allows amplification, detection, monitoring and quantitation in a single step by employing one of several chemistries which are used to detect PCR

products as they accumulate within a closed reaction vessel during the reaction. This methodology has been successfully applied for detection and quantitation or viral targets in insect vectors, such as those developed for *Maize streak virus* (Lett et al., 2002), *Tomato spotted wilt virus* (Boonham et al., 2002), *Barley yellow dwarf virus* (Fabre et al., 2003), *Plum pox virus* (Schneider et al., 2004; Olmos et al., 2004 and 2005) and *Citrus tristeza virus* (Bertolini et al., 2008). This extremely sensitive technology is being applied to basic studies on virus replication as well as in more applied research concerning epidemiology, breeding for resistance, virus-vector relationships, and other fields (Olmos et al., 2007). The use of real-time PCR in plant virology and in epidemiology will open new possibilities to improve control strategies.

1.2.2. Susceptibility of Prunus rootstocks to natural infection by *Plum pox virus*

The PPV hosts are well known (Llácer and Cambra, 2006; James and Thompson, 2006; Polák, 2006) as well as *Prunus domestica* L. and *P. tomentosa* are probably the most susceptible PPV host species. Nevertheless a ranking of *Prunus* susceptibility to PPV never was established in standard conditions (i.e. using a representative collection of the same PPV strains and isolates or in the same ecological area).

The assessment of the susceptibility or resistance of different *Prunus* rootstocks to be infected by PPV-viruliferous aphids in natural conditions is one of the goals of the SharCo project. There are very few previous experience and data on this subject from which only empirical knowledge is available. The evaluation of the resistance to PPV has traditionally been performed in different Prunus rootstocks by graft inoculation (i.e. Rubio et al., 2005), but this system is too drastic in terms of viral charge because the grafted material is a permanent source of PPV-inoculum. In SharCo project a total of six experimental Prunus rootstocks plots were established in Bulgaria, Czech Republic, Poland, Romania, Spain and Turkey, to assess the susceptibility of different Prunus species to the natural PPV infection. The following Prunus rootstocks are being assayed: Nemaguard (*P. persica x P. davidiana*, hybrid seedling), *P. marianna* GF8-1 (Mariana) (*P. cerasifera x P. munsoniana*), Docera 6 (*P. domestica x P. cerasifera*) (PPV-hypersensitive rootstock), Greenpac ((*P. persica*

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x P. davidiana) *x* (*P. dulcis x P. persica*)), Myrobolan 29 C (*P. cerasifera), Garnem (P. dulcis x P. persica*) (*Garfi almond x Nemared peach*), *Wanhgenheim prune* (*P. domestica seedling*), GF677 (*P. dulcis x P. persica*), St. Julien A (*P. insititia*), Adesoto 101 (*P. insititia*). These experimental plots were established in springtime 2008 and still there are not available data, consequently the data referred in this report was originated in previous experimental works done at IVIA in Valencia in 2006 and 2007 (Vidal et al., unpublished data).

2. Detailed description

2.1. Aphid monitoring

The classical monitoring method for aphid species is the direct sampling of established colonies on the young leaves or shoots. This method accurately estimates the aphid species that after visiting the crop, realised different feeding probes and agreeing with the host determined to establish a colony, but escapes to this method the migratory species that occasionally visit the crop for probing and/or feeding. In addition to sampling established colonies, several trapping methods have been used in surveys to determine or to evaluate the aphid species present or visiting an orchard or a single tree. These methods included the conventional suction traps (Taylor, 1955), yellow or green water traps (Moericke, 1951), sticky fishing-line traps (Labonne et al, 1983), and the sticky tree or shoot method using glue-covered bait leaves or shoots/flushes/budsticks (Avinent et al., 1993; Cambra et al., 2000). The later one is the most efficient way for estimating and predicting the numbers of aphids (winged adults) landing on the plants or visiting young shoots and leaves, according to Hermoso de Mendoza et al. (1998) and Derron and Goy (1998). The other methods give and estimation of the aphid species present in the environment of the orchard, but no necessarily visiting the surface of the plants in the crop of our interest.

The sticky tree or shoot method has been extensively used for monitoring aphid species in adult citrus trees (Marroquín et al., 2004; Cambra et al., 2004).

Briefly, young shoots (about 15-20 cm long) are sprayed with an adhesive glue non-phytotoxic (i.e. Souverode aerosol, Scotts France SAS), detached after seven or ten days, and new sticky shoots prepared in trees to complete the scheduled period of survey. The removed sticky shoots with aphids stuck on the surface of the leaves are placed in turpentine to dissolve the glue and then the aphids washed in soapy water to remove the solvent. The collected aphid species are kept in 70% alcohol for later identification and counting (see Annexe 1).

2.2. Sample preparation (viral nucleic acid extraction from aphids and plant material)

The identified aphid species, collected as recommended in Annexe 1, can be analysed for detection of viral targets. The first step is the extraction of the viral targets from individual aphids or for multiple aphid species together. The extraction of targets can be performed by a detergent solution (Singh, 1999), by conventional methods of nucleic acids extraction after extracts preparation (Mehta et al., 1997; Naidu et al., 1998; Fabre et al., 2003) or after immobilization of viral targets on paper (Olmos et al, 1996, 1997) or in nitrocellulose (Singh, 2004) or nylon (positively charged) membranes (Bertolini et al., 2008). The presence of viral targets in individual aphid species can be assessed from fresh as well as from previously collected individuals stored in alcohol and/or squashed on paper (Marroquín et al., 2004). See attached file that contains a video illustrating the process of squashing aphids and extraction of PPV-targets.

The use of previously immobilized targets on filter paper, nitrocellulose, nylon or other materials avoid the preparation of extracts and consequently the release of plant or insect inhibitors as well as potential contamination problems. In addition, can be stored for long time before being used or even mailed, thus allowing their direct preparation in the field if necessary (Olmos et al., 1996). These systems are simpler and much faster than extractions, and allow the manipulation of quarantine viruses without risks (Cambra et al., 1997; Bertolini et al., 2008).

2.3. Real-time RT-PCR for *Plum pox virus* detection and quantitation

Real-time quantitative RT-PCR assay based on TaqMan chemistry (Fabre at al., 2003; Olmos et al., 2005) seems to be more sensitive than intercalating dye SYBR Green I for detection and quantitation of RNA targets from the nonpersistently transmitted PPV (Olmos et al., 2004). The sensitivity afforded by real-time RT-PCR was hundred times higher than nested RT-PCR and one thousand times higher than DASI-ELISA and conventional RT-PCR. The quantities of PPV-RNA targets detected (by real-time RT-PCR) in a single aphid ranged from 40 to more than 2 x 10^3 units (Olmos et al., 2005). More recently, was estimated by real-time RT-PCR that the number of PPV-RNA targets inoculated by a *M. persicae* in a single access probe (about 27,000) was approximately half of the acquired ones. With this number of targets, PPV infection became systemic in 20% in the inoculated GF305 peach seedlings receptor plants (Moreno et al., 2008 and 2009, non published results).

For PPV detection, single aphid species are squashed on paper or positively charged nylon membranes with the rounded end of an Eppendorf tube (Olmos et al. 1999; Olmos et al., 2005). Pieces of membranes harbouring the printed and/or squashed samples are inserted into Eppendorf tubes. One hundred microliters of buffer (0.1 M glycine, 0.05 M NaCl, 1 mM EDTA) (Osman and Rowhani, 2006) are added, incubated at 95℃ for 10 min, vortexed and p laced on ice. Five microliters of this extract are directly used as template for real-time RT-PCR assays. Real-time RT-PCR protocol consist of one step at 48℃ for 30 min and 95℃ for 10 min followed by 45 cycles of amplification (95℃ for 15 s and 60℃ for 1 min). Data acquisition and analysis can be performed with software supplied by different manufacturers of real-time PCR apparatus.

3. Original specifications and actual achievements

The main PPV aphid species caught in Spanish orchards of *Prunus* (Japanese plums, apricots and peaches) in 1999, 2002 and 2003, and their proportions were: *A. spiraecola* (43%), *A. gossypii* (18%), *Hyalopterus pruni* (6%), *Brachycaudus*

prunicola (6%), *A. craccivora* (3%), *M. persicae* (2%) and other species (22%), being the main PPV-vectors *A. spiraecola* and *A. gossypii,* and close to 12% the percentage of PPV-viruliferous aphids after analyses by nested-RT-PCR (Cambra et al., 2004). This is a reference for present studies during SharCo activities in Spain.

In the activities of the SharCo project, a total of 129 individual aphids of *A. spiraecola* (69) and *A. gossypii* (60) species were caught in May 2008 in a plot situated in Llíria (Valencia, Spain). The aphids were caught by the “sticky shoot” method and Moericke water yellow traps. The individuals were analyzed by squash real-time RT-PCR according to Olmos et al. (2005). The average of PPV-viruliferous aphids in the SharCo plot was 15.50%. In the *A. gossypii* species 13.33% of individuals were PPV-viruliferous while in the *A. spiraecola* specie the percentage of aphids PPV- viruliferous was 17.39%. Results from the other experimental plots located in other countries are being analysed and will be soon available.

The ranking of susceptibility of the different *Prunus* rootstocks to PPV natural infection resulted in Spain (period previous to SharCo, during 2006 and 2007): Adesoto 101 (97%), *P. marianna* GF8-1 (96%), Nemaguard (59%), Myrobolan 29C (33%), GF677, Cadaman and Garnem (0%). This data are under confirmation in the experimental plots located in the countries of the different partners. The plants will be analysed in springtime 2009. The available results are in agreement with the very high susceptibility of plums to PPV infection, followed by peach seedlings (Nemaguard) and Myrobolan. Interestingly some *Prunus* rootstocks were not infected after two year cultivation in an area with a high PPV inoculum pressure.

4. Use and dissemination of the results

The detection of viral targets in viruliferous aphids is of great interest not only for basic viral replication studies but for designing of more efficient control strategies of viruses transmitted by aphids. During the SharCo project oil treatments will be sprayed after the detection of the first PPV-viruliferous aphids landing in the experimental plots or caught in Moericke traps, to assay to reduce the natural PPV infection.

It has been demonstrated the possibility of quantitation from fresh individual aphids as well as from aphids previously captured on traps and squashed on paper, without the need of previous RNA extraction. These combined technologies (squash capture and real-time amplification) open possibilities to implement an alert system for nurseries.

An initial ranking of the natural susceptibility of different Prunus rootstocks to PPV infection was established in Spain and will be confirmed in other ecological areas in a next future. The use of less PPV-susceptible rootstocks in nurseries will imply, for sure, the commercialisation of lower numbers of infected plants contributing to reduce the PPV spread at long distances.

Specific recommendations will be disseminated trough SharCo web page and conventional research publications.

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6. Annex

IVIA / SharCo EU project protocol

Prepared by E. Vidal, A. Moreno and M. Cambra

Protocol for "Sticky shoot method"

The aphid species landing on adult trees, rootstock or nursery plants can be accurately determined by the "sticky tree" method (Avinent *et al.*, 1993, Cambra, *et al.*, 2000 and 2004, Marroquín *et al.*, 2004).

Material:

- **Soveurode aerosol** 750 ml/unit from Scotts Bio-systèmes France SAS. With 1 unit it is possible to spray aprox. 55 shoots or flushes.
- **Duct tape** of white color (adhesive tape used to isolate electrical wires)
- **Pruning scissors**
- **Rubbish bags**
- **Pots** (appropriate containers)
- **Turpentine** (solvent)
- **Appropriate mask to manage organic solvents**
- **Fine brush**
- **Straine**r (use a plastic net strainer, because a metal ones can damage the aphids)
- **Falcon tubes** (50 ml)
- **Eppendorf tubes** (1.5 ml)
- **Tap water and wash-dishes detergent**
- **Alcohol ethylic 70%**
- **Filter paper or absorbent paper**

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Method:

1. Spray at aprox. 15 cm with the Soveurode glue a shoot or flush of 15-20 cm long per plant (with minimum 5 fully expanded leaves). Remain the sticky shoots for aprox. one week (maximum 10 days). To facilitate the location of the sticky shoots, it is recommended to mark the shoots with duct tape to find them when you come back to pick them up the next week.

2. Pick up individually the sticky shoots, put in rubbish bags (an individual sticky shoot per bag) and bring them to the laboratory. At the same time spray new flushes or shoots to be collected next week.

3. Introduce individually each sticky shoot in an appropriate pot or container. Each sticky shoots will be considered as an individual sample. If the shoots is too long you can cut it in several pieces.

4. Pour turpentine in each pot to remove the stuck aphids for 1h. Be protected against the use of organic solvents using **mask** and **gloves** from this step. It is recommended the use of turpentine under optimal conditions: extraction hold or in a well ventilated room.

5. After 1h in the solvent, shake the pot and throw out the shoot carefully checking the absence of aphids. Remove with a fine brush any aphid that still remains in the shoot.

6. Pour the liquid through a plastic net strainer (do not use metal net). Collect the insects (aphids, flies…) in the strainer.

Note: The turpentine can be collected in a container to reuse (2-3 times). Do not throw out the turpentine to the sink.

7. Wash (2 or 3 times), in a sink or in a container, the collected insects in the strainer with soap water. Soap water can be easily prepared adding 3 drops of wash-dishes

detergent per liter of tap water. It is very important to eliminate the traces of turpentine.

8. Put the washed insects which are in the strainer in a Falcon tube with alcohol 70%. Use a fine brush to collect the insects from the strainer.

9. Separate the aphids from the rest of insects. Store the aphids until their classification in Eppendorf tubes with alcohol 70%.

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