

SharCo

Containment of Sharka virus in view of EU-expansion

Small Collaborative project of the 7th Framework Programme

Theme 2

Food, Agriculture, Biotechnologies

DE.3.1

**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

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1. General Presentation

1.1. Context

The main goal of the workpackage 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 further information about the epidemiological situation of sharka during plant production and multiplication. This allows us to propose technical methods and cultivation guidelines that help interfere with the natural transmission of PPV by a number of aphid species present in each ecological area (see deliverable DA.2.2).

PPV is a member of the genus *Potyvirus* in the family *Potiviridae*, for which, in recent years, there have been better knowledge on i) the genome organization and expression, ii) function of the viral genome proteins, iii) and on their 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). It is transmitted in the field by aphids in a non-persistent manner (Kunze and Krczal, 1971; Ng and Falk, 2006). In previous studies, the attention focused on *Prunus* colonizing vector species and *Myzus persicae*, *Brachycaudus helychrisi*, *Phorodon humuli*, and *Hyalopterus pruni* were reported as PPV vectors (Jordović, 1963; Kassanis and Šutić, 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, it is suspected that the PPV epidemiology differs from nursery plots to orchards, the main reason being the distinct stages of growth of the host plants and the higher number of succulent shoots attractive for aphid probing and feeding/m² in nurseries. When the goal is to reduce PPV transmission and spreading, both epidemiological situations have to be distinguished. Moreover, illegal traffic and insufficient custom controls of infected yet symptomless propagative plant material has probably been (and might still be) the main source of PPV spreading over long distances (Cambra et al.,



2006c). Despite very significant progress in recent years in accuracy of PPV detection methods (OEPP/EPPO, 2004; IPPC-FAO, 2011), there is still today a significant risk of dissemination of nursery plants with “subclinical” PPV infections, which will ultimately show symptoms once established in the new plantation, after a variable period of latency. Consequently, improved, sensitive control measures of nursery plants are needed, together with the use of less susceptible rootstocks and cultivars that will help control PPV spread.

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; Moreno et al., 2009). 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 viral incidence in the 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 in consequence, to develop any decision making-system aiming at improving virus control (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, initially, indirectly assessed through the observation of symptoms on plants and/or by experimental transmission assays. Later, diagnostics in plant virology were revolutionized with the development of serological ELISA techniques (Clark and Adams, 1977) which were successfully applied to detect circulative (propagative and non-propagative) and non-circulative (semipersistent and non-persistent) 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, the low concentration of non- propagative viruses in single aphids



is close to the normal limit of reliable detection by ELISA assays. In the last decade, due to its high sensitivity, conventional PCR (Polymerase Chain Reaction) 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 technical alternatives were developed, including immunocapture (using crude plant extracts) (Wetzel et al., 1992; Nolasco et al., 1993) or print/squash-capture PCR (using immobilised targets on paper) (Olmos et al., 1996 and 1997). Those techniques enabled the detection of minute quantities of DNA or RNA targets in plant or insect tissues. Nevertheless, when targets are present in very low concentration and/or samples (plant material or insects) contain inhibitors of the polymerase chain reaction, a more sensitive detection is requested. This was achieved by hemi-nested or nested-PCR. However, these two techniques enhance sensitivity but greatly favour the risk of self-contamination when two different tubes are used in subsequent reactions. To avoid this problem, nested-PCR in a single closed tube has been set up, using a compartmentalised Eppendorf tube (Olmos et al., 1999). Usually the PCR products are detected by gel electrophoresis but the 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 non-persistently 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 non-persistently transmitted viruses in individual aphid vectors using conventional 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 is only qualitative and not quantitative.

Nowadays, 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 in the reaction vessel during the reaction. This methodology has been successfully applied for the detection and quantitation of 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; Moreno et al., 2009; Vidal et al., 2010) and *Citrus tristeza virus* (Bertolini et al., 2008; Saponari et al., 2008). In fact, an accurate estimation of the number of PPV virions acquired and transmitted by a single *M. persicae*, was reported (Moreno et al., 2009). In this work, a combination of electrical penetration graph and TaqMan real-time RT-PCR techniques was used to establish the average number of PPV RNA targets inoculated by an aphid in a single probe (26,750), approximately half of the acquired ones. This number of PPV targets is responsible for a success rate of 20% of 'GF305' peach seedlings systemically infected. No significant differences were found between the number of PPV RNA targets acquired after one or five intracellular punctures (pd), but the frequency of efficiently infected plants was higher after 5 pd. The percentage of PPV-positive leaf discs after just 1 pd of inoculation probe (28%/4,603 targets) was lower than after 5 pd (45.8%/135 x 10⁶ targets), demonstrating that only a relatively small number of PPV particles contribute to systemic infection of inoculated woody plants.

This extremely sensitive technology, based on real-time PCR, 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; Moreno et al., 2009). The use of real-time PCR in plant virology and in epidemiology will provide **new opportunities to improve plant protection control measures**. In addition, the application of this technology will produce **relevant information for understanding several aspects of virus-vector-plant interactions** that play a significant role in the transmission of non-persistent viruses.

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

Together use of rootstocks that are less susceptible to natural *Plum pox virus* (PPV) infection and the application of mineral oil treatments are alternative strategies which help reduce viral incidence in nursery plots. Both strategies are being evaluated in the SharCo project, within workpackage WPE3. Concerning rootstocks susceptibility to natural infection, we were already aware that some *Prunus* species are well-known PPV hosts (Llácer and Cambra, 2006; James and Thompson, 2006; Polák, 2006). For example, *Prunus domestica* L. and *P. tomentosa* are probably the most susceptible PPV host species. Nevertheless a ranking of *Prunus* susceptibility to PPV was never established in standard conditions, i.e. using a representative



collection of the same PPV strains and isolates or/and in the same ecological area.

The assessment of the susceptibility or resistance of different *Prunus* rootstocks to aphid-borne PPV infection in natural conditions is one of the goals of the SharCo project. There are very few previous experiments conducted on a similar way and from those, only empirical knowledge is available. The evaluation of PPV resistant *Prunus* rootstocks has traditionally been performed 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.

Vidal et al. (2010) evaluated the susceptibility to PPV natural infection of a number of *Prunus* rootstocks currently used by the Spanish stone fruit nurseries. The evaluation was done in two different locations in Valencia (Spain) under high and low PPV inoculum pressures. Under high inoculum pressure, all rootstocks tested exhibited significant differences in their susceptibility to natural infection. By the end, the most susceptible rootstocks were 'Adesoto 101' and 'Mariana GF8-1', followed by 'Nemaguard' and 'Myrobolan 29C'. The rootstocks 'Cadaman' and 'Garnem' presented the lowest rate of PPV-infected plants, which were detected only by spot real-time RT-PCR but not by ELISA. No differences among the rootstocks tested were found under low PPV inoculum pressure, but still, 'Adesoto 101', 'Mariana GF8-1' and 'Nemaguard' resulted infected.

The available data on susceptibility of *Prunus* rootstocks are in agreement with the very high susceptibility of plums ('Myrobolan 29C') to PPV infection together with the peach 'Nemaguard' seedlings.

In the SharCo project, a total of six experimental rootstock plots were established in Bulgaria, Czech Republic, Poland, Romania, Spain and Turkey, **with the aim of assessing the susceptibility of different *Prunus* species to natural PPV infection in different ecological areas**. The following *Prunus* rootstocks were planted: 'Nemaguard' (*P. persica* x *P. davidiana*, hybrid seedling), 'Mariana GF8-1' (*P. cerasifera* x *P. munsoniana*), 'Docera 6' (*P. domestica* x *P. cerasifera*) (PPV-hypersensitive rootstock), 'Greenpac' ((*P. persica* 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), 'Wangenheim' prune (*P. domestica* seedling), 'GF677' (*P. dulcis* x *P. persica*), 'St. Julien A' (*P. insititia*) and 'Adesoto 101' (*P. insititia*).

The experimental plots were established in Spring 2008 and are still in the field. The last evaluation will be performed in Spring 2011.



2. Detailed description

2.1 Aphid monitoring

The classical method for aphid species monitoring consists in the direct sampling of established colonies on young leaves or shoots. This method accurately estimates the aphid species visiting the crop, probing and then finally establishing a colony,. However, escape to this method the migratory species that occasionally visit the crop only for probing and/or feeding but not for establishment. In addition to the above method, 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 include 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 last 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 estimation on the aphid species present in the environment of the orchard, but not necessarily visiting the surface of the plants or landing on the crop.

The sticky tree (or shoot) method has been extensively used to monitor aphid species in adult trees (Cambra et al., 2000; Marroquín et al., 2004; Cambra et al., 2004; Cambra et al., 2006a; Capote et al., 2008). Briefly, young shoots (about 15-20 cm long) are sprayed with adhesive, non-phytotoxic glue (Souverode aerosol, Scotts France SAS), detached after seven or ten days for laboratory analysis while other sticky shoots are sprayed for the next period of survey. The removed sticky shoots presenting aphids stuck on their surface are then placed in turpentine to dissolve the glue. The aphids are washed in soapy water to remove the solvent and finally collected in 70% alcohol for later identification and counting (see Annexe 1).

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

Once identified, aphid species are preceded through molecular analysis in order to detect viral targets. The first step of this analysis is the extraction of viral targets



from individual aphids or from multiple aphid species together. The extraction of targets is performed either by the use of a detergent solution (Singh, 1999), by conventional methods of nucleic acids extraction after extract 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; Moreno et al., 2009). 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). For more details, see on the SharCo website (<http://www.sharco.eu/sharco/>), a video illustrating the process of aphids squashing and the extraction of PPV targets.

The use of previously immobilized targets on filter paper, nitrocellulose, nylon or other materials permits to avoid extract preparation and consequently the release of plant or insect inhibitors as well as potential contamination problems. In addition, tissue-printed material 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 techniques are simpler and much faster than extractions and allow the manipulation of quarantine viruses without risks (Cambra et al., 1997; Bertolini et al., 2008). The method has also been employed for accurate estimation of the number of PPV virions acquired and transmitted by single aphids (Moreno et al., 2009).

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

Real-time quantitative RT-PCR assay based on TaqMan chemistry (Fabre et 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 non-persistently transmitted PPV (Olmos et al., 2004). The sensitivity afforded by real-time RT-PCR is hundred times higher than nested RT-PCR and one thousand times higher than DAS-ELISA and conventional RT-PCR. For these reasons this method was adopted for sensitive and accurate, viral targets and virions detection in the workpackage WPE3.

For PPV detection, single aphid species are squashed on paper or positively charged nylon membranes with the bottom 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°C for 10 min, vortexed and placed on ice. Five microliters of this extract are directly used as template for real-time RT-PCR assays. The real-time RT-PCR protocol consists of one step at 48°C for 30 min and 95°C for 10 min followed by 45 cycles of amplification (95°C for 15 s and 60 °C for 1 min). Data acquisition and analysis is performed with softwares supplied by different manufacturers of real-time PCR apparatus.

2.4 Evaluation of rootstocks susceptibility to PPV infection

All experimental nursery plots, planted in SharCo, followed a common experimental design which took into account the proximity and position of the PPV inoculum source in each plot.

The SharCo experimental nursery plots were sampled at least once per year, generally in springtime. Rootstock plants were individually sampled by collecting four fully expanded leaves per plant from different parts of the canopy of each individual rootstock plant. Leaves showing PPV-like symptoms were collected when detected. Serological assays for PPV detection were performed by DAS-ELISA using the 5B-IVIA/AMR (Cambra *et al.*, 1994) monoclonal antibody based kit (AMR Lab, Barcelona, Spain) following the EPPO (2004) protocol for PPV detection.

3. Original specifications and actual achievements

3.1. Detection of PPV-viruliferous aphid species

The relative percentage of the main PPV aphid species caught in Spanish orchards of *Prunus* host plants (Japanese plums, apricots and peaches) in 1999, 2002 and 2003 by the “sticky shoot method” were: *A. spiraecola* (43%), *A. gossypii* (18%), *Hyalopterus pruni* (6%), *Brachycaudus prunicola* (6%), *A. craccivora* (3%), *M. persicae* (2%) and other species (22%). It appeared at this time that the most prevalent PPV-vectors are *A. spiraecola* and *A. gossypii*. The analysis of these samples by nested-RT-PCR resulted in approximately 12% of PPV-viruliferous aphids (Cambra *et al.*, 2004). Nevertheless, when real-time RT-PCR is used instead



of nested-RT-PCR, the number of PPV-viruliferous aphids detected is expected to increase due to the higher sensibility of the real-time amplification. Effectively, by real-time RT-PCR, the percentage of PPV-viruliferous aphids that visited an adult plum orchard located in an area with a high PPV inoculum pressure (about 80% infected trees) was estimated to reach 27.5% (Capote et al. 2008). Similarly, Vidal et al. (2010) evaluated the aphid species visiting experimental *Prunus* nursery plots located close to high (about 90% infected trees) and low inoculum (~ 5% infected trees) pressure (Valencia, Spain). The most abundant aphid species were *A. spiraecola*, followed by *A. gossypii* and *H. pruni*. The average percentage of *A. spiraecola* carrying PPV real time RT-PCR-amplifiable targets was 30.4% in the area with high PPV inoculum pressure and 8.00% in the area with low inoculum pressure. Concomitantly, when aphid species caught in Canada from areas with low PPV incidence (Ontario, Canada, where the eradication campaign is conducted), were analyzed by real-time RT-PCR, the percentage of viruliferous aphid species reached 1.70%. Finally, when aphids were caught in *Prunus*-free areas (in the middle of citrus plantations in Castellón, Spain), no PPV-viruliferous aphids were identified.

Consequently, the detection of PPV-viruliferous aphids is an indication of the presence of PPV in a certain area and could be used as an indirect alert-system to assess the PPV-free status of a plot or area in which a nursery is planning to establish. In conclusion, there is a significant correlation between PPV prevalence and the percentage of PPV-viruliferous aphids detected by real-time RT-PCR.

In the course of the SharCo project, the percentage of PPV-viruliferous aphids present in different experimental nursery plots, established in distinct geo-ecological locations, was estimated. In the experimental nursery plot located in Spain, the most abundant aphid species caught during the peak of aphid population was *A. spiraecola*, for two successive years (May 2008-May 2009). The average of PPV-viruliferous *A. spiraecola* landing on the rootstocks was 26.0%. In the experimental nursery plot located in Czech Republic where the experiment was conducted for one year (Spring 2009), the most abundant aphid species was *H. pruni*. The percentage of PPV-viruliferous *H. pruni* collected from established colonies reached 91.00%. In Bulgaria (Spring 2010), the most abundant aphid species was *H. pruni* and the percentage of PPV-viruliferous individuals collected in the plot was 15.00%.

In conclusion, these results are in agreement with the previous indication that it is feasible to assess the presence of PPV in an area just by analyzing the aphid



species visiting the plants present in the plot. **When no PPV-viruliferous aphid species are detected, it indicates, at least, a very low PPV prevalence** in the immediate adjacent area, while we cannot rule out the possibility that PPV is present.

3.2 Susceptibility of different *Prunus* rootstocks to natural PPV infection

The ranking of *Prunus* rootstock susceptibility to PPV natural infection in different ecological areas is displayed in Table 1 (see below).

Table1. PPV natural incidence on different *Prunus* rootstocks in six distinct ecological areas.

PPV infected trees were determined by ELISA-DASI using the 5B-IVIA antibody, two years after establishment of the plots. Data are displayed as PPV-infected plants/total analyzed plants and in brackets, the percent of infected trees.

Rootstock	Partner						Total
	Spain	Poland	Turkey	Czech Republic	Bulgaria	Romania	
Mariana GF8.1	112/144 (77.78%)	12/122 (9.84%)	0/17 (0.00%)	22/186 (11.83%)	96/178 (53.93%)	91/187 (48.66%)	333/834 (39.93%)
Nemaguard	55/145 (37.93%)	26/196 (13.27%)	1/38 (3.80%)	66/170 (38.82%)	97/193 (50.25%)	35/89 (39.32%)	280/831 (33.69%)
Adesoto 101	-	-	-	-	72/169 (42.60%)	91/192 (47.39%)	163/361 (45.15%)
Garmen	1/164 (0.61%)	-	0/47 (0.00%)	-	56/187 (29.94%)	22/175 (12.60%)	79/573 (13.79%)
GreenPac	0/162 (0.00%)	-	-	-	34/123 (27.64%)	-	34/285 (11.93%)
Myrobolan 29C	104/191 (54.45%)	15/198 (7.58%)	7/82 (8.50%)	83/198 (41.92%)	-	88/192 (45.83%)	297/861 (34.49%)
Myrobolan Alina	-	4/192 (2.08%)	-	-	-	-	4/192 (2.08%)
Wangenheim	-	19/196 (9.69%)	-	-	-	-	19/196 (9.69%)
Myrobolan BN4kr	-	-	-	0/51 (0.00%)	-	-	0/51 (0.00%)
GF677	-	-	0/11 (0.00%)	0/150 (0.00%)	-	-	0/161 (0.00%)
St Julien	-	-	-	19/181 (10.50%)	-	-	19/181 (10.50%)
Total	272/806 (33.75%)	76/904 (8.41%)	8/195 (4.10%)	190/936 (20.30%)	355/850 (41.76%)	327/835 (39.16%)	
PPV inoculum	D	D	T	D and Rec	M and Rec	D and Rec	

The above data demonstrates that Mariana 'GF8.1', 'Adesoto 101', 'Nemaguard' and 'Myrobolan 29C' are the most susceptible rootstock varieties to natural PPV infection. On the other hand, 'Garnem', 'Greenpac' and 'Myrobolan BN4kr' resulted in a certain level of resistance to natural PPV infection. In the same experimental plots, 'Docera 6' was also evaluated. Several plants were reported PPV infected but only in Bulgaria and Romania. The final results (evaluation in Spring 2011) should allow to conclude about the high resistance or the immunity of this PPV-hypersensitive rootstock.

4. Use and dissemination of the results

The detection of PPV viral particles in aphids is of great interest not only for basic viral replication studies but also for the design of more efficient control measures targeting aphids transmitted viruses. Spraying oils on nursery plots is another strategy to reduce natural PPV infection once the first PPV-viruliferous aphid is detected. We demonstrated that viral RNA particles can be extracted and quantified from fresh individual aphids as well as from aphids previously captured on traps and squashed on paper or nylon, without the need of previous RNA extraction. Moreover, the combination of those technologies, squash-capture and real-time amplification, opens up 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 different ecological areas. The use of less PPV-susceptible rootstocks in nurseries is also part of our recommendations in order to reduce PPV spreading in nurseries or in commercialised grafted plants.

Specific recommendations will be disseminated through the SharCo web pages (<http://www.sharco.eu/sharco/>), presentations in national and international meetings and conventional research publications. They are included in the Cultivation Guidelines (deliverable DE.2.2).

5. References

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Annex 1.

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**
- **Strainer** (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**

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). Maintain the sticky shoots for aprox. one week (maximum 10 days). To facilitate the location of the sticky shoots, it is recommended to label the shoots with duct tape in order to locate them when it is time to pick them up the next week.



2. Pick up individually the sticky shoots, place them 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 the following week.

3. Introduce one at a time each sticky shoot in an appropriate and individual pot or container. Each sticky shoots will be considered as a separate sample. If the shoot 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 to use turpentine under optimal conditions: extraction hood or in a well ventilated room.

5. After 1h in the solvent, shake the pot and throw out the shoot after carefully checking the absence of aphids. Remove with a fine brush any aphid that remains on 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 be reused (2-3 times). Do not throw out the turpentine into 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 traces of turpentine since it can interfere with the molecular analysis.

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

9. Sort out the aphids from other insects. Store the aphids until their classification in Eppendorf tubes filled with 70% alcohol.



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