

## **Executive Summary:**

Sharka disease significantly impacts the economics and productivity of stone fruit producing countries in EU and worldwide, affecting a wide range of stakeholders from breeders, plant material gardeners, to fruit producers. The FP7 Small Collaborative SharCo (“Sharka Containment”) project focused on helping the stakeholders ameliorate the different types of risks associated with Sharka disease by providing them with tools such as Plum pox virus (PPV) resistant plant materials, accurate and reliable methods of PPV detection, guidelines, warning systems, and a decision-support system. In this regard, in the field of epidemiology, the project surveyed the viral diversity, identified driving factors of PPV spread and diversification and developed novel and highthrough-put detection systems early warning of Sharka outbreaks. In the field of genetics, it provided molecular markers for the implementation of marker-assisted selection of PPV resistant fruit tree varieties. In the field of biology, we assessed innovative biotechnological approaches to broaden resistance to PPV in different fruit tree species. We identified new mechanisms and sources of resistance to PPV that could be combined with the resistant donors currently used in the breeding programs. Finally, to develop a PPV outbreak management scheme, we elaborated: i) guidelines for endusers and policy makers concerning cultivation and risk management, ii) an early warning system coupled with a decision support system. All knowledge and tools developed by the project were widely disseminated in Europe with special attention to PPV-endemic countries.

## **Project Context and Objectives:**

The concept of SharCo was to combine prophylactic and genetic solutions to prevent or limit the spread of the Sharka Plum Pox Virus (PPV). The project scope covered the entire chain from seedling production, grafted material production, to orchard management. It aimed at providing new methods and tools for the containment of Sharka, in orchards and nurseries. For that purpose the project developed specific research activities on a variety of complementary topics: epidemiology, virology, genetic and biotechnological approaches.

The strategic objective of SharCo is to provide the EU with novel methods and tools to face the constant menace of Sharka spreading as well as to reduce the impact of the disease for the various stakeholders, nurserymen and fruit producers. On that purpose, we aimed at

In the field of epidemiology, developing new methods for monitoring and fighting the PPV spread by:

- Identifying the driving factors of the PPV spread and diversification
- Developing novel systems for detecting, assessing and warning Sharka outbreaks
- Defining viral parameters linked to or driving PPV dynamics of dissemination at the field level
- Providing information about the most crucial epidemiological characteristics of PPV in nurseries, in order to design strategies to reduce plum pox incidence in nursery blocks
- Providing information related to the most accurate sampling and testing methods and protocols that would allow analyzing large numbers of nursery plants, thus improving the reliability of PPV detection and consequently the European phytosanitary passport
- Evaluating the effectiveness of mineral oil treatments in reducing PPV infection and spread in nursery blocks.

In the field of biology, develop new genetic tools for selection in view of improving resistance of plants cultivated in orchards by:

- Identifying molecular markers linked to resistance that would help increasing the efficiency of European breeding programs in apricot, peach and plum crop species
- Implementing the marker-assisted selection to speed up the breeding of resistance material
- Identifying new and complementary genetic resistance mechanisms suitable for pyramiding durable resistance to PPV, thus reinforcing plant resistance.

In the field of agricultural management, help the end-users – notably but not exclusively plant protection officers, breeders, nursery gardeners and fruit producers—to take advantage of the project outcomes by:

- Establishing cultivation guidelines aimed at minimising the virus spread likelihood at the levels of nurseries and orchards
- Proposing tools (decision support system) and recommendations for an early warning system
- Delivering a risk management system designed to minimise the entry, establishment and spreading of the virus and potential new variants in EU.

## **Project Results:**

### **1) First Epidemiology work package**

#### *Improving virus typing tools for an early detection of new variant virus outbreaks*

The accurate identification of virus isolates that belong to a particular strain is expected to provide insight into the diversity and evolution of the virus, which is especially interesting when the causal agent displays a broad biological and genetic diversity. Such is the case for Plum pox virus, the viral agent causing the Sharka disease. Therefore, the objective of the WPE1 task TE1.1 was to develop a mini-oligo array for a rapid genome wide analysis of PPV isolates. In the first step, a first generation PPV oligochip was developed in two phases: the first one consisted in the in silico design using the complete sequences of isolates belonging to the main PPV strains using the Array Designer 4 software. The second step consisted in the testing of this first generation array. Subsequently, a second generation PPV oligochip was developed. This second generation contains 10 replicates of 90 probes each constituted of 18 to 22 nucleotides. This second generation oligochip allows the analysis and probing of PPV intra-strain variability within the PPV-D, M and Rec strains. A complete protocol for the use of this PPV mini-oligo array, including spotting of validated probes, post-spotting procedures, hybridization, evaluation of signals using a GenPix 4000B scanner and final signal analysis has been established and assayed in the two distinct laboratories.

In addition, parallel experiments to analyse the variability of PPV isolates were performed using deep sequencing, a more recent and broadest approach, since it analyzes all positions of the viral genome. Deep sequencing was successfully evaluated for its use in the analysis of viral variability and heterogeneity. Several software packages and conditions were checked in order to optimize the de novo assembly and to facilitate the reconstruction of viral genomes from small interfering RNAs (siRNAs sequences). In spite of the success of the mini-oligo array technology to characterize PPV isolates, it presents several limitations when compared with deep-sequencing, mainly for its feasibility, reproducibility and capacity to analyse not only intra-strain but also intra-isolate variability.

#### *Improving our knowledge on Sharka diversity for a better management of the disease outbreaks*

The analysis of genetic diversity and the understanding of the relationships between viral strains or isolates are important aspects in the management of viral diseases and in the analysis of the risk of new emerging viruses. With the aim to provide a precise and broad overview on the current diversity of PPV worldwide, a standardized RT-PCR and sequencing protocol has been established and used by all partners to amplify two highly informative genomic regions (part of the NIb-CP gene and P3-6K1-CI region, respectively). In total, both partial sequences have

been generated from about 800 PPV isolates, originating from 29 countries, among which 16 are European Union Member States. This unprecedented effort has furthered our understanding of the genetic complexity and diversity of PPV as more than twice the sequence information has been generated in comparison with what was previously available in the international Genbank database. Moreover, complete epidemiological data (host, location, period of the year, age of the host tree etc...) have been recorded for each isolate (data which generally was not available for isolates in the public database), allowing unprecedented analyses.

Within this PPV diversity survey, we obtained a range of original results on PPV variability and distribution. Higher than previously envisioned intra-strain variability was observed within PPV-D, indicating that the variability of the most widespread PPV strain had previously been underestimated. Within each main strain, divergent isolates were identified, mostly originating from Central- and Eastern Europe. We confirmed the splitting of the PPV-M strain into two major subgroups (Ma and Mb) and obtained for the first time a wide picture of their geographic distribution. A high prevalence of isolates belonging to the newly discovered PPV-T strain in Turkey was also detected.

Two new strains of PPV were identified, one corresponding to the missing ancestor in the evolutionary history that led to the currently known M strain. The second is a novel cherry-adapted strain identified in Russia, further increasing concern about the potential for impact of PPV in cherry crops.

New information on the distribution of PPV strains has been obtained, e.g. the presence of PPV-Rec in Italy, PPV-M in Spain, PPV-W in Latvia and Russia, PPV-T in Albania, PPV-C in Belarus and Russia.

A total of 52 complete genome sequences belonging to 6 of the 7 previously known PPV strains were determined, together with complete sequences for members of the two newly detected strains. The sequences obtained from European PPV-D isolates counterbalanced the scarcity of information in comparison to the North-American or Japanese data, the sequences of PPV-M, Rec and T isolates considerably enriched the previous limited full-length genome data about these strains. Several genome data have allowed to complete the picture of the evolutionary history of PPV. For example, we performed a complete analysis of the divergent AL-11pl isolate, which originates from Albania. Its complete genome sequence revealed that it represents an "Ancestral" PPV-M isolate from which the current PPV-M strain most probably originated through recombination.

Similarly, we performed a full genome sequence analysis of the PPV-W isolates sampled in Latvia. These isolates appear to be representatives of the "typical" PPV-W strain, which is not affected by recombination events, contrary to the first PPV-W isolate identified in Canada: PPV-W3174. Strikingly, the PPV-W intra-strain variability is substantially higher than for all the other PPV strains. This could impact the ease and reliability of detection of PPV-W isolates, in the near future.

Finally, we also focused our effort on the analysis of PPV diversity on cherry host species. It is indeed acknowledged that PPV only sporadically infects cherry species, with the consequence that at the beginning of the SharCo project, extensive knowledge on the variability and prevalence of the PPV-C strain in Europe, the only strain able to infect (sour and sweet) cherry trees was extremely limited. In the course of the task TE1.1, full genome sequences have been obtained from three PPV isolates found in naturally infected sour cherries in Russia. Those isolates present only ca. 78% of nt identity with other common PPV strains (M, D, Rec), which are not able to infect cherry trees. Complete genome sequencing has confirmed their high divergence from any other known PPV group, PPV-C included. Taking into account their biological properties (ability to infect sour cherry) and genome particularities, the isolates thus represent a new PPV strain, that is able to infect *Prunus cerasus* species. It was later named PPV-CR (Cherry Russian) (see figure 1).

In addition to the classical sequencing approach, the deep sequencing of small RNAs was also assessed. It was successfully applied in the case of the ES-11pe isolate, allowing the determination of its full-length genome sequence from its original host (peach) and confirming the effectiveness of the next-generation sequencing approach for PPV typing and characterization.

#### *Sharing with the public data on PPV diversity analysis and viral collections*

All partial and full-length genome sequences have been transferred to the web-queryable SharCo sequence database (<http://w3.pierroton.inra.fr:8060/>), developed in the course of the project. The database contains sequence data and epidemiological information about the isolates, enables view and export of isolate sequences. It lists full data on PPV isolates including georeferencing, original hosts and location etc... A search procedure using multiple criteria is available, allowing export of results and semi-automatic NCBI import. The current database will be opened to non-SharCo partners by the end of 2012.

The research and practical work in the field of plant virology often requires the availability of a set of reference viruses, strains or isolates. For this purpose, a lyophilized collection of PPV isolates characterized in the frame of the SharCo project has been established. The collection actually contains 654 entries from 22 countries. The isolate naming follows the format used in the SharCo PPV database, allowing easy linkage between sequence and isolate information and the original preserved isolate material. The current number of PPV isolates by country is as follows; Albania (12), Argentina (5), Austria (22), Belarus (15), Bulgaria (73), Chile (1), Cyprus (1), Czech Republic (50), Egypt (1), France (52), Greece (2), Hungary (18), Italy (36), Latvia (3), Moldova Republic (7), Poland (29), Romania (86), Russian Federation (1), Serbia (55), Slovakia (46), Spain (86), Turkey (53). This collection is the most representative of PPV genetic diversity worldwide.

In parallel, we also established a centralized live collection of PPV isolates. This collection is more limited (currently 38 isolates) but it has two main purposes that cannot be achieved easily by the lyophilized collection: i) the maintenance of isolates in their original woody hosts (plum, peach or apricot species) limiting further PPV divergence driven by host adaptation, ii) the access to fully characterized isolates representative of PPV diversity, in a form that can be readily used to inoculate further woody hosts. This material can then be used directly for future analysis of PPV resistance stability and durability, similarly to task TE2.1 in the WPE2 work package. All 38 isolates have been fully sequenced before the establishment of the collection in insect-proof controlled facilities of Partner P5. The collection has been duplicated into an S3 high confinement greenhouse of partner P1, to ensure redundant preservation of the isolates and safeguarding of the collection against potential local incidents.

## 2) Second Epidemiology work package

### *Searching for pertinent indicators of epidemicity that would help to manage PPV outbreaks in the fields*

The increasing knowledge on PPV genetic diversity raises new concerns about the biological properties and related epidemic potential of the identified PPV strains. Indeed, apart from PPV-D and PPV-M, the biological properties of the PPV strains are poorly known. This situation is partially related to the fact that experimental assays aiming at measuring the biological parameters of a given PPV isolate on a given Prunus species are still complicated, long and low throughput. Moreover, the parameters measured in experimental assays are generally not compared with the real epidemiological situation in the field and consequently, the usefulness of the experimental data for risk assessment is rather limited. During the SharCo project, we developed a strategy combining epidemiological surveys in orchards and new experimental assays into a confinement greenhouse to identify and measure pertinent indicators of PPV epidemicity. The experimental assays were designed to gain knowledge about the three successive steps of an infection cycle: (i) the de novo infection of a Prunus tree after aphid inoculation, (ii) virus multiplication and accumulation in the infected tree after a given incubation time (production of inoculum), and (iii) acquisition of the virus by aphids from this infected tree (virus that will be transmitted to another still-healthy tree: dispersal). Three indicators linked to these three steps were defined:

1. The ability for a given strain / variant to infect certain Prunus species after aphid transmission;
2. The dynamics of virus accumulation in Prunus hosts;

3. The efficiency of the infected Prunus as inoculum sources for the aphids. This last indicator may be correlated to virus accumulation but other factors specific to the Prunus species might influence it.

The experimental assays were initially designed to be used in the future as a routine test for every PPV isolate/strain to be characterized. We were thus particularly careful at facilitating the plant material (Prunus) production step and at reducing the duration of the overall assays. A specific methodology that enables quantifying and comparing PPV amounts in the three main stone fruit Prunus species susceptible to PPV (plum, peach and apricot) was developed. The protocol was designed as indicated on the figure 2 and carried out on one isolate of each PPV-D, PPV-M and PPV-Rec strains with a duplication of each experiment. While all the results are not yet available, some preliminary conclusions can be inferred:

- The specific strain / Prunus interactions evidenced under orchard conditions were not clearly detected in the experimental assays. More precisely, we detected very few (PPV-D) or exceptional (PPV-Rec) cases of infection in peach orchards whereas in our biological assays, the PPV-D and PPV-Rec isolates were found able to infect systematically peach seedlings, thus providing a good source of inoculum for further aphid transmission. Such a result may indicate that differences in the ability to infect peach in orchards might not be due to viral factors only but also to environmental and physiological parameters which cannot be easily reproduced in simple experimental assays carried out under high confinement greenhouses.
- The experimental assays evidenced more subtle differences between the strains, and especially in favour of a higher epidemicity of the PPV-M strain. Indeed, the PPV-M isolate used in our biological assays was found to induce faster systemic infections of apricot and was better generalized in peach, generating higher transmission rates by aphids from this Prunus species in some conditions. Those differences might be useful for risk assessment and predictions.
- For those reasons and because of the numerous difficulties that arose while carrying out the experiments, it is not expected that the same experimental protocol can be used to routinely test any new isolates for risk assessment. However, this does not exclude the possibility that parts of the experimental design targeting specifically the parameters that allowed us to differentiate the isolates can be adapted for further risk assessment.



### *Challenging Prunus sources of resistance with the viral diversity*

A set of resistance genitors was challenged with an extended range of PPV isolates that are representative of the viral genetic diversity as defined in the first Epidemiology work package. Among those apricot, peach and almond genitors, only two were truly and stably resistant to infection by eight PPV isolates, apricot cv. 'Harlayne', 'Lito' and in consequence 'SEO', parent of 'Lito'. We are thus recommending them for future breeding programs as described in the Genetic pillar.

### *Identification of genetic determinants of adaptation to Prunus species*

Some PPV strains or isolates are known for their specificity against Prunus or herbaceous host plants. For example, PPV-C is the only strain that infects systemically cherry trees and PPV-Rec has rarely been observed on peach trees. Isolates derived from the PPV-PS isolate (M strain) were shown to have two mutations in the sequence coding for the P1 protein that specifically prevent PPV-PS infection of 'GF305' peach seedlings. Finally, the PPV-D and PPV-R (D strain) isolates are adapted to Prunus and Nicotiana respectively and have specific determinants at the N-terminal region of the capsid protein (CP) involved in adaptation to these hosts. This material was the basis to study genetic determinants of adaptation to Prunus species. For this purpose, we developed PPV-C and PPV-Rec infectious clones, artificial recombinants, site-directed mutagenesis and gene shuffling. Till now, by challenging various hosts with these constructs, we have demonstrated that point mutations in the P1 protein are responsible for symptom expression while other ones in the CP are determining viral systemic infection in peach 'GF305' seedlings and herbaceous plants. A 1.9kb long region was also shown to be essential for PPV-C systemic movement in *P. avium* (cherry trees).

### *Evaluation of competitiveness and disease dynamics of PPV-Rec in orchards*

The prevalence and host distribution of a given PPV strain are driven by its specific biological properties but also by many other factors like the agro-ecological conditions occurring at one location and the historical pathways of PPV strain introductions. A strong geographical effect is thus expected. To disentangle intrinsic properties of the strains from the other factors, surveys using same methodologies and statistical procedures were performed in three distinct countries (Bulgaria, Romania, and Serbia).

As expected, strong geographical effects were recorded both among countries and also among regions of a same country where agronomical conditions differ. PPV-D and PPV-Rec are

commonly found in each of the three country, but PPV-M was not found in any region or *Prunus* species in Romania so that it seems still absent in this country.

Data from Bulgaria and Serbia suggested a strong association between PPV-M and peaches, which is consistent with previous data obtained from Europe, but no preference was evident among the three strains and apricot or plum host plants. Indeed, the three strains appear equally fit on these 2 crop species. Multiple infections were shown both at the level of the orchard and within the same trees, leading to mixed infection, especially for plum (10% of plum trees were mixed infected) and at a lower extent for apricot. Every possible type of mixed infections (involving 2 or even 3 strains) was detected in plum and the hypothesis of independence between strains conditionally to the infection by one strain was not rejected. This result suggested that over-infection by any other PPV strain (PPV-M, PPV-D or PPV-Rec) was possible on this *Prunus* species. Extended follow-up of mixed infections might however evidence specific strain competitiveness. The monitoring of over-infections between PPV-D and PPV-Rec isolates in an experimental orchard in Romania is currently under analysis to confirm part of these results. Overall, these data evidenced the potential role of domestic plum in PPV evolution. Indeed, mixed infection constitutes a pre-requisite for the generation of new variants by genome exchanges (recombination).

Three experimental orchards were newly established at the beginning of the project with the aim to monitor the competitive dynamics of PPV-Rec and PPV-D spread (figure 3). A well-characterized primary inoculum of each strain were introduced in the orchards. Infected trees were detected in plum orchards, but, after three vegetative periods, (nearly) all trees that resulted were infected with contaminant inoculums from outside of the experimental orchards. Phylogeny analyses are currently done after partial sequencing to try to identify the exact origins of first cases and subsequent chains of transmission.

### *Reconstructing the chains of virus transmission*

An experimental and modelling approach was developed to trace back aphid transmission events by combining viral genetic and epidemiological data. The reconstruction of the chains of virus transmission aims at providing information upon the frequency of transmission from one particular source as well as an estimation of the distances of spread. This study was based on a 450 ha area of *Prunus* orchards mixed with other crop, non-host species where every diseased tree (800 in total) was geo-referenced and sampled, giving rise to as many PPV isolates. A previous analysis based on partial viral genome sequencing showed that full-length genome sequencing (10,000 bp) was necessary to get enough resolution at the tree scale. The full-length genomes of 188 PPV isolates corresponding to all diseased trees located in 13 neighbouring orchards were fully sequenced and the genealogical relationships between the sequences were reconstructed. The development of a generic mathematical framework to link genomic and epidemiological information is underway. Expected results should be used in a

second step to feed a simulation model of sharka disease spread at a regional scale (see below).

### *Modelling disease spread at a regional scale*

A spatially-explicit model of sharka disease spread at a regional scale has been constructed. It is based on a large regional geo-referenced database supplemented with data of PPV-M epidemics in 1,600 peach orchards located in South-East of France which were surveyed for 14 years. This database was constructed during the first period of SharCo. It includes parameters related to the orchards (location, structural characteristics and conditions of cultivation), to the monitoring (dates of survey, identity of inspectors) and control of the infected trees (dates of removal) as well as to the dynamics of the disease (number of detected disease cases at each survey date). This hybrid mechanistic-statistical model links real data from the regional database with biological and human processes (dissemination by aphids, latency period, introduction of infected plant material) underlying the observations. The mechanistic part of the model is based on a SEIR model (Susceptible/ Exposed/ Infectious (hidden and detected subclasses)/ Removed) linked to a spatial reference map of the orchards. The statistical component of the model deals with imperfect detection and event-time censoring (figure 4). The core algorithms are the basis of both a simulation model and an estimation model. The estimation model is aimed at assessing the parameter values used in the model (kernel of dissemination, latency period, sources at the origin of the epidemics, ...) following a Bayesian frame. The simulation model has been extracted from this full estimation model and has been adapted to be used under a classical Windows® environment. It will be used to test different strategies of orchard surveillance and disease control.

### 3) Third Epidemiology work package

The main goal of this work package was to develop strategies to contain PPV in nurseries, thus avoiding PPV introduction and later commercialization of infected nursery plants.

### *Towards strategies to limit PPV spreading and establishment in nurseries*

At the beginning of the project (spring 2008), six experimental plots were established in six different ecological areas (Bulgaria, Czech Republic, Poland, Romania, Spain and Turkey), close to well-established PPV inoculum sources, following a common statistical experimental design. Using those newly established experimental nursery plots, we have identified Prunus rootstock species more resistant to natural PPV infection in field conditions. Twelve Prunus

rootstock species were evaluated in six different ecological areas to assess their susceptibility to natural PPV infection (Figure 5). In the experimental nursery plots, PPV infection occurred at random in any of the evaluated areas. *Prunus mariana* GF8.1, Adesoto 101, Nemaguard and myrobalan 29C were the most susceptible rootstock cultivars. Other rootstock genotypes such as Wangenheim and myrobalan Alina showed a clear susceptibility. On the other hand, GF677, Garnem, Greenpac and myrobalan BN4kr showed a certain level of resistance to natural PPV infection. Docera 6, a PPV-hypersensitive rootstock, showed a relatively good level of resistance to natural PPV infection. When grafting PPV susceptible plum cultivars onto hypersensitive rootstocks, no tree death could be clearly associated up to now with Sharka infection, in the experimental conditions.

The efficiency of horticultural mineral oil (HMO) treatments was also assessed in the same experimental nursery plots. Two PPV susceptible rootstocks were compared: *P. Mariana* GF8.1 and the peach Nemaguard cultivar (figure 6). Treatment with 1% emulsion did not avoid PPV natural infection in areas with high PPV-prevalence but significantly reduced the percentage of PPV infected nursery plants, limiting significantly the spread of the disease. Therefore, the use of HMO is highly recommended in nurseries and young plantations in particular during the aphid species peak flight and/or as soon as the first PPV-viruliferous aphids are detected.

*Providing information about the most crucial epidemiological period when nurserymen can interfere with the natural spread of PPV*

We monitored and identified the different aphid species that are landing on nursery plants located in different ecological areas, as well as the peak period of aphid populations. The predominance of certain PPV-vector aphid species is dependent on the European region as follows: *Hyalopterus pruni* in Czech Republic, Turkey, Romania, Bulgaria and Poland, *Aphis spiraeicola* in Spain and Turkey, *Rhopalosiphum padi* in Poland, *Brachycaudus cardui* A. craccivora and *B. helicyrsi* in Turkey, *Phorodon humuli* in Bulgaria and Romania. The maximum peak of aphid flights was scored in springtime in each different ecological area, whereas a more irregular and non-reproducible second peak was observed in autumn, in some areas. PPV-viruliferous aphid individuals were successfully detected by squash real-time RT-PCR. We are thus recommending the use of strategies to limit PPV entry, establishment and spreading in the nurseries during the aphid flight peak and as soon as the first PPV positive aphid is detected.

The variation of PPV-prevalence during the three-year period, revealed a continuous increase in disease incidence in each agro-ecological condition. The use of the same PPV susceptible rootstock cultivars in all scenarios allows comparing PPV prevalence and incidence in different conditions and shows the ability/efficiency of local aphid species in spreading the different PPV strains present in each location. The most efficient combination of parameters for PPV spread was detected in Spain where PPV-D spreads on *P. mariana* GF8.1 through the vector *A. spiraeicola*, followed by Bulgaria where PPV-M and PPV-Rec were efficiently spread onto *P.*

mariana's 'GF8.1' plantations mainly by *H. pruni* and *P. humuli*. In plots of 'Nemaguard' peach seedlings, a similar efficient PPV-spread was estimated in both ecological areas. These results reveal the importance of knowing the vector aphid species prevalent in a specific area. In some situations, the high efficiency of transmission by *A. spiraecola* of PPV-D strain can overcome the potential of PPV-M transmission by *H. pruni* and *P. humuli* (which are less efficient PPV vectors), therefore having significant impact on the theoretical propensity of different PPV isolates to be transmitted in natural conditions.

In addition, the epidemiological role of weed species as PPV reservoirs was evaluated in orchards infected by PPV\_M but no PPV infection was detected on weeds.

#### *Providing information related to the most accurate sampling and testing methods and protocols*

The purpose here is to allow the analysis of larger numbers of rootstocks and nursery plants in order to improve the reliability of PPV detection and consequently of the EU phytosanitary passport. Methods for PPV detection in nursery plants were assessed and compared for their diagnostic parameters. Serological tests (ELISA based on 5B-IVIA monoclonal antibodies) and spot real-time RT-PCR were simultaneously evaluated by latent class models using maximum likelihood functions and a Bayesian approach. The sensitivity and specificity of both techniques did not vary according to the latent model applied. Spot real-time RT-PCR was more sensitive while ELISA was more specific for PPV detection (figure 7). The concordance between both techniques, using the same extract, was almost perfect (Cohen's kappa index of  $0.88 \pm 0.01$ ) after the analysis of 5,379 plants. The results demonstrate that a coincidental result obtained by both techniques leads to a practical accuracy of 100% to rule in or rule out the disease in a specific sample. In addition, spot real-time RT-PCR technique can be successfully applied on composite samples (up to 10 plants, 3-4 leaves or dormant buds/plant) at any vegetative stage or latency period without losing accuracy in detection. By contrast, ELISA showed significant differences in detection accuracy depending on the time of the year and the number of plants pooled together, knowing that spring and summer provided the best sensitivity for detection of PPV. In this case, up to 4 plants (3-4 leaves/plant) could constitute a composite sample. In conclusion, the recommendation is the use of composite samples (4 plants) for accurate PPV detection by ELISA (5B-IVIA based) in spring or summer vegetative periods. However, the use of composite samples (10 plants) is recommended at any vegetative or latency period when analysed by the spot real-time RT-PCR technique using Taqman probe.

We also compared different methods of sampling, individual analyses versus hierarchical methods. The result showed an overestimation of the real PPV incidence in the experimental plot when using the hierarchical method (Table 2). It is worth noting that the hierarchical method is a procedure used widely to detect PPV in an area, starting from a limited number of analyses. Therefore, the cost and time required are reduced when the hierarchical method is used.

#### 4) First Genetic work package

The major goal was to implement efficient, rapid and less cost-effective breeding programs for resistance to PPV in European countries. This implies the availability of large progenies segregating for the trait (resistance) and the development of molecular markers.

##### *To simplify and speed up the procedure of selection of PPV resistant cultivars*

In apricot, the development of molecular tools was based on association mapping between PPV resistant phenotypes and genotypes at one specific locus, named PPVres. This locus is located at the top of linkage group 1 and was constantly linked to PPV resistance in apricot in the last 10 years. Association mapping was achieved through the construction of apricot linkage maps from previously phenotyped families segregating for the trait. In those preliminary steps, two resistant donors were used: Goldrich' and SEO' (Stark Early Orange). Fine mapping was carried out by increasing the number of markers overlapping PPVres and by increasing the number of individuals and families screened for resistance. Progress in mapping allowed narrowing the region containing the locus linked to Sharka resistance down to 2.1 Mb (figure 8). Forty-three SSR markers designed from the peach genome sequence version 1 were tested in several apricot mapping populations that were already phenotyped for PPV resistance. Three markers, namely PGS1.21, PGS1.23 and PGS1.24, were selected as the best, co-segregating tightly with the resistance. Other extra markers (called MP) were developed from other progenies challenged with the virus in other agro-ecological environments. Those markers were then tested in other progenies already phenotyped by several SharCo partners validating those molecular tools as well as the alleles linked to the resistance in progenies either originating from distinct donors of resistance (Harlayne, Harcot) or tested for resistance to PPV in different agro-environmental conditions.

After validation of the markers, transfer of the technology to all other SharCo partners was achieved and implementation of Marker assisted Selection (MAS) was accomplished in the last period of SharCo, both in Western and Eastern European apricot breeding programs. Currently, a total of 8,904 apricot pre-breeding materials are now being screened with the PGS and MP molecular markers. This eliminates the bottleneck of phenotyping thousands of seedlings and speeds up the process of selection of new PPV resistant cultivars, adapted to various European, local environments.

In peach, where no source of resistance was identified, interspecific crosses and progenies using the peach related *P. davidiana* species were used for construction of linkage maps. The quantitative nature of the resistance to PPV lead to the identification of QTL linked to the trait but impeded the development of molecular tools suitable for MAS.

In plum, a resistance trait characterized by hypersensitive reactions that restrict the viral systemic infection was under focus. However, due to the complexity of the European plum

genome, which is hexaploid, the development and identification of molecular markers linked to the trait proved tedious and difficult. A transcript profiling approach was thus applied. It provided several cDNA markers that are up-regulated in PPV-infected, hypersensitive individuals. Primer sets were developed from those transcript markers and validation in other plum progenies is still pending.

#### *Looking for new sources of resistance*

The introduction of natural resistance to breeding programs, while successful, has limitations: a restricted number of sources for resistance have been found, resistance genes cannot always be easily transferred between species and undesirable traits may be transferred along with the resistance locus. Additionally, we cannot rule out that existing resistances may be overcome, in the future, by new virus strains. We thus developed methodologies to test new local germplasm for resistance against the main PPV isolates circulating in Europe. Indeed, local germplasm was surveyed by partners in Romania, Poland, Turkey and Bulgaria. In Romania, the local plum cultivars 'Rival' and 'Miroval' and the apricot varieties Traian, Auras, Orizont, Ovidiu, 'Ceres', 'Euxin', 'Tudor', Augustin, Danubiu, Hristia were found resistant to PPV by artificial inoculation. In Bulgaria and Turkey, none of the landraces and hybrids were found resistant to PPV while in Poland, one single plum cultivar (Vision) presented promising behaviour against PPV natural infection.

#### 5) Second Genetic work package

The purpose of this second work package was to enlarge resistance mechanisms available for gene pyramiding with the above sources of resistance. Part of its tasks was the characterization of plant factors involved in PPV replication or propagation.

#### *Identifying alleles of resistance in the Prunus germplasm*

The EcoTilling approach searched in the natural germplasm of stone fruit trees for rare alleles of translation initiation factors with mutations that interfere with their functions in PPV infection without affecting their function in the host plant. For this purpose, we collected Prunus germplasm all over the world, through the scope of another FP7 European People project, STONE. One thousand thirty five individuals, including cultivars, accessions and wild representatives, were tested looking for mutations expected to affect the interaction domain or the global protein structure and stability of the eIF4E and eIFiso4E proteins (figure 9). Fifty-five presented a suspicious mutation (deliverable DG.2.2). Of these, 24 genotypes were introduced

in the greenhouse for the purpose of PPV resistance tests. One apricot tree, Kostinskij, appeared promising and was thus used to complete deliverable DG.1.5 (Pyramiding different sources of resistance). Six other promising peach or interspecific peach cultivars were transferred in the field in Bulgaria to challenge its resistance to PPV infection in natural conditions.

For the second subtask, intron hairpin DNA constructs were developed both for the different isoforms of the eIF4E and eIF4G genes. A total of six constructs were obtained in the pHANNIBAL or Gateway vectors and then introduced in plums (hexaploid European plum and diploid Japanese plum) by the Chilean partner of a France-CONYCIT program. Ten of the regenerated transformed lines were recently transferred to the high confinement greenhouse in order to challenge them with the virus.

### *Searching for new mechanisms or genes of resistance to PPV*

We also focused on the characterization of plant factors involved in resistance against PPV. A first objective of this task was the cloning of resistance to Plum Pox Virus (rpv) genes in *A. thaliana*. Genes coding for host factors contributing to PPV resistance in *A. thaliana* when depleted were identified through different approaches: i) Positional mapping and cloning and ii) Screening of loss-of-function mutants in host factors shown to interact with viral proteins or implicated in the signalosome interacting with viruses. The first approach resulted in the identification and validation of two distinct genes or gene families coding for host factors contributing to PPV resistance, depending on their allelic form as follows: a) RPV1 gene, which is coding for a cPGK protein that significantly reduces PPV accumulation when silenced and b) SHA3 gene, which belongs to a TRAF like proteins cluster and its depletion results in complete inhibition of PPV systemic infection (figure 10).

The second approach lead to the demonstration that a functional CSN5 host protein is indispensable for early viral infection of *A. thaliana*. CSN5 is part of the COP9 signalosome complex implicating this pathway in the virus infectious cycle. We thus potentially identified a new mechanism of resistance to PPV infection. A patent was deposited in July 2011, covering its use as strategy to fight against viral infection. All these results are fully described in deliverable DG.2.4.

A second objective has been the search for resistance genes activated in herbaceous and woody plants showing a HR to PPV infection. Results related with this objective are also described in the deliverable DG.2.4. We have found four Resistance Gene Analogs (RGA) of the NBS-LRR family in *Chenopodium foetidum* and two in *Nicotiana occidentalis* expressed in plants showing HR after PPV infection. Upregulation after PPV infection has been shown by real time RT-PCR for 5 of these genes, but initial functional analyses with one of them has not revealed antiviral activity. A second approach was taken with the woody host *P. domestica*. A



transcriptomic analysis of Jojo variety plants undergoing HR after PPV infection was performed using RNA deep sequencing. Analysis of the data allowed us to identify 2,794 contigs with expression level variations higher than 2, upon PPV infection and HR induction. One hundred and three of these contigs match genes described in the resistance genes database (pRGDB). In addition, 51 genes with NBS sequences characteristic of the NBS-LRR genes have been identified and at least one of them shows significant variation in expression following infection.

In this work package we also focused on the identification of PPV factors involved in specific resistance against different PPV isolates. For this purpose, we constructed chimeric viruses to identify the determinant for overcoming RTM resistance in Arabidopsis at the N-terminal region of the capsid protein (deliverable DG.2.1).

#### *New strategies that interfere with PPV infection*

New strategies to interfere with the virus were tested in this work package: the evaluation of the antiviral potential of PPV-specific recombinant antibodies; approaches to interfere with the post-translational modifications of the viral CP; and RNA silencing-related strategies. We generated single chain antibodies (scFV) specific for the CP and the RNA replicase NIb of PPV that have been targeted to three different cell compartments. Transgenic *N. benthamiana* lines with reduced sensitivity to PPV have been obtained by targeting the NIb-specific antibody to any of the three compartments. The efficacy of both NIb-specific and CP-specific recombinant antibodies has been also demonstrated in experiments of transient expression by agroinfiltration (figure 11). The best candidates have been selected to transform woody plants.

Another anti-PPV strategy assessed in this task has been the interference with the O-GlcNAcylation and phosphorylation of PPV CP (see deliverable DG.2.4). The O-GlcNAcylation is performed in Arabidopsis by the glycosyl transferase SEC, and PPV infection is delayed in an Arabidopsis mutant deficient in SEC. When downregulated in *N. benthamiana* by RNAi, we observed a drastic reduction in O-GlcNAcylation, but the effect in susceptibility to PPV is low. In contrast with animal systems, it appears that the phosphorylation and O-GlcNAcylation of PPV CP appears not to be reciprocal. Indeed, phosphorylated residues do not coincide with the O-GlcNAcylated ones, and phosphorylation of PPV CP is not enhanced in the O-GlcNAcylation-deficient plants.

Another important part of this work package was devoted to assess the performance of different ways of pathogen-derived resistance mediated by RNA silencing (see deliverables DG.2.3 and DG.2.5). An ihCP-RNA (ihpRNACP) construct, also known as 'B14', was engineered into *Nicotiana* and *Prunus* genomes. Regardless to the plant species utilized, the ihpRNACP construct efficiently produced siRNAs and provided stable anti-viral resistance against all the major PPV strains.

We also assessed the efficiency of a novel PDR strategy based on artificial miRNAs (amiRNAs) alone or in combination with the more classical approach based on viral dsRNA expression. We have thus studied the molecular basis of artificial miRNA (amiRNA) expression and activity (figure 12), allowing us to design amiRNAs with efficient anti-PPV activity in *N. benthamiana*. Several lines of *N. benthamiana* transgenic plants expressing different single amiRNAs and a double construct have been already obtained, and first experiments suggest that some of these lines have high anti-PPV resistance. Prunus transformation with these amiRNA constructs has been started in collaboration with INIA (Chile).

All these results provide a large battery of demonstrated or potential sources of antiviral resistance to be used in future approaches to develop plants with more efficient and durable resistance to Sharka disease.

#### 6) First Application work package

Research activities developed by the SharCo consortium as described above resulted in tools, novel information and expertise. They will be instrumental in containment, and eventually control of the disease. It includes a better view on the diversity of the virus, new knowledge on PPV epidemiology, more sensitive and accurate methods of PPV detection, molecular tools for improvement of the breeding programs, new sources and mechanisms of resistance and new stone fruit cultivars and rootstocks resistant to Sharka. In complement and based on these data, the SharCo consortium prepared the following reports that are expected to support policy-makers, regulatory bodies, extension services and other stakeholders in defining more efficient and realistic measures of Sharka management:

- Cultivation guidelines that would help to restrict the virus spread in mother plant blocks, nurseries and orchards (figure 13).
- Recommendations for the implementation of an early warning system, assessing the risk of occurrence of unknown PPV variants in EU and new PPV outbreaks, mainly in virus-free zones.
- Guidelines for a risk management system targeted on Sharka outbreaks and spread and applicable everywhere in Europe (figure 14).

To facilitate knowledge and tool access to any stakeholder, three decision support systems (for cultivations of mother plants, for nurseries and for fruit orchards, see figure 15) have been developed and implemented in nine EU member states and two associated countries. They are accessible through institutions websites in eleven National languages.

## 7) Second Application work package

Critical for achieving the goal of efficient transfer of knowledge from researchers to major stakeholders was the dissemination work package. Through various activities it focused on Dissemination and transfer, for informing officers of the Plant Protection as well as extension Services, agricultural advisers, and breeders, nurserymen and fruit producers on the know-how, tools and plant material generated by the SharCo project. This was achieved by: i) systematically maintaining the SharCo webpage updated with the project's public milestones and deliverables and providing on-line access; ii) through training workshops that provided an efficient, inter-active communication platform between the SharCo beneficiaries and all potential endusers; iii) disseminating project's results to an enlarged scientific community, including non-European countries, through SharCo Research Workshop or communication(s) to more restricted audience and iv) via the use of the public media and publications in both local and international, peer-reviewed journals.

## 8) Beyond the SharCo project

In this project, we have met and exceeded our original project goals as described above. Consequently, we have gained particular insight into certain questions and research areas that should be given priority for continued investigation in the future (see below). This work will be instrumental in translating our results into sustainable management practice for this important disease of fruit trees.

### *Evaluating new potential risks linked to PPV diversity and spread in un-expected hosts*

Intensive efforts during the four years of SharCo provided original results on PPV variability and distribution, including the discovery of two new PPV strains. The first one is corresponding to the missing ancestor in the evolutionary history that led to the currently known PPV-M strain. The second is a novel cherry-adapted strain identified in Russia, further increasing concerns about the potential for impact of PPV in cherry crops. In addition, our knowledge of the diversity and distribution of PPV-C, the other cherry-infecting strain have been somewhat improved. It seems now possible to try to answer several questions that were outside of the scope of the SharCo project such as: i) what is the extent of the diversity of PPV on cherry host species, ii) what (novel) risk do cherry adapted PPV strains pose to European cherry crops (knowing that those hosts are not included in the annex IV of the 2000/29 directive).

### *Increasing the number of strategies that interfere with PPV infection to promote a more durable resistance to sharka disease*

When trying to manage PPV-related risks, the use of genetic resistance is, beyond doubt, the best solution for long term control because it provides effective protection all along the growing season. It allows the new plantations of stone fruit trees in regions where PPV is established. However, too few natural resistance genes have been found in different cultivated and wild species of Prunoideae: one single source in plum and apricot and none in peach. When challenging new genitors with a range of PPV isolates, representatives of the viral diversity, only two or three donors (Harlayne, Lito and SEO) remained durably resistant. Therefore, with a restricted number of sources of resistance, we cannot rule out that the existing mechanisms may be overcome in the future, by new PPV strains or variants. Pyramiding in a commercial cultivar several complementary resistance factors is an alternative that should result in a better durability of resistance to PPV. Such approach has been initiated in the frame of SharCo (DG1.5), in apricot. It is based on the identification of new mechanisms of resistance that interfere with viral infection, most of them being discovered in model plants (*Arabidopsis thaliana*, *Nicotiana benthamiana*) (DG2.4 and DG2.5). It thus requires transfer to *Prunus* crop species before pyramiding with previous donors of resistance. To be sustainable for the European Fruit production, this should be performed in every major susceptible *Prunus* crop species (peach, apricot and plum). In SharCo, we initiated such approaches (DG1.5) but the unravelling of such new mechanisms of PPV restriction is just beginning and needs to be strongly supported in order to provide enough alternatives that could be easily transferred and combined in all *Prunus* crop species.

On the other hand, more efficient and durable protection will also be achieved by deploying resistant cultivars together with agro technical packages associating diverse phytosanitary measures and cultural practices aimed at preventing, delaying or reducing virus spread in orchards and nurseries (see DA1.2 Cultivation Guidelines). These packages will not only enhance resistance efficiency at the crop level but may also contribute to reducing the risk of occurrence of virulent variants by decreasing the virus inoculum pressure on resistant plants. We thus recommend the future deployment of those PPV resistant commercial varieties only together with the application of the SharCo cultivation guidelines.

### *Understanding the biological parameters of the three main PPV strains and linking them to differential spreading risks*

Despite a large effort devoted in SharCo on this matter, our knowledge of the epidemic properties of the most common PPV strains (PPV-D, -M and Rec) remains very partial. Aphid transmissibility and dynamics of multiplication of one isolate for each of the three PPV strains was evaluated in the most important *Prunus* crop species (plum, peach and apricot), using experimental conditions and by analysis of their respective prevalence in endemic regions. Of

significant concern, we identified rather frequent cases of mixed infection, in particular in plums in some countries. This poses a clear risk of evolution of the pathogen diversity and thus properties through recombination. However, our preliminary analysis of data collected experimentally did not show clear-cut differences in PPV/host interactions between the three strains and the three *Prunus* species (peach, apricot and plum). No pertinent indicators directly linked to the biological cycle of the virus for a given strain in a given host was identified in our conditions, despite the fact that the behavior of the various strains may be markedly different under field conditions. In the prospect of sharka management, it remains crucial to characterize the biological parameters determining the epidemic properties of PPV strains, including transmissibility by aphids and pathogenicity on *Prunus* hosts. Indeed, once identified and validated, these indicators are expected to be used to predict the epidemicity of any PPV variant in a given agro-system, thus modulating the response of the National Plant Protection officers depending on the occurrence or not, of new strains or isolates with novel properties with the potential to further degrade the situation in any given area.

## **Potential Impact:**

*From the characterisation of PPV diversity to the detection of unknown PPV variants and development of early warning tools*

The objective of establishing a current view on the diversity of PPV has been largely achieved. The sequence data generated during the project has widely contributed to update the views of the international scientific community on PPV diversity. This sequence data and the associated biological and geographical data are made available to all parties interested through the web queriable SharCo database. Among other uses, this sequence information can further serve to improve the robustness and specificity of detection tests (designing new primers, etc.). Both PPV collections (centralized lyophilized collection and in vivo grafted collection) include reference isolates, but also variable or highly divergent isolates, thus encompassing the presently known PPV variability. Therefore, their services can be summarized as follows: i) provide well-characterized inoculum maintained in the Prunus hosts, which can be used in the evaluation of resistant germplasm and help to standardize the resistance test procedures, ii) provide well characterized or reference PPV isolates for development and validation of future robust, reliable and efficient detection tools, or specific typing tools targeting particular isolate(s) or PPV strain(s), iii) provide a reference baseline for future monitoring of changes in the pathogen distribution or for further studies of PPV population genetics, variability and evolution (e.g. using next generation sequencing tools), iv) provide well characterized or reference PPV isolates for validation efforts of protocols or detection techniques, including procedures of optimization and demonstration of performance characteristics and evaluation of sensitivity and specificity, v) preservation of the biodiversity of an important plant pathogen.

*From experimental characterisation of PPV variants epidemic properties to the understanding of agro-system-wide spreading factors*

Simple and highly significant indicators of epidemicity of PPV strains on specific Prunus crop species were unfortunately not obtained from the experiments realized under confined conditions. Instead, some subtle differences were set out that constitute the starting point(s) to design better adapted tests. While disappointing, this result implies important consequences. We clearly demonstrated that the three tested PPV strains are able to infect the three tested Prunus species, while real differences were observed in field conditions. These differences are thus not due to an intrinsic property of the virus to infect or not a given Prunus species. Indeed, it points out that field differences can be due to the impact of the physiological state of the trees in field conditions that give subtle but efficient advantages to a given strain and not to another one. The results stress also that none of the present tests are really appropriate at identifying strain specific resistances in Prunus; the classical test used in genetics is probably the best one as it covers several physiological conditions during three vegetation cycles.

The identification of mutations in the P1 and CP proteins of PPV that cause host-specific pathogenicity properties could facilitate the unravelling of host factors interacting with these proteins, which would be excellent targets for antiviral actions. The results point to the existence in *Nicotiana* species of an anti-PPV resistance mechanism targeting the N-terminal region of the CP protein, which potentially could be transferred to *Prunus* hosts once identified. The construction of libraries of PPV clones with random sequences will facilitate the design of experimental approaches for forced evolution allowing the identification of virus factors involved in PPV adaptation to particular hosts. As stressed in the previous paragraph, these approaches are meaningful when host preferences are clear-cut, that seems not to be the case except maybe for PPV-C. But they are very useful to find new ways of hampering host-virus interactions.

The survey of strain prevalence under different agronomical situations points to the risk of emergence of new recombinants through the role of *P. domestica* that seems to be a host suitable for all the strains and which is found with a significant percentage of mixed infection when the situation is favourable. This point is relevant when considering the preparation of cultivation guidelines and early warning systems: the monitoring of plum orchards to quickly detect abnormal situations is a priority.

The simulation model is close to a functional version and will quickly be used at exploring different control scenarios or at comparing different agrosystem situations. As a tool for PPV control strategies, it can be transferred, when validated, to official plant protection agencies. It can be used to test different strategies through the sensitivity to a range of possible human control actions (i.e., detection time, monitoring system, impact of quick removing, orchard structure, inter-orchard distances.), at time and spatial scales that are not accessible to practical experimentation. While useful to test different strategies, the outputs of the model are impacted by the precision of the main biological parameters it includes (i.e., range of dissemination by aphids, latency period, rate of increase). These parameters are still imprecise. Several approaches were employed to improve the precision on these parameters: inference through regional data bases via the estimation from the regional estimation model; reconstruction of the chains of dissemination events on a defined 400 ha area; experimental orchards with a defined PPV strain as primary inoculum. All these approaches will continue to bring practical results in the next two years, particularly on distances of spread and thus possibility to recommend improved regulatory rules.

Many data are still currently under analysis. The first outcomes provided valuable information on PPV epidemiology in nurseries and orchards that were thus included in the cultivation guidelines (deliverable DA1.2) as well as in the risk management system (deliverable DA1.5). Other ones will be added if necessary as the analyses will progress.

### *For the definition of guidelines in support of stone fruit tree propagation in nurseries*

Following SharCo outcomes, significant reduction of the risks of entry and establishment of PPV in nursery blocks can be achieved by the use of proper and accurate protocols for sampling and testing nursery Prunus plants as described in the deliverables DE3.2 and 3.3. By following those procedures, identification as early as possible of contaminated plant material and/or latent PPV infection that is usually overseen during visual inspections is expected. Also it was demonstrated that natural infection of propagated plant material is significantly limited by the use of mineral oils at the peak period of aphid flights. It is thus also recommended to monitor aphid flights, in order to apply adequate means of management of PPV natural vectors.

To reduce the establishment and further spread of the virus in the nursery, we advise using rootstocks less susceptible to PPV infection as the ones described above and in the deliverable DE3.2. At least, nursery blocks of PPV susceptible stone fruit trees should alternate with non-host or less susceptible propagating material.

When applied all together, these measures of PPV management are expected to significantly reduce the occurrence and the incidence of PPV in nursery blocks as well as secure the trade of stone fruit, PPV-free, propagation material. In consequence, all these data served to elaborate recommendations for PPV management in nurseries; they are detailed in the DE3.3 deliverable.

### *From conventional breeding to marker-assisted selection of PPV resistant material*

The use of genetic resistance is, beyond doubt, the best solution for the control of virus-induced diseases because it provides effective protection throughout the growing season. It allows the new plantings of stone fruit trees in areas where PPV is present. However, breeding for resistance to PPV encounters the usual problems of breeding in perennial plants that include extended vegetative periods, high labor cost, large space needed for phenotyping and difficult and lengthy procedures of screening for PPV resistance. Standardization of the resistance tests has proved difficult because of delayed response to inoculation, variability of the virus, physiological state of the host plant and inoculation method. To test an interesting cultivar, one needs 3 years of monitoring after infection to assess the level of resistance or susceptibility. The breeding programs that are focused on the selection of cultivars with agronomic value, fruit quality, local adaptation and PPV resistance, become a long, difficult and expensive procedure. It is therefore of major importance to implement the selection of promising individuals by molecular assisted selection (MAS). Molecular markers linked to the trait have the potential to select the genes of interest (for instance PPV resistance genes) in vegetative tissues, giving information early in the plant development, at an early stage such as young seedlings. The screening of PPV resistant plant material through molecular markers, once they are identified, is a simple, fast and reliable procedure. In the Genetic pillar, markers linked to resistance to PPV in apricot were developed and new breeding programs have been implemented and



supplemented with molecular markers. In consequence, selection of pre-breeding plant material takes now a few months instead of 3 to 4 years. This accelerates the introgression of PPV resistance into locally adapted apricot cultivars, in many agro-ecological environments.

All together these results are increasing the efficiency of the breeding programs, as initially planned, but also impact the deciphering of the inheritance and genetics of Sharka resistance, opening new opportunities for genetic strategies in Sharka containment.

#### *Towards the development of a durable resistance to PPV*

Another of our goals, to increase the collection of potential sources of resistance to PPV, has been largely achieved. These new sources of resistance cover a broad range of mechanisms, largely differing in strength of protection and proximity to practical application.

The stimulation of antiviral RNA silencing by expression of ih (intron hairpin) viral constructs has been proven to provide efficient and durable protection to plum trees against the major PPV strains, and joins to the already approved in the USA Honey Sweet cultivar as an answer to the Sharka problem very close to practical application. Expression of amiRNAs is another way to exploit RNA silencing to engineer antiviral resistance, which lacks the environmental risks associated with transgenic expression of long viral sequences, and is expected to have reduced off-target effects. Our results on the molecular analysis of amiRNA expression and activity could help us to design more efficient anti-PPV amiRNAs. Moreover, we have designed amiRNAs with efficient anti-PPV activity in *N. benthamiana*. If these results are reproduced in *Prunus*, we would have available another interesting source of resistance to confront Sharka disease.

An intense EcoTilling approach applied to a gene essential for PPV infection, the eIFiso4E protein, identified *Prunus* individuals in natural germplasm with promising PPV resistance that are already being tested in natural conditions, and could be introduced in breeding programs in the very near future. Ongoing approaches to silence by RNAi the eIFiso4E and different isoforms of the eIF4G factor, one of which is also essential for PPV infection, in *Prunus* cultivars are expected to yield PPV-resistant plants to be used when European concerns in transgenic plants decline.

Work in *A. thaliana* has allowed the identification of three additional sources of PPV resistance based on host proteins required for PPV infection. These proteins appear to be involved in different steps of the virus cycle, enhancing their utility in pyramiding strategies. If the role of these proteins is conserved in the *Prunus* hosts of PPV, they can be also the targets of EcoTilling approaches to look for functional alleles unable to support PPV infection, and of genetic engineering approaches to silence them in case they are not essential for the host, or to selectively disturb their virus-related activities. Another host target for anti-PPV action is the *Prunus* homolog of the *A. thaliana* SEC protein, which O-GlcNAcylates PPV CP. The results of this work package show that O-GlcNAcylation of PPV CP is not essential but has a fine tuning

effect in PPV infection. It was recently demonstrated that quantitative (partial) resistance traits enhance the durability of protection provided by strong resistance genes. Thus, combining down regulation of SEC-like genes with other antiviral strategies could have a positive effect in protecting plants against Sharka disease. Similarly, the expression of PPV-specific recombinant antibodies, which in the laboratory only partially protects against PPV infection, could be very useful to protect plants against Sharka disease in natural conditions.

Our transcriptomics analysis of herbaceous and woody plants displaying an HR in response to PPV infection did not allow the definitive recognition of host factors involved in this defensive reaction. However, the identification of a set of genes whose expression was altered by the HR reaction should help us to discover genes whose disturbance could potentiate HR-related anti-PPV defense.

#### *For an upgraded and harmonized management of PPV outbreak*

The application of the recommendations and tools we proposed for Sharka containment is expected to significantly reduce the spread and impact of the disease at the European, national and regional level. They should help implement sustainable plant, fruit quality and yield with obvious positive effects on rural socio-economical issues, including job opportunities, maintenance of agricultural activities in many low to mid-income Southern European rural regions, in particular in marginalized lands.

Based on the results obtained in the scope of SharCo, we proposed more acceptable cultivation guidelines (DA1.2), an early warning system (DA1.4) and a risk management system (DA1.5) to European policy makers, national plant protection services and governments. We also formulated recommendations for nurseries gardeners and fruit producers of best-practices all along the fruit tree multiplication and production process. Therefore, stakeholders range from EU policy makers, plant protection services, extension personnel, nursery gardeners, fruit growers, and fruit industry representatives. Likewise, certification standards at the European level are needed to guarantee PPV-free production, transport, and sale of nursery stock and budwood. To facilitate knowledge and tool access to any stakeholder, a decision support system has been developed and several training workshops were organized, targeting information dissemination and transfer to European and PPV endemic regions. They took place successively in Poland, Bulgaria, Turkey, Romania and Czech Republic. A major Research workshop focusing on Sharka was organized also in Sofia (Bulgaria) and gathered over 80 international scientists and officers of the Plant Protection Services from most of the European member states.

**List of Websites:**

[www.sharco.eu](http://www.sharco.eu)