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2010 International *Plum pox virus* Symposium

# Proposals for *Plum pox virus* detection in nurseries

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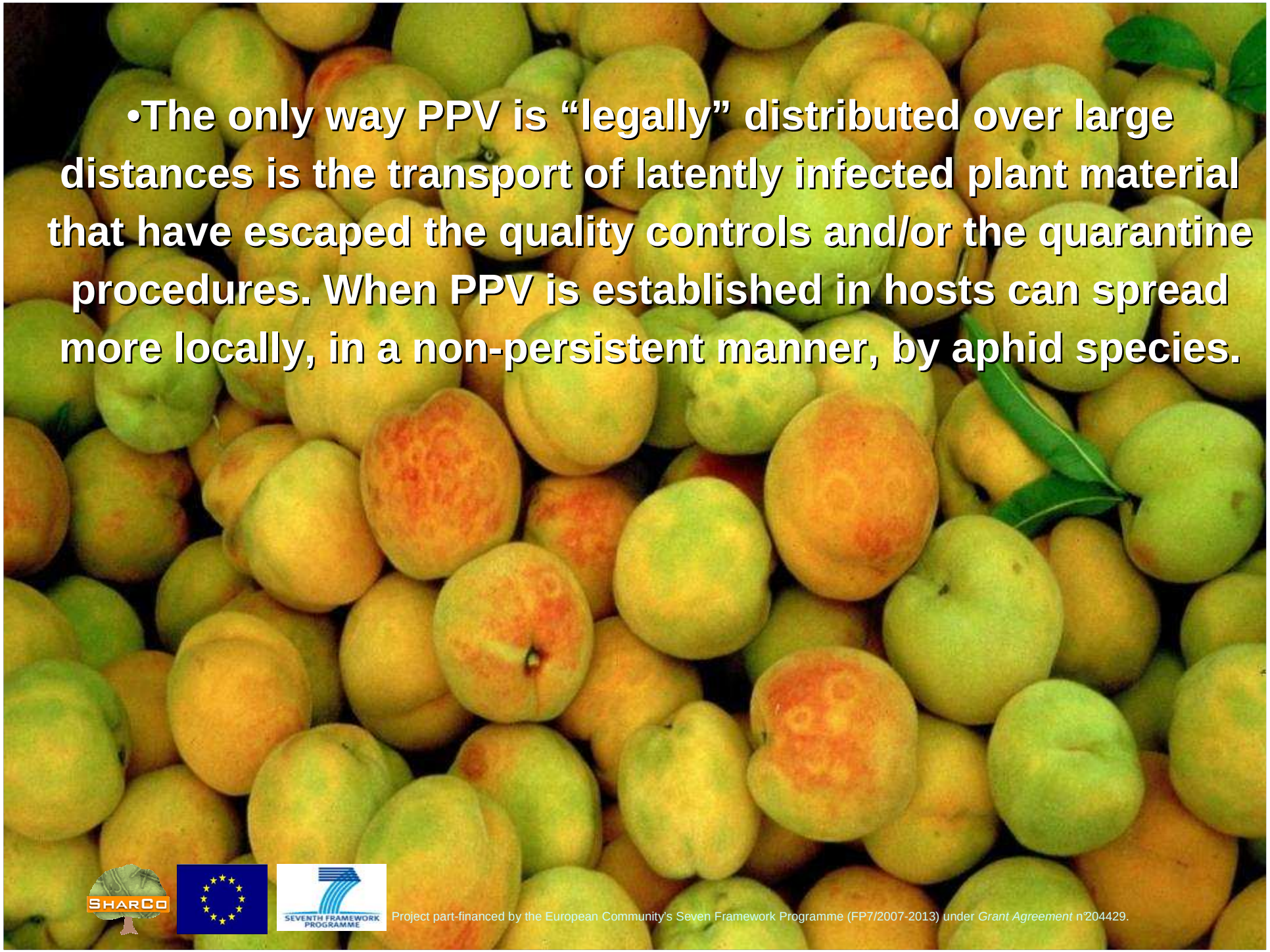
Project part-financed by the European Community's Seven Framework Programme (FP7/2007-2013) under *Grant Agreement* n°204429.

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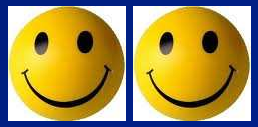
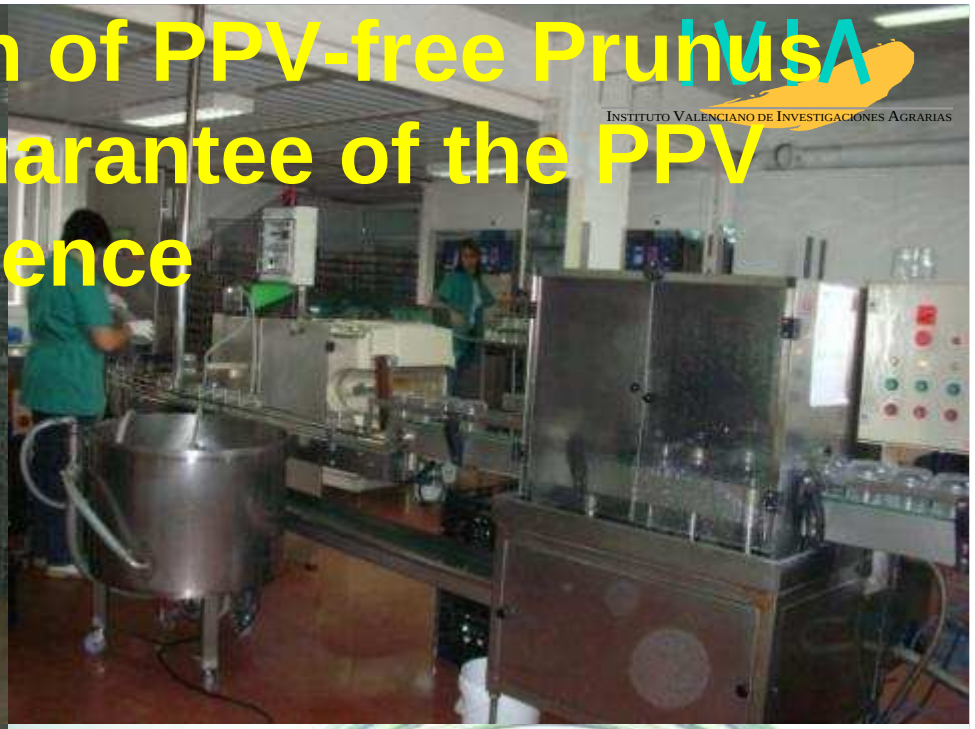
# *Different scenarios for the establishment of Prunus nurseries*

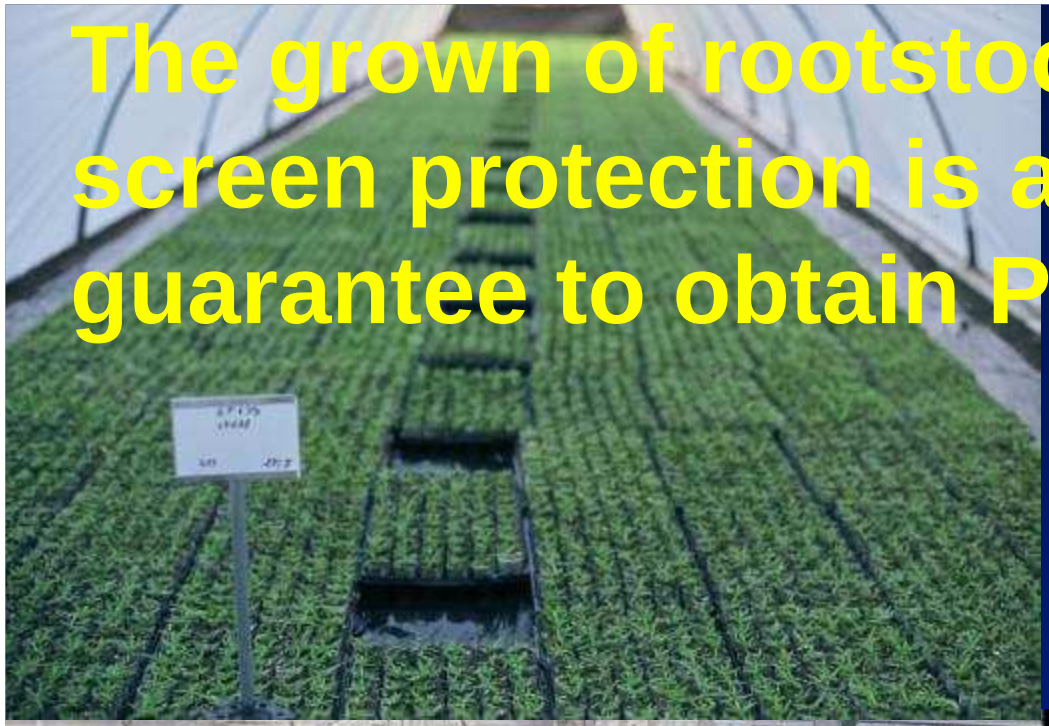
1. Isolated, mother plants protected and based on in *vitro* produced rootstocks 
2. Isolated and mother plants protected 
3. Isolated and mother plants non-protected 
4. Non isolated but mother plants under protection
5. Non isolated and non-protected mother plants

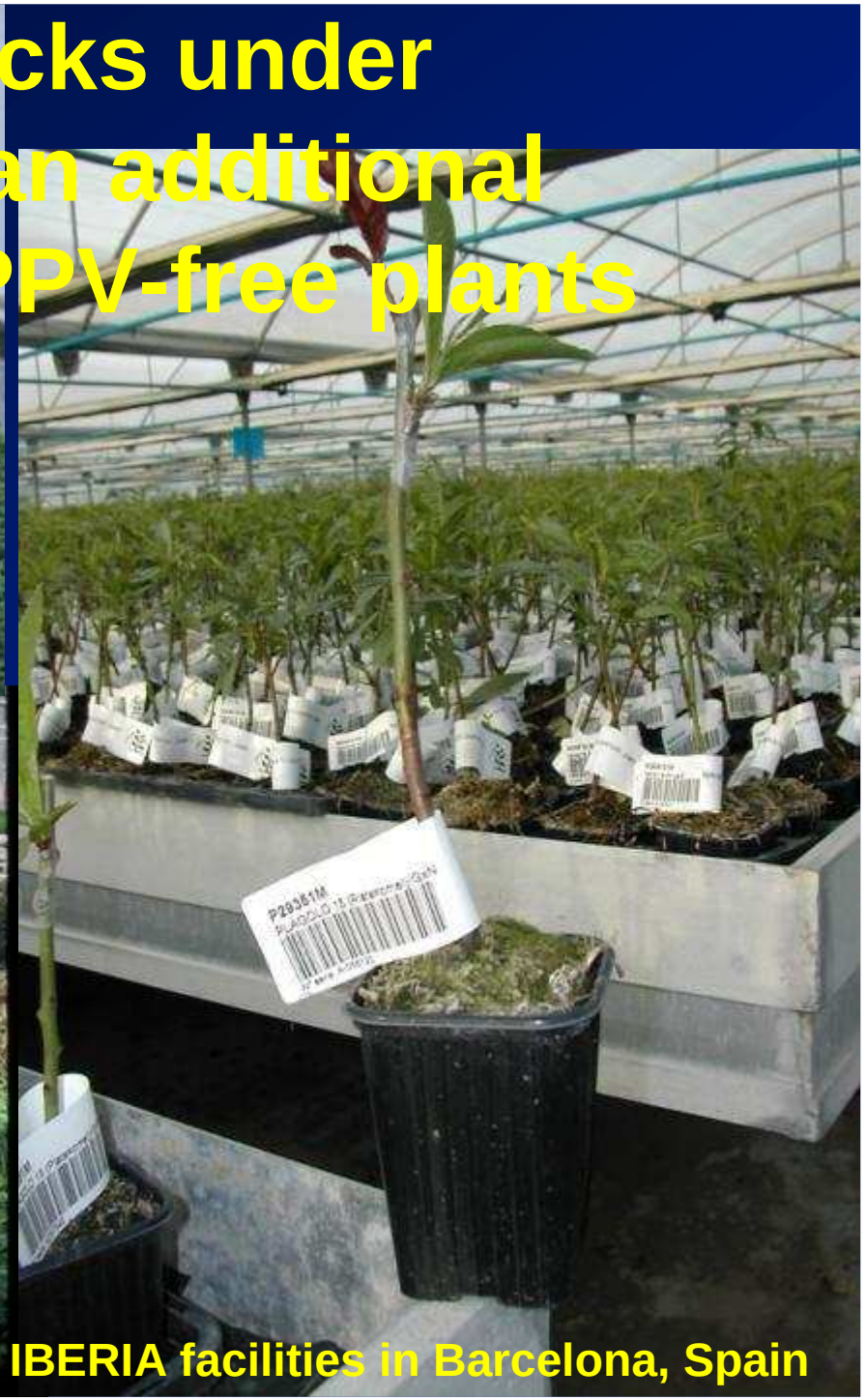
# *In vitro* propagation of PPV-free Prunus rootstocks is a guarantee of the PPV absence



AGROMILLORA IBERIA facilities in Barcelona, Spain

# The grown of rootstocks under screen protection is an additional guarantee to obtain PPV-free plants





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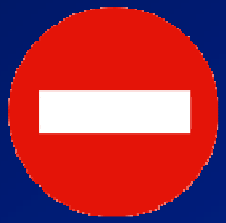
# Production of PPV-free plants is possible under protected facilities

AGROMILLORA IBERIA facilities in Barcelona, Spain

**Only PPV tested plant material coming from mother plants maintained under screenhouse facilities or grown in areas PPV-free have to be grafted in a nursery or use PPV resistant cultivars (obtained by conventional breeding or biotechnological approaches- HoneySweet)**

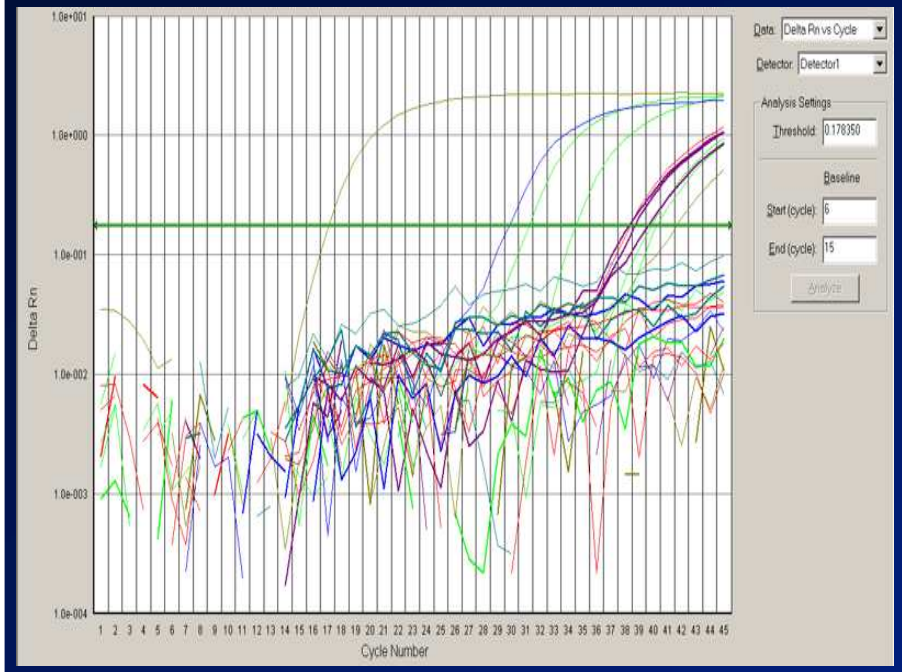
Mother plants maintained under aphid protection

**…it is Imposible to produce PPV-free plants in the proximity of infected Prunus!!!**

**PPV is the only fruit tree virus transmitted by aphid species in a non-persistent manner!!!**

# A careful analysis against PPV of all new material imported / introduced in the nursery have to be performed.




**Nowadays there are powerful (sensitive, specific and accurate) methods and reagents for reliable PPV detection at any vegetative period or in dormant plants.** (Improving in sampling, serological and molecular methods is being evaluated in SharCo project for a more confident PPV detection).

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# Plant material present or introduced in a nursery that has to be tested for PPV detection:

- 1) Mother plants (for cultivars) or new introduced cultivars (budsticks) + + + + +
- 2) Rootstocks + +
- 3) Grafted plants + +

*Sampling and analyses intensity should be proportional to the number of crosses

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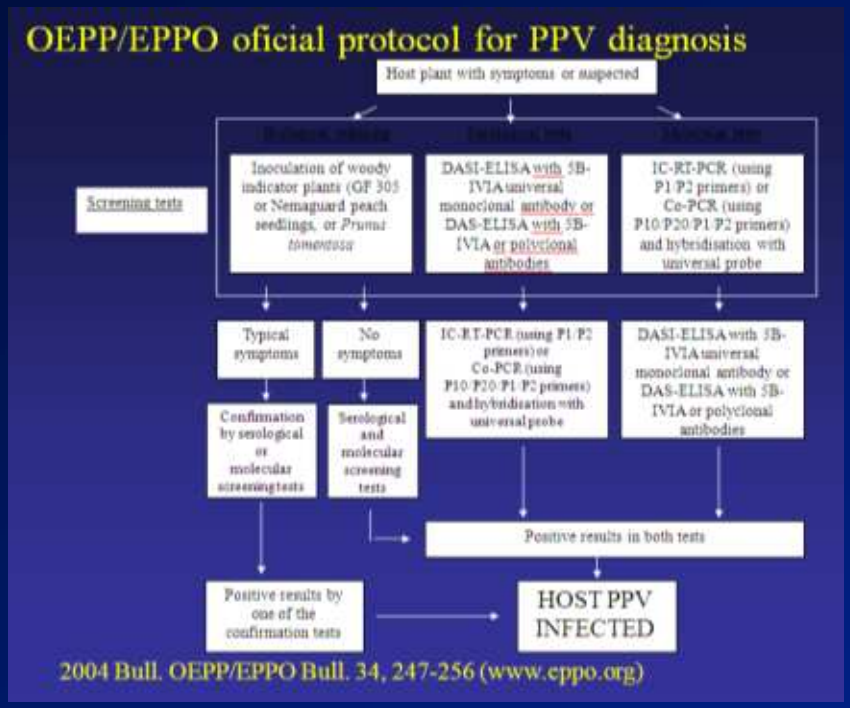
# Currently there are available and validated protocols for PPV detection and identification

## 1) OEPP/EPPO (2004) Standard PM7/32. Bull.OEPP 24: 525-536





## 2) International Plant Protection Convention. INTERNATIONAL. STANDARDS FOR PHYTOSANITARY MEASURES. DRAFT ANNEX to ISPM 27:2010 (in discussion)

# Mother plants analyses

1) Anual analysis of individual or composite trees (100%) by validated ELISA and/or RT-PCR based methods

2) Sampling: Plant samples should be taken from at least 4-5 one-year-old shoots with mature fully expanded leaves collected from the middle of each of the main branches around the canopy. Plant material should preferably be collected from the internal parts of the canopy of the tree. In springtime, samples can be flowers, young shoots with fully expanded leaves (8-12) or fruits. In summer and autumn mature leaves and the skin of mature fruits. In winter dormant buds or bark tissues from the basal part of twigs, shoots or branches, or complete spurs can be selected.

# Rootstocks and grafted plants analyses

- Anual analysis of individual or composite rootstock plants and/or grafted plant samples (from 5% to 25%) by validated ELISA and/or RT-PCR based methods.

- Sampling: Plant samples (3-4 leaves/nursery plant) should be taken from one-year-old shoots with mature fully expanded leaves in springtime, summer and/or autumn. Plant material should preferably be collected from the internal part of the plant. In winter dormant buds or bark tissues from the basal part of twigs, shoots or branches can be selected.

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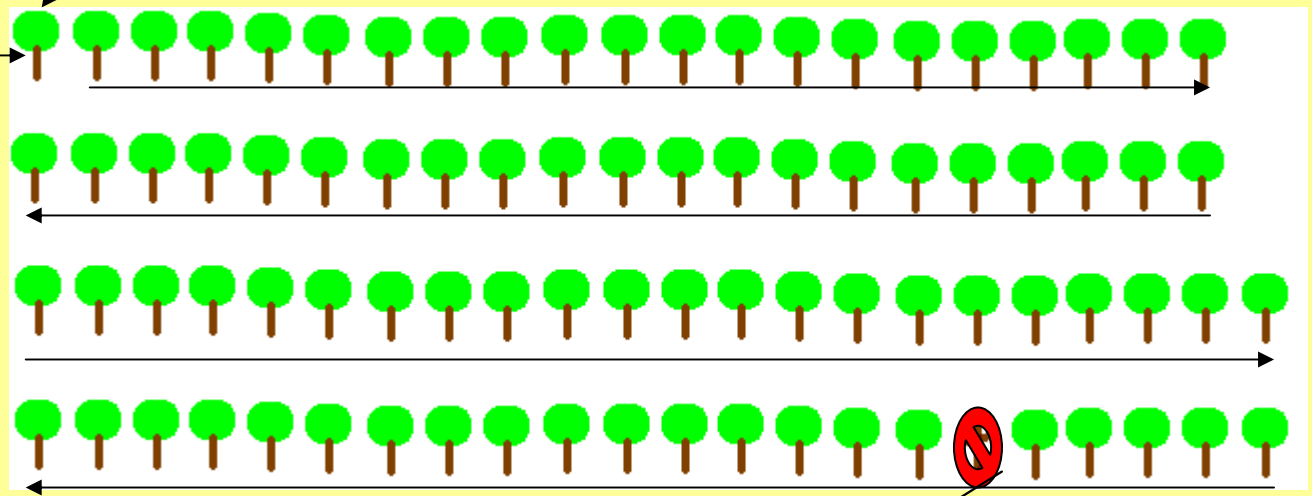
# 100% Sampling Method

3 leaves are collected from each nursery plant.
Every plant is sampled.

Nursery Block

Start Corner



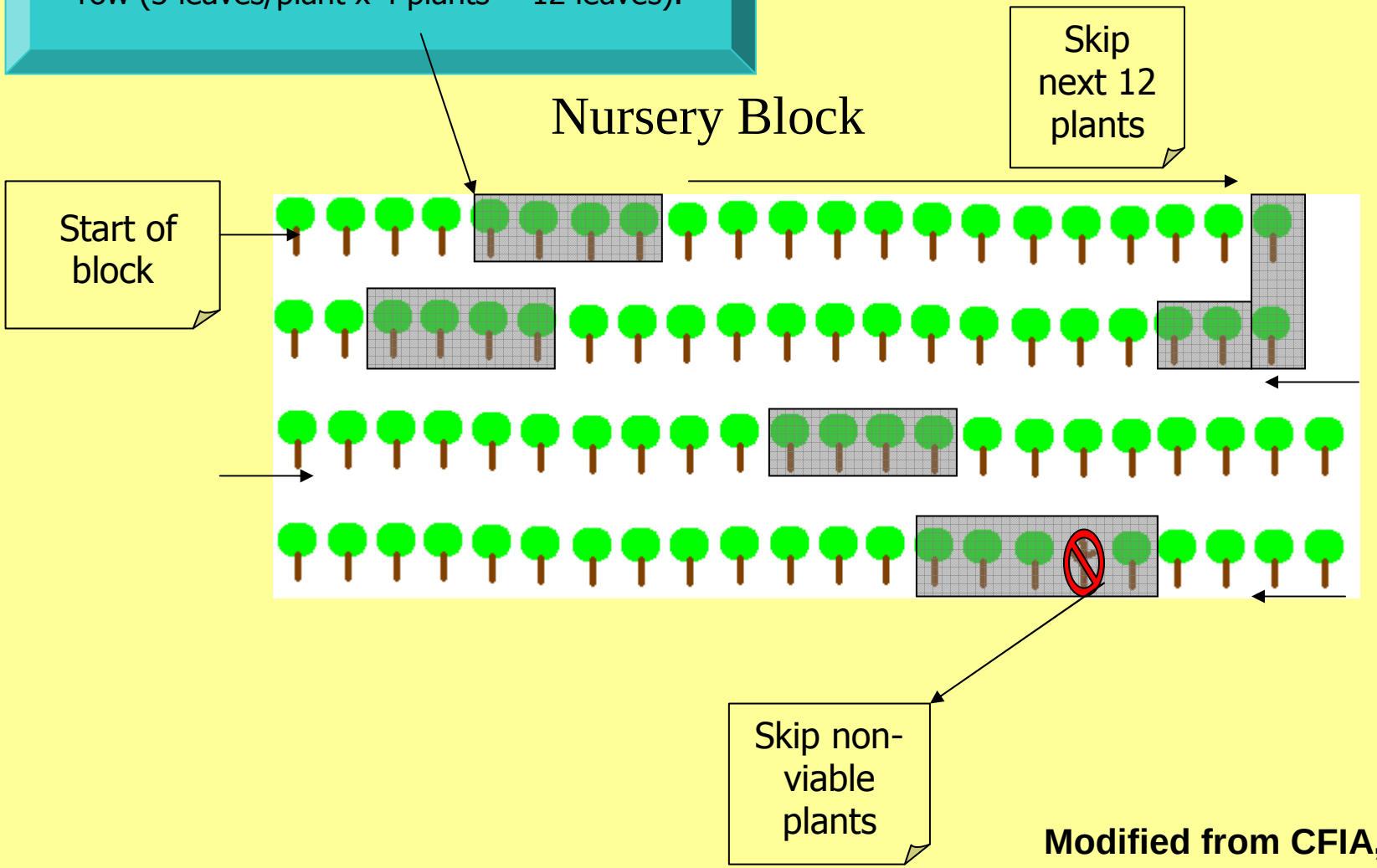
Skip non-viable plants

**Modified from CFIA, Canada**

# R4X12 Sampling ( Hierarchical Method)

**Hughes et al (2002). Plant Dis. 86: 259-263.**

Random the start quadrat in the first nursery row (3 leaves/plant x 4 plants = 12 leaves).




**Modified from CFIA, Canada**

# Autum sampling (block 1)

- 3-4 falling leaves /plant were collected and analysed by single, pairs, quads and hierarchical method by ELISA 5B-IVIA in Carlet (Valencia, Spain) under medium/low PPV-inoculum pressure.

| Analysis | Nº samples | Nº positives (%) |
|---|---|---|
| X1 (100%) | 2,610 | 8 (0.30%) |
| X2 (100%) | 1,305 | 8 (0.30%) |
| X4 (100%) | 653 | 8 (0.30%) |
| 4X12 (25%) | 324 | 3* (0.23%) |

Underestimation *P.low = 1 - (1 – (3/324) $^{1/4}$

# Autum sampling (block 2)

- 3-4 falling leaves /plant were collected and analysed by single, pairs, quads and hierarchical method by ELISA 5B-IVIA in Llíria (Valencia, Spain) under high PPV-inoculum pressure

| Analysis | Nº samples | Nº positives (%) |
|---|---|---|
| X1 (100%) | 1,269 | 74 (5.83%) |
| X2 (100%) | 635 | 74 (5.83%) |
| X4 (100%) | 318 | 74 (5.83%) |
| 4X12 (25%) | 80 | 23* (8.13%) |

Overestimation *P.low = 1 - (1 – (23/80) $^{1/4}$

# Analysis of individual and composite samples by serological and molecular detection methods

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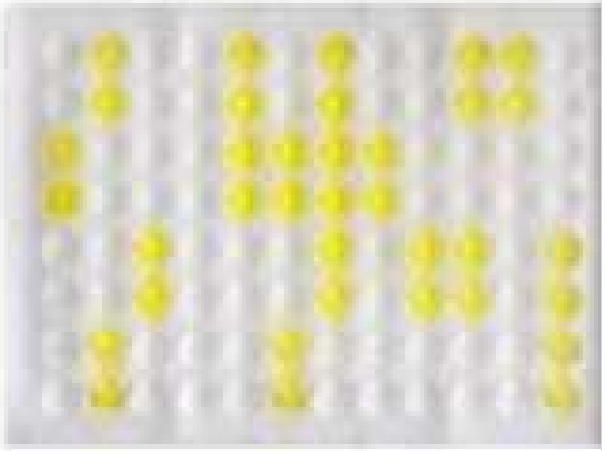
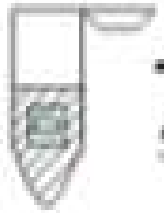
ELISA (5B-IVIA)

Extraction buffer
PBS + 2%PVP + 2% DIECA

5 µl

Spot real-time-RT-PCR

5 µl spotted on nylon membrane

100 ul 0.5% Triton X-100 and vortex

# Spurs *vs* buds for PPV-detection in dormant period

**Comparasion of spot real-time RT-PCR and DASI-ELISA 5B-IVIA from 6 positive apricot mother plants**

| | Spot rt RT-PCR (Ct ) | DASI-ELISA O.D$_{405\ nm}$ |
|---|---|---|
| 4 spurs/tree | 23,27 | 1,634 |
| 8 spurs/tree | 22,98 | 1,442 |
| Apical buds | 27,63 | 0,523 |
| Medium buds | 27,52 | 0,551 |
| Basal buds | 27,04 | 0,766 |

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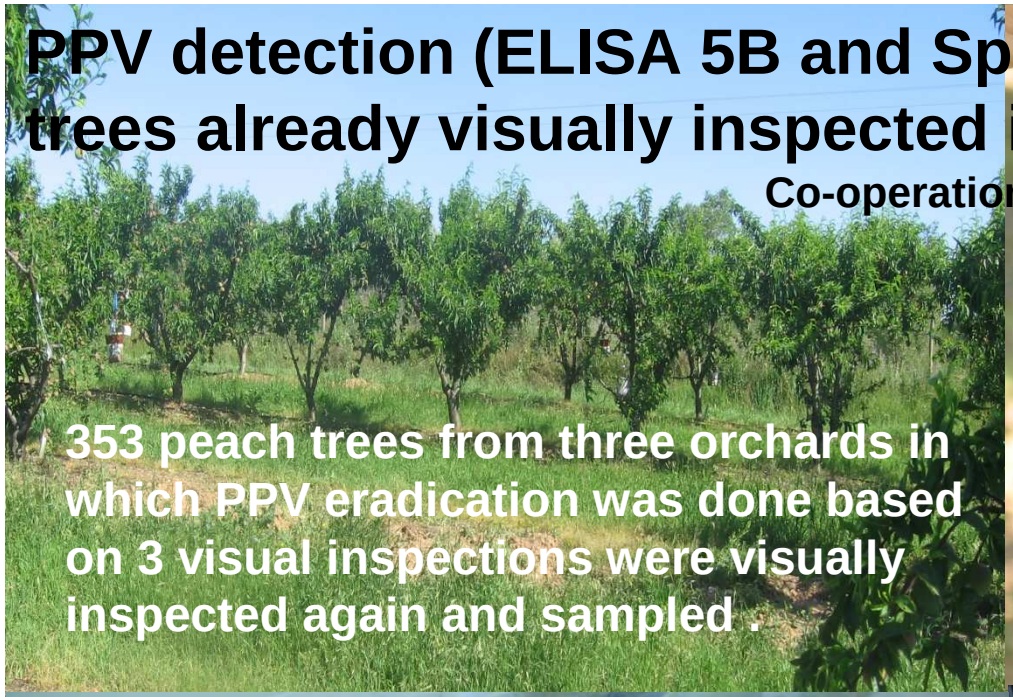
## Analysis of individual and composite samples by serological and molecular detection methods in dormant period

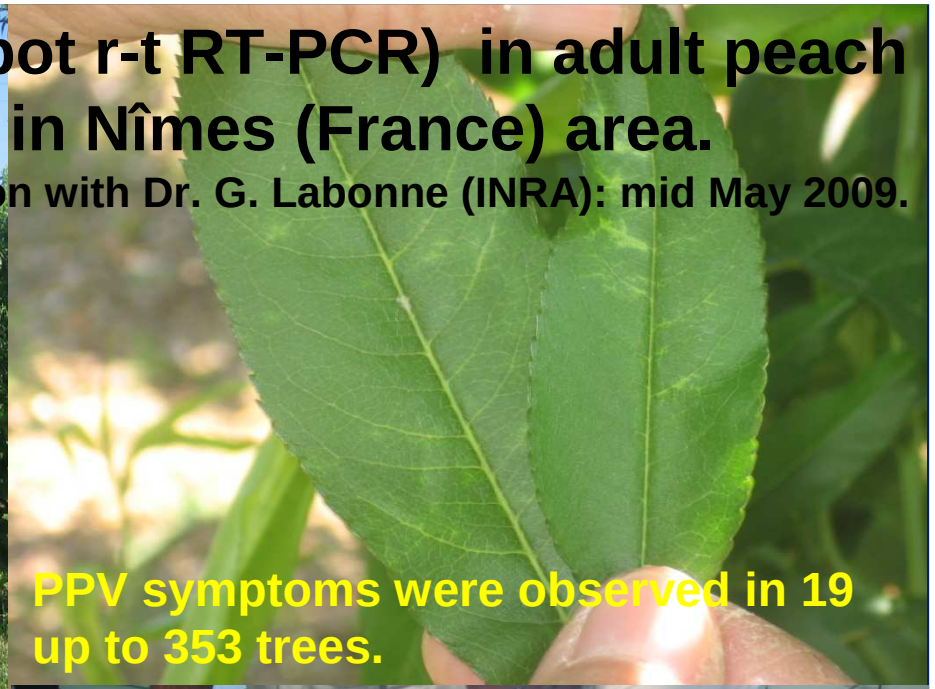
| Composite sample | Buds from PPV infected plant + buds from healthy plant | Nº positive PPV detection by ELISA up to six replicates | Nº positive PPV detection by Spot real-time PCR up to six replicates |
|---|---|---|---|
| Single plant | 3+0 | 2 | 5 |
| Two plants | 3+3 | 4 | 5 |
| Tree plants | 3+6 | 5 | 6 |
| Four plants | 3+9 | 3 | 6 |
| Five plants | 3+12 | 1 | 5 |
| Six plants | 3+15 | 0 | 6 |
| Seven plants | 3+18 | 0 | 6 |
| Eight plants | 3+21 | 0 | 5 |
| Nine plants | 3+24 | 0 | 6 |
| Ten plants | 3+27 | 0 | 6 |

# PPV detection (ELISA 5B and Spot r-t RT-PCR) in adult peach trees already visually inspected in Nîmes (France) area.

**Co-operation with Dr. G. Labonne (INRA): mid May 2009.**



**353 peach trees from three orchards in which PPV eradication was done based on 3 visual inspections were visually inspected again and sampled .**



**PPV symptoms were observed in 19 up to 353 trees.**

**8 to 12 leaves were collected per tree and package together**

**Extracts were prepared using the basal part of the leaves and analyzed**

t part-financed by the European Community's Seven Framework Programme (FP7/2007-2013) under *Grant Agreement* n°204429.

# *Results (May 2009)*

| Spot r-t RT-PCR | ELISA-DASI | Symptoms | % PPV-Incidence |
|---|---|---|---|
| (+) 67 | (+) 27 | (+) 12 | 3.40 % |
| | | (-) 15 | 4.25 % |
| | (-) 40 | (+) 4 | 1.13 % |
| | | (-) 36 | 10.20 % |
| (-) 286 | (+) 0 | (+) 0 | 0.00 % |
| | | (-) 0 | 0.00% |
| | (-) 286 | (+) 3 | 0.85 % |
| | | (-) 283 | 80.17 % |
| **353** | **353** | **353** | **100.00%** |

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A) Analysis (in March 2010) of 33 peach trees that tested only positively by spot real-time RT-PCR in May 2009

B) Analysis (in March 2010) of 32 peach trees that tested negatively by ELISA, spot real-time RT-PCR and symptomless in May 2009

**A**

**Winter**
**DAS-ELISA**

| Spot r-t RT-PCR | + | - |
|---|---|---|
| + | 3 | 5 |
| - | 0 | 25 |

33

**B**

**Winter**
**DAS-ELISA**

| Spot r-t RT-PCR | + | - |
|---|---|---|
| + | 2 | 3 |
| - | 0 | 27 |

32

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# General conclusions

**Visual inspection is not a method for PPV detection especially in nurseries. Laboratory tests must be done !**

1) Accurate analysis of composite sample is possible by r-t RT-PCR (3 leaves/plant). Ten nursery plants can be mixed in a single sample (a maximum of 4-5 plants by ELISA).

2) Reliable PPV detection can be afforded by ELISA 5B or r-t RT-PCR at any time of the year in symptomless plants (including summer and winter periods). Spring is the best period followed by summer and fall.

3) The combination of ELISA 5B and r-t RT-PCR analysis supply practical 100% post-test probability (Olmos et al., 2008; Capote et al., 2009).

4) A detailed guideline for PPV detection in nursery plants is being prepared in the framework of the SharCo project for a more confident PPV detection.


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