Susceptibility of *Prunus* rootstocks to natural infection of *Plum pox virus* and effect of mineral oil treatments

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Keywords
Epidemiology; non-persistent virus; nursery blocks; plant virus control; spray.

Abstract
The use of rootstocks that are less susceptible or resistant to natural *Plum pox virus* (*PPV*) infection and/or the application of mineral oil treatments are two possible strategies to reduce viral incidence in nursery plots. We evaluated the susceptibility of *Prunus* rootstocks used in the Spanish stone fruit industry and the effect of mineral oil treatment (Sunspray Ultrafine at 1%) on the spread of the virus at two different localities in Valencia, Spain, under different natural PPV inoculum pressures (high inoculum pressure, 2006–08; low inoculum pressure, 2006–07). Samples from both plots were analysed by double-antibody sandwich indirect enzyme-linked immunosorbent assay (DASI-ELISA) and spot real-time RT-PCR. Under high inoculum pressure, the assayed rootstocks exhibited significant differences in their susceptibility to natural infection. The most susceptible rootstocks at the end of the experiment were Adesoto 101 and Mariana GF8-1. Cadaman and Garnem rootstocks presented the fewest PPV-infected plants; these infections could be detected only by spot real-time RT-PCR. No differences among the assayed rootstocks were found under low PPV inoculum pressure. Aphid species were monitored using Moericke yellow water traps and sticky-plant methods at both localities in May 2006 and 2007. *Aphis spiraecola* was the most abundant aphid species monitored by both methods at both localities. The average percentage of *A. spiraecola* carrying PPV PCR-amplifiable targets was 30.37% in the plot with high PPV inoculum pressure and only 7.98% in the plot with low inoculum pressure. We found significant differences in PPV incidence between Mariana GF8-1 plots that were treated with mineral oil and those that were not treated after one year under natural conditions and high PPV inoculum pressure.

Introduction
Plum, apricot and peach stone fruit crops are of major economic importance worldwide, yielding approximately 30 244 490 t in 2007. The most important areas of production are China (14 050 000 t), the European Union (6 012 366 t) and USA (1 764 530 t) (FAOSTAT, 2007). The most devastating viral disease affecting these stone fruits is plum pox or sharka, which is caused by *Plum pox virus* (*PPV*), genus *Potyvirus*. The estimated cost associated with sharka management worldwide in the last 30 years exceeds 10 000 million Euros (Cambra et al., 2006b). The disease was first described in Bulgaria (1917–18) in *Prunus domestica* cv. Kjustendil. Since then, it has spread from the European continent towards the most important *Prunus*-growing areas around the world, except Australia, New Zealand, South Africa and California (USA) (García & Cambra, 2007). The main pathway by which PPV spreads over long distances is the introduction of infected propagative plant material (Cambra et al., 2006b). The
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virus is spread locally by aphids in a non-persistent manner (Ng & Falk, 2006).

PPV has different isolates that differ in their epidemiology, symptomatology and aggressiveness. These isolates have been classified into seven types or strains: PPV-D (Dideron), PPV-M (Marcus), PPV-EA (El Amar), PPV-C (Cherry), PPV-W (Winona), PPV-Rec (recombinant between D and M) and PPV-T (Turkish) (a second type of recombinant isolate recently reported) (Candresse & Cambra, 2006; James & Glassa, 2006; Ulubas¸Serçe et al., 2009). Among these strains, the most prevalent are D and M. PPV-D isolates are naturally able to infect apricot and plum and rarely spread from these hosts to and among peach cultivars. PPV-M spreads more readily by aphids to and among peach cultivars than do D isolates (Candresse & Cambra, 2006).

Researchers have endeavoured to generate PPV-resistant cultivars using classical breeding and biotechnological approaches (García & Cambra, 2007). The use of PPV-resistant or PPV-tolerant Prunus rootstocks is an agronomic strategy to reduce the spread of the disease in nurseries and, therefore, in new orchards. PPV resistance has been reported in some rootstocks, such as BN 4Kr myrobalan plum (Minoiu et al., 1998), GF677 (Prunus dulcis × Prunus persica), Myrobalan 29C (Prunus cerasifera) and L2 (Prunus lannesiana) (Rubio et al., 2005). However, the susceptibility of the most important Prunus rootstocks used in the stone fruit industry to natural PPV infection by viruliferous aphid species has not been well evaluated under open field conditions.

The management of viral diseases based on the use of pesticides to eliminate viral vectors is not effective for non-persistent viruses such as PPV (García & Cambra, 2007) due to the very short time between acquisition and inoculation (Perring et al., 1999). Products with the potential to reduce the probing activity of the vector can be effective for managing non-persistent viruses (Irwin, 1999). The continuous use of conventional pesticides may contribute to the spread of viral diseases in nurseries and, therefore, in new orchards. PPV resistance has been reported in some rootstocks, such as BN 4Kr myrobalan plum (Minoiu et al., 1998), GF677 (Prunus dulcis × Prunus persica), Myrobalan 29C (Prunus cerasifera) and L2 (Prunus lannesiana) (Rubio et al., 2005). However, the susceptibility of the most important Prunus rootstocks used in the stone fruit industry to natural PPV infection by viruliferous aphid species has not been well evaluated under open field conditions.

The most environmentally friendly control methods are physical barriers that efficiently interfere with the transmission of non-persistent viruses. Among these systems are barrier crops (Fereres, 2000), ultraviolet (UV)-blocking plastic covers (Díaz et al., 2006), plastic mulch and yellow sticky traps (Budnik et al., 1996), mineral oil treatments (Simons & Zitter, 1980; Lowery et al., 1990; Webb & Linda, 1993; Umesh et al., 1995, Asjes, 2000; Asjes & Blom-Barnhoorn, 2002) and oil treatment combined with barrier crops (Boiteau et al., 2009).

The mechanism by which mineral oil prevents aphid transmission of viruses is still not well understood, but oils seem to influence both acquisition and inoculation processes. For non-persistent viruses, Wang & Pirone (1996) have reported that mineral oil alters the surface structure of the aphid stylet, interfering with the insects’ ability to attach viral particles and reducing their transmission capacity. Newer generations of oils are biodegradable and present low toxicity to humans and wildlife, and insects have not developed resistance to these barriers because of the physical action of oils.

In this study, we evaluated the natural susceptibility to PPV-D of the most widely used Prunus rootstocks in Spain (where approximately 1 500 000 t year−1 are produced) under high and low PPV pressure conditions. In parallel, we evaluated the efficiency of spray oil in reducing the spread of PPV in experimental nursery blocks. The knowledge generated from both aspects of our research could contribute to improved control of PPV in Prunus nurseries.

Materials and methods

Plant material and experimental nursery plots

The assays were conducted in two different irrigated experimental fields located in Llíria (Field 1) and Carlet (Field 2), both in the area of Valencia, Spain, during 3 consecutive years (2006–08) in Field 1 and 2 years (2006–07) in Field 2.

The susceptibility to natural PPV infection and the effect of mineral oil treatment on the reduction of PPV incidence and proliferation were evaluated in the most widely used Prunus rootstocks in Spain: Garnem or GxN 15 (P. dulcis × [(P. persica × Prunus davidiana) × P. persica]), Adesoto 101 (Prunus insititia), Cadaman (P. persica × P. davidiana), Myrobalan 29C (P. cerasifera), Nemaguard (P. persica × P. davidiana, hybrid seedling) and Mariana GF8-1 (P. cerasifera × Prunus munsoniana). In addition, the susceptibility of GF677 rootstock (P. dulcis × P. persica) (107 plants) to natural PPV infection was evaluated in an area of high PPV inoculum pressure after 10 consecutive years (1997–07) of cultivation.

Field 1 was planted with 1360 plants in March 2006 in Llíria, which has a continental moderate climate. The field was located approximately 1 m from an infected orchard of adult Japanese plums that had approximately 90% PPV-D incidence. The field was divided into two experimental blocks. Each experimental plot comprised one
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row that was 35 m in length (two rows of double length for Mariana GF8-1 and Nemaguard, 1.5 m apart, repeated three times within a block). Experimental plots were separated by 5 m. A total of 520 plants (from 40 to 45 plants × double length × 2 rows × 3 replicates) were planted for Nemaguard and Mariana GF8-1 rootstocks. Plots with Nemaguard and Mariana GF8-1 were further subdivided lengthwise in a split-plot manner to accommodate the oil treatment (oil-treated versus non-treated control). A total of 80 plants (40 plants × 2 rows) were used for the other rootstocks. The planting depth was 20 cm.

Field 2 was planted in Carlet, which has a typical Mediterranean climate, in April 2006. A total of 3240 rootstock plants were planted in a split-plot experimental design with six blocks set 4 m apart. Each block contained six paired rows (subplots). Each pair of rows consisted of one of the six rootstock cultivars, and 45 plants were planted in each row; one row was treated and the other left as control. Thus, rootstocks formed the main plots, 40 plants (2 rows) were used for the other rootstocks. The planting depth was 20 cm.

Both experimental fields were grown under standard nursery practices without any phytosanitary treatment except mineral oil. Rootstocks were pruned during the winter in 2007 and 2008 and were not grafted during the experimental period.

**Mineral oil treatments**

The oil spraying treatments began when the rootstocks sprouted at the end of April and ended when the leaves fell in mid-December. The influence of the oil treatments on the phenological development of the rootstocks was evaluated during 1 year. Sunspray® Ultrafine [paraffin mineral oil 85% w/v (EC); Sun Oil Co, Philadelphia, USA] was used as a 1% (v/v) emulsion in water every 10–12 days during the first year of rootstock cultivation. Spraying occurred weekly in April, May and June of the following year; these months saw the maximum peak of winged-aphid species as determined by Moericke yellow traps (data not shown). The oil was applied by a spray gun (pressure of 10 bar and a spray angle of 40°) assisted by a pull-type sprayer with an agitation system.

**Plum pox virus spread monitoring**

The rootstocks in each experimental field were individually sampled at the beginning of the experiment to confirm the absence of PPV in the original plant material. In addition, Field 1 was sampled and tested in autumn 2006 (October), spring 2007 (May) and spring 2008 (April). In the last sampling, performed in April 2008, only non-oil-treated plant blocks were sampled. Experimental Field 2 was tested in autumn 2006 (November) and autumn 2007 (November). The GF677 plants were tested annually in the spring during the 10 years of the experiment.

Rootstock plants were checked for PPV symptoms. Plants were individually sampled by collecting four fully expanded leaves from different parts of the canopy of each individual rootstock plant. Symptomatic leaves were collected when PPV-indicating symptoms were observed.

Serological assays for PPV detection were performed by DASI-ELISA using the 5B-IVIA (Cambra et al., 1994) monoclonal antibody kit (AMR Lab, Barcelona, Spain), following the EPPO (2004) protocol for PPV detection. In addition, rootstocks from Field 1 during the three experimental years and from Field 2 during the second experimental year were analysed by spot real-time RT-PCR with the universal Taqman probe (Olmos et al., 2005) using the same extracts prepared for the enzyme-linked immunosorbent assay (ELISA) tests (Capote et al., 2009). GF677 plants were tested annually by DASI-ELISA from 1997 to 2007, and some plants were tested by spot real-time RT-PCR in 2006 and 2007.

**Serological and molecular characterisation of *Plum pox virus* isolates**

Samples from 11 PPV-positive plants selected at random from both experimental fields were analysed for PPV strain identification according to the EPPO (2004) protocol. Six samples were collected in October 2006 from Field 1 and five samples were collected from Field 2, of which two were collected in November 2006 and three were collected in November 2007. DASI-ELISA tests were performed using the following monoclonal antibodies: 4DG5 for specific detection of PPV-D, AL for specific detection of PPV-M, EA24 for specific detection of PPV-EA and AC and TUV for specific detection of PPV-C. The same plant material was analysed by real-time RT-PCR with a universal Taqman probe (Olmos et al., 2005) and MGB-specific D and M probes (Capote et al., 2006).

**Aphid species monitoring and estimation of the number of *Plum pox virus*-viruliferous aphid species**

Adult winged-aphid populations in the area and those landing on or visiting the rootstock plants in the experimental plots were monitored by Moericke yellow traps (Moericke, 1955) and by the sticky-plant method (Avinent et al., 1993; Marroquín et al., 2004) during May 2006 and 2007, which was the period when the
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aphid populations were greatest in the area (Capote et al., 2008). Insects in the Moericke yellow traps were collected every 10 days. During May 2006 and 2007, 10 rootstock plants from Field 1 and 30 from Field 2 were completely sprayed with glue (Souverode, Scotts, France) and maintained for 10 days. The complete rootstock plants were collected and processed according to Capote et al. (2008) to identify aphid species and estimate their numbers. A sample (at least 100 individuals per year and per experimental field) of Aphis spiraecola Pagenstecher caught in the field from complete sticky rootstock plants and from Moericke yellow traps was used to estimate the number of PPV-viruliferous aphids visiting the experimental nursery. Aphids were squashed individually on nylon membranes using the round bottom of an Eppendorf tube to ensure complete disruption of each aphid. RNA was extracted from the individual squashed aphid specimens using 100 μL buffer [0.1 M glycine, 0.05 M NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA)] (Osman & Rowhani, 2006) and analysed by squash real-time RT-PCR (Olmos et al., 2005).

Statistical analysis

The natural susceptibility to PPV infection of the different assayed rootstock cultivars and the effect of mineral oil treatment in both fields were analysed for each season by a generalised linear mixed model (Molenberghs & Verbeke, 2005) with block as random effect and rootstock plants and from Moericke yellow traps was used to estimate the number of PPV-viruliferous aphids visiting the experimental nursery. Aphids were squashed individually on nylon membranes using the round bottom of an Eppendorf tube to ensure complete disruption of each aphid. RNA was extracted from the individual squashed aphid specimens using 100 μL buffer [0.1 M glycine, 0.05 M NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA)] (Osman & Rowhani, 2006) and analysed by squash real-time RT-PCR (Olmos et al., 2005).

Results

Evaluation of the susceptibility of different Prunus rootstocks to natural Plum pox virus infection

PPV was not detected in the rootstock plants used to establish the experimental plots at the beginning of the experiment. Tables 1 and 2 show the natural incidence of PPV-D under high (Field 1) and low (Field 2) PPV inoculum pressures in the assayed Prunus rootstocks determined by DASI-ELISA and spot real-time RT-PCR.

Table 1 Plum pox virus (PPV) D-type natural incidence in different Prunus rootstocks determined by DASI-ELISA and spot real-time RT-PCR under high inoculum pressure (Field 1) 1

<table>
<thead>
<tr>
<th>Rootstock</th>
<th>October 2006</th>
<th>November 2006</th>
<th>April 2008</th>
<th>May 2007</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mariana GB8-1</td>
<td>24/2226</td>
<td>18/232</td>
<td>221/230</td>
<td>221/230</td>
</tr>
<tr>
<td>(0.108 ± 0.021)a</td>
<td>(0.108 ± 0.021)a</td>
<td>(0.108 ± 0.021)a</td>
<td>(0.108 ± 0.021)a</td>
<td></td>
</tr>
<tr>
<td>Adesoto 101</td>
<td>5/80</td>
<td>5/80</td>
<td>63/65</td>
<td>63/65</td>
</tr>
<tr>
<td>(0.050 ± 0.024)a</td>
<td>(0.050 ± 0.024)a</td>
<td>(0.050 ± 0.024)a</td>
<td>(0.050 ± 0.024)a</td>
<td></td>
</tr>
<tr>
<td>Nemaguard</td>
<td>13/225</td>
<td>13/225</td>
<td>123/210</td>
<td>123/210</td>
</tr>
<tr>
<td>(0.058 ± 0.016)a</td>
<td>(0.058 ± 0.016)a</td>
<td>(0.058 ± 0.016)a</td>
<td>(0.058 ± 0.016)a</td>
<td></td>
</tr>
<tr>
<td>Myrobolean 29C</td>
<td>3/82</td>
<td>3/82</td>
<td>26/80</td>
<td>26/80</td>
</tr>
<tr>
<td>(0.037 ± 0.021)a</td>
<td>(0.037 ± 0.021)a</td>
<td>(0.037 ± 0.021)a</td>
<td>(0.037 ± 0.021)a</td>
<td></td>
</tr>
<tr>
<td>Cadaman</td>
<td>0/80</td>
<td>0/80</td>
<td>0/78</td>
<td>0/78</td>
</tr>
<tr>
<td>(0.000 ± 0.000)c</td>
<td>(0.000 ± 0.000)c</td>
<td>(0.000 ± 0.000)c</td>
<td>(0.000 ± 0.000)c</td>
<td></td>
</tr>
<tr>
<td>Garnem</td>
<td>0/68</td>
<td>0/68</td>
<td>0/66</td>
<td>0/66</td>
</tr>
<tr>
<td>(0.000 ± 0.000)c</td>
<td>(0.000 ± 0.000)c</td>
<td>(0.000 ± 0.000)c</td>
<td>(0.000 ± 0.000)c</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>45/757</td>
<td>261/778</td>
<td>433/729</td>
<td>57/757</td>
</tr>
</tbody>
</table>

1Field 1 located in Llíria, Valencia, Spain in an area with approximately 90% PPV incidence.
2Number of PPV-infected plants/total analysed plants.
3Mean and their standard error (mean ± SE).
4Data in the same column followed by different letter are significantly different according to a binomial generalised linear mixed model (overall P-value < 0.05 using Bonferroni correction).
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Table 2  Natural incidence to Plum pox virus (PPV) D-type and effect of mineral oil treatments on the virus spread determined by DASI-ELISA and spot real-time RT-PCR in different Prunus rootstocks grown under low inoculum pressure (Field 2)

<table>
<thead>
<tr>
<th></th>
<th>November 2006</th>
<th></th>
<th>November 2007</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control Plants</td>
<td>Spray Oil Plants</td>
<td>Control Plants</td>
<td>Spray Oil Plants</td>
</tr>
<tr>
<td></td>
<td>DASI-ELISAb</td>
<td>Spot r-tb</td>
<td>DASI-ELISA</td>
<td>Spot r-t</td>
</tr>
<tr>
<td>Mariana GF8-1</td>
<td>2/22c</td>
<td>n.d</td>
<td>0/255</td>
<td>n.d</td>
</tr>
<tr>
<td>Nemaguard</td>
<td>0</td>
<td>174</td>
<td>n.d</td>
<td>0</td>
</tr>
<tr>
<td>Myrobolan 29C</td>
<td>0</td>
<td>225</td>
<td>n.d</td>
<td>0</td>
</tr>
<tr>
<td>Cadaman</td>
<td>0</td>
<td>261</td>
<td>n.d</td>
<td>0</td>
</tr>
<tr>
<td>Garnem</td>
<td>0</td>
<td>228</td>
<td>n.d</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>2</td>
<td>1366</td>
<td>n.d</td>
<td>0</td>
</tr>
</tbody>
</table>

aField 2 located in Carlet, Valencia, Spain in an area with approximately 5% PPV incidence.
bDASI-ELISA and spot real-time RT-PCR.
cNumber of PPV-infected plants/total analysed plants.
dNon-determined.

rootstocks were found in spring 2008 (Field 1). From the estimated proportions of infected plants and their standard errors, Mariana GF8-1 (0.960 ± 0.020; 0.982 ± 0.009) and Adesoto 101 (0.969 ± 0.025; 0.969 ± 0.022) were the most susceptible rootstocks to natural PPV infection when PPV incidence was determined by DASI-ELISA or by spot real-time RT-PCR, respectively. The high susceptibilities of these rootstocks were followed by those of Nemaguard (0.612 ± 0.103; 0.637 ± 0.052) and Myrobolan 29C (0.313 ± 0.102; 0.384 ± 0.068). Cadaman and Garnem presented the fewest PPV-infected plants, and infection was detected only by spot real-time RT-PCR. Mariana GF8-1 and Adesoto 101 had previously shown the greatest susceptibility to natural PPV infection in the May 2007 analyses. In addition, significant differences in susceptibility were found among the different assayed rootstocks (Table 1). However, no significant differences were found among the different assayed rootstocks in susceptibility to PPV after 7 months of cultivation under high PPV inoculum pressure, except between Cadaman and Garnem, and the rest of the assayed rootstocks as reported by DASI-ELISA analysis.

No differences in PPV incidence among the assayed Prunus rootstocks were found after 1.5 years of cultivation in the experimental nursery established under low inoculum pressure (Field 2; Table 2). PPV was detected only in two Adesoto 101 plants by DASI-ELISA after 6 months of cultivation. This incidence increased at the end of the experimental period in November 2007 when analysed by DASI-ELISA and spot real-time RT-PCR. At that time, PPV was detected only in Adesoto 101 and Nemaguard control plants (Table 2).

All GF677 plants tested during 10 consecutive years were negative for PPV.

Characterisation of Plum pox virus isolates

The 11 PPV isolates collected from the analysed rootstock plants from both experimental fields reacted with 5B-IVIA universal monoclonal antibody but were not recognised by the type-specific monoclonal antibodies AL, EA24, TUV and AC. Eight of the isolates reacted against monoclonal antibody 4DG5 (D-specific); three from Field 1 and five from Field 2. The 11 PPV isolates were molecularly amplified by real-time RT-PCR using the universal Taqman and D-specific MGB probes, but there was no amplification with the M-specific MGB probe.

Effect of mineral oil treatment on the incidence of Plum pox virus in nursery blocks

The effect of mineral oil treatment on the spread of PPV-D under high (Table 3) and low (Table 2) inoculum pressures is shown.

In Field 1, significant differences (P < 0.05) between treated and control Mariana GF8-1 plants were found both by DASI-ELISA and spot real-time RT-PCR analysis in May 2007 (Table 3). No significant differences were detected between treatments in the Nemaguard rootstock. The highest percentage of PPV-infected plants was always detected by spot real-time RT-PCR.

Light phytotoxicity symptoms (sunburn) were observed on the borders of some Mariana GF8-1 leaves as a result of the accumulation of oil drops. No negative effects of the mineral oil treatment were observed in Nemaguard rootstock plants.

In the field grown under low PPV inoculum pressure, no significant differences were found between control and oil-treated plants in either year (Table 2). In this plot, which is located in a classical Mediterranean temperate
of 10 321 aphid individuals were caught during May of 2007 using Moericke yellow traps and sticky plants. A total of 9333 aphid individuals landed in Moericke yellow traps, and 988 aphid individuals landed in sticky plants. Moericke yellow traps caught a total of 5191 aphid individuals in Field 1 (high PPV inoculum pressure) and a total of 4142 in Field 2 (low PPV inoculum pressure). Substantial variations were observed in the number of aphids caught in May of each year (2006–07).

Table 3 shows the number of individuals belonging to A. spiraecola was the most abundant aphid species caught during May of two consecutive years in the experimental plots. A total of 933 aphid individuals landed in Moericke yellow traps, and 988 aphid individuals landed in sticky plants. Moericke yellow traps caught a total of 5191 aphid individuals in Field 1 (high PPV inoculum pressure) and a total of 4142 in Field 2 (low PPV inoculum pressure). Substantial variations were observed in the number of aphids caught in May of each year (2006–07). A. spiraecola was the most abundant aphid species caught in both plots by both type of traps (93.21% of the total captures). The other aphid species listed in Table 4 represented a climate area, an important attack of A. spiraecola was observed in May 2007 only in the non-treated rootstock plants.

Estimation of aphid species, visitors and number of Plum pox virus-viruliferous aphids present in the plot

Table 4 shows the number of individuals belonging to each aphid species and the percentage of total captures for each species caught during the experimental period using Moericke yellow traps and sticky plants. A total of 10 321 aphid individuals were caught during May of May of 2007 and 2008 for each species. A total of 1299 aphid individuals landed in sticky plants. Moericke yellow traps, and 988 aphid individuals landed in sticky plants. Moericke yellow traps caught a total of 5191 aphid individuals in Field 1 (high PPV inoculum pressure) and a total of 4142 in Field 2 (low PPV inoculum pressure). Substantial variations were observed in the number of aphids caught in May of each year (2006–07). A. spiraecola was the most abundant aphid species caught in both plots by both type of traps (93.21% of the total captures). The other aphid species listed in Table 4 represented a climate area, an important attack of A. spiraecola was observed in May 2007 only in the non-treated rootstock plants.
small percentage of the total captures. The number of *A. spiraceola* individuals differed in both types of traps.

The number of aphids landing on or visiting a single rootstock plant in Field 1 during 10 days in May, as estimated by the sticky-plant method, was greater in 2006 than in 2007 (20.30 in 2006 and 5.10 in 2007). The opposite pattern occurred in Field 2 (1.57 in 2006 and 22.90 in 2007). The same results were obtained using the Moericke yellow trap. The total number of individual aphids caught by the Moericke yellow trap in Field 1 during the same 10 days in May 2006 and 2007 were 3946 and 463, respectively, while in Field 2 were 540 and 1890, respectively. The percentage of PPV-viruliferous *A. spiraceola* in Field 1, as estimated by squash real-time RT-PCR, was 51.85% in 2006 and 16.05% in 2007. In Field 2, the percentage of PPV-viruliferous aphids was 8.28% in 2006 and 7.69% in 2007.

**Discussion**

The PPV isolates found in the experimental nurseries were of the PPV-D type, which is reflective of the current situation in Spain (Cambra *et al*., 2006a). All analysed samples reacted with the universal Taqman and D-specific MGB probes and did not react with the M-specific MGB probe, although some of them did not react with the D-type monoclonal antibody 4DG5. This behaviour has been reported previously by Candresse *et al.* (1998). Consequently, PPV-D, which is the most common and frequent PPV isolate worldwide, presented the greatest challenge to the plants we tested under natural field conditions.

In Field 1, under high PPV inoculum pressure, the assayed commercial rootstocks exhibited significant differences in their natural susceptibilities to PPV-D infection (Table 1).

Many authors have reported plum susceptibility since the first descriptions of sharka disease (García & Cambra, 2007). Our data show that Adesoto 101 and Mariana GF8-1 plums are highly susceptible under natural conditions. These data are consistent with those of Rubio *et al.* (2005), who challenged the plants by graft inoculation with a PPV-D isolate. Our data show that Nemaguard rootstock is highly susceptible, but this rootstock is less susceptible than Mariana GF8-1 and Adesoto 101. In fact, the high susceptibility to PPV of Nemaguard and other peach seedlings is well known; these plants are recommended as indicator plants for PPV indexing (EPPO, 2004).

Under natural conditions, Myrobalan 29C is highly susceptible, with more than 30% of plants showing PPV infection in our experiment. Although it is known that *P. cerasifera* is susceptible to PPV infection (James & Thompson, 2006), different clones (BN 4Kr and Myrobalan 29C) have been reported to be resistant to PPV-D (Minoiu *et al*., 1998; Rubio *et al*., 2005).

Cadaman rootstock shows a high degree of resistance, despite sharing the same genotype (*P. persica × P. davidiana*) as Nemaguard. The resistance of *P. davidiana* to PPV has been reported previously (Pascal *et al*., 1998). Nevertheless, Boeglin *et al.* (2006) have reported Cadaman rootstock to be PPV susceptible after challenge by chip-budding inoculation with PPV-D-infected and PPV-M-infected plant materials. Under the natural conditions of our assay, PPV was detected in some Cadaman plants (Table 1) only by spot real-time RT-PCR and not by DASI-ELISA; in addition, rootstock plants did not display symptoms of infection. The discrepancies in the results between DASI-ELISA and spot real-time RT-PCR could be because of different mechanisms of natural resistance in the plant against PPV-D infection, such as the restriction of PPV infection to the inoculated area, thereby preventing systemic infection; impaired spread of the virus in the inoculated organ; restricted interorgan movement of the virus or systemic infection with a reduced virus titre (Bruening, 2006). Consequently, Cadaman can be considered to have a high level of resistance to the PPV-D isolates present in the experimental area. The different PPV susceptibility of Cadaman plants when they are graft inoculated or submitted to aphid inoculation may be explained by the different viral titres in the PPV targets inoculated by the two methods. The high PPV viral titre introduced by chip budding and the continuous supply of viral particles could cooperate to overcome the possible resistance of Cadaman rootstocks. This resistance may be effective against the lower number of viral particles introduced by aphid transmission (Moreno *et al*., 2009).

The same considerations apply to Garnem rootstock (*P. dulcis × [(P. persica × P. davidiana) × P. persica]*)). The results for this rootstock under our experimental natural challenge conditions are consistent with the findings of previous researchers, who have reported that almond germplasm may be a source of resistance to PPV-D (Rubio *et al*., 2003). Similarly, GF677 (*P. dulcis × P. persica*) adult trees were not infected after 10 years of exposure to PPV-D infection under high inoculum pressure. Similar results have been reported by Rubio *et al.* (2005) for PPV-D and by Boeglin *et al.* (2006) for PPV-D and PPV-M isolates after graft inoculation. However, Pascal *et al.* (2002) have reported contrasting results for the PPV-M isolate.

We found no significant differences in PPV susceptibility among the assayed rootstocks under low PPV inoculum pressure. Detecting such differences might require a greater number of plants when inoculum pressure is low. The different PPV inoculum pressures in the two
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Experimental fields were because of a different number of infected hosts in each area, as the numbers of aphid individuals and species visiting the plants were similar in both areas (Table 4). For epidemiological purposes, it is important to take into consideration the aphid vector species as well as the number of PPV-viruliferous aphids visiting the nursery plots. In addition, efforts should be made to interfere with the natural non-persistent manner of transmission of the virus.

Two different monitoring methods have been used in experimental nurseries of Prunus rootstocks (Moericke yellow traps and sticky plants). For both type of traps, the most abundant aphid species was A. spiraecola. This aphid species has been reported previously as the main PPV vector under Mediterranean conditions (Cambra et al., 2006a). However, the percentage of A. spiraecola caught by each method differed substantially. The proportion of A. spiraecola on the sticky plants in May of two consecutive years (55.90% in the high PPV inoculum pressure area; 56.26% in the low PPV inoculum pressure area) was lower than that in the Moericke yellow traps (97.59% and 96.54% in the low and high PPV inoculum pressure areas, respectively). These differences may be explained by the yellow colour of Moericke traps, which attracts certain aphid species such as A. spiraecola (Labonne et al., 1989; Hermoso de Mendoza et al., 1998). This condition may lead to overestimates of the frequency of this aphid species when Moericke yellow traps are used. However, this sampling method is very useful to estimate the population dynamics of aphids within a determined area. Conversely, the sticky-plant method has been successfully used to estimate the number of aphids landing on or visiting adult trees (Marroquin et al., 2004; Capote et al., 2008). This study is the first to apply this method to nursery plants.

To evaluate the role of each aphid species in spreading the virus, it is necessary to know its relative abundance in the area, its landing rate and its ability to transmit the virus. Previous studies conducted under field and laboratory conditions have attempted to establish relationships between the presence of viruliferous aphids in the field and the spread of a virus (Harrington et al., 1986; Peters et al., 1990; Marroquin et al., 2004). Non-persistent virus proliferation is mainly caused by non-colonising aphids that alight on the plant and make brief superficial probes before departing (Raccah et al., 1985; Pérez et al., 1995), allowing the virus transmission (Ng & Falk, 2006).

The average percentage of A. spiraecola individuals caught in May that were PPV viruliferous was 30.37% under high PPV inoculum pressure but only 7.98% under low PPV inoculum pressure. The large number of PPV-viruliferous aphids found under high PPV incidence was probably because of the proximity of an adult Japanese-plum orchard, where 90% of the trees were PPV-D infected. In fact, the experimental nursery was located in this area to ensure a high probability of disease transmission. Therefore, the high PPV infection rate observed for 3 years in Field 1 can be explained by the arrival of a large number of PPV-viruliferous A. spiraecola from the Japanese-plum orchard. In contrast, the small number of viruliferous aphids present in Field 2 is consistent with the low PPV infection level reached at this location and reflects the number of infected hosts in the experimental farm (approximately 5%).

In Field 1 during 2006, only approximately 10% of untreated Mariana GF8-1 rootstocks and 6% of Nemaguard rootstocks were PPV infected, although PPV-viruliferous A. spiraecola were most prevalent during that year (making up 51.85% of the aphid population). During 2007, in contrast, we observed the greatest increase in PPV infection in Mariana GF8-1 and Nemaguard PPV plants (62% and 28% of PPV-infected plants for Mariana GF8-1 and Nemaguard, respectively), despite the low incidence of PPV-viruliferous A. spiraecola (16.05% of the aphid population). One possible explanation is that in most cases, PPV can be effectively detected only one year after inoculation by PPV-viruliferous aphids.

The use of mineral oil as a physical barrier to control PPV incidence and spread in nursery plots is an interesting alternative approach. Significant differences in PPV incidence were obtained between treated and untreated Mariana GF8-1 plots after a one-year challenge under unfavourable conditions (high PPV inoculum pressure) (Table 3). These results encourage the use of the mineral oil under less-stressful natural conditions.

When the inoculum pressure increases, the effectiveness of mineral oil to control the spread of the virus is reduced (Simons & Zitter, 1980; Umesh et al., 1995). Consequently, the high inoculum pressure present in one of the experimental nursery plots may explain the failure to control PPV spread under the assayed conditions. Simon & Zitter (1980) have reported that under ecological conditions present in Florida (USA), mineral oil is ineffective against non-persistent viruses in horticultural crops when the level of infection is between 10% and 20%. Before installing a Prunus nursery, it is critical to verify the absence of PPV-infected hosts in the vicinity of the nursery. Therefore, the combination of low PPV inoculum pressure (separation from PPV foci) and the use of mineral oil treatments could help to reduce the incidence and spread of sharka in the nursery industry.

PPV spread was contained during the first year of cultivation in Field 2 by mineral oil application because PPV infections occurred only in the non-oil-treated block (Table 2). Nevertheless, the lack of significant differences in PPV incidence between control and oil-sprayed
treatments may be because of the small number of PPV-infected plants. The infected sprayed plants in November 2007 were located very close to the two plants that were previously infected in November 2006 (adjacent rows). This phenomenon can be explained by the close proximity of the control and sprayed plots (Simons & Zitter, 1980; Webb & Linda, 1993). The oil-treated plots were constantly exposed to aphids that could have acquired the virus in adjacent untreated plants (Webb & Linda, 1993).

The canopy structure of the plants also modulated the effectiveness of the mineral oil treatments (Vanderveken, 1977; Simons, 1982). Under our experimental conditions, the mineral oil persisted longer in Mariana GF8-1 than in Nemaguard plants in Field 1. This observation may explain the differences in the effect of the mineral oil between the assayed rootstocks. Leaf orientation, foliar characteristics, such as leaf surface, size and shape, and twig flexibility can also influence the uniformity of spray deposition (Furness & Combellack, 2002). Asjes & Blom-Barnhoorn (2002) have reported a difference between two varieties of Lilium in the effect of mineral oil against Lily symptomless virus and Lily mottle virus because of canopy structure. Therefore, the characteristics of the sprayed plants are fundamental to achieve an adequate protection with mineral oil. The high plant densities in a stone fruit nursery block make it difficult to apply mineral oil treatments in an uniform manner. For this reason, it is crucial to implement spray systems that cover the entire surface of the plant; the type of sprayer and the set-up parameters used to determine the film coverage of the mineral oil should be taken into account (Furness & Combellack, 2002; Chueca et al., 2009). A wide variety of technologies are available for mineral oil application (Furness & Combellack, 2002), and these technologies should be assayed in stone fruit nurseries to improve the efficiency of the treatment.

Mineral oil treatment should also be evaluated under different climatic conditions and PPV inoculum pressures, using different rootstock genotypes and challenging the plants with different PPV strains, before it is applied on a larger scale. These very diverse experimental and ecological conditions are currently being tested in the framework of the 7FP European project SharCo-204429 (http://www.sharco.eu).

In conclusion, the combined use of resistant or less-susceptible PPV rootstocks, such as Cadaman, Garmem and GF677, together with environmentally friendly treatments with zero residues, such as mineral oil, could constitute an integrated strategy to reduce PPV spread in the nursery industry. Combined with accurate detection methods, our findings will play an important role in PPV control in nurseries, which are the most frequent sources of long-distance distributions of infected material worldwide. These strategies could be also applied to prevent/reduce the PPV spread in new orchards.

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