Epidemiology of *Plum pox virus* in Japanese Plums in Spain

M. Cambra, M.T. Gorris, N. Capote, M. Asensio, M.C. Martínez, E. Bertolini, C. Collado and A. Hermoso de Mendoza
Instituto Valenciano de Investigaciones Agrarias (IVIA). Moncada, Valencia. Spain

E. Mataix
Estación Experimental Agraria de Llutxent. Luchente, Valencia. Spain

A. López
Estadística e Investigación Operativa. Universidad de Valencia. Burjassot, Valencia. Spain

Keywords: PPV, *Prunus salicina*, viruliferous aphids, sticky shoots, sensitivity, interference, characterisation.

Abstract

The Japanese plum (*Prunus salicina*) industry is economically important in Spain and in other countries with Mediterranean climate. *P. salicina* was described as a natural host of *Plum pox virus* (PPV) in Spain in 1984, where the 'Red Beaut' cultivar become an important source of inoculum and it spread the virus to apricots and plums along the Spanish Mediterranean coast. The spatial and temporal spread of PPV was monitored along a twelve year period in a collection of 41 Japanese plum cultivars, planted in Luchente (Valencia) in 1990. PPV incidence in 1991 was 11% and reached 95% after 13 years. The spread of the virus followed a logistic model without aggregation of the new infected trees around the previously infected ones. In May of 1992, 2002 and 2003 the numbers of aphid species landing on mature Japanese plum trees were estimated by counting the number of shoots (average of different cultivars: 752) and aphids trapped on "sticky shoots". The proportions of the different aphid species captured were: *Aphis spiraecola* (43%), *A. gossypii* (18%), *Hyalopterus pruni* (6%), *Brachycaudus prunicola* (6%), *A. craccivora* (3%) and *Myzus persicae* (2%), and other species (22%). Vigorous Japanese plum cultivars were the most visited with 5,606 aphids landing in May/tree. An average of 667 PPV-viruliferous aphids visited each vigorous Japanese plum tree in May. The percentage of detection of viral RNA in the aphid species that landed was 11.9%. This high incidence of viruliferous aphids is consistent with the high incidence and rapid spread of PPV in Japanese plums in the region. A complete serological and molecular characterisation of the PPV isolates spreading in *P. salicina* in Spain showed that only PPV-D was present. Seven different serogroups and variability in the nucleotide sequence of the NIb and CP genes were found among 21 PPV isolates studied. Pre-inoculation of trees with a typical PPV-D isolate did not cross-protect Japanese plums against the infection with PPV-M inoculated by grafting. The sensitivity to PPV-D of 33 Japanese plum cultivars was evaluated. Unmarketable fruits from infected trees reached as maximum as 15%, making possible economic profit in heavily infected plantations.

INTRODUCTION

The Japanese plum (*Prunus salicina*) industry is economically important in Spain (where approximately 42,000 ha. are grown) and in other countries around the

Mediterranean Basin, as well as in areas with temperate climate as America (USA-California and Chile), South-Africa and Australia. *P. salicina* was described as a natural host of *Plum pox virus* (PPV) in Spain in 1984 (Llácer et al., 1986). PPV-infected 'Red Beaut' cultivar, in an important nursery in Sevilla, was distributed along the Spanish Mediterranean coast, due to the high economical value (0.6 Euros/kg) of the production at this time. This cultivar became an important source of inoculum and the aphids spread the virus to apricots and plums with a high efficiency.

The aim of this work was to study the temporal and spatial spread of PPV-D, the only type currently present and spreading in Spain, in *P. salicina*, as well as to evaluate the sensitivity of different Japanese plum cultivars to PPV by the expression of symptoms in leaves and fruits. The total number of aphids carrying RNA amplified-PPV targets visiting adult Japanese plums was estimated, and the PPV isolates currently spreading in *P. salicina* in the area of Valencia were characterised. The putative protection of pre-inoculed Japanese plums with PPV-D isolates against post-graft inoculation with PPV-M was also analysed.

MATERIAL AND METHODS

Experimental Plot and Temporal and Spatial Spread of PPV

A collection of 182 trees of 41 Japanese plum cultivars grafted on *Prunus marianna* rootstock was planted in 1990 in Luchente, Valencia (Spain). The spatial and temporal spread of PPV was annually monitored from 1991 to 2003. Trees were individually and carefully checked twice along the year (Spring time and beginning of Summer) for the presence of symptoms on leaves and fruits. Five shoots or ten fully expended leaves were collected around the canopy of each individual tree from the middle of each scaffold branch, until the outcome of high temperatures at the beginning of the Summer. Samples were analysed by a DASI-ELISA kit (Durviz, Spain) based on the universal and PPV-specific monoclonal antibody 5B-IVIA (Cambra et al., 1994). The PPV incidence was evaluated and spatial-temporal analysis of the PPV evolution was performed by means of analysis of aggregation and logistic regression, respectively.

Aphid Monitoring

Aphids were monitored in May 1999, 2002 and 2003 to establish the percentage of species visiting *P. salicina* in the experimental plot in Luchente. Aphid occurrence was assessed by the sticky shoot method (Avinent at al., 1993; Cambra et al., 2000). The method used five shoots/trees selected in the upper part of the canopy of three different cultivars (vigorous, medium vigour and compact) using three trees/cultivar (45 shoots in total). The shoots were sprayed with an adhesive (Souverode aerosol), detached after a week and new sticky shoots were prepared. The aphids (winged adults) captured were identified and counted.

Estimation of the Number of Landing Aphids Carrying PPV

Identified winged adult aphids were counted to establish the total numbers of aphids and the species visiting individual shoots of each Japanese plum representative of vigorous ('Sun Gold'), medium vigour ('Fortune') and erected or compact ('Friar') cultivars. The number of shoots or twigs for each cultivar of *P. salicina* was estimated in Winter, at pruning time, by counting the number of twigs (shoots with both flower and vegetative buds) per tree and the number of shoots with vegetative buds more than 10 cm long. More than 50% of the captured and identified aphid species were squashed

individually on Whatman 3MM paper, stored at 4ºC until use and RNA extracted from the individual squashed aphids by Triton X-100 (Olmos et al., 1996 and 1997) and analysed by nested RT-PCR in a single close tube to detect PPV targets (Olmos et al., 2003). The numbers of aphids from which a positive amplification of the PPV RNA was obtained was calculated from each *P. salicina* cultivar type. Total numbers of aphids that landed on a single tree was estimated by multiplying the number of captured aphids/shoot by the number of shoots/tree according to Marroquín et al. (2004).

Serological and Molecular Characterisation of PPV Isolates

A complete characterization of twenty one selected PPV isolates spreading in *P. salicina* was performed by typing with seventeen monoclonal antibodies from different origins by DASI-ELISA (Cambra et al., 1994) and by Immunocapture RT-PCR (Wetzel et al., 1991, 1992; Olmos et al., 1997, 2002). In addition, the nucleotide sequence of the 3'NIb-5'CP fragment corresponding to the most hypervariable region of the *Potyvirus* genome was determined for eight PPV isolates. Total RNA was purified using a RNeasy Kit (Qiagen). The 36-172 pair of primers (36: 5'-GAGGCAATTTGTGCWTC AATGG-3' and 172: 5'-TGCAGGACTGTAATGTGCCAA-3') was used for RT-PCR amplification.

Sensitivity to PPV of Different Japanese Plum Cultivars

The sensitivity to PPV-D types spreading in the experimental plot was evaluated in 33 Japanese plum cultivars. Symptoms on leaves were classified into three groups: obvious symptoms, slight symptoms and very mild or no symptoms. Symptoms evaluation on leaves and fruits was performed by three people that surveyed the infected trees of the collection during the last ten years. Estimation of the percentage of unmarketable fruits from infected trees was also assessed.

Interference Assays between PPV-D and M Isolates

Fourteen Japanese plum trees of 'Sun Gold' and 'Black Diamond' cultivars grown in quarantine facilities at IVIA were graft-inoculated with a PPV-D (3.3 RB/GF) isolate and another 15 with a PPV-M (MS Mp11) isolate, in May 1997. One year later, the infection was confirmed by DASI-ELISA using D and M specific monoclonal antibodies (Cambra et al., 1994; Boscia et al., 1997) and IC RT-PCR using D and M specific primers (Olmos et al., 1997). Some of the pre-inoculated trees were graft-inoculated again. Eight out of 14 previously PPV-D inoculated trees were now inoculated with the PPV-M isolate, and 7 out of 15 trees previously inoculated with PPV-M were now inoculated with the PPV-D isolate. The rest of the trees remained as inoculated controls with the original PPV isolate. Five years after this challenge experiment the trees were analysed as previously to determine the presence of mixed infection or the prevalence of one of the isolates, the original or the post inoculated one.

RESULT AND DISCUSSION

The PPV incidence ranged from 11% of the total trees (182) in 1991 to 96% in 2003. The spread of PPV followed a logistic model without aggregation of the new infected trees around the previously infected ones (Fig. 1).

Table 1 shows the relative percentage of different aphid species captured on Japanese plum trees by the sticky shoot method in the Luchente experimental plot in May 1999, 2002 and 2003. A total of 619 individual aphids was captured. *Aphis spiraecola* and *A. gossypii* were the most abundant species visiting *P. salicina* during the period studied, in agreement with previously reported data in citrus crops in the area

(Hermoso de Mendoza et al., 1997) and in other *Prunus* species (Avinent et al., 1993, 1994). The presence of *Myzus persicae*, the main PPV vector in non-Mediterranean areas, was insignificant (1.5% of the total captured aphids). The percentage of detection of viral RNA in the most abundant vector species ranged from 12.3% for *A. spiraecola* to 7.9% for *A. gossypii*. An average of 11.9% (43 out of 360 analysed individuals) of the aphid species that visited Japanese plums in Valencia resulted PPV-viruliferous in the period studied. The mean numbers of shoots per tree of different *P. salicina* cultivars were: 1,224 for vigorous cultivars, 711 for medium vigour cultivars and 321 for erect or compact cultivars. The average of individual aphids captured per shoot was 4.58. Consequently, the estimated number of aphids visiting vigorous, medium vigour or compact cultivars of adult trees were: 5,606, 3,256 and 1,470, respectively. An average of 3,444 aphids visited any adult *P. salicina* tree in Valencia in May from which 11.9% carried RNA amplifiable-PPV targets (average of 410 individual aphids per tree). This results explains the high incidence and rapid spread of PPV in Japanese plums in the studied area.

Table 2 shows the sensitivity of 22 different Japanese plum cultivars to PPV-D symptoms expression on leaves. PPV-D symptoms on leaves are not correlated with those on fruits. Most *P. salicina* cultivars showed no symptoms or slight ones affecting only the skin. The earliest cultivars usually showed more aggressive symptoms on fruits. Unmarketable fruits from infected trees reached a maximum of 15% in cold Spring years in the 'Red Beaut' cultivar (one of the most sensitive to PPV), making feasible economic profit in heavily infected plantations.

All typed PPV isolates were PPV-D. Atypical D types (negative reaction against D-specific monoclonal antibodies but positive by RT-PCR using D-specific primers, and confirmed by RFLP) were found as have previously described by Candresse et al. (1998). Seven patterns of reaction against 17 monoclonal antibodies were established. The partial nucleotide sequence of the NIb and CP genes was determined for 8 of these PPV-D isolates (data not shown) showing a relatively high variability among the PPV isolates from *P. salicina* in agreement with the serological diversity detected.

Assays to study the interference between PPV-D (the only type currently present in Spain and widely spread in *P. salicina*) and PPV-M (with a high risk of introduction from neighbouring countries) were performed to evaluate if the previous infection of a tree with a PPV-D population could protect the tree against the infection with a PPV-M isolate. No cross protection was assessed. After a five years trial period trees infected firstly with PPV-D and then with PPV-M showed a predominance of PPV-M populations. In 2 out of 8 trees PPV-D was the only type detected, in 3 out 8 trees a mixed infection was assessed, and in 3 out of 8 trees PPV-M was only detected. By the other hand, only in 1 out of 7 trees firstly infected with PPV-M and then challenged with PPV-D, PPV-D was the only type detected. Serological typing and detection was confirmed by molecular methods in all cases. This unsuccessful cross-protection assay performed by graft-challenging would need to be evaluated under natural conditions (aphid transmission). Analysis of recombination and heteroencapsidation will be performed in a next future.

PPV-D is not endangering *P. salicina* industry but due to its relative high incidence in the Mediterranean area of Spain, this host is playing an important role as PPV reservoir. The high efficiency of *A. spiraecola* and other aphid species in transmitting PPV-D and the abundance of individuals of this species visiting *P. salicina* trees, explains the rapid spread of PPV in *Prunus* species in Spain.

ACKNOWLEDGMENTS

This work was financed by grants from INIA projects SC98-060 and RTA03-099 and IVIA project 5901. M. Asensio was recipient of a PhD fellowship from IVIA.

Literature Cited

Avinent L., Hermoso de Mendoza A. and Llácer G. 1993. Comparison of sampling methods to evaluate aphid populations (Homoptera, Aphidinea) alighting on apricot trees. Agronomie 13: 609-613.

Avinent L., Hermoso de Mendoza A. and Llácer G. 1994. Transmission of Plum pox potyvirus in Spain. Bulletin OEPP/EPPO Bul. 24: 669-674.

Boscia D., Zeramdini H., Cambra M., Potere O., Gorris M.T. and Myrta A. 1997. Production and characterization of a monoclonal antibody specific to the M serotype of plum pox potyvirus. European Journal of Plant Pathology 103: 447-480.

Cambra M., Asensio, M., Gorris M.T., Pérez E., Camarasa E., García J.A., López-Moya J.J. López Abella D., Vela C. and Sanz A. 1994. Detection of plum pox potyvirus using monoclonal antibodies to structural and non structural proteins. Bulletin OEPP/EPPO Bul. 24:569–577.

Cambra M., Gorris M.T., Marroquín C., Román M.P., Olmos A., Martínez M.C., Hermoso de Mendoza A., López A. and Navarro L. 2000. Incidence and epidemiology of *Citrus tristeza virus* in the Valencia Community of Spain. Virus Research 71:85-95.

Candresse T., Cambra M., Dallot S., Lanneau M., Asensio M., Gorris M.T., Revers F., Macquaire G., Olmos A., Boscia D., Quiot J.B. and Dunez J., 1998. Comparison of monoclonal antibodies and polymerase chain reaction assays for the typing of isolates belonging to the D and M serotypes of plum pox potyvirus. Phytopathology 88, 198–204.

Llácer G. and Cambra, M. 1986. Occurrence of plum pox virus in Spain in a new natural host: *Prunus salicina* Lindl. (Japanese plum). Plant Disease, 70 (2). Disease note.

Marroquin C., Olmos A., Gorris M.T., Bertolini E**.**, Martínez M.C., Carbonell E., Hermoso De Mendoza A. and Cambra M. 2004. Estimation of the number of aphids carrying *Citrus tristeza virus*, that visit adult citrus trees. Virus Research 100:101-108.

Olmos A., Dasí M.A., Candresse T. and Cambra M. 1996. Print-capture PCR: A simple and highly sensitive method for the detection of plum pox virus (PPV) in plant tissues. Nucleic Acid res. 24:2192-2193.

Olmos A., Cambra M., Dasi M.A., Candresse T., Esteban O., Gorris M.T. and Asensio M. 1997. Simultaneous detection and typing of plum pox potyvirus (PPV) isolates by Heminested-PCR and PCR-ELISA. Journal of Virological Methods 68: 127-137.

Olmos A., Bertoloni E. and Cambra M. 2002. Simultaneous and co-operational amplification (Co-PCR): a new concept for detection of plant viruses. Journal of Virological Methods 106:51-59.

Olmos A., Esteban O., Bertolini E., Cambra M. 2003. Nested RT-PCR in a single closed tube. 153-161. In: Methods in Molecular Biology vol. 226. PCR Protocols. Second Edition. Bartlett, J.M.S., Stirling, D. (Eds.). Humana Press.Totowa, USA. 500 pp.

Wetzel T., Candresse T., Ravelonandro M. and Dunez J. 1991. A polymerase chain reaction assay adapted to plum pox potyvirus detection. J. Virol. Methods 33: 355-365.

Wetzel T., Candresse T., Macquaire G., Ravelonandro M. and Dunez J. 1992. A high sensitive immunocapture polymerase chain reaction method for plum pox virus detection. J. Virol. Methods 39: 27-37.

Figures

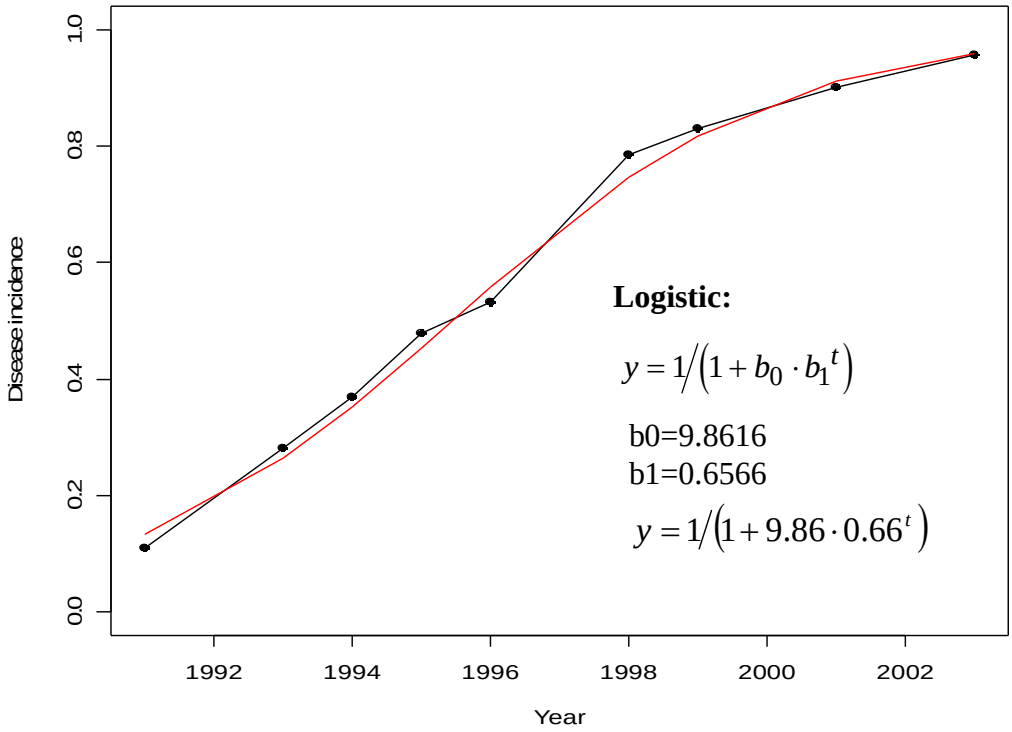

Fig 1: Temporal plum pox disease incidence on a collection of 182 Japanese plum trees during a thirteen years period (1991-2003) in Luchente, Valencia (Spain). Black line: observed results; red line: predicted incidence of *Plum pox virus.*

Tables

Table 1: Relative percentages of different aphid and PPV-viruliferous aphid species captured on Japanese plum trees by the sticky shoot method in Luchente, Valencia (Spain) in May 1999, 2002 and 2003

Aphid species	Relative percentage of captured species (%)[1]	Percentage of viruliferous aphids (%)[2]
Aphis spiraecola	43.4	12.3
Aphis gossypii	18.0	7.9
Hyalopterus pruni	6.0	
Brachycaudus prunicola	6.0	
Aphis craccivora	3.0	12.6
Myzus persicae	1.5	
Other species	22.1	

[1]Relative percentage of a total of 619 individuals captured

[2]Percentage of a total of 360 individuals analysed from which RNA amplifiable–PPV targets were amplified by nested RT-PCR in a single closed tube

Table 2: Sensitivity to PPV-D of thirty three different Japanese plum cultivars evaluated by symptom expression on mature leaves

Obvious symptoms	Slight symptoms	Very mild or no symptoms
Red Beaut	Black Amber	Carolina Harris
606	Delbarazur (Strival)	Catalina
Ambra	Friar	Beauty (Nueva Extremadura)
Black Beaut	Freedom (Larry Ann)	Queen Rosa
Durado	Ozark Premier	Midnight Sun
Santa Rosa	Wickson	Laroda
Sierra Plum	Formosa	Sun Gold
Golden Japan	Simka	Superior Angeleno
Fortune		Superior Black Gold
Autum Giant		Trompellot 1 and 2
		Susy
		Superior Black Diamond
		Royal Diamond
		Pioneer