

EVALUATION OF *PLUM POX VIRUS* INFECTION ON DIFFERENT STONE FRUIT TREE VARIETIES

C. Poggi Pollini¹, L. Bianchi¹, A.R. Babini², V. Vicchi², A. Liverani³, F. Brandi³,
L. Giunchedi¹, C. Rubies Autonell¹ and C. Ratti¹

¹ Dipartimento di Scienze e Tecnologie Agroambientali, sezione di Patologia Vegetale, Università degli Studi,
Via G. Fanin 42, 40127 Bologna, Italy

² Servizio Fitosanitario Regionale, Regione Emilia-Romagna, Via di Saliceto 81, 40128 Bologna, Italy

³ CRA, Centro di Ricerca per la Frutticoltura, sezione di Forlì, Via La Canapona, 1/1, Magliano, 47100 Forlì, Italy

SUMMARY

Fifty-nine peach, 19 apricot and 19 plum varieties, grown in a greenhouse after inoculation by chip budding with the M strain of *Plum pox virus* (PPV), were surveyed from 2003 through 2007 for symptom expression on the leaves, flowers and fruits. A large number of accessions of the three species showed discoloration and mottling of the leaves in spring, and many peach varieties with pink flowers displayed strong colour breaking of the petals. Some peach and apricot and a single plum cultivar produced deformed fruit with typical rings and mottling. Eleven out of 97 inoculated cultivars, showed no apparent symptoms and gave negative responses in ELISA and Real time PCR assays. These plants were re-inoculated with the same PPV isolate and will be the object of further observations.

Key words: Stone fruits, Sharka, Real-Time RT-PCR, PPV-M, ELISA.

INTRODUCTION

Sharka, caused by *Plum pox virus* (PPV), is the most dangerous disease of stone fruit trees, reducing fruit quality and yield. PPV is easily transmitted by aphids and by graft-inoculation, so, despite the considerable efforts made in many countries, Sharka has been reported in all the most important *Prunus* cultivation areas.

There is no cure nor treatment against Sharka and the control of aphid vectors is ineffective (Kegler *et al.*, 1998). The control of PPV is essentially based on the early identification and elimination of the infected trees in the field, and on the use of resistant germplasm (Giunchedi *et al.*, 2007).

The establishment and selection of PPV resistant germplasm have been for a long time the objective of different research projects based on classical breeding

(Vilanova *et al.*, 2003) or on the use of genetically modified plants (GMP) (Malinowski *et al.*, 2006). Different studies have reported that resistance to PPV is strongly connected with the plant genome, the interactions between host and virus, i.e. its concentration and distribution in the host (Kegler *et al.*, 1998) but, at the moment, ethical-legislative limitations to the use of GMP in the European Union, underline the necessity to identify naturally resistant germplasm to Sharka.

In Italy, PPV foci have been found in many stone fruit-growing areas (Giunchedi, 2003). Thus, the use of resistant cultivars represents the only chance to maintain stone-fruit cultivation in these areas. This paper reports the results of 4 years of observations, in a case-control study, the evaluation of new and traditional cultivars on the development of the future germplasm resistant to PPV infection.

MATERIALS AND METHODS

Plant materials. The trial was set up at the experimental station of Martorano V in Cesena (Po Valley, lat. 44°2' lat N, 34 m a.s.l., Italy). At the end of winter 2003/04, 59 peach cultivars grafted on G.F. 677, 19 apricot and 19 plum (European and Japanese) cultivars grafted on myrobalan 29C (Fig. 1A), coming from commercial nursery, were placed in a 300 m² aphid-proof greenhouse. Each genotype line was represented by four potted trees, three of which were inoculated in spring 2004 by grafting buds excised from peach trees infected with an isolate of strain PPV-M whereas the fourth tree was the uninoculated control plant. Symptomless trees were re-inoculated in spring 2005, 2006 and 2007.

All trees were continuously surveyed from 2004 to 2007 and classified for symptoms expression on the leaves, flowers and fruits. In the spring of each year leaf samples were collected from inoculated plants and used for serological (DAS-ELISA) and molecular (Real-Time RT-PCR) analyses.

To evaluate the effects of infection on tree growth, the size of the trunk of each tree was measured annually. Symptomatic fruits and fruits from control trees were

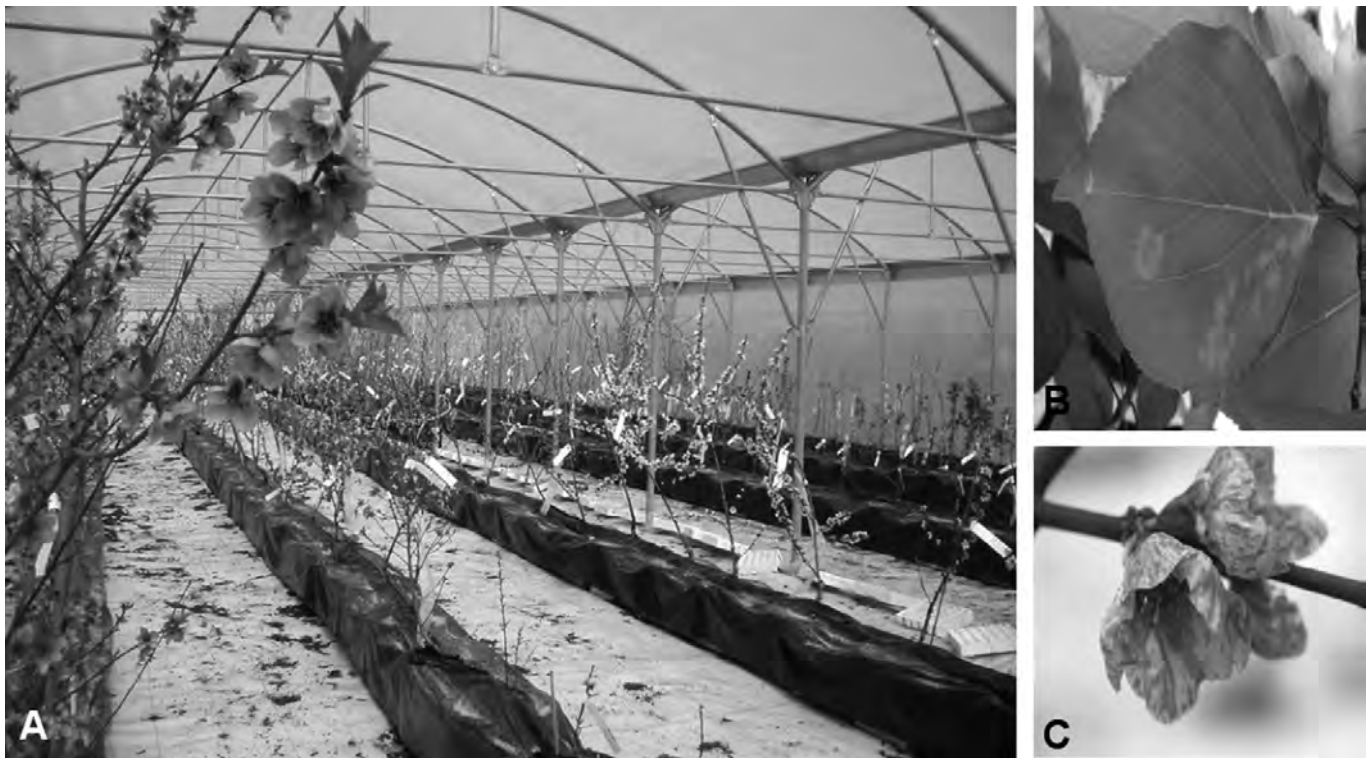


Fig. 1. A. Plants growing in a screenhouse after PPV inoculation. B. Chlorotic mottling on a young apricot leaf. C. Conspicuous colour break of peach petals.

sampled and their weight, soluble solid content and titratable acidity was analysed. In early summer 2006, leaves were sampled from each tree to measure their surface area, fresh and dry weight.

DAS-ELISA. To confirm virus infection in symptomatic and symptomless samples, specific polyclonal antisera and monoclonal antibodies to PPV, *Prunus necrotic ringspot virus* (PNRSV), *Prune dwarf virus* (PDV) and *Apple chlorotic leaf spot virus* (ACLSV) conjugated with alkaline phosphatase (AP) (Bioreba, Switzerland) were used in DAS-ELISA (Clark and Adams, 1977) tests according to the manufacturer's instructions. Polyclonal antisera diluted 1:1000 with coating buffer, were used to coat 96-well polystyrene Costar plates (Corning Incorporated, USA). Samples were homogenised 1:20 (w:v) in extraction buffer and incubated at 4°C overnight before adding AP-conjugated IgGs diluted 1:1000 in conjugate buffer. After adding AP, absorbance at 405 nm was evaluated with an Opsy MR Microplate Reader (Dynex Technologies, USA).

RNA extraction and Real-Time RT-PCR. A CTAB RNA extraction method (Chang *et al.*, 1993) was modified as previously described (Ratti *et al.*, 2004) to extract total nucleic acid. Real-Time RT-PCR analyses were performed using primers PPV-P1 (5' ACCGA-GACCACTACTACTCCC 3') (Wetzel *et al.*, 1991), PPV-

RR (5' CTCTTCTTGTGTTCCGACGTTTC 3') and PPV-U (5' TGAAGGCAGCAGCATTGAGA 3') suitable for universal detection of all PPV isolates (Varga and James, 2005). RT reaction was at 42°C for 1h in a final volume of 5 µl containing 1 µl nucleic acid extract, 1mM deoxynucleoside triphosphates (dNTPs), 50 units reverse transcriptase (M-MLV Promega, USA) and 1.5 µM PPV-P1 reverse primer. Real-Time PCR reaction was made in a final volume of 25 µl using Power SYBR® Green PCR Master Mix (Applied Biosystems, USA), 1µl of 1:5 diluted cDNA, 300 nM of primers PPV-RR and PPV-U. Tubes were cycled at generic system conditions (50°C for 2 min followed by 95°C for 10 min and 40 cycles of 60°C for 1 min plus 95°C for 15 sec) using a 7000 Sequence Detection System (Applied Biosystems, USA) with real-time data collection and dissociation analysis.

RESULTS AND DISCUSSION

Symptom evaluation. Many inoculated trees showed symptoms in the leaves in spring. In a large percentage (60%) of peach varieties, leaf symptoms were very conspicuous on the first leaves of young shoots, which showed mild light green discoloration, chlorotic spots, bands or rings, vein clearing and yellowing or leaf deformation. Severe leaf symptoms, such as pale green

blotches, spots, rings and vein banding appeared also in apricot and plum cultivars. Furthermore, almost all peach varieties with pink flowers (72%) showed discoloration of the petals consisting of mild to severe colour breaking (Fig. 1B and 1C).

After three PPV inoculations, 21 peach, 4 apricot and 3 plum cultivars showed only very slight symptoms consisting of a very mild discoloration of the leaves or flowers, appearing on a few branches for a short time. Moreover, 11 varieties, i.e. Morsiani 90, Summer Lady, Maria Dolce (peaches); Bora, Pieve, Aurora, Pisana, Sungiant, Orange Red, Harval (apricots) and Liablù (European plum), remained symptomless even though inoculated apricots and plums pushed suckers with PPV typical symptoms (Table 1).

Fruit deformations accompanied by typical rings and mottling, appeared on 15% of the inoculated varieties, especially peach and apricot (July Flame, Maria Bianca, Maria Delizia, Springcrest, Kioto, Silvercot, Autumn Giant).

Inoculation did not affect tree growth or leaf size or leaf dry weight/leaf size. Fruit quality was also negligibly influenced by the infection. Of the variables measured, only the soluble solid content appeared to be slightly higher in fruits from control trees compared with the symptomatic fruits of the same genotype.

Serological and molecular analyses. All tests successfully and specifically identified viruses on positive controls, no reactions were observed on healthy samples

Table 1. Reaction of different stone fruit species to inoculation with PPV and their response to serological and molecular assays.

	Severe symptoms, positive response	Light symptoms, positive response	Very light symptoms, positive response	No symptoms, negative response
Peach	Suncrest		Maeba Top	
	Rome Star		Western Red	
	July Flame		Tendresse	
	Orion		Nectaross	
	Fayette	Amiga	Guerriera	
	Tardibelle	Rita Star	Bella di Cesena	
	Red Moon	Venus	Pesca Carota	
	Maycrest	Sanguinella	Redhaven	
	Benedicte	Maria Camilla	Red Star	
	Kaweah	Big Top	Neve	Morsiani 90
	Silver Star	Buco Incavato	Diamond Bright	Maria Dolce
	Jade	Alexa	Sweet Red	Summer Lady
	Crimson Lady	Velvet Sister 1- 19C 16	Percoca di Romagna	
	Springcrest	Guglielmina	Max	
	Maria Delizia	Alix Carson	Rose Diamond	
	Max 7	Andross	Maria Marta	
	Stark Red gold	California	Spring Bright	
	Maria Bianca		Royal Glory	
	Rich May		Rich Lady	
	Amber Sister D93 1/6		Ruby Rich	
	August Flame		Maria Anna	
Apricot	Silvercot			Bora
	Vitillo	Kioto	Marietta	Pisana
	Bergeron	Pallummella	Bella d'Imola	Harval
	Piera	Solaria	Robadà	Sungiant
	Portici		Pinkcot	Orange Red
Plum	Dofi Sandra	Stanley		Aurora
	Helena	Black Sunrise		Pieve
	Fortune	Black Gold		
	Black Glow	Friar	K 801 - 55CC	
	Aphrodite	Black Top	Jojo	Liablù
	President	Firenze 90	Angeleno	
	Anne Gold	Autumn Giant		
Black Amber				

Table 2. Intensity of ELISA reaction to different viruses of stone fruit trees varieties inoculated with PPV, showing very mild PPV symptoms and found PPV-positive by ELISA and Real-Time PCR.

	Variety	Intensity of ELISA reaction				Real Time RT-PCR
		PPV	PNRSV	PDV	ACLSV	
Peach	Maeba Top	+	+++		+++	++
	Western Red		+++			++
	Tendresse	+	+++		+++	++
	Nectaross	+			+++	++
	Guerriera	+	+++			++
	Bella di Cesena	+				++
	Pesca Carota	+				++
	Redhaven	+				++
	Red Star	+				++
	Neve	+				++
	Diamond Bright	+	+++			++
	Sweet Red	+			+++	++
	Percoca di Romagna	+				++
	Max	+				++
	Rose Diamond	+				++
	Maria Marta	+				++
	Spring Bright	+	+++		+++	+
	Royal Glory	+	+++			+
	Rich Lady	+				+
	Ruby Rich	+				+
Maria Anna	+	+++	+++		++	
Apricot	Marietta	+				++
	Bella d'Imola	+				++
	Robadà	+			+++	++
	Pinkcot	+				++
Plum	K 801-55CC	+				++
	Jojo	+				++
	Angeleno	+			+++	+

Intensity of reaction: + = weak; ++ = moderate; +++ = strong.

and no cross reactions were reported against other viruses. Samples from plants with severe and mild symptoms gave strong DAS-ELISA positive reactions, whereas 28 cultivars with very mild symptoms had a very low relative PPV concentration. In addition, 35% of these plants reacted in ELISA to other stone fruit viruses, mainly PNRSV and ACLSV.

Results of Real Time RT-PCR tests allowed to discriminate infected samples, with C_T values ranging between 13 and 30, and negative samples the C_T value of which remained undetermined (Fig. 2a). Moreover dissociation analyses confirmed test specificity showing the presence of no more than one amplicon from positive samples, with $T_m = 75.1^\circ\text{C}$, and absence of amplicons from negative samples (Fig. 2b). All samples showing

very mild symptoms, gave positive results when analysed by Real Time RT-PCR. One of these was the plum variety Jojo, which showed first symptoms on the rootstock and scion and, following re-inoculation, exhibited necrosis of the young leaves, which were also positive in Real-Time RT-PCR (Table 2). Neither ELISA nor Real time PCR was able to detect PPV in samples from symptomless plants of 3 peach, 7 apricot and 1 plum cultivars, but symptomatic suckers developed from some apricots and plum plants gave positive reactions (Table 1).

The results obtained in the course of this study showed that the commercial stone fruit cultivars tested had different level of susceptibility to PPV, which allowed the identification of a few peach and apricot vari-

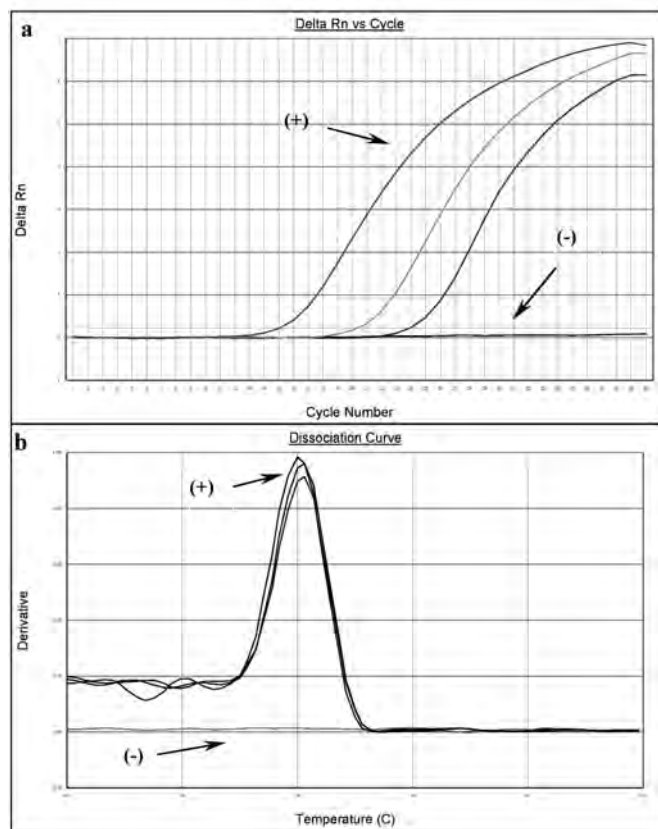


Fig. 2. Examples of Real Time RT-PCR amplification (a) and dissociation (b) curves of positive (+) and negative (-) samples.

eties and, at least, one plum variety resistant to PPV inoculation by grafting. In order to investigate if these genotypes retained their resistance to PPV in the open field, a new study will be conducted, in which this material will be grown in experimental fields located in areas with high PPV pressure, where there is active virus spread by aphids. Concerning the very mild symptoms shown by some varieties that were infected by ACLSV and/or PNRSV prior to PPV inoculation, additional studies will be conducted to investigate whether this may be consequent to a possible interference between PPV and the co-infecting viruses.

ACKNOWLEDGEMENTS

This study was supported by Emilia Romagna Region grant L.R. 28/98-CRPV.

REFERENCES

- Chang S., Puryear J., Cairney J., 1993. A simple and efficient method for isolating RNA from pine trees. *Plant Molecular Biology Reporter* **11**, 113-116.
- Clark M.F. and A.N. Adams, 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for detection of plant viruses. *Journal of General Virology* **34**: 475-483.
- Giunchedi L., 2003. Malattie da Virus, Viroidi e Fitoplasmi degli Alberi da Frutto. Il Sole 24 Ore-Edagricole, Bologna, Italy.
- Giunchedi L., Credi R., Mantovani M., Martini L., Poggi Pollini C., 2007. Considerazioni sulla sharka o vaiolatura della drupacee. *Agronomica* **1**: 42-50.
- Kegler H., Fuchs E., Gruntzig M., Schwarz S., 1998. Some results of 50 years of research on the resistance to *Plum pox virus*. *Acta Virologica* **42**: 200-215.
- Malinowski T., Cambra M., Capote N., Zawadzka B., Gorris M.T., Scorza R., Ravelonandro M., 2006. Field trials of plum clones transformed with the *Plum pox virus* coat protein (PPV-CP) gene. *Plant Disease* **90**: 1012-1018.
- Ratti C., Budge G., Ward L., Clover G., Rubies-Autonell C., Henry C., 2004. Detection and relative quantitation of *Soil-borne cereal mosaic virus* (SBCMV) and *Polymyxa graminis* in winter wheat using real-time PCR (TaqMan (R)). *Journal of Virological Methods* **122**: 95-103.
- Vilanova S., Romero C., Abbott A.G., Llacer G., Badenes M.L., 2003. An apricot (*Prunus armeniaca* L.) F2 progeny linkage map based on SSR and AFLP markers, mapping *Plum pox virus* resistance and self-incompatibility traits. *Theoretical and Applied Genetics* **107**: 239-247.
- Varga A., James D., 2005. Detection and differentiation of *Plum pox virus* using real-time multiplex PCR with SYBR Green and melting curve analysis: a rapid method for strain typing. *Journal of Virological Methods* **123**: 213-220.
- Wetzel T., Candresse T., Ravelonandro M., Dunez, J., 1991. A polymerase chain reaction assay adapted to plum pox potyvirus detection. *Journal of Virological Methods* **33**: 355-365.

