

## The Response of Different *Prunus* genotypes to D and Rec Strains of Plum Pox Virus

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### Abstract

Three genotypes of *Prunus* (Mirololan BN 4Kr – a mutant of *Prunus cerasifera*, Local de Drăgășani – a selection of *Prunus insititia*, and Čačanska Najbolia cv. - *Prunus domestica*) were evaluated for PPV resistance based on their response to PPV-D and PPV-Rec strains, after inoculation by budding. Both PPV-D and PPV-Rec strains were translocated from the inoculum buds to Myrobolan BN 4Kr but the virus was able to induce only a limited infection, indicating a possible inhibition of virus replication. The response of Local de Dragasani to inoculation with the two PPV strains revealed a typical hypersensitivity reaction which could be a promising natural resistance source to PPV. The infection with both D and Rec strains became systemic in Čačanska Najbolia and no evidence of an effective inhibition of virus replication.

**Keywords:** plum, resistance, hypersensitivity, strains, PPV- D, PPV-Rec

### INTRODUCTION

*Plum pox virus* (PPV) is the causal agent of Sharka, one of the most devastating diseases of *Prunus* species (Cambra *et al.*, 2006). In order to restrict the spread of PPV, EPPO recommended measures such as quarantine isolation, nursery and orchard surveys, propagation of virus-free *Prunus* and chemical treatment of trees against aphid vectors. These measures have appeared to be ineffective in halting the spread of PPV which is endemic in many countries. Due to the rapid spread of PPV by aphids and the presence of many potential hosts, Sharka disease is difficult to eradicate once it has become established in an area. Therefore, the use of resistant cultivars represents the most important strategy to control PPV.

There are two approaches to the development of PPV resistant plum. The first one is the conventional breeding which may exploit the natural PPV resistance. Unfortunately, the paucity of natural resistance has hampered the efforts to obtain PPV resistant cultivars by conventional breeding. The second one is the genetic engineering which allows the obtaining of genetically modified plants resistant to PPV (Scorza and Ravelonandro, 2006). Following the Pathogen Derived Resistance (PDR) approach, *Prunus domestica* was transformed with the PPV coat protein (CP) gene (Scorza *et al.*, 1994). One transgenic line, C5, subsequently named 'HoneySweet' (Scorza *et al.*, 2007) was found to be highly resistant to PPV both in greenhouse (Ravelonandro *et al.*, 1997; Scorza *et al.*, 2001; Hily *et al.*, 2004) and field trials

(Malinowski *et al.*, 2006, Zagrai *et al.*, 2008a, b). 'HoneySweet' can be of significant benefit to growers and consumers while providing unique genetic material for use in conventional breeding programs (Scorza *et al.*, 2007). In spite of its benefits, there are still some retentions in Europe regarding to application of genetic engineering strategies to fight against pathogens. Therefore, the utilization of any potential natural sources of PPV resistance remain important for the development of new varieties, although it is difficult and long-term to incorporate such resistance into new stone fruits varieties through conventional breeding

Different potential types of natural PPV resistance were described (Kegler *et al.*, 1998). Since the immunity is characterized as "non - host resistance" (Fraser, 1998) and no interaction between the host genotype and the virus can occur (Kegler *et al.*, 1998) it is difficult to find immune genotypes. To date, no immune genotypes within *Prunus domestica*, *Prunus cerasifera*, *Prunus spinosa* and *Prunus insititia* have been identified (Hartmann and Neumuller, 2006). However, an immune myrobolan (BN 4Kr) was reported (Minoiu *et al.*, 1998).

The lack of the immune genotypes has directed the attention of breeders to the other types of natural PPV resistance: qualitative (complete, absolute) and quantitative (incomplete, relative) resistance. A qualitatively resistant genotype does not get diseased because the virus is localized in the infection site (Kegler *et al.*, 1998). The qualitative resistance to PPV it was supposed to be associated with hypersensitivity. Nevertheless, a multigenic hypersensitive reaction has been reported in *Prunus domestica* and a resistant hypersensitive cultivar 'Jojo' has been released (Hartmann and Petruschke, 2002). The absolute resistance of this cultivar was opposed by Polak *et al.*, 2005 which showed that PPV can be present in the tissue of cv. Jojo because the virus was transferred to the rootstock. Another genotype with hypersensitivity response was found in *Prunus insititia* and denoted Local de Dragasani (Minoiu *et al.*, 1998).

A genotype possessing a quantitative resistance trait get the disease but the infection rate, virus replication and virus invasion can be more or less inhibited (Kegler *et al.*, 1998). Čačanska Najbolia cv. can be considered as quantitatively resistant because the virus concentration within this genotype is significantly lower compared to other varieties (Kegler, 1992). A possible manifestation of the quantitative resistance after inoculation of the trees with virulent aphids was also observed in Čačanska Najbolia and Carpatin cvs. (Zagrai *et al.*, 2005).

In the present experiment it were tested the responses of Myrobolan BN 4Kr, Local de Dragasani and Čačanska Najbolia cvs. to artificial inoculation with the two different strains of *Plum pox* virus reported in Bistrita plum growing area from Romania (Zagrai *et al.*, 2008).

## MATERIALS AND METHODS

Plant material and inoculation of PPV strains. Three genotypes of *Prunus* (Myrobolan BN 4Kr – a mutant of *Prunus cerasifera*, Local de Drăgășani – a selection of *Prunus insititia*, and Čačanska Najbolia cv. - *Prunus domestica*) which showed interest in terms of resistance and another one very susceptible to PPV (Tuleu dulce cv.) used as control were selected for challenge experiment under greenhouse conditions. Two isolates from Bistrita area which were previously typed as PPV-D and PPV-Rec respectively, were used as inoculum sources (Zagrai *et al.*, 2008). Virus-free myrobolan rootstocks were grown in an experimental greenhouse started on April 2007 and double grafted with each genotype and inoculum on August 2007. The transfer of the virus to genotypes was made via rootstock as follow: buds of

genotypes were inserted at the top of grafting area and buds infected with D or Rec strain were inserted at the bottom. Seven plants were used for each genotype-virus combination. Only three plants were used in the case of control.

Virus monitoring. The response of the genotypes was evaluated along of vegetative period of 2008 and 2009 by visual monitoring of symptoms development and by serological and molecular methods. For testing, leaves samples were collected from the basal half and from the top half of the plants developed from genotype buds. In the case of shoots derived from inoculum buds, leaves were also collected to confirm the PPV presence.

Serological detection of PPV was made by DAS-ELISA (Double Antibody Sandwich Indirect - Enzyme Linked Immunosorbent Assay) using 5B-IVIA universal monoclonal antibody (Durviz, Spain) according to the manufacturer's instructions. Molecular detection was done by IC-RT-PCR (Immunocapture-Reverse Transcription-Polymerase Chain Reaction) using a pair of primers (P1/P2) that allows the production of the 243 bp fragment located at the C-terminus of PPV CP gene (Wetzel *et al.*, 1991). PPV immunocapture was trapped with PPV polyclonal antibodies (Bioreba, Switzerland). Qiagen one-step kit (Qiagen, Germany) was used for RT-PCR. The thermal cycling scheme used was the following: RT - 30 min at 50° C, denaturation / RT inactivation - 2 min at 94° C followed by 35 cycles: template denaturation - 30 s at 94° C, primer annealing - 45 s at 61° C and DNA elongation - 60 s at 72° C. Following to the last cycle, amplified DNA was elongated for 10 min at 72° C. An aliquot of the amplified products (10µl) was fractionated onto 1.5 % agarose gel electrophoresis in 1 x TAE buffer. Bands were visualized by ethidium-bromide staining under UV light.

Assays were made before grafting (to check the virus free status of the rootstocks and the genotypes) and after inoculation in July 2008 and June 2009.

## RESULTS AND DISCUSSION

The responses of those three plum genotypes after PPV inoculation using D and Rec strains are summarized in table 1.

Myrobolan BN 4Kr. No PPV symptoms appeared on the leaves of Myrobolan BN 4Kr one and two years after inoculation, and the plants showed a normal vegetative growth. Both PPV-D and PPV-Rec were translocated from the inoculum buds to Myrobolan BN 4Kr but the virus could only be detected at the basal half of the inoculated plants (DAS-ELISA and IC-RT-PCR, 2008). Although PPV was detected one year post inoculation, the infection did not become systemic in the following year and the plants remained symptomless. DAS-ELISA (2009) confirmed the presence of PPV only on the basal half of the plants. This means that the virus is able to induce a limited infection in Myrobolan BN 4Kr indicating a possible inhibition of virus replication.

Although PPV remained close to the inoculation site, the response of Myrobolan BN 4Kr to inoculation with PPV-D and PPV-Rec strains revealed an interaction between the host genotype and the virus. Therefore, this genotype can be characterized at most as resistant to PPV.

No substantial difference was recorded in the response of Local de Dragasani between the two PPV strains used for inoculation.

Local de Dragasani. Shoots from buds of plum genotype started to grow in April 2008, but they grew slowly. In addition, one of the plants inoculated with PPV-D and two plants

inoculated with PPV-Rec began to wilt and they died three weeks later. No PPV symptoms on leaves were observed on the remained plants during the vegetative period of 2008, and the absence of the virus was confirmed in leaves both by DAS-ELISA and IC-RT-PCR. However, the hypersensitivity reaction to PPV was obvious in both PPV-D and PPV-Rec groups. The responses as necrosis on leaves and bark, twisted leaves, gums on stem, wilting tips of shoots and sporadically leaves with large chlorotic regions were observed during the vegetative period of 2008. Although in April 2009 the plant seems to grow, most of the plants suddenly died. Only one plant inoculated with PPV-D and two plants inoculated with PPV-Rec survived. No PPV symptoms were observed in these plants and DAS-ELISA results were negative. Strong leakages of gums were recorded on the basal half of the three plants, especially close to the inoculum site. As the reaction showed, most probably these plants will not survive.

No substantial difference was noticed in the response of Local de Dragasani to the two PPV strains used for inoculation.

According to Hartmann and Neumuller (2006) the use of genotypes hypersensitive to PPV enables fruit growers to avoid the virus spreading. As Polak *et al.*, (2005) showed, in the case of Jojo cultivar, the hypersensitivity did not prevent the transfer of the virus to the rootstock. Therefore, in this case the PPV spreading can be stopped only if the rootstock used is also hypersensitive. In the case of Local de Dragasani this question remained and should be checked if the infected buds directly grafted on this plum genotype will be or not rejected and the infection transferred to the rootstock. Also, a long term trial in endemic areas under high natural infection pressure with PPV is required to verify the potential of Local de Dragasani as a valuable natural resistance source to PPV.

Čačanska Najbolia. The shoots showed normal vegetative growth and the leaves developed typical PPV symptoms. The symptoms were severe on the basal plants and decrease in intensity to the top. The presence of the virus was confirmed both by serological and molecular tests. The infection became systemic and the high viral concentration on the basal half and slightly lower on the top half of the plants suggested an ineffective inhibition of virus replication. The evidence showed that the response of Čačanska Najbolia cv is significantly different when plants were inoculated by budding compared with the case when the virus was transferred by aphids (Zagrai *et al.*, 2005). The low incidence of the symptoms on leaves in Čačanska Najbolia cv, accompanied by relatively low concentration of the virus after controlled infestation with virulent aphids described by Zagrai *et al.*, 2005, could also be explained by a relative resistance of this genotype to the virus vectors. This kind of resistance also can play an important role in protecting stone fruit trees from PPV infection (Kegler *et al.*, 1998).

No substantial differences in symptoms development or PPV spread were observed on Čačanska Najbolia when the inoculation was done with PPV-D or PPV-Rec strain.

Tuleu dulce (control). As expected both PPV-D and PPV-Rec strain readily invaded Tuleu dulce plants inoculated and severe PPV symptoms (chlorotic rings, diffuse spots and bands) appeared. The virus spread throughout the trees was confirmed both by serological and molecular tests.

No substantial differences in symptoms expression or PPV spread were observed between the two strains used for inoculation of Tuleu dulce cv.

Tab. 1

Evaluation of D and Rec strains of *Plum pox* virus in different parts of the graft-inoculated four plum genotypes under greenhouse conditions.

Inoculation: August 2007

Genotype	Inoculum	Analyzed part of plant	2008			2009	
			DASI-ELISA (DO=405nm)	IC-RT-PCR	PPV symptoms intensity	DASI-ELISA	PPV symptoms intensity
Myrobolan 4Kr	PPV-D	bottom half	+++	+	-	++	-
		top half	-	-	-	-	-
	PPV-Rec	bottom half	+	+	-	+	-
		top half	-	-	-	-	-
Local de Dragasani *	PPV-D	bottom half	-	-	-	-	-
		top half	-	-	-	-	-
	PPV-Rec	bottom half	-	-	-	-	-
		top half	-	-	-	-	-
Cacanska Najbolia	PPV-D	bottom half	++++	+	+++	+++	+++
		top half	+++	+	+	++	+
	PPV-Rec	bottom half	++++	+	+++	++++	+++
		top half	+++	+	+	++	+
Tuleu dulce (control)	PPV-D	bottom half	++++	+	+++++	+++++	+++++
		top half	++++	+	+++	++++	+++
	PPV-Rec	bottom half	++++	+	+++++	++++	+++++
		top half	+++	+	+++	++++	+++

\* No PPV symptoms but evidence of hypersensitivity response: necrosis on leaves and bark, gums on stem, wilting tips of shoots and sporadically leaves with large chlorotic regions

## CONCLUSIONS

- Both PPV-D and PPV-Rec strains were translocated from the inoculum buds to Myrobolan BN 4Kr but the virus remained close to the inoculation site indicating a possible inhibition of virus replication.
  - The response of Local of Dragasani to inoculation with PPV-D and PPV-Rec strains revealed a typical hypersensitivity reaction which could be a valuable natural resistance source to PPV.
  - No evidence of an effective inhibition of virus replication was observed in Čačanska Najbolia inoculated with PPV-D and PPV-Rec strains.
  - No substantial differences in the response of each genotype between the two groups of plants (inoculated with PPV-D or PPV-Rec) was observed.

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