Scientific Papers. Series B, Horticulture. Vol. LXIV, No. 1, 2020
Print ISSN 2285-5653, CD-ROM ISSN 2285-5661, Online ISSN 2286-1580, ISSN-L 2285-5653

FURTHER EVIDENCE OF DURABLE RESISTANCE IN HONEYSWEET TRANSGENIC PLUM UNDER NATURAL INFECTION WITH D AND REC STRAINS OF PLUM POX VIRUS

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Abstract

'HoneySweet' is a transgenic plum protected against Plum pox virus (PPV). Its behaviour to natural PPV infection was the subject of different field trials undertaken in several endemic European countries. The first experiment in Romania was performed between 1996-2006 and no 'HoneySweet' tree was found infected under high natural PPV infection pressure. To further asses the durability of resistance to PPV of 'HoneySweet', a new field trial was established in 2013. As inoculum sources of PPV were used plants, previously artificially inoculated with D or Rec strains, grown in pots and placed inside of the blocks. Limited treatments with insecticides were made within the plot in order to stimulate the virus spread by aphids. The monitoring of PPV spreading was made by serological and molecular assays. Strains discrimination was made by RT-PCR. The temporal spread of PPV revealed a continual evolution of infection in conventional plums. No trees of transgenic plum 'HoneySweet' expressed PPV symptoms or were found infected, by serological and molecular assays, confirming its durable resistance to natural PPV infection with D or Rec strains.

Key words*: plum pox virus, D and Rec strains, resistance, transgenic plum.*

INTRODUCTION

In Europe, the plum is highly affected by infections with *Plum pox* virus (PPV) for more than a century, being considered the most dangerous viral pathogen affecting *Prunus* spp. It produces severe symptoms on fruits, and also premature dropping, causing serious yield losses (Cambra et al., 2006).

Plum pox virus, the causal agent of Sharka disease, was described for the first time in Bulgaria, at the beginning of the 20th century (Atanasoff, 1932). Since then, PPV has progressively spread around the Mediterranean basin and Middle East. It has also been found in North and South America, North Africa and Asia (Barba et al., 2011). So far, PPV has not been found in Australia, New Zeeland and South Africa (García and Cambra 2007; EPPO, 2019). The virus is naturally spread by aphids in a non-persistent manner (Labonne et al., 1995).

Ten strains of PPV have been reported so far (Kamenova and Borisova, 2019): Dideron (D) and Marcus (M) - (Kerlan and Dunez, 1976), Recombinant (Rec) - (Glasa et al., 2002), El Amar (EA) - (Wetzel et al., 1991), Cherry (C) - (Kalashyan et al., 1994; Crescenzi et al., 1995; Maxim et al., 2002), Winona (W) - (James and Varga, 2004), Turkey (T) - (Serçe et al., 2009), Ancestor Marcus (An) - (Palmisano et al, 2012), Cherry Russian (CR) (Glasa et al., 2013) and Cherry Volga (CV) - (Chircov et al., 2018). The most common PPV strains are D, M and Rec, which are largely distributed in Europe (Šubr and Glasa, 2013). Although PPV is widespread in all plum growing areas from Romania and causes economic losses, a large-scale study revealed that only two strains (PPV-D and PPV-Rec) are present in this endemic country (Zagrai et al., 2010). PPV-C was also reported in a few sweet cherry trees, in an orchard from Bistrița area (Maxim et al., 2002), which was promptly rooted out, and a survey done ten years later did not found PPV-C in Romania (Zagrai et al., 2012).

Sharka disease strongly reduces the profitability of stone fruits crops in endemic areas, and can compromise the most part of yield of susceptible plum varieties (Minoiu, 1997; Zagrai et al., 2001). An eradication program of *Plum pox* virus in endemic areas is difficult to establish due to a fast spread of the virus via aphids and also, by the presence of

many potential host. Thus, using of resistant cultivars represents the most efficient solution to control PPV infection. In the context of over 80 years of conventional breeding which did not led to the expected results regarding the resistance to PPV, genetic engineering was used as a complementary approach to develop resistant plums by introducing a virus gene fragment into the DNA of *Prunus* host plants. Thus, a transgenic European plum (*Prunus domestica* L.) containing the coat-protein (CP) gene of PPV has been developed inside a cooperation between U.S. and France (Scorza et al., 1994). 'HoneySweet' (Figure 1) is a transgenic plum protected against *Plum pox* virus based on RNA interferance.

Figure 1. Fruits of 'HoneySweet' transgenic plum (original)

It was found highly resistant to PPV both under greenhouse conditions (Ravelonandro et al., 1997; Scorza et al., 2001) and in the field to natural PPV infection in several endemic European countries. Field trials carried out in Poland and Spain (Malinowski et al., 2006), Czech Republic (Polak et al., 2008) and Romania (Zagrai et al., 2011) demonstrated that 'HoneySweet' transgenic plum shows a highly effective and durable resistance to natural PPV infection.

The first experiment in Romania was performed between 1996-2006, and no 'HoneySweet' tree was found infected under high natural PPV infection pressure (Zagrai et al., 2011).

To further asses the durability and stability of resistance to *Plum pox* virus of 'HoneySweet' transgenic plum, a new field trial was established in 2013, and a new cycle of investigation was undertaken until 2019.

MATERIALS AND METHODS

Plant material and experimental field plot. The field trial, established in 2013, was statistically designed in twelve blocks of four trees (two trees of 'Honey Sweet' transgenic plum and two trees of conventional tolerant plum cultivars, used as control, 'Stanley' and 'Reine Claude d'Althan'). Thus, twenty-four trees of transgenic plum and twenty-four trees of conventional, all of these having virus-free status, were in the subject of this study.

The plot was surrounded by a large apple orchard in order to secure a buffer zone of minimum 500 m.

Plants of conventional plum, previously artificially inoculated with D (Čačanska rana 15/16) or Rec (Oneida 10/12) strains of PPV, were grown in pots and used as inoculum sources of PPV. The infected plants were then placed inside of the blocks, one infector per block, alternately each one of the two strains, to ensure a high inoculum pressure of the virus inside the experimental field plot (Figure 2). Limited treatments with insecticides were made within the plot in order to stimulate the virus spreads by aphids.

Virus monitoring. The monitoring of PPV spreading was made three times on each vegetative period by visual observations for potential symptoms of sharka disease, and annually by serological and molecular assays. In the case of trees which expressed typical symptoms of PPV, samples were collected from symptomatic leaves. In all the other cases, samples consisted in asymptomatic leaves taken from different parts of the canopy. All forty-eight trees belonging to transgenic and conventional plums were tested for the presence of PPV by DAS-ELISA (Double Antibody Sandwich-Enzyme Linked Immunosorbent Assay) - (Clark and Adams, 1977) using polyclonal antibody (Bioreba, Switzerland) according to the manufacturer's instructions.

Molecular detection was done by IC-RT-PCR (Immunocapture - Reverse Transcription - Polymerase Chain Reaction) using pairs 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 50ºC, denaturation/RT inactivation - 2 min at 94ºC followed by 35 cycles: template denaturation - 30 s at 94ºC, primer annealing - 45 s at 61ºC and DNA elongation - 60 s at 72ºC. Following to the last cycle, amplified DNA was elongated for 10 min at 72ºC. 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.

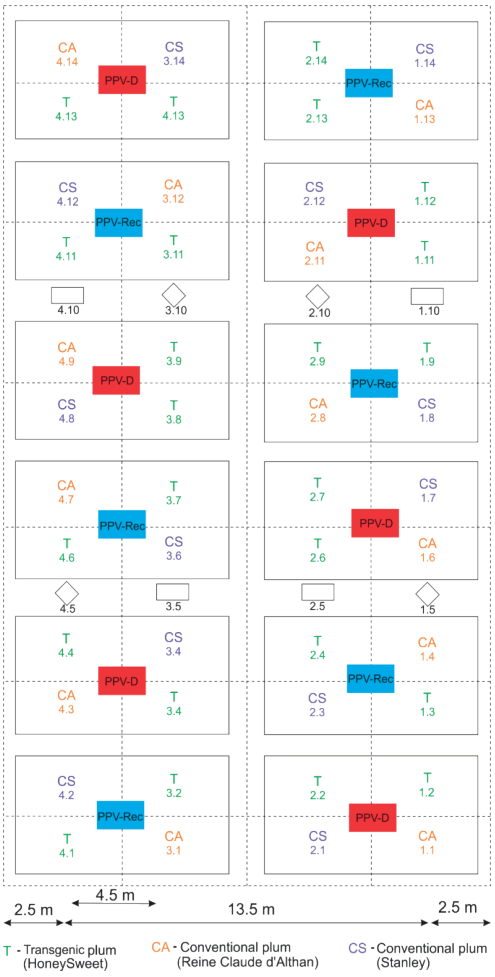

Figure 2. The design of field trial with transgenic and conventional plums (2013-2019)

Molecular discrimination of PPV strains. Infected trees were then analyzed for the presence of PPV-D and PPV-Rec strains. PPV strain discrimination was performed by RT-PCR targeting the C-terminus of CP genomic region using P1/PD and P1/PM primer sets (Olmos et al., 1997) and 6K1-CI genomic region of PPV using CIP-M/CIP-MR and CIP-D/CIP-DR primer pairs (Kamenova et al., 2011). Total RNA was extracted by RNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's instructions.

RESULTS AND DISCUSSIONS

PPV incidence in experimental plot. All artificially infected plants with D or Rec strains of PPV, used as inoculum sources, showed PPV typical symptoms since 2013, ensuring thus a high natural infection pressure of the virus for transgenic and conventional plums grown in the same experimental plot. After three years of study, typical symptoms of sharka has been observed on a tree of conventional plum, 'Stanley' cultivar. The presence of *Plum pox* virus was confirmed by laboratory tests (Figure 3). The first tree of 'Reine Claude d'Althan' cv. became infected with PPV after four years (2017). In the fifth year (2018), two trees belonging to 'Reine Claude d'Althan' and another one of 'Stanley' cvs. confirmed the presence of PPV by serological and molecular tests. In the last year of the study, additional three trees of 'Reine Claude d'Althan' and five of 'Stanley' cvs. proved to be infected by PPV.

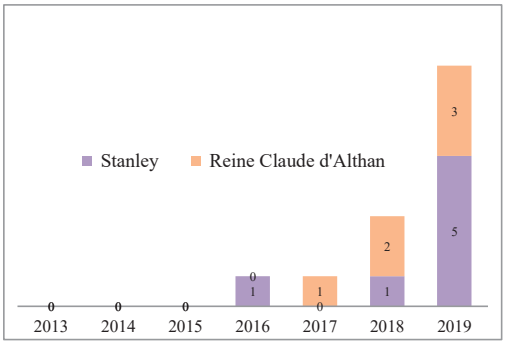

Figure 3. Newly conventional plum trees infected by *Plum pox* virus

In conventional plum, the number of infected trees with PPV has increased year by year approximately similar on both cultivars, 'Reine Claude d'Althan' and 'Stanley'.
Typical symptoms of PPV were expressed on leaves of both conventional plum cultivars and on the fruits of 'Reine Claude d'Althan' (Figure 4).

Figure 4. PPV symptoms on fruits ('Reine Claude d'Althan' plum cv.) - original

During six years of field testing, thirteen out of twenty-four conventional plums, seven trees of 'Stanley' and six of 'Reine Claude d'Althan' cultivars, became infected with PPV (Figure 5).

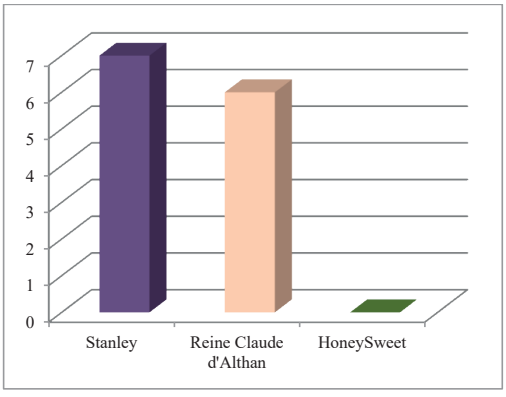

Figure 5. No. of trees infected by PPV (2013-2019)

The results revealed an annually increase of PPV infection inside the plot between 4% to 33% on conventional plum (Table 1), being similar with results obtained by Blazek et. al (2003) that state an over 4% newly PPV infected trees per year on young (three to five years old) plum trees.

Table 1. Newly PPV infected trees per year

Year/ Specification	Conventional plum		Transgenic plum	
	Infected/ total trees	%	Infected/ total trees	%
2013	0/24	0	0/24	0
2014	0/24	0	0/24	0
2015	0/24	0	0/24	0
2016	1/24	4.2	0/24	0
2017	1/24	4.2	0/24	0
2018	3/24	12.5	0/24	0
2019	8/24	33.3	0/24	0
Total	13/24	54.2	0/24	0

Therefore, the temporal spread of *Plum pox* virus revealed a continual evolution of infection in both conventional plum cultivars, from about 4% in 2016 to over 50% in 2019 (Figure 6).

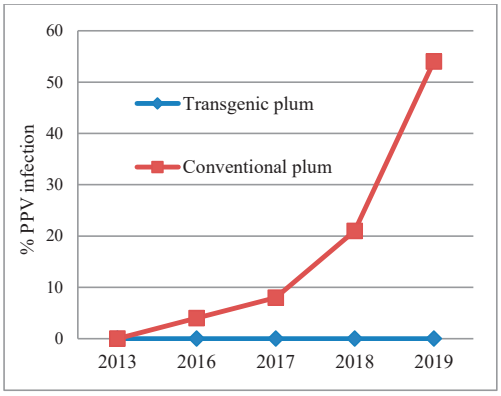

Figure 6. Temporal spread of PPV in experimental plot (2013-2019)

In the same conditions, no PPV symptoms appeared on 'HoneySweet' leaves or fruits, and no infection was found in transgenic plum trees by DAS-ELISA and IC-RT-PCR tests.

PPV strain discrimination. All PPV infected trees, belonging to conventional plum 'Reine Claude d'Althan' and 'Stanley' cultivars, were subjected to PPV discrimination. The results revelead that six out of thirteen trees proved to be infected by PPV-D strain, and the other seven, by PPV-Rec strain (Table 2).

Table 2. Strains discrimination of conventional plums infected with *Plum pox* virus

Plum cultivars/Code	PPV-D	PPV-Rec.
Stanley/1.7	+	-
Stanley/1.8	-	+
Stanley/2.1	+	-
Stanley/3.4	+	-
Stanley/3.6	-	+
Stanley/3.14	-	+
Stanley/4.12	-	+
Reine Claude d'Althan/1.1	+	-
Reine Claude d'Althan/2.8	-	+
Reine Claude d'Althan/2.11	+	-
Reine Claude d'Althan/3.12	-	+
Reine Claude d'Althan/4.3	+	-
Reine Claude d'Althan/4.7	-	+

Three trees belonging to Stanley cultivars were found infected by PPV-D, and four by PPV-Rec strains. Three of each PPV-D or PPV-Rec strain were detected in the six 'Reine Claude d'Althan' infected trees. Thus, the occurrence of the two strains in newly infected trees, along of six vegetative periods of study, revealed a quite similarity of D and Rec strains of PPV rate occurring in the two conventional plum cultivars. No mixed infection (D+Rec) of PPV was found by molecular tests in conventional plums.

Durability of resistance to PPV of transgenic plum. The virus infection in conventional plums suggest a high inoculum pressure of *Plum pox* virus for natural transmission by aphids inside the field plot. In situation in which incidence of PPV in conventional plums increased over 50%, 'HoneySweet' transgenic plum did not express PPV symptoms along six years of field trial. Moreover, no tree of transgenic plum 'HoneySweet' was found infected by PPV in both serological and molecular assays. These results coroborated with those obtained between 1996-2006 in Romania (Zagrai et al., 2011), Poland and Spain (Malinowski et al., 2006), and Czech Republic (Polak et al., 2008) demonstrated the durability and stability of 'HoneySweet' transgenic plum under high inoculum pressure of PPV over time.

CONCLUSIONS

Plum pox virus was rapidly spread by natural transmission in conventional plum cultivars 'Reine Claude d'Althan' and 'Stanley' (over 50% along 6 years).

A similar rate of D and Rec strains of PPV was occurred in the conventional plum cultivars.

In the same conditions, 'HoneySweet' transgenic plum remained uninfected under high *Plum pox* virus inoculum pressure confirming its durable resistance to natural *Plum pox* virus infection with D or Rec strains.

ACKNOWLEDGEMENTS

This research work was carried out with the support of Ministry of Agriculture and Rural Development, financed from Project ADER 4.1.3/2015.

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