Studies of Resistance to Sharka in Several Romanian Apricot Progenies

Ligia Ion[1], Tudora Neagu[1] , Adrian Asănică[1], and Cristina Moale[2]
[1] University of Agronomic Sciences and Veterinary Medicine Bucharest, Marasti Street, no. 59 Romania.
[2] Fruit Research & Development Station Constanta, Romania

Abstract **:***Sharka caused by Plum pox virus (PPV) is considered to be one of the most devastating diseases in Prunus species such as peach, plum, apricot, and cherry. The spreading of PPV might be limited by planting PPV resistant or at least less-susceptible rootstocks on which PPV resistant scions have been grafted. Certain apricot cultivars ('Stark Early Orange', 'Traian', 'NJA 21') display significant levels of resistance to the disease. In the last eighth years, at USAMV Bucharest, Romania, unfolds a breeding program aiming to develop cultivars and rootstocks resistant to PPV and an efficient procedure for the determination of sharka resistance within the progenies was established. The GF305 rootstocks were grafted with apricot individuals originating from crossing between a PPV resistant genitor (e.g. 'SEO', 'NJA2' "NJA 17, NJA 42) and Romanian preferred varieties. Grafted plants were inoculated by chip-budding and monitored by visual inspection and ELISA, completed by RT-PCR for the PPV negative plants. For the selection by molecular markers the DNA simples were isolated.*

After fenotyping respons of the most important romanian progenies were was compared with genotyped results using the implemented the markers PGS 1.21 and PGS 1.252 (which amplifies 193/239 CM and respectively 92/122 CM).

We observed that an important number of hybrids in the combinations MARI DE CENAD ♀ x SEO ♂ - 4 individuals; SIRENA ♀ x NJA 42 ♂ - 7 individuals; CRISTAL ♀ x NJA 21 ♂ - 6 individuals; combination AMIRAL ♀ x NJA 21 ♂ - 6 individuals, and combination NJA 17 ♀ x TRAIAN ♂ - 6 individuals proved to be clearly resistant to PPV infection for identification of genes linked to plant response to PPV infection.

Keywords**:** *Plum pox virus, apricot, cultivars, artificial infection, molecular markers, resistance.*

1. Introduction

Since the late 1980s, the sharka disease has become a serious threat for this species. It is caused by an aphid-borne virus, plum pox virus (PPV), which is included in the genus Potyvirus. Detection of PPV in 1999 in Adams County, PA prompted the implementation of an aggressive eradication program to control its spread [17]. Eradication is an extremely costly way of controlling PPV. In Europe, PPV is the most important virus affecting *Prunus* fruit crops and the most limiting factor for the apricot cultivation in terms of economics [16].

Countries with a low incidence of disease limit PPV spread by removing infected trees and through the use of certified virus-free materials [20]. For the others, the only way to solve this problem is to cultivate resistant varieties [12].

Currently, there are two strategies for obtaining resistant cultivars: (1) identify natural resistance present in *Prunus* germplasm and introduce this resistance into commercial cultivars by standard breeding practices, and (2) engineer resistance through application of transgenic technologies.

Several programs aiming at breeding PPV resistant or partially resistant apricot cultivars are carried out in Europe ([18], [21], [11], [14]). The majority of existing apricot cultivars show different level of susceptibility to PPV. The Romanian cultivar 'Mari de Cenad, Traian, Tabriz' belongs to highly partially resistant cultivars: only few symptoms are observed on leaves and virus particles are present in plant tissues in low concentration ([6], [26], [13]).

The aim of this study was to identify the resistants genotypes that are differentially expressed during PPV infection on apricot Romanian cv. 'Mari de Cenad, Traian, Tabriz' and to screen with putative molecular markers of resistance/tolerance to the virus, useful in breeding programmes.

2. Materials and methods

2.1. Plant material

New apricot crosses - F2 progenies issued from crosses between PPV resistant genitors ('NJA2', NJA 17, NJA 42 and 'Stark Early Orange' –SEO) and local varieties ('Viceroy', 'Mari de Cenad', Traian, Tabriz etc…), was performed.

Crosses were performed by hand pollination without isolation of flowers after removing of petals and anthers from flower buds. The BC1 seeds were stratified at 5oC for 3 months and subsequent seedlings were grown in an insect-proof greenhouse. This plant material was used in BSA.

The plant material consist in 9 hybrids combinations of apricot and two strains of *Plum pox virus* Marcus (M) and Dideron (D). These nine combinations of apricot are presented below:

The crossbreeding scheme is shown below (*for the resistant parent):

Pop 1 = Population 1 obtained by crosses 'Mari de Cenad' x ,SEO*' – 9 individuals

Pop 2 = Population 2 obtained by crosses 'Sirena' x 'NJA 42*' – 43 ind.

Pop 3 = Population 3 obtained by crosses between, NJA 21*' x 'Kesth Pshor' – 46 ind.

Pop 4 = Population 4 obtained by crosses 'Cristal' x ,NJA 21*' – 19 ind.

Pop 5 = population 5 obtained by crosses between Sulmona x 'NJA17*' – 9 ind.

Pop 6 = Population 6 obtained by crosses between "VICEROY x NJA 2*" – 22 ind.

Pop 7= Population 7 obtained by crosses between "WARLEY'S x TABRIZ" – 23 ind.

Pop 8 = Population 8 obtained by self pol. "C41/68" (Danubiu x NJA 21*)-SELF POL – 19 ind.

Pop 9 = Population 9 obtained by crosses between "TRAIAN* x CRISTAL" – 11 ind.

Pop 10= population 10 obtained by crosses between "NJA 42* x VICEROY" – 7 ind.

Pop 11 = Population 11 obtained by crosses between "AMIRAL x NJA 21*" – 19 ind.

Pop 12 = population 12 obtained by crosses between "TRAIAN* x TABRIZ" – 9 ind.

Pop 13 = Population 13 obtained by crosses between "NJA 17* x TRAIAN*" – 7 ind.

PPV isolates **M (Marcus); D (Dideron**) used in these studies were collected from three field experimental plots containing conventional varieties planted at Fruit Research Station,Bistrita, Romania.

2.2. PPV inoculation and evaluation of PPV infection

For fenotyping the apricot genotypes were grafting onto inoculated GF305 (used like susceptible rootstock) and Mirobolan BN 4 Kr used like resistant rootstock, ready for testing to PPV resistance.

Seedlings were pruned directly after grafting to promote the growth of the inoculated bud. Plants without sharka symptoms on shoots growing from the inoculum bud and with negative enzyme-linked immunosorbent assay (ELISA) reaction were re-inoculated. PPV infection was evaluated over five consecutive growth periods through visual symptoms and ELISA [18] Pruning was performed at the beginning of each growth period to induce vigorous new shoots for symptom scoring.

The plants, in which PPV was not detected by ELISA, were tested by reverse transcription polymerase chain reaction (RT-PCR) using the PPV specific primers P1 and P2 [25]. Plants were classified as resistant if they did not show symptoms and positive ELISA or RT-PCR reaction in the last three growth periods evaluated.

The infection process was different for various plant individuals; in some plants the presence of PPV was detected after the first dormancy period, in some in the three vegetative cycles, while some plants were not infected at all.

Molecular detection was performed by RT-PCR (Reverse Transcription-Polymerase Chain Reaction) using a primer pair (Pl/P2) that amplifies a 243 bp fragment located at the C-terminus of the PPV CP gene. PPV was trapped with PPV-polyclonal antibodies adsorbed on an Eppendorf micro tube. Enhanced Avian kit provided by Sigma 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 at72°C. An aliquot of the amplified products (10 µI) was fractionated onto *1.5%* agarose gel electrophoresis in 1x TBE buffer. Bands were visualized by ethidium-bromide staining under UV light.

2.3. DNA extraction

Genomic DNA was isolated from fresh apricot leaves using the hexadecyltrimethylammonium bromide (CTAB) protocol described by [10]. DNA concentrations were measured by a minifluorimeter (TKO100, Hoefer Scientific). Working solutions of genomic DNA at 100 ng/µl in TE buffer (pH 8.0) were prepared for AFLP analysis.

2.4. SSR analysis

The parents and their progenies were screened with two peers of SSR primer combinations from Aranzana et al.[2] and the eight SSR primer combinations developed from INRA Bordeaux associated with PPV resistance. PCR reactions, electrophoresis, and detection of PCR products for the each population were carried out according to conditions specified in Zhebentyayeva et al. [27] SSR primer combinations revealing polymorphism were screened in the entire mapping population.

3. Results and Discussion

Screening the Romanian progenies F1 and F2 in artificial infection conditions for resistance/tolerance to PPV was performed by visual inspection, by ELISA and RT-PCR analysis. [15].

Serological tests were performed by DAS-ELISA (Double Antibody Sandwich-Enzyme Linked Immunosorbent Assay), using polyclonalantibodies according to the manufacturer (Bioreba)

After each cycle of symptom observation, one ELISA– DASI (DASI, double antibody sandwich indirect) test was performed to assess the presence or absence of the virus (Photo1 and Photo 2). In asymptomatic plants, five leaf samples were picked at random, whereas in the symptomatic ones, the leaves showing symptoms were chosen. Absorbance was determined at 405 nm after, 60-min substrate incubation. The samples with optical densities double than of the healthy control were considered ELISA-positive. Individuals showing symptoms and a positive ELISA reaction were considered susceptible. The plants showing no symptoms and ELISA negative after four vegetative cycles were considered resistant. In artifficial infection conditions in the greenhouse the results presented in Photo 3 (in the top of Elisa plate) shows that samples belonging to susceptible GF 305 rootstock were found to be positive compared with most samples of apricot genotypes,(in the bottom of Elisa plate) even if were collected on the same plant. Under these conditions the virus is able to infect susceptible peach rootstock but not a most of part of the apricot genotypes like V3P16, V2P18, V4 P14. [4].

Plants were classified as resistant if they did not show symptoms and/or positive ELISA or RT-PCR reaction in the last three growth periods evaluated.(Table 1) Thirteen individuals were found resistant and 67 susceptible. Resistant individuals were coded as heterozygous for the trait and those susceptible were coded as homozygous recessives (consistent with Vilanova et al. [23]). The segregation ratio 1:5 (resistant/susceptible deviated significantly from the expected for a single dominant locus (1:1) with $\chi 2$ value of 36.5 (resistant/ susceptible)). These results clearly indicate that the resistance is controlled by more than a single gene.[24]

These potential resistants individuals were tested in terms of molecular techniques to confirm the nature of resistance to sharka.

Results concerning the molecular detection performed by RT-PCR (Reverse Transcription-Polymerase Chain Reaction) using a primer pair (Pl/P2) that amplifies a 243 bp fragment located at the C-terminus of the PPV CP gene. proved, that some apricot hybrid genotypes that were found to be negative after Elisa test, were revealed to be positive after molecular testing like V2P18.(Table 1). It supports the sensitivity of molecular testing.[25]

While the genetic determinism of the resistance to PPV is still questioned (one or two or three genes, dominant versus recessive), we observed that an important number of hybrids like: V3P16, V4P18, V2P14, V2P16, V4P19, V1P19, V5P18, V4P19, V2P17**,** V4P16, V3P20 proved to be clearly resistant to PPV infection (Photo. 1 and 2)

This suggests that a putative quantitative trait locus (QTL) for the PPV resistance trait may reside in the region of G1 between 1.5 and 40.9 cM.(Table 2)

SSR marker detection with primers developed from peach, apricot, and almond ([19]; [5]; [22]; [1]) demonstrates the high transportability of SSRs in *Prunus* [8]. 'Stark Early Orange', 'Traian', and progenies were screened with 2 SSR primer combinations. These SSRs were polymorphic.

Due to the co-dominant nature of SSRs along with their high genetic transportability, the development of SSRs associated with PPV resistance in apricot could facilitate the use of MAS in breeding strategies aimed at breeding for natural resistance. Additionally, these markers can serve as landmarks to aid in fine mapping of this region as well as enable positional cloning of genes that may contribute to PPV resistance.

4. Conclusions

We observe an important number of hybrids, like: V3P16, V4P18, V2P14**,** V2P16, V4P19, V1P19, V5P18, V4P19, V2P17, V4P16, V3P20, manifested a resistance to the artificial infection with PPV, the detection of the virus was performed by serological and molecular tools.

Romanian apricot F1 and F2 progenies evaluated were initially classified into three groups: susceptible to PPV, partially resistant and resistant to PPV. After that the most important genotypes were grafted onto infected GF 305 (indicators for susceptibility) and Mirobolan BN 4 Kr (considered to be resistant to sharka) in greenhouse conditions. The rootstocks and also apricots hybrids were monitored by visual inspection, ELISA, completed by RT-PCR for the PPV symptoms.

Concerning the rootstocks, results confirm that, PPV infection was 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, in comparison with GF305 where the virus spread onto the apricot genotypes, but some of them show not symtoms only in their rootstocks. The Roumanian local genotypes used like rootstocks could be a important promise to limited the infection of the virus on to the *Prunus* valuable cultivars.

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Fig. 1 and 2: Apricot response after artificial inoculations with PPV

The resistant progenitors ('Stark Early Orange', "NJA38" and "Traian") were able to transmit PPV resistance to the descendants, in agreement with previous results observed by other authors ([3]; [9]; [7]).


Fig. 3 and 4 and 5: ELISA and RT-PCR analysis of 'GF305' rootstocks and apricot hybrids:

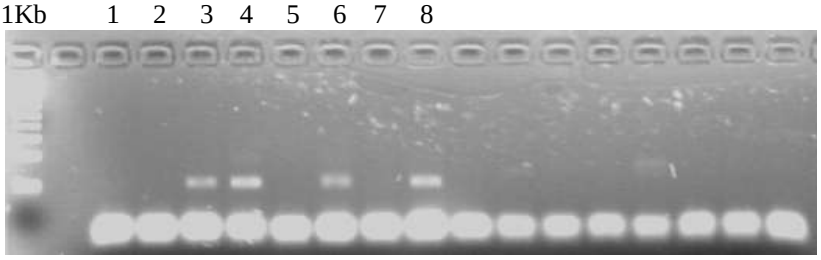

Fig. 5: RT-PCR detection of PPV in apricot progenies

TABLE I: Phenotyping Romanian apricot progenies by artificial inoculation
Resistance/susceptibility to sharka was scored first by visual inspection and then the presence of the virus was checked by ELISA and RT-PCR

Short name of progeny	Cross	Variants	GF 305			Apricot hybrids		
			PPV symptoms intensity	DASI-ELISA (DO= 405nm)	RT-PCR	PPV symptoms intensity	DASI-ELISA (DO= 405nm)	RT-PCR
Pop 6.	Viceroy x NJA2*	V3P16	+	+	+	-	-	-
Pop 10.	Viceroy x NJA42*	V2P18	+	+	+	-	-	-
		V4P18	+	+	+	-	-	-
		V6P20	+	+	+	-	+	+
Pop. 11	Amiral x NJA21*	V4P19	+	+	+	-	+	+
Pop.8	Danubiu x NJA21*	V2P14	+	+	+	-	-	-
Pop12.	Traian X Tabriz	V4C5	++	++	+	+	+	+
Pop9.	Cristal X Traian*	V5P19	+	+	+	-	-	-
Pop 1.	Mari de Cenad x SEO *	V1P19	++	++	+	+	+	-
		V2P16;	+	+	+	-	-	-
		V2P18	+	+	+	-	+	+
		V3P18	++	+	+	++	+	+
		V4P19	+	+	+	-	-	-
		V5P18	+	+	+	-	-	-
		V6P16;	+	+	+	-	-	-
		V6P18	+	+	+	+	+	+
Pop 5	Sulmona X NJA 17*	V2P17	+	+	+	-	-	-
		V4P16	+	+	+	+	-	-
Pop 7.	Tabriz X Warly's*	V3P20	+	+	+	+	+	+
Pop. 13	Traian X NJA 21	V2 P4	-	-	-	-	-	-
Pop 4.	Cristal X NJA 21	V4P7	-	-	-	-	-	-
Pop 3.	NJA 21 X Kesth Pshor'	V3P6	+	+	+	+	+	+

TABLE II: Results concerning the implement of French markers PGS 1,21 and PGS 1,252 in Roumanian progenies.

Individual	Individual	PGS 1,21	PGS 1,252	
1+	NJA42	199/239	90/92	resistant
2+	JT10/79	199/239	92/92	resistant
3+	Moongola	193/215	92/122	recombinant
4+	Danubiu	193/239	92/122	resistant
5+	SEO	193/239	92/122	resistant
6+	Tabriz	193/213	92/122	recombinant
7+	Resth_phor	-	92/92	recombinant
8+	name not in the list	193/193	122/122	susceptible
9+	Viceroy_603_G	193/213	92/122	recombinant
10+	Traiam	193/239	92/122	resistant
1	C2P30V6	193/239	92/122	resistant
2	C3P19V4	193/239	92/122	resistant
3	C2P18V4	193/239	92/122	resistant
4	C2P18V2	193/239	92/122	resistant
5	C8P17V5	193/193	122/122	susceptible
6	C7P18V5	193/239	92/122	resistant
7	C7P13V4	193/239	92/122	resistant
8	C1P16V3	193/239	92/122	resistant
9	C7P16V6	193/239	92/122	resistant
10	C9P20V3	239/239	92/92	resistant
11	C7P16V6	193/239	92/122	resistant
12	C2P18V4	193/239	92/122	resistant
13	C7P16V2	-	92/122	resistant
14	C5C5V4	215/215	92/92	recombinant
15	C7P18V3	193/239	92/122	resistant
16	C9P19V1	239/239	92/92	resistant
17	C7P18V6	193/239	92/122	resistant
18	C8P16V4	193/193	no 92	susceptible
19	C7P18V2	193/239	92/92	resistant

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