SHARCO

SHARKA CONTAINMENT IN VIEW OF EU-EXPANSION

Implementation of Marker-Assisted Selection in Several Romanian Apricot Genotypes

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Plum pox virus (PPV) is a member of the genus Potyvirus (family Potyviridae) and Sharka disease is considered to be one of the most devastating of stone fruit crops in Europe. The spreading of PPV might be limited by using in the Romanian breeding program the PPV resistant genitor (e.g. 'SEO', 'NJA2' "NJA 17, NJA 42) and Romanian varieties

The apricot selection 'R9 P 53' ('NJA 2' ×'Viceroy')♀ was crossed with 'NJA 17'♂ and the selection 'R13 VT 8/57'('Mari de Cenad x NJA 21') ♀resistant to PPV was crossed with 'Kesth Pshor' ♂-susceptible one. For phenotyping this Romanian progenies were grafted on to peach GF 305 previously inoculated with PPV by chip-budding and monitored by visual inspection, ELISA completed by RT-PCR for the PPV negative plants.

SEVENTH FRAMEWORK PROGRAMME
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Objectives

•The goals of the work presented in this communication are the identification of a natural source of resistance to PPV, introduce this resistance into commercial cultivars well adapted in our country, and the implementation of marker-assisted selection (MAS), based on markers tightly associated with resistance, as a measure to substantially streamline the breeding process.

•In Romania at the USAMV Bucharest, in 2008 was started a breeding program using the Romanian cultivars 'Mari de Cenad','Traian', 'Tabriz', 'Viceroy', 'Kesth Pshor' and different sources of resistance like varieties 'Stark Early Orange' (SEO),(NJA 2, NJA 42, NJA 17). Segregation of susceptible and resistant progenies is guided by Mendel's laws. (Dicenta *et al,* 2000).

Materials and methods

- **Plant material**
- **We are worked with 5 apricot populations**:

Pop 1 = Population 1 obtained by crosses ‘Mari de Cenad’ x ,SEO

Pop 2 = Population 2 obtained by crosses ‘Sirena’ x ‘NJA 42’

Pop 3 = Population 3 obtained by crosses between (‘Mari de Cenad, x ,NJA 21’) x ‘Kesth Pshor’

Pop 4 = Population 4 obtained by crosses ‘Cristal’ x ,NJA 21’

Pop 5 = population 5 obtained by crosses between (‘Viceroy’, x ,NJA 2’) x ‘NJA17’

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- **The apricot selection R9 P 53 (‘NJA 2’ ×‘Viceroy’) was crossed as a female parent to ‘NJA 17’ a PPV resistant apricot cultivar .The selection R13 VT 8/57 resistant to PPV (issued from ‘Mari de Cenad x NJA 21) was crossed as a female parent to ‘Kesth Pshor’ (susceptible to PPV) in the frame of the Faculty of Horticulture of University of Agronomical Science and Veterinary Medecine Bucharest Romania in 2008. *Crosses* were performed by hand pollination with isolation of flowers after the petals and anthers removal from the flower buds. The F1 seeds were stratified at 5°C for 3 months and subsequent seedlings were grown in an insect-proof greenhouse.**
- **The young apricot populations sticks were grafted onto inoculated GF305 (used like susceptible rootstock) ready for testing to PPV resistance.**
- **They were inoculated with a chip-bud collected from three experimental field plots containing conventional varieties planted at Fruit Research Station,Bistrita, Romania**

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Phenotyping methods

For phenotyping this Romanian progenies, 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 three consecutive growth periods through visual symptoms ELISA and RT- PCR .

- **DNA extraction**
- **Genomic DNA was isolated from fresh apricot leaves using the hexadecyltrimethylammonium bromide (CTAB) protocol described by Eldredge *et al.* (1992). 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 SSR (Simple sequence repeat) analysis.**
- **The SSRs (Simple sequence repeat) primers were designed from the sequences flanking the SSRs using the online primer design program Primer3 (Rozen and Skaletsky 2000) and purchased from Integrated DNATechnologies**

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- **SSR analysis**
- **'Stark Early Orange', 'R13 VT 8/57' NJA and R9 P 53 were screened with 3 SSR primer combinations from Aranzana *et al.* 2003 associated with PPV resistance, PGS 1.21, PGS 1-24 and ppb22-195-F:CTCTTCTCGCCTCCCAATTT and R:GCTTAGCCCTGGGTACAAG and F:ATCTGCTCTTTCCCTCACCT with**
- **R:GATTATCCCTCAACCCATCC.**
- **SSR primer combinations revealing polymorphism were screened all apricot populations**
- **The mix PCR consit in 10X buffer- 2ul, MgCl2 (25 mM)-1.2 ul, dNTP 10 mM – 0.16 ul, PGS 1.21 -F – 0.6ul, PGS 1.21- R – 0.6 ul, PGS 1.24- F – 0.4 ul, PGS1.24-R – 0.4 ul, ppb22-195-F -0.28 ul,ppb22-195-R – 0.28ul and Taq 0.1 ul. A 2-µl aliquot of the PCR reaction was separated by electrophoresis on an agarose gel 2% in order to confirm the amplification of fragments of the expected size and DNA concentration.**
- **The PCR-products were diluted (45 ul H2O and 5 ul DNA) and used to prepare the plate for sequencing .**

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Results and discussions

- **Results concerning the molecular detection performed by RT-PCR) using a primer pair (PI/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. This, it show us that it supports the sensitivity of molecular testing (Wetzel*. et al.* 1991).**
- **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 that were evaluated. In the population 'R13 VT 8/57' x 'Kesth Pshor' 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*.2003a).The segregation ratio 1:5 (resistant/susceptible) deviated significantly from the expected for a single dominant locus (1:1) with value of 36.5 (resistant/ susceptible). These results clearly indicate that the resistance is controlled by more than a single gene.(Audergon *et al* ,1994) (Fig 4)**

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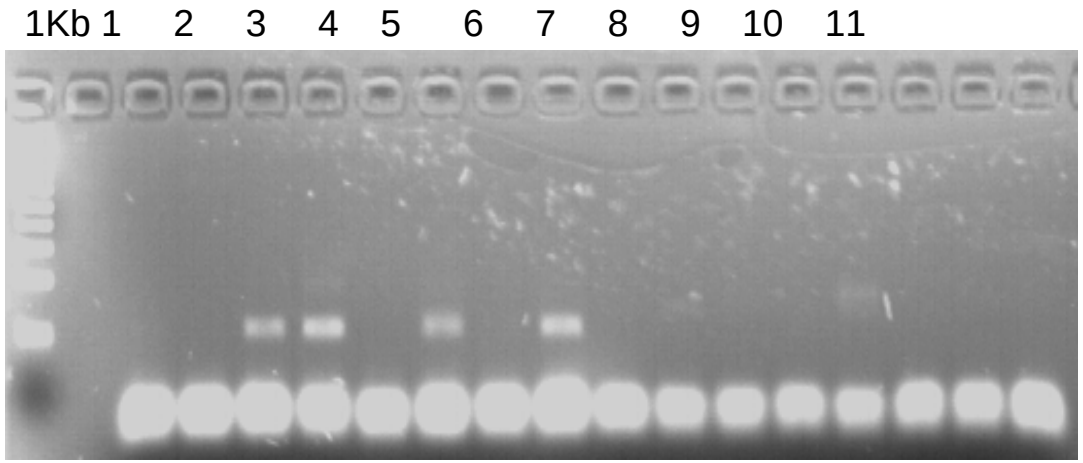
Electrophoresis for RT –PCR to revealed the resistant or susceptible apricot progenies

- **The segregation ratio (1:5)resistant/susceptible) of PPV resistance in the 'R13 VT 8/57' x 'Kesth Pshor' showed that this markers are present in the upper half of linkage group G1. This suggests that a putative quantitative trait locus (QTL) for the PPV resistance trait may reside in the region of G1 between 193 / 239 cM for PGS 1.21 and 92/122 for PGS 1.24**



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Phenotyping Romanian apricot progenies (population 5) in artificial infection conditions for resistance/tolerance to PPV; by visual inspection and ELISA and RT-PCR analysis

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Short name of progeny	Cross	Hybrids	Rootstock 'GF 305'			Apricot hybrids		
			PPV Symptoms intensity	DASI ELISA (DO=405)	RT-PCR	PPV Symptoms intensity	DASI-ELISA (DO= 405nm)	RT PCR
C1	VT 92.02.52 – NJA 17* (female) x R9 [53 (male) (Viceroy x NJA2*)	V3P16	+	+	+	-	-	-
C2	VT 92.01.05 – NJA 17* X R9 P 53 (Viceroy x NJA2*)	V2P18	+	+	+	-	-	-
		V4P18	+	+	+	-	-	-
		V6P20	+	+	+	-	-	-
C3	VT 92.02.95 - NJA 17* X R9 P 53 (Viceroy x NJA2*)	V4P19	+	+	+	-	-	-
C4	VT 92.02.91 - NJA 17* X R9 P 53 (Viceroy x NJA2*)	V2P14	+	+	+	-	-	-

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Short name of progeny	Cross	Hybrids	Rootstock 'GF 305'			Apricot hybrids		
			PPV symptoms intensity	DASI-ELISA (DO= 405nm)	RT-PCR	PPV symptoms intensity	DASI-ELISA (DO= 405nm)	RT-PCR
C5	R10 P79 (Viceroy x NJA2*) X Tabriz	V4P5	+	+	+	-	-	-
C6	R10 P79 X Traian*	V5P19	+	+	+	-	-	-
C7	V5 – VT 30/40 Mari de Cenad x (SEO *)	V1P19	+	+	+	+	+	-
		V2P16; V2P18	+ +	+ +	+ +	- -	- -	- -
		V3P18	++	+	+	+	-	-
		V4P19	+	+	+	-	-	-
		V5P18	+	+	+	-	-	-
		V6P16; V6P18	+ +	+ +	+ +	- -	- -	- -
C8	V6 – VT 12/13 – MOONGOLD X NJA 42*	V5P17	+	+	+	+	+	+
		V4P16	+	+	+	+	+	+
C9	VT 4/73 – VIVAGOLD X NJA 42*	V3P20	+	+	+	-	-	-

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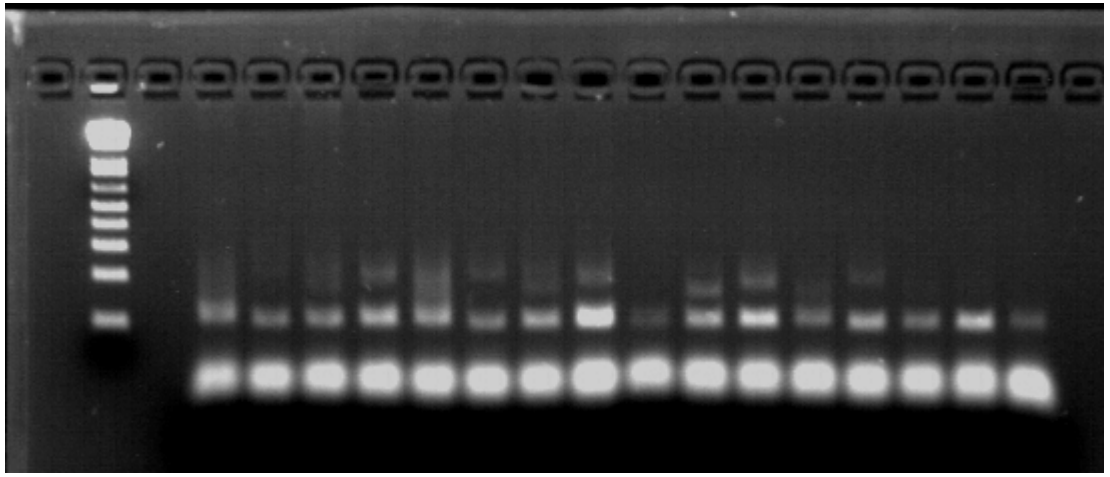
Electrophoresis for PCR with SSR markers in apricot progenies

- Selections,'Kesth Pshor' × 'R13 VT 8/57' and 'R9 P 53' were screened with 3 SSR primer combinations. The products of PCR were separated by electrophoresis on an agarose gel 2% in order to confirm the amplification of fragments of the expected size and DNA concentration.

- 1Kb 1 2 3 3 4 5 6 7



- the SSRs, (PGS1_24)- F:CTCTTCTCGCCTCCCAATTT with R:GCTTAGCCCTGGGTACAAG were significant for the first screening in a lager population and may be useful for starting a MAS in breeding for PPV resistance. Further evaluation of these loci will be necessary to characterize the genetic control of the PPV resistance trait. 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 (Aranzana *et al.* 2003).

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Grafting of the most valuable Romanian varieties, accessions, genitors and local material previously scored for resistance to PPV, within the University collection field.

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- **Results concerning the implement of Spanish markers PGS 1,21 and PGS 1,252 in Romanian progenies**

Individual	PGS 1,21	PGS 1,252	
NJA42	199/239	90/92	resistant
VT10/79	199/239	92/92	resistant
Moongold	193/215	92/122	recombinant
NJA17	193/239	92/122	resistant
SEO	193/239	92/122	resistant
Tabriz	193/213	92/122	recombinant
Resth_phor	-	92/92	recombinant/susceptible
Vivagold	193/193	122/122	susceptible
Viceroy_603_G	193/213	92/122	recombinant
Traiam	193/239	92/122	resistant
C2P20V6	193/239	92/122	resistant
C3P19V4	193/239	92/122	resistant
C2P18V4	193/239	92/122	resistant
C2P18V2	193/239	92/122	resistant
C8P17V5	193/193	122/122	susceptible
C7P18V5	193/239	92/122	resistant
C7P13V4	193/239	92/122	resistant

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Individuals	PGS 1,21	PGS 1,252	Expression of genotype after sequencing
C7P13V4	193/239	92/122	resistant
C1P16V3	193/239	92/122	resistant
C7P16V6	193/239	92/122	resistant
C9P20V3	239/239	92/92	resistant
C7P16V6	193/239	92/122	resistant
C2P18V4	193/239	92/122	resistant
C7P16V2	-	92/122	susceptible
C5P5V4	215/215	92/92	recombinant
C7P18V3	193/239	92/122	resistant
C9P19V1	239/239	92/92	resistant
C7P18V6	193/239	92/122	resistant
C8P16V4	193/193	no 92	susceptible
C7P18V2	193/239	92/92	resistant

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Results concerning the implement of markers PGS 1,24 and Ppb0022-195 in Romanian progenies.

Genotypes	Ppb0022-195 Map distance cM	Ppb0022-195 Map distance cM	PGS1_24 Map distance cM	PGS1_24 Map distance cM	Expression of genotype after sequencing
Kesth_Phor'	112.01		129.53		**sensible**
Viceroy	111.96		100.93		sensible
H2+	107	116.4	98.93	102.9	resistant
	111.98		100.92		susceptible
NJA 17	107.01	112.01	98.9	100.91	resistant
Cristal	111.84		100.78	102.73	susceptible
Sirena	111.81		100.78	127.76	susceptible
Tabriz	112.01		100.88		susceptible
SEO	107.11	111.97	98.9	100.91	resistant
Pop3-37	111.9		100.79		susceptible
Pop3-38	111.83		129.42		susceptible
Pop3-42	111.9		102.73	129.43	susceptible
Pop3-49	111.93		129.42		susceptible
Pop3-64	107.05	111.98	98.89	100.92	resistant
Pop3-65	112		129.62		susceptible
Pop3-68	107.13		98.88	102.93	resistant
Pop3-71					susceptible
Pop3-73	107.08		98.94		resistant
Pop3-76	107.1	112.04	98.94	129.24	resistant
Pop3-77	106.97		98.76		resistant
Pop3-78	111.91		100.81		susceptible
Pop3-79	106.94	111.91	98.81	102.76	resistant
Pop3-80	106.97	111.92	98.78	100.83	resistant
Pop3-81	111.9		100.77		susceptible

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Results concerning the implement of markers PGS 1,24 and Ppb0022-195 in Romanian progenies.

Génotype	Ppb0022-195 Map distance cM	Ppb0022-195 Map distance cM	PGS1_24 Map distance cM	PGS1_24 Map distance cM	Expression of genotype after sequencin
Pop1-20	107.08	112	98.94	100.96	resistant
Pop1-21	111.88		102.75	129.09	susceptible
Pop1-22	111.89		129.18		susceptible
Pop1-55	106.96	111.86	98.8	127.76	resistant
Pop1-72	106.98	11.87	9875	137.97	resistant
Pop2-106	107.01	111.91	102.81	106.46	resistant
Pop2-14	107.03	11.87	98.74	100.78	resistant
Pop2-17	11.92		102.85		susceptible
Pop2-43	11.93		100.89	102.89	susceptible
Pop2-47	107.06	111.99	100.87	106.51	resistant
Pop2-66	111.91		101.81	102.78	susceptible
Pop2-63	111.97		100.85	102.83	susceptible
Pop2-69	107		106.37		susceptible
Pop2-70	111.92		100.81	102.74	susceptible
Pop2-82	111.84		100.75		susceptible
Pop4-104	111.92		102.9	129.28	susceptible
Pop4-19	107.03	111.95	98.88	129.3	resistant
Pop4-45	107.07	111.92	98.83	129.22	resistant
Pop4-46	107.12	111.98	98.94	129.32	resistant
Pop4-48	107.91		98.75		resistant
Pop4-54	106.97	111.91	98.74	129.11	resistant
pop4-59	106.94	111.87	98.74	129.13	resistant
pop5.51	112.3		100.97		susceptible
Pop5-52	112.4		100.95		susceptible
pop5-53	107.1	112.01	98.86	100.94	resistant

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Conclusions

- **The identifying of a natural source of resistance to PPV, using this resistant source into new crosses with Romanian commercial cultivars well adapted in our country, and the implement of marker-assisted selection (MAS), based on markers tightly associated with resistance, as a measure to substantially streamline the breeding process, may be a promising strategy to obtain apricot varieties with natural genetic resistance to PPV.**
- **First couple of markers PGS 1,21 (Reverse and Forward) could be enough for the screening of a lager population of apricot and then start to develop the others SSRs associated with PPV resistance to facilitate the use of MAS in Romanian apricot breeding program**

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