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IDENTIFICAREA UNOR AGENȚI PATOGENI DE TIP VIRAL PRIN TEHNICI SEROLOGICE SI MOLECULARE DE DIAGNOSTIC LA CAIS, PIERSIC ȘI MIGDAL

IDENTIFICATION OF VIRAL PATHOGENS BY SEROLOGICAL TECHNIQUES AND MOLECULAR DIAGNOSTIC TECHNIQUES IN APRICOT, PEACH AND ALMOND

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Abstract

In order to obtain plants of the biological category Pre-basic, plants of the genotypes 'Olimp' and 'De Valu' in apricot, 'Raluca', 'Monica', 'Tomis 1' in peach, 'Mirela' and 'Veronica' in almond were evaluated for the presence of virus-type harmful organisms. Diagnosis was made by the serological DAS-ELISA method for *ACLSV, ApMV, PPV, PDV, PNRSV* and *SLRSV* and by the molecular RT-PCR test for *ESFY, ApLV* and *PLMVd*. No positive samples were found in apricot. In peach the viruses were identified in 'Monica': *ApLV* in 57.15% of the plants tested, *PNRSV* in 14.29% and *PLMVd* in 7.15% of the plants. Two of the positive plants had mixed infections produced by *PNRSV +ApLV* and *PNRSV +PLMVd*. In peach genotype 'Tomis 1', 23.08 % plants were found infected with *ApMV*, 7.7 % with *ACLSV* and 7.7 % with *PPV. ACLSV* and *PPV* infections were identified in the same plant. In almond the diagnosis revealed a mixed infection with *ACLSV + ApMV* (7.7 % of plants from 'Mirela' variety).

Cuvinte cheie: Pre-basic, testare, serologic, molecular, virusuri.
Key words: Pre-basic, testing, serological, molecular, viruses.

1. Introduction

Stone fruit species can be attacked by a very large number of viruses (Nemeth, 1986; Desvignes, 1999; Myrta et al., 2003).

Among the viruses affecting apricot, peach and almond species, those with significant incidence in terms of substantial economic losses and worldwide distribution are *Apple chlorotic leafspot virus (ACLSV), Apple mosaic virus (ApMV), Plum pox virus (PPV), Prune dwarf virus (PDV), Prunus necrotic ring spot virus (PNRSV), European stone fruit yellows phytoplasma (ESFY), Apricot latent virus (ApLV)* in apricot and peach and *Peach latent mosaic viroid (PLMVd)* in peach only (Nemeth, 1986; Desvignes, 1999; Myrta et al. , 2003).

Legislation related to the certification process of biological categories OM 784/2016, OM 119/2020, OM 40/2023, mentions these virus-like pathogens within the organisms from which the biological material must have the status "free from pests".

These pathogens represented by viruses, phytoplasmas and viroids alone or in combination can severely affect fruit yield, fruit maturity and tree growth (Uyemoto et al., 1992).

To date, several serological and RT-PCR-based methods have been reported for fruit tree viruses detections. These methods include the double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (Sertkaya et al. 2006; Rouag et al., 2008; Nourolah et. al., 2013) triple antibody sandwich ELISA (TAS-ELISA) (Elibuyuk et al., 2006), reverse transcription-polymerase chain reaction (RT-PCR) (Rouag et al., 2008, Eichmeir et al., 2016) real time RT-PCR (Eichmeir et al., 2016), reverse transcription loop-mediated isothermal amplification (RT-LAMP), immunocapture RT-PCR (IC-RT-PCR) (Kajic et al., 2018). Because these methods are all time-consuming and require complicated operations and expensive laboratory instruments, they are not suitable for rapid and large scale field surveys.

The aim of this work is to identify the presence of possible harmful organisms of viral and phytoplasma type mentioned in the legislation in force for the production of Pre-basic fruit propagating material in some genotypes of apricot, peach and almond species.

2. Material and methods

Samples were represented by leaves were randomly collected around the canopy from each individual tree and represented one sample. In total 68 leaf samples (one sample/one tree) from 7 different genotypes, including different cultivars and rootstock: **'**Mirela**'**, **'**Veronica**'** cv. (almond), **'**Olimp**'**, **'**De Valu**'** cv. (apricot), **'**Raluca**'**, **'**Monica**'** cv, **'**Tomis 1**'** rootstock (peach).

The samples were collected from selected trees without symptoms. Samples were placed in plastic bags in an ice box and transported to the laboratory.

DAS ELISA (Cark & Adams, 1977). For the initial screening and detection of *ACLSV, ApMV, PPV, PDV, PNRSV, SLRSV,* commercially available DAS ELISA kits (Bioreba AG, Switzerland) were used according to the manufacturer's instructions. The absorbance was read at 405/492 nm with a dual filter microplate reader PR 1200, after 30 min, 1 h and 2 h of incubation. Lyophilised samples supplied in the kit for each virus were used as posi- tive and negative controls. A "cut-off" value was calculated according to manufacturer recommendations (Bioreba AG, Switzerland).

One set of primers was used for molecular diagnostics for *ApLV, ESFY, PLMVd* (Table 1).

The RNA used in the RT-PCR reaction, as well as the DNA used in the PCR reaction for the detection of ESFY phytoplasma, were extracted using the "ISOLATE II Plant RNA / DNA Kit" kit, in accordance with the Bioline manufacturer's protocol.

Amplificarea RT- PCR pentru *ApLV* şi *PLMVd* a fost realizată într-un volum de reacție de amplificare de 20 µl, incluzând următoarele componente în concentrația finală: 13 µl 2x MyTaq One-Step Mix, fiecare dintre primerii F și R: 1 µl de primer (5 µM / µl în volumul final de reacție), 3 µl de ARN (10 ng / µl), 0,2 µl Reverse transcriptase, 0,4 µl RiboSafe RNase Inhibitor și 1,4 µl de apă ultrapură. Amplificarea specifică PCR pentru detectarea fitoplasmei a fost efectuată într-un volum de reacție de amplificare de 15 µl, incluzând următoarele componente în concentrația finală: 11,6 µl MyTaq™ Red Mix, fiecare primer F și R: 0,1 µl primer (0 ,6 µM / µl în volumul final de reacție), 3 µl ADN (10 ng / µl) și 0,3 µl apă ultrapură.

RT-PCR amplification for ApLV and PLMVd was performed in an amplification reaction volume of 20 µl, including the following components in the final concentration: 13 µl 2x MyTaq One-Step Mix, each of primers F and R: 1 µl primer (5 µM/µl in the final reaction volume), 3 µl RNA (10 ng/µl), 0.2 µl Reverse transcriptase, 0.4 µl RiboSafe RNase Inhibitor and 1.4 µl ultrapure water. Specific PCR amplification for phytoplasma detection was performed in a 15 µl amplification reaction volume, including the following components in the final concentration: 11.6 µl MyTaq™ Red Mix, each primer F and R: 0.1 µl primer (0.6 µM/µl in the final reaction volume), 3 µl DNA (10 ng/µl) and 0.3 µl ultrapure water

RT-PCR amplifications were performed in a FastGene PCR analyzer under the following conditions: reverse transcription at 45°C for 20 min, followed by 35 cycles of 45 sec at 95°C, 45 sec at primer annealing temperature (58°C for ApLV and 60°C for PLMVd) , 1 min at 72°C and 10 min at 72°C for final extension), and classical PCR amplification for ESFY under the following conditions: initial denaturation step at 94°C for 2 min, followed by 35 cycles of 2 min. at 94°C, 2 min. at 55°C, 2 min. at 72°C and 10 min. at 72°C final elongation. Evaluation of the PCR product for all primers (ApLV, PLMVd and universal primers used for phytoplasma) was performed using horizontal electrophoresis on 2% agarose gel (Cleaver), 1X TBE buffer and staining with RedSafe Nucleic Acid Staining. The gel was read using a high quality Uvitec Cambridge Essential imaging system using UVITec1D analysis software.

3. Results and discussions

Viral evaluation of apricot varieties did not identify positive samples by the DAS-ELISA serological method for the pathogens *ACLSV, ApMV, PPV, PDV, PNRSV* nor by the molecular method which was used to identify *ESFY* and *ApLV* (Table 2).

In the case of peach varieties (Table 3), the presence of *ACLSV* was identified in the Tomis 1 P1 rootstock following diagnosis. In the same tree, PPV infection was also identified, the tree being therefore affected by mixed *ACLSV +PPV* infection.

ApMV was identified in **'**Tomis 1**'** P2, **'**Tomis 1**'** P8 and **'**Tomis 1**'** P12. Among the varieties tested virus infections were identified only in **'**Monica**'**, two mixed infections **'**Monica**'** P3 *PNRSV+ApLV* and **'**Monica**'** P10 *PNRSV +PLMVd*. *ApLV* virus was also identified in **'**Monica**'** P1, **'**Monica**'** P3, **'**Monica**'** P4, **'**Monica**'** P5, **'**Monica**'** P6, **'**Monica**'** P7, **'**Monica**'** P8, **'**Monica**'** P9.

Although peach was found to be the most infected of the species tested, the diagnostic methods used did not identify plants infected with the pathogens *PDV, SLRSV*, and *ESFY*.

Regarding the degree of infection (%) of the tested peach plants (Fig. 1), it can be seen that the most susceptible variety in peach was the **'**Monica**'** variety against *ApLV* with 57.15 %. This is worrying first of all from the point of view of the speed of spread of the virus being described for the first time in Moldova, in 1993, in apricot variety Silistra 4 introduced from Bulgaria (Grimová, et. al., 2012, Nemchinov, L.G. et. al, 2018) and also identified at RSFG Constanta following these tests. The corresponding amplifications of the 200 bp fragment associated with the viral RNA can be seen in Fig. 2. Our result was in agreement with previous findings indicating the presence of *ApLV* virus by amplification of the same fragment (Nemchinov and Hadidi, 1998; Nemchinov et al., 2000; Abbadi et al., 2003; Pourrahim et al., 2021.

The universal P1/P7 markers used to identify phytoplasma species amplify the full-length 16S rRNA gene, the 16S-23S intergenic region and a small part of the 23S rRNA gene (Guo et al, 2003), and

are efficient for the diagnosis of phytoplasmas of subgroup B, the group (16SrX) to which *'Candidatus Phytoplasma Prunorum/(ESFY)'* belongs, together with *'Candidatus Phytoplasma mali'* and *'Candidatus Phytoplasma pyri'*. Recommendations for the use of PCR testing methods for the amplification of the gene of interest (16S rRNA) are available both in EPPO Bulletins (2008, 2020) and in literature (Pastore et al., 1999; Marcone et al., 2010).

A significant proportion of the plants tested 14.29% were positive with *PNRSV* and 7.15% with *PLMVd*. The Raluca variety was found to be more resistant to the virus, all plants tested were healthy.

In almond, (Table 4) virus tests showed a mixed infection in **'**Mirela**'** P2 with *ACLSV+ApMV*, representing 7.70% infection of the plants tested.

All plants identified as positive in the tests were eliminated don't introduced in certification process for Pre-basic material.

4. Conclusions

A high incidence of *ApL*V infection was identified in peach 57.15% of Pb-Candidate plants tested.

Of the 3 species tested peach was found to be more susceptible, to viral infections compared to apricot and almond.

Peach variety **'**Mirela**'** can be considered as a susceptible variety to *ApLV* infections.

For the biological material tested the highest incidence was *ApLV,* followed by *ApMV, PNRSV, PPV, ACLSV* and *PLMVd.*

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Table and Figures

Table 1. Primers used for molecular diagnosis

No. crt.	Marker name	Primer sequence (F + R)	Tm	Amplified product size (bp)	References
1	*ApLV*	5'-GGAATAGAGCCCCAAGAAG-3' 5'-AGCAAGGTAAACGCCAAC-3'	58°C	200	Nemchinov, et. al. 2000
2	H *PLMVd* /C *PLMVd*	5'-AACTGCAGTGCTCCGAATAGGGCAC -3' 5'-CCCGATAGAAAGGCTAAGCACCTCG-3'	60°C	337	Shamloul, et. al. 2002
3	P1/P7	5'-AAGAGTTTGATCCTGGCTCAGGATT-3' 5'-CGTCCTTCATCGGCTCTT-3'	55°C	1800	Deng et. al, 1991; Schneider et. al,1995

Table 2. Viral status following assessment in apricot cultivars

Assortment	Diagnostic						
	DAS-ELISA					RT-PCR	
	ACLSV	***ApMV***	***PPV***	***PDV***	***PNRSV***	***ESFY***	***ApLV***
Olimp P1	-	-	-	-	-	-	-
De Valu P1	-	-	-	-	-	-	-
De Valu P2	-	-	-	-	-	-	-
De Valu P3	-	-	-	-	-	-	-
De Valu P4	-	-	-	-	-	-	-
De Valu P5	-	-	-	-	-	-	-
De Valu P6	-	-	-	-	-	-	-
De Valu P7	-	-	-	-	-	-	-
De Valu P8	-	-	-	-	-	-	-
De Valu P9	-	-	-	-	-	-	-
De Valu P10	-	-	-	-	-	-	-

Table 3. Viral status following assessment in peach cultivars

Assortment	Diagnostic								
	DAS-ELISA						RT-PCR		
	ACLSV	*ApMV*	*PPV*	*PDV*	*PNRSV*	*SLRSV*	*ESFY*	*ApLV*	*PLMVd*
Raluca P1	-	-	-	-	-	-	-	-	-
Raluca P2	-	-	-	-	-	-	-	-	-
Raluca P3	-	-	-	-	-	-	-	-	-
Raluca P4	-	-	-	-	-	-	-	-	-
Raluca P5	-	-	-	-	-	-	-	-	-
Raluca P6	-	-	-	-	-	-	-	-	-
Raluca P7	-	-	-	-	-	-	-	-	-
Raluca P8	-	-	-	-	-	-	-	-	-
Raluca P9	-	-	-	-	-	-	-	-	-
Raluca P10	-	-	-	-	-	-	-	-	-
Raluca P11	-	-	-	-	-	-	-	-	-
Monica P1	-	-	-	-	-	-	-	+	-
Monica P2	-	-	-	-	-	-	-	-	-
Monica P3	-	-	-	-	+	-	-	+	-
Monica P4	-	-	-	-	-	-	-	+	-
Monica P5	-	-	-	-	-	-	-	+	-
Monica P6	-	-	-	-	-	-	-	+	-
Monica P7	-	-	-	-	-	-	-	+	-
Monica P8	-	-	-	-	-	-	-	+	-
Monica P9	-	-	-	-	-	-	-	+	-
Monica P10	-	-	-	-	+	-	-	-	+
Monica P11	-	-	-	-	-	-	-	-	-
Monica P12	-	-	-	-	-	-	-	-	-
Monica P13	-	-	-	-	-	-	-	-	-
Monica P14	-	-	-	-	-	-	-	-	-
Tomis 1 P1	+	-	+	-	-	-	-	-	-
Tomis 1 P2	-	+	-	-	-	-	-	-	-
Tomis 1 P3	-	-	-	-	-	-	-	-	-
Tomis 1 P4	-	-	-	-	-	-	-	-	-
Tomis 1 P5	-	-	-	-	-	-	-	-	-
Tomis 1 P6	-	-	-	-	-	-	-	-	-
Tomis 1 P7	-	-	-	-	-	-	-	-	-
Tomis 1 P8	-	+	-	-	-	-	-	-	-
Tomis 1 P9	-	-	-	-	-	-	-	-	-
Tomis 1 P10	-	-	-	-	-	-	-	-	-
Tomis 1 P11	-	-	-	-	-	-	-	-	-
Tomis 1 P12	-	+	-	-	-	-	-	-	-
Tomis 1 P13	-	-	-	-	-	-	-	-	-

Table 4. Viral status following evaluation in almond cultivars

Assortment	Diagnostic					
	DAS-ELISA					RT-PCR
	ACLSV	*ApMV*	*PPV*	*PDV*	*PNRSV*	*ESFY*
Mirela P1	-	-	-	-	-	-
Mirela P2	+	+	-	-	-	-
Mirela P3	-	-	-	-	-	-
Mirela P4	-	-	-	-	-	-
Mirela P5	-	-	-	-	-	-
Mirela P6	-	-	-	-	-	-
Mirela P7	-	-	-	-	-	-
Mirela P8	-	-	-	-	-	-
Mirela P9	-	-	-	-	-	-
Mirela P10	-	-	-	-	-	-
Mirela P11	-	-	-	-	-	-
Mirela P12	-	-	-	-	-	-
Mirela P13	-	-	-	-	-	-
Veronica P1	-	-	-	-	-	-
Veronica P2	-	-	-	-	-	-
Veronica P3	-	-	-	-	-	-
Veronica P4	-	-	-	-	-	-
Veronica P5	-	-	-	-	-	-
Veronica P6	-	-	-	-	-	-

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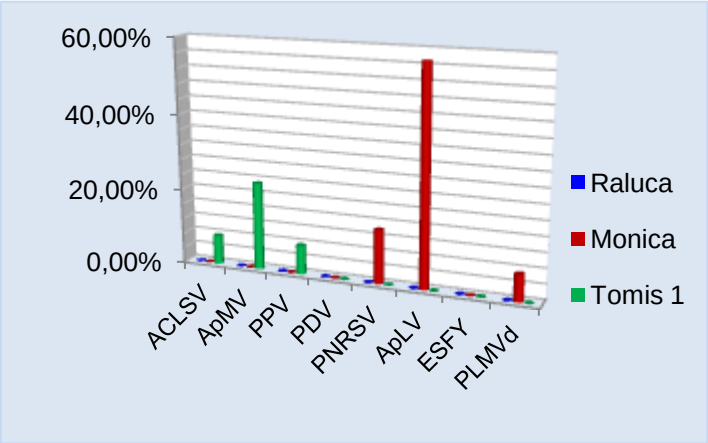

Fig 1. The degree of infection (%) of the tested peach plants

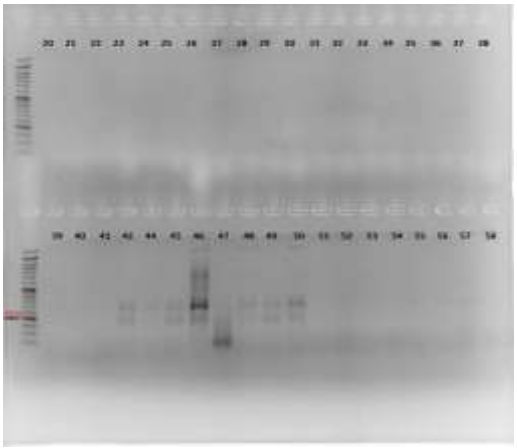

Fig 2. The fragments amplified with the pair of primers used for the diagnosis of *Apricot Latent Virus*

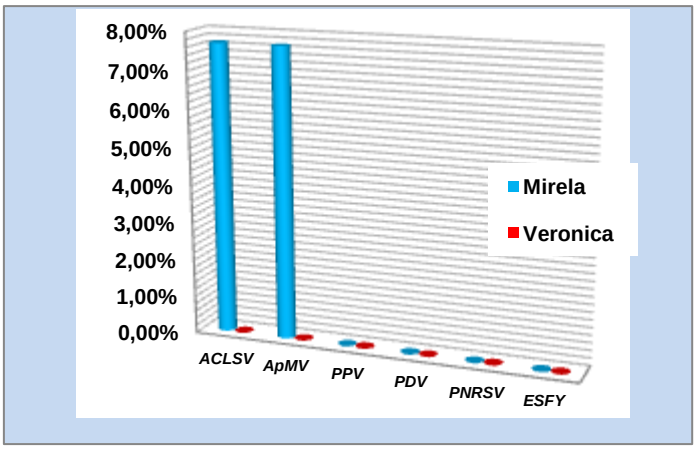

Fig. 3. The degree of infection (%) of the tested almond plants