World Journal of Advanced Research and Reviews
eISSN: 2581-9615 CODEN (USA): WJARAI
Cross Ref DOI: 10.30574/wjarr
Journal homepage: https://wjarr.com/

(RESEARCH ARTICLE)

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The study of the phenotyping process of some Romanian cherry genotypes

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World Journal of Advanced Research and Reviews, 2023, 19(02), 575–582

Publication history: Received on 01 July 2023; revised on 09 August 2023; accepted on 11 August 2023

Article DOI: https://doi.org/10.30574/wjarr.2023.19.2.1606

Abstract

The *Plum pox virus (PPV)*, is considered to be particularly serious in stone species, has appeared in research and documents since 1915 from which revelations emerge about the first symptoms appearing in orchards in central and eastern Europe, where the *Plum pox virus* (PPV) being so viral, it caused serious production losses in species such as plum, nectarine, cherry, almond, sour cherry, nectarine and last but not least apricot, where losses were 100%. The creation of new varieties with natural genetic resistance to *Plum pox virus* remains one of the most solid ways to limit the spread of this quarantine virus. The Elisa serological technique and the molecular PCR technique offer the possibility of highlighting the presence or absence of the virus in the plant.

The present study illustrates the results of phenotyping tests (indexing on different rootstocks, Elisa and PCR) for some autochthonous cherry genotypes. Even if it is difficult to establish a definitive hypothesis regarding the genetic control of resistance to Plum pox virus in cherry, we still observe an important number of graft/rootstock combinations, such as: Margarit / Otesani 11, Mari de Malu / Otesani 11, Rosu de Mai/ Otesani 11, which showed a resistance to the artificial infection with *Plum pox virus*. These genotypes are of interest in future breeding works.

Keywords: Cherry; Phenotyping; Genotypes; Plum Pox Virus

1. Introduction

In terms of implications for actual fruit production, infection of plantations with *Pum pox virus* can lead to considerable losses. Thus, at the level of the whole of Europe, approximately 100 million trees belonging to stone species are infected and, in some sensitive varieties, losses can reach 80-100% [1]. Planting sensitive varieties within each stone fruit species for eastern and central Europe can lead to premature fruit drop and cracks on their surface and automatically to significant production decreases. The first symptoms in cherry were described as manifestations of circular spots of chlorotic or necrotic nature and premature fruit drop. [2].

Limiting the spread of the *Plum pox virus* can only be the result of integrated preventive, ameliorative and curative measures [3]. Control and prevention measures for *Plum pox virus* include careful observation of orchards (detection and removal of infected trees), use of virus-free (certified) propagating material, cultivation of resistant varieties, control of aphid populations, and removal from nurseries and orchards of infected host plants. Once a plantation is infected, the only option to control the spread is clearing and burning the infected biological material [4].

1.1. Detection of *Plum pox virus* infections and removal of affected trees

Plum pox virus strains differ in severity of associated symptoms, efficiency of transmission by aphids, host plant and geographic distribution. To achieve successful eradication or effective management of the associated disease, virus strain testing is required.

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The expression of the symptoms is different depending on the stage of the infection, the variety, the period in which the infection occurs and the growing season [5].

The distribution of the virus in the tree can be irregular. However, obtaining samples from flowers, young leaves, old leaves, fruits, dormant wood and roots is possible. The time of year when the sample is collected is critical and can greatly affect test results. Plum pox virus can be detected by all the mentioned techniques, in the symptomatic parts of the leaves in May and with one exception even at the beginning of August (when due to the high temperatures, the replication rate is low, the viral concentration is lower), generally in the spring or early summer (when temperatures are between 18-28 °C), but may vary from season to season depending on weather conditions. The time of full bloom has been shown to be a good source of Plum pox virus detection [6].

Plum pox virus is more difficult to detect in asymptomatic leaves using field tests, DAS-ELISA and partially also molecular techniques [7], [8].

During the first years after the appearance of the virus in plantations, the number of affected trees remains quite low, which gives the impression that the speed of spread is low, but once the 10% threshold is reached, the number of trees infected annually increases exponentially. That is why it is absolutely necessary to detect and remove infected trees from the plantations in the early stages of the spread of the virus [9], [10].

Using modern methods of variety selection with the help of genetic engineering, new genotypes resistant to some diseases and pests are created. These genotypes require a minimum number of treatments, limit the propagation of viruses, reduce phytotoxicity with important economic advantages. Depending on the genetic characteristics of the plant, some strains of some viruses can circumvent this natural or induced resistance of the varieties [11], [12]. There is the phenomenon of tolerance in the phenotypic and genotypic manifestation, which represents the ability of plants to withstand a severe attack of a disease without registering significant losses in terms of production and quality [13], [14]. The manifestation of a resistant variety, externalizes a good development of the plant and production despite a good infection of the pathogen but whose propagation remains limited to some organs of the plant or at the level of spots in the tissues. The manifestation of the tolerance reaction is a special type of reaction, totally separate from the resistance phenomenon, which cannot be equivalent to the pathogenic or horizontal resistance, although it is polygenically controlled [15].

Obtaining hybrids with resistance to Plum pox virus follows two strategies: resistance derived from the pathogen determined by creating genetically modified organisms and natural resistance obtained through hybridization. The resistance derived from the pathogens uses the virus, its genomic sequences that are introduced into the plum genome [16].

2. Materials and methods

The plant material consists of different autochthonous cherry cultivars were grafted onto different rootstocks, then infected in the parcel area by chip-budding with Plum pox virus-infected material. Samples were collected from both rootstocks and grafts, on which Elisa and RT PCR immunological tests were performed, evaluation in terms of resistance to Plum pox virus.

The plant material consisted of:

The varieties used Genotypes Rival (rootstocks), Mari de Malu, Mărgărit, Iosif, Alex, Ludovic and Oteşani 11 and Mahaleb (rootstocks).

2.1. Work methods

2.1.1. Elisa immunological test

This test or technique was introduced in 1976 by Clark and Adams, and the first test was done to determine the presence of Plum pox virus in pits. There are many variants regarding the name of this type of test, the most common being "double antibody sandwich". The technique allows the detection of viruses even in very small quantities, and if necessary the viruses can be detected even directly from the vectors.

The principle on which this test is based is the possibility of coupling antibodies with an enzyme and fixing them. Thus, to detect the presence of the virus in a plant, we only need to add the antibodies corresponding to the pathogen we want to detect in the presence of a sample from the plant we are interested in.

For the ELISA method the leaves (samples) were mortared in extraction buffer (AFT 0.2% + Dieca 2% + PVP - 10) and were placed in the holes of a special Elisa plate previously coated with conjugated polyclonal antibodies, conjugated immunoglobulins (anti - Plum pox virus) and incubate at 4 0C for 16h. After 3 washes (with AFT-Tween) 200 µI Plum pox virus -specific monoclonal antibodies were added and incubated at 37 0C for 2 hours. The last step was the addition of immunoglobulins conjugated with alkaline phosphatase 1:1000 (200µI) and incubated for 2 h at a temperature of 37 0C. The reading was done at a wavelength of 405 nm, considering the positive values exceed twice the reading value of the negative test (T-x 2). To carry out the ELISA test, a support represented by a polystyrene plate is needed, antibodies specific to the virus we are detecting (immunoglobulins of type G: IgG), the same antibodies with an enzyme and a substrate with this enzyme.

The polystyrene plate has 96 compartments, the ones on the edge not being used for the test, so 60 compartments remain available. Two samples are also used, one from a healthy plant and one from an infected plant (the samples must be extracted from the same type of organ, and the plants from which the samples are extracted must belong to the same plant species to which the plant for which perform the test) as well as a control sample. The samples from the plants to be tested are mixed with a basic BPS-tween buffer solution (specific to Plum pox virus), and then they are centrifuged or filtered (what results from this filtration/centrifugation is used in the actual determination).

2.2. PCR TEST (genetic amplification technique)

PCR (Polymerase Chain reaction), as a technique allows the in vitro amplification of a DNA sequence by repeating an elongation reaction in the presence of DNA polymerase. The discovery of a thermophilic bacterium that lives at temperatures of 70 to 75 degrees C in Yellowstone National Park in the United States of America (Thermus Aquaticus) and the possibility of using its polymerase (which withstands temperatures up to 100 degrees C), is the basis of the development this technique. Previously, total RNA was isolated from cherry samples, the varieties studied and presented in the table below. The QIAshredder kit (under-column extraction) was used for isolation. In parallel, we worked with two techniques IC-RT-PCR and RT-PCR (QHIAGEN kit). The primers used for diagnosis were P1 and P2.

3. Results and discussions

Plum pox virus, is the cause of one of the most destructive and feared diseases of the genus Prunus (Sharka), the main viral disease of fruit trees from the stone group (such as peach, plum, apricot or cherry) with important economic impact on fruit production. The current research proposed the involvement

The graft-rootstock relationship is an interaction relationship through which the two partners influence each other so that in terms of the interaction with Plum pox virus, the resistance of the rootstock can block the propagation of the virus at the level of the graft. The samples were collected at the base, middle and tip of the shoot so that we have a holistic picture of the viral infection.

Following the tests, it was demonstrated that the rootstocks Rival and Oteşani 11 showed forms of resistance to Plum pox virus, which associated with the resistance of the native varieties Mărgărit and Mari de Malu showed an interesting resistance to be studied in terms of the impact on the Plum pox virus.

In the Margărit variety, although weak symptoms were highlighted in the basal area near the grafting point, the virus was no longer detected in the middle or at the tip of the shoot, which means that the plant's resistance barriers to the impact with the virus were efficient and the variety can be tolerant or partially resistant to Plum pox virus under artificial infection conditions.

Table 1 Phenotyping Romanian cerries progenies F1 in artificial infection conditions for resistance/tolerance to Plum pox virus; by visual inspection and ELISA and RT-PCR analysis

Combination	**Varieties/portals**	**Variants**	**Parent stock**			**Cherry varieties**		
			Plum pox virus symptoms intensity	DASI – ELISA (DO= 405nm)	RT-PCR	Plum pox virus symptoms intensity	DASI-ELISA (DO= 405nm)	RT-PCR
C1	Iosif/Mahaleb	V3P1	+	+	+	-	-	-
C2	Alex/Mahaleb	V2P1	+	+	+	-	-	-
		V4P2	+	+	+	-	-	-
		V6P3	+	+	+	-	+	+
C3	Ludovic/Mahaleb	V4P1	+	+	+	-	+	+
C4	Mărgărit/Mahaleb	V2P1	+	+	+	-	-	-
C5	Mari de Malu/Mahaleb	V4P2	++	++	+	+	+	+
C6	Roşu de Mai/Mahaleb	V5P1	+	+	+	-	-	-
C7	Iosif/ Oteşani 11	V1P19	++	++	+	+	+	-
		V2P16; V2P18	+ +	+ +	+ +	- -	- -	- -
		V3P18	++	+	+	-	-	-
		V4P19	+	+	+	-	-	-
		V5P18	+	+	+	-	-	-
		V6P16; V6P18	+ +	+ +	+ +	- -	- -	- -
C8	Alex/Oteşani 11	V2P17	+	+	+	-	-	-
		V4P16	+	+	+	-	-	-
C9	Ludovic/ Oteşani 11	V3P20	+	+	+	+	+	+
C10	Mărgărit / Oteşani 11	V1P1	-	-	-	-	-	-
C11	Mari de Malu/ Oteşani 11	V2P1	-	-	-	+	+	+
C12	Roşu de Mai/ Oteşani 11	V3P1	-	-	-	-	-	-

Plum pox virus infection in grafts and rootstocks was assessed after each dormancy cycle by assessing the presence of symptoms and subsequently confirmed by DASI-ELISA. The inoculation efficiency was very high and more than 95% of the rootstocks "Miroval" and Mahaleb developed symptoms. However, the distribution of symptoms was highly irregular among young shoots, preventing evaluation of viral symptoms in intermediate classes. Plum pox virus resistance phenotype was scored as resistant or susceptible to avoid misclassifications. Most of the young shoots were detected after the first cycle, but about 15-40% (depending on the population) were resistant in cycle 1 and turned out to be susceptible in cycle 2.

Evaluation after additional cycles did not detect significant changes.

Even if the difficulty in establishing a definitive hypothesis regarding the genetic control of resistance to the Plum pox virus in cherries, we observe an important number of combinations, such as: Margarit / Otesani 11, Mari de Malu / Otesani 11, Rosu de Mai / Otesani 11, showed resistance to artificial infection with Plum pox virus.

The native cherry cultivars evaluated were initially classified into two groups: susceptible to Plum pox virus and resistant to Plum pox virus. After that, the most important genotypes were grafted onto infected GF 305 under greenhouse conditions.

The next step of this work is the introduction and development of marker-assisted selection (MAS) rootstock varieties and hybrid offspring obtained after pollination in order to improve natural resistance to Plum pox virus.

Interestingly, among the 6 varieties studied, this aspect of virus resistance revealed that three elite varieties Mărgărit, Roşu de Mai, Iosif, were not detected Elisa positive, which could open new avenues of research and molecular analysis.

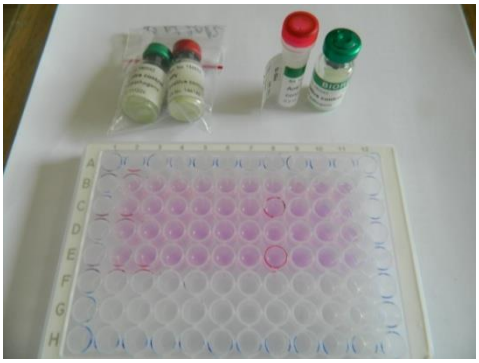

Figure 1 Elisa test reaction results

Figure 2 Elisa test reaction results

Assumptions regarding resistance to viral genetic control, for cherry consider it to be the dominant resistance allele.

The infection process was different for each individual (genotype).

Primers P1 and P2 are used for both diagnosis and differentiation of Plum pox virus strains, Marcus and Dideron P1 – Pd and P2 – Pm.

The temperature gradient PCR results determined that the best recipe for annealing temperature is below i.e. 610C - 45 seconds

- 1. reverse transcription - 50^0C - 40 minutes
- 2. polymerase activation - 95^0C - 15 minutes
- 3. distortion - 94^0C - 45 minutes
- 4. primer attachment - 61^0C - 45 seconds
- 5. extensión - 72C - 1 minute
- repeating steps 3 – 5, 35 times

- 6. final extension - 72^{0}C - 10 minutes
- 7. cooling - 4^{0}C

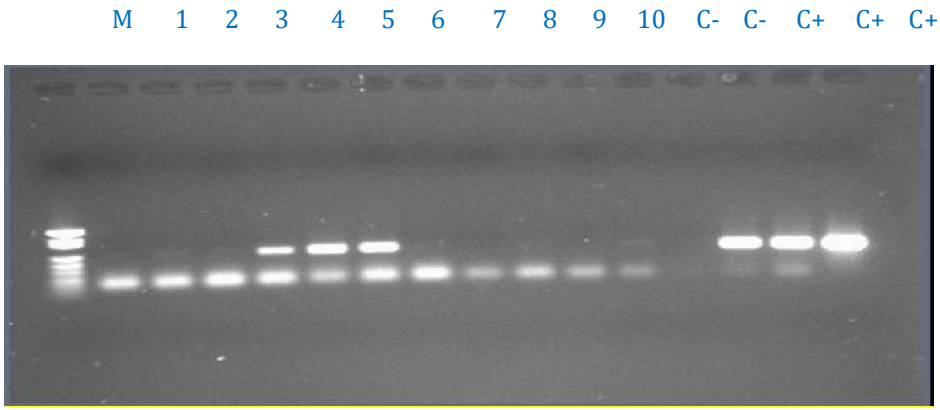

Figure 3 Results regarding agarose gel electrophoresis in cherry cultivars in RT-PCR

- M - waist marker
- 1 – the Iosif variety
- 2 – the Alex variety
- 3 – the Ludovic variety
- 4 – Mahaleb genotype (rootstocks)
- 5 – Miroval genotype ditto
- 6 – Tuleu dulce genotype ditto
- 7 – genotype Oteşani 11 (rootstocks)
- 8 – the Mărgărit variety
- 9 – the Mari de Malu variety
- 10 – the Roşu de Mai variety
- C+ - positive control
- C- - negative control

The results from the photo of the 1.5% agarose gel demonstrate the presence of bands, that is, the presence of the Plum pox virus in Mahaleb, Tuleu Dulce, Miroval rootstocks as well as the Roşu de Mai variety, compared to the two positive control samples.

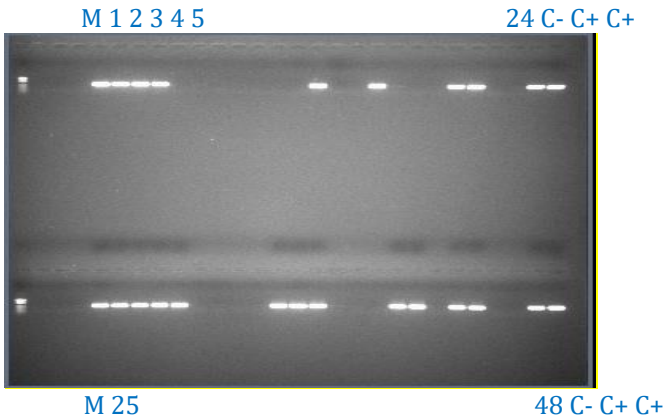

Figure 4 Image with Real Time Polymerase Chain Reaction (RT-PCR)

The genotypes Rival (rootstocks), Mari de Malu, Mărgărit and Oteşani 11 (rootstocks) proved to be resistant to Plum pox virus, which was demonstrated both serologically and molecularly by the PCR method.

4. Conclusions

The obtained results show that initially the viral infection is determined by a visual inspection of the symptoms, followed by Elisa serological tests. For the genotypes that did not show symptoms either visually or after the Elisa test, they can be molecularly tested in PCR, using detection primers P1 and P2.

Among the 6 Romanian varieties studied, this aspect of resistance to viruses and Plum pox virus in particular revealed that three varieties Mărgărit, Mari de Malu and Roşu de Mai as well as the rootstocks Oteşani 11 and Rival were not detected Elisa positive which could open new research perspectives and molecular analyses.

The hypothesis of the gene for the control of virus resistance in cherry species for the Rival (rootstocks), Mari de Malu, Mărgărit and Oteşani 11 (rootstocks) genotypes, which proved to be resistant to Plum pox virus, demonstrated both serologically and molecularly by the PCR method, is perfectly valid and to be determined by molecular tests.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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