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Studying tolerance of prune (*Prunus domestica*) to the plum pox virus (PPV) by criterion "Efficiency of microshoots' regeneration" in controlled *in vitro* conditions

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Abstract

The relevance of the study is determined by the significant economic importance of plum pox virus (PPV) in fruit growing and nursery work. The plum pox disease caused by the PPV is considered to be the most dangerous viral disease of stone fruit crops worldwide. To date, creation of resistant varieties and production of virus-free planting material have remained the only way of fighting this disease. The paper describes such type of resistance as tolerance, where prune varieties preserve normal (close to the mean annual specific variety) productivity of fruits with the proven presence of virus. This paper is aimed at studying efficiency of shoots' regeneration by explants from symptomatic and asymptomatic mother trees of prune that are PPV carriers in the course of microcloning *in vitro.* The leading method of studying this problem was the method of clonal micropropagation of plants. The experiment involved three varieties of prune - Stanley, Donetskaya, and Kubanskaya Early. During the clonal micropropagation, the level of microshoots' regeneration by explants taken from symptomatic and asymptomatic trees that are carriers of PPV had been studied. The virus-carrying nature of prune plants was confirmed by PCR testing. The results of microshoots' regeneration show that explants from asymptomatic plants strike root and regenerate microshoots significantly more efficiently than explants from symptomatic plants. The materials of the article may be useful for virologists, plant breeders, and stone fruit crops nurseries.

Keywords: prune, PPV, tolerance, clonal micro-propagation, regeneration of microshoots.

INTRODUCTION

Modern fruit agrobiocenoses are subject to increasing exposure to viral infections [1-6]. A solution to the problem of wide spreading of viral diseases of fruit crops may be radical quarantine measures, production of improved planting stock, or creation, selection and propagation of varieties, forms and clones that are resistant in varying degrees to the viruses [7], for example, tolerant ones.

In the scientific literature, tolerance is interpreted as a state of resistance to pathogens. Let us remind that the main types of resistance are immunity (complete resistance), tolerance and hypersensitivity [8]. Tolerance of prune (*Prunus domestica*) to the plum pox virus (PPV) is a widespread phenomenon. It is due to tolerance to pox that fruit growing in the South of Russia is dominated by prune varieties Stanley and Krasnodarskaya Early. At the same time, the nature of prune tolerance to viruses has been insufficiently studied. For example, it is not clear how prune tolerance to the PPV correlates with the development of symptoms on leaves of infected trees.

This paper is aimed at studying efficiency of shoots' regeneration by explants from symptomatic and asymptomatic mother trees of prune that are PPV carriers in the course of microcloning *in vitro.* We will examine the form of tolerance which is manifested in the weakening of the symptoms, or their complete absence in infected plants [9]. According to this understanding, tolerance of asymptomatic carrier trees should be expressed stronger than that of symptomatic.

The work has been performed on the example of tolerance to PPV, being the most malicious object of this crop. Relevance of the study is determined by the important economic effect of PPV in nursery work and horticulture.

Studying tolerance of prunes during clonal micropropagation *in vitro* allows conducting analysis in the most controlled conditions with the greatest possible accuracy. Available literature contains no information about studying prune tolerance to PPV by the "efficiency of microshoots' regeneration" criterion in the crop *in vitro;* that is where the novelty of the research lies.

LITERATURE REVIEW

It is known that mass uncontrolled spread of viruses and phytoplasms, and other chronic diseases is one of the major reasons for fruit plantations' degradation. In terms of damage to fruit crops, viruses and phytoplasms take the third position after fungi and pests [10].

In the world and in Russia in particular, the most malicious virus disease of prunes is the PPV [11], which may cause losses up to 85-100% in susceptible varieties' yield due to deterioration of fruit quality and their premature abscission [12-18].

In general, virus diseases affect almost all parameters of the plant: the state and functional activity of the photosynthetic apparatus, the activity of the enzyme systems, consumption and accumulation of mineral elements, architectonics and the rate of passing phenological phases [19, 20].

With that, most viral diseases do not cause lethal changes in plants. Virus diseases in fruit plants usually delay development, stimulate abnormal growth processes, and transformation of organs (from generative into vegetative). However, the hidden, latent course of viral diseases does not pass unnoticed for plants. In comparative study of the intensity of photosynthesis and transpiration, roots and shoots' growth, enzymatic activity, etc., changes are observed in infected plants [21].

It also fully applies to tolerant plants. Prunes have many varieties that show tolerance to infection with the PPV. When infected with pox, these varieties in stable environmental conditions virtually do not lose productivity and fruit quality [22]. Other manifestations of prune tolerance to PPV have been less studied.

The nature of plants' tolerance to viruses has not been completely studied. It is believed that tolerance is determined by changes in plant metabolism caused by penetration and

reproduction of viruses. Upon accumulation of viral particles in the cells of plants, resources of the host are used, and the normal state and functioning of individual cells and their organelles are directly violated. At the same time, various defense mechanisms of the host plant get activated, which limit the spread of the virus, and require some transformation of metabolism [23, 24].

Various forms of tolerance are known. In some cases, the virus in tolerant plants propagates throughout the entire plant and gets accumulated without clear symptoms of the disease [25, 26], in other cases, virus propagation in a plant is inhibited, but the symptoms of the disease are clearly visible, and the third form of tolerance is weakening (until complete absence) of symptoms, and weak virus accumulation [9].

For prune, typical example of tolerant variety is Stanley. PPV causes in it specific mosaic, wrinkling and spots on leaves and mild mosaic on the fruit [22]. Usually, the damage caused by the virus to the Stanley variety is limited to that. The following varieties: Stanley, Kabardinskaya Early, Anna Shpet, and others are known to be tolerant to the PPV.

In these varieties, tolerance to PPV is manifested in preservation of the normal (close to the average for a specific variety) fruit yield with the proven presence of the virus. The symptoms of infection by PPV are typically manifested and transmitted to vegetative offspring [18].

The practice of virological research shows, however, that there are exceptions – the symptoms do not appear in all vegetative offspring (seedlings) grown from the infected nursery plants. *In vitro* cultivation of explants from symptomatic and asymptomatic plants in identical and strictly monitored conditions will allow to establish the relationship between the symptomatic nature and the level of tolerance of experimental plants. Answering the question about whether the asymptomatic nature of prune plants that are PPV carriers is related to strengthening of tolerance in part of the vegetative offspring will provide if not a totally new approach to creating tolerant clones, then, at least, solving the problem of growing healthy planting material of this crop.

MATERIALS AND METHODS

The work was completed in 2011-2017. The research was made on the Donetskaya, Kubanskaya Early, and Stanley (reference) prune varieties. Variety Stanley was used as reference, due to its veracious, stable, and well-studied tolerance to PPV[18]. The source trees are growing at LLC "RPC "Gardens of Chechnya" (settlement Dzhalka). The total of over 10,000 trees of experimental varieties were examined for infection with PPV.

In the collection of plants infected with PPV, symptomatic and asymptomatic samples were isolated with 20 trees of each variety. Both were diagnosed for infection with PPV. Testing was performed at the Laboratory of Genetics and Molecular Biology of the Chechen State University. In testing, a set of reactant for diagnosing PPV by the method of RT-PCR with primers and reaction mixtures prepared at LLC "Agrodiagnostika" was used. Sampling and diagnostics were performed in May – in the period of the maximum accumulation of virus particles in the leaves of prune trees.

Explants of the experimental varieties were introduced to the crop *in vitro* onto the nutrient media prepared by the prescriptions of Murashige and Skoog (MS) [27], Gamborg B_5 [28], modified MS [29]. Growth regulators were 6-BAP (0.1 mg/l for MS and B_5, 0.2 mg/l for modified MS), GK (0.1 mg/l for all variants) and succinic acid 0.1% (4 mg/l for modified MS), medium pH 5.4 to 5.6.

For introduction *in vitro,* apexes of vegetative terminal shoots were isolated with the size of 1-3 mm. The source material – 1 cm long tips of shoots were cleaned of stipules and covering leaves, and washed in the running water for 2 hours. Then they were sterilized in 0.1% solution of mercury iodide for 30 seconds. After washing three times in sterile distilled water apexes of the given size were extracted and used as explants. In each variant of the experiment (variety x growing medium x phytosanitary status (symptomatic or asymptomatic)) 20 explants were introduced into the *in vitro* culture.

The explants planted on the nutrient medium were cultivated on shelves with top-side illumination. The lighting sources were fluorescent lamps LDC-80. Prune explants were cultivated in 16-hour light day, with illuminance of 3.5-5 thousand Luxes, at 23-26° C and relative humidity of 70-75%.

The obtained results were processed using variance analysis. All required calculations were made using applications MS Office (Excel) and Stat Soft STATISTICA 7.0.

The basis of the used methodological approaches were the following publications: Innis, M. A. PCR protocols, a guide to methods and applications [30]; Diagnostics of several quarantine phytopathogens by the PCR method with fluorescent detection of results using diagnostic kits made by LLC "Agrodiagnostika" [31]; Methodical recommendations for using biotechnological methods in working with fruit, berries and decorative crops [29]; Mathematical methods in biology, 2004. [32].

RESULTS AND DISCUSSION

During the study of tolerance to PPV, symptomatic and asymptomatic source trees of this crop were tested for the presence of the virus. By the results of PCR analysis it was found that symptomatic and asymptomatic trees of prune varieties Donetskaya, Kubanskaya Early and Stanley were infected with PPV.

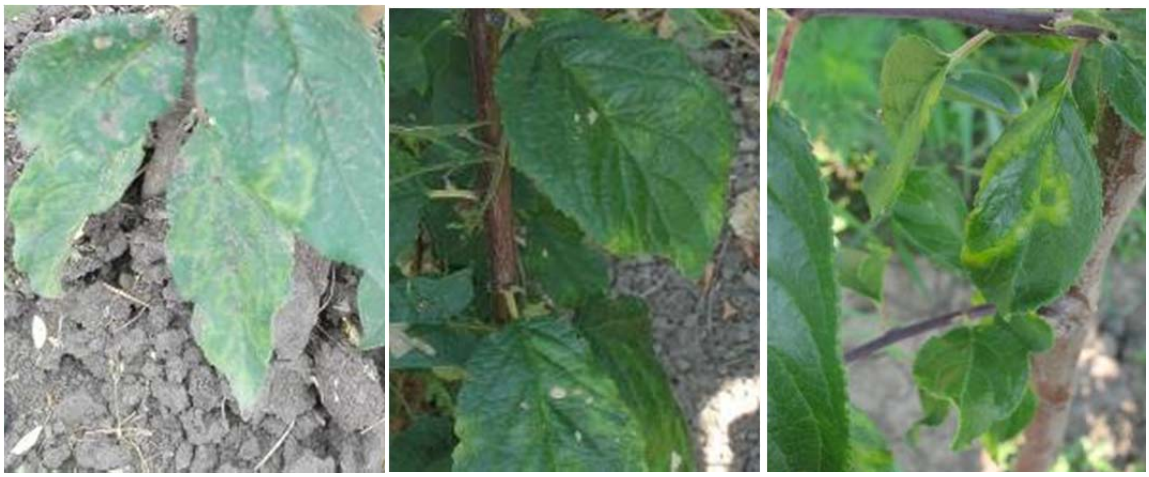
The presence or absence of symptoms of viral infection is itself a symptom of various susceptibility of prune varieties to infection: more resistant varieties (clones) do not show symptoms of infection when infected (reproduction from infected mother trees), less stable varieties show such symptoms [9]. In our case, some trees of the same variety showed symptoms of infection with PPV in the form of specific annular spots (Fig. 1), while some trees did not show any symptoms, which was a symptom of varying susceptibility.

In the next phase of the research (*in vitro*), against the background of other equal conditions (composition of the nutrient medium, humidity, sterility, photoperiod, lighting, etc.), the differences in the success of regenerating microshoots with explants of experimental varieties from symptomatic and asymptomatic source trees that were carriers of PPV were studied.

Results of microshoots' regeneration in the crop *in vitro* are shown in Figure 2 and in Tables 1 to 6.

Analyzing the content of Table 1, we can see that in the Donetskaya variety, out of every 20 apexes planted into various nutrient media, successfully regenerated microshoots of 12-16 (the average of 14.3, or 71%) explants isolated from asymptomatic mother plants. In explants from symptomatic trees, microshoots took root and regenerated 7 to 12 meristems from every 20 planted onto the experimental nutrient media (on the average - 10 pcs. or 50%). The decrease in the efficiency of microshoots' regeneration with symptomatic explants, compared to asymptomatic ones, was 21% (Table 1).

Variance analysis of the results of microshoots' regeneration from explants of variety Donetskaya (Table 2) showed that the phytosanitary status (symptomatic or asymptomatic seedlings) significantly affected the studied parameter (Fact.> Ftab.). The share of influence of the factor is 58.3 (Table 2).



a **b** **c**

Figure 1 – Prune leaves symptomatic with the PPV varieties Donetskaya (a), Kubanskaya Early (b), and Stanley (c)

a **b** **c**

Figure 2 – Regeneration of prune microshoots *in vitro* with explants of varieties Donetskaya (a), Kubanskaya Early (b), and Stanley (C)

Table 1 – Results of microshoots' regeneration *in vitro* with explants from asymptomatic and symptomatic prune trees of the Donetskaya variety that are PPV carriers

Nutrient medium variant	Phytosanitary status	Planted meristems, pcs/%	Successfully regenerated microshoots	
			pcs	%
1. MS	asymptomatic	20/100	15	75
	symptomatic	20/100	11	55
2. Gamborg	asymptomatic	20/100	12	60
	symptomatic	20/100	7	35
3. Modified MS medium	asymptomatic	20/100	16	80
	symptomatic	20/100	12	60
On the average	asymptomatic	20/100	14.3	71
	symptomatic	20/100	10	50

Table 2 - Variance analysis of the results of microshoots' regeneration *in vitro* with explants from source prune trees symptomatized with the PPV, and asymptomatic prune trees that are PPV carriers (factor 1 - medium, factor 2 - presence or absence of symptoms of PPV), variety Donetskaya

Variability	Degrees of freedom	Average square	F-ratio	F st. at 0.05	Dispersion	Share of effect
The number of regenerated meristems						
Between media	2	22.33	3.72	5.14	0.00	0.0
Between symptomatic and asymptomatic (the phytosanitary status)	1	56.33	9.39	5.99	8.39	58.3
Interaction: medium x phytosanitary status	2	0.33	0.056	5.14	0.00	0.0
Residual	6	6.0	-	-	6.00	41.7

Table 3 – Results of microshoots' regeneration *in vitro* with explants from asymptomatic and symptomatic prune trees of the Kubanskaya Early variety that are PPV carriers

Nutrient medium variant	Phytosanitary status	Planted meristems, pcs/%	Successfully regenerated microshoots	
			pcs	%
1. MS (st)	asymptomatic	20/100	16	80
	symptomatic	20/100	7	35
2. Gamborg	asymptomatic	20/100	13	65
	symptomatic	20/100	3	15
3. Modified MS medium	asymptomatic	20/100	13	65
	symptomatic	20/100	8	40
On the average	asymptomatic	20/100	14	70
	symptomatic	20/100	6	30

Table 4 - Variance analysis of the results of microshoots' regeneration *in vitro* with explants from mother prune trees symptomatized with PPV, and asymptomatic prune trees that are PPV carriers (factor 1 - medium, factor 2 - presence or absence of symptoms of PPV), variety Kubanskaya Early

Variability	Degrees of freedom	Average square	F-ratio	F st. at 0.05	Dispersion	Share of effect
The number of regenerated meristems						
Between media	2	13.00	3.54	5.14	0.00	0.0
Between symptomatic and asymptomatic (the phytosanitary status)	1	192.00	52.36	5.99	31.39	89.5
Interaction: medium x phytosanitary status	2	7.00	1.91	5.14	0.00	0.0
Residual	6	3.67	-	-	3.67	10.5

Table 5 – Results of microshoots' regeneration *in vitro* with explants from asymptomatic and symptomatic prune trees of the Stanley variety that are PPV carriers

Nutrient medium variant	Phytosanitary status	Planted meristems, pcs/%	Successfully regenerated microshoots	
			pcs	%
1. MS (st)	asymptomatic	20/100	14	70
	symptomatic	20/100	9	45
2. Gamborg	asymptomatic	20/100	10	50
	symptomatic	20/100	5	25
3. Modified MS medium	asymptomatic	20/100	15	75
	symptomatic	20/100	10	50
On the average	asymptomatic	20/100	13	65
	symptomatic	20/100	8	40

Table 3 shows that in variety Kubanskaya Early, out of every 20 meristems that had been planted into experimental culture media, successfully regenerated 13 to 16 microshoots (on the average 14, or 70%) of explants from asymptomatic mother plants, which was close to the yield of microshoots from asymptomatic explants of variety Donetskaya (70%, Table 3). In the explants from symptomatic trees of early variety Kubanskaya Early, out of every 20 microshoots planted onto the experimental nutrient media of meristems, from 3 to 8 microshoots, on the average 6 pcs, or 30%, stroke root and regenerated, which was significantly less than for variety Donetskaya (50%, Table 1). The decrease in the efficiency of microshoots' regeneration with symptomatic explants, compared to asymptomatic ones of the Kubanskaya Early variety, was 40% (Table 3).

Variance analysis of the results of microshoots' regeneration from explants of variety Kubanskaya Early (Table 4) showed that the phytosanitary status (symptomatic or asymptomatic seedlings) significantly affected the studied parameter (Fact. > Ftab.). The share of influence of the factor was 89.5 (Table 4)

From the data in Table 5, we can see that for variety Stanley (reference), out of every 20 apexes planted onto the experimental nutrient media, the number of symptom-free explants that regenerated microshoots ranged between 10 and 15 (the average being 13 pcs. or 65%), slightly below the level of regeneration of microshoots from the asymptomatic explants of variety Donetskaya (70%, Table 1) and Kubanskaya Early (71%, Table 3). In explants with symptomatic trees of variety Stanley, out of every 20 apexes, 5 to 10 microshoots, on the average, 8 pcs, or 40%, stroke root and regenerated, which was significantly less than that for variety Donetskaya (50%, Table 1), but higher than for variety Kubanskaya Early (30%, Table 3). The decrease in the efficiency of microshoots' regeneration with symptomatic explants, compared to asymptomatic ones of the Stanley variety, was 25% (Table 5).

Variance analysis of the results of microshoots' regeneration from explants of variety Stanley (Table 6) showed that the factors "phytosanitary status" (symptomatic or asymptomatic seedlings) and "nutrient medium" significantly affected the studied parameter (Fact. > Ftab.). For factor "phytosanitary status", the share of influence was 52.0, for factor "medium" - 25.6 (Table 6).

Thus, the results of microshoots' regeneration *in vitro* show that explants from asymptomatic prune plants strike root and regenerate microshoots significantly more efficiently than explants from symptomatic plants (Table 1-5).

During cultivation of prune explants *in vitro*, differences between nutrient media appeared in the results of microshoots' regeneration.

Table 6 - Variance analysis of the results of microshoots' regeneration *in vitro* with explants from mother prune trees symptomatized with PPV, and asymptomatic prune trees that are PPV carriers (factor 1 - medium, factor 2 - presence or absence of symptoms of PPV), variety Stanley

Variability	Degrees of freedom	Average square	F-ratio	F st. at 0.05	Dispersion	Share of effect
The number of regenerated meristems						
Between media	2	28.00	5.6	5.14	5.75	25.6
Between symptomatic and asymptomatic (the phytosanitary status)	1	75.00	15.00	5.99	11.67	52.0
Interaction:medium x phytosanitary status	2	0.00	0.00	5.14	0.00	0.0
Residual	6	5.00	-	-	5.00	22.4

Table 7 – Summary results about the efficiency of the *in vitro* regeneration of microshoots of experimental explants of prune varieties in experimental nutrient media, %

Nutrient medium variant	Phytosanitary status	Successfully regenerated microshoots, %				
		Donetskaya	Kubanskaya Early	Stanley (reference)	On the average	Sum of the values for the variant
1. MS (st)	asymptomatic	75	80	70	75	120
	symptomatic	55	35	45	45	
2. Gamborg	asymptomatic	60	65	50	58	83
	symptomatic	35	15	25	25	
3. Modified MS medium	asymptomatic	80	65	75	73	123
	symptomatic	60	40	50	50	

For assessing the efficiency of nutrient media in *in vitro* microshoots' regeneration with explants of experimental prune varieties, summary table (Table 7) was made.

The best results in terms of efficiency of *in vitro* regeneration of microshoots of prune varieties Donetskaya, Kubanskaya Early, and Stanley were shown by the media prepared according to the Murashige-Skoog recipe – the sums of values for option (asymptomatic + symptomatic explants) was 123% in modified MS medium, and 120% in standard MS. The sum of the values in the variant with Gamborg medium was only 83%.

The significanly higher efficiency of microshoots' regeneration on the media prepared according to the basic recipe MS, compared to Gamborg medium (37-40% of the sum of values for asymptomatic and symptomatic explants) may be explained by the difference in the composition of nutrient medium.

Additionally, the presence of infection in explants mostly affected regeneration of microshoots of the Kubanskaya Early variety, where the difference between asymptomatic and symptomatic samples was 40%, while for variety Stanley it was 25%, and for variety Donetskaya - 21% (Tables 1 to 5). Different efficiency of infected explants' regeneration depending on the variety is the proof of genetic mediation of its nature.

CONCLUSION

In studying the tolerance of prune varieties to PPV, the criterion of study was the explants' ability to regenerate microshoots *in vitro*. The differences in the degree of success of microshoots' regeneration by explants from symptomatic and asymptomatic mother trees that were PPV carriers had been studied against the background of other equal conditions (composition of the nutrient medium, humidity, sterility, photoperiod, lighting, etc.) in the *in vitro* culture.

The results of microshoots' regeneration showed that explants from asymptomatic prune plants stroke root and regenerated microshoots significantly more efficiently than explants from symptomatic plants. The difference between the level of *in vitro* regeneration of microshoots isolated from symptomatic and asymptomatic plants was 21 to 40%, depending on the variety.

Significant excess of the efficiency level of microshoots' regeneration on media prepared according to the MS recipe had been detected, compared to the Gamborg medium B_5.

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