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PRELIMINARY RESULTS REGARDING THE EVOLUTION OF SMALL POTATO MERISTEMATIC EXPLANTS (0.1-0.2 MM)

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Abstract

This study has as its main objective the meristematic regeneration starting from small explants (0.1-0.2) to ensure viral eradication in potato culture. The experiment is monofactorial, the analyzed factor being the variety with 4 graduations: a1: Azaria; a2: Braşov; a3: Cosiana; a4: Cezarina (as control). For Azaria, Brasovia and Cosiana cultivars, the DAS-ELISA test was performed from potato tuber sprouts to determine the viral infection of these cultivars. Thus, the Cosiana variety presented the highest viral load, according DAS-Elisa test, 3 viruses specific to potato culture were identified: PVS, PVM and PLRV; this is followed by the Brasovia variety, which presented the PVS and PVM viruses, and for Azaria variety, the existence of PVS virus was observed. The inability to guarantee complete elimination of viral particles, especially in cases with mixed infections, remains a limitation for these methods based on meristem cultures. After each pass of the meristems, the statistical analysis was performed, regarding the regeneration of the meristems, finding after each pass the decrease in the viability of the meristems, the reason being the reduced sizes of the meristems.

Key words*: potato, virus, in vitro, meristem, regeneration.*

INTRODUCTION

Climate change and human population pressures are leading to rapid changes in agricultural practices and cropping systems that favour destructive outbreaks of viral diseases. Globalization of agriculture and international trade are spreading viruses and their vectors to new geographic regions, with unexpected consequences for food production and natural ecosystems (Jones and Naidu, 2019). Viruses account for nearly 50% of plant disease pathogens worldwide and damage natural vegetation as well as cultivated plants (Jones and Naidu, 2019).

Potato, being a vegetatively propagated crop, is highly affected by seed quality degeneration as more potato viruses accumulate in seed tubers with each generation, leading to reduced yield potential (Sakha et al., 2017).

Obtaining virus-free plants is necessary for successful viral disease management and sustainable propagation activities, including potato germplasm conservation and global exchange of genetic resources (Naik and Khurana, 2003; Volmer et al., 2017; Ellis et al., 2020).

The *in vitro* culture technique represents the most successful strategy for obtaining virus-free plants (Wang et al., 2018).

In methods based on meristem cultures, the size of the explant affects the efficacy of virus eradication. Excision of 0.2 mm shoot tips containing the apical dome with one or two leaf primordia is usually required (Wang et al., 2006; Zhang et al., 2019). For meristematic explant regeneration, the apical tip taken must contain at least 1-2 leaf primordia, which ensure the production of auxins and cytokinins (Bhojwani and Dantu, 2013). Excision of such small shoot tips is laborious, time-consuming and a highly skilled task. Also, results can be variable in terms of shoot growth and frequency of virus eradication (Bettoni et al., 2016; Magyar-Tábori et al., 2021).

The inability to guarantee complete elimination of viral particles, especially in mixed infections, remains a limitation for these meristem culture-based methods (Faccioli and Marani, 1998; Zhang et al., 2019).

All plants obtained by meristem cultures, with or without prior thermotherapy, must be tested to confirm the "virus-free" status. It is sufficient to confirm this status in the case of a single

regenerant in order to obtain a virus-free clone further, through vegetative propagation (Badea and Săndulescu, 2001).

MATERIALS AND METHODS

To determine the capacity to regenerate small meristems, 4 potato varieties (created at National Institute of Research for Potato and Sugar Beet Brasov) were studied in a monofactorial experiment, with 4 gradations: a_1: Azaria; a_2: Braşov; a_3: Cosiana; a_4: Cezarina. As control, the Cezarina variety was established.

In the experiment carried out, for the statistical analysis of the obtained results, the variance analysis method (ANOVA monofact) was used. The statistical analysis was performed after each subculture.

According to the result of the DAS-ELISA test, the following viruses were identified: PVS virus for Azaria potato variety; PVM and PVS viruses for Braşovia variety; PLRV-PVA, PVM and PVS viruses in Cosiana potato variety.

The meristem sampling experience was carried out in the laboratory, under the conditions required for *in vitro* technology; the operations of sampling meristems and their inoculation were carried out in the sterile premises, previously prepared with the necessary materials.

The preparation of biological material for meristematic sampling includes several stages.

In the first stage, tubers were selected, belonging to the studied varieties. The tubers were allowed to sprout in the dark, at a temperature of 18^0C, until the sprouts reached 2-3 cm in length.

The next stage consisted in the preparation of the biological material for inoculation, which was achieved by sterilizing it. Disinfection of the biological material used was carried out by: immersing potato sprouts in a 0.1% sodium hypochlorite solution for 10 minutes; 3 successive washes in sterile distilled water; brushing spouts, on sterile filter paper. Aseptic dissection of the meristem is a delicate process and all operations were performed in the sterilized room, in the niche with laminar flow of sterile air, previously asepticized by irradiation with ultraviolet lamps, for 30 minutes. In the hood with laminar air flow, before the meristematic sampling procedure, it is preferable to start the air flow, 20-30 minutes.

Another stage was the excision of the meristematic explants and their inoculation in culture vessels (test tubes) with the aseptic medium. The excision of the meristems was carried out under a binocular magnifier (x10-40), in hood with sterile air flow and by using sterile equipment, on a surface that was previously disinfected with alcohol 70^0. The removal of the small leaflets surrounding the growth tip was carried out until the meristematic dome with leaf primordia was observed (Figure 1). The meristem must not exceed the size of 0.2, since the further we move away from the meristematic cells, the greater the danger of viral infection.

The initiation of the meristem culture was achieved by explanting the cauline meristems (apical or axillary) and inoculating them on a nutrient medium for plant regeneration (Figure 2).

The meristem represents an identical clone to the mother plant and can be preserved in a test tube, as the offspring of a healthy mother plant with satisfactory sanitary conditions. The advantage of micropropagation *in vitro* is that of the rapid multiplication, to infinity, of a material genetically identical to the plant from which it started, especially "rejuvenated", healthy and much more homogeneous material.

The culture of meristems therefore represents the starting point in obtaining a healthy material. The successful elimination of potato viruses depends both on the type of virus to be eradicated and on the size of the meristematic explant to be inoculated, which is the main factor that conditions the ability to obtain healthy plants.

For potato, the PVS, PVX and PVM viruses are eliminated by the culture of very small meristematic explants, 0.1-0.3 mm, and the chances of survival of these meristems and regeneration are low; PLRV and PVY viruses can be eradicated by sampling meristems larger than 0.5-3 mm.

The first subculture was performed one month after inoculation (Figure 3). As a regeneration agent, naphthyl acetic acid (NAA 0.5 mg/l) was applied to the culture medium. Each meristem receives a number, which when multiplied is called a clone.

From the inoculation stage to the formation of small meristematic buds, 10 subcultures were

carried out every 25 days. Explants inoculated on aseptic media, cultivated under environmental conditions favourable to their development, evolve over time.

Figure 4 shows the development of meristem four to five months after inoculation and formation of small buds (after 6-8 subculture). Compared to the first stage, initiation of the meristematic culture, in the second stage, namely the formation of buds, the culture medium plays a more important role. This stage consists in the maintenance and growth of stock inoculums. In the third stage, plantlets are formed, the buds elongate, becoming shoots; from this stage the *in vitro* multiplication begins. A few days after inoculation on fresh medium and after each subculture, test tubes with necrotic inoculums were selected, following the evolution and development of explants that survived. No infections of the inoculums were observed in test tubes.

Figure 1. Meristem (original photo)

Figure 2. Inoculated meristem (original photo)

Figure 3. Subculturing meristem on the fresh medium (original photo)

Figure 4. Bud formed from the meristematic explant (original photo)

RESULTS AND DISCUSSIONS

The statistical interpretation of meristem regeneration after the first subculture procedure (Table 1), highlights a significant positive difference for Brasovia variety (13.14%) compared to the control variety (Cezarina).

The influence of variety in the process of meristematic regeneration suggests, after the second subculture, the superiority of the Brasovia variety, which differs significantly positively (16.60%) compared to the Cezarina variety (Table 2).

The analysis of the influence of the variety on the survival of the meristems after the third subculture, places the Brasovia variety in first place, with a significant positive difference of 17.64%, compared to the control variety (Table 3).

At the 4th, 5th, 6th and 7th subcultures, it can be seen that values obtained regarding the regeneration capacity of the meristems are close, without significant differences (Tables 4, 5, 6 and 7). At the sixth subculture, Cezarina variety has the highest regeneration capacity (74.92%), followed by Azaria (67.46%), Brasovia (63.10%). In the seventh subculture, in the Brasovia variety, a decrease in viable meristems is observed; of those initially inoculated and passed, 75.89% necrosed.

At the sixth subculture, for Brasovia variety, the formation of plantlets from the meristematic bud was observed (Figure 5). Since each meristem receives a number, which is called a clone, a number that will be respected throughout the multiplication stage, the respective clone, the regenerated plantlet is multiplied by uninodal segmentation into mini cuttings (Figure 6), with the aim of forming and developing new plants.

The clones that generated plantlets are in percentage of 5.56% (relative to the number of meristematic samples) for Brasovia variety, and they were multiplied, going to generate new plantlets (Figure 7). Following the DAS-ELISA test carried out between 7-08.08.2023, the plantlets received the "virus-free" status, and they will be multiplied. Thus, the viruses with which the Brasovia variety was infected, respectively PVM and PVS, were eradicated for the tested clone. For the PVM and PVS viruses, the success of their elimination is difficult, because the meristems must be very small, and there is a risk that they will become necrotic.

At the 7th pass, for Azaria variety, a plantlet was formed from the meristem (in percentage of 4.34% compared to the total number of inoculated meristems for this variety. The plantlet developed from meristem was sectioned at the level of each internode, in mini cuttings, to obtain new plantlets, identical to the mother plant.

According to Table 8, in the 8th subculture, the lowest percentage of meristematic regeneration is observed at Brasovia variety (16.97%, and the difference of 83.03% shows that they have necrotized, the reason being the very size small dimension of meristems when sampling, to ensure the success of eradicating the virus, which is why in this variety, the plantlets developed from the meristem received the "virus-free" status).

In the 9th subculture (Table 9), the lowest percentage of meristematic regeneration (16.97%) is also observed at Brasovia variety. The Cezarina variety presented the highest meristematic regeneration capacity (27.65%), followed by the Cosiana variety (25.28%).

At the tenth pass, it is found that the values obtained regarding the meristem regeneration capacity are close, with no significant differences between the varieties (Table 10).

The losses incurred during the meristem subculturing procedure were due to tissue necrosis. Since meristematic sampling is performed under a microscope and the explant is very small, necrosis is rarely observed in the first subculture. Along the way, necrosis may also occur due to the stress for each explant, by transferring to fresh medium and possible wounding with the instruments, taking into account the small dimensions of the explant.

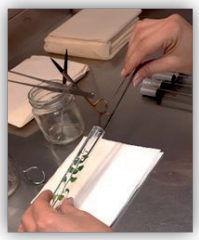

Figure 5. Plantlet developed from meristematic bud

Figure 6. Minicutting

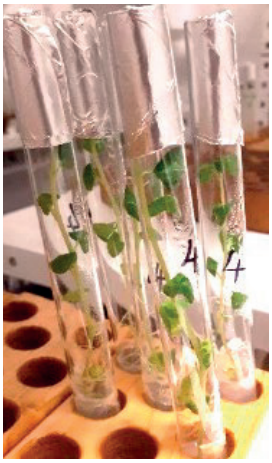

Figure 7. Plantlets developed from mini-cuttings (original photo)

Table 1. Influence of genotype on meristem regeneration after the first subculture

Variety	Survival after the first subculture (%)	Diff.	Sign.	
Azaria	80.95	-5.91	ns	B
Brasovia	100.00	13.14	*	A
Cosiana	87.71	0.84	ns	B
Cezarina (Ct)	86.86	-	-	B

LSD (p 5%) = 9.92%; (p 1%) = 15.03%; LSD (p 0.1 %) =24.14%.
Means found in the same columns followed by the same letters are not significant according to the Duncan test (p≤0.05)

Table 2. Genotype influence on meristem regeneration after 2nd subculture

Variety	Survival after 2nd subculture (%)	Diff.	Sign.	
Azaria	73.81	-9.59	ns	B
Brasovia	100.00	16.60	*	A
Cosiana	81.32	-2.08	ns	B
Cezarina (Ct)	83.40	-	-	B

LSD (p 5%) = 16.33%; (p 1%) = 27.73%; LSD (p 0.1 %) =39.73%.
Means found in the same columns followed by the same letters are not significant according to the Duncan test (p≤0.05)

Table 3. Genotype influence on meristem regeneration after 3rd subculture

Variety	Survival after 3rd subculture (%)	Diff.	Sign.	
Azaria	73.81	-2.30	ns	B
Brasovia	93.75	17.64	*	A
Cosiana	74.38	-1.73	ns	B
Cezarina (Ct)	76.11	-	-	B

LSD (p 5%) = 15.21%; (p 1%) = 23.03%; LSD (p 0.1%) =37.00%. Means found in the same columns followed by the same letters are not significant according to the Duncan test (p≤0.05)

Table 4. Genotype influence on meristem regeneration after 4th subculture

Variety	Survival after 4th subculture (%)	Diff.	Sign.	
Azaria	71.03	-5.08	ns	A
Brasovia	79.17	3.06	ns	A
Cosiana	71.25	-4.86	ns	A
Cezarina (Ct)	76.11	-	-	A

LSD (p 5%) = 10.02 %; (p 1%) = 15.18%; LSD (p 0.1 %) =24.38%. Means found in the same columns followed by the same letters are not significant according to the Duncan test (p≤0.05)

Table 5. Genotype influence on meristem regeneration after 5th subculture

Variety	Survival after 5th subculture (%)	Diff.	Sign.	
Azaria	67.46	-7.46	Ns	A
Brasovia	63.10	-11.82	Ns	A
Cosiana	61.88	-13.04	Ns	A
Cezarina (Ct)	74.92	-	-	A

LSD (p 5%) = 28.11 %; (p 1%) = 42.57%; LSD (p 0.1 %) =68.39%. Means found in the same columns followed by the same letters are not significant according to the Duncan test (p≤0.05)

Table 6. Genotype influence on meristem regeneration after 6th subculture

Variety	Survival after 6th subculture (%)	Diff.	Sign.	
Azaria	67.46	-7.46	ns	A
Brasovia	63.10	-11.82	ns	A
Cosiana	59.10	-15.82	ns	A
Cezarina (Ct)	74.92	-	-	A

LSD (p 5%) = 29.85 %; (p 1%) = 42.50%; LSD (p 0.1 %) =72.61%. Means found in the same columns followed by the same letters are not significant according to the Duncan test (p≤0.05)

Table 7. Genotype influence on meristem regeneration after 7th subculture

Variety	Survival after 7th subculture (%)	Diff.	Sign.	
Azaria	50.40	1.53	ns	A
Brasovia	24.11	-24.76	ns	A
Cosiana	41.04	-7.82	ns	A
Cezarina (Ct)	48.86	-	-	A

LSD (p 5%) = 50.69 %; (p 1%) = 76.76%; LSD (p 0.1 %) =123.31%. Means found in the same columns followed by the same letters are not significant according to the Duncan test (p≤0.05)

Table 8. Genotype influence on meristem regeneration after 8th subculture

Variety	Survival after 8th subculture (%)	Diff.	Sign.	
Azaria	23.61	-15.69	ns	A
Brasovia	16.97	-22.33	ns	A
Cosiana	25.28	-14.02	ns	A
Cezarina (Ct)	39.30	-	-	A

LSD (p 5%) = 34.32%; (p 1%) = 51.97%; LSD (p 0.1 %) =83.49%. Means found in the same columns followed by the same letters are not significant according to the Duncan test (p≤0.05)

Table 9. Genotype influence on meristem regeneration after 9th subculture

Variety	Survival after 9th subculture (%)	Diff.	Sign.	
Azaria	23.61	-4.04	ns	A
Brasovia	16.97	-10.69	ns	A
Cosiana	25.28	-2.37	ns	A
Cezarina (Ct)	27.65	-	-	A

LSD (p 5%) = 37.18%; (p 1%) = 56.30%; LSD (p 0.1%) =90.44%. Means found in the same columns followed by the same letters are not significant according to the Duncan test (p≤0.05)

Table 10. Genotype influence on meristem regeneration after 10th subculture

Variety	Survival after 10th subculture (%)	Diff.	Sign.	
Azaria	23.61	0.72	Ns	A
Brasovia	13.39	-9.50	Ns	A
Cosiana	25.28	2.39	Ns	A
Cezarina (Ct)	22.89	-	Ns	A

LSD (p 5%) = 41.40%; (p 1%) = 62.70%; LSD (p 0.1 %) =100.72%. Means found in the same columns followed by the same letters are not significant according to the Duncan test (p≤0.05)

At the tenth subculture, meristems that survived for each variety developed small buds and a part of them form plantlets (16% for Azaria, 11.11% for Brasovia, 2.56% for Cosiana and 2.17 for Cezarina).
Figure 8 shows the behaviour of each variety regarding meristematic survival.

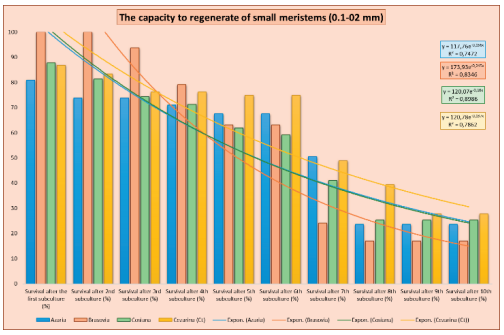

Figure 8. The survival of meristems

CONCLUSIONS

Up to the third subculture, the survival capacity of meristems was influenced by cultivar, but after the fourth subculture, there were no significant differences between cultivars.
Among the four varieties, the Brasovia variety presented the lowest survival capacity from the eighth to the tenth subculture (13.39%).
At the opposite pole is the Cosiana variety, which in the last subculture showed a superior survival capacity (25.28%).
The sampling of very small meristems (0.1-0.2 mm) leads to their loss, not being able to survive.

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