EMBRYO RESCUE IN SEEDLESS GENOTYPES

RECUPERAREA EMBRIONILOR LA GENOTIPURI APIRENE

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Abstract. *Works regarding the optimization of recover paths for immature embryos from 5 seedless grapevine genotypes from the collection of the National Institute for Viticulture and Oenology of the Republic of Moldova were performed. A higher potential of regeneration of immature embryos was obtained for the varieties Apiren roz Basarabean, Apiren extratimpuriu and for the form I-15-15. Viable plants were obtained by cultivating ovules on Nitsch and Nitsch medium (1969) supplemented with AIA and GA_3.A larger ratio of embryos were converted to plants by sectioning the ovules after a period of in vitro cultivation of 90-100 days.*

Rezumat. *S-au efectuat lucrări privind optimizarea căilor de recuperare a embrionilor imaturi de la 5 genotipuri apirene de viță de vie din colecția Institutului Național pentru Viticultură si Vinificație al Republicii Moldova. Un potențial mai mare de regenerare a embrionilor imaturi s-a obținut la soiule Apiren roz Basarabean, Apiren extratimpuriu şi la forma I-15-15. Plante viabile au fost obținute prin cultivarea ovulelor pe mediul Nitsch şi Nitsch (1969) suplimentat cu AIA si GA_3. O cotă mai mare de embrioni au fost convertiți in plante prin secționarea ovulelor după o perioada de cultivare in vitro de 90-100 zile.*

The development of viticulture genetic resources by creating new varieties with high quality and productivity is a major objective of the programs for melioration of grapevine varieties for different uses with raisins of sternospermocarpic type (Emershad, Ramming, 1984; Bouquet, Davis, 1989). That is why an important part of the researches in the grapevine field are oriented towards the improvement of genetic programming by using seedless genitors. The usage of seedless genotypes in the improvement implies the use of methods preventing the embryo abortion. The technique of embryo saving resides in cultivation of sternospermocarpic seeds on nutritive mediums, after which the excised viable embryos or the plants (germinated directly from embryos) are transferred on fresh mediums, and afterwards in hot-houses. The number of obtained plants depends on the cultivation mediums, genotypes used in crossings and the age of embryos in the moment of transfer on mediums (Bouquet, Davis, 1989; Emershad, Ramming, Serpe, 1989; Liu, Sykes, Clingeleffer, 2003). That is why a high attention is allotted to the selection of parental genitors, the terms of grapes harvesting and viable embryos excising.

The importance of genitor's selection is determined by the necessity of including during the work of local genotypes with valuable characteristics (seedless, high productivity and taste features, resistance to abiotic factors) and that can serve as an

excellent source for obtaining new forms with different seedless type and with resistance to low temperatures and humidity deficit. A higher rate of output for descendants with desired characteristics, especially with seedless features, is obtained from combinations seedless x seedless. In these cases the raisins are more natural and the seeds rudiments are smaller (Perl, Sahar, Spiegel-Roy, Gavish, Elyasi, Orr, Bazak, 2000).

In the same time there are very big differences with regard to the *in vitro* germination capacity of zygotic embryos for different types of grapevine (Cain, Emershad, Tarailo, 1983; Goldy, Amborn, 1987). Popescu C.F. and Teodorescu A. (2004) consider that the response reaction at the *in vitro* culture of the ovules obtained by natural pollination of some seedless genotypes can be very useful indicators for selecting varieties of grapevine that can transfer to the descendants a high potential of germination of immature embryos.

The aim of the present work was the optimization of the paths for recuperating plantlets from immature embryo from local genotypes with seedless characters, precocity, winter resistance as well as preliminary testing of those forms and varieties for including them in melioration programs, as well as by interbreeding seedless x seedless.

MATERIALS AND METHODS

Seedless berries of varieties Apiren extratimpuriu, Apiren roz Basarabean, Apiren roz, Apiren alb and hybrid form I-15-15 were harvested about 35-45 days after anthesis from the experimental vineyard at the National Institute for Viticulture and Oenology, Republic of Moldova.

Berries were surface sterilized with 70 % alcohol for 30 sec and 5.2 % calcium hypochlorite for 20 min, and washed three times with sterilized distilled water.

The ovules were aseptically extracted from the berries and put in 9 cm diameter Petri dishes (15-20 per dish), with Nistch and Nistch (1969) medium, supplemented witch 20 g/l sucrose, 2,7 g/l activated charcoal, 2,8 g/l Phytagel (Sigma-Aldrich Chemie Gmbh), 2 mM putrescine, 3 mg/l 3-indolylacetic acid (IAA) and 5 mg/l gibberellic acid (GA_3). The medium pH was adjusted to 5,6 prior to autoclaving. Plates were incubated for 4 weeks in the dark at 25±2^{o}C, and then transferred to growth chambers at 25±2 ^{o}C with 16 hours photoperiod. Ovules were subcultured on fresh medium every 30 days.

Ovules were excised after 90-130 days of culture. Excised embryos were transferred to half strength Murashige and Skoog (1962) medium supplemented with 10 g/l of sucrose and 7 g/l agar. The globular, heart and torpedo stage embryo was transferred to media supplemented with 0.45 mg/l 6-benzyladenine (BAP). When cotyledons become green the embryo is placed to hormone-free media.

Well rooted and elongated plants were transplanted to soil.

Assessments during the experiments performed in the culture with regularity: the eliminations due to infections, the grown ovules, the ovules that formed callus, the ovules that grew and remained without changes. The grown embryos that formed cotyledons and green hypocotyls were considered to be well-grown. The ratio of ovules with viable embryo, or with 2 or more viable embryos, the state of development of the embryos and the number of embryos with different states of necrosis was determined at sectioning.

RESULTS AND DISCUSSIONS

The critical point of this technique is the development stage of the embryo in the moment of ovule excising. It is difficult to cultivate in culture very small embryos, formed from 4-50 cells, or at the beginning phases of development (Ramming, 1990). It is more useful to save the embryos at the stage of seed traces (Ramming, 1983). In favorable mediums the embryo continues its development inside the seeds (Gray, Purohit A., 1991) and have all the chances to reach the maturation phase after the endosperm degeneration (Ledbetter, Ramming, 1989).

The performed works proved that the optimal terms of harvesting biological material for the studied genotypes is of 35-45 days from pollination. In such cases the ratio of ovules that continue to develop and grow in volume was of 54.87 % for the variety Apiren extratimpuriu and of 90.56 – 99.41 % for the other genotypes. A large number of regenerations directly from ovules were obtained, as well, for these terms (table 1).

Table 1

Development of ovules on Nitsch and Nitsch medium (1969) after 90 – 100 days of cultivation

Genotype	Examined ovules	% ovules		
		Developed	With callus	With regenerations
Apiren extratimpuriu	246	54,87	7,72	3,90
Apiren roz Basarabean	265	90,56	57,73	5,60
Apiren roz	341	99,41	46,93	2,64
Apiren alb	409	97,78	1,73	1,36
I-15-15	860	98,37	4,88	0,56

At ovules sectioning after 90-120 days of cultivation on Nistch and Nitsch (1969) medium, the ratio of ovules with viable embryos varied from 7.52% for the variety Apiren alb to 34,48% for Apiren roz Basarabean (table 2). This index was higher for the genotypes Apiren roz Basarabean (30.55-34.48%) and I-15-15 (27.10-32.22 %). These results prove the role of the genotype in expressing the *in vitro* germination capacity of immature embryos. Cultivation duration on medium didn't have a significant impact on the number of ovules with embryos. In the same time the data presented in table 2 prove that at ovule selection after 90 days embryos with browning were attested in a much lower ratio comparatively with the one present at dissected ovules after 100 – 120 days of cultivation on mediums. The highest ratio of embryos on the path to necrotizing was attested for the genotypes I-15-15 at ovules cultivated during 120 days (23.53%) and Apiren roz after a period of keeping on medium for 110 days (21.05%). For the variety Apiren extratimpuriu the percentage of browned embryos was practically on the same level irrespective of the length of keeping the ovules on medium. This can be attributed to the fact that this variety is characterized as very precocious. That is why the embryos mature earlier in comparison with the other varieites. Starting from this fact, the optimal length of cultivation is being established for each genotype separately. For the tested varieties this term is of 90 days for Apiren alb,

Apiren roz Basarabean, I-15-15 and Apiren roz. For Apiren extratimpuriu this term is under 90 days and is expected to be established later. These results are confirmed by the relations from literature according to which an optimal term for excising and transfer of embryos on fresh mediums is considered to be the term after 60-90 days of cultivation (Emershad, Ramming, 1984; Goldy, Amborn. 1987; Gray, Fisher, Mortensen, 1987; Bouquet, Davis, 1989). A special importance in plant development from embryos is played by the size of embryos in the moment of transfer on the conversion medium. A higher effect is obtained when the embryos are transferred to the torpedo stage (Zlenko, Koticov, Troshin, 2005; Liu, Sykes, Clingeleffer, 2003). That is why the ovule selection after 90 – 100 days is suggested also by the fact that in this period the percentage of embryos at the stage of heart – torpedo reaches the highest level, after which it slowly decreases (table 2).

Table 2

Results of ovules sectioning

Genotype	Days on medium	Dissec-ted ovules	% ovules with		Embryos , %		
			Viable embryos	2 and more embryos	Heart - torpedo	Cotyle-dons	Browned
Apiren extratimpuriu	90	235	18,29	2,99	25,67	50,00	5,40
	120	113	18,58	4,42	30,77	53,85	6,67
I-15-15	90	90	32,22	5,55	33,33	38,89	0,00
	120	107	27,10	4,67	32,35	58,82	23,53
Apiren roz Basarabean	90	36	30,55	8,33	26,31	47,37	0,00
	100	116	34,48	11,21	28,45	50,86	9,48
Apiren roz	90	235	18,30	2,55	34,48	34,48	1,75
	110	155	19,35	3,22	20,05	55,26	21,05
Apiren alb	100	133	7,52	0,75	18,18	45,45	0,00
	120	80	10,00	3,75	0,00	85,71	0,00

The appearance of embryos with traces of necrosis can be explained by the toxic effect of the substances produces as a result of the ovules metabolism. Gray D.J. (1992) observed a strict correlation between the ovules browning, caused probably by the tannins that are spread in the medium of culture and the formation of rings around the ovules. This process can result in necrosis of embryos. It is considered that the appearance of additional callus also increases the quantity of metabolic products. Valdez J.G. (2005) observed a decrease of the ratio of saved embryos after 240 days of cultivation for genotypes that produce callus abundantly on exterior teguments. The author emphasizes that, for the studied varieties, this result is due to the accumulation of extra-cellular molecules in the culture medium as a result of additional callus formation and refuses thus the assumptions regarding the toxic effect of phenyl and tannin substances produced as a result of ovules metabolism.

The obtained results in our experiences do not confirm the link of embryo abortion with the ovules predisposition of forming additional calus. Thus, if for the variety Apiren roz the formation of callus was attested at 46.93% from the

cultivated ovules, for the genotype I-15-15 – at 7.72% while the ratio of embryos with traces of necrosis was higher for the form I-15-15. Meanwhile for the varieties Apiren roz Basarabean – the one with the highest percentage of ovules that formed callus (57.73), the ratio of embryos with necrosis constituted 9.48% after 116 days. Probably thus can be explained by the fact that in the culture medium was added 2,7g/l active coal. It is assumed that the toxins produced by the callus are absorbed by the active coal added to the culture medium, that absorbs toxic substances and releases gradually the hormones in the culture medium (Evert, Taylor, 1990). It also has an antioxidant effect and being incorporated in the culture medium prevents the browning caused by phenyl oxidants. That is why active coal adding increases the ratio of germinating embryos (Bouquet, Davis, 1989) and reduces tissue browning, callus formation and medium discoloration (Cain, Emershard, Tarailo, 1983). The gathered data in our experiences suggest the use of active coal at stages of sub cultivation, until the plantlet is formed.

The efficiency of the technique of immature embryos saving is determined by the ratio of plantlets resulted from ovules cultivated on nutritive mediums. The ratio of ovules with direct germination for the used genotypes varied from 0.56% for the variety Apiren alb and 5.60% for Apiren roz Basarabean (table 1). The ratio of plants obtained from excised embryos is exceeding the one resulted from direct germinations (Valdez, 2005).

At ovule sectioning the embryos were transferred on medium for their conversion into plants. A medium containing ½ salts after Murashige Skoog supplemented with 0.45 mg/l BAP or free from growth regulators was used. Better results were obtained by cultivating embryos at the torpedo stage on BAP medium during 7 days with further transfer on medium that is free from hormones (table 3). The percentage of embryos converted in plants varied between 0.00% for the variety Apiren alb and 21.11% for variety Apiren extratimpuriu.

Table 3

Results obtained in culture of immature embryos

Genotype	Regenerates and embryos transferred on			
	Medium without hormones		Medium as chitochinine activity	
	Sum	Converted in plants	Sum	Converted in plants
I-15-15	26	2	60	8
Apiren extratimpuriu	21	1	90	19
Apiren roz	12	0	90	7
Apiren alb	13	0	20	0
Apiren roz Basaraben	48	3	115	18
Total	120	6	375	52
%		5,00		13,86

CONCLUSIONS

A higher potential of regeneration was obtained by cultivating ovules on Nitsch and Nistch medium (1962) supplemented with AIA and GA_3 during 90-100 days, with excising and transfer of embryos at the torpedo stage on the conversion medium.

Starting from the degree of seedless, winter resistance, precociousness and regeneration potential of immature embryos - a higher interest for inclusion in melioration programs in quality of maternal genitors are presented by the following genotypes Apiren extratimpuriu, Apiren roz Basarabean and I-15-15.

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