Features of somatic embryogenesis in the culture *in vitro* in hybrid grape form E-342

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Abstract. Correlation analysis of the influence of media variants with introducing various biologically active substances on the development of somatic embryoids and germinating seedlings in the hybrid form E-342 was carried out during two repeated subculturings on the same variant of media, and during the third subculturing - on different variants of media. The repeated subculturing in media with the same growth regulator BAP or IAA caused the development of abnormal large somatic embryoids without the expressed hypocotyls and cotyledons. Only the use of medium variant with DLPA in two subculturings (the third subculturing into the medium with BAP or IAA) and the third repeated subculturing with DLPA, but to a smaller extent, have led to the development of torpedo-shaped embryoids, to be growing into germinating seedlings. Strong positive correlation dependence of influence was established for: BAP on the development of heart-shaped embryoids; IAA on the increase in the size of globular embryoids and transformation of heart-shaped embryoids into torpedo-shaped ones; DLPA on the development of a great number of normal torpedo-shaped embryoids. After longstanding culturing of suspensions of cells and embryoids, it was not possible to get rid of chlorophyll deficiency in the resulting germinating seedlings due to somaclonal variability.

1 Introduction

In the age of active development of plant biotechnology and scientific inventions in the field of genetic engineering, it becomes relevant to obtain protocols for sustainable plant regeneration from somatic cells subjected to transformation. Positive results on the transfer of resistance genes in grapes have already been achieved [1].

Brand new direction in changing genetically determined “bad” heredity, its “cleansing” from viral and other “foreign” DNA, is Crispr/Cas 9 - indirect genome editing, which promises to make a future breakthrough in medicine (treatment of currently incurable diseases), and in horticulture, in particular, in resistance of apple trees and grapes to powdery and downy mildew [2-4]. The most convenient objects for the use of Crispr/Cas 9, as intermediated targeted genetic editing without introducing foreign genetic information, are single proembryogenic cells cultivated in liquid suspension cultures to prevent the formation of chimeric genotypes in the future.

But since there are restrictions on the use of transgenic plants in agricultural production, it is still relevant to use biotechnology methods to expand genetic diversity due to somaclonal variability, as well as genome mutations under the influence of colchicine on cells in the culture *in vitro* followed by somatic embryogenesis [5-7]. Somatic embryogenesis is one of the most successful methods of recovery from viral infections [8].

But if somatic embryogenesis in the culture *in vitro* comes easily in some plant families and species, then in others it is strictly molecularly and genetically determined, and affected by many factors [7, 9]. The induction of somatic embryogenesis in plants of different species and in grapes from cells of suspension cultures was achieved in a small number of varieties, creating difficulties for application of modern biotechnology methods [7, 8, 10, 11].

This research was aimed at studying the effect of various growth regulators (6-benzylaminopurine (BAP), ß-indoleacetic acid (IAA)) and biologically active substances (D, L-phenylalanine (DLPA) and Na humate) on the development of various stages of somatic embryoids in grapes on example of the hybrid form E-342.

2 Materials and methods

Materials. The proembryogenic callus tissue was taken for the research. It was generated from chlorophyll-defective germinating seedlings (white cotyledons) cultured on a solid medium. The seedling with proembryogenic callus was cultivated after an experiment on somatic embryogenesis and plant regeneration in the hybrid form E-342 [7]. The proembryogenic callus was subcultivated into a liquid medium, followed by subculturing of cell suspensions and small germinal globular embryoids into different variants of media (Fig. 1).

Methods. Suspension culture, containing small globular embryoids, was thoroughly washed with sterile distilled water. For this purpose the suspension was allowed to settle for gravitating globular embryoids to the bottom of culture vessel. Liquid part was poured off, leaving sediment with a small amount of liquid. Then, sterile distilled water was poured into the culture vessel to wash out previous medium. It was drained again after settling. The sediment with embryoids was left at the bottom of culture vessel, after that fresh culture medium was added to it.

Suspension culture with small globular embryoids (<0.2 mm) (about 1000 pcs. of 0.05-0.2 mm in size in 10 ml of suspension) was equally added to culture vessels, in which different variants of nutrient media with the base, contained fourfold diluted mineral elements and vitamins of PG medium [10], and 10 g/l of sucrose were added. The variants of media differed in the presence or absence of growth regulators and biologically active substances. The factors (variants of media) were taken as independent variables x1…x6, all of which were at two levels: 0.5 or 50 in the coded variables (c.v.) and in the listed below actual variables (a.v.), respectively:

- x1 (0 or 0.5 mg/l BAP in a.v.),
- x2 (0 or 0.1 mg/l IAA in a.v.);
- x3 (0 mg/l IAA and 0 mg/l Na humate or 0.1 mg/l IAA and 30 mg/l Na humate in a.v.);
- x4 (0 or 5.0 mg/l DLPA in a.v.);
- x5 (0 mg/l IAA and 0 mg/l DLPA or 0.1 mg/l IAA and 5.0 mg/l DLPA in a.v.);
- x6 (0 mg/l IAA, 0 mg/l DLPA and 0 mg/l Na humate or 0.1 mg/l IAA, 5.0 mg/l DLPA and 30 mg/l Na humate in a.v.).

After two repeated subculturings for each variant of medium, the results were evaluated by the number (pcs.) of developed somatic embryoids: globular (y1), heart-shaped (y2), torpedo-shaped (y3) and germinating seedlings in number, pcs. (y4) and length, mm (y5).

The further third subculturing of embryoid suspensions was carried out only after two repeated subculturings, (subculturings are indicated as factors): x7 (IAA, x2→IAA, x2); x8 (DLPA, x4 → DLPA, x4) and x9 (IAA + DLPA, x5 → IAA + DLPA, x5).

These third subculturings were carried out not only in x1…x6 variants of media, but were supplemented with the following variants (factors):

- x10 (0.5 or 50 in c.v.; 0 mg/l BAP and 0 mg/l Na humate or 0.5 mg/l BAP and 30 mg/l Na humate in a.v.);
- x11 (0.5 or 50 in c.v.; 0 mg/l DLPA and 0 mg/l Na humate or 5.0 mg/l DLPA and 30 mg/l Na humate in a.v.).

After 3 months of culturing on the variants of media (x1-x6, x10, x11), the results were evaluated on every stage by the number (pcs.) of the developed somatic embryoids and germinating seedlings (y1...y4), and by the size of seedlings (mm, y5,). The conditions for conducting of the experiments were presented earlier [10].

Statistical processing of the results. Cultures of somatic embryoids in each variant of liquid medium were presented in triplicate. To establish the influence degree of factors (media variants containing various biologically active substances), as well as to determine the influence dependence on further processes of the factors, present in two previous subculturings, a correlation analysis was carried out with reliability rate of 95% (R = 0.05). The correlation coefficients were calculated in Excel using the correlation analysis method. The absence of a factor (the lowest level, x1…xn), or a result (y…yn) was designated by the value 0.5 in order to eliminate zero values in correlation analysis. The strength of correlation relationship was determined using Chaddock scale of coefficients (average 0.5–0.7, high 0.7–0.9, very high 0.9–0.99. The results of correlation analysis are presented in Table 1.

3 Results and discussion

After long-term culturing of a chlorophyll-defective seedling of the hybrid form E-342 using solid (agar) medium, a proembryogenic callus was formed from its cotyledons. It was transferred (subcultivated) into a liquid medium. After that cell suspension was subcultivated several times, resulting in propagation of proembryogenic cells, which formed small globular embryoids <0.2 mm. It is necessary to choose the optimal scheme for subculturing both suspensions of proembryogenic cells and somatic embryoids into different variants of liquid media for maximum development from torpedo-shaped germinating seedlings: their number (pcs.) and an increase in the size of cotyledons (mm) (Fig. 1).

Correlation analysis was used to establish the effect of different variants of liquid media (factors) with diverse content of biologically active substances on further process of consistent development of various stages of somatic embryoids and their transformation into germinating seedlings (Table). The dependence of this influence after the third subculturing on the presence of a certain factor in two previous subculturings was also revealed.

For these experiments small globular embryoids have already been in the initial suspension culture (Fig. 1). After subculturing in a medium with IAA (x2) → IAA (x2), factor x7, they grew (cell growth by elongation) up to 0.2-1.0 mm. Later on they developed into abnormal embryoids of next stages as unable to turn into germinating seedlings (Fig.2.). But if to start with using of two repeated subculturings with DLPA (x4), factor x8, and then the third subculturing with IAA (x2), or BAP (x1), or (BAP + Na humate) x10, then a positive result for the development of torpedo-shaped embryoids into germinating seedlings with a high correlation dependence of this process is obtained (Table 1). A positive effect of BAP (x1) on the development of heart-shaped embryoids was confirmed

(Table 1), but with inhibition of their further transformation into normal torpedo-shaped embryoids.

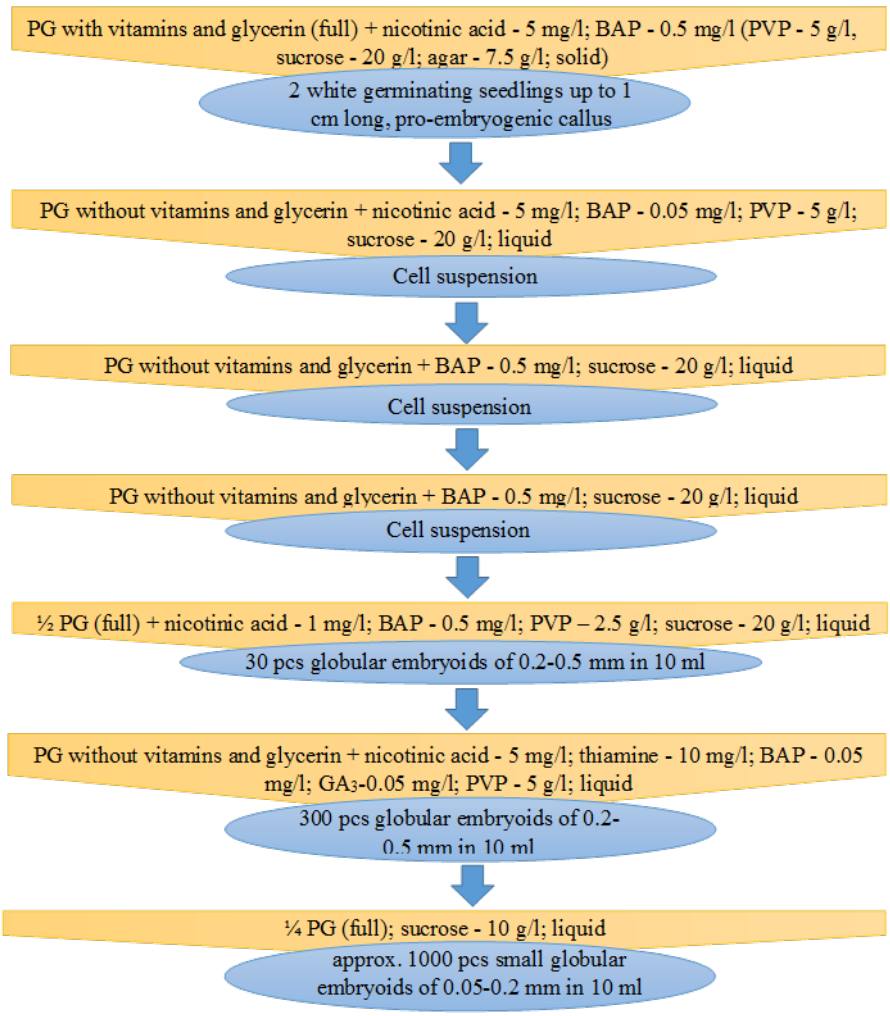
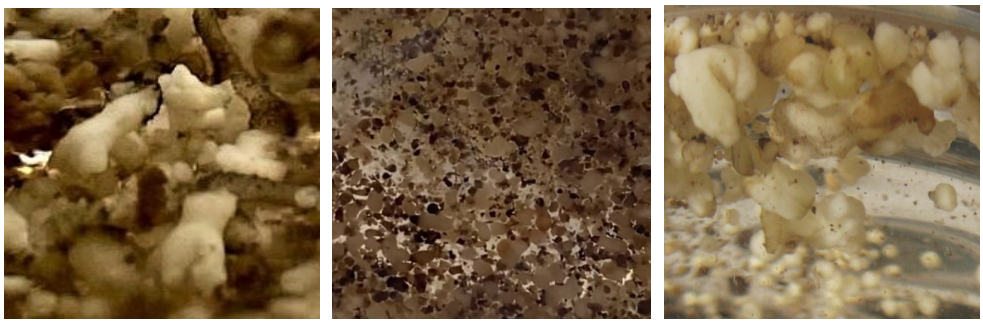


Fig.1. Scheme of subculturing proembryogenic cells of the hybrid form E-342 to obtain the initial suspension of small globular embryoids used in these studies.



a b c

Fig. 2. The development of various stages of somatic embryoids (a - globular (subculturing: x1 (BAP) -> x1 (BAP) -> x2 (IAA)), b - heart-shaped (x1 (BAP) -> x10 (BAP + Na humate) - > x4 (DLPA)), c – torpedo-shaped (x4 (DLPA) -> x4 (DLPA) -> x2 (IAA)) in the hybrid form E-342.

Table 1. The effect (correlation) of growth regulators and biologically active substances, individually or in combination, in different variants of liquid media (factors) after 2 (Table 1, T1) and 3 (T2, T3, T4, T5) repeated subculturings on the development of various stages of somatic embryoids and germinating seedlings of the hybrid form E-342

Factors (variants of media) in actual and coded variables		The effect of factors (variants of media) on the development of various stages of somatic embryoids (number, pcs.) and germinating seedlings (number, pcs. and size, mm) in correlation coefficients. Reliability degree - 95%, R-0,05 (correlation: average 0,50-0,69; high 0,70-0,89; very high 0,90-0,99)																			
	Factor levels in actual (mg/l) variables	Globular, y1					Heart-shaped, y2					Torpedo-shaped, y3					Germinating seedlings				
																	Number, y4			Size, y5	
Factor		T1	T2	T3	T4	T5	T1	T2	T3	T4	T5	T1	T2	T3	T4	T5	T1	T3	T5	T1	T3	T5
x1	BAP – 0.5	0.24	0.34	-0.08	0.04	-0.03	0.64	0.39	0.77	0.8	0.59	-0.39	**0.98**	0.19	0.2	0.18	-0.32	0.38	0.27	-0.31	0.08	0.12
x2	IAA – 0.1	0.34	0.34	**0.91**	**0.91**	0.69	0.07	0.39	0.31	0.22	0.31	0.34	-0.04	0.71	0.02	0.2	-0.32	0.38	0.15	-0.31	-0.16	-0.08
x3	IAA – 0.1, NA humate - 30	0.44	0.34	-0.33	-0.13	-0.16	-0.58	-0.52	-0.61	-0.48	-0.53	-0.35	-0.23	-0.32	-0.4	-0.14	0.57	-0.52	-0.23	0.51	-0.52	-0.23
x4	DLPA – 5.0	0.15	0.34	0.17	0.04	0.12	0.48	0.28	-0.15	-0.25	-0.04	0.84	-0.23	-0.58	-0.4	-0.2	0.69	-0.27	-0.13	0.74	0.56	0.22
x5	IAA – 0.1, DLPA – 5.0	-0.34			-0.13		-0.42			-0.25		-0.15			0.58		0.32			-0.31		
x6	IAA – 0.1, DLPA – 5.0, Na humate - 30	-0.83	-0.57				-0.18	-0.18				-0.29	-0.04				-0.32		-0.1	-0.31		
x7	Two repeated subculturing IAA->IAA, and the third x1-x6, x10, x11					-0.08					-0.12					-0.48			-0.33			-0.33
x8	Two repeated subculturing DLPA -> DLPA, and the third x1-x6, x10, x11					0.29					0.21					0.89		0.4	0.68		0.4	0.68
x9	Two repeated subculturing IAA+ DLPA ->IAA+ DLPA, and the third x1-x6, x10-x11					-0.22					-0.32					-0.43			-0.37			-0.37
x10	BAP - 0.5, Na humate - 30		-0.57	-0.33	-0.48	-0.36		0.28	0.03	0.22	0.17		-0.23	0.32	0.58	0.12		0.55	0.22		0.56	0.22
x11	DLPA – 5.0, Na humate - 30		-0.21	-0.33	-0.25	-0.26		-0.64	-0.34	-0.25	-0.39		-0.23	-0.33	-0.4	-0.14		-0.52	-0.23		-0.52	-0.23

Developed on a solid medium heart-shaped embryoids were turning into torpedo-shaped ones only after their subculturing into a liquid medium without growth regulators. Long-term culturing of cell suspension and embryoids of the rootstock ‘Kober 5BB’ in auxin-free

liquid medium was leading to the release of proteins and glycoproteins into the medium, inhibiting further transformation of heart-shaped embryoids into torpedo-shaped ones. The torpedo-shaped embryoids, capable of further transformation into germinating seedlings with green cotyledons, and regeneration of shoots from them on a medium with BAP + GA_3, were developing in a liquid medium with IAA.

Endogenous auxins are synthesized from DLPA amino acid. A positive effect of DLPA on the development of somatic embryoids and germinating seedlings in these studies can be associated with the synthesis of endogenous auxins in embryoids just when it is necessary for their transformation by various stages of development and synthesis of endogenous abscisic acid for their maturation.

Cytokinin BAP causes transformation of globular embryoids into heart-shaped, but inhibits their further development into torpedo-shaped ones. The action of Na humate is to inhibit uncontrolled processes of cell reproduction under the influence of BAP and their growth by extension (IAA). Its joint introduction with BAP + Na humate (x10) (Table) or with IAA + Na humate (x3) favorably influences the transformation of torpedo-shaped embryoids into germinating seedlings. The formation of endogenous auxins and abscisic acid, as well as their introducing to the nutrient medium, plays a key role in the development of zygotic and somatic embryoids and their germinal cotyledons [9, 11]. A positive effect on somatic embryogenesis is played by stress factors, causing synthesis of endogenous abscisic acid, without which somatic embryoids, capable of further transformation into germinating seedlings with green cotyledons, cannot develop [12-15]. Perhaps, in our case, Na humate was acting as a stress factor.

4 Conclusion

With the use of correlation analysis, we have proved a strong influence of presence of D, L - phenylalanine (DLPA, 5.0 mg/l) in the variant with liquid medium during two subculturings, and at the stage of the third subculturing, regardless of the presence in the medium, on further development of torpedo-shaped embryoids, capable to grow into germinating seedlings.

Aminoacid D, L - phenylalanine (DLPA) does not only precede the synthesis of endogenous IAA, but also influences the activity of antioxidant system enzymes. It has antioxidant properties, promotes the development of normal tissues (vessels and grains of birch and pine), while the activity of peroxidase and polyphenol oxidase is 3, 7 and 2.3 times higher, respectively, in woods containing parenchymal cells. Therefore, DLPA inhibited the formation of proembryogenic undifferentiated cells, but promoted the development of various stages of somatic embryoids and seedlings [6, 10]

In the process of a long-term subculturing of suspensions of proembryogenic cells and embryoids due to somaclonal variability, it was not possible to get rid of chlorophyll deficiency, i.e. the presence of white cotyledons in germinating seedlings resulted from embryogenesis.

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