

Suspension culture formation in red grapevine varieties

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Abstract

That work had the purpose to obtain some stable and productive cell suspensions (shake flask cultures) in a reduced volume (300ml) and also the existent differences establishment between the grapevine varieties from the point of view of cellular increasing in the liquid culture medium. Cell suspension cultured in the Murashige-Skoog culture media supplied with 1-naphthaleneacetic acid (2 mg·l⁻¹) and kinetin (0,5 mg·l⁻¹) it were obtained from callus cultures of tissue internode explants. The measures realized from 3 in 3 days beginning with the 6th day of culture and ending with the 36th day of culture. To all the varieties studied, in our experimental conditions, the cell suspension increasing stopped after 30th days of culture. The rate of increase of the cell culture in suspension registered a maximum medium value to Merlot species (275,10%), superior value to the others species, that being followed by Pinot Noir variety (269,22%), Oporto (251,65%), Negru Tinctorial (247,69%), Cabernet Sauvignon (237,49%) and Burgund Mare (229,73%). Those results could contribute in the future to the possibility of modern techniques usage of cell suspension as alternative method of multiplying in vitro and also in obtaining some secondary metabolites synthesized at cellular level.

Keywords: suspension cell culture, culture medium, *Vitis vinifera*

Introduction

Plant cell culture offers interesting possibilities for the large scale production of pharmaceuticals (SHEN X. & al. [1]), food additives (LARRONDE F. & al. [2]), and somatic embryos (BORNHOFF B.A. & al. [3]). However, knowledge about the effect of the physicochemical environment on plant cell behavior is still limited, and the low productivities usually obtained compromise bioprocess profitability. Until now, the development of plant cell bioprocesses has been carried out in sequential steps, from cell line selection to secondary metabolite production and from static solid to agitated liquid shake flask and bioreactor cultures. Plant species are chosen for their economic potential (PEPIN M.F. & al.[4]).

Cell suspensions constitute a good biological material for studying biosynthetic pathways (DOUGALL D.K.[5]). Cell lines are selected from calli and grown in suspension cultures using standard media, usually MS (MURASHIGE T. & al.[6]). The formulation of growth media, usually developed from shake flask factorial experiments, is aimed at maximizing growth index, for a given culture duration (YAMAKAWA Y. & al.[7]). The shake flask remains the reference culture system. Its main advantages are its low mixing shear and simplicity (PEPIN M.F. & al.[4]).

In vitro reactivity of *Vitis vinifera* L. species had constituted a very interesting subject of study. This is due to the biotechnological implications of this experimental system as well as from the necessity to elucidate the cellular and molecular mechanisms of cell development, the factors involved in the differentiate response regarding proliferative and or morphogenetic potential of different genotypes and tissues (BREZEANU A. & al.[8], TORREGROSA L. [9], LUPŞEA S. & al.[10]). Among the multiple aspects which have been studied, a particular attention is accorded to cellular mass obtained (callus or cell suspension cultures) which could constitute a potential source of secondary metabolites, anthocyanins mainly (CORMIER F. & al. [11], SPARVOLI F. & al. [12], TANAKA N. [13], BELHADJ A. [14], RIEDEL H. & al. [15], CONN S. & al. [16])

Materials and methods

Plant material

The biologic material was constituted from six varieties of grapevine for red wine: Burgund Mare, Cabernet Sauvignon, Oporto, Merlot, Negru Tinctorial and Pinot Noir. For callus cultures initiation it was used internode segments.

The cultures cells in suspension obtaining

To induce the callusing process, the base environment Murashige-Skoog (MURASHIGE T. & al. [6]) it was supplied with 1-naphthaleneacetic acid ($2 \text{ mg}\cdot\text{l}^{-1}$) and kinetin ($0,5 \text{ mg}\cdot\text{l}^{-1}$).

After callus obtaining on a solid culture media it passed to cells cultures in suspension in the liquid culture media.

A callus quantity determined it passed in the liquid culture media. The cultures made in Erlenmayer flask of 300 ml, in the darkness, and in conditions controlled by temperature of (25 C°) and shake (110 rpm).

The measures realized from 3 in 3 days beginning with the 6th day of culture and ending with the 36th day of culture.

The statistics elaboration of experimental data

The data obtained after different analyzing accomplishment and determinations referring to increase rate of cellular weight were statistically processed, determining the average estimative values, standard departure of the average and variability coefficient (average, standard departure of the average, variability coefficient).

After significance determination of differences among studies varieties, the obtained experimental data processing, it had made through variation analysis and test conform the monofactorial experiences methodology (the sum of departures squares, variation, the freedom degrees, the differences error, limit differences).

To establish the dependence among different characters and features it had established the linear and quadric regressions and also the correlation coefficients: linear regression, quadric regression, correlation coefficient (CIULCA S. [17]).

Results and discussions

From the results regarding the variation analysis for the increase rate of cells cultures in suspension (Table 1), it had been observed to Burgund Mare variety the existence of some significant distinct differences among the repetitions concerning the cellular biomass increasing. The significant distinct value of the test F ($F=50,70$) attested the fact that concerning the culture period it existed real differences under the increase rate aspect of cells in suspension.

In base on the results of table 2, it observed that the cellular rate increase to Burgund Mare variety proportionally increased with the cultivation period touching a maximal average value of 229,73% after 30 days of cultivation. Ulterior, the cellular increase rate presented a regress until 36th day of cultivation when registered an average value of 223%, with a difference of 6,73% face to a maximal rate achieved after 30 days.

Table 1. Variance analysis regarding the increase rate of cell suspension culture on Burgund Mare variety

Source of variation	SP	GL	S ²	Test F
Total	104212,21	35		
Repetitions	4590,96	2	2295,48	F =13,35**
Cultivation period	95840,66	11	8712,79	F = 50,70**
Error	3780,60	22	171,84	

Table 2. The significance of differences between different days of culture regarding the increase rate of cell suspension culture on Burgund Mare variety

No.	Cultivation period (days)	Average increase rate (%)		Difference from the blank	Significance
		$\bar{x} \pm s_{\bar{x}}$	$s_{\%}$		
1	0	100,00+0,00	0,00	0,00	Blank
2	6	100,39+0,16	0,27	0,39	-
3	9	101,41+0,57	0,97	1,41	-
4	12	114,10+2,39	3,63	14,10	**
5	15	129,62+3,46	4,63	29,62	***
6	18	149,05+7,42	8,62	49,05	***
7	21	175,39+7,45	7,36	75,39	***
8	24	197,23+9,86	8,66	97,23	***
9	27	218,07+17,11	13,59	118,07	***
10	30	229,73+18,45	13,91	129,73	***
11	33	227,51+16,06	12,23	127,51	***
12	36	223,00+16,68	12,96	123,00	***
DL 5%		DL 1%	DL 0,1 %		
9,20		12,51	16,83		

The increase rate of cell cultures didn't present a significant increasing in the first 9th days of culture, from the 12th day the values being significant distinctly and very significant.

From analysis variation for the increase rate of cellular cultures to Cabernet Sauvignon variety, it also observed, the existence of some significant distinctly differences both between repetitions and cultivation days under the increase rate aspect. (Table 3).

Table 3. Variance analysis regarding the increase rate of cell suspension culture on Cabernet Sauvignon variety

Source of variation	SP	GL	S ²	Test F
Total	111036,35	35		
Repetitions	1175,12	2	587,56	F =8,12**
Cultivation period	108269,95	11	9842,72	F = 136,08**
Error	1591,28	22	72,33	

The rate of cellular cultures in suspension increasing, to Cabernet Sauvignon variety proportionally increase with the cultivation period, touching a maximal average value of 237,49% after 30 days of cultivation. After that date, the increase rate gradually decreased, at 36th days of culture registering a value of 222,62%, the difference being of 14,87% (Table 4). To Cabernet Sauvignon variety, the increase rate of cellular culture registered very significant values beginning with the 15th day of cultivation.

Table 4. The significance of differences between different days of culture regarding the increase rate of cell suspension culture on Cabernet Sauvignon variety

No.	Cultivation period (days)	Average increase rate (%)		Difference from the blank	Significance
		$\bar{x} \pm s_{\bar{x}}$	$s_{\%}$		
1	0	100,00±0,00	0,00	0,00	Blank
2	6	100,78±1,06	1,83	0,78	-
3	9	105,75±1,10	1,80	5,75	-
4	12	114,39±1,67	2,52	14,39	-
5	15	132,89±2,41	3,14	32,89	***
6	18	154,91±4,14	4,63	54,91	***
7	21	186,52±3,97	3,69	86,52	***
8	24	213,39±5,57	4,52	113,39	***
9	27	235,18±11,18	8,23	135,18	***
10	30	237,49±10,47	7,63	137,49	***
11	33	232,01±9,10	6,79	132,01	***
12	36	222,62±8,30	6,46	122,62	***
DL _{5%}	DL _{1%}	DL _{0,1%}			
14,41	19,58	26,35			

To Merlot variety, from the variation analysis for the cell cultures increase it could observe the existence of some significant distinct differences between repetitions concerning the cultivation under the aspect of cellular increase rate (Table 5).

Table 5. Variance analysis regarding the increase rate of cell suspension culture on Merlot variety

Source of variation	SP	GL	S ²	Test F
Total	17987,69	35		
Repetitions	3109,54	2	1554,77	F = 14,71**
Cultivation period	171553,85	11	15595,80	F = 147,61**
Error	2324,30	22	105,65	

Table 6. The significance of differences between different days of culture regarding the increase rate of cell suspension culture on Merlot variety

No.	Cultivation period (days)	Average increase rate (%)		Difference from the blank	Significance
		$\bar{x} \pm s_{\bar{x}}$	$s_{\%}$		
1	0	100,00±0,00	0,00	0,00	Blank
2	6	101,66±1,66	2,24	1,66	-
3	9	110,85±10,85	8,45	10,85	-
4	12	120,59±20,59	9,96	20,59	*
5	15	143,26±43,26	12,91	43,26	***
6	18	169,85±69,85	10,30	69,85	***
7	21	199,29±99,29	12,74	99,29	***
8	24	232,33±132,33	13,31	132,33	***
9	27	272,69±172,69	3,15	172,69	***
10	30	275,10±175,10	0,84	175,10	***
11	33	271,84±171,84	2,05	171,84	***
12	36	260,65±160,65	4,21	160,65	***
DL _{5%}	DL _{1%}	DL _{0,1%}			
17,41	23,67	31,85			

In base of results from Table 6 it observed that the highest values of culture increase rate to Merlot variety (275,10%) registered also after 30 days of culture afterwards the values decreased with a difference of 14,45% until the 36th day of culture.

In case of Merlot species, the increase rate of cellular culture in suspension registered significant values after 12 days of culture.

In case of Oporto variety, from Table 7 of variation analysis for the increase rate of the cells culture in suspension, it observed the existence of some distinct significant differences both among repetitions ($F=14,71$), and concerning the cultivation period ($F=147,61$) under the aspect of cellular increasing rate.

Table 7. Variance analysis regarding the increase rate of cell suspension culture on Oporto variety

Source of variation	SP	GL	S ²	Test F
Total	17987,69	35		
Repetitions	3109,54	2	1554,77	F = 14,71**
Cultivation period	171553,85	11	15595,80	F = 147,61**
Error	2324,30	22	105,65	

The increase rate of cellular culture at Oporto species registered a maximal average value of 251,65% also after 30 days consecutively. After that day and until the 36th day of culture, the increase rate of cellular culture gradually decreased registering a difference of 14,69%.

In case of cells culture in suspension to Oporto species it observed the increase rate registered significant values beginning with the 12 day of culture (Table 8).

Table 8. The significance of differences between different days of culture regarding the increase rate of cell suspension culture on Oporto variety

No.	Cultivation period (days)	Average increase rate (%)		Difference from the blank	Significance
		$\bar{x} \pm s_{\bar{x}}$	s %		
1	0	100,00±0,00	0,00	0,00	Blank
2	6	101,69±0,88	1,50	1,69	-
3	9	111,13±1,64	2,55	11,13	-
4	12	123,96±4,57	6,39	23,96	*
5	15	140,00±5,20	6,44	40,00	***
6	18	163,35±7,21	7,65	63,35	***
7	21	204,61±16,01	13,55	104,61	***
8	24	224,82±13,58	10,46	124,82	***
9	27	244,78±11,82	8,37	144,78	***
10	30	251,65±13,15	9,05	151,65	***
11	33	248,21±12,48	8,71	148,21	***
12	36	236,96±10,00	7,31	136,96	***
DL 5%		DL 1%	DL 0,1%		
18,34		24,92	33,53		

From the variation analysis for the increase cells in suspension to Negru Tinctorial variety (Table 9), it observed the existence of some distinct significant differences only regarding the cultivation period ($F=383,16$).

The cultures of cells in suspension registered an increase rate with maximal average value of 247,69% at Negru Tinctorial species after a period of 30 days of cultivation. Until the 36th day of cultivation, the increase rate decreased registering in value a difference of 12,24% (Table 10).

Table 9. Variance analysis regarding the increase rate of cell suspension culture on Negru Tinctorial variety

Source of variation	SP	GL	S ²	Test F
Total	125182,82	35		
Repetitions	121,28	2	60,63	F = 2,05
Cultivation period	124412,16	11	11310,20	F = 383,16**
Error	649,39	22	29,51	

Table 10. The significance of differences between different days of culture regarding the increase rate of cell suspension culture on Negru Tinctorial variety

No.	Cultivation period (days)	Average increase rate (%)			Difference from the blank	Significance
		$\bar{x} \pm s_x$	s_x	s_x		
1	0	100,00±0,00	0,00	0,00	0,00	Blank
2	6	102,30±2,07	3,50	2,30	2,30	-
3	9	107,89±2,11	3,38	7,89	7,89	-
4	12	122,14±3,06	4,34	22,14	22,14	***
5	15	144,44±3,44	4,12	44,44	44,44	***
6	18	173,14±0,97	0,97	73,14	73,14	***
7	21	208,73±3,66	3,03	108,73	108,73	***
8	24	230,85±2,77	2,08	130,85	130,85	***
9	27	244,14±3,56	2,52	144,14	144,14	***
10	30	247,69±5,62	3,93	147,69	147,69	***
11	33	243,18±3,76	2,68	143,18	143,18	***
12	36	235,45±4,26	3,13	135,45	135,45	***
DL _{5%}		DL _{1%}	DL _{0,1%}			
9,20		12,51	16,83			

In case of cell suspension cultures at Negru Tinctorial variety, the increase rate of culture registered very significant values beginning with the 12th day of culture.

From Table 11 of variation analysis for the increase rate of cells cultures at Pinot Noir variety it could observe the existence of some distinct significant differences both among repetitions (F=4,55) and concerning the cultivation period (F=252,7) under the aspect of cells culture increasing.

Table 11. Variance analysis regarding the increase rate of cell suspension culture on Pinot Noir variety

Source of variation	SP	GL	S ²	Test F
Total	154625,10	35		
Repetitions	501,25	2	250,62	F = 4,55**
Cultivation period	152913,69	11	13901,24	F = 252,71**
Error	1210,17	22	55,00	

In base of Table 12 results, it observed also at Pinot Noir variety as in case of the others species that the rate of culture proportionally increased with the cultivation period touching a maximal average value of 269,22% also after 30 days of cultivation. After that date, the culture increase registered a regress until 36 days where touched the level of 257,64%, with a difference of 11,58% face to the maximal average value registered.

In case of cells in suspension cultures at Pinot Noir variety, the cellular culture increase registered significant values from 9th day of culture and from 12th day of cultivation very significant values had done.

Table 12. The significance of differences between different days of culture regarding the increase rate of cell suspension culture on Pinot Noir variety

No.	Cultivation period (days)	Average increase rate (%)		Difference from the blank	Significance
		$\bar{x} \pm s_{\bar{x}}$	$s_{\%}$		
1	0	100,00+0,00	0,00		Blank
2	6	101,57+0,79	1,34	1,57	-
3	9	113,30+2,19	3,35	13,30	*
4	12	127,45+3,88	5,27	27,45	***
5	15	146,65+4,01	4,74	46,65	***
6	18	168,51+7,46	7,67	68,51	***
7	21	203,28+7,75	6,60	103,28	***
8	24	237,24+5,76	4,20	137,24	***
9	27	255,70+4,13	2,80	155,70	***
10	30	269,22+5,32	3,43	169,22	***
11	33	263,79+4,63	3,04	163,79	***
12	36	257,64+5,75	3,87	157,64	***
DL _{5%}		DL _{1%}	DL _{0.1%}		
12,57		17,08	22,98		

Through calculation of regression equation between the cultivation period and increase rate of cells culture it observed that to all species existed a relation of linear proportionality between the cultivation period and culture increase rate, the registered data grouping enough close around the regression line; the correlation between two variables was direct and positive (Figure 1.A-F).

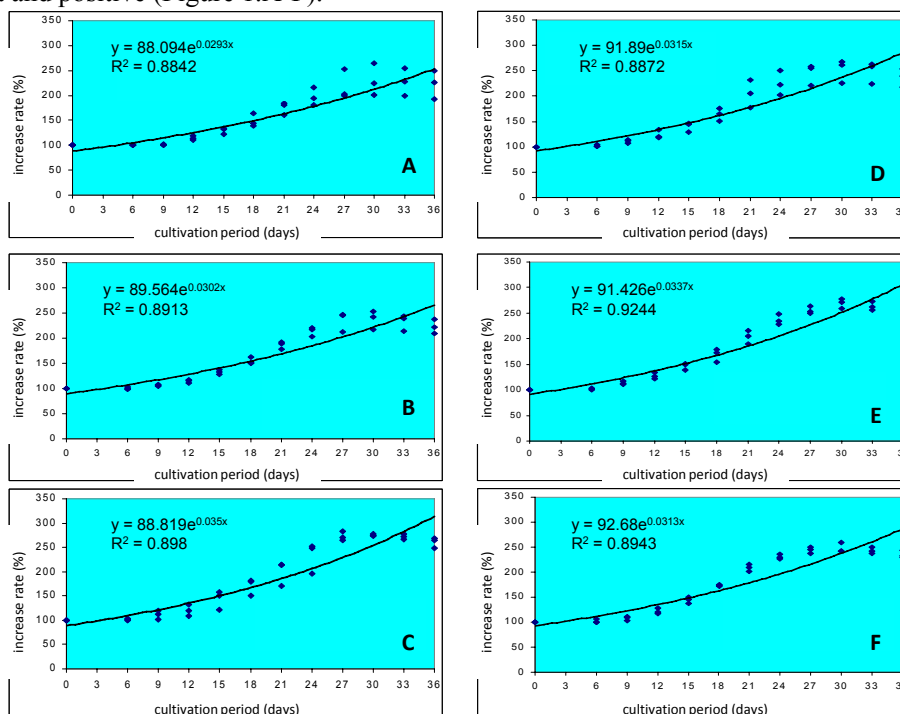


Figure 1. Graphical representation regarding the growth rate of cell suspension culture on grapevine varieties (A – Burgund Mare, B – Cabernet Sauvignon, C – Merlot, D – Oporto, E – Negru Tinctorial, F – Pinot Noir)

Comparing the varieties studied from the point of view of the increasing rate of cell suspensions, it could observe from the variation analysis of every variety that to the major ones existed distinct significant differences among repetitions and about the cultivation

period, exception making the differences among repetitions at Negru Tinctorial variety which weren't statistically assured.

Regarding the increase rate of culture, it observed to all the varieties, that the one proportionally increased with the period of cultivation through a maximal average value registered at 30 days of culture, afterwards, it gradually decreased with a difference of 6,73-14,87% in function of the variety.

The increase rate of cells culture registered a maximal average value at Merlot variety (275,10%). The values registered at Merlot variety, were superior to the others species, being followed by Pinot Noir species (269,22%), Oporto (251,65%), Negru Tinctorial (247,69%), Cabernet Sauvignon (237,49%) and Burgund Mare (229,73%).

In case of the cell suspension cultures, the increase rate registered significant values after 9th days of culture at Pinot Noir variety, 12 days of culture at Burgund Mare, Merlot, Oporto, Negru Tinctorial and after 15 days of culture at Cabernet Sauvignon.

Conclusions

The results of variation analysis of increasing rates of cell suspensions at grapevine varieties studied, it had emphasized the existence of distinct significant differences among repetitions. Exception was Negru Tinctorial variety, at which the differences among repetitions weren't statistically assured.

In our experimental conditions at all the varieties studied, the cell suspension increasing stopped after 30 days of culture.

At Merlot species was registered a maximal average value of 275,10% the increasing rate of cell suspensions. For the others varieties were registered the following values: Pinot Noir- 269,22%, Oporto- 251,65%, Negru Tinctorial- 247,69%, Cabernet Sauvignon- 237,49% and Burgund Mare - 229,73%.

It existed differences among varieties about initiation the phase of exponential increasing. At Pinot Noir variety, increase rate registered significant values after 9 days of culture, at Burgund Mare variety, Merlot, Oporto, Negru Tinctorial, after 12 days, and at Cabernet Sauvignon variety, after 15 days of culture.

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