IN VITRO REACTIVITY OF *CYMBIDIUM HYBRIDUM* L. PROTOCORMS, ON BISTRATIFIED CULTURE MEDIUMS, USING BIDISTILLED WATER OR GLUCOSE SOLUTIONS AS SUPERNATANTS

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ABSTRACT. Knowing the fact that the protocorms multiplication processes are accelerated in their submersion conditions in liquid medium, against the situation that, these protocorms are vitrocultivated on solid (agarized) medium cultures (which prevails in organogenesis processes), our objective is to study the influence exerted by the cultures, practiced in bistratified regime, to *Cymbidium* protocorms vitrocultures. In this interest, we used bidistilled water as supernatant, either on 2% or 5% glucose, which were applied over the inoculated protocorms on agarized medium cultures. The basic medium culture used by us in these experiments was Murashige – Skoog (1962). To this, we added different growth regulators, such as: 2,4-D (2 mg/l), or mixture of BA (2 mg/l) with NAA (1 mg/l), or only BA (2 mg/l), or only NAA (1 mg/l). The witness lot was consisted of vitrocultivated protocorms on agarized medium culture, without growth regulators, cultivated in monolayer. After 90 days from the initiation of double-layer medium cultures, we ascertained that, the application of the second layer (the liquid one) over the agarized medium cultures, strongly stimulated the multiplication of Cymbidium protocorms, mostly if the second layer was bidistilled water; the usage of a solution of glucose 2% as supernatant on medium culture without growth regulators, was better in comparison with the control variant, the neoformed protocorms, being numerical above 1.8 times more.

Keywords: protocorms, *Cymbidium* (orchid), double-layer, glucose, *in vitro*

INTRODUCTION

The purpose followed by us in these experiments consisted of analyzing of the *Cymbidium* protocorms reaction in their vitrocultivation conditions in aseptic regime, on a bistratified medium culture, the agarized medium in the basal zone of the recipients covered whit a liquid layer, water or glucose solutions.

From the specialized literature we know that the *Cymbidium* protocorms, *in vitro*, breed and multiply both on solid substratum and, in submersing regime covered by a liquid medium. Particularly, on a solid medium, on the level of protocorms, a multiplication process of protocorms takes place (Cachiţă, 1987).

Molnár (1982) used the vitroculture in double layer system to various kinds of explants, cultivated *in vitro*, inoculated on the solid medium in the same moment with the supernatant, which had an identical chemical composition with the agarized medium, situated in the basal zone of the culture recipient (from the liquid medium culture missing only the agar).

Cachiţă (1982) used the bilayer culture system, especially to *Cymbidium* protocorms cultivation. But, she placed the protocorm, initially on the surface of the agarized medium, and then, in time, she covered it with liquid medium; the supernatant column, was volumetrically equal to the agarized mass.

In 1985, Molnár used his method, respectively the double layer system culture, with success, over a 26 vegetal genus, in the *in vitro* plant multiplication, through this method, increasing the micropropagation efficiency.

Pătru et al. (2002), observed that the complete replacement of saccharose by 30g/l fructose was efficient, enhancing by 22% the number of neoformed protocorms, and growth by over 100% of their weight, fresh and dry, but only on agarized medium. The increase of fructose concentration to 50 or 70 g/l, did not prove a positive effect of this fructose supplement, especially in the case of 70 g/l concentration, which has influenced in a negative way the protocorms multiplication.

Cymbidium protocorms reaction, in the performed cultures which on solid (agarized) medium, or on submersed regime, was different in the presence of fitoregulators in the cultivated medium (Blidar and Cachiţă, 2002). Blidar and Cachiţă tested the *in vitro* reactivity of the *Cymbidium* protocorms, using, bidistilled water as supernatant sucrose or fructose solution (in varied concentration). They observed that, the *Cymbidium* protocorms, which were covered by bidistilled water, were stimulated in there multiplication, and also in the morphogenetic processes (Blidar and Cachiţă, 2003; Blidar et al., 2008).

The present study represents a continuation of our researches, in the followed morphogenesis direction, and *in vitro* multiplication of *Cymbidium* protocorms, in the usage of supernatant conditions, in bistratified cultures; the supernatant was represented by bidistilled water, or by 2%, or 5% glucose solutions.

MATERIALS AND METHODS

The biological material consisted in *Cymbidium hybridum* protocorms. There were inoculated on a basic Murashige-Skoog solid medium (1962) (MS), modified by us, without: glicine, 3-indolil acetic acid (IAA) or kinetin (K), and with only 20 g/l sucrose in the cultivated medium, instead of 30 g/l sucrose stipulated in the original recipe; for the culture we used liquid or agarized medium, solidified with 7 g/l Difco-Bacto agar (BM). In this experiment we used protocorms derived from a micro propagated culture collection, made in the Vegetal Biotechnological Laboratory, from Oradea University. The control lot of protocorms was vitrocultured on the same kind of medium, without growth regulators.

The present experiment used the organization of four variant series, structured - each of them - on five variants (table 1). In all, it was analyzed the evolution of the cultivated on 20 variants of medium culture. Practically, on 5 cm^3 agarized substratum, after the protocorms' inoculation in aseptic regime, a second layer was administrated, respectively with 5 cm^3 supernatant, consisting of: bidistilled water (H_2O series), or in 2% (G_2), or 5% (G_5) glucose solution. The protocorm cultures were put in glass bottles, with the height of 70 mm, and with interior diameter of 25 mm. The experimental variant was the following:

- series I: V_0M-V_4M – protocorms cultivated on agarized medium (reference series, control);
- series II: V_0H_2O-V_4H_2O – protocorms cultivated on agarized medium, covered with bidistilled water, as supernatant;
- series III: V_0G_2-V_4G_2 – protocorms cultivated on agarized medium, and covered with 2% glucose solution;
- series IV: V_0G_5-V_4G_5 – protocorms cultivated on agarized medium, and covered with 5% glucose solution.

Table 1

THE USED CULTURE MEDIUM VARIANTS IN THE *CYMBIDIUM HYBRIDUM* PROTOCORMS VITROCULTURES

Monolayer culture on solid media Series I	Double layer cultures		
	H_2O as supernatant Series II	**G 2% as supernatant Series III**	**G 5% as supernatant Series IV**
V_0M – solid medium culture (agarized) with basic nutritive elements specific to *Murashige-Skoog* (MS) medium	$V_0M + H_2O$ = **V_0H_2O**	V_0M + 2% glucose solution = **V_0G_2**	V_0M + 5% glucose solution = **V_0G_5**
V_1M – MS basic medium plus 2 mg/l 2,4-D (*2,4- dichlorophenoxyacetic acid*)	$V_1M + H_2O$ = **V_1H_2O**	V_1M + 2% glucose solution = **V_1G_2**	V_1M + 5% glucose solution = **V_1G_5**
V_2M – MS basic medium plus 2 mg/l BA (*benzyladenine*) and 1 mg/l NAA (*α-naphtylacetic acid*)	$V_2M + H_2O$ = **V_2H_2O**	V_2M + 2% glucose solution = **V_2G_2**	V_2M + 5% glucose solution = **V_2G_5**
V_3M – MS basic medium plus 2 mg/l BA	$V_3M + H_2O$ = **V_3H_2O**	V_3M + 2% glucose solution = **V_3G_2**	V_3M + 5% glucose solution = **V_3G_5**
V_4M – MS basic medium plus 1 mg/l NAA	$V_4M + H_2O$ = **V_4H_2O**	V_4M + 2% glucose solution = **V_4G_2**	V_4M + 5% glucose solution = **V_4G_5**

After portioning the agarized medium, the bottles with medium were sterilized in autoclave, at 121^0C, for 20 minutes. The supernatant (liquid medium) was autoclaved separately. In each bottle only a single protocorm has been inoculated. After the inoculation of the protocorms, and the application of the second liquid layer, the bottles were covered with colourless transparent foil of polyethylene, immobilized with rubber rings. Then, the bottles were put in a growth chamber, and were placed on artificially illuminated shelves with white coloured fluorescent tubes (1400 lucs luminous intensity), in photoperiodic regime of 16 hours light / 24 hours, the surrounding temperature oscillated between 24^0C in the light period (the day), and 22^0C in the dark phase.

At 30, 60 and 90 days of vitrocultures, observations and biometric measures were made, concerning the number of protocorms, and the fresh and dried weight of these. The experimental dates obtained to the control variant, respectively on V_0M variant basic medium (BM-MS without growth hormones, and supernatant) was considered as reference lot (control), respectively 100%; the average of the registered values – to each parameter and variant – fractionally – were reported to the average values obtained at similar parameters, to witness variant. The experimental dates were processed statistically, establishing the sense of these, on the basis of the variability values.

The most illustrative appearance concerning the differentiated reactivity of the inoculs, respectively to

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the resulted vitrocultures from *Cymbidium* protocorms, to those 20 experimental variants, the photos from drawing 1 and the objects of tables 2-4.

RESULTS AND DISCUSSION

From each initial protocorm, in the vitroculture period, new protocorms were regenerated, which – in time – constitutes a glomerule (like blackberries fruit), with or without morphogenese manifestation of another nature.

In the case of the observations performed at 30 days from the inoculation of the protocorms, the highest values, as regards the regenerated *number of protocorms* to the level of each protocormic clone, were registered to variant of medium with supernatant consisted of bidistillated water, an exception being evident in the case of using the synthetic auxine 2 mg/l 2,4-D, as growth regulator, in the situation in which the presence of supernatant inducted the lagging of morphogenesis (table 2). In the matter of *fresh weight* of protocorms, the highest values labelled per each culture bottle, were registered on variant of medium culture V_3G_2 (BM-MS with 2 mg/l BA as growth regulator, covered with a 2% glucose solution, as supernatant), the difference against witness (V_0M – agarized BM-MS, devoid of the growth regulator) being enhanced average with 65,4 mg/glomerule, respectively the numbers being with about 132,1% higher that the values obtained to this parameter to the control variant (values which weren't sustained as relevant statistical point of view, because of the variability of the protocorms populations included in the experiences, concerning the morphogenesis, …) (table 3). Also, it has been noticed that the solution of glucose 5% - used as supernatant – favoured the accumulation of *dry substance* in the protocorms glomerule, to all variants of the growth regulators used in this experiment, with the exception of V_3 variant (MB-MS with addition of BA 2 mg/l) and V_4 variant (BM-MS with 1 mg/l NAA), where the best results was registered in case we use as supernatant a solution of 2% glucose) (table 4).

The performed observations made after 60 days from inoculation, emphasized that, the previously described phenomenon increased in the case of the *number of protocorms*, respectively on medium with supernatant consisting of bidistilled water, the single exception been evident in the case of using the 2 mg/l 2,4-D auxine (table 2); in case of *weight* glomerullar mass (both *fresh* and *dry*), the highest registered value to the variants of vitrocultivated protocorms, growth on the medium culture hereupon the supernatant was consisted of 2% glucose solution as the second layer on basal MS agarized medium with 2 mg/l BA and 1 mg/l NAA growth regulators, the difference against witness (V_0M – agarized BM-MS, devoid of the growth regulator) being enhanced average with 135.6 mg/glomerule, respectively the numbers being with about 82% higher for the fresh weight, and 11.7 mg/glomerule, with about 81.8% higher for the dry weight than the values obtained to this parameter to the control variant (table 3 and 4).

The performed observations made at 90 days from inoculation (drawing 1), matter the *number of protocorms*, best results were registered when we used bidistilled water as supernatant, when the solid substratum was consisted from a MS medium with addition of 2 mg/l BA with 1 mg/l NAA, variant of V_2H_2O medium (drawing 1C) meaningful data from statistical point of view). To this variant, we registered 54.7 protocorms/glomerule, against 15.7 marked to the control variant (table 2). The presence in the agarized medium of 2,4-D auxine, in amount of 2 mg/l, in the condition of covering the protocorms with any type of fit supernatant, launched the necrosis of these (drawing 1B); on same type of agarized medium, with a same quantity of 2,4-D in substratum, but without supernatant, protocorms proliferated, but these didn't grow, remaining tiny, presenting the senescence phenomenon (drawing 1B). In the matter of the *fresh weight* evaluation of protocorms glomerules, the best results were registered on V_2H_2O variant, value data closely followed by the sample in which we used as the supernatant the 2% glucose solution, the solid medium being devoided by growth regulators (V_0G_2) (table 3). If on V_2 and on V_4 variants, the *dried weight* of the protocorms were maximum to the covered variants with bidistilled water, as supernatant (meaningfully statistical values – see table 4), in the case of the control variant (V_0M) an on V_3 sample, the highest gravimetric values were observed to variants hereupon the supernatant were constituted from a solution of 2% glucose (V_0G_2, respectively V_3G_2), data sustained as relevant statistical point of view (table 4).

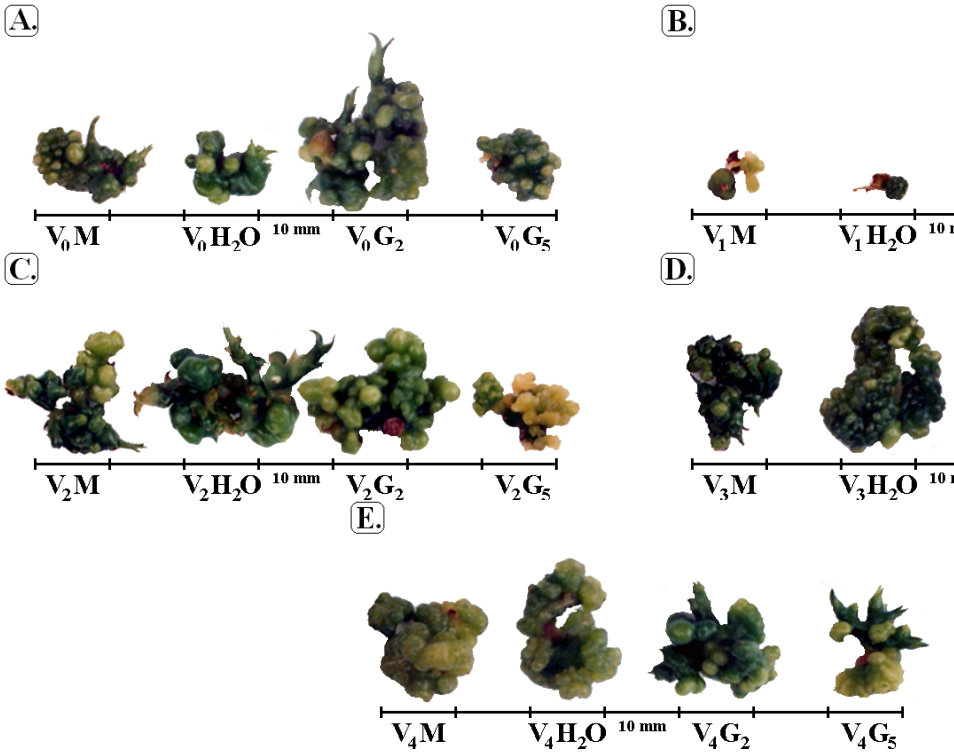
CONCLUSIONS

At 90 days of vitrocultures, except the medium cultures with (2 mg/l) 2,4-D, the neogenesis of *Cymbidium* protocorms was more economic than, in the conditions that, over these – located on agarized medium cultures – was applied bidistilled water, as second layer.

To assure an intense multiplication of *Cymbidium* protocorms, in a subculture program to a distance of 3 months, the use of bidistilled water, as supernatant, applied on an agarized medium culture with a mixture of (2 mg/l) BA and (1 mg/l) NAA, it proved to be the optimal variant of these vitrocultures, the number of protocorms being above 3.5 times higher than the values registered on medium cultures without supernatant and growth regulators (witness medium).

The use of a solution of glucose 5%, as supernatant, was the most inefficient procedure for the micropropagation of *Cymbidium* protocorms, regardless of the quantity of growth regulators existing in the agarized layer of medium cultures.

In double-layer medium cultures, the presence of (2 mg/l) 2,4-D auxine, it was proved that it is an inefficient procedure, matter the multiplication and growth of *Cymbidium* protocorms, regardless of the presence or absence of the second layer, since, already at 30 days of vitrocultures, the protocorms presented severe senescence processes, which, later – at 60 days, but mostly at 90 days of vitrocultures – led to their necrosis.



Drawing 1. The aspects of *Cymbidium hybridum* protocorms, constituted in glomerules, "in vitro" neoformed, on a solid BM Murashige-Skoog (1962) basic medium culture modified (experimental series **"M"**), vitrocultures without supernatant, or covered with a liquid layer, apply across basic medium (solid), the liquid layer being represented on bidistilled water (experimental series **"H_2O"**), of a 2% glucose solution (experimental series **"G_2"**) or of 5% glucose solution (experimental series **"G_5"**); the agarized substratum presented a varied content of growth regulators, as how follows: **V_0** - BM without growth (control lot) (**A**), **V_1** – BM with an adding of 2 mg/l *2,4-dichlorophenoxyacetic acid* (2,4-D) (**B**); **V_2** – BM with an adding of 2 mg/l *benzyladenine* (BA) mixed with 1 mg/l *α-naphthylacetic acid* (NAA) (**C**); **V_3** – BM only with 2 mg/l BA (**D**), **V_4** – BM only with 1 mg/l NAA (**E**), after *90 days* from assembling of the experiments.

Table 2

The statistical sense of the data concerning the protocorms number of *Cymbidium hybridum*, in their vitrocultivation condition on a solid BM Murashige-Skoog (1962) basic medium culture modified, vitrocultures without supernatant (experimental series "M") or covered with a liquid layer, apply across basic medium (solid), the liquid layer being represented on bidistilled water (experimental series "H_2O"), of a 2% glucose solution (experimental series "G_2") or of 5% glucose solution (experimental series "G_5"); the agarized substratum presented a varied content of growth regulators, as how follows: V_0 - BM without growth (lot control), V_1 – BM with an adding of 2 mg/l *2,4-dichlorophenoxyacetic acid* (2,4-D), V_2 – BM with an adding of 2 mg/l *benzyladenine* (BA) mixed with 1 mg/l *α-naphthylacetic acid* (NAA), V_3 – BM only with 2 mg/l BA, V_4 – BM only with 1 mg/l NAA, after *30 days, 60 days* and *90 days* from assembling of the experiments

Hormonal variant (basal layer)	Series	Thesupernatant composition (2nd layer)	The medium number of protocorms (pieces) / flask ± SD			Variant value			Statistical significance (p)		
			30 days	*60 days*	*90 days*	*30 days*	*60 days*	*90 days*	*30 days*	*60 days*	*90 days*
V_0	M	-	4,8 ± 0,51	10,1 ± 0,50	15,7 ± 0,89	0,454	0,49	0,028	*	*	**
	H_2O	H_2O	5,3 ± 0,54	18 ± 0,21	35,7 ± 0,55	0,371	0,384	0,356	*	*	*
	G_2	2% glucose	5,3 ± 0,52	9,7 ± 0,54	35,5 ± 0,41	0,986	0,854	0,448	-	-	*
	G_5	5% glucose	3,2 ± 0,65	8,1 ± 0,85	16,7 ± 0,84	0,402	0,488	0,345	*	*	*
V_1	M	-	3,2 ± 0,63	3,7 ± 0,85	7,4 ± 0,44	0,587	0,802	0,69	-	-	-
	H_2O	H_2O	2,7 ± 0,74	3,6 ± 0,6	3,8 ± 0,84	0,985	0,644	0,741	-	-	-
	G_2	2% glucose	3,7 ± 0,43	5,5 ± 0,74	5,8 ± 0,32	0,421	0,524	0,875	*	-	-
	G_5	5% glucose	2,5 ± 0,51	4 ± 0,91	6,8 ± 0,48	0,917	0,554	0,745	-	-	-
V_2	M	-	4,6 ±	13 ±	22,6 ±	0,385	0,41	0,452	*	*	*

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			0,65	0,41	0,44						
	H_2O	H_2O	5,6 ± 0,15	22,3 ± 0,98	54,7 ± 0,84	0,85	0,548	0,48	-	-	*
	G_2	2% glucose	4,3 ± 0,70	14 ± 0,74	35,6 ± 0,25	0,911	0,68	0,447	-	-	*
	G_5	5% glucose	3,7 ± 0,47	12,7 ± 0,58	31,7 ± 0,71	0,452	0,41	0,32	*	*	*
	M	-	4,2 ± 0,27	12,4 ± 0,47	16,5 ± 0,35	0,389	0,378	0,482	*	*	*
V_3	H_2O	H_2O	5,9 ± 0,73	26,6 ± 0,84	36,9 ± 0,58	0,45	0,345	0,389	*	*	*
	G_2	2% glucose	3,2 ± 0,65	18 ± 0,95	36,9 ± 0,65	0,955	0,712	0,84	-	-	*
	G_5	5% glucose	3,5 ± 0,89	12 ± 0,41	20,2 ± 0,78	0,444	0,112	0,4	*	*	**
	M	-	2,4 ± 0,41	5,4 ± 0,68	12 ± 0,69	0,345	0,441	0,035	*	*	**
	H_2O	H_2O	4,9 ± 0,89	7,8 ± 0,71	24,7 ± 0,64	0,51	0,87	0,489	-	-	*
V_4	G_2	2% glucose	3 ± 0,52	6,9 ± 0,4	15,2 ± 0,77	0,524	0,811	0,511	-	-	-
	G_5	5% glucose	2,5 ± 0,44	3,6 ± 0,88	8,7 ± 0,69	0,079	0,24	0,31	*	*	*

*where: SD – standard diversion; * 0,05 < p ≤ 0,5 – significant values; ** p ≤ 0,05 – very significant values*

Table 3

The statistical sense of the data concerning the fresh weight mass of *Cymbidium hybridum*, in their vitrocultivation condition on a solid BM Murashige-Skoog (1962) basic medium culture modified, vitrocultures without supernatant (experimental series "M") or covered with a liquid layer, apply across basic medium (solid), the liquid layer being represented on bidistilled water (experimental series "H_2O"), of a 2% glucose solution (experimental series "G_2") or of 5% glucose solution (experimental series "G_5"); the agarized substratum presented a varied content of growth regulators, as how follows: V_0 - BM without growth (lot control), V_1 – BM with an adding of 2 mg/l *2,4-dichlorophenoxyacetic acid* (2,4-D), V_2 – BM with an adding of 2 mg/l *benzyladenine* (BA) mixed with 1 mg/l *α-naphthylacetic acid* (NAA), V_3 – BM only with 2 mg/l BA, V_4 – BM only with 1 mg/l NAA, after *30 days, 60 days* and *90 days* from assembling of the experiments

Hormonal variant (basal layer)	Series	The supernatant composition (2nd layer)	The fresh weight mass (mg) / flask ± SD			Variant value			Statistical significance (p)		
			30 days	*60 days*	*90 days*	*30 days*	*60 days*	*90 days*	*30 days*	*60 days*	*90 days*
	M	-	297,4 ± 0,47	992,1 ± 0,62	1965,8 ± 0,87	0,405	0,42	0,26	*	*	*
V_0	H_2O	H_2O	466,9 ± 0,68	1072,1 ± 0,87	3767,1 ± 0,31	0,387	0,369	0,402	*	*	*
	G_2	2% glucose	199,5 ± 0,95	712,2 ± 0,74	7044,8 ± 0,54	1,395	1,021	0,322	-	-	*
	G_5	5% glucose	553,1 ± 0,23	563,5 ± 0,44	1725,8 ± 0,45	0,081	0,289	0,21	*	*	*
	M	-	121,6 ± 0,78	162,6 ± 0,54	696,2 ± 0,51	0,925	0,701	0,888	-	-	-
V_1	H_2O	H_2O	152,2 ± 0,48	197,7 ± 0,96	287,2 ± 0,45	0,524	0,78	0,874	-	-	-
	G_2	2% glucose	154,3 ± 0,96	211,4 ± 0,14	254,8 ± 0,48	0,536	0,994	0,79	-	-	-
	G_5	5% glucose	222,1 ± 0,68	252,7 ± 0,25	282,5 ± 0,47	0,745	0,825	0,741	-	-	-
	M	-	404,4 ± 0,96	724,4 ± 0,62	2836 ± 0,95	0,455	0,37	0,287	*	*	*
V_2	H_2O	H_2O	450,8 ± 0,41	1569,3 ± 0,74	8248,6 ± 0,25	0,554	0,955	0,255	-	-	*
	G_2	2% glucose	541,2 ± 0,85	1805,5 ± 0,25	6753 ± 0,45	0,847	0,7	0,398	-	-	*
	G_5	5% glucose	456,2 ± 0,21	511,8 ± 0,41	2815,4 ± 0,98	0,765	0,48	0,287	-	*	*
V_3	M	-	275,3 ± 0,33	663,2 ± 0,54	1623,2 ± 0,89	0,227	0,386	0,488	*	*	*
	H_2O	H_2O	562,6 ±	1065,8 ±	5911,4 ±	0,389	0,455	0,34	*	*	*

			0,47	0,58	0,4						
	G_2	2% glucose	689,6 ± 0,7	1098,5 ± 0,41	4005 ± 0,92	0,658	1,021	0,489	-	-	*
	G_5	5% glucose	137,9 ± 0,68	382,4 ± 0,62	1327,2 ± 0,45	0,445	0,135	0,041	*	*	**
	M	-	228,4 ± 0,45	586,7 ± 0,66	2043,4 ± 0,45	0,245	0,489	0,035	*	*	**
	H_2O	H_2O	284,6 ± 0,99	884,7 ± 0,79	5823,6 ± 0,48	0,589	0,648	0,43	-	-	*
V_4	G_2	2% glucose	429,7 ± 0,22	987,6 ± 0,98	4346,5 ± 0,42	0,868	0,964	0,682	-	-	-
	G_5	5% glucose	203,4 ± 0,45	264 ± 0,24	1252,6 ± 0,81	0,385	0,339	0,341	*	*	*

*where: SD – standard diversion; * 0,05 < p ≤ 0,5 – significant values; ** p ≤ 0,05 – very significant values*

Table 4

The statistical sense of the data concerning the dry weight mass of *Cymbidium hybridum*, in their vitrocultivation condition on a solid BM Murashige-Skoog (1962) basic medium culture modified, vitrocultures without supernatant (experimental series "M") or covered with a liquid layer, apply across basic medium (solid), the liquid layer being represented on bidistilled water (experimental series "H_2O"), of a 2% glucose solution (experimental series "G_2") or of 5% glucose solution (experimental series "G_5"); the agarized substratum showed a varied content of growth regulators, as how follows: V_0 - BM without growth (lot control), V_1 – BM with an adding of 2 mg/l *2,4-dichlorophenoxyacetic acid* (2,4-D), V_2 – BM with an adding of 2 mg/l *benzyladenine* (BA) mixed with 1 mg/l *α-naphthylacetic acid* (NAA), V_3 – BM only with 2 mg/l BA, V_4 – BM only with 1 mg/l NAA, after *30 days, 60 days* and *90 days* from assembling of the experiments

Hormonal variant (basal layer)	Series	Thesuper-natant composition (2nd layer)	The dry weight mass (mg) / flask ± SD			Variant value			Statistical significance (p)		
			30 days	*60 days*	*90 days*	*30 days*	*60 days*	*90 days*	*30 days*	*60 days*	*90 days*
	M	-	30,1 ± 0,98	86,2 ± 0,94	178 ± 0,98	0,456	0,036	0,022	*	**	**
	H_2O	H_2O	35,1 ± 0,74	65,2 ± 0,62	230,5 ± 0,87	0,345	0,484	0,497	*	*	*
V_0	G_2	2% glucose	19,5 ± 0,48	56,3 ± 0,48	450 ± 0,44	0,748	0,712	0,41	-	-	*
	G_5	5% glucose	65,5 ± 0,55	70,9 ± 0,71	191,2 ± 0,98	0,23	0,495	0,046	*	*	**
	M	-	14,1 ± 0,21	23,1 ± 0,57	83,2 ± 0,41	0,862	0,612	0,58	-	-	-
	H_2O	H_2O	15,9 ± 0,35	23,3 ± 0,54	28,8 ± 0,88	0,947	0,987	0,881	-	-	-
V_1	G_2	2% glucose	14,4 ± 0,39	25,6 ± 0,85	35,3 ± 0,44	0,63	0,941	0,785	-	-	-
	G_5	5% glucose	39,4 ± 0,89	43,7 ± 0,42	56,6 ± 0,74	0,573	0,7	0,658	-	-	-
	M	-	35,9 ± 0,46	66,8 ± 0,56	217,5 ± 0,78	0,45	0,388	0,045	*	*	**
	H_2O	H_2O	31,4 ± 0,89	94,3 ± 0,22	414,1 ± 0,51	0,781	0,745	0,254	-	-	*
V_2	G_2	2% glucose	45,7 ± 0,75	156,4 ± 0,44	356,3 ± 0,74	0,845	0,884	0,445	-	-	*
	G_5	5% glucose	61,5 ± 0,24	65,3 ± 0,60	254,6 ± 0,80	0,877	0,476	0,312	-	*	*
	M	-	24 ± 0,81	53,3 ± 0,74	127,2 ± 0,85	0,477	0,464	0,025	*	*	**
	H_2O	H_2O	37,2 ± 0,57	67,9 ± 0,45	338,9 ± 0,15	0,33	0,21	0,285	*	*	*
V_3	G_2	2% glucose	58,6 ± 0,54	82,4 ± 0,78	607,3 ± 0,35	0,925	0,698	0,42	-	-	*
	G_5	5% glucose	17,8 ± 0,85	40,1 ± 0,56	128,4 ± 0,88	0,096	0,471	0,048	*	*	**
	M	-	27,8 ± 0,48	60,5 ± 0,35	175,8 ± 0,87	0,412	0,41	0,048	*	*	**
	H_2O	H_2O	42,9 ± 0,98	72,6 ± 0,41	352,1 ± 0,35	0,588	0,887	0,401	-	-	*
V_4	G_2	2% glucose	51,6 ±	102,2 ±	350,9 ±	0,658	0,744	0,523	-	-	-

G_5	5% glucose	0,74 25,6 ± 0,10	0,61 34,3 ± 0,89	0,41 143,2 ± 0,58	0,325	0,478	0,029	*	*	**

*where: SD – standard diversion; * 0,05 < p ≤ 0,5 – significant values; ** p ≤ 0,05 – very significant values*

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