THE REACTIVITY OF *CYMBIDIUM HYBRIDUM* L. PROTOCORMS VITROCULTIVATED ON LIQUID MEDIUMS WITH FRUCTOSE AS SUBSTITUTE OF SUCROSE, USING PYLONS/HANGERS OF FILTER PAPER

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Abstract. In this study, we had proposed to dignify the *Cymbidium hybridum* protocorms reactions, in a vitroculture regime/condition, on liquid medium MS (1962), maintained on special filter paper bridges (*Blidar type* [1]), in the conditions of the presence fructose as principal sources of carbon, using various phytohormons. The basic medium culture used by us in these experiments was Murashige – Skoog (1962), without agar. 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 same liquid medium culture, without growth regulators.

At the end of the experimental period, was proved that the stronger multiplication of the protocorms number was registered using medium culture with BA cytochine in a quantity of 2 mg/l, being numerical above 1.7 times more in comparison with the control variant.

Keywords: protocorms, *Cymbidium* (orchid), paper-bridge, fructose, "in vitro"

INTRODUCTION

The protocorms are small green tubers with increased self-sufficient functionality degree and with organogenesis capabilities, being able on their surface to form numerous morphogenetic, vegetative and adventive centers, which subsequently may generate other protocorms, or caulogenesis centers, which can form buds, respectively leafs [7].

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 filtered paper-bridges in cross forms [1] darkling in contact with liquid medium layer.

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 [6].

The influence of different type of saccharides introduced in culture mediums as substitute of saccharose, was studied by many researchers, as like **Pătru** et al. [9], who 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 [2]. **Blidar** and **Cachiţă** tested the *in vitro* reactivity of the *Cymbidium* protocorms, using, bidistilled water, sucrose or fructose solution (in varied concentration) as supernatant. They observed that, the *Cymbidium* protocorms, which were covered by bidistilled water, were stimulated in there multiplication, and also in the morphogenetic processes [3, 4, 5, 7].

The present study represents a continuation of our researches, in the followed morphogenesis direction, and *in vitro* multiplication of *Cymbidium* protocorms, in aered condition, on surface of especially paper-bridge, who was amplased in the culture recipients in direct contact with liquid medium with various growth regulators.

MATERIALS AND METHODS

The *Cymbidium hybridum* protocorms were derived from the vitrophytobase of Laboratory of Biotechnology, University of Oradea, maintained on a basic medium (BM) *Murashige-Skoog* (1962) (MS) [8], classic, without glicine, agar-agar and phytohormones, but with PP, B_6 and B_1 vitamins in a concentration of 1 mg/l. Periodically, at approximate 3 month, it was possible the cloning of these protocorms by subculture.

In this performed experiments, the *Cymbidium* protocorms were cultivated on the same type of MS growth medium, with fructose, as substitute of sucrose (on original recipe) by adding various regulators, resulting the next culture mediums:

- **V_0** – basic medium (BM), without growth regulators;
- **V_1** – BM-MS basic medium plus 2 mg/l 2,4-D (*2,4- dichlorophenoxyacetic acid*)
- **V_2** – BM-MS basic medium plus 2 mg/l BA (*benzyladenine*) and 1 mg/l NAA (*α-naphtylacetic acid*)
- **V_3** – BM- MS basic medium plus 2 mg/l BA
- **V_4** – BM- MS basic medium plus 1 mg/l NAA

After the pH adjustment at 5.7, this was distributed in thermoresistant glass recipients, with 70 mm height and 25 mm diameter (interior). Before inserting the

culture medium in the recipient, was proceed to a positioning of a filter paper bridge, with cross form, for sustain the protocorms [1], assuring an inserted "platform" on the liquid medium surface, being 2-3 mm height of those level. In each bottle was introduced 5 ml culture medium, this assuring a liquid column with 13-14 mm height.

For the aseptisation of the recipients with medium, the phials were obturated with hydrophilic cotton plug, after this there was autoclaved at 121^0C temperature (which correspondent to 1 atm. pressure), for 20 minutes. After the cooling of these, in the perfect aseptic condition (at the box with laminar flux sterile air), was proceed to the protocorms separation from the donor glomerules, derived from the vitrophytobase of laboratory, for inoculations using only green protocorms (the proof of their viability), they having some characteristics as regards their form and diameter. In each bottle, centered on the deck from the filtered paper, were located only one protocorm.

After inoculation, the obturation of the bottles was made with transparent foil, from polyethylene, preliminarily sterilized, for 15 minute, with 70° ethanol.

The incubation and growth of the cultures, was accomplished through exposure bottles on shelves, to white fluorescent light, with an intensity of 1700 lx, the tubes of neon be seated to a distance of 33 cm of vitrocultures, the photoperiod has corresponded to 18 of 24 hours light; temperature in the growth room oscillated between 24° C (the day) and 22° C (the night).

To an interval of 30 of days, respectively to 30, 60 and 90 days from inoculation, were performed prolusion matter the general appearance of vitrocultures, analysing the evolution of three parameters: *the number of neoformed protocorms* from the initially protocorms, the *fresh weight* and the *dry weight* of the biomass at these protocorms.

For each of these parameters, the values registered at 30 days of vitroculture, on the witness medium – without growth regulator (**V_0**), were considered reference value (as 100%), to this reference value retrospect all the other values of the respective parameter, on the period of 90 days from inoculation. The experimental dates were processed statistically, establishing the sense of these, based on the variation values.

The most illustrative appearance concerning the differentiated reactivity of the inoculs, respectively to the resulted vitrocultures from *Cymbidium* protocorms, the photos from drawing 1, the histograms from figure 1, and the objects of tables 1.

RESULTS AND DISCUSSIONS

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.

The performed prolusions at *30 days from inoculation*: of the protocorms, the highest values, as regards the regenerated *number of protocorms* to the level of each protocormic clone, the *fresh* and *dry cumulated substances* of these, were registered to variant of medium with 2 mg/l BA and 1 mg/l NAA (**V_2**), the difference against witness (**V_0** –BM-MS, devoid of the growth regulator), being with 4.5 neoprotocorms/culture bottle (86.5%) superior, concerning the number of the protocorms, respective with 14.5 mg/glomerule (21.3%) and 2.0 mg/glomerule (29.4%) superior in case of the gravimetric parameters *fresh weight* and *dry substance* (**fig. 1 A-C, drawing 1A**), values sustained as relevant statistical point of

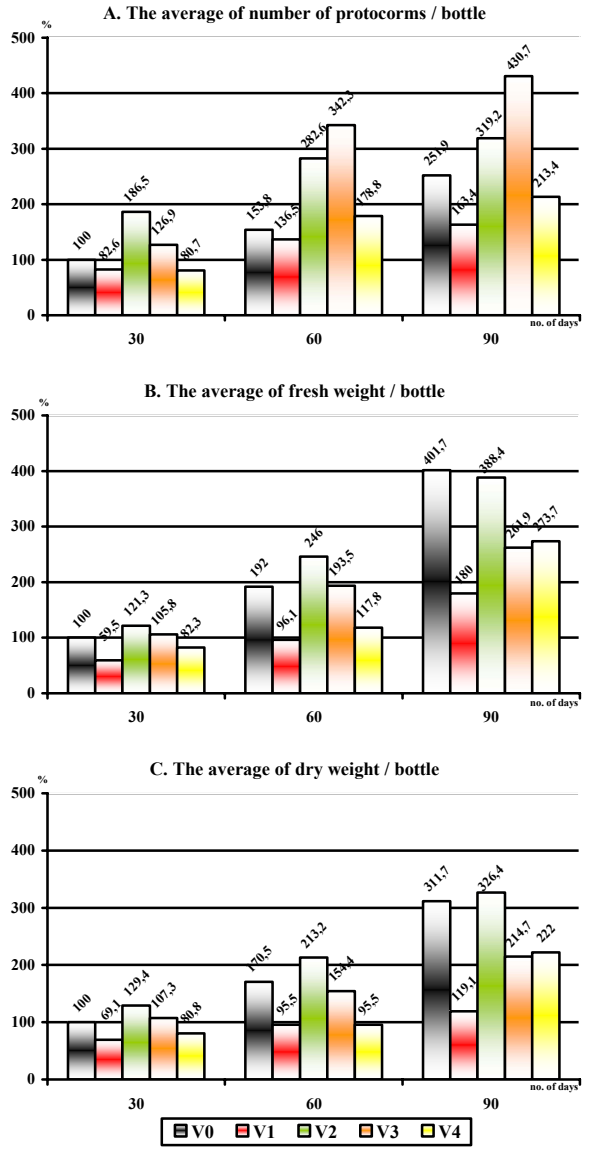


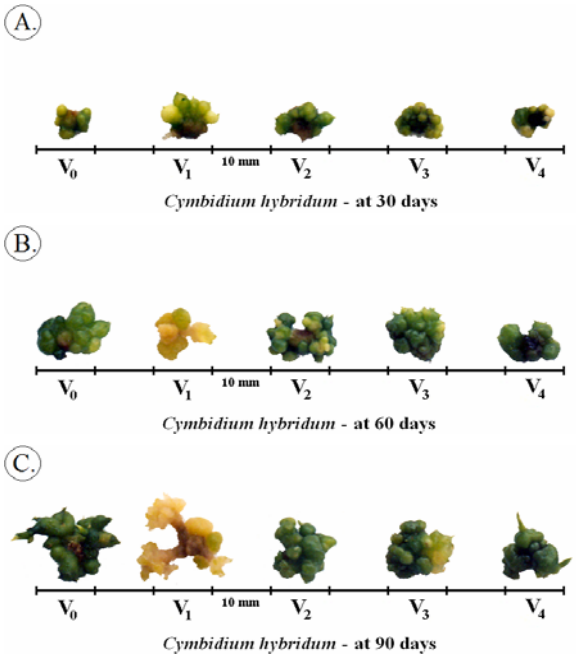
Figure 1. The comparison of the absolute value of average concerning the number (**A**), the fresh weight (**B**) and the dry weight (**C**) of *Cymbidium hybridum* protocorms, in their vitrocultivation condition on filter paper bridge, positioned on surface of liquid culture basic medium BM *Murashige-Skoog* (1962) (MS) with fructose (as substitute of sucrose on original recipe), with or without a varied content of growth regulators, as how follows: **V_0** – BM without regulators (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.

Table 1. The statistical sense of the data concerning the number, fresh weight and dry weight of *Cymbidium hybridum*, in their vitrocultivation condition on filter paper bridge, positioned on surface of liquid culture basic medium BM *Murashige-Skoog* (1962) (MS) with fructose (as substitute of sucrose on original recipe), with or without a varied content of growth regulators, as how follows: **V_0** – BM without regulators (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.

Analised parameter	Variant	The value of parameter / flask ± SD			Variation value			Statistical significance (p)		
		30 days	*60 days*	*90 days*	*30 days*	*60 days*	*90 days*	*30 days*	*60 days*	*90 days*
The number of protocorms (pieces)	V_0	5,2 ± 0,71	8 ± 0,66	13,1 ± 0,98	0,012	0,044	0,02	**	**	**
	V_1	4,3 ± 0,43	7,1 ± 0,74	8,5 ± 0,32	0,521	0,52	0,814	-	-	-
	V_2	9,7 ± 0,70	14,7 ± 0,74	16,6 ± 0,25	0,411	0,28	0,47	*	*	*
	V_3	6,6 ± 0,65	17,8 ± 0,95	22,4 ± 0,65	0,95	1,247	1,854	-	-	-
	V_4	4,2 ± 0,52	9,3 ± 0,4	11,1 ± 0,77	0,324	0,411	0,485	*	*	*
The fresh weight mass (mg)	V_0	67,8 ± 0,98	130,2 ± 0,77	272,4 ± 0,71	0,242	0,35	0,022	*	*	**
	V_1	40,4 ± 0,96	65,2 ± 0,87	122,1 ± 0,52	0,659	0,698	0,745	-	-	-
	V_2	82,3 ± 0,61	166,8 ± 0,78	263,4 ± 0,65	0,48	0,487	0,402	*	*	*
	V_3	71,8 ± 0,78	131,2 ± 0,85	177,6 ± 0,74	0,87	0,554	0,625	-	-	-
	V_4	55,8 ± 0,41	79,9 ± 1,54	185,6 ± 0,78	0,474	0,41	0,456	*	*	*
The dry weight mass (mg)	V_0	6,8 ± 0,54	11,6 ± 0,99	21,2 ± 0,74	0,48	0,044	0,035	*	**	**
	V_1	4,7 ± 0,85	6,5 ± 0,48	8,1 ± 0,68	0,689	0,948	0,71	-	-	-
	V_2	8,8 ± 0,65	14,5 ± 0,41	22,2 ± 0,68	0,379	0,355	0,089	*	*	*
	V_3	7,3 ± 0,87	10,5 ± 0,52	14,6 ± 0,4	0,504	0,698	0,985	-	-	-
	V_4	5,5 ± 0,25	6,5 ± 0,25	15,1 ± 0,45	0,444	0,402	0,395	*	*	*

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

view (**table 1**). We specify that, the presence in the composition of culture mediums of synthetic auxine 2 mg/l 2,4-D, (**V_1** variant), leads to the weaker neorformating processes of the protocorms, fact that has the consequence of the accumulation of fresh and dry substances in lowest proportion of every five experimental variants (**fig. 1**).



Drawing 1. The aspects of *Cymbidium hybridum* protocorms, constituted in glomerules, "in vitro" neoformed, in their vitrocultivation condition on filter paper bridge, positioned on surface of liquid culture basic medium BM *Murashige-Skoog* (1962) (MS) with fructose (as substitute of sucrose on original recipe), with or without a varied content of growth regulators, as how follows: **V_0** – BM without regulators (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.

The performed observations made after *60 days from inoculation*, emphasized that, the previously described phenomenon increased in the case of the *fresh* and *dry weight*, respectively on medium with 2 mg/l BA and 1 mg/l NAA, the difference against witness (**V_0** – BM-MS, devoid of the growth regulator) being enhanced average with 99.0 mg/glomerule, respectively the numbers being with about 146% higher for the fresh weight, and 7.7 mg/glomerule, with about 113.2% higher for the dry weight than the values obtained to this parameter to the control variant values sustained as relevant statistical point of view (**table 1**). Therefore, the spell 30 – 60 days of vitrocultures, excels an emphasised growth of the numbers of protocorms on the culture medium only with BA (**V_3**), comparatively with those culture mediums which contains the hormonal balance made of BA and NAA (**V_2**), respectively with almost 2.7 times higher for V_3, against 1.5 times higher for V_2. Synthetic auxine 2,4-D (2 mg/l), as growth regulator (**V_1**), inducted the lagging of morphogenesis, related with the first senescence phenomena (drawing 1B), fact that has result the marking of the lowest values of all analyzed parameters between all experimental variants (**fig. 1A-C, table 1**).

The performed observations made at *90 days from inoculation* (**drawing 1C**), matter the *number of protocorms*, best results were registered when the liquid substratum was consisted from a MS medium with addition of 2 mg/l BA (**fig. 1C**). To this variant, we registered 22.4 protocorms/glomerule, against 17.2 marked to the control variant (**table 1**). The presence in culture medium of 2,4-D auxine, in amount of 2 mg/l, protocorms proliferated, but these didn't grow, remaining tiny, presenting the senescence phenomenon (**drawing 1C**). In the matter of the *fresh weight* evaluation of protocorms glomerules, the best results were registered on **V_0** variant (BM-MS devoided by growth regulators), value closely followed by the

sample, in which case we used as growth regulator BA (2 mg/l) and NAA (1 mg/l) ($\mathbf{V_2}$) (**fig. 2B**) meaningfully statistical values (table 1); in the case of *dried weight* of the protocorms registered results were reversed against those marked for parameter *fresh weight*, respectively the values was maximum on $\mathbf{V_2}$ variant, followed closely by registered values of vitroculture variants $\mathbf{V_0}$, data sustained as relevant statistical point of view (**table 1**).

CONCLUSIONS

- To assure an intense multiplication of *Cymbidium* protocorms, in a subculture program to a distance of 3 months, the usage of liquid Murashige-Skoog medium culture (1962), modified by us (without glicine, IAA, K and agar-agar) with 2 mg/l BA, it proved to be the optimal variant of these vitrocultures, the number of protocorms being above 1.7 times higher than the values registered on medium cultures without supernatant and growth regulators (witness medium).
- The usage of auxine 2,4-D (2 mg/l) in culture medium, was the most inefficient procedure for the micropropagation of *Cymbidium* protocorms, matter the multiplication and growth of *Cymbidium* protocorms, 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 – to their necrosis.

REFERENCES

[1] Blidar, C.F., (2004). Evoluția protocormilor de *Cymbidium hybridum* cultivați „in vitro" pe medii lichide, pe punți din hârtie de filtru, în funcție de sezonul de inoculare. În: Lucrările celui de al XII-lea Simpozion Național de Culturi de Țesuturi şi Celule Vegetale „Fiziopatologia celulei vegetale în regim de vitrocultură", editori: Cachiță-Cosma Dorina, Ardelean Aurel, Fati Vasile, 5 iunie 2003, Jibou, Ed. Daya, Satu-Mare, pp. 213-227.

[2] Blidar, C.F., Cachiță-Cosma, D., (2002). Evoluția protocormilor de *Cymbidium* pe medii de cultură bistratificate, în regim hormonal variat. In: Manifestare jubiliară "25 de ani de culturi de țesuturi în România", Lucr. celui de al X-lea Simp. Naț. de Cult. de Țes. şi Cel. Veg., Cachiță, C.D., Rakosy, T.L., Ardelean, A. (Ed.), Edit. Risoprint, Cluj-Napoca, pp. 195-201.

[3] Blidar, C.F., Cachiță-Cosma, D., (2003). Reacția protocormilor de *Cymbidium hybridum* pe medii de cultură bistratificate, utilizând soluție de zaharoză ca supernatant. In: Lucr. celui de al XI-lea Simp. Naț. de Cult. de Țes. şi Cel. Veg., (ed.: Cachiță-Cosma D, Ardelean A), Edit. Daya, Satu-Mare, pp. 178-184.

[4] Blidar, C.F., Cachiță-Cosma, D., Petruş-Vancea, A., Pop, L., Marian, M., (2007). The *Cymbidium hybridum* protocorms reaction, cultivated „in vitro" on a bistratified medium, using a fructose solution as supernatant. In: Internat. Conf. „Agricultura land food industries within the context of european integration", Edit. „Lucian Blaga" University of Sibiu, pp. 37-48.

[5] Blidar, C.F., Cachiță-Cosma, D., Petruş-Vancea, A., Szabó, I., Marian, M., (2008). In vitro reactivity of *Cymbidium hybridum* L. protocorms, on bistratified culture mediums, using various supernatant sucroses solution. Analele Ştiințifice ale Universității „Al. I. Cuza" Iaşi (serie nouă), vol. 2, Secțiunea II a. Biologie vegetală, in press.

[6] Cachiță-Cosma D, (1987). *Metode in vitro la plantele de cultură. Bazele teoretice şi practice*. Bucureşti, Edit. Ceres: 274 pp.

[7] Cachiță, C.D., (2003). Dublu jubileu – Împlinirea a 100 de ani de la lansarea teoriei lui Haberlandt referitoare la culturile de celule vegetale şi a 50 de ani de la publicarea cercetărilor lui Morel şi Martin, cu privire la obținerea de plante libere de viroze, prin culturi de meristeme. În: „Lucrările celui de al XI-lea Simpozion Național de Culturi de Țesuturi şi Celule Vegetale – Omagiu dedicat lui G. Haberlandt (100 de ani de la lansarea teoriei totipotențialității celulei vegetale) şi lui Morel şi Martin (50 de ani de vitroculturi libere de viroze)", editori: Cachiță, C. D. şi Ardelean, A., Ed. Daya, Satu-Mare, pp. 1-9.

[8] Murashige, T., Skoog, F., (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiol., 15, pp. 473-479.

[9] Pătru, D.M., Cachiță-Cosma, D., Floriş, C., (2002). Studii referitoare la evoluția „in vitro" a protocormilor de *Cymbidium hybridum* în cazul substituirii zaharozei din mediile de cultură, cu fructoză. Analele Univ. Oradea Fasc. Biologie, Tom IX, pp. 235-240.