# *IN VITRO* REACTIVITY OF *CYMBIDIUM HYBRIDUM* L. PROTOCORMS, ON BISTRATIFIED CULTURE MEDIA, USING VARIOUS SUPERNATANT SUCROSES SOLUTION

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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), we propose to study the influence exerted by the cultures, practiced in bistratified regime, to *Cymbidium* protocorms *in vitro* cultures. In this interest, as supernatant we used bidistilled water, either on sucrose, glucose or fructose solutions, in different concentrations, 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) [13]. To this, we added different growth regulators, like: 2,4-D (2 mg/l), or mixtures 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 consisted of vitrocultivated protocorms on agarized medium culture, without growth regulators, cultivated in monolayer.

After 90 days from the initiation of the double-layered 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, and mostly if the second layer was bidistilled water; the usage of a 5% glucose solution as supernatant, was the most inefficient procedure, matter the micropropagation of *Cymbidium* protocorms, regardless the content of growth regulators existing in agarized layer of medium cultures.

**Keywords:** protocorm, *Cymbidium* (orchid), double-layer, carbohydrates, "in vitro"

## INTRODUCTION

The purpose of our experiments consisted in 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 with a liquid layer, water or carbohydrate solutions.

From the specialty literature is known the fact that the *Cymbidium* protocorms, *in vitro*, breed and multiply as much on solid substratum, as well in submersing regime, covered by a liquid medium. On a solid medium, on the level of protocorms, is happening – in particular – a multiplication process of protocorms [7].

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) [11].

Cachiţă (1982) used the bilayer culture system, especially to *Cymbidium* protocorms cultivation. But, she placed the protocorm, first on the surface of the agarized medium, and then, in time, she covered it with liquid medium; the supernatant column, was volumetric equal, with the agarized mass [6].

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 [12].

Pătru et al. observed that, the complete replacement of saccharose by 30g/l fructose was efficient enhancing by 22% the number of neoformed protocorms, and a growth by over 100% of their weight, fresh and dry, but only on agarized medium. The enhancement of the fructose's concentration to 50 or 70 g/l, did not prove to be a positive effect of this fructose supplement, especially in the case of 70 g/l concentration, which has negatively influenced the protocorms multiplication [14].

*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 [1]. Blidar et al., tested the *in vitro* reactivity of the *Cymbidium* protocorms, using as supernatant the bidistilled water, 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 [2, 3, 5].

The present study represent a continuation of ours 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% sucrose solutions, either glucose, or either fructose solutions.

## MATERIALS AND METHODS

The biological material consisted in *Cymbidium hybridum* protocorms. They were inoculated on a basic Murashige-Skoog solid medium (1962) (MS) [13], 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 micropropagated culture collection, made on the Vegetal Biotechnological Laboratory, from Oradea University. The control lot of protocorms were vitrocultured on the same kind of medium, without growth regulators.

The present experiment behaved the organization of eight variant series, structured - each of them - on five variants (tab. 1). In all, were analyzed the *Cymbidium* protocorms evolution cultivated on 40 variants of medium culture. Practically, on 5 $cm^3$ agarized substratum, after the protocorms inoculation in aseptic regime, was administrated a second layer, respectively with 5 $cm^3$ supernatant, consisting in: bidistilled water ($H_2O$ series), or in 2% ($G_2$), or 5% ($G_5$) glucose solution, 2% ($F_2$), or 5% ($F_5$) fructose solution, or 2% ($Z_2$), or 5% ($Z_5$) sucrose solution. The protocorm cultures were practiced in glass bottles, with the height of 70 mm, and with interior diameter of 25 mm. The experimentally variant were 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 – as supernatant – with bidistilled water;
- 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;
- series V: $V_0F_2$-$V_4F_2$ – protocorms cultivated on agarized medium, and covered with 2% fructose solution;
- series VI: $V_0F_5$-$V_4F_5$ – protocorms cultivated on agarized medium, and covered with 5% fructose solution;
- series VII: $V_0Z_2$-$V_4Z_2$ – protocorms cultivated on agarized medium, and covered with 2% sucrose solution;
- series VIII: $V_0Z_5$-$V_4Z_5$ – protocorms cultivated on agarized medium, and covered with 5% sucrose 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 super-natant Series II** | **G 2% as super-natant Series III** | **G 5% as super-natant Series IV** | **F 2% as super-natant Series III** | **F 5% as super-natant Series IV** | **Z 2% as super-natant Series III** | **Z 5% as super-natant 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 = **$V_0G_5$** | $V_0M$ + 2% fructose solution = **$V_0F_2$** | $V_0M$ + 5% fructose solution = **$V_0F_5$** | $V_0M$ + 2% sucrose solution = **$V_0Z_2$** | $V_0M$ + 5% sucrose solution = **$V_0Z_5$** |
| **$V_1M$** – MS basic medium plus 2 mg/l 2,4-D (*2,4-dichloro-phenoxyacetic acid*) | $V_1M$ + $H_2O$ = **$V_1H_2O$** | $V_1M$ + 2% glu-cose so-lution = **$V_1G_2$** | $V_1M$ + 5% glu-cose so-lution = **$V_1G_5$** | $V_1M$ + 2% fruc-tose solu-tion = **$V_1F_2$** | $V_1M$ + 5% fruc-tose solu-tion = **$V_1F_5$** | $V_1M$ + 2% su-crose so-lution = **$V_1Z_2$** | $V_1M$ + 5% su-crose so-lution = **$V_1Z_5$** |
| **$V_2M$** – MS basic me-dium plus 2 mg/l BA ($N^6$-*benzyl-adenine*) and 1 mg/l NAA (*1-naphthalene acetic acid*) | $V_2M$ + $H_2O$ = **$V_2H_2O$** | $V_2M$ + 2% glu-cose so-lution = **$V_2G_2$** | $V_2M$ + 5% glu-cose so-lution = **$V_2G_5$** | $V_2M$ + 2% fruc-tose so-lution = **$V_2F_2$** | $V_2M$ + 5% fruc-tose so-lution = **$V_2F_5$** | $V_2M$ + 2% su-crose so-lution = **$V_2Z_2$** | $V_2M$ + 5% su-crose so-lution = **$V_2Z_5$** |
| **$V_3M$** – MS basic medium plus 2 mg/l BA | $V_3M$ + $H_2O$ = **$V_3H_2O$** | $V_3M$ + 2% glu-cose so-lution = **$V_3G_2$** | $V_3M$ + 5% glu-cose so-lution = **$V_3G_5$** | $V_3M$ + 2% fruc-tose so-lution = **$V_3F_2$** | $V_3M$ + 5% fruc-tose so-lution = **$V_3F_5$** | $V_3M$ + 2% su-crose so-lution = **$V_3Z_2$** | $V_3M$ + 5% su-crose so-lution = **$V_3Z_5$** |
| **$V_4M$** – MS basic medium plus 1 mg/l NAA | $V_4M$ + $H_2O$ = **$V_4H_2O$** | $V_4M$ + 2% glu-cose so-lution = **$V_4G_2$** | $V_4M$ + 5% glu-cose so-lution = **$V_4G_5$** | $V_4M$ + 2% fruc-tose so-lution = **$V_4F_2$** | $V_4M$ + 5% fruc-tose so-lution = **$V_4F_5$** | $V_4M$ + 2% su-crose so-lution = **$V_4F_2$** | $V_4M$ + 5% su-crose so-lution = **$V_4Z_5$** |

After portioning the agarized medium, the bottles with medium were sterilized by autoclave, at $121^0$C, for 20 minutes. The supernatant (liquid medium) were autoclaved separately. In each bottle has been inoculated only a single protocorm. 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 past in growth chamber, and were seated on artificially illuminated shelfs with fluorescent tubes of white colour (1400 lux luminous intensity), in photoperiodic regime of 16 hours light / 24 hours, the surrounding temperature oscillated between $24^0$C in the light period, and $22^0$C in the course of darkness phase.

At 30, 60 and 90 days of vitrocultures observations and biometric measurers 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 to the similar parameters, to witness variant. The experimental dates were statistically processed, establishment – based on the variability values – the sense of these.

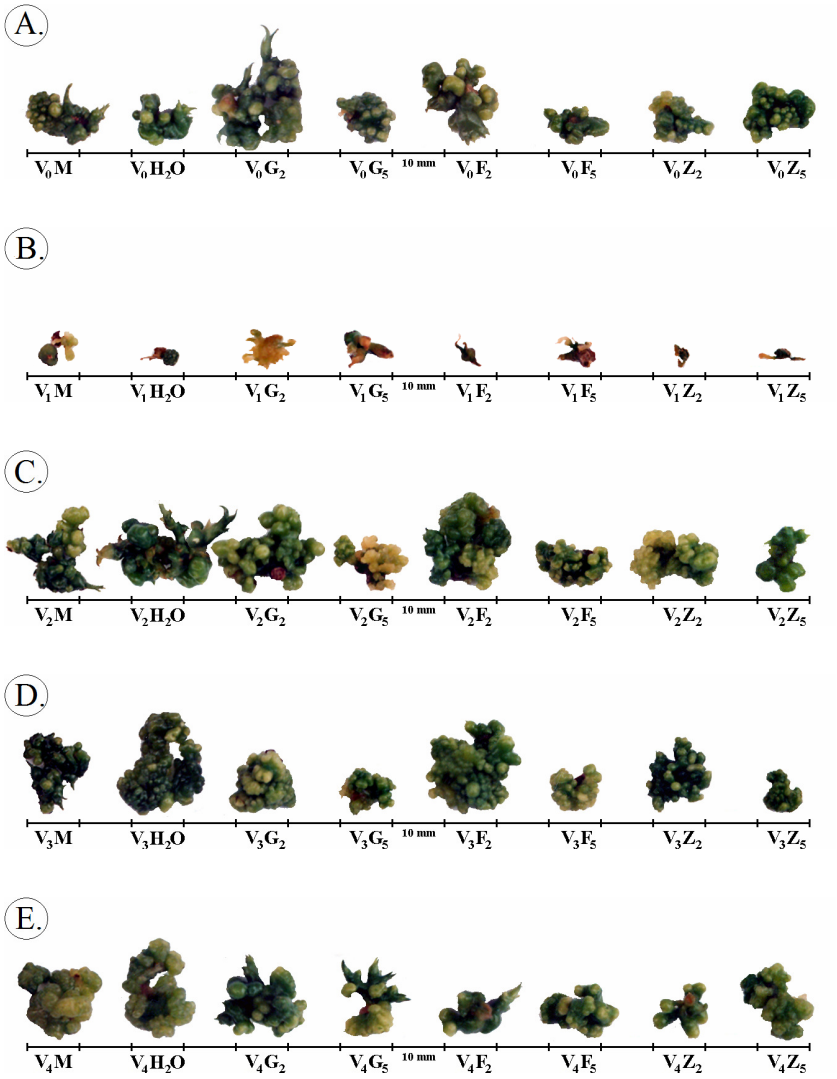
The most illustrative appearance concerning the differentiated reactivity of the inoculs, respectively to the resulted vitrocultures from *Cymbidium* protocorms,

to those 35 experimental variant, do the photos from drawing 1, and the histograms from Figures 1-3.

## RESULTS

From each initial protocorm, in the vitroculture period, were regenerated new protocorms, which – in time – constitutes a glomerule (like blackberries fruit), with or without morphogenesis 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 consisting in bidistilled water, an exception been evident in the case of using the synthetic auxine 2 mg/l 2,4-D, as growth regulator, situation in which the presence of supernatant inducted the lagging of morphogenesis; the second exception was marked on medium variant, in which case the supernatant consisted in 2% fructose solution, the agarized medium culture contained 2 mg/l BA, values sustained as relevant statistical point of view (Fig. 1A). 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, because of the variability of the protocorms populations included in the experiences, concerning the morphogenesis, weren't sustained as relevant statistical point of view) (Fig. 2A). Also, it has been notices that, the solution of glucose 5% - used as supernatant – favoured the accumulation




**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 by bidistilled water (experimental series **"$H_2O$"**), of a 2% glucose solution (experimental series **"$G_2$"**), of 5% glucose solution (experimental series **"$G_5$"**), of a 2% fructose solution (experimental series **"$F_2$"**), of 5% fructose solution (experimental series **"$F_5$"**), of a 2% sucrose solution (experimental series **"$Z_2$"**), or of 5% sucrose solution (experimental series **"$F_5$"**); the agarized substratum presented a varied content of growth regulators, as it follows: **$V_0$** - BM without growth (lot control) (**A**), **$V_1$** – BM with the adding of 2 mg/l *2,4-dichlorophenoxyacetic acid* (2,4-D) (**B**); **$V_2$** – BM with an adding of 2 mg/l *$N^6$-benzyladenine* (BA) mixed with 1 mg/l *1-naphthalene acetic 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 the experiments' assembling.

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 when we use as supernatant a solution of 5% glucose) (Fig. 3A).

The observations performed 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, a single exception has been evident in the case of using the 2 mg/l 2,4-D auxine (Fig. 1B); in case of *weight* glomerular mass (both *fresh* and *dry*), the highest registered value to the variants of vitrocultivated protocorms, growth on the medium culture hereupon the supernatant 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 ($V_0$M – agarized BM-MS, devoid of the growth regulator) being average enhanced with 135.6 mg/glomerule, respectively the numbers being about 82% higher for the fresh weight, and 11.7 mg/glomerule, and about 81.8% higher for the dry weight than the values obtained to this parameter to the control variant (Fig. 2B & 3B).

The observations performed at 90 days from inoculation (Drawing 1), matter the *number of protocorms*, best results were registered when we used the bidistilled water as supernatant, and when the solid substratum consisted of 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) (Fig. 1C). To this variant, we registered 54.7 protocorms/glomerule, against 15.7 marked to the control variant. 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;

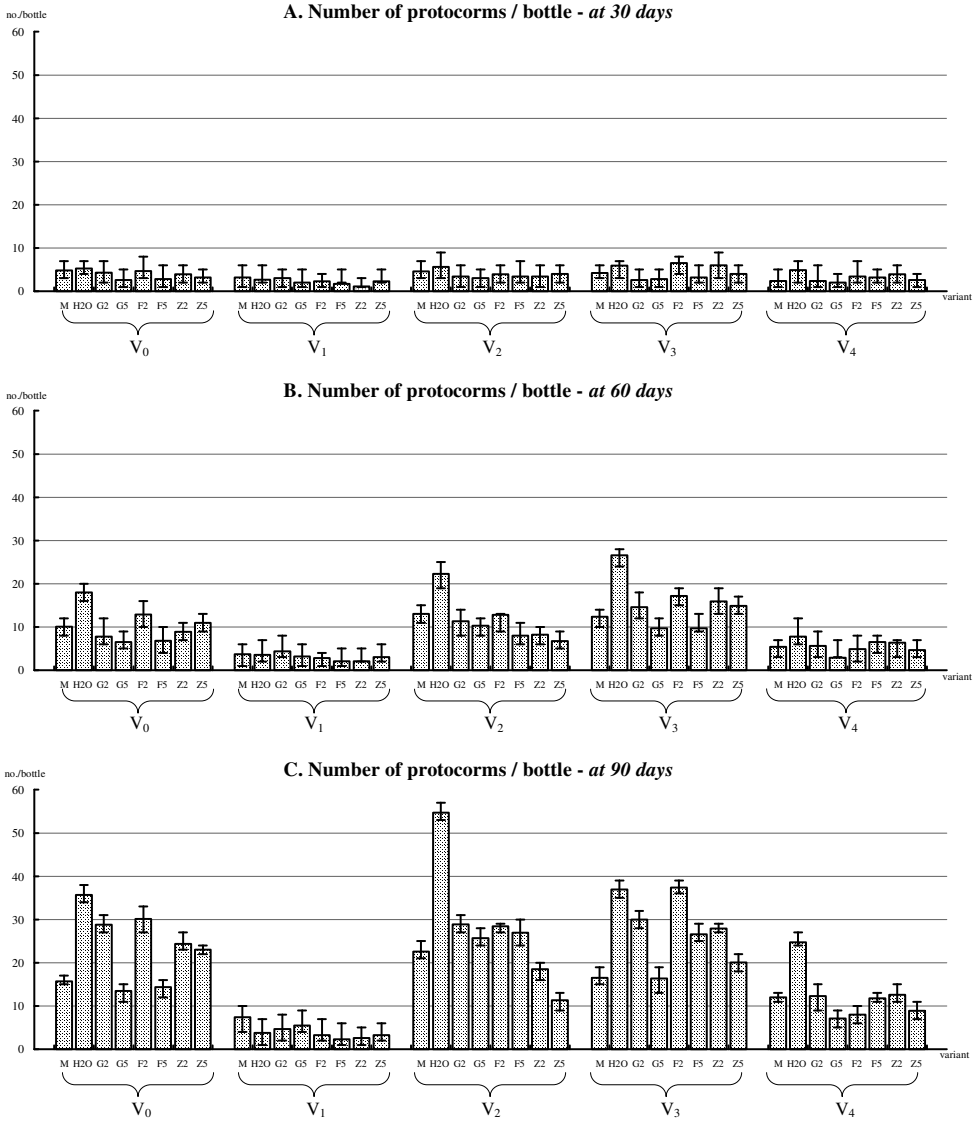



**Figure 1.** The comparison of the absolute value of average concerning the number of *Cymbidium hybridum* protocorms, in their vitrocultivation condition on a solid BM Murashige-Skoog (1962) basic medium culture modified, vitrocultures without supernatant (experimental series **"M"**) or covered with a liquid layer, apply across basic medium (solid), the liquid layer being represented by bidistilled water (experimental series **"$H_2O$"**), of a 2% glucose solution (experimental series **"$G_2$"**), of 5% glucose solution (experimental series **"$G_5$"**), of a 2% fructose solution (experimental series **"$F_2$"**), of 5% fructose solution (experimental series **"$F_5$"**), of a 2% sucrose solution (experimental series **"$Z_2$"**), or of 5% sucrose solution (experimental series **"$Z_5$"**); the agarized substratum presented a varied content of growth regulators, as how follows: **$V_0$** - BM without growth (lot control) (**A**), **$V_1$** – BM with an adding of 2 mg/l *2,4-dichlorophenoxyacetic acid* (2,4-D) (**B**); **$V_2$** – BM with the adding of 2 mg/l *$N^6$-benzyladenine* (BA) mixed with 1 mg/l *1-naphthalene acetic acid* (NAA) (**C**); **$V_3$** – BM only with 2 mg/l BA (**D**), **$V_4$** – BM only with 1 mg/l NAA (**E**), after *30 days* (**A**)*, 60 days* (**B**) and *90 days* (**C**) from the experiments' assembling.

on the 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, the value data closely followed by the variant in which we used as supernatant the 2% glucose solution, the solid medium being devoiced by growth regulators ($V_0G_2$) (Fig. 2C). If on $V_2$, $V_3$ and the $V_4$ variants, the *dried weight* of the protocorms were maximum to the covered variants with bidistilled water, as supernatant (meaningful statistical values), in the case of the control variant ($V_0M$), the highest gravimetric values were observed to variants hereupon the supernatant were constituted from a solution of 2% glucose ($V_0G_2$), dates sustained as relevant statistical point of view (Fig. 3C).

## DISCUSSIONS

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. The same results were obtained by Blidar et al. in similar researches effectuated before, the present results coming as a confirmation of those studies [2, 3, 5].

To assure an intense multiplication of *Cymbidium* protocorms, in a subculture program to a distance of 3 months, the usage 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 most 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).

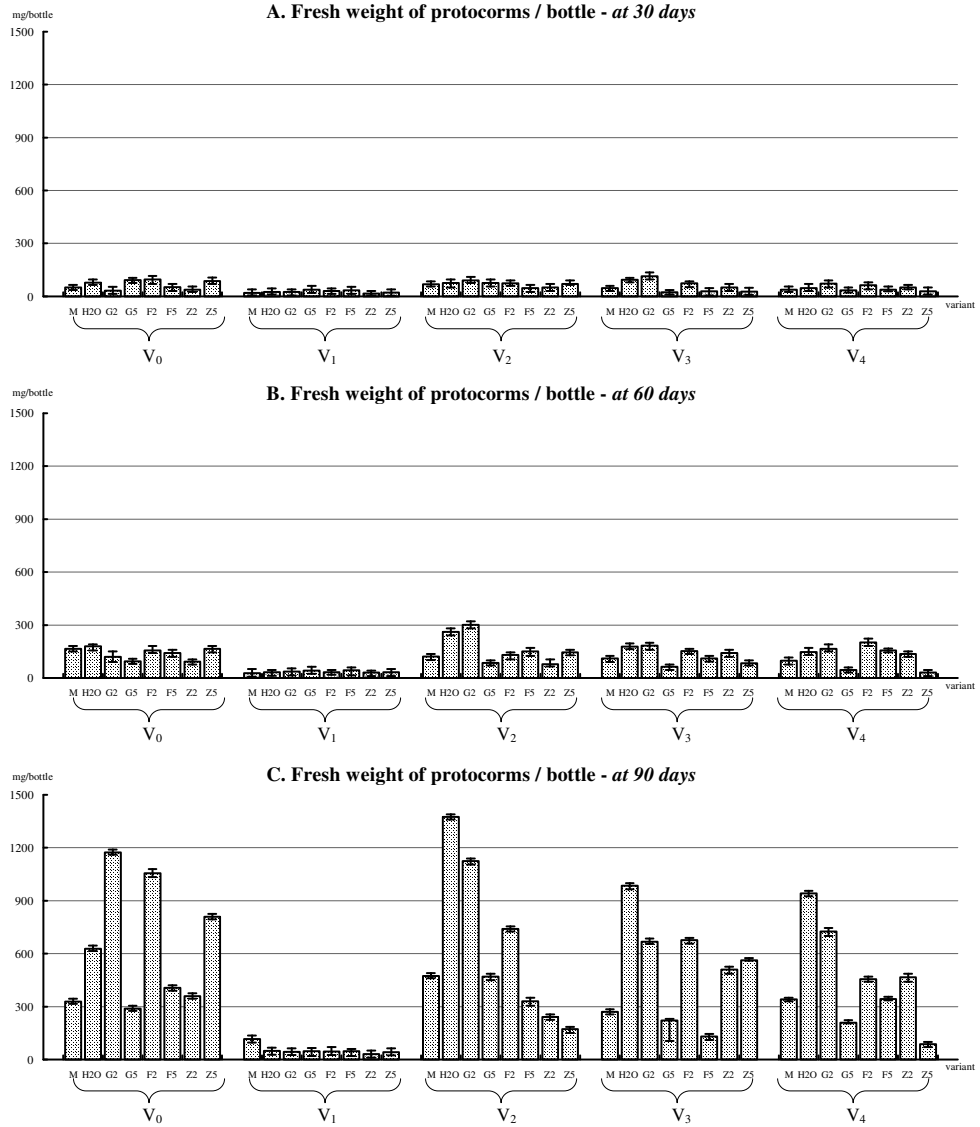



**Figure 2.** The comparison of the absolute value of average concerning the fresh weight of *Cymbidium hybridum* protocorms, in their vitrocultivation condition on a solid BM Murashige-Skoog (1962) basic medium culture modified, vitrocultures without supernatant (experimental series **"M"**) or covered with a liquid layer, apply across basic medium (solid), the liquid layer being represented by bidistilled water (experimental series **"$H_2O$"**), of a 2% glucose solution (experimental series **"$G_2$"**), of 5% glucose solution (experimental series **"$G_5$"**), of a 2% fructose solution (experimental series **"$F_2$"**), of 5% fructose solution (experimental series **"$F_5$"**), of a 2% sucrose solution (experimental series **"$Z_2$"**), or of 5% sucrose solution (experimental series **"$Z_5$"**); the agarized substratum presented a varied content of growth regulators, as it follows: **$V_0$** - BM without growth (lot control) (**A**), **$V_1$** – BM with an adding of 2 mg/l *2,4-dichlorophenoxyacetic acid* (2,4-D) (**B**); **$V_2$** – BM with the adding of 2 mg/l *$N^6$-benzyladenine* (BA) mixed with 1 mg/l *1-naphthalene acetic acid* (NAA) (**C**); **$V_3$** – BM only with 2 mg/l BA (**D**), **$V_4$** – BM only with 1 mg/l NAA (**E**), after *30 days* (**A**), *60 days* (**B**) and *90 days* (**C**) from the experiments' assembling.

If, however, we use as medium culture a liquid one, in which the sucrose was substituted with fructose, the *Cymbidium hybridum* protocorms being sustained by a filter paper bridge, we ascertained that the maximum efficiency concerning the *in vitro* multiplication and organogenesis at this orchid, was marked on experimental variant which contained 2 mg/l BA [4].

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

In double-layered medium cultures, the presence of (2 mg/l) 2,4-D auxine, was proved to be an inefficient procedure, matter the multiplication and growth of *Cymbidium* protocorms, regardless 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 – leaded to their necrosis. Callus culture of orchids, induced by 2,4-D, has seen only limited success due to slow growth and a tendency to become necrotic [8-10].

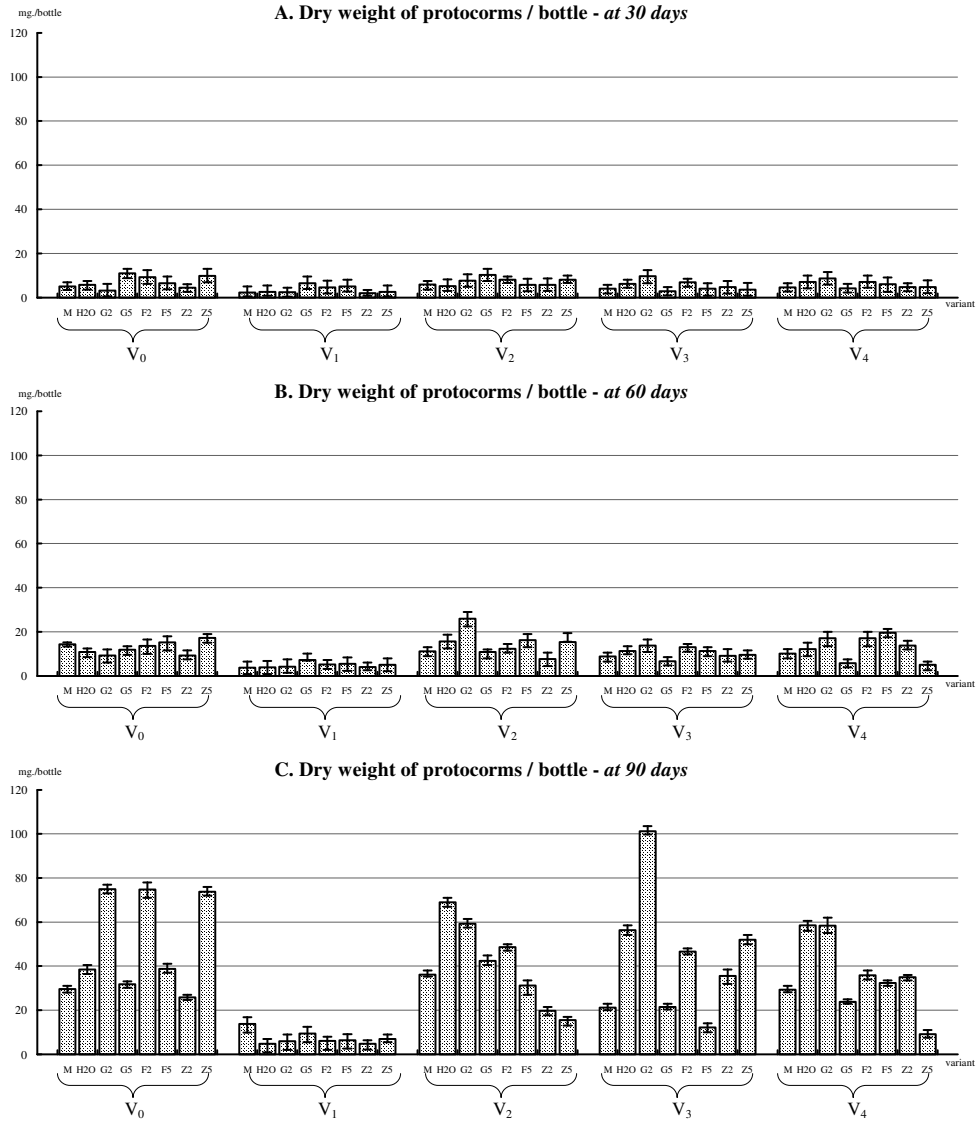



**Figure 3.** The comparison of the absolute value of average concerning the dry weight of *Cymbidium hybridum* protocorms, in their vitrocultivation condition on a solid BM Murashige-Skoog (1962) basic medium culture modified, vitrocultures without supernatant (experimental series **"M"**) or covered with a liquid layer, apply across basic medium (solid), the liquid layer being represented by bidistilled water (experimental series **"$H_2O$"**), of a 2% glucose solution (experimental series **"$G_2$"**), of 5% glucose solution (experimental series **"$G_5$"**), of a 2% fructose solution (experimental series **"$F_2$"**), of 5% fructose solution (experimental series **"$F_5$"**), of a 2% sucrose solution (experimental series **"$Z_2$"**), or of 5% sucrose solution (experimental series **"$Z_5$"**); the agarized substratum presented a varied content of growth regulators, as it follows: **$V_0$** - BM without growth (lot control) (**A**), **$V_1$** – BM with an adding of 2 mg/l *2,4-dichlorophenoxyacetic acid* (2,4-D) (**B**); **$V_2$** – BM with the adding of 2 mg/l $N^6$*-benzyladenine* (BA) mixed with 1 mg/l *1-naphthalene acetic acid* (NAA) (**C**); **$V_3$** – BM only with 2 mg/l BA (**D**), **$V_4$** – BM only with 1 mg/l NAA (**E**), after *30 days* (**A**), *60 days* (**B**) and *90 days* (**C**) from the experiments' assembling.

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