

THE REACTIVITY OF *Cymbidium hybridum* PROTOCOL-LIKE BODY VITROCULTIVATED IN LIQUID MEDIUM, DEPLETED IN MICROELEMENTS

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Abstract: The nature of the chemical compounds, the proportion and concentration in mineral elements, as part of the culture medium's preparation, they influence the growth and the morphogenesis of clones. Starting from these premise, we proposed ourselves to study the *Cymbidium hybridum* protocorms reactivity, in the cultivation conditions of those in submersing regime in liquid medium Murashige-Skoog (1962) (MS) [14], with a complete set of mineral elements, or damaged in one of those microelements as B, Mn, Zn, Mo, Cu, Co or I. Those culture medium contained or not various growth hormones.

After 90 days from inoculation, the most proliferative intense processes of the protocorms, we have registered in the practiced vitrocultures without iodine microelement, but in the presence of BA (2 mg/l) in the liquid medium, as a unique growth regulator (reference lot, considered 100%), the registered increases were 600% in the protocorms multiplication. The absence of iodine in the culture medium has determined the increase of the *fresh* and *dry weight* of the protocorms' biomass, regardless the growth hormones content in the substratum, except the medium with 2 mg/l 2,4-D, from which, the performed observations made after 30 days from the experiment's montage, were noticed senescence processes.

Keywords: protocorm-like body (PLB), *Cymbidium* (orchid), microelements, growth regulators, "in vitro"

INTRODUCTION

The protocorms are small green tubers with increased self-sufficient functionality degree and with organogenesis capabilities, that are able to form on their surface numerous morphogenetic, vegetative and adventive centres, which subsequently may generate other protocorms, or caulogenesis centres, which can form buds, respectively leaves [6].

It is known the fact that the *Cymbidium hybridum* protocorms or other orchids, whether in solid medium, or in liquid medium, suffer – in relation to the used culture medium and culture conditions from the growing room (in condition by understanding the physical situation of the solid or liquid medium, and, also, the temperature and the lightning regime of the cultures) – a proliferation process. If it is used in solid medium, than it will start the organogenesis process [16, 17].

In vitro culture, the clones cannot propagate adequately if it isn't assured the optimal conditions for nutrition – on one hand, and the lightning and temperature conditions – on the other. It is known the fact that the nature of the chemical compounds, the proportion and the concentration of different chemical elements, which enter in the structure of the culture medium, can influence the growing and the morphogenesis at the cloned tissues level. Early studies performed in this direction, that belong to Gautheret (1959), was singularizing the importance of the nitrates, phosphates, sulphates and K, Ca, Mg and Na chloride in the evolution of the carrot explants [12].

A series of other scientists had performed experiments, observing the macro elements' role, but early studies on the morphologic and biochemical effect of the microelements belong to Heller [13], Street [19, 20], Steward [18] and Gautheret [12], scientists which had brought an important contribution

to knowing the role of: Fe, Zn, Mn, B, Mo, Cu and I in the growing of the "in vitro" cultivated tissues [5, 7].

The present study is desired to be a confirmation of the researches undertaken by the illustrious scientists mentioned above, meant to follow the *Cymbidium hybridum* protocorms reaction in those cultivation conditions in aseptic, liquid culture medium, in the presence or absence of growth regulators [1 - 4].

The evolution of the protocorms was followed for 90 days, their reaction being studied in dependence to the presence or the absence, consecutively of the B, Mn, Zn, Cu, Co and I microelement, and the hormonal composition of the culture medium. At the protocorms' level it has been overviewed the estimation of the multiplication and organogenesis process, in a period of 3 months.

MATERIALS AND METHODS

The *Cymbidium hybridum* protocorms, derived from the vitro base of the Biotechnology laboratory of the University of Oradea, vitrocultures sustained in a classic Murashige-Skoog (1962) (MS) medium [14], modified by us, coagulated with 7 g/l agar not 10 g/l like in the original prescription, without glycine, 3 indolilacetic acid and kinetin, but with 30 g/l sucrose, and the medium's pH was adjusted to 5.7 value at autoclavation.

The introduced protocorms used in this experiment came from the same type of basic liquid medium (MB) MS (1962) (MS abbreviated), modified by us in an appropriate manner description mention before, to which was added phytohormones, composing the next experimental variants:

- V_0 – basic medium (MB) (MS) without regulators – witness;
- V_1 – basic medium with an addition of 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D);

- **V₂** – basic medium with an addition of 2 mg/l *N⁶-benzyladenine* (BA) and 1 mg/l *1-naphthalene acetic acid* (NAA);
- **V₃** – basic medium with an addition of 2 mg/l BA;
- **V₄** – basic medium with a surplus of 1 mg/l NAA.

For testing the influence of successive elimination from the culture medium of the microelements, one by one (B, Mn, Zn, Mo, Cu, Co and I) at the growth of the *Cymbidium hybridum* L. protocorms vitrocultures, were organized, in parallel, eight experimental series (Table 1), respectively:

- **S₀** – witness, including all minerals contents in MS (1962) medium prescription;
- **S₁** – because the stock solution of H₃BO₃ wasn't inserted in the culture medium, caused the absence of the **B** microelement ;
- **S₂** – because the stock solution of MnSO₄*4H₂O wasn't inserted in the culture medium, caused the absence of the **Mn** microelement fact that also caused a depletion in sulphur of the culture medium with 5.77%;

- **S₃** – the medium without **Zn** microelement by eliminating ZnSO₄*7H₂O from the culture medium, caused the impoverishment of the culture medium in sulphur with 1.72%;
- **S₄** – the medium without **Mo** microelement by eliminating Na₂MoO₄*2H₂O from the culture medium, caused a depletion of the medium culture in sodium with 1.21%;
- **S₅** – because the stock solution of CuSO₄*5H₂O wasn't inserted, generated the absence of the **Cu** microelement fact that also caused the impoverishment of the culture medium in sulphur with just 0.005%;
- **S₆** – the experimental lot growing on culture medium without **Co** microelement, by eliminating the stock solution of CoCl₂*6H₂O, also caused the depletion of the medium in chlorine with just 0.34%;
- **S₇** – because the KI stock solution wasn't inserted in the culture medium, generated the absence of the **I** microelement fact that also caused the impoverishment of the culture medium in potassium with only 0.02%.

Table 1. The used culture medium variants in the *Cymbidium hybridum* L. protocorms vitrocultures

Experimental series Growth regulators variants		S ₀	S ₁	S ₂	S ₃	S ₄	S ₅	S ₆	S ₇
		MS-BM complete	MS-BM without B	MS-BM without Mn	MS-BM without Zn	MS-BM without Mo	MS-BM without Cu	MS-BM without Co	MS-BM without I
V₀	Liquid <i>Murashige-Skoog</i> (MS) basic medium without growth regulators	V ₀ S ₀	V ₀ S ₁	V ₀ S ₂	V ₀ S ₃	V ₀ S ₄	V ₀ S ₅	V ₀ S ₆	V ₀ S ₇
V₁	MS basic medium plus 2 mg/l 2,4-D (2,4-dichlorophenoxyacetic acid)	V ₁ S ₀	V ₁ S ₁	V ₁ S ₂	V ₁ S ₃	V ₁ S ₄	V ₁ S ₅	V ₁ S ₆	V ₁ S ₇
V₂	MS basic medium plus 2 mg/l BA (<i>N⁶-benzyladenine</i>) and 1 mg/l NAA (<i>1-naphthalene acetic acid</i>)	V ₂ S ₀	V ₂ S ₁	V ₂ S ₂	V ₂ S ₃	V ₂ S ₄	V ₂ S ₅	V ₂ S ₆	V ₂ S ₇
V₃	MS basic medium plus 2 mg/l BA	V ₃ S ₀	V ₃ S ₁	V ₃ S ₂	V ₃ S ₃	V ₃ S ₄	V ₃ S ₅	V ₃ S ₆	V ₃ S ₇
V₄	MS basic medium plus 1 mg/l NAA	V ₄ S ₀	V ₄ S ₁	V ₄ S ₂	V ₄ S ₃	V ₄ S ₄	V ₄ S ₅	V ₄ S ₆	V ₄ S ₇

The culture medium was distributed in temperature-resistant glass recipients, of 70 mm height and 25 mm inner dimension. Each bottle contained 5 ml medium, assuring in the recipient a column of liquid medium with a 10 – 11 mm height, necessary for the nutrition in good conditions of the protocorms, during a period of 90 days, the duration of the experimenting process. The culture recipients were occluded with hydrophilic cotton wool bungs, covered in gauze, the recipients sterilization was made by autoclaving at 121 °C temperature (according at a 1 atm. pressure), for 20 minutes.

After the culture medium's cooling, in perfect sterile conditions, to the box with sterile air flux, we proceeded to the separation of the protocorms derived from the vitro base, by conglomerating glomerules. For inoculations were used just single protocorms, with the same size, green and viable.

For each experimental variants were inoculated each 80 phials, with a single protocorm/culture recipient.

After inoculation, the phials were occluded with colourless, translucent, polyethylene folia, sterilized at 70 °C ethylic alcohol, for 15 minutes.

The incubation and the growth of the cultures were performed by exposure of the recipients containing explants, on illuminated shelves with white fluorescent light, with 1700 lux light intensity, the tubes were installed at a distance of 33 cm above the vitrocultures; the photoperiod was 18 hours light/24 hours; the temperature on the shelves with the explants/clones oscillated between 26 °C ± 2 °C (day) and 24 °C ± 2 °C (night).

To a periodicity of 30 days, respectively 30, 60 and 90 days after protocorms inoculation, observation was performed looking to the general aspect of those, the

number of new formed protocorms, as well as the fresh weight of the whole biomass of the generated protocorms from the promoter protocorm of the culture phial (the protocorms being blotted with filter paper, at their weighing to the obliteration of the liquid medium spots from their surface) and the dry medium weight (the drying of these was made in the cabinet dryer at 105 °C temperature, for 3 days). The experimental dates obtained at the control variant, respectively on V₀S₀ variant basic medium (BM-MS complete, without growth regulators) 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 for the similar parameters, to the witness variant. The experimental dates were statistically processed, establishing – based on the variability values – the sense of these.

The most representative aspects were photographed, and then were presented and discussed in the analysis part of the experimental results (Fig. 1-3).

RESULTS

The performed determinations at 30 days after the protocorms' inoculation, has demonstrated that the presence of some variation of the number, fresh and dry weight of these, in respect to the hormonal balance, introduced in the culture medium and in dependence to the presence or absence of the B, Mn, Zn, Mo, Cu or Co microelements, showed that:

- in the case of the number of the new formed protocorms, the cultivation of the *Cymbidium hybridum* clones in a medium without iodine, in the conditions of using 2 mg/l benzyl adenine (BA), respectively on the variant medium V₃S₇, had lead – related to the entire experiment – to the registration of the best results, because this parameter's value was maximum, over 5.1 times superior to the witness variant (V₀S₀ – liquid BM-MS, devoid of the growth regulator, and with all micronutrients) values sustained as relevant statistical point of view. Contrary, weakest results – regarding the protocorms multiplication – as it was shown in the case of the V₄S₃ variant (BM-MS without Zn and with 1 mg/l NAA). In this last case, the medium number of the new formed protocorms was inferior to the control medium (V₀S₀) with 1.53 times (Fig. 1A);

- the experimental analysis performed on the *Cymbidium hybridum* protocorms evolution in respect to their fresh weight, proves the fact that the most favourable development of the vitrocultures was registered in the condition of the absence of the iodine microelement from the medium, but in the presence of BA (2 mg/l) mixed with NAA (1 mg/l) as growth regulators (V₂S₇ variant), this parameter's value report towards the witness variant (V₀S₀ – BM-MS without regulators, but with all microelements), being superior over 5.8 times, highly meaningful values from the statistical point of view. The protocorms growing on V₂S₁ variant (BM-MS with 2 mg/l BA and 1 mg/l ANA, without boron microelement), constituted the

most inefficient variant of culture medium, from the fresh weight point of view, fact that acknowledges also the smaller values of this parameter (Fig. 2A);

- in the case of the dry weight of the vegetable biomass accumulated level of the *Cymbidium hybridum* protocorms, the usage of the medium culture without the iodine microelement, with 1 mg/l NAA as exclusive phyto regulator (V₄S₇), has lead to superior results in respect to the other variants of medium culture, and toward the control medium variant (V₀S₀), the dry substance being almost 7.12 times superior (sustained values are relevant from the statistical point of view). Contrary, to the case of the gravimetric parameter previously discussed, the lowest accumulation of dry substance was marked in the case of V₂ variant from the same S₁ series (BM-MS with 2 mg/l BA and 1 mg/l NAA, without boron microelement), respectively, being almost 1.9 times inferior to the witness variant (V₀S₀) (Fig. 3A).

After the performed determinations at 60 days of vitroculture, we ascertained the following:

- the number of the new formed protocorms on the V₂S₇ variant was the highest (BM without the iodine microelement, in addition with 2 mg/l BA and 1 mg/l NAA), and the lowest on witness variant (V₀S₀ – BM-MS without phyto regulators and with all microelements), values sustained as relevant from the statistical point of view (Fig. 1B);

- as in the first observations, also at 60 days from assembling of the experiments, the accumulated fresh substance / clone, at the V₂S₇ variant (BM without iodine, in addition with 2 mg/l BA mixed with 1 mg/l NAA), quantitatively was bigger from the entire experimental series, the difference from the control variant (V₀S₀ complete medium without growth regulators) increasing, to this time of observations, the values were over 30.7 times superior to this (meaningful data from the statistical point of view). Finally, at the installation of an easily physiological decline at the *Cymbidium hybridum* protocorms level, cultivated on V₂S₁ medium variant (MB without boron, in addition with 2 mg/l BA and 1 mg/l NAA), was registered the lowest rate of fresh substance accumulation per clone, thus this experimental date, the gravimetrical values of this parameter being over 2.3 times inferior to the witness variant (V₀S₀), values which, because of the variability of the protocorms' populations included in the experiences, concerning the morphogenesis, weren't sustained as relevant from the statistical point of view (Fig. 2B);

- the highest, respectively lowest values of the dry substance, accumulated/clone, was registered – as in case of the fresh weight – to the V₂S₇ variant (BM without iodine, in addition with 2 mg/l BA and with 1 mg/l NAA), respectively V₂S₁ (MB without boron, with an surplus of 2 mg/l BA and 1 mg/l ANA), reports against the control variant (V₀S₀), being over 28 : 1, respectively 1 : 1.8 (Fig. 3B).

At the end of the experimental period, respectively at 90 days of "in vitro" culture, the biometrical and gravimetrical determinations has put in evidence a stronger amplification of the physiological activities on the S₇ series variants (MB without iodine), in bearing

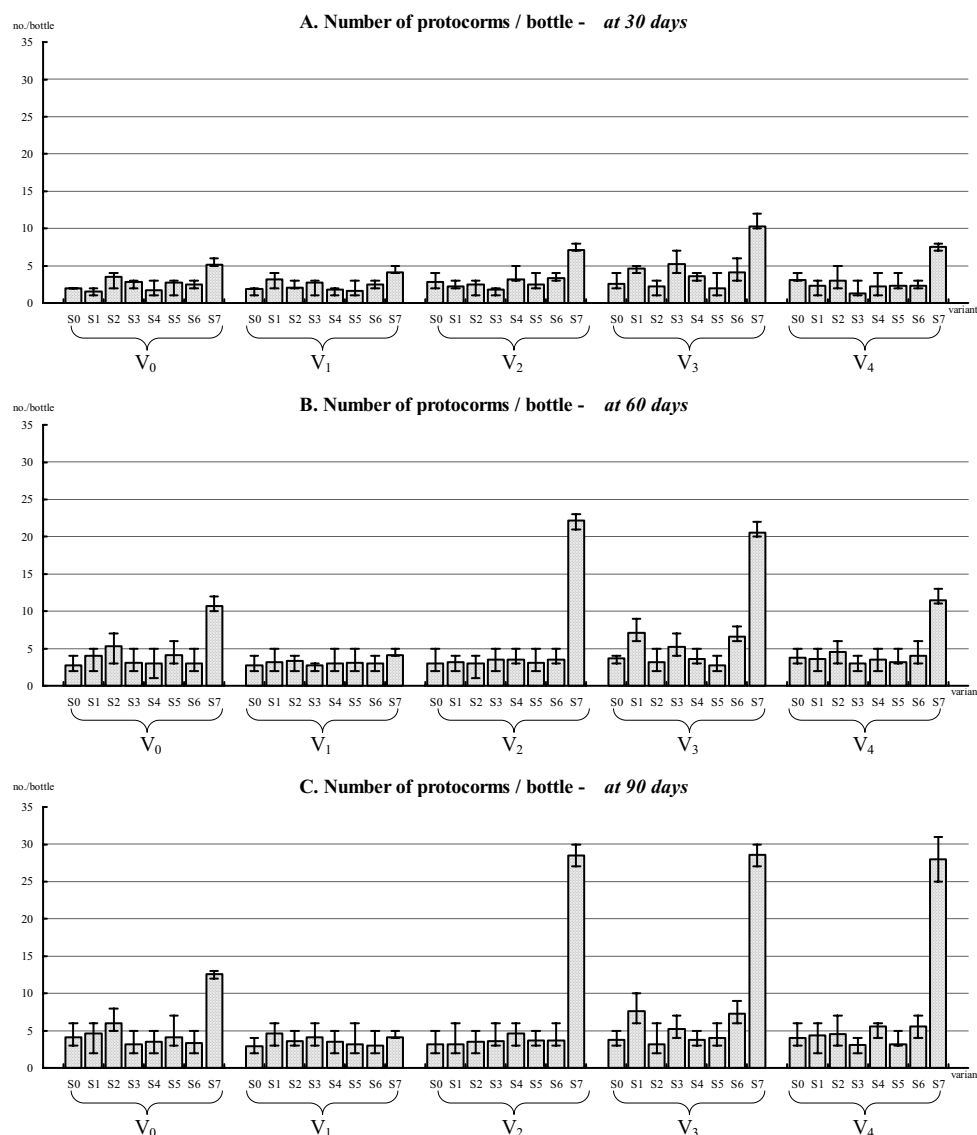


Figure 1. The comparison of the absolute value of average concerning the number of *Cymbidium hybridum* protocorms, constituted in glomerules, “in vitro” new formed, on a basic liquid (BM) Murashige-Skoog (1962) (MS) modified medium, with a content of various growth regulators, as follows: V₀ - BM without growth (lot control), V₁ - BM with an adding of 2 mg/l 2,4- dichlorophenoxyacetic acid (2,4-D); V₂ - BM with an adding of 2 mg/l benzyladenine (BA) mixed with 1 mg/l α-naphthylacetic acid (NAA); V₃ - BM only with 2 mg/l BA, V₄ - BM only with 1 mg/l NAA, cultivated either on S₀ experimental series – witness (complete), S₁ – BM without B microelement, S₂ – BM without Mn microelement, S₃ – BM without Zn microelement, S₄ – BM without Mo microelement, S₅ – BM without Cu microelement, S₆ – BM without Co microelement, or S₇ – BM without I microelement, after 30 days (A), 60 days (B) and 90 days (C) from the assembling of the experiments.

with other experimental series, where these activities were linear increased, showed that:

- the highest number of the new formed protocorms was marked on MS medium depleted in iodine, but with 2 mg/l BA and 1 mg/l NAA (V₃S₇ experimental variant), this parameter's value being superior with almost 7 times to the control variant (V₀S₀ – MB-MS complete, without any growth regulators) (meaningful data from the statistical point of view), respectively, the smaller number of this parameter, was put in evidence by the V₁S₀ variant (culture medium with all micronutrients, with 2 mg/l 2,4-D), but this time was marked a decrease of over 1.4 times, in comparison with the witness variant (Fig. 1C);
- the performed determinations on the *Cymbidium hybridum* protocorms fresh weight, inoculated on culture medium without iodine microelement, they had

set off a good development of these, the highest value was registered by the V₂S₇ experimental variant (MB without iodine microelement, with 2 mg/l BA and 1 mg/l NAA), where, comparatively to the witness variant (V₀S₀), the fresh substance accumulation was almost 43 times higher (meaningful data from the statistical point of view), simultaneously, the smallest quantity of fresh vegetal biomass was dignified on the V₄S₁ experimental variant (MB without boron, in addition with 1 mg/l NAA), the gravimetric values being 2.24 times under those marked at the control variant (lot cultivated on BM-MS complete medium, without growth regulators) (Fig. 2C);

- the data regarding the dry weight accumulated per clone, the higher, respectively the lower values respecting this parameter, were registered – as in the case of the fresh substance – also on the V₂S₇,

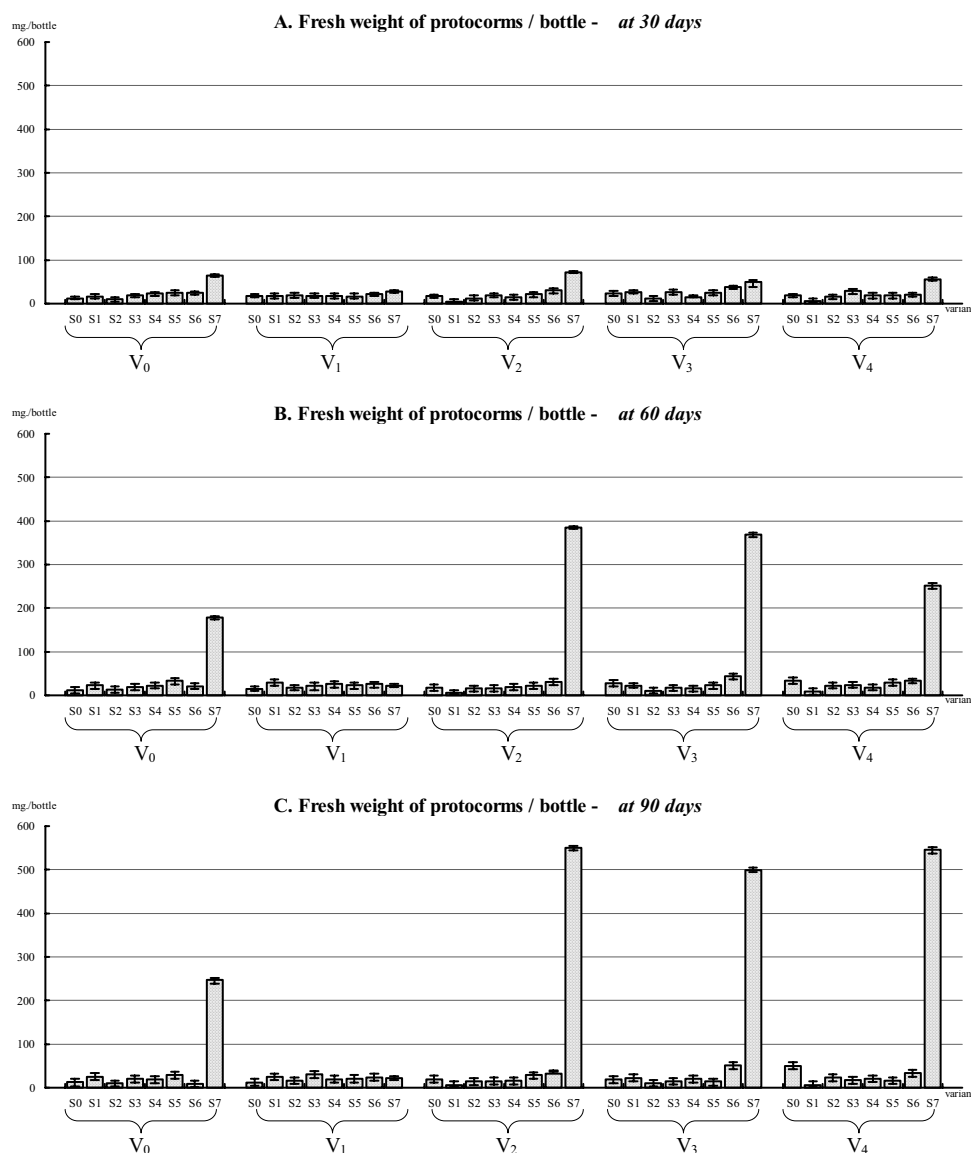


Figure 2. The comparison of the absolute value of average concerning the fresh weight of *Cymbidium hybridum* protocorms, constituted in glomerules, “in vitro” new formed, on a basic liquid (BM) Murashige-Skoog (1962) (MS) modified medium, with a content of various growth regulators, as follows: V_0 - 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, cultivated either on S_0 experimental series – witness (complete), S_1 – BM without B microelement, S_2 – BM without Mn microelement, S_3 – BM without Zn microelement, S_4 – BM without Mo microelement, S_5 – BM without Cu microelement, S_6 – BM without Co microelement, or S_7 – BM without I microelement, after 30 days (A), 60 days (B) and 90 days (C) from the assembling of the experiments.

respectively V_4S_1 variants, the accumulations being superior by 35.2 times, respectively 1.3 times inferior to control variant (Fig. 3C).

DISCUSSIONS

The absence of the microelement *iodine* from the culture medium (respective the non usage of the supply solution of KI in the preparing of medium), led to the marking of the most ample *proliferation* and *growth of protocormial vegetal biomass*, at *Cymbidium*, respectively of those protocorms *dry* and *fresh weight*, regardless of the growth hormones presence or absence from medium, excepting the variant with 2 mg/l 2,4-D 2 mg/l 2,4-D (V_1), to which – from the beginning of the experimental period – we observed the installation of a *senescent process* of

those protocorms, phenomenon, which evolved in those 90 days of vitroculture with an average of 90.9% necrosis. Therefore, to obtain a maximal multiplication of *Cymbidium* PLBs, we recommend the usage of a liquid Murashige-Skoog (1962) medium [14], in submersed cultures, and the elimination from their composition of KI, respectively *iodine*.

The absence of any microelements, as *boron*, *manganese*, *zinc*, *molybdenum*, *copper* or *cobalt* from the culture medium, they affected positively or negatively only a small part of the proliferation processes and the *Cymbidium* protocorms growth, with all these, the submersing of the protocorms in the medium without *cobalt*, but in the presence of BA, comparatively with the complete medium – reference – (containing all minerals, but without growth hormones), has determined a growing of those

protocorms' number and the increase of their protocormial biomass (fresh and dry), increases which were inferior to those obtained in vitrocultures of protocorms, performed in the medium without *iodine*.

A series of studies regarding "in vitro" multiplication of the plants' protocorms from the *Cybidium* genus [8, 9], and also on other orchids – *Onicidium* Gower Ramsey [10] or *Paphiopedilum philippinense* [11] – were realized using the same culture medium Murashige – Skoog (1962) [14] except that they were reduced at a half. The registered results

were satisfactory, only if in the culture medium were included different growth regulators.

An interesting change in the *Cymbidium hybridum* PLBs culture medium's compositions was made by Petruş and collaborators [15] which substituted the distilled water with deuterium depleted water (DDW) (which has just 25 ppm deuterium, no 155 ppm deuterium as distilled water). The authors reported that DDW has a grown inhibitory effect and recommended this water for "in vitro" conservations procedures of *Cymbidium* protocorms or other species.

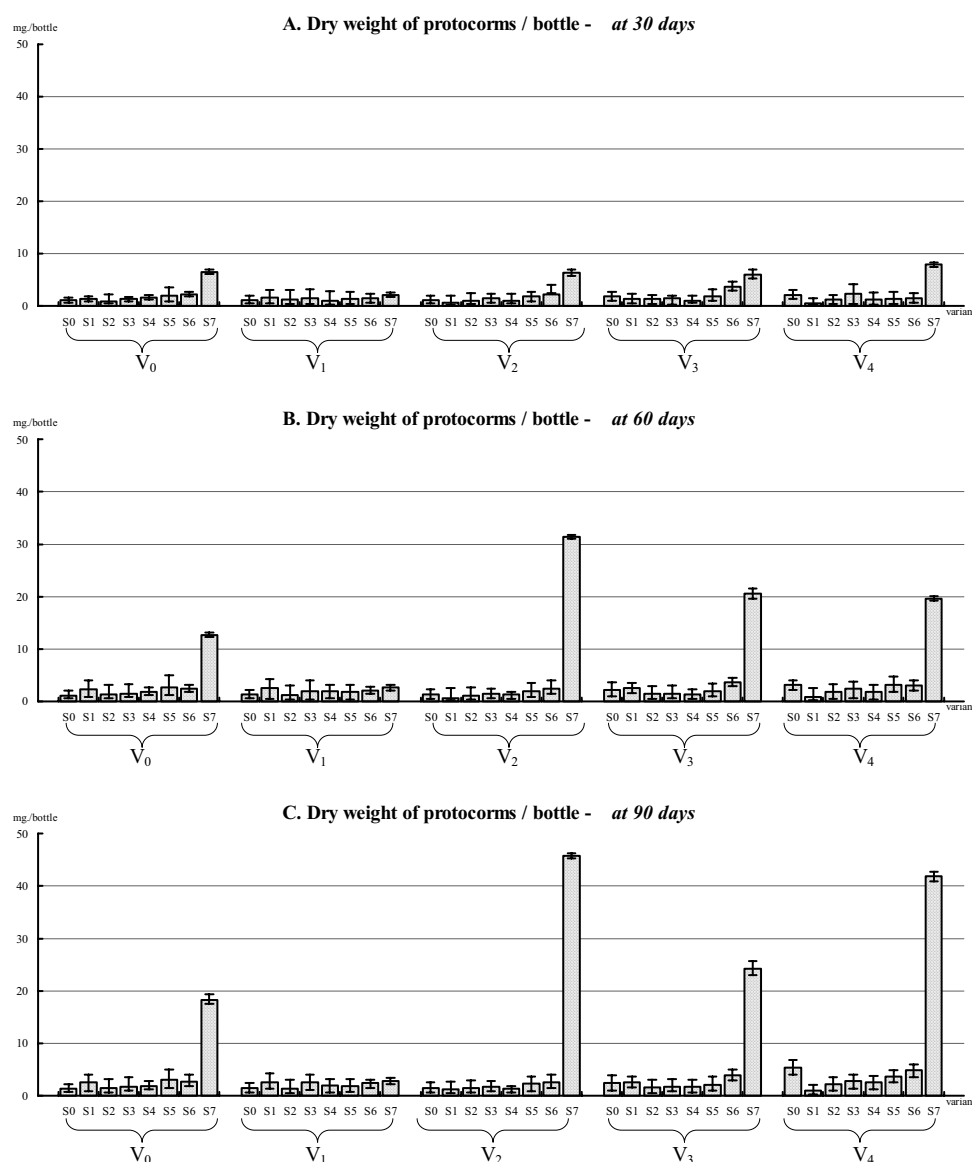


Figure 3. The comparison of the absolute value of average concerning the dry weight of *Cymbidium hybridum* protocorms, constituted in glomerules, "in vitro" new formed, on a basic liquid (BM) Murashige-Skoog (1962) (MS) modified medium, with a content of various growth regulators, as follows: V_0 - 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, cultivated either on S_0 experimental series – witness (complete), S_1 – BM without B microelement, S_2 – BM without Mn microelement, S_3 – BM without Zn microelement, S_4 – BM without Mo microelement, S_5 – BM without Cu microelement, S_6 – BM without Co microelement, or S_7 – BM without I microelement, after 30 days (A), 60 days (B) and 90 days (C) from the assembling of the experiments.

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