THE REACTIVITY OF *CYMBIDIUM HYBRIDUM* PROTOCORMS, SUSTAINED ON LUFFA LIGNO-SKELETON IN VITROCULTURES IN LIQUID MEDIA

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Summary: Is known the fact that the *Cymbidium hybridum* orchid protocorms, submersed in liquid culture medium (in hypoxia conditions) himself multiply with higher speed in comparison to aerated condition, on surface of agarized (solid) culture media. Also, is known the fact that the *Luffa* ligno-skeleton (obtained from ripe pulp of *Luffa cilindrica* fruits) lends successfully at its usage as wick in supply with nutrients of various plant species inoculs, cultivated "in vitro" on liquid media. In this work, we are presenting the role of Luffa ligno-skeleton in maintaining the *Cymbidium hybridum* protocorms, cultivated "in vitro", on culture medium liquid surface (type of Murashige-Skoog, 1962).

Behind these experiments, was proved that the ligno-skeleton of Luffa type of bridges for *Cymbidium hybridum* protocorms, sustained in aerated condition, is more efficient in bearing with those marked on solid culture medium, as in number, as in accumulation of fresh and dry weight, regardless of growth regulators included in media; on liquid culture media containing 1 mg/l AIB or 2 mg/l K as growth regulators, where bearing with the homologue culture variant (solidified media with agar-agar, with same mineral and organic composition), the differences was until 29.3% superior, and bearing with the witness medium (solid – agarized – medium, but without growth regulators) until 30.2% biggest, concerning the number of protocorms.

Keywords: Cymbidium (orchid); growth regulators, "in vitro", lingo-skeleton, Luffa

INTRODUCTION

In micropropagation industry of vegetal species by economic interest, the usage of jellified agents, is necessary for solidifying the aseptic culture media, fated for "in vitro" growth of some various explant types, their sustaining being indispensable for some clones categories, the submersion regime on some clones causing countless negative physiological implications, regardless to provide the tissues with oxygen.

Sometimes, the vitrocultures evolution, morphogenesis, growth and the differentiation depends on the agar quality, this ingredient influence not only the clones, but also the production price, respectively the sales of the plant material generated "in vitro". On these considerations, is benefic the investigation of new replacing ways – totally or partially – of agar, with different layer efficient types for the vitrocultures sustaining, but with a low cost.

The vegetal support, *ligno-skeleton* (Romanian patent act Nr. 110018 B1 from 1995, elaborated by *Cachiţă* et all.), consisted in a sclerenkimatic fiber network, isolated from super matured fruits of *Luffa cilindrica (L.)* Roem. The *ligno-skeleton* networks have the fate to sustain the explants and vitrocultures performed in a liquid medium, without agar-agar, this preventing the clone submersion.

An alternative in sustaining of the vitrocultures on liquid medium, is offered by some various synthetic fibers, which enter in some different materials, the useful condition consisting in the assurance of the clones sustaining in the ventilated medium, keeping out their submersion.

In vegetal vitrocultures, it were used for new purposes, supports as cartridge type, or "folded" bands from filter paper (Gauthered, 1959), and newest filtered paper-bridges in cross forms (Blidar, 2005).

In some preceding paperwork's (Cachiţă et all., 2003, Petruş et all., 2004), we have published our researches with potato and chrysanthemum explants, but also with african violets clones, where we used the luffa *ligno-skeleton* as support for the vegetal material cultivated "in vitro".

In this paperwork, we present our researches concerning the study of the *Cymbidium hybridum* L. reactions, in their "in vitro" culture, on liquid medium, sustained on *Luffa hybridum* L. Roem *ligno-skeleton* (vegetal sponge).

MATERIAL AND METHOD

The biological material consisted in *Cymbidium hybridum* protocorms. They were inoculated on a basic Murashige-Skoog medium (1962) (MS), modified by us, without glicine, 3-indolilacetic acid (IAA) and kinetin (K), and with only 20 g/l sucrose in the culture medium, instead of 30 g/l stipulated in the original recipe; for the culture we used liquid or agarized medium, solidified with 7 g/l Difco-Bacto agar (BM) and the medium pH was adjusted to 5.7 value at autoclavation. In our experiments we used protocorms derived from a micropropagated culture collection, made on the Vegetal Biotechnological Laboratory from Oradea University. The protocorms were vitrocultured on the same type of medium, illuminated with fluorescent tubes of white color (1400 lux luminous intensity), in photoperiodic regime of 16 hours light / 24 hours.

The present experiment behaved the organization of two experimental series, structured – each of them – on five variants in terms of growth regulators (Table 1).

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

Monolayer culture: on solid media (Series I)	**Double layer culture: Luffa lingo-skeleton in contact with liquid medium (Series II)**
V_0C – solid medium culture (agarized) with basic nutritive elements specific to *Murashige-Skoog* (MS) medium (control variant)	**V_0LL** – liquid medium culture with basic nutritive elements specific to *Murashige-Skoog* (MS) medium
V_1C – MS basic medium plus 2 mg/l 2,4-D (*2,4- dichloro-phenoxyacetic acid*)	**V_1LL** – MS + 2 mg/l 2,4-D
V_2C – MS basic medium plus 1 mg/l IBA (*indole 3-butyric acid*) and 2 mg/l K (*kinetin*)	**V_2LL** – MS plus 1 mg/l IBA and 2 mg/l K
V_3C – MS basic medium plus 1 mg/l IBA	**V_3LL** – MS plus 1 mg/l IBA
V_4C – MS basic medium plus 2 mg/l K	**V_4LL** – MS plus 2 mg/l K

In all, were analyzed the *Cymbidium* protocorms evolution cultivated on 10 variants of medium culture. The protocorms cultures were practiced in glass bottles, with the height of 70 mm, and with interior diameter of 25 mm. In every glass bottles was introduced 5 ml medium (agarized or liquid). In case of liquid medium (experimental variants of series II), but before this it was introduced the layer of luffa lingo-skeleton (fig. 1), support, which assured the aeration of the vitrocultures, the protocorms being sustained at the surface of culture mediums, avoiding the hypoxia.

Figure 1. Ligno-skeleton of Luffa

Each bottle was content 5 ml medium, assuring in the recipient a column of liquid medium with a 10 – 11 mm height, necessary for nutrition in good conditions of the protocorms, in a period of 90 days, period of time of the experiment process.

The bottles with medium were sterilized by autoclaving, at 121^0C, for 20 minutes. In sight of effectuate the operations of inoculation, the medium were left to cooled after in each bottle, has been inoculated a single protocorm.

After the inoculation of the protocorms, the bottles were covered with colorless transparent foil of polyethylene, immobilized with rubber rings. Then, this bottles were past in growth chamber and were seated on artificially illuminated shelfs with fluorescent tubes of white color (1400 lux luminous intensity), in photoperiodic regime of 16 hours light / 24 hours. The temperature on the shelfs with the explants/clones have oscillate between 26 ^{0}C ± 2 ^{0}C (day) and 24 ^{0}C ± 2 ^{0}C (night).

To a spell 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 neoformed 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 tamponated 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 etuve at 105 ^{0}C temperature, for 7 days). The experimental dates obtained to the control variant, respectively on V_0C basic medium variant (solid 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 to the similar parameters, to witness variant. The experimental dates were processed statistically, establishing – 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 10 experimental variant, do the photos from drawing 1, and histograms from figures 2-4.

RESULTS AND DISCUSSIONS

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

In the case of the performed observations at 30 days from the inoculation of the protocorms, the highest values, as regards the *number of protocorms*, were registered to variant of agarized medium with 2 mg/l 2,4-D, as growth regulator (fig. 2A), these having small diameter (1-1,5 mm); the difference against witness (V_0C – agarized BM-MS, devoid of the growth regulator) was enhanced average with 2,1 protocorms /glomerule, respectively the numbers being with about 43.7% higher than the values obtained to this parameter to the control variant (values sustained as relevant statistical point of view).

In the matter of *fresh weight* of protocorms, the highest values labeled per each culture bottle, were registered on control variant of culture medium V_0C (agarized BM-MS without growth regulator), closely followed by results marked on V_2LL experimental variant (liquid BM-MS with 1 mg/l IBA and 2 mg/l K – as growth regulators, sustained in aerated condition on ligno-skeleton type of bridge) the difference against witness being less average with only 3.4 mg/glomerule, respectively the numbers being with about 4.6% lower that the values obtained to this parameter to the control variant (fig. 3A) (values sustained as relevant statistical point of view). Also, it has been notices that, the presence of kinetine (1 mg/l) as unique growth regulator, in solid (agarized) culture medium (V_4C)

favored the accumulation of *dry substance* in the protocorms glomerule (fig. 4A).

The performed observations made after 60 days from inoculation, emphasized that, the previously described phenomenon has change in the case of the *number of protocorms*, respectively the highest values (11.4 protocorms / glomerule) has marked on liquid medium without any growth regulators (with ligno-skeleton bridge – V_0LL), the difference against witness (BM-MS agarized, without regulators) was 1.2 protocorms / glomerule (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. 2B). In case of *weight* of glomerullar mass (both, *fresh* and *dry*), the highest values was registered to the variants of vitrocultivated protocorms, growth on the witness medium culture (MS agarized without growth regulators). We remark the fact that in case of existing in culture medium of 2 mg/l kinetin as single growth regulators (V_4 variants), for both case of weight (fresh and dry), the usage of *ligno-skeleton* bridge for sustain of *Cymbidium* protocorms in aerated condition on surface of liquid medium, the dates was highest, reported to agarized medium culture, with 75.6 mg/glomerule (118.8% highest) for fresh weight, and with 3.1 mg/glomerule (30% highest) for dry weight (dates sustained as relevant statistical point of view) (fig. 3B & 4B).

The performed observations made at 90 days from inoculation (drawing 1), matter the *number of protocorms*, *fresh* and *dry weight*, most good result were registered in the conditions in which we used the solid agarized substratum, consisted from a MS medium with addition of 1 mg/l IBA with 2 mg/l BA, (variant V_2C) (drawing 1C) (fig. 2C, 3C & 4C). To this variant (V_2C), we registered 29.3 protocorms/ glomerule, against 15.1 in absolute values (against 106.3%) marked to the control variant supernatant (meaningfully statistical values).

In the matter of the V_3 (MS with 1 mg/l IBA) and V_4 (MS with 2 mg/l K) variants, the most good results were registered on liquid media with *ligno-skeleton* support for sustaining *Cymbidium* protocorms in aerated condition ("LL" series), in bearing with solid (agarized) medium culture ("C" series), the differences against control variant (V_0C – agarized BM-MS medium, without growth regulators), was between 25.3% for V_4LL and 30.2% for V_3LL culture media, in case of *number of protocorms / glomerule*, between 21.3% for V_4LL and 48% for V_3LL in case of *fresh weight* and between 14.5% for V_4LL and 21% for V_3LL in case of *dry weight* (meaningfully statistical values). The presence in the media (either solid, either liquid) of 2,4-D auxine, in amount of 2 mg/l, the protocorms was proliferated, but these didn't grow,

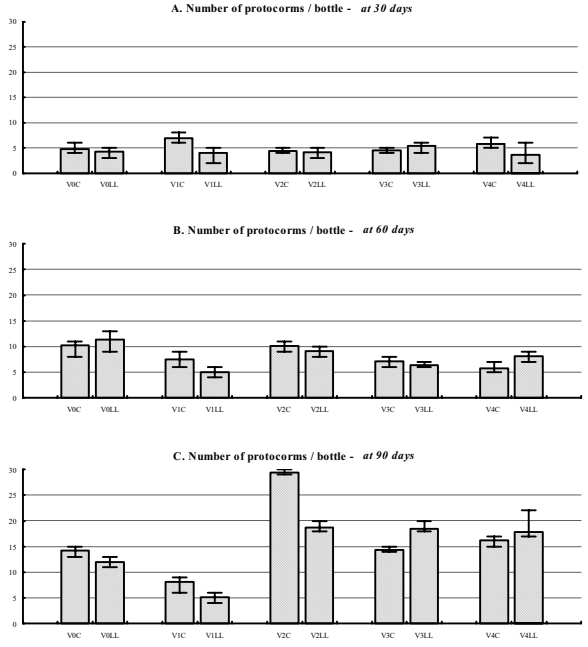


Figure 2. The comparison of the absolute value of average concerning the number of *Cymbidium hybridum* protocorms, constituted in glomerules, "in vitro" neoformed, on a basic (BM) Murashige-Skoog (1962) (MS) modified medium, with a content of various growth regulators, as how follows: **V_0** – BM without 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 1 mg/l *indole 3-butyric acid* (IBA) mixed with 2 mg/l *kinetin* (K); **V_3** – BM only with 1 mg/l IBA, **V_4** – BM only with 1 mg/l K, cultivated either on solid agarized medium (series "**C**"), either on lignoskeleton of luffa being in contact with liquid medium of culture (series "**LL**"), after *30 days* (**A**), *60 days* (**B**) and *90 days* (**C**) from assembling of the experiments.

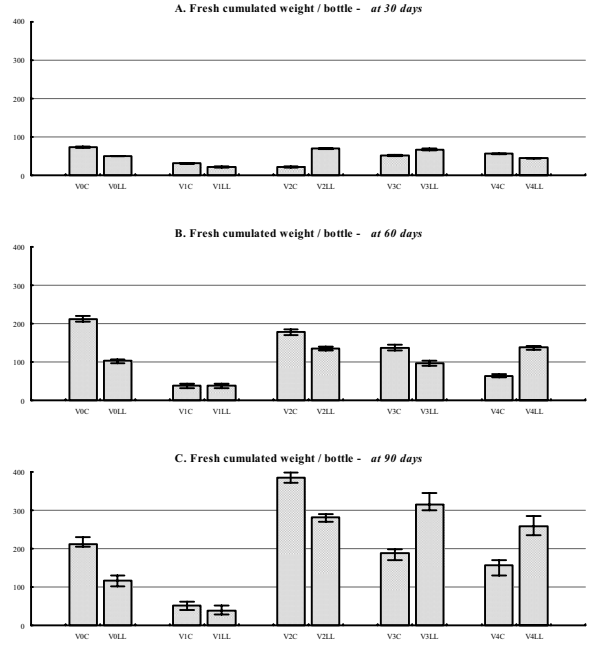


Figure 3. The comparison of the absolute value of average concerning the fresh weight of *Cymbidium hybridum* protocorms, constituted in glomerules, "in vitro" neoformed, on a basic (BM) Murashige-Skoog (1962) (MS) modified medium, with a content of various growth regulators, as how follows: **V_0** – 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 1 mg/l *indole 3-butyric acid* (IBA) mixed with 2 mg/l *kinetin* (K); **V_3** – BM only with 1 mg/l IBA, **V_4** – BM only with 1 mg/l K, cultivated either on solid agarized medium (series "**C**"), either on ligno-skeleton of luffa being in contact with liquid medium of culture (series "**LL**"), after *30 days* (**A**), *60 days* (**B**) and *90 days* (**C**) from assembling of the experiments.

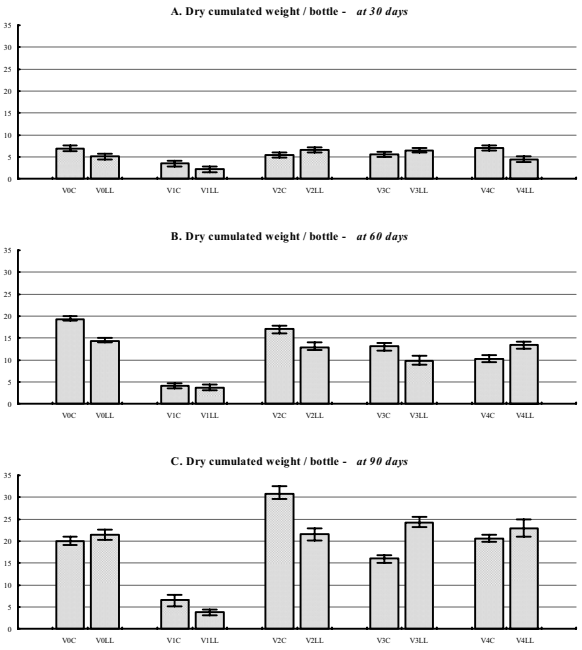
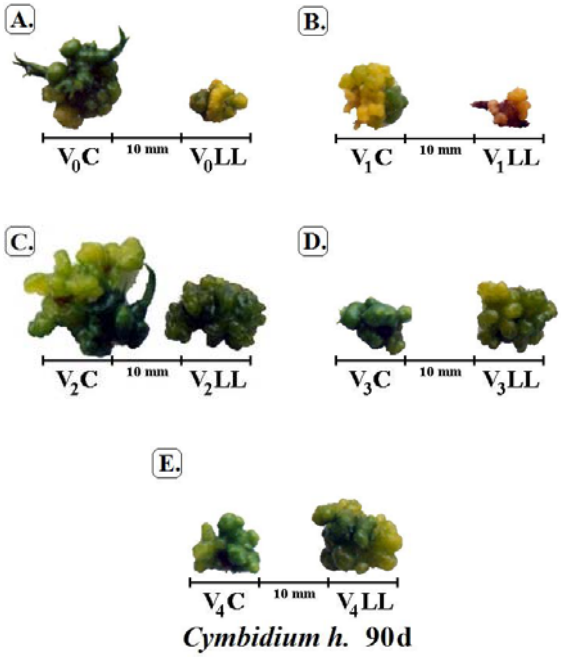


Figure 4. The comparison of the absolute value of average concerning the dry weight of *Cymbidium hybridum* protocorms, constituted in glomerules, "in vitro" neoformed, on a basic (BM) Murashige-Skoog (1962) (MS) modified medium, with a content of various growth regulators, as how follows: $\mathbf{V_0}$ – BM without growth (lot control), $\mathbf{V_1}$ – BM with an adding of 2 mg/l *2,4- dichlorophenoxyacetic acid* (2,4-D); $\mathbf{V_2}$ – BM with an adding of 1 mg/l *indole 3-butyric acid* (IBA) mixed with 2 mg/l *kinetin* (K); $\mathbf{V_3}$ – BM only with 1 mg/l IBA, $\mathbf{V_4}$ – BM only with 1 mg/l K, cultivated either on solid agarized medium (series "**C**"), either on ligno-skeleton of luffa being in contact with liquid medium of culture (series "**LL**"), after *30 days* (**A**), *60 days* (**B**) and *90 days* (**C**) from assembling of the experiments.



Drawing 1. The comparison of the aspects of *Cymbidium hybridum* protocorms, constituted in glomerules, "in vitro" neoformed, on a basic (BM) Murashige-Skoog (1962) (MS) modified medium, with a content of various growth regulators, as how follows: $\mathbf{V_0}$ – BM without growth (lot control) (**A**), $\mathbf{V_1}$ – BM with an adding of 2 mg/l *2,4- dichlorophenoxyacetic acid* (2,4-D) (**B**); $\mathbf{V_2}$ – BM with an adding of 1 mg/l *indole 3-butyric acid* (IBA) mixed with 2 mg/l *kinetin* (K); $\mathbf{V_3}$ – BM only with 1 mg/l IBA, $\mathbf{V_4}$ – BM only with 1 mg/l K, cultivated either on solid agarized medium (series "**C**"), either on ligno-skeleton of luffa being in contact with liquid medium of culture (series "**LL**"), after ***90 days*** from assembling of the experiments.

remaining tiny, presenting the senescence phenomenon (drawing 1B) (fig. 2C, 3C & 4C).

CONCLUSIONS

To assure an intense multiplication of *Cymbidium* protocorms, in a subculture program to a distance of 3 months, the usage of agarized medium culture with a mixture of (1 mg/l) IBA and (2 mg/l) K, was proved as being the most optimal variant of these vitrocultures, the number of protocorms being above with 15.1 protocorms/bottle (with 106.3%) higher than the value registered on medium cultures without supernatant and growth regulators (witness medium).

The usage of Luffa *ligno-skeleton* was proved as being efficient just in the case of culture media either with IBA (1 mg/l), either with only K (2 mg/l), between them, the best results were registered on culture medium with IBA, the reactivity differences concerning the number of protocorms against homologue medium – solidified medium with agar-agar, with same mineral and organic composition – being 29.3%, respective, against the witness medium - without growing hormones – being 30.2%.

The presence of 2,4-D auxine (2 mg/l) was proved as an inefficient procedure, matter the multiplication and growth of *Cymbidium* protocorms, regardless of the type of culture medium (solid agarized, or liquid with *ligno-skeleton* support), since, already at 30 days of vitrocultures, the protocorms presented severe senescence processes, which, latter – at 60 days, but mostly at 90 days of vitrocultures – leaded to their necrosis.

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