

THE TESTING OF CAFFEIN “IN VITRO” REACTION ON *CYMBIDIUM HYBRIDUM* PROTOCORMS SUBCULTURED ON ESPECIALLY BRIDGE OF FILTERED PAPER

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Abstract: The *Cymbidium hybridum* protocorms, in vitro culture regime, on *Murashige-Skoog* liquid medium, were maintained on especially filtered paper bridges, which had responded different, depending on the introduced quantity of caffein on the cultures medium. After 90 days of vitro culture, the maximal number of protocorms, and the *fresh* and *dry* biomass of these, was registered at the variants of culture medium with caffein in a 0,001% concentration. On the lowest or highest concentration of 0,001% caffein, we find out a progressive decrease of the protocorms vitality.

INTRODUCTION

There are numerous compounds which can influence negative the biostructures of the vegetal cell, in this can be engaged the antifuzoriale substances or c-mitotic agents, which can inhibit the performance of the division spindle, the chromatoclastic substances or chromosomal “poisons”, called as radiomimetic substances or alkylates agents, which produce the chromosomes fragmentation, or the stathmodieretic substances which can stop cytodieresis, by inhibiting the fragmoplast differentiation (**Constantinescu**, 1958, 1961, 1965). Based on the studies with a fundamental character in this domain, **Apostol & Niculescu** (1970) were made a series of experiments on experimental models, with the purpose of the investigation of those sensitivity at the action of caffein on this, and of the alkylates agents administrated in low concentrations, same as the stathmodieretic effect of xanthine methyl derivatives. The Romanian school of experimental vegetal cytology, on the researches made from **Constantinescu** et. al (1958, 1961, 1965), becoming the merit to establishing that the methylic derivatives of xanthine, especially of caffein, in M/1000 concentrations, can sensitize the vegetal cells at the action of alkylate agents, condition in which the alterations produced in these, can be identical, till identity with the mitotic figures caused by this substances, or by the ionized radiations, at the culture. Researches in this domain were made by **Kihlman** (1965, 1971 a & b, 1972) which had explained the chromatoclastic action of purinici derivatives, especially of etoxycaffeine.

The investigations of various groups of researchers, has established the correlation between the chemical structure and the stathmodieretic activity in derivatives series of the theobromine, theophylline and caffein, and on the other hand can clear up the mechanism in which the purinic alkaloids and some derivatives can sensitize the meristematic cells at the alkylate cytostatic actions. These researches were made with photonic microscope and with electronic microscope, too.

Our studies in the present work, represent a confirmation of the performed studies by **Constantinescu** at all, by using *Cymbidium hybridum* protocorms as an experimental sample, with the purpose of analysing the effect of this on their growth and morphogenesis level, cultivated on liquid medium which contained various concentrations of caffein.

MATERIAL AND METHOD

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), classic, without glicine, IAA, agar-agar and phytohormones, but with PP, B₆ and B₁ 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 medium culture, by adding various quantity of caffein, resulting the next medium cultures:

- V₀ – basic medium (BM), without caffein;
- V₁ – BM-MS with 0,0001% caffein;
- V₂ – BM-MS with 0,001% caffein;
- V₃ – BM-MS with 0,01% caffein;
- V₄ – BM-MS with 0,1% caffein;

After the pH adjustment at 5,7, this was distributed in thermoresistant glass recipients (ampicillin type), 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 (**Blidar**, 2004), 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 aseptization of the recipients with medium, the phials were obturated with hydrophilic cotton plug, after this there was autoclaved at 121°C 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 the some characteristics looking 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 of lucs, 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 26° 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 neofomed protocorms* from the initially protocorms, the *fresh weight* and the *dry weight* of the biomass at these protocorms.

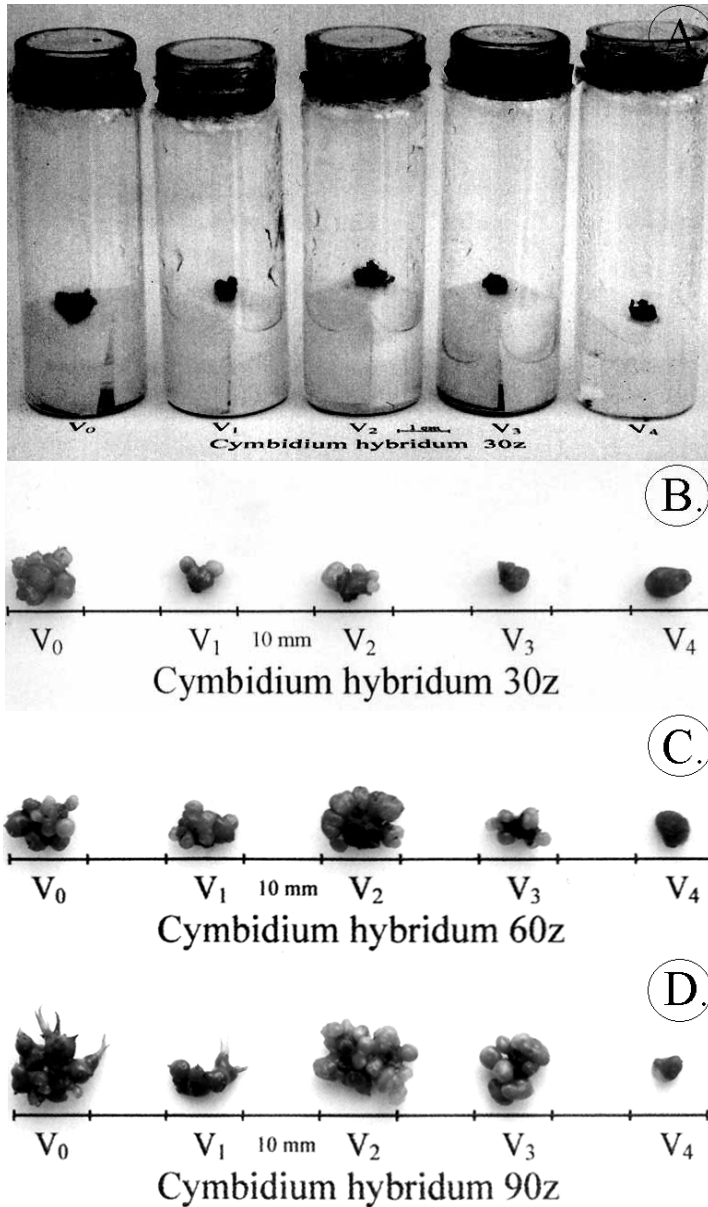
For each of these parameters, the values registered at 30 days of vitroculture, on the witness medium – without caffeine (V_0), were considered reference value (as 100%), to this reference value retrospect all the other values of the respective parameter, on the period of 90 days from inoculation.

RESULT AND DISCUSSIONS

The most illustrative appearance, concerning the reactivity of differentiated protocorms, respectively the resulted vitrocultures from these, on five variants of culture medium, made the object of the photos from Drawing 1, and histograms from Figure 1.

The performed prolusions at *30 days from inoculation*: most higher values in matter the *number of protocorms* regenerated to the level of each inocul, but the *dry cumulated substances* of these, they were registered to witness variant of average (V_0), without caffeine (fig. 1, A & C); with all these, most significant accumulations of *fresh substance* were marked on variant of average with a content of 0,001% caffeine (V_2), a closed value to this, was register on check average, the quantitative report among V_2 and V_0 being only 1,09: 1 (fig. 1, B). We mention the fact that in the conditions of a presences of a higher concentrations of caffeine on the culture medium, respectively of 0,01%, but mostly 0,1% (variants V_3 and V_4), we remarked a strong inhibition on the multiplication and growth of the *Cymbidium* protocorms, the values at all three parameters take in step, were inferior against the witness, direct proportional phenomenon with the growing of the concentration of caffeine from the culture medium (fig. 1, A & C, Drawing 1, A & B).

In behind the performed determinations at *60 days of vitroculture*, we ascertained an increasing of the *number of protocorms* of *Cymbidium*, as much to variant V_2 (BM with 0,001% caffeine), and to the variant V_3 (BM with 0,01% caffeine), the values of this parameter being of 1,21 either, respectively of 1,05 either else bigger than the values registered to the witness lot (fig. 1, A). Instead, just like at 30 days from inoculation, the values of studied gravimetric parameters (*fresh and dry substance*), to this date, they maintained to the maximum value to variant of check average (fig. 1, B & C). From among the variants of medium with caffeine, most good reactivity was registered on the medium V_2 (MB with 0,001% caffeine), as much the appearance, and the number of neoprotocorms generated from the initially protocorm, but concerning the fresh and dry weight, remarking a stimulation of multiplication and growth of these (Drawing 1, C).



Drawing 1. Comparative appearance concerning the reactivity of the *Cymbidium hybridum* protocorms, cultivated on basic medium *Murashige-Skoog* (1962), where: V_0 – witness lot deprived of caffeine; V_1 – medium with 0,0001% caffeine; V_2 – medium with 0,001% caffeine; V_3 – medium with 0,01% caffeine; V_4 – medium with 0,1% caffeine, to 30 days (A & B), 60 days (C) and to 90 days (D) from the inoculation of unique protocorm per bottle.

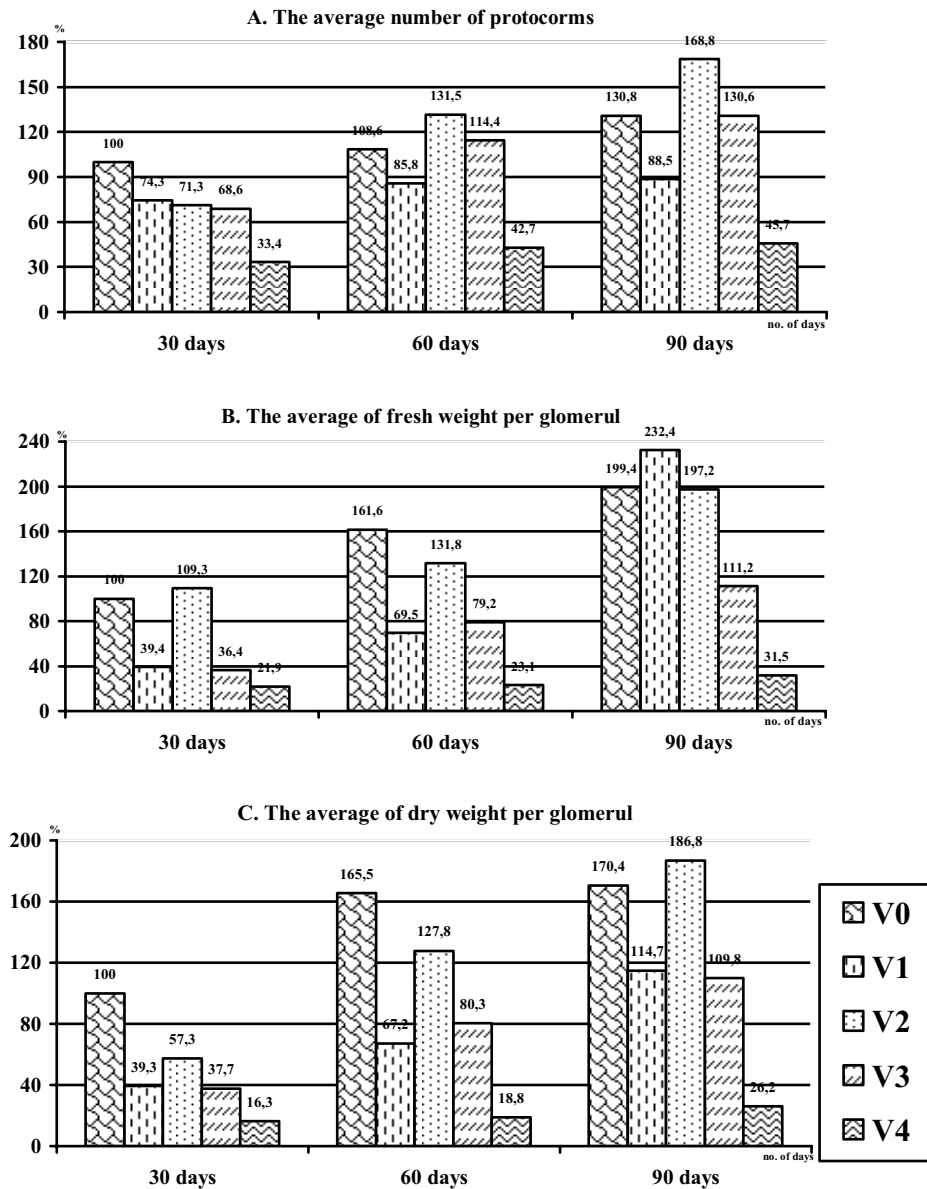


Figure 1. Dynamically growth of vitroculture of *Cymbidium hybridum* protocorm, for 90 days, on filter paper bridges, positioned on average of liquid culture medium *Murashige-Skoog* (1962), with addition of caffeine (where V₁ - BM with content of 0,0001% caffeine; V₂ – BM with content of 0,001% caffeine; V₃ – BM with content of 0,1% caffeine; V₄ – BM with content of 0,1% caffeine); the percentage reports were made taking as reference values (100%) the medium number of protocorms, the *fresh weight* and *dry weight* of these per glomerules, generated from initially protocorms of vitrocultures, after the first 30 days from the initiation of the experiments.

To final experimental period, respectively *at 90 days from inoculation*, the digital determinations sets off an amplification a the proliferation of cultivated protocorms on medium V₂ (BM with a content of 0,001% caffeine), the report of the *number of neoprotocorms* – against of the registered values to witness (V₀ – BM deprives the caffeine) – amplifying from 1,21:1 to 60 of days, to 1,29:1 to this last experimental development (fig. 1, A). We remarked, however, a strong growth of *fresh weight* to *Cymbidium* protocorms on the medium with a content of 0,0001% in caffeine (V₁), forward slip even the gravimetric values putted in evidence to the performed cultures on witness medium (V₀), the reported values being of 1,16:1 (fig. 1, B). Therefore, not but that the *number of protocorms* regenerated on variant of medium V₂ were most raised, from the all experimental series, numbers that illustrates that the *fresh weight* of generated glomerules in the variant of medium V₁ were superior to medium V₂. Concerning the *dry substance*, expectedly, the bigger values brands to medium variant with a content of 0,001% in caffeine (V₂), the report against the same parameter registered on the witness, being of 1, 09:1. Most thin proliferation were observed to protocorms cultivated on culture medium V₄ with 0,1% caffeine, where we distinguished a strong inhibition, in report with the witness, the values registered being inferior of 2,86 either concerning the *number of neoprotocorms*, respectively of 6,33 times and 6,5 either for the *fresh substance* and the *dry substance* (fig. 1, B & C and Drawing 1, D).

The performed biometric analysis about inoculs of *Cymbidium hybridum*, cultivates on liquid medium with different concentrations in caffeine, supported on especially filter paper bridges in sight avoiding hypoxias, we distinguished the following ultimate conclusions:

CONCLUSIONS

1. The bigger *number of neoprotocorms* generated at the surface of the inocul, as much at 60 days, but chiefly at 90 days of vitrocultures, it was registered to variant with 0,001% caffeine (V₂). Concerning this biometric parameter, good result was reflected on the witness variant of average, too, without caffeine, but they were inferior against the results registered to variant with 0,001% caffeine on the medium. On these motives, in sight obtain of a big number of neoprotocorms, after 60 days of vitroculture, we recommend the usage of liquid culture medium *Murashige-Skoog* (1962), with concentrations in caffeine of 0,001%. The presence of a concentration of 0,1% caffeine on the culture medium, it was proved to be inhibitory on the multiplication and growth of the *Cymbidium hybridum* protocorms, which conducted to the installation of a premature senescence on these level, phenomenon selling of with the necrosis of these, as far back as first 30 days from the initiation of the experiments.

2. The resulted *fresh weight* of the total amount protocorms per bottle, were higher on the variant of average with 0,0001% caffeine (V₁), values followed by the results obtained on the protocorms generated on the witness lot (medium MS without caffeine). As a succession of the proliferation of protocorms, as much below the appearance number, same as the volume of these, to variant of average V₂ (MS with 0,001% caffeine), the *fresh weight* registered per glomerules were all high, the values obtained was situated immediately below the values registered on variant V₀. Same as in the case *number of protocorms*, most thin result concerning the *fresh weight* of these, was marked to protocorms cultivated on the medium of culture with a contain of 0,1% caffeine.

3. The most significant accumulation of *dry substance*, therefore the most good evolution of the protocorms *Cymbidium hybridum*, cultivated on liquid culture medium *Murashige-Skoog* (1962), on filter paper bridges, putted in evidence in the conditions of using a concentration in

caffeine of 0,001%, as the addition to this medium, which fact is explained through that on this variant of medium, has generated the higher number of neoprotocorms, what bring us to recommend the including of this compound as growth regulator on the culture medium of fated for micropropagation of the protocorms at this plant species. On the contrary, the presence on the culture medium a concentration of 0,1% caffeine, caused a premature necrosis to these protocorms.

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