THE INFLUENCE OF PARTIALLY DEDEUTERISED WATER ON CHLOROPHYLLIAN PIGMENTS CONTENT IN HYPERHYDRIC *Coleus* VITROPLANTLETS LEAVES

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Abstract. We studded the influence of glucose solution 1.5% concentration, prepared with normal distilled water which normally contain 150 ppm deuterium, and the effect of the same concentration of glucose solution prepared with partially dedeuterised water (with only 87.5 ppm deuterium), on normal or hyperhydric Coleus blumei Benth., C. black Dragon, C. hybridus var. Jupiter, C. hybridus var. Ethna and C. hybridus var. Charteuse vitroplantlets, cultured „in vitro" on Murashige – Skoog (1962) (MS) agarised medium. In the 9th week of vitroculture we covered the basal zone of the hyperhydric or normal Coleus vitroplants with glucoses solutions, as supernatant layer. After 4 weeks of glucose solution administration, the treatment with partially dedeuterised glucose solution stopped the anormal hyperhydric plants growth, and induced on its a normal apex generation. The binodal apexes taken over from normal or hyperhydric vitroplants were subcultivated on MS medium, with different growth regulators addition, and the regenerated unhyperhydric vitroplants contained in leaves normal chlorophyll a, b, and carotenoids pigments concentration. The best results in normal Coleus plants regenerated from minicuttings, which were subcultivated on variant MS medium, were obtained on medium with NAA 1 mg/l exception, was observed on C. black Dragon which manifested higher pigments accumulation on MS medium with BA 2 mg/l mixed with NAA 1 mg/l.

Key wards: *hyperhydricity, Coleus, dedeuterised water, assimilatory pigments.*

INTRODUCTION

Hyperhydricity is a physiological disorder frequently affecting shoots vegetatively propagated "in vitro". Hyperhydric shoots are characterized by a translucent aspect, due to a high water accumulation in the intercellular spaces, a chlorophyll deficiency, a very thin cell wall development, aspects which were considered as a neoplassic manifestations The hyperhydric vitroplantlets suffer morphological transformations which consist in: hypertrophied, recurred and translucent (glassily) leaves, sometimes become curly and casant, with distort growth, normally they have big waist and ethyolled aspect.1, 2, 3

In aseptically conditions, the hyperhydric vitroplantlets appears spontaneously, provoked by the higher temperature, or by the presence in the recipients of higher ethylene concentration.

The anatomy of normal and hyperhydric leaves of *Dianthus caryophyllus* plantlets were studied by Olmos and Hellin (1998), using scanning or transmission electron microscop. The hyperhydric leaves showed a hypertrophy of the mesophylic cells, with larger intercellular spaces and vacuoles. This leaf epiderm lacked cuticular wax, and the chloroplasts presented abundant plastoglobuls. In the hyperhydric leaves epiderm the stomatic guard cells were different in morphology from those which were developed on the normal one, and demonstrated high levels of K^+. The alteration of guard cells structure of stomatal could be a mechanical impediment to stomatal function.6

Some researches made in Hungary, from the group leaded by thy biologist Somlyai, showed that the decreasing deuterium content in the water may influence the metabolism and the function regime in the live cells, especially to the tumoral cells, producing a stop of cellular multiplication, and diminution the neoplassic tumors dimension. Somlyai demonstrate experimentally the fact that the partially dedeuterised water inhibit germination of some seeds species.10

Frequently, some *Coleus* vitroplantlets manifest hyperhidric status manifested by a high transparence degree of shoots and leaves, a hypertrophy growth, a higher number of leaves, nods and stem ramification, but small and rolled leaves (fig. 1A) etc.8

The minicuttings prelevated from hyperhydric vitroplants, after 4 weeks of treatment with partially dedeuterised water, in subculture generated normal vitroplants.7-9

In our experiments, we investigated the effect of partially dedeuterised water, used for 1.5% glucose solution preparation, utilized as a supernatant, in a double layer medium on *Coleus* hyperhydric vitrocultures. The unhyperhydric effect of the 1.5% glucose solution with 87.5 ppm deuterium final concentration,

prepared with partially dedeuterised water, was compared with the evolution of the vitrified vitroplantlets on control lot, covered by distilled water, which, normally had 150 ppm deuterium content.

MATERIAL AND METHOD

Our experiments were made in several steps:

Step 1: *Coleus* minicuttings, prelevated from *C. blumei Benth*., *C. black Dragon, C. hybridus* var. *Jupiter, C. hybridus* var. *Ethna, C.hybridus* var. *Charteuse* vitroplantlets, were cultured "in vitro" on agarised Murashige – Skoog (MS) (1962) basal medium (BM), modified by us, without growth regulators and glicine, but with vitamins (thiamine HCl, pyridoxine HCl, nicotinic acid, each 1mg/l), meso – inositol 100 mg/l, sucrose 20 mg/l (not 30 mg/l in the original recipe), and with 7 g/l agar-agar.5 The trials with culture media were sterilized by autoclavation on 120°C for 25 minutes. After minicuttings inoculation the realized vitrocultures were maintained at white light at 1700 lucks, on 16/24 hours photoperiod.

Step 2: when the shoots had about 5 cm length, after 9 weeks of vitroculture (fig. 1A (b), in each recipients was introduced – as a supernatant - 10 ml of 1.5% glucose solution, prepared in distillated water - at the control variant - or with partially dedeuterised water, composing the next experimental variants:

- H series – 1.5% glucose solution prepared with distilled water which contained *150 ppm deuterium*;
- D series – 1.5% glucose solution prepared with partially dedeuterised water (with 25 ppm deuterium), the final concentration in deuterium being *87.5ppm* (table 1).

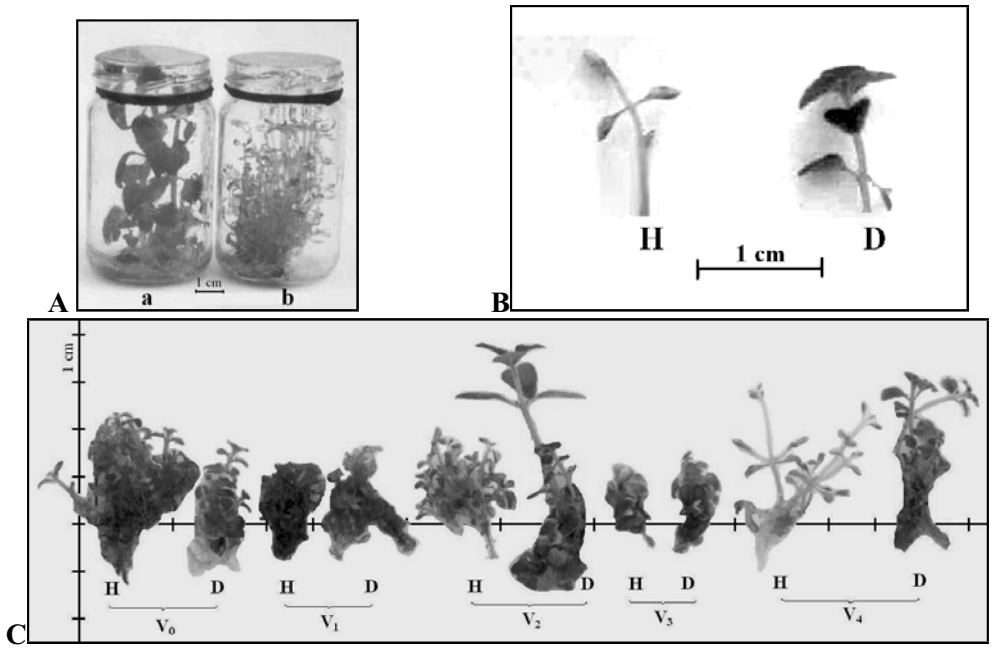

Fig.1. The comparatively aspects of Coleus black Dragon vitroculture, after 9 weeks (**A**) of minicuttings inoculation on MS agarised medium - normal (a) and hyperhidric (b) vitroplantlets (step 1); **B** – the image of the minicuttings after 4 weeks of subculture, being preleved from the double layer system - MS basal agarised medium – the first layer, and the second layer: H series = 1.5% glucose solution prepared in distillated water, having 150 ppm deuterium, and D series = 1.5% glucose solution prepared with partially dedeuterised water, final content being only 87,5% ppm deuterium (step 2); **C** – plants after 4 weeks of „in vitro" binodal apical minicuttings subculture on MS basal medium with different growth regulators: V_0 medium without growth regulators; V_1 – MS – BM with 2,4-D 2 mg/l; V_2 – MS – BM with BA 2 mg/l plus NAA 1 mg/l; V_3 – MS – BM with BA 2 mg/l; V_4 – MS – BM with NAA 1 mg/l (step 3).

After preparation, the glucose solutions were sterilized by autoclaving, at a pression of 1 atmosphere, for 25 minutes. The sterilized glucose solutions were administered to vitrocultures – as supernatant layer - by injection, in sterile condition, through polietilen folia which obtured the recipient culture, over vitroplantlets being developed roots on agarised medium, and with shoots (1/5 of the basal region) submersed in solution, realizing – for 4 weeks – "a double layer culture system".

Step 3: after **4 weeks** from the glucose solution administration, as a supernatant in the double layer system culture, from vitroplantlets were taken over binodal apexes (fig. 1 B), which were subcultured on MS

normal agarised medium, with different growth regulators, the experimental variants are described in the table 2.

Table 1. The preparation of different partially dedeuterised water obtained by mixing up partially dedeuterised water (with 25 ppm deuterium), with distilled water (with 150 ppm deuterium).

Partially dedeuterised water 25 ppm	Distilled water 150 ppm	Final concentration (ppm)
The components proportion in mixing up		
1	9	137.5
2	8	125.0
3	7	112.5
4	6	100.0
5	**5**	**87.5**
6	4	75.0
7	3	62.5
8	2	50.0
9	1	37.5
10	0	25.0

Table 2. The experimental variant used for apexes subculture, after 4 weeks from the administration as supernatant the glucose solution.

The variants on which were subcultured the binodal apexes taken over from **H series** hyperhydric vitroplants – the previous treatment being with 1.5% glucose solution prepared in distillated water, having 150 ppm deuterium (from the step 2).		The variants on which were subcultured the binodal apexes taken over from **D series** hyperhydric vitroplants – the previous treatment being with 1.5% glucose solution prepared with partially dedeuterised water, with only 87.5% ppm deuterium concentration (from the step 2).	
V_0H	BM – MS modified and devoided of growth regulators – control variant	**V_0D**	BM – MS modified and devoided of growth regulators – control variant
V_1H	BM – MS with 2,4 – D 2 mg/l	**V_1D**	BM – MS with 2,4 – D 2 mg/l
V_2H	BM – MS with BA 2 mg/l plus NAA 1 mg/l	**V_2D**	BM – MS with BA 2 mg/l plus NAA 1 mg/l
V_3H	BM – MS with BA 2 mg/l	**V_3D**	BM – MS with BA 2 mg/l
V_4H	BM – MS with NAA 2 mg/l	**V_4D**	BM – MS with NAA 2 mg/l

After 4 weeks of the binodal apexes subcultivation on $V_0 – V_4$ media derived from H or D series, over generated vitroplantlets were made photos (fig. 1C), observations, and assimilatory pigments analyses at the level of leaves, respectively was determined the content in green pigments: a and, b chlorophyll (fig. 2); yellow carotenoids pigments, and total photosynthetic pigments (fig. 3).

The pigments extraction from leaves was made with pure dimethylphormamyde solution (DMP 99.9%). The method consisting in triturating of 50 mg leaves in 5 ml DMP; the realized composition was maintained 72 hours at 4º C temperature; than, the supernatant was decanted; in this was determined the pigments content by photometrization, with Carl Zeiss Jena spectrophotometer SPECOL 11, produced from, using 664 nm wave lengthiness filters, for determining the a chlorophyll, 647 nm for determining the b chlorophyll, and 480 nm filter for determining the carotenoids content. The obtained results (the media of 5 probes / variant) were mathematically calculated after Moran and Porath (1980) proposed formulas:

a chlorophyll (µg/gsp) = $11.65\ A_{644} – 2.69\ A_{647}$·v/sp

b chlorophyll (µg/gsp) = $20.8\ A_{644} – 3.14\ A_{664}$·v/sp

Carotenoids (µg/gsp) = (1000 A_{480} – 1.28 a chlorophyll – 56.7 b chlorophyll)/245 v/sp

where: v – used solution (ml);
sp – µg of vegetal material used for extraction/probe;
a and b chlorophyll – amount in µg calculated in the first two formulas.4

The results were statistically processed and represented graphically in figure 2 and 3; the final dates were expressed in percent values, 100% were considered the registered values to the control variant.

RESULTS AND DISCUSSIONS

We observed that in the step 3 of our experiment the reaction of *Coleus* vitroplantlets was different, in dependence of deuterium concentration being in supernatant 1.5% glucose solution layer, derived from the step 2 of the experiment.

On the **Step 2** of the experiments, after **4 weeks** of glucose solution administered as supernatant, which was prepared with partially dedeuterised water (87.5 ppm deuterium – D variant), was observed an increase of the height of the hyperhydric vitroplantlets, with maximum **3 nodes**, and their apex – developed up to the level of the glucose solution had a normal morphologically status, with the presence of specific pigments in leaves, at the superior nodes level of the *Coleus* stems. So, these leaves were 3 times bigger, without transparency (fig. 1B and table 3), and with more pronounced pigments content, comparatively with the leaves derives from vitroplantlets present on H series variant (V_0H – V_4H), which were partially submersed in 1.5% glucose solution, with 150 ppm deuterium concentration (fig. 1B).

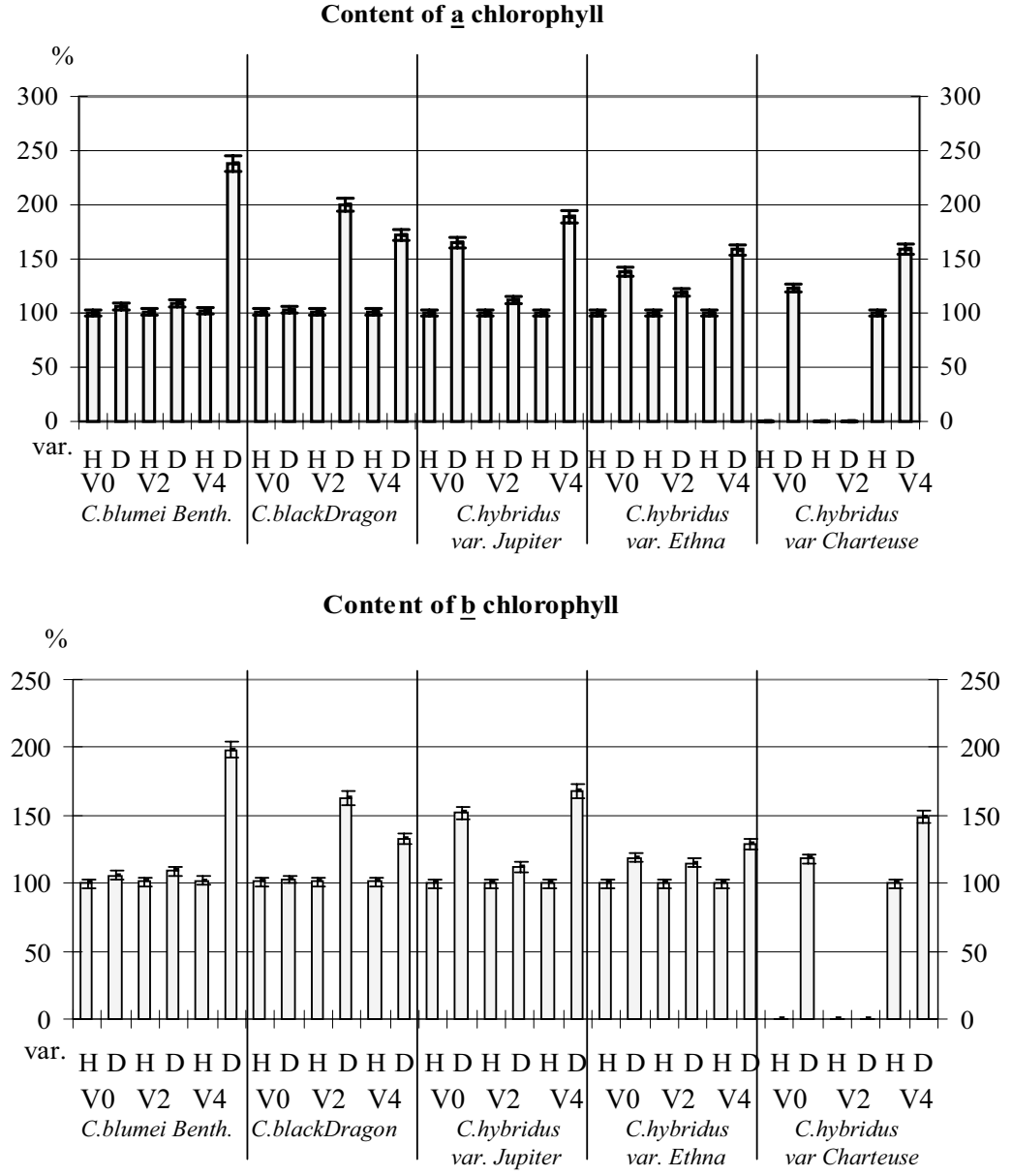

Fig. 2. The comparatively graphical representation of assimilator pigment's content in a and b chlorophyll, in Coleus leafs, after 4 weeks of „in vitro" culture of binodal minicuttings subcultured on medium with different growth regulators (step 3); the variants were: V_1 – MS – BM with 2,4-D 2 mg/l; V_2 – MS – BM with BA 2 mg/l plus NAA 1 mg/l; V_3 – MS – BM with BA 2 mg/l; V_4 – MS – BM with NAA 1 mg/l; minicuttings derived from **H** expertimental series represent plants resulted from cultures in double layer with 1.5% glucose solution prepared with distilled water which contained 150 ppm deuterium, and **D** series, 1.5% glucose solution prepared with partially dedeuterised water with 87,5 ppm deuterium.

In the **Step 3** of our experiments we observed that the apexes which were took over and were subcultured, for other 4 weeks, on fresh medium with growth regulators, had a particularly evolution dependent of the nature of growth substances content in medium in which its were inoculed, also in dependent of the kind of supernatant applied as a double layer culture.

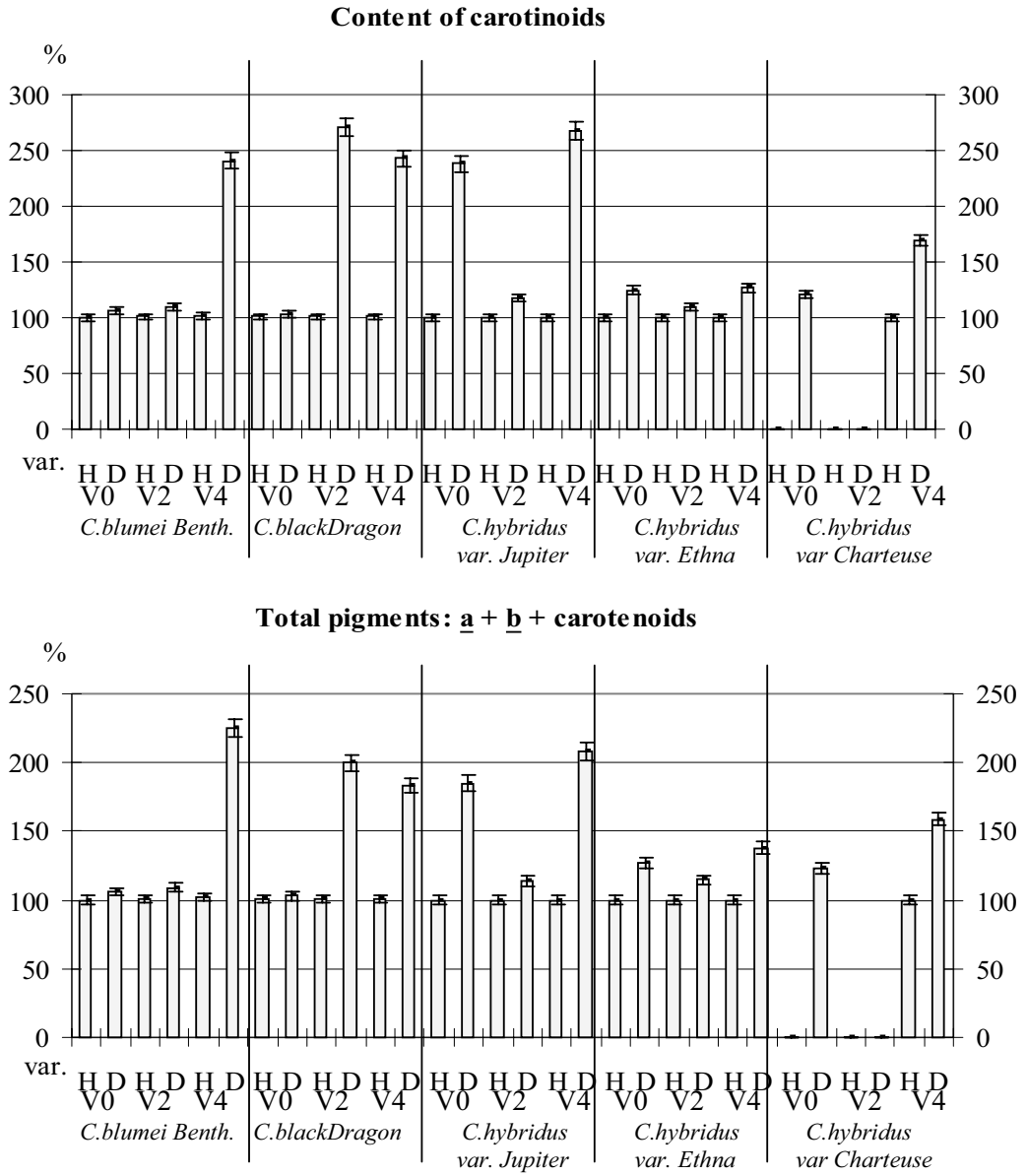

Fig. 3. The comparatively graphical representation of assimilator pigment's content in carotenoid and total pigments (a + b chlorophyll + carotenoids) in Coleus leafs, after 4 weeks of „in vitro" culture of binodal minicuttings subcultured on medium with different growth regulators (step 3); the variants were: V_1 – MS – BM with 2,4-D 2 mg/l; V_2 – MS – BM with BA 2 mg/l plus NAA 1 mg/l; V_3 – MS – BM with BA 2 mg/l; V_4 – MS – BM with NAA 1 mg/l; minicuttings derived from **H** experimental series represent plants resulted from cultures in double layer with 1.5% glucose solution prepared with distilled water which contained 150 ppm deuterium, and **D** series, 1.5% glucose solution prepared with partially dedeuterised water with 87,5 ppm deuterium.

The *Coleus* minicuttings preleved from the *C. blumei Benth*., *C. black Dragon, C. hybridus* vitroplantlets, and from the 3 varieties of *Coleus hybridus* sp. (*C. hybridus* var. *Jupiter, C. hybridus* var. *Ethna, C. hybridus* var. *Charteuse*), inoculated on H series, on MS basal medium with 2,4-D 1 mg/l (V_1), or with BA 2 mg/l (V_3), after 4 weeks of subculture, become senescent, but the minicuttings subcultured on V_0H, V_2H and V_4H variants survived in subculture, but the plantlets regenerated from its were hyperhydric,

and at the shoot and leaves level presented bigger number of leaves, nods and stem ramifications - which is a sign of a high hyperhydricity on *Coleus* vitroplantlets level. The vitroplantlets of D variant (plants resulted from cultures in double layer with 1.5% glucose solution prepared with partially dedeuterised water, with final 87.5 ppm deuterium content) presented normal growth, and assimilatory pigments content.

The necrosis of apexes subcultived on V_1 (V_1H and V_1D) and V_3 (V_3H and V_3D) variants, imposed the elimination of these variants from the results analysis, and in consequence, these variants are not represented in the graphical representation of assimilatory pigments content (fig. 2-3).

1. At ***C. blumei Benth***. vitroplantlets level was observed a significant difference between H and D series, especially at the vitroplantlets resulted on V_4D variant (MS – BM with NAA 2 mg/l); the higher **a chlorophyll** content was registered in the leaves preleved from the plantlets grown on V_4D variant, this results were higher with 136%, comparatively with V_4H variant, and higher with 138%, respectively with 132% in compare with control variant V_0H series, respectively control V_0D series, the result on V_4D variant being the highest in a chlorophyll content, comparatively with all species and varieties taken in study in all experiment (fig. 2).

Also at leaves level of *C. blumei Benth.* was registered the highest **b chlorophyll** content, in compare with the others *Coleus* species and varieties utilized as experimental models in our study, the best result was observed at leaves level on vitroplantlets regenerated from minicuttings derived from the V_4D series, its results were higher by 96% in compare with V_4H variant, and higher with 98%, respectively 92%, in compare with the control variants derived from V_0H and V_0D media (fig. 2).

On **carotenoids** content, the best result was registered also at vitroplantlets derived from D series, on V_4 D variant, at which was obtained higher carotenoid content with 139% comparatively with V_4H variant and higher by 141% and 135%, in compare with the control variants V_0H series, respectively V_0D series (fig. 3).

Table 3. The hyperhydricity degree of Coleus vitroplants, after 4 weeks of subculture on V_0 – V_4 experimental variants (step 3), noted with „+" if are much more, then the transparency degree is higher, and with „-" the hyperhydricity absence, V_0 medium without growth regulators; V_1 – MS – BM with 2,4 – D 2 mg/l; V_2 – MS – BM mixing up with BA 2 mg/l and NAA 1 mg/l; V_3 – MS – BM with BA 2 mg/l; V_4 – MS – BM with NAA 1 mg/l; position **H** represent plants resulted from cultures in double layer with 1.5% glucose solution prepared with distilled water which contains 150 ppm deuterium (the H series from the step 2), and **D** position, 1.5% glucose solution prepared with partially dedeuterised water with only 87.5ppm deuterium (the D series from the step 2); s = senescence.

Species / Variants	V_0		V_1		V_2		V_3		V_4	
	H	**D**	**H**	**D**	**H**	**D**	**H**	**D**	**H**	**D**
C. blumei Benth.	+++	+	s	s	+++	+	s	s	+++	-
C. black Dragon	+++ ++	+	s	s	+++ ++	-	s	s	++++	-
C. hybridus* var. *Jupiter	++	-	s	s	++	+	s	s	++	-
C. hybridus* var. *Ethna	+++ ++	+	s	s	+++ ++	+	s	s	+++ ++	-
C. hybridus* var. *Charteuse	s	+	s	s	s	s	s	s	+++ ++	-

The small content of all assimilatory pigments, in the leaves of vitroplantlets regenerated from minicuttings which were derived from H experimental series, is explainable, generally, by the presence of a high hyperhydricity process at stem and leaves level, at all H variants (V_0H – V_4H); the assimilatory pigments content in regenerated vitroplantlets from minicuttings derived from D series was probably, dependent by the nature of growth regulators present in the culture medium.

2. In the leaves of ***C. black Dragon*** vitroplantlets, a high content of **a chlorophyll** was observed at V_2D and V_4D variants; the best results were registered at V_2D variant, on the vitrocultures grown on a medium with a mixture between BA and NAA in the MS – basal medium, results which was higher by 99%, respectively by 71%, in compare with the result registered at the control V_0D and V_0H variant (fig. 2). This significant results show that the partially dedeuterised 1.5% glucose solution (87.5 ppm deuterium) induced a normal growth of the plantlets regenerated from the binodal minicuttings preleved from hyperhidric *C. black Dragon* vitroplantlets, which were subcultured on medium with BA 2 mg/l plus NAA 1 mg/l, plants which manifested a good pigment content, this species being the most greener plants.

At the D series the same hierarchy of the results was registered in **b chlorophyll** and **carotenoids** pigments content, at V_2D (MS – MB supplemented with BA 2 mg/l plus NAA 1 mg/l), and V_4D (MS – MB with NAA 1 mg/l) variants. Its results were higher with 63%, respectively 33%, comparatively with control variant (V_0) in b chlorophyll content (fig. 2); at V_2D variant and V_4D variants the content of carotenoids

content was higher with 171%, and with 143%, in compare with control variant (V_0H and V_0D variants, the percent values being equal – 100%) (fig. 3).

3. At the ***C. hybridus*** species level, the higher results and the most significant pigments content were observed at the vitroplantlets level regenerated on the V_4D series medium.

So, at ***C. hybridus var. Jupiter*** leaves and stems level the hyperhydricity was less aggressive, the transparency degree was lower comparatively with all *Coleus* species, and varieties, taken in our studies (table 2). The highest **a chlorophyll** content was registered at vitroplantlets level subcultured on V_4D variant, results higher by 89% in compare with the same parameter determined to V_4H variant, and higher by 89% and 24%, comparatively with the control variants V_0H and, V_0D (fig. 2).

The same hierarchy of results, at D series, were registered in **b chlorophyll** and **carotenoids** content in leaves; so, at V_4D variant, the results were higher with 68%, respectively 36%, in compare with the results obtained in b chlorophyll content at the control variant V_0H, respectively V_0D (fig. 2); the **carotenoids** content in the leaves at V_4D variant, was higher by 168% and 60% in compare with the results registered on the control variant V_0H, and respectively V_0D; at this varieties, on the V_4D variant, was measured the highest content of carotenoids, in comparison with all species and varieties taken in our study (fig. 3).

At the ***C. hybridus*** var***. Ethna*** leaves and stems level was observed the highest hyperhydricity degree, in comparison with all species and varieties taken in study in this experiment, which is the explanation, on its results, on its variety for the small pigment content in vitroplantlets leafs at D series, comparatively with the same parameter determined to the control variant – H series, which also stays in a hyperhydric state. So, the highest results in **a chlorophyll** content were registered at vitroplantlets regenerated on V_4D medium, the results were higher by 58%, respectively by 20%, in comparison with those obtained to the control variant V_0H, respectively V_0D (fig. 2), and higher with 58% and 39%, in comparison with V_2H variant, respectively V_2D variant (fig. 2).

The same hierarchy was registered in **b chlorophyll** and **carotenoids** content; in b chlorophyll content the result of V_4D variant was higher by 29%, respectively 10%, in compare with the control variant V_0H (results which dates values were considered as 100% in the percent calculations) and V_0D; the result to the V_4D variant was higher by 29%, respectively 14%, comparatively with V_2H and V_2D variants (fig. 2). At the **carotenoids** content, at V_4D variant the results were higher by 27% and 2% in compare with the control variant V_0H, and respectively V_0D, and in comparison with V_2H variant the results were higher by 27%, and by 17% for V_2D variant (fig. 3).

The most affected by hyperhydricity process are ***C. hybridus*** var***. Charteuse*** vitroplantlets, which minicuttings in subculture had survived only on V_0D, V_4 H and V_4D variants, this is why in the graphics are missing the other variants, which were senescent (fig. 2-3). The highest **a chlorophyll** content in leaves and stems was observed at the vitroplantlets generated on V_4D variant, result which was higher by 59%, in comparison with the same variant, but in H series (V_4H), and higher with 36% comparatively with the control V_0D variant (fig. 2).

The same hierarchy was registered in **b chlorophyll** and **carotenoids** content, in b chlorophyll content the result at V_4D variant was higher with 49%, respectively 31%, in comparison with the control variant at V_4H variant; respectively V_0D variant (fig. 2). The carotenoids level at V_4D variant was higher by 69% at V_4H variant and by 48% comparatively with V_0D (fig. 3).

The **total chlorophyll pigments** level (fig. 3) in *Coleus* species and varieties, in minicuttings subculture, show that the best result generally were observed at the leaves derived from the D series (which in step 2 supported a previous treatment of hyperhydric vitroplants with 1.5% glucose solution, containing only 87.5 ppm deuterium); the vitroplants inoculated on V_4D variant, medium with NAA 1 mg/l, to the majority of species and varieties. For example, of this parameter, on V_4D variant of *C. blumei Benth*, the result was higher with 125%, in comparison with those obtained at the V_0D variant; at *C. hybridus* var. *Jupiter* on V_4D the total pigments content percent was higher by 108%, for *C. hybridus* var. *Ethna*, at V_4D variant, the headway - in compare with those which were obtained to V_0H - was about 38% and at *C. hybridus* var. *Charteuse* was 59% at V_4D in compare with V_0H, with one exception - *C. black Dragon* – to which were obtained the best results in assimilatory pigment content, to V_2D variant – results higher by 99%, and respectively 17%, in comparison with V_0D and V_4D variants (fig. 3).

CONCLUSIONS

The 1.5% glucose solution, prepared with partially dedeuterised water (87.5ppm D) administered over hyperhydric vitrocultures of *Coleus*, in 9 weeks age, in subculture of *C. blumei Benth*., *C. black Dragon, C. hybridus* var. *Jupiter, C. hybridus* var. *Ethna, C. hybridus* var. *Charteuse,* annihilated the hyperhydricity in new generated shoots and leaves. So the shoots and leaves grown up to the partially dedeuterised glucose solution slowed the hypertrophyc growth, favoring the morphological recovery.

The most indicated nutritive medium for the subcultivation of binodal apex taken over from the recovered apexes, after vitrocultures treatment, with a 1.5% glucose solution, prepared with partially

dedeuterised water (87.5ppm deuterium) in double layer culture system: an agarised medium on the bases of the vitroplantlets and a second liquid layer up to its, was with NAA 1 mg/l addition, which induce the risogenesis to the majority of *Coleus* species and varieties taken in our study with one exception: *Coleus black Dragon*, which show the best results on MS basal medium, with BA 2 mg/l and NAA 1 mg/l addition.

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