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Changes in Nucleus, Nucleolus and Cell Size Accompanying Somatic Embryogenesis of *Theobroma cacao L.* 1. Relationship between DNA and Total Protein Content and Size of Nucleus, Nucleolus and Cell

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ABSTRACT

There was a linear relation between an increase in DNA content and size of nuclei, nucleoli and cells in callus and proembryos (*Theobroma cacao L.*). In callus the increase of DNA content was accopanied by proportional increase in nuclear size whereas in proembryos the increase in nuclear size did not match the increasing amount of DNA. The stimulation of embryogenesis by 10^{-2} mg/l 2,4-D was associated with increase in nuclear and nucleolar size and with decrease in cell sizes. Inhibition of embryogenesis by 1.0 mg/l 2,4-D+10% coconut water did not change nuclear size, but increased cell size in relation to the control.

The process of embryo formation was accompanied by changes in relationship between nuclear, nucleolar and cell size and the total (DNFB-stained) proteins content. In callus as well as in proembryo the increase in total protein content in nucleus was not equivalent to the increasing sizes of nuclei which leads to the decrease in nuclear protein concentration. Similar situation was observed for nucleoli. Differences were found in the concentration of cytoplasmic proteins between the callus and proembryo cells.

The stimulation of embryogenesis by low concentration of 2,4-D resulted in decrease in concentration of total proteins in nuclei and nucleoli and the increase in cytoplasm.

KONONOWICZ, H., JANICK, J.: *Zmiany rozmiarów jąder, jąderek i komórek towarzyszące somatycznej embriogenezie u Theobroma cacao L. I. Zależność pomiędzy zawartością DNA i całkowitą zawartością białek a rozmiarami jąder, jąderek i komórek*

W komórkach kalusa i proembrionów (*Theobroma cacao L.*) obserwowano liniową zależność pomiędzy zwiększeniem zawartości DNA a rozmiarami jąder, jąderek i komórek. W kalusie zwiększeniu zawartości DNA towarzyszyło proporcjonalne zwiększenie rozmiarów jąder, natomiast w proembrionach zwiększenie rozmiarów jąder nie dorównywało wzrastającej ilości DNA. Stymulacja embriogenezy pod wpływem

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Following abbreviations have been used: 2,4-D = dichlorophenoxyacetic acid; CW = coconut water; DNFB = Dinitrofluorobenzene; AU = arbitrary units.

10^{-2} mg/l 2,4-D była związana ze zwiększeniem rozmiarów jąder i jąderek oraz obniżeniem rozmiarów komórek. Hamowanie embriogenezy pod wpływem 1.0 mg/l 2,4-D + 10% CW nie powoduje zmian rozmiarów jąder, ale zwiększa rozmiar komórek w odniesieniu do kontroli.

Proces powstawiania embrionów z kalusa był związany ze zmianami zależności pomiędzy rozmiarami jąder, jąderek i komórek a całkowitą zawartością białek (barwiących się DNFB). W kalusie jak w proembrionach zwiększenie całkowitej ilości białek jądrowych nie było tak znaczne jak zwiększenie rozmiarów jąder, czego konsekwencją było obniżenie stężenia tych białek w jądrach. Podobną sytuację obserwowano w jąderkach. Stwierdzono także różnice w stężeniu białek cytoplazmatycznych pomiędzy komórkami kalusa i proembrionów.

Stymulacja embriogenezy pod wpływem niskich stężeń 2,4-D powodowała obniżenie całkowitej ilości białek jądrowych i jąderkowych oraz zwiększenie stężeń białek cytoplazmatycznych.

INTRODUCTION

DNA content is strongly correlated with cell and nuclear volume in plants and animals (1). Nuclear size is regarded as a useful parameter which reflect some nuclear changes during growth and differentiation. The size of the nucleus may change as a result of two different processes: 1) the normal progression of the cell through interphase, and 2) some differentiation events. CAVALIER-SMITH [2] suggested, that if developmental processes were highly sensitive to cell and nucleus sizes, different sizes will be optimal in different tissues. MICHEL and VAN DER PLOEG [8] observed a relationship between nuclear protein content, nuclear size, and tissue differentiation. The variation in nuclear size that has been reported in a number of systems could be due to the variation in major constituents such as DNA, RNA, or proteins.

In a previous paper [4] we found that somatic embryogenesis of cacao is associated with DNA endoreplication, and the increase of DNA template activity and RNA synthesis. Furthermore changes in total (DNFB-stained) protein content in nucleus, nucleolus, and cytoplasm were observed [5]. The aim of this study was to ascertain if there is a relationship between DNA and total protein content with nuclear, nucleolar, and cell sizes, during embryo formation from callus. We previously showed [3] that embryogenic-competent callus of cacao may be shifted towards homogeneous callus production without any evidence of embryogenesis with 1.0 mg/l 2,4-D plus 10% coconut water or to high frequency embryogenesis with 10^{-2} mg/l 2,4-D. The use of double staining technique; a combination of Feulgen for DNA with DNFB for total protein allows for DNA and total proteins quantification in the same cell [12].

MATERIAL AND METHODS

Culture media

Methods for maintenance of stock callus culture originating from somatic embryos were the same as described previously [10]. The basal medium consisted of MURASHIGE and SKOOG [9] salts (in mg/l):0.1 thiamine-HCl; 0.5 pyridoxine-HCl; 100 i-inositol; 0.5 nicotinic acid; 2.0 glycine; 1000 casein hydrolysate; 1500 sucrose; 800 bacto-agar (Difco). Basal medium was supplemented with 2,4-D at concentration either 10^{-2} mg/l or 1.0 mg/l + 100 ml/l coconut water (CW). Media were sterilized by autoclaving after adjusting pH to 5.7.

Plant material

Zygotic embryos of BC 5 clone spontaneously form callus through cotyledonary tissue when cultured on a hormone free medium [3]. The friable white-yellow callus was seperated from the cotyledonary tissue and subcultured on fresh medium. The stock callus was grown in glass jars (7 × 5.5 cm diameter) containing 15 ml basal medium, under low intensity illumination (25—100 μmol s^{-1} m^{-2}) from cool white fluorescent lamp per 19 h daily at 26° C.

Pieces of white-yellow callus 10—15 mm^3 were used to initiate experiments. After three weeks, two stages were distinguished under the stereoscopic microscope: white-yellow callus — stage I callus — and nodular bodies — stage II callus. Our previous histological data showed [3] that white-yellow callus was homogeneous and without any evidence of embryogenesis. On the other hand nodular bodies represented proembryos-early stage of embryogenesis. All experiments were performed on these two developmental stages.

Staining procedure

DNA and protein contents in nucleus, nucleolus and cytoplasm were estimated cytophotometrically — with Zeiss (Jena) histophotometer after staining according to Feulgen-DNFB method [7].

Tissue was stained at 65° C for 4 h in a solution of DNFB obtained by dissolving 0.74 g in 52 ml ethanol followed by the addition of 40 ml water and 8 ml 1N sodium bicarbonate. Unbound stain was removed by rinsing with 50% ethanol and the tissues were stained according to Feulgen procedure (1 h hydrolysis in 4N HCl at room temperature, Shiff's reagent prepared from pararosaniline).

Relative amounts of DNA per nucleus were measured at 560 nm, and nuclear, nucleolar, and cytoplasmic DNFB-stained proteins at 486 nm. Relative content of DNA was determined as an equivalent of 2 C in the thelophase nuclei and, as 4 C equivalent in the prophase nuclei. Nuclear, nucleolar, and cell dimensions were determined stereologically; 100 cells were measured for each experiment series.

RESULTS

Relationship between DNA content and nuclear size

A positive correlation was observed between nuclear DNA content and the sizes of nuclei in callus cells (Fig. 1:1A) and proembryos (Fig. 1:1B). The doubling in the amount of DNA was accompanied by a doubling in a nuclear sizes in callus. However, the increase in nuclear size in proembryos was not equal to the increasing amount of DNA, which was especially significant in cells of higher polyploidy level. For example: the average size of 4C DNA nuclei was 230 μm^2 and that of 8C nuclei only 325 μm^2. 2,4-D used with or without CW did not influence nuclear size in callus in any essential way (Figl: 2A, 3A). However, the cells of proembryos responded to the presence of 2,4-D by substantial increase in the size of polyploid nuclei (Fig. 1:2B).

Relationship between DNA content and nucleolus size

DNA content and nucleolar size were compared in callus and proembryo cells (Fig. 2: 1A, 1B, 2A, 2B). Changes in nucleolar size together with progressive polyploidy were similar in callus and proembryo cells. In both cases the increase in the size of nucleoli was lower than expected on the basis of the doubled amount of DNA. The sizes of the nucleoli in diploid callus and proembryo cells were similar (Fig 2: 1A, 1B). The 2, 4-D (with or

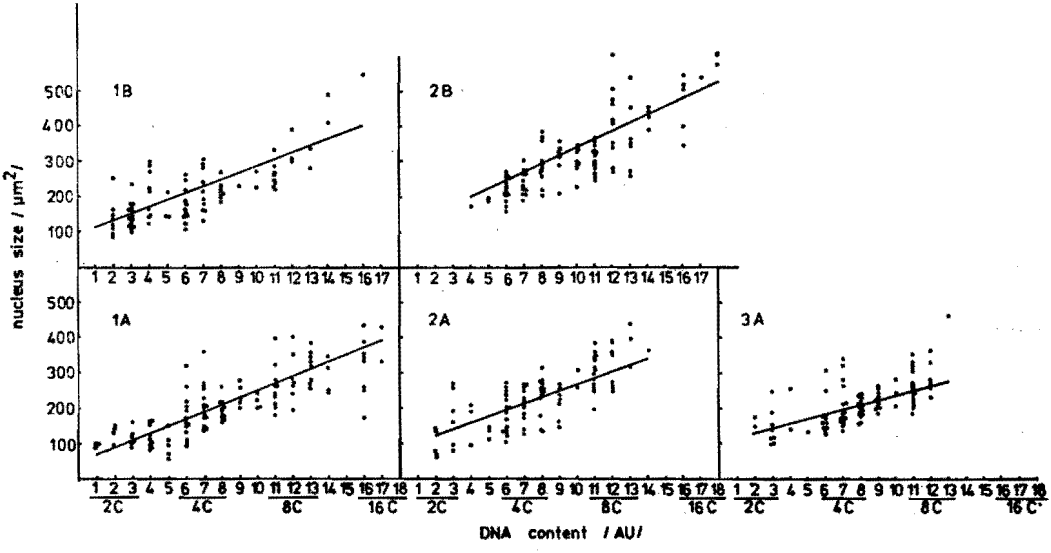

Fig. 1. The relationship between DNA content and nucleus size in callus and early stage of embryo. A = = callus, B = proembryos; 1 = control, 2 = after 10^{-2} mg/l 2,4-D treatment, 3 = after 1.0 mg/l 2,4-D+ +CW treatment. The appropriate regression equations and r^2 values as follows: A) control: $y = 48.66 + 20.11 \times (r^2 = 0.98)$; 2,4-D: $y = 86.14 + 17.99 \times (r^2 = 0.91)$; 2,4-D+CW: $y = 109.81 + 13.41 \times (r^2 = 0.92)$; B) control: $y = 93.41 + 19.20 \times (r^2 = 0.78)$, 2,4-D: $y = 109.81 + 23.40 \times (r^2 = 0.93)$.

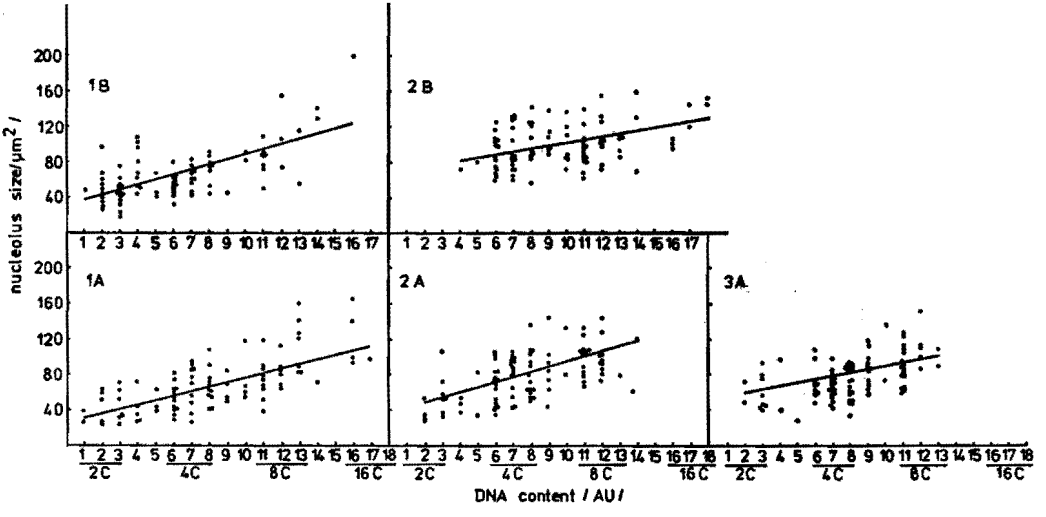

Fig. 2. The relationship between DNA content and nucleolus size in callus and early stage of embryo. A = = callus, B = proembryos; 1 = control, 2 = after 10^{-2} mg/l 2,2-D treatment, 3 = after 1.0 mg/l 2,4-D+CW treatment. The appropriate regression equations and r^2 values as follows: A) control: $y = 24.51 + 5.09 \times (r^2 = 0.9)$; 2,4-D: $y = 40.90 + 0.08 \times (r^2 = 0.91)$; 2,4-D+CW: $y = 51.71 + 3.87 \times (r^2 = 0.96)$. B) control $y = 31.6 + 5.71 \times (r^2 = 0.82)$; 2,4-D: $y = 66.58 + 3.51 \times (r^2 = 0.94)$.

without CW) caused an increase in nucleolar size in diploid and polyploid callus cells (Fig.; 2A, 3A). Similar increase in the sizes of nucleoli after 2, 4-D treatment was observed also in the cells of proembryos (Fig. 2: 2B).

Relationship between DNA content and cell size

The increase in size of endopolyploid cells of callus and proembryos was not equal to the increase in size of nuclei or nucleoli. Essential differences were detected between the 2C cells of callus and proembryos growing in control media. The 2C cells of proembryos were 2.5 times the size of 2C cells of callus (Fig. 3: A and B).

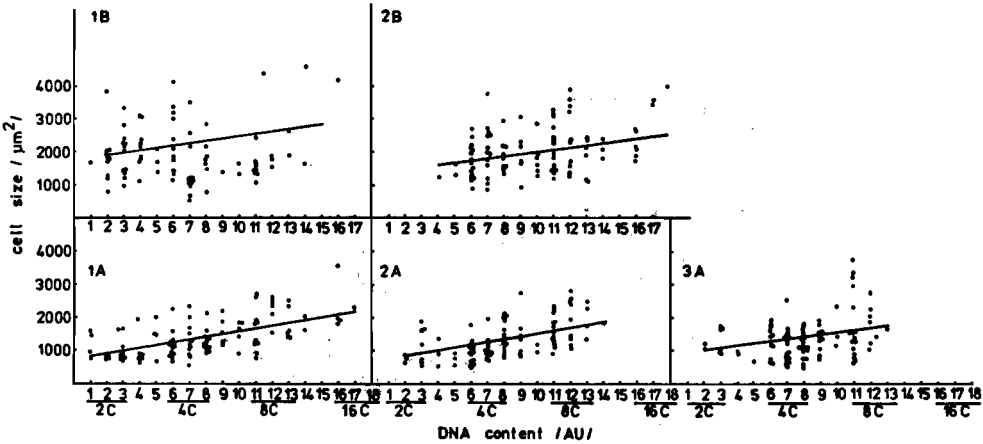

Fig. 3. The relationship between DNA content and cell size in callus and early stage of embryo. A = callus, B = proembryos; 1 = control, 2 = after 10^{-2} mg/l 2,4-D treatment, 3 = after 1.0 mg/l 2,4-D+CW treatment. The appropriate regression equations and r^2 values as follows: A) control: $y = 22.05 + 52.60 \times$ ($r^2 = 0.57$); 2,4-D: $y = 638.41 + 87.16 \times (r^2 = 0.80)$; 2,4-D+CW: $y = 906.1 + 68.60 \times (r^2 = 0.57)$. B) control: $y = 647.57 + 8.81 \times (r^2 = 0.97)$; 2,4-D: $y = 140.7 + 0.03 \times (r^2 \times 0.73)$.

2,4-D at 10^{-2} mg/l did not cause any changes in the size of callus cells as compared to the control (Fig. 3: 1A, 2A), though it evidently inhibited the growth of polyploid cells in proembryos (Fig. 3: 1B, 2B). For example: the average size of 8C DNA cells of proembryos growing on control media was 2550 µm² and 2100 µm² after 2,4-D treatment. Polyploid cells of homogeneous callus originated under the influence of 1.0 mg/l 2,4-D+CW were larger than callus cells growing on control media (Fig. 3: 1A, 3A). The average size of 8C cells growing in the control media was 800 µm² and 1050 µm² on media supplemented with 1.0 mg/l 2,4-D+CW.

Our data indicate that changes in cell size under the influence of 2,4-D were different from the changes in nuclear and nucleolar sizes. The sizes of nuclei and nucleoli increased, especially in proembryo cells whereas cells sizes remained the same or diminished as compared to the control.

Relationship between DNFB-stained nuclear protein content and nuclear size

Although there was a linear relation between DNFB-stained protein content and nuclear size (Fig. 4) the increase in protein content was not equal to the increase in the nuclear size. In cells of callus and proembryos the increase in nuclear sizes was larger than expected

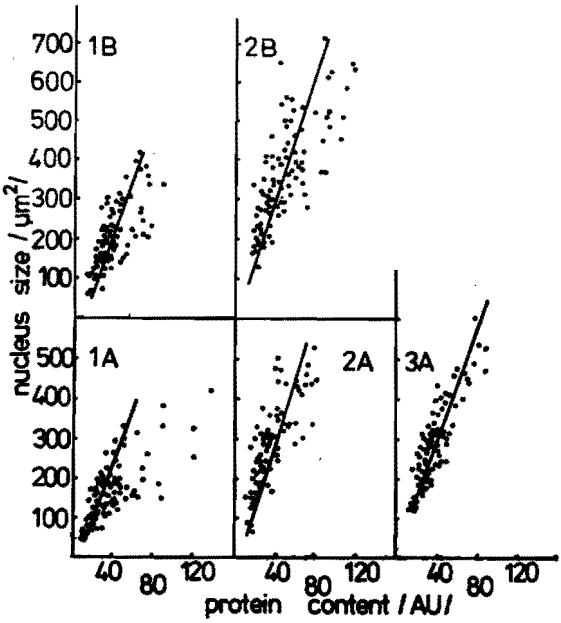

Fig. 4. The relationship between DNFB-stained nuclear protein content and nucleus size in callus and early stage of embryo. A = callus, B = proembryos; 1 = control, 2 = after 10^{-3} mg/l 2,4-D treatment, 3= = after 1.0 mg/l 2,4-D+CW treatment. The appropriate regression equations and r^2 values as follows: A) control: $y = 4.73+0.15\times(r^2 = 0.38)$; 2,4-D: $y = 3.66+0.12\times(r^2 = 0.25)$; 2,4-D+CW: $y =$ $= 1.31+0.14\times(r^2 = 0.59)$. B) control: $y = 9.44+0.15\times(r^2 = 0.46)$; 2,4-D: $y = 3.1+0.13\times(r^2 = 0.5)$

TABLE 1

Effect of the conditions stimulating (2,4-D) or inhibiting (2,4-D+CW) embryogenesis on the concentration (AU/μm²) of DNFB-stained nuclear proteins in callus and proembryos of *Theobroma cacao L.*

Treatment	Stage	Size of nuclei (μm²)			
		50	100	200	400
control	I	0.28	0.22	0.18	0.16
	II	0.36	0.24	0.20	0.17
10^{-3} mg/l 2,4-D	I	0.20	0.18	0.15	0.13
	II	0.18	0.16	0.14	0.13
1.0 mg/l 2,4-D+ 10% CW	I	—	0.12	0.14	0.14

on the basis of the increased in content of DNFB-stained protein (Fig. 4: 1A, 1B). In proembryos cultured on basal medium doubling in nucleus size from 100 to 200 μm² was accompanied by only 1.5 time increase in protein content. It led to the significant decrease in protein concentration in nuclei of larger sizes (Table 1).

There were no differences in the relation between protein content and nuclear sizes after treatment with 2,4-D alone or 2,4-D+CW (Fig. 4: 2A, 2B, 3A). Both treatments, however, slightly decreased nuclear protein content as compared with the same size nuclei of the control.

Relationship between DNFB-stained nucleolar protein content and nucleolus size

The relation between DNFB-stained protein content and nucleolar size is presented in Fig. 5. In callus and proembryos growing on control media the increase in protein content was accompanied by the increase in nucleolar size, but the increase in nucleolar size exceeded that of protein content (Fig. 5: 1A, 1B). As an effect nucleoli of a greater size were characterized by the relatively low protein concentration. For example: for nucleoli of 20 μm^2 protein concentration was determined to be 0.35 AU/μm^2, while for 160 μm^2 only 0.18 AU/μm^2 (Table 2).

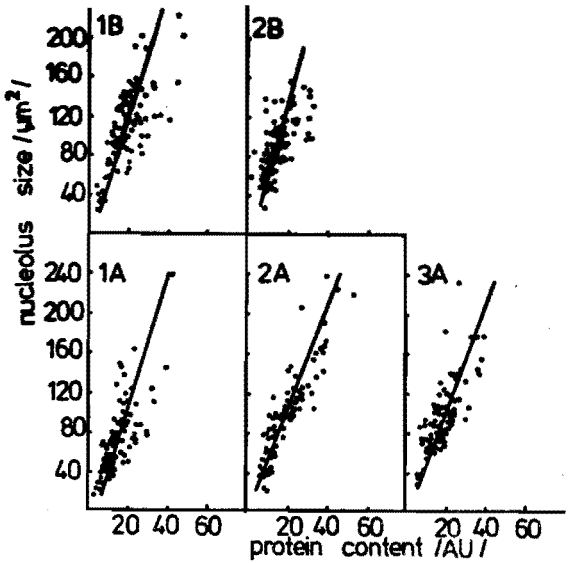

Fig. 5. The relationship between DNFB-stained nucleolar protein content and nucleolus size in callus and early stage of embryo. A = callus, B = proembryos; 1 = control, 2 = after 10^{-2} mg/l 2,4-D treatment, 3 = after 1.0 mg/l 2,4-D+CW treatment. The appropriate regression equations and r^2 values as follows: A) control: $y = 4.33+0.5\times(r^2 = 0.56)$; 2,4-D: $y = 0.11+0.20\times(r^2 = 0.76)$; 2,4-D+CW: $y = 1.09+$ $+0.19\times(r^2 = 0.59)$. B) control: $y = 3.14+0.16\times(r^2 = 0.52)$; 2,4-D: $y = 2.94+0.14\times(r^2 = 0.49)$.

TABLE 2

Effect of the conditions stimulating (2,4-D) or inhibiting (2,4-D+CW) embryogenesis on the concentration (AU/μm^2) of DNFB-stained nucleolar proteins in callus and proembryos of *Theobroma cacao L.*

Treatment	Stage	Nucleoli size (μm^2)			
		20	40	80	160
control	I	0.35	0.25	0.21	0.18
	II	0.30	0.22	0.20	0.18
10^{-2} mg/l	I	0.20	0.20	0.21	0.20
2,4-D	II	0.25	0.21	0.18	0.16
1.0 mg/l 2,4-D +10% CW	I	0.25	0.22	0.20	0.19

The stimulation of embryogenesis with 2,4-D was associated with substantial increase in nucleolar sizes, whereas the protein content increased slightly in the early stage of embryogenesis (Fig. 5: 2B). As an effect of that a decreased protein concentration in nucleoli was found (Table 2). The relation between the nucleolar protein content and nucleolar size did not change in response to 1.0 mg/l 2,4-D+CW (Fig. 5: 3A).

Relationship between DNFB-stained cytoplasmic protein content and cell size

The relation between DNFB-stained protein content and cell size in callus and proembryos is presented in Fig. 6: 1A, 1B. When cytoplasmic protein content was compared in cells of the same sizes both in callus and in proembryos, there was a marked decrease

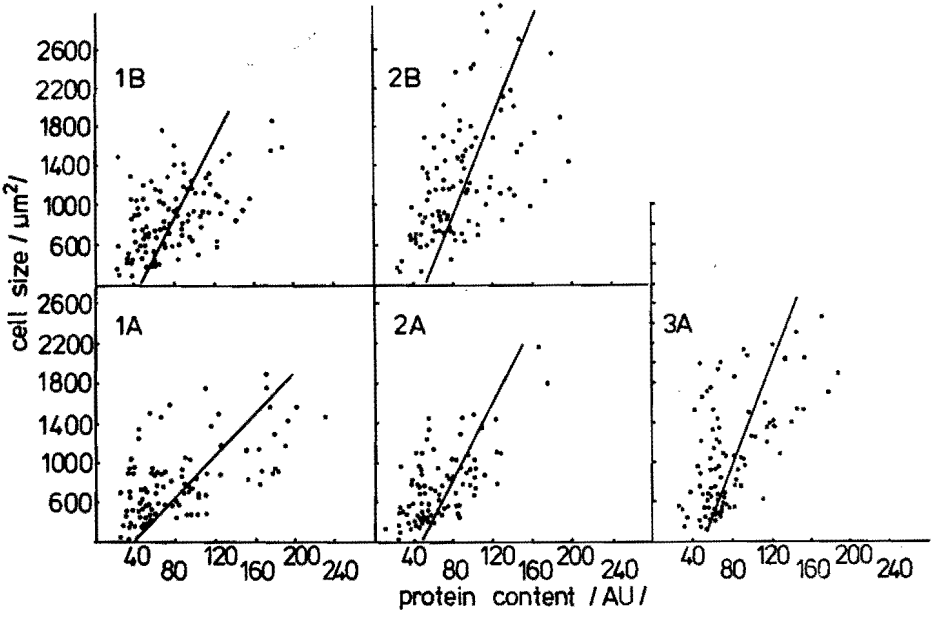

Fig. 6. The relationship between DNFB-stained cytoplasmic protein content and cell size in callus and early stage of embryo. A = callus, B = proembryos; 1 = control, 2 = after 10^{-2} mg/l 2,4-D treatment, 3 = after 1.0 mg/l 2,4-D+CW treatment. The appropriate regression equations and r^2 values as follows: A) control: $y = 2.13+0.09\times(r^2 = 0.28)$; 2,4-D: $Y = 37.7+0.05\times(r^2 = 0.11)$; 2,4-D+CW· $y = 45.57+$ $+0.04\times(r^2 = 0.26)$. B) control: $y = 36.22+0.05\times(r^2 = 0.22)$; 2,4-D: $y = 42.99+0.04\times(r^2 = 0.28)$.

in protein concentration in proembryos (Fig. 6: 1A, 1B) which intensified as cell size increased. For instance, in callus cells of 15000 µm² an average protein concentration was determined to be 0.107 AU/µm² while in proembryo cells of the same sizes 0.075 AU/µm² (Table 3). In both examined stages the increase in cell size led to the decrease in cytoplasmic protein concentration and this effect was especially distinct in proembryos (Table 3).

2,4-D increased cytoplasmic protein content less than cell size which resulted in a decreased cytoplasmic protein concentration as compared to the control. This effect was

TABLE 3

Effect of the conditions stimulating (2,4-D) or inhibiting (2,4-D+CW embryogenesis on the concentration (AU/μm^2) of DNFB-stained cytoplasmic proteins in cells of callus and proembryos of *Theobroma cacao L.*

Treatment	Stage	Cell size (μm^2)			
		500	1000	1500	2000
control	I	0.12	0.11	0.107	—
10^{2}-mg/l	II	0.12	0.088	0.075	—
2,4-D	I	0.12	0.088	0.075	0.071
1.0 mg/l	II	0.12	0.082	0.068	0.060
2.4-D	I	0.12	0.084	0.068	0.061
+10% CW					

especially evident in proembryos (Fig. 6: 2B, Table 3). It was also observed with 1.0 mg/l 2,4-D+CW (Fig. 6: 3A, Table 3) indicating that the increase in cell size is an auxin effect and is not associated with the process of embryogenesis.

DISCUSSION

Our studies indicated a close relation between DNA content and nuclear, nucleolar and cell sizes. This relation differed in callus and proembryos. In callus the increase in DNA content was followed by the proportional growth of the sizes of nuclei (Fig. 1: 1A), whereares in proembryos the growth of nuclei was not equal to the increasing amount of DNA (Fig. 1: 1B). Essential differences were evident in the sizes of 2C DNA cells. The sizes of 2C cells of proembryos were 2.5 times larger than those of analogouse callus cells (Fig. 3: A and B).

The stimulation of embryogenesis by 10^{-2} mg/l 2,4-D was accompanied by an increase in size of proembryo (Fig. 1: 2B) and nucleolar sizes of callus as well as proembryos (Fig. 2: 2A, 2B, 3A), wherease, cell size of proembryos decreased as compared to auxin-free (control) medium (Fig. 3: 1B, 2B). Inhibition of embryogenesis by 1,0 mg/l 2,4-D+CW did not influence nuclear size in homogenous callus (Fig. 1: 3A), but increased cell size in relation to the control (Fig. 3: 1A, 3A). These differences in nuclear size with constant DNA content, associated with callus and proembryos could reflect changes accompanying the transition from nonorganized growth of callus to embryo production.

There are many examples of the existance of the relation between DNA content and a nuclear size [1, 2]. CAVALIER-SMITH [2] suggested that DNA acted as a nucleoskeleton determining nuclear size and that larger cells required larger nuclei and more DNA.

A correlation between the size of cell, the size of nucleus and nuclear DNA content were observed in the cells of *Pisum arvense cotyledons* [11]. The progressive growth of the cells was followed by the increase in nuclear size, but this increase did not keep pace with the increasing sizes of cells.

In vascular tissue of *Pisum sativum* [8] although DNA endoreplication was observed this did not account for increase in nuclear size. There were many Gl nuclei much larger

than some G2 nuclei, and many G2 nuclei much larger than those of 4—8C DNA. These variations in size may be due to an increased hydration of nuclear material or to an increase in some non-DNA nuclear component eg proteins [8].

Our previous studies have demonstrated [4] that increasing concentration of 2,4-D lengthened the cell cycle in cacao callus. The question is whether the observed increase in nuclear size caused by 2,4-D is solely due to the lengthening of the cell cycle. Indeed, the lengthening of the cell cycle from 10 hours for the control to 14 hours by 10^{-2} mg/l 2,4-D was accompanied by an increase in nuclear size although DNA content was constant. However, further lenghening of cell cycle to 18 hours with higher concentration of 2,4-D+ +CW did not increase nuclear sizes in comparison with control. These facts suggest that the changes in nuclear size were not solely due to the lenthening of cell cycle but may be related to the changes connected with the process of embryo formation from callus.

The increase in the size of nuclei with the simultaneous diminishing of the cell size during the stimulation of embryogenesis caused remarkable changes in nucleoplasmic ratio. This ratio may be important in processes leading to the transition from nonorganised growth of callus to embryogenesis.

Our results indicate that embryo formation from callus in cacao involved changes in the relation between the sizes of nuclei, nucleoli, and cells with total protein content (DNFB-stained). A comparison of protein content and sizes of nuclei, nucleoli and cells permits an analysis of protein concentration.

The increase in total protein content was not equivalent to the increasing size of nuclei, which led to the decrease in nuclear protein concentration (Fig. 4). Because this effect was observed in callus as well as in proembryos it suggested that the process of embryo formation from callus did not involve any essential changes in concentrations of DNFB-stained nuclear proteins. Similar situation was observed for nucleoli. No differences were found between the increase in total protein content and the sizes of nucleoli in callus and proembryos (Fig. 5). In both cases the increase in nucleolar size exceeded that of protein content (Fig. 5: 1A, 1B). However significant differences appeared in the concentration of cytoplasmic proteins between cells of callus and proembryos. During embryos formation there was a marked decrease in the concentration of total proteins (Fig. 6: 1A, 1B).

The stimulation of embryogenesis with 10^{-2} mg/l 2,4-D slightly decreased nuclear concentration of total proteins (Fog. 4: 2A, 2B). The process embryogenesis intensification was accompanied by the increase in nucleolar size although total protein content remained constant (Fig. 5: 2B). This resulted in a decrease in concentration of proteins in nucleolus. During embryo formation from callus — in presence of 2,4-D- the increase in cytoplasmic protein content exceeded that of cell size, which accounted for an increase in concentration of cytoplasmic proteins (Fig. 6: 2B).

Our data suggest that DNA as well as proteins are responsible for the increase in nuclear size. However, the fact that the protein content may increase although DNA content remains constant, indicates that the proteins play a distinct role in this process.

During the process of embryos formation from callus, changes in the relation between sizes of nuclei, nucleoli and cell and total protein content have been found. These kind of changes were observed neither during the cell cycle nor during the growth of nonem-

bryogenic callus mass. This fact strongly suggests that changes in relative protein content in nuclei, nucleoli, and cytoplasm are associated with the process of embryogenesis.

In our next paper [6] studies on changes in basic proteins content in relation to nucleus, nucleolus, and cell sizes will be presented.

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