



FACTORS CONTROLLING PHENOL CONTENT ON *Theobroma cacao* CALLUS CULTURE

Factores que controlan el contenido de fenoles en el cultivo de callos de *Theobroma cacao*

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ABSTRACT. *Theobroma cacao* L. is known in folk medicine as an antiseptic, diuretic and antiparasitic. Foods derived from this plant are rich in natural products of high added value, including phenolic compounds. As *in vitro* cultivation handle is an alternative source for the production of these metabolites. The present study was conducted to obtain phenolic compounds from callus culture with embryogenic structures. Culture conditions (agitation, light and glucose) were established to increase the concentration of phenols in calluses and elicitors to achieve the increase in callus and excretion into the culture area. The accumulation of phenolic compounds was favored with the additional supplement of glucose, growth in agitation and darkness. The addition of random hydroxylated cyclodextrins allowed the increase in the specific yield of phenols and biomass.

RESUMEN. *Theobroma cacao* L. se conoce en la medicina popular como antiséptico, diurético y antiparasitario. Los alimentos derivados de esta planta son ricos en productos naturales de alto valor agregado, entre los que se destacan los compuestos fenólicos. Por lo que manejar su cultivo *in vitro* es una fuente alternativa para la producción de estos metabolitos. El presente estudio se realizó con el objetivo de obtener compuestos fenólicos a partir del cultivo de callos con estructuras embriogénicas. Se establecieron las condiciones de cultivo (agitación, luz y concentración de glucosa) para aumentar la concentración de fenoles en los callos y los elicitores para lograr su incremento en callos y excreción al medio de cultivo. La acumulación de compuestos fenólicos se favoreció con el suplemento adicional de glucosa, el crecimiento en agitación y a la oscuridad. La adición de ciclodextrinas hidroxipropiladas al azar permitió el incremento del rendimiento específico de fenoles y la biomasa.

Key words: cacao, *in vitro* culture, glucose

Palabras clave: cacao, cultivo *in vitro*, glucosa

INTRODUCTION

Plants constitute a significant source to isolate natural products, such as drugs, dyes, flavorings, foods, fragrances and other chemicals used in industry (1). Several of these compounds are derived from secondary metabolism, which plants do not need for their growth, but they do need them to interact with the surrounding environment. Secondary metabolites are grouped into four main classes: terpenes, glycosides, alkaloids and phenolic compounds, among which phenolics are very important for plant-environment interaction (2).

These compounds can be of natural complex and are associated with important activities; for example, antioxidant (3), antihypertensive (4), antitumour (5), insecticide (6), nematicide (7) and antimicrobial (8). Extracts from *Theobroma cacao* L. plants, which are rich in phenolic compounds, are used by people as antiseptic, diuretic and antiparasitic (9); therefore, their seeds and derivatives have been deeply studied in the last decade. Their physiological functions on human and animal health have been correlated with antimutagenic, antioxidant and antitumour activities, among others (5, 10, 11).

In the case of *T. cacao*, it is important to look for some alternatives, with the aim of obtaining phenolic compounds, so that plantations devoted

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for commercial products, such as chocolate, cocoa butter and other derivatives are not affected. In this sense, cell and tissue culture is a biotechnological choice studied for several years, so as to get natural products. So far, there are different production strategies through cell culture (callus and cell suspensions) (12).

Active products obtained from cell and tissue culture have a high potential for a large scale and long period production under controlled conditions (13). However, a large amount of plant material is often required (14); thus, different alternatives are developed to increase metabolite yield of *in vitro* plant culture, for instance, by improving crop conditions (15) and elicitation (16, 17).

Tissue culture has been studied in *T. cacao* since the last century and embryogenesis is among the alternatives mostly considered, which was initially described (18,19); up-to-date it is still worked on plant propagation (20). However, cell differentiation reached by embryo formation could also enable phenolic compound production. There are current results on the monitoring of phenol accumulation in *T. cacao* seeds and cell suspensions (12), as well as in embryogenic and non-embryogenic calli (21). But there is poor information about handling growing conditions for obtaining phenolic compounds in this crop.

Based on previous statements and taking into account the significance of phenolic compounds in *T. cacao* as well as the advantages of using *in vitro* culture techniques for these purposes, this research was aimed at evaluating the effect of different factors controlling the synthesis and excretion of phenolic compounds in callus culture with *T. cacao* embryogenic structures.

MATERIALS AND METHODS

The experimental work was carried out at the Metabolic Engineering, Cell and Tissue Culture Laboratories from Bioplant Center, Ciego de Ávila, Cuba, whereas plant material for *in vitro* culture was collected at the germplasm bank from Baracoa Cocoa Experimental Station, pertaining to the Agro-Forest Research Institute, Baracoa, Cuba.

Combined methodologies with modified glucose (166.52 mmol L⁻¹) and 2,4-D (2,4-dichlorophenoxyacetic acid, 18,096 µmol L⁻¹) concentrations was followed for callus formation with embryogenic structures, according to previous experimental results that are not yet shown (22, 23). UF677 clone staminoids, previously selected

for its phenolic compound content (results not shown), were disinfected with sodium hypochlorite at 1 % (v:v) for 20 min and later rinsed with plenty sterile distilled water. Plant material was placed in culture flasks containing 25 mL semi-solid medium (10 explants per flask).

For callus initiation, staminoids were put in a primary callus growth (PCG) medium (22), made up of DKW basal medium salts (24), supplemented with glucose (166,52 mmol L⁻¹), thiamine-HCl (2 mg L⁻¹), nicotinic acid (1 mg L⁻¹), glycine (2 mg L⁻¹), 2,4 D (18,096 µmol L⁻¹), TDZ (N-phenyl-N'-1,2,3-thiadiazol 5-ylurea, 0,023 µmol L⁻¹), L-glutamine (250 mg L⁻¹), myo-inositol (200 mg L⁻¹) and gelrite (2,5 g L⁻¹) (DUCHEFA) for 14 days. Later on, for callus proliferation, they were subcultured in a secondary callus growth (SCG) medium (22) modified by Maximova (23), made up of WPM basal medium salts (25), supplemented with glucose (166,52 mmol L⁻¹), thiamine-HCl (10 mg L⁻¹), nicotinic acid (1 mg L⁻¹), pyridoxine (1 mg L⁻¹), myo-inositol (100 mg L⁻¹), 2,4 D (18,096 µmol L⁻¹), 6-BA (6-benzyladenine; 0,222 µmol L⁻¹) and gelrite (2,5 g L⁻¹) (DUCHEFA) for 14 days.

Finally, they were placed in an embryo developing (ED) medium (22), made up of DKW basal medium salts, supplemented with sucrose (87,64 mmol L⁻¹), glucose (5,55 mmol L⁻¹), thiamine-HCl (2 mg L⁻¹), nicotinic acid (1 mg L⁻¹), glycine (2 mg L⁻¹) and myo-inositol (100 mg L⁻¹), where they were subcultured every 14 days until callus formation with embryogenic structures. Cultures were kept in the dark at a temperature of 25 ± 2 °C. Culture flasks consisted of glass containers of 250 mL capacity with twig-soft plastic caps for callus formation experiments and erlenmeyers of 250 mL for phenol production.

Phenolic compounds obtained by handling callus culture conditions with embryogenic structures

The effect of three factors (glucose concentration, light and stirring) was evaluated in an embryo developing liquid medium, in order to increase phenol content and callus biomass with embryogenic structures. Thus, different glucose concentrations (277.54, 222.03, 166.52, 111.02, 55.08 and 0.00 mmol L⁻¹) were tested, so as to determine its effect on phenol concentration and biomass increase. The experiment was carried out in an orbital platform shaker (RETOMED®) at 120 rpm with a temperature of 25 ± 2 °C and under dark conditions with erlenmeyers covered by black polyethylene.

Light effect was observed under two culture conditions: darkness with erlenmeyers covered by

black polyethylene and light with fluorescent white lamps (Sylvania, F40T12/D 40 W), which provided a photosynthetic photon stream of $60 \mu\text{mol m}^{-2}\text{s}^{-1}$, both at a temperature of $25 \pm 2 \text{ }^\circ\text{C}$ under shaking conditions and with $277.54 \text{ mmol L}^{-1}$ glucose.

Two cultural conditions were chosen to evaluate shaking effect: static and stirring in an orbital platform shaker (RETOMED®) at 120 rpm with a temperature of $25 \pm 2 \text{ }^\circ\text{C}$ under dark conditions, with erlenmeyers covered by black polyethylene and with $277.54 \text{ mmol L}^{-1}$ glucose.

Regarding the three experiments, 1 g calli was used per each treatment, besides evaluating three replicates. Soluble phenol concentration was determined in calluses at 0, 14 and 28 days. Dry mass increase was calculated per each treatment by subtracting the initial mass (0 d) from the final mass (28 d) and three determinations were made per replicate.

Influence of eliciting agents on phenol production and callus mass with embryogenic structures and their excretion to culture medium

A described methodology (15) was used, with the objective of stimulating phenol synthesis in calluses with embryogenic structures and their excretion to culture medium modified by employing Biojas® (Biojas, jasmonic acid, 1 g L^{-1} a.i.), obtained at Cuban Research Institute of Sugar Cane Derivatives (ICIDCA), instead of methyl jasmonate. The effect of three elicitors was evaluated: two cyclodextrins: random hydroxypropylated cyclodextrins (CDHA) and random dimethylated cyclodextrins (CDMA) and Biojas.

Then, 1 g calli with embryogenic structures was transferred to each erlenmeyer. Elicitation treatments were: control (without elicitor); Biojas $100 \mu\text{M}$; CDMA 50 mM ; CDHA 50 mM ; CDHA (50 mM)+Biojas ($100 \mu\text{M}$) and CDMA (50 mM)+Biojas ($100 \mu\text{M}$). Each erlenmeyer contained 25 mL modified ED liquid medium according to the above results.

The liquid culture medium with cyclodextrins was autoclaved at $120 \text{ }^\circ\text{C}$ for 20 min. Biojas was added at experimental initiation, sterilized by filtration with a $0.45 \mu\text{m}$ filter. Increased dry mass (DM) and phenol concentration in calluses with embryogenic structures and culture medium (mg g^{-1} MS) was determined seven days after applying elicitors. Three erlenmeyers were processed per treatment, each one considered as a replicate and three determinations were made per replicate.

Phenolic compound determination

Calluses were lyophilized and macerated until obtaining a fine powder to extract phenolic compounds. Samples were extracted in Soxhlet Büchi, according to a described procedure (26). Therefore, they were previously degreased with hexane (Merk) at $70 \text{ }^\circ\text{C}$

for 3 h (3 g in 30 mL), dried to remove hexane residues and extracted at the same time, temperature and methanol (Merk) ratio. This procedure was repeated three times.

Phenolic compounds excreted into culture medium were extracted by adding the same volume of cold ethyl acetate as culture medium and allowed to stir overnight in the dark. The organic phase was concentrated to dryness at $60 \text{ }^\circ\text{C}$ in a roto-evaporator (Rotadex, Heidolph 94200) and finally resuspended in 1 mL methanol.

Phenols were quantified through spectrophotometer (T70, UV/VIS spectrometer, PG Instruments Ltd., China) following a described procedure (27). Then, $900 \mu\text{L}$ distilled water and $100 \mu\text{L}$ Folin-Ciocalteu reagents were added to $100 \mu\text{L}$ plant extract. The mixture was allowed to settle for five minutes; later on, $600 \mu\text{L}$ of 1 mol L^{-1} saturated NaOH solution with Na_2CO_3 was added and incubated for 1 h at $25 \text{ }^\circ\text{C}$, to allow color development. Absorbances were measured by means of a spectrophotometer at 725 nm. Phenolic compound content was expressed in mg phenols per g dry mass (mg g^{-1} DM) referred to a standard curve of chlorogenic acid.

Statistical analysis

Results were statistically processed through the Statistical Package for Social Sciences (SPSS) version 20 for Windows (28). Parametric tests (T-test, one factor and bifactorial ANOVA) were performed. HSD Tukey tests were applied in those cases where ANOVA showed significant differences. It was previously shown that data of each treatment fulfilled the assumptions of normal distribution and variance homogeneity, according to Kolmogorov-Smirnov and Levene tests for $p \leq 0.05$ respectively. Statistical treatment details appear in each figure or table of the Results and Discussion.

RESULTS AND DISCUSSION

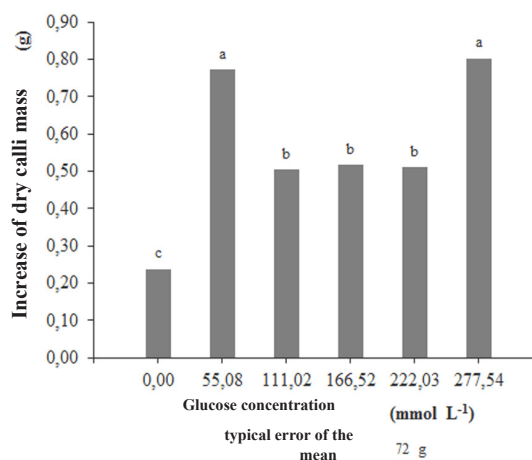
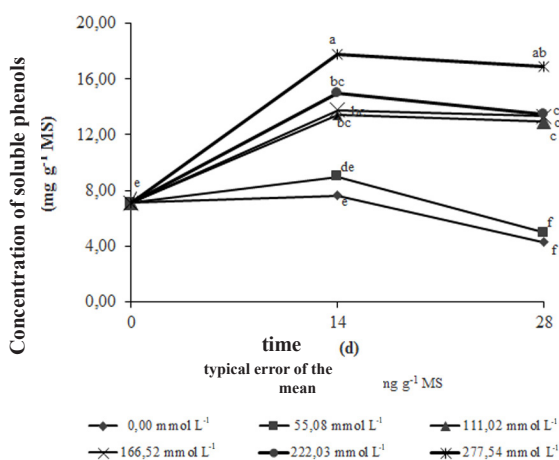
Results about the effect of glucose concentration (Figure 1), light (Figure 2) and stirring (Figure 3) on soluble phenol concentration (A) and increased dry mass of calluses with embryogenic structures (B) are illustrated.

Soluble phenol concentration (Figure 1A) reached a top value (17.79 and 16.87 mg g^{-1} DM) at 14 and 28 days, for the highest glucose concentration treatment ($277.54 \text{ mmol L}^{-1}$), without significant differences at equal times with $222.03 \text{ mmol L}^{-1}$ sugar. Meanwhile phenol concentration was low at $55.08 \text{ mmol L}^{-1}$, without differences with the control. Besides, mass increase was favored by the highest glucose concentration ($277.54 \text{ mmol L}^{-1}$), without differences with the lowest one ($55.08 \text{ mmol L}^{-1}$),

0.3 g above the other treatments and 0,56 g higher than the control. These results may be related to the osmotic stress caused by high sugar concentrations, which could enhance phenolic compound synthesis and accumulation. However, increased mass can be associated either with a higher phenol synthesis or embryo differentiation at the highest glucose treatment. This behavior was not the same with the lowest concentration, where increased mass did not fit to a higher embryo differentiation.

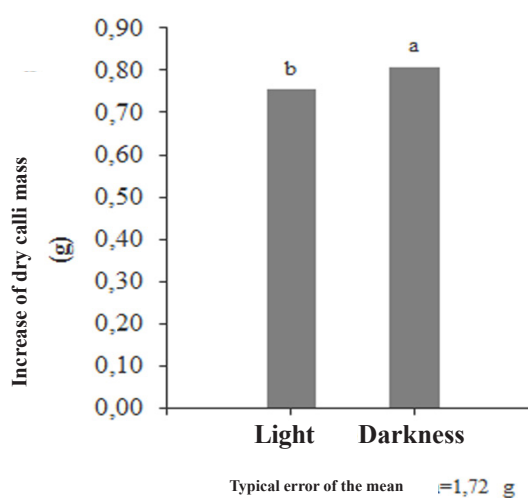
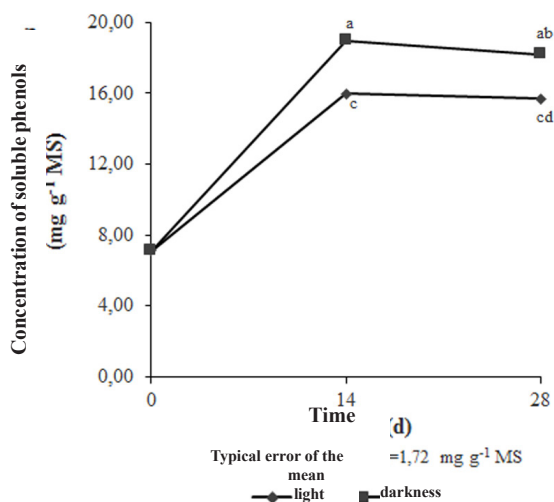
Some phenol production is closely related to sugars (29). Its synthesis improves by employing glucose during *in vitro* culture of *Vitis vinifera* L.,

at concentrations of 58,00; 234.00 and 468,00 mmol L⁻¹ (30). What was associated to these sugars change structural and regulatory gene expression, especially UDP-glucose: anthocyanidin 3-O-glucosyltransferase, together with a massive reprogramming in transduction signal pathways. This gene was correlated with anthocyanin content in *Vitis vinifera* L. fruits (31). It was proved that the synthesis of anthocyanins and some flavonoids is favored by increased glucose (30). Thus, the highest phenolic compounds obtained in this experiment (Figure 1A) could be related to the synthesis of different phenols, such as anthocyanins and flavonoids.



A: Bifactorial ANOVA B: Simple ANOVA Tukey, p≤0,05, n=9 Means with unequal letters differ significantly

Figure 1. Glucose concentration (277.54, 222.03, 166.52, 111.02, 55.08 and 0.00 mmol L⁻¹) effect on soluble phenol concentration (mg g⁻¹ DM) (A) and increased dry mass of calli with embryogenic structures (g) (B)



A: Bifactorial ANOVA B: T-test Tukey, p≤0,05, n=9 Means with unequal letters differ significantly

Figure 2. Light (277.54, 222.03, 166.52, 111.02, 55.08 and 0.00 mmol L⁻¹) effect on soluble phenol concentration (mg g⁻¹ DM) (A) and increased dry mass of calli with embryogenic structures (g) (B)

Soluble phenol concentration (Figure 2A) and increased dry mass of calluses (Figure 2B) were higher under dark conditions compared to light. Phenol concentration reached a top value (18.97 mg g⁻¹ DM) at 14 days and remained without significant differences at 28 days (18.21 mg g⁻¹ DM). Increased dry mass of calluses cultivated in the dark was slightly higher (0.05 g) than those in the light; however, such mass difference contributed to phenol concentration difference.

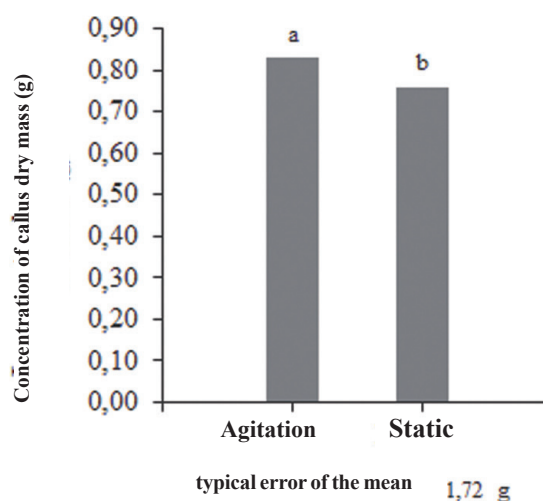
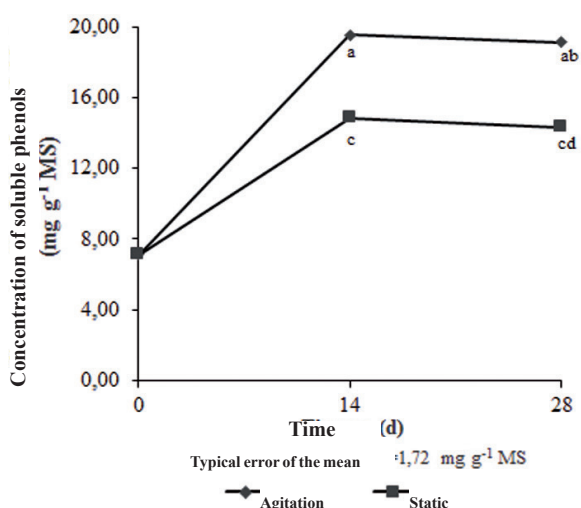
Light effect on tissue and embryogenic structure development has been deeply studied in recent years, as for instance, light influence on crop embryogenesis of *Campanula punctata* var. *rubriflora* (32) and *Agave tequilana* var. *Blue* (33). Nevertheless, there are few studies about light influence on secondary metabolite synthesis. In this sense, it is known that light absence may provoke stressed crops; thereby, some secondary metabolite synthesis is activated. For example, furanocoumarin production is stimulated in the dark during *in vitro* culture of *Ruta graveolens* ssp. *divaricata* outgrowth (34); however, phenolic acid synthesis was encouraged under white and blue light conditions. Regarding soluble phenol concentration in *T. cacao* calluses, the best results were observed under dark conditions, but phenolic compounds were also obtained under light conditions, indicating that different groups of phenolic compounds could have been accumulated under each condition, which should be proved in future investigations.

When analyzing stirring effect on soluble phenol concentration (Figure 3A) and increased dry mass of calluses (Figure 3B), it was found that shaking had a positive influence upon both parameters. Phenol concentration was higher when stirring at 14 days (19.57 mg g⁻¹ DM), without significant differences at 28 days (19.13 mg g⁻¹ DM), besides increasing dry mass at 28 days, which exceeded static culture in 0.1 g. Shaking is a factor that may also influence phenol production.

Phenols were present at *in vitro* culture of *Manguifera indica* L. embryos under static and shaking conditions (35), the latter favoring somatic embryo growth without being affected by toxic compounds, such as some phenols.

Figure 3 shows that the highest phenol concentration did not affect increased mass at the shaking treatment, which is also observed in Figure 4, where calluses with embryogenic structures are observed (mainly globular embryos) (A, B and C), as well as different stages of embryo formation: heart-shaped (D), torpedo (E) and cotyledonar (F) at 28 days of stirring, in the dark and with glucose at 50 g L⁻¹.

In short, culture conditions play a key role, not only for *in vitro* culture establishment, but also for metabolite production. Carbon source, light and shaking are the factors evaluated in this work.



A: Bifactorial ANOVA

B: T-test

Tukey, p≤0,05, n=9

Means with unequal letters differ significantly

Figure 3. Shaking (277.54, 222.03, 166.52, 111.02, 55.08 and 0.00 mmol L⁻¹) effect on soluble phenol concentration (mg g⁻¹ DM) (A) and increased mass of calli with embryogenic structures (g) (B)

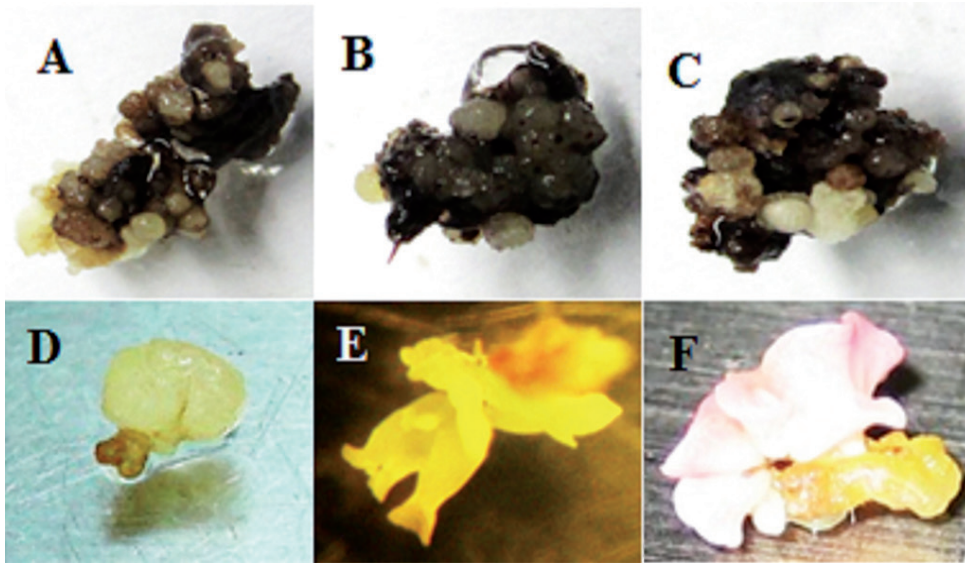


Figure 4. Calluses with embryogenic structures (mainly globular embryos) (A, B and C) and different stages of embryo formation: heart-shaped (D), torpedo (E) and cotyledonar (F) at 28 days of stirring, in the dark and with glucose at 50 g L⁻¹

The best results were obtained for 50 g L⁻¹ glucose, in the dark and with shaking. These treatments increased both dry mass and phenol content. However, only phenol content of calluses has increased so far; therefore, different elicitors were tested to stimulate their excretion to culture medium.

Results of elicitation treatment effect, increased dry mass and soluble phenol concentration in calluses with embryogenic structures of *T. cacao* L. are shown in Figures 5 and 6, respectively. All elicitors increased dry mass of calluses (Figure 5) compared to the control. CDMA + Biojas combination was the greatest promoter of increased mass in calluses with embryogenic structures 1.83 times than the control, whereas CDHA was the lowest promoter of increased mass with only 1.22 times the control value.

Elicitor effect on phenol production was positive during callus culture with embryogenic structures of *T. cacao* (Figure 6). Phenol concentration in calluses with embryogenic structures increased in all cases, except for Biojas treatment that maintained such concentration below the control.

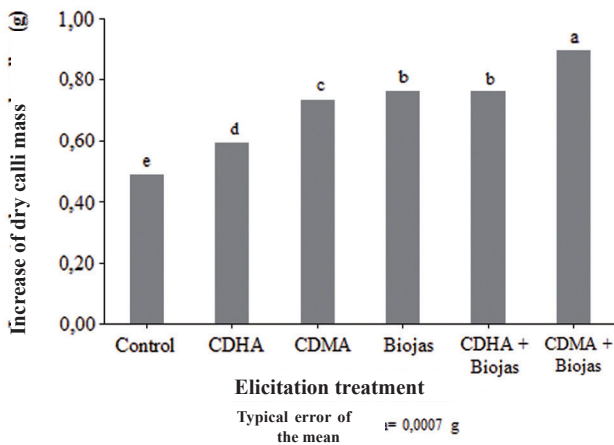
The highest phenol concentration in calluses and culture medium was obtained by CDHA treatment (18.50 and 4.82 mg g⁻¹ DM respectively).

Nevertheless, phenol excretion to culture medium did not show any significant differences with CDHA + Biojas (4.05 mg g⁻¹ DM). The lowest values were recorded in the control and Biojas; therefore, under tested elicitation conditions, CDHA treatment showed the best result alone and combined with Biojas.

Elicitor addition is a common practice to increase secondary metabolite content that have low or null concentration at *in vitro* culture compared to intact plants (14). Cyclodextrins have proved to provide a strong eliciting activity on cell cultures of *V. vinifera* for resveratrol production; this activity is only achieved with cyclodextrins and its combination with methyljasmonate (36, 37).

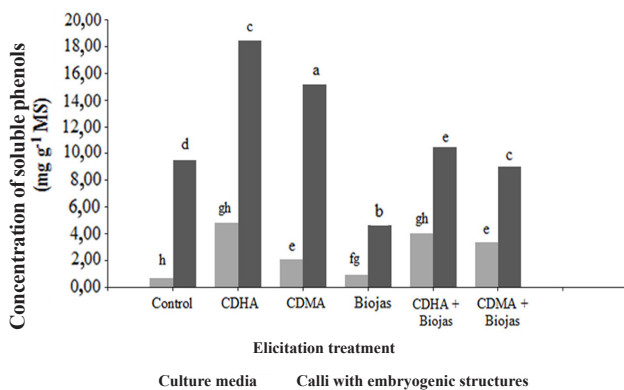
In *Silybum marianum* L. cell suspensions, methyljasmonate, CDHA and CDMA cyclodextrins encouraged the synthesis and excretion to culture medium of flavolignans and coniferyl alcohol (15); the best results were obtained by combining CDMA at 50 mmol L⁻¹ + methyljasmonate 100 μmol L⁻¹. However, in callus culture with embryogenic structures of *T. cacao* (Figure 6), there was a greater phenolic compound excretion to culture medium with CDHA 50 mmol L⁻¹ treatment, without differences with CDHA at 50 mmol L⁻¹ + Biojas 100 μmol L⁻¹.

This research work proves that phenolic compound accumulation in calli was favored by the additional glucose supplement, shaking growth and darkness.



Means with unequal letters differ significantly (Simple ANOVA, Tukey, $p \leq 0,05$, $n=9$)
 Control (without elicitor); Biojas $100 \mu\text{mol L}^{-1}$; CDHA 50 mmol L^{-1} ; CDMA 50 mmol L^{-1} ; CDHA (50 mmol L^{-1}) + Biojas ($100 \mu\text{mol L}^{-1}$); CDMA (50 mmol L^{-1}) + Biojas ($100 \mu\text{mol L}^{-1}$)

Figure 5. Elicitor effect on increased dry mass (g) in calluses with embryogenic structures of *T. cacao* L.



Means with unequal letters differ significantly (Bifactorial ANOVA, Tukey, $p \leq 0,05$, $n=9$)
 Control (without elicitor); Biojas $100 \mu\text{mol L}^{-1}$; CDHA 50 mmol L^{-1} ; CDMA 50 mmol L^{-1} ; CDHA (50 mmol L^{-1}) + Biojas ($100 \mu\text{mol L}^{-1}$); CDMA (50 mmol L^{-1}) + Biojas ($100 \mu\text{mol L}^{-1}$)

Figure 6. Elicitor effect on soluble phenol concentration ($\text{mg g}^{-1} \text{ DM}$) in calluses with embryogenic structures of *T. cacao* L. and excreted to culture medium

CONCLUSIONS

◆ The use of elicitors caused a significant phenolic compound increase and the values obtained in calluses ($9.02\text{-}18.50 \text{ mg g}^{-1} \text{ DM}$) with all treatments, except Biojas, exceeded those obtained in previous studies (data not shown) for flowers of plants under natural environment ($7.44 \text{ mg g}^{-1} \text{ DM}$) and close to leaves ($19.98 \text{ mg g}^{-1} \text{ DM}$).

◆ The excretion to culture medium remained below the one recorded in extracts of plant organs under natural environment and it is yet unknown which phenolic compounds are present in each case.

◆ For future research works, it is necessary to improve phenol excretion levels to culture medium, as well as to identify its chemical composition.

ACKNOWLEDGEMENTS

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Note:

During the editing process it was not possible to access the work of retouching and improvement of images, so they have been inserted with the same quality as the ones sent by their authors.

The editorial