Glutamate Dehydrogenase and Glutamine Synthetase Activities during Somatic Embryogenesis in *Theobroma cacao* L.

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ABSTRACT

The behaviour of staminodes and petals from unopened *Theobroma Cacao* L. flowers was studied in presence of different phytohormon ratios (2.4.D/BAP). Protein contents, glutamate dehydrogenase and glutamine synthetase activities were evaluated in different morphogenetic structures induced by growth regulators. The highest callogenenis rate was observed in the medium containing 1/1 mg.l⁻¹ (2.4-D/BAP) ratio. The appearance of embryogenics structures occured mostly in the medium containing 1/1 mg.l⁻¹ (2.4-D/BAP). The highest frequency of calli forming somatic embryos (27.1%) was obtained with the clone SNK413. The embryos formation was associated with proteins accumulation in calli. It was equally observed that glutamate dehydrogenase and glutamine synthetase activities were positively correlated to protein content. In SNK413 (which shows the highest frequency of somatic embryos) the induction of these structures is characterized by the appearance of 3 bands P5 (32.7kDa), P6 (26.1 kDa) and P7 (20.6 kDa) as revealed by poly acrylamide gel electrophoresis. These results show the influence of genotype, culture medium and morphogenetic process (somatic embryogensis) on protein distribution.

Keys words: Glutamate dehydrogenase, glutamine synthetase, embryogenesis, *Theobroma cacao* L., staminodes, petals.

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INTRODUCTION

In the process of nitrogen assimilation in higher plants, ammonium ions are incorporated to oxoglutaric acid to form glutamic acid by glutamate dehydrogenese (E.C1.4.1.2.) (GDH). Subsequently glutamate is bound to a molecule of ammonium and forms glutamine by glutamine synthetase (EC 6.3.1.2.). In *Zea mays* calli glutamate dehydrogenese has shown to be and indicator of embryogenic activity (Rao *et al.*, 1990).

It seems that glutamine synthetase (GS) is a limiting factor to supply nitrogen compounds that are required for the induction and development of somatic embryos in some species such as carrot (Doderman, *et al.*,1998). *Theobroma cocao* L., the chocolate tree is mainly propagated by seeds. As consequence of this and the high heterozygosity of the crop, wide genetic variation is observed among seed-derived plants, resulting in large proportion of low-yielding trees. Therefore, an efficient protocol for somatic embryogenesis of cocoa would be of great use for breeding, multiplication and distribution of elite genotypes. However, somatic embryogenesis is limited by genotypic variability and low plant conversion rates. In addition, results are inadequate for mass propagation of elite material and genetic transformation for pest resistance. One of the possible ways of improving somatic embryogensis is through the modification of the culture media, plant growth regulation being undoubtedly a determining factor (Collin and Sue, 1998). The identification of proteins closely associated with somatic embryogenesis in *Cocos nucifera* (Ilas-Flores *et al.*, 2000) or *Daucus carota* (Dodeman *et al.*, 2000) has improved this process. Thus, an understanding of the biochemical and molecular determinants of the somatic embryogenesis of cocoa would contribute in improving this process in cocoa.

In this paper, we study the influence of the growth regulator (2, 4-D/BAP) ratios on cocoa somatic embryogenesis induction. In addition, the variation of protein content, glutamate dehydrogenase and glutamine synthetase activities have shed some light on the nature of the compounds governing somatic embryogenesis in *Theobroma cocoa* L.

MATERIALS AND METHODS

Explant material, disinfection and culture media

Tissue culture experiments were conducted on staminodes and petals of immature flower buds, from four different cocoa genotypes (SNK10, SNK64, SNK413 and SCA-6), collected at IRAD (Institut de Recherche Agricole pour le Développement) of Nkolbisson in Yaounde. Immature flowers were harvested in the morning, surface-sterilised by immersion in: 30% (v/v) mercurobutol for 1 hour, 4% (w/v) sodium hypochlorite for 15 minutes, 0,1 % (w/v) mercuric chloride for 2 minutes, and then rinsed two times with sterile water. Culture and subculture media were prepared from the basal medium containing DKW macro and micro-elements (Driver and Kuniyuki, 1984), Morel and Wetmore (1951) vitamin, amino acids, organic acids (malic acid and succinic acid) and agar- agar 0,5 % (w/v). In this basal medium, three ratios (2/0.5, 1/1, 2/2 mg.l^{-1}) of growth regulators (2,4 –D/BAP), were added and tested. All cultured media were adjusted at pH 5,8 with 1M KOH or 0.10M HCl and autoclaved at 115°C for 30 minutes.

Culture, subculture and growth conditions

Surface sterilized flowers were sliced. Staminodes and petals extracted and placed in culture media and incubated in dark at 25± 1°C. After 14 days incubation, explants were transferred in the same medium and in the same conditions. 14 days later, a second subculture was made in a free growth regulator medium in the dark at 25°C. At the 42nd day of culture, we used the same medium where maltose (30 mg.l^{-1}) substituted to sucrose/glucose/fructose (60/10/5 g.l^{-1}) combination; cultures were then placed under 16/8 hour photoperiod at 25±°C. After 30 days (72nd day of culture), a fourth subculture was in made the same medium but maltose (30 g.l^{-1}) was changed into maltose/glucose (20/10 g.l^{-1}). Subcultures were identically, made at 90th and 102nd day of culture as described in 72nd days. Samples of induced morphogenetics structures were harvested, weighted and kept at –4°C for biochemical analysis at 28th, 42nd, 72nd, 90th and 102nd day. Callogenic explants are also evaluated at day 28 (D28).

Proteins extraction

Protein extraction was done according to the method of Gallerdo *et al.*(1988). Morphogenetic structures were weighed and immediately homogenised in buffer (1g/3ml) containing: 2 mM EDTA, 10 mM β-mercaptoethanol, 10 % (v/v) glycerol and 50 mM Tris-HCl (pH 8,0). Homogenates were centrifuged at 22 000g for 30 minutes. Supernatants obtained were used for subsequent analysis.

Table I: Behavior of explants in callogenic media

Media	Clones	Total explants	callogenic explants	Percentage of callogenic explants	Size of calli
I	SNK10	97	84	87,2	+++
	SNK 64	207	199	96	++++
	SNK 413	149	118	78,7	++++
	SCA-6	110	91	82	+++
II	SNK10	372	343	92,2	++++
	SNK64	449	441	98,3	++++
	SNK413	108	96	88,4	++++
	SCA-6	64	51	79,68	++++
III	SNK10	81	65	80	+++
	SNK64	209	158	75,5	+++
	SNK413	97	84	86,5	+++
	SCA-6	60	60	100	+++

Protein contents, glutamate dehydrogenase and glutamine synthetase activities determination and electrophoresis analysis.

Protein contents were determined by the Bradford's (1976) method using bovine serum albumin as standard. The glutamate dehydrogenase activities were determined by measuring the reduction of NAD^+ at 340 nm according to Bulen's method (1956). Glutamine synthetase activities were measured using the method described by Shapiro and Standman (1976). Crude extracts from the morphogenetic structures samples (after 72 days of culture) were electrophoresed on fully denaturing 10% SDS/poly acrylamide.

Data analysis

Correlations between protein content, glutamate dehydrogenase and glutamine synthetase activities were studied using "SPSS statistical package, release 8.0" for Windows. (Spearman correlation)

RESULTS

Effects of growth regulators on morphogenesis

Staminodes and petals explants from immature flowers of four *Theobroma Cocoa* L. genotypes (SNK10, SNK64, SNK413 and SCA-6) were cultured in induction media containing three 2,4-D/BAP ratios: 2/0.5 mg.l^{-1} (medium I), 1/1 mg.l^{-1} (medium II) and 2/2 mg.l^{-1} (medium III). Callus induction was evident within 14 days after culture initiation (Fig. 1A) but with a limited rate. On 28th day, calli are well developed over the entire explants. They were compact, clustered, globular and whitish (Fig. 1B). The evolution of callogenic explants shows a profile depending on the genotype and culture medium. The highest frequency of callogenic explants was obtained in medium II. Thus, the average percentage of explants forming calli is 92, 98, 89 and 79 with respect to SNK10, SNK64, SNK413 and SCA-6 genotypes (Table I). When calli (28 days old) were transferred into expression medium (free-growth regulation medium), all SCA-6 genotype calli necrosed, whereas those of the other genotypes developed embryogenic structures within the 42nd and 90th day. These embryogenic were yellowish, globular, heart-shape or torpedo-shaped (Fig. 1C). The percentage of calli forming embryo and the number of embryos per callus depended on the culture medium, genotype and

Table II: Behavior of calli in expression medium

Media	Clones	Ages (days)	Pourcentage embryogenic calli	Number embryos per callus	Development stages of embryos
I	SNK 10	42	13,56	2-3	Globular
	SNK 64	72	15,3	3	Globular
		90	19,4	4	Globular
	SNK 413	42	11,2	2	Globular and heart shape
II	SNK 10	72	14,3	1-2	Globular, heart shape and cotylédonary
	SNK 64	72	13,1	2-3	Globular and heart shape
	SNK 13	42	3	1	Globular
		72	27,13	4-5	Globular and heart shape
III	SNK 10	72	14,6	2	Globular
		90	7,3	1-2	Heart shape

the age of calli (Table II). In the medium I, embryogenic structure were evident from the 42nd day in SNK10 (13.56%) and SNK413 (11.2%) genotypes; whereas in SNK64, somatic embryos appeared later but with higher frequency: 15.3% (D72) and 19.4% (D90). When calli were from medium II, three genotypes (SNK10, SNK64 and SNK413) presented embryogenic structure at D72; SNK413 having the high-

est percentage (27.13) of calli developing embryos. In medium III, embryogenic expression was seen only in SNK10 genotype at D72 (14.6%) and D90 (7.3%).

Protein contents

Protein contents of morphogenetic structures from the three medium (I, II, III) of three genotypes (SNK10, SNK64 and SNK413) have been measured (Fig. 2). When calli were from medium I, the high-
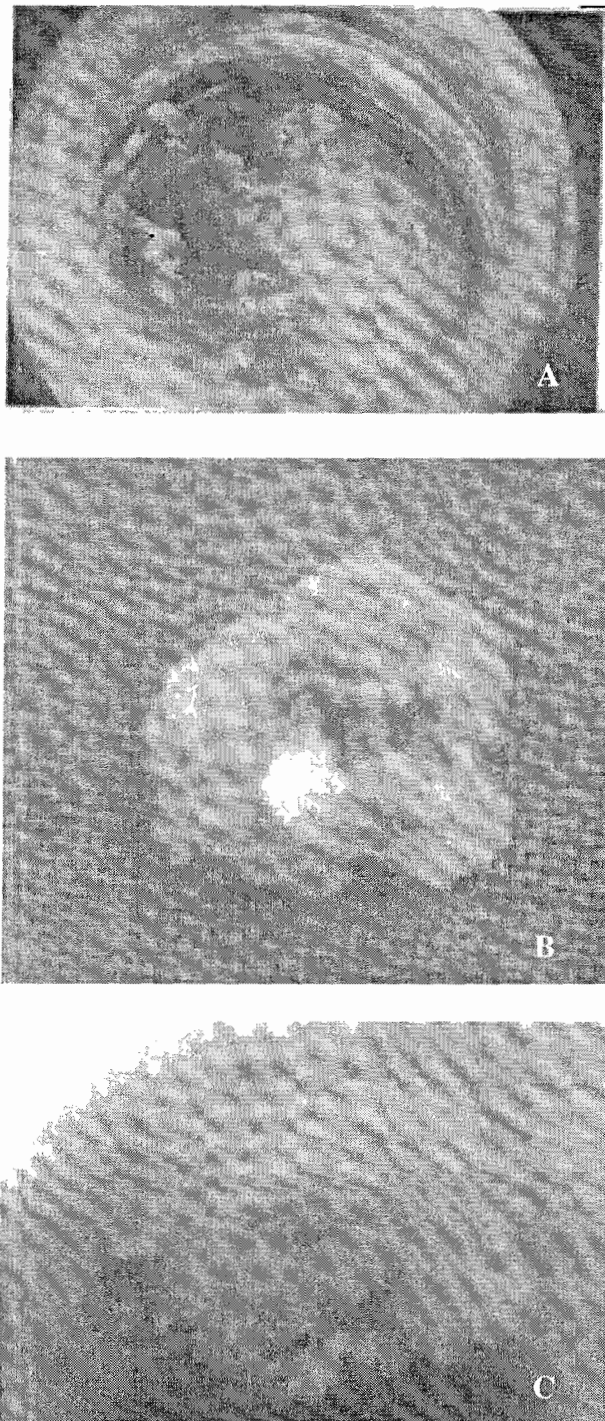
Figure 1: Different stages of morphogenic structures. A : calli at 14 days culture; B: calli at 28 days culture C: embryogenic calli (72 days)
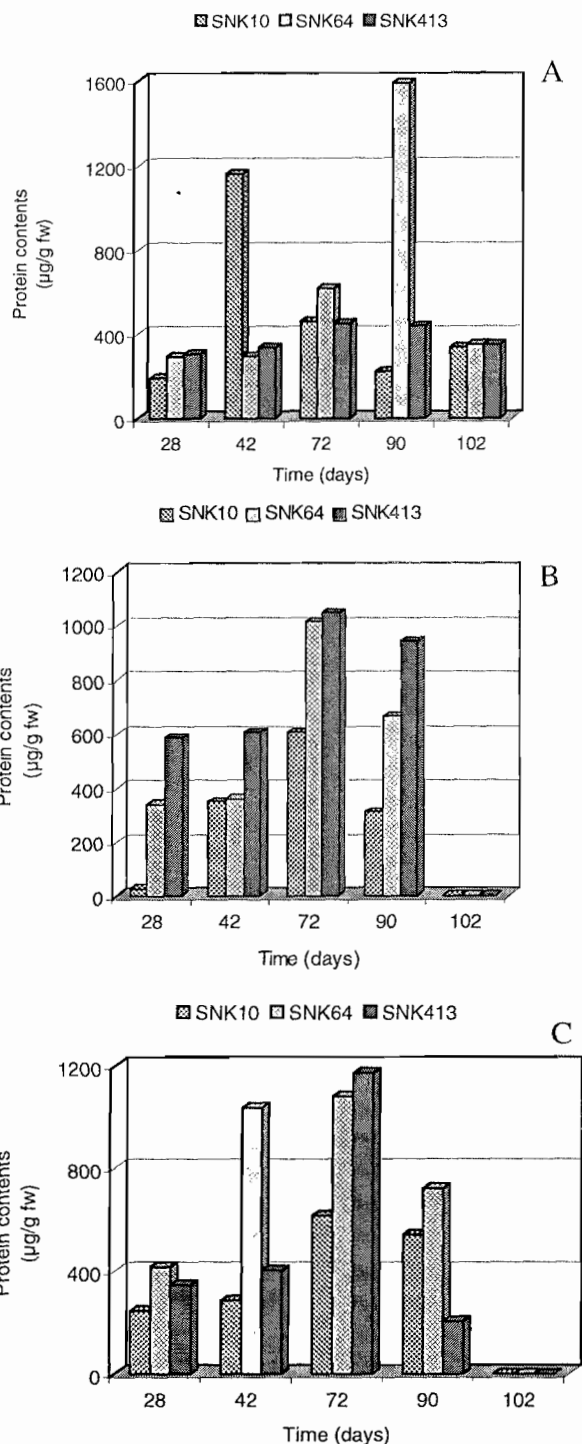

Figure 2: Protein contents in calli from media I (A), II (B) and III (C)

est protein contents was registered in SNK10 (1160±8.2mg/g FW) and SNK64 (1588± 12mg/g FW) at D42 and D90 respectively. The protein content was also important in SNK64 (616±20μg/g FW) at D72. In medium II, independently of genotype, the peaks of protein contents were observed at D72; the highest protein content being measured in SNK413 genotype.

In the media I and II, we noticed that, the peaks of protein contents are coinciding with the periods of embryos appearance on the calli. In addition, the protein contents of calli increased with the number of embryos on the callus. Thus, SNK413 calli from medium II, presenting 1 embryo per callus at D42 had 608± 32μg/g FW; whereas for the same genotype's calli from the same medium, protein content is 1046±14μg/g FW when present 3 – 4 embryo per callus at D72.

When calli were from medium III, the protein contents were significantly higher at D72 for the three genotypes. Embryogenic structures were observed at this point in that genotype.

Glutamate dehydrogenase activity
Glutamate dehydrogenase (GDH) activity was measured (Fig.3) in different morphogenetic structures obtained in various madia (I, II and III) from 3 genotypes (SNK10, SNK64, SNK413) . In medium I, the lowest enzymatic activity (107.11±16.5μmol NADH/min/g FW) was registered at D72 and the highest activity (443.3±34.1μmol NADH/min/g FW) measured at D102 in SNK10 genotype. At D72, GDH activity in SNK64 (457.98±2.44 μmol NADH/min/g FW) was higher than those of SNK10 (107.1±2.1μmol NADH/min/g FW) and SNK413 (107.71±1.1μmol NADH/min/g FW). The highest GDH activity was registered at D90 in SNK64. When calli are from medium II, peaks of GDH activity were observed at D72. This profile is similar to that of protein contents. The highest activity (1057.09 ± 7.8 μmol, NADH/g FW) was measured in the embryogenic calli of SNKJ413 at D72.

When calli were from medium III, the GDH activity profile was similar to that of protein contents. Thus, GDH activity profiles of calli obtained from the three media show a correlation with protein contents and somatic embryos differentiation. Except for SNK10 calli's from medium I, spearman test indicates positive correlations between protein contents and GDH activities. Correlation coefficients varied from 0.7 to 1 at P=0.05.

Gluatmine synthetase activity
In the case of calli obtained from medium I, peaks of glutamine synthetase (GS) activities were measured at D42 and D90 for the clone SNK64, the highest GS activities are observed at D72 (42±0.905 DO/min/g FW) and D90 (69±1.02DO/min/g FW). These peaks coincided with those of protein contents. In medium II, the evolution of GS activity is parallel to that of protein contents except in SNK413 where, the increase in protein contents is associated to decrease of GS activity. Calli from medium II presented high activity between D90 and D102. On the 90th day (D90), the GS activity of SNK10 is (65±0.825 DO/min/g FW) (Fig.4). In most cases, Spearman test shows positive correlations (from 0.1 to 1 at P=0.05) between protein contents and GS activities.

Electrophoresis analysis
A SDS-PAGE analysis of crude extracts from 72 days old calli obtained in the 3 media (I, II, III) from 3 clones (SNK10, SNK64 and SNK413) revealed the presence of 7 polypeptides: P1 (65.3kDa) P2 (62.7kDa), P3 (54.9kDa), (52.4kDa), P5 (32.7kDa), P6 (26.1kDa) and P7 (20.6kDa) (Fig.5). P1 was present in the 3 clones and media except in SNK10 (medium III) . P2, absent SNK10 and SNK64 (medium III) is present in other clones and media. P3 and P4 were simultaneously present only in SNK10 and SNK64 (media I and II). Polypeplides P5, P6 and P7 wee exclusively present in SNK413 (medium II) which has the highest frequency of calli developing embryos.

DISCUSSION
The influence of 2.4-D/BAP ratio on callogenesis and embryogenesis induction from the flowers staminodes and petals has been studied. Whatever the clone and 2.4-D/BAP ratios, the rate of callogenesis was more than 55%. For SCA-6 clone, callogenic explants frequency reached 100%. Best calli development was noticed when explants are cultured in 1/1 mg.l^{-1} (2,4-D/BAP). These finding reveal the influence of auxine/cytokinine ratio over calli formation. Similar facts were reported by Mezzeti et al. (1997) in Rubus fruticosus and R. idaeus. These authors also showed that, the explants response to callogenic stimulus depends both on the genotype and auxine used. According to the same authors, 2,4-D promotes calli formation in R. fruticosus whereas in R. idaeus, this auxine seems to
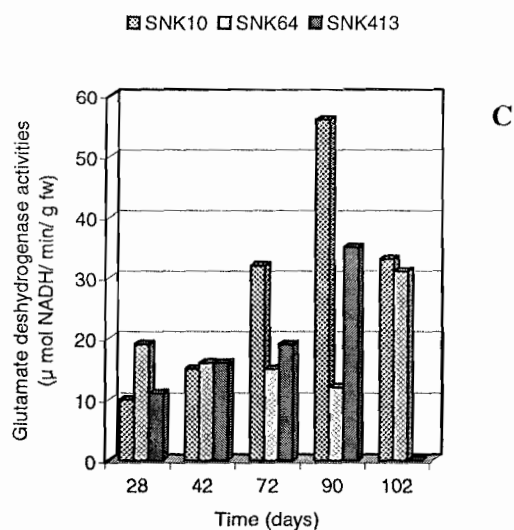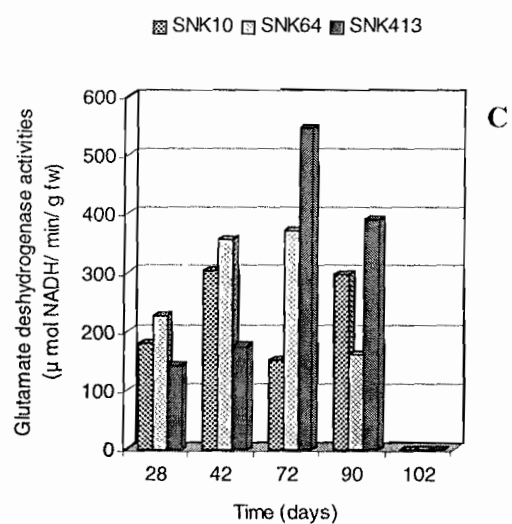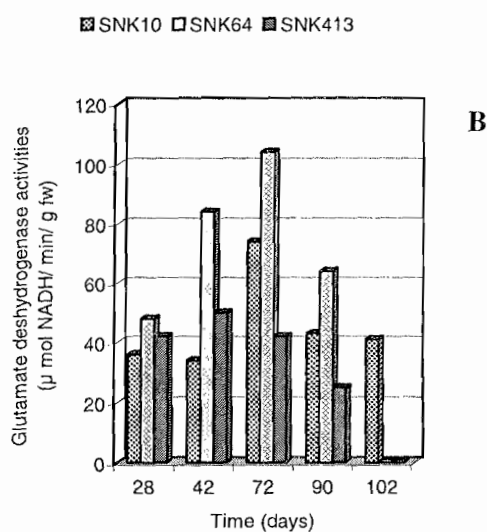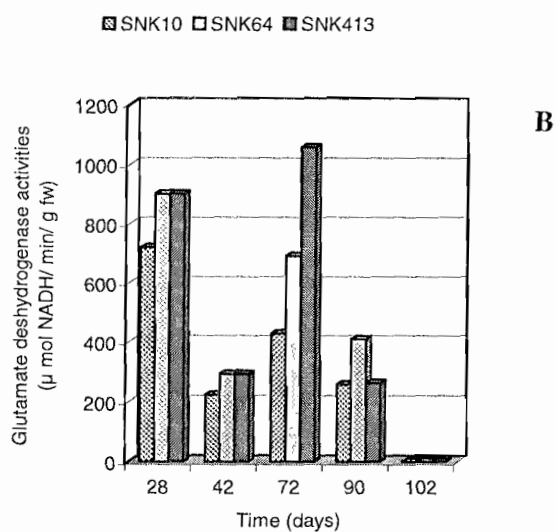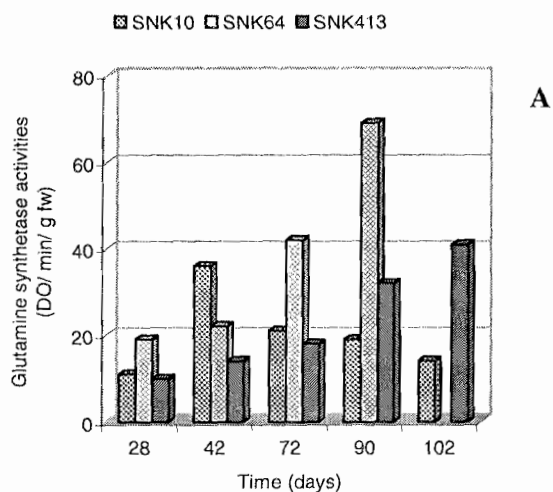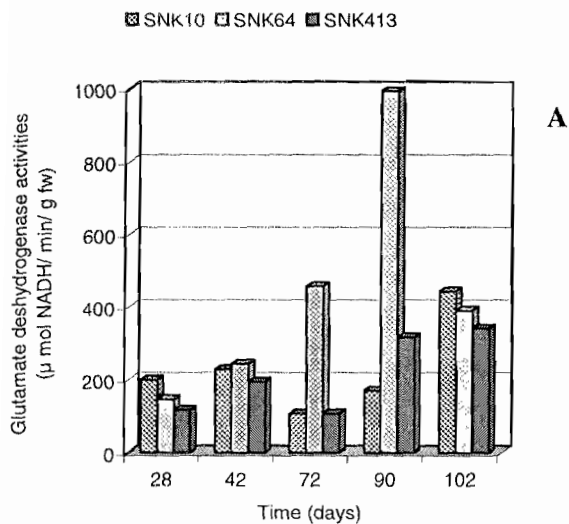
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Figure 3: Glutamate deshydrogenase activity of calli obtained from media I (A), II (B) and III (C)

Figure 4: Glutamine synthetase activity of calli obtained from media I (A), II (B) and III (C)
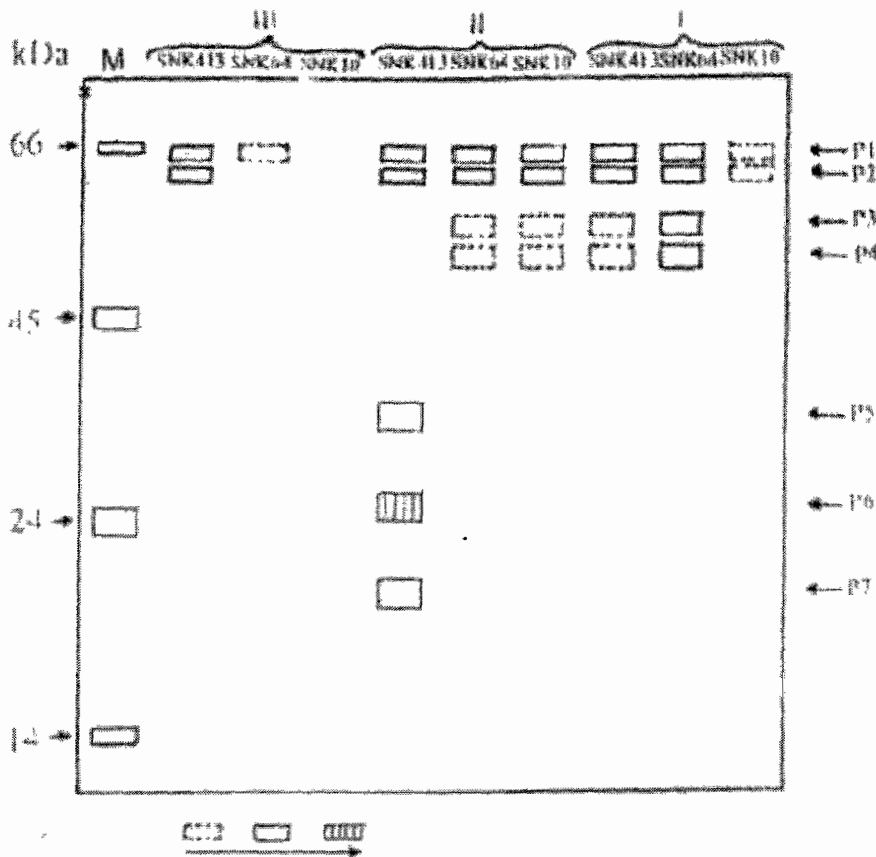
Figure 5: Soluble protein pattern of callus obtained in 72 day's culture exudates of SNK10, SNK64,and SNK413 from medium I, II and III

be toxic.

After calli transfer into free-growth regulators media, they differentiate somatic embryos from the 42nd day. Calli aptitude to express somatic embryogenesis at that date varies from 1.5 % to 13.5 % depending on both clone and culture medium. These results show the influence of genotype and culture medium over the embryogenic expression in *Theobroma cacao* L. These factors are well known to govern the same process in several plants species (Mezzeti *et al.* 1997; Islas-Flores *et al.*, 2000). These authors have reported differences in somatic embryogenesis potential up to 10 folds between certain genotypes. Studies done on carrot, maize and *Citrus grandis* have shown that genotype and auxine medium content, strongly influence the embryos formation process (Islas-Flores *et al.*, 2000). Hence, somatic embryogenesis is not controlled only by one factor. Biochemical and molecular traits could therefore be important in understanding this process in cocoa.

Protein contents of calli show that the peaks of these components coincide with the embryos appearance dates. This suggests that embryos induction is accompanied by the proteins accumulation in calli. This observation could be related to a high metabolic activity necessary not only for embryogenesis establishment *sensus stricto* (ontogenesis), but also for the initiation of embryos maturation.

In conifers (Feirer, 1995), the accumulation of these compounds has been reported during the initiation and maturation of somatic embryos. For the same medium at the same date, our results indicate that, protein contents in calli varies from one clone to another. This difference leads us to believe in differential expression of genes in clones explants put under same culturing conditions. Thus, our results could partially justify the variable responses of the genotypes to embryogenic expression.

Glutamate dehydrogenase activities in the morphogenetic structures obtained show that, enzymatic activities peaks coincide or precedes those of protein contents. This finding defines a positive correlation between protein content and glutamate dehydrogenase activity (as it has been shown using Spearman test). Furthermore, glutamate dehydrogenase activities are higher in embryogenic calli than in non-embryogenic calli. Thus, the regulation of glutamate dehydroganse gene expression could be an important factor in somatic embryos differentia-

tion. Kormutak and Vookova (1997) also reported glutamate dehydrogenase activities are high in embryogenic calli compared to non-embryogenic calli in conifer. Glutamate dehydrogenase was found to participate in stress saline resistance in *Vigna radiata*. This enzyme also intervenes in ammonium ions detoxification in carrot. Glutamine synthetase activity measured in different morphogenetic structures shown in most cases, the existence of parallelism between protein contents and glutamine synthetase activities. The increase of protein contents would be associated to the synthesis of enzymatic proteins during the embryos development. Higashi *et al.* (1998) also realised an increase of glutamine synthetase activities during the first steps of somatic embryos differentiation in carrot. The increase in glutamate synthetase activities could explain the accumulation of amino acids, mainly glutamine, in somatic embryo structures. Moreover, glutamine could be an important metabolite in the embryos development. Also, the increase of glutamine synthetase activities could enable best nitrogen assimilation necessary to somatic embryos development.

Electrophoretic analysis of soluble protein extracts (pH 8.0) shows that polypeptidic bands distribution varies from one clone to another and from one medium to another for the same clone. In SNK413 calli from medium II, where the highest proportion of calli developing embryos is obtained, three polypeptides bands P5(32.7 kDa), P6(26.2kDa) and P7 (20.6 kDa) were stained. These results could indicate, the clone, medium and morphogenetic process (somatic embryogenesis) influences over polypeptide bands distribution. These findings agree with those obtained by Hilbert *et al* (2002) relative to somatic embryogenesis in *Cichrorium*. Beside the influence of genotype and culture medium on polypeptides distribution, these authors that two polypeptides (MW=37.7kDa) of different pHi (4 and 4.5) were associated to somatic embryos formation.

CONCLUSION

The aim of this work was to study the influence of 2.4-D/BAP ratios on calli and somatic embryo induction. Protein content, glutamate dehydrogenase and glutamine synthetase activities were also measured in different morphogenetic structures. This study established that 2.4-D/BAP at 1/1 mg.l^{-1} induce the best production of calli and somatic embryos from explants used. Embryos induction was associated to protein accumulation in calli. Glutamate dehydrogenesis and glutamine synthetase actvities were positively corralated to protein content in calli.

Electrophoretic analysis showed that polypeptides P5, P6 and P7 could be potential markers of somatic embryogenesis in the clones studied.

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