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Implication of Cysteine, Glutathione and Cysteine Synthase in *Theobroma cacao* L. Zygotic Embryogenesis

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Abstract: An investigation on sulfur metabolism during cocoa zygotic embryogenesis was carried out by analysing total amino acids, cysteine, glutathione, cysteine synthase and proteins in the endosperm and in the embryos. Cacao clones SNK10 and Sca6 were used. As the embryo was getting mature, the endosperm became progressively cellularized from the mycropilar zone. Amino acid, cysteine, glutathione and protein contents were always higher in the embryos than in the endosperm in both genotypes. In the embryo, the contents of these molecules were higher in the earlier stages while in the endosperm, their contents were almost constant during maturation. There was a negative correlation (r = -0.623; p<0.01) between cysteine content in the embryo and glutathione content in the endosperm. Meanwhile cysteine content was positively correlated to amino acids (r = 0.883; p<0.01) and protein (r = 0.866; p<0.01) in the embryo. Our findings suggest that cysteine might be mainly provided by the endosperm for embryo development. In the embryo, two cysteine synthase isoforms (A and B) were revealed from stage 5+ to stage 8+ but were not detected from stage 0+ to stage 4+. Reversely, in the endosperm, both isoforms were present only from stage 0+ to stage 3+. Similarity in protein distribution in the endosperm at different embryo stages suggests that embryogenesis takes place through seven steps characterized by their protein patterns.

Key words: Cocoa, cysteine synthase, embryo, metabolism, sulfur

INTRODUCTION

Cocoa (*Theobroma cacao* L.), the chocolate tree, is a tropical crop and an important source of income for the producers. For the cocoaculture improvement and vulgarization among farmers elite genotypes, there is a need for an efficient vegetative propagation method. Propagation by cutting is unsuccessful. Attempts to propagate this species by somatic embryogenesis are not successful for all clones (Esan, 1975; Lopez-Baez *et al.*, 1993; Omokolo *et al.*, 1997; Li *et al.*, 1998; Minyaka *et al.*, 2005). Calli induced from different tissues are sometime converted into somatic embryo. But the growth of the later stops at early stage. The DKW (Driver and Kuniyuki, 1984) basal salt medium in which somatic embryogenesis was positively tested for some genotypes is characterized by a high level of sulfate compare to MS (Murashige and Skoog, 1962) basal salt medium that gives fewer results. Additionally, the analysis of cocoa endosperm during seeds development has revealed high concentration of

sulfate in that tissue (Sossou-Dangou *et al.*, 2002). These findings tend to prove that sulfur metabolism is a key process for the success of somatic embryogenesis. It then seems that the knowledge of the needs of zygotic embryo for its development into a full plant may help for setting nutritional needs for the development of somatic embryo.

Sulfur, is absorbed by plants mainly in the oxidized state (SO_4^{2-}) which is reduced to sulfide before its assimilation into organic material. In the S-assimilation process, the biosynthesis of cysteine from OAS (O-acetyl-L-serine) and sulfide (S^{-2}) is the first reaction of the incorporation of sulfur in organic material in higher plants. This reaction is catalyzed by cysteine synthase. As a point of convergence of the N and S assimilatory pathways, cysteine synthase represents a major regulator point in S-assimilation pathways (Warrilow and Hawkesford, 2002). In a subsequent series of reactions, other sulfurous compounds such as methionine or glutathione (GSH) are produced from cysteine. GSH is a major part of antioxydative system and is considered

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to be involved in tissue development and induction of embyogenesis (Foyer and Rennenberg, 2000). Cysteine is essential for protein biosynthesis. In higher plants, cysteine has two important functions in Iron-Sulfur clusters biosynthesis: mobilisation of the iron centre and providing sulfur for the Iron-sulfur cluster assembly (Ding *et al.*, 2005). In *Arabidopsis thaliana*, Xu and Møller (2004) reported that alteration in Iron-Sulfur clusters biosynthesis leads to the death of zygotic embryo.

This suggests that sulfur metabolism may have an effect on cocoa embryogenesis. But little is known about the implication of sulfurous compounds in cocoa zygotic embryogenesis. The screening of the biochemical events related to sulfur assimilation during zygotic embryogenesis may be helpful to adjust sulfate or cysteine supply in the culture medium in order to improve cocoa somatic embryogenesis.

The purpose of the present investigation is to examine the implication of cysteine, glutathione and cysteine synthase, in cocoa zygotic embryogenesis. Such information is useful in defining sulfur need for the development of cocoa somatic embryogenesis.

MATERIALS AND METHODS

Plant material: Two cocoa genotypes, SNK10 (Trinitario) and Sca6 (Forastero) were used. Eighty pods, at different maturation stages (Fig. 1) were harvested from IRAD (Institut de recherche Agricole pour le Développement) genebank at Nkolbisson, Yaoundé (Cameroon). For each pod, three fragments were considered: apical, median and distal. Pods were fixed on a gallows; sections (2 mm thick) were transversely cut from the distal to the apical end using a blade of lancet. At each transversal section of a pod, morphologic characteristics of a giving ovum (axial diameter), embryo (developmental stages) and endosperm (consistence of the endosperm) were registered. The stages of embryo development were registered in arbitrary symbol, from 0+ to 8+: no visible embryo (0+), globular stage (1+), torpedo stage (2+), hart shape stage (3+) and different cotyledonary stages (4+ to 8+). Endosperm and associated embryos at different maturation stages were collected separately and kept at -20°C in Eppendorf tubes containing 0.25 mL ethanol 80° (extraction of amino acid and cysteine), 0.25 mL Tris-HCl buffer 50 mM, pH 7.4 (extraction of glutathione) or 0.25 mL buffer A (extraction of proteins and cysteine synthase). Buffer A is made of 0.1% (v/v) Triton X-100, 0.1% (w/v) dithiothreitol, 0.2% (w/v) sodium ascorbate in 50 mM sodium phosphate, pH 8. These samples were then used for biochemical analysis.

Fig. 1: *Theobroma cacao* L. pod at different maturation stages, a: 3 weeks; b: 6 weeks; c: 9 weeks; d: 12 weeks; e: 15 weeks

Metabolites extraction and analysis: Amino acids were extracted, using the method of Singer *et al.* (1990). Embryo or endosperm was homogenized in ethanol 80° (2 mL ethanol 80° for 1 g of biological material). The homogenate was then heated at 100°C for 20 min in a reflux system and filtrated through Watman paper N°1. The filtrate was used to assay total amino acids and cysteine. Amino acid content was measured as described by Yemm and Cocking (1955) using cyano-ninhydrine reagent. Cysteine was assayed by adding 0.15 mL of amino acid extract to 0.35 mL acidic ninhydrin reagent (1.3% ninhydrin (w/v) in 1:4 conc. HCl:HOAc). The mixture was heated at 100°C for 10 min, followed by cooling in ice and addition of 1 mL ethanol to allow colour development. The absorbance was measured at 550 nm (Gaitonde, 1967). The standard curve was drawn using cysteine.

Glutathione content, was estimated by DNTB [5,5'-dithiobis (2-nitrobenzoic acid)] method (Ellman, 1959). Frozen embryo or endosperm was ground in a mortar and extracted with a buffer containing Tris-HCl (50 mM pH 7.4) and a pinch of PVPP. The homogenate was then centrifuged at 10,000 g for 20 min. Supernatant was used to determine glutathione content.

Cysteine synthase extraction and analysis: Proteins were extracted using the method of Warrilow (1997). One gram of frozen embryo or endosperm was ground in a mortar at 4°C and extracted with a buffer containing 0.1% (v/v) Triton X-100, 0.1% (w/v) dithiothreitol, 0.2% (w/v) sodium ascorbate in 50 mM sodium phosphate, pH 8 (1 g tissue for 2 mL buffer). After centrifugation at 10,000 g (Centrifuge SELECTA) for 20 min, supernatant was used for protein and cysteine synthase analysis. Proteins content was assayed according to Bradford (1976). Bovin Serum Albumin (BSA) was used as standard.

Soluble protein extract was used for native polyacrylamide gel electrophoresis (PAGE). One hundred microliter proteins extract were mixed with equal volume of

sample buffer (20% (v/v) glycerol, 38 mM Tris, 0.293 M glycine, 0.048 M dithiothreitol and 0.02% (w/v) bromophenol blue). Sample was electrophoreted in 0.050 M Tris and 0.384 M glycine (pH 8.8) at a constant 0.030 A current. The gel was stained for the cysteine synthase activity using the KCN-Pb stain procedure described by Warrilow and Hawkesford (2002). The staining solution contained 1.3% (w/v) Tris, 0.1% (w/v) cysteine-HCl, 0.05% (w/v) lead acetate and 0.02% (w/v) KCN.

Data analysis: The comparison of averages within variables was carried out by the analysis of variances (ANOVA) using the test of Student, Newman and Keuls. Correlations between variable were estimated by the method of Pearson using the software SPSS version 10.0.

RESULTS

Morphologic characteristics of ovum, zygotic embryo and endosperm: A transversal section of pods from SNK10 or Sca6 cocoa genotypes has revealed that, the axial diameters of ovum belonging to the same section were different from one another. The axial diameters of ovum varied from 8 to 22 mm in SNK10 and from 7 to 20 mm in Sca6; but it was not always related to the developmental stage of the embryo (Table 1). The sizes (developmental stages) of embryos at the same section were also different (Fig. 2). The endosperm was condensed or not depending on developmental stage of embryo. However, in both genotypes, endosperm was liquid at the early stage of the embryo (Fig. 3). But as the embryo was getting mature, the endosperm becomes more and more cellularized.

Biochemical analysis
Amino acids: In the embryos, 11.76 folds increase in total amino acid content was observed from stage 0+ (57.71 ± 5.31 µg g^{-1} FW) to stage 1+ (678.85 ± 11.78 µg g^{-1} FW) in SNK10; while in Sca6 embryos there was 2.62 folds increase in amino acid content from 0+ (191.55 ± 12.42 µg g^{-1} FW) to 1+ (501.33 ± 5.13 µg g^{-1} FW). Beyong stage1+, amino acid contents in the embryos decreased substantially up to stage 3+ (229.41 ± 12.63 µg g^{-1} of FW and 177.33 ± 2.08 µg g^{-1} of FW respectively for SNK10 and Sca6). The content in these metabolites in the embryos then remained almost constant from stage 3+ to stage 8+ independently of the genotype. When compared to endosperm, the amino acid content was always higher in the embryos (Fig. 4 and 5).

Cysteine: The quantitative evaluation of cysteine in the embryo from SNK10 revealed an increase of about 10 folds in this amino acid from stage 0+ (4.30 ± 0.32 µg g^{-1}
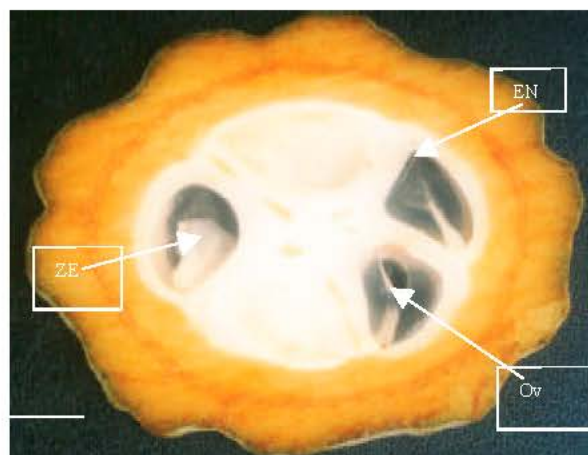

Fig. 2: Transversal section of cocoa pod. Line = 1.5 cm, ZE: Zygotic embryo; EN: Endosperm; Ov: Fertilized ovum

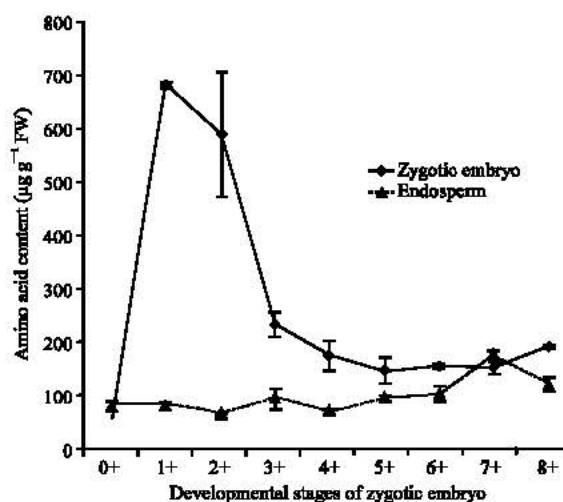Fig. 3: Zygotic embryo of *T. cacao* L. at different developmental stages

Fig. 4: Variation in amino acid content in *T. cacao* genotype SNK10

FW) to stage1+ (48.00 ± 12.00 (µg g^{-1} FW). The content in cysteine in the embryos then decreased significantly from stage1+ to stage 5+ (8.94 ± 1.97 µg g^{-1} FW) and then remained almost constant in the following stages. In the corresponding endosperm, cysteine content was relatively lower and remained constant from stage

Table 1: Axial diameter (mm) of ovum at different developmental stages of embryo

Developmental stages of embryo		0+	1+	2+	3+	4+	5+	6+	7+	8+
Axial diameter of ovum	SNK10	8-15	10-16	11-17	12,5-17,5	13-17,5	14-17,5	15-18	16-20	15-22
	Sca6	7-14,5	9-15,5	12-16,5	11-17	12-17	10,9-18	13-19	15-19,5	15-20
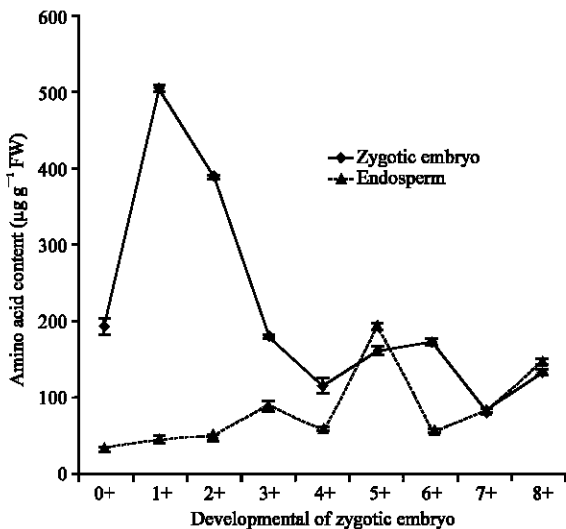
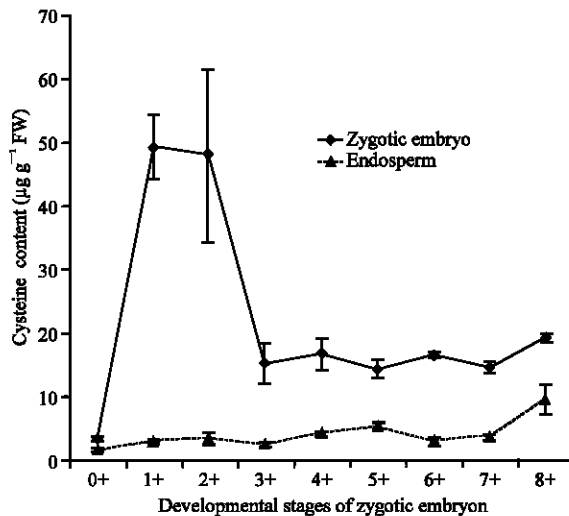Fig. 5: Variation in amino acid content in *T. cacao* genotype Sca-6

Fig. 7: Variation in cysteine content in *T. cacao* genotype Sca-6
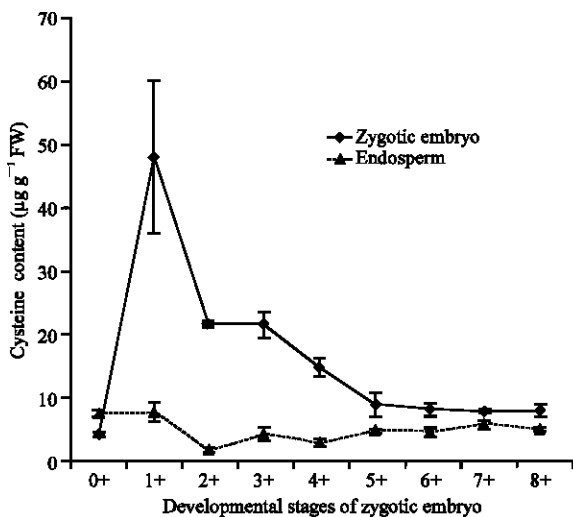

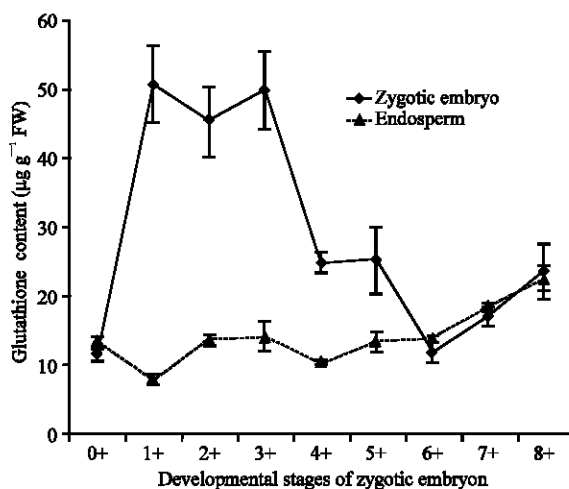Fig. 6: Variation in cysteine content in *T. cacao* genotype SNK10

Fig. 8: Variation in glutathione content in *T. cacao* genotype SNK10

0+ to stage 8+. However, from 5+ to 8+, there was not a significant difference in cysteine content between the embryo and the endosperm (Fig. 6).

The same analysis in Sca6 embryo also indicated an increase (14.83 folds) in cysteine content from stage 0+ (3.31 ± 0.45 µg g^{-1} FW) to stage 1+ (49 ± 3.28 µg g^{-1} FW); then it became constant between stage 1+ to stage 2+ (47.777 ± 2.8 µg g^{-1} of FW). A significant decrease (4.34 folds) was then recorded from stage 2+ to stage 4+

(11.00 µg g$^{-1} \pm 1.40$ FW). There was no significant variation in cysteine content between stage 4+ and 8 stage. As noticed with SNK10, cysteine content in the endosperm of Sca6 was low and almost constant in all developmental stages (Fig. 7).

Glutathione: Independently of the genotype, glutathione (GSH) analysis indicated that, the content in this metabolite was always higher in the embryo as compared
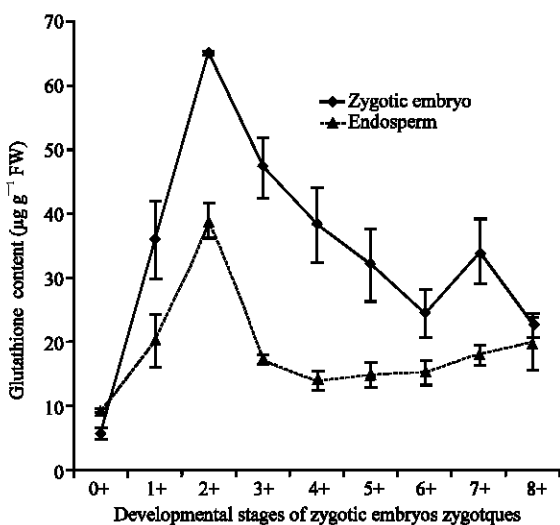
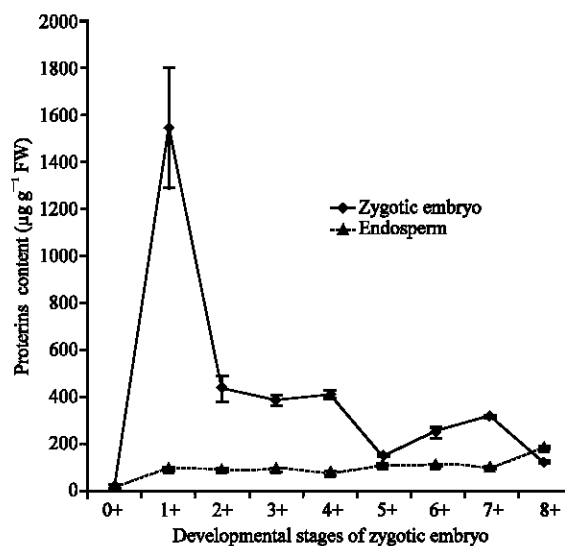Fig. 9: Variation in amino acid content in *T. cacao* genotype SNK10

Fig. 11: Variation in protein content in *T. cacao* genotype Sca-6
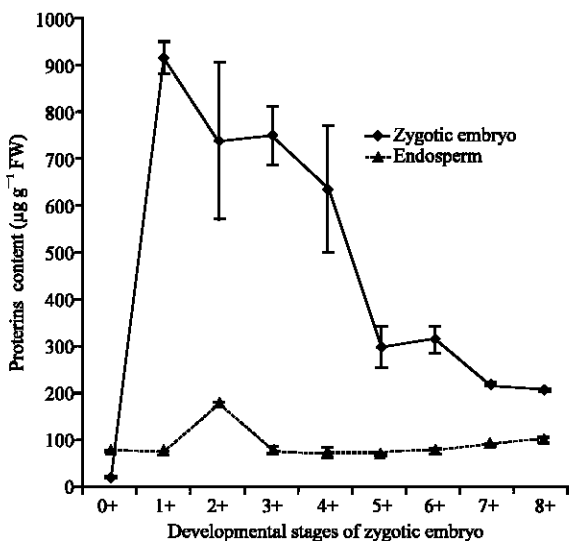

Fig. 10: Variation in protein content in *T. cacao* genotype SNK10

to the corresponding endosperm (Fig. 8 and 9). In SNK10 embryos, GSH content increased from stage 0+ (11.67±0.85 (μg g^{-1} FW) to stage 1+ (50.83±2.45 (μg g^{-1} FW). Beyond this developmental stage, it remained almost constant up to stage 3+. A decrease (76.50%) was then observed between stage 3+ and stage 6+ or 8+ (Fig. 8). GSH content in Sca6 embryos behaved as with SNK10; but the highest GSH content was observed at stage 2+ (Fig. 9).

Proteins: An increase in protein content of 50.72 folds was observed from stage 0+ (18.00±1.73 μg g^{-1} FW)

to 1+ (913.00±15.14 μg g^{-1} of FW) in SNK10. While in Sca6 there was a 809.63 folds increase in proteins content between 0+ (2.00±0.11 μg g^{-1} FW) and 1+ (1619.25±18.34 FW). A significant decrease then followed from stage 1+ to stage 8+. Compared to the embryos, the protein content in the endosperm from both genotypes was almost constant and lower from stage 0+ to stage 8+ (Fig. 10 and 11).

Correlations: Pearson correlation between cysteine, glutathione, amino acids and proteins content of embryos and endosperm of both genotypes (SNK10 and Sca6) was analysed.

In SNK10 a negative correlation (r = -0.623; p<0.01) was observed between cysteine content in the embryos (cysezsnk10) and glutathione content in the corresponding endosperm (gshensnk10). In contrary there was a positive and highly significant correlation (r = 0.883; p<0.01) between amino acid (aaezsnk10) and cysteine content in the same embryo. Protein (prezsnk10) and cysteine content in the embryos were also highly and positively correlated (r = 0.866; p<0.01). A positive and significant correlation (r = 0.795; p<0.01) was found between cysteine content in the endosperm and in the embryos. Cysteine and protein content in endosperm were positively correlated (r = 0.844; p<0.01). Within the same genotype, protein and amino acid contents in the embryo were positively correlated (0.796; p<0.01). Amino acid content in the embryo and glutathione content in the endosperm also displayed a positive correlation (0.673; p<0.01) (Table 2).

Table 2: Correlation matrix between cysteine, glutathione, amino acids and proteins contents in zygotic embryo and endosperm from SNK10

	Cysezsnk10	Cysensnk10	Gshezsnk10	Gshensnk10	Aaezsnk10	Aaensnk10	Prezsnk10	Prensnk10
Cysezsnk10	1							
Cysensnk10	0.465	1						
Gshezsnk10	0.274	0.074	1					
Gshensnk10	-0.623	-0.075	0.148	1				
Aaezsnk10	0.883**	0.795*	0.283	-0.447	1			
Aaensnk10	-0.361	-0.364	0.628	0.673*	-0.371	1		
Prezsnk10	0.866**	0.549	0.248	-0.608	0.796*	-0.465	1	
Prensnk10	0.077	0.844**	0.188	0.184	0.516	-0.175	0.225	1

Cysezsnk10 (cysteine content in zygotic embryo from SNK10); cysensnk10 (cysteine content in endosperm from SNK10); gshezsnk10 (glutathione content in zygotic embryo from SNK10); gshensnk10 (glutathione content in endosperm from SNK10); aaezsnk10 (amino acids content in zygotic embryo from SNK10); aaensnk10 (amino acid content in endosperm from SNK10); prezsnk10 (proteins content in zygotic embryo from SNK10); prensnk10 (protein content in endosperm from SNK10), ** Significance of correlation at 0.01 probability level, * Significance of correlation at 0.05 probability level

Table 3: Correlation matrix between cysteine, glutathione, amino acids and proteins contents in zygotic embryo and endosperm from Sca6

	Cysezsca6	Cysensca6	Gshezsca6	Gshensca6	Aaezsca6	Aaensca6	Prezsca6	Prensca6
Cysezsca6	1							
Cysensca6	-0.157	1						
Gshezsca6	-0.685*	-0.106	1					
Gshensca6	-0.312	0.729*	0.308	1				
Aaezsca6	0.143	-0.299	0.389	0.178	1			
Aaensca6	-0.080	0.697*	-0.075	0.253	-0.391	1		
Prezsca6	0.306	-0.253	0.322	-0.026	0.795*	-0.347	1	
Prensca6	-0.183	0.866**	0.085	0.718*	-0.172	0.620*	-0.055	1

Cysezsca6 (cysteine content in zygotic embryo from Sca6); cysensca6 (cysteine content in endosperm from Sca6); gshezsca6 (glutathione content in zygotic embryo from Sca6); gshensca6 (glutathione content in endosperm from Sca6); aaezsca6 (amino acids content in zygotic embryo from Sca6); aaensca6 (amino acid content in endosperm from Sca6); prezsca6 (proteins content in zygotic embryo from Sca6); prensca6 (protein content in endosperm from Sca6),** Significance of correlation at 0.01 probability level,* Significance of correlation at 0.05 probability level

Similar correlations were observed with Sca6. In fact, negative and significant correlation (r = -0.685, p<0.01) was also noticed between cysteine and glutathione contents in the embryo. As with SNK10, there was a positive and highly significant correlation (r = 0.866; p<0.01) between amino acid and protein contents in the endosperm. A positive and significant correlation (r = 0.795; p<0.01) was observed between amino acid and protein contents in the embryo (Table 3).

Protein pattern: Crude protein extracts from the embryo or endosperm were used for native polyacylamide gel electrophoresis (PAGE). Sca6 endosperm revealed 8 bands: P1, P2, P3, P4, P5, P6, P7 and P8 with respective migration (Rf) value of: 0.02, 0.04, 0.07, 0.09, 0.12, 0.17, 0.19 and 0.75. P1, P6 and P8 were present at all developmental stages of zygotic embryo. P2 was present in all stages except stage 2+. P3 was observed at stage 0+, 1+ and stage 2+ while P4 was absent at stage 5+. P5 was characteristic of stages 5+, 6+ and 7+. P7 was absent at stages 3+ and 8+ (Fig. 12).

The dendrogram of similarity (at 95%) in protein distribution between endosperm at different embryo maturation stages indicated that there was seven groups in proteins distribution in the endosperm during embryo maturation: group I (stages 0+ and 1+); group II (stage 2+); group III (stage 3+); group IV (stage 4+); group V (stage 5+); group VI (stages 6+ and 7+) and
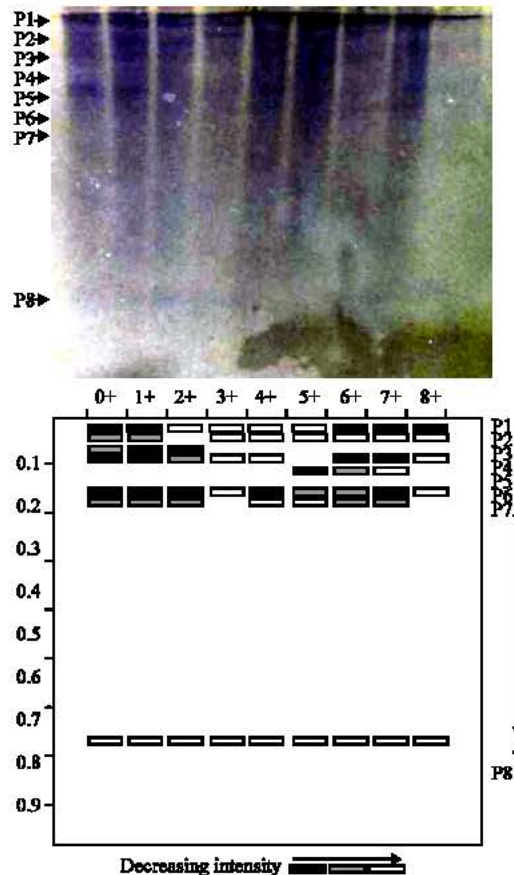

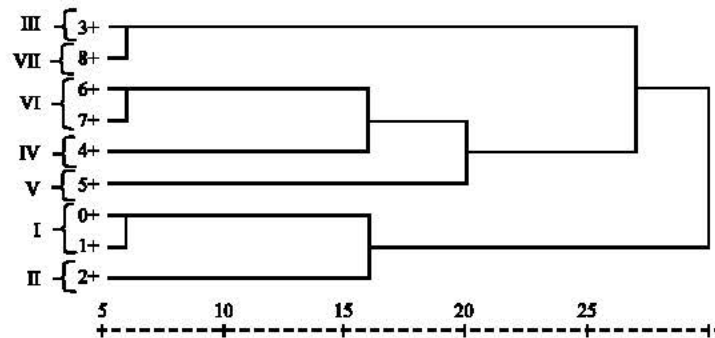Fig. 12: Proteins profile from endosperm of Sca6

Fig. 13: Similarity in proteins distribution between endosperm at different zygotic embryo maturation stages considered
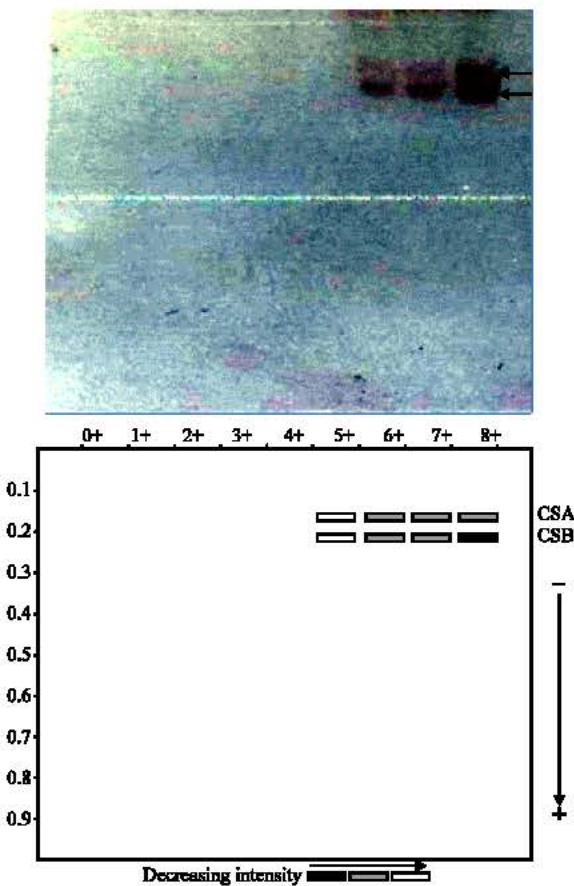

Fig. 14: Profile of cysteine synthase (CS) from zygoytic embryo of Sca-6 Decreasing intensity

group VII (stage 8+). However, there was not a significant difference in protein pattern between stages 3+ and 8+ (Fig. 13).

Specific staining of cysteine synthase from embryos revealed two isoforms of this enzyme: CSA and CSB different from each other by their molecular weight. Both isoforms were present from stage 5+ to stage 8+ and absent in the earlier stages (from stage 0+ to stage 4+) of

the embryo (Fig. 14). The analysis of the endosperm showed different pattern. Both isoforms were present from stage 0+ to stage 3+ and absent in the other stages (data not shown).

DISCUSSION

The implication of sulfur metabolism in *T. cacao* zygotic embryogenesis was analyzed. The investigation was carried out at different maturation stages of the embryo and corresponding endosperm from ovum. Amino acids, cysteine, glutathione, proteins and cysteine synthase were analyzed in the embryo and in the endosperm.

Transversal section of pods showed that at the same pods section, each embryo develops independently. This was supported by the fact that, the axial diameters of ovum and maturation stages of embryos belonging to the same section were different. Embryo at stage 0+ was associated to liquid endosperm. As the embryo matured, endosperm became more and more cellularized. This observation is similar to that in *Arachis hypogea* L. (Zamski, 1995) and *Vicia faba* (Werber *et al.*, 1998). Sossou Dangou *et al.* (2002) have associated this condensation to endosperm degradation. This should mean that biomolecules in the endosperm are progressively used by the embryo for its maturation.

Regardless the genotype, the content in metabolites (amino acids, cysteine and glutathione) evaluated in this study was always higher in the embryo than in the endosperm. Moreover the amount of these metabolites was high in the first stages of the embryo maturation while in the endosperm their contents were always constant during the maturation process of the embryo. It seems therefore that, there is migration of these metabolites from the endosperm to zygotic embryo. But the flux of metabolites should be subjected to a given threshold. The abundance of these metabolites in the embryo may be explained by their needs for optimal

embryo maturation. The negative correlation between cysteine content in the embryo and glutathione content in endosperm indicates that, in the endosperm glutathione is catabolized to liberate cysteine which is used by the embryo. According to the results of Xu and Møller (2004), cysteine is a key metabolite to avoid zygotic embryo lethality in *Arabidopsis thaliana*. These authors reported that deficiency in cysteine or AtNAP7 (a protein implicated in the biosynthesis of Fe-S clusters) prevent zygotic embryogenesis to go beyond globular stage. It was demonstrated that the sulfur atom in Fe-S clusters is directly derived from cysteine (Ding *et al*., 2005). Our results have shown a positive and highly significant correlation between cysteine and total amino acid contents. This might suggest that the high amount of amino acids in the embryo is mainly supported by the uptake of cysteine from endosperm.

Our finding has shown the presence of cysteine both in zygotic embryo and in the endosperm. This result contradicts that of Sossou Dangou *et al*. (2002). These authors reported the absence of sulfur amino acids in *T. cacao* endosperm. This difference may be due to the method used by these authors to analyze this specific amino acid. Furthermore, the sulfate found in endosperm by Sossou Dangou *et al*. (2002) is surely used for the synthesis of sulfur molecules among which cysteine; since our results have revealed the presence of cysteine synthase both in the endosperm and in the embryo. In our assay, native PAGE revealed two cysteine synthase isoforms. This result confirms that of Warrilow and Hawkesford (2002) in *Spinacea oleracea* L. In the embryo, both cysteine synthase isoforms were present from stage 5+ to stage 8+ but absent in the younger stages. Contrarily, in the endosperm, these isoforms were present from stage 0+ to stage 3+. This observation suggests that during the first stages of the embryogenesis, only isoforms from endosperm are substantially active in order to provide cysteine for the embryo maturation. As the embryo is getting mature, the embryo isoforms become active.

CONCLUSION

Our results suggest that within a given pod, zygotic embryos mature independently. The content in metabolites (amino acids, cysteine and glutathione) studied was always higher in the embryo than in the endosperm. The negative correlation between cysteine content in the embryo and glutathione content in the endosperm indicates that, in the endosperm glutathione is catabolized to liberate cysteine which is used by the embryo for its development. Our findings highlight that cysteine for embryo maturation also derived from its synthesis catalyzed by cysteine synthase present in two forms (CSA and CSB) in the endosperm (at the earlier stages) and zygotic embryo (at the later stages). Hierarchical cluster analysis of proteins profile from endosperm indicates that zygotic embryogenesis in cocoa might occur in seven steps different by their protein pattern in endosperm. The data presented in this report suggest that media for somatic embryogenesis in cacao should be supplemented with cysteine and/or with sulfate. Proportions of both sulfur sources in the culture medium are to be determined.

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