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*Full Length Research Paper*

# Sulphur depletion altered somatic embryogenesis in *Theobroma cacao* L. Biochemical difference related to sulphur metabolism between embryogenic and non embryogenic calli

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**Somatic embryogenesis is a useful tool for *Theobroma cacao* improvement and propagation. Depending on culture medium composition, different morphogenetic structures (including somatic embryo) occur in response to alteration of genes expression patterns and biochemical changes. The effect of $SO_4^{2-}$ ion deficiency in culture media on somatic embryogenesis was studied through sequential replacement of $MgSO_4$ and $K_2SO_4$ by $MgCl_2$ and KCl, respectively, at different steps of somatic embryogenesis. It appears that explants gradually lost their embryogenic competence as the period of exposition to sulphur free medium increases. These results suggest that, sulphur availability and the duration to sulphur exposition might modulate the expression of genes involved in somatic embryo differentiation in *T. cacao*. Cysteine, glutathione, reducing sugars, cysteine synthase and cysteine desulfurase activities were analysed in different morphogenetic structures obtained *in vitro*. Cysteine and reducing sugars contents appeared to be higher in embryogenic calli than their non-embryogenic homologues, whereas glutathione content appears to be lower in embryogenic calli. Cysteine synthase activities also discriminate the embryogenic calli from non embryogenic calli. In the embryogenic calli, the ratio cysteine synthase/cysteine desulfurase activities were above unit. The assimilation of exogenous sulphur (sulphate) for the synthesis of cysteine might hence be crucial for somatic embryogenesis in *T. cacao*. This explains the reduction and the absence of somatic embryo response observed during sulphur depletion in culture media. Sulphur nutrition is therefore critical in cacao somatic embryogenesis.**

**Keys words:** Cacao, embryo, sulphate, cysteine synthase, glutathione, deficiency.

## INTRODUCTION

*Theobroma cacao* (chocolate tree) is a tropical plant. It is a source of income for many developing countries. *T. cacao* cultivation still faces many challenges including the lack of elite plant material. Plant tissue culture plays an important role in agricultural biotechnology. It allows *in vitro* regeneration, genetic improvement and large scale multiplication of plants under arsenic conditions. Somatic embryogenesis, tissue culture, vegetative and micro-propagation processes have been studied in *T. cacao*. This crop appears unfortunately to be recalcitrant. The

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type of explants, the genotype and the culture medium are some of the factors influencing *T. cacao* somatic embryogenesis. The medium composition is the most reported and seems to be the preponderant factor (Tan and Furtek, 2003). Li et al. (1998) achieved better somatic response from many cacao genotypes using DKW complex salt than Lopez-Baez et al. (1994) did using Murashige and Skoog complex salt with the same explants (petals and staminodes). DKW complex salt provides a significantly higher concentration of calcium, sulphur and magnesium compare to MS complex salt. Therefore, these elements are probably essential for *T. cacao* somatic embryogenesis and differentiation. A promoting effect of $MgSO_4$ and $K_2SO_4$ supplementation on somatic embryogenesis was reported (Minyaka et al., 2008). The recalcitrance to somatic embryogenesis of many genotypes of *T. cacao* was overcomed with the addition of both sulphate salts in culture media (Minyaka, 2009). The influence of sulphur depletion on *T. cacao* somatic embryogenesis has not been investigated yet. Furthermore, studies dealing with biochemical changes related to sulphur metabolism in embryogenic and non-embryogenic calli of *T. cacao* are inexistent. Sulphur is an essential and one of the most abundant macronutrient in plant (Leustek, 2002; Saito, 2004). In plant sulphurous biomolecules, sulphur not only serves as a structural component, but it also plays roles in catalytic, electrochemical or characteristic functions of these biomolecules in cells (Saito, 2004). In growing conditions, sulphur deficiency challenges plants (explants) to alter the metabolism necessary for growth. Restriction in sulphur not only limits the synthesis of sulphurous amino acids, but will also limit the proteins synthesis and the rate with which all amino acids are incorporate into protein (Leustek, 2002). The present research work is trying first of all to investigate the mechanism that can help to solve the recalcitrance, secondly to understand the importance of sulphur metabolism in cacao somatic embryogenesis.

## MATERIALS AND METHODS

### Explants preparation

The tissue culture system used in this investigation was the same as the system previously described by Minyaka et al. (2008). Flowers buds used in this work were harvested from Sca6 genotype (Forastero) at CNRA (Centre National de Recherche Agronomique) experimental farm in Bengerville (Côte d'Ivoire). Flowers were collected early in the morning in cold water. They were surface-sterilized by immersion for 20 min in 1% (w/v) calcium hypochloride followed by three 2 min rinses in sterilized distilled water. Staminodes and petals were excised with scalpel and placed on culture media (in distinct set) into Petri plate (thirty-five staminodes and thirty-five petals per Petri plate).

### Study of the effect of sulphur deficiency on somatic embryogenesis expression

All media were defined using DKW (Driver and Kuniyuki Walnut medium) basal salts of Driver and Kuniyuki (1984). In order to test the effect of sulphur deficiency on *T. cacao* somatic embryogenesis, sulphur was sequentially added into culture media as $MgSO_4$ and $K_2SO_4$. The explants were first cultured in primary callus growth medium with normal ($K_2SO_4$ and $MgSO_4$) sulphate concentration and without sulphate ($MgSO_4$ and $K_2SO_4$, respectively, replaced by $MgCl_2$ and KCl). Primary callus growth medium was supplemented with 250 $mgL^{-1}$ glutamine, 100 $mgL^{-1}$ myoinositol, 1 ml $L^{-1}$ DKW vitamin stock (100 $mgml^{-1}$ myoinositol, 2 $mgml^{-1}$ thiamine-HCl, 1 $mgml^{-1}$ nicotinic acid and 2 $mgml^{-1}$ glycine), 20 $gL^{-1}$ glucose, 18 µM 2,4 dichlorophenoxyacetic acid (2,4-D) and 45.4 nM thidiazuron (TDZ). Media were dispensed into sterilized Petri plates after autoclaving for 20 min at 1 bar pressure and 121°C. Each Petri plate contained 35 staminodes and 35 petals in two separate sets. Experiments were repeated five times with three replicate Petri plate at each culture initiation. Petri plates were incubated in the dark at 25 ± 1°C for 14 days. After 14 days incubation in primary callus growth medium, the explants from the medium with $MgCl_2$ and KCl were subdivided into two batches. One batch was transferred in secondary callus growth medium with normal (0.3 mM $MgSO_4$ and 8.946 mM $K_2SO_4$) sulphate concentration and the other batch in the secondary callus growth medium with $MgSO_4$ and $K_2SO_4$, respectively, replaced by $MgCl_2$ and KCl. Explants from primary callus growth medium with normal sulphate concentration were transferred in secondary callus growth medium with normal sulphate concentration. Secondary callus growth medium consisted of DKW (except where $MgSO_4$ and $K_2SO_4$ were respectively, replace by $MgCl_2$ and KCl) basal salts, supplemented with 0.5 $mlL^{-1}$ DKW vitamin, 20 $gL^{-1}$ glucose, 9 µM 2,4-D, 250 $\mu gL^{-1}$ kinetin and 0.2% (w/v) phytagel. Cultures were also incubated at 25 ± 1°C for 14 days in darkness.

Explants from secondary callus growth medium without sulphate were subdivided into two batches. One batch was cultured in embryo development medium without sulphate. The other batch was cultured in embryo development medium with 6.0 mM $MgSO_4$ (two fold compared to normal) and 8.946 mM $K_2SO_4$ (normal) concentration. Cultures from secondary callus growth medium were also transferred in embryo development medium with two fold (6.0 mM $MgSO_4$ and 8.946 mM $K_2SO_4$) sulphate concentrations. Embryo development medium was made of DKW basal salt supplemented with 1 ml DKW vitamin, 30 $gL^{-1}$ sucrose, 1 $gL^{-1}$ glucose and 0.2% (w /v) phytagel. Cultures were incubated at 25 ± 1°C in darkness for 21 days. After 21 days, cultures from embryo development medium without sulphate were subdivided in two batches, one was cultured in presence of sulphate (6.0 mM $MgSO_4$ and 8.946 mM $K_2SO_4$), the other in the absence of sulphate. Twenty one days later, the explants cultured in absence of sulphate were also subdivided in two batches, one transferred in presence of sulphate, the other in the absence of sulphate (Table 1). This experience was done five times (cultures) with three replicates each time.

### Biochemical analysis of embryogenic and non embryogenic calli (white calli, necrosis calli, whithe calli)

At the end (91st day) of each experience (a giving culture), each type of mophogenetic structure (embryogenesis calli, white calli, calli with roots and necrosis calli) was collected (in three different samples) and analyzed independently.

### Amino acids and soluble sugar extraction

Amino acids and soluble sugars were extracted according to the modified method of Babu et al. (2002). A gram of biological material (embryogenic, non embryogenic calli) was ground in 3 ml of 80% ethanol and centrifuge at 6000 g for 20 min. The supernatant was collected and used for amino acids and sugar analysis.

**Table 1.** Culture monitoring during sequential supply of sulphate in the cultures media.

| Culture media | PCG | SCG | ED 1st | ED 2nd | EDth |
|---|---|---|---|---|---|
| Incubation time (days) | 14 | 14 | 21 | 21 | 21 |
| Cultures ages (days) | 0 -14 | 14 - 28 | 28 - 49 | 49 - 70 | 70 – 91 |
| *Reference medium (positive control) | $PCG_{1S}$ | $SCG_{1S}$ | $ED_{2S}$ | $ED_{2S}$ | $ED_{2S}$ |
| **Delay of sequential sulphate supply as $MgSO_4$ and $K_2SO_4$ in culture media | | | | | $PCG_{-S}$ - $SCG_{-S}$ - $ED_{-S}$ -$ED_{-S}$ -$ED_{2S}$ |
| | | | | $PCG_{-S}$ - $SCG_{-S}$ - $ED_{-S}$ -$ED_{2S}$ | $ED_{2S}$ |
| | | | $PCG_{-S}$ -$SCG_{-S}$ -$ED_{2S}$ | $ED_{2S}$ | $ED_{2S}$ |
| | | $PCG_{-S}$ -$SCG_{1S}$ | $ED_{2S}$ | $ED_{2S}$ | $ED_{2S}$ |
| ***Reference medium (negative control) | $PCG_{-S}$ | $PCG_{-S}$ -$SCG_{-S}$ | $PCG_{-S}$ -$SCG_{-S}$ - $ED_{-S}$ | $PCG_{-S}$ - $SCG_{-S}$ - $ED_{-S}$ -$ED_{-S}$ | $PCG_{-S}$ - $SCG_{-S}$ - $ED_{-S}$ -$ED_{-S}$ -$ED_{-S}$ |

* $PCG_{1S}$: Primary callus growth medium with normal $MgSO_4$ and $K_2SO_4$ concentration; $SCG_{1S}$: Secondary callus growth medium with normal $MgSO_4$ and $K_2SO_4$ concentration; $ED_{2S}$: Embryo development medium with two fold $MgSO_4$ and $K_2SO_4$ concentration. ** $PCG_{-S}$ -$SCG_{1S}$: $MgSO_4$ and $K_2SO_4$ added into culture medium on the 14th day; $PCG_{-S}$ -$SCG_{-S}$ -$ED_{2S}$: $MgSO_4$ and $K_2SO_4$ added into culture medium on the 28th day; $PCG_{-S}$ - $SCG_{-S}$ - $ED_{-S}$ -$ED_{2S}$ : $MgSO_4$ and $K_2SO_4$ added into the culture medium on the 49th day; $PCG_{-S}$ - $SCG_{-S}$ - $ED_{-S}$ -$ED_{-S}$ -$ED_{2S}$ : $MgSO_4$ and $K_2SO_4$ added into culture medium on the 70th day. *** $MgSO_4$ and $K_2SO_4$ respectively replaced by $MgCl_2$ and KCl during the 91 days of culture.

**Total amino acids, cysteine and reducer sugars analysis**

The amino acids content was assayed using the method described by Yemm and Cooking (1955). 0.5 ml citrate buffer (0.2 M, pH 5.0), 1.0 ml acetone ninhydrin KCN reagent, 50 µl ethanolic extract (sample) and 0.5 ml ethanol 80% were mixed. The mixture was heated (100°C, 15 min) and cooled in ice. 8 ml of distilled water was subsequently added and the absorbance was read at 570 nm. To measure cysteine concentration in extracts, the method of Gaitonde (1967) was used. An aliquot (0.15 ml) of the a sample was added to 0.35 ml acidic ninhydrin reagent (1.3% ninhydrin (w/v) in 1:4 conc. HCl:HOAc). These were heated at 100°C for 10 min to allow colour development, followed by cooling in ice and addition of 0.7 ml ethanol 95%. The absorbance at 550 nm was measured.

Reducing sugars were assayed using Müller reagent (1% 3,5-dinitrosalicilic acid (w/v), 1.6% NaOH (w/v), 30% sodium potassium tartrate (w/v). The reaction mixture contained 200 µl sample, 0.5 ml Müller reagent and 1.5 ml distilled water. The mixture was heated at 100°C for 10 min and cooled at room temperature. The absorbance was measure at 575 nm.

**Glutathione extraction and assay**

Glutathione was extracted by grinding 1 g of morphogenetic structures in 3 ml extraction buffer (Tris-HCl pH 7.4, 50 mM) in presence of 0.1 g polyvinylpyrilidone phosphate. These were centrifuged (10000 g, 10 min, 4°C). The supernatant was collected and used to assay total glutathione using Ellman (1959) method. 50 µl phosphate buffer (100 mM, pH 6.8) supplemented with DTNB (8 mM) and EDTA (19 mM) was added to 100 µl extract (sample) and 1 ml Tris-HCl buffer (pH 8.0, 500 mM). The mixture was homogenized and incubated at room temperature for 25 min. Absorbance was determined at 412 nm.

**Cysteine synthase and cysteine desulfurase extraction and analysis**

For cysteine synthase analysis, morphogenetic structures were collected and cysteine synthase was immediately extracted using the method of Warrilow et al. (1998). A gram of morphogenetic structures (embryogenic calli and non embryogenic calli) was ground in a mortar at 4°C with 2 ml of 50 mM sodium phosphate, pH 8 buffer containing 0.1% (v/v) Triton X-100, 0.1 % (w/v) dithiothreitol, and 0.2% (w/v) sodium ascorbate. After centrifugation at 10,000 g for 20 min, the supernatant was used for cysteine synthase analysis. The cysteine synthase assays contained 1.5 mM O-acetylserine, 3 mM sodium sulphide, 10 mM dithiothreitol, 12.5 mM sodium phosphate, pH 8 (0.2 ml volume) and 100 µl enzyme extract. The reaction was initiated by the addition of sodium sulphide and was incubated for 10 min at 26°C after which, 0.35 ml of acidic ninhydrin reagent (1.3% ninhydrin (w/v) in 1:4 conc HCl:HOAc) was added to determine cysteine concentration (Gaitone, 1967). These were heated at 100°C for 10 min to allow colour development follow by cooling in ice and addition of 0.7 ml ethanol. The absorbance at 550 nm was determined. One unit of enzyme is defined as the formation of 1 µmol of cysteine per min under the state assay conditions.

Cysteine desulfurase was extracted using Riemenschneider et al. (2005) method. The reaction mixture for cysteine desulfurase activity contained Tris-HCl buffer (pH 8.0, 100 mM), 0.25 mM dithiotreitol, 0.8 mM cysteine and 100 µl enzyme extract. After 15 min incubation at 37°C, the remaining cysteine was assayed using Gaitonde method (1967). The enzyme activity is defined as the transformation of 1 µmol of cysteine per min under the state assay conditions.

**Data analysis**

Data were subjected to statistical analysis using SPSS software version 10.0. Analysis of variance was performed where applicable and differences between means were determined using Student Newman and Kell's multiple-range test.

**Table 2.** Influence of sulphur depletion in culture media on the percentage of explants producing callus.

| Non embryogenic calli | Date of sulphate supply (days) | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 14 | 28 | 49 | 70 | 91* |
| % of staminodes-derived non embryogenic calli | 80 ± 17 | 85 ± 14 | 89 ± 18 | 91 ± 12 | 100 | - |
| % of petals-derived non embryogenic calli | 71 ± 15 | 86 ± 11 | 88 ± 13 | 93 ± 19 | 100 | - |

Each value is a mean of five identical experiments. Individual experiment included three replicate Petri dishes with 35 staminodes and 35 petal-derived morphogenetic structures. Significance was determined at $P < 0.05$ using ANOVA. In the same line, values that are significantly different (at $P < 0.05$) are indicated with different letters. *In the absence of sulphate in culture medium (91 days), all explants died (necrosis).

## RESULTS

In order to test the effect of sulphur deficiency on cacao somatic embryogenesis, $MgSO_4$ and $K_2SO_4$ were sequentially replaced by $MgCl_2$ and KCl, respectively, in culture media at different steps of *T. cacao* somatic embryogenesis (induction to expression steps). The results showed that the frequency non-embryogenic morphogenetic structures (white calli, necrosis calli and calli with roots) increase with the duration of sulphur starvation in culture media (Table 2). All explants died (necrosis) when they are cultured for 91 days in the absence of sulphur. White calli without embryo were mostly observed when sulphur was supplied on 49th day of culture. Calli with roots were abundant when sulphur was supplied on the 28th in culture media.

The percentage of explants producing somatic embryo is highly altered by sulphur deficiency in culture media. There was no embryo differentiation when $MgSO_4$ and $K_2SO_4$ were replaced by $MgCl_2$ and KCl, respectively, in culture media during the whole culture period (91 days). The presence of $MgSO_4$ and $K_2SO_4$ in culture media at all steps (91 days) leaded to 29.04 ± 0.4 and 20 ± 3.6% of staminodes and petals producing embryo, respectively. The addition of sulphur later on in culture media progressively and significantly reduced the percentage of explants producing embryo. Hence, when explants stayed in culture medium for the 14 first days of culture followed by their transfer in media with $MgSO_4$ and $K_2SO_4$, just 14% of explants produced embryo. This represents approximately 50% reduction of explants producing embryo compared to the positive control. And when the explants were cultured in absence of sulphate salts for 28 first days (then transferred in presence of sulphate), the percentage of explants producing embryo was 10%. The absence of sulphate in culture media for the 70 days of culture inhibited embryo differentiation (Figure 1). Curves tendency linking the percentage of explants producing embryo to the dates of sulphate supply in culture media presented negative slopes. With staminodes the straight line equation was $y = -4.51x + 24.53$. The determining coefficient was 93.2%. With pe-tals, the straight line equation was $y= -6.55x + 31.96$. Determining coefficient was 91.61%. The comparison of both slopes shows that, as embryo expression is concerned, petals are more sensitive to sulphur defi-ciency than staminodes (Figure 1).

The *in vitro* morphogenesis of *T. cacao* presented different morphogenetic structures including, white calli, necrosis calli, calli with roots and embryogenic calli. The embryos obtained were able to germinate and generate plantlet (Figure 2). Biochemical analyses related to sulphur metabolism were conducted in these structures. Total amino acids, cysteine, reducer sugars, glutathione, cysteine synthase and cysteine desulphurase were analyzed.

The amino acid content in morphogenetic structures varied from 29420.23 ± 496 µg $g^{-1}$ FW (embryogenic calli) to 39501.97 ± 615 µg $g^{-1}$ FW in necrosis calli (data not shown). Cysteine contents in white calli (31.29 ± 1.88 µg $g^{-1}$ FW) and in calli with roots (30.74 ± 1.57 µg $g^{-1}$ FW) were not significantly different. However, cysteine contents in necrosis and embryogeneic calli were significantly higher compared to the content of this metabolite in calli with roots and white calli. Additionally, embryogenic calli presented the highest cysteine content (Figure 3). In the morphogenetic structures analysed, cysteine content pattern was not matched to glutathione content pattern. The glutathione content was lower in em-bryogenic calli (9.94 ± 1.54 µg $g^{-1}$ FW) than the glutathione contents in white calli (12.38 ± 2.68 µg $g^{-1}$ FW), necrosis calli (14.25 ± 3.1 µg $g^{-1}$ FW) and calli with roots (14.50 ± 1.2 µg $g^{-1}$ FW) (Figure 4). Reducing sugars discriminate morphogenetic structures. The lowest redu-cing sugars content was observed in white calli (36.95 ± 2.33 µg $g^{-1}$ FW) while the highest content was measured in embryogenic calli (140.30 ± 4.18 µg $g^{-1}$ FW). Reducing sugars content was significantly higher in calli with roots (104.85 ± 5.78µg $g^{-1}$ FW) than in necrosis calli (71.34 ± 3.77 µg $g^{-1}$ FW). It appears that, cells differentiation is characterized by high reducing sugars synthesis (Figure 5).

Cysteine synthase activities presented patterns related to a type of morphogenetic structure. The activity was 2.0, 1.7 and 4.3 fold higher in embryogenic calli than the activities in white calli, necrosis calli and calli with roots, respectively (Figure 6). Cysteine desulphurase discriminated embryogenic calli from non embryogenic calli. In embryogenic calli, cysteine desulphurase activity presented the highest activity. There was no significant difference between cysteine desulphurase activities in white calli and necrosis calli. However, the cysteine

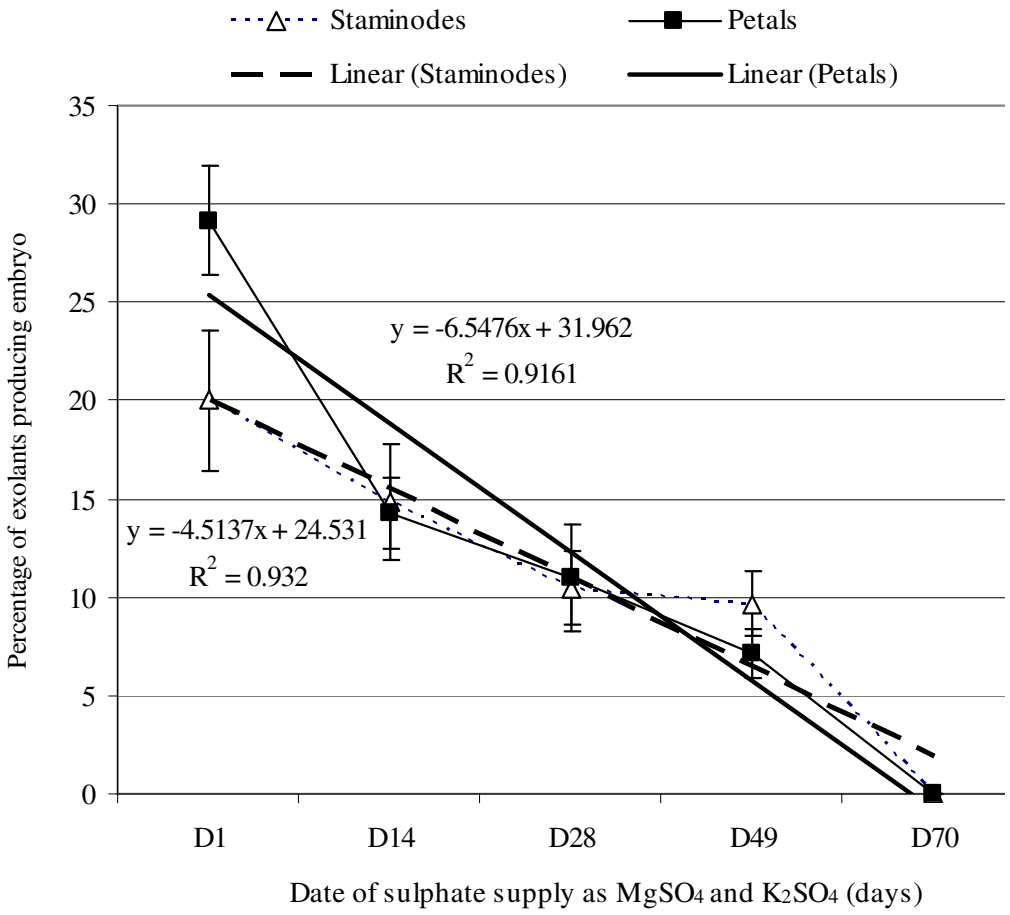


**Figure 1.** Influence of sulphur depletion in culture media on the percentage of explants producing somatic embryos. Vertical bars represent standard error. Each plot was drawn from means of five identical experiments. Individual experiment included three replicate Petri dishes with 35 staminodes and 35 petal-derived morphogenetic structures. Significance was determined at $P < 0.05$ using ANOVA.

de-sulphurase activity was significantly higher in necrosis calli than in white calli and calli with roots (Figure 7). The ratio cysteine synthase/cysteine desulphurase was estimated. This ratio significantly varied from one morphogenetic structure to another. This ratio was not similar to the activity pattern of both enzymes. Calli with roots presented the lowest ratio and embryogenic calli the highest ratio (Figure 8).

## DISCUSSION

Staminodes and petals were sequentially subjected to sulphur deficiency. It appeared that explants gradually lost their potential to differentiate embryo as the period in medium without sulphur increases. These results suggest that, sulphur availability and the duration to sulphur exposition might modulate the expression of genes involved in somatic embryo differentiation in cacao. It is known that somatic embryo differentiation in cacao is a genes controlled process (Santos et al., 2005; Alemanno et al., 2008). Thus, it will be interesting in the future investigations to analyze key genes transcriptome involved in sulphur metabolism and embryogenesis (somatic and zygotic). Additionally, the analysis of sulphur transcriptome related to sulphur deficiency in *Arabidopsis thaliana* revealed the alteration of the expression of genes implicated in sulphur assimilation (Nikiforova et al., 2003). Moreover, there was a difference between slopes of straight lines explaining the loss of embryogenic potential for staminodes and petals. This difference underlines the "explant effect" on embryogenic response of cultured tissues. This set of results suggests that, when genetically a genotype of cacao is embryogenic, the limit factor is the availability of sulphur which control the expression of genes implicated in cacao somatic embryogenesis.

During somatic embryogenesis, different patterns of genes expression are observed from the acquisition to the expression of somatic embryogenic competence (Zimmerman, 1993). The variation of genes expression

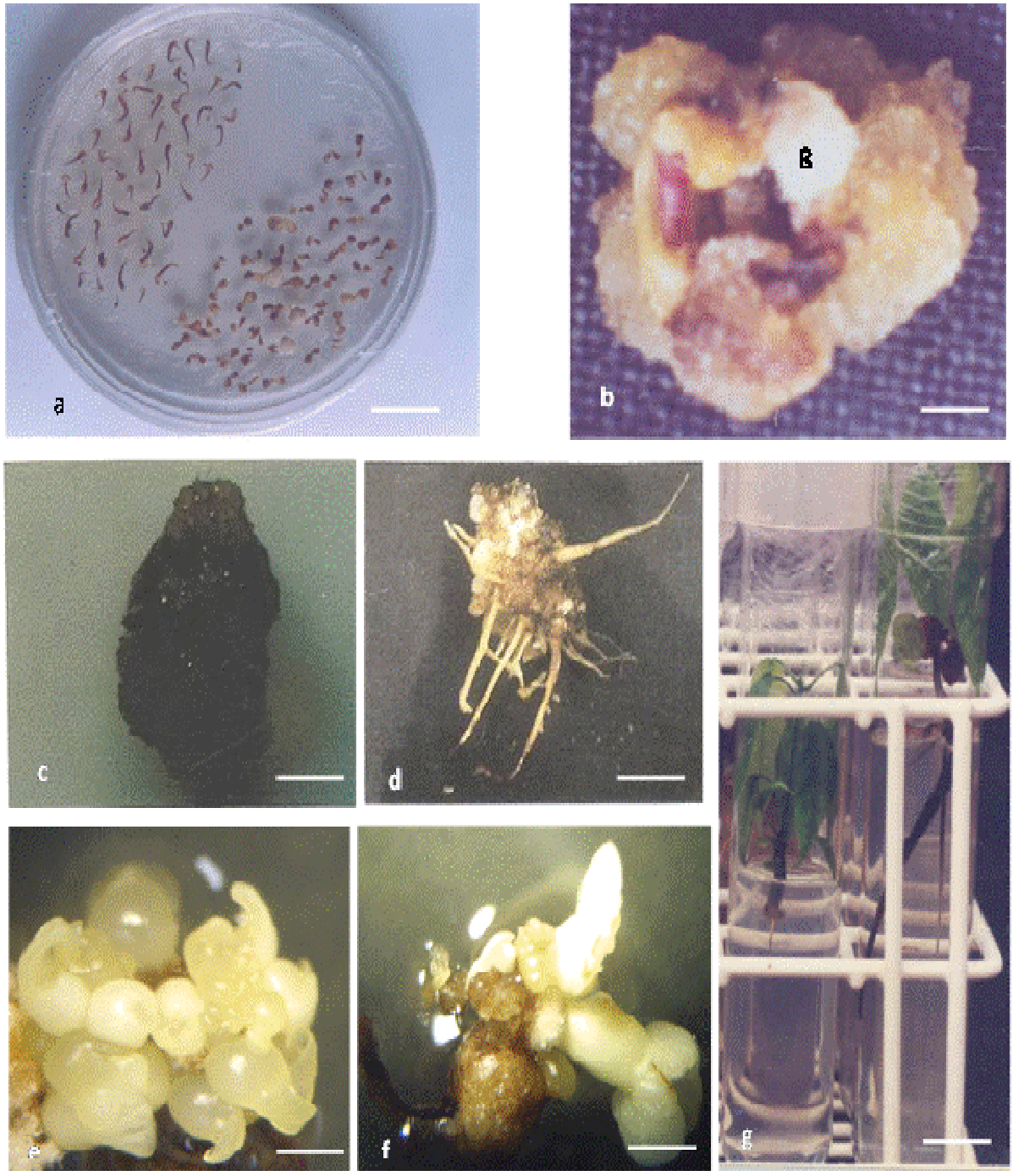


**Figure 2.** Different morphogenetic structures. a) Staminodes and petals in Petri plate; b) callus (ß- white callus); c) necrosis callus; d) callus with roots; e) somatic embryos at globular state; f) somatic embryo at cotyledonary state, and g) plantlets. Bar = 0.5 cm.

pattern, leads to the modification of biochemical (metabolites, enzymes activities) and physiological statutes of explants from which derived morphogenetic structures (Sharp et al., 1980). Amino acids, cysteine, glutathione, reducers sugars, cysteine synthase and cysteine desulfurase activities were analyzed in different morphogenetic structures (white calli, calli with roots,necrosis calli and embryogenic calli). Cysteine content was higher in embryogenic calli than their non embryogenic homologous. This result might underline the implication of this cysteine in cacao somatic embryogenesis. The present result indicates a determinant role of sulphur assimilation somatic embryo differentiation. And, this explains the improvement of somatic embryo response and the increase in somatic

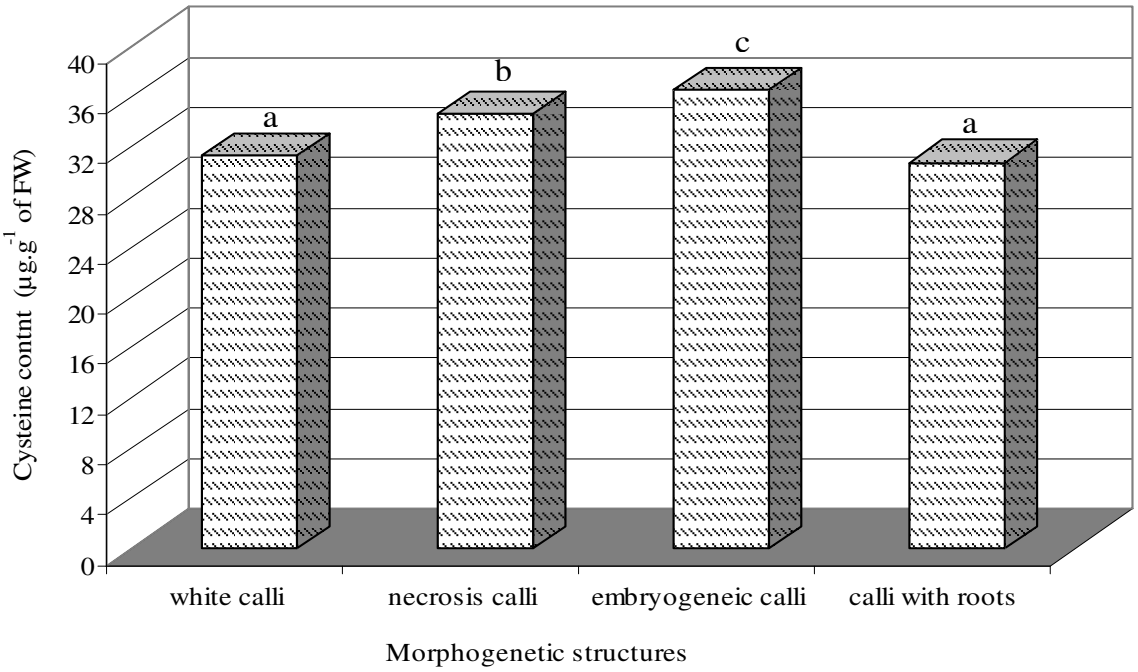


**Figure 3.** Variation of cysteine contents in morphogenetic structures. Data are presented as means of five identical experiments with three replicate. Values that are significantly different at the 5% level of significance are indicated with different letters.

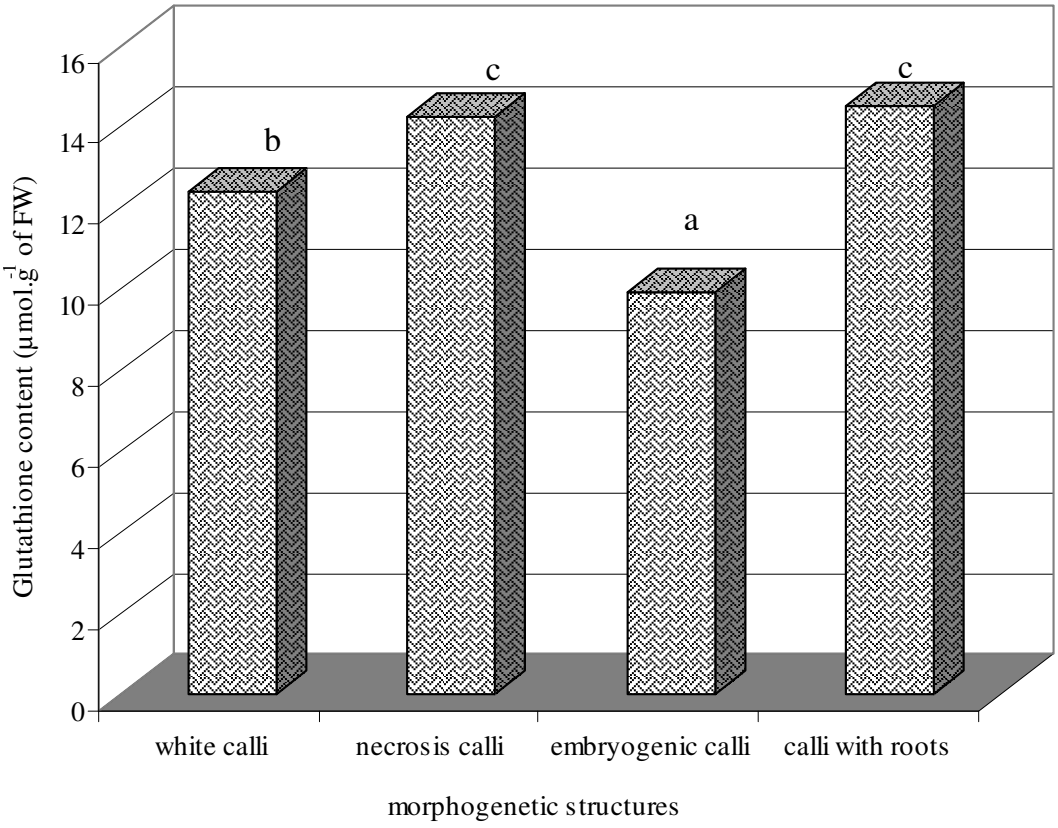


**Figure 4.** Variation of glutathione content in morphogenetic structures. Data are presented as means of five identical experiments with three replicate. Values that are significantly different at the 5% level of significance are indicated with different letters.

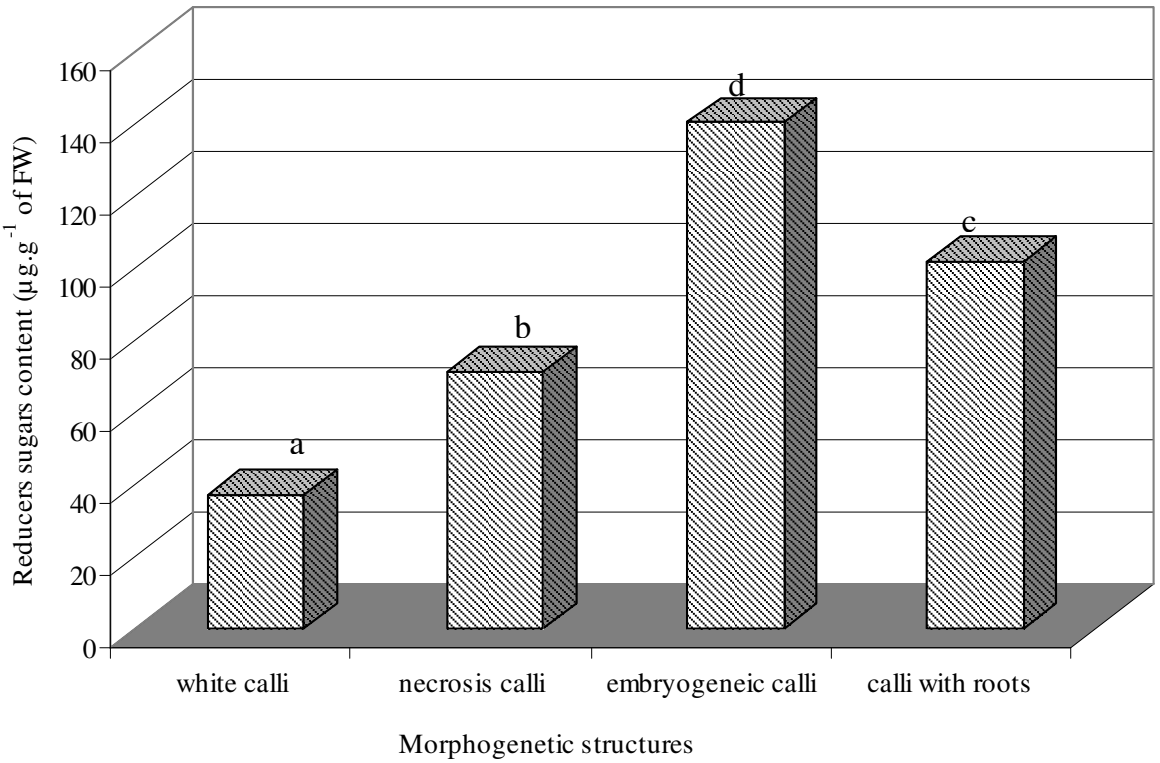


**Figure 5**. Variation of reducing sugars content in morphogenetic structures. Data are presented as means of five identical experiments with three replicate. Values that are significantly different at the 5% level of significance are indicated with different letters.

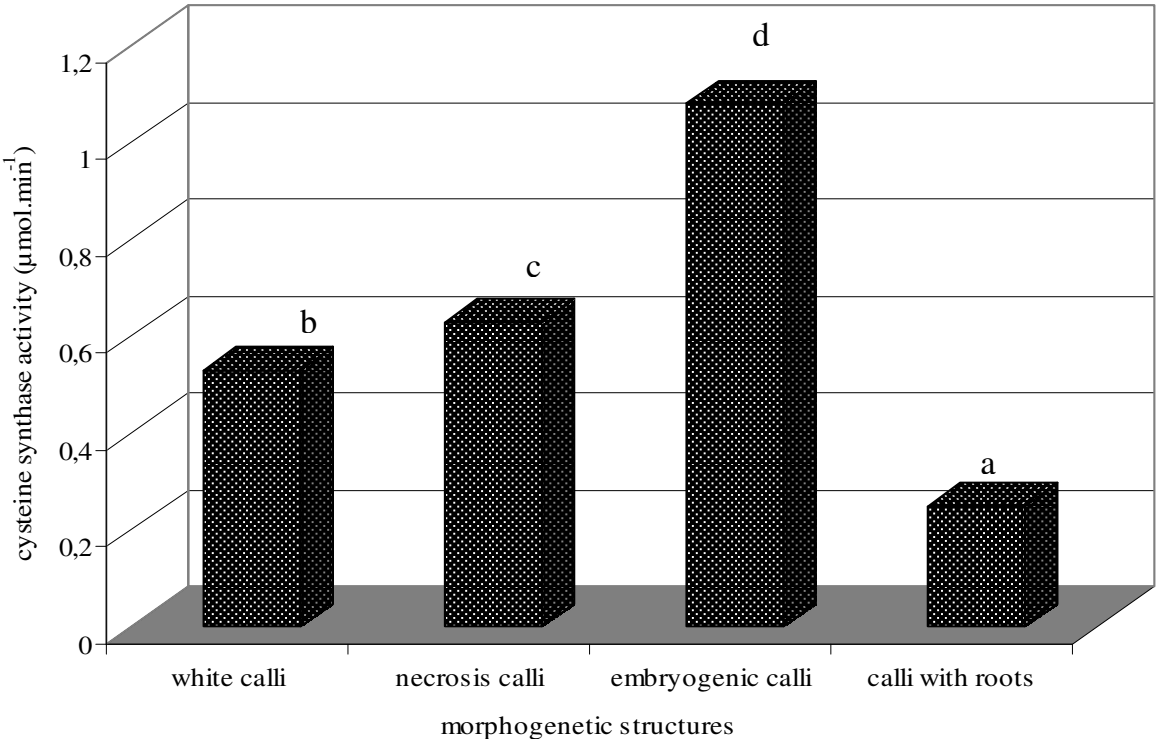


**Figure 6.** Variation of cysteine synthase activities in morphogenetic structures. Data are presented as means of five identical experiments with three replicate. Values that are significantly different at the 5% level of significance are indicated with different letters.

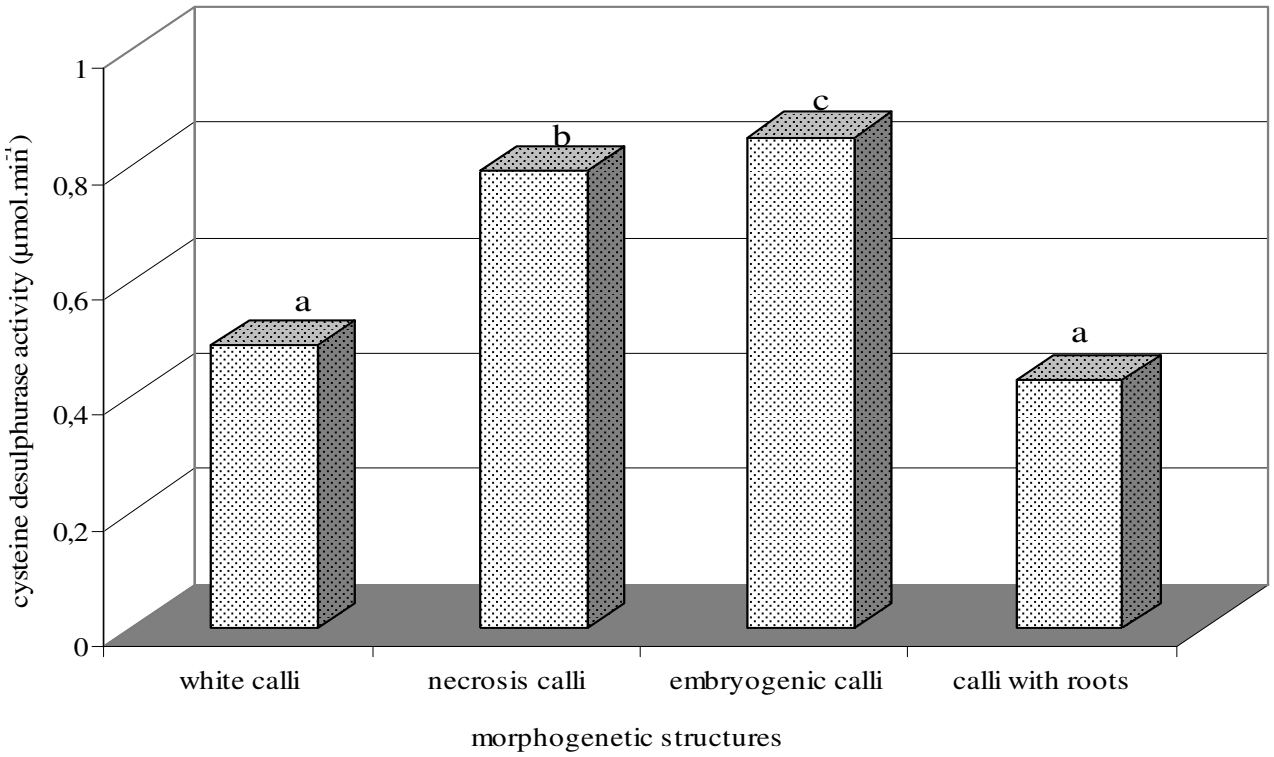


**Figure 7.** Variation of cysteine desulphurase activities in morphogenetic structures. Data are presented as means of five identical experiments with three replicate. Values that are significantly different at the 5% level of significance are indicated with different letters.

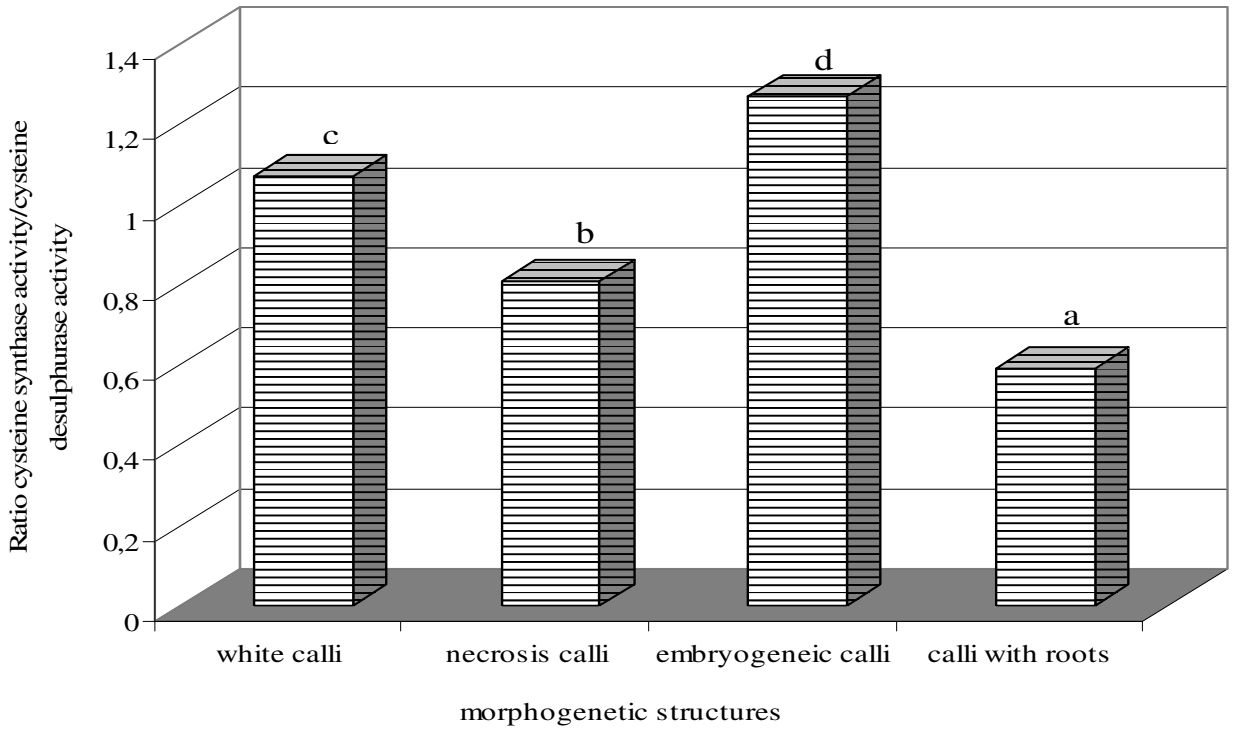


**Figure 8.** Variation of cysteine synthase/cysteine desulphurase ratio in morphogenetic structures. Data are presented as means of five identical experiments with three replicate. Values that are significantly different at the 5% level of significance are indicated with different letters.

embryogenesis of many cacao genotypes observed when the media were supplied with sulphate as reported by Minyaka et al. (2008). Sulphur in culture media was supplied as $MgSO_4$ and $K_2SO_4$. Both sulphate salts were uptaken up by explants cells. Within a cell, sulphate is subsequently activated into adenosine 5'-phosphosulfate (5'-adenylyl-sulfate [APS]) for further conversion. The major assimilatory pathway is reduction of APS to sulfite ($SO_3^{2-}$) and then sulfide ($S^{2-}$). Sulphide is then coupled with O-acetyl- serine (OAS) that is formed from serine, yielding cysteine (Leustek, 2002). Cysteine is the central compound for production of a variety of metabolites containing reduced sulfur, such as methionine, glutathione, phytochelatins, and glucosinolates (Saito, 2000). In contrast to cysteine content, glutathione content was lower in embryogenic calli than non embryogenic calli. It seems that in embryogenic calli, glutathione is catabolised in order to liberate cysteine for embryo development or cysteine synthesized from sulphate is used for another metabolic pathway but not for the synthesis of glutathione. The accumulation of glutathione in white calli, necrosis calli and calli with roots (all non embryogenic) could be explained when we assume that most cysteine is used for the synthesis of glutathione; but no for metabolic sequences necessary for somatic embryo development. Reducing sugars content was particular to each morphogenetic structure. The accumulation of this group of carbohydrate appeared to be associated to the differentiation of roots or somatic embryos. However, the content of reducer carbohydrates was higher in embryogenic calli than calli with roots. This might consolidate the fact that reducer carbohydrates are important for calli formation and cell differentiation (Ana et al., 1997). Hence, it appears that, in cacao, somatic embryogenesis is associated with high synthesis of cysteine, reduction in carbohydrates, and glutathione catabolism. Cysteine synthase activities discriminate embryogenetic calli from their non embryogenetic homologous. The association of embryogenic response, the activities of cysteine synthase and cysteine content in embryogenic calli confirmed the use of exogenous sulphur (sulphate) for the synthesis of cysteine crucial for somatic embryogenesis in cacao. This therefore explains the reduction and the absence of somatic embryo response observed during sulphur depletion in culture media. Xu and Moller (2004) reported that cysteine deficiency inhibits zygotic embryogenesis in *A. thaliana* from the globular state.

Cysteine desulphurase activities were highest in necrosis calli (which frequently differentiate embryo) and embryogenic calli. Additionally, the ratio cysteine synthase activities/cysteine desulphurase activities were determined in each type of morphogenetic structures in order to evaluate the difference between the flux of synthesized cysteine and cysteine used (as precursor for the synthesis of other sulphurous metabolites). This ratio was above one in embryogenic calli. This might suggest that, in embryogenic calli there is very high cysteine synthesis, however, a fraction of cysteine synthesized is used in other biosynthesis pathways. In plant, cysteine is used as precursor of thiamine and biotine (Begley et al., 1999).

## Conclusion

In *in vitro* culture conditions, sulphur supply improves somatic embryogenesis response in cacao. Contrarily, sulphur depletion in culture media gradually and irreversibly inhibits somatic embryogenesis because, sulphur depletion in culture media impairs cysteine (which appears to be crucial for somatic embryogeneis in cacao) synthesis catalyzed by cysteine synthase. And the cysteine synthesized is partially used in other biosynthetic pathways. Sulphur nutrition is therefore crucial in cacao somatic embryogenesis.

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