Plant Cell Tiss Organ Cult (2008) 94:149–160
DOI 10.1007/s11240-008-9398-5

ORIGINAL PAPER

Effect of $MgSO_4$ and K_2SO_4 on somatic embryo differentiation in *Theobroma cacao* L.

Emile Minyaka · Nicolas Niemenak · Fotso · Abdourahamane Sangare · Denis Ndoumou Omokolo

Received: 6 November 2007 / Accepted: 16 May 2008 / Published online: 5 June 2008

Abstract Somatic embryogenesis in cacao is difficult and this species is considered as recalcitrant. Therefore, reformulation of culture media might be a breakthrough to improve its somatic embryogenesis. In cacao, acquisition of somatic embryogenesis competence involves three main stages: induction of primary callus, induction of secondary callus and embryo development. Screening for $MgSO_4$ and K_2SO_4 concentrations for somatic embryo differentiation was conducted on three genotypes (Sca6, IMC67 and C151-61) at the three stages. The effect of these two salts in culture media appears to be most efficient at the embryo development stage. At this stage, high $MgSO_4$ (24 mM) and K_2SO_4 (71.568 mM) in the culture media induced direct somatic embryos on staminodes and petals of the Sca6 and IMC67 genotypes. Media supplemented with 6.0 mM and 12.0 mM $MgSO_4$ enabled high responsive of explants and produced high proportion of embryos. The positive effect of $MgSO_4$ and K_2SO_4 on the acquisition of embryogenesis competence was further tested on seven cacao genotypes reputed as non embryogenic: SNK12, ICS40, POR, IMC67, PA121, SNK64 and SNK10. All these genotypes were able to produce somatic embryos depending on the $MgSO_4$ concentration. Thus, our results showed that the recalcitrance of cacao to somatic embryo differentiation can be overcome by screening for the suitable $MgSO_4$ or K_2SO_4 concentration. Studies of the influence of different K^+/Mg^{2+} ratios (at normal sulphate concentration) on somatic embryo differentiation revealed that sulphate supply was the main factor promoting responsive explants and the proportion of embryos. Cysteine synthase isoforms showed patterns related to morphogenetic structures sustaining that sulphur supply and its assimilation improve somatic embryogenesis in cacao.

Keywords Cacao · Competence · Embryogenesis · Genotypes · Recalcitrance · Sulphate supply

E. Minyaka (✉) · N. Niemenak · Fotso · D. Ndoumou Omokolo
Department of Biological Sciences, Higher Teachers Training College, University of Yaounde I, P.O. Box 47, Yaounde, Cameroon
e-mail: minyakae@yahoo.fr

E. Minyaka · A. Sangare
Laboratoire Central de Biotechnologies, Centre National de Recherche Agronomique, P.O. Box 1740, Abidjan 01, Cote d'Ivoire

E. Minyaka
Laboratoire de génétique, UFR BIOSCIENCES, University of Cocody/Abidjan, P.O. Box 582, Abidjan 22, Cote d'Ivoire

Introduction

Cacao (*T. cacao*) is a perennial and allogam crop. The allogamous character of this tropical crop and the absence of an efficient vegetative propagation system

hinder the vulgarization of elite genotypes among farmers. A go forward was achieved by studying cacao somatic embryogenesis in order to develop an efficient tissue culture system for a rapid and efficient vegetative multiplication of elite genotypes (Esan 1975; Lopez-Baez et al. 1993; Omokolo et al. 1997, Li et al. 1998; Maximova et al. 2002; Minyaka et al. 2004). Current results indicate that many genotypes are recalcitrant whereas somatic embryogenesis response is affected by genotypes and culture medium (Tan and Furtek 2003).

The culture media currently used for cacao somatic embryogenesis are made of MS (Murashige and Skoog 1962) or DKW (Driver and Kuniyuki 1984) basal salts. The core difference between both mineral complexes is their sulphate content. DKW basal salt, where a set of *T. cacao* genotypes were positively tested for somatic embryogenesis, contains higher amounts of sulphate than MS mineral complex which gives low embryo yield. Besides, the analysis of cacao endosperm during seeds development has revealed high concentrations of sulphate (Sossou-Dangou et al. 2002). In a previous study, we reported an implication of cysteine, glutathione and cysteine synthase in cacao zygotic embryogenesis (Minyaka et al. 2007). Sulphate or sulphur metabolism may therefore play a key role in cacao embryogenesis. Furthermore, somatic embryogenesis is known as a cells-stress-response process. The stress conditions can result from pathogenesis (Jabs and Slusarenko 2000), wounding (Fowler et al. 1998), plant hormones (Sessa et al. 1995), cold or salts. Under these stress conditions, plant cells alter their metabolism, growth and development in order to best suit the new environment (Dahleen and Bregitzer 2002). Elemental sulphur (S^6) was reported to be implicated in the resistance against *Verticillium dahliae* in *T. cacao* (Cooper et al. 1996).

In plant, sulphate is the main and primary source of sulphur. The integration of reduced sulphur in the amino acid cysteine (catalyzed by cysteine synthase; EC.2.5.1.47) is positioned at a decisive stage of assimilatory sulphate reduction pathways (Wirtz et al. 2004). The importance of cysteine in *Arabidopsis thaliana* zygotic embryogenesis has been reported (Xu and Møller 2004). Currently, in plant sulphurous biomolecules, sulphur not only serves as a structural component but it is also implicated in the catalytic, electrochemical or characteristic functions of these molecules in cells (Saito 2004). This may highlight a probable implication of sulphur metabolism in biological processes such as embryogenesis in higher plants.

Very little is known about the effect of sulphate supply in culture media on somatic embryogenesis and the implication of cysteine synthase in this process has not yet been reported.

In the present report we focus on the effect of exogenous sulphate on cacao somatic embryogenesis in order to improve media formulation and culture protocols for micropropagation of this plant. Therefore, the effect of $MgSO_4$ and K_2SO_4 supply in cacao somatic embryogenesis was analyzed. Moreover, cysteine synthase was analyzed during cacao somatic embryogenesis in order to unravel at the mechanistic level the role of sulphur during this process.

Material and methods

Explant material

The explants consisted of staminodes (sterile stamens) and petals from immature flower buds of different cacao genotypes. Flower buds from a single tree of each genotype were simultaneously harvested (early in the morning in cold water) at the CNRA (*Centre National de Recherche Agronomique*) experimental farm at Bengerville (Abidjan, Côte d'Ivoire) and at IRAD (*Institut de Recherche Agricole pour le Developpement*) gene-bank at Nkolbisson (Yaounde, Cameroon). They were surface-sterilized by immersion in 1% (w/v) calcium hypochlorite for 20 min, followed by four times rinsing with sterilized-distilled water. Staminodes and petals were then extracted and placed on culture media in Petri dishes (Ø 90 mm).

Screening for the best K_2SO_4 and $MgSO_4$ concentrations for somatic embryo development

The protocol used in this study was adapted from that of Maximova et al. (2005) which encompassed three main steps: induction in primary callus growth medium, maintenance in secondary callus growth medium and embryo expression in embryo development medium. Instead of using woody plant medium (WPM) as mineral complex in secondary callus

growth medium as described by Maximova et al. (2005), all media were defined using DKW basal salts as described by Driver and Kuniyuki (1984). To test the effect of sulphate supply on cacao somatic embryogenesis, K_2SO_4 and $MgSO_4$ (used in DKW mineral complex) were separately used as regulatory factors at each step. One sulphate salt was varied in a given step, while the second sulphate salt was maintained constant and normal. For each salt, six concentrations were used at each step.

The concentration of a given sulphate salt (K_2SO_4 or $MgSO_4$) at each step was given by:

$$C_{n+1} = U_0 \cdot 2^n \quad (0 \leq n \leq 5); n \in IN$$

where C = Concentration (mM); $U_0 = 4.473$ mM (for K_2SO_4); $U_0 = 1.5$ mM (for $MgSO_4$).

Experiment 1: K_2SO_4 and $MgSO_4$ modification in primary callus growth medium

Explants were cultured in primary callus growth medium containing 4.473 (negative control), 8.946 (positive control), 17.892, 35.784, 71.568 and 145.134 mM K_2SO_4 or 1.5 (negative control), 3.0 (positive control), 6.0, 12.0, 24.0 and 48.0 mM $MgSO_4$. Primary callus growth medium was supplemented with 250 mg l^{-1} glutamine, 100 mg l^{-1} myo-inositol, 1 ml l^{-1} DKW vitamin stock (100 mg ml^{-1} myo-inositol, 2 mg ml^{-1} thiamine-HCl, 1 mg ml^{-1} nicotinic acid and 2 mg ml^{-1} glycine), 20 g l^{-1}glucose, 18 μM 2,4 dichlorophenoxyacetic acid (2,4-D) and 45.4 nM TDZ. Media were dispensed into sterilized Petri dishes after autoclaving for 20 min at 1 bar pressure and 121°C. Each Petri dish contained 35 staminodes and 35 petals in two separated sets (Fig. 1a). Experiments were repeated five times with three replicate Petri dishes at each culture initiation. Petri dishes were incubated in the dark at 25 ± 1°C for 14 days.

After 14 days in primary callus growth medium, explants were transferred (for 14 additional days) into a secondary callus growth medium containing normal K_2SO_4 (8.946 mM) and $MgSO_4$ (3.0 mM) concentrations. Secondary callus growth medium consisted of DKW basal salts, supplemented with 0.5 ml l^{-1} DKW vitamin, 20 g l^{-1} glucose, 9 μM 2,4-D, 250 μg l^{-1} kinetin and 0.2% (w/v) phytagel.

Explants from the secondary callus growth medium were then transferred for 21 days in the embryo development medium containing normal K_2SO_4

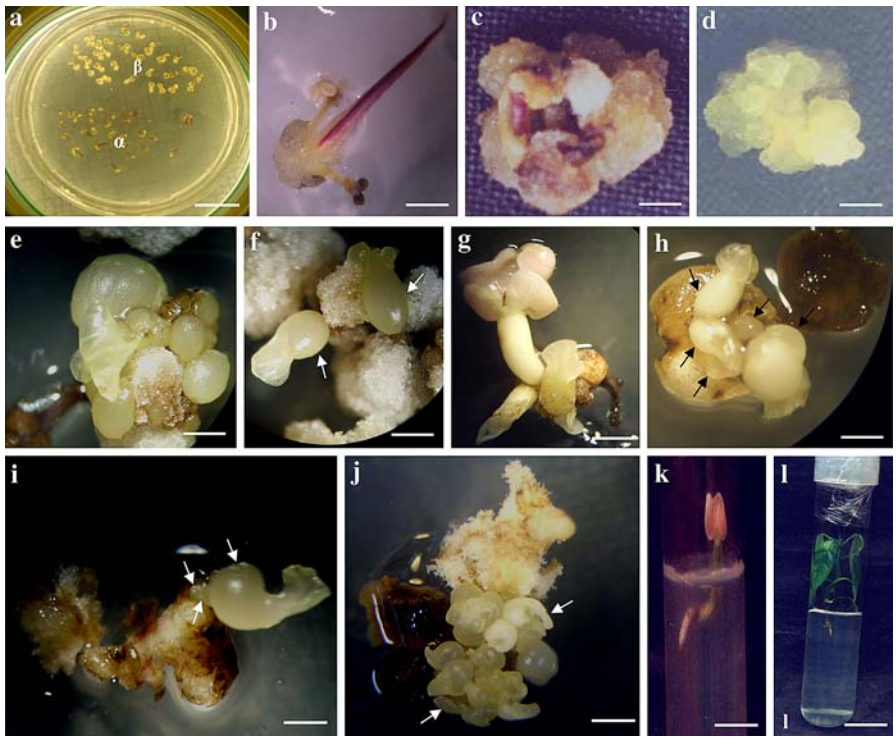

Fig. 1 Different phases of in vitro morphogenetic process in *T. cacao*: (**a**) disposition of staminodes (α) and petals (*β*) explants in a Petri dish; (**b**) staminode explant; (**c**) callus derived from staminode; (**d**) callus derived from petal; (**e**) and (**f**) somatic embryos at different maturation stages derived from well developed calli (indirect somatic embryogenesis); (**g**) and (**i**) direct somatic embryogenesis from staminode-explants; (**h**) direct somatic embryogenesis from petal-explant); (**j**) repetitive secondary somatic embryogenesis; (**k**) and (**l**) regeneration of plantlets. Arrows indicate structures to be shown. Bar = 0.5 cm

(8.946 mM) and $MgSO_4$ (3.0 mM) concentrations. Two successive subcultures of explants were carried out in embryo development medium at intervals of 21 days. Embryo development medium was made of DKW basal salt supplemented with 1 ml DKW vitamin, 30 g l^{-1} sucrose, 1 g l^{-1} glucose and 0.2% (w/v) phytagel.

Experiment 2: K_2SO_4 and $MgSO_4$ modification in secondary callus growth medium

Explants were cultured in primary callus growth medium containing normal K_2SO_4 (8.946 mM) and $MgSO_4$ (3.0 mM) concentrations. After 14 days of incubation, explants were randomly transferred in secondary callus growth medium containing 4.473 (negative control), 8.946 (positive control), 17.892, 35.784, 71.568 and 145.134 mM K_2SO_4 or 1.5 (negative control), 3.0 (positive control), 6.0, 12.0, 24.0 and 48.0 mM $MgSO_4$. Fourteen days later, explants were transferred in embryo development medium containing normal K_2SO_4 and $MgSO_4$ concentrations and incubated for 21 days. Two additional subcultures were done in embryo development medium. The experiment was repeated five times with three replicate Petri dishes at each experiment.

Experiment 3: K_2SO_4 and $MgSO_4$ modification in embryo development

Explants were first cultured in primary callus growth medium with normal K_2SO_4 and $MgSO_4$ concentrations. After 14 days, they were transferred into secondary callus growth medium and incubated for 14 days. To test the effect of sulphate supply in embryo development medium on somatic embryogenesis, 4.473 (negative control), 8.946 (positive control), 17.892, 35.784, 71.568 and 145.134 mM K_2SO_4 or 1.5 (negative control), 3.0 (positive control), 6.0; 12.0; 24.0 and 48.0 mM $MgSO_4$ were used. Explants derived from secondary growth medium were randomly transferred in embryo development medium with the above concentrations of K_2SO_4 or $MgSO_4$. Two additional and successive subcultures of explants were done in embryo development medium at intervals of 21 days. The experiment was repeated five times with three replicate Petri dishes at each experiment.

Experiment 4: Clonal variability of somatic embryogenesis response in the best modified embryo development medium

After screening, 6.0 mM, 12.0 mM and an average concentration of 9 mM of $MgSO_4$ were defined. Seven genotypes (SNK12, ICS40, POR, UPA409, IMC67, Sca6 and PA121) from the CNRA experimental farm at Bengerville-Abidjan in Côte d'Ivoire and two genotypes (SNK64 and SNK10) from IRAD (*Institut de Recherche Agricole pour le Developpement*) gene-bank at Nkolbisson (Yaounde–Cameroon) were tested using these three $MgSO_4$ concentrations in embryo development medium. SNK10, SNK12 and SNK64 are not yet reported to be competent for somatic embryogenesis. ICS40 and IMC67 (Tan and Furtek 2003), PA121 and POR (CNRA/LCB internal experimentation) were reported to be non embryogenic. UPA409 (Alemanno et al. 1996) and Sca6 (Li et al. 1998) were reported to be embryogenic.

Experiment 5: Influence of K^+/Mg^{2+} on the somatic embryo expression of Sca6

Since the variation of K_2SO_4 or $MgSO_4$ altered the K^+/Mg^{2+} ratio and this might influence somatic embryogenesis, this experiment was conducted to evaluate the hypothetical effect of K^+/Mg^{2+} ratio on somatic embryogenesis expression and to dissociate it from that of sulphate (SO_4^{2-}). The embryo development medium was supplemented with different $K_2SO_4/MgSO_4$ ratios (25.281, 14.747, 7.900, 2.107, 0.562, 0.301 and 0.176). These ratios were calculated from 2.3385, 2.7283, 2.9231, 1.559, 0.7795, 0.3898, 0.1949 g l^{-1} K_2SO_4 and 0.0925, 0.1850, 0.3700, 0.7400, 1.3875, 1.2950, 1.1100 g l^{-1} $MgSO_4$, respectively. Sulphate (SO_4^{2-}) concentration in embryo development medium was constant and normal (1.0964 g l^{-1}) while K^+/Mg^{2+} ratio varied. Explants were simultaneously cultured in the seven K^+/Mg^{2+} ratios. The experiment was repeated five times with three replicate Petri dishes per ratio each time.

Cysteine synthase extraction and analysis

For cysteine synthase analysis, morphogenetic structures were collected and cysteine synthase was immediately extracted using the method of Warrilow

and Hawkesford (1998). One gram of morphogenetic structures (staminodes, petals, embryos, embryogenic calli and non embryogenic calli) were 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, 0.2% (w/v) sodium ascorbate. After centrifugation at 10,000 g for 20 min, the supernatant was used for protein and cysteine synthase analysis. Total protein content was assayed using Bradford (1976) method. Soluble proteins were used for native PAGE (polyacrylamide gel electrophoresis). 100 µl protein extract were mixed with an equal volume of sample buffer (20% (v/v) glycerol, 38 mM Tris-base, 0.293 M glycine, 0.048 M dithiothreitol and 0.02% (w/v) bromophenol blue). The electrophoresis was done at an intensity of 30 mA in a solution of 0.050 M Tris-base and 0.384 M glycine (pH 8.8). The gel was stained for the cysteine synthase activity using the KCN-Pb stain method described by Warrilow and Hawkesford (1998). The solution used to stain the native PAGE gels for cysteine synthase activity contained 1.3% (w/v) TRIS-base, 0.1% (w/v) cysteine-HCl, 0.05% (w/v) lead acetate, 0.02% (w/v) KCN and 0.01% (w/v) dithiothreitol.

Data collection

After 91 days of culture initiation, the number of embryos expressed per genotype per medium per Petri dish and type of explant (stimanodes or petals) was recorded. The responsive of genotypes to somatic embryogenesis and the proportion of embryos per culture condition were calculated, thus:

$$\text{Responsive} = \frac{\text{Total staminodes or petals in a given Petri dish producing embryos}}{\text{Total staminodes or petals in a given Petri dish}} \times 100$$

$$\text{Proportion of embryos per culture condition} = \frac{\text{Number of embryos from a given genotype and explant-type in a given } K_2SO_4 \text{ or } MgSO_4 \text{ concentration}}{\text{Total number of embryos from a given genotype and explant-type in all } K_2SO_4 \text{ or } MgSO_4 \text{ concentrations}} \times 100$$

Statistical analysis

All statistical analyses were performed by SPSS (version 10.0) software. Data were subjected to analysis of variance (ANOVA); the standard error was estimated and the means were separated with the test of Student, Newman and Keuls (SNK) at 5% significance level.

Results

Somatic embryogenesis

Callus induction and embryo differentiation

When cultured in primary callus growth medium, staminode (Fig. 1b) and petal explants enlarged within the first 7 days of incubation. Calli were distinguishable between 9th and 13th day in primary callus growth medium. Callus development was well established 5 to 8 days after the subculture of explants in secondary callus growth medium (Fig. 1c and d). By the end of three-week in embryo development medium, some calli differentiated roots or embryos. After 6 weeks in embryo development medium, callus-derived explants substantially differentiated somatic embryos. The date of appearance of the first somatic embryos depended mainly on the sulfur content in embryo development medium. The earliest somatic embryos were observed between 42nd and 58th day of culture on callus-derived staminodes or petals mostly from embryo development medium containing 6.0 and 12 mM $MgSO_4$ (Fig. 1e

and f). High exogenous sulphate (71.568 mM K_2SO_4 and 24.0 mM $MgSO_4$) in ED, inhibited callogenesis and promoted direct differentiation of somatic embryos on staminodes and petals (Fig. 1g and i). When cultured in embryo development medium containing 17.892 mM K_2SO_4, 8.21 ± 1.75% and 10.19 ± 3.2% staminodes produced repetitive embryo clusters from IMC67 and Sca6, respectively (Fig. 1j). The maturation of these somatic embryos led to plantlet regeneration (Fig. 1k and l). Thus in cacao tissue culture, we reported different morphogenetic structures summarized in a diagram (Fig. 2).

Effect of sulphate supply in primary and secondary callus growth media on embryogenesis

Concentrations of K_2SO_4 and $MgSO_4$ were modified in the primary and secondary callus growth media to subsequently evaluate their action on embryogenesis in the embryo development medium. Results showed that when these sulphate concentrations were applied in primary callus growth medium, the explants responsive to embryogenesis did not change compared to the positive control (data not shown). However, in the secondary callus growth medium, 17.892 mM K_2SO_4 (2 fold higher than the positive control), improved the responsiveness of Sca6 and IMC67 explants to embryogenesis (Fig. 3). When $MgSO_4$ was used as sulphate supplying salt in the secondary callus growth medium, a peak of responsive for the three genotypes was observed at $MgSO_4 = 6.0$ mM (data not shown).

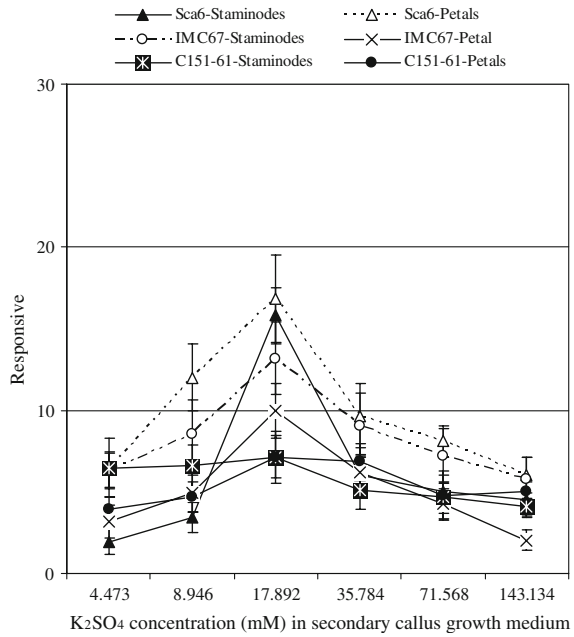

Fig. 3 Influence of K_2SO_4 content on the responsive of Sca6, IMC67 and C151-61 explants. Explants were subcultured during 91 days. Vertical bars represent standard error. Each plot was drawn from means of five identical experiments. Individual experiment included three replicate Petri dishes with 35 staminode and 35 petal-derived morphogenetic structures. Significance was determined at $P < 0.05$ using ANOVA

Effect of sulphate supply in embryo development medium on embryogenesis

Converse to what was observed in the primary and secondary callus growth media, sulphate supply as

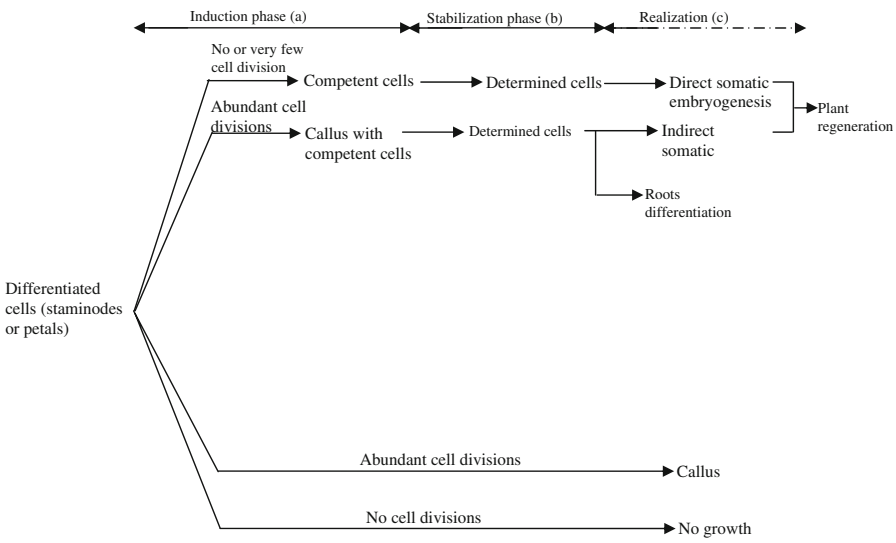

Fig. 2 General sequence of in vitro tissue culture development in *T. cacao* (adapted from De Klerk *et al.*, 1997); a- primary callus growth medium, b- secondary callus growth medium, c- embryo development medium

K_2SO_4 or $MgSO_4$ in embryo development medium significantly affected somatic embryogenesis expression of the three genotypes (Sca-6, IMC67 and C151-61) explants.

There was no embryo expression in 4.473 and 145.134 mM K_2SO_4, for all tested genotypes. The aptitude of Sca6 explants to produce somatic embryos significantly increased with 17.892 and 35.784 mM K_2SO_4 in embryo development medium. With IMC67 genotype, the highest responsive was observed with 17.892 mM K_2SO_4 for both types of explants. However, with this K_2SO_4 content (17.892 mM) petals reacted better than staminodes. With C151-61 genotype, high responsive was also observed in 17.892 mM K_2SO_4. At this K_2SO_4 concentration, the responsive of C151-61 staminodes and petals were not significantly different (Fig. 4).

Unlike what was observed with 4.473 mM K_2SO_4 (the negative control of K_2SO_4), all tested genotypes were embryogenic in 1.5 mM $MgSO_4$ (the negative control of $MgSO_4$). However, for a given genotype, both types of explants were not always simultaneously responsive in embryo development medium containing 1.5 mM $MgSO_4$. Furthermore, the use of $MgSO_4$ as regulatory factor displayed different patterns of somatic embryogenesis. The responsive of Sca6 was highest in embryo development medium with 6.0 mM $MgSO_4$ for both explant types. Hence, 2 and 6 fold (for staminodes and petals, respectively) increase in responsive were registered from 3.0 to 6 mM $MgSO_4$ in embryo development medium. IMC67 staminodes displayed a high responsive ($31.03119 \pm 4.566165\%$) with 6.0 mM $MgSO_4$ in embryo development medium while the petals of this genotype appeared to be most responsive with 12 mM $MgSO_4$ in embryo development medium. $MgSO_4$ at 6.0 mM displayed the highest value of responsive for C151-61 staminodes ($19.247 \pm 1.35\%$) while, for petals ($20 \pm 1.5\%$) 12 mM $MgSO_4$ in embryo development medium was the best medium (Fig. 5). Beside the effect of sulfate supply on genotype and explant response to somatic embryogenesis, this set of results also shows an influence of sulfate salt type on this variable.

The proportion of embryos was evaluated for the three genotypes. The results indicate that, Sca6 staminodes ($72.05 \pm 4.7\%$) and petals ($63.07 \pm 5.7\%$) displayed the highest proportion of embryos when

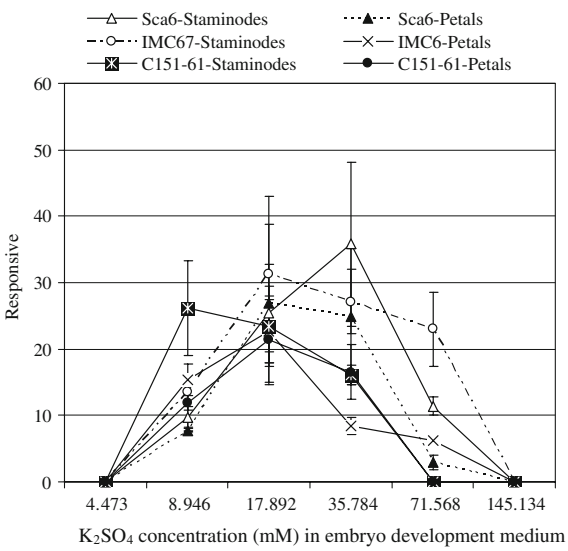

Fig. 4 Influence of K_2SO_4 concentration on the responsive of Sca6, IMC67 and C151-61 explants. Explants were subcultured during 91 days. Vertical bars represent standard error. Each plot was drawn from means of five identical experiments. Individual experiment included three replicate Petri dishes with 35 staminode and 35 petal-derived morphogenetic structures. Significance was determined at $P < 0.05$ using ANOVA

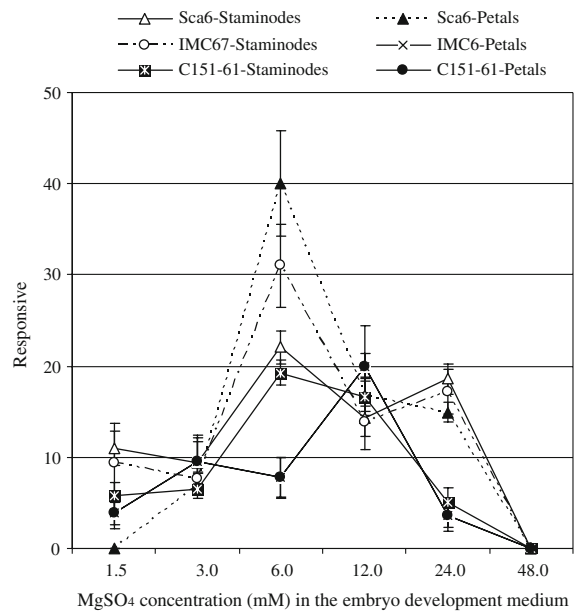

Fig. 5 Influence of $MgSO_4$ concentration on the responsive of Sca6, IMC67 and C151-61 explants. Explants were subcultured during 91 days. Vertical bars represent standard error. Each plot was drawn from means of five identical experiments. Individual experiment included three replicate Petri dishes with 35 staminode and 35 petal-derived morphogenetic structures. Significance was determined at $P < 0.05$ using ANOVA

cultured in embryo development medium with 35.784 mM K_2SO_4. An increase (29.16 fold and 9.89 fold for staminodes and petals, respectively) in the proportion of embryos was observed from embryo development medium with 8.946 mM K_2SO_4 to embryo development medium with 35.784 mM K_2SO_4. A decrease in the proportion of embryos occurred from embryo development medium with 35.784 mM K_2SO_4 to embryo development medium with 145.134 mM. IMC67 staminodes and petals showed the highest proportion of embryos in embryo development medium with 17.892 mM K_2SO_4. Staminodes of C151-61 genotype exhibited the highest proportion of embryos in embryo development medium containing 17.892 mM K_2SO_4 while the petals reached their peak in embryo development medium with 35.784 mM K_2SO_4 (Fig. 6).

Taken as a whole, a significant increase of the proportion of embryos was observed from embryo development medium with 4.473 mM K_2SO_4 to embryo development medium with 17.892 mM K_2SO_4 for the three genotypes and both type of explants. Beyond 17.892 mM K_2SO_4 in embryo development medium, the proportion of embryos significantly decreased for staminodes and petals.

Experiments conducted with $MgSO_4$ as the regulatory factor indicate that, the proportion of embryos of Sca6 staminodes significantly increase from 1.5 mM $MgSO_4$ in embryo development medium (5.77 ± 2.21%) to 24.0 mM $MgSO_4$ in embryo development medium (42.22 ± 3.87%) while petals of this genotype presented the highest proportion of embryos with 6.0 mM $MgSO_4$ in embryo development medium (62.33 ± 5.37%). IMC67 staminodes (64.91 ± 4.87%) and petals (62.90 ± 4.65%) appeared to be most embryogenic when these explants were cultured in embryo development medium with 6.0 mM $MgSO_4$. C151-61 explants also exhibited the highest proportion of embryos in embryo development medium with 6.0 mM $MgSO_4$ (Fig. 7). Thus, sulfate supplied as K_2SO_4 or $MgSO_4$ improves somatic embryogenesis for the three cacao genotypes, and $MgSO_4$ seems to be more effective (in promoting somatic embryogenesis) than K_2SO_4.

Therefore, sulphate content in the embryo development medium, substantially affected the responsive

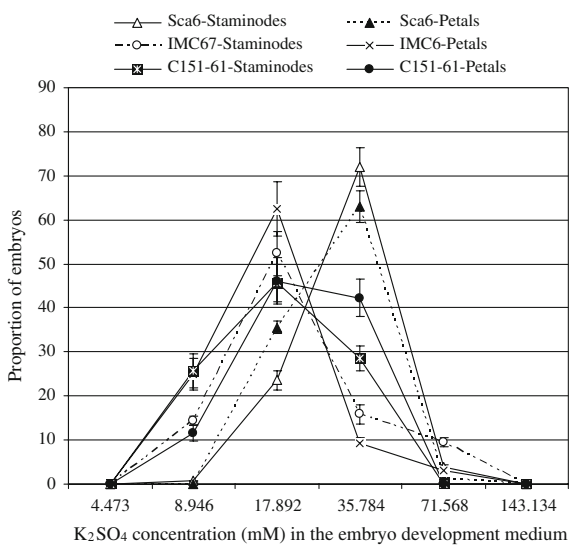

Fig. 6 Influence of K_2SO_4 concentration on the proportion embryos of Sca6, IMC67 and C151-61 explants. Explants were subcultured during 91 days. Vertical bars represent standard error. Each plot was drawn from means of five identical experiments. Individual experiment included three replicate Petri dishes with 35 staminode and 35 petal-derived morphogenetic structures. Significance was determined at $P < 0.05$ using ANOVA

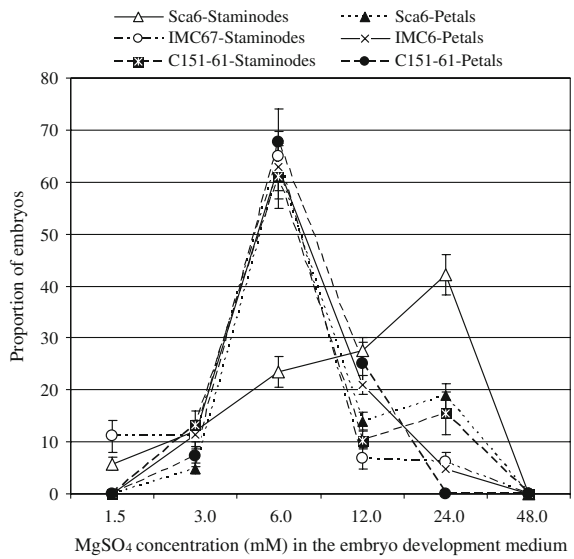

Fig. 7 Influence of $MgSO_4$ concentration on the proportion of embryos of Sca6, IMC67 and C151-61 explants. Explants were subcultured during 91 days. Vertical bars represent standard error. Each plot was drawn from means of five identical experiments. Individual experiment included three replicate Petri dishes with 35 staminode and 35 petal-derived morphogenetic structures. Significance was determined at $P < 0.05$ using ANOVA

and the proportion of embryos of genotypes tested for somatic embryogenesis.

Embryogenic responsive of nine cacao genotypes

To evaluate how far sulphate supply in culture medium can influence cacao somatic embryogenesis, the best sulphate salts concentrations ($MgSO_4 = 6.0$ mM), ($MgSO_4 = 12.0$ mM) and their average ($MgSO_4 = 9.0$ mM) were tested on seven cacao genotypes known to be non embryogenic and two genotypes reported as embryogenic. The results obtained showed significant difference between genotypes and explants in the same medium. The genotypes ICS40, PA121, POR, SNK64, SNK12, SNK10 or IMC67, which have not yet been reported to be embryogenic, differentiated somatic embryos (Table 1). Staminodes from ICS40 differentiated somatic embryos when the embryo development medium was supplied with 9.0 mM $MgSO_4$. PA121, SNK10, SNK12, SNK64 and POR staminodes produced embryos in embryo development medium containing 6.0 mM $MgSO_4$. The IMC67 produced embryos in the three sulfate concentrations tested. The embryogenic potential of the reference genotypes, Sca6 and UPA409, was not altered by any of the sulphate contents in embryo development medium.

Effect of Mg^{2+}/K^{+} ratio on somatic embryo expression

When K_2SO_4 or $MgSO_4$ is added in the embryo development medium, the ratio of K^{+}/Mg^{2+} is modified. To measure the impact of this ratio on somatic embryo expression, their effect was analysed. The results indicated that, the responsive of explants was higher at $K^{+}/Mg^{2+} = 14.747$ and lower in the reverse ratio ($K^{+}/Mg^{2+} = 0.301$). There was no significant difference in the explant responsive between 25.281, 7.900, 2.107 (the control), 0.562, 0.301 and 0.176 ratios. However, the highest proportion of embryos (30.14 ± 3.87%) was observed in the control, indicating that sulphate content in the medium is the most favourable factor of somatic embryogenesis (Table 2).

Cysteine synthase profile

The analysis of cysteine synthase displayed seven isozymes in cacao namely: CS1, CS2, CS3, CS4, CS5, CS6 and CS7. The distribution of these isoforms differed from one morphogenetic structure to another. Necrosed callus, white callus, necrosed callus with embryo, repetitive somatic embryos and rhizogenic callus were characterized by isoforms CS3, CS5 and CS7. The activity of CS3 and CS5 seemed to be very high in repetitive somatic embryos. Somatic embryo with cotyledons displayed five isoforms CS1, CS2, CS3, CS5 and CS7. Somatic embryo with cotyledons presented two isoforms, CS1 and CS2, absent

Table 1 Responsive of different cacao genotypes

$MgSO_4$ concentration in embryo development medium	Genotype	Explant-type responsive (%)	
		Staminode	Petal
6 mM	Sca6	22.85 ± 3.67f	17.62 ± 2.38c
	IMC67	29.03 ± 4.18g	20.08 ± 2.15cd
	UPA409	11.39 ± 2.47d	5.32 ± 1.49b
	ICS40	0a	0a
	POR	2.69 ± 1.51b	0a
	SN12	15 ± 2.43e	0a
	PA121	15.06 ± 4.37e	0a
	SNK10	3.84 ± 1.53bc	0a
	SNK64	11.42 ± 2.85d	5.88 ± 1.76b
9 mM	Sca6	19.33 ± 5.16e	11.92 ± 1.47c
	IMC67	18.53 ± 3.70e	15.23 ± 1.90d
	UPA409	14.52 ± 3.15e	12.33 ± 4.25c
	ICS40	12.3 ± 1.77d	0a
	POR	6.24 ± 1.9b	0a
	SN12	0a	0a
	PA121	0a	0a
	SNK10	7.41 ± 1.32bc	0a
	SNK64	6.66 ± 2.45b	3.44 ± 1.79b
12 mM	Sca6	13.58 ± 2.98bc	10.85 ± 2.25bc
	IMC67	19.04 ± 5.55c	15.73 ± 4.08d
	UPA409	10.43 ± 1.76b	6.53 ± 2.74b
	ICS40	0a	0a
	POR	0a	0a
	SN12	0a	0a
	PA121	0a	0a
	SNK10	0a	0a
	SNK64	0a	0a

Data are presented as means of Explants responsive ± Standard Error. For each $MgSO_4$ concentration and explant-type, values that are significantly different within column at the 5% level of significance are indicated with different letters

Table 2 Responsive and proportion of embryos of Sca6 explants in different K^+/Mg^{2+} ratios

	K^+/Mg^{2+} ratio in the embryo development medium						
	25.281	14.747	7.900	2.107 (control)	0.562	0.301	0.176
Explant responsive (%)	10.63 ± 1.56b	21.10 ± 3.78c	11.88 ± 2.0b	10.80 ± 2.11b	8.85 ± 1.42b	4.28 ± 0.76a	14.376 ± 2.11b
Proportion of embryos (%)	9.56 ± 1.33b	17.22 ± 1.98c	12.91 ± 1.07bc	30.14 ± 3.87d	8.61 ± 1.90b	3.34 ± 1.13a	18.18 ± 4.52c

Data are presented as means of Explant responsive ± Standard Error (SE) and Proportion of embryos ± SE. Values that are significantly different within line at the 5% level of significance are indicated with different letters

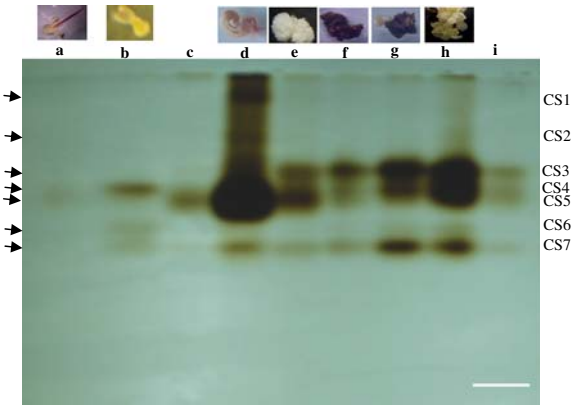

Fig. 8 Cysteine synthase profile of different morphogenetic structures from *T. cacao*. a-staminodes, b-petals, c-endosperm, d-somatic embryo with cotyledons, e-white callus, f-necrosed callus, (g, h)-necrosed callus with embryo, h-repetitive secondary embryogenesis, i-rhizogenic callus. Bar = 1.25 cm

elsewhere. Somatic embryo with cotyledons was also characterized by a high activity of CS5. Endosperm presented CS5 activity similar to staminodes. Petals were characterized by CS4, CS6 and CS7 activity (Fig. 8).

Discussion

Under specific conditions, a somatic plant cell can dedifferentiate to give a totipotent embryogenic cell that has the ability to proliferate and/or regenerate an embryo. In many cases, somatic embryos appear from cells that are induced by either high doses of auxin or stress-related treatments (Halperin 1995). For crops such as cacao, a limiting factor is the lack of a reliable procedure for embryogenesis and plant regeneration from a wide range of genotypes. Studies on the improvement of cacao somatic embryogenesis have so far focused on the effect of growth regulators and basal salt composition (Li et al. 1998; Tan and Furtek 2003). Here we investigated the effect of a single mineral such as K_2SO_4 and $MgSO_4$ on cacao somatic embryogenesis. The screening of K_2SO_4 and $MgSO_4$ supply in three steps has shown that, the influence of sulphate supply on somatic embryogenesis was most effective when sulphate was provided at the embryo development step. According to the level of sulphate in the embryo development medium, direct or indirect somatic embryogenesis can be induced. Indirect somatic embryogenesis (always reported in *T. cacao*) was observed when the K_2SO_4 and $MgSO_4$ concentrations were under 71.568 mM and 24.0 mM, respectively. Direct somatic embryogenesis occurred from cells when the $MgSO_4$ concentration was 24.0 mM. Expression of direct somatic embryogenesis with a high sulphate content implies the inhibition of callogenesis and leads the explant-cells to be directly competent and determined to change their differentiation pathways in order to become embryogenic. This result supports the observation that, stress-related compounds are required for somatic cells to become embryogenic cells (Verdeil et al. 2007). Repetitive primary somatic embryo clusters were observed at high (compared to the positive control) sulphate content.

Sulphate supply up to a limit threshold significantly improves the precocity and the aptitude of the floral explants to express somatic embryogenesis. The explants responsive and the proportion of embryos significantly increased with the K_2SO_4 or $MgSO_4$ content (up to a certain limit) in the embryo development medium. As it is the case with *Arabidopsis thaliana* (Xu and Møller 2004), sulphur appears also to be essential for cacao somatic embryogenesis.

Our results showed that to overcome the recalcitrance of many cacao genotypes, a screening for a suitable K_2SO_4 or $MgSO_4$ concentration is required. Indeed, some recognized recalcitrant cacao genotypes (Tan and Furtek 2003; Alemanno et al. 1996) were able to differentiate somatic embryos at high sulphate content. The present work therefore suggests that, sulphate supply can overcome recalcitrance of cacao genotypes to express somatic embryogenesis. In higher plants, sulphate undergoes reduction-assimilation pathway that leads mainly to cysteine, a pivotal compound in sulphur assimilation (Saito 2004). 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) prevented zygotic embryogenesis to go beyond the globular stage. Moreover, sulphur in Fe–S clusters is directly provided by cysteine and, the synthesis of the latter depends on the sulphate availability (Smith et al. 2001; Ding et al. 2005). In plant, cysteine is used as precursor for the synthesis of other organic sulfurous compounds, such as biotin and thiamine. In a study conducted on date palm (*Phoenix dactylifera* L.), Al-khayri (2001) reported that thiamine and biotin supply improve somatic embryogenesis expression. In addition, these authors reported that, the absence of thiamine resulted in failure of callusing and the absence of somatic embryo differentiation. Thus, exogenous sulphate supply might be necessary for the synthesis of cysteine. This sulfurous metabolite is then used for the synthesis of Fe–S clusters, thiamine or biotin which are involved in somatic embryogenesis process in cacao.

The activity of cysteine synthase, a key enzyme in sulphate assimilation-reduction pathway, was revealed in morphogenetic structures. The cysteine synthase profile showed the patterns linked to the morphogenetic changes that occurred during development. When staminodes were used as explants, CS5 was conserved along the morphogenetic process but its activity changed during the morphogenesis; however there were four other (CS1, CS2, CS3 and CS7) new isoforms that appeared. Two (CS3 and CS7) of them were conserved during the morphogenesis and two (CS1 and CS2) were related to cotyledonary somatic embryos.

The maximum explants responsive observed with $K^+/Mg^{2+} = 14.747$, and the highest proportion of embryos obtained in the control ($K^+/Mg^{2+} = 2.107$), might indicate that besides the preponderant effect of sulphate supply in somatic embryo expression in *T. cacao*, an adequate balance in K^+/Mg^{2+}ratio is also needed for optimal response to somatic embryo stimuli. In *Cucumus melo* L., Kintzios et al. (2004) reported an accumulation of both cations (K^+ and Mg^{2+}) during somatic embryogenesis. Magnesium is usually a cofactor of some kinase enzymes. The *Theobroma* somatic embryo protein kinase (which uses Mg^{2+} as cofactor) has been reported to be an important element for somatic embryo process in cacao (Santos et al. 2005).

Sulphate supply screening therefore, appears as a breakthrough to overcome cacao genotypes recalcitrance to somatic embryogenesis and to improve this process.

Acknowledgements We will like to thank the AUF (Agence Universitaire de la Francophonie) for their fellowship. Authors are also thankful to CNRA/LCB (Centre National de Recherche Agronomique/Laboratoire Central de Biotechnologies) for the chemicals and equipments.

References

Alemanno L, Berthouly M, Michaux-Ferrière N (1996) Embryogenese somatique du cacaoyer a partir de pieces florales. Plant Rech Dev 3:225–233

Bradford MM (1976) A rapid sensitive method for the quantisation of microgram quantities of protein utilizing the principle of proteins binding. Ann Biochem 2:248–254

Cooper RM, Resende MLV, Flood J, Rowan MG, Beale MH, Potter U (1996) Detection and cellular localization of elemental sulphur in disease resistant genotypes of *Theobroma cacao*. Nature 379:159–162

Dahleen LS, Bregitzer P (2002) An improved media system for high regeneration rates from barley immature embryo-derived callus cultures of commercial cultivars. Crop Sci 42:934–938

De Klerk G-J, Arnoldt-Schmitt B, Lieberei R, Neumann K-H (1997) Regeneration of roots, shoots and embryos: physiological, biochemical and molecular aspects. Biol Plant 39(1):53–66

Ding B, Smith ES, Ding H (2005) Mobilization of the iron centre in IscA for the iron-sulphur cluster assembly in IscU. Biochem J 389:797–802

Driver JA, Kuniyuki AH (1984) In vitro propagation of Paradox walnut root stock. Hort Sci 19:507–509

Esan E (1975) Tissue culture studies on cacao (*Theobroma cacao* L.). Int Cacao Research Conference. Ibadan (Nigeria), pp 119–125

Fowler MR, Ong LM, Russinova E, Atanassov AI, Scott NW, Slater A, Elliott MC (1998) Early changes in gene expression during direct somatic embryogenesis in alfalfa revealed by RAP-PCR. J Exp Bot 319(49):249–253

Halperin W (1995) In vitro embryogenesis: some historial issues and unresolved problems. In: Thorpe TA (ed) In vitro embryogenesis in plants. Kluwer Acad Pub, Dordrecht, pp 1–16

Jabs T, Slusarenko AJ (2000) The hypersensitive response. In: Slusarenko A, Fraser R, van Loon L (eds) Mechanisms of resistance to plant diseases. Kluwer Acad Pub, Dordrecht, The Netherlands, pp 279–323

Al-khayri JM (2001) Optimization of biotin and thiamine requirements for somatic embryogenesis of date palm (*Phoenix dactylifera* L.). In Vitro Cell Dev Biol Plant 37:453–456

Kintzios S, Stavropoulou Er, Skamneli S (2004) Accumulation of selected macronutrients and carbohydrates in melon tissue cultures: association with pathways of in vitro dedifferentiation and differentiation (organogenesis, somatic embryogenesis). Plant Sci 167:655–664

Li Z, Traore A, Maximova S, Guiltinan M (1998) Somatic embryogenesis and plant regeneration from floral explant of cocao (*Theobroma cacao* L.) using thidiazuron. In Vitro Cell Dev Biol Plant 34:293–299

Lopez-Baez O, Bollon H, Eskes A, Pétiard V (1993) Embryogenèse somatique du cacaoyer *Theobroma cacao* L à partir des pièces florales. Comptes Rendus Acad Sci Paris 316:579–584

Maximova SN, Alemanno L, Young A, Ferriere N, Traore A, Guiltinan MJ (2002) Efficiency, genotypic variability, and cellular origin of primary and secondary somatic embryogenesis of *Theobroma cacao* L. In Vitro Cell Dev Biol-Plant 38:252–259

Minyaka E, Niemenak N, Nankeu DJ, Boudjeko T, Omokolo ND (2004) Glutamate dehydrgenase and glutamine synthase activities during somating embryogenesis in *Theobroma cacao* L. J Cam Acad Sci 4:306–313

Minyaka E, Niemenak N, Soupi NMS, Sangaré A, Omokolo ND (2007) Implication of cysteine, glutathione and cysteine synthase in *Theobroma cacao* L. zygotic embryogenesis. Biotechnology 6(1):129–137

Murashige T, Skoog F (1962) A revise medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Omokolo N, Tsala N, Niemenak N (1997) Phenol content, acidic peroxides and IAA-Oxides during somatic embryogenesis in *Theobroma cacao* L. Biol Plant 39: 337–347

Saito K (2004) Sulfur assimilatory metabolism. The long and smelling road. Plant Physiol 136:2443–2450

Santos OM, Romano E, Yotoko CSK, Tinoco PML, Dias ABB, Aragão LFJ (2005) Characterisation of the cacao somatic embryogenesis receptor-like kinase (SERK) gene expressed during somatic embryogenesis. Plant Sci 168: 723–729

Sessa G, Yang XQ, Raz V, Eyal Y, Fluhr R (1995) Dark induction and subcellular localization of the pathogenesis-related PRB-1b protein. Plant Mol Biol 28:537–547

Smith AD, Agar JN, Johnson KA, Frazzon J, Amster IJ, Dean DR, Johnson MK (2001) Transfer from IscS to IscU: the first step in iron-sulfur cluster biosynthesis. J Am Chem Soc 123:11103–11104

Sossou-Dangou J, Hocher V, Ferrière N, Fulcheri C, Morard P, Alemanno L (2002) Histological and biochemical characterization of *Theobroma cacao* L. endosperm during seed development. Seed Sci Res 12:91–100

Tan CL, Furtek DB (2003) Development of an in vitro regeneration system for *Theobroma cacao* from mature tissues. Plant Sci 164:407–412

Verdeil J-L, Alemanno L, Niemenak N, Tranbager TJ (2007) Pluripotent versus totipotent plant cells: dependence versus autonomy. Trends Plant Sci 12(6):245–252

Warrilow AGS, Hawkesford MJ (1998) Separation, subcellular localization and influence of sulphur nutrition on isoform of cysteine synthase in spinach. J Exp Bot 327(49): 1625–1638

Wirtz M, Droux M, Hell R (2004) O-acetylserine (thiol) lyase: an enigmatic enzyme of plant cysteine biosynthesis revisited in *Arabidopsis thaliana*. J Exp Bot 404(55): 1785–1798. August 2004

Xu M, Møller S (2004) AtNAP7 is a plastidie sufC-like ATP-buriding casette/AtPase essential for Arabidopsis embryogenesis. Proc Nat Acad Sci 101(24):9143–9148