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Full Length Research Paper

Esterase isoenzymes are linked to embryogenic structures induction in cotton cell suspension cultures

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Esterase activity and isoenzymes pattern of two cultivars of cotton (*Gossypium hirsutum* L.), Coker 312 an embryogenic cultivar and ISA 205N a non embryogenic cultivar, were studied and compared during cell suspension cultures. The use of polyacrylamide gel electrophoresis allowed the identification of isoenzymes that number increased with the successive stages of cell culture of the two cultivars. At the stage of embryogenic structures induction which occurs only in Coker 312 cell suspension, we noted the presence of two isoenzymes (y and z) identified as aryl esterase, while one isoenzyme (x) identified as choline esterase was exclusively found in the cell suspensions of the non embryogenic cultivar ISA 205N. Esterase activity increased in cells of Coker 312 whereas it's remained constant in ISA 205N. These results suggested a great implication of esterase enzyme in the induction of embryogenic structures during cotton cell suspension cultures.

Key words: *Gossypium hirsutum L.*, cultivar, cell suspensions, embryogenic structures, Esterase; isoenzyme.

INTRODUCTION

Genotypic dependence of somatic embryogenesis limits the success of embryos production in many plant species improvement programs (Gawel and Robacker, 1990; El Hadrami and Baaziz, 1995; Zouzou et al., 1997; Feng et al., 1998; Sofiari et al., 1998). Compared to other plants, cotton somatic embryogenesis occurrence is highly reduced by this dependence. First success of cotton somatic embryogenesis was obtained with the American cultivar Coker 312 (Davidonis and Hamilton, 1983). This cultivar was identified, among a hundred cotton varieties known at this time, as the only one that produce higher rate of embryogenic structures in cell suspension cultures (Trolinder and Xhixian, 1989; Firoozabady and Deboer, 1993; Wu et al., 2004). However, recent studies showed the regeneration of few cultivars through somatic embryogenesis by modifying medium and environmental conditions of culture (Gonzalez-Benito, 1997; Sakhanokho et al., 2001; Zhang et al., 2001). But, at the local cultivar level, embryogenic structures production in cell suspension culture of ISA 205N is not effective now due to genotype dependence of embryos induction (Kouakou, 2003; Kouakou et al., 2006). Expression of genotypic characters was made through physiological and biochemical changes in plant metabolism. Many authors pointed out a relationship between biochemical expressions and somatic embryogenesis induction in plant cell cultures. In several plants, embryogenesis induction in cells suspension requires an enhancement of nitrogen assimilation (Bayley et al., 1972; Trolinder and Goodin, 1988; Beck and Renner, 1989; Grimes and Hodge, 1990; Witjaksono and Litz, 1999). In cotton, the formation of somatic embryos occurs after an accumulation of storage protein pattern in the cells (Shoemaker et al., 1987). Kouadio et al. (1999) and Kouakou et al. (2004) showed an important activity of peroxydase and nitrate reductase in calli and cells of the embryogenic cultivar of cotton Coker 312 during embryogenic structures formation. Several authors reported also a relationship between cell embryogenesis and the change in phenolic compounds metabolism (Chibar et al., 1989; Karting et al., 1993; Kato et al.,

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1994; Cvikova et al., 1996; Tchorbadjieva and Odjakova, 2001; Kouakou et al., 2004; Kouakou et al., 2006).

The need of biochemical markers for the early identification of cells capable to undergo embryogenic structures formation has always been a major concern of researchers (Swarnkar et al., 1986; Wann et al., 1987; Rao et al., 1990).

In this study, quantitative and qualitative comparison of esterase at different stages of cell suspension cultures of two cotton cultivars, Coker 312 which is an embryogenic cultivar and ISA 205N an non embryogenic cultivar was made in the aim of biochemical markers of somatic embryogenesis identification.

MATERIAL AND METHODS

Plant material

Two cultivars of cotton (*Gossypium hirsutum* L.), Coker 312 from Centre International pour la Recherche Agronomique et le Développement (CIRAD), France and ISA 205N from Centre National de Recherche Agronomique (CNRA), Côte d'Ivoire, were used for this study.

In vitro seed germination and callus culture

Cotton seeds were first delinted with sulphuric acid and then disinfected by dipping in 70% (v/v) ethanol (1 min) prior to a 20 min exposure to 2.5% sodium hypochlorite (v/v). Seeds were rinsed three times in sterile distilled water and placed on a 10 ml half-strength MS (Murashige and Skoog, 1962) salts medium with vitamins B5 (Gamborg et al., 1968). The medium was supplemented by 30 g/l sucrose, 0.75 g/l $MgCl_2$ and solidified with 2.5 g/l gelrite. Seeds germination was initiated by incubating them in dark for 3 days at the temperature of 28 ± 2°C, followed by their transferred under 24 h photoperiod (16 h light/8 h dark) for 4 days.

3 - 5 mm of length hypocotyl segments of 7 days old from sterile seedlings were used to initiate callus. Six replications of five explants per Petri dish and per cultivar were used. All experiences were repeated three times, that is, 90 explants used per cultivar. Callus induction was made on 30 ml MS basal salts with B5 vitamins containing 30 g/l glucose, 0.1 mg/l 2.4-D and 0.5 mg/l kinetin (MSB), solidified with 2.5 g/l gelrite and 0.75 g/l $MgCl_2$. Calli were maintained and stabilized through three subcultures with 4 weeks intervals on the same medium (MSB). At the end of the third subculture, friable calli were used to initiate cell suspension cultures.

Cell suspension cultures and embryogenesis induction

Cotton suspension cultures were established by transferring approximately 4 g of friable callus into 500 ml Erlenmeyer flasks, containing 100 ml of the above medium (MSB) without gelling agent (MSL1). Suspensions were incubated on an orbital shaker at 110 rpm during 4 weeks (primary culture). The resulting cell suspension was filtered under partial vacuum through a 250 µm mesh sieve and 50 ml of the filtrate were refreshed with 50 ml of MSL1 medium devoid growth regulators containing 1.9 g/l KNO3 and 0.5 mg/l casein hydrolysate and 40 g/l sucrose (MSL2) into 500 ml Erlenmeyer flasks. Cultures were incubated during 4 weeks (first subculture). The second subculture was performed by sieving cells from the first subculture on a 150 µm mesh sieve. Approximately 2 g (fresh weight) of cells collected were resuspended on 50 ml MSL2 medium in 250 ml Erlenmeyer flasks under the same culture conditions previously described. Fractions of cells obtained after sieving on a 100 µm mesh sieve were resuspended in MSL2 medium and incubated in the above mentioned condition cultures to obtain the third subculture. Ten Erlenmeyer flasks were achieved by subculture and by cultivar. All experiences were repeated three times. Ten samples of each subculture were examined with a stereomicroscope to detect the formation of embryogenic structures.

The pH of all media was adjusted to 5.8 by KOH (0.1 N) before autoclaving at 121°C for 30 min. All the cultures were incubated at 28 ± 2°C under a light intensity of approximately 2000 lux. Light was provided by cool white fluorescent lamps with photoperiod (16 h light/8 h dark).

Esterase extraction and enzyme activity determination

Esterase extraction and assay were carried out at 4°C as described by Boerhinger (1975) with modifications. In all assays, 0.5 g of harvested cells was crushed with quartz sand for 10 min in a pre-cooled mortar and mixed with 20 ml of extraction buffer (100 mM sodium borate, pH 8 at 4°C). The mixture was centrifuged at 15 000 g for 20 min at 4°C. The supernatants were kept on ice until used. Esterase activity was determined in a spectrophotometer (Spectronic 601), by measuring the increase in absorbance at 578 nm at 25°C. The reaction mixture contained 2.9 ml of substrate solution and 0.1 ml of prepared enzyme. The substrate solution was prepared with 6 ml of red cresol 5%, 5 ml of 0.1 M sodium borate (pH 8.0) and 0.1 ml of ethyl butyrate. The reference cuvette contained only the substrate solution. The enzyme activity was determined by measuring the slope of the reaction line at zero time (initial rate). The enzyme activity unit was defined as the change in absorbance/min/g FW. Change of 0.001 in the absorbance value under the conditions of the assay was defined as a unit (U). All the measurements were performed in triplicate.

Isoenzyme analysis

The harvested cells of each filtration were washed with mannitol (1 M) and concentrated by centrifugation at 15 000 rpm for 20 min. 300 mg of washed cells were grinded in Tris-HCl buffer (90 mM), pH 8.5, containing β-mercaptoethanol (15 mM) at 4°C. The homogenate was centrifuged for 20 min at 15 000 rpm, and the supernatant used for enzymes analysis after the addition of 0.3 ml of blue bromophenol (0.2 %). Polyacrylamide gel electrophoresis was performed for isoenzyme analysis (Ollitrault, 1989; Kouakou, 2003). Approximately 50 µl of enzyme extract was electrophoresed on 12% polyacrylamide gels (PAGE) under non-denaturing conditions as described by Laemmli (1970). The migration is carried out at 4°C, in Tris borate EDTA buffer (0.1 M), pH 8.3. An electric current of 65 mA was applied first for 1 h on gel without enzyme extract, and then for 5 h after the addition of the enzyme extract on gel until the blue bromophenol front reached the end of the gel. Esterase isoenzymes were detected on gels by using α-naphtyl acetate and β-naphtyl acetate as substrates, and subsequent colour development with fast garnet GBC salt (Lanaud, 1986).

RESULTS AND DISCUSSION

One-month-old cell suspension (primary culture stage in MSL1 medium), 2 and 3 month-old cell suspensions in MSL2 medium (1st and 2nd subculture stage) rarely con-

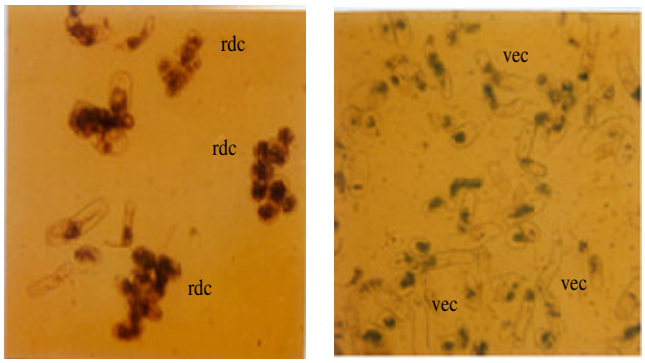

Figure 1. Cotton cell suspensions at third subculture in MSL2 medium. The clusters of round cells with dense cytoplasm (rdc) observed in Coker 312 are indication of cells embryogenic (E) formation. The very vacuolated and elongated cells (vec) observed with ISA 205N are characteristic of non embryogenic cells (NE). x 440.

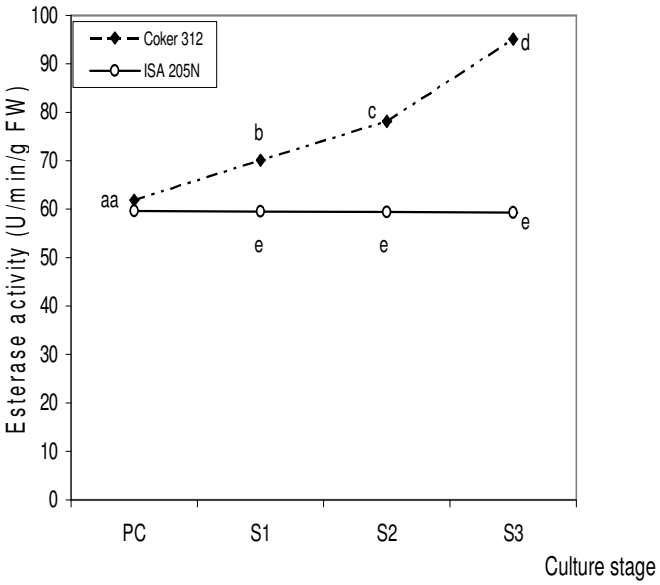

Figure 2. Change of esterase activity in cotton cell suspension at different stages of culture. Values followed by the same letter are not significantly different from each other according to Newman-Keuls test ($P< 0.05$). Each value represents the mean of three replicates ± S.E. (PC) primary culture, (S1, S2 and S3) first, second and third subcultures.

tained embryogenic structures in comparison to 4 month-old cell suspensions in MSL2 medium (3rd subculture stage) that frequently contained numerous embryogenic structures.

However, the development of embryogenic structures was observed only in cultivar Coker 312 cell suspensions but not in ISA 205N (Figure 1). That confirms the genotype dependence of somatic embryogenesis in cotton cell cultures. Only few cotton cultivars are able to form embryogenic structures during cell suspensions (Trolinder and Xhixian, 1989; Gonzalez-Benito, 1997; Zhang et al., 2001). Somatic embryogenesis in cotton required specific cultures conditions, as shown by several authors. Kouadio et al. (2004) showed an embryogenic development in callus from etiolated explants of cultivar ISA 205N. Modification of culture medium also could induce embryogenic structures development. In this study, the beneficial effects of the removal of hormones and the addition of KNO_3 and casein hydrolysate on the embryogenic structures induction in Coker showed by Trolinder and Xhixian (1989) and Wu et al. (2004) was confirmed.

A time course study depicted that a slight enhanced level of esterase activity was detected during cell suspension culture of Coker 312, an embryogenic cultivar. Esterase activity increases rapidly, and the level reached a maximum at third subculture. In non embryogenic cells of ISA 205N, esterase activity subsequently remained fairly constant at the different culture stages (Figure 2). It should be noted that increased esterase activity in cells was linked to induction of embryogenic structures (Rao et al., 1990).

Esterase isoenzymes are identical in cells of the two cultivars at the stage of primary culture, first and second subcultures. However, our results indicated a difference between esterase isoenzymes patterns from the embryogenic cultivar Coker 312 to the non embryogenic cultivar ISA 205N, at the stage of the third subculture. Two groups of esterase isoenzymes, based on their mobility in the gel, using α-naphtyl acetate as substrate, were distinguished; a slow moving group and a fast moving group.

In the slow moving group, an isoenzyme, marked “x”, was found only in the non embryogenic cells of ISA 205N. Contrarily, two isoenzymes, marked “y” and “z”, which belong to the fast moving group, were only present in the embryogenic cells of Coker 312 (Figure 3A). According to several authors β-naphtyl acetate stains both the aryl esterase as well as choline esterase and α-naphtyl acetate is cleaved only by choline esterase (Chibbar et al., 1989; Ollitrault et al., 1989; Tchorbadjieva and Odjakova, 2001). So, the use of α-naphtyl acetate, lead to the identification of esterase “x” present in non embryogenic cells of the cultivar ISA 205N as a choline esterase (Figure 3B), and the group of esterases marked “y” and “z” in the embryogenic cells of Coker 312 was easily identified as aryl esterase. Esterase might play an important rule in the embryogenesis process. According to Rao et al. (1990) the embryogenic cell suspensions reveals a more significant esterase activity compared to non embryogenic ones and the esterase electrophoretic profiles evidenced several isoenzymes which have a positive influence on embryogenic structures induction (Chibbar et al., 1989). Cell development in culture involv-

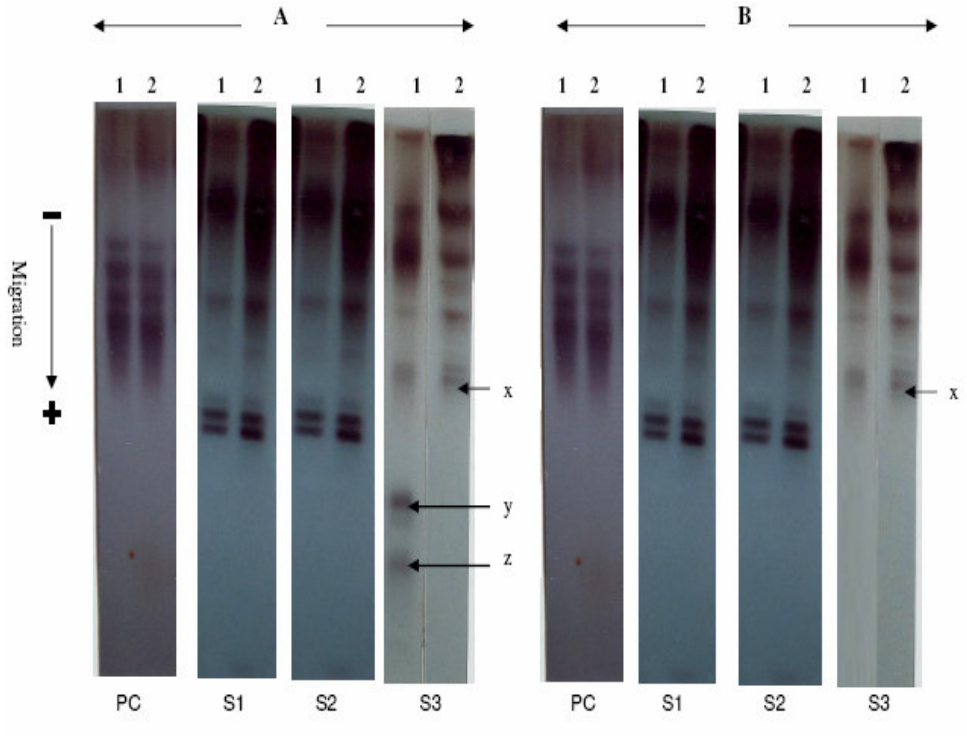

Figure 3. Esterase isoenzymes pattern from different stages of cotton cell suspension cultures, analysed on PAGE. Comparison was made between the embryogenic cells of Coker 312 (1) and the non embryogenic cells of ISA 205N (2). (PC) primary culture, (S1, S2 and S3) first, second and third subcultures. x, y and z represent isoenzymes. Section A is stained with β-naphtyl acetate and section B is stained with α-naphtyl acetate

es the appearance or disappearance of specific protein at particular stages of the culture, regulated by differential gene expression. The isoenzyme analysis at different stages of suspension cultures may unravel the physiological or biochemical changes underlying the process of differentiation for the establishment of embryogenic structures (Scandalios, 1974).

Our results substantiated earlier reports of esterase isoenzymes as potential markers of somatic embryogenesis (Chibbar et al., 1989; Rao et al., 1990; Tchorbadjieva and Odjakova, 2001). The presence of aryl esterases in the embryogenic cells of Coker 312 can be associated with competence of these cells to undergo somatic embryogenesis in suspension culture, while choline esterase in cell suspension of a non embryogenic cotton cultivar could proved their inability to induce embryogenic structures formation. Esterase isoenzymes could be considered as marker of somatic mbryogenesis in cotton cells cultures.

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REFERENCES

Bayley J, King J, Gamborg O (1972). The effect of the source of inorganic nitrogen on growth and enzymes of nitrogen assimilation in soybean and wheat cells in suspensions cultures. Planta 105:15-24.

Beck E, Renner U (1989). Ammonium Triggers Uptake of NO_3^- by *Chenopodium rubrum* suspension culture cells and remobilization of their vacuolar nitrate pool. Plant Cell Physiol. 30 (4): 487-495.

Boerhinger M (1975). Esterase. In Boerhinger (eds) Biochemical information, volume II, West Germany, pp. 64-65.

Chibbar R, Polwick P, Newsted W, Shyluk J, Geoges F (1989). Identification and isolation of a unique esterase from the medium of non-embryogenic cell line of cultured carrot cells. Plant Cell Tiss. Org. Cult. 18:47-53.

Cvikova M, Hrubocova M, Josef E, Binarova P (1996). Change in the levels of endogenous phenolics, aromatic monoamines, phenylalanine ammonia-lyase, peroxydase and auxine oxidase activities during initiation of alfalfa embryogenic and non embryogenic calli. Plant Physiol. Biochem. 34: 853-861.

Davidonis GH, Hamilton RH (1983). Plant regeneration from Callus tissue of *Gossypium klotzchianum* L. Plant Sci. Lett. 32: 89-93.

El Hadrami I, Baaziz M (1995). Somatic embryogenesis and analysis of peroxydases in *Phœnix dactylifera* L. Biol. Plant. 37:197-203.

Feng R, Zhang B, Zhang W, Wang Q (1998). Genotype analysis in cotton tissue culture and plant regeneration. Proceedings of the 4th

Asia-pacific conference on agric. Biotechnol. Darwin, Australia, pp. 13-16.
Firoozabady E, Deboer D (1993). Plant regeneration via somatic embryogenesis in many cultivars of cotton (*G. hirsutum* L.). *In vitro* Cell. Dev. Biol. 29:166-173.
Gamborg OL, Miller RA, Ojima K (1968). Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50: 151-158.
Gawel NJ, Robacker CD (1990). Genetic control of somatic embryogenesis in cotton petiole callus culture. Euphyta 49: 249-253.
Grimes HD, Hodges TK (1990). The inorganic NO_3^- NH_4^+ ratio influences plant regeneration and auxin sensibility in primary callus derived from immature embryos of indica rice (*Oryza sativa* L.*).* Plant Physiol. 136:362- 367.
Gonzalez-Benito ME, Frota-Chagas JM, Peres C (1997). Somatic embryogenesis of an early cotton cultivar. Pesqui. Agric. Bras. 32: 485-488.
Kato Y, Yamanouchi H, Hinata K, Ohsumi C, Hayashi T (1994). Involvement of phenolic esters in cell aggregation of suspension-cultured rice cells. Plant Physiol. 104: 147-152.
Karting T, Kogl G, Heydel B (1993). Production of flavonoids in cells culture of *Crataegenus monogyna*. Plant. Med. 59: 537-541.
Kouadio YJ, Zouzou M, Dogbo D, Kouakou TH, Koné M (1999). Activities of peroxydases, polyphenoloxydases and invertases as physiolgical markers of somatic embryogenesis in cell culture of cotton plant. African Crop Science Conference Proceeding, Casablanca, Morocco. 4: 31-35.
Kouadio YJ, Koné M, Djè Y, D'Almeida MA, Zouzou M (2004). L'étiolement est un facteur d'induction de l'embryogenèse somatique au cours de la callogenèse chez deux variétés récalcitrantes de cotonnier (*Gossypium hirsutum* L.) cultivées en Côte d'Ivoire. Biotechnol. Agron. Soc. Env. 8(3): 155-162.
Kouakou TH (2003). Contribution à l'étude de l'embryogenèse somatique du cotonnier *Gossypium hirsutum* L. (Malvaceae) : évolution de quelques paramètres biochimiques au cours de la callogenèse et de culture de suspensions cellulaires chez trois cultivars (Coker 312, ISA 205N et ISA GL7). Thèse de doctorat N° 367/2003, Université de Cocody, Abidjan, Côte d'Ivoire.
Kouakou TH, Kouadio YJ, Koné M, Zouzou M, Anno AP (2004). Evolution des composés phénoliques au cours de la callogenèse et de la culture de suspensions cellulaires chez *Gossypium hirsutum L.* Bioterre 4(1): 5-13.
Kouakou TH, Decendit A, Waffo TP, Valls J, Kouadio YJ, Mérillon JM (2006). First evidence of *trans*-resveratrol production in cell suspension cultures of cotton (*Gossypium hirsutum* L.). Plant Cell Tiss. Org. Cult. 86(3): 405-409.
Laemmli UK (1970). Cleavage of Structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.
Lanaud C (1986). Utilisation des marqueurs enzymatiques pour l'étude génétique du cacaoyer: Theobroma cacao L. II- étude du polymorphisme de six systèmes enzymatiques. Café Cacao Thé 30 (4): 271-280.
Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant. 15: 473-497.
Ollitrault P, Escoute J, Noyer JL (1989). Polymorphisme enzymatique des sorghos. I- Descriptions de 11 systèmes enzymatiques, déterminisme et liaisons génétiques. Agron. Trop. 44 (3): 203-209.
Rao KV, Syrasanna P, Reddy GM (1990). Biochemical changes in embryogenic an non-embryogenic calli of Zea mays L. Plant Sci. 66: 127-130.
Sakhanokho HF, Zipf A, Rajasekaran K, Saha S, Sharma GC (2001). Induction of highly embryogenic calli and plant regeneration in Upland (*Gossypium hirsutum* L.) and Pima (*Gossypium barbadense* L.) cotton. Crop Sci. 41: 1235-1240.
Scandalios JG (1974). Isoenzymes in development and differentiation. Ann. Rev. Plant Physiol. 25: 225-258.
Shoemaker RC, Christofferson SE, Galbraith DW (1987). Storage protein accumulation patterns in somatic embryos of cotton (*Gossypium hirsutum L.*). Plant Cell Rep. 5: 178-181.
Sofiari E, Raemakers C, Bergervoet J, Jacobsen E, Visser R (1998). Plant regeneration from protoplasts isolated from friable embryogenic callus of cassava. Plant Cell Rep.18:159-165.
Swarnkar PL, Bohra SP, Chandra N, (1986). Biochemical changes during growth and differentiation of the callus of Solanum surattense. J. Plant Physiol. 126: 75-81.
Tchorbadjieva M, Odjakova MK (2001). An acidic esterase as a biochemical marker for somatic embryogenesis in orchadgrass (*Dactylis glomerata* L.) suspension cultures. Plant Cell Rep. 20 (1): 28-33.
Trolinder NL, Goodin JR (1988). Somatic embryogenesis in cotton (*G. hirsutum* L). II-requirements for embryo development and plant regeneration. Plant Cell Tiss. Org. Cult. 12: 43-53.
Trolinder NL, Xhixian C (1989). Genotype specificity of the somatic embryogenesis response in cotton. Plant Cell Rep. 8: 133-136.
Wann SR, Johnshon MA, Noland TL, Carlson JA (1987). Biochemical difference between embryogenic and nonembryogenic callus of Picea abies (L.) Karst. Plant Cell Rep. 6: 39-42.
Witjaksono E, Litz R (1999). Maturation of avocado somatic embryos and plant recovery. Plant Cell Tiss. Organ Cult. 59: 47-56.
Wu J, Zhang X, Nie Y, Jin S, Liang S (2004). Factor affecting somatic embryogeneis and plant regeneration from a range of recalcitrant genotypes of Chinese cottons (*Gossypium hirsutum* L.). *In Vitro* Cell. Biol. 40: 371-377.
Zhang BH, Feng R, Liu F, Wang Q, (2001). High frequency somatic embryogenesis and plant regeneration of an elite Chinese cotton variety. Bot. Bull. Acad. Sin. 42: 9-16.
Zouzou M, Kouakou TH, Koné M, Peeters M, Swennen R (1997). Callogenèse chez le cotonnier cultivé en Côte d'Ivoire : effets position explant hypocotyle, variétés, source de carbone et régime hormonal. *In*: Proceeding of 3rd African Crop Science Conference, Pretoria, Afrique du Sud. pp. 1489-1494.