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Full Length Research Paper

Trans-resveratrol as phenolic indicator of somatic embryogenesis induction in cotton (*Gossypium hirsutum* L.) cell suspensions

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***Trans*-resveratrol is a phytoalexin which was found in some grapes and in many other plants. This phenolic compound has gained much attention, as it was indicated to be associated with fungus and disease resistance. Also, it has been shown that phenols compounds could play an important role in somatic embryogenesis induction of various plants. In cotton, embryogenic structures take place in cell suspension cultures. The need of biochemical indicators for the early identification of cells capable to undergo embryogenic structures formation has always been a major concern of researchers. *Trans*-resveratrol was found only in embryogenic cell suspensions of Coker 312. This phenolic compound started at the first subculture (2.44 µg/g dw) and the maximum level was reached at the third subculture (7.2 µg/g dw) with an increase of nearly 295%. This study showed a correlation between *trans*-resveratrol synthesis in cotton cell and embryogenic structures induction. *trans*-resveratrol may be a phenolic indicator for is induction of cotton somatic embryogenesis.**

Key words: *Trans*-resveratrol, suspension culture, embryogenic structures, cotton, *Gossypium hirsutum* L.

INTRODUCTION

Cotton is one of the most important fiber crops. It has been estimated to contribute US $15 - 20 billion to the world's agriculture economy with over 1 million people depending on it for their livelihood (Benedict and Altman, 2001). Cotton constitutes the principal raw material for textile industries. In addition, cotton seeds are an important source of proteins which can be used in human and animal nutrition. So, several research programs have been conducted to ensure cotton production improve-ment. Somatic embryogenesis has been found to be a possible pathway for cotton improvement. First regenera-tion of cotton plant by cell suspension culture was obtain-ed with Coker cultivar (Davidonis and Hamilton, 1983). On a hundred cotton varieties known currently, Coker is the only cultivar that produces high frequency embryoge-nic structures in cell suspensions (Trolinder and Xhixian, 1989; Firoozabady and Deboer, 1993; Zhang et al., 2001) . Coker is not a cultivar of high agronomic value, but it is used in program selections implying recalcitrant cultivars to give hybrid able of *in vitro* regeneration (Kumar et al., 1998; Sakhanokho et al., 2001). Regeneration through somatic

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Abbreviations: DAD - diode array detector, 2,4-D - 2,4 dichlorophenoxy-acetic acid, TFA – trifluoro acetic acid, HPLC – high performance liquid chromatography, CIRAD (Centre

embryogenesis is preferred over organogenesis because of single-cell origin of the somatic embryos (Liu and Zhang, 2004; Sakhanokho et al., 2004; Sun et al., 2006; Wang et al., 2006; Xie et al., 2007), thus reducing the chimeric transformation events. Somatic embryogenesis in cells is genetically dependent (Gawel and Robacker, 1990).

Genotypic variations in plants are expressed by different metabolic expression in these plants. Somatic embryogenesis can be influenced by many factors. Some workers showed the correlation between specific phenolic compounds presence and induction of embryogenic cells (Ishikura and Teramoto, 1983; Karting et al., 1993; Alemano et al., 1994; Cvikrova et al., 1996; Lozovaya et al., 1996; El Bellaj and El Hadrami, 1998; El Bellai et al., 2000; Malabadi and Nataraja, 2003; Kouakou et al., 2004). Stilbenes are physiologically active secondary metabolites found in numerous families of plants. Among them, *trans*-resveratrol (3,5,4'-trihidroxyestilbene) has been the most widely studied (Jang et al., 1997). Cotton produces a large number of secondary products that often occur in specialized cells or tissues (Lege et al., 1995; Kouakou et al., 2004). The phenolic compounds are a complex mixture, and only a small number of plants have been examined. In cell cultures, phenols compounds could play a very important role in inducing the embryogenic structure of various plants. And 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 (Swarn-kar et al., 1986; Wann et al., 1987; Rao et al., 1990; Kouakou et al., 2007).

In this study, chromatographic analysis of phenolic compounds was compared between Coker 312 which is an embryogenic cultivar and R405-2000 a non embryogenic cultivar; this in the aim to identify phenolic compounds which are linked to embryogenic structures induction in cotton cell suspension cultures.

MATERIALS AND METHODS

Plant material

Two cultivars of cotton (*Gossypium hirsutum* L.) were used. Coker 312 seeds were obtained from CIRAD (France) and R405-2000 seeds from CNRA (Côte d'Ivoire). Seeds were delinted with sulphuric acid. Plump and mature seeds were chosen and disinfected by dipping in 70% (v/v) ethanol (1 min) prior to a 20 min exposure to 2.5 % sodium hypochlorite (v/v), followed by 3 rinses in sterile distilled water. Sterile seeds were placed on half-strength MS (Murashige and Skoog, 1962) salts with vitamins B5 (Gamborg et al., 1968) medium, supplemented by 30 g/l sucrose, 0.75 g/l $MgCl_2$ and solidified with 2.5 g/l gelrite for germination at 28 ± 2°C. The seeds were placed in culture tubes and incubated in the dark for 3 days to initiate germination and then transferred under photoperiod cycle (16 h light/8 h dark) for 4 days.

Callus initiation and maintenance

Hypocotyls of 7-day-old seedlings were cut into segments of 3 - 5 mm length, as explants. Callus induction medium (MSC) included MS basal salts with B5 vitamins containing 30 g/l glucose, 0.1 mg/l 2.4-D and 0.5 mg/l kinetin. The medium was solidified with 2.5 g/l gelrite and 0.75 g/l $MgCl_2$. Explants were placed in Petri dishes and incubated for 4 weeks. Calli were maintained and stabilized through monthly subcultures on the same medium (MSC). At the end of the third subculture, the best friable calluses were used to initiate cell suspensions.

Cell suspension initiation and maintenance

Cotton suspension cultures were established by transferring approximately 2 g of friable callus into 250 ml Erlenmeyer flask containing 50 ml of above medium (MSC) devoid of gelling agent (MSL1). Suspensions were incubated on an orbital shaker at 110 rpm during 4 weeks. The resulting cell suspension was filtered under partial vacuum through a 250 µm mesh sieve and the filtrate was harvested (primary culture). Then, cell suspensions were subcultured 3 times at 4- week intervals on MS liquid without growth regulators, glucose was replaced by 40 g/l sucrose. This medium was supplemented with 1.9 g/l KNO_3 and 0.5 mg/l casein hydrolysate (MSL2). Approximately 2 g (fresh weight) of the fraction collected at the end of each subculture were resuspended in 50 ml of MS liquid2 as described above into 250 ml Erlenmeyer flasks. At the end of each subculture, cell suspensions were respectively filtered through 150 µm, then 100 µm mesh sieves, and samples were examined with a stereomicroscope to observe the evolution of cell suspensions concerning somatic embryogenesis. Cells were harvested at each culture stage by filtration and then frozen until analysis.

Incubation conditions

The pH of all media was adjusted to 5.8 before autoclaving at 121°C for 30 min. All 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).

Sample preparation

For each sample 50 mg of dry biomass were extracted overnight by 5 ml methanol at 4°C with a blender. Samples were centrifuged at 3000 rpm for 10 min; supernatant was collected and filtered through a Millipore membrane with 0.45 µm porosity. The filtrated samples were diluted with a same volume of distilled and filtered water and injected directly into HPLC.

Reagents and solutions

All solvents were of liquid chromatographic grade (Scharlau), except H_2O which was distilled and filtered through a Millipore membrane (0.22 µm). Solutions were degassed before use. Chemically pure standard of *trans*-resveratrol was obtained from Sigma (Sigma chemical Co., St. Louis, Mo, USA).

HPLC conditions

Analyses were performed on a Varian HPLC unit. This HPLC system was equipped with an autosampler (model 410), two pumps (Prostar, model 210), an automated controller gradient (Normasoft software), an automated injector (Alcott, model 708). Detection was carried out with a UV-VIS detector (Kontron, model 430), and a

Table 1. Biomass reached (mg fw/culture flask ± SD) and means biomass increase (%) at the end of each culture cycle (4 weeks) of Coker 312 suspension cultures[a]

Culture stage	Coker 312		R405-2000	
	Biomass reached (mg fw/culture flask)	Biomass increase (%)	Biomass reached (mg fw/culture flask)	Biomass increase (%)
Primary	.3 ± 0.20 a	65 e	2.5 ± 0.30 a	25 h
1st subculture	4.3 ± 0.80 b	115* f	3.1 ± 0.72 a	55 e
2nd subculture	4.9 ± 0.75 b	145* f	3.5 ± 0.84 ab	75 e
3rd subculture	5.6 ± 0.75 c	230* g	4.1 ± 0.84 d	110 f

[a]fw: fresh weight; ± SD (standard deviation); *these suspensions differentiated the cluster cells, the embryogenic structures; data are the means of three replicates. For each flask the inoculum's biomass was 2 g fresh weight. In line and column, values followed by a same letter are not statistically different at 5%. Values are means of three replicates.

diode array detector (Prostar, model 335) was used to check peak purity (at room temperature). Baseline workstation 6.41 software (Varian) and a desk computer were employed for data storage and evaluation. A C18 reverse phase column (Prontosil, 250 x 4.0 mm, 5 µm, Bischoff) was used for analytical and (Prontosil, 250 x 8.0 mm, 5 µm, Bischoff) semi-preparative HPLC with a binary gradient eluent (solvent A, 0.1% TFA in water; solvent B, 0.1% TFA in acetonitrile) . The elution program was 10 to 50% B (0 – 40 min), 50 to 100% B (40 - 41 min), 100% B (41 - 50 min). For analytical purposes, 100 µl of each sample were directly injected at a flow rate of 0.6 ml/min and chromatograms were monitored at 286 and 306 nm. Quantitation was accomplished by comparison with a standard response curve prepared from solutions of pure *trans*-resveratrol (from Sigma chemical Co., St. Louis, Mo, USA). Moreover, *trans*-resveratrol (retention time = 26.0 min) was isolated by semiprep using a flow rate of 2.4 ml/min.

Nuclear magnetic resonance (NMR) spectroscopy

The structure was confirmed by ^{1}H-NMR. NMR measurements were made using 5 mm tubes. ^{1}H-NMR spectra were recorded on a Bruker Avance 300 NMR spectrometer operating at 300 MHz for ^{1}H. For recording ^{1}H-NMR spectra, solutions were prepared by dissolving 10 mg of the sample in 0.5 ml of methanol-d_6. Methanol signal at 3.31 ppm as internal reference. Chemical shifts are given in δ (ppm) value based on the solvent signal.

Statistical analysis

Experimental data were analysed using Statistica software (release 6). These data were subjected to analysis of variance (ANOVA). The means of phenolic compounds contents were tested for significant difference (Newman keuls) at 5%. Data are the average values of three replicates.

RESULTS AND DISCUSSION

Cell suspension cultures

Cotton cell suspensions grew well in MS medium supplemented with KNO_3 (Trolinder and Goodin, 1988; Trolinder and Xhixian, 1989). Wu et al. (2004) have extensively characterized the beneficial effects of the removal of hormone in medium on embryogenic structures initiation. We observed after one month of culture in liquid suspension the clusters of round cells with dense cytoplasm with Coker 312. These cells seem to be to be proembryos in the globular stage. Thus, somatic embryos were observed in different stages of somatic embryogenesis: codiforme, heart and cotyledonous (Profumo et al., 1986; Nomura and Koumamime, 1995; Kassem and Jacquin, 2001). On the other hand, the vacuolated large associated to round cells without cyoptlasm observed with R405-2000 are characteristic of non embryogenic cells.

Davidonis and Hamilton (1983) have reported that KNO_3 increased the number of embryogenic structures. Ragan et al. (1984) suggested that casein hydrolysate was important to embryogenic structures development. We confirmed the beneficial effects of the removal of hormone and the addition of KNO_3 and casein hydrolysate on the induction of embryogenic. The grow rate was lowest for the suspensions established with R405-2000 while for Coker 312 suspensions highest grow rate was obtained (Table 1). We observed that one month old cell suspensions (primary culture) rarely contained embryogenic structures. However, when these cell suspensions are 2, 3 and 4 months old (1st, 2nd and 3rd subculture stage), they frequently contained numerous embryogenic structures.

The formation of embryogenic structures was observed only in Coker 312 cell suspensions but not in R405-2000 ones (Figure 1). The highest grow rate of cell suspendsions obtained with Coker 312 could notify greater reactivity of the cells of this cultivar in medium culture (Trolinder and Goodin, 1988; Kumria et al., 2003; Koné, 2003). These results seem to show that response in cotton tissue culture is highly genotype dependent. That is in agreement with results reported by several authors (Gonzalez-Benito et al., 1997; Sakhanokho et al., 2001; Zhang et al., 2001; Kouadio et al., 2004; Wu et al., 2004; Sun et al., 2006; Xie et al., 2007).

HPLC analysis

To investigate the endogenous factors influencing the

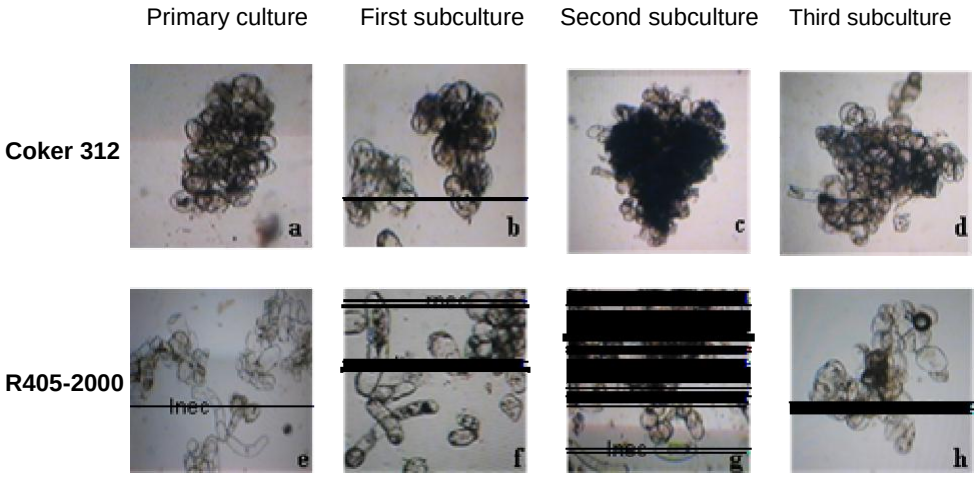

Figure 1. Cells suspension of cotton at different culture stages (x 440). Embryos in stage of (a) globular, (b) codiforme, (c) heart and cotyledonous (d) were observed with Coker 312; in cell suspensions of R405-2000 we observed large vacuolated cells (e), large and round vacuolated cells (f), large vacuolated cells and a pile of small rounded cells in intensive division (g), and round cells without cytoplasm (h). These cells are characteristic of non embryogenic cells; lnec and rmec: large and round non embryogenic cells.

behavior of Coker 312 and R405-2000 with regard to somatic embryogenesis, we analyzed the phenolic compounds accumulated by cells during suspension culture. Indeed, although no study has ever connected embryogenic structures and polyphenols production in cotton cell suspensions. Many authors have reported that polyphenols could play an important role during embryogenic structures induction or somatic embryogenesis recalcitrance of certain plants. There have been some reports concerning production of polyphenols by cell cultures of other plants. Thus, according to Cvikrova et al. (1996), embryogenic calli of *Medigo sativa* were characterized by a more important accumulation of phenolic acids in the cell walls compared to non-embryogenic calli. In the same way, Kanji et al. (1993) reported polyphenol production in the cell cultures of *Cornus kousa*. Lozovaya et al. (1996) revealed that regenerating calli contained more ferulic acid than no regenerating calli in *Fragaria xananassa*. Baaziz et al. (1994) and El Hadrami and Baaziz (1995) also showed that embryogenic capacity acquisition by cells of *Phoenix dactylifera* was accompanied by an increase of synthesis of phenolic acids. Thus, the important rule occupied by polyphenols in competence acquisition of somatic embryogenesis in many plants can be observed. In order to study the polyphenol production in our cotton cell suspensions, we used HPLC coupled with UV detection. The majority of these phenolic compounds are phenolic acids which have already detected in cotton leaves (Lege et al., 1992; Lege et al., 1995) . We founded thirty phenolic compounds in our cotton cell suspensions (Kouakou et al., 2007b). One of these was identified as *trans*-resveratrol, a phenolic compound never previously detected in cotton. *trans*-Resveratrol (3,5,4'-trihydroxystilbene) is a phytoalexin, a class of antibiotic compounds (Celotti et al., 1996), found in various plants including grapes, and food products containing them are considered as an important dietary source (Soleas et al., 1997; Cassady et al., 2000; Jian et al., 2004; Vitrac et al., 2005). *trans*-Resveratrol has major biological activities and is thought to be beneficial for human health (Docherty et al., 1999; Fremont et al., 1999). The chromatogram of standard solution using UV-Vis detector at 280 nm was presented in Figure 2.

The analysis of phenolic profiles of two cultivars of cotton cell suspensions has permitted to detect a peak (x) in Coker 312 (embryogenic cultivar) samples at 26.0 min of retention time which was not detected in any R405-2000 (non embryogenic cultivar) samples (Figure 3). However, no relationship was never established between *trans*-resveratrol and somatic embryogenesis induction.

This phenolic compound could be identified as *trans*-resveratrol, by chromatography, comparing of its spectra and retention time with authentic standard *trans*-resvera-trol using HPLC. However, much phenolic compounds can appear at this same retention time. So, to confirm this result, the peak (x) was isolated by semi -preparative HPLC and analyzed by ^{1}HNMR (Pandiarajan et al., 2007; Shangwu et al., 2006). The ^{1}H-NMR data (Figure 4; Table 2) was revealed that the compound isolated was *trans*-resveratrol (Mattivi et al., 1995; Martınez-Ortega et al., 2000).

The results of the calibration are shown in Table 3 where the regression coefficient, the limits of detection and quantification are indicated. Each point of calibration

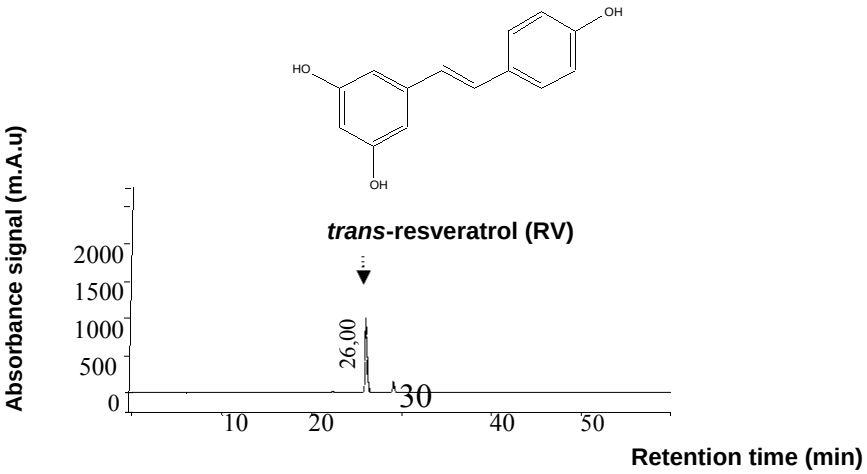

Figure 2. Chromatogram of a standard solution of *trans*-resveratrol using 286 nm absorbance detection (retention time: 26.00 min).

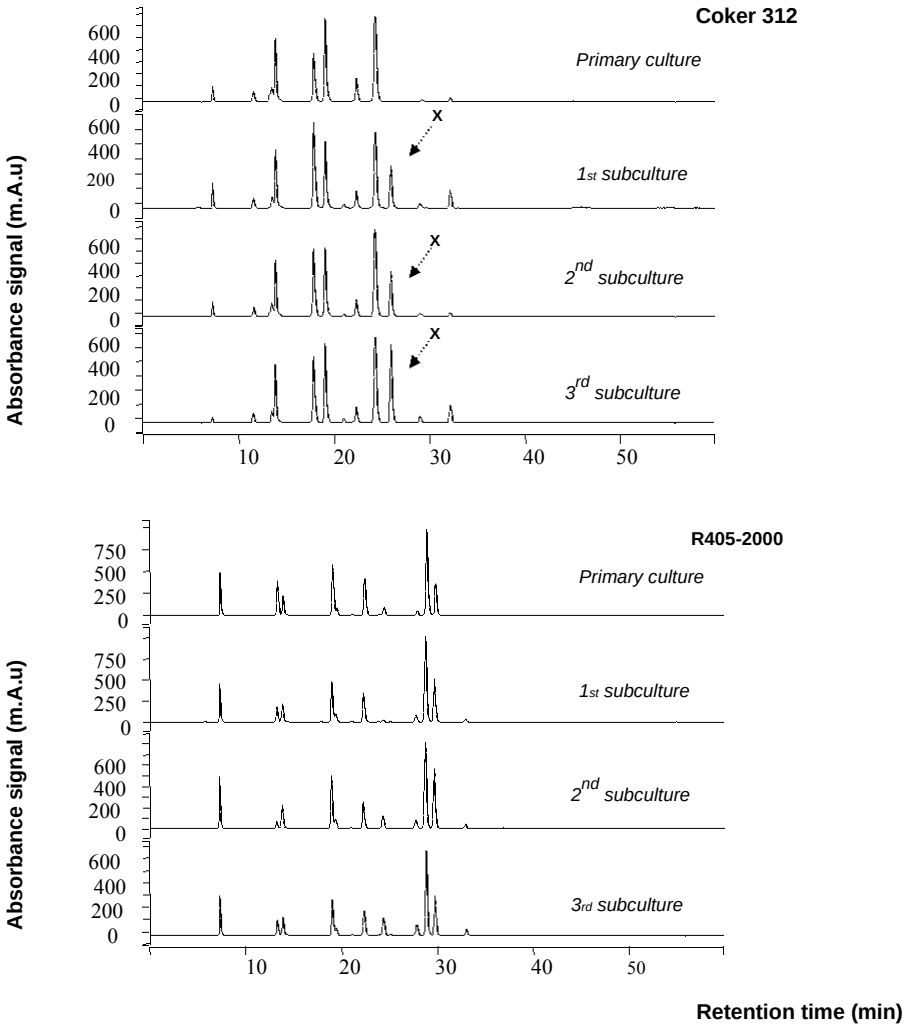

Figure 3. Chromatogram of polyphenol extracts of cell suspensions using 286 nm absorbance detection.

Table 2. Proton chemical shiffs (ppm) of phenolic compound (x) isolated in Coker 312 cell suspension cultures.

Node	Shiff	Base + Inc.	ppm relation to methanol
CH	6.05	7.26	1- benzene
		-0.53	1- C
		-0.53	1- C
		-0.15	1- C=C-1:C*C*C*C*C*C*1
CH	6.45	7.26	1- benzene
		-0.53	1- C
		-0.44	1- C
		0.16	1- C=C-1:C*C*C*C*C*C*1
CH	6.45	7.26	1- benzene
		-0.44	1- C
		-0.53	1- C
		0.16	1- C=C-1:C*C*C*C*C*C*1
CH	7.25	7.26	1- benzene
		0.00	1- C=C-1:C*C*C*C*C*C*1
		-0.53	1- C
CH	6.73	7.26	1- benzene
		0.00	1- C=C-1:C*C*C*C*C*C*1
		-0.53	1- C
CH	6.73	7.26	1- benzene
		0.16	1- C=C-1:C*C*C*C*C*C*1
		-0.17	1- C
CH	7.25	7.26	1- benzene
		0.16	1- C=C-1:C*C*C*C*C*C*1
		-0.17	1- C
OH	5.0	5.00	Aromatic C-OH
OH	5.0	5.00	Aromatic C-OH
OH	5.0	5.00	Aromatic C-OH
H	6.99	5.25	1- ethylene
		1.38	1 -1:C*C*C*C*C*C*1 gem
		0.36	1 -1:C*C*C*C*C*C*1 cis
H	6.99	5.25	1- ethylene
		1.38	1 -1:C*C*C*C*C*C*1 gem
		0.36	1 -1:C*C*C*C*C*C*1 cis

is the mean value of three independent area measurements. The quantification and detection limits were calculated as the concentrations giving signals 10 times and 3 times as high as the standard deviation of the blank value value, respectively. The procedure was applied to four series of cotton cell suspensions stage culture of Coker 312 and R405-2000 cultivars. According to certain authors, *trans*-resveratrol is produced by plant cells in response to exogenous stimuli, fungal infection or UV radiation (Douillet-Breuil et al., 1999; Cantos et al., 2001; Jeandet et al., 2002; Wang et al., 2002). *Trans*- resveratrol synthesis induced in cotton cell suspensions could

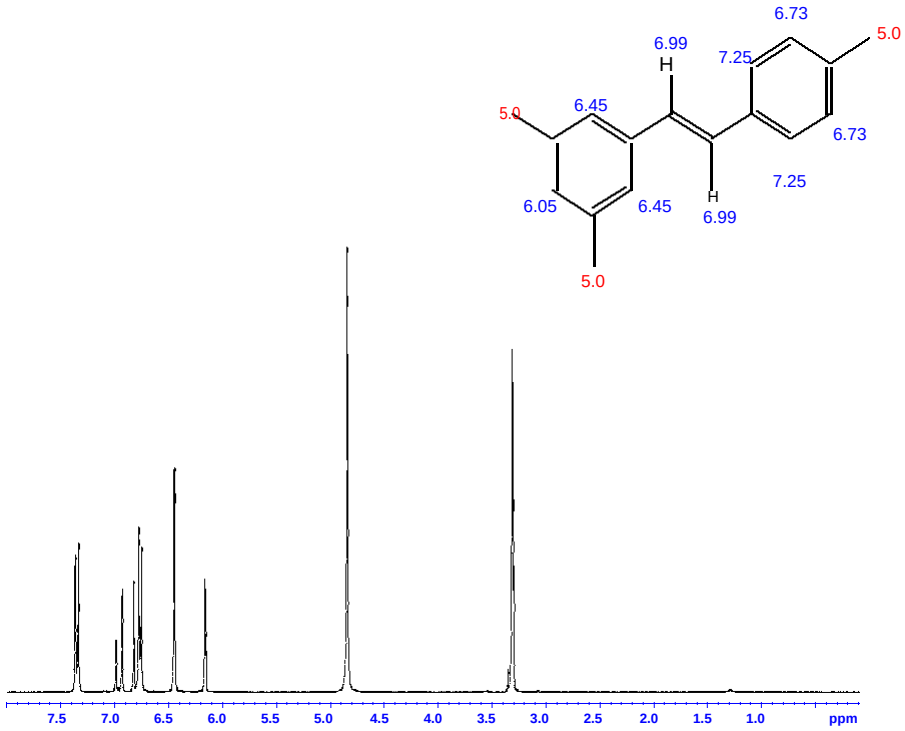

Figure 4. ^{1}H-NMR spectrum of phenolic compound (x) isolated in Coker 312 cell suspension cultures.

Table 3. Calibration results for determining *trans*-resveratrol in cell suspensions of cotton [a]

Detector	Linear equation	r	LOD (µg/g dw)	LOQ (µg/g dw)
Area	$Y= 3\ 10^{6}\ x - 0.273065\ 10^{6}$	0.9998	0.063	0.112

[a] r: correlation coefficient; LOD: limit of detection; LOQ: limit of quantification

depend on the cultivar because it occurs only in embryogenic Coker 312 cell suspensions. *trans*-Resveratrol could support the induction of embryogenic structures in these cell suspensions. Thus, there might be a relationship between *trans*-resveratrol production and the acquisition of embryogenic capacity by cotton cells. To study *trans*-resveratrol accumulation in relation to subculturing, Coker 312 cell suspensions were analyzed during the embryogenic induction period from primary culture to third subculture. Production of *trans*-resveratrol started at the first subculture (2.44 µg/gdw) and the maximum level was reached at the third subculture (7.2 µg/gdw) with an increase of nearly 295 % (Figure 5). *Trans*- resveratrol accumulation in Coker 312 cell suspensions increased markedly during all subcultures wherever induction of embryogenic structures took place (1st, 2nd and 3rd subculture stage). This result indicated that *trans*-resveratrol could play an important physiological role in the induction of cotton embryogenesis structures. Its role in somatic embryogenesis remains to be determined. However, the presence of *trans*-resveratrol in the cells during suspension cultures seems to be an indicator of somatic embryogenesis induction.

Conclusion

We observed a positive relationship between the presence of *trans*-resveratrol and somatic embryogenesis. *trans*-Resveratrol could be considered as a good phenollic indicator of embryogenic structures induction in cell suspension cultures of cotton. The absence of *trans*-resveratrol in cell suspensions of R405-2000 which is unable to produce somatic embryos reinforces our hypothesis. Further investigations are in progress to study the presence of *trans*-resveratrol in embryogenic calli of different cultivars of cotton. This study would allow confirming the relationship between *trans*-resveratrol synthesis and somatic embryogenesis. We will also study the addition of *trans*-resveratrol or elicitors, known to enhance stil-bene production in plant cells, in non embryogenic sus-

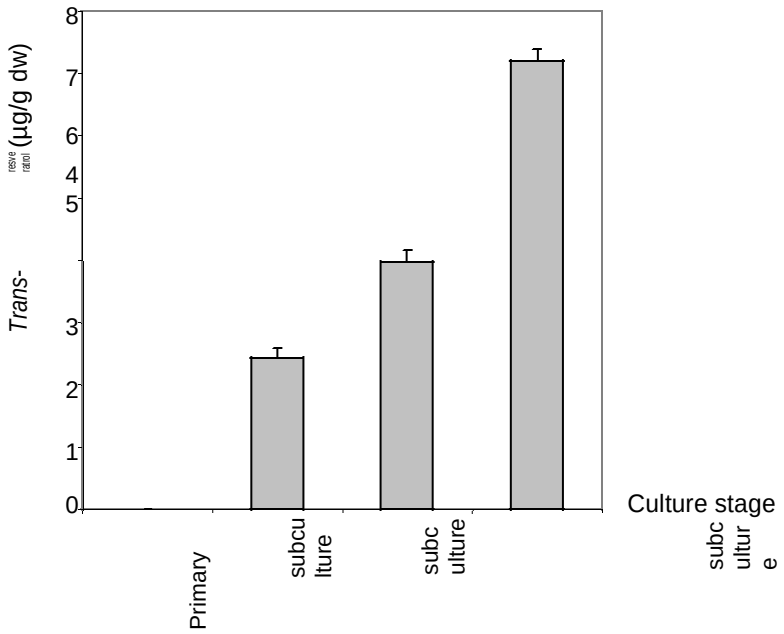

Figure 5. Concentration of *trans*-resveratrol in cell suspensions of cotton (Coker 312) dw: dry weight; data are the means of three replicates, vertical bars represent standard deviation.

suspension cultures. This could induce the formation of somatic embryos in unable cell suspensions.

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