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Characterization and Quantification of Polyphenols in Callus and Cells Suspension of Embryogenic and Non Embryogenic Cultivars of Cotton (*Gossypium Hirsutum* L.)

Kouakou Tanoh Hilaire[1,2]*, Ayolié Koutoua[3], Yapo Sopie Edwige S.[3], Kouakou Kouakou Laurent[2], Mérillon Jean-Michel[1]

[1]Groupe d'Etude des Substances Végétales à Activités Biologiques, EA 3675, UFR des Sciences Pharmaceutiques, Université de Bordeaux 2,146, rue Léo-Saignat F-33076 Bordeaux cedex, France.

[2] Laboratoire de Biologie et d'Amélioration des Productions végétales, UFR Sciences de la Nature, Université Nangui Abrogoua, 02 BP 801 Abidjan 02, Côte d'Ivoire.

[3]Laboratoire de Physiologie Végétale, UFR Agroforesterie, Université Jean Lorougnon Guédé, BP 150 Daloa, Côte d'Ivoire

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Abstract: Polyphenols were identified in calli and cells suspension of embryogenic and non embryogenic cotton cultivar. Polyphenols were separated by HPLC methods. Coker 312 developed better callogenesis and produced embryogenic structures contrarily to R405-2000 which was found to be a non embryogenic cultivar. We demonstrate relationship between different polyphenols synthesis, their level and the induction of callus and embryogenic structures. Coker 312 was highly linked respectively to a higher content of caffeic, ferulic and salicylic acids and the appearance presence of *p*-coumaric, benzoic acids, *trans*-resveratrol, quercetin and catechin. On the other hand, the presence of *trans*-cinnamic, *trans* and *cis*-methoxycinnamic acids in calli, genistein, *trans* and *cis*-methoxycinnamic acids in cell suspensions of cultivar R405-2000 is an indicator of formation of non embryogenic structures in this cultivar. Polyphenols can be considered as markers of the somatic embryogenesis induction in cotton

Keywords: Callus; Cell suspension; Cotton; *Gossypium hirsutum* L.; Polyphenols.

INTRODUCTION

Cotton is one of the most important fibre crops in the world[1]. Among species, *Gossypium hirsutum* L. is main species currently cultivated, primarily for its fibbers, which is the principal raw material using in textile industries. In addition, cotton seeds are an important source of proteins which can use in human and animal nutrition[2]. Cotton represents thus an interesting income source for developing countries where several research programs were purchased to ensure cotton production improvement. Somatic embryogenesis was found to be a possible pathway of cotton improvement. Cotton somatic embryogenesis was first observed by Price and Smith in *Gossypium klotzchianum*[3] and Davidonis and Hamilton[4] first described plant regeneration from two years old callus of *Gossypium hirsutum* L. cv. Coker 310 *via* somatic embryogenesis. Although significant progress has been reported in cotton tissue culture[5-11], the embryogenesis genotype dependent on cotton limited the numbers of varieties which could produce somatic embryos and thus reduced the effectiveness of somatic embryogenesis use in cotton improvement[12-14]. The most responsive lines to embryogenesis are Coker varieties[15,16].

Many other factors influenced the efficiency of somatic embryogenesis process[1,14,17] showed a difference between metabolites produced during cell suspension cultures of Coker 312 an embryogenic cultivar and ISA 205N a non embryogenic one. Indeed, total polyphenols content was higher in Coker 312 cell suspensions. Therefore, phenolic compounds could play important rules the somatic embryogenesis process. However, only a small number of plants have been examined for their polyphenols content and the possible roles of these compounds in plant improvement *via* somatic embryogenesis induction in various plants such as *Fragara xananassa*[18], *Phoenix dactylifera*[19], *Triticum aestivum*[20], *Oriza sativa*[21], *Vitis vinifera*[22] and *Phaseolus vulgaris*[23]. With particular regard to cotton, data are limited to some studies, which pointed out phenolic acids, are phenolic acids, primarily caffeic acid, *p*-coumaric acid, *o*-coumaric acid, vanillic acid, ferulic acid and salicylic acid as major phenolic compounds in cotton plants[24-27]. Currently, in our knowledge, no study concerning polyphenols in cotton tissues and cells during *in vitro* culture and their implication in the somatic embryogenesis induction has been carried out. The aim of the present study was to compare the polyphenols identified and quantified in callus and cell suspensions of embryogenic and non-embryogenic cotton. The correlation between polyphenols, callus and cells suspension leading to somatic embryogenesis was evaluated in order to identify specific polyphenols as markers in the induction of somatic embryogenesis in cotton.

MATERIALS AND METHODS

Plant material and aseptization: Cotton seeds were obtained from CIRAD, France (cultivar Coker 312) and CNRA, Cote d'Ivoire, West Africa (cultivar R405-2000). The seeds were first delinted with sulphuric acid to discard the fibbers and sterilised as follows: seeds were surface sterilised with 2.5% sodium hypochlorite for 20 min after a pre-treatment for 1min in 70% ethanol. After three times, rinsing the sterilized seeds are kept in sterile water for one day to soften the seed coats. Sterile seeds without coats were then sown in tubes containing a halft-strengh MS[28] medium with B_5 vitamins[29], supplemented with 30 g/l sucrose, and solidified with 2.5 g/l gelrite and 0.75 g/l $MgCl_2$ (MS1) for germination. Cultures were maintained in growth room at 28 ± 2°C in the dark for 3 days to initiate germination and then transferred on a 16 h light/8 h dark cycle for 4 days.

Callus culture: Hypocotyls of 7 days-old sterile seedling were cultivated in 250 ml, Pyrex flask containing MS medium, including B_5 vitamins, 30 g/l glucose, 0.5 mg/l kinetin, 0.1 mg/l 2,4-D (2,4 dichlorophenoxy-acetic acid) and solidified with 2.5 g/l gelrite and 0.75 g/l $MgCl_2$ (MS2). Callus was stabilized through monthly subcultures on the same medium (MS2). Tissue from each subculture were dispersed in water in Petri dish and examined with stereomicroscope for detection of embryogenic

structures. The dry biomass of the callus obtained from each culture, was used for the study of polyphenols production.

Cells suspension culture: Friable and well grown callus of third subculture were used to initiate cell suspensions. Approximately 2 g of callus was placed into 250 ml Erlenmeyer flask containing 50 ml of the above medium, deprived gelling agent (MS3). The suspensions were placed on an orbital shaker at 110 rpm, for 4 weeks (primary culture). The resulting cell suspension was sieved through a 60 mesh sieve and the filtrate refreshed with MS3 medium containing 40 g/l glucose, 1.9 g/l KNO_3 and 0.5 mg/l casein hydrolysate (MS4). The second subculture was made by sieving cells from the first subculture on a 50 mesh sieve. Cells collected were suspended again in MS4 medium at 40 mg/ml of cells concentration under the same conditions cultures previously described. Fraction cells obtained after sieving on 30 mesh sieve were re-suspended in MS4 medium and incubated on the above same condition cultures to obtain third subculture. The samples were examined of each subculture with a stereomicroscope to detect the formation of embryogenic structures. Cells of each culture were harvested and frozen for polyphenols analysis. Medium for all callus and cell cultures were adjusted to pH 5.8 by KOH (0.1 N) before autoclaving at 121°C for 30 min. The cultures were kept in a room at 28±2°C under 24h photoperiod (16h light/8h dark). Illumination was supplied by cool white fluorescent tubes approximated 2000 lx light intensity.

Growth determination: Calli and cells growth was measured after each subculture. Cells were sieved and weighed. The growth index was defined as w/w_o, where w_o and w represent fresh weigh of cells respectively before and after the cultivation.

Chemicals and reagents: Gallic acid (1), protocatechuic acid (2), hydroxy-3 benzoic acid (3), catechin (4), hydroxy-4 benzoic acid (5), vanillic acid (6), gentisic acid (7), syringic acid (8), caffeic acid (9), epicatechin (10), ellagic acid (11), isovanillic acid (12), vanillin (13), *p*-coumaric acid (14), synapic acid (15), ferulic acid (16), veratric acid (17), rutin (18), benzoic acid (19), piceatannol (20), *o*-coumaric acid (21), piceatannol (22), salicylic acid (23), *trans*-resveratrol (24), trans cinnamic acid (25), *trans*-methoxycinnamic acid (26), quercetin (27), naringenin (28) and genistein (29) were purchased from Sigma-Aldrich (Steinheim, Germany). The phenolic compounds were selected according to their usual occurrence in cotton and their availability as commercial chemical standards. All reagents used were of analytical grade.

Apparatus: The analyses were carried out in Bischoff liquid chromatograph equipped with two Varian Prostar pumps (model 210), an automated gradient controller (Normasoft software) and an automated injector (Alcott, model 708). Detection was carried out with a UV-VIS detector (Kontron, model 430) and Varian Prostar diode array detector (model 335) at room temperature.

Polyphenols extraction and samples preparation: Frozen dry biomass of calli and cells (50 mg) was ground at 4°C with a blender and kept overnight in 5 ml methanol. The samples were centrifuged at 3000 rpm for 10 min, and supernatants were collected and filtered through a 0.45 µm Millipore membrane. The filtrates were diluted with an equal volume of distilled and filtered water and used for quantitative analysis and polyphenols identification.

High-performance liquid chromatography (HPLC): The HPLC analysis of polyphenols was performed on a Varian ProStar HPLC system with a Prontosil C-18 analytical column (250 mm × 8 mm i.d., 5-µm particle size) (Bischoff). The mobile phase was composed of water (A) and acetonitrile (B) in 0.1% TFA (trifluoroacetic acid). The gradient was as follows: 10-50% solvent B (0-40 min), 50-100% solvent B (40-41 min), 100% solvent B (41-50 min), 100-10% solvent B (50-51 min) and 10% solvent B (51-60 min), flow rate of 0.6 ml/min. The temperature of the column was maintained at 40°C, and the effluent was monitored at 280 nm. Detailed descriptions are available elsewhere[30].

The peaks were identified by congruent retention time and spectral matching, and compared with those of the standards.

Calibration curves: Calibration curves: Eleven-point calibration curves for each polyphenol were constructed. Working solutions at concentrations of 0, 5, 10, 15, 20, 25 and 30 µg/ml were used. This operation was repeated about 3 times with freshly prepared calibration standards.

Limits of detection and quantification: The limit of detection and limit of quantification of the analysis method was determined as the analyte concentrations giving rise to signal-to-noise ratios of 3 and 10, respectively. For this purpose, the baseline noise was also evaluated by the injection of 20 µl of the mobile phase A (blank) in triplicate. Correlation coefficient R^2 was > 0.9993.

Statistical analysis: Experimental data were subjected to analysis of variance (ANOVA) using statistical software (release 6.0). Differences between the means of polyphenols contents were compared using Newman-Keuls test. Differences at $P < 0.05$ were considered as significant.

RESULTS AND DISCUSSION

Callus and cell suspension cultures: Callus aspect and growth depended on cotton cultivar used. Calli of R405-2000 were green partially friable, while those of Coker 312 were grey-green and friable (Figure 1). Callus and cells biomass increased progressively during the different subculture and no significant difference of callus was observed between Coker 312 and R405-2000. However, Tre cells suspension growth index of Coker 312 was found to be far higher than R405-2000 ones (Figure 2).

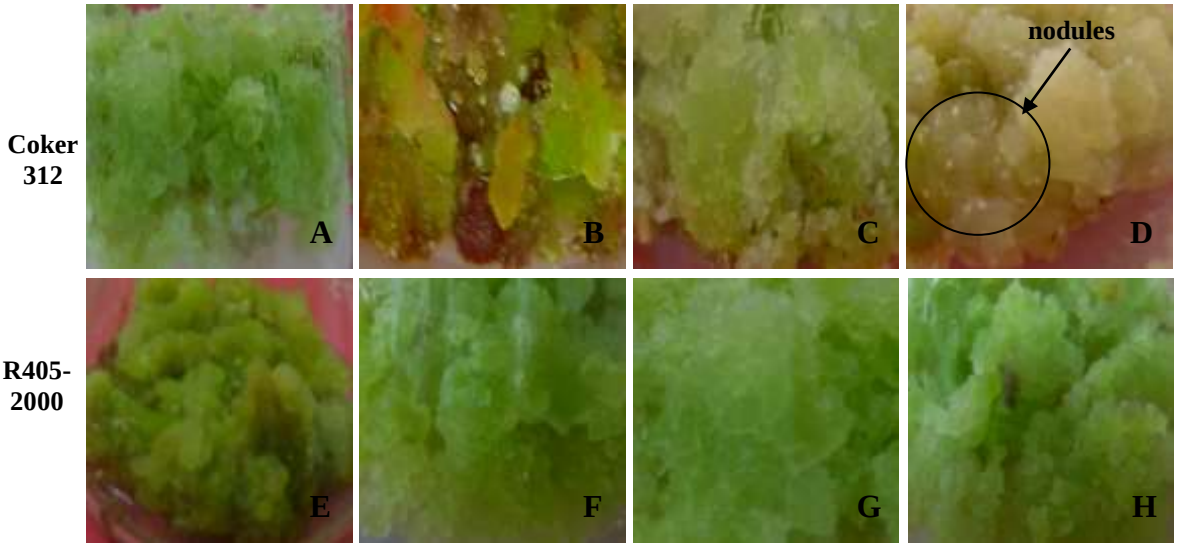

Fig. 1: Aspects of cotton callus formed in different cultures (x 10)

Callus of Coker 312 is grey-green in primary culture (A), turns yellow-green in subculture 1 (B), brownish-yellow in subculture 2 (C) and whitish in subculture 3 (D); callus of R405-2000 is yellow-green R405-2000 in primary culture (E) and grey-green in subcultures 1 (F), 2 (G) and 3 (H). The appearance of nodules (nd) in callus of Coker 312 is characteristics of the presence of embryogenic structures.

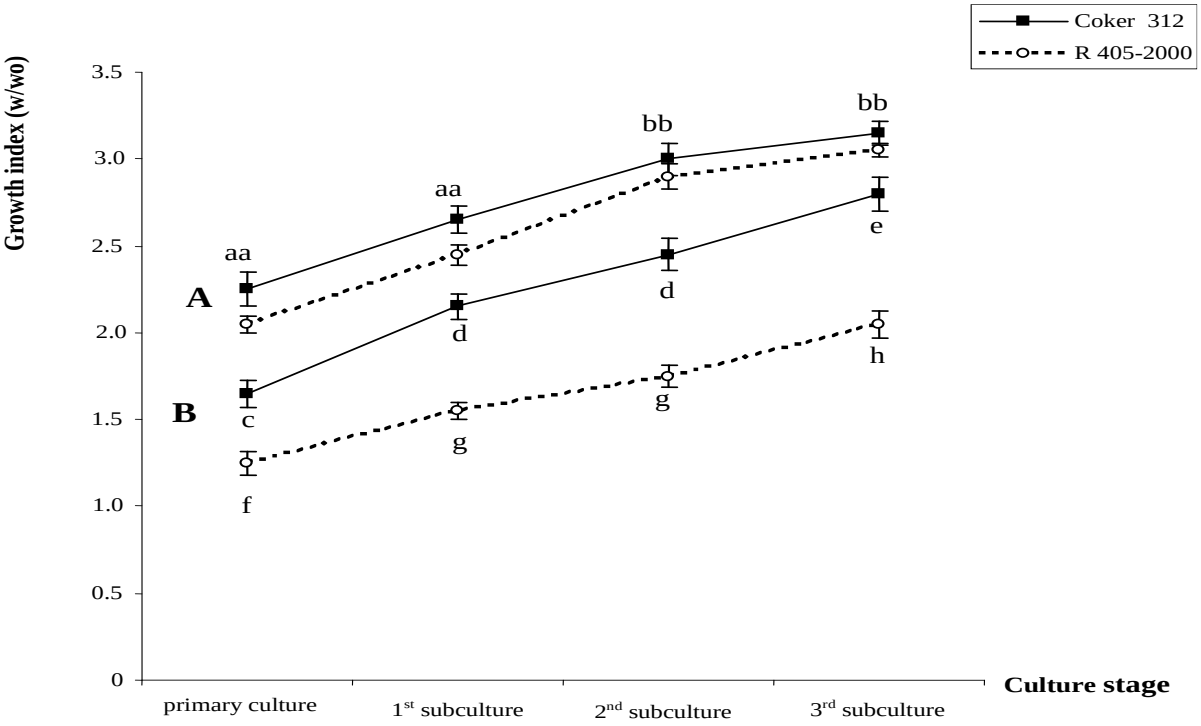

Fig. 2: Tissue growth in cotton calli (A) and cells suspension (B)

Data are the means of three replicate; for each flask, the inoculation biomass was 2 g fresh weight; each culture stage lasts for weeks; Bars in the figure represent standard deviation of three replications; values followed by a same letter are not statistically different (test of Newman keuls at 5%).

Cells suspension was obtained from callus in subculture 3. One-month-old cell suspensions (primary culture) rarely contained embryogenic structures whereas 2, 3 and 4-month-old cells suspensions (subculture 1, 2 and 3) frequently contained numerous embryogenic structures. We observe the formation of embryogenic structures only in Coker 312 cells suspension but not in R405–200 ones (Figure 3).

Identification of polyphenols: To know better endogenous factors that can influence the different behaviour of Coker 312 and R405–2000 considering somatic embryogenesis, we decided to analyse polyphenols compounds accumulated by callus and cells suspension culture. Baseline resolution of polyphenols was achieved with the chromatographic conditions described.

Embryos at the stage of (A) globular, (B) codiforme, (C) heart and cotyledons (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 rnec: large and round non embryogenic cells.

Whenever available, the reference standard of polyphenols (Table 1) was used to substantiate the identification of peaks. The identification of each compound was based on a combination of retention time and spectral matching (HPLC and ^{1}HNMR). Several polyphenols could be identified in cotton callus however cells suspension chromatograms contained major peaks (polyphenols).

Figure 4 shows the HPLC polyphenols profiles of callus and cells suspension of Coker 312. We identified eight polyphenols in callus and twelve in cells suspension in the three subcultures.

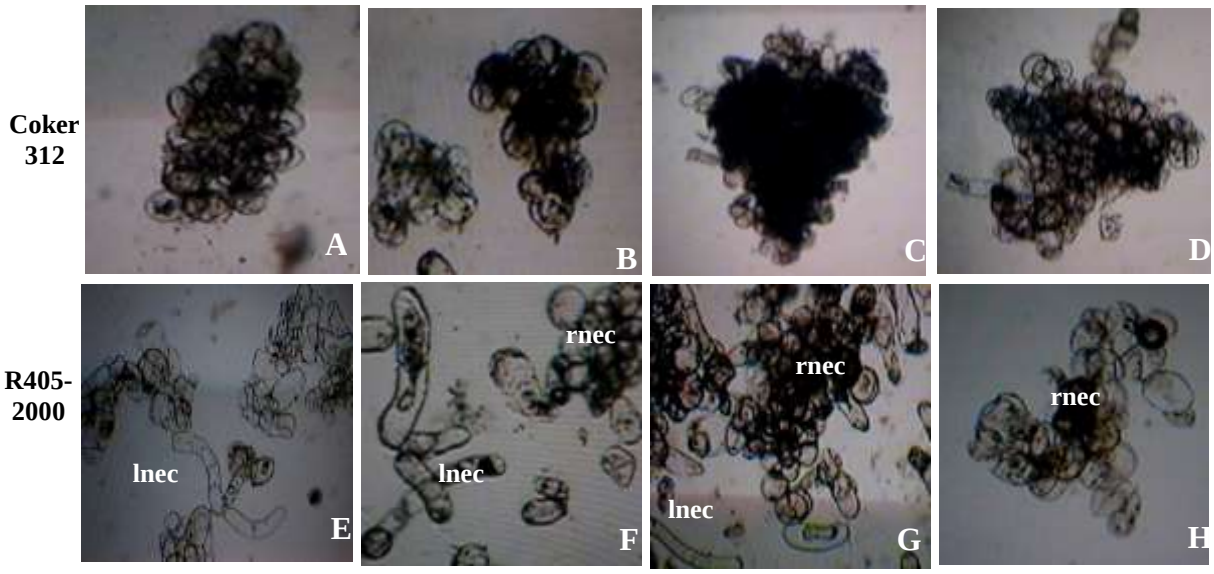

Fig. 3: Cells suspension of cotton at different culture stages (x 440)

The polyphenolic profile of callus did not differ significantly between the different stages of callogenesis (Figure 4A). The compounds revealed in callus were phenolic acids: gallic acid (1), gentisic acid (7), caffeic acid (9), ferulic acid (16), rutin (18), *o*-coumaric acid (21) and salicylic acid (23), except for compounds 4 respectively identified as catechin. On the other hand, the polyphenolic profile of cell suspensions showed the presence of twelve polyphenols at the end of third subculture. In addition to the polyphenols identified in callus, we observed the appearance to five news compounds: *p*-coumaric (14), benzoic acid (19), *trans*-resveratrol (24), *trans*-cinnamic acid (25) and naringenin (27). However, it is significant to note that from the polyphenols identified in callus, only rutin disappeared in the cells suspension.

Table 1: Retention time of standard polyphenols at 280 nm

Standard polyphenols	No. of peaks	Retention time (min)
Gallic acid	1	7.17
Protocatechuic acid	2	9.16
Hydroxy 3-benzoic acid	3	10.30
Catechin	4	11.54
Hydroxy 4-benzoic acid	5	11.98
Vanillic acid	6	13.07
Gentisic acid	7	13.46
Syringic acid	8	13.68
Caffeic acid	9	13.79
Ellagic acid	10	13.81
Epicatechin	11	13.83
Isovanillin	12	13.94
Vanillin	13	16.94

p-coumaric acid	14	17.80
Synapic acid	15	18.81
Ferulic acid	16	18.93
Veratric acid	17	19.00
Rutin	18	19.50
Benzoic acid	19	20.67
Piceatannol	20	21.50
o-coumaric acid	21	22.24
Quercitrin	22	22.29
Salicylic acid	23	24.27
trans-Resveratrol	24	26.00
trans-Cinnamic acid	25	28.71
trans-Methoxycinnamic acid	26	29.62
Quercetin	27	31.97
Naringenin	28	32.27
Genistein	29	32.90

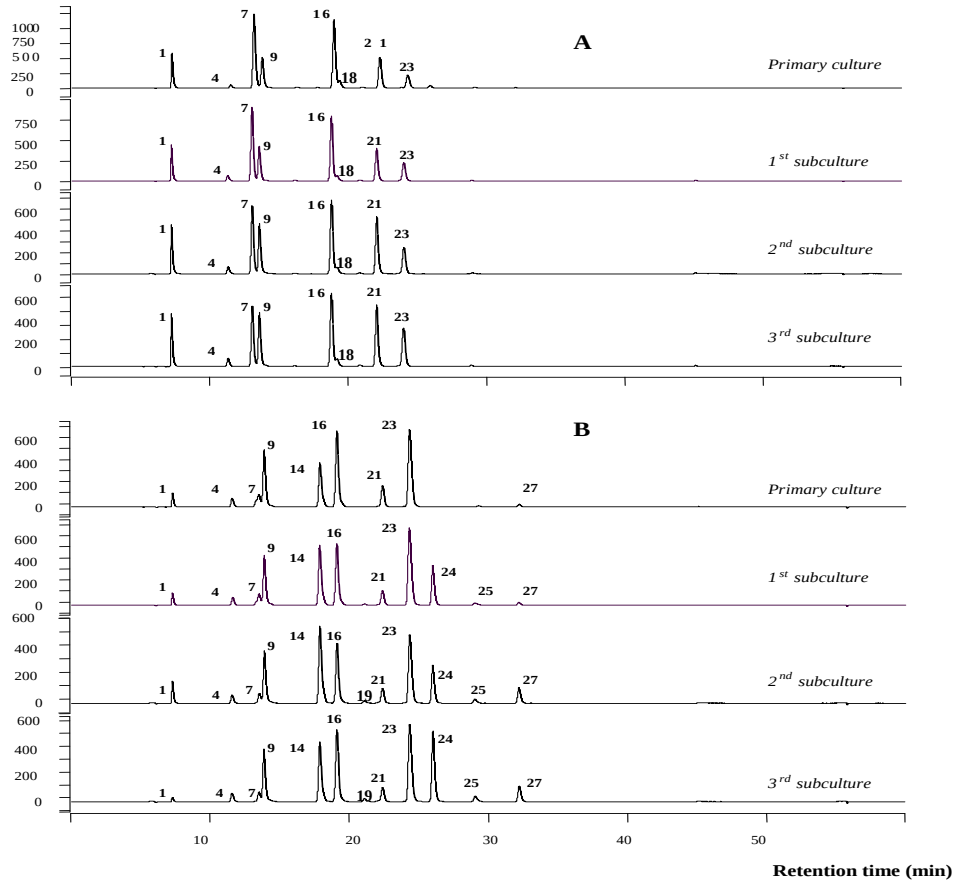

Fig. 4: HPLC profiles of polyphenols from callus (A) and cells suspension (B) of cultivar Coker 312.

Detection is shown at 280 nm; peaks were identified by comparison with reference standards when available or by ^{1}H-NMR data; numbering of peaks refers to their retention time as shown in Table 1; gallic acid (1), catechin (4), gentisic acid (7), caffeic acid (9), *p*-coumaric (14), ferulic acid (16), rutin (18), benzoic acid (19), *o*-coumaric acid (21), salicylic acid (23), *trans*-resveratrol (24), *trans*-cinnamic acid (25) and naringenin (27).

Concerning R405-2000, figure 5 showed HPLC profile of polyphenols identified in callus and cells suspension. Eleven polyphenols compounds were revealed in callus and cells suspension, including nine phenolic acids:

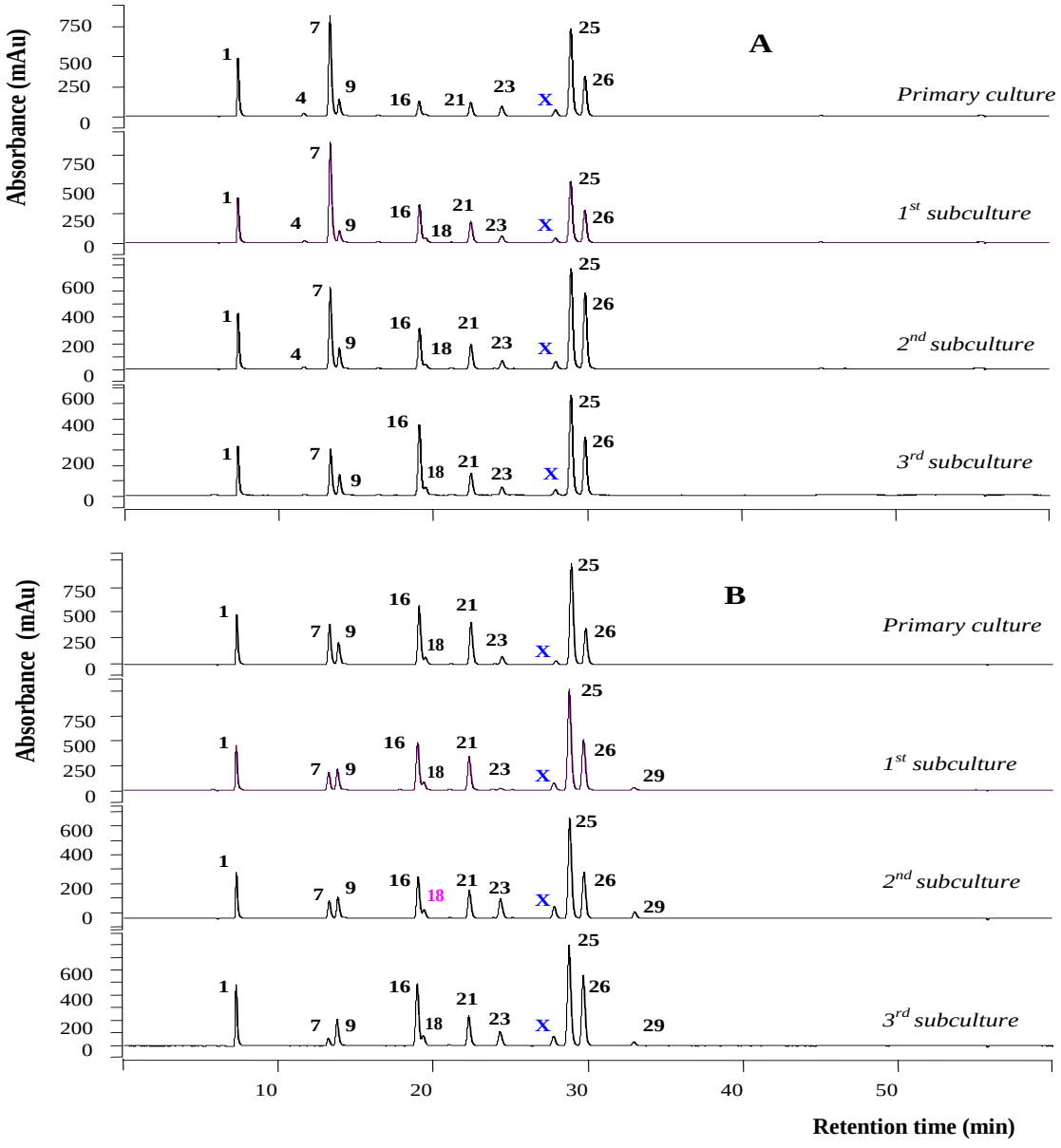

Fig. 5: HPLC profiles of polyphenols isolated in cotton callus (A) and cells suspension (B) of cultivar R405-2000.

Detection is shown at 280 nm; peaks were identified by comparison with reference standards when available or by ^{1}H-NMR data; numbering of peaks refers to their retention time as shown in Table 1;

gallic acid (1), catechin (4), gentisic acid (7), caffeic acid (9), ferulic acid (16), rutin (18), *o*-coumaric acid (21), salicylic acid (23), *trans*-cinnamic acid (25), *trans*-methoxycinnamic acid (26), genistein (29) and *cis*-methoxycinnamic acid (x).

gallic acid (1), gentisic acid (7), caffeic acid (9), ferulic acid (16), *o*-coumaric acid (21), salicylic acid (23), *trans*-cinnamic acid (25), *trans*-methoxycinnamic acid (26) and an unknown compound x whose retention time was found to be different from the reference standard of phenolic compounds. ^{1}H-NMR analyses identified this compound as a *cis* form of methoxycinnamic acid. Two flavonoids were also identified: rutin (18) and genistein (29). Moreover, by comparing the polyphenols synthesized, we observed the disappearance of catechin (4) and the appearance of genistein (29) in the cells suspension.

With regard to these both cotton cultivars, we remarked that *trans*-methoxycinnamic acid (26) *cis*-methoxycinnamic acid (x) and genistein (29) are present in callus of R405-2000 and not that of Coker 312. In cells suspension, Coker 312 product benzoic acid (19), *trans*-resveratrol (24) and naringenin (27) while R450-2000 synthesized *trans*-methoxycinnamic acid (26), *cis*-methoxycinnamic acid (x) and genistein (29).

Quantitative analysis: Table 2 showed the content of polyphenols in callus and cells suspension from primary to third subculture of Coker 312. Regarding to mean content of each polyphenol identified, we notice that among the compounds identified in callus, gentisic acid was the major polyphenol (337.95 µg/g dw). Ferulic acid (21.01 µg/g dw) and salicylic acid (18.14 µg/g dw) were presented with intermediate content while caffeic acid (6.72 µg/g dw), followed by *o*-coumaric acid (6.26 µg/g dw), gallic acid (6.21 µg/g dw) and catechin (5.64 µg/g dw) had the lowest amounts. In cells suspension, the most abundant polyphenols detected were gentisic acid (46.98 µg/g dw) and salicylic acid (46.80 µg/g dw). *p*-Coumaric acid (27.60 µg/g dw) and ferulic acid (21.20 µg/g dw) presented the intermediate amounts. On other hand caffeic acid (7.60 µg/g dw) and catechin (6.35 µg/g dw), followed by *trans*-resveratrol (4.54 µg/g dw), naringenin (2.90 µg/g dw), *o*-coumaric acid (2.29 µg/g dw), benzoic acid (1.69 µg/g dw**),** gallic acid (1.35 µg/g dw) and *trans*-cinnamic acid (0.70 µg/g dw) had the lowest rate.

With regard to R405-2000 (Table 3), gentisic acid (295.88 µg/g dw) was found to be the major polyphenol in callus, followed by *trans*-cinnamic (10.52 µg/g dw), *cis*-methoxycinnamic (7.52 µg/g dw), ferulic (6.83 µg/g dw), salicylic (5.64 µg/g dw) and gallic (5.14 µg/g dw) acids. *trans*-Methoxycinnamic acid (3.52 µg/g dw), rutin (2.65 µg/g dw), *o*-coumaric acid (2.32 µg/g dw), caffeic acid (1.94 µg/g dw) and catechin (1.68 µg/g dw) presented lower contents. Concerning cells suspension, gentisic acid (88.02 µg/g dw) was found again as the major polyphenol. *trans-Cinnamic* acid (10.70 µg/g dw), *cis*-methoxycinnamic acid (10.02 µg/g dw), ferulic acid (7.69 µg/g dw), salicylic acid (7.66 µg/g dw) and gallic acid (5.10 µg/g dw) showed intermediate amounts unlike *o*-coumaric acid (4.13 µg/g dw), *trans*-methoxycinnamic acid (4.05 µg/g dw), caffeic acid (2.35 µg/g dw) and genistein (2.37 µg/g dw) which presented the least important contents. In general, the polyphenols contents were highest in Coker 312 than in R405-2000. Indeed, ratio analysis showed that catechin, ferulic, *o*-coumaric and salicylic acids were thrice more important in callus of Coker 312 than R405-2000 whereas caffeic acid and rutin ratios are respectively four and two more important. In suspension, *o*-coumaric decreases twice while salicylic acid increases until six times and, caffeic and ferulic acids remain to thrice. Moreover, *trans*-cinnamic acid which appeared suddenly in Coker 312 cells suspension was eleven times lesser amounts than R405-2000 ones.

Table 2: Content (µg/g dw ± SD) of polyphenols detected in different culture stages of cotton cultivar Coker 312 during somatic embryogenesis

		Gallic acid	Catechin	Gentisic acid	Caffeic acid	*p*-Coumaric acid	Ferulic acid	*o*-Coumaric acid	Salicylic acid	Benzoic acid	*trans*-resveratrol	*trans*-cinnamic acid	Naringenin
CALLUS	P	5.13±0.01a	4.66±0.03a	343.10±1.92b	5.51±0.02a	nd	21.36±0.70c	4.13±0.01a	9.72±0.07e	nd	nd	nd	nd
	S1	5.45±0.05a	5.65±0.02a	341.65±1.69b	5.95±0.09a	nd	20.32±0.80c	4.46±0.03a	13.83±0.20e	nd	nd	nd	nd
	S2	6.86±0.05a	5.96±0.02a	338.10±1.52b	7.51±0.09a	nd	21.14±0.40c	7.91±0.06a	19.50±0.10c	nd	nd	nd	nd
	S3	7.39±0.05a	6.28±0.04a	328.94±1.31b	7.91±0.03a	nd	21.23±0.70c	8.55±0.07a	29.50±0.30c	nd	nd	nd	nd
	ΔΣ	**6.21±0.04a**	**5.64±0.06a**	**337.95±1.45b**	**6.72±0.03a**	**nd**	**21.01±0.50c**	**6.26±0.05a**	**18.14±0.30c**	**nd**	**nd**	**nd**	**nd**
CELLS SUSPENSION	P	1.42±0.02e	6.30±0.08a	50.04±1.05f	7.80±0.09a	20.19±0.1c	21.40±0.60c	3.31±0.05e	46.62±0.40f	nd	nd	nd	0.29±0.001d
	S1	1.51±0.01e	6.37±0.03a	46.46±0.20f	7.64±0.04a	28.14±0.10c	21.10±0.40c	2.05±0.02e	46.86±0.50f	nd	2.44±0.10e	0.27±0.005d	0.37±0.001d
	S2	1.68±0.03e	6.39±0.03a	45.74±0.13f	7.55±0.02a	30.80±0.14c	21.08±0.60c	1.98±0.01e	46.76±0.30f	1.66±0.01e	3.97±0.10e	1.16±0.02e	5.41±0.20a
	S3	0.79±0.01e	6.35±0.06a	45.67±0.12f	7.42±0.01a	29.85±0.09c	21.20±0.80c	1.82±0.01e	46.95±0.50f	1.70±0.05e	7.20±0.20a	1.35±0.05e	5.53±0.10a
	ΔΣ	**1.35±0.02e**	**6.35±0.04a**	**46.98±0.20f**	**7.60±0.04a**	**27.25±0.10c**	**21.20±0.60c**	**2.29±0.03e**	**46.80±0.40f**	**1.69±0.04e**	**4.54±1.60a**	**0.70±0.03e**	**2.90±0.02e**

Analysis performed in triplicate; nd: not detected; values are means of triplicate determination ± standard deviation (SD); P: primary culture; S1, S2 and S3: 1st, 2nd and 3rd subculture; ΔΣ: mean of each isolated polyphenol; peaks were identified by comparison with reference standards when available or by ^{1}H-NMR data; in a line and a column, values followed of a same letter are not statistically different (test of Newman-Keuls at 5%).

Table 3: Content (µg/g dw ± SD) of polyphenols detected in different culture stages of cotton cultivar R405-2000 during somatic embryogenesis

		Gallic acid	Catechin	Gentisic acid	Caffeic acid	Ferulic acid	*o*-Coumaric acid	Salicylic acid	*trans*-cinnamic acid	*trans*-methoxy cinnamic acid	Genistein	*cis*-methoxy cinnamic acid
CALLUS	**P**	5.17±0.06a	1.85±0.02b	342.22±1.63c	1.89±0.03b	4.40±0.02a	1.36±0.01b	5.86±0.05a	10.58±0.10g	2.38±0.02b	nd	7.53±0.07a
	S1	5.06±0.04a	1.67±0.02b	340.83±1.09c	1.80±0.02b	6.13±0.05a	2.57±0.01b	5.68±0.03a	10.20±0.08g	2.29±0.01b	nd	7.40±0.09a
	S2	5.23±0.08a	1.53±0.02b	334.98±1.33c	2.03±0.02b	6.21±0.08a	2.63±0.02b	5.59±0.05a	10.68±0.06g	4.77±0.03a	nd	7.67±0.05a
	S3	5.09±0.03a	nd	165.50±1.71d	2.06±0.03b	10.60±0.10g	2.71±0.02b	5.43±0.04a	10.63±0.05g	4.64±0.02a	nd	7.69±0.05a
	ΔΣ	**5.14±0.05a**	**1.28±0.03b**	**295.88±1.30c**	**1.94±0.05b**	**6.83±0.04a**	**2.32±0.01b**	**5.64±0.05a**	**10.52±0.03g**	**3.52±0.05ab**	**nd**	**7.57±0.03a**
CELLS SUSPENSION	**P**	5.12±0.04a	nd	154.44±1.23d	2.10±0.04b	10.44±0.10g	4.20±0.04a	5.30±0.03a	10.67±0.07g	3.81±0.02ab	nd	7.67±0.04a
	S1	5.07±0.04a	nd	84.95±0.55e	2.14±0.03b	7.01±0.07a	4.18±0.03a	1.96±0.01f	10.70±0.04g	3.89±0.02ab	1.52±0.01b	9.49±0.08ag
	S2	5.10±0.03a	nd	79.38±1.03e	2.17±0.03b	6.41±0.08a	4.10±0.02a	9.72±0.09g	10.71±0.05g	3.68±0.04ab	3.01±0.01b	11.43±0.11g
	S3	5.14±0.06a	nd	33.32±0.84f	3.01±0.02b	6.90±0.05a	4.05±0.01a	13.66±0.06g	10.73±0.06g	4.84±0.02a	2.57±0.02b	11.50±0.09g
	ΔΣ	**5.10±0.03a**	**nd**	**88.02±1.10e**	**2.35±0.03b**	**7.69±0.06a**	**4.13±0.02a**	**7.66±0.05a**	**10.70±0.06g**	**4.05±0.04a**	**1.78±0.02b**	**10.02±0.07g**

Analysis performed in triplicate; nd: not detected ; values are means of triplicate determination ± standard deviation (SD); P: primary culture; S1, S2 and S3: 1st,2nd and 3rd subculture; ΔΣ: mean of the determined phenolic compound content; peaks were identified by comparison with reference standards when available or by ^{1}H-NMR data; in a line and a column, values followed of a same letter are not statistically different (test of Newman-Keuls at 5%).

Callus and cell suspension cultures: The whitish and friable callus obtained with Coker 312 represented an efficient material for embryogenic structure formation in suspension culture[14,31-33]. Callus and cells suspension fresh biomass, increase recorded at the end of the third subculture. That led us to determine the growth profile of callus and cells suspension between primary and third subculture. The stairs-like growth profile showed is consistent with high number of cells dividing synchronously[34-37]. No significant difference between the callus growth index of Coker 312 and R405-2000 showed that callus color and texture have no influence on the growth. However, many authors reported a relation between grey-green color at the beginning of callogenesis and whitish color at the end of callogenesis culture as well as friable aspect of callus and callus growth in somatic embryogenesis induction[3-6,12,18,36]. The higher growth index of Coker 312 cell suspensions compared to that of R405-2000 confirms these authors' findings. 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 cells suspension but not in R405-2000 ones. The highest grow rate of cells suspension obtained with Coker 312 could notify greater reactivity of the cells of this cultivar in medium culture. 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[1,3-5,10,15-16]. Thus, induction of somatic embryogenesis in cotton was genotype dependant and favourable in suspension cultures[6,11,13].

HPLC analysis: Extraction was performed with a methanol. Methanol has a protective role because it can prevent polyphenols from being oxidized by enzymes, such as phenoloxidases[34]. During cotton somatic embryogenesis process significant changes took place in the composition and the content of callus and cell suspensions polyphenols. The phenolic profiles differed significantly between Coker 312 and R405-2000 during the different subcultures, some either culture type achieved. That usual differences observed with embryogenesis which was genotype-dependent species[28-33,35,38-39] often exploited in chemotaxonomy[10-13,40]. Polyphenols founded in callus and cells suspension can be grouped into for classes of polyphenols: phenolic acids (gallic acid, gentisic acid, caffeic acid, *p*-coumaric, *o*-coumaric acid, ferulic acid, benzoic acid, salicylic acid, *trans*-cinnamic acid, *trans*-metoxycinnamic and *cis*-metoxycinnamic acid), flavonoid (rutin, genistein and naringenin), tannin (catechin) and stilbene (*trans*-resveratrol). Thus, phenolic acids were the dominant polyphenols of cotton callus and cells suspension. Indeed, among the fifteen polyphenols identified, only four are not phenolic acids. This result has also been mentioned by others who founded thirteen phenolic acids out of fourteen polyphenols identified in cotton leaves[29]. Gentisic acid is the major polyphenol in the cultivars of cotton. This is in agreement with the several works which found the same result in cotton leaves[27,41]. Gentisic acid is sometime considered as major polyphenol in cotton and was regularly degraded during somatic embryogenesis as above indicated[27,29,41,42]. Indeed, gentisic acid content in embryogenic Coker 312 cell suspensions is twice less significant than of R405-2000. So, it could exist a correlation between a low level of gentisic acid and embryogenic structures induction in cotton cells.

The analysis of phenolic compounds of callus showed significant differences between Coker 312 and R405-2000. Indeed, callus of R405-2000 produced three phenolic acids: *trans*-cinnamic, *trans*-methoxycinnamic acid and *cis*-methoxycinnamic acid in addition to common phenolic compounds found in both cotton cultivars. These three compounds are cinnamic acids which had a favorable effect on cotton callogenesis. These results are contrary to those of certain authors which revealed had a beneficial effects of cinnamic acids on callus induction[43,44]. The quantity of polyphenols in callus is influenced by cultivar genotype[18,20,30,31]. Thus, callus of cotton cultivar Coker 312 contained of polyphenols the highest amounts. The amount of revealed polyphenols also seem to link to callus induction and growth as already reported above, we found that the level of rutin is twice more important, ferulic, *o*-coumaric, salicylic acids and catechin in that of three times and finally caffeic

acid four times more elevated in Coker 312 callus than R405-2000 when gallic acid and gentisic acid contents are identical. These results confirm the implication of polyphenols in callus induction in cotton[18,27,41,42].

The phenolic profiles of cells suspension showed a significant difference between Coker 312 which develops embryogenic structures and R405-2000 which does not produce embryos. Indeed, we noted in addition to polyphenols in callus, a novo synthesis of *p*-coumaric, benzoic, *trans*-cinnamic acids, *trans*-resveratrol and naringenin in Coker 312 cells suspension when R405-2000 synthesized only genistein in addition. Among these compounds, only *trans*-cinnamic acid is produced in common by the two types of cotton cells suspension. Catechin remained present in cells suspension of Coker 312 while disappeared in R405-2000 cells. We observed also the disappearance of rutin in Coker 312 and catechin in R405-2000. Polyphenols had specific actions on the cellular development[45]. Diphenols would stimulate the cell multiplication while monophenols such as coumaric acid inhibits it[20,46]. In the case of cotton, rutin would be responsible for the inhibition of embryogenic structures induction in cell suspensions as reported by several authors[18,29,46,47]. Embryogenic capacities acquirement of Coker 312 cell suspensions could be due to remain of the inhibition by thee reducing of rutin rate or its disappearance in cells. That could also be attributed to the presence of *p*-coumaric acid, benzoic acid, *trans*-resveratrol, naringenin and catechin in cells suspension. The synthesis of these compounds in cotton cell suspensions depends on cultivar, since it occurred only in the cell suspensions of the embryogenic cultivar Coker 312. Thus, there might be a relationship between *p*-coumaric acid, benzoic acid, *trans*-resveratrol, narigenin and catechin synthesis with embryogenic capacities acquirement in cotton cells which lead to consider these polyphenols as biochemical indicators of cotton somatic embryogenesis. Moreover, this study reported the presence of *trans*-resveratrol in cotton cells suspension for the first time since had never been detected in a cotton organ. *trans*-Resveratrol (3,5,4' trihydristilbene) is a phytoalexin present in various plants[48,49] and considered as the most important dietary source[50,51]. It could promote differentiation of human cells and activates sirtuins which are involved in embryogenesis of metazoans[52,53]. It could also associate to fungus and disease resistance in plant[27,48]. Several works reported the implication of polyphenols content in formation of embryogenic structure in cell suspensions[30-35,43]. Our results showed also a possible relation between phenolic level and the induction of embryogenic structures in cotton. Indeed, in cell suspensions of embryogenic cultivar Coker 312, the levels of salicylic acid is six times more, caffeic acid and ferulic acid are three time more and *o*-coumaric acid is twice higher than those of no embryogenic cultivar R405-2000 where gallic acid and *trans*-cinnamic acid levels are respectively twice and eleven lower. The high amounts of caffeic acid, ferulic acid and salicylic acid in embryogenic cells, contrarily to the non embryogenic ones had been announced by authors in several plants[20,31,44,47]. However, the decrease of gallic acid in our knowledge during the induction of somatic embryogenesis was been never observed. On the contrary, many studies reported an increase of phenols contents and particularly gallic acid content in the embryogenic cells[19,20,30,43,44]. In fact, certain phenols levels were inhibitors and others would be activators of the somatic embryogenesis induction as mentioned by certain authors[18,20,29,46,47]. Caffeic acid, ferulic acid and salicylic acid accumulation and gallic acid degradation in Coker 312 cells seems also to be linked with embryogenic structures induction.

CONCLUSION

Polyphenols quality and amount produced by nonembryogenic R405-2000 and embryogenic Coker 312 cotton cultivars are significant different. Although polyphenols production depend on multiple factors such as culture type, culture stage and genotype, their synthesis during callogenesis and cells suspension culture seems to promote embryogenic structures induction. *trans*-Cinnamic, *trans*-methoxycinnamic and *cis*-methoxycinnamic acids were indicators of cotton callogenesis which did not

lead to embryogenic cells development. On the other hand, ferulic acid, *o*-coumaric acid, salicylic acid, caffeic acid and catechin high levels were indicator with cotton callogenesis of subsequent development of embryogenic cell suspensions. *p*-Coumaric acid, *trans*-resveratrol, naringenin and catechin presence in cells suspension associated to high levels of caffeic, ferulic and salicylic acids and low levels of gallic and *trans*-cinnamic acids were indicators for the induction of embryogenic structures in cotton. Further investigations are in progress to study the addition of these polyphenols in the non-embryogenic cells and tissue cultures of recalcitrant cotton cultivars in the aim of inducing embryogenic structures formation in these cultivars.

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Corresponding author: Kouakou Tanoh Hilaire;

Groupe d'Etude des Substances Végétales à Activités Biologiques, EA 3675, UFR des Sciences Pharmaceutiques, Université de Bordeaux 2,146, rue Léo-Saignat F-33076 Bordeaux cedex, France.