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GC-MS Analysis of Induced Metabolites in Cell Suspensions of Cotton (*Gossypium hirsutum* L.) under Methyl Jasmonate and Ethephon Treatment

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Abstract The study aims to determine the phytochemical constituents present in the cell suspensions of cotton elicited by MeJA and ethephon. Elicitors were added alone or in combination. The freeze-dried cells were soaked with 10 mL of methanol 96% and purified through a mini-column of C18. After concentration of filtrate, the dry extract was dissolved in methanol with chromatographic grade and filtered through a Millipore membrane with 0.22 µm porosity. Then, each of the extracts was further subjected to Gas Chromatography-Mass Spectrometry (GC-MS). Results showed six compounds in control, seven in MeJA, eight in ethephon and nine in co-treatment MeJA/Ethephon. In total, 22 different compounds were detected in the cell suspensions by elicitor or not. Thirteen of these compounds were identified with three in control, four in Ethephon, six in MeJA and four in MeJA/Ethephon co-treatment. Most of identified compounds are lipids and involved in endogenous MeJA metabolism, including *3-oxo-2-(2'-pentenyl)-cyclopentane-*1*-octanoic acid* by the octanoic pathway. This compound predominates with a rate of 100% under MeJA/Ethephon treatment and 75% under MeJA alone treatment. The findings suggest that Nitro-tert-butyl-acetate, 4-O-α-D-glucopyranosyl-D-glucose, Octanoic acid ethyl ester and Monolinoleoylglycerol trimethylsilyl ether under Ethephon treatment, n-decanoic acid, hexanoic acid, D-glycero-D-ido-Heptose, 6-Hepatadecyl-5,6-dihydro-2H-pyran-2-one and Bicyclo [3.3.1] nonan-9-one 1,2,4-trimethyl-3-nitrile under MeJA treatment and, 4,5-diethyl-3,6-dimethyl-3,5-octadiene under concomitant application of MeJA and Ethephon may be potential biomarkers of the response to cotton cell suspensions elicitation by MeJA and ethephon. This study demonstrates that elicitors application can affect highly the biosynthesis of compounds in cotton using cell suspensions as model.

Keywords Cotton; Cell suspension; Ethephon; chromatography mass spectrometry; methanol; Methyl jasmonate

Introduction

Cotton is a tropical plant of which four species are currently cultivated. The most cultivated *Gossypium hirsutum* L. provides nearly 95% of the world cotton production [1]. Cotton is grown for its fibers, which are the main raw material in the textile industry [2]. Seeds contain 18-20% oil, the meal contains 40% protein, which can satisfy 5-6%

of the world's protein requirement [3]. It is therefore both an industrial and subsistence plant and plays an important role in the economy and food security. In Côte d'Ivoire, cotton production represents 1.7% of GDP and is the fourth largest export product after cocoa, rubber and cashew nuts. It is provided for this purpose the main speculation that provides income masses peasant masses of the north of the country which is a marginal zone in Cote d'Ivoire. Thus, it makes it possible to fight the rural exodus and against poverty. Unfortunately, cotton is attacked by a wide range of pests and diseases that limit its production. Among these phytosanitary constraints, fungal diseases play a very detrimental role in reducing production levels.

In Côte d'Ivoire, cotton diseases are generally the main cause of production losses, which is estimated at between 15 and 20%. In the cotton plant, pathogens responsible for diseases are viruses, bacteria, mycoplasmas and especially fungi [4]. Fusarium wilt caused by FOV is the disease that causes the most damage in cotton. In addition, most cultivated cotton varieties are highly susceptible to Fusarium wilt. The incidence of parasitism on cotton crops in tropical climates is such that chemical control, which represents the dominant control strategy, causes problems of toxicity, pollution of the environment, health and even biodiversity [5]. Also, abuse of crop protection products leads to the emergence of resistance which renders them ineffective [6]. Thus, despite the correct application of fungicides, Fusarium wilt persists in the fields and many authors have even advocated abandoning farms or rotating crops as a solution to this disease [7-8]. Moreover, use of transgenic plants and biological control by means of antagonistic organisms which has been proposed as a control strategy also has limits due to the high parasitic pressure [9-10]. Faced with these constraints, researchers have turned to more effective alternatives. They consist in giving plants the means to defend themselves, or to strengthen their own means of defense, rather than to directly combat the aggressor. In this category are the stimulators of natural defenses (SND) of plants that induce the defense reactions of the plant and then mobilizes its own means. They are completely biodegradable and have a generally good eco-toxicological profile [11] and are therefore very environmentally friendly molecules. SNDs are most often analogues or derivatives of natural molecules, among which salicylic acid, ethylene and methyl jasmonate (MeJA) are the most widely used [12-13]. Furthermore, many studies reported that MeJA and ethylene can act synergistically on certain defense genes, leading to increased resistance to pathogens [14-17]. This relies on antifungal compounds stimulation in plant cells or tissues. In cotton, according to Kouakou *et al.* [18], such compounds are likely to belong to the family of phenolic compounds. MeJA and ethylene has been used to induce phenolic metabolites that reduce the incidence of FOV attack in cotton by 90-100% [19-20]. In addition, several studies have reported the induction of compounds of diverse nature with antifungal action by elicitors [22-23]. In addition, non-phenolic compounds have been demonstrated in the stimulation of natural defenses in several plants [24-26].

Secondary metabolites are complex organic molecules with various structures. They generally accumulate in small amounts in specialized cells or tissues and are derived from a highly complex cellular metabolism which process is well regulated. As a result, plant cell culture offers many benefits for the production of these metabolites and is a preferred material for studying their metabolism [27]. One of the major advantages of plant cell culture is the simplicity of this biological tool. The cells are grown under controlled and reproducible environmental conditions. They constitute a homogeneous material, i.e. having the same physiological stage. The addition of substance is easy to achieve and gives better absorption efficiency. All cells will respond, unlike the plant where the reaction is more limited to a site or tissue. Thus, the molecular events leading to the cellular response are easier to study. In addition, secondary metabolism is very active, which facilitates the study of biosynthetic pathways.

The present study aims to characterize metabolites synthesized by cotton cells in response to elicitation with MeJA, ethylene and co-treatment MeJA/ethylene, using cell suspensions as a culture model.

Material and Methods

Plant material and *in vitro* seed germination

Cotton (*Gossypium hirsutum* L.) seeds of cultivar R405-2000 were obtained from Centre National de Recherche Agronomique (CNRA) of Côte d'Ivoire (West Africa). The germination conditions were those described previously [18;28]. Briefly, Seeds were delinted with sulphuric acid. Plump and mature seeds were chosen and pre-treated for 1

min using 70% ethanol, surface-sterilized in a 2.5% aqueous solution of sodium hypochlorite for 20 min. After three successive rinses of 5 min with sterile distilled water, the sterile seeds were soaked and for 24 hours for the softening of the integuments. The seeds which the radicle has emerged were in test tubes containing half-strength MS [29]. salts with Gamborg vitamins B5 [30] medium. The medium contained 30 g/L sucrose solidified with 2.5 g/L gelrite and 0.75 g/L $MgCl_2$. The pH of the media was adjusted to 5.8 with 1 M NaOH before autoclaving. Ten (10) mL of MS medium was poured into test tubes (Pyrex) and one seed was germinated in each test tube. The seeds were incubated in dark for three days to initiate germination and then transferred in culture room during four days at 28 ± 2°C.

Callus Cultures

Hypocotyls of 7-day-old sterile seedlings were cut into segments of 5 mm length and were used as explants for callus initiation. Explants were cultivated in Petri dishes containing MS medium including B5 vitamins, 30 g/L glucose, 0.5 mg/L kinetin and 0.1 mg/L 2,4-D. This callus medium (CM) was solidified with 2.5 g/L gelrite after addition of 0.75 g/L MgCl2. The Petri dishes were hermetically sealed and placed in culture room during one month. Calli were maintained and stabilized through sub-culturing on the same medium (CM) condition for three times with intervals of four weeks. At the end of the third subculture, friable and well-grown calli were used to initiate cell suspensions cultures (Figure 1).

Cell Suspensions Cultures

Cotton suspension cultures were established by transferring approximately 2 g of friable callus into 250 mL Erlenmeyer flask containing 50 mL CM without gelling agent. Callus placed in this cell suspension culture (CSC1) was incubated on an orbital shaker at 110 rpm for one month. Two monthly subcultures were performed on CSC1 medium devoid growth regulator (CSC2).

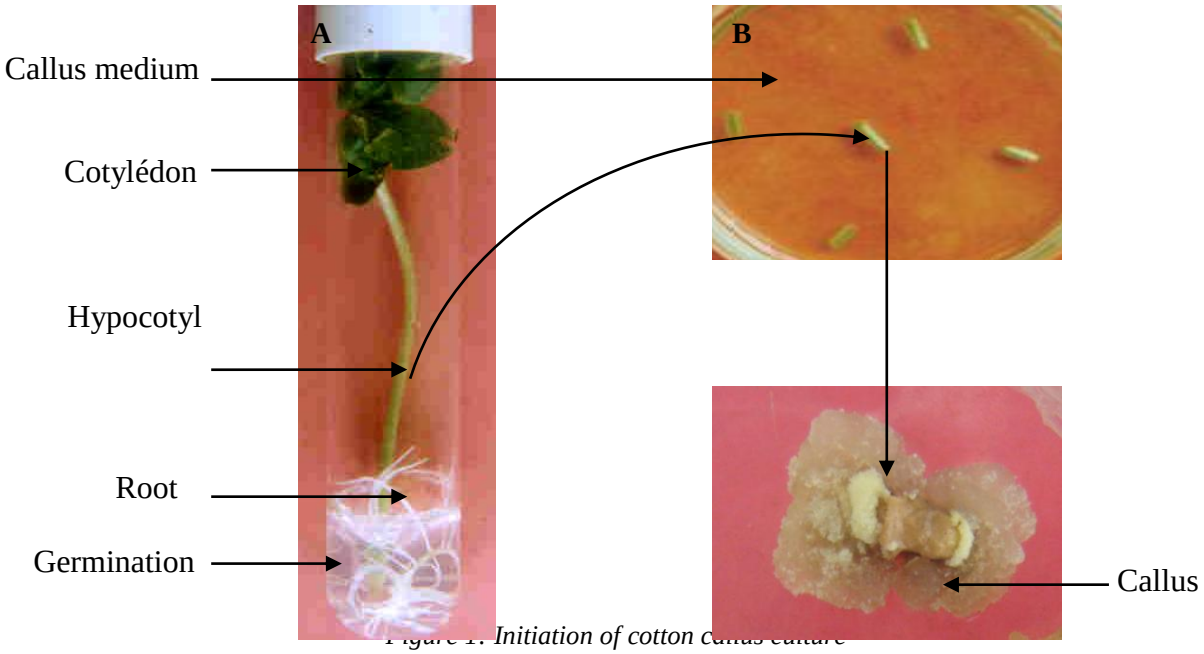

Figure 1. Initiation of cotton callus culture

A, 7-days of cotton vitroplant; B, hypocotyl segments on the callus initiation medium; C, callus formed from the hypocotyls after 4-weeks culture.

To promote cell defense reactions, cells are transferred to the third subculture on the medium CSC2 whose glucose concentration is 40 g/L (CSC3) instead of 30 g/L as previously. Inoculum (cells) is diluted to 1/8th, i.e. 25 mg/mL of CSC3 medium (Figure 2).

Figure 2: Cotton cell suspension in Erlenmeyer flask placed on an orbital shaker turning at 110 rpm

Two (2) g of friable callus were placed into 250 mL Erlenmeyer flask containing 50 mL liquid medium

Incubation conditions

The pH of all media was adjusted to 5.8 prior to autoclave sterilization at 121 ° C for 30 min. All cultures were incubated culture room at 28 ± 2°C under a light intensity of 2000 lx. Light was provided by cool white fluorescent lamps with photoperiod (16 h light/8 h dark).

Treatment of cell suspensions

MeJA (25 mM) is dissolved in 1% ethanol and ethylene, used as ethephon (0.5 g/L) was dissolved in distilled water. Ethephon is a precursor of ethylene. These eliciting substances are prepared aseptically under the laminar flow hood using solvents sterilized by filtration (0.22 µm). They were added to the cell suspension, alone or in combination (co-treatment). A control without eliciting substances was carried out. Cell suspensions were finally placed under on orbital shaker at 110 rpm at 25 ± 2°C under a 16 h photoperiod for two weeks. After the various treatments, the cells are harvested by filtration (cloth to blutter, porosity 30 µm), stored at -20°C and then lyophilized.

Chemical analysis

The solvents employed in GC-MS (Gas Chromatography-Mass Spectrometry) analysis were nanopure grade purchased from Merck (Darmstadt, Germany). The solvents or compounds employed in other analyses were of analytic grade and obtained from Sigma-Aldrich (St Louis, MO, USA).

Preparation of cell extracts

Fifty (50) mg of freeze-dried cells were placed in hemolysis tubes containing 10 mL methanol 96% overnight at 4 °C in a blender. After a sonication for 5 min in Bonsonic ultrasound type 220, the whole was centrifuged at 3000 rpm during 10 min. Supernatant was collected and concentrated by evaporation of solvent using the SpeedVac Automatic evaporation system (Savant System, Holbrook, NY). Freeze-dried extract was then dissolved in 30% methanol (1 mL) and purified through a mini-column of C18 (Sep pack®) grained silica in Supelco Visiprep™ system. Before, columns were conditioned by successive washing with 100% methanol (2 mL), 50% methanol (2 mL) and distilled water (6 mL). After deposit of extract, the mini-column containing extract was washed with 2 mL distilled water and cholorphyll-depleted extract was eluted with methanol 90% (4 mL). Methanolic filtrate was

concentrated with Speed Vac concentrator and the dry extract was dissolved in 1 mL of methanol pure chromatographic methanol. The mixture was filtered through a Millipore membrane with 0.22 µm porosity and filtrate was stored at -20 °C before analysis.

Gas chromatography-mass spectrometry (GC-MS) analysis

Purify methanolic extracts of cell suspensions of cotton were analyzed for the presence of metabolites by Gas chromatography-Mass spectroscopy (GC-MS) technique. GC analysis of the extracts was performed using a GC-MS (Model; Thermo Trace GC Ultra Ver.5.0) equipped with a DB5-MS fused silica capillary column (30 m length x 0.32 µm internal diameter, 0.1 µm film thickness). Gas chromatograph interfaced to a Mass Selective Detector with Hewlett Packard 5989 B.05.02 MS Chemstation software. For GC-MS detection, an electron ionization system with ionization energy of -70eV was used. Helium gas 6.0 was used as a carrier gas at a constant flow rate of 1 mL/min and the sample injected was 1 µL. Injector temperature was 250 °C; Ion source temperature was 200 °C. The oven temperature was programmed from 40 to 200 °C at the rate of 6 °C/min, held isothermal for 1.0 min and finally raised to 250 at 10 °C/min. Interface temperature was kept at 250 °C. The MS scan parameters included an electron impact ionization voltage of 70 eV, a mass range of 29-350 m/z and a scan interval of 0.5 s. The relative percentage of each extract constituent was expressed as percentage with peak area normalization.

Identification of compounds

The identity of the compounds in the extract was assigned by the comparison of their retention time and mass spectra fragmentation patterns with those stored on the computer library and also with published literatures. Wiley 9.0 and NIST libraries sources were also used for matching the identified compounds from the cell suspensions [31;32].

Statistical analysis

Statistical analyzes were carried out using SAS 6.0 software. The means of percentage compounds detected in cell suspensions was compared between the applied treatments. Before any analysis, the test of Kolmogorov-Smirnov was carried out to check the normal distribution of the means. When this distribution is not significant ($P > 0.001$), no transformation was conducted. Then, the test of Kruskal-Wallis was performed to determine the significant differences ($P < 0.05$) between treatments.

Results and Discussion

GC-MS running time was 15.60 minutes. GC-MS chromatogram clearly shows twenty peaks distributed as follows: six peaks in control (1 to 6), seven in MeJA (1, 2, 13 to 17), eight in ethephon (1, 2, 5, 7 to 11) and nine in co-treatment MeJA/Ethephon (2, 3, 6, 15, 18 to 22). Peaks indicating the presence of phytocompounds or metabolites (Figure 3).

Peak Details: 1 (5.75 min); 2 (6.38 min); 3 (6.75 min); 4 (7.16 min); 5 (7.80 min); 6 (8.50 min); 7 (4.98 min); 8 (6.77 min); 9 (9.68 min); 10 (13.41 min); 11 (14.77min); 12 (6.38 min); 13 (7.18 min); 14 (9.57 min); 15 (9.69 min); 16 (10.16 min); 17 (11.78 min); 18 (9.57 min); 19 (9.92 min); 20 (11.79 min); 21 (13.06 min); 22 (13.55 min). GC-MS, gas chromatography coupled to mass spectrometry; MeJA, methyl jasmonate (Refer Figure 3).

The peak number of compounds was given based on their counts per treatment. The compounds distribution with respect to treatment of cell suspensions are presented in the Table 1. Their presence or absence was reported as well as their retention time and mass to charge ratio. Thus, in comparison with control (compounds 1 to 6), the application of ethephon causes the disappearance of compounds 3 and 4 and a synthesis of five new compounds (7 to 11).

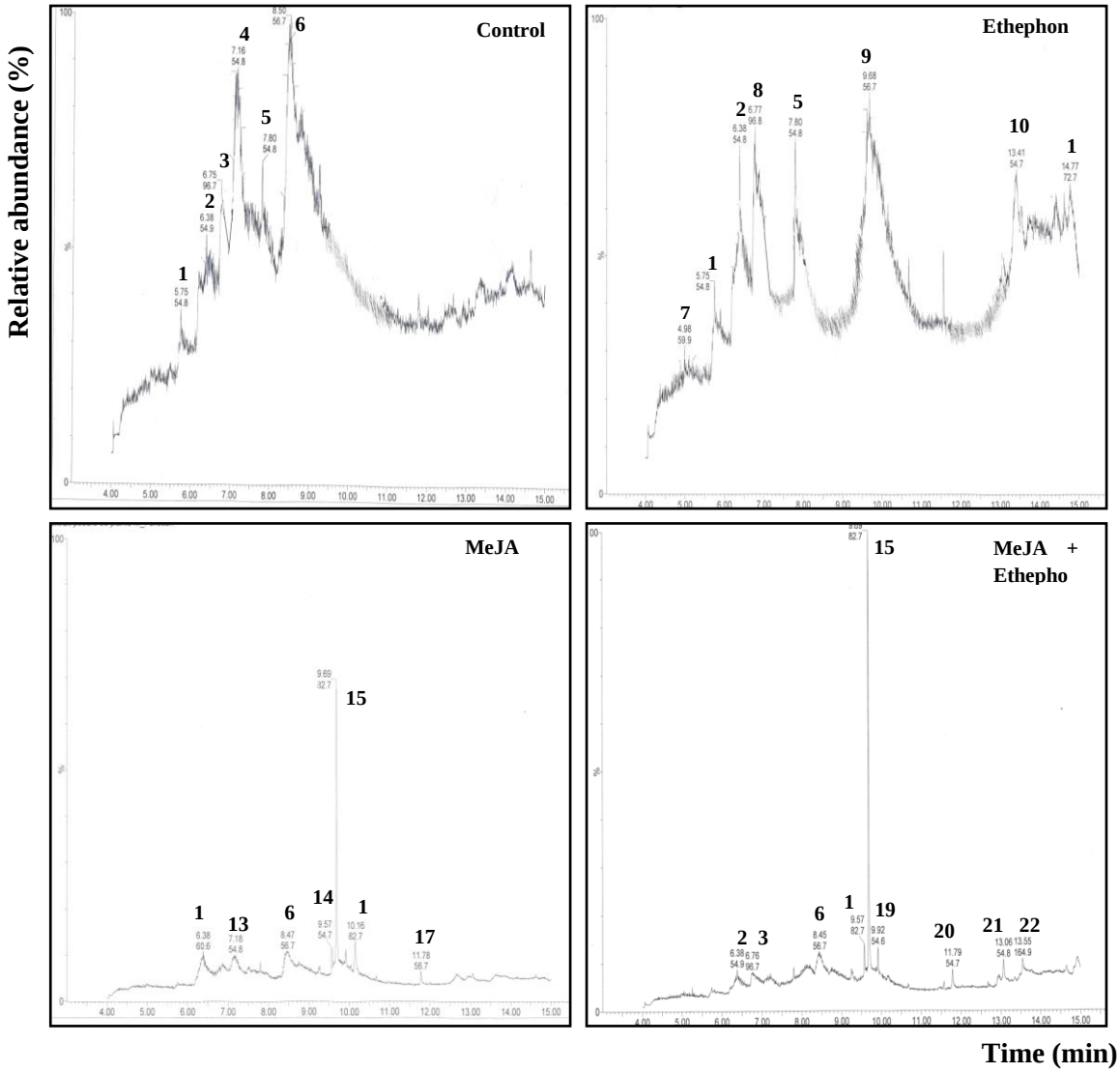

Figure 3: GC-MS chromatogram of methanolic extract of cotton cell suspensions treated with elictors

Table 1: Distribution of compounds detected by GC-MS in cotton cell suspensions treated with elicitors

Peak	RT (min)	Mass to charge ratio (m/z)	Treatments of cotton cell suspensions			
			Control	Ethephon	MeJA	MeJA + Ethephon
1	5.75	54.8	+	+	-	-
2	6.38	54.9	+	+	-	+
3	6.75	96.7	+	-	-	+
4	7.16	54.8	+	-	-	-
5	7.80	54.8	+	+	-	-
6	8.50	54.9	+	-	+	+
7	4.98	59.9	-	+	-	-
8	6.77	95.8	-	+	-	-
9	9.68	56.7	-	+	-	-

10	13.41	54.7	-	+	-	-
11	14.77	60.6	-	+	-	-
12	6.38	72.7	-	-	+	-
13	7.18	54.8	-	-	+	-
14	9.57	54.7	-	-	+	-
15	9.69	82.7	-	-	+	+
16	10.16	82.7	-	-	+	-
17	11.78	56.7	-	-	+	-
18	9.57	54.6	-	-	-	+
19	9.92	82.7	-	-	-	+
20	11.79	54.7	-	-	-	+
21	13.06	54.8	-	-	-	+
22	13.55	164.9	-	-	-	+

(-), absent; (+):, presence; GC-MS, gas chromatography coupled to mass spectrometry; MeJA, methyl jasmonate.

In addition, with MeJA, only compound 6 is present followed by six *de novo* synthesis of compounds. The results also reveal the disappearance of compounds 1, 4 and 5, whereas six new compounds (15, 18 to 22) are synthesized in co-induction treatment (MeJA/Ethephon). Therefore, the presence of compounds seems to depend on application of elicitor types. These results suggest an influence of the treatment on phytoconstituents in cell suspensions of cotton. Similar results have been reported for abiotic stress and induction of metabolites due to elicitors by several authors [33-36]. With respect to control, peaks No. 7 to 22 are therefore synthesized under the influence of elicitor. They are induced compounds as opposed to the preformed or constitutive compounds present in control [37;38]. Furthermore, it is worth noting the following phytocompounds present in control are also present in cotton cell suspensions treated with Ethephon (peaks No. 1, 2 and 5), MeJA (peak No. 6) and MeJA/Ethephon (peaks No. 2, 3 and 6). The peak area percentage is represented in Table 2. It reveals that compound No. 15 present in cell suspensions treated with MeJA/Ethephon was predominant (100%) followed by MeJA alone (75%), compound No. 6 in control (45%) and compound No. 9 in Ethephon (43%). The elicitor treatments reduce the proportion of compound No. 6 (6-7%) compared to control (45%). Moreover, Ethephon increases the level of compound No. 2 (20%) against 9% in control. The lowest levels were obtained with compounds No. 13-14 (3-5%), 16-18 (5-8%) under MeJA treatment and 19-22 (6-8%) under treatment MeJA/Ethephon. Similarly authors were reported the GC-MS analysis of plants extract in many plants [39-41]. Present investigation deals with the determination of constituents from the cell suspensions of cotton treated with MeJA and Ethylene in form of Ethephon, alone or combined, using GC-MS and their comparative analysis. The GC-MS analysis revealed the presence of total 22 compounds (six from control, seven from MeJA, eight from Ethephon and nine from MeJA/Ethephon) with valuable retention time and ratio mass/charge (m/z). Some of the compounds were found to be difficult to identify because they are absent in the referenced database. However, thirteen of the twenty-two compounds present in cell suspensions have been identified. The name of chemical compounds observed in cotton cell suspensions elicitated with MeJA, Ethephon and MeJA/Ethephon was presented in Table 3. These compounds are those which has been identified using the study's identification methods.

Table 2: Percentage of compounds detected in cotton cell suspensions

Peak No	**Relative abundance of compound (%)**			
	Control	**Ethephon**	**MeJA**	**MeJA + Ethephon**
1	10^{a}	13^{a}	ni	ni
2	09^{a}	20^{b}	ni	05^{c}
3	14^{c}	nd	ni	ni
4	33^{d}	nd	ni	ni
5	17^{b}	22^{b}	ni	ni

6	45[e]	nd	07[c]	06[c]
7	ni	05[c]	ni	ni
8	ni	25[b]	ni	ni
9	ni	43[e]	ni	ni
10	ni	12[a]	ni	ni
11	ni	09[a]	ni	ni
12	ni	ni	03[c]	ni
13	ni	ni	04[c]	ni
14	ni	ni	05[c]	ni
15	ni	ni	75[f]	100[g]
16	ni	ni	08[a]	ni
17	ni	ni	04[c]	ni
18	ni	ni	ni	05[c]
19	ni	ni	ni	08[c]
20	ni	ni	ni	06[c]
21	ni	ni	ni	07[c]
22	ni	ni	ni	06[c]

MeJA, methyl jasmonate; ni, not identified; GC-MS, gas chromatography coupled to mass spectrometry. In the line and column, means followed by the same letter are not significantly different (Kruskal-Wallis test at 5%); experiments are triplicates.

Table 3: Compounds identified in the methanolic extract of cotton cell suspensions in GC-MS

Peak	**RT (min)**	**Mass to charge ratio (m /z)**	**Name of the compound**	**Molecular formula**	**Molecular weight**
2	6.38	56.7	Nonylaldehyde (Nonanal)	$C_9H_{18}O$	142.23
4	7.16	54.8	D-glucuronic Acid	$C_6H_{10}O_7$	194.14
6	8.50	54.9	n-decanoic Acid	$C_{10}H_{20}O_2$	172.27
7	4.98	59.9	Nitro-tert-butyl-acetate	$C_6H_{12}O_2$	116.16
8	6.77	95.8	4-O-α-D-glucopyranosyl-D-Glucose (Maltose)	$C_{12}H_{22}O_{11}$	342.29
10	13.41	54.7	Octanoic acid ethyl ester	$C_{10}H_{20}O_2$	172.26
11	14.77	60.6	Monolinoleoylglycerol trimethylsilyl ether	$C_{27}H_{54}O_4Si_2$	498.89
12	6.38	72.7	hexanoic Acid (*caproic Acid)*	$C_6H_{12}O_2$	116.16
13	7.18	54.8	D-glycero-D-ido-Heptose	$C_7H_{14}O_7$	210.18
15	9.69	82.7	*3-oxo-2-(2'-pentenyl)-cyclopentane-1-octanoic Acid*	$C_{18}H_{29}O_3$	293.42
16	10.16	82.7	Bicyclo [3.3.1] nonan-9-one 1,2,4-trimethyl-3-nitrile	$C_{12}H_{21}NO$	195.30
17	11.78	56.7	6-Hepatadecyl-5,6-dihydro-2H-pyran-2-one	$C_{22}H_{40}O_2$	336
22	13.55	164.9	4,5-diethyl-3,6-dimethyl-3,5-Octadiene	$C_{14}H_{26}$	194.36

RT: retention time; GC-MS: gas chromatography coupled to mass spectrometry.

Thirteen compounds were identified with three in control (compound No. 2, 4, 6), four in Ethephon (compound No. 2, 8, 10 11), six in MeJA (compound No. 6, 12, 13, 15, 16, 17) and, four in MeJA and Ethephon co-induction (compound No. 2, 6, 15, 22). Their peak No., name and retention time are (2) Nonylaldehyde or nonanal (6.38 min), (4) 3,4,5,6-tetrahydroxy tetrahydropyran-2-carboxylic acid or D-glucuronic Acid (7.16 min), (6) n-decanoic Acid or capric Acid (8.50 min), (7) Nitro-tert-butyl-acetate (4.98 min), (8) 4-O-α-D-glucopyranosyl-D-Glucose or Maltose (6.77 min), (10) Octanoic acid ethyl ester (13.41 min), (11) Monolinoleoylglycerol trimethylsilyl ether (14.77min), (12) hexanoic Acid (6.38 min); 12 (6.38 min), (13) D-glycero-D-ido-Heptose (7.18 min), (15) *3-oxo-2-(2'-pentenyl)-*

cyclopentane-1-*octanoic Acid* (9.69 min), (16) Bicyclo [3.3.1] nonan-9-one 1,2,4-trimethyl-3-nitrile (10.16 min), (17) 6-Hepatadecyl-5,6-dihydro-2H-pyran-2-one (11.78 min) and (22) 4,5-diethyl-3,6-dimethyl-3,5-Octadiene (13.55 min). The compounds No. 1, 3, 5, 9, 14, 18, 19, 20 and 21 have not been able to be identified with the methods used in this study. These are unidentified compounds which have not been taken into account for further investigations.

Compounds 3 and 4 (D-glucuronic Acid) present only in the control seem to indicate an inhibition of its biosynthesis by the application of elicitors. This result would show that these compounds do not intervene in the defense mechanisms put in place by elicitation in the cotton plant. In addition, compounds No. 8 and 11(D-glucose 4-O-α-D-glucopyra-nosyl ether trimethylsilyl monolinoleoylglycerol ether) are synthesized by the Ethephon-treated cell suspensions as well as 12 (hexanoic Acid), 13 (D-glycero-D-ido-Heptose), 16 (Bicyclo [3.3.1] nonan-9-one 1,2,4-trimethyl-3-nitrile), 17 (6-Hepatadecyl-5,6-dihydro-2H-pyran-2-one) synthesized under MeJA seem to indicate clearly that Ethephon and MeJA induce mechanisms of biosynthesis of the compounds by different routes. Moreover, a microarray analysis revealed that ethylene (Ethephon) and MeJA have few target genes in common [42]. Indeed, in this study, no compound was synthesized jointly by Ethephon and MeJA.

Induction of defense mechanisms requires the synthesis and perception of defense signals whose jasmonic acid or its methylated derivative MeJA and ethylene are among the best characterized at present [43]. These compounds are powerful inducers of a wide range of defense genes, and are responsible for the implementation of a good part of the defense responses in the plant. In fact, each elicitor would control a particular spectrum of defense responses. Ethylene induces the synthesis of enzymes of biosynthesis pathways of aromatic compounds for the lignification and production of phytoalexins. Finally, a second type of defense signal is represented by jasmonic acid and/or its methylated derivative MeJA. They would generate oxylipines that are effective in activating the synthesis of phytoalexins regulating defense responses [44;45]. Moreover, it is now established that these two elicitors are produced in a coordinated manner in the inducible response and orchestrate complex signaling networks leading to the stimulation of the natural defenses of plants. Jasmonates and ethylene regulate each of the specific responses [46]. In addition, a growing number of examples show that positive and negative (cross-talk) interactions exist between these metabolic pathways and allow a good response regulation [47]. This would explain the unfavorable effect of co-treatment MeJA/Ethephon on biosynthesis of compound No. 3 and decanoic Acid (compound No. 6). Therefore, there are a negative interaction or amensalism between MeJA and Ethephon for the synthesis of certain compounds. On the other hand, the increase in *3-oxo-2-(2'-pentenyl)-cyclopentane*-1-*octanoic Acid* in MeJA/Ethephon co-treated cell suspensions compared to those treated with MeJA alone , suggests a positive interaction of these two elicitors on the biosynthesis of this compound. Moreover, compounds 18 to 21 and 4,5-diethyl-3,6-dimethyl octa-3,5-diene (compound No. 22), synthesized only in the cell suspensions co-treated with MeJA and Ethephon also shows their cooperative effect on the biosynthesis of the compounds in the cotton plant.In addition, the compounds identified in the cell suspensions treated with the elicitors are for the most part lipid derivatives (Nonanal, n-decanoic Acid, Octanoic acid ethyl ester, Monolinoleoylglycerol trimethylsilyl ether, hexanoic Acid (caproic Acid), *3-oxo-2-(2'-pentenyl)-cyclopentane*-1-*octanoic Acid*, Bicyclo [3.3.1] nonan-9-one 1,2,4-trimethyl-3-nitrile, 4,5-diethyl-3,6-dimethyl-3,5-octadiene, 6-hepatadecyl-5,6-dihydro-2H-pyran-2-one), certainly oxylipines [48]. Indeed, oxylipines constitute a vast family of compounds derived from fatty acids taken from structural glycerolipids (phospholipids and/or galactolipids) of cell membranes and are synthesized in response to various and intervene in the mechanisms of defense put in place by the stressed plants [49-51]. Their biosynthesis generally begins with the intervention of a lipoxygenase on a polyunsaturated fatty acid, the most common precursors being linolenic acid (C18: 3), linoleic acid (C18:2), or hexadecatrienoic acid (C16:3). The formed lipid hydroperoxides are at the crossroads of several metabolic branches which will lead to end products that are generally more stable and endowed with various biological activities. Chloroplast envelope membranes have been shown to be a cellular site of metabolism of these hydroperoxides [52] .The regulation of these various enzymatic pathways would constitute an important control point since the signatures of oxylipines vary considerably according to the plant and the stress applied. Detection in cell suspensions treated with Ethephon such as Monolinoleoylglycerol trimethylsilyl ether, a compound close to α-linoleic acid, would indicate the existence of a biosynthetic activity from

fatty acids found in plant cell membranes composition [26]. These fatty acids, according to some authors, activate the expression of phenylalanine ammonia lyase, stilbene synthase and chalcone synthase genes as well as defensin antifungal peptides synthesis in several plants [53;54]. Cell suspensions treated with MeJA revealed the presence of Nonanal, hexanoic Acid, D-glycero-D-ido-Heptose, Bicyclo [3.3.1] nonan-9-one 1,2,4-trimethyl-3-nitrile and 6-Hepatadecyl-5,6-dihydro-2H-pyran-2-one but most preferably a major compound. This predominant presence would be synonymous with an intense bio-activity. Indeed, 3-oxo-2-(2'-pentenyl)-cyclopentane-1-octanoic Acid is from the group of cyclopentanones to which jasmonic acid and its methylated derivative, MeJA, belongs. This compound would be very involved in jasmonic acid biosynthesis. Indeed, the pathway leading to jasmonate-like molecules (octadecanoic pathway, derived from C18:3) has been particularly studied [23] and presents analogies with the biosynthesis of prostaglandins from arachidonic acid (C20:4) biosynthesis in animals during inflammatory response [55]. Hydroperoxide generated by a 13-LOX is converted into an unstable oxide allene, which is cyclized stereospecifically by an allen oxide cyclase (AOC) to 12-oxo-phytodienoic acid (OPDA). The double bond in the pentacycle of OPDA is then reduced by an OPDA reductase (OPR). Finally, the side chain is shortened by three cycles of α-oxidation to lead jasmonic acid [56]. Thus, 3-oxo-2- (2'-pentenyl)-cyclopentane-1-octanoic Acid would be a jasmonic acid precursor. In addition, an acid carboxylic acid participation to this biosynthesis would probably justify the presence of D-glycero-D-ido-Heptose, a probable isomer of D-glucuronic acid detected in untreated cell suspensions. Bicyclo [3.3.1]-1,2,4-trimethyl-3-cyanononan-9-one detection confirmed a biosynthesis activity of jasmonic acid by the octadecanoic pathway, but above all to clarify its nomenclature. Indeed, this compound would be a bicyclic precursor of MeJA. Indeed, Bicyclo [3.3.1]-1,2,4-trimethyl-3-cyanononan-9-one would be a bicyclic precursor of MeJA [26]. Thus, MeJA is likely to act on its own metabolism since it induces the accumulation of precursors (3-oxo-2-[(2'Pent-2 "-enyl)-cyclopentane] octanoic acid and Bicyclo [3.3.1] nonan-9-one 1,2,4-trimethyl-3-nitrile) of cis-jasmonic acid or MeJA biosynthesis pathway. Jasmonic acid and its derivatives are very diversified translating the plants potential to respond to different endogenous or exogenous stimuli. The signaling pathway of jasmonic acid is distinguished by its ability to self-regulate [47]. Thus, genes of jasmonic acid biosynthesis pathway can be activated by jasmonates, suggesting positive feedback that involves calcium channels [57] . Cell suspensions co-treated with MeJA and Ethephon revealed the n-decanoic Acid, 4,5-diethyl-3,6-dimethyl-3,5-Octadiene and a majority compound which is also 3-oxo-2-(2'-pentenyl) -cyclopentane-1-octanoic Acid with a proportion of 100%. The addition of Ethephon and MeJA increased this compound rate in cell suspensions (33%). The addition of Ethephon and MeJA in cell suspensions increased this compound rate by 33%. Thus, octadecanoic pathway which led to synthesis of *cis*-jasmonic acid or MeJA was amplified under the joint action of the both elicitors, with in addition a synthesis *de novo* of 4,5-diethyl-3,6-dimethyl-3,5-octadiene. This synergistic effect, which induces the synthesis of jasmonate, has also been observed in plants such as tobacco and vines [5;58]. The detection of hexanoic Acid, 3-oxo-2-(2'-pentenyl)-cyclopentane-1-octanoic Acid, Bicyclo [3.3.1] nonan-9-one 1,2,4-trimethyl-3-nitrile, 6-Hepatadecyl-5,6-dihydro-2H-pyran-2-one and 4,5-diethyl-3,6-dimethyl-3,5-octadiene clearly indicates the existence of an active endogenous synthesis of jasmonic acid in cotton cells under MeJA and MeJA/Ethephon application. These results suggest that the octadecanoic pathway is also involved in defense mechanisms in cotton. On the other hand, the absence of these compounds in untreated cell suspensions and those treated with Ethephon supposes that cotton cells would have an endogenous MeJA deficiency and, ethylene through its precursor (Ethephon) seems to have no influence on octadecanoic pathway activation which leads to of jasmonates synthesis in cotton.

The results obtained although constituting a first approach in the knowledge of interaction between ethylene and jasmonates in cotton remain unexpected. The aim of these experiments was to investigate the effect of MeJA and ethephon used alone or in combination on phytoalexins type-phenol accumulation in cell suspensions by GC. However, any compounds of this nature were detected in cotton cell suspensions. This disappointment would be explained in the chromatographic technique used. GC although an effective tool in complex mixtures separation has a major disadvantage. Indeed, although CG is an efficient tool in the separation of complex mixtures, it would have a major disadvantage that the compounds to be analyzed must be volatile and thermostable [56]. What has not been the case in our study. Thus, phytoalexins synthesized in cotton cell suspensions would not volatile and thermostable. However, several authors have reported the presence of volatile polyphenols type-terpenoid in cotton leaves after an

exogenous application of MeJA [59]. Therefore, nature of the sample seems to have an influence on detcetion of volatile secondary metabolites synthesized by plants. This result shows the crucial role of chromatographic analysis technique in volatile compounds detection in plants. Indeed, although the chromatographic technique used is identical, variables such as the column (length and diameter), detection of the wavelength of compounds, solvent nature as well as elution gradient and rate may heavily impact on the nature of volatile compounds detected [18;60]. In reality, there are many volatile compounds in cotton cell suspensions, few of which are phenolic and not thermostable. In this study, most of volatile compounds identified in cell suspensions with GC are lipids. Indeed, application of elicitor would lead to specific molecules production such as systemin or parieta1 oligogalacturonides that would activate a lipase. This enzyme would release, from the plasma membrane of cells, linolenic acid [23;52]. Then, there would be several lipid intermediates induction which would activate the octadecanoic pathwaye and leads to jasmonic acid synthesis *via* lipoxygenases [24;25]. This study made it possible to highlight the presence of lipid derivatives in cell suspensions which could be considered as specific markers of the stimulation of cotton defenses. Thus, GC-MS would be an interesting tool for detection and quantification of specific markers of plant defense [47]. Nitro-tert-butyl-acetate, 4-O-α-D-glucopyranosyl-D-glucose, Octanoic acid ethyl ester and Monolinoleoylglycerol trimethylsilyl ether may be specific markers of cotton defense when ethephon is applied alone. Similarly, n-decanoic Acid, hexanoic Acid, D-glycero-D-ido-Heptose, 6-Hepatadecyl-5,6-dihydro-2H-pyran-2-one and Bicyclo [3.3.1] nonan-9-one 1,2,4-trimethyl-3-nitrile would be specific markers under MeJA used alone. Finally, with co-treatment of MeJA and Ethephon, 4,5-diethyl-3,6-dimethyl-3,5-octadiene would be a specific marker for the stimulation of cotton defenses.

Conclusion

The use of cell suspensions has made it possible to develop a system-model which can constitute a material choice for the study of defense mechanisms regulation under the elicitor's action. CG is an interesting tool in compounds detection that are the basis of plant defense mechanisms induction. Moreover, knowledge of molecular foundations of the regulatory mechanisms controlled by elicitors is still very fragmentary and constitutes a very fertile field of investigation to be explored.

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