Original Research Article

Effect of methyl jasmonate and ethephon exogenous application on phenolic compounds accumulation in cotton [*Gossypium hirsutum* L. (Malvaceae)]

ABSTRACT

Aims: The study had for objective to estimate the effect of the stimulating ones of natural defense in the defense of plants.
Place and Duration of Study: Laboratory of Biology and Improvement of Crop Production (Nangui Abrogoua University, Abidjan, Côte d'Ivoire), between February 2018 and August 2018.
Methodology: Thus, the effect of the exogenous application of methyl jasmonate and ethephon on the accumulation of phenolic compounds in cotton [*Gossypium hirsutum* L. (Malvaceae)] grown *in natura* was tested.
Results: The results showed the ability of both stimulators to induce an accumulation of phenolic compounds in cotton. However, the treatment combining the two molecules (MeJA + ETH) was more effective compared to that with MeJA, followed by ETH. Qualitative analysis by HPLC showed the de novo synthesis of protocatechic acid, piceid, pterosilbene and chicoric acid, which can be considered as phenolic markers of the precondition state of cotton. The exogenous application of MeJA and ETH allowed an amplification of the level of synthesis of phenolic compounds.
Conclusion: The stimulation of cotton defense systems by the use of SDN is therefore an interesting alternative to chemical control. Its application in the agricultural sector could contribute to the development of a reasoned and sustainable agriculture that is therefore more respectful of the environment and human health.

Keywords: Methyl Jasmonate (MeJA), ethephon (ETH), natural defense stimulator, cotton, phenolic compounds

1. INTRODUCTION

The fight against plant diseases is a major concern in agriculture. It is estimated that in the world 30 % of crops are destroyed in the field or during storage by phytopathogenic agents. The application of pesticides or fungicides is currently the main means of protection of plants [1]. Pesticide consumption in agriculture is about a little over 3 million tons a year. China (1.8 million tons), Argentina (0.207 million tons), Ukraine (0.078 million tons) and France (0.075 million tons) are the largest users in the world [2]. In Côte d'Ivoire, pesticide consumption is nearly 10,000 tones [3]. The cotton sector is one of the first users of pesticides in the world. In the United States and India, 50 % of the pesticides used are for cotton farming [4, 5]. This strategy is certainly effective, but the problems of diffuse pollution and the possible risks to human health that are linked to it are less and less tolerated by society [6].

In this context, it appears necessary to look for more effective alternatives for the development of sustainable agriculture. One of these is to give plants the means to defend themselves, or to strengthen their own defenses, rather than fighting the attacker directly [7, 8]. In this category are the stimulators of the natural defenses of plants (SDN). Indeed, plants can most often naturally resist their aggressors. However, some plants are more sensitive to pathogens and disease establishment than others by a slow defense response or a low level of compound synthesis rather than an absence of a defense mechanism [9, 10]. Among the natural defense mechanisms that plants develop is the biosynthesis of compounds belonging to the family of polyphenols [11]. These phenolic compounds accumulate in tissues adjacent to necrotic areas suggesting that these compounds may be defensive [12-13]. Cotton produces a large number of phenolic compounds that are critical for disease resistance [8, 14, 15]. The biosynthesis of these compounds can be stimulated by SDN. These are

most often analogs or derivatives of natural molecules among which methyl jasmonate and ethylene. The objective of this work is to estimate the effect of the stimulating of natural defense in the defense of plants. Specifically, it aims to evaluate the effect of the exogenous application of methyl jasmonate and ethephon on the accumulation of phenolic compounds in cotton.

2. MATERIAL AND METHODS

2.1 Plant material

The plant material consists of cotton seed (*Gossypium hirsutum* L.) from cultivar Y764G3, originating in Côte d'Ivoire (West Africa). It is an improved cultivar, resulting from the cross between local lines and introduced lines [16]. The seeds were provided by the Ivorian Textile Development Company.

2.2 Chemicals

All chemicals used were at least analytical grade. Gallic acid, ethanol, methanol, sodium carbonate, triton X-100 and Folin-Ciocalteu reagent were purchased from Sigma-Aldrich (Natick, MA, USA). Methyl jasmonate (MeJA) and ethephon (ETH) produced by Aldrich (Natick, MA, USA).

2.3 Site study

This experiment was carried out in the field on the experimental plot of the Nangui Abrogoua University (UNA) in Abidjan (Côte d'Ivoire). The geographical coordinates of this site are: 5°17 and 5°31' North latitude between 3°45' and 4°2' West longitude [17]. The forest relic of this University contains numerous plant species such as *Chrysophyllum albidu*m G. Don (Sapotaceae), *Synsepalum afzelii* (Engl.) T.D. Penn. (Sapotaceae), *Palisota hirsut*e (Thunb.) K. Schum. (Commelinaceae). The soil is derived from sedimentary formations of the ferralitic type [18]. These sedimentary formations have a clay-sandy texture that is favorable to cotton growing. The mean annual rainfall and temperature are 1,642 mm and 27.16 °C [19].

2.4 Implementation of experimental design

The experimental device used consists of four plots, separated by 100 m from each other. Each plot consists of three ridges 3 m long and 1 m wide. On each ridge, the pockets are separated by 30 cm and 20 cm from those of another ridge.

2.5 Sowing seeds and obtaining cotton vivoplants

The seeds were sown on the ridges at the rate of three seeds per pouch at 5 cm depth. At emergence, the plants were demigrated. Each ridge contains a row of 10 cotton plants, thus 30 cotton plants per basic plot. Plant growth was monitored for two months (size and number of leaves).

2.6 Preparation and application of stimulators

2.6.1 Methyl jasmonate

Methyl jasmonate (MeJA) was prepared at the optimal concentration of 5 mM [19]. Thus, 600 µL of methyl jasmonate was dissolved in 800 µL of 80% ethanol in the presence of 0.5 mL of 1% Triton X-100, and the final volume was then added to 500 mL with water. distilled.

2.6.2 Ethephon

Ethephon (ETH) was prepared at the optimum concentration of 5 g/L [19]. So, 2000 µL of ethephon was dissolved in 4000 µL of 80% ethanol in the presence of 0.5 mL of Triton X-100.
1%, then the final volume was made up to 500 mL with distilled water.

In every elementary plot of land, 10 plants were handled with three repetitions, which is all in all 30 handled plants of the cotton plant. During a treatment, plastic bags were used to separate the treated plants from the others, in order to avoid their contact with the solution. The treatment was carried out by spraying and each plant received 50 mL of solution. The control plants were sprayed with a solution

containing 400 µL of 80% ethanol in the presence of 0.5 ml of 1% Triton X-100, and the final volume was then added to 500 ml with distilled water. After the treatment, an incubation time of 72 h was observed. The leaves were then harvested and freeze-dried for quantitative and qualitative analysis.

2.7 Quantitative analysis of phenolic compounds

2.7.1 Extraction and determination of total phenols in cotton leaves

Phenolic compounds were extracted following the method of [20, 21]. A sample of 100 mg of freeze-dried leave derived from elicited plants was placed in 20 mL of pure methanol and then placed at 4 °C for 12 h. After centrifugation of the mixture at 2000 rpm for 10 min, the supernatant was filtered through a Millipore membrane (0.45 µm) and represented crude phenolic extract. The total phenol content of crude extract was determined using Folin-Ciocalteu's reagent according to the method of [22]. Briefly, an aliquot of crude extract (0.1 mL) was mixed with 0.9 mL of distilled water and 0.5 mL of Folin-Ciocalteu's reagent. The mixture added to 1.5 mL of sodium carbonate 17 % was incubated at 25 °C for 35 min in the dark. The intensity of coloration which is proportional to phenolic compound concentration was monitored with a spectrophotometer at 765 nm a standard curve was prepared using gallic acid (0-100 µg/mL). Total phenol content was calculated from the calibration plot and expressed as mg gallic acid equivalents (mg GAE) of phenol/g of freeze-dried extract (g FDE). The calibration equation for gallic acid was y=0.586x; R^2= 0.998, where y is absorbance and x is the concentration of gallic acid in mg/mL. All measures were performed in triplicate.

2.8 Qualitative analysis of phenolic compounds by high performance liquid chromatography (HPLC)

2.8.1 Extraction and purification of phenolic compounds in cotton leaves

Extraction of the total phenols was carried out as in the previous experiment. For purification, 4 mL of the crude phenol extract was evaporated at Speed Vac (Savant, USA). The sample was taken up in 1 mL of methanol/water (30/70, v/v) and then chromatographed on a mini-column of C18 (Sep pack®) scraped silica in the Supelco Visiprep™ system. Beforehand, the conditioning of the columns is carried out by successive washing with 100 % methanol (2 mL), with 50 % methanol (2 mL) and with distilled water (6 mL). After the sample was removed, a wash with 2 mL of distilled water was performed and the phenolic compounds were eluted with 4 mL of methanol / water (90/10, v/v). The eluate obtained is evaporated at Speed Vac, taken up in 1 mL of methanol/water (50/50, v/v) and then filtered on a Millipore membrane (0.45 µm) before being injected into high performance liquid chromatography (purified phenolic extract).

2.8.2. Analysis conditions

High performance liquid chromatography (HPLC) is performed according to the modified method of [12]. It is used for the separation and quantification of the various phenolic compounds of cotton leaves treated with the fungal fraction.

The analysis of the samples is carried out on two HPLC chains; the first chain (Agilent LC 1100 series) is equipped with a degasser, an automatic injector, a high pressure binary pump and a UV-visible detector. The second chain (Agilent LC 1200 series) includes a quaternary pump and is connected to an iodine array detector and a nuclear magnetic resonance spectrometer (Bruker Avance III, 600 MHZ). The column used with the two chains was a reverse phase C18 (Prontosil, 250 x 4.0 mm, 5 µm, Bischoff). Elution is carried out with a binary gradient composed of :

- solvent A: trifluoroacetic acid (TFA) 1% / water (2.5 / 97.5; v / v)

- solvent B: acetonitrile / solvent A (80/20, v / v)

The profile of the elution gradient is shown in table 1. The chromatograms were detected at 254 nm with a flow rate of 0.8 ml / min. The phenolic compounds used in this study are selected based on their availability in the trade and their possible presence in cotton [14, 21]. These phenolic compounds are presented as follows: caffeic acid, cinnamic acid, ferulic acid, gallic acid, *p*-coumaric acid, salicylic

acid, astringin, catechin, epicatechin, genistein, gossypin, naringenin, piceatannol, piceide, pterostilbene, quercetin, quercitin, resveratrol and rutin.

Table 1. Elution gradient of phenolic compounds extracted from cotton leaves

Time (min)	Solvent A (%)	Solvent B (%)
0-5	85	15
5-10	80	20
10-15	55	45
15-25	40	60
25-40	30	70
40-45	0	100
45-50	85	15

HPLC: High Performance Liquid Chromatography; solvent A (0.1% TFA in filtered distilled water); solvent B (0.1% TFA in acetonitrile); TFA = trifluoroacetic acid

2.8.3 Separation and identification of phenolic compounds by HPLC

The separation and the determination of the phenolic compounds are carried out in HPLC whose control is managed by microcomputer (Workstation system). About 10 µL of the hydromethanic extract was injected into the chromatograph and the detection of the chromatograms was carried out at 254 nm, with a flow rate of 1 mL/min. Each analysis was repeated three times. A reference library of phenolic compounds was made with compounds purified and identified by nuclear magnetic resonance (RMN).

This library contains the retention times and RMN spectra of these compounds. The chromatograms obtained were used for the identification of the compounds contained in the injected samples. The structure of the phenolic compounds was verified by RMN.

2.9 Statistical analysis

Experiments were performed using a completely randomized design. Data were subjected to analysis of variance (ANOVA) were carried out for the experiment using Statistica software (release 7.1). Means of data were compared by Newman-Keuls's Multiple Range Test. Differences at $P \leq 0.05$ were considered as significant.

3. RESULTS

Fig 1 shows total phenol contents in cotton leaves treated by stimulators. The analysis of the figure shows that cotton leaves treated with the combination of methyl jasmonate and ethephon (MeJA+ETH) yielded the highest total phenol content (94.65 mg/g FDE), followed by those treated with MeJA (84.16 mg/g FDE). While those treated with ethephon resulted in a total phenol content of 71.46 mg/g FDE, compared to 37.12 mg/g FDE in the control leaves.

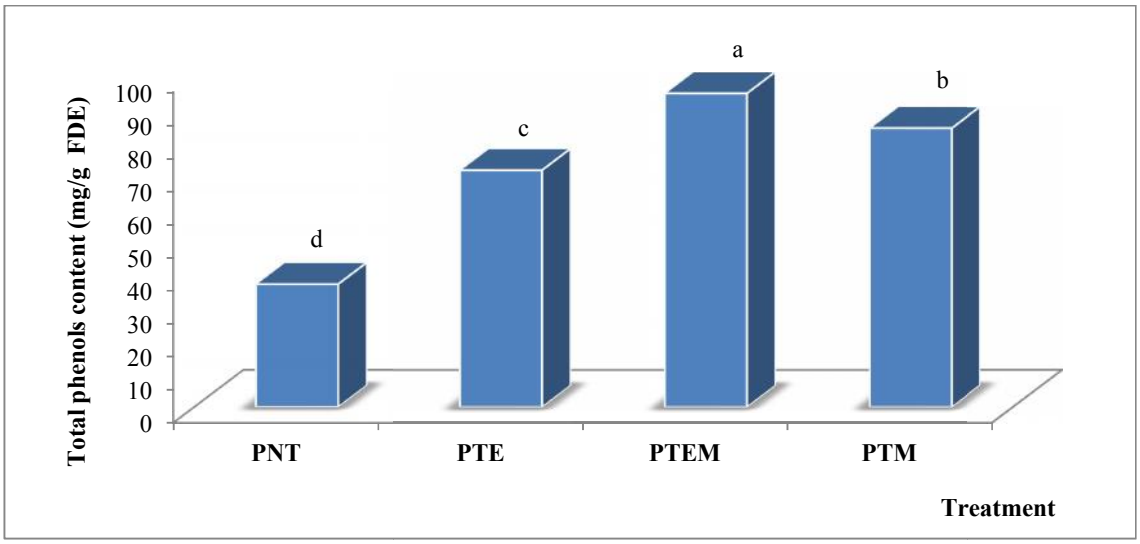

Fig 1. Total phenol content in cotton leaves treated with stimulators

PNT: untreated plant (control), PTE: ethephon treated plant, PTEM: plant treated with the combination of methyl jasmonate and ethephon, PTM: plant treated with methyl jasmonate. The values followed by the same letter are not significantly different (Newman-Keuls test at 5%); the values represent the average of three repetitions.

HPLC analysis of the samples allowed accurate comparison and identification of phenolic compounds in cotton leaves treated with SDN. Before sample analysis, 19 phenol standards were chromatographed under the same conditions as the samples. This made it possible to determine the different retention times of the phenolic controls (Table 2). Thus, by comparing the retention time of each chromatogram with those of the standards, the various phenolic compounds could be identified. This was made possible by a reference library made with commercially available or purified phenolic compounds. This contains the retention time and the RMN spectra of the phenolic standards.

Table 2. HPLC retention times of phenolic standards detected at 254 nm

Phenolic compounds	Retention time (min)
Gallic acid	05,496
Gossypin	07,113
Genistein	11,544
Epicatechin	12,341
Catéchin	13,595
Querctrin	15,963
p-coumaric acid	17,616
Férulic acid	18,525
Piceid	18,816
Rutin	19,301
Salicylic acid	19,617
Caffeic acid	20,816
Piceatannol	21,546
Naringenin	21,905
Astringin	22,496
trans-Cinnamic acid	24,730
Quercetin	24,855
trans-Resveratrol	26,992
Pterostilbene	28,345

HPLC (High Performance Liquid Chromatography)

The analysis in fig 2 shows that the chromatographic profile of cotton leaves treated with ethephon (PTE), methyl jasmonate (PTM), the combination of methyl jasmonate and ethephon (PTEM) and leaves untreated (PNT) has similarities and differences. In fact, the PTE sheets synthesized seven phenolic compounds, as well as the PNT sheets. The results revealed that the compounds 4, 7, 8, 9, and 10 are synthesized by both the PTE and PNT sheets. Compounds 5 and 10 disappeared after the ethephon treatment, while there was de novo synthesis of compounds 6 and 14. As for PTM sheets, they synthesized 10 compounds. Compounds 2, 6 and 13 were synthesized de novo with respect to PNT sheets. The treatment associating the two molecules (PTEM) induced the synthesis of 11 phenolic compounds. It allowed the appearance of compounds 2; 6; 11 and 14, relative to PNT leaves. It resulted in the appearance of compounds 2; 11 and 14 compared to the PTE sheets, whereas compared to the PTM leaves, they are the compounds 11 and 14. This treatment has therefore allowed an increase in the number of compounds, compared to each of the two molecules used separately. The results also showed that all the compounds identified after treatment with SDN show large phenolic peaks.

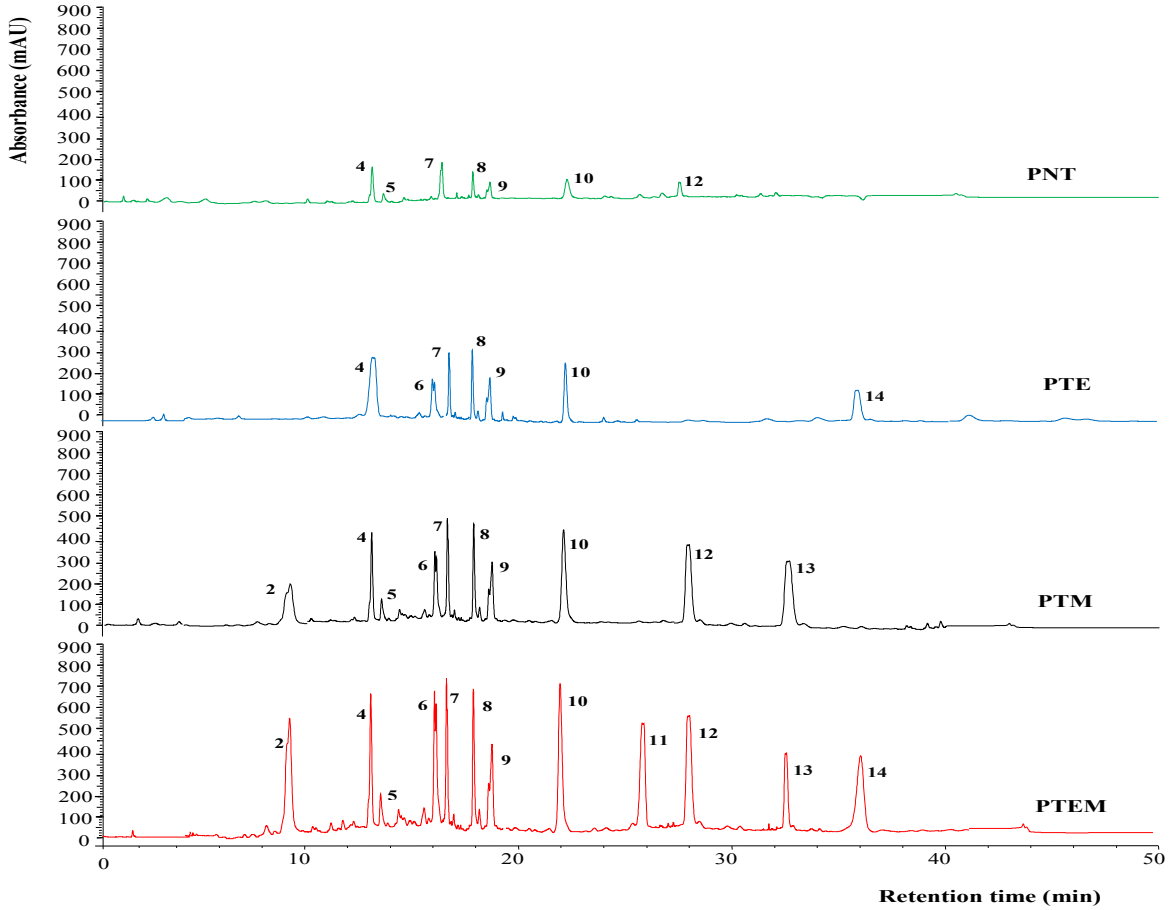

Fig 2. Chromatographic profile of phenolic compounds extracted from cotton leaves treated with natural defenses stimulators at 254 nm

The analysis is performed by high performance liquid chromatography; the chromatograms are detected at the wavelength of 254 nm; identification of phenolic compounds is achieved by retention times and NMR spectra compared to those contained in a reference library of pure compounds; ; MeJA: methyl jasmonate; PNT: untreated plant (control); PET: plant treated with ethephon; PTM: plant treated with MeJA; PTEM: plant cotrested by ethephon and MeJA; 1: gallic acid (3.241 min); 2: protocatechic acid (9.211 min); 3: Gentisic acid (11.538min); 4: Caffèyol-D-glucose (13.605 min); Catechin (14.187 min); 6: Quercetrine (17.201 min); 7: 3-carbamoylquinic acid (17.499 min); 8: Ferulic acid (17.698 min); 9: Gossypetin (18.461 min); 10: Piceatannol (22.215 min); 11: Piperide (25.822 min); 12: Resveratrol (28.101 min); 13: Pterosilbene (32.658 min); 14: Chicory acid (36.075 min).

3. DISCUSSION

The results showed that the exogenous application of the stimulators induced an increase in the total phenol content. Thus, the MeJA allowed inducing the highest content of total phenols, followed by ETH. This increase was more accentuated by the treatment associating the two stimulators. MeJA is therefore the stimulator that induces the production of phenolic compounds the most. These results are in agreement with those of Belhadj *et al*. [12] who reported an accumulation of polyphenols after spraying grapevine plants with MeJA.

Such results have also been obtained by Onil [6] in cotton farmers grown and treated under glass. These authors have shown that the application of MeJA induces an increase in the content of total phenols. In addition to MeJA, the exogenous application of ethephon resulted in an increase in total phenol content. These results suggest that ethylene in the form of ethephon also induces the biosynthesis of phenolic compounds. This stimulator would also be involved in the natural defense of cotton against pathogens. Indeed, ethephon would be involved in the stimulation of phenolic compounds belonging to large phenolic groups such as hydroxycinnamic acid, terpenoid and flavonoids, which are very involved in the protection of cotton according [22; 23]. Moreover, the combination of methyl jasmonate and ethephon (MeJA+ETH), allowed to obtain a total phenols content much higher than that obtained by each of them taken separately. The concomitant application of MeJA and ethephon on the leaves thus seems to have a supra-additive or potentiating effect on the accumulation of phenolic compounds in cotton. This synergistic or cooperative effect of MeJA and ethephon on the accumulation of phenolic compounds has also been reported in cress, grapevine and tobacco [24-27].

HPLC analysis isolated and identified 14 phenolic compounds in the cotton leaves. These are stilbenoids (pterostilbene, piceide, resveratrol and piceatanol), hydroxybenzoic acids (gallic acid, protocatechic acid and genistein acid), hydroxy-cinnamic acids (chicoric acid, ferulic acid and caffeol-D-glucose, *p*-coumaric acid) and flavonoids (catechin, quercetin and gossypetine). This plurality phenolic metabolites biosynthesis has already been reported by Kouakou *et al.* [28] in cotton grown *in vitro* under hormonal stress. Comparison of the chromatographic profiles of the leaves revealed the presence of seven phenolic compounds in both PNT and PTE, ten with PTM and eleven with PTEM. This result clearly indicates that the application of the stimulators has caused a de *novo* synthesis of phenolic compound. The SDN are essential molecules of the defense and plant growth [29]. In addition, the treatment made it possible to increase the level of synthesis of the compounds. However, a plant falls ill due to lack of compounds but a low level of compound synthesis [30]. This seems to suggest that the application of SDN is an effective way that allows the plant to defend itself. The treatment of cotton plants by the combination of methyl jasmonate and ethephon made it possible to identify more compounds with high amplitudes of phenolic peaks compared to those induced by each of the two stimulators taken separately. MeJA and ethylene in the form of ethephon seem to fit into the same complex cascade of cotton signals that lead him to mobilize his own defenses. The combination of these two stimulators seems to be the best for triggering more enhanced defense mechanisms. Thus, joint cotton treatment with these two molecules could increase resistance gains and protect the plant against pathogens such as *Fusarium oxysporum* f. sp. vasinfectum. This association would thus induce a series of defense genes whose implementation and responses would lead to a more effective protection of cotton against pathogens.

4. CONCLUSION

This study showed that MeJA (5 mM) and ETH (5 g/L), after 72 h of incubation, induce an accumulation of phenolic compounds. This ability of MeJA and ETH to better induce the biosynthesis of phenolic compounds was more pronounced after a joint treatment of the two stimulators. The exogenous application of MeJA made it possible to synthesize 10 compounds and ethephon allowed seven. In contrast, the treatment associating the two molecules made it possible to identify 11 compounds. The two stimulators also allowed an increase in the amplitude of the phenolic peaks of the compounds, therefore their level of synthesis. The association of MeJA and ethephon is therefore best indicated for the treatment of cotton plants. Protocatechic acid, piceid, pterosilbene and chicoric acid *de novo* induced by the stimulators are thus the phenolic markers of the cotton plant state. Thus, cotton plants will be equipped with phenolic compounds able to anticipate possible attacks of fungi or other pathogens.

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