Original Research Article

Effect of the exogenous application of methyl and ethephon jasmonate on the accumulation of phenolic compounds and efficacy against Fusarium wilt 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. The efficacy of methyl jasmonate and ethephon was subsequently inoculating the cotton plants with a virulent strain of FOV.
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 Gossypine, Pterosilbene and Resveratrol, 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. Inoculation experiments with FOV, a causal agent for Fusarium wilt in cotton, confirmed the efficacy of methyl jasmonate and ethephon. These two molecules conferred protection of cotton plants against FOV.
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 evaluate the effect of the exogenous application of methyl and ethephon jasmonate on the accumulation of phenolic compounds for the natural defense of cotton.

2. MATERIAL AND METHODS

2.1 Biological Material

2.1.1 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.

2.1.2 Fungal material

The fungal material consists of Fusarium oxysporum f. sp. vasinfectum (FOV). It was provided by the phytopathology laboratory of the Higher School of Agronomy (ESA) of the Houphouet Boigny Polytechnic Institute (INPHB) of Yamoussoukro in the Petri dishes containing the PDA medium.

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 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, quercitrin, resveratrol and rutin.

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

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

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 Fungus culture

2.9.1 Transplanting and purifying FOV

The transplantation of the strain FOV was carried out according to the method described by Vakili [22]. Thus, agar fragments of about 0.5 cm in diameter containing the fungus were collected and then put into test tubes. They were then transferred to the hood in petri dishes containing the 8 % PDA medium and sterilized in autoclave at 120 °C for 30 min. The petri dishes thus sown were incubated for 14 days in a culture room at a temperature of 25 °C with a 12 h photoperiod.

2.9.2 Pathogenicity of *Fusarium oxysporum* f. sp. vasinfectum

The purpose of this pathogenicity test was to confirm the special form "vasinfectum" of our isolate of *Fusarium oxysporum*. The aggression of its specific host (the cotton) is manifested by the appearance of the external symptoms: yellowing of the leaves, convexity of the limb (epinasty), yellowing of the ribs beginning either by the circumference of the ribs. Yellowing and necrosis progress between the ribs and, at the end of the evolution, the leaves wilt and fall. The fall of the leaves as you went along was caused by the dieback of the plant due to the alteration of the libéroligneux vessels which are no longer able to drive the water from the roots to the leaves [23].

2.9.3 Preparation of inoculum

Agar fragments of FOV 14 days old were collected and then crushed under sterile conditions in the presence of 5 mL of sterile distilled water. The maceration was filtered on sterile gauze, which retains the mycelium fragments and allows the spores to pass through. About 1 mL of this filtrate was deposited by Petri dish containing the PDA medium and incubated for seven days under the same conditions as before. The resulting FOV colonies were then submerged with 5 mL of sterile distilled water containing a drop of tween 20. The culture surface was gently scraped with a curved, sterile Pasteur pipette to obtain a spore suspension. This method of obtaining the inoculum allows to avoid possible morphological variations. The concentration of the inoculum was determined using a cell of improperly and then adjusted to 104/mL.

2.9.4 Inoculation of 14-day-old cotton vivoplants

The pathogenicity of FOV was demonstrated in the inoculant of 14-day-old cotton and two-to three-leaf stages. Seedlings were obtained by in vivo germination of cotton seeds in pots. On these seedlings, stings were made at the roots using a sterile needle and scarifications using a sterile scalpel blade. Subsequently, inoculum volumes of 50; 1,00; 5,00 and 1,000 µL were deposited at the base of each cotton plant to investigate the dose of inoculum capable of causing the disease. The follow-up of the symptoms of the disease caused by FOV was achieved by observation with the naked eye. The experiment was stopped when dead plants were obtained for developing the disease and the mortality rate of the vivoplants was estimated. 10 vivoplants were used for each dose of inoculum and the experiment was repeated three times.

2.9.5 Reisolating the fungus

To confirm the presence and virulence of the inoculated isolate, the vitroplants used for the pathogenicity test and with symptoms of the disease, including the (uninfected) control, were recovered to perform a reisolation of the pathogen. The reisolation was made from stem fragments because the fusarium wilt caused by FOV is a vascular disease, the presence of the fungus is much more pronounced in the stem [23]. The transplantation of 0.5 cm stem fragments was made in petri dishes on the 8 % PDA Medium, previously sterilized in autoclave at 120 °C for 30 min. Petri dishes

were then incubated for seven days in a culture room at a temperature of 25 °C with a 12 h photoperiod. The fungal cultures obtained are compared to the parent strain.

2.9.6 Characterization and identification of isolated fungal strains

The different fungal strains obtained after isolation and transplantation were identified after a week of culture on a PDA medium using the Botton *et al.* [24].

2.10 Inoculation of cotton plants pretreated in the field

Inoculation of cotton seedlings in the field was carried out 72 h after treatment with stimulators. This time corresponding to the best incubation time of the stimulators for the biosynthesis of phenolic compounds. During inoculation, wounds were made at the neck of the seedlings by punctures with a sterile needle and scarifications with a sterile scalpel blade. Second, the 30-day cotton previously treated by stimulators are inoculated with FOV by depositing about 2 mL of spore suspension at the base of each plant [25]. In this experiment, only the dose of inoculum with the best pathogenicity rate in 14-day-old cotton was retained. Five treatment modalities were carried out:

-Plant not treated by SDN and not inoculated with FOV (PNT);
-Plant not treated by the SDN and inoculated with FOV (PNTi);
-Plant treated with methyl jasmonate and inoculated with FOV (PTMi);
-Plant treated with ethephon and inoculated with FOV (PTEi);
-Plant treated by the association of methyl jasmonate and ethephon, then inoculated with FOV (PTEMi).

For each treatment modality, 20 cotton were used. The experiment was repeated three times. The incidence of the disease caused by FOV was evaluated every 10 days up to the 150th day after inoculation.

2.10.1 Evaluation of the efficacy of FDS against Fusarium wilt

Cotton protection conferred following pacemaker treatment was assessed by determining the mortality rate of FOV treated and inoculated plants compared to untreated and uninoculated controls (PNT) and untreated and inoculated controls. (PNTi). This procedure requires waiting, in untreated and inoculated controls, for the onset of symptoms of the disease characterized by progressive loss of leaves caused by alteration of the liberalising vessels leading water from the roots to the leaves, wilting leaves, yellowing leaves that eventually dry out and wilt announcing the death of the plant.

The severity of the disease (mean wilt score) was determined using the following arbitrary scale:

- score 1: no faded leaves attached or fallen;

score 2: 1 to 15% faded leaves attached or fallen (FFAT);

score 3: 15 to 25% of FFAT;

score 4: 25 to 35% of FFAT;

- score 5: 35 to 45% of FFAT;

score 6: 45 to 55% of FFAT;

- score 7: 55 to 100% of FFAT.

Another way to assess the degree of cotton protection against Fusarium wilt has been to determine the growth parameters. It is a question of measuring the height of the stem and the diameter of the stem, to count the living leaves.

2.11 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

3.1 Effect of methyl jasmonate and ethephon on total phenol content in cotton leaves

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.

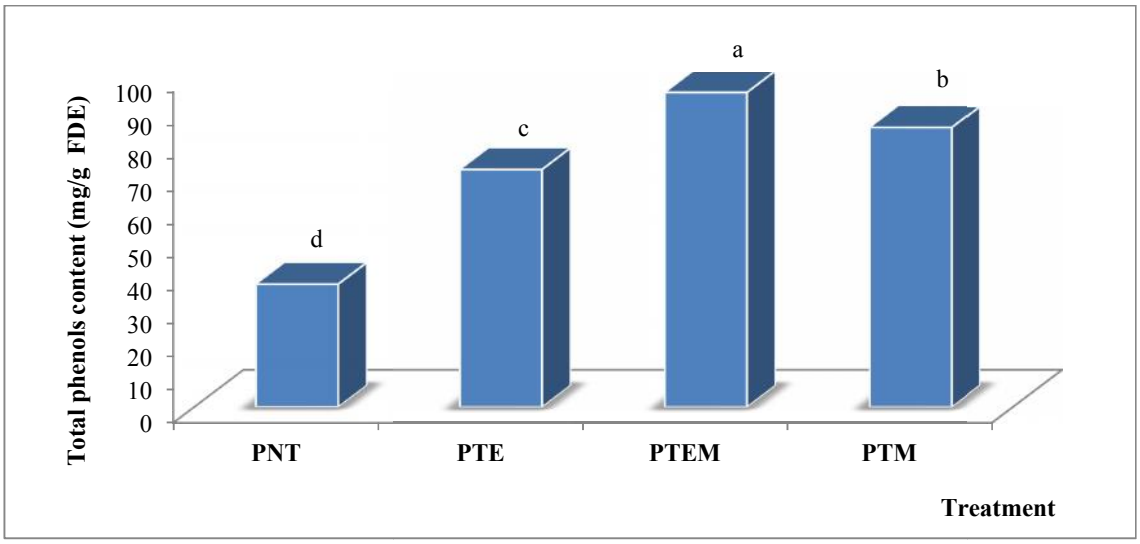

Fig 1. Total phenol content in cotton leaves treated with stimulators

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.

3.2 Identification of phenolic compounds in cotton leaves

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.

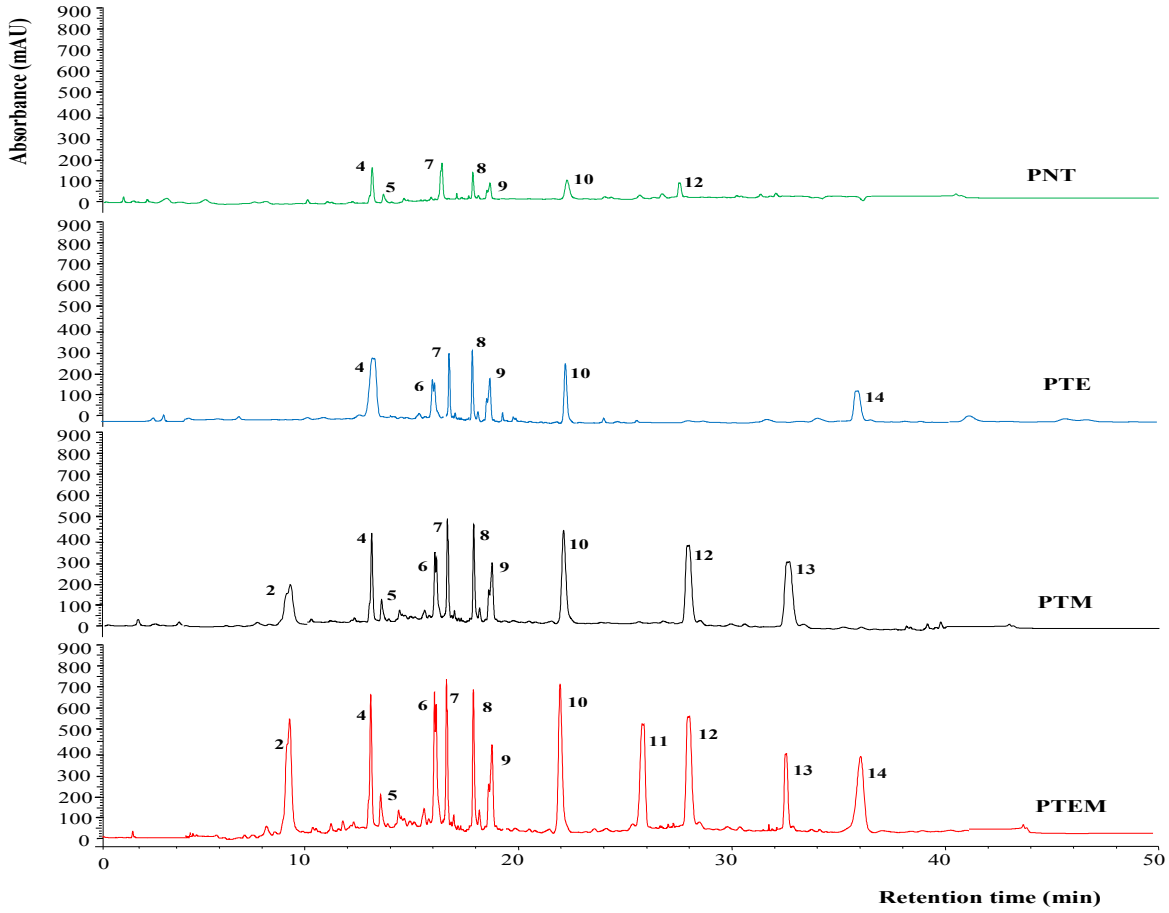

Fig 2. Chromatographic profile of phenolic compounds extracted from cotton leaves treated with natural defenses stimulators at 254 nm

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).

3.3 Evaluation of cotton protection against Fusarium wilt FOV mortality rate

The evaluation of the mortality rate caused by FOV is reported in Table IX. It was carried out every 10 days after the inoculation of the cotton plants to FOV to end on the 150th day when no live leaf was observed on the plants not treated by the SDN and inoculated with FOV (PNTi).

Thus, from day 0 until the 30th day after the incubation, no wilting was observed on all the leaves (score 1: no withered leaf attached or fallen). From 40 days to 50 days after inoculation, a local wilting symptom (less than a quarter of the leaf area) was observed (score 2, 1 to 15% faded leaves) in untreated and inoculated plants. at FOV (PNTi) while plants treated with pacemakers and inoculated, as well as untreated and uninoculated plants are intact.

On day 60, the ethephon-treated and inoculated plants (PTEi) began to exhibit wilting symptoms such as PNTi. In the 70th, these symptoms began to appear also on the plants treated with methyl jasmonate and inoculated (PTMi). From 80th to 110th day, wilt intensified in all treated plants except untreated and uninfected (PNT) plants, which still showed a wilt of score 1.

On the 120th day after inoculation, symptoms of wilting began to decline in plants treated with the combination of methyl jasmonate and ethephon and then inoculated with FOV (PTEMi) and remained at a severity level of disease score 1 as in the PNT until the 150th.

For other treatments, symptoms of wilting persisted up to 130 days in PTMi and up to 140 days for IPTs. After this time, it stabilizes at a severity level of the disease at score 1 until the 150th day. On the other hand, in PNTi, the severity of the disease is accentuated with a more pronounced wilting of the leaves which results in the total death of the plants at the 150th day (score 7: 55 to 100% of faded leaves). PNT plants showed no wilting of leaves during this study (Figure 22).

Table 3. Effect of the type of SDN treatment on the severity of FOV-Induced disease in cotton

Incubation time (day)	Severity of the disease (score 1-7)				
	Treatment				
	PTMi	PTEi	PTEMi	PNTi	PNT
0	1g	1g	1g	1g	1g
10	1g	1g	1g	1g	1g
20	1g	1f	1g	1g	1g
30	1g	1g	1g	1g	1g
40	1g	1g	1g	2f	1g
50	1g	1g	1g	2f	1g
60	1g	2f	1g	2f	1f
70	2f	2f	1g	2f	1g
80	2f	2f	2f	3e	1g
90	3e	2f	2e	3e	1g
100	3e	2f	2f	4d	1g
110	3e	3e	2f	5c	1g
120	2f	3e	1g	6b	1g
130	1[f]	2f	1g	7a	1g
140	1g	1g	1g	7a	1g
150	1g	1g	1g	7a	1g

FOV: Fusarium oxysporum f. sp. vasinfectum; MeJA: methyl jasmonate; PNT: untreated and uninoculated plant; PNTi: untreated and infected plant; PTEi: plant treated with ethephon and then infected; PTMi: plant treated with MeJA and then infected; PTEMi: plant treated by the association of MeJA and ethephon then inoculated. After treatment, the plants were inoculated with 1000 µl of FOV inoculum and the symptoms of the disease were monitored for 150 days. The severity of the disease was evaluated every 10 days using an arbitrary scale: score 1: no faded leaf attached or fallen (FFAT); score 2: 1 to 15% of FFAT; score 3: 15 to 25% FF; score 4: 25 to 35% FF; score 5: 35 to 45% FF; score 6: 45 to 55% FF; score7: 55 to 100% FF. The severity of the disease was evaluated on each plant, and the average of the scores of each trial. The experiment was repeated three times. On a row and column,
the values followed by the same letter are not significantly different (Newman-Keuls test at 5%).

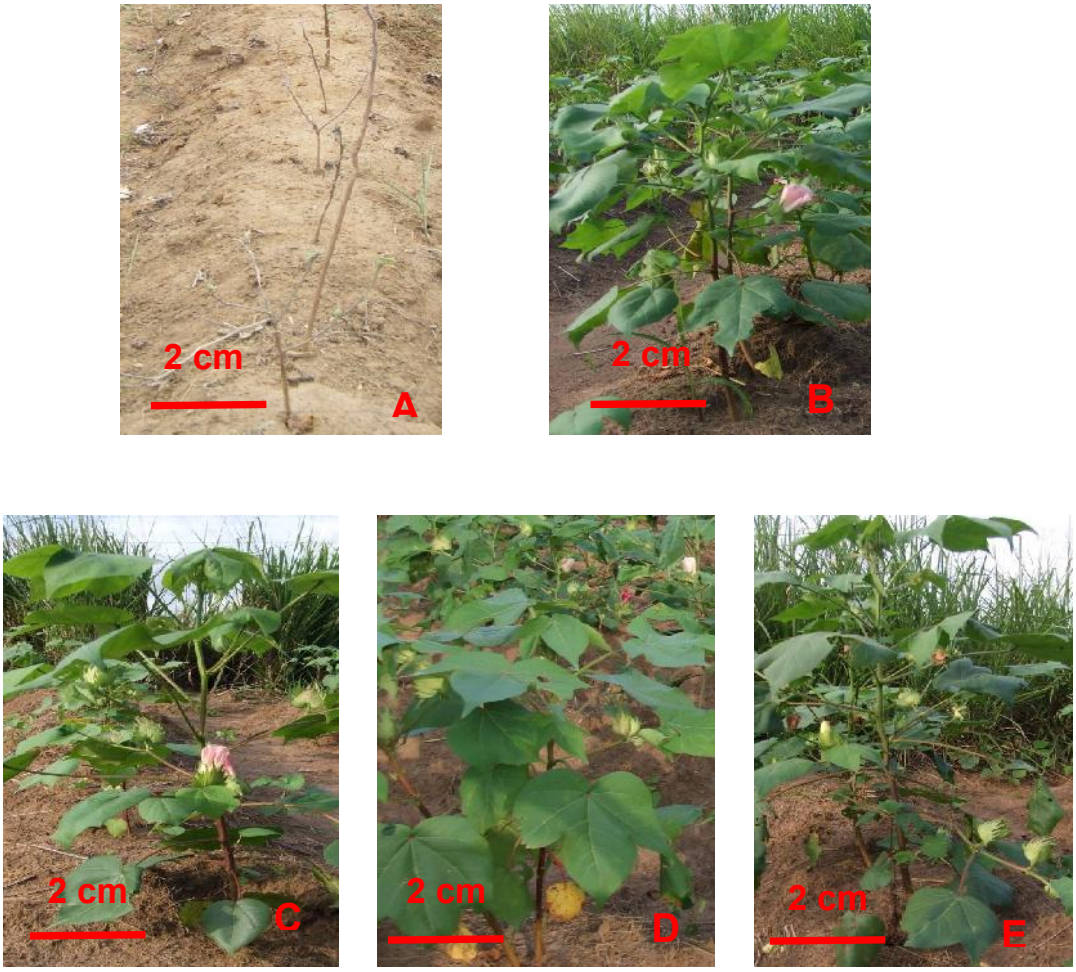

Figure 3. Appearance of cotton plants treated with stimulators then inoculated with fov after 150 days of inoculation

FOV: fusarium oxysporum f.sp. vasinfectum; meja: methyl jasmonate; a: untreated and inoculated plant; b: untreated and uninoculated plant; c: plant treated with ethephon and inoculated; d: plant treated with meja and not inoculated; e: plant treated by the association meja and ethephon then inoculated

3.4 Effect of stimulators on the growth parameters of inoculated cotton plants

Figure 23 shows the influence of the exogenous application of SDN on the evolution of the number of live leaves in cotton inoculated with FOV. The analysis of this figure reveals an increase in the number of living leaves in all cotton plants from the first day of inoculation (D0) to the 10th day (D10). After this time, the number of live leaves remained constant until the 40th day in PNTi cotton plants. But beyond the 40th day, the leaves began to die regularly until the total loss on the 150th day (D150) after FOV inoculation.

As for treated and inoculated cotton and untreated and uninoculated cotton (PNT), the number of live leaves increases until the 70th day (D70) after inoculation. Beyond this time, the plants produce more leaves until the 150th day (D150). In all the cotton plants treated and inoculated, the number of living leaves begins to fall from the 70th to the 110th day.

Beyond this time, in the treated and inoculated cotton plants, the number of living leaves increases until the 150th day. The number of live leaves at the 150th day is significantly higher in PTEMi cotton plants.

Figure 24 shows that up to the 20th day of incubation after FOV inoculation, all cotton plants show the same growth in stem height. After this time, although the PNTi height remains constant, it remains lower than those of the treated plants and inoculated until the 150th day of incubation after inoculation.

The height of the stems of the treated and inoculated plants remained almost constant from the 40th day after inoculation up to the 110th day, and then increased gradually until the 150th day after infection, however the height of the PTEMi stems remained the highest. In PNT, the height of the stems remains almost constant from the 60th day to the 150th day.

Figure 25 shows the effect of exogenous application of SDN on the increase in stem diameter of FOV-inoculated cotton. From the beginning of the inoculation until the 50th day, there is an increase of the diameter of the stem in all the cotton plants. This increase continues progressively in the NTPs until the 150th day, whereas in the treated and inoculated cotton plants and the PNTi, the diameter of the stems decreases regularly. This decline stops on the 110th day for treated and inoculated plants. Then after this time, the diameter of their stem increases gradually until the 150th day. This increase is more accentuated in PTEMi.

As for the PNTi, the decrease in the diameter remains pronounced, to cancel at the 110th. Subsequently, the plants wither with lower stem diameters than initially, thus showing a negative increase in stalk diameter until the 150th day.

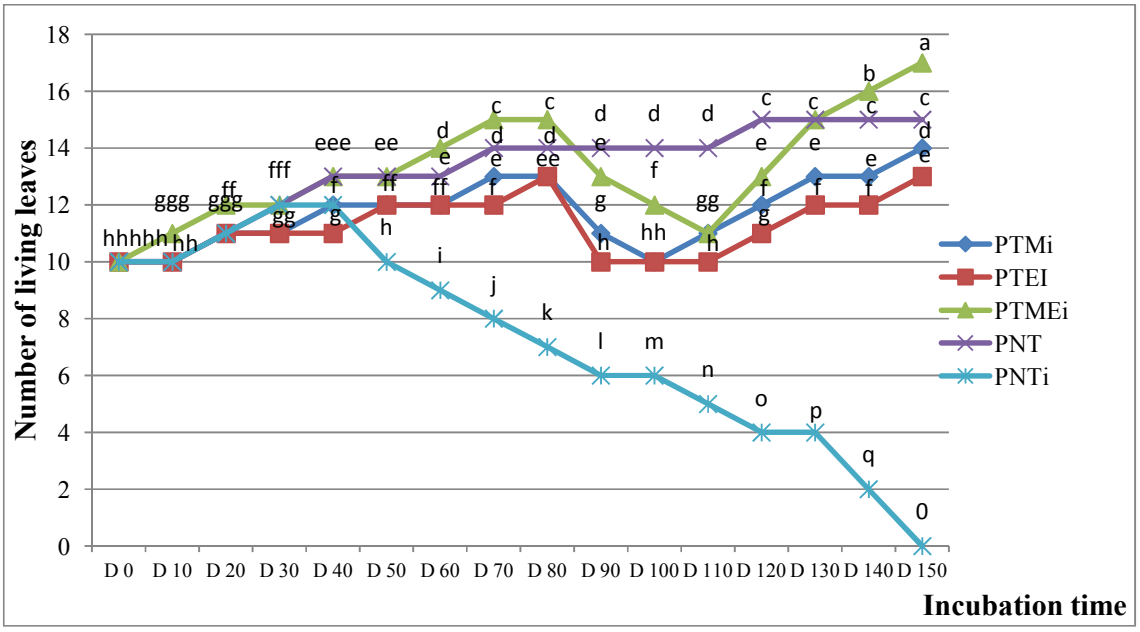

Figure 4. Evolution of the number of live leaves of cotton plants treated with natural stimulators and inoculated with FOV

FOV: Fusarium oxysporum f. sp. vasinfectum; PTMi: plant treated with MeJA and inoculated; PTEi: plant treated with ethephon and inoculated; PTEMi: plant treated by the association of MeJA and the ethephon then inoculated; PNT: untreated and uninoculated plant; PNTi: untreated and inoculated plant. The experiment was repeated three times. Values followed by the same letter are not significantly different (Newman-Keuls test at 5%).

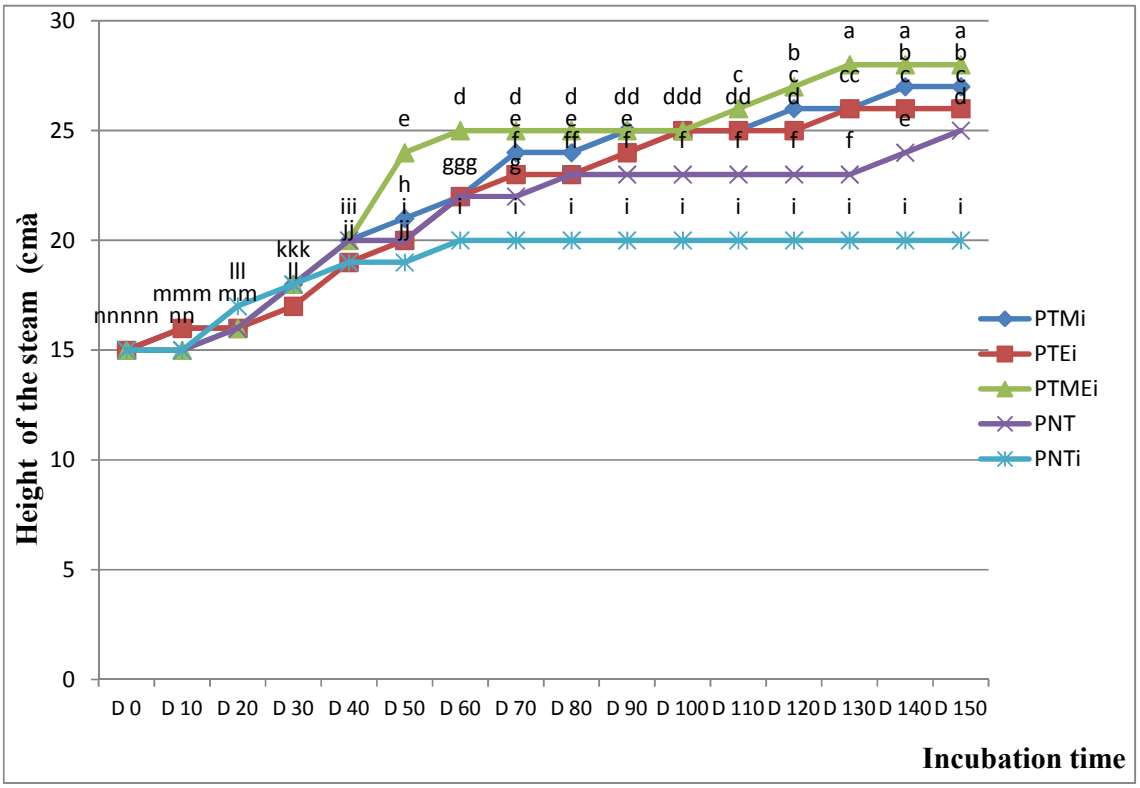

Figure 5. Variation in height growth of cotton plants treated with natural stimulators and inoculated with FOV

FOV: Fusarium oxysporum f. sp. vasinfectum; PTMi: plant treated with MeJA and inoculated; PTEi: plant treated with ethephon and inoculated; PTEMi: plant treated by the association of MeJA and the ethephon then inoculated; PNT: untreated and uninoculated plant; PNTi: untreated and inoculated plant. The experiment was repeated three times. Values followed by the same letter are not significantly different (Newman-Keuls test at 5%).

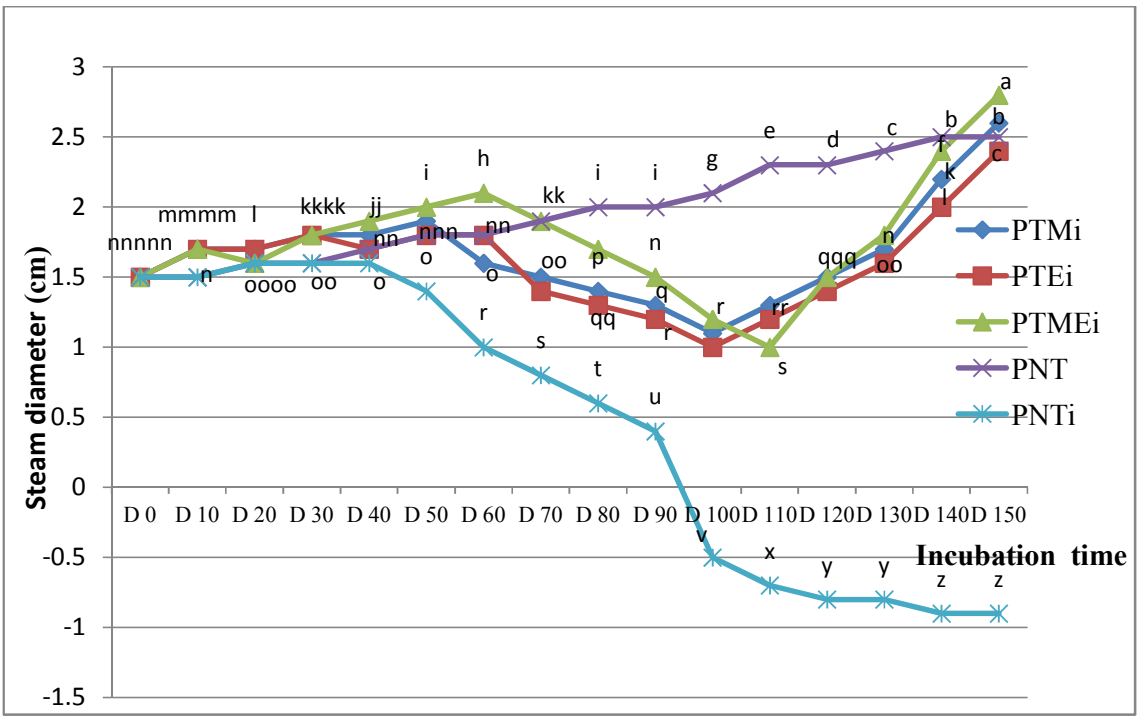

Figure 6. Variation of stem diameter of cotton plants treated with natural stimulators and inoculated with FOV

FOV: Fusarium oxysporum f. sp. vasinfectum; PTMi: plant treated with MeJA and inoculated; PTEi: plant treated with ethephon and inoculated; PTEMi: plant treated by the association of MeJA and the ethephon then inoculated; PNT: untreated and uninoculated plant; PNTi: untreated and inoculated plant. The experiment was repeated three times. Values followed by the same letter are not significantly different (Newman-Keuls test at 5%)

4. 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 [26; 27]. 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 [28-29].

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 gossypine). 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.

The treatment of cotton by the SDN has shown a positive influence on the protection against fusariosis caused by FOV. Indeed, the treatment associating MeJA and ethephon allowed a better protection against FOV compared to MeJA or ethephon applied alone. The symptoms of Fusarium wilt caused by the inoculation of FOV in treated cotton plants decreased, disappearing completely on the 150th day. These results seem to show a reversible effect of the symptoms of fusariosis under the action of different SDNs. The treated cotton plants therefore behaved like PNTs and better in the PTEMi. The combination of methyl jasmonate and ethephon appears to provide a complementary effect by increasing the efficacy of MeJA or ethephon alone. This association would have allowed the establishment of natural defenses by inducing a gain of resistance against FOV. Such results have been reported by Konan et al [31]. greenhouse cotton growers. Indeed, these authors showed the synergistic effect between MeJA and ethephon in phytoprotection against FOV. As for the PNTi, 100% mortality was recorded, thus showing the low level of compounds for the defense of the plant.

The efficacy of SDN in cotton protection was also assessed through a few plant growth parameters such as the number of healthy leaves, the height and stem diameter of the plants after inoculation with FOV. Thus, the results showed an increase in the number of live leaves of cotton plants treated and inoculated with FOV. While among the PNT, the number of leaves remained virtually constant throughout the evaluation period. In contrast, PNTi lost almost all of their leaves. It was the same for the diameter of the stems. Indeed, the fall of the leaves materialized by the decrease of the number of living leaves would be responsible for the die-off of the plants, thus a diminution of the diameter of the stems. FOV will cause browning followed by obstruction of the liberalising stem vessels that carry water from the roots to the leaves. Thus, the plants would become unable to respond to the foliar water appeal as mentioned by Delattre [23]. and Konan [31]. The vital functions of the seedlings, ie primary metabolism (photosynthesis) and secondary metabolism, would be affected or even completely stopped, which resulted in widespread wilting of the plants followed by death of the plants due to lack of nutrients [32].

In addition, the height growth of the PTEMi stems was greater than that of the PNT followed by the PTMi and the PTEi respectively. The height of the PNTi stems remained stable and was the lowest. These results suggest that FOV attack reduced plant growth and development. However, cotton stimulation eventually overcame the progression of the pathogen [33]. The association of MeJA and ethephon had a synergistic effect on the number of healthy leaves as well as the growth in height and the diameter of the stems. This treatment confers a better resistance of the plants against FOV.

5. 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. Gossypine, Resveratrol and Pterosilbene 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. Field inoculations confirmed the properties of MeJA, ethephon, and their association on the protection of cotton against Fusarium wilt. MeJA and ethephon have shown their ability to induce better resistance of the cotton plant to Fusarium oxysporum f. sp. vasinfectum through increased growth and development of cotton plants. Phenolic compounds are therefore an effective means of self-defense of the plant.

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