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Research Article

Effect of Methyl Jasmonate on Phytoalexins Biosynthesis and Induced Disease Resistance to *Fusarium oxysporum f. sp. Vasinfectum* in Cotton (*Gossypium hirsutum* L.)

Yao Kouakou François Konan,[1] Kan Modeste Kouassi,[2] Kouakou Laurent Kouakou,[1] Edmond Koffi,[2] Koffi Nazaire Kouassi,[2] Diabaté Sekou,[2] Mongomaké Kone,[1] and Tanoh Hilaire Kouakou[1]

[1] *Laboratoire de Biologie et Amélioration des Productions Végétales, UFR des Sciences de la Nature, Pôle de Recherche Productions Végétales, Université Nangui Abrogoua, BP 801 Abidjan 02, Cote D'Ivoire*
[2] *Laboratoire Central de Biotechnologies, Centre National de Recherche Agronomique (CNRA), BP 1740 Abidjan 01, Cote D'Ivoire*

Correspondence should be addressed to Tanoh Hilaire Kouakou; tanohilaire@yahoo.fr

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The effect of methyl jasmonate (MeJA) sprayed on cotton healthy leaves was evaluated in terms of inherent bioactive chemicals induction. The total phenolic content significantly increased after MeJA 5.0 mM treatments compared to the other tested concentrations (0; 2.5; 10; 15; 20 mM). Among the eleven phenolic compounds which were found except for ferulic acid, gossypetin, gossypol, 3-*p*-coumaroylquinic acid, and piceatannol were identified as major phenolic constituents of cotton. Their content also significantly increased after the MeJA treatment. In addition, gossypol increased 64 times compared to the control, in the 5.0 mM MeJA treatment. Furthermore, cichoric acid, chlorogenic acid, and pterostilbene are synthesized *de novo* in leaves of MeJA-treated plant. Treatment of cotton leaves with MeJA 5.0 mM followed 72 h of incubation hampered the expression of *Fusarium* wilt caused by *Fusarium oxysporium f. sp. vasinfectum* (FOV). MeJA efficiency was concentration and incubation time dependent. Disease severity on MeJA-treated leaves was significantly lower as compared to the control. Therefore, the high content of gossypetin, gossypol, 3-*p*-coumaroylquinic acid, ferulic acid, and piceatannol and the presence of cichoric acid, chlorogenic acid, and pterostilbene in plants treated with MeJA, contrary to the control, are essential to equip the cotton compounds with defences or phytoalexins against FOV.

1. Introduction

Cotton (*Gossypium hirsutum* L.) is the major textile plant worldwide. Many diseases such as gray mold and powdery, damping, and *Verticillium* and *Fusarium* wilt affect the development of cotton. *Fusarium* wilt is a very widespread disease in tropical zone. Parts of cotton leading water upward turn brown and are unable to perform their function, which causes dieback of the plant. The leaves turn yellow between the veins and the fall progressively. No effective control exists currently to fight this disease, except soil fumigation which is prohibitively expensive. Wilt of cotton is a very important problem in Sub-Saharan Africa [1]. In cotton crop, this disease is caused by the telluric fungus *Fusarium oxysporum f. sp. vasinfectum*. The cotton varieties cultivated such as R405, Coker, ISA 205, GL7, Deltapine, MacNair, Guazuncho, X449, and W766 are very susceptible to *Fusarium* wilt. The disease leads to important yield losses if no protective measures are taken. To limit damage, an intensive use of phytochemical compounds is required. Although these chemicals are relatively effective when applied as part of a strategic spray program, the cost for the grower and the environmental impact of their residues are generally considered undesirable. Moreover, pesticide resistant pathogen strains appear; therefore, efforts are being made to develop alternative protection strategies. An approach to prevent plant diseases consists of

inducing natural plant defences by using elicitors. A variety of pathogenic plant-derived molecules can act as elicitors, including polysaccharides, peptides, and lipids [2]. Elicitor perception triggers the formation of ion fluxes, oxidative burst, protein phosphorylation, and the synthesis of signal molecules such as salicylic acid, ethylene, and jasmonic acid [3, 4].

To limit pathogen development, plants possess physical barriers and inducible defence mechanisms like programmed cell death (hypersensitive response), cell wall reinforcement, production of low molecular weight molecules with high antimicrobial properties, phytoalexins, and production of pathogenesis-related proteins (PR proteins), which, like phytoalexins, possess antimicrobial activities [5, 6]. Some of these defence mechanisms seem to be under the control of two phytohormones, ethylene and methyl jasmonate (MeJA).

MeJA plays an important regulatory role in the coordination of plant growth and defence [7]. Exogenous application of MeJA has been shown to elicit responses in plants that are typically induced by insect herbivory and fungal infestation [8]. Treatment of spruce saplings and mature trees with MeJA resulted in increased terpenoid biosynthesis and the formation of polyphenolic parenchyma cells and traumatic resin ducts [9]. For many plants, the foliar application of MeJA induced the generation of a wide range of secondary plant metabolites, like alkaloids, terpenoids, flavonoids, coumarins, stilbenoids, hydroxycinnamic acids, and so forth [6, 10]. Some of these compounds have positive actions protecting plants against pathogens and thus were considered as phytoalexins [11, 12]. Treatment with MeJA induces the accumulation of phytoalexins and was also found to provide a potential plant defence activator that has been shown to induce resistance against fungal attack [8, 9, 13]. Similarly, other studies reported that in grapevine MeJA strongly stimulated the biosynthesis of phenolic acids, flavonoids, and stilbenes which are phytoalexins having an efficient action on pathogens [6, 14, 15]. Thus, MeJA application led to an increase in the resistance including powdery mildew in *Vitis* [16] and *Botrytis* in *Arabidopsis* [17].

The aim of this study was to investigate effects of MeJA on phytoalexins biosynthesis associated with resistance of *Gossypium hirsutum* to *Fusarium oxysporum f. sp. vasinfectum*, causal agent of *Fusarium* wilt. This will permit us to suggest an alternative solution to chemical struggle in cotton culture for better protection of the environment and human health.

2. Materials and Methods

2.1. Plant Material. Seeds of cotton (*Gossypium hirsutum* L.) were obtained from CNRA (Centre National de Recherche Agronomique, Cote D'Ivoire, West Africa). The cultivar R405 which has a high sensitivity to *Fusarium* wilt was used in this study.

2.2. Fungal Material. Growth and spore harvesting of the fungi *Fusarium oxysporum f. sp. vasinfectum* (strain CBS-116616; Centraalbureau voor Schimmelcultures, Baarn, The Netherlands) were provided by the Phytopathology Laboratory of the Ecole Supérieure d'Agronomie (Félix Houphouët-Boigny National Polytechnic Institute, Yamoussoukro, Cote D'Ivoire).

2.3. Seed Germination. Cotton seeds were delinted with sulphuric acid. Plump and mature seeds were chosen and surface sterilized by dipping in 70% (v/v) ethanol for 30 s prior to a 20 min exposure to 2.5% sodium hypochlorite (v/v). After rinsing three times with sterile distilled water, two seeds per test tubes were soaked in approximately 35 mL of sterile distilled water and germination was initiated in the dark during 48 h. Seedlings were cultivated in 500 mL pots containing substrate (soil) previously sterilized and incubated in a greenhouse during two months.

2.4. Preparation and Application of MeJA. MeJA solutions with concentrations of 2.5, 5.0, 10.0, 15.0, and 20.0 mM were made up in 1% ethanol containing 0.1% Triton X-100 as a surfactant. MeJA solutions (10 mL) were applied on 4-week-old cotton seedlings as a foliar spray until runoff. Inoculated seedlings were maintained in the greenhouse. Humidity was maintained at 90% through regular water spraying system in the enclosure. Water-treated leaves were used as controls. Watering of the seedlings was ensured according to the moisture of the substrate. Thereafter, plants were incubated during 24, 48, and 72 h. Each treatment consisted in three replicates, and the entire experiment was performed twice.

2.5. Phenol Extraction and Quantification in Leaves

2.5.1. Phenol Extraction. The leaves were removed and freeze-dried. 100 mg of freeze-dried leaf was dissolved overnight with 10 mL of methanol at 4°C in a blender. Sample was centrifuged at 2000 g for 10 min. Supernatant was collected and filtered through a Millipore membrane (0.45 μm) and represents the phenolic extract.

2.5.2. Total Phenolic Content. The total phenolic content (TPC) of extract was determined using Folin-Ciocalteu's reagent according to the method of Siriwoharn et al. [18]. Sample 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 20 min in the dark. The absorbance was measured at 765 nm, and the standard curve was prepared using the gallic acid (10–100 μg/mL). TPC 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 $y = 0.021x + 0.053$, $R^2 = 0.999$, where y is absorbance and x is the concentration of gallic acid in mg/mL. All measures were performed in triplicate.

2.5.3. Isolation and Identification of Phytoalexins. In this experiment, leaves from better concentration of MeJA (5.0 mM) and the best time of incubation (72 h) were used. The samples were dried/evaporated and extracts were dissolved in 700 μL of H_2O/MeOH (50/50, v/v). Modified

HPLC analysis was conducted using the method described by Belhadj [15]. Analysis was performed by HPLC (Agilent, model-LC 1100 series) on a 250 × 4 mm Prontosil C18 (5 μm) reverse-phase column (Bischoff, Leonberg, Germany) protected by a guard column of the same material. Separation was performed at a flow rate of 0.8 mL/min with a mobile phase composed of (A) H_2O/TFA 1% (97.5/2.5, v/v) and (B) acetonitrile/solvent A (80/20, v/v). The run was set as follows: 10% B (0–40 min), 10–50% B (40–41 min), 50–100% B (41–50 min), 100–10% B (50–51 min), and 10% B (51–70 min). The chromatogram was monitored at 284 and 306 nm using a UV detector (Kontron 430, Germany).

NMR spectra were recorded on LC-NMR (Agilent 1200 series PLC/Bruker Avance III spectrometer operating at 600 MHz for proton). A reference library of compounds was performed previously with purified compounds and identified by NMR in laboratory and also with commercially available compounds such as *trans*-resveratrol, *trans*-piceatannol, *trans*-pterostilbene, gallic acid, caffeic acid, chlorogenic acid, ferulic acid, *p*-coumaric acid, salicylic acid, *trans*-cinnamic acid, catechin, epicatechin, genistein, gossypin, naringenin, quercetin, quercitrin, and rutin. This database contains the retention time of these compounds and can be compared with those obtained from unknown samples and thus proceeds to their identification. Compound contents were evaluated from calibration curves prepared with standards.

2.6. Pathogen Inoculation and Disease Symptom Measurement

2.6.1. Preparation of Inoculums. The approximately 0.5 cm diameter of agar fragments with *Fusarium oxysporum f. sp. vasinfectum* (FOV) mycelia was taken from test tubes containing inclined potato dextrose agar (PDA) medium. The transfer was carried out under a sterilized laminar flow cabinet in Petri dishes containing PDA medium beforehand sterilized by autoclaving at 121°C for 30 min. The Petri dishes were incubated during 14 days at 25 ± 2°C under a 12 h photoperiod.

The mycelia fragments taken in Petri dish containing cultures of FOV were crushed under sterile conditions in the presence of 5 mL of sterile distilled water. The mixture was filtered through two layers of sterile cheesecloth to remove mycelia. The filtrate was plated in Petri dishes containing a new PDA medium (1 mL/Petri dish) and incubated under the same conditions as above during 7 days. Each Petri dish was flooding with 5 mL of sterile distilled water containing a drop of 0.1% Tween 20. A FOV spore suspension was prepared. Indeed, conidia were dislodged by gently rubbing the surface of the fungus colony with a Pasteur pipette curved. Conidial concentration was adjusted to 10^4 conidia/mL using a haemocytometer.

2.6.2. Fungal Inoculations. Fungal inoculations were performed by infecting cotton plants spores suspension of FOV. Many prick wounds have been made on the root base with a sterile needle and 2 mL of inoculums was deposited at the base of each cotton plant. The three treatments carried out were the following: (i) no-MeJA/no-infected plants (NTNI); (ii) no-MeJA/infected plants (NTI); (iii) MeJA/infected plants. Five replicates of six plants per each treatment were performed. Fungal inoculations were made 72 h after treatment with MeJA (5.0 mM) and water (MeJA 0 mM). Thereafter, plants were incubated in the greenhouse. Humidity was maintained at 100% through regular water spraying system in the enclosure. Disease severity was evaluated during 38 days.

2.6.3. Diseases Assessment. Disease assessment was started two days after artificial inoculation of two-month-old plants. It stopped when diseased plants have only one living leave or no living leaves. Disease severity (mean wilt score) was evaluated using the following arbitrary scale: 0, no wilt attached or detached leaves; 1, 1–15% wilt attached or detached leaves (WADL); 2, 15–25% WADL; 3, 25–35% WADL; 4, 35–45% WADL; 5, 45–55% WADL; 6, 55–100% WADL. Disease severity was assessed on each plant, and the mean wilt score for each replicate was calculated.

2.6.4. Plant Growth and Physiology. Fusarium wilt of the cotton appears through the symptoms of withering of the leaves and the plant, foliar chlorosis, and necrosis followed by the death of the plant. According to Delhove et al. [19], the progressive loss of leaves caused by the decline of the plant due to the alteration of conducting vessels of water from the roots to the leaves is considered typical symptoms of *Fusarium* wilt of cotton. One-month-old plants were treated with 0.0 or 5.0 mM MeJA. Seedling physiology was monitored every two days after fungus inoculation over a 38-day period following the initial MeJA treatment. The number of healthy green attached leaves, that is, having a good photosynthetic activity and therefore the vigor of the seedling, has been estimated. Increasing of height and diameter of the stem which expresses the plant growth were measured using slide caliper or a ribbon meter.

Total chlorophyll was determined using a modification of the Harborne method [20]. Fresh leaves were cut and placed in 5 mL of acetone for 2 h in the dark. Leaves were crushed in acetone and residue was washed twice with acetone. Thereafter, the filtrate was centrifuged to 5 000 rpm at 4°C during 15 min and the supernatant obtained constitutes the chlorophyll extract. The absorbance of the sample was measured at 646 and 663 nm on a Milton Roy spectrophotometer (Spectronic 601) against a blank sample extract. The total chlorophyll content (mg/g FW) was then calculated using the following formula: Total chlorophyll = $(17.3 \times A_{646}) + (7.18 \times A_{663})$.

2.7. Statistical Analysis. Experiments were performed using a completely randomized design. Data were subjected to analysis of variance (ANOVA) using SAS software (release 6.0) and differences between means were compared using Newman-Keuls test. Differences at $P < 0.05$ were considered as significant.

3. Results and Discussion

3.1. Effect of Methyl Jasmonate (MeJA) on Phenolic Compounds in Cotton. MeJA-treated leaves exhibited early and produced

Table 1: Effect of concentration and timing of methyl jasmonate (MeJA) treatments on phenolic content in cotton leaves.

MeJA concentration (mM)	Incubation time (h)	Total phenolic contents (mg/g FDE)
0	24	20.50 ± 0.51^{a}
	48	26.75 ± 0.45^{b}
	72	34.25 ± 0.25^{c}
2.5	24	44.20 ± 0.90^{d}
	48	46.50 ± 0.25^{d}
	72	54.00 ± 1.05^{e}
5	24	46.75 ± 0.26^{d}
	48	70.50 ± 0.25^{f}
	72	78.75 ± 0.85^{g}
10	24	53.15 ± 0.80^{e}
	48	68.75 ± 0.22^{f}
	72	71.50 ± 1.08^{f}
15	24	55.50 ± 0.56^{e}
	48	44.50 ± 0.55^{d}
	72	38.75 ± 0.25^{c}
20	24	40.20 ± 0.20^{cd}
	48	38.15 ± 0.15^{c}
	72	23.50 ± 0.35^{a}

FDE: freeze-dried extract; data are expressed as mean of three replicates; ±SD: standard deviation; means followed by a different letter are significantly different according to Duncan's multiple range test at $P = 0.05$ level.

more phenols, as compared to untreated leaves (Table 1). Phenols production gradually increased in control leaves and reached 20.50, 26.75, and 34.25 mg/g FDE at 24, 48, and 72 h after MeJA treatment, respectively. MeJA-treated leaves showed a steep increase in phenols production on day 3 (72 h), 2.30-fold higher than that of control.

The results showed that total phenolic contents of the cotton incubated 24 h after MeJA were significantly the greatest with MeJA 5.0 mM (55.50 mg/g FDE). At advanced stages (48 h of leaves incubation), total phenolic contents were higher with MeJA 5 mM (70.50 mg/g FDE). The same trend was observed after 72 h of leaves incubation where the same trend was observed after 72 h of leaves incubation where the level of total phenolic synthesis was more significant with MeJA 5.0 mM. Although total phenolic concentration in 5.0 mM MeJA-treated plants was greater than that in control plants, the phenolic concentration significantly dropped in plants treated with the highest MeJA concentrations. In most cases of our study, plants treated with 20.0 mM MeJA showed symptoms of stress or toxicity in form of chlorosis that might alter total phenolic concentration in the needles. The phytotoxicity effect of MeJa at high concentrations has been previously reported in many plants [21, 22]. In addition, although the total phenolic content of the cotton increased after the MeJA treatment, it was observed that, compared to the plants such as raspberry, the cotton was less affected by higher MeJA concentrations [23]. Similar results have also already been obtained with *Cupressus lusitanica* and *Vitis vinifera* [6, 13]. The higher total phenolic contents observed with the treated plants unlike control showed that 5.0 mM MeJA was the optimal concentration to safely stimulate the metabolism and the development of natural defence in cotton [24]. Similarly, several researchers demonstrated that the exogenous application of MeJA on annual plants induced plant chemical defence responses such as the increased phenolic synthesis and accumulation of phytoalexins [5, 14]. 5.0 mM MeJA and an incubation time of 72 h after MeJA application were considered to be the best methods to continue the experimentations.

To investigate the effect of MeJA on each phenolic compound in the cotton, the methanolic extract was analyzed by using reversed-phase HPLC (Figure 1). Eleven phenolic compounds were detected. Identification and peak assignment of phenolic compounds were based on comparison of their retention time with that of standards such as *trans*-resveratrol, piceatannol, *trans*-pterostilbene, *trans*-piceid, gallic acid, caffeic acid, chlorogenic acid, ferulic acid, *p*-coumaric acid, protocatechuic acid, salicylic acid, *trans*-cinnamic acid, catechin, epicatechin, genistein, gossypin, naringenin, quercetin, quercitrin and rutin, and polyphenols previously purified and identified by NMR. The retention time of these compounds was stored in a reference library of our database.

Comparing their retention times with standards, peaks 3, 5, 7, 8, 9, and 10 were identified as catechin (13.597 min), ferulic acid (18.524 min), chlorogenic acid (20.995 min), piceatannol (21.545 min), *trans*-resveratrol (26.993 min), and pterostilbene (28.344 min), respectively. In addition, NMR data indicated that peaks 1, 2, 4, 6, and 11 were gossypol (4.237 min), caffeoyl-D-glucose (12.770 min), 3-*p*-coumaroylquinic acid (17.895 min), gossypetin (19.704 min), and cichoric acid (34.083 min), respectively. We identified ten phenolic compounds in MeJA-treated leaves and eight in control leaves. Figure 1 showed clearly the disappearance of peak 9 (*trans*-resveratrol) and *de novo* synthesis of peaks 7 (chlorogenic acid), 10 (pterostilbene), and 11 (cichoric acid) in MeJA-treated leaves. Moreover, seven phenolic compounds (gossypol, caffeoyl-D-glucose, catechin, 3-*p*-coumaroylquinic acid, ferulic acid, gossypetin, and piceatannol) were common to both leaves.

All identified compounds were quantified and Table 2 shows the comparison of phenolic contents in MeJA-treated and untreated leaves. Among the various phenolic compounds, 3-*p*-coumaroylquinic acid (55.42 μg/g FDE), gossypetin (33.25 μg/g FDE), ferulic acid (11.08 μg/g FDE), and piceatannol (8.31 μg/g FDE), reported to have some bioactive properties such as antifungal and antimicrobial activities [25], were found to be major phenolic compounds in cotton treated with MeJA. It was reported that these compounds were produced through the phenylpropanoid pathway initiated by phenylalanine ammonia lyase (PAL) [26]. Many phenolic compounds produced through this pathway can be induced by stresses, elicitors, and MeJA. These phenolic compounds were found to be induced and accumulated in some other plants such as grapevine [6, 15]. With regard to catechin and caffeoyl-D-glucose, no significant difference was observed between MeJA-treated and untreated plants. Therefore, it was suggested that these two

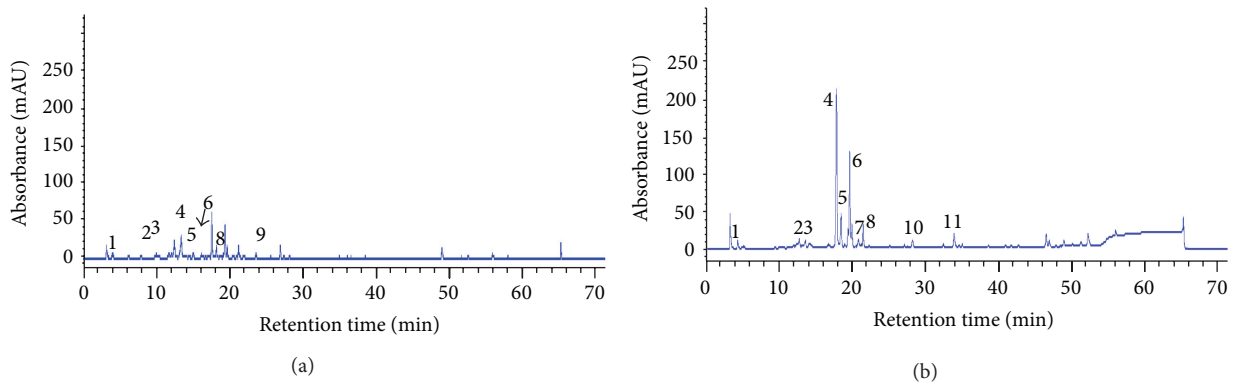

Figure 1: HPLC profile of phenols from leaves extract of *Gossypium hirsutum* treated with methyl jasmonate (MeJA). Detection is shown at 284 nm. Peaks were identified by comparison with reference standards when available or by HNMR data (retention time). (1) Gossypol (4.237 min); (2) caffeoyl-D-glucose (12.770 min); (3) catechin (13.597 min); (4) 3-*p*-coumaroylquinic acid (17.895 min); (5) ferulic acid (18.524 min); (6) gossypetin (19.704); (7) chlorogenic acid (20.995 min); (8) piceatannol (21.545 min); (9) *trans*-resveratrol (26.993 min); (10) pterostilbene (28.344 min); (11) cichoric acid (34.083 min). (a) No-MeJA treated plants. (b) Plants treated with MeJA 5.0 mM.

Table 2: Phenolic composition of cotton leaves treated with methyl jasmonate at 72 h posttreatment.

Chemical group	Compounds	Phenolic content of leaves (μg/g FDE)	
		No-MeJA-treated plants (control)	Plants treated with MeJA (5.0 mM)
Stilbenoids	Pterostilbene (10)	nd	02.21 ± 0.01^{a}
	trans-Resveratrol (9)	01.84 ± 0.01^{d}	nd
	Piceatannol (8)	02.14 ± 0.01^{e}	08.31 ± 0.17^{h}
Hydroxycinnamic acids	Cichoric acid (11)	nd	03.45 ± 0.02^{f}
	Ferulic acid (5)	0.16 ± 0.002^{c}	11.08 ± 1.09^{d}
	Caffeoyl-D-glucose (2)	03.21 ± 0.02^{f}	03.51 ± 0.02^{f}
Chlorogenic acids	Chlorogenic acid (7)	nd	02.58 ± 0.01^{a}
	3-*p*-Coumaroylquinic acid (4)	07.39 ± 0.06^{h}	55.42 ± 1.33^{j}
Flavonoids	Catechin (3)	03.31 ± 0.02^{f}	03.65 ± 0.02^{f}
	Gossypetin (6)	04.06 ± 0.03^{g}	33.25 ± 1.31^{i}
Terpenoids	Gossypol (1)	0.092 ± 0.001^{a}	05.91 ± 0.12^{b}

FDE: freeze-dried extract; nd: nondetected; data are expressed as mean of three replicates; ±SD: standard deviation; within row and column means, numbers followed by a different letter are significantly different according to Duncan's multiple range test at $P = 0.05$ level; MeJA: methyl jasmonate was used at 5.0 mM.

phenolic compounds production could not be stimulated by MeJA treatment in cotton. In our study, catechin and caffeoyl-D-glucose do not seem to influence cotton defence against pathogen although studies have shown their involvement in the defence of several other plants [27, 28]. Gossypol maximal amount (5.91 μg/g FDE) was observed in the MeJA-treated cotton, and this amount was 64 times higher than that of the control. This result seems to confirm the important role played by this compound in the cotton defence. Indeed, gossypol is a terpenoid aldehyde found in plants and conferring resistance against pathogens [29]. This compound was strongly stimulated by MeJA treatment in cotton and at a weak rate compared to the majority of the compounds. Tao et al. [30] reported that a small dose of gossypol would be efficient against the pathogens. The strongest increase in terpenoid appeared in plants that were treated with MeJA, a ubiquitous plant hormone known to mediate defence responses to biotic and abiotic stresses [31]. A previous study on cotton showed that the application of MeJA induced the synthesis and emission of volatile terpenes, a response also observed for herbivore-damaged plants [32]. Obviously, MeJA plays an important role in mediating damage-induced signalling in cotton. These same observations were made by Opitz et al. [33] in cotton after exogenous application of jasmonic acid whose MeJA is a methylated derivative. However, several terpenoids were synthesized by plants in contrast to our case. The chromatographic analysis technique seems to be crucial in the detection of secondary metabolites produced. Indeed, we used the HPLC technique while those authors used gas chromatography (GC). Furthermore, cichoric acid (3.45 μg/g FDE), chlorogenic acid (2.58 μg/g FDE) and pterostilbene (2.21 μg/FDE) were synthesized de novo in MeJA-treated plants unlike with control. This result suggested that these phenolic compounds have a relationship with the stimulation of natural defences of cotton. Indeed, cichoric acid is a caffeic acid derivative. This compound as most hydroxycinnamic acids possesses some functional properties such as antifungal, antiviral, and

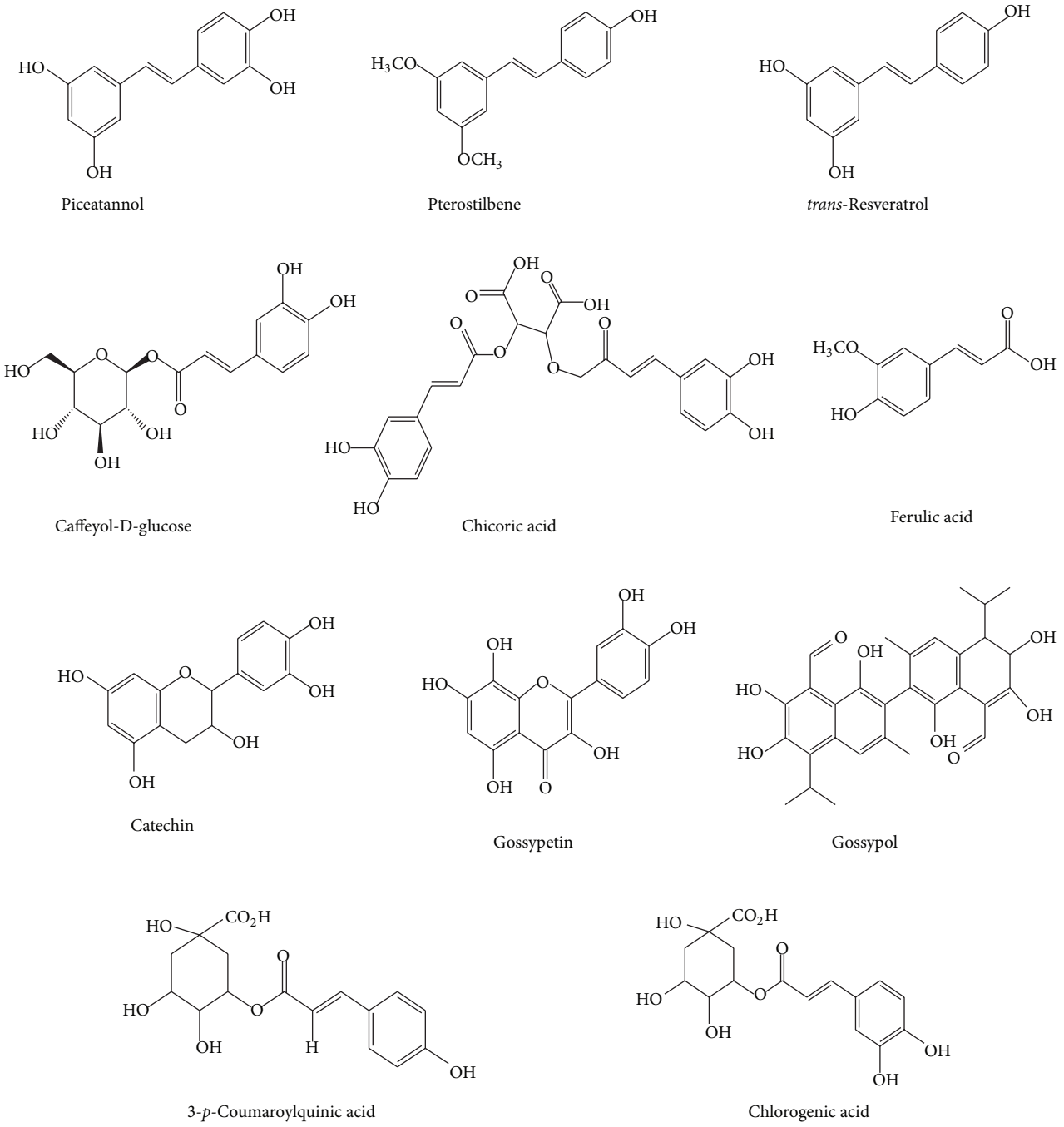

Figure 2: Phytoalexins isolated from cotton leaves. The compounds can be classified into five phenolic groups as follows: (i) stilbenoids: piceatannol, pterostilbene, and *trans*-resveratrol; (ii) hydroxycinnamic acids: caffeoyl-D-glucose, cichoric acid, and ferulic acid; (iii) chlorogenic acids: 3-*p*-coumaroylquinic acid and chlorogenic acid; (iv) flavonoids: catechin and gossypetin; (v) terpenoid aldehyde: gossypol.

antioxidant activities [34]. Functionally, chlorogenic acid is a polyphenol having antifungal activity, and, according to several authors, it would be involved in the defence of plants [35, 36]. Our data show that pterostilbene is also detected in leaves of MeJA-pretreated plants, even if the concentrations seemed low compared to the other analyzed compounds. Pterostilbene is indeed a biologically very active phytoalexin usually found in low quantities in plants [5]. Otherwise, its beneficial effect in protecting against mildew, *Botrytis*, and other pathogens is well established [6, 14, 17]. Besides, *trans*-resveratrol present at the beginning in the plants (control) would be hydroxylated and methylated under the action of MeJA to give piceatannol and pterostilbene. Indeed, several studies reported that resveratrol and its derivatives are involved in the resistance to various pathogens [15, 37, 38]. They also significantly inhibit conidial germination and mycelial growth of fungi [5]. All identified compounds (Figure 2) could be classified into five phenolic groups

as follows: (i) stilbenoids (piceatannol, pterostilbene, and *trans*-resveratrol), (ii) hydroxycinnamic acids (caffeoyl-D-glucose, cichoric acid, and ferulic acid), (iii) chlorogenic acids (3-*p*-coumaroylquinic acid and chlorogenic acid), (iv) flavonoids (catechin and gossypetin), and (v) terpenoid aldehyde (gossypol). These phenolic groups have positive actions on plant protection against pathogens and were considered as phytoalexins [6, 11, 12]. These results lead us to the hypothesis that, in certain plants, notably in the cotton, MeJA would be part of a network which regulates the expression of genes known to be elicitor inducible, and this could activate the accumulation defensive compounds such as stilbenoids, phenolic acids (hydroxycinnamic and chlorogenic acids), flavonoids, and terpenoid phytoalexins which are regulated by pathogen attacks. These compounds stored in MeJA-treated plants behave as antibodies that will defend the plant against possible aggressors and are relevant to resistance to cotton fungal diseases.

3.2. Disease Incidence. Disease symptoms (i.e., disease severity) on inoculated plants were observed after two-day incubation period (Table 3). Disease symptoms on MeJA-treated plants were significantly ($P < 0.05$) lower as compared to untreated control plants throughout the four-day incubation period. Greatest differences in disease symptoms between MeJA-treated plants and untreated and infected plants with FOV (no-MeJA/infected plants) were recorded at 14 days of incubation. At this stage, disease severity of MeJA-treated plants was hopeless, and no plants showed fading leaves or loss of leaves, as compared to the no-MeJA/infected plants. Wilt scores on days 2, 4, 6, 8, 10, and 12 for MeJA-treated plants were significantly ($P < 0.05$) lower than those of the untreated and noninfected plants with FOV (no-MeJA/no-infected plants). No symptom was observed in plant leaves with no-MeJA/no-infected plants. Thereafter, MeJA-treated plants did not present disease symptom with respect to FOV like no-MeJA/no-infected plants.

For tolerance induction, analysis of the results indicated that the untreated and inoculated plants with FOV (no-MeJA/infected plants) have abundant falling leaves, dieback of the stem, and a slowdown in stems growth. These symptoms are similar to those of *Fusarium* wilt caused by FOV. In contrast, plants treated with MeJA and inoculated with FOV after losing a few leaves during the first 12 days of infection showed an identical growth with untreated and noninoculated plants (control 2). It is clear from this analysis that MeJA induces significant tolerance to FOV. Treatment with MeJA seems to generate more efficient defence reactions against FOV. Actually, the application of MeJA on leaves induced defensive responses in the plants as mentioned by several authors [5, 6, 9]. As MeJA generally did not show direct *in vitro* antifungal effect [22, 36], it is suggested that FOV disease incidence and severity on cotton were reduced by activating natural defence responses. It is well documented in the literature that application of exogenous MeJA can induce sets of defence genes and synthesis of defence compounds that are also activated by pathogen infection [16, 21]. The genes include those encoding plant defensins, phytoalexins, PR proteins, and proteinase inhibitors [8, 11, 16].

Table 3: Effect of methyl jasmonate treatment on *Fusarium* wilt severity of cotton plant.

Incubation time (day)	Disease severity (score 0–6)		
	MeJA/infected plants	No-MeJA/infected plants	No-MeJA/no-infected plants
0	0^a	0^a	0^a
2	1^b	1^b	0^a
4	1^b	2^c	0^a
6	1^b	2^c	0^a
8	2^c	3^d	0^a
10	1^b	3^d	0^a
12	1^b	3^d	0^a
14	0^a	3^d	0^a
16	0^a	5^e	0^a
18	0^a	6^f	0^a
20	0^a	6^f	0^a
22	0^a	6^f	0^a
24	0^a	6^f	0^a
26	0^a	6^f	0^a
28	0^a	6^f	0^a
30	0^a	6^f	0^a
32	0^a	6^f	0^a
34	0^a	6^f	0^a
36	0^a	6^f	0^a
38	0^a	6^f	0^a

FOV: *Fusarium oxysporum f. sp. vasinfectum*; leaves were treated with MeJA 5.0 mM, inoculated at 72 h (day 0) with FOV, and incubated at 25°C for 38 days. Disease severity (mean wilt score) was evaluated every two days using the following arbitrary scale: 0, no wilt attached or detached leaves; 1, 1–15% wilt attached or detached leaves (WADL); 2, 15–25% WADL; 3, 25–35% WADL; 4, 35–45% WADL; 5, 45–55% WADL; 6, 55–100% WADL. Disease severity was assessed on each plant, and the mean wilt score for each replicate was calculated. data are expressed as mean of three replicates; within row and column, numbers followed by a different letter are significantly different according to Duncan's multiple range test at $P = 0.05$ level; MeJA: methyl jasmonate.

MeJA plays an important regulatory role in the coordination of plant growth and defence. Exogenous application of MeJA has been shown to elicit responses in plants that are typically induced by fungal infection according to [8, 22]. MeJA is involved in several plants biological aspect such as defence reaction against fungi pathogen; its signalling is important in limiting the growth of certain pathogens such as FOV and seems to have systemic effects resulting in gene expression throughout the plant [11, 12]. MeJA defence responses are based on ability to produce secondary metabolites such as terpenoids, flavonoids, stilbenoids, and phenol acids which are considered as phytoalexins [6, 13]. In this study, the phytoalexins responsible for the induction of resistance to *Fusarium oxysporum f. sp. vasinfectum* (FOV) are known and they are associated with the pretreatment of cotton leaves by MeJA. Thereby, gossypetin, gossypol, 3-*p*-coumaroylquinic acid, ferulic acid, and piceatannol, which have important concentration, and cichoric acid, chlorogenic acid, and pterostilbene synthesized *de novo* in plants treated with MeJA compared to untreated and infected plants treated

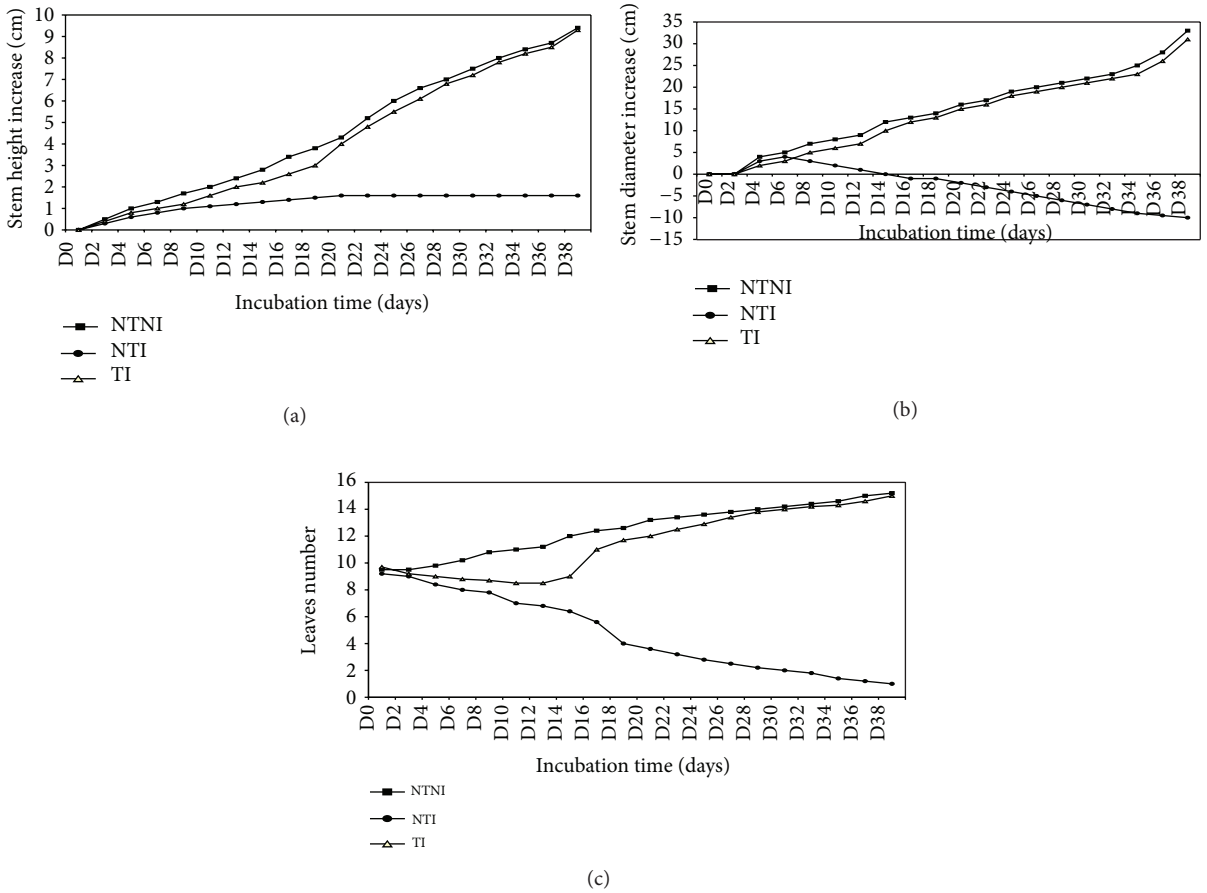

Figure 3: Protective effect of exogenously applied MeJA on cotton against fusarium wilt caused by *Fusarium oxysporum f. sp. vasinfectum*. Prior to inoculation, leaves were treated with MeJA 5.0 mM and were incubated at 72 h (0 day) at 25°C for 38 days. Disease severity was evaluated by noting every two days the stem height (a) and diameter increase (b) as well as the number of healthy attached leaves (c). TI: MeJA/infected plants; NTI: no-MeJA/infected plants; NTNI: no-MeJA/no-infected plants.

are phytoalexins or phytoanticipins that are able to protect against FOV causal agent of *Fusarium* wilt in cotton. This study has demonstrated that treatments with MeJA on leaves can suppress FOV in cotton. MeJA is an established elicitor of enhanced natural host defences in plants [8, 10, 14]. However, it is important to note that the presence of weak symptoms of *Fusarium* wilt in the 12 first days after infection in plants treated with MeJA and FOV infected shows that the induction of phytoalexins is not done in a systematic way after the application of the elicitor (MeJA). The cotton is certainly devoid of endogenous MeJA as reported by Belhadj [15] in grapevine. The evaluation of the biosynthesis of phytoalexins after MeJA treatment and study tests protection against FOV allowed us to assert that MeJA induced a biosynthesis of phytoalexins that give an increased tolerance to *Fusarium* wilt of plant. MeJA is an effective elicitor of defence reactions in the cotton. However, the confirmation of these tests in field conditions will be of great interest for a sustainable crop of cotton and management of the environment.

3.3. Plant Growth and Physiology. Plant stress following application of MeJA was observed in a number of the physiological traits measured (Figure 3). The height and diameter of the stem, as well as the number of leaves, were strongly influenced by the *Fusarium* wilt. Indeed, the stem height of infected plants did not evolve; it remained stationary (Figure 3(a)). On the other hand, stem height increased by about 9 cm in MeJA-treated plants on day 38 compared to day 0 and is significantly identical with noninfected plants with FOV. These same observations were reported at the level of stem diameter (Figure 3(b)).

Indeed, the increase of stem's diameter was about 30 mm in the plants treated with MeJA on day 38 compared to day 0 as shown in no-MeJA/infected plants. However, from the 16th day, the stem diameter became lower than baseline (day 0), which clearly indicates a decline of plants suffering from *Fusarium* wilt. These results suggest that the FOV attack reduces the growth and development of plants whereas a pretreatment of plants with MeJA seems to protect plants against pathogens [17, 39, 40]. Furthermore, for infected plants with FOV, the number of leaves decreases significantly compared to no-MeJA/no-infected plants for the first 14 days. The number of leaves was subsequently increased in pretreated plants with MeJA to reach that of no-MeJA/no-infected plants starting from day 20 up to day 38. On the other hand, the number of leaves decreased significantly, and

Table 4: Effect of methyl jasmonate treatment on chlorophyll content of cotton leaves after treatment and fungus inoculation.

Incubation time (day)	Chlorophyll content (mg/g FW)		
	MeJA/infected plants	No-MeJA/infected plants	No-MeJA/no-infected plants
0	0.35 ± 0.01^{a}	0.37 ± 0.02^{a}	0.33 ± 0.01^{a}
12	0.33 ± 0.01^{a}	0.28 ± 0.01^{b}	0.54 ± 0.03^{e}
20	0.69 ± 0.02^{b}	0.22 ± 0.01^{c}	0.77 ± 0.02^{b}
30	1.10 ± 0.04^{c}	0.15 ± 0.01^{e}	0.99 ± 0.06^{c}
38	1.32 ± 0.09^{d}	0.05 ± 0.00^{f}	1.27 ± 0.08^{d}

FOV: *Fusarium oxysporum f. sp. vasinfectum*; FW: fresh weight; leaves were treated with 5.0 mM of MeJA, inoculated at 72 h (day 0) with FOV, and incubated at 25°C for 38 days. data are expressed as mean of three replicates; within row and column, numbers followed by a different letter are significantly different according to Duncan's multiple range test at $P = 0.05$ level; MeJA: methyl jasmonate.

from the 38th day most of the plants have no leaves and die thereafter (Figure 3(c)). These results demonstrate that FOV causes the wilting of the leaves, which justifies their downfall. These observations were confirmed by the determination of chlorophyll content in cotton leaves (Table 4). Following the no-MeJA/infected plants, FOV attack was significant for the reduction in chlorophyll content with increasing incubation time. No significant difference was observed between MeJA-treated plants and no-MeJA/no-infected plants. MeJA was efficient in conferring protection against FOV. MeJA-treated and inoculated plants showed an increase in chlorophyll content like no-MeJA/no-infected plants. Such an increase in chlorophyll content may result in an increase in photosynthesis and enhance good activity of growth and development of plants. These results show that the plant is healthy and seems to be related to phytoalexins biosynthesis. The decrease in chlorophyll content with untreated and infected plants has involved a reduction in the photosynthetic capacity, depriving plants of nutrients needed for growth, plus the possible inhibition of genes involved in the photosynthesis process [7, 40]. This would explain the reduction in height and diameter of stem observed in plants suffering from *Fusarium* wilt which is known as one of the most devastating agriculture diseases in cotton [1]. MeJA would stimulate the biosynthesis of the phytoalexins responsible for the plants defence against pathogens. These compounds which are phytoanticipins would equip the plants against the future aggressors such as FOV. This seems to explain the good growth and good development of plants treated with MeJA. Therefore, pretreatment with MeJA protects cotton against FOV causal agent of *Fusarium* wilt.

4. Conclusion

This is the first work reporting the effect of MeJA on cotton protection against FOV. Despite the reduced size of our samples and the difficulties associated with the use of seedlings, we were able to detect a significant effect of the MeJA application on the phenolic content in the leaves, consequently promoting the phytoalexins biosynthesis. Plant is equipped with compounds able to anticipate fungal and other attacks. In addition, we also demonstrated that the application of MeJA on leaves induced defensive responses in the cotton. This result may have relevance for forthcoming studies aimed at understanding systemic resistance induced by MeJA in cotton.

Conflict of Interests

The authors declare that they have no conflict of interests regarding the publication of this paper.

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