Impact of Elicitation by Methyl Jasmonate and Ethephon on Cotton (*Gossypium Hirsutum* L.) Protection Against *Fusarium Oxysporum* F. Sp. Vasinfectum

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Abstract

The objective of the study was to evaluate the effect of methyl jasmonate and ethephon on cotton against Fusarium wilt. As well, cotton plants were treated in the field by methyl jasmonate (MeJA), ethephon (ETH) and the association of methyl jasmonate and ethephon (MeJA + ETH) and inoculated by *Fusarium oxysporum* f. sp. Vasinfectum (FOV). The incidence of the disease caused by FOV was evaluated every 10 days up to the 150th day after inoculation. The protection afforded to cotton following treatment by stimulators was assessed by determining the mortality rate of plants treated and inoculated FOV compared with untreated and inoculated controls. The degree of protection of cotton from Fusarium wilt was also determined through growth parameters, foliar pigment content (Chlorophylls and carotenoids) and an evaluation of the rate of lines. The results showed better protection of the cotton plants treated by the stimulators and inoculated by FOV through an increase in the number of healthy leaves, stem height, and stem diameter. This protection was also confirmed by an increase in photosynthetic activity of plants and a reinforcement of cell walls. The combination of these two molecules (MeJA + ETH) was better to induce this resistance against FOV compared to each of the two taken separately. The use of natural defence stimulators (SDN) is therefore an effective means of protecting cotton from FOV. Their application in the agricultural environment could contribute to the development of a well-reasoned, sustainable agriculture that is more respectful of the environment and human health.

Indexing terms/Keywords: cotton, methyl jasmonate, ethephon, natural defence stimulator, Fusarium oxysporum f. sp. vasinfectum

Subject Classification: Plant Physiology and Physiopathology

Introduction

Cotton is a tropical plant belonging to the genus *Gossypium*, of which four species are currently cultivated. *G. hirsutum* is the most cultivated species, accounting for almost 95 % of world cotton production (Munro, 1994). African cotton-producing countries account for only 8 % of world production (Estur, 2005). Cotton plays important, economic and social role in Africa. Cotton represents a considerable economic and social importance in Africa. For example, it constitutes 7 % of agricultural exports in Côte d'Ivoire and contributes to 1.7 % of the annual gross domestic product. Thus, cotton is the fourth most important export product after cocoa, rubber and cashew and generates about 100 billion CFA franc currency in Côte d'Ivoire (Coulibaly, 2014). It provides substantial income for this purpose to the peasant masses in the central and northern parts of the country which are the main cotton regions. However, cotton is threatened by pests and various diseases that reduce the production and quality of fibres and seeds (Vaissayre, 1994). Of all the diseases of fungal origin, fusarium wilt caused by FOV seems to be the most formidable. It invades the interior of the plant and blocks the vessels where

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the sap circulates, which causes a generalized fanaison of the plant starting with the aerial parts. The attack is more intense when some nematodes such as *Meloidogyne incognita* are abundant in the soil (Delattre, 1973). The fungus can develop actively in the soil and persist for many years, even in the absence of the host plant. Fusarium wilt is one of the main causes of production loss in cotton cultivation. Thus, without chemical phytosanitary protection, there is no room today for profitable cotton production. Unfortunately, the use of pesticides, including fungicides, is increasingly on the spot for toxicity and environmental pollution issues (Faurie *et al.*, 2013).

In this context, it appears necessary to look for more effective alternatives for the development of sustainable agriculture. One is to give plants the means to defend themselves, or to strengthen their own defences, rather than to directly combat the aggressor (Amari *et al.,* 2014; Konan *et al.,* 2014). In this category are the stimulators of the natural defenses (SDN) of the plants. Indeed, plants can most often naturally resist their aggressors through natural molecules called natural defence stimulators (Amari, 2012). SDN are effective at very low doses, inactive on pathogens as acting on the plant (Lyon *et al.*, 1995). They are completely biodegradable and have a generally interesting eco-toxicologic profile. So they are very environmentally friendly molecules. Among which are methyl jasmonate and ethylene. The objective of this work is to evaluate the effectiveness of natural defence stimulators in the defence of cotton. Specifically, it aims was to evaluate the impact of methyl jasmonate and ethephon on cotton against Fusarium wilt caused by FOV.

MATERIALS AND METHODS

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 (Hau, 1988). The seeds were provided by the Ivorian Textile Development Company.

Fungal material

The fungal material consists of a strain of Fusarium oxysporum F. Sp. Vasinfectum (FOV). It was provided by the laboratory of plant pathology of the Higher School of Agronomy (ESA) This part should contain sufficient detail that would enable all procedures to be repeated. It can be divided into subsections if several methods are described. of the Polytechnic Institute Houphouët Boigny (INPHB) in Yamoussoukro in test tubes containing the PDA medium.

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).

Site study

This experiment was carried out in the field on the experimental plot of the Nangui Abrogoua University (UNA) in Abidjan (southern Côte d'Ivoire). The geographical coordinates of this site are: 5 ° 17' and 5 ° 31' North latitude between 3 ° 45' and 4 ° 22' West longitude (Koffi *et al.,* 2009). The forest relic of this university contains numerous plant species such as *Chrysophyllum albidum* G. Don (Sapotaceae), *Synsepalum afzelii* (Engl.) T.D. Penn. (Sapotaceae), *Palisota hirsute* (Thunb.) K. Schum. (Commelinaceae). The soil is derived from sedimentary formations of the ferralitic type (Perraud, 1971). These sedimentary formations have a clay-sandy texture that is favorable to cotton growing. The mean annual rainfall and temperature are 1642 mm and 27.16 °C (SODEXAM, 2017).

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.

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).

Preparation and application of stimulators

Methyl jasmonate (MeJA) was prepared at the optimum concentration of 5 mM and ethephon (ETH) at 5 g/L (Konan *et al*., 2015). The two stimulators were previously dissolved in 80 % ethanol in the presence of 0.5 mL of 1 % Triton X-100, and then supplemented with distilled water. About 500 mL of each stimulator solution was prepared. A second preparation consisting of an equal volume mixture of the two stimulators (MeJA+ETH) was performed. In every elementary plot of land, 10 plants were handled with three repetitions, that 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 distilled water containing 1 % Triton X-100.

Fungus culture

Transplanting and purifying FOV

The transplantation of the strain FOV was carried out according to the method described by Vakili (1968). 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.

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 (Delattre, 1973).

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.

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, 100, 500, 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.

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 (Delattre, 1973). 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.

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.* (1990).

Action of methyl jasmonate and ethephon on *Fusarium oxysporum* f. sp. vasinfectum

The purpose of this test is to evaluate the non-fungal effect of methyl jasmonate and ethephon on the growth of mycelia of *Fusarium oxysporum* f. sp. vasinfectum (FOV). Agar fragments approximately 0.5 cm in diameter containing the fungus were transferred to the hood in petri dishes containing the PDA medium to which a volume V (0.5; 1 and 2 mL) of methyl jasmonate and ethephon was added. 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. In the witness, the PDA medium does not contain stimulators. The growth of FOV grown on enriched media in stimulators is compared with that of the control (without stimulators).

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 [15]. 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 (NTP);

-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.

Evaluation of the effectiveness of the SDN on Fusarium wilt

Another way to assess the degree of protection of cotton from fusarium wilt was to determine the growth parameters. It is to measure the height and diameter of the stem, to count the healthy leaves (intact), the content of foliar pigments (chlorophylls and carotenoids). This makes it possible to assess the photosynthetic activity and an evaluation of the lignin rate was carried out.

Evaluation of photosynthetic activity

Evaluation of photosynthetic activity of treated and inoculated cotton leaves at FOV was conducted every 30 days.

Extraction and dosage of leaf pigments

The extraction and the determination of the leaf pigments were carried out according to the method described by Lichtenthaler (2001). Thus, 200 mg of treated and inoculated leaves were cut into small pieces and placed in test tubes containing 5 mL of 20 % acetone. It was kept in the dark at 4 ° C for 2 hours. These pieces of leaves were then crushed in acetone in the presence of a pinch of calcium carbonate and Fontainebleau sand. The extraction residue was washed twice with acetone. The ground material was centrifuged at 5.000 rpm for 15 min at 4 ° C. The supernatant obtained constituted the crude extract of chlorophylls. The mass of the crushed leaves and the volume of the crude extract were determined and the absorbance, measured spectrophotometer at 470 nm, 647 nm and 663 nm against a control sample made with acetone. The chlorophyll contents a, b and total (Chl a, Chl b and Chl t) and carotenoid leaves expressed in µg/mL was then calculated using the following formulas:

$$Chl\ a\ (µg/mL) = [12, 25 \times DO663 - 2, 79 \times DO647] \times V/1000\ m$$

$$Chl\ b\ (µg/mL) = [21, 5 \times DO647 - 5, 10 \times DO663] \times V/1000\ m$$

$$Chl\ t\ (µg/mL) = [7, 15 \times DO663 + 18, 71 \times DO647] \times V/1000\ m$$

$$Car\ (µg/mL) = [1000 \times DO470 - 1, 82 \times chl\ a - 85, 02 \times chl\ b]/198 \times V/1000\ m$$

Where V is the volume of the crude extract (mL) and m is the mass of the fresh leaves used (g).

Production gain in foliar pigments

The gain in leaf pigment production (GPF), induced by the application of SDN, was determined relative to the control. It was expressed as a percentage and calculated according to the formula:

$$GPF\ (\%) = [(P\ test - P\ control) / P\ control] \times 100$$

Where P is the content of leaf peppers (chlorophylls a, b, total and carotenoids).

Evaluation of lignin content in treated and inoculated leaves

For this study, the effect of SDN on the lignification of cotton plants was evaluated in roots, stems and leaves. These samples were harvested 90 days after inoculation with the pathogen, when the first symptoms of FOV started to appear on untreated and inoculated cotton plants (PTNI).

Grinding of cotton samples

The harvested cotton samples were freeze-dried. Then, 10 g of each sample was weighed and ground in a mortar to obtain powder samples on which lignin extraction and assaying was performed (Figure 1).
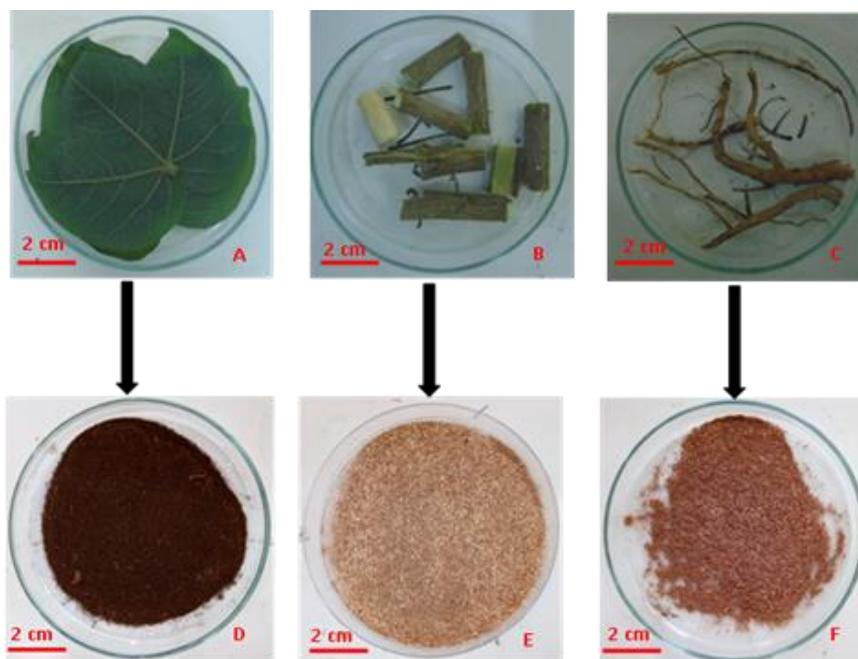
Figure 1. Cotton Explants Treated with Natural Defense Stimulators (A, B and C) and inoculated with FOV and their respective homogenates (D, E and F)

FOV: Fusarium oxysporium f.sp. vasinfectum; SDN: natural defense stimulators; A: cotton leaves; B: Cotton stems; C: cotton roots; D: cotton leaf powder; E: cotton stalk powder; F: cotton root powder

Extraction and dosage of lignins

The extraction and the determination of the lignins were carried out according to the method of Ellis (1946). The extraction of lignins took place in four essential stages.

First step: 0.9 g of powder from each sample were weighed and put into cotton fabrics and tied with a thread. These wood powder samples successively underwent two hot extractions with 96 % ethanol and with water. The organic solvent was used to remove single phenolic acids and polyphenols while the aqueous solvent allowed the removal of water-soluble compounds such as proteins and tannins. This analytical step was aimed at extracting the soluble compounds present in the cottonwood powder which would subsequently interfere with the lignins during their dosages. The samples were then dried in an oven for 18 hours at 105 ° C.

Second step: After cooling, the dry powder samples were each poured into a two-liter Erlenmeyer flask containing one liter of 96 % ethanol. The Erlenmeyer flask is placed on a heated magnetic stirrer. When the ethanol began to boil, the mixture was stirred for 30 min using a magnetic stirrer to remove the maximum of phenolic compounds. Then the ethanol was emptied and replaced with one liter of water; which allowed the extraction of more polar compounds. This step was repeated successively twice with ethanol and once with water.

Third step: the samples were then recovered and put in an oven for drying for 18 h at 105 ° C; then cooled. The powders of samples thus dried were used for the determination of the lignins.

Fourth step: this step allowed to degrade the cellulose still contained in the samples. Thus, 0.2 g of powder of each sample was weighed (mass of the test portion). Each sample was placed in a crucible (250 mL glass bottle) previously put in the oven and weighed after cooling (anhydrous mass crucible). Then 5 mL of 72 % sulfuric acid was added to each crucible. Then, they were placed on a magnetic stirrer for stirring at room temperature for two hours. After this etching time, 115 mL of distilled water was added to each crucible to effect hydrolysis with 3 % sulfuric acid. The crucibles were then autoclaved at 120 ° C for one hour; which made it possible to burst the maximum number of cells that were not in the previous step, in order to let out all the lignins of the cells. For the determination of the lignins, the contents of each crucible were filtered and the pellet was washed several times with distilled water in order to remove the cellulose which would still be retained in the wood fibers. After washing, the pellet was dried in an oven at 105 °C for 18 h, then returned to the crucibles and weighed (mass crucible with residue). The lignin content was expressed as a percentage and calculated according to the formula:

$$\text{Lignin content (\%)} = \frac{\text{Mass of residue}}{\text{Mass of the test sample}}$$

Where, Residue mass = Crucible mass with residue - Anhydrous crucible mass

Statistical analysis

Statistical analysis were performed using the STATISTICA 7.1 software. An analysis of variance (ANOVA) was performed on all applied treatments. When this analysis shows a difference between the means, the Newman-Keuls test is performed in order to determine the significant differences between treatments at the 5 % threshold. For percent values, the Kruskal-Wallis test was used to determine significant differences ($P < 0.05$) between treatments.

Results

Characterization of the purified isolate

After purification of the isolate by monospore culture, only one morphological group was observed among the offspring. The aerial mycelium had a whitish downy appearance with a purplish, sparse, and sparse background. Microscopic observation revealed the presence of a compartmentalized thallus with short microphialids on which many microconidia are found as well as macroconidia and chlamydospores (Figure 2). These characteristics indicate the presence of *Fusarium oxysporum*.
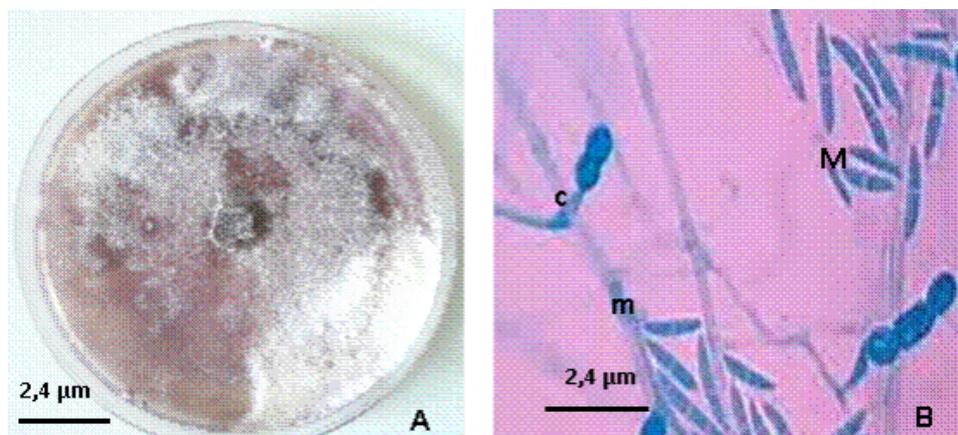
Figure 2. Morphological description of a virulent FOV strain

A: macroscopic appearance; B: microscopic appearance; c: chlamydospore; m: microconidia; M: macroconidia; FOV: *Fusarium oxysporum* f. sp. vasinfectum

Pathogenicity of FOV

The pathogenicity test for FOV, the causal agent of Fusarium wilt, was carried out on 14-day-old vivoplants. The results obtained are shown in Table I. All the amounts of inoculum used (50; 100; 500 and 1.000 μL) induced wilting of the leaves and stems after seven days. However, the aggressiveness of FOV or the severity of the disease varied with the dose of inoculum. In fact, although the cotton plants inoculated with 50 μL of spore suspension showed symptoms of wilting, they remained alive. On the other hand, cottonwood inoculated with 100; 500 and 1.000 μL of inoculum had wilted and necrotic leaves and stems. However, the 500 and 1.000 μL doses of FOV inoculum were more pathogenic, with symptoms occurring 21 days after inoculation resulting in the death of almost all the seedlings (97.84 % mortality by 500 μL and 100 % mortality per 1.000 μL). The seedlings inoculated with 100 μL of inoculum developed less severe symptoms with 10.47 % mortality rate. On the control seedlings, there was no manifestation of symptoms of the disease (Figure 3).

Table 1. Pathogenicity Testing of FOV inoculum

Volume of inoculum (μL)	Leaf yellowing	Leaf wilt	Withering from the stem	Rate of mortality (%)	Profile
0	-	-	-	0	non pathogenic
50	-	-	-	0	non pathogenic
100	+++	+++	+	10.47	pathogenic
500	+++++	+++++	++++	97.84	pathogenic
1.000	+++++	+++++	++++	100	pathogenic

FOV: *Fusarium oxysporum* f. sp. vasinfectum; (-): absence; (+): presence
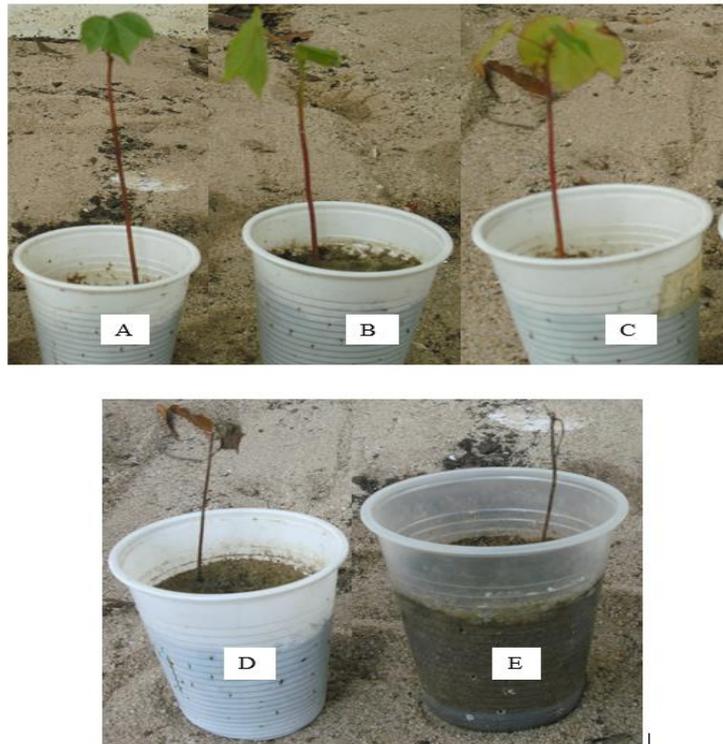
Figure 3. Manifestation of the external symptoms of Fusarium wilt on cotton plants inoculated with different doses of FOV inoculum

FOV: *Fusarium oxysporum* f.sp. vasinfectum; A: witness; B: 50 μL of FOV inoculum after 21 days of incubation; C: 100 μL of FOV inoculum after 21 days of incubation; D: 500 μL of FOV inoculum after 21 days of incubation; E: 1,000 μL of FOV inoculum after 21 days of incubation

Reisolation of the fungus was performed with inoculated cotton seedling fragments and uninoculated vivoplant stem fragments (control). After seven days of incubation, the inoculated stem fragments developed fungal mycelia on the PDA medium (Figure 4) contrary to the control.

The mushroom colonies showed a whitish downy appearance with a purplish, sparse, and sparse background. Microscopic observation showed numerous ovoid and group macroconidia and more or less curved fusoid microconidia. These morphological characteristics of the reisolated fungus strain showed no variation compared to those of the parent culture (purified isolate). After characterization, the key of Botton *et al.* (1990) identified isolated fungal strains as Fusarium oxysporum f. sp. In addition, the isolate (1000 μL of inoculum) that was able to cause 100 % mortality in all inoculated cotton seedlings confirmed the special form "vasinfectum" of our species *Fusarium oxysporum*.

Effect of methyl jasmonate and ethephon on Fusarium oxysporum f. sp.vasinfectum

Methyl jasmonate and ethephon were added to the culture medium of *Fusarium oxysporum* f. sp. vasinfectum (FOV) to see their effect on the growth of FOV mycelium. The results showed that the stimulators do not have a negative effect on the growth of the FOV mycelium. Whatever stimulator is used, mycelium growth occurs. Compared to the control, growth of the mycelium was better in the presence of the stimulators. Thus, the evolution front is 1.7 cm in Petri dishes containing ethephon; 1.8 cm in petri dishes containing methyl jasmonate and 1.9 cm in those combining the two stimulators against 1.6 cm in the control. On the other hand, aerial mycelium having a whitish downy appearance with a purplish, shallow, sparse bottom is observed in all petri dishes (Figure 5). In addition, microscopic observation showed the presence of a compartmentalized thallus with short microphialids on which many microconidia are found as well as macroconidia and chlamydospores (Figure

6). These characteristics clearly indicate according to the key of Botton *et al.* (1990) that the fungus grown on PDA medium containing SDN is indeed FOV.
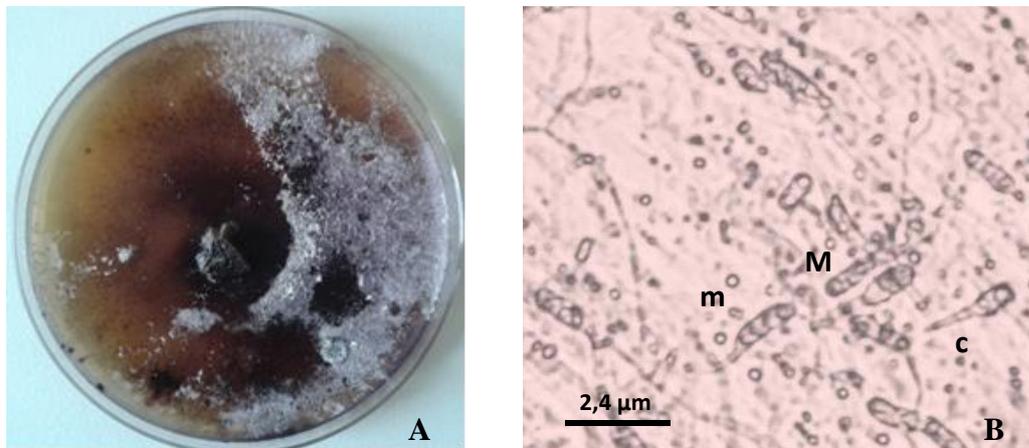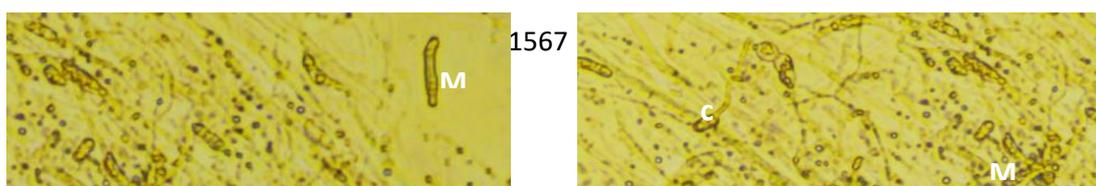

Figure 4. Macroscopic and microscopic appearance of the virulent FOV strain extracted from cotton buds inoculated with 1.000 µL of inoculum

FOV: *Fusarium oxysporum* f. sp. vasinfectum; A: macroscopic appearance; B: microscopic appearance; C: chlamydospore; m: microconidia; M: macroconidia
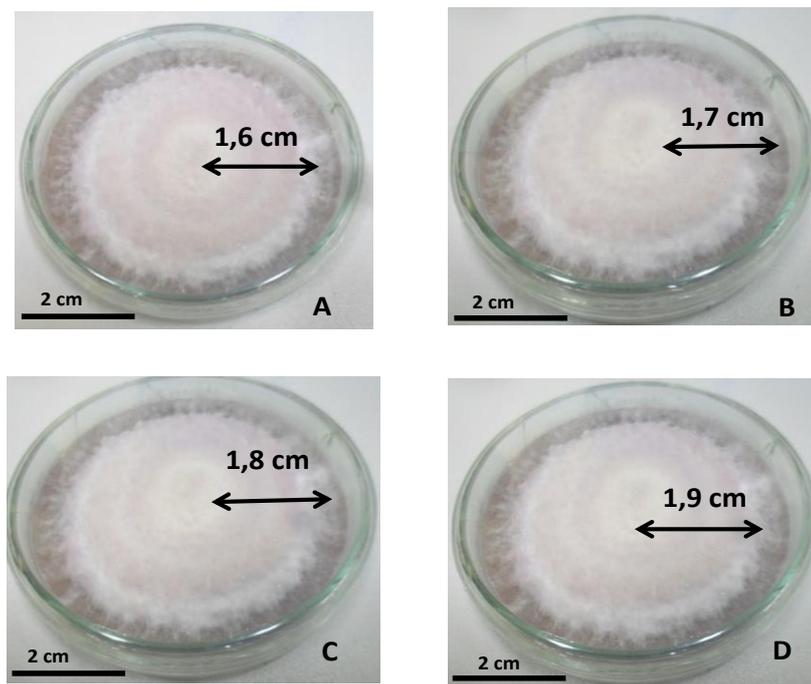
Figure 5. Macroscopic appearance of FOV strain grown on PDA medium containing stimulators

MeJA: methyl jasmonate; FOV: *Fusarium oxysporum* f. sp. vasinfectum; PDA, potato dextrose agar; A: containing medium containing no stimulators (control); B: medium containing ethephon; C: medium containing MeJA; D: medium containing the association of MeJA and ethephon;   : forehead of evolution of the mycelium

Figure 6. Microscopic appearance of the strain of FOV grown on PDA medium

FOV: *Fusarium oxysporum* f. sp. vasinfectum; PDA: potato dextrose agar; A: PDA medium containing no stimulator (control); B: PDA medium containing the stimulator; m: microconidia; M: macroconidia; c: chlamydospore.

Evaluation of physiological status of treated and inoculated cotton plants

The degree of protection of the cotton plant against FOV or the physiological state of the treated plants was evaluated by determining the leaf pigment content.

Chlorophyll Pigments and Carotenoids

The evaluation of the physiological state of the cotton plants was carried out by determining the content of chlorophyll a, b, total and carotenoids in the leaves. The effect of exogenous stimulator application on total chlorophyll a, b and carotenoid content in FOV-inoculated cotton leaves is shown in Tables II. The results showed that at D0, total chlorophyll content is almost identical for all treatments, as well as for control cotton. From J30 to J60, total chlorophyll content decreased for all treatments and for PNTi. This decrease is more pronounced in the J90 for all treatments and PNTi, while for PNT, it remains almost invariable. From J120 to J150, total chlorophyll content increases for all treatments. This content is higher in PTEMi (479.07 µg/mL, a gain of 96.52 %). In the PTMi, the content is 396.23 µg/mL, a gain of 65.52 %, while in the PTEi this content is 325.22 µg/mL for a gain of 33.74 %. On the other hand, for PNTi, the drop in content that began on day 30 continues and increases more to become zero in J150. In PNT, the content remains almost identical to that of J0 (244.07 µg/mL).

Concerning the variation of the content of chlorophylls (a) and of chlorophylls (b), the contents are almost identical at the level of the treatments as well as, in the control plants at day 0. However, the content of chlorophylls (a) is higher than that of chlorophyll (b). From J30 to J60, the chlorophyll content (a) decreases for all treatments and in PNTi, while the chlorophyll content (b) increases. In PNT, they remain almost identical to those of J0. This drop in chlorophyll content (a) is more pronounced in J90. From J120 to J150, chlorophyll (a) and chlorophyll (b) levels increase for all treatments. Thus, in J150 in PTEMi, the chlorophyll content (a) is 300.04 µg/mL against 162.03 µg/mL for chlorophyll (b), in PTMi, the chlorophyll a content is 246.19 µg/mL compared with 150.04 µg/mL for chlorophyll (b), while for chlorophylls the chlorophyll content is 197.10 µg/g

MF versus 128.12 µg/mL for chlorophylls (b). On the other hand, in PNTi, the chlorophyll (a) and chlorophyll (b) contents become nil. However, in PNT, these levels remained almost identical.

The variation of the carotenoid content in the cotton leaves is similar to that of the chlorophyllian pigments Thus, from day 0 to J150, in the PTEMi, the carotenoid content is passed from 27.09 to 128.50 µg/mL, a gain of 374.34 %, followed respectively by PTMi (from 26.73 to 119.16 µg/mL, a gain of 345.79 %) and PTEi (from 28.04

to 91.80 μg / mL, a gain of 227.38 %). In PNT, the carotenoid content increased from 27.07 to 71.10 μg / mL, a gain of 162.65 %, while in PNTi the carotenoid content decreased from 28.53 to 0 μg/mL, a loss of 100 %.

Table 2. Effect of stimulators on variation of leaf pigment content in cotton leaves inoculated with FOV from D0 to D150

TI (hour)	Nature of pigment	Leaf pigment content (µg/mL)				
		Treatement				
		PNTi	PNT	PTEi	PTMi	PTEMi
0	Chla	123,25± 0,24a	123,05± 2,11a	125,10± 2,03a	121,29± 2,21a	124,50± 1,16a
	Chlb	87,30± 1,09a	89,02± 0,21a	91,06± 1,17a	88,48± 1,07a	87,37± 2,02a
	Chlt	242,55 ± 1,52a	243,07 ± 0,93a	243,16 ±1,55a	243,77 ± 2,11a	241,87 ±1,40a
	Car	28,53 ± 2,71a	27,07 ± 0,93a	28,04 ±1,22a	26,73 ± 1,05a	27,09 ±2,40a
30	Chla	122,47± 1,09e	140,58± 1,09d	150,78± 1,09c	166,47± 1,09b	188,58± 1,09a
	Chlb	115,53± 1,09c	104,49± 1,09b	132,27± 1,09a	125,64± 1,09a	113,44± 1,09b
	Chlt	239,17 ± 0,84d	245,07 ± 0,88c	283,05 ±1,02b	292,11 ± 0,65a	300,02 ±1,37a
	Car	24,21 ± 2,35c	25,24 ± 0,27c	40,26 ±2,02b	50,22 ± 0,13a	57,11 ±1,31a
60	Chla	111,42± 1,09d	136,56± 1,09b	145,48± 1,09b	148,48± 1,09b	174,56± 1,09a
	Chlb	103,81± 1,09c	103,51± 1,09c	111,88± 1,09b	129,55± 1,09a	113,57± 1,09b
	Chlt	215,23 ± 0,71f	240,07 ± 0,21d	257,36 ±1,31c	278,03 ± 1,22b	288,13 ±2,21a
	Car	20,08 ± 2,22c	26,12 ± 1,15c	36,32 ±1,16c	48,16 ± 2,41a	51,35 ±2,36a
90	Chla	65,02± 1,11c	136,03± 1,16c	70,28± 0,29b	74,54± 1,22b	86,48± 0,15a
	Chlb	69,05± 0,02c	104,04± 2,55b	126,20± 1,37a	120,40± 0,11a	121,20± 1,31a
	Chlt	134,07 ± 1,03d	245,07 ± 0,01lc	198,48 ±1,13b	231,94 ± 0,33a	235,68 ±0,34a
	Car	20,08 ± 2,22c	26,12 ± 1,15c	36,32 ±1,16b	48,16 ± 2,41a	51,35 ±2,36a
120	Chla	45,48± 1,09f	135,48± 1,09d	186,25± 1,09c	232,48± 1,09b	255,48± 1,09a
	Chlb	36,59± 1,09f	103,66± 1,09c	114,07± 1,09b	146,1± 1,09b	203,09± 1,09a
	Chlt	82,07 ± 1,33f	255,14 ± 2,66d	300,32 ±0,22c	378,58 ± 2,16b	458,57 ±1,72a
	Car	14,11 ± 2,08g	25,17 ± 1,21d	49,78 ±1,17b	57,36 ± 1,23b	74,30 ±0,22a
	Chla	-	124,05± 1,13e	197,10±1,23c	246,19 ± 0,28b	300,04 ±0,93a
	Chlb	-	110,02± 0,56e	128,12± 0,09c	150,04 ± 0,19b	162,03 ± 0,49a

| 150 | Chlt | - | 250,07 ± 2,40d | 325,22 ±1,07c | 396,23 ± 1,47b | 479,07 ±2,42a |
| | Car | - | 71,10 ± 1,24d | 91,80 ±1,24c | 119,16±1,04b | 128,50 ±2,12a |

FOV: *Fusarium oxysporum* f. sp. vasinfectum; TI: incubation time; PNT: untreated and uninoculated plant; PNTi: untreated and inoculated plant; PTEi: plant treated with ethephon and inoculated; PTMi: plant treated with MeJA and inoculated; PTEMi: plant treated by the association of MeJA and ethephon; ± S: standard error; on the same line, the values followed by the same letter are not significantly different (Newman-Keuls test at 5%); the values represent the average of three repetitions.

Effect of natural defense stimulators on the variation of lignin

Figure 28 shows the influence of exogenous application of SDN on the evolution of lignin levels in cotton roots, stems and leaves after 150 days of FOV inoculation. Thus, the lignin content increased in the roots of treated cotton plants compared to controls. Cotton trees treated with the combination of methyl jasmonate and ethephon, then inoculated (PTEMi) had the highest lignin level (76.87 %), followed by cotton plants treated with methyl jasmonate and inoculated (PTMi) with a rate of 64.23 %, while those treated with ethephon and inoculated (PTEi) had a rate of 56.47 %. As for controls, lignin levels remained statistically identical (14.56 % for PNTi and 15.66 % for PNT).

In the stems, the results also showed an increase in the lignin content in treated cotton compared with controls. This evolution of the lignin level in the stems is similar to that obtained in the roots. Thus, PTEMi had the highest lignin level (41.87 %), followed by PTMi with a rate of 38.23 %, while PTEi had a rate of 34.48 %. As for the PNTi and PNT, the rates remained statistically identical (4.06 % for PNTi and 5.62 % for PNT).

The report is the same at the leaf level. The controls had the lowest levels (1.78 % for PNTi and 2.52 % for PNT), while in treated and inoculated plants, an increase was observed. Thus, PTEMi had the highest lignin level (18.26 %), followed by PMTi (12.08 %) and PTEi (9.17 %).
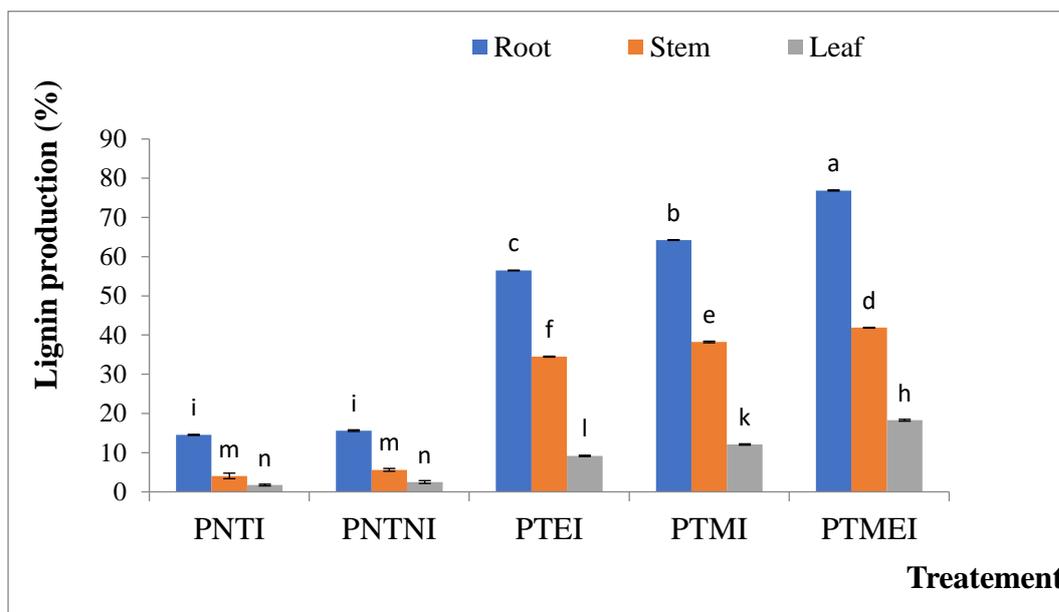
Figure 7. Evolution of lignin content in cotton explants treated with stimulators and inoculated with FOV

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

Discussion

The whitish, feather-white, shallow, low-density appearance of the mycelium obtained after purification of the fungal isolate, as well as the presence of microconidia, macroconidia and chlamydospores, led to the conclusion that this is *Fusarium oxyspum* type (Bacchetta *et al.*, 1995, Henni *et al.,* 1998). Inoculation of two-week-old young cotton farmers, followed by almost total mortality, confirmed its virulence. This fact proves the special form "vasinfectum" of our mushroom, responsible for Fusarium wilt in cotton.

Analysis of inoculum concentration variation (50; 100; 500 and 1000 µL) showed that doses between 500 and 1.000 µL were more pathogenic. The dose of 100 µL of inoculum that allowed 10.47 % mortality rate was described as non-pathogenic, because according to Djerbi (1990), the pathogenicity test of a fungal isolate is valid when the final mortality of plants exceeds 20 %.

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 SDN. The treated cotton plants therefore behaved like PNTs and better in the PTEMi. The combination of methyl jasmonate and ethephon seems to provide a complementary effect by increasing the efficiency of MeJA or ethephon applied 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* (2014) in 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.

In addition, the analysis of leaf pigment levels in treated and inoculated cotton leaves confirms this. Chlorophylls, which are the pigments responsible for the green color of the leaves, represent the "blood of plants" because their molecular structure is close to that of hemoglobin, one of the main constituents of human blood (Stassiaux, 2010). Chlorophylls give plants the power of photosynthesis, that is, the biosynthesis of carbohydrates from the light energy captured by chlorophylls. Thus, under the action of FOV, total chlorophyll content fell in PTNi to cancel at J150. On the other hand, under the action of SDN, the total chlorophyll content has increased steadily showing a positive effect on the biosynthesis of chlorophylls. The total chlorophyll content of the PTEMi was the highest compared to the other treatments and even higher than that of the PTN. This shows a beneficial effect of treatment associating MeJA and ethephon on the biosynthesis of chlorophylls. In addition, the work of Couderchet *et al.* (2003) showed a strong link between PR proteins, induced phytoalexins and protoporphyrogen oxidase, an enzyme involved in chlorophyll synthesis. However, the combination of MeJA and ethephon stimulates the production of phytoalexins more than that of MeJA or ethephon taken separately. This could explain the significant increase in total chlorophyll content seen in PTEMi compared with other treatments. In addition, the significant decrease in total chlorophyll content in PTNi that vanishes at J150 revealed the influence of FOV on chlorophyll degradation. Indeed, the plant needs sugars to ensure its growth, its development and the establishment of defense mechanisms. The sugars are synthesized in leaves and then transported via the stem to the organs wells of the plant (roots, flowers, leaves). For its part, the heterotrophic fungus for carbon will draw sugars from the tissues it infects. It thus establishes a competition for the carbon resources. The pathogen can then represent a new well that will divert to its profit assimilas flows within the plant. In this competition between the plant and the mushroom, the mobilization of sugars is a major issue for both opponents, cotton trying to defend itself and the fungus to develop (Afoufa-Bastien *et al.*, 2010; Konan, 2014).

The application of SDN also resulted in an increase in chlorophyll content (a) and a decrease in chlorophyll content (b). Indeed, under the action of SDN, there would have been a bioconversion of chlorophyll (b) to chlorophyll (a), thus increasing the content of chlorophyll a and therefore a good photosynthetic activity (Folly, 2000, Djapic, 2012). As for carotenoid levels, they increase slightly, similarly to those of chlorophyll content b. These results show that the exogenous application of SDN has a beneficial effect on the synthesis of chlorophyll (a). Indeed, chlorophyll (a) is the main pigment, that is to say the most active during photosynthesis because it allows the conversion of light energy into chemical energy in the form of ATP while other pigments (Chlorophyll (b) and carotenoids) that transmit energy to it by resonance are called accessory pigments (Olson, 2006, Raven *et al.,* 2007, N'cho *et al.,* 2018).

,The evaluation of the effect of the SDNs on the reinforcement of the cotton cell walls of FOV-inoculated cotton plants through the quantification of lignins in the roots, stems and leaves, showed an increase of their level in the treated and inoculated plants compared to the witnesses. Indeed, lignins are essential constituents of any wood because they are at the base of the rigid and durable nature of a tree by being deposited in the secondary wall of certain plant cells. Thus, a high lignin level would reduce the chances of a pathogen entering a plant (Maladier, 2014). The results showed significant levels of lignin in the roots, followed by stems and then leaves. Indeed, FOV is a soil fungus, which under favorable conditions invades the roots following the penetration of the epidermis; this promotes the development of disease symptoms in the plant (Bacchetta *et al.,* 1995). The roots are therefore the first organs to be in contact with FOV. Thus, a high level of lignin in it will therefore ensure the strengthening of the cell walls and prevent the penetration of the fungus into the plant. The roots serve as a sort of "shield" to the whole plant. In addition, lignin levels are the same in roots stems and leaves in PTNi and PTN. This shows that the presence of the fungus in the plant does not influence lignin production and metabolism. Therefore, the increase in the level of lignins in the plant would be exclusively due to the exogenous application of SDN on the cotton leaves.

Conclusion

MeJA and ethephon have shown their ability to induce better resistance of the cotton plant to *Fusarium oxysporum* f. sp. vasinfectum through the increase in photosynthetic activity and the strengthening of the constitutive defense of the plant by an increase in the lignin content. This protection is more effective after a treatment combining the two molecules.

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