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Effect of elicitation of cotton [(*Gossypium hirsutum* L.) cv. Y331B] with methyl jasmonate and ethephon in relation to secondary metabolites

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Abstract

Cotton is a versatile plant grown for the fibers and these seeds. However, its culture dating numbers constraints productions including pest attacks. Faced with these attacks certain control methods prove ineffective. The research will be directed on stimulation of the natural defenses of the plant by applying elicitors to find an effective alternative to chemical control. Thus, the study of the influence of two elicitors (Ethephon and methyl jasmonate) on the biosynthesis of phenolic compounds in the cultivar Y331B showed that the phenolic metabolism is significantly altered after treatment of the leaves by the elicitors. Two elicitors cause an increase in the production of phenolic compounds. However, the methyl jasmonate has the greater accumulation of phenolic compounds with respect to ethephon. Moreover, the co-processing of sheets by MeJA and ethephon has permit to obtain a significant increase in the levels of phenolic compounds.

Keywords: Cotton, Gossypium hirsutum, elicitor, ethephon, methyl jasmonate, phenolic compound

1. Introduction

The cotton plant is a multipurpose textile and protein-oil plant (Sement, 1998) [29]. It is grown in many hot countries for its fibres, which are the main raw material for the textile industry and for its seeds, which contain 18-20% lipids and 35-40% protein. Gossypium hirsutum is the most widely cultivated species, providing nearly 95% of the world's cotton production (Mergeai, 2003) [22].

Cotton cultivation occupies about 2.5% of the world's arable land and its global production is about 12.7 million tonnes, accounting for 60% of the raw material in the textile industry (FAOSTAT, 2021) [16]. The United States, China, Uzbekistan, India and Pakistan are the largest producers. Africa accounts for only 8% of world production (Estur, 2005) [12]. Côte d'Ivoire has become the 2nd largest African producer (55,800 tonnes) behind Benin (750,000 tonnes) but ahead of Burkina Faso (49,600 tonnes) and Mali (147,200 tonnes) (Olopo, 2021) [24]. Cotton represents a very important source of income as it supports more than 3.5 million people in Côte d'Ivoire (FIRCA, 2019) [15]. In Côte d'Ivoire, cotton is the 3rd largest export product after cocoa and coffee. Côte d'Ivoire's seed cotton production provides 8-10% of the country's operating revenue and contributes 1.8% of gross domestic product (FIRCA, 2019) [15].

However, the cotton plant is threatened by parasites including viruses, bacteria, fungi and various diseases that reduce production by up to 25% and even the quality of the fibres and seeds (Vaissayre, 1994) [31]. Of all the diseases of cryptogamic origin, fusariosis caused by Fusarium oxysporum f. vasinfectum (FOV) seems to be the most dreaded. Indeed, classical fungicides are not able to control this

disease which causes enormous damage in cotton farms (Demol *et al*., 1992; Optiz *et al*., 2008) [8, 24]. In this context, the search for more efficient alternatives for the development of sustainable agriculture is necessary. One of them is to provide plants with the means for self-defence, rather than directly fighting the aggressor (Amari, 2012; Konan *et al*., 2014) [2, 19].

Thus, in this study we will first evaluate the effect of these two elicitors and their co-treatment on the total phenol content of cotton leaves. Then, we will evaluate their action on the activities of the biosynthesis and degradation enzymes of the phenolic compounds.

2. Materials and Methods

2.1. Plant material

The plant material consisted of cotton seeds (Gossypium hirsutum L.cv. Y331B). These seeds come from the Korhogo region (north of Côte d'Ivoire) and were supplied by the Ivoirian Company of textile development (CIDT).

2.2. Elicitors

The elicitors were methyl jasmonate and ethephon used at 5 mM respectively.

2.3. Preparation of cotton seeds

The seeds are first washed with concentrated sulphuric acid (Figure 1). After several rinses, the seeds are immersed in water to separate the good seeds from the bad ones. After drying, these seeds were then sterilised under a laminar flow hood by a quick soak (1 min) in 70% ethanol, followed by a 20 min immersion in sodium hypochlorite (3.6% active chlorine). After three (3) successive rinses with sterile distilled water for 3 to 5 min, they were soaked in a jar containing sterile distilled water and placed in the dark in a culture room. After 48 hours, the seeds that have developed a pointed radicle are transferred to the prepared pots. The whole set is then grown under a shelter covered with a transparent plastic film where the light and temperature conditions are close to natural conditions.

A B

Fig 1: Fibre-bearing cotton seeds (A) and de-fibred or de-shedded seeds (B)

2.4. Culture medium and condition

The culture medium for our study is soil placed in polystyrene pots and placed under shelters (figure 2). This soil was previously sterilised in an autoclave for 30 min at a temperature of 121°C under a pressure of 1 bar.

Fig 2: Cotton seedlings in cultivation

2.5. Treatment of cotton leaves with MeJA

At the 5-6 leaf stage, reached after 4 weeks of cultivation, 30 cotton plants were sprayed (using a hand sprayer, plant by plant) with 5 mM MeJA (Konan *et al*., 2014) [19] previously prepared and dissolved in 1% ethanol, then added to an aqueous solution containing triton X-100 (1%). The latter will act as an adjuvant and will allow a longer retention of the product on the leaves while conferring a penetrating power in the leaves. After spraying the plants with a hand sprayer, the incubation time of the cotton plants was 72 h (Konan *et al*., 2014) [19]. Each plant received approximately 50 ml of MeJA solution. Watering of the seedlings was provided depending on the moisture content of the substrate.

2.6. Leaf treatment with ethephon

Ethephon (5 mM), an ethylene precursor derived from phosphonic acid (C2H6O3Pcl, chloro-2-ethylphosphonic acid), was diluted in distilled water and an aqueous solution containing triton X-100 (1%) was added. Each of the 30 cotton plants received 50 ml of 5 mM ethephon and then the plants were incubated 72 h (Konan *et al*., 2014) [19].

2.7. Leaf Co-treatment with MeJA/Ethephon

At the 5-6 leaf stage, 30 cotton plants were sprayed with a solution consisting of an equal volume mixture of MeJA (5 mM) and ethephon (5 mM). MeJA previously dissolved in 1% ethanol and ethephon were added to an aqueous solution containing 1% Triton X-100 and sprayed onto the leaves using a hand-held sprayer. Each plant received approximately one 50 ml application of each solution. After spraying, the incubation time of the cotton plants was 72 h. To promote the opening of the stomata, the humidity was maintained at 90% by spraying water under the enclosure. After spraying, the incubation time of the cotton plants was 72 h. The concentrations used were chosen according to the work of Konan *et al*. (2014) [19]. Untreated cotton plants numbering 30 served as controls for each condition tested.

2.8. Analysis of phenolic compounds

2.8.1. Extraction of phenolic compounds

The extraction of total phenols is done according to the method of Singleton *et al*. (1999) [30]. Indeed, 0.5 g of leaves taken from each batch are cold ground in 5 ml of 80% ethanol. The crushed material was centrifuged at 5000 rpm for 10 min. The supernatant obtained constitutes the crude

extract on which the determination of total phenols will be performed.

2.8.2. Determination of phenolic compounds

The determination of total phenols was done according to the method of Singh *et al*. (2002) [30] modified and adapted to our plant material. The reaction mixture is mainly composed of phosphotungstic acid and phosphomolibdic acid which will be reduced in alkaline medium, in parallel with the oxidation of phenols. The presence of the phenols is revealed by the addition of 0.5 ml of Folin Ciocalteu 1 N reagent; 1.5 ml of 17% sodium carbonate and 0.5 ml of crude extract. The intensity of the coloration produced by this reaction, in proportion to the concentration of phenolic compounds in the extract, is monitored by spectrometer at 765 nm. During the assay, a control is carried out where the phenolic extract is replaced by distilled water. The content of total phenols, expressed in milligrams of gallic acid equivalents per gram of fresh matter extract (mg/g.MF), was determined using a calibration line performed with 200 µg/ml of gallic acid (y = 0.021 x + 0.053; R2 = 0.99, where y is absorbance and x is the concentration of the gallic acid)

2.9. Analysis of the enzymes of phenolic metabolism

2.9.1. Extraction of enzymes

The buffer required for the extraction of the enzymatic substances will depend on the enzymes studied and will therefore be specified later for each one. The extraction of the enzymes is carried out at a cold temperature of 4°C (to prevent the inhibitory actions of phenolic compounds and proteolytic enzymes) by grinding 0.5 g of cold-dried plant material (cotton leaves pulverised with elicitors) in extraction buffer. During grinding 1.2 ml of an extraction medium solution consisting of 0.5 ml polyethylene glycol 6000 (PEG 6000) is used to bind the phenolic compounds, 0.25% sodium thiosulphate to protect the enzyme sites, 15% glycerol for stability of the enzyme systems, 1 mM EDTA as complexing agent and 15 mM mercaptoethanol is added as reducing compounds to prevent enzyme oxidation. After centrifugation, at 5000 rpm for 20 min, the resulting supernatant represents the crude enzyme extract.

2.9.2. Determination of phenolic biosynthesis enzymes

The determination of these two enzymes was carried out using the method described by Regnier. The base buffer used was 0.2 M sodium borate at pH 8.8. The reaction mixture contained 0.1 ml of enzyme extract; 1 ml of 0.1 M phenylalanine for PAL or 0.1 M tyrosine for TAL and 1.9 ml of 0.2 M sodium borate buffer at pH 8.8. After 10 min incubation at room temperature, the activity of PAL and TAL, which is proportional to the amount of cinnamic acid and p-coumaric acid respectively, is monitored by spectrophotometer at a wavelength of 290 nm. In the assay, a control test is performed for each extract in which phenylalanine or tyrosine is replaced by 0.2 M sodium borate buffer at pH 8.8. The activity of PAL and TAL is respectively expressed in millimoles of cinnamic acid or p-coumaric acid formed per minute per gram of fresh material, considering that the molar extinction coefficient of cinnamic acid is equal to 19600 cm-1 mol-1 and that of p-coumaric acid is equal to 17600 cm-1 mol-1.

2.9.3. Enzymes for the degradation of phenolic compounds

Peroxidase (POD) activity was determined according to the technique described by Santimone (1973) [27]. The base buffer used was 0.1 M sodium phosphate at pH 7.5. The reaction mixture consisted of 0.2 ml of enzyme extract and 2.8 ml of substrate consisting of 10-2 M guaiacol and 10-2 M hydrogen peroxide solution (V/V). After shaking, the mixture is incubated for 10 min. in the dark to prevent partial destruction (by light) of the red-brown oxidation product formed from guaiacol in the presence of hydrogen peroxide. A delay of one minute is allowed between tubes when adding the enzyme extract to the substrate. The oxidation of guaiacol is monitored with a spectrophotometer at a wavelength of 470 nm, always respecting the one-minute delay between tubes. A control is made where the substrate is replaced by 0.1 M sodium phosphate buffer at pH 7.5. Peroxidase activity is expressed in millimoles of product formed per minute per gram of fresh material. The molar extinction coefficient of the product formed at the wavelength of 470 nm is 26.6 cm-1 mol-1 (Santimone, 1973) [27].

The determination of polyphenoloxidase (PPO) activity was done according to the method of Joslyn and Ponting (1951) [18], modified and adapted to our plant material. The base buffer used was 0.1 M phosphate citrate at pH 6.5. Before adding DOWEX 50, two volumes (2 x Vml) of 100% acetone (to precipitate the enzymes contained in the supernatant) are added to the volume (v) of supernatant. A final centrifugation under the same conditions as above yields a supernatant which represents the crude PPO extract. The reaction mixture, incubated for 10 min at room temperature (25 °C), is composed of 0.1 ml of enzyme extract and 1 ml of 130 mM pyrocatechol. The oxidation of pyrocatechol is monitored by spectrophotometer at a wavelength of 500 nm against a control assay in which pyrocatechol is replaced by 0.1 M phosphate citrate buffer at pH 6.5. PPO activity is expressed as millimoles of product formed per minute per gram of fresh material.

2.9.4. Assessment of enzymatic activities

The rate of participation of each enzyme in phenolic metabolism was assessed relative to the control (untreated) and defined as follows

$$TP = \frac{AE}{AET} X\ 100$$

TP = Rate of participation of enzymes in phenolic metabolism
AE = Enzyme activity of elicited plants
AET = Enzyme activities of control plants

2.10. Statistical analysis

The data obtained were analysed using Statistica 7.1 software. An analysis of variance with two classification criteria was performed to determine significant differences. In case of significant differences, the means were classified into homogeneous groups using the Newman-Keuls test at the 5% level. All experiments were repeated three times.

3. Results

3.1 Effect of elicitors on the total phenol content of treated cotton leaves

The analysis in Table 1 shows that the phenol content which was initially 18.54 mg/g MF in the untreated leaves (control) increased to 68.78 mg/g MF in the leaves sprayed with MeJA

and that of the leaves treated with ethephon increased to 48.43 mg/g MF. On the other hand, the phenol content in the leaves co-treated with MeJA/etherphon increased to 101 mg/g MF. Furthermore, the phenol content increased more in the leaves co-treated with MeJA/ ethephon compared to the phenol content of the leaves treated separately with MeJA and ethephon.

Table 1: Total phenol content of treated cotton leaves according to elicitors

Elicitors	Control	MeJA	ETH	MeJA/ETH
Concentration	0 Mm	5 Mm	5 Mm	5 mM
Phenols Compounds (mg/g MF)	18,54±1,22e	68,78 1,46c	48,43±3,23b	101±1,16a

Values followed by the same letter are statistically identical at 5% (Newman-Keuls test)±S: standard error

3.2. Levels of phenolic biosynthesis enzymes (PAL and TAL)

Table 2 shows the activity of some phenolic metabolism enzymes. In general, the activity of the biosynthesis enzymes increases with the different treatments compared to the control. Indeed, PAL activity, which is 160.24 mmol/min/g MF in untreated leaves, increases to 190 mmol/min/g MF after ethephon treatment. This activity increases by 50 mmol/min/g MF in the case of MeJA treatment (210 mmol/min/g MF) compared to the untreated control. PAL activity in MeJA-treated leaves is higher than that observed in ETH-treated leaves (190 mM/g MF). However, the MeJA/etherphon co-treatment stimulated the most PAL activity, which was 245 mmol/min/g MF. TAL activity follows the same trend as PAL but with lower values than PAL. Indeed, in untreated leaves the TAL activity is 95.02 mmol/min/g MF. This activity increases after treatment with MeJA to 145.07 mmol/min/g MF. After ethephon treatment, the TAL activity increased compared to the control but remained lower than after MeJA treatment. The MeJA/etherphon co-treatment further increases TAL activity to a maximum of 155 mmol/min/g MF.

Table 2: Levels of enzymatic Compounds (PAL, TAL) in elicitor-treated leaves

Enzyme (mmoles /min/g MF)	Control	MeJA (5 mM)	ETH (5 mM)	MeJA/ETH (5mM)
PAL	160,24±4,56e	210,51±7,41d	190±3,56e	245±2,66d
TAL	95,02±2,03g	145,07±14,9f	135±8,25f	155±11,32a
PAL + TAL	225±4,23d	355±8,07 b	325±7,25b	400±15,47a

Values followed by the same letter are statistically identical at 5% (Newman-Keuls test);±S: standard error

3.3. Activities of phenolic degradation enzymes (PPO and POD)

The activity of the degradation enzymes decreased with the treatments. Indeed, the activity of POD which is 1.5 mmol/min/g MF in untreated leaves decreases to 0.75 mmol/min/g MF in ethephon treated leaves. However, this activity is slightly higher in the case of MeJA treatment (0.85 mmol/min/g MF) compared to ethephon treatment. However, after MeJA/etherphon co-treatment, the POD activity decreases and becomes very low (0.56 mmol/min/g MF) compared to the untreated control (1.5 mmol/min/g MF). PPO activity evolves in a similar way to that of POD but with higher values. Indeed, in untreated leaves, the PPO activity of 2.84 mmol/min/g MF decreases after MeJA treatment (1.81 mmol/min/g MF). This activity is lower in the case of ethephon treatment (1.71 mmol/min/g MF) compared to MeJa treatment. However, after co-treatment with MeJa/etherphon, the PPO activity decreases and becomes lower (1.36 mmol/min/g MF) compared to treatment with the two elicitor molecules separately. It should be noted that the activity of PPO remains higher than that of POD whatever the treatment.

Table 3: Levels of phenolic degrading enzymes (PPO, POD,) in elicitor-treated leaves

Enzyme (mmoles /min/g MF)	Control	MeJA (5 mM)	ETH (5 mM)	MeJA/ETH (5mM)
PPO	2,84±0,73b	1,81±0,57c	1,71±0,45e	0,74±0,45f
POD	1,5±0,83d	0,85±0,08f	0,75±0,25f	0,56±0,41f
PPO+POD	4,34±1,01a	2,66±0,23b	2,46±0,14b	1,3±0,23c

Values followed by the same letter are statistically identical at 5% (Newman-Keuls test);±S: standard error

3.4. Participation rate (%) of biosynthetic enzymes

Table 4 shows the participation rate of enzymes in phenolic metabolism. The analysis of the table shows that the participation rates of PAL decrease compared to the control. Indeed, the participation rate of phenylalanine ammonia-lyase (PAL) in the biosynthesis of phenolic compounds, which is 54% in the untreated control leaves, decreases to 51.67% in the ethephon-treated leaves, i.e. a decrease of 2.3%. When the leaves are treated with MeJA, this rate drops to 47%, a sharp decrease of 7%. But when the leaves are co-treated with MeJA/etherphon, this rate reaches 53.26%, a decrease of 0.74%. On the other hand, the participation rates of the TAL increased compared to the control. Indeed, the participation rate of tyrosine ammonia-lyase (TAL) in the biosynthesis of phenolic compounds, which was initially 31% in the untreated control leaves, rose to 60.61% in the ethephon-treated leaves, i.e. an increase of 29.61%. When the leaves are treated with MeJA this participation rate increases to 58%, i.e. an increase of 27%. But when the leaves were co-treated with MeJA and ethephon, the participation rate of TAL in the biosynthesis of phenolic compounds reached 76.54%, i.e. an increase of 45.54%. TAL is therefore the enzyme that participates most in the biosynthesis of phenolic compounds.

Table 4: Participation rate (%) of biosynthetic enzymes in polyphenol metabolism after elicitor application

Biosynthetic enzyme	Control	MeJA (5 mM)	ETH (5 mM)	MeJA/ETH (5mM)
PAL	54	47	51,67	56,26
TAL	31	58	60,61	76,54
PAL + TAL	85	105	112,28	128,8

3.5. Participation rate (%) of degradation enzymes

The participation rate of POD degradation enzymes in untreated leaves is 65.43% and decreases to 60.24% in ethephon-treated leaves, a decrease of 5.19%. On the other hand, the participation rate of POD degradation enzymes increased in MeJA-treated leaves (68.04%), i.e. an increase of 2.61%, and in MeJA/etherphon-treated leaves (71.21%), i.e. an increase of 5.78%. The participation rate of PPO degrading enzymes in untreated leaves is 3.45% and increases to 35.14% in ethephon treated leaves, i.e. an increase of 31.69%. This rate increases to 31.95% in MeJA-treated leaves, i.e. an increase of 28.5%, and 42.22% in MeJA/etherphon sprayed leaves, i.e. an increase of 38.77%. Thus, polyphenoloxidases (PPO) participate more in the degradation of phenolic compounds than peroxidases (POD) because they present higher participation rates than peroxidases.

Table 5: Participation rate (%) of biosynthetic enzymes in polyphenol metabolism after elicitor application

Enzymes of dégradation	Control	MeJA (5 mM)	ETH (5 mM)	MeJA/ETH (5mM)
POD	65,43	68,04	60,24	71,23
PPO	3,45	31,95	35,14	42,22
PPO+POD	68,88	99,99	95,38	113,45

4. Discussion

The results obtained show that the phenol content of the leaves of cotton Gossypium hirsutum L. cv. Y331B varies significantly following the application of elicitors. These results are in agreement with those of El Bellaj *et al*. (2006) [10] who showed that treatment with methyl jasmonate and ethephon increases phenol levels in grapevine leaves. Thus, the applied MeJA seems to give the maximum phenols. However, the application of ethylene in the form of ethephon, mobilises less phenolic compounds than MeJA. However, the work of Diaz *et al* (2002) [9] showed that ethephon stimulates the natural defences of tomato by inducing resistance to Botrytis cinerea. Thus, our study clearly shows that ethephon seems to activate the natural defences of cotton less than MeJA as already reported by Faurie (2009) [14] in grapes. Furthermore, co-treatment of leaves with MeJA and ethephon resulted in an exponential increase in the content of all synthesized phenolic compounds compared to that obtained with MeJA or ethephon alone. Thus, the concomitant application of MeJA and ethephon to the leaves seems to have an additive effect (synergy or potentiation) on the accumulation of phenolic compounds in cotton. This synergistic or cooperative effect of MeJA and ethephon on the biosynthesis and accumulation of phenolic compounds has also been reported in tobacco (Xu *et al*., 1994) [32], Arabica (Penninckx *et al*., 1998) [26] and grapevine (Larronde *et al*., 2003; Faurie *et al*., 2009) [14, 21]. The gains in phenolic compounds conferred by MeJA (68.78 mg/g.MF) and ethephon (48.43 mg/g MF) separately, represent only (53.13%) compared to the phenolic pool obtained by co-treatment with these two elicitors (101 mg/g MF). MeJA and ethephon seem to be part of a complex cascade of signals that lead the cotton plant to mobilise defence mechanisms, notably polyphenols. The addition of these elicitors would trigger the most important defence mechanisms. These results are in line with those of Framer *et al* (2003) [13] who showed that methyl jasmonate and ethylene could act in synergy on certain genes coding for the biosynthesis of phenolic compounds. The results obtained with regard to the activity of enzymes showed an intense activity of PAL and TAL in leaves. These results are in agreement with those of Bouarab *et al*. (2004) [5] who showed that MeJA stimulates PAL and TAL activity in Cripus algae. For some authors, the inducing effect of MeJA is often preceded by an activation of the synthesis of enzymes involved in the biosynthesis of various metabolites such as PAL (Ellard-Ivey and Douglas, 1997; Constanbel and Ryan, 1995) [6, 11]. Furthermore, our results showed an increase in the rate of participation of TAL in polyphenol metabolism compared to that of PAL which decreased in MeJA-treated leaves compared to the control (untreated). These results suggest that the Tyrosine pathway is more involved than the Phenylalanine pathway in the accumulation of polyphenols in cotton leaves following exogenous (spray) application of MeJA. This preponderance of the TAL pathway has already been demonstrated in vitro plants and in FOV-inoculated cotton callus (Konan, 2006) [19]. In addition, analysis of phenol degrading enzyme activities shows a decrease in POD and PPO activity in sprayed leaves compared to controls. This decrease in activities is thus opposite to the increase in PAL and TAL content in the treated leaves. This correlation had already been reported by Ziouti *et al*. (1996) [16], who suggested that the increase in Peroxidase (POD) and Polyphenoloxidase (PPO) activities coincided with the decrease of PAL and TAL contents in date palm roots and vice versa. In this degradation activity our results showed an increase in the participation rate of Polyphenoloxidases activity compared to that of PODs whatever the treatment. This would mean that PPOs would have a higher degradation activity than peroxidases in cotton leaves sprayed with MeJA. All these results could constitute a contribution to the study of the stimulation of natural plant defences.

5. Conclusion

The study showed that the application of MeJA stimulated the synthesis of several phenolic compounds and caused a significant increase in the content of existing phenolic compounds in the untreated control leaves. The participation rate indicates that the application of ethylene in the form of ethephon mobilised fewer phenolic compounds than MeJA.

In contrast, co-treatment of leaves with MeJA and ethephona revealed a high phenolic content compared to leaves treated separately with ethephon and MeJA. The addition of the phenolic pools conferred by the application of MeJA and ethephon separately represented only 53.13% of the phenolic pool obtained with the joint application of these two elicitors. The combination of MeJA and ethephon has a supra-additive effect on the accumulation of phenolic phytoalexins in cotton and thus on the production and accumulation of phenolic compounds as well as on the activities of TAL and PPO.

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