

EVALUATING THE RESPONSE TO *FUSARIUM MONILIFORME* VAR. SUBGLUTINANS INOCULATION IN PINEAPPLE (*ANANAS COMOSUS* VAR. COMOSUS) ELICITED BY TWO BIOCONTROLS AND IMPACT ON FRUITLET CORE ROT (BLACK SPOT) DISEASE PROTECTION

^{1*}YEO Navigué Abou, ²KONAN Yao Kouakou François,
³KOUADIO Oi Kouadio Samuel, ⁴N'goran Kouakou Désiré,
⁴ABEDA Zagadou Herman, ⁴KOUAKOU Tanoh Hilaire, ¹ZOUZOU Michel

¹Université Félix Houphouët Boigny, UFR Biosciences, 22 BP 582 Abidjan 22, Côte d'Ivoire

²Université Jean Lorougnon Guédé, UFR Agroforesterie, BP 150 Daloa, Côte d'Ivoire

³Université Péléforo Gon Coulibaly, UFR Sciences Biologique, BP 1328 Korhogo, Côte d'Ivoire

⁴Université Nangui Abrogoua, UFR Sciences de la Nature, 02 BP 801 Abidjan 02, Côte d'Ivoire

*Corresponding Author

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ABSTRACT

The natural defense of pineapple was stimulate to evaluate the tolerance to *Fusarium moniliforme* var. subglutinans (FMS), agent of fruitlet core rot (FCR). For this purpose, vivoplants of pineapple varieties (MD2 and Smooth Cayenne) were treated with methyl jasmonate (MeJA) and Calliete® (Ca) as well as combination of both biocontrols (MeJA + Ca). The plants were then inoculated with the pathogen. The incidence of FCR was estimated after inoculation of the flower and opening the fruit at harvest. Protection of pineapple by natural defense stimulators (NDS) against FCR was appraised in fruits of plants treated and inoculated with FMS compared to untreated and inoculated plants. Growth parameters (stem diameter) and physiological parameters such as leaf pigments (chlorophylls and carotenoids) and lignin content were evaluated. The results showed that the elicitors mobilized a wide range of defense molecules in pineapple to undermine the FCR. However, the combination of both elicitors (MeJA + Ca) has a potentiating effect on the tolerance of pineapple to FMS compared to the

elicitors taken individually. The use of natural defense stimulators (NDS) is therefore an effective way to protect pineapple against FMS. Their application in the agricultural environment could contribute to the development of a sustainable agriculture that respects the environment and human health.

Keywords: Pineapple, *Fusarium moniliforme*, natural defenses stimulator, tolerance, fruitlet core rot disease, Smooth Cayenne, MD2.

1. INTRODUCTION

Pineapple plays an important role in the economy, health and food security of populations. It is both an industrial and a food crop. It accounts for 40 % of the world's production of major tropical fruits with exports climbing to 3.1 million tons in 2019 compared to 2018 [1]. However, pineapple is threatened by many pests, parasites and various diseases that contribute to the reduction of production and fruit quality [2]. Fruitlet core rot disease (FCR) is one of the main postharvest disease of pineapple that causes of production loss in pineapple [3]. To control this disease, several strategies were designed to eliminate symptoms. Unfortunately, no control method has been found to be effective so far. These control methods which, by not addressing the cause or the mechanisms of the disease, give only moderate success [4; 5]. In addition, chemical control is increasingly in the spotlight because of the negative consequences on the environment and human health.

In this context, it seems necessary to seek more efficient alternatives for the development of sustainable agriculture. According to Rees and Harborne [6], the ability of a plant species to resist attack by pathogens is most often correlated with the secondary metabolites content such as lignin and leaf pigments that gives a glimpse of plant vitality. Elicitors are generally molecules secreted by microorganisms, derived from the cell wall of the fungus, bacteria and / or the host plant [7]. These molecules have varied modes of action, but instead of mimicking the aggressor, causes a complex cascade of signals, which will lead the plant to induce defense molecules and therefore mobilize its own means of defense [8].

In this study, we focused on host-pathogen interactions through pineapple natural defense systems stimulation in order to increase tolerance to FMS, responsible for fruitlet core rot disease (FCR). First, we performed pathogenicity tests to assess the virulence of the *Fusarium moniliforme* var. *subglutinans* (FMS) inoculum on pineapple plants. Second, we described the model of plant elicitation with biocontrols and investigated the plants associated of infection with FMS using two pineapple cultivars (Smooth cayenne and MD-2). Finally, biochemical analyzes of chlorophylls, carotenoids and lignin after inoculation were performed to assess methyl jasmonate and Calliete® as well as their combination effects on the pineapple protection

against FCR.

2. MATERIAL AND METHODS

2.1. Plant material

The plant material used for this study consisted of pineapple shoots of the Smoot Cayenne and MD2 varieties. The suckers of these both varieties, whose mass is between 350 and 500g, were collected from the farmer's pineapple plantations at Bonoua (Côte d'Ivoire). These cultivars show a very high level of susceptibility to FCR disease caused by FMS.

2.2. Fungal material

The fungus *Fusarium moniliforme*, the causal agent of FCR, was used for inoculations. It was isolated from necrotic areas of pineapple pulp harvested in a plantation at Bonoua, an intensive pineapple production area in Côte d'Ivoire.

2.3. Experimental design

The experimental design was consisted of two blocks, A (MD2) and B (Smooth Cayenne), with 5 m apart. Block A was composed of three plots (A, B, C) and block B four plots (A, B, C and D). These plots were spaced 2 m apart. Each plot contained ridges 6 m long and 1 m wide, spaced 1 m apart. Each ridge consisted of two rows of 10 pineapple plants separated by 50 cm. Experimental plots (A, B, C) were made up of 10 ridges and 4 ridges for plot D (**Fig 1**), where PNTNI is plant not treated with an elicitor and not infected by the fungal strain, PNTI is plant not treated with an elicitor and infected by the fungal strain, PTMI is Methyl jasmonate-treated plant infected with the fungal strain, PTCaI is Calliote-treated plant infected with the fungal strain and PTM+CaI is plant treated with Methyl jasmonate and Calliote combination but uninfected by the fungus.

2.4. Fungi cultivation

Fusarium moniliforme strains were isolated from black spots on pineapple fruits showing characteristic symptoms. Under a laminar flow hood, samples collected were placed in Petri dishes containing PDA (Potato Dextrose Agar) medium supplemented with 20 g/L potato mash, 20 g/L D- glucose and 20 g/L agar. Subculturing of *F. moniliforme* was carried out according to the method described by Vakili[9]. Under a hood, agar fragments of about 0.5 cm in diameter containing the fungus were taken from the Petri dishes. They were then transferred to a new PDA medium of identical composition to the previous one. The medium was sterilized in autoclave at 120 °C for 30 min. The inoculated Petri dishes were incubated for 14 days in a culture room at a temperature of 25 ± 2 °C under a 12 h photoperiod. Identification of *Fusarium moniliforme* was performed according the key of Botton [10] based on macroscopic and microscopic observations.

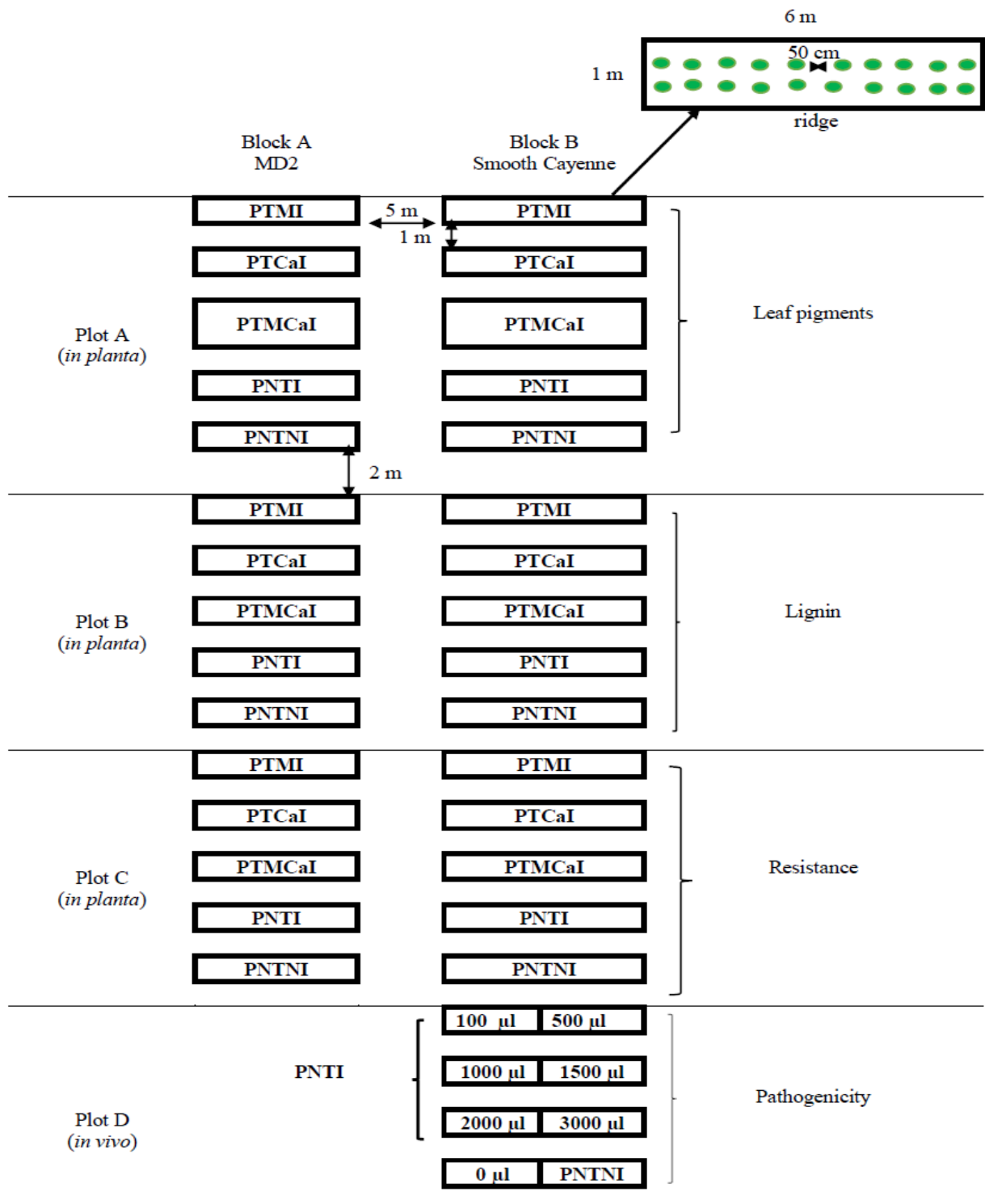


Figure 1: Experimental design

2.5. Preparation of pineapple plants for inoculation

Smooth Cayenne plants were used in this study because this variety was the most sensitive to *F. moniliforme*. The rejects were washed with tap water and disinfected by soaking in a solution containing a mixture of Chlorpyrifos® 0.03 %; Mefenoxan® 0.02 %; Carbosulfan® 0.03 %; ethanol 70 % and bleach 2.5 %, for 30 min. After rinsing three times with tap water. The seedlings were planted in polyethylene bags containing 3000 cm³ of soil from the experimental site of Nangui Abrogoua University, previously autoclaved at 121°C for 45 min. The plants were then kept for 13 months in a greenhouse until the end of the fruiting period, where the light and temperature conditions were close to natural conditions (60 to 70 % humidity, 27 to 28 °C during the day and 23 to 25 °C at night). The experiment was triplicate.

2.6. Preparation of the fungal inoculum

Agar fragments of 14-day-old *F. moniliforme* were taken from the previous culture medium and ground in the presence of 5 ml of sterile distilled water under a laminar flow hood. The ground material was filtered through sterile gauze which retains the mycelial fragments and allows the spores to pass. About 1 ml of filtrate was placed on each Petri dish containing PDA medium and incubated for seven days under the same conditions as above. Then, the resulting *F. moniliforme* colonies were 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 avoids possible morphological variations. The concentration of the inoculum is determined using a Malassez cell and then adjusted to 10⁴ spores/ml.

2.7. Fungal inoculation of pineapple plants

The virulence of *F. moniliforme* was demonstrated by inoculating pineapple plants at the floral stage (after 8 month of planting) of their development cycle, in a greenhouse. Wounds were made on the flowers at eyes level and in the intermediate areas of the eyes, by punctures with a sterile needle connected to a micropipette. To investigate the dose of fungal inoculum having the capacity to induce disease, inoculum volumes of 100, 500, 1000, 1500, 2000 and 3000 µl were deposited inside each blooming pineapple flower whose D-leaf have not been treated with biocontrols or elicitors. Plants whose flowers were not inoculated were considered as controls. The symptoms of the disease caused by *F. moniliforme* were monitored by visual observations. The experiment was stopped when the fruits were mature.

The obtained fruits were cut to observe the development of the disease and to estimate the black spot rate. Ten Smooth Cayenne plants were used per inoculum volume and the experiment was

triplicate.

2.8. Re-isolation and identification of the fungus

To confirm the presence and virulence i.e. the pathogenicity of the fungal inoculum isolate, the pineapple plants used for pathogenicity test and which were showed symptoms of the disease, including the control (uninfected), were recovered for a re-isolation of the pathogen. Subculture of necrotic tissues in pineapple pulp was done in Petri dishes on PDA medium as before. After one week of incubation on PDA medium, identification of the fungal strain was performed using the key of Botton *et al.* [10].

2.9. Evaluation of the virulence of the fungal inoculum on elicited vivoplants

2.9.1. Study of the resistance to *Fusarium moniliforme* of elicited plants

The objective of this study was to evaluate *in situ* the tolerance of pineapple elicited-plants (Smooth Cayenne and MD2) by methyl jasmonate and calliete® to black spot disease, following inoculation with the fungal strain of *F. moniliforme*.

2.9.2. *In situ* production of pineapple plants

Pineapple plants were produced from seedlings of the Smooth Cayenne and MD2 varieties from the Bonoua farmers' plantations. The shoots were first sorted according to size and then trimmed. Surface disinfection was carried out by soaking in a solution containing a mixture of insecticide (Chlorpyrifos® 0.03%), fungicide (Mefenoxan® 0.02%), bactericide and nematicide (Carbosulfan® 0.03%) for 30 min. After three rinses with sterile distilled water, the rejects were immersed in 70% ethanol for 5 min and then in 2.5% active chlorine bleach for 30 min. After three rinses with distilled water, seedlings were transferred to the experimental plot.

2.9.3. Elicitation of pineapple plants

Seven months after cultivation, pineapple D-leaves were sprayed with 50 ml of MeJA (10 mM/72 h), calliete® (5 mM / 48 h) and the MeJA/Ca combination (10 mM and 5 mM / 72 h). The pineapple D-leaves not sprayed with the elicitors were used as controls. For each variety and for each treatment 10 plants were used. This experiment was triplicate.

2.9.4. Inoculation of pineapple elicited-plants

After elicitation of pineapple D-leaves, floral induction treatment (FIT) was performed to stimulate flowering. Forty-five days after FIT, flowers were inoculated with 2.5 mL of *F.*

moniliforme inoculum at 104 ml/spore. Inoculation was carried out by microinjection into the eyes and/or in the intermediate eye areas using a sterile needle and micropipette. Experiment was triplicate. Five treatments modality groups were constituted:

- plant not treated with an elicitor and not infected by the fungal strain (PNTNI);
- plant not treated with an elicitor and infected by the fungal strain (PNTI);
- Methyl jasmonate-treated plant infected with the fungal strain (PTMI);
- Calliete-treated plant infected with the fungal strain (PTCaI);
- (Methyl jasmonate + Calliete)-traeted plant and uninfected (PTM+CaI).

2.9.5. Protection of pineapple elicited-plants against fruitlet core rot disease

To evaluate the effect of exogenous application of elicitors on plants inoculated with *Fusarium moniliforme*, three parameters were measured. These were the rate of black spots in the fruits, the content of leaf pigments (carotenoids, chlorophyll) and the rate of lignin.

2.9.6. Fruitlet core rot or Black spot rate in the fruit

After 12 months of culture, the fruits from treated and non-treated with biocontrols plants were harvested and peeled. Then, a longitudinal section and several horizontal sections of 2 cm thickness were made. For each of the treatment modalities described above, the number of black spots was counted. Then, the protection of the pineapple after treatment by elicitors was evaluated by the rate of black spots in the fruits according to the following formula:

2.9.7. Black spot rate in the fruit

After 12 months of culture, the fruits from treated and non-treated with biocontrols plants were harvested and peeled. Then, a longitudinal section and several horizontal sections of 2 cm thickness were made. For each of the treatment modalities described above, the number of black spots was counted. Then, the protection of the pineapple after treatment by elicitors was evaluated by the rate of black spots in the fruits according to the following formula:

Black spot rate = [number of contaminated eyes in fruit/number of total eyes in fruit] x 100

Then, the intensity of the disease was estimated through a five-level severity scale:

Degree 0: no black spots in the fruit;

Degree 1: 1-20% of eyes are spotted in the fruit;

Degree 2: 20-40% of eyes are spotted in the fruit;

Degree 3: 40-60% of eyes are spotted in the fruit;

Degree 4: 60-80% of eyes are spotted in the fruit;

Degree 5: 80-100% of eyes are spotted in the fruit.

2.9.8. Extraction and determination of leaf pigments

Extraction of chlorophyll pigments and carotenoids was carried out according to the method described by [11], modified and adopted to our plant material. Thus, for each treatment, 200 mg of D leaves were incubated in the dark, at 4 °C in a test tube containing 10 mL of 20 % acetone for 48 h. The whole was ground in the presence of a pinch of calcium carbonate and Fontainebleau and then centrifuged at 5,000 rpm for 15 min at 4 °C. The supernatant obtained was the crude leaf pigment extract. Untreated and inoculated or uninoculated plants served as a control.

The determination of leaf pigments was carried out according to [12]. The optical density (DO) was measured with a spectrophotometer at 663 nm and 647 nm for chlorophyll and 470 nm for carotenoids, against a control sample made with acetone. The chlorophyll a, b and total content of the leaves (mg/g FM) is then calculated using the following formula:

$$\text{Chlorophyll a} = ((12.25 \times \text{DO } 663) - (2.79 \times \text{DO } 647) \times V) / 1000 \text{ m}$$

$$\text{Chlorophyll b} = ((21.5 \times \text{DO } 647) - (5.1 \times \text{DO } 663) \times V) / 1000 \text{ m}$$

$$\text{Total Chlorophyll} = ((7.15 \times \text{DO } 663) + (18.71 \times \text{DO } 647) \times V) / 1000 \text{ m}$$

$$\text{Carotenoid} = [(1000 \times \text{DO } 470) - (1.82 \times \text{Chl a} - 85.02 \times \text{Chlb}) / 198] \times V / 1000 \text{ m}$$

Where m is mass of ground leaf, V is final volume of extract, FM is fresh matter, Chl is chlorophyll, Car is carotenoids and DO is optical density.

2.9.9. Extraction and determination of lignin

The aim was to evaluate the impact of elicitor on lignification in D leaves, stems, roots and flowers. For each treatment modality, ten pineapple plants were used. Samples were collected on day 60 after inoculation with the fungal strain. The experiment was triplicate.

Lignin extraction was performed according to the modified method of Ellis [13]. The collected

samples were lyophilized and then, ground in a mortar. Then, 10 g of powder from each sample was weighed and homogenized in an Erlenmeyer flask containing 20 mL of 96 % ethanol. The mixture was boiled under magnetic stirring for 30 min. The ethanol was drained and replaced with 25 mL of distilled water. The organic solvent was used to remove simple phenolic acids and polyphenols while the aqueous solvent was used to remove water-soluble compounds such as proteins, tannins and polar molecules. This step was successively repeated twice with ethanol and once with water. The samples were collected, and oven dried for 18 h at 105 °C.

The assay was also carried out according to the method described by Ellis[13]. Thus, 0.2 g of powder of each sample was weighed (mass of the test sample) and then homogenized in 5 mL of 72 % sulfuric acid contained in a crucible previously placed in the oven and weighed after cooling. The mixture was placed on a magnetic stirrer at room temperature for 2 h. After homogenization, 115 mL of distilled water was added to each crucible to perform hydrolysis with 3 % sulfuric acid. The crucible was then autoclaved at 120 °C for 1 h to induce the bursting of the maximum number of cells to release the lignin from the cells. The contents of each crucible were filtered, and the pellet was washed several times with distilled water to remove the cellulose retained in the wood fibers. After washing, the pellet was dried in an oven at 105 °C for 18 h, then put back into the crucibles and weighed (crucible mass with residue). The lignin content was expressed as a percentage and calculated according to the formula:

$$\text{Lignin} = [\text{Residue mass} / \text{Set mass}] \times 100$$

where, Residue mass = Crucible mass with residue - Anhydrous crucible mass.

2.9.10. Statistical analysis

Statistical analyses were performed using Statistica 7.1 software. An analysis of variance (ANOVA) was performed on all treatments applied. When this analysis showed a difference between the means, Duncan's test was carried out to determine the significant differences between the treatments at the 5 % level. For data in percentages, the Kruskal-Wallis test was used to determine significant differences ($P < 0.05$) between treatments.

3. RESULTS

3.1. Evaluation of the pathogenicity test of *F. moniliforme* on the Smooth Cayenne plants

The results of the pathogenicity tests for *F. moniliforme* are given in TABLE1. All the quantities of inoculum used (0, 100, 500, 1000, 1500, 2000 and 3000 µl) did not induce black spots in the fruit (Fig 2). Indeed, no symptoms were observed in the fruits of plants inoculated with 0 and 100

µl of spore suspension. Symptoms of fruitlet core rot or black spot disease appeared from 500 µl. Eye discoloration, gum exudation, leaf yellowing and taste alteration were observed from 1500, 2000 and 3000 µl, respectively. However, the plant became diseased from 1000 µl of spores. The doses of 2000 and 3000 µl of *F. moniliforme* inoculum were more pathogenic. Black spots were the characteristic symptoms of *Fusarium moniliforme* manifestation. Control plants showed no symptoms of the disease.

Table 1: Pathogenicity test of *Fusarium moniliforme* inocula

Quantity of inoculum (ml)	Black spots in the fruit	Discoloration of the eyes	Exudation of a gum	Yellowing Of the leaf	Taste alteration	Plant health status
0	-	-	-	-	n	Not sick
100	-	-	-	-	n	Not sick
500	+	-	-	-	n	Not sick
1000	++	-	-	-	o	Sick
1500	+++	+	-	-	o	Sick
2000	++++	++	++	-	o	Sick
3000	+++++	+++	+++	+	o	Sick

(-): absence; (+): presence; (n): no; (o): yes;

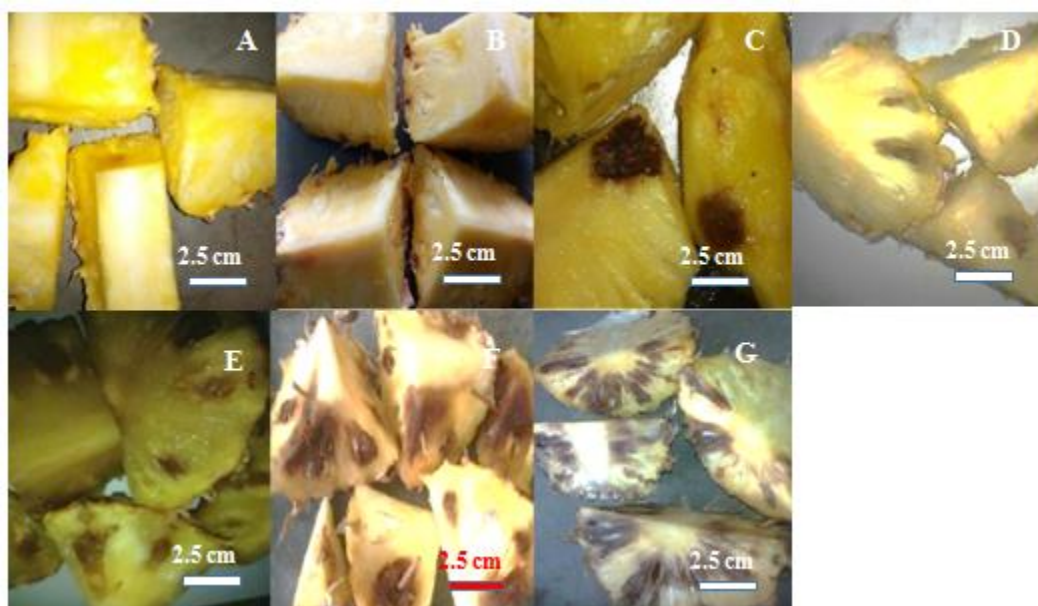


Figure 2: Internal fruit symptoms of Smooth Cayenne at harvest after inoculation with different amounts of *Fusarium moniliforme* inoculum

A: control; B: 100 µl inoculum; C: 500 µl inoculum; D: 1000 µl inoculum; E: 1500 µl inoculum; F: 2000 µl inoculum; G: 3000 µl inoculum of *F. moniliforme*

3.2. Re-isolation and identification of the fungus

Agar fragments obtained from pineapple black spot tissues (Fig 2) previously cultured on PDA medium were replicated again. After two weeks of incubation, the inoculated tissues developed fungal mycelia on the PDA medium (Fig 3). The result showed a white to peach thallus fungus with dense and flaky aerial mycelium with a powdery appearance. The microconidia are arranged in chains, and are spindle-shaped, unicellular or sometimes uniseptate. Macroconidia are rare, spindle-shaped and septate. There are no chlamydospores (Fig 3). After characterization, the key of Botton *et al.* [10] identified the isolated fungal strain as *Fusarium moniliforme* and variety var. *subglutinans* the causal agent of pineapple fruitlet core rot disease.

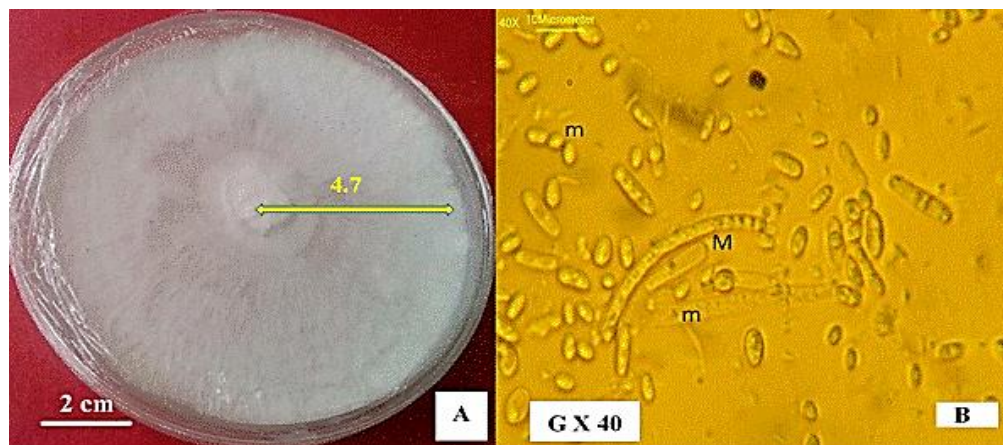


Figure 3: Morphological description of a strain of *Fusarium moniliforme* extracted from infected pineapple pulp

A: macroscopic appearance; B: microscopic appearance; m: microconidia; M: macroconidia; my: mycelium

3.3. Evaluation of the severity index or degree of disease in fruit

The evaluation of the severity or degree of the disease was reported in **TABLE 2**. The results show that the disease severity index differs according to the type of cultivar (**Fig 4**). Indeed, untreated and inoculated plants (PNTI) showed the highest levels of contamination, 4 for MD2 against 5 for Cayenne. Untreated and uninfected plants (PNTNI) and those of plants co-treated with MeJA and Ca (PTMCAI) showed no symptoms of the disease regardless of variety. Plants treated with MeJA and infected (PTMI) had a severity index of level 1 for each cultivar, while those treated with Calliete® and then infected (PTCAI) had a severity index of level 1 for MD2

and a level 2 for Smooth Cayenne.

Table 2: Disease severity index of *Fusarium moniliforme*

	Disease severity index				
	PNTNI	PNTI	PTCaI	PTMI	PTM+CaI
MD2	0	4	1	1	0
Smooth Cayenne	0	5	2	1	0

plant not treated with an elicitor and not infected by the fungal strain (PNTNI); plant not treated with an elicitor and infected by the fungal strain (PNTI); Methyl jasmonate-treated plant infected with the fungal strain (PTMI); Calliete-treated plant infected with the fungal strain (PTCaI); plant treated with Methyl jasmonate + Calliete and uninfected (PTM+CaI).

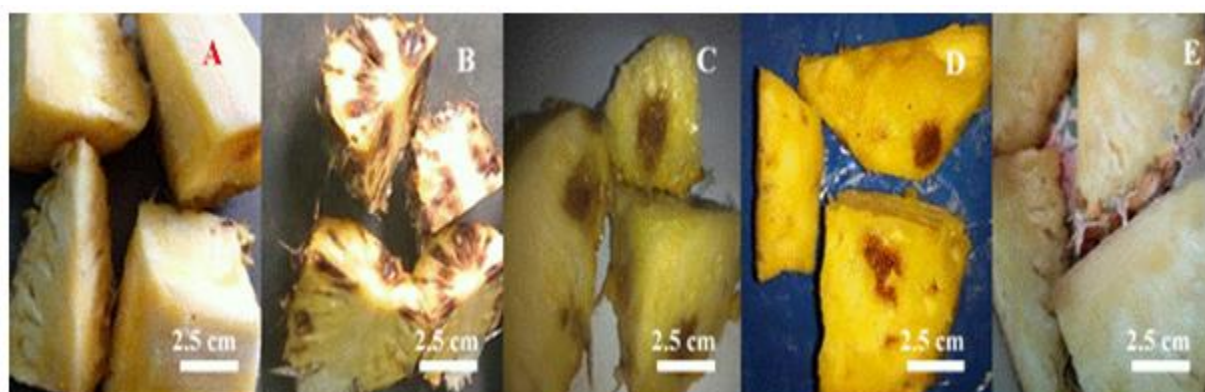


Figure 4: Pulp appearance of Smooth Cayenne pineapple from plants treated with biocontrols and then inoculated with *F. moniliforme*

MeJA: methyl jasmonate; A: untreated and non-inoculated plant; B: untreated and inoculated plant; C: plant treated with calliete® and inoculated; D: plant treated with MeJA and inoculated; E: plant treated with the MeJA and calliete® combination and then inoculated

3.4. Effect of biocontrols on leaf pigment content of elicited pineapple plants

The physiological status of the plants was assessed through the content of leaf pigments. Thus, chlorophylls (a, b and total) and carotenoid contents in D-leaves harvested 60 days after fungal inoculation were evaluated. The analysis of the results recorded in **TABLE 3**, show that the chlorophylls content varies according biocontrols and pineapple variety. Thus, these results reveal that in both varieties (MD2 and Smooth Cayenne), chlorophyll a and total chlorophyll contents increase with the exogenous application of biocontrols compared to the control

(PNTNI). However, chlorophyll a and total chlorophyll levels were higher in MD-2 than in Smooth Cayenne, regardless of the applied treatment. In these two varieties, the methyl jasmonate (MeJA) and Calliete (Ca) co-treatment (PTM+Ca) induced the highest chlorophyll contents (Chl a and Chl total), followed by MeJA (PTMI) and Calliete® (PTCaI) treatment. As for the chlorophyll b content, the results show that it decreases in the presence of elicitors in MD-2 while it increases in the presence of elicitors in Smooth Cayenne. On the other hand, carotenoid levels showed no significant difference with the application of biocontrols compared to the control (PNTNI: 0.27 mg/g FM) but PNTI induce a significant decrease (0.21 mg/g FM).

Smooth Cayenne variety has evolved in the same direction as the MD-2 variety. Thus, the chlorophyll a and total chlorophyll content is higher in biocontrols-treated and inoculated plants than in untreated and uninoculated plants. Chlorophyll content varied with treatment. However, chlorophyll content was higher in MeJA-treated and inoculated plants (PTMI: 0.69 mg/g FM) than in Calliete®-treated and inoculated plants (PTCaI: 0.58 mg/g FM), but their joint effects (PTM+CaI: 0.93 mg/g FM) increased more than when they were treated alone. No significant difference was observed on chlorophyll b content between PTMI (0.25 mg/g FM) and PTCaI (0.22 mg/g FM). On the other hand, their additive effects induced the increase of chlorophyll b content (0.32 mg/g FM). Total chlorophyll was higher with the exogenous application of the stimulators especially in PTMI: 0.94 mg/g MF; PTCaI: 0.81 mg/g FM; PTM+CaI: 1.25 mg/g FM compared to the control (PNTNI: 0.61 mg/g FM). The biocontrols do not show a significant effect on the accumulation of carotenoid levels. With PNTI, chlorophyll a, b, total and carotenoids were low amount compared to controls.

Table 3: Effect of exogenous application of elicitors on chlorophyll a and b and carotenoid content of D-leaves of pineapple plants 60 days after inoculation with *Fusarium moniliforme*

Cultivar	Chlorophyll and carotenoid contents (mg/g FM)					
	PNTNI	PTNI	PTMI	PTCaI	PTM+CaI	
MD2	Chl a	0.70 ± 0.02d	0.28 ± 0.01e	0.92 ± 0.05b	0.83 ± 0.08c	0.98 ± 0.05a
	Chl b	0.26 ± 0.01b	0.40 ± 0.05a	0.20 ± 0.01c	0.18 ± 0.02c	0.11 ± 0.10d
	Chl t	0.96 ± 0.09d	0.68 ± 0.07e	1.12 ± 0.05b	1.02 ± 0.01c	1.20 ± 0.07a
	Car	0.27 ± 0.10a	0.21 ± 0.01b	0.29 ± 0.03a	0.28 ± 0.05a	0.28 ± 0.03a
	Chl a	0.43 ± 0.04d	0.05 ± 0.02e	0.69 ± 0.08b	0.58 ± 0.16c	0.93 ± 0.08a
Smooth Cayenne	Chl b	0.17 ± 0.06c	0.10 ± 0.09d	0.25 ± 0.01b	0.22 ± 0.01b	0.32 ± 0.01a
	Chl t	0.61 ± 0.04d	0.15 ± 0.01e	0.94 ± 0.03b	0.81 ± 0.05c	1.25 ± 0.04a
	Car	0.17 ± 0.02b	0.11 ± 0.05b	0.19 ± 0.02a	0.17 ± 0.01a	0.20 ± 0.01a

Values represent the mean of three replicates; ±S: standard deviation; within the same line, values followed by the

sameletter are not significantly different (Newman-Keuls test at 5%). PNTNI: untreated plants not inoculated with *Fusarium moniliforme*; PNTI: untreated plants inoculated with *F. moniliforme*; PTMI: plants treated with MeJA and inoculated with *F. moniliforme*; PTCaI: plants treated with calliete® and inoculated with *F. moniliforme*; PTM+CaI: plants treated with Methyl jasmonate + Calliete and uninfected. Chla: chlorophyll a;Chlb: chlorophyll b; Chlt: total chlorophyll; Car: carotenoid; FM: Fresh Mater.

3.5. Effect of biocontrols on the lignin content of treated pineapple plants

The results show that treatment of pineapple plants with biocontrols has a positive impact on lignin synthesis in roots, stem, D-leaf and flowers of the cultivars. However, the roots produced the highest levels of lignin regardless of treatment and cultivar followed by the stem, D-leaf and finally the flowers. Moreover, the plants treated with the combination of methyl jasmonate and calliete® and inoculated (PTCa+MI) showed the highest lignin content(95 %) in Smooth Cayenne and 97.01 % in MD-2 in the roots followed by plants treated with methyl jasmonate and inoculated (PTMI) with a rate of 75.50 % in Smooth Cayenne and81.41 % in MD-2. On the others hand, plants treated with Calliete® and inoculated (PTCaI) recorded the lowest lignin content 30.50 % in Smooth Cayenne and 36.03 % in MD-2. As for the untreated and non-inoculated plants (PNTNI), the lignin content was 14.30 % in Smooth Cayenne and 15.25 % in MD-2. Untreated and inoculated seedlings (PNTI) induced 7.01 and 08.54 % in Smooth Cayenne and MD-2, respectively (Tab4).

Table 4: Effect of exogenous application of elicitors on lignin content in roots, stem, D-leaves and flower of pineapple plants 60 days after inoculation with *Fusarium moniliforme*

Cultivar		Chlorophyll and carotenoid contents (mg/g FM)				
		PNTNI	PNTI	PTMI	PTCaI	PTM+CaI
MD2	Root	15.25 ±0.01d	08.54 ±0.08e	36.03 ±0.02c	81.41 ±0.04b	97.01 ±0.02a
	Stem	11.32 ±0.02d	06.18 ±0.01e	30.70 ±0.03c	76.02 ±0.01b	86.10 ±0.09a
	Sheet	09.02 ±0.04d	03.01 ±0.02e	24.32 ±0.05c	55.12 ±0.05b	77.20 ±0.05a
	Flower	07.50 ±0.02d	02.21 ±0.05e	20.44 ±0.01c	38.06 ±0.03b	68.04 ±0.04a
Smooth Cayenne	Root	14.30 ±0.03d	07.01 ±0.07e	30.50 ±0.08c	75.50 ±0.06b	95.03 ±0.03a
	Stem	10.45 ±0.04d	04.48 ±0.06e	21.80 ±0.01c	72.22 ±0.08b	83.16 ±0.04a
	Sheet	08.69 ±0.08d	02.70 ±0.02e	20.04 ±0.04c	30.54 ±0.02b	72.01 ±0.01a
	Flower	08.17 ±0.01d	02.14 ±0.05e	10.08 ±0.01c	21.60 ±0.01b	58.60 ±0.03a

Values represent the mean of three replicates; ± S: standard deviation; within the same line, values followed by the same letter are not significantly different (5 % Kruskal-wallis test). MeJA: methyl jasmonate; PNTNI: untreated plants not inoculated with *F. moniliforme*; PNTI: untreated plants inoculated with *F. moniliforme*; PTMI: MeJA-treated plants inoculated with *F. moniliforme*; PTCaI: calliet®-treated plants inoculated with *F. moniliforme*

4. DISCUSSION

Purification of the fungal isolate revealed the presence of only one morphotype among the progeny. The whitish, cottony appearance of the mycelium and the presence of isolated or chain-like microconidia, macroconidia and the absence of chlamydospores would indicate that the purified fungal isolate is of the *Fusarium moniliforme* type [14]. The plants used for the pathogenicity test including the control were recovered for re-isolation of the pathogen. Only vivoplants inoculated with the pathogen showed disease symptoms. This result was confirmation of the presence and virulence of the inoculated isolate. Thus, the relationship between the inoculum and the occurrence of black spot disease could be established verifying Koch's postulate [15]. Based on the appearance and color of the developed mycelium, the fungus strain reisolated in the black spot of pineapple pulp showed no morphological variation with that of the parent crop. Microscopic observation revealed that the isolated strain did not exhibit chlamydospores but produced microconidia and macroconidia of the same morphotype as those of the purified strain. In addition, the fact that the fungus isolate was able to cause the disease in inoculated pineapple *in vitro* plants would be evidence that *Fusarium moniliforme* is indeed responsible for fruitlet core rot or black spot disease [16; 17]. However, subglutinans variety is the one that is pathogenic for the pineapple in Côte d'Ivoire [18; 19; 20]. Thus, the pathogenic fungus responsible for pineapple fruitlet core rot disease (FCR) in Côte d'Ivoire would be *Fusarium moniliforme* var. subglutinans.

The severity of the disease induced mainly in pineapple fruits by *Fusarium moniliforme* var. subglutinans (FMS) was measured on untreated and uninoculated plants (PNTNI), elicitor-treated and inoculated plants (PNTI), MeJA-treated and inoculated plants (PTMI), Calliete®-treated and inoculated plants (PTCaI), and MeJA-treated and Calliete®-treated and inoculated plants (PTM+CaI). Elicitation of the plants showed that these biocontrols promote protection against fruitlet core rot disease caused by FMS. Moreover, the low rate of black spot ($\leq 10\%$), suggests that MeJA-treated pineapples can be considered 90 % tolerant to the pathogenic form of FMS for both varieties (MD-2 and Smooth Cayenne) while those treated with Calliete® were considered 78 % resistant to the pathogen for the Smooth Cayenne cultivar and 85 % for MD-2. Indeed, the pathogenicity test of a fungal isolate is valid when the black spot rate of the plants exceeds 20 % [21]. Our results are similar to those reported by Belhadj *et al.* [22] on resistance to *Erysiphe necator*, the causal agent of powdery mildew in grapevine. These authors showed that plants treated with MeJA developed increased resistance against the disease. In addition, Konan *et al.* [23] reported induced disease resistance to *Fusarium oxysporum* in cotton treated with MeJA.

Then, Natali [24] also showed the role of Calliete® against *Phytophthora*, the downy mildew

disease in pineapple. Thus, the treatment with stimulators allowed a considerable reduction of the intensity of the fungal attack and an effective resistance against the pathogens. Plants co-treated with MeJA and Calliete® did not show black spots in the fruits. This suggests 100 % resistance to FMS. This treatment was therefore effective against FMS-induced FCR in pineapple. The co-treatment seems to provide a complementary effect by increasing the efficacy of MeJA applied alone. Indeed, the action of MeJA would be enhanced by co-treatment with Calliete® *via* ethephon (ethylen) as already reported by several studies [25; 26; 27]. According to Natali[24], fosethyl-aluminium, the active molecule of Calliete®, induces the production of defense molecules by ethylene pathway. Thus, ethylene in the form of ethephon improved the increase in tolerance to FMS induced by methyl jasmonate in pineapple. The role of co-treatment of MeJA and Calliete® *via* ethephon in plant defense had already been reported in many plants as activator, inducer and accumulator of phytoalexins and PR proteins. Similarly, protective effect of biocontrol against pathogens has already been demonstrated in several plants [28; 27; 29; 30].

Furthermore, under the action of FMS, it was observed in both cultivars a degradation of leaf pigments (Chlorophyll a, carotenoids and total Chlorophyll) with the exception of Chlorophyll b, which increased in cultivar MD-2. However, MeJA application induces an increase in Chlorophyll a and total chlorophyll. The increase in total chlorophyll content was only due to chlorophyll a. Indeed, MeJA, would induce a bioconversion of Chlorophyll b into Chlorophyll a thus increasing the content of D-leaves in Chlorophyll a and thus a good photosynthetic activity [31; 32]. Application of biocontrols in pineapple has a beneficial action on the synthesis of secondary metabolites such as Chlorophyll a. It is a good indicator of the vitality or vigor of the plants. Indeed, chlorophyll a is the main photosynthetic pigment. It allows the conversion of light energy into chemical energy (in the form of ATP) while the other pigments (chlorophyll (b) and carotenoids that transmit energy to it by resonance are called accessory pigments [33;34; 35]. Moreover, Couderchet *et al.* [36] reported a close relationship between PR proteins, induced phytoalexins and protoporphyrin oxidase, an enzyme involved in chlorophyll synthesis. Yet, MeJA has been shown to stimulate the production of phenolic compounds as well as PR proteins [37;38; 39]. This could also explain the significant increase in chlorophyll a contents observed in MeJA- and Calliete-elicited susceptible seedlings compared to non-elicited ones. This result suggests that the increase in chlorophyll caused good photosynthetic activity in MeJA and Calliete® treated plants. Thus, the induction of the antifungal molecules PR proteins and phytoalexins, correlated with the increase in chlorophyll a levels under the action of MeJA and Calliete® seems to be the basis for the good physiological condition and tolerance of pineapple plants.

The decrease in chlorophyll (Chl a and Chl total) content in the PNTI showed that the inoculated

fungus strain destroys or degrades these chlorophylls. Indeed, the plant needs sugars to ensure its growth, its development and the implementation of defense mechanisms. Sugars are synthesized in the leaves and then transported *via* the stem to the plant's sink organs (roots, flowers, leaves, fruits). For its part, the fungus, which is heterotrophic for carbon, draws sugars from the tissues it infects. There is therefore competition for carbon resources. The pathogen can then represent a new sink that will divert assimilate flows within the plant to its own benefit. In this competition between the plant and the fungus, the mobilization of sugars is a major issue for both adversaries, the pineapple plant trying to defend itself and the fungus to develop [40; 23]. The plant will not be able to feed properly, loses its vigor and becomes susceptible to attack. In addition, *Fusarium moniliforme* blocks the activation of magnesium chelatase and chlorophyllase, enzymes involved in chlorophyll biosynthesis [41]. As for carotenoids, the content does not change between plants treated and plants not treated with elicitors. This could be explained by the fact that photosynthesis does not depend on the carotenoid content [42].

The evaluation of the effect of biocontrols on the reinforcement of the cell walls of plants inoculated with *F. moniliforme* through the quantification of lignins in roots, stems and leaves on the 60th day after fungus inoculation, showed an increase in their level in treated and inoculated plants compared to controls. These results suggest that lignins are essential constituents of any xylem vessels because they are the basis of the rigidity of organs [43; 23; 35]. In fact, it is deposited in the wall of plant cells, preventing the pathogen from entering the cell. Lignin participates in the formation of the first barriers against the invasion of pathogens into the cell to induce disease. Thus, a high level of lignins would reduce the chances of a pathogen to penetrate a plant [13]. To this effect, the work of some researchers reported that the strategy implemented by the plant in case of attack against any biotic or abiotic aggressor as soon as possible and which is common to all plants is the reinforcement of the cell wall [8]. This technique aims to prevent the entry of the aggressor into the cells by thickening the wall at the site of pathogen penetration. Kauffmann *et al.* [44] also approved that the plant synthesizes several molecules such as proteins, polysaccharides or aromatic polymers to reinforce the wall after an infection. Moreover, many authors have shown that these modifications occur at several levels and involve several substances that vary from plant to plant [45; 46]. Lignin content was correlated with the frequency of pathogen infection [47]. Lignin participates in plant defense mechanisms by making the cell wall rigid and impermeable, and protecting plants from microbial invasion [48]. Studies have proven the involvement of biocontrols including MeJA in lignin biosynthesis [49; 50; 51].

Furthermore, results also showed high levels of lignins in roots, followed by stems, leaves and flowers in all treatments. Roots are the first organs to come into contact with *Fusarium moniliforme* var. *subglutinans* (FMS) because it survives well on plant debris deposited on the

soil surface or slightly buried [52; 53]. Thus, a high lignin content in the roots will strengthen the cell walls and slow the penetration of the fungus into the plant. In this case, being heterotrophic, FMS becomes a parasite of the aerial parts and was dispersed by wind and insects [2]. Thus, the application of elicitors would be beneficial to the plant in order to carry out a generalized counter-attack. The results also showed a low lignin content in the PNTI. This shows that the presence of the fungus in the plant negatively influences the production and metabolism of lignins. Indeed, Hüchelhoven [54] reported that blocking lignin biosynthesis enzymes with inhibitors decreased lignin content and favored pathogen penetration. Therefore, the increase in lignin level that acts as a physical barrier in the plant due to exogenous application of biocontrol on pineapple D-leaves would be necessary. The MeJA and Calliete® (ethephon) co-treatment or separately made it possible to increase the level synthesis of lignin in all the organs of the plant conferring complete tolerance to FMS [45; 13; 27].

5. CONCLUSION

The pathogenicity of *Fusarium moniliforme* var. *subglutinans* (FMS) on pineapple plants confirms the ability of biocontrols to induce natural defense mechanisms. In fact, the biocontrol treatment induced tolerance to FMS. However, the combination treatment of MeJA and Calliete® resulted in greater protection against FMS, the causative agent of fruitlet core rot disease or black spot disease in pineapple. Thus, there was a gain in resistance, which was distinguished by a total disappearance of the parasite pressure. This protection of pineapple through biocontrols was also manifested by a strengthening of the cell walls by an increasingly high lignification at the level of roots, stems, leaves and flowers. This treatment also stimulates photosynthetic activity for vigor plant growth and may therefore be a significant and promising alternative to chemical control of FMS. Its introduction in agricultural practices can contribute to the development of sustainable agriculture.

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