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Biocontrol of *Mycosphaerella fijiensis* Morelet, the Causal Agent of Black Sigatoka of Banana Tree (*Musa* spp.) Using Biopesticides in Côte d'Ivoire

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Abstract: Black Leaf Streak Disease (BLSD) is the most restricting leaf disease to banana tree cultivation around the world. In order to control this disease, synthetic fungicides are extensively used. However, these products pose a real danger to environmental pollution and the health of applicators and consumers. Faced with this situation, alternative solutions must be considered to overcome their systematic use. This study was initiated in this context so as to assess the effectiveness of 20 biopesticide formulations on *Mycosphaerella fijiensis* conidia stemming from banana tree leaf samples originating from village plantations and showing the typical symptoms of stage 2 or 3 black Sigatoka. The assessment method used was that of dispersion in solid medium. Observations were made under an optical microscope equipped with a camera and consisted in determining the inhibition rates of conidia germ tube growth. A pathogenicity test was performed with 8 *Mycosphaerella* spp. isolates according to an inoculation technique under controlled conditions on whole plants of 5 banana tree cultivar vivoplants. The assessment of biopesticide protection effectiveness against BLSD was conducted on cultivar "Orishele" (very susceptible) with the most aggressive and virulent strain selected during isolate pathogenicity test. The results obtained show that all biopesticide formulations have significant antifungal activity on *M. fijiensis* conidia germ tube elongation. The average inhibition rate ranged from 83.31 to 99.89% for all biopesticides. The 8 *M. fijiensis* isolates used have all raised symptoms characteristic of black leaf streak disease regardless of the cultivar. In contrast, no isolate caused symptoms characteristic of Sigatoka disease (Yellow Sigatoka). Biopesticides and synthetic fungicides significantly reduced disease development rate compared to inoculated and untreated controls, but at varying degrees. Preventive treatment of seedlings is found to be much more effective than curative treatment. However, in order to protect banana and plantain tree varieties against *M. fijiensis*, both types of treatments are necessary.

Keywords: Banana Trees, *Mycosphaerella spp.*, Biopesticides, Inoculation; Preventive, Curative

1. Introduction

Banana tree (*Musa* spp.) is a perennial monocotyledonous giant herb of the Musaceae family. Its cultivation is increasingly threatened by numerous biotic and abiotic constraints which considerably reduce its yield. Among the biotic constraints, fungal leaf diseases are the most damaging [1]. Black Sigatoka or black leaf streak disease (BLSD), caused by the ascomycete fungus *Mycosphaerella fijiensis* Morelet, is currently the major constraint in dessert banana and plantain tree yield [2, 3]. It causes leaf necrosis, leading

to yield losses, but also and above all, early fruit ripening [4].

Effective control of *Mycosphaerella fijiensis* pathogen requires the systemic application of contact fungicides and preventive and curative systemic fungicides from the benzimidazole, triazole and strobilurin family [5]. The intensive use of these fungicides, especially systemic fungicides, has resulted in the emergence and quick development of resistant strains [6] and strains more virulent than the original ones [7].

Faced with all these difficulties, many actions are being taken in order to prevent the development of resistance to fungicides in banana tree plantations so as to reduce their use and their effects on the environment and living organisms. Monitoring or tracking the effectiveness of the molecules used is therefore essential [8, 5]. Semi-annual monitoring is necessary in order to achieve a good assessment of molecule effectiveness. The use of biopesticides is an effective and less restrictive alternative [9, 10]. This study aims at promoting the use of 20 plant extract-based biofungicides from the Ivorian flora for black Sigatoka ecological management. The effectiveness of these biofungicides will be assessed both under *in vitro* cultivation conditions on *Mycosphaerella fijiensis* conidia germination and under controlled conditions on black Sigatoka symptom evolution.

2. Material and Methods

2.1. Material

2.1.1. Plant Material

Vivo plants of five (5) dessert banana and plantain tree cultivars were used for inoculation tests under controlled conditions (Table 1). These cultivars consisted of three (3) local cultivars ["Orishele" (AAB), very susceptible; "Corne 1" (AAB), susceptible and "Figue Sucrée" (AA) partially resistant] and two (2) tetraploid hybrids [PITA 3 (AAAB) and FHIA 21 (AAAB) of disease-tolerant cultivars].

Table 1. *Characteristics of dessert banana and plantain tree cultivars.*

Varieties	Cycle duration	Yield (t/ha)	Resistance to pathogens	Human consumption
Orishele	11 - 12 months	15 – 31.5	Very susceptible to leaf diseases	Foutou, Foufou, Alloco, Claclo, Akpecie, Chips
Corne 1	11 - 12 months	12 - 25	Susceptible to leaf diseases	Foutou, Foufou, Alloco, Claclo, Akpecie, Chips
Figue Sucrée	-	-	Susceptible to Cladosporiosis	Dessert
PITA 3	9 - 10 months	25	Resistant to leaf diseases	Foutou, Foufou, Alloco, Claclo, Akpecie, Chips
FHIA 21	10 - 12 months	40	Resistant to leaf diseases	Foutou, Foufou, Alloco, Claclo, Akpecie, Chips

Sources: [11]; -: Undetermined

2.1.2. Biopesticides Used for Biological Control

A total of twenty (20) biopesticides formulated by the Industrial Research Unit (URI) on biopesticides of the University Félix HOUPHOUËT-BOIGNY of Cocody (Côte d'Ivoire) were used in this study. These biopesticides were formulated from plant extracts of the Ivorian flora (Table 2). Biopesticide NECO 50 EC was used as a reference [10].

Table 2. *Biospesticides formulated and tested.*

Majority active ingredients (V/V)	Formulation name
Thymol and Eugenol (100)	NECO® (Reference)
Carvacrol and 1.8-Cineole (100)	NORDINE 50 EC
Geranial and Neral (100)	ASTOUN 50 EC
β-Caryophyllene and Sabinene (100)	DOCUS 50 EC
Citronellal and Citronellol (100)	FERCA 50 EC
1.8-Cineole and Terpineol (100)	TUSEL 50 EC
β-Caryophyllene and Sabinene / Citronellal and Citronellol (50/50)	TEBAYE 50 EC
β-Caryophyllene and Sabinene / 1.8-Cineole and Terpineol (50/50)	YESOLI 50 EC
β-Caryophyllene and Sabinene / Thymol and Eugenol (50/50)	RINEVES 50 EC
Thymol and Eugenol / Citronellal and Citronellol (50:50)	WACHET 50 EC
β-Caryophyllene and Sabinene / Carvacrol and 1.8-Cineole (50/50)	LOGUY 50 EC
Thymol and Eugenol / Carvacrol and 1.8-Cineole (50/50)	RHOSO 50 EC
Thymol and Eugenol / Geranial and Neral (50:50)	NOSTAG 50 EC
Carvacrol and 1.8-Cineole / 1.8-Cineole and Terpineol (50/50)	KAGNI 50 EC
Thymol and Eugenol / 1.8-Cineole and Terpineol (50/50)	HACADO 50 EC
β-Caryophyllene and Sabinene / Citronellal and Citronellol (25/75)	FIRCHE 50 EC
β-Caryophyllene and Sabinene / 1.8-Cineole and Terpineol (25/75)	SECARI 50 EC
β-Caryophyllene and Sabinene / Thymol and Eugenol (25/75)	REBRACI 50 EC
Thymol and Eugenol / Citronellal and Citronellol (25/75)	PRORALY 50 EC
Thymol and Eugenol / β-Caryophyllene and Sabinene (25/75)	VOGOH 50 EC
Thymol and Eugenol / 1.8-Cineole and Terpineol (25/75)	ARIMA 50 EC

2.1.3. Fungal Material

The fungal material consisted of *Mycosphaerella* spp. conidia and isolates, taken from the leaves of dessert and plantain banana trees from different production areas in Côte d'Ivoire. Eight (8) *M. fijiensis* isolates were used for

pathogenicity and susceptibility tests of local cultivars and hybrids (Table 3). They were chosen on the basis of their phenotypic and morphological characteristics.

Table 3. *Mycosphaerella spp. isolates used for pathogenicity tests.*

Isolate codes	Town of origin	Type of cultivar	Year of acquisition
AZEP6	Azaguié-Ahoua	Plantain	2014
AFEP18	Afouavame/Bondoukou	Plantain	2014
KGOD34	Promafolo/Korhogo	Dessert	2014
KDUP35	Koudougou/Bouaflé	Plantain	2014
BUID47	Baoubli/Duékoué	Dessert	2014
ZTAP57	Zatta/Yamoussoukro	Plantain	2014
BROD63	Broukro/Tiassalé	Dessert	2015
BCED65	Banacomoé/Abangourou	Dessert	2014

2.1.4. Synthetic Fungicides Used as Controls

The fungicides used at the approved doses were all unisite fungicides and belonged to three families (Table 4). These fungicides were chosen on the basis of their frequency of use in industrial banana tree plantations in Côte d'Ivoire.

Table 4. *Different fungicides used for Mycosphaerella spp. control in Côte d'Ivoire.*

Trade name	Families	Active ingredients	Recommended dose		Distributor
			g/l	ppm	
Tilt	Triazoles	Propiconazole	250	25 0000	Callivoire
Opal	Triazoles	Epoxiconazole	75	75 000	Tech Agro International
Junior	Triazoles	Tebuconazole	250	250 000	ALM Afrique de l'Ouest
Comet Plus	Morpholines +Strobilurins	Fenpropimorph +Pyraclostrobin	375 + 100	375 000 +100 000	Tech Agro International

2.2. Methods

2.2.1. Effect of Biopesticides on Mycosphaerella spp. Conidia Germination

(i). Culture Media Preparation and Amendment

The assessment method used was that of dispersion in a solid medium. For this purpose, an agar medium was used for the inhibition test of conidia germination by the formulated biopesticides. The effect of biopesticides was compared to that of four (4) synthetic fungicides (Tilt, Opal, Junior and Comet Plus) whose respective active ingredients are Propiconazole, Epoxiconazole, Tebuconazole and Fenpropimorph plus Pyraclostrobin. The agar medium was prepared with 20 g of Difco Bacto®. The volume was adjusted with distilled water to 1 L and then sterilized in an autoclave at 121°C under a pressure of 1 bar for 20 min. The medium was then cooled to a temperature of 45°C then amended with biopesticides or synthetic fungicides under magnetic stirring.

The concentrations 100, 200, 400, 600, 800 and 1000 ppm of biopesticides were selected for the comparative study of their effectiveness on the inhibition of *Mycosphaerella* spp. conidia germination. However, only one concentration of these biopesticides (100 ppm) was selected for comparison with synthetic fungicides (Propiconazole, Epoxiconazole and Tebuconazole at 0.1 ppm and Fenpropimorph + Pyraclostrobin at 5 ppm).

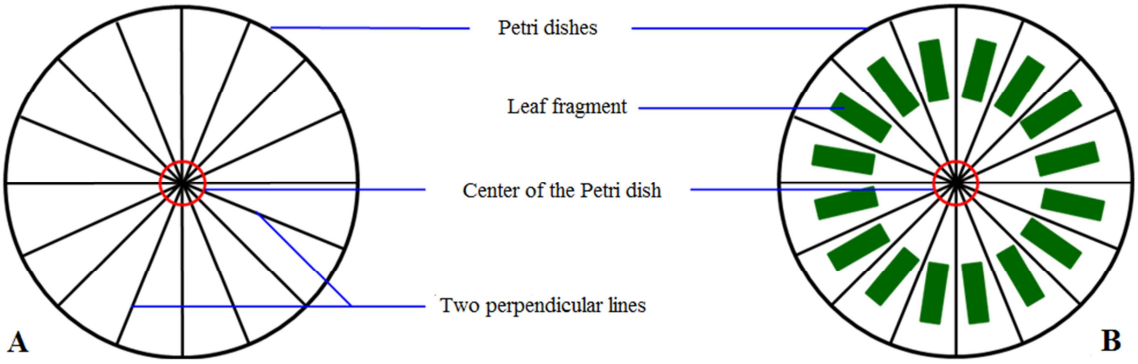
As for biopesticide combination, the concentration of 50 ppm was used to assess their synergistic effects. For this purpose, biopesticide dilutions were made from a stock solution with a concentration of 100 µl/ml. The different mixtures of biopesticides (v/v) were prepared from different volumes taken from the stock solution of each biopesticide tested. Each concentration of synthetic biopesticides and fungicides was emulsified in agar culture medium cooled to 45°C using a sterile micropipette.

The biopesticide was dispersed in the medium thanks to tween 20 which acts as surface active agent. Just twenty (20 ml) of amended medium was supplied per 90-mm diameter Petri dish. The effect of tween has been tested beforehand in order to ensure that it is harmless to the pathogen. For each concentration, three repetitions were performed.

(ii). Seeding of Conidia on Amended Culture Media

Eight (8) lines perpendicular to each other in pairs were drawn on the back of each Petri dish after solidification of the culture medium in sterile condition, their point of intersection indicating the center of the dish (Figure 1A). They made it possible to determine then 16 angular sectors. These Petri dishes containing 20 ml of the culture medium (agar-biopesticide or frozen synthetic fungicide) were seeded. In each angular sector, a piece of leaves showing symptoms of stage-2 Sigatoka was used at a rate of 16 sections per sample (Figure 1B). In control Petri dishes containing only the agar medium, conidia were seeded by depositing them in each angular sector. Thus, the underside of leaf fragments was applied to the surface of the culture medium freshly prepared and amended with biopesticide and/or synthetic fungicide or not. The leaf fragments were sixteen per 90-mm diameter Petri dish (Figure 1B). Two (2) Petri dishes were seeded per treatment (concentration and per biopesticide and/or per synthetic fungicide as well as for the control). Petri dishes were sealed with paraffin and incubated at 25 ±

2°C at 12-h photoperiod for 2 days (48 h).



A: Unseeded Petri dish; B: Seeded Petri dish

Figure 1. *Arrangement mode of leaf fragments in the Petri dish.*

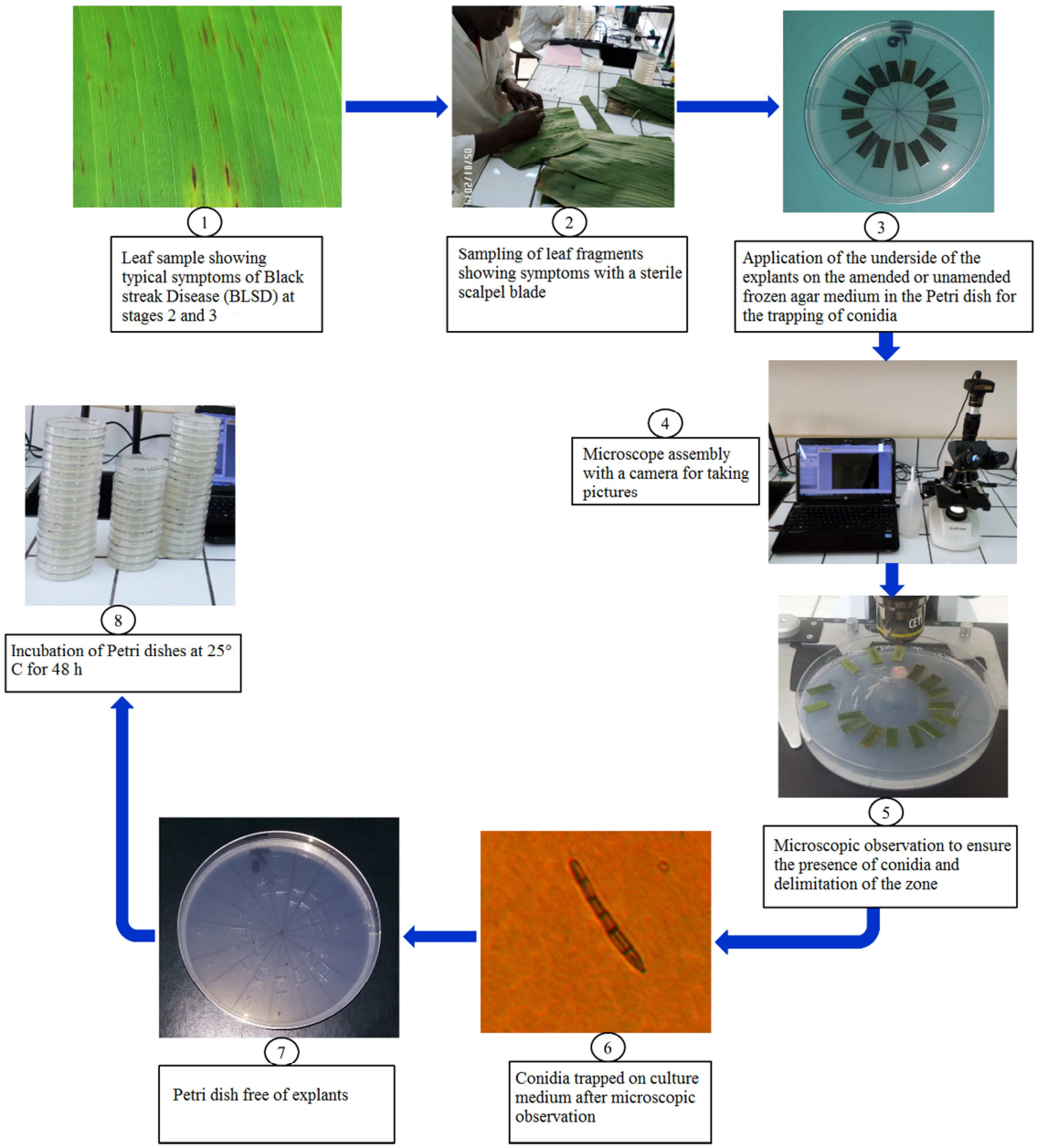


Figure 2. *Methods of trapping and bringing into germination Mycosphaerella spp. conidia on agar culture medium, amended or not.*

(iii). Determination of Biopesticide in vitro Antifungal Activity

The *in vitro* assessment of biopesticide antifungal activity was carried out on the basis of different parameters. These included inhibition rate (TI), growth rate (TC), IC_{50} and IC_{90} corresponding respectively to inhibiting concentrations at 50% and 90% of conidia germ tube growth. After 48 h of incubation of the seeded Petri dishes, the culture media were mounted on a microscope for observations. Microscopic photos of conidia germ tubes were taken at G x 10 magnification (Figure 2). Conidia germination measurements in micrometers were carried out following germ tube elongation using an optical microscope equipped with an "Amscope" brand camera and software which allowed the measurements to be made. A total of 32 views and measurements were performed for each concentration.

Germination inhibition rate and conidia growth rate of the treatments compared to the control were calculated respectively according to the formulas of Hmouni *et al*. [12]:

$$\text{Taux d'inhibition (TI en \%)} = \frac{(Lo-L)}{Lo} x\ 100$$

and

$$\text{Taux de croissance (TC en \%)} = 100 - TI$$

Where L_O: Average length of germ tubes (controls) and L: average length of germ tubes of the different treatments.

The laboratory threshold for declaring resistance was set, for triazoles, at 35% growth rate at 0.1 ppm and at 75% for strobilurins according to FRAC recommendations [13].

The inhibitory concentrations (IC_{50} and IC_{90}) were determined graphically from the linear relationship between the decimal logarithm of essential oil concentration on the abscissa and the probit values derived from conidia germination inhibition percentages on the ordinate.

2.2.2. In vivo Study of Mycosphaerella spp. Isolate Pathogenicity and Biopesticide Effectiveness

(i). Bringing Mycosphaerella spp. Colonies into Sporulation and Producing Inoculum

Strain storage tubes removed from the refrigerator were placed in the incubation room (25 ± 2°C) for 7 days in order to restart mycelium growth.

For producing conidia, small fragments of mycelium taken from colonies growing on agar medium were subcultured on PDA medium for three weeks before being subcultured on modified V8 medium. The composition of this V8 medium was as follows: 200 ml of V8 vegetable juice; 0.03 g of calcium carbonate ($CaCO_3$); 20 g of Agar-agar and 800 ml of distilled water. The pH of this mixture was adjusted to 6 using a pH meter and sterilized in an autoclave under a pressure of 1 bar at 120°C for 20 min. Then 350 µL of lactic acid, 250 mg/L of ampicillin and 10 mg/L of rifampicin were added to the medium cooled to 45°C. Finally, the latter was supplied to 90-mm diameter sterile plastic Petri dishes [1].

Mycelial fragments taken from colonies rejuvenated on PDA medium were ground in a mortar in the presence of previously sterilized lagoon sand. A quantity of 10 ml of sterile distilled water was added to the ground material in the mortar. The mixture was stirred using a magnetic stirrer and carefully poured onto the V8 medium in the Petri dishes. These were sealed with stretch film and stored at 25 ± 2°C under permanent white light. After 14 days of incubation, the mycelium was removed and crushed in sterile distilled water. The ground material was filtered through an 80-µm mesh diameter sieve so as to remove large fragments. The conidia were observed under an optical microscope and counted using a Malassez cell with a minimum of 3 counts. The inoculum was calibrated at 2.10^5 conidia per ml [1].

(ii). Pathogenicity Test and Biopesticide Assessment

The pathogenicity test was carried out with 8 *Mycosphaerella* spp. strains according to an inoculation technique under controlled conditions on whole plants belonging to the 5 cultivars stemming from *in vivo* culture. These plants were 3 months old after separation.

1) Banana Tree Inoculation

In order to test strain aggressiveness and virulence, 9 vivo banana plants of each cultivar bearing at least 3 leaves (noted 1 for the youngest leaf after the cigar and 3 for the oldest) were selected to be inoculated. A total of 45 plants were used for this study. Inoculations were performed using a sterile micro-sprayer. Two (2) ml of conidial fungal suspension at 2.10^5 per ml was sprayed. The sprays were made on the underside of the leaves along the midrib. The micro-sprayer was calibrated using tap water so as to estimate the amount sprayed at one pressure. The leaves were kept 50 cm perpendicular to the inoculum stream for the distribution to be homogeneous. The inoculations were carried out crosswise; each fungus strain was sprayed six times, once on leaf 1 of two plants, once on leaf 2 of two other plants and once again on leaf 3 of two other plants. Control plants were inoculated with sterilized distilled water. The inoculated banana trees were then placed under controlled conditions according to a completely randomized design (Figure 3) with a hygrometric degree of 100% the first three days then an alternation of 12 hours at 100%, 12 hours at 80% for 4 days.. The hygrometry was then maintained at 80% throughout the experiment.

Figure 3. *Young dessert and plantain plants inoculated with different isolates of Mycospharella spp. then placed under controlled conditions in a greenhouse.*

2) Plant preparation for biopesticide assessment

The assessment of biopesticide protective effectiveness and their combinations against BLSD was carried out on the

cultivar "Orishele" (very susceptible) with the most aggressive and virulent strain AZEP6 selected during isolate pathogenicity test. For this study 48 banana trees were used. On each plant, the first two leaves after the cigar, noted 1 for the youngest leaf and 2 for the oldest one, were inoculated. The inoculation and incubation of the plants were carried out under the same conditions as before according to a completely randomized design.

3) Application of biopesticides to banana plants under controlled conditions

Twelve (12) treatments with biopesticides and synthetic fungicides were carried out crosswise. Each product was sprayed 3 times 72 hours before on the one hand, and on the other hand 72 hours after inoculation, once on leaf 1 of one plant, once on leaf 2 of another plant and another time on leaves 1 and 2 of another plant (Figure 4). For each treatment, four (4) plants were inoculated.

Positive control plants were inoculated without having been treated with biopesticides or synthetic fungicides. As for the negative control plants, the inoculum was replaced by sterile distilled water and they received no application of biopesticide or synthetic fungicides.

Figure 4. *Young plants of the cultivar "Orishele" treated with biofungicides 72 h after their inoculation with an isolate of M. fijiensis and exposed under controlled conditions.*

4) Symptom and development rate monitoring after inoculation

Banana tree monitoring was carried out by considering two (2) variables, namely: incubation period (TI) and disease development time (TDM). TI expresses virulence or number of days between inoculation and the onset of the first symptoms of the disease. As for TDM, it reflects aggressiveness or the number of days between the onset of the first symptoms and the appearance of dry spots in their center. For this purpose, the plants were observed every 2 days and the yellowish depigmentation of the underside of the leaf was considered as the first symptom [14]. For each variable, the date of observation of the corresponding step was noted. The durations of the different phases were obtained by taking away the date of inoculation.

2.3. Data Analysis

Statistical analyses of the data obtained were carried out with the STATISTICA version 7.1 software. Analyses of variance were carried out to assess the yield of essential oils of the different plant species; germination inhibition rates of *Mycosphaerella* spp. conidia; agronomic and phytopathological parameters of BLSD control trials.

In the event of significant differences, the Newman-Keuls test made it possible to classify the average values at α risk of 5% and compare them.

3. Results

3.1. Effect of Pure and Mixed Biopesticides on M. fijiensis Conidia Germination

3.1.1. Effect of Pure Biopesticides on M. fijiensis Conidia Germination

The nature of biopesticides and the effect of concentration on *M. fijiensis* conidia germination during 48 h of incubation are shown in Figure 5. For concentrations from 100 to 1000 ppm, the average inhibition rate varied from 83.31 to 96.08% for FERCA 50 EC, from 87.99 to 97.63% for TUSEL 50 EC, from 88.55 to 97.81% for ASTOUN 50 EC, from 90.40 to 98.34% for NORDINE 50 EC, from 94.70 to 99.02% for NECO 50 EC and from 95.28 to 99.89% for DOCUS 50 EC (Figure 5). These data, represented in the form of curves, show that their overall appearance is increasing and expresses the progressive inhibition of *M. fijiensis* conidia *in vitro* germination. This inhibition is dependent on the concentration and the biopesticides. In fact, the biopesticides of all the plant species tested significantly reduced *Mycosphaerella fijiensis* conidia germination. For each biopesticide, the effect was greater especially as the concentration was high. They caused a reduction of more than 80% of conidia germination whatever the concentration and the biopesticide. In fact, the inhibition percentages all varied between 83.31 and 99.88% for all the biopesticides. DOCUS 50 EC was most effective in inhibiting *M. fijiensis* conidia germination, followed by NECO 50 EC. However, the lowest average inhibition rates were obtained with FERCA 50 EC for all concentrations compared to other biopesticides.

However, it should be noted that the concentration of 100 ppm which gave an inhibition of more than 80% with all the biopesticides was used for the comparative study with synthetic fungicides.

The experimental data represented in the form of curves made it possible to determine the antifungal parameters, which are the inhibitory concentrations at 50 and 90%. Of the different biofungicides tested (Table 5). The IC_{50}s of these biofungicides were between $3.16.10^{-10}$ and 2.10^{-1} ppm and the IC_{90}s between 6.76 and 338.84 ppm. The low values of IC_{50} (IC_{50} < 0.1 ppm) and IC_{90} (IC_{90} < 400 ppm) of all biofungicides demonstrate their significant antifungal power. However, by comparing the IC_{50} and IC_{90} of all these biofungicides with each other, it appeared that DOCUS 50 EC and NECO 50 EC biofungicides were ten to fifty times more active on *M. fijiensis* conidia germination than the other biofungicides tested.

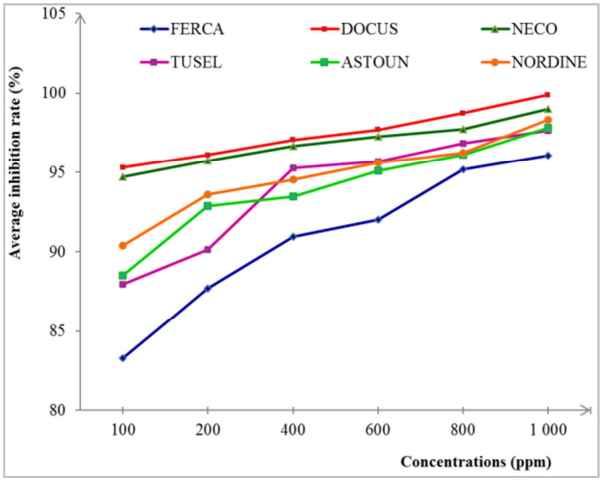


Figure 5. *Average inhibition rate of Mycosphaerella fijiensis conidia germination depending on biopesticides and concentration.*

Table 5. *Biopesticide IC_{50} and IC_{90} values after 48 hours of Mycosphaerella fijiensis conidia incubation at 25 ± 2°C.*

Biopesticides	Conidia inhibition concentration (ppm)	
	IC_{50} (ppm)	IC_{90} (ppm)
FERCA 50 EC	2.10^{-1} a	338.84 a
DOCUS 50 EC	$2.63.10^{-9}$ e	6.92 e
NECO 50 EC	$3.16.10^{-10}$ f	6.76 e
TUSEL 50 EC	2.10^{-2} b	162.18 b
ASTOUN 50 EC	2.10^{-3} c	128.82 c
NORDINE 50 EC	$1.05.10^{-4}$ d	77.62 d

NB: The inhibitory concentrations of the same column with the same letter do not differ significantly from each other at 5% probability level.

3.1.2. Biopesticide Synergistic Effect on M. fijiensis Conidia Germination

The assessment of the antifungal activity by synergistic action carried out from the different formulations combining biopesticides, showed variable effectiveness. The results reported in Table 6 give the average inhibition rate of *M. fijiensis* conidia germination at a concentration of 50 ppm after 48 h incubation at 25 ± 2°C. Analysis of this table made it possible to observe a highly significant inhibition (< 0.0001) of the germination of *M. fijiensis* conidia exposed to the vapors of pure biopesticides and regardless of the type of combination adopted. Overall, all biopesticide mixtures showed a significant inhibitory effect on the pathogen *M. fijiensis*. The strongest inhibition was obtained with biopesticide mixtures. Pure biopesticides at a concentration of 50 ppm reduced the germ tube elongation of the pathogen less compared to mixtures (Table 6). Furthermore, FERCA 50 EC was the least effective in inhibiting *M. fijiensis* conidia germination (55.06% inhibition rate). However, the most effective biopesticides in inhibiting *M. fijiensis* conidia germination were the combinations ARIMA 50 EC, VOGOH 50 EC, PRORALY 50 EC, REBRACI 50 EC, KAGNI 50 EC, NOSTAG 50 EC and LOGUY 50 EC. However, the pure biopesticide FERCA 50 EC, although very little active (% I = 55.06), when combined with NECO 50 EC or DOCUS 50 EC showed an increase in its effectiveness. In general, the lower its proportion in the mixture, the more effective this mixture was.

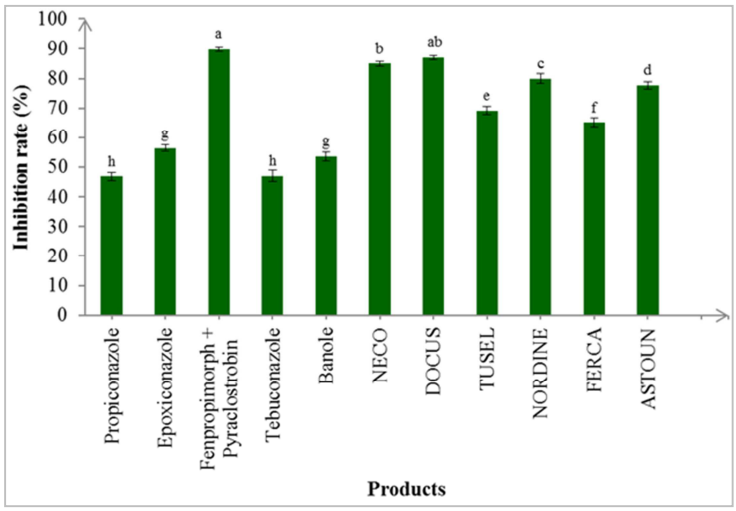
Table 6. *Effect of biopesticides and their combinations at 50 ppm on M. fijiensis conidia germination.*

Treatments	Formulation (V/V)	Inhibition rate (%)
T_1	NORDINE 50 EC	65.83 ± 1.88 de
T_2	ASTOUN 50 EC	60.62 ± 1.69 e
T_3	NECO 50 EC	71.52 ± 1.43 d
T_4	DOCUS 50 EC	72.62 ± 1.50 d
T_5	FERCA 50 EC	55.06 ± 0.97 f
T_6	TUSEL 50 EC	60.32 ± 2.56 e
T_7	TEBAYE 50 EC	88.25 ± 2.17 c
T_8	YESOLI 50 EC	90.76 ± 1.06 bc
T_9	RINEVES 50 EC	93.79 ± 0.37 b
T_{10}	WACHET 50 EC	93.84 ± 0.69 ab
T_{11}	LOGUY 50 EC	96.07 ± 0.41 a
T_{12}	RHOSO 50 EC	91.06 ± 1.87 bc
T_{13}	NOSTAG 50 EC	96.77 ± 0.39 a
T_{14}	KAGNI 50 EC	95.59 ± 0.76 a
T_{15}	HACADO 50 EC	93.06 ± 0.38 bc
T_{16}	FIRCHE 50 EC	85.54 ± 1.85 cd
T_{17}	SECARI 50 EC	92.50 ± 1.68 bc
T_{18}	REBRACI 50 EC	96.16 ± 0.82 a
T_{19}	PRORALY 50 EC	95.01 ± 0.78 a
T_{20}	VOGOH 50 EC	96.42 ± 0.76 a
T_{21}	ARIMA 50 EC	94.72 ± 0.75 a
Overall average		85.02 ± 0.99
CV (%)		16.91
P		< 0.0001

NB: Averages assigned the same letter do not have statistically different antifungal activity at $p < 0.05$ probability threshold of the Newman-Keuls test. The data in the last column represent the average values of the inhibition percentages of 3 repetitions compared to the control ± standard error.

3.1.3. Comparative Effect of Biopesticides and Synthetic Fungicides on M. fijiensis Conidia Germination

Figure 6 shows the average inhibition rate of *M. fijiensis* conidia germination under the biocidal action of different biopesticides, mineral oil and synthetic fungicides, 48 hours after seeding. On examination of this figure, at a concentration of 100 ppm the biopesticides tested as well as the mineral oil Banole and the synthetic fungicides exerted varying depressive effects on *M. fijiensis* conidia germination. The differences in antifungal activity between biopesticides, mineral oil and synthetic fungicides were highly significant ($P < 0.01$). Biopesticides had a stronger effect than mineral oil and synthetic fungicides except the one made up of Fenpropimorph and Pyraclostrobin with an average inhibition rate of 89.80%. Propiconazole and Tebuconazole-based fungicides were the least effective in inhibiting *M. fijiensis* conidia germination with inhibition rates (below 50%) of 46.82 and 47.09%, respectively (Figure 6).

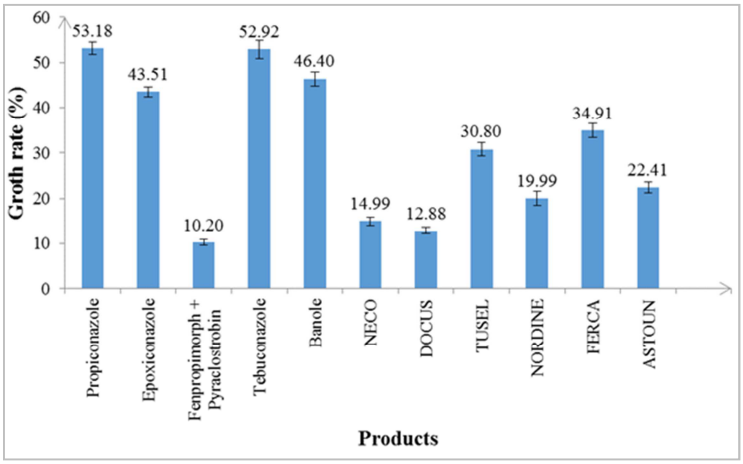


NB: The histograms followed by the same letter are not significantly different (Newman-keuls test at 5% threshold)

Figure 6. *Inhibition rate of Mycosphaerella fijiensis conidia germination depending on biopesticides and synthetic fungicides.*

3.1.4. M. fijiensis Growth Rate on Amended Media

The growth rates observed with triazoles (Propiconazole, Epoxiconazole and Tebuconazole) compared with the controls were all greater (Figure 7) than the threshold which was 35% for declaring resistance. The results obtained with these triazoles were respectively 53.18% for Propiconazole, 43.51% for Epoxiconazole and 52.92% for Tebuconazolele. As for strobilurin (Fenpropimorph + pyraclostrobin), the samples showed germ tube growth levels below the resistance threshold (75%). The growth rate with this molecule was 10.20%. All biopesticides showed germ tube growth levels below the resistance thresholds for synthetic fungicides (35% and 75%) except FERCA 50 EC which gave a 34.91% growth rate approaching that of triazoles (35%). Figure 8 shows the effect of synthetic fungicides on *M. fijiensis* conidia growth rate on agar medium, amended or not, 48 hours after inoculation.



NB: The histograms followed by the same letter are not significantly different (Newman-keuls test at 5% threshold)

Figure 7. *Mycosphaerella fijiensis conidia germination rate depending on biopesticides and synthetic fungicides.*

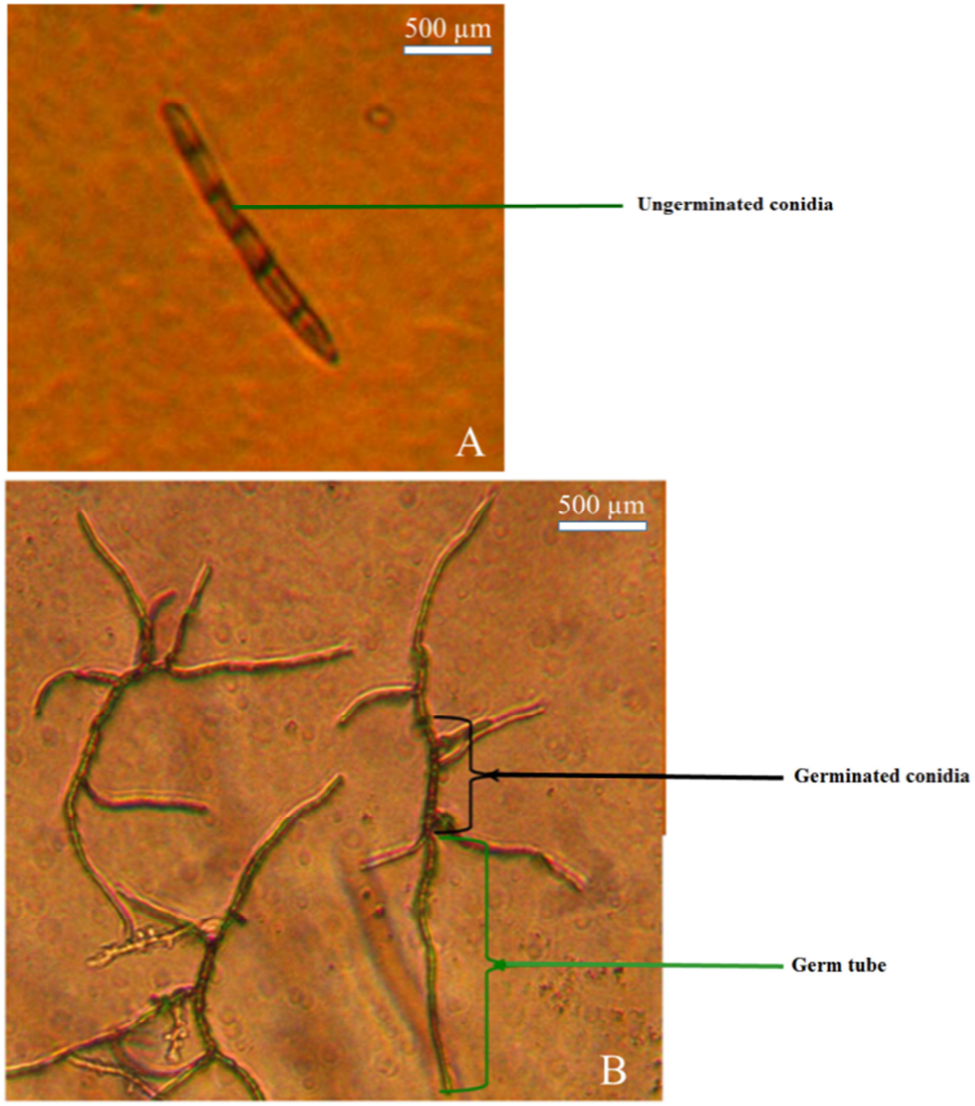


Figure 8. *Mycosphaerella fijiensis conidia growth rate on agar medium, amended or not, 48 h after inoculation.*

A: Amended agar medium (Trials)
B: Unamended agar medium (Control)

3.2. In Vivo Biopesticide Action

3.2.1. Virulence of M. fijiensis Isolates Tested and Susceptibility of Cultivars

The pathogenicity of the 8 isolates (AFEP18, KDUP35, ZTAP57, AZEP6, BCED65, BUID47, KGOD34 and BROD63) of *M. fijiensis* tested on the five (5) cultivars ("Orishele", "Corne 1", "Figue Sucrée", PITA 3 and FHIA 21), under conditions controlled by inoculation with conidial suspensions, is reported in Tables 7 and 8. The values in these tables show that all of the isolates used caused symptoms characteristic of black leaf streak disease regardless of the cultivar. In contrast, no isolate caused symptoms characteristic of Sigatoka disease (yellow Sigatoka). All the isolates therefore showed pathogenicity towards the cultivars tested. However, the time to onset of the first visible lesions, small brown dots and dark brown leaf necrosis varied from one cultivar to another and from one isolate to another (Figure 9). Indeed, the first visible lesions, small brown dots, appeared between the 12th and 30th day after inoculation in all cultivars (Table 7), and dark brown leaf necrosis was observed between the 26th and the 56th day after inoculation (Table 8). The first typical symptoms of black Sigatoka appeared in local cultivars ("Orishele", "Corne 1" and "Figue Sucrée"), approximately two (2) weeks after inoculation and in hybrids (FHIA 21 and PITA 3), three (3) weeks after inoculation (Table 7). Differences in susceptibility between cultivars were observed depending on the origin of isolates. Overall, hybrids FHIA 21 and PITA 3 had longer disease development times (Table 8) compared to local cultivars ("Orishele", "Corne 1" and "Figue Sucrée"). This duration was approximately one month (29 to 33 days) in cultivars "Orishele" and "Corne 1" while in hybrids FHIA 21 and PITA 3, it reached one month and a half (43 to 46

days).. The duration was 37.58 days in dessert cultivar Figue Sucrée. The more aggressive isolates (BROD63, BCED65, KGOD34, AFEP18, BUID47 and AZEP6) favored shorter disease development times. It varied between 34 and 38 days (Table 8). This means that they can be considered more virulent. Among them, two were very virulent, namely isolates AZEP6 and BCED65 which caused symptoms 12 and 13 days after inoculation in the susceptible cultivar "Orishele".

Table 7. *Disease incubation time caused by different isolates of Mycosphaerella fijiensis inoculated on the 5 cultivars of banana and plantain trees tested.*

Cultivars	Disease incubation time (d) Mycosphaerella fijiensis isolates								Overall average	CV (%)
	AFEP18	KDUP35	ZTAP57	AZEP6	BCED65	BUID47	KGOD34	BROD63		
Orishele	16.15 ± 0.21 c (a)	13.88 ± 0.12 c (bc)	15.08 ± 0.17 d (b)	12.33 ± 0.22 c (c)	13.69 ± 0.11 d (bc)	16.48 ± 0.20 c (a)	16.57 ± 0.24 d (a)	16.37 ± 0.41 bc (a)	15.07	7,83
Corne 1	17.53 ± 0.09 bc (a)	15.63 ± 0.15 bc (bc)	16.12 ± 0.23 cd (b)	16.77 ± 0.18 bc (b)	16.06 ± 0.07 bc (b)	14.83 ± 0.33 d (c)	17.11 ± 0.14 c (a)	14.46 ± 0.70 c (c)	16.06	4,53
Figue Sucrée	16.37 ± 0.28 c (cd)	17.47 ± 0.25 b (c)	19.55 ± 0.11 c (b)	20.55 ± 0.32 b (a)	15.38 ± 0.09 c (d)	17.18 ± 0.17 c (c)	18.15 ± 0.28 bc (bc)	19.07 ± 0.33 b (b)	17.97	6,75
PITA 3	24.54 ± 0.13 a (b)	21.27 ± 0.27 a (c)	29.45 ± 0.40 a (a)	26.12 ± 0.20 a (ab)	20.69 ± 0.41 b (d)	23.22 ± 0.18 a (bc)	21.71 ± 0.32 b (c)	26.23 ± 0.45 a (ab)	24.15	8,95
FHIA 21	22.25 ± 0.11 b (c)	20.24 ± 0.18 a (cd)	26.14 ± 0.21 b (b)	27.47 ± 0.12 a (ab)	23.66 ± 0.19 a (bc)	20.33 ± 0.26 b (cd)	28.45 ± 0.33 a (a)	19.29 ± 0.16 b (d)	23.48	11,17
Overall average	19.37	17.70	21.27	20.65	17.90	18.41	20.40	19.08		
CV (%)	19.62	17.44	29.59	30.70	23.11	18.20	24.15	23.41		

NB: In the same column, the figures followed by the same letter (outside brackets) are statistically identical at the threshold α = 5% and also on the same row, the figures followed by the same letter (in brackets) are statistically identical at the threshold α = 5% (Newman-Keuls test); Average ± standard error

Table 8. *Disease development time caused by different isolates of Mycosphaerella fijiensis inoculated on the 5 cultivars of banana and plantain trees tested.*

Cultivars	Disease development time (d) Mycosphaerella fijiensis isolates								Overall average	CV (%)
	AFEP18	KDUP35	ZTAP57	AZEP6	BCED65	BUID47	KGOD34	BROD63		
Orishele	32.12 ± 0.35 cd (a)	30.18 ± 0.23 b (ab)	32.51 ± 0.28 d (a)	28.65 ± 0.13 c (b)	26.23 ± 0.22 c (c)	28.78 ± 0.34 c (b)	27.33 ± 0.44 d (bc)	30.12 ± 0.43 c (ab)	29.49	5,25
Corne 1	28.14 ± 0.12 d (d)	29.75 ± 014 b (c)	40.22 ± 0.31 c (a)	37.58 ± 0.24 b (b)	30.71 ± 0.37 bc (c)	29.17 ± 0.60 c (c)	30.85 ± 0.31 c (c)	35.87 ± 0.31 b (bc)	32.79	10,38
Figue Sucrée	33.27 ± 0.44 c (d)	39.51 ± 0.61 ab (ab)	48.78 ± 0.47 b (a)	37.12 ± 0.33 b (c)	31.25 ± 0.28 bc (cd)	40.66 ± 0.54 b (ab)	38.74 ± 0.70 b (b)	31.33 ± 0.26 bc (cd)	37.58	10,26
PITA 3	45.35 ± 0.25 b (b)	48.11 ± 0.24 a (ab)	55.12 ± 0.29 a (a)	42.71 ± 0.29 ab (c)	47.28 ± 0.20 a (ab)	43.65 ± 0.24 ab (bc)	42.13 ± 0.32 a (c)	39.84 ± 0.36 a (d)	45.52	6,80
FHIA 21	50.12 ± 0.54 a (a)	47.85 ± 0.27 a (ab)	40.33 ± 0.17 c (bc)	44.12 ± 0.41 a (b)	39.54 ± 0.15 b (bc)	46.80 ± 0.13 a (ab)	38.56 ± 0.59 b (c)	37.72 ± 0.24 ab (c)	43.13	8,43
Overall average	37.80	39.08	43.39	38.04	35.00	37.81	35.52 ±	34.98		
CV (%)	21.03	18.66	15.78	11.31	19.22	18.70	14.49	9.72		

NB: In the same column, the figures followed by the same letter (outside brackets) are statistically identical at the threshold α = 5% and also on the same row, the figures followed by the same letter (in brackets) are statistically identical at the threshold α = 5% (Newman-Keuls test); Average ± standard error

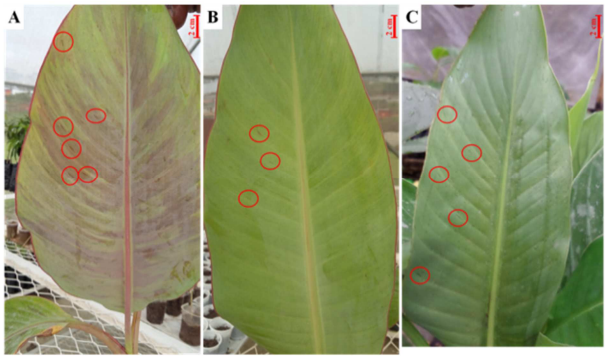


Figure 9. *Black leaf streak disease symptoms on the underside of leaves, 22 days after inoculation with BCED65 isolate in plantain tree cultivars.*

(A: "Orishele"; B: "Corne 1" and C: PITA 3)
Red circles show black leaf streak disease symptoms

3.2.2. In vivo biopesticide Synergistic Action on Sigatoka Development

In vivo trials under controlled biological control conditions carried out on young plantain plants of the cultivar "Orishele" inoculated with *M. fijiensis* isolate AZEP6, showed a significant reduction of disease incidence in the types of experiment (preventive and curative control). For the curative treatment of plants with synthetic fungicides, Sigatoka development rates ranged from 62.5 to 75%, but for plants treated with pure biopesticides, this rate fluctuated from 12.50 to 50%. However, with their mixtures, disease development rates were zero. In contrast, they varied from 0 to 62.50% with all the preventive treatments tested. Strong attacks often followed by necrosis were observed on young untreated banana trees (positive control). As for uninoculated plants, they did not develop typical Sigatoka symptoms. The analysis of variance with a single classification criterion

(treatment) showed significant differences at 5% threshold for the two types of treatment (Table 9).

Synthetic biopesticides and fungicides significantly reduced disease development rate compared to inoculated and untreated controls, but to varying degrees. Indeed, the different biopesticides and synthetic fungicides tested did not behave in the same way with respect to this pathogen. Preventive treatment of seedlings was found to be much more effective than curative treatment. This is because disease development rates were low or even zero with preventive treatments.

Table 9. *Biopesticide effectiveness on disease development on plantain tree "Orishele" leaves inoculated with isolate AZEP6.*

Treatments	Attack rate (%)	
	Preventive	Curative
TN: Negative control	0 f	0 h
TP: Positive control	100 a	100 a
T_1: Banole (Mineral oil)	62.50 b	100 a
T_2: NECO 50 EC	0 f	25.00 f
T_3: DOCUS 50 EC	0 f	12.50 g
T_4: FERCA 50 EC	37.50 c	50.00 d
T_5: NORDINE 50 EC	0 f	25.00 f
T_6: TUSEL 50 EC	12.50 e	37.50 e
T_7: PRORALY 50 EC	0 f	0 h
T_8: REBRACI 50 EC	0 f	0 h
T_9: SECARI 50 EC	0 f	0 h
T_{10}: Propiconazole	25.00 d	75 b
T_{11}: Epoxiconazole	37.50 c	62.50 c

NB: Averages of the same column with the same letter do not differ significantly from each other at 5% probability threshold of Newman-Keuls test.

4. Discussion

Increasingly, for the protection of crops against diseases caused by pathogens and in order to reduce the intensive use of synthetic chemicals, increased emphasis is being placed on essential oils, aqueous extracts and their constituents. Thus, in this study, the antifungal activity of twenty (20) formulations of biopesticides based on essential oils extracted from aromatic plants of the Ivorian flora was studied *in vitro* and *in vivo* for controlling *Mycosphaerella fijiensis*, causal agent of black Sigatoka of banana trees. The results obtained *in vitro* show that all biopesticide formulations exert an antifungal effect on *M. fijiensis*. This antifungal effect differs from one biopesticide formulation to another and also from one concentration to another for a product.

4.1. Effect of Biopesticides on M. fijiensis Conidia Germ Tube Growth

The influence of all biopesticide formulations on *M. fijiensis* conidia germination was assessed *in vitro*. All biopesticides and their mixtures were found to be remarkably effective on *in vitro M. fijiensis* conidia germination.

In fact, fungus conidia germination was inhibited by all the biopesticides with variability. The positive correlation observed between the inhibition rate and the different concentrations for each biopesticide demonstrates the inhibitory power (antifungal properties) of the latter on the fungus.

The maximum inhibition threshold (more than 50%) is exceeded at minimum inhibitory concentrations at 100 ppm for all pure biopesticides and at 50 ppm for mixtures. The inhibition of conidia germination to more than 80% of the fungus at all concentrations regardless of the biopesticide reflects a fungicidal activity of the biopesticides formulated and tested. The inhibition of *M. fijiensis* conidia germination by pure biopesticides and their mixtures varied depending on the type of biopesticide and the concentrations tested. In fact, at the same concentration, there were differences in activity between the biopesticides. This could be due to their differences in respective chemical profiles. Indeed, it has been established that certain constituents of biopesticides can positively influence their overall activity. All the biopesticides tested offered a very marked effectiveness on *M. fijiensis* conidia germination. This result is in agreement with the work of Sartoratto *et al*. [15], Camara *et al*. [16], Mawussi [17], Camara [18] and Soro *et al*. [19] who showed that essential oils have antimicrobial, insecticidal, fungicidal and bactericidal activities because they might have a capacity to stimulate defense reactions of plants. According to these authors, the activity of essential oils might depend on the nature and chemical structure of their constituents. Thus, it should be pointed out that the antifungal effects of essential oils are very often linked to simultaneous actions of their constituents [20]. The fact that all the mixtures were found to be more active than the pure biopesticides suggests the existence of a complementarity of action which might exist between the different constituents found in these mixtures. These biopesticides could effectively replace certain synthetic fungicides either in curative and/or preventive process within the framework of a biological fight against this fungus since it presents a very great variability and an aptitude to bypass the inhibitory effect of the latter [8].

With regard to synthetic fungicides, different reduction and growth rates of the germ tube were observed. The growth rates observed with triazoles (Propiconazole, Epoxiconazole and Tebuconazole) compared with the controls were all above the threshold which was 35%. Indeed, according to FRAC recommendations [13], the laboratory threshold for declaring resistance was set at 35% of germ tube growth rate at 0.1 µl/L and at 75% for strobilurins (5 µL/L) of the control. The loss of effectiveness observed with triazoles against the strains of the fungus tested seems to be explained by the use of these sterol biosynthesis inhibitors for treatments in industrial banana tree plantations [21].

Regarding *Mycosphaerella* spp conidia germ tube growth rates on agar media amended with Fenpropimorph + pyraclostrobin, it was found that this strobilurin in the laboratory was the most effective fungicide with a growth rate of 10.20%. This germ tube growth rate is well below the resistance threshold (75%). These results confirm those of Lynton *et al*. [22] who showed that fungicides from the strobilurin family (trifloxystrobin, pyraclostrobin and azoxystrobin) have been shown to be more effective in controlling leaf lesions (Sigatoka disease) than conventional industrial products such as propiconazole and mancozeb.

4.2. Virulence of M. fijiensis Isolates Tested and Susceptibility of Cultivars

It appears from the pathogenicity tests carried out that the 8 isolates of *Mycosphaerella* spp. (AFEP18, KDUP35, ZTAP57, AZEP6, BCED65, BUID47, KGOD34 and BROD63) tested under controlled conditions by inoculation with conidial suspensions, all caused symptoms characteristic of black Sigatoka or black leaf streak disease (BLSD) on the five (5) cultivars ("Orishele", "Corne 1", "Figue Sucrée", PITA 3 and FHIA 21). In contrast, no isolate caused symptoms characteristic of Sigatoka disease (yellow Sigatoka). All the isolates therefore showed pathogenicity towards the cultivars tested. These results are in agreement with those of Onautshu *et al*. [23], who showed that all the strains of *Mycosphaerella fijiensis* from the Kisangani region in the Democratic Republic of Congo, had a pathogenic power vis-à-vis the Cavendish cultivar "Grande Naine". The pathogenicity of the 8 isolates of *Mycosphaerella* spp. also showed that the time to onset of the first visible lesions, small brown dots and dark brown leaf necrosis is variable from one cultivar to another and from one isolate to another. Indeed, the first visible lesions, small brown dots, appeared between the 12^{th} and 30^{th} day after inoculation in all cultivars, and dark brown leaf necrosis was observed between the 26^{th} and 56^{th} day after inoculation. The development of disease symptoms for these 8 isolates closely corresponded to descriptions by Fullerton and Olsen [24] on vitro plants of the cultivar Grande Naine inoculated with *Mycosphaerella fijiensis* conidia. The first typical symptoms of black Sigatoka appeared in local cultivars ("Orishele", "Corne 1" and "Figue Sucrée"), approximately two (2) weeks after inoculation and in hybrids (FHIA 21 and PITA 3), three (3) weeks after inoculation. Differences in susceptibility between cultivars were observed depending on the origin of the isolates. Overall, hybrids FHIA 21 and PITA 3 had longer disease development times compared to local cultivars ("Orishele", "Corne 1" and "Figue Sucrée"). This duration was approximately one month (29 to 33 days) in cultivars "Orishele" and "Corne 1" while in hybrids FHIA 21 and PITA 3, it reached one month and a half (43 to 46 days). The duration was 37.58 days in dessert cultivar Figue Sucrée. According to Dzomeku *et al*. [25], banana tree hybrids are generally tolerant to black Sigatoka caused by *M. fijiensis*. The more aggressive isolates (BROD63, BCED65, KGOD34, AFEP18, BUID47 and AZEP6) favored shorter disease development times. It varied between 34 and 38 days. This means that they can be considered more virulent. Among them, two were very virulent, namely isolates AZEP6 and BCED65 which caused symptoms 12 and 13 days after inoculation in the susceptible cultivar "Orishele".

4.3. Effect of Biopesticides on the Expression of Disease Symptoms Under Controlled Conditions

Black Sigatoka treatment under controlled conditions (*in vivo*) of banana trees with biopesticides whose active ingredients are compounds of essential oils, has shown their effectiveness. Biopesticides, as well as their mixtures, were applied to banana tree leaves as a preventive and curative measure. The effectiveness noted *in vitro* with these biopesticides and their mixtures was observed *in vivo*. These results are contrary to those of Soro *et al*. [19] who showed that the inhibitory effect observed *in vitro* with the essential oil of *Xylopia Aethiopica*, on *Fusarium oxysporum* f. sp *Radicis-lycopersici* (Forl), a parasitic fungus of tomato crops has not been observed *in vivo*. However, no mortality was observed on plants inoculated and treated with this essential oil. Indeed, this essential oil has been supplied as an amendment to the culture medium of tomato plants. One could then think that the active molecules of *Xylopia Aethiopica* essential oil, under *in vitro* conditions might have been metabolized once this essential oil is applied as an amendment to the substrate on tomato plants. It should be noted that some bioactive compounds are unstable in heat and therefore rapidly degradable under the effect of environmental factors such as the sun. But for some authors, fungi do not react in the same way to biopesticides [26].

5. Conclusion

The results of this study show that the biopesticides assessed *in vitro* made it possible to reduce the lengthening of *Mycosphaerella fijiensis* conidia germ tube depending on the doses supplied and the formulations. The pathogenicity of the 8 isolates of *M. fijiensis* based on incubation time (TI) demonstrated their virulence and aggressiveness. About 75% of the isolates tested were found to be very virulent and more aggressive. Inoculated, hybrids PITA 3 and FHIA 21, with longer incubation times and slower disease progression, were found to be more resistant than the reference cultivar "Orishele". Thus, the preventive treatment of plants with biopesticides is effective against Sigatoka development. However, in order to protect banana and plantain tree varieties against *M. fijiensis*, both types of treatments are necessary. With a view to enhancing the value of these biopesticides, their effect on black leaf streak disease control under conditions of natural infestation in plantations should be compared with those of triazoles (Propiconazole and Epoxiconazole).

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