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Research Article
Effect of Some Synthetic Fungicides on the *in vitro* Growth of *Colletotrichum gloeosporioides*, Causative Agent of Cashew Tree Anthracnose in Côte d'ivoire

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Abstract

Background and Objective: Anthracnose is the most common disease of tropical and sub-tropical cultures. It is the main disease in cashew orchards of Côte d'Ivoire. The objective of the study was to screen *in vitro* effective chemicals fungicides to control the disease. **Materials and Methods:** The synthetic fungicides Azoxystrobin, Carbendazim, Prochloraz, Propiconazole Mancozeb, Thirame, Cymoxanil+Mancozeb and Metalaxyl-M+Copper oxide have been assessed for mycelial growth, sporulation and spore germination of *Colletotrichum gloeosporioides*, the fungus causative agent of cashew tree anthracnose in Côte d'Ivoire. Each Fungicide was incorporated into a Potato Dextrose Agar (PDA) medium at six concentrations (0.1, 1, 5, 10, 25 and 50 ppm) and data collected were submit to one way analysis of variance (ANOVA) with Statistica software 7.1 version. **Results:** These fungicides and combinations of fungicides have shown *in vitro* fungitoxic activity more or less significant on the three life stages of the pathogen. Among the fungicides tested, Carbendazim and Prochloraz were more effective on fungus growth with similar properties. Both fungicides showed very toxic activity by significantly reducing mycelial growth at the lowest dose of 0.1 ppm. Carbendazim had fungistatic and fungitoxic properties, respectively at doses of 1 and 5 ppm while for Prochloraz they were, respectively 5 and 10 ppm. Carbendazim and Prochloraz have been highly toxic to *Colletotrichum gloeosporioides* compared to other fungicides because they have IC_{50} less than 0.1 ppm. **Conclusion:** All fungicides tested can be therefore regards as a prospective means of limiting mycelial growth, spores germination and protecting cashew from *Colletotrichum gloeosporioides* with Carbendazim, Prochloraz, Propiconazole and Azoxystrobin as the bests.

Key words: Anthracnose, cashew tree, *Colletotrichum gloeosporioides*, IC_{50}, synthetic fungicides

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

In Côte d'Ivoire, cashew tree cultivation is increasingly spreading thus moving the country into the world's biggest producers of cashew nut. However, yields remain low[1] and range between 300-400 kg ha^{-1} while in Brazil more than 1 t ha^{-1} is recorded for some dwarf varieties[2]. In the past, cashew tree was cited among crops that were attacked neither by serious diseases nor by insect pests[3,4]. The extension of its cultivation zone has certainly increased parasitic pressure without, however, causing economic losses. Today, in some areas of cashew nut production, diseases have caused enormous yield losses[5]. Anthracnose is one of the most common diseases found in almost all cashew nut production areas[6]. The losses caused by this pathology are considerable in certain production areas such as Brazil and Mozambique[7,8].

Anthracnose, one of the diseases encountered in all cashew nut-producing areas in Côte d'Ivoire[9]. It infects almost all the organs of the plant and in particular the reproductive organs (flowers and fruits) and therefore, can reduce yields.

Chemical control is the most effective practice for crop protection in the short term. In some cashew nut production areas, synthetic fungicides such as copper oxychloride, captafol, benomyl, dithianon, anilazine, bitertanol, triadimenol have been used successfully for disease control[10-12]. As well as dithiocarbamates (Propineb and Mancozeb) gave adequate control against cashew anthracnose[13] two strobilurins fungicides (azoxystrobin and trifloxystrobin) were report to be effective against cashew plant anthracnose[14].

In Côte d'Ivoire, the literature on the identification of cashew nuts pathogens and adapted control methods is practically non-existent. This study, aimed at screening *in vitro* effective synthetic fungicides against *Colletotrichum gloeosporioides*, the most common fungus found in cashew nut orchards, has been conducted within this framework.

MATERIALS AND METHODS

This fungicides screening were carry out from February to March, 2017 in Plant Physiology Laboratory of Félix Houphouët Boigny University.

Fungal material: An isolate (SEKA76) of *Colletotrichum gloeosporioides* provided by the mycological culture collection of the Laboratory of Plant Physiology of the University Félix Houphouët Boigny of Abidjan was used for this study. This isolate, characterized by high virulence, was obtained from leaves showing symptoms of anthracnose and harvested from cashew trees in the region of Séguéla in Northern Côte d'Ivoire.

Chemical material (synthetic fungicides): Eight synthetic fungicides showing different characteristics were used in this study (Table 1).

Methods

Preparation of culture media: For each of the fungicides or combination of fungicides, a stock solution of 1000 ppm was prepared by solubilizing them in sterilized distilled water. The medium use in this study is the PDA (potato extract 20 g L^{-1}, D-glucose 20 g L^{-1} and agar 20 g L^{-1}) one. To prepare the medium, 20 g of each component is weighed and solution volume adjusted to 1 L with distilled water and then homogenized. The medium was autoclaved at a temperature of 121°C under a pressure of 1 bar for 30 min. After cooling the medium to a temperature of 45°C, the fungicides from the stock solutions were incorporated into the PDA medium in order to obtain concentrations of 0.1, 1, 5, 10, 25 and 50 ppm. The PDA media amended with fungicides were homogenized and dispensed in 90 mm-diameter petri dishes at a rate of 18 mL/dish. A fungicide-free control was carried out under the same culture conditions.

Table 1: Characteristics of the fungicides tested

Active ingredient	Trade name	Chemical family	Type of formulation
Azoxystrobin	Ortiva	Strobilurin	250 SC
Carbendazime	Carhino	Benzimidazole	50 WP
Cymoxanil+Mancozeb	Cyman	Acetamide+dithiocarbamate	72 WP
Mancozeb	Mancozan	Dithiocarbamate	80 WP
Metalaxyl-M+Copper oxide	Fongis extra	Phenylamide+acid alanine	66WP
Prochloraz	Mirage	Imidazole	45 EC
Propiconazole	Reference	Triazole	50 EC
Thirame	Banguard	Dithiocarbamate	250 SC

SC: Suspension concentrate, WP: Wettable powder, EC: Emulsifiable concentrate

Culture of the fungus: Using a punch, 7 mm-diameter mycelial slices were taken from 7 day old cultures and deposited in the center of the petri dishes containing the fungicide-PDA mixture. The cultures were incubated at a temperature of 27±2°C under a 12 h photoperiod. Each treatment was carried out in 5 petri dishes. The experiment was repeated 3 times.

Assessment of mycelial growth: Colony mycelial growth was assessed every 3 days until the petri dishes were filled with the controls, that is, 15 days after the culture was set up. Measurements of the radial growth of the mycelium were made according to two perpendicular lines drawn on the back of each petri dish and which intersect at a point in the middle of the mycelial disk. The effect of fungicides on the fungus was determined from the inhibition rate (Ic) of the mycelial growth calculated using the following equation[15]:

$$Ic = \frac{D_o - D_c}{D_o} \times 100$$

The D_o being the diameter of the fungicide-free control and D_c the growth diameter of the fungus at a concentration (c) of the fungicide.

From the linear regression equation between the decimal logarithms of the fungicide concentrations and the mycelial growth inhibition percentages transformed into probit values, the concentrations reducing 50% (IC_{50}) mycelial growth were deduced.

After calculating the IC_{50}, the fungicides and isolate SEKA76 of *Colletotrichum gloeosporioides* were classified according to the following scale[16]:

- IC_{50}<1 ppm, highly effective fungicide and very sensitive strain
- 1<IC_{50}<10 ppm, moderately effective fungicide and moderately sensitive strain
- 10<IC_{50}<50 ppm, poorly effective fungicide and weakly sensitive strain
- IC_{50} 50 ppm, ineffective fungicide and insensitive strain

Preparation of spore suspension and sporulation assessment: In order to assess sporulation, after 28 days of culture on the different media, each petri dish according to the concentration of the fungicide was scraped using a curved Pasteur pipette in the presence of 10 mL of sterilized distilled water. A drop of this solution was taken and mounted between slide and cover glass and then observed under an optical microscope so as to check the presence of spores.

Assessment of spore germination: The spore suspension harvested from the 28 days cultures of the control was adjusted to 10^3 spores mL^{-1} by successive dilutions using a Malassez cell. A volume of 200 µL of the suspension was spread on an agar medium to which the fungicides were incorporated in order to obtain the following final concentrations: 0.1، 1، 5، 10، 25 and 50 ppm. Five petri dishes were cultured by concentration and the experiment was repeated 3 times. The cultures were incubated at 27±2°C for 20 h. Over a total of 100 counted spores, the number of germinated spores was determined and then the rate of germination inhibition (Ig) was calculated using the following equation[15]:

$$Ig = \frac{N_o - N_c}{N_o} \times 100$$

where, N_o being the number of germinated spores on a fungicide-free PDA medium and Nc the number of germinated spores on a medium containing a concentration c of the fungicide.

Statistical analysis: Data collected were analyzed using one way analysis of variance (ANOVA-1). The $p \leq 0.05$ and averages were compared through Newman-Keuls multiple range test at 5% threshold with statistica software 7.1 version[17].

RESULTS

Effect of fungicides on mycelial growth: All the fungicides tested had a fungi toxic activity on the mycelial growth of isolate SEKA76 of *Colletotrichum gloeosporioides*. The effectiveness of these fungicides in reducing the mycelial growth of the fungus depended on the nature of the chemical compound and its concentration (Fig. 1-8).

Carbendazim and Prochloraz have proved to be very effective on the mycelial growth of isolate SEKA76 of *Colletotrichum gloeosporioides*. They were strongly fungitoxic at the concentration of 0.1 ppm with inhibition rates of 75.85 and 87.39%, respectively, for Carbendazim and Prochloraz. With these two fungicides, growth was totally inhibited from the concentration of 1 ppm for Carbendazim (Fig. 1) and 25 ppm for Prochloraz (Fig. 2). Prochloraz at 0.1 and 1 ppm resulted in low mycelium growth beginning on the sixth day of cultures with inhibition rates of 96.10 and 96.62%, respectively. With Prochloraz, concentrations of 5 and 15 ppm delayed mycelium growth which started 15 days after the cultures (Fig. 2). Mycelial growth inhibition rates at these concentrations were 97.44 and 97.86%, respectively, at 5 and

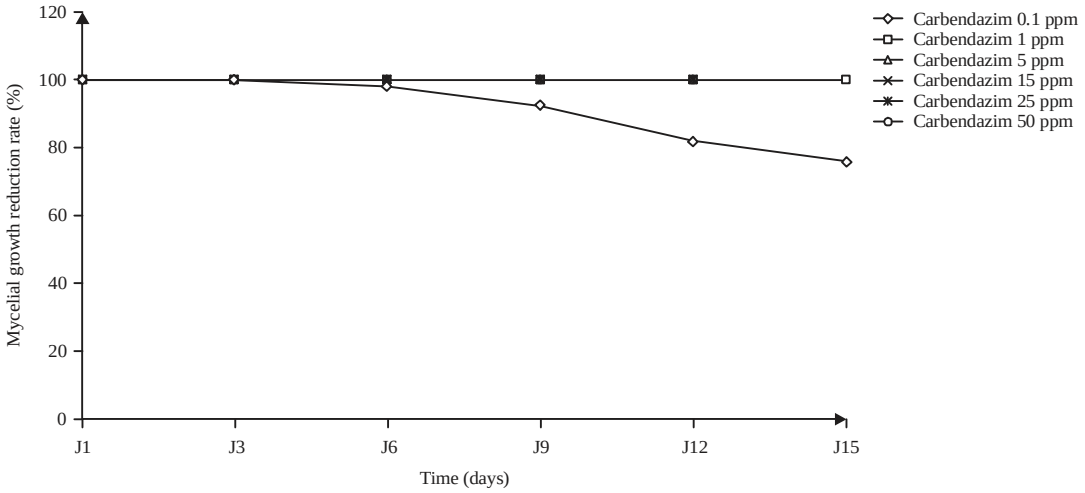

Fig. 1: Reduction of the mycelial growth of *Colletotrichum gloeosporioides* depending on the period and concentrations of Carbendazim

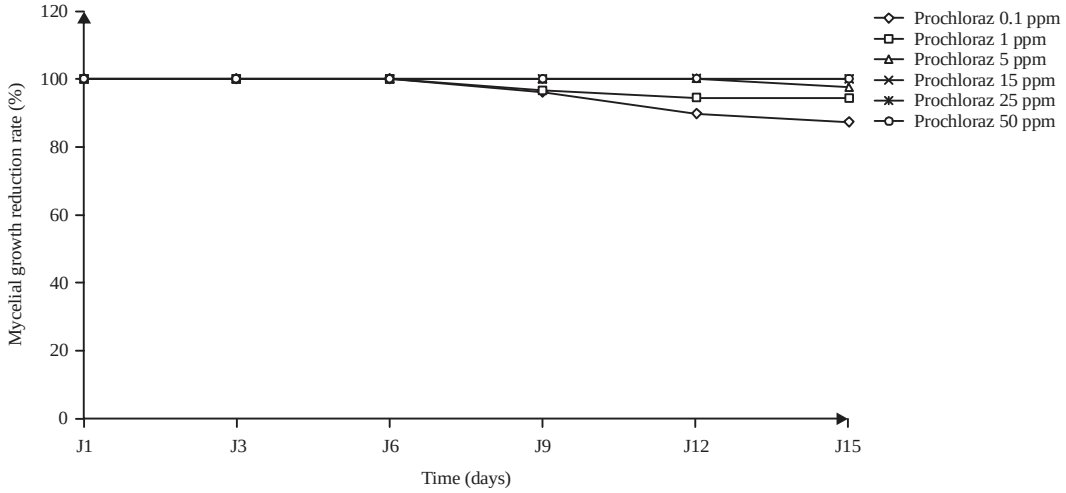

Fig. 2: Reduction of the mycelial growth of *Colletotrichum gloeosporioides* depending on the period and concentrations of Prochloraz

Table 2: Concentration of synthetic fungicides reducing by 50% the mycelial growth on the 15th day of *Colletotrichum gloeosporioides* cultures

Fungicides	IC_{50} (ppm)
Azoxystrobin	5.30
Carbendazim	<0.10
Cymoxanil+mancozeb	9.00
Mancozeb	45.05
Metalaxyl+Copper oxide	8.23
Prochloraz	<0.10
Propiconazole	4.20
Thirame	47.25

15 ppm. The concentration which reduced mycelial growth at 50% (IC_{50}) with these two fungicides was less than 0.1 ppm after 15 days culture (Table 2). Thus, according to the Edgington *et al.*[16] scale, Carbendazim and Prochloraz might be very effective because of the very high sensitivity of isolate SEKA76 of *Colletotrichum gloeosporioides* as these fungicides caused at 1 ppm a reduction rate of mycelial growth higher than 50%.

Propiconazole, Azoxystrobin, Metalaxyl-M+Copper oxide and Cymoxanil+Mancozeb combinations inhibited the growth of the fungus with inhibition rates of 92.74, 67.31, 64.32 and 68.80%, respectively on the 15th day of cultures. However, only Propiconazole completely inhibited the growth of the pathogen at 50 ppm concentration, which was the highest concentration used in this study (Fig. 3). Azoxystrobin at 25 and 50 ppm delayed mycelial growth until the 6th day

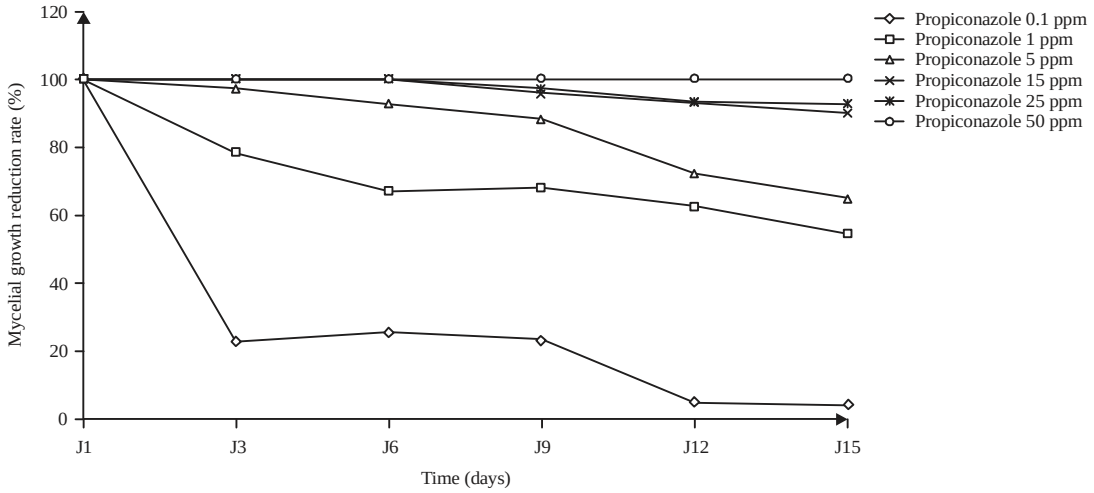

Fig. 3: Reduction of the mycelial growth of *Colletotrichum gloeosporioides* depending on the period and concentrations of Propiconazole

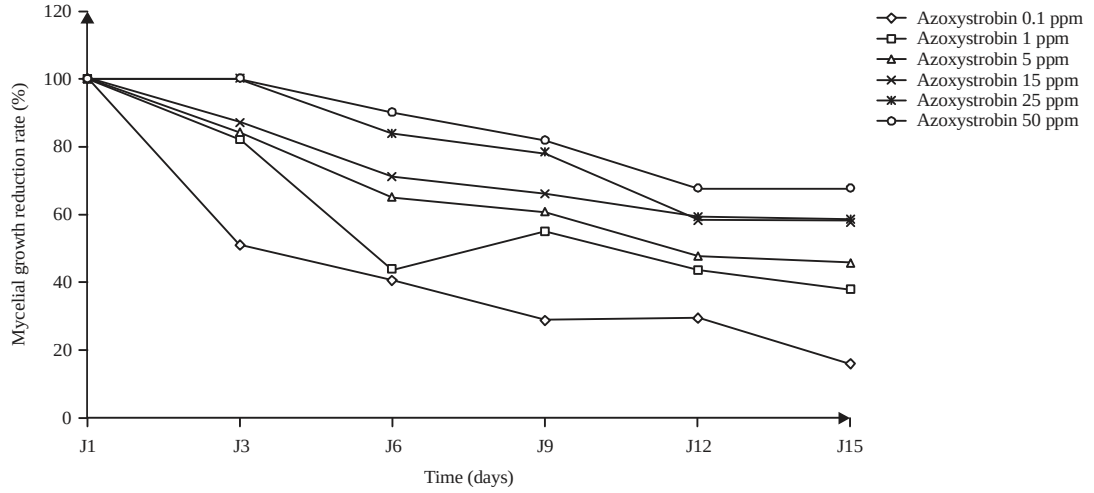

Fig. 4: Reduction of the mycelial growth of *Colletotrichum gloeosporioides* depending on the period and concentrations of Azoxystrobin

of cultures (Fig. 4) and concentration inhibiting 50% mycelial growth is 5.3 ppm. With the combination cymoxanil+mancozeb the growth of the fungus was delayed and started on the 6th day after culture for all concentrations except the 1 of 0.1 ppm. Concentrations of 15, 25 and 50 ppm impacted similarly the growth of the fungus. The same applied to concentrations of 0.1; 1 and 5 ppm (Fig. 5). All the concentrations of the combination métalaxyl+cooper oxide delayed mycelial growth until the 6th day of cultures with the exception of 0.1 ppm (Fig. 6). With this concentration, mycelial growth were recorded at third day of cultures.

Among the fungicides used, Mancozeb and Thirame were the least effective on the *in vitro* mycelial growth of the fungus. With these fungicides, inhibition of mycelial growth required higher concentrations than the other fungicides tested. Inhibition rates were nil (0.00%) on the 15th day of cultures at the concentration of 0.1 ppm with both fungicides. Mycelial growth reduction rates were 44.87 and 52.35%, respectively, for Mancozeb and Thirame at 50 ppm concentration on the 15th day of cultures (Fig. 7, 8). The concentrations required to reduce mycelial growth by 50% were 45.05 and 47.25 ppm, respectively for Mancozeb and Thirame (Table 2). These fungicides were poorly effective on the growth of isolate SEKA76 of *Colletotrichum gloeosporioides* which showed a low sensitivity to Mancozeb and Thirame.

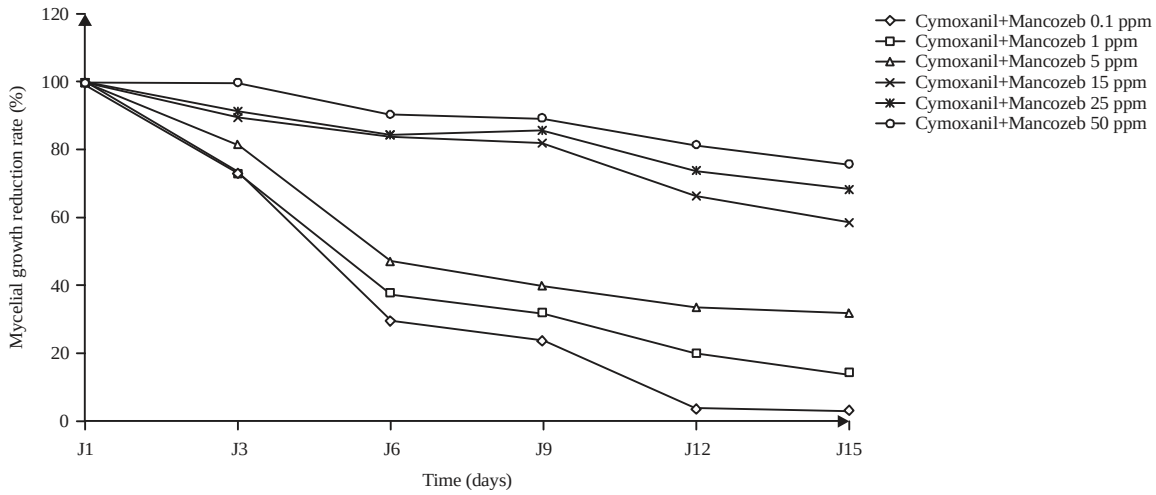

Fig. 5: Reduction of the mycelial growth of *Colletotrichum gloeosporioides* depending on the period and concentrations of Cymoxanil+Mancozeb

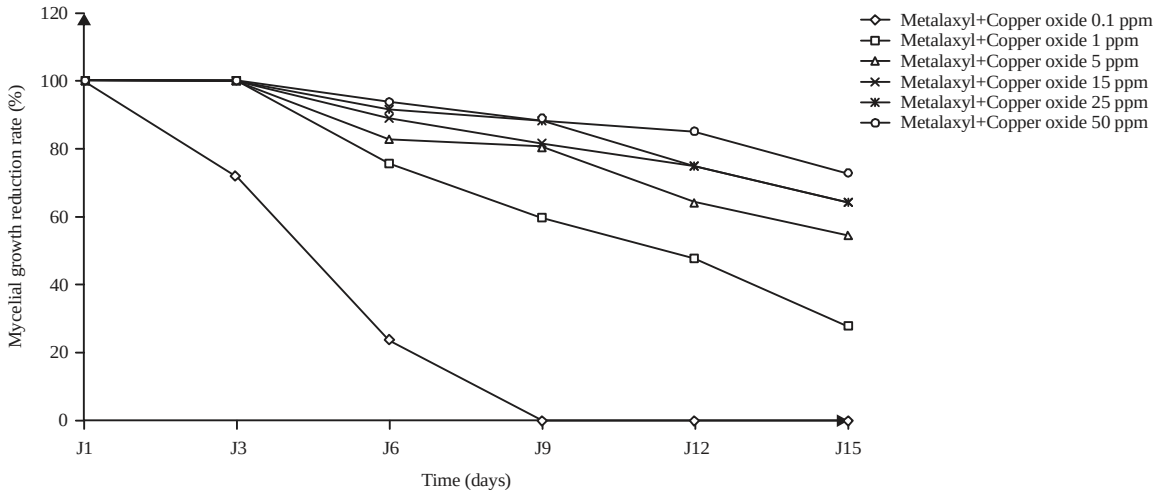

Fig. 6: Reduction of the mycelial growth of *Colletotrichum gloeosporioides* depending on the period and concentrations of Metalaxyl+Copper oxide

Effect of the different concentrations of fungicides on *Colletotrichum gloeosporioides* spores production: For all fungicide concentrations where fungus growth occurred, the presence of spores was noted. Spores were very abundant in media amended with 0.1 ppm of Azoxystrobin, Mancozeb, Cymoxanyl+Mancozeb and Metalaxyl+Copper oxide at concentrations of 0.1 and 1 ppm (Table 3). With Thirame the concentrations of 0.1, 1 and 5 ppm were the ones that made it possible to obtain a very great number of spores. Spores were produced in small quantities with Azoxystrobin and Metalaxyl+Copper oxide at 50 ppm, Carbendazim (1 ppm), Propiconazole (25 ppm) and Prochloraz at concentrations of 1.5 and 5 ppm. The absence of spores was noted with concentrations of Carbendazim, Prochloraz and Propiconazole which totally inhibited the growth of the fungus.

Effect of fungicides on spores germination: The results of the effect of fungicides and their concentrations on spore germination are summarized in Table 4. Generally, the rate of spore germination decreases with increasing concentration for all fungicides tested.

The combination of Cymoxanil+Mancozeb and Mancozeb were the most effective active ingredients. With these two fungicides, the rates of inhibition of germination were, respectively 69.29 and 50% at the concentration of 1 ppm.

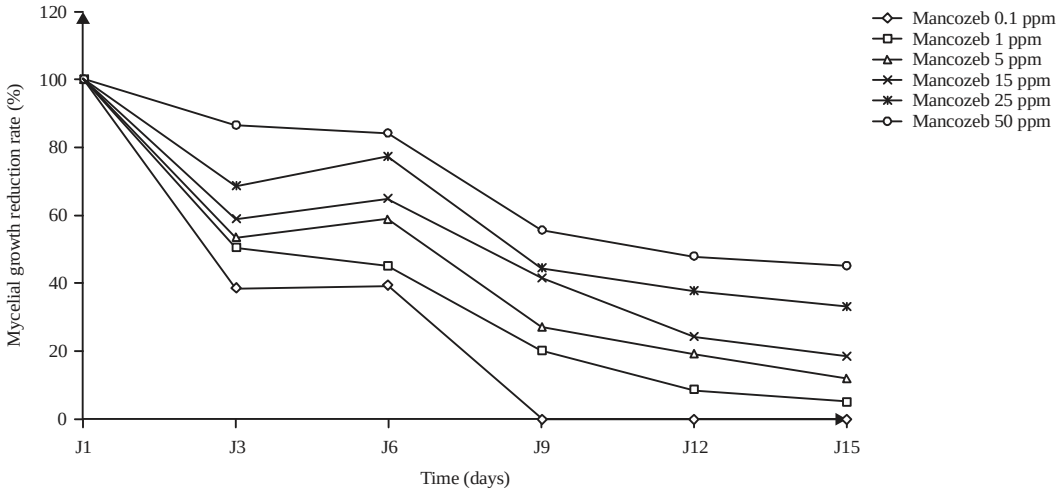

Fig. 7: Reduction of the mycelial growth of *Colletotrichum gloeosporioides* depending on the period and concentrations of Mancozeb

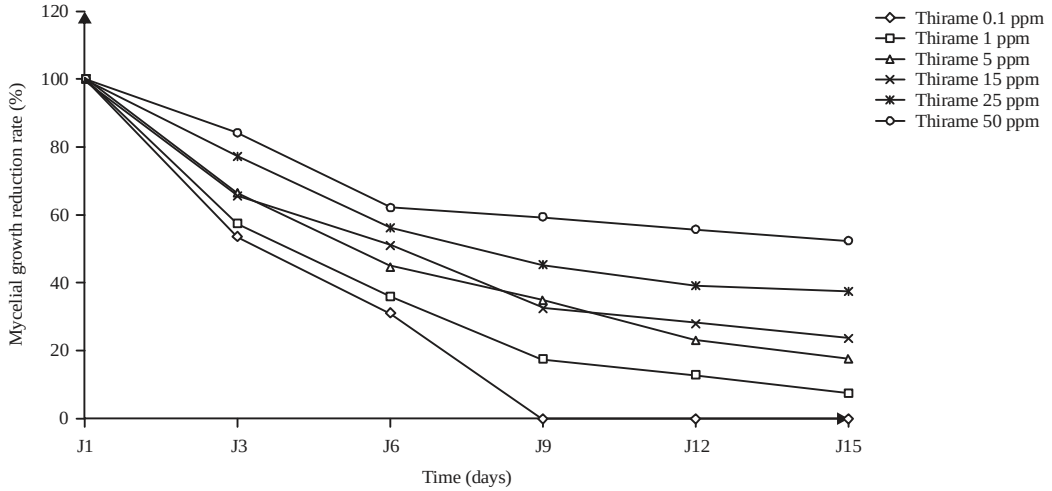

Fig. 8: Reduction of the mycelial growth of *Colletotrichum gloeosporioides* depending on the period and concentrations of Thirame

Table 3: Production of spores after 28 days of culture on PDA medium containing the synthetic fungicides at different concentrations

	Production of spores/concentration of fungicides					
Synthetic fungicides	0.1 ppm	1 ppm	5 ppm	15 ppm	25 ppm	50 ppm
Azoxystrobin	+++	++	++	++	++	+
Carbendazim	++	+	-	-	-	-
Cymoxanil+Mancozeb	+++	+++	++	++	++	++
Mancozeb	+++	+++	++	++	++	++
Metalaxyl+Copper oxide	+++	+++	++	++	++	+
Prochloraz	++	+	+	+	-	-
Propiconazole	++	++	++	++	+	-
Thirame	+++	+++	+++	++	++	++

+++: Very abundant, ++: Abundant, +: Less abundant, -: Absence of spores

From 5 ppm, all fungicides significantly inhibited spore germination. At a concentration of 10 ppm, the Cymoxanil+Mancozeb combination totally inhibited spore germination whereas, with Carbendazim the inhibition was

Table 4: Effect of different fungicides and combinations of fungicides on the germination of *Colletotrichum Gloeosporioides* spores

	Germination inhibition rate (%) per concentration of fungicides					
Synthetic fungicides	0.1 ppm	1 ppm	5 ppm	10 ppm	25 ppm	50 ppm
Azoxystrobin	15.00^{b}	21.43^{d}	32.86^{d}	48.57^{e}	62.14^{e}	83.57^{b}
Carbendazim	22.14^{b}	39.29^{bc}	50.00^{c}	65.71^{c}	100.00^{a}	100.00^{a}
Cymoxanil+Mancozeb	61.43^{a}	69.29^{a}	86.43^{a}	100.00^{a}	100.00^{a}	100.00^{a}
Mancozeb	25.71^{b}	50.00^{b}	75.00^{b}	89.29^{b}	95.71^{a}	100.00^{a}
Metalaxyl-M+Copper oxide	28.57^{b}	39.29^{bc}	47.86^{c}	64.29^{cd}	74.29^{c}	82.86^{b}
Prochloraz	23.57^{b}	39.29^{bc}	50.00^{c}	61.43^{cd}	82.86^{b}	100.00^{a}
Propiconazole	24.29^{b}	34.29^{c}	44.29^{c}	59.29^{d}	67.14^{d}	83.57^{b}

In the same column, the means followed by the same letter show no significant difference at 5% threshold according to the Newman-Keuls test

total at 25 ppm. Except for Azoxystrobin, Metalaxil-M+Copper oxide and Propiconazole, the concentration of 50 ppm was sufficient to completely inhibit spore germination with all the other fungicides.

DISCUSSION

All the fungicides tested had an effect on the three life stages of the fungus (mycelial growth, sporulation and spore germination). Carbendazim, Prochloraz, Propiconazole and Azoxystrobin were the most effective by inhibiting the mycelial growth and production of fungal spores. However, Carbendazim and Prochloraz have greatly reduced the growth and production of *in vitro* spores of *Colletotrichum gloeosporioides*. These two fungicides belong to the family Imidazoles which block cell division (mitosis) of the fungus by preventing the formation and proper functioning of the mitotic spindle[18]. These results has been in accordance with those of Filoda[19] and Chand *et al.*[20] who showed the effectiveness of Carbendazim in the control of *Colletotrichum gloeosporioides*. Indeed, the author in a study of the impact of three fungicides (Carbendazim, Azoxystrobin and Chlorothalonil) on the mycelial growth of *Colletotrichum gloeosporioides* causative agent of anthracnose in *Hypercium perforatum* L., showed that Carbendazim and Azoxystrobin at very low concentrations had a mycelial growth inhibition rate of more than 80%. Similar result were found by Sepiah[21], who reported that Prochloraz and Propiconazole were more effective than Benomyl and Thiabendazole on the control of *Colletotrichum* sp., from papaya and Chacko and Gokulapalan[22], who proved the efficacy of Propiconazole against *Colletotrichum capsici*.

Carbendazim inhibits the synthesis of beta-tubulin (a component of the cytoskeleton that imparts certain rigidity to cells). In the absence of beta-tubulin, fungi can no longer reproduce. This fungicide hinders thus the development of germinal tubes, appressorium formation and mycelium growth. However, authors reported resistant of *Colletotrichum gloeosporioides* developed under conditions of repeated Carbendazim application[23].

For Azoxystrobin, these findings are on the one hand contrary to those of Wong *et al.*[24], who reported an *in vitro* resistance of *Colletotrichum cereale* isolates and on the other hand consistent with many studies which proved the efficacy of the fungicide against *Colletotrichum* species[25-27].

The study of the influence of fungicides on the germination of *Colletotrichum gloeosporioides* spores revealed the effectiveness of the combination Mancozeb+Cymoxanil and Mancozeb which belong to the dithiocarbamate family. With these two fungicides, the inhibition rates obtained at 25 and 50 ppm concentrations were identified and showed no statistical difference. This family of fungicides (dithiocarbamates) impacts fungi by blocking thiol groups and by disrupting the metabolism of fungi by inhibiting either glucose oxidation or nucleic acid synthesis or by inhibiting degradation of fatty acids. Similar results were obtained by Mbaye *et al.*[28], who showed the effectiveness of contact fungicides including Mancozeb on spore germination as well as mycelial growth of *Colletotrichum gloeosporioides* isolated on mango as also Moses *et al.*[29] recommended in Pest management guid, the dithiocarbamates family fungicides to control cashew anthracnose. Also, Amin *et al.*[30] showed that combinations of Mancozeb seed treatment and Carbendazim foliar spray reduced anthracnose disease severity and increased seed yield of common bean.

The dithiocarbamates show a broad spectrum of antifungal activity. These fungicides might be moderately effective in the control of isolate SEKA76 of *C. gloeosporioides*[16]. However, spores germination inhibition require low concentrations while mycelial growth rate reduction need high concentrations of the fungicides.

CONCLUSION

In the search for effective cashew tree anthracnose control strategies in Côte d'Ivoire, the *in vitro* screening of different families of synthetic fungicides was carried out on the three stages of life of the fungus (*Colletotrichum gloeosporioides*) causative agent of the disease. Carbendazim, Prochloraz, Propiconazole and Azoxystrobin have proved to be more effective on mycelial growth and sporulation. However, Mancozeb and the combination Mancozeb+Cymoxanil and also Carbendazim gave the best results on the inhibition of spore germination. None of the fungicides tested specifically prevented one of the three stages of life (mycelial growth, sporulation and spore germination) from occurring. *In vivo* tests in greenhouse and plantation should be carried out to complete the results obtained.

SIGNIFICANCE STATEMENT

This study discovers that cashew anthracnose can be efficiently controlled with reasonable spray program of these fungicides.

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