



## **Control of Sugarcane Smut Disease: Evaluation of the *In vitro* Fungitoxicity of Three Essential Oils**

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### **Authors' contributions**

This work was carried out in collaboration among all authors. Authors YKJE and KKD designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors KKG, KKFJM and KD managed the analyses of the study. Authors NAC and CB managed the literature searches. All authors read and approved the final manuscript under the leadership of author KD.

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### **ABSTRACT**

The sugarcane smut disease caused by *Sporisorium scitamineum* occurs in all production areas. In order to contribute to the management of this disease, a study was carried out *in vitro* conditions. The aim of this study was to evaluate the antifungal efficacy and to determine the inhibitory concentration of the essential oils of *Ocimum gratissimum*, *Zingiber officinale* and *Cymbopogon citratus* upon two strains B and Z of *Sporisorium scitamineum*. These oils were tested on mycelial growth of colonies, spores' production and teliospores germination. The results showed a variable fungitoxic effect of the oils according to doses. Mycelial growth and spores production were totally inhibited (100%) from 1000 ppm by all the oils. The 90% inhibitory concentrations (IC90) obtained were 880.37 ppm, 847.83 ppm and 805.75 ppm for *Ocimum gratissimum*, *Zingiber officinale* and *Cymbopogon citratus*, respectively. The germination of

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teliospores was completely reduced by the three essential oils from 500 ppm upwards. Propiconazole had strong inhibitory effects at 400 ppm with a very low IC<sub>50</sub> and the IC<sub>90</sub> ranged from 305 ppm to 323.36 ppm. Moreover, at the level of the strains, no difference in resistance was observed. These oils could provide a good means of controlling sugarcane smut.

**Keywords:** *Essentials oils; in vitro; Sporisorium scitamineum; Côte d'Ivoire; Sugarcane.*

## 1. INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) is a monocotyledonous field crop cultivated in the tropical and subtropical regions of the world, mainly for its ability to accumulate a high concentration of sucrose. World production of sugar has reached 162 million tons from 5 countries such as Brazil, India, China, United States and Thailand [1]. It is coveted for markets such as biofuels, paper, chemicals, furniture and packaging [2]. In Côte d'Ivoire, sugarcane is cultivated on an area of 25,400 ha by four agro-industrial complexes specializing in planting and sugar production. Sugar production is estimated at 214,000 tons/year. Despite its contribution to the economy, many biological constraints contribute to limitate its production. Among these constraints the smut disease caused by *Sporisorium scitamineum* [3], a fungus belonging to the class Basidiomycetes [4]. Smut disease is spread in plantations by wind-borne teliospores dissemination. The development of the disease starts with the germination of teliospores to give spores [5], in contact with the stomata, leaf epidermis and axillary buds [6]. These spores constitute the dicaryotic phase that develops systemically throughout the stem and rapidly reach the apical meristem [7]. Subsequently, a long peripheral tissue is formed like a blackish whip causing the death of the plant [8]. However, complete suppression of the disease was not achieved [9]. Therefore, the search for an alternative control solutions at low cost without health risk for users and environment protection is necessary. Natural non-polluting substances have clear advantages over chemicals, as they are less harmful to the ecosystem, biodegrading *in situ* [10]. This study was carried out in the laboratory in order to highlight the antifungal effect of essential oils *in vitro* conditions on the development cycle of *Sporisorium scitamineum*, the causal agent, with a view to developing an effective control strategy.

## 2. MATERIALS AND METHODS

### 2.1 Extraction of Essential Oils

The plant material used for the production of the essential oils has been kept at the ambient

temperature of the laboratory. The oils were obtained from the fresh plant materials, by saturated steam training carried out with the Clevenger type device for 2 h [11]. This method consists of a conventional distillation in which the plant materials was not in direct contact with water. During the passage of steam, the cells are torn and release the essential oil. The oil is dragged to the condenser and then to the tank. At the end of the 2 h, the separation is done by decantation.

### 2.2 Effect of Essential Oils on Mycelial Growth

#### 2.2.1 Preparation of different doses and fungal cultures media

Four doses of the following essential oils and synthetic fungicide: 50, 100, 400 and 1000-ppm were tested. The propiconazole doses were taken from a 10000-ppm stock solution. The essential oils and synthetic fungicide were previously dissolved in Tween 20. The culture medium PDA (Potato Dextrose Agar) was used for inhibition tests. Then, after autoclaving at 121°C, 1 bar for 30 min, the essential oils or the synthetic fungicide used were incorporated after cooling. The medium was dispensed at a rate of 17 ml in 90 mm diameter Petri dishes. From a 7-day old monosporic culture of *Sporisorium scitamineum*, 6 mm explants were taken with a punch and seeded. For each dose, five Petri dishes were used. The control was carried out under the same conditions without essential oil or synthetic fungicide. All Petri dishes were incubated in the dark at room temperature (25°C±2) in the laboratory.

#### 2.2.2 Effects of essential oils on mycelial growth

The tests were carried out with two *Sporisorium scitamineum* strains, coded strains B and Z, from Borotou-Koro and Zuénoula respectively. These strains were collected from NCo376 and R570 respectively. Mycelial growth was assessed every 24 hours by measuring the 2 perpendicular diameters passing through the middle of the puck. These two diameters were averaged. Then

the mycelial growth inhibition rates (MIG) compared to the control were calculated according to the following formula:

$$\text{MIG (\%)} = \frac{(C - E) * 100}{C}$$

**C:** average growth (mm) in the control medium.  
**E:** average growth of the fungus (mm) in the culture medium at concentration of the synthetic essential oil or fungicide. The minimum inhibition concentrations reducing 50 p.c (IC50) and 90 p.c (IC90) were determined using ED50v10 software.

### 2.2.3 Fungitoxicity of the essential oils

The doses of essential oils for which no mycelium growth was observed, were subjected to a fungitoxicity test. A new PDA culture medium was prepared without a single addition of essential oils or fungicide. The explants used for seeding in the efficacy tests were recultured and incubated under the same growth conditions. Mycelial growth was assessed for seven days. If growth was resumed, the dose is said to be fungistatic, otherwise it is said to be fungitoxic for the pathogen.

### 2.2.4 Effects of essential oils on spores' germination

From an identified strain, a culture was made for 21 days on PDA medium. The mycelial thallus was scraped off using a sterilized Pasteur pipette. The solution was placed in a test tube and vortexed to release the spores. A counting was performed using the Malassez hematimeter. The counting was reduced to a concentration of 106 spores/ml, after this dilution series. This suspension (1 ml) was sampled and spread in a Petri dish containing a PDA medium with the various concentrations (50, 100, 400 and 1000-ppm). Three replicates were made for each product concentration. The control was carried out under the same conditions without essential oils or synthetic fungicide. Using a microscope, the number of germinated spores was evaluated on 100 spores. The counting was carried out after 6, 12 and 24 hours. The germination inhibition rate (GIR) was calculated by the following formula:

$$\text{GIR (\%)} = \frac{(S0 - S) * 100}{S0}$$

**S0:** Germination rate in the control medium;  
**S:** Germination rate in the media with the different doses.

## 2.3 Effect of Essential Oils on Spores' Production

After 21 days, three mycelial explants were taken from the boxes used for the mycelial growth tests. These explants were placed in a test tube containing 15 ml of sterile distilled water and vortexed for 15 seconds. The suspensions obtained were used for spores counting. Five counts per suspension were made using the Malassez hematimeter. The counting was brought back per unit area (mm<sup>2</sup>) in 1 ml. The sporulation inhibition rate was thus determined by the formula [12]:

$$\text{SIR (\%)} = \frac{(\text{NSo} - \text{NSt}) * 100}{\text{NSo}}$$

**NSo:** Average number of spores in the control;  
**NSt:** Average number of spores in treated boxes.

## 2.4 Assessment of the Teliospores' Germination

### 2.4.1 Preparation of the growth medium

An agar growth medium was used for germination tests [13]. After sterilization at 121°C, 1 bar for 30 min, the medium was dispensed at 17 ml in 90 mm diameter Petri dishes. After cooling, essential oils or synthetic fungicide were then incorporated.

### 2.4.2 Preparation of the teliospores suspension

The smutted whips collected seulement on the cultivar NCo376 and stored at the same room temperature for 5 days. A whip fragment (2 cm) was collected and placed in a tube containing 5 ml of sterile distilled water. The tubes were vortexed in a 5-second sequence for 15 seconds to detach the teliospores. They were then counted with the Malassez hematimeter to determine the initial density. This density was adjusted to 5.10<sup>5</sup> teliospores/ml after dilution with sterilized distilled water.

### 2.4.3 Effects of essential oils on the teliospores germination

A suspension of 0.1 ml with a density of 5.10<sup>5</sup> teliospores/ml was spread in Petri dishes at five repetitions per dose. The oils and synthetic fungicide were evaluated at 200 ppm, 500 ppm and 1000 ppm. Five control Petri dishes were also made under the same conditions, but

without fungicide and essential oils. Petri dishes were incubated in the dark at room temperature. The evaluation of germination rate of teliospores was done according to Santiago method [14] with slight modifications. The numbers of germinated teliospores was counted every 24 hours using Amscope software, connected to a computer. The trial was conducted over a 1 month period. The teliospores germination rate (TGT) was calculated using the following formula :

$$TGT = \frac{(T - S) * 100}{T}$$

T: number of teliospores on the medium ;  
S: number of germinated teliospores

### 3. RESULTS

#### 3.1 Average Inhibition Rate of Three of the Essential Oils on Mycelial Growth

Analysis of variance showed a highly significant effect ( $P = 0.05$ ) between the inhibition rates, but no difference in susceptibility was observed between the two strains (B and Z). Thus, inhibition rates varied with doses and test material. From the 1000-ppm dose onwards, the three essential oils and propiconazole completely (100%) inhibited the mycelial growth of the strains (Fig. 1). At 400-ppm, *Cymbopogon citratus* essential oil and propiconazole had the highest inhibition rates with 82 p.c and 100 p.c respectively. However, *Ocimum gratissimum* and

*Zingiber officinale* oils had lower levels with 67.86 p.c and 64 p.c respectively. At 100-ppm and 50-ppm, the three essential oils had statistically identical rates varying 50 p.c between 62.86 p.c and 100-ppm and between 44.65 p.c and 49.94 to 50-ppm (Fig. 1). However, at the same dose, propiconazole showed a high level of inhibition well above the essential oils with values of 77 p.c and 74 p.c respectively.

#### 3.2 Mycelial Growth Inhibition Concentration

The analysis showed a significant difference between the IC50 and IC90 of the products (Table 1). Propiconazole obtained low IC50 and IC90 values ranging between  $10^{-4442}$ -ppm and  $10^{-4252}$ -ppm for the IC50 and between 305-ppm and 323.36-ppm for the IC90, respectively. The three essential oils obtained statically identical inhibition concentrations (IC50 and IC90). In *Ocimum gratissimum* oil, the IC50 values ranged from 140.76-ppm to 218.0-ppm and the IC90 values ranged from 821.69-ppm to 880.37-ppm in strains Z and B respectively. With essential oil of *Cymbopogon citratus*, the IC50 values are between 149.6 ppm and 173.8 ppm and the IC90 values are between 805.75 ppm and 813.9 ppm in strains B and Z respectively. In *Zingiber officinale* oil, the IC50 values ranged from 191.08-ppm to 238.23-ppm for strains B and Z and the IC90 values ranged from 847.83-ppm to 861.80-ppm in strains B and Z.

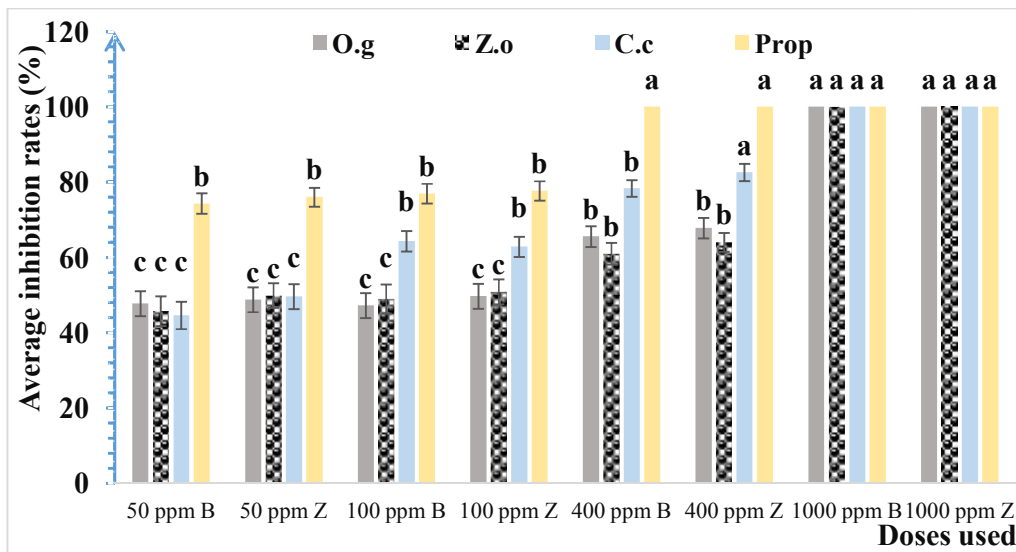


Fig. 1. Mean mycelial growth inhibition rates

\*C.c = *Cymbopogon citratus* ; O.g = *Ocimum gratissimum* ; Z.o = *Zingiber officinale*

**Table 1. IC50 and IC90 comparison for mycelial growth of propiconazole and three essential oils**

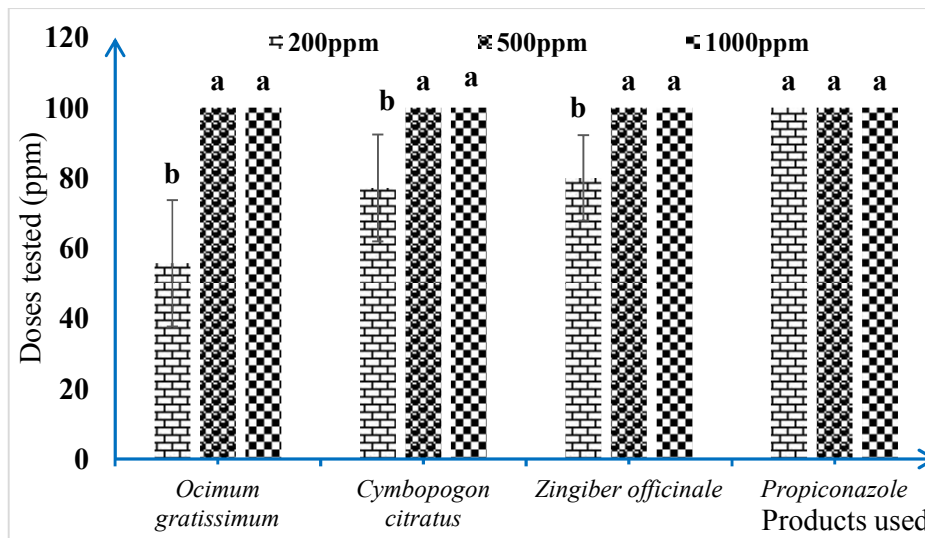
Products	Strain B		Strain Z	
	IC50 (ppm)	IC90 (ppm)	IC50 (ppm)	IC90 (ppm)
<i>Ocimum gratissimum</i>	218,01b	880,37b	140,76b	821,69b
<i>Zingiber officinale</i>	238,23b	847,83b	191,089b	861,80b
<i>Cymbopogon citratus</i>	149,67b	805,75b	173,82b	813,9b
Propiconazole	10 <sup>-4252</sup> a	323,36a	10 <sup>-4442</sup> a	305,30a

Means followed by the same letters in the same column are not significantly different at P=0.05% according to Newman-Keuls Post-hoc test

**Table 2. Spores production as a function of essential oils and propiconazol**

Products	50-ppm	100-ppm	400-ppm	1000-ppm
<i>Ocimum gratissimum</i>	51,4.105 ±11,49 c	42,25.105 ±11,63 b	30,4.105 ±15,8 c	0a
<i>Zingiber officinale</i>	42,6.105 ± 4,96c	40,2.105 ±11,12 b	37,6.105 ± 11,63 c	0a
<i>Cymbopogon citratus</i>	39,6.105 ± 15,82c	14,5.105 ± 3,34 a	12,0.105 ± 1,68 b	0a
Propiconazole	13,5.105 ± 2,64 a	11,85.105 ± 1,14 a	0a	0a
Témoin	58,25.105 ±6,18c			

Means followed by the same letters in the same column are not significantly different at p=0.05 according to Newman-Keuls Post-hoc test



**Fig. 2. Inhibition rates of teliospores germination of *Sporisorium scitamineum***

### 3.3 Effect of the Products on Spores Production

The analysis revealed a very significant difference between the numbers of spores depending on the oils and tested doses (Table 2). With *Ocimum gratissimum* oil, spores production was reduced. The spores number decreased from 51.4\*10<sup>5</sup> spores/ml to 30.4\*10<sup>5</sup> spores/ml, respectively for concentrations from 50-ppm to 400-ppm before nulling at 1000-ppm. In *Cymbopogon citratus* essential oil, spores

production was highly reduced. Spores number decreased from 39.6\*10<sup>5</sup> at the 50-ppm concentration to 12.0\*10<sup>5</sup> at 400-ppm. Spores concentration was zero at 1000-ppm. The same effects were observed with *Zingiber officinale*, the spore number increased from 42.6\*10<sup>5</sup> to 37.6\*10<sup>5</sup> for 50-ppm and 1000-ppm respectively. At 1000-ppm, spores production was stopped. The synthetic fungicide strongly inhibited spores production from the 50-ppm onwards (13.5\*10<sup>5</sup> spores/ml) and was cancelled at 400ppm (Table 2).

### 3.4 Effect of Essential Oils on the Teliospores Germination

There was a highly significant effect of oils ( $p=0.05$ ) on germination rates. Doses of 500 and 1000-ppm of essential oils resulted in total inhibition of teliospores germination. But at the dose of 200-ppm, the inhibition rates of teliospores germination varied according to the essential oils (Fig. 2). Rates of 55.71, 77.14 and 80 p.c were recorded for *Ocimum gratissimum*, *Zingiber officinale* and *Cymbopogon citratus* oils, respectively. With propiconazole, teliospores germination was totally (100%) inhibited with the three doses of 200-ppm, 500-ppm and 1000-ppm (Fig. 2.)

### 4. DISCUSSION

The essential oils of the plant species were subjected to efficacy test under *in vitro* conditions on *Sporisorium scitamineum* strains. The results showed that the three essential oils of *Ocimum gratissimum*, *Cymbopogon citratus* and *Zingiber officinale* totally inhibit mycelial growth and germination of teliospores of *Sporisorium scitamineum* from of 500-ppm. In addition, at the same dose, the oils also reduced the sporulation of the pathogen. These oils acted on all the different stages of development of *Sporisorium scitamineum* the causal agent of smut disease at low doses. This shows that the essential oils have antifungal effects and would be effective against smut disease at the field. Our results corroborate those obtained by [15], under *in vitro* conditions on this same pathogen. Moreover, the fungicidal effect of *Cymbopogon citratus* oil had been demonstrated by [16] and [17]. Regarding the oil of *Zingiber officinale*, its effect had been proven by the work of [18] by tests on the causal agent of anthracnose disease in mango fruits. As for the efficacy of *Ocimum gratissimum* essential oil, it has been reported by several authors [19,20,12] on different pathogens. During its development cycle on culture media, teliospores germinate to produce a promycelium with four haploid cells [21]. Each of these cells forms a sporidium. Essential oils alter the permeability of the membranes, denature and precipitate the proteins of these cells [22]. These oils have similar effects to the synthetic fungicide. This demonstrates that chemicals can be substituted by essential oils for the control of this pathogen. Indeed, chemicals are widely used for the control of smut disease [23]. Yet these products are expensive and contribute to an increase in the cost of sugarcane production. Also, they are

found to be carcinogenic and dangerous for users [24]. The use of oil-based formulations would be an effective and sustainable option in the control of sugarcane smut disease with the advantage of being economical and non-toxic to farmers, consumers and the environment [25]. It could replace propiconazole, which remains expensive and with high persistence.

### 5. CONCLUSION

The plant species used showed a richness in essential oils. These essential oils showed a strong fungicidal effect, at low concentrations, on mycelial growth, spores production and germination of teliospores of *Sporisorium scitamineum*. Therefore, this oil can be exploited as an ideal alternative to synthetic fungicides against smut disease. However, further work on the sanitation of cuttings under controlled conditions using essential oils should be carried out in order to justify the actual use of these essential oils.

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### COMPETING INTERESTS

Authors have declared that no competing interests exist.

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