Evaluation of a biopesticide containing essential oils from Ocimum gratissimum L., Cymbopogon citratus L., and Zingiber officinale Roscoe, and propiconazole, on the growth of strains of Sporisorium scitamineum, the causative agent of smut disease, collected in Côte d'Ivoire.

Abstract

Sugarcane is a tropical plant cultivated for its sugar. It is an important crop in Côte d'Ivoire, where it is used for the production of sugar, ethanol, and other byproducts. Despite its contribution to the country's economy, its cultivation is subject to numerous biological constraints that limit its production. Among these threats is charcoal disease. The objective of this study is to determine the effect of essential oils (*Ocimum gratissimum* L., *Cymbopogon citratus* L., and *Zingiber officinale Roscoe*) compared to the fungicide propiconazole on the life cycle of S. scitamineum in order to identify the doses that could help control charcoal disease. The various essential oils were extracted by hydrodistillation using a Clevenger apparatus for 3 hours. The oils were subsequently stored in a refrigerator at a temperature of 12°C. Four doses of essential oil and synthetic fungicide—50, 100, 400, and 1000 ppm—were tested. During this study, the effect of the oils and synthetic fungicide on mycelial growth, spore production activity, and teliospore germination was evaluated. At the conclusion of this study, it should be noted that the essential oils of Ocimum gratissimum, Cymbopogon citratus, and Zingiber officinale offer real potential for controlling sugarcane smut in Côte d'Ivoire.

Keywords: biopesticide; essential oil; fungicide; anthracnose; Côte d'Ivoire

1. Introduction

Sugar cane (*Saccharum officinarum*) is cultivated in Côte d'Ivoire on an area of 25,400 hectares by four agro-industrial complexes specializing in sugarcane planting and sugar production. It accounts for 0.9% of GDP, 3.3% of the agricultural sector, and provides more than 5,000 jobs (Kouame et al., 2010). It ranks 53rd globally and 16th in Africa (FAO, 2005) with an estimated production of 214,000 tons of sugar. However, despite its contribution to

the economy, numerous biological constraints limit its production. Among these threats is charcoal disease, first reported in Natal, South Africa (McMartin, 1961). The causative agent of this disease is *Sporisorium scitamineum* (Pipenbring, 2002). It belongs to the class Basidiomycetes, subclass *Ustilagimoncetidae*, order *Ustilaginales*, family *Ustilaginaceae*, genus *Sporisorium* (Thaung 2005). It causes significant damage in Africa (Wada, 2003; Yoseph et al., 2008). This fungus disrupts growth in the host plant, leading to severe yield losses. Losses due to smut thus range from 20 to 30% of the final yield (Frison and Putter, 1993). The disease reduces sugarcane yield (Singh et al. 2004) and affects both plant growth and juice quality (Martínez et al. 2000). The fungus spreads from one plant to another through the dispersal of teliospores. Subsequently, when environmental conditions are favorable, the teliospores germinate to produce spores (Singh et al. 2004). These spores then form dikaryotic mycelia, which constitute the parasitic phase (Agnitori 1990; Moosawi-Jorf et al. 2006). The main entry points into the plant are the stomata, the leaf epidermis (Santiago et al. 2008b), and the buds (Alexander and Ramakrishnan 1980). From these entry points, the pathogen spreads systemically throughout the stem and rapidly reaches the apical meristem (Alexander and Ramakrishnan 1980). However, teliospores are formed only in the peripheral tissues of the long, whip-like structure. Pest control methods were the subject of studies by Agnihotri (1983) and Ferreira and Comstock (1989). They demonstrated that treating cuttings in hot water and spraying fungicides reduce charcoal disease. Srinivasan and Rao (1968) reported that hot water treatment causes bud germination losses due to temperature. Bhuiyan et al. (2009) demonstrated that the use of propiconazole reduces the spread of the disease. However, methods for controlling fungal diseases have been developed using natural substances. The studies by Camara et al. (2007) and Kassi et al. (2014) demonstrated that essential oils possess antifungal properties against parasites. The study by Kouame et al. (2018) showed that essential oils inhibit the mycelial growth of *Sporisorium scitamineum*. Furthermore, Sánchez Rodriguez et al. (2002) demonstrated that essential oils are biodegradable, non-polluting, and non-harmful to the ecosystem. The objective of this study is to determine the effect of essential oils on the life cycle of S. *scitamineum* in order to identify the doses that could help control anthracnose.

2. Materials and Methods

2.1. Materials

2.1.1. Fungal material:

Two strains of *Sporisorium scitamineum* (strains B and Z) were isolated from anthracnose-infected shoots of the varieties NCO376 and R570 originating from Borotou-Koro (08 30 881 and 007 16 578) and Zuénoula (07 36 516 and 006 11 713), respectively.

2.1.2. Products Used

The products used in this study include a synthetic fungicide and three essential oils. The fungicide contains the active ingredient propiconazole (250 g/L), which belongs to the triazole family, and is tested and marketed by Callivoire. It is used as a curative treatment. The three essential oils used were extracted from the leaves of *Ocimum gratissimum* L. (*Lamiaceae*) and *Cymbopogon citratus* L. (*Poaceae*) harvested in Abidjan (Ivory Coast) and from the rhizomes of Zingiber officinale Roscoe (*Zingiberaceae*) harvested in Divo (Ivory Coast).

2.2. Methods

2.2.1. Extraction and evaluation of essential oil yield

The plant material used for oil production was stored in the laboratory at room temperature. The mass of the plant material was measured using a balance before filling the pressure cooker. The essential oils were obtained by hydrodistillation using a Clevenger apparatus for 3 hours (Oussou, 2009). The oils were collected in vials by decantation every 25 minutes (Laghchimi et al., 2014). At the end of the 3-hour period, the essential oil yield (Yield) was determined by calculating the ratio of the mass of the oil obtained to the mass of the plant material using the following formula (Kouame et al., 2018):

$$Rdt\ (\%) = \frac{(Mass\ of\ oil\ *\ 100)}{mass\ of\ plant\ material}$$

The oil vials were covered with aluminum foil to protect them from any adverse effects of light. The oils were then stored in the refrigerator at a temperature of 12°C. The tests were repeated five times under the same conditions.

2.2.2. Preparation of fungal cultures and different doses for the tests

Four doses of essential oil and synthetic fungicide—50, 100, 400, and 1000 ppm—were tested. The propiconazole doses were prepared from a 10,000 ppm stock solution. The essential oils and synthetic fungicide were first dissolved in Tween 20 before being added to the culture medium and then stirred. The essential oils or synthetic fungicide used were incorporated into the PDA (Potato Dextrose Agar) culture medium after autoclaving at 121°C, 1 bar for 30 min. They were dispensed at a volume of 17 ml into 90-mm diameter Petri dishes. For each concentration, five Petri dishes were inoculated with a 6-mm diameter disc taken from the margin of a 7-day-old monospore culture of *Sporisorium scitamineum*.

The inoculated Petri dishes were incubated at 28°C under a 12/12 light-dark cycle. The control was conducted under the same conditions without essential oils or synthetic fungicides.

2.2.3. Effect of oils and synthetic fungicides on mycelial growth:

Mycelial growth was assessed every 24 hours by measuring the two perpendicular diameters passing through the center of the disc. The average of these two diameters was calculated. Growth inhibition rates (GIR) relative to the control were then calculated using the following formula : $$TIC\ (\%) = \frac{(T - E) * 100}{T}$$

T: average growth (mm) in the control medium. E: average growth of the fungus (mm) in the culture medium at concentration (c) of the essential oil or synthetic fungicide.

2.2.4. Effect of oils and synthetic fungicides on spore production activity

From each Petri dish used to measure mycelial growth, three 6-mm-diameter explants were taken and placed in a test tube containing 5 mL of sterile distilled water. The tubes were shaken in 3-second intervals for 15 seconds to detach the spores from the conidia. The resulting suspensions were filtered through muslin to remove mycelial fragments. Spore counts were performed using a Malassez slide, with ten counts per suspension. The number of spores per unit area (mm^2) was counted, followed by the number of particles per 1 ml.

2.2.5. Effect of oils and synthetic fungicides on teliospore germination

A smut whip collected from the NCO376 variety grown in a greenhouse in Bingerville. A piece of the whip was taken and placed in a tube containing 3 mL of sterile distilled water. The teliospores were released after agitation and counted using a Malassez hemometer to determine the initial density, which was then adjusted to 105 spores/ml after dilution. A 0.1-ml suspension was spread in Petri dishes containing a gelled culture medium (Rhayour et al., 2003), distributed at a rate of 17 ml in 90-mm-diameter Petri dishes. Three doses of essential oil and synthetic fungicide—200, 500, and 1000 ppm—were tested.

Propiconazole solutions were prepared from a stock solution of 10,000 ppm. The essential oils and synthetic fungicide were first dissolved in Tween 20 before being added to the culture medium and mixed. The essential oils or synthetic fungicide used were incorporated into the culture medium. Five replicates were conducted for each concentration. Control plates were also prepared without fungicides or oils. The Petri dishes were incubated in the dark at 28°C. The germination rate of the teliospores was assessed according to the method of Santiago et

al. (2010) with slight modifications. The number of germinated teliospores was counted every 24 hours using Amscope software installed on a computer. For each concentration, 500 teliospores were counted. The experiment lasted for 1 month. The teliospore germination rate was calculated using the following formula: $TGT = \frac{(T - S) * 100}{T}$

T: number of germinating teliospores in the control medium; S: number of germinating teliospores in the culture medium at concentration (c) of the essential oil or synthetic fungicide.

3. Results

3.1. Essential oil yields and proportions obtained over time

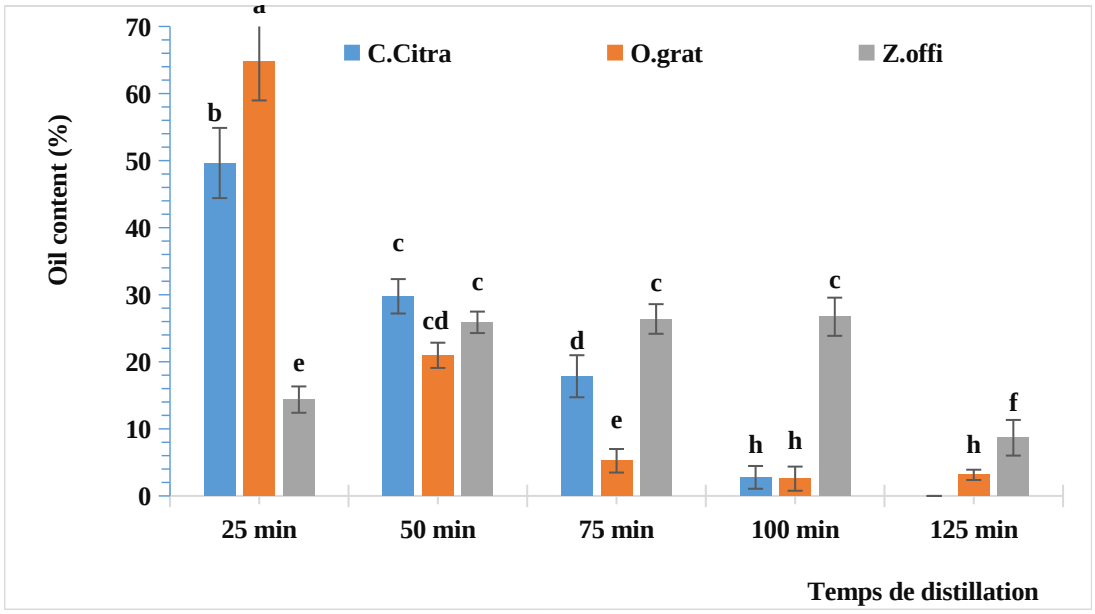
Table I shows the yields of the essential oils obtained. The highest yields were obtained from Ocimum gratissimum and Cymbopogon citratus, at 0.86% and 0.64%, respectively. The lowest yield was obtained from Zingiber officinalis, at 0.4%. Figure 1 shows the oil yields as a function of time. The analysis showed that the volumes of oil collected decrease over time. The essential oil of Ocimum gratissimum yielded the highest volumes of oil after 25 and 50 minutes of distillation, representing yields of 63% and 25%, respectively. From 75 to 125 minutes of distillation, the volumes obtained were low and essentially identical, ranging from 10% to 2%. The quantities of Cymbopogon citratus oil obtained after 25 minutes, 50 minutes, and 75 minutes were the highest, with proportions of 50%, 25%, and 20%, respectively. Low proportions were recorded after 100 and 125 minutes of distillation, at 3% and 2%, respectively. The amounts of Zingiber officinalis oil obtained after 50, 75, and 100 minutes were statistically identical, representing a proportion of 26%. A proportion of 8% was recorded at 125 minutes.

Table I: Essential oil yield from three plant species

Plant species used (%)	Average yield (%)
Cymbopogon citratus	0,64 ± 0,134 a
Ocimum gratissimum	0,86± 0,166 a

Zingiber offinalis	0,40± 0,024 b

Means followed by the same letters in the same column and row are not significantly different at the 5% level according to the Newman-Keuls test



Histograms marked with the same letters in the same column and row are not significantly different at the 5% significance level according to the Newman-Keuls test

Figure 1. Proportion of the three essential oils collected by distillation at different time points

3.2. Effect of essential oils on mycelial growth

The sensitivity of Sporisorium scitamineum varied depending on the doses of the products used and the time elapsed. Ocimum gratissimum reduced growth in a dose-dependent manner. The 1000 ppm dose completely inhibited mycelial growth. The 400ppm dose showed inhibition below 65% until the 5th day. As for the 50 and 100 ppm doses, they achieved low inhibition rates that decreased over time, from 50% to 36%. All doses of Z. officinalis Roscoe reduced mycelial growth. The 1000 ppm dose had the highest inhibition rate of 100%. At a concentration of 400 ppm, mycelial growth was reduced by 50%. However, the 50 and 100 ppm doses showed low inhibition rates. The doses of Cymbopogon citratus used demonstrated the ability to reduce mycelial growth. Cymbopogon citratus oil induced a significant reduction in growth at 400 and 1000 ppm. The inhibition rates obtained at 400 ppm ranged from 80% to 66% between the 2nd and 8th day, and at 1000 ppm, the reduction was complete (100%) up to the 8th day. The 50- and 100-ppm doses maintained a reduction rate of over 50% until the fourth day, before decreasing to 23% and 31%, respectively.

Propiconazole showed high inhibition rates at all doses tested. At 400 and 1000 ppm, the inhibition rate was 100%; at concentrations of 50 and 100 ppm, the inhibition rate remained above 80% until the 8th day.

Jour means day

Figure 2: Changes in the inhibition rates of essential oils and propiconazole on mycelial growth over time.

The figure presents the inhibition rates of five doses of four products on the mycelial growth of two strains of Sporisorium scitamineum under in vitro conditions. Analysis of variance revealed a highly significant effect ($p \leq 0.01$), and the Newman-Keuls test allowed for comparison of inhibition rates across different groups. The highest inhibition rates for the four products were observed at 1000 ppm; the three essential oils and the fungicide completely inhibited mycelial growth of both strains, resulting in a 100% inhibition rate. The lowest inhibition rates were obtained at 50 and 100 ppm for the essential oils. Ocimum gratissimum had inhibition rates ranging from 47.79% to 48.82% and 47.27% to 49.75%, respectively, for strains B and Z at 50 and 100 ppm.

Zingiber officinalis oil yielded concentrations ranging from 45.81% to 49.94% and from 49.04% to 50.93% ppm, respectively, for strains B and Z at these same doses. As for Cymbopogon citratus, the recorded levels ranged from 44.65% to 49.64% and from 64.37% to 62.86%. Propiconazole had the highest levels at 50 and 100 ppm, with values of 74.36% and 77.73%, respectively. At 400 ppm, the rates for Ocimum gratissimum and Zingiber officinalis

oils were nearly identical, ranging from 65.06% to 67.86%; with 61.04% and 64.06% respectively for each strain, while Cymbopogon citratus achieved the highest rates at 78.38% for strain B and 82.63% for strain Z. The fungicide propiconazole exhibited 100% inhibition at a dose of 400 ppm.

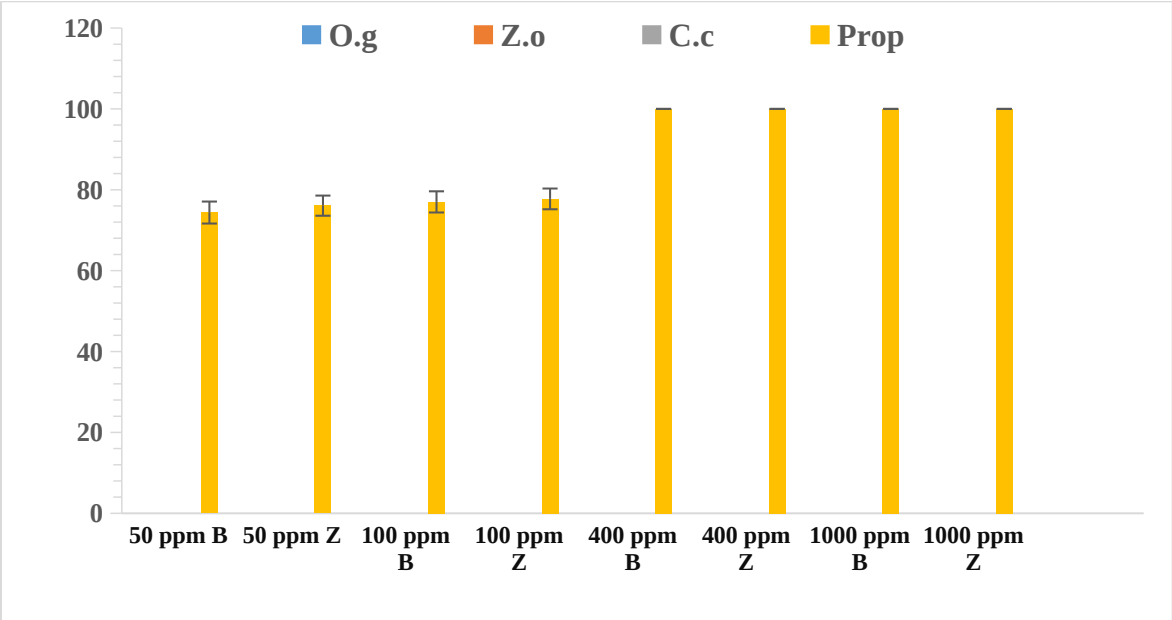


Figure 3: Average inhibition rates of three essential oils and propiconazole on two strains of Sporisorium scitamineum

3.3 Effect on spore production

Ocimum gratissimum oil produced the highest spore concentration at 50 ppm, with 51.4 × 10^5 spores/ml, and was completely inhibited at 1,000 ppm. Intermediate concentrations were obtained at 100 and 400 ppm, with 42.25 × 10^5 spores/ml and 30.4 × 10^5 spores/ml, respectively. Zingiber officinalis doses induced the highest spore production at 50 ppm and 100 ppm, with concentrations of 42.6 × 10^5 spores/ml and 40.2 × 10^5 spores/ml, respectively, and none at 1000 ppm. The 400 ppm dose produced an intermediate concentration of 37.6 × 10^5 spores/ml. Cymbopogon citratus had the highest spore concentration at 50 ppm with 39.6 × 10^5 spores/ml and was negligible at 1000 ppm. The concentrations obtained at 100 and 400 ppm are roughly equal, at 14.5 × 10^5 spores/ml and 12 × 10^5 spores/ml, respectively. As for propiconazole, it yielded low spore concentrations compared to the control at 50 and 100 ppm, with 13.5 × 10^5 spores/ml and 11.85 × 10^5 spores/ml, respectively, and was ineffective at 400 ppm.

Table II : Pore volume of the product as a function of the oils and the concentrations of three essential oils and propiconazole.

	50 ppm.	100 ppm.	400 ppm.	1000 ppm.
Ocimum gratissimum	51,4 ±11,49 c	42,25 ±11,63 b	30,4 ±15,8 b	0
Zingiber officinale	42,6 ± 4,96 b	40,2 ±11,12 b	37,6 ± 11,63 b	0
Cymbopogon citratus	39,60 ± 15,82 b	14,5 ± 3,34 a	12,0 ± 1,68 a	0
Propiconazole	13,5 ± 2,64 a	11,85 ± 1,14 a	0	0
Témoin	58,25 ± 6,18 c	58,25 ± 6,18 c	58,25 ± 6,18 c	58,25 ± 6,18 c

3.4 Changes in Germination Rates of Three Essential Oils and Propiconazole Over Time

The figure shows changes in germination rates over time for three essential oils and propiconazole compared to the control. Germination rates varied depending on the doses and products tested. Compared to the control, doses of 500 and 1000 ppm of Cymbopogon citratus oil showed zero germination for a period of 30 days. However, the 200 ppm dose showed zero germination until 72 hours after 120 hours of incubation; the teliospores then germinated, reaching an 80% germination rate by the 30th day. Zingiber officinalis oil showed zero teliospore germination at concentrations of 500 ppm and 1000 ppm compared to the control. The 200 ppm dose of Zingiber officinalis showed a germination rate of 0% for 120 hours, but after that time, the teliospores began to germinate and reached a rate of 90% by the 30th day. As for Ocimum gratissimum, the 500 ppm and 1000 ppm doses inhibited germination, maintaining a 0% rate until the 30th day. After 72 hours of incubation, the teliospores germinated and reached a germination rate of 95% on the 30th day of incubation. With propiconazole, the three doses of 200, 500, and 1000 ppm maintained a germination rate of zero until the 30th day.

3.5 Teliospore Germination Inhibition Rates

At concentrations of 500 and 1000 ppm, all three essential oils exhibited a 100% germination inhibition rate. However, at the 200 ppm dose, the inhibition rates varied, with rates of 55.71%, 77.14%, and 80% respectively for the oils of Ocimum gratissimum, Zingiber officinalis, and

Cymbopogon citratus. Propiconazole achieved a 100% inhibition rate at concentrations of 200, 500, and 1000 ppm.

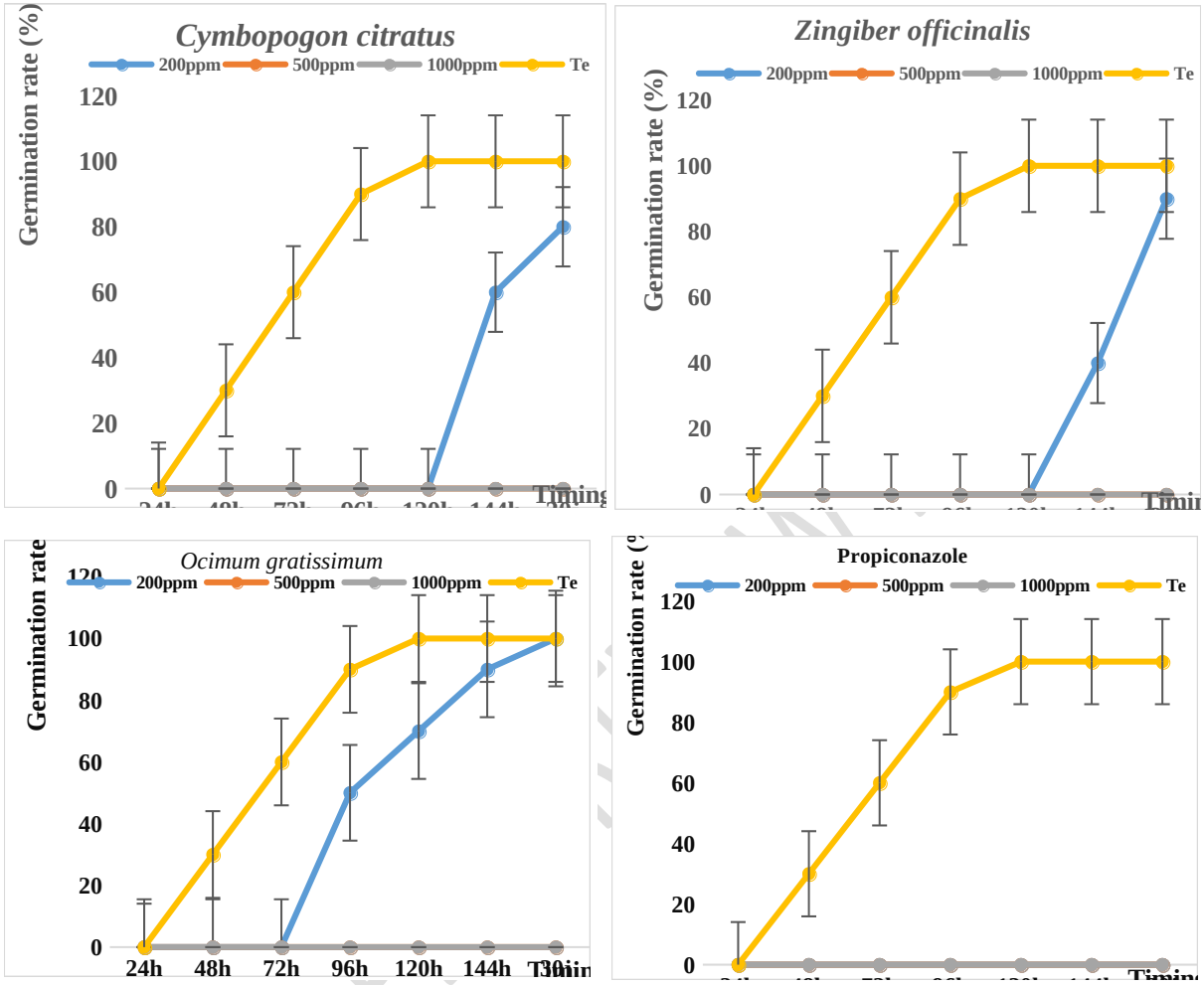
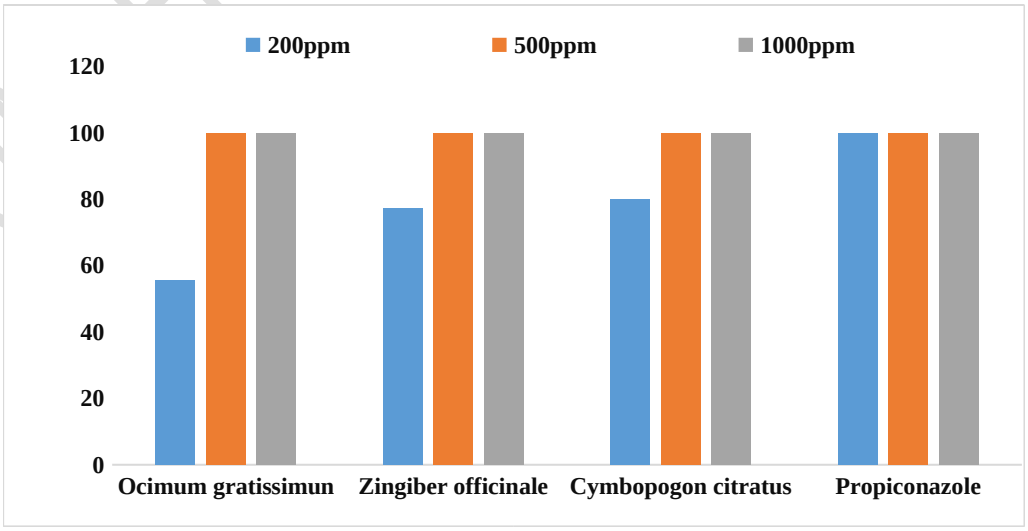


Figure 4 : Teliospore Germination Inhibition Rates



Discussion

The essential oil yields from the leaves of Ocimum gratissimum, Cymbopogon citratus, and Zingiber officinalis were 0.64%, 0.86%, and 0.10%, respectively. The essential oil yield from Ocimum gratissimum obtained in this study is identical to that reported by Camara et al. (2009) and lower than that reported by Kouame et al. (2018) in Côte d'Ivoire, which were 0.8% and 1.2%, respectively. A yield of 0.60% was reported by Tchoumbougnang et al. (2009) in other studies on Ocimum gratissimum from Cameroon. As for the oil yield of Zingiber officinalis, our results are lower than those obtained by Massada (1976) and Kouame et al. (2018), which were 0.85% and 0.7%, respectively. The oil yield of Cymbopogon citratus is lower than those obtained by Oliveira et al. (2011) and by Costa et al. (2013) via hydrodistillation, at 1.04% and 1.39%, respectively. All these varying yields indicate that there are factors influencing oil yield in aromatic plants. Indeed, plant species exhibit significant activity in the summer, which is reflected in high yields of secondary metabolites such as essential oils. This is thought to be due to the maximum photoperiod conducive to essential oil synthesis, as well as a prolonged dry period that promotes the biosynthesis of numerous other secondary metabolites (Boukhebti, 2011). The results of the time-dependent distillation monitoring provided insight into the extraction kinetics of essential oils using the conventional hydrodistillation method for the three plant essential oils. Between 25 and 100 minutes, we observed a decrease in the volume of oil from Ocimum gratissimum and Cymbopogon citratus, which virtually leveled off. This corresponds to the time marking the end of the extraction process for these oils. The extraction time for Ocimum gratissimum and Cymbopogon citratus is 1 hour and 40 minutes. As for Zingiber officinale, the distillation time is 2 hours and 25 minutes. Essential oils offer significant potential as biodegradable substances with antimicrobial activity (Tripathi et al., 2008). The results of this study showed that various concentrations of essential oils extracted from Ocimum gratissimum, Cymbopogon citratus, and Zingiber officinalis, as well as the synthetic fungicide, inhibited mycelial growth, sporulation, and teliospore germination of Sporisorium scitamineum. The essential oil of Ocimum gratissimum exhibited fungicidal effects on mycelial growth and teliospore germination. The results are similar to those of previous studies describing O. gratissimum oil as an inhibitor of mycelial growth and spore germination of Sporisorium scitamineum (Kouame et al., 2018). These findings are consistent with those of Nguefack et al. (2004) and Camara et al., (2004). They also found significant antifungal activity of O. gratissimum oil against other plant pathogens such as

Aspergillus flavus, A. fumigatus, Fusarium moniliforme, and Deightoniella torulosa. Indeed, the antifungal properties of essential oils are related to their content of antimicrobial compounds (Dikbas et al., 2009). Seri-Kouassi (2004) reported a higher thymol content (43.1%) in O. gratissimum essential oil from Côte d'Ivoire compared to 9.80% thymol. In fact, due to its dual nature as both an alcohol and a lipid, it promotes the degradation of fungal cell membranes (Kwazou et al., 2009). This may explain the inhibitory activity of Ocimum gratissimum on the germination of teliospores and the mycelial growth of Sporisorium scitamineum at 500 ppm. The essential oils of Z. officinalis were effective against teliospore germination at 500 ppm and inhibited mycelial growth and sporulation at 1000 ppm. The efficacy of Z. officinalis oil has already been demonstrated in the work of El-Baroty et al. (2010), who found that Z. officinalis oil strongly inhibited the mycelial growth of four pathogenic fungi. These results also corroborate those of Kouame et al. (2015) regarding post-harvest treatment of anthracnose. The work of Anwar et al. (2009) demonstrated through in vitro tests that Zingiber officinalis exhibits remarkable antifungal activity. As for Cymbopogon citratus oil, it demonstrated remarkable antifungal effects by inhibiting mycelial growth and teliospore germination at 400 and 500 ppm, respectively. This result allows us to conclude that this oil possesses antifungal properties against Sporisorium scitamineum. These findings are consistent with those of Kouame et al. (2018). Our study revealed effects similar to those of propiconazole. Indeed, the work of Bhuiyan et al. (2015) showed that fungicidal treatments with flutriafol and propiconazole have highly effective fungicidal effects against Sporisorium scitamineum. The literature summarizing disease control efforts indicates that very few studies have been conducted on control methods using essential oils. The results of this study therefore allow us to consider implementing sustainable control methods using these essential oils. They can be used for treating cuttings as well as for treating sugarcane regrowth.

Conclusion

At the conclusion of this study, it should be noted that biopesticides based on essential oils of Ocimum gratissimum L., Cymbopogon citratus L., and Zingiber officinale Roscoe effectively inhibit the growth, sporulation, and germination of Sporisorium scitamineum, sometimes as effectively as the fungicide propiconazole. These biopesticides therefore represent a sustainable ecological alternative for controlling sugarcane smut disease in Côte d'Ivoire.

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