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Blondo P Metsebing
1. Mycological Laboratory, University of Yaoundé 1, Yaoundé, Cameroon
2. Microbiology Laboratory, University of Yaoundé 1, Yaoundé, Cameroon

Romuald Oba
1. Mycological Laboratory, University of Yaoundé 1, Yaoundé, Cameroon
2. Department of Biomedical Sciences, University of Bertoua, Bertoua, Cameroon

Landry G Kengne
1. Microbiology Laboratory, University of Yaoundé 1, Yaoundé, Cameroon
2. Department of Biochemistry, University of Yaoundé I, Yaoundé, Cameroon

Nkodo Ekeme
1. Mycological Laboratory, University of Yaoundé 1, Yaoundé, Cameroon
2. Microbiology Laboratory, University of Yaoundé 1, Yaoundé, Cameroon

Regine G Mboubou
1. Mycological Laboratory, University of Yaoundé 1, Yaoundé, Cameroon
2. Microbiology Laboratory, University of Yaoundé 1, Yaoundé, Cameroon

Maximillienne A Nyegue
Microbiology Laboratory, University of Yaoundé 1, Yaoundé, Cameroon

Choupo Guifo
Mycological Laboratory, University of Yaoundé 1, Yaoundé, Cameroon

Fabrice T Tsigain
Mycological Laboratory, University of Yaoundé 1, Yaoundé, Cameroon

Rosemary T Kingue
Department of Plant Sciences, Faculty of Sciences, University of Bamenda, Bambili, Cameroon

Astride C Djeuani
1. Mycological Laboratory, University of Yaoundé 1, Yaoundé, Cameroon
2. Microbiology Laboratory, University of Yaoundé 1, Yaoundé, Cameroon

Dominique C Mossebo
1. Mycological Laboratory, University of Yaoundé 1, Yaoundé, Cameroon
2. Microbiology Laboratory, University of Yaoundé 1, Yaoundé, Cameroon

Correspondence:
Dr. Blondo P Metsebing
Mycological Laboratory, University of Yaoundé 1, P.O. Box 1456, Yaoundé, Cameroon
Email: bmetsebing@yahoo.com

Study of the antifungal activity of *Citrus sinensis* (Rutaceae) essential oil on *Ganoderma resinaceum* (Ganodermataceae), a parasitic and wood-decaying fungus of mango and oil palm trees in Cameroon

Blondo P Metsebing*, Romuald Oba, Landry G Kengne, Nkodo Ekeme, Regine G Mboubou, Maximillienne A Nyegue, Choupo Guifo, Fabrice T Tsigain, Rosemary T Kingue, Astride C Djeuani, Dominique C Mossebo

ABSTRACT

Background: *Ganoderma resinaceum* is a parasitic fungus of oil palms and fruit trees in Africa in general and Cameroon in particular, where it often affects almost half of the plantation stands, causing destruction on several levels. **Aims and Objectives:** In order to solve the problems of trunk base rot commonly encountered in palm groves and fruit crop fields, which is responsible for enormous losses to human food supplies and the economy, *Citrus sinensis* essential oil was tested on *Ganoderma resinaceum* mycelium and its inhibitory and fungicidal activity was evaluated. **Material and Methods:** The mycelium of *G. resinaceum* is obtained from cultures on PDA medium with fragments of carpophores collected from the trunks of fruit trees. The essential oil of *C. sinensis* was extracted by hydrodistillation method using a Clevenger apparatus. This essential oil is tested on the mycelial growth of *G. resinaceum* by microatmosphere and direct contact method. **Results:** The yield of essential oil extracted from the pericarp of *C. sinensis* is 0.84%. This oil, slows the growth of *G. resinaceum* mycelium at concentrations of 0.125, 0.25 and 0.5 µL/mL and shows inhibitory activity at a concentration of 1 µL/mL and fungicidal activity at concentrations of 1, 1.25 and 2 µL/mL with respective inhibition percentages of 27. 55, 32.37, 68.83, 100, 100 and 10.59, 15.22, 69.94, 100, 100 % at different methods. *C. sinensis* essential oil has a significantly positive effect on the parasitic and wood-decaying fungus *G. resinaceum* due to its high content of more than 50% monoterpenes, which are volatile compounds with antibacterial, antiviral and antifungal properties. **Conclusion:** Essential oils are most often used against microscopic pathogenic fungi in food, the environment and crops, particularly *Aspergillus*, *Fusarium*, *Candida*, *Phytophthora* and *Pythium*. This study shows that they can also be applied in the same way as natural extracts to macroscopic parasitic fungi that cause several destructions in fruit trees such as mango and oil palm.

Keywords: Essential oil, Wood-decaying fungus, *Citrus sinensis*, *Ganoderma resinaceum*, Antifungal activity, Mycelial growth inhibition, Plant pathology.

INTRODUCTION

The genus *Ganoderma* Karst., with more than 428 species, of which *Ganoderma lucidum* as the most studied [1], is a cosmopolitan genus found throughout the world in both tropical and temperate climates and belonging to the family Ganodermataceae [2,3]. Its main distinguishing features are its basidiospores with a hard double membrane, the inner membrane having transverse ridges [3-5]. Several species are found in tropical Africa [6,7] and several studies are being conducted on this genus given its importance in various scientific fields such as medicine, forestry and agriculture.

In medicine, in addition to the species *Ganoderma lucidum*, several other species have been examined and reported by many authors as having various pharmacological properties or being used in traditional Chinese medicine throughout life [8,9]. The carpophores of species of the *Ganoderma* genus are used to treat several diseases, particularly in traditional Asian pharmacopoeia. Several authors have reported the presence of substances with antioxidant properties in various species [10-12], antibacterial [12,13], antiviral [12,14] and anti-tumour substances [12,15,16].

However, in forestry, *Ganoderma* is known as a major wood decomposer causing white rot on dead and living wood of several species [17,18]. In agriculture, Most of the species belonging to this genus cause root and stem rot in many cash crops and trees, notably oil palms in several countries in Africa and

Asia [19-21], where they cause yield losses and are sometimes responsible for the death of several plants in the fields [22]. The fight against fungi through the application of natural fungicides has become very important in alternative strategies to synthetic fungicides, since the use of these products has disastrous consequences not only on the environment, but also on human health through acute or permanent poisoning, allergies and even cancer [1,13,23,24]. These natural fungicides have already been the subject of several studies [25,26] due to their antimicrobial properties [27,28], particularly in plants.

Indeed, the therapeutic properties of medicinal and aromatic plants have been tested for centuries, and the value of their essential oils (EOs) in various applications, particularly as anti-inflammatory, antiseptic, antifungal, antibacterial, antioxidant, antitoxic, insecticides and insect repellents, tonics, stimulants and calming agents [25,29-31]. EOs can be used as an alternative to synthetic pesticides, insecticides and fungicides in antifungal control programmes [32,33]. In general, plant EOs have been recognised as an important natural antimicrobial resource [27,34]. It is within this context that the objective of this study is to develop a new strategy to combat *Ganoderma resinaceum*, a parasitic fungus of oil palms and mango trees, through the use of essential oil from *Citrus sinensis*, a plant with proven medicinal properties.

MATERIAL AND METHODS

Mushroom Collection and mycelium cultivation

Mushroom sample were made of carpophores of *Ganoderma resinaceum*. These carpophores were mostly collected on tree stumps of mangos and oil palm trees in Yaoundé and their outskirts in Cameroon, were identified and preserved in the Mycological Herbarium of the Faculty of Science of the University of Yaoundé 1 under the numbers HUY1-DM 1506. After collection and identifacation, fresh flesh fragments were taken from the context and trama and transfered in the PDA medium. Petri dishes sealed with parafilm were incubated in the dark at room temperature. Mycelium developed from the explant and reached sufficient growth after 14 days. The mycelium was purified by successive subculturing of fragments taken from the mycelial growth front to the fresh media until pure cultures were obtained.

Extraction of *Citrus sinensis* essential oil (EO)

Citrus sinensis pericarps were collected at the Mfoundi market (Yaoundé, Cameroon). Essential oil extractions were carried out by hydrodistillation method using a Clevenger apparatus [30,31,35]. This involves directly immersing the plant material to be treated (intact or crushed) in a still containing water and bringing everything to a boil. The volatile compounds are carried away by the water vapour and then condensed in the condenser. For this work, the plant material was placed in a Clevenger-type apparatus (still) with a sufficient volume of water to keep it afloat. The mixture was brought to a boil for 5 hours and the volatile components of the essential oil (EO) were carried by the steam along a coil cooled in a water circuit. The EOs were collected in opaque bottles and stored in a low-temperature refrigerator. The extraction yield [Rdt (%)] of each EO was calculated in relation to the weight of the plant material using the following formula :

$$[\text{Rdt}(\%)] = \frac{\text{mass of essential oil (g)}}{\text{mass of plant material (g)}} \times 100$$ [35].

Determination of the Minimal Inhibitory Concentration (MIC) of EO

This was done using the microatmosphere method and the direct contact method described respectively by Delespaul *et al* [36] and Laghchimi *et al* [37].

Microatmosphere method

In Petri dishes containing 20 mL of PDA agar sterilised in an autoclave for 30 minutes at 120 °C and thus providing 80 mL of air, a 6 mm disc-shaped mycelium explant taken from a 14-day-old pure strain of *G. resinaceum* is placed in the centre of the dish. Filter papers with a diameter of 80 mm were placed at the bottom of the lid of each Petri dish and impregnated with different quantities of EO, namely 0 (control), 10, 20, 40, 80 and 100 µL to obtain concentrations of 0, 0.125, 0.25, 0.5, 1 and 1.25 µl/mL of air, respectively. Three replicates are performed for each concentration. The boxes are sealed with parafilm and incubated at room temperature in the dark. Observations are made on the 3rd, 5th, 7th, 10th and 14th day after incubation and focus on the average mycelial growth measured according to two perpendicular diameters of the Petri dish [36,37].

Medium dilution method

13.5 ml of liquid PDA are introduced into 6 test tubes numbered 1-6, sterilised in an autoclave for 30 minutes at 120 °C and kept supercooled at 45 °C in a water bath. 1.5 mL of 5% DMSO is also added to 6 other test tubes numbered 1-6, which have been sterilised in an autoclave at 120 °C for 1 hour. In the tubes containing 5% DMSO, volumes of 0 (control -), 1.875, 3.75, 7.5, 15 and 30 µL are taken, replaced with EO and shaken using a vortex to obtain the EO dilutions. These dilutions are introduced into the supercooled PDA culture media, then shaken appropriately to obtain the respective concentrations of 0 (control -), 0.125, 0.25, 0.5, 1, 2 µL/mL and poured into 90 mm Petri dishes. Three replicates are performed for each EO concentration. 6 mm disc-shaped explants of *G. resinaceum* are taken from the 14-day-old pure strain and placed in the centre of the Petri dishes. These dishes were then sealed with parafilm and incubated at room temperature in the dark [36,38]. Observations were made on the 3rd, 5th, 7th, 10th and 14th days after incubation and focused on the average mycelial growth measured according to two diameters of the Petri dish. It is calculated using the modified formula of Singh *et al* [39].

$$D = \frac{d1+d2}{2} - do,$$

where d_1 and d_2 represents the perpendicular diameters of the pathogen and d_0 represent the diameter of the explant.

Percentage of inhibition of essential oil

The inhibition percentages (I%) of the different concentrations of EO are calculated after 14 days of incubation and mycelial growth of *G. resinaceum* by averaging the three replicates for each concentration. This inhibition, expressed as a percentage (%), is determined using the formula of Pandey *et al*. [40]

$$\%I = \frac{Dt-Di}{Dt} \times 100,$$

where D_t represents the average diameter of fungal growth in the control box and Di represents the average diameter of fungal growth in the test box.

Determination of minimum inhibitory concentrations

The minimum inhibitory concentration (MIC expressed in µl/mL of air or µl/mL) represents the lowest concentration of EO that completely inhibited the growth of *G. resinaceum* mycelium. It correspond to the concentration at which no mycelial growth is observed [30,31,35,41].

Evaluation of the fungicidal or fungistatic activities of the different treatments

After incubation, the Petri dishes in which the growth of *G. resinaceum* mycelium was completely inhibited are counted and the explants are transferred to new PDA culture media without EOs. If mycelial growth resumes in this new medium, the essential oil is fungistatic; if there is no resumption of growth, the essential oil is fungicidal [31,35,36,40].

RESULTS

The essential oil extraction yield obtained from *Citrus sinensis* peel in this study was 0.84%. This oil is orange-yellow in colour. The *G. resinaceum* mycelium, transferred to fresh PDA media, colonised the 90 mm Petri dish in 14 days. Overall, the results obtained show that *Citrus sinensis* essential oil significantly reduced the mycelial growth of *Ganoderma resinaceum* from the third to the fourteenth day of incubation at the different concentrations tested. This reduction was observed in both methods used (Table 1 and 2). The results in Table 1 and Figure 1 also show that in the presence of *Citrus sinensis* EO and under micro-atmospheric conditions, *G. resinaceum* mycelium grows in Petri dishes at concentrations of 0.125, 0.25 and 0.5 µL/mL with respective diameters of 6.02, 5.62 and 2.59 cm on day 14. However, during the incubation period, no mycelium growth was observed in Petri dishes at concentrations of 1 and 1.25 µL/mL. Using the direct contact method (Table 2 and Figure 2), mycelial growth of *G. resinaceum* was also observed at essential oil concentrations of 0.125, 0.25 and 0.5 µL/mL with respective diameters of 4.64, 4.40 and 2.34 cm on day 14, and no mycelial growth at concentrations of 1 and 2 µL/mL during the incubation period (Table 2).

Percentage of inhibition of *Citrus sinensis* EO on *G. resinaceum* mycelium growth

The *Citrus sinensis* EO used proved to be very effective against the mycelial growth of *Ganoderma resinaceum* (Table 3 and 4, Figure 3 and 4). Inhibitions of 100% were obtained with concentrations of 1 and 1.25 µL/mL (microatmosphere method) as well as concentrations of 1 and 2 µL/mL (direct contact method) with the EO. However, inhibitions of 27.55, 32.37 and 68.83% (microatmosphere method) were observed for concentrations of 0.125, 0.25 and 0.5 µL/mL of *Citrus sinensis* EO, respectively, while inhibitions of 10.59, 15.22 and 69.94% (direct contact method) were obtained at concentrations of 0.125, 0.25 and 0.5 µL/mL, respectively.

Minimum inhibitory concentration (MIC) and fungicidal concentration (MFC) of *C. sinensis* EO on *G. resinaceum* mycelium

The MIC was obtained at a concentration of 1 µL/mL using the microatmosphere and direct contact methods. For the fungicidal test, only concentrations that completely inhibited mycelial growth were considered. Thus, concentrations of 1 and 1.25 µL/mL showed fungicidal activity (Table 5).

Table 1: Effect of *Citrus sinensis* EO on the mycelial growth of *Ganoderma resinaceum* using the microatmosphere method

Micro-atmospheric concentration of *C. sinensis* EO (en µL/mL of air)	Average mycelium growth after incubation (in cm/day)				
	3 days	5 days	7 days	10 days	14 days
Témoin -	1.07	2.74	3.16	4.82	8.31
0.125	0.27	1.39	1.53	3.23	6.02
0.25	0	1.02	1.37	2.92	5.62
0.5	0	0.21	0.28	1.06	2.59
1	0	0	0	0	0
1.25	0	0	0	0	0

Table 2: Effect of *Citrus Sinensis* EO on the mycelial growth of *Ganoderma resinaceum* using the direct contact method

Direct concentration of *C. sinensis* EO (in µL/mL)	Average mycelial growth after incubation (in cm/days)				
	3 days	5 days	7 days	10 days	14 days
Témoin -	1.31	2.52	3.01	4.14	5.19
0.125	1.27	2.15	2.91	3.59	4.64
0.25	1.05	1.84	2.66	2.71	4.40
0.5	0	0	1.07	1.36	2.34
1	0	0	0	0	0
2	0	0	0	0	0

Table 3: Percentage of inhibition *Citrus sinensis* EO on *Ganoderma resinaceum* mycelial growth using the microatmosphere method

Micro-atmospheric concentration of *C. sinensis* EO (in µL/mL of air)	Inhibition of mycelial growth during incubation (in %)
0.125	27.55
0.25	32.37
0.5	68.83
1	100
1.25	100

Table 4: Percentage of inhibition *Citrus sinensis* EO on *Ganoderma resinaceum* mycelial growth using the direct contact method

Direct concentration of *C. sinensis* EO (in µL/mL of air)	Inhibition of mycelial growth during incubation (in %)
0.125	10.59
0.25	15.22
0.5	69.94
1	100
1.25	100

Table 5: Minimum inhibitory and fungicidal activity of EO

Strain	essential oil	MIC (µL/mL)	MFC (µL/mL)	Method	Activity
Ganoderma resinaceum	EOCS	1	1 et 1,25	Microatmosphere	Fungicide
		1	1 et 2	Medium dilution	Fungicide

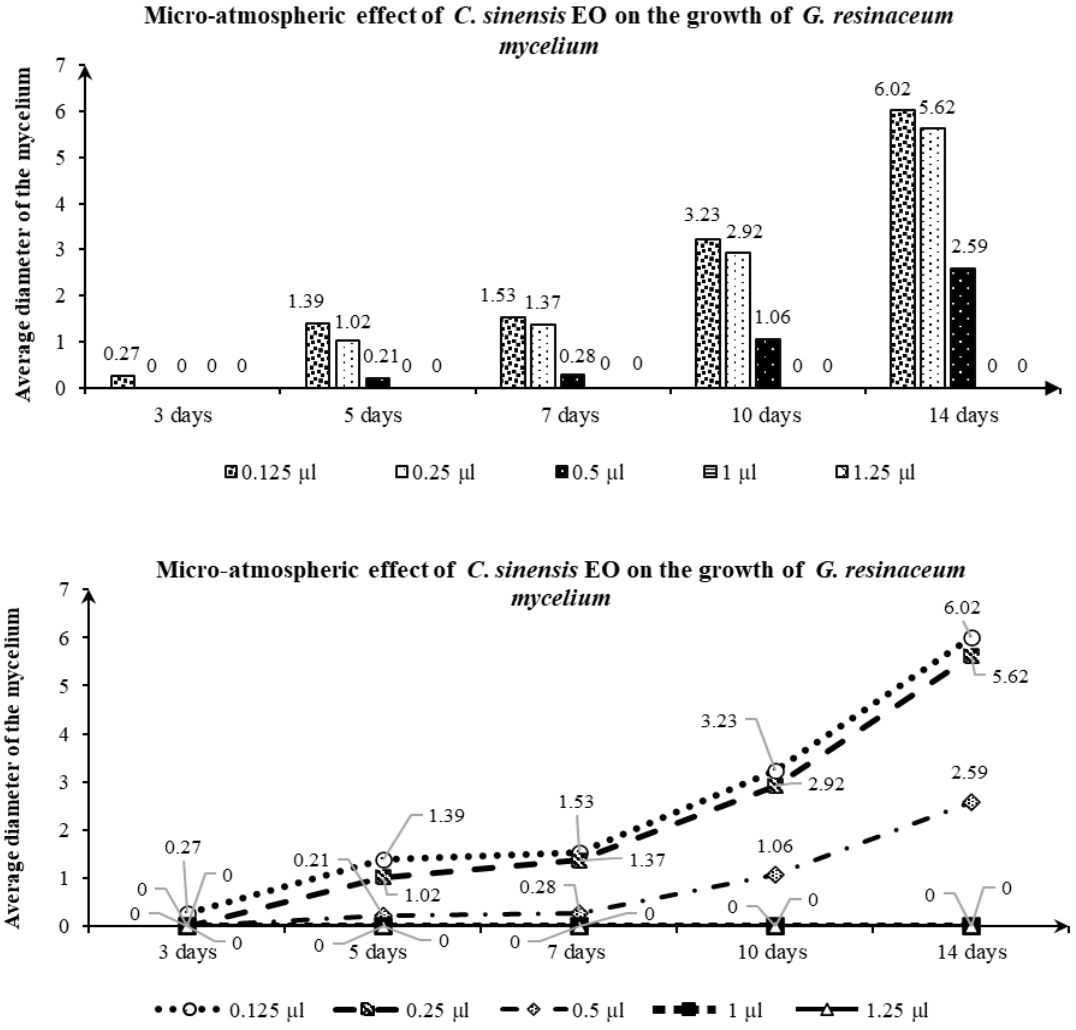

Figure 1: Histogram and curve comparing the growth diameter of *G. resinaceum* mycelium according to different concentrations of *C. sinensis* EO using the micro-atmosphere method

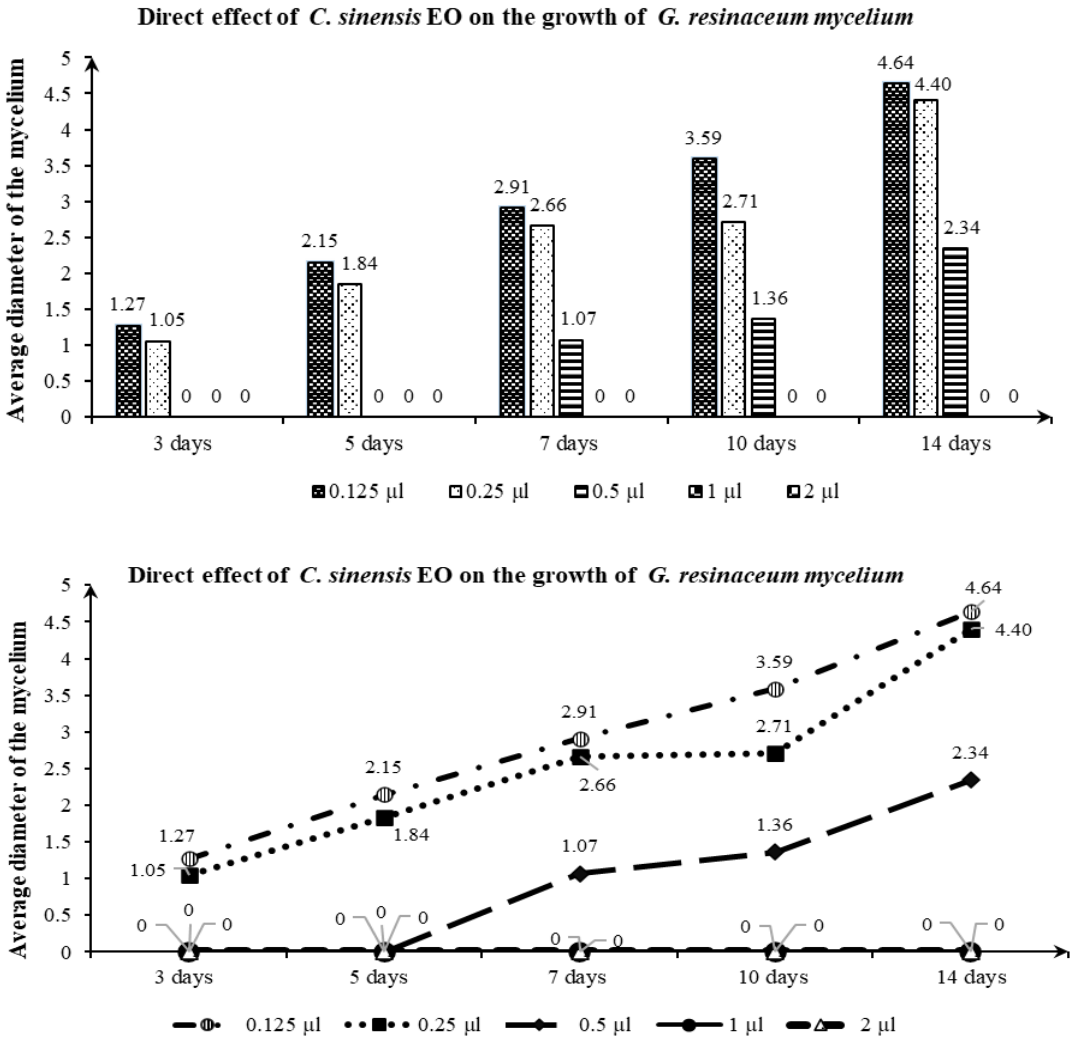

Figure 2: Histograms and curves comparing the growth diameter of *G. resinaceum* mycelium according to different concentrations of *C. sinensis* EO using the direct contact method

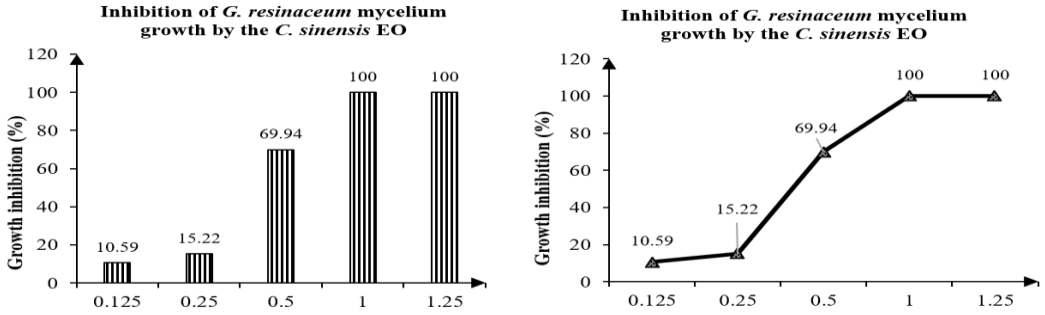

Figure 3: Histogram and curve comparing the growth inhibition of *G. resinaceum* mycelium according to different concentrations of *C. sinensis* EO using the micro-atmosphere method

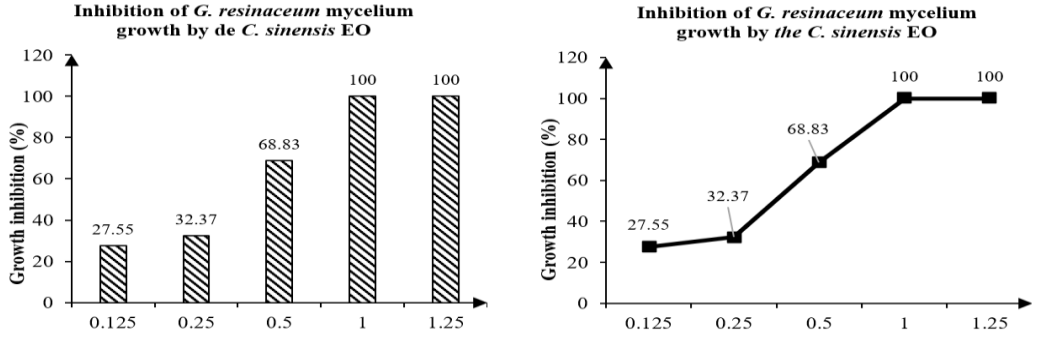

Figure 4: Histogram and curve comparing the growth inhibition of *G. resinaceum* mycelium according to different concentrations of *C. sinensis* EO using the direct contact method

DISCUSSION

This study was based on the extraction of essential oil from *Citrus sinensis* and the evaluation of its activity on the mycelial growth of *Ganoderma resinaceum*. An extraction yield of 0.84% was obtained from the pericarp of *C. sinensis*. This yield is lower than that obtained (0.93%) by Hamdani & Allem [42] from fresh leaves. This observed difference shows that the yield of essential oil varies depending on the part of the plant used and can also be influenced by other factors such as climate, harvest area and harvest period [43].

Citrus sinensis essential oil, applied by microatmosphere during the incubation period, slows down the mycelial growth of *G. resinaceum*. The mycelial diameters decrease from 9 cm to 6.02 cm, 5.62 cm and 2.59 cm, respectively, at concentrations of 0.125 µL/mL, 0.25 µL/mL and 0.5 µL/mL, with total inhibition observed at 1 µL/mL and 1.25 µL/mL. This effect is greater with the direct contact method at the same concentrations, where the mycelial diameters are reduced to 4.64 cm, 4.4 cm and 1.56 cm after 14 days of incubation, with total inhibition at 1 µL/mL and 2 µL/mL. This proves the fungicidal effect of *C. sinensis* EO on the wood-decaying fungus *G. resinaceum* at a minimum inhibitory concentration (MIC) of 1 µL/mL. The fungicidal activity of volatile compounds results from a combined effect between direct absorption of vapours by fungi and indirect absorption of vapours by the culture medium [30]. Several studies have highlighted the fungicidal effect of the volatile constituents of EOs on various microorganisms. The work of Laghchimi *et al* [37] showed that the EOs tested in the gas phase have a lethal effect on the moulds responsible for apple rot.

The *C. sinensis* essential oil tested in this study had a significantly positive effect on the parasitic and lignivorous fungus *G. resinaceum*. This could be explained by the high content of monoterpenes in the EOs (more than 50%), which are volatile compounds with antibacterial, antiviral and antifungal properties [44-46, 31].

Eos are most often used against microscopic fungi that are pathogenic to food, the environment and crops, including *Aspergillus*, *Fusarium*, *Candida*, *Phytophthora*, *Pythium* and bacteria [31,44,46]. This study shows that they can also be applied in the same way as raw natural extracts [47-50]. on macroscopic parasitic fungi that cause enormous losses by destroying fruit trees such as mango and oil palm.

CONCLUSION

Essential oil of *C. sinensis* tested on *G. resinaceum* mycelium shows inhibitory and fungicidal activity at a concentration of 1 µL/ml, proving that this oil can be used to combat basal rot in oil palm and mango tree trunks, which are commonly found in many palm groves and fruit fields, where they cause enormous losses, particularly in terms of food and the economy.

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Conflict of interest

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ORCID ID

Blondo-Pascal Metsebing: https://orcid.org/0009-0000-1434-7900

Romuald Oba: https://orcid.org/0009-0005-5794-0022

Landry Gounmadje Kegne: https://orcid.org/0000-0002-0973-513X

Nkodo Ekeme: https://orcid.org/0009-0003-2548-1891

Regine G Mboubou: https://orcid.org/0009-0002-4696-1432

Maximillienne A Nyegue: https://orcid.org/0000-0002-9642-9930

Choupo Guifo: https://orcid.org/0009-0004-4158-4070

Fabrice Ts Tsigain: https://orcid.org/0009-0005-8536-4464

Rosemary T Kingue: https://orcid.org/0000-0002-5402-1021

Astride Carole Djeuani: https://orcid.org/0000-0002-4299-8759

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