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Microbicidal Effect of Thyme (*Thymus vulgaris*) Essential Oil against *Phytophthora megakarya*, the Causative Agent of Cocoa Black Pod Disease

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Abstract In subtropical countries, black pod caused by *Phytophthora megakarya* is one of the main diseases causing drastic losses of cocoa. The synthetic chemicals used to control the pathogen, although effective, could be harmful to Human and environment. The use of natural substances such as essential oils (EO) is necessary. This work aimed to determine the chemical composition of thyme essential oil and its antimicrobial potential against *Phytophthora megakarya*. Essential oil was extracted by hydrodistillation and the chemical composition was analyzed by gas chromatography couple to mass spectrometry (GC/MS). The evaluation of the antimicrobial activity was done *in vitro* by agar incorporation and liquid dilution. *In situ* tests were carried out by spraying the EO on the cocoa cortex after and before infected by the pathogen respectively, for the preventive test and curative healing. Results showed that, the yield of thyme EO was 0.42% with thymol (32.41%), γ-terpinene (19.65%) and p- cymene (19.43%) as major components. At 225µl/l, the oil completely inhibited the mycelial growth of *P. megakarya* while at 400µl/l this oil completely inhibited the germination of zoospores. At 1125 µl/l, thyme EO completely inhibited the necrosis on cocoa pods for preventive test. At the same concentration, the oil significantly reduced (40 %) the necrosis during the curative healing. There was significant correlation between EO concentration and the necrosis formation. These results showed that, thyme essential oil could be used as alternative to fight against black pod disease. However, the formulation of oil and fields essay are needed.

Keywords: *Theobroma cacao, Cocoa black pod, Phytophthora megakarya, Essential oil, Thymus vulgaris*

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1. Introduction

Cocoa (*Theobroma cocoa* L) is a subtropical culture which is a source of income for many small farmers and the raw material for chocolate industries. Africa contries provides more than 70% of the world's cocoa production [1], mainly in four of the five largest producing countries in the world, namely: Ivory Coast (32%), Ghana (24%), Nigeria (9%) and Cameroon (8%). Despite their importance, cocoa cultivation faced many problems including pest pressure, insufficient of selected genotypes, and low soil fertility [2]. Among them, black pod disease caused by *Phytophthora sp.* is the main cause of significant losses of cocoa. In absence of any treatment, losses could reach to 50 - 100% [3]. In Cameroon, *P. megakarya* is the causal agent of cocoa pod and the losses could be estimated at 50-80% [4,5].

To overcome the problem, farmers have embarked on the use of chemical pesticides, which have so far been of great help. However, many risks are associated with their use. Hazardous pesticides used could behave undesirable effects such as environmental pollution related to their toxicity, their non-biodegradability, the presence of residues in soils and waters affecting the health of populations [6]. Indeed, for some years now, the problems linked to the abusive use of chemical pesticides have affected many populations and have made an urgent appeal to the scientific world. It has been estimated that about 2.5 million tons of chemical pesticides are used in fields each year worldwide, and the damage associated with this use reaches $100 billion [7]. Thus, the awareness of the environmental cost of these practices and consumers' fears of the danger that pesticide residues may pose to human health are giving rise to a growing interest in other control alternatives. According to the requirement of European Union Regulation 2009/128/EC,

it is urgent to reduce pesticide residues in food and in the environment for better human and animal health. It is therefore important to use chemicals that cause few problems in the control of crop diseases [8]. The solution could be reside in the use of pesticides based on natural products, thus giving birth to the concept of "biopesticide", referring to any type of natural products that can contribute to the reduction of plant pests and thus, to the increase of agricultural production and productivity [9,10].

Many studies have been carried out on a new plant diseases treatment based on the use of plant essential oil [11,12]. Essential oils (EOs) are volatile secondary metabolites with complex chemical composition and possess antimicrobial properties. This biological activity can be exploited in order to protect crop against several pathogens and parasites [13]. Moreover, the studies of Alves-silva *et al*. [14] reveled that, EOs are biodegradable natural substance and could be possess eco-friendly antimicrobial properties against several pathogens. Thyme (*Thymus vulgaris*) is a plant herb, shrub and annual. The aerial parts of this plant are used in traditional medicine and as spices and condiments in food [15]. Although the chemical composition of the EO of thyme is well known [16], its occurrence in different climate and edaphic zones could significantly change its chemical composition [17]. Recently, several studies showed that plant EOs including thyme EO strongly inhibited the microbial growth of different plant pathogens [18]. In Cameroon, *Thymus vulgaris* is largely cultivated and used in food as spices and condiment. In certain agro-ecological zones, farmers used fresh and dried whole plant of thyme to protect plant against plant pathogens.

The objective of this work was to determine the chemical composition of the EO of *Thymus vulgaris* and to evaluate its antimicrobial potential against *P. megakarya*.

2. Materials and Methods

2.1. Plant Material

Fresh leaves and stems of *Thymus vulgaris* have been collected on 28 November 2019 in Dschang (5°06´28"North, 10°26´10"East) sub-division of West region - Cameroon. The specimen was identified at the Cameroon National Herbarium under the number 25746/SRF/Cam. The leaves and stems have been air dried and hydro-distillated by using a Clevenger-type apparatus during five hours. Essential oil collected has been air dried with Na_2SO_4 (anhydrous sodium sulfate), added into dark flask and keep at 4°C into freeze until used.

Cocoa pods (Forestaro variety) with symptoms of black pod that have been used to isolate the pathogen were harvested from the farmer field at Njombe (4° 35′ 00'' North, 9° 40′ 00'' East), Subdivision of Littoral region of Cameroon.

2.2. Chemical Analysis of the Essential Oil

The essential oil was analyzed by gas chromatography couple with mass spectrometry (GC/MS). The chromatograph was equipped with ionization flame detector. The temperature of the detector and injector was temperature 200°C. The temperature domain was programmed from 60 to 250°C with a rate of 5°C/min. The linear retention indices of the compounds were determined in regards to the retention times of a homologous series of n-alkanes (C8-C24). The percentage compositions were obtained directly from the electronic integration measurements. The identification of the compounds was done by the comparison of their retention indices with those given in the literature [19], and by comparison of their mass spectral fragmentation patterns with those of similar compounds in the reference mass spectra library (NIST02.LIB).

2.3. Plant Pathogen

Isolation of the pathogen from the tissues of cocoa pods showing the black pod symptoms was carried out. Cortexes of 2 to 4 mm were taken from the margin of the pod with a sterilized knife. They were washed several times with tap water and then disinfected in a 70% alcohol solution for 5 minutes and rinsed 3 times with sterile distilled water and placed on blotting paper for 20 minutes to dry. These fragments were placed in the center of 90mm diameter Petri dishes containing PDA medium supplemented with antibiotics (ampicillin 250mg/l and penicillin 250mg/l) in the vicinity of the Bunsen burner flame using forceps. The inoculated plates were incubated at room temperature for 48 to 72 hours and observed daily. Colonies from primary isolations were sub-cultured several times on freshly prepared culture media to obtain a single pathogen stain (pure colonies) per Petri dish. The identification of the pathogen was done by using the key of Brotton *et al*. [20].

2.4. Inhibition of Mycelial Growth

The Effect of thyme essential oil on the mycelial growth of *P. megakarya* was evaluated by the agar incorporation method as described by Lahlou [21]. The test consisted of supplementing the culture medium (PDA) with essential oil at different concentrations, pouring it into Petri dishes and placing a microbial culture disc in the centre of each dish to monitor its growth. A 5 mm diameter mycelial disc cut with a scapel at the end of a 3-days pre-culture was seeded in the centre of the dishes. These dishes were sealed with the film and incubated in an inverted position at 28 ± 2°C. Plates containing PDA and the microorganism were used as negative control; those with essential oil replaced by DMSO were used as negative control. The plates containing the PDA supplemented with Ridomil 66 WP solution were the positive control. All tests were performed in triplicate under sterile conditions in a microbiological hood and in the vicinity of a Bunsen burner flame and the experiment was repeated twice. Mycelial growth was monitored by measuring the growth diameters after 7 days. The Inhibition Percentage (%I) was recorded as follow: %I = ((Dc – D)/Dc) x 100 where Dc is the diameter of mycelial on the plates with pathogen and D the diameter of mycelial growth on the plate with pathogen supplemented with EO. The microbicidal or

microbistatic activities were evaluated by transferring the pathogen disc from the treated plates where the growth was completely inhibited by the used oil into non-supplemented fresh PDA plates.

2.5. Inhibition of Zoospores Germination

The effect of the EO on the zoospore germination was tested using a liquid dilution method in Potato Dextrose Broth (PDB) adapted from Madjouko et al. [22]. The oil was added separately into micro-cupules containing 100 µl Potatoes Dextrose Broth (PDB) in serial dilution following a geometrical progression with a common ratio of two in order to obtain the final concentrations of 112.5; 225 and 450 µl/L. At the same time, aliquots (100 µl) of the zoospores suspensions (10^5spores/ml) of the pathogen were added to each micro-cupule. All the treatments were carried out in triplicates, and each experiment was repeated twice. After 6 days of incubation at 28 ± 2°C, at least 100 spores were observed per replicate under light microscopy using a haemocytometer to determine the germination rate. The percentage inhibition was calculated from the obtained data with the formula: Ig (%) = ((Gc – G) x 100), where Gc is the zoospores germination in the control plates, G being the zoospores germination in the treated plates.

2.6. *In situ* Test Evaluation

The cocoa pods (Forestaro variety) were harvested at the mature stage and arranged based on size and the disease infection absence. Their cortex was disinfected with sodium hypochlorite 1% for 5 min, rinsed with tap water and then air - dried. The cocoa were distributed into groups, each with 8 exemplars, three replicates being used for each treatment. The emulsion of the EO was prepared by diluting a given volume in DMSO and adjusting to 5 ml with sterilized distilled water to obtain various concentrations from 450 to 1125 µl/L. The EO was applied by spraying 10 ml of oil emulsion on each cocoa group. The infection was achieved by inoculating 10µl of a fresh suspension of *P. megakarya* zoospores (10^5 spores/ml) at three different sites under the cortex of each cocoa; one hour after and 2 days before the oil application, for the prevention and the disease treatment, respectively. The controls were the cocoa pods inoculated with only sterile distilled water. The treated cocoa were put in plastic boxes together with five filter papers and about 25 ml of sterile water in order to maintain a high relative humidity (90%–95%). The cocoa were stored in the dark, at 25°C, for 14 days. After storage, the reduction in disease occurrence RDO) by the EO was evaluated using the following formula: % RDO = ((Do – D)) x 100 where Do is the diameter of the necrosis surface without EO and D the diameter of the necrosis surface with the EO.

2.7. Statistical Analysis

The data were entered into Excel (Microsoft Excel 2013) and analyzed using SPSS, 22.0 version. The results were expressed as means ± standard deviation (SD). The degree of significance of each test is determined at the 5% level ($p<0.05$). The Pearson correlation was used to establish the link between parameters.

3. Results

3.1. Yield and Chemical Composition of Thyme Essential Oil

The extraction yield of *T. vulgaris* EO was 0.42%. GC/MS analysis of EO identified 29 compounds with the preponderance of monoterpene (57.45%), and aromatic (35.52%) compounds. Thymol (32.41%), ɣ-terpinene (19.65%) and p-cymene (19.43%) were the major components (Table 1).

Table 1. Chemical composition of *T. vulgaris* EO

N°	Compounds	RT (min)	MW (g/mol)	RP (%)
Monoterpenes				**57.45**
Hydrocarbon monoterpens				**49.06**
1	α -Thujene	3.63	136	1.35
2	α -pinene	4.36	136	3.85
3	Camphene	3.93	136	1.35
4	α –terpinene	4.76	136	2.42
5	P-cymene	4.88	134	19.43
6	Limonene	4.92	136	1.01
7	ɣ-terpinene	5.34	136	19.65
8	HM	7.89	164	1.75
Oxygenated monoterpenes				**8.39**
9	Linalol	5.84	154	2.50
10	L-α-Terpinéol	7.19	154	0.30
11	Borneol	6.88	154	3.18
12	Terpinen-4-ol	7.02	154	1.50
13	2-Bornanone	6.59	152	0.31
14	Neryl propionate	10.86	210	0.17
15	Borneol propionate	9.73	210	0.18
16	Geraniol	8.03	154	0.25
Sesquiterpenes				**3.56**
17	Muurolene	11.53	204	0.17
18	ʌ-Cadinene	11.61	204	0.48
19	Oxyde de Carophyllene	12.41	220	0.83
20	ß-caryophyllene	10.38	204	1.35
21	HS_1	12.83	222	0.19
22	HS_2	15.77	150	0.18
23	HS_3	17.08	222	0.36
Aromatic compounds				**35.52**
24	Thymol	8.56	150	32.41
25	Carvacrol	8.67	150	2.63
26	3,4-Dimethylbenzol	5.74	136	0.34
27	Secoxanthoplanine	4.68	355	0.14
Aliphatic compounds				**1.59**
28	1-Octèn-3-ol	4.18	128	1.32
29	Ethyl 2-pyridylacétate	4.60	165	0.27
	Total			**98.12**

RT: Retention Time; **MW**: Molecular Weight; **RP**: Relative Percentage; **HM**: Hydrocarbon Monoterpens; **HS:** Hydrocarbon Sesquiterpenes

3.2. Effect of Thyme Essential Oil on Mycelial Growth and Zoospore Germination of *P. megakarya*

The inhibition of the *P. megakarya* mycelial growth increased significantly ($p < 0.05$) with the increasing concentrations of the EO. The minimal inhibitory concentration (MIC) of the oil was 225µl/L, while the MIC of ridomil was 500µl/L (Table 2). Moreover, both EO and ridomil exhibited microcidal activities on the mycelial growth of the pathogen.

Table 2. Effect of thyme essential oil on the mycelial growth of *P. megakara*

Essential oil (µl/l)	Mycelial growth (mm)	Inhibition (%)
0	90±0.00^{a}	0±0.00^{e}
125	60±0.17^{b}	33.33±0.15^{d}
175	20±0.29^{c}	77.77±0.25^{c}
200	10±0.45^{d}	88.88±0.18^{b}
225	0±0.00^{e}	100±0.00^{a}
Ridomyl (500 µl/l)	0±0.00^{e}	100±0.00^{a}
	MIC (µl/l)	MMC (µl/l)
Thyme EO (µl/l)	225 ±0,00	225 ±0,00
Ridomyl (µl/l)	500±0,00	500±0,00

According to Duncan's test, in the same column, means with same letters are not significantly different at $p < 0.05$. MIC: Minimum Inhibition Concentration; MMC: Microbicidal Minimum Concentration.

The essential oil of thyme exhibited inhibitory effect on the zoospores germination of *P. megakarya* with a MIC at 450 µl/L, while the MIC of ridomil was 1000 µl/L (Table 3). When the zoospores of *P. megakarya* was removed and transferred to a fresh PDA medium, the pathogen was killed. These results showed that *P. megakarya* mycelial are more sensitive to the activity of thyme EO compare to their zoospores.

Table 3. Inhibition percentage obtained with different concentrations of essential oil and Ridomil on zoospore germination of *P. megakarya*

Essential oil (µl/l)	Inhibition (%)
0	0.00^{d}±0.00
112,5	62.84^{c}±5.03
225	89.46^{b}±4.49
450	100.00^{a}±0.00
Ridomil 1000	100.00^{e}±0.00

According to Duncan's test, in the same column, means with same letters are not significantly different at $p < 0.05$.

3.3. Effect of Thyme Essential Oil on Necrosis Cocoa Black Pod Disease

The inhibition of necrosis on the cocoa black pod cause by *P. megakarya* was evaluated after 7 days. The cocoa treated with oil was better maintained and had lower necrosis degree while the control suffered an increased deterioration. The first symptom of disease appeared on the non-treated cocoa 2 days after incubation while in treated cocoa, the first symptom appear 4 days depending of the oil concentration. The inhibition of black pod increased significantly with the oil concentration for both preventive and curative tests (Table 4). The reduction of the disease was significantly higher ($p = 0.0001$) for the preventive test than for the curative test. In fact, for the preventive treatment, the complete inhibition of black pod was obtained at 1125 µl/L while, for the curative healing, at the same concentration of the oil, the reduction of necrosis was 40 %. The data revealed a significantly ($p = 0.001$) and positive correlation ($R^2 = 0.95$) between the concentration of thyme oil.

Table 4. Inhibition percentage obtained with different concentrations of essential oil on necrosis cocoa black pod disease

	Inhibition (%)	
Essential oil (µl/l)	Preventive test	Curative test
0	0,00^{e}±0,00	0,00^{e}±0,00
450	21,42^{d}±1,22	10,71^{d}±1,21
675	39,28^{c}±4,73	17,85^{c}±2,10
900	68,92^{b}±3,20	26,78^{b}±1,22
1125	84,64^{a}±5,74	37,50^{a}±0,48

According to Duncan's test, in the same column, means with same letters are not significantly different at $p < 0.05$.

4. Discussion

In this study, yield extraction of thyme EO was 0.42% with thymol, ɣ-terpinene and p-cymene as major components. For the same plant harvested in Bafoussam-Cameroon, Nguefack et al. [23] obtained 0.65% as yield EO with thymol (57.9%), P-cymene (10.3%) and linalool (6.9%) as predominant components. From the chemical analysis of thyme EO harvested in Morocco, El-Akhal et al. (2015) had reported thymol (50%), p-cymene (20%), and γ-terpinene (18%) as major compounds. These differences could be explained by the nature of the plant, its age, the site and period of harvesting, the post-harvest treatment, including the extraction time as well [24]. The differences could also due to the existence of a large number of chemotypes of each of these species. Indeed, according to Pibiri [25], the chemotypes of the same botanical species allow to obtain EO of different chemical compositions. However, thymol remains the predominant compound in the essence of *T. vulgaris* and would constitute the marker of this one.

Many studies have shown the efficacy of plant EO to inhibit the growth of different plant pathogen [26,27]. Our findings revealed the effectiveness of the EO isolated from Thyme on the mycelial growth and the germination of *P. megakarya* zoospores. This EO had been previously reported as good sources of antimicrobial components [15,18]. Bosquez-Molina *et* al. [28] showed that at 60ppm *T. vulgaris* EO total inhibits the mycelial growth of *Colletotrichum gloeosporioides* and *Rhizopus stolonifer* at a MIC of 60ppm.The findings of Riccioni and Orzali. [29] revealed that, *T. vulgaris* EO at 50ppm had a significant effect on the mycelial growth of *Colletotrichum lindermuthianum*. Abd-Alla *et al.* [30] revealed that *T.vulgaris* EO inhibits *Fusarium semitectum* conidia germination with a MIC of 1000ppm and this effect had been related of the chemical composition of oil. The results could be explained by a synergy between the compounds of the EO, or their different chemical composition. It could also be linked to the difference in sensitivity of the germs used. Indeed, because of the complexity of the chemical composition of essential oils,

Alonso-Gato et al. [31] showed that the observed antimicrobial activity would be due to the presence of interactions between the compounds found in majority and in minority. Furthermore, several authors reveal that the activity of an essential oil is related to its chemical composition, structural configuration of its components and its functional groups [32,33]. Meepagala et al. [34] showed that the various compounds of an EO including alcohols, aldehydes, terpenoids and phenolic compounds would act together and/or independently to contribute to the overall action of an EO. In addition, these authors showed that the antimicrobial activity of an EO on the germination of conidia could be a function of its proportion in monoterpenes and aromatic compounds. Indeed, MTOs such as limonene and phenolic compounds such as thymol, mainly represented in the sample studied here, are known for their strong activity on phytopathogenic fungi because of the interactions they establish with their cell membrane. They lead to the alteration of membrane permeability, loss of homeostasis and death of the microorganism. However, although the antimicrobial activity of EO on the germination of *P. megakarya* zoospores could be attributed to these constituents, the synergistic effect of the compounds in low proportions could significantly influence this activity [35].

Due to the nature of the antimicrobial compounds, they would have diffused through the tissues to confer systemic protection to the fruit. The preventive test (almost complete inhibition at 1125 µl/L) is more effective than the curative test. This would be due to the difficulty of the EO to diffuse through the plant tissue and to induce the stimulation of the defence mechanisms. Thus, the effectiveness of an EO would be more interesting when it is in direct contact with the pathogen. Indeed, according to Rao *et al*., [35], the intrinsic 00.0.0.00properties of the food (lipid, protein, water) as well as the extrinsic properties (temperatu re, characteristic of the microorganism) can influence the antimicrobial activity of the Enjuè=_O. The results obtained do not corroborate with those of Abd-Alla *et al.* [30] who showed that *T. vulgaris* EO and *Cinnamomum zeylanicum* applied at 4000 ppm can inhibit 100% of the incidence of banana crown rot of Williams's variety, disease due to *Fusarium semitectum*. This observed difference could be related to the germ used, the choice of fruit, as well as the physiological state of the fruit including the age at harvest (young fruits are more sensitive than older ones), the filling of the fruit called grade, which are the parameters that influence the susceptibility of cocoa pods to post-harvest diseases In fact, Perumal et al. [36] cites some factors that influence the incidence of post-harvest diseases: the physiological state of the fruit in relation to the stage of harvesting, climatic conditions during the growth phase and hydro-mineral nutrition and mechanical stresses on the fruit during harvesting and transport.

5. Conclusion

These studies showed that, thyme essential oil significantly inhibit the myecelial growth, the zoospores germination of *P. megakara* and reduced the necrosis on cocoa pod. This oil could be used as biopesticide to control cocoa black pod disease. However, to promote it field application, the formulation is needed.

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