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Antifungal effects of clove (*Syzygium aromaticum*) essential oil against *Colletotrichum gloeosporioides*, the fungus associated with papaya (*Carica papaya* L.) fruit anthracnose

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ABSTRACT

The present study was carried out to determine the chemical composition of clove essential oil and evaluate its potential to control *Colletotrichum gloeosporoides*, the causal agent of fruit rots in *Carica papaya* L. Hydro-distillation method was used to extract essential oil from clove and its chemical composition was analyzed by gas chromatography (GC) and gas chromatography coupled with mass spectroscopy (GC/MS). The antifungal potential of clove oil was evaluated *in vitro* in two life stages of the pathogen and *in vivo* using artificially infected fruits. The results obtained showed that, the yield of extraction of oil was 9.66%. The GC analysis revealed the presence of 28 volatile compounds with eugenol (87.62%), β-caryophyllene (5.88%) and β-bisabolene (4.41%) being major components. Clove oil significantly inhibited the mycelial growth and conidia germination of *C. gloeosporoides* with minimum inhibition concentration (MIC) at 375 and 100 µl/l, respectively. On papaya fruits, 3000 µl/l of essential oil was required to completely inhibit (100%) the necrosis caused by *C. gloeosporoides* both for preventive and curative assay. These results show that clove oil is a potential candidate for bio-pesticide development to manage papaya fruit anthracnose disease.

INTRODUCTION

Papaya (*Carica papaya* L.) is one of the most consumed fruits with paramount importance for nutrition (Sharma, 2015) both in tropical and subtropical countries including Cameroon. Papaya is a good source of glucose, minerals (K^+, Mg^{2+} and Ca^{2+}), vitamins (C, B_3, B_5) and fibers. It has good attractive sensory characteristics and presents a variety of health-related bioactive components (Lakshmi et al., 2011).

Papaya fruits are susceptible to many diseases, including anthracnose caused by *Colletotrichum gloeosporioides* (Rampersad, 2011). This phyto-pathogen is responsible for the main fungal disease damaging papaya fruits both in field and post-harvest. The disease alters the nutritional and organoleptic qualities of fruits. In case of absence of any control measure, yield loss could reach 70 to 100% (Rampersad, 2011). The management of papaya anthracnose disease is a serious issue since the treatment by the repetitive use of fungicides not only alters

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the ripening process of fruits but may lead to human and environmental toxicity as well as the development of fungicide resistance in *Colletotrichum* strains (Dos Santos et al., 2019). Then, eco-friendly alternative strategies are needed to control the occurrence of post-harvest disease agents such as anthracnose disease.

The use of plant extracts as food preservative and postharvest disease management have received most attention since many decades (Souza, 2016). Among plant extracts, essential oils are often used due to their strong and large spectrum antimicrobial activity, less toxicity and low resistance effects in targets microorganisms. Essential oils are defined as a complex mixture of low weight secondary metabolites extracted from different organs of aromatic plants.

Cameroon is a country which possesses a very wide range of species of aromatic plants endowed with numerous biological properties (Amvam et al., 1998). Among them, clove is commonly used as spice and medicinal plants. The available literature showed that, essential oils extracted from clove have biological properties including antifungal. These antifungal activities are related to the composition of the oil which varies with the ecological area. Although antifungal activities of different essential oils studied against pathogen (Barrera et al., 2008), to the best of our knowledge, there are no previous works reporting the antimicrobial potential of clove essential oil against *C. gloeosporoides* in Cameroon.

The aim of this study was to evaluate *in vitro* and *in vivo* antifungal potential of essential oil from clove against *C. gloeosporoides*, the pathogen associated with papaya fruit anthracnose disease.

MATERIALS AND METHODS

Plant material

The floral buds of clove (*S. aromaticum*) were harvested in the locality of Penja–Cameroon and identified at the National Herbarium (Yaoundé-Cameroon) where voucher specimens are available.

Essential oil (EO) analysis

Essential oil was extracted by hydro-distillation of the floral buds of plant by using Clevenger apparatus during 10 h. Collected oil was dried in anhydrous sodium sulphate and stored in dark containers at 4°C until use.

The chemical composition of oil was determined by gas chromatography and gas chromatography coupled by mass spectroscopy. Oil was analyzed on a Varian CP-3380 GC with flame ionization detector fitted with a fused silica capillary column (30 m × 0.25 mm coated with DB-5, film thickness 0.25 µm); temperature program 50-200°C at 5°C/min, injector temperature 200°C, detector temperature 200°C, carrier gas N_2 1 ml/min. The linear retention indices (RI) of each component were determined by using the retention times corresponding to a series of n-alkane classes. The percentage compositions were thereafter obtained from electronic integration measurements without taking into account relative response factors. The identification of the constituents of oil was assigned on the basis of comparison of their RI with those given in the literature (Adams, 2007).

Pathogen

C. gloeosporoides used in this study was obtained from core culture collection of the Laboratory of Biochemistry of Faculty of Science, University of Douala. The fungus was previously isolated from the papaya fruits having distinctive symptoms of anthracnose. The pathogen was maintained on potato dextrose agar (PDA) plate and stored at 4°C until use.

In vitro test

The fungicidal effect of the essential oil was evaluated *in vitro* on mycelia growth and on the germination of conidia.

The food poisoning method was used to evaluate the mycelia growth inhibition as described by Lahlou (2004). Essential oil was first dissolved in dimethyl sufoxide at a rate of 1:9. Agar plate supplemented with this oil solution at different concentrations ranging from 125 to 4000 µl/l. A 5-mm diameter mycelial disc from the active growing area of 3-days old colony was placed on the center of each PDA plate and incubated in the dark at 28°C. The diameter of mycelial growth was recorded during 7 days. Dimethyl methoxylsulfate sodium (DMSO) was used as control. The mycelial growth inhibition (MGI) was evaluated according to the formula

$$MGI = [(Dc – De)/Dc] \times 100$$

Where Dc is the mean diameter of mycelial growth in the control and De is the mean diameter of mycelial growth in the treatment with oil. Each treatment was evaluated in three replicates and the experiment was repeated twice. MIC and minimum fungicidal concentration (MFC) of essential oil was determined according to the method of Wang et al. (2005).

The inhibitory effect of essential oil on conidia germination was evaluated by micro dilution liquid method as described by Sharma and Tripathi (2006). The conidia suspension obtained from 14 days old colony was diluted with distilled water and adjusted to 10^5conidia/mL. 100 µl of prepared suspension was then introduced in potato dextrose broth (PDB) previously supplemented with EO to

obtain final concentrations ranging from 25 to 200 µl/l. The PDB medium with distilled water served as control. The microplates were then covered and incubated at 28 ± 2°C. 24 h after incubation, the number of germinated conidia was evaluated using an optical microscope (x 10) with a minimum of 100 spores in each treatment counted. The averaged results were converted in percent inhibition (%I) according to the formula:

$$\%I = [(Gt - Gx)/Gt] \times 100$$

Where Gt is average number of germinated conidia in the control tubes and Gx average number of germinated conidia in oil supplemented test tube. Each treatment was maintained in triplicate and the experiment was repeated twice.

In vivo test

The *in vivo* inhibitory effect of essential oil on disease symptoms was performed on whole green papaya fruits as described by Madjouko et al. (2019). Basically, healthy fruits were harvested and washed using running tap water. The fruits were then disinfected with 70% alcohol during 5 min, rinsed 3 times with distilled water and dried using absorbent sterile towel paper. A bores measuring 1 cm in diameter and depth were made on fruits surface using a sterile cork borer.

One hundred and fifty microliters of conidia suspension (10^5 conidia/ml) of *C. gloeosporoïdes* was introduced into the wells to promote infection. Essential oil was tested at concentrations ranging from 375 to 3000 µl/l by vaporizing 1000 µl of prepared concentrations in the bores 3 h before and after infection for the preventive and curative test, respectively.

After processing the papayas were placed on trays containing blotting paper soaked with sterile distilled water. The trays were sealed with para film and incubated for 8 days at room temperature. The percentage of necrosis inhibition (%I) was calculated by the following formula:

$$\%I = [(Dt - De)/Dt] \times 100$$

Where Dt is the mean diameter of necrosis developed in the control, De is the mean diameter of necrosis developed in the test. Each treatment carried out on 10 papaya fruits and the experiment was repeated twice.

Statistical analysis

The data were analyzed using Statview version 5.0 for Windows (SAS, Institute, Inc., USA). The significance threshold was set to p-value <0.05. The correlation of Pearson was used to evaluate the correlation between the parameters.

RESULTS AND DISCUSSION

Yield and chemical composition of clove essential oil

The clove EO obtained was a-yellow pale in color with a yield of 9.66 % (Table 1). The chemical composition of oil obtained by gas chromatography revealed the presence of 28 components with eugenol (87.62%), β-caryophyllene (5.88%) and β-bisabolene (4.41%) being the principal components (Table 1). Chemical composition of clove EO from different countries was previously analyzed. The yield and the composition of oil differed according to organ, agro-ecological zones and physiological factors including age and development stage of the plant. Sameza et al. (2016) and Nana et al. (2015) had each one analyzed clove essential oil of another locality of Cameroon and obtained respectively, a yield of 6.34 and 10.54%. The major components found were eugenol (79.4%; 83.02%), eugenylacetate (9.2%; 9.15%) and isocaryophyllene (7%; 7.04%). Razafimamonjison et al. (2014) compared the composition of clove oil from Madagascar, Indonesia and Zanzibar and obtained eugenol, eugenyl acetate and β-caryophyllene as main components with more or less significant differences in terms of percentages. In the same way, other some results also identified these 3 constituents as major components in clove oil (Ranasinghe et al., 2002; Lopez-Malo et al., 2002; Xing et al., 2012). In this study, the different results obtained (yield and major constituents) corroborate the fact that the level of secretion of secondary metabolites by plants could vary depending on the site and the chemotypes of the plant.

Mycelial growth and conidia germination inhibition

The results presented in Table 2 and 3 showed that, clove EO significantly inhibited ($p \leq 0.05$) the mycelial growth and the conidia germination of *C. gloeosporoides*. The inhibition increased along with the concentration of oil. The MIC was 375 and 100 µl/l respectively, for mycelial growth and conidia germination. When fungal disc was removed from MIC plate and transfered into fresh medium, no growth occurs, showing that the pathogen was killed. Therefore, oil was found to be fungicidal at the MIC. Previous studies have investigated the effects of plant essential oil and major pure environmental friendly compounds on the growth inhibition of some important post-harvest plant pathogens (Soylu et al., 2010; Kara and Soylu, 2020). The results have shown the effectiveness of oils to inhibit the mycelial and conidial growth of pathogens. In this way, the efficacy of clove oil as a good source of antifungal components has been previously reported (Ranasinghe et al., 2002; Park et al., 2007; Xing

Table 1. Chemical composition of clove (*Syzygium aromaticum*) oil.

N°	KI	Components	Relative percentatage (%)
1	1019	P- cymene	0.01
2	1087	Terpinolene	0.03
3	1027	1.8- cineole	0.03
4	1079	Fenchone	0.03
5	1104	Camphenol	0.03
7	1212	Carveol	0.01
8	1237	Geraniol	0.35
9	1353	Eugenol	87.62
10	1367	Dihydro-eugenol	0.21
11	1445	Isoeugenol	0.67
12	1521	Eugenol acetate	0.05
13	1338	δ-elemene	0.09
14	1412	β-caryophyllene	5.88
15	1463	Epi-(E)- Caryophyllene	0.05
16	1465	Ɣ-gurjunene	0.02
17	1483	Germacrene-D	0.02
18	1489	β-sélinene	0.08
19	1511	β-bisabolene	4.41
20	1514	Δ-cadinene	0.05
21	1545	Elemol	0.02
22	1576	Spathulenol	0.14
23	1602	Guaiol	0.02
24	1618	Cubenol	0.04
25	1627	Ɣ-eudesmol	0.01
26	1674	β-bisabolol	0.04
27	844	3(E)-hexenol	0.01
28	884	4-heptanol	0.02
Yield (%)			9.66

KI, Kovats index.

Table 2. Effect of clove oil on mycelial growth of *C. gloeosporoides* during 7 days.

Oil concentration (µl/l)	Inhibition (%)
0	0.00^{a}±0.00
125	40.05^{b}±4.90
250	58.01^{c}±3.51
300	65.30^{c}±3.80
325	75.55^{d}±4.03
350	88.61^{e}±4.71
375	100.00^{f}±2.81
400	100.00^{f}±2.79

Each value is the average of three and the treatment was repeated twice. According to DUNCAN'S test, means with the same letter are not significant different at p≤0.05.

Table 3. Effect of clove oil on conidia germination of *C. gloeosporoides* during 24 h.

Oil concentration (µl/l)	Inhibition (%)
0	0.00[a]±0.00
25	43.15 [b]±5.90
50	77.40[c]±4.55
100	100.00[d]±2.79
200	100.00[d]±2.79

Each value is the average of three and the treatment was repeated twice. According to DUNCAN'S test, means with the same letter are not significant different at p≤0.05.

Table 4. Effect of clove oil on the necrosis of papaya fruit anthracnose.

Oil concentration (µl/l)	Inhibition (%)	
	Preventive test	Curative test
0	0.00[a]±0.00	0.00[a]±0.00
375	35.63[b]±2.85	28.35[b]±5.85
750	56.25[c]±3.10	48.85[c]±2.90
1500	78.61[f]±3.61	76.55[d]±3.14
3000	100.00[d]±2.76	100.00[f]±2.76

According to DUNCAN'S test, in the same line, means with the same letter are not significant different at p≤0.05.

et al., 2012). Sameza et al. (2016) showed that clove oil completely inhibited the mycelial growth of *Rhizopus stolonifer* and *Fusarium solani*. Nana et al. (2015) reported that clove oil had a significant antimicrobial activities against mycelia growth and spore germination of *Phytophthora megakarya*. Barrera et al. (2008) revealed that at 50 µg/ml, EO of *S. aromaticum* inhibited the mycelial growth and the germination of conidia of *C. gloeosporoides* and *Fusarium* sp.

Globally, the antimicrobial activity of EO is related to its chemical composition. In this study, the deleterious effect of *S. aromaticum* EO could be assigned to the presence of its major compounds eugenol which antimicrobial properties have already been clearly reported. Cakir et al. (2004) showed that hydrocarbon mono-terpenes and sesquiterpenes have a strong inhibitory activity against fungi. Moreover, Mohammad et al. (2013) showed that the most active compounds against fungi belonging to the close group of phenols, aldehydes and alcohols. These groups of compounds possess antifungal properties which are partly related to their hydrophobicity. The activity of the EO could be due either to the action of compounds found in higher amount or to a synergy between components found in higher and fewer quantities including those present in trace (Burt, 2004). Burt (2004) suggested that the antimicrobial effect exerted by EOs could be explained by inhibition of some important enzymatic systems occurring in metabolic pathways of pathogen metabolism. Those enzymatic systems including those involved in the production of cellular energy and structural compounds.

In vivo antifungal assay

Inhibition of the necrosis caused by *C. gloeosporioides* on papayas rot fruits was evaluated 7 days after inoculation. Results showed that clove essential oil significantly ($p \leq 0.05$) inhibited the fruits rot for both preventive and curative tests (Table 4 and Figure 1). Total inhibition (100 %) was recorded at 3000 µl/l both for disease preventive and curative. Statistical analysis revealed that, there was strong and positive correlation ($p = 0.001$; $r = 0.89$) between the reduction of necrosis and the concentration of clove oil for both preventive and healing test. These results corroborated the findings from previous works on the antifungal potency of clove essential oil. Sameza et al. (2016) tested the activity of EO *S. aromaticum* on *R. stolinifer* and *F. solani* and indicated that this oil could protect yam against tuber rot both during preventive and curative test. In the same way, Hong et al. (2015) suggested that clove oil could prevent pepper rot fruits caused by *C. gloesporioides*. Moreover, Nana et al. (2015)

Figure 1. Effect of clove oil concentration on necrosis of papaya fruits causing by *C. gloeosporoides*. **A**, Preventive test. **B**, curative test.

demonstrated the effectiveness of clove essential oil to control cocoa black pod disease. Indeed, EOs are able to induce direct resistance and/or antimicrobial activity in plants. This phenomenon of resistance is characterized by a swift synthesis of reactive oxygen species (H_2O_2) or oxidative stress, which is a reaction that occurs in all plants that have undergone biological disturbance or physical degradation.

Conclusion

This work showed that clove essential oil significantly inhibit the mycelial growth and conidia germination of *C. gloeosporioides in vitro.* It was able to significantly reduce the necrosis symptoms of papaya fruit rot. Clove oil could serve as alternative to reduce the damages caused by *C. gloeosporioides* on papaya fruits. However, the formulation of oil as biopesticide for large scale use is needed.

Conflict of interest

The authors declare that they have no competing interests.

REFERENCES

Adams R. P. (2007). Identification of essential oil components by gas chromatography mass spectrometry. Illinois: Allured Publishing Corporation. 495p.

Amvam Z. P. H., Biyiti L., Tchoumbougnang F., Menut C., Lamaty & Bouchet P. (1998). Aromatic plants of tropical central Africa: Chemical composition and antifungal activity of thirteen essential oils from aromatic plants of Cameroon. Flav. Frag. J. 13: 1-8.

Barrera N. L. L., Bautista B. S., Flores-Moctezuma H. E. & Estudillo A. R. (2008). Efficacy of essential oils on the conidial germination, growth of *Colletotricum gloeosporioides* (Penz.) and control of postharvest diseases in papaya (*Carica papaya* L.). Plant Pathol. J. 7:174-178.

Burt S. (2004). Essential oils: Their antibacterial properties and potential applications in foods a review. Int. J. Food Microbiol. 94:223-253.

Cakir A., Kordali S., Zengin H., Izumi S. & Hirata T. (2004). Composition and antifungal activity of essential oils isolated from *Hypericum hyssopifolium* and *Hypericum heterophyllum*. Flav. Frag. J. 19:62-68.

Dos Santos V., Dos Santos A. N., Veloso S. J., Balbino O. V., Da Silva C. A., Gomes M. A. A., Doyle P. V. & Saraiva P. M. (2019). *Colletotrichum truncatum* causing anthracnose on papaya fruit (*Carica papaya*) in Brazil. Aust. Plant Dis. Notes. 15:2.

Hong K. J., Yang J. H., Jung H., Sang K. M. & Yong-Chull J. (2015). Application of volatile antifungal plant essential oils for controlling Pepper fruit anthracnose by *Colletotrichum gloeosporioides*. Plant Pathol. J. 31:269-277.

Kara M. &, Soylu E. M. (2020). Assessment of glucosinolate-derived isothiocyanates as potential natural antifungal compounds against citrus sour rot disease agent *Geotrichum citri-aurantii*. J. Phytopathol. 168:279-289.

Lahlou M. (2004). Methods to study the phytochemistry and bioactivity of essential oils. Phytother. Res. 18:435-448.

Lakshmi S. L., Abirami R., Pushkala R. & Srividya N. (2011). Enhancement of storage life and quality maintenance of papaya fruits using *Aloe vera* based antimicrobial coating. Indian J. Biotechnol. 10:83–-89.

Lopez-Malo A., Alzamora S. M. &, Palou E. (2002). *Aspergillus flavus* dose response curves to selected natural and synthetic antimicrobials. Int. J. Food Microbiol. 73:213-218.

Madjouko M. A., Tchameni N. S., Tchinda S. E., Jazet D. P. M., Kamsu

N. P., Kamga Souop M. V. A., Sameza M. L., Tchoumbougnang F. &, Menut C. (2019). Inhibitory effects of essential oils from *Ocimum basilicum* and *Ocimum gratissimum* on *Colletotrichum musae*: The causal agent of bananas anthracnose. J. Phytopathol. 167:257-264.

Mohammad M., Pourbaige M., Tabar H. K., Farhadi H. K. & Hosseini S. M. A. (2013). Composition and antifungal activity of peppermint (*Mentha piperita*) essential oil from Iran. J. Essent. Oil Bear. Plants. 16:506-512.

Nana Wakam L., Eke P., Fokom R., Issakou B., Begoude D., Tchana T., Tchameni N. S., Kuate J., Menut C. & Fekam F. B. (2015). Antimicrobial activity of *Syzygium aromaticum* and *Zanthoxylum xanthoxyloides* essential oils against *Phytophthora megakarya*. J. Phytopathol. 167:632-641.

Park M. J., Gwak K. S., Yang I., Choi W. S., Jo H. J., Chang J. W., Jeung E. B. & Choi I. G. (2007). Antifungal activities of the essential oils in *Syzygium aromaticum* (L.) Merret Perry and *Leptospermum petersonii* bailey and their constituents against various dermatophytes. J. Microbiol. 45:460-465.

Rampersad S. N. (2011). Molecular and phenotypic characterization of *Colletotrichum* species associated with anthracnose disease of papaya in Trinidad. Plant Dis. 95:1244-1254.

Ranasinghe L., Jayawardena B. &, Adeywickrama K. (2002). Fungicidal activity of essential oils of *Cinnamomum zeylanicum* (L) and *Syzygium aromaticum* (L) against crown rot and anthracnose pathogens isolated from banana. Lett. Appl. Microbiol. 35:208-211.

Razafimamonjison G., Jahiel M., Duclos T., Ramanoelina P., Fawbush F. &, Danthu P. (2014). Bud, leaf and stem essential oil composition of *Syzygium aromaticum* from Madagascar, Indonesia and Zanzibar. Int. J. Basic Appl. Sci. 3:224-233.

Sameza M. L., Bedine Boat M. A. B., Tchameni N. S., Nguemnang M. L., Bedine B. A., Tchoumbougnang F., Jazet D. P. M., Fekam B. F. (2016). Evaluation of clove essential oil as a mycobiocide against *rhizopus stolonifer* and *Fusarium solani*, tuber rot causing fungi in yam. J. Phytopathol. 164:433-440.

Sharma N. & Tripathi A. (2006). Effects of *Citrus sinensis* (L.) Osbeck epicarp essential oil on growth and morphogenesis of *Aspergillus niger* (L.) Van Tieghem. Microbiol. Res. 163:337-344.

Sharma V. (2015). Evaluation of incidence and alternative management of post-harvest fungal diseases of papaya fruits (*Carica papaya* L.) in Western U.P. Int. J. Theor. Appl. Sci. 7(1):6-12.

Souza E. L. (2016). The effects of sublethal doses of essential oils and their constituents on antimicrobial susceptibility and antibiotic resistance among food related bacteria: a review. Int. J. Food Microbiol. 56:1-12.

Soylu E. M., Kurt, Ş. & Soylu, S. (2010). *In vitro* and *in vivo* antifungal activities of the essential oils of various plants against tomato grey mould disease agent *Botrytis cinerea*. Int. J. Food Microbiol. 143:183-189.

Wang M., Bohmanm D. & Jasper H. (2005). Extends life span and limits growth by antagonizing cellular and organin- wide responses to insulin signaling. 121(1): 115-125. Working Paper 34.

Xing Y., Xu Q., Li X., Che Z., & Yun J. (2012). Antifungal activities of clove oil against *Rhizopus nigricans, Aspergillus fl Asp* and *Penicillium citrinum in vitro* and in wounded fruit test. J. Food Safety. 32:84-93.