In vitro study of selected essential oils against *Colletotrichum* sp. and *Lasiodiplodia* sp. causing postharvest diseases in papaya

Y.A.S. Samithri, K.O.L.C. Karunanayake and A.A. Kulasinghe

Highlights

- Citronella oil at 750 $\mu\text{l l}^{-1}$ and Cardamom oil at 1000 $\mu\text{l l}^{-1}$ significantly inhibit the *in vitro* growth of *Colletotrichum* sp. and *Lasiodiplodia* sp. isolated from papaya fruit.
- Higher percentages of antifungal geraniol and geranyl acetate are present in citronella oil
- Higher percentages of antifungal α -terpinyl acetate and 1, 8-cineole are present in cardamom oil.

RESEARCH ARTICLE

***In vitro* study of selected essential oils against *Colletotrichum* sp. and *Lasiodiplodia* sp. causing postharvest diseases in papaya**

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Abstract: Anthracnose caused by *Colletotrichum* sp. and stem-end rot (SER) caused predominantly by *Lasiodiplodia* sp. are common postharvest diseases affecting papaya in Sri Lanka and are currently managed by synthetic chemicals which are hazardous. Use of essential oils (EOs) is considered a non-hazardous alternative. This *in vitro* study investigated the antifungal potential of selected EOs against anthracnose and SER pathogens isolated from papaya var. 'Red Lady'. A poisoned food bioassay was carried out to evaluate the antifungal effect of EOs (Cardamom, citronella, orange, mustard and lemon). Colony diameter was taken as a measure of growth. Bioassays consisted of EOs at 500, 750, 1000 $\mu\text{l l}^{-1}$ with untreated control, three replicates and three separate trials. Cardamom (1000 $\mu\text{l l}^{-1}$) and citronella (750 $\mu\text{l l}^{-1}$) significantly ($P < 0.05$) inhibited *Colletotrichum* sp. and *Lasiodiplodia* sp. from papaya. According to GC-MS, higher percentages of antifungal geraniol, geranyl acetate are present in citronella while α -terpinyl acetate, 1, 8-cineole are present in cardamom oil.

Keywords: papaya, anthracnose, stem-end rot, essential oils, poisoned food bioassay.

INTRODUCTION

Papaya (*Carica papaya* L.) occupies a prominent place in the local and international market as a much sought-after tropical fruit. During ripening, papaya fruits are subjected to numerous diseases, mostly of fungal origin, and a large percentage of fruits are lost at the postharvest stage. Anthracnose (*Colletotrichum gloeosporioides*), stem-end rot (*Lasiodiplodia theobromae*) and Phomopsis rot (*Phomopsis caricae-papayae*) are major postharvest diseases of papaya in Sri Lanka, resulting in relatively higher postharvest losses of up to 45% (Abeywickrama *et al.*, 2012; Sarananda *et al.*, 2004). Further, postharvest diseases caused by fungi reduce the shelf life and market value of freshly harvested produce, including papaya fruits (Espitiya *et al.*, 2012), and make them unhealthy for human consumption (Kumar *et al.*, 2008). Currently, synthetic fungicides are used world-wide to prevent and control fungal decay. Yet, synthetic fungicides may lead to the development of fungicide-resistant strains, environmental contamination and chemical residues on fruit surfaces which pose a potential health hazard (Alemu *et al.*, 2014; Maqbool *et al.*, 2011). Due to these concerns, use of

certain chemical fungicides is restricted in some countries (Ramezani *et al.*, 2002).

Therefore, as an alternative method of disease management, essential oils (EOs) with fungicidal properties have gained much attention (Imelouane *et al.*, 2009; Meepagala *et al.*, 2002; Wilson *et al.*, 1997). EOs are naturally occurring substances which are generally recognized as safe (GRAS) by the U.S. Food and Drug Administration (Lopez *et al.*, 2007) and are known to contain a variety of chemical constituents, including terpenoids which are known to be involved in the plant defense mechanisms against plant pathogens (Lee *et al.*, 2001). Nychas (1995) reported that the EOs may affect the metabolic pathways of microorganisms. Further, the hydrophobic nature of EOs and their components, reportedly enables the compounds to penetrate the lipid in the fungal cell membrane and mitochondria (Cox *et al.*, 2000).

Use of EOs for the control of postharvest disease has been tried with promising results on fruits such as mandarin, kiwi and rambutan (Arras, 1988; Thanassouloupoulos and Yanna, 1997; Sivakumar *et al.*, 2002) and locally on crown rot and anthracnose of banana (Anthony *et al.*, 2004). Anthracnose of mango has been effectively controlled by use of EOs such as basil oil (*Ocinum basillicum*), orange oil (*Citrus sinensis*), lemon oil (*Citrus medica*) and mustard oil (*Brassica juncea*) (Abd-alla and Haggag, 2013). Extensive work has been carried out by Espitiya *et al.* (2012) on the efficiency of EOs in retarding postharvest diseases of papaya.

The present study aimed investigation of the antifungal potential of selected EOs against anthracnose and SER pathogens, *Colletotrichum* sp. and *Lasiodiplodia* sp. respectively, isolated from ripe papaya fruits showing disease symptoms, under *in vitro* conditions, with the view of using the selected EOs in future *in vivo* for the control of related postharvest diseases.

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MATERIALS AND METHODS

Essential oils

Pure-graded EOs of cardamom (*Elettaria cardamomum*), citronella (*Cymbopogon nardus* L.), lemon (*Citrus limon*), mustard (*Brassica juncea* L.) and orange (*Citrus sinensis*) were obtained from 'Herble Exotics' Pugoda, Sri Lanka and their quality and stability were certified by the commercial supplier. Essential oils used (cardamom and citronella) were analyzed by Gas Chromatography Mass Spectrometry (GC-MS) at the Industrial and Technology Institute, Colombo, Sri Lanka for their chemical composition.

Isolation of postharvest fungal pathogens

Papaya fruits cv. "Red lady" showing characteristic symptoms of anthracnose and stem-end rot were obtained from the Nugegoda and Matara markets. The symptoms were verified using the colour atlas of postharvest diseases and disorders by Snowdon (1990). Fungal pathogens were isolated on to Potato Dextrose Agar (PDA) (Meron Bacteriological Agar, superfine grade).

Fungal pathogens were isolated from symptomatic fruit tissue following a standard protocol (Wanigasekara, 2009). First, pieces of diseased fruit tissues (1 cm²) were cut under aseptic conditions and were surface disinfected by washing the sections in freshly prepared 1 % sodium hypochlorite solution for 30 sec and washed in three changes of sterile distilled water. The segment of tissue was blotted dry on sterile tissue paper and cut in to four pieces. These pieces were placed (4 pieces per plate) on Potato Dextrose Agar (PDA) medium and the plates were incubated at room temperature (28±2 °C) in the laboratory of the Department of Botany, The Open University of Sri Lanka. After the emergence of mycelia growth, each fungal colony was transferred to fresh PDA plates and incubated at room temperature (28±2 °C) for 2-4 days to obtain pure cultures. Sub-culturing was done every two weeks and maintained on PDA at 28±2 °C (Wijesundara *et al.*, 2015). Ten isolates from each fungal species of each commodity were identified in pure culture following 7-10 days incubation based on fungal / conidial morphology (CMI descriptions).

Koch's postulates were performed to confirm the pathogenicity of isolates. First, papaya fruits showing typical symptoms of anthracnose and stem-end rot were selected. Next the causal agents of these diseases were isolated on to PDA using the standard protocol described above. After the emergence of mycelia, each fungal colony was transferred to fresh PDA and the plates were incubated at room temperature (28±2 °C) for 2-4 days to obtain pure cultures. Petri-plates with sporulating mycelia were flooded with sterile distilled water and the mycelium was scraped with a sterile bent glass rod to dislodge conidia. The resulting suspension with dislodged conidia was filtered through glass wool to obtain a suspension of conidia. The concentration of the suspension was adjusted to 10⁵ conidia ml⁻¹. This conidial suspension was used to inoculate healthy papaya fruits. Inoculation was done by placing 25µl drops of the conidia suspension along the fruit peel for anthracnose and on the stem-end of fruit for stem-

end rot. Inoculated fruits were kept in moist chambers for symptoms to appear. After disease symptoms appeared, they were compared with the symptoms that were originally observed. Then pathogens were re-isolated from the newly infected tissues of the host on to PDA following standard protocol as described above and were identified in pure cultures based on conidial morphology (CMI descriptions) to confirm the pathogenicity.

In vitro screening of EOs against fungal pathogens *Colletotrichum* sp. and *Lasiodiplodia* sp. of papaya

A poisoned food bioassay was carried out to evaluate the antifungal effect of EOs [cardamom (*E. cardamomum*), citronella (*C. nardus* L.), orange (*C. sinensis*), mustard (*B. juncea* L.) and lemon (*C. limon*)] by assaying mycelial growth inhibition of *Colletotrichum* sp. and *Lasiodiplodia* sp. from papaya fruits.

The pure-grade EOs in the selected concentrations were dispersed individually as an emulsion using Tween 80 (0.05%) and added to autoclaved PDA (40-45 °C) immediately before pouring into Petri dishes (Pitarokili *et al.*, 2003). The three concentrations of oils tested were 500, 750, 1000 µl l⁻¹ and the untreated controls included the same quantity of sterile distilled water added to autoclaved PDA (40-45 °C). A disk (5 mm diameter) of the test fungus, cut with a sterile cork borer from the periphery of actively growing cultures, was placed in the middle of each plate. The prepared plates were incubated at room temperature (Pitarokili *et al.*, 2003).

Linear diameter of the fungal colony (2 pairs – each pair at right angles to each other) was measured daily until the mycelium in the control completely filled the Petri plate. Three replicate plates were used per EO treatment and the experiment was repeated three times. The percentage mycelial inhibition was calculated by the formula used by Tripathi *et al.* (2008),

$$\text{Percentage mycelial growth inhibition} = \frac{(dc - dt)}{dc} \times 100$$

where, dc - mean colony diameter of the control sets, and dt - mean colony diameter of treatment sets.

Composition of Essential oils

The composition of the EOs was analyzed as described by Herath *et al.*, (2017) using a Trace 1300 Gas Chromatograph coupled with single MS (Model: ISO QD, Make: Thermo Scientific) at ITI, Colombo.

Statistical analysis

Data were analyzed using the statistical package, IBM SPSS version 20.0. Analysis of variance (ANOVA) among means was performed using one-way ANOVA.

RESULTS

Isolation of fungal pathogens

The fungi isolated from papaya anthracnose and SER showed typical morphological characteristics of *Colletotrichum* sp. and *Lasiodiplodia* sp. *Colletotrichum*

Figure 1: Colonies of A - *Colletotrichum* sp. and B - *Lasiodiplodia* sp., isolated respectively from an anthracnose lesion and SER in papaya (cv. Red Lady) fruit.

Figure 2: Mycelial inhibition of *Colletotrichum* sp. isolated from papaya on PDA enriched with different essential oils, 2 days after incubation.

sp. isolated from papaya anthracnose gave a colony which was initially white and later became light greyish and the mycelium covered the entire PDA surface within 2 days (Figure 1 A). The fungus produced numerous hyaline, one-celled, ovoid to oblong shaped conidia characteristic of *Colletotrichum* species.

The fungus *Lasiodiplodia* sp., isolated from papaya SER, gave a colony which was initially white and became grayish (Figure 1 B) and then dark grey and greyish-black after 2 weeks of incubation. Mature conidia were

uniseptate, oval-shaped and brown in colour with the presence of irregular longitudinal striations typical of conidia of *Lasiodiplodia* sp.

Table 1: Percentage mycelial inhibition of *Colletotrichum* sp. isolated from papaya on PDA Petri plates enriched with different essential oils at the time when control reached its maximum growth (90 mm) at 48 h (day 2) after inoculation.

EOs	Inhibition (%) at different concentrations ($\mu\text{l l}^{-1}$)		
	500	750	1000
Cardamom	31.62 ^a	31.53 ^a	53.70 ^b
Citronella	43.19 ^a	86.06 ^b	94.44 ^b
Lemon	3.803 ^c	14.77 ^c	13.00 ^c
Orange	1.15 ^c	9.68 ^c	2.27 ^c
Mustard	4.77 ^c	9.68 ^c	6.62 ^c

*Values followed by the same letters within each column are not significantly different at ($p \leq 0.05$) ($n = 9$). Inhibition % = Mean inhibition of nine replicates as a percentage.

Figure 3: Mycelial growth inhibition of *Lasiodiplodia* sp. isolated from papaya on PDA Petri plates enriched with different essential oils, 2 days after incubation.

Based on colony morphology and conidial morphology, the two fungi isolated from anthracnose and SER affected tissues, were identified to generic level as *Colletotrichum* sp. and *Lasiodiplodia* sp. respectively.

***In vitro* screening of EOs against *Colletotrichum* sp. and *Lasiodiplodia* sp. in papaya fruit**

Results indicated that the EOs, cardamom and citronella, significantly ($P < 0.05$) inhibited the mycelial growth of *Colletotrichum* sp. compared with the other EOs tested. Lemon, orange and mustard oils did not cause a significant inhibition of mycelial growth. Citronella was the most effective out of the EOs studied at 48 h (day 2). All replicates in the control sets reached maximum mycelial growth (90 mm colony diameter). The extent of inhibition of mycelial growth of *Colletotrichum* sp. varied with the concentration of the EOs used (Figure 2).

After 48 h (day 2), citronella oil at 750 $\mu\text{l l}^{-1}$ caused 86% inhibition while the highest inhibition (94.4%) was

observed at 1000 $\mu\text{l l}^{-1}$. On the other hand, cardamom oil showed more than 50% mycelial growth inhibition only at the highest concentration (1000 $\mu\text{l l}^{-1}$) (Table 1).

In vitro bioassays showed cardamom and citronella to be significantly ($P < 0.05$) more effective in inhibiting the growth of *Lasiodiplodia* sp.

The EOs, lemon, mustard and orange, inhibited less than 30% of mycelial growth of *Lasiodiplodia* sp. at all concentrations tested and their effect was not significant (Table 2). After 48 h (day 2), citronella oil showed more than 50% of mycelial growth inhibition under all tested concentrations. At 500 $\mu\text{l l}^{-1}$ citronella oil caused 79.63% inhibition in fungal growth, while at 750 $\mu\text{l l}^{-1}$ and 1000 $\mu\text{l l}^{-1}$ citronella caused 85.18% and 100% inhibition of growth respectively. At 500 $\mu\text{l l}^{-1}$ and 750 $\mu\text{l l}^{-1}$, cardamom oil showed a moderate (28-33%) inhibitory effect on fungal growth whereas at the highest concentration of 1000 $\mu\text{l l}^{-1}$ cardamom oil showed 62.96% significant reduction in

Table 2: Percentage mycelial growth inhibition of *Lasiodiplodia* sp. isolated from papaya on PDA, enriched with different essential oils, at the time when controls reached their maximum growth (90 mm colony diameter) at 48 h (day 2) after inoculation.

EOs	Inhibition (%) at different concentration (µl/l)		
	500	750	1000
Cardamom	33.80 ^a	28.24 ^a	62.96 ^b
Citronella	79.63 ^a	85.18 ^b	100 ^b
Lemon	15.74 ^c	15.28 ^c	25.92 ^c
Orange	24.53 ^c	21.76 ^c	37.5 ^a
Mustard	8.33 ^c	7.40 ^c	2.78 ^c

Values followed by the same letters within the column are not significantly different at ($p \leq 0.05$) ($n=9$). Inhibition% = Mean inhibition of nine replicates as a percentage

Table 3: Principal constituents of citronella oil and their relative percentages of total chromatogram area.

Compound	Retention time	Area %
α - Pinene	3.16	0.04
α -Phellandrene	4.94	0.28
α - Terpinolen	5.17	0.08
D-Limonene	5.49	2.77
β -Phellandrene	5.68	0.36
Trans- β -Ocimene	6.09	0.09
β -Ocimene	6.42	0.08
p-Cymene	6.86	0.18
4-Nonanone	8.02	0.31
5-Hepten-2-one, 6-methyl	8.62	0.04
Citronellal	11.47	5.47
Linalool	12.98	1.71
Isopulegol	13.59	0.46
Trans- α -bergamotene	13.79	0.83
Caryophyllene	14.1	5.63
Citronellol acetate	15.6	0.98
Humulene	15.75	0.71
α -citral	16.16	2.75
β -citral	17.28	4.1
Geranyl acetate	17.7	10.27
Citronellol	17.9	2.63
Geraniol	19.66	54.09
Caryophyllene oxide	22.48	0.64

mycelial growth of *Lasiodiplodia* sp. (Table 2).

GC-MS analysis of essential oils

GC-MS analyses of EOs, carried out in the present study, showed the presence of higher percentages of antifungal compounds, geraniol, geranyl acetate in citronella oil and α -terpinyl acetate, 1,8-cineole in cardamom oil (Table 3 and 4).

Table 4: Principal constituents of cardamom oil and their relative percentages of total chromatogram area.

Compound	Retention time	Area %
α - Pinene	3.2	3.89
β -Pinene	4.2	3.74
β -Phellandrene	4.36	2.81
β -Myrcene	4.94	1.4
4-Terpinenyl acetate	5.26	0.27
D-Limonene	5.59	5.31
1,8-Cineole	5.81	16.02
γ -Terpinene	6.44	0.39
p-Cymene	6.97	2.76
L-a-Terpineol	11.18	1.04
Fenchyl acetate	11.35	0.53
Linalool	13.14	4.42
Linalyl acetate	13.35	4.53
2-Carene	13.68	2.07
Bornyl acetate	14.06	1.62
4-Terpinenyl acetate	14.46	2.06
p-Menth-8-en-1-ol, acetate	15.96	1.57
2-Carene	16.23	5.74
α -Terpinyl acetate	16.61	21.51
2,6-Octadien-1-ol, 3,7-dimethyl-, (Z)-4-Hexen-ol	17.86	0.84
Geraniol	19.82	1.26
trans-Nerolidol	23.7	1.68

DISCUSSION

The present study evaluated the ability of five EOs to inhibit the growth of *Colletotrichum* sp. and *Lasiodiplodia* sp. isolated from ripe papaya fruits cv. Red Lady showing anthracnose and stem-end rot symptoms. According to literature, the mechanisms of action of EOs against fungi are varied. Nychas (1995) reported that the EOs may affect the metabolic pathways of microorganisms. According to Cox *et al.* (2000), the hydrophobic nature of EOs and their components enable them to penetrate through fungal cell membrane lipids and mitochondria and these compounds accumulate in the cell membrane of pathogen causing energy depletion. Li *et al.* (2013) reported citronella oil to have a greater inhibitory effect on conidia of *Aspergillus niger* possibly by rupturing the cell wall and then affecting the sporoplasm.

Lozada *et al.* (2019) reported that the EOs of thyme, lemongrass and citronella at a concentration of 2000 ppm completely inhibited mycelial growth of *Colletotrichum* sp. on onion seeds. *In vitro* studies by Sellamuthu *et al.* (2013) revealed that in the vapor phase, thyme oil at 5 μ l/plate, as opposed to peppermint and citronella oils, completely inhibited the radial mycelial growth of two postharvest fungal pathogens of avocado, *Colletotrichum*

gloeosporioides (anthracnose), *Lasiodiplodia theobromae* (stem-end rot) and three peach pathogens, *Monilinia fructicola* (brown rot), *Rhizopus stolonifer* (Rhizopus rot) and *Penicillium expansum* (blue mould rot). However, in contrast, the present study showed that citronella oil was significantly inhibitory to *Colletotrichum* sp. from papaya. Concentration of 750 μ l l⁻¹ of citronella oil were required to inhibit completely the fungus isolated from papayas. In addition to the tested EOs, others such as cinnamon oil, clove oil (*Syzygium aromaticum*) (Barrera-Necha *et al.*, 2008), thyme and Mexican lime EOs (Combrinck *et al.*, 2011; Bosquez-Molina *et al.*, 2010) are reported to have an inhibitory effect on *C. gloeosporioides* from papaya.

According to literature (Nigam and Purohit, 1960; Lawrence, 1970; Patra *et al.*, 1982; Hussain *et al.*, 1988), 1, 8-cineole is the principal component of cardamom essential oil. In the present study, the highest percentage constituent in cardamom was α -terpinyl acetate followed by 1, 8-cineole. The slight variation in composition could be due to environmental, developmental or genetic factors. Results of the GC-MS analysis of the present study revealed that α -terpinyl acetate (21.51%) and 1,8-cineole (16.02%) to be principal constituents of cardamom oil. The antifungal properties in cardamom oil (Gilani *et al.*, 2006; Rahman *et al.*, 1999) would have contributed towards inhibition of

fungal pathogens of papaya in the present study.

According to GC-MS analysis, the principal components of citronella oil used in the study were geraniol, geranyl acetate followed by citronellal. As previously reported by Toledo *et al.* (2016) and Nakahara *et al.* (2003), these constituents have antimicrobial properties. The antimicrobial effect these three constituents in citronella oil would have contributed towards inhibition of both anthracnose and SER pathogens from papaya in the present study.

CONCLUSIONS

EOs, citronella and cardamom, have a potential to be used as alternatives to chemical fungicides in controlling growth of *Colletotrichum* and *Lasiodiplodia* sp. in papaya fruit. The method of application should be determined by *in vivo* studies.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest.

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