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Activity of Some Plant Oils and Extracts Against *Colletotrichum gloeosporioides*

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Abstract: The antifungal effects of a range of plant extracts and oils were studied in a series of *in vitro* experiments against *Colletotrichum gloeosporioides* isolated from black pepper (*Piper nigrum* L.). Spore germination of the fungus was completely inhibited by cinnamon (*Cinnamomum zeylanicum* Blume.) oils as well as by water or ethanol extracts from galangal (*Alpinia galanga* L. Willd.) rhizomes and cardamom (*Elettaria cardamomum* Maton.) leaves. Ethanol extracts were more efficient in inhibiting spore germination than water extracts. Phytotoxicity symptoms were not observed or were minimal on pepper leaves and berries or red pepper fruits when treated with cardamom extract and galangal extract and a group of oils of cardamom, Eucalyptus, lesser galangal, lemon grass, lemon myrtle, neem, pepper black and tea-tree, but were pronounced with cinnamon oils. Cardamon oil was non toxic, but was required in higher concentrations to completely inhibit germination.

Key words: *Colletotrichum*, oils, extracts, phytotoxicity, inhibition

INTRODUCTION

Black berry disease is one of the most common fungal diseases on pepper and is caused by *Colletotrichum capsici* (Syd.) E.J. Butler and Bisby and *C. gloeosporioides* Penz (Kumar and Anoop, 2003; Sarma *et al*., 2000). It limits berry production and causes significant yield losses in north Queensland (L. Campagnolo, *personal communication*) and the disease has been reported as a major fungal disease on black pepper in other tropical areas (Weber, 1973). Costly synthetic fungicides are utilised on pepper for effective control of diseases in several countries (Weiss, 1997). However, these fungicides may exert harmful effects so that alternative methods of control have been sought through the use of plant extracts and oils. The potential biological activity of plant extracts and oils has been assessed against a wide range of fungal pathogens on plants (Carson and Riley, 1998; Fiori *et al*., 2000; Letessier *et al*., 2001; Mahmoud, 1999; Northover and Scheider, 1993; Ranasinghe *et al*., 2002; Suganda and Yulia, 1998). In the present study, we investigated the antimicrobial activity and phytotoxicity of a s election of plant extracts and oils on *C. gloeosporioides* isolated from black pepper.

MATERIALS AND METHODS

Plant extracts, oils and fungicide preparation: Fresh leaf and rhizome samples were taken from selected plants collected from Silkwood, Nambour and Townsville, Queensland (June, 2003 to September, 2004). These were processed in the laboratories of the School of Veterinary and Biomedical Sciences, James Cook University. Aqueous extracts were prepared by blending 50 g of fresh weight of plant material in 100 mL sterile distilled water in a Waring blender (Waring International, New Hartford, CT, USA) for 10 minutes. Ethanol extracts were prepared similarly in 50% ethanol. The macerate was filtered through four layers of cheesecloth, Whatman filter paper No.1 and then, for water extractions, sterilised by filtration through a bacteriological filter (0.2 μm pore size,). The extracts were preserved aseptically in bottles at -18°C temperature until needed.

Oil suspensions were prepared by adding purchased oils to sterile, distilled water containing 0.01% Tween 80 emulsifier (Letessier *et al*., 2001) to obtain concentrations of 0.1, 0.2, 0.5, 1 and 3%. Synthetic fungicides chosen for the study were Amistar™ (systemic fungicide; 250 g L^{-1} azoxystrobin) and Dithane™ M-45 (contact fungicide;

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800 g kg^{-1} Mancozeb) with concentrations used selected above and below the levels used in commercial practice. These concentrations were 12.5, 25 and 100%, 2 and 5 times current usage patterns.

C. gloeosporioides, isolated from infected leaves and berries, was grown on Czapek-Dox Yeast Extract agar medium (Warcup, 1955). The isolation procedure involved serial washings in sterile, distilled water with a non-ionic detergent (Tween 80) added (Shipton *et al*., 1981).

Spore germination tests: To study the effects of the extracts or oils on spore germination, a spore suspension (1 x 10^5 conidia mL^{-1}) was diluted (1:1) with 3% water agar held at 50°C. The resulting mixture was poured immediately into 9 cm plates (11.4 mL) to give a uniform thickness (2 mm) of agar on culture plates. After drying, 10 mm diameter agar discs were cut from the agar-spore plate with a sterile cork borer and four discs per treatment were placed on sterile slides.

The treatment solutions were applied in volumes of 5 μL to the agar-spore surface. As a control, sterile distilled water was used instead of the treatment solutions. The inoculated slides were suspended in sterile plastic boxes containing moist, sterile blotting paper. The boxes were incubated at room temperature (28±2°C) for 24 h. Germinated spores were observed using a light microscope at 100x magnification. Germination ability was assessed from 3 replicate samples observed in the area enclosed by a 100x1 mm^2 grid cell inserted in the ocular of the microscope.

Effect of plant extracts and oils on *in vitro* fungal growth: To study the effect of the extracts or oils on disease development, pepper berries (*Piper nigrum*) and red capsicum fruits (Capsicum annum L.) were inoculated by the fungus in the presence of the extract or oil.

Pepper berries were inoculated with a spore suspension. Mycelial plugs (0.5 cm diameter) of fungi were used for red capsicum fruit inoculation. A spore suspension (5x 0^4 spores mL^{-1}) in 0.01% Tween 80 was applied in 1 μL aliquots to berries followed by a 1 μL application of treatment solutions (0.1-0.5% oil concentrations or 500 mg mL^{-1} extract concentration). Red capsicum (*C. annum*) fruits were treated with 10 μL of each solution per experimental site. The berries and red pepper fruits were incubated at room temperature (28°C) in covered containers to provide moist conditions. Disease development was assessed 7 days after inoculation by estimating the percentage of the berries that was spotted, or by measuring the diameter of the necrotic lesions on red capsicum fruits.

RESULTS

Potency groups for extracts and oils: The potency of oils was grouped into several categories. Those giving greater than 50% inhibition with any of the oil concentrations (0.1-3%) tested included cardamom, cinnamon, clove, lesser galangal, lemon grass and lemon myrtle. Cinnamon bark and leaf oils completely inhibited the growth of *Colletotrichum* at concentrations equal to or greater than 0.1%. Clove bud and leaf oils were less effective than cinnamon leaf oil, with concentrations equal to or above 0.5% being required to completely inhibit germination. Cardamom oil inhibited germination completely at concentrations greater or equal to 1%. Eucalyptus, neem, tea tree, black pepper, garlic, onion, tumeric and rosemary oils were largely ineffective in limiting germination. At the highest concentration of these oils tested (3%), germination was commonly greater than 84%, except for tumeric oil which allowed only 71% of the spores to germinate.

Meanwhile, water and ethanol extracts of galangal rhizome and cardamom leaf inhibited the germination of *C. gloeosporioides* by 60-100%. Water extracts of galangal rhizome and cardamom leaf were slightly less effective than the corresponding ethanol extracts (e.g. complete inhibition by ethanol extracts of galangal rhizome started at 100 mg mL^{-1} concentration rather than at 400 mg mL^{-1} concentration with water extracts; no inhibition was shown by water extracts of cardamom leaf at concentrations lower than 500 mg mL^{-1} (Table 1).

Table 1: Germination of *C. gloeosporioides* in ethanol extracts of galangal rhizome and cardamom leaf over the range of concentrations 5-500 mg mL^{-1} (controls are included)

Conc.	Galangal rhizome		Cardamom leaf	
(mg mL^{-1})	Germ. (%)	SE	Germ. (%)	SE
Water	100.0	0.0	100.0	0.0
Amistar™	30.2*	1.6	30.2*	1.6
Dithane™	0.0*	0.0	0.0*	0.0
500	0.0*	0.0	0.0*	0.0
400	0.0*	0.0	60.6*	0.9
300	0.0*	0.0	62.5*	1.0
200	0.0*	0.0	66.9*	1.8
100	0.0*	0.0	64.8*	0.5
50	58.0*	4.0	81.5*	3.6
10	97.9	1.3	79.3*	0.1
5	100.0	0.0	81.3*	3.7

* Significant difference from the water ($p<0.05$, df = 10)

Table 2: LC_{50} values of several extracts and oils on germination of *C. gloeosporioides*

Treatments	Concentration level
Extract (mg mL^{-1})	
Cardamom leaf (ethanol)	357.00
Galangal rhizome (ethanol)	42.30
Cardamom leaf (water)	510.00†
Galangal rhizome (water)	53.40
Oil (%)	
Cardamom	0.47
Cinnamon bark	0.04
Cinnamon leaf	0.07
Clove bud	0.10
Clove leaf	0.11
Lemon grass	0.57
Lemon myrtle	0.13
Lesser galangal	0.62

†Extrapolated value

Table 3: Phytotoxicity levels of treatment solutions applied to pepper leaves in concentration of 0.5% oils and 500 mg mL^{-1} extracts

Phytotoxicity level for treatment	
Non-toxic	Low toxicity
Distilled water	Lemon myrtle oil
Amistar™ fungicide	Lesser galangal oil
Dithane™ fungicide	Lemon grass oil
Cardamom extract	Galangal rhizome extract
Cardamom oil	
Eucalyptus oil	
Neem oil	
Pepper oil	
Tea-tree oil	

The synthetic fungicide Amistar™ was less effective than Dithane™ and the galangal rhizome extract in inhibiting germination (Table 1), although the relative difference was variable in replicate tests.

Lethal concentration (LC_{50}): Galangal rhizome extract, cardamom leaf extract, cinnamon oils and clove oils were tested at low concentrations to determine their lethal concentrations on *C. gloeosporioides*. The concentration at which 50% of the spores fail to germinate (LC_{50} value) was determined by regression analysis. The oils were effective at reasonably low concentrations (<0.65%). In contrast, relatively large amounts (~360 mg mL^{-1}) of cardamom leaf extract were required to produce inhibition (Table 2).

Effect of plant extracts and oils on *in vitro* fungal growth and phytotoxicity: Inoculation of detached pepper (*P. nigrum*) leaves with test solutions and with fungal inoculum gave rise to phytotoxicity symptoms. Clove bud and leaf oils had medium toxicities and severe toxicity was experienced from the application of cinnamon bark and leaf oils. The phytotoxicity levels of other treatment solutions that exerted mild effects or were non-toxic to the pepper host at 0.5% (Table 3). Many of these oils were ineffective in limiting germination.

Table 4: Disease developments (pathogenicity) of *C. gloeosporioides* on pepper berries two weeks post-inoculation after the application of 0.1-0.5% oils or 500 mg mL^{-1} extracts

Treatment	Infected population (%)†	Disease development
Untreated	0.0	No symptoms
Distilled water	83.3	Whole black lesion with sporulation
Dithane™	0.0	No symptoms
Amistar™	33.3	Whole black lesion with sporulation
Oil		
Clove bud	16.7	Whole black lesion, no sporulation
Clove leaf	33.3	Whole black lesion, no sporulation
Lemon myrtle	16.7	Whole black lesion with sporulation
Lemon grass	50.0	Whole black lesion, no sporulation
Pepper	66.7	Whole black lesion with sporulation
Tea tree	16.7	Black sunken lesion, no sporulation
Extract		
Cardamom (water)	50.0	Whole black lesion, no sporulation

† Calculated from three replications with two berries per replication

Table 5: Anthracnose lesion diameter on red capsicum in oil (0.1-0.5%) treatments at seven days post-inoculation

Treatments	Diam. (mm)	SE
Distilled water	16.3	0.9
Dithane™	0.0*	0.0
Amistar™	6.0*	2.5
Tea tree oil	1.8*	1.8
Cardamom oil	0.0*	0.0
Galangal lesser oil	0.0*	0.0
Lemon myrtle oil	0.0*	0.0
Pepper oil	7.3*	1.0
Lemon grass oil	0.0*	0.0
Clove bud oil	4.8*	1.7
Clove leaf oil	0.0*	0.0
Cinnamon bark oil	3.0*	1.8

* Significant difference from the water ($p<00.5$, $df = 12$)

C. gloeosporioides failed to infect pepper leaves over the period of incubation, but caused anthracnose symptom on berries 6 days after inoculation. At this time, symptoms appeared on berries treated with distilled water, lemon grass oil and clove bud oil treatments. As incubation was extended, the small black spots were enhanced and the fungus sporulated within 10 days after inoculation with distilled water. All extracts and oils treatments were assessed two weeks after inoculation (Table 4).

Plants inoculated with the test fungus together with the following oils and extracts showed no symptoms of infection: cardamom, cinnamon bark or leaf oils and lesser galangal oil, together with galangal rhizome (water and ethanol extracts) and cardamom leaf (ethanol extract). Clearly, these were the most effective against the disease organism, but some also were phytotoxic, such as cinnamon bark and leaf oils.

Red capsicum (*C. annum*) fruit is more sensitive to fungal infection than pepper. On these fruits, *C. gloeosporioides* caused sunken, anthracnose symptoms 7 days after inoculation. Significant differences in lesion diameter were found between

Table 6: Red pepper anthracnose lesion diameter in plant extract (500 mg mL^{-1}) treatments at seven days post-inoculation

Treatments	Diam. (mm)	SE
Distilled water	16.3	0.9
Dithane™	0.0*	0.0
Amistar™	6.0*	2.5
Galangal rhizome (ethanol)	0.0*	0.0
Galangal rhizome (water)	1.5*	1.5
Cardamom leaf (ethanol)	0.0*	0.0
Cardamom leaf (water)	8.5*	2.2

* Significant difference from the water ($p<0.05$, df = 6)

distilled water treatments and plant extract or oil treatments (Table 5 and 6). The oils of cardamom, lesser galangal, lemon myrtle, lemon grass and clove leaf were highly effective, as were the ethanol extracts from cardamom leaf and galangal rhizome. Disease development occurred when cinnamon and clove bud oils were used. This may have been due partly to phytotoxic damage caused by some of the oils and extracts, rendering the host more susceptible to fungal infection.

DISCUSSION

The potential of plant extracts and oils to inhibit *C. gloeosporoides* is particularly interesting owing to the significance of the fungus as a pathogen.

The tests on fungal spore germination and disease development showed the efficacy of galangal rhizome extract, cardamom leaf extract, cinnamon oils, clove oils, lesser galangal oil, lemon grass oil and lemon myrtle oil against the fungus (Table 5). Those extracts and oils were shown to be more efficient than the synthetic fungicide Amistar™ at field concentrations. However, several extracts, such as ginger, black pepper leaf, lemon grass and turmeric were not efficient in inhibiting spore germination. The ineffectiveness of such extracts cannot be generalized (Fiori *et al*., 2000; Guynot *et al*., 2003).

The highest inhibition of spore germination provided by cinnamon and clove oils is in accordance with the Ranasinghe *et al*. (2002) who showed that these two oils were fungicidal against anthracnose and crown rot pathogens on bananas. However, the concentration of the oils required to achieve complete inhibition of germination was higher than those of the present study. Concentrations of oils that gave significant inhibition of spore germination varied, but many inhibited germination at the lowest concentration tested (0.1%). This concentration is well within the range noted for fungal inhibition by oils from medicinal plants (Soliman and Badeaa, 2002; Singh *et al*., 2003). Galangal extract (both solvents) had an LC_{50} of around 50 mg mL^{-1} whereas cardamom leaf extract (ethanol solvent) had an LC_{50} of around 350 mg mL^{-1}. Other medicinal plant extracts have been shown to be effective against fungi at the relatively low concentration noted for galangal (Valsaraj *et al*., 1997). Galangal has a number of active constituents including 1, 8-cineole and various sesquiterpenoids (Jantan *et al*., 2004).

Preparations derived from plants by ethanol extraction methods were much more effective than water extracts in terms of ability to inhibit spore germination. Ethanol extractions resulted in thicker liquid, in greater oil production and stronger colour when compared with the corresponding water extracts. The extraction efficiency displayed by solvents is dependent, however, on the nature of the active ingredients, so that ethanol extracts are not always superior (Somchit *et al*., 2003).

The phytotoxic effects of various oils, such as cinnamon and clove oils, removed them from further consideration. Complementary trials conducted on papaya seedlings indicated that of the oils and extracts performing well on pepper berries, both cardamom oil and galangal rhizome water extract were inhibitory to spore germination and were not phytotoxic. Phytotoxicity may have rendered some hosts more susceptible to infection. For example, the development of disease symptoms following the application of cinnamon oils to red capsicum fruits suggests this possibility, as do some of our results on papaya. Oh *et al*. (1999) were of the opinion that destroyed host tissue on red capsicum fruits play a significant role in fungal infection by *C. gloeosporioides*. Anthracnose development was found to be more vigorously on younger or smaller pepper berries compared to older or harder berries.

Several of the plant extracts and oils tested in this study (cinnamon and cardamom oils and galangal and cardamom extracts) were shown to contain antifungal compounds against *C. gloeosporioides* and phytotoxicity levels were within acceptable limits, except for cinnamon oil.

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