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Postharvest antifungal activity of extracts and compounds from Cinnamomum zeylanicum, Boesenbergia pandurata and Syzygium aromaticum against Colletotrichum gloeosporioides and Botryodiplodia theobromae

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Abstract

The objective of this study was to investigate the efficacy of three crude extracts on the infection of two important postharvest pathogens of mango fruit. The *in vitro* the activity of eugenol oil against *Colletotrichum gloeosporioides* and *Botryodiplodia theobromae* and also the possibility of integrating crude extracts to reduce postharvest decay of mango was investigated. Lipophilic extracts of three spices; *Cinnamomum zeylanicum, Boesenbergia pandurata* and *Syzygium aromaticum* were selected for investigation of their antifungal properties. Antifungal compounds against *Cladosporium cladosporioides* were detected using silica TLC plates in bioautography. Comparative studies of all the extracts showed clear inhibition zones on TLC plates against *C. cladosporioides*. In a micro dilution bioassay, the trunk bark extract of *C. zeylanicum* showed the highest fungicidal activity with a minimum inhibitory concentrations (MIC) value at 1.2 µg/mL against *C. gloeosporioides* and *B. theobromae* at 24 48 and 120 hr after infection. In addition a separated fraction on TLC revealed clear differences for the antifungal activity in the bioautography bioassays. The spore germination of *C. gloeosporioides* was also inhibited with MIC values of 100 µg/mL at 24 hr. However the three crude spice extracts (5,000 and 10,000 ppm) were not able to control anthracnose on 'Nam Dok Mai' mango. In addition, the application of the extracts resulted in the development of skin disorder on the surface of fruits.

Keywords: anthracnose, stem end rot, mango, crude extract

Introduction

Mango (*Mangifera indica* L.) is an important tropical fruit. However it is susceptible to a number of biotic and abiotic stresses that leads to rapid deterioration and large postharvest losses, estimated to be over 50% in some developing countries (Yahia, 1998; Kader, 2002). Fungal disease is one of the most important causes of postharvest losses in mango. The observable external symptoms often only become apparent after ripening, by which it is usually equated with the edibility of the fruit and causing serious losses during storage. The economic costs of such postharvest losses are higher than the field losses (Jeger and Plumbley, 1988; Johnson et al., 1993). The most serious postharvest diseases of mango fruits are anthracnose (*Colletotrichum gloeosporioides*) and stem-end rot (*Lasiodiplodia theobromae)* (Barkai-Golan, 2001). Stem-end rot can cause heavy losses of mangoes during transit and storage (Johnson *et al.,* 1993) and explains why many studies have been conducted to reduce the fruit ripening rate using physical and chemical methods**.**

Plants contain a range of phytochemicals which have numerous functions. Aromatic spicy and medicinal plants such as cinnamon (*Cinnamomum zeylanicum* Blume, syn C. verum, family Laureceae) is a widely used spice and has many applications in perfumery, flavoring and pharmaceutical industries. *C. zeylanicum* produces various oils which contain antioxidant (Mathew and Abraham, 2006), antifungal and antibacterial properties (Tabak *et al*., 1999; Hsieh *et al.*, 2001; Soliman and Badeaa, 2002; Velluti *et al*., 2003). The major component of the leaf volatile oil and oleoresin is eugenol (87.3% and 87.2%, respectively). The analysis of cinnamon bark volatile oil showed the presence of 13 components with (E)-cinnamaldehyde being the major component (Singh *et al.,* 2007).

Syzygium aromaticum buds (clove) has been used extensively in folk medicine. Volatile oils from species of *Syzygium* exhibit antibacterial activity (Nassar, 2006). The isolation and structural elucidation of a eugenol glucoside gallate, a chromone-C-glycoside, galloyl and hexahydroxydiphenoyl esters of 2,4,6-trihydroxyacetophenone 3-C-fl-D-glucopyranoside, have been reported from the leaves of clove. The major phenolic constituent of this species is eugeniin, which was accompanied by many structurally and biosynthetically related ellagitannins (Tanaka *et al.,* 1996).

Boesenbergia pandurata is a perennial herb belonging to the Zingiberaceae family. It is commonly used in Southeast Asia where its rhizomes contain the essential oil, pinostrobin, cardamonin, boesenbergin, 5,7-dimethoxyflavone, 1,8-cineole and panduratin. B. pandurata also exhibits antifungal activities. (Cheenpracha et al., 2006; Tuchindaa et al., 2002). The aim of this study was to explore the possible anti-plant pathogens properties by study antifungal activity of extracts and compounds of C. zeylanicum, B. pandurata and S. aromaticum against C. gloeosporioides and B. theobromae of mango fruit. This study examined the efficacy of three crude extracts against two important postharvest pathogens in mango fruit. Specific objectives of this experiment were to evaluate in vitro activity of eugenol oil against C. gloeosporioides and B. theobromae and the possibility of integrating crude extracts into postharvest handling practices to reduce postharvest decay of mango.

Materials and Methods

*Plant Material***:** 520 g stem bark of *Cinnamomum zeylanicum* (with $CHCl_3$ extract: 148.52 mg) from commercial sources, 545 g flower bud of *Syzygium aromaticum* (with $CHCl_3$ extract: 647.84 mg) from commercial sources and 840 mg roots of *Boesenbergia pandurata* ($CHCl_3$

extract: 107.85 mg) from Thung Kai Botanic Garden Trang, Thailand. Crude extracts and fractions were storage at -18^{o}C in MeOH.

*Extraction and Isolation***:** Dried plants were ground and extracted with MeOH at room temperature for three days. The extracts were then filtered, concentrated and the aqueous residue was extracted with $CHCl_3$. The obtained fraction was subject to preparative TLC (Merck, Si gel 60, 0.5 mm; Solvent systems: Hexane:EtO_2 3:2) which was used to purify the compounds (compare Brader et al., 1998). Known compounds were identified by their retention times and UV spectra.

*Bioautography***:** This technique was used to detect active compounds of the crude extracts. The extracts were dissolved and spotted on two TLC plates (Merck 60 F 254, 0.25 mm) using a disposable glass micropipette for each sample. The developed plates were dried and observed under a CAMAG UV-Lamp (254 nm). One plate was sprayed with a MeOH–HOAc–H_2SO_4–anisaldehyde reagent (85:10:8:0.5) and activated at 100-110 ^{0}C for 10 min to visualize UV-invisible compounds in the extracts. The other plate was used for the bioautography assay system to detect any antifungal activity that had occurred directly on the developed TLC plate. The silica gel plates were sprayed with spore suspensions of *Cladosporium cladosporioides* adjusted to a final concentration of 10^5 conidia/mL in a 1.7% malt extract broth (Merck). The inoculated plates were then placed in a humid chamber to monitor the activity of single compounds after 3 days at 25 ^{o}C in darkness. Clear zones of fungal growth inhibition indicated the presence of antifungal constituents.

Preparations of spore suspensions were conducted using a tissue transplanting method. The spore suspension was adjusted to a density of 10^5 spores/mL, whereby spores were counted per CFU system.

*Micro dilution assay***:** A standardized 96-well microtiter plate assay developed for the discovery of natural products of fungicidal activity (Hadacek and Greger, 2000) was used to evaluate naturally occurring antifungal agents against *C. gloeosporioides* and *B. theobromae*. Each fungus was challenged in a dose-response format using test compounds with the final treatment concentrations of 1- 2500 µg/mL for crude extracts. Each dose dilution was repeated four times for evaluation. Microtiter plates (Greiner) were incubated at room temperature**.**

*Data analysis***:** Minimum inhibitory concentrations (MIC) were determined as the lowest compound concentration completely inhibiting spore germination by compound microscope at 24, 48 and 120 hr (Hadacek and Greger, 2000).

*In vivo assay on natural infected mango fruit***:** Trials were conducted in 2007 on mango (Nam dok mai variety) from local commercial packinghouses near Chaing Mai, Thailand. Fruit with uniform size, shape, maturity and free from defects were sorted, cleaned with hot water 52 ◦C 5 min, air-dried and sprayed with 5,000 and 10,000 ppm of each crude extract on wound inoculated with *C. gloeosporioides* on surface. Fruits were stored in plastic baskets at room temperature for 7 days and the disease severity was evaluated by estimating the level of disease development on the fruit.

*Toxicity of crude extract on mango fruit***:** Three different crude extracts of *C. zeylanicum, B. pandurata* and *S. aromaticum* were tested for their phytotoxicity on the mango peel. Phytotoxicity is defined as the extent of the apparent burning damages (discoloration) induced at 10,000 ppm. Mango (Nam dok mai variety) were surface washed with tap water, dried and painted the treatment solution. Fruits were stored in plastic baskets at room temperature for 7 days and phytotoxicity was evaluated using a scale indicating the extent of surface on which discoloration is observed.

Results and Discussion

Antifungal activity and Microdiution assay

Air-dried samples of *C. zeylanicum, B. pandurata* and *S. aromaticum* were extracted in methanol. Bioautography on silica TLC plates with the test fungus *C. cladosporioides* demonstrated a series of inhibition spots from the extracts. There were further separated by preparative TLC. The bioautography of the lipophilic fraction of three plant extracts showed clear inhibition zones indicating the presence of antifungal compounds (Figure 1). Subsequent HPLC-UV analysis of the lipophilic chloroform *C. zeylanicum* extract showed an accumulation of different classes of compounds. Three compounds were detected from which the majority could be identified by co-chromatography with authentic samples. More detailed data on the antifungal properties of crude extracts were obtained by a micro dilution spore inhibition tests system employing *C. gloeosporioides* and *B. theobromae* as test organisms (Table 1). In these micro dilution bioassays, the trunk bark extract of *C. zeylanicum* exhibited strongest fungicidal activity with a MIC value at 1.2 µg/mL against *C. gloeosporioides* and *B. theobromae* at 24 48 and 120 hr. Three inhibition zones on the bioautography plates lead to the separation of these compounds using preparative TLC dividing into three fractions (Table 2). Fraction3 at Rf 0.70 revealed clear differences for the antifungal activity in bioautography bioassays and the spore germination of *C. gloeosporioides* was inhibited with MIC values at 100 µg/mL at 24 hr. According to the HPLC-profile of Fraction3 at Rf 0.70 there was no characteristic long wave length UV absorption, but this could be detected at 19 min at short wavelength absorption (HPLC profile data not shown). In addition, the antifungal Fraction3 at Rf 0.70 (10 mg) was further isolated after separation. *S. aromaticum* showed activity against *C. gloeosporioides* and *B. theobromae* resulting in a MIC of 1.2 and 7.8 µg/mL, respectively at 24 hr. The crude extracts of root of *B. pandurata* showed high fungicidal activity with MIC values from 1.2 µg/mL against *C. gloeosporioides* and *B. theobromae* at 24 hr. Antifungal effects have also been reported for the essential oil of the bark of *C. zeylanicum* and three of its main components, eugenol, (E)-cinnamaldehyde, and linalool (representing 82.5 % of the total composition) (Chericoni *et al*., 2005). Chericoni *et a*l. (2005) also showed this oil exhibited fungitoxicity against strains of *Stachybotrys chartarum* causing stachybotryotoxicosis at 400 ppm. More detailed chemical investigations have revealed that eugenol and benzaldehyde are the active constituents (Misra et al., 2000). Furthermore the antifungal effects of cinnamon leaf and clove essential oils (0-1,000 ppm) against *Eurotium* spp., *Aspergillus* spp., and *Penicillium* spp. have been demonstrated (Guynot *et al*., 2005 and Simic *et al*., 2004).

In liquid bioassays, cinnamon leaf, bark, and clove oils were tested against anthracnose and crown rot pathogens *(B. theobromae, C. musae* and *Fusarium proliferatum*). The test oils were fungistatic and fungicidal against the test pathogens within a range of 0.03-0.11% (v/v). Eugenol extracted from cloves and thymol from thyme caused the complete inhibition of the growth of both *A. flavus* and *A. versicolor* at 0.4 mg/mL or less (Hitokoto et al., 1980). Antifungal activity was also detected in the oil of clove by the agar well diffusion method. The *in vitro* antifungal potency was characterized by the minimum fungicidal concentration (MFC). MFC of the essential oils tested were in the range of 0.06-0.75% against *A. niger* and *A. alternata.* These observations indicated that these essential oils can be exploited as antifungal agents in the management of plant infectious diseases and postharvest spoilage of crops (Farrukh et al., 2001).

In vivo assay on infected mango fruit and toxicity of crude extract on mango fruit

Wound inoculated mango fruits were treated with three different crude extracts of *C. zeylanicum, B. pandurata* and *S. aromaticum* at 5,000 and 10,000 ppm to observe disease severity. No significant difference of lesion diameter and disease incidence was observed between the two treatments and control (Table 3). Toxicity *C. zeylanicum S. aromaticum* and

B. pandurata occurred at 10,000 ppm of crude extract on mango fruit. The fruit surface showed 6-10% severe damage in all treatments, but did not effect the odor nor taste of the treated fruit with *S. aromaticum* and *B. pandurata*. Mangoes were treated with *C. zeylanicum* demonstrated an off odor.

In this study, three crude extracts were effective to control the development of the two important pathogens that cause damages on mangoes but affected the appearance of the mangoes. Investigating the potential of the vapor phase application and its applicability in the storage room may facilitate the application of *C. zeylanicum* , *S. aromaticum* and *B. pandurata* as a control agent for long periods and may avoid the problems of phytotoxicity induced by some liquid formulations.

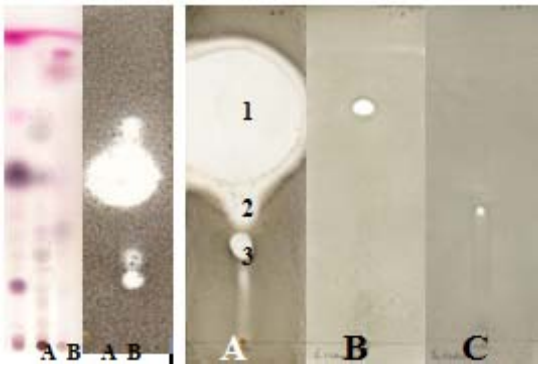

Figure 1: Antifungal activity observed by direct spraying of a conidia suspension of *C. cladosporioides*. **A**: *C. zeylanicum* **B**: *S. aromaticum* and **C**: *B. pandurata* Solvent system: hexane–Et_2O (3:2).

Table 1: MIC values (µg/mL) of crude extract against *C. gloeosporioides* and *B. theobromae* determined by microdilution bioassay.

Crude extract	Minimum inhibitory concentration (MIC) µg/ml		
	24 hr.	48 hr.	120 hr.
Colletotrichum gloeosporioides			
Control (Acetone pa. + Malt broth + Tween80)	1.2	>2,500	>2,500
C. zeylanicum	1.2	1.2	1.2
Control (Acetone pa. + Malt broth + Tween80)	78.1	>2,500	>2,500
S. aromaticum	1.2	156.3	312.5
Control (Acetone pa. + Malt broth + Tween80)	>2,500	>2,500	>2,500
B. pandurata	1.2	1,250	2,500
Botryodiplodia theobromae			
Control (Acetone pa. + Malt broth + Tween80)	4.9	>2,500	>2,500
C. zeylanicum	1.2	1.2	1.2
Control (Acetone pa. + Malt broth + Tween80)	>2,500	>2,500	>2,500
S. aromaticum	7.8	312.5	625
Control (Acetone pa. + Malt broth + Tween80)	1.2	>2,500	>2,500
B. pandurata	1.2	>2,500	>2,500

MIC (Minimum inhibitory concentration) defined the lowest concentration of the dilution series, which completely inhibited spore germination.

Table 2: MIC values (µg/mL) of *C. zeylanicum* fractions against *C gloeosporioides* determined by microdilution bioassay.

Crude extract	Minimum inhibitory concentration (MIC) µg/ml		
	24 hr.	48 hr.	120 hr.
Control (Acetone pa. + Malt broth + Tween80)	>200	>200	>200
C. zeylanicum (Fraction+ Acetone pa. + Malt broth + Tween80)			
Fraction1 Rf = 0.30 (8 mg)	>200	>200	>200
Fraction2 Rf = 0.42(16 mg)	>200	>200	>200
Fraction3 Rf = 0.70 (10 mg)	100	>200	>200

MIC (Minimum inhibitory concentration) defined the lowest concentration of the dilution series, which completely inhibited spore germination.

Table 3: *In vivo* assay on natural infected on mango fruit.

Crude extract	ppm	Disease severity area (mm)
Control (Methanol)		4.73
C. *zeylanicum*	5,000	8.48
	10,000	10.30
S. aromaticum	5,000	11.45
	10,000	10.00
B. pandurata	5,000	6.43
	10,000	9.50

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