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Antifungal activity of plant-based extracts against *Colletotrichum musae, Botryodiplodia theobromae* and *Rhizopus stolonifer* and potential application as a fruit coating

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Abstract

Postharvest diseases of fruits due to fungi are known to be the main factor that limits the storage period of the fruits. Synthetic chemical fungicides are commonly applied to control postharvest losses of fruits after harvesting. Natural antifungal plant extracts are less hazardous alternatives for the synthetic fungicides due to their safety and nontoxicity. Thus, a study was planned to determine the antifungal activity of the ethanol extract of vetiver root (*Chrysopogon zizanioides*), hot distilled water (80^{o}C) extracts of soursop (*Annona muricata*) leaves, mustard (*Brassica nigra*) roots, *Aloe vera* gel matrix, and papaya (*Carica papaya*) latex against *Colletotrichum musae, Botryodiplodia theobromae* and *Rhizopus stolonifer*. Standard agar-well diffusion method was used for testing the antifungal activity of the plant extracts. The Minimum Inhibitory Concentration (MIC) was determined by using different concentrations of plant extracts. Banana fruits (*Kolikuttu*) were coated only with vetiver oil, melted bee wax, and vetiver oil and bee wax (2 g/L) mixture, then inoculating 50 µL of *C. musae* spore suspension (200 spores) on selected patches on the coated and uncoated (control) fruit surfaces. Disease severity was determined by the lesion length. Antifungal activity of vetiver oil against *C. musae, B. theobromae*, and *R. stolonifer* which resulted in 25.72 ± 1.22, 19.33 ± 0.00, 27.00 ± 0.00 mm of inhibition zones respectively ($p<0.05$). *Aloe vera* and soursop showed antifungal activity (16.21 ± 0.59 and 16.64 ± 0.13 mm inhibition zones respectively) against *C. musae* only. Minimum inhibitory concentrations for vetiver oil, *Aloe vera*, and soursop leaf extract were 100%, 25% and 0.1 g mL^{-1} respectively. Vetiver oil and bee wax mix coating showed the least (9.12 ± 0.59 mm) ($p<0.05$) lesion length followed by vetiver oil and bee wax alone coating (17.41 ± 0.57 and 28.4 ± 0.39 mm respectively) compared to the control (30.16 ± 0.36 mm) which resulted in the highest ($p<0.05$) after five days. The vetiver oil-bee wax coating provides promising protection against *C. musae* in bananas.

Keywords: Agar-well diffusion, Bee wax, Inhibition zone, Plant extracts, Banana fruit

1. Introduction

Due to high respiration rate and moisture content (80-90%), tropical fruits are highly perishable. Therefore, the postharvest pathogen invasion is also high. Fungal pathogens cause high postharvest losses from all microorganisms and result high economic losses worldwide due to postharvest spoilage, reduction of storage time and shelf life [1]. Anthracnose, stem-end rot, and watery white rot are common diseases caused by fungi for mango, banana, papaya, and avocado like tropical fruits [2]. Fungicides are the most effective method of preventing postharvest fungal diseases. However, because of their hazardous effects on environment and

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human health, use of synthetic chemicals in postharvest disease management has been limited [3].

Currently, people have interested in foods with natural additives and synthetic-preservative free foods, to maintain good health conditions. Therefore, food researchers have tended to move towards natural substances to improve the postharvest quality of fruits. It is well known that some plant extracts have antimicrobial and antioxidant properties, hence have a greater potential to use in non-synthetic postharvest disease management [1,4]. Therefore, the present study was planned to evaluate the antifungal activity of plant-based extracts against selected postharvest pathogenic fungi and potential application of them as a banana fruit coating. The Minimum Inhibitory Concentration (MIC) of some plant extracts against selected postharvest fungal pathogens and the antifungal activity of coated fruits compared to control at the ambient storage were also evaluated.

2. Materials and Methods

2.1 Location

All the experiments were conducted at Food Microbiology Laboratory, Faculty of Agriculture, Rajarata University of Sri Lanka from October 2020 to April 2021.

2.2 Materials:

Vetiver (*Chrysopogon zizanioides*) roots were collected from Kandy and Bandarawela areas and soursop (*Annona muricata*) leaves, *Aloe vera* leaves and papaya (*Carica papaya*) latex were collected from Faculty of Agriculture, Rajarata University of Sri Lanka. Banana (*Kolikuttu* variety) was collected at three localities from Anuradhapura and bee wax was collected from Kandy in Sri Lanka.

2.2.1 Preparation of plant extracts:

Soursop leaves were washed with tap water, dried under shade and ground into a powder using a grinder (CML-7360065-Jaipan). The ground sample was sieved through 1 mm sieve. Then 10 g of sieved sample was dissolved in 100 mL of sterile hot distilled water (80°C) and the extract was prepared. The mixture was swirled continuously at 120 rpm in an orbital shaker (VRN-360) for one hour. Extracts were filtered through Whatman filter paper No: 01 (150 mm). The filtrate was concentrated by rotary evaporator (CVP-13-1) under reduced pressure at 40 °C temperature and stored at 4 °C for further analysis [5].

Mustard roots were washed with tap water and dried under shade and chopped with 100 mL of sterile hot distilled water using motor and pestle in order to prepare the extract. Extract was filtered through Whatman filter paper No: 01 (150 mm) and the filtrate was concentrated by rotary evaporator under reduced pressure at 40 $^{\circ}$C temperature. The concentrated filtrate was stored at 4 $^{\circ}$C for further analysis [4].

Thirty grams of vetiver roots were placed in Soxhlet apparatus and 500ml of ethanol was kept in round bottom flask. Extraction was carried out at boiling temperature of ethanol for 5h and ethanol was evaporated [6].

Aloe vera was freshly harvested and the gel matrix was separated from the outer cortex and ground in a blender. The resulting suspension was filtered and was pasteurized at 70 $^{\circ}$C for 45 minutes. It was then cooled at ambient temperature immediately [7]. Papaya latex was obtained by wounding the immature papaya fruits.

2.3 Isolation and identification of postharvest pathogenic fungi:

The method specified by Anthony et al. [8], was followed with modifications. Infected fruits (banana, papaya and mango) were collected from market in Anuradhapura. The surface of the fruits was disinfected with 70% ethanol using cotton swab. Diseased fruit tissues (1/3 area) were cut aseptically and were placed in potato dextrose agar (PDA) plates. Plates were incubated at 32 $^{\circ}$C for 3 days. In order to obtain pure cultures, fungal colonies that emerged were sub-cultured. Cultures were identified by macro and microscopic (sticky tape method) features.

All fungal suspensions were made by suspending 24 h fungal culture in sterile saline (0.89% NaCl w/v) solution. The turbidity of the fungal suspensions was adjusted 1.0 McFarland standard (equivalent to 1.5×10^8 CFU/ml) for fungal strains.

2.4 Testing plant extracts for antifungal activity:

Antifungal activity of plant extracts against test pathogens were assessed by Agar Well Diffusion method in triplicates [9]. Each sterile Petri plate was filled with approximately 20 ml of PDA and allowed to solidify. A sterile cork borer of 7 mm diameter was used to make four wells in each plate and 50 μL of test plant extracts were added to each well. Commercial fungicide (Mancozeb) and hot sterile distilled water (80 °C) were used as positive and negative controls respectively and 50 μL of each were dispensed into the other wells of the same plate. With using sterile cork borer, 6 mm diameter plugs from pure cultures of isolated pathogenic fungi were placed separately in the center of each plate and were kept at 32°C for 3 days [10]. The diameter of the inhibition zone (mm) was measured to determine the zone of inhibition. The average values were tabulated after taking readings in three different directions.

2.5 Determination of Minimum Inhibitory Concentration (MIC):

The Crude extracts were prepared as 100%, 75%, 50% and 25% different concentrations for vetiver oil. *Aloe vera* concentrations were 0.2 g/ mL, 0.15 g/ mL, 0.1 g/ mL, and 0.05 g/ mL for soursop leaf extracts and stored in -20 $^{\circ}$C. To determine the MIC of different extracts, Agar Well Diffusion method as previously described was followed [10].

2.6 Banana fruit coating:

Fresh banana fruits (*Kolikuttu* variety) free from physical damage and diseases were purchased in three localities from Anuradhapura area. Banana fruits were used in nine replicates (nine fingers) for each treatment. All fruits were artificially wounded (1 cm x 1 cm area) by using sterile needle. Then fruit coating was applied and four replicates were used [11].

Table 01: Treatments for fruit coating

Treatments	**Concentrations**
Control	-
Coated with melted bee wax	2 gL^{-1}
Coated with vetiver oil	100% (Pure oil)
Coated with bee wax and vetiver mixture	4 ml L^{-1} and 2 g L^{-1}

Fruits were coated according to the treatments (Table 01) by dipping them. Spore suspension of *C. musae* were prepared and counted by using hemacytometer. Inoculation of coated and uncoated fruits were carried out by giving 50 μL spore suspension (200 spores) of *C. musae* using sterile syringe to specific area (wounded place) and then air dried at room temperature. All replicates were stored in corrugated cardboard boxes for 5 days at room temperature and the most prominent first lesion length was measured after 5 days and recorded as disease severity [12].

2.7 Statistical analysis:

The experimental design used was Completely Randomized Design (CRD). Results were analyzed in one way ANOVA, using SAS program (Version 9.0, SAS Institute Inc. USA). Means were compared using Tukey's simultaneous test set at p <0.05. All experiments of antifungal activity testing were done in triplicate (n = 3) and the inhibition zone diameter was expressed as means ± standard error. All treatments of fruit coating were done in nine replicates (n = 9) and the disease severity (lesion length) was expressed as means ± standard error.

3. Results and Discussion

3.1 Antifungal activity of plant extracts

Five plant extracts such as vetiver oil, *Aloe vera* gel extract, soursop leaf extract, mustard root extract and papaya latex were investigated to evaluate their antifungal activity against some postharvest fungi including *C. musae, B. theobromae* and *R. stolonifer* using well diffusion method. Vetiver (*C. zizanioides*) oil has the highest antifungal activity against all three tested fungi ($p<0.05$).

Table 02: Antifungal Activity (Diameter of the inhibition zone) of plant extracts

Plant extracts	**Antifungal Activity (DIZ) (mm)**		
	Colletotrichum musae	***Botryodiplodia theobromae***	***Rhizopus stolonifer***
Vetiver (*Chrysopogon zizanioides*) oil	$25.72^{a} \pm 1.22$	$19.33^{a} \pm 0.00$	$27.00^{a} \pm 0.00$
Aloe vera leaves extract	$16.21^{b} \pm 0.59$	$8.00^{b} \pm 0.00$	$8.00^{b} \pm 0.00$
Soursop (*Annona muricata*) leaves extract	$16.64^{b} \pm 0.13$	$8.00^{b} \pm 0.00$	$8.00^{b} \pm 0.00$
Mustard (*Brassica nigra*) root extract	$8.00^{c} \pm 0.00$	$8.00^{b} \pm 0.00$	$8.00^{b} \pm 0.00$
Papaya (*Carica papaya*) latex	$8.00^{c} \pm 0.00$	$8.00^{b} \pm 0.00$	$8.00^{b} \pm 0.00$

Note: Within column, means followed by a different letter differ significantly at $p< 0.05$.

Antifungal activity of vetiver oil against *C. musae, B. theobromae, and R. stolonifer* which resulted in 25.72 ± 1.22, 19.33 ± 0.00, 27.00 ± 0.00 mm of inhibition zones respectively ($p<0.05$). However, *Aloe vera* and *soursop* showed antifungal activity (16.21 ± 0.59 and 16.64 ± 0.13 mm inhibition zones respectively) against *C. musae* only. Other plant extracts have minimum or may not have the antifungal activity against the tested fungi (Table 02).

Essential oils contain aldehydes (cinnamaldehyde), alcohols, phenols (eugenol), methoxy derivatives (anethole, estragole) and methylene deoxy compounds (myristicine, apiole) might be active against postharvest pathogens [13]. The bioactive compounds of khusimol, β-vetivone, α-vetivone present in *C. zizanioides* [13]. These compounds might inhibit all tested postharvest pathogens.

Aloe vera has been used for medicinal and therapeutic purposes for centuries [14]. *Aloe vera* extract was only inhibited the growth of *C. musae*. *Aloe vera* is rich in many chemical substances. There are 75 of nutrients and 200 of active compounds including sugars, vitamins, enzymes, minerals, anthraquinones, saponins, lignin, salicylic acid and amino acids confirmed to be present in *Aloe vera* [15]. Aloine is an anthraquinone, main active constituent in *A. vera* may possess antifungal characteristics 16].

Several parts of *A. muricata* tree is used in traditional medicine [1]. Among the tested pathogens soursop leaf extract was inhibited only the growth of *C. musae*. The leaves of *A. muricata* consisted of alkaloids, flavonoids, tannin, saponins and phenols as active compounds [1]. These active compounds may inhibit the growth of the *C. musae*.

Previous studies also revealed the antifungal activity of mustard root extract [17,18] and papaya latex [19]. Bioactive compounds of mustard root extract and papaya latex are isothiocyanate [18] and two enzymes (α-D-mannosidase and N-acetyl-β-D-glucosaminidase) [19], respectively. However, there were no any growth inhibition observed for all tested pathogens.

3.2 Minimum Inhibitory Concentration (MIC):

The MIC was determined for plant extracts that had antifungal activity against *C. musae, B. theobromae and R. stolonifer*. According to the MIC results of all effective plant extracts, vetiver oil was inhibited the growth of all tested fungi at the concentration of 100%. While *Aloe vera* and soursop leaf extracts were inhibited the growth of *C. musae* only at the concentrations of 25% and 0.1 g/mL respectively.

3.3 Fruit Coating:

Disease severity of *C. musae* on coated and uncoated banana fruits were determined by using the first lesion prominent length (Table 02).

Table 03: Effect of vetiver/ bee wax mixture (V+B) or vetiver (V) on anthracnose disease severity in inoculated banana fruits

Treatments	**Disease severity (Lesion length, mm)**
Vetiver + Bee wax (V+B)	$9.12^{c} \pm 0.59$
Vetiver (V)	$17.41^{b} \pm 0.57$
Bee wax (B)	$28.4^{a} \pm 0.39$
Control (C)	$30.16^{a} \pm 0.36$

Note: Within column, means followed by a different letter differ significantly at $p < 0.05$.

Vetiver oil and bee wax mix coating showed the least (9.12 ± 0.59 mm) ($p<0.05$) lesion length followed by vetiver oil and bee wax alone coating (17.41 ± 0.57 and 28.4 ± 0.39 mm respectively) compared to the control (30.16 ± 0.36 mm) which resulted in the highest ($p<0.05$) after 5 days. There is no significant difference observed between banana coated with melted bee wax and control (Table 03).

4. Conclusion

Among all the tested plant extracts, vetiver oil had the highest antifungal activity against *C. musae, B. theobromae,* and *R. stolonifer. Aloe vera* and soursop possess antifungal activity against *Colletotrichum musae* only. Furthermore, MIC could be 100% for vetiver oil to inhibit the growth of above fungi. Hence, vetiver oil can be used as a potential natural antifungal agent. Vetiver oil -bee wax mixed coating on banana fruits provides promising protection against *C. musae*.

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