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Antifungal Activity of Some Plant Extracts against (*Colletotrichum Musae*) the Cause of Postharvest Banana Anthracnose

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Abstract

The present study was conducted to determine the efficacy of extracts of some plant species against *Colletotrichum musae*. Methanolic extracts of 21 plant species were screened for their inhibitory effect against *C. musae* using paper disc method and spore germination assay. Among them, extract of *Prosopis juliflora* exhibited superior antifungal activity (30.7 mm), followed by *Acacia albida* (19 mm) as compared to nill in the control. On the other hand, carbendazim, which was used as a standard chemical check, demonstrated by far the highest inhibition zone of 51.7 mm diameter. Extracts from *A. albida, Dovalis abyssinica* and *P. juliflora* reduced conidial germination to 0.2, 0.5 and 0.3%, respectively, which didn't vary statistically from 1.2% in Carbendazim. Six plant species, viz., *A. albida, Azadirachta indica, Argemone mexicana, D. abyssinica, P. juliflora* and *Vernonia amygdalina*, that showed high to moderate antifungal activity in the preliminary screening, were further tested for their thermal stability at 60°C and for the efficacy of their aqueous extracts against *C. musae.* Extracts of the tested plant species were found to be heat stable and aqueous extracts of *A. albida* showed the highest antifungal activity (18 mm), followed by *P. juliflora* (12.3 mm). Further studies need to be undertaken to isolate the active compounds from those extracts with fungicidal potential.

Keywords: Antifungal; Banana anthracnose; *Colletotrichum musae;* Plant extracts

Introduction

Anthracnose caused by the fungus *Colletotrichum musae*, is the most important postharvest disease of banana that can result in 30-40% losses of marketable fruit [1]. Recently, plant products have attracted high attention to search for some phytochemicals for their exploitation as antimicrobials; such plant products would be easily degradable less phyto-toxic and safe to human health [2]. However, no efforts have been made in Ethiopia to screen plants that have potential antimicrobial properties against *C. musae.* Several investigations have evaluated spices and medicinal plants with the aim of using them for plant disease management [3-5]. However, although many such plants have shown antimicrobial activity, their practical use for disease management has been limited because of the high price they fetch for other purposes. Thus, attention should be focused on plants with limited known economic values.

It has been suggested that combining plant extracts with non-chemical postharvest treatments, such as heat treatment, might control postharvest anthracnose diseases effectively [6]. However, the antifungal substances that are present in the plant extracts may be unstable at higher temperatures [7]. Accordingly, there is a need for investigating a heat stable plant extract that could be integrated with hot water treatments. This study was undertaken to determine the inhibitory effects of extracts of plants with limited economic value against *C. musae*.

Materials and Methods

Experimental materials

Fresh leaves of 21 plant species were collected from local sources. The identity of these plant species was confirmed at Haramaya University Herbarium where voucher specimens are deposited. The plants included *Acacia albida, A. seyal, Argemone Mexicana, Azadirachta indica, Calotropis procera, Cassia occidentalis, Cynodon dactylon, Datura stramonium, Dovalis abyssinica, Lantana camara, Melia azedarach, Moringa olifera, Olea europaea, Petiveria alliacea, Plantago lanceolata, Prosopis juliflora, Ricinus communis, Schinus molle, Solanum incanum* and *Verbasacum sinaiticum*.

Isolation of the pathogen and inoculum preparation

Banana fruits showing anthracnose disease symptoms were collected and used for isolation of *Colletotrichum musae*. Pure culture was maintained on PDA medium. Inoculum was prepared by dislodging the surface of 10 days old culture with sterile distilled water and adjusted to 105 ml^{-1} using a hemacytometer.

Extraction of plant samples

Extraction was done using methanol and water. The methanolic extracts were used for preliminary screening of the plant specimens for their potency against the fungal pathogen, while aqueous extracts were used for further testing. Fifty gram of powdered samples of each of the plant specimens was extracted with 250 ml solvent (methanol or water). The methanolic and aqueous extracts were filtered and reduced to dryness on rotary evaporator under vacuum at 40°C and 60°C water bath temperatures, respectively [8,9]. The extract of each plant was weighed, re-dissolved and then tested for antifungal activities.

In vitro assay of plant extracts

Paper disc method: The method as described by [9] was used.

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Briefly, sterile filter paper discs (6 mm in diameter) were impregnated with 10 µl plant extract (at a concentration of 100 µg/µl). Five milliliter of a culture suspension of *C. musae* with a concentration of 1×10^6 conidia ml^{-1} was added to 250 ml of PDA medium which had been previously cooled down to ca. 50°C. The medium was then poured into Petriplate and allowed to solidify. The impregnated discs were dried and placed with forceps aseptically on the surface of the pre-inoculated PDA medium. Paper discs that were similarly handled but without plant extract served as untreated control and paper discs impregnated with commercial fungicide carbendazim (at the concentration of 2 µg/µl) served as a positive control. Four days of after incubation (at 28°C), the diameter of inhibition zone around the test discs was measured and the degree of inhibition of fungal growth expressed on a 0-4 scale was recorded [9], where: 0=no inhibition zone visible, 1=inhibition zone barely distinct, fungal growth and sporulation only slightly inhibited, 2=inhibition zone well distinct fungal growth ca. 50% of the control, slight sporulation, 3=inhibition zone with sparse (ca. 25% of the control) fungal growth, 4=inhibition zone free of visible fungal growth.

Spore germination assay: In this assay, about 0.5 ml of methanolic plant extract was pipetted into sterile test tubes and the solvent was allowed to evaporate at room temperature. About 4.5 ml volume of potato dextrose broth was pipetted into the test tubes and mixed with extracts. At the same time an aliquot (100 µl) of spore suspension (adjusted to 10^6 conidia ml^{-1}) was added into each tube. After 24 hr of incubation at 28°C on a rotary shaker, a drop of the mixture from each tube were placed in a microscope slide and slides were fixed in lactophenol cotton blue and observed microscopically for spore germination. The same volume of methanol was added in place of plant extracts in the control samples and carbendazim at the concentration of 2.5 mg/ml served as a positive control. A conidium was considered as germinated if the length of the germ tube was at least half the length of the conidium. The number of germinated conidia was counted out of 100 randomly selected conidia in three replicate slides. Percentage spore germination was calculated according to the following formula:

$$\text{pore Germination (\%)} = (\text{Germinated Spores (No.)}/\text{Total Spores (No.)})\times 100$$

Thermal inactivation of plant extracts: Plant extracts showing potential antifungal activity in the preliminary evaluation were further tested for thermal stability. About 1 ml of methanolic extracts in glass tubes were exposed to 60°C in a water bath for 10 min and cooled to room temperature. Afterwards, the paper disc method was employed to assess their effect on antifungal activity against *C. musae* as described under Section 2.4.1.

Statistical data analysis: Percentage data on spore germination inhibition was arcsine transformed before statistical analysis. The data were subjected to analysis of variance (ANOVA) using SAS software version 9.2 [10]. Means were separated using Duncan's Multiple Range test (DMRT) at $P \leq 0.05$.

Results and Discussion

Antifungal activity of methanolic extracts

Preliminary screening of 21 plant species was conducted for antifungal activity against *Colletotrichum musae* using mycelial inhibition test and spore germination assay.

Mycelial inhibition test: Out of the 21 plant species tested, Prosopis juliflora exhibited the highest antifungal activity with inhibition zone of 30.7 mm diameter against *C. musae*. The methanolic extract of *A. albida* showed the second best antifungal activity (19 mm), followed by *D. abyssinica* (11.7 mm) and A. mexicana (11.0 mm). Methanolic extract from *V. amygdalina* exhibited inhibitory activity of 9.7 mm, while *A. indica* showed antifungal effect in the test with inhibition zone of 7.3 mm. Regarding the degree of inhibition of fungal growth, expressed on a 0-4 scale, extracts of *A. albida, A. mexicana, D. abyssinica, P. juliflora* and *V. amygdalina* resulted in strong activity presenting zones of inhibition free of visible fungal growth. On the other hand, extracts of *A. indica* showed moderate activity against the test fungus.

Methanolic extracts of the remaining plant species and methanol, which served as negative control, produced no zones of inhibition. On the other hand, carbendazim, which was used as a standard chemical check, demonstrated by far the highest inhibition zone of 51.7 mm diameter.

The present study has demonstrated the antifungal potential of methanolic extract of *P. juliflora* that corresponded with the work done by [11] who reported methanolic extract of *P. juliflora* leaves to have excellent in vitro antifungal activity against soil mycoflora i.e. Fusarium oxysporum, Rhizoctonia solani, Alternaria alternata and Curvularia lunata. Extracts from *A. albida* showed potent activity and this result is in agreement with the previous finding of [12]. Similarly, antifungal effect of *A. mexicana* was previously reported by [13]. The antifungal activity of methanolic extract of *D. abyssinica* has also been reported [14].

Spore germination assay: Methanolic extract from *A. albida, D. abyssinica* and *P. juliflora* showed potent antifungal activity in reducing the spore germination to 0.2, 0.5 and 0.3%, respectively. Carbendazim, used as a standard chemical check, markedly reduced spore germination percentage to 1.2% which is not significantly different (P>0.05) from the above most effective plant extracts (Tables 1 and 2). The extracts

Plant species	Zone of inhibition (mm)[a]	Scale
Carbendazim	51.7[a]	4
Prosopis juliflora	30.7[b]	4
Acacia albida	19[c]	4
Dovalis abyssinica	11.7[d]	4
Argemone mexicana	11.0[e]	4
Vernonia amygdalina	9.7[f]	4
Azadirachta indica	7.3[g]	3.5
Acacia seyal	0.0[h]	0
Calotropis procera	0.0[h]	0
Cassia occidentalis	0.0[h]	0
Cynodon dactylon	0.0[h]	0
Datura stramonium	0.0[h]	0
Lantana camara	0.0[h]	0
Melia azedarach	0.0[h]	0
Moringa olifera	0.0[h]	0
Olea europaea	0.0[h]	0
Petiveria alliacea	0.0[h]	0
Plantago lanceolata	0.0[h]	0
Ricinus communis	0.0[h]	0
Schinus molle	0.0[h]	0
Solanum incanum	0.0[h]	0
Verbasacum sinaiticum	0.0[h]	0
Control	0.0[h]	0
CV (%)	6.87	

[a]Values are means of three replications and means within a column followed by the same letter are not significantly different (P<0.05), DMRT

Table 1: *In vitro* antifungal activity of methanolic extracts of 21 plant species against *Colletotrichum musae* four days after incubation

Plant species	Conidial germination (%)[a,b]	
Prosopis juliflora	0.3	(2.0)[k]
Acacia albida	0.2	(2.2)[k]
Dovyalis abyssinica	0.5	(3.8)[k]
Carbendazim	1.2	(4.6)[k]
Argemone mexicana	5.6	(13.6)[j]
Vernonia amygdalina	8.7	(17.1)[j]
Azadirachta indica	13.0	(21.1)[i]
Acacia seyal	18.7	(25.6)[h]
Plantago lanceolata	19.7	(26.3)[h]
Melia azedarach	23.3	(28.9)[gh]
Cynodon dactylon	27.3	(31.5)[g]
Petiveria alliacea	34.1	(35.7)[f]
Moringa olifera	34.9	(36.2)[f]
Ricinus communis	78.5	(62.4)[e]
Datura stramonium	85.7	(67.8)[d]
Verbasacum sinaiticum	85.9	(68.0)[d]
Cassia occidentalis	91.0	(72.4)[c]
Lantana camara	91.8	(73.4)[bc]
Solanum incanum	93.3	(75.2)[abc]
Olea europaea	94.3	(76.3)[abc]
Schinus molle	95.0	(77.5)[ab]
Untreated control	95.4	(77.9)[a]
Calotropis procera	95.7	(78.1)[a]
CV (%)		5.70

[a]Percentage of germinated spores 24 h after incubation (mean of three replications)
[b]Values in the parenthesis are arcsine transformed
Means followed by the same letters within the column are not significantly different (P<0.05), DMRT

Table 2: Effect of methanolic extracts of 21 plant species on conidial germination of *Colletotrichum musae*.

of *A. mexicana* and *V. amygdalina* reduced germination percentage to 5.6 and 8.7%, respectively, and were statistically at par with each other. On the other hand, the extracts of *C. procera, O. europaea, S. incanum* and *S. molle,* were observed to be least effective in reducing conidial germination of *C. musae* and were at par with the conidial germination in the untreated control (95.4%).

In the present study, spore germination was reduced to below 1% by methanolic extracts of *A. albida, D. abyssinica* and *P. juliflora*. Previous study conducted by [15] revealed that extracts of *P. juliflora* completely inhibited the germination of conidia of Phaeoisariopsis personata.

Thermal stability and antifungal activity of aqueous extracts: Extracts of *A. albida, A. indica, A. mexicana, D. abyssinica, P. juliflora,* and *V. amygdalina* that showed high activity against *C. muase* in the preliminary screening were further tested for their thermal stability and for the potency of their aqueous extracts. The results showed that there was no significant difference (P>0.05) between heated (at 60°C) and unheated plant extracts in their efficacy against the test pathogen (Table 3). This indicates that temperature slightly above the hot water treatment (HWT) range had no effect on the antifungal activity of the plant extracts. The result of the study is supported by previous works [16,17] that natural extract components are heat stable under moderate (50-80°C) heat treatment. It is worthy to note that heat treatment slightly but insignificantly increased inhibition zone diameter (Table 3). In addition, the degree of inhibition of fungal growth was increased from 3.5 to 4 scales in the case of *A. indica*. This is in agreement with the earlier findings [18,19] that heat treatments increased the activity of plant extracts. This may be due to an increase in the release of active compounds and free radicals [20].

Similarly, among the six plant species extracts tested, the highest zone of inhibition was exhibited by *A. albida* (18 mm) followed by *P. juliflora* (12.3 mm) (Table 4). Extracts of *A. mexicana* and *D. abyssinica* showed low antifungal activity with an inhibition zone of 7 and 6.7 mm diameter, respectively. On the other hand, aqueous extracts of *A. indica* and *V. amygdalina* showed no antifungal activity and the pathogen grew even on the paper discs. Distilled water, used as negative control, showed no zone of inhibition as expected. The fungi-toxic effect of aqueous extracts of *P. juliflora* against *Aspergillus* species [21] and *Fusarium* species [22] have been reported, which is in line with present result.

It is important to note that the activity of methanolic extracts of *P. juliflora*, *A. indica*, A. mexicana and *D. abyssinica* showed greater activity than their aqueous extracts (Tables 1 and 4). This is in harmony with the work of [11] that showed methanolic extracts have greater activity than the aqueous extract. This might be due to better solubility of the active components in organic solvents [23]. Furthermore, [24] indicated that such observations can be rationalized in terms of the polarity of the compounds being extracted by each solvent and, in addition to their intrinsic bioactivity, by their ability to dissolve or diffuse in the media.

Temperature (°C)	Plant species	Zone of inhibition (mm)[a]	Scale
Room temperature (ca. 22)	*Prosopis juliflora*	29.7[b]	4
	Acacia albida	18.7[c]	4
	Dovyalis abyssinica	10.7[d]	4
	Argemone mexicana	9.3[e]	4
	Vernonia amygdalina	8.7[e]	4
	Azadirachta indica	6.5[f]	3.5
60	*Prosopis juliflora*	30.7[b]	4
	Acacia albida	19.0[c]	4
	Dovyalis abyssinica	11.0[d]	4
	Argemone mexicana	9.7[e]	4
	Vernonia amygdalina	9.0[e]	4
	Azadirachta indica	7.0[f]	4
Control	Carbendazim (ca. 22°C)	51.0[a]	4
	Distilled water (ca. 22°C)	0[g]	0
CV (%)		3.53	

[a]Values are means of three replications.
Means within a column followed by the same letter are not statistically different (P<0.05), DMRT.

Table 3: Thermal inactivation of *in vitro* activity of six plant extracts against *Colletotrichum musae* four days after incubation.

Plant species	Zone of inhibition (mm)[a]	Scale
Carbendazim	50.5[a]	4
Acacia albida	18[b]	4
Prosopis juliflora	12.3[c]	4
Argemone mexicana	7[d]	3
Dovyalis abyssinica	6.7[d]	3
Azadirachta indica	0[e]	0
Vernonia amygdalina	0[e]	0
Untreated control	0[e]	0
CV (%)	4.14	

[a]Values are means of three replications.
Means within a column followed by the same letter are not significantly different (P<0.05), DMRT

Table 4: *In vitro* antifungal activity of aqueous extracts of six plant species against *Colletotrichum musae* four days after incubation.

Conclusions

In the preliminary screening of methanolic extracts of 21 different plant species against *Colletotrichum musae*, *Acacia albida* and *Prosopis juliflora* were the most effective in inhibiting mycelial growth of the test fungus (19.0 and 30.7 mm, respectively), while *Argemone mexicana, Dovalis abyssinica* and *Vernonia amygdalina* showed good antifungal activity (11.0, 11.7 and 9.7 mm, respectively). Spore germination was affected by some of the plant extracts but complete inhibition was not achieved by any of them. In the second stage screening, the results of heat inactivation test showed that all plant extracts tested were thermally stable at 60°C. Aqueous extracts of *A. albida* and *P. juliflora* showed the highest activity in the second stage screening (18.0 and 12.3 mm, respectively). This is the first report of *A. albida* and *P. juliflora* activity against *C. musae*. Further studies are warranted on isolating the active components found in the plant extracts for chemical characterization and possible use for postharvest management of *C. musae*.

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