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Bio-Treatment of Spoilage Fungi Associated with Postharvest Deterioration in *Citrullus lanatus* Thumb

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Abstract: Watermelon is a highly perishable fruit due to its high water content and several other factors which result in great loss. This study, therefore, investigated the antifungal activities of methanol and ethanol extracts of *Zingiber officinale* (ginger), *Vernonia amygdalina* (bitter leaf) and *Acalypha wilkesiana* (copper leaf) on spoilage fungi of watermelon. Spoilt watermelon samples were inoculated into Potato Dextrose Agar for isolation of spoilage fungi associated with watermelon. The fungi obtained were identified using macroscopic and microscopic characteristics. Pathogenicity test was carried out by inoculating healthy watermelon fruit with the isolated fungi. The methanolic and ethanolic extracts of *Z. officinale* rhizome*, V. amygdalina* and *A. wilkesiana* leaves were tested against the isolated fungi. The isolates were identified as *Aspergillus niger*, *A. flavus and Rhizopus* sp. and they were proven as the causal agents of spoilage with *A. niger* being the most virulent on day 11 (54.77mm) compared with *A. flavus* and *Rhizopus* sp which had (43.13mm and 40.63mm). All tested plant extracts significantly reduced mycelial growth of the isolated fungi with ethanol being the more effective solvent especially at 750mg/mL (0.88mm) concentration of extract. *Rhizopus* sp. and *A. niger* on day 4 (0.59mm and 0.62mm) through day 10 (1.16mm and 1.22mm) were more sensitive to the plant extract while *A. flavus* was found to be more resistant across the days of observation. The extracts of these indigenous plants can therefore serve as a natural fungitoxicant to ensure the availability of fresh healthy watermelon.

Key words: Plant Extract · *Citrullus lanatus* · Antifung

INTRODUCTION

Watermelon *(Citrullus lanatus* Thumb*)* is an economically important fruit crop, valuable for its water content especially in desert areas as well as low calories [1, 2]. It is also known to contain minerals, vitamins, lycopene and citruline [3]. It is a trailing, annual, monoecious and herbaceous plant classified in the family *Cucurbitaceae* [4], thought to originate from Southern Africa and now grown in more than 96 countries worldwide [5]. The fruit is a berry with an exocarp, mesocarp and endocarp. The fruit colour varies from pale green to yellow based on the type of varieties [6].

In Nigeria, watermelon production has increased significantly in the last decade with the major production areas in the Sahel, Sudan and Guinea agro ecological zones [7]. In these regions, the production of fruits has been hampered by postharvest loss as a result of deterioration caused by microbial attack which result from handling, contact with soil, water and mechanical injuries at harvest or during postharvest processing, reducing the quantity and quality of fruits directly. Biological control such as plant extract is host specific, economically feasible, cheap, easily degradable and has little or no side effects and could serve as an alternative means to the use of synthetic chemical (fungicides) which has negative impacts on man and the environment. Therefore, this study aims to investigate the antifungal effects of extracts of *Vernonia amygdalina* (bitter leaf), *Acalypha wilkesiana* (copper leaf) and *Zingiber officinale* (ginger) on spoilage fungi of watermelon.

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MATERIALS AND METHODS

Sample Collection: Fresh and healthy leaves of *Acalypha wilkensiana* and *Vernonia amygdalina* were obtained from the Botanical Garden, University of Ibadan, Nigeria and rhizomes of *Zingiber officinale* were purchased from Bodija market, Ibadan, Nigeria. The plant materials were authenticated in the Herbarium unit of Botany Department, University of Ibadan. Samples of spoilt watermelon fruits purchased from Bodija and Oje markets, leaves of *Acalypha wilkensiana* and *Vernonia amygdalina* as well as rhizomes of *Zingiber officinale* were washed under a running tap, surface sterilized with 70% ethanol to remove surface contaminants, rinsed in three changes of distilled water and air dried at room temperature. The rhizomes were milled into coarse powder with crusher (manufacturer by British Jeffery Diamond Limited) in Chemistry Department, University of Ibadan, Nigeria while the leaves were ground into fine powder with a sterile electric blender and stored in air-tight plastic containers until required [8].

Isolation and Identification of Fungi: The infected parts of the watermelon fruits were cut into small fragments and plated directly onto freshly prepared potato dextrose agar (PDA). The plates were incubated at 28 ± 2°C for 3-5days and were examined daily [8]. The fungi were characterized using the method of Barnett and Hunter [9].

Pathogenicity Test: Using a modification of the method of Oyeyipo [10], healthy watermelon fruits were surface sterilized as described above. A hole of 5mm diameter was aseptically bored into each fruit using a 5mm diameter cork borer. Mycelium plug of 5-days-old culture from the fungal isolates were cut from the active growing part of the colony and inserted into the hole in the watermelon. The wound was sealed with petroleum jelly while control samples were not inoculated with the isolate. The treated and control samples were placed individually in sterile polyethylene bags each moistened with wet balls of absorbent cotton wool to create a humid environment and incubated at 28±2°C. The samples were sectioned through the site of inoculation and examined for lesion development. Infected portions were aseptically transferred onto freshly prepared Potato Dextrose Agar to confirm the infection was caused by the inoculants. The extent of damage of each fungus on the inoculated samples was determined by measuring the lesion development at the site of inoculation at 2 days intervals from the 3rd day till the 11th day.

Extraction Procedure: The powdered rhizomes and leaves (100g each) of each plant were weighed separately into conical flasks containing 250ml of 90% ethanol and 100% methanol respectively [11]. These were covered and stirred every 24 hours using a sterile glass rod for 5 days after which it was filtered using muslin cloth. The filtrates were concentrated in a rotary evaporator at 45°C until slurry was formed and stored in bottles in a refrigerator at 4°C prior to use.

***In-vitro* Bio-control of the Isolated Fungi:** Different concentrations (100mg/mL, 250mg/mL, 500mg/mL and 750mg/mL) of the extracts of each plant were prepared and aseptically introduced into sterile Petri dish using a pipette. This was overlaid with 10mL of molten potato dextrose agar and mixed thoroughly. After the agar solidified, mycelial plug of 5-day-old culture was cut from the active growing portion of the colony and placed in an inverted position on the already prepared media. The culture plates were incubated at 28±2°C in an incubator. The diameter of growth of the pathogen was measured daily for 10 days. All the treatments were carried out in triplicates. Ethanol and methanol were both used as control in the determination of radial growth of the fungal isolates [12].

Analysis of Data: All data obtained in this study were analysed using Statistical Analysis System (SAS Version 9.1) software and subjected to the analysis of variance, while means were separated at 5% confidence interval, using Duncan Multiple Range Test (DMRT).

RESULTS

The fungal pathogens isolated from spoilt watermelon were identified as *Aspergillus niger*, *Aspergillus flavus, Rhizopus oryzae.*

All the fungi isolated reproduce spoilage symptoms when re-inoculated into fresh and healthy watermelon fruits (Table 1). At day 3, *Rhizopus* sp. recorded a more significant ($p<0.05$) growth (21.30mm) while the radial growth of *A. niger* and *A. flavus* as indicated by spoilage were not significantly different (10.20mm and 10.27mm) from the control (14.27mm). There was no significant difference in the growth of all the pathogens at days 5, 7 and 9. *A. niger* had the most significant growth at day 11 (54.77mm) while mycelial growth of *A. flavus* and *Rhizopus oryzae.* (47.13mm and 46.63mm) were not significantly different from the control (40.40mm).

Table 1: Pathogenicity of isolates re-inoculated in healthy fruits

	Level of spoilage induced (mm)				
Pathogen	Day 3	Day 5	Day 7	Day 9	Day 11
A. niger	10.20[b]	33.37[a]	39.60[a]	41.83[a]	54.77[a]
A. flavus	10.27[b]	34.50[a]	36.50[a]	38.50[a]	47.13[b]
Rhizopus sp.	21.30[a]	34.57[a]	37.53[a]	40.27[a]	46.63[b]
Control	14.27[ab]	30.50[a]	32.40[a]	34.43[a]	40.40[b]
LSD	8.29	9.61	9.23	9.19	6.57
EMS	17.22	23.12	21.34	21.15	10.82

Means with different letters are significantly ($p<0.05$) different across each column.

EMS = Error mean square

Table 2: Inhibitory effect of plant extracts at varying concentration against fungi associated with watermelon spoilage

		Radial mycelial growth (mm)								
Parameters	Variables	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
Extracts	Bitter leaf	0.41[a]	0.50[a]	0.63[a]	0.72[b]	0.87[a]	0.97[a]	1.07[a]	1.17[a]	1.27[a]
	Ginger	0.43[a]	0.56[a]	0.75[a]	0.91[a]	1.06[a]	1.16[a]	1.21[a]	1.28[a]	1.37[a]
	Acalypha	0.41[a]	0.51[a]	0.69[a]	0.78[ab]	0.90[a]	1.00[a]	1.10[a]	1.18[a]	1.26[a]
	LSD	0.13	0.16	0.17	0.16	0.19	0.19	0.20	0.20	0.21
Concentration	125	0.39[b]	0.48[b]	0.61[b]	0.72[b]	0.86[b]	0.96[b]	1.01[b]	1.10[b]	1.19[b]
	250	0.42[b]	0.48[b]	0.68[b]	0.79[b]	0.93[b]	1.07[b]	1.18[b]	1.30[b]	1.42[b]
	500	0.40[b]	0.57[b]	0.71[b]	0.82[b]	0.92[b]	0.99[b]	1.10[b]	1.19[b]	1.30[b]
	750	0.15[c]	0.20[c]	0.34[c]	0.42[c]	0.50[c]	0.58[c]	0.64[c]	0.66[c]	0.71[c]
	Control	0.73[a]	0.88[a]	1.13[a]	1.28[a]	1.51[a]	1.62[a]	1.70[a]	1.79[a]	1.89[a]
	LSD	0.17	0.21	0.22	0.22	0.24	0.25	0.25	0.26	0.27
Error mean square		0.20	0.30	0.33	0.33	0.42	0.42	0.45	1.97	0.52

Means with different letters are significantly ($p<0.05$) different across each column.

Table 3: *In-vitro* pathogenic effect of fungi associated with deterioration of watermelon

	Radial mycelia growth (mm)								
Pathogens	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
A. niger	0.42[ab]	0.50[ab]	0.62[b]	0.71[b]	0.86[b]	0.96[b]	1.05[b]	1.11[b]	1.22[b]
A. flavus	0.51[a]	0.64[a]	0.87[a]	0.97[a]	1.11[a]	1.24[a]	1.31[a]	1.43[a]	1.52[a]
Rhizopus sp.	0.33[b]	0.43[b]	0.59[b]	0.74[b]	0.86[b]	0.94[b]	1.01[b]	1.08[b]	1.16[b]

Means with different letters are significantly ($p<0.05$) different across each column

The *in-vitro* experiments showed that there was no significant difference recorded in the mycelial inhibitory effect of *Acalypha wilkensiana, Vernonia amygdalina* and *Zingiber officinale* against fungi causing spoilage of watermelon. At different concentration level of the extracts, the most mycelia inhibition was recorded at 750 mg/mL across the days of observation, while the concentrations of 125, 250 and 500 mg/mL did not show any significant difference from one another. However, all the concentrations significantly inhibited the radial mycelial growth of the fungi as compared to the control set up for the experiment (Table 2)

It was also observed that *Rhizopus oryzae* followed by *A. niger* were the most significantly inhibited by the extracts as observed in days 4 to 10, whereas *A. flavus* was least inhibited, thus had the highest mycelial growth ($p<0.05$) (Table 3).

It was also observed that ethanol extracts of all the botanicals had significant inhibitory action on all the pathogens than the methanol extracts as observed in all the days of data collection (Table 4).

Table 4l: Effect of different solvents on the fungal growths

	Radial mycelia growth (mm)								
Solvents	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
Methanol	0.62[a]	0.78[a]	0.96[a]	1.09[a]	1.29[a]	1.41[a]	1.49[a]	1.60[a]	1.72[a]
Ethanol	0.22[b]	0.26[b]	0.42[b]	0.51[b]	0.59[b]	0.68[b]	0.76[b]	0.82[b]	0.88[b]

Means with different letters are significantly ($p<0.05$) different across each column

DISCUSSION AND CONCLUSION

In this study, the fungi isolated from spoilt watermelon fruits were identified as *Aspergillus niger*, *A. flavus* and *Rhizopus* sp. suggesting that these fungal isolates might be responsible for spoilage of watermelon. Similar organisms have been isolated from pawpaw, watermelon, orange, tomato and pineapple as reported by Chukwuka *et al.* and Mailafia *et al.* [13,14]. These pathogens may have infected the fruit from the field with latent symptoms but cause severe damage under favourable environmental conditions such as high temperature, humidity and light obtainable in storage as observed by Jay [15]. Fruits may also encounter mechanical injury or wound during processes such as harvesting, transportation and storage which could be source of contamination for the organisms thereby resulting in the reduction of its quantity and quality for consumption and the profits obtained from the sales.

Pathogenicity test carried out proved that *Rhizopus* sp., *A. niger* and *A. flavus* were responsible for the symptoms observed in spoilt watermelon. This finding is consistent with previous reports [16, 17] where it was reported that *Aspergillus* species, *Penicillium* species, *Mucor* species and *Rhizopus* species were able to cause spoilage on re-infection with healthy orange fruits. Similarly, Kassali1, Aremu and Shittu [18] reported *A. niger,* as one of the causal agents of fruit rot of watermelon in storage and fruit stalls in Maiduguri, Nigeria. Generally, spoilage fungi such as *Aspergillus* spp are considered toxigenic or pathogenic and these have also been isolated from spoilt fruits as found in this research [17, 19]. However, as a proof of its virulence, among the three fungi isolated in this study, *A. niger* was the most virulent of all the isolates. This agrees with the work of Udoh *et al.* [20] as well as Kassali1, Aremu and Shittu [18]. The least pathogenic fungal isolates were *A. flavus* and *Rhizopus* sp. that caused little damage to the fruits after inoculation.

In this study, the *in vitro* screening of the extracts against the spoilage pathogens, *Zingiber officinale, Veronia amygdalina* and *Acalypha wilkensiana* significantly reduced the mycelia growth of all the fungal pathogens at all concentrations but the inhibitory action was most effective at 750mg/mL. This is germane since [21, 22] reported that plant extracts are safe and effective in the control of plant diseases. The antifungal effect of *Zingiber officinale* against various pathogens is well documented [23-25]. Similarly, it was reported that the extract of *Vernonia amygdalina* leaves can inhibit the growth of various fungal pathogens [26-28]. Furthermore, the antagonistic effect of *Acalypha wilkensiana* leaves on various fungal pathogens is well known [29-31]. In addition, radial mycelial growth of *Rhizopus* sp. and *Aspergillus niger* were significantly inhibited by extracts of the plant materials. This agrees with previous studies which reported the fungistatic and fungicidal activates of different botanicals on the named organisms [27, 32-34]. However, ethanol extracts of all the medicinal plants were very effective in the inhibition of the radial mycelial growth of the tested organisms than the methanol extracts. This affirms the efficiency of ethanol as an extraction solvent for phytochemical compounds in the botanicals better than methanol. Ekwenye and Elegalam [35] obtained similar results while working on garlic. They opined that as an organic solvent it will effectively dissolve organic compounds thereby liberating the active compounds (phytochemical) required for antifungal activity.

In conclusion, this study has shown that the extracts of the three indigenous plants used have the potential of protecting watermelon fruit against spoilage fungi especially spoilage caused by *Rhizopus* sp. and *Aspergillus niger*. Hence, it can serve as a natural fungitoxicant to replace hazardous synthetic chemicals (fungicides) which are used for prolonging its shelf-life to ensure the availability of fresh healthy watermelon fruit nationwide**.**

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