

Control of Rot of Kolanut caused by *Botrydiploia Theobromae* using some Plant Leaf Extracts

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Abstract: Water and ethanol leaf extracts of five plant species significantly ($P \geq 0.05$) reduced the *in vitro* radial growth, sporulation, fresh weight and dry weight of *Botrydiploia theobromae* as well as the development of rot disease in Kolanut seeds during storage. Ethanol extract of *Terminaliasuperba* gave significantly ($P > 0.05$) highest rot reduction of 96.3% when it was used to treat unwounded kolanut seed for 12 hours. This was followed by ethanol extract of *Pycathus angonensis* that gave a rot reduction of 91.4%. In the control untreated kolanut seed zero percent rot reduction was observed. Ethanol extract of all tested plants significantly inhibit the growth and sporulation of *Botrydiploia theobromae* as well as subsequent rot development. *Terminaliasuperba* and *Pycathus angonensis* appears to have the potentials to be used for managing kolanut seed rot during storage.

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1. Introduction

Kolanut is an important crop mainly cultivated in the South Western part of Nigeria. There are many species of kolanut in West Africa, but the Nigeria kolanut farmers recognize only two species and gave them local names (Oladokun, 2000). These are: *Cola nitida* Yoruba call it 'Obi Gbanja, Hausa call it 'Goro', Ibo ('Ojo') and *Cola acuminata*. Each fruit pod contains between six and twelve nuts that can be broken into two halves in the case of *Cola nitida* and three or rarely four lobes each for *Cola acuminata* (Agbeniyi, 2004; Agbeniyi and Ayodele, 2013).

Kolanut is eaten by all Nigerian's culturally diverse population while virtually all Nigerians to some extent appreciate the fruits peacemaking, friendship and sympathy values. The nut has varying degrees of importance to the three major Nigerian ethnic groups, the Hausa, Igbo and Yoruba. A Nigerian saying goes that the Hausa cultivate the kola for food, the Yoruba for commerce and the Igbo out of reverence (Opeke, 1996). Kolanut contains a substantial amount of *Caffeine*, *Theobromine*, *Kolatin* (a heart trinculant) and *Thophylline* as the active ingredients. The properties of these constituents make eating kola to induce strength, alertness and concentration in an individual (Oladokun, 2000; Saliuet *al.*, 2013).

Kolanut rot incited by *Botrydiploia theobromae* is an important storage disease of kolanut and could result in fatal crop loss during storage (Opeke, 1996; Agbeniyi and Ayodele, 2013). Different physical, cultural, biological and chemical

treatments have been suggested to control the pathogen (Opeke, 1996). Apart from hazards involved in using these chemicals, some of them are beyond the reach of resource poor farmers who produce over 98% of the food consumed (Olayideet *al.*, 1980; Okigbo and Ogbonnaya, 1994; Amadioha and Obi, 1997; Agbeniyi, 2004). Development of pesticides of plant origin will be cheap and readily available to resource poor farmers. Investigations by some workers have shown the importance of natural chemicals as a possible source of non-phytotoxic systemic and easily biodegradable alternative pesticides (Beye 1978; Singh 1994; Qasam and Abubalan 1996; Mason and Matthew, 1996; Amadioha and Obi, 1997; Fu *et al.*, 2007). Some workers have confirmed through *in vitro* investigations the antifungal potentials of the extracts of some plants species (Qasam and Abu-Blan, 1996; Amadioha and Obi 1997; Agbeniyi and Ayodele, 2013). Much of the plant kingdom still remains unexplored for possible exploitation against major fungal pathogen such as *Botrydiploia theobromae* the causal agent of kolanut rot. Leaves of five plant species *Cytopogon citratus*, *Ficus thonnigii*, *Funtomiaelastica*, *Pycathus augonensis* and *Terminaliasuperba*. Which were abundantly available in the South western Nigeria were selected for the present study to evaluate their efficacy both *in vitro* and *in vivo* against *Botrydiploia theobromae*.

2. Material and Methods

2.1 Source of Pathogen

Botrydiplochia theobromae was isolated in the laboratory from an infected kolanut demonstrating typical rot disease in the storage. The infected portion were cut into small pieces (about 2mm) with a flamed scalpel and two pieces were placed per dish containing potato dextrose agar (PDA) medium and then incubated at 25°C for three days. The pathogen was subcultured to obtain a pure culture. Pure cultures were maintained in PDA until needed.

2.2 Plant Leaf Extraction

Water and alcohol extracts were obtained from fresh leaves of *Cytopogon citratus*, *Ficus thonnigii*, *Funtomiaelastica*, *Pycathus augonensis* and *Terminaliasuperba*. The leaves were thoroughly washed in running tap water and sterile distilled water. The leaves were air dried at 30°C for 1h before being ground separately obtain 500g paste of each of the plant species. Water extract was obtained by adding each paste (100g) to 100ml of sterile distilled water in a 250ml beaver separately, stirred vigorously and allowed to stand for 1hr. The supernatant liquid was passed through a whatman No.1 filter paper, then a membrane filter (0.2µm) to avoid any bacterial or fungal contamination for alcohol leaves of each plant species which was then extracted with 95% ethanol and separately concentrated through a rotary vacuum pump flash evaporator to a syrupy form weighing 125g from each powder. The syrupy residue thus obtained was diluted to 2.5gl⁻¹ and used in all experiments.

2.3 Effect of Extract on Spore Germination and Radial Growth

The extracts obtained as describe above were evaluated for inhibition of the *sclerotium* germination and the mycelial growth of pathogen. One ml of each extract or sterile distilled water or alcohol was sprayed with a syringe on the solidified surface of 2% malt agar (MA) in Petri dishes spread and allowed to stand for 4h. One drop (0.1ml) of spore suspension (5x10⁴ spore/ ml of distilled water) was placed at the centre of each of the four sectors in each Petri dish. Five replicates were set up for each treatment. The plates were incubated at 30°C for at least 6h and then fixed with formalin acetic acid (FAA).

The spore germinating and those not germinating from each of the four inoculated areas in each plate were carefully counted under the low power (x10) of the microscope. The percentage inhibition was calculated from the data obtained following the formula of Amadioha (1997):

$$\% \text{ inhibition} = \frac{sc - st}{sc} \times 100$$

Where *Sc* = Spore Germination in the control

St = Spore Germination in the treated plants

The effect of the extracts on the mycelia growth of the rot- causing organism was investigated on PDA. One ml each of the extracts or sterile distilled water alone or alcohol alone was sprayed with a syringe onto the solidified surface of PDA in each of a series of Petri dishes. Each plate was inoculated at the centre with fungal disc (5mm diam) of 8-day-old culture and incubated at 30°C. The growth inhibition was determined after 7, 14, 21 and 28 days by measuring the growth of the fungal colonies along two preset diametrical lines. Fungitoxicity was expressed in term of percentage of mycelial growth inhibition and calculated according to the

$$\% \text{ growth inhibition} = \frac{dc - dt}{dc} \times 100$$

Where *dc* = colony diameter of control
dt = colony diameter of treated plates.

2.4 Evaluation of Extract against Rot Development

The healthy (uninfected) kolanut seeds were mechanically wounded with a sterile scalpel and dipped in each of the extracts or sterile distilled water alone or alcohol alone for 2h or 12h. The treated kolanut seeds were allowed to air-dry for 1h before spraying with a spore suspension (5x10⁴ spore/ ml) of pathogen. Inoculated Kolanut seeds wounded or unwounded were each placed in micro humidity chambers (sterile wet polythene bags) for 30 days at 30°C. The Kolanut seeds were then examined for rot development. The rot reduction index (RRI) was to determine the percentage rot reduction according to Amadioha (1997)

$$RRI = \frac{\%RC - \%RT}{\%RC} \times 100$$

Where *RC* = rot in control
RT = rot in treatment extract.

The data collected for each treatment was analyzed using analysis of variance (ANOVA) and the means were separated using LSD (Least Significant Difference).

3. Results

The results of the effect of the plant extracts on radial growth of *Botrydiplochia theobromae* shows that the extracts of *Terminaliasuperba* were the most effective of the five plant species tested and this was significantly different (P≥5%) at all days of incubation from all other plant extracts. (Table1). Infact, all the five plant extracts tested were significantly (P≥0.5) different from the controls.

Table 1: Antifungal effect of plant extracts on radial Growth of *Botrydiplodia theobromae* at different days of incubation

Plant Extracts	Radial growth inhibition (%) at different days of incubation							
	Water extract				Alcohol extracts			
	7	14	21	28	7	14	21	28
<i>Cytopogon citratus</i>	43.8 ^e	48.1 ^d	53.4 ^d	59.2 ^d	49.3 ^d	53.2 ^d	58.7 ^d	66.3 ^d
<i>Ficus thonnigii</i>	50.1 ^d	56.3 ^c	65.6 ^c	71.9 ^c	55.9	61.8	70.6	79.0 ^c
<i>Funtumia elastic</i>	57.0 ^c	64.9 ^b	70.3 ^b	76.7 ^c	63.8 ^c	69.0 ^c	76.1 ^c	85.1
<i>Pycathus angonensis</i>	63.6 ^b	68.8 ^b	75.5 ^b	83.0 ^b	69.0 ^b	73.4 ^b	81.2 ^b	89.7 ^b
<i>Terminaliasuperba</i>	69.4 ^a	75.9 ^a	81.2 ^a	87.8 ^a	75.2 ^a	80.8 ^a	88.5 ^a	95.6 ^a
Control I-water	0 ^t	0 ^e	0 ^e	0 ^e	-	-	-	-
Control II- Alcohol	-	-	-	-	3.8 ^t	5.4 ^t	8.7 ^t	9.6 ^e

Data are average of five replicates from four separate experiments. Values in the same column followed by a similar letter are not significantly different using Duncan Multiple Range Tested (DMRT) at 5% level of probability.

Table 2: Antifungal effect of plant extracts on sporulation of *Botrydiplodia theobromae* isolated from Kolanut rot at different days of incubation.

Plant extracts	Sporulation inhibition (%) at different days of incubation							
	Water extract				Alcohol extracts			
	7	14	21	28	7	14	21	28
<i>Cytopogon citratus</i>	48.2 ^a	54.8 ^d	59.1 ^d	65.4 ^d	53.5 ^e	59.0 ^c	64.7 ^e	70.9
<i>Ficusthonningii</i>	55.4 ^d	61.2 ^d	67.0 ^d	72.1 ^d	61.2 ^d	66.8 ^d	72.0 ^d	77.6 ^d
<i>Funtumia elastic</i>	63.0 ^c	69.3 ^c	75.1 ^c	80.5 ^c	69.4 ^c	75.0 ^c	81.3 ^c	87.0 ^c
<i>Pycathus augonensis</i>	68.1 ^b	73.0 ^b	79.3 ^b	86.9 ^b	73.8 ^b	79.1 ^b	86.9 ^b	92.1 ^b
<i>Terminaliasuperba</i>	74.7 ^a	79.7 ^a	85.7 ^a	91.6 ^a	79.9 ^a	85.3 ^a	90.8 ^a	96.4 ^a
Control I-water	0 ^t	0 ^t	0 ^t	0 ^t	-	-	-	-
Control II- Alcohol	-	-	-	-	5.6 ^t	8.3 ^t	11.4 ^t	13.8 ^t

Data are average of five replicates from four separate experiments. Values in the same column followed by a similar letter are not significantly different using Duncan Multiple Range Tested (DMRT) at 5% level of probability.

Table 3: Antifungal Effect of Plant extracts on Fresh Weight of *Botrydiplodia theobromae* Different Days of Incubation

Plant extracts	Fresh weight (g) of <i>Botrydiplodia theobromae</i> at Different Days of Incubations							
	Water extract				Alcohol extracts			
	7	14	21	28	7	14	21	28
<i>Cytopogon citratus</i>	38.3 ^b	65.0 ^b	77.2 ^b	91.6 ^b	25.7 ^b	40.3 ^b	61.5 ^b	79.9 ^b
<i>Ficusthonningii</i>	25.7 ^c	49.2 ^c	65.4 ^c	80.7 ^c	18.3 ^c	35.5 ^c	52.7 ^c	64.3 ^c
<i>Funtumia elastic</i>	17.4 ^d	37.9 ^d	59.6 ^d	71.1 ^d	10.6 ^d	29.8 ^d	48.8 ^d	67.9 ^d
<i>Pycathus augonensis</i>	10.8 ^e	23.5 ^e	35.0 ^e	60.5 ^e	5.7 ^e	17.1 ^e	30.2 ^e	50.3 ^e
<i>Terminaliasuperba</i>	5.5 ^t	10.7 ^t	14.9 ^t	18.3 ^t	2.0 ^t	5.3 ^t	9.5 ^t	13.0 ^t
Control I-water	66.4 ^a	78.2 ^a	87.9 ^a	96.5 ^a	-	-	-	-
Control II- Alcohol	-	-	-	-	51.3 ^a	58.7 ^a	75.1 ^a	90.7 ^a

Data are average of five replicates from four separate experiments. Values in the same column followed by a similar letter are not significantly different using Duncan Multiple Range Tested (DMRT) at 5% level of probability

There was a significant difference ($P \geq 0.05$) between the water extracts of all the plant species that of the alcohol extracts (Table 1). Alcohol extracts of the plant species significantly ($P \geq 0.55$) inhibited the mycelial growth more than that water extract (Table 1). The antifungal activity of the plant extracts was observed to have increased significantly ($P \geq 0.05$) with incubation period (Table 1). The extracts

showed an increased inhibition of radial growth of the pathogen as the period of culture increases.

The result of the antifungal effects of the plant extracts on spore germination of *B. theobromae* is presented in Table 2. it showed that the extracts of *Terminaliasuperba* were the best inhibitor of the spore germination of the kolanut seed rot causing (Table 2). In addition, all the extracts of plant species tested were significantly different from that of

the controls. Alcohol extracts of the plant species inhibited spore germination significantly ($P \geq 0.05$) than water extracts (Table 2). pathogen and this was significantly different ($P \geq 0.05$) from all other plant extract at all days of incubation

The results of the effect of the plant extracts on fresh weight of *B. theobromae* mycelium at different days of incubation is presented in Table 3.

The result showed that the extracts of all plant species tested gave significantly lowest ($P \geq 0.05$) fresh weight of the pathogen mycelium compared to the controls (Table 3). While *Terminaliasuperba* extracts was the most significantly ($P \geq 0.05$) effective in reducing the fresh weight of pathogen mycelium compared to all other plant species extracts (Table 3).

Table 4: Antifungal effect of plant extracts on Weight of *Botrydiplodia theobromaemycelium* at different Days of incubations

Plant extracts	Dry weight (g) of <i>Botrydiplodia theobromae</i> at Different Days of Incubation							
	Water extract				Alcohol extracts			
	7	14	21	28	7	14	21	28
<i>Cybopogon citratus</i>	20.11 ^b	34.81 ^b	46.23 ^b	58.1 ^b	12.9 ^b	21.1 ^b	32.1 ^b	41.7 ^b
<i>Ficusthonningii</i>	14.34 ^c	26.00 ^b	34.16 ^b	47.30 ^b	07.1 ^b	18.3 ^b	27.3 ^b	35.1 ^b
<i>Funtumia elastic</i>	8.91 ^d	19.23 ^d	28.19 ^d	39.35 ^d	05.3 ^d	15.3 ^d	25.1 ^d	38.8 ^d
<i>Pycathus augonensis</i>	5.40 ^c	14.41 ^c	22.56 ^c	30.11 ^c	02.4 ^c	08.5 ^c	15.3 ^c	25.8 ^c
<i>Terminaliasuperba</i>	2.70 ^t	5.60 ^t	11.20 ^t	17.24 ^t	01.1 ^z	2.7 ^t	4.8 ^t	06.7 ^t
Control I-water	43.2 ^a	60.24 ^a	71.32 ^a	80.30 ^a	-	-	-	-
Control II- Alcohol	-	-	-	-	27.1 ^a	30.3 ^a	39.5 ^a	46.4 ^a

Data are average of five replicates from four separate experiments. Values in the same column followed by a similar letter are not significantly different using Duncan Multiple Range Tested (DMRT) at 5% level of probability.

Table 5: Effect of plant extracts on Kolanut seed rot development by *Botrydiplodia theobromae* Rot reduction %

Plant extract	Water extract				Alcohol extract			
	2h dip		12h dip		2h dip		12h dip	
	Wounded		Unwounded		Wounded		Unwounded	
<i>Cybopogon citratus</i>	31.6 ^c	43.4 ^c	36.2 ^c	48.1 ^c	35.7 ^c	40.2 ^c	48.1 ^c	59.7 ^c
<i>Ficus thonnigii</i>	38.3 ^d	56.8 ^d	44.5 ^d	60.3 ^d	43.4 ^d	49.7 ^d	56.8 ^d	68.2 ^d
<i>Fontomiaelastica</i>	45.1 ^c	63.8 ^c	49.4 ^c	78.1 ^c	51.2 ^c	60.5 ^c	71.1 ^c	83.1 ^c
<i>Pycathus angonensis</i>	58.4 ^b	79.6 ^b	64.6 ^b	84.4 ^b	60.8 ^b	70.9 ^b	82.0 ^b	91.4 ^b
<i>Terminaliasuperba</i>	67.9 ^a	85.5 ^a	72.4 ^a	90.9 ^a	69.9 ^a	81.7 ^a	90.5 ^a	96.3 ^a
Control I – water	0	0	0	0	-	-	-	-
Control II - Alcohol		-	-	-	8.5	17.9	13.3	28.5

Data are average of five replicates from four separate experiments. Values in the same column followed by a similar letter are not significantly different using Duncan Multiple Range Tested (DMRT) at 5% level of probability.

The significantly ($P \geq 0.05$) highest mycelium fresh weight of this pathogen was recorded in the controls. It is clearly revealed that the alcohol extracts of all the plant species tested give significantly ($P \geq 0.05$) lowest fresh weight of the mycelium compared to the water extract. Table Similar trend was observed for the results on the effect of extracts of plant species on the dry weight of the mycelium of the pathogen (Table 4).

Investigations conducted *in vivo* to test the efficacy of the extracts in the control of storage rot disease incited by *B. theobromae* showed that kolanut rot caused by the pathogen was reduced by 67.9%

and 72.4% when the wounded kolanut seeds were dipped in water extract of *T. superba* for 2h and 12h respectively. Whereas 67.9% and 90.5% rot reduction was recorded for alcohol extract in 2h and 12h respectively (Table 5). This was followed with water extracts of *Pycathus angonensis* with rot reduction of 58.4% and 64.6% when the wounded kolanut seeds were dipped in it for 2h and 12h respectively while 60.8% and 82.0% rot reduction was recorded for alcohol extract in 2h and 12h respectively (Table 5). The significantly ($P \geq 0.05$) lowest rot reduction percentage was noticed in that of control. Indeed, in all the tested plant extracts significantly ($P \geq 0.05$)

least percentage rot reduction was recorded with extracts of *Cytopogon citratus*. Unwounded kolanut were effectively protected against the fungal attack after 2h dip in alcohol extract of *Terminaliasuperba*. The alcohol extracts of plant species were more effective in protecting the kolanut seeds from rot than that water extracts.

4. Discussion

Botrydiplodia theobromae has been reported to cause serious damage to kolanut seed particularly during storage. (Opeke, 1996; Suleiman and Ogundana, 2010). This pathogen infects the follicle, which develops a black nut and subsequently attacks the nut. Control of the pathogen through chemical, physical and cultural methods have been suggested (Opeke, 1992; Agbeniyi and Ayodele, 2013). Investigation on the antifungal properties of tropical plant extracts on the growth of *B. theobromae* isolate shows that the plant extracts possess some inhibitory components which cause significant reduction in mycelial growth, spore germination, fresh weight, dry weight, and rot reduction in kolanut seed. The current investigations on exploit in pesticides of plant origin were undertaken with view of countering obvious pollution problems in the environment well as avoiding the toxic effects of synthetic chemicals on non-target organisms. Studies carried out by some workers through *in vitro* investigation have confirmed the fungicidal potential of the extracts of some plant species such as Piper beetle, *Ocimum sanctum*, *Chenopodium murale*, *Azadiracta indica*, *Cytopogon citrates*, *Datura*, *Lawsonia inermis*, *Carica papaya*, *Acalypha ciliate*, *Vernonia amygalina*, *Mangifera indica*. (Tewari and Nayak, 1991 Okigbo and Ogbonnaya, 1994 Amadioha and Obi, 1997; Agbeniyi, 2004).

It was observed in this study that alcohol extracts of the plant species were relatively more effectively than water extracts and that the potency of each extract differs from one another, suggesting that the toxicity of different extracts may be due to their solubility in extracting solvents and or the presence of inhibitors to the fungi toxic principle (Qasam and Abu-Blan, 1996; Bankole *et al*, 1996). The result might have been influenced by the solubility of the active compound(s) in extracting solvents, with high solubility of *T. superba*. Compounds than other plant species tested.

The persistence of fungi toxic activity of the extracts appear to the same among extracts, since all showed increasing inhibition of the radial growth of the rot-causing organism with time. This indicates that the active compounds were absorbed or diffused on the medium which acted against the pathogen for the whole period of culture.

Rot development by the pathogen in infected seeds was significantly reduced by the extracts, over the control, suggesting that the extracts could be used as protectant fungicide. Extracts of these plant species as a natural fungicide offer broad-spectrum activity, rapid degradation and low mammalian toxicity (Tewari and Nayak, 1991; Agbeniyi and Ayodele, 2013; Bankole and Adebayo, 1997) and may therefore, be preferable to synthetic fungicides. Infact, Cardellina (1988) submitted that they poses little environmental risk because they do not bio accumulate. Also, these natural product pesticides are readily available and cost-effective in countries like Nigeria where synthetic pesticides are expensive and difficult to obtain by resource-poor farmers who produce over 98% of food consumed.

The above results suggest that extracts of these tropical plants has potential for use as bio fungicides, as alternative to the recommended synthetic fungicides. These tropical plants are widely available on most farms in the tropics, hence availabilities are ensured. The technology of preparation and application is simple.

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