Pathogenicity and Control of *Phomopsis sp*. Associated with Yam Rot Using Bark Extracts of *Mangifera indica* L. and *Parinari polyandra* Benth.

[*1]Ahmed, O., [1]Baba, H.S., [1]Yusuf, S.Y., [2]Lawal, R.A., [1]Adebayo, O.V. and [1]Ojomu, J.O.

[1]Department of Crop Protection, Faculty of Agriculture, University of Ilorin, Ilorin, Nigeria
[2]Nigeria Stored Product Research Institute, Ilorin, Nigeria

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Abstract
The antifungal property of *Mangifera indica* and *Parinari polyandra* bark extracts against *Phomopsis* sp. isolated from rotting yam tubers was evaluated. Stock solutions prepared by dissolving 1 g of each extract in 100 ml of sterile water (i.e. 1% w/v) was applied at 1:2, 1:4 and 1:6 (i.e. 1 part of the stock to 2, 4 and 6 parts of the culture medium v/v) *in-vitro*. The extracts were later applied at 0.5%, 1% and 1.5% w/v as coating on the yam tubers. The plant extracts significantly suppressed the growth of the fungus *in vitro* and reduced rot development in healthy yam tubers. The percentage inhibition of growth of the test fungus on medium treated with *Parinari polyandra* bark extract ranged between 10.50 – 21.35 , 19.52 – 43.15 , 29.41 – 50.14 , 33.18 – 51.92 and 27.71 – 47.30 for concentration range between 1:6v/v – 1:2v/v of the stock solution at days 3 , 5 , 7 , 9 and 11 of plating respectively. Percentage inhibition of growth of the fungus on *Mangifera indica* extract treated medium also ranged between 11.02 – 39.06, 34.93 – 49.32, 34.45 – 52.94, 38.37 – 54.18 and 31.88 – 50.42 for the same concentration range and days after plating respectively. The study showed the potentials of these plant extracts in the control of post harvest rots of yam tubers.

Keywords: Yam rot fungus, Solvent extracts, Botanicals, *in-vitro* test, Pathogenicity

1.0 Introduction

Yam (*Dioscorea sp.*) is the second most important tropical root crop in West Africa after cassava. In Nigeria, it is produced both for household consumption and as a cash crop [1]. Yam also plays a significant role in the socio- cultural lives of people in West Africa. According to FAO statistics [2], 48.7 million tonnes of yam were produced worldwide in 2005, and 97% of this was in sub-Saharan Africa. West and Central Africa account for about 94% of the world production. Nigeria is the leading producer with 34 million tonnes followed by Cote d'Ivoire (5 million tonnes), Ghana (3.9 million tonnes), and Benin (2.1 million tonnes).

There are several constraints to the cultivation of yam in Nigeria. Of these constraints, diseases contribute greatly to high yield losses before and after harvest. Yam plants are prone to infection by fungi, bacteria and viruses at all stages of growth and also during storage of tubers. The disease causing agents reduce the quantity/yield of yam produced and also reduce the quality thereby making them unappealing to the consumer.

Fifty percent of the yam tuber produced and harvested in Nigeria is lost in storage [3]. Losses due to post-harvest rot significantly affect farmers' and traders' income, food security and seed yams stored for planting. Most rots of yam tubers are caused by pathogenic fungi. In Nigeria, over 60% of white yam varieties get rotten when stored for more than six months [4]. Various methods have been used to control yam rot although the use of chemicals has been the most effective so far even though it has its limitations. The environment gets polluted when pesticides accumulate without bio-degradation. There have also been reports of pesticide poisoning when treated produce are consumed [5].

***Corresponding Author: Tel: +234(0)8038365315, E-mail: ahmelad2007@yahoo.com, ahmelad@unilorin.edu.ng**

These and other problems have resulted to the use of natural products. Compared to synthetic products, natural substances are biodegradable, readily available, cheaper and environmentally friendlier than synthetic chemicals. They are also easy to apply and are usually less or non-toxic to man and his livestock. This work was therefore carried out to investigate the antimicrobial effect of extracts from the bark of *Mangifera indica* and *Parinari polyandra* on yam rot fungus (*Phomopsis* sp) *in vitro* and *in vivo*.

2.0 Materials and Methods

2.1 Collection of Yam Tubers

Healthy and rotten yam tubers were collected from three major markets in Ilorin, Nigeria to increase the chances of isolating the rot fungus – *Phomopsis* sp. The yams were collected at the onset of the harvesting season around August 2015. The yams were packed in polythene bags and taken to the Biotechnology Laboratory of the Faculty of Agriculture, University of Ilorin, Nigeria where the study was carried out.

2.2 Isolation of Fungal Pathogens from Rotten Yam Tubers

The fungus was isolated from the yam tubers by direct tissue plating on Potato Dextrose Agar (PDA) medium using the modified rules for seed health testing [6]. Briefly, the rotten yam tubers were rinsed in sterile water, surface sterilized with 70% ethanol and cut open. Five millimeter diameter and two millimeter thick of infected tissues were picked with flame sterilized forceps and plated on solidified PDA medium amended with streptomycin in 90 mm petri dishes. The inoculated plates were incubated at room temperature and observations were made daily for emergence of colonies. Sub-culturing was done to obtain pure cultures of the isolate.

2.3 Preparation of Pure Cultures from the Isolated Fungi

The spores of the pathogen was transferred aseptically unto fresh sterile media with ethanol flamed inoculating needle to prepare the pure cultures from the fungal isolate. The plates were incubated for seven days at room temperature. Pure cultures are needed for pathogenicity test and identification of the fungal isolate.

2.4 Identification of the Fungal Isolates

The pure cultures of the isolates were observed for morphological characteristics. Three-day-old cultures were then sent to the Pathology laboratory of the International Institute for Tropical Agriculture (IITA) Ibadan, Nigeria for confirmation of identity.

2.5 Pathogenicity Test

A fresh, healthy tuber yam was washed with tap water followed by distilled water, and thereafter sterilized with 70% ethanol. Cylindrical discs were removed from the tuber with a sterile 5 mm cork borer. A four disc of five days old cultures of the isolates was used to plug the holes created in the tuber and the disc of the tuber in the cork borer was replaced, then candle wax was applied on the point of inoculation. The inoculated yam was then incubated for 10 days and thereafter observed for the development of symptoms of rot.

2.6 Collection of Plant Materials and Extraction from the Barks

The bark of *Mangifera indica* tree was collected from the premises of Faculty of Agriculture, at the University of Ilorin while that of *Parinari polyandra* was collected from the surrounding of the University Senate Building. Both plants were identified at the Herbarium of Department of Plant Biology University of Ilorin, Ilorin, Nigeria where voucher specimen was deposited and voucher number obtained (UILH/002/969 for *M. indica* and UILH/003/582 for *P. polyandra*). The barks were washed with sterile water and air dried on the laboratory bench of the Department of Chemistry, University of Ilorin for two weeks at ambient temperature and then milled to powder form using mortar and pestle. The powdery bark material was then soaked separately in ethanol for 72 hours after which it was decanted, filtered and concentrated under reduced pressure in a rotary film evaporator (BORO-G GRFE2). The concentrated extracts were left to dry to constant weight in order to ensure that the ethanol evaporates completely.

2.7 Phytochemical analysis of the plant extracts.

Qualitative screening was carried out on the plant extract to determine the presence of secondary metabolites using standard procedures [7, 8].

2.7.1 Test for Flavonoids

Dilute ammonia solution (5 ml) was added to aqueous filtrate of the plant extract followed by addition of concentrated H_2SO_4 (1 ml). A yellow precipitate confirmed the presence of flavonoids.

2.7.2 Test for Phenolic Compounds and Tannins

Two milliliter (2 ml) of the extract was boiled in 10 ml of water in a test tube and filtered. About 6 drops of feric chloride was added. A dark blue colouration confirmed the presence of phenolic compounds and tannins.

2.7.3 Test for Glycosides

Small quantity of the extract was diluted with 5 ml distilled water. Glacial acetic acid (2 ml) was added containing one drop of ferric chloride solution (3.5%). This was then underlain with 1ml of concentrated H_2SO_4. Formation of a reddish brown ring indicated the presence of cardiac glycosides.

2.7.4 Test for Saponins

Distilled water (10 ml) was added to 2.5 ml of the filtrate and the mixture was shaken vigorously for 2 minutes. Frothing indicated the presence of saponin.

2.7.5 Test for Carbohydrates

The presence of carbohydrates was detected by Molisch's test. Briefly, 2 ml of the crude extract was added to about 5 drops of molisch reagent followed by the addition of 1 ml of concentrated sulphuric acid carefully inside a test tube, so that the acid forms a layer beneath the aqueous solution without mixing it. The mixture was allowed to stand for two minutes. A positive reaction is indicated by the formation of a purple ring at the interface between the acid and the test layers.

2.7.6 Test for alkaloid

Each extract (1 ml) was mixed with 5 ml of 1% aqueous hydrochloric acid on water bath, this was divided into two equal parts. Meyer’s reagent was added to the first part while Dragendoff's reagent was added to the second part. Precipitation with either of the reagent confirmed the presence of alkaloids.

2.7.7 Test for Terpenoids

Each extract (1 ml) was dissolved in 2 ml ethanol, this was concentrated a little on the water bath, then 1 ml of 2,4-dinitrophenyl hydrazine (0.4 g of 2, 4-dinitrophenyl hydrazine in 100 ml, 2 M HCl) was added. A deep yellow precipitate indicated the presence of terpenoids.

2.7.8 Test for Anthraquinones

Each extract (1 ml) was shaken with 3 ml petroleum ether, and then 2 ml of 25% ammonia solution was added to the mixture and was shaken. Formation of red color indicates the presence of anthraquinone.

2.8 Effect of the Extracts on Fungal Growth in vitro

One percent stock solution was prepared from each of the extract by dissolving 1 g of the evaporated extract first in 5 ml of ethanol and then sterile water was added to make 100 ml solution. The culture medium (PDA) was amended with the stock solutions at the ratio of 1:6, 1:4 and 1:2 v/v of the stock in the medium. The method of Amadioha and Obi [9] was used to determine the effect of the extract on fungal growth in which a disc (5 mm diameter) of the pure culture of *Phomopsis* spp was placed at the centre of 9 cm petridish containing the culture medium amended with the extracts in three replicates per treatment. Control was made up of the medium not amended with the plant extracts. Fungal growth inhibition was recorded as percentage colony inhibition and calculated in accordance with Pandey *et al*. [10].

Growth inhibition (%) = [(DC-DT)/DC] X 100. Where
DC = average diameter of control, and
DT = average diameter of fungal colony with treatment

Measurement of the change in diameter of the fungus commenced three days after plating and was repeated five times at the interval of fourty eight hours i.e at 3DAP, 5DAP, 7DAP, 9DAP and 11DAP (Days After Plating).

2.9 Effect of Plant Extracts on Fungal Growth in vivo

Fresh healthy yams were washed with sterile water and sterilized with 70% ethanol. The yam tubers were bruised on the surface, and the extract treatments at 0.5%w/v, 1.0%w/v and 1.5%w/v were spray-applied using hand spray. Three replicates were created for each level of the plant extracts. The yam tubers were left to dry for about 3 hours to ensure that the solution of the extracts are well absorbed into the tuber. Inoculum suspension prepared by blending 10 g of the mycelia mat of the organism (grown on Potato dextrose broth) in 1 litre of sterile water was then applied also by spraying on the bruised surface. Control experiments were set up by spraying with only sterile distilled water. The yam tubers were packed in polyethene bags and incubated in the laboratory for 30 days under ambient condition. The diameter of fungal growth was recorded within the interval of five days after the first ten days i.e. 10DAT, 15DAT, 20DAT, 25DAT and 30DAT (DAT Days after Treatment).

2.10 Data Collection and Analysis

Data were collected on the diameter of growth of test fungus on plates and on the yam tubers. These were subjected to analysis of variance in the Completely Randomized Experimental Design using IBM SPSS version 21 statistical package. Mean values were separated using the Duncan's New Multiple Range Test at 5% level of significance.

3.0 Results

3.1 Identification of the Fungal Isolates

The cultural and microscopic features of the isolated fungus are shown in Figs. 1 and 2. The identity of the fungus was confirmed at the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria to be *Phomopsis* sp.

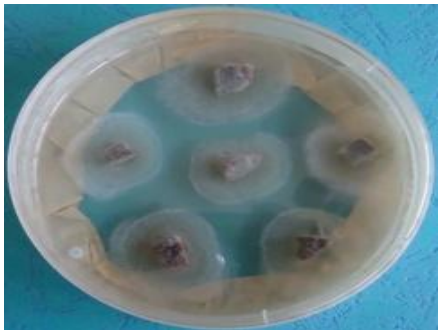

Fig. 1: *Phomopsis* sp. growing on yam tissue 2 days after plating

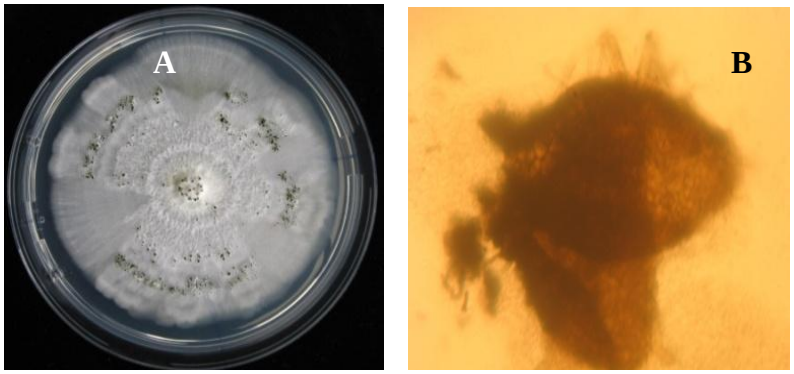

Figure 2: Five-day-old Pure Culture of *Phomopsis* sp. (A) Pycnidia of *Phomopsis* sp. (B)

3.2 Pathogenicity of Phomopsis sp.

The pathogenicity test conducted on the fungus showed that the organism produced rot lesions on healthy yam tubers tested within the time of exposure. The lesions were similar to those observed on the previously rotted yam. When the organism on the lesion spots was re-isolated, it was identified as *Phomopsis* sp.

3.3 Phytochemical Screening of the Plant Extracts

The result of the Phytochemical screening of the plant extracts is presented in Table 1. The only compound not present in *Mangifera indica* bark extract out of the eight tested was anthraquinone while glycoside was lacking in *Parinari polyandra* extract.

Table 1: Phytochemical Screening of *Mangifera indica* and *Parinari polyandra* Bark Extracts

Plants	Tanin	Saponin	Glycoside	Flavonoid	Anthraquinone	Terpenoid	Alkaloid	Carbohydrates
M. indica	+	+	+	+	-	+	+	+
P. polyandra	+	+	-	+	+	+	+	+

+ = Present; – = Absent

3.4 In-vitro Antifungal Activity of the Plant Extracts

The percentage inhibition of *Phomopsis sp*. *in vitro* was significantly high ($p<0.05$) for the different plant extracts. Generally, percentage reduction in diameter of the test fungus was significantly higher ($p<0.05$) for *Mangifera indica* than *Parinari polyandra*. The values for percentage reduction in growth diameter of the fungus also decreased with decreasing concentration and increased with increasing concentration of the plant extracts. The highest percentage inhibition was observed in 1:2 v/v concentration and the least was observed in 1:6 v/v concentration (Table 2).

Table 2: Effect of Different Concentrations of *Parinari polyandra* and *Mangifera indica* Bark Extracts on Percentage Inhibition of *Phomopsis sp. in vitro*

Levels of application	3DAP	5DAP	7DAP	9DAP	11DAP
1:6 v/v of *P. polyandra*	10.50^{d}	19.52^{d}	29.41^{c}	33.18^{b}	27.71^{c}
1:4 v/v of *P. polyandra*	12.60^{cd}	29.79^{cd}	35.29^{bc}	37.70^{b}	28.13^{c}
1:2 v/v of *P. polyandra*	21.35^{b}	43.15^{ab}	50.14^{a}	51.92^{a}	47.30^{a}
1:6 v/v of *M. indica*	11.02^{ab}	34.93^{bc}	34.45^{bc}	38.37^{b}	31.88^{bc}
1:4 v/v of *M. indica*	22.92^{b}	40.75^{ab}	43.13^{ab}	44.02^{ab}	40.63^{ab}
1:2 v/v of *M. indica*	39.06^{a}	49.32^{a}	52.94a	54.18^{a}	50.42^{a}

Values followed by the same superscript(s) are not significantly different at p=0.05 by Duncan's New Multiple Range Test at 5% level of significance.
DAP = Days after Plating

3.5 In-vivo Antifungal Activity of the Plant Extracts

Figs. 3 and 4 show the effect of the extracts on growth of *Phomopsis* sp. in healthy yam tubers. Compared to the control, the change in growth diameter of the fungus decreased with increasing concentration of the plant extracts. Both extracts performed better at higher concentrations and the effectiveness decreased as the concentration decreased. *Mangifera indica* however showed better performance than *Parinari polyandra* in reducing the fungus growth. The diameter of rot in *Mangifera indica* treated yams at 30DAT decreased from 0.83 to 0.50 and then 0.20 when concentration of the extract increased from 0.5% through 1.0% and 1.5% respectively. Similarly, the rot diameter in *Parinari polyandra* treated yam tubers decreased from 1.20 to 1.0 and 0.8 as concentration of the extract increased from 0.5% through 1.0% and 1.5% respectively at 30DAT.

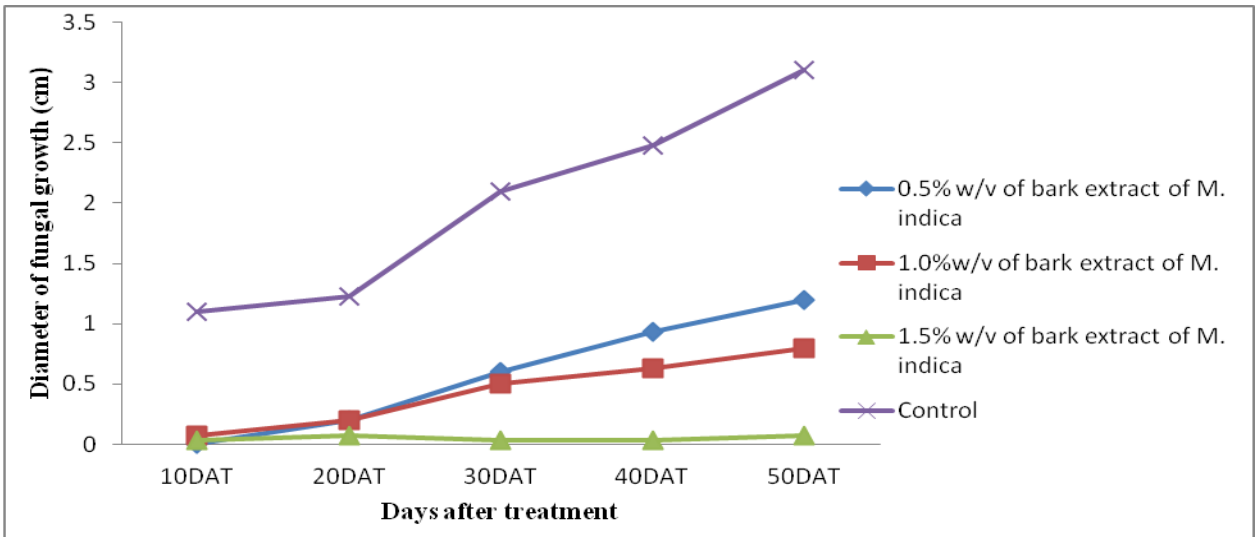

Figure 3: Effect of different concentration of *Mangifera indica* bark extract on inhibition of Phomopsis in yam tuber

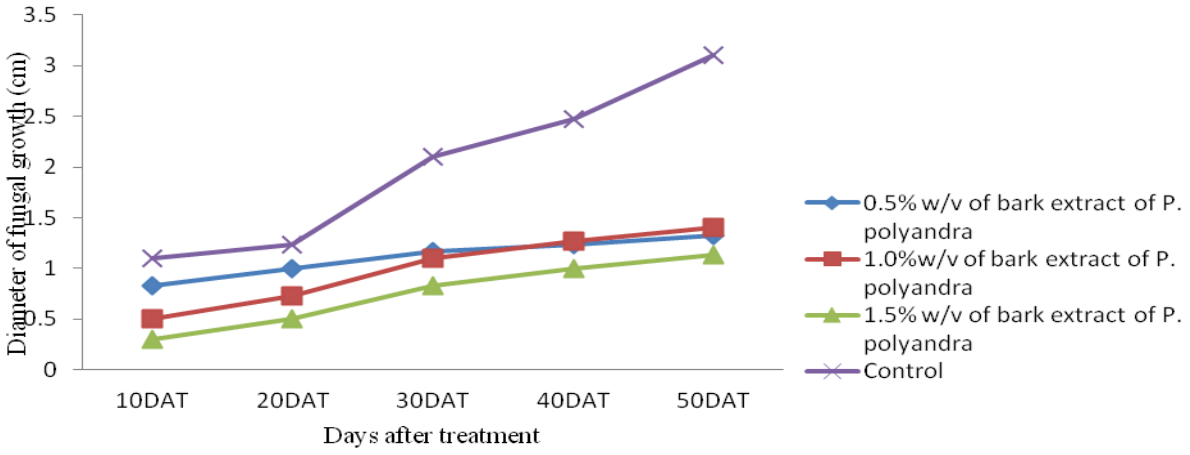

Figure 4: Effect of *Parinari polyandra* bark extract on inhibition of *Phomopsis* sp. in yam tuber

4.0 Discussion

Studies have been conducted on tuber rots of yam occurring either on the field or in the store [11] and many rot causing fungi have been identified [12, 11]. A few of the fungi isolated from the rots have been shown to be appreciably pathogenic [13, 14, 15]. *Phomopsis*, sp. is not notable among the fungi associated with yam rots both in Nigeria [16] and in Ghana [17]. The fungus is more popularly associated with rots in fruits and grains especially grain legumes like soybeans than in yams. The fungus may have become pathogenic in yams by developing the ability to use the nutrient in yams as substrate for its growth and development especially in the guinea savannah where post harvest rots of grain legumes and fruits caused by *Phomopsis* sp. are prevalent. Yam tubers at the time of harvest may already have been infected by pathogens derived from diseased foliage, roots or mother tubers.

Tubers which are already attacked by rotting pathogens when harvested get spoilt to greater extent in storage. It was observed that the soil adhering to the tubers contain many organisms, indicating that potential pathogens are ubiquitous on the surface of normal freshly harvested yam tuber [15].

This work also showed that fungitoxic compounds were present in *Mangifera indica* and *Parinari polyandra* since they were able to suppress the growth of the tested fungus. This agrees with earlier reports by some workers on effects of *Mangifera indica* on bacteria and fungi [18] and of *Parinari polyandra* on bacterial and viral pathogens [19].

5.0 Conclusion and Recommendation

Nigeria is a major producer of yam in the world accounting for about 65% to 71% of the World total production. The crop contributes significantly to the country's food security. Income generated from yam production helps improve the living standards of farmers. Post-harvest storage of yam tubers is therefore an important component of its production to ensure continuous availability of the tubers in the market.

Fungi are the principal organisms of yam tuber rots. Results from this study indicated that the bark extracts of *Mangifera indica* L. and *Parinari polyandra* Benth can be used in controlling yam tuber rot if applied as determined in this study. Surface coating of white yam tubers with this plant extracts prior to storage in pits or barns would reduce the incidence of rot diseases and increase shelf life of yam tubers. Additionally, seed yams for next cropping season, which are expensive, can be protected against rots with the use of these plant extracts.

The extracts showed anti-fungal activities against *Phomopsis* sp. which caused yam tuber rot and can be applied at 0.5%w/v, 1.0%w/v or 1.5%w/v as determined in this study. These extracts have proven to be effective against the rot fungus both *in vitro* and *in vivo*. The extracts are therefore recommended for use as treatment for yam in storage. The concentration with the best performance in this study (1.5%w/v) is also recommended for use.

6.0 References

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