THE EFFICACY OF *Alternanthera brasiliana* LEAF EXTRACT IN CONTROLLING FUNGAL PATHOGEN ASSOCIATED WITH BAMBARA NUT (*Vigna subterranean* (L.) Verdz) IN STORAGE

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M.N. Suleiman[1]* and E.O. Anyika[2]

[1]Department of Biological Sciences, Federal University, Lokoja, Kogi State, Nigeria
[2]Department of Biological Sciences, Kogi State University, Anyigba, Nigeria
*Corresponding author: nasirums69@gmail.com

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Abstract: Using direct plating method, seeds of four (4) bambara nut (*Vigna subterranea)* cultivars were tested for seed borne mycoflora in the laboratory. Phytopathogenetic fungi viz: *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus clavatus* and *Alternaria alternata* were isolated from these samples. The percentage of fungal incidence on seeds of four cultivars ranged from 40% to 75% with cream white having recorded the highest percentage incidence, while red nut had the least. The different percentages of fungal infection on seeds by the isolated fungi were equally determined. It ranged from 0.00 – 9.25%. The fungitoxic effects of the aqueous leaf extracts of *Alternanthera brasiliana* to control the radial mycelia growth of the isolated fungi was evaluated at four concentrations (10%, 20%, 30% and 40%). Results show that the aqueous leaf extracts of the plant had general retardation on the vegetative growth of the isolates; with the higher retardation at 40% concentration. The highest inhibitory effects of the extract observed on *A. alternata* at 40% (0.40 ± 0.44).

Keywords: Mycoflora, *Vigna subterranea*, leaf extracts, inhibition, *Alternanthera brasiliana*

Introduction

Bambara nut (*Vigna subterranea* (L.) Verdc) is a leguminous crop in the family Fabaceae (Bamishaiye *et al.*, 2011). The crop has its origin in Africa and is widely distributed throughout south of the Saharan zones (Hillocks *et al.*, 2011). The plant, much like cowpea (*Vigna unguiculata*), and ground nut (*Arachis hypogea*), is grown for its underground seeds and ripen its pods underground (Oyeleke *et al*., 2012). Bambara nut is an annual crop which produces three leaflets that are low, flat, and compound. It has an erect body form like cowpea and groundnut. The seeds are enclosed in the pods which are formed on or just below ground level. The pods are round, wrinkled and many contain between one to three seeds. The seeds are smooth and very hard when dried. The seeds are of different types basically in colour; it may be black eyed, brown, cream, red and mettle. Being a widely distributed crop, it is now ranking high among other legumes. The crop has many common names like "cokon", also known as "Jugo" beans or in Swahili, "njugumawe" (Hillocks *et al*., 2011). In the Republic of Zambia, it is called "ntoyo". In Hausa language in Nigeria, is variously referred to as gurjiya" or "kwaruru". It is called "Kwam" by Goemi people of Plateau state while the Kanuries call it "ngamala". It is popularly called "Okpa" by Igbo and Igala people of Kogi State. The crop is very essential in food security. It contains almost all the necessary dietary elements and very palatable (Mkandawire, 2007).

Bambara nut has its domains in tropical Africa but grown world wild in Australia, South and Central America. Most of the world's production comes from West Africa (Yao *et al.*, 2005), where it plays a key role in the traditional food and culture of the people. It is regarded as the third most important food legume after cowpea (*Vigna unguiculata*) and groundnut (*Arachis hypogea*) (Mkandawire, 2007). Bambara nut among its contemporaries has a high nutritive value and aroma which is reflected in them and makes them pleasant to eat. The composition of the seeds from the point of view for human nutrition is very well balanced, as they contain 65% soluble carbohydrate, a relative high protein content of about 18% and 8% of fats (Ncube and Twomlow, 2007). The carbohydrate fraction of Bambara nut is predominantly composed of starch and non-starch polysaccharides with lesser amount of reducing and non-reducing sugar (Bamishaiye *et al*., 2011). It also contains the vitamins, thiamine, riboflovin, niacin and carotene, but very low inascorbic acid (Abdulsalami and Sheriff, 2010). It is high in protein but very little in oil content unlike groundnut (Tweneboah, 2000). At maturity seeds of Bambaranut are too hard to be eaten raw they could be eaten when boiled with its pods or roasted when removed from the pods (Alopo, 1999). It can also be milled into flour which can be used to make different dishes like Okpa, which is made into paste and wrapped in banana leaves, boiled in most part of the eastern Nigeria.

The crop is diseased and pest free in its production stage, however, they are susceptible to various fungal diseases in damp condition mostly in storage (Bamdoin and Mergeai, 2001). The tough shell of the seeds is said to protect it against infestation (Hillocks *et al.*, 2011). This view is not without limitation as Ayaundoo *et al.* (2013), said that bambara nut suffers deterioration both in the field and in storage. Fungi diseases are serious cause of deterioration, especially in humid condition. Among the fungal infestation associated with bambara nut are cercospora leaf and pod spot (*Cercospora* sp); Powdery mildew (*Erysiphepolygoni*) and Fusarium wilt (*Fusariumoxysporum*) (Ayandoo *et al*., 2013). Other common storage fungi are *Aspergillus*, *Penicillium*, *Mucor*and *Rhizopus* (Ayandoo *et al*., 2003). *Alternanthera brasiliana* (L) Kuntze is of Amaranthaceae family. Several species are grown for their ornamental leaves which are variegated green and yellow, or bronze and green, or red and pinkish brown (Saawan *et al*., 2011). The plant is a Brazilian plant occurring in several regions, being known as "Penicilina" or "terramicina" widely used by rural communities as medicinal agent to cure different diseases. The secondary metabolites such as riboflavin, flavonoids, niacin, sterols contained in the plant accords them the antimicrobial activities against some microorganisms (Coelho *et al*., 2004).

Materials and Methods

Seed samples

This study was carried out on seeds of four (4) cultivars of Bambara nut in Nsukka zone of Enugu state. The diseased ones of the four varieties namely; mettle, red, cream white and black were collected from Nsukka, Orba, Obollo – Affor, Eke – Ozi, Adani, Ibagwa – Aka and Umulokpa in Enugu state. The apparently diseased ones were taken to the laboratory for further studies.

Testing procedure

Seeds of all varieties were analyzed for their association of seed – borne mycoflora by standard blotter paper method, as modified by Suleiman *et al.* (2013). Samples of 100 infected seeds were taken at random from each variety, plated on 9 cm diameter sterilized petri-dishes. In each of the dish 10 seeds were placed on three – layered blotter paper soaked with sterilized distilled water. The seeds were disinfected with 0.1% Hypochlorite ($HgCl_2$) for 30 seconds and subjected to three washings with distilled sterilized water before plating. Serial dilution method was used as testing procedure. Ten (10) seeds of bambara nut were soaked in 10 ml of distilled water in a test tube. Serial dilution was carried out as described by Suleiman and Emua (2009) and potato dextrose agar plates inoculated with the serially diluted samples and incubated for seven days at 27± 2^oC and observed for fungal growth.The emerging fungi were sub-cultured on potato dextrose Agar medium (PDA) and examined under compound microscope for specific identification. The seeds were classified as infected and non-infected to determine the fungal incidence. The experiment was repeated once.

Leaves of *Alternanthera brasiliana* plant was used for the preparation of the plant extract. From the fresh samples, crude methanolic extraction was used (Epidi *et al.*, 2005) and a modified method of Suleiman (2012). The plant leaf sample was washed thoroughly in cold running tap water and allowed to dry drying for seven days. Exactly (500 g) sample was homogenized using warring blender, and placed in 1000 ml flask containing 50 ml methanol and thoroughly mixed together using glass rod and left for 24 h for proper extraction of the active ingredients as described by Wokocha and Okereke (2005). Hot organic solvent extraction was equally carried out by weighing the same quantity of samples (500 g), washed and soaked in 500 ml of methanol in a 1000 ml conical flask. The filtrate was concentrated using the vacuum evaporator so as to regenerate the methanol. It was filtered using Buckner funnel and dried solidified extract weighed and dissolved in 100 ml distilled water to give the final concentrations of 100% (stock), subsequent concentration of 10%, 20%, 30% and 40% were prepared by serial dilution method as described by Epidi and Alamene (2005), and a modified method of Suleiman (2012). Potato Dextrose Agar was prepared according to manufacturer's specifications and sterilized at 1.2 kg/cm^3 pressure for 15 min in the autoclave at 121^oC. Six millilitres (0.1%) streptomycin was added to the 1 litre of the sterile medium just before pouring into petri dishes, to prevent bacterial growth and kept for the *in vitro* assay.

In vitro assay of plant extracts

The bioassay of the plant extract was carried out by determining the effects of their concentration on radial growth inhibition. The PDA/crude extract medium was prepared by spreading 1 ml of the extract on the surface of the solidified PDA in the petri dishes as described by Suleiman *et al.* (2013). The control was PDA on which 1ml of sterile distilled water was spread on the surface. With the aid of sterile cork borer 5 mm diameter discs of seven – day old culture of the isolated fungi were cut, each placed at the centre of the petri dish containing the PDA/crude extract media at different concentrations stated earlier were inoculated. Similarly, plant extract – free PDA plates inoculated with mycelia discs served as controls. All dishes were incubated for seven days at 27 ± 2^oC. All the experiments were repeated three times with three replications each.

The whole set-up was arranged in a completely randomized design. The incubation was carried out at 27 ± 2^oC and terminated at 7 days when the control mycelia had reached the edge of the petri dish. The growth rate was measured along 80mm radii using the site of the inoculums as the centre, minus the inoculums. Two perpendicular lines intersecting at right angles were drawn at the bottom of each plate. Percentage inhibition of mycelia growth was calculated using the formula of Amadioha (2003) as follows:

$$\% \text{ inhibition} = \frac{dc - dt}{dc} \; x \frac{100}{1}$$

Where: dc = average diameter of fungal colony in control plated, while dt = average diameter of fungal colony in treated plates.

Data analysis

Data collected on each treatment were analyzed using Analysis of Variance (ANOVA), while means with significant differences were separated using Duncan Multiple Range Test (DMRT). All analyses were carried out using SPSS version 16 software package.

Results and Disscusion

From the survey in the seven locations, white cream variety was more susceptible to fungal attack, while red nut variety had the least. Fungal incidence ranged from 40.00 to 75.00% on varieties of bambara nut. Fungal incidence was more on cream white nut (75.00%); while black nut had 60.00%. More than 40% fungal incidence was found on mettle (49.00%) and red nuts (41.00%), respectively (Table 1). Four (4) fungal species were isolated and were found associated with bambara nut seeds samples. They include *Aspergillusniger*, *Aspergillusflavus*, *Aspergillusclavatus* and *Alternari aalternata*; with varied percentage infection (Table 2). These fungi were found almost in all the varieties. Cream white recorded the highest percentage of fungal infection; with *A. niger* (10.3%), *A. flavus* (2.50%), *A. clavatus* (7.50%) and *A. alternate* (15.10%). Red nut however, showed the least in term of fungal infection.

Table 1: Fungal incidence on four varieties of bambara nut seeds

Varieties	Normal seeds*	Infected seeds*	Fungal incidence (%)
Mettle nut	51	49	49.00
Cream white nut	25	75	75.00
Black nut	40	60	60.00
Red nut	59	41	41.00

***Out of 100 seeds each variety**.

Table 2: Percentage of fungal infection on four varieties of bambara nut

	Mettle	Cream white	Black nut	Red nut
Aspergillus niger	7.20	10.30	6.60	0.00
Aspergillus flavus	6.40	2.50	0.00	5.00
Aspergillus clavatus	5.10	7.50	2.20	0.00
Alternaria alternata	8.20	15.10	9.25	2.20

The results showed *A. niger* (0.00%), *A. flavus* (5.00%), *A. clavatus* (0.00%) and *A. alternata* (2.20%), respectively.

The results equally showed that Black nut and red nut were more resistant to fungal attack, most especially *A. flavus* and *A. niger*, having 0.00% infection respectively. In addition to that, fungal infections percentage was high in *Aspergillus niger* and *Alternaria alternata* on the infected nuts. It represents 7.2% mettle, 10.3% cream white, 6.60% black and 0.00% red on *Aspergillus niger*, while *Alternaria alternata* represents 8.20% mettle, 15.10% cream white, 9.25% black and 2.20% red *Aspergillus flavus* and the least fungal infections percentage with mettle (6.40%), cream white (2.50%), Black (0.00%) and red (5.00%).

Leaf aqueous extract of *Alternanthera brasiliana* used for the study only accorded retardation of mycelia growth of the fungi *in vitro*. There is a significant difference at different concentrations and the control at ≤0.05 level of significant difference with P value of 0.033.

On *Aspergillus niger*, leaf aqueous extracts of *Alternathera brasiliana* was found inhibitory to mycelia growth during the first two days of inoculation. On the fourth day, however, their effects had reduced especially at 10% concentration, there was a significant difference; P<0.05, there was however no significant difference between 10% and 20%. Similarly, the effect of the extract on *A. flavus* and *A. clavatus* showed retardations on mycelia growth with some levels of significant differences when compared. The crude leaf extract of *Alternanthera brasiliana* on *Aspergillus flavus* showed significant reduction in mycelia growth at different levels of concentration. High fungitoxicity*in vitro* was observed at 40% concentration. There was complete inhibition of mycelium at this concentration for the first three days of inoculation, but their inhibitory effects had worn off by the fourth day of inoculation. A significant difference was noticed in all the concentration compared with the control. Between 20% and 30% there was no significant difference. However, there was a significant difference at 40% concentration compared with control (P 0.04<0.05). A significant difference was also observed between 10% and 20% concentration, respectively. The crude leaf extract from *Alternanthera brasiliana was* effective in inhibiting the mycelia extension of *Aspergillus clavatus* at all concentration tested. Beside the inhibition of radial mycelia growth of the fungus, the extract also affected the growth habit. The inhibitory effects showed some level of significance at 0.05% at all levels of concentration compared with the control. The results between 10% and 20% showed no significant difference (P>0.05). A significant difference was observed between 30% and 40% and between 20% and 30%, respectively (Table 4). The inhibitory effects of the extract on the four fungi isolated showed that fungicidal effects was more noticed on *Alternaria alternata* compared to other fungi (Tables 3 and 4).

Table 3: Inhibition effect of aqueous leaf extracts of *Alternanthera brasiliana* on the isolated fungi (mm)

Conc of extract (%)	*A. alternata*	*A. niger*
10	1.50 ± 0.00^{a}	1.90 ± 0.00^{a}
20	1.30 ± 0.14^{a}	1.60 ± 0.21^{a}
30	0.70 ± 0.43^{b}	1.30 ± 0.36^{b}
40	0.44 ± 0.40^{b}	0.80 ± 0.37^{b}
Control	4.50 ± 0.94^{c}	4.50 ± 0.92^{c}

Mean ± Standard error; Mean followed by the same letters are not significantly difference

Table 4: Inhibition effect of aqueous leaf extracts of *Alternanthera brasiliana* on the isolated fungi

Conc of extract (%)	*A. flavus*	*A. clavatus*
10	1.80 ± 0.00^{a}	1.50 ± 0.00^{a}
20	1.60 ± 0.22^{b}	1.73 ± 0.12^{a}
30	1.60 ± 0.28^{b}	1.10 ± 0.28^{b}
40	0.90 ± 0.36^{c}	0.85 ± 0.43^{c}
Control	4.50 ± 0.72^{d}	4.50 ± 1.02^{d}

Mean ± Standard error; Mean followed by the same letters are not significantly difference

Hillocks *et al*. (2011) noted that the stored bambara nut seed is particularly prone to attack by diseases. Ayandoo *et al*. (2013) viewed also that loss of bambara nut as a result of the effects of pests and diseases at all level involving producers and sellers, i.e. both in the field and in storage. The present study confirms the reports. The study was also to ascertain if the problem is location specific (within the area) or species specific (within four types). Despites little knowledge of the production practices in the three localities sampled, storage for the immediate and longtime uses is very well practiced, hence the biodeterioration of the nuts with fungi. Bambara nut deterioration is and with time will be a major constraint in the marketing of the produce in the region if not checked as the sellers cannot do without storage. The experimental research work in the area confirmed the findings of Taylor (1997) who reported *Aspergillus* species as the common diseases of the nut at all levels. The present results obtained have revealed that *Alternaria alternata*, *Aspergillus niger*, *Aspergillus flavus* and *Aspergillus clavatus*are associated with bambara nut (*Vigna subterranean*) deterioration in storage. In addition to these, *Alternaria alternata* was observed and isolated from cream white variety. This started with a slow growth but after two days of shooting out, a bushy whitish growth which later turned dark black carpet and slimy developed. It is characterized by large conidiophores mostly simple, light brown to brown or dark, septate typically bearing a simple or branched chain of conidia. However, the findings of higher percentage of *Alternaria alternata* on the cream white type of the nut may have resulted from the area of cultivation or the soft nature of the type at its storage place (Quaya and Kanda, 2014).

The percentage of fungal incidence on seeds of four cultivars ranged from 40% to 75% with cream white having recorded the highest percentage incidence, while red nut had the least. Percentage fungal incidence was 49.00% and 60.00% in mettle nut and black nut, respectively. The different percentages of fungal infection on seeds by the isolated fungi were equally determined. It ranged from 0.00% to 9.25% (Table 2). *Alternaria alternata* was found on all varieties. However, the three species of *Aspergillus* showed comparatively high fungal infection percentage in seeds of mettle and cream white. The differences in isolation frequency and percentage incidence may be due to variation in the moisture content of the nuts, varietal differences, seed susceptibility to infection and environmental conditions of the seeds during storage.

Many synthetic fungicides had shown promise in the control of fungal diseases both *in vitro* and *in vivo*, but the high cost of some of these chemicals had led to the search for an alternative, cheap and eco-friendly (biodegradable) plant extracts. The present study showed that the plant extract evaluated significantly reduced or inhibited the mycelia growth of the fungi *in vitro*, which could lead to reduction in the incidence of the disease in storage. Leaf aqueous extract of *Alternanthera brasiliana* used for the study only accorded retardation of mycelia growth of the

fungi *in vitro*. There is a significant difference at different concentrations and the control at ≤0.05 level of significant difference with P value of 0.033. Suleiman (2010), while working on the fungitoxic activity of Neem and pawpaw leaves extracts on *Alternaria solani*, reported the water – soluble antifungal principles in some plants as being responsible for the anti – fungal activities. The present result on *Alternaria alternata* showed that there is significant difference at different concentrations and the control at ≤0.05 level of significant difference with P value of 0.033. The mean percentage inhibition of mycelia growth in *Alternaria alternata*in plates containing extract was 90.70% at 40% concentration. The extract was inhibitory to mycelia growth of *Alternaria* during the first day and partially the second day of inoculation. However, their effects reduced, especially at 10% and 20% concentration on the third and fourth day of inoculation. There was however a significant difference (P<0.05) between 40% and other concentrations, suggesting high toxicity of the extract against the mycelia growth of the fungus. Treatments containing leaf extract of *Alternanthera brasiliana* showed mean percentage inhibition of 81.40%, 79.07% and 80.23% at 40% concentrations on*Aspergillusniger*, *Aspergillus flavus* and *Aspergillus clavatus*, respectively at the end of incubation period. In all of them, appreciable growth could only be observed on the third day of inoculation most especially on the 10% and 20% concentration where the effect of the extract had reduced. High fungitoxicity*in vitro* with a level of significance was observed (P< 0.05) on *Aspergillusniger*. Similarly, a significance levels of (P<0.05) was observed on *Aspergillus flavus* while the results between 10% and 20% contractions on *Aspergillus clavatus* showed no significant difference (P>0.05).

Conclusion

This study has revealed the potential of botanicals in the control of seed – borne mycoflora of bambara nut in storage caused by *Alternaria* and *Aspergillus*. This will go a long way in providing better alternative to over dependency on synthetic fungicides. The use of plant products, such as *Alternanthera brasiliana* in fungal disease control could reduce over reliance on chemicals, as well as cut down production cost. The facts that the leaf extract applied and used in this study are easily available, with easy method of extraction; can be exploited in the control of seed – borne mycofloral of bambara nut.

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