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Postharvest Management of Fungal Rot Agents of Groundnut (*Arachis hypogea* L.) Using Leaf Extracts of Neem (*Azadirachta indica*)

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

This study was carried out to manage groundnut seed rot in Mubi, Adamawa State Nigeria. Groundnut Rot was reported in all the three location surveyed and the average rot incidences in the market was 21%. Mubi New Market showed 25% as the highest percentage, followed by Mubi Old Market with 20% and MubiKuturu Market with 18%. Four organisms were isolated from the lesions on Potato Dextrose Agar (PDA) as follows; *Aspergillus niger*, *Fusariumsolani*, *Rhizopus stolonifer* and *Aspergillus flavus*. The pathogen most frequently occurring was *Rhizopus stolonifer* (67.22%),

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followed by *Aspergillus niger* (15.56%), *Aspergillus flavus* (15%) and the least was *Fusarium solani* (2.22%). Pathogenicity test confirmed that all four fungal isolates from groundnut seed were pathogenic and the level of severity was assessed with *Aspergillus flavus* being most severe with 83.3%, followed by *Aspergillus niger* with 50%, *Rhizopus stolonifer* 50% and *Fusarium solani* 33.33%. Plant extracts employed to control the vegetative mycelial growth was from aqueous and ethanolic leaf extracts of neem at 20%, 40% and 60% concentration. In vitro application of extracts for the control showed that neem aqueous controlled the mycelial growth better as compared with neem ethanol extracts. There was complete inhibition at 60% on *Rhizopus stolonifer* and *Fusarium solani* while neem ethanol on *Aspergillus flavus* and *Aspergillus niger* showed progressive inhibition on mycelial growth. There was a significant difference on vegetative growth of the fungi on both neem extracts with increase in concentration at p= 0.0001 as compared with the control. It is recommended that the use of antimicrobials commonly found in populated areas of rural areas can be used to reduce chemical costs and smallholder farmers' over-reliance on agricultural chemicals reduce.

Keywords: Aqueous; extracts; fungi; pathogenicity; mycelial growth; isolation.

1. INTRODUCTION

Groundnut (*Arachis hypogea L*.) is an annual, self-pollinated, moist season developing plant found in lots of tropical, subtropical and temperate nations of the world. It is now grown in approximately 108 nations of the world [1]. Asia with 63–65% land mass produces 71.72% of global groundnut observed via way of means of Africa with 18.6% manufacturing and North-Central America with 7.5% [2]. Groundnuts are considered a valuable legume grown in Pakistan on an area of 994 hectares with a production of about 1019 kg per hectare or 101 tons between 2001 and 2002 [3]. Groundnut seeds contain 50% oil [4]. The seeds are rich in fat, protein, vitamin B1, B2, B6, nicotinic acid and other vitamins [5]. Recently, groundnut consumption has been linked to metabolic disorders leading to obesity and metabolic syndrome [6]. It is an important seed with great global economic importance [6]. It is found in a wide variety of edible products, its shells are used in the manufacture of plastic, drywall, abrasives and fuel [7]. Stored foods are severely affected by various groups of fungi damaged, including *Aspergillus spp., Fusarium spp. and Penecillium spp.* [4]. Aspergillus is a common mold in tropical and subtropical countries and causes aflatoxin contamination as a result of molds in poorly stored staple foods such as peanuts, grains and cottonseed [4]. Fungi such as *Aspergillus niger, Aspergillus flavus, Alterneria dianthocola, Curvularia lunata, Curvularia pellesecens, Fusarium oxysporum, Fusarium quiseti, Microphomina phasiolina, Rhizopus stolonifer, Penecillium digitatum and Penecillium chrysogenum* caused discoloration, rot, shriveling, seed necrosis, loss of germination and toxicity to oilseeds [eleven]. These fungi are associated with large losses of seeds, fruits, grains, vegetables, and other plant products during harvest, transportation, and storage, rendering them unfit for human consumption, even through the production of mycotoxins and compromising their overall nutritional value [4]. Tropical climates with high temperature and relative humidity combined with unscientific storage conditions affect the shelf life of grains and oilseeds and lead to a complete loss of seed quality [8]

2. MATERIALS AND METHODS

2.1 Study Area

Isolation and control of pathogens was carried out in the laboratory and botanical garden of the Department of Plant Sciences from May to July 2015 at Modibbo Adama Yola Technological University. According to [9], the state of Adamawa is located in the north-eastern part of Nigeria and lies between the 70^{th} and 110^{th} degrees of latitude N of the equator and between the 100^{th} and 140^{th} degrees of longitude E of the Greenwich meridian. It is bordered to the south and west by the state of Taraba and by Gombe in its Northern Guinea Savanna ecozone.

2.2 Incidence of Rot of Groundnut Seeds in Storage

The incidence and degree of spoilage of stored groundnut seeds were determined. Samples purchased from markets were taken by subtracting the number of spoiled groundnut seeds from the total number of groundnut seeds

purchased at the market. The incidence of groundnut blight was expressed as a percentage using the formula from [10,11].

$$\frac{\text{Spoilage groundnut seeds}}{\text{total groundnut seeds}} \text{ X } 100\%$$

2.3 Isolation of Pathogens

The method of [12] was used. Diseased tissue (DT) from the periphery of rotten peanut seeds was cut into 5 mm 2 pieces using a sterilized scalpel after the seeds were sterilized in 0.1% mercuric chloride solution for 30 seconds and washed three times with sterile distilled water. The pieces were collected with sterilized forceps.

The sterilized portions were dabbed dry between sterile filter papers. Using cold forceps, a sterilized piece of seed was plated onto sterile solidified Potato Dextrose Agar (PDA) and incubated at a temperature of 27 ± 2^0C for 5 – 7 days and constant observation for any growth for sub-culturing.

Pure isolates of fungal species turned into received by repeated sub-culturing on solidified sterile Dextrose Agar and natural cultures had been preserved in agar slants in McCartney bottles. This turned into appropriately labelled in keeping with organisms. The slants turned into to begin with corked loosely to allow the content material fungus to develop after which tightly corked and saved at a temperature variety of 0 – 100C in a fridge to function as stock cultures.

2.4 Identification of Isolated Fungi

The colony characteristics such as colony colour (front and reverse) and growth pattern on media was examined using Microscope. A little portion of the hyphae containing spores was taken using a sterile needle on to the glass slide which was stained with Lectophenol cotton blue and observed under the light microscope with power objective lens X 40 for the structures of the fungi [13]. Morphological structures such as septation of mycelia and nature of spores was observed under the microscope and compared with the structures in [14].

2.5 Collection and Preparation of Leaf Extracts

The method of [15,16] was used to prepare both the aqueous and ethanol extracts. Fresh leaves of *Azadirachtaindica* plants were collected from Sangere village, Girei Local Government, Adamawa State. The plant was taken to the Plant Sciences Department of Modibbo Adama University of Technology, Yola.

Collected plant was washed thoroughly and allowed to air dry under shade for 7 days and grinded. Thirty grams of the sample was added to 150ml of distilled water in conical flask shaken vigorously and left to stand for 24 hours. The sample was filtered with three layers cheese cloth. The aqueous filtrate was used at 60, 40, and 20 per concentrations. The same procedure was used for 60, 40 and 20 per ethanol extracts.

2.6 Effect of Extracts on Fungal Mycelia Growth

The effect of the extracts on fungal growth was evaluated using the approach of [15] by creating four equal sections on each plate by drawing two perpendicular lines at the bottom of the plates. The point of interception shows the centre of the plates. This was done before dispensing PDA into each of the plates. The extracts were poured into the flask plug with cotton wool and kept at room temperature [16].

Poisoned food method was used, about 2ml of extracts of *Azadirachtindica* was separately introduced into the Petri-dish containing the media and pure isolates. Control experiment was without addition of any plant extract but sterile distilled water. Fungi growth inhibition was determined in terms of percentage spore germination [17].

$$\text{Inhibition percentage (\%)} = \frac{\text{DC}-\text{DT}}{\text{DT}} \text{ X } 100$$

Where; DC – Average diameter for fungi spore germination in control
DT- Average diameter of fungal spore germination with treatment.

2.7 Statistical Analysis

All the data was analysed using one-way and two-way analysis of variance (ANOVA) according to [18]. Least Significant Difference (LSD) according to [19] was used to separate the means where there was significant difference. The statistical package used to analyse the result was Statistical Analysis Software (SAS) version 7.

3. RESULTS

3.1 Incidence of Ground nut Rot and Identification of Rot Pathogens in Mubi

Rot of groundnut seed was observed in all locations. The incidence of rot showed that Mubi New Market with the highest percentage of infected samples (25%), followed by Mubi Old Market with 20% and MubiKuturu Market with the lowest (18%), and the average rot was 21% (Table 1).

Four fungi were isolated from groundnut seed rot and identified as *Aspergillus niger, Fusarium solani, Rhizopus stolonifer and Aspergillus flavus*. Pathogenicity test showed that the four isolates were pathogenic to groundnut seeds.

Table 1. Incidence of Rot of Groundnut in Mubi Markets

Source of Samples	Incidence of Rot (%)
Mubi New Market	25
Mubi Old Market	20
MubiKuturu Market	18
Mean	21

3.2 Incidences of Groundnut rot Disease in Mubi

The frequency of fungi isolated from Mubi New Market showed *Rhizopus stolonifera* had the highest frequency of occurrence (60%), followed by *Aspergillus niger* with 26.67% while *Fusarium solani and Aspergillus flavus* had the least frequency of 6.67% each. The highest frequency of occurrence in Mubi Old Market was *Rhizopus stolonifer* with 75%, followed by *Aspergillus flavus* (25.00%) and there was no occurrence of *Aspergillus niger* and *Fusarium solani.* In MubiKuturu Market, *Rhizopus stolonifer* had the highest frequency of occurrence (66.67%), followed by *Aspergillus niger* with 20% and there was no occurrence of *Fusarium solani.* The overall total showed *Rhizopus stolonifer* with the highest frequency of occurrence (66.67%), followed by *Aspergillus niger* with 16.67%, *Aspergillus flavus* had 14.29% and the least was *Fusarium solani* with 2.38% (Table 2).

3.3 Virulence of the Pathogens

Pathogenicity test was carried out the four fungal isolates from groundnut were confirmed to be pathogenic (Table 3). They produced rot lesions, and upon re-isolation they were identified to be same fungi initially isolated, thus fulfilling Koch's postulates. The weight of ground seeds before inoculation was 68.8g and the weight of seed after inoculation was 35.00g. The severity of fungi isolates from groundnut seeds showed that *Aspergillus flavus* was the most virulent group, *Aspergillus niger* and *Rhizopus stolonifer* were classified as highly virulent group while *Fusarium solani* was classified as moderately virulent group respectively (Table 3).

3.4 Effect of Plant Extract on Fungi Mycelial Growth

Analysis of variance was carried Statistical Analysis Software (SAS) version 7 for the *in vitro* control of the pathogens using aqueous and ethanol leaf extracts of neem shows that there was a significant difference between mycelial growth of the treatments (extract had lower mycelial growth compared to control at p=0.0001).

Table 2. Frequency of Fungi Isolated from Groundnut Seed in Mubi

Pathogen	Frequency of Pathogens (%)			Average
	MNM	MOM	MKM	
Aspergillus niger	26.67	--	20.00	15.56
Fusarium solani	6.67	--	--	2.22
Rhizopus stolonifer	60.00	75.00	66.67	67.22
Aspergillus flavus	6.67	25.00	13.33	15.00
Total	100	100	100	100

Key: MNM= Mubi New Market; MOM= Mubi Old Market; MKM= Mubi Kuturu Market

Table 3. Virulence of Fungi Isolated from Groundnut Seed Using Modified Ratanacherdchail *et al.* (2010)

Visual Scale	*Aspergillus niger*	*Fusarium solani*	*Rhizopus stolonifer*	*Aspergillus flavus*
Low virulent group	-	3	2	-
Moderately virulent group	-	4	-	-
High virulent group	4	4	3	-
Very high virulent group	3	-	5	5
Totally virulent group	-	2	3	1

Key: 1-20% - Low virulent group; 21-40% - Moderately virulent group; 41-60% - High virulent group; 61-80% - Very high virulent group; More than 80% - Totally virulent group

Table 4. Mean Effect of Concentration of Aqueous Leaf Extracts on the Mycelial Growth of Pathogens

Concentration (%)	Mean effect (mm)			
	Aspergillus niger	*Fusarium solani*	*Rhizopus stolonifer*	*Aspergillus flavus*
20	3.60	1.88	2.81	1.99
40	3.13	1.79	1.29	1.45
60	1.06	0.93	0.91	1.13
Control	72.14	16.21	72.43	18.40
LSD (p=0.0001)	2.39	0.60	2.36	0.46

The concentration effect of the aqueous extract on mycelial growth of fungal isolates revealed significant differences at p=0.0001.The level of growth inhibition increased as concentration of the aqueous extracts increases. There was a minimum level of inhibition on *Aspergillus niger* at 20% (3.60mm) while, at 60% showed maximum level of inhibition (1.06mm) as compared with control (72.14). Treatment of *Rhizopus* at 20ml had the mean of 2.81mm while at 60% had 0.91mm as compared with control (72.43mm). On *Fusarium solani*, at 20% had the mean of 1.88mm, while at 60% had 0.93mm as compared to control (16.21mm). In the case of *Aspergillus flavus,* at 20% had mean of 1.99mm, while at 60% had 1.13mm as compared with control (18.40mm) (Table 4).

The mean effect of concentration of the ethanol extracts on mycelial growth of fungal isolates showed significant differences at p=0.0001. As the level of concentration with ethanol increases, the level of inhibition also increased. There was a minimum level of growth inhibition on *Aspergillus niger* at 20% (1.60mm) while, at 60% showed maximum level of inhibition (1.20mm) as compared with control (66.43mm). Treatment with *Rhizopus* at 20ml had the mean growth of 3.75mm while at 60% had 2.05mm as compared with control (72.43mm). On *Fusariumsolani*, at 20% had the mean growth of 3.41mm, while at 60% had 1.80mm as compared to control (16.43mm). In the case of *Aspergillus flavus*, at 20% had mean growth of 3.60mm, while at 60% had 0.85mm as compared with control (17.29mm) (Table 5). Control of fungal mycelial growth with leaf extracts of *Azadirachtaindica* supressed the growth of pathogens at different concentration. As the level of concentration with *Azadirachta*increases, then the level of growth inhibition also increases on *Aspergillus niger* and *Fusariumsolani*. There was a minimum level of growth inhibition on *Aspergillus nig*er at 20% (3.92mm) while at 60% showed maximum level of growth inhibition (1.55mm) as compared with control (69.29mm). Control of *Rhizopus* at 20% had the mean of 5.36mm while at 60% had 1.75mm as compared with control (72.43mm). On *Fusariumsolani* at 20% had the mean growth of 3.77mm, while at 60% had 2.51mm as compared to control (16.32mm) in the case of *Aspergillus flavus* at 20% had the mean growth of 3.96mm, while at 60% had 1.65mm as compared with control (17.84mm) (Table 6).

Table 5. Mean Effect of concentration of Ethanol Leaf Extracts on the Mycelial Growth of Pathogens

Concentration (%)	Mean effect (mm)			
	Aspergillus niger	***Fusarium solani***	***Rhizopus stolonifer***	***Aspergillus flavus***
20	1.60	3.41	3.75	3.60
40	1.37	2.33	3.35	1.05
60	1.20	1.80	2.05	0.83
Control	66.43	16.43	72.43	17.29
LSD (p=0.0001)	2.39	0.60	2.36	0.46

Table 6. Mean Effect of Concentration of *Azadirachtaindica* on the Mycelial Growth of the Fungal Isolates

Concentration (%)	Mean effect (mm)			
	Aspergillus niger	***Fusarium solani***	***Rhizopus stolonifer***	***Aspergillus flavus***
20	3.92	3.77	5.36	3.96
40	2.92	2.61	3.85	1.82
60	1.55	2.51	1.75	1.65
Control	69.29	16.32	72.43	17.84
LSD (p=0.0001)	2.39	0.60	2.36	0.46

Table 7. Mean Effect of *Azadirachtaindica* Leaf Extracts and Solvent (Aqueous and ethanol) on the Mycelial Growth of Fungal Isolates

Pathogens	Mycelial growth (mm)	
	Aqueous	**Ethanol**
Aspergillus niger	3.05	2.54
Fusariumsolani	1.42	4.25
Rhizopusstolonifer	2.07	5.23
Aspergillus flavus	1.64	3.32
Control	51.60	51.60
LSD (p=0.0001)	1.45	1.45

The interaction of fungi mycelial growth with leaf extracts and solvents (aqueous and ethanol) minimized the growth of pathogens. The mean effect of neem with ethanol on *aspergillus niger* is 2.54mm and with aqueous is 3.05mm, for *Aspergillus flavus* neeem with ethanol is 3.32mm and with aqueous is 1.64mm compared with control (51.60mm) (Table 7).

4. DISCUSSION

Postharvest rot of groundnut is caused by fungal pathogens. The incidence of rot showed Mubi New Market with the highest percentage of infected samples (25 %), followed by Mubi Old Market with 20 % and MubiKuturu Market with the lowest (18 %), and the total percentage of infected sample is 21 % (Table 1). [20] reported that groundnuts stored in different storage facilities are susceptible to fungi, insects and other microorganisms under favorable conditions. The difference in seed storage potential at different locations may be due to the difference in initial seed quality under different environmental conditions and fungal invasion [21].

Four fungi were isolated from groundnut and identified as *Aspergillus niger*, *Fusarium solani*, *Rhizopus stolonifer* and *Aspergillus flavus* and pathogenicity test showed that the four isolates were responsible for the rot and pathogenic to the groundnut. This result is in accordance with the result obtained by [22] who found 28 species of fungi to be associated with groundnut rot in India. [23] Isolated nine species of fungi from seeds of different groundnut during storage. [24] revealed that the species of *Aspergillus, Penicillium, Fusarium, Rhizopus* and *Alternaria* were commonly occurring postharvest moulds in

storage condition. According to [25], groundnut seeds are highly susceptible to disease because of the presences abundant nutrients useful for numerous fungi such as *Rhizopus* spp, *Penicillium* spp, *Aspergillus niger* and *Aspergillus flavus*.

The results of this investigation indicatess that the radial growth of fungi was inhibited *in vitro* by aqueous and ethanolic leaf extracts of *Azadirachtaindica,* indicating the presence of antifungal substances which include Phenols, Alkaloids, Saponins, Flavonoids and Glycoside in the plant tissue, which is in accordance with the results reported by other workers on different pathogens and plants [26,11,27,28,25,29]. The aqueous extract from leaves was more effective than the ethanolic extracts from neem. The extent of inhibition was determined by the concentration level. The degree of inhibition also increased with increasing concentration. The differences in the toxicity of the extracts could be attributed to the presence of the active substances extracted with different solvents, which can be influenced by the extraction method and the type of extraction solvent [30,31]. The greater effectiveness of aqueous as compared with ethanol extract of the neem leaf may be due to differences in constituent extraction [32]. It has been previously reported that the active ingredients of neem constitute mostly of triterpenoides, e.g Azadirachtin [33,34]

5. CONCLUSION

It may be concluded from this study that *Aspergillus niger*, *Fusarium solani*, *Rhizopus stolonifer* and *Aspergillus flavus* are common pathogenic fungi which cause rot of groundnut seeds in the study area. The sensitivity of groundnut to fungal spoilage is related to its poor conservation of places of production and storage is a big problem for national operators. This alteration is particularly important as the storage conditions and storage are inadequate.

Higher inhibition of fungal growth observed at higher concentrations of the ethanolic extracts was recorded. The result also indicates that ethanolic *Azadirachta indica* has more inhibitory compounds than aqueous. This show a clear indication of the potentials of plant extracts in control of fungal pathogens. It seems that the antifungal effects are as a result of many compounds acting synergistically. This can be formulated and successfully used to produce fungicides with local technology, which can be applied at both pre and post-harvest seed management.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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