Research Article ISSN 2770-2928

International Journal of Agriculture and Technology

Mycobiota Incidence of Cowpea (*vigna unguiculata* l. Walp) Seeds in Nigeria

Akintunde FC[1]*, Ugoh Sylvanus Chukwudi[2], Anjorin TS[3] and Olasunkanmi TC[4]

[1]*Department of Biological Sciences, Tennessee State Univerity, 3500 John A Merritt Blvd Nashville, TN 37209.*

[2]*Department of Microbiology, Faculty of Sciences, University of Abuja.*

[3]*Department of Crop Protection, Faculty of Agriculture, University of Abuja.*

[4]*National Agricultural Seed Council, Km 29, Abuja-Lokoja Express Way, Sheda Abuja.*

***Correspondence:**
Akintunde FC, Department of Biological Sciences, Tennessee State Univerity, 3500 John A Merritt Blvd Nashville, TN 37209.

Received: 17 Jun 2023; **Accepted:** 24 Jul 2023; **Published:** 01 Aug 2023

Citation: Akintunde FC, Chukwudi US, Anjorin TS, et al. Mycobiota Incidence of Cowpea (*vigna unguiculata* l. Walp) Seeds in Nigeria. Int J Agriculture Technology. 2023; 3(2): 1-5.

ABSTRACT

The study was conducted to identify fungal pathogens associated with Cowpea (Vigna unguiculata [L.] Walp) seeds obtained from the South Western States of Nigeria. Three hundred seed samples of cowpea were collected from twenty (20) local governments across five States in the South West of Nigeria. The seeds were surface sterilized using 10% sodium hypochlorite, plated on Potato Dextrose Agar (PDA), and incubated at 25°C. The colonial, morphological, and molecular characteristics were used for the identification of the pathogens. For molecular identification, ITS Universal Fungi primer was used for the PCR analysis to amplify the extracted DNA. The results showed that A. niger, A. flavus, and B. theobromae were the most common with values of 7.77, 7.6,1, and 6.59% respectively. Ondo State had the highest level of disease incidence of 76.61%. This call for an integrated approach toward mitigating seed-borne fungi during post-harvest storage.

Keywords

Cowpea, Fungi, Pathogen, Molecular identification, Vigna unguiculata.

Introduction

Cowpea (*Vigna unguiculata* [L.] Walp) is a food and animal feed crop grown in the semi-arid tropics covering Africa, Asia, Europe, the United States, and Central and South America [1,2]. It originated and was domesticated in Southern Africa and was later moved to East and West Africa and Asia [3]. The grains contain 25% protein, and several vitamins and minerals [4]. Cowpea is the most important source of plant protein consumed in Nigeria with major supplies coming from the northern part of the country. However, the reduction in supplies due to insurgency resulted in sharp increases in price, especially in Southwest Nigeria where cowpea is relished in different delicacies. Cowpea is one of the most economically important crops and a veritable source of plant protein considered crucial for the reduction of malnutrition among children and poor households [5].

The major economic diseases of cowpea in the humid agroecology of South-western Nigeria include brown blotch, anthracnose, Cercospora leaf spot, canephora pod rot, false smut, web blight, and sclerotium stem blight [3]. The diseases have been found to be seed borne. More so a fall in cowpea production in Nigeria will result in a serious food security crisis in the whole of West and Central Africa because Nigeria does not only harbor all the agro-ecological zones; it is also the largest producer and consumer of cowpea in the region [6].

Seed-borne fungi are the limiting factors among others in the production of cowpea. Seed-borne fungi invade cowpea grains

while still in the field or during storage causing seed rotting, mycotoxin contamination, and loss of seed viability. The seed infection leads to low germination of seeds and thus reduces yield loss both quantitatively and qualitatively [7]. This study set out to determine the incidence and identify the various fungal pathogens associated with cowpea seeds grown in the South-Western States of Nigeria.

Materials and Methods

Collection of Samples

Samples were taken from Five (5) South-Western states namely Osun, Oyo, Ogun, Ondo, and Ekiti. In each State, samples were taken from four local government areas, and three (3) farms were visited in each local government area. Five hundred grams (500g) of cowpea seed samples of three different varieties each were obtained from farmers' fields across the local governments of the five states. The choice and number of farms visited were based on the number of commercial farms that were available during the time of sample collection. A total of 300 samples were used for this study.

Preparation and sterilization of media

According to the method described by Al-Sadi et al. [8], Potato Dextrose Agar (PDA) SIGMA was prepared following the manufacturer's instructions. Forty grams (40g) of PDA was weighed and distilled water was added to make 1000 ml. The medium was then autoclaved at 121°C for 15 minutes, and allowed to cool to about 45°C before the addition of 0.1g streptomycin. This mixture was stirred by gently swirling the flask before pouring it into 9 mm diameter sterile Petri dishes and allowed to solidify before inoculation.

Isolation of fungi from seed samples

According to Omaima et al. [9], the seed samples were surface sterilized by dipping seeds in 10% aqueous sodium hypochlorite solution for 2 minutes, followed by three successive rinses in sterile distilled water. The seeds were blotted dry in between sterile Whatman No. 1 filter paper and plated on Potato Dextrose Agar at the rate of 10 seeds per plate. This was done in four (4) replicates. Plates were sealed with parafilm wax and incubated at a temperature of 25 ± 2°C for 5-7 days.

Molecular Identification

Fungal cultures were harvested by scraping mycelia from 10-day-old PDA plates into a sterile Eppendorf tube and kept at -80°C as described by Castro de Souza et al. [10].

DNA Extraction

According to Omaima et al. [9], the fungal mycelium and spores were grounded in a mortar and pestle with sand and then 600 µl of lysis buffer was added. The mixtures were then mixed and incubated at 65 °C for 1 hr. After 1hr, 600 µl of phenol: chloroform: isoamyl alcohol (25:24:1) was added, vortexed, and centrifuged for 15 min at 10,000 g. About 300 µl of supernatant was transferred to new Eppendorf tubes and 10 µl of sodium acetate and 180 µl of isopropanol were then added. The mixture was incubated at −20°C overnight. The mixtures were centrifuged for 2 minutes at 10,000 g and the supernatant was discarded. The pellet was washed using 600 µl of 70% ethanol, followed by centrifugation for 2 min at 10,000 g. Then, the pellets were dried for 10–15 min. Pellets were suspended in 100 µl of autoclaved sterile distilled water and DNA concentrations of samples were measured with a spectrophotometer and stored at −20°C until required for use.

DNA Quantification

According to Al-Sadi et al. [8], the concentration and purity of the extracted DNA was monitored using NanoDrop ND-1000 Spectrophotometer at a wavelength of 260 and 280 nm. The DNA was then stored at -20°C until used for DNA-PCR.

Polymerase Chain Reaction

According to the method adopted by Iyanyi and Ataga [11], the fungi were characterized by the amplification of their Internal Transcribed Spacers (ITS) the forward (ITS-1F) and reverse (ITS-4R) primers were used to amplify the ITS region of the rRNA operon. Each PCR mixture contained 10 µl of Red taq ready mix, 0.5µl of each primer pair, 8µl of analytical grade sterile water (Sigma-Aldrich) and 5µl of genomic DNA in a total volume of 24µl. The thermocycling program used was in initial denaturation (94°C for 5 minutes), 30 cycles of denaturation (94°C for 1 minute), annealing (60°C for 1 minute), and elongation (72°C for 1 minute), then the stabilization (72°C for 5 minutes). The genetic materials were electrophoresed on 2% agarose gel in Tris-acetate EDTA buffer, and the gel was stained with ethidium bromide before observation in a UV detector. Approximated molecular sizes of the amplicons were determined using a molecular weight marker 1kb Plus DNA ladder (Invitrogen Carlsbad, California USA).

Primers and PCR Chemicals for Analysis

The Internal Transcribed Spacer (ITS) gene for the characterization of fungi using the ITS universal primer set which flank the ITS1, 5.8S, and ITS4 region was used according to Trindade et al. [12];
ITS 1F: 5' TCC GTA GGT GAA CCT GCG G 3'
ITS 4R: 5' TCC TCC GCT TAT TGA TAT GC 3'
PCR conditions included a cycle of initial denaturation at 94°C for 5 min, followed by 35cycles of each cycle comprised of 30secs denaturation at 94°C, 30secs annealing of primer at 55°C, 1.5 min extension at 72°C and a final extension for 7min at 72°C.

Purification of Amplified PCR Product

According to Ongom et al. [13], the amplified fragments were purified using ethanol to remove the PCR reagents. 2.5 Volume (125 µl) of 95% ethanol was added to each about 50µl PCR amplified product in a new sterile 1.5 µl tube Eppendorf, mixed by vortexing or by simple inversion and kept at -20°C for at least 30 min/1hr. Centrifugation for 10 min at 12000g, followed by removal of supernatant after which the pellet was washed by adding 500 µl of 70% ethanol, mixed, and centrifuged for 5 min at 12000g. Again, all supernatant was removed and washed, and the procedure was repeated and stored in the incubator at 37°C for 25-30min until it dried. It was re-suspended with 30 µl of sterile distilled water and stored in a -20°C freezer prior to quantification

by Nanodrop and separation on agarose gel electrophoresis before being dispatched for sequencing. The purified fragment was checked on a 2% Agarose gel run on a voltage of 120V for about 45 minutes, to confirm the presence of the target gene.

Gene Sequencing

The amplified fragments were sequenced using a Genetic Analyzer 3130xl sequencer from Applied Biosystems using the manufacturers' manual while the sequencing kit used was that of BigDye Terminator v3.1 cycle sequencing kit. Bio-Edit software and MEGA 6 were used for all genetic analyses.

Aflatoxin detection using the HPLC method

The laboratory analysis was carried out at the Central Research and Diagnostic Laboratory -Ilorin, Kwara State. The analysis text that was carried out was the Determination of Aflatoxin B1 and B2 load in cowpea seeds using High-Performance Liquid Chromatography.

Using HPLC (high-performance liquid chromatography) the analysis was carried out for the quantification of the aflatoxin load present in all the samples using the method described by AOAC official method 990.33 was adopted for the analysis using HPLC. Water and methanol were used as reagents. UV detection was carried at an excitation wavelength of 365nm and maintained at an emission wavelength of 440nm. The HPLC technique makes use of a stationary phase confined to either a glass or a plastic tube and a mobile phase comprising aqueous/organic solvents, which flow through the solid adsorbent. When the sample to be analyzed was layered on top of the column, it flowed through and distributed between both the mobile and the stationary phases. This was achieved because the components in the samples to be separated had different affinities for the two phases and thus moved through the column at different rates.

Results

Incidence of fungi in the cowpea seeds

The mean values for the percentage incidence of disease recorded from seed samples collected used in the study across the states were 61.18%, with Ondo State having the highest disease incidence of 76.61% and Ekiti having the lowest value of 36.67% (Table 1).

Identification of Fungal Pathogen

In this study, twenty-four fungi pathogens were identified from the seed samples. These include: *Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger, Aspergillus temarii, Botryodiplodia theobromae, Cladosporium cladosporioides, Cladosporium cladosporioides, Corynespora cassiicola,, Curvularia lunata, Fusarium equiseti, Fusarium oxysporum, Fusarium solani, Fusarium verticilioides, Lasicodiplodia theobromae, Macrophomina phaseolina, Penicillium oxalicum, Penicillium chyrosogenum, Pseudomonas syringae pv phaseolicola, Rhizopus stolonifera, Rhizotonia solani, Rhizopus oligosporus, Septoria vignae, Xanthomonas vignicola, Xanthomonas axonopodis.* In general, *Aspergillus flavus, Aspergillus niger,* and *Botryodiplodia theobromae* were the most common.

The seeds collected from Ondo State had the highest fungal incidence of 76.61%, followed by Oyo State with 67.96%, Osun State with 67.62 seed infection, Ogun State with 57.06 and Ekiti State with 36.63%. For pathogen occurrence, out of 24 fungi pathogens intercepted, Ondo State had the highest number of fungal occurrence of 19 pathogens while Ekiti had the least number of 10 pathogens (Table 2).

Table 1: Prevalence of fungal infection on cowpea seed samples collected across five States in the southwestern region of Nigeria.

State frequency (24)	Mycobiota Incidence (%)
Ekiti 10	36.67 ± 0.03
Oyo 16	67.96 ± 1.01
Osun 19	67.62 ± 0.9
Ogun 16	57.06 ± 0.82
Ondo 19	76.61 ± 1.20
Total 80	305.92 ± 2.11
Mean 16	61.18 ± 1.11

Table 2: Incidence of the different pathogens associated with cowpea seed lots across the Southwestern region of Nigeria.

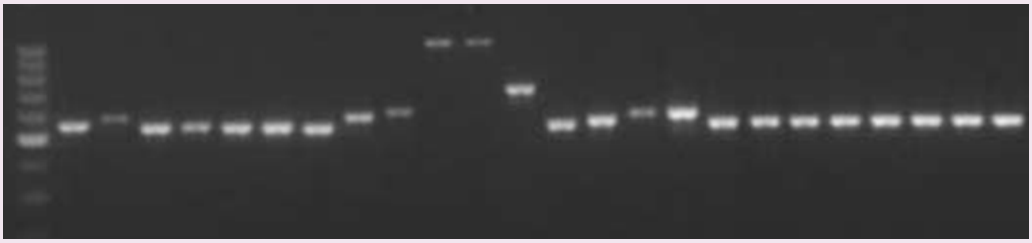
Mycobiota	Level of Incidence by State (%)				
	Ekiti	Oyo	Osun	Ogun	Ondo
Aspergillus flavus	9.72	6.12	5.41	9.13	7.66
Aspergillus fumigatus	0.00	3.40	6.09	2.85	3.06
Aspergillus niger	9.00	4.76	6.76	6.85	11.49
Aspergillus temarii	0.36	1.36	3.38	1.71	6.89
Botryodiplodia theobromae	3.60	8.16	6.09	5.14	9.96
Cladosporium cladosporioides	2.16	6.80	5.41	3.42	4.60
Colletotrichum gloeosporioides	0.00	1.36	3.38	0.57	1.53
Corynespora cassiicola	0.00	0.68	0.68	0.57	0.00
Curvularia lunata	1.08	1.36	0.68	0.00	4.60
Fusarium equiseti	0.00	0.00	0.00	0.00	0.77
Fusarium oxysporum	3.24	6.80	6.09	6.28	9.96
Fusarium solani	0.00	5.44	4.73	0.00	0.00
Fusarium verticilioides	1.80	2.72	2.70	1.71	8.43
Lasicodiplodia theobromae	0.00	0.00	0.00	1.14	0.00
Macrophomina phaseolina	0.00	0.00	1.35	0.00	1.53
Penicillium chyrosogenum	0.00	0.00	0.00	1.71	3.83
Penicillium oxalicum	2.88	5.44	4.73	4.56	6.13
Pseudomonas syringae pv phaseolicola	0.00	0.00	0.68	4.56	0.00
Rhizopus oligosporus	0.00	0.00	0.00	2.85	0.77
Rhizopus stolonifera	0.00	4.76	2.03	0.00	6.13
Rhizotonia solani	2.52	6.12	4.06	3.99	6.13
Septoria vignae	0.00	0.00	1.00	0.00	0.00
Average ± S.E	36.63	67.96	67.62	57.06	76.61
Total occurrence	10/24	16/24	19/24	16/24	19/24

Molecular Analysis

The SSR marker detected polymorphism for the fungi pathogens. This primer amplified 24 alleles with product sizes ranging from 345bp in *Fusarium oxysporum* to *Xanthomonas axonopodis* 740bp in (Plate 4.1). This reflects a considerable difference in the number of repeats between the different alleles.

The SSR primer established the DNA profile for fungi pathogens thus establishing the differences that exist among pathogens. It was apparent that allele bands for *Aspergillus, Fusarium, and*

Plate 4.1: DNA fingerprints of 24 fungi pathogens identified through PCR analysis.

	M	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
100bps																									

M =	100 bps	I=	*Pseudomonas syringae*	S=	*Fusarium equisetti*
A =	*Fusarium oxysporum*	*K=*	*Septoria vignae*	T=	*Aspergillus fumigatus*
B=	*Cladosporium cladosporiodes*	*L=*	*Botrydiplodia theobromae*	U=	*Aspergillus temari*
C=	*Rhizotonia solani*	*M=*	*Penicillium chyrosogenum*	V=	*Aspergilus flavus*
D=	*Colletotrichum gloeosporioides*	*N=*	*Macrophomina phaseolina*	W=	*Aspergilus niger*
E=	*Rhizopus stolonifera*	*O=*	*Corynespora casiicola*		
F=	*Curvularia lunata*	*P=*	*Lasidiploida theobromae*		
G=	*Rhizopus oligosporus*	Q=	*Fusarium verticiloides*		
H=	*Penicillium oxalicum*	R=	*Fusarium solani*		

Lasoidiplodia species were uniform in size while variabilities became more pronounced among the *Macrophomina, Corynespora,* and *Xanthomonas* species (Plate 4.1).

Discussion

Generally, the level of fungal contamination recorded in this study was high for the whole region, which corroborates the report of Olisa et al. [14], who reported that the warmer southwestern part of the country is always prone to seed pathogens infestation because of the prevailing environmental conditions. This region is associated with high relative humidity and high temperature. These environmental conditions are conducive for the growth of pathogens and disease development. The result obtained from Oyo state again signifies that the production environment is more prone to fungi disease development and distribution. Cowpea seeds produced in Ekiti state will have higher quality and lower risk of disease infestation when compared with other states particularly Ondo state, which had the highest level of fungal contamination.

Houssou [15] and Van den Berg [16] in West Africa and southwestern Asia respectively, studied cowpea genotypes and revealed that many cowpea seeds are prone to fungal infestation. Also, Khare [7] isolated eight fungal species belonging to five genera which include *Aspergillus, Penicillium, Fusarium, Rhizopus,* and *Cylindrocarpon* from seeds of three cultivars of cowpea. This implies that the pathogen could be varietal-specific. However, the present study established that twenty-four fungal species belonging to fourteen genera: *Aspergillus, Botryodiplodia, Cladosporium, Corynespora, Curvularia, Fusarium, Lasicodiplodia, Macrophomina, Penicillium, Pseudomonas, Rhizopus, Rhizoctonia, Septoria, Xanthomonas* were associated with cowpea seed lots in the southwestern region of Nigeria. Out of which, *Aspergillus flavus, Aspergillus niger,* and *Botryodiplodia theobromae* were dominant.

Aspergillus flavus, which is one of the dominant fungi isolated in this study, is a saprophytic soil fungus that infects and contaminates pre-harvest and postharvest seed crops with the production of carcinogenic secondary metabolite aflatoxin. The fungus is also an opportunistic animal and human pathogen causing aspergillosis with incidence increasing in the immune-compromised population. Few fungi have had as broad an economic impact as *A. flavus*. According to Maren [17], *A. flavus* is a pathogen of plants, animals, and insects and causes storage rots in numerous crops and it produces aflatoxin $B_{1.}$

Aspergillus niger which is a filamentous ascomycete has the ability of fast growth and pH tolerance is the most important cosmopolitan fungi associated with postharvest decay of different substrates. This organism is a soil saprobe with a wide array of hydrolytic and oxidative enzymes involved in the breakdown of plant lignocelluloses. Because of their ability to produce extracellular organic acids some of them are commonly used in the food industry. These features of *A. niger* enable them to cause the decay of various organic substances including fruits, vegetables, nuts, beans, cereals, herbs, wood, and herbal drugs. *A. niger* has been found to be an opportunistic pathogens of humans. If inhaled, in large quantities it can cause severe lung problems i.e., aspergillosis in humans. It is also associated with various plant diseases resulting in huge economic loss. Gautam et al. [18].

Conclusion

The level of fungi infection across the states in the South Western part of Nigeria is very high. This could result in high levels of aflatoxin in cowpea seeds across the region if not mitigated.

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