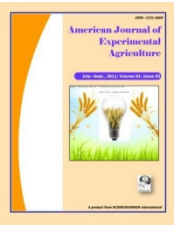

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Incidence of *Lasiodiplodia theobromae* and other Fungi in Kolanuts (*Cola nitida* and *Cola acuminata*) in Nigeria

Sunday O. Agbeniyi[1*]

[1]Crop Protection Division, Cocoa Research Institute of Nigeria, P.M.B. 5244, Ibadan, Nigeria.

Author's contribution

The sole author designed, analyzed and interprets and prepared the manuscript.

Original Research Article

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ABSTRACT

Aim: A study was conducted to determine the frequency of occurrence of fungi associated with kolanuts *(Cola nitida and Cola acuminata)* at processing and storage.
Methodology: Healthy and infected kolanuts (*Cola nitida* and *Cola. acuminata*) collected randomly during processing and storage from various locations in Nigeria were used in this study. Infected kolanut samples were plated on potato dextrose agar (PDA). The plates were incubated at 25^0C and the incidence of associated fungi was recorded after 5-10 days depending on when growth could be observed. The fungal colonies emerging from the tissue piece were hyphal-tip-transferred onto new PDA plates to obtain pure cultures.
Results: *Lasiodiplodia theobromae*, *Fusarium pallidoroseum*, *Fusarium moniliforme*, *Fusarium cacispermum*. *Aspergillus niger*, *Aspergillus fumigatus*, *Aspergillus flavus* and *Paecilomyces variotii* were obtained from the infected kolanuts. The means frequency of occurrence of *Lasiodiplodia theobromae* on *Cola nitida* and *Cola acuminata* ranged between 40-47% and 33.5-40% respectively. The mean frequency of occurrence of other isolated fungi ranged between 3-14% on both species of *Cola*. The pathogenesis tests established *Lasiodiplodia theobromae* as the causal pathogen of black rot disease of kolanuts. When kolanuts were artificially wounded before inoculation with *Lasiodiplodia theobromae,* 79.2% infection was recorded compared to 33.3% infection recorded in unwounded nuts. A highly significant positive correlation (r^2 = 0.8844) existed between wounding of nuts and incidence of black rot diseases.

**Corresponding author: Email: remiagbeniyi@yahoo.com;*

Conclusion: The present study established that kolanuts were susceptible to fungal infections. This study confirmed the occurrence of storage rot fungi on kolanuts in all the locations of sampling, which represent the rain forest and guinea savanna zones of Nigeria.

Keywords: Incidence; Lasiodiplodia theobroamae; Kola nuts; Nigeria.

1. INTRODUCTION

Cola popularly called *Kola nuts* is a genus of the family *Sterculiacea*e in the order *Malvales*. It is indigenous to tropical rain forest of West Africa, West Indies, Brazil and Java [1]. *Cola nitida* and *C. acuminata* are the most common Cola species and Cola of commercial value in Nigeria.

The effects of chewing the odourless nut with an astringent taste have enhanced its continued use for about 1000 years now in Nigeria. The habit if not comparable to the tobacco smoking of Western civilization or opium usage of Far Eastern societies certainly shows similarity to the Betel nut chewing of Asiatic communities [2].

Its role in Nigeria societies is not entirely socio-religious. The dried sliced nut is a commercial export commodity for kola-chocolate and the liquors and laxatives to which *kola nut* has become an important ingredient. *Kola nuts* contains alkaloids and such as caffeine, kolanin and thebromine, which are used for pharmaceutical purposes [3].

Storage rot caused by *Lasiodiplodia theobromae* is considered the most serious post-harvest disease of *Kola nut* [4,5]. These fungi can also initiate infections on nuts in the field when harvest is delayed and then cause rot during storage [3] infected nuts decay rapidly and the surrounding nuts are covered with masses of fungal spore. The contamination of healthy nuts with conidia from rotted nuts is termed spoilage and is often a greater economic problem than decayed nut in *Cola* producing areas [6], *Kola nut* traders often control spoilage by removing diseased nuts at intervals during the storage period. Thus, this study was designed to fill the gaps in our understanding of storage not development and spread with a view to evolving scientific control measures.

The specific objectives of this study were to isolate fungi associated with *Cola nitida* and *C. acuminata* during processing and storage and to establish the pathogenicity of the fungi isolated.

2. MATERIALS AND METHODS

Healthy and infected kolanuts (*Cola nitida* and *C. acuminata*) collected randomly during processing and storage from various locations in Nigeria were used in this study. The nuts were obtained from Ibadan (Oyo State), Ogunmakin, Sagamu, Ishara (Ogun State), Ile-Ife, Osogbo, Garage-Olode (Osun State). Okenne, Ayangba (Kogi State). Otupko (Benue State), Onitsha (Anambra State) Ondo, Owena (Ondo State), Ikom (Cross Rivers State) and Kola processing unit of the Cocoa Research Institute of Nigeria (CRIN), Ibadan.

Both skinned and unskinned nuts were collected randomly at the on-set processing and each type was kept in separate polyethylene bags appropriately labeled and stored under room temperature on laboratory bench.

The culture media used for the fungal isolations and counts were potato dextrose agar (PDA) (Difco, Detroit, USA) These media were sterilized by autoclaving for 15min at 1.05kg cm^{-2} (121ºC). The media were acidified by adding 70% lactic acid to suppress bacterial growth.

2.1 Isolation of Fungi Associated with Nuts, Testa and Pod

Infected Kola samples (nut, testa and pod) were cut to pieces of 4 mm diameter and surface sterilized by immersion in 10% sodium hypochlorite for 60 sec followed by two rinses in sterile distilled water and blotted dry with sterile Whatman filter papers (Grade 41, ashless 150mm, Kent, England), the samples were then plated on potato dextrose agar (PDA).

The plates were incubated at 25ºC and the incidence of associated fungi was recorded after 5-10days depending on when growth could be observed. The fungal colonies emerging from the tissue piece were hyphal-tip-transferred onto new PDA plates to obtain pure cultures.

2.2 Frequency of Occurrence of Fungal Isolates

Frequency of occurrence of fungal isolates encountered on pod, testa and nuts of *C. nitida* and *Cola acuminata* were determined. The percentage frequency of occurrence for each fungal isolate was calculated as described by [7].

$$\text{Frequency of Occurrence} = \frac{\text{Number of plates containing species X} \quad \text{x } 100}{\text{No of plats in sample}}$$

2.3 Pathogenicity Tests

For inoculcation purposes spore suspensions of isolated fungi grow on PDA for 7-12 days were obtained by adding 10-20mL of sterile distilled water to the Petri dish cultures and scraping the mycelia mat of the fungi with a heat sterilized scapel.

The suspensions were filtered through an 8 layer cheese cloth. The inoculum was agitated before and during inoculation in order to maintain uniform conidia distribution. The number of conida in the suspension was estimated by using Malassez hemocytometer (France).

The suspension was adjusted to a concentration of 2 $x10^5$ conida per mL. A volume of 10μL of this suspension was inoculated to the wounded portion of nuts, which were obtained by using a 5mm diameter sterile cork-borer. The control nuts were wounded and inoculated with 10μL of sterile distilled water. They were incubated for 10 days in the dark at 25ºC.

2.4 Effect of Artificial Wounding and Incubation Temperatures on the kolanuts Infection

Samples of kolanut were obtained from kola processing unit at CRIN. The samples were either subjected to surface wounds or were left unwounded.

Following this initial treatment, *Kola nuts* were placed in 11cm Petri dishes containing wet Whatman filter paper (Grade 41, ashless 150mm, Kent, England) and inoculated with 10μL of conidia suspension of *L. theobromae*. Inoculated samples were incubated at 10, 20 and 30ºC for 10 days. For each treatment, there were three replications and the percentage of *Kola nuts* that developed infection was recorded.

3. RESULTS

Twenty storage fungi were isolated from each of *Cola nitida* and *Cola acuminata*. The fungi isolated were *Lasiodiplodia therobramae*, *Fusarim pallidorosunm, F. moniliforme, F. cavispemum, Curvularia sp, Chrysogenum, Mucor spinosus, Paecilomyces variotii, Chlamydomyces sp* (Table 1). The incidence of these moulds was established across the kola growing belt of Nigeria. The frequency occurrence of fungal species in *Kola nuts* in the fourteen locations were listed in Table 2. *L. theobromae* was isolated in 10 locations while *F. pallidoroseum* occurred in 8 locations of the sampling sites (Table 1).

The result obtained across the kola-growing belt of Nigeria established *L. theobromae* as the two most frequently encountered fungi associated with *Kola nuts*.

The percentage frequency of occurrence observed for *L. theobromae* and *F. pallidoroseum* was 44 and 47% respectively on C*. nitida* and 34 and 20%, respectively on *C. acuminata* (Table 3).

Fusarium pallidoroseum has the highest frequency of occurrence in *C. nitida* while *L. theobromae* has the highest frequency of occurrence in *C. acuminata. Paecilomyces variotii*. *Chlamydomyces* sp and *Curvularia* sp. were not isolated on *C. acuminata* but were isolated on *C. nitida* (Table 2).

Lasiodiplodia theobromae, F. pallidoroseum and *A. niger* has the highest frequency of occurrence in infected pods, testa and nuts (Table 3).

Higher frequency of occurrence of *L. theobromae* (69%) and *F. pallidoroseum* (53%) were recorded on infected pods (Table 3) similar trend was observed on the kolanut testa whereas *Curvularia* sp and *Paecilomyces variotii* were isolated from nuts, they were not isolated from pod. *Chlamydomyces* on the other land was encountered on infected pods, but was not isolated from nuts.

There was a significant difference (P=0.05) in the occurrence of *L. theobromae* on kola pod when compared with *F. pallidoroseun* for instance *L. theobromae* was frequently encountered from nut extracted from completely necrotic pod than from symptomless pod.

From the values obtained for each of the fungi isolated from pods, testa and nuts in Table 3, *L. theobromae* and *F. pallidorosum* can be regarded as primary invaders of kolanuts irrespective of the sampling site in Nigeria Similar. *A. flavus. Aspergillus niger, Penicillum chrysogenum* can be said to be the secondary invaders of kolanuts while *Curvularia* sp, *Paecilomyces variotiii* and *Chlamydomyces* can be termed the tertiary invaders of kolanuts produce in Nigeria (Table 2). *Kola nuts* attacked by these fungi are discolored and are of lower commercial value, the pathogenicity tests of the isolate established *L. theobromae* as the causal pathogen of the brownish spot on the cotyledons, which after sometime turned dark and eventually became black and shrunken. The surface of the invaded part of the *Kola nut* remains dry and often compact and coherent making the nut to become mummified.

Table 1. Isolation of the fungal species associated with kolanut across growing belt of Nigeria

Fungi	Ibadan	Ife	Ogunmakin	Sagamu	Ishara	Sabogida	Owena	Olode	Osogbo	Okenne	Ayangha	Otukpo	Onitsha	Ikom
L. theobromae	+	-	+	+	+	-	+	+	+	-	-	+	+	+
F. moniliforme	+	+	+	+	+	-	+	-	+	+	-	-	-	+
F. pallidoroseum	+	-	+	+	+	+	-	+	+	-	-	+	-	-
Curvularia sp.	+	+	+	+	+	-	-	+	+	-	+	+	+	-
Aspergillus niger	+	+	-	+	+	+	-	+	+	-	-	-	-	+
A. fumigates	-	+	-	+	+	+	-	-	+	+	+	-	-	-
Penicillium sp.	+	-	-	+	+	-	-	+	+	+	+	-	-	+
F. cavispermum	-	-	-	+	+	-	-	-	-	-	-	-	-	-
Mucor spinosus	+	-	-	+	+	-	-	+	+	-	-	-	-	-
A. flavus	-	-	-	+	+	-	-	-	-	-	+	-	-	-
P. variotii	+	-	+	+	+	-	-	-	-	-	-	-	-	-
Chlamydomyces	+	-	-	+	+	-	-	-	-	-	-	-	-	-

**+ve = Present.*
-ve = Absent.

Table 2. Percentage frequency occurrence of the most common fungal species isolated from *C. nitida* and *C. acuminat*a collected across the kola grown zones

Genus	Frequency of occurrence (%)	
	C. nitida	***C. acuminata***
L. theobromae	31.7a	34.0b
F. pallidoroseum	22.3b	39.3a
F. moniliforme	11.6c	7.8d
F. cavispermum	1.3e	0.00f
A. flavus	7.6d	4.7e
A. niger	10.9cd	9.7d
A. fumigatus	0.00g	8.6d
P. chrysogenum	11.3c	21.0c
P. variotii	11.3c	2.0e
Chlamydomyces sp.	0.03f	0.00f
Curvularia sp.	0.05f	0.00f
M. spinosus	9.3d	9.0d

Data were nominalised by log (X + 1). Means not followed by the same letter in the same column are significantly different (p = 0.05) according to Duncan's Multiple ranged test

Table 3. The frequency of occurrence of the most common fungal genera obtained from *C. nitida* collected at CRIN Ibadan headquarters

FUNGI	POD	TESTA	NUT
B. theobromae	0.69a	0.4a	0.5a
F. pallidoroseum	0.53b	0.3b	0.4bc
A. niger	0.32c	0.3b	0.3c
P. chrysogenum	0.28d	0.2c	0.1d
C. lunata	0.10c	0.2c	0.1d
P. variotii	0.00f	0.1d	0.1d
M. spinosus	0.00f	0.1d	0.1d
Chlamydomyces sp.	0.10c	0.01c	0.0c

Data were nominalised by log (X + 1). Means not followed by the same letter in the same column are significantly different (p = 0.05) according to Duncan's Multiple ranged test

Kola nuts artificially wounded and inoculated had a maximum of 33.3% infection when kept at 10ºC (Fig. 1) whereas when *Kola nuts* that were not wounded but inoculated and incubated at 10ºC the percentage infection drastically reduced to 16.7%. Similarly, higher infection percentages were recorded for artificially wounded and incubated at 20 and 30^0C, respectively. The mycelia growth of the fungus was apparent on the wounded portion of the nut than unwounded parts.

The highest infection percentage of 79.2% was recorded for *Kola nuts* that were wounded inoculated and incubated at 30ºC when *Kola nuts* were wounded and held at 20ºC, 50% of the nuts developed diseases symptoms, in contrast to 26.6% infection percentage. There was a significant difference (p =0.05) in the level of infection of *Kola nuts* for both wounded and unwounded nuts.

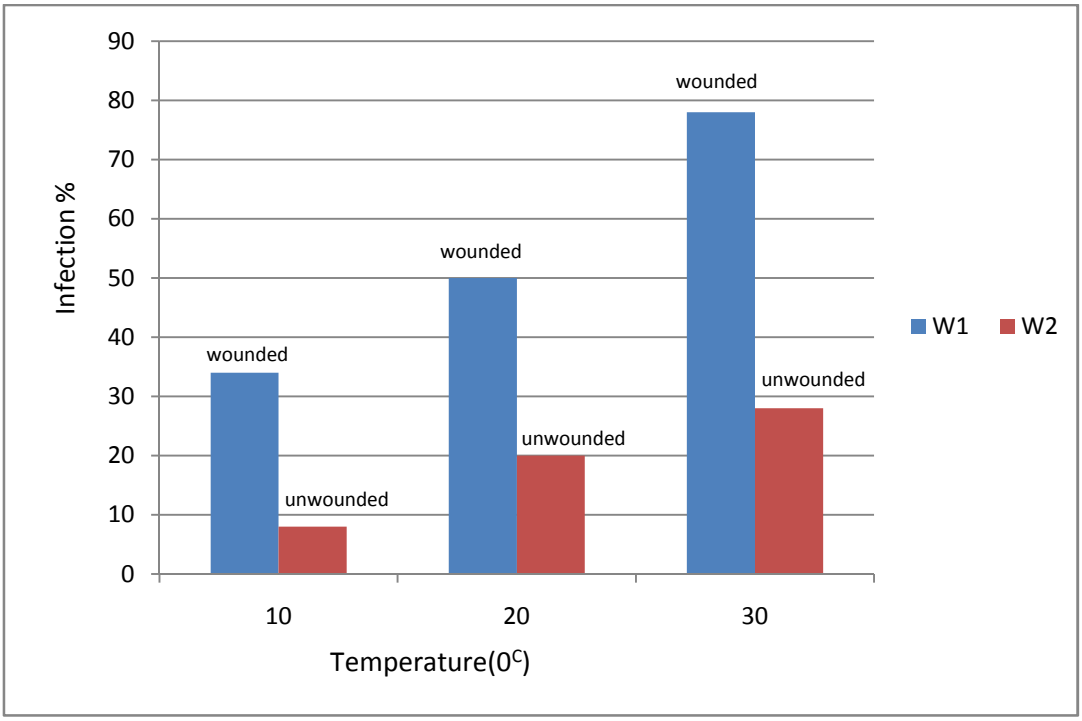

Fig. 1. Effect of artificial wounding and incubation temperatures on the infection of *Kola nut* (*Cola nitida*)

4. DISCUSSION

The high number of fungi isolated from *Kola nut* in this study suggests the vulnerability of healthy *Kola nut* to infestation by pathogenic fungi. These fungi caused damage to *Kola nut* in the form of discoloration of nut, shrinking of nuts, seed rot and physiological alterations in nuts. Subsequently, these damages lower the quality of *Kola nut* by causing defects which seriously depreciate the commercial value of the nut. Similar result have been reported in wheat carrot, barley, Sorghum, maize and pearl millet [8,9]. Fungi such as *L. theobromae*, *Aspergillus sp. Fusarium sp, Penicillium sp. Mucor spinosus, P. variotii* are known to cause seed rot in many crops [10]. This assertion has been corroborated in this study in respect of the pathogenicity of these fungi on kolanut. The extent of the nut discoloration depends on the nut mycoflora, environmental conditions and nut physiology [11,12] Coatings caused by mycelium and sporulating structures such as *Fusarium pallidoroseum* and *L. theobromae* were observed on *Kola nut*.

For instance in the infection caused by *F. pallidoroseum* on *Kola nut*, infected nuts had a mouldy appearance, which developed copious whitish mycelia on nuts. This result is in agreement with the earlier reports made by [13,3,4].

The fact that *L. theobromae* and *F. pallidoroseum* were isolated on *C. acuminata* may be partially explained by either similar ecological or substrate preference or similarity in the bio-chemical composition of the two *Cola s*p. The present study confirmed that incidence of *L. theobromae* on *Kola nut* occurred primarily at site of injury or wounds that occurred during

skinning, (removal of testa) of nuts, subsequent contact of the wound sites with inoculum during washing result in the rapid spread of diseases.

In the absence of wounding, *L. theobromae* was incapable of penetrating the epidermical layer of the *Kola nut*. It is probable that the pathogen could not easily degrade the intact cells of *Kola nut*, or that they need to establish first on injured cells which provide exposed nutrient before they could colonise healthy tissue [3] The fact that not only *L. theobromae* was isolated during storage suggests that other pathogens can only attack *Kola nut* if the nut has been injured or modified by the environment so as to be more susceptible to invasion by orgarnisms which might not otherwise attack it. Thus, when nuts were wounded before inoculation, infection was usually favoured, wounding therefore, constitutes a type of predisposition. The incidence of several other post harvest diseases on crops such as potatoes, apples, carrot and stone fruits had been shown to be enhanced by injury incurred during various harvesting and grading processes [14,15,9].

Possibility of wounds cannot be ruled out during skinning and this present study had demonstrated that more fungi were isolated in the wounded nuts than in the unwounded nuts. Therefore introduction of a wounding with a protectant chemical or botanicals could prevent such wounds from becoming infected since most of the wounds were incurred during removal of testa, development of appropriate technology that could facilitate easy removal of testa and subsequent reduction in wounding of nuts is recommended. However, such technology should be simple enough for adoption.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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