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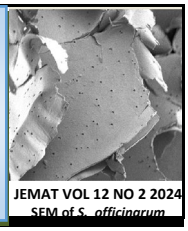

Control of Fungal Contaminants on Cocoa Pods Obtained from Akungba and Ogbagi Akoko

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ABSTRACT

A cocoa pod has a thick, rough, and tough surface, which is usually filled with mucilaginous pulp that coats and protects cocoa beans. This mucilaginous pulp is sweet and allows growth of fungal contaminants. This study determined antifungal activity of some fungi obtained from some cocoa pods. Isolated organisms were cultured, identified using their macroscopic and microscopic characteristics, compared with those of compendium of soil fungi, pictorial atlas of soil, and seed fungi. Antifungal activity assay was conducted using agar-well diffusion method containing nystatin, fluconazole, ketoconazole or griseofulvin at 200 mg/mL, 100 mg/mL, and 50 mg/mL concentrations. The results revealed the presence of twelve fungal genera that include *Aspergillus, Aureobasidium, Byssochlamys, Chrysosporium, Cladosporium, Colletotrichum, Curvularia, Epicoccum, Fusarium, Geomyces, Syncephalastrum,* and *Trichoderma. Aspergillus* species had the highest percentage of occurrence (14.3%). The findings showed that all tested antifungal agents had varied degrees of inhibition against isolated organisms and that fluconazole had the highest inhibition zone of 40 mm against *A. niger* and *S. racemosum* (at a concentration of 200 mg/mL). Nystatin had the least antifungal activity against *C. xerophilum* (10 mm at 50 mg/mL) while griseofulvin had little or no activity against tested organisms. The findings underscore the need for proper monitoring to safeguard cocoa quality and to prevent likely cocoa beans spoilage via fungal contamination. The study recommends the use of nystatin, ketoconazole, and fluconazole for the control of fungal contaminants.

INTRODUCTION

Theobroma cacao L is a tree with green leaves that belongs to the class Magnoliopsida, order Malvales, family Malvaceae, genus, *Theobroma* and species *cacao*. The seeds (cocoa beans) contain fat and are used to produce chocolate [1]. Cocoa beans are encompassed by a fragrant pulp that emerges from the outer layers of the beans. This pulpy substance consists of soft, sponge-like cells that contain a liquid rich in sugars, citric acid, and salts. Once the mature pods are gathered, they are opened, and the beans are taken out and put into baskets to be transported to fermentation facilities. During the handling process, the pulp gets bruised, and the weight of the beans causes some of the pulp's liquids to be released. These liquids seep from the accumulated beans as a slightly cloudy, pale liquid. This liquid is referred to as cocoa pulp juice or "sweatings" within the industry. It's a common practice for workers and even children to gather these drippings in make shift containers and enjoy them as a revitalizing beverage [2]. The cacao plant, whose fruit is a pod holding up to 50 beans and covered in a white mucilage, produces the seeds (beans) that are used to make cocoa [3]. The plant's composition includes various components, each contributing to its overall characteristics and uses. Cocoa Beans are the seeds of the cocoa pod and are the most well-known part of the cocoa plant. They are rich in fats, specifically cocoa butter, which gives chocolate its smooth texture. Cocoa beans also contain alkaloids such as theobromine and caffeine, which contribute to the stimulant properties of cocoa [4].

Cocoa is a significant economic crop for countries such as Ghana, Ivory Coast, Nigeria, Indonesia, and Malaysia. Evidence from numerous ancient cultures, including the Olmec, Maya, and Mexica (Aztec), indicates that cocoa was used medicinally. The use of cocoa in the treatment of some liver disorders, such as infirmities and heat distempers, and cancers, such as stomach cancer and hemorrhoid tumors, has also been explained in a number of documents [5]. According to Adejumo [6], one of the

agricultural products that generate the largest foreign exchange revenues in Nigeria is cocoa. Nigeria is ranked fifth with 174 000 tonnes, or 4.6% of global production in 2007 [4]. In Nigeria, the "cocoa belt" region's rainforest is where the majority of the country's cocoa is produced. Ondo, Osun, Ogun, Delta, Edo, Cross Rivers, Akwa-Ibom, and Ondo State produce more than 50% of the nation's annual production of cocoa [6,7].

Cocoa (*Theobroma cacao*) is susceptible to various diseases that can significantly impact its production; most notably black pod disease caused by various species of the *Phytophthora* genus, which affect the production and quality of cocoa pods, is a major constraint on Agricultural production. Other diseases of cocoa are witches' broom disease caused by *Moniliophthora perniciosa*, which leads to the formation of abnormal vegetative structures on the cocoa tree, resembling broom-like clusters. Ceratocystis Wilt (*Cephalosporium* wilt) caused by *Ceratocystis cacaofunesta* affects vascular system of cocoa trees, which can lead to wilt and death. Frosty pod rot of cocoa is caused by a fungus *Moniliophthora roreri,* the fungus affects pods and is characterized by a whitely, powdered spore masses on surface of cocoa [8]. Also, *Oncobasidium theobromae* cause vascular-streak dieback which affects the vascular system of cocoa trees, leading to wilting, leaf necrosis, and dieback.

Black pod of cocoa is the most common cause of cocoa disease, which has led to more than 30% loss of world cocoa production [8,9]. The production of cocoa in Nigeria is severely hampered by black pod disease (pod rot) where *P. palmivora, P. megakarya,* and *P. capsici* are mostly implicated. *P. megakarya* is the most significant species in Nigeria and the West African sub-region [10]. The main ways that *Phytophthora* spreads include through infected soil, animals, water, plants, and plant materials, with a few species being transferred via aerial transmission [11].

MATERIALS AND METHODS

Sample collection

Cocoa pod samples were obtained from cocoa farmlands in Ogbagi-Akoko and Akungba-Akoko, Ondo State, Nigeria. Samples were immediately transported to the laboratory of Adekunle Ajasin University in sterile zip lock bags and stored at 4°C throughout the study period.

Isolation of fungal species

Potato dextrose agar (PDA, 39 g/L) was prepared, autoclaved at 121ºC for 15 minutes; the medium was supplemented with an appropriate antibiotic (chloramphenicol) and left for 1 hour to solidify [12]. The Pod(s) surface was pre-washed with tap water and subjected to a series of disinfections with 95% ethanol for 30 seconds, in 10% sodium hypochlorite, for 2 minutes to remove external contaminants. The pods were then rinsed three times with sterile distilled water to remove traces of disinfectant [13,14]. Sterile surgical knife was used to remove sections of infected pod (5 g), mashed in a laboratory-type mortar and pestle to form a paste. A 1 g of each mashed sample was added to a test tube containing 9 mL of sterile water (stock) and was serially diluted before inoculation onto a PDA medium. The plates were incubated for 5 days at 28°C. After incubation, each discrete colony was aseptically picked with a sterile inoculation needle and subcultured.

Identification of fungi isolates

A drop of lactophenol cotton blue stain was placed on a clean, grease-free slide with the aid of a mounting needle, where a small portion of the aerial mycelia from each fungal culture was used. The mycelia were well spread on the slide with sterile inoculating wire. A cover slip was gently placed with little pressure to eliminate air bubbles. The slide was then mounted and viewed under x40 objectives lens. The micrograph of each fungus was compared with those of compendium of soil fungi, pictorial atlas of soil, and seed fungi. Identification of the isolated fungi was determined on the basis of the macroscopic and morphological characters of the mycelium (septate or non-septate) and fruiting bodies (conidia) observed under a compound [15].

Antifungal activity assay

Fourteen fungal isolates obtained from the cocoa pod samples were tested. Pure culture of each filamentous fungus (9 mm mycelial mat containing conidia and hyphal fragments) was suspended in 4 mL of sterile saline in a test tube. The mixture was vortexed and particles were allowed to settle. A sterile cotton swab was dipped into the prepared mixture, pressed firmly against the inside wall of each tube to remove excess liquid, and then used to streak the entire agar surface (3 times) with rotation of the plate approximately 60º each time to ensure that the inoculum is evenly distributed across the plate. After each plate had been dried for at least 5 minutes, a flamed cork borer (9 mm) was used to bore four holes adjacent to each other on each plate. Stock solutions of nystatin (NYS), fluconazole (FLU), ketoconazole (KET), and griseofulvin (GRI) were prepared in test tubes containing sterile distilled water to make different concentrations viz; 200 mg/mL, 100 mg/mL, and 50 mg/mL. Then, 3 drops of each antifungal agent of a specific concentration was placed into the hole [16]. The set up was incubated for 3 days at 28°C. After incubation period diameter of clear zone for each antifungal agent was measured in millimeter [17].

Statistical analysis

All experiments were conducted in triplicate and analysed using one-way ANOVA. Error bars were represented as the standard errors of the means (±SEM).

RESULTS

Enumeration of fungi showed that mould counts on cocoa pods ranged between $3.8x10^2$ CFU/g and $3.2x10^3$ CFU/g (**Table 1**). Sample AO recorded the least fungal counts ($3.8x10^2$ - $4.4x10^2$). *Aspergillus* genus was the most predominant fungal species with a percentage occurrence of 14.3%, constituting a significant portion of the isolated fungi from the cocoa pod samples (**Table 2**). Fourteen (14) fungal isolates were obtained from the cocoa pod samples and were identified based on their macroscopic and microscopic characteristics (**Table 3**). The identified fungal species include; *Fusarium oxysporum, Trichoderma harzianum, Byssochlamys fulva, Aspergillus nidulans, Chrysosporium xerophilum, Aspergillus niger, Epicoccum nigrum, Syncephalastrum racemosum, Aspergillus clavatus, Colletotrichum gloeosporioides, Cladosporium cladosporioides, Aureobasidium pullulans, Geomyces pannorum* and *Curvularia geniculata* (**Table 3**).

Table 1. Fungal counts on cocoa pod samples.

Sample	Count (CFU/g)
Sample AA1	$2.5x10^3$
Sample AA2	$3.0x10^3$
Sample AA3	$3.2x10^3$
Sample OA1	$4.4x10^2$
Sample OA2	$4.1x10^2$
Sample OA3	$3.8x10^2$

Key: Aa = Akungba-Akoko; Oa = Ogbagi-Akoko

Table 2. Percentage occurrence of fungal genera obtained from cocoa pods.

Organism	Frequency of Occurrence	Occurrence (%)
Aspergillus clavatus	2	5.7
Aspergillus nidulans	3	8.6
Aspergillus niger	5	14.3
Aureobasidium pullulans	2	5.7
Byssochlamys fulva	2	5.7
Chrysosporium xerophilum	3	8.6
Cladosporium cladosporioides	2	5.7
Colletotrichum gloeosporioides	3	8.6
Curvularia geniculata	1	2.9
Epicoccum nigrum	2	5.7
Fusarium oxysporum	2	5.7
Geomyces pannorum	2	5.7
Syncephalastrum racemosum	3	8.6
Trichoderma harzianum	3	8.6
Total	35	100

Also, results of commonly used antifungal drug such as NYS, FLU, KET, and GRI (**Figs. 1, 2, 3,** and **4**) were elucidated. Antifungal susceptibility testing demonstrated varying degrees of susceptibility among the isolated species (**Plates 1 – 4**). Notably, most of the fungal species were susceptible to nystatin and ketoconazole while some fungal species showed no sign or little sign of susceptibility to commonly used antifungal agent (griseofulvin) as shown in **Fig. 3**.

All the fungal species were susceptible to nystatin. Fluconazole had highest inhibition zones against *A. niger* and *S. racemosum* (40 mm) with no effect on *F. oxysporum, B. fulva, E. nigrum, G. pannorum,* and *C. geniculata* (**Fig. 2**). Nystatin had moderate activity against all organisms and least antifungal activity only against *C. xerophilum* (10 mm at 50 mg/mL) while ketoconazole had no effect on *F. oxysporum*, *A. niger, E. nigrum, G. pannorum,* and *C. geniculata* (**Figs. 1** and **4**). Also, Griseofulvin had no effect on all the fungal isolates except on *C. cladosporioides* and *G. pannorum*, 15 mm at 200 mg/mL concentration (**Fig. 3**).

Table 3. Morphological characteristics of fungal isolates obtained from cocoa pod.

Isolate code	Appearance of colony on PDA	Nature of Hyphae	Spore bearing/producing structure	Organism
OA1	Velvety and bluish-gray green in colour	Septate and hyaline	Conidia bearing conidiophores were elongated and club-shaped. Conidia supported phialides over the entire surface.	*Aspergillus clavatus*
AA4	Powdery, dark green in centre with pale olive green near the margins	Septate and hyaline	Conidiophores were short and each consisted of a stalk terminating in a swollen end. Phialides were with long chains of conidia. Vesicles were mostly hemispherical in shape.	*Aspergillus nidulans*
AA6	Powdery, slightly brown.	Septate and hyaline	Conidiophores were long, each ending in a bulbous head (vesicle). Conidia were very rough and globular in chains formed at the end of the tubular phialides.	*Aspergillus niger*
OA4	Smooth faint pink with yeast-like colonies	Septate and hyaline	Conidiophores were unbranched bearing conidia that were hyaline, smooth, ellipsoidal, and one-celled.	*Aureobasidium pullulans*
AA3	Velvety, yellowish white	Septate	Conidia were cylindrical. Conidiogenous cells are phialides with long, tapered ends.	*Byssochlamys fulva*
AA5	Floccose, white	Septate and hyaline	Conidiophores were unbranched. One-cell conidia produced directly on vegetative hyphae by non-specialised conidiogenous cells.	*Chrysosporium xerophilum*
OA3	Velvety, olive-grey	Septate	Conidiophores were unbranched that supported elliptical conidia. Conidiophores and conidia were pigmented.	*Cladosporium cladosporioides*
OA2	Floccose, pinkish yellow	Septate	Conidiophores were erected (unbranched) with hyaline, one-celled, ovoid and dumbbell shaped conidia.	*Colletotrichum gloeosporioides*
OA6	Cottony, cream	Septate	Conidiophores produced 4-septate conidia, they were branched and bent at the points where the conidia originated. Conidia were curved with dark brown central section.	*Curvularia geniculata*
AA7	Velvety, yellow, orange	Septate	Conidia were formed singly on densely compacted, non-specialised, slightly pigmented conidiophores on a sporodochium.	*Epicoccum nigrum*
AA1	Floccose, dark purple	Septate	Conidiophores were unbranched and monophialides were observed.	*Fusarium oxysporum*
OA5	Powdery, yellowish brown	Septate	Conidiophores were branched and hyaline which bore small, wedge-shaped conidia with a flat base.	*Geomyces pannorum*
AA8	Fluffy, light gray	Aseptate	Cylindrical merosporangia were observed. Sporangiophores were erected, stolon-like, and showed sympodial branching.	*Syncephalastrum racemosum*
AA2	Wooly, yellowish green	Septate and hyaline	Conidiophores were hyaline and branched. Conidia were globose to subglobose and loosely arranged. Ellipsoidal phialides were observed.	*Trichoderma harzianum*

KEY: AA = Akungba-Akoko, OA = Ogbagi-Akoko

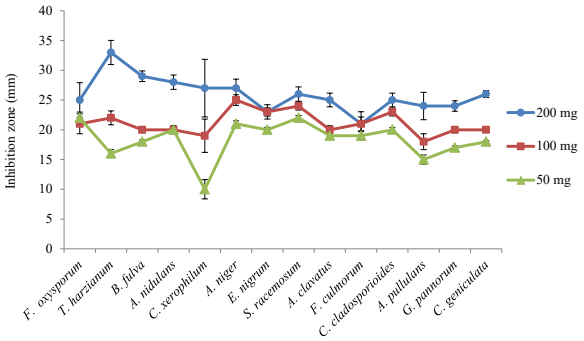

Figure 1: Nystatin against fungal species obtained from cocoa pods

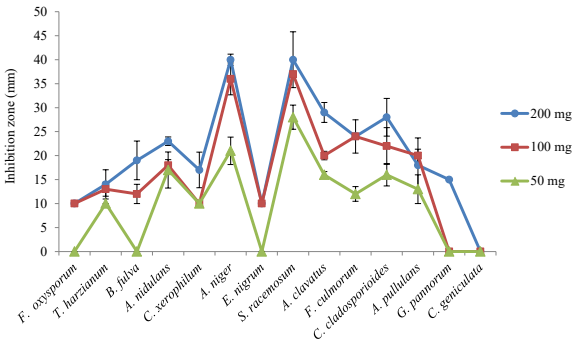

Figure 2: Fluconazole against fungal species obtained from cocoa pods

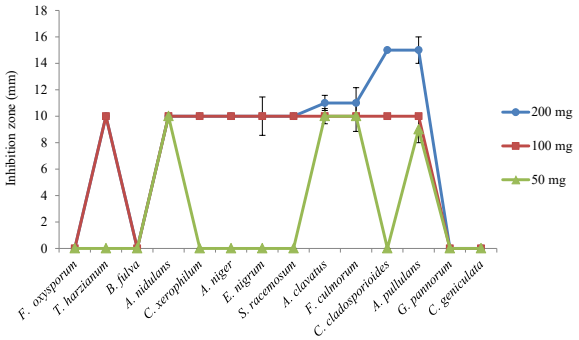

Figure 3: Griseofulvin against fungal species obtained from cocoa pods

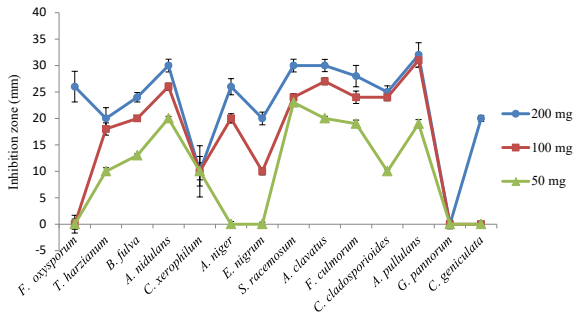

Figure 4: Ketoconazole against fungal species obtained from cocoa pods

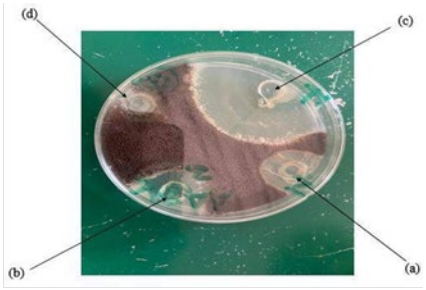

Plate 1. Nystatin (a), Ketoconazole (b), Fluconazole (c), and Griseofulvin (d) against *Syncephalastrum racemosum*

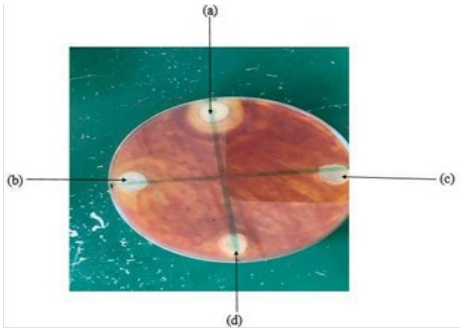

Plate 2. Nystatin (a), Ketoconazole (b), Fluconazole (c), and Griseofulvin (d) against *Epicoccum nigrum.*

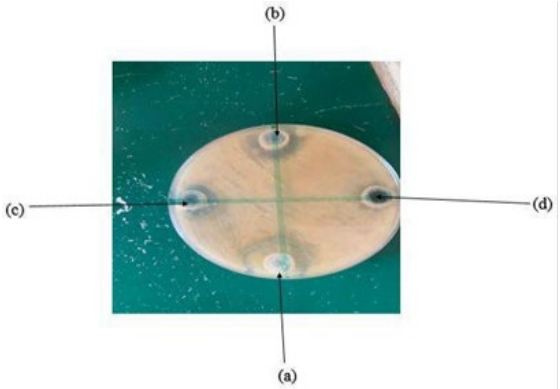

Plate 3. Nystatin (a), Ketoconazole (b), Fluconazole (c), and Griseofulvin (d) against *Byssochlamys fulva.*

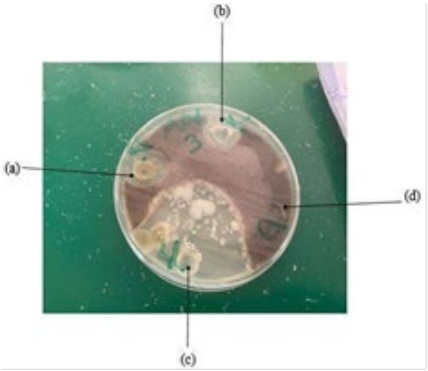

Plate 4. Nystatin (a), Ketoconazole (b), Fluconazole (c), and Griseofulvin (d) against *Aspergillus niger*

DISCUSSION

Fungal species obtained from cocoa pod samples underscore the complexity of the microbial contaminants on cocoa pods. Prevalence of these fungi in cocoa pods has significant implications on cocoa quality and yield [8]. *Aspergillus* genus was predominant with a percentage occurrence of 14.3%, constituting a significant portion of the isolated fungi from the cocoa pod samples [18]. Some of the identified fungal species such as *A. niger, S. racemosum, C. gloeosporioides,* and *F. oxysporum* are known to be associated with spoilage and may contribute to the deterioration of cocoa pods and beans [19,20]. Additionally, certain fungi isolated from cocoa pod samples such as *F. oxysporum* can produce "deoxynivalenol" (DON); a mycotoxin during their fungal growth which can pose risks to human health [21]. Also, *A. clavatus* is a known producer of patulin producer, a mycotoxin which causes haemorrhages of lung and brain with other immunological, neurological and gastrointestinal outcomes [22].

Fungal species such as *F. oxysporum, T. harzianum, A. niger, S. racemosum,* and *C. gloeosporioides* obtained in this findings corroborate the results of Fapohunda *et al.* and Medjap *et al.* [19,20] while other fungal species such as *C. cladosporioides*, *C. xerophilum, A. clavatus, C. geniculata, G. pannorum,* and *E. nigrum* isolated from cocoa pods in this study are similar to the organisms obtained in previous studies on cocoa [18,23,24,25,26,27]. In another study conducted by Fapohunda *et al.* [19]*, A. niger* and *S. racemosum* were isolated, which highlight the need for proper disease management.

The emergence of reduced susceptibility and very little susceptibility to certain antifungal agents (ketoconazole, fluconazole and griseofulvin) raises concerns about the long-term sustainability of these treatment options [28]. Several factors can contribute to the development of reduced susceptibility and very little susceptibility to antifungal agents in fungal populations associated with cocoa pods. The indiscriminate use of antifungal agents in agriculture, suboptimal dosing practices, and environmental factors all play roles in shaping resistance patterns [29].

Fungal contamination extends beyond economic considerations for cocoa farmers; they also have broader implications for the cocoa supply chain and the global chocolate industry [8]. Contaminated cocoa pods can infect cocoa beans which can lead to lower-quality chocolate products, affecting consumer satisfaction and brand reputation [19]. Improved agricultural practices, such as proper drying and storage conditions, may contribute to reducing fungal growth and mycotoxin production [30].

CONCLUSION

The identification of fungal species obtained from cocoa pods sheds light on the diversity of fungi associated with cocoa. This study contributes to the broader understanding of fungal ecology in cocoa pods and lays the foundation for future initiatives aimed at improving cocoa quality, ensuring food safety, and sustaining the livelihoods of cocoa farmers. Regular surveillance and monitoring of fungal contaminants on cocoa pods in Ogbagi and Akungba Akoko should be established. This will provide valuable data for early detection of emerging resistance and inform timely intervention strategies. Outreach programmes and training sessions for cocoa farmers will enhance awareness of fungal diseases affecting cocoa pods. Strategies to manage antifungal resistance in cocoa-related fungi should be explored, including the development of alternative bio-control methods and the implementation of integrated pest management practices. Finally, the findings underscore the importance of antifungal agents in cocoa farming to prevent the spread of spoilage organisms.

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