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Research Paper

Mycoflora associated with cocoa (*Theobroma cacao*) pods obtained in the field and their effects on seed nutritional contents

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Abstract. Mycoflora associated with the pod rot disease of cocoa (*Theobroma cacao*) and their effect on the nutrient composition of cocoa beans were investigated. The fungal pathogens implicated in the pod rot disease were *Phytophthora palmivora*, *Ceratocytis paradoxa*, *Botryodiplodia theobromae* and *Rhizopus stolonifer*. The results of proximate analysis in mg/100 g of fungal infected and non-infected (control) cocoa beans that has been carried out showed that there was an increase in moisture and carbohydrate content of the fungal infected cocoa beans relative to the healthy ones (control). Meanwhile, there was a decrease in protein, fat, fibre and ash contents of the fungal infected cocoa beans relative to the healthy ones (control). It has been observed that the moisture content had increased from 46.53 ± 0.15 in the non-infected cocoa beans to 50.17 ± 0.12 in the infected samples, and carbohydrate content had also increased from 56.37 ± 0.24 in the non- infected to 67.00 ± 0.29 in the infected beans. The following parameters were found to be decreased in the infected than in the non-infected cocoa beans viz protein content 13.10 ± 0.06, fat 12.93 ± 0.26, crude fibre 4.6 ± 0.09 and ash content 2.30 ± 0.12. The caloric value for the infected cocoa beans was higher 436.80 ± 1.42 than in the non-infected 430.07 ± 0.38. However, the nutrient compositions between the infected and non-infected cocoa beans are not significantly different ($p > 0.05$).

Keywords: Mycoflora, cocoa seeds, effects, nutritional contents.

Abbreviations: CHO, Carbohydrate; **C/V,** caloric value.

INTRODUCTION

Theobroma cacao also known as the Cacao tree and cocoa tree, is a small (4 to 8 m) (13 to 26 ft) tall evergreen tree in the family Malvaceae (Juan et al., 2008) native to the deep tropical regions of central and South America. Its seeds, cocoa beans, are used to make cocoa mass, cocoa powder and chocolate (Copetti et al., 2010). The fruit or cocoa pod is in ovoid shape, 15 to 30 cm (5.9 to 11.8 inch) long and 8 to 10 cm (3.1 to 3.9 inch) wide, ripening yellow to orange, and weighing about 500 g (1.11 lb) when ripe. The pod contains 20 to 60 seeds, usually called “beans”, embedded in a white pulp. The seeds are the main ingredients of chocolate, while the pulp is used in some countries to prepare refreshing juice, smoothies, jelly and natal (Terry et al., 2007). Each seeds contains significant amount of fat (40 to 50%) as cocoa butter. Their most noted active constituent is leobromine, a compound similar to caffeine.

Cacao is cultivated on roughly 17,000,000 acres (27,000 sqmi, 69,000 km^2) worldwide (FAO, 2005). Cacao production has increased from 1.5 million tonnes

in 1983-1984 to 3.5 million tonnes in 2003-2004, almost entirely due to the expansion of the production area rather than to yield increases. Cacao is grown both by large agro industrial plantations and small procedures, the bulk of production coming from millions of farmers who have a few trees each (Henderson, 2007).

A cocoa tree begins to bear when it is four or five years old. A mature tree may have 6,000 flowers in a year yet develop only about 20 pods. There are about 1,200 seeds (40 pods) are required to produce 1 kg (2.2 lb) of cocoa paste.

Historically, chocolate makers have recognised three main cultivars groups of cacao beans used to make cocoa and chocolate (Henderson, 2007). The rarest prized, and expensive is the criollo group, the cocoa bean used by the Maya. Only 10% of chocolate is made from criollo, which is less bitter and more aromatic than any other bean. The cacao bean in 80% of chocolate is made using beans of the forastero group. Forastero trees are significantly hardier than criollo trees resulting in cheaper cacao beans. Trinitario, a hybrid of criollo and forastero, were used in about 10% of chocolate. The criollo cacao beans from chuao in Aragua, Venezuela are widely regarded as some of the finest in the world. In November, 2000, the cacao beans coming from said region have been awarded an appellation of origin under the title “Cacao de chuao (from Spanish –Cacao of chuao (Terry et al., 2007) effectively making this bean one of the most expensive and sought after types of cacao (Xavier et al., 2011).

Cacao bean is the dried and fully fermented fatty bean of *Theobroma cacao,* from which cocoa solids and cocoa butter were extracted (Henderson, 2007). They are the basis of chocolate, as well as many Mesoamerican foods such as mole sauce and tejate. A cocoa pod (fruit) has a rough and leathery rind about 3 cm thick (these varies with the origin and variety of pod). It is fitted with sweet, mucilaginous pulp inclosing 30 to 50 seeds that are fairly soft and white to a pale lavender colour. While seeds are usually white, they become violet or reddish brown during the drying process (Henderson, 2007).

Over the years there have been reports of fungal attack on cocoa pods rendering the seeds (beans) unfit for human consumption. Fungi such as *Phytophthora palmivora*, *P. capsici, P. kevea* causative agents of (black pod rot), *Lasiodiplodia* spp (*Lasiodiplodia* pod rot) *Macrophoma spp (Macrophoma* pod rot), *Phytophthora citropthora* and *P. megakarya* (*Phytophthora* pod rot) have been reported to cause depletion of pods/seeds value in the field (APS, 2011).

Due to the adverse effect of fungal infection on cocoa pods as observed in the field in Ikom and Etung Local Government Areas of Cross River State, Nigeria (the major cocoa producers in the South South/South East region of Nigeria), it has became necessary for them to isolate and identify the fungal pathogens responsible for the spoilage and rot of the cocoa pods as well as evaluating the effect of pods mycoflora on the nutritional contents of the seeds. Information on the effects of fungal infection to the nutritional contents of pods/seeds of cocoa obtained in the field and in storage was scanty. This paper seeks to breach this gap.

MATERIALS AND METHODS

Sample collection and pathogen identification

Matured infected and uninfected cocoa pods were obtained from the field in different locations in Ikom and Etung Local Government Areas of Cross River State, Nigeria and transported to the Laboratory for analysis. The two Local Government Areas are the major producers of cocoa in the South South/South East region of Nigeria. Proximate (Nutrient) analyses of infected and uninfected cocoa pods were carried out in the Department of Biochemistry, University of Calabar, Calabar, Nigeria. Cocoa seeds (beans) (about 5mm in diameter) from the symptomatic and asymptomatic cocoa pods were removed following surface sterilisation with 70% ethanol for 10secs, blotted dry with sterile paper towel, and plated onto chloramphenicol-amended Potato Dextrose Agar (PDA). After three days of incubation at 28˚C microbial growth has been assessed by microscopy. Cultures of the isolates were transferred to new PDA-containing plates, from where axenic cultures were generated (Gevens et al., 2008). Identification of the isolates was based on morphological characteristics, described in the 1998 illustrated genera of fungi by Barnett and Hunter (1998) and with literature on the identification of pathogenic fungi by Dugan (2006). Confirmation was made by comparing the cultures identified by International Mycological Institute, Egham, UK.

Koch’s postulates and pathogenicity test

To confirm pathogenicity of isolates from cocoa pods/seeds, axenic cultures of these isolates were used to inoculate twenty cocoa pods with 5 mm-diameter mycelia agar plugs of a 4-day old culture. On appearance of symptoms, the tissues at the margins of the healthy and diseased parts were surfaced-sterilised, excised and plated onto molten PDA for incubation at 28°C for 4 days. At the end of this period, morphological characteristics and growth patterns observed in each case were compared with the ones of the original isolates (Markson et al., 2010).

Effect of fungal infection on proximate composition of cocoa beans

To ascertain the effects of fungal infection on nutritional

composition of cocoa beans, mature cocoa pods showing signs of fungal infection obtained were cracked open to remove the beans (seeds). The seed coat of the beans was removed and the moisture content of the beans were determined. The beans were then oven dried at 60°C for 24 h and grounded into fine powder using mortar and pestle. The powdered samples were stored in plastic container for laboratory analysis. Most of the methods adopted in this research work are those recommended by Association of Official Analytical Chemist (2002).

Proximate analysis (procedure)

Moisture content

A clean 100 ml beaker was dried in an oven to constant weight (a). A known amount of the 5 g sample was introduced in the beaker and weighed (b). The samples were then fried in a ventilated electrically heated atmosphere oven at 75°C for about 24 h cooled in a desiccator until constant weight was obtained (c). The Percentage Moisture content was calculated from the formula:

$$\% \text{ moisture content} = \frac{b - c}{b - a} \times \frac{100}{1}$$

The experiment was carried out in triplicates.

Ash content

5 kg sample were accurately weighed into the crucible. This was ignited at 55°C for about 24 h in a dessicator and weighted. This step was repeated until a constant weight was obtained. The parentage ash content was calculated from:

$$\% \text{ Ash content} = \frac{\text{Wt of ash}}{\text{Wt of sample}} \times \frac{100}{1}$$

Determination was made in triplicate.

Crude fat

5 g samples were accurately weighed into a thimble. About 120 ml petroleum ether was poured into a previously dried and weighted round bottom flask. The Soxhlet extractor into which the thimble with content had been introduced was then filled into the round bottomed flask and the condenser and extraction apparatus set up with a cramp and stand. Gentle heat has been applied then the heater evaporated and as it condensed, it drooped into the thimble where it extracted ether soluble constituents into the round bottomed flask. The extraction then continued for about 8 hours. The thimble was then removed and air-dried) later far free extract was used for fibre determination). The petroleum ether in the flask was distilled off and collected in the Soxhlet extractor tube. The flask was then fried in an air circulating desiccators for 48 h.

The round bottomed flask and the lipid extract was then weighted. The flask and content was again dried and weighed till a constant weight was obtained. The amount of lipid extracted was obtained from the difference between the weight of the flask before and after extraction.

$$\text{Calculation} = \frac{\% \text{ Ether extract wt of extraction}}{\text{Wt of sample}} \times \frac{100}{1}$$

Crude fibre

5 g far free material was weighted and quantitatively transferred into 400 ml beaker, which had been previously marked at 200 ml level. 50 ml of 1.25% sulphuric acid were added and the mixture was made up to 200 ml mark with distilled water. The contents of the beaker were heated to boiling point for 30 min.

Crude protein (Micro Kjedahl method)

40% sodium, hydroxide pellets (40 g pellets carbonates free were dissolved in 100 ml distilled water). Concentrated sulphuric acid, Selenium Kjedahl Catalyst (each tablet containing 1 g sodium sulphate, and 0.05 g copper sulphate was dissolved in 0.1N% hydrochloric acid. Methyl red-ethylene blue indicator was prepared by mixing the equal volume of 0.2% twice recrystallised methyl red and 0.0% methylene blue made up in absolute ethanol. This sample was then stored in a dark brown bottle in a refrigerator.

Digestion (Micro Kjedahl)

1 g sample was weighed out into a 50 ml Kjedahl digestion flask. 20 ml of antidumping chips were added. The mixture was incinerated to gentle boiling on a digestion rack and then heated strongly until the digest became clear. The digest was removed cooled quantitatively transferred to a 100 ml volumetric flask and made up to mark. An Erlenmeyer flask containing 10 ml of boric acid indicator solution was placed at the tip of the condenser extended below the surface of the solution. 10 ml of the sample digest was introduced into quick fit micro Kjedahl flask and steam heated. 10 ml of 40% Sodium hydroxide solution was added to the digest and the digested steam distilled into the Erlenmeyer's flask until the contents become more than double of its original

Table 1. Proximate composition of infected and non-infected cocoa beans mg/100 g (dry matter).

Sample	Moisture	Protein	Fat	Crude fibre	Ash	CHO	C/V
Non-infected	46.53 ± 0.15	18.60 ± 0.06	14.47 ± 0.15	6.10 ± 0.06	4.47 ± 0.15	56.37 ± 0.19	430.07 ± 0.38
Infected	50.17 ± 0.12	13.10 ± 0.06	12.98 ± 0.26	4.67 ± 0.09	2.30 ± 0.12	67.00 ± 0.29	436.80 ± 1.421

Note: CHO = Carbohydrate, C/V = Caloric Value. Source: Effiong, 2014.

volume as the ammonia changed to green. A blank determination was carried out in a similar manner as described above except 1 g digestion sample was replaced by 1 ml of distilled water.

Titration

The content of the Erlenmeyer flask was titrated with 0.1% hydrochloric acid to a pink end point.

Calculation

$$\% \text{ Protein} = \frac{1 \text{ ml of HCl (Test)} - \text{ml of HCl (Blank)} \times \text{NX} \times 100}{1000 \times 10 \times 1}$$

N = Normality of the acid
10 = Ml of digest use
I = Gram of sample used

RESULTS

Sample collection and pathogen identification

Following isolations from the rotting tissues (pods) and beans of cocoa, four organisms (*Ceratocystis paradoxa*, *Rhizopus stolonifer*, *Botryodiplodia theobromae* and *Phytophthora palmivora*) have been obtained. Fungal colonies that emerged on the culture medium (PDA) were identified. On the PDA, colonies of *C. paradoxa* were initially grayish white and eventually turned to dark olivaceous green with smooth margin. The growth was radial in pattern from the centre of the plate outwards. *R. stolonifer* exhibited profuse growth with their whitish thread-like mycelia dotted with grey coloured sporangiospores after three days of growth. The colonies of *B. theobromae* were initially white, fluffy and feathery, becoming grey and eventually black. The growth was radial in pattern from the centre of the plate outwards. *P. palmivora* produced abundant sporangia, usually papillate and ovoid with mycelia masses.

Literature on the identification of pathogenic fungi (Rossman et al., 1997) and illustrations by Barnett and Hunter (1998) corroborate these observations and the appearance of *C. paradoxa* fitted the description of *Ceratocytis* (Dade) C. Moreau. (*Thielaviopsis thielaviodes* (Peyr.) Paulin, Harr & Mcnew) as presented by Dugan (2006).

The true identity of each of these fungi was confirmed by comparing their cultures with those identified by International Mycological Institute, Egham, UK.

Koch's postulates and pathogenicity test

The *C. paradoxa, R. stolonifer*, *B. theobromae* and *P. palmivora* isolates were pathogenic on the cocoa pods/seeds used for each pathogen for the test. Symptoms of decay (rot) caused by *C. paradoxa* was seen as soft black rot. *R. stolonifer* produced soft rot symptoms, *B. theobromae* and *P. palmivora* caused black dry rot on the cocoa pods/seeds tested. On re-isolation, the four isolates exhibited similar patterns of growth as observed in the original isolates.

Proximate analysis

The results of proximate analysis in mg/100 g of fungal infected pod/beans of cocoa showed that there was an increase in the moisture and carbohydrate content of the fungal infected beans of cocoa relative to the healthy ones (control), while there was a decrease in the protein, fat, fibre and ash content of the fungal infected cocoa beans relative to the healthy ones (control). Moisture content increased from 46.53 ± 0.15 in the non-infected cocoa beans to 50.17 ± 0.12 in the non-infected to 67.00 ± 0.29 in the infected beans, while the following parameters were found to decrease in the infected than in the non-infected beans viz protein content 13.10 ± 0.06, fat 12.93 ± 0.26, crude fibre 4. 67 ± 0.09 and ash content 2.30 ± 0.12. The caloric value for the infected cocoa beans was higher 436.80 ± 1.42 than in the non-infected 430.07 ± 0.38 as presented in Table 1.

DISCUSSION

In this study, a total number of four fungal pathogens were isolated as the causative agent of pod rot of cocoa obtained in the field, namely *Phytopthora palmivora, Ceratocystis paradoxa, Rhizopus stolonifer* and *Botryodiplodia theobromae* in the infected cocoa pods. This result is in agreement with the findings of Fagbohun (2012) who isolated *P. palmivora* as the causative agent of pod rot in cocoa. Fanny et al. (2000) have also reported the isolation of *P. palmivora and R. stolonifer* as the causative agent of pod rot of cocoa. Ndife et al. (2013) reported the isolation of toxigeneric genera of

Phytopthora spp and *Ceratocystis* spp in cocoa pods. Fungal contamination of cocoa pods and its contents (beans and pulp) must not exceed the level that could adversely affects the shelf life of the pods. If it does, it renders that the pods and beans are useless.

The result of proximate analysis (mg/100 g) of non-infected and infected cocoa beans revealed that moisture content increased from 46.53 ± 0.15 in the non-infected to 50.17 ± 0.12 in the infected cocoa beans, and carbohydrate content had also increased from 56.37 ± 0.24 in the non-infected to 67.00 ± 0.29 in the infected. This result is in agreement with the findings of Falaye and Fagbohun (2012) who reported an increase in moisture content from 5.09 in the non-infected to 6.13 in the infected and carbohydrate (5.01 to 5.53) of groundnut (*Arachis hypogea*) infected with *R. stolonifer* and other moulds. Similarly, Nweke and Ibiam (2012) reported an increase in the moisture and ash content of (*Anonia muricata)* fruits infected by *Colletotrichum gloeosporoides* and *R. stolonifer.*

However, Ndife et al. (2013) reported that carbohydrate (61.74%) and moisture content (5.81%) of non-infected cocoa beans were higher, but were seriously depleted when infected with *P. Capsici and P. Megarkaya* (43.97 and 3.13) respectively. Omokolo et al. (1996) have also found that carbohydrate content of non-infected pods (91.0) have decreased to (13.2) in infected cocoa pods. Onifade and Jeff-Agboola (2003) reported that moisture decreased from 36.49 to 10.4 g/100 g in infected samples. The decrease in protein content (13.0 ± 0.06), fat (12.93 ± 0.26), crude fibre (4.67 ± 6.09) and ash content (2.30 ± 0.12) in the infected sample is in agreement with the findings of Ndife et al. (2013) who reported that protein (81.14%), fibre (3.59), ash (5.81) and fat (5.23%) of non-infected cocoa beans were higher, but were seriously depleted when infected with *P. capsici and P. megakarya* to 4.80, 1.80, 2.13 and 4.02%, respectively. Shehu and Aliero (2010) have also reported that the infected onion leaf showed a significant decrease in the quantity of the crude protein, fat, fibre and ash content. Opayemi (2012) reported that ash content of non-infected pods (10.7) and beans (8.0) were depleted when infected with *P. palmivora* to 9.3 and 7.8 in cocoa pods and beans, respectively. It could therefore be deduced that the relative increase of moisture and carbohydrate in the infected beans may be caused by the digestion, degradation and dissolution of the beans tissue into a mush (water rot) by the pathogens. These degradation activities by pathogens might have also resulted to the relative reduction in the protein, fat, fibre, and ash contents of the infected beans. The protein, fat, fibre and ash might have been broken down by the fungi into smaller molecules that they absorbed (Nweke and Ibiam 2012). Bonner (1997) reported that complex molecules such as polysaccharide and protein are required by fungi to build the hyphal wall (chitin, glucan and cellulose) and for respiration to obtain energy. This suggests that these pathogens might have denied the man of these essential nutrients upon consumption through their degradation activities, thereby causing some great damaging effects to human health. Van Duyn and Pivanka (2000) stated that the deficiency of fibre in our diet leads to diverticular diseases and intestinal cancer. However Fanny et al. (2000) who also reported a decrease in fat, ash and protein content of cocoa infected by fungi, stated that the nutrient depletion in entire test plant sample might have been as a result of the internal defence system of the host tissue. The caloric value of the infected cocoa beans was found to be higher (435.50 + 1.42) than that of the non-infected beans (430.07 + 0.38). This result is in disagreement with Akpabio (2012) who reported higher caloric value in healthy almond seeds (*Terminata catappa*).

CONCLUSION

The results of proximate analysis of fungal infected and non-infected cocoa beans showed that there was an increase in the moisture and carbohydrate content of the fungal infected beans of cocoa relative to the healthy ones (control), while there was a decrease in the protein, fat, fibre and ash contents of the fungal beans relative to the healthy ones (control). Meanwhile, the caloric value for the infected cocoa bean was higher than in the non-infected. It is possible that the isolated fungal pathogens might be resident on the leaves and stems of the plant from where they were dispersed into the fruits to initiate infection spore during rainfall. To this effect, timely spraying of cocoa tress with fungicides during flowering and fruiting will reduce the damaging activities of the fungal pathogens and contamination with mycotoxins and other related fungal metabolites that might be hazardous to human health.

RECOMMENDATION

The damaging activities by plant pathogens could be reduced by the use of plant extracts. Plant extracts, particularly offer potentially simple environmentally safe alternative to be used as botanical fungicides and could be exploited for the effective management of pre and post harvest disease of tropical fruits. The added advantages to these are that the plant extracts are cheaper and non-toxic to man if the appropriate concentrations are used. Further research will be carried out by these authors using different plant extracts in controlling pod rot of cocoa in the field.

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