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MYCOFLORA OF *Dioscorea rotundata* TUBERS IN CALABAR METROPOLIS AND THEIR EFFECT ON THE NUTRIENT COMPOSITION OF THE TUBERS

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ABSTRACT

Dioscorea rotundata Poir is a primary source of carbohydrate in most African homes, where it is consumed in various forms. This research focuses on the identification of the prevalent fungal isolate responsible for the rot of yam in Calabar metropolis and the effect of these fungi on the nutritional composition of white yam. A survey of the major markets (Akim, Marian, Mbukpa and Watt) showed that the major fungi responsible for the soft rot of yam included *Aspergillus flavus*, *A. niger*, *Fusarium oxysporium*, *Penicillium notatum* and *Rhizopus stolonifer*. *Aspergillus niger* recorded the highest incidence, occurring in all the samples from various locations. The result of the pathogenicity test, proved that *Aspergillus niger*, *Penicillium notatum* and *Rhizopus stolonifer* were the major causes of rot in Calabar metropolis. The proximate analysis of the healthy tubers and those inoculated with *Aspergillus niger*, *Penicillium notatum* and *Rhizopus stolonifer* showed that the infection by these fungal isolates caused significant changes in the nutrient composition of the tubers. The total moisture content of the inoculated tubers increased within a range of 65.43±0.35 – 70.01±0.05% as compared to 59.59±0.12% in the control experiment. The carbohydrate content decreased significantly from 27.87±0.50% to a range of 18.11±0.36 – 22.68±0.28%.

KEYWORDS: Microflora, nutrient composition, white yam, pathogenicity and tubers

INTRODUCTION

White yam (*Dioscorea rotundata Poir*) is widely cultivated in west and central Africa, in Asia and South American countries (FAO, 2007). In West African, White yam is one of the most important tuber crops in terms of area coverage and a key staple food, particularly in Nigeria. More than 90% of the global yam production (40 million tons fresh tubers per year) is produced in West Africa. Nigeria has been consistent in the lead of yam producing countries worldwide. White yam can be stored longer than most other fresh farm products, and in most communities, especially in northern Cross River State, Nigeria, stored yam represents stored wealth. Statistics have shown that Nigeria accounted for over 65% (38 million tons) of the total yam production (58.8 million tons) in 2012 (IITA, 2013).

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Many important cultural values are attached to white yam, especially during weddings, religious (as thanksgiving items in churches) and other socio-cultural ceremonies. Izekor and Olumese (2011) reported that due to the importance attached to yam, many communities celebrate the new yam festival annually in Nigeria. Traditionally, yam is a prestige crop that is viewed and received with high respect, prominently during special gatherings such as new yam festivals in most communities in Cross River State (Nahanga and Bečvařova, 2015).

White yam (*Dioscorea rotundata*) is one of the major staple food crop in Nigeria and has potential for livestock feed and industrial starch production (Ayanwuyi *et al*., 2011). Nutritionally, yams are mainly carbohydrate food, but contain about 1-2% dietary protein, which is high compared with other tropical root crops (Ogaraku and Usman, 2008). Yam tuber stores proteins that have been observed to have significant antioxidant capacities. In one study, these proteins had about one-third of the antioxidant activity of glutathione one of the body's most impressive internally produced antioxidants. Most of starch industries also make use of yam as one of their important raw materials (Ibitoye and Onimisi, 2013). Yam is rich in carbohydrate (75.5 - 83.3%), amino acid and vitamins (thiamine, riboflavin and ascorbic acid). Yam contains a high value of protein (2.4%) and substantial amount of vitamins and minerals than some other common tuber crops (Ekunwe *et al*., 2008). White yam contribute about 200 dietary calories daily for more than 95 million people in Nigeria and as an important source of income and livelihood security to more than 55% of Nigerians (Amusa *et al*., 2018; Omohimi *et al*., 2918).

Udemezue and Nnabuife (2017) confirmed that irrespective of the growing attentions given to yam production in Nigeria, its production is still below average and this could be as a result of some limitations occasioned by the activities of yam production coupled with pests and diseases that could retard its growth. Yam production is generally influenced by several problems such as weeds, decline in soil fertility, storage pests, high cost of labour, cost of land preparation and maintenance, staking and barn making, and most importantly, field and storage pests and diseases (Okon *et al*., 2022). The causes of storage losses of white yam tubers include: sprouting, transpiration, respiration, rot due to mould and bacteria, insects, nematodes and mammals. One major constraint to yam production worldwide is post-harvest rot which may be either physiological or microbial (Otoo *et al.,* 2001). Jeff-Agboola and Jeff-Agboola (2019) estimated microbial post-harvest losses in yam at 40% while Okigbo and Ikediugwu (2000), indicated that between 20 and 39-5% of stored tubers may be lost to decay. The principal factors responsible for yam losses during storage are: the natural metabolic processes of the dormant tubers, which result in the conversion of starch of the tuber into carbon (IV) oxide and water; evaporation of water from the tuber; sprouting; and infection by various fungi which decay the tuber (Markson *at al*., 2010). Losses in stored white yam mostly due to rot are considered to be heavy in Nigeria, with FAO (1998) estimating these losses to be up to 56% after 6 months in the barn. Most of the pathogens of white yam tuber are soil borne, but manifestations of the tuber disease are observed mostly during storage. It has been estimated that an average of over 25% of the yield of yam is annually lost due to disease (Ezeh, 1998). Rot causing fungi are of particular importance because they reduce vigor and subsequently cause a reduction in tuber yield and quality (Amusa *et al.*, 2011).

Microbial agents causing rot of white yam are mainly fungi (Osai *et al*., 1996). Several pathogenic fungi have been found associated with white yam, causing diseases such as anthracnose, leaf spots and blight, as well as rotting of yam tubers (IITA, 2009). Many fungal pathogens have been associated with deterioration of white yam during storage. The implications of *Fusarium oxysporium, Fusarium solani, Penicillium* and *Aspergillus* species as common pathogens in yam storage have earlier been reported. (Morse *et al*., 2000). Yam tubers are subjected to several diseases caused by fungi, bacteria and viruses (Yeni, 2011). It has been reported that microbial infection of yam leaves resulted to low yam tuber productivity Garrido *et al*. (2003). *Colletotrichum gloeosporioides* (Penz.) and *Curvularia eragrostides* has been resported to be secondary pathogen to yam leaves, which caused dark brown leaf lesions with irregular borders and yellowish halo (Garrido *et al*., 2003).

Fungi have been reported to be significantly responsible for low yam tuber productivity. The fungi infection renders yam tuber unfit for human consumption by reducing their nutritive value and often by producing mycotoxins (Okigbo, 2004; Yao *et al.,* 2003; Navi *et al.,* 2005; Pestka and Smolinski, 2005). The reduction of fungi infestation of yam leaves which reduces yam tuber productivity will be a right step to improve or enhance food security and improvement on the economy of the yam growers and farmers worldwide. The need for effective and cheap antifungal agents will be the right step in this direction (Aiko et al., 2013). The aim of this paper is to evaluate the extent of nutrient depletion in *Dioscorea rotundata* due to the presence of fungi.

MATERIALS AND METHODS

Collection of Samples

The sample area (Calabar metropolis) was surveyed using cluster sampling method. Samples of yam tubers (*Dioscorea rotundata*) were collected from different markets in Calabar. A total of five markets were sampled, and the sections where yam are sold were divided into sections (units). In each market about about 3 to 4 stalls in each section were visited.

Samples where then inserted into sterile plastic bags, labelled and then taken to the laboratory and stored under suitable temperature prior to analyses.

Inoculum preparation and isolation from samples
Potato dextrose agar (PDA) medium supplemented with chloramphenicol was employed for the isolation of fungi from samples. Samples were cut into slices and were washed in alcohol and then rinsed with sterile distilled water before direct plating was employed using a sterile dissecting forceps. Plates were incubated on sterile laboratory bench for 5-7 days at room temperature (27^0C±1). The fungi cultures were sub-cultured until pure colonies were obtained by successive hypha tip transfer. The cultures were examined under the microscope for fruiting bodies, hyphae to determine the common fungi present.

Pathogenicity test of the isolated fungi
Aspergillus niger isolates obtained from the rotted tubers were inoculated into healthy tubers of yam. The fresh healthy yam tuber was washed under running tap water and surface-sterilized in 10% sodium hypochlorite (NaOCl) solution for 2 minutes after which they were rinsed twice in sterile distilled water. Cylindrical discs (5mm diameter) were removed from two tubers (one per tuber) with a sterile cork borer. Mycelial discs (4mm diameter) were made from 7 day-old cultures of the fungi and each fungal disc was put into a hole in each of the two tubers. The yam discs were replaced and inoculation sites smeared with petroleum jelly. The remaining yam tuber served as the control. The inoculated tubers were place separately in sterile polythene bags containing cotton wool soaked in sterile distilled water. The bags were properly labelled and incubated at 28°C for 14 days. Disease symptoms produced by artificial inoculation after the incubation period were compared with those observed on the contaminated tubers. The fungi were re-isolated from the inoculated diseased yam tubers and cultured on PDA plates. The morphology of each pathogenic fungi was compared with that of the original culture.

Morphological characterization of fungi (Microscopy).
The process of microscopy took the following steps.
i. A drop of lactophenol in cotton blue was placed on a glass slide.
ii. Small portion of fungal growth on the Petri dish was picked and smeared in the lactophenol blue on the slide using a sterile needle.
iii. The smeared portion was covered with a cover slip.
iv. The prepared slide was placed on a compound microscope and viewed under x40 objective lens.

Identification of fungi
This was done by comparing the features such fruit bodies, shape, colour and arrangement of conidia, presence or absence of septa, colour of mycelia, as established by Bannet (2009)

Proximate analysis
Moisture content
Weights of dry sample with crucible were recorded (W_1), samples were then dried and reweighed till a constant weight (W_2) was obtained. Moisture content was computed using the formula

$$\% \text{ Moisture} = \frac{W_1 - W_2}{W_1 - W_0} \times 100 = \frac{\text{weight of moisture}}{\text{weight of sample}} \times \frac{100}{1}$$

Where: W_0 – weight of empty crucible
W_1 – weight of crucible + wet sample
W_2 – weight of crucible + dry sample.
(Udo *et al*., 2009).

Dry matter content
Total solid (dry matter content) was derived from subtraction of percent moisture content from 100 percent.
% Total solid (dry matter content) = 100 - % moisture (Onwuka, 2005)

Ash content
Weight of empty crucibles was recorded (W_0). Two grams (2 g) of the test sample was added in each of the crucibles and weight recorded (W_1). Samples were transferred to a pre heated muffle furnace at 550^0C and left for 5 hours. They were thereafter cooled in desiccators, weight of the ashed sample and crucible taken (W_2).
Ash content was computed using the formula

$$\% \text{ Ash} = \frac{W_2 - W_0}{W_1 - W_0} \times 100 = \frac{\text{weight of ash}}{\text{weight of sample}} \times \frac{100}{1}$$

(Udo *et al.,* 2009).

Where
W_0 – weight of empty crucible
W_1 – weight of crucible + wet sample
W_2 – weight of crucible + ashed samples

Fat content
Two grams of sample were wrapped in filter paper, secured each with clips and their weights recorded (W_1). They were then placed in a soxhlet apparatus. 200ml of ethanol was put in boiling flask and placed on a hot plate with the extraction thimble plunged tightly, and the soxhlet apparatus allowed to reflux for about 12 hours (until the solvent in the extractor became colourless and the distillate yellowish). The wrapped samples were then removed from the apparatus and dried for 3 hours at 105^0C in an oven. They were then cooled in desiccators, and the weight taken (W_2).

Fat content was computed thus

$$\% \text{ Fat} = \frac{W1 - W_2}{W_0} \times 100 = \frac{\text{weight of fat}}{\text{weight of sample}} \times \frac{100}{1}$$

Where
W_0 – weight of sample
W_1 – weight of wrapped sample before de fatting
W_2 – weight of wrapped sample after de fatting.

(Udo *et al*., 2009)

Crude fibre content

The de fatted samples were then placed in 200 ml of 1.25 % H_2SO_4 and left to boil for 30 minutes, then they were filtered through muslin cloth stretched over 9 cm Buchner funnel, rinsed with hot water and scraped into another flask containing 200 ml 1.25 % NaOH and allowed for 30 minutes. They were filtered again and rinsed thoroughly with hot distilled water. They were allowed to drain, and then scraped into pre weighed crucible (W_0) and weighed (W_1). They were then dried overnight at 105^0C in an oven, and then placed in a pre-heated muffle furnace at 550^0C for 3 hours. The samples were then cooled in desiccators. Weight of crucible and ashed sample was recorded (W_2), and crude fibre content was computed thus:

$$(W_1 - W_0) - (W_2 - W_1)$$
$$W_3 - W_4 = W_5$$
$$\% \text{ Crude fibre} = W_5 \times 100$$
$$= \text{loss of weight after incineration} \times 100$$

(Onwuka, 2005)

Where
W_0 – weight of crucible
W_1 – weight of crucible and hydrolysed sample
W_2 – weight of crucible and ashed sample
W_3 – weight of hydrolysed sample
W_4 – weight of ashed sample
W_5 – loss of weight after incineration (weight of fibre)

Determination of protein content

Determination of protein content was done by first of all determining the total organic nitrogen using the macro- Kjeldhal method. This involved digestion, distillation and titration.

a. Digestion

Samples measuring 0.2 grams were placed in labelled digestion flask and a gram of selenium catalyst added to each. A few drops of distilled water were added to dissolve the catalyst. This was allowed to stand for 30 minutes before adding 5 ml of concentrated H_2SO_4. The sample was digested by placing on a digester in a fume cupboard. Digestion continued until a clear solution was obtained, thereafter the set up was left to cool. Particles at the neck of the flask were washed down using distilled water which resulted in a turquoise blue solution.

b. Distillation

The Markham distillation apparatus was allowed to steam for 15 minutes, and then 10 ml of digest was pipette into the flask and used for the distillation process. For distillation, thee flask was set up and connected using a Liebig condenser to a beaker (receiving flask) containing 10 ml off boric acid with drops of Mazuazaga indicator. The condenser was submerged in the boric acid by use of discharge tube. 10 ml of sulphuric acid was then pipette into the Markham apparatus and distillation was achieved by heating the mantle. The distillation continued until the boric acid changed from blurred red to army green.

c. Titration

The boric acid mixture containing the ammonium borate complex formed during distillation (army green solution) was titrated with 0.02N H_2SO_4 till a pink end point was reached and the titre noted too. A blank experiment was run alongside, and titre noted. The total organic nitrogen was then calculated using:

$$\% \text{ TON} = \frac{K \times X \times Nacid}{W} \times \frac{100}{1}$$

Where
X – Titre value – blank
Nacid – Normality of acid – 0.02
W – Weight of sample used – 0.2
K – 0.07 (constant)

$$\% \text{ Crude protein} = \% \text{ TON} \times 6.25$$

(Udo *et al*., 2009; Onwuka, 2005).

Soluble carbohydrate (Nitrogen free extract - NFE)

The nitrogen free extract (NFE) referred to as soluble carbohydrate was not determined directly, but obtained by subtracting all the other components (except fat and dry matter) from 100%.

NFE = 100 – (% ash + % crude fibre + % crude protein + % moisture)

(Onwuka, 2005).

RESULTS

Isolated fungi

The fungi isolated from the tubers of *Dioscorea rotundata* were characterized morphologically and microscopically. Table 1 shows the frequency of incidence of these isolates in the various markets.

Table 1: Incidence of fungi in *Dioscorea* species in Calabar metropolis

ISOLATED FUNGI	LOCATION (MARKETS)							
	AKIM		MARIAN		MBUKPA		WATT	
	Frequency	%	Frequency	%	Frequency	%	Frequency	%
Aspergillus flavus	1	7.1	-	-	2	25.0	1	11.1
Aspergillus niger	6	42.9	4	50.0	2	25.0	5	55.6
Fusarium oxysporium	4	28.6	2	25.0	1	12.5	2	22.2
Penicillum notatum	1	7.1	2	25.0	-	-	-	
Rhizopus stolonifer	2	14.3	-	-	3	37.5	1	11.1
Total	14	100	8	100	8	100	9	100

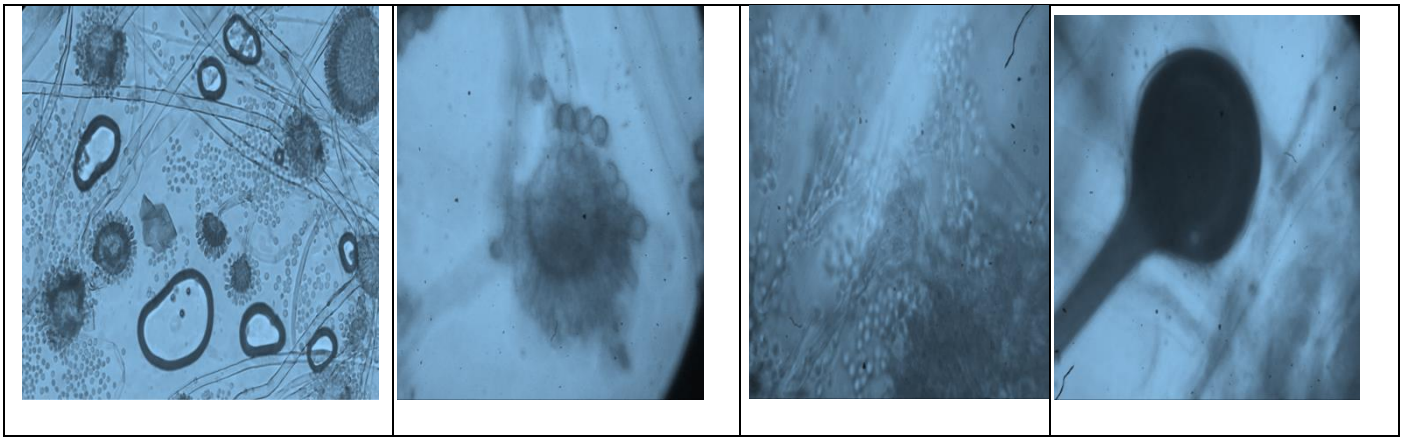

Figure 1: *Penicillium notatum* grows by producing white, round and fine mycelia.

The photomicrograph of *Aspergillus flavus* shows colonies that are plain green in colour or yellow-green becoming green with age. Hyphae are septate and hyaline. Conidal head are short columnered and biseriated.

The mycelia of *Aspergillus niger* are yellow to blackish brown with a moderately rapid growth rate. Reverse side is creamish-yellow to yellow in colour. Septated hyphae, long smooth and colourless conidiophores biseriatephalides, globose conidial head and presence of dark conidia on the conidia head as seen in figure 1.

The macroscopic appearance of *Rhizopus stolonifer* shows that its growth rate is very rapid and colonies are typically cotton, the surface colony color is initially white becoming grey to yellowish brown in time while reserve is white to pale and pathogenic *Rhizopus stolonifer* can grow well at a temperature of 37^{o}C. It has a non-septate broad hyphae; sporangiosphores are usually un-branched, brown in color and appear in clusters

Effect of fungal isolates on the nutrient composition of *Dioscorea rotundata*

Table 2 shows the result of the proximate analysis of uninfected yam tuber (control) and the infected samples that were inoculated with *A*. *niger, R*. *stolonifer* and *P*. *notatum* respectively.

There was an increase in the moisture of the tubers of *Dioscorea rotundata* inoculated with *A*. *niger, R*. *stolonifer* and *P*. *notatum* respectively after two weeks of incubation. Also, the fibre content of the inoculated tubers increased.

There were reductions in the carbohydrate, protein and fat contents of the fungi inoculated tubers; the ash content was relatively unchanged. *A. niger* caused an increase in the moisture and fat content of the inoculated tubers. The carbohydrate and protein content were reduced due to the presence of the pathogen. There were no significant changes in the fat and ash contents of both the control and the inoculated tubers. The most significant increase in moisture was seen in tubers inoculated with *P. notum*, while the carbohydrate and protein content reduced significantly, as was the case with the tubers inoculated with *Rhizopus stolonifer*.

The analysis of variance showed that the change in nutrient content of *D. rotundata* inoculated with the different fungal isolates was significantly different at P≤0.

Table 2: Effect of fungal isolates on the nutrient composition of *Dioscorea rotundata*

	Nutrient composition (%)					
	Moisture	**CHO**	**Protein**	**Fibre**	**Fat**	**Ash**
Control	59.58±0.12	27.87±0.50	5.74±0.02	2.07±0.04	0.49±0.02	4.25±0.03
A. niger	65.43± 0.35	22.68±0.28	4.23± 0.02	3.06± 0.04	0.42± 0.02	4.18±0.03
R. stolonifer	67.52±0.10	20.96±0.31	4.14±0.02	2.78±0.03	0.36±0.02	4.24±0.02
P. notatum	70.01± 0.05	18.11±0.36	4.12± 0.07	2.98± 0.06	0.39± 0.02	4.39±0.02

Values are means of triplicate determinations ± the standard error of mean

DISCUSSION

In this study, the fungal isolated were identified to be *Aspergillus niger, Aspergillus flavus, Fusarium oxysporium, Penicillium notatum* and *Rhizopus stolonifer.* This agrees with the report of Morse *et al.* (2000), Okigbo, 2004; Yao *et al.,* 2003; Navi *et al.,* 2005; Minervini*et al.,* 2005; Pestika and Smolinski, 2005 who reported this species of fungi as being the major fungal contaminants of stored yam tubers. Also, Fagbohun & Faleye (2012) implicated species of the genus *Aspergillus*, *Fusarium* and *Penicillium* in most cases of fungal incidence in tropical crops.

Several changes were noticed in the nutrient composition of tubers of *D. rotundata* inoculated with *A*. *niger, R*. *stolonifer* and *P*. *notatum* respectively. Previous studies have shown that the incidence of plant pathogenic fungi in food crops causes noticeable changes in the nutritional composition of such crops (Mba and Akueshi, 2001).

There was an increase in moisture and fibre content of the fungi inoculated tubers. Stephen and Olajuyigbe (2006) reported biochemical changes in plant products due to the presence of storage fungi including *Penicillium* spp. The increase in moisture content may be due to the degradation of the cellulose and pectic components of the cell wall by the fungal isolates.

There was a decrease in the carbohydrate, protein and fat contents. These may be due to the utilization of these nutrients by the fungus for the production of mycotoxins. The reduction in carbohydrate content conforms to the report of Fagbohun and Faleye (2012). The reduction in protein content may be due to the utilization of the protein molecules for the metabolic needs of the tuber. Also, the reduction in fat may be due to the fact that the fungi utilized the fatty acids for its metabolic activity and mycotoxin production.

Nutrient depletion in the infected tubers may also be due to the utilization of the nutrients by the internal defense system of the host tissue. It may equally be due to the production of metabolic products such as mycotoxins which might either add or reduce the amount of the nutritional component of the infected tubers (Tripathi and Mishra, 2009).

CONCLUSION

The fungal pathogens isolated and identified in this study included A*pergillus flavus, Aspergillus niger, Rhizopus stolonifer* and *Penicillium notatum*. These shows that *Dioscorea rotundata* is a suitable substrate for the growth and proliferation of these rot causing fungi.

The result of the proximate analysis shows that *Dioscorea rotundata* has a very high nutrient composition. But, most of these nutrient components appeared to influence the susceptibility of the tuber crop. The fungal isolates were able to induce several biochemical changes in the plant, while using these nutrients for its growth and metabolic activity.

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