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MYCOFLORA AND AFLATOXIN LEVELS IN WALNUT SAMPLES STORED IN DIFFERENT PACKAGING MATERIALS IN OGUN STATE

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ABSTRACT

Aflatoxin levels in nuts and fruits pose a great threat to food storage and availability. This study aimed at determining the mycoflora and aflatoxin level in stored walnut under various conditions. Freshly harvested walnuts were stored for 90 days in three different media: jute bags, Plastic containers and sterile polythene bags under room (37°C) and refrigeration (4°C) temperature. After 90 days of storage, the stored walnuts were examined for fungal growth at one month interval after which colonial and morphological characterization were carried out to identify the fungi present. Proximate analysis and aflatoxin content of the walnut samples were determined by standard methods. Fungi isolated from the walnuts include; *Aspergillus niger, Saccharomyces cerevisae, Penicillum notatum, Aspergillus sydowi, Fusarium oxysporium,* and *Rhizopus stolonifer.* Walnuts stored in plastic container at room temperature had the lowest aflatoxin level of 0.002 ng/kg while that stored in polythene bags had the highest (0.054 ng/kg). Proximate analysis also revealed that walnuts stored in polythene bag reduced significantly ($p<0.05$) in protein, ash content, fat, fibre, moisture and carbohydrates while samples stored in jute bag and plastic container remained unchanged. This study shows that storage methods contributed to the overall quality, shelf life and aflatoxin content of walnut and hence care should be taken during post harvesting process.

Keywords: Aflatoxin, Fungi, Mycoflora, Proximate, Walnut

INTRODUCTION

The global demand for food security and availability has become an issue of consideration in recent years and several researches are ongoing in the preservation of foods and fruits. Walnut is known to be rich in oil, vitamins, minerals and proteins (Ozcan, 2009; Özcan, *et al*., 2010). For many years, Walnut has been regarded as a healthy food that is nutritious and delicious as well. The nutritional value of walnut shows that it is rich in calorie, essential fatty acid, quality protein, fiber and pectic substances (Milind and Deepa, 2011). The consumption of two to three servings of walnut per day consistently decreased total cholesterol (Özcan, *et al.,* (2010). Walnuts contain free radical scavenging compounds like ellagic acid, juglone

and certain phytosterols that support the immune system and appear to have anti – cancer properties (Milind and Deepa, 2011). It has also been stated that walnut possesses a high shelf life. Nuts are generally prone to contamination by fungi during growth, harvesting and storage under various climatic conditions, agricultural and storage practices especially during storage (Bankole *et al*., 2005). This is further enhanced by adverse temperature and relative humidity, which are quite conducive for fungal growth and toxin production (Njobeh *et al*., 2009).

Iqbal *et al.* (2014) opined that aflatoxin levels increase under storage conditions such as excessive heat, high humidity, lack of aeration and insect or rodent damages, which are usually common conditions in the tropics (Kaaya and Kyamuhangire, 2006). However, the most important factors influencing fungal development during nut storage are the storage temperature, moisture content and aeration (Markuszewski and Kopytowski, 2015). Nuts in the shell have 25-50% longer shelf-life than the nutmeats alone; this percentage can vary considerably depending on the particular nut and whether the packaging provides a moisture barrier and/or a low oxygen concentration. Pieces of nutmeats have about half the shelf-life of the intact nutmeats. Some roasted nuts have a shelf-life of one fourth than that of the raw nutmeats (Bruhn *et al*., 2010). Mexis and Kontominas (2010) estimated that the nut meat of walnut can stay up to 12 months when refrigerated and 3 months at room temperature (37°C). In addition, storage of walnut can also affect the proximate content of the fruit. Christopoulos and Tsantili (2011) stated that storage at 1°C had a positive effect on saving total phenol compound content, total antioxidant content, and colour of the cultivars stored for 12 months compared to samples stored at 20 °C.

Since walnut is a seasonal fruit, it is therefore imperative to set the levels of mycoflora and aflatoxin contamination in walnut and also device a means of having the fruit all year round. It is also necessary to assess and prevent the risk of aflatoxin in nuts in order to minimize food safety concerns and consequently boost the economy because the economic losses due to nut contaminated with fungi and its toxins are difficult to estimate. It leads to direct nut losses, human illness and reduced productivity; consequently, food availability all year round will be impaired. Thus this study was aimed at isolating and identifying mycoflora associated with walnut, quantification of aflatoxin in stored walnut as well as determining the best packaging material for walnut storage.

MATERIALS AND METHODS

Sample collection and storage

A total of 200 freshly harvested walnuts were purchased at a local market in Ile – Ife, Osun State. South West, Nigeria. Twenty pieces of unshelled walnuts were then stored in sterile jute bags, plastic containers and polythene bags under two different temperature conditions [room temperature (37°C) and refrigeration temperature 4°C]. All the packaging materials were sterilized with (10%) sodium hypochloride solution prior to usage.

Fungal isolation from stored samples

The stored walnuts were examined for any sign of spoilage or fungal growth at one month interval and the storage was terminated after 90 days. One gram of the walnut was pulverized in a sterile blender and cultured on Potato dextrose agar (supplemented with Chloramphenicol) using the pour plate

technique and incubated at 27°C for 3 – 4 days. The fungal colonies observed were thereafter identified using their cultural and morphological characteristics as described by Barnett and Hunter (1972).

Preparation of walnut and quantification of aflatoxin

Walnut samples were prepared for analysis by the modified method of Stroka *et al*. (2004). Twenty five grams (25 g) each of the stored walnut samples were weighed out with 5 g sodium chloride and placed in a separate jar for each storage type. Methanol:water (150 ml) in the ratio 80:20 was added to each jar and the samples were blended at a high speed for 30 mins. Fifty milliliters (50 ml) of the walnut were passed through an immunoaffinity cartridge/ column. The column contains specific antibodies bound on to solid support materials. As the sample goes through the column, antibodies selectively bind with any aflatoxin (antigen) contained in the sample matrix. The column was prewashed with 10 ml of distilled water twice, using a disposable 25 ml syringe. The walnut samples were loaded onto a cartridge and eluted at a flow rate of 10 ml/min after which the elute was discarded.

The extracts were pooled and concentrated to about 0.5 mL under a gentle stream of nitrogen gas. A volume of 200 µl was injected and analyzed for aflatoxin by HPLC. Extracted walnut samples were analyzed by an HPLC system consisting of a Waters 6000 A solvent delivery system and a WISP 710B sample processor for sample injections (Waters Associates India). Samples were eluted isocratically on a radically compressed 10 µm octadecylsilane cartridge (Waters Associates India) with a mobile phase of acetonitrile: methanol: water (15:15: 70) at a flow rate of 0.8 ml/min. a prefilter was placed between the injector and the cartridge. The aflatoxin was detected fluorometrically (excitation wavelength, 365 nm; emission wavelength, 425 nm) with a fluorescence detector (model 420 C, Water Associates). The HPLC chromatograms were recorded on a Water Data Module (Waters Associates) at a chart speed of 1.0 cm/min. The concentration of aflatoxin in walnut samples was determined by peak area and comparison with samples containing known concentrations of aflatoxin (spike sample).

Phytochemical analysis of Walnut

The extracts were analyzed to test for the presence of the active chemical constituents such as alkaloid, tannin, saponins and flavonoids. The phytochemical analysis was done on the stored walnuts samples using acetone, ethanol and methanol solvent extract according to the method of AOAC, (1990).

RESULTS

Table 1: Mycoflora isolated in walnut under different storage conditions

Walnut and the packaging materials	Day 1	Day 30	Day 60	Day 90
Walnut inside Jute bag	Nil	*A. niger* + *A. Cerevisiae* +	*A. niger* ++ *Mucor* + *S. Cerevisiae* ++	*A. niger* +++ *Mucor* + *S. Cerevisiae* +
Walnut inside Polythene bag	Nil	*A. niger* + *Penicillium* +	*A. niger* +	*A. niger* +
Walnut inside Plastic Container	Nil	Nil	Nil	*S. Cerevisiae* +
Refrigerated Walnut inside Polythene bag	Nil	*A. flavus* + *A. niger* ++ *Penicillium* +	*A. flavus* + *A. niger* +++	*A. flavus* ++ *A. niger* +++
Refrigerated Walnut inside jute bag	Nil	*A niger*+ *Fusarium* +	*A. niger* +++ *A. flavus* + *Fusarium* +	*A. sydowi* ++ *A. niger* + *Fusarium* + *Rhizopus* +
Refrigerated Walnut inside Plastic Container	Nil	*Rhizopus* +	*Rhizopus* +	*Rhizopus* ++
Walnut exposed to air at room temp (37 ^{o}C)	Nil	Nil	*Penicillium* +	*Penicillium* + *S. Cerevisiae* ++

The microflora isolated from the stored walnut samples over the 90 days period of storage include *Aspergillus niger*, *S. cerevisiae*, *Penicilliun* spp., *A flavus*, *Fusarium* spp., *A sydows*, *Mucor* spp and *Rhyzopus* spp. (Table 1). Over the 90 days of storage, walnut kept in plastic container had no fungal spores at the first 60 days and minimal fungal presence after the 90 days of storage, while the samples exposed to air at ambient temperature showed no fungal growth until 60 days. All the samples were confirmed as having no fungal isolate at the start of storage on day 1 (table 1).

From the distribution of mycoflora in stored walnut over a period of 90 days, it could be deduced that the walnut stored in plastic containers at room temperature had the longest shelf life of 60 days compared with other storage types used in this study. Also, this study also shows that refrigeration does not hinder the growth of fungi as all the ones refrigerated in different storage containers showed minimal fungal growth (table 1)

Table 2: Aflatoxin progression of walnut stored under different conditions

Aflatoxin level of freshly purchased walnut = 0.001ng/kg

Walnut and Storage conditions	Day 1 (n/kg)	Day 30 (ng/kg)	Day 60 (ng/kg)	Day 90 (ng/kg)
Walnut inside Jute bag	0.001	0.001	0.004	0.006
Walnut inside Polythene bag	0.001	0.006	0.015	0.036
Walnut inside Plastic Container	0.001	0.001	0.001	0.002
Refrigerated Walnut inside Jute bag	0.001	0.005	0.009	0.014
Refrigerated Walnut inside Polythene bag	0.001	0.0013	0.029	0.054
Refrigerated Walnut inside Plastic Container	0.001	0.004	0.006	0.011
Walnut exposed to air at room temp (37^{o}C)	0.001	0.001	0.002	0.002

The aflatoxin content of the walnut in the plastic container at room temperature also showed the least concentration while the one stored in polythene bag and refrigerated had the highest aflatoxin content (table 2)

Proximate analysis of walnut stored under different conditions

Proximate analysis of the stored walnut samples showed that protein, ash, fat, fibre, moisture and carbohydrate contents of the samples kept in the plastic container was well preserved over 90 days period of storage. However, samples preserved in jute and rice sacks depleted in nutrient content over the same period of storage (figure 1).

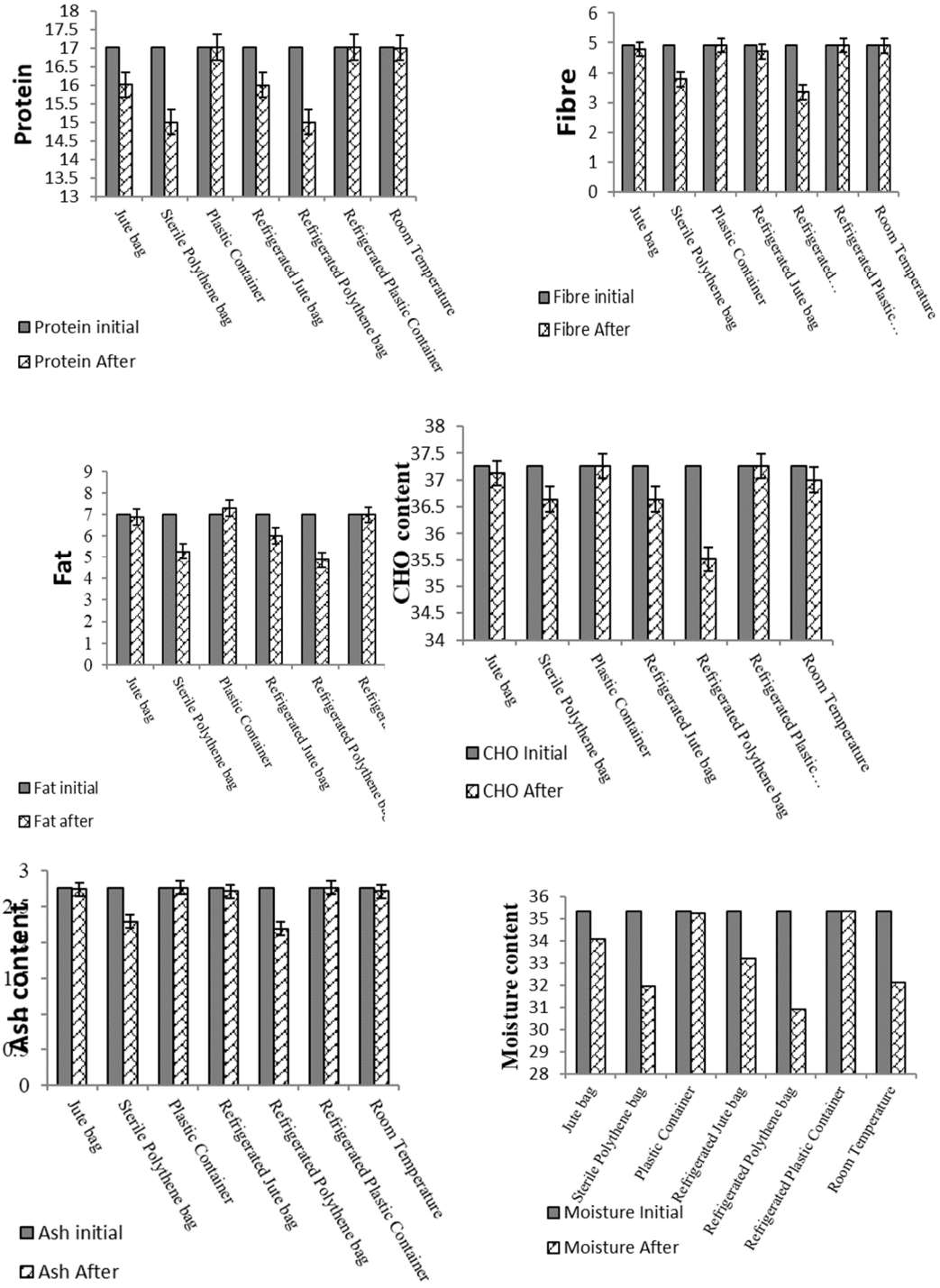

Figure 1: Proximate composition of the walnuts before storage and 90 days after storage

DISCUSSION

The result of this present study indicates that the mycoflora present in walnut is a factor of its packaging material. The microrganisms isolated mostly comprises of fungi of the *Aspergillus* and *Rhizopus* genera. They have been also known to be the major cause of aflatoxin production in nuts (Bankole *et al.,* 2005). It can also be deduced that the rate of aflatoxin production is greatly affected by packaging material.

From this result, it was observed that the walnuts kept in plastic containers at room temperature (37 °C) have the least aflatoxin level after 90 days of storage.

Aspergillus flavus, Aspergillus niger, Aspergillus paraticus, Aspergillus nominus produce toxins which are detrimental to human health (Oluwafemi 2012). Although some toxins can be inhaled but most times these toxins are often introduced into the person by ingestion of food containing these moulds and these toxins are very powerful (Gong *et al .,* 2002).

The isolation of moulds from the walnut after storage is in accordance with the study of Amnah and Alsuhaibani (2018) that fungal contamination can occur in field, during harvest, during transport and also storage.
Aflatoxins were detected in the walnuts which is in agreement with findings of (Reddy, 2010; Iqbal *et al*., (2014). It is also in agreement with previous reports of contamination of food items within and outside Nigeria (Jimoh and Kolapo 2008). The isolation of moulds from the walnut after storage and fungal contamination during harvest, transportation and also storage is in accordance with the study of (Kader and Hussein in 2009).

CONCLUSION

In view of the fluctuating availability of walnut all round the year, this study has been able to show that packaging materials play a vital role in preservation of the nuts. Hence, the study recommended that walnut must be stored in a controlled proper storage (temperature) as soon as possible after harvesting to control contamination by *Aspergillus spp*.

Aflatoxin has been a great detriment to both economy and health so any means of ameliorating its proliferation in nuts should be ensured. Walnuts are highly nutritional but seasonal, hence the reason for this study so as to have it all year round

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