INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
1560–8530/2004/06–6–955–959
http://www.ijab.org

Diversity of Fungal Species Associated with Peanuts in Storage and the Levels of Aflatoxins in Infected Samples

GACHOMO E.W.†, E.W. MUTITU AND O.S. KOTCHONI†[1]
Department of Crop Protection, Faculty of Agriculture, University of Nairobi, P.O. Box 29053 Nairobi, Kenya
†Department of Molecular Biochemistry, Institute of Plant Molecular Physiology and Biotechnology, University of Bonn, Kirschallee 1, D–53115 Bonn, Germany
[1]Corresponding author's e–mail: skotchoni@yahoo.com

ABSTRACT

The threat of aflatoxin contamination in food commodities and its association with health risks in both animals and humans continues to raise increasing concern over years. In this report, fungal species found in association with peanuts in storage and their potential to produce aflatoxin in collected samples was determined. About 60 to 70% of collected peanut samples were infected with various moulds including *Rhizopus stolonifer*, *Fusarium* sp., *Aspergilus flavus*, other *Aspergillus* sp., *Penicillium* sp., *Eurotium repens*, *Sclerotium* sp., *Rhizoctonia* sp., and *Aspergillus Parasiticus*. *Eurotium repens*, *Aspergillus Parasiticus*, and *A. flavus* were found to be the most patent aflatoxigenic strains. The average levels of aflatoxins detected in the seed samples were far above 100 ppb. This level of toxicity is more than five times higher than the acceptable dosage (20 ppb: US Standards) in edible peanuts. This report points out the health risks associated with aflatoxin contamination in edible food commodities despite enormous efforts to control this mycotoxin. Current research efforts to control or minimize the intake of aflatoxins especially in warmer regions of the world are hereby included.

Key Words: Aflatoxins; Food commodities; Fungal infection; Kenya; Peanuts

INTRODUCTION

Fungal infection of seeds before and after harvest remains a major problem of food safety in most parts of Africa. Problems associated with this infection include loss of germination, mustiness, mouldy smell (Sauer *et al.*, 1992; Frisvad, 1995) and aflatoxin contamination (McAlpin *et al.*, 2002; Bankole & Adebanjo, 2003). These problems are however dealt with, in most developed world where a careful commodity screening and improved storage conditions are provided (Ito *et al.*, 2001; McAlpin *et al.*, 2002; Wilson *et al.*, 2002). However, fungal species that produce mycotoxins are more common in the warmer, subtropical and tropical areas than in temperate areas of the world. Validated methods of analysis exist but an internationally accepted sampling plan for aflatoxin control for each commodity is still a targeted goal despite years of various contributions (Coker, 1989; Cunnif, 1995) and recently a so–called update on worldwide regulation for mycotoxin contamination was published by FAO under the title Food and Nutrition (van Egmond, 2002). In this edition, only 77 countries were reported to have specific regulations for mycotoxins, 13 countries were known to have no specific regulations, whereas no data were available for about 50 other countries many of them in Africa (van Egmond, 2002).

Aflatoxins are secondary metabolites produced by some isolates of *Aspergillus flavus*, *A. parasiticus*, and several unnamed fungi belonging to a non–classified taxon from Africa (Ito *et al.*, 2001). Developing grains, nuts and nut products such as peanut butter, roasted shelled peanuts and peanut oil are the most vulnerable to aflatoxigenic fungal infections (Rachaputi *et al.*, 2002). It has been a serious concern to control the increasing incidence of fungal infection and aflatoxin contamination of valuable commodities. Aflatoxins are among the most potential mutagenic and carcinogenic substances known (Bankole & Adebanjo, 2003). Therefore, setting of internationally agreed tolerance levels of aflatoxins in foods and feedstuffs is of global importance. Currently aflatoxin B_1 is the major contaminant of foods in tropical regions of Africa and this, has been linked with hepatitis B and C infections and, to the high incidence of liver cancer in these regions (Montalto *et al.*, 2002; Elegbede & Gould, 2002). The high mortality rate of liver cancer patients in these regions indicates the seriousness of the issue (Montalto *et al.*, 2002). In addition, Li *et al.* (2001) found that the level of aflatoxin B_1, B_2 and G_1 in corn were significantly higher in the area with high incidence of human hepatocellular carcinoma, and the average daily intake of aflatoxin B_1 from the high–risk area was 184.1μg. Therefore, means of tackling this problem should be a priority. Molecular characterization of microbial genes involved in the regulation of aflatoxin biosynthesis pathway could provide ways to produce genetically modified organisms to permanently inhibit aflatoxin synthesis, especially during interactions between aflatoxin–producing fungi and plants.

In this study, the correlation of occurring fungal infection in stored peanuts and the level of aflatoxin content in the collected samples was investigated in different fresh–produce markets of Nairobi, Kenya (Eastern Africa). We present here evidence of various identified fungal infections and a very high detectable level of aflatoxins in the samples. In addition, proper monitoring programs, recommendations for minimizing the rate of aflatoxigenic fungal infection in food commodities and the risk of toxicity to the consumers are herein discussed.

MATERIALS AND METHODS

Reagents and chemicals. Unless stated otherwise, all reagents and chemicals used in this work were from Sigma and Merck's Company, Roch (Germany).

Sample collection. Unshelled peanuts were obtained from five fresh–produce markets within Nairobi (Kenya). The markets were identified as V, W, X, Y and Z. Two peanut varieties (Valencia Red: VR & Homa Bay local: HbL) were sampled from each of the markets V, W, and X while only one variety (VR) was available from the markets Y and Z. An average weight of 2000 g of peanuts per variety and per market was considered. Fungi were isolated from the peanuts under laboratory conditions using agar and blotter test methods according to Dhingra and Sinclair (1994).

Isolation of fungi using blotter and agar test. Under blotter test, sterile filter papers were aseptically placed in petri dishes and moistened with sterile distilled water to serve as moist chambers. All experiments were carried out under sterile conditions to avoid contamination. Eight hundred peanuts per variety and per market were considered for this test. Half of the seeds (400 seeds per variety) was surface–sterilized in 3% (v/v) sodium hypochlorite for 3 min and rinsed in three changes of sterile distilled water. The seeds were then placed on the filter papers and incubated at room temperature (23°C±2) for 14 days. The second half of the samples (400 seeds) were not sterilized but were incubated under similar conditions. The results recorded represent means (±SD) of triplicate experiments.

For agar test, the set up was similar to that of blotter test except petri dishes containing 10 ml potato dextrose agar (PDA) were used as moist chambers. After incubation, colony characteristics (colour, shape) of different types of fungi that grew were recorded. The number of seeds infected with the same type of fungus was recorded. The individual isolates were transferred to new PDA plates in order to obtain pure cultures. All isolates were maintained on PDA and kept at 4°C for further analysis. The fungal identification was performed using microscopic observations, identification keys and illustrated manuals (Raper & Fennell, 1965; Klich & Pitt, 1998). Synoptic keys were used to identify different fungal genera.

Qualitative and quantitative analysis of aflatoxins produced by the isolates. The seeds were qualitatively analysed for the presence of aflatoxins according to Cunnif method adopted for aflatoxin analysis by the Association of Official Analytical Chemists (AOAC) (Cunnif, 1995). Basically, the method consists of an extraction phase, followed by a column clean–up phase and finally by a qualitative assay via a one–dimensional thin layer chromatography (TLC), which uses a silica gel adsorbent and an acidic solvent system as described by Kuiper–Goodman and Scott (1989).

For the quantitative estimation of aflatoxins, scanning densimetric analysis was carried out. The TLC plates were scanned according to the instructions of the manufacturer using CD 60 Desaga computer program. The program is set up to analyse the intensity of the spots developed by TLC. The peak areas of the samples were compared to those of the standards to quantify the aflatoxin content in the samples.

In order to identify the most toxigenic fungi, uninfected peanut samples were infected with the isolated fungi under laboratory controls and screened for aflatoxin production after two weeks of incubation as described above.

RESULTS

Isolation of the fungi from peanut samples. To better isolate the various fungal species from the infected fresh peanut samples, simple growth analyses made of agar plates and sterile moistened filter papers were carried out. Several fungal species became obvious from the growth media after seven days of incubation. From the seven days onwards, infected samples displayed fungal species, which were obvious enough for isolation. Fig. 1 presents an illustration of fungi screening by a blotter test showing different types of fungi growing from the peanut samples. From these tests, it was easy to evaluate the percentage of various fungal occurrences in the collected fresh peanuts. Table 1 shows sum total occurrence (in %) of fungal species in different markets samples using both agar and blotter methods. Of the several fungal species isolated from the peanuts, *R. stolonifer* was the most predominant with 80% occurrence followed by *Fusarium* sp. (45%) and *Aspergillus* species (24%). The other species accounted for 5 to 10% (Table I). The *Aspergillus* species isolated were *Aspergillus flavus*, *A. parasiticus*, *A. niger*, *A. ochraceous*, and the *Fusarium* species isolated include *F. oxysporum*, *F. equiseti* and *F. torulosum* (results not shown). The highest occurrence of *Aspergillus* species (*A. parasiticus*, *A. flavus* & the other *Aspergilli*) was in the markets V and X. Relatively high percentage of occurrence for *Rhizopus* sp. and *Fusarium* sp. was reported in all the samples collected, while the occurrence of *Penicillium* sp. and *Aspergillus* sp. was moderate and that of *Eurotium repens*, *Sclerotium* sp., and *Rhizoctonia* sp. was low (Table I).

To ascertain whether the fungal infection is on the surface or within the peanut seeds, the collected samples were surface–sterilized prior to incubation, and a

Fig. 1. *In vitro* screening of different fungal infections in peanut samples using blotter test; (a): *Penicillium* sp., (b): *Fusarium* sp., (c): *Aspergillus* sp.

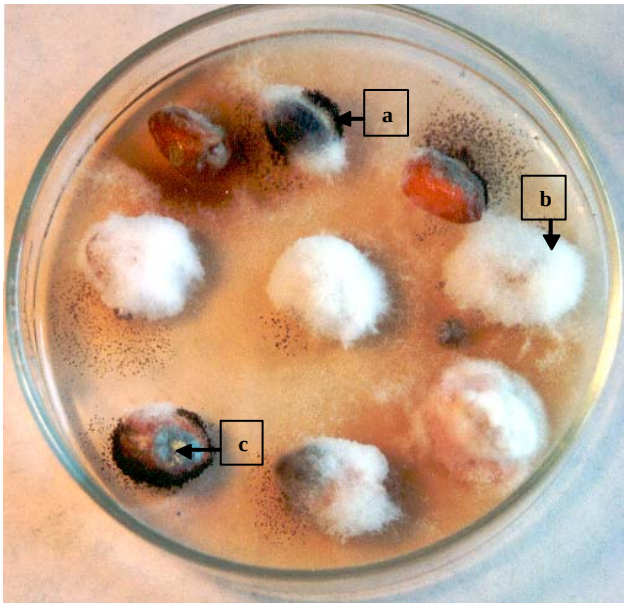
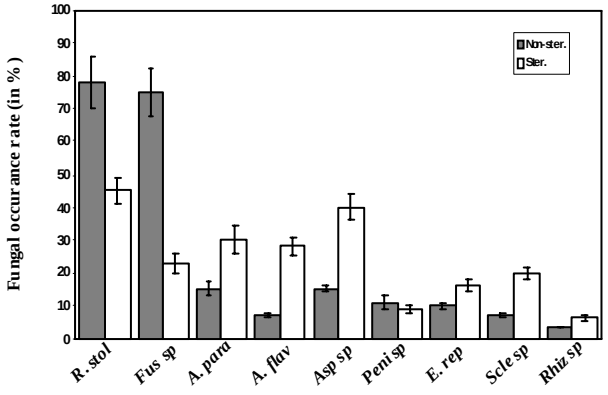


Fig. 2. Detection of fungal infections on surface-sterilized and non-sterilized peanut samples: *R. stol* = *Rhizopus stolonifer*, *Fus sp* = *Fusarium* sp., *A. para* = *Aspergillus parasiticus*, *A. flav* = *Aspergillus flavus*, *Asp sp* = other *Aspergillus* species including *A. niger* and *A. ochraceous*, *Peni sp* = *Penicillium* sp., *E. rep* = *Eurotium repens*, *Scle sp* = *Sclerotium* sp., *Rhiz sp* = *Rhizoctonia* sp.



comparative analysis was carried out with the non–sterilized sample cultures. Fig. 2 shows the rates of occurrence of different fungi on the surface–sterilized and non–sterilized peanut samples. *Rhizopus stolonifer* and *Fusarium* sp. had the highest occurrence rate in both surface–sterilized and non–sterilized peanuts but this occurrence was higher in the non–sterilized samples. *Aspergillus* sp. (*A. flavus*, *A. parasiticus* & the other *Aspergilli*) had markedly higher incidence of occurrence in the surface–sterilized samples than the non–sterilized ones. A similar pattern of occurrence was observed for *Eurotium repens*, *Sclerotium* sp. and *Rhizoctonia* sp. (Fig. 2). However, the occurrence rate of *Penicillium* sp. was found to be almost equal (10%) in both surface–sterilized and non–sterilized samples.

Detection and estimation of aflatoxins in peanut samples. All samples were tested for aflatoxin contents. We carried out also in vitro fungal infection experiments of healthy seeds in order to identify the aflatoxigenic strains from the isolated fungal population. Fig. 3 shows the levels of aflatoxins in the peanuts from the sampling locations (Fig. 3a) coupled with the identification of the most aflatoxigenic fungi in the samples (Fig. 3b). Peanut VR was found to contain high levels of aflatoxins B_1, B_2 and traces of aflatoxin G_1. The peanut HbL however had relatively lower detectable levels of aflatoxin B_1 and B_2 (Results not shown). The aflatoxin contamination of the samples was generally associated with the isolation of strains such as *Aspergillus flavus*, *Aspergillus parasiticus*, and *Eurotium repens*. Fig. 3b shows the estimation of aflatoxins produced by the different toxigenic fungi. Among the species of *Aspergillus*, *A. flavus* produced 151.26 ppb and 130.5 ppb of aflatoxin B_1 and B_2, respectively, while *A. parasiticus* produced 159.3 ppb and 110.9 ppb of aflatoxin B_1 and B_2 respectively. Both *Aspergillus* sp. produce traces of aflatoxin G_1 (4.0 ppb) as shown in Fig. 3b. *Eurotium repens* on the other hand produced larger amounts of aflatoxin B_1 (160, 19 ppb), aflatoxin B_2 (140 ppb) and aflatoxin G_1 (75.26 ppb) and considered therefore as the most aflatoxigenic fungus in this work.

DISCUSSION

Interest in aflatoxin contamination of food and feedstuff arose from its association with disease and mortality in humans and animals. Up to date, practical strategies to control this mycotoxin are still under investigation. Mycotoxins of the greatest concern are aflatoxins, ochratoxin A, and fumonisins produced by *Aspergillus* sp., *Penicillium* sp. and *Fusarium* sp., respectively (Bullerman, 2002). These toxins are a major threat for public health particularly in warmer and tropical regions of the world, like in Africa where proper and accurate screening methods are lacking. We point out in this report, the ever–permanent concern of aflatoxin contamination for rural and urban communities of Africa. Aflatoxin content above 20 ppb in peanuts is considered very dangerous for human health worldwide (Coker, 1989; Cunnif, 1995; Wilson *et al.*, 2002). According to the Kenya Bureau of Standards, the total levels of aflatoxin content in peanuts intended for human consumption should not exceed 20 ppb, but in this report, the detection levels of aflatoxins in peanut samples were found to be about four to five times higher than the acceptable 20 ppb value (Fig. 3a, b). The presence of aflatoxigenic fungi in surface–sterilized samples (Fig. 2) demonstrates that a simple clean–up precaution before consumption would never safeguard the consumers from the risk of contamination. Using this study as an example, probably several other food commodities

Fig. 3. Estimation of aflatoxins in peanut samples per markets (a) and identification of aflatoxigenic fungal species (b): *A. para* = *Aspergillus parasiticus, A. flav* = *Aspergillus flavus, E. rep* = *Eurotium repens*: —: Limit of estimation accuracy, *: Aflatoxin content above limit of accuracy.

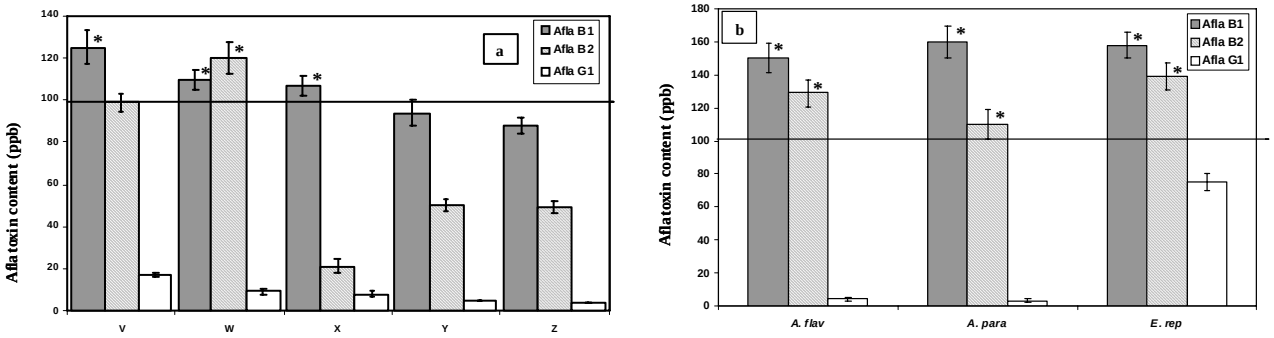


Table 1. Occurring rate of fungi isolated from peanut samples per market

	Identification rate of Fungal species (%) in peanut samples per market				
Fungal species	**V**	**W**	**X**	**Y**	**Z**
Rhizopus stolonifer	80 ± 9	90 ±10	85 ± 7	82 ± 6	88 ± 9
Fusarium sp.	50 ± 4	40 ± 6	20 ± 3	12 ± 2	30 ± 2
Aspergillus parasiticus	15 ± 4	5 ± 1	3 ± 1	2 ± 0	2 ± 0
Aspergillus flavus	3 ± 0.5	5 ± 1	50 ± 5	5 ± 0	3 ± 0
Aspergillus sp.	50 ± 6	20 ± 3	40 ± 5	10 ± 1	30 ± 3
Penicillium sp.	10 ± 2	11 ± 2	7 ± 2	5 ± 1	3 ± 0
Eurotium repens	5 ± 1	2 ± 0	3 ± 1	2 ± 0	2 ± 0
Sclerotium sp.	25 ± 2	30 ± 7	5 ± 0	2 ± 0	2 ± 0
Rhizoctonia sp.	2 ± 0	2 ± 0	2 ± 0	10 ± 1	2 ± 0

Aspergillus sp. including other Aspergillus such as *A. niger* and *A. ochraceous*. Data represent the mean value of triplicate experiments (± SD).

susceptible to aflatoxin contamination, such as cereal grains and tree nuts in the region although not investigated in this study, may contain high level of aflatoxins as well. The aflatoxin quantitative methods used here were not accurate above 100 ppb. Therefore, we were not able to ascertain the aflatoxin quantities exceeding 100 ppb in the samples (Fig. 3). However, these data were more than convincing to draw a conclusive remark i.e. consumers of fresh peanuts (non–processed) in Africa are exposed to the risk of high mycotoxin intake. The contamination may also result indirectly from consumption of animal products such as milk from livestock exposed to contaminated feed (Bankole & Adebanjo, 2003). These are broadening effects of aflatoxin contamination that one should take into account for accurate risk evaluation of aflatoxin contamination in a given region.

Several contributions about aflatoxin detoxification using dietary clay and isothermal adsorption of aflatoxin contamination have been documented (Grant & Phillips, 1998; Phillips, 1999). However, it is far much better to minimize or avoid contamination of products if possible, rather than to depend on detoxification. In addition biological control processes such as competitive exclusions of toxigenic fungi by use of different *Aspergillus* mutants are of tremendous contributions to the control of aflatoxin accumulation both in pre– and post–harvest seeds (Wilson *et al.*, 1986; Cotty & Bayman, 1993). However, several of such controls are expensive for farmers to implement profitably and accurately especially in developing world. Cotty and Bayman (1993) reported that atoxic *Aspergillus* species competed successfully with toxic isolates in a mixed culture condition, but the competition mechanism is still not well elucidated. Some atoxic aflatoxigenic fungi may be potential producers of several other toxins, which might be harmful both to humans and animals. For these reasons more information should be rather generated about the storage conditions repressing the aflatoxin contamination worldwide and especially in regions where farmers are still holding tightly to the traditional methods of storage. In other words, resources should be oriented into making scientific findings more adaptable for the traditional farmers.

Developing post–harvest strategies for sorting or any other aflatoxin control measures in warmer, tropical and subtropical regions should be therefore highly welcomed. Aflatoxin formation in peanuts is favoured by prolonged period of drought associated with soil–elevated temperature (Wilson *et al.*, 2002; Rachaputi *et al.*, 2002; Bankole & Adebanjo, 2003). Irrigation of the peanuts while still in the fields especially in warmer and tropical regions of the world could be therefore an effective option in reducing aflatoxin contamination. It was also suggested that late season irrigations could increase soil moisture and decrease soil

temperature and thereby be used as a promising way to lower aflatoxin content in mature seeds (Wilson *et al.*, 2002). Moreover, sorting the peanuts to remove damaged seeds before storage could also be a fairly effective and cheaper way to control aflatoxin contamination. Udoh *et al.* (2000) demonstrated that aflatoxin contamination of stored commodities in five agro–ecological zones of Nigeria (West–Africa) was strictly related to storage practices. Seed integrity could therefore be maintained by observing proper storage conditions. It is imperative to avoid a long–term storage system in warmer regions. As demonstrated by Goel and Sheoran (2003) in stored cottonseeds, after 18 months of storage, the germination ability of the seeds decreased and the membrane deterioration increased with storage period, which could lead to a high probability of fungal infection.

On the other hand, several other mycotoxins such as ochratoxin A, and other potent toxic metabolites (not investigated in this study) are also thought to be present in the collected samples. The reason being that *A. flavus*, *A. parasiticus*, *A. ochraceus*, *A. niger* (all isolated in this study) were known as source of ochratoxins, cyclopiazonic acid, patulin, sterigmatocystin, gliotoxin, citrin production (Wilson *et al.*, 2002). *Penicillium* species isolated also from the collected samples are reported to produce cyclopiazonic acid, ochratoxins and sterigmatocystin (Wilson *et al.*, 2002). The principal reason for such recurring situation is mainly the lack of awareness in these regions. For the safety of human food and the welfare of consumers, it is imperative to educate the population on the danger of aflatoxin contamination and to screen for all possible mycotoxin contaminants in any given commodity before allowing it to be marketed. These screening steps should strictly receive a higher priority over any economical aspects. It is in this respect that the danger and risks of toxicity could be greatly minimized in years to come. The ever–present health risks to which the unsuspecting and ignorant public (especially in most of Africa regions) is exposed to is here clearly evident. The need for interdisciplinary cooperation involving governments, non–governmental organizations and scientists in this area in order to establish monitoring and regulatory risk management procedures has never been timelier.

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(Received 01 April 2004; Accepted 20 September 2004)