FUNGAL AND MYCOTOXIN CONTAMINATION OF SORGHUM DURING STORAGE

A thesis submitted to

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for the degree

of

MASTER OF SCIENCE

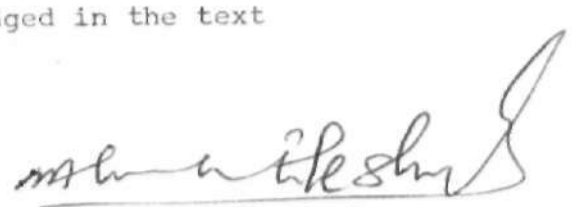
by

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November, 1978

This thesis is my own work. Any collaboration with colleagues is acknowledged in the text



J. A. Elegbede

To the memory of

Michael

Alice

Elijah

Christianah.

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SUMMARY

The studies described in this thesis have been mainly concerned with an investigation of the fungal and mycotoxin contamination of sorghum during storage. The effect of rainfall on the moisture content, the relationship between the moisture content and the mycoflora of stored sorghum were also examined. Sorghum samples were collected from villages in Giwa District of Kaduna State in March, August and November/December 1977.

The moisture content of stored sorghum was related to the rainfall distribution. When the rainfall was heavy the moisture content became high, and when the rainfall decreased, the moisture content also fell. While the moisture content of samples collected at different periods showed significant differences, the moisture content of samples collected from different farmers at the same period did not show such differences.

Field and storage fungi were; identified from surface-sterilised sorghum grains. The storage fungi replaced the field flora as the moisture content of sorghum increased during storage.

Aflatoxins were not detected in the samples. Other mycotoxins detected were ochratoxins (A,B,C), zearalenone and sterigmatocystin. The level of mycotoxin contamination detected was generally low. The infrequent occurrence of, and the decline in number of samples showing mycotoxin contamination as storage

progressed led to the suggestion that fungal activity in the stored sorghum was minimal. This was probably due to the good storage method; allowing good aeration but keeping water out. Thus the mycotoxins detected were believed to be from the field fungi rather than from the storage fungi.

Therefore, if sorghum is the principal source of dietary mycotoxins, the risk of exposure of Nigerians to mycotoxins is minimal.

INTRODUCTION

Sorghum (Sorghum spp., guinea corn; Hausa dawa) is a staple food for many people in Nigeria. It is widely cultivated throughout the country though to a greater extent in the Northern part due to climatic and other factors. Sorghum, like other cereal grains, is susceptible to fungal contamination both in the field and during storage. When conditions of temperature and moisture are suitable, these fungal contaminants produce secondary metabolites toxic to man and animals. Such substances are referred to as mycotoxins.

The mycotoxins show acute toxicity in large doses, and cause liver cancer and other diseases in sublethal doses in a wide range of animal species. It is therefore evident that fungal (and, invariably, mycotoxin) contamination of food and feeds could be a potential public health hazard.

In this study, emphasis has been directed at the mycoflora and mycotoxin contamination of sorghum developing during storage and its possible consequences for human and animal health.

1. The Problem of Mycotoxicosis.

Fungal and mycotoxin contamination of food and feeds is not a recently observed phenomenon. Ergotism, produced by ingestion of ergot derived from Claviceps purpurea growing on rye, was referred to as St. Anthony's Fire in the Middle Ages (Davidson et al., 1975, van Rensburg and Altenkirk, 1974).

It is known to affect man, other mammals and birds (see van Rensburg and Altenkirk, 1974; Martin and Gilman, 1976). Toxicoses due to a variety of other mycotoxins have also been reported (Mitchell, 1918; Theiler, 1927; Schwarte et al., 1937; Schwarte, 1938; Biester et al., 1940; Morgan, 1940).

In the early 1960s, renewed interest in mycotoxicoses was generated when thousands of young turkeys died in the south of England from a previously unknown disease which was referred to as"Turkey X-disease" (Blount, 1961). The cause of the disease was traced to the metabolite of a mould, Aspergillus flavus Link ex Fries (Sargeant, et al., 1961), which was present in the feedstuff. Since then, a number of other outbreaks of toxicoses have been reported in pigs (Forgacs and Carll, 1962; Noskov and Sharapov, 1965; Pelhate, 1966) and cattle (Meshkov, 1962; Aust et al., 1963; Albright et al., 1964). In the last 17 years or so, a large and expanding literature has developed on the subject of mycotoxin and fungal contamination of food and feeds.

Mycotoxicoses have been defined as intoxications of animals and men caused by ingestion of mycotoxins produced by fungi (Krogh, 1969). It therefore follows that a consideration of mycotoxin contamination of food must also include the fungal contamination of the food. The presence of one does not guarantee the presence of the other at the same time. However, as stated by Bullerman (1974), "while occurrence of toxin-

producing moulds in a product does not necessarily mean that mycotoxins are present, knowledge of the contamination of foods with these types of moulds is of interest, and can be significant when making an assessment of potential public health hazards associated with the flora of a product."

2. Occurrence of Fungi in Foods and Feeds.

Traditionally, the focus of attention on fungal contamination of cereals was directed towards problems of plant pathology and grain storage. However, since 1960, fungal contamination has been viewed in a wider context. Various workers have looked at the types of fungi invading crops before and after harvest and during storage, and the conditions that each fungus requires to survive at different times on different crops. Fungi can be classified into field and storage fungi (Christensen, 1965) although it has been realised that such a classification cannot be too rigid (Christensen et al., 1971).

The natural occurrence of fungi in cereal grains has been investigated. Tuite (1961), working on undamaged corn seeds taken from the fields in Indiana, found that Cephalosporium acremonium and Fusarium moniliforme were the most common fungi isolated from the samples. Other fungi isolated but at a lower frequency included Gibberella zeae, Alternaria spp. Penicillium spp., Cladosporium spp., Nigrospora oryzea, Mucor spp., and Diplodia maydis. On the other hand, Christensen et al. (1971) isolated Aspergillus and Penicillium spp., as the dominant fungi from corn damaged in the field.

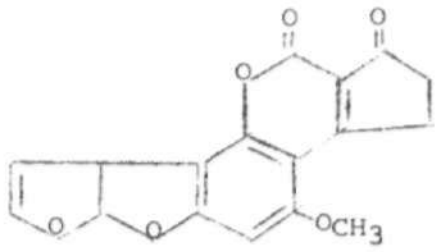
A number of workers have studied and identified the types of fungi developing during the storage of various grains (Machacek and Wallace, 1952; Mishra et al., 1969; Mislivec and Tuite, 1970a; and Scott et al., 1972). Species of Penicillium, Aspergillus Alternaria, Helminthosporium, Curvularia, Rhizopus and Gloeocercospora have been implicated as the main contaminants.

Factors and conditions that influence the invasion of cereals by fungi during storage have also been investigated (Lutey and Christensen, 1963; Gonen and Calderon, 1968; Christensen, 1970; Mislivec and Tuite, 1970b; Burroughs and Sauer, 1971). It is agreed that the moisture content and the temperature of storage play important roles; there is a critical moisture content and particular temperature below which cereals can be stored without the danger of invasion by storage fungi. Length of storage has been implicated as a factor in the survival of some common fungi (Lutey and Christensen, 1963; Machacek and Wallace, 1952). If the conditions of storage (moisture content and temperature) are conducive to invasion by storage fungi, the field fungi which had invaded the crop before harvest would die out; some rapidly (Alternaria spp., Fusarium spp., and certain species of Helminthosporium) while other species of Helminthosporium may die out slowly (Machacek and Wallace, 1952; Lutey and Christensen, 1963).

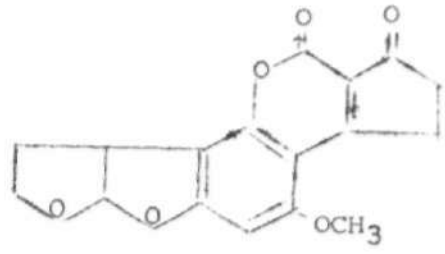
3. Natural Occurence of Mycotoxins.

As mentioned earlier, mycotoxicoses have been recognised for a long time dating back to the historical reports of ergotism. The next mycotoxin isolated and characterised was penicillic acid (Alsberg and Black, 1913) which was found in Penicillium puberulum. The list of naturally occurring mycotoxins is now very extensive. They have a worldwide distribution among cereals, legumes and their products (Scott, 1965).

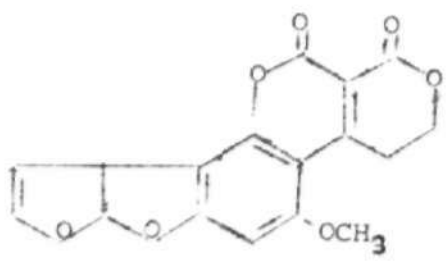
(a) Aflatoxin: Aflatoxins (Figure 1) have been the most extensively studied mycotoxins. They were first isolated from groundnut (Blount, 1961) as a metabolite of Aspergillus flavus. This mould has been demonstrated to have particular affinity for groundnuts as a substrate both for growth and aflatoxin production (Lafont and Lafont, 1971). However, it is generally agreed that aflatoxin is either totally absent from crops like groundnut harvested at the normal time (Ashworth, et al., 1965), or, if present, in minimal concentrations (Joffe, 1970; Barnes and Young, 1971) but that the level rises if the crop is harvested late and also during subsequent storage (McDonald and Harkness, 1967). Cereals and other foodstuffs have been reported contaminated with aflatoxin. Sorghum, sorghum flour, millet, wheat, corn, oats, soyabean, cassava and other items have been reported to contain aflatoxin to a varying extent (Coady, 1965, Shotwell et al., 1969a,b; Serck-Hanssen, 1970; Alpert et al., 1971; Richard and Cysiewski, 1971; Lillehoj et al., 1976).



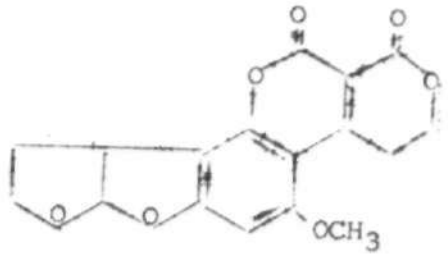
Aflatoxin B_1



Aflatoxin B_2

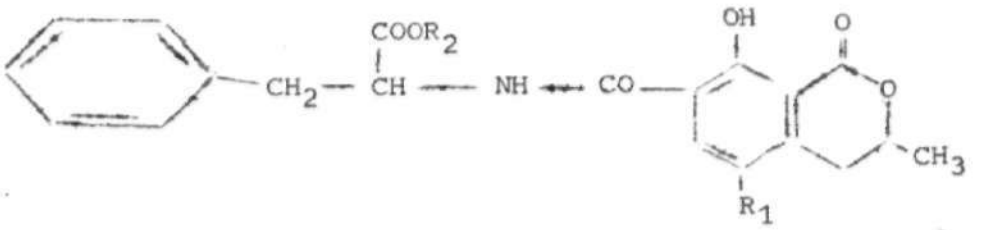


Aflatoxin G_1

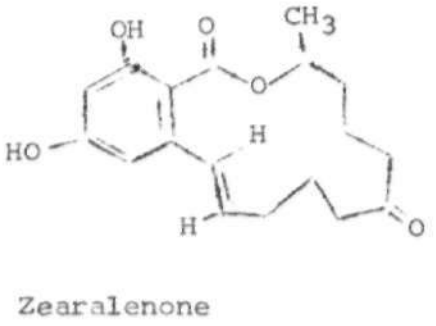


Aflatoxin G_2

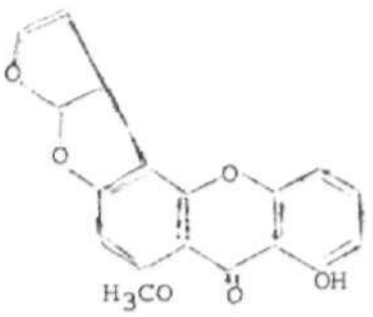
Figure 1. Structures of mycotoxins investigated in this study



Ochratoxin



Zearalenone



Sterigmatocystin

Figure 1. Structures of mycotoxins investigated in this study (contd.)

Ochratoxin A: R_1 = Cl; R_2 = H

Ochratoxin B: R_1 = H; R_2 = H

Ochratoxin C: R_1 = Cl; R_2 = C_2H_5

Other food items found contaminated included milk, milk powder, loaves of bread, cotton and groundnut products, cabbage fried with pork and garlic (Purchase and Vorster, 1968; Ashworth et al., 1971; Halliday, 1966a,b,c; Shank, 1971; Shank et al, 1972; Halliday and Kazaure, 1967).

(b) Other mycotoxins: Reports of natural occurence of other mycotoxins (zearalenone, ochratoxins and sterigmatocystin) are not as common as for aflatoxin. This may be due partly to the fact that their occurence has not been intensively investigated as has been aflatoxin or it could probably be that they do not occur frequently under natural conditions.

Zearalenone (Figure 1), a fusarial toxin producing hyperoestrogenism, has been found in a number of feedstuffs in North America, Europe and Japan. For example, zearalenone was detected in Japan in 43% of samples of swine feed which were submitted for analysis because they showed toxic effects (Mirocha and Schauerhamer, 1973; Mirocha et al., 1974); in 17% of samples from the 1972 corn crop in the US (Eppley et al., 1974); and in South Africa in samples of malted sorghum, 'sour' fermented porridge, beer made from maize and sorghum and mouldy maize off the cob (Martin, 1974).

Ochratoxin A (Figure 1) was first isolated from a strain of Aspergillus ochraceus Wilh. by van der Merwe et al. (1965). It was later isolated from maize offered for sale in the United States of America (Shotwell et al., 1969c).

It has been found in wheat in Canada. The first report was in rolled wheat for livestock feed which accidentally became wet (Scott et al., 1970b) and later it was found in 18 out of 29 samples of heated grain from Saskatchewan farms at levels between 0.03 to 27mg/kg (Scott et al., 1972). Similar results were found in Denmark where an outbreak of mycotic nephropathy in pigs was associated with the isolation of ochratoxin A from 54% of samples of feed barley (Krogh, 1973).

Sterigmatocystin (Figure 1) has been reported in samples of wheat by Scott et al. (1972) but it is not widely found.

4. Production of mycotoxins.

Mycotoxin production has been investigated extensively. However, care must be taken when interpreting the results obtained because the conditions under which the fungus grows and produces toxin in the laboratory may not be the same as those found in the field. Such studies, however, have been useful in indicating the substrate preference of each fungus and the shortest time in which toxins can be produced when conditions are optimal.

Several culture media have been tested for their ability to support fungal growth and mycotoxin production. Scott et al. (1970a) and Bullerman (1974) have tested yeast extract-sucrose, potato-dextrose-agar, Czapek-Dox broth plus yeast extract and others, and found them suitable to support the growth of different fungal species and production of mycotoxins to

varying degrees between 4 and 7 days. In a study involving the conversion of versicolorin to aflatoxin B_1 by Aspergillus parasiticus, Lee et al. (1976) developed a growth medium and a replacement medium suitable for this conversion after only 3h incubation.

The suitability of various foods as substrates for fungal growth has also been tested. Bassir (1964) examined some Nigerian foods as substrates and found that, under optimal conditions, gari (derived from Cassava farina) on its own supported the growth of Aspergillus flavus and the production of aflatoxin. Similarly, substrates such as wheat breakfast cereal (Nesheim, 1969), sterilised maize meal (van der Merwe et al., 1965) and cowpeas (El-Hag and Morse, 1975) have been found to support fungal growth and mycotoxin production.

The growth of Fusarium spp. and the production of zearalenone in stored wheat, maize, oats and barley were markedly influenced by the moisture content of the grain (Sherwood and Peberdy, 1974). When the moisture content was 24%, fungal growth and mycotoxin production occured even when the temperature was as low as 7°C. However when the moisture content was between 15 and 18%, the growth of the fungus and production of zearalenone increased as the storage temperature increased (Sherwood and Peberdy, 1974). Species of Penicillium, Aspergillus and Fusarium are the major mycotoxin producing fungi (Table 1).

TABLE 1. Major mycotoxin-producing fungi and their respective mycotoxins*

Fungus	Strain	Mycotoxin produced
Penicillium patulum	ATC 18172	Patulin
P. urticae	NRRL 1953	Patulin
P. terlikowski	HLX 136	Gliotoxin
P. viridicatum	69-22	Ochratoxin A; citrinin
Aspergillus flavus	MRE 2	Aflatoxins B_1,B_2,G_1,G_2; aspertoxin
	4 strains	Aflatoxins B_1,B_2; kojic acid
A. sulphureus	ATCC 18413	Aflatoxins B_1,G_1; ochratoxin A penicillic acid
A. versicolor	CM1 49124	Sterigmatocystin
A. versicolor	CM1 16139 and 64-45	Sterigmatocystin
Fusarium spp.	Mapleton 10	Zearalenone
	69-15 and 69-17	T-2 toxin
	66-57-3	Diacetoxyscirpenol

* Scott et al., 1970a

5. Effects of Mycotoxin Consumption on Animals.

Aflatoxins, which are most studied of all the mycotoxins in this regard, have been shown to be toxic to virtually all species of animal tested (Wogan, 1966; 1968). The toxicity can be either chronic or acute and the aflatoxins are also known to be carcinogens. Some animals are particularly susceptible such as turkey poults, ducklings, rainbow trout and guinea pigs whilst others such as mice and sheep are more resistant (Butler, 1973).

The nature and severity of toxicity depends on a number of factors such as the strain, sex, age and the nutritional and health status of the animals (Alpert and Davidson, 1969; Butler, 1971; 1973; Wogan, 1966). Males are more susceptible than females, young animals are more susceptible than older animals and low protein diets, cirrhosis of the liver and the concurrent ingestion of other hepatotoxins have been found to enhance aflatoxin toxicity (Wogan, 1966; Wogan and Newberne, 1967; Madhavan and Gopalan, 1965; Newberne et al., 1966).

The type of aflatoxin, dose and duration of exposure are also important. Aflatoxin B_1, which is the most common of the aflatoxins, is also the most toxic. It has an LD_{50} of less than 0.5mg/kg body weight in duckling which are particularly susceptible (Wogan, 1966). Aflatoxin G_1 is about half as toxic as B_1 followed by B_2 and G_2 respectively.

When the ingestion of sub-lethal doses of aflatoxin is prolonged, histopathological changes are observed. Liver damage occurs to a varying degree in ducklings (Wogan, 1966), rats (Lancaster et al., 1961) and monkeys (Tulpule et al., 1964; Alpert and Serck-Hanssen, 1970). Aflatoxin B_1 is also the most carcinogenic of known hepatocarcinogens. It produces cancer in rats when a diet containing as little as 15µg/kg of food is fed continuously (Wogan and Newberne, 1967).

The toxicity of ochratoxins, zearalenone and sterigmatocystin in experimental animals has also been demonstrated. The ochratoxins are acutely toxic to ducklings, rats, chicks and rainbow trout producing kidney and liver damage in these animals (Purchase and Theron, 1968; Scott et al., 1972). Ochratoxin A also induces foetal death and resorption in pregnant rats (Still et al., 1971) and, when fed with the co-carcinogen sterculic acid, it produces liver tumours in rats (Doster et al., 1971).

Zearalenone has been shown in swine to produce vulvar hypertrophy and occasional vaginal eversion in females, preputial enlargement in castrated males and prominent mammary glands in both sexes (Stob et al., 1962). A similar oestrogenic response is also observed when white weanling virgin female rats are injected with purified zearalenone (Mirocha et al., 1965).

Sterigmatocystin, which is structurally related to the aflatoxins (Birkinshaw and Hammady, 1957; Davis et al., 1960; Bullock et al., 1962), produces liver and kidney damage in rats leading to necrosis. It is not as powerful a carcinogen as aflatoxin, but the production of tumours with sterigmatocystin in rats has been reported (Purchase and van der Watt, 1968; Nel et al., 1971; Purchase and Vorster, 1970).

6. Implications for Human Health.

All evidence linking mycotoxins to diseases of the liver and other organs in humans, so far, has been indirect. The fact that the mycotoxins, particularly the aflatoxins, are toxic to experimental animals including primates, led to the suggestion that they might also be toxic to humans. The first definite statement implying a link between high concentration of mouldy diets, liver injury and liver carcinoma was made by Kraybill and Shimkin in 1964 although other workers had earlier suggested such relationships (Le Breton et al., 1962; Oettle, 1956, 1964).

It has been difficult to investigate the effects of mycotoxins on man because it is not possible to feed people with toxic substances to discover their effects. Indirect methods of investigation have, however, been employed. These include:

- studying the effect of mycotoxins in human tissue cultures
- examining post-mortem and biopsy specimens obtained from cases of mycotoxicosis and liver cancer, and

- studying the statistical data on liver cancer in different parts of the world in relation to eating habits and the concentration of mycotoxins in food.

Aflatoxins (particularly, B_1) have been studied extensively.

The mitotic division of human embryonic lung cells has been shown to be affected by as little as 0.01µg of aflatoxin B_1 in tissue culture. Application of higher levels produced abnormal morphological patterns with the production of giant cells (Legator and Withrow, 1964).

Post-mortem examinations of tissues from patients suspected to have died of cancer or liver disease has been another source of information linking aflatoxin to liver cancer. From such data on children who died in Thailand from an acute disease of unknown aetiology, aflatoxin B_1 was detected in one or more specimens from 96% of the cases. The autopsy specimens were liver, stool, stomach and intestinal contents, bile, brain, kidney and urine (Shank et al., 1971).

Workers in this laboratory have examined the aflatoxin B_1 concentration in samples of liver obtained from patients at post-mortem (Addy et al., 1977; Oloke and West, unpublished observations). Aflatoxin was found in 1 out of 4 samples obtained from patients who died from hepatoma (concentration, 3µg/kg fresh weight of liver) and in 5 out 69 samples obtained at random (3, 3, 10, 16 and 1080µg/kg). Of the samples obtained at random which contained aflatoxin, 1 died of carcinoma of the

bladder and at least 2 of the other 4 showed evidence of liver disease including enlargement and fatty infiltration. A report from the United States has also shown a high concentration of aflatoxin B_1 in a liver biopsy specimen obtained from a patient suffering from carcinoma of the rectum and liver (Phillips et al., 1976). Such findings are suggestive of an association between aflatoxin and primary liver-cell carcinoma (PLCC). However, it is difficult to come to any firm conclusions from such data especially as there would be a delay between the ingestion of aflatoxin and the development of PLCC.

The geographic distribution of cancer has some interesting correlations with the incidence of high fungal and mycotoxin contamination of the diet of the people. Studies carried out in Ethiopia, Kenya and Thailand have established that the high incidence of liver cancer in the populations corresponds to their high dietary aflatoxin intake (Coady, 1965a,b; Peers and Linsell, 1973; Shank, 1971; Shank et al., 1972).

In Uganda the incidence of hepatoma has been shown to be highest in the tribes that had the highest incidence of aflatoxin contamination in their diets (Alpert et al., 1971). Similarly in Swaziland, the frequency of aflatoxin contamination was found to be particularly high in provinces with a high hepatoma incidence (Keen and Martin, 1971a,b; Peers et al., 1976).

As for the other mycotoxins, the evidence has also been indirect. The similarity in the histopathology of primary liver cancer lesions produced artificially in the rat by sterigmatocystin

to that in human patients surveyed in Mozambique has been pointed out by a number of workers (Purchase and Vorster, 1970; Torres et al., 1970).

Cervical cancer, similar to that observed in some women in Lesotho and Swaziland, has been induced in rats and mice by oestrogens and by zearalenone. This similarity and the detection of zearalenone in beer and maize, led Martin and his co-workers to suggest that there could be a link between ingestion of zearalenone and cervical cancer in humans (Martin and Gilman, 1976). Although there is, as yet, no direct evidence, it has been suggested that a variety of other mycotoxins may have neoplastic potential in humans (Martin and Gilman, 1976; Alpert and Davidson, 1969).

Mention must be made here of the unresolved issue of whether the prevalence of cirrhosis and hepatoma in the Third World is due to nutrition or infective agents. Nutrition plays a role as a consequence of natural toxin ingestion, with or without an unbalanced intake of essential food elements; while the infective agent is the hepatitis B virus (see Coady, 1976). There are arguments for and against each aetiology and it has been suggested that an interplay between the two is involved. The evidence in support of a role for nutrition is, however, overwhelming.

7. Methodology

Several methods have been developed for the extraction and identification of mycotoxins. Many of such methods were developed mainly for the aflatoxins but have been extended, where possible, to include other mycotoxins. Generally, the methods involve the extraction of mycotoxins from the samples with organic solvents, purification of the extract, separation of the mycotoxins on thin-layer chromatographic (t.l.c.) plates developed in appropriate solvent systems and identification by visualizing under u.v. light. Modifications are introduced to cater for each mycotoxin.

The methods for the identification of mycotoxins in this project were developed from those used by Vorster (1969) for the analysis of cereals and groundnuts. Confirmation of identity of the mycotoxins was by comparison with standards on t.l.c. plates in different solvent systems and by applying different spray reagents specific for each mycotoxin (Scott et al., 1972). Other t.l.c. methods (Stoloff et al., 1971; Lee, 1965; Pons and Goldblatt, 1965), apart from that of Vorster (1969), were tried. Although satisfactory for some mycotoxins, the recoveries of sterigmatocystin and zearalenone were found to be poorer in these methods than in the method of Vorster (1969). Hence the latter was chosen.

The method used for screening for fungal contaminants was based on that used at the Department of Crop Protection Institute for Agricultural Research, ABU (IAR). The method

has been tested and proven over a long period of time.

8. Sorghum.

(a) The Nigerian races of sorghum: In 1958, a survey was carried out by workers at IAR in which different sorghums cultivated in Nigeria were collected. The sorghums were classified into seven 'races' (Curtis, 1966). In a definition similar to that of Anderson and Cutler (1942), the term 'race' was used to define a "group of populations in which there appears to be a constant association of characters".

The races of sorghum were found to be related to the common agronomic groupings and the 'species' as proposed by Snowden (1936) (Curtis, 1967). The races also corresponded broadly to certain of the subseries defined by Snowden although Curtis did not think that Snowden's classification at the species level could be applied to them. As he expressed it, "a broad division into seven races appears to correspond to the taxonomic realities of the cultivated sorghums in Northern Nigeria" (Curtis, 1967).

The characteristic varieties or races of sorghum grown in Giwa District (Figure 2) fall into three main categories. These are Farafara, Kaura and Mori (Table 2).

(i) Farafara (Figure 3): This is the name given to all white-grained sorghum varieties in Nigeria. Curtis (1966) restricted the use of this name to define a race that includes those sorghums with large, round grains protruding from adpressed

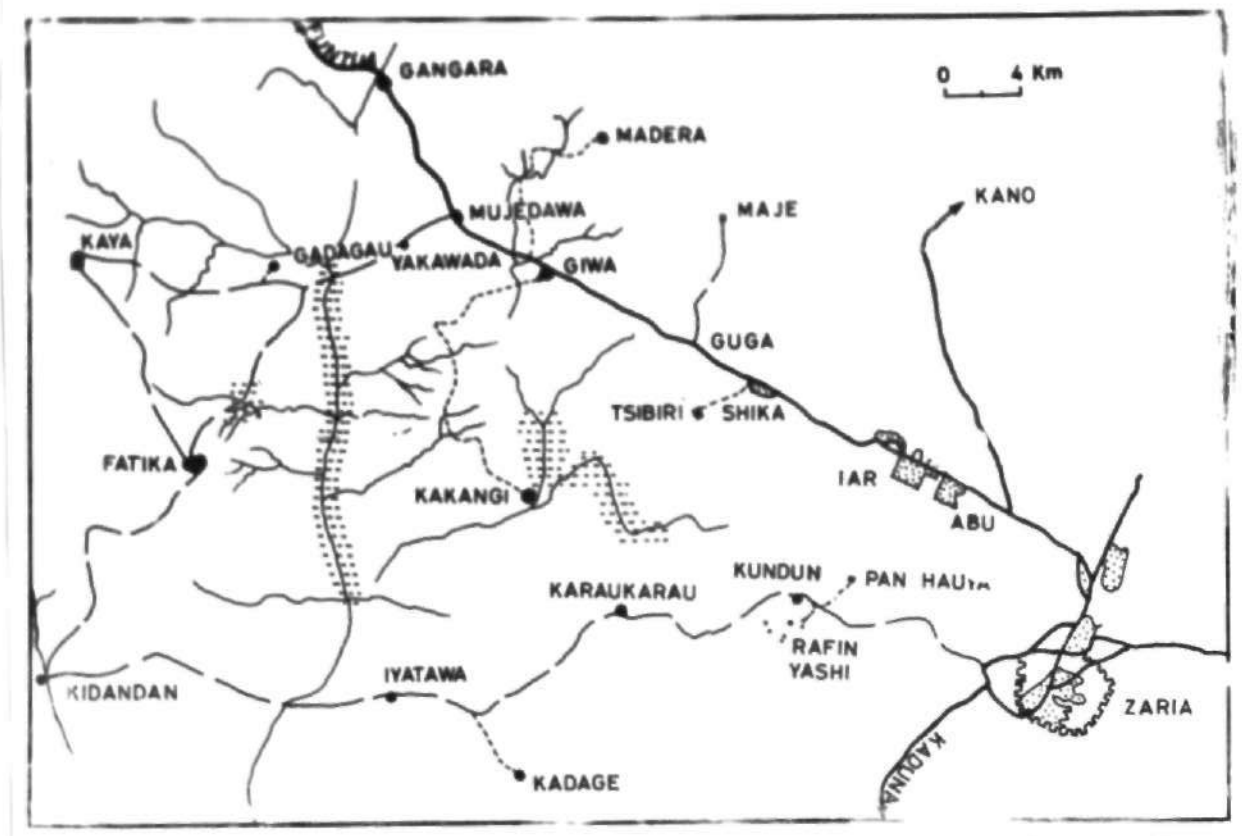


Figure 2. Map of Giwa District showing villages* where samples of sorghum were collected for study in 1977

* The villages are Gangara, Maje, Guga, Kadage and Pan Hauya

TABLE 2. Varieties of sorghum in each sample collected for study in 1977

Sample number	Month of sample collection		
	March	August	November/December
1	Mori	Mori	
2	Farafara	Kaura	
3	Kaura	Kaura	
4	Mori	Mori	
5	Kaura	Kaura	Jan dawa(red sorghum)
6	Mori	Mori	
7	Mori+Farafara	Farafara*	Mori*
8	Kaura	Mori	
9	Mori+Farafara	Mori+Farafara	Mori
10	Mori+Farafara	Mori	
11	Farafara	Mori	Farafara
12	Farafara	Farafara	Farafara
13	Kaura+Farafara	Kaura+Farafara	
14	Mori+Farafara	Mori+Farafara	
15	Mori+Farafara	Farafara	
16	Farafara+Kaura	Farafara+Mori	
17	Mori+Farafara	Farafara	Mori
18	Farafara	Farafara	Mori*
19	Mori	Farafara	Mori*
20	Mori	Mori	Mori

* Sample supplied by a different farmer than who supplied previous sample although from same village.

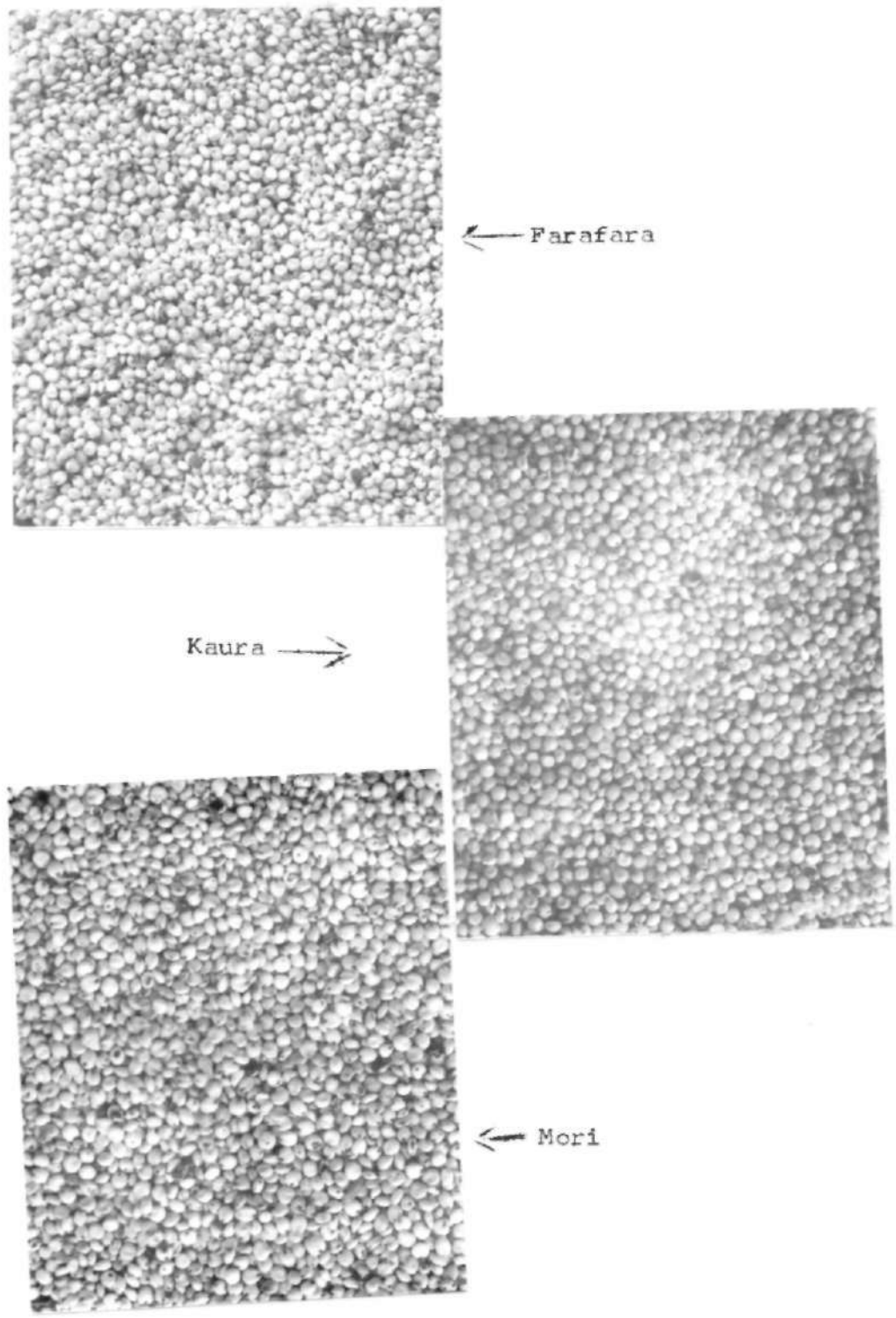


Figure 3. Grain of the principal varieties of sorghum collected from Giwa District in 1977

glumes. The grain has no dorsal hump, the base is not markedly wedge-shaped and the endosperm is never yellow.

(ii) Kaura (Figure 3): Sorghums belonging to this race have large, round grains which protrude from tightly adpressed glumes. The pericarp and endosperm are usually yellow. The grain is either biconvex or planoconvex with a dorsal hump. It is broadest above the middle, has rounded apex and wedge-shaped base.

(iii) Mori (Figure 3): It is believed that sorghums in this group do not belong to a stable variety, but are descendants from crosses involving Kaura varieties, thus they are not included as a race in the classification of Curtis, 1967. They are regarded as hybrids because they resemble segregants obtained at Samaru from crosses between Kaura and Guinea races (Curtis , 1967). However, the grains have no dorsal hump and the endosperm is yellow.

The other races of sorghum cultivated in Nigeria are Guinea, Sokoto, Chad, Umbellate and Tunicate.

(b) Planting and Harvesting: Traditional sorghum is grown as a rain-fed crop in most parts of Nigeria. In areas near Lake Chad, it is grown during the dry season because the fields are usually flooded at the end of the rainy season and the moisture is sufficient to support a crop without irrigation (Clayton, 1954; Curtis, 1967). In most parts of Northern Nigeria, traditional sorghum is planted in June at the onset

of the rainy season. It matures in October and is harvested in November/December.

During harvesting, sorghum stalks are cut and left in the furrows. After a few days, the sorghum heads are cut, left further in the sun to dry while the stalks are collected and used for other purposes. On drying, the heads are either tied into bundles or the grains are threshed from them before storage.

(c) Storage: Food storage constitutes a problem of great magnitude especially in the tropics. Several methods are employed by farmers and Government agencies for grain storage. Some of these methods are considered below.

(i) Rumbu: The rumbu (Hausa) is an elevated storage bin constructed of mud with zana matting or mud roof. It is usually constructed in the open within the family compound. It is used mainly to store unthreshed guinea corn or millet, which are tied in bundles or for groundnuts which are just put in the bin. The rumbu is constructed in such a way that rain cannot enter but so it is adequately aerated. This method is used mainly by farmers.

(ii) Underground pit: Usually, threshed grains are stored in underground pits. This method is used by some farmers and has been investigated for large scale use. A pit is dug, prepared and filled with sorghum or any other grain and then sealed off.

In a report, based on work carried out by the West African Stored Product Research Unit (WASPRU), Prevett (1962) suggested that sorghum intended for seed purposes should not be stored in underground pits for more than 15 months. Otherwise, deterioration in viability occurs. It was also observed that though the moisture content did not increase greatly, there is some doubt regarding the suitability of this method of storage because of an appreciable decrease in thiamine content of the sorghum. Other disadvantages of this method are that air and water leakages are usually experienced. These may contribute to a rise in moisture content of the grain, survival of pests and possible invasion of the sorghum by fungi.

(iii) Store-rooms: Threshed and bagged sorghum is usually stored in such rooms. The floor of the store-room is kept clean by sweeping periodically. Activities of insects or pests are controlled by fumigating with insecticide. This, however, has no direct control over invasion by fungi if other conditions are suitable. This method is widely used by farmers and government agencies to store their grains.

(iv) Other methods of storage: Various storage methods have been studied to evaluate their suitability for large scale storage programmes. So far, only storage in bags is practised on a large scale (Ozburn et al., 1960; Halliday,

1966c). The threshed grain is bagged and the bags are stacked and covered with plastic sheet against rain. Other methods recommended are bulk storage in silos and hermetic storage (Ozburn et al., 1960; Caswell, 1962). Storage in silos has been found to be good over a short period, the grains still being in good condition, while hermetic storage in 44-gallon drums has been recommended for storage over a long period of time (Caswell, 1962).

9. Aims of the Project.

This research project is part of a broadly based research programme into the mycotoxin contamination of cereal grains and other foods and the possible consequence for human health. This investigation focuses attention upon sorghum as it is the most widely grown cereal in Northern Nigeria. Attention has been directed at toxins developing during storage as the development of toxins in the field has been extensively studied by workers in IAR.

10. The Raison d'etre for the Research.

The need for such a research cannot be over-emphasised. The acute toxicity of mycotoxins to animals and man was mentioned earlier. Mycotoxins and other causative agents have also been implicated in producing cancer in experimental animals. Moreover, the incidence of primary liver-cell carcinoma (PLCC) is high in tropical regions of Africa and Asia (Wogan, 1975).

The incidence of PLCC as a proportion of total carcinomas in Northern Nigeria has been calculated as 20.2% from biopsy and clinical data collected at the teaching hospitals in Zaria and Kaduna (Edington and Greenwood, 1976). This value is one of the highest in the world compared with 3% in temperate countries, 10% in Uganda, 20% in South Africa but with amazingly over 60% in Mozambique (Stewart, 1965; Doll, 1969; 1977). There is evidence from work in Kenya, Uganda, Thailand and Mozambique that the high incidence of PLCC is correlated with a high ingestion of aflatoxin (Peers and Linsell, 1973; Alpert et al., 1971; Shank, 1971; Shank et al., 1972). However, this does not, ipso facto, demonstrate that aflatoxin produces PLCC.

The discovery of aflatoxin in groundnut in the early 1960s has tended to overshadow the problem of mycotoxins in other foods. As groundnuts were a major export crop from countries such as Nigeria, much work was carried out in Nigeria to protect export earning and in Europe to protect consumers from potential hazard. Thus crops such as sorghum, which are grown mainly for local consumption, have been neglected.

In the early 1970s the drought in the Northern parts of Nigeria and neighbouring countries of the Sahel region resulted in failure of much of the sorghum crop. In order to overcome such problems workers at the Institute for Agricultural Research, Samaru have selected short-season varieties of

sorghum which would be suitable in such drought conditions. These mature earlier than the traditional varieties, have higher yields and protein content and many have good resistance to mould and insect infestation during the growing season. The short-season varieties have a certain degree of acceptance with local farmers (El-Rouby, personal communication 1977).

However, during 1976, the rains in Northern Nigeria continued until late in October which is about one month later than average. Consequently both the traditional and short-season varieties matured during the rains; the short-season varieties almost being ready for harvest before the rains stopped. Thus both types of sorghum became infected with Phoma and Rhizopus causing head mould disease (Figure 4); the short-season varieties particularly so (Salifu, 1979). In addition, the short season varieties became infected with other species of fungi including Aspergillus, Penicillium and Fusarium which resulted in the production of aflatoxin, zearalenone and patulin (Salifu, 1979).

As fungi and mycotoxins can develop during storage of sorghum as well as in the field, it was decided to investigate their development during storage. The investigation was carried out during 1977 in villages in the Giwa district of Kaduna State.

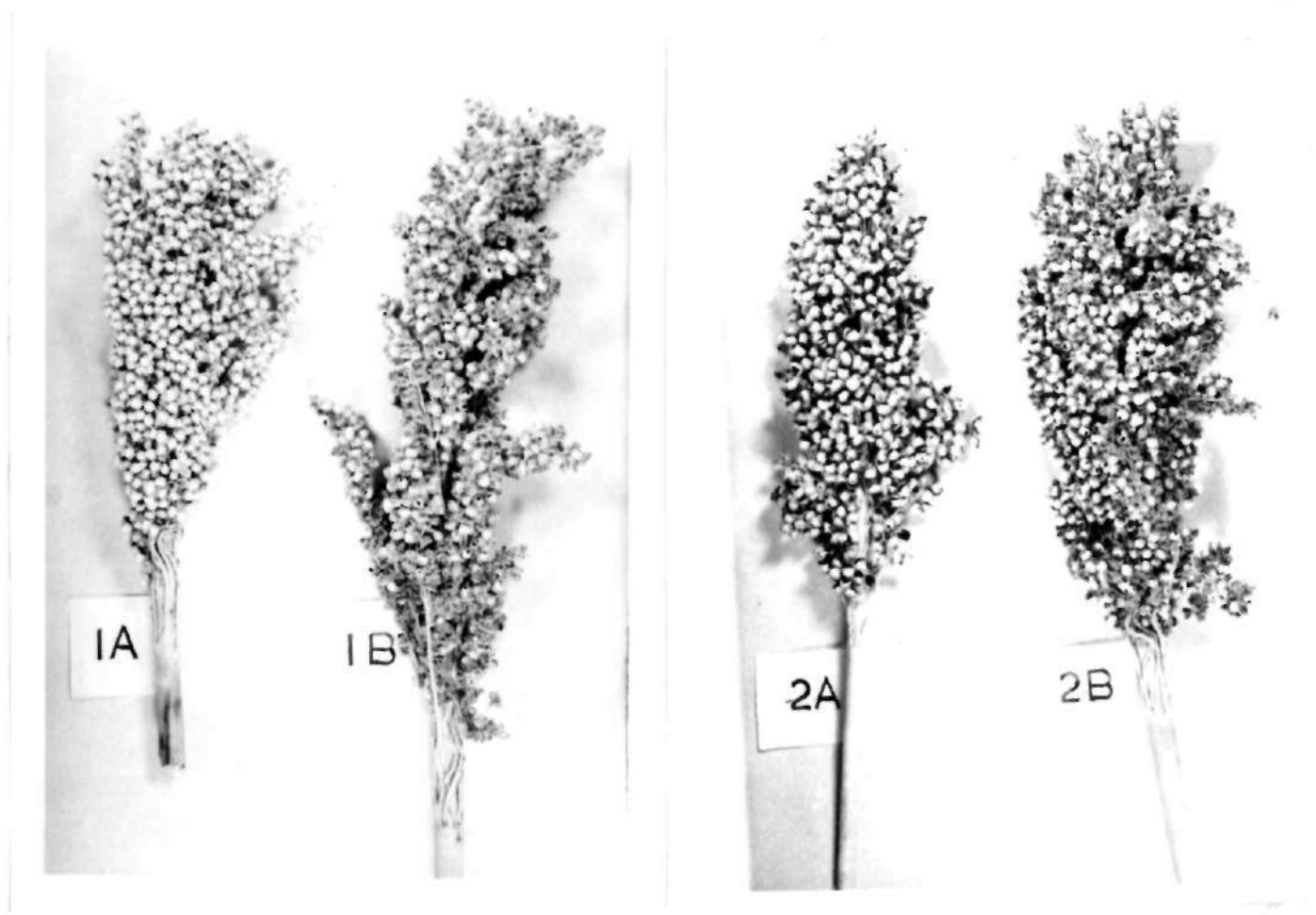


Figure 4. Headmould disease in sorghum

A = clean sorghum head; B = infected with headmould

1 - race, Farafara; strain, MN 1056

2 - race, Farafara; strain, Wad-Abr 51/3 Sudan

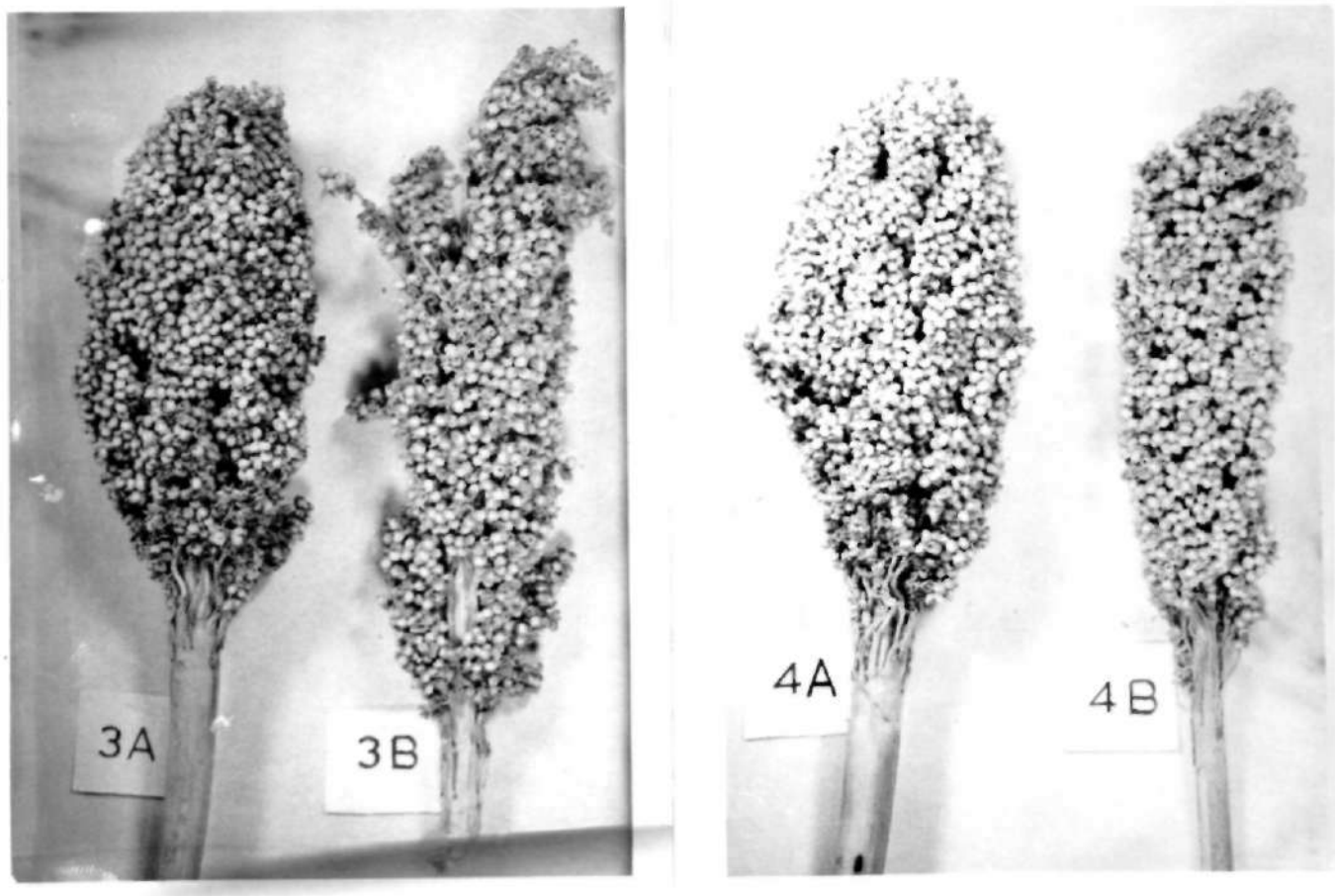


Figure 4• Headmould disease in sorghum (contd)

A = clean sorghum head; B = infected with headmould

3 - race, Mori; strain, Senegal synthetic

4 - race, Mori; strain, Ramada

MATERIALS AND METHODS

1. Chemicals.

Analytical reagent grade chemicals were used without further purification. Other chemicals, in particular solvents, were redistilled before use. Silica gel G-HR for thin-layer chromatography was obtained from CamLab Limited, Cambridge, England. All other chemicals except the mycotoxin standards were supplied by BDH Chemicals, Poole, Dorset, England and Hopkins and Williams, Chadwell Heath, Essex, England.

Mycotoxin standards were obtained from Calbiochem, Bishops Stortford, Herts, England (aflatoxins B_1,B_2,G_1,G_2, and Ochratoxin A), Dr. S. Nesheim, Department of Health Education and Welfare, Washington D.C., USA (aflatoxins B_1,B_2,G_1,G_2; ochratoxins A,B and C), Dr. L. Leistner, Federal Meat Research Institute, 865 Kulmbach, Germany (aflatoxin B_1) and I.M.C. Chemical Group Inc., USA (zearalenone). The National Institute of Public Health, The Netherlands, kindly supplied quantitative (aflatoxin B_1) and qualitative (aflatoxins B_1,B_2,$G_1$and G_2) standards.

2. Collection of Samples.

Samples of sorghum, 2 measures (Hausa, mudu) or 3kg, were purchased from each of 20 farmers from 5 villages (Maje, Guga, Kadage, Pan Hauya and Gangara) in the Giwa District of Kaduna State (Figure 2). Three main varieties of sorghum were collected; Farafara, Kaura and Mori (Table 2). Sample collection was carried out in March, August, and November/December, 1977.

The method of collection was simple, involving the farmer taking the sample from the container in current use by the family. As outsiders are not allowed to enter the compounds, the collections could not be supervised to comply with standard methods. The farmers were, however, instructed to make the samples as representative as possible by mixing thoroughly before collection.

The first series of samples, collected in March, was obtained mainly from unthreshed bundles in the external, elevated covered storage bin (Hausa, rumbu) where it had been stored for 4-5 months. The grain was then threshed and mixed before sampling.

The second and third series of samples were collected in August and November/December after being stored for about 9 and 12 months respectively. The sorghum was being stored as grain in bags held in the rumbu or in store-rooms. Only 9 out of 20 farmers were able to supply samples at the third collection as the other farmers had sold off their grain after harvesting the new crop. The samples obtained were put in polythene bags, securely tied, labelled and transported to the laboratory.

On arrival of the sample at the laboratory, about 500 seeds were taken for mycoflora screening. A portion (about 500g) of the sample was ground in a mechanical mill and used for the determination of moisture and mycotoxins. The remainder of each sample and the ground material were transferred to glass bottles fitted with plastic screw caps and stored at -20°C.

3. Screening for Mycoflora.

Samples were screened for mycoflora immediately after collection. Saprophytes, which are the main surface contaminants of grain sorghum (Semeniuk, 1954; Majunder et al., 1965) were removed by the methods of Christensen (1970) and Burroughs and Sauer (1971).

About 500 grains of sorghum were shaken for 60 seconds in a solution of sodium hypochlorite (2%, w/v) and then rinsed twice in sterile water. One hundred kernels were picked randomly and placed on 10 plates (9cm diameter) of Potato Dextrose Agar (Irish potato, peeled and boiled, 200g; sucrose or glucose, 20g; commercial agar, 20g; distilled water, 1000ml) and incubated at room temperature (27-32°C) until the fungi grew out and could be identified in about 6 days. The plates were kept a further 4-6 days and examined occasionally to detect additional fungi. This was necessary because Aspergillus candidus, when present along with other species of fungi, often did not appear until the plates had been incubated for about 10 days (Christensen, 1970; Burroughs and Sauer, 1971).

The fungi were identified to genus by the colour and growth pattern of their colonies and by microscopic examination of their spores where in doubt. Plating and incubation of the seeds were carried out in a hood— an enclosed space with limited access for hands which could be sealed up after manipulations were carried out. Prior to use, the hood was

disinfected with a few grains of potassium iodide (approx. 1g) in ethanol (50% v/v, approx., 5ml) and sprayed with a solution of Dettol (Trade mark, chloroxylenol 4.8% w/v, Reckitt and Colman Pharmaceutical Division, Hull, England, 5% v/v). These procedures destroyed any fungal spores that might be present in the hood.

4. Determination of Moisture Content.

The moisture content of the samples was determined using a method based on those developed by Christensen (1970) and Burroughs and Sauer (1971). Modifications introduced included grinding of samples to increase surface area, use of a vacuum oven and a lower temperature. A sample (2.0-2.5g) of ground sorghum was transferred to a beaker, previously dried to constant weight, and dried at 80-85°C in a vacuum oven (50mm Hg residual pressure, LTE Qualivac, Laboratory Thermal Equipment, Greenfield Nr., Oldham England, connected to a Speedivac high vacuum pump, Model 2SC 20, W. Edwards and Co. Limited Manor Royal, Crawley, Sussex, England).

After 5h beaker plus sample were weighed. Drying was continued until a constant weight was achieved on three consecutive weighings at intervals of 3h. Prior to each weighing the samples were cooled for 15 min in a vacuum desiccator over sulphuric acid (sp.gr.1.84). The moisture content (%) was calculated as follows:

$$100. - \frac{\text{Loss of weight, g}}{\text{Initial weight of sample, g}}$$

5. Determination of Mycotoxins.

(a) Preparation of samples:

(i) Extraction: Samples (50g) of ground sorghum were extracted for 30 min with chloroform-methanol (4:1 v/v, 200ml) using a wrist-action shaker (Griffin and George Ltd., England). The resultant suspension was filtered through filter paper (Whatman No.1) using diatomaceous earth (Grade 1, Sigma Chemical Company, St. Louis , Missouri 63118, USA) as a filter aid. A clear pink-coloured filterate was obtained.

(ii) Clean-up of extract: The clarified extract was shaken with sodium bicarbonate (0.1M, 50ml), in a separatory funnel. The lower chloroform layer was drained into another separatory funnel and re-extracted with sodium bicarbonate (50ml). The lower chloroform layer was drained into a flask, labelled A and retained for further processing. The top aqueous layers were combined and acidified with hydrochloric acid (2M, 6ml). The pH changed from 9 to about 1.5 and the colour changed from pink to yellow/orange.

The acidified solution was extracted with chloroform (3 x 50ml). The lower chloroform layer was filtered through a bed of anhydrous sodium sulphate (5-10g) which was subsequently washed with fresh chloroform (20ml). The chloroform extract was evaporated to dryness under feduced pressure at 40^{o}C (Magnetic stirrer regulator hotplate, A. Gallenkamp and Co. Ltd., London EC2P 2ER, England) using a rotary evaporator

(Buchler Instruments, Fort Lee, New Jersey, USA) fitted with a vacuum pump (Model 1206, Lacy-Hulbert Croydon, England) and a water circulating pump (Model C16/300,Charles Austen Pumps Ltd., Byfleet, Surrey, England). The residue (Fraction I) was dissolved in benzene-acetonitrile (98:2 v/v, 1.0ml) and transferred quantitatively to a test-tube (4ml) fitted with a glass stopper (B10) prior to analysis by t.l.c.

The chloroform extract (A) was evaporated under reduced pressure at 40°C and the oily residue was dissolved in aqueous methanol (85% v/v, 100ml). The solution was transferred to a separatory funnel and the aqueous methanol solution extracted three times with petroleum ether (b.p. 60-80°C, 40ml). The combined petroleum ether extracts were further extracted with aqueous methanol (100ml). The methanol extracts were combined and the water content adjusted to 50% (v/v) by the addition of distilled water (140ml). The solution was extracted with chloroform (3x50ml)(Vorster, 1969).

In order to break the intractable emulsion formed, anhydrous sodium sulphate (2g) was added to the mixture. The chloroform extracts were filtered through a bed of anhydrous sodium sulphate (approx.15g) which was subsequently washed with fresh chloroform (20ml). The combined filtrates were evaporated to dryness under reduced pressure at 40°C and the residue (Fraction II) dissolved in benzene-acetonitrile as for Fraction I.

(b) Thin-layer Chromatography of Samples:

(i) Preparation of chromatoplates: Kieselgel G-HR (50g) was shaken with distilled water (90ml) for 2 min in a glass-stoppered flask. The resultant slurry was used to prepare thin layers (500µ) on five glass plates (20cmx20cm) using a spreader (Shandon Scientific Co. Ltd., London N.W10, England) and plate leveller (Quickfit Model CR 7/1, Gallenkamp and Co. Ltd, London EC2P 2ER, England). The plates were dried at room temperature for 30min taking care to reduce the amount of dust that could contaminate the plates. They were then activated for 2h at 100°C (Shotwell et al., 1969a), allowed to cool for 30min and then stored in a plate cabinet over self-indicating silica gel.

(ii) Qualitative analysis of mycotoxins: Aliquots (30ul) of Fractions I and II were spotted on separate plates together with qualitative standards as outlined in Table 3. The following amounts of mycotoxin standards were applied at each spot: aflatoxins B_1 and G_1(0.005µg), $B_2$and G_2 (0.0013µg); ochratoxins A,B and C (0.05µg); sterigmatocystin (0.1µg) and zearalenone (0.5µg). Spots were applied 2cm from the bottom of the t.l.c. plate using a spotting template as a guide. Plates were developed in a tank containing toluene-ethyl acetate-formic acid (5:4:1, by volume). In addition, plates spotted with Fractions I or II were developed in benzene-acetic acid (3:1 v/v, Steyn and Merwe, 1966; Vorster, 1969) and

TABLE 3. Spotting programme for qualitative analysis of mycotoxins on t.l.c. plates

PLATE 1.									
Spot number	1	2	3	4	5	6	7	8	9
Sample number	S_1		S_2	S_3		S_4	S_5		S_6
Ochratoxins (A,B,C)		x			x			x	

PLATE 2.									
Spot number	1	2	3	4	5	6	7	8	9
Sample number (Fraction II)	S_1		S_2	S_3		S_4	S_5		S_6
Aflatoxins (B_1,B_2,G_1,G_2)		x			x			x	
Sterigmatocystin		x			x			x	
Zearalenone		x			x			x	

other plates spotted with Fraction II were developed in chloroform-methanol (98:2 v/v, Vorster, 1969). After the solvent had advanced 15cm from the origin the plates were removed from the tank.

After the solvent had evaporated, the plates were viewed under longwave (375nm) and shortwave (254nm) u.v. light. Spots in the samples corresponding in R_f and colour to the standards were noted (see Table 9). Plates containing Fraction II of the samples were sprayed with potassium hydroxide (20% w/v) and examined immediately under longwave u.v. light for the presence of sterigmatocystin (Vorster, 1969). Sterigmatocystin was further confirmed by spraying with $AlCl_3.6H_2O$ (20% w/v, Stack and Rodricks, 1971; Stoloff et al., 1971) While the ochratoxins were confirmed by treatment with ammonia fumes (Scott et al., 1972)

(iii) Quantitative estimation of Mycotoxins: Mycotoxins were quantitatively estimated by the "comparison of standards" method (Official Methods of Analysis, 1970). Analysis was carrid out on those samples which had been shown to contain mycotoxin by the qualitative procedure.

A series of aliquots from the sample extracts and relevant standard mycotoxin solutions were applied to thin-layer plates (Table 4). The amount of material applied was estimated to produce spots with intensities of fluorescence which would enable comparison between sample and standard. The plates were developed and viewed as previously described. The toxin

TABLE 4. Spotting programme for quantitative estimation of mycotoxins by "comparison of standards" method on t.l.c. plates

Spot number	1	2	3	4	5	6	7	8	9
Sample extract, µl*	10		15	15		20	25		30
Mycotoxin standard, µl‡	5		5	5			5		

* The starting volume of extract spotted depended on result obtained in section 5b(ii).

‡ The amount of mycotoxin standard (e.g. ochratoxin B) spotted was sufficient to produce fluorescence at the level of precision by visual estimation.

concentration in the sample was calculated as follows:

Mycotoxin concentration (µg/kg sample)

$$= \frac{S \cdot Y \cdot V}{W \cdot Z}$$

where S = volume (µl) of mycotoxin standard spotted to give fluorescence intensity equal to that of material being evaluated on the plate

Y = concentration of mycotoxin, µg/ml

V = final volume of extract, µl

W = weight of sample extracted, g

Z = volume (µl) of sample extract spotted to give fluorescence intensity equal to S, the mycotoxin standard.

6. Recovery, Precision by Visual Estimation and Limit of Detection of Mycotoxins.

Ground sorghum samples (50g) free of mycotoxins were contaminated with known amounts of a mycotoxin. The sample was then subjected to the extraction procedure of each method. The mycotoxin recovered was quantitated (see Section 5b (iii). The method of Vorster (1969) gave better recoveries than the other tested methods.

The precision by visual estimation (Eppley, 1966) was investigated to determine the degree of accuracy of visual estimation of mycotoxin concentration on t.l.c. plates. Different quantities of a mycotoxin were spotted on t.l.c. plates,

developed in appropriate solvent and visualized under u.v. light. The concentrations of any two spots that showed identical fluorescence intensity were used in the calculation thus:

$$\text{Precision (\%)} = 100.\frac{G-H}{G}$$

where G = spot with higher concentration

H = spot with lower concentration but equal fluorescence intensity as G.

The limit of visual detectability of mycotoxins was evaluated by determining the minimum quantity of each mycotoxin detectable on t.l.c. plate. This was carried out by spotting different quantities of a mycotoxin on a t.l.c. plate. The spot where fluorescence was just visible under u.v. light after development was noted and the concentration of that spot is the smallest detectable amount of the mycotoxin.

7. Determination of the Concentration of Mycotoxin Standards.

The concentration of mycotoxin standard solutions were determined using u.v. absorption spectrophotometers (Rodricks and Stoloff, 1970; Stoloff et al., 1971; Jones, 1972) The spectrophotometers (Pye Unicam SP6-400, Pye Unicam Ltd., York Street, Cambridge, England; DB-GT spectrophotometer, Beckman Instrument Inc., Fullerton CA 9263, USA) were calibrated as described below.

(a) Calibration of the Spectrophotometers: Concentrated sulphuric acid (96% H_2SO_4, sp.gr.1.84, 1.0ml) was diluted and made up to 21 with distilled water to give a solution of H_2SO_4 (approx.0.018N). Potassium dichromate (125mg) was weighed, dissolved and made up to 1 litre with the sulphuric acid solution. The molarity (about 0.4mM) of this solution was calculated to three significant figures. Two accurate successive serial dilutions of the acid dichromate solution were made to prepare solutions of approximately 0.2mM and 0.1mM respectively. The absorbance of the three solutions (0.4, 0.2 and 0.1mM) was determined at 350nm in 1cm quartz cell against H_2SO_4(0.018N) as solvent blank.

The molar extinction coefficient (ε) for each solution was calculated using the equation:

$$\varepsilon = \frac{1000.A}{C}$$

A = absorbance

C = concentration in mM

The average of the three ε values gave $\bar{\varepsilon}$. The correction factor, CF, which must be between 0.95 and 1.05 for particular instrument and cells used, was determined by applying the equation

$$CF = \frac{3160}{\bar{\varepsilon}}$$

(b) Preparation of mycotoxin standard solutions: Mycotoxin standards received as dry films or crystals were dissolved in appropriate solvents (see Table 5) to give the nominal concentration. The mycotoxin standards received as solutions were either used directly or diluted with appropriate solvent to give the required concentration where necessary.

(c) Determination of Mycotoxin Concentration: The u.v. spectrum (330-370nm) of each mycotoxin solution was recorded against a suitable solvent blank. The concentration of each mycotoxin was determined by measuring absorbance (A) at wavelength of maximum absorption (usually 350nm) and using the following equation

$$\text{Concentration, ug/ml} = \frac{1000.A.\ MW.\ CF}{\varepsilon}$$

MW = molecular weight of the mycotoxin

CF = correction factor

ε = molar extinction coefficient of the mycotoxin.

TABLE 5. The concentration of mycotoxin standard solutions determined by spectrophotometry

Mycotoxin	Solvent	Nominal concentration (ug/ml)	λ_{max} (nm)	Absorbance (A)	Molecular weight	ε	Actual concentration (ug/ml)
Calibration of equipment using $K_2Cr_2O_7$ solution	$0.018N-H_2SO_4$	0.107mM	350	0.328		3062	
		0.213mM	350	0.645		3030	
		0.426mM	350	1.275		3015	
Aflatoxin B_1	Benzene-acetonitrile (98:2,v/v)	10	348	0.605	312	19800	9.72
Aflatoxin B_2		10	348	0.646	314	20900	9.27
Aflatoxin G_1		10	348	0.415	328	17100	8.149
Aflatoxin G_2		10	348	0.393	330	18200	7.25
Ochratoxin A	Benzene	40	333	0.410	403	5550	30.80
Ochratoxin B	"	40	320	0.105	369	6000	6.72
Sterigmatocystin	Chloroform	10	327	0.445	324	16220	9.25

Zearalenone was treated as described by Stolòff et al. (1971)

RESULTS AND DISCUSSION

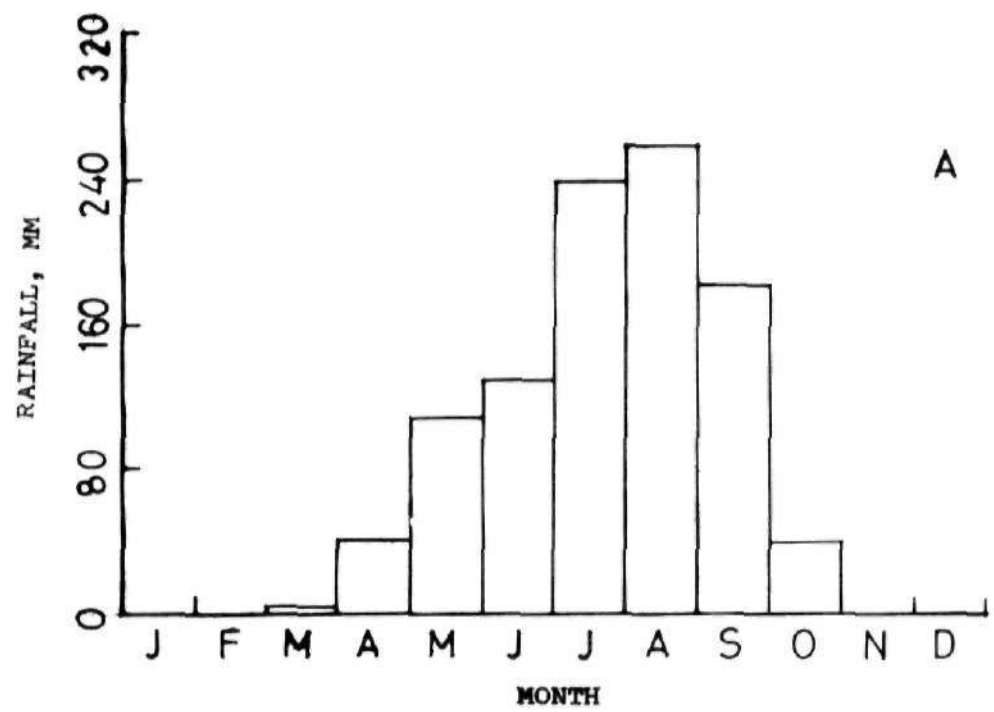
1. Effects of Rainfall on Moisture Content of Stored Sorghum.

Atmospheric humidity at any particular time is dependent on the extent of rainfall. This fact has been documented by several workers (McDonald and Harkness, 1963; 1964; McDonald, 1969; McDonald, Harkness and Stonebridge, 1964; Harkness et al., 1967). At the onset of the rainy season, the atmospheric humidity rises sharply. This may lead to an increase in the moisture content of stored products with a cosequent increase in fungal activity.

(a) Results

(i) Rainfall: Data, collected by the Meteorology Section of IAR at Samaru, were obtained for the years 1968 to 1977. The pattern for all years was similar. The rains started in late March/early April, and ended in October; July and August were usually the wettest months (Figure 5A, Appendices 1-3).

However in 1976 and 1977, the rainy season started late. In 1976, the first recorded rain at Samaru was in April while in 1977, it was in May (Figure 5B). The rain each month was higher in 1976 than in 1977, except for August and September (Figure 5B). An unusual feature of the 1976 rainfall was that in October, when the rain is usually low and drawing to an end, rain fell on 15 days extending to the 28th day of the month (see Appendices 2 and 3). There were also reports, though unconfirmed by rainfall registrations, that the rainy season in some parts of the Zaria area extended to 10th November.



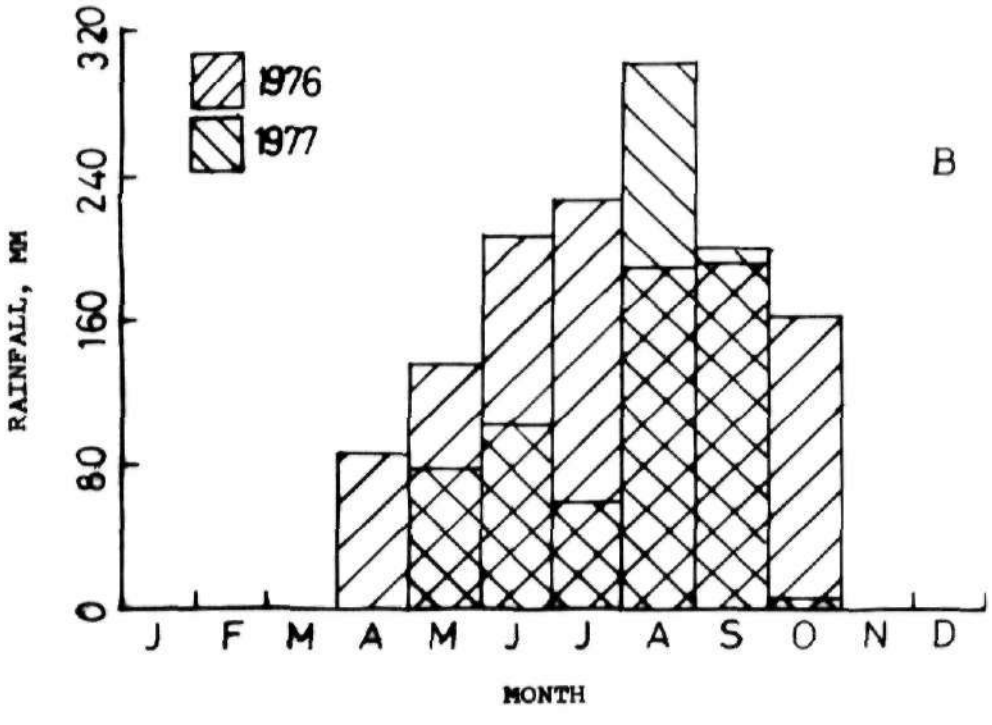


Figure 5. A = Mean monthly rainfall in Samaru between 1968 and 1977.
B = Mean monthly rainfall in Samaru in 1976 and 1977.

(ii) Moisture content: The moisture content increased from 8% in March to 12% in August and then fell to 9.5% in November/December (Table 6). These changes in the moisture content followed the same pattern as the rainfall (compare the data in Figure 5 with that in Table 6). However, the moisture content of sorghum samples collected from different villages at the same period showed no significant differences (Table 6, Appendix 4).

(b) Discussion

As would be expected, the moisture content of the stored grain followed the rainfall pattern. The rainfall distribution, as distinct from the total annual rainfall, is important in determining the period of time when the humidity will be increased thus leading to an increase in the moisture content of the grain and consequently, a higher risk of fungal contamination. For example, it has been observed in Thailand that aflatoxin contamination of foodstuffs was better correlated with local flooding than with overall rainfall or humidity (Shank, 1971; 1973).

2. *Grain Moisture Content and the Mycoflora of Sorghum during Storage*.

Cereals are subject to invasion by a great variety of fungi before harvest, after harvest and during storage. In the preceeding section, the variation of moisture content of stored sorghum grain during the period under study was

TABLE 6. Moisture content of sorghum samples collected during 1977

Month	Moisture content, % wet weight
March	7.95±0.160
August	12.06±0.115***
November/December	9.51±0.220*** ###

Results are mean ±S.E. In November/December only 9 samples were collected compared with 20 for March and August respectively.

Comparisons by Student t-test

with March *** $P < 0.001$

with August ### $P < 0.001$.

investigated. In this sedtion, the relationship between the types and quantity of fungi present in surface-sterilised sorghum and the grain moisture content will be examined.

(a) Results

The main fungal genera identified from surface-sterilised sorghum grains were Aspergillus, Fusarium, Penicillium, Curvularia, Phoma, Alternaria, Cheatomium and Helminthosporium (Tables 7 and 8). Other genera represented were Colletotrichum, Periconia, Rhizopus, Mucor, Trichotecium, Cephalosporium, as well as some bacterial colonies (Appendices 5-7).

Comparisons between contaminations found in March, August and November/December showed that the colonies of Curvularia, Cheatomium, Alternaria and Helminthosporium decreased as the storage period increased. Alternaria and Helminthosporium recorded higher numbers of colonies in August than in March. However, the numbers for November/December were lower than either March or August. On the whole, a pattern of disappearance of these fungi was established.

Conversely, the number of colonies identified of the genera Fusarium, Penicillium, Phoma and Aspergillus increased from March to November/December as the storage period lengthened. Aspergillus showed unusual behaviour because the number of colonies identified in August was lower than in either March or

TABLE 7. Major fungi, known to produce mycotoxins, identified from surface-sterilised sorghum seeds collected during 1977

Fungi	March	August	November/December
Aspergillus	3.7±0.99	1.8±0.46*	10.9±4.59*‡‡
Fusarium	12.9±1.44	13.3±1.49	18.2±2.97*
Penicillium	1.6±0.38	2.3±0.91	3.3±0.94*

Results expressed as numbers (mean ± S.E.) of colonies of fungi developing per 100 seeds.

Comparisons by Student t-test

with March * $P < 0.05$

with August ‡‡ $P < 0.01$.

TABLE 8. Fungi, other than those producing mycotoxins, identified from surface-sterilised sorghum seeds collected during 1977

Fungi	March	August	November/December
Curvularia	32.5±2.76	30.7±2.84	13.3±2.29*** ###
Phoma	26.4±2.50	35.6±4.47	39.2±7.72*
Alternaria	0.7±0.32	5.2±1.77	0###
Cheatomium	1.2±0.39	1.2±0.31	0.7±0.55
Helminthosporium	0	1.9±0.94*	0

Results expressed as numbers (mean ± S.E.) of colonies of fungi developing per 100 seeds.

Comparisons by Student t-test

with March * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$

with August # $P < 0.05$ ### $P < 0.001$.

November/December. However, the overall contamination increased because the contamination in November/December was greater than in the two previous collections.

(b) Discussion

The classification of fungi identified from cereals, introduced by Christensen (1965), was mentioned earlier. Fungi are divided into field, storage and advanced decay fungi. While Christensen regarded these as distinct groups, Pelhate (1968; 1969) recognised some flexibility. He subdivided the mycoflora of grain into three groups based on an ability to persist: ephemeral, mesobiontic and persistent. The first two groups comprise the field flora while the second and third groups comprise the storage flora, sensu Christensen (1965). However, the problem of too rigid a classification was later recognised by Christensen et al., (1971) when they observed the prevalence of storage fungi in harvested maize that had been damaged.

The division into field and storage fungi will be applied. Caution will, however, be exercised bearing in mind that field fungi can persist into storage while storage fungi have been found in freshly harvested cereal damaged in the field.

Field fungi: Fungi that have been classified into this group are Alternaria spp., some species of Fusarium and Helminthosporium (Lutey and Christensen, 1963; Christensen, 1970); others are species of Cheatomium, Curvularia and Cladosporium

(Burroughs and Sauer, 1971; Pelhate, 1968). These fungi invade cereals in the field hence the name; but they are also known to persist for a long time after harvest. For example, Fusarium has been found to remain viable in some seeds for 4 years while Alternaria and Helminthosporium spp. have been identified from barley 7 years after being stored dry in the laboratory (Lutey and Christensen, 1963).

Storage fungi: The fungal genera classified into this group are Aspergillus, Penicillium, Phoma and some other species of Fusarium, (Christensen, 1970; Lutey and Christensen, 1963; Pelhate 1968). These fungi have been identified from stored products and are not usually found contaminating crops before harvest. The classification should not, however, be too rigid as mentioned earlier. For example, a species of Fusarium (F. moniliforme) which is known as a field fungus, was found to rapidly contaminate harvested maize with a high moisture content (Martinez et al., 1970).

A comparison of the results show that while the number of colonies of field fungi (Curvularia, Cheatomium, Alternaria and Helminthosporium) decreased as storage progressed, the number of colonies of storage fungi (Penicillium, Phoma, Fusarium and Aspergillus) tended to increase (Tables 7 and 8). The moisture content also rose to a peak in August and then fell to a lower value (but higher than in March) in November/December (Table 6). There appears to be a relationship between the fungal contaminants

and the moisture content. This can be explained: at the onset of the rainy season, the moisture content of stored sorghum rose to a level that facilitated invasion by storage fungi. Hence the field flora started to die out. This result agrees with reports of other workers (Lutey and Christensen, 1963; Martin and Gilman, 1976).

The comparatively slow disappearance of Curvularia from stored grain showed that it probably belongs to the type of fungi which die out slowly. It has been reported that some field fungi die out rapidly while others do so comparatively slowly from stored products even after the development of storage fungi (Machacek and Wallace, 1952). Some of the fungi identified belong to the genera known to produce mycotoxins (see Tables 1 and 7).

3. Mycotoxin Contamination of Sorghum.

The presence of some known mycotoxin-producing fungi was mentioned in the preceeding section. As mentioned earlier, this is neither a confirmation nor a rebuttal of the presence of mycotoxins in the sorghum grain. However, the presence of these fungi and the changes in the environment (temperature and humidity) which could enhance mycotoxin production in stored products (Sherwood and Peberdy, 1974), necessitated the investigation of stored sorghum for possible mycotoxin contamination.

Thin-layer chromatographic analysis was employed for this investigation. There are several methods which can be used. Preliminary tests were carried out to justify the choice of

method and to establish criteria for the identification of mycotoxins.

(a) Results

(i) Thin-layer chromatography of mycotoxins: The mycotoxins investigated were aflatoxins (B_1,B_2,G_1,G_2), ochratoxins (A,B,C), zearalenone and sterigmatocystin. The criteria for their identification (R_f in different solvent systems, colour of fluorescence under shortwave and longwave u.v. light before and after spray treatments) were established (Table 9). These criteria were used in the subsequent identification of the mycotoxins.

(ii) Recovery, precision and limit of visual detectability: The recovery of the method used (based on that of Vorster, 1969) for each mycotoxin is presented in Table 10. This method showed better recovery than other methods with which it was compared.

The precision by visual estimation on t.l.c. plates was found to be $\pm$20% for most mycotoxins (Table 10). This showed that the evaluation of the mycotoxins is accurate to only about $\pm$20%. The minimum quantity of each mycotoxin which would give detectable fluorescence under u.v. light was given as the limit of detection (Table 10). Thus if less than the indicated amount of a mycotoxin was present in the volume of extract spotted on t.l.c. plates (Tables 3 and 4), the mycotoxin would not be detected.

Sterigmatocystin was difficult to quantitate because of interference from lipid which caused the spots to spread or

TABLE 9. The relative mobility and fluorescence characteristics of mycotoxins investigated

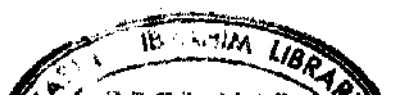
Mycotoxin	R_f Solvent system		Colour Type of u.v. light		Colour under longwave u.v. light after spray treatment		
	TEF	BA	Longwave	Shortwave	20% KOH	20%$AlCl_3{}^{6H_2O}$	Ammonia fumes
Aflatoxin B_1	0.51	0.55	faint blue	faint blue	bright blue		
Aflatoxin B_2	0.50	0.49	"	"	"		
Aflatoxin G_1	0.38	0.41	blue-green	faint-green	green		
Aflatoxin G_2	0.37	0.35	"	"	"		
Ochratoxin A	0.71	0.78	green	blue-green	bright green		bright blue
Ochratoxin B	0.67	0.71	faint green	blue-green	"		"
Zearalenone	0.73	0.73	blue-green		faint blue-green	blue	
Sterigmatocystin	0.83	0.77			dull yellow	brick-red	

TEF = toluene - ethylacetate-formic acid (5:4:1, by volume)
BA = benzene - acetic acid (3:1, v/v).

TABLE 10. The recovery, precision by visual estimation and limit of visual detectability of mycotoxins determined by t.l.c.

Mycotoxin	Recovery (%)	Precision (%)	Limit of detection (µg)
Aflatoxin B	85	±20	0.001
Aflatoxin G	85	±20	0.002
Ochratoxin A	100	±20	0.01
Ochratoxin B	95	±20	0.01
Zearalenone	80	±18	0.1

Each result is the mean of three or more determinations.



elongate. However, sterigmatocystin did give a better recovery with this method.

(iii) Qualitative analysis of mycotoxins: The mycotoxins detected were ochratoxins (A,B,C), sterigmatocystin and zearalenone (Table 11). The aflatoxins were not detected in any of the samples. However, some sample extracts yielded substances which showed blue fluorescence at R_f values close to the aflatoxins. When alternative solvent systems and spray reagent were employed, these substances behaved differently from the standards, showing they were not aflatoxins.

Some substances in a few extracts also behaved similar to ochratoxins A and B (R_f and fluorescence) in the TEF solvent system. In a different solvent system (BA), these substances showed different mobilities from those of the standards (see Table 9). Extracts that were actually contaminated by ochratoxins were thus identified. Contrary to expectations, the number of samples showing mycotoxin contamination decreased as the storage progressed.

(iv) Quantitative estimation of mycotoxins: Mycotoxin contamination was estimated in samples that showed positive results in Section 3a (iii). The level of contamination was found to be below 50ug/kg except as follows:

two samples in March contained 100 and 143µg/kg of zearalenone

TABLE 11. Results of the qualitative analysis for mycotoxins in sorghum samples collected in 1977

Mycotoxin	March	August	November/December
Aflatoxin B	0	0	0
Aflatoxin G	0	0	0
Ochratoxin A	3	4	1
Ochratoxin B	8	4	1
Ochratoxin C	1	0	1
Zearalenone	2	0	0
Sterigmatocystin	3	3	0

Results are expressed as total number of samples, from each collection, which showed detectable levels of the mycotoxin.

one in August contained 52µg/kg of ochratoxin B, and one in November/December contained 52µg/kg of ochratoxin A and 60µg/kg of ochratoxin B (Table 12, Appendix 8)

Zearalenone contaminations were found only in the March sample while the highest level of ochratoxin contamination was recorded in one of the samples collected in November/December.

(b) Discussion

The failure to detect aflatoxins in any of the samples is comforting considering their acute toxicity and reported widespread detection in many foodstuffs. This probably points to good farm and storage practices by the farmers (see Stoke's report on 'Ferrumbu', 1978).

The presence of ochratoxins, zearalenone and sterigmatocystin, albeit, in a few samples is a matter for concern. Although these mycotoxins were detected in relatively low concentrations, their presence indicated the activities of fungi in the grain .

It is strongly suspected that the mycotoxin contamination was from the field rather than during storage. There is evidence in support of this postulate. The samples under study were affected by the unusually heavy and protracted rainfall of the 1976 planting season which resulted in headmould disease as earlier discussed. Furthermore, the number of mycotoxin-contaminated samples decreased as the storage progressed. The

TABLE 12. Quantitative estimation of mycotoxins* detected in sorghum samples collected in 1977

Concentration (µg/kg)	Ochratoxin A			Ochratoxin B			Zearalenone		
	M	A	ND	M	A	ND	M	A	ND
<20	0	0	0	0	1	0	0	0	0
21-30	3	3	0	0	2	0	0	0	0
31-40	0	1	0	5	0	0	0	0	0
41-50	0	0	0	3	0	0	0	0	0
>50	0	0	1	0	1	1	2	0	0

Results expressed as number of samples that showed a level of mycotoxin contamination within the given range.

M = March; A = August; ND = November/December.

* Sterigmatocystin and ochratoxin C were analysed qualitatively only.

fact that mycotoxins are not destroyed even though the fungi that produced them might have disappeared lends more weight to this argument.

CONCLUSION

The principal conclusions drawn from the work presented in this thesis concern the changes in the moisture content of stored sorghum and the relationship of such changes to the development of the fungal and mycotoxin contamination of sorghum.

In this study, the effect of rainfall distribution on the moisture content of stored sorghum was examined. The moisture content was found to follow the rainfall distribution pattern: low when rainfall was low and high when rainfall was high, since rainfall affects humidity. Although samples collected at different periods had different moisture content, the difference between moisture content of samples collected at the same time was not significant.

In the screening for fungal contaminants both field and storage fungi were identified. It was found that as the moisture content became high enough to allow invasion by storage fungi, the field fungi started to disappear from the stored sorghum. However, the moisture content did not rise to such a level to allow the proliferation of storage fungi as the number of colonies detected was quite low. This indicated that the method of storage was adequate.

The presence of mycotoxins in the stored sorghum was also investigated. The mycotoxins detected were ochratoxins (A,B and C), sterigmatocystin and zearalenone. These were detected at

comparatively low levels and in very few samples. Moreover, such mycotoxins are believed to have been produced by the fungi prior to harvest rather than by the storage fungi. Although some storage fungi known to produce mycotoxins were detected, no mycotoxins were produced probably because the conditions for such development were not optimal. The aflatoxins which are the most toxic of the mycotoxins and which have been reported to contaminate a variety of foodstuffs, were not detected.

What then is the risk of exposure of people in this area to mycotoxin ingestion when consuming sorghum? Sorghum is a staple food of many Nigerians with individuals consuming up to 500g per day. However, with the low level and infrequent occurrence of mycotoxin contamination together with the absence of aflatoxins, the danger to health would appear to be minimal. Furthermore, the practice of rejecting mouldy and discoloured seeds by housewives during food preparation also helps in minimising exposure. However, during periods of drought, famine or localised pressure on food resources, people often have little choice but to eat mouldy grain which may contain considerable amounts of mycotoxins. Even so, under normal conditions, the risk of exposure is minimal.

Much has been written on the possible role of mycotoxins in primary liver-cell carcinoma in humans. Mycotoxins may be a

causative factor as animal studies have shown that aflatoxins and sterigmatocystin can produce liver cancer (Barnes, 1970; Purchase and Vorster, 1970; Nel et al., 1971). However it is possible that other factors such as other food toxins and viruses may be more important in the development of the disease (Coady, 1976). Much more work needs to be done in the Nigerian scene to elucidate the possible role of mycotoxins in primary liver-cell carcinoma. Such studies should include an examination of:

i) the geographical distribution of PLCC in Nigeria as obtained from Hospital records as it is known that the incidence of PLCC is high in the region

ii) urine and faeces for the presence of mycotoxin metabolites in areas with high incidence of PLCC

iii) the level of mycotoxin contamination of diets in such areas of high incidence.

" Unlike East Africa and South-East Asia, West Africa as a 'cancer ecology' has not hitherto been systematically studied with respect to mycotoxin exposure of human populations" (Coady, 1976). It is hoped that by the time the areas suggested for further investigation are studied, the challenge of Coady's statement would have been met in part. Although small, this present work is a contribution to determining the involvement of mycotoxins in PLCC.

APPENDICES

APPENDIX 1. Monthly rainfall (mm) recorded in Samaru between 1968 and 1977

	1968	1969	1970	1971	1972	1973	1974	1975	1976	1977
JAN	0	0	0	0	0	0	0	0	0	0
FEB	0	0.25	0	0	0	0	0	0	0	0
MAR	0.25	0	6.86	3.56	0.25	0.51	11.2	0.3	0	tr
APRIL	85.85	43.18	1.78	0	51.31	21.59	30.0	85.8	86.2	0
MAY	143.51	116.84	131.32	116.84	152.91	46.99	33.80	130.1	135.5	78.3
JUN	124.97	184.66	104.90	31.24	95.76	191.77	114.2	135.5	206.9	101.7
JULY	257.30	445.26	259.08	230.89	172.47	191.76	253.0	303.1	226.4	59.0
AUG	302.01	233.68	262.89	298.70	325.12	227.57	328.7	113.4	188.1	302.7
SEPT	139.19	89.92	174.75	195.07	85.34	292.61	246.9	212.2	191.0	199.5
OCT	6.35	104.39	6.60	7.62	24.13	1.50	67.6	8.3	162.2	4.3
NOV	0	0	0	0	0	0	0	0	0	0
DEC	0	0	0	0	0	0	0	0	0	0
	1059.43	1218.18	948.18	883.92	907.29	974.30	1085.4	988.7	1196.3	745.5

tr = trace amount

APPENDIX 2. Total number of days in which rain was recorded at Samaru in each month between 1968 and 1977

	1968	1969	1970	1971	1972	1973	1974	1975	1976	1977
JAN	0	0	0	0	0	0	0	0	0	0
FEB	0	1	0	0	0	0	0	0	0	0
MAR	1	0	2	1	1	1	1	1	0	0
APRIL	9	4	1	0	5	2	4	9	6	0
MAY	13	11	11	8	11	6	7	11	9	12
JUN	13	16	10	7	10	10	9	10	14	13
JUL	16	16	18	18	13	13	24	22	16	12
AUG	18	21	10	22	18	22	20	14	13	19
SEPT	13	13	14	13	12	14	20	19	15	13
OCT	2	11	1	2	5	1	4	2	15	1
NOV	0	0	0	0	0	0	0	0	0	0
DEC	0	0	0	0	0	0	0	0	0	0
TOTAL	85	93	76	71	75	69	89	88	88	70

APPENDIX 3. First and last days of each month between 1968 and 1977 on which rain was recorded at Samaru

	1968	1969	1970	1971	1972	1973	1974	1975	1976	1977
JAN	-	-	-	-	-	-	-	-	-	-
FEB	-	27/27	-	-	-	-	-	-	-	-
MARCH	12/12	-	6/7	29/29	18/18	27/27	11/11	23/23	-	-
APRIL	13/26	2/24	15/15	-	4/29	17/19	15/28	16/28	9/29	-
MAY	3/29	8/31	6/30	13/31	4/31	4/27	3/31	2/30	3/28	1/31
JUN	4/29	1/29	4/30	7/26	2/29	4/29	4/26	3/29	2/29	2/30
JUL	2/31	1/31	2/31	1/30	1/30	5/31	1/31	1/30	1/31	1/28
AUG	2/30	3/31	2/31	1/31	2/30	1/30	1/29	4/30	1/28	5/31
SEPT	3/30	1/29	2/25	1/21	5/30	2/27	1/28	1/29	1/28	1/31
OCT	3/11	1/28	23/23	1/17	2/16	3/3	1/14	4/18	1/28	11/11
NOV	-	-	-	-	-		-	-	-	-
DEC	-	-	-	-	-		-	-	-	-

The numerator and denominator in this table indicate the first and last days respectively of each month when rainfall was recorded.

APPENDIX 4. Moisture content of sorghum samples collected in March, August and November/December, 1977

Village	Sample number	Moisture content, % wet weight		
		March	August	Nov/Dec
Guga	1	8.03	12.29	
	2	8.86	11.52	
	3	9.08	12.26	
	4	8.22	12.03	
Maje	5	8.17	11.53	10.25
	6	7.09	11.41	
	7	9.04	12.94	8.95
	8	6.89	11.22	
Pan Hauya	9	7.99	12.53	8.39
	10	7.53	12.38	
	11	8.08	12.02	9.28
	12	8.10	12.35	9.07
Kadage	13	8.72	12.60	
	14	6.38	12.13	
	15	7.95	13.06	
	16	8.18	12.27	
Gangara	17	7.36	11.82	10.47
	18	7.39	11.42	9.73
	19	8.42	11.81	9.59
	20	7.48	11.67	9.88

Each figure represents the average of three determinations.
The variation from the mean was $< \pm 2.5\%$ of the value.

APPENDIX 5. Fungi identified from surface-sterilised sorghum grains collected in March 1977

Sample number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Fungi																				
Alternaria	5		3		2	1		3												
Aspergillus	3		12	1	6		1		2	3	2	1	1	3	1	2	9	2	15	11
Cheatonium				5			2	3	4	5		2	1			1				
Colletotrichum								2				2								
Curvularia	6	25	41	43	10	22	45	27	30	37	40	35	48	19	35	28	41	54	38	26
Fusarium	2	11	23	21	18	16	8	16	9	23	17	14	13	10	22	8	3	11	7	6
Mucor							1					1	3	3						2
Penicillium	3	2						1	3	2	2		1	4	4	2	2	1		6
Periconia															4	5	2			
Phoma	17	33	14	26	33	61	15	31	34	22	25	12	31	15	35	18	32	18	29	26
Bacteria	62	4	2		33			4			4									1
S. M.	1	2				1	14		7		1		2	17				1		1

Each figure represents the number of colonies of fungi developing per 100 seeds.

S. M. = sterile mycelia

APPENDIX 6. Fungi identified from surface-sterilised sorghum grains collected in August 1977

Sample number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Fungi																				
Alternaria	25	26	9	4	6	13	6		3	8	3									
Aspergillus	3		1		1		1	1	1	3	1	8	2	2	6		3		2	2
Cheatomium	2				1			5	2	1		3	1	3			3	1	1	1
Colletotrichum				2					1	3	1								2	2
Curvularia	13	21	54	54	27	46	52	16	31	24	43	38	20	21	23	24	31	25	26	25
Fusarium	5	9	10	14	12	9	8	5	5	4	19	13	16	24	21	23	18	12	25	14
Helminthos-porium	18	7	2		3	4		2		2										
Mucor								2					1	1		2		1	4	11
Penicillium					5			15	2	4		10	7			1		1		1
Phoma	23	34	23	17	20	24	33	9	27	11	14	16	61	59	67	56	54	68	51	45
Rhizopus													2							
S.M													2	1			4		10	1
Trichotecium		3																		
Bacteria								2		4	4					1	1			

Each figure represents the number of colonies of fungi developing per 100 seeds.
S.M. = sterile mycelia

APPENDIX 7. Fungi identified from surface-sterilised sorghum grains collected in November/December 1977

Sample number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Fungi																				
Aspergillus									36		24	2					1	1	25	9
Cheatomium							5		1											
Colletotrichum									1											
Curvularia					18		7		14		6	27					15	13	5	15
Fusarium	31		11		18		25		8								7	29	14	21
Mucor	4								3									3		
Penicillium	1				2		6		5									5	3	8
Phoma	41		73		25		15		24								78	50	21	53
Rhizopus							1												1	
S.M.	4								10								1		1	1
Trichotecium																			30	

Each figure represents the number of colonies of fungi developing per 100 seeds.

S.M. = sterile mycelia

APPENDIX 8. Mycotoxins detected from sorghum samples collected for study in 1977

	Sample number	Mycotoxin contamination (µg/kg)		
		Ochratoxin		Zearalenone
		A	B	
March	1	ND	33.5	ND
	2	25	38.3	ND
	3	28.5	44.7	ND
	4	ND	33.5	ND
	5	25	38.3	ND
	6	ND	44.7	ND
	7	ND	33.5	ND
	8	ND	44.7	ND
	12	ND	ND	100.4
	13	ND	ND	143.5
August	4	40	52	52
	5	30	26	ND
	6	30	26	ND
	8	30	13.4	ND
Nov/Dec	9	52	60	ND

In March sterigmatocystin was detected in samples 9,11 and 13; ochratoxin C in sample 16.

In August sterigmatocystin was detected in samples 2,4 and 5.

In November/December ochratoxin C was detected in sample 9.

ND = not detected.

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