IDENTIFICATION OF MICROFLORA ASSOCIATED WITH GROUNDNUTS IN STORAGE AND DETERMINATION OF THE EFFECT OF ANTAGONISTIC MICRO-ORGANISMS ON GROWTH AND PRODUCTION OF MYCOTOXINS.

BY

GACHOMO, E. W.

A THESIS SUBMITED TO THE DEPARTMENT OF CROP SCIENCE, UNIVERSITY OF NAIROBI IN PARTIAL FULFILLMENT OF REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN AGRICULTURE (PLANT PATHOLOGY)

1997

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DECLARATION

I hereby declare that this thesis is my original work and has not been presented for a degree in another University

Signed :----------

Emma Gachomo

(Candidate)

This thesis has been submited for examination with my approval as University supervisor.

Signed :---------- 1/9/97

Dr E.W. Mutitu

(University Supervisor)

DEDICATION

TO

My parents :Mr W. G. Gachomo Karwega and Mrs Rachel Muthoni Gachomo.

ACKNOWLEDGEMENT

I owe much to DAAD for the scholarship granted me through the University of Nairobi, which enabled me to start and successfully complete my M.Sc. programme.

I am also grateful to my supervisor Dr. E. W. Mutitu for her consistent guidance, reading and correction of this thesis. To the technical staff of crop protection, department of crop science Mr. Musyimi, Mr. Gathuma and Mr. Mathenge I am grateful. I acknowledge the help accorded to me by the Kenya Bureau of Standards (KBS): for letting me use their laboratory facilities and the assistance from their staff. I'm grateful to the following KBS staff members for their personal assistance; Mrs Catherine Nyagah, Mr. J. Gikubu, Mr. P. Ngobia, Mr. Maru, Mr. Wachira, Mr. Mureithi, Mr. Musa and all the others whose names I have not mentioned.

I sincerely appreciate the assistance of my family members: my parents, Karwega, Wanjeri, Nyambu, Wanji, Kamau, Gitonga, Mwangi, and Gachomo. I also thank Mr. Kiama for the help he offered in whichever way.

I would also like to acknowledge the assistance of my friends. I thank Mr. L. K. Rutto and Mr. J. Wesonga for their commitment in typing and editing my work, Mr. H.P. Ng'ang'a for taking the photographs, Mr. Masinde and Mr. L.S. Wamocho for helping in all the ways that they did.

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ABSTRACT

Several micro-organisms were found to be associated with groundnuts in storage: *Rhizopus* spp., *Fusarium equiseti, F. torulosum* and F *.oxysporum*, *Aspergillus niger, A. flavus, A. ochraceous* and *A. parasiticus*, *Eurotium repens, Rhizoctonia* spp., *Sclerotium* spp., *Penicillium* spp. and *Cheatonium* spp. The aflatoxigenic ones were found to be *A. parasiticus, A. flavus* and *Eurotium repens*.

Two groundnut samples out of 8 from the markets were found to contain detectable levels of aflatoxin ranging from 126.8 ppb to 436.8 ppb. The permitted total aflatoxin content in any edible sample is 20.0 ppb (Kenya Bureau of Standards)which is many times lower than what was found in the two samples.

Three isolates;- *Trichoderma* isolate 13 (T_{13}), *Aspergillus niger* isolate 1 and 2 were tested for their antagonism to growth of and aflatoxin production by the toxigenic strains. The antagonists *Trichoderma* iso.*13* and *A. niger* 1 were found to be effective in reducing aflatoxin production by the toxigenic strains. The greatest reduction (100%) was observed when the antagonists were inoculated into the groundnuts 2 days before these aflatoxigenic isolates. Trichoderma isolate 13 (T_{13}) was found to be less effective in reducing aflatoxin production than *A. niger* 1.

The radial growth of the aflatoxigenic strains grown on PDA was significantly ($P=0.05$) reduced by T_{13}, *A.niger* 1 and *A. niger* 2. Trichoderma isolate 13 (T_{13}) was more effective in suppressing the radial growth than the *A. niger* isolates. There was no significant difference in radial growth reduction by *A. niger* 1 and *A. niger* 2.

1.0 INTRODUCTION

1.1. GROUNDNUT PRODUCTION

In Kenya groundnut is an important legume crop. In 1990 the production was 9000 tonnes from 15000 ha with a productivity of 621 Kg/ha (Mahmoud *et al.*, 1992). Western and Nyanza provinces of western Kenya are the major groundnut-producing areas, with scattered pockets of production in Rift valley and Eastern provinces.

1.2. USES AND IMPORTANCE OF GROUNDNUTS

Among the crops standing between mankind and starvation, peanuts rank thirteenth in importance (McGill, 1973).

Groundnuts is a high calorific value food stuff and is added to diets to improve dietary proteins and supply vitamins of the ß-complex. Groundnuts foods are simple to prepare and there are a multiplicity of forms in which they can be prepared.

Groundnuts produce vegetable oils and fats. Dry legume seeds are frequently the most practical source of storable and transportable proteins in regions lacking refrigeration facilities. Grain legume proteins are the least expensive protein source for both rural and urban populations of Africa.

Groundnut seeds can be eaten raw, lightly roasted or boiled, sometimes salted and made into a paste known as peanut butter. Groundnut kernels can be decuticled and pressed into edible cake which can be ground into flour or from which oil can be extracted using a solvent to give a defatted meal which can then be ground into flour.

In Senegal, leaves are used as a vegetable in soup. Groundnuts can be used as a substitute or to supplement other sources of proteins in animal feeds. Plant nutritionists, say

groundnut as a legume has beneficial effects in the soil due to its association with nitrogen fixing bacteria. Groundnuts are a source of cash and food.

1.3. PROBLEMS ASSOCIATED WITH GROUNDNUT PRODUCTION

The major problems facing groundnut production in Kenya are, lack of certified planting seed, diseases, pests and drought. Studies in Western province of Kenya indicate that poor weeding was possibly one of the limiting factors to increased production in late 1960s (Goldson, 1967)

The major diseases affecting groundnut production are early leaf spot by *Cercospora arachidicola* Hori and Late leaf spot caused by *Phaeoisariopsis personata* (Berk & Curt)v. Arx which are rated as the most important diseases of groundnuts world wide (Garren and Jackson, 1973). Other diseases include stem rust caused by *Sclerotium rolfsii.* Saccardo, *Rhizoctonia* diseases by *R. solani.* Kunn, *Aspergillus* crown rot caused by *Aspergillus niger.* van Tieghem, and *A. pulverulentus.* (McAlpini) Thom, Pepper spot and leaf scorch by *Leptosphaerulina crassiasca* (Sechet)Jackson & Bell; yellow mold by *Aspergillus niger* among others. The most important bacterial disease of peanuts is bacterial wilt caused by *Pseudomonas solanacearum.* E.F. Smith. The viral diseases of groundnuts are peanut rosette and peanut stunt. Nematode damage to peanuts is caused by root-knot nematode *Meloidogyne arenaria(* Neal, 1889), (Chitwood, 1949) and *M. hapla* Chitwood, 1949, root-lesion nematode (*Pratylenchus brarchyvirus).* Godfrey, 1929/ Filip & Steak, 1941), and sting nematode (*Belonalaimus longicaudatus* , Rau, 1958).

The diseases named above occur in the field, but most of the diseased causing these micro-organisms do not persist in storage. It is generally accepted that most mold infection of peanut in storage occurs after the peanuts are dug (Sanders, 1983). The microflora associated with groundnuts in storage are mainly fungi and are called storage fungi.

After digging, the field fungi tend to die out during the curing period and species of saprophytic and weakly parasitic fungi proliferate at the lower kernel moistures and soon become dominant. This second group of fungi that is associated with peanuts during curing, picking and storage consists principally of species of *Aspergillus* and *Penicillium* but *Rhizopus* spp, *Sclerotium bataticola* and *Fusarium* spp are frequently found (Borut and Joffee, 1966; Joffee, 1968; Joffee, 1969; Joffee and Lisken, 1968).

Of 173 species of field and storage fungi of groundnuts only *A. flavus* and *A. parasiticus* produce aflatoxin (Sanders, 1983). Aflatoxin is a naturally occuring carcinogenic metabolite found in peanuts when they become contaminated with toxigenic strains of *Aspergillus flavus / A. parasiticus*. (Sanders, 1983). Contamination of groundnuts by aflatoxins is a serious quality problem in many parts of the world. According to ICRISAT reports (1989), some work has been done in a number of African countries viz: Zambia, Zimbabwe, Malawi, Nigeria, Tanzania, Mozambique, Cote d'Ivoire, Egypt and Sudan. Available literature indicates only one study on the content of aflatoxins in two samples of groundnuts in Kenya (Muraguri *et al* 1981).

Also work on isolation and identification of the groundnut microflora associated with groundnuts in storage has not been done in Kenya (Mutitu personal comm., Siboe personal comm.). Storage pathogens on foodstuffs may be killed using certain chemicals but the rates at which these are effective to kill the micro-organisms are not safe for human consumption. Therefore alternative methods for control of storage pathogens must be sought which are safe both for human consumption and to the enviroment.

Thus the objectives of this project were

(a) to isolate and identify the microflora found on groundnuts seeds in storage.

(b) to determine the toxigenic fungi found on the groundnut seeds in storage.

(c) to determine effects of antagonistic fungi on growth of toxigenic fungal isolates isolated from groundnuts in storage.

(d) to determine the effects of antagonistic fungi on the aflatoxin production by toxigenic fungi on groundnut seeds.

2.0 LITERATURE REVIEW

2.1. STORAGE FUNGI OF GROUNDNUTS

Storage fungi are the dominant molds associated with stored seeds. They are usually only superficially present on seeds at harvest, but some invasion of these microorganisms into dead plant material such as blossom ends, leaf fragments, or straw etc. does occur. These fungi principally include species of the genera *Aspergillus* and *Penicillum*. They are the microorganisms primarily responsible for post-harvest spoilage, and are active in stored grain with a moisture content in the range of 13.2 to 18% (Christensen, 1965). Molding of peanuts is a problem of storage resulting in damage that may vary from discolouration of the seed coats to complete destruction of the kernels (Thompson *et al.*, 1951). Most storage fungi preferentially attack the germ on the embryo of seeds causing discolouration and finally outright decay (Golumbic and Laudani, 1966) and in severe cases the peanuts mat together (Woodroof, 1966)

Many types of molds and other soil microorganisms are present on the nuts when harvested, and during shelling and handling the seed may become infected with organisms (Woodroof, 1966)

The microflora of freshly dug and stored peanuts has been studied by several investigators. Jackson (1965a) investigated the microflora of soil adhering to peanut pods, using dilution methods. *Aspergilus niger, A. flavus, A. terreus, Rhizopus spp.* and *Sclerotium bataticola* were found in relatively small numbers. Conversely *Penicillium funiculosum, P. rubrum, P. citrinum* and *Fusarium* spp. were present in large numbers. *A. niger, A. flavus, S. batalicola* and *Rhizopus* spp. extensively penetrated pods and kernels when dry, infested pods were permitted to hydrate for 6 days at 26°C, 32°C or 38°C. Infection by *A. flavus* and *A. niger* increased as the temperature increased. Garren (1966) analysed the endogeocarpic flora of Virginia peanuts and reported that "*Trichoderma viride* seems dominant and *Penicillium*

spp. seem sub- dominant in the climax endogiocarpic community of sound and rotting peanut pods; and *A. flavus* and *A. niger*, which have a potential of causing trouble are quantitatively minor but persistent species in the flora".

Diener (1960) made a comprehensive study of Georgia farmers' stock (uncleared and unshelled) peanuts that had been in storage for 8 to 56 months. The predominant flora was found to consist of certain species of *Aspergillus glaucus* group (*A. amstelodani*, *A. chevalieni*, *A. repens* and *A. ruber*, *A. restructus*, *A. kamarii)*, *Penicillium citrinum*, *Cladosporium* spp. *Torula sacchari*, and members of the mucorales. Large numbers of fungi were directly associated with kernel moisture contents of 12.5% or higher at the time the seeds were placed in storage.

The number and kind of fungi associated with peanut seed stored for 1-6 months were studied by Welty and Cooper in 1969. In the intial period of storage *A. repens* was isolated from 54% of seed, *Penicillium* spp. from 80% and *A. flavus* from 7%. After 2-3 months of storage, the percentage of seed with *A. repens* and *Penicillium* spp. dropped to about 10% and then gradually increased to 40% after 6 months. *A. flavus* remained at 7% for the first 3 months, but after 4 to 6 months it was isolated from 18% of the seed.

In Israel, *A. niger* was the most common species in 114 seed groundnut samples from the 1963 and 1964 crops (Borut and Joffee, 1966). *A. niger* occurred in 97.4% of the 1963 samples and in 63.5% of the 1964 samples. However, *A. niger* occurred in large numbers (40% of the total colonies) in 114 samples, whereas *A. flavus* made up only 5.7% of the mycoflora of stored seed. *A. niger* was the dominant species in the mycoflora of 419 samples of stored seed examined over a 5 year period in Israel (Joffee, 1969), and *A. flavus* was present in relatively small quantities in comparison with *A. niger*. The high incidence of *A. niger* in stored seed that were disinfected with mercuric chloride was considered to be attributable to selective removal of antagonists (Joffee, 1968)

In Egypt, groundnut seed were adjusted to 8.5, 13.5, 17.5 and 21% moisture levels and stored for 6 months at 5, 15, 28, and 45°C (Moubasher *et al.*, 1980) *A. fumigatus* was the dominant fungus followed by *A. flavus, A. niger, A. terreus* and *P. funiculostum.*

2.2.0. GROUNDNUT TOXICOLOGICAL PROBLEMS:

Aflatoxin is a naturally occurring carcinogenic agent found associated with peanuts when they become contaminated with toxin producing strains of *A. flavus* or *A. parasiticus*. *A. flavus* and *A. parasiticus* are present in the soil and air throughout the world, especially in tropical and sub-tropical areas. It has been found more prevalent when peanuts follow peanuts on the same piece of land (Sanders, 1983).

The moisture condition under which peanuts are grown has great influence on the invasion of peanut fruit by *A. flavus*, especially prior to digging. Drought stress just before digging peanut is associated with greater aflatoxin contamination (Sanders, 1983). Sanders (1983) adds that peanut are more likely to be contaminated when the soil moisture levels in the pod zone approach levels at which moisture moves from pod into the soil, or when the seed moisture is below 31%

The most vulnerable time for peanut pod contamination after harvest is when seed moisture is 12-30%. Adverse weather conditions or rain that extends the drying and curing time may result in increased contamination. There is greater contamination in over mature peanuts, those from dead plants, or in peanuts attacked by insects or otherwise damaged in the field or during harvesting (Sanders, 1983)

There has been no practical fungicidal application or treatment schedule found to be effective in preventing aflatoxin contamination; however, pesticide applications and rapid curing and drying after harvest have been effective.

2.2.1. Factors affecting mold growth and mycotoxin production:

The main factors influencing the growth of *A. flavus* and other storage fungi in groundnuts are moisture (relative humidity), temperature, time and gaseous composition of the atmosphere. High mycoflora counts have been associated more often with high initial moisture contents of groundnuts going into storage than with any other factor (Diener, 1960).

A. flavus probably the most important toxigenic mold, can invade peanuts having a moisture content greater than 8% and stored in a temperature range of 12°C - 47°C. However, it has been reported that the optimal temperature for production of aflatoxin by *A. flavus* was 25°C on sterilized peanuts or in a liquid medium (Diener and Davis, 1966a). Schroeder and Hien (1967) grew four strains of the *A. flavus* group on acid-delinted cotton seeds, shelled spanish peanuts and rough rice at temperatures which ranged from 10°C to 40°C. Aflatoxin was produced in small amounts at 10°C and 40°C but the optimal temperature range was between 20°C and 35°C. These investigations suggest that accelerated growth of the fungus leads to increased aflatoxin production. However, this increase is offset because the fungus metabolizes the toxin at a faster rate than it produces it.

Diener and Davis (1967) investigated the influence of temperature and relative humidity on production of aflatoxin in sound, mature peanut kernels, broken mature kernels and unshelled nuts inoculated with spores of *A. flavus*. The limiting relative humidity for aflatoxin production by *A. flavus* was 85 ± 1% RH for 21 days at 30°C. The limiting low temperature for visible growth and aflatoxin production by the fungus was 13°C ± 1°C for 21 days at 97-99% RH. Damaged kernels, however developed some aflatoxin in 21 days at 12°C. The maximum temperature for aflatoxin production was 41.5°C ± 1.5°C for 21 days at 97-99% RH". Schindler *et al.* (1967) grew two toxigenic isolates of *A. flavus* for 5 days on wort media at temperatures ranging from 2°C to 52°C. Maximal production of aflatoxin occurred at 24°C. Maximal growth of *A. flavus* isolates occurred at 29°C and 35°C. The ratio

of production of aflatoxin B_1 to aflatoxin G_1 varied with temperature. Aflatoxin production was not related to growth rate of *A. flavus;* one isolate at 41°C, at almost maximal growth of *A. flavus*, produced no aflatoxins. At 5 days, no aflatoxins were produced at temperature lower than 18°C or higher than 35°C.

Dickens and Pattee (1966) found that aflatoxin did not develop in high moisture peanuts, inoculated with toxigenic strain of *A. flavus*, after prolonged periods in the windrow in cool weather. Austwick and Ayerst (1963) studied the growth of *A. flavus* and *A. chivalieri* of the *A. glaucus* group at different levels of relative humidity and temperature, they were unable to grow at less than 80% RH equilibrium.

Studies on peanuts in Nigeria in co-operation with the Tropical Products Institute, London (McDonald and Harkness, 1964), yielded the following information concerning production of aflatoxin in peanuts before storage.

(i) production of aflatoxin was not associated with a given location.

(ii)kernels from damaged pods were highly contaminated with *A. flavus* but this was not always associated with toxin production.

(iii) *A. flavus* made better growth on senescent or dead peanuts.

(iv) aflatoxin was not found in any peanuts at the time of digging except in kernels from broken pods.

(v) production of aflatoxin did not occur before 5 days after digging.

(vi) damaged pods favoured contamination by *A. flavus* and were more likely to be toxic.

(vii) the level of toxin production in peanuts grown in given areas varied from year to year.

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In summary *A. flavus* infection and aflatoxin contamination may increase in groundnuts during storage until their moisture content drops below 9%. Increases in moisture from rewetting in storage or exposure to high humidity for extended periods of time result in rapid invasion by the aflatoxigenic *A. flavus* with consequent aflatoxin contamination. Natural accumulation of carbon dioxide (CO_2) and decreased levels of oxygen (O_2) in closed storage reduce mycofloral development (Landers *et al.,* 1967, Jackson and Press, 1967) Low temperature and uniform moisture distribution reduce mold growth and insect activity. According to Diener and Davis (1977), high relative humidity and temperatures, rain water leakage, condensation, and insect infestation are all important factors that contribute to aflatoxin contamination of groundnuts in storage.

2.2.2. IMPORTANCE OF AFLATOXINS

A. flavus and *A. parasiticus* often infest groundnuts and develop secondary metabolites popularly called aflatoxins. Aflatoxins are known to be hepatotoxic, carcinogenic and teratogenic. For historical reasons, the problem of aflatoxins has been commonly associated with groundnuts although it is much more of a problem in maize (Bhat, 1989).

2.2.2.1 Aflatoxins in groundnuts and their association with various diseases in human

During the past two decades, there have been several attempts to correlate the consumption of foodstuffs contaminated with aflatoxin with human diseases.

a.) Aflatoxins and occupational diseases among groundnut workers:

Workers engaged in postharvest activities related to shelling, bagging, storage, transport etc. may be exposed to aflatoxin through the respiratory route. There is evidence from epidemiological studies to indicate that aflatoxins in respirable particles pose a potential occupational hazard. A chemical engineer involved in sterilizing Brazilian groundnut meal contaminated by *A. flavus* developed alveola cell carcinoma and died within a year. Aflatoxin

B_1 was detected in his lung tissue. His colleague had also developed aveolar cell carcinoma (pulmonary adenonatosis); (Dvorackova *et al.,* 1976).

b.) Association between aflatoxin and protein energy malnutrition:

Aflatoxins have been recognised to be more harmful to malnourished than well nourished animals and humans. Thus, malnourished children in developing countries are more susceptible to aflatoxin toxicity (Bhat, 1989)

c.) Aflatoxin in groundnut and Indian childhood cirrhosis (ICC):

ICC is a liver disorder found only among children in the Indian subcontinent. Toxins (including aflatoxin), virus, and hereditary factors have been put forward as possible etiologic agents. According to Amea *et al.* (1969), there is sufficient circumstantial evidence to indicate that children exposed to aflatoxin through breast milk and dietary items such as unrefined groundnut oil and parboilded rice may develop ICC.

d.) Aflatoxin and liver cancer

Studies carried out in Kenya, Swaziland, the Transkei region of South Africa, Mozambique and Thailand have found a positive correlation between hepatocellular carcinoma (HCC) and aflatoxin ingestion by man.

Consumption of contaminated groundnuts has been associated with occurrence of endemic oesteoarthroasis: several chronic, progressively disabling, polyarthritic diseases with fairly similar features have been described from different countries during the last decade (Bhat, 1989).

2.2.2.2 Livestock

There are also hazards to livestock consuming aflatoxin contaminated groundnut meal. The high content of aflatoxins in groundnut meal in African countries has serious implications

for livestock feeding. According to Reed and Kasali (1989), the risks depend on the level and type of aflatoxin in the diet, the strain of animal and its nutritional status. Subclinical aflatoxicosis is characterized by reduced feed intake and poor productivity, but may not be associated with overt clinical symptoms. Chronic problems occur when aflatoxins are present in the diet at less than 1000 μgkg^{-1} but the lower limits for effects on productivity are not certain.

The principal lesions of aflatoxicosis occur in the liver and maybe classified as toxic hepatitis. Natural cases usually result from repeated ingestion of the toxin. One of the most constant responses to aflatoxin B is bile ductile hyperplasia at the periphery of hepatic lobules, changes in hepatocytes (vacuolization, fatty change) leading to necrosis, are usually localized in one part of the hepatic lobule, depending on the species (Allcroft, 1969). Hepatic veno-occlusive lesions are also common. The immunosuppressive effect of aflatoxin, coupled with high exposure to disease and poor nutrition are detrimental to increased livestock production in Africa.

2.3.0. MANAGEMENT OF GROUNDNUT MICROFLORA

The mold that produces aflatoxin has the potential of invading healthy peanut tissue as the pod approaches maturity and during curing and drying period. For management of *A. flavus* infection and aflatoxin contamination of groundnuts both preventive and curative procedures may be necessary. Aflatoxin management therefore starts in the farmer's field, continues through crop produce handling, marketing, storage and processing and ends with the consumer.

In general, cultural practices and use of crop protection chemicals are preventive in nature as are provision of effective storage procedures and care during transportation and processing. Curative measures concentrate on isolation and segregation of toxic groundnuts

and various systems of detoxification. Varietal resistance is a desirable component of any integrated aflatoxin management system.

Since the factors influencing the growth of *A. flavus* and other storage fungi in groundnuts are similar, their management procedures are also similar.

2.3.1. Cultural Control

Removal : Goldblatt (1970) distinguished between removal by separation of contaminated kernels, and removal of aflatoxin by extraction from contaminated kernels. It has been demonstrated that aflatoxins levels are correlated with the proportion of broken shells in the lot and the number of shrivelled, rancid, or discolored kernels. When the latter are discarded, the remaining high quality peanuts are relatively free of aflatoxin. The vast majority of aflatoxin in contaminated seeds resides in a relatively small number of kernels. Culling of these seeds is accomplished by screening for size at shelling plants; by removing discoloured kernels manually on picking tables, and by utilizing various mechanical or electronic sorting devices which pass or reject each kernel on basis of colour when scanned by a photoelectric cell (Goldblatt 1970, Dollear, 1969).

After harvesting, control of deterioration of sound pods is primarily one of moisture. During curing it is accomplished by promptly drying pods and kernels to a safe storage moisture level. Most mold development that affects quality of peanuts occurs when the kernel moisture content is between 11% and 35%; at 10% and below, mold growth is inhibited. Within the critical range mold development increases with time and temperatures. To dry them, pods are exposed to direct sunlight and air currents and they dry rapidly and effectively (Pettit *et al.*, 1971). The use of artificial drying (forced air and supplemental heat) when windrow conditions are unfavourable for rapid drying reduces the possibility of aflatoxin accumulation (Pettit and Taber, 1968).

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Once the drying process is started, the rate of drying is the most important factor in minimizing mold growth. Once begun the drying process should be carried non-stop to 10% kernel moisture content as quickly as possible while staying within recommended drying practices; highest heat without damage to kernels, low humidity, and fast air flow over to kernels, (Sander, 1981). It should be borne in mind that very rapid drying may cause the skin to split and production of off-flavours in the seeds (Mehan *et al.* 1991).

2.3.2. Chemical Control

There has been no practical fungicidal application or treatment schedule found to be effective in preventing aflatoxin contamination. Several attempts have been made to control or reduce *A. flavus* infection of pods and seeds by applying fungicides to the soil, to groundnut foliage or to freshly lifted groundnut pods. Jackson (1967a) found that use of soil fumigants did not increase yield, reduce pod mycoflora or indirectly suppress the formation of aflatoxins in Argentine groundnuts. The possibility of chemical control of fungi invading pods and seeds of groundnuts by spraying fungicides onto freshly dug pods in the windrows was studied by Jackson(1967b). The fungicides applied were,

i) Captafol - 0.67 Kg

ii) triphenyltin hydroxide (Du Ter) - 0.67 Kg

iii) Tri - basic copper sulphate (TBCS) - 0.67 Kg

iv) Sodium propionate - 1.78 Kg and 3.57 Kg

v) Sulfur - 3.57 Kg

vi) Tap water (control)

Difolatan and TBCS reduced development of pod surface fungi. Aflatoxin contents of kernels from slowly dried pods, which had been treated previously with various fungicides in the windrow, were not related closely to observed efficacy of fungicides in controlling pod surface

fungi or fungi from kernels. Hence the fungicides had no definitive results in terms of their effects on subsequent fungal invasion and aflatoxin contamination of seeds.

However Mixon *et al.* (1984) found that PCNB -fensulfothion or CGA 64250 reduced seed infection by *A. flavus*. Bell and Doupnik (1971, 1972) found that aflatoxin contamination of windrowed groundnuts could be substantially reduced by treatment with Manzate, Benlate or Botran. Madaan and Chohan (1978) reported prevention of *A. flavus* invasion of seeds by spraying freshly lifted groundnut pods with propionic acid, sorbic acid or Chlorothalonil. No fungicide, combinations of fungicides or other chemical treatments have been adopted for practical control of *A. flavus* infection and subsequent aflatoxin contamination of groundnuts in the field.

Biocides applied to the soil may have direct or indirect effects on the populations of the toxigenic fungi. For instance they may affect other components of the soil microflora and fauna and stimulate biological control systems. Such interactions are likely to be complex and difficult to unravel. Similarly, addition of organic substances to soil e.g. green manure, crop residues and farm yard manure is likely to have complex effects upon the soil microflora

Some farmers store a portion of their crop in grain bins on the farm until the new year for tax purposes, and hope for a high price. They may be stored as farmers stock or after they are cleaned. If insects are controlled there is little deterioration of in-shell peanuts in storage during the winter months (Sanders, 1983)

Beetles, moths, and weevils that attack stored peanuts may be controlled with microencapsulated pyrethrin insecticide. This is a controlled release insecticide approved for use in food areas, after diluting with water (Sanders, 1983).

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2.3.3 Genetic Resistance

Resistance of aflatoxin production:

Rao and Tulpule (1967) first reported varietal resistance in groundnut to aflatoxin production. In Laboratory inoculation tests they found that the cultivar US 26 (P1 246388) did not support aflatoxin production when seeds were colonized by aflatoxin- producing strains of *A. flavus*. These findings were not confirmed by other workers (Mehan *et al.*, 1991). A laboratory method to screen live groundnuts for resistance of aflatoxin production was used at ICRISAT (Mehan and McDonald, 1980) to test 502 genotypes. None was totally resistant to aflatoxin production but highly significant differences in aflatoxin production were found (Mehan *et al.*, 1986). Recently, two wild *Arachis* species, *A. candenosii* and *A. duranensis*, have been reported to support production of only trace levels of aflatoxins (Ghewande *et al.*, 1989). These *Arachis* species were also found to be highly resistant to in vitro seed colonizition by *A. flavus*. It is important that these findings should be confirmed and wild *Arachis* species accessions tested should be fully specified. A limited search has been made for groundnut genotypes that do not support, or support only very low levels of aflatoxin production following seed infection by aflatoxigenic strains of *A. flavus* or *A. parasiticus*.

Resistance to *A. flavus* infection

Mixon and Rogers (1973a) first suggested that use of groundnut cultivars resistant to seed invasion and colonization by the aflatoxin producing fungi could be an effective means of preventing aflatoxin contamination. They developed a laboratory inoculation method for screening groundnut genotypes for resistance to *A. flavus*/ *A. parasiticus* invasion and colonization of rehydrated, mature, sound, stored seeds. Mixon and Rogers (1973a) reported that two Valencia type genotypes PI 337394F and PI 337409, had high levels of resistance to *in vitro* seed colonization by *A. flavus* and *A. parasiticus*. Six more breeding lines (GFA1,

GFA2, ARI, AR3, and AR4) were later reported resistant (Mixon 1986) According to the 1989 ICRISAT annual report, five lines of nuts (ICGS 1859, 1994, 10020, 10094, ICG 10933) when tested had consistently low levels of seed infection (2%) by *A. flavus*. Other workers have used Mixon and Rogers method or modification of it, in screening genotypes for resistance to seed colonization by aflatoxin producing strains of *A. flavus* and *A. parasitocus* to give on the whole, comparable results (Mehan *et al.*, 1991). A total of 37 genotypes have now been reported to have resistance of this type.

Resistance to *A. flavus* invasion and colonization of rehydrated, stored, dried seed has relevance when aflatoxin contamination is largely postharvest. The resistance is likely to be of value to groundnuts dried in the field or when groundnuts in storage are wetted, or absorb moisture from the atmosphere. The resistance is of less value for decorticated seed that may have suffered damage to the testa in processing (Mehan *et al.*, 1991).

2.3.4. Biological control:

Aspergillus flavus is frequently found associated with several other fungi in groundnut pods and seeds (Hanlin, 1970). Based on such observations it has been hypothesised that interactions between fungi as they compete for the substrate might under, unfavourable environmental conditions restrict invasion of groundnuts by *A. flavus* and/or contamination with aflatoxin. Microbial competition or microbial breakdown may be responsible for lower levels of aflatoxin in parasite-damaged pods than in seed from mechanically broken pods.

It has been reported that several fungi can break down aflatoxin in groundnuts and in aflatoxin containing liquid media. *A. niger* and *Rhizoctonia solani* appeared to limit the development of *A. flavus* and aflatoxin production in substrate (Diener, 1973). In Israel Joffee (1969) observed that a large number of viable propagules of *Aspergillus niger* in the geocarposphere and moderate invasion of seeds by this species was associated with very limited invasion of seeds by *A. flavus*, *Fusarium solani* and *Penicillium* spp. Frequent

invasion of seeds by *A. niger* has been suggested to be important in preventing the development of *A. flavus* (Joffee, 1969). *A. niger* has been shown to inhibit growth of *A. flavus* and aflatoxin production *in vitro* (Burnett *et al.*, 1972).

Presence of normal endogeocarpic mycoflora, including *A. niger* and *Macrophomina phaseolina*, appears to inhibit invasion of groundnuts by *A. flavus*. This notion is supported by studies of Lindsey (1970) who found that *A. flavus* penetrated and colonized a high percentage of shells of living, attached, immature and mature pod under gnotobiotic conditions.

A number of micro-organisms are capable of degrading aflatoxin B_1 by transforming it to aflatoxicol (oflatoxin Ro) and other yet to be characterized compounds. These microbes include *Corynebacterium rubrum, Aspergillus niger, Trichoderma viride, Mucor ambiguus, Dactylum dennoides Mucor griseo-cyanus, Absidia repens, Helminthosporium sativum, Mucor alternans, Rhizopus arrhizus, Rhizopus oryzae, Rhizopus stolonifer* and the protozoan *Tetrahymena pyriformis* (Brakett and Marth, 19..). The studies by Mann and Rehm (1976) to demonstrate ability of *C. rubrum, A. niger, T. viride and M. ambiguus* were done using extracted aflatoxins but not with aflatoxin producing micro-organisms.

3.0 MATERIALS AND METHODS

3.1 COLLECTION OF GROUNDNUTS

Unshelled groundnuts were obtained from five open air markets within Nairobi; namely Kawangware, Kangemi, Gikomba, Nyamakima and Nyayo. Three kilogrammes of each variety available in the market were obtained. Two varieties (differentiated by colour of seed) were available from three markets (Kawangware, Kangemi and Nyamakima) while only one variety was available from each of the other two markets (Nyayo and Gikomba). Six sellers were chosen at random and from each seller a half kilogramme of each variety was obtained. These were then bulked according to variety and market to form a sample. The samples were placed in brown paper bags and stored in the refrigerator at 4°C.

3.2 ISOLATION OF FUNGI ASSOCIATED WITH GROUNDNUTS.

The fungi associated with groundnut were isolated using blotter test and agar plate methods as given by Dhingra and Sinclair (1985).

3.2.1 Blotter test

Nine cm diameter filter papers were soaked in distilled water, drip drained, wrapped in aluminium foil and autoclaved at 121°C, 15psi for 15-20 minutes. Clean dry petridishes were placed in metallic canisters and sterilized in an oven at 160°C for 2 hrs. The sterile filter papers were then aseptically placed into the sterile petri dishes - two papers per petri dish.

Eight hundred groundnut seeds per variety per market were taken, half of them surface sterilized in 5% sodium hypochlorite for 5 minutes, rinsed in 3 changes of sterile distilled water and placed in the cooled sterile petridishes with filter papers. Ten seeds were placed in each plate under aseptic conditions and well spaced to avoid cross infection. The filter paper was kept moist by periodic moistening with sterile distilled water (excess water was avoided).

The plates were incubated at room temperature 23°C ± 2. The set up was replicated three times and the design was split-plot completely randomized block design: the markets were the main plots while the varieties were the sub-plots.

Observations on the type and number of microorganisms that grew on the different seeds were taken from the second day of incubation until the seventh day. A similar experiment was repeated with the other half of groundnut that were not surface sterilized. All the micro-organisms that grew were purified on potato dextrose agar (PDA) medium, then identified using the standard procedures described below.

3.2.2 Agar plate

Eight hundred groundnut seeds per variety per market were taken, half of them surface sterilized in 5% sodium hypochlorite for five minutes and rinsed in 3 changes of sterile distilled water. Excess water was dried on a sterile filter paper. The groundnuts were placed on petri dishes containing 15 ml of potato dextrose agar (PDA) medium, 10 seeds per plate and well spaced to avoid cross infection were used. The groundnuts were incubated at room temperature 23°C ±2.

Three replicates per variety per market were set up in a split-plot completely randomized block design, where the markets were the main plots while the varieties were the sub-plots. The same experiment was repeated with the unsterilized groundnuts. Observations for the types of fungi that grew, colony characteristics (colour, shape, form etc), number of seeds infected with one type of fungus were taken from the second day of incubation. All fungi that grew were purified on PDA and identified using the methods given below.

3.3 IDENTIFICATION OF FUNGAL ISOLATES.

Identification was done based on cultural and morphological characteristics of pure cultures of the isolates. First visual and microscopic examinations were done to determine the genus later identification keys and illustrated manuals were used for species separation.

3.3.1 Visual and microscopic examination

This was done on one week old pure cultures of isolates grown on PDA. Visual observation of mycelial colour, growth form, smell and other variable characteristics was done as preliminary identification. Microscopic slides were prepared from each isolate and mounted under a stereo compound microscope. The specimens were mounted using cotton blue in lactophenol under medium power magnification (400x) and, oil immersion lens magnification (1000x) and examined for reproductive structures. The type, arrangement and how the spores are borne on mycelium were noted. Other vegetative structures were also recorded. Identification keys were used to separate the genera to species level.

3.3.2 Use of synoptic identification keys

(a) Identification of Aspergilli

Pure cultures of *Aspergillus* were used to inoculate into dishes containing czapek agar (CZ). The composition of czapek agar (CZ) was:

Sucrose	30.0g	
$NaNO_3$	3.0g	Trace metal solution
K_2HPO4	1.0g	($ZnSO_4.7H_2O$ 1.0g
KCal	0.5g	$CuSO_4.5H_2O$ 0.5g
$MgSO_4.7H_2O$	0.5g	water 100ml)
$FeSO_4.7H_2O$	0.01g	

Agar 15.0g

distilled water 1000 ml

trace metal solution 1.0ml

The inoculation was done using a cool flame sterilised inoculating needle dipped into a spore suspension of the test pathogen. The petridish containing the medium was held upside down and spot inoculated. The dishes were incubated at 25^{0}C for 7 days in complete darkness in an upright position. Colony characteristics were recorded and slides made using cottonblue in lactophenol and clear lactophenol for examination of morphological characters under stereo compound a microscope.

The following observations were recorded:-

Diameter of colony in cm

Obverse: Colour of conidia in mass as seen by the naked eye in day light/artificial light.

Reverse: Colour produced in the mycelium in contact with the agar medium and seen in daylight/artificial light.

Head: clavate, column or radiate

stipe: long or short, smooth or rough

vesicle: shape, big or small, fertile area

Metulae: present or absent

Phialide: shape (ampuliform or puriform), type of neck

Conidia: Globose, subglobose or ellipsoidal/eliptical, smooth or rough and as seen under a compound microscope.

For Eurotium spp.the following characteristics were included:

Ascomata: colour

Ascospore: shape (lenticular or ellipsoidal), equatorial crest present or absent, smooth or rough and colour

(b) Identification of *Fusaria*

Pure *Fusarium* isolates were examined after culturing in potato sucrose agar (PSA) and spezieller nahrstoffarmer agar (SNA).

The composition of PSA was -

Potato extract(P.E.)	500ml
Sucrose	20.0g
Agar	15.0g
Trace metal solution	1.0ml
Distilled water	500ml

Adjust pH to 6.7 ± 1

PE: Weigh out 200 g potato cubes - wash and place in cheese cloth (one layer) then in 1 litre of water and boil for 1 hour until soft mash and squeeze as much of the pulp as possible through a fine sieve

SNA

Agar	15.0g
Sugar solution (see below)	10ml
Salt solution (see below)	10ml
Distilled water	1000ml

Sugar solution

Glucose (anhydrous)	10.0 g
Sucrose	10.0 g
Distilled water	500 ml

Salt solution

KH_2PO_4	50.0g
KNO_3	50.0g
$MgSO_4.7H_2O$	25.0g
KCl	25.0g
Distilled water	500ml

Composition of trace metal as given earlier.

When the SNA medium had set, a sterile filter paper (10 x 30 mm) was placed in each petridish to enhance sporulation.

Freshly prepared SNA was inoculated in triplicate with different species of *Fusarium* from pure cultures. The plates were held upside down during inoculation to avoid spores dropping on other parts of the medium. They were incubated at 25°C for 7 days under alternating cycles of 12 hours light and 12 hours darkness in upright position. The dishes were observed directly under the microscope for morphological characters; sporodochial colour, type of conidiospores (monophialidic, or polyphialidic), microconidia (in chains, or false heads), microconidia, macroconidia(shape) and chlamydospores. Slides were also prepared and examined to get more details.

Colony characters were obtained from cultures grown on PSA medium. The dishes were incubated at 25°C for 4 days and colony diameter recorded. The same plates were re-incubated until 7 days when pigmentation in agar and other colony characters were checked. On other plates containing freshly prepared PSA the different pure cultures were inoculated and incubated at 37°C for 7 days to check for growth of the isolate at this temperature.

(c) Identification of the other fungal species

The fungal species that were not in great numbers or which had few isolates were identified using their colony characters on PDA medium and Ridell slide. The Ridell slide was set by placing a sterile microscope slide in a moist chamber then aseptically placing a small block of agar on the slide. The pathogen being studied was then inoculated into this block of agar and covered with a coverslip. The pathogen quickly finished the available food and formed fruiting structures which could be easily seem by mounting the microscopic slide or mounting the coverslip on another slide.

3.3.3 Maintenance of all fungal isolates

The fungal isolates were preserved using Booth's method (Booth, 1971). A universal bottle was filled (two third full) with seived loam soil. The bottle and soil were steam autoclaved twice (121^{0}C, 15 Psi, 15 minutes) at two days interval. A spore suspension was prepared by flooding the petridish of a pure culture with 5 ml sterile distilled water and rubbing the surface of the colony gently with a sterile glass rod to dislodge the spores. Two milliliters of the suspension was aseptically poured into the sterilized soil and caps with rubber liners fitted on loosely for two weeks at room temperature to allow the fungus to grow. The caps were then tightened and the universal bottles stored in refrigerated conditions at 4^{0}C.

All subsequent cultures were retrieved by sprinkling a few particles of the soil on solidified medium under aseptic conditions. Sub-culturing was done as soon as the fungus started to grow out of the soil particles.

3.4 SIMPLE SCREENING METHOD FOR MOLDS PRODUCING INTRACELLULAR MYCOTOXINS IN PURE CULTURE

To identify the toxigenic strains of the fungi isolated from groundnuts, a modification of a simple screening method developed by Filtenborg *et al.* (1983) was used.

The fungal isolates obtained from peanuts were maintained on PDA. Seven day old cultures of fungi associated with aflatoxin production grown on PDA at room temperature were obtained. Several plugs were cut out of these mold colonies near the centre using a sterile pasteur pipette (inner diameter 0.5 cm). The plugs were removed using a flame sterilized scalpel blade and a drop of extraction liquid chloroform was placed directly on the mycelium side of the plug. While the plug was still wet, the mycelium side of the plug was pressed gently on a spot along the application line on a pre coated thin layer chromatography (TLC) plate (fig 1) and then removed immediately. After the spot was dry the procedure was repeated with other plugs from different cultures on different points on the application line/spotting line.

Using a precision syringe (Hamilton Co.) different quantities of mixed standards of aflatoxin B_1, B_2, G_1 and G_2 were applied in quick succession at different spots on the application line keeping spots small and uniformly sized. This operation was carried out in subdued incandescent light and as rapidly as possible to avoid aflatoxin breakdown by light. A hundred millilitres of acetone- chloroform (1:9 [v/v]) was placed in an unlined developing tank (rectangular developing chamber with glass edging and all glass cover). The plate was immediately placed in the tank and sealed with a glass cover coated with petroleum jelly at the sides to make it airtight. The plates were developed in subdued light because exposure of mycotoxin on absorbent surfaces to (uv) light may lead to decomposition particularly in presence of solvents. The plates were developed until the solvent reached the solvent front

which was 100mm (fig 1) The plates were then removed from the tank and the solvent evaporated in a hood at room temperature (23 ± 2^0C) for 20 minutes.

The plates were placed with the coated side up and illuminated from above, using 366 nm uv lamp in a darkened room. The pattern of the 4 fluorescent spots of the standards were observed. In order of their decreasing relative front they were B_1, B_2, G_1 and G_2. The B_1 and B_2 fluoresced bluish which contrasted with slightly green $G_1$and G_2aflatoxins.

The fluorescent spots from the sample cultures were examined to identify those having Rf values similar to those of aflatoxin standards and similar coloured flourescence. From these plates one was able to determine the aflatoxins the different molds produced. And therefore the toxigenic molds could be identified.

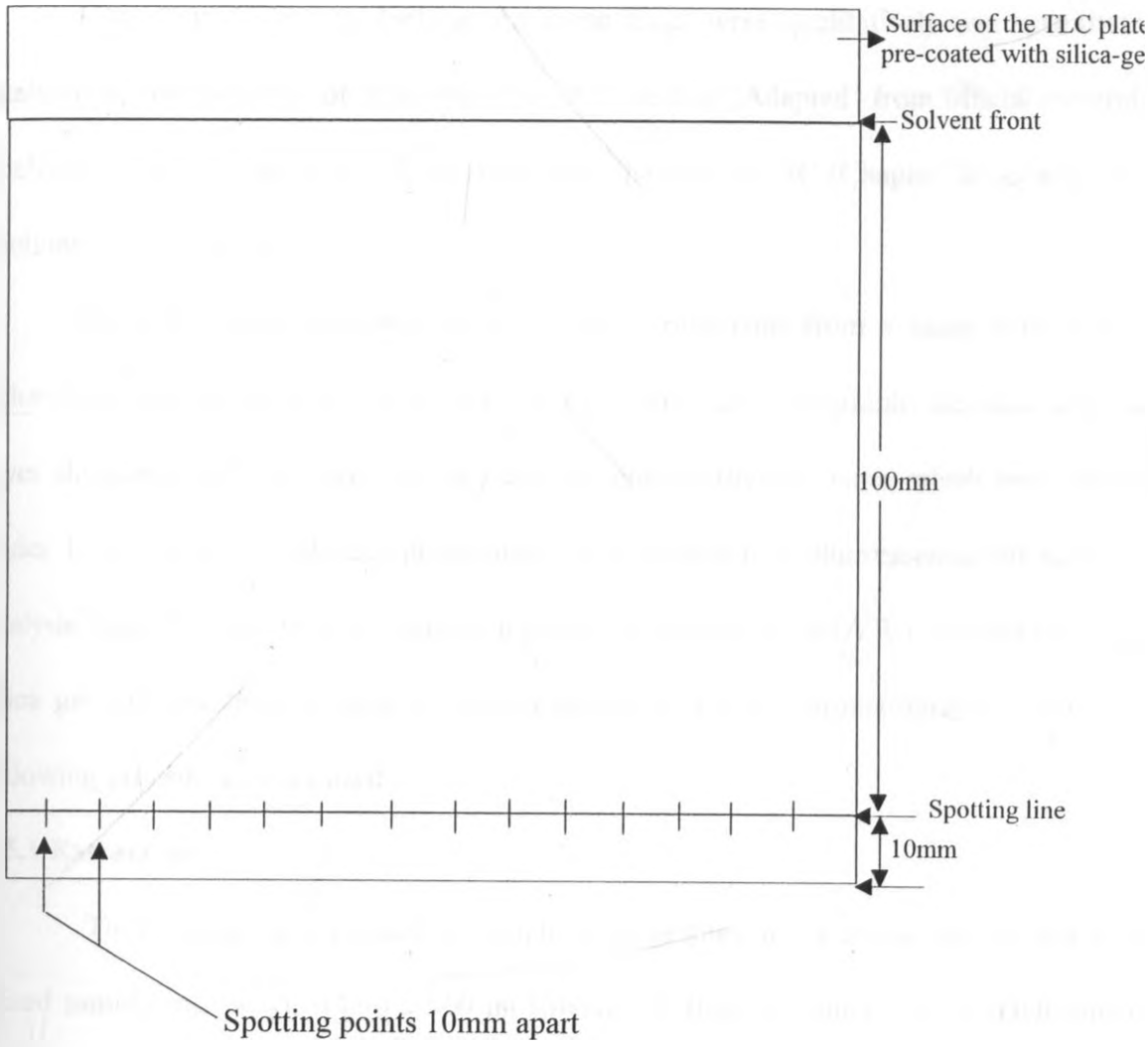

Fig. 1. Thin layer chromatography (T.L.C.) plate.

3.5 QUALITATIVE/QUANTITATIVE ANALYSIS FOR DETECTION AND ESTIMATION OF AFLATOXIN

Groundnuts found to harbour toxigenic fungi were qualitatively and quantitatively analysed for the presence of aflatoxins using CB method (Adapted from official methods of analysis of the Association of official Analytical Chemists AOAC (Chapter 26 separate, 1980, sections 26-026-26.031).

The CB method involved the extraction of aflatoxins from a moist substance with chloroform and the cleaning of the extract by column chromatography on silica gel. Thin layer chromatography on silica gel was used to separate the aflatoxins, which were identified under U.V. 366nm wavelength illumination by comparison of fluorescence intensities. The analysis basically consists of an extraction phase using chloroform (A.R.), column clean up on silica gel and quantitative assay by unidimensional thin layer chromatography (TLC). The following procedures were used.

3.5.1 Extraction

Thirty grams of a groundnut sample were ground in a warring blender and a 20g mixed sample was weighed into a 500 ml Erlenmeyer flask, to which 20g of 'Hyflosupercel' (celite) was added and mixed well. Then 200ml of chloroform and 20 ml distilled water were added avoiding splashing. The flask was well stoppered with aluminium foil and shaken for 30 minutes on a mechanical shaker at room temperature.

The contents of the flask were filtered using a 24cm whatman filter paper No. 41 or 541 into a graduated 100 ml measuring cylinder. One hundred millilitres of the filtrate was transferred into a 250 ml round bottomed flask and evaporated to near dryness in a rotary evaporator at 40°C.

3.5.2 Column clean up

3.5.2.1 Column preparation

1. Several hours before preparing the column 5g and 15g anhydrous Sodium sulphate (Na_2SO_4) were weighed into porcelain dishes and glowed in an oven for approximately 6 hours at 600°C.

2. Shortly before preparing the column, 10g silica gel for chromatography - gram size 0.063-0.200 mm were weighed, deactivated by addition of 1% by weight of distilled water and dried in an oven for 1 hour at 105°C.

3. The column size was filled up to approximately 2/3 of its height with chloroform. Then 5g anhydrous Na_2SO_4 transferred to the column (a sieve was used to prevent clotting and formation of air bubbles), the surface of the material in the column was maintained flat. Then 10g of silica gel was transferred to the column under the same precautions as those above. The tap of the column was then opened and the flow rate adjusted till it was similar to that wanted during extraction i.e. 1-2 drops per sec. Then 15g anh Na_2SO_4 was added to the column under the same precautions as those mentioned above. Finally some defatted glasswool was placed onto the upper layer using a pair of forceps taking care not to inhale the glasswool or touch it with bare hands (fig. 2).

4. Prior to transferring the extract of sample to the column the chloroform was let to drain through the adsorbent till the surface of fluid was at the glasswool level. Taking care not to let the adsorbent dry out.

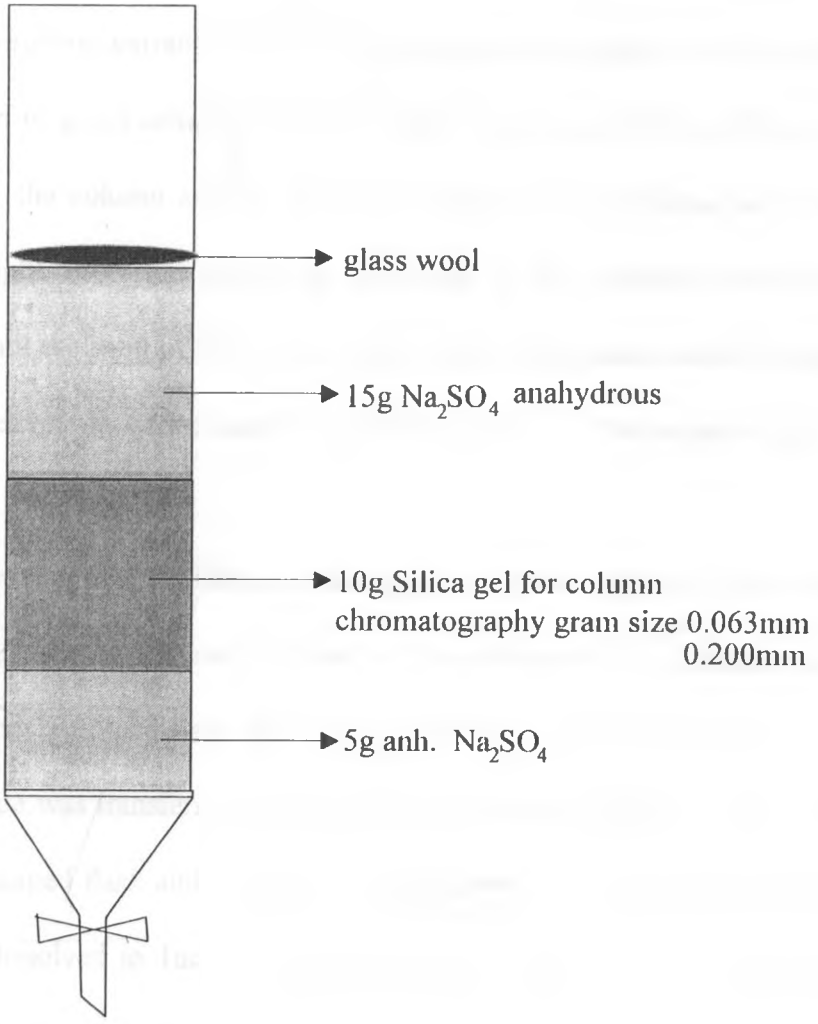

Fig. 2. Thin layer chromatography (T.L.C.) column.

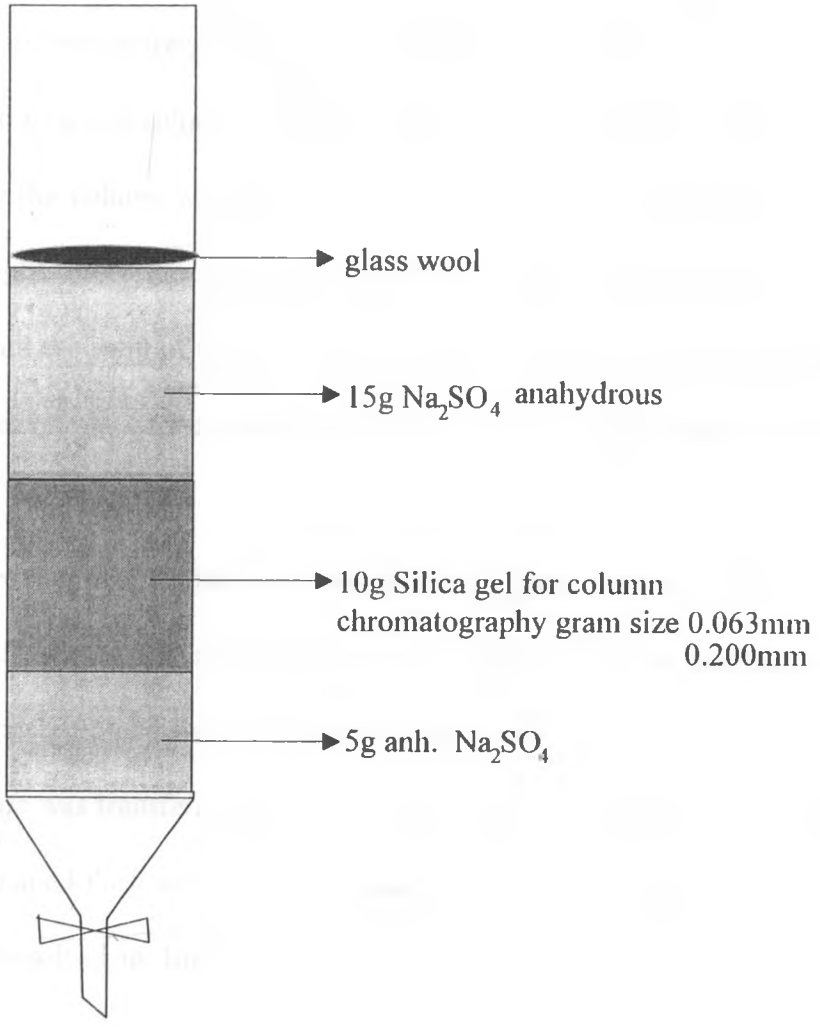

Fig. 2. Thin layer chromatography (T.L.C.) column.

The residue from extraction phase was dissolved in small volumes of chloroform with several washings totalling up to 15ml of chloroform and the mixture transferred into the column. This step is not quantitative but the total volume of solvent should be as little as possible. The chloroform-extract mixture was allowed to drain into the silica gel adsorbent and care was taken to avoid column drying up. One hundred and fifty millilitres of n-hexane was transferred to the column and the flow rate adjusted to 1-2 drops per second. High pressure which might affect the separation properties of the adsorbent was avoided. The column was run until the level of the solvent was at that of the glasswool in the column, then 150 ml of diethylether was added and run just like n- hexane. The eluate in both cases was discarded.

One hundred and fifty millilitres of chloroform-methane mixture (145.5ml chloroform plus 4.5ml methanol) was added into the flask to elute aflatoxin. This fraction was collected into a 250 ml round bottomed flask and evaporated to near dryness in a rotary evaporator (40°C). The residue was transferred quantitatively by several washings with chloroform to a 10 or 25 ml pear shaped flask and evaporated using a rotary evaporator (40°C) - to dryness. This residue was dissolved in 1ml of chloroform and transferred into a stoppered vial and protected from light by wrapping the vial in aluminium foil. This was stored in a refrigerator until use for unidimensional thin layer chromatography (T.L.C.) on precoated Kieselgel 'G' plates or plates prepared from silica gel GHR or equivalent.

3.5.3 Qualitative TLC

The vial was shaken to dissolve the residue and using a precision microsyringe two spots of 10 µl sample extract were placed on the application line 1 cm from the bottom edge of the TLC plate as rapidly as possible. The remainder of the solution was retained in the vial for quantitative analysis.

On the same plate two 0.5 µl, two 2.0 µl and two 6.0 µl spots of the mixed aflatoxin reference standard solution (containing B_1, B_2, G_1 and G_2 standards) were placed on the application line. The volumes of sample extract and standard solutions spotted were varied depending on the concentration of aflatoxin the investigator expected the sample to contain. If the sample was expected to have high amounts of aflatoxin then low volumes of sample extract and high volumes of reference standard solution were spotted. When the sample was expected to have low levels of aflatoxin the reverse was done. This was carried out in subdued incandescent light as rapidly as possible keeping the spots small and uniformly sized.

After spotting the plates were developed in the same manner as those in the simple screening method for molds producing intracellular mycotoxin in culture. The plates were then let to dry for 20 mins in a hood and viewed under U.V. lamp at 366 nm wavelength. The aflatoxin in the extract were located on the plate with the help of the co-developed standards with similar colour of fluorescence and Rf-values. In the case where interpretation of the chromatogram was hampered by the presence of other spots at similar Rf-value as of the presumed toxin spot, or when there were doubts about the identity of a "presumed" toxin spot, supplementary chromatography was done.

In such supplementary chromatography, the T.L.C. procedure was repeated, now with an internal standard superimposed on the extract spot before developing the plates (this is called spiking). After completion of TLC the superimposed standard and the "presume" toxin spot from the sample must coincide. After viewing the plates, the chromatogram was interpreted to establish whether or not the mycotoxins of interest (aflatoxins B_1, B_2, G_1 and G_2) were present in the sample. To confirm that the spots obtained contained aflatoxins, the plate was sprayed with concentrated sulfuric acid. If the aflatoxin was present the spots turned yellow and if absent they remained blue in the case of aflatoxins B_1 and B_2 for instance

From the preliminary plate one was able to determine the aflatoxins present in the sample and to establish suitable extract concentrations for quantitative T.L.C. analysis.

3.5.4 Quantitative analysis

If the preliminary plate showed that a different concentration of sample extract was required to match the standard spot, the sample extract was evaporated to dryness on a steam bath using the rotary evaporator and residue dissolved in known volume of chloroform or diluted to the concentration required. In either situation, the volume of extract initially removed was taken into account.

The sample extract was then spotted on another plate using the required volumes known amounts of the standards were also spotted and co-developed with the sample extract. Detection was again done visually to confirm the presence of the aflatoxin and by a densitometer to quantify the aflatoxin. In densitometric determination, the intensities of sample and standard spot(s) were scanned according to the instructions of the manufacturer: the required computer programme (CD 60 Desaga) was installed into the computer and the TLC plate in the densitometer (which was connected to the computer) scanned along the development path of each spot one length at a time. The peak areas were printed using a printer connected to the computer. The peak areas of the sample(s) spot was compared to the closest of the standard spot. The calculation for the aflatoxin content microgrammes/kilogram of sample in the sample was done according to the formula

$$W = \frac{V_1 V_2 P_s B V_5}{m A V_3 V_4 V_6}$$ Micrograms/kilogram

Where:

m =mass of sample from which aflatoxins were extracted (g).

P_s=mass concentration of mixed aflatoxin standard (mg/L)

V_1=volume of spot of mixed standard with the same intensity as sample spot (µl)

V_2=volume of final dilution of sample extract (ml)

V_3=volume of sample spot whose aflatoxin intensity matches that of mixed stardard spot - V1 (µl)

V_4= volume of chloroform extract chromatographed (ml)

V_5=total volume of chloroform employed during extraction(ml)

w=content of the aflatoxin in the sample in µg/Kg of sample (ppb)

V_6=volume of chloroform-extract filtrate obtained after extraction (ml)

B=average area of mycotoxin peak from sample.

A=average area of mycotoxin peak from standard.

3.6 BIOLOGICAL CONTROL

Antagonism by antibiosis in culture

The antagonistic isolates used were all obtained from Dr. Mutitu E. W., Department of Crop Science, University of Nairobi, Kenya.

The antagonists used were: *Trichoderma* isolate 13 (T_{13}), *Aspergillus niger* isolate 1 (*A. niger* 1), and *A. niger* isolate 2 (*A. niger* 2) Three identified toxigenic strains were used as test pathogens and they were: *Aspergillus flavus* (A_1), *A. parasiticus* (A_5), *Eurotium repens* (A_9).

Three different petridishes containing 10ml potato dextrose agar (PDA) were point inoculated each with one of the 3 antagonists at four equidistant points 3 cm from the centre of the petridish. A flame sterilised inoculating needle was used. The plates were incubated for two days at room temperature 23°C±2 then in the same petridishes the toxigenic strains were stab inoculated at the centre and incubated again at room temperature.

The following treatment combinations of antagonist and toxigenic strains were set up.

$T_{13}A_1$	An_1A_1	An_2A_1
$T_{13}A_5$	An_1A_5	An_2A_5
$T_{13}A_9$	An_1A_9	An_2A_9

There were three replicates for each combination. Measurements of the colony radius of the toxigenic isolates were taken on the seventh day for all the replicates.

Two more experiments were set up at the same time by inoculation of toxigenic strain two days before the antagonist and the other at the same time with the potential antagonist and incubated for 7 days after which results were recorded. The statistical design was a completely randomized design (CRD) with 3 replicates. (Table 1)

b) Determination of effects of antagonists in mycotoxin production

Seven day old fungal cultures of potential antagonists and toxigenic strains maintained on potato dextrose agar (PDA) at room temperature were used to make spore suspensions. Five millilitres of cool sterile distilled water was poured on the colony of each isolate and the colony rubbed gently with a sterile glass rod to dislodge the spores. This was passed through one layer of cheese cloth. The spore count was determined under a compound microscope using a haemocytometer and adjusted to 1 x 10^5 spores/ml with sterile distilled water.

Groundnuts autoclaved at 121°C, 15 psi for 15 mins and cooled were sprayed with a spore suspension (1x 10^5 spores/ml) of the potential antagonist Trichoderma iso.13 and *A. niger* iso 1. The groundnut seeds were then plated -ten seeds per petridish containing 15ml of PDA and incubated at room temperature. After 2 days the groundnuts in the petridishes were divided into 3 and one batch inoculated with one of each of the toxigenic strains (A_1, A_5, A_9) by spraying with their spore suspension 1x10^5 spores/ml using an atomizer. The groundnuts were then plated ten seeds per petri dish incubated at room temperature in petridishes containing fresh 10 ml of solidified PDA. After 5 days the groundnuts were harvested in brown paper bags and placed in the refrigerator for mycotoxin analysis using the CB method as described earlier

A similar experiment was done by inoculating the toxigenic fungi 2 days before the antagonist and in a third set at the same time with the potential antagonist. Each experiment was replicated 3 times and after five days of incubation at room temperature all replicates were bulked together to form one experimental unit due to limitation of funds for aflatoxin analysis.

For the control the groundnuts were inoculated only with the toxigenic fungus. A sample of the autoclaved groundnuts was analysed for the presence of aflatoxin before inoculation with the toxigenic fungi using the CB method given earlier. This was done to establish the initial aflatoxin content in the groundnuts before inoculation with aflatoxigenic

strains. The samples were coded using alphabetical letters at random and then analysed systematically according to the order of the codes to enhance randomness.

Table: 1 : Treatment combinations and their code letters.

Test Pathogen	**Antagonist seeded 2 days after the pathogen**	**code letter**
A. flavus (A1)	A. niger 1	L
	T13	I
A. parasiticus (A5)	A. niger 1	K
	T13	B
E. repens (A9)	A. niger 1	F
	T13	C
Test pathogen	**Antagonist seeded at the same time with the pathogen**	**Code letter**
A1	T13	M
	A. niger 1	J
A5	T13	D
	A. niger 1	H
A9	T13	N
	A. niger 1	P
Test pathogen	**Antagonist seeded 2 days before the pathogen**	**Code letter**
A1	T13	V
	A. niger 1	G
A5	T13	Y
	A. niger 1	A
A9	T13	R
	A. niger 1	E

4.0.RESULTS

4.1 ISOLATION OF FUNGI ASSOCIATED WITH GROUNDNUT SEEDS.

A range of fungi were isolated from the market groundnut samples using the blotter and the agar plate method. *Rhizopus* sp. and *Fusarium* sp. were isolated from groundnut samples from all markets, while *Aspergillus* sp. were isolated from all markets except Gikomba (Table 2). *Penicillium* spp. was isolated from four (Kawangware, Kangemi, Nyamakima and Gikomba) markets and was absent in one (Nyayo) market. *Eurotium repens* was only present in Nyamakima and Kawangware market samples while *Sclerotium* spp was isolated from Nyamakima and Kangemi market samples. *Rhizoctonia* sp was isolated from seeds obtained from Gikomba and Nyayo markets only (Table 2).

Nyamakima market samples had the highest number of fungi isolated while Gikomba market sample had the least. The *Aspergillus* species isolated were *:A.flavus, A. niger, A. parasiticus* and *A. ochraceous* while the *Fusarium* sp were *F. oxysporum, F. equiseti* and *F. torulosum*. (Table 2,Plate 1 and 2).

Table 2: **Species of fungi found in association with groundnuts from different open markets in Nairobi.(isolation based on agar and blotter plate methods).**

Source/ Market	Fungi isolated						
	Rhizo	Rhiz	Pen	Euro rep.	Asper	Scler	Fus.
Gikomba	+	+	+	-	-	-	+
Nyamakima	+	-	+	+	+	+	+
Nyayo	+	+	-	-	+	-	+
Kawangware	+	-	+	+	+	-	+
Kangemi	+	-	+	-	+	+	+

Rhizo.= *Rhizopus* spp., Rhiz.=*Rhizoctonia* spp. Pen. =*Penicillium*, Euro. rep. = *Eurotium repens*, Asper. = *Aspergillus* spp., Sclero. = *Sclerotium* spp., Fus. = *Fusarium* spp

4.1.1 Fungal isolation from white groundnut seeds using agar plate method

Rhizopus spp. was common in all white seeded samples. However in Nyamakima and Kawangware markets this species was only isolated from surface non-sterilised groundnuts. In the case of Kangemi market it was isolated from both non-sterilised and surface sterilised samples but the percentage numbers of seeds associated with it were higher in the non-sterilsed-40% than in the sterilised-10%. *Penicillium* spp. was also found in the white groundnut seed samples. In Nyamakima and Kawangware markets this species was only isolated from non-sterilised peanuts while in Kangemi market sample it was only in the surface sterilised ones. *Fusarium oxysporum* was found in all the samples of white groundnuts. In Kawangware market it was isolated from non- sterilised peanut seeds (10%) while in Kangemi and Nyamakima it was isolated from the surface sterilised seeds.

Sclerotium spp was isolated from surface sterilised peanuts from Nyamakima and Kangemi markets only. *Aspergillus parasiticus* was isolated from surface sterilised samples

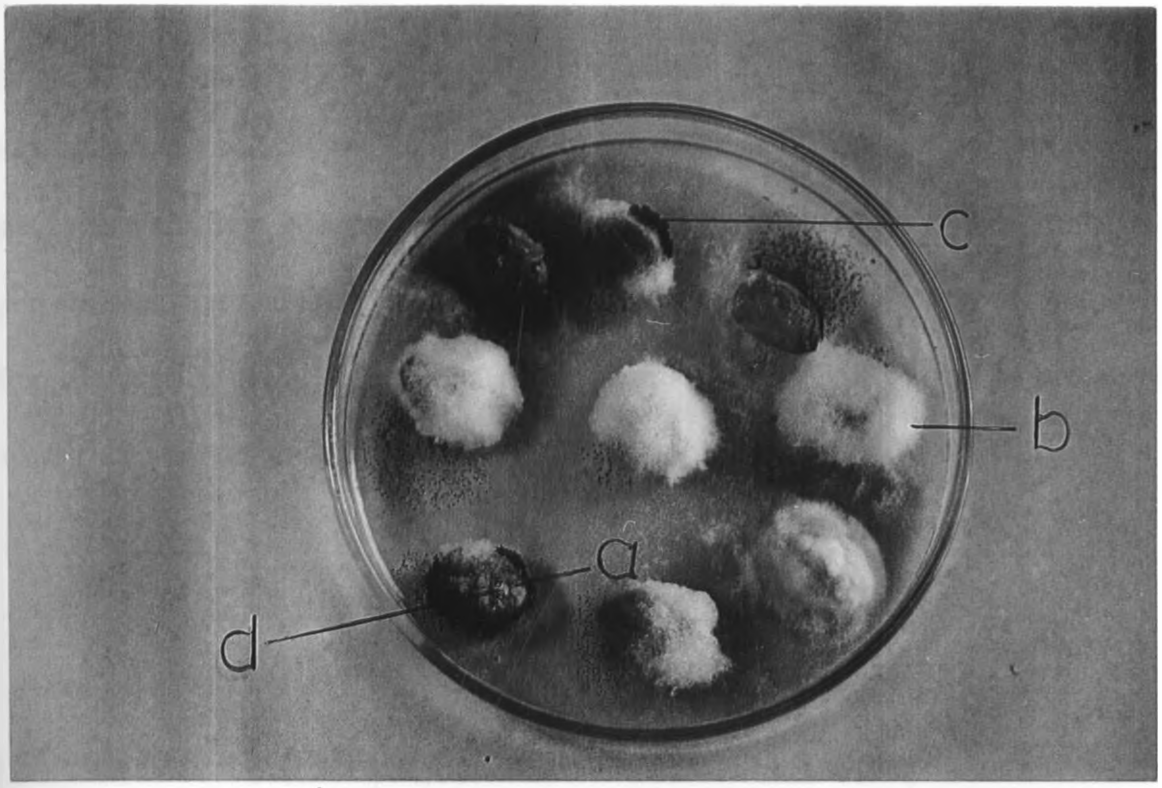

Plate 1: Some fungi isolated from groundnuts from Nairobi markets
(a.) *Penicillium* spp (b.) *Fusarium* spp (c.) *Aspergillus niger* (d.) *A. niger* at an early stage

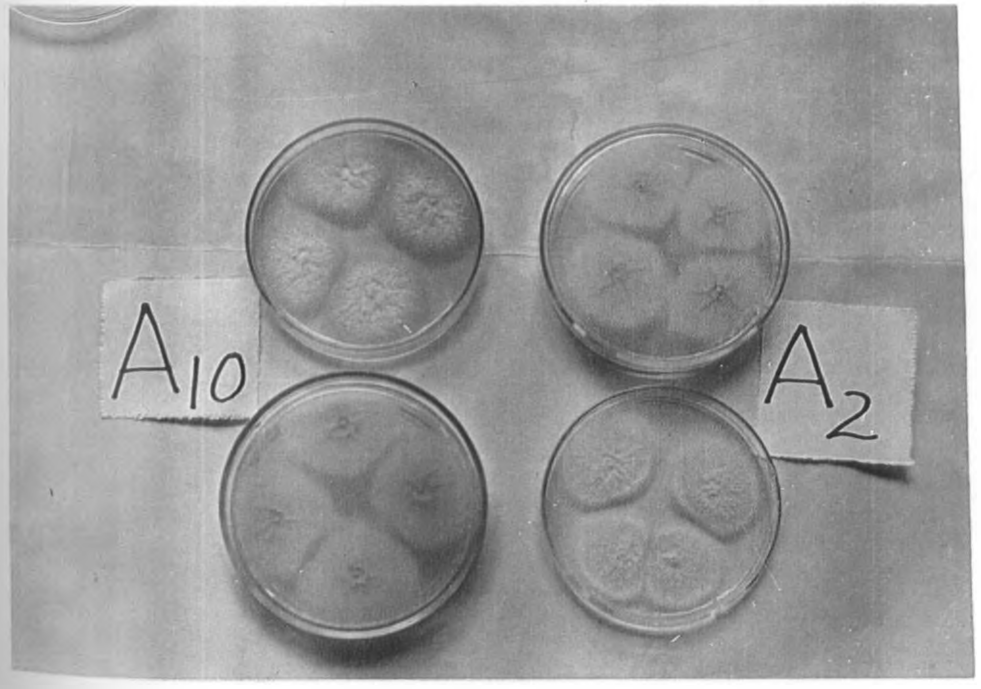

Plate 2. Isolates of *Aspergillus* spp from Nairobi groundnuts.
A_{10} -*Aspergillus ochraceous* A_2 - *Aspergillus flavus*

from Kawangware market and *A. niger* only from Kangemi market. A higher incidence of same type of fungus was in the sterilised seed than in the non- sterilised ones. *Fusarium torulosum* was isolated from 3.3% of non- sterilised seeds as opposed to 33.3% of surface sterilised seeds from Kangemi market. *Eurotium repens* was present in 3.3% of surface non-sterilised nuts and in 46.6% of surface sterilised seeds from Kawangware. *Rhizopus* sp. from Kangemi market gave different results whereby a higher percentage of contaminated seeds were non- sterilised (Table 3).

TABLE 3: **Different fungi (in %) isolated from white groundnut seeds from different markets using agar plate method.**

Source	**Nyamakima**	**Nyamakima**	**Kawangware**	**Kawangware**	**Kangemi**	**Kangemi**
state of seed	**non-sterilised**	**surface-sterilised**	**non-sterilised**	**surface- sterilised**	**non-sterilised**	**surface sterilised**
Micro-organism	%	%	%	%	%	%
Rhizopus SPP.	100.0	-	80.0	-	40.0	10.0
Sclerotium SPP.	-	6.7	-	-	-	33.3
Fusarium oxysporum	-	6.7	10.0	-	-	16.7
Penicilium SPP.	3.3	-	20.0	-	-	6.7
Eurotium repens			3.3	46.6		
A. parasiticus				20.0		
A. niger						6.7
A. flavus						
F. torulosum					3.3	33.3

4.1.2 : **Fungi isolated from white peanut seeds using blotter method:**

Rhizopus spp. was isolated from all the samples. It was associated with 80% non-sterilised peanuts from Kawangware and 30% from Kangemi markets while in Nyamakima it was also isolated from surface sterilised seeds. The number of seeds that yielded *Rhizopus* spp. in the Nyamakima sample were higher in non-sterilised (16%) than in surface sterilised seeds (3.3%). *Aspergillus niger* was isolated from nuts from Kangemi and Kawangware markets. In the case of Kangemi peanuts 63.3% non sterilised and 13.3% surface sterilised ones yielded *A. niger*. *Aspergillus flavus* was only found in Kangemi market nuts where 3.3% of both non-sterilised and surface sterilised nuts were contaminated. *Aspergillus parasiticus* was found in Kawangware market in 6.7% of the non-sterilised peanuts and in 36.7% of surface sterilised ones. *Eurotium repens* was isolated from Nyamakima and Kawangware samples while *Sclerotium* spp. was only isolated from Kangemi peanuts. *Penicillium* spp. and *Fusarium oxysporum* were found in Kangemi and Kawangware nuts. (Table 4)

Table 4: **Fungi isolated from white peanut seed using blotter method.**

Source	**Nyamakima**	**Nyamakima**	**Kawangware**	**Kawangware**	**Kangemi**	**Kangemi**
state of seed	**non- sterilised**	**surface sterilised**	**non- sterilised**	**surface sterilised**	**non- sterilised**	**surface sterilised**
Micro- organism	%	%	%	%	%	
Rhizopus sp.	16	3.3	80	-	30	
Scler..	-	-	-	-	-	13.3
F. o.	-	-	6.7	-	-	46.7
Pen.	-	-	3.3	6.7	-	6.7
E. repens	3.3	-	-	10	-	-
A. par.	-	-	6.7	36.7	-	-
A. niger	-	-	-	30	63.3	13.3
A. flavus	-	-	-	-	3.3	3.3

Scler.=*Sclerotium* spp., A. par.=*A.parasiticus* Pen.=*Penicillium* F.o.=*Fusarium oxysporum*

More fungi were isolated from Nyamakima white groundnuts seeds using the agar plate method than the blotter method. *Penicillium* sp. ,*Sclerotium* sp. and *Fusarium oxysporum* were isolated from Nyamakima market sample only using agar method but not blotter. While *A. flavus* was only isolated using blotter method. The percent of non-sterilised seeds from Nyamakima market associated with *Rhizopus spp* was higher in agar plate method (100%) than in the blotter method (16%). In the case of Kawangware market sample: *A. niger* was only isolated using blotter method. From Kangemi market samples, *A. flavus* was only isolated using blotter method while *F. torulosum* was only isolated by agar plate method.

4.1.3: Fungi isolated from red groundnut seeds from different markets using agar plate method:

Rhizopus spp. were present in all samples in both non-sterilised and sterilised seeds except in the Kawangware peanuts where they were not isolated from surface sterilised seeds. A hundred percent of non-sterilised seeds yielded *Rhizopus* spp. from Nyayo, Kangemi and Kawangware markets. *Rhizoctonia* spp. and *Penicilium* spp. were isolated from Gikomba market samples. *Fusarium torulosum* and *F. equiseti* were isolated from 6.7% of surface sterilised seeds from Gikomba and Nyamakima markets respectively. *F. oxysporum* was isolated from surface sterilised seeds from Nyayo market where 10% of the seed was contaminated. *Aspergillus* spp. were only isolated from surface sterilised nuts: *A. flavus* from 60% of Nyamakima groundnuts and 3.3% Kangemi; *A. parasiticus* was isolated from 20% of Kawangware nuts and *A. ochraceous* from 3.3% of Nyayo market nuts. *A. niger* was isolated from 3.3% of Nyamakima nuts, 23% of Nyayo nuts and 46.7% of Kawangware nuts. *Sclerotium* spp. was found in surface sterilised groundnuts : 3.3% of Nyamakima nuts and 36.7% of Kawangware nuts. (Table 5)

TABLE 5 **Fungi (in %) isolated from red peanut seeds from different markets using agar plate method.**

Source	Gik.	Gik.	Nyam.	Nyam.	Nyayo	Nyayo	Kaw	Kaw.	Kan .	Kan.
State of seed	n-s	s-s	n-s	s-s	n-s	s-s	n-s	s-s	n-s	s-s
M.o	%	%	%	%	%	%	%	%	%	%
Rhizopus sp.	56.7	20.0	46.7	33.3	100.0	6.7	100.0	-	100.0	36.7
Rhiz.	6.7	10.0	-	-	-	-	-	-	-	-
Pen.	10.0	3.3	-	-	-	-	-	-	-	-
F.t.	-	6.7	-	-	-	-	-	-	-	-
F.e	-	-	-	6.7	-	-	-	-	--	-
F.o.	-	-	-	-	-	10.0	-	-	-	-
A.f	-	-	-	60.0	-	-	-	-	-	3.3
A.p	-	-	-	-	-	-	-	20.0	-	-
A.o	-	-	-	-	-	3.3	-	-	-	-
A.n	-	-	-	3.3	-	23.0	-	46.7	-	-
Scler.	-	-	-	3.3	-	-	-	36.7	-	-

Key : Gik.=Gikomba, Nyam.=Nyamakima, Kaw.=Kawangware, Kan.=Kangemi, Rhiz.=*Rhizoctonia* spp., Pen.=*Penicilium* spp. F.t.=*Fusarium torulosum*, F.e.=*F.equiseti*, F.o.=*F. oxysporum* A.f.=*Aspergillus flavus*, A.p.=*A. parasiticus*, A.o.=*A.ochraceous*, A.n.=*A. niger* Scler.=*Sclerotium* spp., n/s=non sterilised, s/s=surface sterilised

Where *Rhizopus* spp. had contaminated 100% of the seeds, no other fungus was able to grow. The non-sterilised seeds were associated with a narrower range of micro-organisms compared to the surface sterilised ones of the same sample (Table 4). In Gikomba non-sterilised seeds yielded 3 types of micro-organisms while surface sterilised ones yielded 4 types. Non-sterilised seeds from Nyamakima, Nyayo, Kawangware and Kangemi samples were contaminated only with *Rhizopus* spp. surface sterilised seeds from the same markets had more than one type fungus (Table 5)

4.1.4 Different fungi isolated from red peanuts seeds using blotter plate method:

Rhizopus spp. was isolated from all the market samples. In Gikomba, Nyamakima and Nyayo market seeds *Rhizopus* was in both non-sterilised and surface sterilised seeds while in Kangemi and Kawangware samples it was only in non-sterilised seeds. *Penicilium* spp. was in 4 of the 5 markets in relatively low numbers; 3.3% of surface sterilised seeds from Gikomba, Nyayo and Kawangware but in 5% of those from Nyamakima. *Fusarium oxysporum* was found in 46.7% of surface sterilised seeds from Kawangware and in 66.7% of Kangemi seeds. *Aspergillus niger* was found in 75% surface sterilised seeds from Nyamakima, 20% of seed from Nyayo, 26.7% of seed from Kawangware and 6.7% of seed from Kangemi market. *A. ochraceous* was only found in 6.7% of seeds from Nyayo market. *A. flavus* was isolated from 30% of Nyamakima seeds and 3.3% of seeds from Kangemi sample. (Table 6)

TABLE 6: **Different fungi (in %) isolated from red peanut seeds from different markets using blotter test method:**

Source	Gik.	Gik.	Nyam	Nyam	Nyayo	Nyayo	Kaw	Kaw	Kan	Kan
State of seed	n-s	s-s	n-s	s-s	n-s	s-s	n-s	s-s	n-s	s-s
M.o	%	%	%	%	%	%	%	%	%	%
Rhiz.	56.7	16.7	50	10	33.3	10	40	-	33.3	
Pen.	-	3.3	-	5	-	3.3	3.3	3.3	-	-
F.o.	-	-	-	-	-	-	-	46.7	-	66.7
A.f.	-	-	-	30	-	-	-	-	3.3	-
A.o.	-	-	-	-	-	6.7	-	-	-	-
A.n.	-	-	-	75	-	20	-	26.7	-	6.7

Key : Gik=Gikomba Nyam=Nyamakima Kaw=Kawangware Kan=Kangemi, Rhiz.=*Rhizopus* spp. Pen.=*Penicillium* spp. F.O.=*Fusarium oxysporum*, A.f.=*Aspergillus flavus*, A.o.=*A.ochraceous*, A.n.=*A.niger*.

More fungi were isolated using agar method than blotter method from Gikomba market sample. *Rhizopus* spp., *Rhizoctonia* spp., *Penicillium* spp. and *F. torulosum* were isolated using agar method while only *Rhizopus* and *Pecillium* spp. were isolated from the same sample using blotter plate method. From Nyamakima market samples, *Rhizopus* spp. *A. niger* and *A. flavus* were isolated by both methods while *F. equiseti* and *Sclerotium* spp. were isolated only by the agar method.

In the case of Nyayo market, *F. oxysporum* was isolated using agar method only while *Penicillium* spp. was isolated with the blotter method only. *Rhizopus* spp. *A. niger* and *A. ochraceous* were common in both methods but a higher percentage of seeds were found to have *Rhizopus* spp. when agar method was used. In Kawangware market sample *Rhizopus* spp. and *A. niger* were isolated using both methods although the percentages were higher in agar method. *A. parasiticus* and *Sclerotium* spp. were only observed under agar method while *Penicillium* spp. and *F. torulosum* were isolated with the blotter method. In Kangemi market sample, blotter method brought out more fungi than agar method. *Rhizopus* spp. and *A. flavus* were isolated using both methods while *F. torulosum* and *A. niger* were isolated using blotter method only. (Table 6)

Many fungi were obtained using the different methods from the different market varieties. A mean calculated for all the fungi isolated from all the markets using the 2 methods gave the following percentage occurrence: *Rhizopus* spp-49%, *A.niger*- 15% , *F. oxysporum*-10%, *Sclerotium* spp.-5%, *A. parasiticus*-5%, *A. flavus*-4%, *E. repens*-4%, *Penicillium* spp.-4% and others-4%.Others included : *F. torulosum, F. equiseti, Rhizoctonia* spp. and *A. ochraceous* (Fig.3).

The 3 markets (Nyamakima, Kawangware and Kangemi) from which the two groundnut varieties were available, were analysed for variance and were not found to be significantly different (appendix 1).

Key

F. oxy. -	*Fusarium oxysporum*
Scler. -	*Sclerotium* spp.
A. par. -	*Aspergillus parasiticus*
E. repens -	*Eurotium repens*
Peni. -	*Penicillium* spp.
Rhizo -	*Rhizopus* spp.
Others -	*F. torulosum*
	F. equiseti
	Rhizoctonia spp.
	A. ochraceous

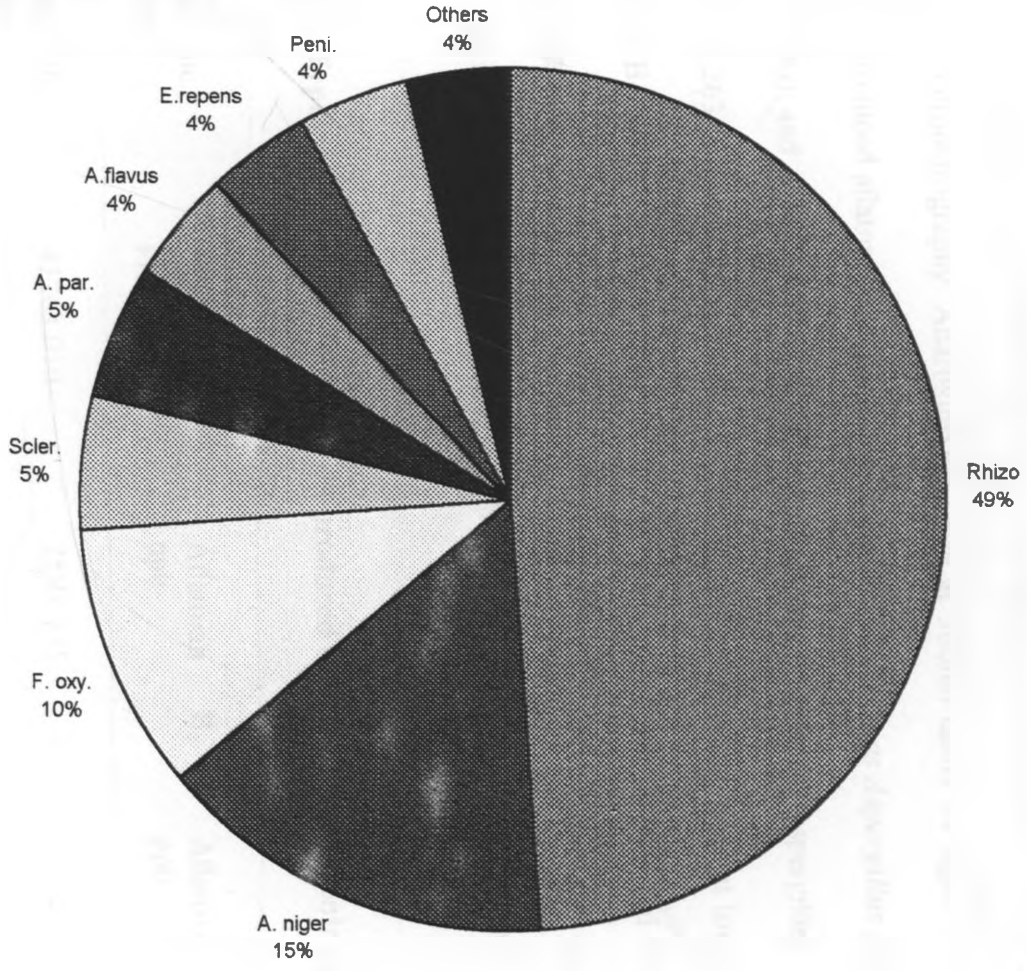

Fig. 3 Fungi isolated from groundnuts in Nairobi.

4.2 SIMPLE SCREENING METHOD FOR MOLDS PRODUCING INTRACELLULAR MYCOTOXINS IN CULTURE

The screening method used for molds producing intracellular mycotoxin in culture was by thin layer chromatography. Aflatoxins flouresce at 366nm under uv light. The toxigenic strains that produced aflatoxin in culture were identified (Plate 3) *as Aspergillus flavus*(A_1), *A. parasiticus*(A_5) and *Eurotium repens*(A_9). Among the species of Aspergillus; isolate A_1 produced 41,263 ppb while A_5 produced 67,266.785 ppb of aflatoxin B_1. But lower quantities of aflatoxin B_2 were detected where ;isolate A_1 produced 2,401.02 ppb and A5 produced 2,597.375 ppb. *E. repens* on the other hand produced large amounts of B_1 (57,145.881 ppb), equally high amounts of B_2(46,502.787 ppb) and low quantities of G_1 (75.265 ppb). (Table 7)

TABLE 7: **Types and quantities of aflatoxin produced by different toxigenic fungi**

Toxigenic fungus	Aflatoxin B_1 ppb	Aflatoxin B_2 ppb	Aflatoxin G_1 ppb
Aspergillus flavus	41263.010	2597.375	-
Aspergillus parasiticus	67267.795	2401.020	-
Eurotium repens	571416.881	46503.787	75.265

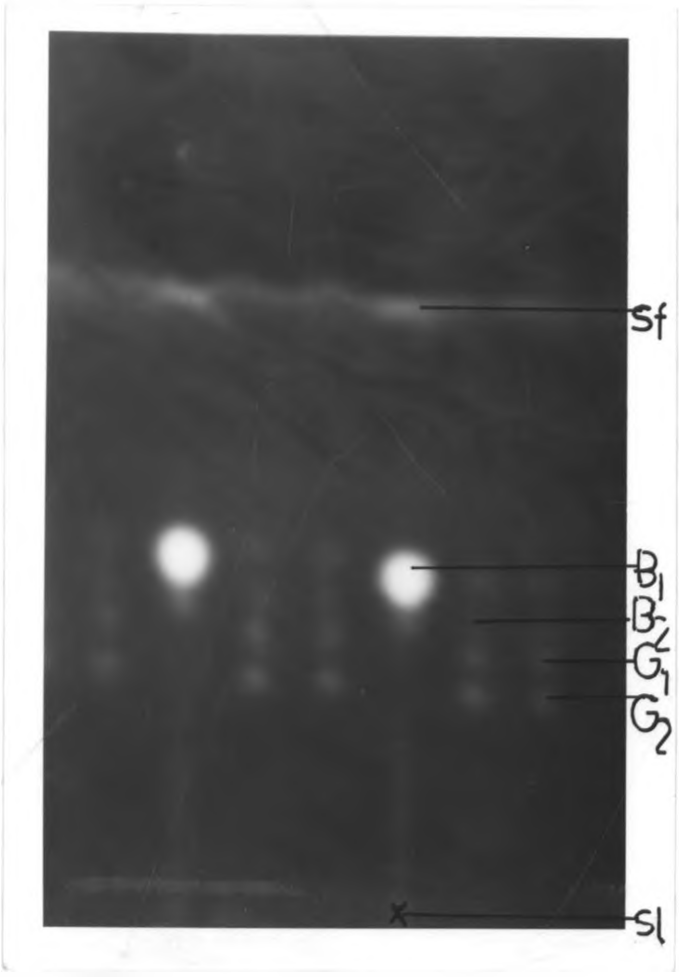

Plate 3
Developed TLC plate showing the relative positions of aflatoxins B_1, B_2, G_1, and G_2 spotting line (sl) and solvent front (sf). X shows points of spotting the sample

4.3 QUALITATIVE /QUANTITATIVE ANALYSIS FOR DETECTION AND ESTIMATION OF AFLATOXIN.

The groundnut samples found to harbour toxigenic fungal strains were: Red groundnut varieties from Nyamakima, Kawangware and Kangemi markets, white varieties from Kangemi and Kawangware markets. There were only two samples: Nyamakima and Kangemi red variety that were found to contain detectable levels of aflatoxin after analysis before inoculation with toxigenic strains. These levels were quantified and Nyamakima red variety found to contain 125.216 ppb of aflatoxin B_1 and 1.55 ppb of B_2 while Kangemi red variety had 415.466 ppb of B_1 and 21.364 ppb of B_2 (Table 8).The permitted total aflatoxin content in any edible sample is 20.0 ppb (Kenya Bureau of Standards) which is many times lower than the sample aflatoxin levels found in these two red variety samples.

TABLE 8: Aflatoxin content in the initial groundnut samples before inoculation with the toxigenic strains.

Sample/Variety	Aflatoxin B_1 (ppb)	Aflatoxin B_2(ppb)
Nyamakima red	125.216	1.550
Kangemi red	415.466	21.364
Kangemi white	Not detected	Not detected
Kawangware red	Not detected	Not detected
Kawangware white	Not detected	Not detected

4.4 BIOLOGICAL CONTROL.

4.4.1 Effects of antagonists on mycotoxin production by toxigenic fungi.

The groundnuts used in this experiment were part of the red groundnut sample from Kawangware market. This sample was not found to contain any initial detectable levels of aflatoxin.

(a)Antagonistic effects of *Trichoderma* isolate 13 (T_{13}).

(i)Effects of T_{13} inoculated 2 days after toxigenic isolate.

When the antagonist *Trichoderma* (T_{13}) was inoculated on to the groundnuts 2 days after the toxigenic fungus *Aspergillus flavus* (A_1) both fungi colonized well on the inoculated groundnut seeds and there was significant reduction in the levels of aflatoxin produced compared to the control. The level of aflatoxin B_1 was reduced from 41,263.01ppb in the control to 33,029.062 ppb this was a 19.95% reduction , the decrease of B_2 was from 2,597.375 ppb to 450 ppb an 82.67% decrease. In the case of *A. parasiticus* (A_5) aflatoxin was decreased from 67,266.795 to 7515.369 ppb (88.83% reduction) and B_2 from 2,401.02 ppb to 66.986 ppb (a 97.21% reduction). In the case of *Eurotium repens* (A_9) the reductions were greater :B_1 was reduced by 97.99% from 57,145.866 to 1151.183 ppb, while B_2 was decreased from 46,502.787 ppb to non-detectable levels (100% reduction) and G_1 was also reduced by 100% from 75.265 ppb to non-detectable levels (Table 9 and Fig 4).

TABLE 9: Effects of antagonist *Trichoderma* isolate 13 (T_{13}) on mycotoxin production by toxigenic isolates in peanut seeds when T_{13} was inoculated two days after the toxigenic strains.

		Aflatoxin levels ppb and % reductions					
Ant	Path	B1	% (B1)	B2	% (B2)	G1	%(G1)
T13	A1	33029.062	19.95	450	82.67	-	-
T13	A5	7515.369	88.83	66.986	97.21	-	-
T13	A9	1151.183	97.99	ND	100	ND	100

Key: Path.=Pathogen, Ant.=Antagonist, ND=not detectable

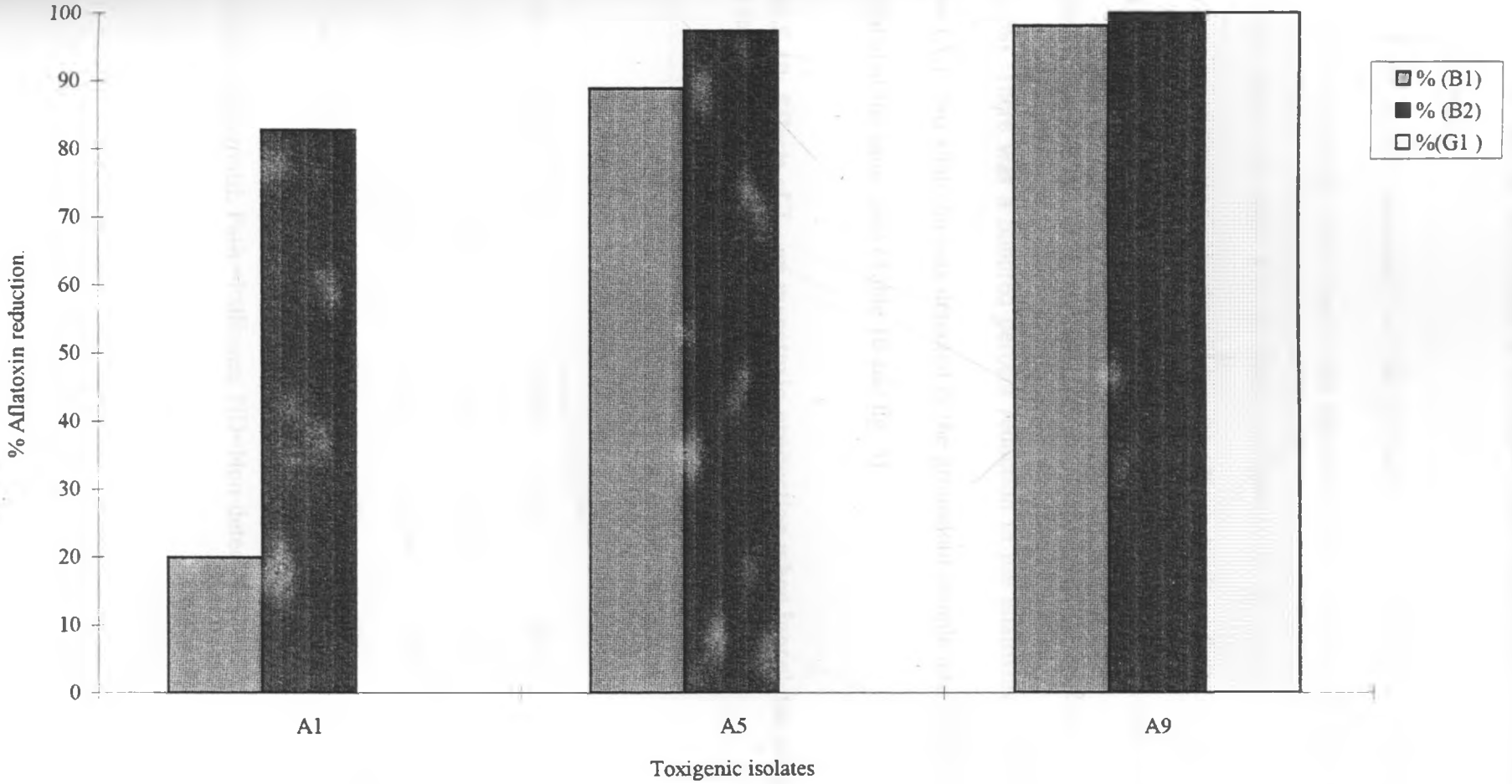

Fig. 4. % reduction of aflatoxin produced by toxigenic isolates when antagonisT_{13} was inoculated 2 days after the aflatoxigenic isolate. Where A_1 is *Aspergillus flavus*, A_5 *Aspergillus parasiticus* and A_9 *Eurotium repens*.

(ii) Effects of T_{13} on mycotoxin production when inoculated at the same time with toxigenic isolates;

When T_{13} was inoculated into the groundnut seeds at the same time with the toxigenic isolates, the percentage reductions of the aflatoxin levels was much greater than when it was inoculated after two days. For *A. flavus* (A_1) aflatoxin B_1 was reduced by 56.54% from 41,263.01 to 17,933.416 ppb, there was a 91.65% reduction of aflatoxin B2 from 2,597.375 to 216.972 ppb. In the case of *A. parasiticus* (A_5) aflatoxin B_1 was reduced to 442.657 from 67,266.795 ppb a 99.34% reduction, B_2 was decreased to 21.206 from 2,401.02 ppb (99.12%). There was a hundred percent reduction in the aflatoxins produced by *Eurotium repens* (A_9). No aflatoxin was detected in the groundnut sample into which A_9 and T13 were inoculated at the same time (Table 10 and fig. 5).

TABLE 10: Effects of T_{13} on mycotoxin production when inoculated at the same time with toxigenic isolates.

		Aflatoxin levels ppb and % reductions					
Ant	Path	B_1	% (B_1)	B_2	%(B_2)	G_1	% G*1*
T13	A1	17933.42	56.54	216.97	91.65		
T13	A5	442.67	99.34	21.206	99.12		
T13	A9	ND	100	ND	100	ND	100

Key: Ant.=Antagonist, Path.=Pathogen, ND=Non-detectable

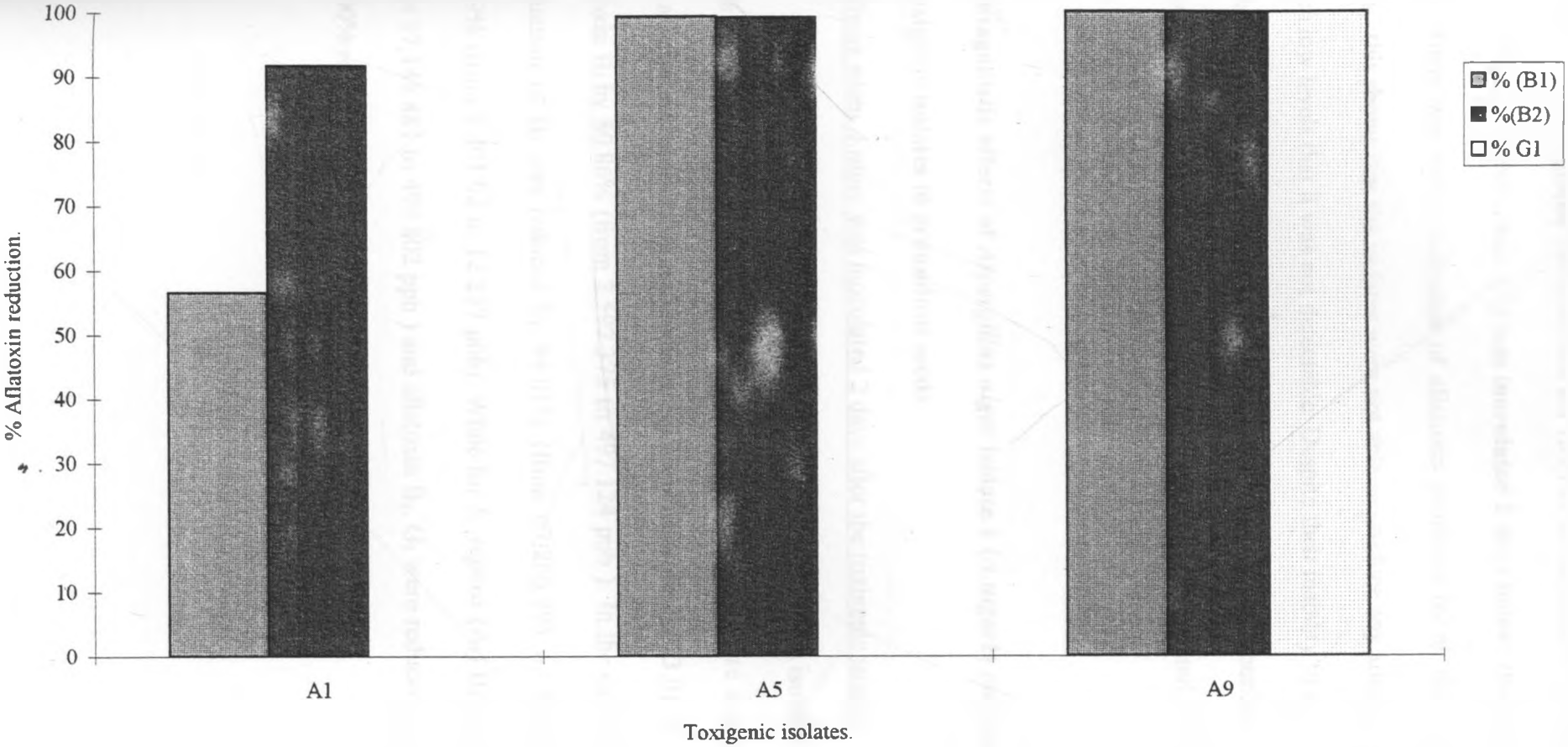

Fig.5 % reduction of aflatoxin produced by toxigenic isolates when antagonist T_{13} was inoculated at the same time with these isolates. Where A_1 - *Aspergillus flavus*, A_5 - *Aspergillus parasiticus* and A_9- *Eurotium repens*.

(iii) Effects of antagonist *Trichoderma* iso 13 (T13)on mycotoxin production by toxigenic isolates in groundnuts when T13 was inoculated 2 days before the toxigenic isolates.

There was 100% reduction of aflatoxin produced by all the toxigenic isolates, this shows, this shows that the isolates were not able to produce any aflatoxin or if they did it was at such low levels that it was not detectable. Despite their inability to produce detectable levels of aflatoxins, the micro-organisms were observed to grow on groundnuts. Their growth was however not as much as when the toxigenic isolates were inoculated 2 days before and at the same time with T13.

(b)Antagonistic effects of *Aspergillus niger* isolate 1 (*A.niger*1) on mycotoxin production by toxigenic isolates in groundnut seeds

(i)Effects when *A.niger* was inoculated 2 days after the toxigenic isolate:

When *A. niger* was inoculated two days after the toxigenic isolates there was reduction in the aflatoxin produced by the isolates. For the seeds that were inoculated with *A. flavus* (A_1) aflatoxin B_1 production was reduced by 45.14% (from 41263.01 to 22637.399 ppb) and aflatoxin B_2 by 80.86% (from 2,597.375 to 497.124 ppb). In the case of *A. parasiticus* (A_5) production of B_1 was reduced by 94.01% (from 67,266.795 to 4030.16 ppb)and B_2 by 99.49% (from 2,401.02 to 12.237 pbb). While for *E. repens* (A_9) B_1 was reduced by 99.13% (from 57,145.881 to 499.802 ppb) and aflatoxin B_2, G_1 were reduced to non-detectable levels (a 100% reduction).(Table 11 and fig 6).

TABLE 11: **Effects of antagonist *A.niger* isolate 1 on mycotoxin production by toxigenic isolates in peanut seeds when the antagonist was inoculated 2 days after the toxigenic strain:**

		Aflatoxin levels ppb and % reductions					
Atagonist	Pathogen	B_1	% (B_1)	B_2	% (B_2)	G_1	%(G_1)
A.niger 1	A_1	22637.4	45.14	497.124	80.86		
A.niger 1	A_5	4030.16	94.01	12.237	99.49		
A.niger 1	A_9	499.802	99.13	ND	100	ND	100

KEY: ND - non-detectable

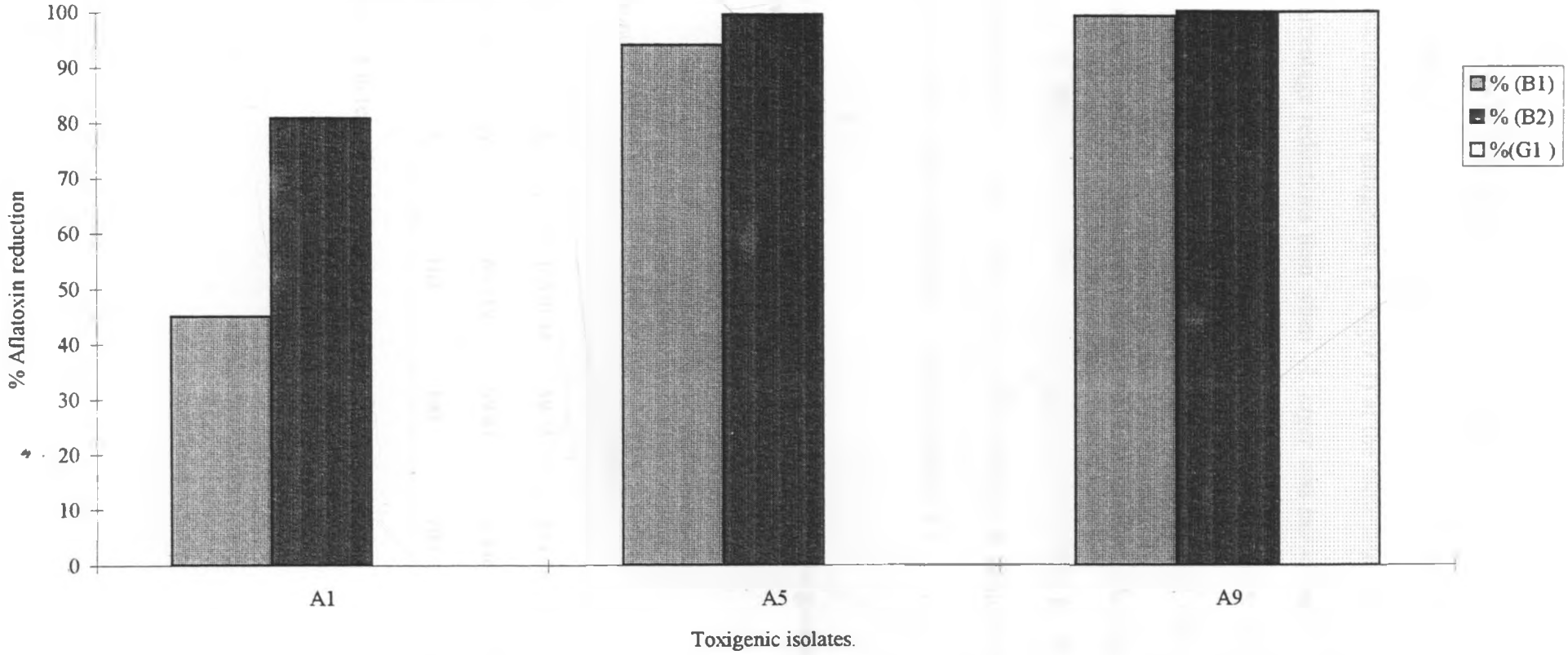

Fig 6. % reduction of aflatoxin produced by toxigenic isolates when antagonist *A. niger* 1, was inoculated 2 days after these isolates. Where A_1- *Aspergillus flavus* , A_5-*Aspergillus parasiticus* and A_9- *Eurotium repens*

(ii) Effects on aflatoxin production when *A. niger 1* was inoculated at the same time with the toxigenic isolates:

Inoculation of antagonist (*A. niger* 1) at the same time with the toxigenic isolates caused higher percentage reductions than when *A. niger* was introduced two days after the toxigenic isolates. In the case of *A. flavus* (A_1) production of aflatoxin B_1 was reduced from 41,263.01 ppb to 16,610.335 ppb a59.15% reduction and B_2 from 2,597.375 to 194.787 ppb a 92.50% reduction. In case of the seeds inoculated with *A. parasiticus* (A_5) the production went down by 99.89% for B_1 (from 67,266.795 to 86.219 ppb) and 99.898% for B_2 (from 2,401.02 to 2.446 ppb).In the case of *E. repens* there was 100% reduction in aflatoxin production i.e none of the aflatoxins were detected in the sample. (Table 12 and fig 7)

TABLE 12 : **Effects of *A.niger* 1 on mycotoxin production in groundnut seeds by toxigenic isolates when the two were inoculated at the same time**

		Aflatoxins in ppb and % reduction					
Antagonists	Pathogen	B_1	%(B_1)	B_2	%(B_2)	G_1	%(G_1)
A.niger 1	A_1	16610.34	59.75	194.79	92.5		
A.niger 1	A_5	86.219	99.87	2.446	99.898		
A.niger 1	A_9	ND	100	ND	100	ND	100

Key: ND=not detected

(ii) Effects on aflatoxin production when *A. niger 1* was inoculated at the same time with the toxigenic isolates:

Inoculation of antagonist (*A. niger* 1) at the same time with the toxigenic isolates caused higher percentage reductions than when *A. niger* was introduced two days after the toxigenic isolates. In the case of *A. flavus* (A_1) production of aflatoxin B_1 was reduced from 41,263.01 ppb to 16,610.335 ppb a59.15% reduction and B_2 from 2,597.375 to 194.787 ppb a 92.50% reduction. In case of the seeds inoculated with *A. parasiticus* (A_5) the production went down by 99.89% for B_1 (from 67,266.795 to 86.219 ppb) and 99.898% for B_2 (from 2,401.02 to 2.446 ppb).In the case of *E. repens* there was 100% reduction in aflatoxin production i.e none of the aflatoxins were detected in the sample. (Table 12 and fig 7)

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A.niger 1	A_1	16610.34	59.75	194.79	92.5		
A.niger 1	A_5	86.219	99.87	2.446	99.898		
A.niger 1	A_9	ND	100	ND	100	ND	100

Key: ND=not detected

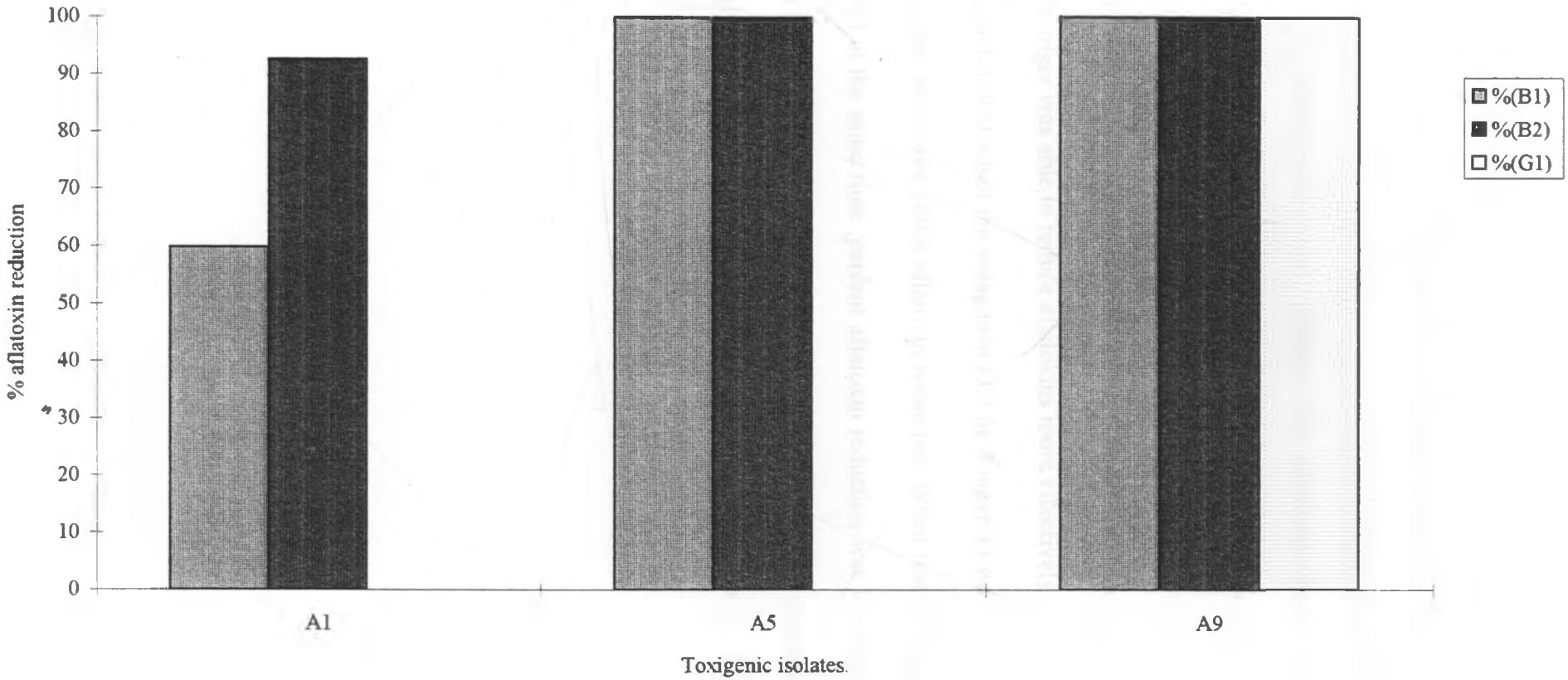

Fig.7 % reduction of aflatoxin produced by toxigenic isolates when antagonist *A. niger* 1 was inoculated at the same time as the isolates.
Where A_1- *Aspergillus flavus*, A_5-*Aspergillus parasiticus*, A_9- *Eurotioum repens*

(iii) Effects of *A. niger* 1 on aflatoxin production in groundnut seeds when inoculated two days before the toxigenic fungi;

When *A. niger* 1 was inoculated into the peanut seeds 2 days before the *A. niger* and *A. parasiticus*, there was a hundred percent reduction in aflatoxin production in all the cases. Although no pathogen was able to produce any detectable levels of aflatoxin, their growth was observed. However this growth was less compared to the other cases when *A. niger* 1 was introduced 2 days after and at the same time with the pathogen. The observation was similar to that of *Trichoderma* inoculated two days before the toxigenic strains.

A. niger was able to reduce aflatoxins more effectively than T_{13} (figures 8, 9 and 10). It was found to that when the antagonist (T_{13} or *A.niger* 1) was inoculated 2 days before the test pathogen, there was 100% aflatoxin reduction. When test pathogen and antagonist were inoculated at the same time ,percent aflatoxin reduction was less than when the antagonist was inoculated 2 days before. The least percent aflatoxin reduction occurred when the antagonist was inoculated 2 days after the test pathogen (figures 8, 9 and 10).

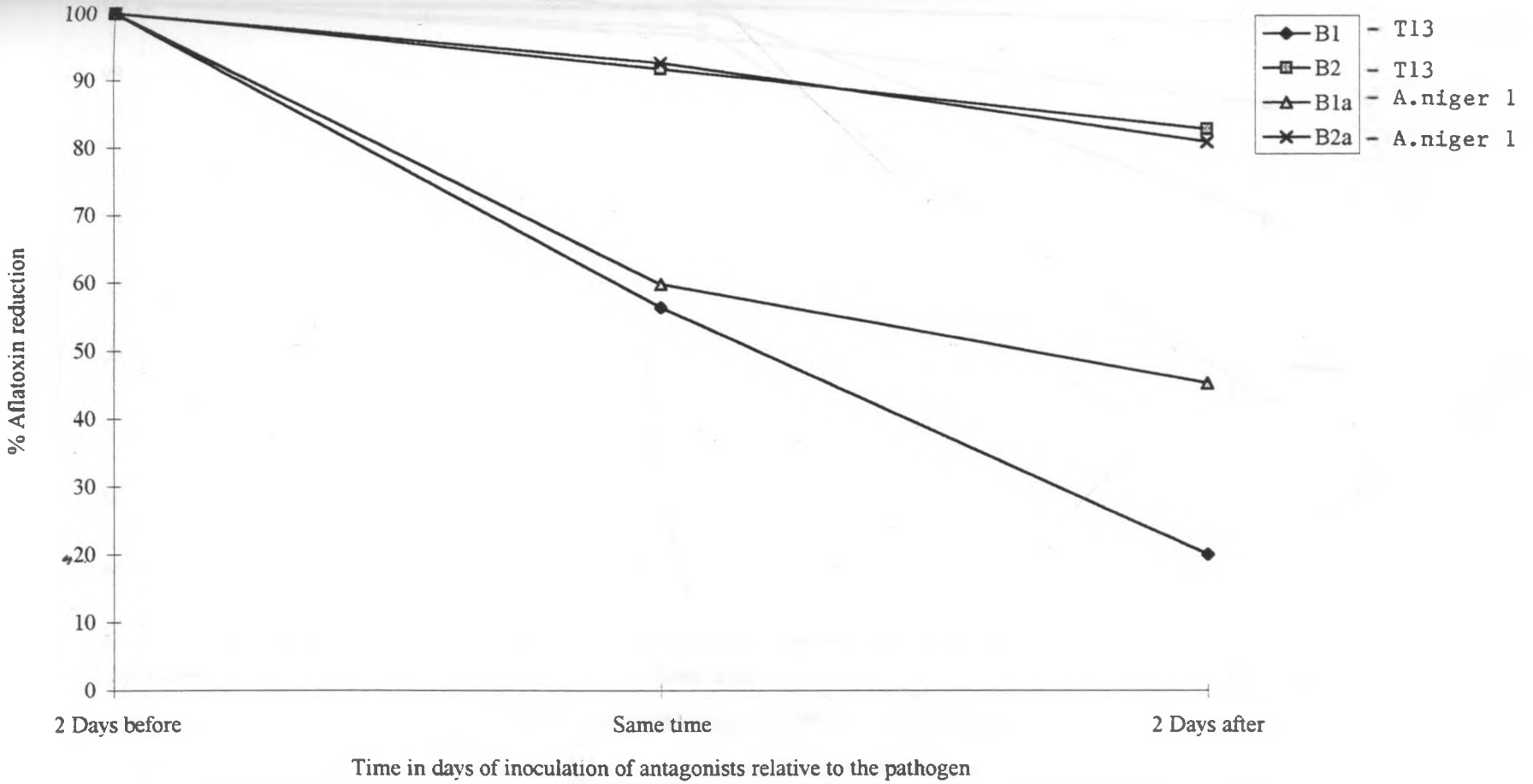

Fig 8. Aflatoxin reduction by antagonists in groundnut seeds inoculated at different times relative to the pathogen (*A. flavus*)

Where B1 - aflatoxin B1 B2 - aflatoxin B2
T13 - Trichodeima isolate 13 B1a - aflatoxin B1
B2a - aflatoxin B2

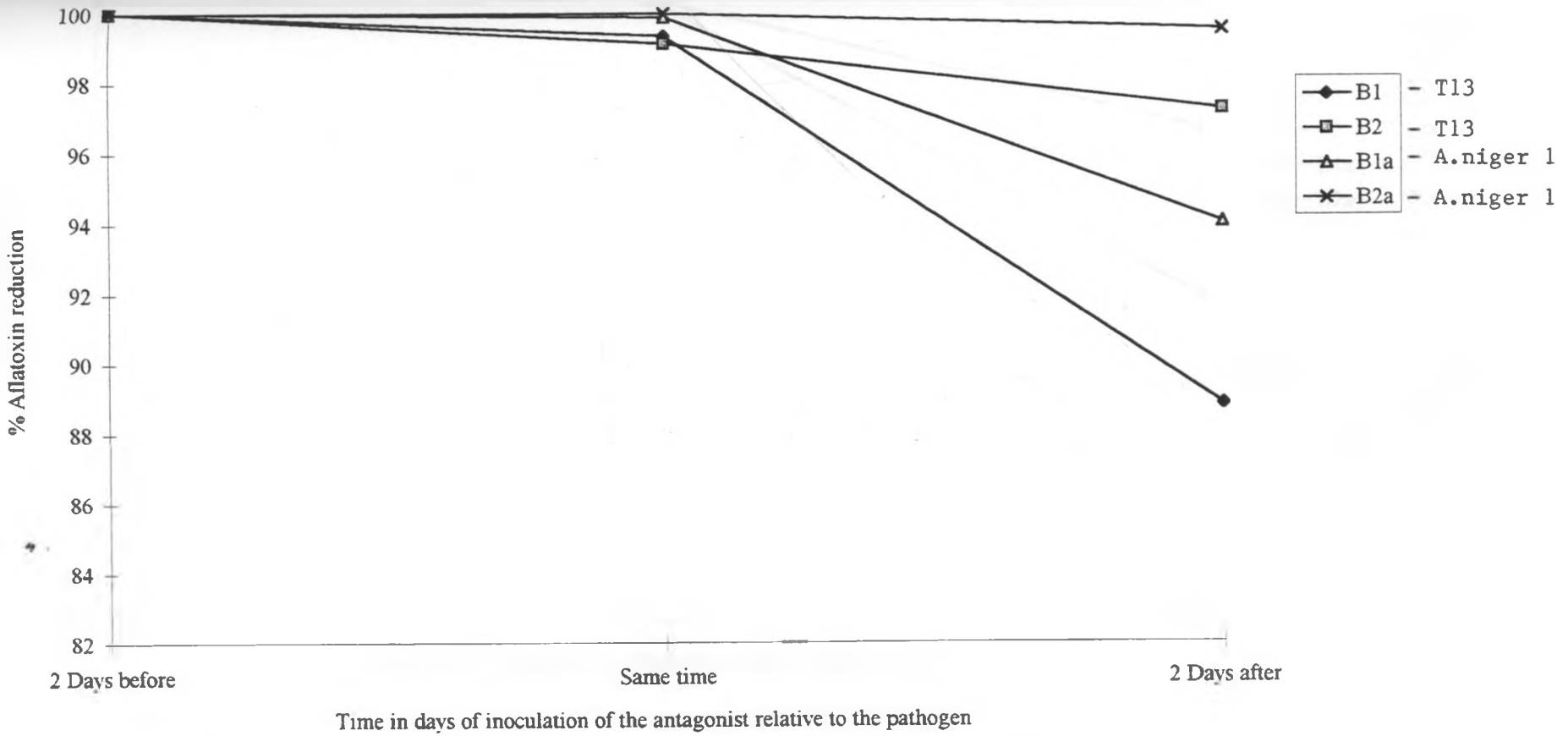

Fig 9. Aflatoxin reduction by antagonists in groundnut seeds inoculated at different times relative to the pathogen (*A. parasiticus*)

Where B1 - aflatoxin B1
B2 - aflatoxin B2
T13 - Trichodeima isolate 13
B1a - aflatoxin B1
B2a - aflatoxin B2

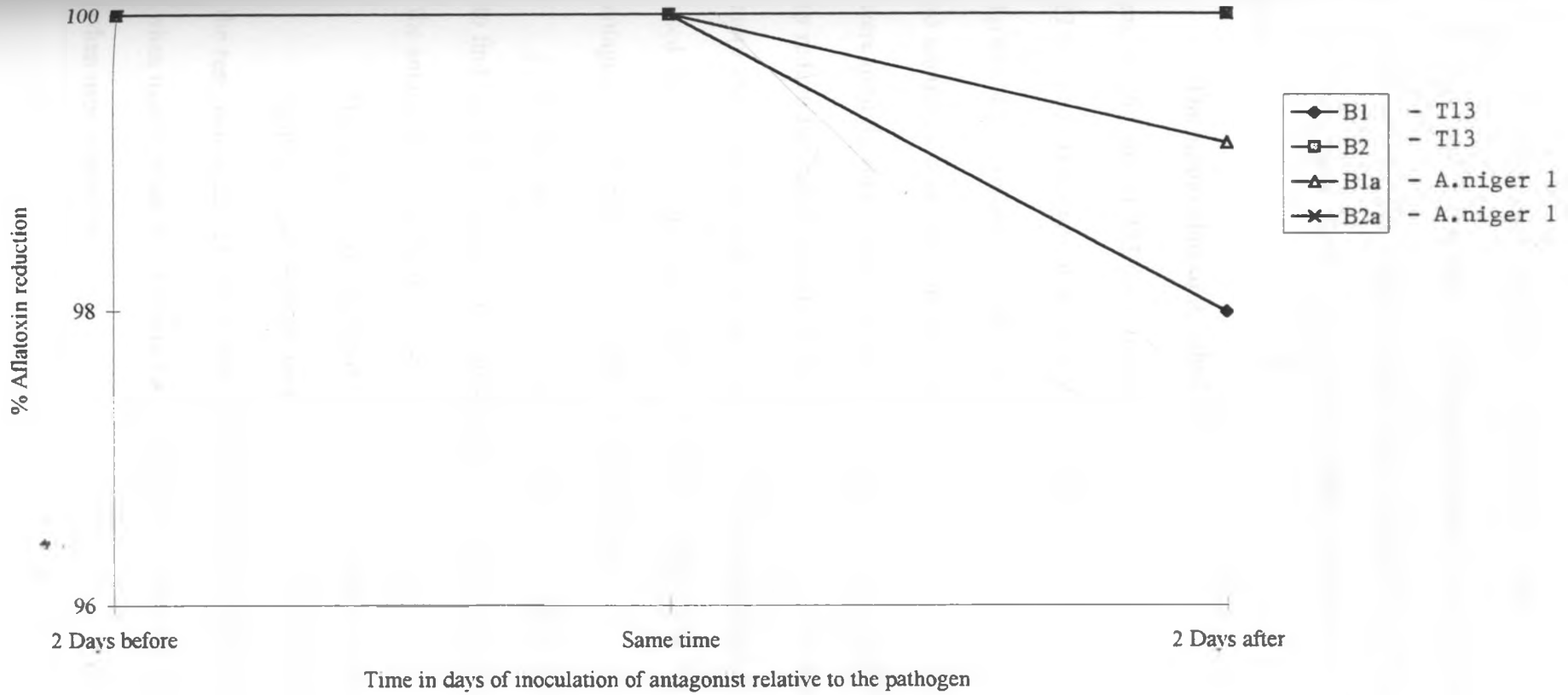

Fig 10. Aflatoxin reduction by antagonist in groundnut seed inoculated at different times relative to the pathogen (*E. repens*)

Where B1 - aflatoxin B1 B2 - aflatoxin B2
T13 - Trichodeima isolate 13 B1a - aflatoxin B1
B2a - aflatoxin B2

4.4.2 ANTAGONISM BY ANTIBIOSIS IN CULTURE (ON GROWTH):

The antagonists used were *Trichoderma* isolate 13 (T13), *Aspergillus niger* isolate 1 *(A.niger 1)* and 2 *(A.niger 2)*. The test pathogens were *A. flavus* (A1), *A parasiticus* (A5) and *E. repens* (A9). The test pathogens were inoculated on the second day after the antagonists. The colony radius of pathogens was measured on the fifth day after seeding the pathogen on the agar.

The mean radius of A_1 when antagonists T_{13}, *A.niger* 1 and *A.niger* 2 were used was 7.33 cm, 10.667 and 11.333 cm respectively. When no antagonist (control) was used the radius was 27.667 cm. This showed that the antagonists were effective in reducing the radial growth of A_1. T_{13} was able to reduce the radial growth more than the *A.niger* isolates. In the case of A_5 when no antagonist was used the mean radius was 31.333 cm. When T_{13}, *A.niger* 1 and *A.niger* 2 were used the mean radius was 8, 11.333 and 11.667 cm respectively. The antagonists were able to reduce the radial growth of the test pathogen (A_5). T_{13} was able to reduce the radius of A_5 more effectively than the *A.niger* isolates. When antagonists T_{13} A. niger 1 and A. niger 2 were used against A_9, the mean radius was 10, 10.667 and 10.667 cm respectively. When no antagonist was used the mean radius was 29.667 cm.

The measurements of the radial growth were analyzed for variance (Appendix 2,3, and 4) to find out if the effects of the antagonists were significantly different (Table 13). The effects of the antagonists were found to be significantly different at 0.1%.

The means were separated using Duncan's multiple range test at 5% level of significance.

Both *A. niger* isolates were not significantly different from each other when used on all the test pathogens. However they were found to be significantly different from T_{13} and control when used against A_1. T_{13} was also significantly different from the control (no antagonist used) when used against A_1.

When the antagonists were used against A_5, antagonist T_{13} was significantly different from the *A.niger* isolates and control. *A. niger* isolates were also significantly different from the control. When the antagonists were used against A_9 their effects were all significantly different from the control but there was no significant difference between T_{13}, *A. niger* 1 and 2

Table 13. Radial growth means (in cm)of test pathogens A_1, A_5, A_9, measured on the 5th day after antagonists T_{13}, *A.niger* 1, *A. niger* 2 and control were introduced.

Test pathogen	**T_{13}**	***A.niger* 1**	***A.niger* 2**	**Control**
A_1	7.33x	10.667y	11.333y	27.667z
A_5	8x	11.333y	11.667y	31.333z
A_9	10y	10.667y	10.667y	29.667z

Means followed by different letters are significantly different at 5% level.

This means that in the case of A_1 the three antagonists were able to reduce the radial growth of A_1. The reductions effected by T_{13} were significantly different from those of the *A. niger* isolates at 5% level. In the case of A_5 the effects of T_{13} were also different from those of the *A. niger* isolates at 5% level. However the effects of the 3 antagonists were not different from each other in the case of A_9. Inspite of the antagonists reducing the radial growth of A_9 their effect was not significantly different at 5% level. The reductions by the 3 antagonists were however significantly different from the control for all the test pathogens.

5.0 DISCUSSION

Microflora associated with groundnuts.

A number of micro-organisms were found to be associated with groundnut seeds from five Nairobi markets: Nyamakima, Nyayo, Kawangware, Kangemi and Gikomba. These microflora included: *Fusarium* spp.; *F. oxysporum, F. equiseti* and *F. torulosum, Aspergillus* spp.; *A. niger, A. flavus, A. parasiticus* and *A. ochraceous, Rhizoctonia bataticola; Sclerotium* spp., *Penicillium* spp., *Rhizopus* spp., *Eurotium repens* and *Cheatonium* spp. Norton *et al.* (1956) found similar species *: A. niger, A. flavus, Sclerotium* spp. and *Fusarium* spp. to be associated with unblemished spanish peanuts. A Study by Diener *et al.* (1965) gave similar findings. The micro-organisms included *Chaetonium* spp., *Fusarium* spp., *Penicillium* spp., *Aspergillus* spp., and *Rhizoctonia* spp. A Study done by ICRISAT (1989) led to the conclusion that several pathogens cause seed and seedling diseases of groundnut and may severely damage the crops, *Aspergillus niger, A. flavus, Fusarium* spp., *Rhizopus* spp and *Pythium* spp have been frequently observed in West Africa.

Work done earlier in the 20th century had implicated the above mentioned species and a few others to be associated with peanuts and to be responsible for concealed damage. Evans and Poole in 1938 isolated *Fusarium* spp., *Rhizoctonia solani* Kuhn, *Rhizopus* spp., *Botrytis* spp., *Pythium* spp., *Sclerotium bataticola* Taub,*S. rolfsii* Sacc., *Trichoderma* spp., *Penicillium* spp. and *Aspergillus* spp. from lesions on peanut seedcoats and shells.

Prince (1945) listed *Fusarium* spp., *Alternaria* spp., *Sclerotium bataticola, S. rolfsii, Diplodia natalensis, Trichoderma* spp., *Penicillium* spp., *Rhizoctonia solani* and *Rhizopus* spp., as fungi isolated from peanut seeds, some of which appeared to have concealed damage. Work done by Garren and Higgins in 1947 showed that *Diplodia* spp., *Sclerotium bataticola* and *Fusarium* spp., were prevalently and consistently associated with peanut fruits. In a study by Garren and Porter (1970) the dominant micro-organisms isolated from peanuts were:

Aspergillus flavus, Alternaria tenuis, Cheatonuim globosum, Cylindrocladum spp., *Diplodia gossypina, Fusarium* spp., *Penicillium* spp., *Rhizoctonia bataticola, R. solani* and *Rhizopus stolonifera.* In this study *Rhizopus* spp. was isolated from all (100%) seed samples from the five markets using both isolation methods (agar plate and blotter methods). Apart from being common in all markets *Rhizopus* spp was associated with the highest percentage of seeds compared to the other fungi.

In Israel *A. niger* was the most common species in 114 groundnut samples from the 1963 and 1964 crops (Borut and Joffee 1966) *A. niger* occurred in 97.4% of the samples, while *A. flavus* was detected in 78.4% of the 1963 samples and in 63.5% of the 1964 samples. They also isolated species of *Rhizoctonia* and *Rhizopus* .

Moubasher *et al.* (1980) found that in Egyptian groundnut seeds adjusted to various moisture levels and stored for 6 months at various temperatures; *Aspergillus fumigatus* was the most dominant fungus followed by *A. flavus, A. niger, A. terreus* and *Penicillium funiculosum.* Other fungi isolated included *Aspergillus terreus, A. ochraceous, Fusarium* spp., *Mucor* spp., *Rhizopus* spp., *Gibberella* spp., and *Drechslera* spp.

The number and kind of fungi associated with peanut seeds stored for 1-6 months were studied by Welty and Cooper in 1969. Initially *A. repens* was isolated from 54% of seed, *Penicillium* spp from 80% and *A. flavus* from 7%. After 2-3 months of storage, the percentage of seed with *A. repens* and *Penicillium* spp dropped to about 10% and then gradually increased to 40% after 6 months *A. flavus* remained at 7% for the first 3 months, but after 4-6 months it was isolated from 18% of the seed. *Fusarium* spp. and *A. amstelodami* were also present in the peanuts.

Antagonism or other factors may result in temporary elimination of one or more of these prevalent fungi. In this study incidence of *A. niger* was higher in surface sterilised groundnuts than in non-sterilsed ones. This observation was also made by Joffee in 1968 and he attributed

this high incidence to selective removal of antagonists by surface sterilisation. This tends to indicate selective removal of antagonists of this species by surface sterilisation. Some of the fungi isolated from the peanuts and identified in this study were the same genera and species that literature reviews (Borut and Joffee 1966, Joffee 1968, Joffee 1969, Joffee and Lisken 1968) cited as causing deterioration of groundnuts and leading to aflatoxin production. These are *Aspergillus flavus* group (*A. flavus* and *A. parasiticus)* and *Eurotium repens.* Presence of micro-organisms capable of producing aflatoxin in surface sterilised seeds indicated that these micro-organisms had penetrated the seeds and that they could have produced aflatoxins inside the seeds. All the micro-organisms (toxigenic and pathogenic) found in association with groundnut seed are supposedly transmitted through seeds from one cropping season to another.

Detection and estimation of aflatoxin :

Out of the five samples found to harbour toxigenic strains of fungi (red groundnut seeds from Kangemi, Kawangware and Nyamakima markets, white peanuts from Kangemi and Kawangware markets) only two samples Nyamakima and Kangemi red varieties were found to contain detectable levels of aflatoxin after analysis before inoculation with the toxigenic molds. This findings are similar to those obtained by Muraguri et al 1981, whereby of the two groundnut samples (from Nairobi) analysed for aflatoxins, one had trace levels of B_1 and 40µg/kg of B_2, while the other had 50µg/kg of B_1 and 1000µg/kgof B_2.

The two samples found to contain detectable levels of aflatoxin in this study were only found associated with *A. flavus* and not any other toxigenic strain in both isolation methods. In the case of Nyamakima red variety, *A. flavus* was only isolated from surface sterilised seeds: 60% of the seeds using agar plate method and 30% using blotter method. From Kangemi red variety this species was found in 3.3% of surface sterilised seeds and a similar percentage of non sterilised seeds. This indicates that only *A. flavus* out of the 3 toxigenic strains produced

detectable levels of aflatoxin under the conditions prevailing in Kenyan markets. Isolates of *A. flavus* and *A. parasiticus* (*A. flavus* group) vary widely in the amount of aflatoxin produced on groundnuts and in capacity to produce different aflatoxins. Some isolates produce no aflatoxin (Codner *et al.* 1963, Diener and Davis 1966, Taber and Schroeder 1967).

Despite the fact that *Aspergillus parasiticus* and *Eurotium repens* did not produce any detectable levels of aflatoxins in market groundnuts this study showed that the two micro-organisms had potential to produce more aflatoxins than *A. flavus invitro*. A study by Garren *et al.* (1963) in the U. K. in which they tested aflatoxin producing ability of 5 isolates of *A. flavus* and one of *A. parasiticus* had similar results: where *an isolate of A. parasiticus* isolate produced 265 microgrammes /gram of a mixture of all four aflatoxins (B_1, B_2, G_1 and G_2) while *A. flavus* isolates produced from 14- 162 micrograms/gram. In general *Aspergillus flavus* isolates have been found to produce 8 to 10 times more aflatoxin B_1 than B_2. Codner *et al.* (1963) found that no isolate produced either G_1 or G_2. In this study *A. flavus* produced approximately 16 times more B_1 than B_2 and no G (G_1 or G_2) aflatoxins were produced.

BIOLOGICAL CONTROL

Aspergillus niger isolate 1 decreased aflatoxin production by toxigenic strains in groundnuts. The reduction was greater if *A. niger* 1 had been in the groundnuts for a longer period i.e. aflatoxin produced decreased with increasing length of incubation with *A. niger*. This is shown by the fact that percent reduction of aflatoxin B_1 increased from 45.14% to 59.75% and finally to 100% when *A. niger* was inoculated 2 days after pathogen A_1 through to when they were inoculated at the same time and finally to when *A. niger* 1 was inoculated 2 days before pathogen A_1. Similar trends were observed with Aflatoxin B_2. The other two toxigenic isolates (A_5 and A_9) showed a similar pattern for both types of B aflatoxins. The ability of *A. niger* to reduce aflatoxin production was also reported by Burnett *et al.* (1972).

They found that the aflatoxin produced on autoclaved groundnuts decreased with increasing length of incubation with *A. niger*. *A. niger* produced a metabolite which inhibited aflatoxin production by *A.flavus*. This could also have been the reason for reduced aflatoxin production in my study.

In this study no aflatoxins were detected in any groundnuts into which *A. niger* had been inoculated 2 days before *A. flavus*. Burnett *et al.* (1972) found that no aflatoxin was detected when *A. flavus* was grown on a 9 day old culture filtrate of *A. niger*. *Trichoderma* isolate 13 designated as T_{13} was also found to be effective in reducing aflatoxin production. However *A. niger* 1 was found to be slightly more effective in reducing aflatoxin production than T_{13}.

Other pathogens reported to show antagonism to mycotoxin production by *A. flavus* include *Streptococcus* spp. The findings of Idziak and Coollier (1984) indicated that *Streptococcus lactis* produced an extracellular compound which inhibited the production of aflatoxin by *A flavus*. This compound was found to be a low molecular weight (<500) phosphoglyco-lipid containing an aromatic ring structure. Studies by Nixon *et al.* (1984) in which chemical and biological soil amendments were investigated for effects on seed infection by fungi of the *Aspergillus flavus* group (*A. flavus* and *A. parasiticus)* and in some instances aflatoxin contamination of one or more genotypes of groundnut indicated that gypsum had effect on aflatoxin production. No aflatoxin was detected in groundnuts harvested from gypsum treated plots but it was occasionally found in groundnuts from non gypsum treatments resulting in a highly significant treatment x genotype interaction.

Dorner *et al.* (1990) revealed that a biocompetitive agent was useful in an effective management strategy for pre harvest aflatoxin contamination. The strategy involved the incorporation of a non aflatoxin producing strain of *Aspergillus parasiticus*. This treatment resulted in a significant reduction in aflatoxin in edible grade groundnuts compared to non treated controls. Soil populations of the biocompetitve agent were not higher than

populations of wild strains of *A. flavus/parasiticus*. Doyle *et al.* (1982) reported that "mycelia of *Aspergillus parasiticus* can degrade aflatoxin possibly via fungal peroxidase". Such degradation was said to be affected by the strain of *A. parasiticus*, amount of mycelium, temperature, pH and concentration of aflatoxin. They also found that Patulin can be degraded by fermenting yeasts and that rubratoxin can be degraded by the mycelium of *Penicillium rubrum*.

The reduction in radial growth of the toxigenic strains (A1, A5, A9) was significant at 5% level of significance for the 3 antagonists *(A. niger* 1, *A. niger* 2 and T_{13}) used. However T_{13} was more effective in reducing the radial growth of the toxigenic strains than the *A. niger* isolates were

The antagonistic effects of *A. niger* against *A. flavus* were also reported by Burnet *et al.* (1972). They showed that when *A. flavus* was grown on a 9 day old culture filtrate of *A. niger* there was some reduction in mycelial growth of *A. flavus*. Conidia of *A.flavus* germinated more slowly in *A. niger* culture filtrate than in the control, but the percentages of germinated spores were similar in both. Joffee in 1969 reported pronounced antagonism between *A. flavus* and *A. niger*.

Antagonism between *A. flavus* and *A. niger* had been reported earlier (Koehler and Woodworth, 1938) where in *Zea mays A. niger* was found to reduce virescence caused by *A. flavus* in maize.

Trichoderma species have been widely used in biological control both in the field and under laboratory conditions. A study by Mixon *et al.* (1984) showed that *T. harzianum* in combination with PCNB (10%), fensulfothion (3%) granules and CGA 64250 (2.5%) Ciba Geigy experimental granules were effective in reducing colonization of peanut pods by *A. flavus* in gypsum treated soils in the field. Preparations of *T. harzianum* have been found to control *Sclerotium rolfsii* and protect peanuts under field conditions.

On other crops apart from peanuts *Trichoderma* spp. have been found to be equally effective. Application of conidia isolates of *T. harzianum* or *T. koningii* to pea seed reduced the incidence of pre-emergence damping off induced by *Pythium* spp. (Lifshitz *et al.* 1986). Several isolates of *T. harzianum* were found to reduce the growth and build up of populations of *Rhizoctonia solani* and to a lesser extent *Fusarium solani* in sterilised soil (Cole *et al.* 1988).

In vitro tests carried out to determine the ability of *T. viride* to control growth of various fungal species specific to citrus fruits in storage showed that *T. viride* exhibited a potent antagonistic effect upon; *Cladosporium herbanum*, a lower effect upon *Penicillium digitatum*, *Alternaria* spp., *Geotrichum candidum, Fusarium oxysporum* and *Phytophthora citrophtora* and a slight effect upon *Fusarium roseum* (diaz-Borras and Vila, 1988).

In this study *Trichoderma* isolate (T13) was found to be more effective in reduction of the radial growth of the strains (*Aspergillus flavus, A. parasiticus and Eurotium repens*) than the *A. niger* isolates (1&2). Similar findings were reported by Gaun and Sharma (1991) whereby *Trichoderma viride* was found to be a more effective antagonist than *A. niger* in control of *Fusarium udum* on nutrient agar medium. The direct parasitism of *Trichoderma* hyphae on other fungi is a significant mechanism which can explain the antagonistic activity of *Trichoderma* species (Dennis and Webster 1971 c). The ability of *Trichoderma* to excrete extracellular lytic enzymes: b-1,3 glucanase and chitinase (Elad *et al.* 1982 b) antibiotic compounds (Dennis and Webster 1971 a,b) as well as competition (Ahmed and Baker, 1986) may play a role in biological control of plant pathogens by *Trichoderma* species.

Biological control is a potentially sound method of pest suppression because; it is self perpetuating; it has a high level of effectiveness at low costs following initial costs; it is harmless to man, his cultivated plants, domesticated animals, wildlife and other beneficial organisms on land or in the sea; and some types are used as biotic insecticides. It is difficult

to come up with safe chemicals for control of storage pathogens beacause those available are not safe for human consumption at the levels of application that are effective to kill the microbes.Fumigation is usually applied on stored grain but other effective biological methods must be sought to ensure a clean environment in future. Biological control has a great deal of undiscovered potential in control of plant pathogens.

CONCLUSION AND RECOMMENDATIONS

1. The groundnuts in Kenyan markets were found to harbour many fungi of which were aflatoxigenic and were found to contain unacceptable levels of aflatoxins.
2. Antagonistic micro-organisms like *Trichoderma* isolate 13, *Aspergillus niger* isolates 1 and 2 reduced aflatoxin production by aflatoxigenic strains : to even non-detectable levels depending on the time the antagonist was introduced relative to the aflatoxigenic pathogen.
3. The antagonists T_{13}, *A. niger* 1 and 2 also reduce the radial growth of the aflatoxigenic isolates significantly.

Based on the above conclusions it would be of use to make groundnut consumers aware of the fact that there are micro- organisms associated with the groundnuts in the Kenyan markets, Some of which are aflatoxigenic. The consumers should also be aware of the risks of aflatoxin ingestion, inhalation and dermal contact in both humans and livestock.

Farmers with groundnuts in stores should have them periodically checked to ensure that they are clean. Farmers and other people handling groundnut after harvest should be educated on post harvest handling of this product to ensure minimal spoilage by molds.

More research needs to be done to find out the methods and formulation in which this antagonists can be used in storage.

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APPENDICES

Appendix 1

Isolation of micro-organisms from groundnut seeds obtained from 3 open markets(Nyamakima, Kawangware,and Kangemi) in Nairobi.

ANOVA TABLE

Source	ss	df	ms	F	P
Markets	0.52	2	0.26	1.09	ns
micro-organism	16.58	2	8.29	34.46	**
Error	0.96	4	0.24		
Total	18.06	8			

ns-not significant

**-significant at 1%

Appendix 2

Effect of antagonists *Trichoderma* isolate 13(T_{13}),*A.niger* 1,and *A.niger* 2 on the radial growth of the pathogen *A.flavus* (A_1)

ANOVA TABLE

source	ss	df	ms	F	P
Main effects					
treatments	747.583	3	249.194	427.19	.0000***
Error	4.667	8	0.583		
Total	752.25	11			

***-significant at 0.1%

Appendix 3

Effects of antagonists T_{13}, A.niger 1 and A.niger 2 on radial growth of the test pathogen A. parasiticus.

ANOVA TABLE

Source	ss	df	ms	F	p
Main effects					
Treatments	1016.917	3	338.072	451.963	.000***
Error	6	8	0.75		
Total	1022.917	11			

***- significantly different at 0.1%

Appendix 4

Effect of antagonists T_{13}, *A.niger* 1 and 2 on radial growth of the test pathogen *Eurotium repens*.

ANOVA TABLE

Source	ss	df	ms	F	P
Main effects					
Treatments	825.25	3	277.417	277.417	0.000***
Error	8	8	1		
Total	840.25	11			

***-Significant at 0.1% level