ASPECTS OF THE MICROBIAL ECOLOGY OF AFLATOXIN PRODUCTION IN NIGERIAN STAPLE FOODS

BY

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DEDICATION

This PhD Thesis is

DEDICATED TO

DR (SIR) EMMANUEL CHUKWUKA EZEKWESILI-
A father of great esteem
A renowned educationist and administrator,
who has till this day been the great relentless force behind my achievements.

AND

A MOST DEDICATED MOTHER

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Who as a great teacher, gave me a solid start in education.

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ABSTRACT

The examination of the influence of seasonality on the ocurrence pattern of fungi, bacteria and aflatoxins in the various varieties of eighteen (18) Nigerian staple foods and their products; the clarification of the effects of microbial interaction and the establishment of the influence of the bivalent metals, zinc (Zn^{2+}), iron (Fe^{2+}),manganese (Mn^{2+}) and copper (Cu^{2+}) on aflatoxin production and microbial distribution both qualitatively and quantitatively, was carried out within a two year period - 1996 to 1998 in Enugu and its environs. The results show a significant difference in seasonality of fungal genera ($P<0.05$) but none for bacteria ($P>0.05$). Fungal isolations were made in 39.0%, 26.5% and 24.0% of food samples during the rainy, hot-dry and cold harmattan periods, respectively, while that of bacteria was 9.3%, 5.5% and 4.6% for the hot dry, rainy and cold harmattan seasons, respectively. Of the 15 mould groups encountered in the study, *Aspergillus flavus* group (*A.flavus*) occurred most frequently, accounting for 34.8% of all fungal isolates in the study, being followed by *Penicillium* species (16.8%) and *A.niger* (12.0%) among the commonest ones with; *Bacillus subtilis* accounting for 14.7%, *Streptococcus faecalis* (14.3%) and *Escherichia coli* (13.9%) amongst the commonest bacteria. The *A. flavus* group also showed the highest incidence during the rainy seasons being isolated in 14.4% of the samples in that season. The incidence declined considerably in samples taken in the hot dry seasons (9.43%) and in the cold harmattan seasons (7.25%). Most other fungi also showed a rainy season prepondence, including *A.niger, Cladosporium, Trichoderma, Curvularia, Geotrichum, Saccharomyces* and *Candida* species. *A.fumigatus* and *Mucor mucedo* however showed a hot season

preponderance while *Penicillium* and *Rhodotorula* species occurred mostly in the cold harmattan periods of the study. For the bacteria however, 48.1% of all isolates were recorded in the hot dry season, with 28.1%and 23.8% in the rainy seasons and cold harmattan seasons, respectively. Maize (252 samples) and peanuts (234 samples) were the foodstuffs most frequently contaminated by fungi while acha (*Digitaria exilis* Stapf) grains (47 samples) and oil bean seeds (sliced and fermented 42 samples) were those most frequently contaminated by bacteria. Differences were observed in terms of microbial and aflatoxin contamination according to food varieties (seed colour, class and cultivation practice). Thus eg., white maize (49.6%) was more contaminated by moulds than the yellow variety (43.5%). White testae peanuts (68.8%) were significantly more contaminated by moulds than those with red testae (23.1%), ($P<0.05$). The Abakaliki (swamp) rice variety (57.8%) was significantly more contaminated by moulds than the Ugbawka (upland rice) (42.2%) ($P<0.05$). Also, white peanuts had a mean aflatoxin B_1 (AFB_1) level of 876 ppb while the red variety had a mean of 510 ppb; Abakaliki rice had 193 ppb against 38ppb for Ugbawka rice. Of all the classes of foods, the highest level of natural contamination with total aflatoxins was recorded in oil seeds, eg. coconut seeds (2,732.1ppb); followed by the cereal grains, eg white sorghum (416.4ppb); Protein foods, eg. white cowpeas (70.5ppb) as against a load of 11.2ppb for the carbohydrate foods, eg. unripe plantain. Of the 369 *A.flavus* isolates encountered in the study, 126were aflatoxigenic with more numbers of the aflatoxigenic strains being encountered in hot dry seasons (42.1%), followed by the cold harmattan seasons (29.4%) and the rainy seasons (28.6%) of the study period even though 46.3% of the total *A.flavus* was actually

encountered in the rainy season as against 30.3% and 29.4% in the hot dry and cold harmattan seasons, respectively. Also, though more food

numbers were contaminated by aflatoxins in the hot dry seasons, generally higher concentrations of aflatoxins were recorded in these foodstuffs during the rainy season. Again, varietal differences in seed crops were also recorded in the distribution patterns of both total and aflatoxigenic *A. flavus* eg, white peanuts significantly contained more (61) total *A.flavus*, than the red counterpart (23), but only 29.0% of these were aflatoxigenic. The red peanuts, 43.5% were aflatoxigenic. Similarly, the white maize again contained more total *A.flavus* organisms (42), from which 19.0% were aflatoxigenic while the yellow maize contained 33, from which 48.5% were aflatoxigenic. Some foodstuffs,eg. pigeon peas, oil bean seeds, and cassava tubers contained aflatoxigenic *A.flavus* but contained no aflatoxins, while some, eg. moin-moin and okpa cake (wet-milled steamed cowpeas and bambara peanuts) contained no toxigenic *A.flavus* but contained some detectable levels of aflatoxins. It was also observed that the majority of food crops associated with the soil environment, eg. cocoyam, yam, peanuts produced largely both the B and G toxins while others that are not associated with this environment, eg. maize, sorghum, millet, produced largely only the B toxins. In comparison with *A.flavus* in monoculture, co-culture studies showed marked reduction of aflatoxin levels by some fungi eg. *Rhizopus* and total inhibition by others, eg. *Trichoderma*, still other groups eg. *Cladosporium*, gave a mixed trend of either stimulation or no effect depending on the food substrate. Some bacterial species, eg. *Pseudomonas* and *Escherichia coli* reduced while others like *Acetobacter* stimulated. *Proteus* spp generally reduced aflatoxin levels in the solid media (foodcrops) and in the synthetic liquid medium (low salt sucrose

medium) but stimulated in the semi-synthetic medium (yeast extract sucrose medium-YES). Also *Streptococcus lactis*, reduced aflatoxin levels in the synthetic medium but showed no effects in the semi-synthetic medium,while significantly reducing aflatoxin levels ($P<0.05$), in all foodcrops (solid media). Some moulds also gave differing responses according to the substrate eg. *Fusarium* spp which significantly reduced aflatoxin levels in the YES medium and the general foodstuffs ($P<0.05$) completely inhibited the production of aflatoxins in the synthetic medium. Generally, Zn^{2+} and Mn^{2+} at concentration ≤ 550ug/g of food stimulated Af production, both the level of stimulation varied from one food to the other except that with Zn^{2+} in oil seeds, a progressive inhibition of aflatoxin production was rather recorded while for the protein foods and oil seeds with Mn^{2+} levels, the stimulation started at metal levels of 100ug and 25ug/g respectively and continued till the highest metal concentration (1000ug Mn^{2+}/gram of food substrate). A rather constant level of aflatoxins was maintained in all cereals and carbohydrate foods at all concentrations of Fe^{2+}, while in the protein foods, a slight increase in aflatoxin levels occurred till Fe^{2+} concentration of 25ug/g of food substrate, and then a decrease thereafter till the highest metal level, but in oil seeds, after the initial increase in aflatoxin levels, at Fe^{2+} concentration of 10ug/g of food, a decrease followed thereafter till 250-400ug iron concentration. This started increasing again till the highest metal level. With copper in the cereals and carbohydrate foods, after the initial increase in toxin levels up to 250ug metal concentration, a progressive decrease till the highest metal level was recorded, while the progressive decrease with increasing metal concentration occurred from metal concentration of 25-50ug/g protein foods. For the oil seed, after the initial decrease in aflatoxin levels recorded at Cu^{2+} level of 25-100ug/g of

food, another drop was recorded again at 550ug copper levels which increased again till the highest metal level for coconut seeds, continue to decrease as in peanuts or levels up at a constant lower values as in melon seeds. With metal studies in the liquid media (semi synthetic and the synthetic media), at Zn^{2+} levels 1-25ug/ml, aflatoxin production increased with a slight depression at 25ug/ml and mat weight of the fungus also increased. A similar event occurred with Mn^{2+} though the mycelial mat weight of the *A.flavus* was essentially constant. With iron in the range 1-25ug/ml, aflatoxin production decreased progressively with mat weight almost constant and with copper levels from 1-25ug/ml,a depression of Af production progressively occurred even though the mat weight was unaffected. Because of the toxicological and economic implications of aflatoxin contamination in these food crops, control programme focusing on the knowledge of seasonality, microbial interactions and probably bivalent metal applications to reduce the incidence in these crops is hereby advocated.

CHAPTER 1

1.0 INTRODUCTION

Over three decades have passed since the discovery of aflatoxins (AF), one of the most potent hepato-carcinogens known. The first fifteen years were described as the era of 'mycotoxin gold rush', which led to the discovery of a large number of mycotoxins.

Mycotoxins are toxic secondary metabolites of moulds that contaminate a wide variety of foods and feeds (Sweeny and Dobson 1998). They are produced when moulds grow on any suitable substrate including foodstuffs. The ingestion of such foodstuff have been implicated in a range of toxic responses, which could present either in the form of acute toxicity or chronic health disorders collectively referred to as mycotoxicoses (Fernandez *et al.* 1996; Bhat 1996; Miele *et al.* 1996; Le-Bars 1996; Matsumura and Mori 1998).

Although mycotoxins have been experienced by man for centuries, it was not until the early 1960s that they received worldwide attention following the death of an estimated 100,000 young turkeys in England in 1960. The deaths were later traced to the consumption of a groundnut meal infested with *Aspergillus flavus* (Blount 1960). The toxin which was elaborated by the mould responsible for the death of the birds was then named aflatoxins

Aflatoxins constitute a family of toxic metabolites with low molecular weight produced by some strains of *A.flavus* and related organisms including *A.parasiticus, A.ruber, A.nominus* and *A.tamarii* (Leitao *et al.* 1989; Goto *et al.* 1996). Aflatoxins are based on the coumarin nucleus (1, 2-benzopyrone) consisting of structurally related bifurano-coumarin-like derivatives or highly oxygenated heterocyclic compounds which are designated as aflatoxin

(AF) B_1, B_2, G_1, G_2 in the order of decreasing Rf value on thin layer chromatography (Faraj *et al.*1993; Moss 1997). They are distiguished on the basis of their fluorescent colour, B standing for blue and G for green with subscripts relating to the relative chromatographic mobility. Aflatoxins B_2 and G_2 are the dihydroderivatives of the parent compounds while M_1, M_2, P_1 and Q_1 are the hydroxylated derivatives of B_1 and B_2 which are excreted in milk, urine and faeces as metabolic products following their consumption by mammals. B_{2a}, G_{2a}, and GM_1 are their closely related compounds (Hoult and Paya 1996; Faraj *et al.*1993).

Aflatoxin B_{2a} differs from B_2 in the presence of a hydroxyl group at the 2-position of the terminal furan. Some compounds have been found to oxidize AFB_1 to the reactive aflatoxin B_1-8,9 epoxide (AFBO) in the human liver microsomes (Gallagher *et al*,1996).

The presence of aflatoxins in foodstuffs and feeds is highly undesirable because they constitute potential hazards to the consumer. Under conditions where foodstuffs and feeds form an appreciable part of the diet, the harmful effects of these toxins on the consumer may be severe. According to Moss (1989), Gabal and Dimitri (1998); Neal et al.(1998), Dhir and Mohandas (1998) and Groopman and Kensler (1999),these toxins are characterized by a diversity of biological activity, including carcinogenicity, nephrotoxicity, hepatotoxicity, immunotoxicity with oestrogenic, dermatitic, haemorrhagic,mutagenic, immunosupressive and teratogenic activities. Other various hazardous effects of aflatoxins in both animals and man have been widely reviewed (Fernandez *et al.*1997; Oyelami *et al.*1997; Yu et al.1997)

The type of mycotoxins found contaminating foodstuffs and feedstuffs vary from one geographical area to another since the mycoflora of any area depends on its climate. Nigeria lies within two major climatic zones; the rainforest and savannah. Within these major zones

are many subzones like the derived savannah and Guinea savannah and within the subzones are microecological enclaves like the cool highland enclaves climate of Jos, Obudu hills, shere hills etc (Okoye 1991). It is therefore logical to expect that a wide variety of mycoflora, many of them toxigenic would infect crops grown in Nigeria and that an equally wide range of mycotoxins may contaminate Nigerian staple foodstuffs.

Certain mycotoxins are also more prevalent in certain geographical zones than others. According to records, the aflatoxins occur more frequently in hot humid areas as is found in Nigeria, whereas the trichothecenes are more common in colder climates (McDonald 1976; Ueno 1985). Toxigenic strains of fungi occur widely in nature thoughout the world. They are present in the soil, air, water and on crops and according to Lovelace and Albergsberg (1989), all food commodities can therefore be attacked by fungi leading to the production of these mycotoxins. Mycotoxins will therefore continue to be important wherever there is agriculture (Moss 1996). Thus, the spoilage of food and stored products pre and post harvest involves a wide range of fungi that differ greatly in their ecological determinants. They range from plant pathogens, weak pathogens and superficial saprophytes that disfigure and damage foods before harvest to moulds that cause losses of dry matter and quality in store (Lacey 1989; Sweeny and Dobson 1998).

Aflatoxins may occur in foodstuff if the *Aspergillus flavus* group develop on them under approriate conditions. Numerous surveys have shown that many commodities will support the growth of toxigenic strains if inoculated, including various dairy products, bakery products, fruit juices, cereals, nuts and forage crops (Awuah and Kpodo 1996; Fernandez-Pinto and Vaamonde 1996). In most cases, the growth of a toxigenic strain and the subsequent

elaboration of aflatoxin occurs following harvesting or formulation of the product while in the case of other foodstuffs such as peanuts (groundnuts), cotton seeds and corn, fungal invasion, growth and toxin production could occur prior to harvesting (Doster and Michailides 1994). Generally, contamination and the potential for aflatoxin production in these crops is related to insect damage, humidity, weather conditions and agricultural practices (Ciegler 1975; Imwidthaya *et al.*1987; Wood 1989; Bauduret 1990; Mishra and Daradhiyar 1991; Moss 1996).

Some countries have currently enacted or proposed regulations for the control of mycotoxins, primarily aflatoxins in foods and animal feeds. Various factors ,however, influence the establishment of limits for certain mycotoxins in foods. One of the major problems in setting legislative limits is understanding the distribution of a mycotoxin in a bulk commodity especially if it is particulate. Moulds rarely grow uniformly throughout a commodity and a mycotoxin will not have an even distribution (Van-Egmond 1995; F A O 1997).The enforcement of the regulations therefore requires monitoring of the suspected commodities. Components of this monitoring and control programme include the full understanding of these mycotoxins.

The chemistry of various aflatoxins and their interrelationships are well at hand, but the biosynthesis and other chemical features have been a topic of much conflicting speculation. However, it is now evident that severe environmental, nutritional and genetic factors considerably influence the variations in types and amounts of aflatoxins produced by moulds of the genus Aspergillus (Lacey 1989).

Perhaps for the two major species of the aflatoxigenic fungi, *A. flavus* and *A.*

parasiticus, the optimal nutritional availability of certain trace metals is a crucial factor that is yet to be completely understood. Obidoa and Ndubuisi (1981) attributed the resistivity or susceptibility of natural commodities to aflatoxin production to the presence or absence of adequate amounts of trace elements, especially zinc. Metal ions are involved as micronutrients in many biological processes and the stimulatory effect of zinc(Zn^{2+})on aflatoxin production has been confirmed both directly (*Tiwari et al.* 1986), and indirectly by correlating the concentration of Zn^{2+} with aflatoxin contamination in such commodities as maize (Failla *et al.*1986) and chicken feed (Jones *et al.*1984).

Tyagi and Venkitasubramanian (1981) attributed the observed enhanced aflatoxin yields with some particular peanut fractions to the presence of inorganic constituents like copper, manganese, zinc and iron. Of all the trace elements, zinc seems to play a key role in the biosynthesis of many secondary fungal metabolites including the aflatoxins. It is a relevant fact that at least 20 zinc dependent enzymes have been detected, all of them being very important in the aflatoxin biosynthesis (Tyagi and Venkitasubramanian 1981) which may partly account for its keyrole. Futhermore,the high yields of aflatoxins obtained on some substrates like papaya, sugarcane, bananas and carrots, were attributed to the presence of magnesium, iron and zinc in them apart from the carbohydrates (Maggon *et al.*1977). Magnesium for instance, appears to be a multi functional metal in the metabolism of numerous fungal and bacterial systems. It is involved in cell wall synthesis as well as nucleic acid and fatty acid synthesis (Maggon *et al.*1977). Detroy *et al.*(1971), however,did not observe any effect of manganese on aflatoxin formation while Maggon *et al.*(1977) observed different effects ranging from inhibitory to stimulatory with varying concentrations of the metal in the

medium. Copper and iron were reported to exert little influence on aflatoxin formation though Maggon *et al.*(1977) reported reduced aflatoxin formation in their presence. Also, depending on the concentration of the added metals, salts of iron, manganese and copper were observed to inhibit aflatoxin formation either partly or totally without influencing the mycelial weight.

Trace elements including Cu^{2+}, Zn^{2+} and Mn^{2+} were shown to inhibit aflatoxin degradation by *Flavobacterium aurantiacum* NRRL B-184 which possesses the ability to degrade aflatoxin B_1 in solution and in several food items (D'Souza and Brackett 1998). Also, in guinea pigs experiments, copper was found to increase the capacity of aflatoxin B_1 to induce liver injury (Schiller *et al.*1998). Literature covering the general role of metals in the regulation of primary as well as secondary metabolism in fungi abound (Agarwala *et al.*1986; Tiwari *et al.*1986; Failla *et al.*1986; Fernandes *et al.*1997). Though many of such reports are conflicting, apparently, the optimal availability of trace metals in the food substrate is a decisive factor to aflatoxin production in amounts and types (Jones *et al.*1984; Niehaus and Failla 1984).

The discovery that *A.flavus* also produces aflatoxins in grains before harvest (Hill *et al*.1985) has forced mycologists and plant pathologists to abandon the previously held viewpoint that mycotoxigenic Aspergilli, Penicillia and other moulds are limited to the post harvest stages of a cereal agroecosystem. Regional surveys have also shown that there is considerable variation in the occurrence of *A. flavus* and aflatoxins among individual fields, rows, ears, kernels and seasons (Lillehoj 1979; Lacey 1989; El-kady *et al*.1996).

Extensive research need therefore be conducted in various parts of the world to provide biological explanations for such variations. Many workers have done quite a lot in this regard.

Such studies include the role of host genotype and genetic factors of resistance (Zuber *et al.* 1978); arthropod relationships (Wicklow *et al.*1984; Moss 1989) and moisture stress during drought (Lillehoj and Hesseltine 1977; Hill *et al.*1985; Moss 1996). An important missing component in these ecological investigations is the failure to recognize that *A.flavus* co-exists or competes with the other microbiota of the general foodstuffs and that the outcome of such species interactions may be important in determining if, and to what extent, aflatoxins are produced in these foods (Coallier-Ascah and Idziak 1985; Cuero *et al.*1987; Moss and Frank 1987).

If *A.flavus*, for instance, is principally introduced into developing grains by arthropods originating in stored cereals, then one should expect that the inoculum of other so-called storage moulds is likely to be introduced as well. The type of individual fungal and bacterial colonists as interference competitiors and the sequence in which these colonists become established within the grain/foodstuff, may contribute to the considerable variation in aflatoxin contamination among field samples (Diener *et al.*1987; Chelkowski 1991; Smith and Henderson 1991).

When stored under harvest, cereal grains develop a succession of microorganisms determined by environmental conditions especially water activity, temperature and composition of the intergranular air. Components of this microflora will certainly interact, giving transient or permanent changes within the population; the interacting organisms may either be inhibited on contact or at a distance, stimulated or even unaffected. Generally, at harvest, most grains are contaminated by a variety of moulds, including *A.flavus*, yeasts and bacteria. The production of aflatoxins can certainly be influenced by the competitive growth

among different micro-organisms (Kirilenko and Egorova 1985; Kharchenko 1986; Cuero *et al* 1987; Ono *et al*.1997; Gourama and Bullerman 1997; Munimbazi and Bullerman 1997; Munimbazi and Bullerman 1998; Ali *et al*. 1998). Growth of other micro-organisms may change available nutrients or produce volatile and/or no volatile end products, which may stimulate, inhibit, detoxify or have no influence on the growth of *A.flavus*. In the developing nations of the world, information available on the probable factors responsible for fungal growth leading to toxin production on specific agricultural commodities are generalized, inconsistent, scanty and in many instances non existent. Most reports have concentrated largely on non tropical foods. In Nigeria today, aflatoxin contamination of food resources is a major problem due principally to the prevalent weather/high R.H, temperature and unsanitary conditions which favour the growth of *A.flavus*.

Compounding these problems is the crude agricultural practices of uninformed farmers which often result in high levels of physical damages to the grains and the general food items. Reviews indicate that the aflatoxin contamination problem is more frequent in poverty stricken and illiterate human communities (Mishra and Daradhiyar 1991). According to Wood (1989) and Moss (1996), these toxins cannot be entirely avoided or eliminated from foods and feeds by current agronomic and manufacturing processes and are therefore considered unavoidable contaminants in the developing world.

From the fore-going, it would appear that the aflatoxin problem is an endemic one in our region and therefore deserves closer research emphasis with a view to curtailing its adverse health effects on the populace. The present study therefore seeks to achieve the following:-

1.1 AIMS AND OBJECTIVES:

1. Screen some Nigerian staple foods and local beverages for the natural occurrence of aflatoxins
2. Isolate and screen *Aspergillus* species for their aflatoxigenic potentials.
2. Establish the effects of seasonality on the distribution of micro-organisms/aflatoxins in the food samples studied.
4. Indentify the micro-organisms that occur together with the aflatoxigenic moulds.
5. Monitor the influence of these micro-organisms on the types and levels of aflatoxins produced on the food substrates, synthetic media and semi-synthetic media.
6. Trace the influence of bivalent metals (Zinc, copper, manganese and iron) on the production of aflatoxins as applied to (5) above.

Chapter 2

2.0 LITERATURE REVIEW

2.1 Historical background

The toxicity of certain mushrooms has been known for a long time. However, the toxicity of other fungi and the mycotoxins they produce were only retrospectively identified as the toxic agents of these diseases in animals and man in different parts of the world beginning from 1853. For instance, there has been a number of dramatic events of human mycotoxicoses in the past. In the middle ages, egortism, caused by the ergot alkaloids produced by *Claviceps purpurea* undoutedly claimed many lives as recorded by Ramsbottom (1953) and Groger (1972).

At the beginning of the century, alimentary toxic aleukia in Russia, associated with the toxins of *Fusarium* species (Joffe, 1986) and yelow rice disease in Japan, associated with the toxins of *Penicillium* (Ghosh *et al*. 1977), caused considerable sufferings and mortality.

Table I summarizes the historical incidence of mycotoxicoses and their association with mycotoxins. Despite these historical episodes of fungal associated diseases in farm animals and man, the seriousness of mycotoxins as a real threat to public health was not realised until in 1960 when over a hundred thousand turkey poults died in Britain within a short period of a disease epidemic of unknown aetiology called 'turkey-X-disease'. Subsequent systematic investigations led to the discovery of the aflatoxins produced by the fungus, *Aspergillus flavus* found to have contaminated a consignment of Brazillian groundnuts used as a constituent of the feed fed to the turkey poults (Blount 1960). A clear and tragic demonstration of the acute toxicity of aflatoxin to humans was reported in India in 1974 and involved nearly 1000 people of whom nearly 100 died (Tandon and Tandon 1988).

(Table 1)Historically important mycotoxicoses of man and animals

S/N	Year	Disease Syndrome	Fungus	Food source	Mycotoxin	Affected species	Country
1.	1853	Ergotism	*Claviceps purpurea*	Grains eg. rye used in bread flour	Ergot alkaloids	Man and livestock	Europe
2.	1908	Facial eczema	*Pithomyces chartarun*	Plant fodder	Sporide-smins	Sheep and cattle	New Zealand
3.	1927	Nephropathy	*Aspergillus ochraceus; Penicillium viridicatum*	Grains, Feeds	Ochratoxins	Man and Livestock	Denmark
4.	1931	Alimentary toxic aleukia (ATA); skin Inflamma-tion	*Stachybotrys alternans*	grains, fodder	Trichothe-cenes	Man and horses	USSR U.K
5.	1943 1947	ATA	*Fusarium species, Sporotrichoi-des species*	Grains	Trichothe-cenes	Man	USSR
6.	1948	Liver diseases, Liver cancer	*Aspergillus islandicum*	yellow rice	Luteoskyrin ; Islandtoxin cyclochorio tine	Man	Japan
7.	1952	Haemorrhagic hepatitis	*Aspergillus flavus*	Maize	Aflatoxin	Swine, cattle	USA
8.	1954	ATA; Circulatory disease; Dermatitis	*Myrothecium roridum*	Grains	Trichothece nes	Man, livestock	USSR, New Zealand
9.	1958	Acute hepatitis	*Aspergillus flavus*	Canned mouldy corn	Aflatoxin	Dogs	USA

2.2. The major mycotoxins in foods

A considerable bibiliography has accumulated covering the major aspects of mycotoxin research, reflecting the multidisciplinary nature of this research. Several monographs on the subject include those of Kurato and Ueno (1984), Smith and Moss (1985), Betina (1989), Lacey (1989), Moss (1989), Chelkowski (1991), Frazier and Westerhoff (1991), Smith and Henderson (1991), Van-Egmond (1995) and Diprossimo and Malek (1996). About two hundred thousand species of fungi have been described and between fifty and hundred of these are known to produce mycotoxins according to literature. In numerical terms, there are over two hundred known mycotoxins today since most of the identified toxigenic fungi can produce more than one mycotoxin, some, as many as ten chemically different mycotoxins depending on the nature of the food substrate, temperature, humidity and aeration. It is also known that one mycotoxin can be produced by several species of fungi.

Apart from aflatoxins, other major mycotoxins of significant occurrence include ochratoxins also produced by species of *Aspergillus* including *A.ochraceus* Wilhelm, in warmer parts of the world but in temperate climates by mostly *Penicillium verrucosum* Dierckx, which may occur on cereals like barley. Ochratoxin A is a nephrotoxin for pigs as well as being acutely toxic probably to man. Others are patulin produced by species of *Penicillium*, *Aspergillus* and *Byssochlamys* in apples and other fruits; the *Fusarium* trichothecenes - (T-2 toxin, Deoxynivalenol and Zearalenone) which are either cytotoxic as well as immunosuppressive, a vomitoxin or an oestrogenic toxin respectively. Fumonisins are other metabolites of *Fusarium* species found to be carcinogenic (Yoshizawa *et al*.1994). Other mycotoxins of significant occurrence include citrinin, citreoviridin, penicillinic acid and

tremorgenic mycotoxin (Park 1995; Kedera *et al.*1999).

2.3. Aflatoxins

By far the most notorious of the known mycotoxins in the food trade are aflatoxins. The term 'aflatoxins' denote toxic metabolites produced by *Aspergillus flavus* and other related species (Lacey 1989). The genus *Aspergillus* is divided into morphologically distinguishable groups which are ubiquitous and the potential for contamination of foodstuff and animal feed is widespread (Strzelecki *et al.*1988; Wood 1992; El-Kady *et al.*1995; Resnik *et al.*1996; Awuah and Kpodo 1996). They will grow on a wide variety of substances over a mesophilic range of temperatures (Ellis *et al.*1991). Optimal conditions for the production of aflatoxins would be a water activity (Aw) of 0.85 and a temperature range of 25-40°C. They belong to the family Ascomycetes and are characterized by a felted, septate branching mycelium, having a conidial apparatus developing on specialized hyphae (sporangiophores) which terminate in a vesicle on which are borne chains of conidia (McDonald and Castle 1996). The mycelia are usually uncoloured, with colonies that are usually zonate, the conidiophore or stalk which may be septate or aseptate, arising from a footcell. According to Frazier and Westerhoff (1991) and Coulombe (1993), the conidia which are in chains are green, brown or black more commonly than other colours.

The aflatoxins (FigA), of which AFB_1 is the most potent, are intensely fluorescent when exposed to long wave ultra voilet (UV) light. This makes it possible to detect these components at extremely low levels on thin layer chromatograms. They are freely soluble in moderately polar solvents like chloroform and methanol and especially in dimethylsulphoxide and as pure substances they are highly stable at high temperatures when heated in air. Little

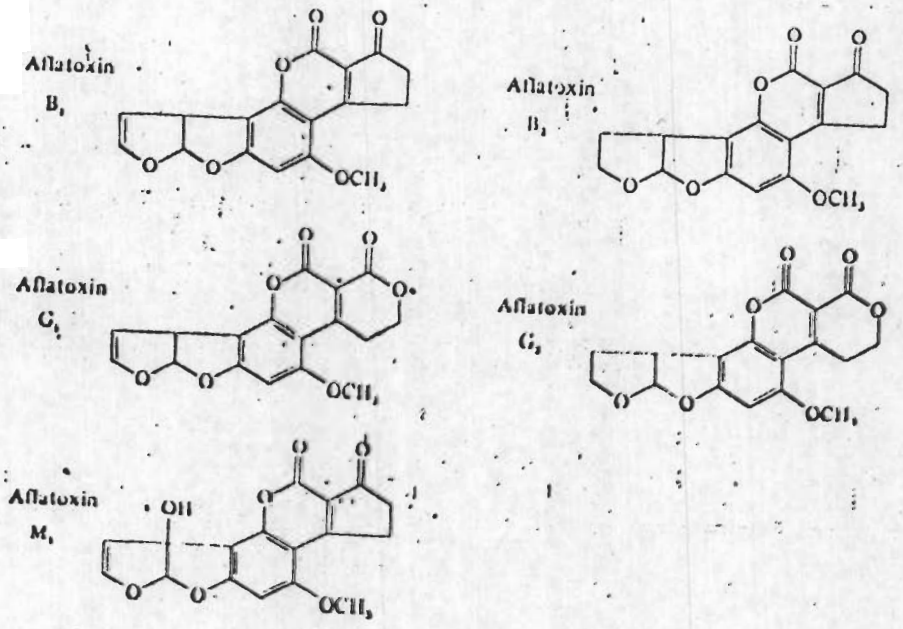


Fig A Biochemical strutures of aflatoxins

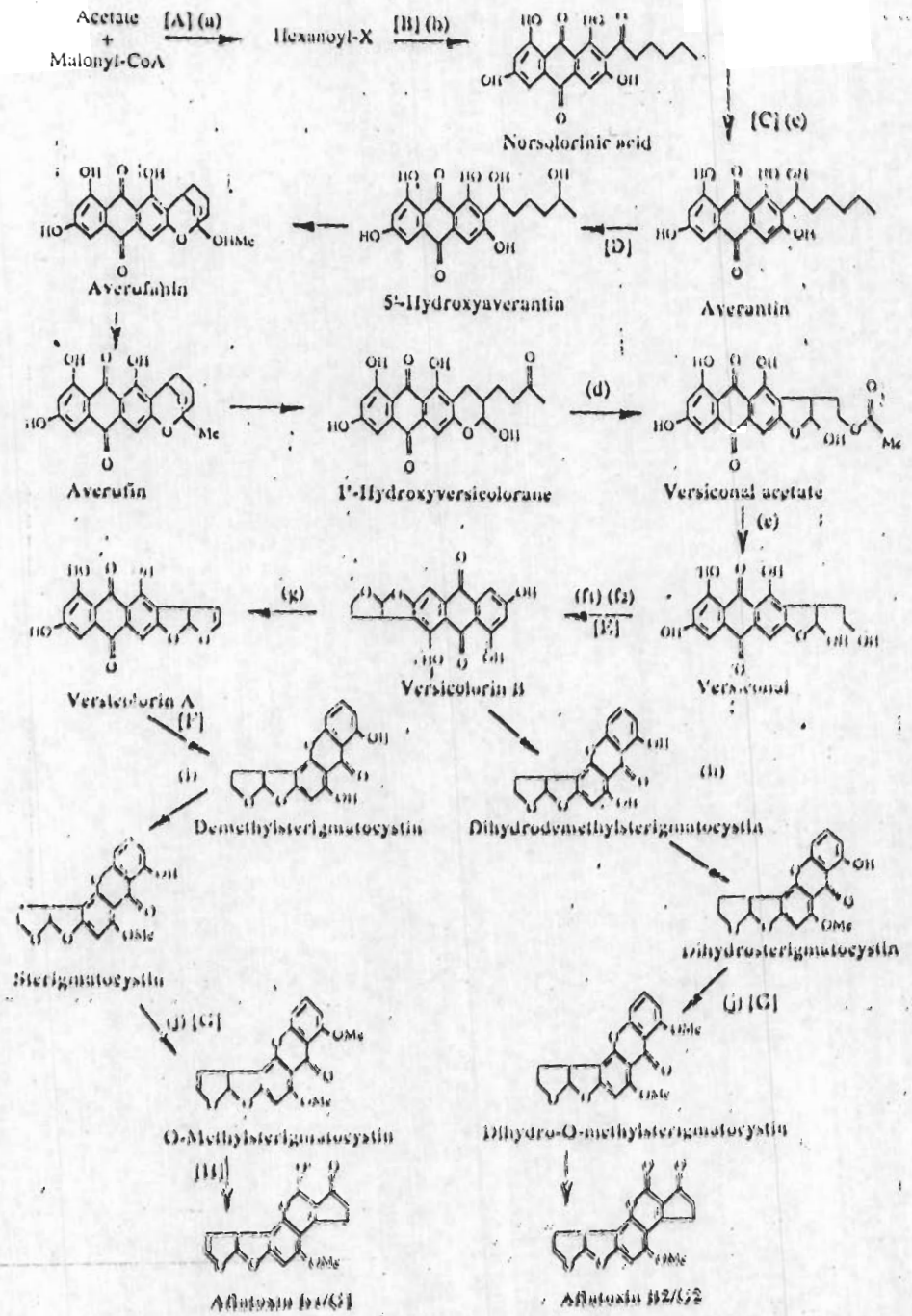


Fig. 8. Aflatoxin biosynthetic pathway. Enzymes involved: (a) fatty acid synthase, (b) polyketide synthase, (c) norsolorinic acid reductase, (d) versiconal hemiacetal acetate reductase, (e) esterase, (f_1) versicolorin B synthase, (f_2) versiconyl cyclase, (g) desaturase, (h) O-methyltransferase (MT-II), (i) O-methyltransferase, (j) O-methyltransferase (MT-I). Genes involved: [A] *fas-1A* and *fas-2A*, [B] *pksA*, [C] *norA*, *norA*, [D] *avnA*, [E] *ver-1*, *ver-1*, [G] *omtA* and [H] *ord-1*. (Compiled from Trail et al., 1995a; Bennett et al., 1997 and Minto and Townsend, 1997).

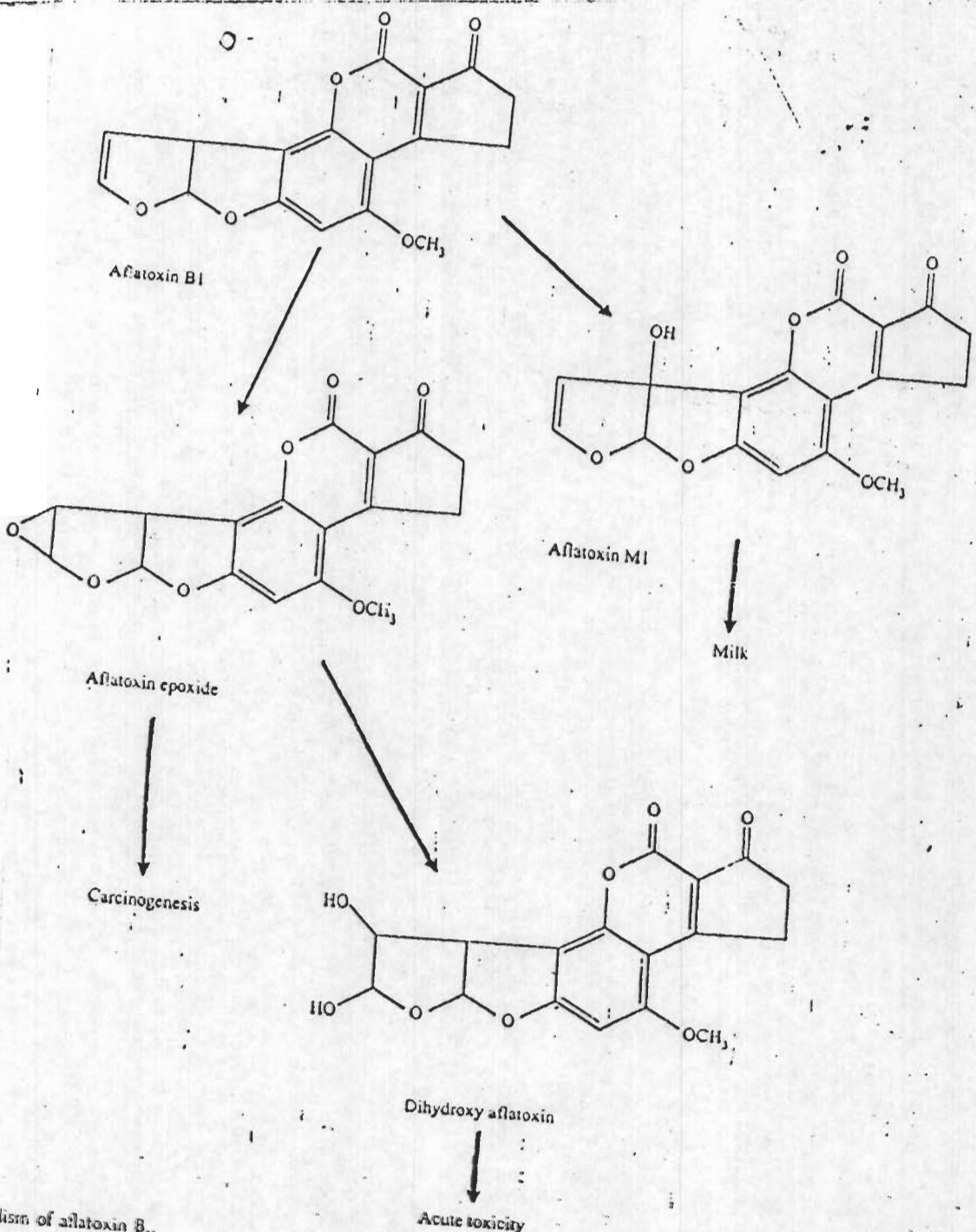


Fig C Metabolism of aflatoxin B_1.

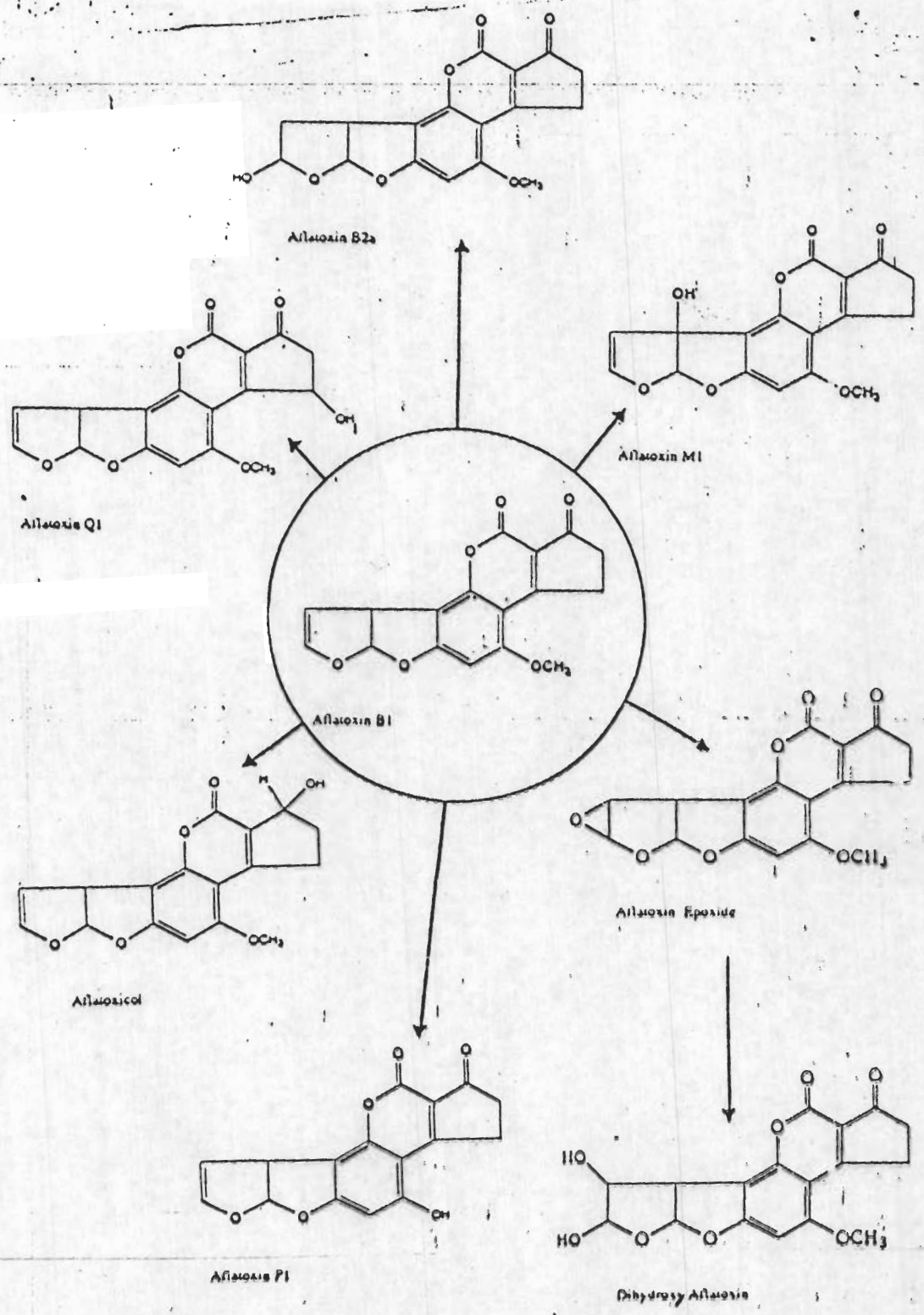


Fig D *Metabolites of aflatoxin B1*

or no destruction of aflatoxins occurs under the ordinary cooking conditions and heating for pasteurization. It is about 60% destroyed by frying in oil. It is highly susceptible to the UV component of sunlight. That is why exposure to sunlight is the easiest method for destroying aflatoxin in foods. The presence of a lactone ring in the aflatoxin molecule makes them susceptible to alkaline hydrolysis.

2.3.1 Aspects of aflatoxin biosynthesis

Metabolites essential for growth which are formed during cell multplication of micro organisms e.g proteins, nucleic acids, lipids and carbohydrates are termed primary metabolites. On the other hand, substances formed during the end of the exponential growth phase of the fungus with no apparent significance to the fungal growth or physiology are termed secondary metabolites. Whereas primary metabolism is basically the same for all living systems, secondary metabolism is essential to lower forms of life and is strain specific. The factors controlling secondary metabolism in fungi are complex and are not fully understood at present. The great chemical diversity of secondary metabolites include such products as quinones, antibiotics, alkaloids, mycotoxins and other pigments (Moss 1989; Prieto *et al.*1996).

The most commonly accepted postulates suggested that secondary metabolites are formed when large amounts of primary metabolic precursors accumulate such as acetate, malonate, pyruvate and amino acids. The fungus gets rid of these precursors by diverting them into secondary metabolites. Secondary metabolites also serve as defence agents, and attractants like in flower flavours etc (Wicklow *et al.*1984). Exhaution of nitrogen or phosphorus in the medium at the end of the exponential growth phase is accompanied by inhibition of nucleic acid and protein synthesis and accumulation of the primary metabolic

precursors. Alpha-Keto acids particularly pyruvic acid accumulate to toxic levels, thereby inhibiting cell division and growth. During the transition phase from exponential to stationary, the synthesis of these macromolecules (proteins, nucleic acids etc) continue but at a reduced rate. The enzymes of aflatoxin biosynthesis are derepressed. The activity of the tricarboxylic acid cycle continues unabated and reaches a maximum then and the fungus thus responds to the toxic levels of pyruvate and other intermediates by shifting to the stationary phase of growth. This is the period of onset of aflatoxin production. The synthesis of nucleic acid and ptoteins is terminated and the tricarboxylic acid cycle activity declines. Lipid synthesis continues unabated during the stationary phase, thereby showing the close relationship with aflatoxin biosynthesis (Ellis *et al*.1991; Bhatnagar *et al*.1994).

Moss (1996) has characterized the stationary phase or idiophase as a period of decline in the rate of growth, carbohydrate utilization and oxygen uptake and a period of secondary metabolite production. Generally, the mycelial dry weight during this phase remains more or less constant. Bu 'lock (1975) describes some cases where the mycelial dry weight continues to increase during the stationary phase. The rate of growth is however lower than during the exponential phase and this increase has been attributed to the accumulation of polyols, polyphosphates, lipids and non structural carbohydrates and secondary products. The main activity metabolically of the fungus therefore is to synthesize secondary metabolites during the stationary phase, pathways of protein and nucleic acid biosynthesis being blocked. This phase continues till the exhaustion of the carbon source.

The biosynthetic pathway of the aflatoxins is quite well understood and has recently been described in a number of reviews (Bhatnager *et al*.1994; Trail *et al*.1995, Bennett *et al*.

1997; Minto and Townsend 1997; Kelkar *et al.* 1997). Firstly, acetate and malonyl CoA are converted to a hexanoyl starter unit by a fatty acid synthase, which is subsequently extended by a polyketide synthase to the decaketide norsolorinic acid, the first stable precursor in aflatoxin biosynthesis. The polyketide then undergoes approximately 12 to 17 enzymatic transformations, through a series of pathway intermediates, which are summarized in Fig.B; including averantin, 5[1]-hydroxyaverantin, averufanin, averufin, 1'hydroxyversicolorone, versiconal acetate, versiconal and versicolorin E. The pathway then branches to form AFB_1 and AFG_1 which contain dihydro bisfuran rings and are produced from demethylsterigmatocystin. (DMST) and in turn the other branch forms AFB_2 and AFG_2, containing tetrahydrobisfuran rings which are produced from dihydrodemethylsterigmatocystin (DHDMST). The proposed pathway is based mainly on evidence from enzymatic and genetic analysis.

2.3.2. Toxicology of aflatoxins

Broiler chicks show depressed weight gains and liver lesions after 10wks on a diet containing 200ppb AFB_1 but laying hens are said to be unaffected after 33 weeks on a diet containing 610ppb. For weanling swine, the no effect chronic level was found to be 140 ppb in the diet and concentrations greater than this had demonstrable effects. Beef cattle are said to show no effects at concentrations in diet below 440ppb but liver damage is demonstrable at 700ppb. The shasta strain of rainbow trout shows liver tumours when exposed to a diet containing as little as 0.5ppb after 20months; whereas the coho salmon is apparently unaffected by as much as 20ppb. Differences in toxicity of AFB_1 to the male and female of a single species and differences between species arise because the mycotoxin is metabolized

by the liver and expression of toxicity according to Moss (1996) depends on the dynamics of this process.

The physico-chemical properties of aflatoxin B_1 are suited for the effective delivery of the toxin from the diet to the cytoplasm of the liver cells. Once there, it may be converted to the epoxide, a good candidate for the carcinogen and known to react with guanyl residues in DNA. The epoxide, itself may be further metabolized by an epoxide hydratase to form a dihydroxy compound which is a good candidate for the acute toxin and known to react with proteins. The implications of aflatoxin metabolism for the expression of disease have been reviewed by Neal (1987) (Fig C).

A diverse range of responses to the toxic effects of a compound may occur because the compound is metabolized in the animal body and the resulting toxicity is influenced by this metabolic activity. This is certainly the case with AFB_1 from which a very wide range of metabolites are formed in the livers of different animal species. Thus, the cow is able to hydroxylate the molecule and secrete the resulting aflatoxin M_1 in the milk, hence affording a route for the contamination of milk and milk products in human foods even though these products have not been moulded (Neal 1987; Egmond 1989; Aksit *et al.* 1997) (Fig D).

The formation of an epoxide could well be the key to both acute and chronic toxicity and those animals which fail to produce it are relatively resistant to both. Those animals which produce the epoxide, but do not effectively metabolize it further, may be at the highest risk to the carcinogenic activity of AFB_1 because the epoxide is known to react with DNA. Those animals which not only produce the epoxide but effectively remove it with a hydrolase enzyme, thus producing a very reactive hydroxy acetal, are most sensitive to the acute toxicity.

The hydroxyacetal is known to react with proteins.

The parent molecule may thus be seen as a very effective delivery system having the right properties for absorption from the gut and transmission to the liver and other organs of the body. It is,however,the manner in which the parent molecule is subsequently metabolized in vivo which determines the precise nature of an animal's response (Moss 1996).

2.3.2.1. Aflatoxins and Primary liver cancer (PLC) in man

Aflatoxins are one of the most carcinogenic compounds known for rat and rainbow trout (Moss 1989; Ramos and Fernandez 1996). Epidemiological studies of PLC in man indicate that aflatoxins are also carcinogenic to man (Saracco 1995; Methenitou *et al.*1996;

Groopman and Kensler 1999). Liver cancer in such parts of the world as the African continent is complex and it is probable that aflatoxin is only one factor in the appearance of the disease. There is a good correlation between the presence of hepatitis B virus and PLC in man and there is an increasing concensus that these two agents act synergistically to elicit hepatocarcinoma (Hsieh 1986; Olubuyide *et al.*1993; Blum 1994; Chao *et al.*1994; Diallo *et al.*1995; Bhat 1996; Yu *et al.*1997; Dhir and Mohandas 1998).

2.3.2.2. Other toxic effects due to aflatoxin

a Humans:

Aflatoxins have been shown to be risk factors either experimentally or by circumstantial evidence in many disease entities in man. For example, several studies have shown significant levels of aflatoxins and aflatoxicols in the blood and other body fluids of children in the tropics and it has been proposed that aflatoxin in the diet of children is

associated with Kwashiorkor (Hendrickse 1984; Coulter *et al.*1986). Very young children can be exposed to aflatoxins even before they are weaned because mothers consuming AFB_1 in their diet can secrete AFM_1 and AFM_2 in the breast milk (Coulter *et al.*1986; Moss 1996). Coulter *et al.*(1984) detected both toxins in the breast milk of Sudanese women, and also demonstrated same in the breast-milk samples of mothers attending maternity hospitals in Abu Dhabi.

Aflatoxins have been shown to exhibit several other toxic effects such as inhibition of protein synthesis, inhibition of mitochondrial energy metabolism and the induction of blood clotting disorders. Thus, according to Hendrickse (1991), the inhibition of protein synthesis is believed to account for the reported role of aflatoxins in the genesis of kwashiokor. Aflatoxins have also been reported as a cause of acute hepatitis in a number of cases from developing countries (Krogh 1989). In Zaria and Ekpoma, Nigeria, in two prospective studies, a possible relationship was shown between perinatal aflatoxin exposure and neonatal jaundice, though no relationship was shown between the severity of hyperbilirubinaemia and serum aflatoxin levels (Ahmed *et al.*1995; Abulu *et al.*1998). In Ibadan, Nigeria, Sodeinde *et al.* (1995) observed that the presence of any serum aflatoxin was a risk factor for neonatal jaundice.

Jonsyn *et al.*(1995) in Seirra Leone observed that the presence of aflatoxins in maternal blood samples contributed to the low birth weight of their infants. De-Vries *et al.*(1989) noted that the mean birth weight of infants born to aflatoxin-positive mothers in Kenya was significantly lower (255g) than those born to aflatoxin-free mothers. Two still births were also recorded, and these results coincided with the adverse-effects of prenatal aflatoxin exposure

recorded in animal experiments.

Dietary exposure to aflatoxins in Benin-City, Nigeria, has been associated with human male infertility. Infertile men with aflatoxins in their semen showed a higher percentage of spermatozoal abnormality (50.0%) than fertile men (10.0-15.0%) (Ibe *et al.*1994). Aflatoxins have also been revealed to act as potent genotoxins causing some forms of genetic alterations, including chromosomal abberations, micronuclei and sister chromosomal abberations and other forms of genetic damages (Harrison *et al.*1993; Miele *et al.*1996). Hollstein *et al.*(1997) also noted gene P53 alterations in human tumours induced by aflatoxins.

Increases in prothrombin time, decreases in total plasma proteins and albumin levels, and increases in red blood cell counts and haematocrit have been demonstrated by Fernandez *et al.*(1995). Robens and Richard (1992) also demonstrated acute hepatic failure and Reye's syndrome due to aflatoxins in their patients. Further researches have also shown that aflatoxins increase childhood susceptibility to infections, compromise immune responses to prophylactic immunisations and also may generally have the potential of immunosuppression and immunotoxicity (Hendrickse 1991; Neal *et al.*1998; Gabal and Dimitri 1998). Aflatoxin involvement in digestive tract cancers and lung lesions have also been reported (Oyelami *et al.*1997; Dhir and Mohandas 1998; Matsumura and Mori 1998) and also in renal problems in Nigeria (Oyelami *et al.*1997).

b. Animals:

In all species of domestic animals, aflatoxicoses has been well documented in which the evidence of disease is a general unthriftiness and reduction in weight gain, feed efficiency, immunity and production (Robens and Richard 1992; Moss 1996). More conclusive evidence

in aflatoxin involvement in animal diseases includes acute to chronic liver diseases with concomitant increases in specific liver enzymes in the serum. In cattle, milk production is affected, but of greater significance is that the aflatoxins in feeds can be rather efficiently converted to toxic metabolites readily detectable even in small amounts in milk. The poultry industry probably suffers greater economic loss than any other livestock industry because of the greater susceptibility of their species to aflatoxins than other species (Robens and Richard 1992).

Fernandez *et al.*(1996) observed significant reduction in body weight and in the average daily weight gain in the intoxicated group of lambs when compared with a control group. In his experiments, lambs fed with aflatoxins had relatively smaller liver weights and higher kidney and spleen weights than lambs from the control group. Blood parameter, urea levels, alkaline phosphatase and glutamate dehydrogenase enzymatic activities were also affected as a result of the intoxication. Changes in the cogulation profile of the lambs intoxicated with aflatoxins were also observed. Similar observations were also noted for hens and broiler chickens including changes in prothrombin time, total plasma and albumin levels, haematological values and red cell counts (Fernandez *et al.*1995).

In further studies in lambs, Fernandez *et al.*(1997) observed that aflatoxins cause a failure in the acquired immunity system of lamb by decreasing antibody production and altering serum profile proteins.

2.3.2.3. Epidemiology - based accounts of aflatoxin induced disease

Descriptive epidemiological studies, elucidating aflatoxin induced disease distribution in time and space havebeen conducted in both man and farm animals. The most convincing

evidence in establishing causal association between factors and disease is however usually obtained from analytical epidemiological studies, from which the necessary quantitative expressions of association can be obtained (Lilienfeld and Lilienfeld 1980).

Lye *et al.*(1995) reported an incidence among thirteen Chinese children who died of acute hepatic encephalopathy in the North western state of Perak Peninsular- Malaysia. The acuteness of the illness differed from previously reported outbreaks. Epidemiological investigations determined that the children had eaten a Chinese noodle, Loh see fun, hours before they died. Aflatoxins were also confirmed in post mortem samples from patients. Harrison *et al.*(1993) using both immunological and HPLC methods examined human DNA from a variety of tissues and organs to identify and quantify aflatoxins - DNA adducts. They detected AFB_1 - DNA adducts in formalin fixed tissues from acute poisoning incident in Southern Asia. Many aetiological relationships between aflatoxins and stomach, colon, cervix, breast, pancreas and liver neoplasms was revealed from adduct studies (Yu *et al.*1997).

Several other epidemiologically based accounts of aflatoxin induced diseases have been reported, including the presence of AFB_1 in lungs of people with lung cancers where attempts were made to detect aflatoxins from culture filtrates of those isolates and the tissue extract of the lung lesion through thin layer chromatography (TLC), densitometry and high performance liquid chromatography (HPLC). Aflatoxins B_1, B_2 and M_1 were demonstrated in all these materials qualitatively and quantitatively from these people with aspergillosis (Oyelami *et al.*1997; Matsumura and Mori 1998).

2.4. Environmental and biological factors influencing fungal attack and the production of aflatoxins

An important aspect of the control of mycotoxins is to identify the factors that contribute to contamination of crops. These factors are outlined below broadly.

2.4.1. Nature of the crop substrate

The substrate is a determining factor in the development of most fungi, some commodities are more susceptible to contamination by certain moulds than others while most moulds can however contaminate many substrates. The grains are considered to be an ideal substrate for the growth of fungi providing the necessary carbohydrates for energy and fats for additional energy (F A O 1997). Of all the agricultural crops studied in Nigeria, the most susceptible to aflatoxin contamination are groundnuts (Crowther 1973; Adebajo 1994) maize (Nwokolo and Okonkwo 1978; Opadokun 1990; Adebajo *et al.*1994) and copra (Obidoa and Gugnani 1990). Maize appears to be one of the most susceptible crops to contamination by various fungi and mycotoxins internationally (Lovelace and Aalbersberg 1989; Wood 1989; Price *et al.*1993; Arim 1995). Even with the same crop, certain cultivars appear to be more resistant to fungal attack than others. These include groundnuts (McDonald 1976), cowpeas and maize (El-Kady *et al.*1996) .

2.4.2. Condition of the crop

Mature, whole, unbroken kernels are less susceptible to mould attack than immature, broken and defective kernels (Haq-Elamin *et al.*1988). Adebajo *et al.* (1994) observed that the incidence of mould contamination was generally higher in shelled water melon seeds than

for unshelled seed samples. Doster and Michailides (1994) got more than three times higher aflatoxin load and *A.flavus* contamination in the early splits of Pistachio nuts than from normal nuts with intact hulls. The early splits are atypical nuts that have split hulls exposing the kernel to invasion by moulds.

2.4.3. Strain of fungus

Fungus may attack food crops without necessarily producing any mycotoxin. This is because not all strains of fungus that attack crops produce toxin. Moss (1996) noted that on a worldwide basis, only about 35% of strains of *A.flavus* produce aflatoxins, while F A O (1997) earlier noted that only 58% of all strains produce aflatoxin. Many other workers have also observed the same trend (Mishra and Daradihiyar 1991). Lacey (1989) in his studies noted that only 42.3% of all his *A.flavus* isolates produced aflatoxins. It has also been shown that isolates of *A.parasiticus* produce aflatoxins in higher concentrations than *A.flavus* which contains a greater percentage of non-aflatoxigenic strains and produces only B aflatoxins (Pitt 1993).

2.4.4. Temperature

Fungi can only attack crops successfully within certain temperature limits (between 0°C and 60°C). In general, low temperatures reduce the rate of fungal growth. All fungi have characteristic minimum, optimum and maximum temperature for growth. Certain fungi like *Fusarium* species and *Cladosporium* species grow and produce toxins at rather low temperatures (10°C and 20°C), while *A.flavus* grows at temperatures between 12°C and 42°C but aflatoxin production is maximum at temperatures between 25°C and 32°C (Koehler *et al.*

1985; Pitt and Hocking 1997). These tempertures approximate the ambient temperature in most parts of Nigeria most of the year. Most fungi growing on plants before harvest (the field environment) grow well in the temperature range 0 to 30°C (Lacey 1989), while most fungi found in the storage environment, however thrive within the range 10-40°C (optima 25-35°C). *Aspergillus fumigatus* has an exceptionally wide range from 10-55°C while some Ascomycetes have ascospores which are very resistant and able to survive ten minutes heating at 90°C.

2.4.5. **Moisture**

Based largely on moisture requirements for growth, fungi that attack crops can be grouped into field fungi that attack grains at moisture levels exceeding 30% (e.g *Alternaria*, *Fusarium* and *Cladosporium* species), and storage fungi that predominate in grains in the 13% to 18% moisture range (eg *Aspergillus* and *Penicillium* groups). The moisture content of the grains which is in equilibrum with air is referred to as 'safe' moisture content for which Agboola (1990) has enumerated for the storage of some Nigerian food crops at 27°C as follows:

Crop	Safe moisture content at 27°C
Cowpea	15.0
Maize	13.0
Millet	16.0
Paddy	15.0
Rice	13.0
Sorghum	13.0

Wheat	13.0
Cocoa beans	7.0
Copra	7.0
Cotton seed	9.0
Groundnut (shelled)	7.0
Ground nut (unshelled)	9.0
Palm kernels	5.0
Soybeans	8.0
Gari	12.0

Lacey (1989) has also shown that even when dry grains are put into store, there is a possibility of exchange of moisture in the vapour phase due to temperature gradients produced by fluctuating external temperatures. This can contribute to even a higher fungal growth during storage.

2.4.6. **Biological agents**

The principal role played by insects in toxin contamination of agricultural crops is that of predisposing the crop to fungal invasion. Insects damage the crops which they attack, rendering them more liable to fungal attack. They also carry spores of fungi into the crops they infest. Their metabolic activities also leads to increase in moisture and temperature of the crop, providing excellent conditions for the growth of the fungi. *A.flavus* has been identified as the dominant fungus associated with rice weevil (*Stophilus* oryzae) in stored wheat and rice, (Ragunathan 1974). Julian *et al.*(1995) in Honduras also associated heavy contamination of pre and post harvest maize crops with *A.flavus* with insect damage.

Insects can also inhibit fungal growth by the secretion of quinones (eg by *Tribolium castaneum*) or by feedings on fungi (Lacey 1989). Some fungal species can also attract insects (eg. *Penicillium* species), (Dunkel 1988). Rodents also damage crops which they infest making them more readily attacked by fungi while their droppings and hair may serve as inoculum for fungal growth and can also act as carriers of fungal spores into crops. Birds, too, by contaminating the crops with their droppings, feather and debris for making nests can similarly contribute to fungal damage in stored crops (Doster and Michailides 1994).

2.4.7. **Nutrients and trace metals**

Besides environmental factors, several metabolic promoters and inhibitor of aflatoxins biosynthesis have been reported (Gupta *et al.*1976). Similarly, some of the chelating agents that selectively bind with zinc or other stimulatory metals have been reported to inhibit aflatoxin production in *A. parasiticus* and *A.flavus*. These include phytic acid, dimethyl sulphoxide, 2-mercaptoethanol and tolnaftate; and may also inhibit the incorporation of acetate into aflatoxin. Many workers reported enhanced aflatoxin production in the presence of amino acids including thiamine, methionine, proline, tryptophane, asparagine and alanine (Luchese and Harrigan 1993).

The stimulatory effects of the various amino acids may be due to their effect on the growth and general primary metabolism of *A.parasiticus* and *A.flavus* or even a direct effect on aflatoxin biosynthesis. The effects of antifungal agents from suppression to stimulation on levels of aflatoxins have also been reported (Moss and Frank 1987).

Reports on the inhibition of aflatoxin production by benzoate and thioglycerol (Buchanan *et al.*1986) and certain formulations of ammonium propronate (Magan and Lacey

1986) abound. Unsaturated but not saturated longer chain fatty acids have also been found to stimulate aflatoxin biosynthesis (Tiwari *et al.* 1986). The inhibitory effect of caffeine against aflatoxin bisynthesis while having no effect on growth of *A.parasiticus* explains why this mould grows on ordinary and decaffeinated coffee beans but only produces aflatoxin on the later (Durakovic *et al.* 1985). Also, the stimulatory and inhibitory effects of carbon sources and nitrogen sources on aflatoxin production have been widely discussed (Obidoa and Onyeneke 1980; Ogundero 1987; Luchese and Harrigan 1993).

2.4.7.1. **Effects of metals**

The role of metals in the regulation of secondary metabolism as well as primary metabolism in microorganisms is well documented. Metals function as cofactors of enzymes to either stimulate or inhibit enzymatic reactions (Vasavada and Hsieh 1988). They are also important in modulating cell wall permeability and in regulating many pre and post transcriptional events (Luchese and Harrigan 1993). Numerous secondary metabolites of fungi are affected very much either resulting in inhibitory or stimulatory effects, with manganese (Mn^{2+}), iron (Fe^{2+}) and Zinc (Zn^{2+}) being the most important metals involved (Agarwala *et al.* 1986; Zaika and Buchanan 1987; Fernando *et al.* 1993). Though it has been established that zinc is especially required for the biosynthesis of aflatoxins in solid substrates like corn (Failla *et al.*1986; Tiwari *et al.* 1986). Obidoa and Ndubuisi (1981) found no correlation between aflatoxins production and zinc content of tropical foodstuffs. A mixed trend was however observed depending on salt concentrations with molybdenum, magnesium and manganese while iron, copper and cadmium salts decreased the aflatoxin production to different levels (Tiwari *et al.* 1986; D'Souza and Brackett 1998).

A new elastinolytic proteinase from *A.flavus* has been shown to be markedly inhibited by numerous metal ions (Sweenyand Dobson1998). Resistant cultures of cowpea and garden pea were found to contain lower levels of sodium and higher levels of phosphate and potassium (El-Kady *et al.*1996).

2.4.8. **Microbial interactions and ecosystems**

Fungi rarely occur in a monospecific culture in stored products but often as a group of interacting species of fungi and bacteria (Lacey 1989). Many studies have shown that organisms growing with *A.flavus* and *A.parasiticus* can influence the final yield of aflatoxin either positively or negatively (Kirilenko and Egorova 1985; Kharchenko 1986; Cuero *et al.* 1987; Salunkhe *et al.*1987; Basappa and Shantha 1996; Sweeny and Dobson 1998). Thus microbial comptetion between different fungi and other microorganisms for substrate under favourable conditions may restrict or reduce the amounts of toxins produced by a particular fungal species. For example, *A.flavus* was apparently capable of suppressing the accumulation of AFG_1 and AFG_2 by *A.parasiticus* when in dual culture with *A.flavus* (Wilson and King 1995).

Weckbach and Marth (1977) observed that *Rhizopus nigricans* and *Saccharomyces cerevisiae* inhibited growth and aflatoxin production by *A.parasiticus* in mixed cultures, while *Brevibacterium linens* caused slight inhibition and *Acetobacter aceti* stimulated growth and toxin formation in the mixed culture. Zhu *et al.*(1989) showed that *Rhizopus delemar* has intensive capacity of inhibiting the toxic damage and carcinogenicity of the liver by AFB_1 in experimentally induced hepatocarcinogenesis in Wistar rats. When *A.flavus* was grown with the yeast, *Candida guilliermondii* on freshly harvested corn, detectable levels of aflatoxins

were recorded while none was detected when *A. flavus* was paired with *A.niger* or *Trichoderma viride* (Bol and Smith 1990; Knol *et al*,1990). The inhibition and removal of aflatoxins were observed with lactic acid bacteria and also *Bacillus pumilus* when in dual culture with *Aspergillus parasiticus* (Rasic *et al*,1991; Muninbazi and Bullerman 1997; el-Nezami *et al*. 1998). Aflastatin A, a novel inhibitor of the production of aflatoxin by aflatoxigenic fungi has been isolated from the solvent extract of mycelial cake of *Streptomyces* specie (Ono *et al.* 1997). Also in an experiment conducted with soil isolates of filamentous fungi to determine potential inhibitors of *A.flavus*, twelve taxa, including several strains of *Fusarium solani, Penicillium vinaceum* and *A.auricomus* produced compounds inhibitory to *A.flavus* (Klich 1998).

The growth rates of *Streptococcus agalactiae, Staphylococcus aureus* and *Yersinia enterocolitica* in the presence of aflatoxin B_1 (AFB_1) were slowed down as shown in an experiment conducted by Ali-Vehmas *et al*, (1998). The bacterium *Flavobacterium aurantiacum* has been shown to be capable of metabolizing AFB_1 in various foods (Lime *et al*.1994)

2.4.9. **Food processing**

Fermentation has been associated with a decrease in aflatoxin yield like the fermented melon seeds for making the local soup condiment 'Ogiri' (Ogunsanwo *et al*,1989). Jespersen *et al.*(1994) and Kpodo *et al.(1996)* however observed no effect in the fermentation of maize dough in the preparation of a West African traditional food 'kenkey' though the microbial load was drastically reduced within twenty four hours of fermentation. During the fermentation of *A.flavus* spiked maize and sorghum for the production of 'tuwo' and 'Ogi' by Adegoke *et al.*

(1994) and Oyelami *et al.* (1996), reductions of about 72.5% and 71.4% of the aflatoxin levels were obtained respectively. In the same study, a reduction of about 68.0% and 80.8% respectively in AFB_1 levels were observed for pastes boiled for 30 minutes and 60 minutes in the production of 'tuwo'. Reconstitution of Ogi paste into a porridge (akamu, pap) recorded also a considerable reduction in the aflatoxin levels.

The fate of aflatoxins when raw cowpea were processed into a porridge or 'moimoin', or fried in oil to form 'akara' balls was studied and reduction of aflatoxin content was also noticed (Ogunsanwo *et al.* 1989). The effect of processing on the mycoflora and AFB_1 level of a cassava based product was also studied by Adegoke *et al.* (1994). In one of these studies, cassava bread was prepared by pre-gelling, battering and baking cassava flour to which moderate amounts of sugar, yeast solution and edible oil were added. Baking was done at 215°C for 40 minutes. Although the raw cassava contained *Penicillium oxalicum*, *A.flavus*, and *Fusarium* species and some unidentified fungi, no moulds were isolated after the baking process. AFB_1 levels of 1.91 micrograms/kg in the cassava was drastically reduced to 0.03 micrograms/kg in the bread. Faraj *et al.* (1993) also observed that microwave heating reduced aflatoxin levels in foodstuffs. However, no reduction with cooking was observed for spiced sauces (MacDonald and Castle 1996). Reductions during fermentation and baking was also reported by Hassanin and Kheirella (1995).

2.4.10. **Other factors**

Both *A.flavus* and *A.parasiticus* grow well over a pH range of 2.1 to 11.2 with an optimum pH between 3.5 and 8 (Wheeler *et al.* 1991). Aflatoxins can also be produced between pH 3.5 and 8.0 with an optimum at about pH 6 (Buchanan and Ayres 1976). Fungi

causing detorioration of stored foods are usually considered obligate aerobes but increases in carbondioxide concentration by 5-10% can stimulate growth of some species especially when water activity is high (Lacey 1989). Mert and Ekmecki (1987) studied the effect of salinity and osmotic pressure of the medium on the growth of *A.flavus* and observed that the negative growth increased with an increase in the sodium chloride content of the nutrient medium while the osmotic pressure increases were inhibitory to growth.

2.5. Pre-and Post harvest ecology of fungi

The colonization of aerial parts of plants by microorganisms starts almost as soon as leaves or inflorescences are exposed to the air. Bacteria usually appear first but are soon followed by yeasts and then by pathogenic and saprophytic filamentous fungi which continue to develope thoughout the plant growth but especially as the plant senesces and seed ripens (Lacey 1989). Harvest profoundly disturbs the ecosystem and marks the transition from the extremes of the field environment to the relatively stable conditions of storage. This is accompanied by a profound change.

Fungi present on plants before harvest have been traditionally referred to as field fungi and may persist on fresh foods after harvest and may be the cause of disfiguring lesions on fruits and vegetables. In stored products, they persist if the grains are dry enough to prevent the growth of typical 'storage' fungi. These usually occur in small numbers before harvest and are chiefly species with *Aspergillus* and *Penicillium* anamorphs. As water content increases, fungal growth becomes more vigorous leading to spontanneous heating up of the substrate and the growth of more thermotolerant fungi including species of *Absidia, Rhizomucor* and *Humicola* often accompanied by thermophilic actinomycetes.

The concept of field and storage fungi should not be pressed too far. The recognition that field species could sometimes grow in storage led to the designation of an intermediate group. More recently, it has been recognized that storage species especially *A.flavus*, often occur in the field in humid tropical areas (Sinha 1987; Julian *et al*,1995; Resnik *et al*.1996). Thus it is important to recognise that some species of moulds, which are commonly associated with post-harvest spoilage may, also be able to proliferate and produce toxic metabolites in the growing crop before harvest. Thus, both *A.flavus* and *A.parasiticus* can establish an epiphytic relationship with crops such as maize and goundnuts (Hill *et al*.1985). The spores of these moulds are able to germinate on the stigma of the plants, apparently mimicking the activity of pollen, and establish themselves in the developing seed. This epiphytic relationship seems normally to be benign but if the plant is stressed (usually by drought), then the mould mycelium becomes more active and produces aflatoxin. Under these circumstances, aflatoxin may occur in apparently undamaged maize or groundnuts at harvest (Moss 1996).

The complex ecology of *A.flavus* and *A.parasiticus* have been reviewed by Diener *et al*.(1987) and it is now appreciated that *A.parasiticus* is well adapted to a soil environment and is therefore prominent in peanuts whereas *A.flavus* seems to be adapted to active development in the aerial parts of plants, such as leaves and flowers and is dominant in maize, cotton seed and tree nuts. That invasion from the soil rather than via aerial parts of the plant is important in the pre-harvest contamination of goundnuts with aflatoxins was demonstrated by Cole *et al*. (1986) using colour mutants of *A.flavus* and *A.parasiticus*. In contrast, natural openings in the plant tissues such as cotyledonary nodes and nectaries at or near the time of anthesis, have been shown to be important in the infection of cotton seed (Klich 1986). Soil moisture and

drought stress are also important factors in the contamination of what appear to be normal, undamaged products post-harvest (Lacey 1989). Thus as much as 244 micrograms per kg of aflatoxins could be found in sound, mature kernels from a groundnut crop which has suffered drought stress during growth. It has also been confirmed that water stress could be a major factor influencing aflatoxin contamination of maize (Moss 1996).

2.6. Occurrence of aflatoxins and fungi in general foodstuffs and animal feeds

In all parts of the world, reports abound on the distribution of moulds and their consequent production of aflatoxins in foodstuffs.

Johnsyn (1988) isolated toxigenic species of *A.flavus*, *A.ochraceus*, *A.tamarii*, *Penicillium citrinum* and *Fusarium* species from sesame seeds in Sierra Leone and AFB_1 and AFG_1 and other fungal toxins were positively identified. In Sudan, Hag.Elamin *et al.*(1988) observed that levels of aflatoxins were distributed according to geographical climatic feature. They also showed that from the samples analysed, that damaged pods had more levels of aflatoxins and mould contamination when compared with intact sound pods. According to them also, gray and red roasted pods also showed higher amounts of aflatoxins while groundnut paste was the least contaminated. A total of 4532 samples of Soviet union domestic and imported cereals and other foods were sampled for aflatoxin contamination. The results showed that 26.9% of peanuts, 2.2% of corn and 28.3% of cotton seeds were contaminated with aflatoxins at levels exceeding the maximum tolerated levels established in the U.S.S.R. (5mg/kg) for AFB_1 in all foodstuff (Tutelyan *et al.*1989). Munir *et al.*(1989) in Pakistan observed that only 41.6% of maize samples and 25% of red chillies assessed were contaminated with levels of 11.12-82.33mg/kg for AFB_1 and AFB_2 41.6mg/kg. In Fiji and

Tonga islands, local copra, cassava and maize samples were found to be contaminated, with maize samples only at a serious level. In the United States of America, food commodities including peanuts, peanuts products, tree nuts, cotton seeds, milk, spices and other miscellaneous foods were analysed by Wood (1989). Correlations were highest between aflatoxin contamination and geographical areas for corn and corn products and cotton seed/cotton seed meals.

Zohri and Saber (1993) isolated 59 species and one variety belonging to twenty-five genera of fungi in Egypt from twenty-nine samples of *coconut*. The fungi included *A.flavus*, *A.niger*, *P.chrysogenum*, *C.cladosporoides*, *Alternaria alternata*, *Rhizopus stolonifer*, *Trichoderma hamatum* and *Eurotium chevaleri*. Analysis showed that five samples tested of the twenty-nine were naturally contaminated with AFB_1 (15-25mg/kkg) and three samples were contaminated with ochratoxin A (50-205mg/kg). Price *et al.* (1993) detected that 10 out of 644 (1.6%) domestic corn samples and 7 of 106 (6.6%) domestic cotton seed samples contained aflatoxins at levels greater than 300ppb in a survey of naturally occurring toxins in feedstuff in Rockville. Russell *et al.* (1991) also noted that *Fusarium* species were the most predominant in maize samples in the United States, however, results from Beijing, where a total of 5,000 food samples classified into 104 kinds of 16 types showed that the natural occurrence of AFB_1 in foods in Beijing was not serious (Jia 1991), though foods seriously contaminated with moulds were encountered.

In a study carried out in Mexico, the 41 samples of corn analysed showed AFB_1 and G_1 465.31ng/g and 1.59ng/g to 57.1ng/g respectively, and of the 89.8% of samples contaminated with aflatoxins, 59.5% contained levels above the Mexican legal limits of

20ng/g (Torres-Espinosa *et al.* 1995). In Sao Paulo, Brazil, out of 130 samples of post-harvest and stored corns analysed, *Fusarium* species was again the most predominant (83.8%), *Aspergillus* species (40.7%) and 11 other fungi (Pozzi *et al.* 1995). In Honduras during October 1992 and November 1993, *Fusarium* species, *Penicillium*, *Stenocarpella*, and *Acremonium* species were

encountered in a decreasing order of frequency from sixty-nine corn samples collected from pre-harvest standing crops and aflatoxins were detected only in the 2 samples heavily contaminated with *A.flavus* (Julian *et al.* 1995).

Jespersen *et al.* (1994) studied the maize dough fermentation for the production of 'kenkey', a West African traditional food from Ghana; a mixed flora comprising *Candida, Saccharomyces, Trichosporon, Kluyveromyces and Debaryomyces* species were isolated from the raw maize during steeping and fermentation and high levels of aflatoxins were also observed in the raw maize.

Maize in flooded areas of the Bhagalpur district of India demonstrated heavy infestation with *A.flavus* and aflatoxins. Sixty samples collected from various field lots were positive in the bright greenish yellow fluorescence test. However, only 42 were found to be contaminated with deleterious levels of aflatoxins. Other fungi on the maize kernels included *A.niger, A. ochraceus, P. citrinum* and *Fusarium* species and the AFB_1 ranged from 1 to greater than 1000μg/kg (Sinha 1987).

In the Phillipines, a survey on the aflatoxin contamination of various foods indicated that corn and peanuts were the two commodities that contained toxic levels of aflatoxins (Arim 1995).

A study was carried out to investigate the microbial and aflatoxin contamination of cereals and cereal products in Bangkok, Thailand. Results showed that rice and rice products were free from bacteria, one sample was contaminated withAFB_1 (10ppb) and four with AFG_1 (20-50ppb). Two of the four samples cultured for fungus revealed *A.niger*. Out of the 30 samples of peanut products, bacterial contamination included only *Bacillus* species and 13(43%) contained aflatoxins (40-780ppb) with a mean of 130ppb and AFG_1 (130-160ppb). *A. flavus* was found in 8 samples and *A.niger* in 2 of the 13 samples. The soybeans samples, 10 soybean oil examined were safe, 4 of 10 samples of fermented soybeans (tao-chiew) were contaminated with AFB_1 level varying from 10-20ppb and AFG_1 20-100ppb and 2 of the 4 samples revealed *A.niger*. Beans samples which included garden pea (Lantao bean), Fava bean, Pigeon pea, red bean, black bean and green bean were studied. One sample of lantao bean contained 50ppb AFG_1. Twenty corn samples studied showed that four contained aflatoxins, the amount varied from 30-140ppb of AFB_1 and 40ppb of AFG_1 (1mwidthaya *et al*,1987)

Stored and cooked samples of pearl millet (*Pennisetum typhoides*) were analysed in Bihar State, India. Of the 22 fungal species isolated, *A.flavus* and *A.parasiticus* were the predominant species (63.8%) during the rainy seasons, followed by other species of *Aspergillus, Penicillium, Fusarium, Rhizopus, Helminthosporium* and *Curvularia* and reveal an alarming level of naturally produced AFB_1 levels between 17 and 2,110ppb in stored and 18 and 549ppb in cooked samples (Mishra and Daradhiyar 1991). Samples of cereals and cereal products, seeds, spices and herbs, sauces and a variety of canned vegetables were analysed in the United Kingdom. Trace levels of mycotoxins including aflatoxins were detected in

samples containing cereals such as rice, corn flour, noodles and pitta bread (*Patel et al.*1994).

In a survey carried out to detect the natural occurrence of aflatoxins and zearalenone in maize samples from Buenos Aires and Santa Fe provinces in Argentina, among 2,271 samples analysed, 1,214 (53.3%) were contaminated with mycotoxins; AFB_1 in 445 samples (19.6%); AFB_2 in 92(4.1%) while zearalenone was identified in 676 samples (29.8%). AFG_1 was detected only in one sample and none of the samples contained detectable amounts of AFG_2. Aflatoxins generally were not detected in the 1988, 1993 and 1994 harvests and in the other years, the contamination levels were low with the exception of 1989 (Resnik *et al.*1996).

The level of toxigenic moulds and mycotoxins were also analysed in 62 samples of medicinal plant materials and 11 herbal tea samples. The most predominant fungi detected were; *Aspergillus, Penicillium, Mucor, Rhizopus, Absidia, Alternaria, Cladosporium* and *Trichoderma* species. *Aspergillus flavus* was present in 11 or 18% of the 62 medicinal plant samples and ochratoxin was found in one of the seven samples analysed (Halt 1998). Air-borne aflatoxins generated in rice and maize processing plants was assayed by an indirect competitive enzyme-linked immunoabsorbent assay and high levels of air-borne aflatoxins were detected in processing plant elevator, the loading/unloading area and the mill (Ghosh *et al.*1997).

Groundnut samples from 21 selected markets in the 10 regions of Ghana yieled high levels of aflatoxigenic *A.flavus* and was associated with 31.7 and 12.8% respectively of all damaged and undamaged kernels assayed. Other fungi detected included *A.niger* 34%, *A.candidus* 1.45%, *A.tamarii* 3.93%, *A.ochraceus* 5.26%, *Fusarium* species 1.7%, *Penicillium* species 5.19%, Mucor spp 2.3%, Trichoderma spp 0.2%, *Rhizopus stolonifer* 12% and certain

unidentifiable fungi (11.72%). Aflatoxin levels of 168ppb were identified with damaged samples while none was detected in 50% of undamaged samples tested and very low levels of aflatoxins ranging from 0.1 to 12.2ppb were associated with these undamaged samples (Awuah and Kpodo 1996) .

Maize appears to be more susceptible to aflatoxin contamination than other cereals sample in Nigeria. Studies conducted showed that only 3.7% of millet, 6.9% of sorghum, 4.7% of rice samples have detectable aflatoxins (Opadokun *et al.* 1979). A study of Nigerian corn and corn-based snacks revealed that 31 fungal species mostly toxigenic belonging to 11 genera were isolated. *Aspergillus, Penicillium* and *Fusarium* species accounted for 10, 6, and 3 of the species and all together they constituted 90, 94 and 88% of all the total fungi in the corn and corn products respectively. Total aflatoxin levels recorded were in the range 25-770ppb for corn; 15-1070ppb for corn cake and 10-160ppb for corn roll snacks respectively (Adebajo *et al.* 1994). Elegbede (1978) sampled sorghum during storage in Zaria, Nigeria and detected no aflatoxins and *Fusarium* mycotoxins although *Aspergillus* and *Fusarium* moulds were isolated in the samples. From a total of 48 samples of maize-based gruels used as weaning foods at Ilesha, Nigeria, 12 samples (25%) were positive for aflatoxins though at relatively low levels (Oyelami *et al.* 1996). Opadokun *et al.* (1979) analysed cowpeas and noted that contamination of these legumes with aflatoxins appears to be low. Ogunsanwo *et al.* (1989) detected similar levels in cowpea samples analysed in Western parts of Nigeria. Popoola and Akueshi (1986) in separate studies on soybeans in Northern Nigeria, no *A. flavus*, no aflatoxins and other mycotoxins were reported. In the work of Ibeh *et al.* (1991) in Benin City, Nigeria, fifty percent of yamflour, 40% of

cassava flour, 30% of gari, 20% of bean and melon and 10% of rice analysed yielded aflatoxin at varying levels.

Nwokolo and Okonkwo (1978) showed that aflatoxin are present at a high level in most common foods stored poorly for long periods in parts of Eastern Nigeria. Aflatoxin ranges of 600 to 1,100ppb for groundnuts, 200-350ppb for sorghum, 100-200ppb for maize, 140-250ppb for rice, 10-125ppb for beans, 2-45ppb for cassava were revealed in their study. Adebajo and Idowu (1994) detected appreciable levels of aflatoxins and ochratoxin A in stored tubers of cassava in Nigeria while Ogunsanwo *et al.*(1989) detected no aflatoxins in 26 market samples of 'Ogiri', a west African fermented melon seed condiment.

Agbanlahor *et al.* (1997) worked on the microbial burden of gari and cassava in Edo State, Nigeria and got *Bacillus* species 75%, *Pseudomonas aeruginosa* 40%, and others, while for fungi, *Aspergillus* species were the commonest, followed by *Rhizopus* and *Pencillium* species. Of the 94 gari samples examined by Opadokun *et al.* (1976), none had aflatoxins, though there was an intense fluorescence which has been identified as scopuletin by several workers including Obidoa and Obasi (1991). Opadokun (1990), recorded mean aflatoxin B_1 levels at ranges between 0 and 40ppb and 0-5ppb for sorghum examined in the North and Southern Nigeria respectively, groundnuts 151-767ppb and 0-216ppb for North and South samples respectively, melon seed crop in the North with 0-24ppb, in the South, 0-5ppb; rice in the North 0-56ppb, in the South 0-152ppb and cowpea in the North 0-7ppb and in the South, 44ppb. Other foodstuff like soyabean, gari and yam flour recorded no aflatoxins in this work.

Amongst the oilseeds, groundnuts are easily the most susceptible to aflatoxin contamination in all samples analysed (Crowther 1973). In Nigeria, studies show that over

60% of the crops examined showed aflatoxin levels higher than the allowable limit by the FAO (Food and Agriculture Organization 1997), ie 30ppb. According to Obidoa (1975), the food crops most commonly infested by aflatoxigenic fungi include groundnuts, cereals, cocoa, coconuts, fruits and rootcrops, with aflatoxins being detected with highest levels in the oil seeds and nuts such as groundnuts and copra.

Obidoa and Obasi (1991) reported the aflatoxin levels of table foods sold in public eating places at Nsukka including gari, egusi, ora and bitterleaf soups and other foods as ranging between 99.84 to 268ppb for AFB_1, while beans and stew ranged between 31-87.36ppb. Atawodi *et al.* (1994) analysing animal feeds noted that groundnut containing materials were the most heavily contaminated, the highest value being 1862ppb in groundnut cakes. Eighty percent of feeds based on maize were found to be highly contaminated with aflatoxins (480mg/kg) and zearalenone (40mg/kg) in Egypt. AFB_1 and AFB_2 were found in 50% of cottonseed cake feeds, wheat bran meal 40% while soybean meal appeared to be a poor substrate for aflatoxin formation (Mahmoud 1993).

In Reunion Island, out of a total of 150 samples of noted poultry feeds and raw materials analysed, feeds based on white corn and Brazilian Soybean meals seemed to present a better microbiological quality than those based on yellow corn and U.S. soybean meal. Mixed protein feeds presented a high total mould count reflecting the mould flora of the raw materials. The most frequent and abundant fungi were *A. flavus*, *A. glaucus* group, *Fusarium* species, *Penicillium* species, *A. candidus*, *Mucor* species, *A. restricutus*, *Scopulariopsis* species, *Cladosporium* species and *A. versicolorin* in order of decreasing frequency (Bauduret 1990).

Occurrence of mycotoxins in raw ingredients used for making animal feed stuffs in the United Kingdom was reviewed by Scudamore *et al.*. (1997). Aflatoxin B_1 was the mycotoxins found most frequently, occurring in most samples of rice bran, maize products, palm kernels and cotton seed but not in 3 out of 20 samples of sunflowers, in none of the 20 samples of soya and in no samples of peas, beans or manioc. Maize products frequently contained an average of 41μg/kg of AFB_1. Out of a total of 180 samples of poultry feeds collected during 1996 and 1997 from different factories in the South of the province of Cordoba - Argentina analysed for moulds and aflatoxin production, the predominant species were *A. flavus* and *A. parasiticus* for *Aspergillus* and *P. oxalicum, P. purpurogenum* and P. *brevicompactum* for *Penicillium* species. Less frequently isolated were *A. candidus, A. fumigatus, A. niger, A. oryzae, A. terreus, A. tamarii, P. expansum, P. funiculosum* and others. The mean value counts ranged from I x 10^3 to 9.5x10^4 CFU/g for the *Aspergillus* spp. and 21 of the 45 assayed strains (47%) produced aflatoxins. From them, 24% of the isolates produced AFB_1 and AFB_2 with levels from 181 to 14,545 and 6 to 3,640 micrograms/kg respectively (Magnoli *et al.* 1998).

Analysis of sorghum and maize used for feeds in Botswana showed a concentration of aflatoxins ranging from 0.1 to 64 microg/kg for sorghum. Aflatoxins were generally detected in 40% of the samples analysed. No aflatoxins were detected in maize (Siame *et al.* 1998).

Thus, from the review, these toxic metabolites (aflatoxins) are produced in association with foods, animal feeds and forages which may be ingested to cause health problems to humans or farm animals. They are diverse in their structure, their biosynthesis and their toxicity and the fungi (*A. flavus* group) producing them have diverse ecologies, ranging from obligate plant pathogens and endophytes to saprotrophs active in the field and post-harvest spoilage fungi active on stored commodities.

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 . Sample collection

Samples of various cereals/grains rootcrops, oilseeds and their products serving as staple foods were randomly bought from stalls in the open market places located in three different areas of Enugu and its environs of Enugu State, Nigeria, during January 1996 through March 1998 for laboratory studies.

Sampling periods corresponded to the dry harmattan cold seasons (November - February); Dry hot seasons (March - May) and the wet humid seasons (Rainy season), June - October) of the entire research period.

For the three groups of samples distributed in time and space, each sample was collected from three dealers in each of the three markets to cover a wider spectrum of the aflatoxins and microbial flora distribution. Thus a total of nine samples of each food item were collected from each of the three markets per season 3(3), making a sum total of 9(3), twenty seven samples for the three seasons of the year. For each food item therefore, 2(27), fiftyfour (54) samples were collected and analysed for the two years of research. This was also done for the sac and basin (display) samples for more chances of microbial isolation, thus, making a total of (54 x 2) 108 samples per food item for the two years of study (Table 2).

Samples were carefully collected in sterile, well labeled brown envelopes and taken back to the Department of Medical Laboratory Sciences of the University of Nigeria, Enugu Campus for studies. Where studies could be done the same day, samples were stored at 4^0C

TABLE 2
LOCAL STAPLE FOODSTUFFS ASSAYED FROM 1996 - 1998

S/N	COMMON NAMES	SPECIES, AND NUMBER SAMPLED FOR THE PERIOD	DESCRIPTION OF COMMODITY USED.
1.	MAIZE	*Zea mays* Linn	
	a. Maize (Yellow variety)	27	Maize Grains
	b. Maize (White variety)	27	Maize Grains
	c. Fermented Maize Flour (Akamu) Yellow variety.	27	Fermented Flour
2.	**Peanuts (Ground Nuts)**	*Arachis hypogea Linn.*	
	a. White testa variety	27	Shelled nuts.
	b. Red testa variety	27	
	c. Roasted (Red)	27	
3.	**ACHA GRAINS (FINDI)(HUNGARY RICE)**	*Digitaria exilis Stapf.*	
	a. Grains	27	Grains
	b. Kunnu	27	Fermented beverage.
4.	**COWPEA (BLACK-EYED BEANS)**	*Vigna unguiculata Walp.*	
	a. White variety	27	Potiskum variety
	b. Brown variety	27	Jos variety
	c. Moi-Moi	27	Wet - milled and steamed in leaves.
5.	Soy - Beans	*Glycine max Merr.* 27	Grains
6.	OIL BEAN SEED (UGBA)	*Pentaclethra macrophylla* 27	Fermented sliced boiled and wrapped in green leaves.

S/N	COMMON NAMES	SPECIES AND NUMBER SAMPLE FOR THE PERIOD	DESCRIPTION OF COMMODITY USED.
7.	MELON	*Cucumis melo*	
	a. Egusi	27	Shelled seeds.
	b. Ogiri	27	Shelled fermentaed paste product.
8.	CASSAVA	*Manihot esculenta*	
	a. Tubers	27	Peeled tubers
	b. Gari	*Manihot utilissima Pohl.* 27	Fermented fried grated cassava
9.	RICE	*Oryza sativa Linn*	Milled dehaulled Upland variety Swamp rice
	a. Ugbawka variety	27	
	b. Abakaliki variety	27	
10.	PIGEON PEA (Fio - fio) (Non-eye pea, Congo pea)	*Cajanus cajan Druce.*	White or brown Seeds (Ex. Ibadan and Ex. Trinidad).
	a. White variety	27	
	b. Yellow variety	27	
11.	SORGHUM (Guinea corn)	*Sorghum bicolor*	Cereals, Yellow and White varieties
	a. White variety	27	
	b. Yellow variety	27	
	c. Burukutu (PITO)	27	Alcoholic fermented beverage.
12.	AFRICAN (Ukwa) Bread fruit	*Treculia africana* 27	Perboiled shelled Seeds.
13.	BAMBARA PEANUTS	*Voandzia geocarpa Thouars.*	
	a. (Okpa - seed).(Stone groundnut, Madagascar pea)	27	Shelled nuts
	b. Okpa – Cake	27	Well - milled, steamed in leaves.

S/N	COMMON NAMES	SPECIES AND NUMBER SAMPLED FOR THE PERIOD	DESCRIPTION OF COMMODITY USED.
14.	**YAM**	***Dioscorea rotudata Poir.*** 27	Peeled pieces.
15.	**MILLET (JORO)** (Bulrush grain).	***Pennisetum typhoides*** (Stapf & Hubbard) 27	Cereals (Pearl millet).
16.	**PLANTAIN** a. Riped b. Unriped.	***Musa sapientum*** 27 riped 27 unriped	Riped fruits (peeled) Unriped fruits (peeled)
17.	**COCONUT SEED**	***Cocos nucifera Linn*** 27	Shelled seeds
18.	**COCO YAM (COCO INDIA).**	***Colocasia esculentus Schott*** 27	Peeled

in the refridgerator. Attempts were made to associate the products like Okpa (steamed pulverized bambara peanuts); moin-moin (steamed pulverized cowpea); Akamu; (fermented maize flour), beverages like burukutu (pito) and Kunnu (Slightly alcoholic drink and non-alcoholic drink from sorghum and acha respectively) and Ogiri (shelled fermented melon paste) with the raw foods where applicable.

Each food sample was divided into three subsamples, A, B and C as follows:-

A - for Mycological studies

B - for Bacterial studies

C - for the analysis of the natural occurrence of aflatoxins.

For some foodstuffs, different varieties were examined for possible disparity in the microbial flora and aflatoxin loads. Examples are: white and yellow varieties each of maize (corn) and sorghum, (Guinea corn); white and brown varieties of beans (cowpea); pigeon pea (fio-fio) *Cajanus cajan* and groundnuts (peanuts) with white and red testae.

3.2: Mycological Studies

3.2.1 Isolation of fungi:

Samples, (5g) each from subsample A, were soaked in 5ml of antibiotic solution, (Penicillin G. Sodium 5,000 units and Streptomycin sulphate 1mg/ml) for thirty minutes to eliminate bacterial contamination. The samples were then rinsed in sterile water before culturing on to the media listed below.

(1) Sabouraud-chloramphenicol agar medium (Difco) SAB-C, containing dextrose (40g), peptone (10g), agar (20g), distilled water (1000mls) and chloramphenicol (3ml/100ml).

The media were then sloped in tubes.

(ii) Aspergillus differential (ADM) medium of composition: -

Tryptone (1.5%), yeast extract (1.0%), Ferric citrate (0.5%), agar (1.5%), distilled water (1000ml) and tetracycline (30.0ppm). The tetracycline was added after autoclaving the rest of the media, after which the medium was poured into sterile disposable petri dishes and tubes.

(iii) Czapek-Dox agar (Difco), with the following composition: Distilled water (1000ml), $NaCo_3$ (Sodium carbonate) (3.0g), K_2HPo_4 (Potassium hydrogen phosphate) (1.0g), $MgSO_4.\ 7H_2O$ (Magnesium sulphate) (0.5g), Kcl (Potassium chloride) (0.5g), $FeSO_4.\ 7H_2O$ (Ferrous sulphate) (0.01g), Sucrose (30.0g) and agar (15.0g).

(iv) Sabouraud agar (SAB - DIFCO).

All chemicals used were obtained from Sigma Chemicals Company and were of 'Analar' grade. Media were cultured with the pre-sterilized and rinsed food samples by pressing the samples on the surfaces of the slopes using pre-sterilized inoculating wire loops and were incubated for five to seven days at room temperature (28^0C). A loopful of the liquid commodities were also streaked on to the agar media surfaces. Methods for isolation were according to Collee *et al.*(1989). Fungal colonies growing on seed surfaces or on the culture media were each isolated and transfered to fresh Czapek– Dox media incubated to get pure colonies.

3.2.2 Identification of fungal isolates

Identification of the various fungal growth was according to Raper and Fennell (1965); Haley and Callaway (1978); Kwong-Chung and Bennett (1992) and Frazier and Westerhoff (1991). The methods were entirely based on the macroscopic and microscopic

appearances. Macroscpically, they were provisionally identified from their gross colonial morphology and distinctive colonial colouration, while microscopically, it was based on the nature of the hyphae, conidiophore, columella, sterigmata and conidia through the needle mount technique.

3.2.2.1 Needle mount:

Using a sterile needle, small bits of cultures of the fungal isolates were carefully lifted from the plates and placed on a microscope slide with a drop of 95% alcohol. The preparation was then gently teased out in the alcohol with the needles. When it had been satisfactorily spread, the alcohol was then allowed to evaporate and a drop of lactophenol cotton blue stain added. A cover slip was then applied, avoiding air bubble formation. A gentle pressure was exerted with finger tips if the fungus fragments did not lie flat. The stain was then allowed to penetrate before being examined microscopically.

3.3 Bacteriological Studies

3.3.1. Isolation of bacteria

Food samples from sub sample B of about 5g amounts each, were placed in to nutrient broth, selenite-F broth and alkaline peptone water and incubated at 37^0C for 18hrs. The broth culture of each item were then subcultured on to freshly prepared plates of the following media:-

(i) Blood agar (Oxoid) for the isolation of all microorganisms

(ii) Nutrient agar (Oxoid) for the isolation of some bacteria.

(iii) Salmonella-Shigella agar (SS agar-Oxoid) for the isolation of *Salmonella* and *Shigella* species.

(iv) Mackonkey agar (Difco) for the isolation of enteric bacteria.

(v) Thoisulphate citrate Bile salt sucrose agar (TCBS - Oxoid) for the isolation of vibrios.

All plates were incubated overnight at 37^0C under aerobic conditions, then examined for microbial growth. Isolated colonies were checked microscopically for purity. After the purity of culture was assured, identification was then carried out according to Collee *et al.* (1989).

3.3.2 Identification of bacteria

Macroscopically, colonies were examined for the following characteristics: colonial morphology, colour, size, presence of haemolysis, and microscopically after Gram-staining. Motility and biochemical tests including catalase, coagulase, oxidase, Voges-Prauskaeur, Methyl red, indole and sugar fermentation tests were also performed

3.4 Assay for the natural occurrence of aflatoxins in foodstuffs

3.4.1 Direct ultraviolet (UV) viewing:

Samples of the various grains, seeds, and other foodstuffs were subjected to the bright greenish yellow fluorescence (BGYF) presumptive test under the ultravoilet (UV) light at 360nm, using the UV lamp (Shimadzu Corp. Kyoto, Japan). Fluorescence indicates the presence of aflatoxins (Food and Agriculture Organisation, 1997).

3.4.2. Preparation of Foodstuffs for Analysis

Methods used were according to Imwidthaya *et al.* (1987). Twenty grams of each food sample analysed was blended with 2.0g citric acid and 8.4ml of saturated sodium chloride solution for 1 min. in a Waring blender. Subsequently, 60ml acetone and 2g celite

were added and reblended for another two minutes and then filtered through Whatman No.40 filter paper. The filtrate was shaken with 16ml lead acetate solution, 60ml water and 5.6g ammonium sulphate for 1min and filtered. Pigments and other interferring substances were removed by this treatment.

Neutral lipids were removed by extraction of each 125ml of filtrate with 40ml petroleum ether (SIGMA) using a separatory funnel. The aqueous portion was then extracted with chloroform ($CHCl_3$) on a wrist-action shaker for about 2h. The organic phase (chloroform extracts) was evaporated to dryness under vacuum in a rotary evaporator and kept for use (Fig E) .

3.4.3. Screening the **Aspergillus flavus group of isolates for their aflatoxigenic potentials:**

Three hundred and sixty-nine (369) *Aspergillus flavus* isolates from the food samples were each subcultured in sterile rice flour liquid medium in 5ml amounts per tube according to Mishra and Daradhiyar (1991) and Sinha and Misra (1979). Each culture tube was incubated for seven days at 28^0C. Each culture filtrate (10ml) was extracted by using equal volumes of chloroform twice. The chloroform extracts were again evaporated under vacuum in a rotary evaporator and kept for use.

3.4.4 Detection of aflatoxins

The two dried chloroform extracts from 3.4.2 and 3.4.3 respectively were reconstituted in 1.5ml chloroform and filtered. Fifty µl aliquots of each extract were then applied to thin layer chromatography plates (TLC). As standards on the same plates, were spotted 50µl each of aflatoxins B_1 and G_1 (10µg/ml), B_2 and G_2 (5µg/ml). These

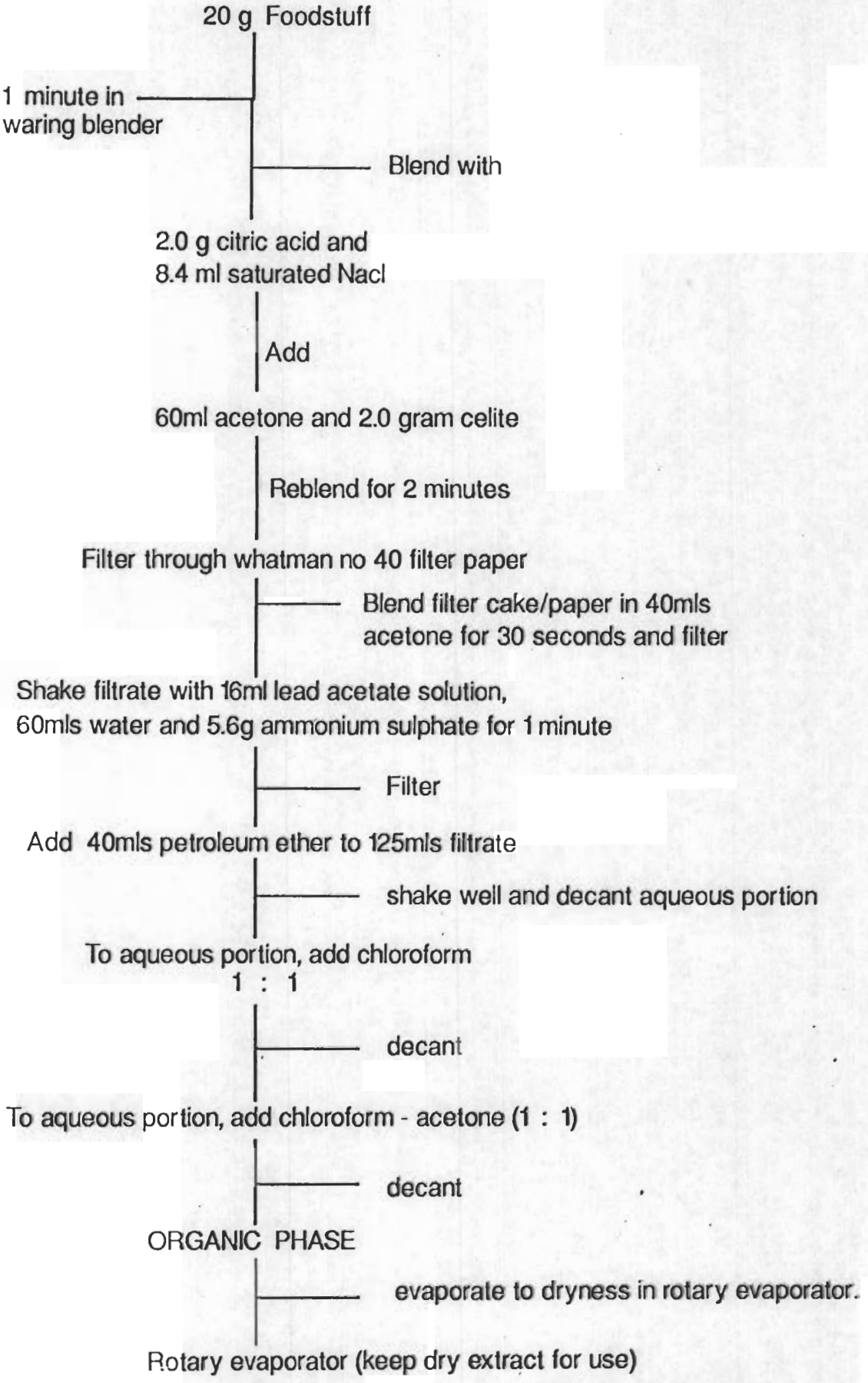
standards were obtained courtesy of Y. Ueno of the Department of Microbial Chemistry, University of Tokyo, Japan.

Thin layer chromatography plates (0.2mm thick, 20 by 20cm) consisting of aluminium sheets coated with silica gel, G60(merck) were used in the study

Prior to the application of these samples, a pencil line was drawn parallel to one edge and a suitable distance from it. A number of small crosses were marked on the line (equal distances apart) corresponding to the number of samples to be applied on the chromatoplate. Fifty microlitres of each sample were spotted on to the appropriate position with a short length carpillary tube. The spots were then allowed to dry. Drying was quickened by the use of a hair dryer or by directing a fan towards the plates.

Fifty millilitres. quantity of acetone - chloroform (4:96) was introduced in a trough of unlined tank, just enough to cover the tank with about 1cm of solvent depth. After about thirty minutes time lag, to allow equilibrium to be reached in the tank, the lid was kept firmly in place while the chromatoplate developed. Plates were removed from the tanks when the solvent fronts have reached 10cm above the origin marked carefully with a pencil. Running time was about one and half hours.

The plates were again allowed to dry and observed under UV lamp at 360nm wavelength. Aflatoxins when present, appeared as four spots or less in the order of mobility B_1, B_2 (blue), G_1 and G_2 (green). Sample spots were compared with standards. Identification was confirmed by spraying the plates with 25% sulphuric acid according to Moss (1996). The sulphuric acid was sprayed uniformly over the surface of the chromatogram using an improvised atomiser. Any aflatoxins present including the standards turned yellow.


20 g Foodstuff
1 minute in waring blender
Blend with
2.0 g citric acid and 8.4 ml saturated Nacl
Add
60ml acetone and 2.0 gram celite
Reblend for 2 minutes
Filter through whatman no 40 filter paper
Blend filter cake/paper in 40mls acetone for 30 seconds and filter
Shake filtrate with 16ml lead acetate solution, 60mls water and 5.6g ammonium sulphate for 1 minute
Filter
Add 40mls petroleum ether to 125mls filtrate
shake well and decant aqueous portion
To aqueous portion, add chloroform 1 : 1
decant
To aqueous portion, add chloroform - acetone (1 : 1)
decant
ORGANIC PHASE
evaporate to dryness in rotary evaporator.
Rotary evaporator (keep dry extract for use)

3.4.5 Quantitative estimation of the aflatoxins

Quantitative estimation of the aflatoxins detected was done following the methods of Nabeny and Nesbitt (1965). Each aflatoxin spot was located with UV lamp and circled with a pencil. The circled zone was then cut out with a pair of scissors and the aflatoxin eluted from the paper with 3ml. methanol. The process was repeated thrice. The eluates were then made up to 10ml each with methanol and the quantity of the aflatoxins determined by optical density measurement at 363nm using a Bausch and Lomb Spectrophotometer (BL Spectronic 2000). The optical density measurement at 363nm was also determined for the standard aflatoxins.

The spectrophotometer was equipped with an indication for the concentration, transmittance and absorbence factors and the results were expressed as microgram per kilograms (µg/kg) or parts per billion (ppb) or (µg/ml). Crosschecking of results was also done using the following formulae.

$$\text{Concentration of unknown} = \frac{\text{Absorbence of unknown x concentration of standard}}{\text{Absorbence of standard}}$$

3.5 EFFECT OF MICROBIAL ECOSYSTEM ON AFLATOXIN PRODUCTION

3.6 3.5.1 Microbial cultures and preparation of inoculum.

A representative of each fungal and bacterial isolate from the screened food items was included in the study. Each fungus was grown as Sabouraud agar slopes at 28^0C for ten days. Spores/cells were then harvested in sterile distilled water using a sterile inoculation loop accompanied by gentle agitation. The suspensions were shaken briefly and then filtered through four layers of sterile muslin cloth into sterile centrifuge tubes. The spores suspensions were then washed by centrifugation in sterile distilled water and the

concentrations standardized at 10^6 cells per ml using the improved Neubauer haemocytometer (Marienfeld Germany). Yeast isolates were grown on yeast extract agar slopes at 28°c for 72h, harvested in sterile distilled water, mixed well and was also standardized to 10^6 cells/ml by using counting chamber.

Each bacterial isolate used in the study was grown on nutrient agar slopes at 37^0C for three days and harvested with sterile distilled water. Number of cells was determined by diluting 1ml of the suspension with 0.1% sterilized peptone water and plating on to Nutrient agar (Difco). After 24hr incubation, the number of cells in the inoculum was estimated from the number of colonies, the volume of inoculum used and the degree to which the sample was diluted. The suspension was also standardized to 10^6 cells/ml

3.5.2 Cultural conditions for the microbial ecosystem studies

3.5.2.1 Production of aflatoxin in semi-synthetic media

Flasks containg 100ml of yeast extract (Difco); (20g), Sucrose (200g) in one litre of water, (YES) broth were sterilized and inoculated with 1ml of a suspension of conidia (10^6/ml) of each of the selected aflatoxigenic *A.flavus* isolate and an equal volume of spores of each of the moulds isolated. Similar sets of inoculations in fresh media were also done using the *A.flavus* and cells of the yeasts (*S. cerevisiae*, and *Candida* species) as well as the bacterial cells respectively. For the bacterial studies, the sucrose concentration was reduced to 100g per litre of water to enable the bacteria to grow. Methods were according to Weckbach and Marth (1977). Each trial consisted of duplicate flasks of the specified cultures and control flasks of each culture with only the *A flavus*..

Inoculated flasks were then incubated at 28^0C for a total of 14 days. After 3,5, 7 and 14 days incubation, aflatoxin concentration, amount of growth and PH of the broth

were determined. Cell concentration was estimated as earlier described. Mould growth was determined as dry weight of mycelium by filtering through Whatman No1 filter paper, washing twice with sterile water, drying at 50^0C for twenty four hours before weighing. PH was determined with the aid of a digital Ph meter (Consort) or Universal indicator paper (PH 1 - 10 Merck). Aflatoxin in 10ml of filtrate from each culture was extracted using the already described methods in earlier experiments.

3.5.2.2 Production of aflatoxin in synthetic media

Sucrose-low-salt medium of Gupta and Venkitasubramanian (1975) was used for this study. The composition was as follows: Sucrose, (85g), Asparagine (10g), $(NH_4)_2$ SO_4 (3.5g), KH_2PO_4 (10g), Mg SO_4. $6H_2O$,(2g), $CaCl_2$.$2H_2O$ (75mg), Zn SO_4. $7H_2O$ (10mg), $MnCl_2$. $4H_2O$ (5mg), Ammonium molybdate (2mg), $Na_2B_4O_7$ (2mg) and Fe SO4.$7H_2O$ (2mg) made up to 1,000ml with double distilled water and steamed sterilized at 121^0C for 15minutes. Medium was adjusted to 4.5.

Inoculations and extractions including determination of aflatoxins both qualitatively and quantitatively were as described for 3.5.2.1.

3.5.2.3 Production of aflatoxin in food substrates

The experiment was designed to investigate the potential effects of different fungal and bacterial co-cultures with aflatoxigenic *A.flavus* in the various foodstuffs on aflatoxin development and on types and quantities formed in sterilized food items. Prior to that study, all food items used in this test were certified as aflatoxin free according to the method of FAO (1997).

For each co-culture experiment, 12-20 undamaged grains, kernels or seeds of the various food items were placed on a double layer Whatman No1 filter paper in a glass

petridish. Five ml. of distilled water were added and the foods allowed to soak for 2h. in each of the dishes before autoclaving for 30min at 121^{0}C stacked in a canister.

A small wound was made in each kernel, grain, seed or food with a sterile microscapel. In one series of test plates, aflatoxigenic *A.flavus* was simultasneously inoculated on to the individual foods with each of the fungi and bacteria as the sole partner. This was accomplished by micropipetting 1 drop (0.05ml) of each pairing consisting of mixed spore/cell suspensions on to the wounded area of the food item. Equal volumes of the earlier standardized cells and spores of the bacterial and fungal isolates used in 3.5.2.1 were mixed with *A.flavus* spores of a similar volume and inoculated and incubated for fourteent days.

A second series consisted of incubation of some seeds, kernels and other food items inoculated with each of the selected fungal species and bacteria, followed by inoculation with *A.flavus* spores after incubating them for five days each. The test plates were then incubated an additional eight days. Controls consisted of food items inoculated with *A.flavus* alone in all cases. Test plates were all placed in a closed plastic container (Eleganza food flasks) to prevent moisture loss and incubated at 28^{0}C for 3,5,7 and 14 days. Food items were then transferred at the end of the incubation periods to a waring blender, item by item and treated as per the earlier experiments for the extraction and determination of aflatoxins both qualitatively

and quantitatively.

3.6 Role of metal ions in the aflatoxigenic potentials of the Aspergillus isolates

The study was designed to examine the correlation between trace metal content and aflatoxin containation of foodstuffs analysed. The trace metals studied were Zinc (Zn^{2+}), iron (Fe^{2+}), Manganese (Mn^{2+}) and Copper (Cu^{2+}) all being bivalent metals.

3.6.1. Trace metal levels in naturally contaminted foodstuffs

Foods items (Kernels, grains, seeds and other foods) from where aflatoxins were earlier detected were selected for the study. Elemental analyses of endogenous metals of the food items were carried out by flame atomic absorption as described by Garcia *et al* (1972). For the analyses, 2.0g of each food item after thorough washing with de-ionized water and blotting dry with sterile filter paper, was decomposed by wet-ashing in test tubes with 5ml nitric acid to yield a clear solution.

From this solution, the presence and quantity of the metals were then determined by atomic absorption techniques using the atomic absorption spectrophotometer (Buck model) of the Science and Training Center (STC) of the University of Nigeria, Nsukka. Calibration and sample data were derived from absorption readings as indicated on the recorder in the equipment.

Single element hollow cathode lamps at the recommended current rating were used for all determinations. A three-slot burner was used for all the elements with fuel (acetylene) and oxidizer (air) at optimum flow rates for each elememt.

Primary standard stock solutions were prepared from metals of high purity. Secondary working standard solutions were prepared from primary stock solutions

covering a concentration range for each element. De-ionized water was used for all the solutions used in the work.

Ashing was achieved by transfering 2.0g of each sample into a 50ml beaker and 5ml of concentrated nitric acid (HNO_3) added. Each beaker was then well covered with a cover glass and placed on a hot plate at low heat for about one hour and a half, (90minutes) until the solution clears. As the HNO_3 is evaporated, dropwise additions of more of the acid are made as the sample starts to char, until a white ash appeared in each beaker. The beakers were then allowed to cool and sides rinsed with the de-ionized water and the mixture was then evaporated to dryness on low heat.

Two ml. of concentrated hydrochloric acid (Hcl) was added to each beaker and warmed still being covered. The walls of the beakers were once more rinsed with de-ionized water the mixture evaporated to dryness. More concentrated Hcl was added to each beaker once more and then each mixture evaporated to dryness on low heat.

Two ml. of concentrated Hcl was again added to each beaker and warmed. Fifteen ml of the water was then added to each sample and heated for about 15min. The solutions were allowed to cool and transfered each to a 50ml volumetric flask, made up to volume with de-ionized water and stored for use. Ashing helps to oxidize all organic materials in the samples.

3.6.1.1 Atomic absorption techniques

The aqueous standard solutions earlier prepared were each aspirated. De-ionized water was also aspirated alternatively between standards of various concentrations and also between samples to compensate for noise signal and instrument zeroing. A composite standard calibration curve was prepared from each series of single element standard

solutions aspirated before and after the aspiration of samples at the wavelengths indicated for each metal.

Absorption readings were converted to absorbence values for linear presentation. Concentrations of elements analysed were read off from the standard curve of concentrations against absorbence.

3.6.2 **Aflatoxin production in bivalent metal enriched media**

3.6.2.1. Studies with food substrates

To each gram sample of the food substrates in 50ml Erlenmeyer flask, was added 1ml of the appropriate solution of a specific trace element. Aqueous solutions of the following compounds were used as trace element additives; $ZnSO_4$. $7H_2O$; $CuSO_4.5H_2O$, $FeCl_3$ $MnSO_4.H_2O$ in graded amounts. (0-1000μg/g). Methods were in accordance with Lillehoj *et al.*(1974).

Test flasks were then autoclaved for 20 minutes at 121^{0}C and the sterile substrates inoculated with 0.5ml of spore suspension (10^6/ml) of the aflatoxigenic *A.flavus*. Flasks were then incubated at 25^{0}C for 1-14 days and 10ml amount of each culture filtrate was extracted and aflatoxins detected qualitatively and quantitatively according to methods used in earlier experiments. Experiments were performed in duplicates and their means calculated.

3.6.2.2 **Studies with synthetic media**

A basal medium as described in Marsh, *et al.*(1975) was used. The components of the basal medium were glycerol (20g/L); NH_4NO_3 (0.7g/L); KH_2 PO_4 (0.9g/L); K_2HPO_4 (0.7g/L); and $MgSO_4$. $7H_2O$ (0.75g/L). Aqueous solutions of the trace elements additive

were used in the experiment in graded amounts (0-25μg/ml) after being adjusted to PH5.2. Twenty five milliliters of medium was then dispensed into serveral 250ml flasks. The flasks were stoppered with cotton plugs and autoclaved for 15min at 121^{0}C. After cooling, they were needle inoculated with spores of the aflatoxigenic *A.flavus*, 1ml (10^6/ml) and incubated at 28^{0}C for periods ranging between 1-14days. After incubation, the pH of the medium, the fungal mycelial dry weight and aflatoxin levels were determined as earlier described

3.6.2.3. Studies with semi-synthetic media

Two percent yeast extract sucrose media (YES) was prepared according to Weckbach and Marth (1977). The medium composition was as follows: Yeast extract (Difco) (20g), Sucrose (200g), distilled water (1L) and pH. 6.4. Twenty five millilitres of the medium was dispensed into each 250ml flasks and autoclaved and similar experiments and determinations conducted as in 3.6.2.1.

Af levels are reported as the mean of the total aflatoxins of triplicate studies in the various experiments.

CHAPTER FOUR

RESULTS

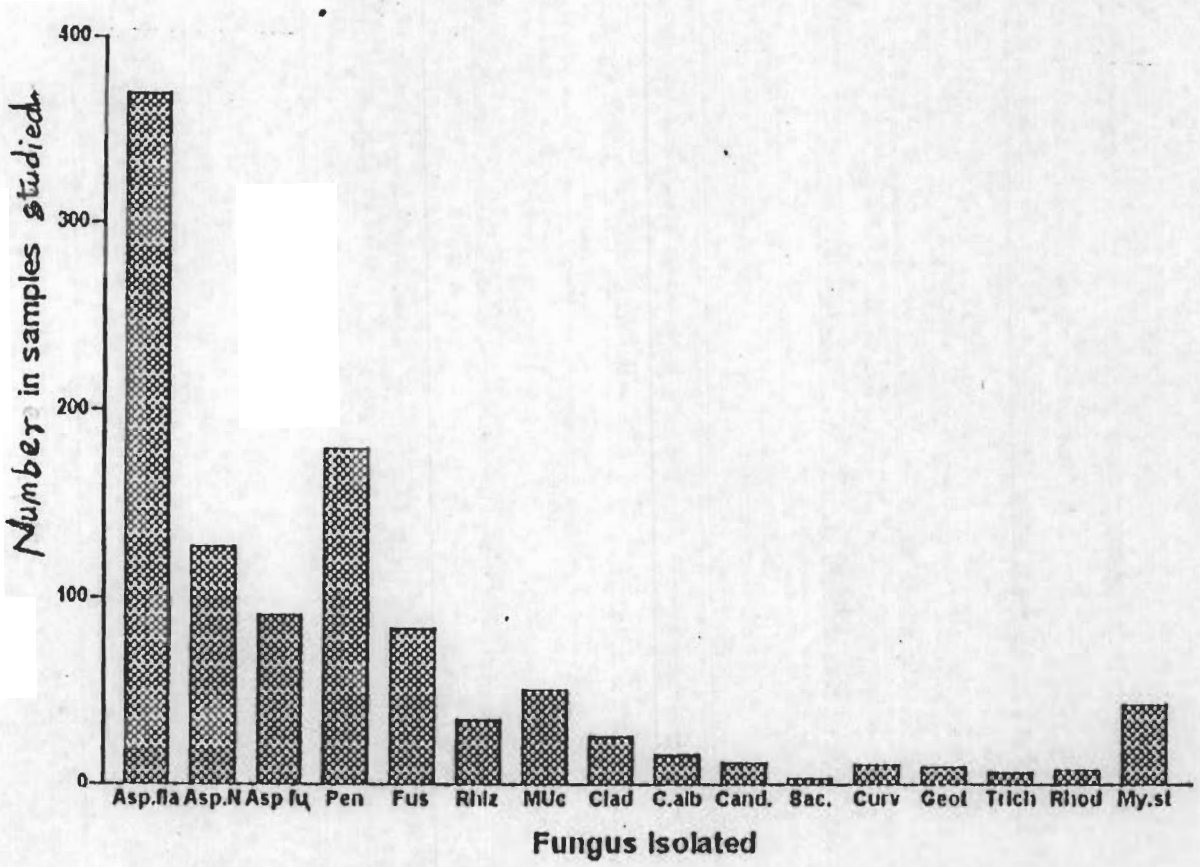
4.1 ISOLATION OF MICROORGANISMS FROM FOODSTUFFS.

4.1.1 Fungi:

The overall incidence of fungal organisms isolated from samples of the 18 different varieties of food stuffs analysed for the two year period is presented in Fig.I.

Of the fifteen identifiable fungal groups encountered, *Aspergillus flavus* group showed the highest incidence (34.8%), followed by *Penicillium* species (16.8%), *A.niger* (12.0%), *A.fumigatus* (8.6%), *Fusarium* spp. (7.8%), *Mucor* spp. (4.7%), Rhizopus *nigricans* (3.2%) *Cladosporium* spp. (2.4%), *Candida albicans (1.5%), Candida* spp. (1.04%), *Curvularia* spp. (0.94%), *Geotrichum* spp. (0.84%), *Rhodotorula* spp. (0.7%), *Trichoderma viride* (0.6%), *Saccharomyces cerevisiae* (0.3%) and *Mycelia sterilia* (4.10%).

Table 3 shows the number of food items and products contaminated by fungi. Maize was the most commonly contaminated food item with an overall contamination rate of 25%, followed by peanuts (22.4%), cowpea and rice (6.1%) each; while the least commonly contaminated foods were Pigeon pea (0.9%) and coconut (0.5%).



Asp.fla=Aspergillus flavus group
Asp.N=Aspergillus niger
Asp fum=Aspergillus fumigatus
Pen=Penicillium species
Fus=Fusarium species
Rhiz=Rhizopus nigricans
Muc=Mucor mucedo
Clad=Cladosporium species

C. alb=Candida albicans
Cand=Candida species
Sac.=Saccharomyces cerevisiae
Curv.=Curvularia species
Geot.=Geotrichum species
Trich.=Trichoderma viride
Rhod.=Rhodotorula species
Myst.=Mycelia sterilia

Fig. 1: Overall incedence of mycoflora in foodstuffs analysed in Enugu during 1996-1998.

TABLE 3
NO OF FOOD ITEMS AND THEIR PRODUCTS CONTAMINATED BY FUNGI ACCORDING T'O SEASONS IN THE SAMPLING PERIOD.

	FOOD COMMODITY	SEASONS SAMPLED, n = 1188			
		HOT DRY SEASONS	RAINY SEASONS	COLD HARMATTAN SEASONS	TOTAL %
1.	Maize	70	119	73	262 (25.0)
2.	Peanuts	72	87	75	234 (22.4)
3.	Acha	8	27	27	62 (5.9)
4.	Cowpeas	29	16	19	64 (6.1)
5.	Soyabeans	8	20	9	37 (3.5)
6.	Oil beans	18	10	5	33 (3.2)
7.	Melon	20	24	16	60 (5.7)
8.	Cassava	4	13	6	23 (2.2)
9.	Rice	11	41	12	64 (6.1)
10.	Pigeon pea	3	5	1	9 (0.9)
11.	Sorghum	28	21	12	61 (5.8)
12.	Breadfruit	8	15	2	25 (2.4)
13.	Bambara Peanuts	4	6	0	10 (1.0)
14.	Yam	8	13	6	27(2.6)
15.	Millet	9	19	6	34 (3.3)
16.	Plantains	6	8	7	21 ((2.01))
17.	Coconut	0	5	0	5 ((0.5))
18.	Cocoyam	1	10	4	15 ((1.4))
	TOTAL	307 ((29.3))	459 ((43.9))	280 ((26.8))	1044 ((29.3))

The variety-wise distribution of mould contaminated food items shows that white maize (49.6%) was more contaminated than the yellow variety (43.5%). Peanuts of the white variety (68.8%) was significantly more contaminated by moulds than those with red testae (23.1%), ($P<0.05$). White cowpeas (54.7%) were also more contaminated by fungal organisms than their brown counterparts (37.5%).

The Abakaliki (swamp) rice variety (57.8%) was significantly more contaminated by moulds when compared with the Ugbawka (upland) rice (42.2%) ($P<0.05$). Brown pigeon peas (88.9%) were also significantly ($P<0.05$) more contaminated than the white variety (11.1%). While ripe plantain (66.7%) clearly doubled the unripe variety (33.3%) in terms of fungal contamination, there was no difference in this regards between the white and yellow sorghum analyzed (Fig.2).

4.1.2 The Bacteria

The bacterial flora encountered in the 18 different foodstuffs are presented in Table 4.

The most predominant bacterial species out of the 231 bacterial isolates encountered were *Bacillus subtilis*, (14.7%), *Streptococcus faecalis* (14.3%), *Escherichia coli* (13.9%), *Pseudomonas aeruginosa* (10.8%), *Proteus* spp (10.0%) and *Staphylococcus aureus* (9.5%) while the least encountered was *Serratia* spp (0.9%).

Figure 3 shows the number of food items and their products contaminated by bacteria. Acha (22%) was the most commonly contaminated foodstuff, followed by oil bean (ugba) (19.6%), maize and melon (10.3%) each, while the least level of contamination (0.5% each), was noted in soybeans, pigeon pea and millet.

The varietal analysis of the bacterial contamination of foods are shown in Fig.4.

Fig. 2 :Varieties of fungi contaminated foodstuffs analyzed

TABLE 4
GENERAL INCIDENCE OF BACTERIAL FLORA ENCOUNTERED IN THE STUDY

	INCIDENCE (% IN FOOD SAMPLES)			
BACTERIA ISOLATED	**HOT DRY SEASONS**	**RAINY SEASONS**	**COLD HARMATTAN SEASONS**	**OVERALL TOTAL n =3564**
Bacillus subtilis	21(18.9)	3. (4.6)	10 (18.2)	34 (14.7)
Bacillus cereus	7. (6.3)	3 (4.6)	2 (3.6)	12 (5.2)
Pseudomonas aeruginosa	12 (10.8)	6 (9.2)	7 (12.7)	25 (10.8)
Lactobacillus spp.	8 (7.2)	3 (4.6)	5 (9.1)	16(6.9)
Staphylococcus aureus	9 (8.1)	7 (10.8)	6 (10.9)	22 (9.5)
Streptococcus faecalis	17 (15.3)	8 (12.3)	8 (14.5)	33 (14.3)
Acetobacter aceti	3 (2.7)	5 (7.8)	-	8 (3.5)
Streptococcus lactis	6 (5.4)	8 (12.3)	2 (3.6)	16 (6.9)
Salmonella typhimurium	3 (2.7)	3 (4.6)	2 (3.6)	8 (3.5)
Serratia spp.	1 (0.9)	-	1 (1.8)	2 (0.9)
Proteus spp.	9 (8.1)	10 (15.4)	4 (7.3)	23 (10.0)
Escherichia coli	15 (13.5)	9 (13.8)	8 (14.5)	32 (13.9)
TOTAL	111 (48.1)	65 (28.1)	55 (23.8)	231 (6.48)

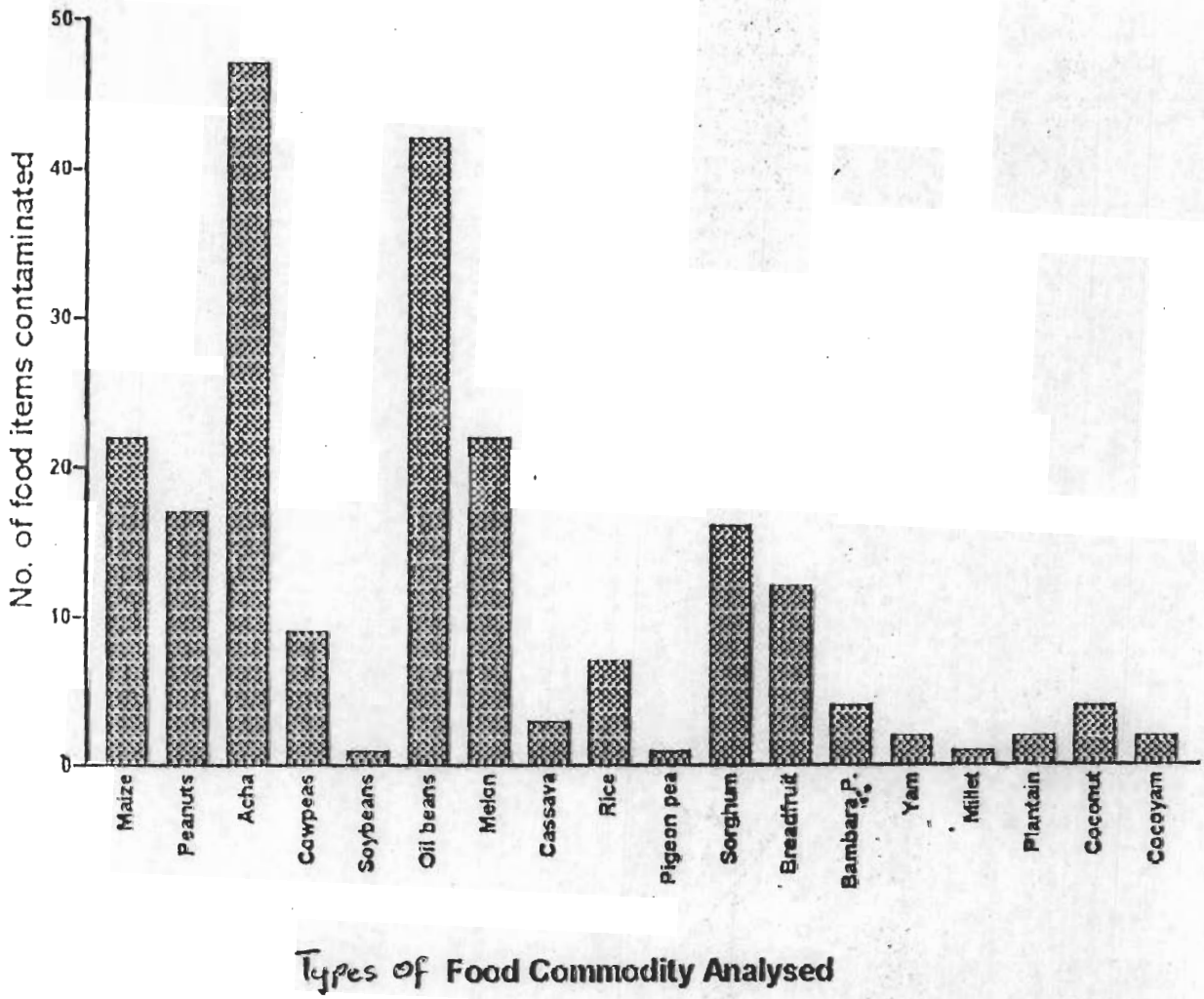


Fig 3: Number of food items and their products contaminated by bacteria

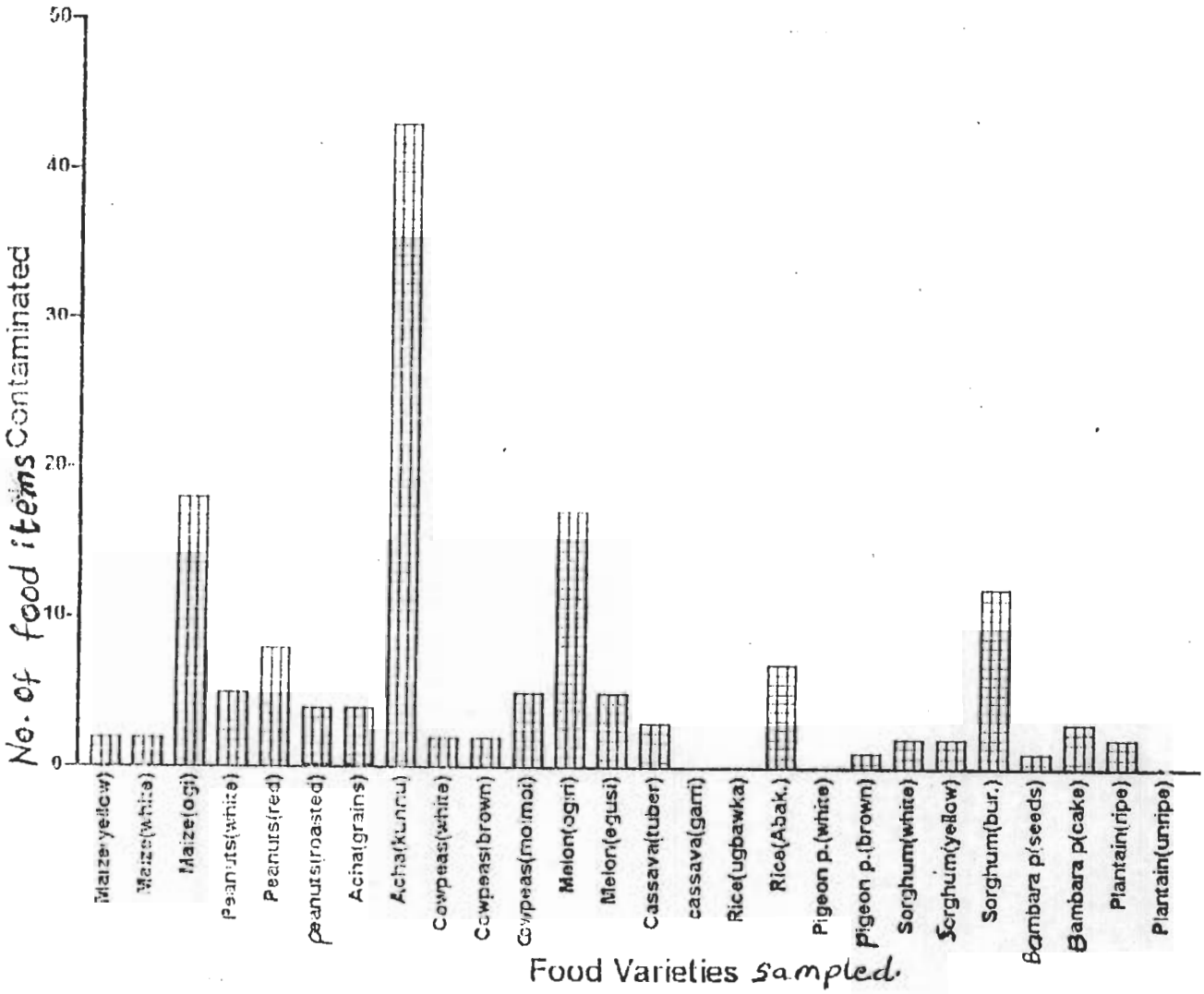


Fig. 4: Varieties of bacteria contaminated foodstuffs analyzed

KEY

Pigeon P - Pigeon Pea.
Bamb ara p.- Bambara Peanut.
Rice Abak- Rice Abakaliki.
Sorghum(bur)- Sorghum burukut

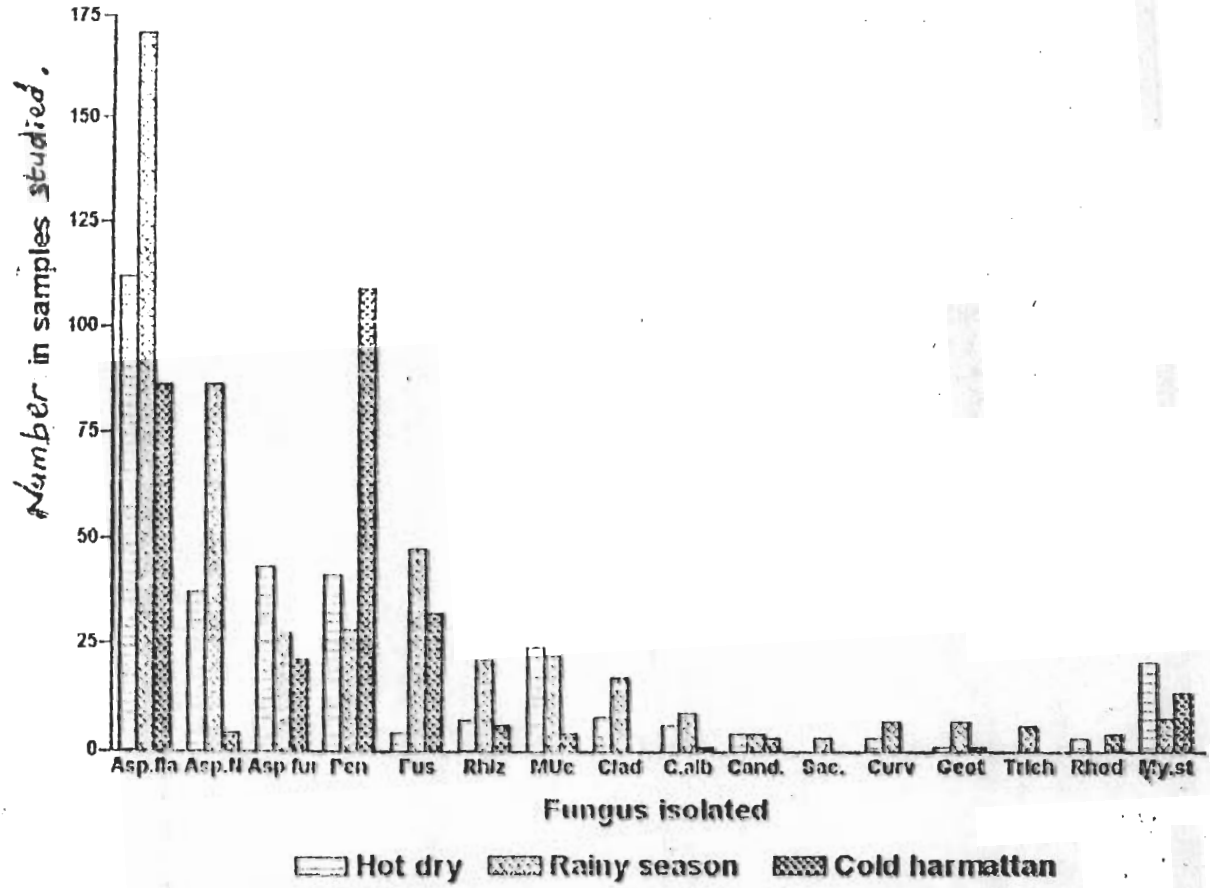
Outside plantain and rice that recorded bacterial contamination only in the ripe samples, and Abakaliki varieties, there was no actual difference in the distribution of these organisms in sorghum, cowpeas, peanuts and maize in the different varieties.

4.2 Seasonal distribution of isolates during the study period

The mould flora isolated from samples of the 18 different varieties of foodstuff taken during the hot dry season (March - May); the rainy seasons (June-October) and the cold harmattan seasons (November-February) over a two-year period are presented in Fig. 5.

Of the fifteen mould groups isolated, *A.flavus* group showed the highest incidence during the rainy seasons, making up 36.9% of all fungal isolates encountered during that season and being isolated in 14.4% of the 1188 samples in that season. The incidence of this group of fungi (9.45%) declined considerably in samples taken in the hot dry seasons, and in the cold harmattan seasons (7.25%). In the hot dry season, *A.flavus* accounted for 35.7% of all fungal isolates in that season and for 30.2% of those of the cold harmattan seasons.

Other fungal agents with preponderance during the rainy season include *Aspergillus niger* which accounted for 18.6% of all fungal isolates, 11.8% of the hot dry season, and 1.4% of the cold harmattan isolates. It was isolated in 7.25%, 3.11% and 0.32% of the 1188 samples of foods analysed during the rainy, hot dry season and cold harmattan seasons each respectively. *Fusarium* spp were also encountered during the rainy season having been isolated in 3.95% of the samples analysed in that season, but only in 0.32% and 2.70% of samples during the hot dry seasons and cold harmattan seasons respectively.



Asp.fla=Aspergillus flavus group
Asp.N=Aspergillus niger
Asp fum=Aspergillus fumigatus
Pen=Penicillium species
Fus=Fusarium species
Rhiz=Rhizopus nigricans
Muc=Mucor mucedo
Clad=Cladosporium species

C. alb=Candida albicans
Cand=Candida species
Sac.=Saccharomyces cerevisiae
Curv.=Curvularia species
Geot.=Geotrichum species
Trich.=Trichoderma viride
Rhod.=Rhodotorula species
Myst.=Mycelia sterilia

Fig. 5: General incidence of mycoflora in foodstuffs analysed in Enugu during 1996-1998

Similar seasonality patterns were observed for *Candida albicans, Saccharomyces cerevisiae* and *Trichoderma* spp. (which did not occur in other seasons at all), *Curvularia* spp., *Geotrichum* spp., *Cladosporium* spp. and *Rhizopus* spp.

Aspergillus fumigatus, however, showed a preponderance for the hot dry seasons of the research period, being isolated in 3.62%, 2.25% and 1.75% of the samples analysed in the hot dry, rainy and cold harmattan seasons each respectively. A similar pattern was also observed for *Mucor mucedo* and most of the unidentified fungi (*Mycelia sterilia*) were encountered in the hot dry seasons. For the cold harmattan seasons, only *Penicillium* spp. and *Rhodotorula* spp. showed pre-eminence when compared with other seasons. *Penicillium* spp. showed an incidence of 9.20% in all the samples analysed in that season and only3.45% and 2.33% in the hot dry and rainy seasons respectively.

Generally, however, the results show a significant difference in seasonality in the general incidence of mycoflora ($P<0.05$). Fungal isolations were made in 39% of all samples (1188) during the rainy seasons, 26.5% during the hot dry seasons and 24% during the cold harmattan period. Out of all the fungi isolated in the study (1062), the rainy seasons again accounted for43.6%, the hot dry seasons29.6% and the cold harmattan seasons 26.8%.

Figure 6 summarizes the seasonality pattern for bacterial distribution in the study. In terms of the general incidence, the results show no significant difference in seasonality ($P>0.05$). However, 48.1% of all the bacterial isolates (231) in the study was recorded in the hot dry seasons, while for the rainy seasons, 28.1% was observed and 23.8% for the cold harmattan period. *Bacillus subtilis* was isolated from 1.78% of all the samples analysed in the hot dry seasons with 0.25% and 0.84% in the rainy seasons and cold harmattan periods

harmattan periods respectively. Apart from *Streptococcus lactis*, *Acetobacter aceti* and *Proteus* spp, all other bacterial organisms showed a preponderance for the hot dry seasons. *Salmonella* spp and *Serratia* spp did not show marked seasonality differences.

4.3 Natural aflatoxin contamination of foodstuffs.

Figure 7 shows the mean aflatoxin levels encountered in food naturally contaminated with aflatoxigenic strains of *A.flavus*. Coconut seeds gave the highest level (2,732.1 ppb), followed by white testa peanuts (2,204.5 ppb), roasted red testa peanuts (1,817.7 ppb), melon seeds (1,176.4 ppb), red testa peanuts (808.6 ppb), white sorghum (416.4 ppb), yellow sorghum (297.2 ppb), millet (296.7 ppb) and Abakaliki rice (282.5 ppb). Comparatively low levels were found in acha grains (21.5 ppb), unripe plantain (11.2 ppb) and soybean (10.3 ppb).

Some food samples in which aflatoxins were detected were either free of *A.flavus* or contained non-toxigenic strains.

Table 5 summarizes the distribution and concentration of the various aflatoxins according to food items. Maize and its products had a total of 28 samples (out of 324) contaminated with aflatoxins; 85.7% of this figure contained aflatoxigenic strains of *A.flavus* while the remaining 14.3% contained either non-toxigenic *A.flavus* or none at all. Two samples,however,that contained toxigenic *A.flavus* contained no aflatoxins.

Peanuts (43 samples out of 324) were naturally contaminated by aflatoxins; 83.8% contained aflatoxigenic *A.flavus* while 16.3% contained non-toxigenic *A. flavus* strains or none at all.

Breadfruits (8 samples out of 108) were naturally contaminated by aflatoxins with 75% habouring aflatoxigenic *A.flavus* while 25% contained none. Melon (11 samples out

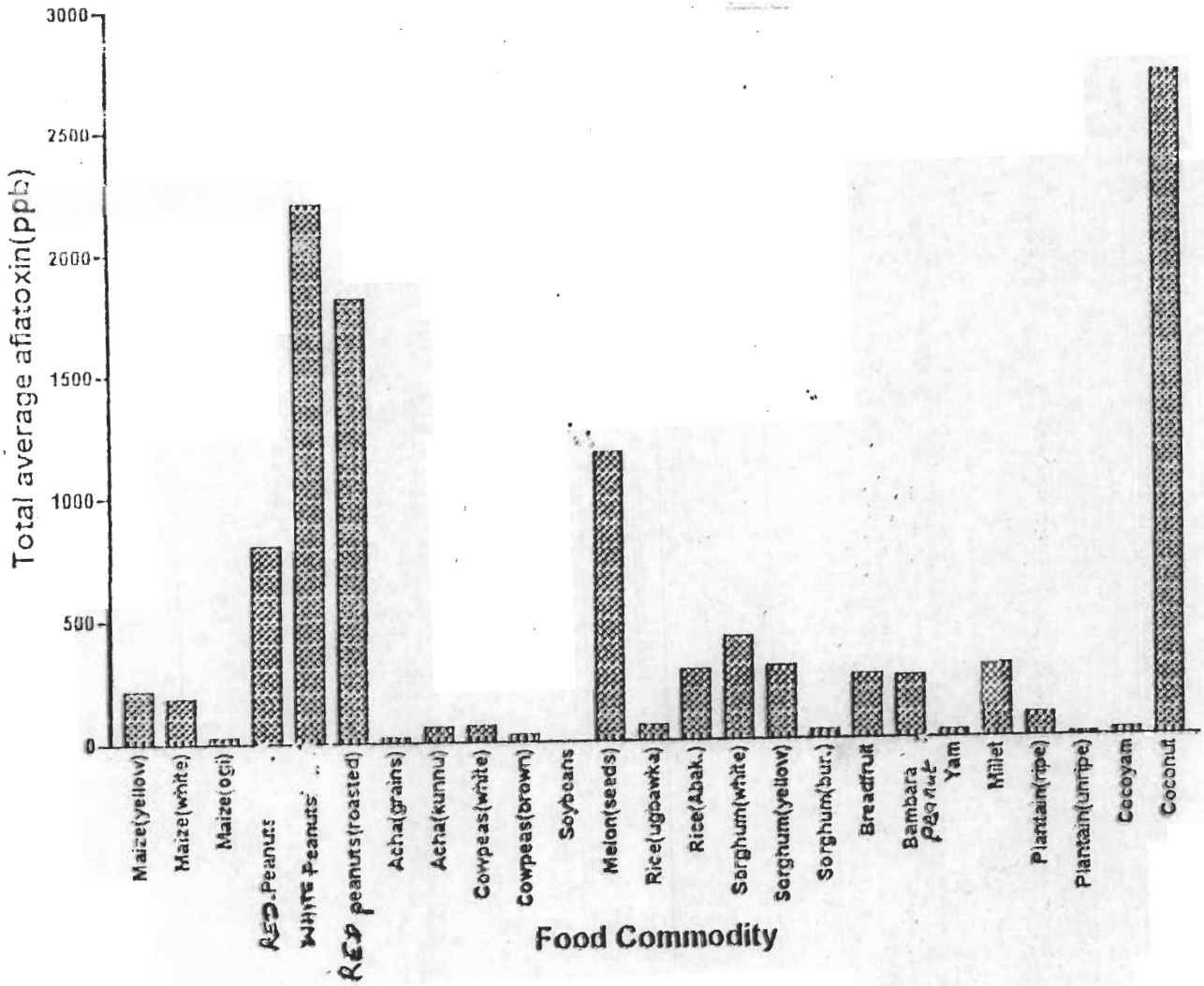


Fig. 7: Natural Average aflatoxins levels encountered in samples with aflatoxigenic strains of *A. flavus*.

TABLE 5

Distribution and concentration of Aflatoxins according to Food items and types.

Aflatoxin content and type		No. Of Samples contaminated (% of contaminated sample)	
1)	Maize and Maize prodacts combination	(n = 28) No. With aflatoxigenic A. flavus	Non aflatoxigenic or No. A. flavus
	B1 only -	5 (17.8)	3 (10.7)
	B1 + B2	17(60.7)	1 (3.57)
	B1 + B2 + G1	2 (7.2)	
	B1 + B2 + G1 + G2	0 (0)	
	B1 + G1	0 (0)	
	B1 + G2	0 (0)	
	ND (2)		
		24 (85.7%)	4 (14. 33)
	B1 range (ppb)	Avg. amount of AFB1	Avg. amount of AFB1
	< 20 0	-	3 (15.3)
	21 - 50 3	43.3	1 (32)
	51 - 100 7	63.9	-
	101 - 200 3	153.2	-
	201 - 500 11	261.1	-
	501 - 1000 ND	-	-
	1001 - 2000 ND	-	-
	> 2000 ND	-	-
2)	Peanuts	n = 43	
	B1 only	1 (0.32)	-
	B1 + B2	0 (0)	-
	B1 + B2 + G1	16 (37.2)	3(7)
	B1 + B2 + G1 + G2	13 (30.2)	2(4·7)
	B1 + G1	6 (14)	2 (4·7)
	B1 + G2	0 (0)	-
	ND (0)	36 (83. 8)	7 (16.3)
	B1 range (ppb)	Avg. amount of AFB1	Avg. amount of AFB1
	20 0	-	-
	21 - 50 0	-	-
	51 - 100 0	-	2 (80·9)
	101- 200 4	147.3	3 (120·7)
	201- 500 5	317.7	-
	501- 1000 11	790.4	-
	1001 - 2000 16	1334.5	2 (1376.1)
	> 2000 ND	-	-
3)	BREADFRUIT	(n = 8)	
	B1 Only	0	0
	B1 + B2	0	0
	B1 + B2 + G1	0	0
	B1 + B2 + G1 + G2	0	0
	B1 + G1	0	0
	B1 + G2	6 (75)	2 (25)
	ND	0	
		6 (75%)	2 (25%)

B1 range (ppb)		Avg. amount of AFB1			
< 20		0		0	
21 - 50		0		0	
51 - 100		0		0	
101 - 200		6	146.6	0	
201 - 500		0		2	243.8
501 - 1000		0		0	
1001- 2000		0		0	
> 2000		0		0	

4)	MELON	(n = 11)			
	B1 only	0			
	B1 + B2	2	18.2		
	B1 + B2 + G1	0		0	
	B1 + B2 +G1 +G2	4	36.4	4	36.4
	B1 + G1	1	9	0	
	B1 + G2	0		0	
	ND (1)				
			7 (63.6)	4 (36.4)	

B1 range (ppb)		Avg. amount of AFB1	Avg. amount of AFB1	
< 20	-	-	-	-
21 - 50	-	-	-	-
51 - 100	-	-	-	-
101 - 200	-	-	2	113.4
201 - 500	2	205	2	378.1
501 - 1000	-	-	-	-
1001 - 2000	5	1377.6	-	-
> 2000	-	-	-	-

5)	COWPEAS		n = 7		
	B1 only	3 (42.9)		1 (14.3)*	
	B1 + B2	0		0	
	B1 + B2 + G1	1 (14.3)		0	
	B1 + B2 +G1 +G2	0		0	
	B1 + G1	2 (28.6)		0	
	B1 + G2	0		0	
	ND (0)				
			6 (85.7)	1 (14.3). *Moi-moi- (White-variety)	

B1 range (ppb)		Avg. amount of AFB1	Avg. amount of AFB1	
< 20	-	-	1	16.9
21 - 50	6	36.9	-	-
51 - 100	-	-	-	-
101 - 200	-	-	-	-
201 - 500	-	-	-	-
501 - 1000	-	-	-	-
1001 - 2000	-	-	-	-
> 2000	-	-	-	-

B1 only	0		3 (75)
B1 + B2	1	(25)	-
B1 + B2 + G1	0		-
B1 + B2 +G1 +G2	0		-
B1 + G1	0		-
B1 + G2	0		-
ND (0)			
	1 (25)		3 (75)
B1 range (ppb)	Avg. amount of AFB1		Avg. amount of AFB1
- 20			
21 - 50			1 39.2
51 - 100			1 98.1
101 - 200	1	157.5	1 117.4
201 - 500			
501 - 1000			
1001 - 2000			
+ 2000			

7)	SORGHUM	n = 12	
B1 only	2 (16.7)	1 (8.3)	
B1 + B2	7 (58.3)	1 (8.3)	
B1 + B2 + G1	1 (8.3)	-	
B1 + B2 +G1 +G2	0	-	
B1 + G1	0	-	
B1 + G2	0	-	
ND (0)	-		
	10 (83.3)	2 (16.7)	
B1 range (ppb)	Avg. amount of AFB1	Avg. amount of AFB1	
- 20	1 17.7	- -	
21 - 50	ND -	2 36.6	
51 - 100	ND -	- -	
101 - 200	1 140.9	- -	
201 - 500	8 261.9	- -	
501 - 1000	ND -	- -	
1001 - 2000	ND -	- -	
+ 2000	ND -	- -	

8)	RICE	n = 9	
B1 only	0	-	
B1 + B2	9 (100)	-	
B1 + B2 + G1	0	-	
B1 + B2 +G1 +G2	0	-	
B1 + G1	0	-	
B1 + G2	0	-	
ND (2)	-	-	
	9 (100)		
B1 range (ppb)	Avg. amount of AFB1	Avg. amount of AFB1	
- 20	- -	-	
21 - 50	2 24.2	-	
51 - 100	1 65.7	-	
101 - 200	2 125.7	-	
201 - 500	4 323.2	-	
501 - 1000	- -	-	
1001 - 2000	- -	-	
+ 2000	- -	-	

9)	ACHA AND KUNNU		n = 7		
	B1 only	4	57.1	-	
	B1 + B2	1	14.3	2	28.6
	B1 + B2 + G1	0		0	
	B1 + B2 +G1 +G2	0		0	
	B1 + G1	0		0	
	B1 + G2	0		0	
	ND (2)	-		-	
		5	(71.4)	2 (28.6)	
	B1 range (ppb)	Avg. amount of AFB1		Avg. amount of AFB1	
	20	2	16.8	2	13.3
	21 - 50	3	32.9	-	-
	51 - 100	-	-	-	-
	101 - 200	-	-	-	-
	201 - 500	-	-	-	-
	501 - 1000	-	-	-	-
	1001 - 2000	-	-	-	-
	> 2000	-	-	-	-
10)	COCONUT		n = 4		
	B1 only	0		2 (50)	
	B1 + B2	0		1 (25)	
	B1 + B2 + G1	1	(25)		
	B1 + B2 +G1 +G2	0			
	B1 + G1	0			
	B1 + G2	0			
	ND (0)	0			
		1	(25)	3 (75)	
	B1 range (ppb)	Avg. amount of AFB1		Avg. amount of AFB1	
	20	ND		-	-
	21 - 50	ND		-	-
	51 - 100	ND		-	-
	101 - 200	ND		-	-
	201 - 500	ND		-	-
	501 - 1000	ND		3	701.4
	1001 - 2000	1	1.605	-	-
	> 2000	ND			
11)	CASSAVA		n = 2		
	B1 only	0		-	
	B1 + B2	0		-	
	B1 + B2 + G1	0		-	
	B1 + B2 +G1 +G2	0		-	
	B1 + G1	0		2(100)	
	B1 + G2	0		-	
	ND (0)	0		-	
		0		2 (100)	
	B1 range (ppb)	Avg. amount of AFB1		Avg. amount of AFB1	
	20	ND		2	8.3
	21 - 50	ND		-	-
	51 - 100	ND		-	-
	101 - 200	ND		-	-
	201 - 500	ND		-	-
	501 - 1000	ND		-	-
	1001 - 2000	ND		-	-
	> 2000	ND		-	-

of 216) were found to be naturally contaminated with aflatoxins, 63.6% harboured the toxigenic strains of *A.flavus* while 36.4% had none. For cowpeas, out of the 7 samples (out of 324) naturally contaminated by aflatoxins, only one sample (moin-moin) which is a milled and steamed cowpea product contained no *A.flavus* at all. An almost similar trend was observed for some other foodstuffs, though some like rice (11 samples out of 216) were found to harbour aflatoxigenic strains of *A.flavus* from which aflatoxins were detected in all but 2 samples and no sample without *A.flavus* at all, or with non-toxigenic strain were found to contain aflatoxins.

For maize, out of the 28 samples harbouring toxigenic *A.flavus* strains, 17.8% contained aflatoxin B_1, 60.7% contained $B_1 + B_2$ while 7.2% contained $B_1 + B_2 + G_1$. All the millet samples contained either AFB_1 or $AFB_1 + AFB_2$ only. All the rice and acha samples contained $AFB_1 + AFB_2$. For sorghum, out of the 12 samples containing aflatoxin, 11 had aflatoxin $B_1 + B_2$ while only one sample had the AFG_1 toxin in addition.

Most peanut, melon, cowpea, cassava, cocoyam and yam samples contained the B and AFG_1 and or AFG_2 toxins while all the eight samples of breadfruit contained B_1 and G_2.

4.3.1 Natural aflatoxin load according to food class

In the whole results, both for those samples containing aflatoxigenic *A.flavus* strains and those containing non-toxigenic strains or none at all from which natural aflatoxins were detected, the mean aflatoxin B_1 (AFB_1) load is presented in Figs. 8-11 according to the class of foods.

Figure 8 shows is the breakdown for cereal/cereal products. White sorghum yielded the highest value with a mean AFB_1 of 221ppb, millet (203.5ppb), yellow sorghum (200.4ppb), Abakaliki rice (193ppb), yellow and white maize (155ppb and 114.8ppb) respectively.

Figure 9 represents the mean AFB_1 values in protein foods. Breadfruits ranked highest with a mean AFB_1 of 170.9ppb, bambara peanuts (124.3ppb) and cowpea (white variety and brown variety) with mean AFB_1 values of 39.7ppb and 31.2ppb respectively.

No aflatoxin was detected in oil bean seed (ugba) while soybean was the least with only a mean AFB_1 value of 9.4ppb.

Figure 10 represents the mean AFB_1 values in lipid foods. The highest value was recorded for roasted red peanuts(1,388.3ppb), coconut seed (927.3ppb), white testa peanuts (876ppb), melon (690.8ppb) and red testa peanuts (510.5ppb).

Figure 11 shows the mean AFB_1 values in carbohydrate foods. Ripe plantain had the highest level of mean AFB_1 in this group with a value of 95.3ppb, yam (21ppb), cocoyam (15.2ppb), cassava (8.3ppb), unripe plantain (6.6ppb) while gari had none at all.

Generally, the lipid foods ranked highest among all food classes in terms of aflatoxin levels. For the 43 peanut samples found to be naturally contaminated with aflatoxins, 67.4% had an average AFB_1 content of >500ppb, 27.9%, (>100ppb) and 4.7% contained less than 100ppb (80.9ppb). Of the 11 samples of melon seeds found to be naturally contaminated with aflatoxins, 9(81.8%) contained aflatoxin B_1 average value of >200ppb, out of which 5(55.6%) were found to contain AFB_1 mean value of >1000ppb. The remaining 2 samples contained AFB_1 mean of >1000ppb. Similarly, of the 4 samples of coconut, with natural aflatoxin contamination, all contained mean AFB_1 of >500ppb. Carbohydrate foods seemed to be poor substrates for aflatoxin production. Only one

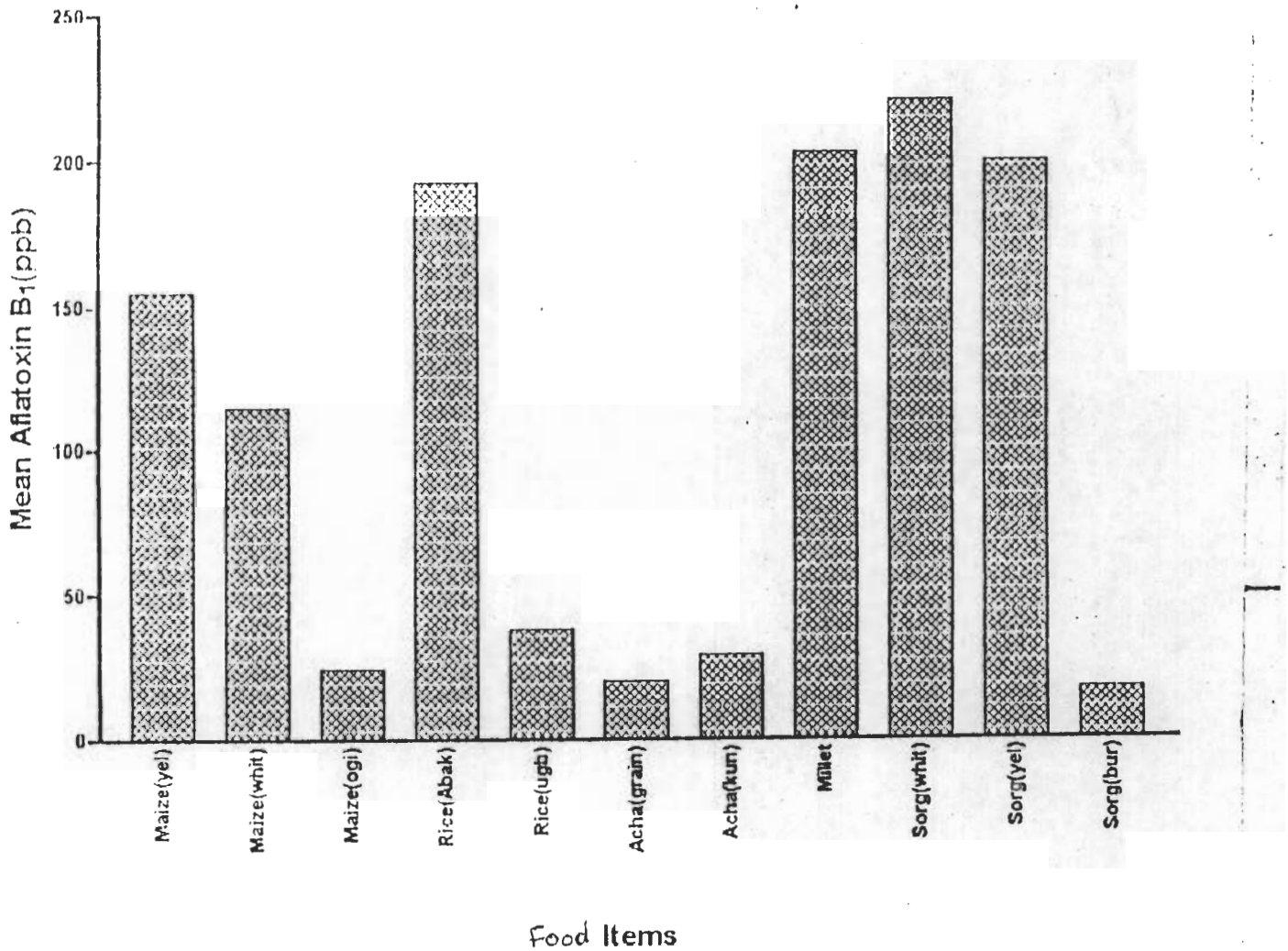


Fig. 8 : Screening of foods for aflatoxin B_1 content according to class of food (Cereals).

Maize (yellow) - (yel)
Maize (white) - (whit)
Rice (Abakaliki) (Abak)
Rice (ugbawka) - (ugb)
Acha (kunnu) - (kun)
Sorghum (white) - (sorg Whit)
Sorghum(yellow) - (sorg yel)
Sorghum (burukutu) - (sorg bu)

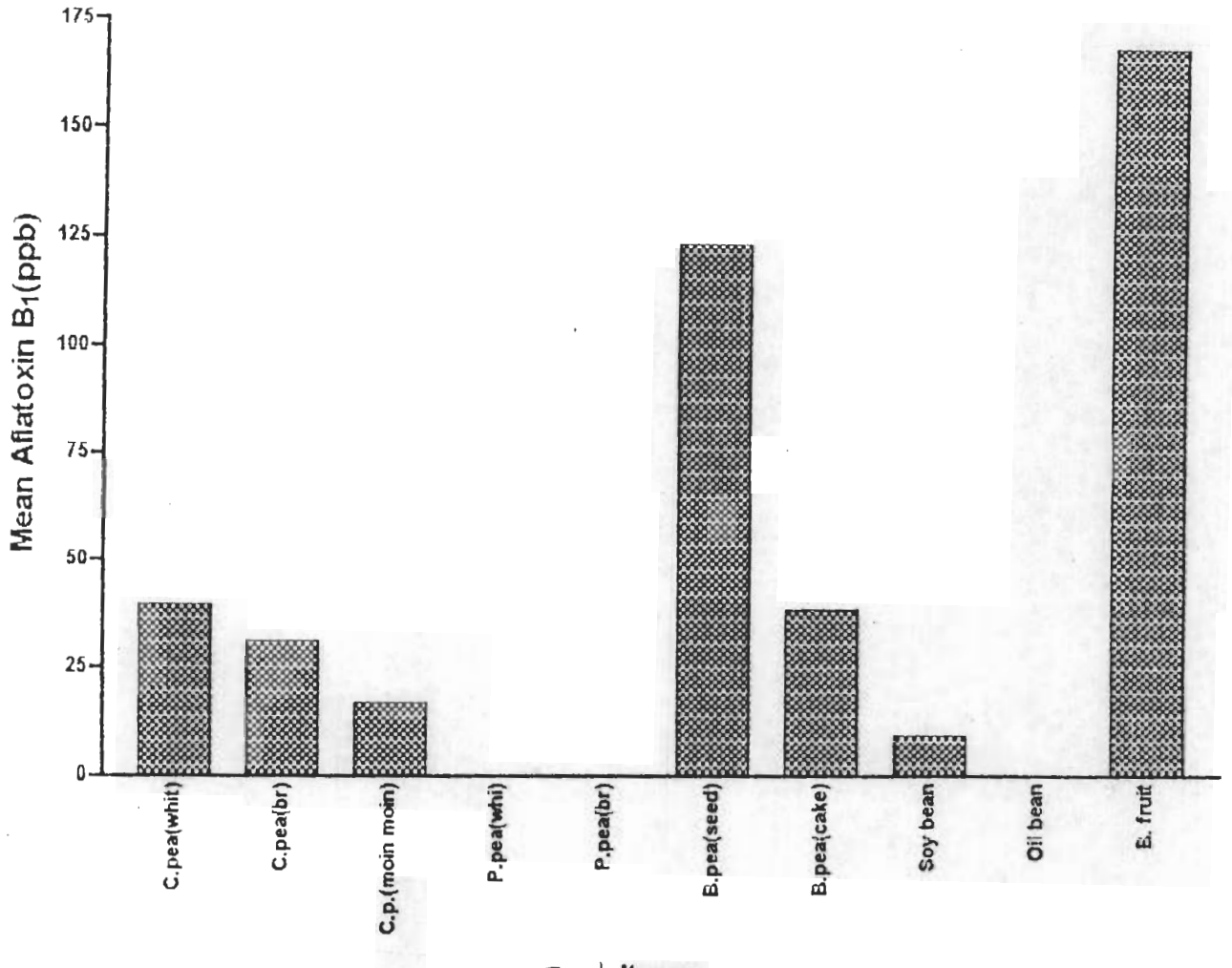


Fig. 9 : Screening of food for aflatoxin B1 content according to class of food (protein foods).

Cowpea white - C. pea (white)
Cow pea brown - C. pea (brown)
Cow pea moimoin - (moinmoin)
Pigeon pea white -P.pea(white)
Pigeon pea brown - P.pea (brown)
Bambra peanuts seed - B.pea (sd)
Bambara peanuts cake -B.(cake)
Bread fruits - B. fruit

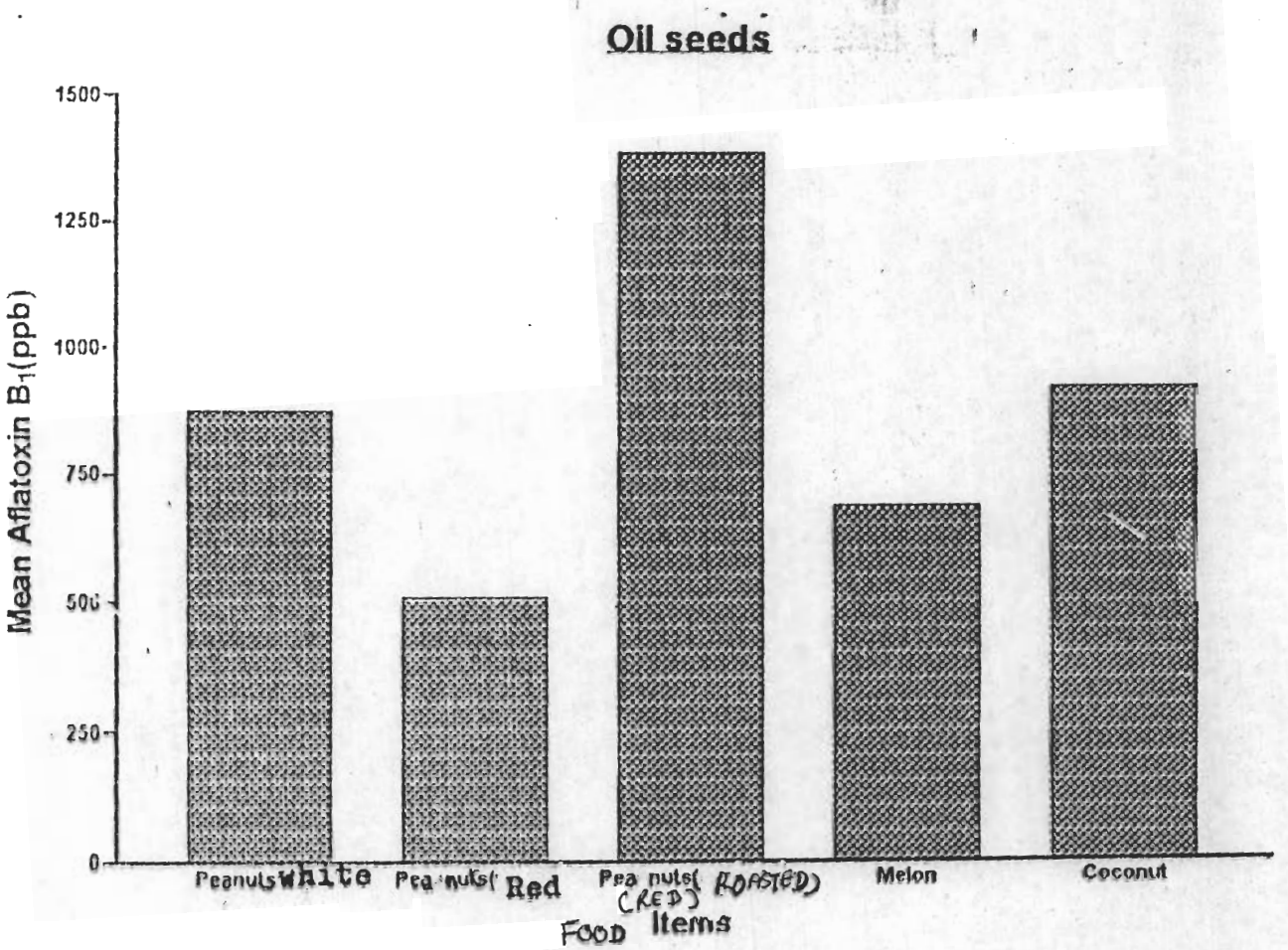


Fig: 10 : Screening of foods for aflatoxin B 1 content according to class of food (Lipid foods).
Oil seeds

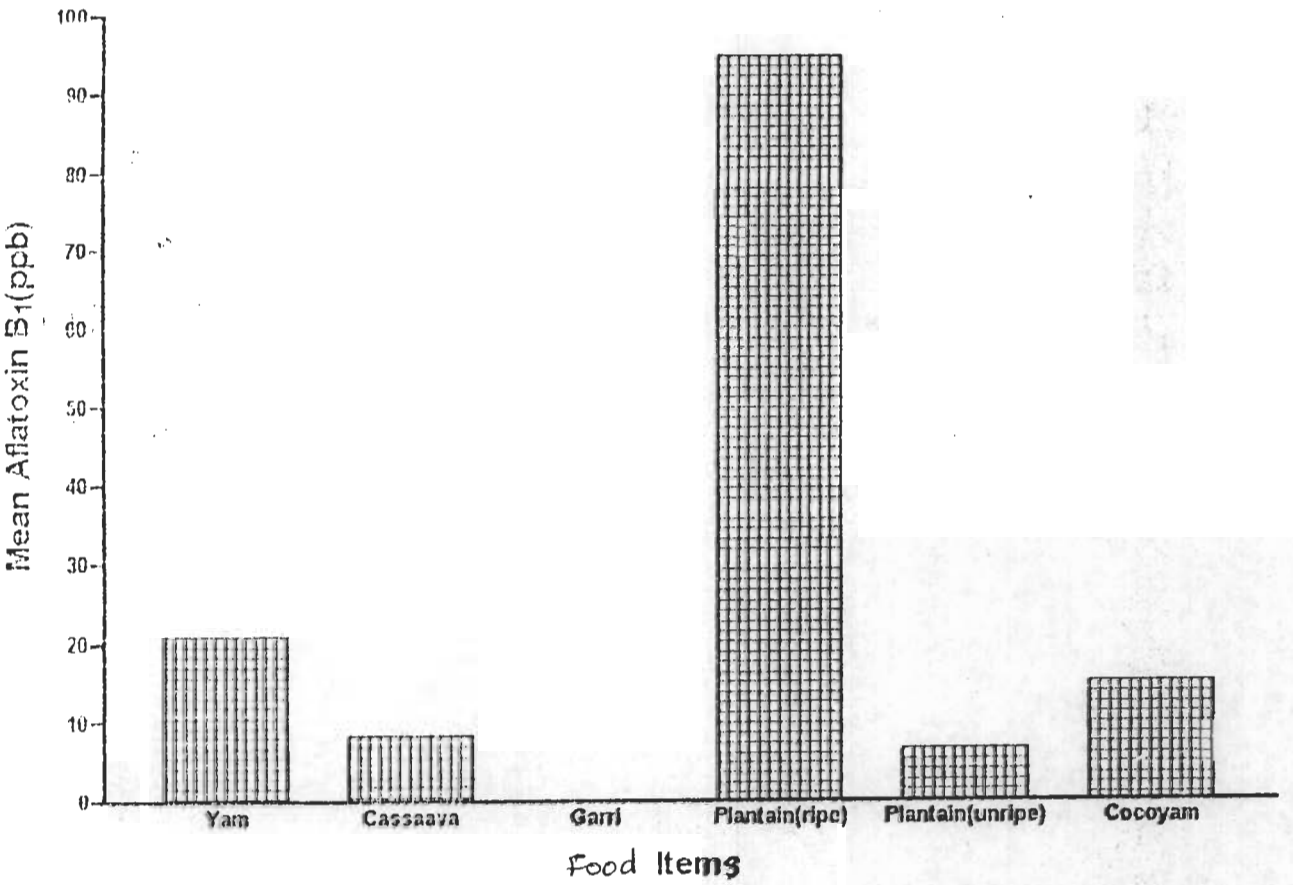


Fig. II : Screening of foods for aflatoxin B1 content according to class of food (carbohydrate foods)

sample (ripe plantain) contained AFB_1 mean value of >50ppb while most other carbohydrate foods had a mean AFB_1 <20ppb. This quality is shared almost with the protein foods where outside some breadfruit samples and some bambara peanut samples that contained mean AFB_1 values >100ppb, all others were <40ppb.

Next to Lipid foods are the cereals, where quite a good number of samples including maize, millet, sorghum and Abakaliki rice had AFB_1 mean values >200ppb. Acha (Findi) and Ugbawka rice were generally poor in AFB_1 yield. Products of the various foods including those of maize (Akamu or Ogi), (24.7ppb); Acha (Kunnu beverage) (29ppb), white sorghum (Burukutu beverage) (17.7ppb), white cowpea (Moin-moin) (16.9ppb), bambara peanuts (steamed cake-okpa) (39.2ppb), melon (Ogiri fermented condiment), oil bean seed (Ugba), (fermented sliced pieces), cassava (Garri), were all found to be very poor in aflatoxin yield, with very low levels of aflatoxin detected or none at all.

4.4. Aflatoxin production by *Aspergilus flavus* isolates:

The results of the 369 isolates of *A.flavus* group screened for aflatoxin production are shown in Table 6. Only 34.1% were found to be aflatoxigenic while the remaining 65.9% were non-aflatoxigenic strains. Of the toxigenic strains, 24.6% produced only AFB_1, 24.6% produced AFB_1+B_2, 27.0%, $AFB_1+B_2+G_1$; 11.10%, $AFB_1+B_2+G_1+G_2$, while the remaining 12.7% produced AFB_1+G_1.

Table 7 shows the results of the analysis of the aflatoxigenic strains for AFB_1 production range.

Eighteen isolates produced AFB_1 in the range of <50μg/ml, nine, within the range 51 to 100ug/ml and thirty three in the range 101 to 150μg/ml. Forty five were in the range of 151 to

TABLE 6

SCREENING OF A . FLAVUS STRAINS ISOLATED FROM VARIOUS FOODSTUFFS FOR AFLATOXIN PRODUCING POTENTIAL.
(N = 369)

Aflatoxin production	No (%) of isolates
B1 only	31 (24.6)
B1 + B2	31 (24.6)
B1 + B2 + G1	34 (27.0)
B1 + B2 + G1 + G2	14 (11.11)
B1 + G1	16 (12.7)
TOTAL aflatoxigenic strains	126 (34.1)
Non aflatoxi-genic strains	243 (65.9)

TABLE 7

ANALYSIS OF THE 126 TOXIGENIC A. FLAVUS STRAINS FOR AFLATOXIN B1 PRODUCTION RANGE (µg / ml).

AFB_1 range	No of isolates	Average AFB1 production (µg/ml)
0 - 50	18	46.2
51 - 100	9	80.9
101 - 150	33	130.6
151 - 200	45	176.3
201 - 250	16	237.8
251 - 300	5	285.1

200µg/ml, with sixteen in the range of 201 to 250, and the last five, in the range 251 to 300µg/ml.

Figure 12 represents the distribution of *A.flavus* organisms/toxigenic strains in the various food varieties.

White peanuts contained the highest number of *A.flavus* organisms (61) representing 16.5% of the entire 369 *A.flavus* organisms encountered in the study. Twenty-nine percent of these were found to be aflatoxigenic. Next was white maize with 42 *A.flavus* organisms, which is11.4% of the entire 369 isolates and from which 19% were aflatoxigenic. Yellow maize (33) ranks third being 8.9% of the entire *A.flavus* isolates and from which 48.5% were toxigenic. Next in a decreasing order of frequency is red peanuts (23 or 6.2%) with 43.5% being aflatoxigenic; white cowpeas and melon seeds (17 or 4.6%each) with 23.1% (for white cowpea) and 47.1% (for melon seeds) being toxigenic; Abakaliki rice (15 or 4.1%) with 53.3% being aflatoxigenic. Breadfruits contained 14, being 3.8% of the total a.flavus with 42.9% of these isolates being aflatoxigenic, while yellow sorghum and roasted red peanuts had 12 each being 3.3% of the entire *A.flavus* isolates with 41.7% and 66.7% being aflatoxigenic in each sample respectively.

All the processed products including akamu (ogi), kunnu, moin-moin, garri, burukutu, ogiri and bambara cake (okpa) had the least numbers of *A.flavus* as well as the aflatoxigenic strains, with moi-moi, ogiri and garri having no toxigenic strains at all.

Though oil bean seeds contained nine *A.flavus* organisms,only one was found to be aflatoxigenic. Soybeans also had only three *A.flavus* organisms out of which only one strain was aflatoxigenic.

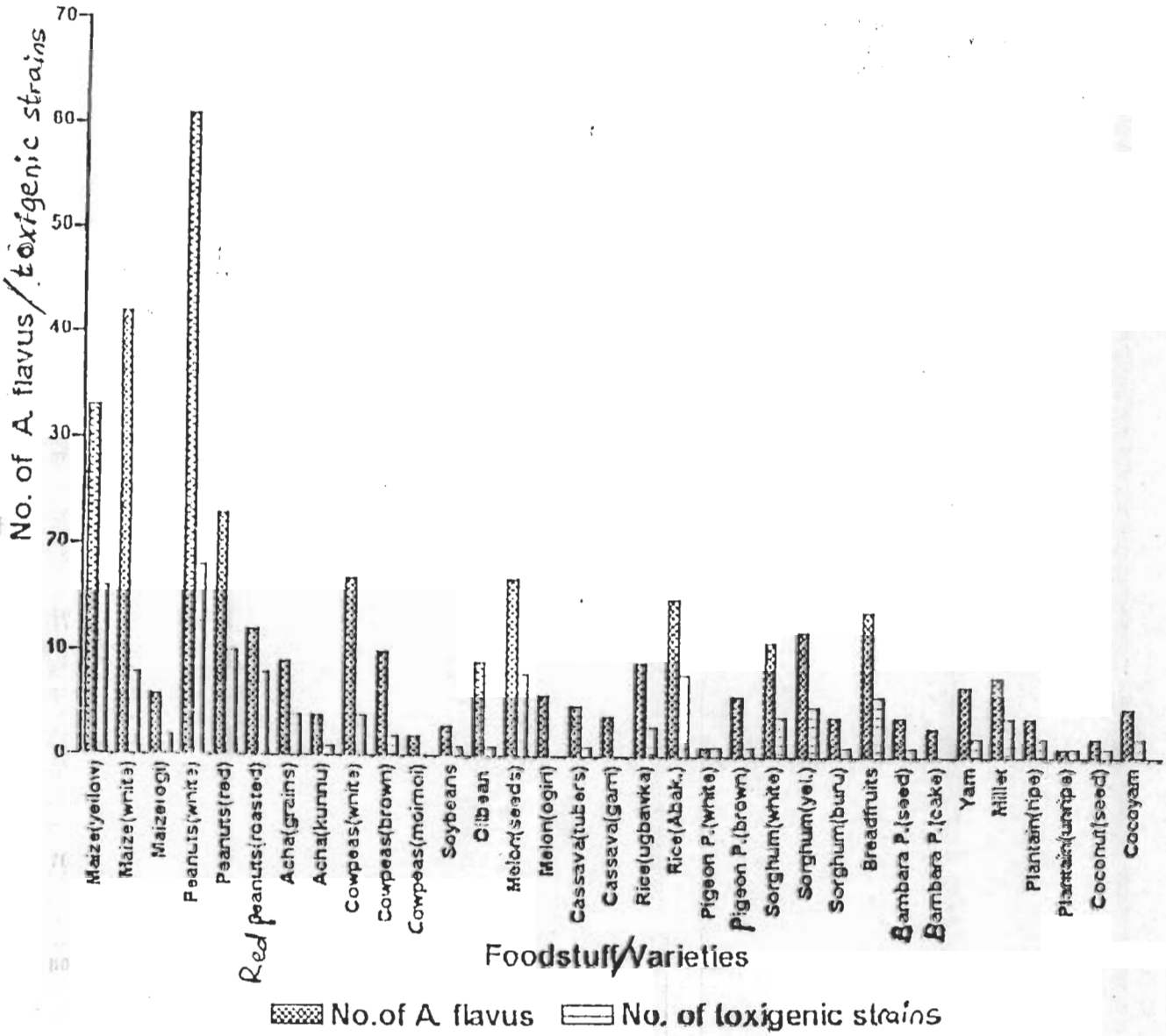


Fig. 12: Distribution of A. flavus organisms and the aflatoxigenic strains encountered in different foodstuffs analysed in Enugu during 1996-1998.

Rice Abak - Rice Abakaliki
Pigeon p - Pigeon pea
Sorghum yel - sorghum yellow
Sorghum buru - sorghum buru-kutu
Bambara P - Bambara peanut

Varietal differences were equally observed in the distribution of aflatoxigenic strains of *A.flavus*. Thus, yellow maize contained more toxigenic strains than the white variety; white testa peanut had more toxigenic strains than the red testa variety; white cowpea more than the brown type and Abakaliki rice more than the Ugbawka variety. There were however no differences between the contents of toxigenic strains of *A.flavus* in the white and brown pigeon peas, white and yellow sorghum, and ripe and unripe plantain.

4.5 EFFECT OF MICROBIAL ECOSYSTEM ON AFLATOXIN PRODUCTION. (FUNGAL ECOSYSTEM STUDIES)

The qualitative and quantitative effects of microbial ecosystem on aflatoxin Production in the various food substrates are shown in figures 10 – 51 and Tables 8 – 11. Each set of experiments involved different food classes with *A. flavus* in monospecific culture and then in partnership with other organisations encountered.

4.5.1 The cereals:

The cereals include the following: Maize and sorghum (white and yellow varieties), acha grains, rice (Abakaliki and Ugbawka varieties) and millet.

(1) ***A. flavus monoculture.***

The results with *A. flavus* in monoculture in the cereals are shown in Fig. 13.

From here, a similar trend was obtained for all cereal grains, each yielding only the 'B' aflatoxins (both B_1 and B_2) which levels gradually decreased over the days of incubation. On the third day of incubation, the highest levels of aflatoxins were observed for millet (480.3 ppb), being followed in a decreasing order by white sorghum (367.9ppb), yellow sorghum (290.6ppb) with acha recording the least

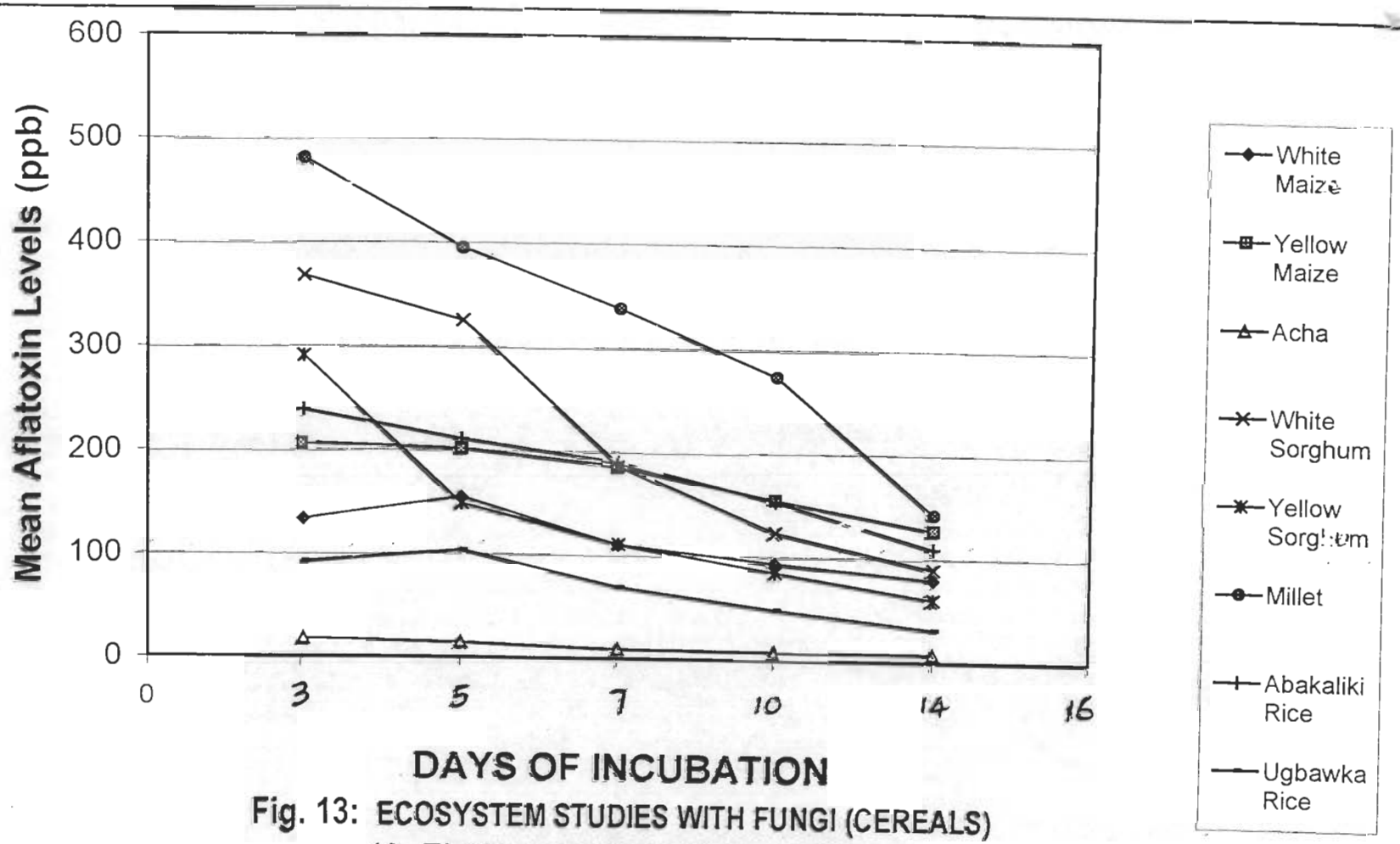


Fig. 13: ECOSYSTEM STUDIES WITH FUNGI (CEREALS)
(A. FLAVUS IN MONOCULTURE)

(17.3ppb). The decrease over the days of incubation from day three to fourteen was statistically significant for all the cereal grains, ($P<0.05$). Yellow maize had higher AF levels than the white variety.

The Abakaliki rice produced almost three times the amount of aflatoxins in the Ugbawka rice. The differences in the AF levels over the days of incubation in the two rice varieties were also statistically significant ($P<0.05$), though the decrease from the third day to the fourteenth day was more for the Ugbawka type. White sorghum also yielded more AF levels in all the days of incubation than the yellow variety, though the decrease over the days of incubation was more in the yellow variety.

(ii) **Dual culture experiments.**

When *A. flavus* was in dual culture in all the cereal grains with *Rhizopus* spp., AF levels were significantly inhibited over the days of incubation ($P<0.05$) with the exception of Ugbawka rice and acha grains when compared with the monoculture results. The decrease was more in the yellow maize when compared with the decrease in the white variety and about five fold in the white sorghum but less so in the yellow variety. Comparing the two varieties of rice, the *Rhizopus* spp. inhibited AF production more signiicantly in the Abakaliki variety than the Ugbawka variety ($P<0.05$) (Fig. 14).

A general reduction in aflatoxin levels was observed with *Saccharomyces* spp. in dual cultures in all the cereal grains. This reduction was, however, significant ($P<0.05$) only in the white sorghum, millet and Abakaliki rice. With acha grains, the reduction became significant only after day five of incubation (Fig. 15). Figure 16

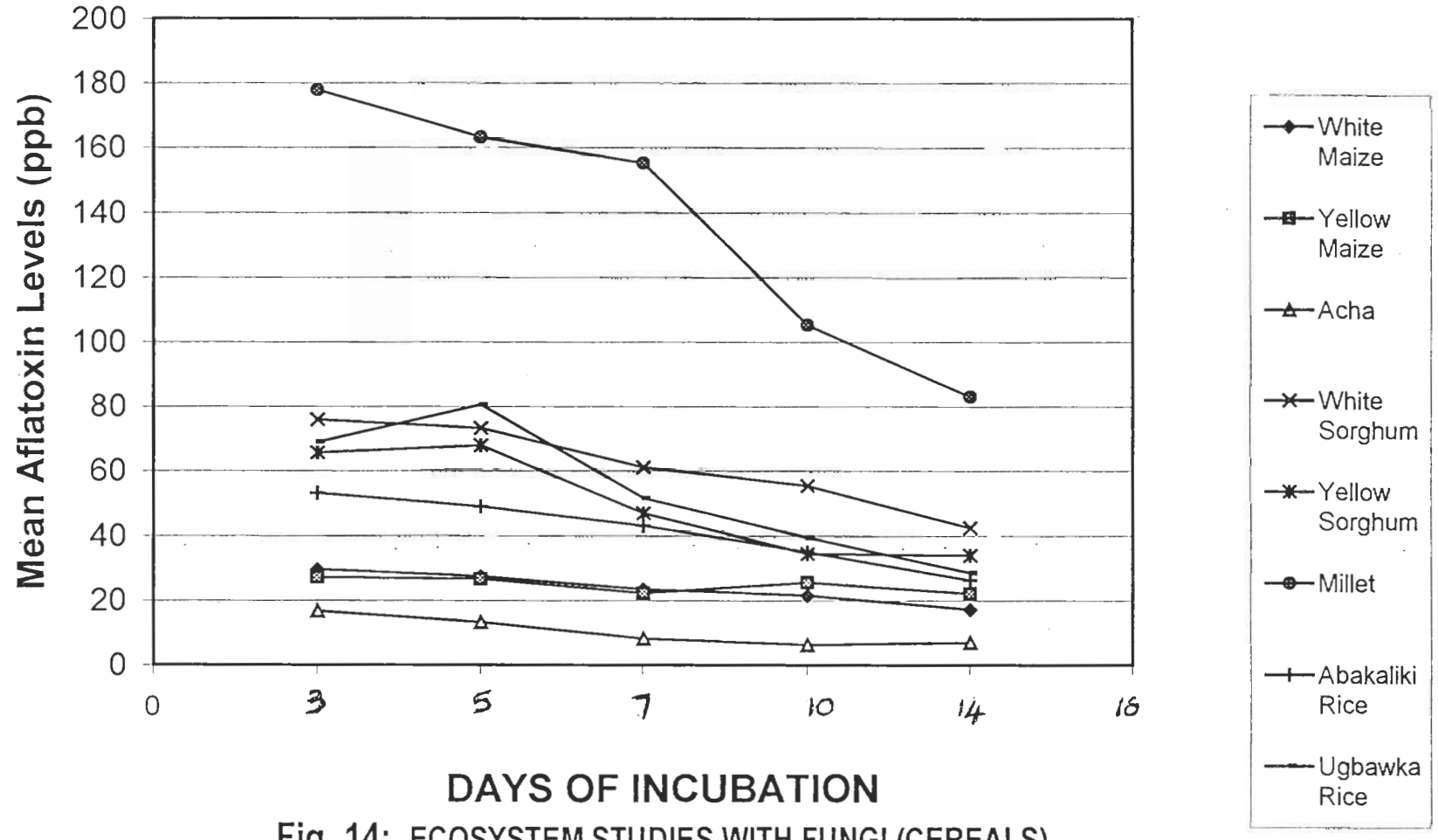


Fig. 14: ECOSYSTEM STUDIES WITH FUNGI (CEREALS)
(A. FLAVUS AND RHIZOPUS spp)

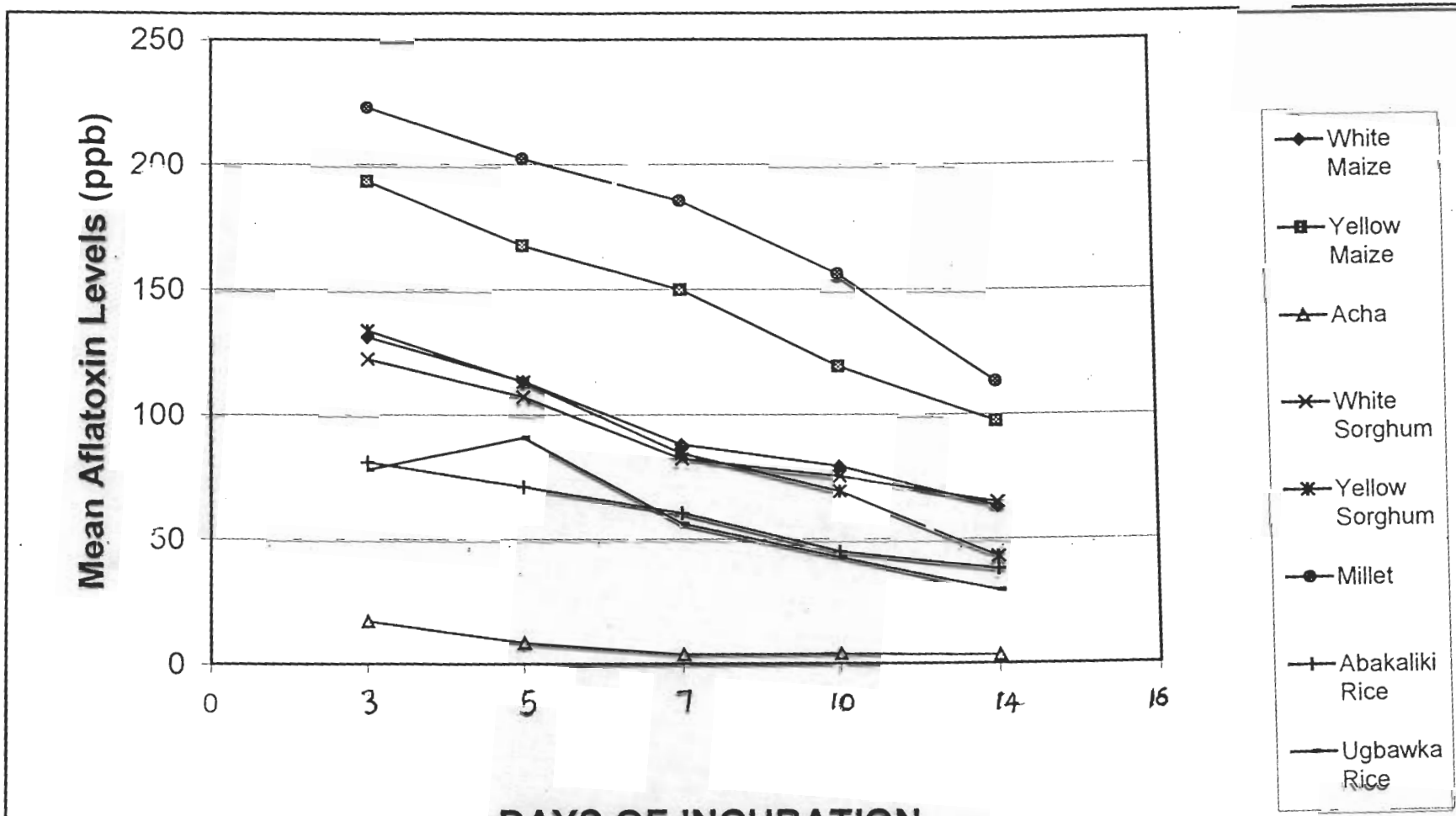


Fig. 15: ECOSYSTEM STUDIES WITH FUNGI (CEREALS)
(A. FLAVUS AND SACCHAROMYCES CEREVISIAE)

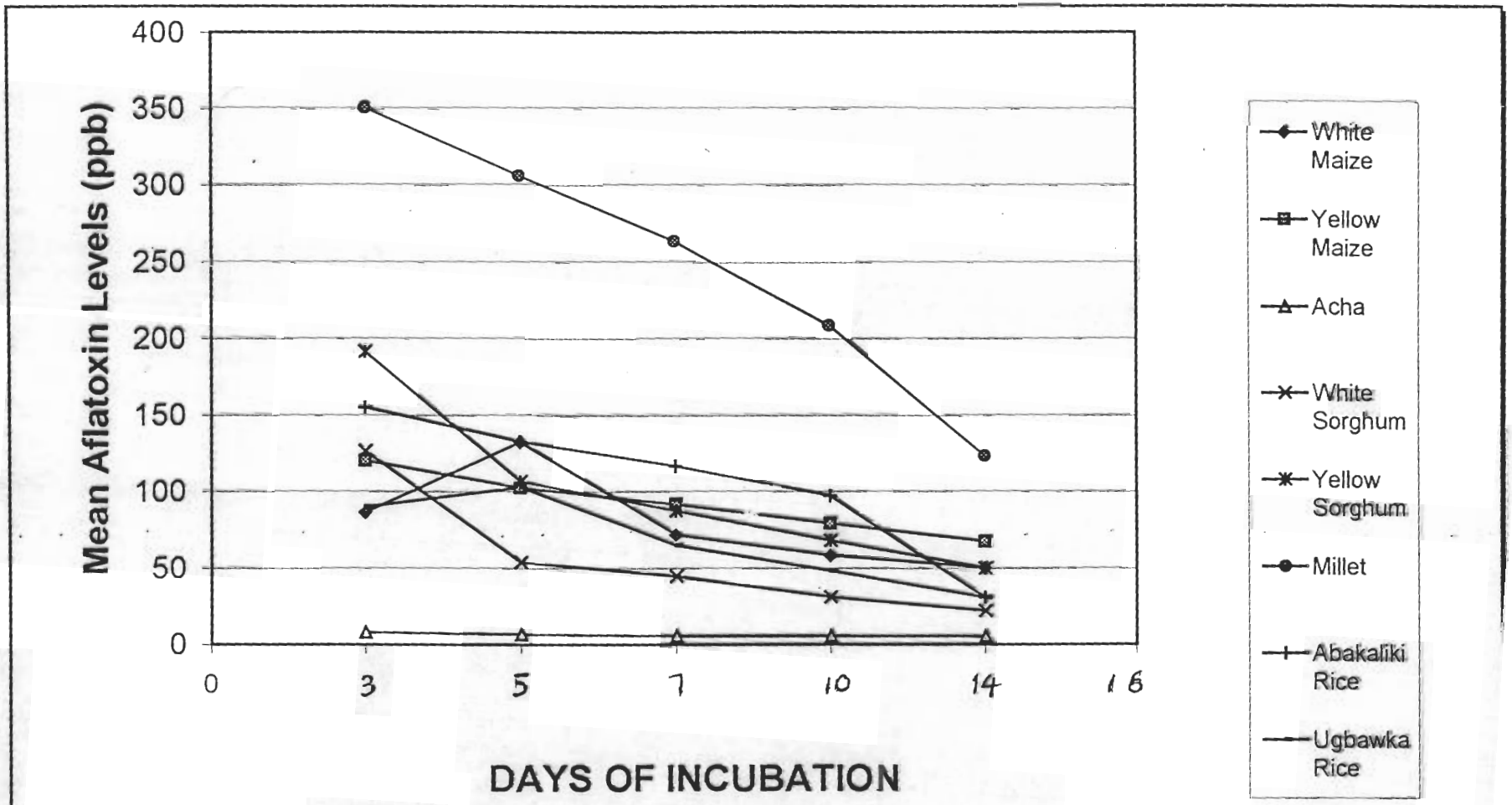


Fig. 16: ECOSYSTEM STUDIES WITH FUNGI (CEREALS)
(A. FLAVUS AND PENICILLIUM spp)

shows the effects of *Penicillium* spp, when in dual culture with *A. flavus* in the cereals. Generally also, there was a reduction in AF levels in all the cereal grains over the days of incubation with the reductions observed for yellow maize and white sorghum being significant ($P<0.05$). In the Abakaliki rice, AF levels were significantly reduced only on the fourteenth day of incubation, though generally more significantly reduced in all the days of incubation when compared with the Ugbawka counterpart ($P<0.05$).

Specifically, aflatoxin B_1 was significantly reduced in all the cereals grains with a subsequent slight increase of AFB_2 except in the Ugbawka rice. In acha grains, a significant reduction of AFB_1 was observed too and a production of AFB_2 that was not formed when *A. flavus* was in monoculture in the grains.

When *Cladosporium* spp. were in dual culture with *A. flavus* in the cereal grains, there were no differences in the AF levels both in the single and dual culture experiments, though a slight stimulation was recorded for the maize varieties and acha grains (Fig. 17). *Curvularia* spp in the dual culture experiments increased the levels of aflatoxins in all the cereal grains with the stimulation being significant in the white maize, yellow sorghum and millet seeds ($P<0.05$) (Fig. 18).

Figures 19 and 20 show the results of the dual culture experiments with *Fusarium* and *Rhodotorula* species. In all the cereals, both moulds significantly reduced the AF levels ($P<0.05$) with the effects of *Rhodotorula* species being much more significant. However, *Fusarium* spp. affected the Ugbawka rice more than the *Rhodotorula* spp.

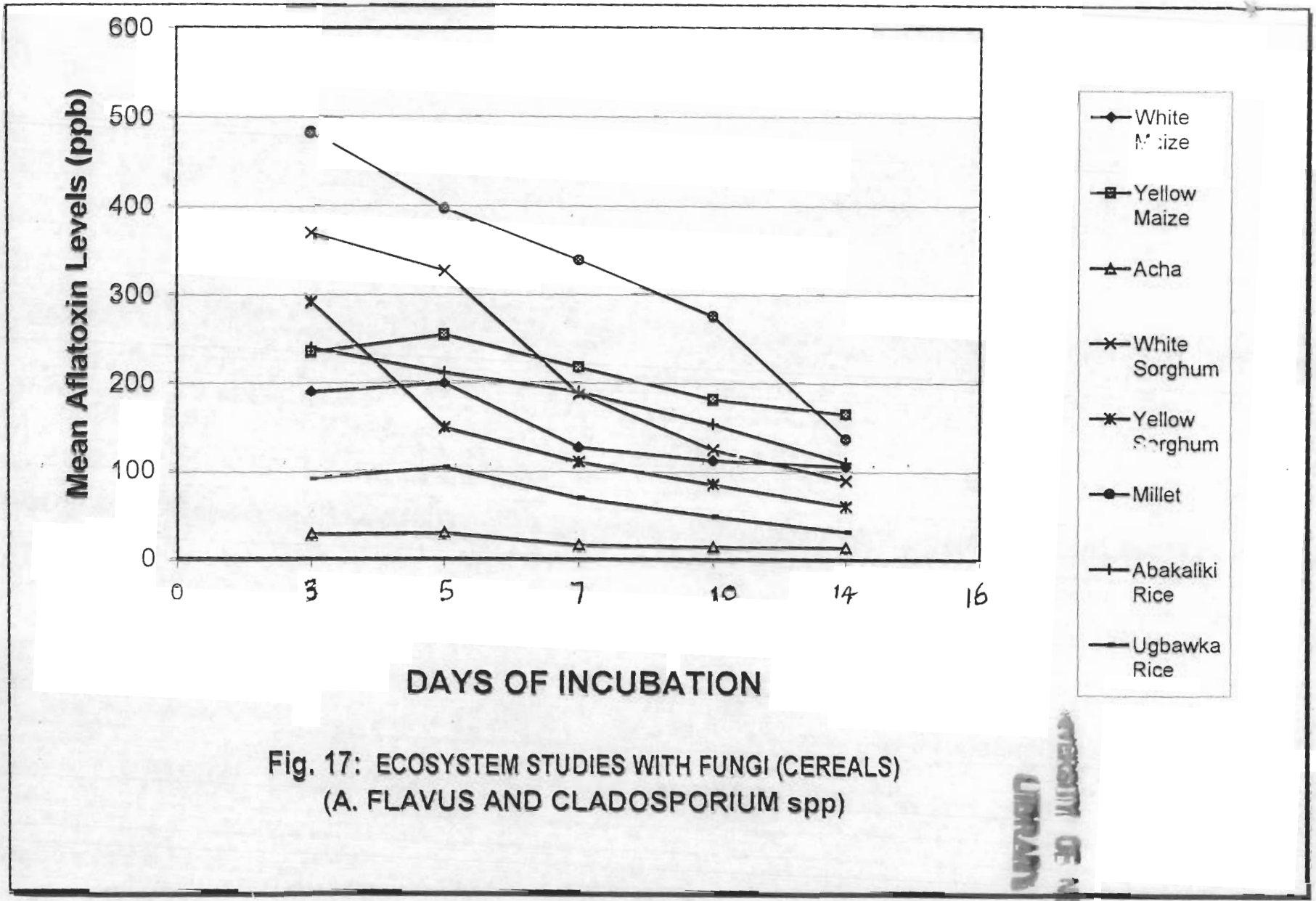


Fig. 17: ECOSYSTEM STUDIES WITH FUNGI (CEREALS)
(A. FLAVUS AND CLADOSPORIUM spp)

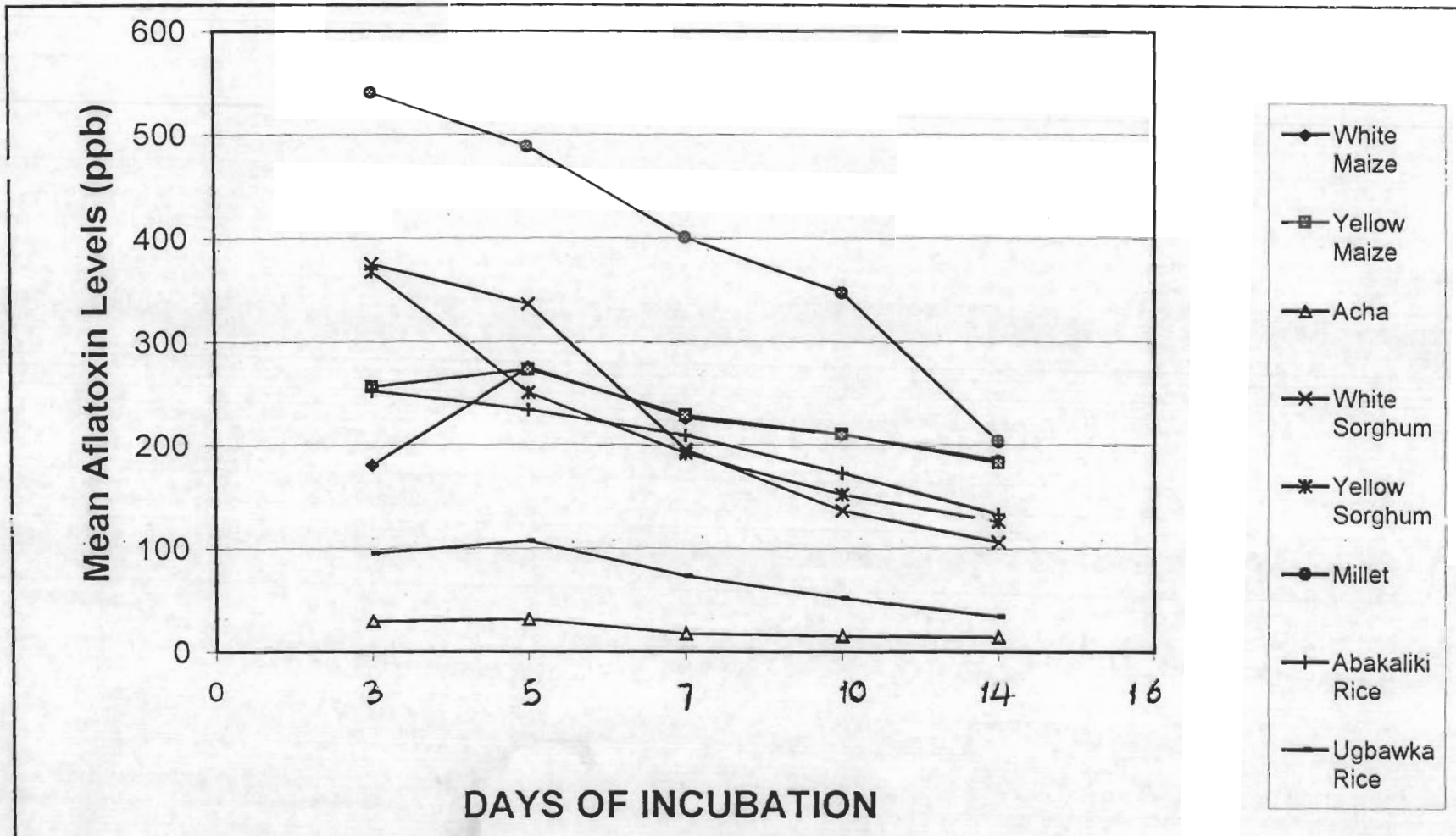


Fig. 18: ECOSYSTEM STUDIES WITH FUNGI (CEREALS)
(A. FLAVUS AND CURVULARIA spp)

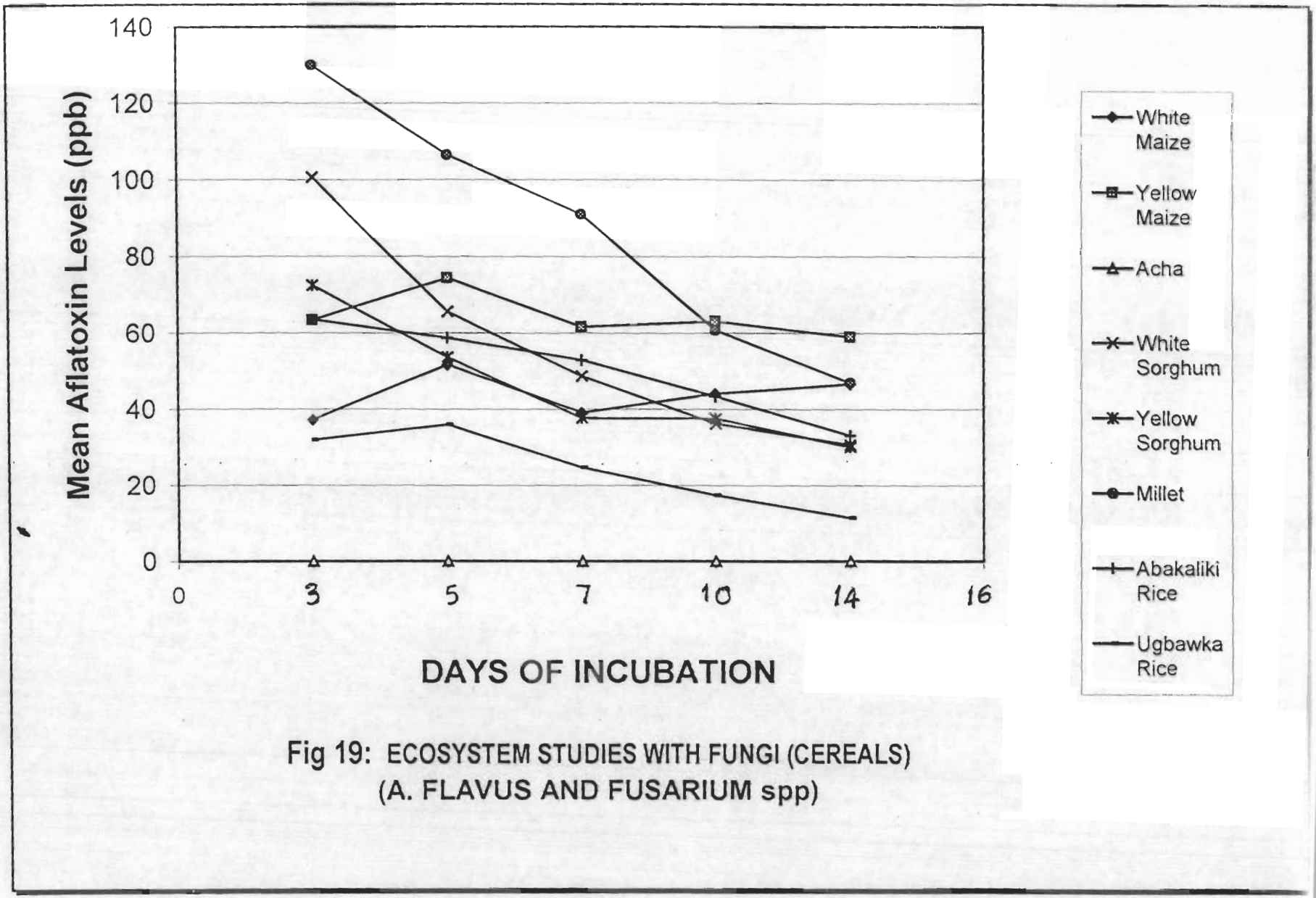


Fig 19: ECOSYSTEM STUDIES WITH FUNGI (CEREALS)
(A. FLAVUS AND FUSARIUM spp)

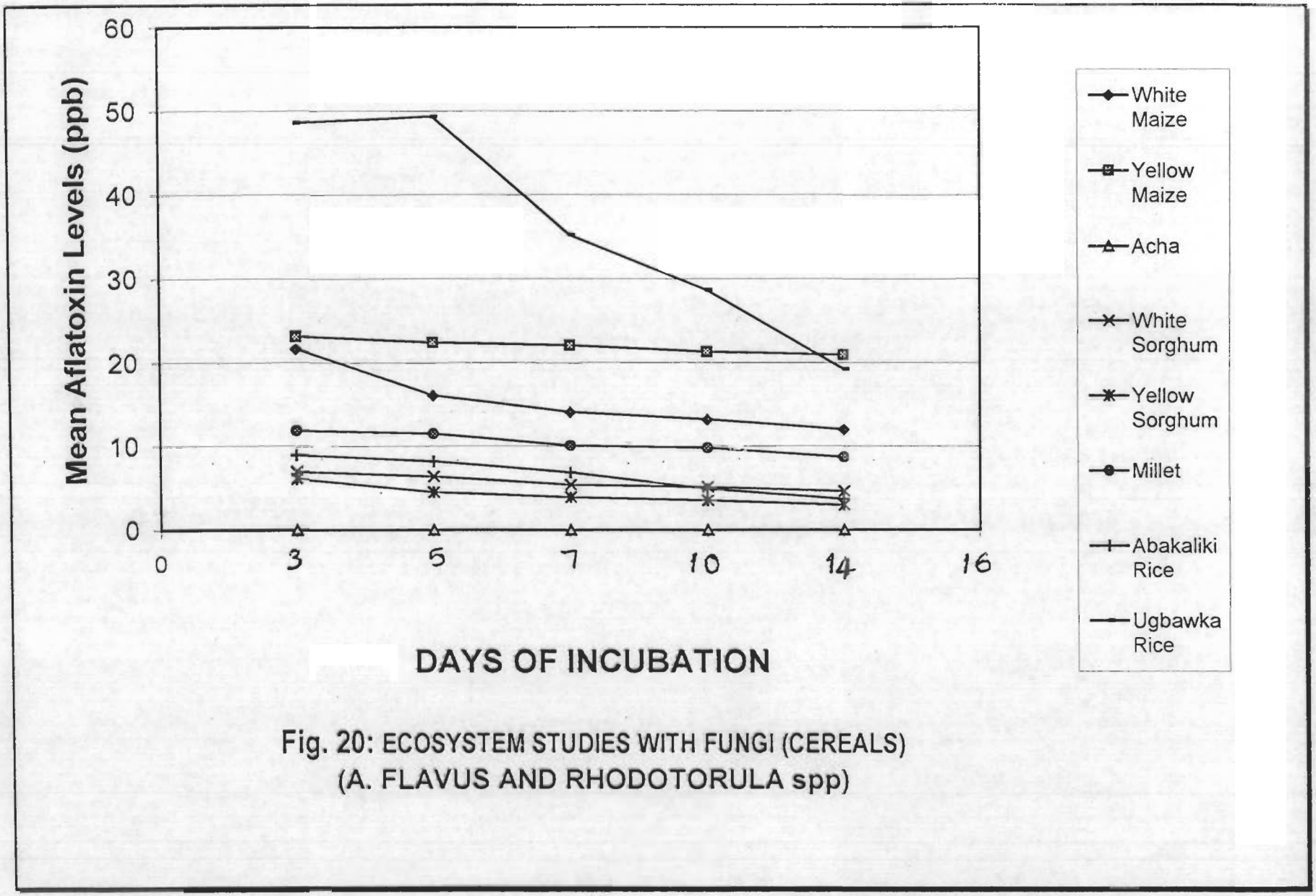


Fig. 20: ECOSYSTEM STUDIES WITH FUNGI (CEREALS)
(A. FLAVUS AND RHODOTORULA spp)

Both moulds in dual cultures with *A. flavus* completely abolished AF in acha grains.

Geotrichum spp. when in dual culture with *A. flavus* had no effects at all on AF levels when compared with the levels when *A. flavus* was in monoculture in all the cereals (Fig. 21), while *Candida albicans* stimulated AF production in all when compared with the results when *A. flavus* was in single culture, (Fig. 22).

Mucor species in the dual culture experiments significantly reduced AF levels in all the cereals ($P<0.05$), except the Ugbawka rice and acha grains. The reduction was also more significant in the yellow maize when compared with the white maize ($P<0.05$) (Fig. 23). The dual cultures with *A. fumigatus* and *Trichoderma* spp. with *A. flavus* on all the cereals completely abolished the production of aflatoxins.

4.5.2 The oil seeds

The oil seeds include the following: Peanuts (white and red testae varieties); melon seeds and coconut seeds.

(1) ***A. flavus in monoculture.***

Figure 24 shows the levels of aflatoxins produced over the days of incubation when *A. flavus* was in single culture in the oil seeds. From the results, it could be seen that a similar trend was followed by all the oil seeds. Generally, high levels of aflatoxins were produced by the oil seeds which increased by day five of incubation and generally dropped by day seven, increased again by the tenth day and still higher by the fourteenth day of incubation for the two varieties of peanuts.

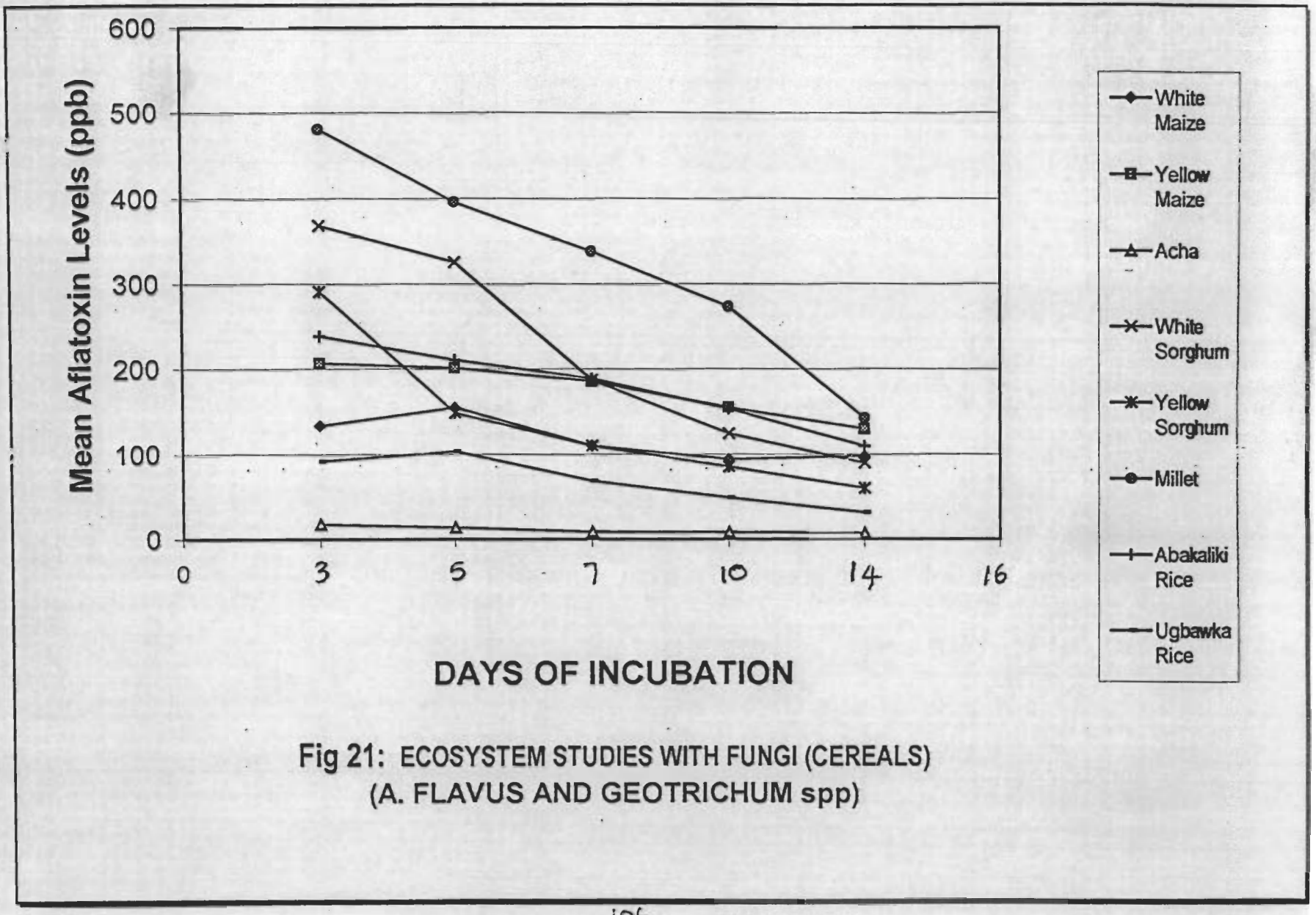


Fig 21: ECOSYSTEM STUDIES WITH FUNGI (CEREALS)
(A. FLAVUS AND GEOTRICHUM spp)

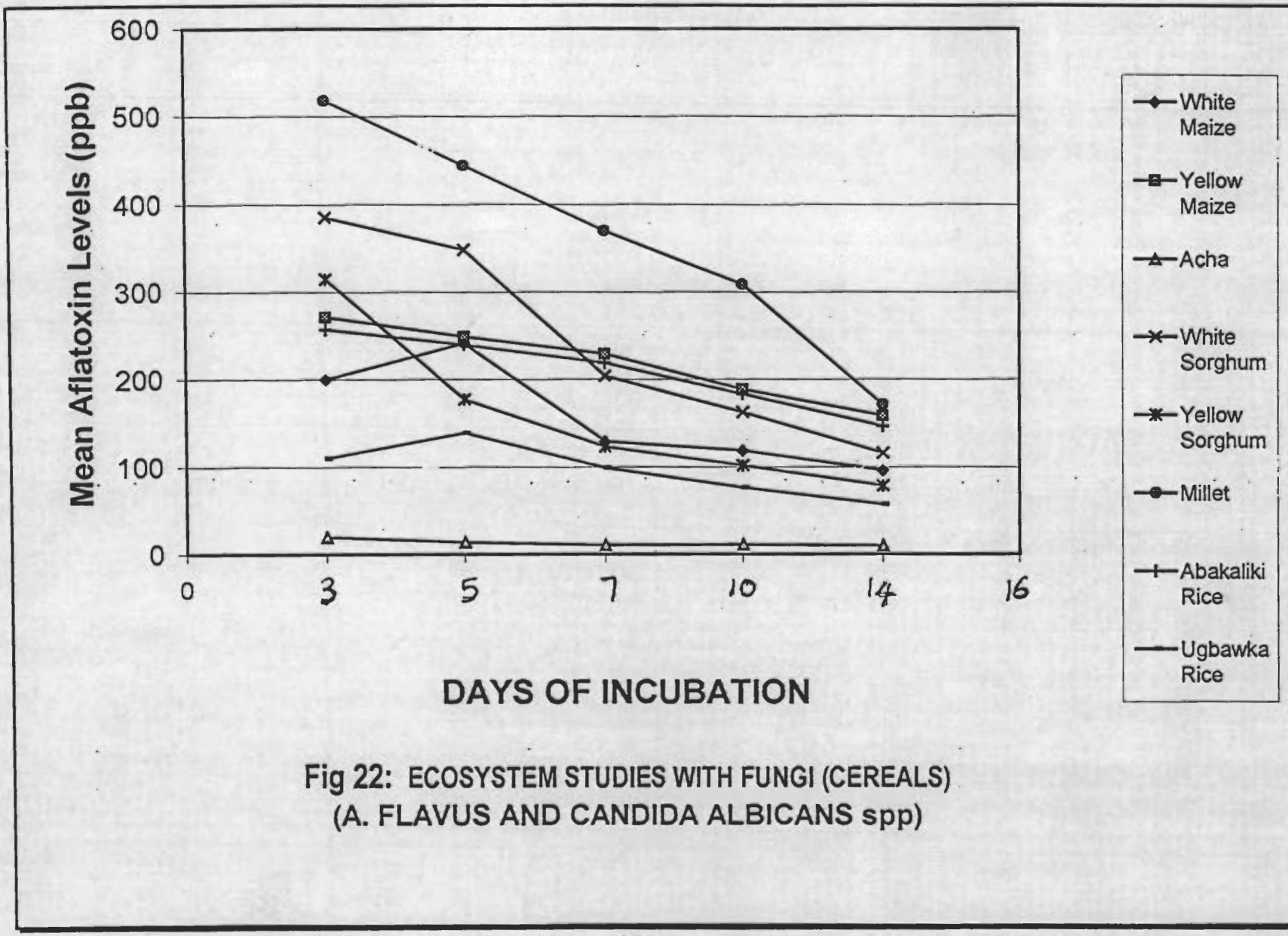


Fig 22: ECOSYSTEM STUDIES WITH FUNGI (CEREALS) (A. FLAVUS AND CANDIDA ALBICANS spp)

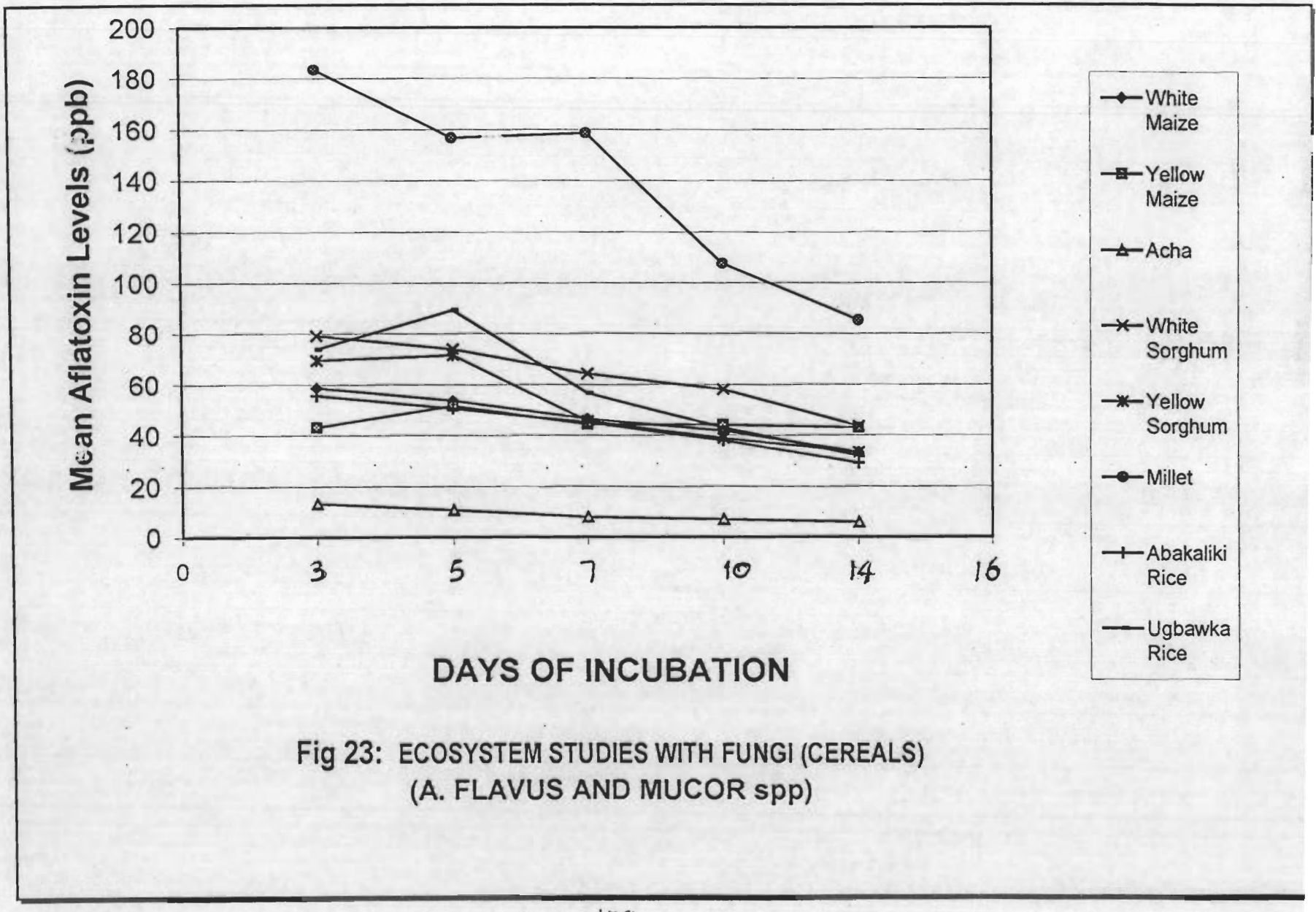


Fig 23: ECOSYSTEM STUDIES WITH FUNGI (CEREALS)
(A. FLAVUS AND MUCOR spp)

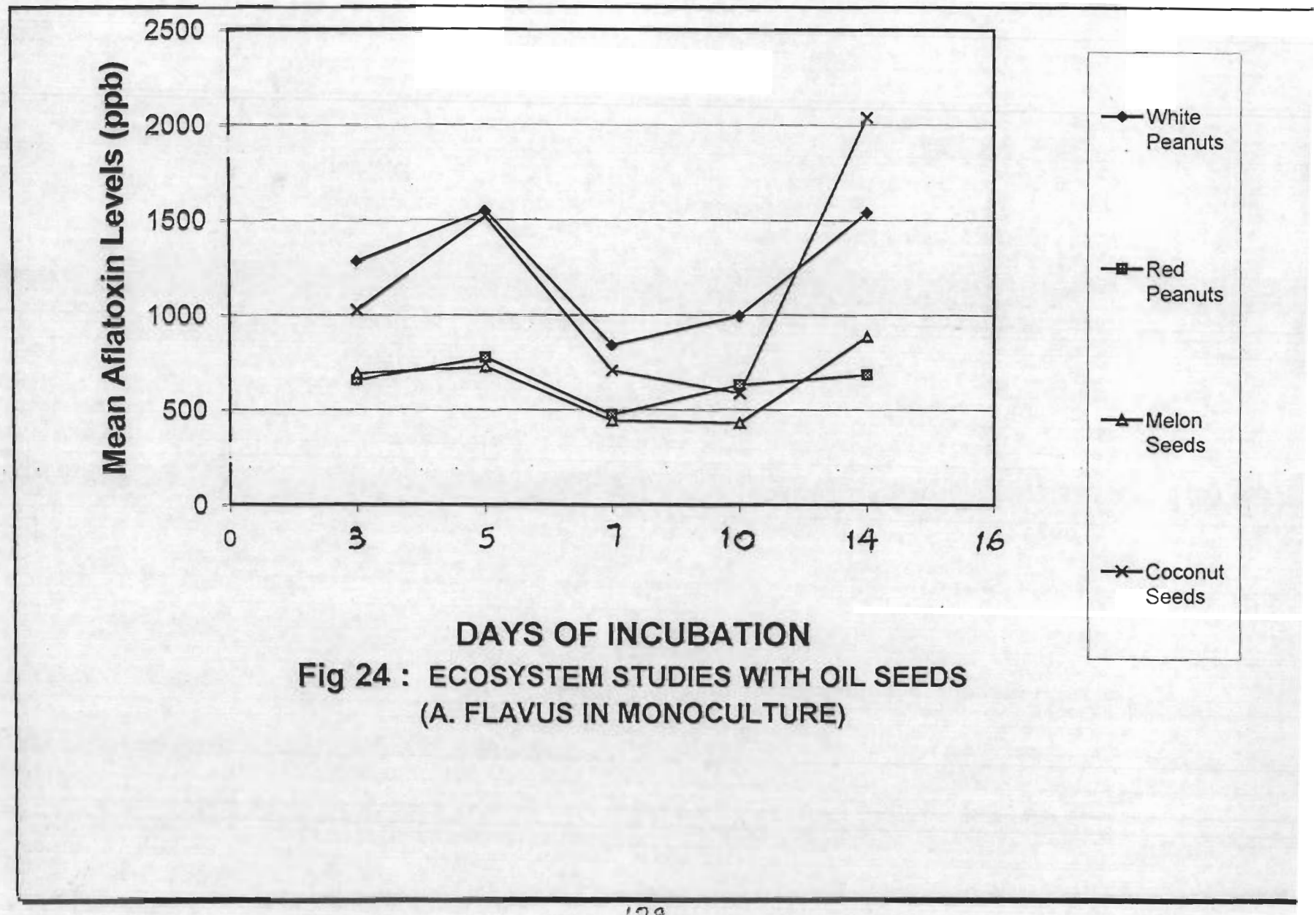


Fig 24 : ECOSYSTEM STUDIES WITH OIL SEEDS (A. FLAVUS IN MONOCULTURE)

For the melon seeds and coconut seeds, the decrease continued into day ten and then got much increased by the fourteenth day.

Coconut seeds yielded the highest AF levels, up to 2036.6 ppb by day fourteen, followed by the white peanuts (1538 ppb); melon seeds, (884.3ppb); with red peanuts (682.9ppb) yielding the least. Thus, the oil seeds generally gave a biphasic curve pattern in aflatoxin prouction over the days of incubation. The oil seeds yielded both AFB_1, B_2, G_1 and G_2.

Dual culture experiment.

Rhizopus spp. in dual culture with *A. flavus* in these seeds, very significantly inhibited AF production ($P<0.05$) (Fig 25). *A. niger* and *A. fumigatus* also significantly reduced AF levels in all the oil seeds ($P<0.05$), though less so than *Rhizopus* spp. for day three but by the fifth day of incubation, a more serious reduction was recorded which got completely abolished by the seventh day till day fourteen, though very minute traces were left for melon seed and coconut seeds (Figs. 26 & 34).

Saccharomyces and *Fusarium* species both also significatly decreased AF levels in all the oil seeds ($P<0.05$), though the reduction was less with *Fusarium* spp. *Penicillium* spp. in dual culture with *A. flavus* in the two peanuts varieties, also significantly reduced AF levels ($P<0.05$), though more so in the red variety than white type. More specifically, almost a similar pattern of reduction was observed as in the cereals. The aflatoxin B_1, G_1 and G_2 were significantly reduced ($P<0.05$) while the AFB_2 was slightly stimulated in the two peanuts. However, for the other oil seeds, melon seeds and coconut seeds, there was only a

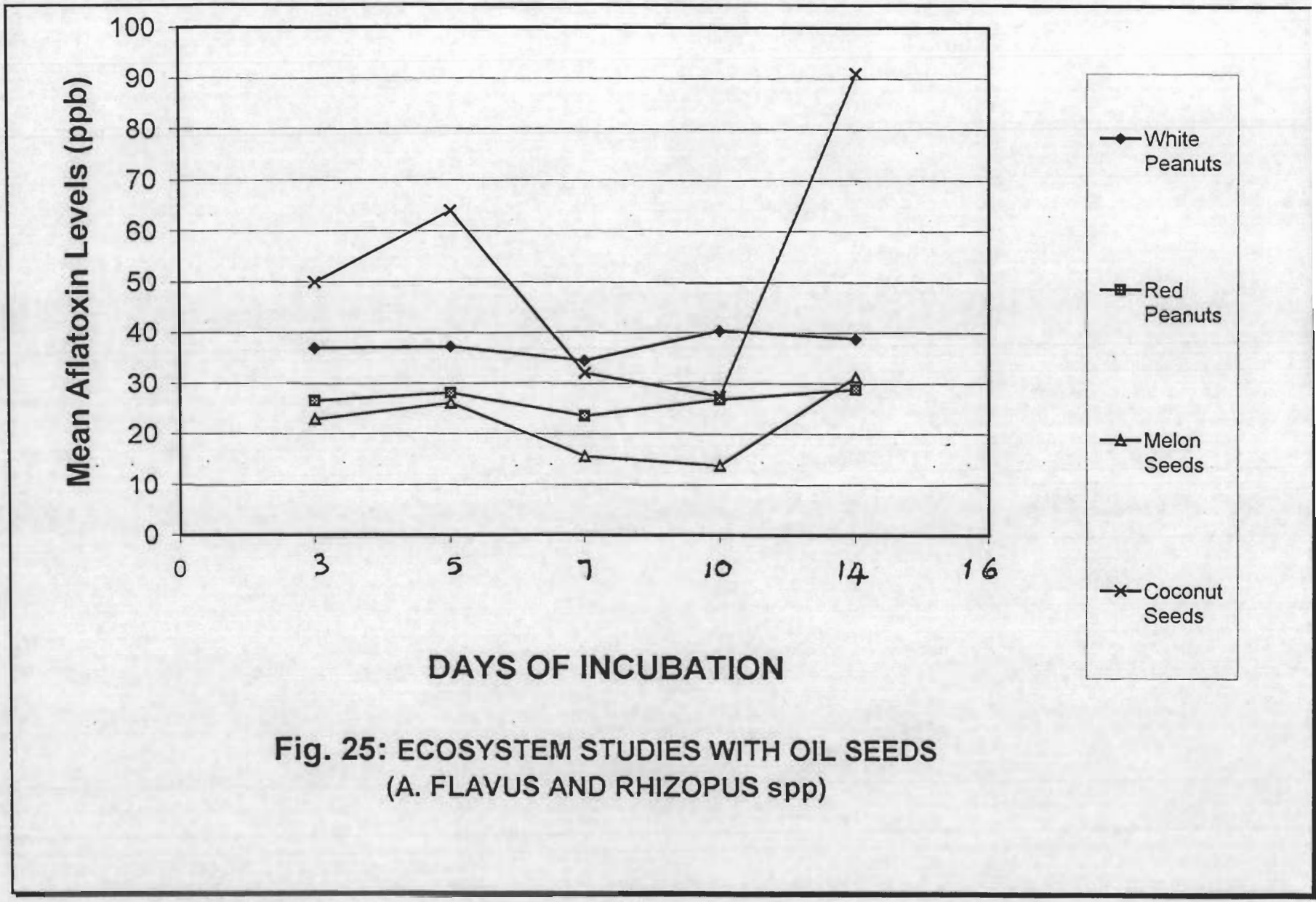


Fig. 25: ECOSYSTEM STUDIES WITH OIL SEEDS
(A. FLAVUS AND RHIZOPUS spp)

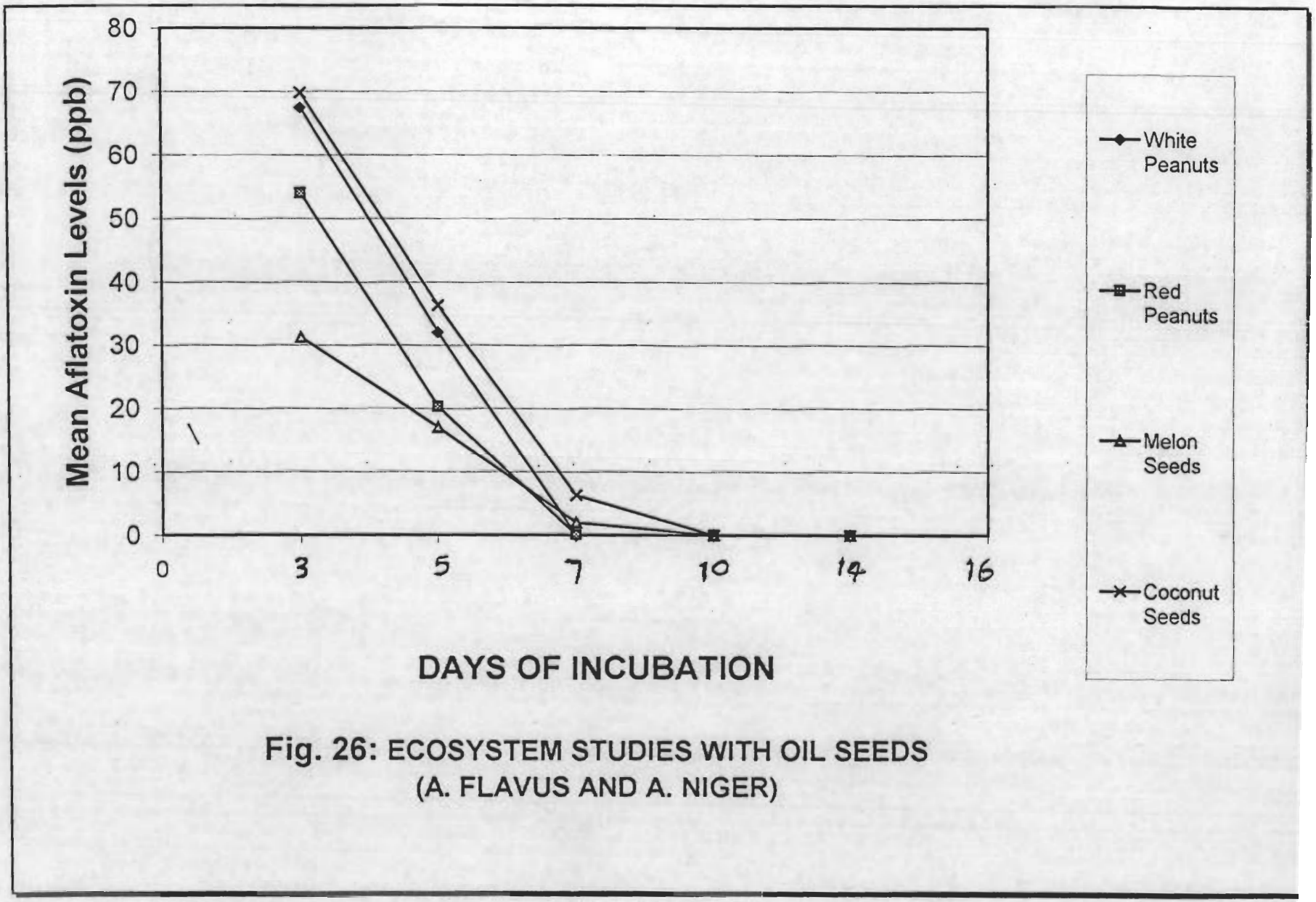


Fig. 26: ECOSYSTEM STUDIES WITH OIL SEEDS
(A. FLAVUS AND A. NIGER)

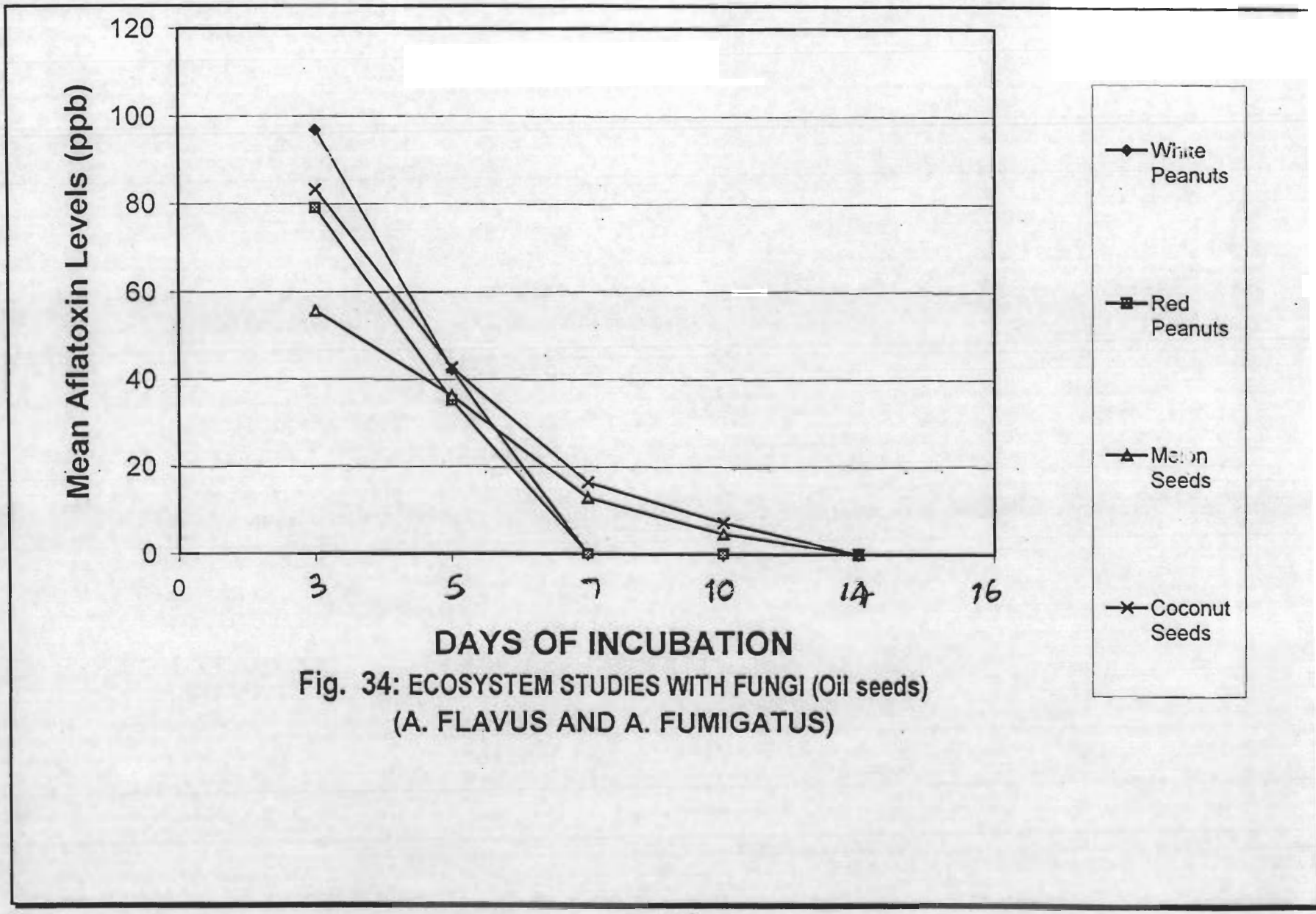


Fig. 34: ECOSYSTEM STUDIES WITH FUNGI (Oil seeds)
(A. FLAVUS AND A. FUMIGATUS)

slight increase in the levels of aflatoxins in all the days of incubation ($P<0.05$). Specifically, the AFB_1, B_2, G_1 and G_2 levels were all slightly stimulated (Figs.27 28, 31).

Cladosporium spp. had a mixed trend on the oil seeds while in dual culture with *A. flavus*. While AF levels in the two peanut varieties were reduced, levels in melon seeds and coconut seeds were increased (Fig. 29). *Curvularia* spp. on the other hand, increased AF levels in all the oil seeds throughout the incubation periods when in dual culture with *A. flavus*, when compared with the levels in the *A. flavus* monoculture experiments (Fig. 27). Figs 29 and 33 show the effects of *Rhodotorula* and *Mucor* species on aflatoxin production when in dual culture with *A. flavus* respectively. It could be seen from the results that in both experiments, AF levels were significantly reduced when compared with the levels in the monoculture experiments with *A. flavus* ($P<0.05$). However, the *Rhodotorula* spp. reduced aflatoxin levels in the two varieties of peanuts more than the Mucor spp while the reverse was the case for melon seeds and coconut seeds.

Both *Geotrichium* spp. and *Candida albicans* stimulated the production of aflatoxins in the oil seeds though the stimulation was more with *Geotrichum* spp. than with *Candida* spp. More specifically though, the increase in the AF levels in the two peanut varieties with both yeasts was less in all the days of incubation than with melon seeds and coconut seeds (Figs 30 and 32). *Trichoderma* spp. again completely inhibited aflatoxin production in all oil seeds when in dual culture with *A. flavus*.

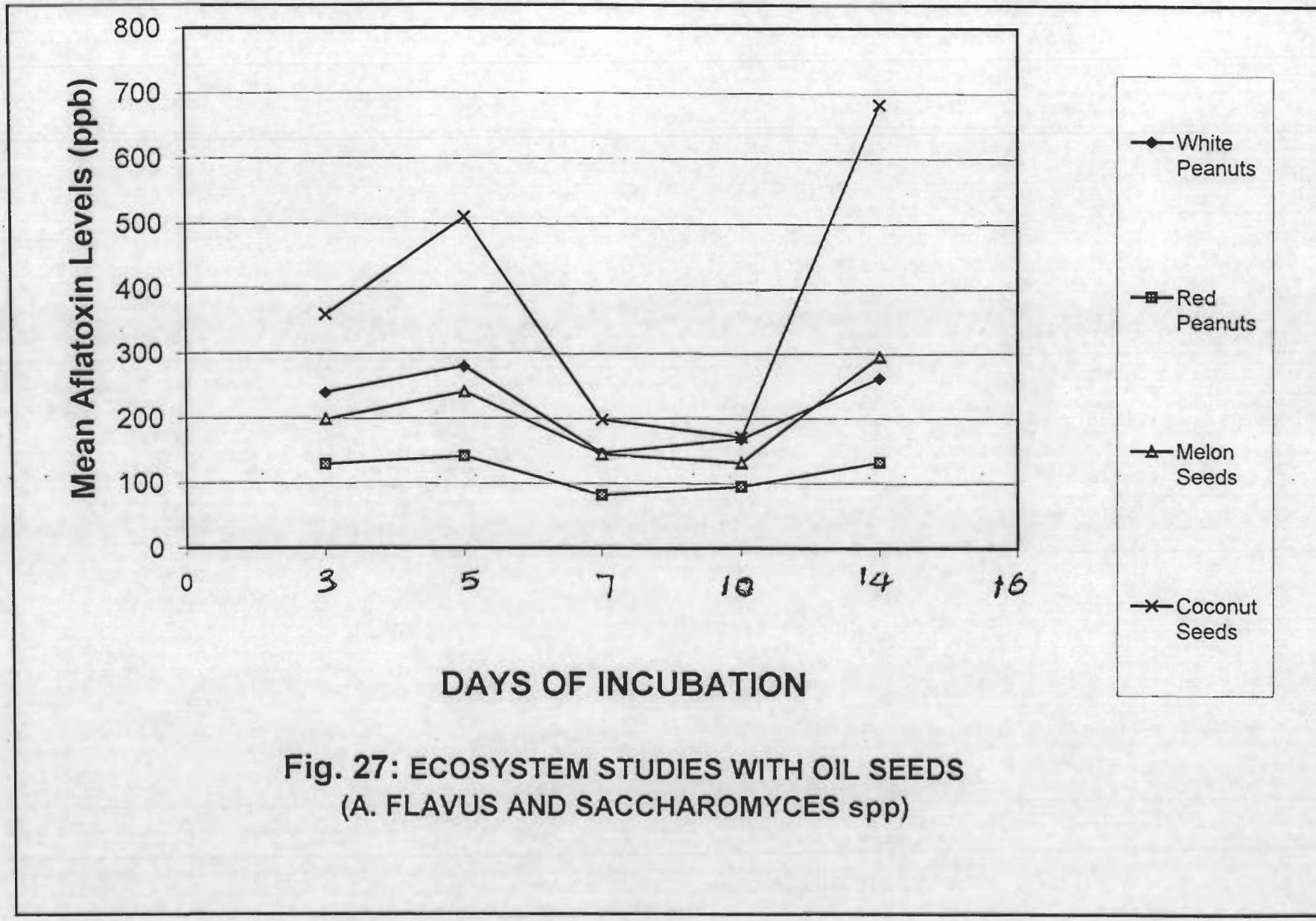


Fig. 27: ECOSYSTEM STUDIES WITH OIL SEEDS
(A. FLAVUS AND SACCHAROMYCES spp)

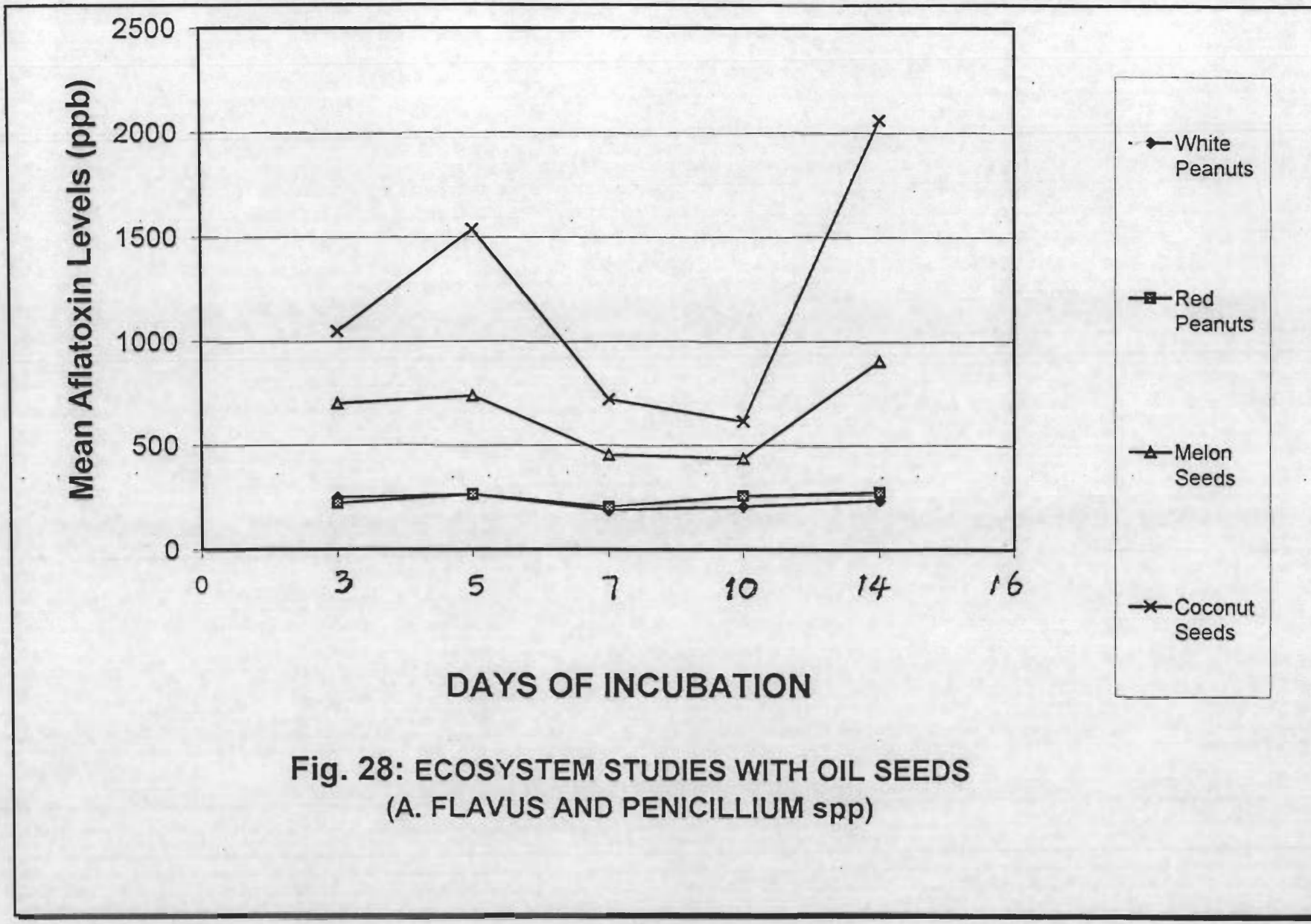


Fig. 28: ECOSYSTEM STUDIES WITH OIL SEEDS
(A. FLAVUS AND PENICILLIUM spp)

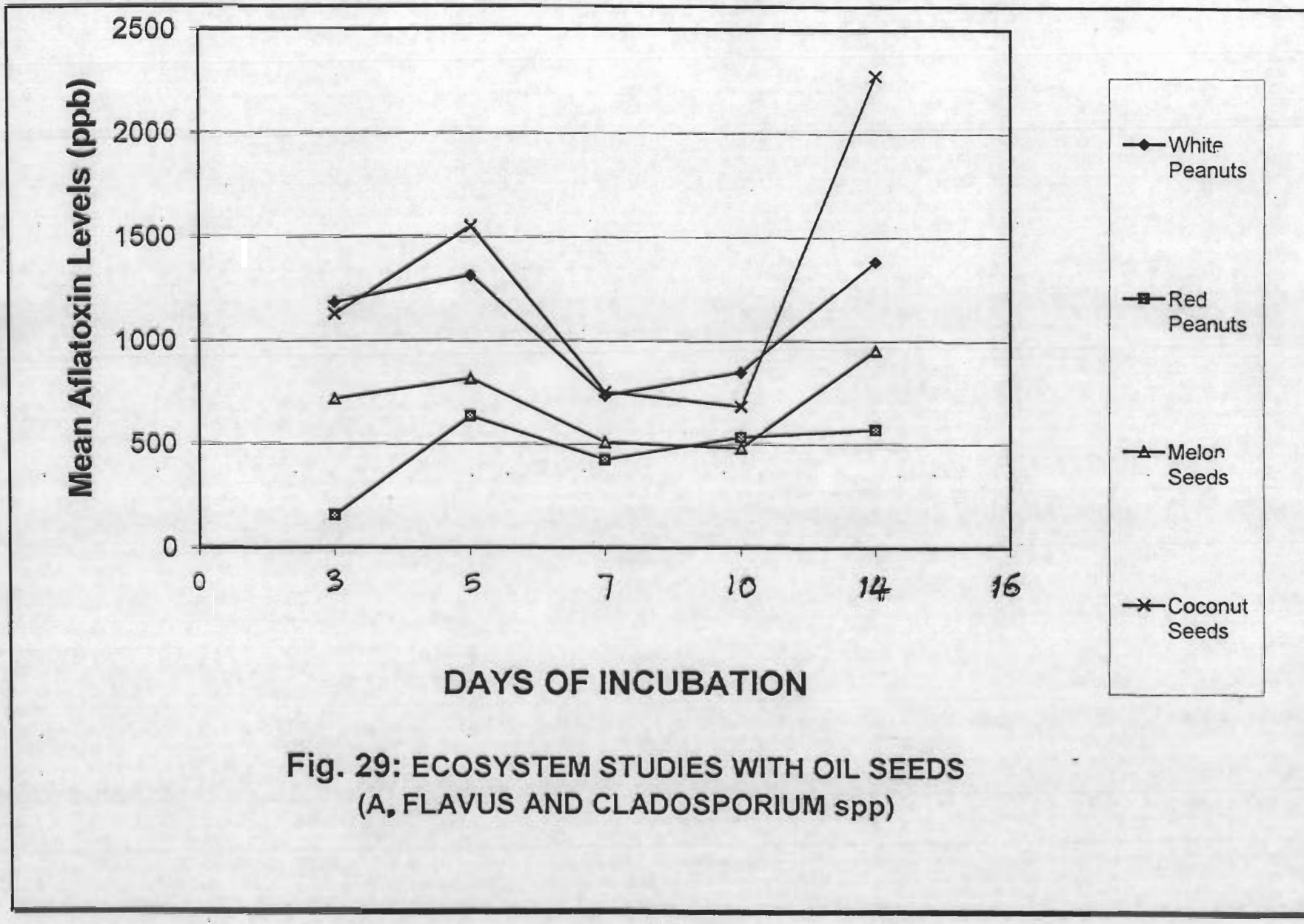


Fig. 29: ECOSYSTEM STUDIES WITH OIL SEEDS (A, FLAVUS AND CLADOSPORIUM spp)

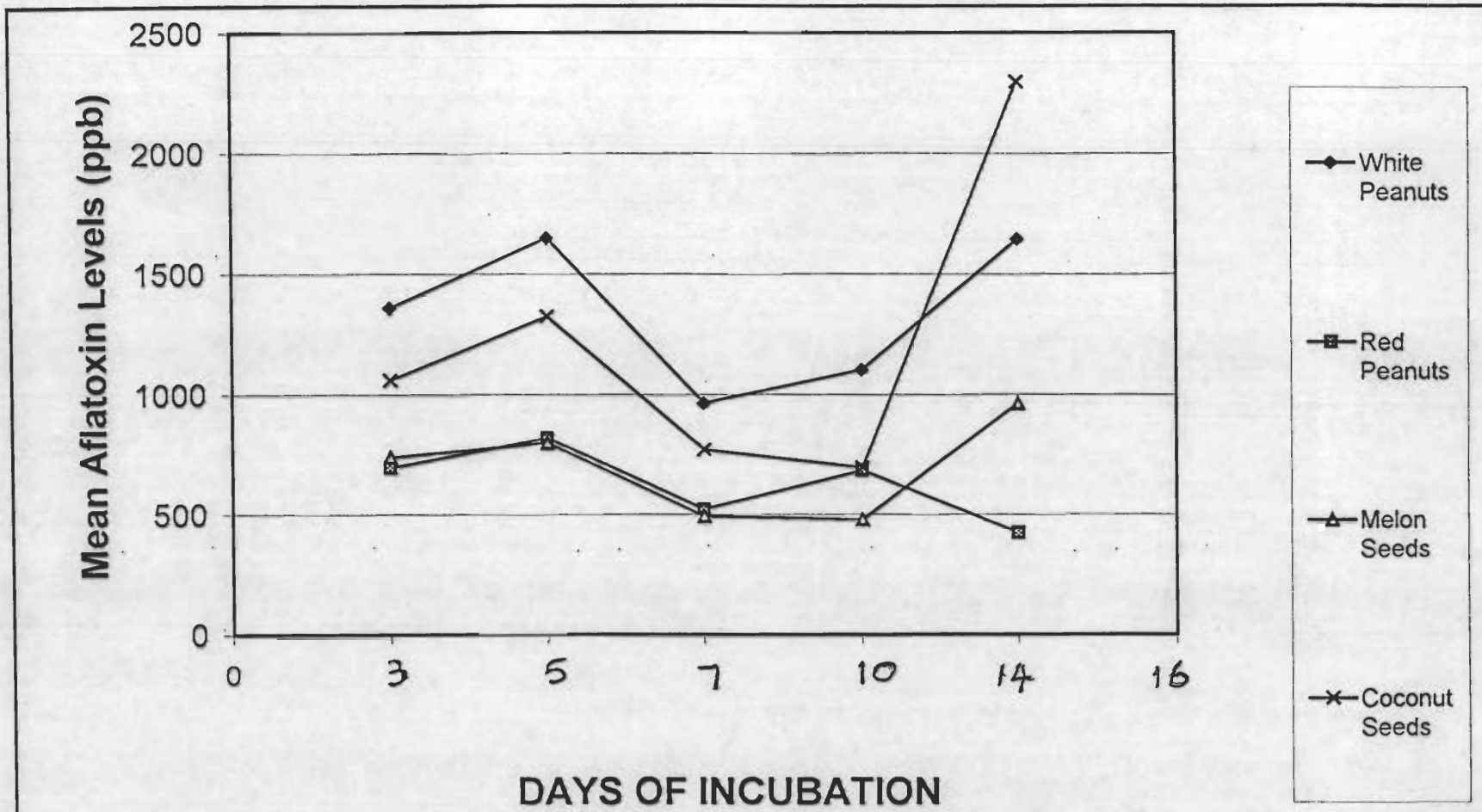


Fig. 30: ECOSYSTEM STUDIES WITH OIL SEEDS
(A. FLAVUS AND CURVULARIA spp)

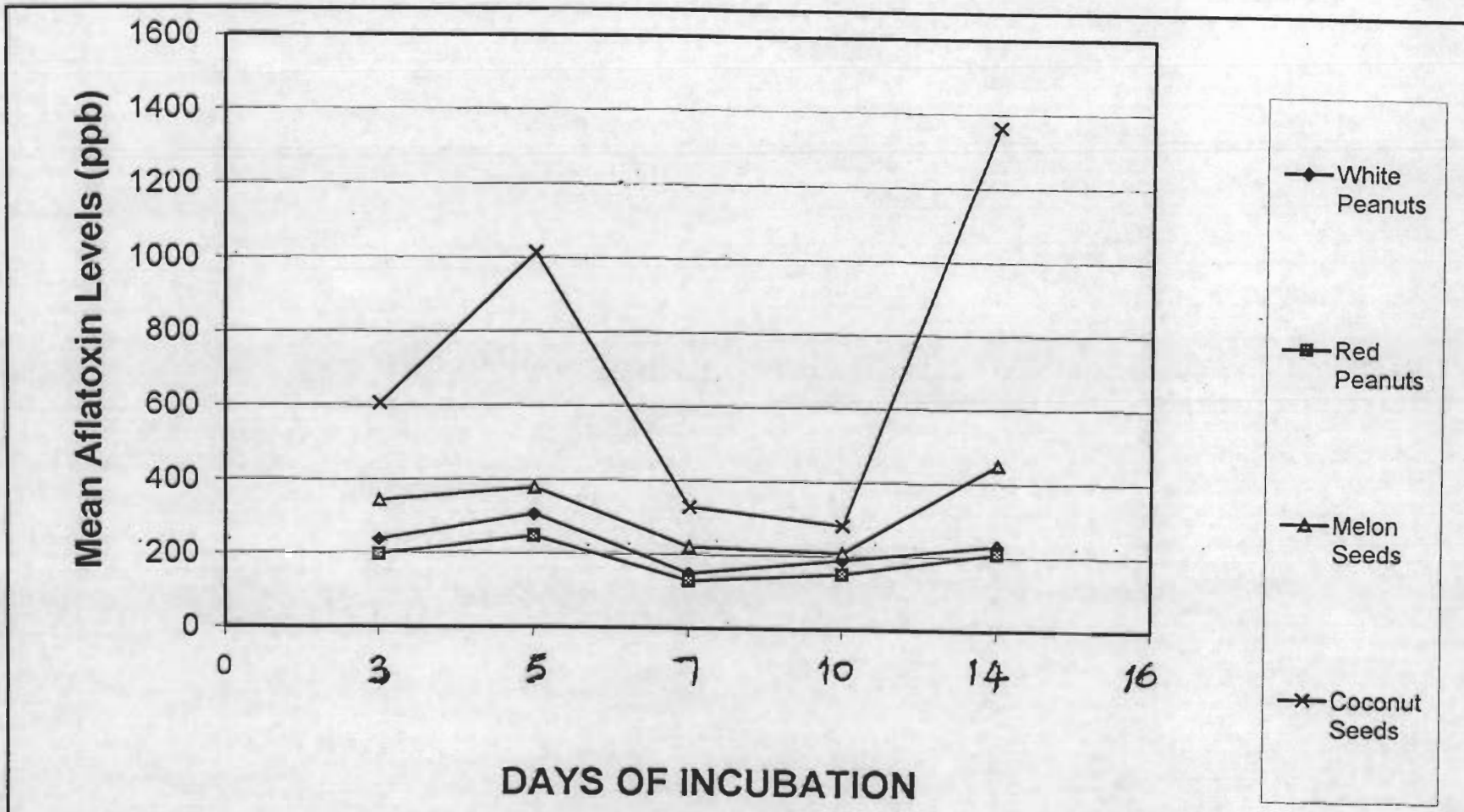


Fig. 31: ECOSYSTEM STUDIES WITH OIL SEEDS (A. FLAVUS AND FUSARIUM spp)

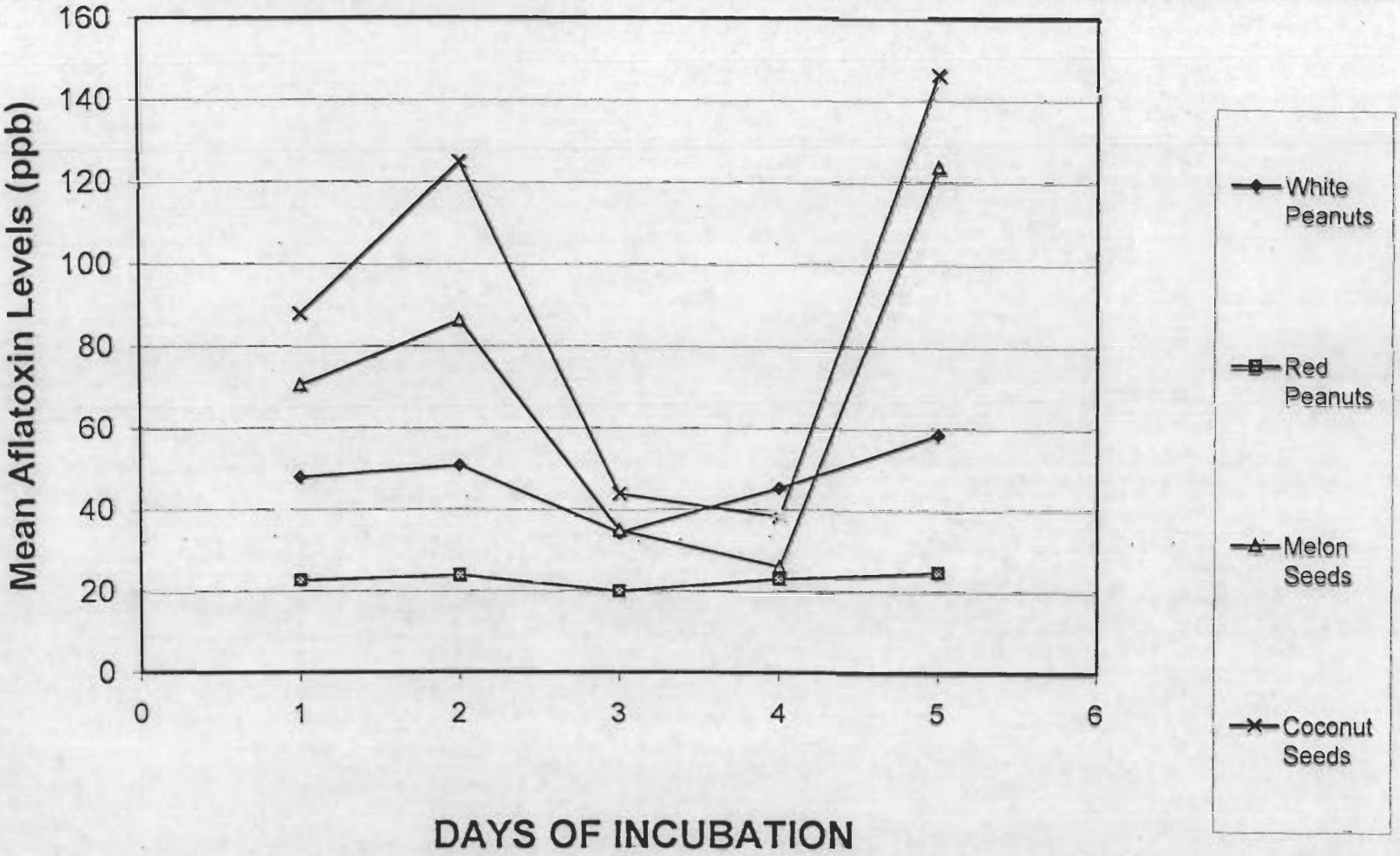


Fig 32: ECOSYSTEM STUDIES WITH FUNGI (Oil seeds)
(A. FLAVUS AND RHODOTORULA spp)

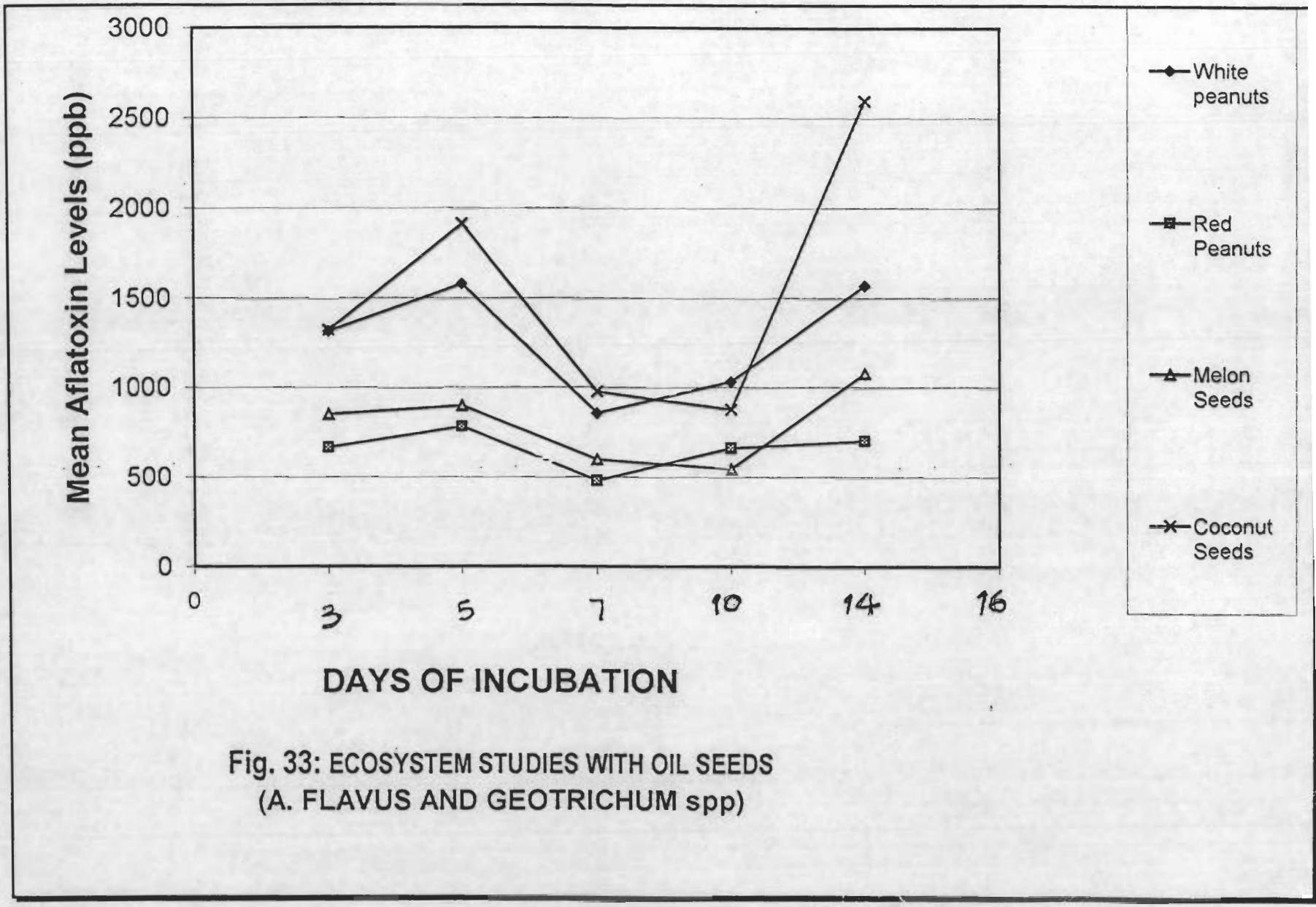


Fig. 33: ECOSYSTEM STUDIES WITH OIL SEEDS
(A. FLAVUS AND GEOTRICHUM spp)

4.5.3 The protein foods

The protein foods include cowpeas (white and brown varieties); soyabeans, breadfruits, bambara peanuts, pigeon peas (white and brown varieties) and oil bean seeds.

(i) *A. flavus in monoculture.*

Figure 37 shows the levels of aflatoxins produced when *A. flavus* was in single culture in all the protein foods. Specifically, aflatoxins B_1, B_2 and G_1 and /or G_2 were all produced depending on the food item.

The white cowpeas by day three of incubation, yielded AF levels of 84.4ppb which showed no differences up to the fourteenth day of incubation. The brown cowpeas, on the other hand, yielded significantly lower levels of aflatoxins by the third day of incubation (28.7 ppb) and similarly showed no differences again up to day fourteen of incubation. AF levels in soyabeans was quite low (3.8ppb) by the third day of incubation, then continued to increase significantly by days five, seven and about trippling by the tenth day (10.2 ppb) with a slight decrease (7.3 ppb) by the fourteen day. The pigeon peas of both varieties yielded about a similar level of aflatoxins like the brown cowpeas (22.4 – 24.2 ppb) by day three of incubation and which levels showed no differences all though the incubation periods. There was also no differences in AF yields between the brown and white pigeon peas.

Breadfruit yielded the highest AF levels among all the protein-rich foods, having 246.3 ppb by the third day and this continued to increase gradually over the days of incubation with the highest value (327.4 ppb) being observed on

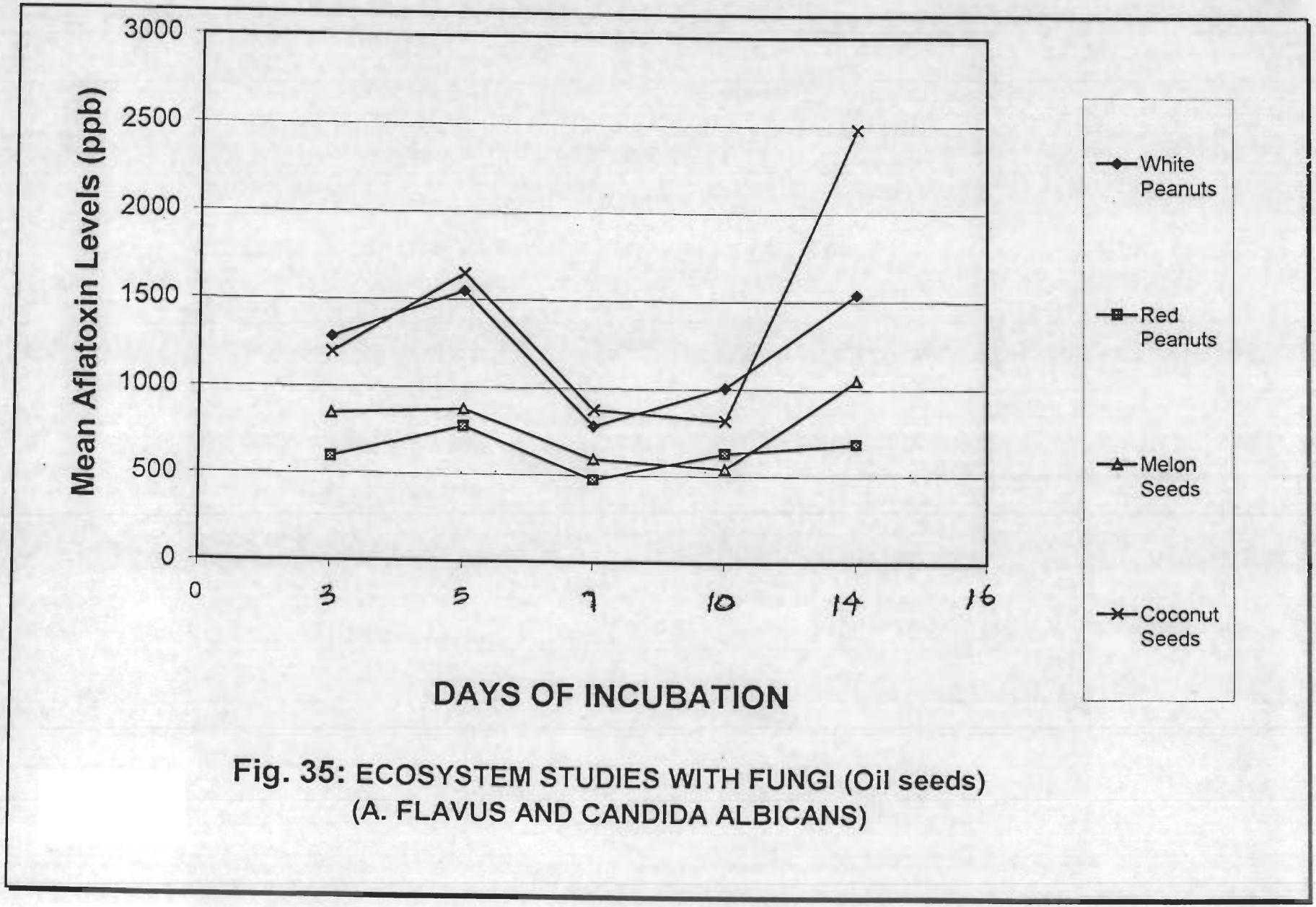


Fig. 35: ECOSYSTEM STUDIES WITH FUNGI (Oil seeds)
(A. FLAVUS AND CANDIDA ALBICANS)

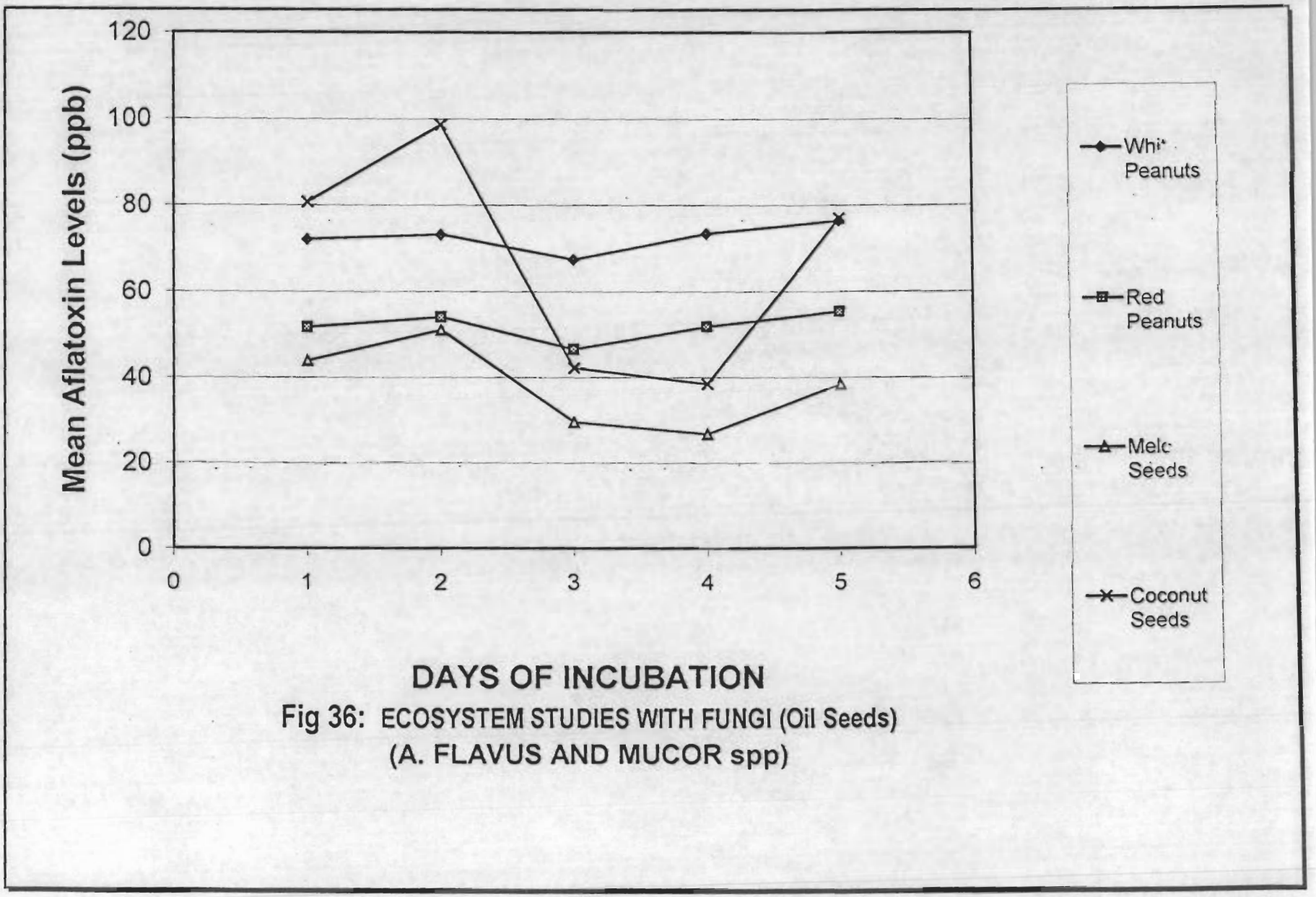


Fig 36: ECOSYSTEM STUDIES WITH FUNGI (Oil Seeds)
(A. FLAVUS AND MUCOR spp)

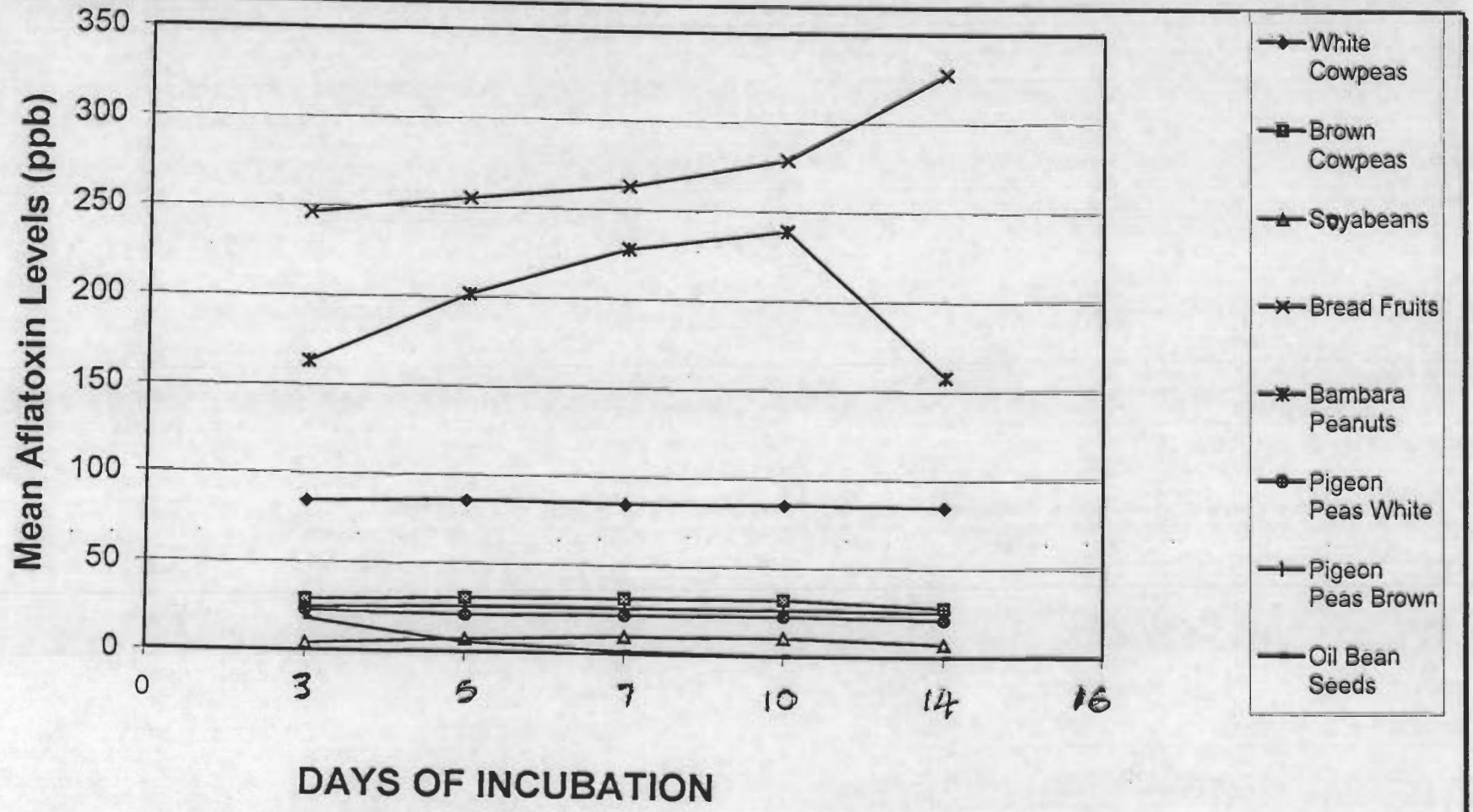


Fig. 37: ECOSYSTEM STUDIES WITH PROTEIN FOODS
(A. FLAVUS IN MONOCULTURE)

day fourteen. Bambara peanuts ranked next to breadfruits in the level of aflatoxins produced; yielding about 162.9 ppb by day three, which increased gradually typical of most of the protein foods with a drop to 157.6 ppb by day fourteen of incubation.

The oil been seeds recorded only 17.8 ppb by day three and this rather decreased significantly ($P<0.05$) over the days of incubation and getting completely abolished by the tenth day of incubation. Specifically, for the protein foods, a pattern of gradual increase was recorded in AF levels over the days of incubation, with a slight drop on the fourteenth day of incubation with the exception of breadfruits which levels continued to increase till day fourteen and the oil bean seeds that got abolished with time.

(ii) **Dual culture experiments**

Figures 38 and 39 show the effects of *Rhizopus* spp. and *Saccharomyces* spp. on AF production when in dual cultures with *A. flavus*. Both organisms decreased AF levels in all the foodstuffs except breadfruits in which there were no effects on AF levels when compared with the levels when *A. flavus* was in monospecific cultures . Both fungi also completely abolished the aflatoxins in soyabeans.

Rhizopus spp. significantly reduced the AF levels in brown cowpeas ($P<0.05$) AF levels in bambara peanuts, white and brown pigeon peas were also significantly decreased by both fungi ($P<0.05$), though *Rhizopus* spp. decreased the aflatoxins more. Aflatoxin production in the oil bean seeds were also reduced by both fungi though *Rhizopus* spp. effected it more and even, abolished the little trace of aflatoxins by day seven.

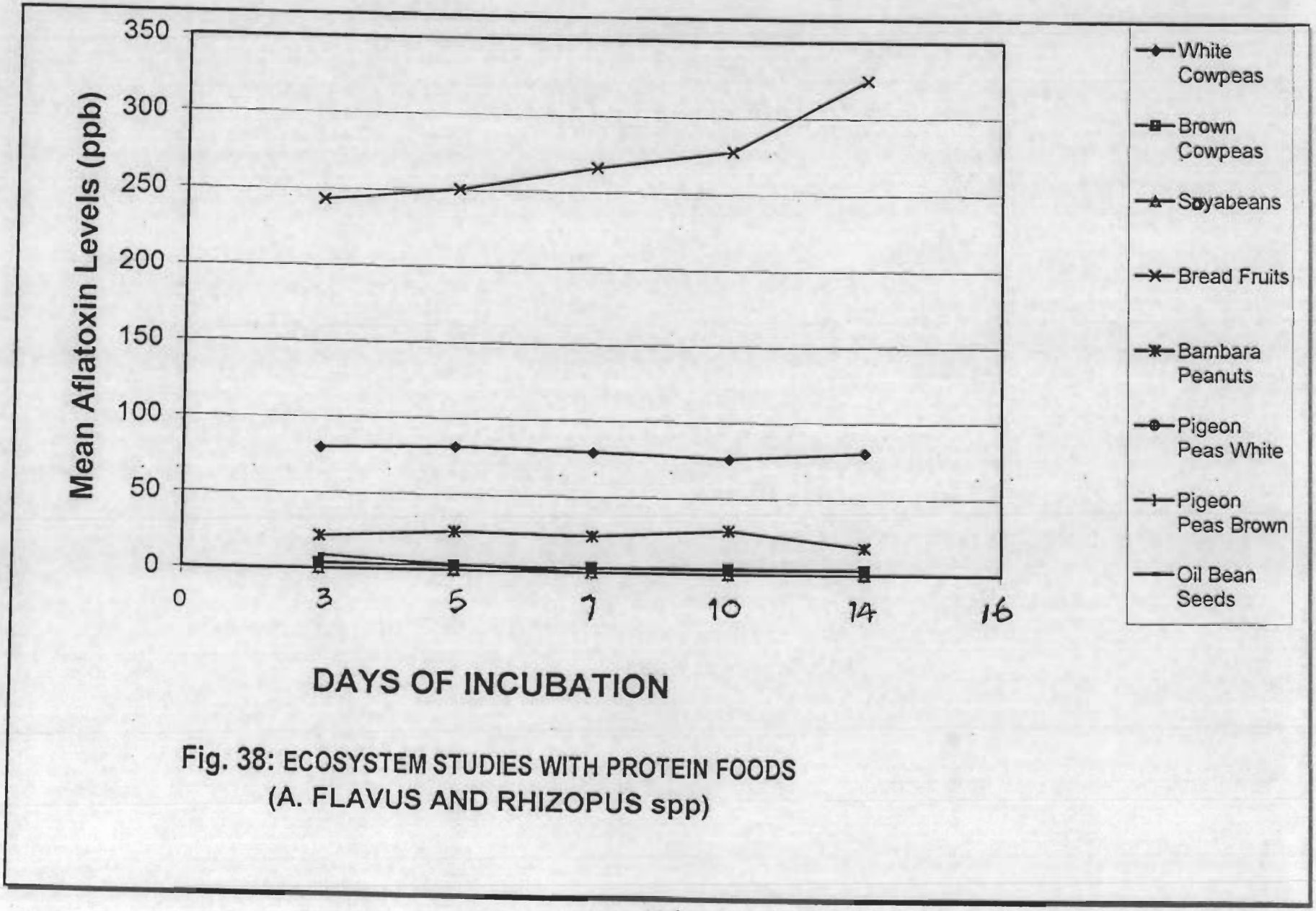


Fig. 38: ECOSYSTEM STUDIES WITH PROTEIN FOODS
(A. FLAVUS AND RHIZOPUS spp)

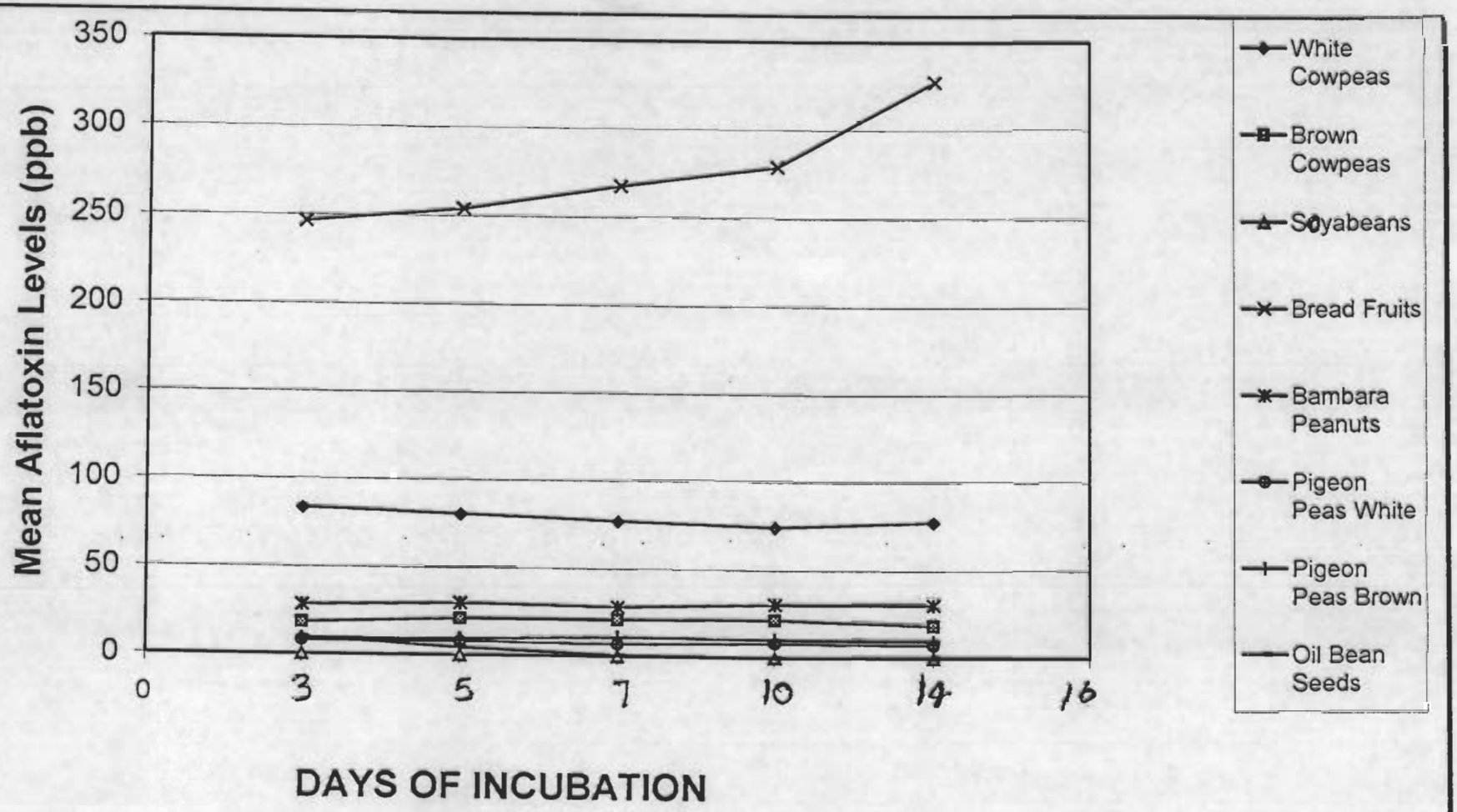


Fig. 39: ECOSYSTEM STUDIES WITH PROTEIN FOODS
(A. FLAVUS AND SACCHAROMYCES spp)

There were no differences in the aflatoxin levels in all the protein foods when *Penicillium* spp was in dual culture with *A. flavus* and when *A. flavus* was in monoculture except the slight stimulation recorded in bambara peanuts from day ten to fourteen and the higher increase recorded for the breadfruits. However, the AF was completely abolished in the oil bean seeds in the dual culture experiment. No differences in AF level were also recorded in all the protein foods except the slight increase in the white cowpeas, with *Cladosporium* spp. when in dual culture with *A. flavus.* However, AF levels in the breadfruits were significantly inhibited ($P<0.05$). The levels went down from 246.3 ppb on the third day of incubation in the monoculture study to only 32.5ppb by the third day in the dual culture study (Fig. 40 and 41).

Curvularia spp. generally increased AF levels in all the protein foods in the dual culture experiments when compared with the monoculture studies, though this increase was statistically significant in the two pigeon peas varieties and breadfruits ($P<0.05$).

There were however, no differences in the AF levels in the oil bean seeds in both studies. *Fusarium* and *Rhodotorula* species significantly decreased ($P<0.05$) AF levels in all the protein foods with the exception of the breadfruits. However, *Rhodotorula* reduced the aflatoxins more than the *Fusarium* spp. in all the food items with the exception of bambara peanuts, and even completely abolished AF production in the two pigeon peas (Figs.39, 40, 41).

Geotrichum and *Candida* species had a mixed trend on the production of aflatoxins in the different protein foods. Both yeasts stimulated AF production in

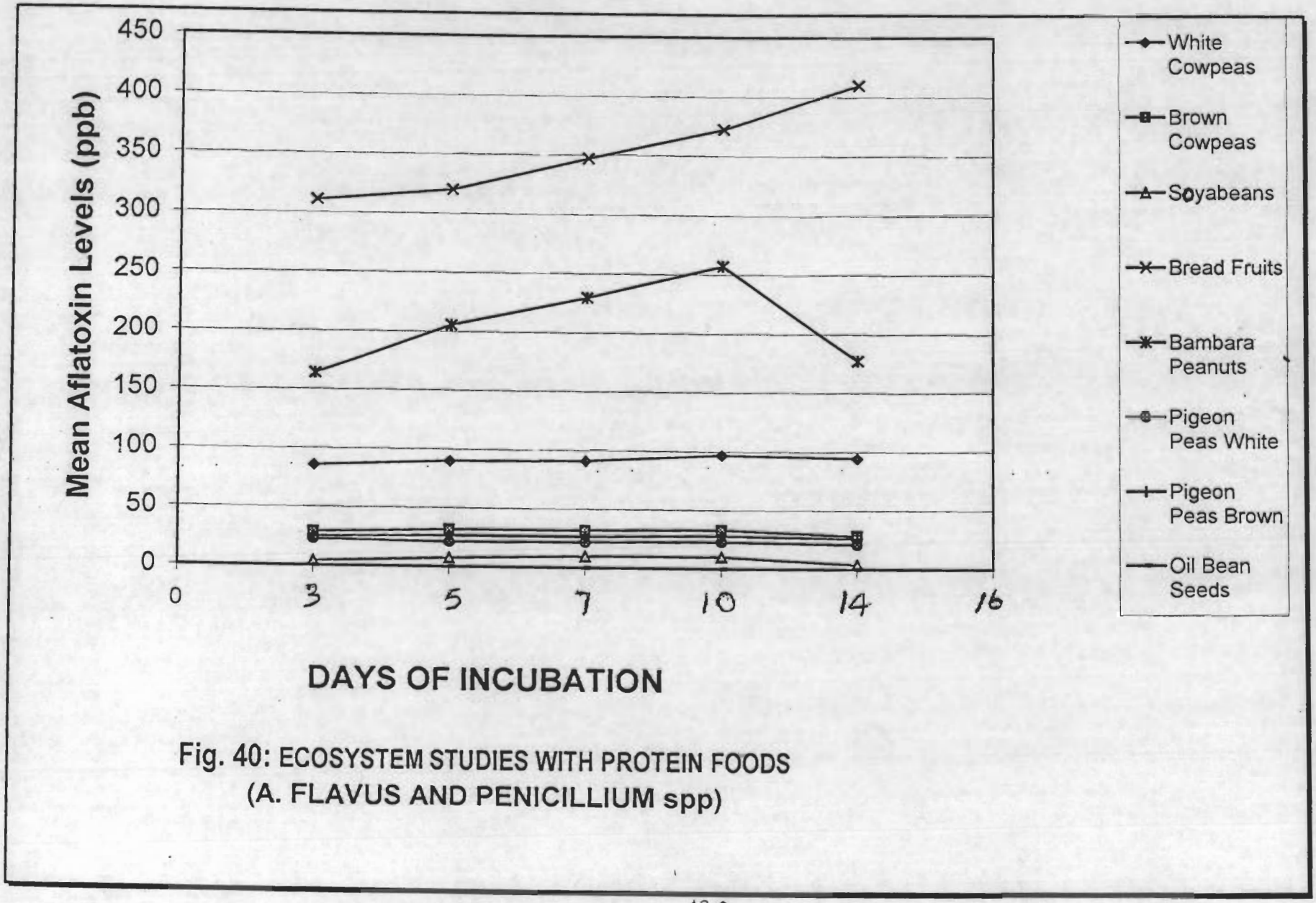


Fig. 40: ECOSYSTEM STUDIES WITH PROTEIN FOODS
(A. FLAVUS AND PENICILLIUM spp)

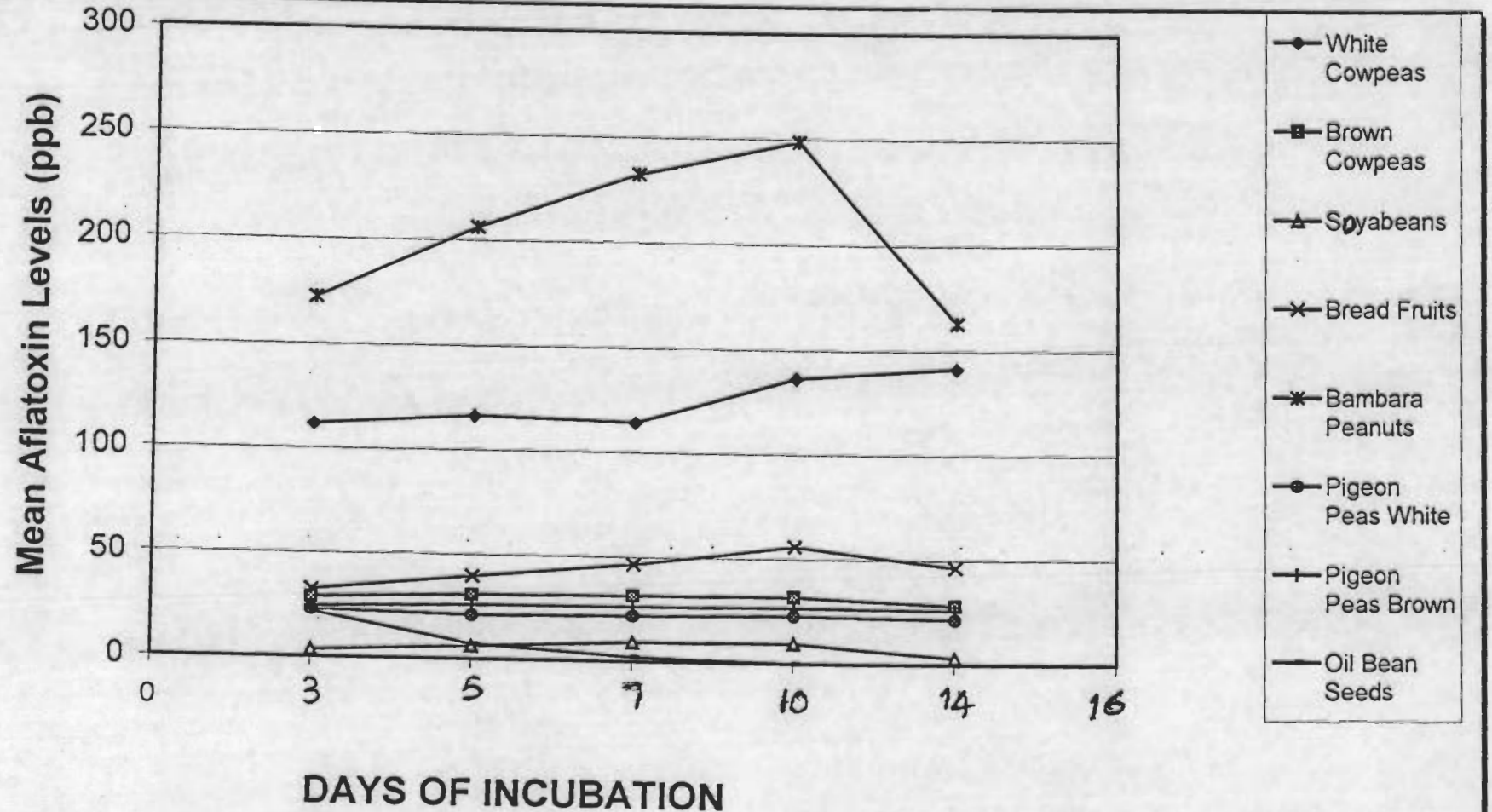


Fig. 41: ECOSYSTEM STUDIES WITH PROTEIN FOODS
(A. FLAVUS AND CLADOSPORIUM spp)

the two varieties of pigeon peas while having no effects in the oil been seeds and brown cowpeas. *Geotrichum* spp., however, also had no effects in bambara peanuts while *Candida* spp. stimulated AF production in this foodstuff. Both yeasts also decreased aflatoxin levels in the breadfruit though significantly with *Geotrichum* spp. ($P<0.05$). *Candida* spp. had no effects on AF production in soyabeans while *Geotrichum* spp. slightly increased the levels. Also, while the levels of aflatoxins were stimulated in the white cowpeas by *Candida albicans, Geotrichum* species decreased it (Figs 45, 46).

Mucor spp. when in dual culture with *A. flavus* in white cowpeas and breadfruits, stimulated the production of aflatoxins but significantly decreased same in bambara peanuts, the pigeon peas and the brown cowpeas ($P<0.05$) while completely abolishing same in soyabeans, and oil bean seeds (Fig. 47).

Trichoderma spp., *A. niger* and *A. fumigatus* all abolished the production of aflatoxins in all the protein-rich foods in this study.

4.5.4 The carbohydrate foods

The carbohydrate foods include plantain (ripe and unripe varieties), cassava, yam and cocoyam.

(i) ***A. flavus* in monocluture**

Cassava, yam and cocoyam all produced aflatoxins B_1 and /or B_2, G_1 and /or G_2. Ripe plantain produced the highest aflatoxin levels about 112.5ppb by the third day of incubation, followed by yam (98.6ppb), cocoyam (30.8 ppb), cassava (13.5ppb) and unripe plantain (12.8ppb). A similar trend was established

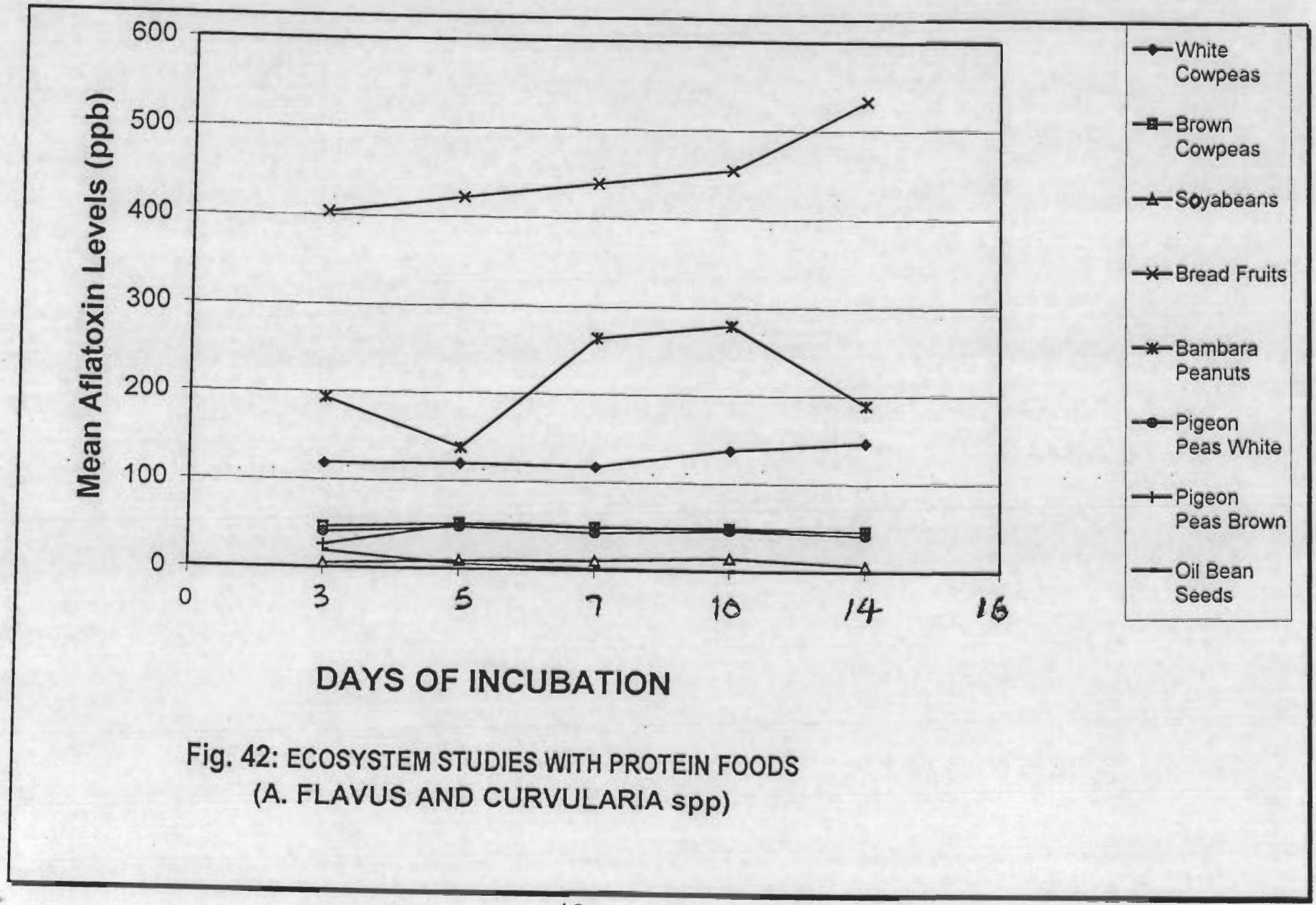


Fig. 42: ECOSYSTEM STUDIES WITH PROTEIN FOODS
(A. FLAVUS AND CURVULARIA spp)

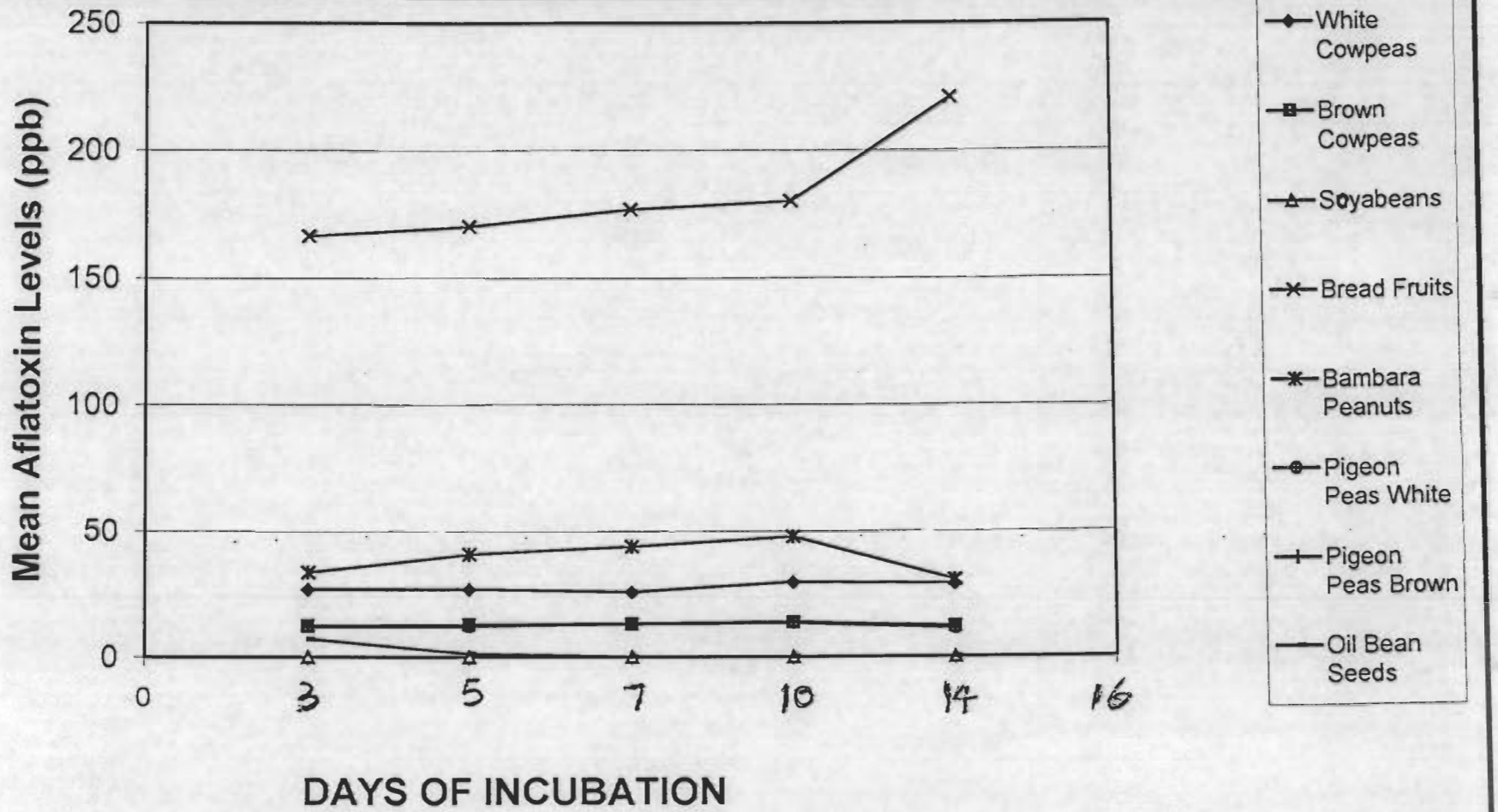


Fig. 43: ECOSYSTEM STUDIES WITH PROTEIN FOODS
(A. FLAVUS AND FUSARIUM spp)

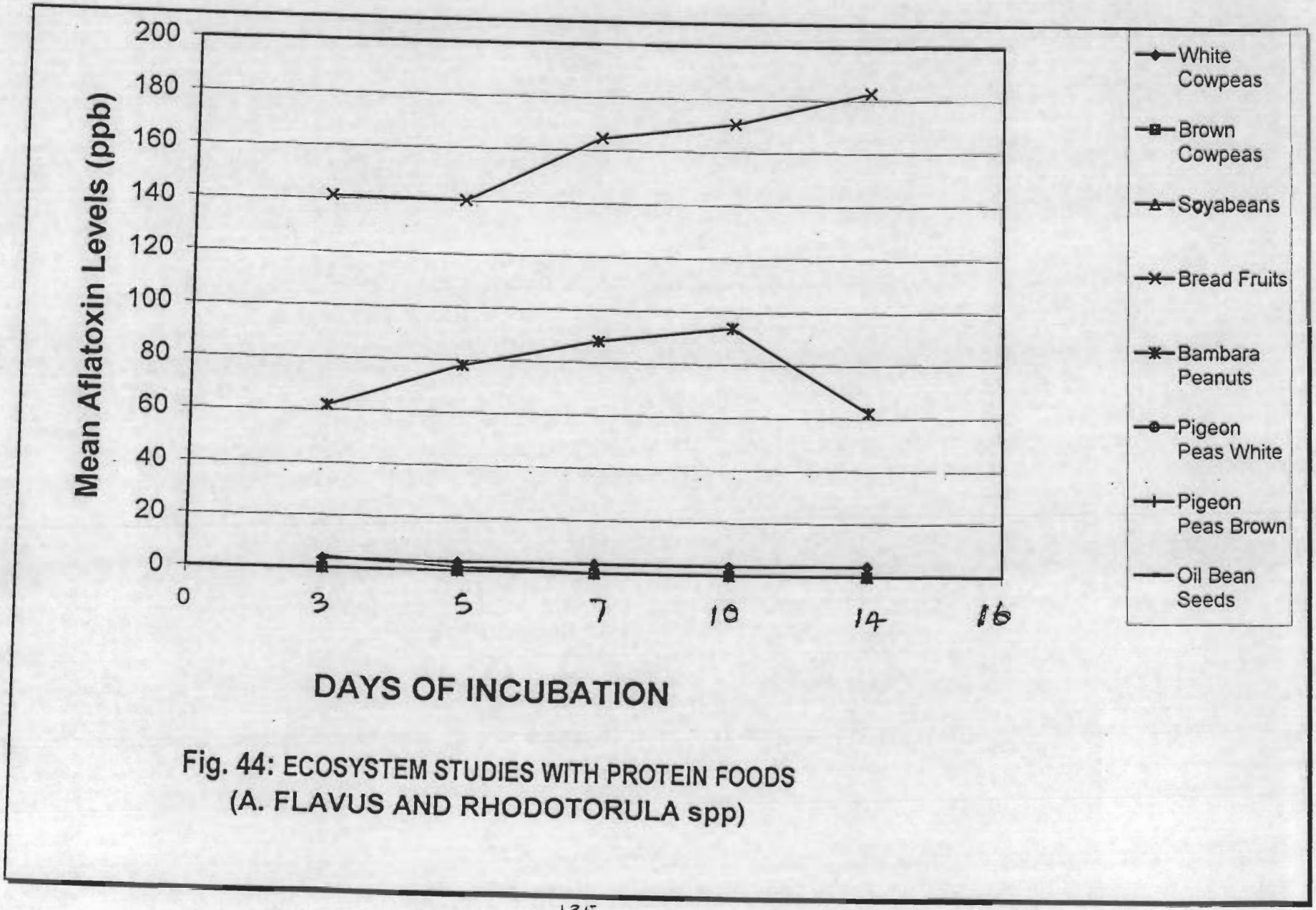


Fig. 44: ECOSYSTEM STUDIES WITH PROTEIN FOODS
(A. FLAVUS AND RHODOTORULA spp)

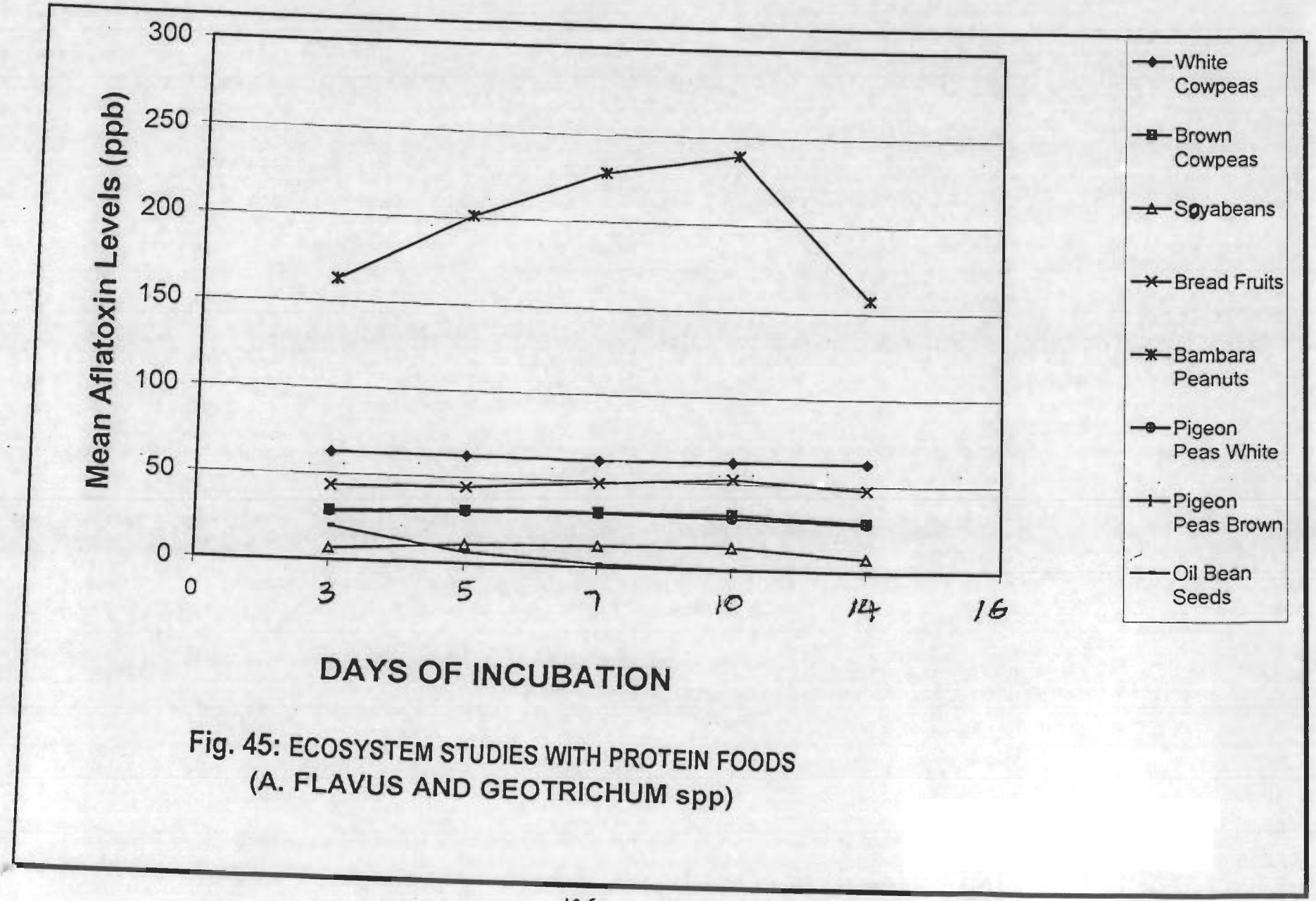


Fig. 45: ECOSYSTEM STUDIES WITH PROTEIN FOODS
(A. FLAVUS AND GEOTRICHUM spp)

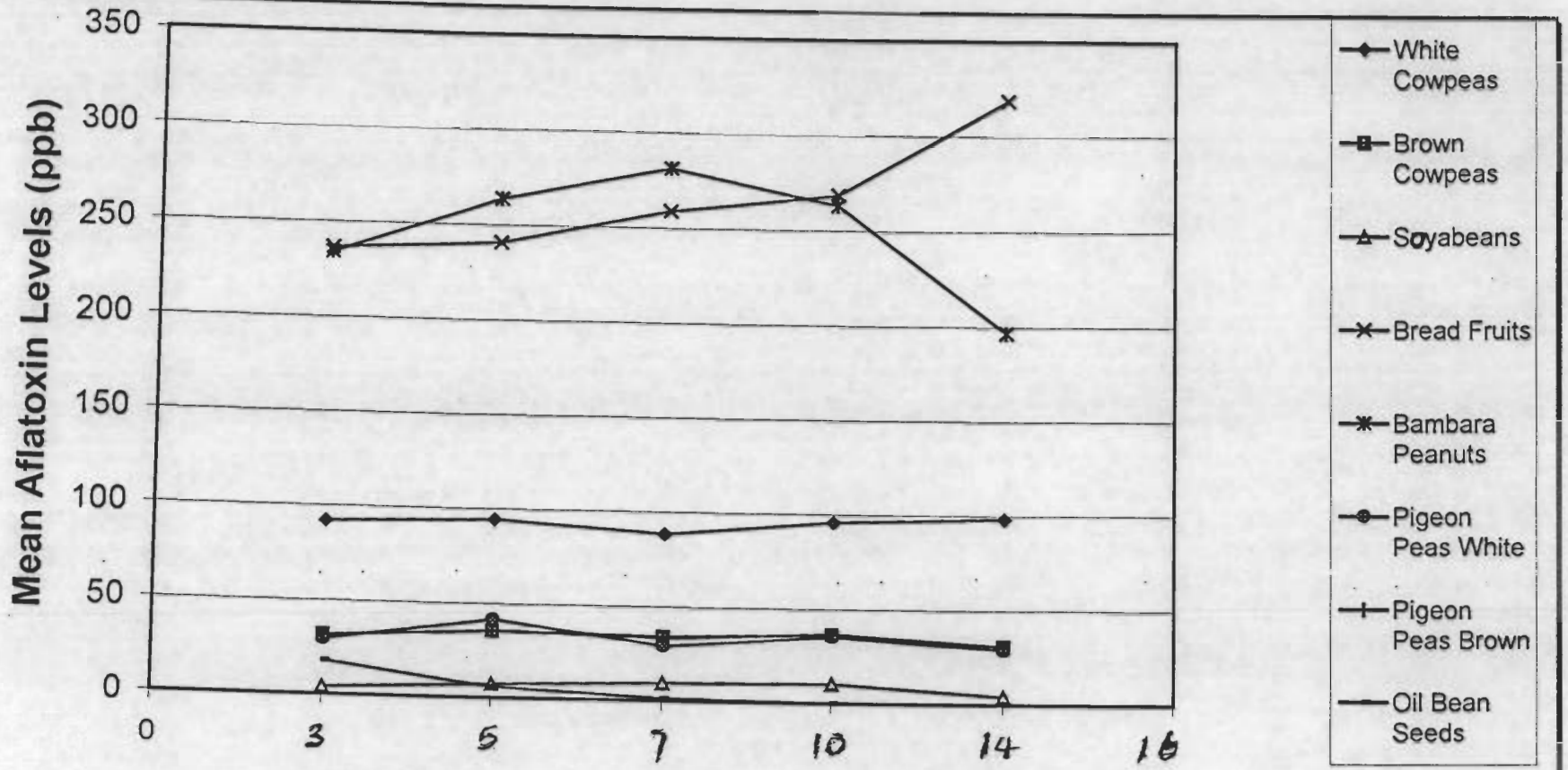


Fig. 46: ECOSYSTEM STUDIES WITH PROTEIN FOODS
(A. FLAVUS AND CANDIDA ALBICANS)

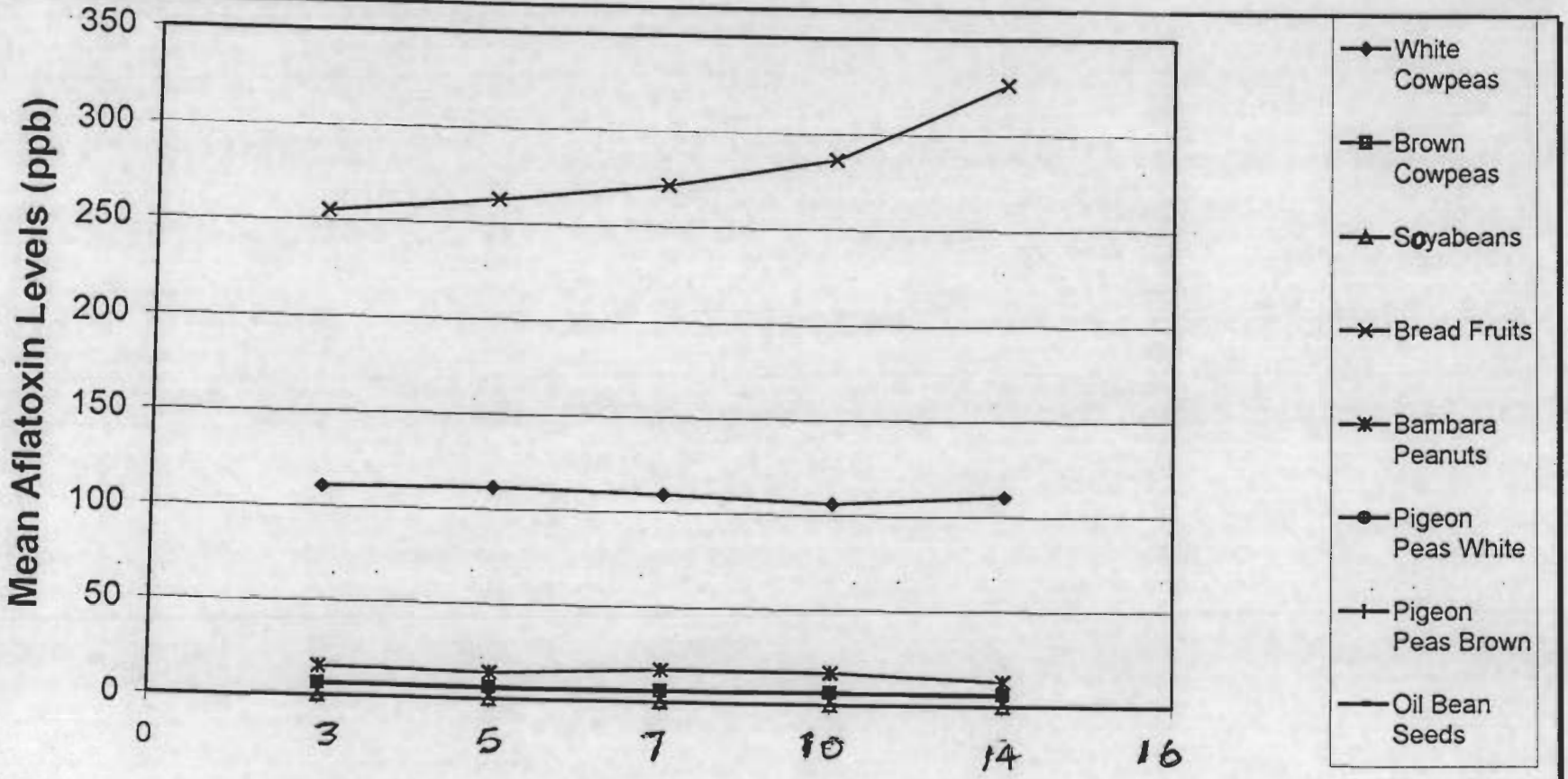


Fig. 47: ECOSYSTEM STUDIES WITH PROTEIN FOODS (A. FLAVUS AND MUCOR spp)

as for the cereal grains. The aflatoxins decreased over the days of incubation, yielding the least levels on the fourteenth day of incubation (Fig.48).

(ii) **Dual culture experiments.**

When *A. flavus* was in dual culture with *Rhizopus* spp., *Saccharomyces, A. fumigatus* and *Mucor* spp. respectively in the carbohydrate foods:, AF production was completely abolished in cassava, yam and cocoyam. *A. fumigatus* and *Mucor* spp. additionally abolished aflatoxin production in unripe plantains also. These four fungi also significantly decreased AF levels in the remaining carbohydrate foods (ripe and /or unripe plantains), ($P<0.05$).

Penicillium spp. significantly ($P<0.05$), decreased AF levels in all the carbohydrate foods while completely inhibiting AF production in cassava and cocoyam. Specifically too, the AFB_1 were either completely inhibited like in the yams or significantly reduced like in the plantains, while the AFB_2 was slightly increased. The AFG_1/G_2 were also reduced (Fig. 46). Figs 47 and 48 show the effects of *Cladosporium* and *Curvularia* species on aflatoxin production when in dual cultures with *A. flavus* in the foodstuffs. Both fungi had stimulatory effect on the aflatoxin production in the two plantains and yam especially with *Curvularia* spp. However, no effects were recorded for cassava and cocoyam.

Fusarium and *Rhodotorula* species both reduced aflatoxin levels in all the carbohydrate foods significantly ($P<0.05$) and completely abolished same in cassava, though *Rhodotorula* spp. also abolished aflatoxins in cocoyam too. Comparatively, however, *Rhodotorula* spp. had more serious effects on AF than the *Fusarium* spp.

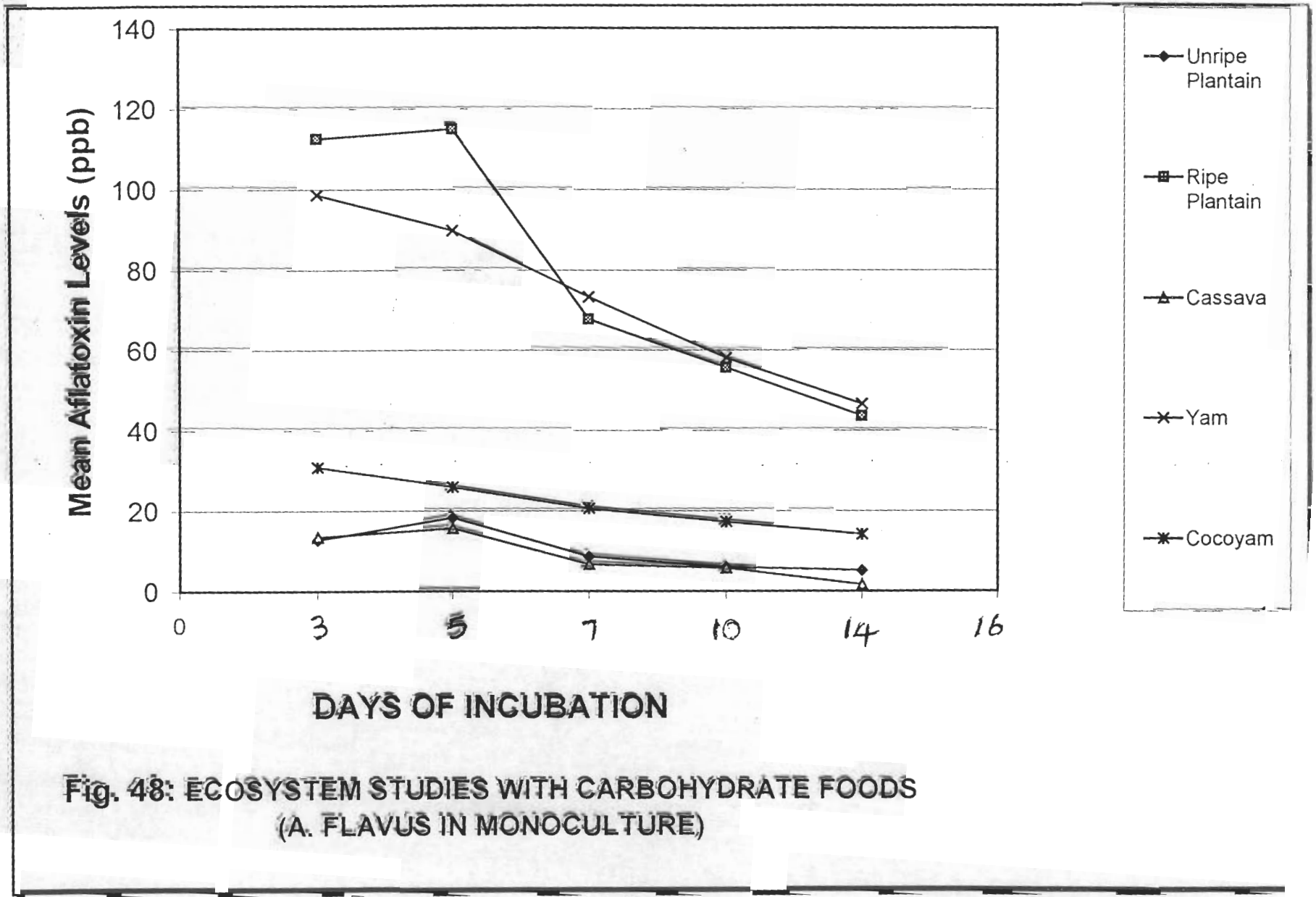


Fig. 48: ECOSYSTEM STUDIES WITH CARBOHYDRATE FOODS
(A. FLAVUS IN MONOCULTURE)

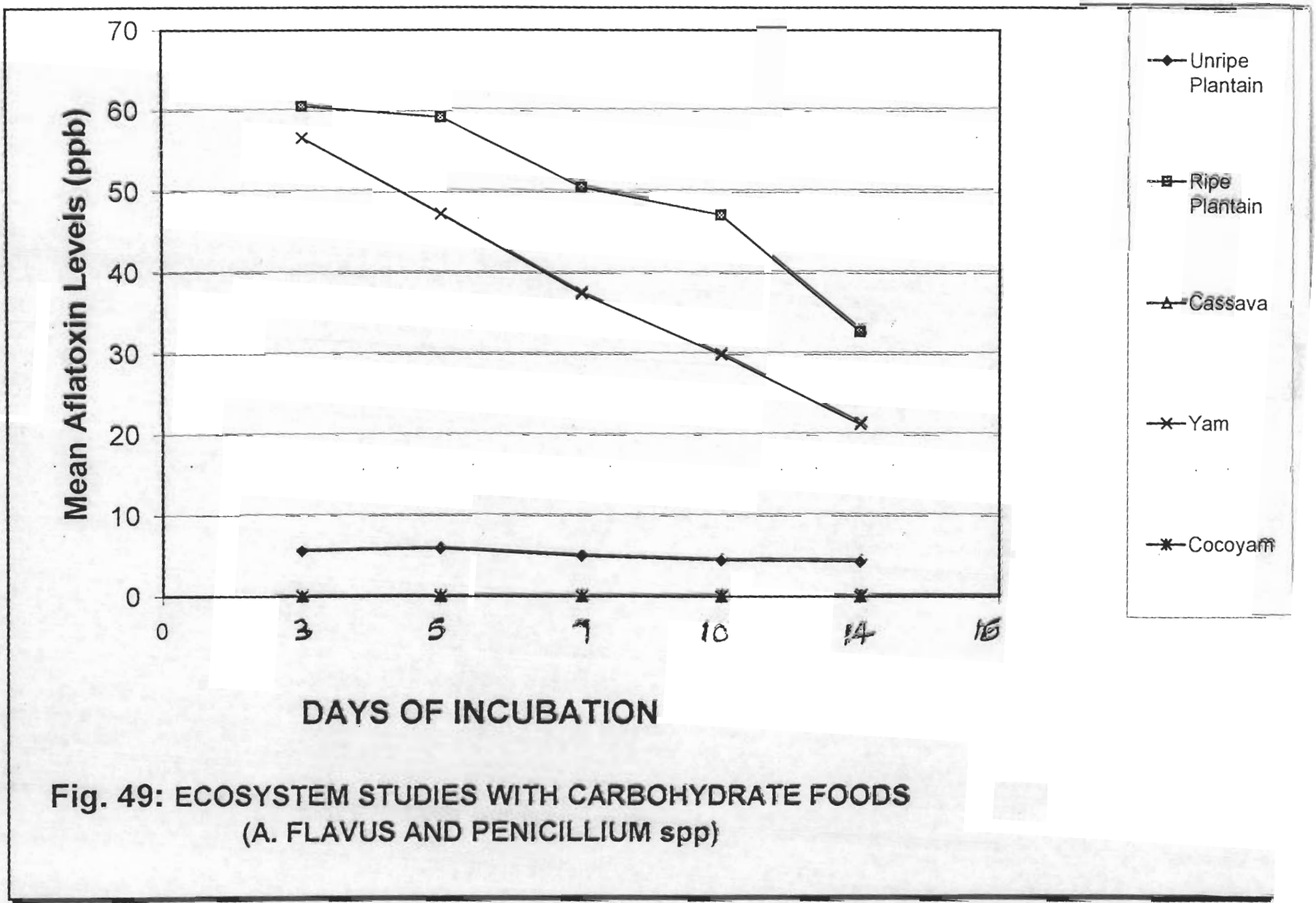


Fig. 49: ECOSYSTEM STUDIES WITH CARBOHYDRATE FOODS (A. FLAVUS AND PENICILLIUM spp)

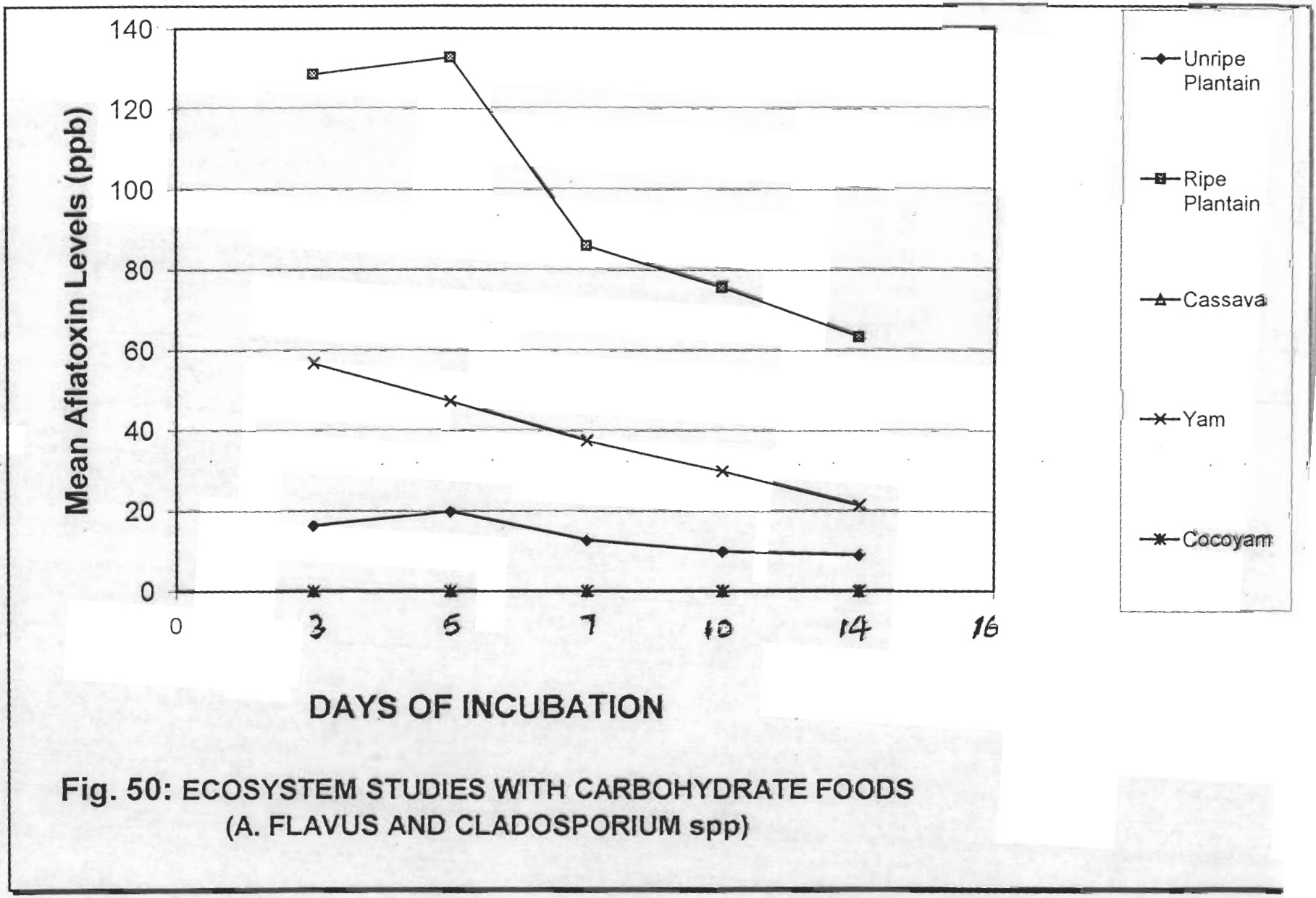


Fig. 50: ECOSYSTEM STUDIES WITH CARBOHYDRATE FOODS (A. FLAVUS AND CLADOSPORIUM spp)

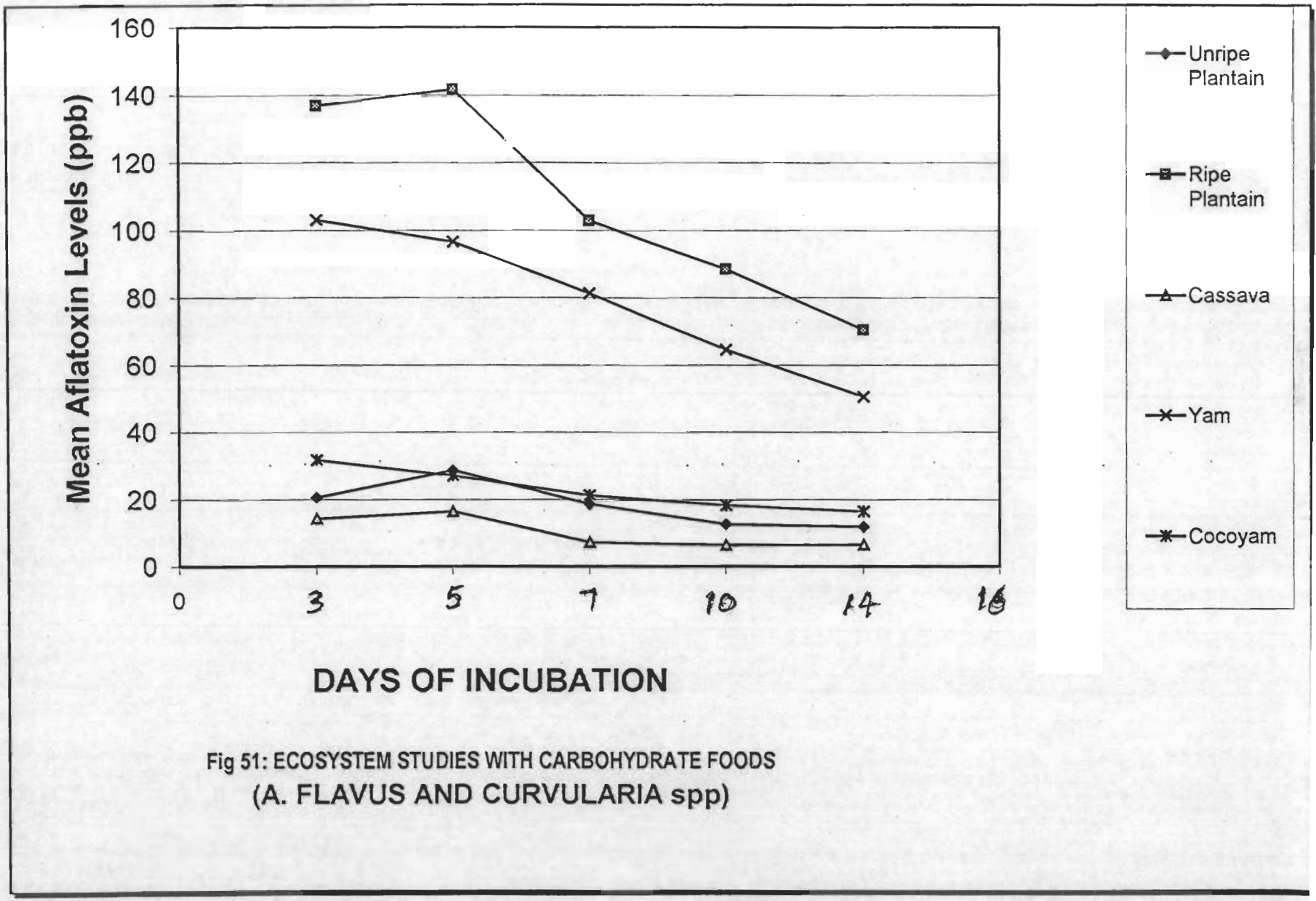


Fig 51: ECOSYSTEM STUDIES WITH CARBOHYDRATE FOODS
(A. FLAVUS AND CURVULARIA spp)

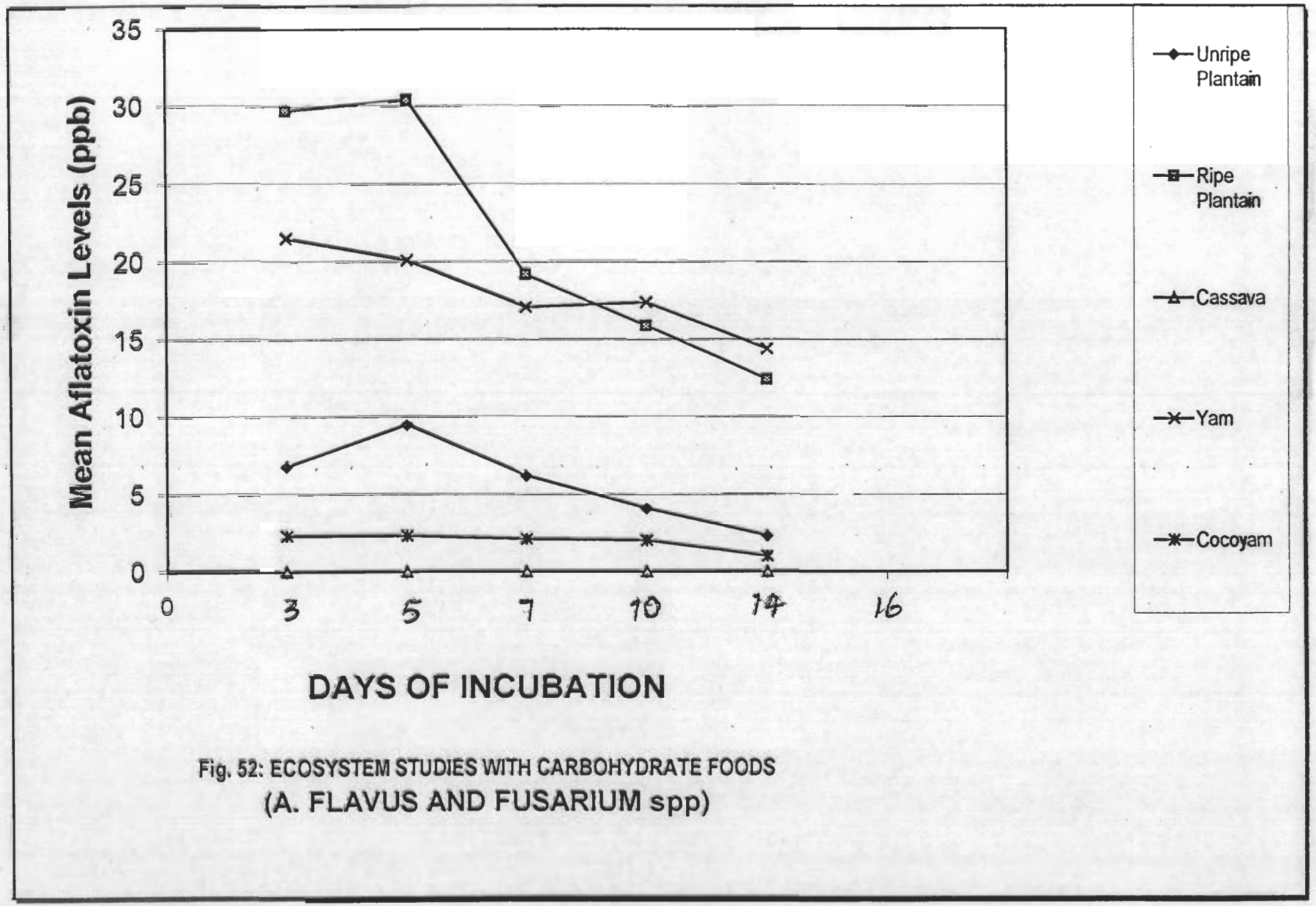


Fig. 52: ECOSYSTEM STUDIES WITH CARBOHYDRATE FOODS
(A. FLAVUS AND FUSARIUM spp)

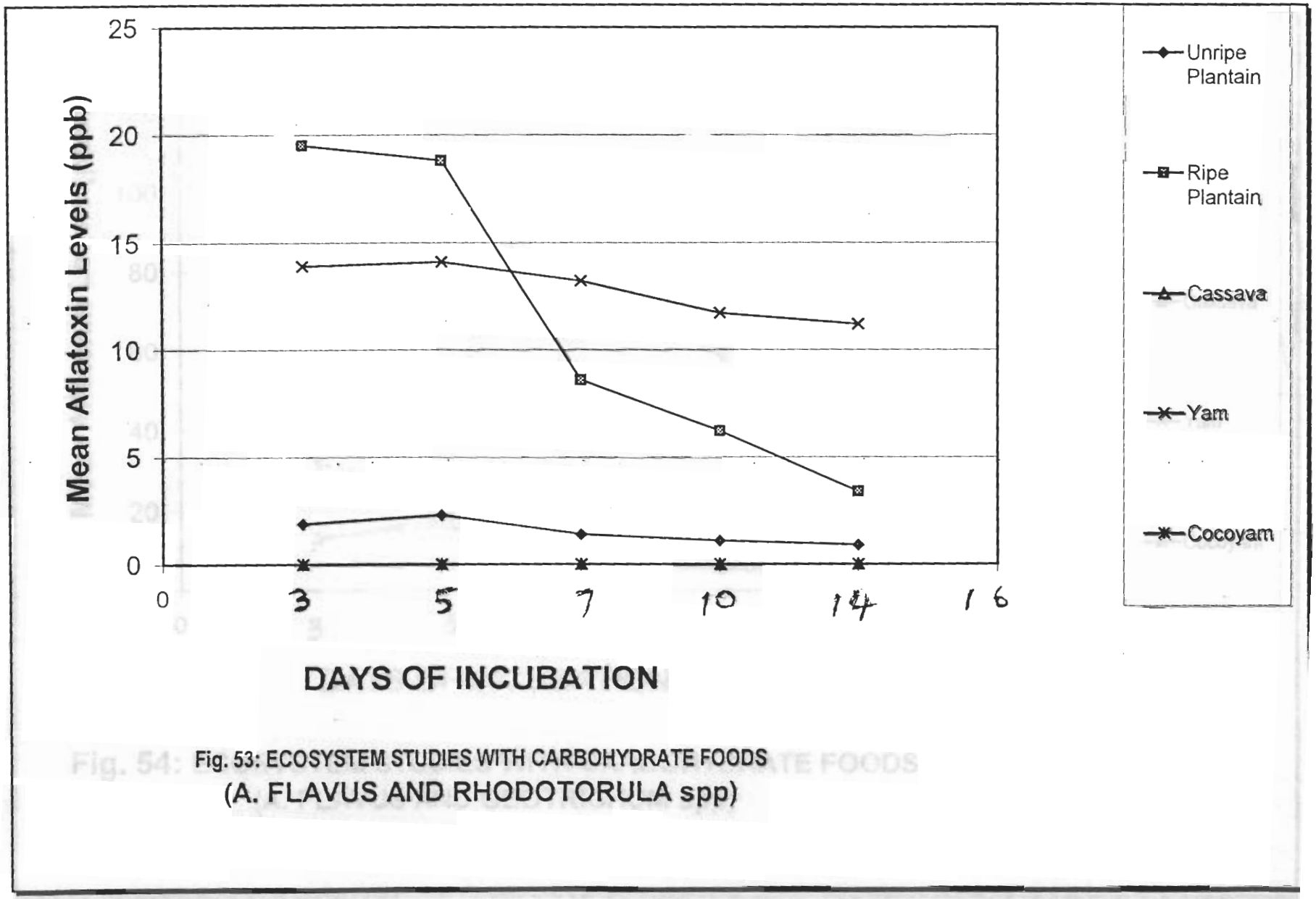


Fig. 53: ECOSYSTEM STUDIES WITH CARBOHYDRATE FOODS
(A. FLAVUS AND RHODOTORULA spp)

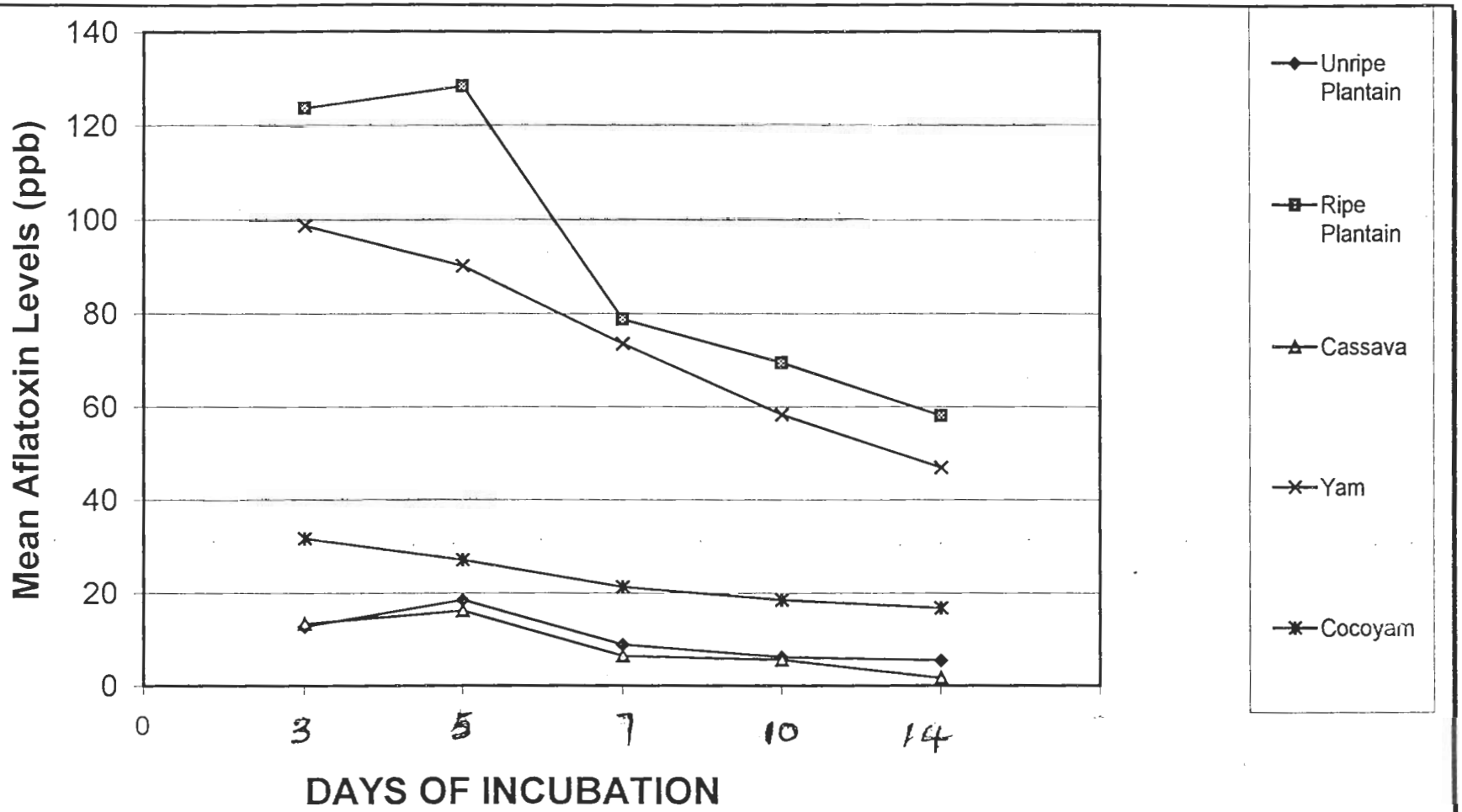


Fig. 54: ECOSYSTEM STUDIES WITH CARBOHYDRATE FOODS
(A. FLAVUS AND GEOTRICHUM spp)

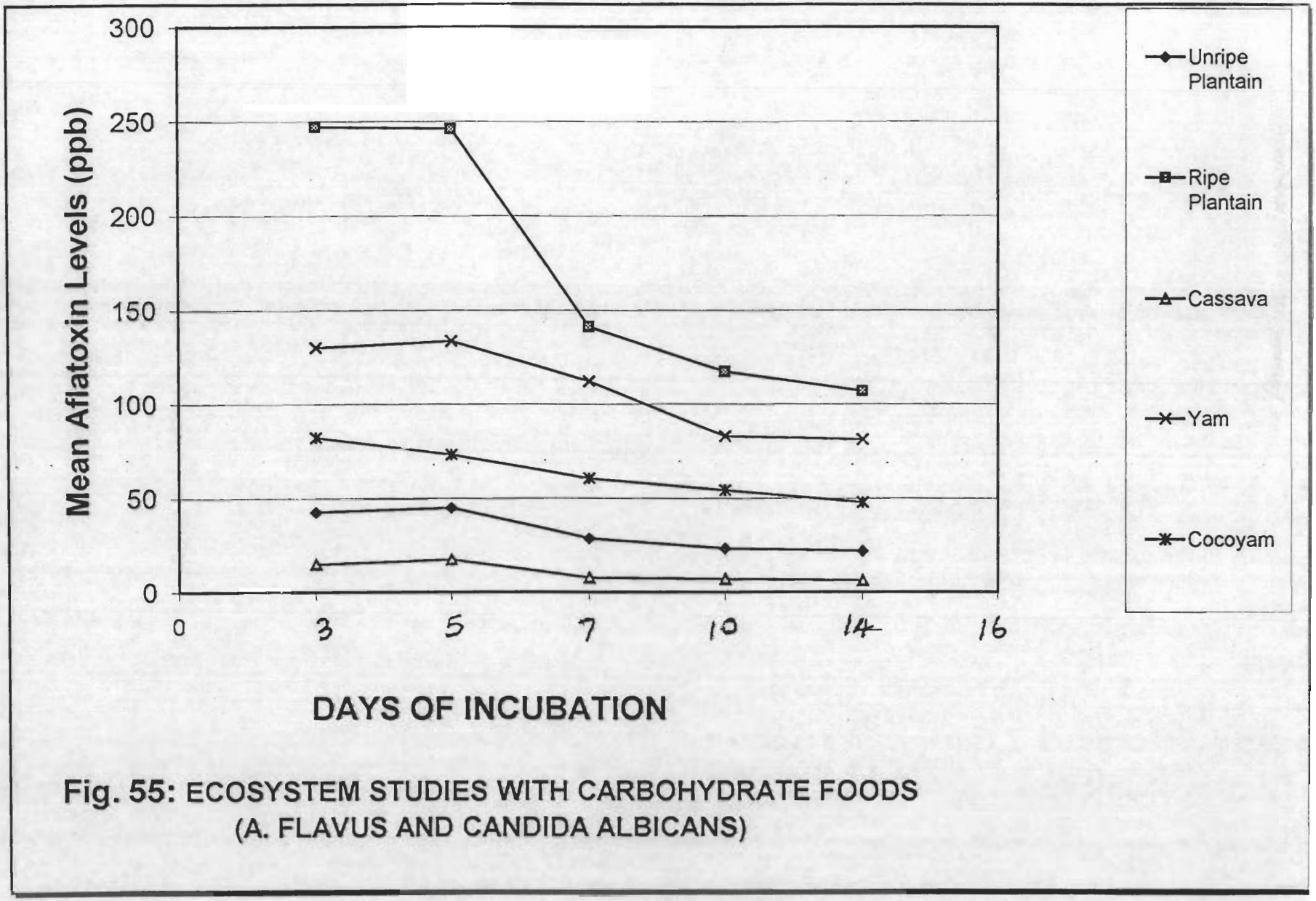


Fig. 55: ECOSYSTEM STUDIES WITH CARBOHYDRATE FOODS
(A. FLAVUS AND CANDIDA ALBICANS)

Geotrichum spp. on the other hand, had no effects on aflatoxin production in all the carbohydrate foods, except the ripe plantains where there was a slight stimulation. (Figs 52, 53, 54).

Candida albicans in dual culture with *A. flavus* when compared with *A.flavus* in monospecific cultures stimulated aflatoxins production in all the carbohydrate foods but this increase was significant in the two plantain varieties and cocoyam ($P<0.05$) (Fig 55). *Trichoderma* species and *A. niger* both completely inhibited aflatoxin production in all the carbohydrate foods.

4.6 FOOD SUBSTRATES AND BACTERIA

Each set of experiment involved different food classes (cereals, oil seeds, protein foods, and carbohydrate foods) with *A.flavus* in monospecific culture and then *A. flavus* and a bacterium, each in a dual culture in the individual foods.

4.6.1 The cereals

The cereals in the study are made up of maize (white and yellow varieties) acha grains, sorghum (white and yellow varieties), millet and rice (Abakaliki and Ugbawka varieties).

(i) *A.flavus* in monoculture:

Table 8 shows the results of the ecosystem effects in the cereals. A general pattern was established with the *A. flavus* monoculture studies in the cereals. AF levels decreased with time, with the lowest levels detected on day fourteen as in the previous experiments with fungi.

TABLE 8 EFFECTS OF MICROBIAL ECOSYSTEM STUDIES WITH BACTERIA ON AFLATOXIN PRODUCTION IN CEREALS (MEAN AFLATOXIN LEVELS IN PPB).

S/N	FOOD	DAYS OF INCUBATION					BACTERIA
		3	5	7	10	14	
1	White Maize	133.1	154.4	109.4	93.5	79.5	Plus
2	Yellow Maize	206.6	201.3	185.6	154.2	126.9	Aspergillus
3	Acha	17.3	13.9	8.9	8.0	7.2	flavus alone
4	White Sorghum	367.9	325.3	186.9	123.3	89.2	(control)
5	Yellow Sorghum	290.6	148.4	110	84.7	59.9	
6	Millet	480.3	395.1	337.8	273.3	142	
7	Abakaliki Rice	238.2	210.6	189.5	152.8	109.3	
8	Ugbawka Rice	91.8	103.1	68.9	48.6	31.4	
1	White Maize	155.8	160	116.9	100	96.1	Plus Bacillus
2	Yellow Maize	216	225.3	2105	169.5	156.6	subtilis
3	Acha	17.5	14.2	9.2	8.3	7.5	
4	White Sorghum	374.7	341.7	195.2	146	116.6	
5	Yellow Sorghum	316	158.5	125.6	99.1	69	
6	Millet	576.5	512.6	404.8	372.6	215.2	
7	Abakaliki Rice	259.3	241.3	214.8	171.1	131.2	
8	Ugbawka Rice	95.8	111.8	75.1	57.1	40.4	
1	White Maize	132.8	128.3	95.3	73.1	58.5	Plus
2	Yellow Maize	176.2	155.1	144.2	132.2	106.8	Pseudomonas
3	Acha	17.0	13.8	8.9	8.0	7.2	spp.
4	White Sorghum	355.9	284.1	145.5	93.7	58.9	
5	Yellow Sorghum	289.9	141.9	102.6	79.9	56.6	
6	Millet	473.4	389.9	333.2	270.4	138.8	
7	Abakaliki Rice	209.7	105.4	87.7	61.7	45.9	
8	Ugbawka Rice	77.5	38.8	26.7	21.5	15.5	
1	White Maize	172.3	207.2	226.2	220.3	218.2	Plus
2	Yellow Maize	315.9	330.3	341.8	349.8	363.11	Acetobacter
3	Acha	35.2	36.8	43.7	40.3	40.1	aceti
4	White Sorghum	437.8	401.2	238.9	190	144.4	
5	Yellow Sorghum	346.9	186.1	155.3	123	95.2	
6	Millet	543.2	483.9	401.2	373.2	201.4	
7	Abakaliki Rice	254.6	227.8	215.9	179.6	246.5	
8	Ugbawka Rice	271	282.9	243.7	229.6	186.1	

1	White Maize	22.5	24.9	19.1	14.6	11.9	Plus
2	Yellow Maize	31.1	33.9	21.5	20.5	17.2	Lactobacillus
3	Acha	7.3	4.5	3.1	3.0	2.9	species.
4	White Sorghum	117.2	94.9	69	59.8	49.4	
5	Yellow Sorghum	103.6	77.8	66.9	50.4	41.6	
6	Millet	170.9	137.3	119.3	80.7	62.9	
7	Abakaliki Rice	34.9	29.3	24.5	18	13.1	
8	Ugbawka Rice	24.2	19.2	16.9	13.1	9.4	
1	White Maize	124.1	137.6	101.4	86.9	69.7	Plus
2	Yellow Maize	198.6	191.7	177.6	143.6	121.4	Staphylococcus
3	Acha	15.3	10.4	7.2	7.0	6.7	aureus
4	White Sorghum	350.9	316.4	179.5	117.2	83.2	
5	Yellow Sorghum	282.2	139.6	106.3	75.4	55.9	
6	Millet	469.7	386.6	327.0	267.4	136.3	
7	Abakaliki Rice	111.5	95.6	83	74.1	49	
8	Ugbawka Rice	84.6	98.5	65	41	27.8	
1	White Maize	131.8	153.6	108.9	93	78.7	Plus
2	Yellow Maize	205.2	200.8	185.1	153.5	126.3	Streptococcus
3	Acha	12.8	10.6	8.0	5.7	4.9	faecalis
4	White Sorghum	367.2	324.4	186.1	122.7	88.4	
5	Yellow Sorghum	289.8	146.7	109.3	83.4	59.1	
6	Millet	475.5	393.6	335.8	270.4	138.7	
7	Abakaliki Rice	233.6	208.2	186.8	151.9	106.8	
8	Ugbawka Rice	89.7	101.5	67..9	47.9	30.3	
1	White Maize	132.7	154.4	109	93	79.4	Plus Proteus
2	Yellow Maize	206.4	200.9	185.4	154	126.8	spp.
3	Acha	17.1	13.9	8.7	8.0	7.0	
4	White Sorghum	367.6	324.7	186.6	122.7	88.7	
5	Yellow Sorghum	290	147.2	109.2	83.5	59.2	
6	Millet	479.4	394.3	336.5	272.4	141.6	
7	Abakaliki Rice	237.5	208.6	189.1	152.1	108.3	
8	Ugbawka Rice	91.1	102.6	68.2	47.7	30.7	

S/N	FOOD	DAYS OF INCUBATION					BACTERIA
		3	5	7	10	14	
1	White Maize	132.9	154.3	109.2	93.2	79.4	Plus Bacillus
2	Yellow Maize	206.4	201.1	185.4	154.2	127.7	cereus
3	Acha	17.2	13.8	8.9	8.0	7.2	
4	White Sorghum	367.1	323.4	186.4	122.3	93.5	
5	Yellow Sorghum	289.7	147.2	109	83	59.1	
6	Millet	479.1	394.1	336.3	272.2	141.2	
7	Abakaliki Rice	237.1	208.1	188.3	151.4	107.5	
8	Ugbawka Rice	90.6	101.7	67.5	47.2	29.6	
1	White Maize	51.9	53.4	44.5	43.7	36.2	Plus
2	Yellow Maize	64.5	63.4	61.5	59.6	52.8	Escherichia
3	Acha	ND	ND	ND	ND	ND	coli
4	White Sorghum	97.8	79.8	60.5	40.9	32	
5	Yellow Sorghum	64.6	53	41.1	29.5	20.6	
6	Millet	114.4	103.5	93.3	78.5	49.9	
7	Abakaliki Rice	13.1	15.5	12.7	10.6	7.8	
8	Ugbawka Rice	31.9	27.7	21.5	17.2	13	
1	White Maize	25.6	30.1	22	16.7	14.6	Plus
2	Yellow Maize	40.6	39.5	31.7	25.8	18.4	Streptococcus
3	Acha	3.9	3.0	2.8	2.2	ND	lactis
4	White Sorghum	91.8	84.7	56.4	46.6	32.5	
5	Yellow Sorghum	66.1	60.2	43	35.6	25.8	
6	Millet	101.6	95.5	77.9	68.9	43	
7	Abakaliki Rice	12.4	12.9	10.7	7.4	5.7	
8	Ugbawka Rice	6.3	5.8	4.8	4.3	4.0	
1	White Maize	132.9	154.2	109.4	93.6	79.1	Plus Serratia
2	Yellow Maize	206.5	200.1	185.3	154.3	126.6	species
3	Acha	17.3	13.9	8.9	8.0	7.0	
4	White Sorghum	367.5	324.6	186.3	123	88.6	
5	Yellow Sorghum	290.2	148.3	109.5	84.3	59.5	
6	Millet	480	394.9	337.2	272.8	141.5	
7	Abakaliki Rice	237.6	209.6	189.7	152.6	108.6	
8	Ugbawka Rice	91.4	102.7	68.6	48.4	31.1	

1	White Maize	113	136.4	52.4	69.7	63	Plus
2	Yellow Maize	191.8	179.1	161.6	128.5	105.4	Salmonella
3	Acha	4.1	3.4	3.0	2.6	2.1	spp.
4	White Sorghum	348	316.7	177.8	115.5	83.6	
5	Yellow Sorghum	281.9	139.3	103	79.3	55.9	
6	Millet	472.1	387	330.9	266.2	130.3	
7	Abakaliki Rice	66.6	64.9	57.2	50	42.4	
8	Ugbawka Rice	32.7	34.8	23.9	17.4	10.5	

(ii) **Dual culture experiments:**

Bacillus subtilis when in dual culture with *A. flavus* in the various cereal grains, generally stimulated the production of aflatoxins, though very slightly in acha grains, and much more than all others, in millet grains. *Acetobacter aceti* also stimulated AF production in these foodstuffs though more when compared with that of *Bacillus subtilis*. AF production in acha grains and the Ugbawka rice for example increased significantly when compared with the levels in the *A. flavus* monospecific cultures ($P<0.05$), and in the two maize varieties after the eleventh day of incubation. ($P<0.05$).

However, with *Pseudomonas* spp, there was a general reduction in AF levels in all the days of incubation for all the cereal grains though there were no effects in acha grains when compared with the levels when *A. flavus* was in single culture. The reduction of the AF level was significant in the two rice varieties after the third day of incubation ($P<0.05$). *Lactobacillus* spp. also significantly reduced AF production in all the cereal grains when in dual culture with *A. flavus* ($P<0.05$).

The reduction was significantly more in yellow maize and in Abakaliki rice when compared with the reduction in the white maize variety and Ugbawka rice variety respectively ($P<0.05$) in all the days of incubation. *E. coli* and *S. lactis* both, reduced AF levels significantly in the dual culture studies with each of them and *A. flavus* ($P<0.05$), though the decrease was more with *S. lactis* than with *E. coli* except with the two varieties of sorghum where the levels were slightly more in the *S. lactis* study. However, in the *E.coli* experiment, the production of aflatoxins in

acha grains was completely inhibited in all the days of incubation but only on day fourteen with *S. lactis.*

With *Streptococcus faecalis, Proteus* spp, *Bacillus cereus* and *Serratia* spp, there were no differences recorded in the levles of aflatoxins in the dual cultures and monoculture experiments for all cereals except the very slight reduction observed in acha with *S. faecalis.*

Staphylococcus aureus and *Salmonella* spp. generally also reduced AF levels in all the cereals grains in all the days of incubation. The reduction was more with *Salmonella* spp. being significant ($P<0.05$), for acha grains and the two varieties of rice, while with *S. aureus,* the reduction was significant only in the Abakaliki rice ($P<0.05$).

4.6.2 The oil seeds

The oil seeds include white and red testae peanuts, melon seeds and coconut seeds.

(i) ***A. flavus* in monoculture:**

Table 9 shows the result of the ecosystem studies in the oil seeds. A general pattern was established as with fungal studies for the monoculture experiments with oil seeds.

(ii) **Dual culture experiments.**

When *B. subtilis* was in dual culture with *A. flavus* in the oil seeds, there were no differences in the aflatoxin levels when compared with the levels when *A. flavus* was in monoculture.

With *B. cereus, E. coli, S. lactis, Salmonella* spp, *Serratia* spp, *S.aureus,*

TABLE 9 EFFECTS OF MICROBIAL ECOSYSTEM STUDIES WITH BACTERIA ON AFLATOXIN PRODUCTION IN OIL SEEDS (MEAN AFLATOXIN LEVELS IN PPB).

		DAYS OF INCUBATION					
S/N	FOOD	3	5	7	10	14	BACTERIA
1	Red Peanuts	655.1	775.2	473.4	630.6	682.9	Plus
2	White Peanuts	1282.4	1546.9	841.4	995.5	1538	Aspergillus
3	Melon seeds	687.9	726.1	440.4	429.3	884.3	flavus
4	Coconut seeds	1023.7	1516.7	706.7	587.6	2036.3	(control)
1	Red Peanuts	654.9	771.6	475.7	634.6	682	Plus
2	White Peanuts	1284.9	1548.7	841.4	999.9	1537.8	Bacillus
3	Melon seeds	688.1	727.2	441.4	431.6	875.1	subtilis
4	Coconut seeds	1030.9	1532.8	709.9	600.8	2047.4	
1	Red Peanuts	188.2	189.9	179.9	190.8	196.4	Plus
2	White Peanuts	1195.8	1533.1	833.3	971.2	1526.1	Pseudomonas
3	Melon seeds	653.4	656.8	393.7	381.2	737.2	aeruginosa
4	Coconut seeds	320.9	457.6	224.1	193.3	658.7	
1	Red Peanuts	665	791.6	519.7	668.3	727.5	Plus
2	White Peanuts	1621.6	1831	1083.5	1230	1681.1	Acetobacter
3	Melon seeds	828.6	878.4	603.5	570.7	969.5	aceti
4	Coconut seeds	1059.3	1566.8	736	665.3	2100.4	
1	Red Peanuts	585.4	756.6	436.5	559.5	674.9	Plus
2	White Peanuts	1280	1538.7	833.2	985.1	1529.7	Lactobacillus
3	Melon seeds	152.7	189.6	99.2	82.5	198	species
4	Coconut seeds	86.3	100.5	36.8	32.2	206	
1	Red Peanuts	591.4	727.9	429.3	521.8	645.8	Plus
2	White Peanuts	1246.9	1501.8	805	968.6	1474.8	Staphylococcus
3	Melon seeds	667.8	710.8	430.2	409.5	868.6	aureus
4	Coconut seeds	513.2	601.8	270	239.8	850.5	
1	Red Peanuts	571.7	678.1	408.9	545.1	585.4	Plus
2	White Peanuts	1072.3	1211.3	750.1	910.8	1320.1	Streptococcus
3	Melon seeds	394.1	518.1	266.8	242.8	568.5	faecalis
4	Coconut seeds	486.8	517.4	241.6	203.1	578.5	
1	Red Peanuts	640.4	759	448.4	623.5	665.4	Plus
2	White Peanuts	1255.3	1521.3	828.4	976.3	1519.3	Proteus
3	Melon seeds	682.2	719.8	433.6	409.7	817.4	spp
4	Coconut seeds	397.5	529.7	238.5	211.4	647.8	

		DAYS OF INCUBATION					
S/NO	FOOD	3	5	7	10	14	BACTERIA
1	Red Peanuts	636.8	757.2	433.8	618.8	672.8	Plus
2	White Peanuts	1238.6	1509.8	807	966.4	1508.7	Bacillus
3	Melon seeds	675.3	709.8	425.1	391.6	857.4	cereus
4	Coconut seeds	1011.6	1511.8	694.1	576.4	2001	
1	Red Peanuts	443.2	455.7	282.2	412.4	423.2	Plus
2	White Peanuts	1084.4	1292.2	684.2	920.9	411.1	Escherichia
3	Melon seeds	634.9	694.6	415.7	386.4	828.7	coli
4	Coconut seeds	1020.5	1502	679.2	587.2	2000.2	
1	Red Peanuts	572.5	730.2	395.2	440	594.6	Plus
2	White Peanuts	1154.6	1359.7	639.4	925.8	1315.9	Streptococcus
3	Melon seeds	187.5	198.3	143.7	132.4	228.9	lactis.
4	Coconut seeds	237.6	338.4	145.8	128.9	568.4	
1	Red Peanuts	92.3	97.8	56.3	75.7	87.8	Plus
2	White Peanuts	117.6	120.6	89.8	103.7	116.4	Serratia
3	Melon seeds	193.7	202.5	143.1	133.5	213.7	species
4	Coconut seeds	1339.1	1650.1	795.3	685.9	2305.2	
1	Red Peanuts	408	450.6	256.9	372.8	458.7	Plus
2	White Peanuts	1137.4	1346	652	930.6	1256.5	Salmonella
3	Melon seeds	274.7	289.3	186.5	174.7	339.5	spp
4	Coconut seeds	1020.4	1538.4	619.5	568	1899.2	

Lactobacillus spp, *S. facecalis, Pseudomonas* spp, and *Proteus* spp, each in dual culture with *A. flavus,* a decrease in AF levels was recorded. Specifically though, with *Pseudomonas* spp, the decrease was only significant in red peanut and coconut seeds ($P<0.05$); with *Lactobacillus* spp and *S. lactis* the reduction was significant only in melon seeds and coconut seeds ($P<0.05$) and with *S. faecalis* and *Proteus* spp, only in coconut seeds ($P<0.05$). *Seratia* spp recorded a mixed trend of events. While AF levels produced in coconut seeds were increased, there were signifcant decrease of same in the two peanut varieties and melon seeds ($P<0.05$). *Salmonella* spp, recorded significant reduction of aflatoxin levels only in the melon seeds ($P<0.05$).

4.6.3 The protein foods:

The protein foods include cowpeas (white and brown), soyabeans, breadfruit, bambara peanuts, pigeon peas (white and brown) and oil bean seeds.

(i) ***A. flavus* in monoculture:**

Table 10 shows the results of the ecosystem effects in the protein rich foods. A general pattern was established as with fungal studies for the monoculture experiments with protein foods.

(ii) **Dual culture experiments:**

Apart from the breadfruits and the two pigeon peas varieties, *B. subtilis* had no effects in all other protein foods when in dual culture with *A. flavus* in such food items. AF levels were however slightly increased in the breadfruits while there were reductions in AF levels in the pigeon peas which was statistically significant in the brown variety when compared with the levels in the *A. flavus* monospecific

TABLE 10 EFFECTS OF MICROBIAL ECOSYSTEM STUDIES WITH BACTERIA ON AFLATOXIN PRODUCTION IN PROTEIN FOODS (MEAN AFLATOXIN LEVELS IN PPB).

S/N	FOOD	DAYS OF INCUBATION					BACTERIA
		3	5	7	10	14	
1	White Cowpeas	84.4	85.8	84.6	85.4	84.5	Plus
2	Brown Cowpeas	28.7	30.6	31.2	31.9	28.0	Aspergillus
3	Soyabeans	3.8	7.2	9.7	10.2	4.3	flavus
4	Bread fruits	246.3	255.1	263.1	279	327.4	(CONTROL)
5	Bambara peanuts	162.9	201.5	228.1	239.3	157.6	
6	Pigeon pea (white)	22.4	20.8	21.7	22.4	20.9	
7	Pigeon Pea (Brown)	24.2	25.6	25.8	26.0	25.2	
8	Oil bean seeds	17.8	5.6	1.4	0	0	
1	White Cowpeas	84.3	85.4	84.9	85.5	85.2	Plus
2	Brown Cowpeas	28.7	30.4	31.0	31.0	29.3	Bacillus
3	Soyabeans	3.8	7.2	9.7	10.1	4.5	subtilis
4	Bread fruits	251.4	263.4	276.2	290.2	342.6	
5	Bambara peanuts	162.9	201.6	228.3	240.1	160.8	
6	Pigeonpea (while)	48.8	49.1	51.9	54.5	48.4	
7	Pigeon Pea (Brown)	53.9	53.9	57	58.9	55.8	
8	Oil bean seeds	17.8	5.0	1.0	0	0	
1	White Cowpeas	45.3	46.4	43.2	44	42.7	Plus
2	Brown Cowpeas	13.6	15.1	15.8	16.2	13.2	Pseudomonas
3	Soyabeans	0	0	0	0	0	spp.
4	Bread fruits	87.9	95.8	108.4	117.6	132.6	
5	Bambara peanuts	133	137.1	147.2	156.4	94.6	
6	Pigeon pea (white)	9.4	9.0	9.2	9.5	8.7	
7	Pigeon Pea (Brown)	10.8	10.6	10.3	10.3	10.4	
8	Oil bean seeds	0	0	0	0	0	
1	White Cowpeas	86.1	87.5	88.1	89.3	90.2	Plus
2	Brown Cowpeas	28.9	30.9	31.7	32.3	28.6	Acetobacter
3	Soyabeans	7.2	10.6	12.2	15.8	8.0	aceti
4	Bread fruits	359.8	370.4	415.6	454.6	513.8	
5	Bambara peanuts	170.1	210.2	233.8	247.2	161.9	
6	Pigeon pea (white)	49.8	41.1	44.6	49.7	41.1	
7	Pigeon Pea (Brown)	53.1	53.9	56.0	60.9	55.3	
8	Oil bean seeds	18.0	7.3	2.3	0	0	

S/N	FOOD	DAYS OF INCUBATION					BACTERIA
		3	5	7	10	14	
1	White cowpeas	13.3	14.1	11.8	12	11.8	Plus
2	Brown Cowpeas	6.2	8.1	4.9	7.6	4.0	Lactobacillus
3	Soya beans	0	0	0	0	0	species
4	Breadfruits	241.8	246.1	265.9	274.4	321.9	
5	Bambara Peanuts	70.6	86	99.4	108.1	63.9	
6	Pigeon Peas (white)	9.9	10.9	11.1	11.5	10.7	
7	Pigeon Peas (Brown)	10.3	10.8	11.8	11.9	10.6	
8	Oil bean seeds	0	0	0	0	0	
1	White cowpeas	76.4	76.3	75.9	75	76.2	Plus
2	Brown Cowpeas	26.3	28.5	28.9	30.4	26.9	Staphylococcus
3	Soya beans	3.4	7.0	9.3	9.8	4.0	aureus
4	Breadfruits	240	245.7	262.4	271.8	317.2	
5	Bambara Peanuts	160.5	200.8	224.9	236.4	155.1	
6	Pigeon Peas (white)	22.1	20.9	21.7	22.6	20.3	
7	Pigeon Peas (Brown)	24.2	25.2	25.7	25.8	25.1	
8	Oil bean seeds	17.8	3.6	0.4	0	0	
1	White cowpeas	0	0	0	0	0	Plus
2	Brown Cowpeas	0	0	0	0	0	Streptococcus
3	Soya beans	0	0	0	0	0	faecalis.
4	Breadfruits	39.2	43	47.4	50.5	5.5	
5	Bambara Peanuts	33.2	37.2	42.4	45.3	25.3	
6	Pigeon Peas (white)	0	0	0	0	0	
7	Pigeon Peas (Brown)	0	0	0	0	0	
8	Oil bean seeds	0	0	0	0	0	
1	White cowpeas	5.1	5.7	5.6	5.7	5.7	Plus Proteus
2	Brown Cowpeas	2.8	3.0	2.3	3.3	2.2	spp.
3	Soya beans	2.4	3.4	4.0	4.6	2.6	
4	Breadfruits	236.8	243.7	258.7	270.2	317.4	
5	Bambara Peanuts	81.4	89.1	96.1	101.8	78.4	
6	Pigeon Peas (white)	4.8	4.8	4.5	4.3	3.0	
7	Pigeon Peas (Brown)	6.3	6.7	5.7	6.1	6.3	
8	Oil bean seeds	0	0	0	0	0	

S/N	FOOD	DAYS OF INCUBATION					BACTERIA
		3	5	7	10	14	
1	White cowpeas	0	0	0	0	0	Plus
2	Brown cowpeas	0	0	0	0	0	Bacillus
3	Soya beans	1.8	3.5	4.2	4.8	2.8	cereus
4	Breadfruits	231.4	238.6	250.3	262.8	312.3	
5	Bambara Peanuts	69	80.5	87.2	91.8	73.0	
6	Pigeon Peas (white)	3.0	2.9	2.7	2.8	2.6	
7	Pigeon Peas (Brown)	3.9	4.2	3.7	4.0	3.8	
8	Oil bean seeds	0	0	0	0	0	
1	White cowpeas	83.8	83.5	84.6	82.8	86	Plus
2	Brown cowpeas	28.2	30.3	30.8	31.4	26.3	Escherichia
3	Soya beans	1.9	3.6	4.4	4.9	2.0	coli
4	Breadfruits	190.5	200.3	211.6	218	266	
5	Bambara Peanuts	162.1	199.9	223.9	238.1	155.3	
6	Pigeon Peas (white)	22.2	20.3	21.3	22.0	19.3	
7	Pigeon Peas (Brown)	23.6	23.8	24.2	24.7	24.0	
8	Oil bean seeds	17.3	4.4	1.0	0	0	
1	White cowpeas	3.3	3.4	3.4	3.0	2.7	Plus
2	Brown Cowpeas	0	0	0	0	0	Streptococcus
3	Soya beans	0	0	0	0	0	lactis
4	Breadfruits	138.9	148.4	151.8	149.4	157	
5	Bambara Peanuts	24.1	25.2	26	26.8	23.3	
6	Pigeon Peas (white)	7.6	8.7	9.0	9.5	9.0	
7	Pigeon Peas (Brown)	9.7	10.6	10.8	11.3	10.3	
8	Oil bean seeds	8.2	2.0	0.8	0	0	
1	White cowpeas	84.1	85.3	83.2	83.4	84.6	Plus
2	Brown Cowpeas	28.1	30.2	31.0	31.3	26.4	Serratia
3	Soya beans	0	0	0	0	0	Species
4	Breadfruits	128.6	129.5	132.3	134.9	142.9	
5	Bambara Peanuts	162.3	201.1	227.9	239.1	157.2	
6	Pigeon Peas (white)	22.1	20.2	20.9	22.0	20.3	
7	Pigeon Peas (Brown)	24.0	25.1	25.4	25.7	24.9	
8	Oil bean seeds	17.6	4.1	0.8	0	0	

1	White cowpeas	36.6	37.7	40.2	41.1	51.3	Plus
2	Brown cowpeas	14.3	15.3	16.1	16.5	14.0	Salmonella
3	Soya beans	1.1	3.0	3.5	3.8	2.2	spp.
4	Breadfruits	116.3	123.4	128.5	133.9	144.6	
5	Bambara Peanuts	83.9	91.4	97.8	104.8	80.8	
6	Pigeon Peas (white)	10.4	10.7	11.2	11.7	10.8	
7	Pigeon Peas (Brown)	11.4	12.1	12.4	12.9	12.0	
8	Oil bean seeds	17.2	5.4	1.0	0	0	

culture results ($P<0.05$). In the dual culture studies also, *Pseudomonas* spp. generally significantly reduced AF levels in all the protein foods ($P<0.05$), except in the bambara peanuts, while abolishing same in soyabeans and oil beans seeds. *Lactobacillus* spp. also significantly reduced AF levels in all protein foods ($P<0.05$), though there was no effects in the breadfruits. AF production was also completely inhibited in soyabeans and oil bean seeds. Comparatively, the effects of *Lactobacillus* spp. was more than that of *Pseudomonas* spp.

Staphylococcus aureus generally had no effects on aflatoxin production in these food items, though some slight decreases were recorded. *Streptococcus faecalis* had more serious effects on AF production in the protein foods. AF production was completely abolished in all food items while being significantly reduced in bambara peanuts and breadfruits ($P<0.05$). *Proteus* spp. also very significantly inhibited the production of aflatoxins in the protein foods ($P<0.05$), though there was a complete inhibition in the oil bean seeds, while there were just slight reductions in AF levels in the breadfruits. Specifically too, the effects were less in the bambara peanuts when compared with the other protein foods.

A similar effect on AF production was also recorded with *Bacillus cereus* on the protein foods as with the *Proteus* spp., though AF production were completely abolished in the two cowpeas as well as the oil bean seeds. *Streptococcus lactis* also recorded similar events though the reduction was more this time around in the bambara peanuts and breadfruits when compared with the other two previous organisims. Here also, AF production was completely abolished in soyabeans and brown cowpeas and significantly inhibited in the oil bean seeds, ($P<0.05$).

E. coli and the *Serratia* spp. both had no effects on aflatoxin production in the two varieties of cowpeas, pigeon peas and the bambara peanuts. Both organisms reduced AF levels in the oil bean seeds. *E. coli* also reduced AF levels in soyabeans and breadfruits while the *Serratia* spp. significantly ($P<0.05$) reduced the AF levels in breadfruits while completely abolishing same in soyabeans. On the other hand, *Salmonella* spp. significantly reduced AF levels in all the protein-foods ($P<0.05$), while having no effects on AF production in the oil bean seeds.

Acetobacter aceti when in dual culture with *A. flavus* generally stimulated AF production in all the protein foods though significantly in the two varieties of pigeon peas ($P<0.05$), when compared with the levels in the *A. flavus* single culture experiments.

4.6.4. The carbohydrate foods:

The carbohydrate foods include plantains (ripe and unripe), cassava, yam and cocoyam.

(i) ***A. flavus* in monoculture.**

Table 11 shows the results of the ecosystem studies with the carbohydrate foods. Also, a general pattern was established as with fungal studies for the monoculture experiments with carbohydrate foods.

(ii) **Dual culture experiments.**

When in dual cultures with *A. flavus* in the carbohydrate foods, *B. subtilis* stimulated AF production in both varieties of plantain though significantly in the ripe variety ($P<0.05$). It however significantly inhibited AF production in the remaining carbohydrate foods ($P<0.05$), though less so in cocoyam. *Proteus spp., S. faecalis, B. cereus, Serratia* spp. and *Pseudomonas* spp. however had no effects on

TABLE 11 EFFECTS OF MICROBIAL ECOSYSTEM STUDIES WITH BACTERIA ON AFLATOXIN PRODUCTION IN CARBOHYDRATE FOODS (MEAN AFLATOXIN LEVELS IN PPB).

S/N	FOOD	DAYS OF INCUBATION					BACTERIA
		3	5	7	10	14	
1	Unripe plantain	12.8	18.3	8.6	6.0	5.2	Plus
2	Ripe Plantain	112.5	114.9	67.8	55.7	43.6	Aspergillus
3	Cassava	13.5	15.7	6.7	5.8	1.6	flavus
4	Yam	98.6	89.7	73.3	58.1	46.6	
5	Cocoyam	30.8	25.7	20.6	17.3	14.1	
1	Unripe plantain	23.8	23.6	14.4	10.7	9.1	Plus
2	Ripe Plantain	224.7	220.9	136.1	116.8	98.1	Bacillus
3	Cassava	6.2	5.1	2.2	2.0	0	subtilis
4	Yam	27.1	17.2	12.2	6.9	4.6	
5	Cocoyam	14.0	13.5	10.2	6.7	5.5	
1	Unripe plantain	12.5	17.9	8.2	5.7	5.0	Plus
2	Ripe Plantain	112	113.8	66.7	54.4	42.9	Pseudomon
3	Cassava	10.8	12.3	4.3	3.8	1.0	Spp
4	Yam	97.3	88	65.5	57.2	45.6	
5	Cocoyam	29.4	24.8	19.7	16.2	13.4	
1	Unripe plantain	21.3	27.5	22.4	16.9	15.1	Plus
2	Ripe Plantain	171.4	171	113.1	104.4	70.7	Acetobacte
3	Cassava	22.8	24.7	19.5	19.0	16.1	aceti
4	Yam	120	112.9	97.9	82.2	66.5	
5	Cocoyam	38.7	34.6	30.8	27.5	23.5	
1	Unripe plantain	3.8	4.2	2.8	0	0	Plus
2	Ripe Plantain	26.1	27.1	14	9.7	8.4	Lactobac
3	Cassava	0	0	0	0	0	species.
4	Yam	43.3	40.4	34.1	28.3	21.4	
5	Cocoyam	12.6	10.1	7.2	5.8	5.0	
1	Unripe plantain	10.3	12.7	6.5	4.2	4.0	Plus
2	Ripe Plantain	98.9	98.3	49.1	44.3	36.4	Staphyl
3	Cassava	5.2	5.8	0	0	0	aureus
4	Yam	93.4	85.6	70.2	57	45	
5	Cocoyam	19.6	17.1	14.6	11.4	9.8	

S/N	FOOD	DAYS OF INCUBATION					BACTERIA
		3	5	7	10	14	
1	Unripe plantain	12.8	18.3	8.4	5.9	5.2	Plus
2	Ripe Plantain	112.1	114.6	67.3	55.3	43.3	Streptococcus
3	Cassava	3.4	3.6	2.2	0	0	faecalis
4	Yam	96.4	90.4	71.1	57.3	44.9	
5	Cocoyam	29.8	25.2	19.9	16.5	13.4	
1	Unripe plantain	12.8	18.2	8.5	6.0	5.2	Plus
2	Ripe Plantain	112.2	114.2	67.5	55.1	43.3	Proteus
3	Cassava	13.4	15.4	5.8	5.5	1.9	Spp.
4	Yam	97.1	88.6	72.3	57.4	45.7	
5	Cocoyam	30.5	25.5	19.7	16.5	13.6	
1	Unripe plantain	12.7	18.1	8.3	5.9	5.0	Plus
2	Ripe Plantain	111.8	114.6	67.3	54.7	42.9	Bacillus
3	Cassava	13.2	15.2	5.4	5.0	1.3	cereus
4	Yam	96.4	88.5	71.8	57.3	44.8	
5	Cocoyam	30.4	25.3	19.2	16.2	13.5	
1	Unripe plantain	0	0	0	0	0	Plus
2	Ripe Plantain	18.3	20.4	10.8	8.1	6.5	Escherichia
3	Cassava	0	0	0	0	0	coli
4	Yam	37.9	39.2	33.7	26.4	23.0	
5	Cocoyam	15.8	12.6	10.2	7.9	4.6	
1	Unripe plantain	4.6	5.3	2.2	2.0	1.9	Plus
2	Ripe Plantain	71.9	72.1	46.4	38	35	Streptococcus
3	Cassava	0	0	0	0	0	lactis
4	Yam	24.6	24.1	23	21.1	19.8	
5	Cocoyam	11.6	9.2	6.5	5.4	4.7	
1	Unripe plantain	12.5	18.0	8.1	5.7	5.0	Plus
2	Ripe Plantain	108.3	110.5	64.8	53.4	42.8	Serratia
3	Cassava	8.3	9.5	3.1	3.1	1.2	Species
4	Yam	96.4	89.1	72.6	57.5	46	
5	Cocoyam	28.0	25.4	20.3	16.4	13.4	
1	Unripe plantain	12.5	18.0	8.1	5.5	5.2	Plus
2	Ripe Plantain	99.5	104.5	59.5	48.4	37.1	Salmonella
3	Cassava	8.2	9.4	3.2	3.0	1.3	Species
4	Yam	73.7	73.5	64.5	50.3	38.9	
5	Cocoyam	26.4	20.6	15.7	14.3	11.5	

AF production in these food items though the levels in cassava were generally reduced by *Serratia* spp, *Pseudomonas* spp and *S faecalis.*

A. aceti generally stimulated the production of aflatoxins in all the food items, with the increase in the plantains and cassava being statistically significant after the fifth days of incubation ($P<0.05$).

Lactobacillus spp significantly decreased AF levels in all the carbohydrate foods ($P<0.05$), the effects being most in the ripe plantain, while a complete inhibition was recorded in the cassava tubers throughout the days of incubation and in the unripe plantains from the tenth day of incubation. *S. aureus* generally decreased AF levels in all the food items. It however affected the cassava tubers most, where the AF levels were significantly reduced ($P<0.05$), by days three and five and being completely abolished thereafter. There were also no differences in the aflatoxin levels in yam when compared with the levels in the monoculture studies. With *Salmonella* spp, AF levels in the dual culture experiments were also generally reduced except the unripe plantain when compared with the monoculture studies.

Escherichia coli and *Streptococcus lactis* both significantly inhibited the production of aflatoxins in all the carbohydrate foods ($P<0.05$). Both organisms completely abolished AF production in cassava. *E. coli* reduced AF levels more than *S. lactis* in the ripe variety of plantains, while *S. lactis* in turn reduced same more in yam and cocoyam tubers respectively.

TABLE 12: Effects of Microbial ecosystem on AF production in YES medium with fungal isolates.

Days of Incubation	3	5	7	10	14	
A. flavus alone	0.25	0.77	1.29	1.76	1.65	AF LEVELS µg/ml
	251	370	389	400	417	Mycelial dry wt. (mg/100ml)
	4.36	3.97	3.94	4.36	4.39	pH
Plus Rhizopus Spp.	0.02	0.02	0.01	0.02	0.02	"
	151	239	287	306	331	
	6.36	5.05	5.00	3.58	3.18	
Plus A. niger	0.25	0.77	0.26	ND	ND	
	171	270	300	340	347	"
	6.40	5.08	3.37	3.17	3.17	
Plus Trichoderma Spp.	ND	ND	ND	ND	ND	
	99	92	78	66	64	"
	6.60	6.32	6.30	5.48	5.07	
Plus Saccharomyces Spp.	0.22	0.38	0.45	1.32	1.29	
	261	368	383	398	406	"
	4.38	3.93	3.99	4.57	4.66	
Plus Penicillium Spp.	0.13	1.03	1.20	1.35	0.78	
	248	322	349	396	410	"
	6.37	5.07	3.38	3.19	3.21	
Plus Cladosporium Spp.	0.24	0.59	1.17	1.62	1.75	
	250	370	380	401	442	"
	4.24	3.91	3.98	4.15	5.16	
Plus Curvularia Spp.	0.27	0.79	1.40	1.79	1.68	
	255	379	389	419	445	"
	4.25	3.90	4.18	4.64	5.22	
Plus Fusarium Spp.	0.003	0.01	0.01	0.01	0.01	
	135	121	160	196	194	"
	6.38	3.36	4.24	5.71	5.76	
Plus Rhodotorula Spp.	0.02	0.10	0.44	0.59	0.45	
	111	193	196	232	286	"
	6.36	5.07	3.34	3.19	3.21	
Plus Geotrichum Spp.	0.23	0.61	0.99	1.47	1.00	
	261	344	368	392	400	"
	4.28	3.94	3.98	4.21	5.17	
Plus A. fumigatus	0.01	0.01	0.01	0.02	0.01	
	173	280	310	351	359	"
	6.48	5.14	5.05	3.45	3.19	
Plus C. albicans	0.30	0.72	1.30	1.77	1.70	
	264	388	401	447	449	"
	4.28	3.97	4.20	5.70	5.70	

ND - NOT DETECTED

TABLE 13: Effects of microbial ecosystem on AF production in semi-synthetic medium with fungal isolates

Days of Incubation	3	5	7	10	14
A. flavus alone	0.30 232 4.46	0.70 340 4.07	1.40 382 4.09	1.15 402 4.57	1.02 405 4.58
Plus Rhizopus Spp.	0.26 170 6.32	0.02 270 5.07	0.01 309 3.35	0.01 341 3.17	0.01 344 3.19
Plus A. niger	0.30 168 6.38	0.35 265 5.09	0.28 271 3.38	ND 296 3.17	ND 300 3.16
Plus Trichoderma Spp.	ND 91 6.48	ND 89 5.09	ND 74 3.40	ND 61 3.17	ND 61 3.11
Plus Saccharomyces Spp.	0.30 240 4.48	0.48 338 4.03	0.22 382 4.13	0.80 399 4.72	0.81 401 4.81
Plus Penicillium Spp.	1.29 201 6.48	0.70 196 5.31	0.36 151 5.18	0.09 100 4.99	0.06 92 4.96
Plus Cladosporium Spp.	0.32 251 4.21	0.51 376 3.89	0.75 398 4.29	0.31 423 5.10	0.29 423 5.13
Plus Curvularia Spp	0.67 258 4.23	0.95 348 3.88	1.31 385 4.13	0.85 407 4.73	0.78 417 4.94
Plus Fusarium Spp.	ND 151 6.48	ND 190 6.33	ND 200 5.09	ND 209 5.46	ND 241 4.78
Plus Rhodotorula Spp.	0.18 122 6.38	0.15 104 6.25	0.46 113 5.18	0.30 98 5.08	0.24 93 5.00
Plus Geotrichum Spp.	0.60 240 4.27	0.79 256 3.37	1.61 320 4.25	0.86 337 5.02	0.10 392 5.11
Plus A. fumigatus	0.02 172 6.40	0.01 241 5.16	0.02 279 4.84	0.02 300 4.29	0.02 349 3.73
Plus C. albicans	0.29 244 4.45	0.54 336 4.02	0.87 381 4.10	0.84 395 4.76	0.82 400 4.84

AF LEVELS µg/ml.
MYCELIAL DRY WT. (mg/100mL.)
pH

not affected as evidenced by the mycelial dry weight. Co-cultures with *C.albicans* showed an increase in the mean Af levels and also in mycelial dry weights.

Cladosporium spp. also reduced Af levels except on day 14 when a slight rise was recorded. No differences were recorded for the mycelial dry weights in the dual culture and single culture for *A.flavus*. A slight increase in Af levels and mycelial dry weights was observed with *Curvularia* spp. up to day 14 when compared with the monoculture results but *Geotrichum* spp. and *Saccharomyces* spp. on the other hand decreased Af mean levels in the competitive environments. As evidenced by the mycelial dry weights, the growth of *A.flavus* was also not affected in dual cultures with *Geotrichum* spp. *Saccharomyces* spp. and *A.fumigatus*, though Af production was affected.

Almost a similar trend of events was observed in the synthetic media as seen in the semi-synthetic media with *Trichoderma* spp, *Rhizopus* spp, *Saccharomyces* spp, *A.niger* and *Rhodotorula* spp. However, in the synthetic media, *Fusarium* spp. completely inhibited the production of aflatoxins while *Penicillium* spp. more significantly inhibited Af production except for AFB_1. Aflatoxins levels were higher when *A.flavus* was in dual culture with *Curvularia* spp. than when in single culture up to day five and then dropped thereafter up to day fourteen.

Geotrichum spp. in dual culture, *A.flavus* increased the mean Af levels up to day seven and then inhibited thereafter.

In the YES medium, *A.flavus* alone in single culture, produced a higher level of mean Af when compared with the pattern in the synthetic medium. In the YES medium also, the aflatoxins produced increased gradually with time and then got inhibited on day fourteen while in the synthetic medium, it increased with time till the seventh day of

incubation and then started decreasing over the tenth and fourteenth days of incubation. Specifically, the AFB_1 was less affected than AFG_1 by *Saccharomyces cerevisiae* in the two liquid media. AFG_1 was more drastically inhibited by this organism than AFB_1.

4.7.2 Effects of bacterial ecosystem on aflatoxin production in YES and synthetic media

In the YES medium co-cultivation experiment with *A.flavus* and *B.subtilis*, mean Af levels were significantly increased in all the days of incubation when compared with *A.flavus* in single culture as evidenced by both mycelial dry weight and pH of the medium ($P<0.05$). The same events were observed also for *Acetobacter aceti, Proteus* spp, and *B.cereus*. However, *Pseudomonas* spp, *Lactobacillus* spp, *E.coli*, *Serratia* spp, *S.aureus* and *Salmonella* spp decreased Af levels to varying degrees, but with *S.lactis* there was no difference in the Af mean levels as compared with *A.flavus* in single culture. With *S.faecalis*, mean Af level was lower than in single culture till the fifth day,then higher thereafter. With *Salmonella* spp and *Lactobacillus* spp, Af levels were significantly inhibited in the dual cultures ($P<0.05$).

In the synthetic media with bacterial co-cultures, *S.lactis* and *Proteus* spp reduced Af mean levels. Otherwise a similar trend of events were observed in both types of media.{Tables 14 and 15}.

4.8 EFFECTS OF THE ADDITION OF SALTS OF BIVALENT METALS TO FOOD SUBSTRATES AND LIQUID MEDIA ON AFLATOXIN PRODUCTION

4.8.1 Bivalent metal concentration in food substrates

Table 16 shows the result of the analysis of the bivalent metal concentrations in the 18 foodstuffs analysed by atomic absorption spectrophotometry. From the results, yellow and

TABLE 14: Effects of microbial ecosystem on AF production in YES medium with bacterial isolates

Days of Incubation	3	5	7	10	14	
A. flavus alone	0.25	0.77	1.29	1.76	1.65	Aflatoxin level µg/ml
	251	370	389	400	417	Mycelial Dry wt Mg/100ml
	4.36	3.97	3.94	4.36	4.39	pH
Plus B. subtilis	0.40	1.20	1.90	1.85	2.28	"
	270	376	399	424	446	
	4.19	3.86	4.08	5.14	5.21	
Plus Pseudomonas Spp.	0.14	0.55	1.00	1.15	1.08	"
	223	328	348	374	381	
	4.48	4.60	5.41	6.10	6.97	
Plus Lactobacillus Spp.	0.07	0.36	0.60	0.60	0.52	"
	219	324	343	370	378	
	4.48	4.60	6.10	6.96	6.98	
Plus Acetobacter aceti	0.29	1.32	1.76	2.17	1.79	"
	271	376	399	423	447	
	4.19	4.88	4.68	6.12	6.21	
Plus S. aureus.	0.18	0.61	1.08	1.54	1.44	"
	251	369	388	402	408	
	4.20	3.89	4.09	5.14	5.20	
Plus S. faecalis	0.22	0.64	1.31	1.92	1.78	"
	223	341	339	379	390	
	4.30	3.91	3.99	4.30	4.41	
Plus Proteus Spp.	0.27	0.81	1.47	1.83	1.68	"
	253	376	392	403	421	
	4.36	3.91	3.95	4.29	4.43	
Plus B. cereus.	0.26	1.22	1.57	2.07	1.80	"
	257	372	400	420	427	
	4.48	4.70	6.12	6.80	6.96	
Plus E. coli	0.22	0.65	1.16	1.56	1.46	"
	234	352	361	376	387	
	4.40	4.00	5.15	5.92	6.84	
Plus S. lactis	0.25	0.77	1.30	1.76	1.63	"
	253	370	390	401	420	
	4.36	3.96	3.92	4.38	4.40	
Plus Serratia Spp.	0.22	0.42	0.83	0.99	0.97	"
	255	345	380	408	417	
	4.44	4.59	5.15	6.10	6.95	
Plus Salmonella Spp.	0.07	0.24	0.47	0.66	0.60	"
	1.62	1.72	1.89	1.08	1.19	
	6.30	5.09	5.06	3.36	3.17	

TABLE 15: Effect of microbial ecosystem on AF production in semi-synthetic medium with bacterial isolates.

Days of Incubation	3	5	7	10	14	
A. flavus alone	0.30	0.70	1.40	1.15	1.02	AF LEVELS ug/ml.
	232	340	382	402	405	Mycelial dry wt. (mg/100ml.)
	4.46	4.07	4.09	4.57	4.58	pH
Plus B. subtilis	0.29	0.77	1.64	1.45	1.06	
	241	353	398	422	423	"
	4.21	3.98	4.23	5.20	5.20	
Plus Pseudomonas Spp.	0.13	0.43	0.88	0.82	0.48	
	229	335	379	390	389	"
	4.49	4.04	4.14	4.74	4.77	
Plus Lactobacillus Spp.	0.12	0.31	0.63	0.48	0.41	
	227	328	348	374	377	"
	4.28	3.98	4.13	4.70	4.72	
Plus Acetobacter aceti	0.57	1.34	1.32	1.40	1.38	
	258	371	396	423	426	"
	4.29	3.96	4.14	5.02	5.12	
Plus S. aureus.	0.27	0.66	1.34	1.11	0.99	
	237	338	380	399	399	"
	4.48	4.03	4.13	4.70	4.72	
Plus S. faecalis	0.27	0.68	1.38	1.11	1.005	
	239	337	380	397	399	"
	4.48	4.01	4.11	4.68	4.70	
Plus Proteus Spp.	0.25	0.66	1.36	1.10	0.98	
	240	334	376	395	395	"
	4.47	3.99	4.10	4.66	4.66	
Plus B. cereus.	0.29	1.01	1.72	1.62	1.38	
	240	374	402	425	428	"
	4.20	3.88	4.08	5.11	5.12	
Plus E. coli	0.20	0.49	1.29	0.89	0.78	
	239	333	371	382	384	"
	4.48	4.01	4.12	4.70	4.72	
Plus S. lactis	0.28	0.68	1.39	1.13	1.005	
	232	339	381	402	404	"
	4.46	4.06	4.09	4.56	4.57	
Plus Serratia Spp.	0.28	0.69	1.21	1.13	1.008	
	231	340	380	401	401	"
	4.45	4.07	4.08	4.56	4.56	
Plus Salmonella Spp.	0.07	0.20	0.60	0.43	0.26	
	171	270	309	340	341	"
	6.25	5.11	5.09	3.38	3.16	

TABLE 16

Bivalent metals concentrations per gram of food items analysed. (Endogenous metal levels) in PPM. (Ug/g)

FOOD SUBSTRATES	ZINC (Zn^{2+})	COPPER(Cu^{2+})	MANGANESE (Mn^{2+})	IRON(Fe^{2+})
1 MAIZE (White)	11.35	4.49	6.37	24.33
2. MAIZE (Yellow)	22.5	4.48	6.83	24.84
3. PEANUTS (White)	36.98	7.61	16.67	21.0
4. PEANUTS (Red)	34.81	7.97	16.49	19.06
5. ACHA Grains	12.72	2.24	4.48	30.7
6. COWPEA (White)	8.88	8.09	22.0	56.0
7. COWPEA (Brown)	10.86	8.4	20.80	40.8
8. SOYA BEANS	0.02	8.5	22.0	70.0
9.OIL BEAN (Ugba)	21.46	7.2	20.4	30.66
10. MELON (Egusi)	21.81	9.23	21.09	9.06
11. CASSAVA	8.75	3.11	7.25	47.1
12. RICE (Abakaliki)	16.72	3.60	10.00	19.17
13 RICE (Ugbawka)	16.50	3.48	10.14	20.0
14. SORGHUM (yellow)	30.0	7.0	27.0	44.0
15. SORGHUM (white)	34.0	3.0	16.0	44.0
16. BAMBARA PEANUTS	20.80	8.0	20.94	40.0
17. BREADFRUITS	18.07	4.0	9.3	9.76
18. MILLET	19.0	5.0	14.0	41.0
19. YAM	8.73	3.7	9.0	48
20. PLANTAIN (Unripe)	9.68	2.0	6.04	6.0
21. PLANTAIN (Ripe)	10.92	2.0	8.02	5.5
22. COCONUTS	6.38	2.0	3.0	3.0
23. PIGEON Peas(White)	20.84	8.42	21.02	58.0
24. PIGEON Peas(Brown)	20.80	8.33	21.18	58.6
25. COCOYAM	10.47	3.6	10.0	43

Table 16 shows the result of the analysis of the bivalent metal concentrations in the 18 foodstuffs analysed by atomic absorption spectrophotometry. From the results, yellow and white maize varieties had no apparent differences in their endogeneous bivalent metal contents except in zinc where the yellow had about double (22.5ppm) that of the white variety (11.35ppm). The red and white varieties of peanuts do not differ, generally speaking, in their endogeneous bivalent metals analysed, while the major difference in the cowpea varieties lie on the iron levels (56 ppm and 40.8 ppm) for the white and brown varieties respectively. Soybean was quite high in its iron content but extremely low (0.02ppm) in the zinc content. The two varieties of rice showed an overall similarity in their bivalent metal contents while the varieties of sorghum on the other hand showed some slight differences in their copper and manganese levels.

The carbohydrate foods (yams, cocoyams, plantain and cassava) were generally poor in the distribution of these bivalent metals except that they were quite high in iron excluding the plantain varieties. The plantains were similar except that the ripe ones showed some superiority in their zinc and manganese content. Soybean and cowpeas differ from other pulses in their zinc content being much lower than in others; otherwise, the pulses were generally rich in these bivalent metals except copper.

4.8.2 Effects of addition of salts of bivalent metals to YES and synthetic medium on aflatoxin production

Tables 17 and 18 show the results obtained with the progressive addition of Zn^{2+}, Mn^{2+}, Cu^{2+} and Fe^{2+} salt each to YES and synthetic media with a constant level of each of the other remaining elements in the medium at seven and fourteen days incubation respectively.

Table 17

EFFECTS OF ADDITION OF SALTS OF BIVALENT METALS TO A SYNTHETIC MEDIUM AFTER 7 DAYS INCUBATION WITH A . FLAVUS ISOLATES

EFFECTS OF ADDITION OF THE BIVALENT METAL SALTS IN YES MEDIUM AFTER 7 DAYS INCUBATION WITH A. FLAVUS ISOLATES.

LEVEL OF Added metals (µg/ml)	mean toxin levels (µg/ml) medium.	Mycelial dry wt. (mg/100ml)	pH	METALS ADDED	aflatoxin levels (µg/ml) Medium	mycelial dry wt (mg/100 ml)	pH
0.0	0.002	86	3.0	Zn^{2+}	0.004	94	3.1
1.0	2.31	215	6.9		2.73	231	7.0
5.0	2.51	220	6.9		2.86	236	7.0
10.0	2.71	227	6.9		3.55	244	7.0
25.0	1.19	230	6.9		1.49	248	7.0
0.0	0.09	230	6.5	Mn^{2+}	0.12	248	6.5
1.0	0.18	226	6.6		0.22	249	6.7
5.0	0.59	229	6.6		0.69	253	6.7
10.0	0.54	233	6.7		0.63	256	6.6
25.0	0.31	230	6.6		0.65	250	6.6
0.0	1.05	139	4.8	Fe^{2+}	1.20	165	4.5
1.0	1.11	213	6.9		1.21	237	6.8
5.0	0.59	210	6.9		0.71	234	6.9
10.0	0.55	216	7.0		0.62	239	7.0
25.0	0.07	214	7.1		0.11	238	7.2
0.0	1.16	208	6.5	Cu^{2+}	1.30	222	6.5
1.0	0.63	212	6.6		0.74	224	6.5
5.0	0.004	200	6.7		0.01	213	6.6
10.0	ND	196	6.7		ND	208	6.7
25.0	ND	205	6.8		ND	217	6.7

ND – not detected .

Table 18

EFFECTS OF ADDITION OF SALTS OF BIVALENT METALS TO A SYNTHETIC MEDIUM AFTER 14 DAYS INCUBATION WITH A. FLAVUS ISOLATES.

EFFECT OF ADDITION OF BIVALENT METALS TO A SEMI SYNTHETIC MEDIUM AFTER 14 DAYS INCUBATION WITH A. FLAVUS ISOLATES.(YEAST EXTRACT SUCROSE MEDIA YES).

µg/Ml levels of added metals	Mean toxin levels (µg/ml) medium.	Mycelial Dry wt (mg/100ml)	PH	METALS	Mean toxin level (µg/ml)	Mycelial dry wt. (mg/100ml)	PH
0.0	0.001	82.5	3.1	Zn^{2+}	0.001	79	3.0
1.0	1.59	212	5.7		2.14	215	6.5
5.0	1.14	213	5.8		1.46	218	6.7
10.0	1.12	216	6.0		1.20	220	6.8
25.0	0.28	218	6.2		0.35	223	6.8
0.0	0.04	230	6.2	Mn^{2+}	0.05	235	6.3
1.0	0.15	219	6.3		0.18	223	6.4
5.0	0.30	219	6.3		0.33	220	6.4
10.0	0.20	221	6.4		0.21	222	6.4
25.0	0.08	222	6.4		0.10	225	6.4
0.0	0.31	147	4.5	Fe^{2+}	0.35	151	4.4
1.0	0.90	189	6.5		0.93	220	6.3
5.0	0.32	176	6.5		0.34	211	6.6
10.0	0.16	181	6.8		0.21	215	6.8
25.0	0.02	178	6.8		0.03	213	7.0
0.0	0.35	200	6.2	Cu^{2+}	0.38	208	6.4
1.0	0.56	203	6.4		0.58	209	6.3
5.0	0.003	183	6.5		0.003	197	6.3
10.0	ND	175	6.4		ND	190	6.5
25.0	ND	170	6.5		ND	199	6.5

ND, not detected.

At zero level of zinc, Af production was very low in YES medium, mycelial mat dry weight low and finai pH also low. At zinc levels 1-25µg/ml, Af production increased steadily with increasing concentration, with a moderate depression at 25µg/ml dose of the metal in the medium, whereas both mat weight and final pH were higher and nearly constant. The increase in the Af from addition at zero level Zn^{2+} to 1.0µg/ml Zn^{2-} was significant ($P<0.05$).

With added Mn^{2+} from 0-25µg/ml, Af production increased to its highest levels at 5 and 10µg/ml levels of metals and then decreased again very slightly at 25µg/ml, whereas mat weight and pH were essentially constant throughout the experiments. With iron salts at 0 level, Af production was relatively high; mat weight slightly lower than at higher concentrations with final pH, 4.5. However, with iron in the range 1-25µg/ml, production decreased progressively with increasing iron concentration, mat weight being almost constant and final pH being nearly constant at pH average of 7.0. With Cu^{2+} levels from 1-25µg/ml, a depression of Af production occurred even though mat weights and final pH were unaffected.

Generally, a similar pattern was obtained with experiments in the synthetic media but levels of aflatoxins were much lower and the reduction more than in the YES medium experiments. Also Af levels were generally much lower in both sets of experiments for those incubated over a period of fourteen days when compared with those incubated over a period of only seven days.

4.8.3 Effects of addition of salts of bivalent metals to food substrates on aflatoxin production:

Figs 55 to 151 show the results obtained when graded doses of the bivalent metals each were added into the food substrates analysed.

(i) Zinc:

The addition of 10-250µg of zinc per gram of cereals increased AF yield gradually with a maximum increase occurring at 250-550µg/gm of the foodstuff and then decreasing gradually till 1000µg/g of zinc per gram of food substrate. In all experiments a similar trend was followed when the incubation was for fourteen days but usually with reduced level of aflatoxins. In protein foods generally, the addition of Zn^{2+} also gradually stimulated AF production by 1-2 folds approximately till 400µg/g of zinc when a further increase occurred till 550µg/g then declined again up to 1000µg/g zinc per gram of food substrate. However, for the breadfruits, this increase was observed at a lower concentration, 250µg of Zn^{2+} per gram of food.

In the oil seeds, a progressive inhibition in AF production was rather observed with increase in concentration (1000µg/g) of the metal ions. The carbohydrate foods had a similar pattern as the cereals; with gradual increase in AF levels with increasing metal concentrations with a peak at 250µg metal per gram of food and a decrease after 550µg Zn^{2+} concentration per gram of food substrate till 1000µg zinc concentration.

There were no actual differences in the AF levels at day seven and day fourteen incubation for the oilseeds and breadfruits in the zinc experiments. For the cereals, protein and carbohydrate foods, AF levels at day seven were generally higher than levels at day fourteen.

(ii) Manganese:

Manganese stimulated AF production almost 2 folds or more from 10µg metal per gram of food, and continued in a constant rate with a very mild drop at 250-400µg Mn^{2-}

Fig-56: Effects of graded levels of bivalent metals(Zinc) on aflatoxin production in Maize(white)

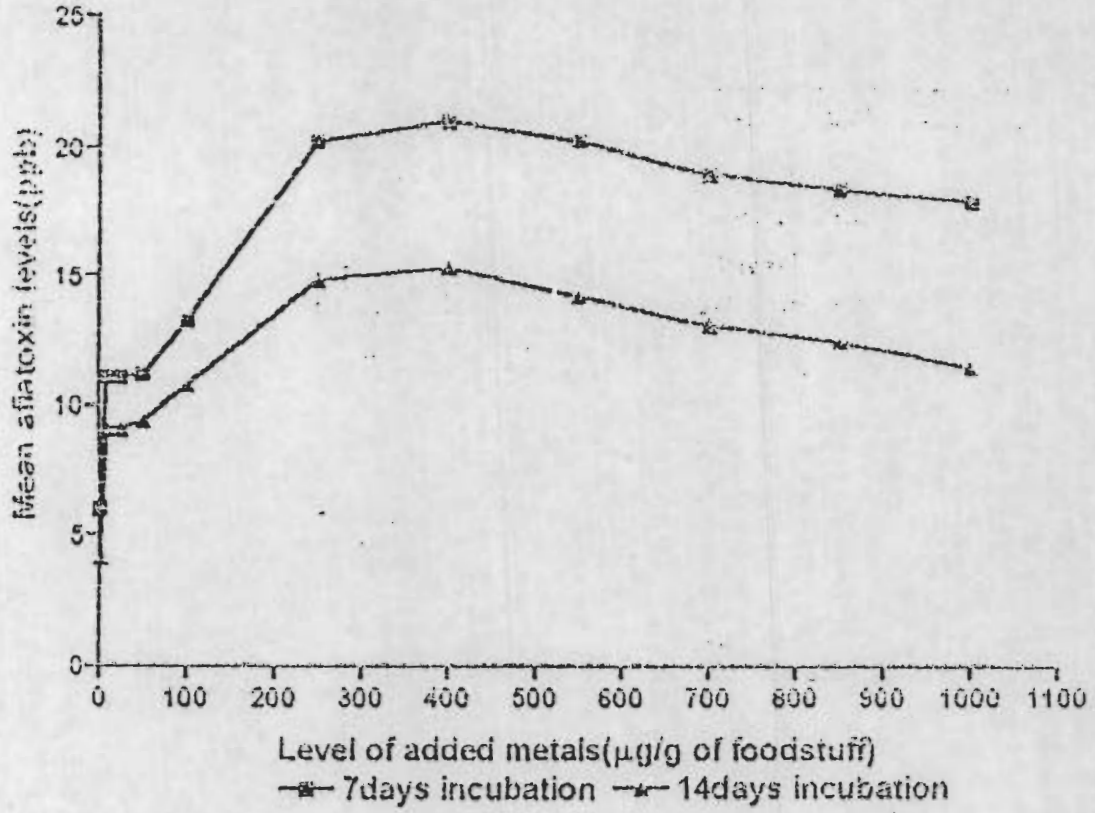


Fig.57: Effects of graded levels of bivalent metals(Zinc) on aflatoxin production in Maize(yellow)

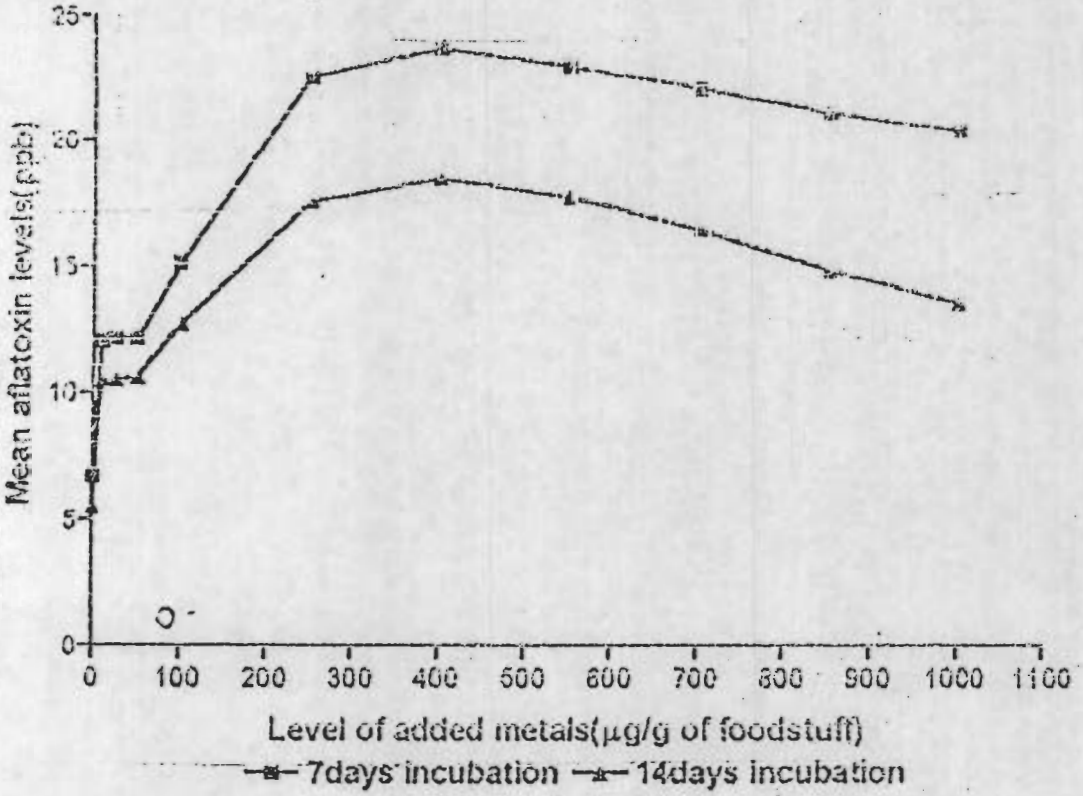


Fig.58: Effects of graded levels of bivalent metals(Zinc) on aflatoxin production in Acha (grains) findi

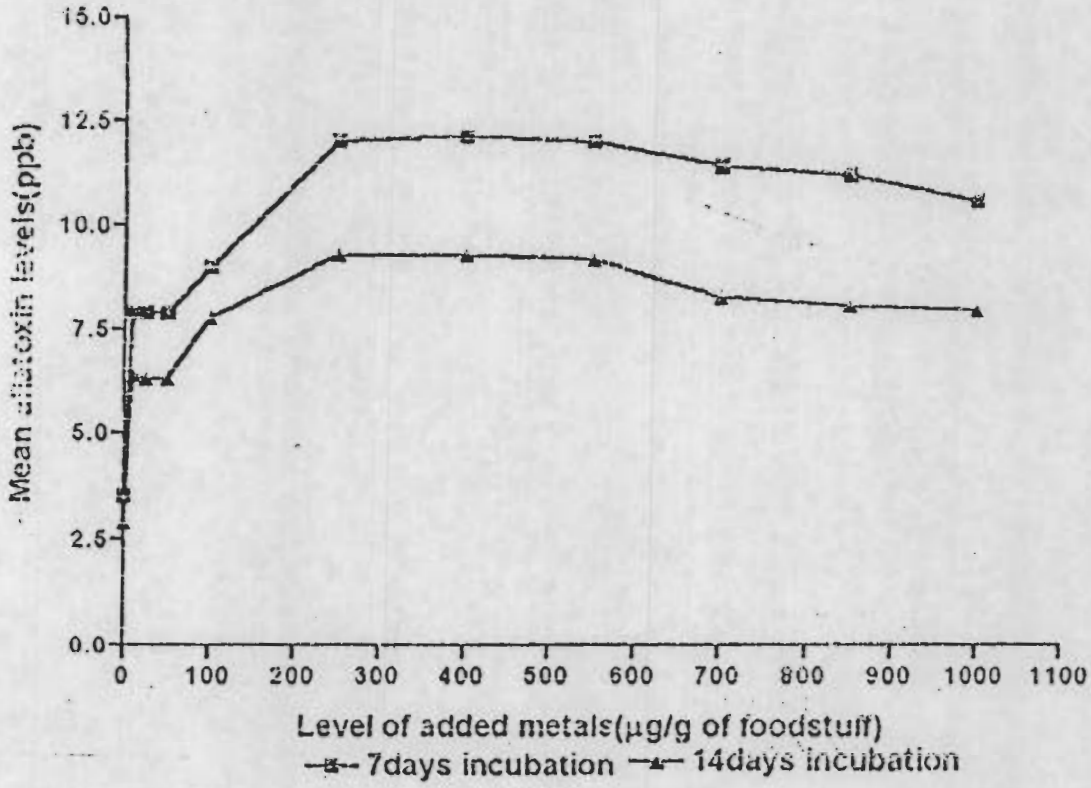


Fig.59: Effects of graded levels of bivalent metals(Zinc) on aflatoxin production in Millet

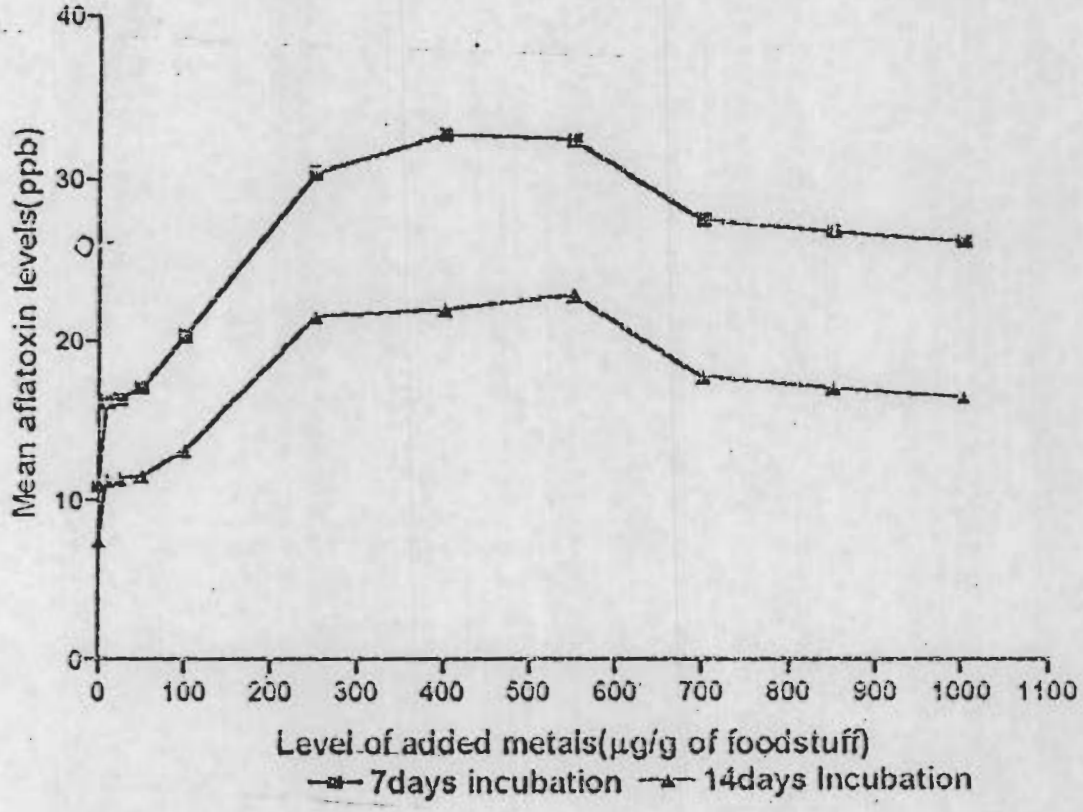


Fig.60: Effects of graded levels of bivalent metals(Zinc) on aflatoxin production in Rice(Abakaliki)

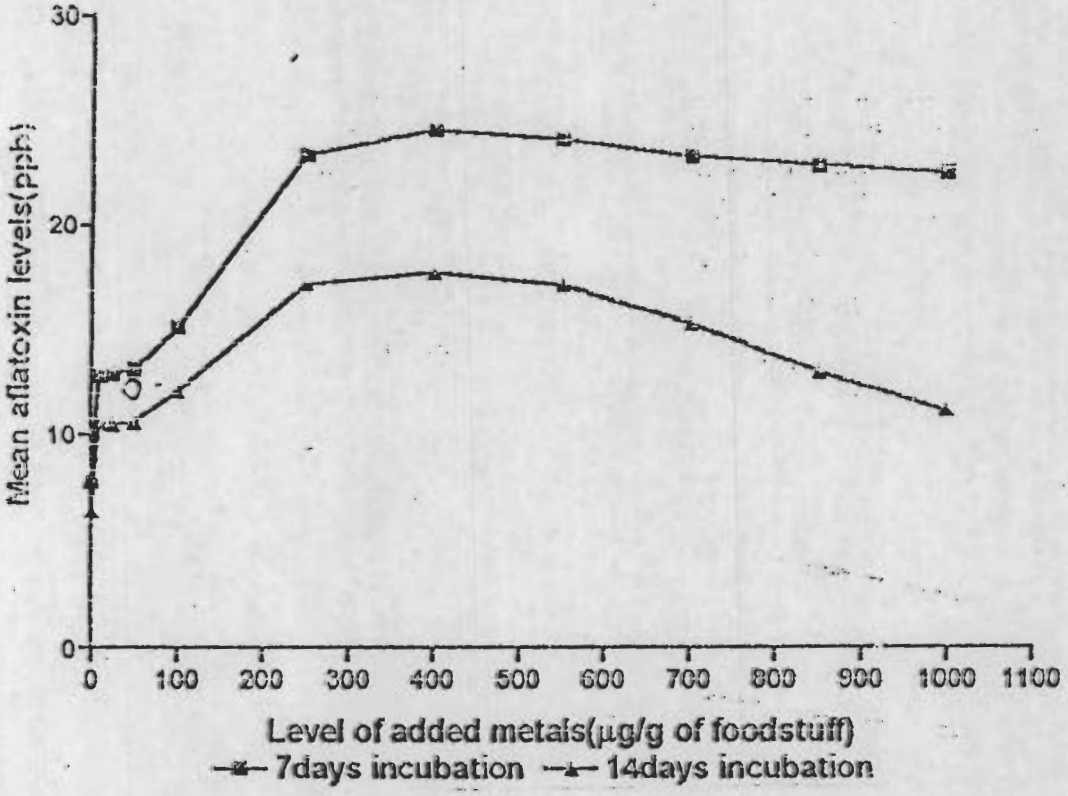


Fig.61 :Effects of graded levels of bivalent metals(Zinc) on aflatoxin production in Rice(Ugbawka)

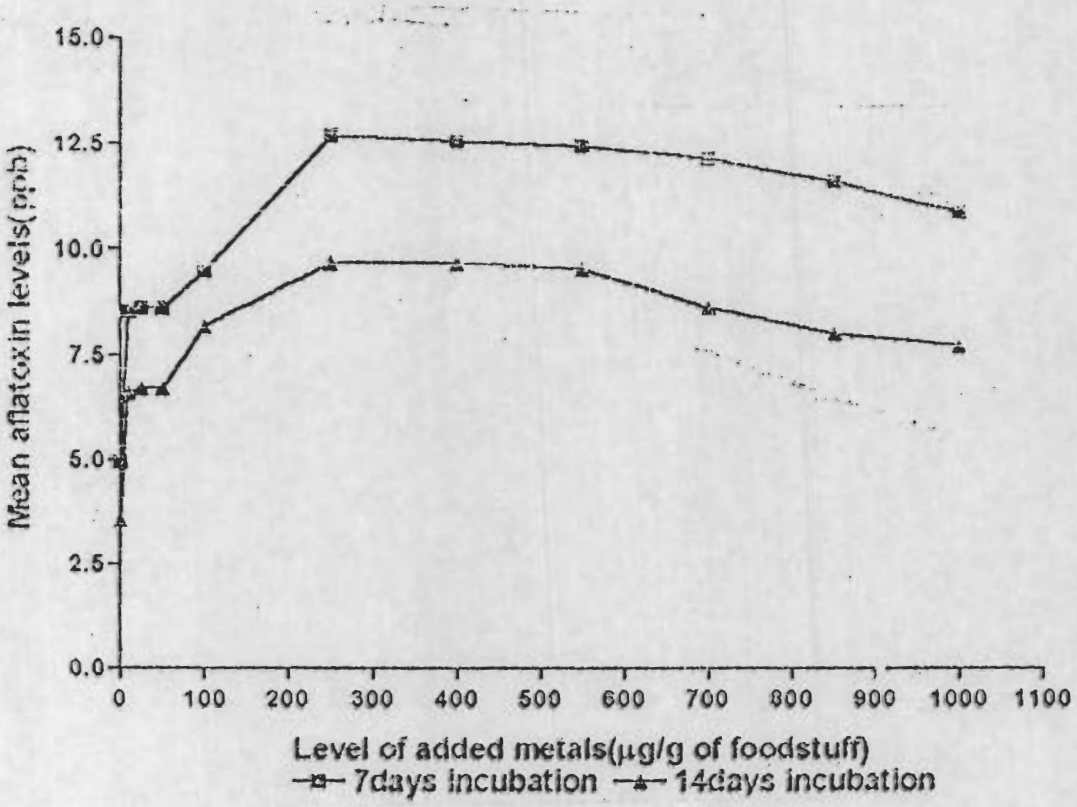


Fig62: Effects of graded levels of bivalent metals(Zinc) on aflatoxin production in Sorghum(yellow)

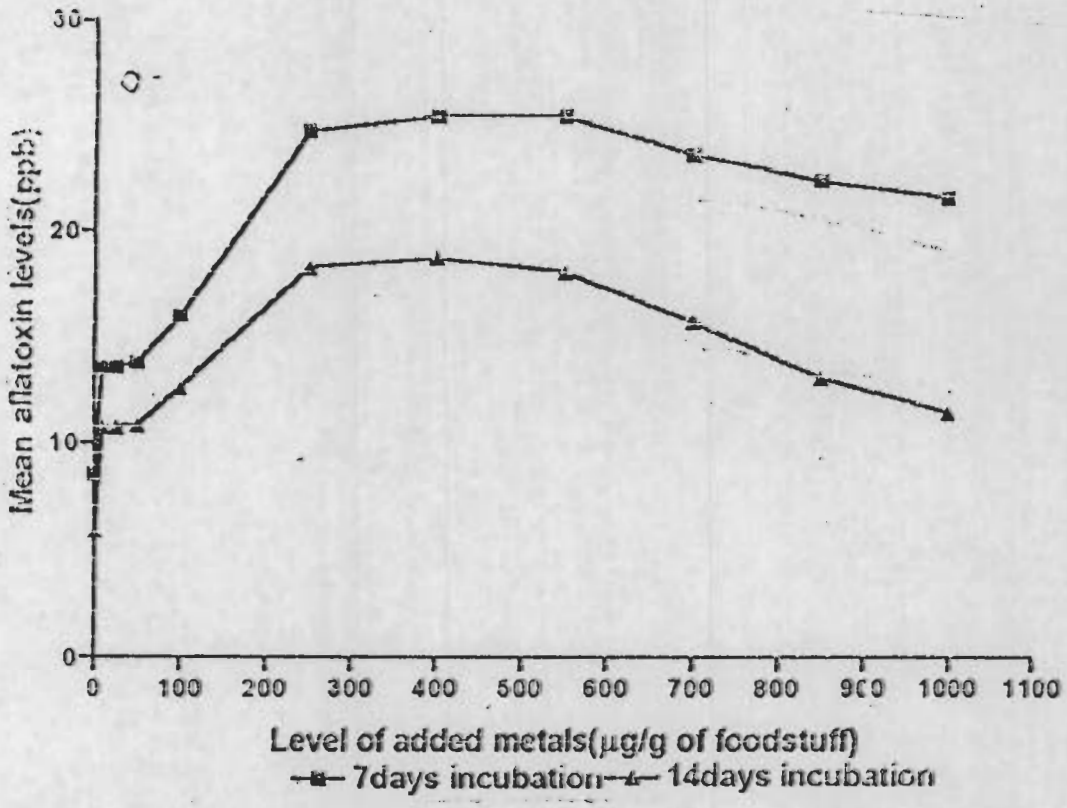


Fig.63:Effects of graded levels of bivalent metals(Zinc) on aflatoxin production in Sorghum(white)

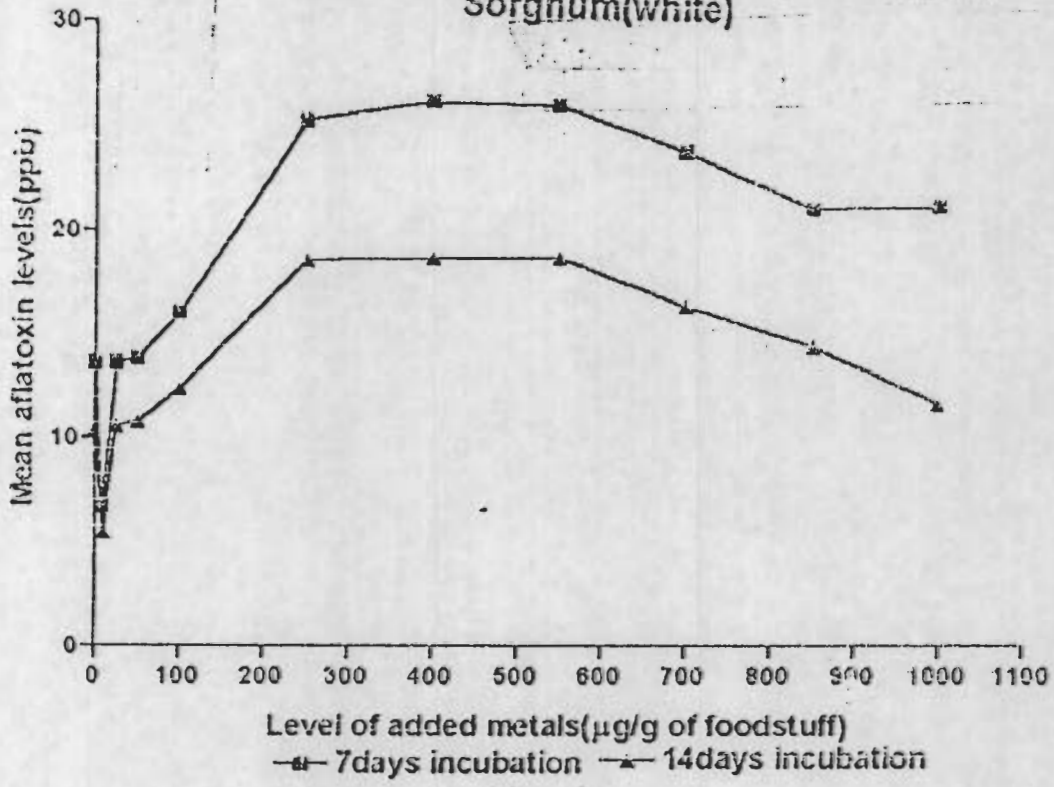


Fig.64: Effects of graded levels of bivalent metals(Zinc) on aflatoxin production in Cowpea(white)

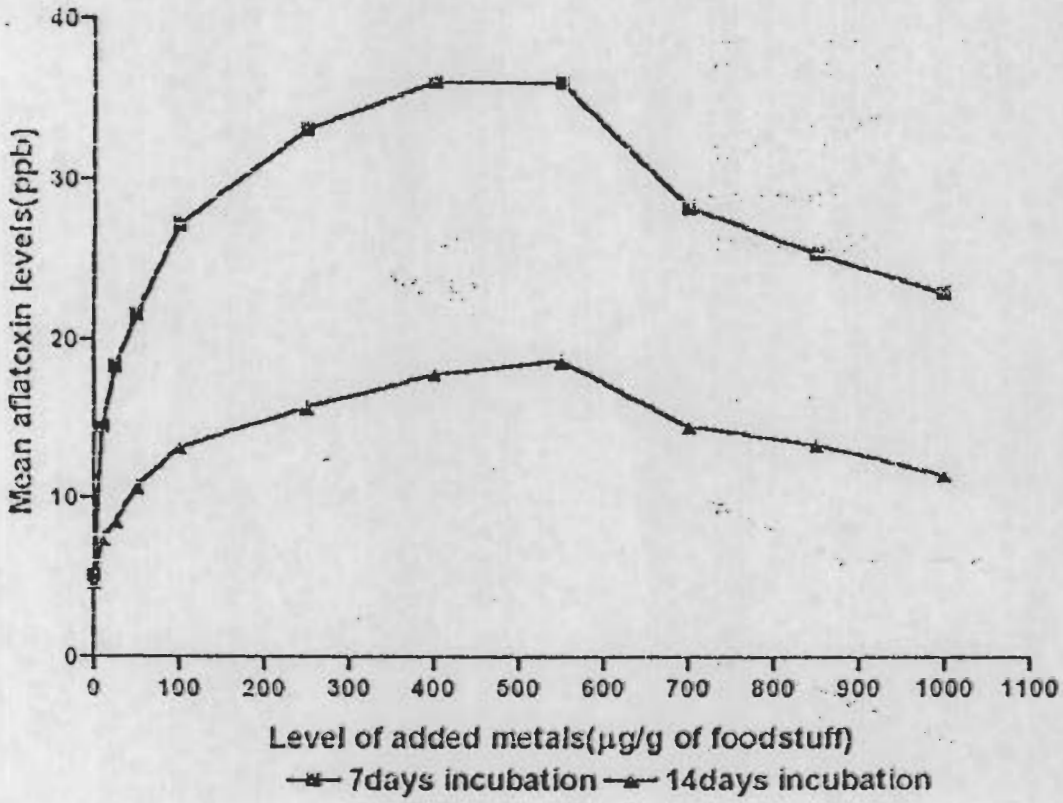


Fig.65: Effects of graded levels of bivalent metals(Zinc) on aflatoxin production in Cowpea(brown).

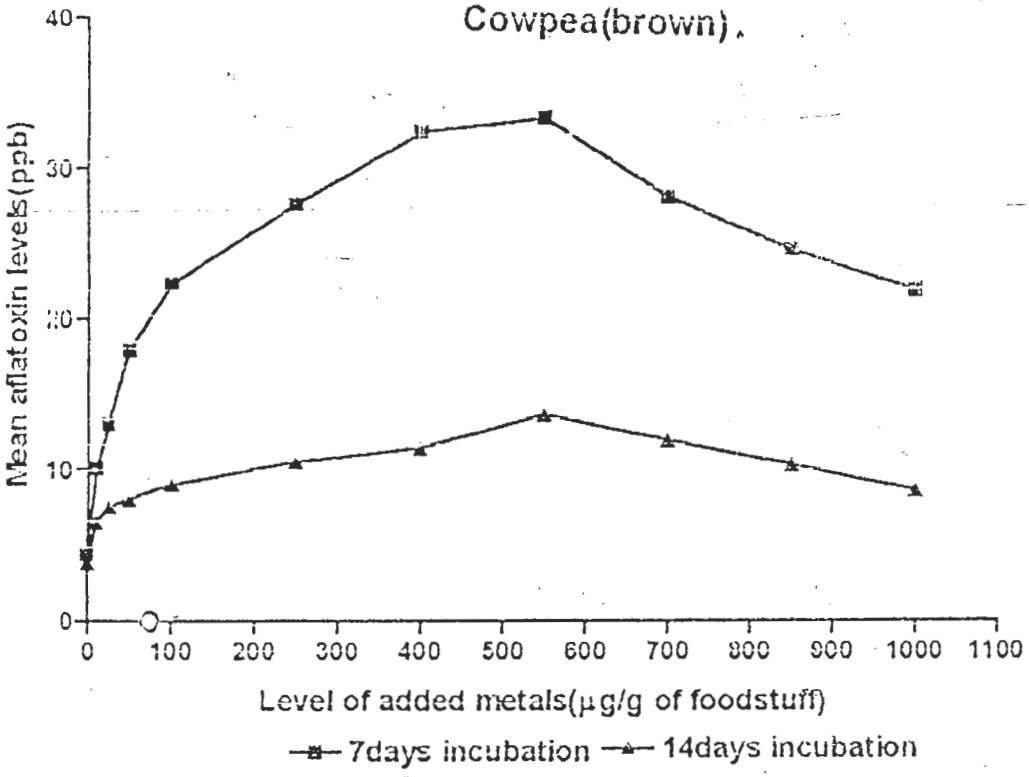


Fig.66; Effects of graded levels of bivalent metals(Zinc) on aflatoxin production in Pigeon pea(white)

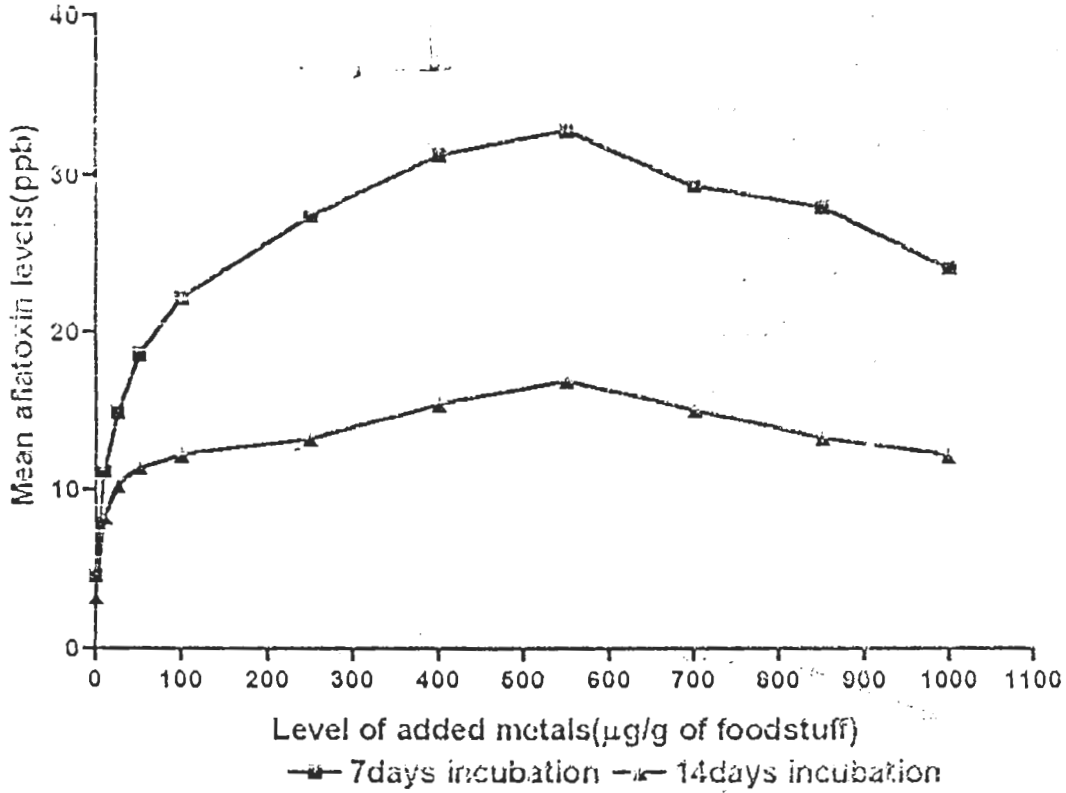


Fig.67: Effects of graded levels of bivalent metals(Zinc) on aflatoxin production in Pigeon pea(brown)

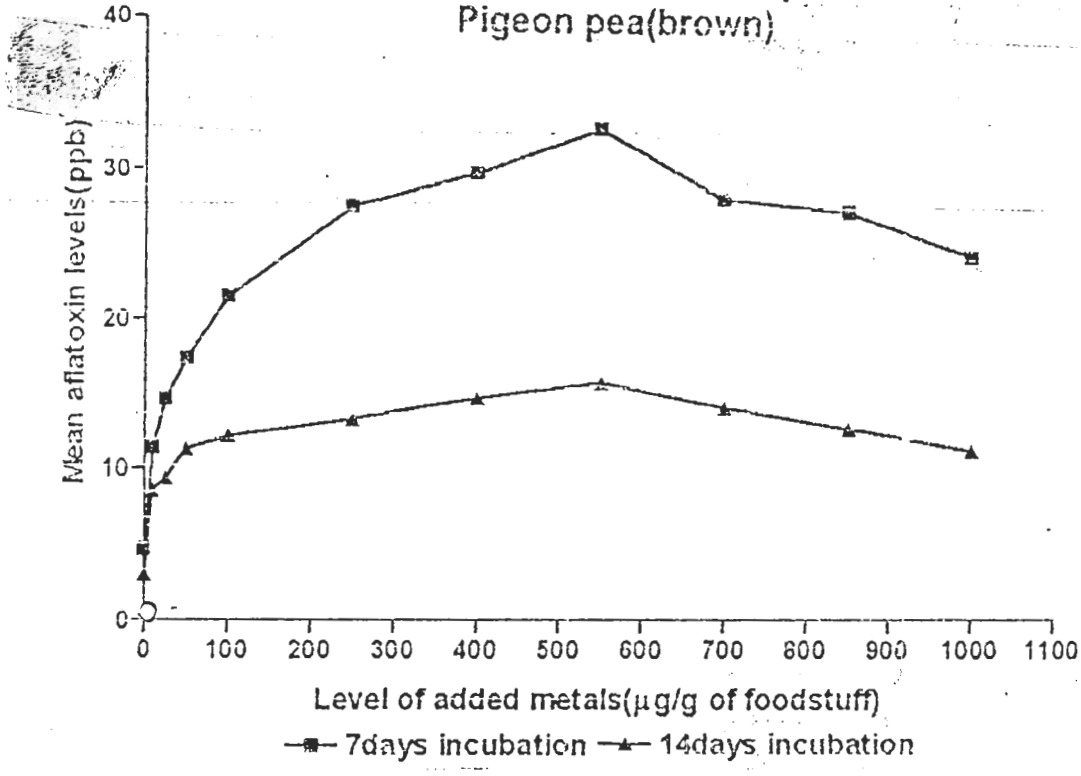


Fig.68: Effects of graded levels of bivalent metals(Zinc) on aflatoxin production in Bambara peanut seed

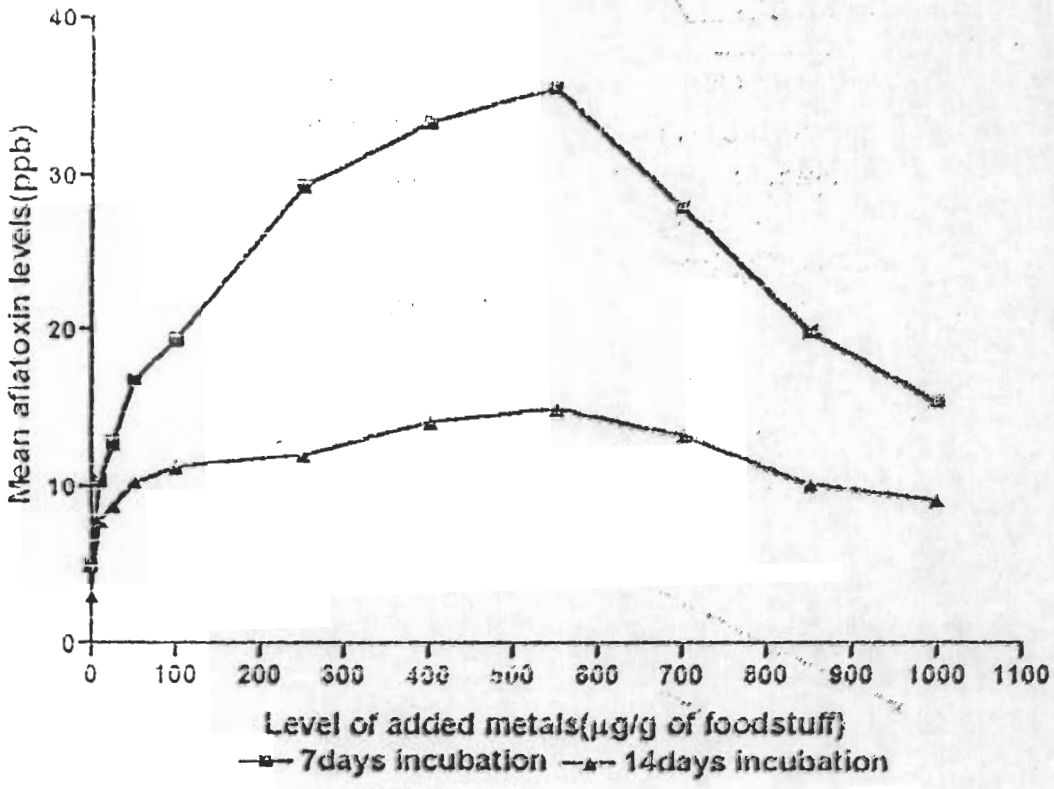


Fig. 69: Effects of graded levels of bivalent metals(Zinc) on aflatoxin production in Soya beans

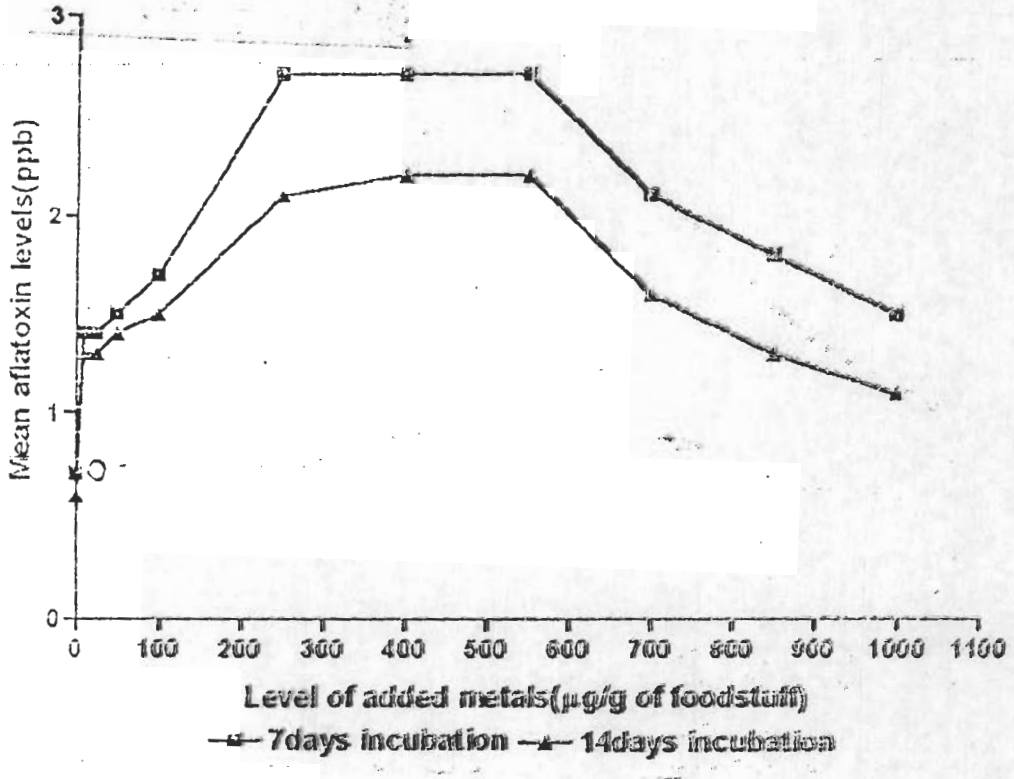
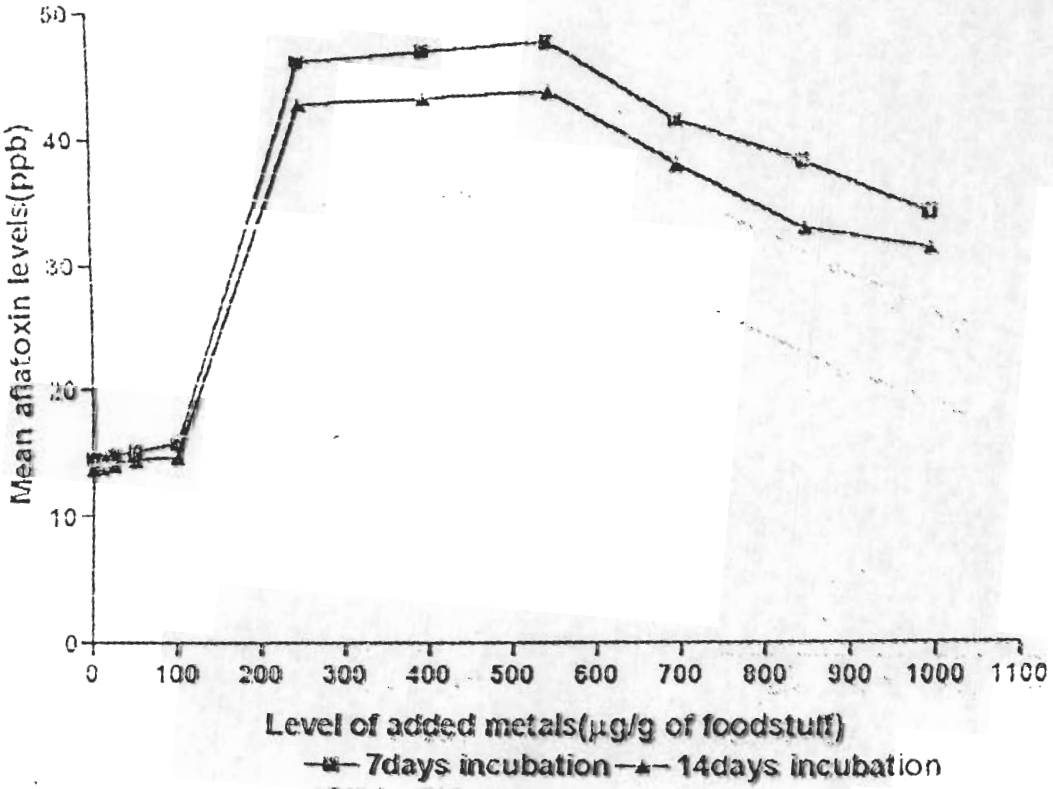


Fig. 70: Effects of graded levels of bivalent metals(Zinc) on aflatoxin production in Bread fruits



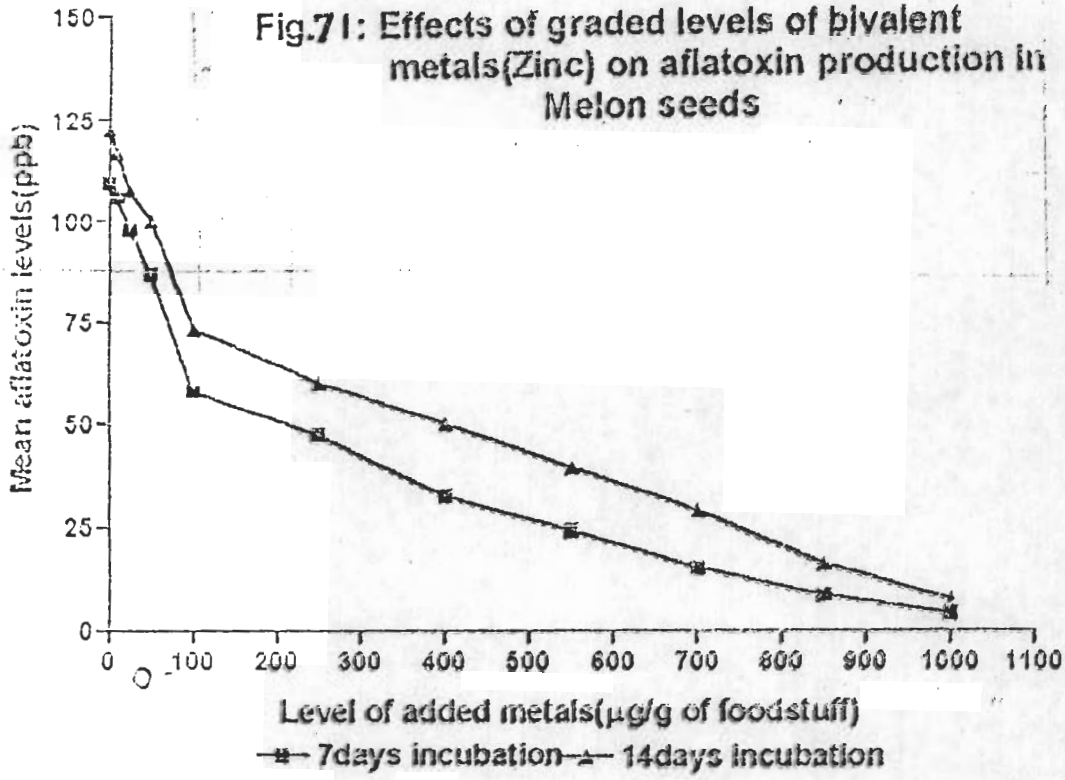


Fig.71: Effects of graded levels of bivalent metals(Zinc) on aflatoxin production in Melon seeds
Mean aflatoxin levels(ppb)
150
125
100
75
50
25
0
0
100
200
300
400
500
600
700
800
900
1000
1100
Level of added metals(µg/g of foodstuff)
7days incubation
14days incubation

Fig.72: Effects of graded levels of bivalent metals(Zinc) on aflatoxin production in Peanuts(white)

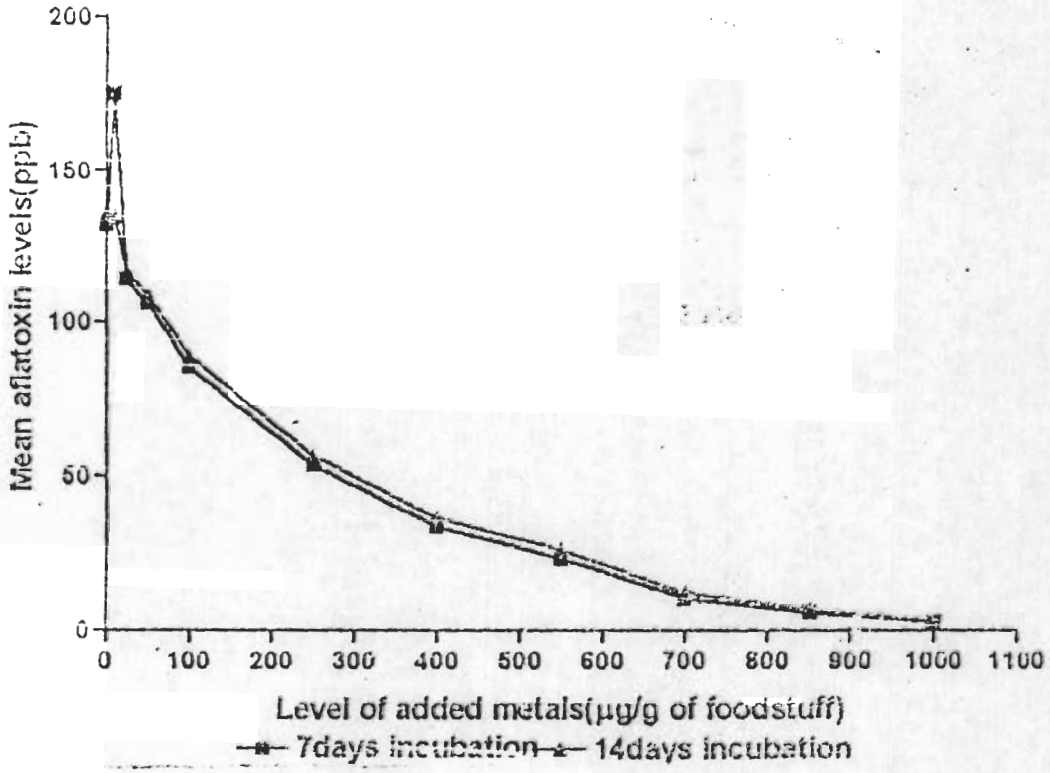


Fig. 73: Effects of graded levels of bivalent metals(Zinc) on aflatoxin production in Peanuts red testa

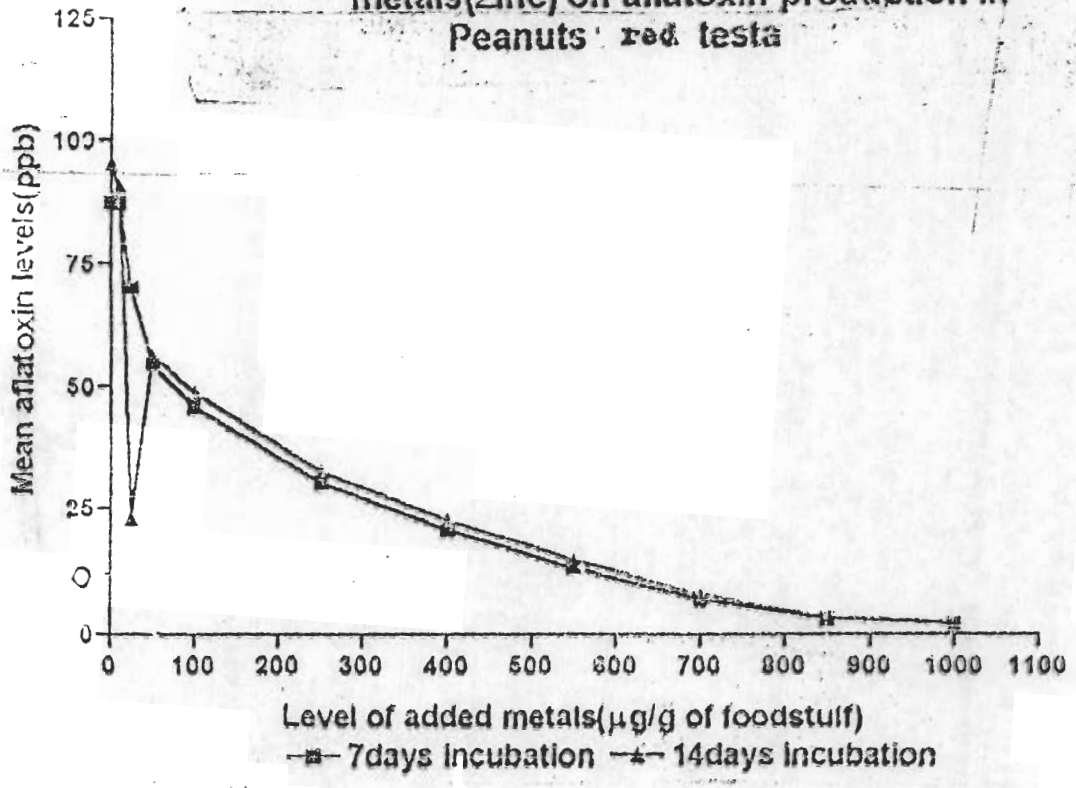
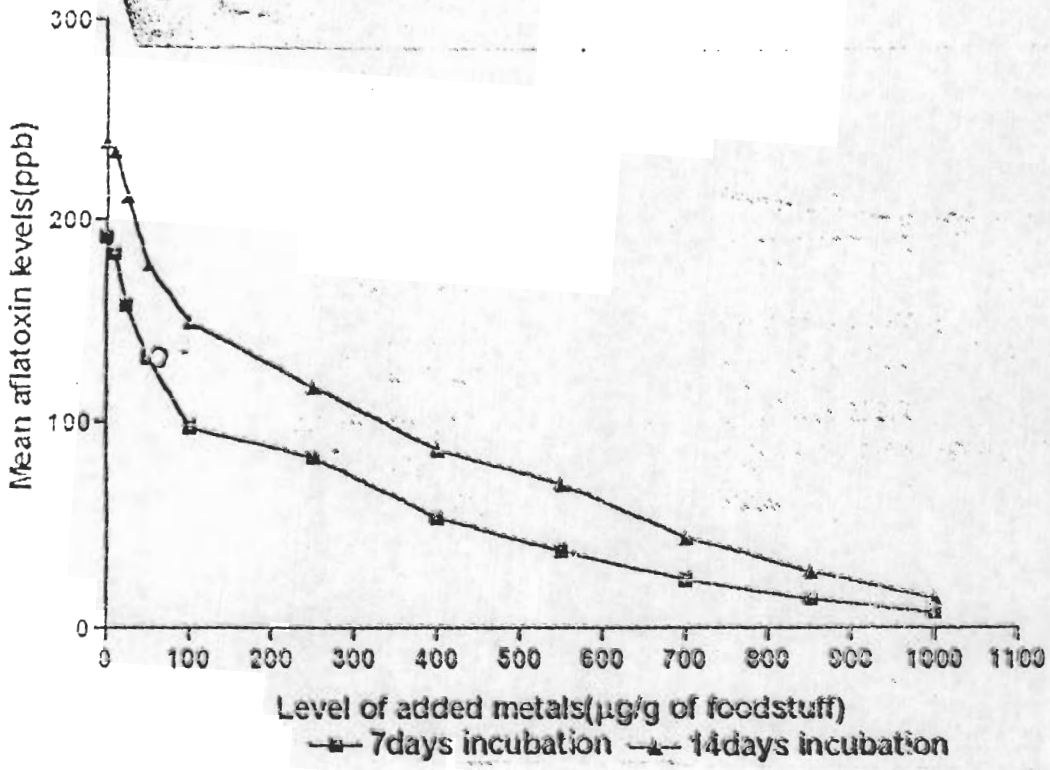


Fig. 74: Effects of graded levels of bivalent metals(Zinc) on aflatoxin production in Coconut seeds



Fig·75: Effects of graded levels of bivalent metals(Zinc) on aflatoxin production in Cocoyam

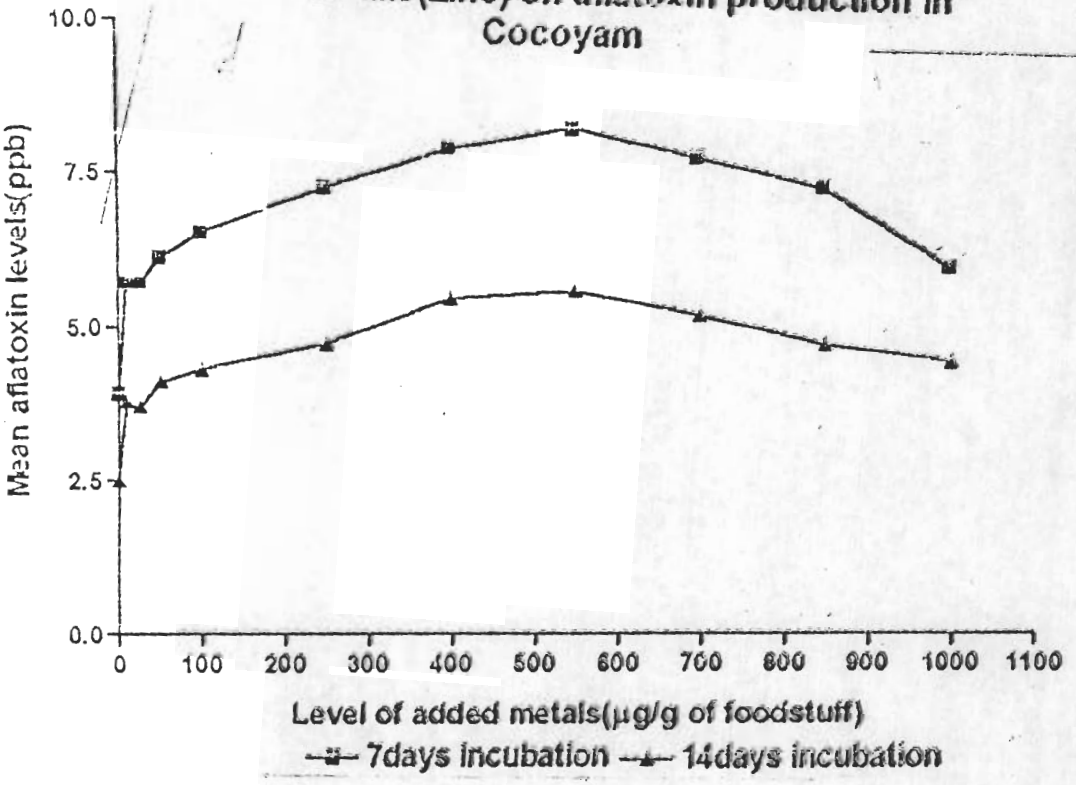


Fig. 76: Effects of graded levels of bivalent metals(Zinc) on aflatoxin production in Yam tuber

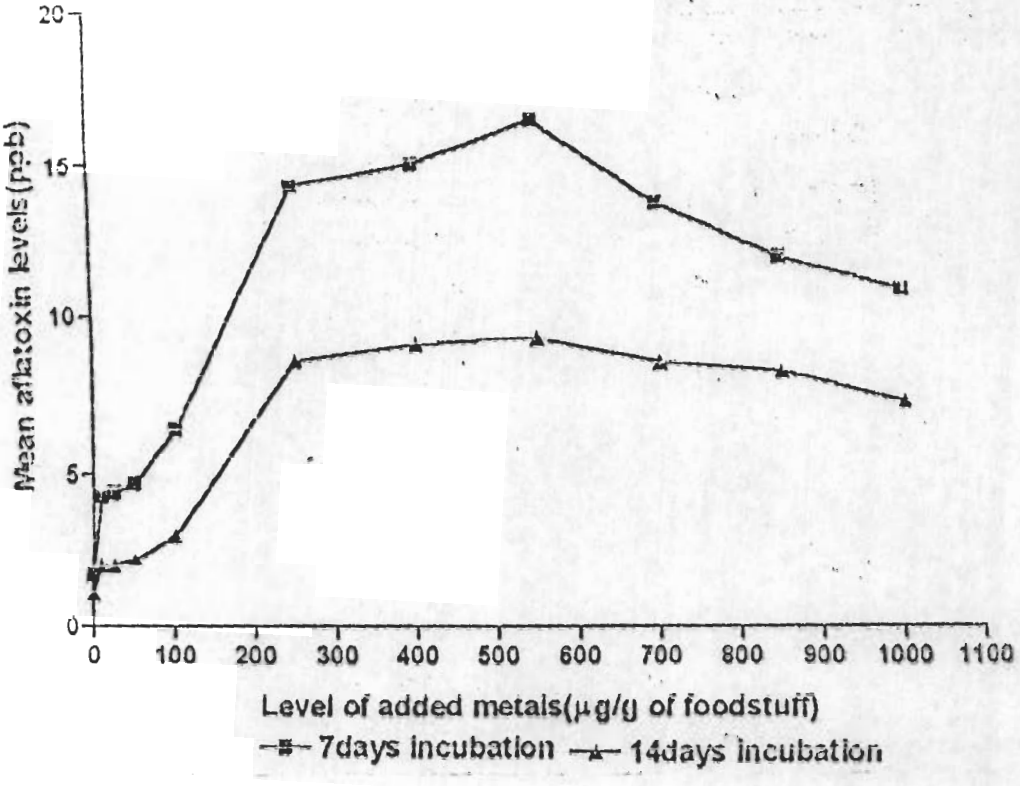


Fig.77: Effects of graded levels of bivalent metals(Zinc) on aflatoxin production in Cassava tubers

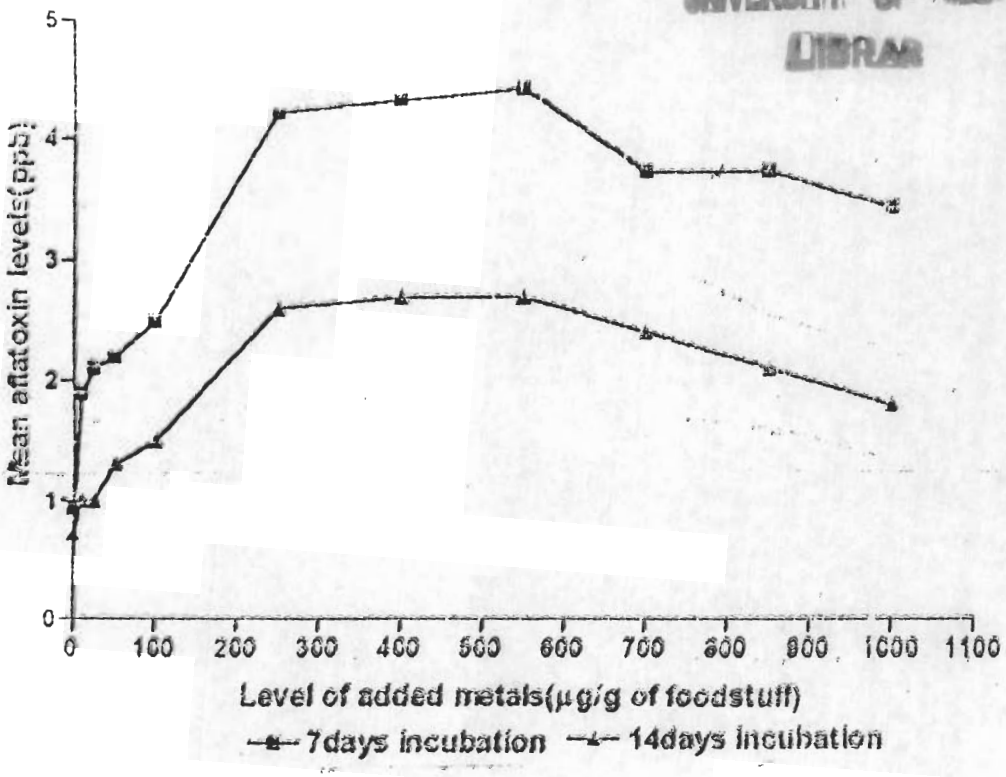


Fig. 78: Effects of graded levels of bivalent metals(Zinc) on aflatoxin production in Plantain (ripe)

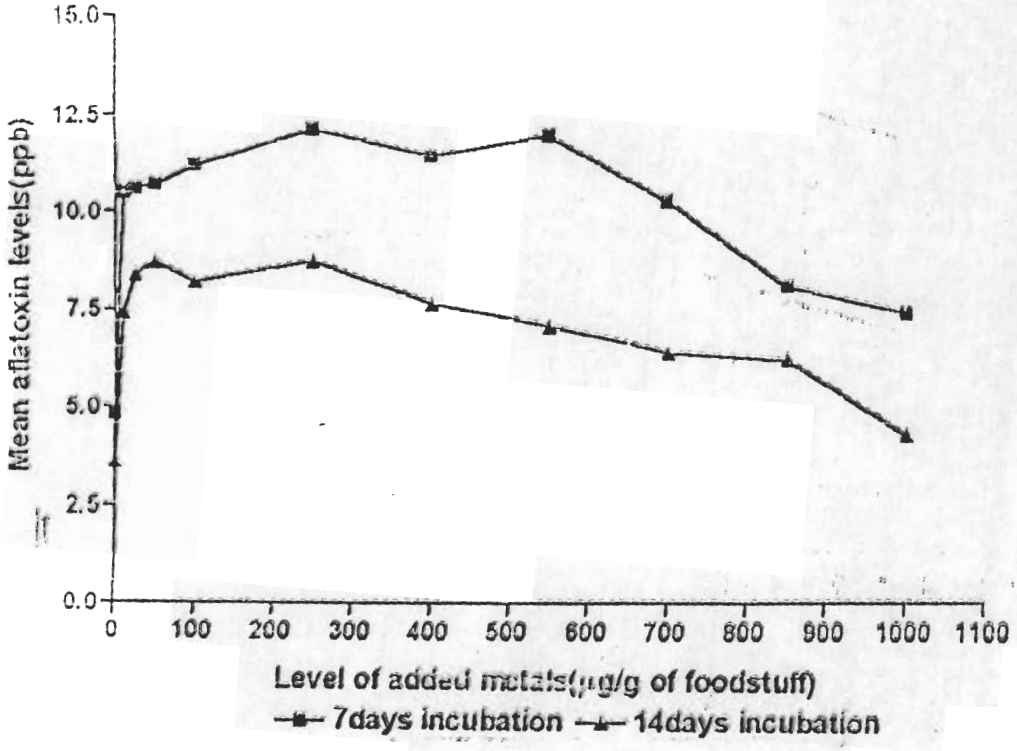
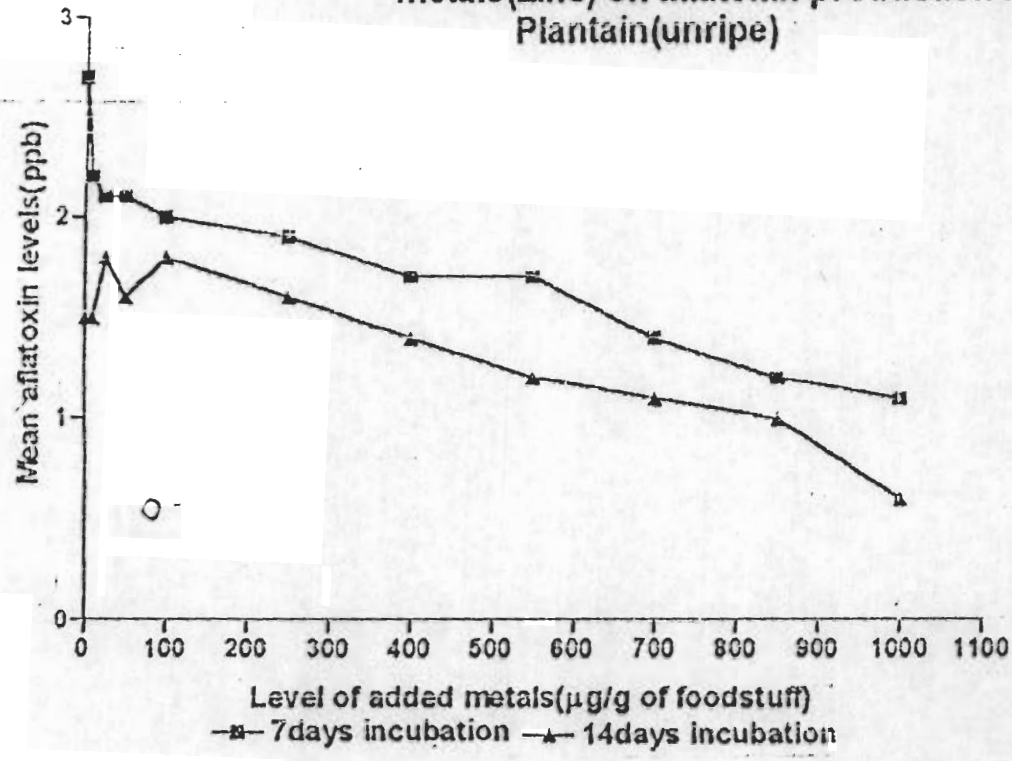


Fig-79: Effects of graded levels of bivalent metals(Zinc) on aflatoxin production in Plantain(unripe)



concentration per gram of cereals which again became constant till the highest metal concentration. A similar trend was also recorded for the carbohydrate foods. However, instead of the slight drop observed in all other food substrates in these food classes at 250-400μg Mn^{2+}, plantain rather increased and maintained same till the highest metal concentration though this effect was more noticeable at seven day incubation rather than fourteen days. In all the carbohydrate foods and cereals with the exception of the sorghum varieties and the Abakaliki rice variety, the difference between the AF levels analysed at 7 days incubation and 14 days incubation were statistically significant ($P<0.05$).

The AF levels in the protein foods decreased from 10μg per gram food substrate of the manganese added, and started increasing at 100μg Mn^{2+} per gram of substrate till the highest metal concentration. However, a different pattern was observed in the breadfruits in which AF levels continued to increase with increasing metal concentration.

For the oilseeds, a drop in toxin production occurred at 10μg metal per gram of food, started picking up again at 25μg/g and increased at 100μg metal concentration per gram of food and rose very significantly from 700μg metal per gram of food till the highest metal concentration ($P<0.05$). There were no differences in AF levels at day seven and fourteen incubation periods for the oil seeds, but for the protein foods, this difference was statistically significant ($P<0.05$) only at higher levels of metal concentration (from 700μg metal per gram of food to 1000μg metal per gram of food substrates) except brown cowpea and soyabean.

AF levels in bambara peanuts seed and soybeans were almost similar for days seven and fourteen analysis.

Fig. 80: Effects of graded levels of bivalent metals(Manganese Mn^{2+}) on aflatoxin production in Maize(white)

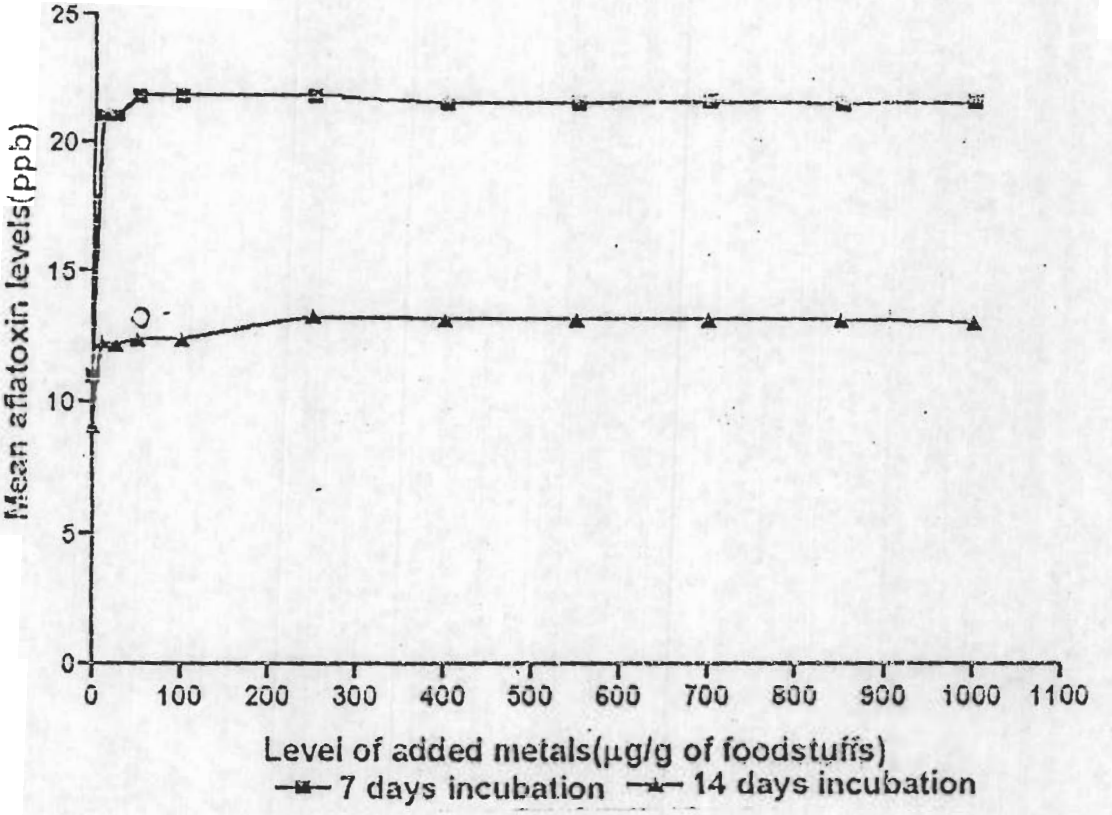


Fig. 8l: Effects of graded levels of bivalent metals(Manganese Mn^{2+}) on aflatoxin production in Maize(yellow)

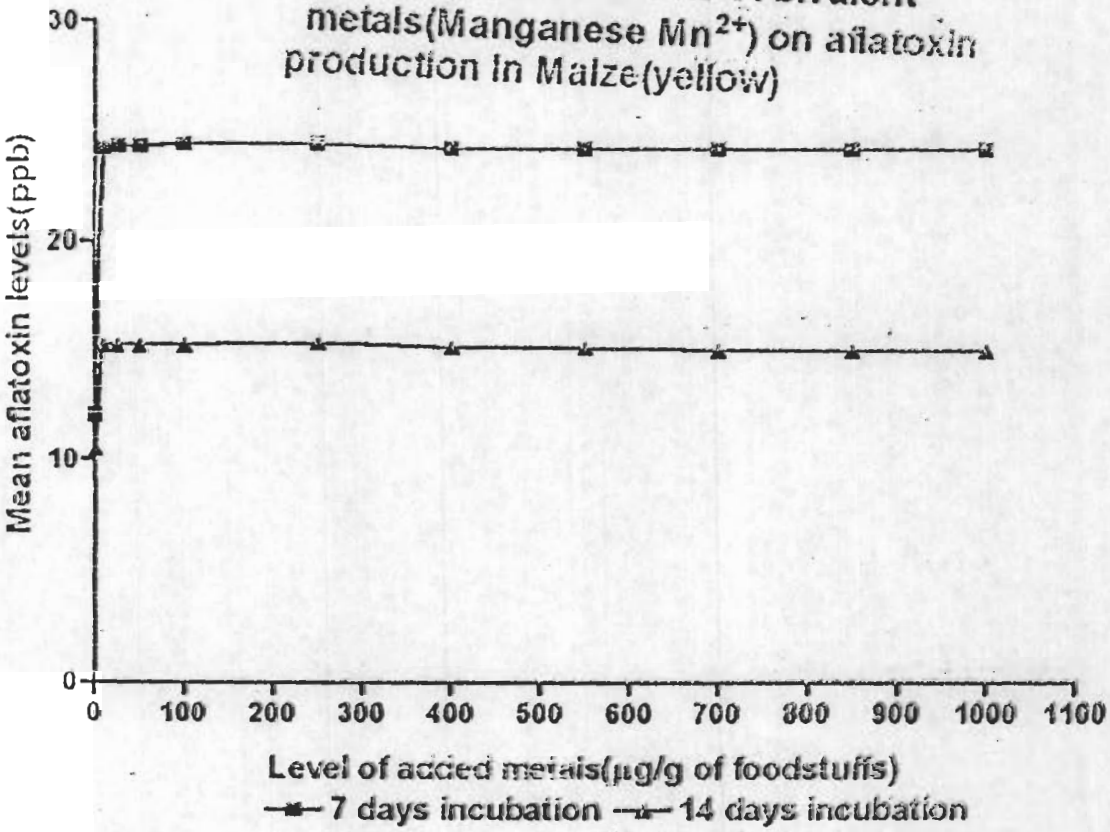


Fig..82:Effects of graded levels of bivalent metals(Manganese Mn^{2+}) on aflatoxin production in Acha (grains) Findi

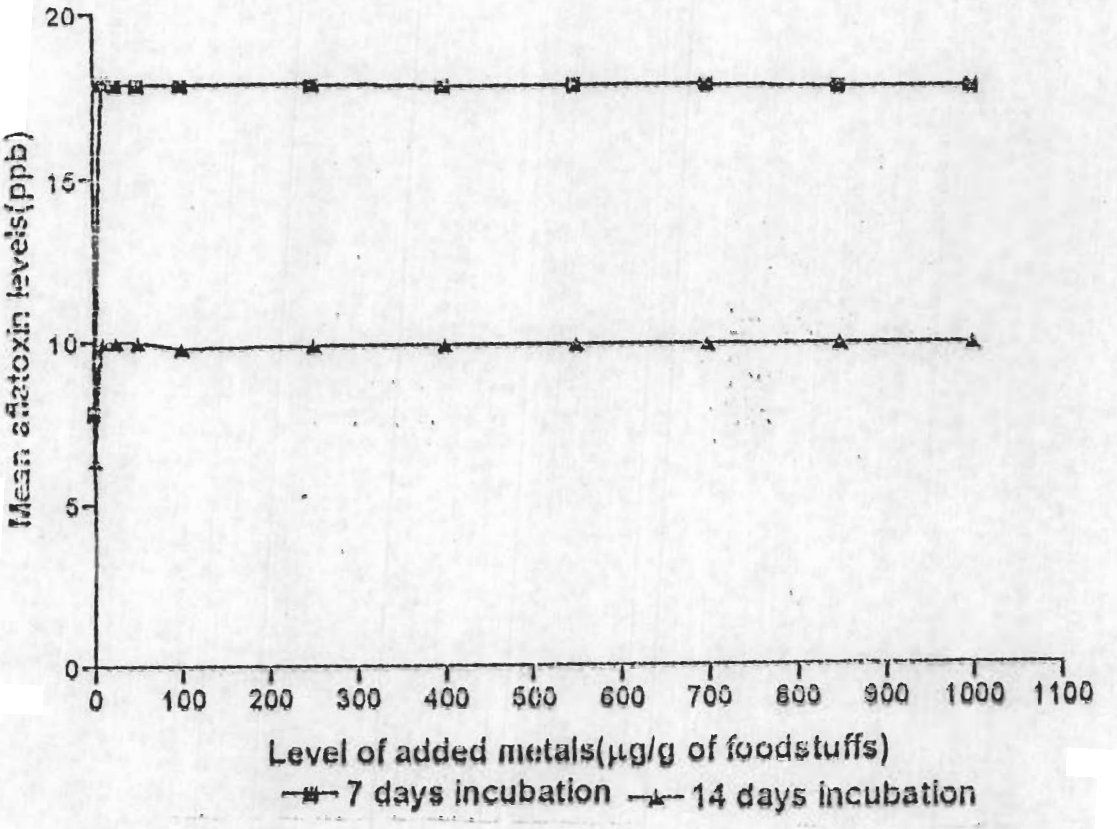


Fig 83 Effects of graded levels of bivalent metals(Manganese Mn^{2+}) on aflatoxinproduction in Millet

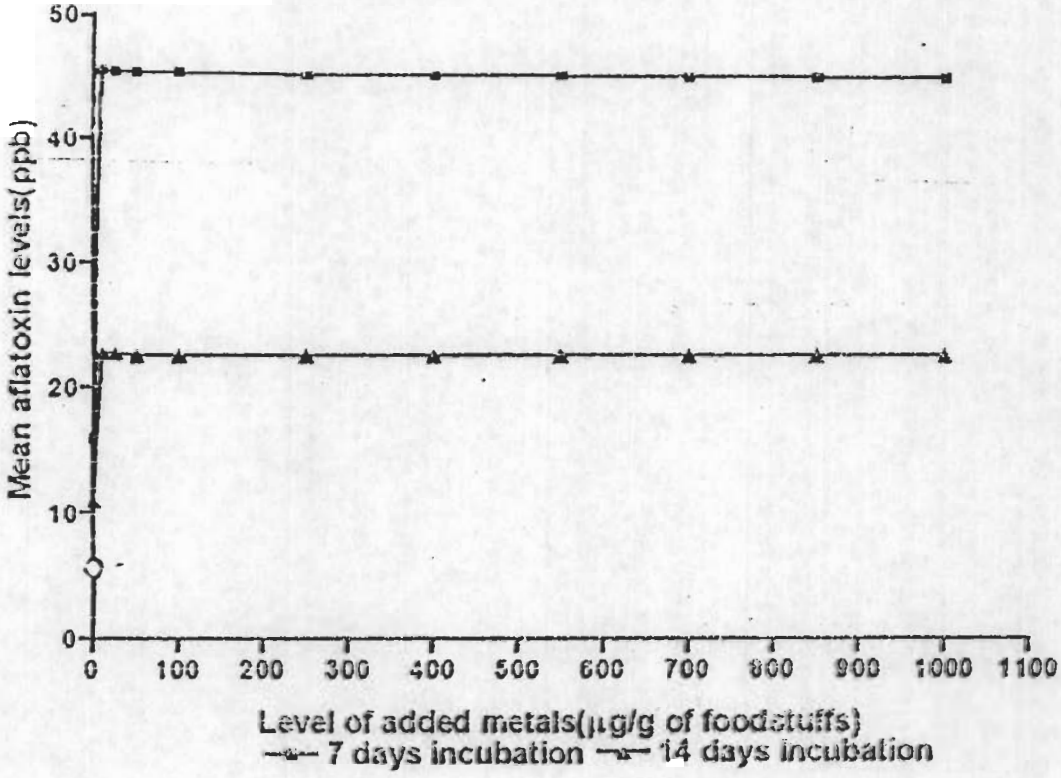


Fig. 8/: Effects of graded levels of bivalent metals(Manganese Mn^{2+}) on aflatoxin production in Rice(Abakaliki)

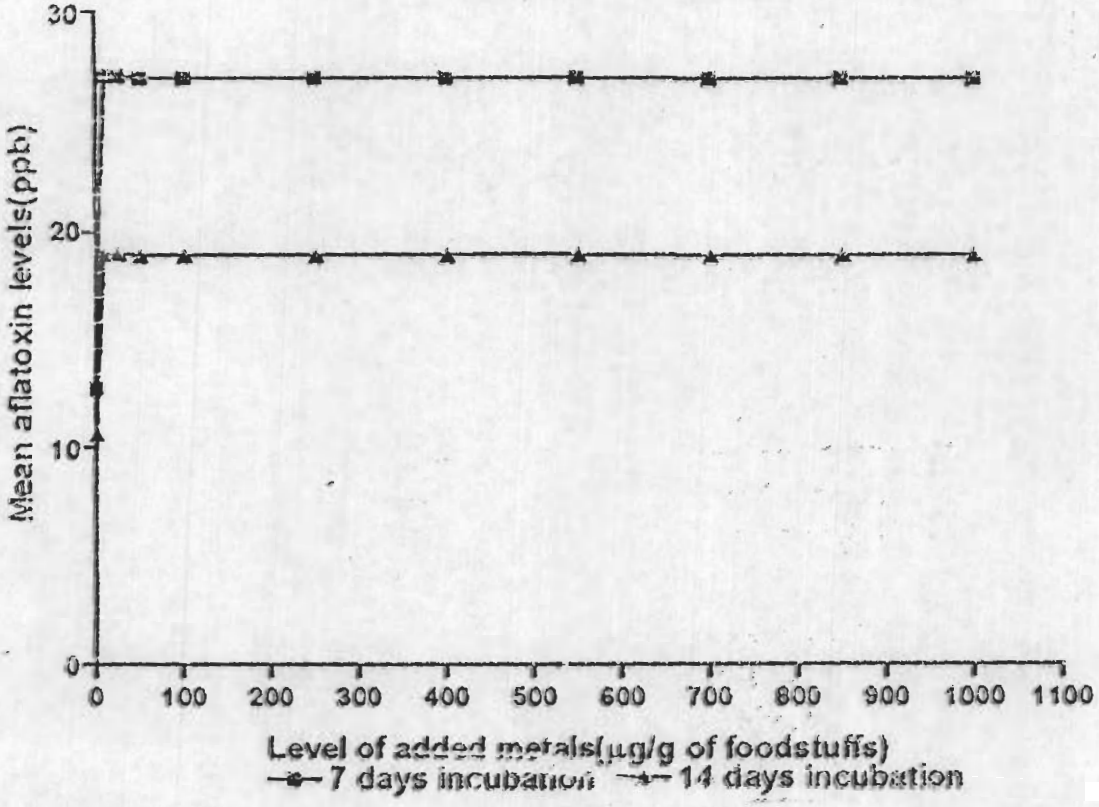


Fig 85: Effects of graded levels of bivalent metals (Manganese Mn^{2+}) on aflatoxin production in Rice (Ugbawka)

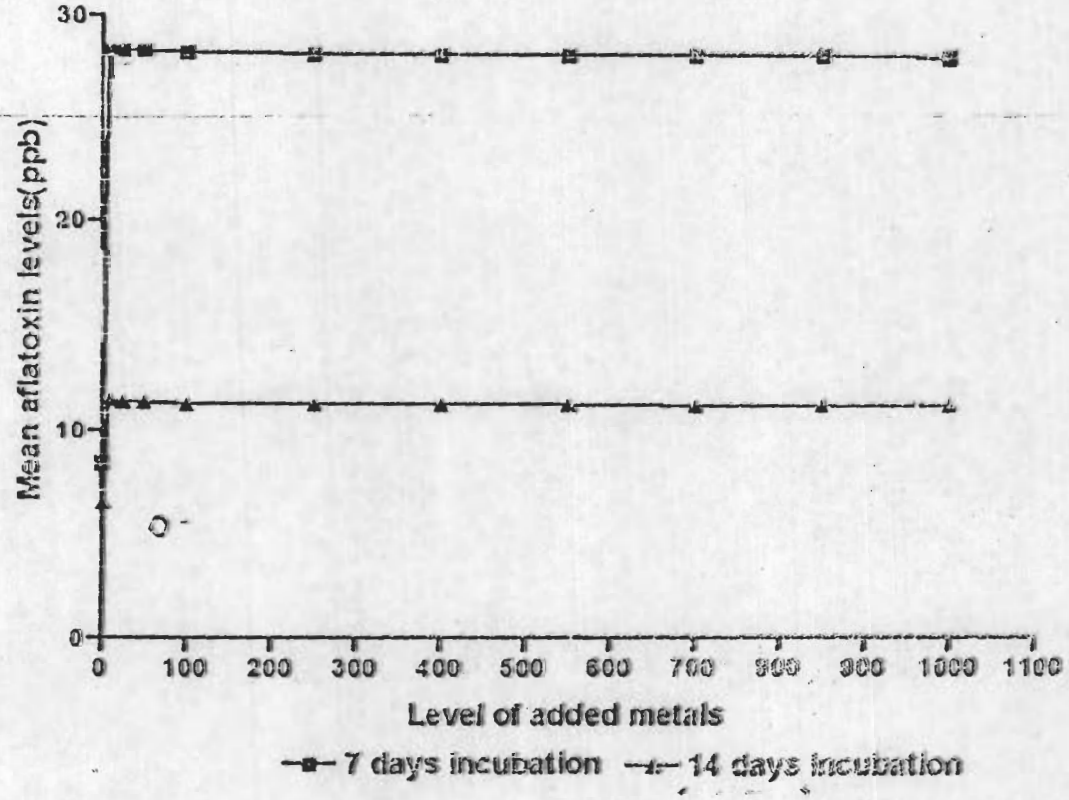


Fig. 86:Effects of graded levels of bivalent metals(Manganese Mn^{2+}) on aflatoxin production in Sorghum(yellow)

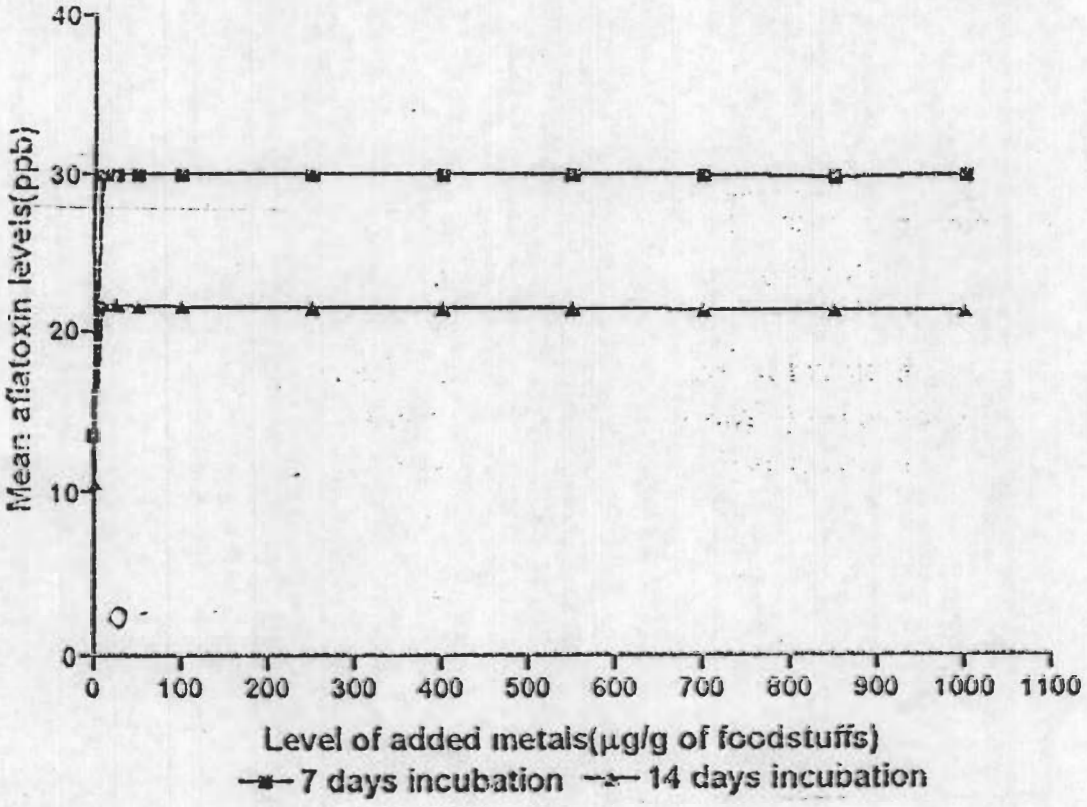


Fig 87: Effects of graded levels of bivalent metals(Manganese Mn^{2+}) on aflatoxin production in Sorghum(white)

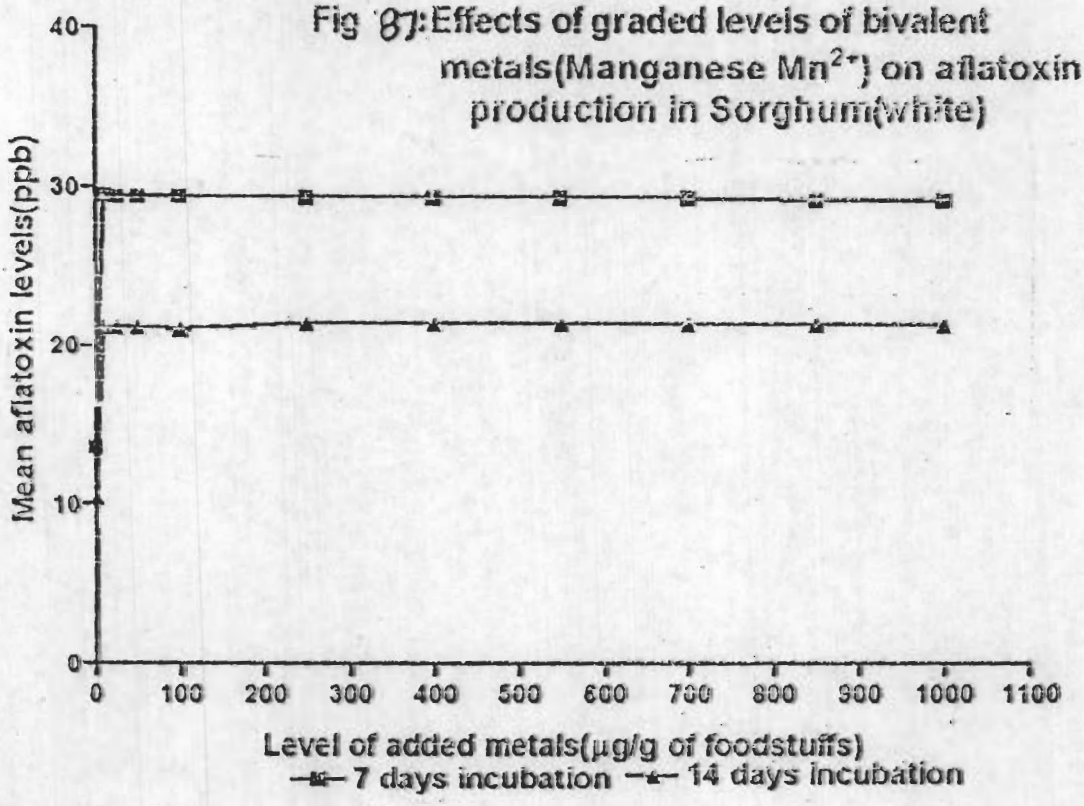


Fig. 88:Effects of graded levels of bivalent metals(Manganese Mn^{2+}) on aflatoxin production in Cowpea(white)

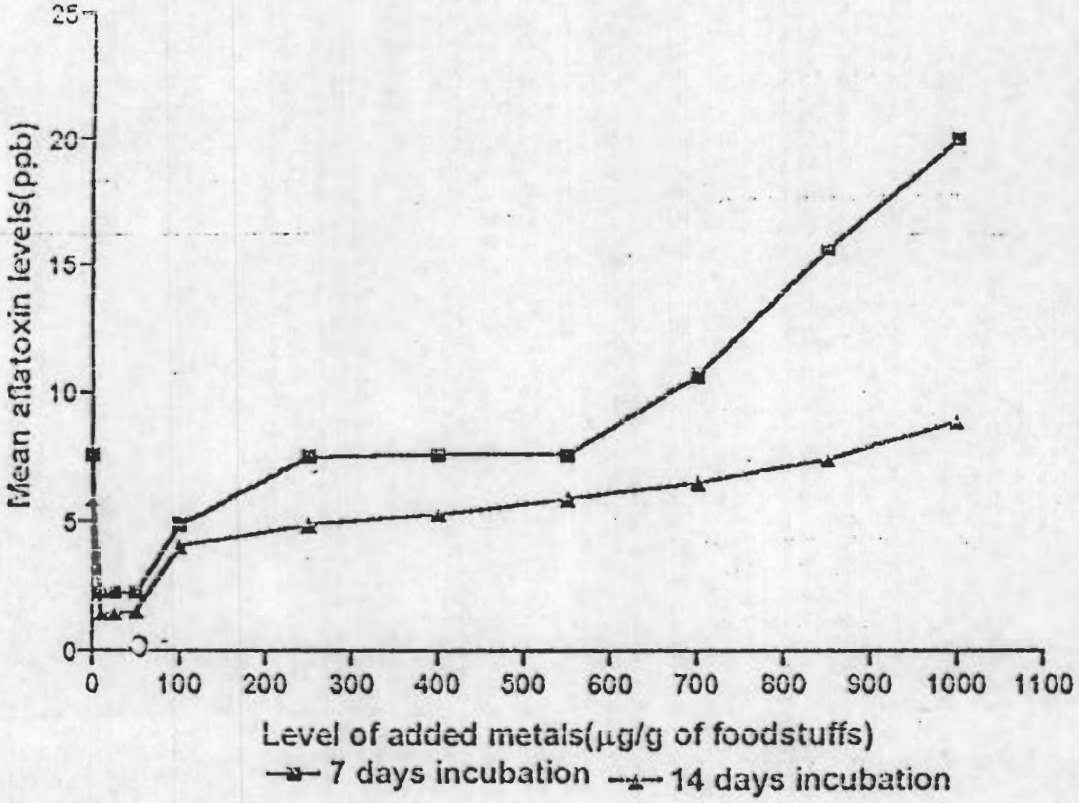


Fig. 89: Effects of graded levels of bivalent metals(Manganese Mn^{2+}) on aflatoxin production in Cowpea(brown)

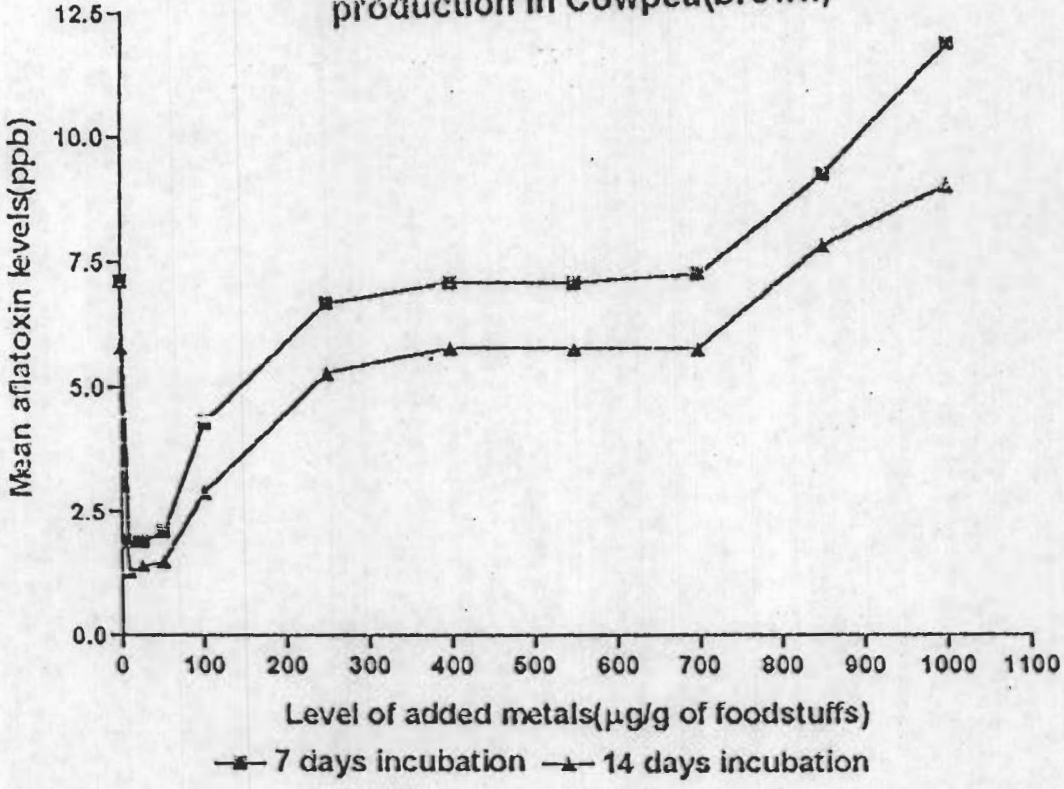


Fig. 90: Effects of graded levels of bivalent metals(Manganese Mn^{2+}) on aflatoxin production in Pigeon pea(white)

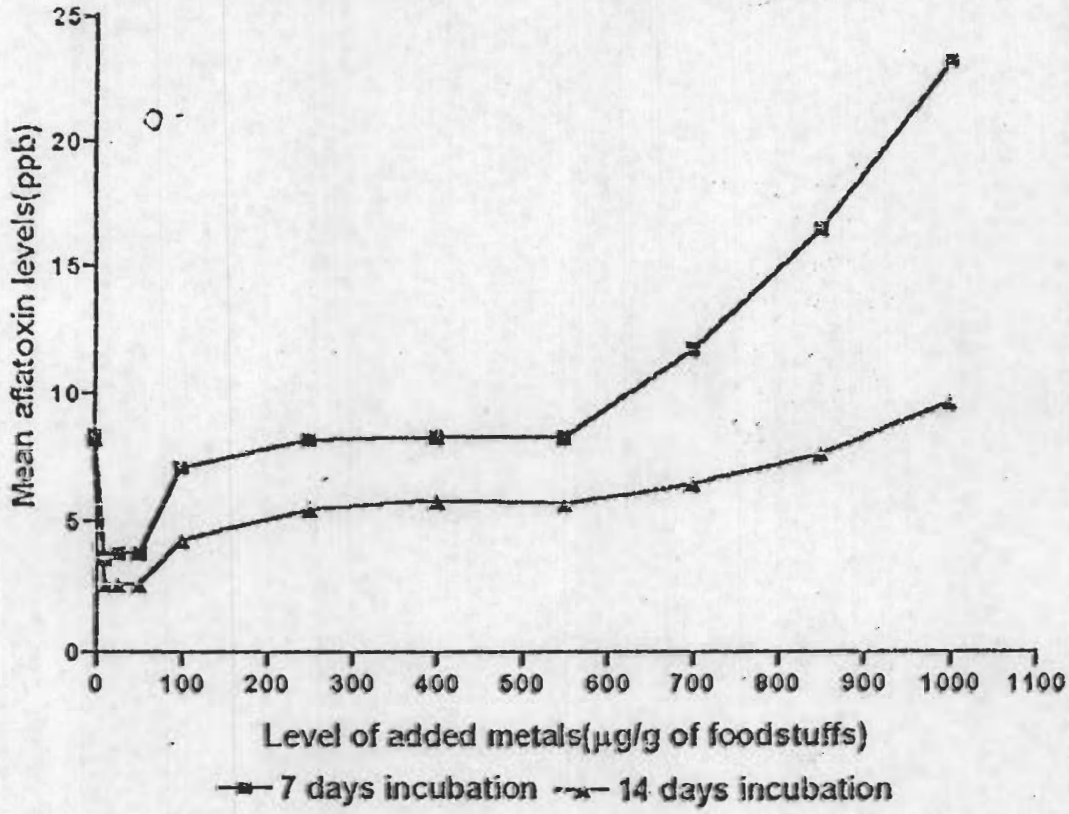


Fig. 9: Effects of graded levels of bivalent metals(Manganese Mn^{2+}) on aflatoxin production in Pigeon pea(brown)

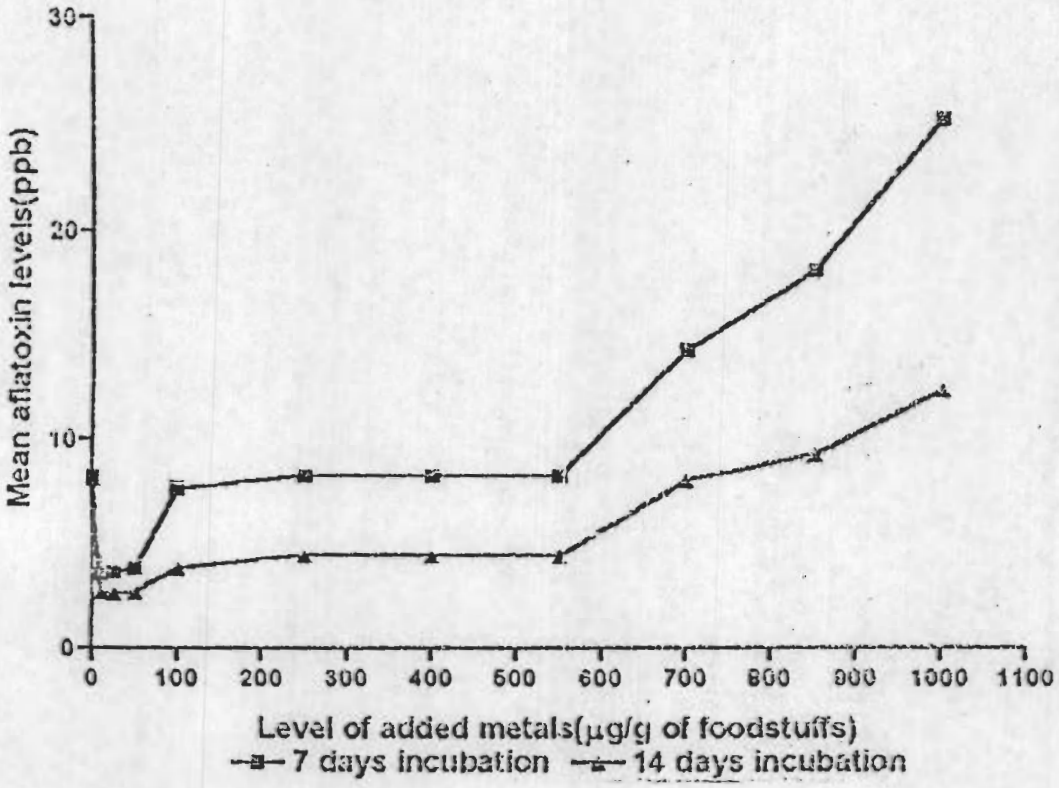


Fig. 92:Effects of graded levels of bivalent metals(Manganese Mn^{2+}) on aflatoxin production in Bambara peanut seed

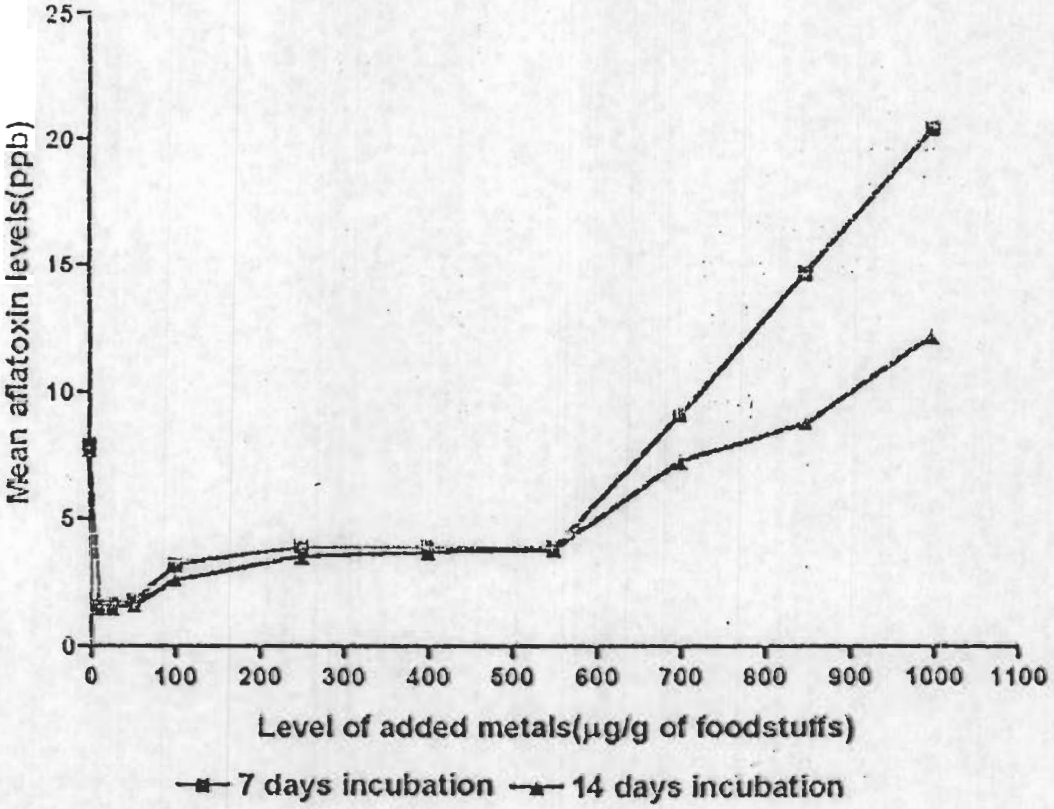


Fig. 93: Effects of graded levels of bivalent metals(Manganese Mn^{2+}) on aflatoxin production in Soya beans

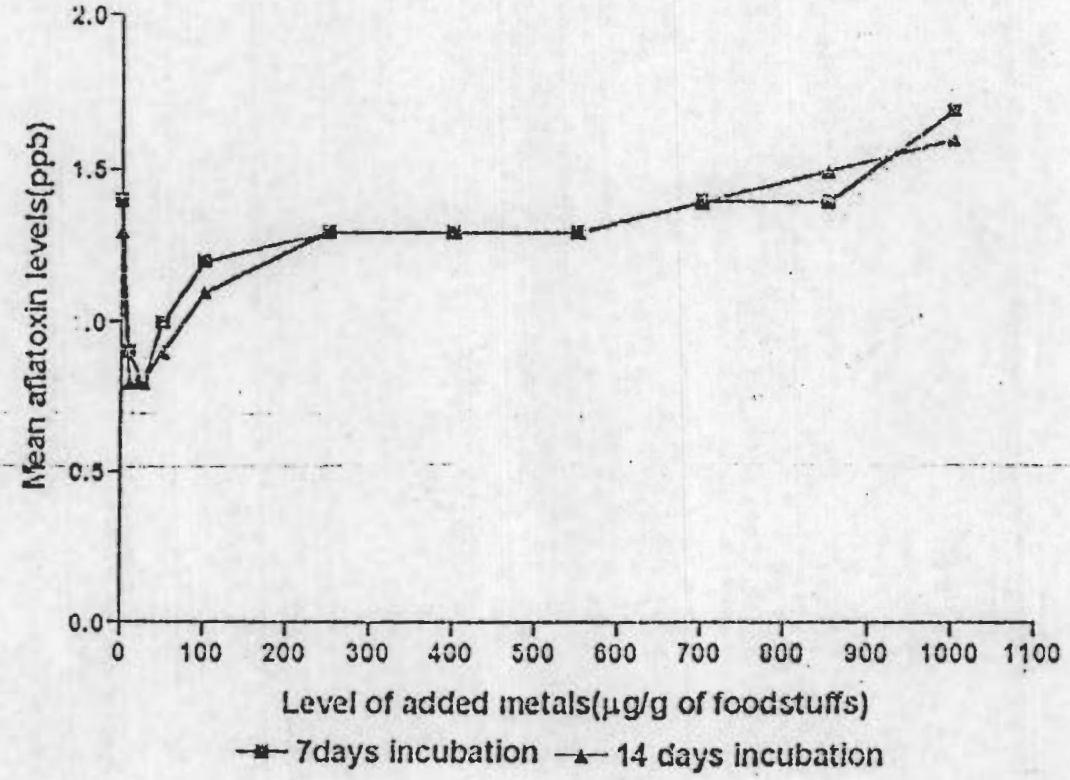


Fig. 94:Effects of graded levels of bivalent metals(Manganese Mn^{2+}) or aflatoxin production in Bread fruits

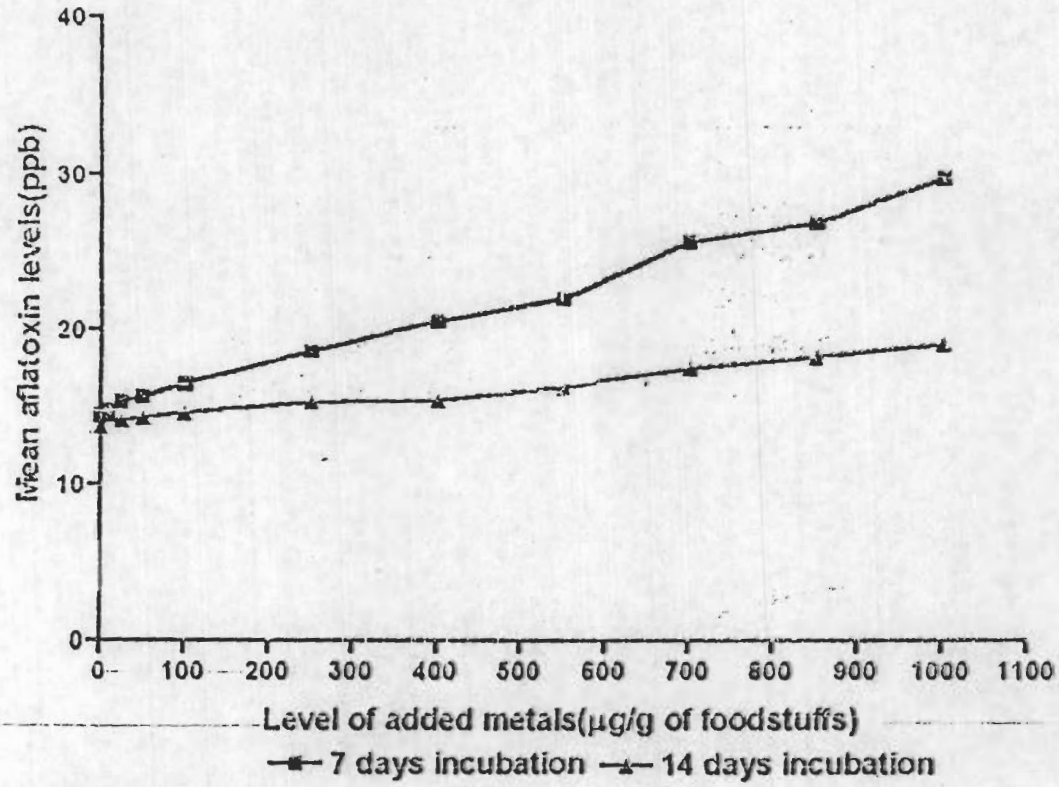


Fig. 95: Effects of graded levels of bivalent metals(Manganese Mn^{2+}) on aflatoxin production Melon seeds

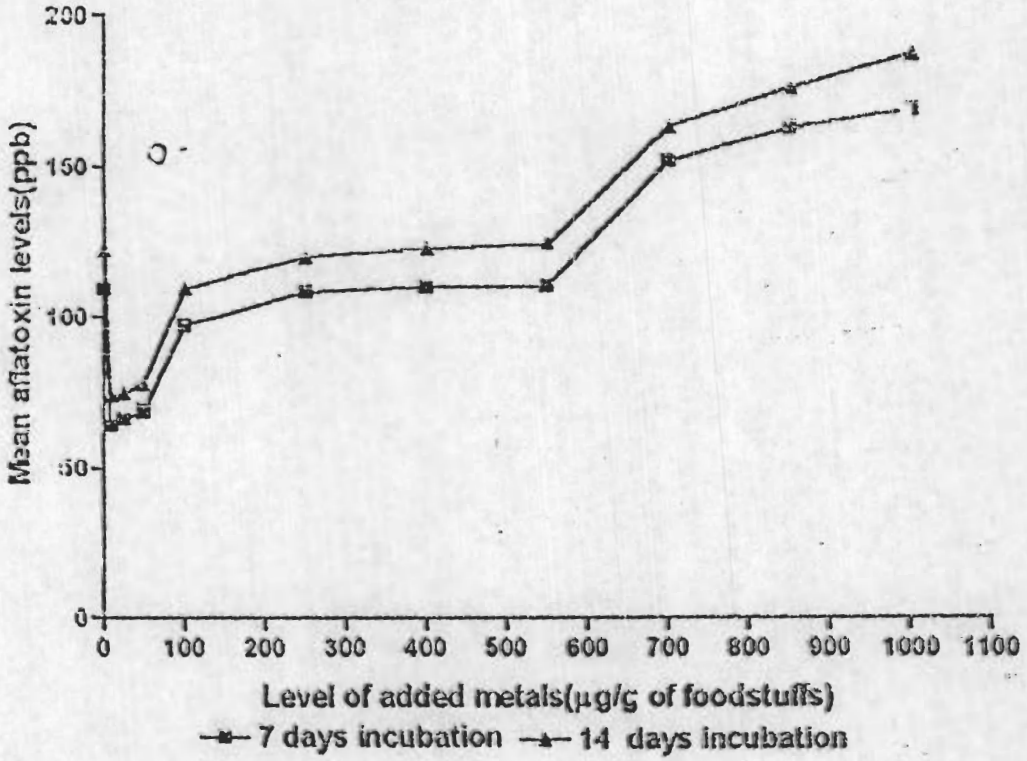


Fig 96: Effects of graded level of bivalent metals(Manganese Mn^{2+}) on aflatoxin production in Peanuts (white)

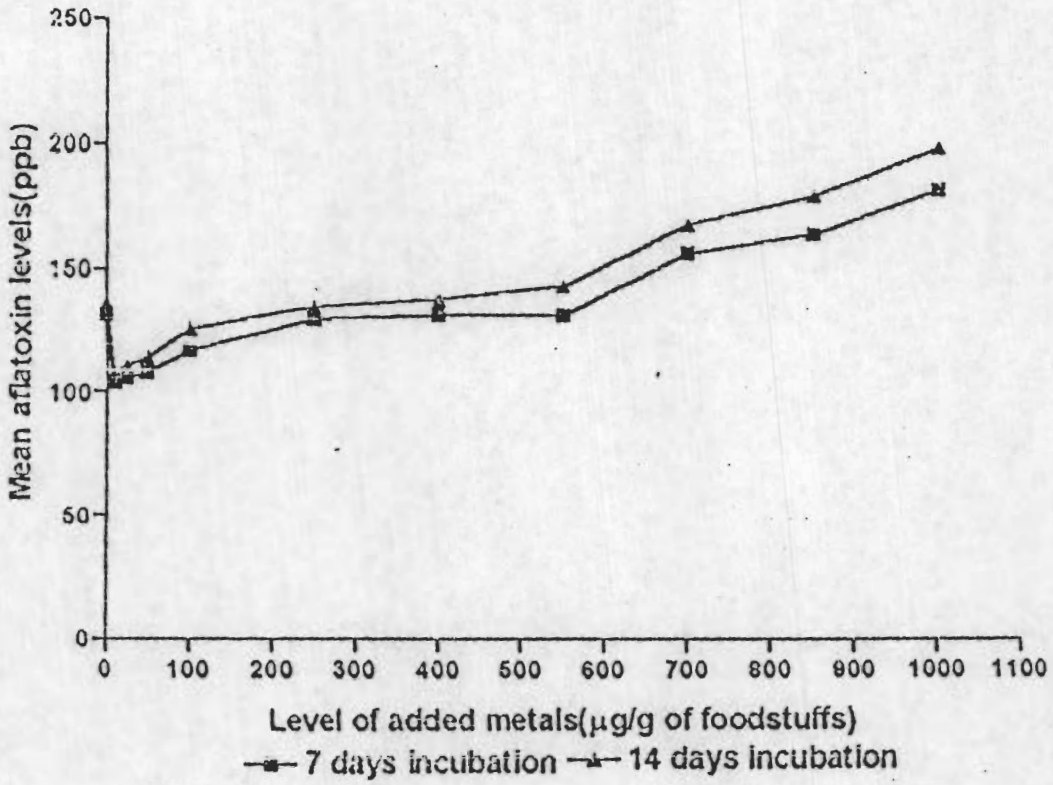


Fig. 97 Effects of graded levels of bivalent metals(Manganese Mn^{2+}) on aflatoxin production in Peanuts, Red testa

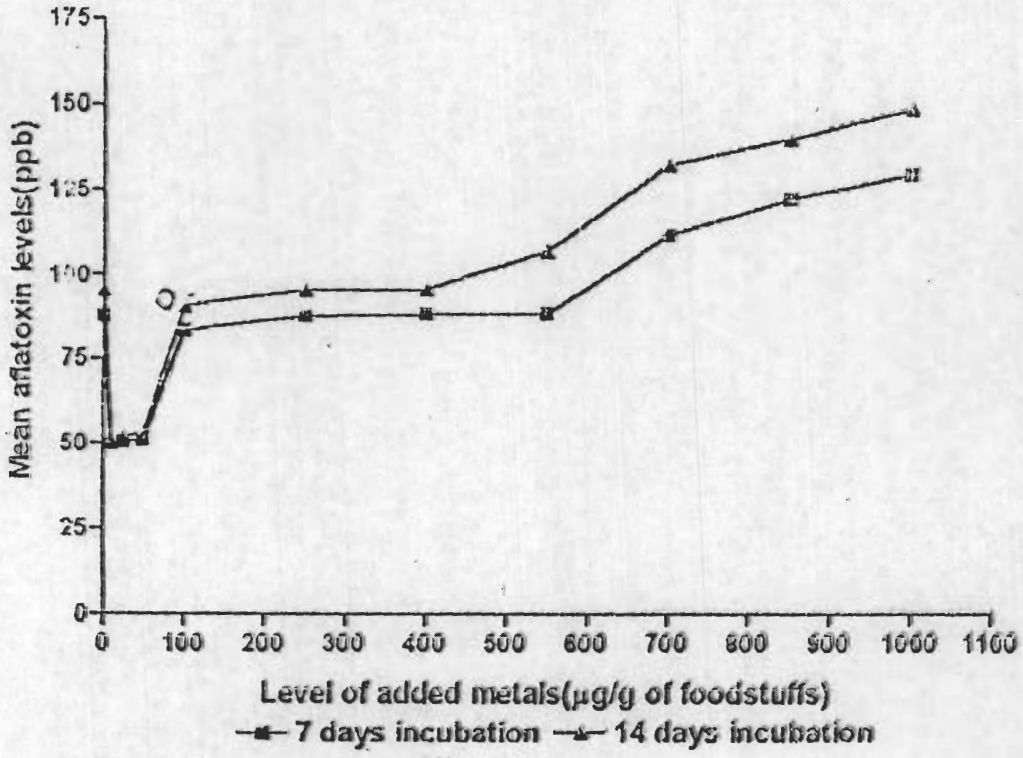
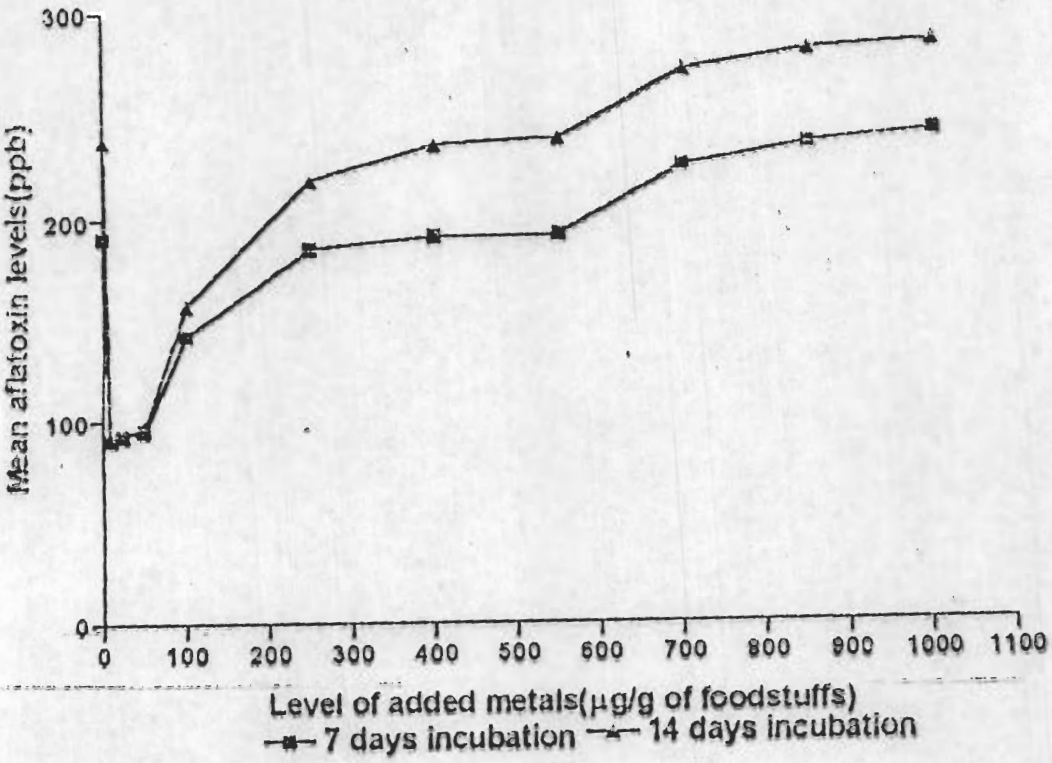


Fig. 98:Effects of graded levels of bivalent metals(Manganese Mn^{2+}) on aflatoxin production Coconut seeds



graded levels of bivalent metals(Manganese Mn^{2+}) on aflatoxin production in Cocoyam

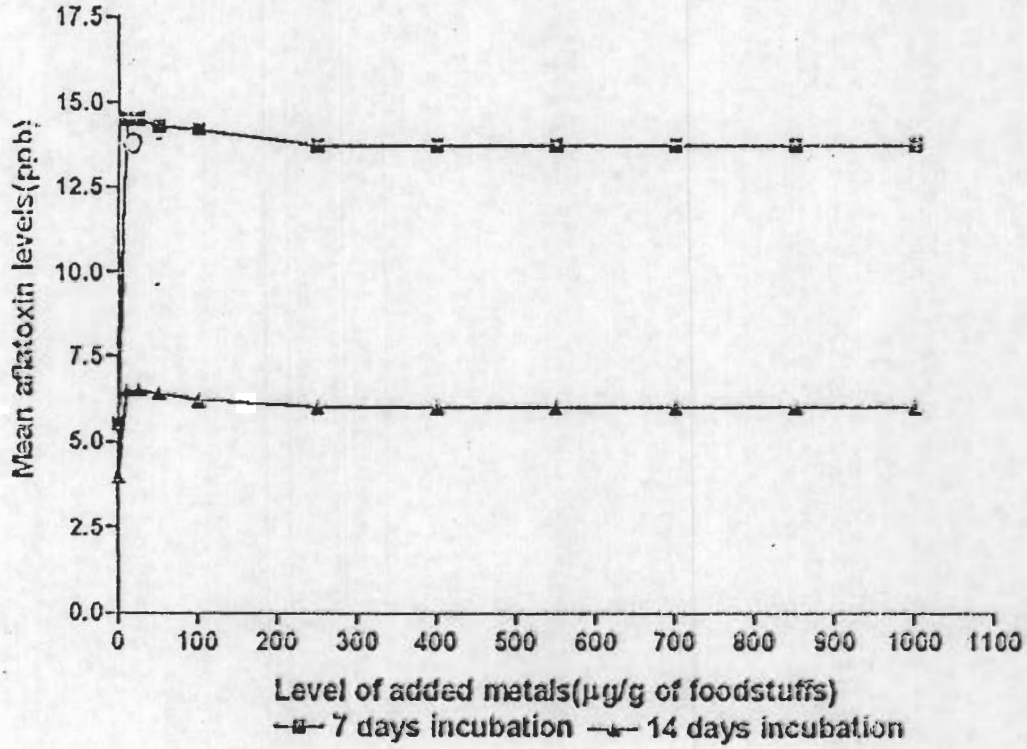


Fig. 100: Effects of graded levels of bivalent metals(Manganese Mn^{2+}) on aflatoxin production in Yam tuber

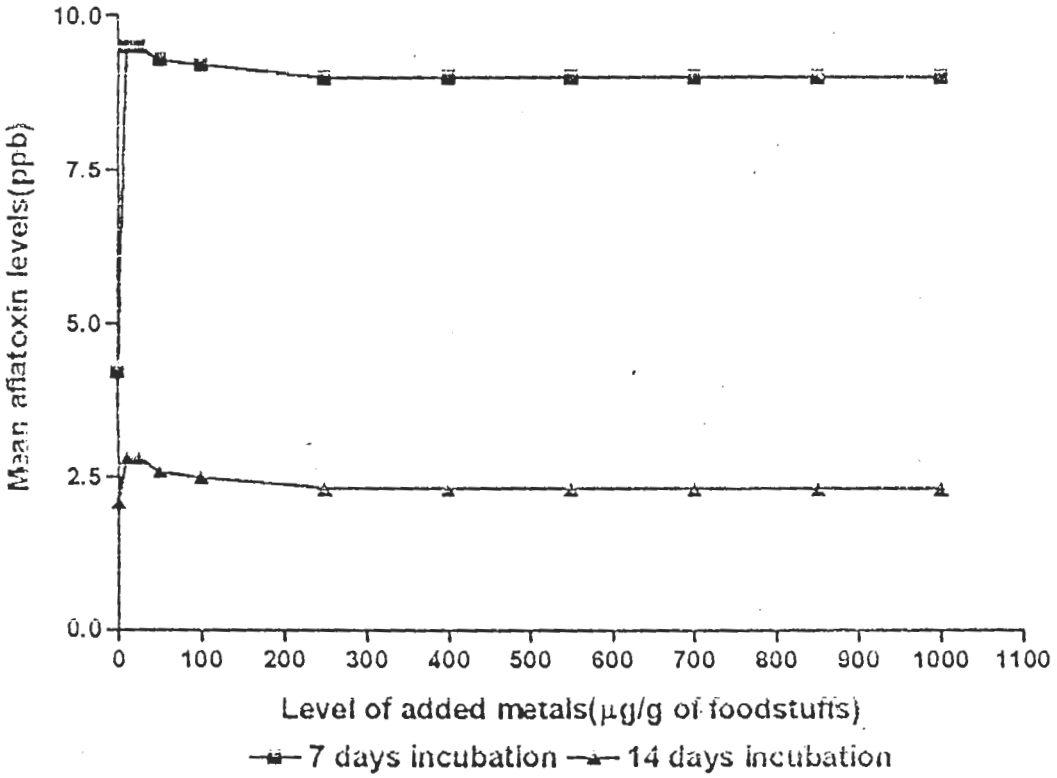


Fig.10: Effects of graded levels of bivalent metals(Manganese Mn^{2+}) on aflatoxin production in Cassava tuber

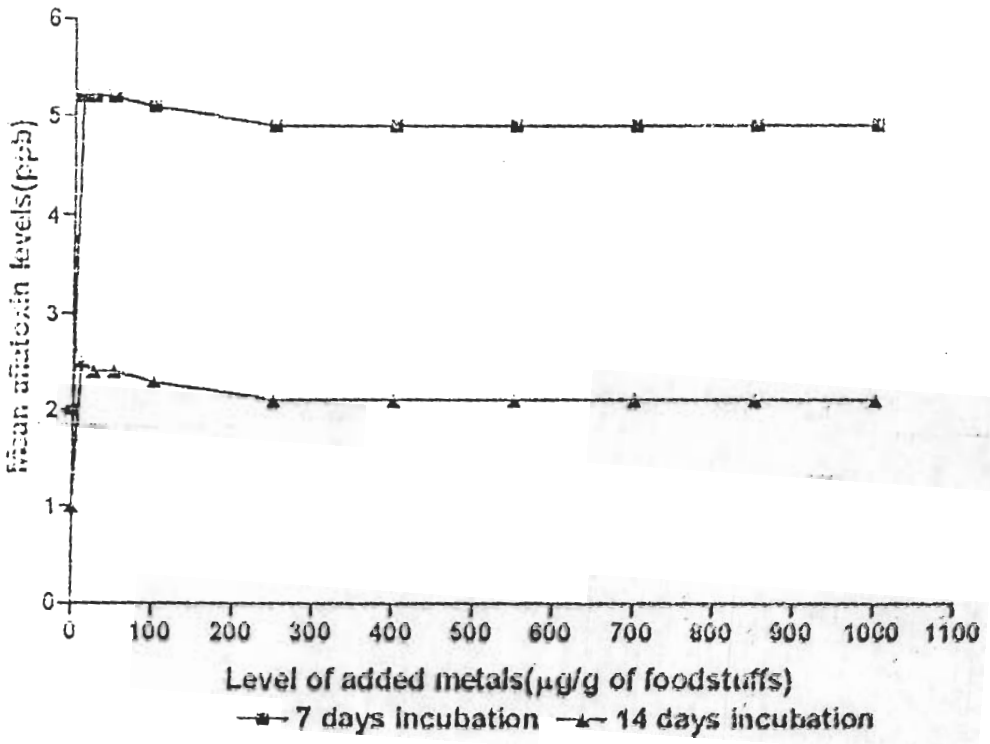


Fig. 102: Effects of graded levels of bivalent metals(Manganese Mn^{2+}) on aflatoxin production in Plantain (ripe)

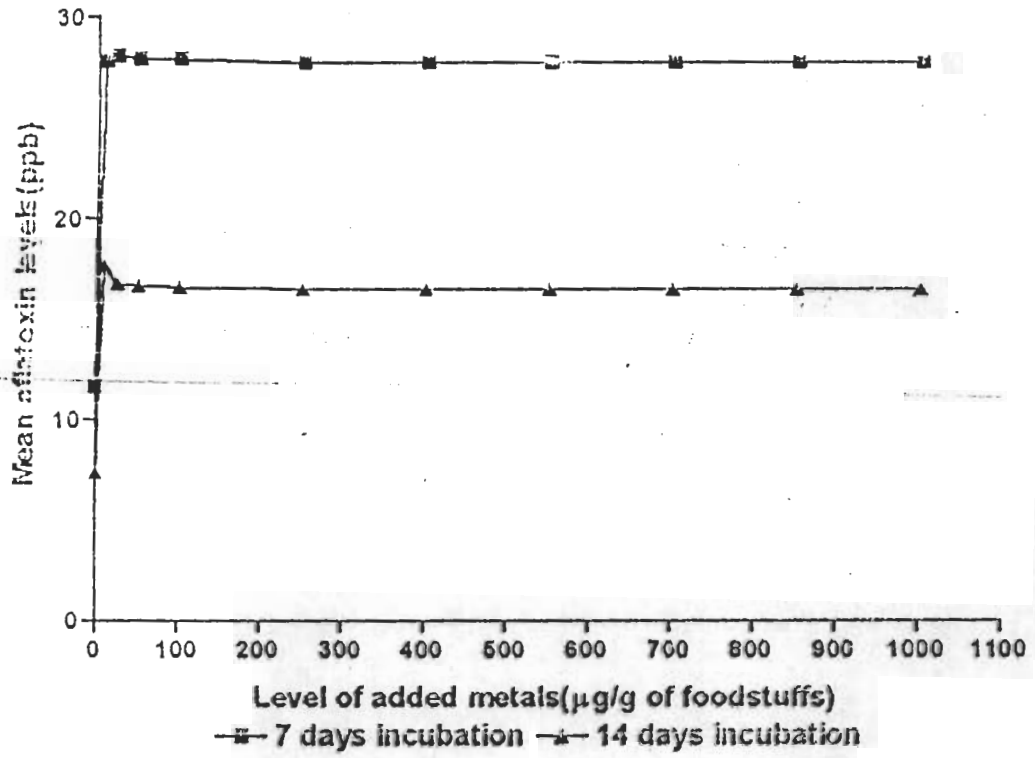
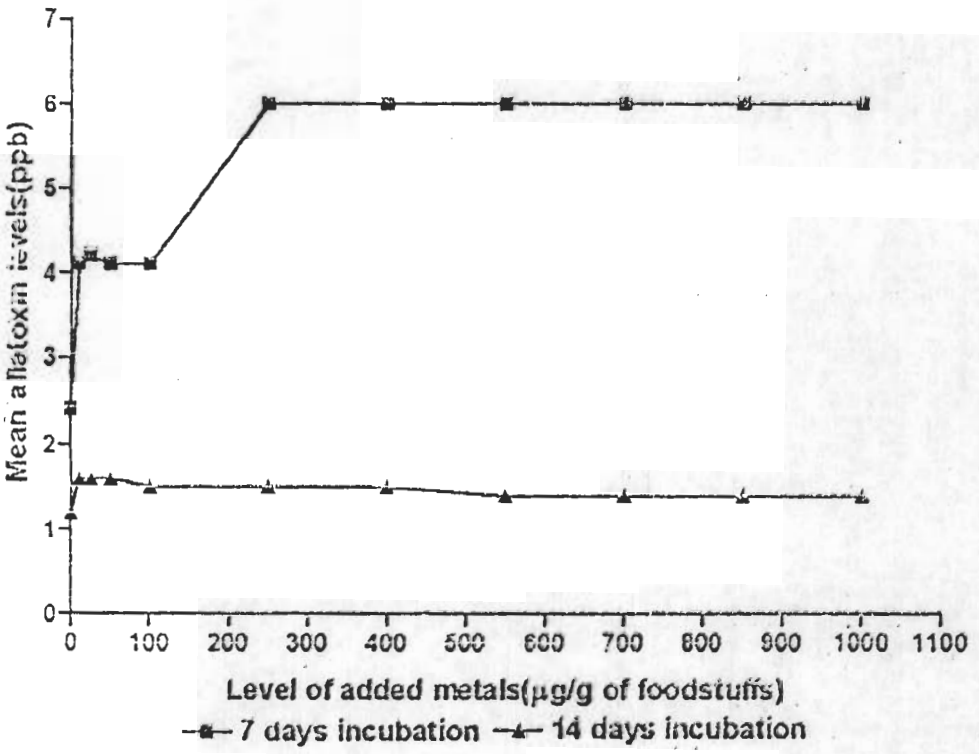


Fig. 103: Effects of graded levels of bivalent metals(Manganese Mn^{2+}) on aflatoxin production in Plantain(unripe)



(iii) Iron:

A constant level of aflatoxins was sustained at all iron concentrations for all the cereals and carbohydrate foods. In the protein foods, a slight increase in aflatoxin levels occurred up to 25µg of iron per gram of food, and then, a drop at 50µg metal which continued till the highest metal concentration per gram of food substrate, even being completely inhibited in some foods at higher metal levels. In some like pigeon peas, brown cowpeas and bambara peanuts, however, the drop occurred earlier at 25µg of iron per gram of food substrate.

For the oil seeds, a significant increase in Af level was observed at 10µg of iron per gram of food substrate ($P<0.05$), and then started declining gradually with a significant drop at 250-440µg iron per gram of food ($P<0.05$), and then thereafter started rising gradually till the highest metal concentration. There was also no difference in the level of Af at day seven and day fourteen of incubation for the cereals, protein foods and oil seeds while this difference was statistically significant for the cereal (yellow sorghum) and carbohydrates ($P<0.05$) except cocoyam.

(iv) Copper:

Added copper increased Af levels from 10µg metal per gram of food substrate of cereals until 250µg metal concentration after which a progressive decrease was recorded till 1000µg/ per gram of food substrate. A similar pattern was obtained for the carbohydrate foods too. In the protein foods, the increase at 10µg metal per gram of food was sustained till between 25 and 50µg metal concentration and then gradually decreased with increasing metal concentration.

In the oil seeds, a significant increase in AF level occurred at 10µg metal per gram of food substrate ($P<0.05$), and dropped again by 25-100µg metal per gram of food depending on the oil seed concerned. It rose sharply again after a drop at 550µg copper for coconut seeds, but for the other oil seeds, it continued to decrease with increasing metal concentration as in the peanuts; or leveled up at a constant lower value as in melon seeds.

In all the oil seeds, protein foods and cereals,there were no differences in AF levels in samples analysed at day seven and day fourteen of incubation.

This differences were however statistically significant for the carbohydrate foods ($P<0.05$) with the exception of cocoyam. AF levels were also generally higher at seven days incubation than at fourteen days incubation in all food items.

Statistical analysis:

Statistical analysis summaries show that the means of aflatoxins in the eight types of cereals studied for effects of iron were significantly different using the one way analysis of variance (ANOVA TEST) ($P<0.0001$) though the variances did not differ significantly with metal concentrations using the Bartlett's test. However, using the Tukey's multiple comparison tests, there were statistically significant differences when the mean AF values per food item was compared with each other in all the cereals ($P<0.001$). In the same iron studies with protein foods, the AF mean were also significantly different with the ANOVA test, ($P<0.05$) and the variances also differed significantly ($P<0.0001$) with metal concentrations.

The Tukey's multiple comparison tests however showed that there were no differences when the means AF values per food item was compared with each other except soybean and breadfruits that were statistically significant ($P<0.05$).

In iron studies with oil seeds, the one way analysis of variance showed that the AF means were not different, though the variances differed significantly ($P<0.05$). There was also no actual mean differences when each food is compared with the other in the multiple comparison test.

In the copper_ studies with cereals, the AF means were not different and the variances also did not differ significantly ($P>0.05$) while the Tukey's test also showed no significant mean differences ($P>0.05$). For the Protein foods, the AF means were significantly different ($P<0.05$), variances also differ significantly using the Bartlett's tests while the Tukey's multiple comparison test showed no significant mean differences among food items except between soybean and breadfruits that differed significantly ($P<0.01$). With oil seeds, again the AF means differed significantly ($P<0.05$), the variances also differed significantly ($P<0.05$) while with the Tukey's comparison test, mean differences were not significant when white and red testa peanuts; white peanuts and melon seeds; red peanuts and melon seeds; melon seeds and coconut seeds were compared respectively, but significant when white peanuts and coconut seeds ($P<0.01$); Red peanuts and coconut seeds were compared respectively ($P<0.0001$).

With carbohydrate foods, AF means were significantly different ($P<0.0001$) while the multiple comparison tests showed significant mean differences in yam and ripe plantain; cassava and ripe plantain; ripe plantain and unripe plantain; ripe plantain and cocoyam pairings respectively ($P>0.05$).

In the manganese studies with cereals, the ANOVA tests reveal that the AF means were not significantly different ($P>0.05$), however, using the Bartlett's tests, the variances differed significantly ($P<0.0001$), while the Tukey's multiple comparison test also showed

no significant mean differences (P>0.05) in all the food samples being compared with each other. The one way analyses of variance for the protein rich foods showed that the Af mean levels were significantly different (P<0.0001) and also the variances differed significantly too (P<0.0001), while the multiple comparison tests showed that when white cowpea was compared with brown cowpeas; white cowpeas and white pigeon peas; with brown pigeon peas, with bambara peanuts, respectively; also when brown cowpeas were compared with each of white pigeon peas, brown pigeon peas, bambara peanuts, soyabeans; when white pigeon pea was compared with each of brown pigeon peas, bambara peanuts, and when brown pigeon peas was compared with bambara peanuts, and when bambara peanut was compared with soyabeans, there were no in Af levels. However, they differed significantly with the following pairs: white cowpeas and soyabeans; white cowpeas and breadfriuts, brown cowpeas and breadfruits; white pigeon pea and soyabeans and breadfruits respectively; brown pigeon pea and soyabeans, and breadfruits respectively, Bambara peanut and breadfruits; soyabean and breadfruits (P <0.001).

In the zinc experiments with cereals, the ANOVA tests show that the AF means were significantly different (P <0.0001) and the variances also differed significantly (P <0.05), while the multiple comparison test showed that Af mean differences were significant in the following pairings (P <0.05): white maize and millet; yellow maize and acha grains; Acha grains and Abakaliki rice, acha and yellow sorghum, white sorghum, and millet respectively; Abakaliki rice and Ugbawka rice, and also ugbawka rice and yellow sorghum, white sorghum and millet respectively.

However the mean differences were not significant (P >0.05) in the following pairings when compared with each other: white maize and each of the followings; yellow

maize, acha grains, Abakaliki rice, Ugbawka rice, yellow sorghum and white sorghum; yellow maize and each of the following: Abakaliki rice, Ugbawka rice, yellow and white sorghum millet; acha grains and Ugbawka rice, Abakaliki rice and each of the following: yellow sorghum, white sorghum, millet; yellow sorghum and white sorghum and millet; then white sorghum and millet.

With Protein foods, the AF means differed significantly ($P < 0.0001$) using the ANOVA tests and the variances also differed significantly ($P < 0.0001$), while the multiple comparison tests among the different foods revealed that mean differences were not significant in the following pairings: white cowpeas versus each of the following - brown cowpeas, white pigeon peas, brown pigeon peas, soyabeans, Brown cowpeas with white and brown pigeon peas and soyabeans, Brown pigeon peas and soyabeans, while pigeon peas and soyabeans, Brown pigeon peas and soyabeans, while the mean differences were significant in the following pairings: white cowpeas and bambara peanuts; white pigeon pea and bambara peanuts, brown pigeon pea and bambara peanuts; bambara peanuts asnd soyabeans.

In the oil seeds. The AF means and variances did not differ significantly ($P > 0.05$). The multiple comparison tests showed that the mean differences between the various foodstuffs were not significant ($P > 0.05$).

In the carbohydrate foods, the AF means were significantly different ($P < 0.0001$). The variances also differed significantly ($P < 0.05$). The multiple comparison tests showed that mean difference were not significant in the following pairings when compared with each other: Yam and ripe plantain, Yam and cocoyam; cassava and unripe plantain; while in the following pairings, the mean differences were significant: Yam and cassava; yam

and unripe plantain, cassava and ripe plantain, cassava and cocoyam; ripe plantain and unripe plantain, ripe plantain and cocoyam, unripe plantain and cocoyam. (Appendix).

Fig. 104: Effects of graded levels of bivalent metals(Iron) on aflatoxin production in maize(white)

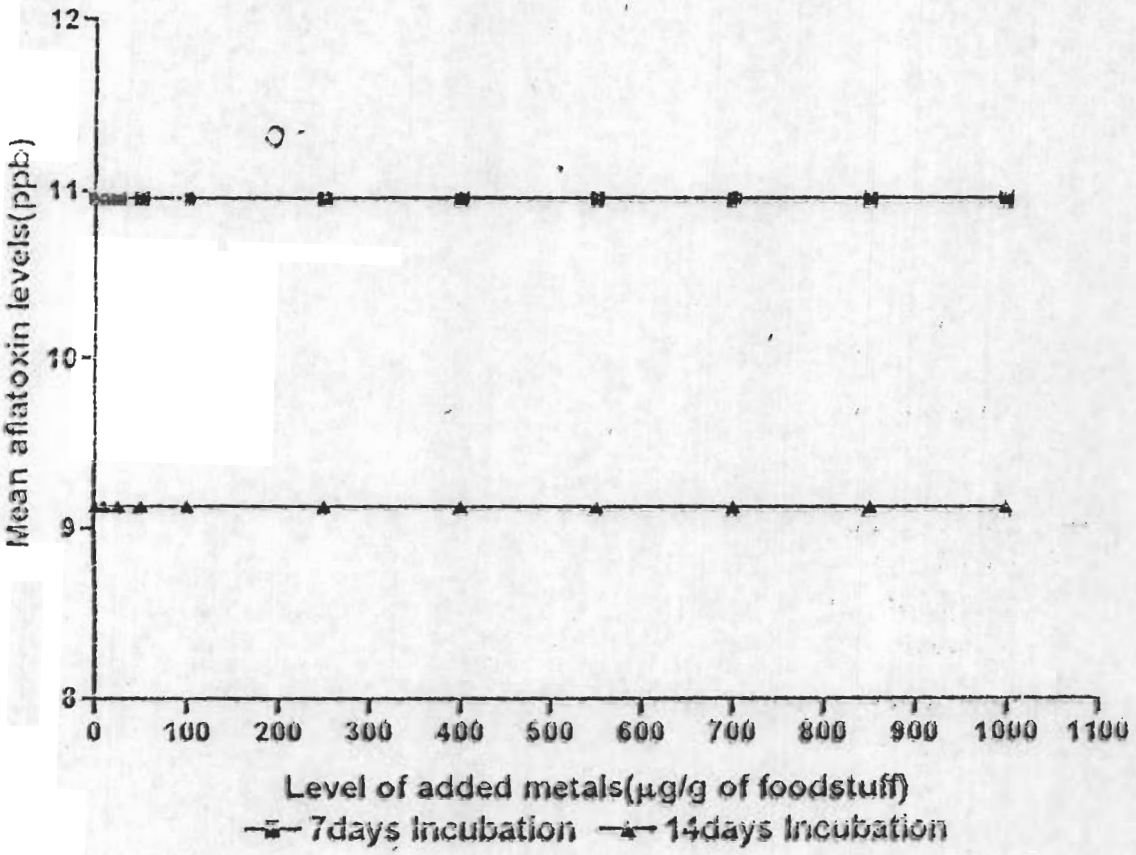


Fig. 105: Effects of graded levels of bivalent metals(iron) on aflatoxin production in maize(yellow)

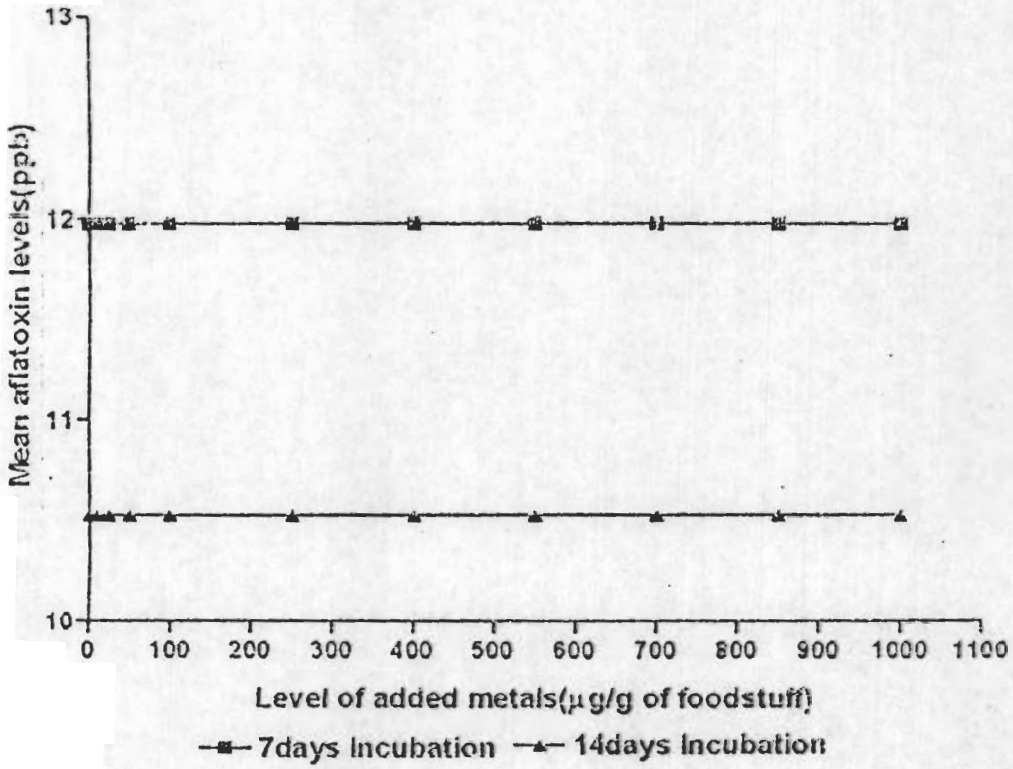


Fig.106:Effects of graded levels of bivalent metals(Iron) on aflatoxin production in Acha

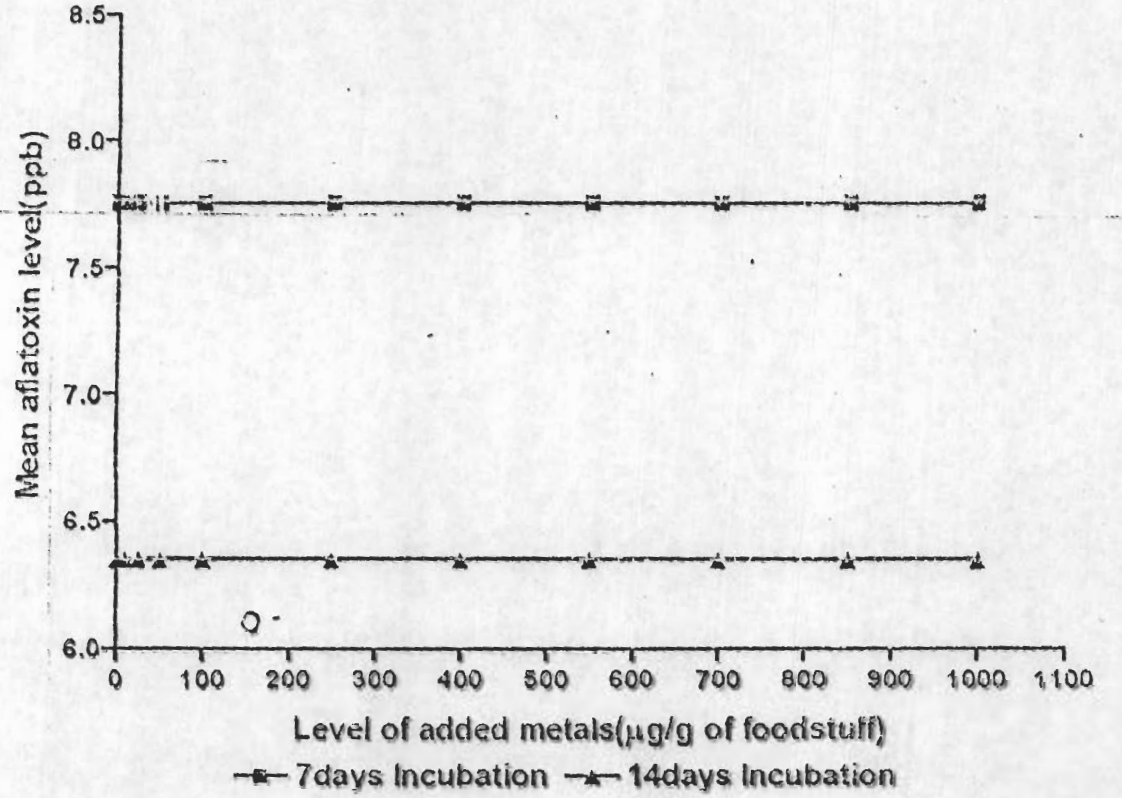


Fig. 10: Effects of graded levels of bivalent metals(Iron) on aflatoxin production in millet

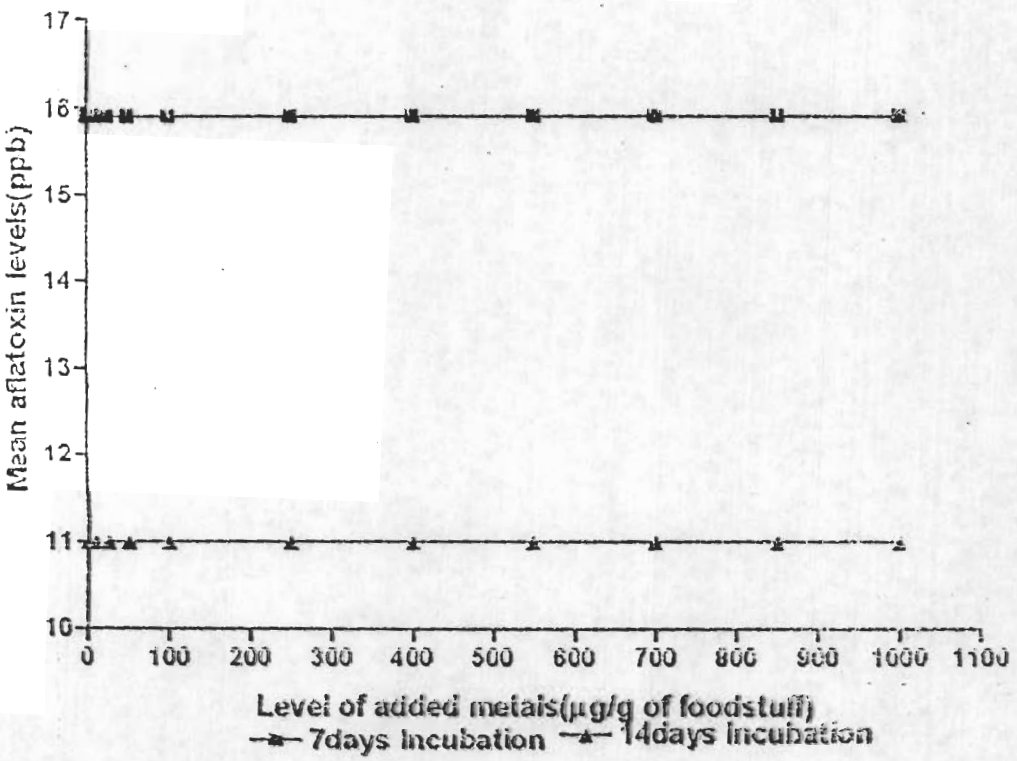


Fig. 108: Effects of graded levels of bivalent metals(Iron) on aflatoxin production in Abakaliki Rice

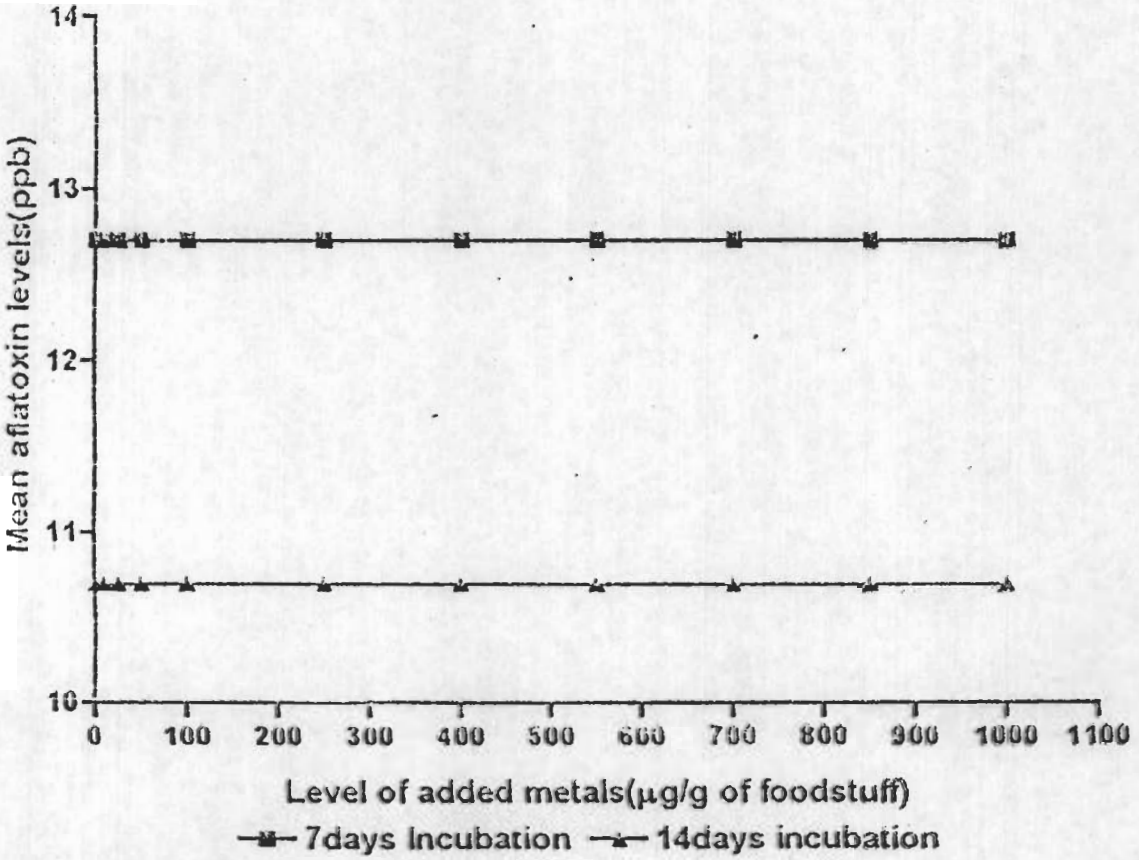


Fig. 10: Effects of graded levels of bivalent metals(Iron) on aflatoxin production in Ugbawka rice

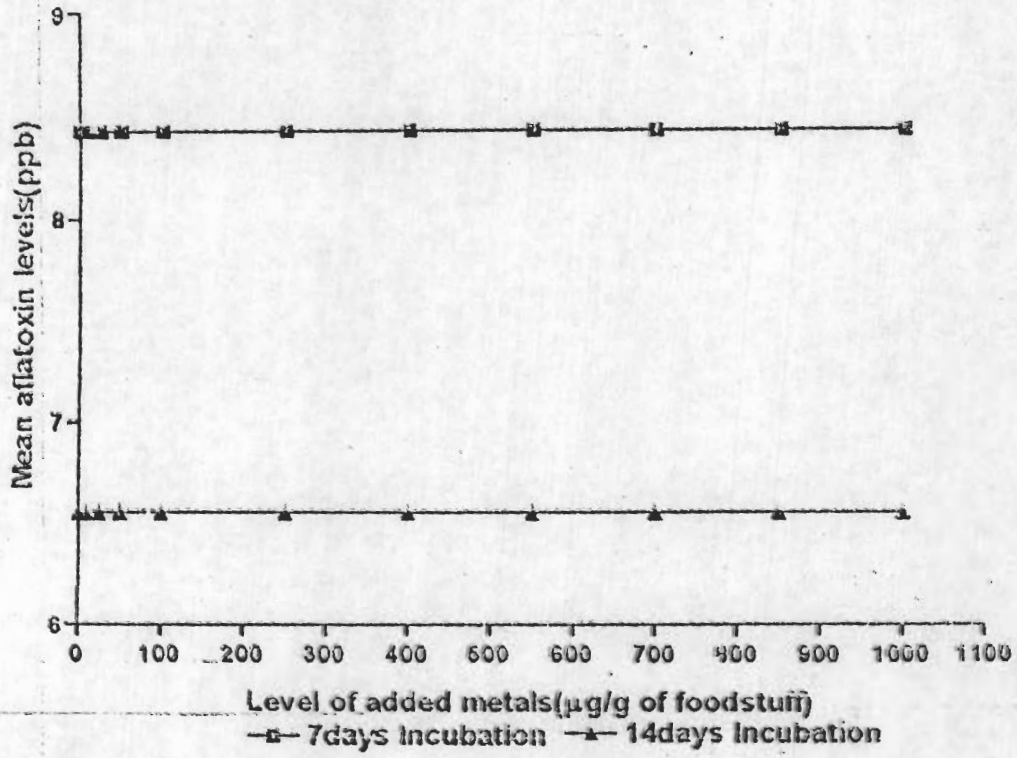


Fig. 110: Effects of graded levels of bivalent metals(Iron) on aflatoxin production in sorghum(yellow)

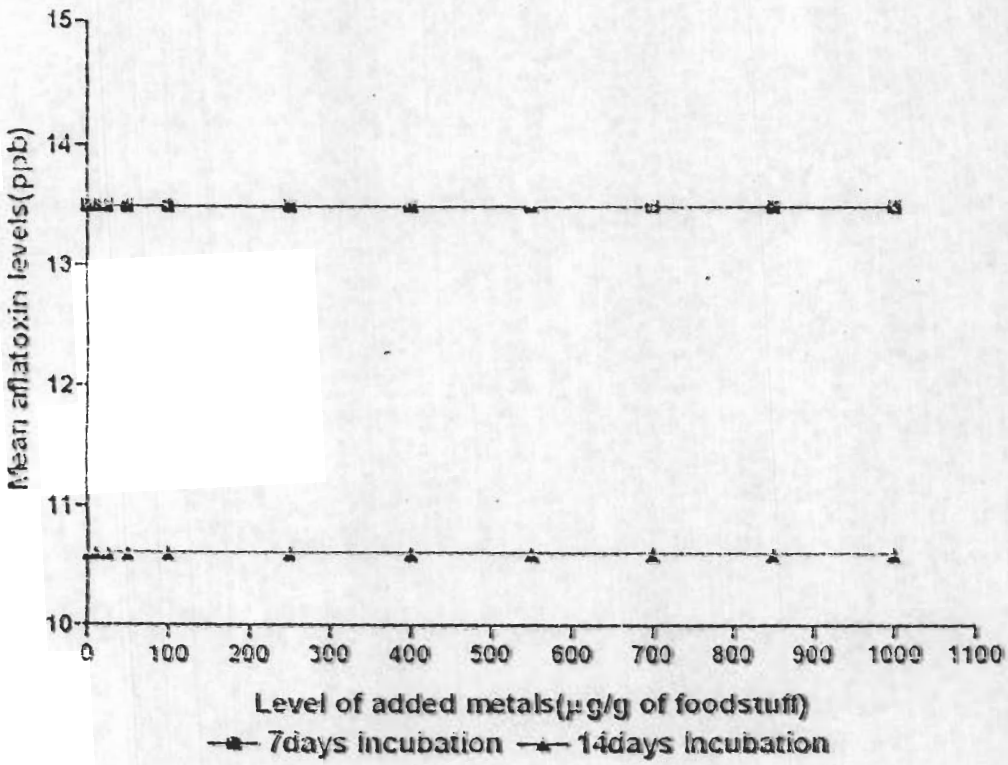


Fig. III: Effects of graded levels of bivalent metals(Iron) on aflatoxin production in sorghum(white)

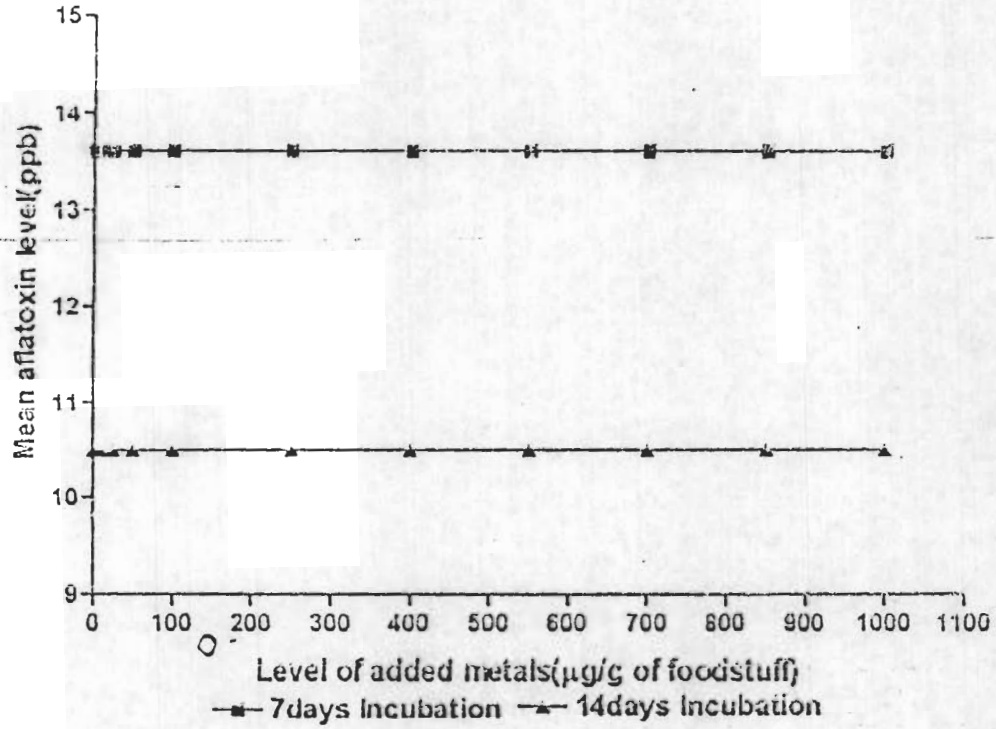


Fig. 112:Effects of graded levels of bivalent metals(iron) on aflatoxin production in cowpea(white)

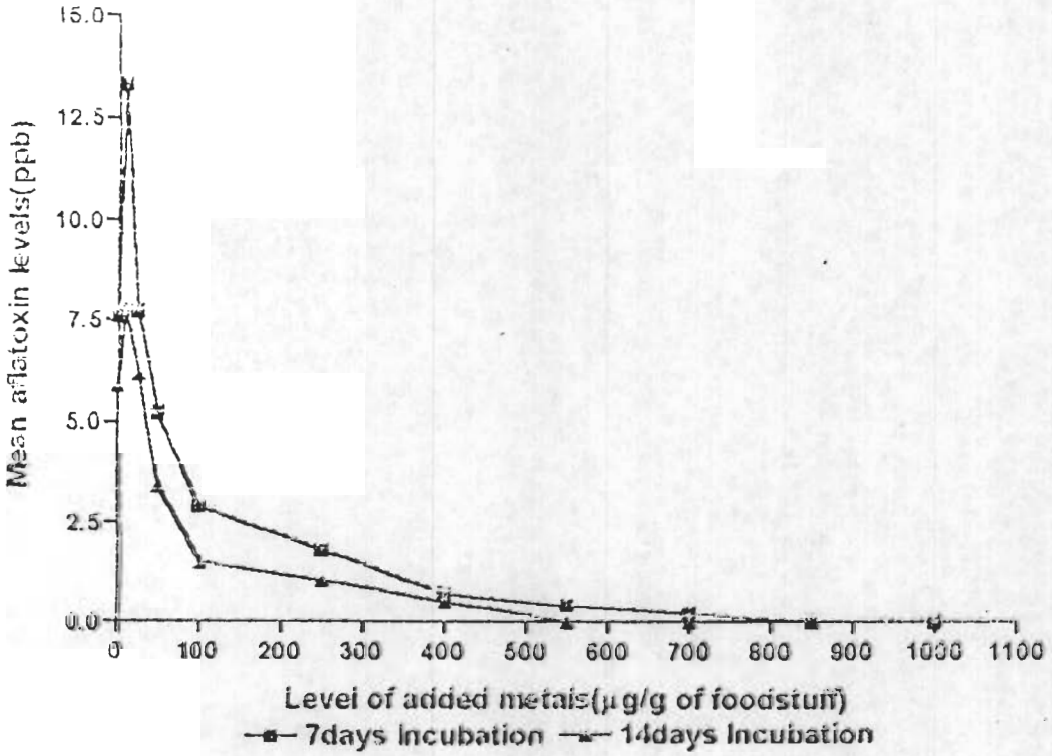


Fig. 113: Effects of graded levels of bivalent metals(iron) on aflatoxin production in cowpea(brown).

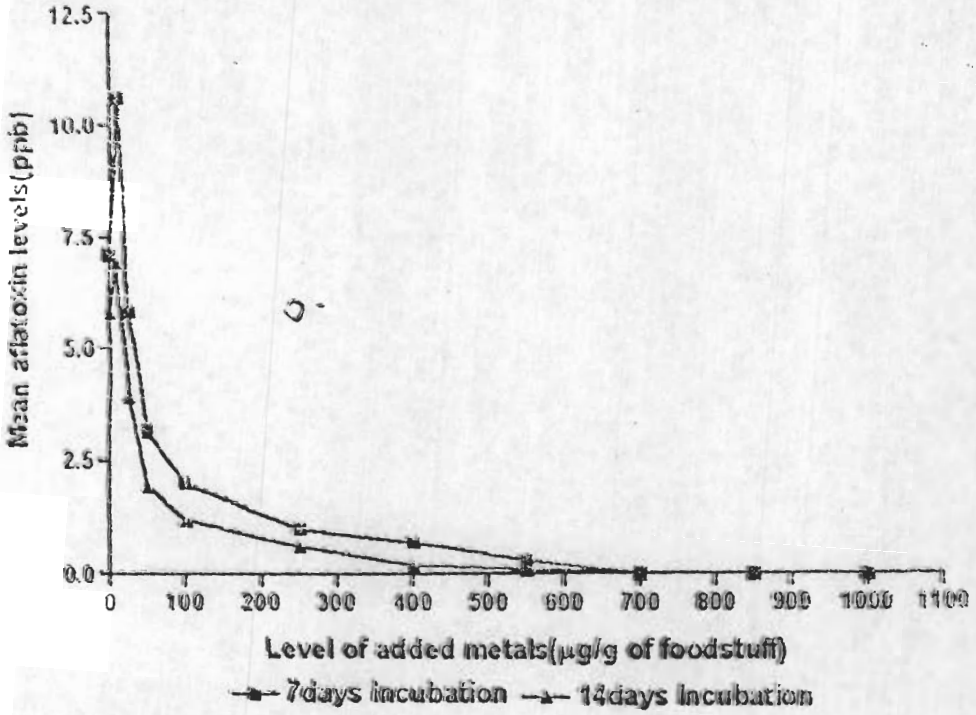


Fig. 114: Effects of graded levels of bivalent metals(Iron) on aflatoxin production in pigeon pea(White)

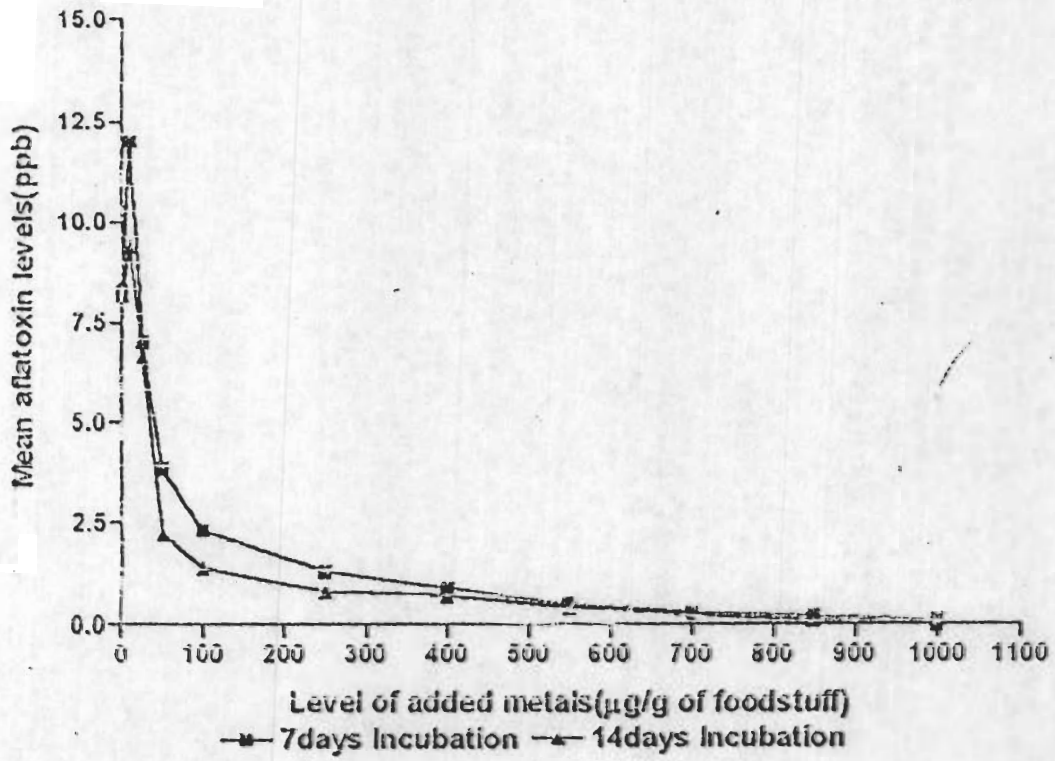


Fig. 115 Effects of graded levels of bivalent metals(Iron) on aflatoxin production in pigeon pea(brown)

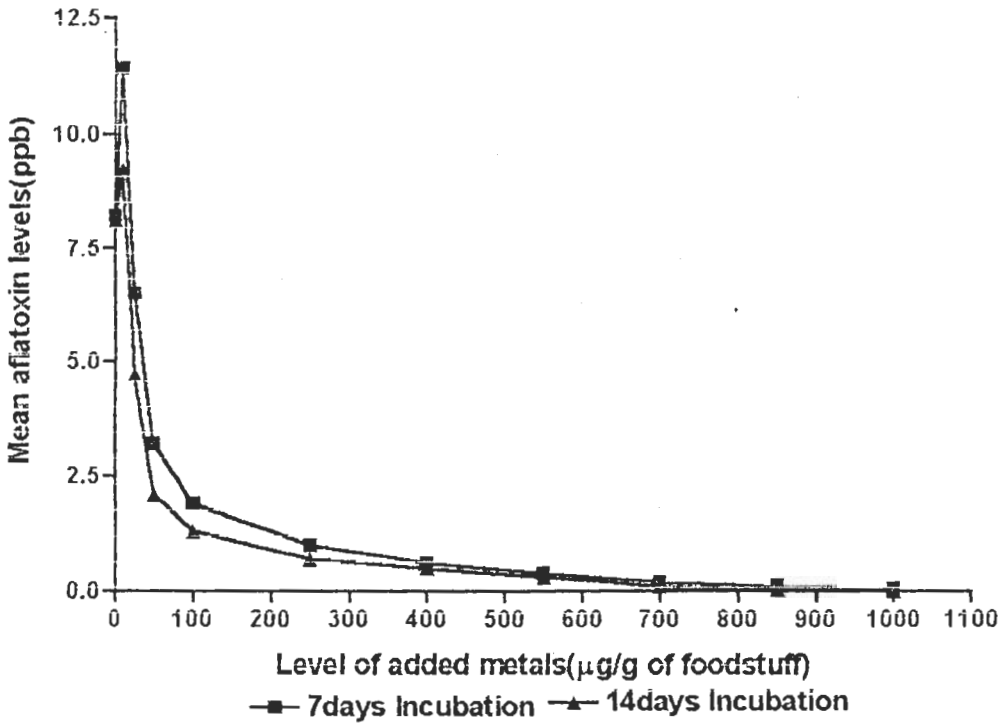


Fig.116:Effects of graded levels of bivalent metals(Iron) on aflatoxin production in bambara peanut seed

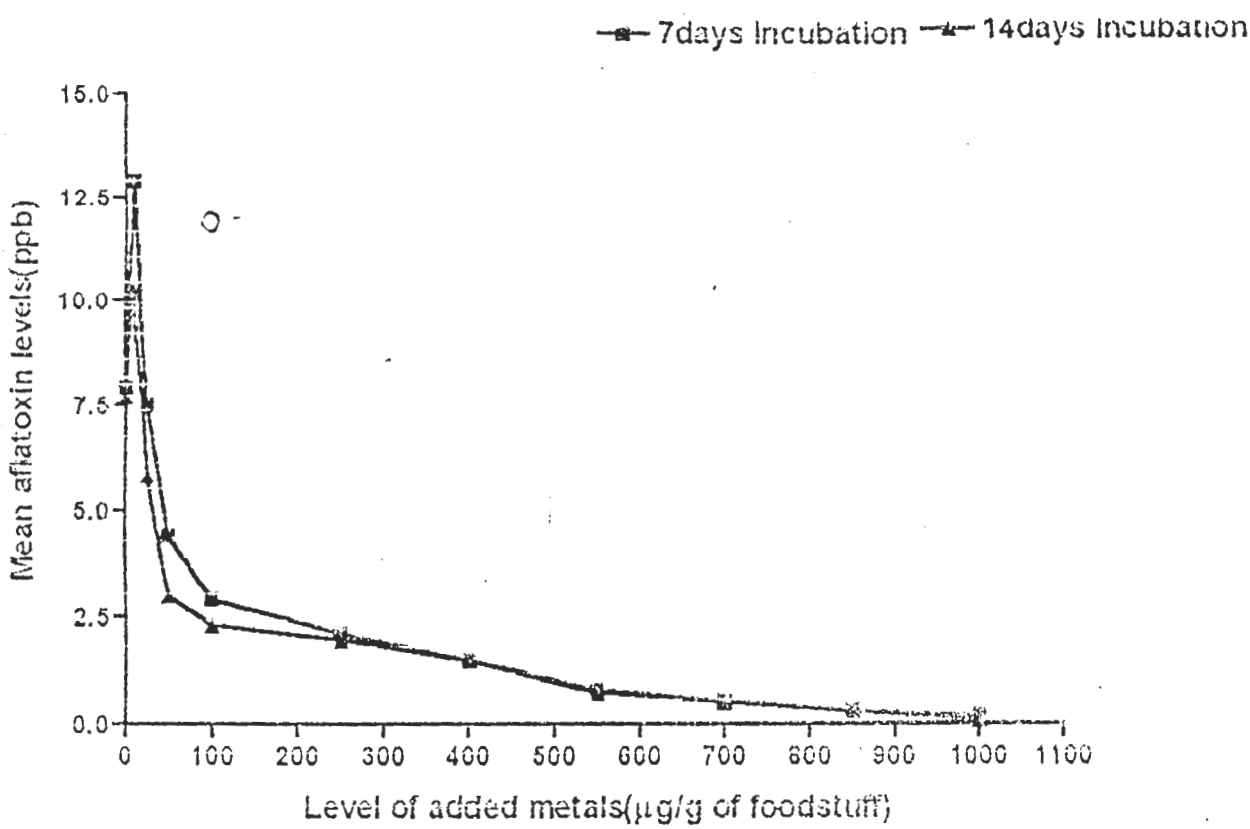


Fig. 17: Effects of graded levels of bivalent metals(Iron) on aflatoxin production in soya beans

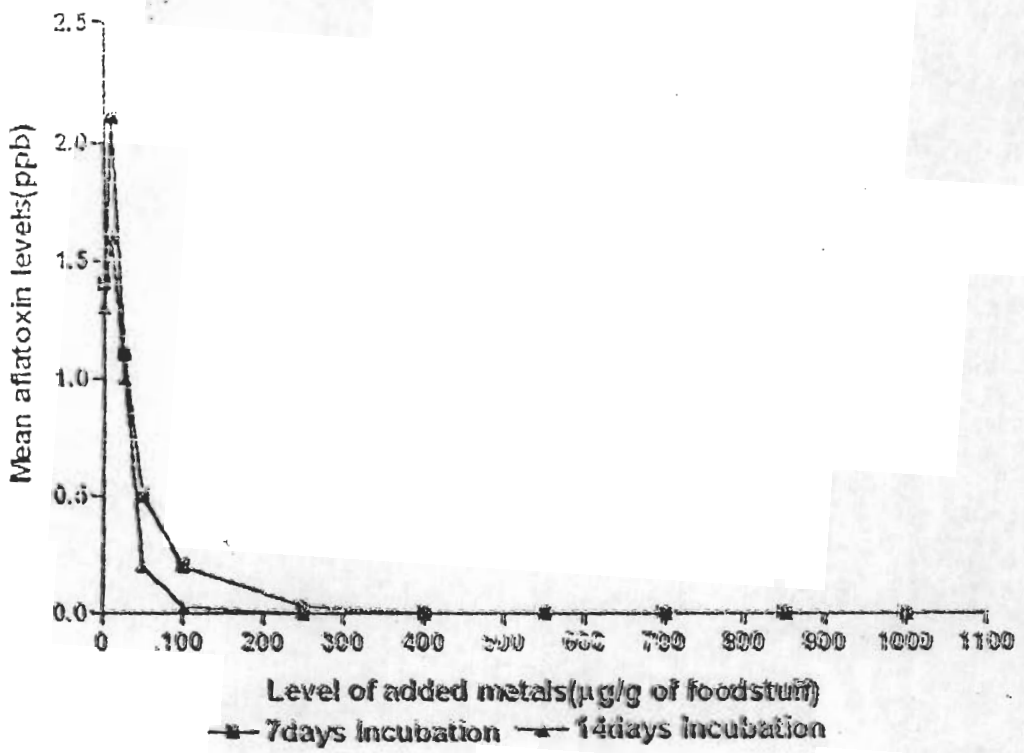


Fig. 118:Effects of graded levels of bivalent metals(Iron) on aflatoxin production in bread fruits

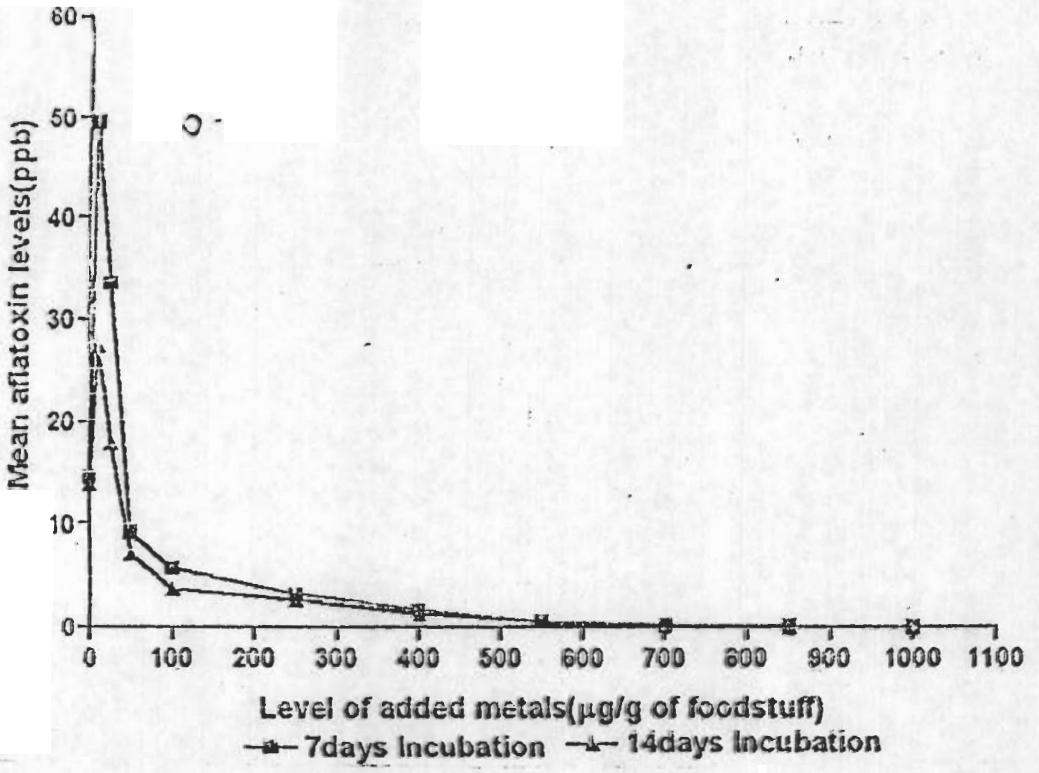


Fig. 19: Effects of graded levels of bivalent metals(Iron) on aflatoxin production in melon seeds

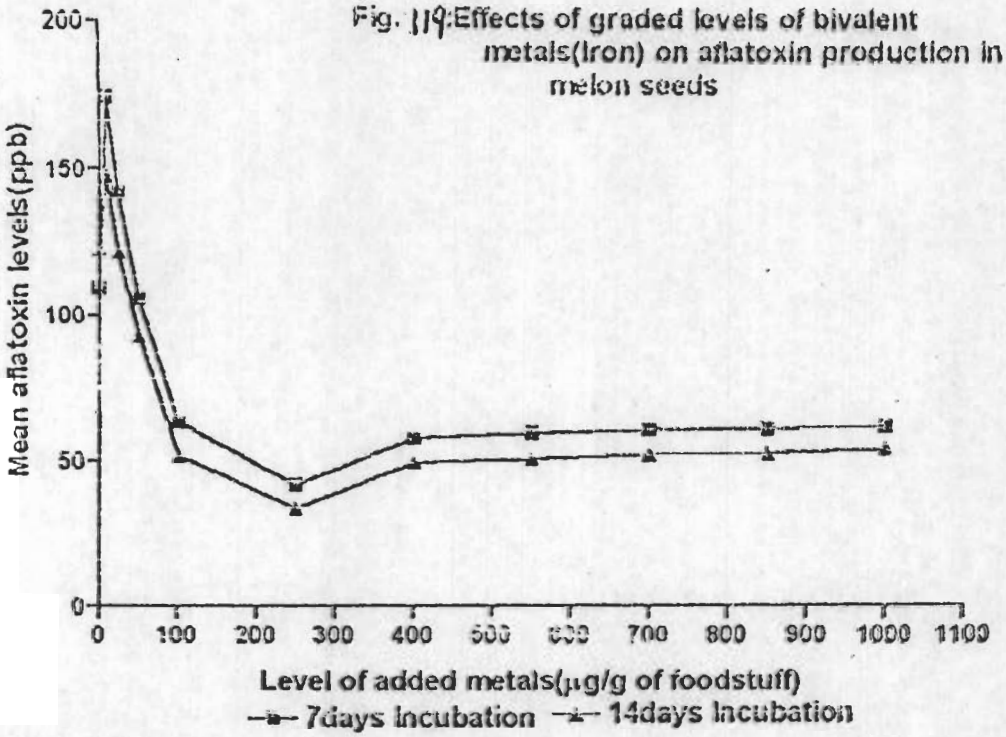
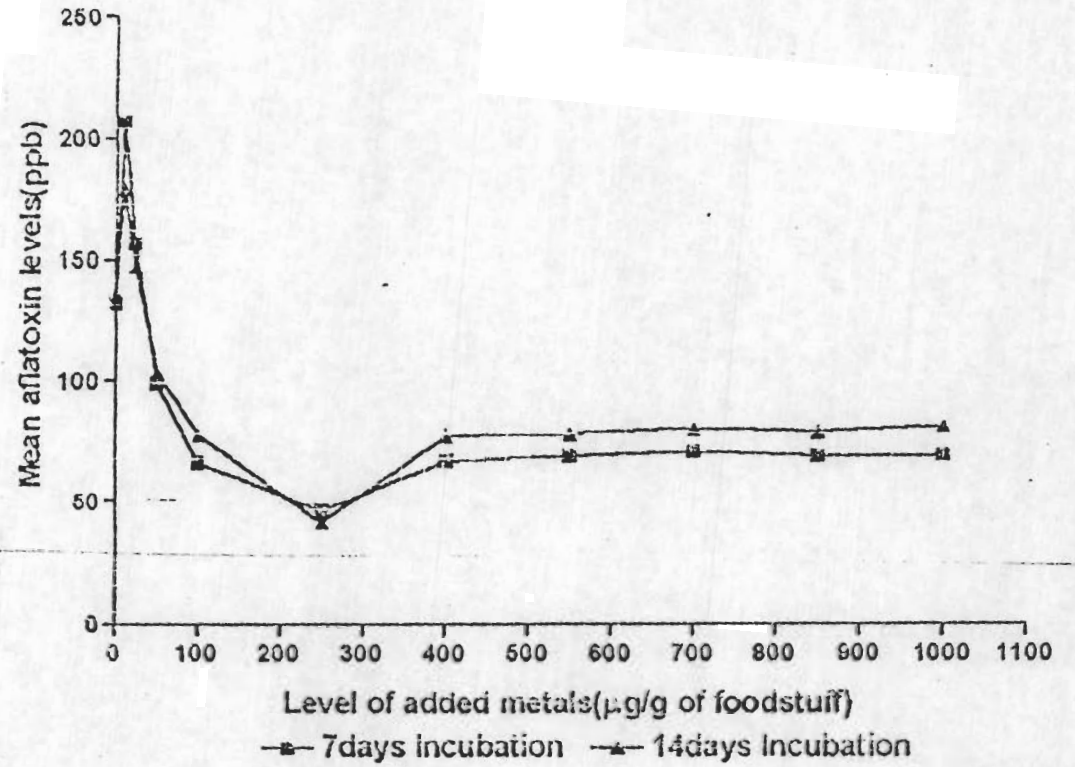


Fig.120: Effects of graded levels of bivalent metals(Iron) on aflatoxin production in peanuts Waito



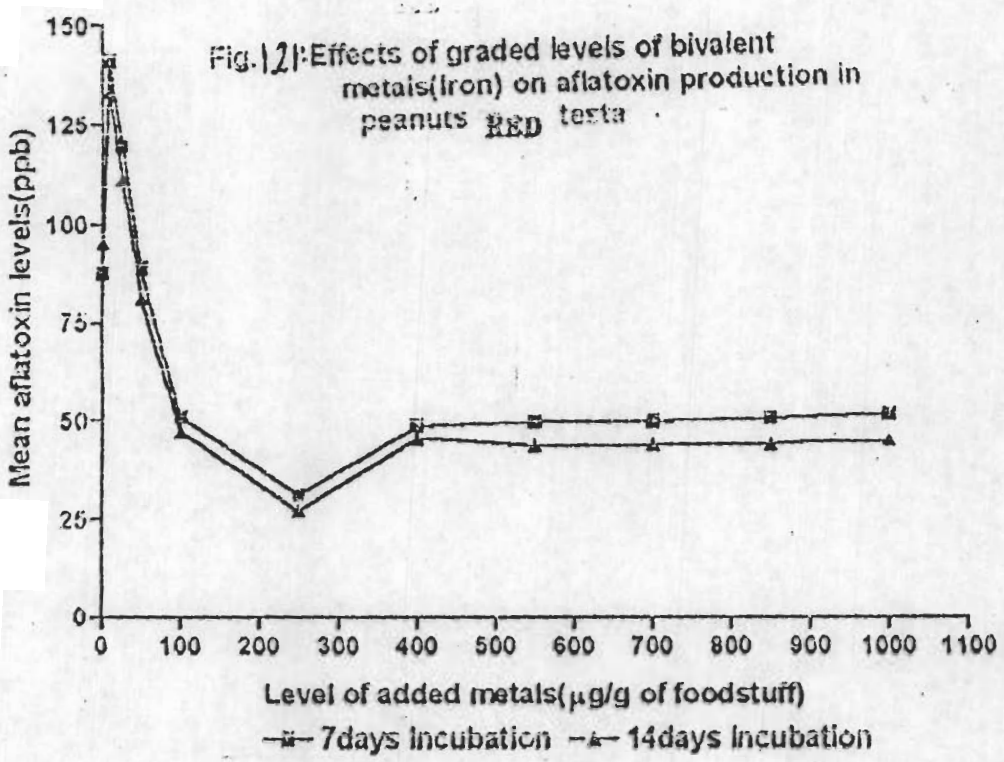


Fig.12: Effects of graded levels of bivalent metals(iron) on aflatoxin production in peanuts RED testa

Fig. 122: Effects of graded levels of bivalent metals(Iron) on aflatoxin production in coconut seeds

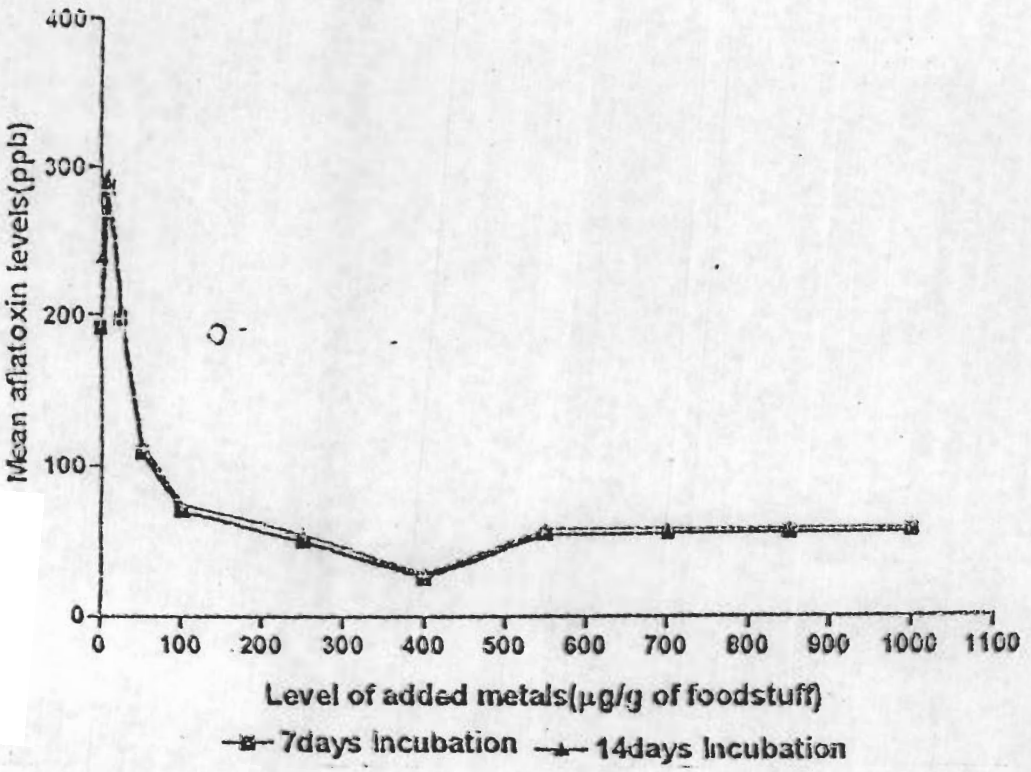


Fig. 123 Effects of graded levels of bivalent metals(Iron) on aflatoxin production in cocoyam

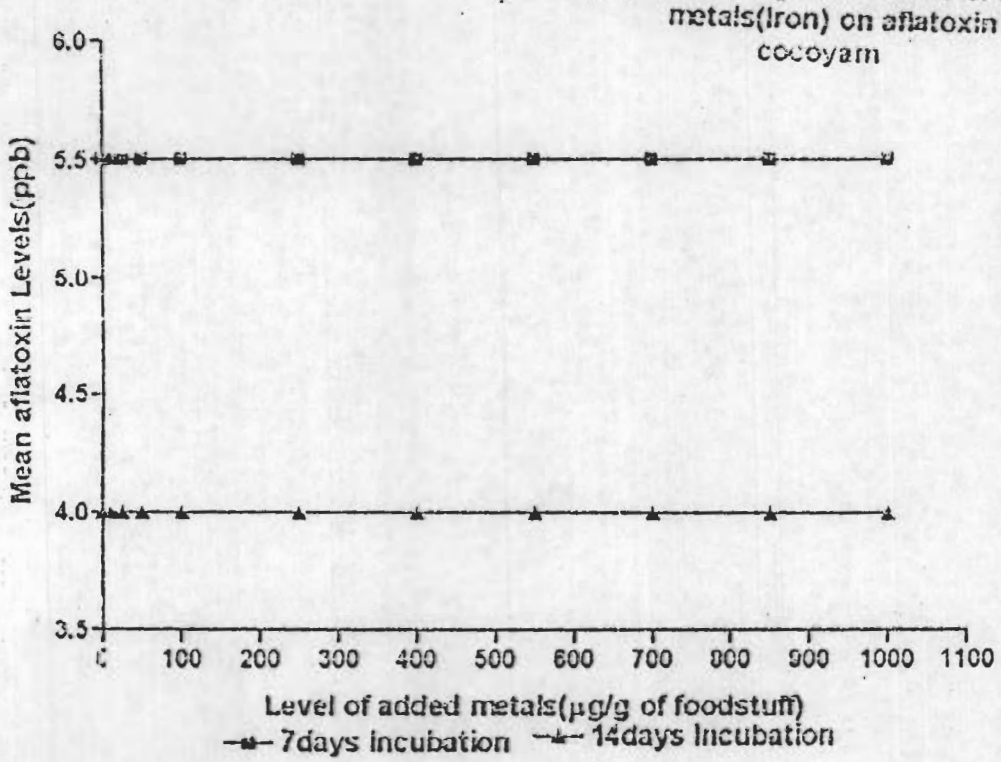


Fig. 124: Effects of graded levels of bivalent metals(Iron) on aflatoxin production in yam tuber

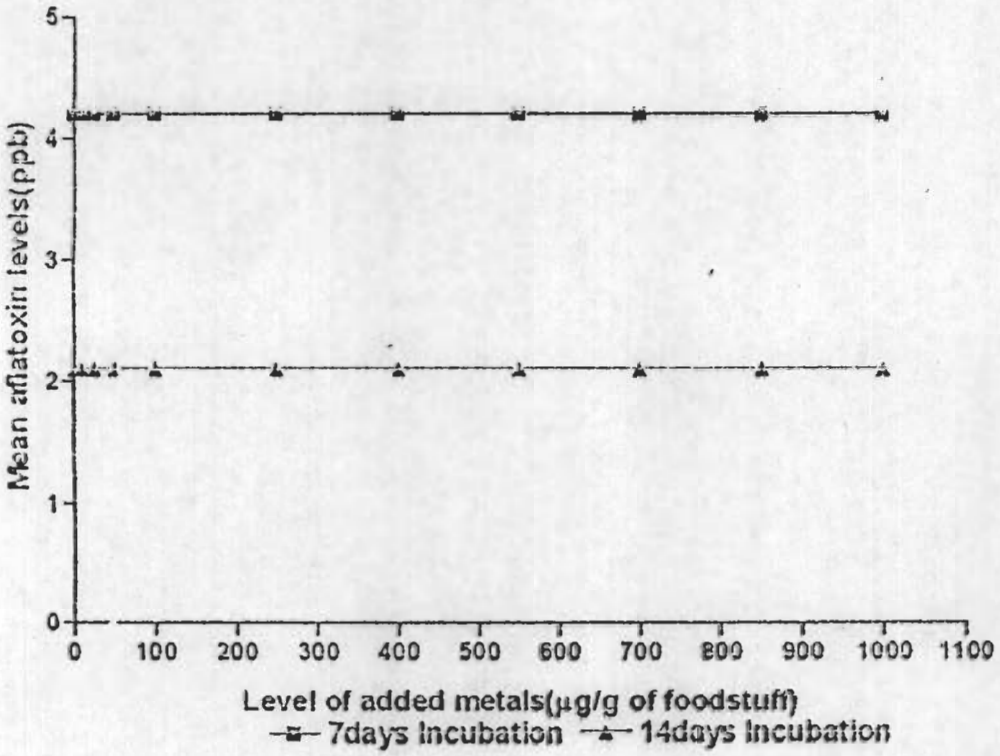
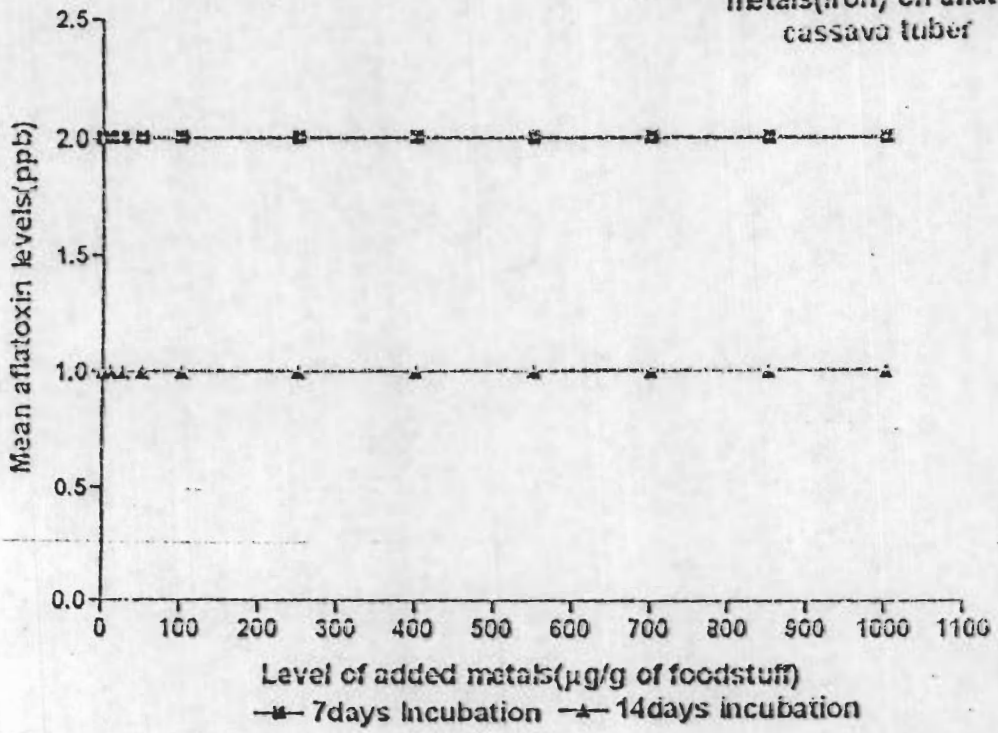


Fig.125 Effects of graded levels of bivalent metals(Iron) on aflatoxin production in cassava tuber



1.126: Effects of graded levels of bivalent metals(Iron) on aflatoxin production in plantain(ripe)

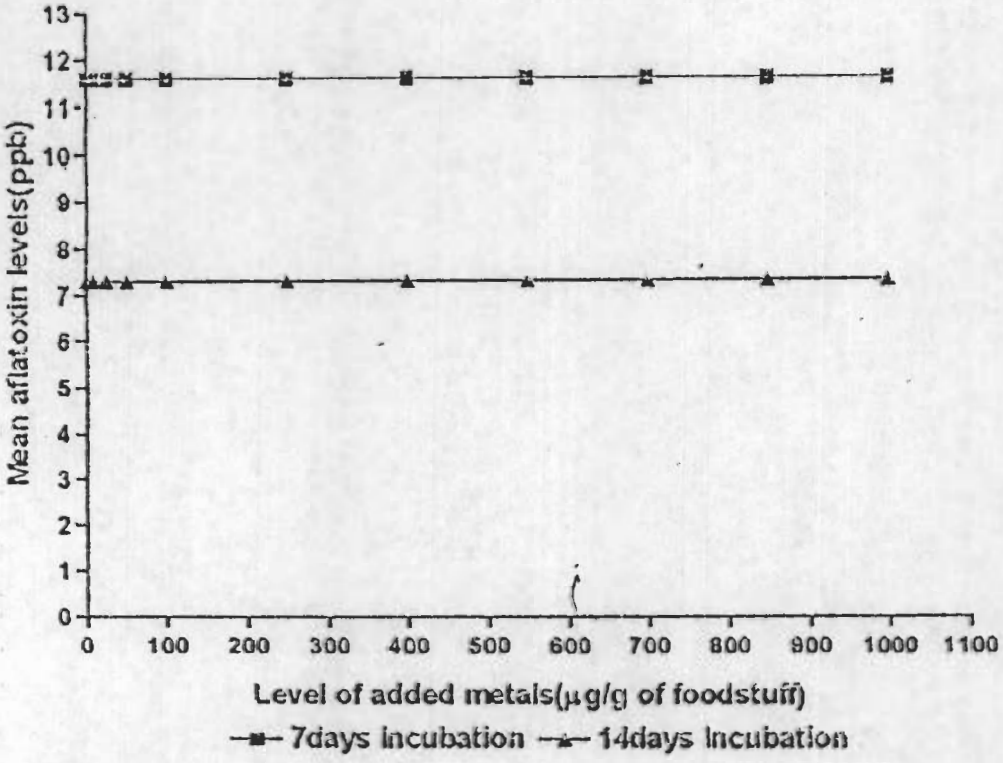


Fig. 127 Effects of graded levels of bivalent metals(Iron) on aflatoxin production in plantain(unripe)

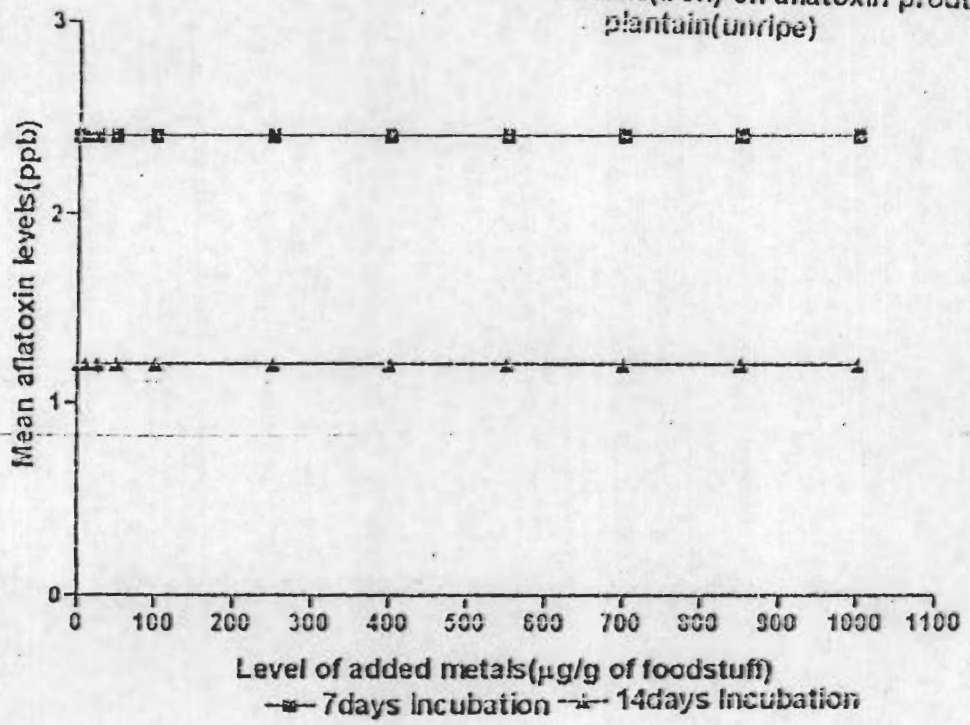


Fig. 128: Effects of graded levels of bivalent metals(Copper Cu^{2+}) on aflatoxin production in maize(white)

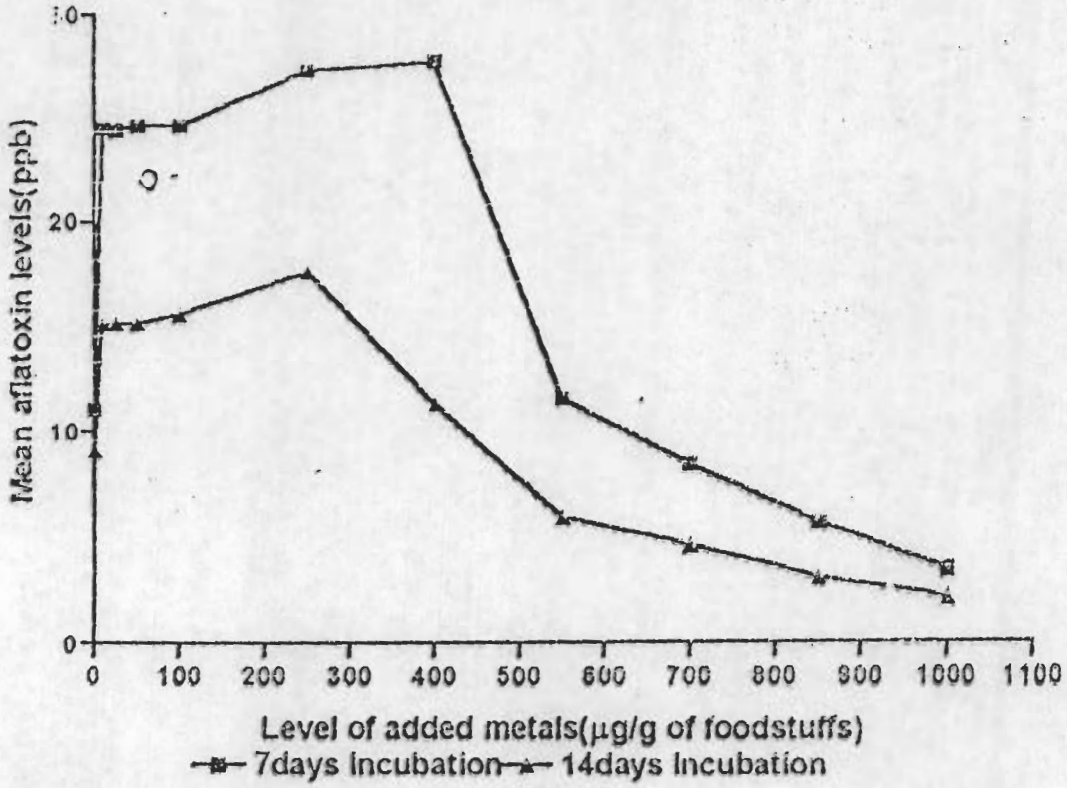


Fig.12: Effects of graded levels of bivalent metals(Copper Cu^{2+}) on aflatoxin production in maize(yellow)

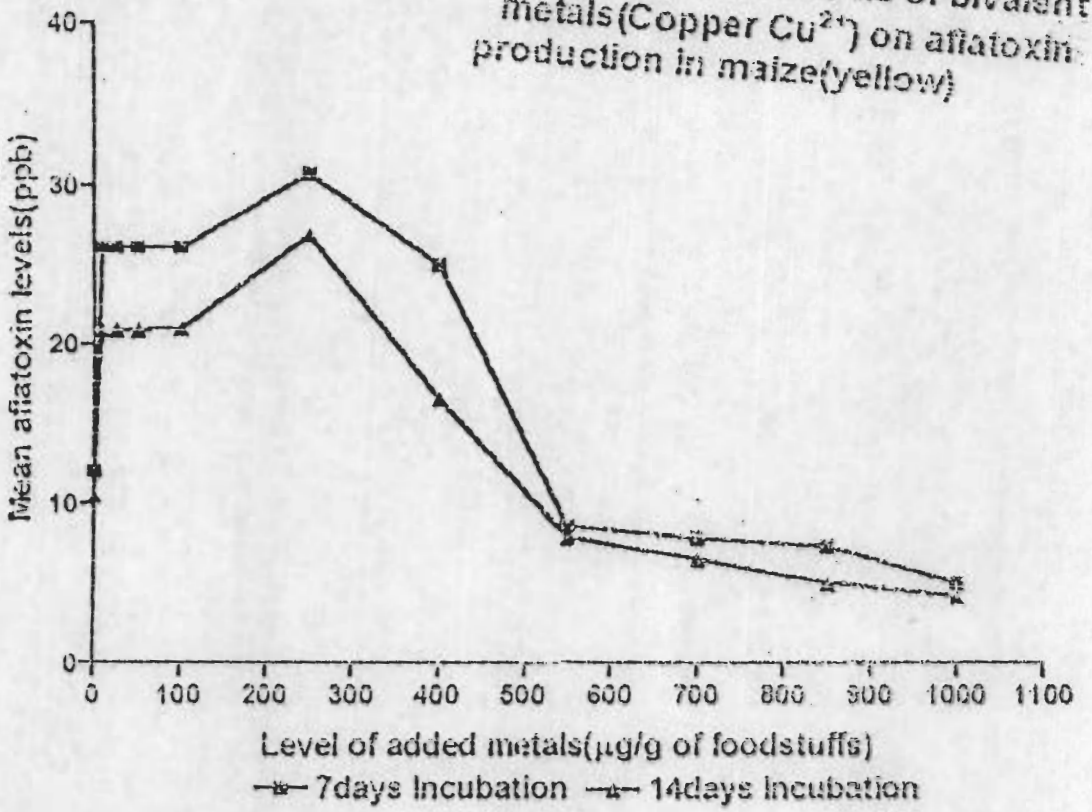


Fig.130:Effects of graded levels of bivalent metals(Copper Cu^{2+}) on aflatoxin production in Acha(findi)

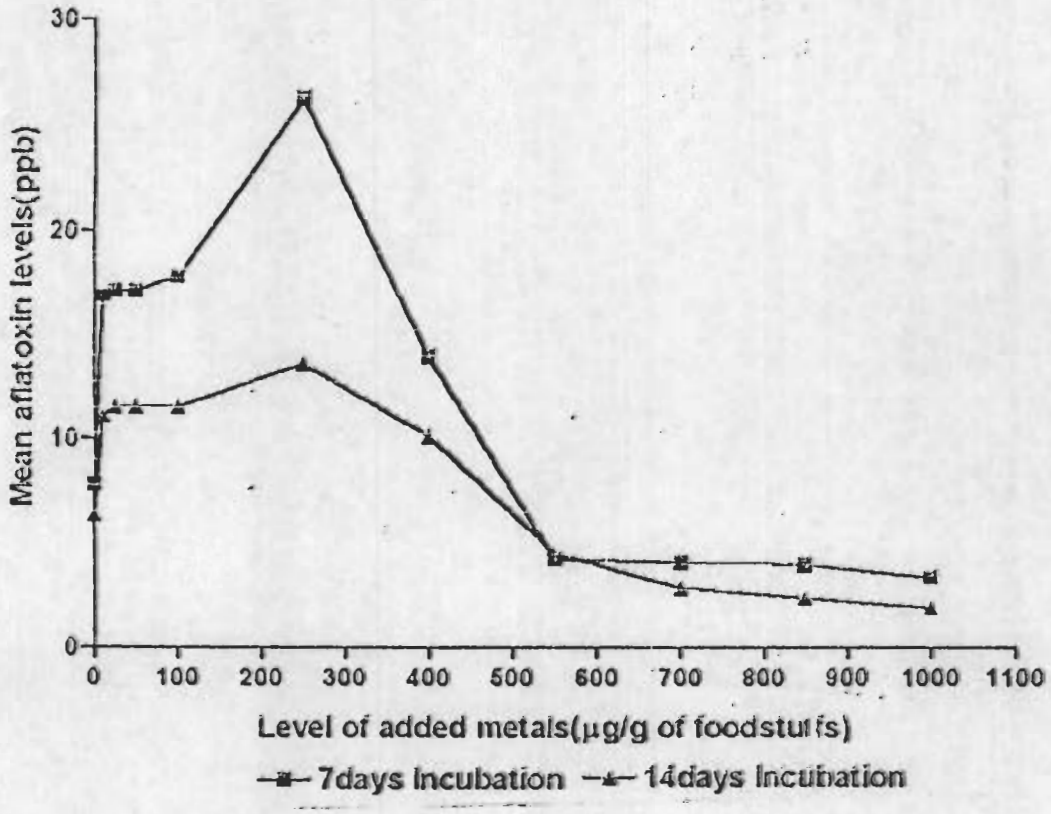


Fig. (3): Effects of graded levels of bivalent metals(Copper Cu^{2+}) on aflatoxin production Miliet

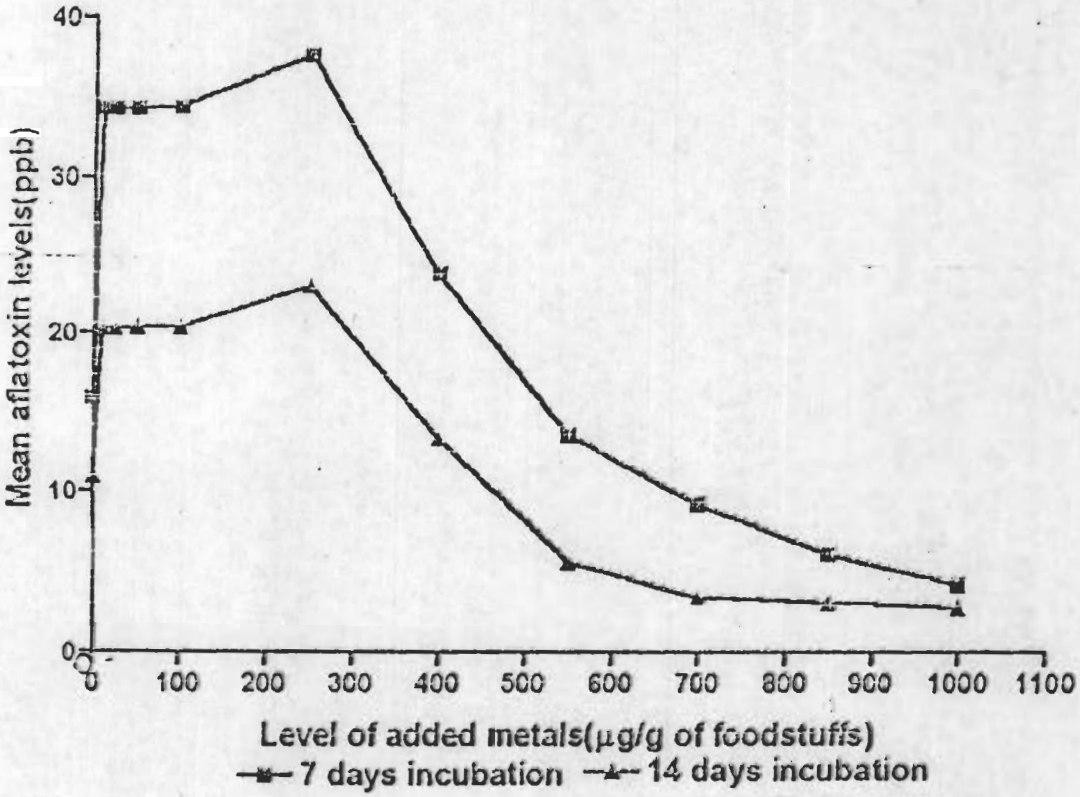


Fig. 32: Effects of graded levels of bivalent metals(Copper Cu^{2+}) on aflatoxin production in Abakaliki Rice

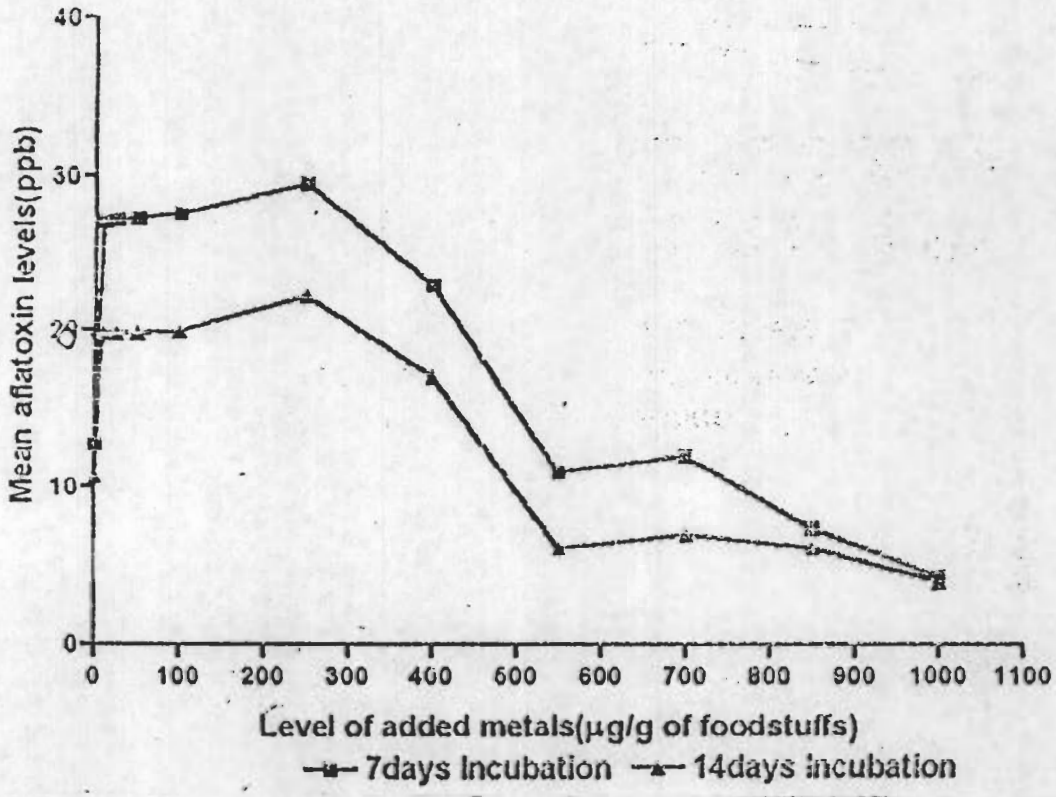


Fig. 33: Effects of graded levels of bivalen metals (Copper Cu^{2+}) on aflatoxin production in Ugbawka Rice

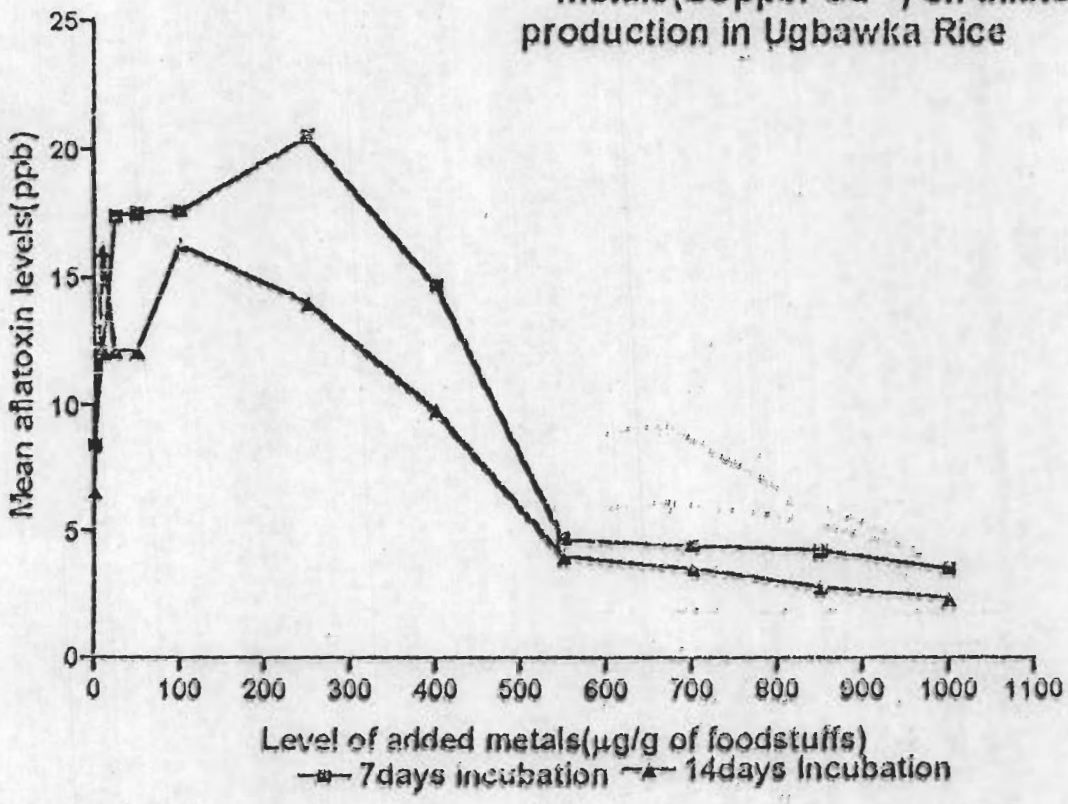


Fig. 134: Effects of graded levels of bivalent metals(Copper Cu^{2+}) on aflatoxin production in Sorghum(yellow)

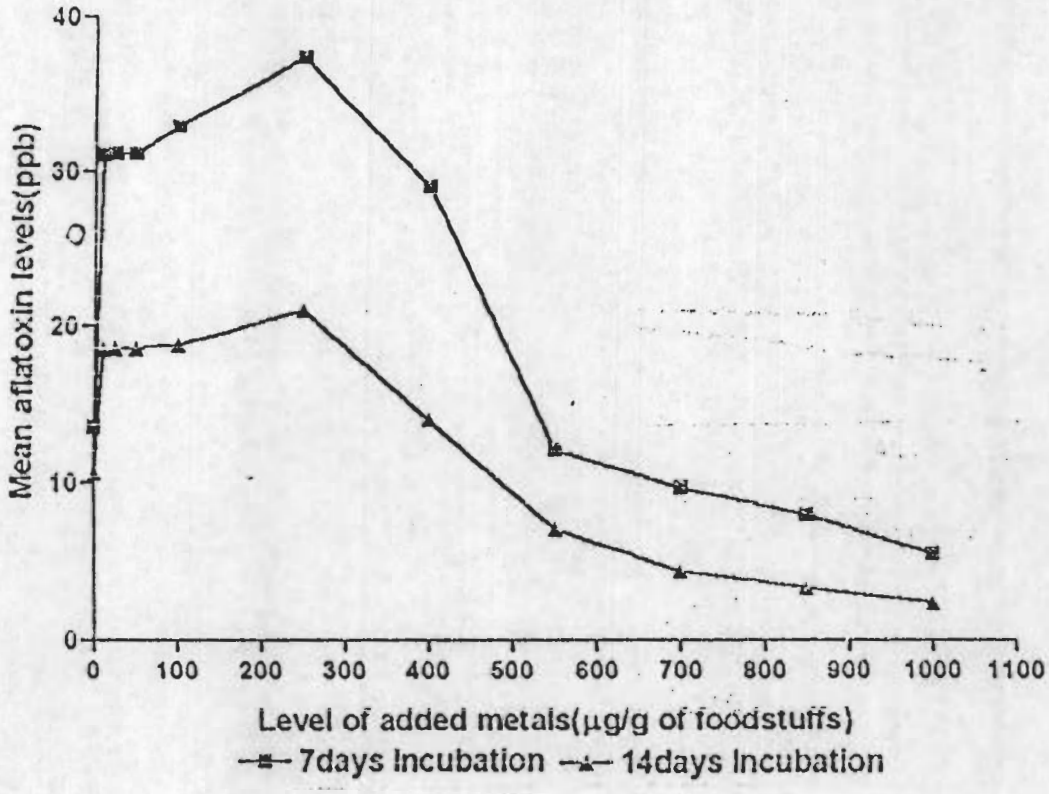


Fig. 135: Effects of graded levels of bivalent metals (Copper Cu^{2+}) on aflatoxin production in Sorghum (white)

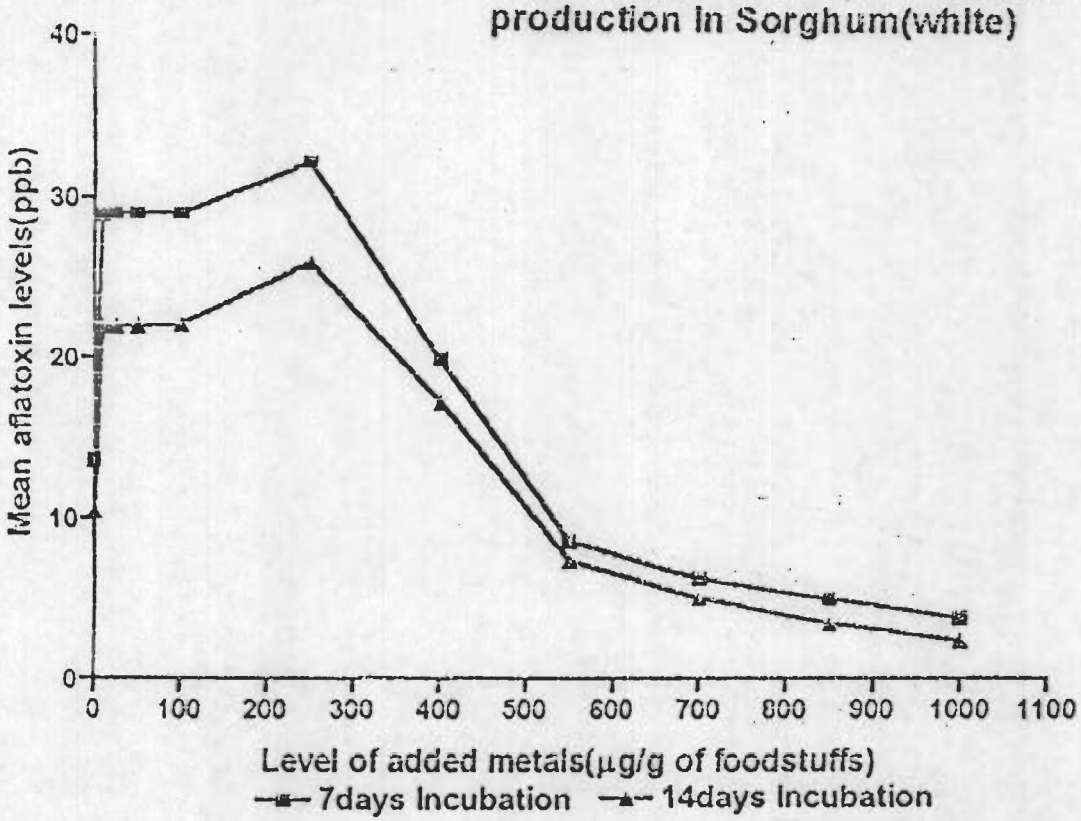


Fig. 136 Effects of graded levels of bivalent metals(Copper Cu^{2+}) on aflatoxin production in Cowpea(white)

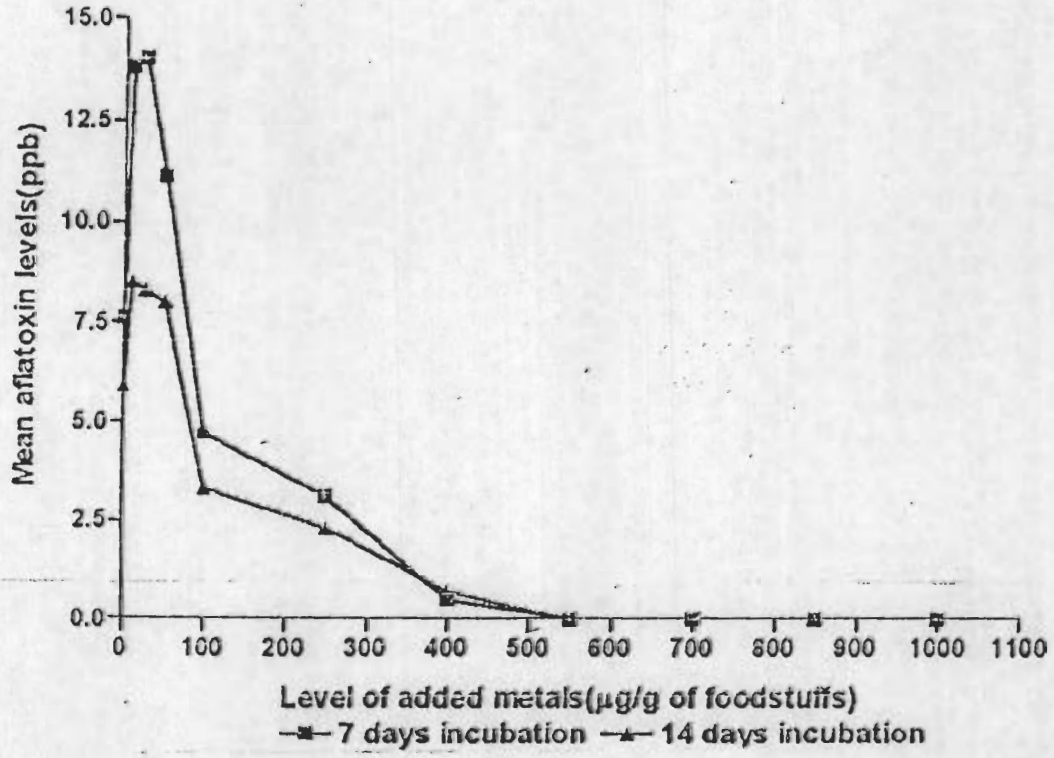


Fig. 137: Effects of graded levels of bivalent metals(Copper Cu^{2+}) on aflatoxin production in Cowpea(brown)

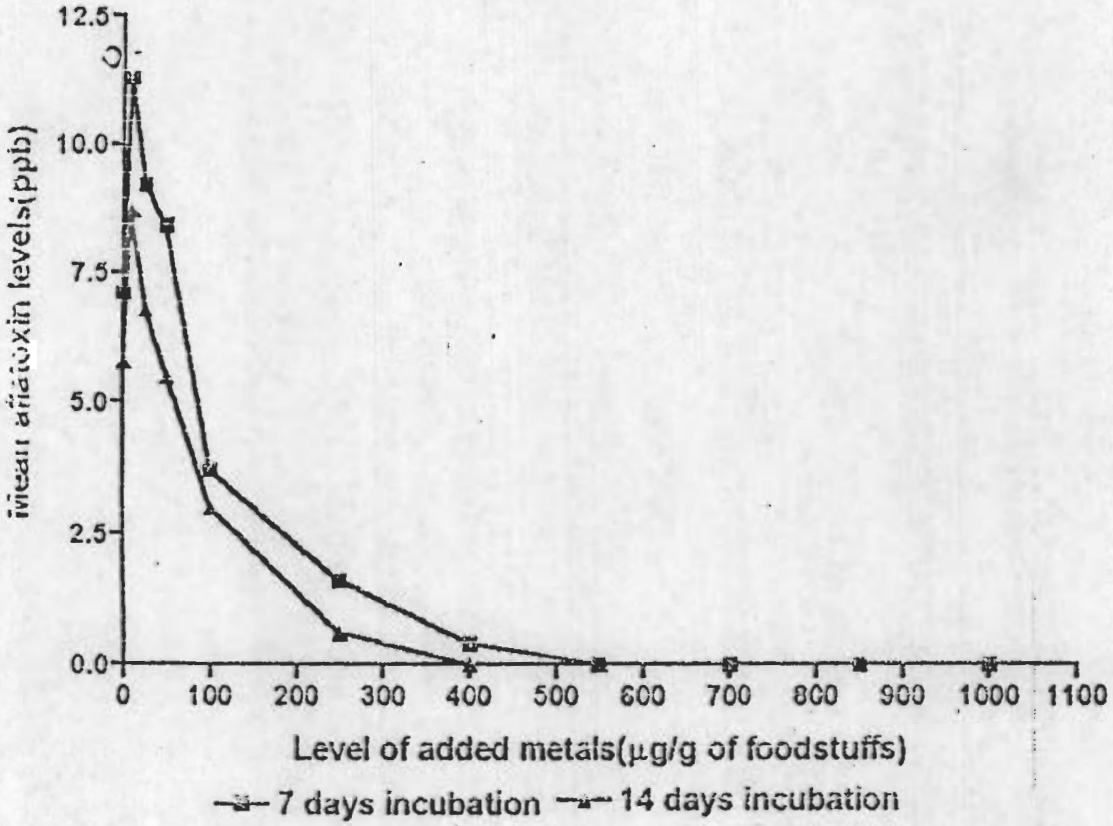


Fig.138 Effects of graded levels of bivalent metals(Copper Cu^{2+}) on aflatoxin production in Pigeon pea(white)

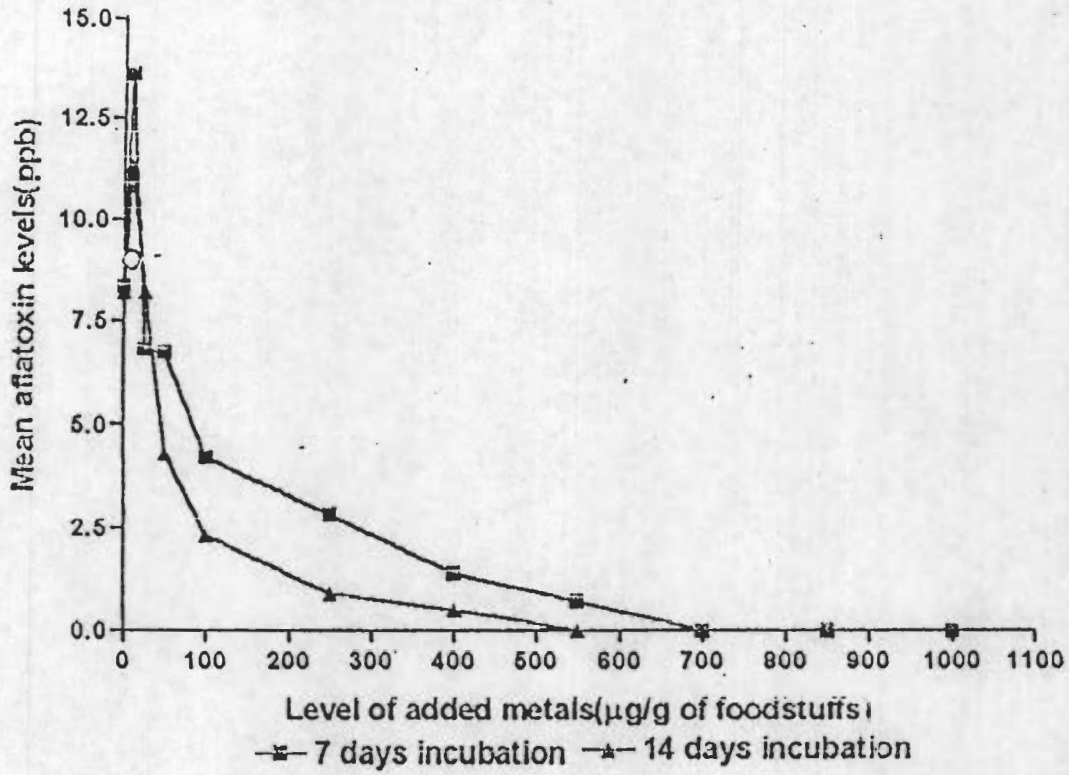


Fig. 139:Effects of graded levels of bivalent metals(Copper Cu^{2+}) on aflatoxin production in Pigeon pea(brown)

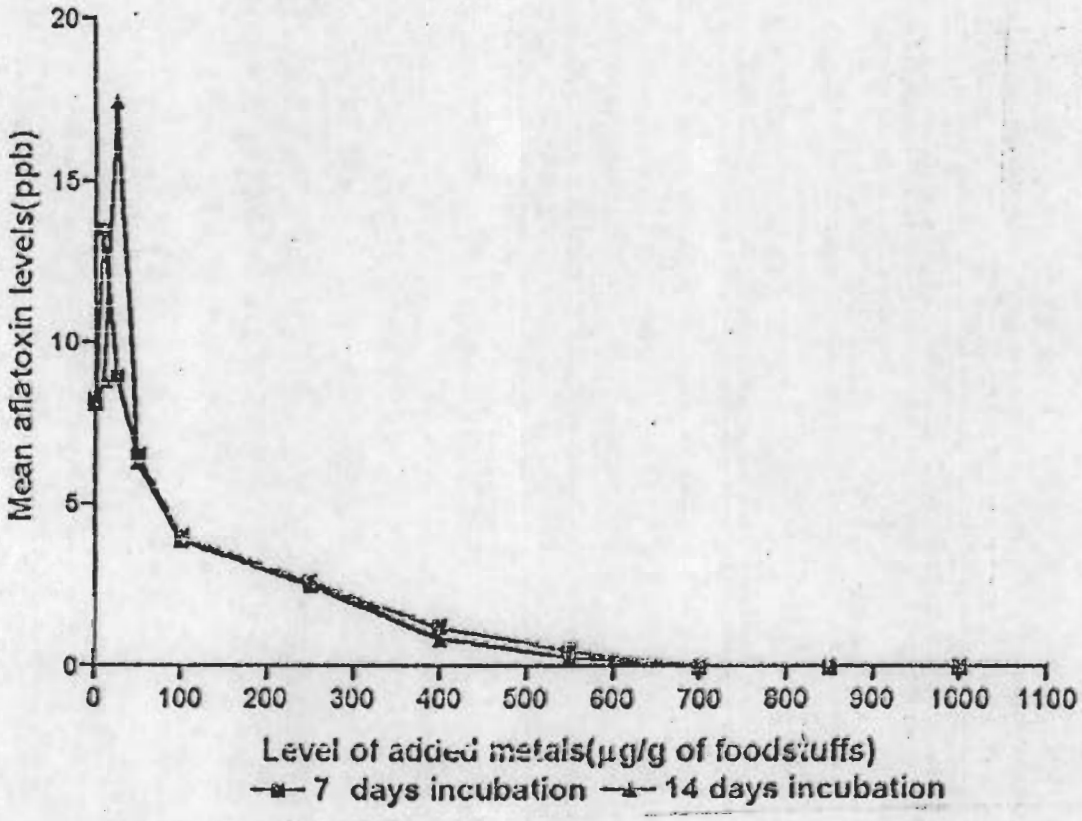


Fig. 40: Effects of graded levels of bivalent metals(Copper Cu^{2+}) on aflatoxin production in Bambara peanuts seed

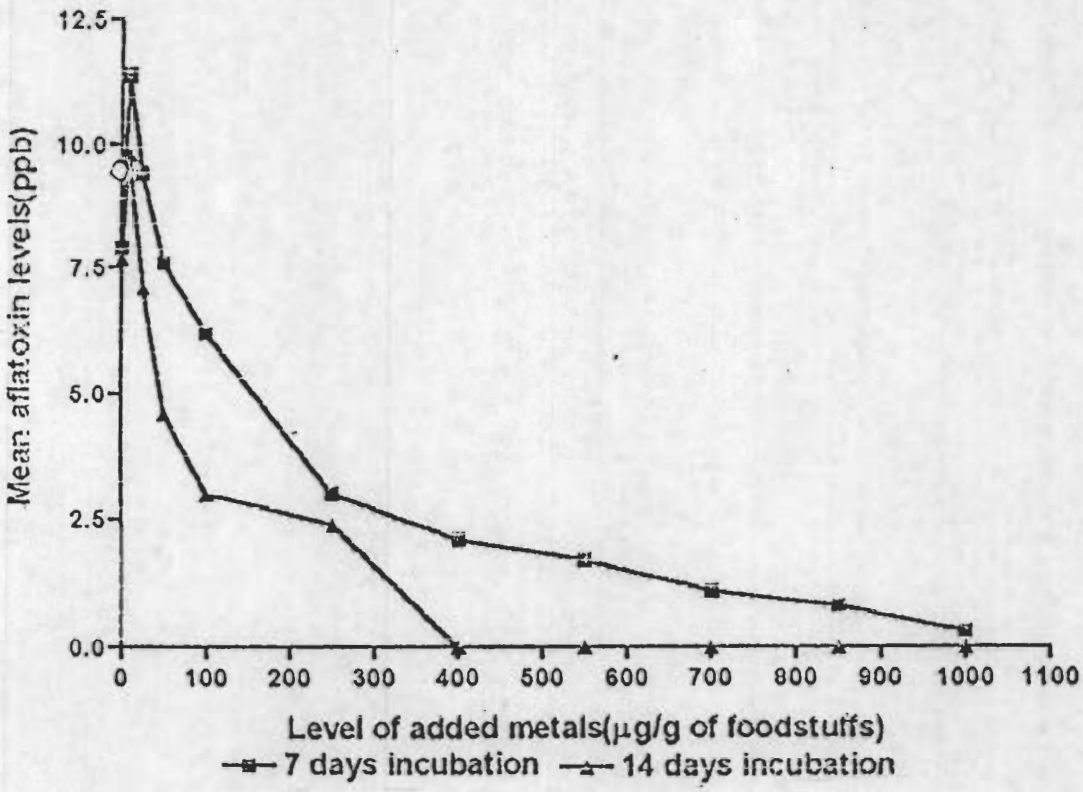


Fig. 14: Effects of graded levels of bivalent metals (Copper Cu^{2+}) on aflatoxin production in Soya beans

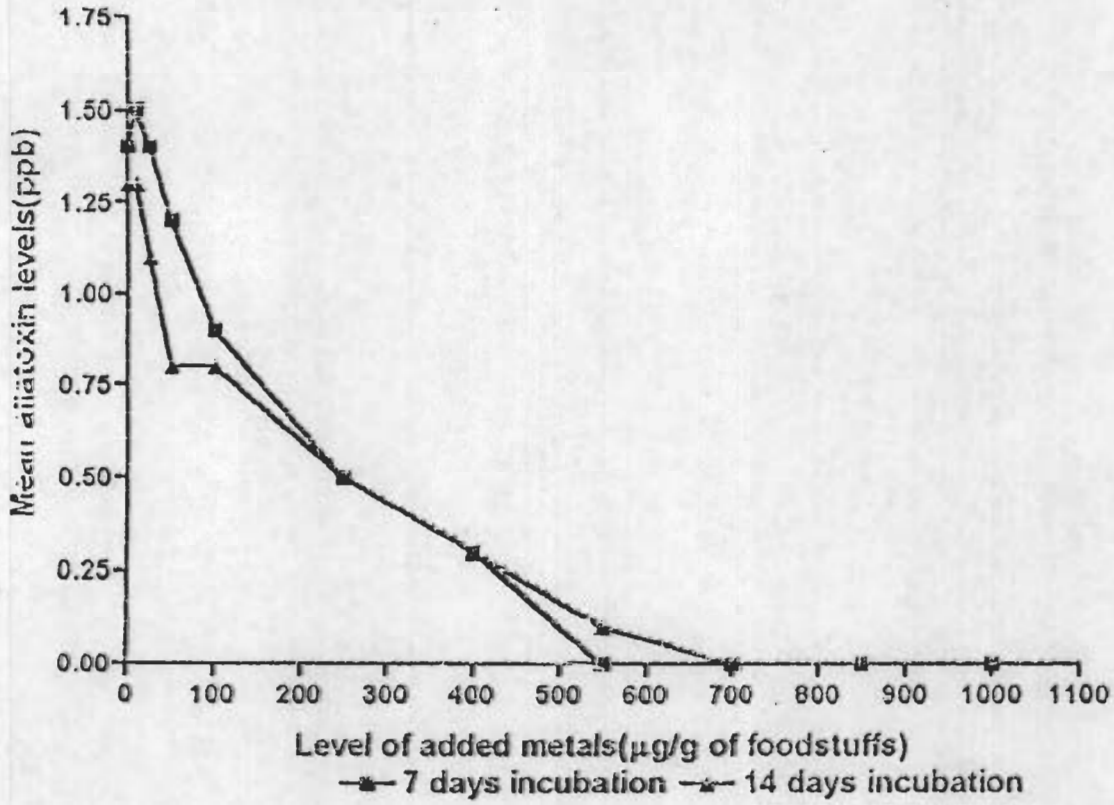


Fig.42: Effects of graded levels of bivalent metals(Copper Cu^{2+}) on aflatoxin production in Bread fruits

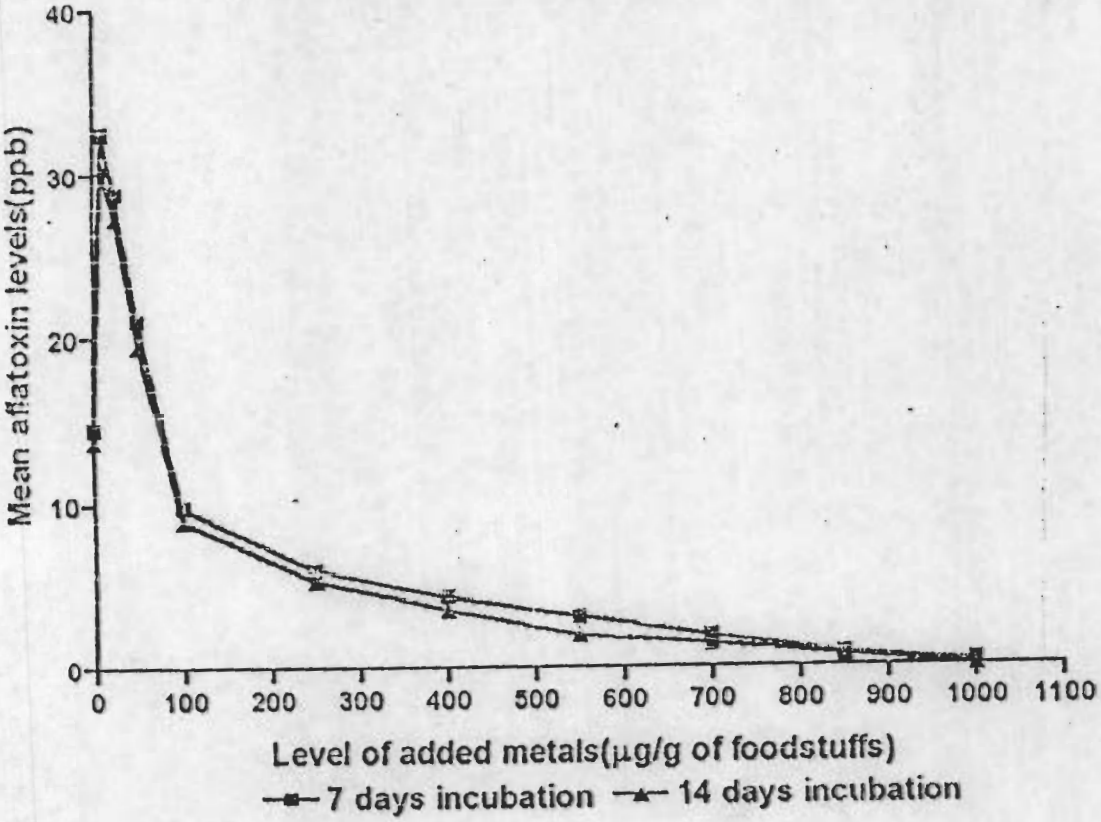


Fig.143:Effects of graded levels of bivalent metals(Copper Cu^{2+}) on aflatoxin production in Melon seeds

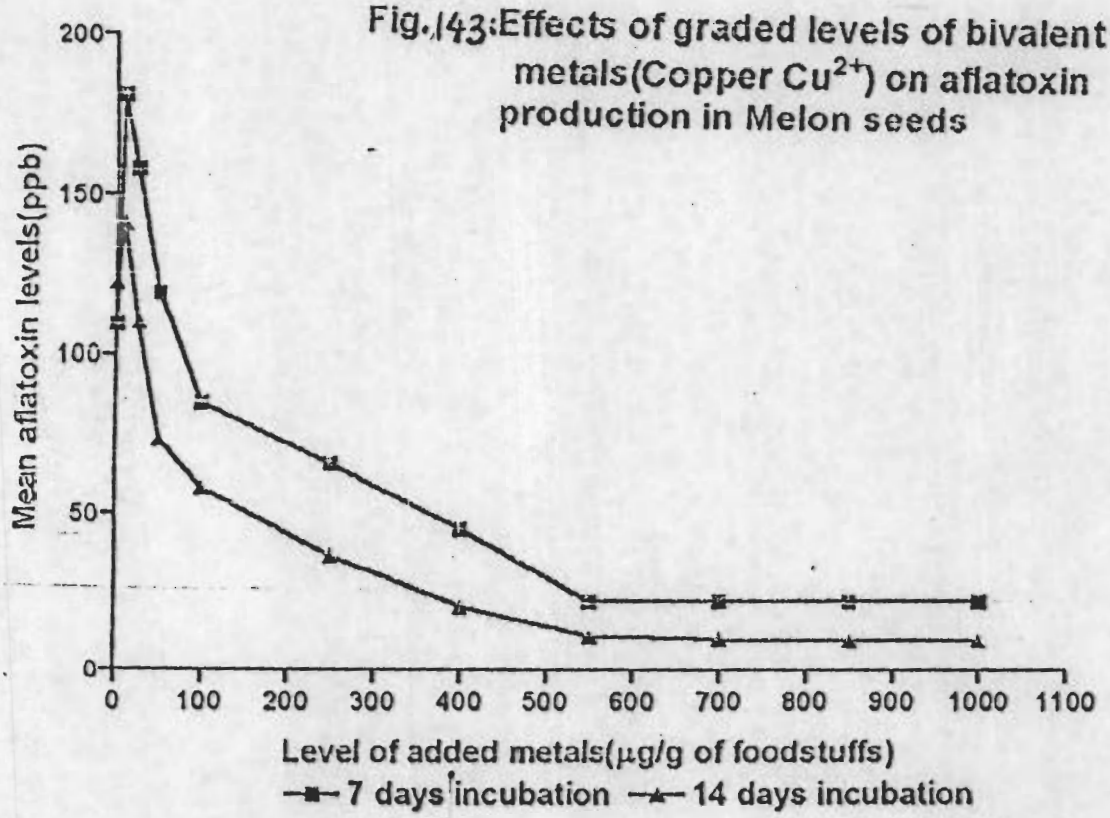


Fig.144: Effects of graded levels of bivalent metals(Copper Cu^{2+}) on aflatoxin production in Peanuts White

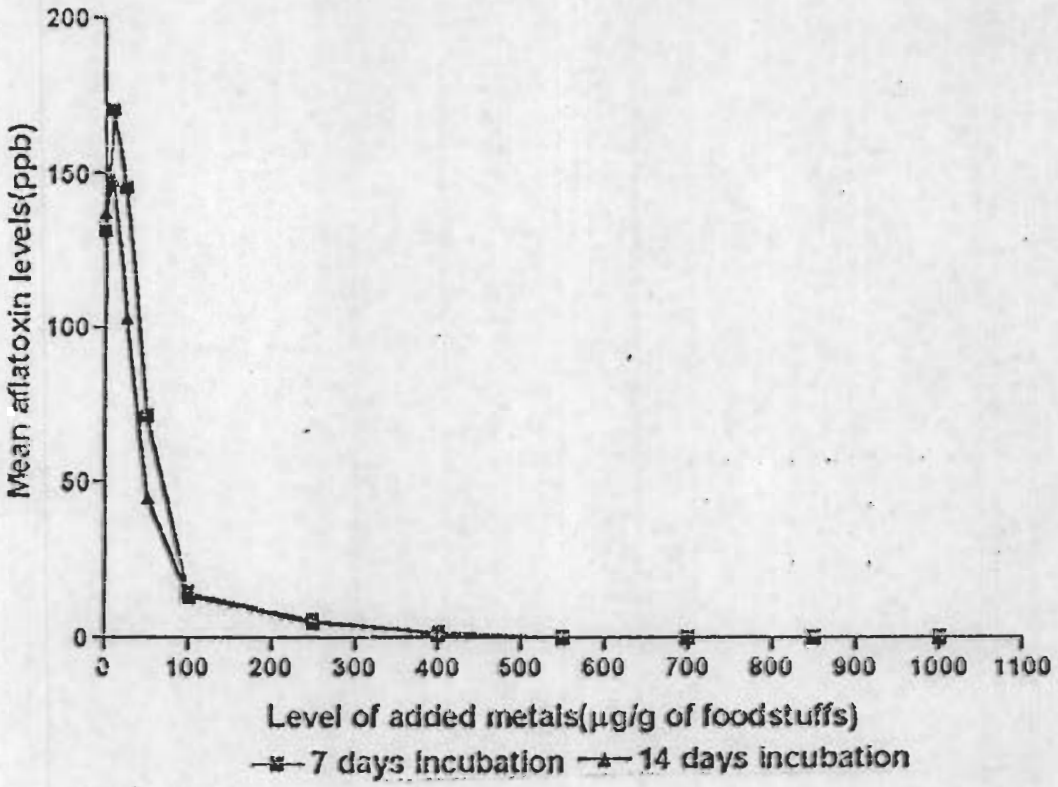


Fig.145:Effects of graded levels of bivalent metals(Copper Cu^{2+}) on aflatoxin production in Peanuts

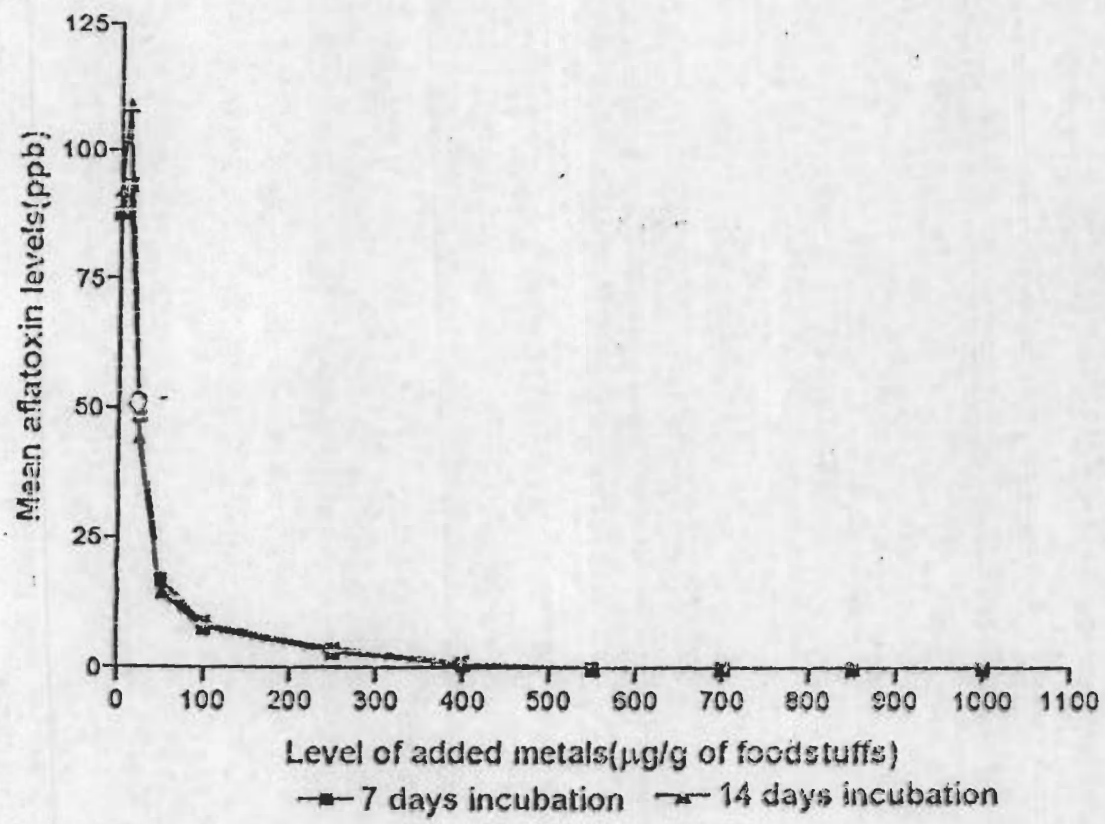


Fig.146: Effects of graded levels of bivalent metals(Copper Cu^{2+}) on aflatoxin production in Coconut seeds

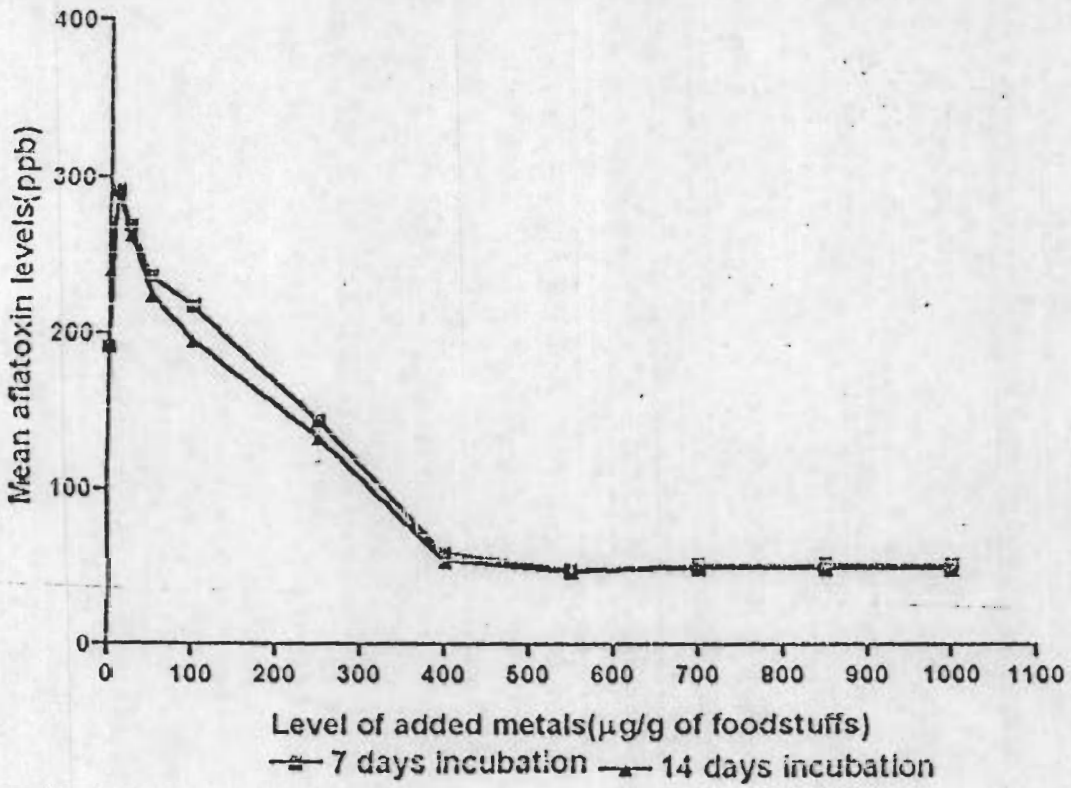


Fig.47: Effects of graded levels of bivalent metals(Copper Cu^{2+}) on aflatoxin production in Cocoyam

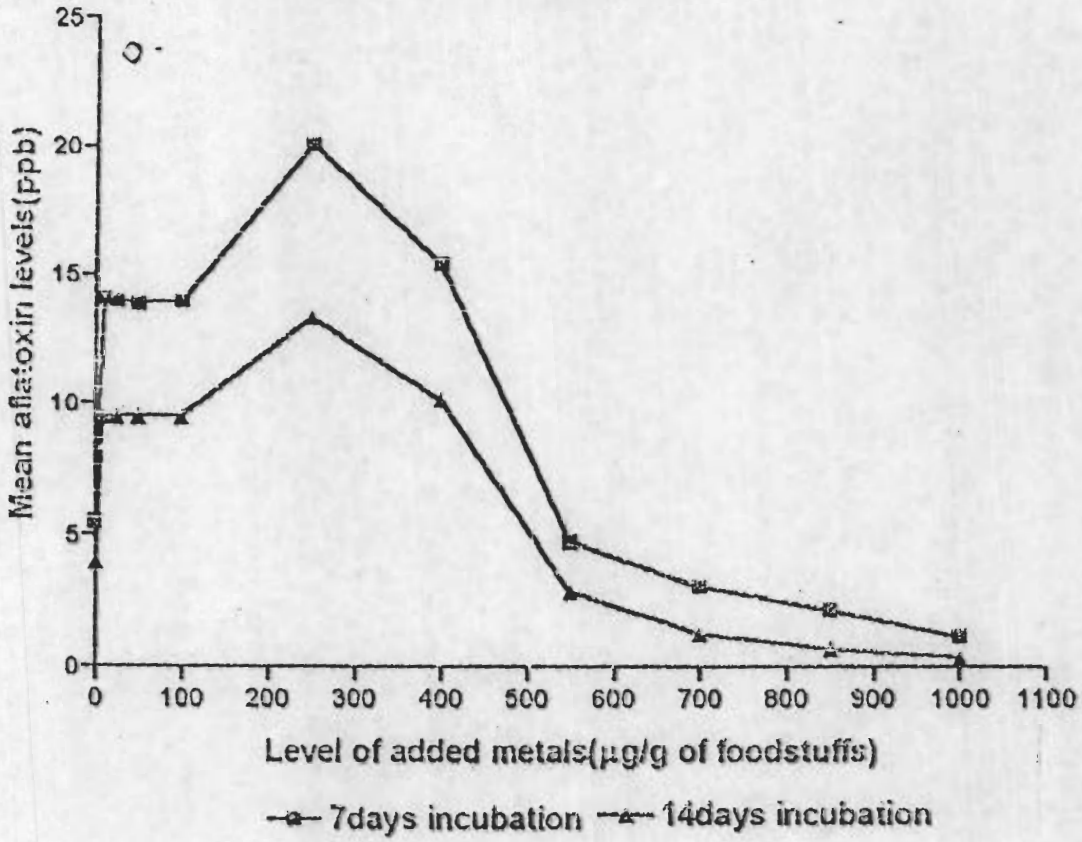


Fig.148:Effects of graded levels of bivalent metals(Copper Cu^{2+}) on aflatoxin production in Yam tuber

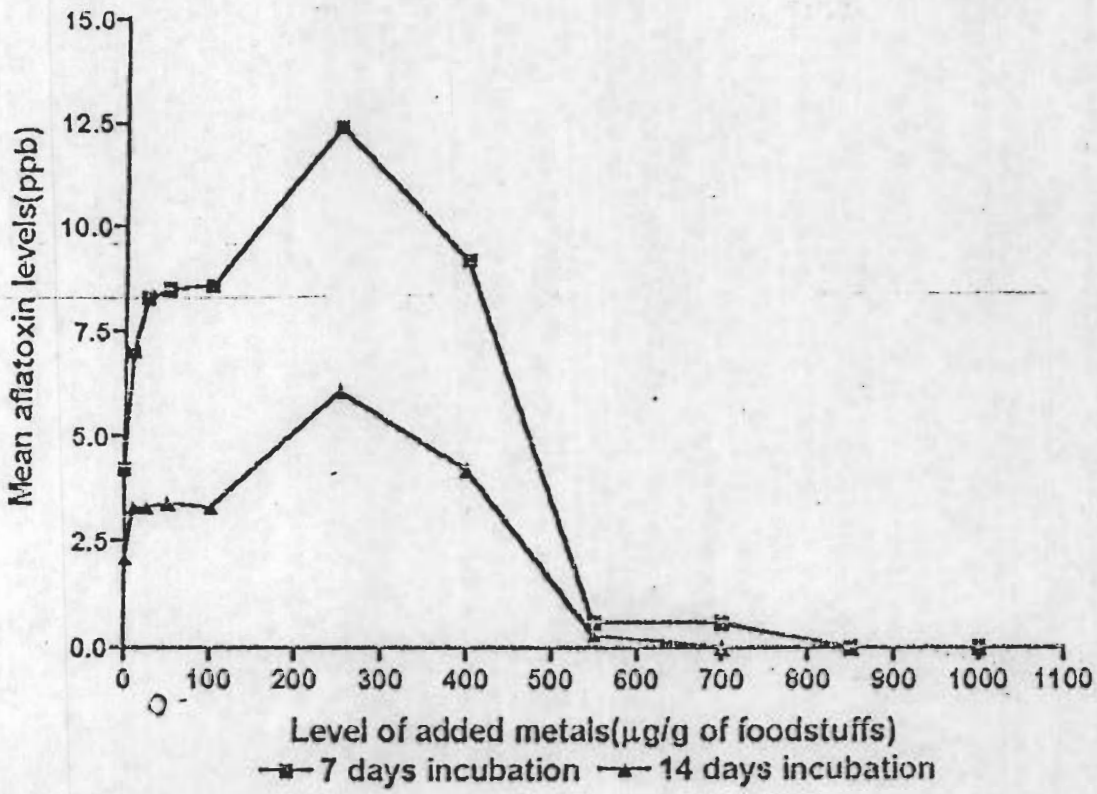


Fig.149:Effects of graded levels of bivalent metals(Copper Cu^{2+}) on aflatoxin production in Cassava tuber

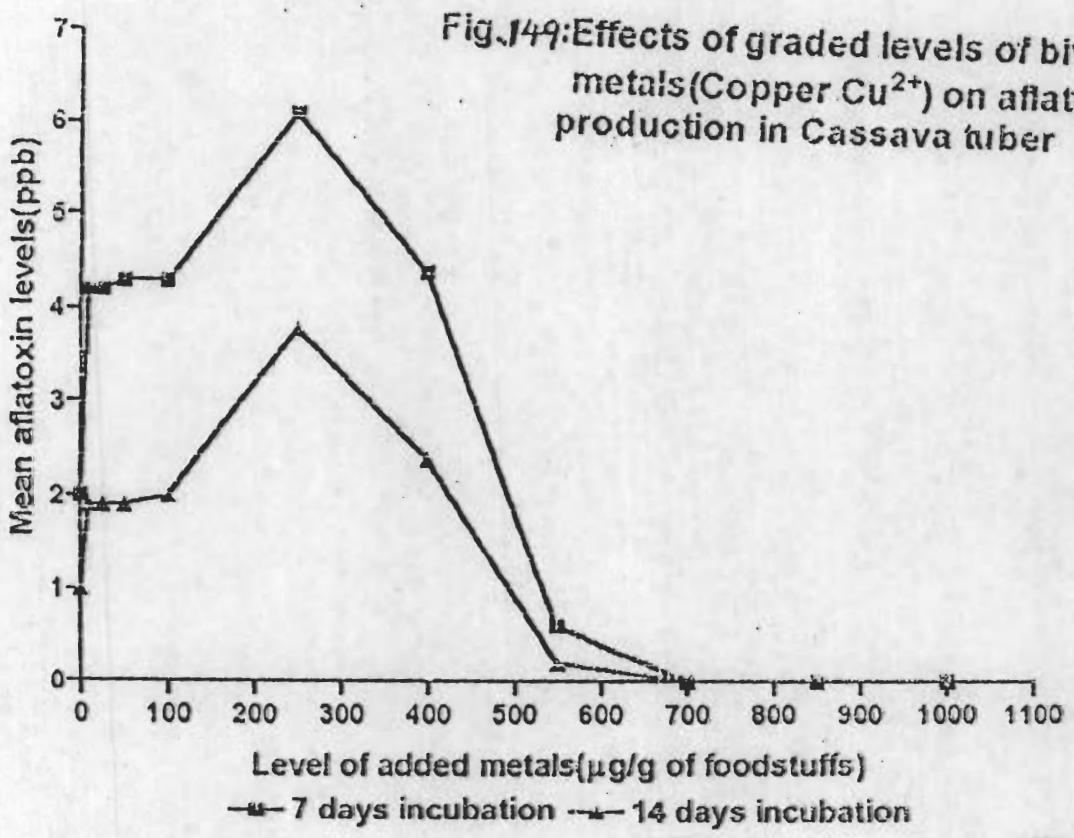
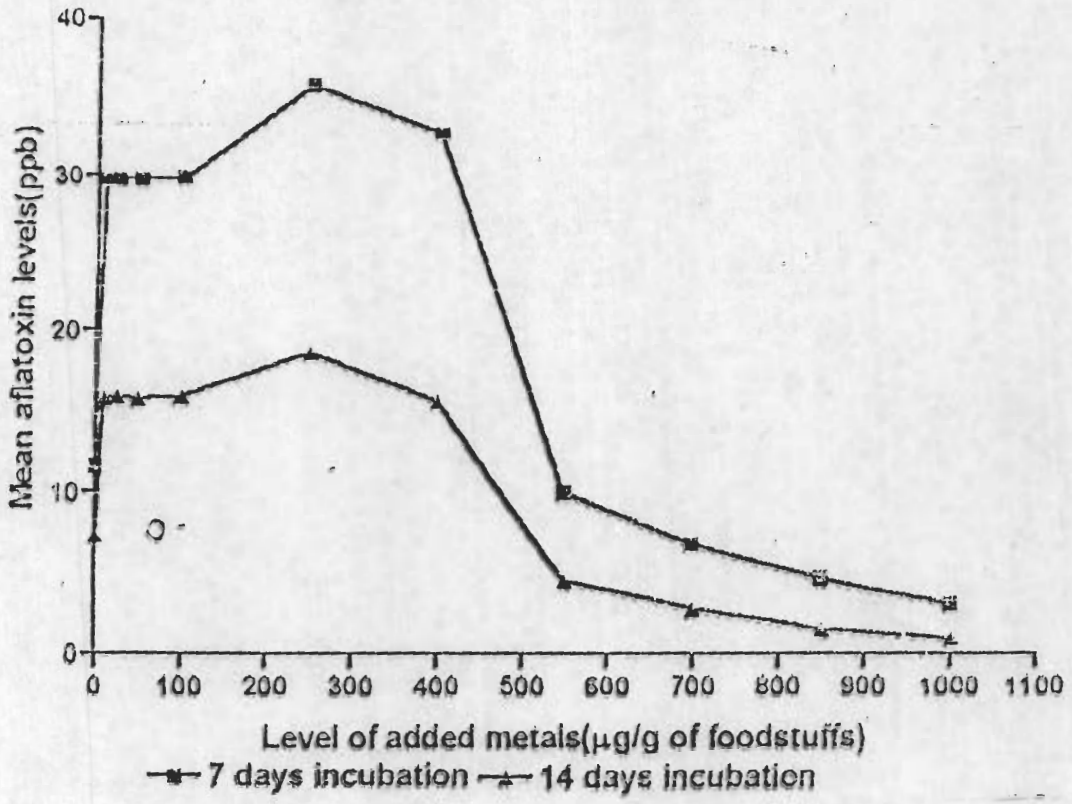


Fig.150: Effects of graded levels of bivalent metals(Copper Cu^{2+}) on aflatoxin production in Plantain (Ripe)



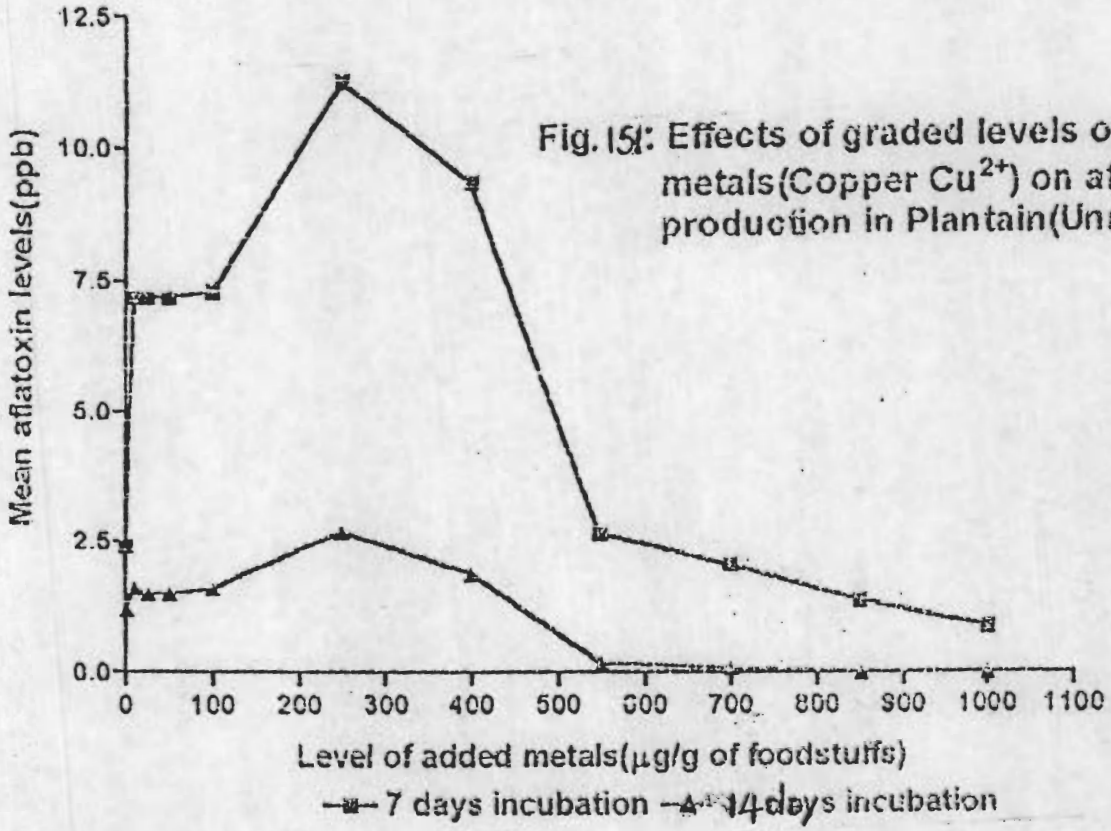


Fig. 15: Effects of graded levels of biv metals(Copper Cu^{2+}) on aflatox production in Plantain(Unripe)

CHAPTER 5.

DISCUSSION:

The results indicate that all the tropical foodstuffs analyzed are subject to mould and bacterial contamination with a dominance of the *A. flavus* group out of the sixteen mould groups isolated with an incidence of 34.8% being followed closely by *Penicillium* species (16.8%), *A. niger* (12.0%), *A. fumigatus* (8. 6%) and *Fusarium* species (7.8%) among others. It does appear from previous works worldwide, that out of the many genera of moulds involved in food contamination, three stand out as especially important (Aspergillus, Penicillium and Fusarium) (Adams and Moss 1996).

In surveys worldwide, the three genera of fungi have been consistently prominent in food samples and animal feeds (Mahnoud 1993; Adebajo *et al.* 1994; Awuah and Kpodo 1996; Fernandez – Pinto and Vaamonde 1996) as found in Egypt, Nigeria, Ghana and Argentina respectively. However, in the temperate areas of the world, reports show that *Fusarium* species seem to be the most incriminated genus out of the three genera (Pozzi *et al.* 1995; Julian *et al.* 1995). The difference appears to go in line with weather conditions – temperate versus tropical climates.

From the results as shown (Fig. 1 and Table 4), 16 mould groups and 12 bacterial groups were encountered. Many previous studies have implicated such groups in various combinations in different foods (Moss 1996; Sweeny and Dobson 1998; Kedera *et al.* 1999). According to Lacey (1989), the spoilage of food and stored products pre-and post-harvest involves a wide range of fungi that differ greatly in their ecological determinants. The large variety and forms of micro-organisims isolated in the current studies is not

surprising with the crude agricultural practices in Nigeria rendering many of these seeds and grains grossly damaged by the hoes and other primitive agricultural tools, poor drying and storage facilities. A huge amount of these food items are spread in the open on roadsides, on top of roofs and other exposed environments for drying which is often largely compounded by the high level of humidity in the country. Pre-and post-harvest attack of food items by insects and birds is also a common feature. As a sequealae to this, fungal infection of these commodities is inescapable.

According to Lacey (1989), most fungal contamination occur during drying and develops further during storage with species of *Aspergillus* being largely dominant. A marked seasonality pattern was noted in the study. As shown in Fig. 5, the findings indicate that temperature, relative humidity and moisture content of the foods play important roles in determining the types of microbial contamination and probably the extent. Thus, while the *A. flavus* group dominated in the hot dry seasons and rainy seasons of the study periods, *Penicillium* spp.took over in the cold harmattan periods. Similar patterns of seasonality distribution have been recorded in other tropical parts of the world (Mishra and Daradhiyar 1991).

Generally, more isolates were observed during the rainy seasons with the least in the cold harmattan periods of this study. In Northern Nigeria, Gbodi *et al.* (1986), also recorded higher isolation rates in the rainy seasons. In the current study also, *Fusarium* spp. were rather increased in the rainy seasons. Lacey (1989) and Lacey and Magan (1991), showed that *Fusarium* spp. are considered `field` fungi becoming of major concern above 25% moisture content and noted that infestation of wheat in Britain and Canada, is

favoured by wet weather and the chief specie is *Fusarium*. Thus, the higher incidence of *Fusarium* spp in the rainy season may be expected. *Penicillium* spp. ranked highest among all isolates made during the cold harmattan period and third in the hot dry seasons. According to Lacey (1989), *Penicillium* spp. have generally greater tolerance of low aw. Ciegler (1975) showed that the occurrence of any given toxin can vary from year to year, depending to a considerable extent on weather conditions. Okonkwo and Obionu (1981) in the South-eastern parts of Nigeria also observed a clear seasonality in aflatoxin levels, being highest during the peak rainy seasons in various tropical food items analyzed with a rainy season/dry season ratio in aflatoxin mean values being 2.80 to 1.65. In the current study; fungal isolations were made in 39% of all samples during the rainy season (1188); 26.5% during the hot dry seasons and 24% during the cold harmattan seasons with similar sample sizes. Also, out of the 1062 fungal organisms encountered in the study, 43.6% were isolated in the rainy seasons, 29.6% in the dry seasons and 26.8% in the cold harmattan seasons.

The clear seasonality disparity favouring the warm rainy seasons is not surprising because it is a known fact that a warm humid environment favours the proliferation of *A. flavus* and other moulds (Lacey 1989, Moss 1989). Additionally, in Nigeria, the rainy season is the planting periods and foods sold in the market at that time comes from the previous harvest. The foods in storage are bound (with all existing conditions) to develop toxigenic moulds. Two factors – prolonged storage and warm humid environment may therefore contribute to the higher values of mycoflora and aflatoxin levels in the rainy season market foods. *A. fumigatus* in the current studies was mostly isolated during the hot dry seasons – ranking highest only after *A. flavus*. Lacey (1989) observed that *A.*

fumigatus can thrive up to 55°C or more. This organism many therefore naturally prefer hotter climatic conditions as shown in this study.

Bacteria were also isolated from the foodstuffs analyzed but with much less frequency than fungal isolates and also being mostly encountered in the dry seasons of the research period (Fig. 6). *Bacillus subtilis* was the commonest bacterial isolate in this season and also in the overall bacterial organisms encountered. The predominance of *B. subtilis* in the hot dry season is not surprising since as spore formers, they are normally associated with dry soil and can normally thrive at elevated temperatures of the soil decaying vegetations from where they can contaminate foodstuffs.

In the work of Imwidthaya *et al.* (1987) in Bangkok, Thailand, only *Bacillus* spp. were the bacteria isolated from cereals and cereal products collected from the local markets. Moss (1989), also observed that *Bacillus* spp. are frequent as surface contaminants of maize seeds. Lacey (1989) stressed that fungi rarely occur in a monospecific culture in stored products but more often as a group of interacting species of fungi and bacteria.

Strzelecki *et al.* (1988) in Poland observed a 94% incidence of proteolytic bacteria contaminating feed and food over a period of about 15 years. Proteolytic bacteria according to Frazier and Westerhoff (1991) include *Bacillus cereus, Pseudomonas* spp. and *Proteus* spp. and these genera were among the most important bacteria in this study where they were involved in the protein foods sampled. It is also not very surprising that *Escherichia coli* and *Streptococcus faecalis* ranked next to *Bacillus* spp. in frequency in this study (Table 4). These organisms, being indicators of faecal pollution as they widely occur in the human colon, can contaminate the foodstuffs during harvesting, storage or in

the field or even when displayed in the market places for sale. The overall preponderance of moulds in the rainy seasons and bacteria in the hot dry seasons supports the views of Frazier and Westerhoff (1991) that fungi tolerate much more moisture than bacteria which also have the ability to survive more in dry environments especially the spore forming genera.

From results obtained in this study (Figs. 5, 7 and 8), it can be shown that susceptibility of agricultural commodities to mycofloral and aflatoxin contamination varies from crop to crop, from one area to another and from season to season. Generally, the cereal grains appear to be ideal substrates for growth of most fungi and also for aflatoxin production. Maize, in the study appears to be the most susceptible cereal to mycofloral and aflatoxin contamination (Table 3). This finding is consistent with other results from Nigeria (Okonkwo and Obionu 1981, Adebajo *et al.* 1994); in the United States (Wood 1981; Russell *et al.* 1991, Price *et al.* 1993); in Thailand and Russia (Imwidthaya *et al.* 1987, Tutelyan *et al.* 1995) and in Kenya and Botswana (Siame *et al.* 1998; Kedera *et al.* 1999).

In the current studies, a total of 24 (85.7%) of the 28 samples of maize with detectable levels of aflatoxins had AFB_1 levels higher than the WHO/FAO recommendation (30 ppb) while only 4(14.3%) had levels below this (FAO 1997). However, the levels of aflatoxins obtained in this study for white maize (mean AFB_1 114.8ppb) and yellow maize (mean 155ppb) agree with a previous work done in the savanna and forest regions of the southern part of Nigeria with AFB_1 range of 100 – 200ppb (Okonkwo and Nwokolo 1978) and in western Nigeria with a mean value of 200ppb (Adebajo *et al.*1994). Studies in other parts of the world also reveal toxic levels of

aflatoxins in maize samples (Sinha 1987; Lovelace and Aalbersberg 1989; Arim 1995; Dhavan and Choudary 1995).

From this work (Fig. 7 and 8), other cereals had higher aflatoxin levels than maize apart form acha grains eg. sorghum had a mean AFB_1 of 221.4ppb and 200.4ppb for the white and yellow varieties respectively; millet (203.5ppb) on the Abakaliki rice (193ppb). Okonkwo and Nwokolo (1978) working in different parts of the south got the following AFB_1 mean values; millet (262.5ppb); sorghum (280ppb), maize (150.75ppb) and acha, with only 10ppb. Though the exact variety of the cereals were not mentioned, other cereals yielded higher Af levels when compared with maize with the exception of acha grains. These results however conflict with Opadokun *et al.*(1979) who detected only a mean AFB_1 values of 58ppb and 40ppb for millet samples from the north while southern samples only yielded 5.0ppb AFB_1 mean values; sorghum, 40ppb and only 5ppb for northern and southern samples respectively.

Acha grains in this work yielded only a mean AFB_1 level of 20.3ppb. This observation rhymes with the Okonkwo and Nwokolo (1978) who detected even lower mean levels of 10.00ppb in their studies in different areas of southern Nigeria. According to Oyenuga (1988), acha (findi) is slightly inferior in protein content to the other cereals (millet, sorghum, maize and rice) and also very low in ash. Opadokun (1990) also got low AFB_1 mean levels for rice (5.ppb and 40ppb) for their southern and northern samples respectively. This however disagrees with the findings of Okonkwo and Nwokolo (1978) who reported a mean AFB_1 of 182.5ppb for their rice samples from the southern part of the country. However, in the present investigation, two varieties of rice were analyzed – the Abakaliki (swamp) rice and the Ugbawka (upland) rice with mean AFB_1 values of 193ppb

and 38ppb respectively. Works done on rice elsewhere also recorded low AFB_1 values. Imwidthaya *et al.* (1987) in Thailand recorded only 10ppb for AFB_1 mean and 20ppb for AFG_1; Patel *et al.* (1996) in the U.K., recorded only trace levels of AF in rice. The two values are consistent with the values obtained for the upland rice in this study, though there was no information on the variety and handling in these studies.

The two rice varieties studied in the current investigation show that the Abakaliki variety was greater than the Ugbawka counterpart in terms of microbial contamination and aflatoxin level. The two rice varieties differ in many ways which could account for these results. The Abakaliki rice for example, which has to be flooded for a period of from 60 to 90 days during growth is unique in being able to thrive on flooded land while the Ugbawka rice can be grown without irrigating or flooding the land. It is not therefore surprising that the Abakaliki (swamp) rice will favour more microbial growth and the greater capacity to support aflatoxin by the invading *A. flavus* when compared with the drier upland variety. Oyenuga (1978) also observed that samples of the upland rice were lower in oil, fibre, ash and minerals and these factors may influence aflatoxin production and microbial contamination as observed in the current study.

Total aerobic bacteria and moulds were higher in the white variety of maize with 42 (11.4%) and 33 (8.9%) being *A. flavus* organisms in the white and yellow maize varieties respectively. However, 12.7% and 6.3% of these *A. flavus* were aflatoxigenic from the yellow and white maize respectively (Fig. 2,3,7,). In a work done in Reunion Island, a French tropical country, Bauduret (1990) observed that white maize seemed to present a better microbial quality than yellow maize but that also yellow maize samples were more frequently contaminated with aflatoxigenic strains of *A. flavus* (54.5%) as against 45% in

the white variety. Also Oyenuga (1978) observed that the yellow maize was a hybrid product originating from the USA, with desirable qualities such as higher carbohydrates, protein and carotene. These factors may favour the choice of *A. flavus* for yellow maize especially for aflatoxigenic potential and higher AF levels as observed in this study. It is of considerable interest that the genome of the plant can itself influence levels of AF contamination during growth and development of the plant (Zuber 1978). There are certain significant strain differences in inbred lines of maize even for aflatoxin levels in inoculated wounded kernels (Lacey 1989). Some correlation has been found between the antifungal effects and lecithin activity of protein extracts from highly susceptible (Huffman) and highly resistant (yellow creole) strains of maize (Lacey 1989).

From this study, most maize crops and other cereals yielded only the `B` aflatoxins while the majority of peanuts and other crops associated with the soil yielded both the B and G aflatoxins. The complex ecology of *A. flavus* and *A. parasiticus* has been reviewed by Diener *et al.* (1987) and Pitt (1993). According to them, it is now well appreciated that *A. parasiticus* is well adapted to a soil environment and is therefore prominent in peanuts whereas *A. flavus* seems to be adapted to active development on the aerial parts of plants such as leaves and flowers and is therefore dominant in maize, general cereals, cotton seeds and tree nuts. They noted that a biochemical distinction between isolates of the two species is that *A. parasiticus* may largely produce AFB and AFG toxins whereas *A. flavus* usually largely produces B1 and B2. Thus, in their reviews, over 90% of contaminated maize samples contain the `B` toxins only whereas the majority of contaminated peanuts contain both the B and G toxins. It is thus considered that the air temperature for maize and others and the gecarposphere temperature for peanuts and others during the seed

development of these crops is an important factor in determining colonization and subsequent aflatoxin contamination by *A. flavus* and *A. parasiticus* (Hill *et al.* 1985). The selective nature of microbial ecosystem in food stuffs was studied by Wilson and king (1995) who showed that in a mixed growth of *A. flavus* and *A. parasiticus,* the former apparently suppresses the accumulation of the `G` aflatoxins with an increase in the `B` toxins. This is consistent with the aflatoxin distribution both qualitatively and quantitatively in the current study.

Acha grains are also used to produce a non-alcoholic beverage, kunnu, which is largely supplemented with sugar, sweet potatoes and other sugar sources for an improved taste. These supplements may further favour higher colonisation and subsequent AF production when compared with the grains themselves as observed in this study. Thus in this study, acha grains yielded a total average aflatoxin levels of 21.5ppb while the kunnu beverage produced from the batch yielded a total of 67.4 ppb. Sorghum (Guinea corn) is also widely used in the production of an alcoholic beverage (burukutu) or pito. The low AFB_1 mean of 17.7ppb observed in this study in Pito can be explained by the fact that fermentation has been largely associated with a decrease in both mycofloral and mycotoxin levels (Ogunsanwo *et al.* 1989; Jespersen *et al.* 1994; Adegoke *et al.* 1994). Thus the beverage which was actually also produced from the same batch of white sorghum analyzed in this study, yielded much reduced AFB_1 mean levels (17.7ppb) as against the level in the grains (221.4ppb) (Fig. 8). According to Sweeny and Dobson (1998), some bye products in fermentation reactions may be lactic acid which has been shown to convert AFB_1 to AFB_2a or degrade the aflatoxins as they are formed. The same may also be true of ogi (akamu), the fermented product of maize analyzed. While the mean AFB_1 level

(155ppb) or total average (216.2ppb) from the yellow maize batch from which it was produced was quite high, the ogi had only a total average of 24.7ppb showing a serious reduction after fermentation. This agrees with Adegoke *et al,* (1994); who observed a reduction of 72.5% and 71.4% respectively in the levels of aflatoxins after the preparation of ogi from contaminated corn and sorghum.

Also in the samples of a fermented condiment (ogiri) made from the melon seeds analyzed in this study, no aflatoxins were detected. This is consistent with the results of Ogunsanwo *et al.* (1989) who also did not detect any aflatoxins from his ogiri samples by the fourth day of fermentation. It is also probable that the succession of various microbial groups during fermentation as detected by Jespersen *et al.* (1994), may account for some competitive interaction which may affect aflatoxin before or after production. In the current analysis, also, various groups of fungi and bacteria including proteolytic forms were detected in the ogiri samples.

For the oil seeds, peanuts were the most contaminated generally and also by *A. flavus* group as well as with the aflatoxigenic strains and with very high levels of aflatoxins being next only to coconut seeds. According to previous works, peanuts are easily the most susceptible of the oil seeds to aflatoxin contamination (Atawodi *et al.* 1994; Awuah and Kpodo 1996; Moss 1996; Siame *et al.* 1998). A mean AFB_1 of 876; 510.5 and 1,388.3ppb were recorded in the white, red and roasted (Red) varieties of peanuts respectively in the current study. The high levels of aflatoxins detected in peanuts in this study has been reported in previous studies (Okonkwo and Nwokolo 1978; Opadokun 1990; Atawodi *et al.* 1994; Awuah and Kpodo 1996). This is however not unexpected since peanuts grow and mature in the soil where there are great potentials for damage by

soil pest such as insects, nematodes, termites and harvesting methods (practices) all of which can predispose this crop to fungal attack before or after harvest.

From the study, a significant difference was observed ($P< 0.05$) in aflatoxin contamination and levels as well as rate of contamination by these micro-organisms for the two peanut varieties studied (Fig. 2, Fig 7, Fig. 9). Thus, the peanuts with white testae were consistently found to favour both in types and in quantity, more moulds when compared with the red testae. The white testa variety also yielded more *A. flavus* group as well as more aflatoxigenic strains 61 (16.5%) and 18 (14.3%) of the total *A. flavus* group and aflatoxigenic strains respectively while the red type yielded only 23(6.2%) and 10(7.9%) of these respectively. Mc Donald (1986) found that groundnuts with red testa were more resistant to invasion by *A. flavus* and other fungi than were seeds with white testa. Dorner *et al.* (1989) in the USA, observed that mature peanuts possessed additional resistance to contamination which may be associated with the production of phytoalexins. It may also be probable that the red varieties possess more phytoalexins than their white counterparts. Similar findings were also reported by Ogundero (1987) and Ellis *et al.* (1991).

The results further reveal that the roasted red peanuts yielded the highest aflatoxins levels among all the peanuts sampled. Haq-Elamin *et al.* (1988) in Sudan observed that gray (white) and red roasted groundnut pods showed higher amounts of aflatoxins. An association between the loss of the capacity of kernels to produce phytoalexins and the appearance of aflatoxins contamination has been shown by Dorner *et al.* (1989). They explained that kernel water activity appeared to be the most important factor controlling the capacity of kernels to produce phytoalexins which seems to endow them with the capacity for resistance to contamination. According to them, roasting appears to reduce

the water activity level to such an extent that it will affect the production of phytoalexins. This observation however conflicts with some workers who noted that roasting groundnuts appreciably reduces the levels of aflatoxins and that about 50% of the aflatoxins are altered to such an extent that they can no longer be detected (Waltking 1971; Stoloff and Trucksess 1981). However, the details of the complexity accounting for the observations in the current study needs further elucidation.

Coconut seeds, in this study were found to have very high levels of aflatoxins in comparison with other oil seeds with an AFB_1 range of 527 –1, 605ppb (mean 927.3ppb) (Fig 7, Fig. 10). Obidoa (1975), and Zohri and Saber (1993) confirmed that coconut is one of the best substrates for the production of large and high quantities of aflatoxins. Zohri and Saber's work recorded AFB_1 range of 1,500 – 2,500ppb for their coconut samples. According to Arseculeratne *et al.* (1969); Davis and Diener (1987)and Lovelace and Aalbersberg (1989), coconut is superior to peanut and other oil seeds for the preparation of aflatoxins. According to them, this may be due to the nature and content of neutral fat in the mature coconut kernel which are predominantly the $C_{12} - C_{14}$ fatty acids, lauric and myristic acids, whereas in peanuts, are mainly the C_{18} acids, oleic and linoleic acids. This according to them results in a proportionately greater yield of glycerol from the hydrolysis of the neutral fats from coconut oil than peanut oil which has been shown to enhance growth of *A. flavus* and subsequent aflatoxin production.

The melon seeds analysed in this study also gave high aflatoxin yields ranging between 104 – 1, 490.3ppb with a mean AFB_1 level of 690.8ppb thus coming mid way between red peanuts and coconut seeds. Isolates including *Aspergillus* spp, *Penicillium* spp., *Fusarium* spp. and *Rhizopus* spp. encountered in this work in melon seeds were also

recorded by Adebajo *et al.* (1994). Opadokun (1990) however detected a much lower aflatoxin AFB_1, mean level (53ppb) than in this work in melon seeds in northern Nigeria, (53ppb) mean AFB_1 level. Many complex factors like moisture, (aW), microbial ecosystem, temperature, seasonality, genetic differences and other undisclosed factors may be responsible for this remarkable difference.

Apart form bambara peanuts and breadfruits with mean AFB_1 of 124.3ppb and 170.9ppb respectively, the protein foods were generally low in aflatoxin levels (Fig. 9).

Oil bean seeds did not contain any detectable levels of aflatoxins. The oil bean seeds which were fermented for 72 hours became soft and slimy on the surface with a characteristic strong aroma. Bacteria were more encountered than moulds in all samples analyzed. The organisms isolated from these fermented sliced oil bean seeds included *Bacillus cereus, Pseudomonas aeruginosa, Proteus* spp, *Strept. faecalis, E. coli* and *S. aureus,* among the bacteria, while moulds included *A. flavus* both toxigenic and non toxigenic strains. Obeta (1982) also obtained similar organisms in his study of oil bean fermented seeds in Nsukka, Nigeria. Ogunsanwo *et al.* (1989); Fardiaz (1991); Hassanin and Kheirella (1995) have shown that activities and products involved in fermentation may inhibit aflatoxin production. Adegoke *et al.*(1994) has identified some volatile products which he thaught to be responsible for the changes in texture and characteristic strong aroma to possibly affect aflatoxin production in oil been seeds during fermentation.

The levels of aflatoxins in cowpeas were also low in this study with AFB_1 range of 23.4 – 46.6ppb (mean 39.7ppbb) for the white variety while the brown type had a mean AFB_1 of 31.2ppb. This falls within the values obtained in previous works (Okonkwo and

Nwokolo 1978; Imwidthaya *et al.* 1987 ; Opadokun 1990). From the results also, the white cowpeas were superior to the brown beans in the levels of aflatoxins produced as well as in the levels of microbial contamination including *A. flavus* both toxigenic and non toxigenic strains. In Egypt, el – Kady *et al.* (1996) showed that aflatoxin production varied between different cultivars of cowpeas which differed morphologically and histologically, in colour, shape and size of seeds though the chemical analyses of the different constituents from their seed coats and kernels also revealed that the resistant cultivars contained lower levels of sodium and higher levels of phosphate and potassium.

The moin-moin (wet-milled and steamed cowpea wrapped in fresh leaves) prepared from the white variety yielded no aflatoxins except a sample that yielded a mean AFB_1 of 16.9ppb even though no *A.flavus* was isolated from the moin-moin samples. There was however a significant reduction in the initial AFB_1 level (46.6ppb) from the raw white cowpeas used in their preparation ($P< 0.05$). Ogunsanwo *et al.* (1989) investigated the fate of aflatoxins when raw cowpeas are prosessed into moin-moin and observed a reduction in the aflatoxin levels. Similar findings were noted in the production of `tuwo` and `ogi`, two cereal based products from sorghum and corn after boiling the pastes to prepare porridge (Adegoke *et al.* 1994).

Soyabeans in this study, were contaminated by only a mean AFB_1 of 9.4ppb (range 8.5 – 10.3ppb). Literature abound supporting the fact that soyabean appears to be a poor substrate for aflatoxin formation (Stossel 1986; Imwidthaya *et al.* 1987; Mahmoud 1993). Many conflicting proposals have been forwarded by many workers attempting to explain the reasons behind this, including seed coat integrity, trypsin inhibitors, limiting role of the carbon source and the availability of zinc said to be bound to phytic acid in the soyabean

(Richardson 1977; Erdman 1979; Obidoa and Onyeneke 1980; Stossel 1986). The view supporting seed coat integrity seems very likely in the current study, and may also be used to explain the lack of detection of aflatoxins in pigeon pea samples in this study. Naturally too, both soyabeans and pigeon peas are rarely prone to insect/weevil attack hence the resultant poor colonization by moulds and consequent aflatoxin contamination as actually observed in the current work. Imwidthaya *et al.* (1987) in Thailand detected no aflatoxins in pigeon pea samples analyzed. Ciegler *et al.* (1966) demonstrated that peroxidized methyl esters of soyabean oil could degrade aflatoxins in vitro. However, further work would be required to elucidate the various factors operating in pigeon peas. The bambara peanuts supported higher aflatoxin yields though less invasion by moulds (AFB_1 mean level of 124.3ppb). These food items also have very hard seed coats but may have other factors supporting aflatoxin formation once invasion occurs. The low levels of aflatoxins detected in okpa (wet-milled and steamed bambara peanuts wrapped in fresh leaves) must not be unconnected with factors already discussed for moin – moin (Ogunsanwo *et al.* 1989; Adegoke *et al.* 1994).

De-coated per boiled breadfruits supported the highest levels of both microbial growth and aflatoxin formation among the protein-rich foods. The striking feature here includes the production of only AFB_1 and AFG_2 throughout the study. Possibilities including quality of the carbon source available to the toxigenic mould, presence and quality of oils in the food substrate and other undisclosed factors may affect aflatoxin production both qualitatively and quantitatively in the breadfruits. Cassava, yam and cocoyam (the carbohydrate foods) analyzed were all shown to be low in aflatoxin yield in this study, having AFB_1 mean levels of 8.3ppb, 21ppb and 15 .2ppb respectively also with

both the B and G toxins being detected. The low levels of aflatoxins observed are consistent with previous reports (Opadokun *et al.* 1976; Okonkwo and Nwokolo, 1978). Though these crops are prone to attack by boring insects which can introduce fungal spores into the root tubers or also through wounds inflicted by diggers and matchets during harvesting, the study surprisingly also reveals poor microbial contamination of these crops generally. Though not investigated in the current study, Onyenuga (1978) attempted to explain the reasons behind his observed poor invasion rates by insects/nematodes in these crops by the fact that the peels of cassava and cocoyam corm samples he analyzed contained 3 – 5 times the amount of glycosides found in the edible portions of the roots for his cassava samples and an acid substance for his cocoyam samples. These he thinks may be responsible for warding off these agents since they were found to be irritating and even poisonous to them.

The garri samples analysed in this study did not contain detectable aflatoxins as noted by Adegoke *et al.* (1994). The finding also agrees with other previous reports on garri (Opadokun 1976; Obidoa and Gugnani 1990). An intense blue colour fluoresced on the thin layer chromatogram at the Rf of AFB_1 but confirmation tests showed that the fluorescing compound was not AFB_1. Similar observations have been recorded by Obidoa and Guguani (1990). This blue fluorescent spot, according to them was shown to be a non fungal coumarin glycoside and their corresponding aglycones (viz scopolin, esculin and esculetin aglycones). Organisms isolated from cassava and other root crops included *A. flavus, A. fumigatus, Penicillium* spp, *Fusarium* spp. and *Rhizopus* spp. in this study. These however differ from the Uganda report by Essers (1995) where *Neurospora* spp, *Geotrichum* spp and *Rhizopus* spp were prominent but however agrees with that of

Agbonlahor *et al.* (1997) in Edo State, Nigeria. This difference may be related to soil types and different geocarposphere temperature and features.

The ripe plantain pulp samples analysed in the current study yielded a mean AFB_1 of 95.25 ppb while the unriped one yielded a mean AFB_1level of 6.6ppb. Okonkwo and Nwokolo (1978) reported a mean AFB_1 of 5.00ppb though the variety was not mentioned but this seems to rhyme with the results obtained in this study for the unripe plantains. The results obtained in the current series is not very surprising though, since the plantain (ripe) seemed over-ripe and were normally thrown down the loaded trucks for the dealers and this practice is likely to encourage the infliction of wounds on the already damaged peels which will encourage the easy invasion of insects/spores into the already softened pulp. To further encourage mould growth and subsequent aflatoxin production, these plantain bunches are normally packed in market stalls for sales where the heat and humidity abounding there will be favorable for such. This category of over ripe plantain is normally cheaper and easily purchased especially for Ukpo Ogede (a steamed –wet milled pudding wrapped in fresh leaves). In this grade of plantain, Oyenuga (1978) showed that the higher availability of reducing sugars in the ripe pulp and the increased moisture content will favour mould growth and aflatoxin production.

In this study, out of the total of 369 *A. flavus* isolates encountered from various foodstuffs analyzed, only 126 (34.1%) were found to be toxigenic using the rice flour liquid medium technique of Mishra and Daradhiyar (1991). This is consistent with previous reports. According to Moss (1996), on a world wide basis, only about 35% of strains of *A. flavus* produced aflatoxins. Magnoli *et al.* (1998) observed that only 47% of the strains encountered in Argentina produced aflatoxins. Pitt (1993) also showed in his

studies that only a small proportion of *A. flavus* produce aflatoxins. The general findings in this study concerning microbial colonization and toxin production stresses the importance of substrates as governing factors in secondary metabolites synthesis. It was observed that the substrate may play a role in selecting for or against toxin producing strains of a given species. According to Ciegler (1975), there is a higher proportion of toxin producing strains of *A. flavus* isolated from peanuts and cotton seeds than from rice and sorghum in his work. Thus, from the current results, out of the total *A. flavus* isolates per food substrate, only a fraction appeared to be toxigenic; with the peanuts having the highest toxigenic isolates (Fig. 12), followed by maize. Consistent with previous reports therefore, different food substrates tend to select for or against the toxigenic strains quite differently from each other. According to Ciegler's findings, factors that influence mould growth on a commodity also influence toxin production, the most important being moisture, type of substrate and temperature. From the current study, the least number of toxigenic strains were encountered in the rainy seasons being followed very closely by the cold harmattan periods, with the highest isolation recorded during the hot dry seasons of the entire research period.

From the results obtained in the analysis of AFB_1 production in rice liquid media by the toxigenic *A.flavus* strains, the amounts ranged between 46.2 to 285.1 µg/ml and is consistent with the findings of Moreno-Romo and Suarez-Fernandez (1986); Bauduret (1990); Mishra and Daradhiyar (1995) and Bsseler *et al* (1995) though the values were much lower than the results of el-Kady *et al*.(1995) in Egypt. The results obtained from the natural occurrence of aflatoxins in the food samples further substantiate the probability of aflatoxin ingestion by the Nigeria people. The high incidence of naturally produced

aflatoxins in these foods illustrates the hazards with which this country is confronted. The actual amounts of aflatoxin levels obtained for most foodstuffs when compared with the tolerance limit of 30ppb decided by the FAO (FAO 1997), leaves no doubt regarding the existence of aflatoxins contamination in the country. Many foodstuffs that did not contain any *A. flavus* at all, or contained only non aflatoxigenic strains of *A.flavus* also yielded appreciable levels of aflatoxins (Table 5). It is probable that the aflatoxigenic *A. flavus* strains involved may have died by the time of the analysis but the aflatoxins themselves were preserved. Some foodstuffs also contained aflatoxigenic strains of *A. flavus* but no aflatoxins were detected in such food items. A lot of factors may account for this observation including the environmental factors, the substrates, as well as the effects of microbial ecosystem. Findings in this study generally show the superiority of lipid foods over all other food classes in terms of aflatoxin formation. This is consistent with observations elsewhere (Moss 1989, Mishra and Daradhiyar 1991; Russell *et al.* 1991; el – Kady *et al.* 1994; Julian *et al.* 1995).

In the ecosystem studies, the experiments show that dual cultures of *Rhizopus* spp and *Saccharomyces* spp.with *A. flavus* either significantly ($P<0.05$) inhibited aflatoxin production as in most cereals, all oil seeds, some carbohydrate foods e.g the plantains, and most protein rich foods, or completely inhibited AF production as in soyabeans, cassava, yam and cocoyam, or had no effects as in acha grains, Ugbawka rice, white cowpeas and breadfruits. *Saccharomyces* spp also had no effects on the two maize varieties.

Rhizopus migricans was found to inhibit growth and AF production by Weckbach and Marth (1977). Zhu *et al.* (1989) were able to decrease hyperplastic and pathological enzymic foci in AFB_1 induced hepatocellular carcinoma in wistar rats fed with *Rhizopus*

spp mixed with rations. *Rhizopus* spp. have also been shown by several workers to be capable of degrading aflatoxins and reducing their inherent toxicity and potential mutagenicity (Bol and Smith 1990; Knol *et al.*1990). Lacey (1989) was also able to suppress the growth of *A. flavus* by interactions with *Rhizopus stolonifer*, while Faraq *et al.* (1993) recorded a 70% aflatoxin degradation when *A. flavus* was in dual cultivation with *Rhizopus oryzae* in maize in Thailand. Zhu *et al.*(1989); Stanley *et al.*(1993) also showed the inhibitory effect of *Saccharomyces* spp. on aflatoxin production by *A. flavus* when in dual culture on peanuts, maize, rice, and also in liquid media.

With *A. niger*, *A. fumigatus* and *Mucor* spp. as co-partners to *A. flavus* each in all food classes analyzed, aflatoxins were completely inhibited except in the oil seeds and ripe plantain where the AF levels were significantly reduced ($P<0.05$). *Mucor* spp. also slightly increased AF levels in white cowpeas while having no effects in breadfruits, acha grains and Ugbawka rice. This finding is consistent with many previous reports. Thus, in a study of low attitude desert cotton fields, soil fungi analyzed for their ability to inhibit *A. flavus* revealed that several fungi including *Fusarium solani. Penicillium* spp. and *Aspergillus* spp. produced compounds inhibitory to *A.flavus* (Klich 1998). Other workers have shown experimentally that interactions of *A.flavus* with other moulds can dramatically influence aflatoxin production and showed the inhibitory effects of *A.niger* for toxin production (Faraq *et al.*1993; Moss 1996).

The lack of detection of aflatoxins in any food item when *Trichoderma* spp. was the partner to *A.flavus* agrees with previous findings (Wicklow 1980; Cuero *et al.* 1987). In the pairing with *Trichoderma* spp. in the current study, there was no visual evidence like sporulation and also the bright greenish yellow fluoresence (B.G.Y.F.) that *A.flavus* was

able to colonize these kernels and seeds. In the pairings with *A. niger*, results of the experiments in this study revealed that both *A.niger* and *A. flavus* grew in harmony on all food substrates as evidenced by the sporulations of both moulds – being about equal to each other. It was therefore noted that both *A. niger* and *Trichoderma* spp interacted quite differently with *A.flavus*. Ciegler *et al*, (1966), observed that occasional degradation or modification of aflatoxins by members of *A. niger* was noted but long incubation, up to 11 days were required. It is therefore likely that *A.niger* in dual culture with *A. flavus* on the food items allowed aflatoxins to form and also the *A.flavus* to grow well, but then degraded the toxins immediately upon formation or graudally as in the oil seeds or might have as well converted them into a modified product with an entirely different Rf value from aflatoxins and so could not be detected by the routine methods in this work.

Studies have shown that *Trichoderma viride* is a strong antagonist of *A.flavus* and could be expected to restrict or prevent the establishment of the later in individual kernels (Wicklow 1980). Ingold (1978) observed that world wide, fungi live as saprophytes on organic matter mainly of vegetable origin and in the soil, and a fairly characteristic flora has been recognized in which species of *Mucor, Penicillium, Aspergillus* and *Trichoderma* are particularly prominent, and observed that *T. viride* is capable of killing the mycelia of other fungi in such ecological niches. Similar observations were also recorded for *A.fumigatus* in dual culture with *A.flavus* on the various food items as observed for *A. niger* and it seems probable that both moulds might have similar mechanisms of action. Ciegler *et al.* (1966) had a similar result with spores of two other *Aspergillus* species – *A. terreus* and *A. luchuensus* which they observed to have absorbed aflatoxins non specifically or partially transformed AFB_1 into a series of pale blue fluorescing compounds

with different Rf values. Lacey (1989) studying interactions of organisms observed that the most competitive or antagonistic species to *A. flavus* included *Penicillium* species, *A. fumigatus* and *A. nidulans* each giving a high I_D (indices of dominance) against *A. flavus* in his experiments. Hill *et al.*(1985) also found that *A. niger* can inhibit aflatoxin production in groundnuts contaminated with aflatoxigenic species of *A. flavus.*

Dual culture experiments of *A. flavus* with each of *Fusarium* spp. and *Rhodotorula* spp, when compared with *A. flavus* in mono-culture in each food stuff in this study, show that these moulds inhibited aflatoxin production by the aflatoxigenic *A. flavus* when grown together on these foodstuff to varying degrees. Studies on the effects of these moulds when in dual cultures with *A. flavus* on peanuts, maize, rice and also in liquid media on aflatoxin production revealed an inhibitory effect (Zhu *et al.* 1989; Stanley *et al.* 1993; Cuero and Osuji 1995; Basappa and Shantha 1996). In the current experiments, the seed crops with the dual cultures of *A.flavus* and *Rhodotorula* spp. were observed to be overtaken by the intense coral pigmentation from the *Rhodotorula* spp. with no visual signs of sporulation by the *A.flavus* was observed. This was mostly noticeable in some protein and carbohydrate foods, though very slight sporulation was observed in the cereals and oil seeds when compared with the observations in these foods with *A.flavus* in mono- culture. It may be possible that the carbon source for the aflatoxin production by *A. flavus* in dual culture with *Rhodotorula* spp. may have been directed towards pigment production.

In the current studies, *Penicillium* spp. either inhibited or stimulated aflatoxin production depending on the food substrate in question when in dual culture with aflatoxigenic *A. flavus* when compared with the toxin levels with *A. flavus* in mono-culture in similar foodstuffs. There was however no effects in the Ugbawka rice for the

cereals, and melon and coconut seeds for the oil seeds. With the protein rich foods, apart from the increase in AF levels recorded for the breadfruits and complete abolishing of AF production in the oil bean seeds, there were generally no differences in the dual culture and mono culture AF levels. In the carbohydrate foods, a significant ($P<0.05$) reduction in AF levels were recorded for the two plantains and yam while same was completely inhibited in cassava and cocoyam. Specifically in the carbohydrate food, AFB_1 production was completely inhibited with a subsequent increase in the AFB_2 levels.

Ciegler *et al.*(1966) observed a reduction in aflatoxin production in cereals and some oil seeds when *Penicillium* spp. was in dual culture with *A flavus.* Kirilenko and Egorova (1985), and Smith *et al.*(1988) observed a complete inhibition of aflatoxin production while Cuero *et al.*. (1987) reported a stimulatory effect with *Penicillium* isolates. Ciegler *et al.*(1966) also observed that *Penicillium* species gave a partial conversion of AFB_1 to acompound that on TLC had an Rf similar to AFB_2.

When compared with *A. flavus* in monoculture, *Geotrichum* spp, *Cladosporium* spp, *Curvularia* spp. and *Candida albicans* each in dual culture with aflatoxigenic *A. flavus* in the foodstuffs sampled, either stimulated or had no obvious effect on aflatoxin production. With the cereal grains, *C. albicans* and *Curvularia* spp. stimulated AF production, *Cladosporium* spp. stimulated same only in the two maize varieties and acha grains while having no effects in the other cereals. There were also no differences in the levels of aflatoxins produced when *A. flavus* was in monospecific culture and when in dual culture with *Geotrichum* spp. in all the cereals.

Cladosporium spp. and *Curvularia* spp. when in dual culutres in the carbohydrates foods slightly increased AF levels in the two plantains and yam tubers while having effects

in the other carbohydrate foods. *Geotrichum* spp. also had no effects in all the carbohydrates foods expect the slight increase recorded in the ripe plantain. *C. albicans* stimulated the production of aflatoxin in all the foodstuffs though with no effects in cassava.

There were no differences in AF levels in the monoculture and dual culture studies in the protein rich foods with *Cladoporium* spp. except the slight increase recorded for the white cowpeas and significant decrease in the breadfruits ($P<0.05$). With *Curvularia* spp, a general stimulation in AF production was observed in these foodstuffs which was significant ($P<0.05$) in the breadfruits and the two pigeon peas.

Geotrichum spp. in dual culture in the protein rich foods, did not have much effects on AF levels except in the breadfruits where AF levels were significantly decreased ($P<0.05$) and the slight decrease in the white cowpeas. However, a slight increase in AF levels were observed in the two pigeon peas and soyabeans. An almost similar trend was followed by *Candida albicans* except that the decrease in the breadfruits was slight while AF levels were also slightly increased in the white cowpeas.

In the oil seeds, AF levels in the two peanut varieties were slightly decrease while being slightly increased in the melon and coconut seeds with *Cladosporium* spp. *Curvularia* spp. generally stimulated AF production in all the oil seeds. The AF levels in the two peanut varieties were unaffected in the dual culture studies with *Geotrichum* spp. and *Candida albicans* while there was a stimulation of same in the other oil seeds. Similar observations of general stimulatory effects by these organisms on aflatoxin production in several types of foodstuffs have also been reported (Wicklow *et al.* 1980; Cuero *et al.* 1987; Lacey 1989; Klich 1998). Wicklow *et al.* (1980), also noted that these fungi in

serveral seed crops including peanuts and maize were less antagonistic than *A.flavus*, and generally did not influence its growth and aflatoxin production.

The bacterial ecosystem studies generally revealed that *Pseudomonas* spp, *Lactobacillus* spp, *Staphylococcus aureus, Streptococcus lactis, Serratia, E. coli* and *Salmonella* species inhibited AF production by toxigenic *A. flavus* or had no effects when in co-culture with this organism. Others also had no effects depending on food type or stimulated AF production. *Pseudomonas* spp, *Proteus* spp and *Bacillus cereus* are all proteolytic bacteria and to an extent also lipolytic according to Frazier and Westerhoff (1991). By virtue of these properties, these bacteria are capable of attacking and metabolising the protein foods and oil seeds as observed in the current study. It is therefore probable that certain metabolites produced in there metabolic processes are inhibitory to either the growth or aflatoxin production by *A. flavus* in these food categories.

Escherichia coli and *Lactobacillus* spp are saccharolytic organisms (Frazier and Westerhoff 1991). According to these workers, these organisms are able to produce acids bye products during the metabolic processes. They are therefore capable of attacking the cereal grains and carbohydrates to produce acid bye products which can be inhibitory to aflatoxins produced in such food substrates. A similar trend was observed for *S. lactis* and *S. faecalis* which Frazier and Westerhoff (1991) have shown to have some proteolytic abilities, producing acid as bye products of proteolytic activities on protein rich substrates. Thus, it is not very surprising that these organisms significantly inhibited aflatoxin production in protein foods in this study ($P<0.05$). *S. lactis* also have some saccharolytic properties according to Frazier and Westerhoff (1991), and so in the current study, this organism also significantly inhibited AF production in the cereal grains as well as the

carbohydrate foods (P< 0.05), when compared with the levels when *A. flavus* in was in monoculture in such foodstuffs. It is possible that acids and other bye products produced in these processes may be inhibitory to AF production. Moss (1989) have demonstrated the inhibitory effects of some metabolites of bacteria and fungi from some foodstuffs on the growth and mycotoxin production of *A. flavus* organisms. *Bacillus subtilis* when in dual culture with *A. flavus* on the cereal grains and some carbohydrate foods stimulated AF production when compared with the AF production in *A. flavus* in monospecific cultures on these foods, with the stimulation in the two varieties of plantain being statistically significant (P<0.05). The oil seeds and protein foods were not affected by this organism, except the two varieties of pigeon peas and some carbohydrate foods (casava, yam and cocoyam) in which the AF production were significantly inhibited (P<0.05) when compared with the levels when *A. flavus* was in monoculture on these food items. According to Frazier and Westerhoff (1991), *B. subtilis* has saccharolytic properties and it is likely that some breakdown products in the cereal grains and plantains may have stimulated AF production while others including acids may have caused some inhibition of the production of aflatoxins in yam, cassava, cocoyam and pigeon peas as observed in this study.

Staphylococcus aureus inhibited AF production in all categories of foodstuffs with the inihibtion in coconut (oil seed) and Abakaliki rice (cereal) being significant (P<0.05), while having no effects in protein foods. *Salmonella* spp. when in dual culture with *A. flavus* significantly inhibited the production of aflatoxins in the protein foods, some cereal grains (acha, and the two rice varieties) and melon seed (P< 0.05) and in the remaining foodstuffs, when compared with the levels when *A. flavus* was in monoculture

in these food items. Kirilenko and Egorova (1985) also observed that *Staphylococcus* spp, *Bacillus cereus* and *Escherichia coli* inhibited AF production in foodstuffs generally. Lacey (1989) noted that with *S. lactis*, inoculation three days before *A. parasiticus* decreased growth of the mould while inoculation afterwards stimulated the growth , though in our studies, the decrease observed was when *S. lactis* and *A. flavus* were inoculated simultaneously. However, according to Coallier-Ascah and Idziak (1985), and Rasik *et al*. (1991). *S. lactis* was found not only inhibiting aflatoxin biosynthesis but also degraded preformed toxins in their own study. This organism also produces lactic acid as one of the end metabolic products and its been widely established that acids can directly degrade aflatoxins and may also catalyse the conversion of AFB_1 to AFB_{2a} (Ciegler *et al.* 1966; Lindenfelser and Ciegler 1970. El – Gendy and Marth 1980, Matsushima *et al* . 1996). Kubo and Kazuhiro (1996) also observed that a *Bacillus subtilis* strain had aflatoxin degrading ability in some food stuffs just as observed in the current studies though Cuero *at al.* (1987) and Lacey (1989) demonstrated that *Bacillus* spp. in rice and maize stimulated Af production. In a study on bacteria-mycotoxins interactions, using the genera *Escherichia, Streptococcus, Staphylococcus, Yersinia, Salmonella , Erysipelothrix* and *Lactobacillus* as test organisms, AFB_1 affected the growth of *Yersinia enterocolitica* only (Ali – Vehmas *et al.* 1998). This shows as illustrated in the current studies, that members of these genera could survive in the presence of competitive growth with *A. flavus* and even affect the growth or production of aflatoxins by the mould. When compared with the Af levels detected in foodstuffs with *A. flavus* in single culture, *A. aceti* stimulated Af production either significantly or non significantly in these foodstuffs. This agrees with Lacey (1989) who observed that the growth of *A. flavus* is stimulated by *A. aceti*. The

major role of acetate – a metabolic product of *Acetobacter aceti* as a starter molecule in the aflatoxin biosynthetic pathway has also been demonstrated (Trail *et al.* 1995; Bennett *et al.* 1997; Minto and Townsend 1997, Sweeny and Dobson 1998).

Also when compared with *A. flavus* in monoculture in semi-synthetic media (YES), *Rhizopus* spp. in dual culture with *A. flavus* , significantly inhibited the production of aflatoxins ($P<0.05$) in all the days of incubation. The mycelial dry weight was also reduced while the pH was lowered to 3.17 by the tenth and fourteenth days of incubation. With *A. niger* in dual culture with *A. flavus*, there was not effects on aflatoxin production till day seven, when it was significantly inhibited ($P < 0.05$) and then completely inhibited by days ten and fourteen of incubation. The mycelial dry weights in the dual culture of the *A. flavus* was also reduced while the pH was also lowered over the days.

With *Trichoderma* spp. in co-culture with *A. flavus*, aflatoxin production was completely inhibited and the mycelial dry weight of the *A. flavus* significantly reduced ($P < 0.05$). *Saccharomyces, Penicillium, Cladosporium,* and *Geotrichum* species all inhibited AF production when compared with *A. flavus* in monospecific culture. *Fusarium,* and *Rhodotorula* species including *A. fumigatus* all significantly reduced both the mycelial dry weight of the *A. flavus* as well as inhibiting AF production significantly ($P < 0.05$) though to a greater extent by *A. fumigatus* and *Fusarium* spp. Curvularia spp. and *Candida albicans* on the other hand stimulated the production of aflatoxins when compared with the AF levels when *A. flavus* was alone in the YES media over the fourteen days of incubation. In both instances also, the mycelial dry weight were also increased. Similar observations were recorded in the tests in the synthetic media except that in the synthetic media tests, *Fusarium* spp. completely inhibited the production of

aflatoxins, while *Curvularia* spp. stimulated Af production only up to day five and then inhibited this thereafter. *Geotrichum* spp. in dual cultures in synthetic medium experiments in comparison with the results in the *A. flavus* monospecific cultures stimulated Af production to day seven of incubation and thereafter inhibited the Af production up to day fourteen. *Candida albicans* on the other hand rather inhibited the production of AF in the synthetic media.

Certain strains of *Rhizopus* spp have been shown to metabolize pre-formed AFB_1 and AFG_1 or possibly convert them to isomeric hydroxy compounds (Bol and Smith 1990; Knol *et al.* 1990). In the dual culture tubes with *A. niger* in the semi synthetic and synthetic media and also from the mycelial dry weights, there were visual evidence of growth of both the *A. flavus* and the *A. niger* though extraction and analysis showed very low levels of aflatoxins when compared with the level in the monospecific culture tubes. This finding may be that the *A. niger* actually degrades aflatoxins as they are formed rather than affect the growth of *A. flavus* in the competitive environment as demonstrated by Ciegler *et al.* (1966) and Faraq *et al.* (1993). In both culture media (Yes and Synthetic), tubes with *Trichoderma* spp. in co-culture with *A, flavus* , there was no visual evidence of the growth of *A. flavus.* According to Wicklow *et al.* (1980) and Ingold (1978), *Trichoderma* spp. have been shown to be very antagonistic towards *A. flavus.* In both media also, *Penicillium* spp. inhibited AFB_1 production either partially or completely while stimulating the production of AFB_2. This is consistent with the reports of Ciegler *et al.* (1966). Tubes with *Rhodotorula* spp. in both media showed no evidence of growth of the *A. flavus* but were rather overtaken by the coral red pigmentation of the *Rhodotorula* spp. It seems probable that the carbon source available for aflatoxin production were

exhausted in the pigment production. However further work to elucidate the details of this finding is needed.

The differences observed in the production of aflatoxins in the YES and synthetic meduim in the dual culture experiments with *Fusarium* spp, *Curvularia* spp and *Candida albicans* may not be unconnected with the nature of the carbon source as well as exhaustion of available nutrients and probably accumulation of toxic wastes that may all affect the production of aflatoxins in the two liquid environments. *Geotrichum* spp. inhibited Af production in the YES medium but rather stimulated the AF production in the synthetic media up to day seven and thereafter inhibited it significantly. It is likely that in the YES medium, *Geotrichum* spp. specifically produced a metabolite that was unfavourable to AF production or growth of *A. flavus* while in the synthetic media, the metabolite accumulated to toxic levels by day ten of incubation.

In a study conducted by Klich (1998), twelve taxa of fungi produced compounds inhibitory to *A. flavus* and these included *Fusarium* and *Penicillium* strains. Lacey (1989) found *Penicillium* and *A. fumigatus* to be highly antagonistic to *A. flavus* in liquid media. Also in the current study, it seems that the production of AFG_1 was retarded by the presence of *Saccharomyces* spp. but the production of AFB_1 was not much affected. The production of more AFB_1 than AFG_1 was expected depending on the favourable pH of the medium (Tables 12 and 13).

For bacterial cultures in dual cultures with *A. flavus* in YES medium (Table 14), when compared with *A. flavus* in monospecific cultures, *B. subtilis, A. aceti, Proteus* spp. and *B. cereus* stimulated AF production by *A. flavus*, while others like *Pseudomonas* spp,

Staphylococcus aureus, E. coli, and *Serratia* spp, inhibited AF production. *Salmonella* spp, and *Lactobacillus* spp however inhibited AF production significantly ($P < 0.05$), while *S. faecalis* had a mixed trend. *S. faecalis* inhibited aflatoxin production up to day five and then stimulated it thereafter up to day fourteen, while *S. lactis* had no effects on aflatoxin production throughout the periods of incubation. Mycelial dry weights were also higher in the sets that stimulated toxin production and was either less or equal to the weight in the monoculture (control) studies in all the sets that inhibited AF production. In the same experiments in the synthetic media, *Proteus* spp, rather inhibited AF production when compared with the levels in the control (ie *A. flavus* in monoculture), *S. lactis* and *S. faecalis* also inhibited AF production. Also *Salmonella* spp. and *Lactobacillus* spp. both significantly inhibited AF production as in the YES experiments.

Differences in some results in the two liquid environments may not be unconnected also with the carbon source or factors available for the *A. flavus* in the two environments in the competitive growth. In the liquid environments and in the presence of *B. cereus, B. subtilis* and *A. aceti,* AF production increased and the growth of *A. flavus* was generally better when both organisms grew together. Results indicate that the increase in total aflatoxins production probably resulted from enhanced growth of *A flavus* as evidenced by the mycelial dry weight. This increase in growth of *A. flavus* could have been caused by initial growth of these bacteria creating conditions which favoured the subsequent growth of the mould. Also according to Faraq *et al,* (1993), *A. aceti* can produce acid from sucrose and in most instances, will produce 5 – ketogluconic acid and gluconic acid which might be utilized for growth by *A. flavus* more readily than sucrose.

A regular pattern of variation in the yield of aflatoxins with age of the *A. flavus* monoculture was observed with different food types. In cereals and carbohydrates foods, it was observed that as the days of fungal culture increase the AF levels generally decreased. According to Maggan *et al.* (1977) decreasing activities of the glycolytic enzymes with culture age may indicate depletion of the carbon source in the medium. The higher activities of the glycolytic enzymes indicate a higher breakdown of glucose for energy and metabolic activities and accumulation of pyruvate which is assumed to be essential for the on set of aflatoxin biosynthesis.

For the oil seeds, biphasic curves were obtained. The variations in aflatoxin content are larger than can be accounted for by any errors arising in the assay procedure. It may probably reflect actual differences in the net content of aflatoxin arising from alterations in the relative rates of synthesis and degradation of these compounds within the culture. The initial drop in aflatoxin content observed on the seventh day to about the tenth day might conceivably be due to a degrading mechanism or possibly also due to breakdown of aflatoxin by an enzyme appearing during this phase of growth of the organism. The appearance of the second peak might be due to an increased production of AF arising as a result of glycerol now being liberated by lipolysis, in addition to a possible exhaustion of the degrading reactions. Ciegler *et al.* (1966) had demonstrated that peroxidized methyl esters could degrade aflatoxins and it is possible that compounds of this type might arise in medium containing unsaturated fatty acids . Arseculeratne *et al.* (1969) also demonstrated that the hydrolysis of neutral fats yields glycerol which has been shown to be good carbon source for growth and aflatoxin production by *A. parasiticus* and *A. flavus* which also have a high content of lipolytic enzymes.

With the protein foods, there was a general steady but gradual increase in AF levels but this dropped by the tenth to the fourteenth day depending on the food crop. In the breadfruits however, AF levels continued to increase unabated up till the fourteenth day of incubation. Details responsible for these observations need further studies. The drop on day ten to fourteen may be related to the exhaustion of the carbon source. Patterns of general increase or decrease in AF levels in the dual culture studies with bacterial and fungal organisms also took a similar pattern as with *A. flavus* in single culture in the entire foodstuffs studied.

In the bivalent metal studies, aflatoxin yields plotted against bivalent metals added 0 – 1000 µg/g of food substrates at seven days incubation, showed that zinc levels between 10 – 400 µg/g of food substrate (cereals) stimulated AF yields gradually with the maximum yield being recorded at metal level 400 µg/g of food substrate. However, the maximum yield for Ugbawka rice was at 250 µg/g of food substrate. Levels of aflatoxins then started to decline thereafter till the highest metal concentrations. There was a stastistically significant difference between the mean AF levels at zero metal concentration and at the levels at the metal concentration (400µg/g) the peak AF yield for the cereals ($P < 0.05$), but not for the Ugbawka rice and acha grains. A similar trend was observed for the carbohydrate foods except that the peak yields were recorded at metal concentration 550 µg/g of food though for the unripe plantain, the drop in AF levels started right from 10 µg/g of food, metal concentration, while the peak for the ripe plantain was recorded at metal level of 250 µg/g of food substrate. The differences in AF levels at zero metal concentration and the levels at peak yield were statistically significant in yam and cassava only ($P < 0.05$).

In the protein foods, AF levels rose sharply though not significantly from the level at zero metal concentration to the level at 10 µg/g of food substrate ($P > 0.05$), though it was significant in white cowpeas ($P < 0.05$) especially for the AFB_1. There was no difference between the levels of AF in the two metal concentrations for soyabeans and breadfruits. For zero µg/g of food substrate metal concentration, the AF levels increased continuously till the peak at metal level of 550 µg/g of food substrate. Thereafter, the AF levels decreased continuously till the highest metal concentration. In all the protein foods, the difference between the levels of AF at zero metal concentration and the peak AF levels at metal concentration of 550 µg/g food substrate was statistically significant ($P < 0.05$). For the oil seeds, a completely different trend was recorded. The AF levels rather decreased continuously with increase in metal concentration with a significant decrease in all oil seeds by the highest metal concentration (100 µg/g) ($P < 0.05$).

The stimulatory effects observed in the zinc experiments in all food substrates apart from oil seeds agree with the works of Faila *et al.* (1986) who observed that AF levels positively correlated with zinc and copper levels in corn simples in USA. Many other reports exist supporting the stimulatory effects of zinc on certain food substrates (Lillehoj *et al.* 1974, Obidoa and Ndubuisi 1981, Stossel 1986, Tiwari *et al.* 1986). Metal ions are involved as enzyme cofactors in many biological processes. Lacey (1989) showed that zinc is required for AF formation and infestation of maize germs may be associated with the increased trace elements levels in the germ fraction. However, the accompanying high levels of phosphate (phytate – inositol – hexaphosphate) in the germ strongly binds several elements particularly zinc and render them biologically unavailable to the fungus. This may be true for all other seeds and food crops. The typical requirements of food

stuffs for zinc salts especially as seen for cereals and carbohydrate foods supports the findings of Maggan *et al.* (1977), that zinc is involved in carbohydrate metabolism in the fungi, and that the glycolytic enzymes of *A. parasiticus* and others are zinc dependent. They also showed that zinc deficiency impaired fungal growth and metabolism of nucleic acids and proteins and activity of enzymes of the glycolytic pathways.

Thus in *A. parasiticus*, Venkitasubramanian and Gupta (1977) and Sweeny and Dobson (1998) noted that an impaired glycolytic cycle tends to reduce the level of primary metabolites such as pyruvate, citrate, and oxaloacetate which tigger off aflatoxin formation and that inorganic phosphates accumulated due to zinc deficiency and may be unfavourable for aflatoxin biosynthesis. The decrease observed at higher zinc levels might be due to an adverse effect of excess zinc on the fungal macromolecular biosynthesis, as reported previously by Gupta *et al.* (1977) and Chulze *et al.* (1987). The higher yields of aflatoxins in the oil seeds at lower Zn^{2+} levels and the serious decrease at higher Zn^{2+} levels may be due to many factors as discussed above, though it does agree with Chulze *et al.* (1987) in their oil seed (sunflower seeds) experiments.

It is also noteworthy that soyabeans which have generally not supported good AF production gave a distinct rise in the level of aflatoxins with increasing zinc levels. Zinc, being an essential trace element for AF production has been used to explain the higher production of aflatoxins on autoclaved soyabeans (Stossel 1986). According to Gupta and Venkitasubramanian (1975), zinc is bound to phytate in soyabeans, but may be released during cooking and therefore become more biologically available. Thus, in this study, the addition of extraneous supply of zinc definitely increased AF production in soyabean; though Stossel (1986) noted that autoclaving soyabean flakes and protein for 30 minutes

destroyed less than 5% of the phytate and that autoclaving the growth, media with and without phytate, apparently did not improve zinc availability, so that other factors must be involved and needs further studies in soyabeans and other tropical foodstuffs. Maggan *et al.* (1977) also showed that zinc deficiency in the medium resulted in altered morphology, growth and metabolism in the fungus, *Rhodotorula gracilis.*

Apparently, from the result in the current series, the optimal availability of zinc is a decisive factor in Af biosynthesis. As Obidoa and Ndubuisi (1981) noted, the resistance and susceptibility of natural food commodities to Af production has been explained in terms of the presence or absence of adequate amounts of trace elements especially zinc. It is a relevant fact that at least 20 zinc dependent enzymes have been detected, all of them being very important in Af biosynthesis. (Tyagi and Venkitasubramanian 1981). Zinc has been observed to stabilize nucleic acids, ribosomes, lysosomes, microtubules and cell membranes in various fungal groups (Maggan et. al. 1977).

Manganese added at 10 µg/g of cereals and carbohydrate foods double or tripled the levels of aflatoxin produced at zero level of metal and continued at such levels with a very slight decrease at the highest metal concentrations. The increase in AF levels in all these foodstuffs were statistically significant ($P < 0.05$). However instead of decreasing as observed generally at higher metal concentrations, AF levels were rather increased in unripe plantain after 100 µg/g metal per food substrate. The Ugbawka rice was more significantly stimulated at 10 µg/g of food by Mn^{2+} more than that of Abakakili rice ($P < 0.05$) while there was no difference with the two varieties of sorghum. For the protein foods, a significant inhibition was recorded in aflatoxin production ($P < 0.05$) at metal

levels concentration 10μg/g of food and this continued till metal concentration 100μg/g of food, and then started increasing thereafter with increasing metal concentration with a significant stimulation by the highest metal concentration ($P < 0.05$), when compared with the level of AF at zero metal levels.

However, there were no differences in AF production at all metal concentrations for soyabeans, while in the breadfruits, AF concentration gradually increased with increasing metal concentration. With the oil seeds, AF levels were high at zero metal levels and then decreased significantly in all the oil seeds ($P < 0.05$) with the exception of the white peanuts at metal level 10 μg/g of food. AF levels then started increasing gradually thereafter with increasing metal concentration from metal level 25 μg/g of food substrate. Agreeing with the current findings for cereals and carbohydrate foods, Lillehoj *et al.* (1974) also observed that the synthesis of aflatoxin on defatted germ of corn was increased by the addition of managanese at 10 μg/g of foodstuff, with no significant inhibition after the addition of 10 – 500 μg per gram of the corn germ.

Managanese appears to be a multifunctional metal in the metabolism of numerous fungal and bacterial systems being involved in cell wall synthesis as well as nucleic acid and fatty acid synthesis (Maggan *et al.* 1977). A mixed trend was observed depending on salt concentrations with Mn^{2+}in the works of Tiwari *et al.*(1986) and this supports the different results at different concentrations in different foodstuffs obtained in the current study. However some conflicting previous results exist. Mateles and Adye (1965), Lee *et al.* (1966) and Detroy *et al.* (1971) did not observe any effects of Mn^{2+} on aflatoxin production though they noted some slight reduction in growth of the organisms in some instances. They also observed in some of their experiments that Mn^{2+} deficiency caused

incomplete or altered cell wall synthesis in *A. parasiticus* resulting in a change of fungal morphology to a yeast-like form.

At copper levels of 10 µg/g of the cereals and the carbohydrate foods analysed in this study, aflatoxin levels were significantly stimulated when compared with the Af levels at zero Cu^{2+} concentration ($P < 0.05$). This increase continued gradually reaching its peak at copper levels of 250 µg/g of the food substrate after which a gradual decrease in Af concentration started, becoming significant at Cu^{2+} concentrations of 550 µg/g of food substrate when compared with the peak concentrations ($P < 0.05$). Thereafter, the decrease in Af levels continued gradually till the highest metal concentration and even being completely inhibited in many of the carbohydrate foods. No varietal differences were observed in the stimulation of Af production at Cu^{2+} levels of 10 µg/g food substrate among all the carbohydrate foods and cereals (Colour, method of cultivation, ripe or unripe). For the protein foods however, at copper levels of 10 µg/g of food, a slight stimulation of Af production was observed when compared with the levels at zero metal concentration but this was however, significant for breadfruits ($P < 0.05$). A slight decrease in Af concentration or production was then recorded thereafter till the highest metal concentration per gram of food substrate. This decrease was significant again for breadfruits and both cowpeas varieties when AF levels at metal concentrations of 50 µg/g and 100 µg/g of food substrates were compared since there was another sharp drop in Af levels at this point ($P < 0.05$). Also after metal levels of 550 ug/g of food, a complete inhibition in Af production was recorded for the protein foods with the exception of bambara peanuts and breadfruits where some traces of aflatoxins could still be detected.

For the oil seeds, at copper levels of 10 µg/g of the food substrate, AF production were increased for both peanut varieties and melon seeds though significantly for coconut seeds ($P < 0.05$). Thereafter a decrease in AF levels were recorded for the melon seeds and coconut seeds but significantly in the two peanuts ($P<0.05$). Interestingly too, the production of aflatoxins became completely inhibited at copper levels of 550 µg/g of food substrate till the highest Cu^{2+} concentration, while for the melon seeds, AF levels remained constant till the highest Cu^{2+} concentration. In the coconut seeds however, after the decrease at Cu^{2+} level of 550 µg/g of the food substrates, a slight increase in AF level occurred again at metal level of 700 µg/g of food and remained constant till the highest Cu^{2+} concentration.

The findings in the current study agrees with Lillehoj *et al.* (1974) in maize. Failla *et al.* (1986) also noted that AF levels positively correlated with Zn^{2+} and Cu^{2+} levels in maize, but in the absence of biochemical data linking copper levels with polyketide synthesis, they believe that this correlation may be incidental. The inhibition of AF production at highest levels of Cu^{2+} (> 550) µg/g of these food substrates analysed may be because, copper as one of the heavy metals like mercury and silver are known to be fungicidal at higher concentrations.

Apparently, from the results in this study, iron does not seem to have any effect on AF production in cereals and carbohydrate foods at all levels of concentration. This is in line with the findings of Lillehoj *et al.* (1974) who also observed no effects on the AF levels in maize. For the protein foods in the current study, a slight increase in AF production and level was observed at Fe^{2+} concentration of 10 µg/g of the foodstuff when compared with the AF levels at zero Fe^{2+} level for bambara peanuts,

soyabeans ,both cowpea and pigeon pea varieties. However, this increase was significant for breadfruits ($P < 0.05$). Thereafter, a significant ($P < 0.05$) decrease in AF levels was recorded down the Fe^{2+} concentrations to the highest levels and becoming completely inhibited in the cowpeas and soyabeans at Fe^{2+} levels of $\geq$ 400 µg/g of food substrate with only traces in the other foods. By Fe^{2+} level 1000 µg/g of breadfruits, AF production was completely inhibited.

For the oil seeds, a completely different pattern was observed. At Fe^{2+} levels of 10 µg/g of the oil seeds, a significant increase in AF levels were recorded as against the levels at zero Fe^{2+} ($P < 0.05$). Thereafter, a decrease was recorded in aflatoxin levels with the greatest decrease at Fe^{2+} levels of 250 µg/g of white peanuts, red peanuts and melon seeds but at Fe^{2+} levels of 400 µg/g of coconut seeds. Thereafter again, a slight increase occurred in AF levels which remained more or less constant till the highest Fe^{2+} levels. Tiwari *et al.* (1986) and Maggon *et al.* (1977) using peanut fractions observed that iron and copper decrease AF production to certain levels.

From the studies in the YES and synthetic media, using the bivalent metals, the fact that at Zn^{2+} levels 1.0 µg/ml of YES media (Table 17) the AF production was increased about 1000 times when compared with the AF level at zero zinc concentration, whereas mat weight was increased only 2.5 times suggest in accordance with previous studies in submerged liquid media (synthetic and non synthetic) (Lee *et al.* 1966; Marsh *et al.* 1975), that zinc has a stimulatory effect or influence on AF production apart from its essentiality for overall growth. In general also, in the current study, in the two liquid media, $AFB_1 > G_1 > B_2 > G_2$ throughout all zinc concentration is consistent with most other works using liquid media (YES and synthetic) and also food substrates (rice, maize,

beans and peanuts) (Lee *et al* . 1966; Marsh *et al.* 1975; Moss 1989; Lacey 1989). The observed depression of AF yield at 25 µg/ml of YES and the synthetic media in the current work, agrees with Chulze *et al*. (1987), that Zn^{2+} could be a stimulating factor in the AF production up to a certain concentration, as it happens with all essential elements. Also from the study, there seems to be a clear distinction in the way *A. flavus* responded to zinc addition and other bivalent metals in the different food substrates studied as compared with the response in the liquid media. According to Chulze *et al.*(1987), the importance of the substrate and strain in toxin production is remarkable. In their sunflower seed experiments, the influence of zinc was not so evident as was observed in the synthetic media. It is evident that in the solid substrates (food items) various factors besides bivalent metals like zinc interact to promote AF biosynthesis.

With the addition of Mn^{2+} at 5 µg/ml of the synthetic medium, and of YES medium, aflatoxin levels increased to their highest levels (Table 17). A decrease again occurred at Mn^{2+} levels of 25.0 µg/ml of the synthetic medium but not in the YES medium. The fact that the mycelial mat weights of the two liquid media remained essentially on the increase throughout – from 230mg/100ml at Mn^{2+} zero level to 233 mg/100ml at Mn^{2+} level of 10.0µg/ml of the synthetic media, and from 248 mg/100ml at zero level of Mn^{2+} to 256 µg/100ml at Mn^{2+} level of 10.0 µg/ml of the YES media, points to the fact that Mn^{2+} is essential for growth rather than for AF production. The mycelial dry weights in both media were however lowered at Mn^{2+} levels of 25.0 µg/ml of media – in the synthetic medium, dropping back to 230 mg/100ml and in the YES 250 mg/100ml. However, the results conflict with Marsh *et al*. (1975) who observed a constant mycelial mass weight throughout their Mn^{2+} experiments in synthetic media. Maggon *et al.*(1977) observed

that Mn^{2+} can be interchanged as cofactors for a number of enzymes as well as in the production of ATP.

Although this study and some other previous ones (Lee *et al*.1966; Marsh *et al*. 1975, Maggon *et al*.1977), have shown that Af yields in liquid media fermentations can be restricted by low levels of manganese or give lower yields, the synthesis of Af in some food substrates (cereals, carbohydrates and oil seeds) was increased by the addition of 10µg of the Mn^{2+} per gram of food with no real inhibition, after the addition of up to 1000µg of the Mn^{2+} per gram of the food substrate. Clearly, the response of the *A. flavus* to manganese differs in the two media (food substrates versus liquid media). A similar observation was also made by Chulze *et al*. (1987).

With iron at zero level in both the synthetic and YES media, Af production was relatively high though the mycelial mat weight were lower than in the higher Fe^{2+} concentrations. Thereafter, Af levels decreased with increasing Fe^{2+} levels. The mycelial dry weights increased at Fe^{2+} levels of 1.0µg/ml of both the synthetic and YES media and fluctuated around a constant value throughout (Table 17). From the results, iron definitely had a stimulatory effect on growth but may not be affecting Af production. Copper, according to the observations in the current studies, depressed Af production in both liquid media (the synthetic and YES media), though the mycelial dry weight of the *A. flavus* was more or less constant, having no actual differences in their values at different Cu^{2+} levels. By the Cu^{2+} levels at 10.0µg – 25µg/ml of both the synthetic media and YES media, Af production was completely inhibited with only traces of Af being detected at Cu^{2+} levels of 1 – 5 µg/ml of both media. The fungicidal action of copper has already

been emphasized, but the lack of correlation between this and the mycelial dry weight makes the explanation more complicated.

The general decrease of AF levels between seven and fourteen days of incubation in both the food substrates and the liquid media was in accordance with literature (Goldblatt 1969; Arseculeratre and Bandunatha 1972; Sweeny and Dobson 1998). In laboratory studies also, differences in toxin production by *A. flavus* have been attributed to heterogeneity of the fungal substrate. Synthetic media have routinely supported minimal toxin production whereas maximum yields occur in such commodities as autoclaved wheat, rice, corn among others (Detroy *et al.* 1971).

According to Reddy *et al.* (1971), the yields of AF are generally low in synthetic media normally used for fungal growth but AF production is higher in crude or synthetic media supplemented with crude extracts like yeast extract (YES medium). It is not therefore surprising that in the current studies, high levels of AF yield were recorded in solid food substrates, followed in a decreasing order by YES medium and lastly the synthetic basal medium. Chulze *et al.* (1987) though, recorded higher levels in sucrose low salts medium (SLS) contrasting with sunflower seeds which he observed had much more complex carbon and nitrogen sources.

Many other previous studies abound, demonstrating a relationship between the addition of several metals and significant differences in toxin yields (Lacey 1989; D'souza and Brackett 1998; Schiller *et al.* 1998). They have also described an enzyme elastinolytic proteinase of *A. flavus* which activity is markedly inhibited by numerous metal ions.

CHAPTER 6

6.0 CONCLUSION

Major factors therefore based on the findings in this work found to influence mould growth on a food commodity and toxin production are summarized as follows:

a) **Seasonality factors:-** Mould growth and aflatoxin production was directly correlated with the time of the year. The rainy season favoured both factors most and the cold harmattan periods favoured them least.

b) **Type of substrate:-** whether oil seeds, protein-rich foods, carbohydrate foods or cereal grains. Results showed that oil seeds supported highest aflatoxin yields, while cereal grains supported greater microbial contamination. The results also showed that substrates may also play a role in selecting for or against toxin producing strains of *A.flavus.*

c) **Agricultural practices:-** whether upland or swamp, where seed crops are faced with either drought or moisture stress as typified by the Ugbawka and Abakaliki rice.

d) **Varieties of seed crops:-** eg Red and white testae peanuts (ie colour variations) or state of the seed crop (eg ripe or unripe plantains).

e) **Growth environment:-** The study showed that seed crops associated with the soil environment eg. peanuts,yam,cocoyam, melon, breadfruits, produced largely the B and G aflatoxins, while those growing above the ground environment, produced largely the B toxins eg, maize, sorghum, millet, acha etc.

f) **Microbial interactions:-** Findings in this study suggest that the successful establishment of *A.flavus* in a food substrate and subsequent aflatoxin contamination, may depend upon the

sequence in which the mould reaches the seed crop in relation to their colonization by other fungi or bacteria. The results indicate the great variability in Af production by *A.flavus* in a competitive environment, even when the substrate is favourable for mould growth and aflatoxin production. It also probably indicates the potential importance of co-occurring fungal/bacterial species in determining which biosynthetic pathways are available to the *Aspergillus flavus*. Thus, a great variability has been observed in Af production and growth of the *A.flavus* during the competitive growth with other fungi and bacteria, but this effect is also largely dependent on the seed crop in question.

g) **Influence of metal ions ie metal salts:-** depending largely on concentration and nature of food crop.

It is therefore hoped that the application of the less harmful microbial strains shown to inhibit or decrease Af production eg. *Saccharomyces* spp, as active ingredients in a livestock feed product or as a microbial consortium in human rations may assist an eradication goal.

Conclusively therefore, results from the current study show that the susceptibility of agricultural commodities to aflatoxin contamination varies from crop to crop (ie its variety, state, and method of cultivation), and from season to season, being largely influenced by the microbial fauna associated with the crop, and this may be affected by the availability of certain metal ions.

Thus, the presence of aflatoxins in agricultural commodities represent an extremely complex series of interactions between:

1. The causative fungi (*A.flavus* group)
2. The contaminated product and
3. The physicochemical environmental factors.

The various components involved still need to be unravelled further; at best, will probably never be fully understood since laboratory studies, where interacting variables are of necessity limited, can only approximate the infinite number of variables manifested in nature eg.

(1) the chemical composition of the commodity.

(2) the complex interactions of the microbial fauna and

(3) weather conditions.

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	X Labels	A	B	C
	Parameter	Value	Data Set-B	Data Set-C
	X	Y	Y	Y
1	Table Analyzed			
2	Data Table-38			
3	One-way analysis of variance			
4	P value	P<0.0001		
5	P value summary	****		
6	Are means signif. different? (P < 0.05)	Yes		
7	Number of groups	8		
8	F	72210000000000		
9	R squared	1.000		
10				
11	Bartlett's test for equal variances			
12	Bartlett's statistic (corrected)			
13	P value			
14	P value summary	ns		
15	Do the variances differ signif. (P < 0.05)	No		
16				
17	ANOVA Table	SS	df	MS
18	Treatment (between columns)	574.7	7	82.10
19	Residual (within columns)	0.000000000091	80	0.000000000001
20	Total	574.7	87	
21				
22	Tukey's Multiple Comparison Test	Mean Diff.	q	P value
23	Column A vs Column B	-1.030	3204000	P < 0.001
24	Column A vs Column C	3.200	9954000	P < 0.001
25	Column A vs Column D	-1.750	5444000	P < 0.001
26	Column A vs Column E	2.520	7839000	P < 0.001
27	Column A vs Column F	-2.550	7932000	P < 0.001
28	Column A vs Column G	-2.650	8243000	P < 0.001
29	Column A vs Column H	-4.950	15400000	P < 0.001
30	Column B vs Column C	4.230	13160000	P < 0.001
31	Column B vs Column D	-0.7200	2240000	P < 0.001
32	Column B vs Column E	3.550	11040000	P < 0.001
33	Column B vs Column F	-1.520	4728000	P < 0.001
34	Column B vs Column G	-1.620	5039000	P < 0.001
35	Column B vs Column H	-3.920	12190000	P < 0.001
36	Column C vs Column D	-4.950	15400000	P < 0.001
37	Column C vs Column E	-0.6800	2115000	P < 0.001
38	Column C vs Column F	-5.750	17890000	P < 0.001
39	Column C vs Column G	-5.850	18200000	P < 0.001
40	Column C vs Column H	-8.150	25350000	P < 0.001
41	Column D vs Column E	4.270	13280000	P < 0.001
42	Column D vs Column F	-0.8000	2488000	P < 0.001
43	Column D vs Column G	-0.9000	2800000	P < 0.001
44	Column D vs Column H	-3.200	9954000	P < 0.001

Iron effects on cereals. Appendix Ia

	X Labels	A	B	C	D
	Parameter	Value	Data Set-B	Data Set-C	Data Set-D
	X	Y	Y	Y	Y
1	Table Analyzed				
2	Data Table-28				
3	One-way analysis of variance				
4	P value	0.0470			
5	P value summary	*			
6	Are means signif. different? (P < 0.05)	Yes			
7	Number of groups	7			
8	F	2.264			
9	R squared	0.1625			
10					
11	Bartlett's test for equal variances				
12	Bartlett's statistic (corrected)	81.46			
13	P value	P<0.0001			
14	P value summary	***			
15	Do the variances differ signif. (P < 0.05)	Yes			
16					
17	ANOVA Table	SS	df	MS	
18	Treatment (between columns)	660.5	6	110.1	
19	Residual (within columns)	3404	70	48.63	
20	Total	4065	76		
21					
22	Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
23	Column A vs Column B	0.8345	0.3969	P > 0.05	-8.210 to 9.880
24	Column A vs Column C	0.2800	0.1332	P > 0.05	-8.765 to 9.325
25	Column A vs Column D	0.5682	0.2702	P > 0.05	-8.477 to 9.613
26	Column A vs Column E	-0.1109	0.05275	P > 0.05	-9.156 to 8.934
27	Column A vs Column F	3.132	1.490	P > 0.05	-5.913 to 12.18
28	Column A vs Column G	-7.065	3.360	P > 0.05	-16.11 to 1.980
29	Column B vs Column C	-0.5545	0.2637	P > 0.05	-9.600 to 8.490
30	Column B vs Column D	-0.2664	0.1267	P > 0.05	-9.311 to 8.779
31	Column B vs Column E	-0.9455	0.4497	P > 0.05	-9.990 to 8.100
32	Column B vs Column F	2.297	1.093	P > 0.05	-6.748 to 11.34
33	Column B vs Column G	-7.900	3.757	P > 0.05	-16.94 to 1.145
34	Column C vs Column D	0.2882	0.1371	P > 0.05	-8.757 to 9.333
35	Column C vs Column E	-0.3909	0.1859	P > 0.05	-9.436 to 8.654
36	Column C vs Column F	2.852	1.356	P > 0.05	-6.193 to 11.90
37	Column C vs Column G	-7.345	3.494	P > 0.05	-16.39 to 1.700
38	Column D vs Column E	-0.6791	0.3230	P > 0.05	-9.724 to 8.366
39	Column D vs Column F	2.564	1.219	P > 0.05	-6.481 to 11.61
40	Column D vs Column G	-7.634	3.631	P > 0.05	-16.68 to 1.411
41	Column E vs Column F	3.243	1.542	P > 0.05	-5.802 to 12.29
42	Column E vs Column G	-6.955	3.308	P > 0.05	-16.00 to 2.090
43	Column F vs Column G	-10.20	4.850	P < 0.05	-19.24 to -1.152

Iron effects in protein foods. Appendix 1b

	X Labels	A	B	C	D
	Parameter	Value	Data Set-B	Data Set-C	Data Set-D
	X	Y	Y	Y	Y
1	Table Analyzed				
2	Data Table-29				
3	One way analysis of variance				
4	P value	0.4320			
5	P value summary	ns			
6	Are means signif. different? (P < 0.05)	No			
7	Number of groups	4			
8	F	0.9347			
9	R squared	0.05991			
10					
11	Bartlett's test for equal variances				
12	Bartlett's statistic (corrected)	10.81			
13	P value	0.0128			
14	P value summary	*			
15	Do the variances differ signif. (P < 0.05)	Yes			
16					
17	ANOVA Table	SS	df	MS	
18	Treatment (between columns)	7984	3	2661	
19	Residual (within columns)	125300	44	2847	
20	Total	133300	47		
21					
22	Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
23	Column A vs Column B	25.30	1.573	P > 0.05	-35.52 to 86.12
24	Column A vs Column C	17.07	1.139	P > 0.05	-39.55 to 73.69
25	Column A vs Column D	-8.052	0.5005	P > 0.05	-68.87 to 52.77
26	Column B vs Column C	-8.233	0.5497	P > 0.05	-64.85 to 48.39
27	Column B vs Column D	-33.35	2.073	P > 0.05	-94.17 to 27.47
28	Column C vs Column D	-25.12	1.677	P > 0.05	-81.74 to 31.50

Iron effects in oil seeds. Appendix Ic

	X Labels	A	B	C	D
	Parameter	Value	Data Set-B	Data Set-C	Data Set-D
	X	Y	Y	Y	Y
1	Teble Analyzed				
2	Data Table-30				
3	One-way analysis of variance				
4	P value	0.1085			
5	P value summary	ns			
6	Are means signif. different? (P < 0.05)	No			
7	Number of groups	8			
8	F	1.750			
9	R squared	0.1286			
10					
11	Bartlett's test for equal variances				
12	Bartlett's statistic (corrected)	7.254			
13	P value	0.4029			
14	P value summary	ns			
15	Do the variances differ signif. (P < 0.05)	No			
16					
17	ANOVA Table	SS	df	MS	
18	Treatment (between columns)	1262	7	180.2	
19	Residual (within columns)	8546	83	103.0	
20	Total	9808	90		
21					
22	Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
23	Column A vs Column B	-3.290	1.138	P > 0.05	-16.04 to 9.462
24	Column A vs Column C	2.910	1.007	P > 0.05	-9.842 to 15.66
25	Column A vs Column D	-3.977	1.376	P > 0.05	-16.73 to 8.776
26	Column A vs Column E	2.701	0.9342	P > 0.05	-10.05 to 15.45
27	Column A vs Column F	-6.999	2.421	P > 0.05	-19.75 to 5.753
28	Column A vs Column G	-3.699	1.280	P > 0.05	-16.45 to 9.053
29	Column A vs Column H	-7.599	2.629	P > 0.05	-20.35 to 5.153
30	Column B vs Column C	6.200	2.026	P > 0.05	-7.296 to 19.70
31	Column B vs Column D	-0.6864	0.2243	P > 0.05	-14.18 to 12.81
32	Column B vs Column E	5.991	1.958	P > 0.05	-7.505 to 19.49
33	Column B vs Column F	-3.709	1.212	P > 0.05	-17.20 to 9.787
34	Column B vs Column G	-0.4091	0.1337	P > 0.05	-13.90 to 13.09
35	Column B vs Column H	-4.309	1.408	P > 0.05	-17.80 to 9.187
36	Column C vs Column D	-6.886	2.251	P > 0.05	-20.38 to 6.609
37	Column C vs Column E	-0.2091	0.06834	P > 0.05	-13.70 to 13.29
38	Column C vs Column F	-9.909	3.239	P > 0.05	-23.40 to 3.587
39	Column C vs Column G	-6.609	2.160	P > 0.05	-20.10 to 6.887
40	Column C vs Column H	-10.51	3.435	P > 0.05	-24.00 to 2.987
41	Column D vs Column E	6.677	2.182	P > 0.05	-6.818 to 20.17
42	Column D vs Column F	-3.023	0.9880	P > 0.05	-16.52 to 10.47
43	Column D vs Column G	0.2773	0.09063	P > 0.05	-13.22 to 13.77
44	Column D vs Column H	-3.623	1.184	P > 0.05	-17.12 to 9.873
45	Column E vs Column F	-9.700	3.170	P > 0.05	-23.20 to 3.796
46	Column E vs Column G	-6.400	2.092	P > 0.05	-19.90 to 7.096
47	Column E vs Column H	-10.30	3.367	P > 0.05	-23.80 to 3.196
48	Column F vs Column G	3.300	1.079	P > 0.05	-10.20 to 16.80
49	Column F vs Column H	-0.6000	0.1961	P > 0.05	-14.10 to 12.90
50	Column G vs Column H	-3.900	1.275	P > 0.05	-17.40 to 9.596

	X Labels	A	B	C	D
	Parameter	Value	Data Set-B	Data Set-C	Data Set-D
	X	Y	Y	Y	Y
1	Table Analyzed				
2	Data Table-31				
3	One-way analysis of variance				
4	P value	0.0071			
5	P value summary	**			
6	Are means signif. different? (P < 0.05)	Yes			
7	Number of groups	7			
8	F	3.251			
9	R squared	0.2180			
10					
11	Bartlett's test for equal variances				
12	Bartlett's statistic (corrected)	53.81			
13	P value	P<0.0001			
14	P value summary	***			
15	Do the variances differ signif. (P < 0.05)	Yes			
16					
17	ANOVA Table	SS	df	MS	
18	Treatment (between columns)	667.8	6	111.3	
19	Residual (within columns)	2396	70	34.23	
20	Total	3064	76		
21					
22	Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
23	Column A vs Column B	1.191	0.6751	P > 0.05	-6.398 to 8.780
24	Column A vs Column C	0.9364	0.5308	P > 0.05	-6.652 to 8.525
25	Column A vs Column D	0.8636	0.4896	P > 0.05	-6.725 to 8.452
26	Column A vs Column E	0.2991	0.1695	P > 0.05	-7.290 to 7.888
27	Column A vs Column F	4.327	2.453	P > 0.05	-3.261 to 11.92
28	Column A vs Column G	-6.255	3.546	P > 0.05	-13.84 to 1.334
29	Column B vs Column C	-0.2545	0.1443	P > 0.05	-7.843 to 7.334
30	Column B vs Column D	-0.3273	0.1855	P > 0.05	-7.916 to 7.261
31	Column B vs Column E	-0.8918	0.5055	P > 0.05	-8.481 to 6.697
32	Column B vs Column F	3.136	1.778	P > 0.05	-4.452 to 10.73
33	Column B vs Column G	-7.445	4.221	P > 0.05	-15.03 to 0.1432
34	Column C vs Column D	-0.07273	0.04123	P > 0.05	-7.661 to 7.516
35	Column C vs Column E	-0.6373	0.3613	P > 0.05	-8.226 to 6.951
36	Column C vs Column F	3.391	1.922	P > 0.05	-4.198 to 10.98
37	Column C vs Column G	-7.191	4.076	P > 0.05	-14.78 to 0.3978
38	Column D vs Column E	-0.5645	0.3200	P > 0.05	-8.153 to 7.024
39	Column D vs Column F	3.464	1.963	P > 0.05	-4.125 to 11.05
40	Column D vs Column G	-7.118	4.035	P > 0.05	-14.71 to 0.4705
41	Column E vs Column F	4.028	2.283	P > 0.05	-3.561 to 11.62
42	Column E vs Column G	-6.554	3.715	P > 0.05	-14.14 to 1.035
43	Column F vs Column G	-10.58	5.999	P < 0.01	-18.17 to -2.993

	X Labels	A	B	C	D
	Parameter	Value	Data Set-B	Data Set-C	Data Set-D
	X	Y	Y	Y	Y
1	Table Analyzed				
2	Data Table-32				
3	One-way analysis of variance				
4	P value	0.0010			
5	P value summary	**			
6	Are means signif. different? (P < 0.05)	Yes			
7	Number of groups	4			
8	F	6.574			
9	R squared	0.3302			
10					
11	Bartlett's test for equal variances				
12	Bartlett's statistic (corrected)	9.021			
13	P value	0.0290			
14	P value summary	*			
15	Do the variances differ signif. (P < 0.05)	Yes			
16					
17	ANOVA Table	SS	df	MS	
18	Treatment (between columns)	92600	3	30870	
19	Residual (within columns)	187800	40	4695	
20	Total	280400	43		
21					
22	Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
23	Column A vs Column B	25.48	1.233	P > 0.05	-52.84 to 103.8
24	Column A vs Column C	-28.34	1.372	P > 0.05	-106.7 to 49.99
25	Column A vs Column D	-97.35	4.712	P < 0.01	-175.7 to -19.02
26	Column B vs Column C	-53.82	2.605	P > 0.05	-132.1 to 24.51
27	Column B vs Column D	-122.8	5.945	P < 0.001	-201.2 to -44.50
28	Column C vs Column D	-69.01	3.340	P > 0.05	-147.3 to 9.315

Copper effects in oil seeds. Appendix 2C

	X Labels	A	B	C	D
	Parameter	Value	Data Set-B	Data Set-C	Data Set-D
	X	Y	Y	Y	Y
1	Table Analyzed				
2	Data Table-33				
3	One-way analysis of variance				
4	P value	P<0.0001			
5	P value summary	***			
6	Are means signif. different? (P < 0.05)	Yes			
7	Number of groups	5			
8	F	10.95			
9	R squared	0.4669			
10					
11	Bartlett's test for equal variances				
12	Bartlett's statistic (corrected)	33.12			
13	P value	P<0.0001			
14	P value summary	***			
15	Do the variances differ signif. (P < 0.05)	Yes			
16					
17	ANOVA Table	SS	df	MS	
18	Treatment (between columns)	2159	4	539.9	
19	Residual (within columns)	2466	50	49.31	
20	Total	4625	54		
21					
22	Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
23	Column A vs Column B	2.664	1.258	P > 0.05	-5.823 to 11.15
24	Column A vs Column C	-15.03	7.097	P < 0.001	-23.51 to -6.541
25	Column A vs Column D	0.01818	0.008587	P > 0.05	-8.468 to 8.504
26	Column A vs Column E	-4.455	2.104	P > 0.05	-12.94 to 4.032
27	Column B vs Column C	-17.69	8.355	P < 0.001	-26.18 to -9.205
28	Column B vs Column D	-2.645	1.249	P > 0.05	-11.13 to 5.841
29	Column B vs Column E	-7.118	3.362	P > 0.05	-15.60 to 1.368
30	Column C vs Column D	15.05	7.106	P < 0.001	6.559 to 23.53
31	Column C vs Column E	10.57	4.993	P < 0.01	2.086 to 19.06
32	Column D vs Column E	-4.473	2.112	P > 0.05	-12.96 to 4.014

Copper effects in carbohydrate foods. Appendix 2d

	A Labels	A	B	C	D
	Parameter	Value	Data Set-B	Data Set-C	Data Set-D
	X	Y	Y	Y	Y
1	Table Analyzed				
2	Data Table-34				
3	One-way analysis of variance				
4	P value	0.0871			
5	P value summary	ns			
6	Are means signif. different? (P < 0.05)	No			
7	Number of groups	8			
8	F	1.861			
9	R squared	0.1400			
10					
11	Bartlett's test for equal variances				
12	Bartlett's statistic (corrected)	232.9			
13	P value	P<0.0001			
14	P value summary	****			
15	Do the variances differ signif. (P < 0.05)	Yes			
16					
17	ANOVA Table	SS	df	MS	
18	Treatment (between columns)	11270	7	1610	
19	Residual (within columns)	69210	80	865.2	
20	Total	80480	87		
21					
22	Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
23	Column A vs Column B	-2.466	0.2780	P > 0.05	-41.62 to 36.69
24	Column A vs Column C	3.759	0.4239	P > 0.05	-35.40 to 42.91
25	Column A vs Column D	-5.009	0.5648	P > 0.05	-44.16 to 34.16
26	Column A vs Column E	-5.682	0.6407	P > 0.05	-44.84 to 33.47
27	Column A vs Column F	-32.35	3.647	P > 0.05	-71.50 to 6.809
28	Column A vs Column G	-7.218	0.8139	P > 0.05	-46.37 to 31.94
29	Column A vs Column H	-21.77	2.455	P > 0.05	-60.93 to 17.38
30	Column B vs Column C	6.225	0.7019	P > 0.05	-32.93 to 45.38
31	Column B vs Column D	-2.543	0.2868	P > 0.05	-41.70 to 36.61
32	Column B vs Column E	-3.216	0.3626	P > 0.05	-42.37 to 35.94
33	Column B vs Column F	-29.88	3.369	P > 0.05	-69.03 to 9.275
34	Column B vs Column G	-4.752	0.5359	P > 0.05	-43.91 to 34.40
35	Column B vs Column H	-19.31	2.177	P > 0.05	-58.46 to 19.85
36	Column C vs Column D	-8.768	0.9887	P > 0.05	-47.92 to 30.39
37	Column C vs Column E	-9.441	1.065	P > 0.05	-48.60 to 29.71
38	Column C vs Column F	-36.10	4.071	P > 0.05	-75.26 to 3.050
39	Column C vs Column G	-10.98	1.238	P > 0.05	-50.13 to 28.18
40	Column C vs Column H	-25.53	2.879	P > 0.05	-64.69 to 13.62
41	Column D vs Column E	-0.6727	0.07586	P > 0.05	-39.83 to 38.48
42	Column D vs Column F	-27.34	3.082	P > 0.05	-66.49 to 11.82
43	Column D vs Column G	2.209	0.2491	P > 0.05	-41.36 to 36.95
44	Column D vs Column H	-16.76	1.890	P > 0.05	-55.92 to 22.39
45	Column E vs Column F	-26.66	3.007	P > 0.05	-65.82 to 12.49
46	Column E vs Column G	-1.536	0.1732	P > 0.05	-40.69 to 37.62
47	Column E vs Column H	-16.09	1.814	P > 0.05	-55.25 to 23.06
48	Column F vs Column G	25.13	2.833	P > 0.05	-14.03 to 64.28
49	Column F vs Column H	10.57	1.192	P > 0.05	-28.58 to 49.73
50	Column G vs Column H	-14.55	1.641	P > 0.05	-53.71 to 24.60

Managanese effects on ccreals. Appendix 3a

	X Labels	A	B	C	D
	Parameter	Value	Data Set-B	Data Set-C	Data Set-D
	X	Y	Y	Y	Y
1	Table Analyzed				
2	Data Table-35				
3	One-way analysis of variance				
4	P value	P<0.0001			
5	P value summary	***			
6	Are means signif. different? (P < 0.05)	Yes			
7	Number of groups	7			
8	F	13.41			
9	R squared	0.5348			
10					
11	Bartlett's test for equal variances				
12	Bartlett's statistic (corrected)	54.14			
13	P value	P<0.0001			
14	P value summary	***			
15	Do the variances differ signif. (P < 0.05)	Yes			
16					
17	ANOVA Table	SS	df	MS	
18	Treatment (between columns)	2163	6	360.5	
19	Residual (within columns)	1882	70	26.88	
20	Total	4045	76		
21					
22	Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
23	Column A vs Column B	1.991	1.274	P > 0.05	-4.734 to 8.715
24	Column A vs Column C	-1.300	0.8316	P > 0.05	-8.025 to 5.425
25	Column A vs Column D	-1.827	1.169	P > 0.05	-8.552 to 4.897
26	Column A vs Column E	1.500	0.9596	P > 0.05	-5.225 to 8.225
27	Column A vs Column F	6.809	4.356	P < 0.05	0.08460 to 13.53
28	Column A vs Column G	-11.93	7.630	P < 0.001	-18.65 to -5.203
29	Column B vs Column C	-3.291	2.105	P > 0.05	-10.02 to 3.434
30	Column B vs Column D	-3.818	2.443	P > 0.05	-10.54 to 2.906
31	Column B vs Column E	-0.4909	0.3140	P > 0.05	-7.215 to 6.234
32	Column B vs Column F	4.818	3.082	P > 0.05	-1.906 to 11.54
33	Column B vs Column G	-13.92	8.904	P < 0.001	-20.64 to -7.194
34	Column C vs Column D	-0.5273	0.3373	P > 0.05	-7.252 to 6.197
35	Column C vs Column E	2.800	1.791	P > 0.05	-3.925 to 9.525
36	Column C vs Column F	8.109	5.188	P < 0.01	1.385 to 14.83
37	Column C vs Column G	-10.63	6.798	P < 0.001	-17.35 to -3.903
38	Column D vs Column E	3.327	2.129	P > 0.05	-3.397 to 10.05
39	Column D vs Column F	8.636	5.525	P < 0.01	1.912 to 15.36
40	Column D vs Column G	-10.10	6.461	P < 0.001	-16.82 to -3.375
41	Column E vs Column F	5.309	3.396	P > 0.05	-1.415 to 12.03
42	Column E vs Column G	-13.43	8.590	P < 0.001	-20.15 to -6.703
43	Column F vs Column G	-18.74	11.99	P < 0.001	-25.46 to -12.01

	X Labels	A	B	C	D
	Parameter	Value	Data Set-B	Data Set-C	Data Set-D
	X	Y	Y	Y	Y
1	Table Analyzed				
2	Data Table-29				
3	One way analysis of variance				
4	P value	0.4320			
5	P value summary	ns			
6	Are means signif. different? (P < 0.05)	No			
7	Number of groups	4			
8	F	0.9347			
9	R squared	0.05991			
10					
11	Bartlett's test for equal variances				
12	Bartlett's statistic (corrected)	10.81			
13	P value	0.0128			
14	P value summary	*			
15	Do the variances differ signif. (P < 0.05)	Yes			
16					
17	ANOVA Table	SS	df	MS	
18	Treatment (between columns)	7984	3	2661	
19	Residual (within columns)	125300	44	2847	
20	Total	133300	47		
21					
22	Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
23	Column A vs Column B	25.30	1.573	P > 0.05	-35.52 to 86.12
24	Column A vs Column C	17.07	1.139	P > 0.05	-39.55 to 73.69
25	Column A vs Column D	-8.052	0.5005	P > 0.05	-68.87 to 52.77
26	Column B vs Column C	-8.233	0.5497	P > 0.05	-64.85 to 48.39
27	Column B vs Column D	-33.35	2.073	P > 0.05	-94.17 to 27.47
28	Column C vs Column D	-25.12	1.677	P > 0.05	-81.74 to 31.50

Iron effects in oil seeds. Appendix Ic

	X Labels	A	B	C	D
	Parameter	Value	Data Set-B	Data Set-C	Data Set-D
	X	Y	Y	Y	Y
1	Table Analyzed				
2	Data Table-7				
3	One-way analysis of variance				
4	P value	P<0.0001			
5	P value summary	***			
6	Are means signif. different? (P < 0.05)	Yes			
7	Number of groups	8			
8	F	7.708			
9	R squared	0.4028			
10					
11	Bartlett's test for equal variances				
12	Bartlett's statistic (corrected)	17.98			
13	P value	0.0120			
14	P value summary	*			
15	Do the variances differ signif. (P < 0.05)	Yes			
16					
17	ANOVA Table	SS	df	MS	
18	Treatment (between columns)	1660	7	237.2	
19	Residual (within columns)	2462	80	30.77	
20	Total	4122	87		
21					
22	Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
23	Column A vs Column B	-1.757	1.051	P > 0.05	-9.141 to 5.627
24	Column A vs Column C	5.955	3.560	P > 0.05	-1.430 to 13.34
25	Column A vs Column D	-2.827	1.690	P > 0.05	-10.21 to 4.557
26	Column A vs Column E	5.288	3.162	P > 0.05	-2.096 to 12.67
27	Column A vs Column F	-3.382	2.022	P > 0.05	-10.77 to 4.002
28	Column A vs Column G	-3.327	1.989	P > 0.05	-10.71 to 4.057
29	Column A vs Column H	-7.857	4.698	P < 0.05	-15.24 to -0.4732
30	Column B vs Column C	7.712	4.611	P < 0.05	0.3277 to 15.10
31	Column B vs Column D	-1.070	0.6398	P > 0.05	-8.454 to 6.314
32	Column B vs Column E	7.046	4.213	P > 0.05	-0.3386 to 14.43
33	Column B vs Column F	-1.625	0.9713	P > 0.05	-9.009 to 5.760
34	Column B vs Column G	-1.570	0.9387	P > 0.05	-8.954 to 5.814
35	Column B vs Column H	-6.100	3.647	P > 0.05	-13.48 to 1.284
36	Column C vs Column D	-8.782	5.251	P < 0.01	-16.17 to -1.398
37	Column C vs Column E	-0.6664	0.3984	P > 0.05	-8.050 to 6.718
38	Column C vs Column F	-9.336	5.582	P < 0.01	-16.72 to -1.952
39	Column C vs Column G	-9.282	5.550	P < 0.01	-16.67 to -1.898
40	Column C vs Column H	-13.81	8.258	P < 0.001	-21.20 to -6.428
41	Column D vs Column E	8.115	4.852	P < 0.05	0.7314 to 15.50
42	Column D vs Column F	-0.5545	0.3316	P > 0.05	-7.939 to 6.830
43	Column D vs Column G	-0.5000	0.2990	P > 0.05	-7.884 to 6.884
44	Column D vs Column H	-5.030	3.007	P > 0.05	-12.41 to 2.354
45	Column E vs Column F	-8.670	5.184	P < 0.05	-16.06 to -1.286
46	Column E vs Column G	-8.615	5.151	P < 0.05	-16.00 to -1.231
47	Column E vs Column H	-13.15	7.860	P < 0.001	-20.53 to -5.761
48	Column F vs Column G	0.05455	0.03261	P > 0.05	-7.330 to 7.439
49	Column F vs Column H	-4.475	2.676	P > 0.05	-11.86 to 2.909
50	Column G vs Column H	-4.530	2.709	P > 0.05	-11.91 to 2.854

Zinc effects on cereals. Appendix 4a

	X Labels	A	B	C	D
	Parameter	Value	Data Set-B	Data Set-C	Data Set-D
	X	Y	Y	Y	Y
1	Table Analyzed				
2	Data Table-40				
3	One-way analysis of variance				
4	P value	P<0.0001			
5	P value summary	***			
6	Are means signif. different? (P < 0.05)	Yes			
7	Number of groups	6			
8	F	10.51			
9	R squared	0.4668			
10					
11	Bartlett's test for equal variances				
12	Bartlett's statistic (corrected)	46.65			
13	P value	P<0.0001			
14	P value summary	***			
15	Do the variances differ signif. (P < 0.05)	Yes			
16					
17	ANOVA Table	SS	df	MS	
18	Treatment (between columns)	4879	5	975.7	
19	Residual (within columns)	5573	60	92.88	
20	Total	10460	65		
21					
22	Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff.
23	Column A vs Column B	3.136	1.079	P > 0.05	-8.961 to 15.23
24	Column A vs Column C	2.618	0.9010	P > 0.05	-9.479 to 14.72
25	Column A vs Column D	3.927	1.352	P > 0.05	-8.170 to 16.02
26	Column A vs Column E	22.67	7.802	P < 0.001	10.58 to 34.77
27	Column A vs Column F	-4.845	1.667	P > 0.05	-16.94 to 7.252
28	Column B vs Column C	-0.5182	0.1783	P > 0.05	-12.62 to 11.58
29	Column B vs Column D	0.7909	0.2722	P > 0.05	-11.31 to 12.89
30	Column B vs Column E	19.54	6.723	P < 0.001	7.439 to 31.63
31	Column B vs Column F	-7.982	2.747	P > 0.05	-20.08 to 4.115
32	Column C vs Column D	1.309	0.4505	P > 0.05	-10.79 to 13.41
33	Column C vs Column E	20.05	6.901	P < 0.001	7.957 to 32.15
34	Column C vs Column F	-7.464	2.568	P > 0.05	-19.56 to 4.633
35	Column D vs Column E	18.75	6.451	P < 0.001	6.648 to 30.84
36	Column D vs Column F	-8.773	3.019	P > 0.05	-20.87 to 3.324
37	Column E vs Column F	-27.52	9.470	P < 0.001	-39.62 to -15.42

Zinc effects on protein foods. Appendix 4b

3	One-way analysis of variance				
4	P value	0.1482			
5	P value summary	ns			
6	Are means signif. different? (P < 0.05)	No			
7	Number of groups	4			
8	F	1.882			
9	R squared	0.1237			
10					
11	Bartlett's test for equal variances				
12	Bartlett's statistic (corrected)	6.158			
13	P value	0.1042			
14	P value summary	ns			
15	Do the variances differ signif. (P < 0.05)	No			
16					
17	ANOVA Table	SS	df	MS	
18	Treatment (between columns)	15220	3	5074	
19	Residual (within columns)	107900	40	2696	
20	Total	123100	43		
21					
22	Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
23	Column A vs Column B	29.11	1.859	P > 0.05	-30.25 to 88.46
24	Column A vs Column C	13.74	0.8774	P > 0.05	-45.62 to 73.09
25	Column A vs Column D	-21.49	1.373	P > 0.05	-80.85 to 37.86
26	Column B vs Column C	-15.37	0.9819	P > 0.05	-74.73 to 43.98
27	Column B vs Column D	-50.60	3.232	P > 0.05	-110.0 to 8.753
28	Column C vs Column D	-35.23	2.250	P > 0.05	-94.58 to 24.13

Zinc effects on oil seeds. Appendix 4c

	X Labels	A	B	C	
	Parameter	Value	Data Set-B	Data Set-C	Data Set-D
	X	Y	Y	Y	Y
1	Table Analyzed				
2	Data Table-37				
3	One-way analysis of variance				
4	P value	P<0.0001			
5	P value summary	***			
6	Are means signif. different? (P < 0.05)	Yes			
7	Number of groups	5			
8	F	21.15			
9	R squared	0.6285			
10					
11	Bartlett's test for equal variances				
12	Bartlett's statistic (corrected)	52.49			
13	P value	P<0.0001			
14	P value summary	***			
15	Do the variances differ signif. (P < 0.05)	Yes			
16					
17	ANOVA Table	SS	df	MS	
18	Treatment (between columns)	566.8	4	141.7	
19	Residual (within columns)	335.0	50	6.701	
20	Total	901.8	54		
21					
22	Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
23	Column A vs Column B	6.134	7.859	P < 0.001	3.005 to 9.262
24	Column A vs Column C	-0.7182	0.9202	P > 0.05	-3.846 to 2.410
25	Column A vs Column D	7.336	9.400	P < 0.001	4.208 to 10.46
26	Column A vs Column E	2.664	3.413	P > 0.05	-0.4646 to 5.792
27	Column B vs Column C	-6.852	8.779	P < 0.001	-9.980 to -3.724
28	Column B vs Column D	1.203	1.541	P > 0.05	-1.925 to 4.331
29	Column B vs Column E	-3.470	4.446	P < 0.05	-6.598 to -0.3418
30	Column C vs Column D	8.055	10.32	P < 0.001	4.926 to 11.18
31	Column C vs Column E	3.382	4.333	P < 0.05	0.2536 to 6.510
32	Column D vs Column E	-4.673	5.987	P < 0.001	-7.801 to -1.545