Production of Aflatoxin by *Aspergillus parasiticus* and its Control

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by

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TO:

* The Spirit of my parents

* My beloved wife and family for their patience and encouragement

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Abbreviations

AFB_1	=	Aflatoxin B_1
AFL	=	Aflatoxicol
AOAC	=	Association of Official Analytical Chemists
ATA	=	Alimentary Toxic Aleukia
a_W	=	Water activity
B_2	=	Aflatoxin B_2
BGYF	=	Bright Greenish-Yellow Fluorescence
CDA	=	Czapek's-Dox Agar medium
DH	=	Dehydrogenase
EDTA	=	Ethylenediaminetetraacetic Acid
EEC	=	European Economic Community
ELISA	=	Enzyme-Linked Immunosorbent Assay
ERH	=	Equilibrium Relative Humidity
FAO	=	Food and Agriculture Organization
FDA	=	Food and Drug Administration
G_1	=	Aflatoxin G_1
G_2	=	Aflatoxin G_2
HPLC	=	High Performance Liquid Chromatography
M_1	=	Aflatoxin M_1
M_2	=	Aflatoxin M_2
MFO	=	Microsomal Mixed Function Oxidase
NLSD	=	New Least Significant Difference
PDA	=	Potato-Dextrose Agar Medium
PPP	=	Pentose-phosphate Pathway
P_1	=	Aflatoxin P_1
Q_1	=	Aflatoxin Q_1
RIA	=	Radioimmuno Assay
S.D.	=	Standard Deviation
SDA	=	Sabouraud-Dextrose Agar medium
TLC	=	Thin Layer Chromatography
TYG	=	Tryptone-Yeast extract-Glucose medium

WHO	=	World Health Organization
UNEP	=	United Nations Environmental Programme
UV	=	Ultra violet

ABSTRACT

The aim of the present work was to investigate aflatoxin levels in various food commodities and to study its production by *Aspergillus parasiticus* in culture to find out the possible ways to control it. Of 40 food samples collected from Abha region, Saudi Arabia, only 25% were contaminated with aflatoxins. Oil-rich commodities had the highly contaminated commodities by fungi and aflatoxins while spices were free from aflatoxins. The optimal pH for the growth of *A. parasiticus* and its productivity of aflatoxin B_1 was found at 6.0, while the best incubation conditions were found at 30°C for 10 days. D-glucose was the best carbon source for fungal growth, as well as aflatoxin production. Corn steep liquor, yeast extract and peptone were the best nitrogen sources for both fungal growth and toxin production. $(NH_4)_2HPO_4$ (1.55 gL^{-1}) and $NaNO_2$ (1.6 gL^{-1}) reduced fungal growth and toxin production with 37.7% and 85%, respectively. Of ten amino acids tested, asparagine was the best for aflatoxin B_1 production. Zn^{2+} and Co^{2+} supported significantly both fungal growth, as well as aflatoxin B_1 production at the different tested concentrations. Zn^{2+} was effective when added to *A. parasiticus* growth medium at the first two days of the culture age. The other tested metal ions gave variable effects depending on the type of ion and its concentration. Water activity (a_w) was an important factor controlling the growth of *A. parasiticus* and toxin production. The minimum a_w for the fungal growth was 0.8 on both coffee beans and rice grains, while a_w of 0.70 caused complete inhibition for the growth and aflatoxin B_1 production. H_2O_2 is a potent inhibitor for growth of *A. parasiticus* and its productivity of toxins. Incubation with $NaHCO_3$ and C_6H_5COONa converted aflatoxin B_1 to a water-soluble form which returned to aflatoxin B_1 by acid treatment. Black pepper, ciliated heath, cuminum and curcuma were the most inhibitory spices on toxin production. Glutathione, quinine, EDTA, sodium azide, indole acetic acid, 2,4-dichlorophenoxy acetic acid, phenol and catechol were inhibitory for both growth, as well as, aflatoxin B_1 production. Stearic acid supported the fungal growth and decreased the productivity of AFB_1 gradually. Lauric acid is the most suppressive fatty acid for both fungal growth and aflatoxin production, but oleic acid was the most potent supporter. Vitamin A supported the growth but inhibited aflatoxin B_1 production. Vitamins C and D_2 were also repressive particularly for aflatoxin production. The present study included determining the activities of some enzymes in relation to aflatoxin production in *A. parasiticus* culture during 20 days. Glycolytic enzymes and pyruvate-generating enzymes seems to be linked with aflatoxin B_1 production. Also, pentose-phosphate pathway enzymes may provide NADPH for aflatoxin B_1 synthesis. The decreased activities of TCA cycle enzymes particularly from 4th day of growth up to 10th day were correlated with the increase of aflatoxin B_1 production. All the tested enzymes as well as aflatoxin B_1 production were inhibited by either catechol or phenol.

CHAPTER 1

INTRODUCTION

Fungal metabolism:

Metabolism serves two general functions for the fungus. First, the anabolic functions whereby nutrients are changed into the structural and functional components (metabolites) of the organism. Second, the catabolic functions whereby the chemical energy is produced and used for anabolic reactions.

Anabolism is dependent on catabolism not only for energy in the form of ATP, NADH, and NADPH, but also for the production of key intermediates for the biosynthesis of the functional macromolecules of the hyphal structures (Griffin, 1981).

There are two types of metabolites. First, the primary metabolites which are essential for growth and are formed during cell multiplication. Second, the secondary metabolites which are formed during the end of the growth phase of the fungus and have no apparent significant role in fungal growth or physiology (Griffin, 1981).

The functions of secondary fungal products have been discussed by Weinberg (1971) and Bu'Lock (1975). The most commonly agreed postulate suggests that secondary metabolites are formed when large amounts of primary metabolic precursors are accumulated. The fungus can get rid of these precursors by diverting them into secondary metabolites (Demain, 1973).

Secondary metabolites are characterised by three main characteristics. First, their production is frequently confined to one species or one strain within a species. Second, they have no obvious function in the life of the producer organism. Third, they are produced by cells whose growth is restricted. In filamentous fungi, however, secondary metabolites may accumulate throughout the growth of a colony because different cells are involved in secondary metabolism and growth (Deacon, 1980).

Some secondary metabolites are of immense commercial value, e.g., the antibiotics; plant hormones like gibberellins which are produced by *Gibberella fujikuroi* and several flavour components of foods. However, other secondary metabolites of some moulds such as mycotoxins are extremely toxic and can produce pathological or undesirable physiological responses in man.

Significance of mycotoxins:

Mycotoxins as secondary metabolites of fungi are capable of producing acute toxic, carcinogenic, mutagenic, teratogenic and oestrogenic effects on animals at the levels of exposure. Toxic syndromes resulting from the intake of mycotoxins by man and animals are known as 'mycotoxicoses' (Pier, 1981).

Mycotoxicoses have been known for a long time. The first recognised mycotoxicosis was probably ergotism (Tulasne, 1953), a disease characterised by necrosis and gangrene of the limbs and better known in the middle ages in Europe as 'Holy Fire'. This disease was caused by the ingestion of grain contaminated with sclerotia of *Claviceps purpurea*, that contained toxic metabolites. Another mycotoxicosis, recognised to have seriously injured human populations is Alimentary Toxic Aleukia (ATA) produced by *Fusarium* species (Mayer, 1953).

Despite the aforementioned examples of mycotoxin-caused diseases in man, mycotoxicoses remained the 'neglected diseases' (Forgacs & Carll, 1962) until the early 1960s, when this attitude was changed drastically due to the outbreak of Turkey X-disease in Great Britain (Stevens *et al.*, 1960). Within a few months, more than 100,000 Turkeys died, mainly in East Anglia and southern England. In addition, the death of thousands of ducklings and young pheasants was reported (Asplin & Carnaghan, 1961). The problem of Turkey X-disease led to a multidisciplinary

approach to investigate the cause of the disease. These efforts were fruitful and the cause of the disease was traced to a toxic factor occurring in the Brazilian groundnut meal which was used as a protein source in the feed of the affected poultry. The toxic factor seemed to be produced by two fungi: *Aspergillus flavus* and *Aspergillus parasiticus*, and hence the name 'Aflatoxin' was coined for it (Nesbitt *et al.*, 1962).

In the period following the outbreak of Turkey X disease, a wealth of information about aflatoxins has been produced and many other mycotoxins have also been isolated and characterised. Of these, patulin, ochratoxin A, deoxynivalenol, T-2 toxin and zearalenone are the more important ones. Some of the mycotoxins produced by various fungi are listed in Table 1.

Patulin may occur in fruits and fruit juices. It has been used in the past as an antibiotic, but later it became known as a mycotoxin because it caused haemorrhages and oedema in experimental animals. Nowadays, patulin is considered rather as an indicator of bad manufacturing practices (use of mouldy raw materials) than a serious threat to human and animal health, as suggested from the results of recent sub-acute and semi-chronic toxicity studies (Speijers & Franken, 1988).

Ochratoxin A, a mycotoxin mainly occurring in grains was shown to be a potent nephrotoxin in all species of tested animals including birds, fish and mammals (Krogh, 1977). Reports indicating the carcinogenicity of ochratoxin A in mice and rats have also been published (Bendele *et al.*, 1985; Boorman, 1988 and Ueno, 1993).

Deoxynivalenol and T-2 toxin belong to the trichothecene group. The trichothecenes mainly occur in grains. These compounds exhibit a wide range of toxic effects in experimental animals including feed refusal, vomiting, diarrhoea and severe intestinal haemorrhage. They have also teratogenic and immunotoxic properties. The

Table 1: Some Mycotoxins which are produced by some fungi (Gorst Allman & Steyn, 1979).

Mycotoxin	Some fungal species which produce mycotoxins
Aflatoxin B_1	*Aspergillus flavus, A. parasiticus*
Patulin	*Penicillum patulum, P. cyclopium, Aspergillus clavatus, A. giganteus, A. terreus*
Ochratoxin A	*Aspergillus ochraceus, A. melleus, Penicillum viridicatum, P. variable*
Penicillic acid	*Penicillum puberulum, P. thomii, Aspergillus ochraceus, A. melleus*
Citrinin	*Penicillium citrinum, P. fellutanum, P. viridicatum, Aspergillus niveus, A. flavipes*
T-2 toxin	*Fusarium tricinctum, F. roseum, F. solani*
Deoxynivalenol (Vomitoxin)	*Fusarium culmorum, F. graminearum F. roseum*
Zearalenone	*Fusarium graminearum, F. moniliforme*

occurrence of deoxynivalenol in foodstuffs for swine may lead to economic loss, because these animals may refuse to feed and loose weight and suffer from vomiting and diarrhoea. T-2 toxin is a mycotoxin that has attracted particular attention not only as a mycotoxin occurring in foodstuffs but also because of its alleged use in biological warfare in Southeast Asia (yellow rain) (Anonymous, 1982; Mirocha *et al.*, 1982).

Zearalenone is a mycotoxin occurring particularly in maize and wheat, and often found together with deoxynivalenol. Zearalenone is related to the anabolic zearanol. It has oestrogenic properties and causes reproductive problems in farm animals, especially swine (Scott, 1989; Luo, *et al.*, 1990).

The mycotoxin sterigmatocystin is produced by several species of *Aspergillus*, *Penicillium luteum*, and *Bipolaris* species. Sterigmatocystin has been detected at low concentrations in green coffee, mouldy wheat, and in the rind of hard Dutch cheese (Bullerman, 1981; Scott, 1985; Vesonder and Horn, 1985).

Citrinin is a yellow-coloured mycotoxin that is produced by several *Penicillium* and *Aspergillus* species including *P. viridicatum* strains that produces ochratoxin A. Like ochratoxin A, citrinin causes kidney damage in laboratory animals similar to swine nephropathy, and may interact synergistically with ochratoxin A in cases of swine nephropathy as found in Denmark (Krogh, 1977).

Fungi capable of producing tremorgenic mycotoxins belong to the genera *Penicillium*, *Aspergillus*, *Claviceps*, and *Acremonium*. The disease caused by these mycotoxins in cattle is called staggers (Cole, 1986).

Penicillium roqueforti and *P. caseicolum*, used to produce mould-ripened cheeses, have been shown to produce several toxic compounds, including penicillic acid, roquefortine, isofumigaclavines A and B, and cyclopiazonic acid (Scott, 1981).

Mycotoxins are of low molecular weight and synthesised by a sequence of enzyme-catalysed reactions from a few simple intermediates of primary metabolism, e.g., acetate, mevalonate, malonate and certain amino acids (Turner, 1971 and Steyn, 1980). The main biosynthetic reactions include condensation, oxidation/reduction, alkylation and halogenation steps which create a remarkable range of secondary compounds.

The production of mycotoxins on foodstuffs depends on: (a) a suitable substrate for growth of the mould, (b) the physical presence of the toxigenic mould, and (c) an environment suitable for the growth of the mould. Mycotoxin production will only occur when all these conditions are fulfilled. Furthermore, each condition will involve many interrelating factors which collectively or individually can affect mycotoxin formation.

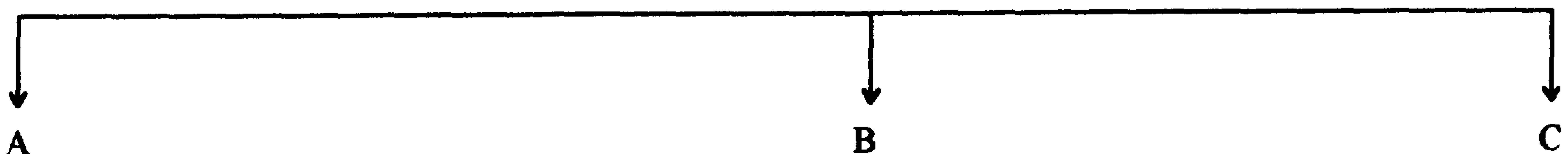
As illustrated in Fig. 1 and according to Hesseltine (1976), mycotoxins can be produced by fungi growing on the living plant by field fungi, stored plant material by storage fungi and decaying plant material by advanced decay fungi.

Biological effects of mycotoxins:

Mycotoxins can enter either human or animal foods by direct or indirect contamination and cause mycotoxicoses by ingestion. In direct contamination the food materials support the toxigenic mould growth. Almost all foods will be susceptible to mould growth at some stages during their production processing, transport and storage. By contrast, indirect contamination will occur when a food ingredient is contaminated with a mycotoxin (Jarvis, 1976). The possible routes for mycotoxin entry into human or animal foods are shown in Fig. 2.

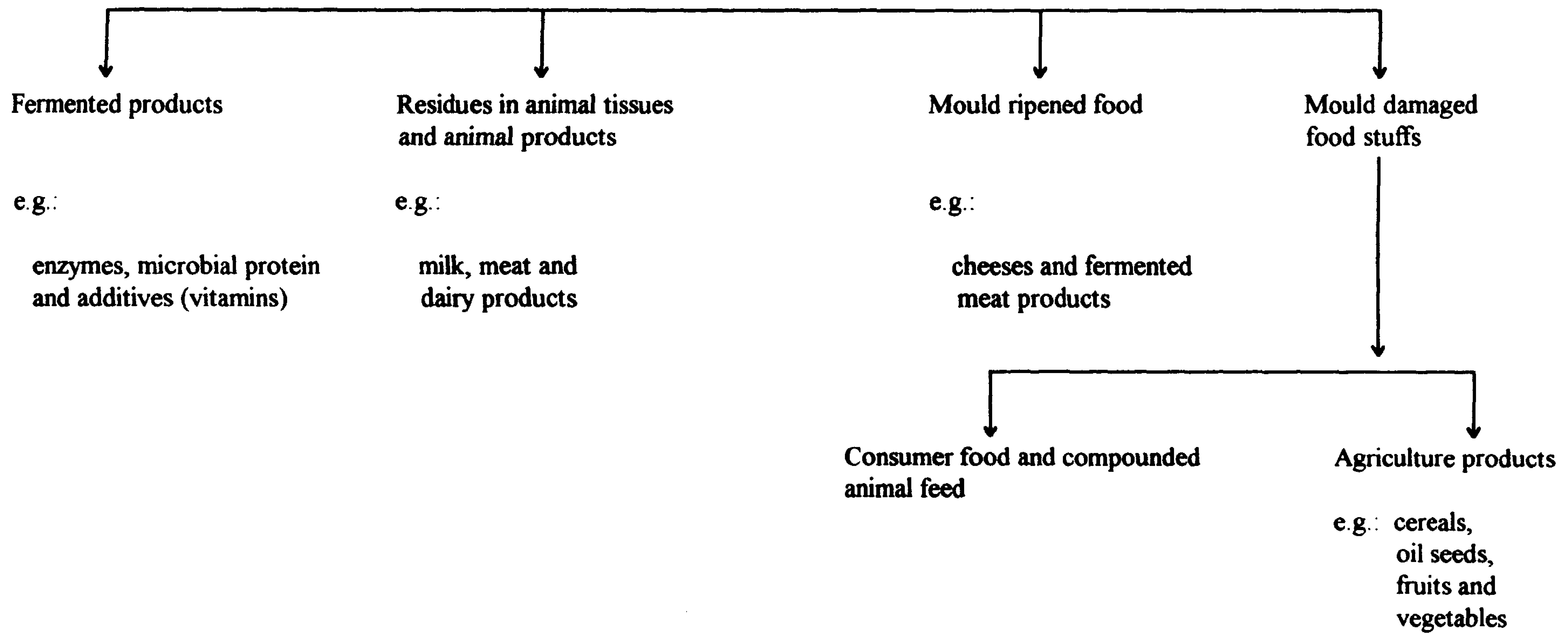
Fig. 1: Groups of fungi producing mycotoxins according to habitat (Hesseltine, 1976).

Groups



A	B	C
Field fungi	**Stored fungi**	**Advanced decay fungi**
* Grow on living plants	* Grow on stored plant material	* Grow on decaying plant material
e.g.:	e.g.:	e.g.:
Aspergillus flavus	*Aspergillus flavus*	*Pithomyces chartaram*
Claviceps purpurea	*A. parasiticus*	*Cladosporium sp.*
Fusarium graminearum	*A. versicolor*	*Alternaria longipes*
Rhizoctonia leguminicola	*Penicillium citrinum*	*Stachybotrys atra*
Helminthosporium biseptatum	*P. rubrum*	*Myrothecium verrucaria*

Fig. 2: **Suggested routes of mycotoxin contamination of human and animal foods (Jarvis, 1976).**


Routes of mycotoxin contamination
Fermented products
e.g.:
enzymes, microbial protein and additives (vitamins)
Residues in animal tissues and animal products
e.g.:
milk, meat and dairy products
Mould ripened food
e.g.:
cheeses and fermented meat products
Mould damaged food stuffs
Consumer food and compounded animal feed
Agriculture products
e.g.: cereals, oil seeds, fruits and vegetables

The Food and Agriculture Organization (FAO) reported that 25% of the world's food crops are affected by mycotoxins (Mannon and Johnson, 1985). Mycotoxin contamination leads to loss of important export markets. As an example, consumption of peanut feedcake in European Economic Community (EEC) countries dropped by over 60% in a four-year period (from 988 million metric tons in 1979 to 368 million metric tons in 1982) as a direct result of economic losses to livestock producers associated with high levels of aflatoxins in imported feedcake. Serious economic losses were sustained by exporting countries as a consequence of reduced demand. The United States annually rejects imported goods for noncompliance of standards, with mould and mycotoxin contamination cited as the primary violation. A 1983 Joint FAO/WHO Committee on Food Safety reported that between October 1979 and September 1980, the FDA rejected products valued at $206 million. Similarly, in the period of October 1980 to September 1981, products valued at $253.5 million were rejected (WHO, 1983; Jammali, 1987; Council for Agric. Sci. and Tech. 1989).

In addition, mycotoxins can reduce the growth rate of young animals and can even interfere with native mechanisms of resistance and impair immunologic responsiveness, making the animals more susceptible to infection (Pier *et al.*, 1980).

Early evidence indicated that high concentrations of mould toxins in the diet of animals led to death related to acute liver and kidney damage (Carnaghan & Sargent, 1961; Hayes, 1980; and Dvorackova, 1990).

The historical record provides evidence that under some circumstances, toxic residues from mould growth in food can be deleterious to human health (Table 2). The recent spate of interest in mould toxins, generated by the hepatocarcinogenicity of the

Table (2): Diseases of humans commonly recognized as having been caused by mouldy food, the moulds involved, and the mycotoxins that have been implicated.

Disease	Moulds involved	Toxins	Reference
Aflatoxicosis	*Aspergillus flavus* *A. parasiticus*	Aflatoxins	Stoloff, 1986
Ergotism	*Claviceps purpurea* *C. paspali* *C. fusiformis*	Ergot alkaloids	Barger, 1931
Alimentary toxic aleukia	*Fusarium sporotrichioides* *F. poae*	T-2 toxin	Joffe, 1986
Stachybotryotoxicosis	*Stachybotrys atra*	Various macrocyclic Trichothecenes	Rodricks & Eppley, 1974
Yellow rice (cardiac beri beri)	*Penicillium citreoviride* *P. citrinum* *P. islandicum*	Luteoskyrin, Islanditoxin, Cyclochloritine, Citrinin, and Citreovirdin	Uraguchi, 1978

aflatoxins, has resulted in the isolation and characterisation of over 100 compounds (Busby and Wogan, 1981). Types of mycotoxicoses associated with the mycotoxins of the Aspergilli are illustrated in Fig. 3.

AFLATOXINS:

The aflatoxins are secondary metabolites produced by *Aspergillus flavus*, *A. parasiticus* and *A. nomius* (Heathcote & Hibbert, 1978; Wei & Hsieh, 1980; and Cotty *et al.*, 1994). The first two species are widely distributed in nature and are able to grow on a wide variety of natural substances including foodstuffs and animal feeds causing serious economic losses.

Structure and chemistry of aflatoxins:

Among the major known types of aflatoxins are aflatoxin B_1; aflatoxin B_2, aflatoxin G_1 and aflatoxin G_2 (Nesbitt *et al.*, 1962 and Betina, 1989). Two other mould-produced aflatoxins M_1 and M_2 were also isolated and identified as mammalian metabolites of aflatoxins B_1 and B_2 (Allcroft *et al.*, 1966 and Saad *et al.*, 1995). The chemical structures of these aflatoxins are shown in Fig. 4.

Aflatoxin B_1 (AFB_1) is the most toxic and is usually produced in the greatest quantity. Most biosynthetic studies have focused on AFB_1 and assumed that the other aflatoxins are metabolically related to it by a direct interconversion process (Bennett and Christensen, 1983).

Natural occurrence of aflatoxins:

Aflatoxins (primarily B_1) have been found in most staple foods, e.g., cereal grains (maize, wheat, oats, rice, etc.), ground nuts, peanut butter, mung beans, Brazil nuts, almonds, cottonseed and meal, cayenne pepper, Indian chili powder, bread, eggs and meat (Stoloff, 1976; Tseng, 1994; Halt, 1994 and Hafez, 1996).

Fig. 3: Diversity of symptoms caused by mycotoxins from the genus *Aspergillus* (Moss, 1977).

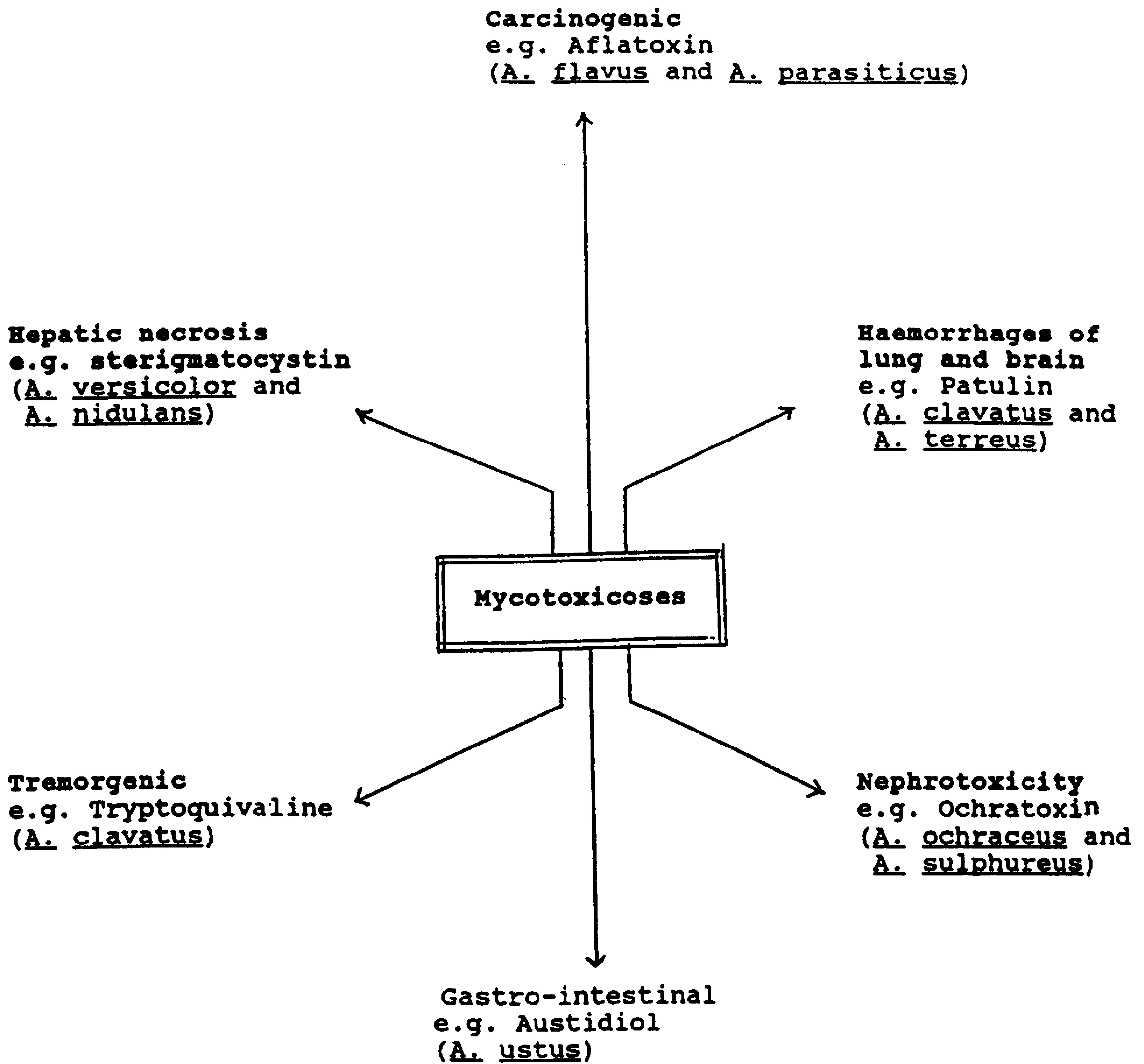
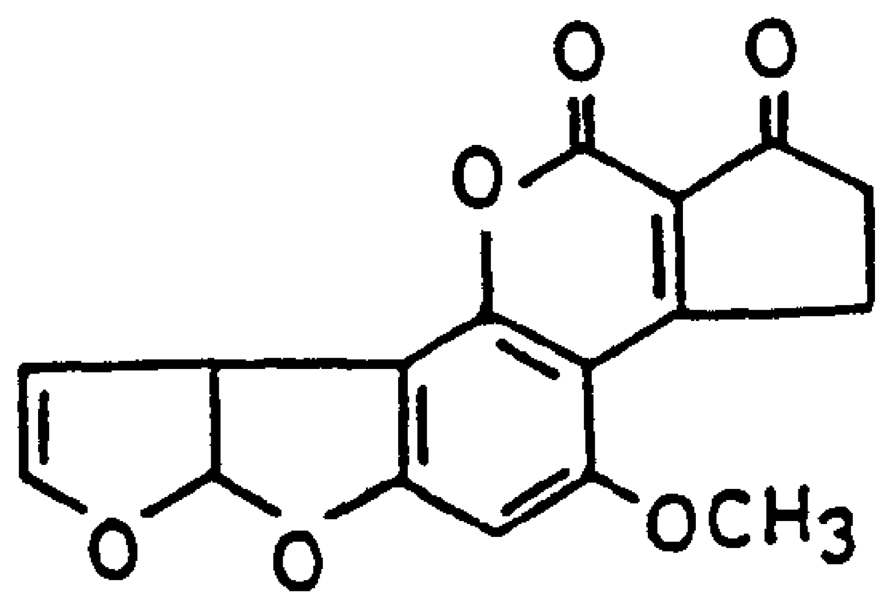
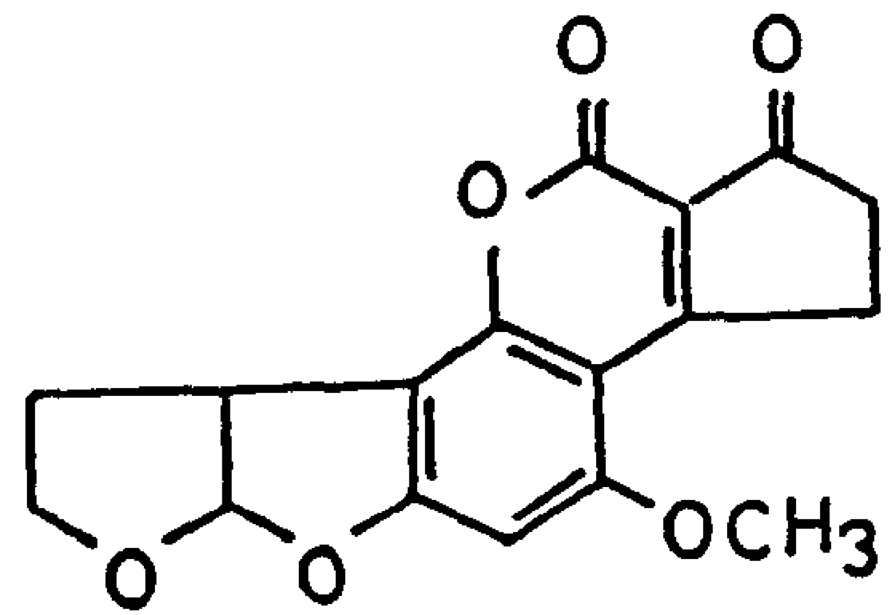

Carcinogenic
e.g. Aflatoxin
(A. flavus and A. parasiticus)
Hepatic necrosis
e.g. sterigmatocystin
(A. versicolor and
A. nidulans)
Haemorrhages of
lung and brain
e.g. Patulin
(A. clavatus and
A. terreus)
Mycotoxicoses
Tremorgenic
e.g. Tryptoquivaline
(A. clavatus)
Nephrotoxicity
e.g. Ochratoxin
(A. ochraceus and
A. sulphureus)
Gastro-intestinal
e.g. Austidiol
(A. ustus)

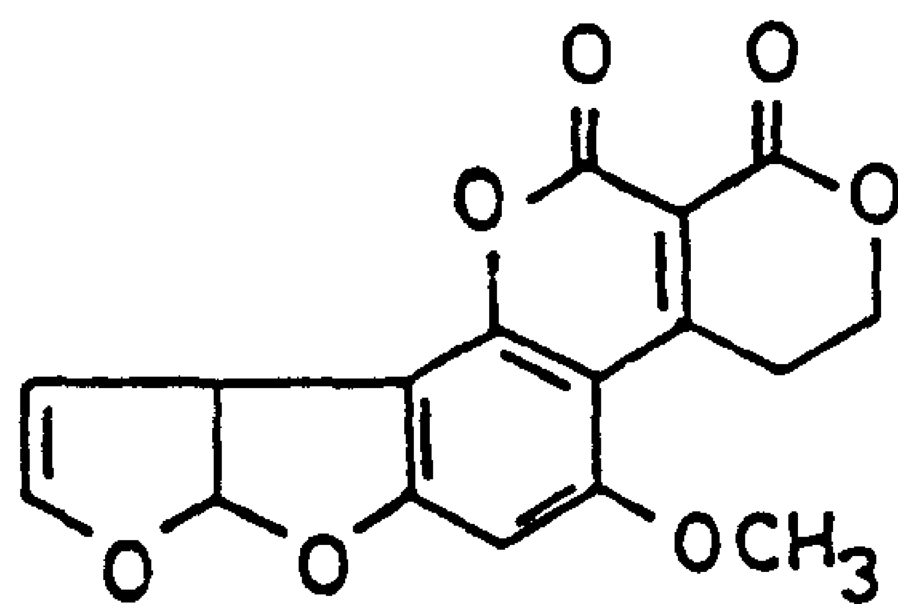
Fig. 4: Structure and nomenclature of natural occurring aflatoxins.



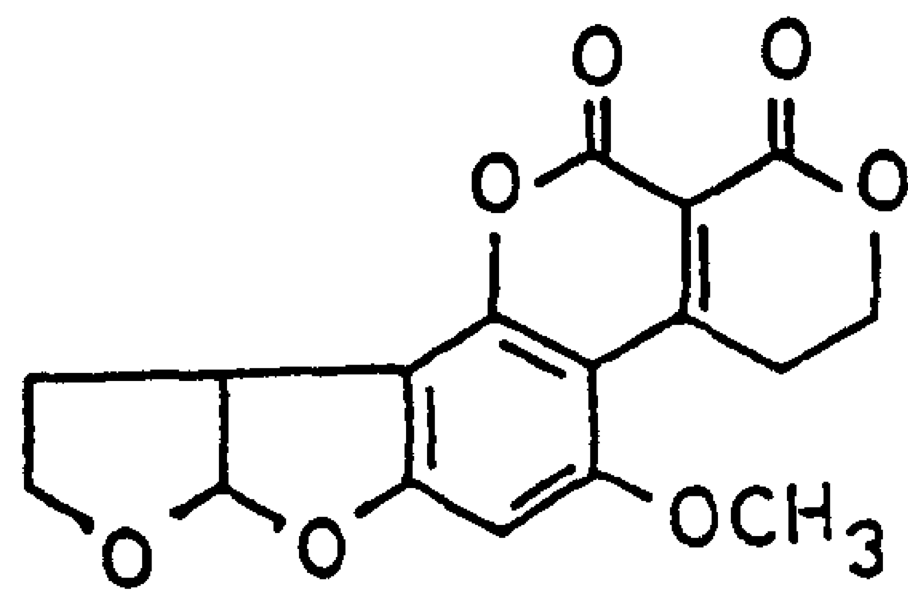
Aflatoxin B_1



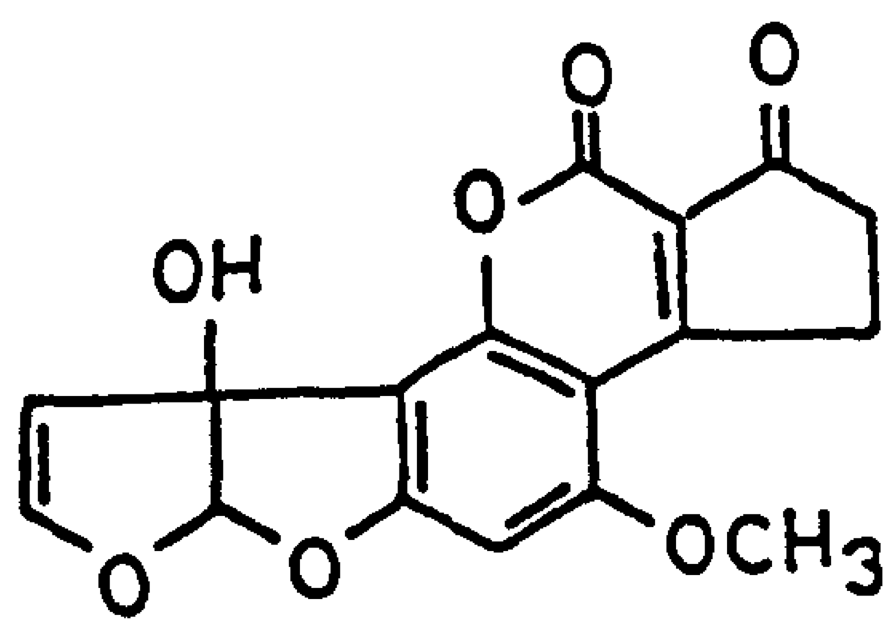
Aflatoxin B_2



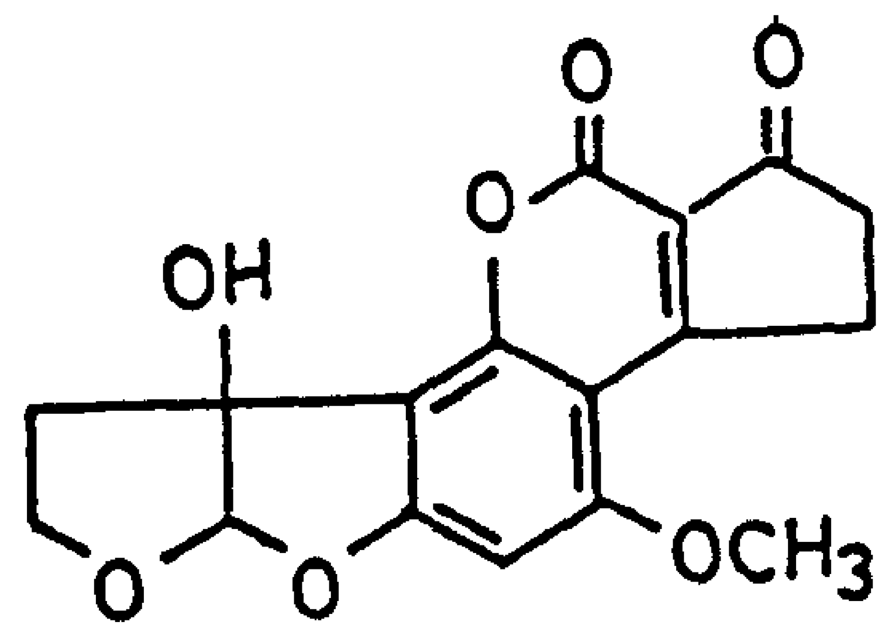
Aflatoxin G_1



Aflatoxin G_2



Aflatoxin M_1



Aflatoxin M_2

The presence of aflatoxin B_1 in food is of special concern because not only has it been directly responsible for human illness, such as the outbreak of hepatitis (Krishnamachari *et al.*, 1975a), but also it is metabolised in the liver to a number of other aflatoxins (Neal, 1987).

The production of aflatoxins B_1 and B_2 by *A. flavus* was reported by Van Welbeek *et al.* (1968) and Trenk & Hartman (1970). In addition, Schroeder and Carlton (1973) found that a strain of *A. flavus* isolated from ground black pepper produced aflatoxin B_1 on several natural substrates. Flannigan and Hui (1976) isolated 24 strains of *A. flavus* from 14 samples of spices and found that only 7 strains produced aflatoxins *in vitro*.

Hesseltine *et al.* (1968) suggested that toxigenic strains of *A. parasiticus* may be the predominant aflatoxin-producing fungi in tropical countries. This observation was supported by Krogh and Hald (1969) who detected aflatoxin B_1 and G_1 in peanuts and peanut products imported to Denmark from Brazil, Nigeria, Senegal, Kenya, Argentina, Uganda and Ghana. In contrast, strains of *A. flavus* that produce aflatoxin B_1 are the predominant aflatoxin producers isolated from peanuts and rice in sub-tropical regions (Bollar & Schroeder, 1966 and Taber & Schroeder, 1967).

Biological activity of aflatoxins:

Regarding the biological activity of aflatoxins, it could be stated that they are toxic to all living organisms but species sensitivity varies enormously. The toxic response of animals to AFB_1 depends on age, strain, and dietary factors as well as drug-treatment. For example, male rats are more susceptible to aflatoxin B_1 than females (Purchese *et al.*, 1973 and Ellis *et al.*, 1991). Patterson and Allcroft (1970) divided different species of animals into two groups namely: a susceptible group

including calf, chick, duckling, guinea pig, and pig as well as a relatively resistant group including goat, sheep, rat and mouse. Their effects on animals vary with dose, length of exposure, and diet or nutritional status. These toxins might be lethal when consumed in large doses. Sublethal doses produced a chronic toxicity, and low levels of chronic exposure could result in cancer (Wogan & Newberne, 1967 and Sinnhuber *et al.*, 1977), primarily liver cancer, in some animal species (Wogan, 1973).

Acute aflatoxicosis in cattle has been thoroughly described. Clinical signs consisted of reduced feed consumption, dramatic drops in milk production, weight loss, and liver damage (Bodine and Mertens, 1983).

Another characteristic of aflatoxin exposure in dairy cattle and human is the conversion of aflatoxin B_1 to the hydroxylated metabolite, aflatoxin M_1, and the excretion of aflatoxin M_1 in milk (Applebaum *et al.*, 1982, Van Egmond, 1989 and El-Nezami *et al.*, 1995).

Aflatoxins have been shown to affect rumen function *in vitro* and *in vivo* by decreasing cellulose digestion, volatile fatty acid formation, and proteolysis (Fehr & Delage, 1970 and Dvorak *et al.*, 1977).

The toxicity of aflatoxins has been reported in suckling piglets, growing and finishing swine, and breeder stock. Clinical and pathological signs included decreased rate of weight gain, decreased feed conversion efficiency, toxic hepatitis, nephrosis, and systemic hemorrhages (Hoerr & D'Andrea, 1983).

Aflatoxicoses have produced severe economic losses in the poultry industry, affecting ducklings, broilers, turkey and quail. Clinical signs of intoxication included anorexia, decreased weight gains, decreased egg production, hemorrhage, embryo-

toxicity, and increased susceptibility to environmental and microbial stressors (Edds & Bortell, 1983).

Aflatoxins have potent carcinogenic, teratogenic and mutagenic activity in many species and have been classified by the International Agency for Research on Cancer (1993) and Anwar (1993) as human carcinogens. Also, increased risk of hepatocellular carcinoma in individuals exposed to both aflatoxin and positive for hepatitis B surface antigen, suggests an interaction between these two risk factors (Dvorackova, 1990; Ross, *et al.*, 1992; Wild, 1993 and Hollstein *et al.*, 1993).

Histopathologic changes, including fatty liver, necrosis, and bile duct hyperplasia, were found in chickens given a high level of dietary aflatoxins (Carnaghan *et al.*, 1966).

Evidence of acute aflatoxicosis in humans has been reported from Taiwan and Uganda (Shank, 1977). The syndrome was characterised by vomiting, abdominal pain, pulmonary edema, and fatty infiltration and necrosis of the liver. More extensive documentation of an outbreak of putative aflatoxin poisoning was provided in 1974 from western India, Philippines and Nigeria (Krishnamachari *et al.*, 1975 a & b; Denning *et al.*, 1995 and Oyelami *et al.*, 1995).

Aflatoxins are also considered to be antifungal agents. It has been found that the crude aflatoxin inhibited sporulation in *Aspergillus niger*, *Cladosporium herbarum*, *Thamnidium elegans*, *Mucor hiemalis* and *Rhizopus nigricans* (Reiss, 1977). Moreover, aflatoxin B_1 suppressed photo-induced conidiation in the deuteromycete, *Trichoderma virida* (Betina & Spisiakova, 1976).

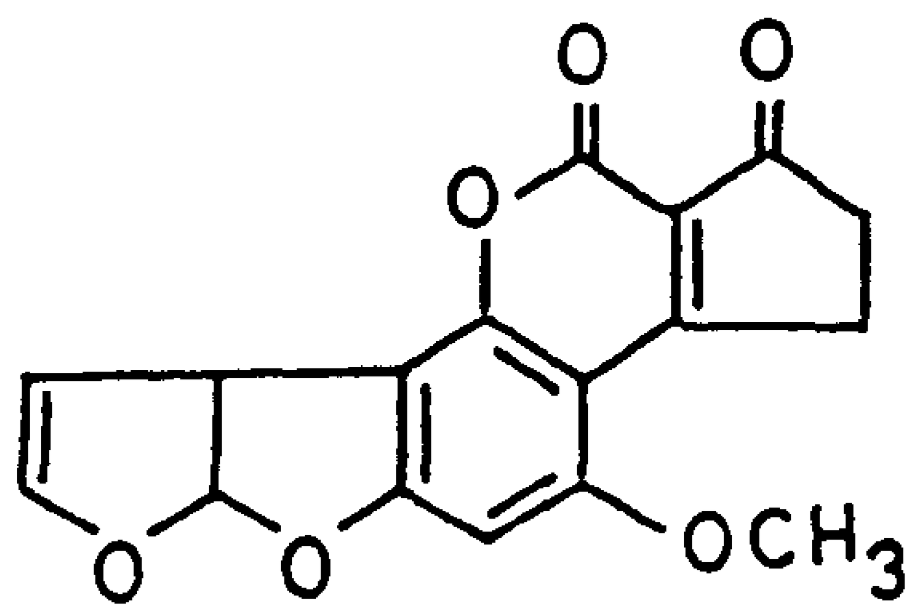
In addition, aflatoxins can produce changes in plant tissues. It has been reported that the germination of cress seeds (*Lepidium sativum*) is inhibited by aflatoxin treatment (Schoental & White, 1965).

Aflatoxin B_1 is also known to be mutagenic in *Neurospora crassa* (Ong & de Serres, 1972) and in *Drosophila melanogaster* (Lamb & Lilly, 1971).

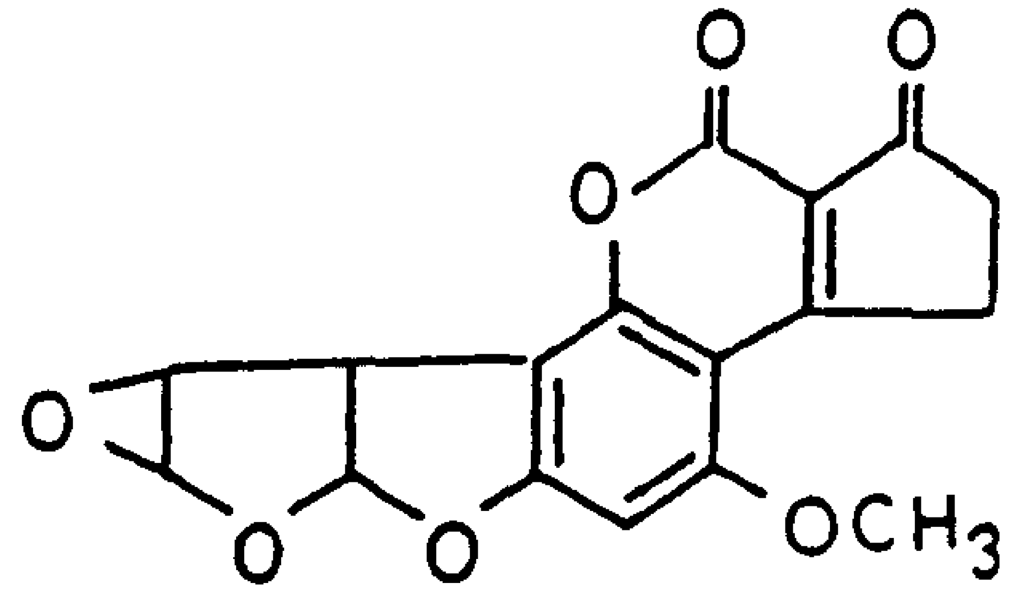
Unusual properties of AFB_1 make it an interesting model for examining mutagenesis by bulky DNA adducts. AFB_1 reacted with DNA at the guanine N-7 position (Wyllie & Morehouse, 1977; O'Brien *et al.*, 1983 and Putt *et al.*, 1995) (Fig. 5).

It is generally believed that aflatoxin B_1 requires metabolic activation to exert its toxicity and there are several competing pathways in the target hepatocyte which ultimately determine its toxicity. With one exception, all primary transformations of aflatoxin B_1 involve its conversion by the microsomal mixed function oxidase (MFO) system to hydroxylated metabolites which are conjugated with sulphate or glucuronic acid and readily excreted in urine or bile. These mechanisms usually detoxify the parent compound. The exception is the conversion of aflatoxin B_1 to aflatoxicol (AFL), a reversible reaction catalysed by cytoplasmic enzymes. The reversibility of this reaction provides a store of intracellular aflatoxin B_1 which enhances its toxic potential (Hendrickse, 1985). Reactive metabolites formed during aflatoxin metabolism have the capacity to react covalently with DNA, RNA and proteins. The covalent incorporation of an aflatoxin moiety into nucleic acids and protein is considered to be an important mechanism by which toxicity and carcinogenicity are initiated. The known and postulated metabolic transformations of aflatoxin B_1 are shown in Fig. 6.

Fig. 5: Reaction of metabolised Aflatoxin B_1 with DNA (guanine) (according to Wyllie & Morehouse, 1977 and O'Brien *et al.*, 1983).



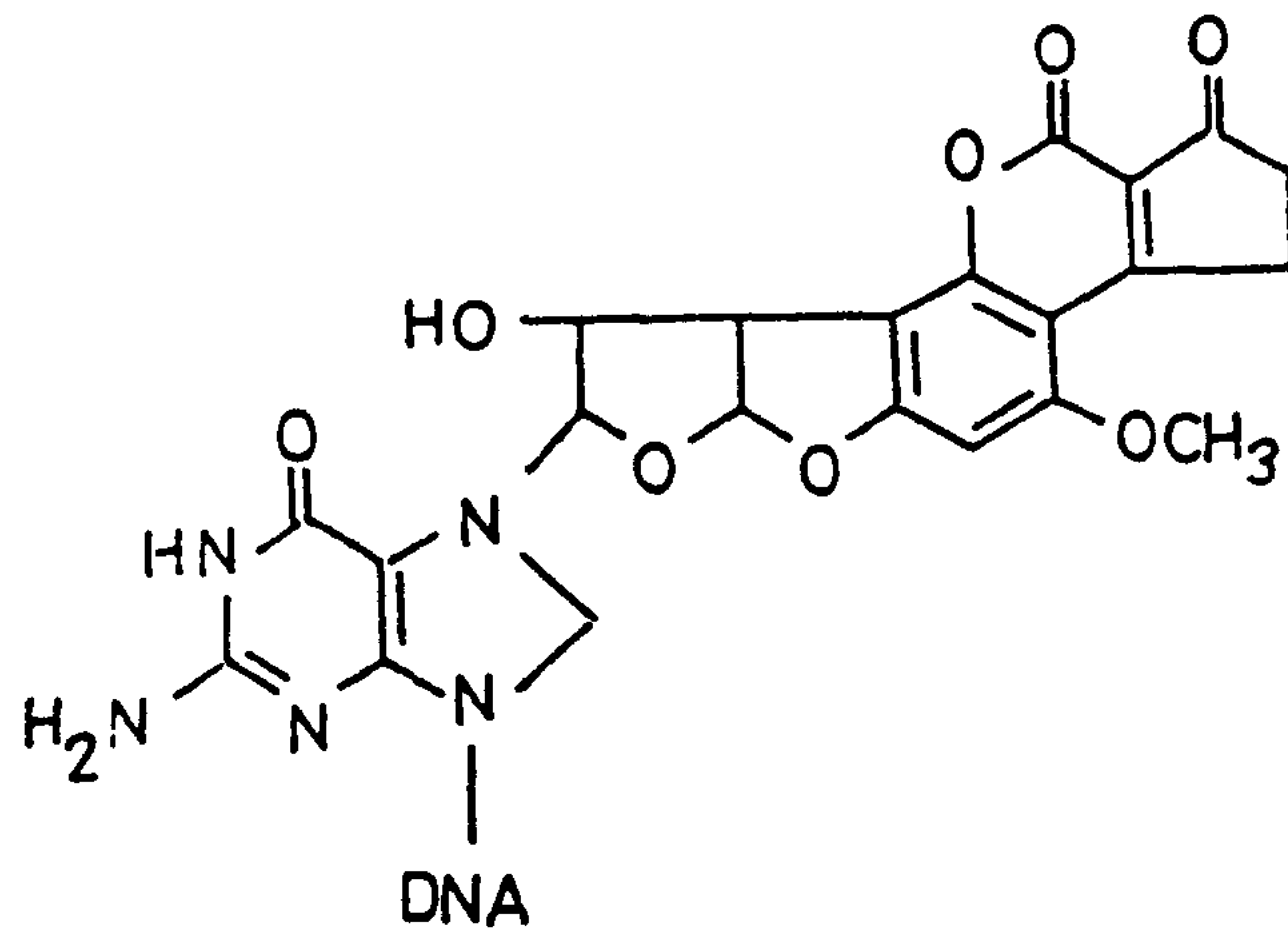
Oxidation



Aflatoxin B_1
(AFB_1)

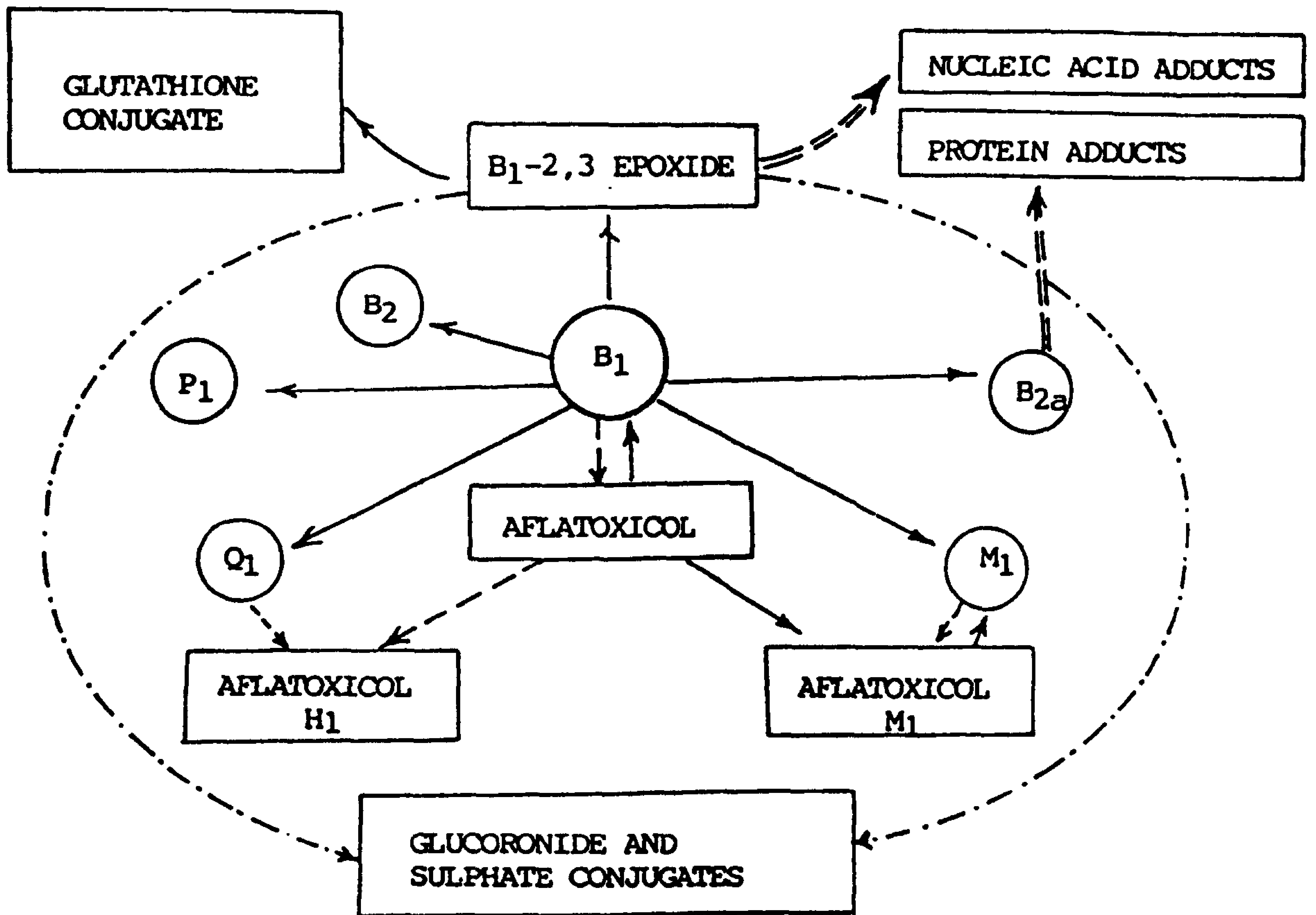
AFB_1-8,9-Epoxide

DNA
(Guanine)



AFB_1-N_7-Guanine

Fig. 6: Metabolic transformations of Aflatoxin B_1 (Hendrickse, 1985).



MICROSOMAL ENZYME REACTION

CYTOPLASMIC ENZYME REACTION

OTHER ENZYME REACTION

DIRECT CHEMICAL REACTIONS

Detection and assay of aflatoxins:

Since mycotoxin contamination is difficult to avoid, the most effective control measures depend on a rigorous program to monitor their presence in foods and feeds. Consequently, sensitive and accurate methods for analysis of mycotoxins in foods are essential for decreasing the risk of human exposure (Langone & Vunakis, 1976).

Different methods have been used for the qualitative and quantitative analysis of aflatoxins as follows:

1. Physical screening:

An example of an indirect physical field test is the so-called BGYF-test (Bright Greenish-Yellow Fluorescence). This rapid method is applicable for corn, wheat, rice, oats and barley but not for soybeans and peanuts (Bothast & Hesseltine, 1975). Under longwave U.V. light, the fluorescence spectrum that is characteristic of aflatoxin contamination depends not on the toxin itself but on the presence of Kojic acid which is also produced by *A. flavus*. This test is not absolute, however, it is only 50-80% reliable; moreover a positive test will only indicate that more definitive tests are required (Shotwell & Hesseltine, 1981).

2. Chemical analysis:

A minicolumn technique is a simple direct chemical assay. The first minicolumn was introduced by Holaday (1968) for the detection of aflatoxins in peanuts. After a simple extraction step, the extract is washed through a composite column, with one or more solvents. Then the miniature chromatography column is examined under longwave UV and intensity of fluorescence of the toxin band is compared with a separate column containing a known amount of standard aflatoxin.

The method does not distinguish between the different aflatoxins and it is, at the best, semi-quantitative.

Many quantitative analyses of food and feed for aflatoxin need appropriate extraction and clean up steps followed by thin layer chromatography (TLC) for the final detection and measurement of the toxins. Following isolation of aflatoxins, quantitative estimation relies upon visual comparison of the UV fluorescence with known standards and serial dilution to extinction.

As further information was obtained from the use of pure aflatoxins, more accurate methods of measurements were developed depending upon flourodensitometric measurements on the elute of TLC plates (Beljaars *et al.*, 1973 and Betina, 1985).

The Association of Official Analytical Chemists (AOAC) and the American Association of Cereal Chemists recommend the contaminants branch method, which is sensitive for aflatoxin B_1 and G_1 down to 1-10 μg Kg^{-1} (Seitz & Mohr, 1977).

Recently, new analytical methods using high performance liquid chromatography (HPLC) have been introduced. In general, HPLC has the advantage of being easier, faster and giving more reproducible results than comparable analysis using TLC, but has the disadvantage of being much more costly than TLC (Applebaum *et al.*, 1982 and Lee *et al.*, 1991).

3. <u>Biological assays</u>:

These assays are carried out when it is necessary to confirm the biological activity of aflatoxins that have been detected by physico-chemical methods. One of the earliest bioassays for a mycotoxin was the duckling test for aflatoxins. For several years following the discovery of aflatoxins, it was the only assay available and based on

the specific dose-related response of the duckling liver to aflatoxins (Sargeant *et al.*, 1961).

The chick embryo bioassay, the only one to be included in the AOAC official methods of analysis (1984), is sensitive to about 10 µg aflatoxin B_1.

Various other biological test systems have been used to screen for the presence of aflatoxins and other mycotoxins. These systems include plant seedlings, algae, bacteria, protozoa, tissue culture, shrimp larva and several laboratory animals. The brine shrimp assay (Harwig & Scott, 1971) has been widely used.

4. Immunological screening methods:

Recently, new methods have been described by Pestka *et al.* (1981) which show great promise. These methods employ radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA). The detection limits are 0.5 ng of aflatoxin M_1 ml^{-1} and 0.25 ng of aflatoxin M_1 ml^{-1}, respectively.

The ELISA method is superior to RIA in that radioactive compounds need not be used and analysis can be completed in only three hours. In addition, ELISA requires no extraction or clean-up. Thus the potential exists for development of commercially available kits which would permit routine surveillance for aflatoxin M_1 by dairies or regulatory agencies (Applebaum *et al.*, 1982 and Bacigalupo *et al.*, 1994).

Physical and nutritional factors influencing fungal growth and aflatoxin B_1 production:

1. Effect of pH:

Most fungi are grown over a broad pH range (3-8). However, as pH moves away from the optimum, the effect of other growth limiting factors may become apparent when superimposed on pH. Aflatoxin formation is completely inhibited

below pH 2.8-3.0 irrespective of the system used for pH adjustment (Horn & Wicklow, 1983 and Bullerman, 1985).

Basappa *et al.* (1970) and Gunasekaran (1981) studied the effect of pH on growth and aflatoxin production by *A. flavus*. The authors found that the optimum pH values for growth and aflatoxin production were 5-6 and 5-4.5, respectively. It seems that pH is an influential factor. High yields of aflatoxin by *A. parasiticus* were obtained with an initial pH value near neutrality (Jarvis & Mason, 1971).

2. Effect of incubation:

Temperature and incubation period are important factors affecting mycotoxin formation. Aflatoxins are produced by *A. flavus* and *A. parasiticus* in high amounts when incubated at 25-30°C for 7 to 21 days (Diener & Davis, 1966; Varma & Verma, 1987 and Salama *et al.*, 1989). When *A. flavus* was incubated at 12-13°C for 5 days, no aflatoxins were produced but when incubation extended to 3 weeks at the same degree, aflatoxins were excreted (Schindler *et al.*, 1967). Mirocha and Christensen (1984) has reported that incubation of *A. flavus* at 40-42°C inhibited toxin production.

3. Effect of carbon sources:

Carbohydrates are usually the major source of carbon for fungi. Most fungi can utilise a range of monosaccharides, oligosaccharides and polysaccharides, although uptake is normally restricted to monosaccharides (Romano & Kornberg, 1968 and Biollaz *et al.*, 1970). Mateles and Adye (1965) reported that the type of carbohydrate employed as the sole carbon source of a synthetic medium strongly influenced the synthesis of aflatoxin. They found that glucose, sucrose, and fructose yielded the greatest aflatoxin production, while lactose, maltose, xylose, sorbose, and glycerol

supported growth but little aflatoxin accumulation. Davis and Diener (1968) reported that glucose, ribose, xylose, and glycerol supported abundant growth and aflatoxin production (Detroy *et al.*, 1971). Moreover, Shih and Marth (1974b) found that maximum yield of aflatoxin from *A. parasiticus* was obtained with glucose.

4. **Effect of inorganic and organic nitrogen sources:**

High yields of aflatoxins have been obtained on media containing crude natural extracts, like corn steep liquor, peptone, yeast extract or malt extract (Wogan *et al.*, 1963; Wildman *et al.*, 1967; Hayes & Wilson, 1968; Diener & Davis, 1969; Venkitasubramanian, 1977; and El-Bazza, 1983).

It was found that the total aflatoxin production by *A. parasiticus* in Czapek's broth fortified with corn steep liquor was increased proportionately by increasing the concentration of corn steep liquor from 0.5% to 8.0%. Although both the fungal growth and aflatoxin production were stimulated by the addition of corn steep liquor to the basic medium, the stimulation of aflatoxin production was much greater than fungal growth (Schroeder, 1966).

Mateles and Adye (1965) studied the influence of other nitrogen sources on aflatoxin production and reported that toxin production was supported by ammonium sulphate and potassium nitrate but potassium nitrate was the better inorganic nitrogen source. However, Shih and Marth (1974a & b) reported that neither aflatoxin B_1 nor aflatoxin B_2 were detected from *A. parasiticus* grown on a medium containing 0.05% ammonium sulphate which was inoculated with spores. The results of Bhatnagar *et al.* (1986a) showed that when *A. parasiticus* was grown on a chemically defined medium containing ammonium sulphate and asparagine as nitrogen sources, ammonium ions are assimilated most rapidly from the medium.

Although *A. flavus* failed to produce aflatoxins in the presence of ammonium carbonate or ammonium chloride as a sole nitrogen source, it grew well and increased its productivity in presence of sodium nitrate, urea and ammonium nitrate (Salama *et al.*, 1989).

5. Effect of some amino acids:

Several workers have related rapid aflatoxin production to the presence of amino acids in the medium. The stimulatory action of methionine, proline and tryptophan on aflatoxin biosynthesis by *A. parasiticus* and *A. flavus* were also reported (Naik *et al.*, 1970). In addition, asparagine, alanine and aspartate were found necessary for higher toxin production by *A. parasiticus* (Reddy *et al.*, 1971 & 1972).

It has been found that, when proline and asparagine were used as sole nitrogen sources, they supported more growth of both *A. flavus* and *A. parasiticus* as well as aflatoxin production than tryptophan or methionine (Payne & Hagler, 1983). These authors added that, proline stimulated more toxin production in stationary cultures than the other tested amino acids, including asparagine, which is generally recognised as supporting good aflatoxin production.

6. Effect of different metal ions:

Trace metal elements play an important role in the regulation of secondary metabolite biosynthesis (Maggon *et al.*, 1977). Although several hypotheses have been put forward to explain the mineral element control of secondary metabolism, the mechanism of action has not yet been established.

Davies *et al.* (1967) examined the effect of some trace elements on synthesis of aflatoxin by *A. flavus*. They found that zinc was required for optimal toxin yield.

Also, Reddy *et al.* (1972) reported the necessity for zinc to achieve maximum aflatoxin elaboration.

Of all the trace elements, zinc seems to play a key role in the biosynthesis of many secondary fungal metabolites including the aflatoxins. At least twenty enzymes have been found to be zinc-dependent (Parisi & Vallee, 1969) which may partly account for its key role. Numerous workers have reported that zinc is essential for aflatoxin production and have noted that a relatively high level of zinc has a stimulatory effect on aflatoxin production (Diener & Davis, 1969). Zinc can have an inhibitory effect on toxin production when supplied at higher concentrations (Mateles and Adye, 1965). However, the low zinc requirement of the fungus reported by earlier workers may be due to the preexisting levels of zinc in the unrefined ingredients such as yeast extract, malt extract and peptone, that were used. Bassir and Adekunle (1972) stated that the absence of zinc completely blocks fungal growth and aflatoxin production. They added that the resistance or susceptibility of natural food commodities to aflatoxin production has been explained in terms of the presence or absence of adequate amounts of zinc .

The content and optimal biological availability of zinc, which may be essential and stimulatory, are not direct indices of the ability of substrates to support fungal growth with maximum aflatoxin production. Apparently, the Zn^{++} requirement for the biosynthesis of the same amount of aflatoxin will vary with the natural food substrate on which the fungus grows. This may explain the widely varying requirements for optimal toxin production reported by different investigators (Reddy *et al.*, 1972 and Maggon *et al.*, 1973).

Obidoa and Ndubuisi (1981) examined the production of aflatoxin (B_1, B_2, M_1, and M_2) in ten tropical foodstuffs inoculated with *A. flavus* and reported that aflatoxin production was not linearly correlated with the zinc levels of the food substrates. Their data indicated that optimal zinc requirement for maximal aflatoxin production was substrate specific.

Jones *et al.* (1984) studied the correlation between aflatoxin production and zinc content of chicken feed taken from feed containers at chicken farms, and found that stricter control of zinc levels during manufacture could reduce aflatoxin contamination of feed consumed by chickens.

Co^{2+}, another metal ion, was reported to stimulate the growth of *A. parasiticus* and its productivity of aflatoxin. In parallel with this, Tulpule (1969) observed that the absence of Co^{2+} from the medium reduced the yield of aflatoxin. However, Maggon *et al.* (1973) found that Co^{2+} had no significant effect on aflatoxin production.

Concerning the effect of Mn^{2+}, it was reported that this slightly reduced the growth of *A. flavus* as well as aflatoxin formation (Mateles & Adye, 1965; Lee *et al.*, 1966 and Davis *et al.*, 1967). However, Detroy and Ciegler (1971) did not observe any effect of Mn^{2+} on aflatoxin production.

Regarding the effect of ferric ion, it was found that it is required for maximum productivity of toxin by *A. flavus* (Davis *et al.*, 1967). Also, it was reported that its omission from the growth medium of *A. flavus* (Davis *et al.*, 1967 and Maggon *et al.*, 1973) resulted in great reduction of the fungal growth and its productivity of toxin. In contrast, Reddy *et al.* (1972) did not find any increase in the toxin production by the same fungus on adding iron to the growth medium.

Nickel and aluminium are known to affect the function of several metabolic pathways (Underwood, 1962). The effect of nickel and aluminium was studied on aflatoxin production by two strains of *A. flavus* (Malini *et al.*, 1984). At lower concentrations, aluminium stimulated aflatoxin production, whereas nickel increased the total aflatoxin production at higher concentrations.

Mg^{2+}, another metal ion, was found to enhance fungal growth as well as aflatoxin production (Maggon *et al.*, 1973 and Tiwari *et al.*, 1986b). Omission of Mg^{2+} from the growth medium decreased growth but increased aflatoxin production (Davis *et al.*, 1967). The reason for such behaviour is not well understood.

7. **<u>Effect of water activity</u>:**

The availability of water to microorganisms can be measured by the water activity (a_w). Scott (1957) showed that a_w effectively quantified the relationship between moisture in foods and the ability of microorganisms to grow on them. The water activity is defined as the ratio of the water vapour pressure of the substrate to the vapour pressure of pure water at the same temperature and under the same pressure. It is an expression of the amount of water available in grams.

Water activity is numerically equal to equilibrium relative humidity (ERH) expressed on a scale 0-1. If a sample of food is held at constant temperature in a sealed container until the water in the sample equilibrates with the water vapour in the enclosed air space then:

$$a_w \text{ (food)} = \text{ERH (air)}/100.$$

Ascomycetous fungi comprise most of the organisms capable of growing below 0.9 a_w. Fungi capable of growing at low a_w, in the presence of extraordinarily high solute concentrations both inside and outside must be ranked among the most highly

evolved organisms on earth (Pitt & Hocking, 1985). Even among the fungi, this evolutionary path must have been of the utmost complexity. The ability to grow at very low a_w is confined to only a handful of genera (Pitt, 1975).

The degree of tolerance to low a_w is most simply expressed in terms of the minimum a_w at which germination and growth can occur. Fungi which are able to grow at low a_w are termed xerophiles (Pitt, 1975).

With respect to the optimum value of a_w required for the production of aflatoxin B_1 by *A. flavus* it was found that its value was 0.99 (Northolt *et al.*, 1976) whereas Diener & Davis (1967) obtained a_w values of 0.95 and 0.99 depending on the substrate.

On studying the production of aflatoxin B_1 in peanuts, Diener and Davis (1967) reported an optimum value of 0.95 a_w whereas no significant quantity of aflatoxin was found at 0.85 a_w. A minimal value of 0.84 a_w for aflatoxin production in corn was reported by Hunter (1969) but a lower a_w value than 0.84 suppressed aflatoxin production.

8. Effect of some food preservatives:

The presence of toxigenic fungi in a product does not automatically mean the presence of mycotoxins, especially if growth has not occurred (Ray & Bullerman, 1982). Thus, it is obvious that if the growth of toxigenic fungi can be prevented, subsequent contamination with mycotoxins will also be prevented.

Various chemical agents such as acids, bases and oxidising agents have been known as inactivators of aflatoxins. It was noticed some years ago that certain vegetables and fruits such as the cranberry, which contains benzoic acid, are easily preserved (Tooley, 1971). Also, the same acid was found to occur naturally in prunes,

cinnamon and cloves (Reddis, 1957 and Chichester & Tanner, 1972). As a result, benzoic acid and benzoate salts have become widely used as chemical preservatives. They are quite harmless and tasteless at the small concentrations permitted in food. Benzoic acid can act as preservative in many beverages such as cordials, fruit juices, cider, and in coffee extract. In addition, sodium benzoate (C_6H_5COONa) has been widely employed as an antimicrobial agent in foods, and it is generally considered to be most active against yeasts and bacteria but less so against moulds. It is most suitable for use in foods and beverages. Sodium benzoate has the advantage of low cost (Chichester & Tanner, 1972). It is used in carbonated and still beverages, syrups, fruit salads, icings, preservers, etc. Since the sodium salt of benzoic acid is more soluble in water than the acid, the former is generally used (Furia, 1972). Little is known regarding the effect of sodium benzoate on growth of Aspergilli and their production of aflatoxins, but further investigation would be worthwhile.

An alternative strategy would be to destroy mycotoxins that have formed by using oxidising agents as hydrogen peroxide (H_2O_2) which is considered an effective compound in detoxifying contaminated foods (Sreenivasamurthy *et al.*, 1967 and Tabata *et al.*, 1994). Hydrogen peroxide is also used as a preservative for milk in some tropical countries where refrigeration is either unavailable or is too expensive. It is also used as an additive in such countries to improve the quality of milk to be used for cheesemaking. El-Gazzer and Marth (1988) found that hydrogen peroxide at a concentration of 0.3 or 0.5% completely prevented growth and aflatoxin production by *A. parasiticus* up to 90 days at 28°C.

Other chemicals have antimycotic properties, e.g. sorbic acid and sorbate appeared to have an inhibitory effect on the growth of toxic moulds as wells as on

mycotoxin production. The degree of inhibition is dependent on the amount of the chemical. Bullerman (1983) found that in liquid media containing potassium sorbate, growth and aflatoxin production by *A. flavus* and *A. parasiticus* were completely inhibited. Propionic acid has been shown to inhibit *A. flavus* naturally present in corn (Herting & Drury, 1974 and Chourasia, 1993). Buchanan and Ayres (1976) reported that at concentration as low as 0.2% propionic acid completely inhibited growth and aflatoxin production by *A. parasiticus*.

9. **Effect of some spices:**

The antimycotic and antiaflatoxigenic effects of some spices were reported by several investigators. It has been known since ancient times that essential oils of certain spices have preservative qualities. The use of these oils by the ancient Egyptians in the processes of mummification of their dead is well documented. Cinnamon powder can inhibit the toxin production and the growth of toxigenic fungi such as *A. flavus, A. parasiticus, A. ochraceus, A. versicolor* and *Fusarium solani* (Hitokoto *et al.*, 1977 and 1980; Mabrouk & El-Shayeb, 1979; and Patkar *et al.*, 1993 and 1994). Pepper and red chilli were also found to prevent aflatoxin production and inhibited the growth of *A. flavus* as well (Scott & Kennedy, 1973 & 1975; Hitokoto *et al.*, 1977; and Masood *et al.*, 1994). Mabrouk & El-Shayeb (1979) stated that, on the addition of low concentrations of cardamon on *A. flavus*, aflatoxin production was decreased.

Whole ginger, Jamaica, red and white pepper could support the growth of *A. flavus* and production of aflatoxins (Flannigan & Hui, 1976). Clove had fungistatic activity, while the activities of black pepper, pepper mint, cumin and ginger were

considered antiaflatoxigenic rather than fungistatic (Mabrouk & El-Shayeb, 1980; Garrido, *et al.*, 1992; and Ito *et al.*, 1994).

10. Effect of fatty acids:

There have been sporadic reports regarding the effect of fatty acids on aflatoxin production. The saturated fatty acids (stearic and palmitic) were found to have no effect on, or slightly depressed aflatoxin B_1 production while unsaturated fatty acids (oleic and linoleic) considerably increased the synthesis of toxin (Jemmali *et al.*, 1974 and Chulze *et al.*, 1991). Also, a 34-fold increase in aflatoxin production was observed with linoleic acid, with an inverse relationship between aflatoxin production and mycelium mass (Tiwari *et al.*, 1986a). However, Priyadararhini & Tulpe (1980) reported the inhibitory effect of linoleic acid on aflatoxin production.

Other fatty acids were found stimulators for toxin production by fungi (Jemmali *et al.*, 1974).

11. Effect of some vitamins:

Vitamins are usually required by organisms in micromolar concentration or less and function as coenzymes. The literature on the vitamin requirements of fungi has been reviewed in detail by Fries (1965) and Griffin (1981). A fungus that requires a vitamin for growth may be described as auxotrophic; however, the one that can synthesise the vitamin is prototrophic. Fungi, like all other organisms so far known, require minute amounts of specific organic compounds for growth. Most of the known vitamins have a catalytic function in the cell as coenzymes or constituent parts of coenzymes.

Little information regarding the effect of vitamins on aflatoxin production were available.

12. Enzymes and their relation to aflatoxin production:

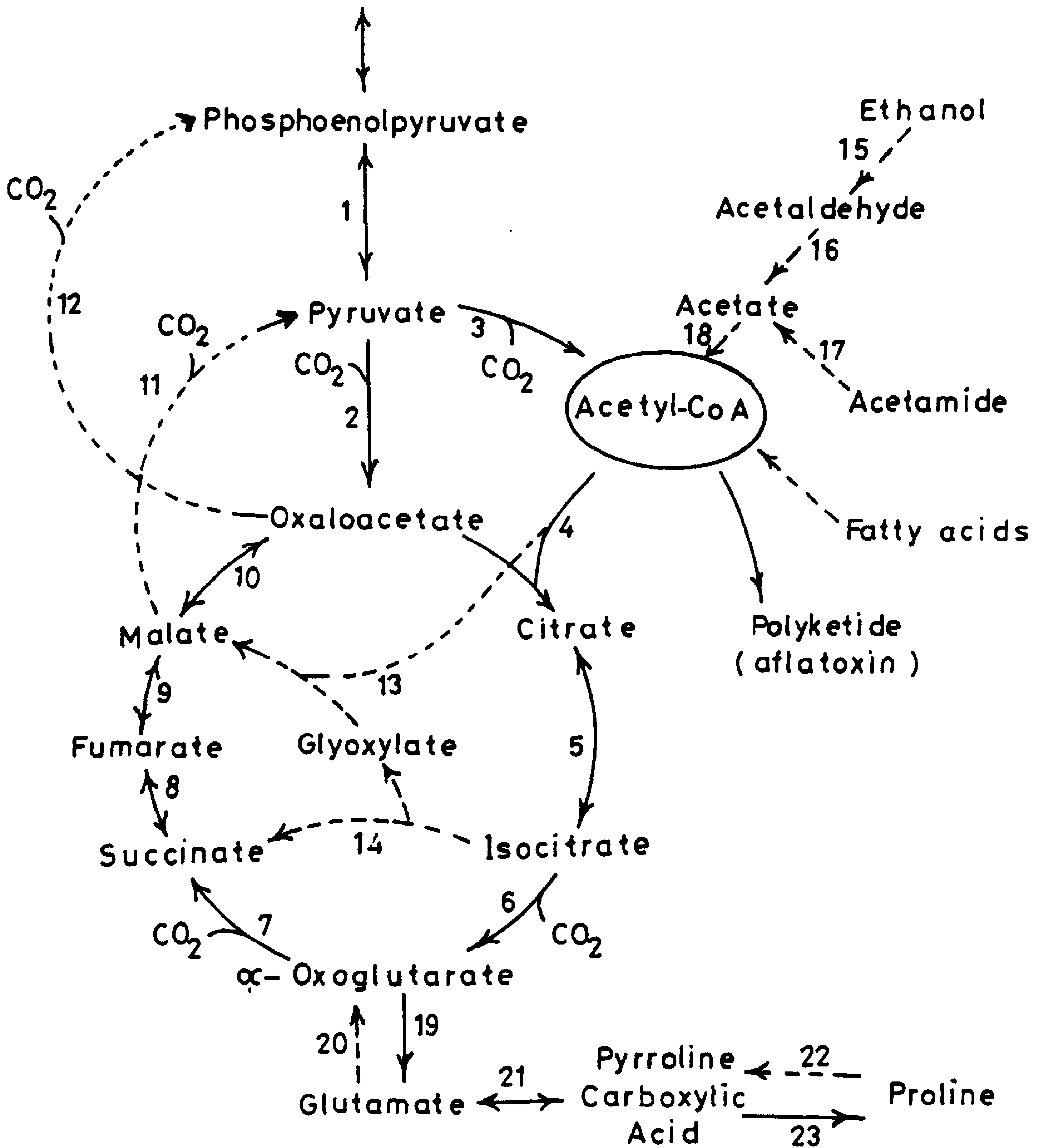
Hsieh and Mateles (1970) concluded that aflatoxins are synthesized from acetyl CoA derived from oxidation of pyruvate by pyruvate dehydrogenase complex. In addition, it has been reported that pentose phosphate pathway is the primary sites for NADPH-generation of *A. parasiticus* (Buchanan & Lewis, 1984). Singh & Hsieh (1976) have demonstrated that *in vitro* conversion of sterigmatocystin to aflatoxin B_1 is NADPH-dependent. Furthermore, Shih & Marth (1974a) have suggested that elevated levels of NADPH stimulate aflatoxin production. However, Niehaus & Dilts (1982) proposed that an elevated NADPH/NADP ratio favours fatty acid synthesis, whereas a depressed NADPH/NADP ratio favours polyketide formation. Thus, this contradiction needs to be resolved.

It was reported that aflatoxin synthesis occurs during a period of decreased tricarboxylic acid cycle activity (Buchanan & Lewis, 1984). In addition, Gupta *et al.* (1977) and Maggon *et al.* (1977) hypothesized that reduced TCA cycle activity leads to an accumulation of TCA cycle intermediates, which leads to a shunting of acetyl CoA to aflatoxin synthesis. Fig. 7 shows the proposed scheme by McCullough *et al.* (1977) which explains the relationship between TCA cycle and polyketide synthesis. Also, Maggon *et al.* (1977) observed differential changes among selected TCA cycle enzymes in *A. parasiticus* as a function of fungal growth.

Generally, little is known about the enzymatic processes involved in aflatoxin biosynthesis and the concept was developed largely by organic chemists, particularly by Birch (1967) as a result of an analysis of the structures of natural products.

Fig. 7: Scheme which explain the relationship between TCA cycle and polyketide (aflatoxin) synthesis (McCullough *et al.* (1977).

The enzyme indicated are: 1, pyruvate kinase; 2, pyruvate carboxylase; 3, pyruvate dehydrogenase; 4, citrate synthase; 5, aconitase; 6, isocitrate dehydrogenase; 7, oxoglutarate dehydrogenase; 8, succinate dehydrogenase; 9, fumarase; 10, malate dehydro-genase; 11, malic enzyme; 12, PEP carboxykinase; 13, malate synthase; 14, isocitrate lyase; 15, alcohol dehydrogenase; 16, aldehyde dehydrogenase; 19, NADP-glutamate dehydrogenase; 20, NAD-glutamate dehydro-genase; 20, NAD-glutamate dehydrogenase; 21, pyrroline-5-carboxylate dehydrogenase; 22, proline oxidase; 23, pyrroline-5-carboxylate reductase.


Phosphoenolpyruvate
Ethanol
15
CO_2
Acetaldehyde
1
16
12
Acetate
CO_2
Pyruvate
3
18
17
CO_2
CO_2
11
Acetyl-CoA
Acetamide
2
Oxaloacetate
4
Fatty acids
10
Malate
Citrate
Polyketide
(aflatoxin)
9
13
Fumarate
Glyoxylate
5
8
14
Succinate
Isocitrate
6
CO_2
7
CO_2
α- Oxoglutarate
20
19
Pyrroline
22
21
Glutamate
Carboxylic
Proline
Acid
23

AIM OF THE WORK

There is a critical need for investigations of contamination of foodstuffs by *Aspergillus* in general, and Abha market was selected as a suitable location for examining this problem

The present investigation highlights the critical importance of indicating the levels of contamination of the most consumable commodities particularly coffee and rice, since coffee is the most common hot drink of Arabs and rice represents the main food diet for the people in Saudi Arabia, and both are consumed in great quantities.

Consequently, if contamination is widespread it would represent a significant health risk to many of the population. As far as my knowledge is concerned, the present work is the first attempt to study the fungal contamination and aflatoxins production in commons foods in Saudi Arabia, particularly at Abha region.

In order to achieve this goal, a plan of study was conducted to tackle the following points:

I. Collection and measurements of levels of contamination by fungi and aflatoxins in the foods.

II. Few workers had looked at the production of mycotoxins by *A. parasiticus*. Therefore, it is aimed in the present work to get more information about the factors affecting aflatoxin B_1 production by this fungus. These factors are: pH, temperature, incubation period, carbon and nitrogen sources, amino acids, metal ions and water activity.

III. Exploring the control of aflatoxin contamination by testing the effect of chemical preservatives, spices, known promoters and inhibitors of aflatoxin B_1 production, fatty acids and vitamins on growth of *A. parasiticus* and its productivity of aflatoxin B_1. This included testing a number of compounds (sodium benzoate, sodium bicarbonate, glutathione, indole-3-acetic acid, phenol, catechol, stearic acid and vitamins C and D_2).

IV. Measuring the activities of some enzymes thought to participate in production and regulation of aflatoxin during the metabolism of fungal growth, hopefully to know which group of enzymes play the critical role in aflatoxin B_1 production and how could we control their activity.

The aim was to provide results which would be valuable, not only in calling for more serious attention to be paid to aflatoxins, especially in Saudi Arabia, but also in suggesting improved methods for controlling aflatoxin contamination.

CHAPTER II

MATERIALS AND METHODS

1. Collection of food samples:

Forty samples of cereals and spices were collected from different public markets in Abha region, South-West of Saudi Arabia. These samples were classified into 4 categories namely, carbohydrates-rich commodities, protein-rich commodities, oil-rich commodities and spices (Table 3) (Robinson, 1978 and Paul & Southgate, 1978). All samples were collected during October 1988 (100 g of each) and stored in refrigerator until use.

2. Microorganisms:

Aspergillus parasiticus NRRL 2999 was obtained from Northern Regional Research Laboratories NRRL, Peoria, Illinois, U.S.A. The strain was subcultured on potato-dextrose-agar for 14 days at 25°C and stored at 4°C until needed.

3. Preparation of spore suspensions:

A. parasiticus NRRL 2999 was used throughout the study. All cultures were grown on slants of potato dextrose agar medium and incubated for 7 days at 28 ± 2°C. Spores were harvested by adding sterilised Tween 80 solution (0.02% v/v) and filtered through several layers of sterilised cheese cloth. The number of spores was estimated by haemocytometer and the suspension adjusted to contain approximately 10^6 spore ml^{-1}. The suspension was kept at 4 to 5°C until needed.

4. Media used:

Three different media were used in the present investigation to isolate and count fungi. These media were the following:

Table (3): List of food samples collected from Abha markets

Group	Common Name	Scientific name
(A) Carbohydrate-rich commodities	1. Barley	*Hordeum distichum*
	2. Coffee beans	*Coffea arabica*
	3. Coffee cortex	*Coffea arabica*
	4. Date (I)	*Phoenix dactylifera*
	5. Date (II)	*Phoenix dactylifera*
	6. Date (III)	*Phoenix dactylifera*
	7. Garlic (dry)	*Allium sativum*
	8. Millet	*Penicillaria spicata*
	9. Onion (dry)	*Allium cepa*
	10. Maize	*Zea mays*
	11. Rice	*Oryza sativa*
	12. Wheat (seeds)	*Triticum vulgare*
	13. Wheat (flour)	*Triticum vulgare*
	14. Poultry diet	-
(B) Protein-rich commodities	1. Chick-pea	*Cicer arietinum*
	2. Cow pea	*Vigna sinensis*
	3. Fenugreek	*Trigonella foenum-graecum*
	4. Haricot	*Phaseolus vulgaris*
	5. Horse-bean (broad bean)	*Vicia faba*
	6. Lentil	*Lens esculenta*
	7. Lupin seed	*Lupinus termis*
	8. Pea	*Pisum sativum*
(C) Oil-rich commodities	1. Catechu nut	*Acacia catechu*
	2. Hazel nut	*Corylus avellana*
	3. Mahaleb	*Cerasus mahaleb*
	4. Pea nut	*Arachis hypogea*
	5. Pistachio nut	*Pistacia vera*
	6. Sesame	*Sesamum orientale*
	7. Walnut	*Juglans nigra*
(D) Spices	1. Anise	*Pimpinella anisum*
	2. Black pepper	*Piper nigrum*
	3. Cardamon	*Elettaria cardamomum*
	4. Chili	*Capsicum frutescens*
	5. Cinnamon	*Cinnamomum zeylanicum*
	6. Clove	*Eugenia aromatica*
	7. Coriander	*Coriandrum sativum*
	8. Cumin	*Cuminum cymimum*
	9. Turmeric	*Curcuma longa*
	10. Garam Masala powder	-
	11. Ginger	*Zingiber officinale*

Sabouraud-Dextrose Agar Medium (SDA):

Ingredient	Amount
Peptone	10 g
Glucose	40 g
Yeast extract	5 g
Agar	15 g
Distilled water	1.0 L
pH	5.6

(Oxoid, 1982)

Potato-Dextrose Agar Medium (PDA):

Ingredient	Amount
Potato	200 g
Dextrose	20 g
Agar	15 g
Distilled water	1.0 g
pH	5.6

(Oxoid, 1982)

Czapek's-Dox Agar Medium (CDA):

Ingredient	Amount
Sucrose	30.0 g
K_2HPO_4	1.0 g
KCl	0.5 g
$Mg\ SO_4$	0.5 g
$Na\ NO_3$	2.0 g
$Fe\ SO_4$	0.01 g
$CuSO_4$	0.005 g
$ZnSO_4$	0.01 g
Agar	15.0 g
Distilled water	1.0 L
pH	7.0

(Oxoid, 1982)

5. Isolation of fungi from food samples:

The dilution plate method adopted in the present investigation was that described by Johnson *et al.* (1959). Twenty-five grams from each ground food sample were transferred to aliquots of 250 ml sterile distilled water in 500-ml Erlenmyer flasks fitted with rubber stoppers. The flasks were then shaken using a rotary shaker for 30

min. Each suspension was then subjected to serial dilution and three replicates were prepared from the suitable dilution which permit the growth of countable plates.

One ml aliquots of each dilution were placed in sterile Petri dishes. Approximately 15 ml of the desired medium previously melted and cooled down to 45°C were then added. Chloramphenicol (0.05 gl^{-1}) was added to the medium to suppress any bacterial growth. Plates were incubated at 28 ± 2°C for 7 days and were then examined. Fungal isolates were classified and identified according to Thom & Raper (1945), Raper & Thom (1949), Gilman (1957), Barnett & Hunter (1972) and Nelson *et al.* (1983). The identification of fungal isolates was carried out in Mycotoxin Lab., the National Research Centre, Cairo, Egypt. Pure cultures were maintained on potato-dextrose agar slants at 5 ± °C as stock cultures.

6. Extraction of aflatoxins:

6.1. Extraction from cultures on chemically defined media:

One ml aliquot of spore suspension (about 10^6 spore ml^{-1}) prepared as described above was used to inoculate 250 ml flasks containing 50 ml Czapek's-Dox medium (liquid culture) without agar. The incubation time used in the present study was 10 days at 28°C. At the end of incubation period, the culture was filtered, the volume of filtrate was measured and an equal volume of chloroform added and mixed thoroughly. The chloroform containing aflatoxins were separated using a separating funnel. The upper aqueous layer was re-extracted several times with chloroform for complete separation. The combined chloroform layers were drained through a column filled with anhydrous sodium sulphate, and evaporated to dryness. The residue was transferred into a screw cap vial containing 1 ml of acetone and dried with steam bath (50°C). The dried extract was kept in the refrigerator at -5°C until analysis.

6.2. Extraction from food samples:

Aflatoxins were extracted from food samples according to the method described by Schuller & Van Egmond (1983). Fifty grams of each ground sample was added to a 500-ml conical flask containing 25 ml distilled water and 250 ml chloroform. The flasks were shaken for 30 min on a rotary shaker and the suspension was filtered. The resulting extract was treated as described above and used for analysis.

7. Aflatoxin purification:

Purification of the final extracts was carried out according to Takeda *et al.* (1979) with some modification. Thirty ml chromatographic tube (22 × 330 mm) was plugged with glass wool. Two-thirds of the tube was filled with chloroform, then 5 g anhydrous sodium sulphate, 10 g of silica gel (particle size 0.05 to 0.20 mm) were added and packed carefully to eliminate air bubbles. The column was left standing for 15 min. An additional amount (15 g) of sodium sulphate was then added. The amount of the chloroform added to the column was adjusted to just above the upper surface layer of the sodium sulphate. Fifty ml of the extracted aflatoxins were mixed with 100 ml n-hexane and transferred to the column. After the addition of each solvent, the liquid was adjusted to be just above the surface layer of the sodium sulphate. Finally, 100 ml of diethyl ether were added to the column. For ensuring good separation, the flow rate of the liquid was about 8-12 ml min^{-1}. The eluate was then evaporated to dryness on a steam bath. The dry extract was dissolved and stored in 1 ml chloroform at -5°C for chromatographic analysis.

8. Detection and determination of aflatoxin by TLC method:

Thin-layer chromatography (TLC) was used for identifying and estimating the quality and quantity of aflatoxin in the sample extract (FAO & UNEP, 1989; and FAO, 1990). A mixture of chloroform: acetone (9:1, v/v) was used to separate the four aflatoxins B_1, B_2, G_1, and G_2 on 20 × 20 cm TLC-plastic sheets of silica gel-60. The sample extract containing aflatoxins was loaded on the silica gel plates. Standard aflatoxins B_1, B_2, G_1, and G_2 (supplied from Sigma Chemical Company, U.S.A.) were used for comparison with the unknown samples. The plates were developed in a glass jar containing the chloroform: acetone mixture as solvent. The plates were removed from the jar and the aflatoxins were examined under long wave UV light (365 nm). Sample Rfs were compared to those of the standards. Aflatoxins B_1 and B_2 showed blue fluorescence while G_1 and G_2 gave a greenish-blue fluorescence. For confirmation, a plate was sprayed with a fine mist of H_2SO_4 50% (v/v) solution in water and viewed under long wave UV. Aflatoxins B_1 and B_2 as expected were found to have yellow fluorescence and G_1 and G_2 yellow-blue fluorescence. The quantities of aflatoxins (B_1 and B_2, G_1 and G_2) were determined by fluorodensitometer (TLD-100 vitatron), following the method described by Shannon *et al.* (1983) and FAO (1990). Scanning on densitometer with settings of 365 nm for excitation and 445 nm for emission was carried out. The concentration of aflatoxin in samples were calculated as follows:

$$\mu g Kg^{-1} = \frac{B.Y.S.V.}{Z.X.W.}$$

where:

B = average area of aflatoxin B_1 peak from sample spots.
Y = concentration of aflatoxin B_1, standard, $\mu g\ ml^{-1}$.
S = µl aflatoxin B_1, standard spotted.
V = final volume of sample extract (µl).
Z = average area of aflatoxin B_1 peaks from standard spots.
X = µl sample extract spotted.
W = mass of sample (g or ml) represented by final extract.

9. Bioassay of aflatoxins:

9.1. Microorganism cultures:

The following bacterial strains were provided by the Cairo Microbiological Resource Centre (CAIM), Ain Shams University, Egypt:

Bacillus megaterium	CAIM 1057
Bacillus cereus	CAIM 1283
Escherichia coli	CAIM 1319

9.2. Aflatoxin standards and sample extracts:

Pure aflatoxins B_1, B_2, G_1, and G_2 were diluted with chloroform to give a concentration of 0.5 $\mu g\ \mu l^{-1}$. The mixture of chloroform extract of food samples containing aflatoxins were examined for the toxic effect by microbiological assay to confirm the presence of aflatoxins B_1, B_2, G_1, and G_2 using bacteria such as *B. megaterium*, *B. cereus* and *E. coli*.

9.3. Preparation of bacterial inoculum:

Tryptone-yeast extract-glucose (TYG) broth and agar were used for this investigation. The cultures were prepared by inoculating a tube of TYG broth with the appropriate bacterium and incubated at 37°C for 24 h. One ml of the inoculum preparation was added to each 100 ml of melted and cooled (50°C) TYG agar when assaying with *B. megaterium*, *B. cereus* and *E. coli*. After inoculation, the TYG agar was swirled to ensure uniform cell distribution and 10 ml of the seeded agar were then aseptically pipetted into sterile glass Petri dishes, and allowed to solidify.

9.4. Assay procedure:

The methods of Brace *et al.* (1970) and El-Sayed *et al.* (1989) were used in the present study. Discs prepared from filter paper were placed upon a wire support to ensure that the disc absorbed all the solvent that was delivered into it. To determine sensitivity limits and the response of each studied microorganism, discs were prepared from the standard solutions containing 2, 4, 6, 8, 10, 15 and 20 µg of aflatoxins B_1, B_2, G_1, and G_2 per disc. Chloroform extracts of samples was added at the rate of 1, 2, 4, 6, 8, 10, 15, and 20 µl per disc. Discs containing 10 or 20 µl of only solvent were prepared and used as control. Solvent controls, standard toxin and chloroform extract of samples were slowly applied to the disc dropwise using microliter syringe.

The discs were allowed to dry for approximately 10 min and then four discs in each case were evenly spaced on the agar surface of each of the plates seeded with bacteria. The plates were then inverted and pre-incubated at 10°C for 30 min to allow uniform diffusion into agar. After pre-incubation, plates were incubated for 24 h at 37°C. The resulting inhibition zones around the bacterial mass were then measured.

10. Determination of fungal growth:

10.1. Dry weight method:

In liquid culture experiments, the dry weight method was used to determine fungal growth. This method was adopted from Cochrane (1965) and Kane & Mullins (1973). The grown mat of the fungus was collected by filtration, dried to a constant weight at 70-80°C for 24 h.

10.2. Estimation of mycelium by chitin measurement:

Chitin analysis for the estimation of mycelium growth on coffee and rice samples was performed to study the effect of water activity on the growth of *A. parasiticus* (for details see section 11.9). The method used was adopted from that of Thornton *et al.*; (1991). *Aspergillus parasiticus* dry spores were spread out using a clean sterile brush onto the surface of both the coffee beans and rice grains. Apparatus was resealed and the samples were further incubated for 6 weeks, after which samples were taken out. Aflatoxin B_1 production was determined in seeds of coffee beans and rice grains after extraction as described in section 6.2.

The mycelial growth was measured by determining the chitin content. One gram of either coffee beans or rice grains on which the fungus has been grown was taken and ground in 70% acetone. The extract was centrifuged at 1000 r.p.m. for 10 min. The pellet was collected and washed with a small volume of distilled water and the supernatant was discarded. The pellet was suspended in 3 ml of 21.4 M KOH and autoclaved for 1 h at 121°C to deacetylate any chitin present into chitosan. The suspension was cooled and 8 ml of 75% (v/v) ethanol added and chilled at -5°C for 15 min. An amount (0.9 ml) of celite suspension (1 g celite 545 + 20 ml 75% v/v ethanol) was allowed to stand for 2 min, then layered onto the top of the KOH-containing suspension and centrifuged as mentioned above. The pellet obtained was washed with 40% (v/v) ethanol followed by washing with distilled water two times. The pellet was suspended in 1.5 ml distilled water. Then, 1.5 ml of each of 5% (w/v) $NaNO_2$ and 5% (w/v) $KHSO_4$ were added to this suspension and mixed for 15 min followed by centrifuging at 1000 r.p.m. for 2 min. An aliquot (1.5 ml) of the resulting supernatant was used for colorimetric assay.

Colorimetric assay:

Aliquots (0.5 ml) of 12.5% (w/v) ammonium sulphamate was added to 1.5 ml of the above prepared supernatant and mixed rapidly for 5 min. Aliquots (0.5 ml) of MBTH (3-methyl-2-benzothiazolinone-1-hydrazone hydrochloride) was then added and the mixture was heated at 100°C in a water bath for 3 min. It was then cooled down to room temperature and 0.5 ml of 0.5% (w/v) $FeCl_3$ (0.83 g $FeCl_3 6H_2O$ in 100 ml distilled water) was added for converting chitosan into glucosamine and left to stand for 30 min. The absorbance was measured at 650 nm.

Standard curve:

Different concentrations of glucosamine, namely 20, 40, 60 and 100 µg, were prepared. To 0.5 ml of each concentration, half ml of 5% (w/v) $NaNO_2$ and 5% (w/v) $KHSO_4$ were added. Samples were then treated as described above and a standard curve was graphically obtained.

11. Physical and nutritional factors influencing the growth of *A. parasiticus* and aflatoxin B_1 production:

11.1. Kinetics of growth and aflatoxin B_1 production:

To determine the optimal incubation time of growth and aflatoxin production, *A. parasiticus* was grown on Czapek's-Dox liquid medium adjusted to pH 6.0. The mycelial dry weight and aflatoxin B_1 production were determined after 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 days of incubation at 30°C.

11.2. Effect of pH:

To find out the optimal pH value for the fungal growth and aflatoxin production, each of them was measured under different pH values namely 3, 4, 5, 6, 7,

8, and 9 in Czapek's-Dox liquid medium. Citrate-phosphate buffer (citric acid + dibasic sodium phosphate) was used to obtain pH values 3-7 (Mcilvaine, 1921). Phosphate buffer (monobasic sodium phosphate + dibasic sodium phosphate) (Sorenson, 1909) was used for pH 8 and borate buffer (boric acid + borax) for pH 9.0 (Holmes, 1943). Equal amounts of double concentration of both Czapek's-Dox liquid medium and the buffer solutions were sterilised by autoclaving and mixed aspectically. After inoculation with 1 ml spore suspensions (10^6 spores ml^{-1}), the flasks were incubated at 30°C for 10 days. Triplicates of each pH were prepared. Aflatoxin B_1 production and mycelial dry weight were determined as described above.

11.3. Effect of incubation temperature:

To find out the optimal temperature for fungal growth and aflatoxin production, *A. parasiticus* was grown on Czapek's-Dox liquid medium, adjusted to pH 6.0 and incubated at a temperature range from 5 to 45°C. After 10 days the mycelial dry weight and aflatoxin B_1 production were determined.

11.4. Effect of different carbon sources:

To find out the best carbon source for both growth and aflatoxin production, *A. parasiticus* was grown on sucrose-free Czapek's-Dox liquid medium in which 3% (w/v) sucrose was substituted by an equivalent amount of one of the following carbohydrates: D-xylose, D-glucose, D-fructose, lactose, maltose, mannitol and starch. The latter carbon source was used at 10 g L^{-1}. The pH was adjusted to 6.0 and the incubation was carried out at 30°C for 10 days, after which mycelial dry weight and aflatoxin B_1 production were determined as described before.

11.5. **Effect of various nitrogen sources:**

To find out the best nitrogen source for the fungal growth and aflatoxin production, *A. parasiticus* was grown on $NaNO_3$-free Czapek's-Dox liquid medium supplemented with 329.4 mg nitrogen L^{-1} which is equimolecular to the nitrogen in 0.2% (w/v) $NaNO_3$ L^{-1} present in Czapek's-Dox medium. Except as indicated the inorganic nitrogen sources tested were: $NaNO_2$, NH_4NO_3, $(NH_4)_2SO_4$, $(NH_4)H_2PO_4$, $(NH_4)_2HPO_4$ and the organic nitrogen sources used were: urea, 0.3% peptone, 0.75% (w/v) yeast extract, 0.5% (w/v) soluble casein and 2.5% (v/v) corn steep liquor obtained from Tura factory, Tura, Egypt (containing 330 mg nitrogen L^{-1}). In all cases the pH was adjusted to 6.0 and incubation was carried out at 30°C for 10 days. Aflatoxin B_1 production and mycelial dry weight were determined as above.

11.6. **Effect of some amino acids:**

The effect of some amino acids on the growth of *A. parasiticus* and aflatoxin B_1 production was examined. These amino acids were: glycine, alanine, leucine, threonine, asparagine, glutamic acid, arginine, phenylalanine, tryptophan and proline. The amino acids were added to nitrogen-free Czapek's-Dox medium at equimolecular amount of nitrogen that exists in 0.2% (w/v) $NaNO_3$. The media were inoculated with *A. parasiticus* spore suspension and incubated at 30°C for 10 days. The mycelial dry weight and aflatoxin B_1 production were determined as described above.

11.7. **Effect of various metal ions:**

The effects of each of the following ions: Fe^{3+}, Cd^{2+}, Cu^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Al^{3+} and Ni^{2+} on aflatoxin B_1 production and fungal mass of *A. parasiticus* were

studied. The experiment was carried out by adding various salt concentrations namely: 5, 10, 20, 50, and 100 mg L^{-1} to Czapek's-Dox medium. After inoculation by a spore suspension of the fungus, the flasks were incubated at 30°C for 10 days then the mycelial dry weight and aflatoxin B_1 production were determined as mentioned before.

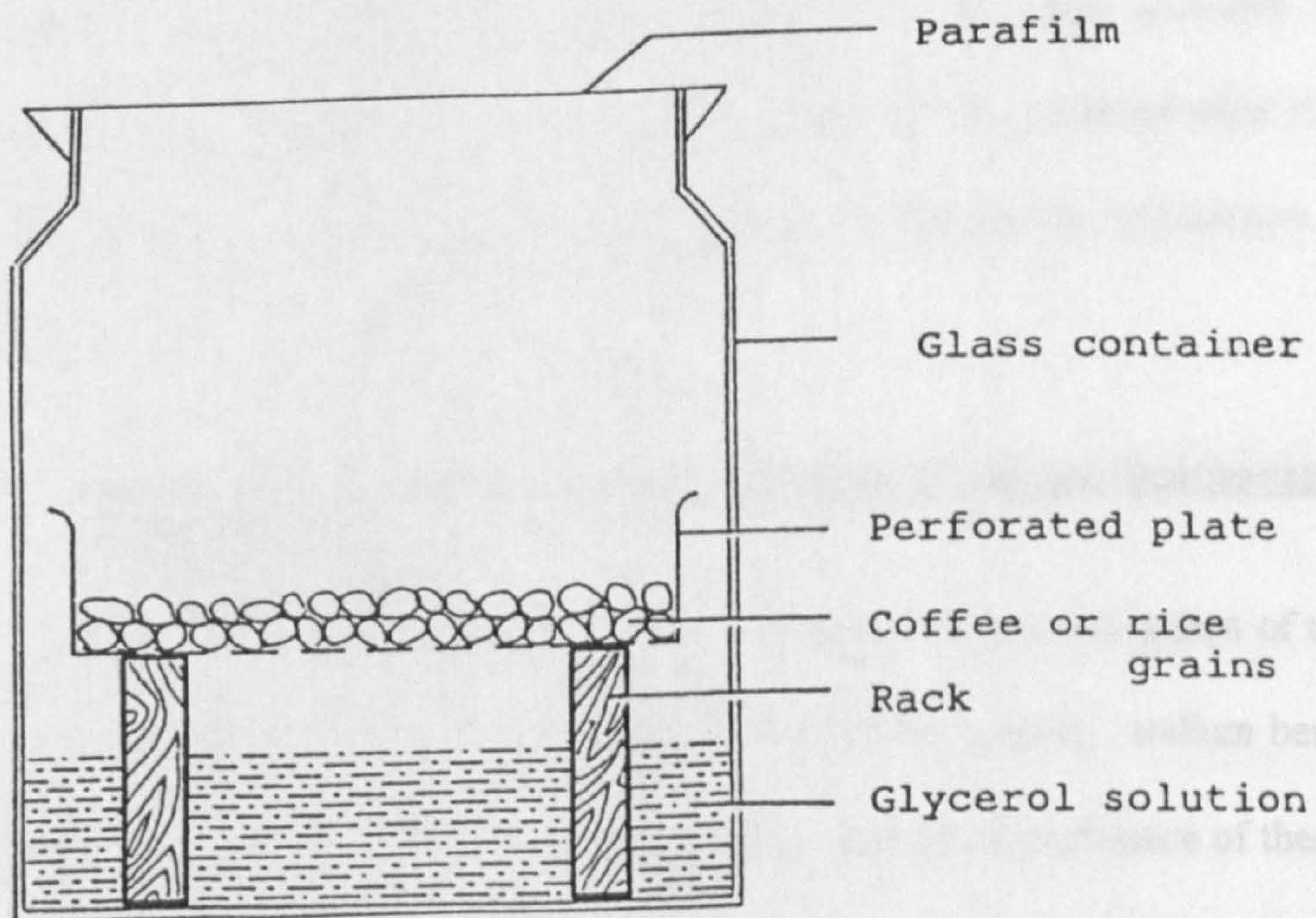
11.8. Zn^{2+}-responsive period:

To find out the Zn^{2+}-responsive period, Zn^{2+} at concentration of 20 mgL^{-1} was added to Czapek's-Dox liquid medium to various times after incubation (0, 2, 4, 6 and 8 days). Mycelial dry weight and aflatoxin B_1 production were quantified after 10 days of incubation at 30°C. The control was Czapek's-Dox Zn^{2+}-free medium.

11.9. Effect of water activity on growth of *A. parasiticus* NRRL 2999 and aflatoxin B_1 production on coffee beans and rice grains:

To achieve the aim of this experiment, solutions of glycerol at different water activities (a_w) namely: 0.88, 0.86, 0.83, 0.80 and 0.77 were prepared according to Wolf *et al.* (1973). These a_w values were chosen from those in the lower range for *Aspergillus* growth (Corry, 1987). The apparatus shown in Fig. 8 consisted of a glass container filled with a solution of glycerol and has a perforated plastic plate fixed some distance above the solution. Twenty gram of either coffee beans or rice grains were put onto the plastic plate. The container was covered tightly with parafilm. The apparatus was incubated at constant temperature (25°C). Coffee and rice samples were taken out every two days and weighed until each sample reaches a constant weight. At this time, it could be said that the water activity of the sample equals that of glycerol solution. The samples were then inoculated with *A. parasiticus* spores.

Fig. 8: The apparatus used for determination of the water activity.


Parafilm
Glass container
Perforated plate
Coffee or rice grains
Rack
Glycerol solution

After 6 weeks of incubation the fungal growth was assessed by chitin measurement and aflatoxin B_1 was determined as mentioned before.

12. Analysis of effect of possible inhibitors on growth of *A. parasiticus* and aflatoxin B_1 production:

12.1.1. Effect of some food preservatives:

To find out the best effective food preservatives, *A. parasiticus* was grown on Czapek's-Dox liquid medium supplemented with different concentrations (0.2-0.8% w/v) of some chemical food preservatives such as: sodium benzoate, ammonium bicarbonate, sodium bicarbonate, sodium chloride, and hydrogen peroxide (v/v). Charcoal was also used but at 2-8% (w/v). The fungal mat was collected after 10 days incubation at 30°C. The mycelial dry weight and aflatoxin B_1 production were determined as described before.

12.1.2. The stability of aflatoxin B_1 in the presence of sodium bicarbonate and sodium benzoate:

An experiment was designed in order to estimate the possible action of two of the tested food preservatives on the produced aflatoxin B_1 namely, sodium benzoate and sodium bicarbonate. This experiment aimed at knowing the influence of these two preservatives on the stability of aflatoxin B_1 produced by *A. parasiticus*.

One ml aliquot of each spore suspension (about 10^6 spore ml^{-1}) was used to inoculate 50 ml Czapek's-Dox liquid medium. Cultures were incubated at 30°C for 10 days. At the end of the incubation period, the culture was filtered, its volume was measured and divided into two equal parts. The first part was used for the determination of aflatoxin B_1 in the filtrate and used as control. The second part was used for examining the stability of aflatoxin B_1 as follows: $NaHCO_3$ or C_6H_5COONa

was added to the second part of the culture filtrate in the rate of 0.8% w/v (which gave maximum inhibition in the previous experiment). Aflatoxin B_1 was measured after 1, 2, 5, 10, 15 and 20 hour periods to determine the percentage of the residual aflatoxin B_1.

Another experiment was carried out in order to estimate the action of acidity on the inactivation characters of $NaHCO_3$ or C_6H_5COONa on aflatoxin B_1. In this experiment, the pH of the culture filtrate containing inactivated aflatoxin was adjusted at a pH 4 level using 0.5 N HCl. The residual aflatoxin B_1 was then remeasured after 2 hours of acidification.

12.2. Effect of some spices:

The effect of some spices such as coriander, red pepper, black pepper, ciliated heath, ginger, cardamon, cuminum, curcuma and cinnamon on the growth of *A. parasiticus* as well as aflatoxin B_1 production was studied. Twenty gram of each spice were ground and then mixed with 100 ml distilled water and autoclaved at 121°C for 5 min to obtain the water extract of the spices. The suspension was sterilised by filtration using Millipore filter (0.45 μm). The filtrate was added to Czapek's-Dox liquid medium at various concentrations namely, 2, 4, 8, 16, and 20% (v/v).

Aspergillus parasiticus was cultivated in the presence of the various concentration of spices under the optimal environmental conditions. After 10 days incubation, the fungal mats were harvested for the determination of their dry weight and aflatoxin B_1 analysis was determined in the supernatant of the culture.

12.3. **Effect of certain metabolic inhibitors:**

The effects of eight compounds on the growth of *A. parasiticus* and aflatoxin B_1 production were investigated. These compounds were glutathione, sodium azide, sodium cyanide, 2,4-dichlorophenoxy acetic acid, indole-3 acetic acid, EDTA, quinine, phenol and catechol. Each compound was added at either 5 mM or 10 mM in Czapek's-Dox liquid medium, except phenol and catechol were added at either 0.5 mM or 1.0 mM and EDTA which was used at 0.25 and 0.5 ml per 100 ml of the medium. After inoculation by *A. parasiticus* spore suspension and incubated at 30°C for 10 days, aflatoxin B_1 and mycelial dry weight were determined.

12.4. **Effect of some fatty acids:**

The effect of either saturated fatty acids, e.g., lauric, palmitic and stearic or unsaturated acids, e.g. oleic on the growth of *A. parasiticus* and aflatoxin B_1 production was examined. The effect of each of the above mentioned fatty acids was tested at various concentrations namely, 1, 5, 10, 15, and 20 mM in Czapek's-Dox liquid medium.

12.5. **Effect of some vitamins:**

The effect of five vitamins on the growth of *A. parasiticus* and aflatoxin B_1 production were tested. These vitamins were vitamin A, riboflavin (vitamin B_2), pyridoxine hydrochloride (vitamin B_6), L-ascorbic acid (vitamin C), calciferol (vitamin D_2) and folic acid. Each vitamin was added at either 5 mM or 10 mM in Czapek's-Dox liquid medium except vitamin A in which 0.25 and 0.5 ml containing 1,700,000 units ml^{-1} was used. The medium was inoculated with *A. parasiticus* spore suspension and incubated at 30°C for 10 days.

13. Determination of the catalytic activity of some fungal enzymes:

13.1. Enzyme extraction:

A. parasiticus mycelium was separated from Czapek's-Dox liquid medium during 20 days of incubation in 2 days intervals and washed with cold 100 mM Tris-HCl (pH 7.5). The washed mycelium was washed with acid-washed sand in the same buffer in a precooled mortar and centrifuged at 5,000 rpm for 10 min. The resulting supernatant was used as crude extract for measuring the enzyme activity. Samples of 0.1-0.2 ml were used to measure the enzyme activity.

13.2 Enzyme assays:

The following spectrophotometric assays were conducted after 15 min as incubation time at 25°C in a total volume of 3 ml. The absorbance was measured at 340 nm (except for fumarase) and the reactions were started by the addition of the enzyme substrate.

1. Phosphoglyceromutase (EC 2.7.5.3) (Ap Rees *et al.*, 1975); 50 mM glycylglycine (pH 7.5), 0.3 mM ADP, 0.08 mM NADH, 5 mM $MgSO_4$, 1 unit enolase, 3 units pyruvate kinase, 0.2 units lactate dehydrogenase, 3 mM 3-phosphoglycerate.
2. Pyruvate Kinase (EC 2.7.1.40) (Ap Rees *et al.*, 1975); 50 mM glycylglycine (pH 7.5), 0.08 mM NADH, 2 mM $MgSO_4$, 0.3 mM ADP, 0.2 units lactate dehydrogenase, 3 mM phosphoenolpyruvate.
3. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) (Wong and Ap Rees, 1971); 50 mM glycylglycine (pH 8.5), 10 mM $MgCl_2$, 0.3 mM NADP, 1 unit 6-phosphogluconate dehydrogenase, 5 mM glucose 6-phosphate.

4. Phosphogluconate dehydrogenase (EC 1.1.1.44) (Ap Rees *et al.*, 1976); 100 mM glycylglycine (pH 8.5), 10 mM $MgCl_2$, 3.6 mM NADP, 2 mM 6-phospho-gluconate.

5. Enolase (EC 4.2.1.11) (Ap Rees *et al.*, 1975); 50 mM glycylglycine (pH 7.5), 0.2 mM ADP, 0.08 mM NADH, 2 mM $MgSO_4$, 3 units pyruvate kinase, 0.15 units lactate dehydrogenase, 3 mM 2-phosphoglycerate.

6. Pyruvate dehydrogenase (El-Shora, 1994); 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1.6 mM NAD, 0.2 mM thiamine pyrophosphate, 2 mM dithiothreitol, 0.1 mM CoA, 2 mM cysteine-HCl and 1.5 mM pyruvate.

7. Fructose-1,6-bisphosphate aldolase (EC 4.1.2.13) (Horecker, 1975); 50 mM glycylglycine (pH 7.5), 0.2 mM NADH, 10 μg glyceraldehyde-3-phosphate dehydrogenase/triosephosphate isomerase, 2 mM fructose,1,6 bisphosphate.

8. Phosphofructokinase (EC 2.7.1.11) (Wong and Ap Rees, 1971); 100 mM glycylglycine (pH 7.5), 0.5 mM ATP, 0.2 mM NADH, 1 mM $MgCl_2$, 2 mM cysteine, 0.2 units fructose 1.6 diphosphate aldolase, 0.1 units triosephosphate isomerase, 0.4 units α-glycerophosphate dehydrogenase, 20 mM fructose-6-phosphate.

9. NAD (NADP) isocitrate dehydrogenase (Cox and Davies, 1967); 2.9 mM NAD (NADP), 0.63 mM $MnCl_2$, 5.8 mM DL-isocitrate, 80 mM tris-(hydroxymethyl) methyl-amine (Tris) HCl, pH 7.5.

10. Malate dehydrogenase (Cooper and Beevers, 1969); 0.13 mM NAD, 0.3 mM oxaloacetate, 50 mM glycylglycine (pH 7.5), 10 mM sodium malate.

11. Aconitase (Cooper and Beevers, 1969); 0.25 mM NADP, 1 mM $MnCl_2$, 7.4 mM aconitate, 0.2 units NADP isocitrate dehydrogenase, 50 mM MOPS (pH 7.4).

12. Malic enzyme (El-Shora, 1988); 21 mM MOPS (pH 7.1), 0.4 mM NADP, 7.5 mM sodium malate, 1.7 mM $MnCl_2$.

13. Fumarase (Cooper and Beevers, 1969); 50 mM sodium malate, 50 mM KH_2PO_4/NaOH (pH 8.1). The reaction was started by the addition of malate and was followed at 240 nm.

N.B. The enzyme activities are expressed as nmol $min^{-1} g^{-1}$ mycelium fresh weight.

13.2 Effect of phenol and catechol on enzyme activities:

Five concentrations of both phenol and catechol were tested for their possible effect on enzyme activities and inhibitory action on both fungal growth and aflatoxin B_1 production namely, 0.2, 0.4, 0.6, 0.8 and 1 mM. Each concentration was added to Czapek's-Dox liquid medium. After inoculation by *A. parasiticus* spores and incubation at 30°C for 10 days, aflatoxin B_1, mycelium dry weight and enzyme activities were determined.

RESULTS & DISCUSSION

CHAPTER III

Isolation & Identification of Fungi Producing Aflatoxin

Orientation experiments:

(a) Fungal density in food samples and selection of fungal media:

Attempts were made to determine the level of natural fungal contamination and amounts of aflatoxins in forty food samples. Sampling was carried out at Abha region (the south-western part of the Kingdom of Saudi Arabia) in order to ascertain the extent of aflatoxin exposure for human consumption in the studied region. Selection of samples was based on the most consumed commodities in Abha, and especially most of those are used freshly without cooking such as nuts, spices, dates, etc. The samples were taken only once, rather than periodically as not all of them are cultivated in Saudi Arabia but imported from different countries. Three different media were used to test the level of general contamination with fungi of the collected samples, namely, (a) Czapek's-Dox agar, (b) Potato-dextrose agar, and (c) Sabouraud-dextrose agar medium. The objective of using three different media was to cover all possibilities for the growth and detection of any fungal species as well as to select the most suitable one for all subsequent tests.

The results presented in Tables 4 & 5 and illustrated in Figs. 9, 10, 11 and 12 indicate that when the mean growth count was calculated on the three media used, the oil-rich commodities promote high fungal growth than the other three food groups (mean 8.8×10^3 colonies g^{-1} food on the three media used) while protein-rich commodities have the lowest fungal growth (mean 2.0×10^3 colony g^{-1} food). The data also showed that Czapek's-Dox medium gave the highest fungal counts for all groups. Accordingly, Czapek's-Dox medium was chosen for almost all the subsequent tests.

Table 4: Total colonial counts ($\times 10^3$ g^{-1}) of fungal isolates from 40 different commodities grown on 3 different culture media.

Name of Samples	Visible growth	Total fungal colony count g^{-1}* Czapek's-Dox medium	Potato-Dextrose medium	Sabouraud-Dextrose medium
A) Carbohydrate-rich commodities				
Barley	-	2.3	4.0	2.1
Date (I)	-	1.9	0.23	0.41
Date (II)	-	1.5	2.1	1.9
Date (III)	-	0.82	0.79	0.41
Rice	-	2.9	2.4	0.84
Wheat (seeds)	-	1.9	3.1	6.4
Wheat (flour)	-	3.9	5.9	3.0
Coffee beans	-	2.3	1.1	2.1
Coffee cortex	+	3.1	4.2	0.99
Garlic (dry)	-	3.5	2.9	2.1
Onion (dry)	-	2.8	3.7	0.21
Millet	-	0.14	0.32	1.1
Maize	-	9.3	8.7	3.2
Poultry diet	+	930	770	840
B) Protein-rich commodities				
Chick-pea	-	1.1	1.4	0.2
Cow pea	-	3.1	0.94	0.32
Fenugreek	-	8.9	2.3	6.1
Haricot	-	0.29	0.40	3.2
Horse-bean	-	0.78	0.71	0.94
Lantil	-	2.3	0.91	2.1
Lupin seed	-	0.12	0.18	0.19
Pea	-	5.1	3.6	3.9
C) Oil-rich commodities				
Catechu nut	-	6.1	4.5	0.41
Hazel nut	-	3.5	2.7	0.24
Mahaleb	-	2.9	3.2	2.7
Pea nut	-	6.8	4.1	0.29
Pistachio nut	-	6.2	4.3	5.9
Sesame	-	2.1	2.2	1.3
Walnut	-	45	31	51

Table 4 (continued)

D) <u>Spices</u>

Anise	-	3.1	3.3	2.1
Black pepper	-	2.1	1.9	0.52
Cardamon	-	7.0	5.9	7.8
Chili	-	1.9	1.2	1.3
Cinnamon	-	3.8	4.1	3.2
Clove	-	4.1	2.8	3.1
Coriander	-	3.1	2.3	4.1
Cumin	-	2.8	3.8	5.1
Turmeric	-	5.3	4.2	6.3
Garam Masala powder	-	2.1	1.2	1.9
Ginger	-	4.1	0.30	0.31

* Mean for three replicates.

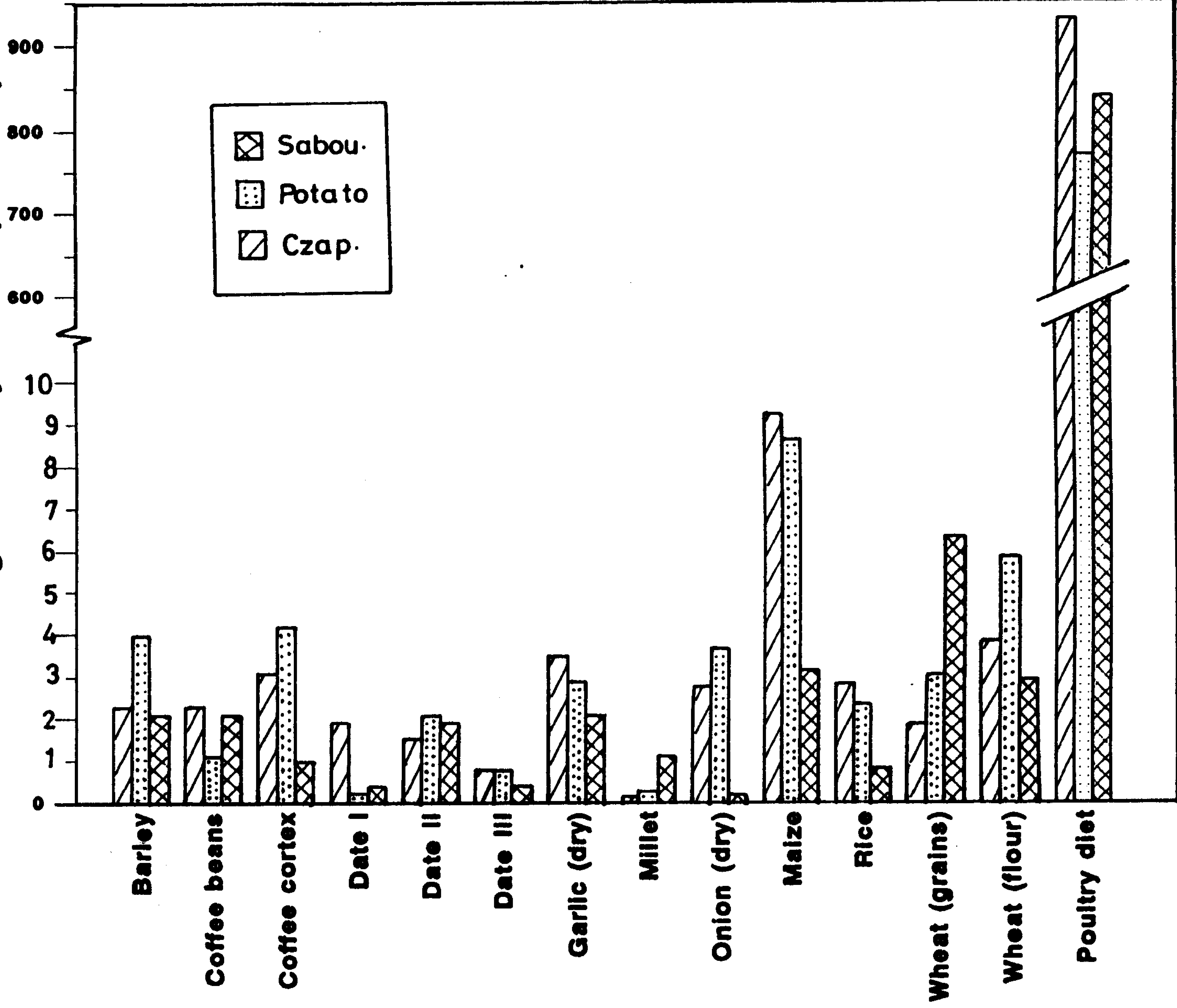
Table 5: Mean fungal colonial counts ($\times 10^3 g^{-1}$)[+] of the 4 food groups when grown on Potato-Dextrose agar medium, Sabouraud-Dextrose medium and Czapek's-Dox medium.

Media	Carbohydrate-rich commodities*	Protein-rich commodities	Oil-rich commodities	Spices	Mean
Potato-Dextrose	3.0	1.3	7.4	2.8	3.6
Sabouraud-Dextrose	1.9	2.1	8.8	3.2	4.0
Czapek's -Dox	2.8	2.7	10.4	3.6	4.9
Mean	2.6	2.0	8.8	3.2	

[+] Taken from Table 4.

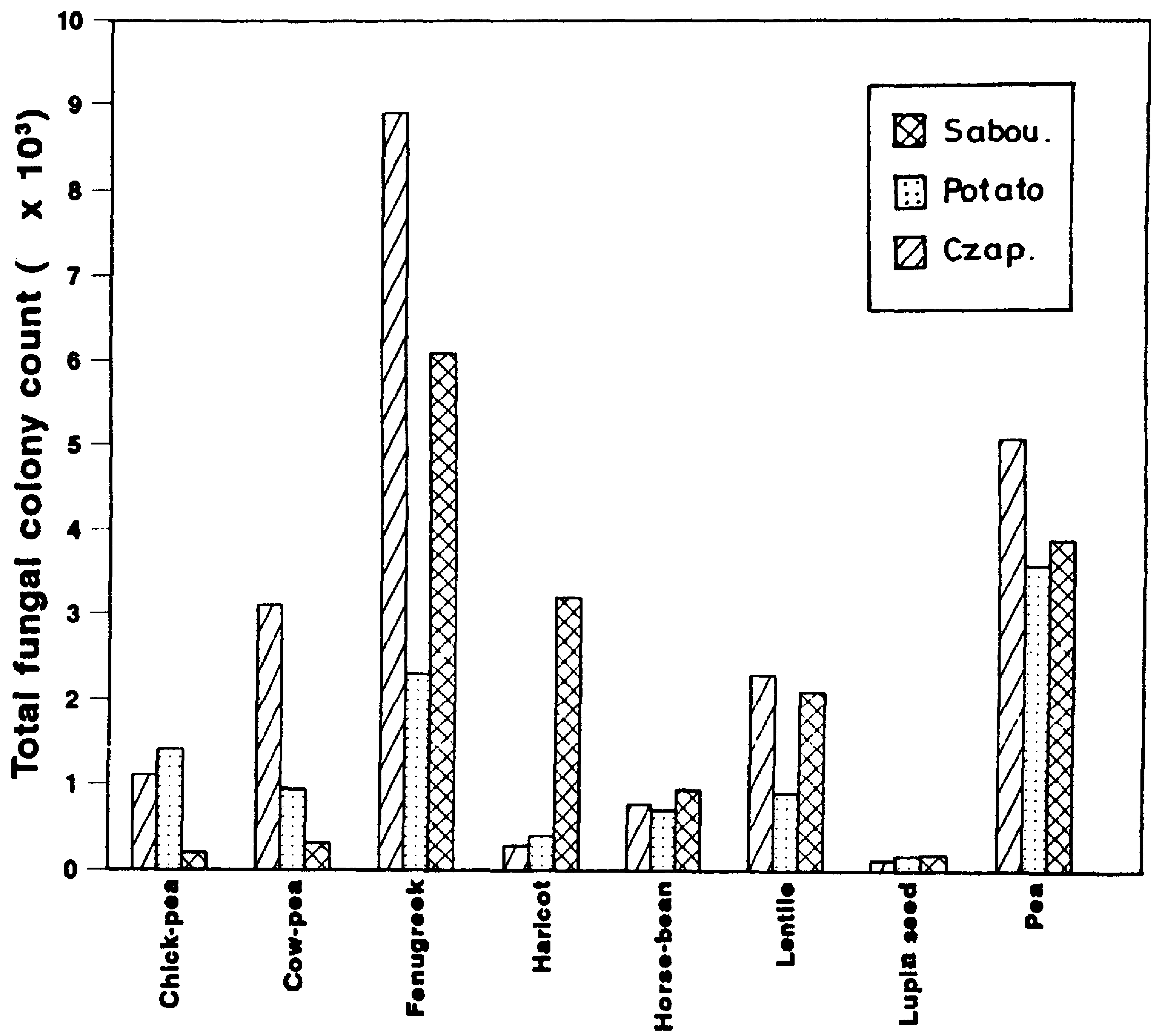
* Poultry diet is not included.

Fig. 9: Colony count of fungal contamination of carbohydrate-rich commodities. (Taken from Table 4).



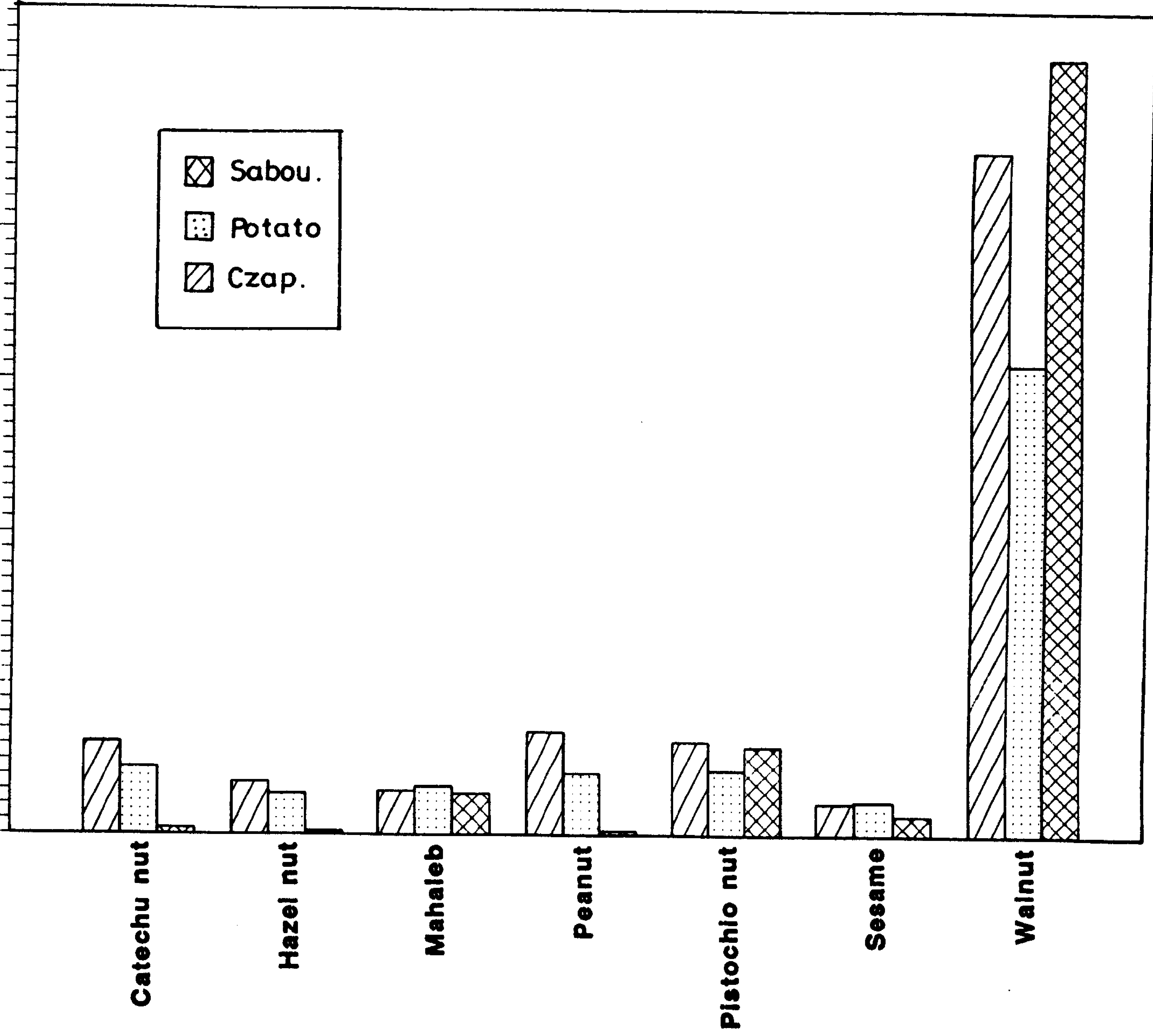
Total fungal colony count (x 10³)
900
800
700
600
10
9
8
7
6
5
4
3
2
1
0
Sabou.
Potato
Czap.
Barley
Coffee beans
Coffee cortex
Date I
Date II
Date III
Garlic (dry)
Millet
Onion (dry)
Maize
Rice
Wheat (grains)
Wheat (flour)
Poultry diet
Carbohydrate-rich commodities

Fig. 10: Colony count of fungal contamination of protein-rich commodities. (Taken from Table 4).



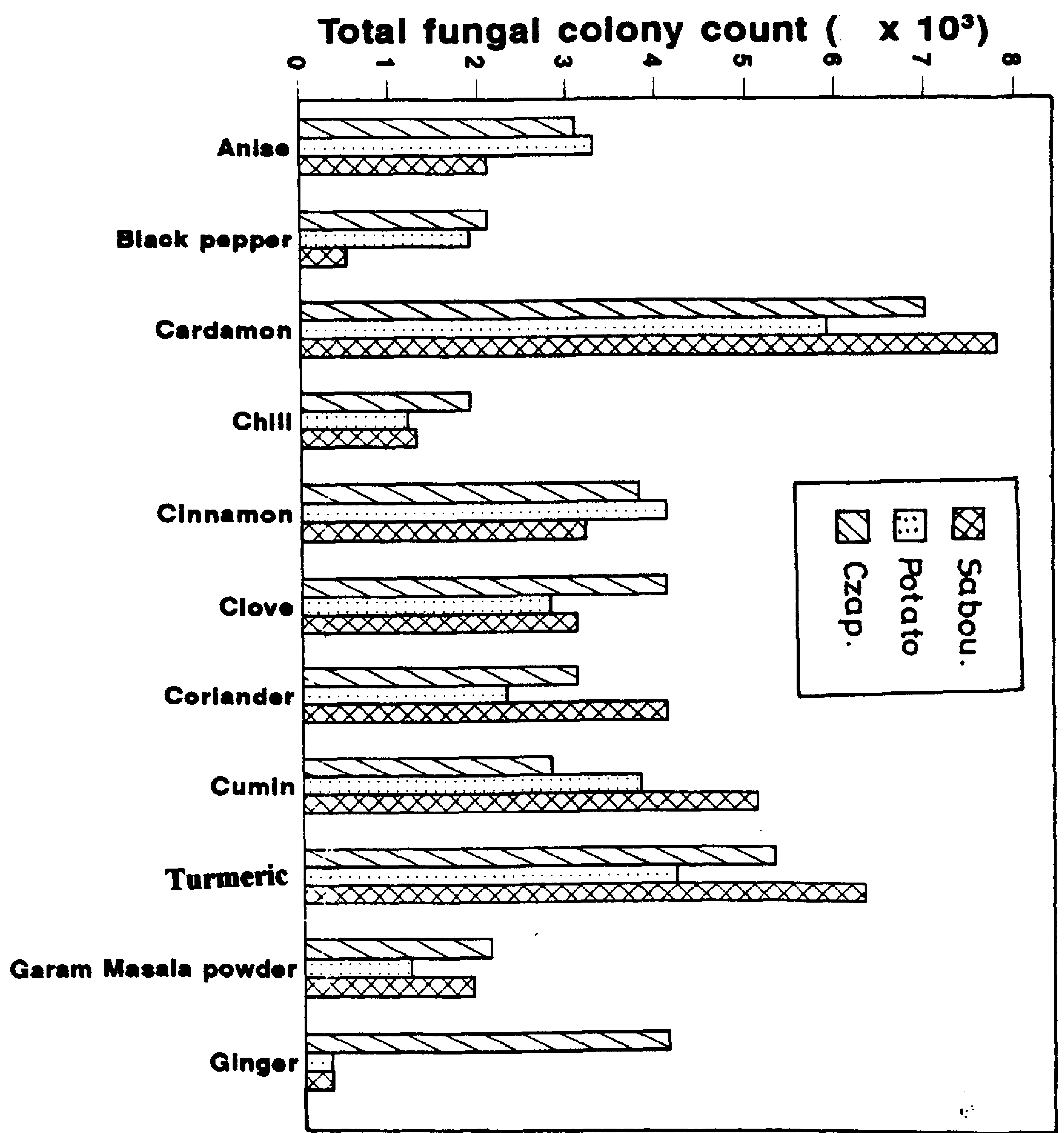
Total fungal colony count (x 10³)
10
9
8
7
6
5
4
3
2
1
0
Sabou.
Potato
Czap.
Chick-pea
Cow-pea
Fenugreek
Haricot
Horse-bean
Lentile
Lupin seed
Pea
Protein-rich commodities

Fig. 11: Colony count of fungal contamination of oil-rich commodities. (Taken from Table 4).



Total fungal colony count (x 10^3)
0
10
20
30
40
50
Sabou.
Potato
Czap.
Catechu nut
Hazel nut
Mahaleb
Peanut
Pistochio nut
Sesame
Walnut
Oil-rich commodities

Fig. 12: Colony count of fungal contamination of spices. (Taken from Table 4).



Total fungal colony count (x 10³)
0
1
2
3
4
5
6
7
8
Anise
Black pepper
Cardamon
Chili
Cinnamon
Clove
Coriander
Cumin
Turmeric
Garam Masala powder
Ginger
Spices
Sabou.
Potato
Czap.

(b) The fungal isolates:

A total of 185 isolates belonging to 7 genera and 23 species were isolated from the forty different food samples (Tables 6 & 7). One hundred isolates of *Aspergillus spp.* were represented by 9 species. The dominant species were *A. parasiticus* (31 isolates) and *A. flavus* (28 isolates) as presented in Table 6. Of the 59 isolates of *A. parasiticus* and *A. flavus*, only 18 were toxicogenic (30%), 7 of *A. parasiticus* and 11 of *A. flavus*.

The results also show that poultry diet was the sample contaminated with the most fungi whereas curry powder, ginger, and rice were contaminated with the least number.

(c) Aflatoxins from food samples:

Generally, *A. flavus* and *A. parasiticus* are the only two common fungi known of producing aflatoxins. In the previous experiments, *A. flavus* and *A. parasiticus* were isolated from the surveyed foodstuffs. The forty food samples were examined for their aflatoxin content and only ten (25%) were found containing aflatoxins. The results summarised in Table 8, show the natural occurrence of aflatoxins (B_1, B_2, G_1 and G_2). The range was 20-312 μgKg^{-1}, which range was beyond the safe limits for human consumption of up to 20 μgKg^{-1}, depending on the kind of commodities, countries and regulations (WHO, 1979; Schuller & Van Egmond, 1983)

Peanut, maize, poultry diet, pistachio nut and rice contained high levels of aflatoxins (312, 191, 150, 89 and 72. μgKg^{-1}, respectively). The lowest amounts were observed in wheat and lentil (25 and 20 μgKg^{-1}, respectively).

Table 6 : Numbers of occurrence of A. parasiticus and A. flavus isolated from different food samples obtained from Abha markets (Saudi Arabia).

	Anise	Barley	Black Pepper	Cardamon	Catechu Nut	Chicken Pea	Chili	Cinnamon	Clove	Coffee Beans	Coffee Cortex	Coriander	Cow Pea	Cumin	Curry Powder	Data (I)	Data (II)	Data (III)	Fenugreek	Garam Masala
A. parasiticus	-	+	+	+	+	-	-	+	+	+	+	+	+	-	-	+	+	-	+	+
A. flavus	+	+	-	+	+	+	+	-	-	+	+	-	+	+	+	+	+	+	+	+

	Garlic	Ginger	Haricot	Hazel nut	Horse-bean	Lentil	Lupin seed	Mahaleb	Maize	Millet	Onion	Pea	Peanut	Pistocio nut	Poultry diet	Rice	Sesame	Walnut	Wheat grains	Wheat (flour)	No. of occurrence
A. parasiticus	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	-	+	+	31
A. flavus	+	-	-	+	+	-	+	-	+	+	+	-	+	-	+	+	-	+	+	-	28

Table (7): Numbers of occurrence of other fungal species isolated from different food samples obtained from Abha markets (Saudi Arabia).

	Anise	Barley	Black Pepper	Cardamon	Catechu Nut	Chicken Pea	Chili	Cinnamon	Clove	Coffee Beans	Coffee Cortex	Coriander	Cow Pea	Cumin	Curry Powder	Date (I)	Date (II)	Date (III)	Fenugreek	Garam Masala	Garlic	Ginger	Haricot	Hazel nut	Horse-bean	Lentil	Lupin seed	Mahaleb	Maize	Millet	Onion	Pea	Peanut	Pistocio nut	Poultry diet	Rice	Sesame	Walnut	Wheat grains	Wheat (flour)	No. of occurrence
<u>Aspergillus aspercens</u>	-	+	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	6
<u>A. awamori</u>	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	4
<u>A. candidus</u>	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	6
<u>A. fumigatus</u>	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
<u>A. niger</u>	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	+	-	+	+	-	+	9
<u>A. oryzae</u>	-	-	-	-	-	+	-	+	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	7
<u>A. nidulans</u>	-	+	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	+	7
<u>Alternaria alternata</u>	-	-	+	-	-	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	8
<u>Cunninghamella elegans</u>	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	4
<u>Mucar sp.</u>	+	-	+	+	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	7
<u>Penicillium citrinum</u>	-	-	-	-	-	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	+	-	-	-	-	+	-	-	8
<u>P. chrysogenum</u>	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	5
<u>P. commune</u>	-	-	-	+	-	-	-	+	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	5
<u>P. cryophilum</u>	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	3
<u>P. cyclopium</u>	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	+	6
<u>P. miczynskii</u>	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	3
<u>P. notatum</u>	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	6
<u>P. terrestre</u>	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	+	-	-	-	+	-	+	8
<u>P. waksmani</u>	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	3
<u>Rhizopus nigricans</u>	-	-	-	-	-	+	+	+	-	-	-	+	+	-	-	-	-	+	-	-	+	-	-	-	-	-	-	+	+	+	-	+	-	-	+	-	-	+	+	-	14
<u>Trichoderma lignorum</u>	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	5
Total No. of Species	2	3	5	4	3	6	2	4	6	1	6	5	2	2	1	2	7	1	1	1	5	1	2	2	2	2	3	3	1	3	1	5	1	5	8	0	3	5	4	6	126

Table 8: Total colony counts of fungi and aflatoxin levels in samples of foodstuff collected from public markets of Abha (Saudi Arabia).

Foodstuffs	Total fungal colony count ($\times 10^3 g^{-1}$)	Aflatoxins (μgKg^{-1})*				Total aflatoxins (μgKg^{-1})
		B_1	B_2	G_1	G_2	
Maize	9.3	78.06± 4.5	60.57±10.5	30.96±4.5	21.72±3.9	191.31
Peanut	6.8	150.78±17.0	90.45± 9.0	40.29±3.9	30.85±6.0	312.37
Rice	2.9	25.95± 1.0	20.19± 0.9	15.71±1.1	10.28±0.5	72.13
Coffee cortex	3.1	17.94± 2.5	15.14± 2.3	10.82±2.5	8.59±2.3	52.49
Sesame	2.1	9.18± 3.0	14.61± 1.5	5.99±1.0	2.79±0.5	32.57
Poultry diet	930	80.19± 7.3	28.62± 4.0	22.55±2.9	18.97±1.5	150.33
Horse-bean	0.78	20.91± 2.0	9.57± 3.0	18.14±3.7	12.93±4.6	61.55
Wheat grains	1.9	6.97± 2.2	10.21± 3.5	4.76±2.2	3.85±1.0	25.79
Lentil	2.3	5.93± 1.9	4.65± 2.4	6.43±1.4	3.17±1.8	20.18
Pistachio nut	6.2	40.15± 6.0	19.15± 2.0	16.97±5.1	12.75±3.5	89.02

* Means ± S.D.

The most common aflatoxin in maize, poultry diet, horse bean, peanut, rice, coffee cortex and pistachio nut was B_1. The highest B_1 and B_2 values were recorded in peanut, those for G_1 and G_2 in peanut and maize, respectively. In support of the present results, international surveys were conducted by FAO/WHO/UNEP from 1976 to 1983, to assess the level of aflatoxin in both domestic and imported corn, peanuts and peanut butter, which revealed high levels of contamination by aflatoxin than the recommended level (Brazil, 30-5000; Ireland, 300-4000; U.K. 38-535; Kenya, 30-1920 and U.S.A., 10-700 $\mu g\ Kg^{-1}$) (Ellis *et al.*, 1991). Also, Tabata *et al.* (1993) found that rice products, corn, peanut products, pistachio nuts, sesame products, white pepper, red pepper and mixed spices, which were collected in Tokyo from 1986 to 1990, were contaminated by aflatoxins. They found also that the highest level of aflatoxin B_1 was observed in pistachio nut (1382 ppb).

(d) <u>Effect of nutritional composition of the tested samples on the general fungal growth and aflatoxin production</u>:

The results in Table (9) indicate that oil-rich commodities followed by spices promoted the highest fungal growth on Czapek's-Dox medium (mean 10.4×10^3 and 3.6×10^3 colony g^{-1} food, respectively), while carbohydrate-rich commodities and protein-rich commodities promoted the lowest fungal growth on the same medium (mean 2.8×10^3 and 2.7×10^3 colony g^{-1} food, respectively). The results also indicate that the oil-rich foodstuffs had a high percentage of samples contaminated aflatoxins (43%) with a high toxin quantity mean of (62.0 μgKg^{-1}). In contrast, all the examined samples of spices were found to be aflatoxin free.

Table 9: Effect of composition of examined foodstuffs on general fungal growth and aflatoxins production.*

Food groups	% of samples contaminated with aflatoxins	Mean of aflatoxins in contaminated samples ($\mu g Kg^{-1}$)	Mean of colony count ($\times 10^3 g^{-1}$) on Czapek's-Dox medium
Carbohydrate-rich commodities	36	35.1	2.8
Protein-rich commodities	25	10.2	2.7
Oil-rich commodities	43	62.0	10.4
Spices	00	00	3.6

*Taken from Tables 5 & 8.

The promotion in oil-rich commodities of fungal growth as well as aflatoxin production may be due to the highest content of these commodities of oils which are broken down by the specific fungal enzymes to 2-C units (ketides) which incorporated directly into the aflatoxin biosynthetic pathway. Also, these commodities contain the highest amount of some metal ions like Zn^{2+} (Paul & Southgate, 1978) which stimulates the fungal growth as well as toxin production.

The present results are in contrast with those reported by Farag *et al.* (1986) who found that contaminated starchy seeds (wheat) had a higher amount of aflatoxin than oily seeds (peanut and sesame) and protein-rich seeds (soybean). This contradiction may be due to the fact that Farag *et al.* (1986) used only one type of starchy seed (wheat), while in the present work, about 13 starch-rich samples were used. In addition, Farag *et al.* (1986) used an artificial inoculation while in the present work, natural contamination was studied.

The type of nutrients affected the rate of fungal growth as demonstrated by colonial count experiments. This was evident from testing the fungal growth from 40 food samples on the three tested culture media, namely, Sabouraud-Dextrose agar, Potato-Dextrose agar and Czapek's-Dox agar medium. The promotion or inhibition of fungal growth on the contaminated food materials may be related to the different components (chemical contents) which are present in each type of food (Table 7).

It may be assumed that the variation of fungal species on different food materials is due to certain growth factors present in different nutritional material. Similar observations and explanations were reported by Mislivec & Tuite (1970); Lillehoj *et al.* (1983); Naguib *et al.* (1983); Tabata *et al.* (1993); and Halt (1994). The current survey of food samples revealed the domination of genus *Aspergillus*, followed

by genus *Penicillium*. However, the effect of food materials seemed to be confined to the production of aflatoxin rather than fungal growth as demonstrated by the two most frequent species (16.7% & 15.1%), namely, *A. parasiticus* and *A. flavus*. Out of the 59 *Aspergillus spp.* contaminated on the surveyed food samples, only 18 (30%) species could produced aflatoxins.

It could be assumed that the production of aflatoxin is not necessarily on accompaniment with *Aspergillus* food contamination. Stoloff (1976), Wood (1989) and Ellis, *et al.* (1991) have reported almost similar findings.

The levels of aflatoxin production in the contaminated foodstuff were variable (Table 8). In certain plant samples such as peanut, the aflatoxin levels were found to be 15 times more than in other sample as lentil. The variation of aflatoxin levels seems to be due to the concentration, presence and/or absence of certain ingredients in the foodstuff. These differences in aflatoxin levels could be due to the concentration of carbohydrates and fatty acids in the substrates which enhance toxin production, as observed in the high yield of aflatoxin from peanut (contains 8.6 g carbohydrates and 49 g fat per 100 g, Paul & Southgate, 1978). Substrates high in proteins and low in carbohydrates did not enhance aflatoxin production by *A. parasiticus*. Also, the resistance or susceptibility of natural commodities to aflatoxin production has been explained as the presence or absence of adequate amounts of trace metals. These explanations are supported by those of Maggon *et al.* (1977); Ellis *et al.* (1991); and Viquez *et al.* (1994).

(e) <u>Bioassay of aflatoxins</u>:

The microbiological assay technique was recently adapted to supplement the results of thin layer chromatographic (TLC) detection and confirmed the presence of

mycotoxins in food samples. Clements (1968) found that it is possible to use *Bacillus megaterium* NRRLB.1368 in developing a rapid confirmatory test and quantitative bioassay for aflatoxin B_1.

The microbiological assay technique of aflatoxins was used to confirm TLC detection and the presence of aflatoxins in all food samples. Three bacteria namely, *Bacillus megaterium*, *B. cereus* and *Escherichia coli* were used for microbiological assay of aflatoxins B_1, B_2, G_1 and G_2 in the present investigation. Furthermore, the positive extract samples containing aflatoxins were confirmed. The assay is rapid (16-18 h), simple and inexpensive.

Results presented in Table 10 indicate that *B. megaterium* and *B. cereus* were more sensitive to the standard samples of aflatoxins B_1 and B_2 than to G_1 and G_2. However, *E. coli* was only affected slightly by the standard aflatoxin B_1 and B_2 but was most resistant against G_1 and G_2. Also, the extract of aflatoxins from contaminated food samples inhibited the three organisms (Table 11) with *E. coli* being the most resistant organism. Therefore, the three tested organisms could be arranged according to their resistance to the tested aflatoxins as *E. coli* > *B. cereus* > *B. megaterium*.

According to the present results, it could be assumed that *B. megaterium* and *B. cereus* were suitable for microbiological assay of aflatoxin B_1 and B_2, while *E. coli* was favourable only for aflatoxin B_1.

Variable inhibitory action of aflatoxin on the growth of *Bacillus megaterium*, *B. cereus* and *E. coli* was detected. The different responses of the tested bacteria to aflatoxin may be related to the inhibitory mode of action of the toxin on the tested bacteria, which is known to be activated by the different exoenzymes derived from bacteria. The varied effects of aflatoxin B_1 on bacteria may be also attributed to the

Table 10: The inhibition zone (mm)* of aflatoxins (B_1, B_2, G_1 and G_2) and extract of positive samples.

Microorganisms	Aflatoxins ug disc^{-1}																												Positive samples extract ul disc^{-1}							
	B_1							B_2							G_1							G_2														
	2	4	6	8	10	15	20	2	4	6	8	10	15	20	2	4	6	8	10	15	20	2	4	6	8	10	15	20	1	2	4	6	8	10	15	20
<u>B. megaterium</u>	6	7	8	8.5	9	10	11	0	0	6	7	7.5	8	9	0	0	0	4	5	6	8	0	0	0	4	4.5	5	6	0	0	0	5	6	6.5	7	9
<u>B. cereus</u>	0	0	5	6	8	9	12	0	0	5	6	6.5	7	9	0	0	0	6	7	8	9	0	0	0	0	4	6	8	0	0	0	0	6	7	8	10
<u>E. coli</u>	0	0	0	4	5	6	8	0	0	0	0	0	5	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	7	8	8.5	9

0 = not detected.

* = values represent means for triplicate assayed.

Table 11: Minimum concentration of Aflatoxins (B_1, B_2, G_1 and G_2) and sample extracts giving a detectable inhibition zone.*

Microorganism	Standard Aflatoxins (µg)*				Sample* extract (µl)
	B_1	B_2	G_1	G_2	
B. megaterium	2	6	8	10	6
B. cereus	6	6	8	10	8
E. coli	8	15	-	-	10

*The results in this table are extracted from Table 10.

reduction of DNA-to-protein ratio, aberrant all formation; mRNA transcription inhibition, inhibited incorporation of precursors into DNA, RNA and proteins and blocked induction as well as production of various enzymes at various levels. Accordingly, bacteria and other microorganisms could be used for the detection and quantification of aflatoxin production. The present findings as well as their explanations are in agreement with those reached by Nezval & Bosenberg (1970), Reiss (1975) and Ellis *et al.* (1991).

The present results indicate that the promotion or inhibition of fungal growth as well as the presence of aflatoxins on the contaminated food materials may be related to the different chemical contents which are present in each type of food. This hypothesis led to an examination of the effect of different components which may be present in the food samples on the growth of *A. parasiticus* and its productivity of aflatoxin. The components examined are: some carbon sources, some nitrogen sources, some metal ions, vitamins, fatty acids and the water activity of the two important commodities used in large amounts in Saudi Arabia namely: rice and coffee beans.

For the examination of these substances in Czapek's-Dox liquid medium it is necessary to find out the best incubation conditions (pH, temperature and incubation period) for the growth of selected fungus *A. parasiticus* and its productivity of aflatoxin B_1.

CHAPTER IV

Physical and Nutritional Factors Influencing the Growth of *A. parasiticus* and Aflatoxin B_1 Production

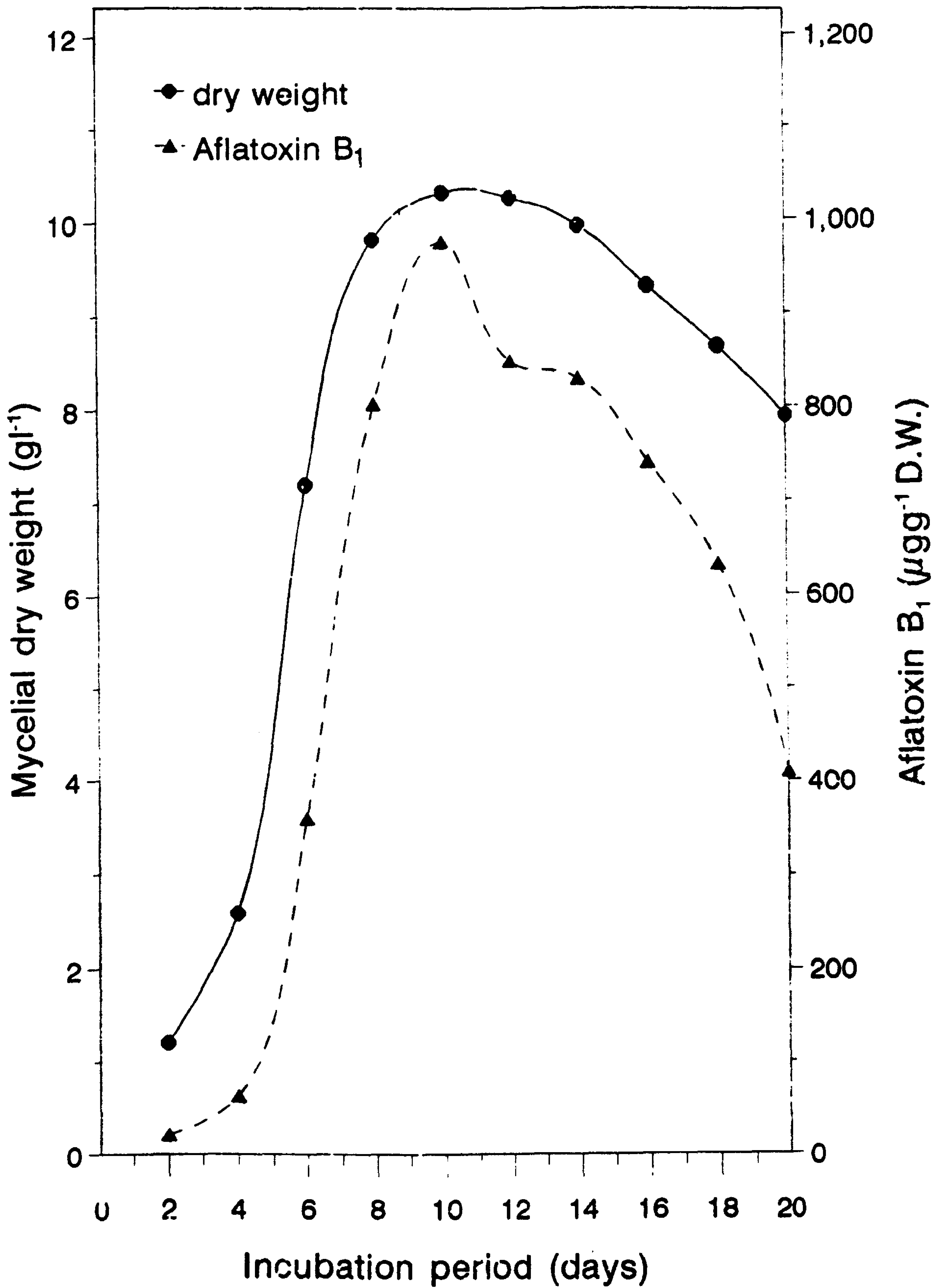
The work in this chapter aimed to study the physical and nutritional factors which could affect the growth of *A. parasiticus* and its productivity of aflatoxin B_1. These factors include kinetics of growth and aflatoxin production, pH, incubation temperature, carbon and nitrogen sources, metal ions and water activity.

(a) Kinetics of growth and aflatoxin B_1 production:

The fungal growth and the productivity of aflatoxin B_1 were determined throughout 20 days at 2 days intervals at 30°C (Fig. 13). The peak yield of AFB_1 was obtained in 10 days. The optimal incubation time was 10 days for both fungal growth and aflatoxin production. Also, in the present work, the fungal growth which is determined as increase in dry weight per liter was studied up to the optimal time (10 days) after which the fungal biomass decreased gradually at 7.99 g dry weight l^{-1} at the end of the incubation time. On the other hand, the aflatoxin B_1 level increased continuously with time until it gave 978 µg g^{-1} dry weight at 10 days (optimal time) after which it declined gradually until it reached a value of 409.5 µg g^{-1} dry weight after incubation of 20 days which represents approximately 41.8% of that observed at 10 days.

Generally, the findings indicate that the rate of toxin formation is proportional to the rate of production of new cell mass. The present results are in agreement with those of Hayes *et al.* (1966), Schroeder (1966), Doyle & Marth (1968), Varma & Verma (1987), Singh *et al.* (1992) and Lohani *et al.* (1994). However, Mashaly & El-Deeb (1983a) found that the optimum growth and maximum aflatoxin production were attained at the 7th day for *A. flavus* which is less than the optimal time for the two processes in the present work. On the other hand, Kheiralla *et al.* (1992) reported

Fig. 13: Effect of incubation period on the growth of *A. parasiticus* and Aflatoxin B_1 production.



dry weight
Aflatoxin B_1
Mycelial dry weight (gl^{-1})
Aflatoxin B_1 (μgg^{-1} D.W.)
Incubation period (days)
0
2
4
6
8
10
12
1,200
1,000
800
600
400
200
0
2
4
6
8
10
12
14
16
18
20

highest incubation period of 14 days for *A. flavus* isolated from corn. Thus, it seems likely that the optimal time for fungal growth and its productivity to the toxin depends on the fungus activity itself as well as the medium of growth rather than the incubation temperature.

The decrease of fungal biomass and aflatoxin production after the optimum incubation time (10 days) may be attributed to the lysis of mycelial cells. The coincidence of mycelial lysis with more rapid toxin degradation may be due to the fact that the toxin degradation is affected by enzymes released during mycelial lysis. Almost similar results and explanations were presented by Bacon *et al.* (1977) and Montani *et al.* (1988), suggesting that degradation of F-2 toxin produced by *Gibberella zeae* and zearalenone produced by *Fusarium graminearum* were the result of enzymatic action.

Aflatoxin decay after the 10th day of incubation may be explained as follows:

(1) The accumulation of the produced aflatoxin leading the fungi to degrade a part of the produced toxin in order to survive. It is thought that these degradation reactions of aflatoxin occur through enzymatic activity and that these enzymes produce end-products or by-products that react with aflatoxins. Peroxidase was speculated to be one such enzyme since it catalyzes the decomposition of hydroperoxides to produce free radicals (Richardson, 1976) which may then react with aflatoxins. Doyle & Marth (1979) have shown that *A. parasiticus* is capable of producing peroxidase and degrading aflatoxin B_1, and also showed a direct correlation between the amount of peroxidase produced and the amount of aflatoxin degraded. A decrease in aflatoxin levels from the peak of production corresponded to a parallel increase in peroxidase activity.

(2) Aflatoxin could be utilized as a nutrient substrate by the fungus after the exhaustion of nutrients in the growth medium.

(3) Aflatoxin B_1 itself is a precursor for a series of reactions leading to the formation of further other types of aflatoxins, e.g., B_2, G_1, G_2, etc. These explanations are supported by various authors such as Hayes *et al.* (1966), Doyle & Marth (1968), Mashaly & El-Deeb (1983a), Smith & Moss (1985); Ellis *et al.* (1991), Pitt (1993), and Smith & Harran (1993). However, further studies are needed to clarify this area since the possibility of producing aflatoxin-degrading enzymes would have considerable commercial interest and application.

(b) <u>Effect of pH</u>:

The effect of pH on growth of *A. parasiticus* and its aflatoxin B_1 production was studied to find out the optimum pH value for both criteria. Different buffers were used to cover a range of pH's 3-9, namely citrate-phosphate (pH 3-7), phosphate (pH 8.0) and borate buffers (pH 9). After inoculation, the cultures were incubated at 30°C for 10 days. The obtained results are listed in Table 12 as well as graphically represented in Fig. 14. Data indicate that the pH for fungal growth ranges between 3 and 9, whereas the pH for aflatoxin B_1 production confined between 4 and 8. As pH value increased, there was a corresponding increase in both fungal growth and aflatoxin B_1 production up to pH 6 which seemed to be the optimal pH for *A. parasiticus* growth and its productivity of aflatoxin B_1, after which a gradual decrease was observed in mycelial dry weight and toxin production.

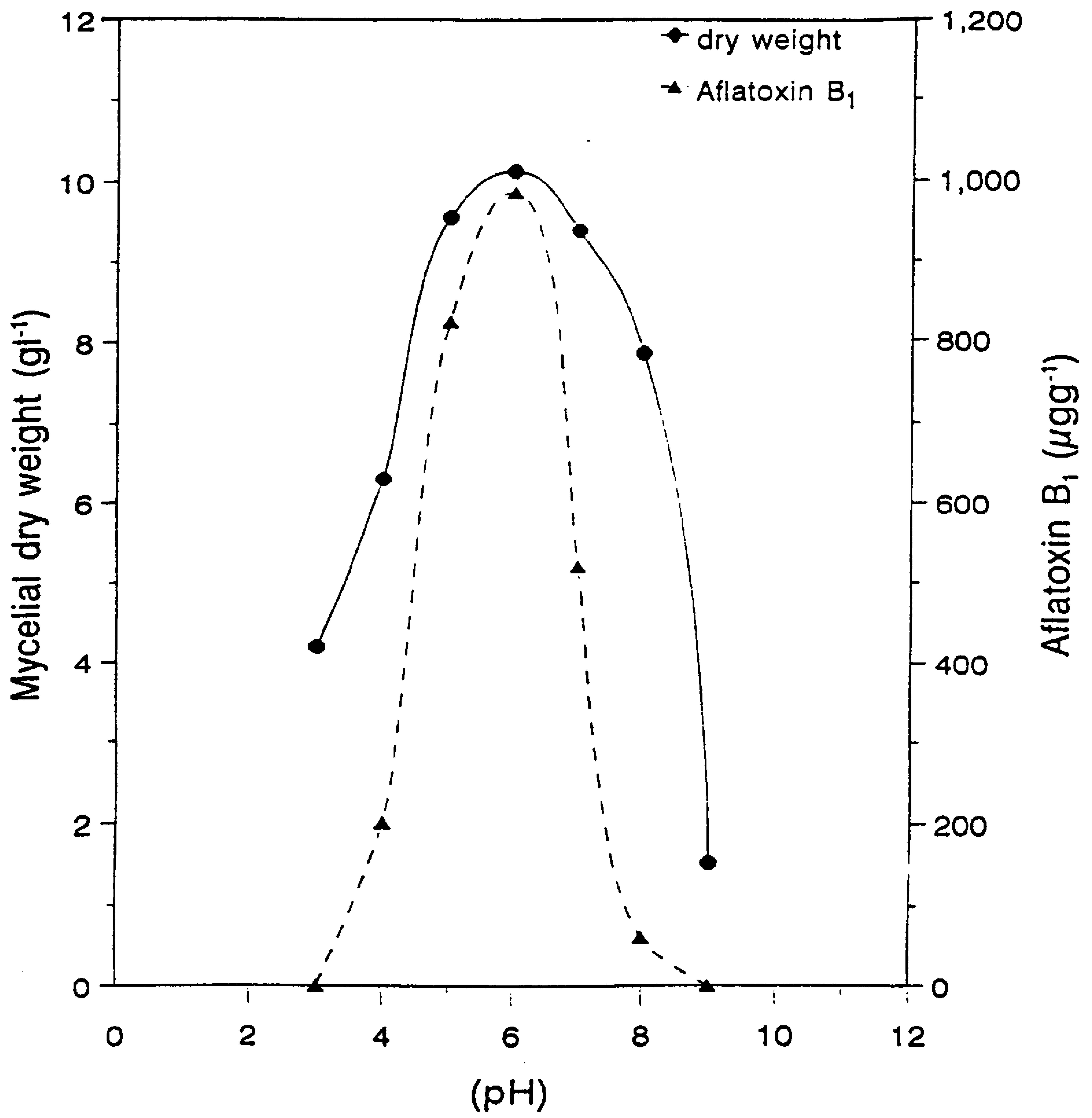
The fungal growth at pH 9.0 was 14.9% of that recorded at pH 6.0. Moreover, the toxin productivity at pH 8.0 was about 6% of that observed at pH 6.0. So, it

Table 12: Effect of pH on the growth of *A. parasiticus* NRRL 2999 and aflatoxin B_1 production.* (The growth period was 10 days at 30°C)

pH	Mycelial dry weight (gl^{-1})	Aflatoxin B_1/dry weight (μgg^{-1})
3.0	4.2 ± 0.0	00.0
4.0	6.3 ± 0.0	201 ± 5
5.0	9.5 ± 0.1	825 ± 12
6.0	10.1 ± 0.0	986 ± 20
7.0	9.4 ± 0.1	520 ± 13
8.0	7.8 ± 0.1	61 ± 3
9.0	1.5 ± 0.0	00.0

* Means of three replicates ± S.D.

Fig. 14: Effect of pH on the growth of *A. parasiticus* and Aflatoxin B_1 production. (Taken from Table 12).



dry weight
Aflatoxin B_1
Mycelial dry weight (gl^{-1})
Aflatoxin B_1 (μgg^{-1})
(pH)
12
10
8
6
4
2
0
1,200
1,000
800
600
400
200
0
0
2
4
6
8
10
12

seems likely that the decrease in the fungal growth at pH 9.0 is more or less similar to that recorded for toxin production at pH 8.0 compared to the values at pH 6.0.

The present results are running parallel with those reported by Basappa *et al.* (1970); Mashally & El-Deeb (1983a) and Garcia et al. (1994). Other workers reported lower acidic optimal pH values, e.g. 4.5 when *A. flavus* was grown in cow casein medium (Ismail *et al.*, 1983) or when the same organism was grown in yeast extract glucose broth medium (Lohani, *et al.*, 1994) and pH 3.5 for *A. flavus* and *A. parasiticus* when other conditions were used (El-Gazzar *et al.*, 1987). This variation in optimal pH values can be explained by the fact that pH is not a unitary factor, any factor in the environment may change the shape of the pH-growth curve. Such factors include fungal species, temperature, time of incubation, gross changes in the medium, growth factor supply and nitrogen sources.

Hydrogen ion concentration in the medium affects the ionization of salts in solutions and hence the availability of ions to the fungus. Ion uptake may also be influenced by the effect of hydrogen ion concentrations on the permeability of the plasmalemma. Enzyme activity is pH-dependent and at non-optimal pH the efficiency of extracellular enzyme catalysis will be reduced. The effect of pH on fungal growth is therefore likely to be rather complex and any changes in the growth responses of fungi to shifts in pH are usually difficult to ascribe to any single factor (Dix & Webster, 1995). Since the aflatoxins are secondary metabolites, this may support the explanation that they are only formed when suitable pH conditions exist where the mycelia are active and thus aflatoxin production seems to be dependent on the permeability properties of the fungal cellular membrane which is affected by the pH of the growth medium.

(c) Effect of incubation temperature:

One of the factors which seemed to play an important role in fungal growth and aflatoxin production is the incubation temperature. The effect of temperature on the fungal growth and aflatoxin B_1 production by *A. parasiticus* was estimated by testing a range of temperature ranged between 5-45°C. After 10 days, aflatoxin B_1 was determined and the mycelial dry weight was measured as a criterion of the fungal growth.

The data presented in Table 13 and in Fig. 15 show the effect of incubation temperature on mycelial growth and the amount of aflatoxin B_1 produced by *A. parasiticus*. The data reveal that the fungal growth was limited in the range of 10-40°C, below or above which there is no detectable growth. However, aflatoxin B_1 production is limited within the range 15-35°C which is narrower from that observed for the fungal growth.

The results also show that the optimal temperature for both the growth of *A. parasiticus* and its productivity of aflatoxin B_1 is 30°C. It is worth mentioning, that fungal growth at 30°C was 1.4 times of that observed at 40°C, whereas the productivity of aflatoxin at 30°C was 1.3 times of that noted at 35°C. In addition, it was observed that a temperature of 10°C was generally too low for aflatoxin production, although the mould was able to grow at this temperature. Aflatoxin production was approximately the same at 25°C and 30°C. No fungal growth or aflatoxin production was observed at 5°C. According to the present findings, it may be assumed that temperature is one of the most critical environmental factors influencing mould growth and aflatoxin production. The present results showed an optimal temperature for fungal growth and aflatoxin B_1 production which

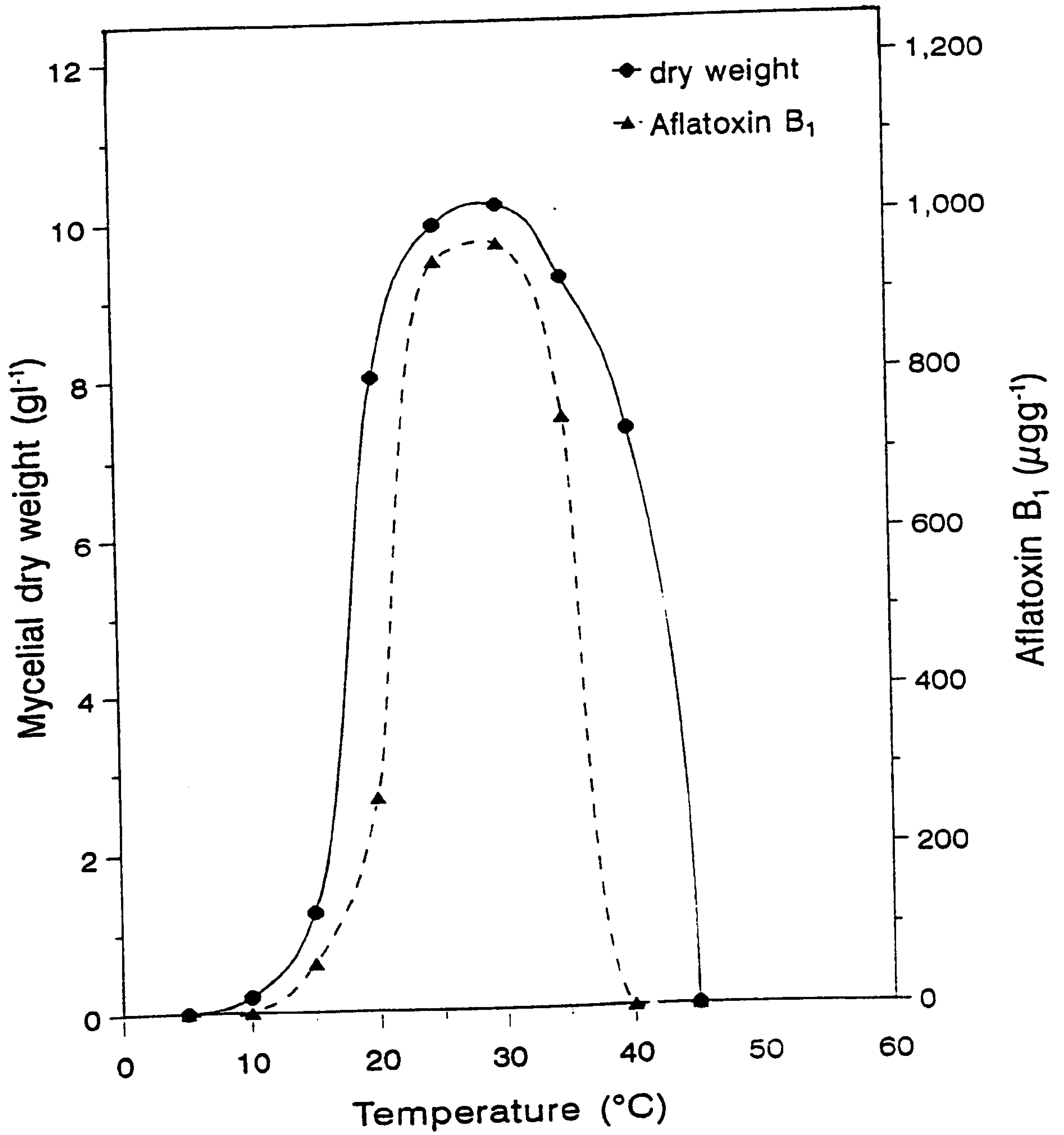
Table 13: Effect of incubation temperature on the growth of *Aspergillus parasiticus* NRRL 2999 and productivity of Aflatoxin B_1.*

(The culture time was 10 days)

Temperature (°C)	Mycelial dry weight (gl^{-1})	Aflatoxin B_1/dry weight (μgg^{-1})
5	00.0	00.0
10	0.1 ± 0.1	00.0
15	1.2 ± 0.1	61 ± 2
20	8.0 ± 0.0	266 ± 23
25	9.9 ± 0.4	945 ± 37
30	10.1 ± 0.3	965 ± 20
35	9.2 ± 0.2	746 ± 13
40	7.3 ± 0.1	00.0
45	00.0	00.0

* Means of three replicates ± S.D.

Fig. 15: Effect of incubation temperature on the growth of *A. parasiticus* and Aflatoxin B_1 production. (Taken from Table 13).



dry weight
Aflatoxin B_1
Mycelial dry weight (gl⁻¹)
Aflatoxin B_1 (μgg⁻¹)
Temperature (°C)
0
2
4
6
8
10
12
200
400
600
800
1,000
1,200
10
20
30
40
50
60

was 30°C. These results are in accord with several workers, such as Rabie & Smalley (1965), Schindler *et al.* (1967), Koehler *et al.* (1985), Kheiralla *et al.* (1992), Lohani *et al.* (1994) and Garcia *et al.* (1994), which revealed that the optimum temperature for fungal growth and aflatoxin production was between 25 and 30°C. However, they differ from those of Mashaly and El-Deeb (1983a) which revealed that the optimal temperature for the growth of *A. flavus* did not coincide with that of aflatoxin production.

(d) Effect of carbon sources:

The effect of different carbon sources on the growth and aflatoxin production was studied to determine the best carbon source required for both criteria. *A. parasiticus* was grown on sucrose-free Czapek's-Dox liquid medium in which 3% (w/v) sucrose was substituted by the equivalent amount of one of the following carbon sources: D-xylose, D-glucose, D-fructose, sucrose, lactose, maltose, mannitol and starch which was used at concentrations of $10 gl^{-1}$. The pH was adjusted to 6.0 and incubation was carried out at 30°C for 10 days. The results obtained are demonstrated in Table 14 and Fig. 16, and indicate that D-glucose was the best carbon source for fungal growth as well as the production of aflatoxin B_1. Comparing the results obtained with glucose to those recorded with other carbon sources, it could be seen that sucrose was the second source to support the fungal growth and aflatoxin B_1 production with 83.9% and 95.7% respectively of that expressed by glucose. It becomes clear that although starch came in the third order regarding fungal growth, it represented the seventh best carbon source for supporting toxin production (33.8% comparing with glucose).

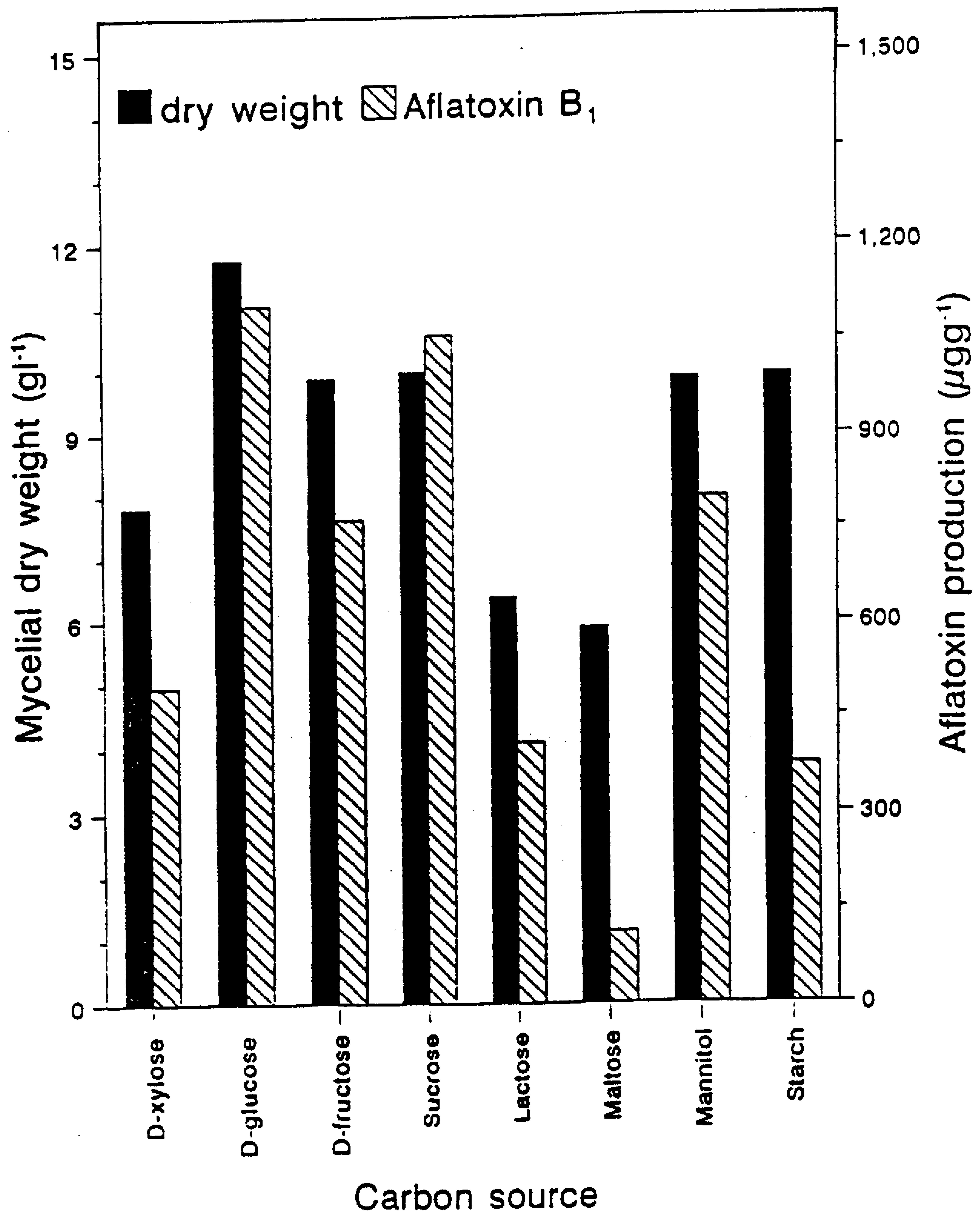
Table 14: Effect of carbon sources on the growth of *Aspergillus parasiticus* NRRL 2999 and aflatoxin B_1 production.*

A. parasiticus was grown on sucrose-free Czapek's-Dox medium in which 3% (w/v) sucrose was substituted by an equivalent amount of one of the tested carbohydrates except starch which was used at $10gL^{-1}$ and the growth period was 10 days at 30°C.

Carbon-Source	Mycelial dry weight (gl^{-1})	Aflatoxin B_1/dry weight (μgg^{-1})
Monosaccharides		
D-xylose	7.8 ± 0.03	494 ± 20
D-glucose	11.8 ± 0.10	1103 ± 65
D-fructose	9.9 ± 0.05	762 ± 42
Hexitols		
Mannitol	9.9 ± 0.05	796 ± 50
Disaccharides		
Sucrose	9.9 ± 0.08	1056 ± 70
Lactose	6.4 ± 0.15	408 ± 35
Maltose	5.9 ± 0.02	112 ± 10
Polysaccharides		
Starch	9.9 ± 0.01	373 ± 27

* Means of three replicates ± S.D.

Fig. 16: Effect of carbon sources on Aflatoxin B_1 production and growth of *A. parasiticus*. (Taken from Table 14).


dry weight
Aflatoxin B1
Mycelial dry weight (gl-1)
Aflatoxin production (µgg-1)
15
12
9
6
3
0
1,500
1,200
900
600
300
0
D-xylose
D-glucose
D-fructose
Sucrose
Lactose
Maltose
Mannitol
Starch
Carbon source

Furthermore, the results indicate that maltose was the poorest carbon source for supporting fungal growth as well as aflatoxin production (50% and 10% of growth and toxin production, respectively).

The results of the present experiment clearly demonstrated that mono-saccharides were much better carbon sources for growth and aflatoxin B_1 production than disaccharides and indicated that sucrose was the best disaccharide for the two processes.

Generally, the various carbon sources examined which supported growth could be arranged as follows: D-glucose > sucrose > starch > D-fructose > mannitol > D-xylose > lactose > maltose. However, regarding their influence on aflatoxin production, they could be arranged as follows: D-glucose > sucrose > mannitol > D-frucose > D-xylose > lactose > starch > maltose.

The various carbon sources examined supported widely differing amounts of aflatoxin B_1 production. Glucose, sucrose, mannitol and fructose supported high levels of toxin production, while low levels of toxin were observed with xylose, lactose, starch and maltose. The reason for these differences is not known so far, but could reflect variations in the mechanisms or relative rates of catabolism among the compounds. These results and explanations are in agreement with those of Mateles & Adye (1965), Davis & Diener (1968), Detroy *et al.* (1971), and Hansa & Saxena (1988). In contrast, Luchese (1991) noticed that under some circumstances, the presence of glucose (or other compatible carbohydrates) was not necessary for the synthesis of aflatoxin. According to the utilization of disaccharides (sucrose, lactose and maltose) and polysaccharides (starch) in the present work by *A. parasiticus*, this utilization may be due to that these substrates are broken down by hydrolytic enzymes,

which are either secreted into the surrounding medium or are present at the fungal cell surface, e.g. amylases, invertase, etc. In addition, free monosaccharides don't accumulate in the fungal cell during uptake but most hexoses are converted to glucose-6-phosphate or fructose-6-phosphate before being metabolized in glycolysis. These explanations are in agreement with those of Berry (1975). In supporting the present results, Buchanan *et al.* (1985) and Luchese & Harrigan (1993) reported that reduced activity of tricarboxylic acid cycle (TCA) which is dependent on the catabolism of suitable carbohydrates, leads to an accumulation of TCA cycle intermediate and/or pyruvate resulting from depressed TCA cycle activity, leading to shunting of acetyl-CoA to aflatoxin synthesis. In addition, glucose may be the inducer of one or more of the enzymes associated with aflatoxin synthesis. Another explanation is that the utilization of readily metabolizable carbohydrates may result in an elevated energy status, which in turn induces the aflatoxin biosynthesis pathway in a process analogous to, but opposite from, catabolite repression. These explanations are in accordance with those of Abdollahi and Buchanan (1981 a & b).

(e) Effect of different nitrogen sources:

This experiment was designed to find out the best nitrogen source for the growth as well as the aflatoxin B_1 production by *A. parasiticus*.

Among the tested eleven nitrogen sources (Table 15 and Fig. 17), yeast extract, corn steep liquor and peptone were the best organic nitrogen sources for both fungal growth and aflatoxin B_1 production. The supplementation with 0.75% yeast extract was more effective in the enhancement of both *A. parasiticus* growth and its toxin productivity. The stimulatory effect of yeast extract may be due to the influence of particular combination of amino acids, minerals, vitamins and other growth factors

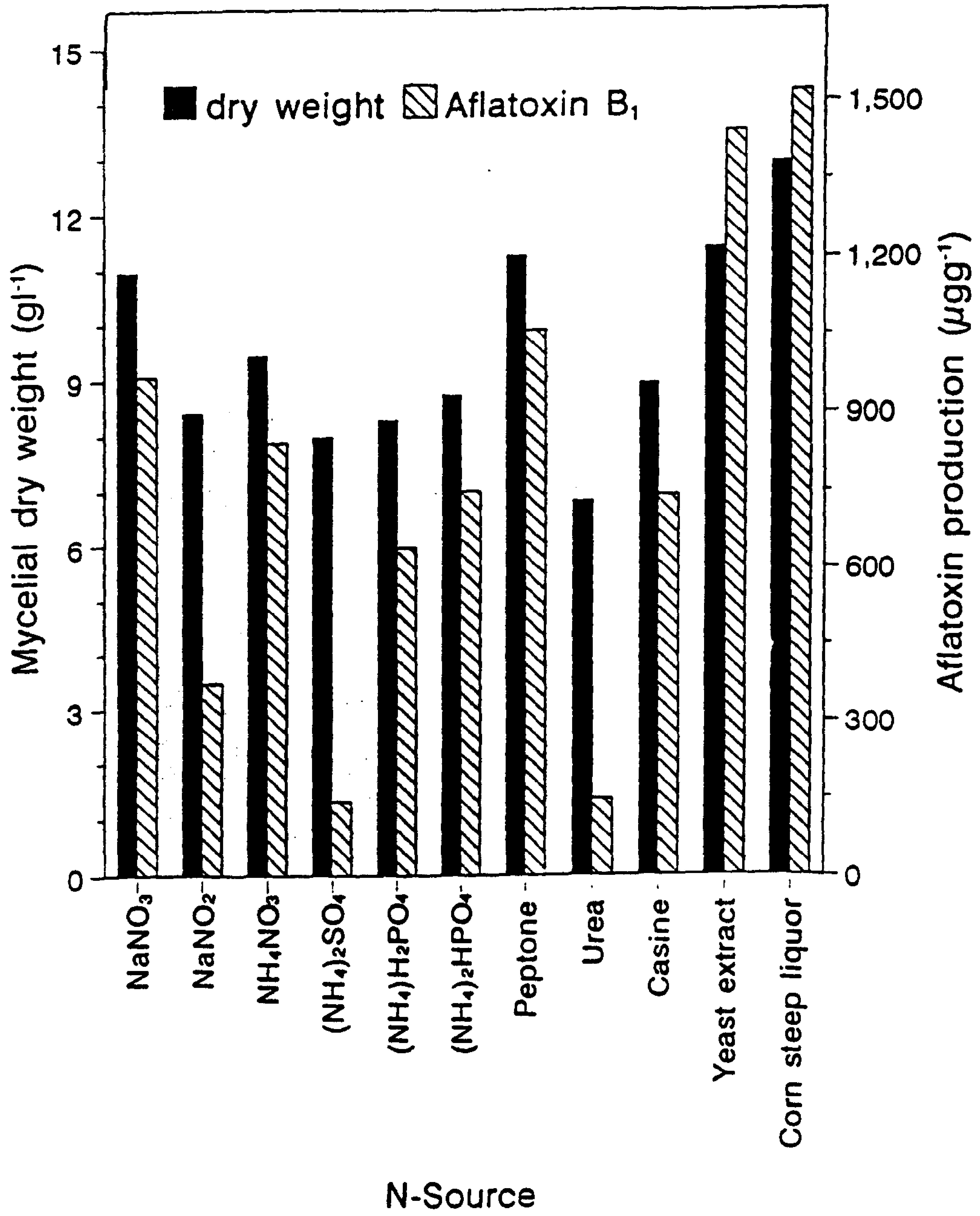
Table (15): Effect of supplying amounts of organic and inorganic nitrogen sources equivalent of NO_3^- on the growth of *Aspergillus parasiticus* NRRL 2999 and its productivity of Aflatoxin B_1.

The fungus was grown on $NaNO_3$-free Czapek's-Dox liquid medium supplemented with 329.4 mg nitrogen L^{-1} which is equimolecular to the nitrogen in 2 g $NaNO_3$ L^{-1}. The organic nitrogen sources used were 0.3% (w/v) peptone, 0.75% (w/v) yeast extract, 0.5% (w/v) casein and 2.5% (v/v) corn steep liquor. The growth period was 10 days at 30°C.

N-Source	Mycelial dry weight (gl^{-1})**	% of control	Aflatoxin B_1/ dry weight ($\mu g g^{-1}$)**	% of control
Inorganic N-sources:				
$NaNO_3$	10.9	100.0	967	100.0
$NaNO_2$	8.4	76.8	373	38.6
NH_4NO_3	9.4	86.1	842	87.0
$(NH_4)_2SO_4$	7.9	72.8	140	14.5
$(NH_4)H_2PO_4$	8.3	75.6	638	66.0
$(NH_4)_2HPO_4$	8.7	79.6	749	77.5
Organic N-sources:				
Peptone	11.3*	102.6	1055*	109.0
Urea	6.8	62.3	145	15.0
Casein	8.9	81.5	740	76.5
Yeast extract	11.4*	104.0	1441*	149.0
Corn steep liquor	12.9*	117.9	1519*	157.0

- NLSD for fungal growth at 5% = 0.04
  at 1% = 0.05
- NLSD for aflatoxin B_1 production at 5% = 7.72
  at 1% = 10.47
- * Highly significant increase.
- Not labeled = highly significant decrease.
- ** Values represent means for triplicate assayed.
- $NaNO_3$ was used as a check N-source material (standard in Czapek's-Dox liquid medium).

Fig. 17: Effect of supplying amounts of organic and inorganic nitrogen sources equivalent of NO_3 nitrogen on the growth of *A. parasiticus* and Aflatoxin B_1 production. (Taken from Table 15).



dry weight
Aflatoxin B1
Mycelial dry weight (gl-1)
Aflatoxin production (μgg-1)
15
12
9
6
3
0
1,500
1,200
900
600
300
0
NaNO3
NaNO2
NH4NO3
(NH4)2SO4
(NH4)H2PO4
(NH4)2HPO4
Peptone
Urea
Casine
Yeast extract
Corn steep liquor
N-Source

required for growth of mould and subsequent production of aflatoxin. These findings are in accordance with the observation of Davis *et al.* (1966), Alderman & Marth (1976) and Varma & Verma (1987).

Regarding corn steep liquour, it may be considered an excellent supplement to defined media such as Czapek's-Dox medium for the stimulation of mycelial growth and this may be attributed to the presence of various elements, amino acids and vitamins that could stimulate aflatoxin production. Further investigations are needed in order to determine whether or not corn steep liquor contains unidentified substances which stimulate the synthesis of aflatoxin.

Peptone, being a complex of nitrogen sources may be the reason of enhancing the fungal growth and aflatoxin B_1 production. Inactivation of fungal growth and aflatoxin B_1 by casein as a nitrogen source could be possibly attributed to the hydrolysis of casein by proteases of *Aspergillus parasiticus* into simple amino acids and amines may affect growth and aflatoxin production (Ismail *et al.*, 1983).

However, when compared with $NaNO_3$ the other tested inorganic nitrogen sources gave less growth and toxin production. The lower fungal growth (23%) and aflatoxin B_1 production (61.4%) with $NaNO_2$ supported the observations of Bullerman *et al.* (1969) and Meir & Marth (1977). In contrast, Strzelecki (1973) observed that $NaNO_2$ enhanced aflatoxin biosynthesis in yeast extract-sucrose broth. Thus, it seems likely to assume that the enhancement or inactivation of aflatoxin depends on the components of the growth medium. Niehaus & Jiang (1989) observed an inactivation of aflatoxin by nitrite and they attributed this inactivation to the elevation of the cytoplasmic ratio NADPH/NADP, resulting in increased conversion of malonyl coenzyme A to fatty acid rather than to polyketide (aflatoxin).

The decreased effect of ammonium ion is consistent with the results of Mashally *et al.* (1983) who found that ammonium was the most effective and promising reagent for chemical inactivation of aflatoxin in contaminated peanut and cottonseed meals. Major studies were carried out on the use of ammonium to decontaminate aflatoxin contaminated feeds, corn, cottonseeds and peanut products and the reaction products have been identified (Coker *et al.* 1985 and Kane *et al.*, 1993).

The effect on secondary metabolite synthesis by nitrogen sources is usually mediated by repressing a number of enzymes involved in the assimilation of ammonium ions or metabolism of amino acids (Martin & Demain, 1980).

The relationship between nitrogen assimilation metabolism and aflatoxin formation may be due to the assimilation of ammonia from the medium via NADPH-requiring glutamate dehydrogenase. It may be assumed also that α-ketoglutamate, the product of NAD-glutamate-dehydrogenase, stimulate acetate incorporation into aflatoxin by inhibition of the tricarboxilic acid cycle. These assumptions are supported by those of Bhatnager *et al.* (1986 a, b) and Dutton (1988).

The importance of corn steep liquor, yeast extract and peptone as organic nitrogen sources for the growth of *A. parasiticus* and its productivity of aflatoxin B_1 led to an investigation of the role of amino acids singly supplied in the subsequent experiments.

(f) <u>Effect of amino acids</u>:

The effect of different amino acids on the growth and the aflatoxin production of *A. parasiticus* was investigated. Ten amino acids were tested, namely: threonine, arginine, glutamic acid, phenylalanine, leucine, proline, alanine, tryptophan, glycine and

asparagine. These amino acids were added to the growth medium in amounts calculated to provide a final nitrogen amount of 0.329 gl^{-1}. The pH was adjusted at 6.0 and the incubation was carried out at 30°C for 10 days.

The results in Table 16 and Fig. 18 show that all the tested amino acids supported growth. Asparagine supported significantly more growth of *A. parasiticus* (126% of control), followed by arginine, (120% of control), whereas alanine and phenylalanine recorded the least effect on growth (101% and 108% of control). Of the ten examined amino acids, proline and asparagine supported higher toxin production (132.3% and 128.7% of control), whereas glutamic acid and leucine (94.8% and 92.8% of control) supported reduced toxin production (Table 16 and Fig. 18). Tryptophan and glutamic acids supported the fungal growth with 118% and 115%, respectively. In contrast, tryptophan supported aflatoxin production with 108.4% but glutamic acid retarded 5.2% of the productivity.

The results show that asparagine was the best amino acid for fungal growth but proline was the best for aflatoxin B_1 production. In addition, tryptophan as an aromatic amino acid was better than phenylalanine another aromatic amino acid in supporting fungal growth as well as the productivity of aflatoxin B_1.

Studying the effect of amino acids on the fungal growth and aflatoxin B_1 production demonstrated that the two processes were variably influenced by the tested amino acids. Proline supported greater toxin production compared to the other amino acids like asparagine which expressed high productivity of aflatoxin as well. Proline and asparagine were reported to be good nitrogen sources supporting aflatoxin production (Reddy *et al.*, 1979; Payne & Hagler, 1983). Furthermore, asparagine was

Table 16: Effect of different amino acids on the growth of *Aspergillus parasiticus* NRRL 2999 and Aflatoxin B_1 production.

The amino acids were added to nitrogen-free Czapek's-Dox medium at equimolecular amount of nitrogen that exists in 0.2% (w/v) $NaNO_3$. The growth period was 10 days at 30°C.

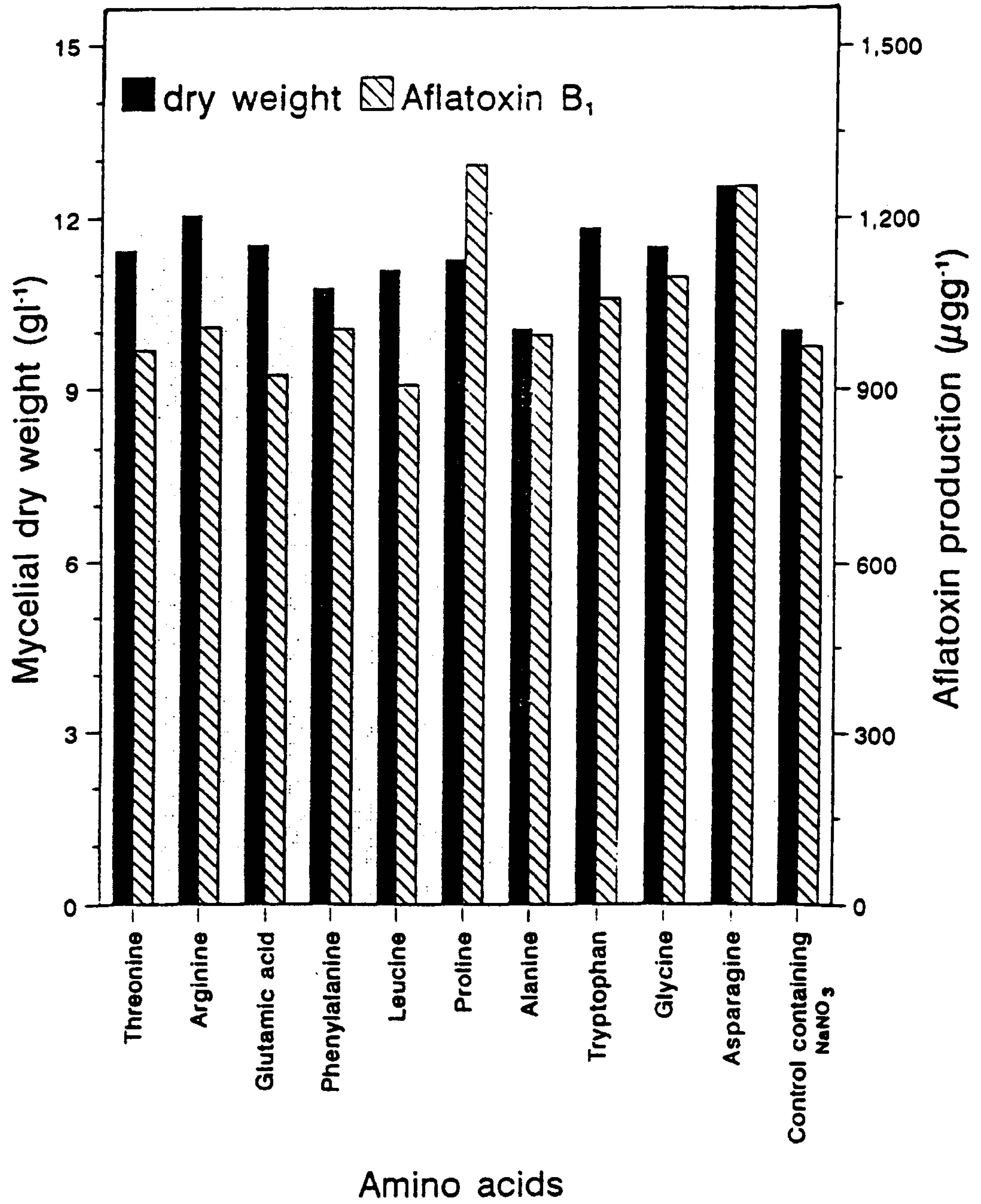
Amino acid	Mycelial dry weight (gl^{-1})**	% of control	Aflatoxin B_1/ dry weight (μgg^{-1})**	% of control
Threonine	11.4**	114	967	99.1
Arginine	12.0**	120	1008	103.3
Glutamic acid	11.5**	115	925	94.8
Phenylalanine	10.8**	108	1005	103.0
Leucine	11.1**	111	906	92.8
Proline	11.3**	113	1291**	132.3
Alanine	10.1	101	995	102.0
Tryptophan	11.8**	118	1058*	108.4
Glycine	11.5**	115	1095**	112.3
Asparagine	12.6**	126	1256**	128.7
Control containing $NaNO_3$	10.0	-	976	-

NLSD : for dry weight at 5% = 0.3
at 1% = 0.4
for aflatoxin production at 5% = 70.6
at 1% = 93.9

* = Significant.

** = Highly significant.

Fig. 18: Effect of amino acids on the growth of *A. parasiticus* and Aflatoxin B_1 production. (Taken from Table 16).



dry weight
Aflatoxin B1
Mycelial dry weight (gl-1)
Aflatoxin production (μgg-1)
15
12
9
6
3
0
1,500
1,200
900
600
300
0
Threonine
Arginine
Glutamic acid
Phenylalanine
Leucine
Proline
Alanine
Tryptophan
Glycine
Asparagine
Control containing NaNO3
Amino acids

found to be necessary for high toxin production by *A. parasiticus* (Reddy *et al.*, 1971). In addition, it was shown that lack of asparagine resulted in a drastic reduction of aflatoxin production by *A. parasiticus* (Rao *et al.*, 1980).

The mechanism(s) by which proline and asparagine stimulate toxin production is still unknown. However, it may be assumed that these compounds could be involved in the biosynthesis pathway of aflatoxin production as carbon sources. More likely, these amino acids influenced toxin production through an effect on primary or secondary metabolism. This assumption is supported by that reached by Payne & Hagler (1983).

The present results showed that the aromatic amino acids tryptophan and phenylalanine supported both fungal growth and aflatoxin production. These results are in agreement with those of Reddy *et al.* (1971) and Payne & Hagler (1983) for *A. parasiticus*.

Alanine, a pyruvate-derived amino acid, did not affect the fungal growth. However, it stimulated the aflatoxin production. These results are consistent with those of Naik *et al.* (1970).

Glutamic acid caused an increase in the fungal growth but inhibited slightly aflatoxin production. These results are in agreement with those of Mateles & Adye (1965) who reported that glutamate supported the fungal growth but little aflatoxin accumulation.

Other amino acids were reported to support a good fungal growth and aflatoxin production, e.g. methionine, tyrosine and histidine (Naik *et al.*, 1970; Shoukry *et al.*, 1992). Generally, the stimulatory effect of various amino acids may

either be due to their effect on growth and general primary metabolism of *A. parasiticus* or a direct effect on aflatoxin biosynthesis.

It may be concluded that organic nitrogen compounds are necessary for the production of high levels of aflatoxin and complex nitrogen sources, such as yeast extract and corn steep liquor gave higher yields than a single amino acid. The effect of secondary metabolite synthesis by nitrogen sources is usually mediated by inhibiting a number of enzymes involved in assimilation of ammonium ions, or amino acid metabolism, or inhibition of enzymes involved in nitrogen catabolism by rapidly used nitrogen sources such as ammonia.

(g) Effect of metal ions

The effect of some selected metal ions namely, Zn^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+}, Al^{3+}, Cd^{2+}, Mg^{2+}, Cu^{2+}, and Fe^{3+} on the growth of *A. parasiticus* as well as its productivity of aflatoxin B_1 was studied. The metal ions were tested as chloride salt except Ni^{2+} and Cd^{2+} which were used as $NiNO_3$ and $CdSO_4$.

A. parasiticus was grown on Czapek's-Dox medium at pH 6.0, containing different concentrations, namely, 5, 10, 20, 50 and 100 mgl^{-1} of the above mentioned ions and incubated for 10 days at 30°C. The results obtained are listed in Tables 17 and 18 representing growth and aflatoxin B_1 production, respectively and illustrated in Figs. 19, 20, & 21. The results indicate that the presence of metal ions in the growth medium of *A. parasiticus* expressed variable effects on the fungal growth and aflatoxin B_1 production. The effect appeared to be dependent on the type of metal ion and its concentration.

Table 17: Effect of different concentrations of some metal ions on the growth of *Aspergillus parasiticus* NRRL 2999.
(The incubation period was 10 days at 30°C)

Metal ions	Cation Concentration (mgl^{-1})									
	5		10		20		50		100	
	dry weight (gl^{-1})	% of control	dry weight (gl^{-1})	% of control	dry weight (gl^{-1})	% of control	dry weight (gl^{-1})	% of control	dry weight (gl^{-1})	% of control
$ZnCl_2$	13.30**	131.7	18.40**	182.2	19.50**	193.1	24.60**	243.6	11.20**	110.9
$CoCl_2$	12.20**	120.8	17.20**	170.3	19.90**	197	23.40**	231.7	12.20**	120.8
$MnCl_2$	11.20**	110.9	9.90	98	9.90	98	9.00	89	9.00	89
$AlCl_3$	10.20^NS	101	10.10^NS	100	9.90	98	9.90	98	9.80	97
$MgCl_2$	10.00^NS	99	10.20^NS	101	10.00^NS	99	10.00^NS	99	9.90	98
$CuCl_2$	9.22	91.3	9.90	98	9.90	98	9.80	97	9.80	97
$FeCl_3$	3.40	33.7	3.30	32.7	3.20	31.7	3.10	30.7	3.10	30.7
$CdSO_4$	10.20^NS	101	10.80**	106.9	10.30**	102	10.30**	102	10.30**	102
$NiNO_3$	10.10^NS	100	10.90**	107.9	11.30**	111.9	11.00**	108.9	9.90	98

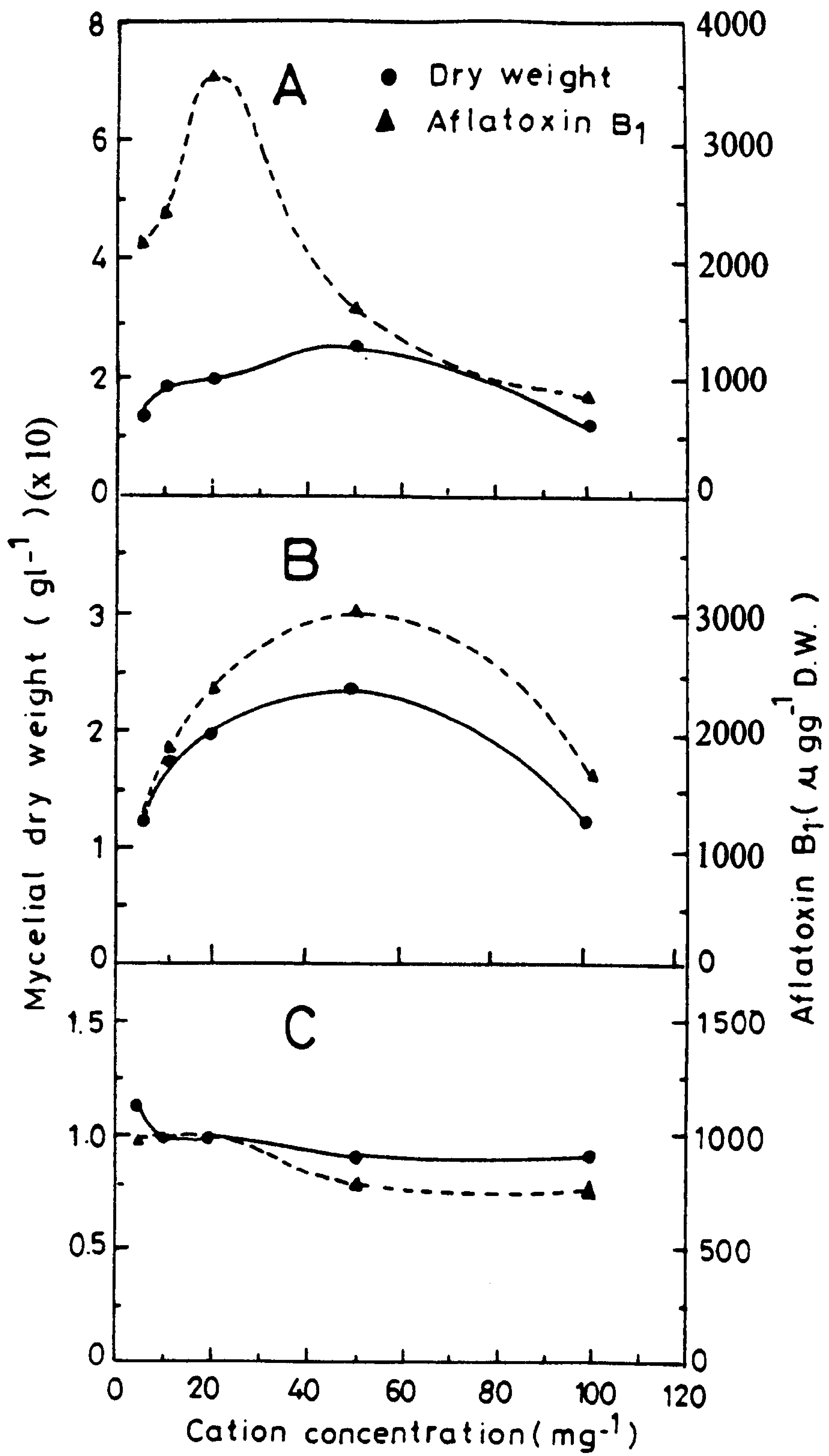
Control value: 10.1 gl^{-1}
NLSD: at 5% = 0.11
at 1% = 0.15
** = Highly significant increase.
Not labeled = Highly significant decrease.
NS = Not significant.

Table 18: Effect of different concentrations of some metal ions on aflatoxin B_1 production ($\mu g g^{-1}$ mycelial dry weight) by *Aspergillus parasiticus* NRRL 2999. (The incubation period was 10 days at 30°C)

Metal ions	Cation Concentration ($mg l^{-1}$)									
	5		10		20		50		100	
	AFB_1	% of control	AFB_1	% of control	AFB_1	% of control	AFB_1	% of control	AFB_1	% of control
$ZnCl_2$	4265**	440.6	4775**	493.3	7097**	733.2	3119**	322.2	1654**	170.9
$CoCl_2$	1229**	127	1864**	192.6	2389**	246.8	3013**	311.3	1609**	166.3
$MnCl_2$	977 NS	101	982 NS	101.4	992*	102.5	781	80.7	759	78.4
$AlCl_3$	1001**	103.4	997**	103	904	93.4	900	93.0	858	88.6
$MgCl_2$	959 NS	99.1	936	96.7	886	91.5	868	89.6	728	75.2
$CuCl_2$	735	75.9	729	75.3	719	74.3	644	66.5	606	62.6
$FeCl_3$	259	26.8	294	30.4	288	29.8	275	28.4	275	28.4
$CdSO_4$	880	90.9	757	78.2	694	71.7	660	68.2	576	59.5
$NiNO_3$	1085**	112.1	1183**	122.2	1427**	147.4	1021**	105.5	677	70

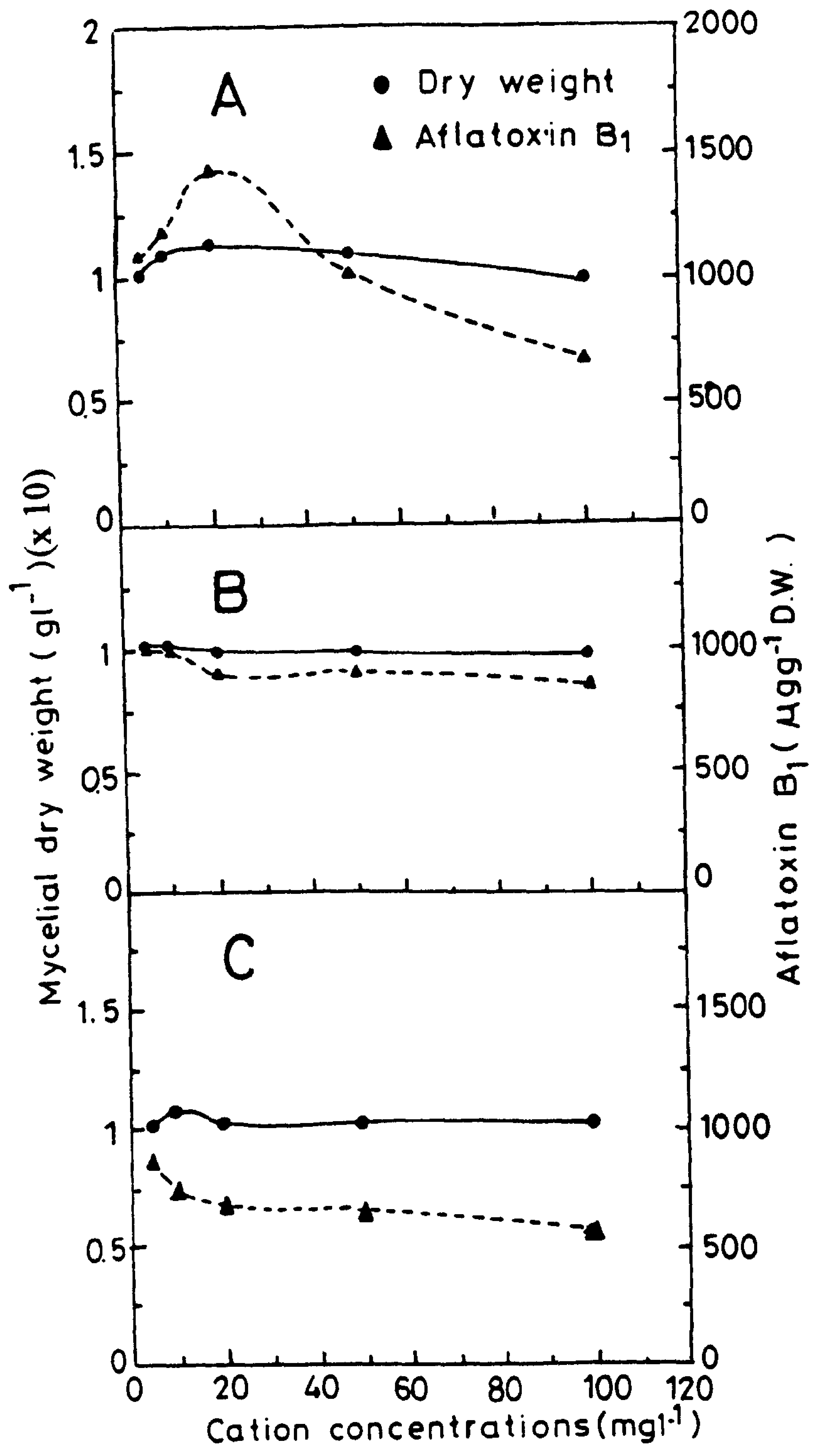
Control value: 968 $\mu g l^{-1}$
NLSD: at 5% = 21.66
at 1% = 28.28
* = Significant increase.
** = Highly significant increase.
Not labeled = Highly significant decrease.
N.S. = Not significant.

Fig. 19: Effect of different concentrations of some metal ions $ZnCl_2$ (A), $CoCl_2$ (B) and $MnCl_2$ (C) on the growth of *A. parasiticus* and Aflatoxin B_1 production. (Taken from Tables 17 & 18).



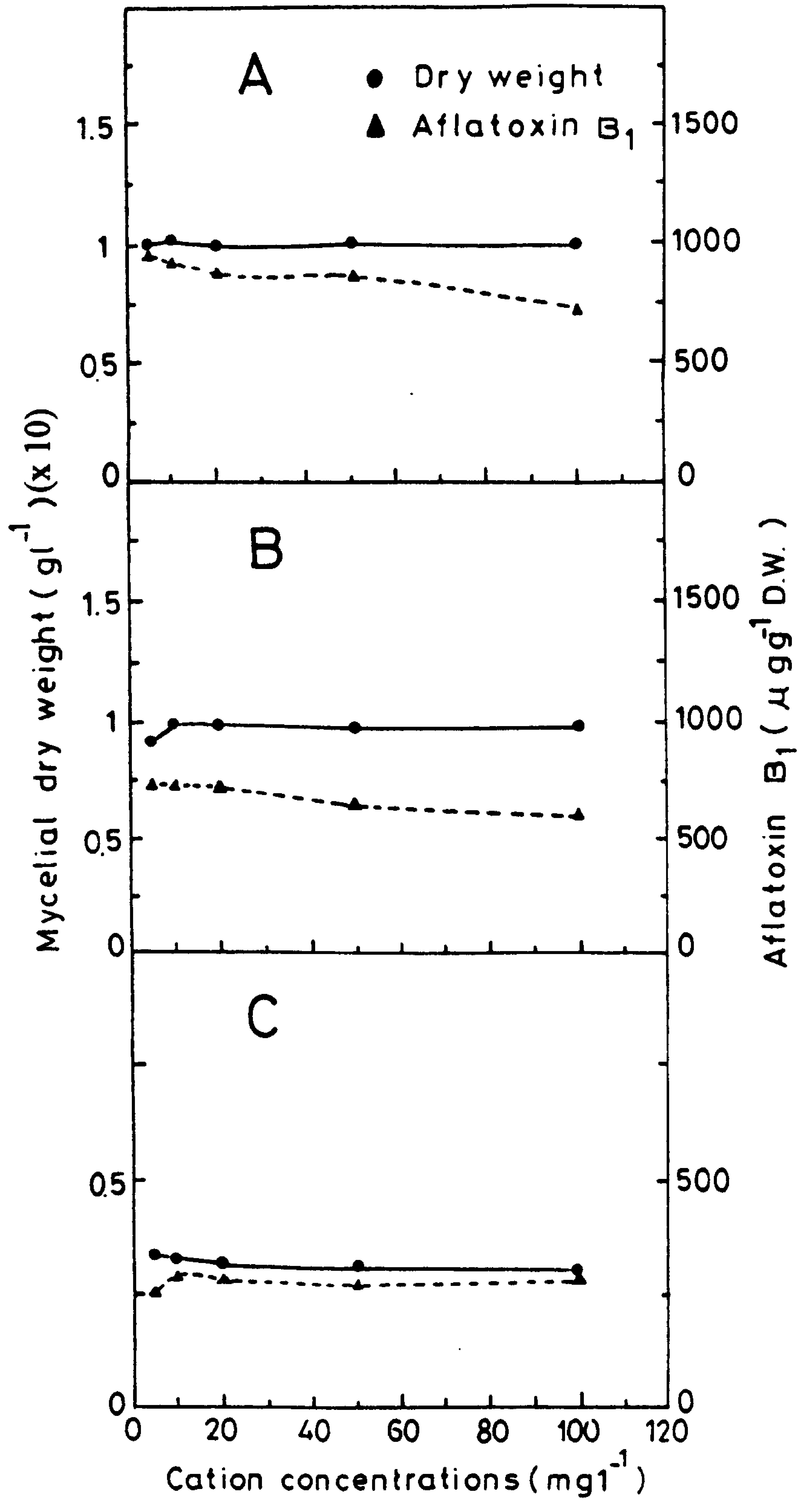
A
B
C
Dry weight
Aflatoxin B1
Mycelial dry weight (gl-1)(x 10)
Aflatoxin B1 (μg g-1 D.W.)
Cation concentration (mg-1)
0
20
40
60
80
100
120
8
6
4
2
0
3
2
1
0
1.5
1.0
0.5
0
4000
3000
2000
1000
0
3000
2000
1000
0
1500
1000
500
0

Fig. 20: Effect of different concentrations of some metal ions $NiNO_3$ (A), $AlCl_3$ (B) and $CdSO_4$ (C) on the growth of *A. parasiticus* and Aflatoxin B_1 production. (Taken from Tables 17 & 18).



A
B
C
Dry weight
Aflatoxin B1
2
1.5
1
0.5
0
2000
1500
1000
500
0
Mycelial dry weight (gl-1)(x 10)
Aflatoxin B1 (μg g-1 D.W.)
0
20
40
60
80
100
120
Cation concentrations (mgl-1)

Fig. 21: Effect of different concentrations of some metal ions $MgCl_2$ (A), $CuCl_2$ (B) and $FeCl_3$ (C) on the growth of *A. parasiticus* and Aflatoxin B_1 production. (Taken from Tables 17 & 18).


A
Dry weight
Aflatoxin B1
B
C
Mycelial dry weight (g l-1)(x 10)
Aflatoxin B1 (µg g-1 D.W.)
1.5
1
0.5
0
1500
1000
500
0
0
20
40
60
80
100
120
Cation concentrations (mg l-1)

Zn^{2+} and Co^{2+} expressed a stimulatory effect which was highly significant on both fungal growth as well as aflatoxin B_1 production at the different concentrations.

The optimum concentration of Zn^{2+} required for the maximum growth of *A. parasiticus* was 50 mgl^{-1}, whereas that for aflatoxin B_1 production was 20 mgl^{-1}. However, in case of Co^{2+}, the optimal concentration required for both maximal growth and aflatoxin B_1 production was 50 mgl^{-1}.

In addition, Mn^{2+}, Al^{3+}, and Cd^{2+} ions expressed a stimulatory effect on the fungal growth but only at the lowest concentration (5 mgl^{-1}). On the other hand, Cd^{2+} caused an 8% increase in the fungal growth apparently at 10 mgl^{-1}, above which there was a 3% increase within the range 20-100 mgl^{-1}.

Regarding the effect of the metal ions on aflatoxin B_1 production (Table 18), the results indicated that Mn^{2+} and Al^{3+} were of stimulatory effect up to 20 and 5 mgl^{-1}, respectively, after which any further increase in its concentration led to an inhibition, while Cd^{2+} which at all tested concentrations toxin production was decreased and the decrease paralleling the increasing concentration.

Tables 17 & 18 clearly shows that Mg^{2+}, Cu^{2+} and Fe^{3+} caused a decrease in the fungal growth and aflatoxin production at the various concentrations. The degree of inhibition was found proportional with the tested concentrations. Ni^{2+} was a stimulatory ion up to 20 mgl^{-1} for fungal growth as well as toxin production, after which any further increase in its concentration led to decrease the two processes.

From the results in Tables 17 and 18 it seems that there is a wide variation between the effects of the different cations on growth and toxin production. This variation could be summarized in the following points:

(a) Zn^{2+} and Co^{2+} expressed higher stimulatory effect on aflatoxin production than on growth rate at all concentrations tested.

(b) Ni^{2+} and Al^{3+} increased aflatoxin production over the growth rate up to 20 mgl^{-1} and 5 mgl^{-1} respectively.

(c) Cd^{2+} resulted in the stimulation of growth rate but decreasing aflatoxin production at all concentrations.

It appears that the various metal ions tested in the present work could be arranged regarding to their effect on fungal growth as:

$$Zn^{2+} > Co^{2+} > Mn^{2+} > Ni^{2+} > Cd^{2+} > Al^{3+}$$

But for their effect on aflatoxin production they are arranged as:

$$Zn^{2+} > Co^{2+} > Ni^{2+} > Al^{3+} > Mn^{2+}.$$

Also, it was found that the ion effect is dependent on the metal type as well as its concentration. The inhibitory effects of iron, as shown in the present results, agree with those of Marsh *et al.* (1975) and Tiwari *et al.* (1986b) who reported a decrease in aflatoxin production when ferric chloride was added to *A. parasiticus* medium and this inhibition varied from 18 up to 37% according to the concentration used. On the other hand, these results disagree with those of Lillehoj *et al.* (1974) who reported that the addition of iron to the medium did not affect aflatoxin production. However, a stimulatory effect was reported by several authors due to the presence of iron in the media (Davis *et al.*, 1967; Maggon *et al.*, 1973 and Mashaly & El-Deeb, 1983b). This contradiction may be attributed to the different strains and culture conditions.

In addition, several workers have found that Zn^{2+} is essential for aflatoxin production and have observed that a relatively high level of zinc has a stimulatory effect on aflatoxin production (Diener & Davis, 1969). In the present work, Zn^{2+} expressed the most striking effect on growth rate and aflatoxin production. These results agree with those of Nesbitt *et al.* (1962), Maggon *et al.* (1973 & 1977) and Steele *et al.* (1973). This stimulatory effect of Zn^{2+} increased with increasing concentrating up to intermediate levels in the growth medium. The highest rates of growth and aflatoxin B_1 production were recorded at concentration of 50 mgl^{-1} and 20 mgl^{-1}, respectively. In support, Prasad (1972) found that maximum aflatoxin production occurred when Zn^{2+} concentration in growth media was 22 mgl^{-1}. However, Maggon *et al.* (1973) found higher Zn^{2+} requirement (50 mgl^{-1}) for aflatoxin production by *A. flavus*.

The stimulatory effect of Cu^{2+} on the production of aflatoxin and growth rate was reported by Maggon *et al.* (1973) which is contradicting with the present results, in which Cu^{2+} did not affect the growth or toxin production. However, the present results agree with those of Davis *et al.* (1967) who reported little or no effect of Cu^{2+} on fungal growth and decreased aflatoxin production.

The present results showed that Co^{2+} expressed a stimulatory effect on growth rate and aflatoxin production of *A. parasiticus*, which disagree with those of Lee *et al.* (1966) and Maggon *et al.* (1973). The stimulatory effects of Co^{2+} in the present results could be attributed to its involvement as a co-factor in a number of enzymes or in altering or regulating the biosynthesis of aflatoxins in some way and enhanced incorporation of acetate into aflatoxins.

Mn^{2+} supported slightly both toxin production and the fungal growth at the lower concentrations but at the higher concentrations inhibited both particularly fungal growth. Mateles & Adye (1965), Lee *et al.* (1966) and Maggon *et al.* (1973) observed that manganese exerted no effect or slightly reduced growth and aflatoxin formation. However, the presence of Mn^{2+} seems to be a variable factor for the growth and toxin production as reported by several authors. However, Park & Han (1988) reported that the growth of *A. flavus* was inhibited when it was grown on Mn-depleted medium. These contradictions could be attributed to different species and different media.

The present results showed that Ni^{2+} and Al^{3+} supported aflatoxin production. This could be attributed to their activation of pyruvate carboxylase and acetyl-CoA carboxylase. In the same context the activation of pyruvate carboxylase may result in an increased production of intermediates of the citrate cycle which are known to stimulate aflatoxin production. The presence of two cations may also result in an increased aflatoxin production through an increased production of malonyl-CoA by activating acetyl-CoA carboxylase. Malonate has been suggested to be an intermediate in aflatoxin biosynthesis. Furthermore, Al^{3+} may activate phosphoglucomutase which leads to increase levels of glycolytic intermediates and hence increased aflatoxin production. These explanations are in agreement with those reached by Dixon & Webb (1958), Underwood (1962), Gupta *et al.* (1975) and Tyagi & Venkitasubramanian (1981).

(h) <u>Zn^{2+}-responsive period</u>:

As shown from the previous experiment, Zn^{2+} had the greatest stimulatory action on aflatoxin B_1 biosynthesis at concentration of 20 mgL^{-1}. Thus, the aim of the present experiment was to find out the Zn^{2+}-responsive period during the culture age.

In this experiment, 20 mgl^{-1} of Zn^{2+} was added to Czapek's-Dox liquid medium at various times after inoculation (0, 2, 4, 6 and 8 days). Mycelial dry weight and aflatoxin B_1 production were quantified after 10 days of incubation at 30°C. The results in Fig. 22 indicate that when the Zn^{2+} was added before 2 days, full stimulation of aflatoxin B_1 synthesis was observed. However, when Zn^{2+} was added after 4 days, no stimulation was observed.

The results, also shown in Fig. 23, reveal that the fungal biomass was increased in presence of Zn^{2+} and the increase was remarkable particulary after Zn^{2+} was added at 0, 2, 4 and 6 days of inoculation.

Weinberg (1978, 1982) reported the effect of trace elements on secondary metabolism. He and others have found that the trace elements were required for secondary processes. The increase in aflatoxin B_1 production after adding Zn^{2+} at 0 and 2 days of inoculation indicate that Zn^{2+} required for aflatoxin B_1 biosynthesis by *A. parasiticus* during early days of growth which permits formation of secondary metabolites. The stimulatory Zn^{2+} could activate enzymes or might function at the genomic (transcriptional) level to facilitate the expression of genes that control formation of secondary metabolites (Weinberg, 1970, and Obidoa & Ndubuisi, 1981).

(i) <u>Effect of water activity</u>

Water activity (a_w) has taken the place of moisture as the most useful expression of the availability of water for growth of microorganisms. This experiment was carried out to determine the lower range of a_w at which *A. parasiticus* be able to grow and produce aflatoxin B_1.

Fig. 22: Effect of addition of 20 mgL^{-1} Zn^{2+} at different periods on the aflatoxin B_1 production by *A. parasiticus*.

Control experiment was carried out without the addition of Zn^{2+} to the growth medium.

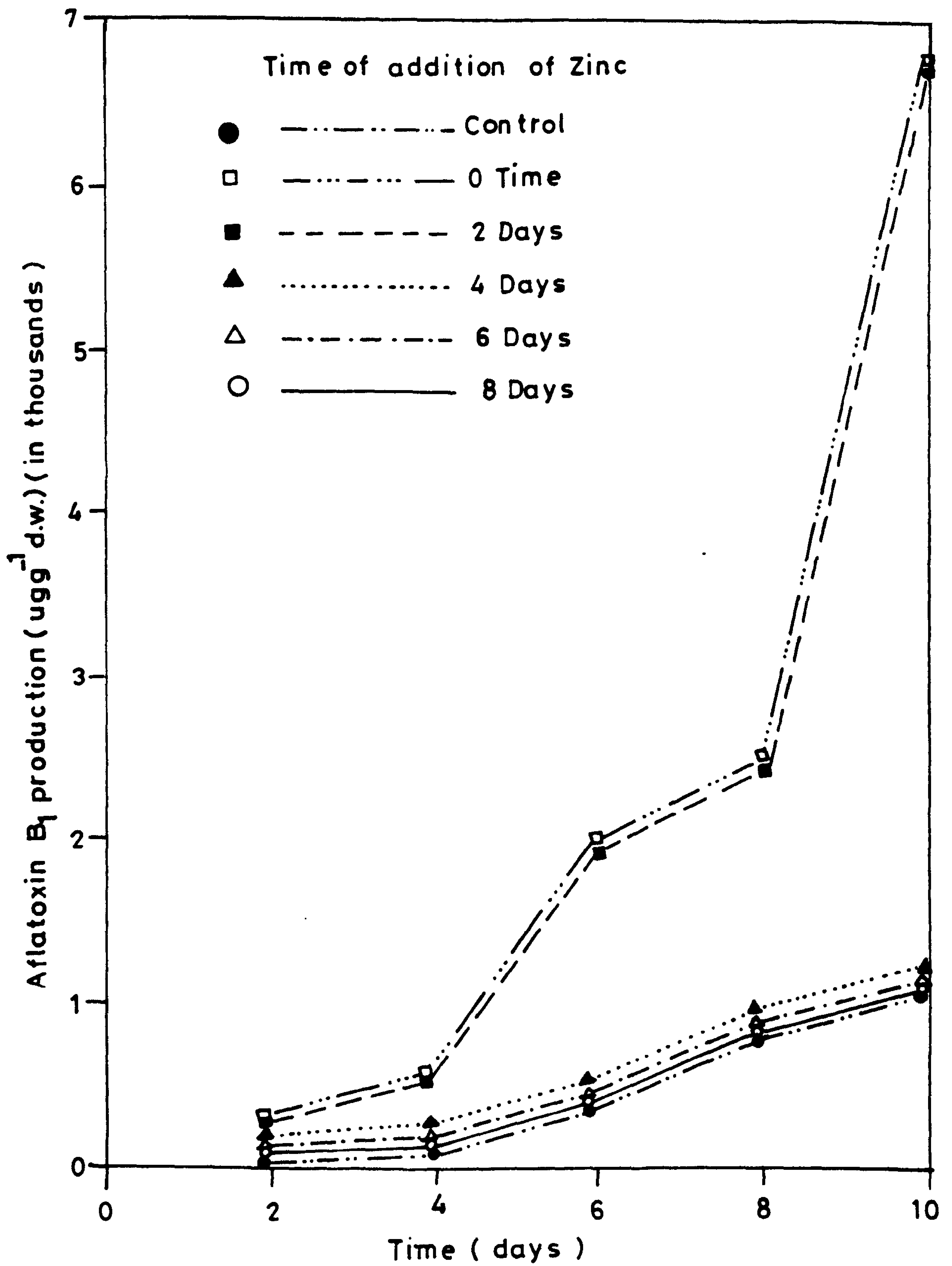

Time of addition of Zinc
Control
0 Time
2 Days
4 Days
6 Days
8 Days
Aflatoxin B1 production (ugg-1 d.w.) (in thousands)
Time (days)
0
1
2
3
4
5
6
7
0
2
4
6
8
10

Fig. 23: Effect of addition of 20 mgL^{-1} Zn^{2+} at different periods on the biomass of *A. parasiticus*.

Control experiment was carried out without the addition of Zn^{2+} to the growth medium.

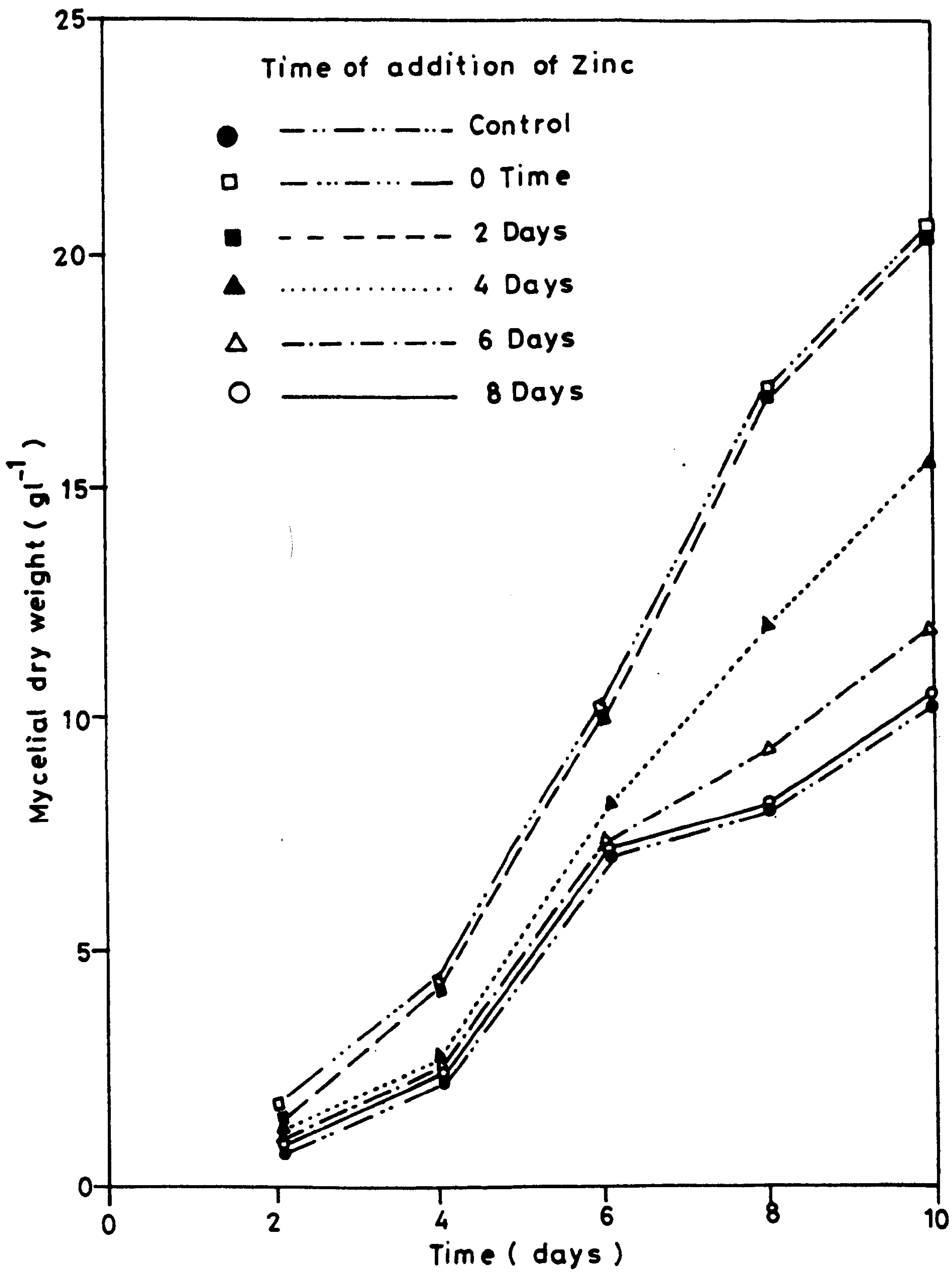

Time of addition of Zinc
Control
0 Time
2 Days
4 Days
6 Days
8 Days
Mycelial dry weight (gl^{-1})
Time (days)
0
5
10
15
20
25
0
2
4
6
8
10

Table 19 includes the results obtained from studying the effect of different water activity (0.77, 0.80, 0.83, 0.86 and 0.88) on the growth of *A. parasiticus* and aflatoxin B_1 production at 25°C, for 6 weeks, on coffee beans and rice grains. The fungal growth on coffee beans and rice grains was determined by measuring glucosamine (as a result of alkaline hydrolysis of fungal cell wall chitin) concentration (Figs. 24 & 25). The results indicate that there is a relationship between a_w (coffee beans and rice grains) and the amounts of glucosamine. The maximal amount of glucosamine (22.1-30.2 and 19.5-23.2 $\mu g g^{-1}$) in both coffee and rice occurred in the range of 0.86-0.88 a_w. Furthermore, the lowest contents of glucosamine at a_w = 0.80 on coffee and rice were 4.0 and 2.9 $\mu g g^{-1}$, respectively .

The highest toxin production recorded at a_w = 0.88 in coffee beans and rice grains were 490 and 565 $\mu g Kg^{-1}$, respectively. The lowest amounts of aflatoxin B_1 were 30 and 10 $\mu g Kg^{-1}$ in coffee and rice at a_w 0.83 and 0.80, respectively. However, neither growth nor aflatoxin production could be detected in coffee beans and rice grains at a_w = 0.77. Generally, it seems likely that toxin production was correlated with growth - maximum at highest a_w value and the fungus grows and produces toxin over a range of 0.8-0.88 a_w.

Based on the present results, water activity could be considered as an important factor. The present results agree well with those of Hunter (1969). It may be assumed that the lower the moisture content, the slower the fungal invasion and the lower glucosamine content of seeds. It is a well known fact that the mycelial growth and aflatoxin B_1 production are dependent on the water activity. This assumption is supported by Northolt *et al.* (1976 & 1977), Holmquist *et al.* (1983) and Ellis *et al.* (1994). However, certain authors reported different results which may be attributed to

another factor that involves with water activity which is temperature. The inhibition of growth and aflatoxin production at low a_w may be due to that water is bound by salts, sugars, proteins and other solutes. Thus growth of *A. parasiticus* cannot take place when water is not present in an available form.

In support of the present results, Stevens & Relton (1983) reported that the lower of water activity to which the organism can grow may depend on three factors; first, on its ability to take up water either from the high osmotic strength substratum on which it grows or direct from the surrounding water vapour, second, the minimum amount of available water within the mycelium which is necessary for cell metabolism to occur, and third, the water content of unimbibed resting seeds and of fungal spores range between 5 and 25%. In both cases metabolic activity is very low and this may be due in part to the fact that enzyme activities and the diffusion of metabolites are much reduced when the water content of cells and cell extracts is reduced below 20% (Stevens & Stevens, 1979). These factors probably combine together with others to limit the growth rate at low water activity.

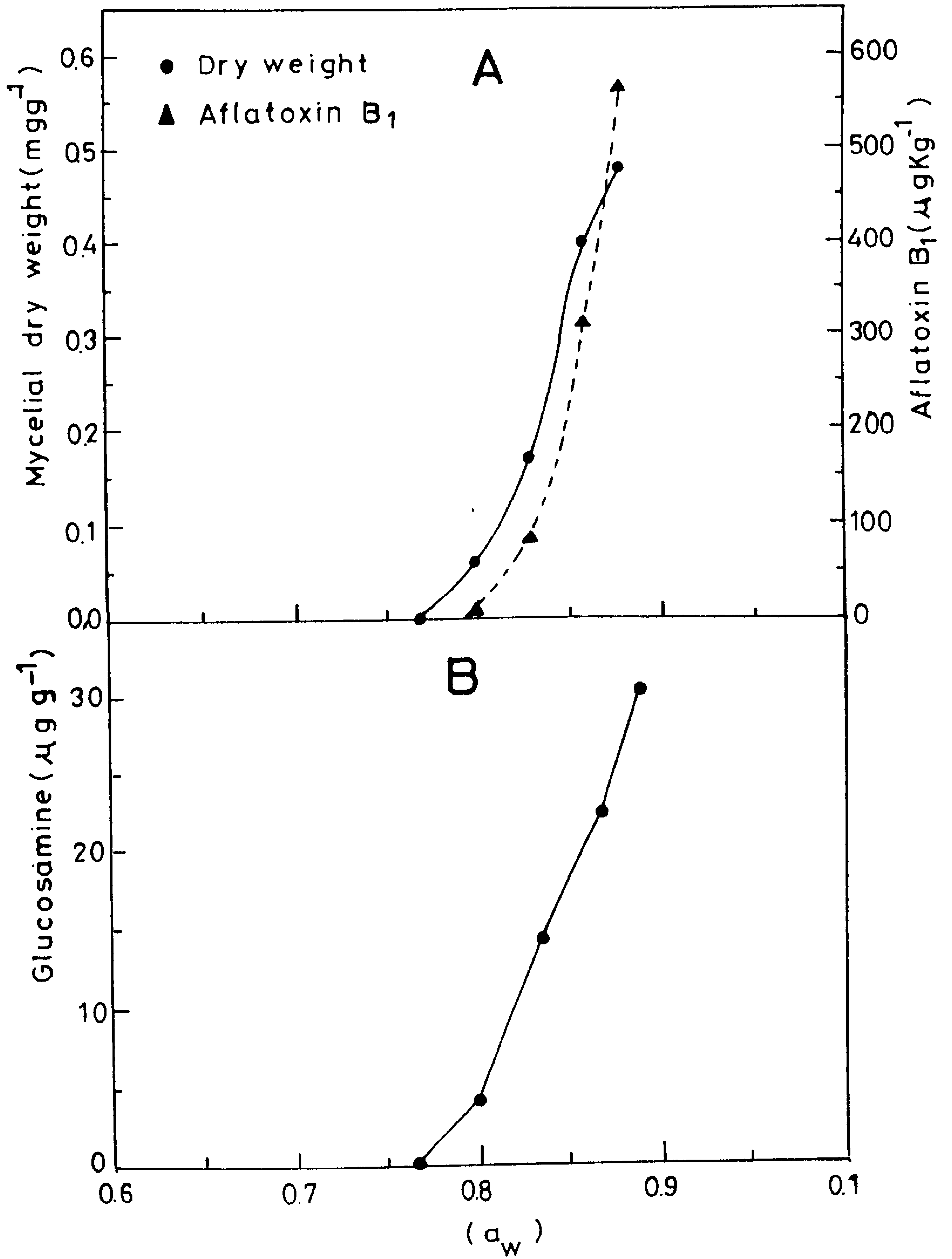
Table 19: Effect of water activity on the growth of *Aspergillus parasiticus* NRRL 2999 expressed as μg glucosamine and Aflatoxin B_1 production on rice grains and coffee beans. (The incubation period was 6 weeks at 25°C)

a_w	Glucosamine ($\mu g g^{-1}$)		Aflatoxin B_1 ($\mu g Kg^{-1}$)	
	Rice	Coffee	Rice	Coffee
0.88	23.2 ± 4	30.2 ± 5	565 ± 30	490 ± 75
0.86	19.5 ± 3	22.1 ± 6	314 ± 22	205 ± 42
0.83	8.0 ± 4	14.1 ± 3	85 ± 12	30 ± 10
0.80	2.9 ± 1	4.0 ± 2	10 ± 5	N.D.
0.77	N.D.*	N.D.	N.D.	N.D.

N.D. = Not detected.

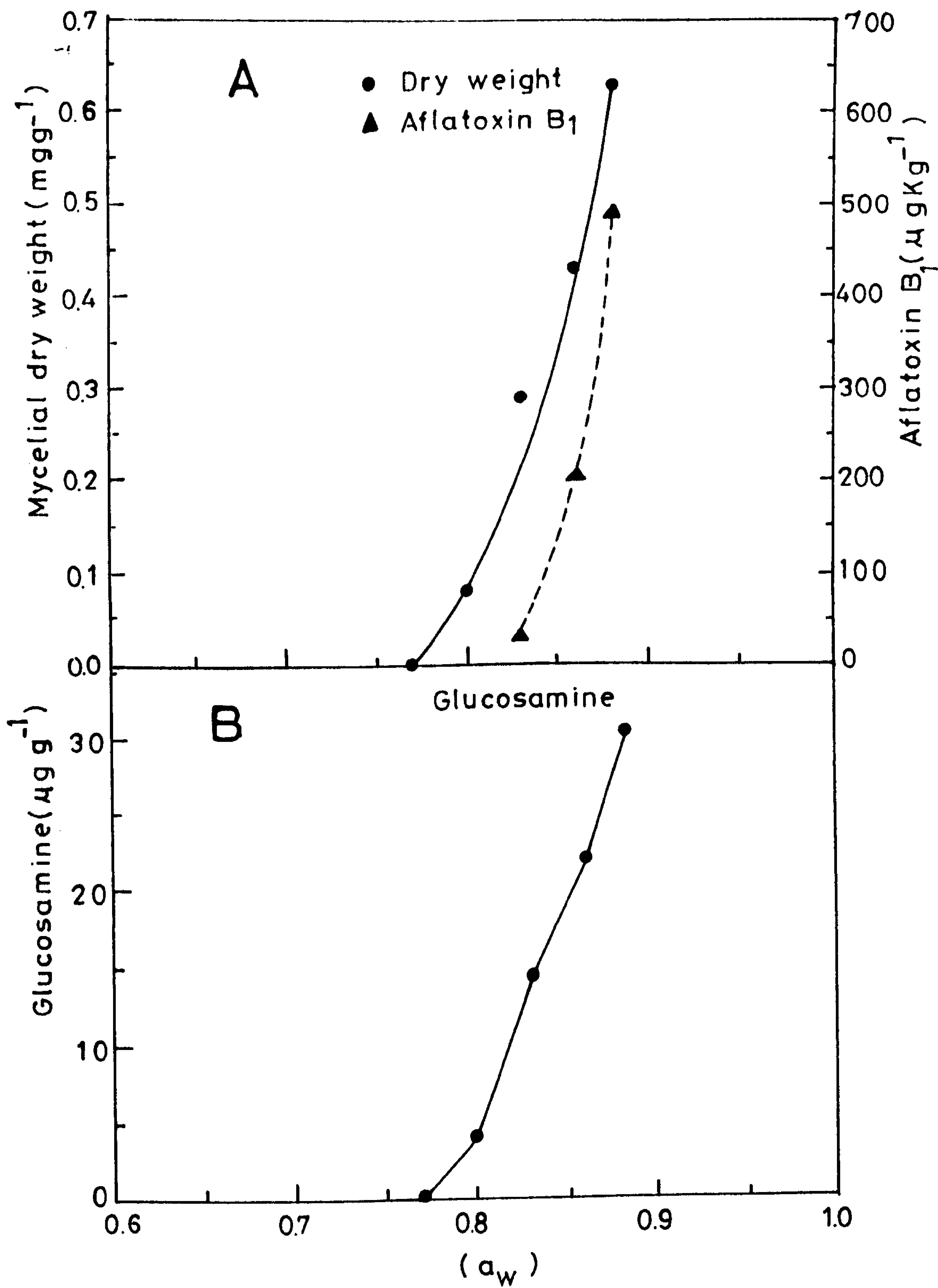
* Means of three replicates ± SD.

Fig. 24: Effect of water activity (a_w) on the growth of *A. parasiticus* and Aflatoxin B_1 production (A) and glucosamine value (B) at 25°C on rice grains. (Taken from Table 19).



Dry weight
Aflatoxin B_1
A
B
Mycelial dry weight ($mg g^{-1}$)
Aflatoxin B_1 ($\mu g Kg^{-1}$)
Glucosamine ($\mu g g^{-1}$)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0
100
200
300
400
500
600
0
10
20
30
0.6
0.7
0.8
0.9
0.1
(a_w)

Fig. 25: Effect of water activity (a_w) on the growth of *A. parasiticus* and Aflatoxin B_1 production (A) and glucosamine value (B) at 25°C on coffee beans. (Taken from Table 19).



A
Dry weight
Aflatoxin B_1
Mycelial dry weight ($mg g^{-1}$)
Aflatoxin B_1 ($\mu g Kg^{-1}$)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
700
600
500
400
300
200
100
0
B
Glucosamine
Glucosamine ($\mu g g^{-1}$)
30
20
10
0
0.6
0.7
0.8
0.9
1.0
(a_w)

CHAPTER V

Analysis of the Effect of Possible Inhibitors on Growth of *A. parasiticus* and Aflatoxin B_1 Production

Some chemical compounds used and known as food preservatives and some natural substances, e.g., spices, vitamins and fatty acids were examined as anti-fungal and/or anti-toxicogenic agents. Thus, the aim of the work in this chapter was to test:

(1) some common chemical preservatives of foods such as: sodium chloride, sodium benzoate, sodium bicarbonaate, hydrogen peroxide and also charcoal;

(2) some common spices used in food processing and flavouring;

(3) some known promoters and inhibitors of aflatoxin biosynthesis (sodium azide, sodium cyanide, EDTA, glutathione, indole-3-acetic acid and 2,4-dichlorophenoxy acetic acid, phenol and catechol;

(4) some saturated (lauric, palmitic and stearic acids) and unsaturated (oleic acid) fatty acids as well as some vitamins (A, B_6, B_2, C, D_2) and folic acid on fungal growth as well as aflatoxin B_1 production.

(a) <u>Effect of some food preservatives</u>:

Since toxins produced by fungi have serious effects on human beings, it was of interest to test the effect of some food preservatives, e.g. C_6H_5COONa, NH_4HCO_3, $NaHCO_3$, NaCl, and H_2O_2 on *Aspergillus parasiticus* and its productivity of aflatoxin. Each chemical was added to Czapek's-Dox liquid medium at concentrations in the range 0.2 to 0.8% (w/v). Also, charcoal was tested at 2-8% (w/v) as additional agent. It should be stressed that all these concentrations are acceptable in foods (Robinson, 1978). The incubation was carried out at 30°C for 10 days. From these data (Tables 20 and Figs. 26 & 27), it seems that all of these chemicals (except NaCl) repressed fungal growth. The repression was proportional to the concentration used. H_2O_2 decreased the fungal growth by 70.7% and 88.0% at concentrations of

Table 20: Effect of different concentrations of some food preservatives on the growth of *Aspergillus parasiticus* NRRL 2999 and aflatoxin B_1 production. (The incubation period was 10 days at 30°C).

Chemical	Chemical Concentration (% w/v)							
	0.2		0.4		0.6		0.8	
	Dry wt. (gl^{-1})	Aflatoxin B_1 (μgg^{-1})	Dry wt. (gl^{-1})	Aflatoxin B_1 (μgg^{-1})	Dry wt. (g^{-1})	Aflatoxin B_1 (μgg^{-1})	Dry wt. (gl^{-1})	Aflatoxin B_1 (μgg^{-1})
C_6H_5COONa	9.64	885^*	7.8	600	3.5	150	N.D.	N.D.
NH_4-HCO_3	9.81	310	5.6	54	1.8	N.D.	0.99	N.D.
$NaHCO_3$	9.90	908^{NS}	5.8	726	2.6	118	2.1	10
NaCl	10.89	943^{NS}	10.8^{NS}	943^{NS}	10.8^{NS}	943^{NS}	10.6^{NS}	941^{NS}
H_2O_2	3.18	N.D.	1.3	N.D.	N.D.	N.D.	N.D.	N.D.

Control: Fungal growth = 10.88 gl^{-1}
Aflatoxin B_1 = 945.42 μgg^{-1} dry weight

N.D. = Not detected.

NLSD: for fungal growth = at 5% = 0.71
at 1% = 0.94
for aflatoxin B_1 = at 5% = 53.30
at 1% = 70.68

* = Significant.
Not labeled = Highly significant.
NS = Not Significant.

Fig. 26: Effect of different concentrations of some food preservatives, C_6H_5COONa (A) and NH_4HCO_3 (B) on the growth of *A. parasiticus* and Aflatoxin B_1 production. (Taken from Table 20).

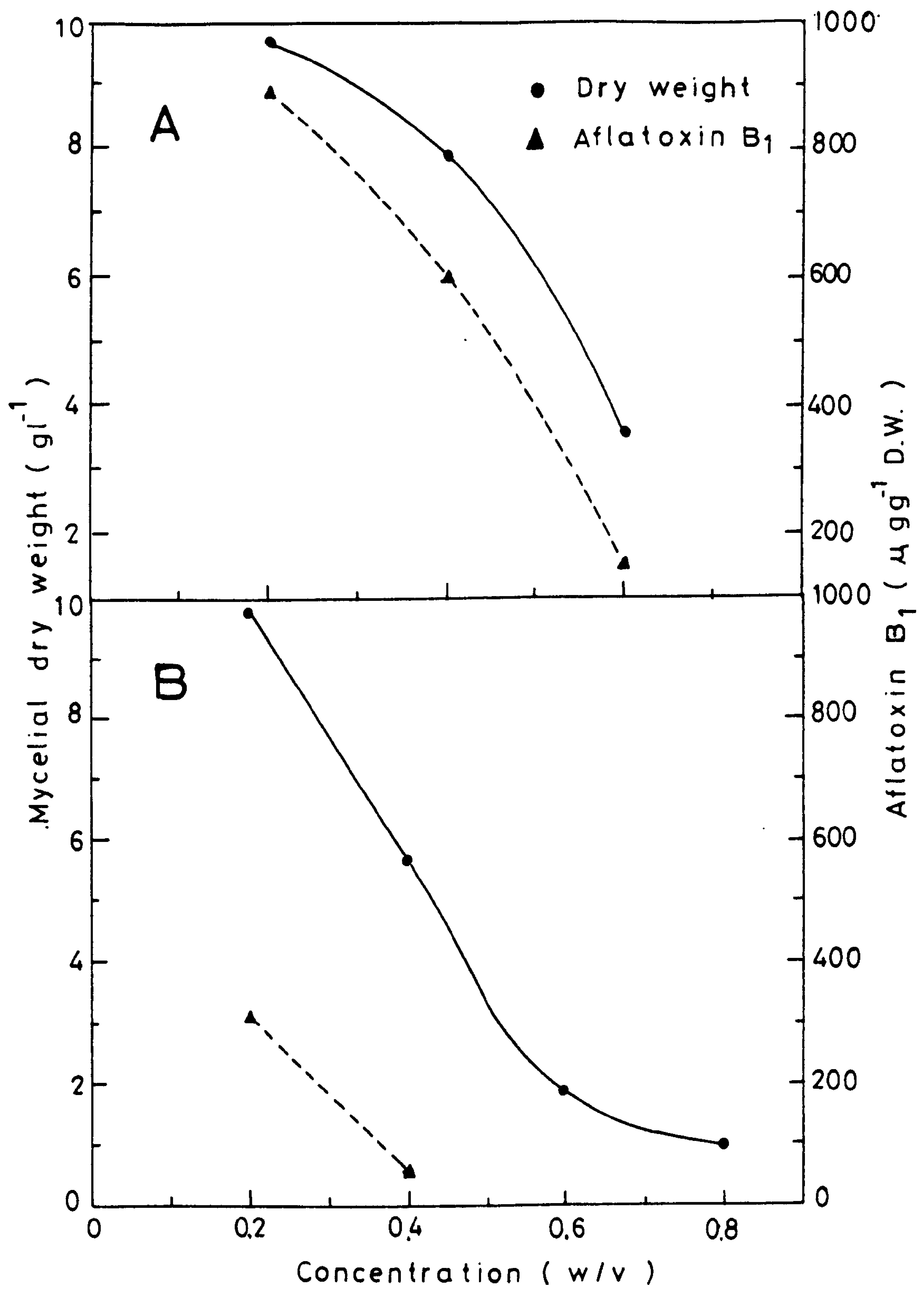
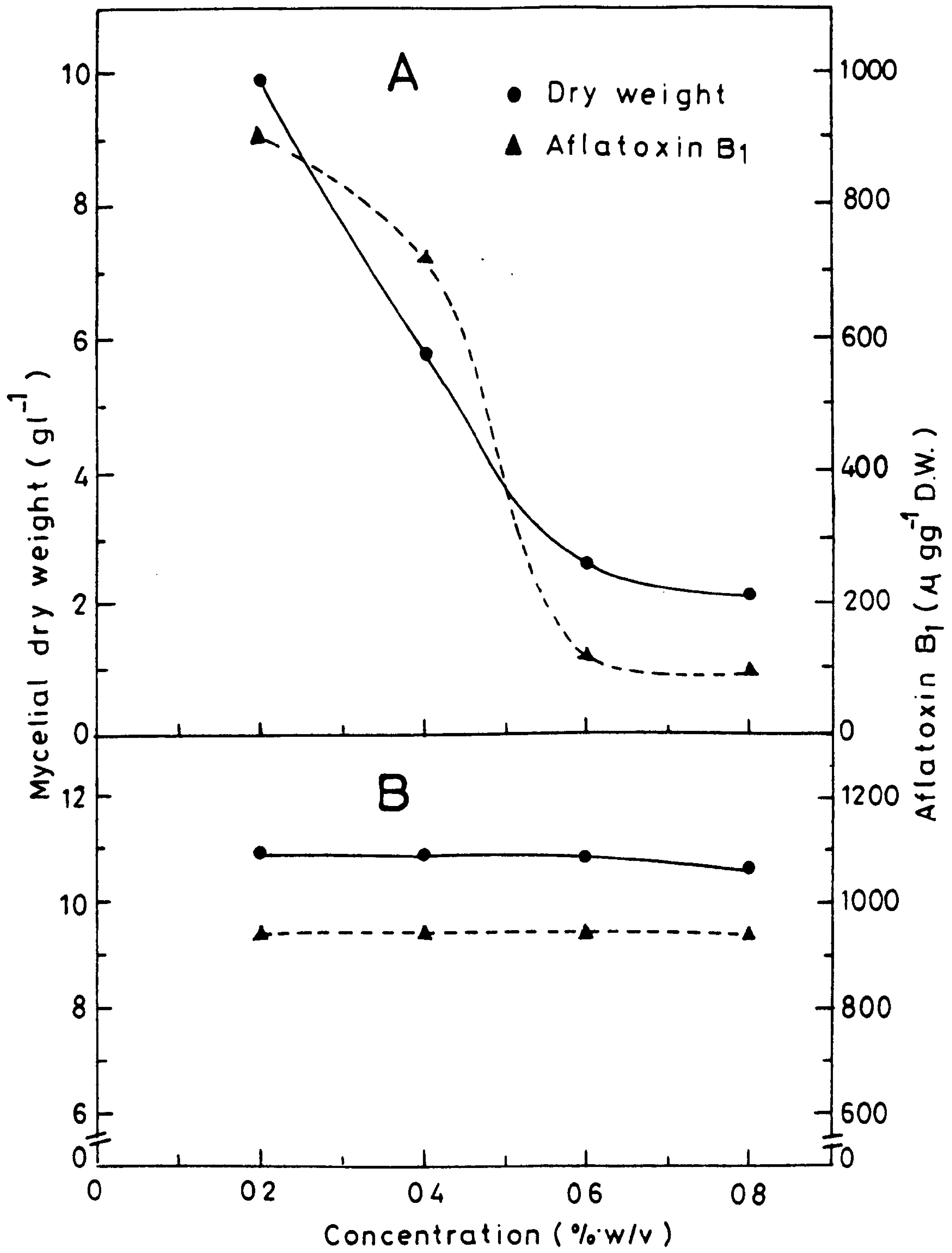

A
B
Dry weight
Aflatoxin B_1
Mycelial dry weight (gl^{-1})
Aflatoxin B_1 ($\mu g g^{-1}$ D.W.)
Concentration (w/v)
0
0.2
0.4
0.6
0.8
2
4
6
8
10
200
400
600
800
1000

Fig. 27: Effect of different concentrations of some food preservatives, $NaHCO_3$ (A), and NaCl (B) on the growth of *A. parasiticus* and Aflatoxin B_1 production. (Taken from Table 20).


A
B
Dry weight
Aflatoxin B1
Mycelial dry weight (gl-1)
Aflatoxin B1 (µ gg-1 D.W.)
Concentration (% w/v)
0
0.2
0.4
0.6
0.8
2
4
6
8
10
12
200
400
600
800
1000
1200

0.2% and 0.4% respectively, after which no growth was observed. No aflatoxin B_1 was detected at any concentration of H_2O_2. Sodium benzoate was also effective at retarding fungal growth as well as toxin production. Similar results for mould inhibition by sodium benzoate were observed by Uraih & Chipley, 1976; Uraih *et al.*, 1977; Masimango *et al.*, 1978; Chipley & Uraih, 1980; Uraih & Offonry, 1981; and Chipley, 1983.

Ammonium bicarbonate is a chemical preservative because of its inhibitory effect on fungal growth. The inhibitory effect of NH_4HCO_3 on the growth of *A. parasiticus* was associated with the retardation of aflatoxin production which disappeared completely at 0.6%. $NaHCO_3$ was found to inhibit fungal growth. The inhibitory effect of $NaHCO_3$ on growth was accompanied with the inhibition of aflatoxin B_1 production.

The present results demonstrated that H_2O_2 gave almost no detectable aflatoxin B_1. The results are consistent with those of El-Gazzar & Marth (1988) and Tabata *et al.* (1994). The mechanism of inhibition by H_2O_2 as a chemical compound is still obscure, but it is thought that H_2O_2 affects the toxin productivity, also this compound may be complexed with aflatoxins to form antitoxic compounds or it may cause O-demethylation of aflatoxin (Sreenivasamurthy *et al.*, 1967 and Tabata *et al.*, 1994).

Sodium chloride in the present investigation was found to have no effect on either fungal growth or aflatoxin production at the various tested concentrations. There are many reports on the effect of sodium chloride on aflatoxin production (Bullerman *et al.*, 1969; Buchanan & Ayres, 1977; El-Gazzar *et al.*, 1986). Most of these reports indicated that sodium chloride stimulated aflatoxin production at low concentrations (1-3%). In addition, Shih & Marth (1972) found that *A. flavus* and

A. parasiticus can grow and produce aflatoxins at concentrations of up to 8 to 12% NaCl in a liquid medium.

An experiment was performed to test the effect of charcoal at concentration of 2-8% (w/v) on both fungal growth and aflatoxin B_1 production. It was found that charcoal did not affect the fungal growth but in its presence no aflatoxin B_1 was detected at the various tested concentrations (Table 21).

Regarding the effect of activated charcoal, the present results are in agreement with those of Dalvi & McGowan (1984) who found that activated charcoal, an effective and non-toxic adsorbent, has been found to be considerably useful in reducing aflatoxin B_1. This may be due to its adsorptive power for toxins. Taking advantage of this adsorption, it would be possible to set up a convenient technique which would eliminate aflatoxin from liquid media.

(b) The stability of aflatoxin B_1 in the presence of sodium bicarbonate and sodium benzoate:

The addition of either $NaHCO_3$ and C_6H_5COONa at 0.8% to the culture medium reduced aflatoxin B_1 levels (Table 20). This observation may be due to a direct inhibitory activity of $NaHCO_3$ on fungal growth and its consequent quantitative effect on the production of aflatoxin B_1 and/or the transformation of the aflatoxin itself.

To clarify this observation, an experiment was designed in order to estimate the direct action of $NaHCO_3$ and C_6H_5COONa on aflatoxin B_1 alone. Therefore, $NaHCO_3$ or C_6H_5COONa (0.8% w/v) was added to the filtrate of fungal culture which was incubated for 10 days. The residual activity of aflatoxin B_1 was examined after 1, 2, 5, 10, 15 and 20 hours. Results presented in Fig. 28 showed that the two tested food

Table 21 Effect of different concentrations of charcoal on the growth of *Aspergillus parasiticus* NRRL 2999 and aflatoxin B_1 production ($\mu g g^{-1}$ dry weight).** (The incubation period was 10 days at 30°C).

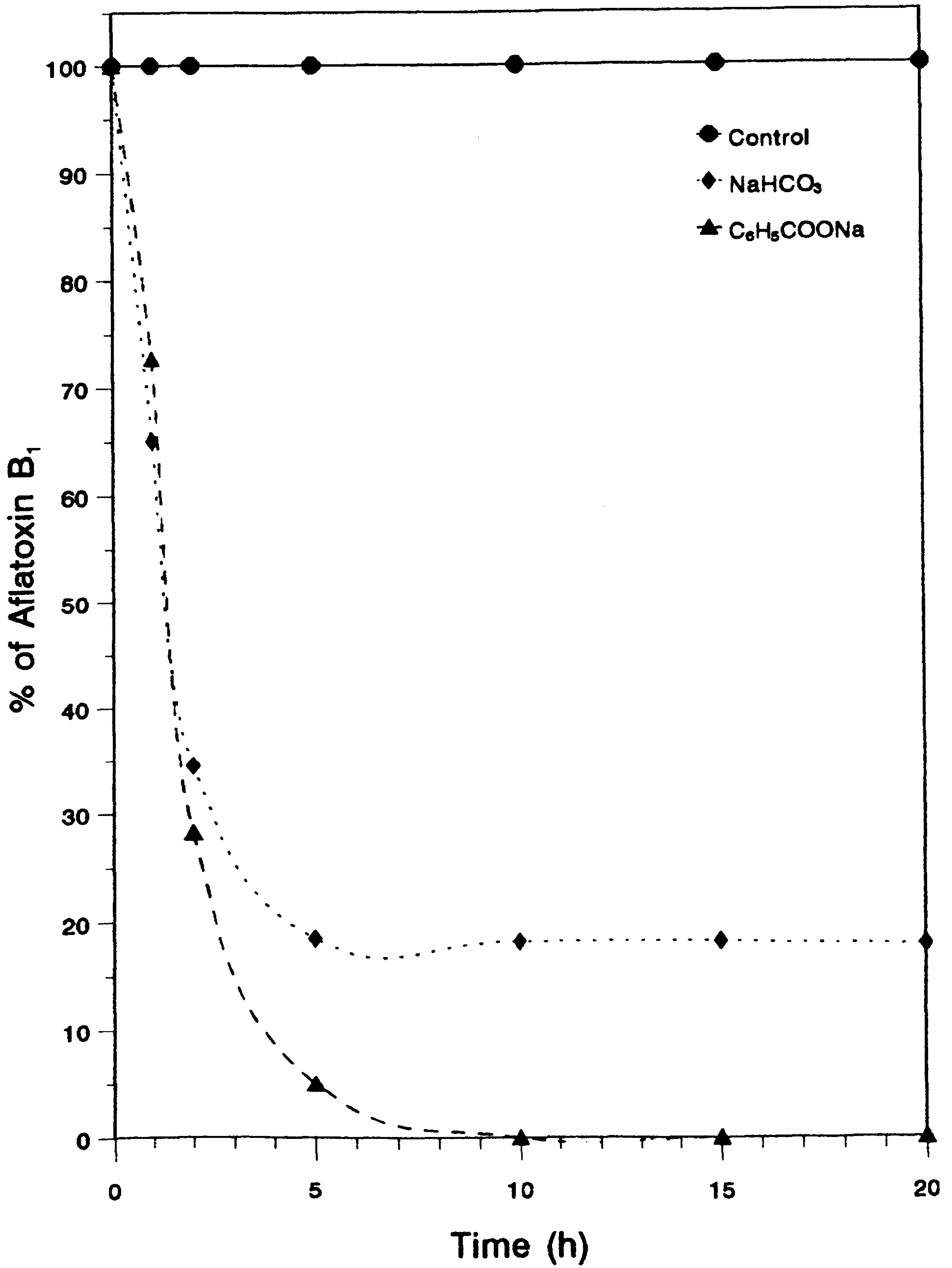
Chemical	Chemical Concentration (% w/v)							
	2		4		6		8	
	Dry wt. (gl^{-1})	Aflatoxin B_1 ($\mu g g^{-1}$)	Dry wt. (gl^{-1})	Aflatoxin B_1 ($\mu g g^{-1}$)	Dry wt. (g^{-1})	Aflatoxin B_1 ($\mu g g^{-1}$)	Dry wt. (gl^{-1})	Aflatoxin B_1 ($\mu g g^{-1}$)
Charcoal	10.9 ± 0.1	N.D.	9.9 ± 0.1	N.D.	9.9 ± 0.1	N.D.	9.90±0.09	N.D.

Control: Fungal growth = 10.98 gl^{-1}
Aflatoxin B_1 = 945.42 $\mu g g^{-1}$ dry weight

N.D. = Not detected.

** Mean of three replicates ± S.D.

Fig. 28: Effect of $NaHCO_3$ and C_6H_5COONa (0.8 w/v) on Aflatoxin B_1 presented in filtrate culture medium of *A. parasiticus*.
Control without any treatment of either $NaHCO_3$ or C_6H_5COONa.



% of Aflatoxin B_1
Time (h)
Control
$NaHCO_3$
C_6H_5COONa
0
5
10
15
20
0
10
20
30
40
50
60
70
80
90
100

preservatives transform the toxin. This transformation was found to increase by exposure time and reached a maximum after 5 hours. No more influence was observed with increased period of exposure even after 20 hours.

Regarding the effect of $NaHCO_3$, the present results agree with those of Mashaly *et al.* (1983) and Tabata *et al.* (1994). However, as far as the author is aware no available information was found regarding the effect of sodium benzoate on aflatoxin. The mechanism(s) of inactivation of aflatoxin B_1 by $NaHCO_3$ or C_6H_5COONa may be due to the in alkaline conditions (initial pH was 8.5 with $NaHCO_3$ and 8.9 with C_6H_5COONa) which transform the coumarin ring of aflatoxin B_1 to a water soluble form (β-keto acid) as indicated in Fig. 29 (Crawford & Mshaw, 1953 and Feigal, 1955).

When a filtrate containing β-keto acid of aflatoxin B_1 was acidified to pH 4, about 90% of aflatoxin B_1 was re-formed after 2 h (Table 22). Incomplete recovery of aflatoxin B_1 by acidification of the alkaline medium could be due to photolysis of cis-cumarinic acid to trans-cumarinic acid in which ring closure is difficult (Marle & Lyons, 1950 and Crawford & Mshaw, 1953).

The opening of the coumarin ring by alkali had been investigated by Marle & Lyons (1950); Krishnaswamy *et al.* (1968); Ali *et al.* (1976) and Balayan *et al.* (1983).

The formation of disodium salt can be confirmed by recovering aflatoxin by the acidification of the medium. These explanations are parallel with those reported by Kane *et al.* (1993) and Tabata *et al.* (1994). On the other hand, Mashaly *et al.* (1983) suggested that the degradation of aflatoxin by alkali was due to opening the furan ring of aflatoxin. Ring opening of furan ring has been misplaced due to the stability of

Table 22: Effect of 2 h of acidification (pH 4.0) on aflatoxin inhibited with 0.8% (w/v) of either $NaHCO_3$ or C_6H_5COONa.**

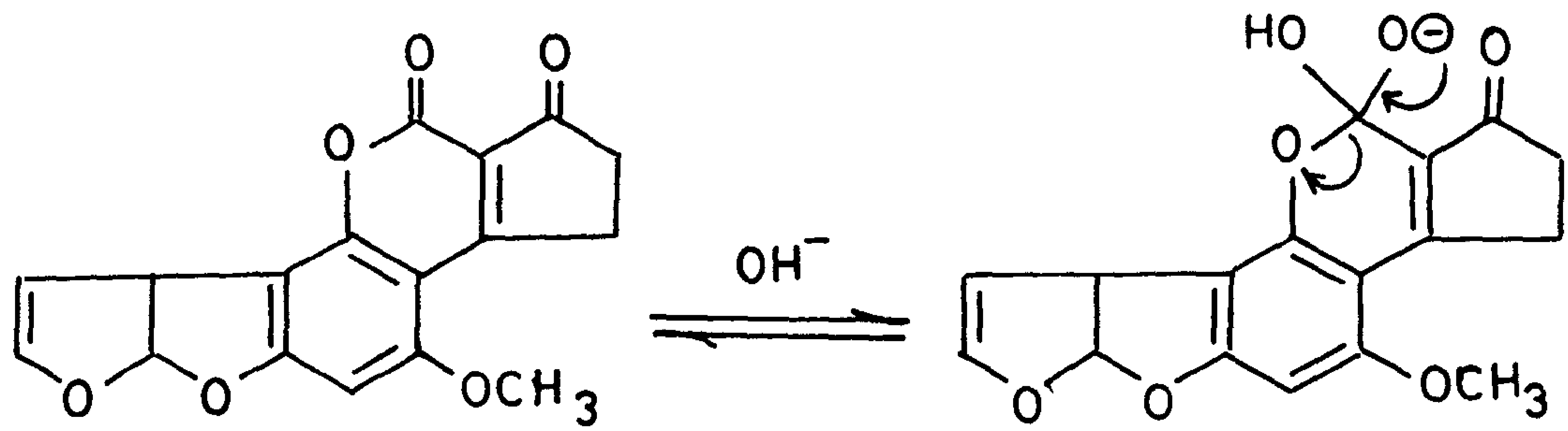
Treatment	Residual aflatoxin B_1 (%)*
$NaHCO_3$	92 ± 5
C_6H_5COONa	88 ± 3
Control***	100 ± 0

* Mean of three replicates ± S.D.

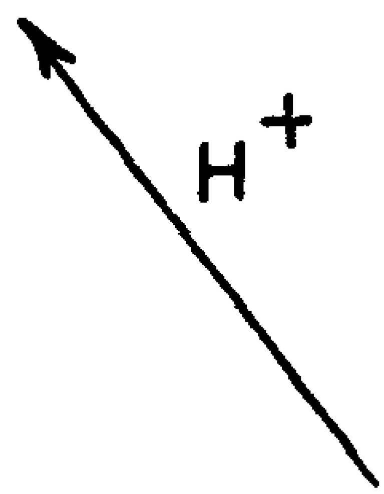
** The filtrate containing β-keto acid of aflatoxin B_1 was acidified by 0.5 N HCl to pH 4 followed by measuring aflatoxin B_1 content after 2 hr. of acidification.

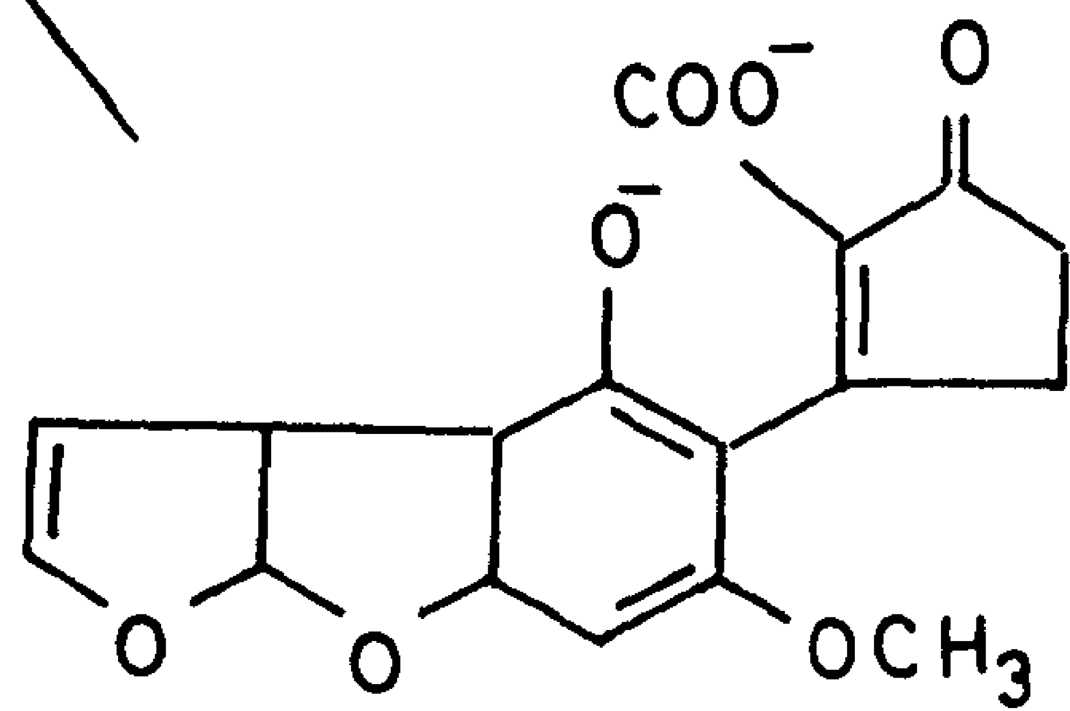
*** Control was carried out by acidified aflatoxin B_1 solution (pH 4.0) without any treatment by either $NaHCO_3$ or C_6H_5COONa.

Fig. 29: Possible hydrolysis mechanism of Aflatoxin B_1 in alkalis.



Aflatoxin B_1





β-Keto acid of aflatoxin B_1
(presented as disodium
salt and soluble in water)

furan ring towards alkalies (Dunlop & Peters, 1953; Bosshard & Eugster, 1966, and Jouly & Smith, 1987).

Based on the aforementioned explanations, it may be assumed that aflatoxin can easily be removed by washing with water after treatment with $NaHCO_3$ or C_6H_5COONa. The transformation of aflatoxins to water soluble compounds with alkali, then eliminating them by washing with water, can be applied during the processing of foods.

(c) Effect of some spices:

The purpose of carrying out this experiment was to evaluate the suitability of a number of major and common spices used in Saudi Arabia on the growth and aflatoxin production of *A. parasiticus*. A preliminary experiment was carried out to find the effective concentration of spices to be used. Four spices namely, black pepper, ciliated heath, cuminum and curcuma were tested as a water extract at different concentrations (0.02-2% v/v). The results are summarized in Table 23. These results indicate that 2% of each individual spice extract was the most effective on the fungal growth and aflatoxin B_1 production. It was then decided to test higher concentrations 2-20% (v/v) with a variety of spices. These spices were coriander, red pepper, black pepper, ciliated heath, ginger, cardamon, cuminum, curcuma and cinnamon. The water extract of each spice was added to Czapek's-Dox liquid medium at concentrations, namely, 2, 4, 8, 16 and 20% (v/v), and incubated for 10 days at 30°C. According to the results listed in Table 24 and Figs. 30, 31, & 32 it could be observed that coriander caused slight inhibition of the growth of *A. parasiticus*. On the other hand, high degree of

Table 23: Effect of different concentrations of black pepper, ciliated heath, cuminum and curcuma (as a water extract) on the growth of *A. parasiticus* NRRL 2999 and aflatoxin B_1 production ($\mu g g^{-1}$ dry weight)*. (The incubation period was 10 days at 30°C).

Spices	Concentration (% v/v)											
	2		1.5		1		0.5		0.1		0.02	
	Dry wt. (gl^{-1})	Aflatoxin B_1	Dry wt. (gl^{-1})	Aflatoxin B_1	Dry wt. (gl^{-1})	Aflatoxin B_1	Dry wt. (gl^{-1})	Aflatoxin B_1	Dry wt. (gl^{-1})	Aflatoxin B_1	Dry wt. (gl^{-1})	Aflatoxin B_1
Black pepper	9.1	00	9.3	132.0	9.8	396.0	10.0	890.0	10.0	951.0	10.0	968.0
Ciliated heath	9.0	00	9.3	340.0	9.7	525.0	10.0	936.0	10.0	968.0	10.0	968.0
Cuminum	8.7	00	9.0	73.0	9.6	321.0	9.9	775.0	10.0	946.0	10.0	961.0
Curcuma	9.9	00	10.0	212.0	10.0	621.0	10.0	951.0	10.0	967.0	10.0	967.0

Control: Mycelial dry weight = 10.0 gl^{-1}
Aflatoxin B_1 = 968 $\mu g g^{-1}$ dry weight

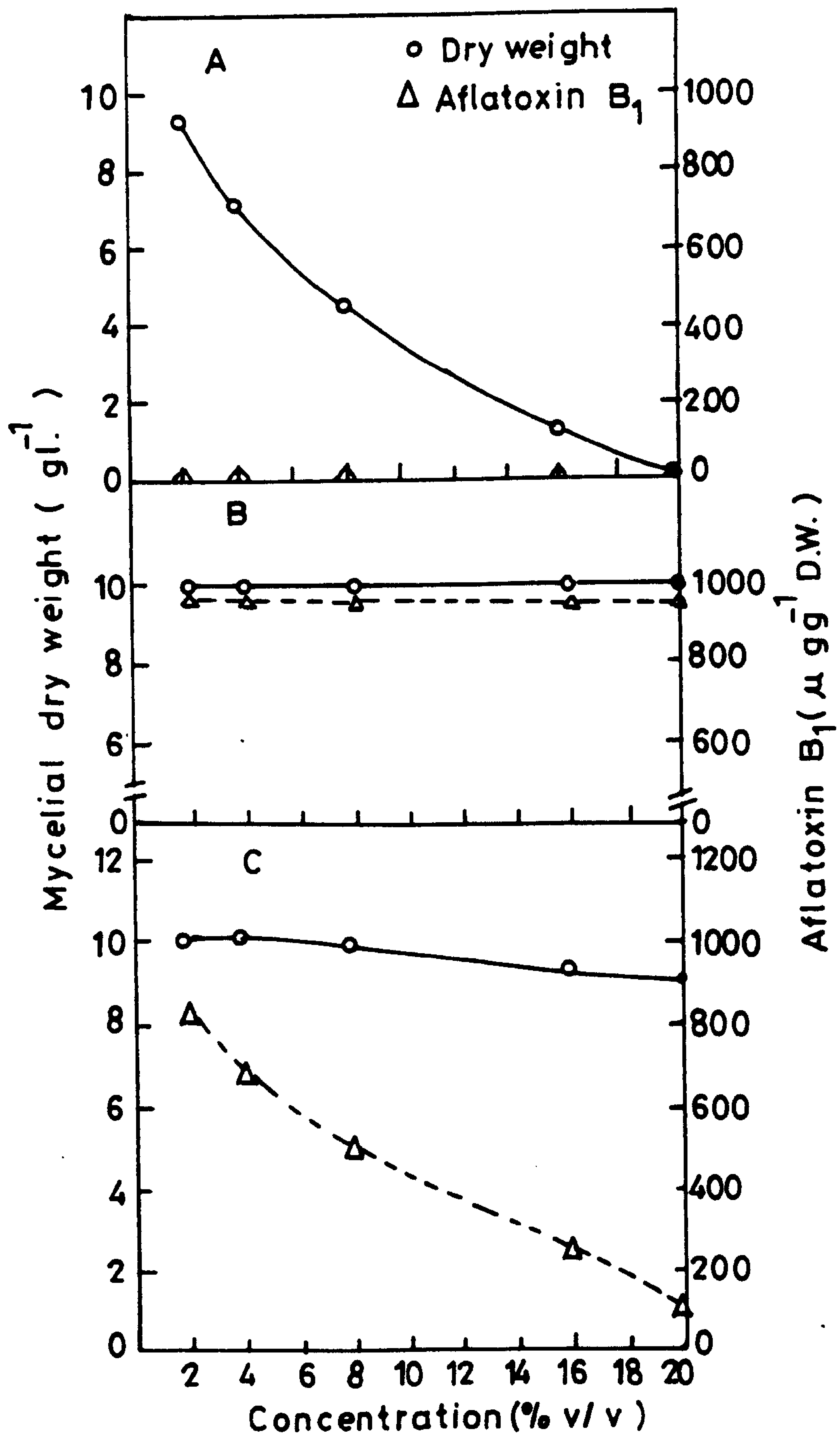
* Values represented means for triplicate assay.

Table 24: Effect of different concentrations of some spices (as a water extract) on the growth of *Aspergillus parasiticus* NRRL 2999 and aflatoxin B_1 production ($\mu g g^{-1}$ dry weight). (The incubation period was 10 days at 30°C).

Spices	Concentration (% v/v)									
	2		4		8		16		20	
	Dry wt. (gl^{-1})	Aflatoxin B_1	Dry wt. (gl^{-1})	Aflatoxin B_1	Dry wt. (gl^{-1})	Aflatoxin B_1	Dry wt. (gl^{-1})	Aflatoxin B_1	Dry wt. (gl^{-1})	Aflatoxin B_1
Coriander	10.00*	820	10.00*	682	9.82	500	9.25	260	9.09	124
Red pepper	10.01*	968*	10.00*	960*	10.00*	950*	9.96*	950*	9.96	950
Black pepper	9.07	N.D.	6.98	N.D.	4.47	N.D.	1.16	N.D.	0.00	N.D.
Ciliated heath	9.04	N.D.	8.83	N.D.	8.26	N.D.	8.04	N.D.	7.77	N.D.
Ginger	9.41	651	8.67	490	6.97	206	3.46	82	1.12	N.D.
Cardamon	10.02*	967*	10.00*	967*	9.15	900**	8.04	708	7.45	622
Cuminum	8.66	N.D.	5.86	N.D.	1.15	N.D.	0.00	N.D.	0.00	N.D.
Curcuma	9.94	N.D.	9.62	N.D.	7.81	N.D.	5.12	N.D.	4.83	N.D.
Cinnamon	9.46	877	9.17	734	7.09	506	5.63	72	3.33	N.D.

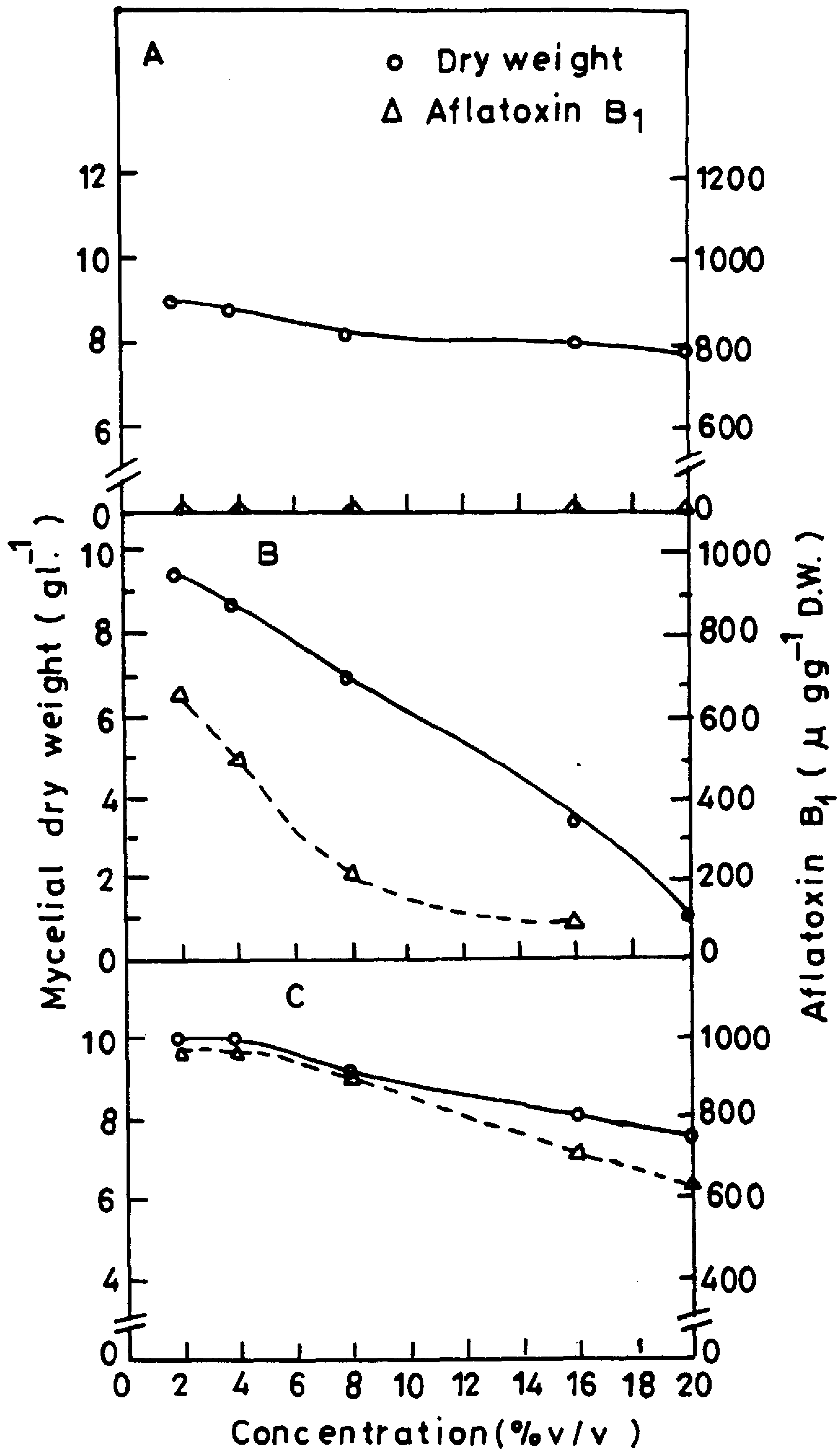
Control: Mycelia dry weight = 10.02 ± 0.02 gl^{-1}
Aflatoxin B_1 = 968 ± 30 $\mu g g^{-1}$ dry weight
Values represented means for triplicate assay.
N.D. = Not detected.
NLSD: for dry weight at 5% = 0.14
at 1% = 0.18
: for aflatoxin production at 5% = 64
at 1% = 84
Not labeled = Highly significant.
* = Not significant.
** = Significant.

Fig. 30: Effect of different concentrations of extracts of spices Black pepper (A), Red pepper (B), and Coriander (C) on the growth of *A. parasiticus* and Aflatoxin B_1 production. (Taken from Table 24).



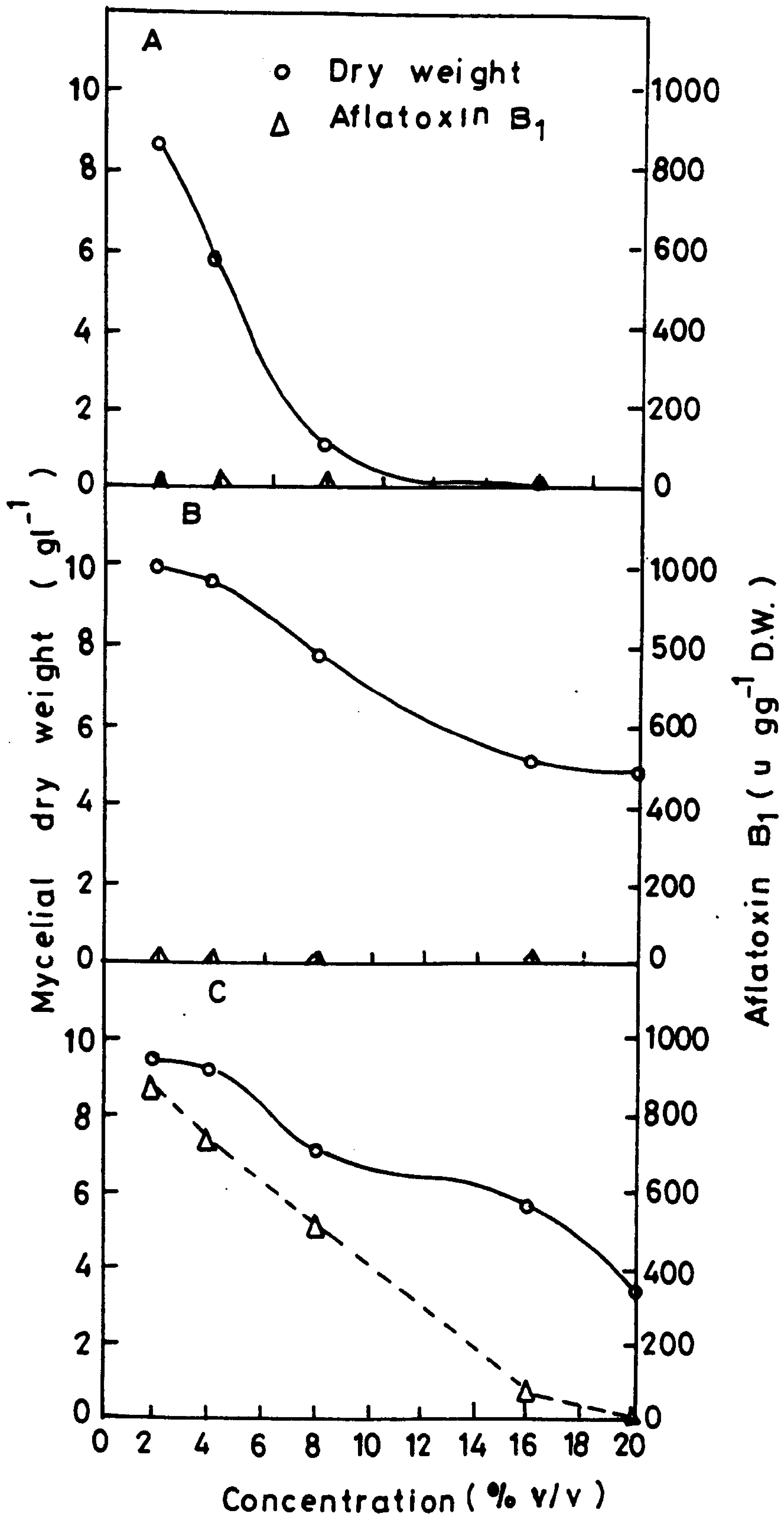
o Dry weight
Δ Aflatoxin B_1
A
B
C
Mycelial dry weight ($gl.^{-1}$)
Aflatoxin B_1 ($\mu g g^{-1}$ D.W.)
Concentration (% v/ v)
0
2
4
6
8
10
12
200
400
600
800
1000
1200
14
16
18
20

Fig. 31: Effect of different concentrations of extracts of some spices Ciliated heath (A), Ginger (B), and Cardamon (C) on the growth of *A. parasiticus* and Aflatoxin B_1 production. (Taken from Table 24).



A
B
C
o Dry weight
Δ Aflatoxin B_1
Mycelial dry weight ($gl.^{-1}$)
Aflatoxin B_1 (μ gg^{-1} D.W.)
Concentration(%v/v)

Fig. 32: Effect of different concentrations of extracts of some spices, Cuminum (A), Curcuma (B), and Cinnamon (C) on the growth of *A. parasiticus* and Aflatoxin B_1 production. (Taken from Table 24).



A
Dry weight
Aflatoxin B_1
B
C
Mycelial dry weight (gl^{-1})
Aflatoxin B_1 (u gg^{-1} D.W.)
Concentration (% v/v)
0
2
4
6
8
10
12
14
16
18
20
200
400
600
800
1000
500

inhibition of toxin production was observed with increasing the concentration of the coriander reaching nearly 87% inhibition at concentration 20%.

Red pepper expressed a little effect on both fungal growth and aflatoxin B_1 production. Black pepper expressed a remarkable effect on both fungal growth and aflatoxin B_1 production, particularly on aflatoxin production which was not detected even with a spice concentration of 2%. There was a gradual inhibition of fungal growth which was complete at 20%.

Ciliated heath, cuminum and curcuma showed a similar effect to that of black pepper. By increasing the concentration up to 20% there was a gradual reduction in fungal growth but no detection of aflatoxin B_1 was found throughout all the tested concentrations.

The last three tested spices namely: ginger, cardamon and cinnamon had similar results in both fungal growth and aflatoxin B_1 production. The ginger and cinnamon expressed severe effect on the production of aflatoxin B_1 where no detection of the toxin was recorded at concentration 20%.

Generally from the above results the following categories of effect were apparent:

(i) Little effect on growth, no aflatoxin production or severely affected as shown by cilited heath and coriander (Fig. 30C; Fig. 31A).

(ii) Inhibition of growth, no aflatoxin production as observed by black pepper, cuminum, and curcuma (Fig. 30A; Fig. 32 A & B).

(iii) Little effect on either as in case of red pepper and cardamon (Fig. 30B; Fig. 31C).

(iv) Inhibition of aflatoxin production and growth and thus aflatoxin production follows growth as in case of ginger and cinnamon (Fig. 32C; Fig. 31B).

The obtained results indicated that black pepper, ciliated heath, cuminum and curcuma were the most inhibitory spices particularly against toxin production. In support, black pepper prevented growth and aflatoxin production by *A. flavus* (Scott & Kennedy, 1973 & 1975; Hitokoto *et al.*, 1977; Mabrouk & El-Shayeb, 1980; and Ito *et al.*, 1994). However, the present results were contradicted with those of Madhyastha & Bhat (1985) who reported that black pepper supported the two processes.

According to the effect of both ciliated heath and curcuma on the growth *of A. parasiticus* and its productivity of aflatoxin B_1, the present work may be considered the first attempt for testing these two spices as antiaflatoxigenic and fungistatic according to the available literature.

Cardamon was the least suppressive spice. In support, Mabrouk & El-Shayeb (1979) found that addition of low concentration of cardamon to the growth medium of *A. flavus* resulted in decreasing the production of aflatoxin, although *Aspergillus spp.* were isolated from cardamon earlier (Pal & Kundu, 1972 and Flannigan & Hui, 1976). Cinnamon retarded aflatoxin production as well as fungal growth. In support, cinnamon was reported to prohibit toxin production (Flannigan & Hui, 1976 and Bullerman *et al.*, 1977). In addition, Hitokoto *et al.* (1978) found that chloroform extract of cinnamon was inhibitory to growth of *A. parasiticus*, *A. flavus*, *A. ochraceus*, *A. versicolor*, and *Fusarium solani*. In addition, Webb & Tanner (1945) showed that water extract of ground cinnamon prevented growth of yeasts. Frazier (1967) stated that oil of cinnamon is an effective inhibitor of yeasts and bacteria. Hartung *et al.* (1973) reported that raisin bread containing cinnamon did not support growth and aflatoxin production by *A. parasiticus* to the extent observed with other

breads which did not contain cinnamon. Cinnamon oil in concentrations as low as 0.02% was shown to inhibit mould growth and aflatoxin production (Bullerman, 1974 and Ryu & Holt, 1993). According to Merory (1960), cinnamon contains 0.5-1.0% volatile oil which is composed of approximately 75% cinnamic aldehyde, 8% eugenol, cinnamic acid and various other compounds. The major constituents of these products, cinnamic aldehyde and eugenol apparently are the active compounds responsible for this action (Bullerman *et al.*, 1977). The interactive effects of the other compounds present in lesser quantities with these major constituents may also be a possibility. Thus, the present data indicate that cinnamon may provide some fungistatic benefit, and hence a health protection against possible aflatoxin development. However, the degree of this benefit and the duration of fungistatic activity would be expected to be quite dependent on the usage level of the spices.

Ginger was a good suppressive for both growth and aflatoxin production and these results are consistent with those reported by Mabrouk & El-Shayeb (1980) for *A. flavus*. However, the present results disagree with those of Madhyastha & Bhat (1985) who reported that ginger was a better substrate for fungal growth as well as toxin production.

Coriander also caused partial inhibition of toxin production. Other spices, e.g. cloves and star anise were found also as inhibitors for growth and toxin production of toxigenic Aspergilli (Hitokoto *et al.*, 1980).

Aflatoxin production in presence of different spices was considerably less if compared to other agricultural commodities such as cereals and oil seeds (Madhyastha & Bhat, 1985; Llewellyn *et al.*, 1992). Furthermore, Hitokoto *et al.* (1978) studied

various kinds of spices and commercial dry condiments and showed that these samples inhibited the growth and toxin production of toxigenic fungi.

The mechanism by which spices could prevent the growth and productivity of aflatoxin by mould is not yet understood. However, it could be said that moulds have certain essential functions to carry out, such as when they come in contact with spices it seems likely that they are forced to perform certain other cellular functions that prevent them from performing those functions necessary for growth and development. Consequently, their growth is considerably slowed or suppressed completely.

The inhibitory effect of spices could be attributed to their content of essential oils which possess antimicrobial activities (Frazier, 1967; Shelef, 1983; Farag *et al.*, 1989a and Mahmoud, 1994). However, antimicrobial activity of spices depends not only on their components of essential oils but also in the chemical structure of these compounds. Most of these antimicrobials are phenolic compounds with M.W. of 150-160, and their inhibitory effect can be attributed to the presence of an aromatic nucleus and phenolic OH group which is known to be reactive and forms hydrogen bonds with active sites of target enzymes (Katayama & Nagai, 1960; Shelef, 1983 and Farag *et al.*, 1989b).

Generally, a renewed interest in the use of spices as antimicrobials in food is evident today for several reasons. (1) At a time when safety of synthetic food additives is questioned, natural substances of plant origin appeal to the public. (2) Reduction of salt and sugar in foods for dietary reasons tends to enhance the use of seasonings, which are low in sodium and contribute to negligible quantity of calories at the commonly added amount. Blends of spices are now frequently recommended as salt replacement. Increasing numbers of low salt preserved foods are marketed today,

and such foods will contain increased levels of spices as flavorings to compensate for the bland taste; (3) It has been suggested that increased spice consumption may produce a bacterial shift in the intestinal tract, and that this alteration can reduce cancer incidence (Shelef, 1983). Consequently, popular and professional literature is replete with recommendations and suggestions of how to incorporate various spices into the diet (Wylie-Rossett, 1982).

(d) Effect of certain metabolic inhibitors:

These experiments were carried out to find the effect of some available inhibitors on the growth of *A. parasiticus* and its productivity of aflatoxin B_1. In a preliminary experiment, two known inhibitors of fungal growth namely, sodium cyanide and 2,4-dichlorophenoxy acetic acid were tested at concentrations range between 0.1 and 5 mM as in Table 25. The results in this table demonstrate that 5 mM is the most effective concentration on fungal growth and toxin production. It was then decided to test other inhibitors namely, glutathione, quinine, EDTA, sodium azide, sodium cyanide, indole acetic acid, 2,4-dichlorophenoxy acetic acid, phenol and catechol. Each compound was used at either 5mM or 10mM except phenol and catechol which were used at 0.5 and 1.0 mM and EDTA which was used at 0.25 and 0.5 ml per 100 ml of the Czapek's-Dox medium. After inoculation by *A. parasiticus* spores, the incubation was carried out at 30°C for 10 days. According to the obtained results presented in Table 26 and illustrated in Figs. 33, 34 and 35, it appears that all these compounds were of inhibitory action on both growth as well as aflatoxin pro duction. From the results, the most effective chemicals on productivity of aflatoxin

Table 25: Effect of sodium cyanide and 2,4-dichlorophenoxy acetic acid on the growth of *A. parasiticus* and aflatoxin B_1 production. (The incubation period was 10 days at 30°C).

Inhibitors	Conc. (mM)	Mycelial dry weight (gl^{-1})*	% of control	Aflatoxin B_1/ dry weight (μgg^{-1})*	% of control
Sodium cyanide	0.1	10.0 ± 0.0	99.6	960.0 ± 35	98.1
	0.25	10.0 ± 0.0	99.6	861.0 ± 18	87.1
	1.0	9.5 ± 0.2	94.6	281.0 ± 23	28.7
	2.5	6.9 ± 0.0	68.7	95.0 ± 7	9.7
	5.0	5.4 ± 0.2	53.8	00	00
2,4-Dichlorophenoxy acetic acid	0.1	10.0 ± 0.2	99.6	961.0 ± 25	99.1
	0.25	9.8 ± 0.1	97.6	900.0 ± 32	92.0
	1.0	8.2 ± 0.3	81.2	615.0 ± 19	62.8
	2.5	7.3 ± 0.1	72.7	205.0 ± 20	20.9
	5.0	6.2 ± 0.2	69.3	00	00

N.B. Control: Aflatoxin B_1 = 978 ± 35.5 μgg^{-1}
Mycelial dry weight = 10.04 ± 0.01 gl^{-1}

* = Means of three replicates ± S.D.

Table 26: Effect of some inhibitors on the growth of *Aspergillus parasiticus* NRRL2999 and aflatoxin B_1 production. (The incubation period was 10 days at 30°C).

Compound	Conc. (mM)	Mycelial dry weight (gl^{-1*})	% of control	Aflatoxin B_1/ dry weight (μgg^{-1*})	% of control
Glutathione	5	4.1 ± 0.01	41.2	525.0 ± 15	53.6
	10	3.4 ± 0.04	33.9	466.0 ± 25	47.6
Quinine	5	9.6 ± 0.1	95.6	782.4 ± 19.0	77.9
	10	8.2 ± 0.03	81.2	710.0 ± 22.0	72.5
Sodium azide	5	N.D.	00.0	N.D.	00.0
	10	N.D.	00.0	N.D.	00.0
Sodium cyanide	5	5.3 ± 0.05	52.6	N.D.	00.0
	10	4.5 ± 0.02	45.2	N.D.	00.0
Indole acetic acid	5	8.0 ± 0.2	80.1	245.0 ± 24	25.0
	10	6.1 ± 0.01	60.7	N.D.	00.0
2,4 dichlorophenoxy acetic acid	5	6.1 ± 0.08	61.2	N.D.	00.0
	10	4.9 ± 0.03	48.7	N.D.	00.0
Phenol	0.5	7.1 ± 0.1	70.7	72.0 ± 9	7.4
	1.0	1.0 ± 0.0	9.96	N.D.	00.0
Catechol	0.5	9.8 ± 0.0	97.6	905.0 ± 22	92.5
	1.0	9.3 ± 0.1	92.6	610.0 ± 30	62.4
EDTA^{+}	0.2	9.3 ± 0.02	92.9	810.0 ± 30	82.7
	0.5	8.2 ± 0.01	81.2	775.0 ± 13.0	79.2

Control: Mycelial dry weight = 10.04 ± 0.01 gl^{-1}
Aflatoxin B_1 = 979.0 ± 36.0 μgg^{-1}

N.D. = Not detected.

* = Means for three replicates ± S.D.

+ = Concentration of EDTA ml per 100 ml medium.

Fig. 33: **Effect of different concentrations of some inhibitors, Glutathione (A), Quinine (B), and EDTA (C) on the growth of *A. parasiticus* and Aflatoxin B_1 production. (Taken from Table 26).**

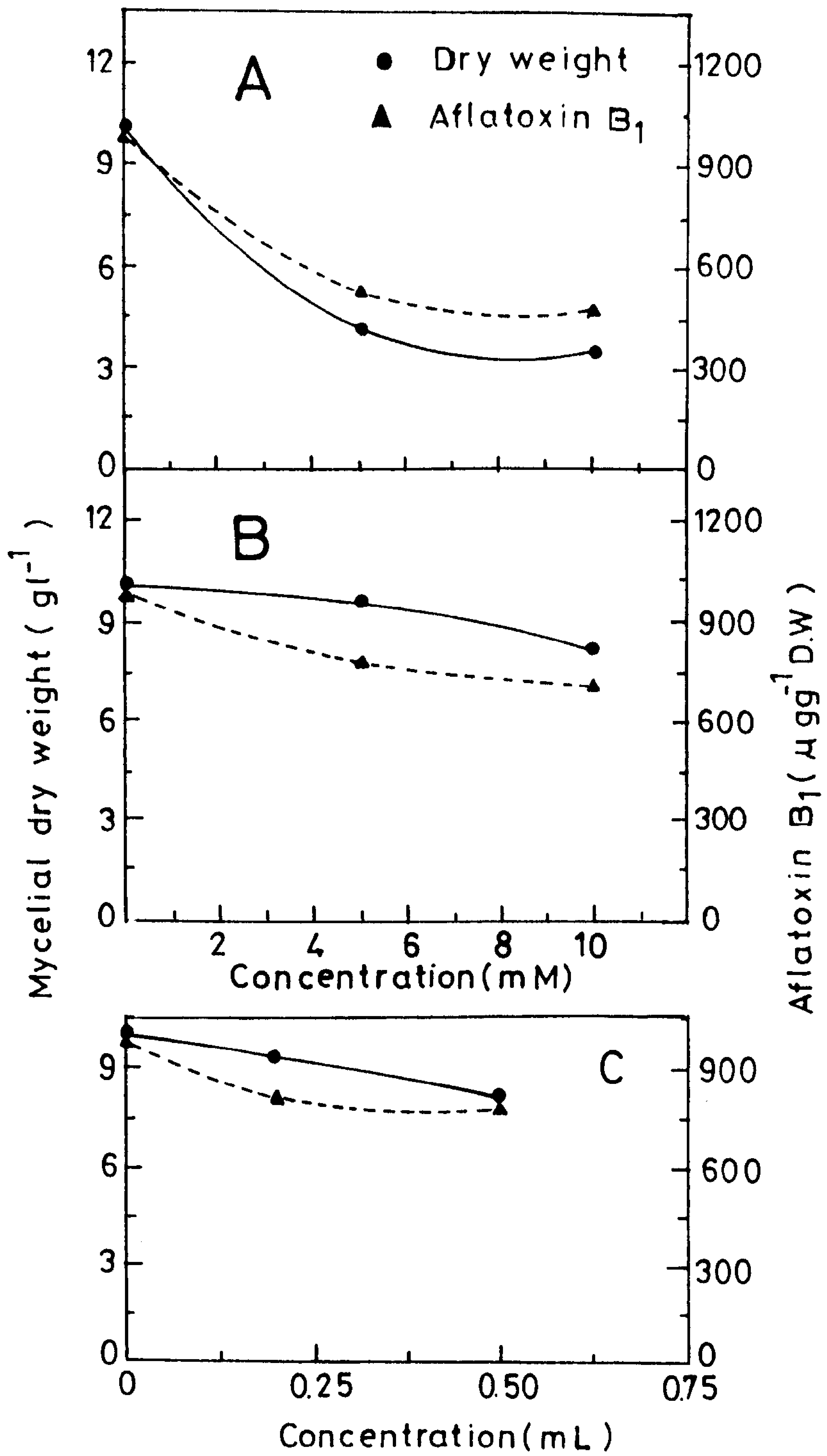

A
Dry weight
Aflatoxin B1
B
C
Mycelial dry weight (gl-1)
Aflatoxin B1 (μg g-1 D.W)
Concentration (mM)
Concentration (mL)
0
3
6
9
12
300
600
900
1200
2
4
8
10
0.25
0.50
0.75

Fig. 34: Effect of different concentrations of some inhibitors, Sodium cyanide (A), Indole acetic acid (B), and 2,4-dichlorophenoxy acetic acid (C) on the growth of *A. parasiticus* and Aflatoxin B_1 production. (Taken from Table 26).

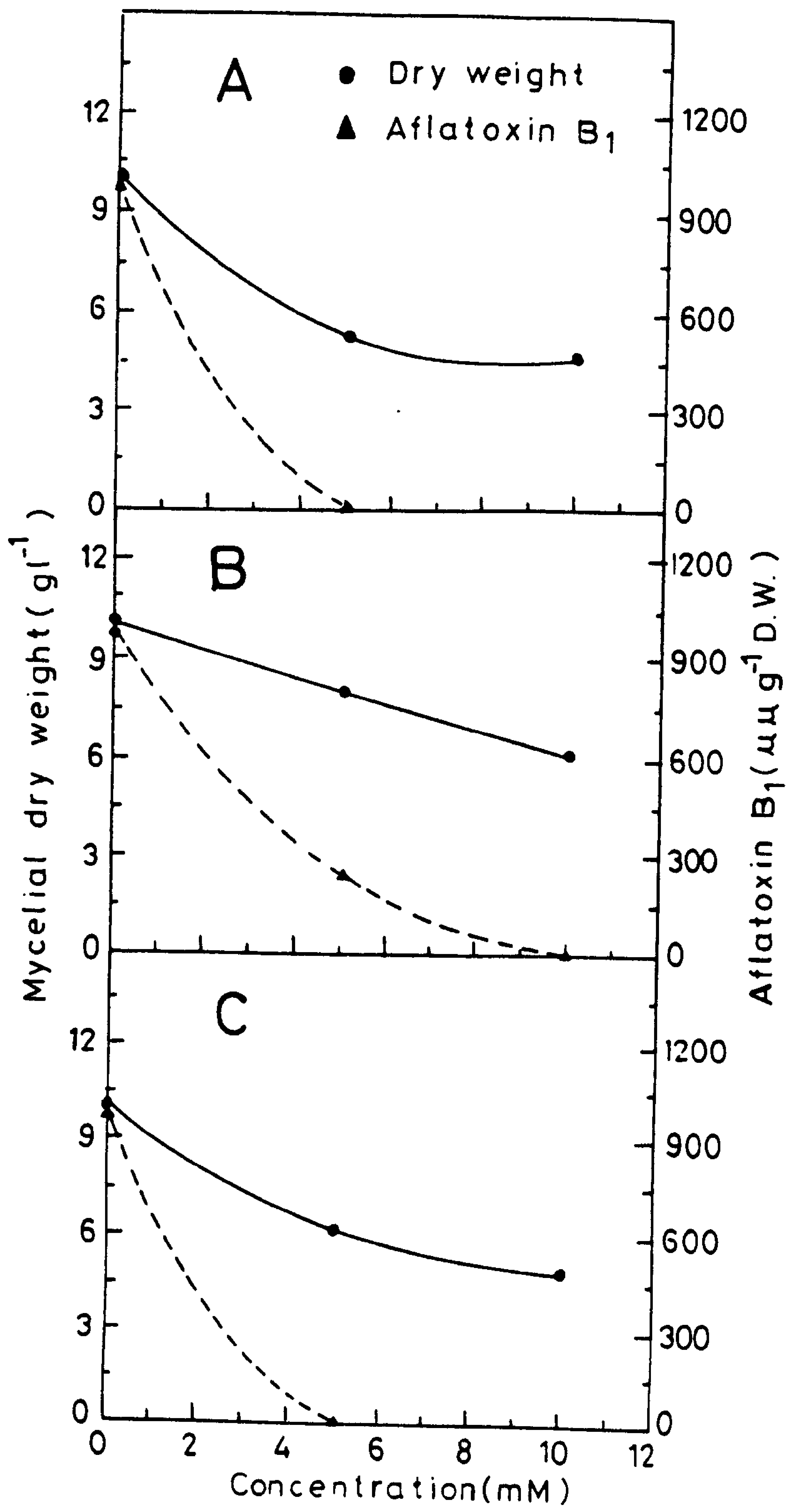
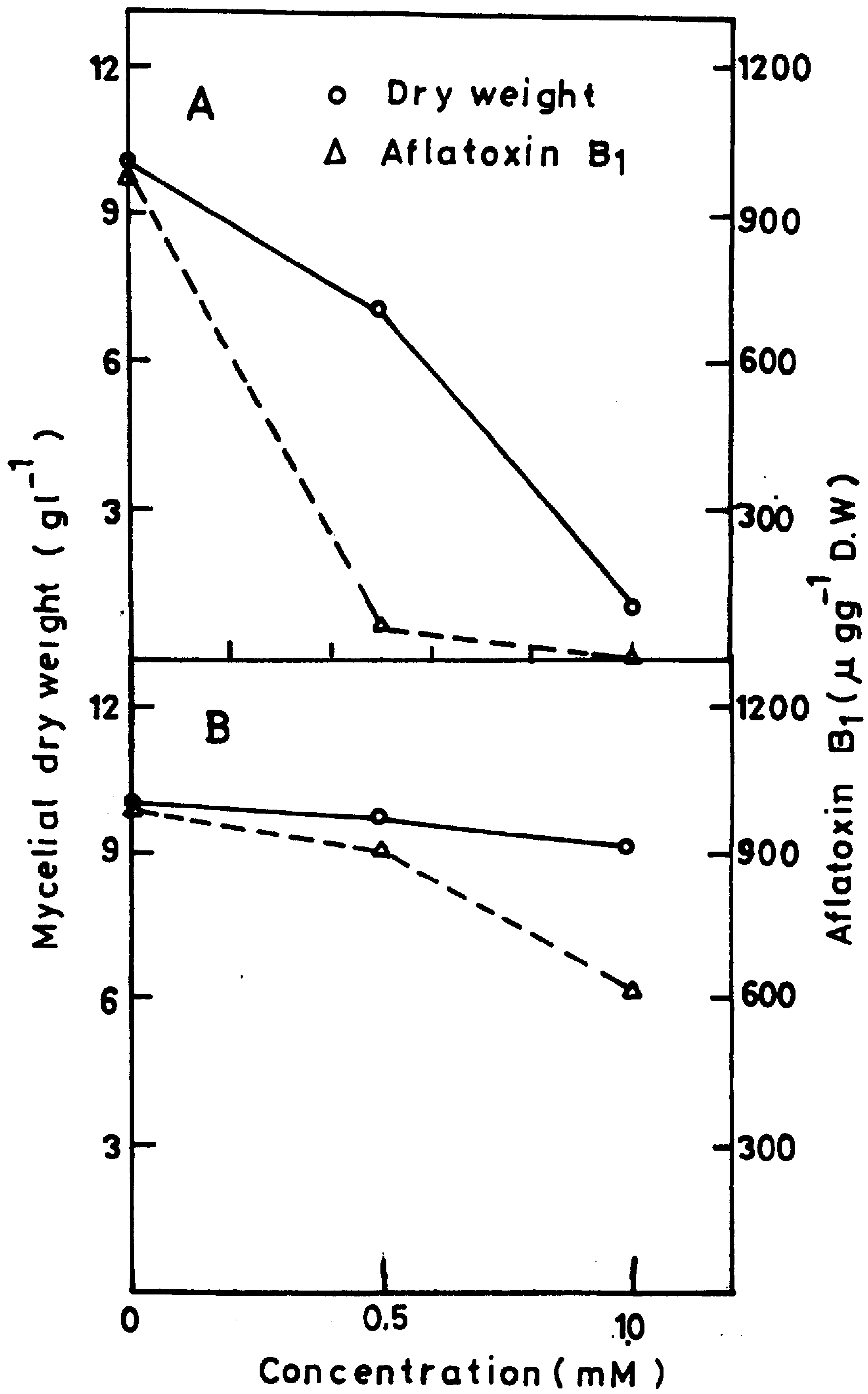

A
B
C
Dry weight
Aflatoxin B1
Mycelial dry weight (g l-1)
Aflatoxin B1 (μg g-1 D.W.)
Concentration(mM)
0
3
6
9
12
0
300
600
900
1200
0
2
4
6
8
10
12

Fig. 35: Effect of different concentrations of some inhibitors, Phenol (A), and Catechol (B) on the growth of *A. parasiticus* and Aflatoxin B_1 production. (Taken from Table 26).



A
B
o Dry weight
Δ Aflatoxin B1
Mycelial dry weight (gl⁻¹)
Aflatoxin B1 (μ gg⁻¹ D.W)
Concentration (mM)
0
0.5
1.0
3
6
9
12
300
600
900
1200

B_1 were sodium azide, sodium cyanide and 2,4-dichlorophenoxy acetic acid. On the other hand, sodium azide was the most inhibitory compound for fungal growth.

Thus, from the results of Figs. 33, 34 and 35, the following categories could be designated:

(i) Little effect on both fungal growth and aflatoxin production as in case of quinine, EDTA and catechol (Fig. 33B, 33C and 35B).

(ii) Little effect on growth, aflatoxin severely affected as in case of sodium cyanide, indole acetic acid and 2,4-dichlorophenoxy acedic acid (Fig. 34A, B, and C).

(iii) Aflatoxin production is inhibited slightly than growth as shown by glutathione (Fig. 33A).

(iv) Severe inhibition of both aflatoxin production and fungal growth as in case of phenol (Fig. 35A).

In contrast, glutathione, quinine, and EDTA were suppressive for fungal growth as well as aflatoxin B_1 production but it was observed that glutathione was more suppressive for both fungal growth and AFB_1 production particularly at 10 mM (33.9% & 47.6%). EDTA as chelating agent had little effect on the growth as well as aflatoxin B_1 production. Indole acetic acid was suppressive at 10 mM for fungal growth (60.7%) but aflatoxin B_1 was not detected.

Shih and Marth (1974a) attributed the suppressive effect of sodium azide to its inhibition of the terminal respiration of *A. parasiticus*. The inhibition by EDTA is in agreement with the results of Maggon *et al.* (1977). The inhibitory effect of EDTA may be due to its chelating effect on the enzymes responsible for growth and aflatoxin formation (El-Shora, 1993; El-Shora & Khalaf, 1994). The inhibitory effect of glutathione on fungal growth and toxin production supports the previous reports of

Firozi *et al.* (1986). The inhibitory effect of sodium cyanide, indole acetic acid, quinine and 2,4-dichlorophenoxy acetic acid could possibly be due to their interference with the tricarboxylic acid cycle or with glycolysis of *A. parasiticus*. The inhibitory effect of phenol on the growth of *A. parasiticus* was associated with the retardation of aflatoxin B_1 production which disappeared completely at concentration 1.0 mM. Catechol showed a slight decrease in fungal growth while it decreased aflatoxin B_1 production by 38% at concentration 1.0 mM. In support, Fajardo *et al.* (1995) found that some phenolic compounds at 1 and 0.1 mM completely inhibited the production of aflatoxin B_1 at 4 days of incubation and slightly inhibited mycelial growth of *A. flavus* and reported that the mode of action of phenolics might be on the secondary pathway for aflatoxin B_1 poduction and not on the primary metabolism for fungal growth. O'Neill and Mansfield (1982) also reported that a mechanism of action of phenols can involve an interaction between the fungal receptor and the structural configuration of phenolics in a membrane-mediated process. The inhibitory effect of phenolic compounds on mycelial growth is attributable to phenol polymerization or phenol oxidation into melanin or lignin. These products are then incorporated into the fungal cell wall causing increased rigidity. As a result, cell wall expansion is likely hindered, thus limiting growth. Also, phenolic compounds have been shown to inhibit enzymes such as phosphorylases, cellulases, transaminases, and decarboxylases, thus causing a virtual cessation on mycelium growth (Vance and Garraway, 1973).

However, there remains a need to learn more about these inhibitory substances from the standpoint of their mechanisms of action, effects on mycotoxin production and effects of interactions with environmental conditions. More work is needed to define the conditions under which inhibitors are most effective in preventing growth of

toxic mould and mycotoxin production. Possibly combined effects of commercial inhibitors and certain active substances, such as spices, might enhance the inhibitory activity of each, or might result in a combined effect that is greater than either alone.

(e) Effect of fatty acids:

The effect of fatty acids on the growth of *A. parasiticus* and its productivity of aflatoxin B_1 was studied. These fatty acids were stearic acid, lauric acid, palmitic acid and oleic acid. They were used at five different concentrations: 1, 5, 10, 15 and 20 mM added to Czapek's-Dox liquid medium, after inoculation with *A. parasiticus* spore cultures were incubated at 30°C for 10 days. It should be mentioned that these tested concentrations were chosen after carrying out orientation experiments. Therefore, higher concentrations than 1 mM were used (1-20 mM). The results are shown in Table 27 and Figs. 36 & 37. From the results it appears that stearic acid promoted growth slightly up to 20 mM, however, it decreased the productivity of aflatoxin B_1 gradually. Lauric acid decreased both the fungal growth and productivity of aflatoxin B_1 with increasing concentration. Palmitic acid gave in a similar way as stearic acid. Both acids increased the fungal growth but decreased the production of aflatoxin B_1. On the other hand, oleic acid was different, it stimulated both fungal growth and aflatoxin B_1 production.

Therefore, from the above results the following points emerged:

(i) Stimulation of growth, decrease of aflatoxin production as in case of stearic and palmitic acids (Fig. 36A, 37A).

(ii) Inhibition of both growth and aflatoxin production by lauric acid (Fig. 36B).

(iii) Both growth and toxin production were increased by oleic acid (Fig. 37B).

Table 27: Effect of some fatty acids on the growth of *Aspergillus parasiticus* NRRL 2999 and productivity of aflatoxin B_1. (The incubation period was 10 days at 30°C).

Compound	Conc. (mM)	Mycelial dry weight (gl^{-1*})	% of control	Aflatoxin B_1/ dry weight (μgg^{-1*})	% of control
Stearic acid	1	10.1 ± 0.0	100.6	978 ± 50	100.00
	5	10.1 ± 0.0	100.6	978 ± 60	100.00
	10	10.3 ± 0.0	102.6	961 ± 24	98.3
	15	11.0 ± 0.1	109.6	946 ± 36	96.7
	20	11.7 ± 0.0	116.5	908 ± 55	92.8
Lauric acid	1	10.1 ± 0.1	100.6	941 ± 40	96.2
	5	9.2 ± 0.1	91.6	910 ± 51	93.0
	10	7.8 ± 0.2	77.7	675 ± 30	69.0
	15	5.8 ± 0.1	57.8	461 ± 36	47.1
	20	4.1 ± 0.2	40.8	344 ± 65	35.2
Palmitic acid	1	10.2 ± 0.1	101.6	978 ± 50	100.0
	5	10.4 ± 0.1	103.6	971 ± 39	99.3
	10	10.9 ± 0.3	108.6	955 ± 75	97.6
	15	11.1 ± 0.1	110.6	949 ± 29	97.0
	20	11.9 ± 0.0	118.5	917 ± 46	93.8
Oleic acid	1	10.2 ± 0.1	101.6	978 ± 21	100.0
	5	10.4 ± 0.2	103.6	978 ± 31	100.0
	10	10.9 ± 0.1	108.6	989 ± 25	101.1
	15	11.2 ± 0.0	111.5	1105 ± 36	112.9
	20	11.9 ± 0.1	118.5	1128 ± 61	115.3

Control: Mycelial dry weight = 10.04 ± 0.01 gl^{-1}
Aflatoxin B_1 = 978 ± 35 μgg^{-1} D.W.

* = Means of three replicates ± S.D.

Fig. 36: Effect of different concentrations of some fatty acids, Stearic acid (A), and Lauric acid (B) on the growth of *A. parasiticus* and Aflatoxin B_1 production. (Taken from Table 27).

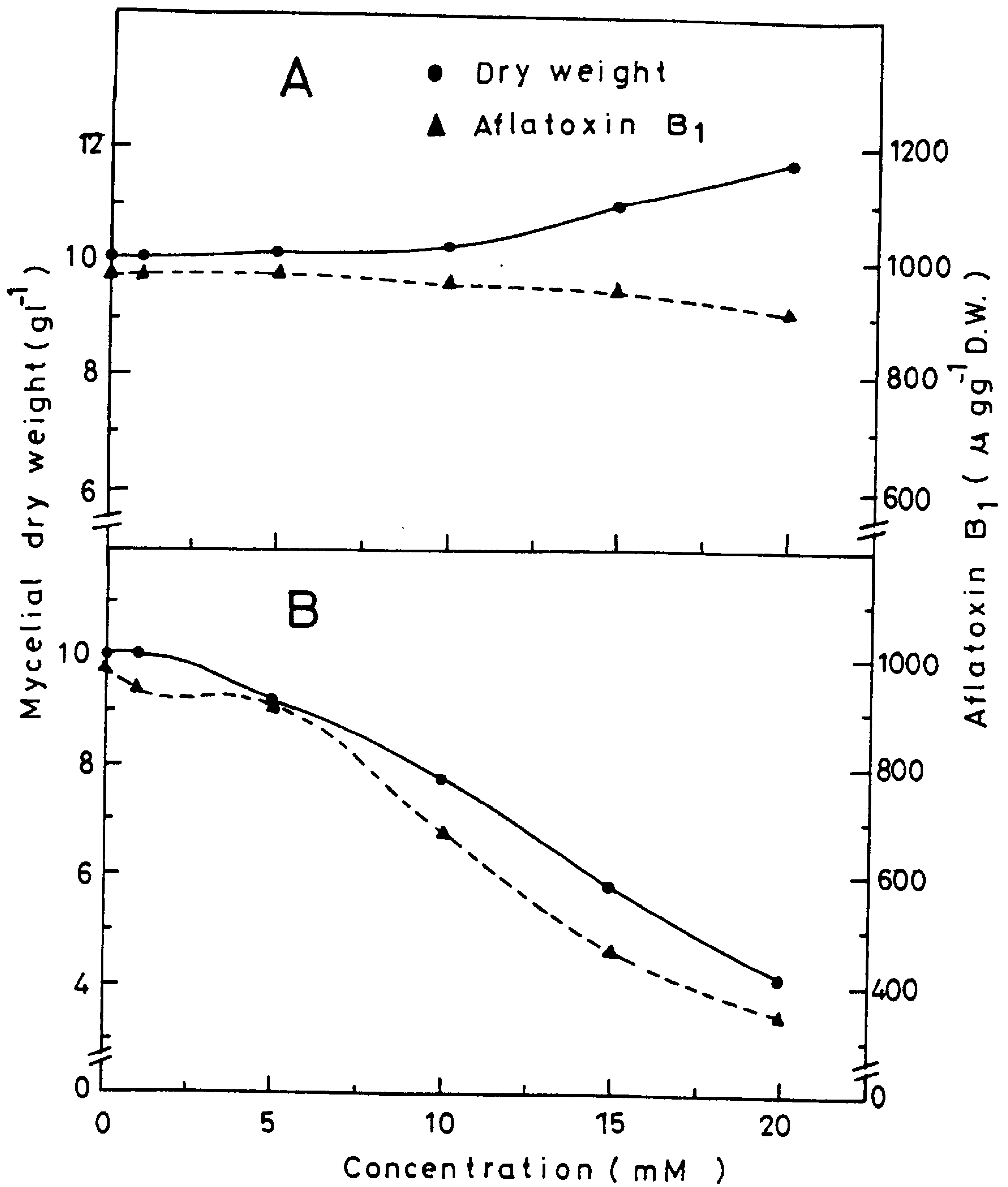
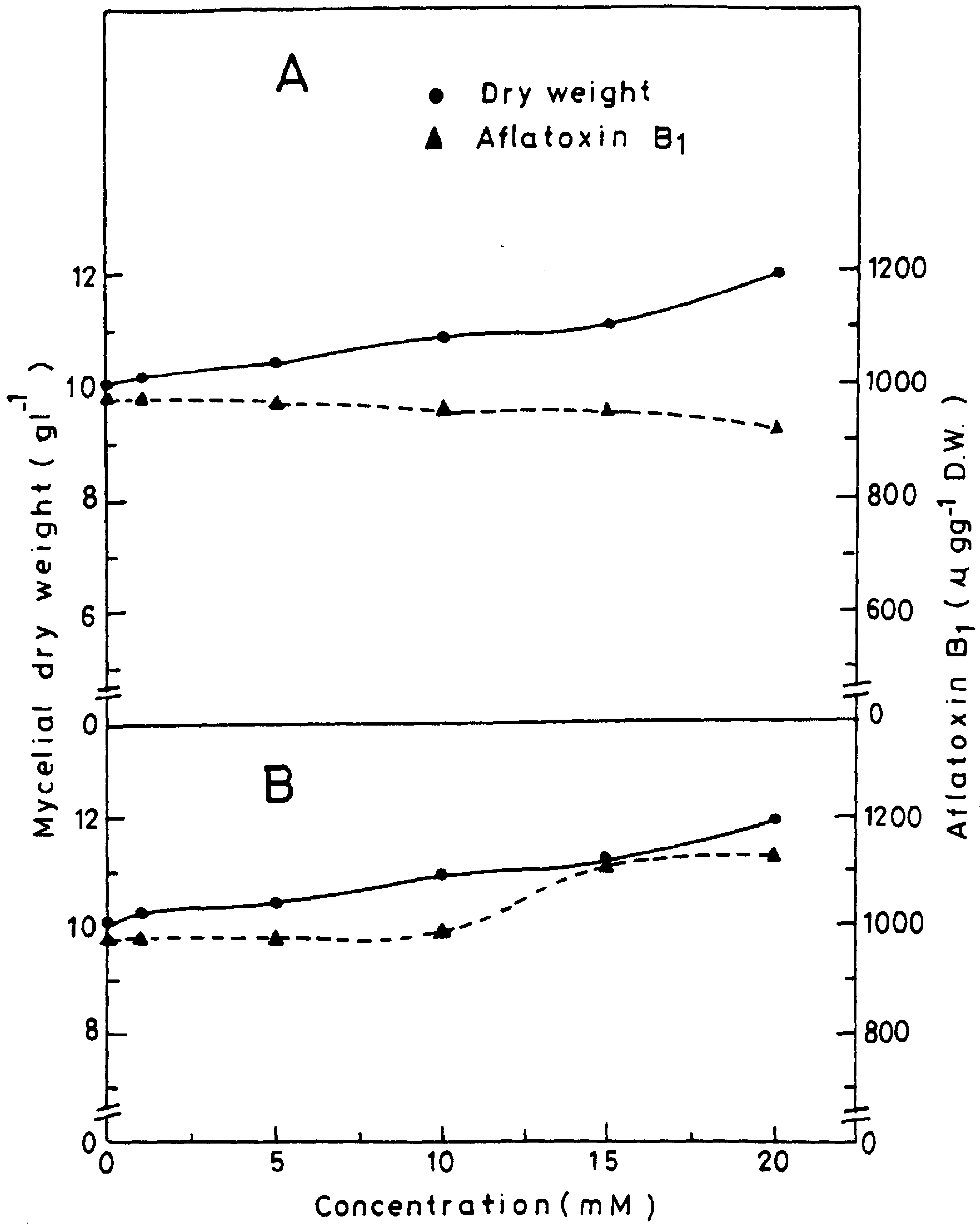

A
B
Dry weight
Aflatoxin B1
Mycelial dry weight (gl-1)
Aflatoxin B1 (µg g-1 D.W.)
Concentration (mM)
0
5
10
15
20
4
6
8
10
12
400
600
800
1000
1200

Fig. 37: Effect of different concentrations of some fatty acids, Palmitic acid (A), and Oleic acid (B) on the growth of *A. parasiticus* and Aflatoxin B_1 production. (Taken from Table 27).



A
B
Dry weight
Aflatoxin B1
Mycelial dry weight (gl-1)
Aflatoxin B1 (µg g-1 D.W.)
Concentration (mM)
0
5
10
15
20
6
8
10
12
600
800
1000
1200

Generally, it seems that lauric acid is the most suppressive fatty acid for fungal growth and aflatoxin production. However, oleic acid as unsaturated fatty acid promoted both the fungal growth and aflatoxin B_1 production (118.5% and 115.3% of the control).

The mechanism of microbial growth inhibition by lauric acid may be due to an inhibition of membrane transport (Freese, 1978) resulting in nutritional starvation of cells. The inhibition of transport results from the destruction of the proton motive force caused the movement of the negative lipophilic ion through the cell membrane. The same mechanism might also apply to secretion of aflatoxin. Another possibility might involve the direct inhibition of aflatoxin biosynthesis by the acid.

Also, the two saturated fatty acids namely, stearic and palmitic, slightly depressed the aflatoxin production which is consistent with results of Mashally & El-Deeb (1983a) and Mayura *et al.* (1985) for *A. flavus* and *A. parasiticus*, but it disagrees with those of Hamid & Smith (1987) who reported that *A. flavus* growth was enhanced by stearic acid.

In fact, the antifungal activity of such fatty acid has been recognised for many years and has been shown to be dependent on chain length and pH of the medium (Chipley *et al.*, 1981).

However, in the present investigation, oleic acid stimulated both fungal growth and aflatoxin production which is in agreement with the results of Tiwari *et al.* (1986a) but against those of Hayatsu *et al.* (1981) who reported that this acid was an inhibitor for the two processes. The stimulation by oleic as unsaturated acid could be possibly attributed to the fact that *A. parasiticus* could use this acid as a carbon source to grow well and produce a pronounced amount of aflatoxin. In such case the amount of

aflatoxin produced and the growth rate could be related to the concentration of this fatty acid in the media, the higher the concentration the greater the effect.

Also, it was reported that aflatoxin production by *A. flavus* is higher on oil-seeds than on starchy-seeds (Fabbri *et al.*, 1980). In addition, Fanelli & Fabbri (1981) found that the added fatty acids in the growth medium of *Aspergillus sp.* did not have an important role in the production of toxins but the other lipid fractions isolated from oil-seeds were more likely to be utilised for supporting production of toxins. This was partially confirmed with other experiments in which toxin production varied according to the organic fractions used (Fabbri *et al.*, 1980).

Generally, to date, it is not known at which step or steps in the biosynthesis of aflatoxin the fatty acids exert their influences. However, it is clear that they play a significant role in aflatoxin production as indicated by their stimulatory and/or inhibitory effects. It is thus possible that these fatty acids in some way, contribute to the formation of double bonds present in the aflatoxin molecule. As reported earlier, aflatoxin and lipid biosynthesis are inverse to each other (Detroy & Hesseltine, 1970). Therefore, the energy used for fatty acid (lipid) synthesis is made available for aflatoxin biosynthesis. Lipids and aflatoxin were found to be synthesised during the stationary phase (Maggon *et al.*, 1977).

(f) <u>Effect of some vitamins</u>:

The possible antifungal growth and antitoxin production due to vitamins was investigated using 6 different vitamins namely, vit. A, riboflavin (vit. B_2), pyridoxine hydrochloride (vit. B_6), L-ascorbic acid (vit. C), calciferol (vit. D_2) and folic acid. Two vitamins (L-ascorbic acid and calciferol) were first tested in order to select the most appropriate concentration. Results presented in Table 28 indicate that 5 mM of these

two tested vitamins could gave total inhibition of aflatoxin production. Accordingly, two concentrations (5 & 10 mM) were chosen for testing the selected vitamins except vitamin A in which 0.25 and 0.5 ml containing 1,700,000 unit ml^{-1} was used). After inoculated with *A. parasiticus* spore suspension, the medium was incubated at 30°C for 10 days.

The results as presented in Table 29 and Figs. 38 & 39 show that vitamin B_2 at either 5 or 10 mM could stimulate both the growth and aflatoxin B_1 production. It is worth mentioning that vitamin A did not affect the growth but inhibited aflatoxin B_1 production at either concentration. Folic acid increased the growth only at 5mM and decreased it at 10 mM, however, it was suppressive for aflatoxin B_1 production at both concentrations. Both vitamin C and D_2 were suppressive for the growth and no aflatoxin B_1 was detected at either 5 mM or 10 mM of these vitamins. Also, vitamin B_6 was repressive for both fungal growth and aflatoxin B_1 production, however, its action at 5 mM was severe on fungal growth (52.8%) compared with aflatoxin B_1 production (70%) but at 10 mM the effect on fungal growth was 46.8% which seems to be much less (42.5%) on aflatoxin production.

It seems from the results presented in Figs. 38 and 39 that there are four categories:

(i) Inhibition of both toxin production and growth as in case of Vit. C, Vit. B_6 and Vit. D_2 (Fig. 38B, 39 A & B).

(ii) Inhibition of aflatoxin production, little or no effect on the fungal growth by Vit. A and Folic acid (Fig. 38A, 39C).

(iii) Stimulation of both aflatoxin production and fungal growth by Vit. B_2 (Fig. 38C).

Table 28: Effect of Vit. C and Vit. D_2 on the growth of *A. parasiticus* and aflatoxin B_1 production. (The incubation period was 10 days at 30°C).

Vitamins	Conc. (mM)	Mycelial dry weight (gl^{-1})*	% of control	Aflatoxin B_1/ dry weight (μgg^{-1})*	% of control
L-ascorbic acid	0.10	10.0 ± 0.1	100.0	978 ± 40	99.9
(Vit. C)	0.25	10.0 ± 0.1	100.0	978 ± 29	99.9
	1.00	10.0 ± 0.1	100.0	891 ± 18	88.9
	2.50	9.8 ± 0.1	98.1	580 ± 26	59.3
	5.00	7.3 ± 0.0	72.6	00	00
Calciferol	0.10	10.0 ± 0.0	99.6	975 ± 56	99.6
(Vit. D_2)	0.25	10.0 ± 0.0	99.6	959 ± 23	98.9
	1.00	9.8 ± 0.0	99.6	621 ± 30	63.4
	2.50	7.3 ± 0.1	97.1	240 ± 19	24.5
	5.00	5.9 ± 0.0	72.7	00	00
Control		10.04 ± 0.05		979 ± 30	

* = Means of three replicates ± S.D.

Table 29: Effect of some vitamins on the growth of *Aspergillus parasiticus* NRRL 2999 and aflatoxin B_1 production. (The incubation period was 10 days at 30°C).

Compound	Conc. (mM)	Mycelial dry weight $(gl^{-1})^*$	% of control	Aflatoxin B_1/ dry weight $(\mu gg^{-1})^*$	% of control
Vitamin A^+	0.25	10.1*	100.6	541	55.3
	0.50	10.2*	101.6	461	47.1
L-ascorbic acid	5	7.6	75.7	N.D.	00.0
	10	5.4	53.8	N.D.	00.0
Riboflavin	5	10.8	107.6	1625	166.2
	10	11.6	115.5	1700	173.8
Pyridoxine hydrochloride	5	5.3	52.8	685	70.0
	10	4.7	46.8	416	42.5
Calciferol	5	6.1	60.8	N.D.	00.0
	10	4.8	47.8	N.D.	00.0
Folic acid	5	10.9	108.4	241	24.6
	10	9.4	93.6	225	23.0

Control: Mycelial dry weight = 10.04 ± 0.01 gl^{-1}
Aflatoxin B_1 = 978 ± 35 μgg^{-1} dry weight

N.D. = Not detected.

NLSD: for fungal growth at 5% = 0.16
at 1% = 0.21
for aflatoxin production at 5% = 47.36
at 1% = 62.79

Not labeled = Highly significant.

* = Not significant.

+ = Concentration of Vit. A ml per 100 ml medium.

Fig. 38: Effect of different concentrations of some vitamins, Vit. A (A), Vit. C (B) and Vit. B_2 (C) on the growth of *A. parasiticus* and Aflatoxin B_1 production. (Taken from Table 29).

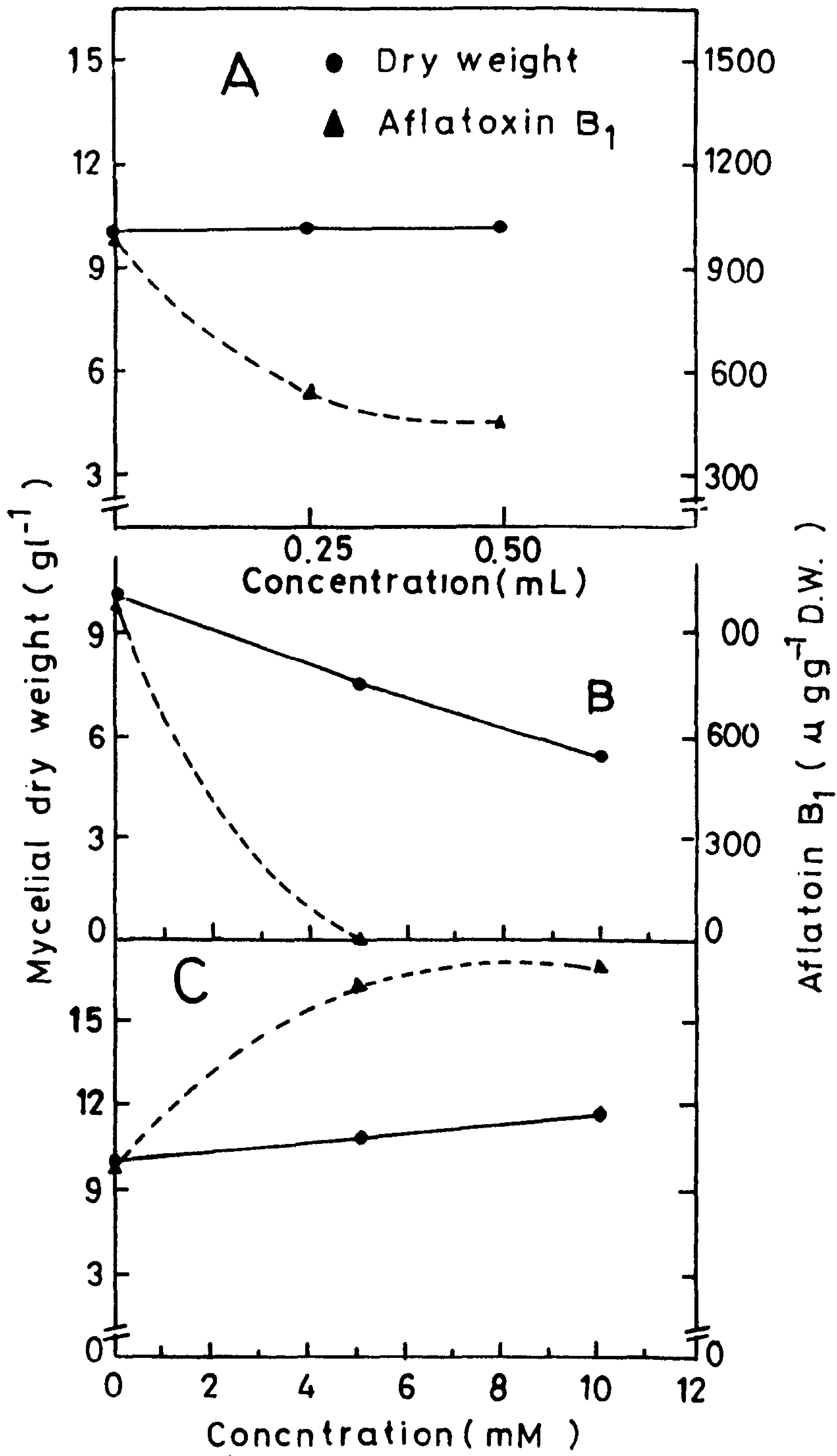
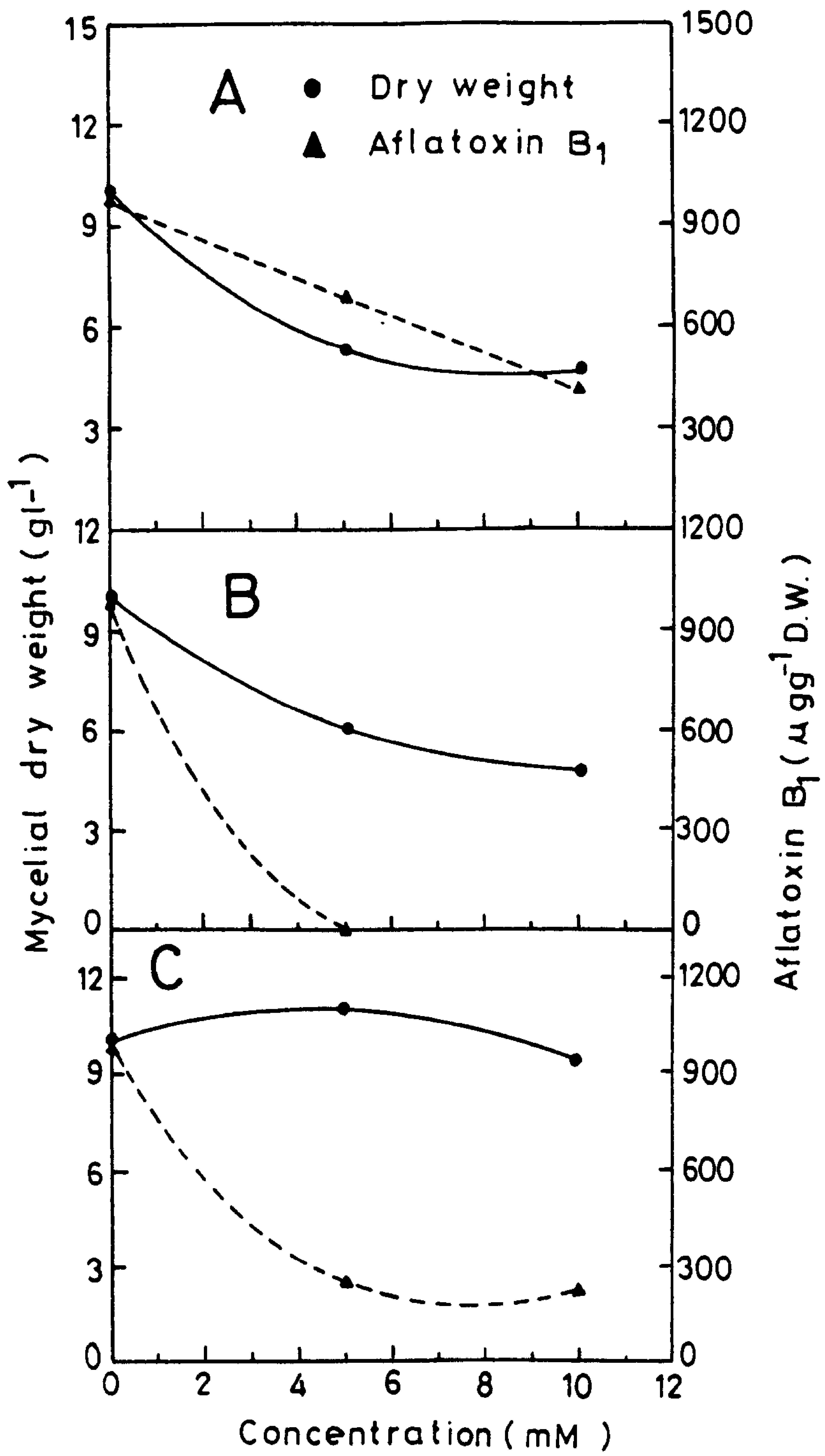

A
Dry weight
Aflatoxin B_1
15
12
9
6
3
1500
1200
900
600
300
0.25
0.50
Concentration(mL)
B
9
6
3
0
00
600
300
0
C
15
12
9
3
0
0
2
4
6
8
10
12
Concntration (mM)
Mycelial dry weight (gl^{-1})
Aflatoin B_1 (μ gg^{-1} D.W.)

Fig. 39: **Effect of different concentrations of some vitamins, Vit. B_6 (A), Vit. D_2 (B) and Folic acid (C) on the growth of *A. parasiticus* and aflatoxin B_1 production. (Taken from Table 29).**



A
B
C
Dry weight
Aflatoxin B_1
Mycelial dry weight (gl^{-1})
Aflatoxin B_1 ($\mu g g^{-1}$ D.W.)
Concentration (mM)

Generally, vitamin C and vitamin D_2 seem to be the most effective vitamins on aflatoxin production. Vitamin B_6 at 10 mM was the most suppressive for the fungal growth.

The vitamin requirements of fungi is reviewed by Janke (1939), Robbins & Kavanagh (1942), and Schopfer (1934).

The present study revealed that riboflavin was the only vitamin stimulating both fungal growth and toxin production. The present results are consistent with those of Clevstrom *et al.* (1983) who reported that addition of riboflavin to *A. flavus* medium caused approximately 15-fold increase in the amount of aflatoxin. The stimulatory effect of riboflavin could be attributed to its possible use as coenzyme by *A. parasiticus* in many enzymatic reaction, several of which are involved in producing energy required for fungal growth and toxin production. On the other hand, vitamins C, B_6, D_2 were found to repress both fungal growth and toxin production. The antitoxigenic effect of vitamin C was supported by the work done by Brackett and Marth (1979) who reported that the addition of 5% of vitamin C to apple juice decreased mycotoxin patulin by 95%.

CHAPTER VI

Enzyme Activities in *A. parasiticus* in relation to Aflatoxin B_1 Production

Introduction:

It is generally accepted that rapid fungal growth under normal conditions results in the accumulation of precursors that are subsequently diverted towards biosynthesis of secondary metabolites like mycotoxins at the end of exponential phase (Demain, 1973). The molecular events that initiate and abruptly terminate secondary metabolism are poorly understood.

Aflatoxins have been among the most known variety of polyketide compounds synthesized by various fungal species. Polyketide biosynthesis is highly characteristic of the fungi. More fungal secondary metabolites are produced by this route than by any other biosynthetic pathway (Turner, 1971).

Little is known about the enzymatic processes involved in polyketide biosynthesis. Hsieh and Mateles (1970) concluded that aflatoxins are synthesized from acetyl-CoA derived from oxidation of pyruvate by the pyruvate dehydrogenase complex. The required pyruvate is the product of glucose catabolism. Therefore, it was thought to measure the activities of pyruvate-generating enzymes namely, phosphoglycero-mutase, enolase and pyruvate kinase. Also, it was decided to measure pyruvate dehydrogenase as it plays the major role in production of acetyl-CoA. Since pyruvate results from the catabolism of glucose through glycolysis, it was decided to measure the two glycolytic enzymes: phosphofructokinase and fructose,1,6-bisphosphate aldolase.

The TCA cycle provides intermediates for synthesis of secondary metabolites. The role of TCA cycle in microbial metabolism in the anabolic synthesis of biosynthetic precursors has been described (Paigan and Williams, 1970). Gupta *et al.* (1977) and Maggon *et al.* (1977) hypothesized that reduced TCA cycle activity leads to an

accumulation of TCA cycle intermediates, which leads to a shunting of acetyl CoA to aflatoxin synthesis. Also, it was reported that aflatoxin synthesis occurs during a period of decreased tricarboxylic acid cycle activity (Buchanan & Lewis, 1984 and Dutton, 1988). Therefore, it was decided to measure the activities of some important enzymes involved in TCA cycle and comparing with those of pyruvate-generating enzymes.

The relative concentrations of NADP and NADPH have been suggested as a factor of primary metabolism that can affect aflatoxin synthesis. Shih and Marth (1974b) have suggested that elevated levels of NADPH stimulate aflatoxin synthesis. In addition, Singh and Hsieh (1976) have demonstrated that *in vitro* conversion of sterigmatocystin to aflatoxin B_1 is NADPH dependent. Accordingly, it was decided to measure the activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase as NADPH-producing enzymes.

The enzyme activities were measured in relation to aflatoxin B_1 production throughout twenty days of fungal growth at intervals of two days. The study of enzyme activity in this part was a preliminary one with the limited aim to find out whether there was any possible relationship between aflatoxin B_1 production and the measured activities.

It was reported that phenolic compounds inhibited mitochondrial respiration (Cheng and Pardini, 1978 & 1979), mevalonate phosphate kinase activities (Shama Bhat and Ramasarma, 1979) and have been shown to inhibit enzymes such as phosphorylases, cellulases, transaminases and decarboxylases, thus causing a virtual cessation on mycelium growth (Vance and Garraway, 1973). Therefore, the extent to which aflatoxin production could be associated with the activities of the measured

enzymes was investigated in order to gain some idea about the relationship between the productivity of aflatoxin and the activity of enzymes in *A. parasiticus*. Thus, it was decided to grow the organism on the usual growth medium but in presence of various concentrations (0.2-1.0 mM) of two inhibitors, namely, phenol or catechol and determining the fungal growth and the productivity of both aflatoxin and the activity of the enzymes. It should be stressed that these two compounds were chosen since they proved in the previous chapter to be good inhibitors for fungal growth and toxin production (see Table 26). Also, these two compounds were chosen since they found in plant as natural compounds (Dix & Webster, 1995 and Fajardo *et al.*, 1995).

Results:

The results (Table 30 & Fig. 40) showed that the activities of pyruvate-generating enzymes as well as pyruvate-dehydrogenase complex in *A. parasiticus* were increased continuously up to 6th day of culture age. These results suggest that pyruvate-generating enzymes may provide pyruvate dehydrogenase with pyruvate for its oxidation and forming acetyl-CoA.

The observed increase in the activities of the two glycolytic enzymes: phosphofructokinase and fructose 1,6-bisphosphate aldolase up to the 6th day with pyruvate-generating enzymes (Table 30 & Fig. 41) may indicate accelerated glycolysis for glucose catabolism into pyruvate. The decreasing activities of glycolytic enzymes after 6 days may indicate depletion of the carbon source in the medium. On the other hand, the higher activities of these enzymes indicate a higher breakdown of glucose for energy production leading to the accumulation of pyruvate, which is essential for the onset of aflatoxin biosynthesis. These explanations are in agreement with those of

Gupta *et al.* (1976), Venkitasubramanian (1977), Maggon *et al.* (1977) and Smith & Moss (1985).

The decreased activities of enzymes of the TCA cycle particularly from 4th day of growth up to 10th day (Table 30 & Fig. 42) are associated with the increase of aflatoxin B_1 production. This may suggest that most of acetyl-CoA in *A. parasiticus* could be used in the synthesis of aflatoxin B_1. These results are in agreement with those of Buchanan & Lewis (1984), Buchanan *et al.* (1985) and Luchese & Harrigan (1993). Also, these results are consistent with those of Jechova *et al.* (1969) who observed that when *Streptomyces auerofaciens* starts to form chlorotetracycline, the activity of malate dehydrogenase decreased significantly. Anyway, the presence of a functional TCA cycle in a number of *Aspergillus* species has been documented (Ainsworth & Sussman, 1965 and Kobr & Vanderhaeghe, 1973).

The observation of higher activities of both aconitase and fumarase between 4th to 10th day compared to those of isocitrate dehydrogenase may indicate that TCA cycle is reduced while sufficient activity is maintained to meet the anabolic needs of the fungus. This respiratory enzyme aconitase was found in the fungal spores with higher activities if compared with other enzymes in the vegetative cells (Zalokar, 1959 and Gottlieb & Caltrider, 1963). In addition, Maggon *et al.* (1977) observed differential changes among selected TCA cycle enzymes in *A. parasiticus* as a function of fungal growth.

The activities of the two enzymes of pentose pathway, namely, glucose-6-P-dehydrogenase and 6-phosphogluconate dehydrogenase were at optimal on 10th day of fungal growth (Table 30 & Fig. 41). These results are in agreement with those of Scott & Abramsky (1973) and Buchanan and Lewis (1984). Thus, it seems likely that

pentose phosphate pathway possibly provides NADPH for *A. parasiticus* which could stimulate aflatoxin B_1 production.

The lower activities of NADP-malic enzyme under *in vitro* testing were not consistent with aflatoxin B_1 production (Table 30 & Fig. 41) and this rules out the possibility that this enzyme is a source of NADPH for aflatoxin production.

The results in Tables 31 and 32 indicate that the fungal growth was decreased in presence of phenol particularly at high concentration but it also decreased slightly with the higher concentration of catechol. However, aflatoxin production was suppressed in the presence of either phenol or catechol in the growth medium. It was found that catechol was less suppressive. It was seen also that the degree of inhibition was dependent on the concentration of either compounds.

The results in Figs. 43 and 44 show that the activities of pyruvate-generating enzymes were lower after growth with either phenol or catechol at all tested concentrations. Pyruvate kinase seemed to be the enzyme most affected by the two compounds but enolase and phosphoglyceromutase were the least affected enzymes. The effect of either compound was dependent on the concentration. Moreover, phenol was more effective than catechol. Although pyruvate dehydrogenase expressed the least activities if compared to pyruvate-generating enzymes, it was the most resistant to phenol at the highest concentration.

The results in Figs. 45 and 46 show the activities of both phosphofructokinase and fructose-1,6-bisphosphate aldolase in *A. parasiticus* were reduced by phenol and catechol while fructose-1,6-bisphosphate aldolase is less susceptible enzyme. The five measured enzymes of TCA cycle (Figs. 47 and 48) were reduced by the various concentrations of phenol or catechol. Isocitrate dehydrogenase was the least affected

enzyme but aconitase and fumarase were the most susceptible although these two enzymes expressed the highest initial activities. The results (Figs. 49 and 50) indicate that the two enzymes of pentose phosphate pathway and NADP-malic enzyme were reduced in level by the two compounds.

These results indicate that pyruvate-generating enzymes (Figs. 43 & 44), the glycolytic enzymes (Figs. 45 and 46), and the enzymes of pentose phosphate pathway (Figs. 49 and 50) showed comparable activities with the exception of phospho-glyceromutase and enolase which expressed appreciable higher activities. It was observed also that TCA cycle enzymes (Figs. 47 and 48) and NADP-malic enzymes (Figs. 49 and 50) were the most affected enzymes by either phenol or catechol. As the case in aflatoxin production, the enzyme activities were reduced increasingly by increasing the concentration of phenol or catechol. Also, phenol was much inhibitor than catechol. The effect of the phenolic compounds on aflatoxin production may be due to depressing the rate of glucose utilization and the reduction of the enzyme activities involved in aflatoxin production. These explanations are in agreement with those of Buchanan & Lewis (1984) and San & Chan (1987).

Reduction of aflatoxin production, pyruvate-generating enzyme as well as the two enzymes of pentose phosphate pathway by either phenol or catechol may add a further piece of evidence to support the idea that the productivity of aflatoxin is correlated with the previously mentioned enzymes. On the other hand, the higher reductive effect of either phenol or catechol on TCA cycle enzymes support the emerged idea, of the present results, that aflatoxin synthesis occurs during a period of decreased tricarboxylic acid cycle activity.

Table 30: Aflatoxin B_1 production ($\mu g g^{-1}$ D.W.) in relation to activities of metabolic enzyme* (nmol min^{-1} g^{-1} mycelium).

Measured Enzymes	Culture age (days)									
	2	4	6	8	10	12	14	16	18	20
Aflatoxin B_1 production	26	63	359	805	978	850	831	741	632	409
<u>Pyruvate-generating enzymes</u>:										
Phosphoglyceromutase	15.2	65.4	185.0	160.0	101.0	82.5	60.4	27.5	20.6	16.4
Enolase	13.7	48.0	117.5	85.5	48.2	42.8	34.3	27.3	14.8	11.2
Pyruvate kinase	21.4	32.4	89.3	76.2	72.0	65.6	64.4	21.2	18.4	10.7
Pyruvate-DH complex	10.9	16.6	38.8	36.0	32.7	31.0	24.4	16.7	11.0	7.8
<u>Glycolytic enzymes</u>:										
Phosphofructokinase	48.8	97.9	108.7	95.6	77.7	74.4	59.7	30.1	23.9	22.5
Fructose 1,6 bisphosphate aldolase	21.4	62.2	79.4	44.5	38.0	26.8	21.4	17.9	17.4	14.9
<u>TCA cycle enzymes</u>:										
NADP-isocitrate-DH	26.7	51.0	45.9	39.8	32.8	31.0	27.4	22.9	16.4	11.5
NAD-isocitrate-DH	12.4	33.7	27.2	26.4	21.9	20.8	16.6	16.4	9.7	6.8
Malate DH	19.3	43.7	37.8	36.4	33.4	30.4	26.5	22.0	20.1	17.1
Aconitase	16.4	67.5	55.5	48.9	41.8	32.7	27.5	19.9	18.4	15.3
Fumarase	15.0	62.5	51.0	45.8	44.0	38.3	31.0	27.4	21.1	15.6
<u>Pentose phosphate pathway enzymes</u>:										
Glucose-6-phosphate-DH	34.0	41.0	62.0	73.7	90.4	86.6	79.7	65.4	41.2	24.0
6-Phosphogluconate-DH	28.5	32.2	49.4	61.0	84.3	71.0	31.0	18.1	15.2	9.7
<u>Other enzymes</u>:										
NADP-malic enzyme	11.0	12.4	16.5	19.0	23.7	29.9	36.5	42.8	19.7	17.4

* Mean of three replicates.

Fig. 40: Relation between aflatoxin B_1 production and pyruvate-generating enzymes and pyruvate-dehydrogenase complex activities during 20 days culture of *A. parasiticus*.

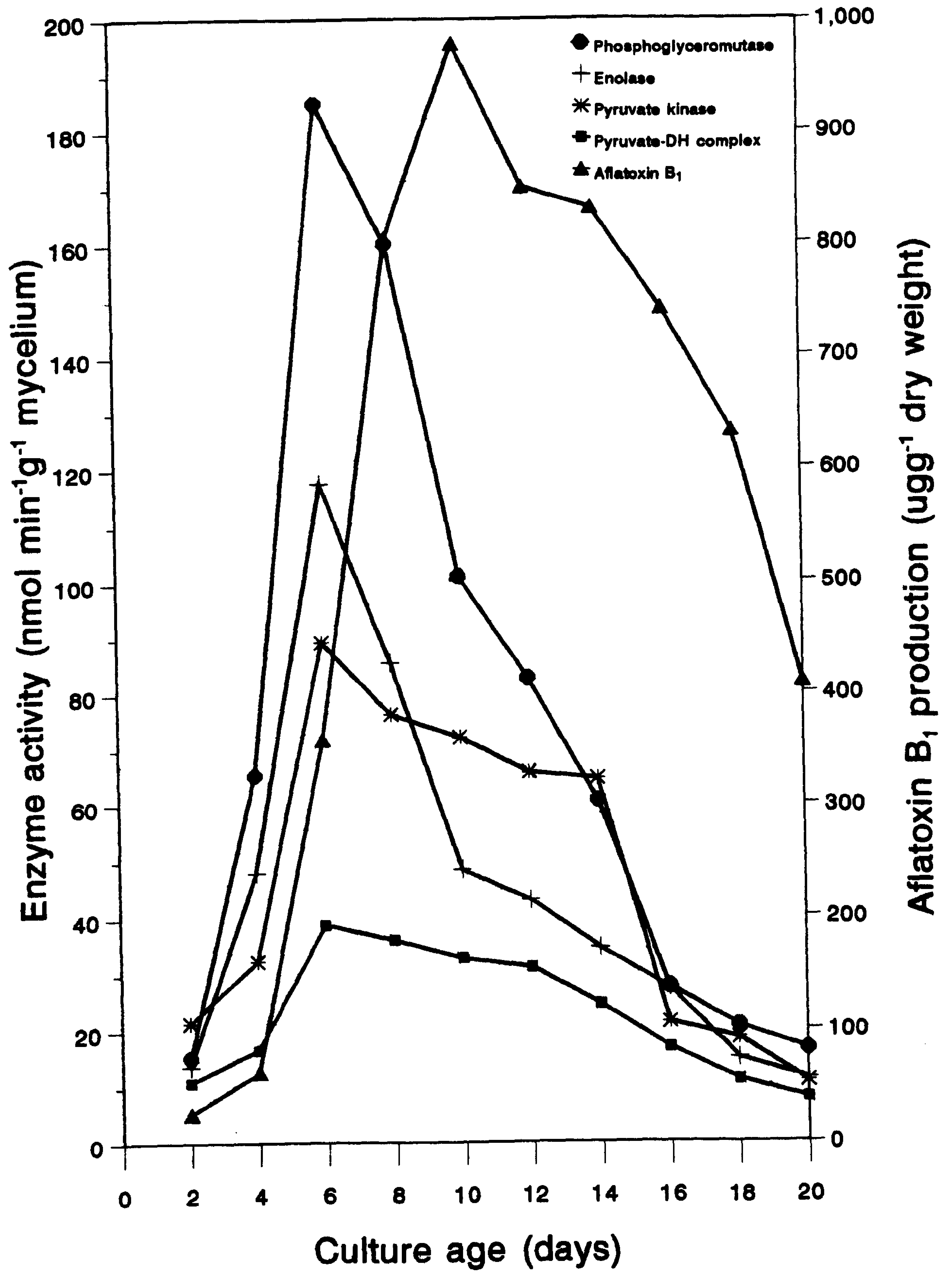
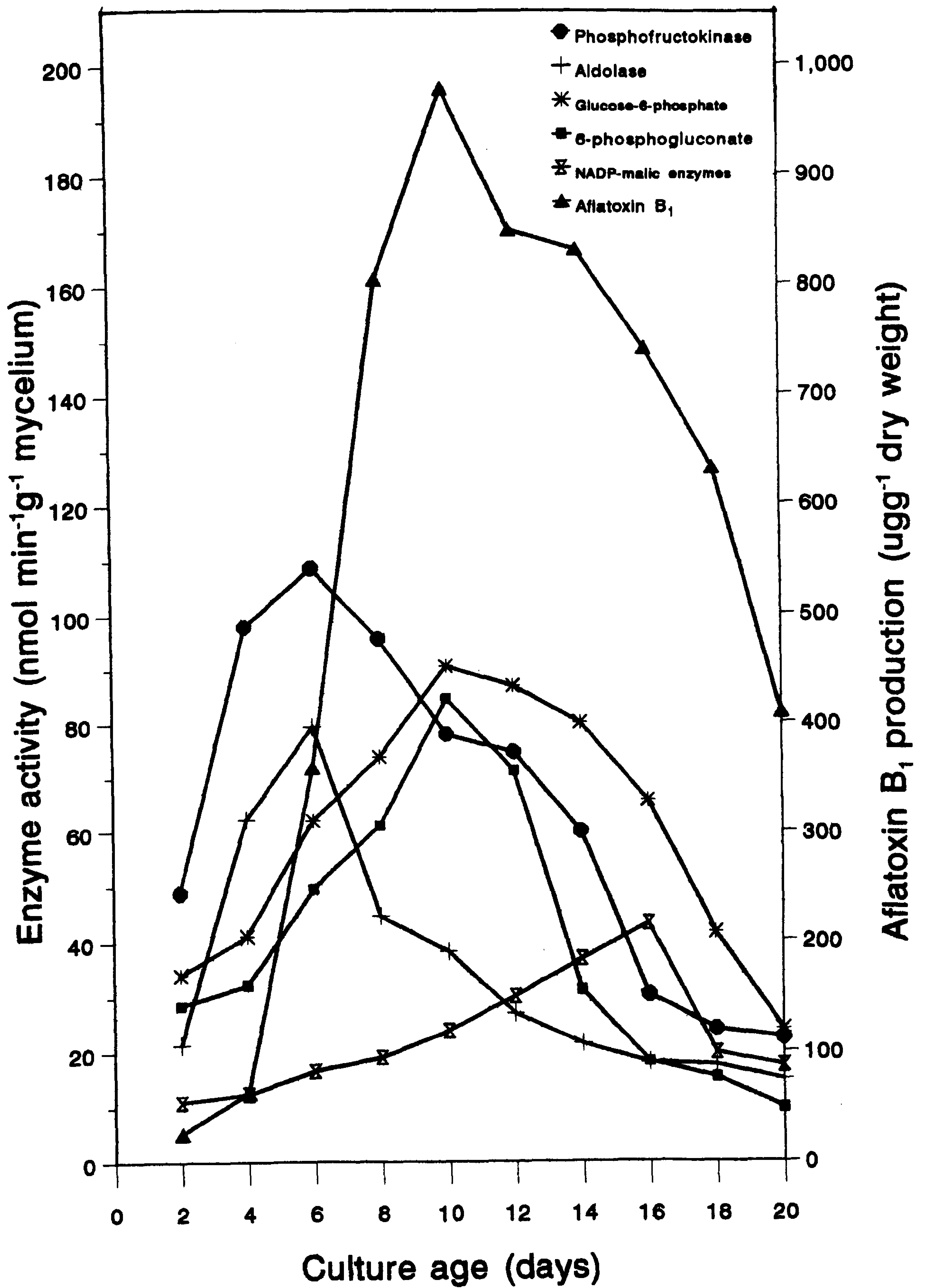
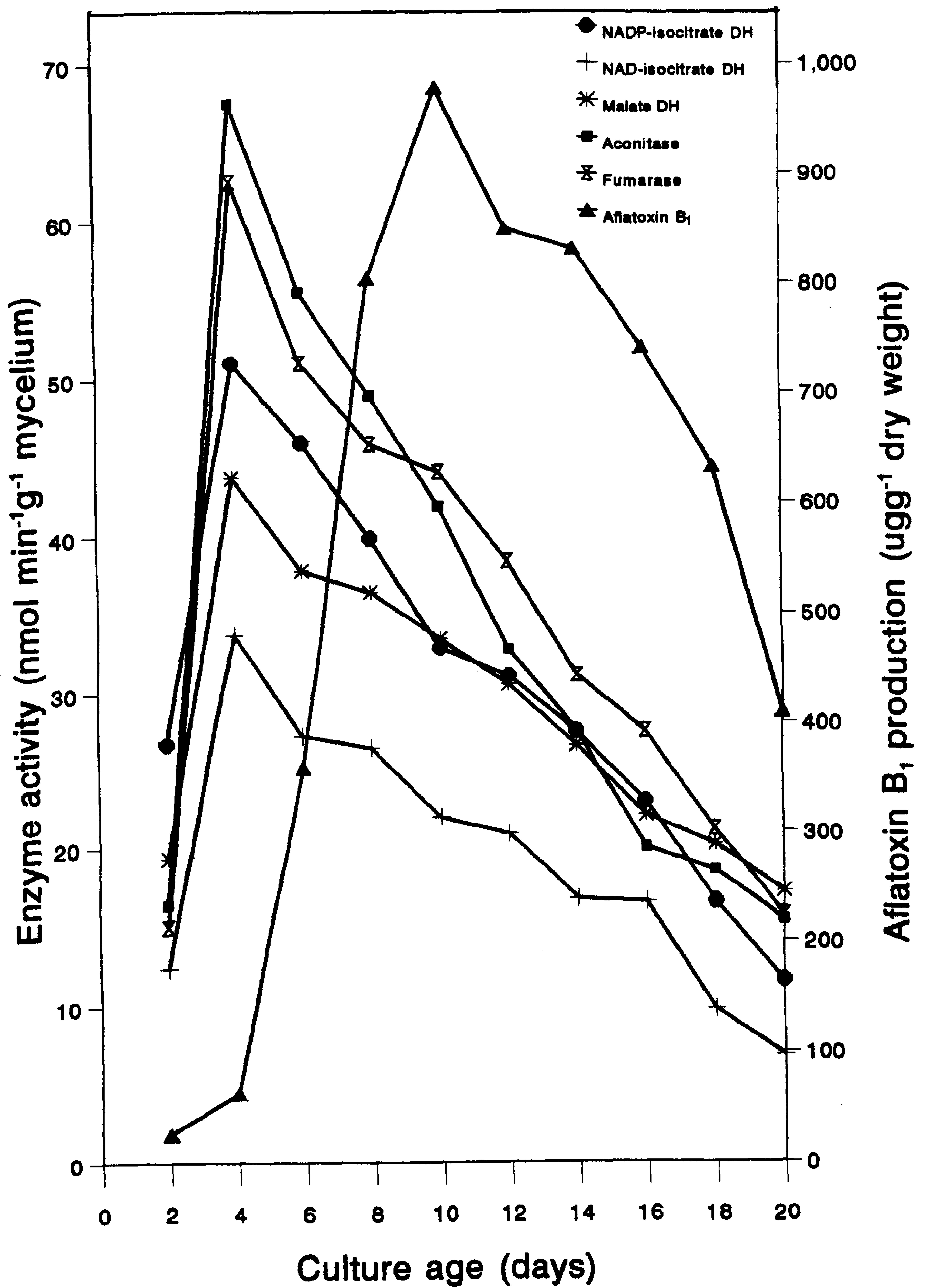

Phosphoglyceromutase
Enolase
Pyruvate kinase
Pyruvate-DH complex
Aflatoxin B1
Enzyme activity (nmol min-1g-1 mycelium)
Aflatoxin B1 production (ugg-1 dry weight)
Culture age (days)
0
20
40
60
80
100
120
140
160
180
200
100
200
300
400
500
600
700
800
900
1,000
2
4
6
8
10
12
14
16
18
20

Fig. 41: Relation between aflatoxin B_1 production and glycolytic enzymes, pentose phosphate pathway enzyme and NADP-malic enzyme activities during 20 days culture of *A. parasiticus*.



Phosphofructokinase
Aldolase
Glucose-6-phosphate
6-phosphogluconate
NADP-malic enzymes
Aflatoxin B1
Enzyme activity (nmol min-1g-1 mycelium)
Aflatoxin B1 production (ugg-1 dry weight)
Culture age (days)
0
20
40
60
80
100
120
140
160
180
200
2
4
6
8
10
12
14
16
18
20
100
200
300
400
500
600
700
800
900
1,000

Fig. 42: Relation between aflatoxin B_1 production and TCA cycle enzyme activities during 20 days culture of *A. parasiticus*.



NADP-isocitrate DH
NAD-isocitrate DH
Malate DH
Aconitase
Fumarase
Aflatoxin B_1
Enzyme activity (nmol min^{-1}g^{-1} mycelium)
Aflatoxin B_1 production (ugg^{-1} dry weight)
Culture age (days)
0
10
20
30
40
50
60
70
100
200
300
400
500
600
700
800
900
1,000
2
4
6
8
12
14
16
18

Table 31: The biomass of *A. parasiticus* grown on the growth medium contained either phenol or catechol.
(The incubation period was 10 days at 30°C)

Chemical	Control		Concentration (mM)									
			0.2		0.4		0.6		0.8		1.0	
	Mycelial dry weight (gl^{-1})*	%	Mycelial dry weight (gl^{-1})	% of control	Mycelial dry weight (gl^{-1})	% of control	Mycelial dry weight (gl^{-1})	% of control	Mycelial dry weight (gl^{-1})	% of control	Mycelial dry weight (gl^{-1})	% of control
None	10.8	100	-	-	-	-	-	-	-	-	-	-
Phenol	-	-	9.8	90.7	7.5	69.4	6.0	55.6	2.5	23.1	0.9	8.3
Catechol	-	-	10.8	100	10.8	100	10.4	96.3	10.0	92.6	9.9	91.7

* The values are the mean of three measurements.

Table 32: Aflatoxin B_1 production* by *A. parasiticus* grown on either phenol or catechol in the growth medium.
(The incubation period was 10 days at 30°C)

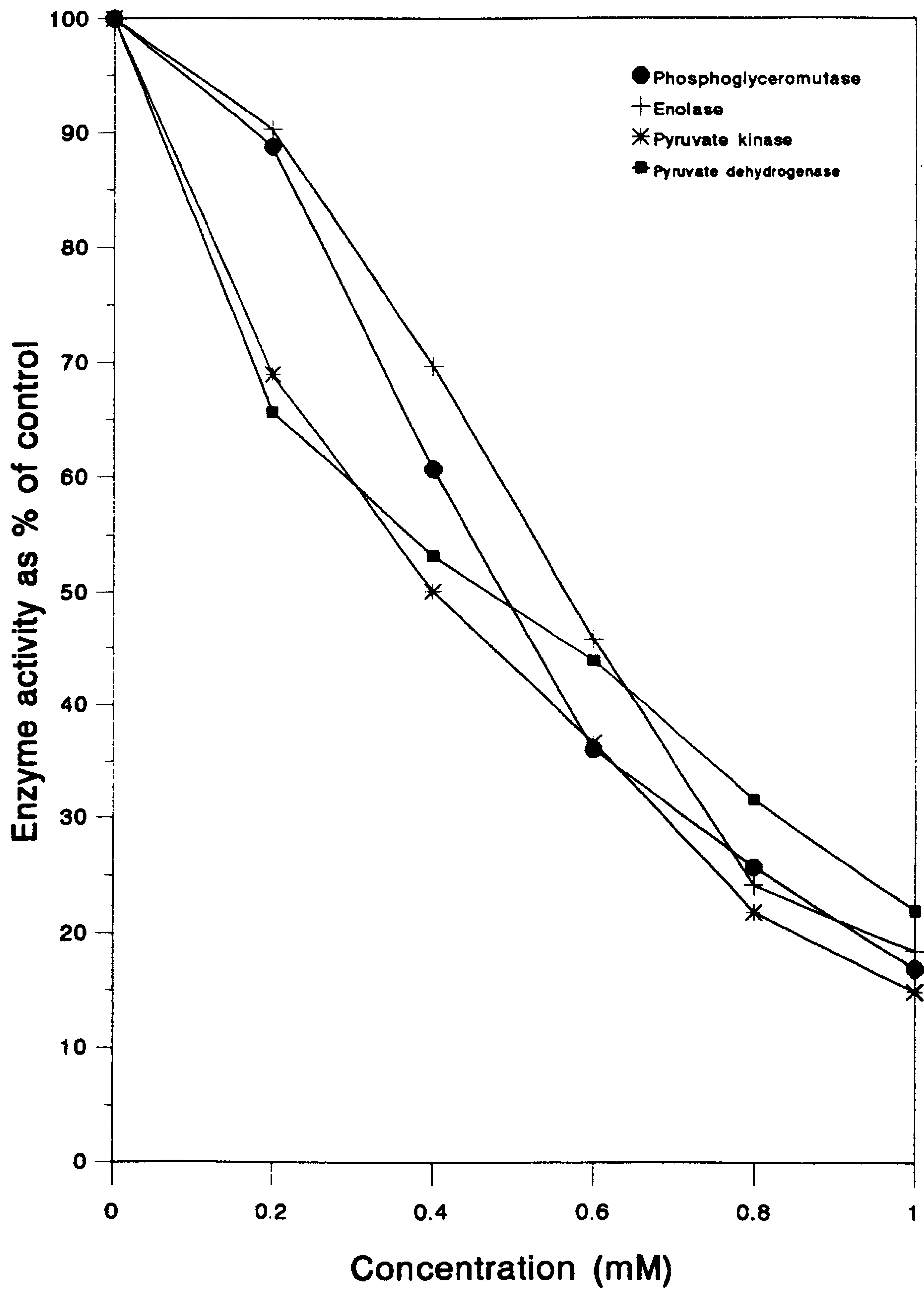
Chemical	Control		Concentration (mM)									
			0.2		0.4		0.6		0.8		1.0	
	Aflatoxin***	%	Aflatoxin	% of control	Aflatoxin	% of control	Aflatoxin	% of control	Aflatoxin	% of control	Aflatoxin	% of control
None	931.7	100	-	-	-	-	-	-	-	-	-	-
Phenol	-	-	410.0	44.0	116.5	12.5	ND**	-	ND**	-	ND**	-
Catechol	-	-	931.0	99.9	931.0	99.9	890.7	95.6	760.0	81.6	620.0	66.5

* Aflatoxin B_1 is expressed as $\mu g\ g^{-1}$ dry wt.

** ND = Not detected.

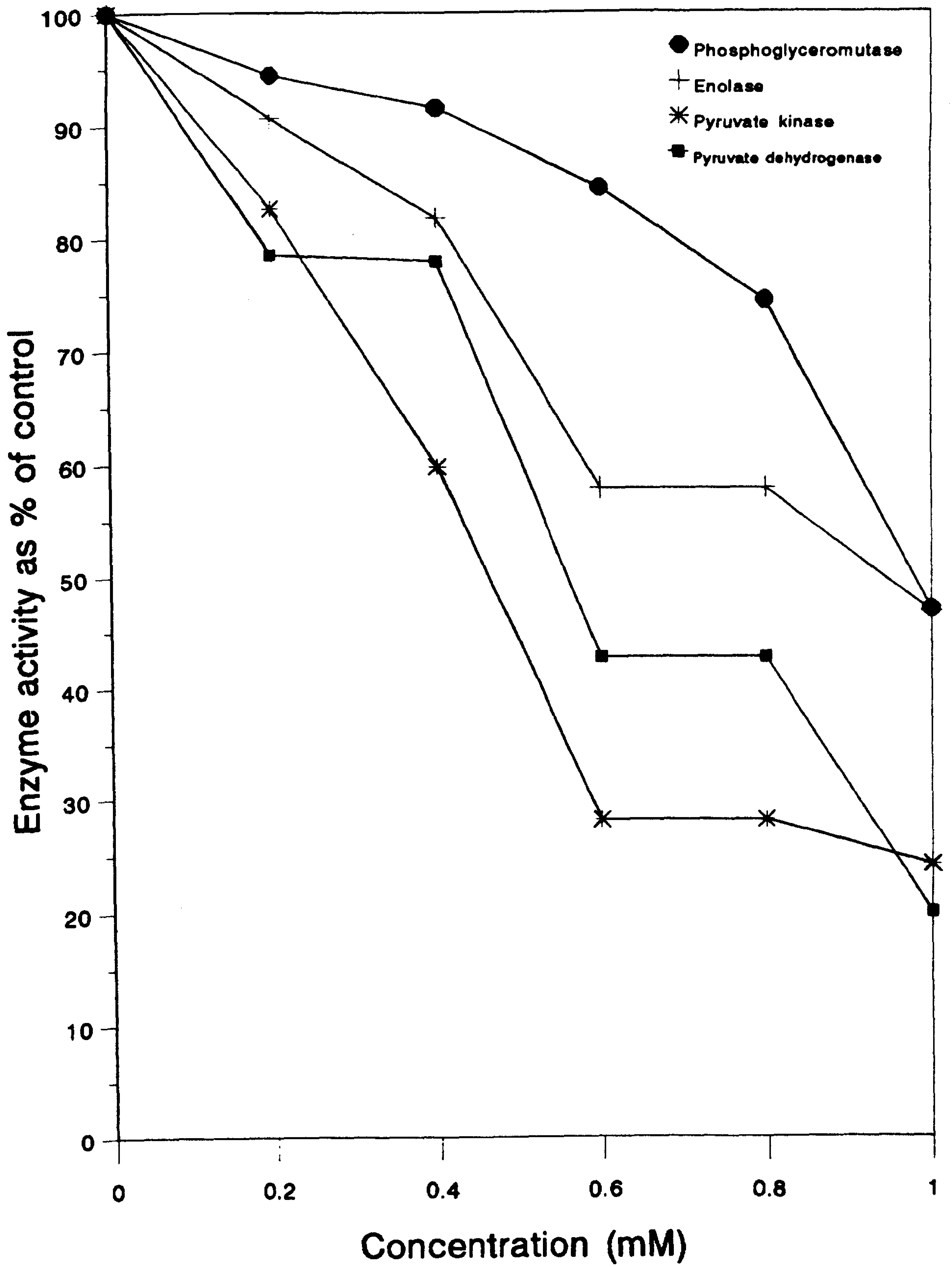
*** The values are the mean of three measurements.

Fig. 43: **Effect of various concentrations of phenol added to the growth medium of *A. parasiticus* on the level of pyruvate-generating enzymes and pyruvate dehydrogenase after 10 days culture.**



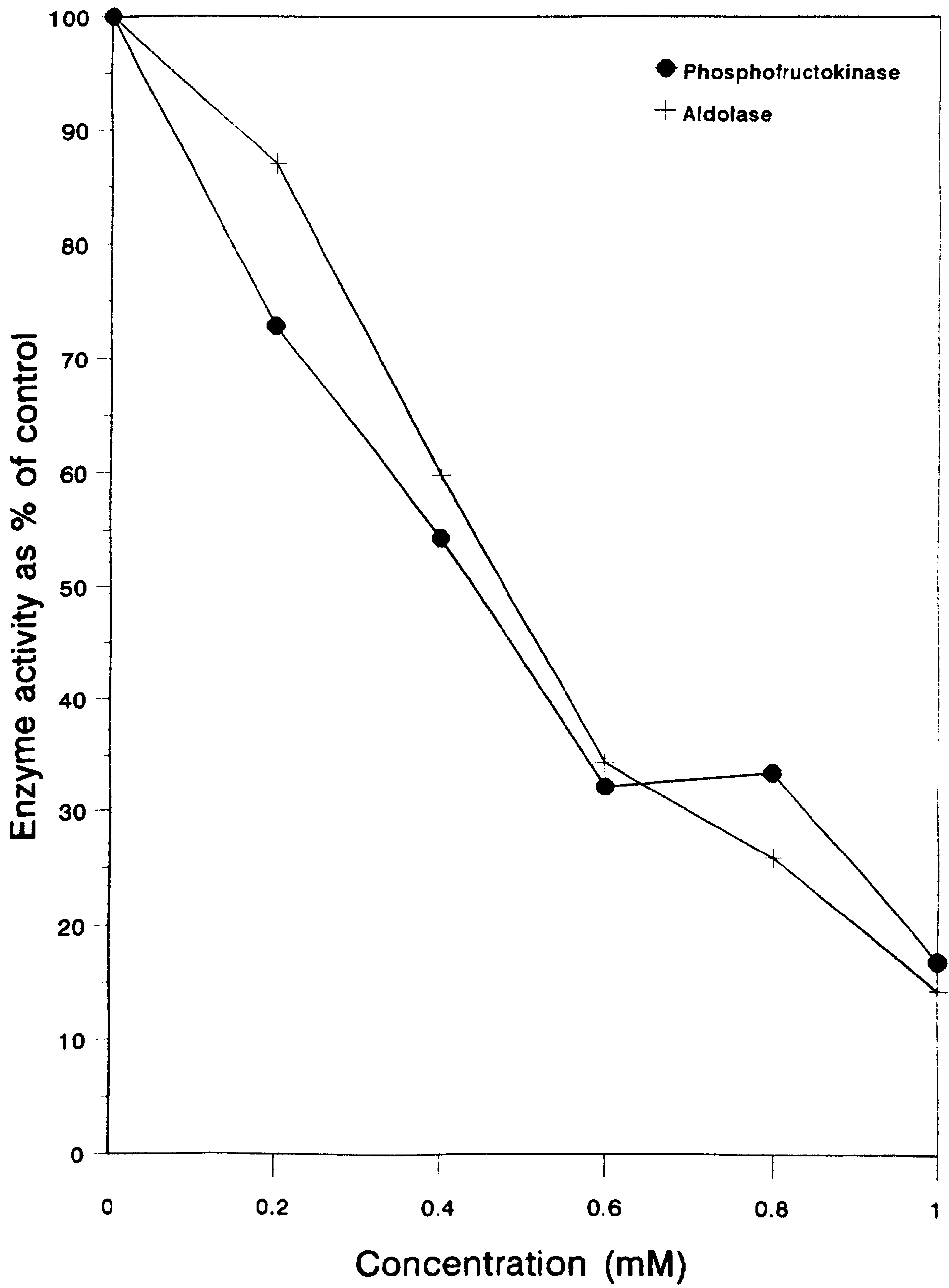
Phosphoglyceromutase
Enolase
Pyruvate kinase
Pyruvate dehydrogenase
Enzyme activity as % of control
Concentration (mM)
100
90
80
70
60
50
40
30
20
10
0
0
0.2
0.4
0.6
0.8
1

Fig. 44: **Effect of various concentrations of catechol added to the growth medium of *A. parasiticus* on the level of pyruvate-generating enzymes and pyruvate dehydrogenase after 10 days culture.**



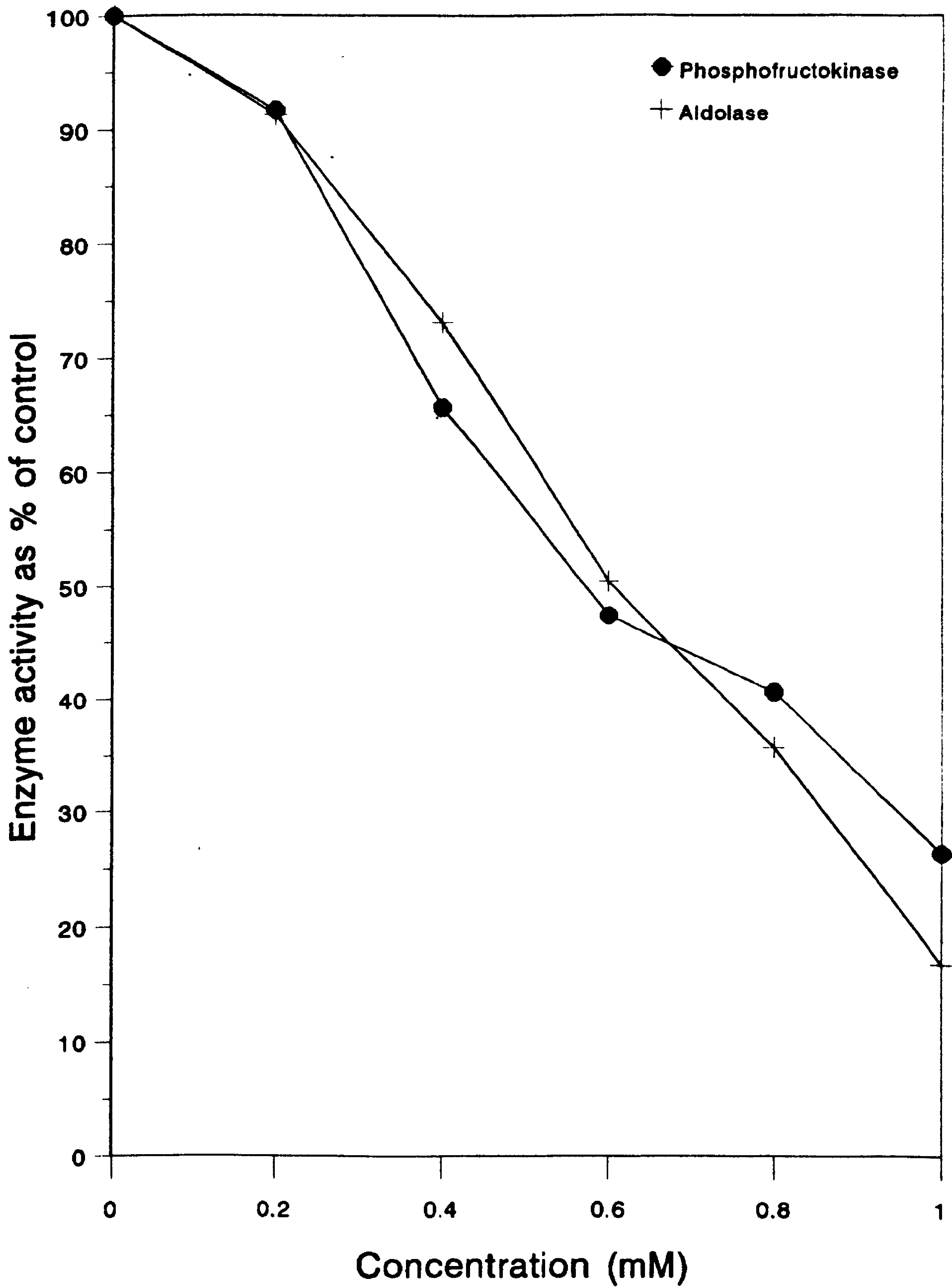
Phosphoglyceromutase
Enolase
Pyruvate kinase
Pyruvate dehydrogenase
Enzyme activity as % of control
100
90
80
70
60
50
40
30
20
10
0
0
0.2
0.4
0.6
0.8
1
Concentration (mM)

Fig. 45: **Effect of various concentrations of phenol added to the growth medium of *A. parasiticus* on the level of glycolytic enzymes with 10 days culture.**



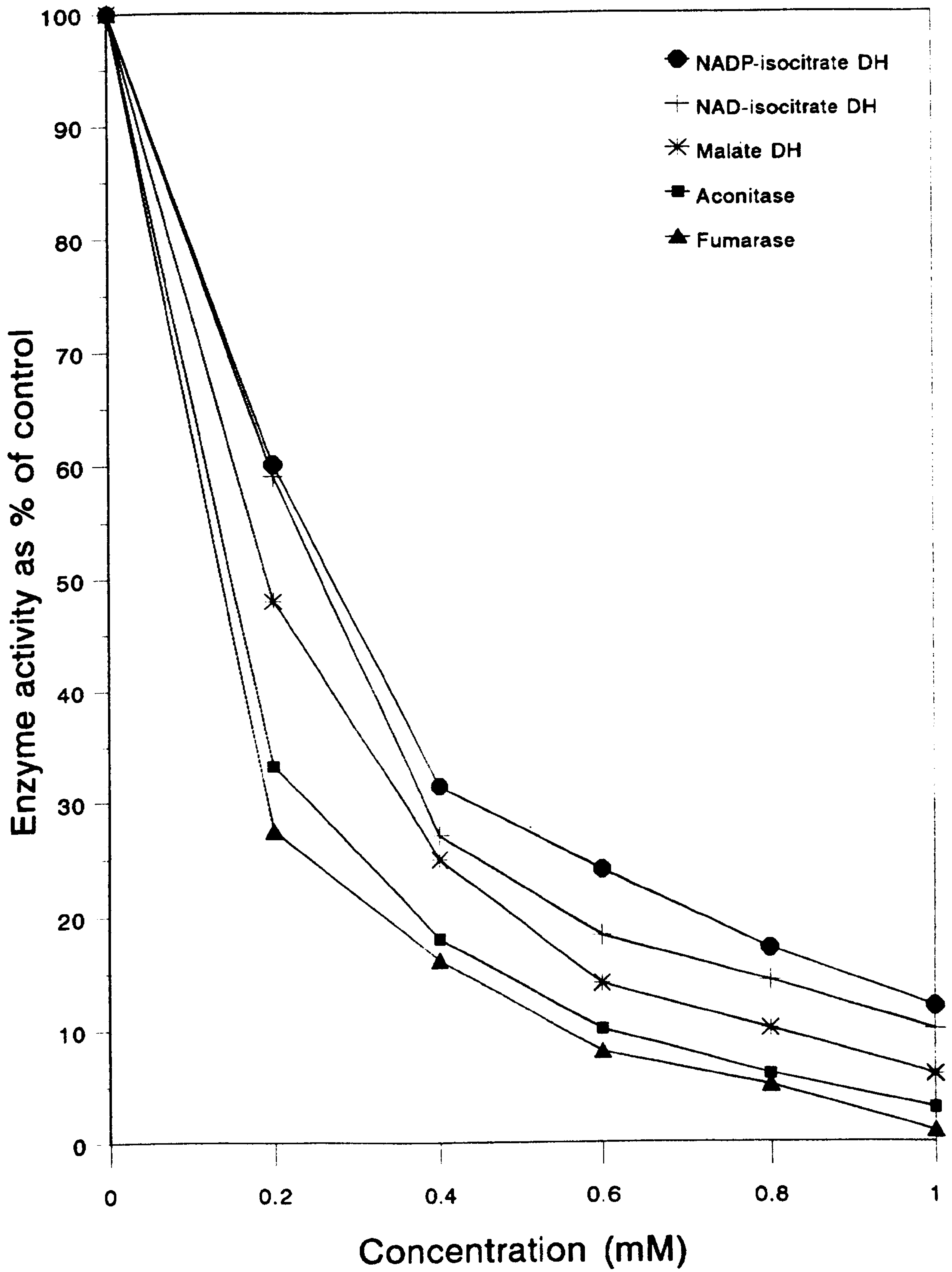
Phosphofructokinase
Aldolase
Enzyme activity as % of control
100
90
80
70
60
50
40
30
20
10
0
0
0.2
0.4
0.6
0.8
1
Concentration (mM)

Fig. 46: **Effect of various concentrations of catechol added to the growth medium of *A. parasiticus* on the level of glycolytic enzymes after 10 days culture.**



Phosphofructokinase
Aldolase
Enzyme activity as % of control
Concentration (mM)
0
10
20
30
40
50
60
70
80
90
100
0
0.2
0.4
0.6
0.8
1

Fig. 47: Effect of various concentrations of phenol added to the growth medium of *A. parasiticus* on the level of the enzymes of TCA cycle after 10 days culture.



NADP-isocitrate DH
NAD-isocitrate DH
Malate DH
Aconitase
Fumarase
Enzyme activity as % of control
100
90
80
70
60
50
40
30
20
10
0
0
0.2
0.4
0.6
0.8
1
Concentration (mM)

Fig. 48: Effect of various concentrations of catechol added to the growth medium of *A. parasiticus* on the level of the enzymes of TCA cycle after 10 days culture.

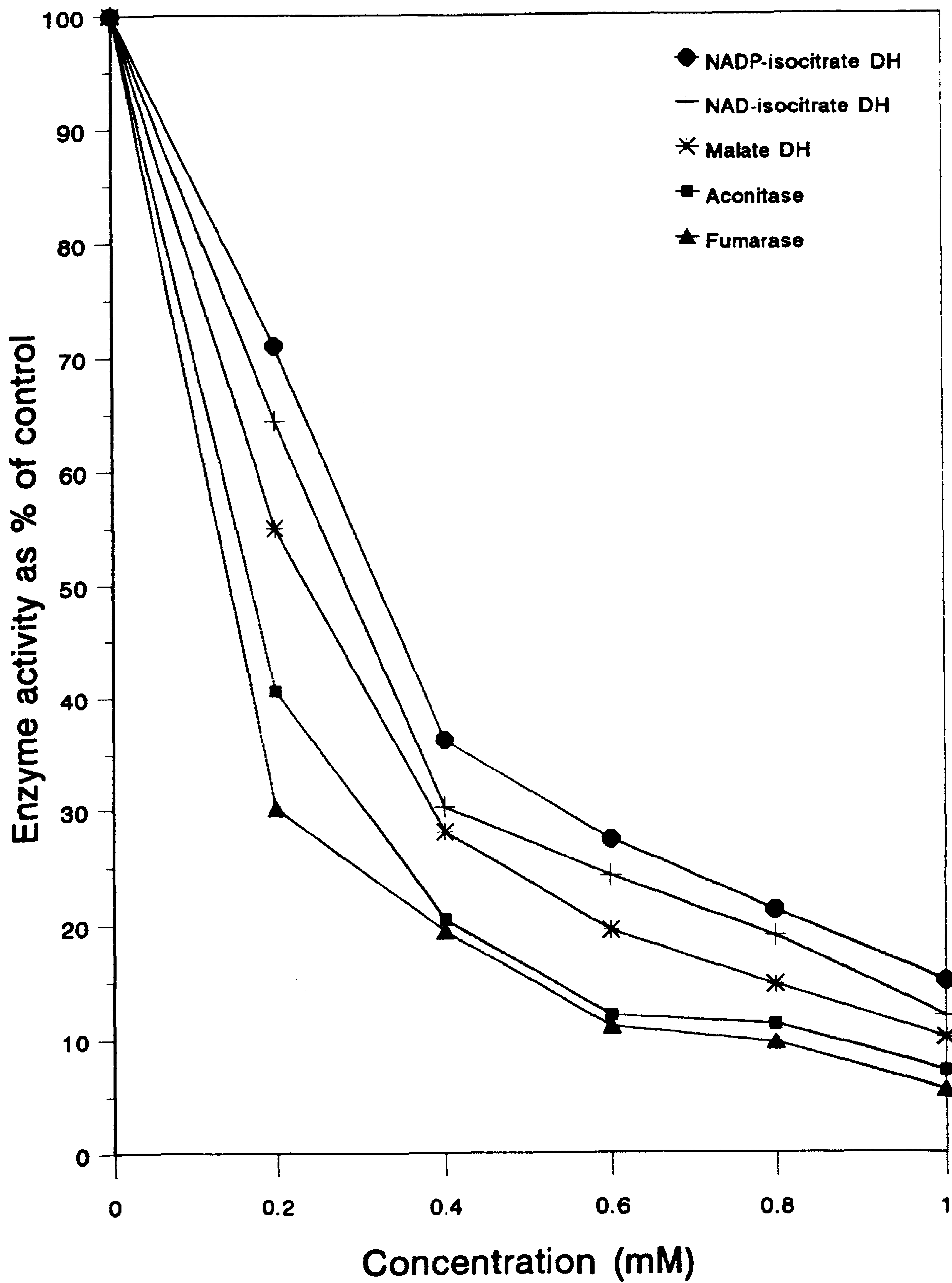
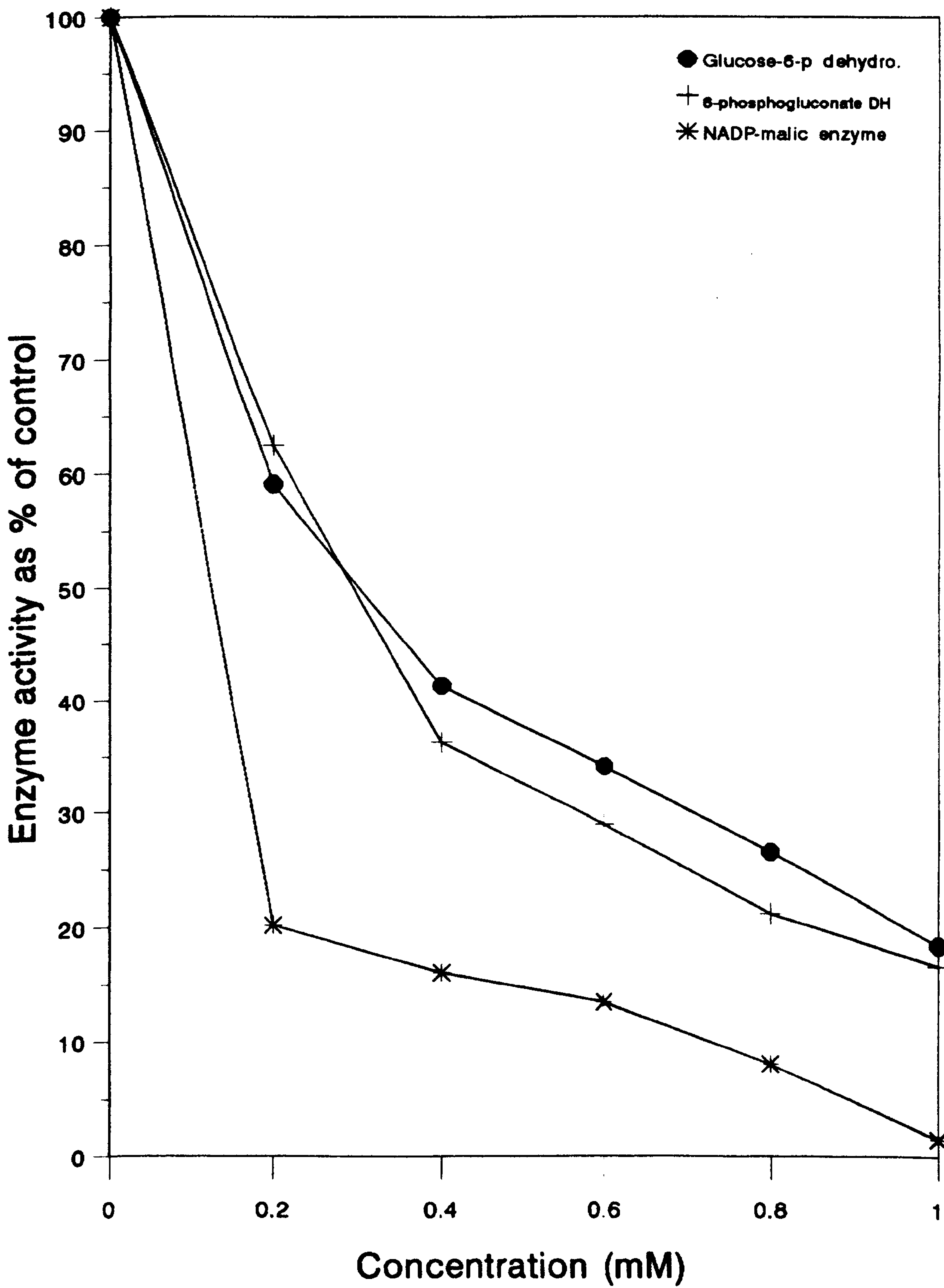
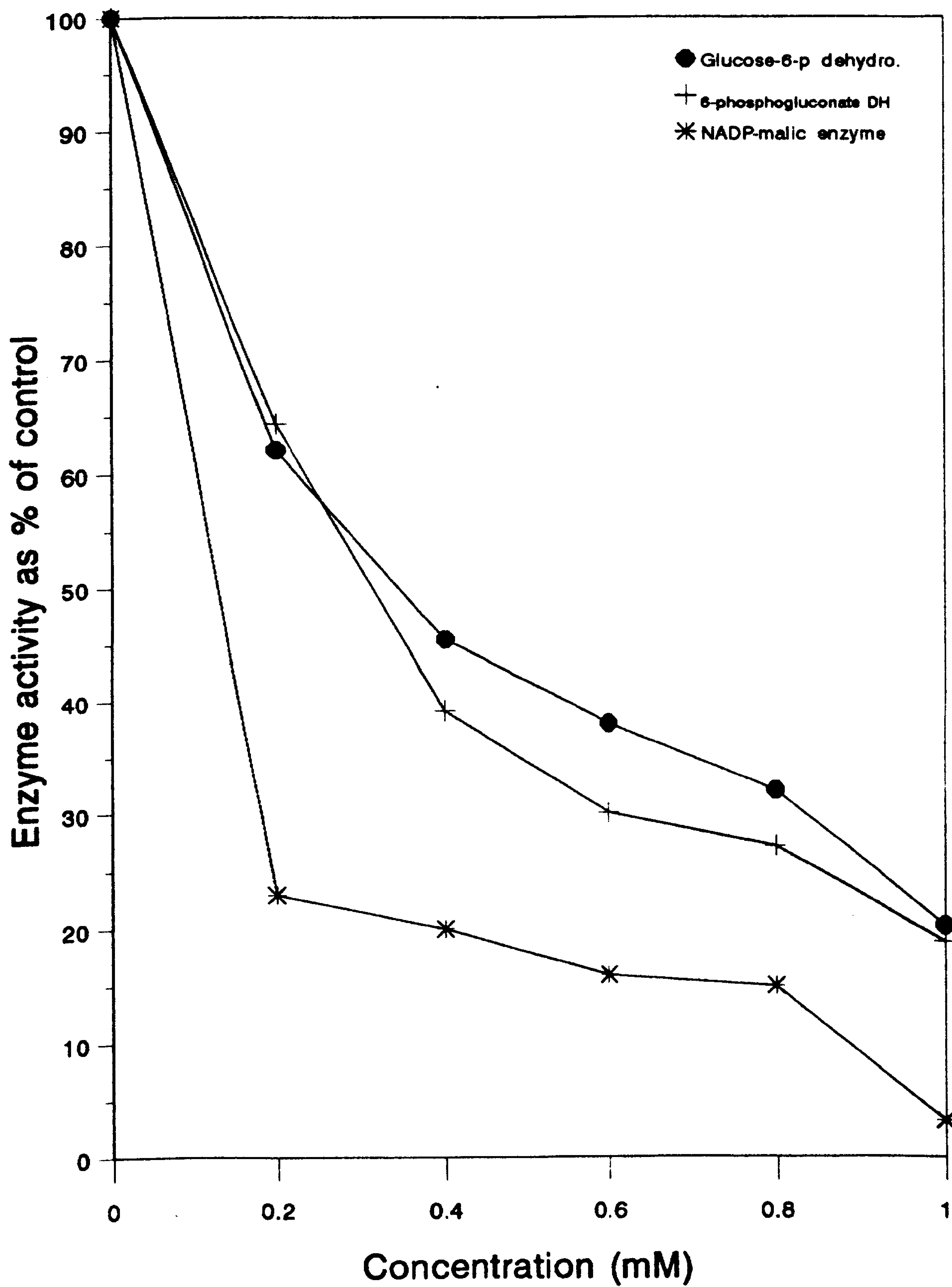

NADP-isocitrate DH
NAD-isocitrate DH
Malate DH
Aconitase
Fumarase
Enzyme activity as % of control
Concentration (mM)
0
10
20
30
40
50
60
70
80
90
100
0.2
0.4
0.6
0.8
1

Fig. 49: **Effect of various concentrations of phenol added to the growth medium of *A. parasiticus* on the level of the enzymes of pentose phosphate pathway (PPP) and NADP-malic enzyme after 10 days culture.**



Glucose-6-p dehydro.
6-phosphogluconate DH
NADP-malic enzyme
Enzyme activity as % of control
Concentration (mM)
0
10
20
30
40
50
60
70
80
90
100
0.2
0.4
0.6
0.8
1

Fig. 50: Effect of various concentrations of catechol added to the growth medium of *A. parasiticus* on the level of the enzymes of pentose phosphate pathway (PPP) and NADP-malic enzyme after 10 days culture.



Glucose-6-p dehydro.
6-phosphogluconate DH
NADP-malic enzyme
Enzyme activity as % of control
Concentration (mM)
100
90
80
70
60
50
40
30
20
10
0
0
0.2
0.4
0.6
0.8
1

CHAPTER VII

GENERAL CONCLUSIONS

GENERAL CONCLUSIONS

The objective of the present investigation aimed to evaluate the contamination levels of the fungal toxin "aflatoxin B_1" in some kinds of human food, and to find out possible ways to minimize such contamination of foods. The importance of the present results may be useful from the public health point of view, as the food samples which were tested during this study are commonly consumed by the inhabitants of Saudi Arabia and elsewhere. Generally, the main source of human exposure to aflatoxins is contaminated food. Two pathways of dietary exposure have been well known: (1) Direct ingestion of aflatoxins (mainly B_1) in contaminated food of plant origin such as maize, etc.; (2) Ingestion of aflatoxin contaminating milk and milk products including cheese and powder milk. According to the achieved results, the following main points may be concluded:

(1) Czapek's-Dox medium was found most suitable for the detection of any possible fungal contamination on the screened food samples.

(2) *Aspergillus parasiticus* was the most frequent fungal contaminant (31 isolates) followed by *A. flavus* (28 isolates) out of 40 examined samples.

(3) Aflatoxin B_1 presence is not necessarily associated with fungal growth.

(4) Oil-rich commodities gave the highest fungal growth as well as the maximum aflatoxin production, followed by carbohydrate-rich commodities regarding aflatoxin production. Spices were found contaminated with fungi but not with aflatoxins. These results indicate that the presence of aflatoxigenic moulds on a substrate does not necessarily mean the presence of aflatoxin.

(5) The occurrence of aflatoxins in the food commodities at levels greater than the recommended level of 20 μg Kg^{-1} of product may pose a potential public health risk to humans through direct and/or indirect consumption of these contaminated food products. The present work may be considered as the first attempt for surveying the fungal contamination and aflatoxins in foods and foodstuffs in Saudi Arabia, particularly at Abha region.

The variation of fungal species on different food materials is attributed to certain growth factors present in different nutritional materials. The effect of food materials seems to be confined to the fungal growth more than the production of aflatoxin B_1.

The variation of aflatoxin B_1 level seems to be due to concentration, presence and/or absence of certain ingredients in the foodstuff. These differences in aflatoxin level could be due to the concentration of carbohydrates and fatty acids in substrate which enhance toxin production.

(6) *Bacillus megaterium* was found most sensitive to the inhibitory action of aflatoxin B_1. Accordingly, it was considered a standard organism for assaying the potency of the toxin.

(7) Aflatoxin production are only formed when suitable pH conditions exist.

(8) The best environmental conditions (incubation) for the growth of *A. parasiticus* as well as its productivity of aflatoxin B_1 were found at 30°C for 10 days and the decrease of fungal biomass and toxin production after 10 days may be attributed to lysis of mycelial cells.

(9) The variations in the effect of different carbon sources on aflatoxin B_1 production could reflect variations in the mechanisms or relative rates of catabolism among the tested compounds.

(10) Ammonium salts seemed to be effective chemicals against contamination by aflatoxins. However, it can not be applied to human food but may be used in case of foodstuffs.

(11) Reduction of the moisture content of the susceptible crops below the level necessary for spore germination seems to be the most important factor for controlling the mould growth and aflatoxin production.

Thus, for a food to be contaminated with aflatoxins, several conditions must be met: (a) There must be an adequate carbon source, nitrogen source and metal ions (these supplied by food) and moisture; (b) There must be present the spore of a species of fungus that produces a toxic secondary metabolite; (c) conditions must allow for the fungus to enter its idiophase and allow secondary metabolism to occur. This latter condition may cover several conditions including nutrition, temperature, pH, length of growth, presence of inhibitory substance, etc. Once these conditions have been met then there may be aflatoxins present.

(12) Sodium benzoate, ammonium bicarbonate, sodium bicarbonate and hydrogen peroxide could control fungal growth and aflatoxin production. Aflatoxin B_1 was eliminated from the medium when treated with sodium benzoate or sodium bicarbonate which convert the toxin to a water soluble form. These simple and inexpensive methods for eliminating aflatoxins from the contaminated materials may be recommended. Charcoal adsorbed the toxin from the liquid media without affecting the fungal growth. The adsorption of aflatoxins on activated

charcoal seems to be a much milder and more practical method of decontaminating aflatoxins. If the results of these experiments are confirmed by more vigorous testing, adsorption of aflatoxins by activated charcoal may be found efficacious in: prevention of system adsorption of aflatoxins via the lungs in workers exposed to contaminated foodstuffs; prevention of systemic adsorption of ingested aflatoxins in man and domestic animals; treatment of individuals poisoned by aflatoxins; and decontamination of foodstuffs containing aflatoxins.

(13) The different tested spices expressed considerable inhibitory effects on both the fungal growth and aflatoxin production. This observation recommend the use of such spices as food preservatives, particularly black pepper, ciliated heath, cuminum, curcuma and cinnamon. The present work may be considered the first attempt for testing ciliated health and curcuma as antifungal and antitoxicogenic agents according to the available literature.

(14) The tested inhibitors, namely, glutathione, quinine, EDTA, sodium azide, sodium cyanide, indole acetic acid, 2,4-dichlorophenoxy acetic acid, phenol and catechol, reduced the growth and aflatoxin production, and the reduction rate was dependent on the type of the inhibitor and its concentration.

(15) Some fatty acids, particularly lauric acid, could be used as a food preservative since it depressed effectively the fungal growth and aflatoxin production and it should be stressed that the concentration used was higher than that needed to support growth.

(16) Vitamins, particularly C and D_2, suppressed both fungal growth and aflatoxin production, therefore, it is suggested to be added as food preservatives.

(17) Glycolytic enzymes and pyruvate-generating enzymes seems to be linked with aflatoxin B_1 production by breakdown of glucose for energy production leading to the accumulation of pyruvate, which is essential for aflatoxin biosynthesis. Also, pentose phosphate pathway enzymes may be providing NADPH for aflatoxin synthesis. TCA cycle enzyme activities were reduced after the 4th day of growth. These lead to the accumulation of acetyl-CoA which provide aflatoxin synthesis. The activity of the tested enzymes: the pyruvate-generating enzymes, glycolytic enzymes, pentose phosphate pathway enzymes, TCA cycle enzymes and NADP-malic enzyme, as well as the growth of *A. parasiticus* and its productivity of aflatoxin B_1 were reduced by growth with either catechol or phenol.

Fig. (51) shows the possible ways to control aflatoxin production in foods as proposed from the present work. The effects of food preservatives, spices, inhibitors, fatty acids and vitamins are summarized in Table (33).

For applying any decontaminating agent or aflatoxin inhibitor, the following should be considered: (1) destroy or inactivate aflatoxin; (2) not produce toxic or carcinogenic/mutagenic residues in the final products, or in food products obtained from animals decontaminated feed; (3) retain the nutritive value and acceptability of the product; (4) not significantly alter technological properties of the product; (5) render spores or mycelia incapable of further growth and toxin production; and (6) have governmental approvals.

Thus, there is, at present, a sufficient evidence to justify the implementation or strengthening of national aflatoxin control programmes, specially in the Third World countries. It is impractical to insist that staple foodstuffs be aflatoxin-free but the level

of aflatoxin contamination should be reduced gradually by programmes involving the following:

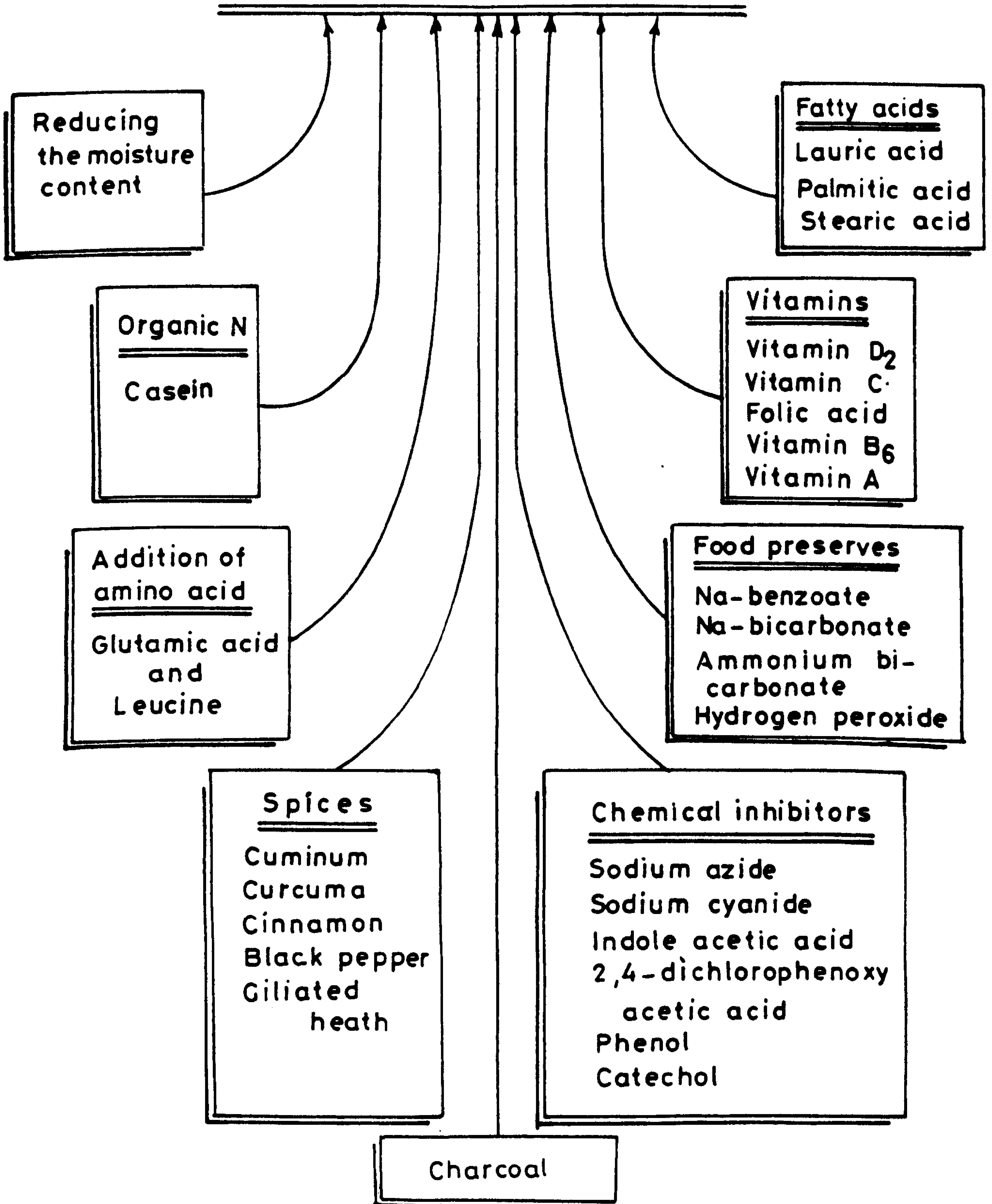
(A) Education of farmers to improve crop quality and consumers to store the foods by the proper way to avoid contamination.

(B) Surveillance of foodstuffs and animal feeds for the presence of aflatoxins particularly the exported one.

(C) Application of appropriate food-processing technology to separate contaminated from non-contaminated food elements.

(D) Statements of Governmental regulations concerning the standard levels of food contamination of aflatoxin for human and animal consumption, particularly for the developing countries.

Fig. 51: Possible ways to control aflatoxin production in foods as proposed from the present work.



Possible ways to control of Aflatoxin B1 production
Reducing the moisture content
Organic N
Casein
Addition of amino acid
Glutamic acid and Leucine
Spices
Cuminum
Curcuma
Cinnamon
Black pepper
Giliated heath
Charcoal
Chemical inhibitors
Sodium azide
Sodium cyanide
Indole acetic acid
2,4-dichlorophenoxy acetic acid
Phenol
Catechol
Food preserves
Na-benzoate
Na-bicarbonate
Ammonium bi-carbonate
Hydrogen peroxide
Vitamins
Vitamin D2
Vitamin C
Folic acid
Vitamin B6
Vitamin A
Fatty acids
Lauric acid
Palmitic acid
Stearic acid

Table 33: Comparison of the effects of the various treatments on both fungal growth and aflatoxin production.

Treatment	No effect on growth No effect on aflatoxin	Effect on growth No parallel effect on aflatoxin	No effect on growth Effect on aflatoxin	Effect on growth Effect on aflatoxin
Food preservative	NaCl		Charcoal	H_2O_2 C_6H_5COONa $NaHCO_3$ NH_4HCO_3
Spices	Red pepper		Coriander Black pepper Ciliated heath	Cardamon Ginger Cuminum Carcuma Cinnamon
Inhibitors				Glutathion Sodium azide Quinine Sodium cyanide IAA 2,4-dichlorophenoxy acetic acid EDTA Phenol Catechol
Fatty acids		Palmitic acid Stearic acid		Lauric acid Oleic acid (+)
Vitamins		Folic acid	Vit. A	Vit. C. Vit B_2 (+) Vit. B_6 Vit. D_2

(+) = Stimulating action.

CHAPTER VIII

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