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FUSARIUM AND *FUSARIUM* TOXINS IN MAIZE IN SOME REGIONS OF ETHIOPIA.

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A Thesis Submitted in (part) Fulfilment for the Degree of Master of Science in Biology in the Addis Ababa University

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by

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DEDICATED TO MY FATHER

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ABSTRACT

Maize grain samples were collected from stores and markets in Shashemene and Alemaya regions. Samples were rated as damaged, normal and malted. Normal and damaged samples were rated based on percentage of kernel discolouration, wrinkledness, floating of kernels in water and attack by insects.

Mycofloral study of the three sets of samples showed that the three toxigenic fungal genera: *Fusarium*, *Aspergillus* and *Penicillium* were common in maize in Ethiopia. Among these toxigenic fungi *Fusarium* was the most common genus, comprising 17.5 % of the total fungi isolated and was recovered from 80.5 % of the samples investigated.

Species of fusarium were most prevalent in damaged and malted samples than in normal samples. The fungus was represented in 91.7 % and 28 % of the total fungal isolates in damaged and malted samples respectively. In the normal samples, however, *Aspergillus* and *Penicillium* spp were more prevalent and they were represented in all the samples examined.

Preliminary test result of screening toxigenic fusarium isolates using the brine shrimp (*Artemia salina*) bioassay showed that 93 % of them were toxigenic. The frequently occurring toxigenic fusarium were identified to be *Fusarium proliferatum*, *Fusarium graminearum*, *Fusarium subglutinans* and *Fusarium anthophilum*.

Isolates of these frequent toxigenic species were grown on sterile shredded maize grain and their extracts were assayed for toxicity on the brine shrimp larvae. The results indicate production of methanol and/or chloroform soluble toxic metabolites. Bioassay directed isolation of toxic principles from chloroform extract of *F. graminearum* isolates showed production of zearalenone and trichothecene compounds (TA and TB). Based on their physico-chemical data four compounds that belong to the fumonisins were isolated from methanol extracts of *F. proliferatum* isolates.

The results of the study on screening of toxigenic fusarium and bioassay directed isolation and purification of toxic principles indicated the importance of brine shrimp bioassay in screening toxigenic fusarium and detection of fusarium toxins.

Toxicity assay results from extracts of selected grain samples of the three sets of samples indicated that 93 % of the tested sample extracts were toxic. In the natural occurrence study of the toxins, zearalenone and the trichothecenes (TA and TB) were detected in 50, 50, and 75 % of the damaged samples examined respectively. The estimated concentration ranges from 0-0.42 µg/g for zearalenone, 0-1.5 µg/g for TA and 0-2.3 µg/g for TB. The fumonisins, on the other hand, were detected in all the samples examined with the highest concentrations in the malted and damaged samples.

The results of this study showed that toxigenic fusarium are associated with maize samples and their toxins are found on the grain. The biological effect and thermostability of some of the toxins indicates the health hazard of consuming mould infected grains, particularly damaged and malted grains. This necessitates the quality control of maize grain destined for human consumption.

1 INTRODUCTION

Maize (*Zea mays* L.) is a new world graminaceous crop which is second to wheat in production among the world's cereal crops. However, among the developing countries maize ranks first in Latin America and Africa (Dowswell *et al.*, 1996). Because of its world wide distribution and relatively lower price, maize has a wider range of uses than any other cereal as food, animal feed and industrial crop (Dowswell *et al.*, 1996; FAO,1992; Singh, 1987).

In Africa maize production has surpassed all other crops. The land cultivated to maize has increased from 17 to 21 million hectares from 1981 to 1993 (FAO, 1993). Ethiopia is one of the important maize producing countries ranking third among Eastern African countries (CIMMIT, 1992).

Maize has been known to Ethiopia for the last 500 yrs (Mulatu *et al.*, 1993). It grows in all parts of the country from sea level to over 2400 m above sea level, but the major producing zones lie between 1000 m and 2000 m (Aregai, 1989). In Ethiopia maize is exceeded only by tef and sorghum and is first in yield per unit area (Mekonen, 1989; Mulatu *et al.*, 1993). It is a staple food crop in some parts of Ethiopia (Mulatu *et al.*, 1993). According to CIMMIT (1992), percapita total maize consumption is 38 Kg/yr (1982-90). The growth rate of percapita maize consumption has increased from 1.3 %/yr in (1970-80) to 2.2 %/yr in (1981-90). In Ethiopia maize is harvested manually and shelled with sticks, mortar and pestle or cattle and stored in various ways traditionally. In Southern and Western maize producing regions including Shashmene, Awasa and Bako area, maize is stored in above ground storage barns made of interwoven sticks or bamboo placed inside the house or out side the house with a grass roof.

Shelled maize is often stored out side the house in 'gotera' or in side the house in 'Dibignet', brocken clay pots, sacks or 'silicha'. In Eastern part of the country storage in under ground pits is a common form of traditional storage system.

Almost all maize produced is directly used for human consumption as food or for preparing local drinks (Mulatu *et al.*, 1993).It is consumed as bread, porridge, 'injera' and 'nifro'. Maize is also used in the preparation of local drinks 'tela', 'areke' or spirit and 'borde'.

Fungi are one of the most important agents for deterioration of maize seeds. Spoilage resulting from fungal invasion and subsequent mycotoxin accumulation are of great concern (Samson, 1992; Wheeler *et al.,* 1992). Several species of *Aspergillus*, *Penicillium* and *Fusarium* are important producers of mycotoxins (Blaney, 1992; Miller, 1992; Samson, 1992).

Members of the genus *Fusarium* and the toxins elaborated by them are highly associated with maize (Logrieco & Bottalico, 1988; Luo *et al.*, 1990; Marasas *et al.*, 1981; Miller, 1992; Sydenham *et al.*, 1990a, b, 1991). It has been proved that ingestion of food & feed contaminated with fusarium toxins results in food and feed borne intoxication in man and farm animals (Miller, 1992).

Natural occurrence of fusarium toxins in maize and maize based products have been reported (Hopmans & Murphy, 1993; Sydenham *et al.,* 1990a, b, 1991). The wide spread infection of maize and maize products destined for human consumption raises series concerns for human health.

Trichothecene fusarium toxins, for instance, have been responsible for a number of large scale human toxicoses in modern times. In china, trichothecenes are suggested to have a role in human oesophageal carcinogenesis (Luo *et al.*, 1990). Zearalenone has been demonstrated to have oestrogenic syndrome in pigs and other animals (Kruger, 1989). Fumonisins have been reported to be hepatotoxic and hepatocarcinogenic in rats (Miller, 1992). Fumonisins are also associated with epidemiology of oesophageal carcinoma in humans in South Africa and China (Moss, 1996).

In Ethiopia, *Fusarium* was reported to be the most prevalent fungi in maize (Abate, 1982; Gilman, 1968). However, there is very little awareness of the problem of fusarium toxins. Furthermore, monitoring and prevention of mycotoxin contamination of maize grain and its products requires basic knowledge of the distibution and identification of the frequently occurring associated fungal flora and their potential to produce mycotoxins. Such information allow to set recommendations on how to protect fungal contamination of maize grain and get safe maize grain for consumption.

Thus, this study was designed to fulfil the following objectives:

1. To determine post-harvest mycoflora associated with maize grain samples and to isolate and identify *Fusarium* spp.
2. To study the mycotoxin producing ability of the common fusarium isolates.
3. To assay and characterise the common fusarium toxins in maize and to discuss on the potential hazard of consumption of toxin contaminated maize and its food derivatives.

2 LITERATURE REVIEW

2.1 Mycoflora Associated with Maize Grain

Fungi are the most important agents of seed deterioration. Many fungi are parasites of seed and they reduce yield of seed both qualitatively and quantitatively (Christensen & Kaufman, 1969; Neergaard, 1977). In general fungi associated with maize grain are classified into field or storage fungi on the basis of their ecological requirements (Christensen and Kaufman, 1969).

2.1.1 Field Fungi

Field fungi are those which invade kernels before harvest while the plants are growing in the field or after the grain is harvested but before it is threshed. If maize is stored in cobs in cribs being exposed to the weather, there is a possibility of invasion of maize kernels by field fungi.

In Ethiopia maize could be infected by field fungi during harvesting and storage. Temporary storage of maize with the husk on the ground immediately after harvest, storage of unshelled dehusked maize cob in above ground stores in and out side of a house and threshing activities might facilitate infection and further growth of field fungi.

The major field fungi that invade kernels are *Alternaria*, *Cladosporium*, *Helminthosporium* and *Fusarium*. The predominant field fungi may differ according to the crop, the geographic location and weather (Christensen and Kaufman, 1969; Neergaard, 1977). *Fusarium* is a common field fungus frequently isolated from maize grain world wide (Abate, 1982; Abbas *et*

al., 1988a, b; Blaney *et al.*, 1986; Gilman, 1968; Marasas *et al.*, 1979, 1981).

Field fungi require a moisture content of 24 to 25 percent on a wet weight basis. However, they may live for years in grain stored at low moisture content, their survival being favoured by low moisture content and low temperature (Christensen & Kaufman, 1969). Abbas *et al.* (1986) isolated *Fusarium graminearum* and *Fusarium moniliforme* from maize samples stored at -8° C and -4° C for 13 years.

2.1.2 Storage Fungi

Storage fungi are those which grow on stored grains and seeds. Storage fungi are mainly species of *Aspergillus* and *Penicillium*, which are active at low moisture content of grains. They invade seeds during harvesting, threshing, and storage (Christensen & Kaufman, 1969; Neergaard, 1977).

Ethiopian farmers store dried and shelled maize grain in traditional storage systems at least until the next harvest. The reduced grain moisture content of dried maize grain and the traditional storage conditions could facilitate dominance of storage fungi over the field fungi. Storage fungi are frequently isolated from maize kernels (Abate, 1982; Blaney *et al.*, 1986; Marasas *et al.*, 1979).

2.1.3 Mould Damaged Maize Grain

Fungi in maize grain causes deterioration of grain quality. Mould damaged kernels generally exhibit seed discoloration, wrinkldness, decrease in germination percentage, damaged embryo, loss of weight, loss of nutritive value, poor millmg quality, unpleasant flavours. Moreover, fungi in grain produces toxic compounds called mycotoxins.

Fusarium spp causes seed rot in cereals resulting in discoloration of seeds. For instance *Fusarium moniliforme* causes pink discoloration in maize seeds while *Fusarium graminearum* produces purple discoloration (Blaney *et al.*, 1986; Neergaard, 1977). However, *Fusarium* spp have little influence on germination of seeds (Rheeder *et al.*, 1990)

Aspergillus and *Penicillium* spp causes discoloration, shrinkage of seeds and decreased germination. Maize kernels artificially inoculated with *Aspergillus candidus* and stored at 18% moisture content at 25° C develop a dark brown discoloration at the germ ends after 4.5 months while non-inoculated seeds appear normal with out loss in germination (Qasem & Christensen, 1958).

2.2 The Genus *Fusarium*

Toxigenic species can be found in all major taxonomic groups of fungi. However, the three genera *Aspergillus*, *Penicillium* and *Fusarium* are considered to be the most significant toxigenic moulds associated with grains, foods and feed (Betina, 1984a; Smith & Moss, 1985).

Fusarium is a form genus in the class Hyphomycetes subdivision Deuteromycotina and Division Eumycota. The Genus is characterised by the production of multiseptate hyaline macroconidia with a foot-shaped basal cell, that may not be distinct for some species. Several teleomorph genera have been associated with the anamorph genus *Fusarium*. The teleomorph stage, if formed, is in order Hypocreales, class pyrenomycetes, subdivision Ascomycotina (Nelson *et al.*, 1983; Webster, 1980). The genera *Nectaria*, *Calonectaria*, *Giberrella*, *Plectosphaerella* and *Monographella* are recognised as perfect states of the form genus *Fusarium* (Nelson *et al.*, 1983).

There is usually little difficulty in recognising a mould as belonging to the genus *Fusarium*, if it has produced macroconidia. It is often much more difficult to identify to species level because of the variability between isolates and because not all features, particularly the macroconidia and chlamydospores, develop fully (Nelson *et al.*, 1983; Windels, 1992).

The taxonomic situation in the form genus *Fusarium* is still in a state of flux and reliable identification is difficult. In the genus 9 to over 90 species and varieties are recognised depending up on the taxonomic system used. Nelson *et al.* (1983), for instance, divide the genus in to 12 sections and 30 species.

The most important characters in the identification of *Fusarium* species are: shape and size of macroconidia, production of microconidia and terminal or intercalary chlamydospores, the extent of growth and pigment production on a defined medium (Nelson *et al.*, 1983; Smith & Moss, 1985).

Most species of *Fusarium* are soil fungi with cosmopolitan distribution and are active in decomposing cellulose rich substrates (Domsch *et al.*, 1980). Some species are plant parasites causing root and stem rot, vascular wilt, fruit rot and ear rot (Domsh *et al.*, 1980; Nelson *et al.*, 1983; Walker, 1969; Wheeler, 1969; Windels 1992).

Certain species of fusarium are also associated with stalk rot, ear rot and kernel rot of maize (Kommendahl *et al.*, 1987; Marasas *et al.*, 1979). In Ethiopia *Gibberella fugikuroi* (= *F. moniliforme*) is reported to cause pink ear rot and *Gibberella zeae* (= *F. graminearum*) to cause red ear rot and stalk rot (Woldekidan, 1985). Almost all species are adapted to high moisture content as occurring in living plants. Grain crops continue to support growth in storage if they are not dried (Blaney, 1992; Mirocha & Christensen, 1974). However, some *Fusarium* species provide a notable exception being generally prevalent not only in growing maize plant but also occurring widely in the seed (Marasas *et al.*, 1979) and persisting during storage at relatively low moisture contents of storage seeds (Russel *et al.*, 1982; Russel & Berjack, 1983).

Most fusarium species are capable of producing mycotoxins. However, some are more important in terms of human and animal disease, because of their prevalence in the edible portion of crops and their capacity for production of high concentration of highly toxic mycotoxins (Blaney, 1992; Scott, 1991; Smith & Moss, 1985).

2.3 Mycotoxin Contamination of Maize Grain

Maize can support fungal growth while still in the field, during storage and after processing into food products or animal feed. *Fusarium* attacks seedlings from infested seeds or directly

from soil. Infection of seed can occur during ripening period, harvesting and threshing processes and could continue during storage (Kruger, 1989). *Aspergillus* and *Penicillium*, on the other hand, contaminates maize grain during post harvest activities. Contamination of maize grain by toxigenic fungi results in the production and accumulation of mycotoxins under favourable conditions.

Aflatoxins are carcinogenic compounds produced by *Aspergillus flavus* and *Aspergillus parasiticus*. They have got a series human health implications and their effect relates to liver damage. There have been several reports on a definite association of liver cancer in man and ingestion of aflatoxin contaminated groundnuts and cereals (Bhat, 1992).

Penicillium toxins citrinin and ochratoxin A have been suggested as the cause of porcine nephropathy. The nephropathy produced in swine by these toxins shows structural and functional similarities to the fatal human renal disorder (Betina, 1984 b). Fusarium toxins, which belong to different groups of chemical compounds, have been implicated to be causal agents of different diseases of man and farm animals.

2.3.1 Toxigenic Fusarium Species

About 24 toxigenic species of fusarium are known (Miller, 1992) and on the basis of their prevalence and toxicity, the most important once were regarded as: *F. graminearum*, *F. moniliforme*, *F. equiseti*, *F. poae* and *F. sporotrichioides* (Blaney, 1992).

Toxigenic fusarium species and the toxins elaborated by them are known to be common in maize (Blaney *et al.*, 1992). Natural occurrence of fusarium toxins in food and feed materials in different parts of the world have been reported (Gelderblom *et al.*, 1984b; Mirocha *et al.*, 1976; Ramakrishna *et al.*, 1990; Sydenham *et al.*, 1990a, b; Thiel *et al.*, 1982).

The ability of most toxigenic fusaria to produce more than one toxin and the co-existence of more than one toxigenic fusarium in a given sample results in co-occurrence of several fusarium toxins (Miller, 1992; Jelink *et al.*, 1989; Sydenham *et al.*, 1990a, b) in a sample.

Fusarium toxins also occur together with toxic metabolites from other genera. The co-occurrence of aflatoxins with fusarium toxins have been reported (Hagler *et al.*, 1984; Miller, 1992; Ramakrishna *et al.*, 1990).

2.3.2 Fusarium Toxins, their Chemical Structure and Biological Effects

The principal naturally occurring fusarium toxins commonly reported elsewhere are: the trichothecenes (deoxynivalenol and nivalenol), zearalenone (Patey and Gilbert, 1989; Tanaka *et al.*, 1988) fumonisins (Sydenham *et al.*, 1990a, b) and the less important fusarium toxins, moniliformin & fusarin C. The chemical structure and biological effects of these toxins are discussed below.

2.3.2.1 Trichothecenes

Chemical Structure

Trichothecenes are sesquiterpene compounds named after the fungus *Trichothecium roseum,*

from which the first trichothecene was isolated. More than 50 derivatives are known that are characterised by the 12,13-epoxy-trichothec-9-ene ring system (Fig. 1). The epoxide at C-12 and C-13 is known to be essential for the toxicity of this group of mycotoxins (Desjardins, *et al.*, 1993).

Trichothecene fusarium toxins are grouped into two groups mainly by the presence or absence of a carbonyl functional group at C-8 position. Type A trichothecenes are those without a carbonyl functional group and type B are those with a carbonyl functional group. Acetylation or methylation of hydroxy groups produces many related trichothecenes (Blaney, 1992; Cole and Cox, 1981).

Producing Organisms

Trichothecenes are known to be produced by a number of toxigenic fusarium species (Table 1). The trichothecene producing strains are often capable of producing more than one toxin, though, there is a variation between isolates of the same species in the production of trichothecene compounds (Ichinoe *et al.*, 1983; Mirocha *et al.*, 1989; Sugiura *et al.*, 1990).

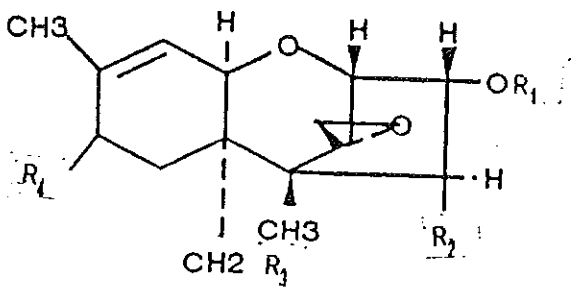


Fig. 1. Basic Trichothecene Ring.

Table 1. Chemical structure, formula and list of producing organisms of some trichothecene compounds.

Trichothecene compound	Chemical formula	Structure				Producing organisms	References
		R_1	R_2	R_3	R_4		
Deoxynivalenol	$C_{15}H_{20}O_6$	OH	H	OH	=O	*F. graminearum* *F. roseum*	Mirocha *et al.*, 1989 Vesonder *et al.*, 1982
3-acetylDON	$C_{17}H_{22}O_6$	OAC	H	H	=O	*F. graminearum*	Mirocha *et al.*, 1989
15-acetylDON	$C_{17}H_{22}O_6$	OH	H	OAC	=O	*F. graminearum*	Mirocha *et al.*, 1989
Nivalenol	$C_{15}H_{20}O_7$	OH	OH	OH	=O	*F. nivale* *F. Crookwellense*	Cole and Cox, 1983 Golinski *et al.*, 1988
Fusarenone-X	$C_{17}H_{22}O_6$	OH	OAC	OH	=O	*F. graminearum* *F. equiseti* *F. avenacium* *F. nivale* *F. solani* *F. laterium* *F. crookwellense*	Cole and Cox, 1983 Golinski *et al.*, 1988
HT-2	$C_{22}H_{32}O_8$	OH	OH	OAC	X	*F. culmorum* *F. poae* *F. sporotrichioides*	Cole and Cox, 1983
T-2	$C_{22}H_{32}O_8$	OH	OAC	OAC	X	*F. tricinctum* *F. culmorum* *F. solani* *F. poae* *F. sporotrichioides*	Cole and Cox, 1983
T-2 tetraol	$C_{15}H_{22}O_6$	OH	OH	OH	OH	*F. poae* *F. sporotrichioides*	Cole and Cox, 1983

X = $OCOCH_2CH(CH_3)_2$

Biological Effect

Trichothecenes are not host-specific in their toxicity and are known to inhibit protein synthesis in a wide range of eukaryotic organisms (Cole & Cox, 1981; Desjardins *et al.*, 1993). For example, trichothecenes HT-2, T-2 and nivalenol inhibit the initiation step of protein synthesis in polyribosomes (Cole & Cox, 1981).

They are also potent phytotoxins and produce wilting, chlorosis, necrosis and other symptoms in a wide variety of plants (Desjardins, *et al.*, 1993; Hergeson *et al.*, 1973; Marasas *et al.*, 1971). Trichothecenes T-2 toxin and deoxynivalenol, inhibit protein synthesis in maize leaf disks and kernel sections (Casale & Hart, 1988).

A wide range of toxicity in vertebrate animals was also reported, though, the toxicological potency varies strongly among individual toxins. Some of the symptoms reported to be associated with trichothecene toxicosis include: refusal of feed stuffs; inflammation of gastrointestinal tract and possible haemorrhaging in the gastrointestinal tract; oedema and diarrhoea; degeneration of bone marrow; reduced egg production; thin egg shell; death and abortion (Cole and Cox, 1981; Kruger, 1989).

Deoxynivalenol (DON) and nivalenol (NIV) are the most common naturally occurring trichothecenes (Patey and Gilbert, 1989). The swine refusal factor vomitoxin (DON) is much less toxic and causes decreased feed intake and weight gain in pigs at >2 mg/Kg feed; vomiting and feed refusal at very high concentrations (>20 mg/Kg diet) (Trenholm *et al.*, 1992). LD_{50} value of DON in male mice dosed IP was 70 mg/Kg; females, 76.7 mg/Kg (Cole and Cox, 1981). DON is also known to exhibit immunosuppressive activity (Moss, 1996).

The acute toxicity of NIV is several times higher than that of DON. Long-term feeding of NIV results in malnutrition in mice. LD_{50} in mice dosed IP was 50 mg/10gm (injures proliferating cells). Minimum SC dosage to induce vomiting in ducklings was 1.0 mg/Kg. Dermal toxicity in rabbit and guinea pig (Cole and Cox, 1981).

Other trichothecenes, T-2, HT-2 and diacetoxyscerpenol (DAS), are much more toxic than DON. T-2 toxin causes diarrhoea and due to its emetic and feed refusal actions pigs refuse to eat diets containing more than 16 mg T-2 toxin/Kg. In pigs DAS is more toxic than T-2 toxin, pigs fed 2 to 10 mg DAS/Kg diet in its purified form exhibit reduced feed in take and weight gain and complete feed refusal occurred at the 10 mg/Kg level (Trenholm *et al.*, 1992).

Prolonged feeding of mouldy maize contaminated with T-2 toxin at a concentration of 2 mg/Kg resulted in death of 7 out of 35 cows. The animals at necropsy had extensive haemorrhage on the mucosal surfaces of all viscera. Highest frequency of abortion was also seen in the herd (FAO, 1982). In poultry T-2 toxicoses causes reduced feed consumption and growth in broiler chicks and reduced egg production in laying hens (Trenholm *et al.*, 1992).

2.3.2.2 Zearalenone

Chemical Structure

One of the commonest naturally occurring fusarium toxin, zearalenone (also known as F-2), is a phenolic macrolide (Fig.2) first isolated and identified from cultures of *Fusarium roseum* "*graminearum*" (*Gibberella zeae*) (FAO, 1982; Stob *et al.*, 1962).

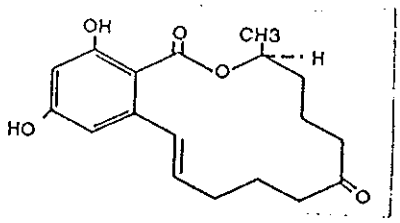


Fig. 2. Chemical Structure of Zearalenone.

Producing Organisms

Zearalenone has been reported to be produced by *F. graminearum*, *F. roseum*, *F. nivale*, *F. tricinctum*, *F. sportrichioides*, *F. oxysporum*, and *F. moniliforme* (Betina, 1984c; Mirocha & Christensen, 1974). *F.sambucinum*, *F. culmorum* (Scott, 1991), *F. crookwellense* (Golinski *et al.*, 1988).

Biological Effect

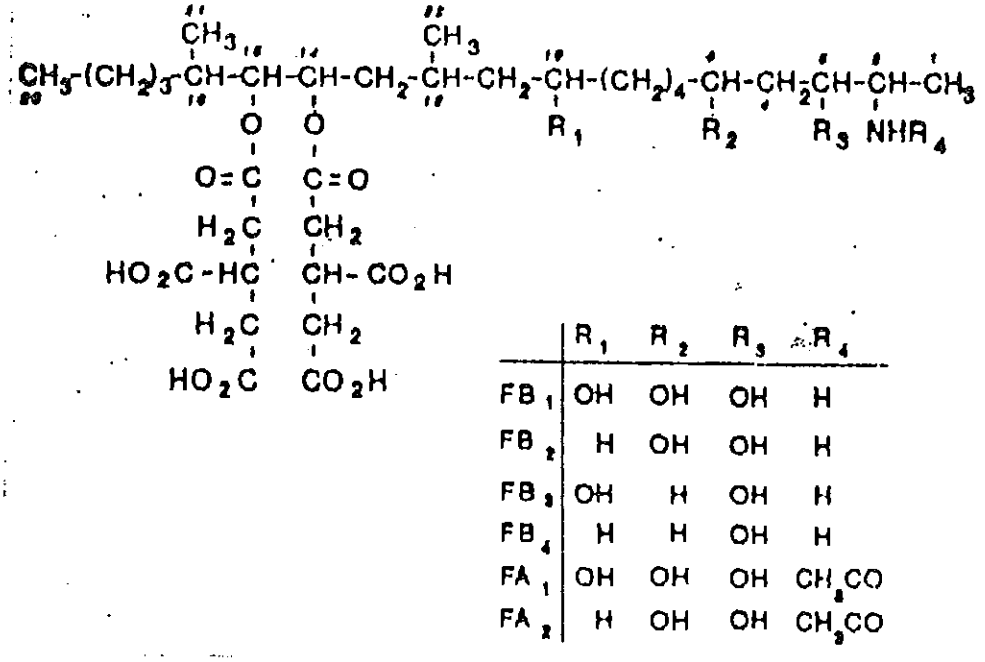
Zearalenone is known to cause a rather specific and sometimes serious oestrogenic syndrome in pigs and other animals, infertility and young males may develop feminisation. The oestrogenic effect involves enlargement of the uteri and mammary glands, vulvar swelling and weight increase, ovaries form cysts, testicular atrophy and vaginal prolapse in some animals, and low weight of pigs (Cole & Cox, 1981; FAO, 1982; Kruger, 1989).

2.3.2.3 Fumonisins

Fumonisins are a group of structurally related mycotoxins relatively recently isolated from maize culture of *F. moniliforme* strain MRC 826 (Cawood *et al.*, 1991; Gelderblom *et al.*, 1988) obtained from maize in Transkei, South Africa (Marasas *et al.*, 1988).

Six structurally related fumonisin compounds (FB_1, FB_2, FB_3, FB_4, FA_1 & FA_2) have been purified and structurally elucidated (Fig. 3). The A series of fumonisins are amides, acetylated on the amine group at C-2, while the B series of fumonisins have a free primary amine at C-2. Fumonisins A_1 and A_2 are N-acetyl derivatives of FB_1 and FB_2 respectively (Bezuidenhout *et al.*, 1988; Cawood *et al.*, 1991; Gelderblom *et al.*, 1992).

Of the six metabolites, fumonisins B_1, B_2, and B_3 are the major compounds usually present in both culture and naturally contaminated maize (Cawood *et al.*, 1991; Hopmans & Murphy, 1993; Visconti & Doko, 1994).



	R_1	R_2	R_3	R_4
FB_1	OH	OH	OH	H
FB_2	H	OH	OH	H
FB_3	OH	H	OH	H
FB_4	H	H	OH	H
FA_1	OH	OH	OH	CH_3CO
FA_2	H	OH	OH	CH_3CO

Fig. 3. Chemical Structures of the Fumonisin Compounds

Producing Organisms

To date strains of *F. moniliforme* have been shown to produce fumonisin mycotoxins (Bezuidenhout, *et al.*, 1988; Nelson *et al.*, 1991; Ross *et al.*, 1990; Thiel *et al.*, 1991). Fumonisins are also produced by strains of *F. proliferatum* (Nelson *et al.*, 1992; Ross *et al.*, 1990;, Thiel *et al.*, 1991), *F. anthophilum*, *F. dlamini*, *F. napiforme* (Nelson *et al.* 1992) and *F. nygami* (Nelson *et al.*, 1992; Thiel *et al.*, 1991).

Biological Effect

Toxicological investigations of *F. moniliforme* cultures or naturally infected maize revealed that fumonisins, particularly fumonisin B's to be causal agents of several animal disease (Kellerman *et al.*, 1990; Kriek *et al.*, 1981; Marasas *et al.*, 1988; Ross *et al.*, 1991; Thiel *et al.*, 1991). Fumonisin B_1 (FB_1), which is the major fumonisin present in both culture and naturally contaminated samples (Sydenham *et al.*, 1990a, b; Thiel *et al.*, 1991), has been shown to have cancer promoting activity in rats (Gelderblom *et al.*, 1988) and be responsible for the major toxicological effects of the fungus in rats, horses and pigs (Kriek *et al.*, 1981; Gelderblom *et al.*, 1992).

The neurotic disease Equine Leukoencephalomalacia (LEM) has been reproduced in horses by both intravenous (IV) and per oral (OS) administration of FB_1 (Marasas *et al.*, 1988; Kellerman *et al.*, 1990), while IV administration of FB_1 reproduced pulmonary edema syndrome in pigs (Gelderblom *et al.*, 1992). A study of the toxicological effects of FB_1 fed to rats at a dietary concentration of 50 mg/g over a period of 26 months showed that the liver was the main target organ in the FB_1-treated rats and FB_1 was responsible for the hepatotoxic and carcinogenic effects of rats (Miller, 1992).

Short term carcinogenesis studies in a rat liver bioassay indicated that over a period of 15 to 20 days at dietary levels of 0.05 - 0.1 %, FB_2 and FB_3 closely mimic the toxicological and cancer initiating activity of FB_1. In contrast, under identical experimental conditions no biological activity was detected for FA_1 (Gelderblom *et al.*, 1992).Fumonisin B_1 also exhibited phytotoxicity on the growth and development of corn callus (van Ash *et al.*, 1990, 1992) and formed necrotic lesions on tomato leaves (Mirocha *et al.*, 1990).

2.3.2.4 Less Important Fusarium Toxins

Fusarin C

Chemical Structure and Biological Effects

Fusarin C is a polyketide that was first isolated from extracts of a North American strains of *Fusarium moniliforme.* The structures of fusarin C and of two related non-mutagenic metabolites fusarins A and D (Gelderblom *et al.*, 1984a; Gaddamidi *et al.*, 1985) are shown in Fig. 4. The biosynthetic pathway was also partially worked out (Steyn & Vleggaar, 1985; Jackson *et al.*, 1989).

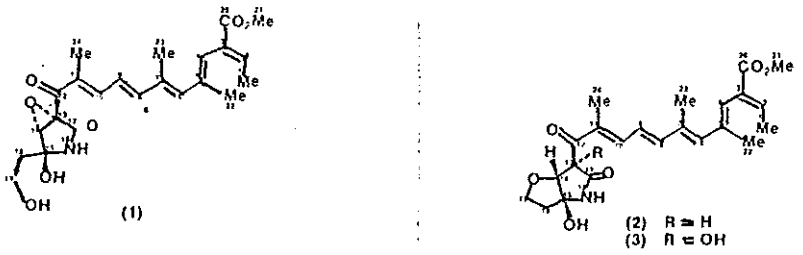


Fig. 4. Chemical Structure of Fusarin A(1) C(2) and D(3).

Fusarin C is a mutagenic metabolite that exhibit mutagenicity to *Salmonella typhimurium* in the Ames test. It's mutagenic activity has been shown to be comparable with that of aflatoxin B_1, and sterigmatocystin (Gelderblom *et al.*, 1984b).

Producing Organisms

Fusarin C is produced by *F. moniliforme*, *F. graminearum* (Farber & Sanders, 1986; Gaddamidi *et al.*, 1985; Gelderblom *et al.*, 1984 a, b) *F. avenaceum*, *F. culmorum*, *F. poae*, *F. sambucinum*, *F. sporotrichioides* (Farber & Sanders, 1986) and *F. crookwellense* (Golinski *et al.*, 1988).

Moniliformin

Chemical Structure and Biological Effects

The mycotoxin moniliformin, sodium or Potassium salt of 1 - hydroxycyclobut-1-ene-3, 4-dione (Fig. 5), is a water soluble toxin first purified by Cole *et al.* (1973) from *F. moniliforme* isolated from maize kernels in U S A.

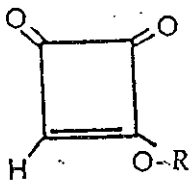


Fig. 5. Chemical Structure of Moniliformin, R = Na or K.

Moniliformin is a highly toxic compound with a profound effect in both plants and animals. It has an oral medium lethal dose of 40 mg/kg in 1 day old cockerels. This toxin also produce plant growth regulating and phytotoxic effects on plant systems (Cole *et al.*, 1973). The 50% lethal dose for chicken embryo was 2.8 mg per egg. For 1 day old chicks dosed with moniliformin by crop intubation and for female and male mice injected intraperitonially, the 50% lethal doses were 5.4, 20.9 and 29.1mg per kg of body weight respectively (Burmeister *et al.*, 1979).

Producing Organisms

Moniliformin has also been reported to be produced by *F. proliferatum* (Logreico & Bottalico, 1988; Marasas *et al.*, 1988;), *F. anthophilum* (Marasas *et al.*, 1986), *F. moniliforme var subglutinans* (Marasas *et al.*, 1979, 1986; Rabie *et al.*, 1982; Thiel *et al.*, 1982), *F. fusarioides* (Rabie *et al.*, 1978, 1982), *F. avenaceum, F. oxysporum, F. acuminatum, F. concolor, F. equiseti* and *F. semitectum* (Rabie *et al.*, 1982). However, the species differ with respect to the percentage of toxic isolates that produces moniliformin and also in the relative amounts of the toxin produced (Logrieco & Bottalico, 1988; Marasas *et al.*, 1986; Rabie *et al.*, 1982).

2.3.3 Methods of Detection

Mycotoxins from various agricultural commodities have been discovered through chemical screening routes and biological assay methods. A variety of chemical screening and bioassay methods have been developed to study the mycotoxins of toxigenic fusaria as well.

2.3.3.1 Chemical Screening

Chemical screenings have been used extensively for isolation and identification of mycotoxins directly from a suspected commodity or fungal culture. In chemical screening different chromatographic methods are used. The most widely and routinely used are Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC).

TLC is very specific and readily adapted to screening large number of samples but it lacks sensitivity. HPLC has become increasingly used for the analysis of mycotoxins because it offers increased sensitivity and improved accuracy over TLC methods.

Gas Liquid Chromatographic (GLC) methods are very specific and are the most sensitive methods available. Since most mycotoxins are non-volatile this method of analysis has not been widely used. For the trichothecene mycotoxins which do not lend themselves easily to TLC and HPLC techniques, however, GLC combined with mass spectrometry has been a most effective method for identification and quantitation (Gorst & steyn, 1984; Smith & Moss, 1985).

Structural elucidation of novel toxic compounds is carried out using mass and NMR spectrometric methods. Mass spectrometric analysis gives the molecular weight and nature of fragments of the chemical compound. NMR spectrometric data are of two types, proton-NMR and ^{13}C-NMR. From proton-NMR data, integration tells the number of protons and the chemical shift about the nature of the protons. ^{13}C-NMR data shows the number of carbon atoms, and their chemical shift indicates the nature of the carbon atoms (Silverstein *et al.*, 1981). Thus, mass and NMR spectrometric analysis allows identification and structure

elucidation of purified mycotoxins as other secondary metabolites.

2.3.3.2 Biological Assays

Chemical screening methods are limited to known mycotoxins where authentic standards and analytical methodology are available. Thus, unless previous information points to a particular mycotoxin(s), the identification of toxins from toxigenic fungi can be facilitated by utilising an appropriate bioassay system.

Several methods have been developed for bioassay of fusarium toxins. Isolation of a variety of fusarium toxins are monitored using cultured tumour cells or cultured normal human and mouse fibroblasts (Abbas *et al.*, 1984) and chick embryo bioassay methods (Sonson *et al.*, 1989). Various vertebrate animals such as rats, horses, pigs, sheep and ducklings were also used as model animals (Gelderblom *et al.*, 1988; Kellerman *et al.*, 1990; Kriek *et al.*, 1981; Marasas *et al.*, 1988; Smith & Moss, 1985). Plant bioassays were also used (Cole *et al.*, 1973; Mirocha *et al.*, 1990; Van Ash *et al.*, 1992).

Among the lower animals, larvae of brine shrimps (*Artemia salina*) have been used as a convenient and cheap test organism for screening fungal toxins. Larvae of *A. salina* demonstrate high sensitivity against a wide range of toxic compounds of both fungal and plant origin (Harwig & Scott, 1971; Michael *et al.*, 1956). Brine shrimp larvae have been widely used as test organisms in screening toxic metabolites from toxigenic fusarium isolates (Ali & Salleh, 1992; Cole, 1984; Logrieco *et al.*, 1990; Munoz *et al.*, 1990; Smith & Moss, 1985; Visconti *et al.*, 1989, 1992).

2.4 Implication of Fusarium Toxins as Health Hazard

Invasion of grain and food stuffs by fusaria and their toxins have been responsible for a number of large-scale human toxicoses. Alimentary toxic aleukia (ATA), described prior to 1900, was associated with the ingestion of over wintered grain. This disease has been recorded in Russia during 1942-1947 famine and else where periodically since the 19th century (Miller, 1992; Smith & Moss, 1985). This syndrome has been shown to have been caused by T-2, neosolaniol and T-2 tetraol toxicosis, derived from *F. sporotrichioides* (Miller, 1992).

In Japan, there was the so called "red mould poisoning" due to contamination of wheat and rice by *Fusarium* species predominantly by *F. graminearum*. Disease symptoms such as nausea, vomiting and diarrhoea were recorded. This was found to be as a result of the toxic effect of nivalenol, deoxynivalenol (DON) and related trichothecenes (Miller, 1992; Smith & Moss, 1985). Large number of the population of Kashmir valley in India were also affected by DON toxicosis in 1987 as a result of the consumption of wheat and wheat products contaminated by it (Ramakrishna *et al.*, 1989).

Consumption of *F. moniliforme* contaminated maize have been associated with high incidence of human oesophageal cancer in Southern Africa (Marasas *et al.*, 1981; Rheeder *et al.*, 1992; Sydenham *et al.*, 1990a, b) and in China (Blaney, 1992). In Transkei, Southern Africa, high prevalence of *F. moniliforme* and significantly higher concentration of fumonisins both from mouldy and healthy corn samples were reported from high-oesophageal cancer rate areas than the corresponding samples from the low incidence areas (Rheeder *et al.*, 1992; Sydenham *et al.*, 1990a). In China frequent occurrence of trichothecene mycotoxins,

DON and 15-acetyl DON, in staple foods especially corn was also associated associated with high incidence of human oesophageal cancer (Luo *et al.*, 1990).

In Ethiopia, preliminary reports on the mycoflora of maize by Abate (1982) and Gilman (1968) indicate the genus *Fusarium* to be highly associated with maize. It was observed that deteriorated maize grain and malted maize are commonly sold in markets. Furthermore, the traditional harvesting, drying, storage methods eventually makes maize susceptible to fusarium infection and contamination by fusarium toxins.

Animals feed on fusarium infected stalks, cobs and deteriorated maize grains. Nearly all maize produced in Ethiopia, including deteriorated or damaged grains, are utilised for human consumption. This indicates the health risk associated with consumption of maize grains contaminated with fusarium toxins both in man and domestic animals in Ethiopia.

3 MATERIALS AND METHODS

3.1 Sample Collection

Maize samples were collected from farmer stores and market places in Shashemene and Alemaya regions in 1995. In this study samples were rated as damaged, normal and malted using the following criteria.

Samples with greater than 50% kernel discolouration, greater than 40% of wrinkled seeds and greater than 30% of the kernels floating in water, and/or above 50% of kernels attacked by insects were designated as damaged (Fig. 6). The damaged samples are also known as 'Yetela Ihele' by the population in Shashemene area. During harvesting and threshing processes farmers select mould infected highly discoloured maize cobs, separately thresh them and the mould damaged maize kernels are sold in the market and also utilised by the producers themselves as 'Yetela Ihele'. Moreover, grains damaged by mould infection and insect attack during storage, that are separated from the normal maize kernels are also used as 'Yetela Ihele".

Samples with less than 50% kernel discoloration, less than 40% wrinkled seeds and less than 30% of kernels floating in water and below 50% of kernels attacked by insect were considered to be normal samples (Fig. 7).

Malted samples are germinated maize grains. In the traditional malting process maize seeds are socked in water for 24-48 hrs. and imbibed seeds were allowed to germinate covered by enset leaves on the ground for 5-7 days. Germinated seeds are then dried naturally by spreading the germinated seeds on bare ground or on sacks (to minimise contact with the soil) exposing to the sun and wind. Malted maize (Bikil) (Fig. 8) is used for local beer (tela) making.

All samples were placed in bags of cotton cloth and kept in the refrigerator until used for mycoflora and mycotoxin investigations.

3.2 Moisture Content Determination

Aliquots of 20 g were taken from each maize sample, dried in an oven at 60° C until constant weight was recorded. The moisture content of samples were expressed as a percentage on a wet weight basis.

3.3 Determination of Percentage of Seed Germination

Percentage seed germination of normal and damaged samples was recorded after 7 days of incubation on PDA and mCDA plates in the dark at 25° C.

3.4 Isolation of Seed Mycoflora

Aliquots of 50 g of each of the normal and damaged samples were surface sterilised in 0.1 % mercuric chloride solution for 1 min. The same amount of sub samples from malted samples

were surface sterilised in 0.5 % mercuric chloride for 5 min. The samples were then rinsed with sterile distilled water three times. A total number of 100 kernels per sample were plated, five kernels per plate, on Potato Dextrose Agar (PDA) and modified Czapek Dox Agar (mCDA).

PDA Ingredients:

Dextrose	20 g
Agar	20 g
Potato extract	1000 ml.

(200 g of sliced potato boiled for 30 min in 1000 ml of water and filtered through cheese cloth and diluted with water to make 1000 ml).

mCDA Ingredients:

Dextrose	20 g
KH_2PO_4	0.5 g
$NaNO_3$	2 g
$MgSO_4$	0.5 g
Yeast extract	1 g
1% Ferrous sulphate solution	1 ml
Agar	20 g
Distilled water	1000 ml

The plates were incubated in the dark at 25 ° C for 7 days and the fungal colonies that

developed from the kernels were counted. Colonies were transferred to plates of PDA for identification and in agar slants for maintenance.

3.5 Identification and Grouping of Fusarium Isolates

3.5.1 Identification of Fusarium Isolates

The species of fusarium were further characterised using the manuals by Barnett and Hunter (1972) and Nelson *et al.* (1983). .To facilitate sporulation pure cultures were also transferred to KCL medium (Fisher *et al.*, 1983; Nelson *et al.*, 1983) and a modified medium S1. Cultures on PDA were incubated in the dark at 25 ° C and those on KCL and medium S1 were incubated at room temperature under diffused day light.

The isolates were determined to be members of the genus *Fusarium* based on the presence of macroconidia and/or microconidial chains. Macroconidia production was seen on water mount microscopic preparations from PDA and medium S1 cultures, while microconidial chain formation was seen under low power objective from cultures on KCl and medium S1.

S1 Ingredients:

KNO_3	1 g
KH_2PO_4	1 g
$MgSO_4$	0.5 g
KCl	0.5 g
$FeSO_4.7H_2O$	0.01 g
Corn grit	20 g
Agar	20 g

Distilled water	1000 ml
(Boiled before sterilisation).	

KCL Ingredients:

KCL	4 g
Agar	20 g
Distilled water	1000 ml

3.5.2. Grouping of Fusarium Isolates

Frequently occurring fusarium isolates were grouped based on their similarity in their cultural and morphological characters in order to make identification of commonly occurring toxigenic fusarium species easier. Information used were presence or absence of microconidial chains on KCL and medium S1; size and shape of macroconidia and microconidia grown on medium S1, and colony colour (obverse and reverse plate agar) and growth rate of isolates on PDA plates after dark incubation at 25 °C for 10 and 3 days respectively. Chlamydospore formation was observed placing a small piece of the agar culture with some of the PDA medium on which it is growing on sterile distilled water according to Nelson *et al.*(1983) for 15 days. Spore size was measured using eyepiece micrometer fitted microscope (Francis, 1991).

Frequently occurring toxigenic fusarium isolates were grouped into two based on presence or absence of microconidial chain. Those isolates without microconidial chain or microconidia produced only on false heads were further grouped into three groups on the basis of size and shape of macroconidia, presence or absence of microconidia, size and shape of microconidia,

colony colour and growth rate. The rest of the fusaria isolates without toxigenic activity and those with low frequency of occurrence and different morphological characters makes the fifth group.

3.5.3. Maintenance of fusarium isolates

Fusarium isolates were maintained in SNA (Spezieller Nahrstoffarmer Agar) slants (Samson, 1992; Singh *et al.*, 1991) and cultures are available in the Department of Biology, Addis Abeba University.

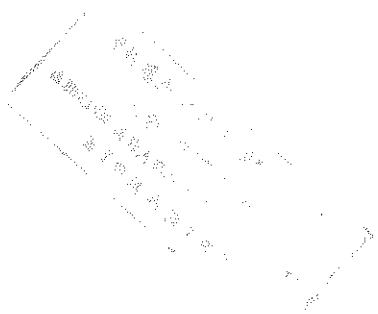
SNA Ingredients:

KNO_3	1 g
KH_2PO_4	1 g
$MgSO_4.7H_2O$	0.5 g
KCl	0.5 g
Glucose	0.2 g
Sucrose	0.2 g
Agar	20 g
Distilled water	1000 ml

3.6 Screening for Toxigenic Fusaria

3.6.1 Preliminary Screening

Culture Preparation



Fusarium isolates were grown on Malt extract Corn grit Agar (MCA) slants and incubated at room condition under diffused day light. Spore suspension prepared by adding 2 ml of sterile water to 7 day old slants served as the inoculum to 100 ml of broth B1 in 500 ml Erlenmeyer flask.

MCA Ingredients:

Malt extract	15 g
Corn grit	20 g
Agar	20 g
Distilled water	1000 ml

(Boiled before autoclaving).

B1 Ingredients:

Sucrose	30 g
$(NH_4)_2HPO_4$	1 g
KH_2PO_4	3 g
$MgSO_4$	0.5 g
NaCl	2 g
$FeSO_4.7H_2O$	0.2 g
Yeast extract	2 g
Malt extract	1 g
Peptone	1 g
Maize extract	1000 ml

(100 g shredded maize boiled in 1000 ml water for 30 min and filtered through cheese cloth and made to 1000 ml by adding water).

Broth Cultures

Broth cultures were incubated on a rotary shaker at 120 rpm for 5 to 7 days. The broth culture was then filtered and 50 ml of the filtrate was extracted with equal volume of chloroform. The chloroform extract was dehydrated by adding anhydrous Na_2SO_4, filtered and dried on a rotary evaporator.

To investigate for more polar toxins, 15 ml of the broth filtrate was freeze-dried and the dried material was extracted with 4 ml of methanol and concentrated to 2 ml of methanolic extract.

Toxicity Test on the Brine Shrimp (Artemia salina) Larvae

Toxigenic fusaria were screened by testing crude chloroform and methanol extracts against the brine shrimp (*Artemia salina*) larvae.

Brine shrimp (*Artemia salina*) eggs were left to hatch for 36 hrs in a two sector plastic dish with artificial sea water prepared from sea salt (Aquarium systems, USA). It was prepared by adding 3.8 g of sea salt per 100 ml of distilled water and incubated at 27° C. The hatching chamber was aerated with an aquarium pump. The dark and light exposed sectors communicate through the space at the bottom of the compartment allowing the active brine shrimp larvae to move to the light exposed sector. Active brine shrimp larvae were collected with a Pasteur pipette in small beaker containing sea water.

Bioassays of crude extracts were performed using, 200 µg/ml and 500 µg/ml concentrations of chloroform extracts and 200 µl/ml and 500 µl/ml concentrations of the concentrated methanolic extract in a 24 cell microliter plates containing about 10-20 brine shrimp larvae in

1 ml of sea water.

Crude extracts that exhibited > 50 % kill in 48 hrs. at 500 µg/ml of the chloroform extract and/or 500 µl/ml of the methanol extract were considered as toxic and the producing fusarium isolates were designated as toxigenic.

3.6.2 Production of Toxic Principles on Shredded Maize

Grain Culture Preparation

Hand selected 'good' maize grain was shredded to half grain size using a laboratory mill., 100 g of Shredded maize (100 g), was taken in 500 ml Erlenmeyer flask, brought to a moisture content of about 25% on a wet weight basis and sterilized for 30 min at 120° C and 121 pa.

Fusarium isolates from each of the four groups were transferred into PDA plates and incubated at 25° C for 7 days in the dark. Agar blocks of PDA cultures (1cm x 1cm, 20-30 pieces) were inoculated into the sterilized maize substrate. Maize cultures were shaken to aid in even distribution of the inoculum. Cultures were incubated in the dark for three weeks at room temperature.

Extraction of Grain Culture

Cultures of group-I isolates were extracted with methanol (MeOH)/water (H_2O) (3:1, 200 ml) according to Cawood *et al.* (1991) to search for the production of the polar and water soluble fumonisin mycotoxins. Cultures of other groups, group-II, group-III and group-IV were extracted with MeOH/H_2O (40:60, 200 ml) according to Vesonder *et al.* (1981) to see trichothecene and zearalenone production.

MeOH/H_2O extracts were solvent partitioned with chloroform (50 ml x 3). The MeOH/H_2O extracts were dried on a rotary evaporator at 50° C and the methanol soluble part was collected and dried. Chloroform extracts were dehydrated with anhydrous Na_2SO_4, filtered and dried using a rotary evaporator.

Toxicity Test

Both methanol and chloroform extracts of representative isolates of each group were tested for toxicity to brine shrimp larvae at 200 and 500 µg/ml concentrations as described above. Those isolates or groups whose methanol and/or chloroform extract(s) exhibit 50% death at 500 µg/ml in 48 hours were considered as toxigenic.

3.7 Toxicity Test of Natural Maize Samples

Sample Selection

Four samples with highest percentage of fusaria isolates among the 12 samples of each sample group were selected and labelled A to D in decreasing order of percentage fusaria isolated.

Extraction

Aliquots of 150 g were ground finely by a laboratory mill and 120 g of it was extracted with 200 ml of MeOH/H_2O (3:1). The MeOH/H_2O extract was solvent partitioned with chloroform (50 ml twice). MeOH/H_2O extracts were freeze-dried and the methanol soluble part was collected and dried on a rotary evaporator at 50° C. Chloroform extracts were dehydrated with anhydrous Na_2SO_4, filtered and dried on a rotary evaporator.

Toxicity Test

Toxicity was tested against *A. salina* at 200 and 500 µg/ml concentrations of both methanol and chloroform extracts as described above.

3.8 Purification and Structural Elucidation

3.8.1 Bioactivity Directed Isolation of Toxic Metabolites

Bioactivity directed isolation of toxic metabolites was done for grain culture of group-II fusarium isolate.

Extraction

Fusarium culture (group-II isolate) was grown on 1 Kg of sterilised shredded maize substrate, whose moisture content was adjusted to about 25% on a wet weight basis. Shredded maize cultures were incubated in the dark for 3 weeks at room temperature. Mouldy shredded maize culture was then dried, ground on a laboratory mill and extracted with MeOH/H_2O (40:60, 2 ml/g) according to Vesonder *et al.* (1981) for 3 hours on a rotary shaker. The MeOH/H_2O filtrate was concentrated and then extracted with chloroform. The combined chloroform phases were dehydrated with anhydrous Na_2SO_4 and concentrated to dryness on a rotary evaporator.

Isolation of Toxic Principles

All the toxic chloroform extract was applied to a silica gel column pre packed with chloroform as follows. In a 28 x 365 µm chromatographic glass tube, chloroform was added

until tube is 1/2 full, then 80 g silica gel (Silica Gel, 0.063-0.2 E, MercK, Darmstadt) was added and sides of the tube was washed with chloroform. The chloroform was drained to the top of the silica gel and chloroform dissolved extract was added. The column was eluted with chloroform (150 ml), chloroform/MeOH (95:5) (250 ml) and MeOH (150 ml). Fractions were tested against the brine shrimp larvae after drying and active fractions were pooled, dried and further chromatographed on a second silica gel column pre packed with chloroform (40 g silica gel in 22 x 440 mm chromatographic tube) as described above. The second column was eluted with chloroform (150ml), chloroform/MeOH (94:6) (200 ml) and MeOH (150 ml). Fractions active against brine shrimp larvae were pooled and dried.

The semi-purified toxic fractions were dissolved in chloroform/MeOH (9:1), spotted on thin layer chromatography (TLC) plates (TLC Aluminium sheets, silica gel 60 F^{254} pre-coated, 5 cm x 7.5 cm, 0.2 mm thick, E, Merck, Darmstadt) and developed in Toluene/ Ethyl acetate/ 90% formic acid (TEF) (5:4:1) solvent system. Presence of trichothecene and zearalenone was seen under short (254nm) and long (366nm) wave uv lights before and after the TLC plate was treated with 20% $AlCl_3$ in ethanol and heated at 110 °C for 5-10 min according to Ichinoe *et al.* (1983). Those compounds which fluoresce blue under long wave uv light (366nm) were separated by preparative thin layer chromatography (PTLC) based on their Rf value.

Structural Elucidation

Chemical structure determination of the three PTLC separated toxic metabolites, toxic to *A. salina* larvae, was carried out by Nuclear Magnetic Resonance (NMR) and Mass Spectroscopy by Dr. Wolf Rainer Abraham at Gesellschaft Fur Biotechnologische Forschung mbH (GBF), Braunschweig, Germany.

3.8.2 Chemical Screening Method of Toxic Metabolite Isolation

Chemical screening of toxic metabolite isolation was carried out for group-I fusarium isolates to isolate fumonisin compounds.

Grain Culture and Chemical Screening

Representative isolate of group I fusaria suspected of fumonisin production was grown on 1 Kg of sterilised shredded maize substrate, whose moisture content was adjusted to 25% on a wet weight basis. Shredded maize cultures were incubated in the dark for 3 weeks at room temperature.

Isolation of fumonisin mycotoxins was carried out using the chemical data and the procedures of Cawood *et al.* (1991) with slight modification. Mouldy shredded maize was dried, ground on a laboratory mill and extracted with MeOH/H_2O (3:1, 2 ml/g) for 3 hours on a rotary shaker. The MeOH/H_2O filtrate was concentrated and solvent partitioned with equal volume of chloroform.

The aqueous phase was freeze-dried, dissolved in MeOH/H_2O (1:3, 5 ml), pH was adjusted to 3.5 using 1N HCl and applied to an Amberlite XAD-1180 column (22 X 440 mm) previously equilibrated with MeOH/H_2O (1:3). The column was washed with MeOH/H_2O (1:3, 200 ml), MeOH/H_2O (1:1, 200 ml) and MeOH (200 ml).

Samples were taken from each of the three sets of eluates, concentrated and analysed by silica gel thin layer chromatography using $CHCl_3/CH_3OH/H_2O/CH_3COOH$ (55:36:8:1) and $CHCl_3/CH_3OH/CH_3COOH$ (6:3:1) as developing solvent systems. The plates were developed

by spraying with ninhydrin (0.2 % in ethanol) solution and heating at 120° C until purple colour developed.

TLC analysis showed that, the compounds of interest were found in MeOH/H_2O (1:3) fraction after eluting through amberlite XAD-1180. Thus, the MeOH/H_2O (1:3) fraction was freeze-dried and compounds of interests were separated on PTLC based on their Rf value using $CHCl_3$/CH_3OH/H_2O/CH_3COOH (55:36:8:1) as developing solvent. Four ninhydrin positive compounds were separated and were sent to Dr. W. R. Abraham, GBF, Germany for confirmation of fumonisins.

3.9 Natural Occurrence of the Isolated Toxic Metabolites

The isolated pure toxins, zearalenone and trichothecenes TA and TB, and semi-purified extracts of selected grain samples (A to D) from each sample type were developed on TLC plates using TEF (5:4:1) solvent system. TLC plates were treated with 20% $AlCl_3$ in ethanol and heated at 110° C for 5-10 min. Natural occurrence was qualitatively determined by comparing the Rf values of the isolated compounds and the compound in the sample extracts under long wave uv light (366 nm).

The amount of toxic substance in the samples was estimated by visual comparison of florescence after developing equal amounts of pure toxic substances and semi-purified grain sample extracts on TLC plates.

Natural occurrence of the fumonisin compounds was studied by comparing the presence of the purified fumonisins in the methanol extracts of selected grain samples using chloroform/

methanol/ water/ acetic acid (55:36:8:1) as developing solvent. Amount of the toxic metabolites was estimated as described above.

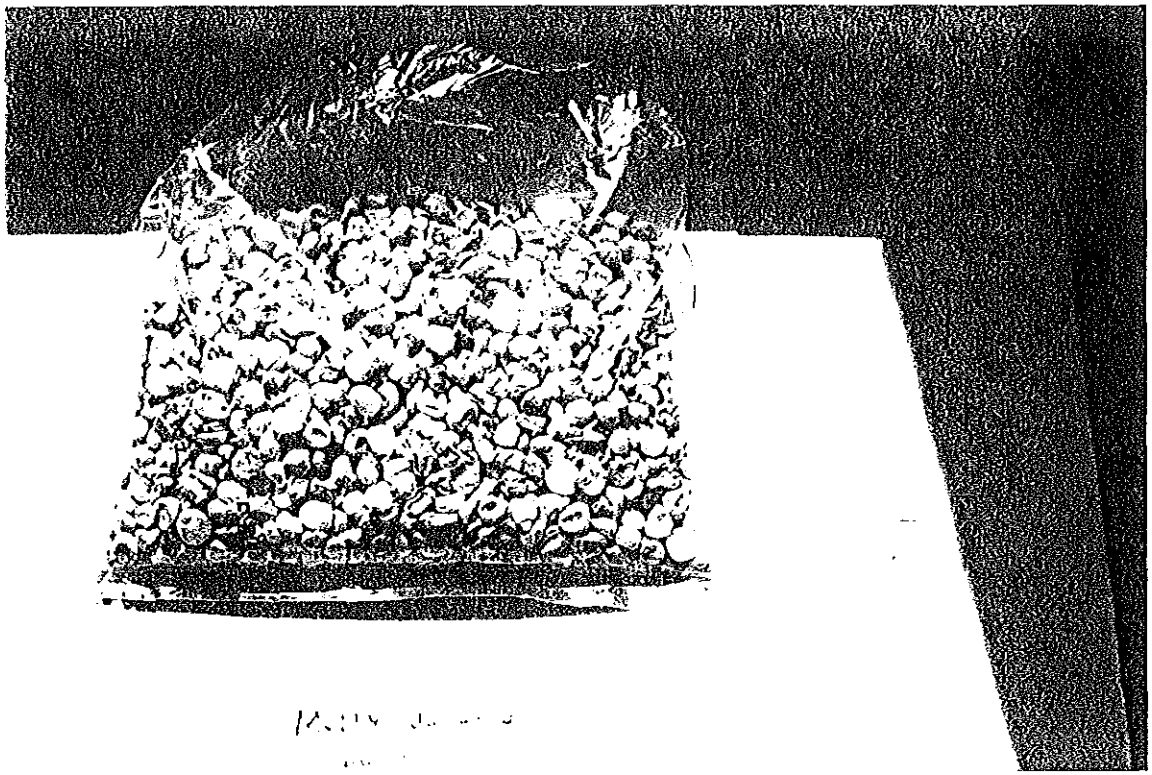


Fig. 6. Damaged Sample

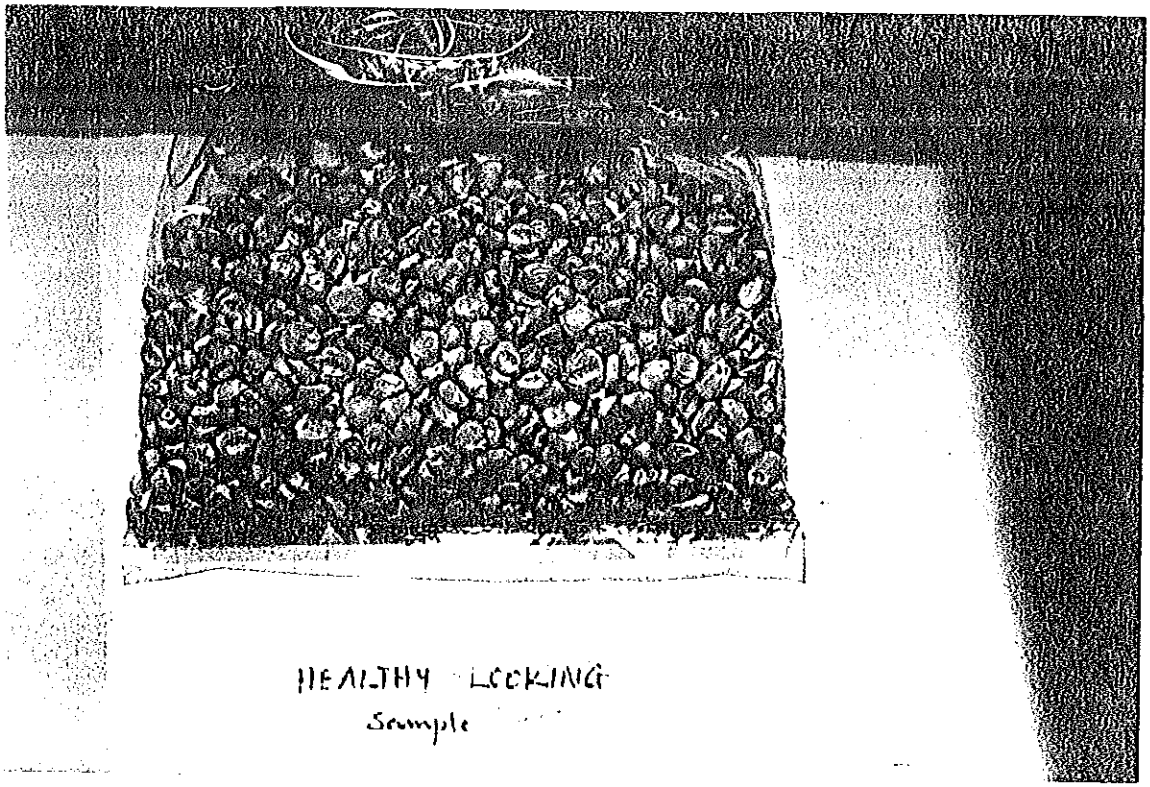


Fig. 7. Normal Sample

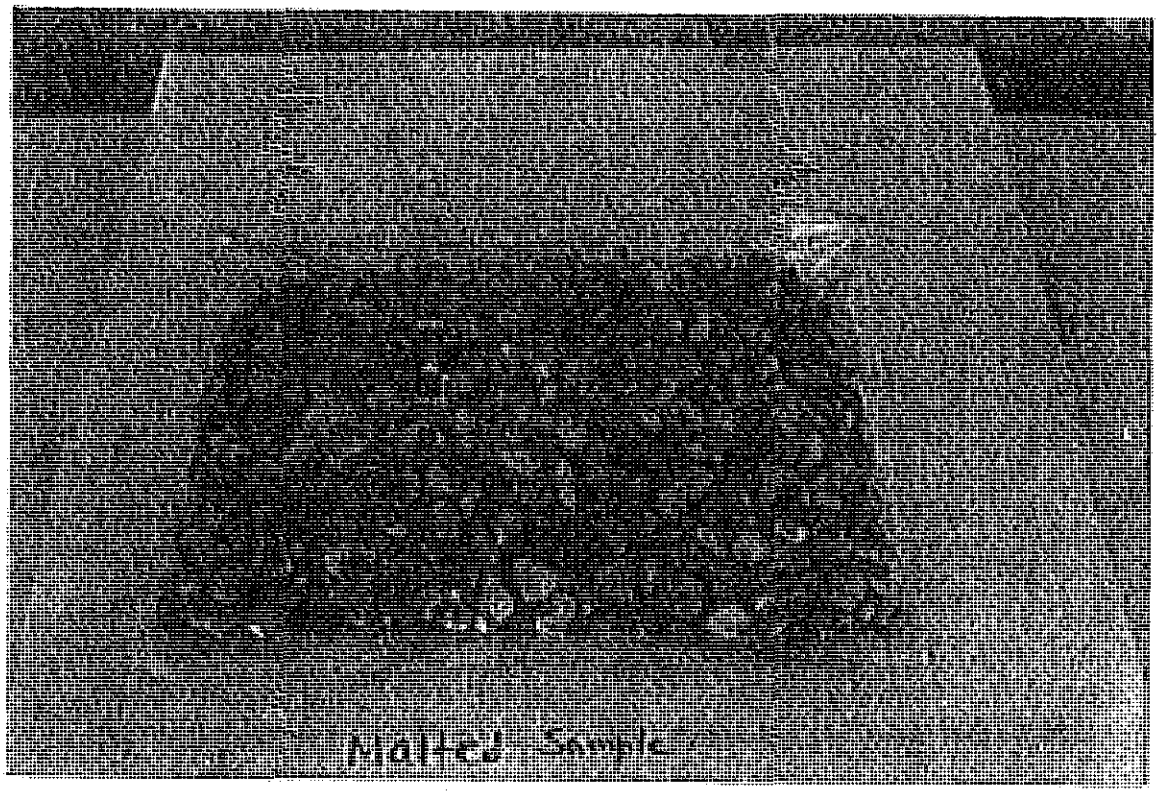


Fig. 8. Malted Sample

4 RESULTS

4.1 Mycoflora Distribution

Thirty-six samples of maize: 12 normal (N), 12 damaged (D) and 12 malted samples were subjected to isolation and identification of fungi. The fungal species isolated were *Aspergillus* spp, *Fusarium* spp, *Penicillium* spp, *Mucor* spp, *Rhizopus* spp and other unidentified moulds (Table 2).

Among the three known toxigenic fungal genera, *Fusarium* was found to be the most common genus comprising 17.5 % (Fig. 9) of the total fungi isolated from all the three groups of samples. Species of *Fusarium* were recovered from 80.5 % of the total samples. *Aspergillus* spp was the second most common genus isolated, which comprises 14 % of the total fungi and represented in 66.7 % of the samples (Table 2). *Penicillium* spp was the third common fungi and was recovered from 72.2 % of the samples comprising 13.8 % of the total fungi isolated (Fig. 9).

In damaged and malted samples the genus *Fusarium* was the most common toxigenic fungi isolated. The genus was represented in 91.7 % (Table 4) of the damaged samples comprising 28 % of the fungal isolates (Fig. 9). In the malted samples *Fusarium* spp was represented in 66.7 % of the samples and comprises 15.3 % of the fungi. In normal samples, however, *Fusarium* spp was low represented in 91.7 % of the samples comprising 9.5 % of the fungi isolated.

Aspergillus spp and *Penicillium* spp were the most prevalent fungi in the normal samples and were isolated from all the samples (Table 4). The genus *Aspergillus* and *Penicillium* comprises 23.8 % and 23.7 % of the fungi isolated respectively (Fig. 9). In the damaged samples *Aspergillus* was the second common genus comprising 14.5 % of the fungi and was represented in 75 % of the samples. *Penicillium* spp comprises 12.5 % of the fungi and were represented in 100 % of the samples. In the malted samples, however, percentage recovery of *Aspergillus* spp and *Penicillium* spp was found to be very low, 1.5 % and 3.4 % of the fungi respectively, from 25 % of the samples examined.

The variation in moisture content of the samples ranged from 5.5 % to 14.5 %. Higher percentage of kernel infection was recorded in the normal samples (71.5 %) followed by damaged (67.75 %) and malted samples (59.3 %). However, % seed germination was relatively higher in normal (49.6 %) than damaged (40.6 %) samples (Table 4).

Mean percentage of kernel infected by *Penicillium* spp and *Aspergillus* spp was higher in normal samples (16.9 % and 17 %) and was low in malted samples (2.0 % and 0.9 %) respectively. On the other hand, 9.1 % of kernels in malted samples and 6.83 % of kernels in normal samples were infected by *Fusarium* spp. Mean percentage of kernel infection by *Fusarium* spp was highest in damaged samples (18.9 %) (Table 4).

Table 2. Fungal genera isolated from three groups of maize samples: Normal (N), Malted (M) and Damaged (D) on Potato Dextrose Agar (PDA) and modified Czapek Dox Agar (mCDA) media.

Fungal genera	TFI (a)	PDA						mCDA						TNI
		N		D		M		N		D		M		
		FI (b)	NI	FI	NI	FI	NI	FI	NI	FI	NI	FI	NI	
Fusarium	29 (80.5)	8 (66.7)	36	11 (91.7)	101	6 (50)	51	10 (83.3)	46	8 (66.5)	126	6 (50)	58	418
Penicillium	26 (72.2)	11 (91.7)	162	9 (75)	54	3 (25)	15	12 (100)	102	10 (83.3)	48	2 (16.7)	9	329
Aspergillus	24 (66.7)	12 (100)	83	7 (58.3)	40	3 (25)	10	10 (83.3)	121	9 (75)	78	1 (8.3)	78	333
Rhizopus	32 (88.9)	10 (83.3)	77	10 (83.3)	140	12 (100)	497	8 (66.5)	61	9 (75)	136	12 (100)	48	959
Mucor	22 (61.2)	6 (50)	9	8 (66.5)	33	4 (33.3)	13	4 (33.3)	18	7 (58.3)	23	2 (16.7)	9	329
Other fungi	17 (47.2)	7 (58.3)	86	5 (41.7)	20	1 (8.3)	5	8 (66.5)	118	4 (33.3)	14	-	-	243

TFI - Total Frequency of Incidence (number of samples from which members of the genera were recorded regardless of the type of media and sample).

FI - Frequency of Incidence (number of samples in which members of the genera were recorded from each sample type on each type of media).

NI - Number of Isolates (number of species belonging to the genera from each sample type isolated on each type of medium).

TNI - Total Number of Isolates (total number of members of the genera isolated from the three sample types on each type of media).

(a) - a corresponds to percentage of samples from which members of the genera isolated from the three set of samples, out of 36 samples.

(b) - b corresponds to percentage of samples from which members of the genera isolated from each sample type, out of 12 samples.

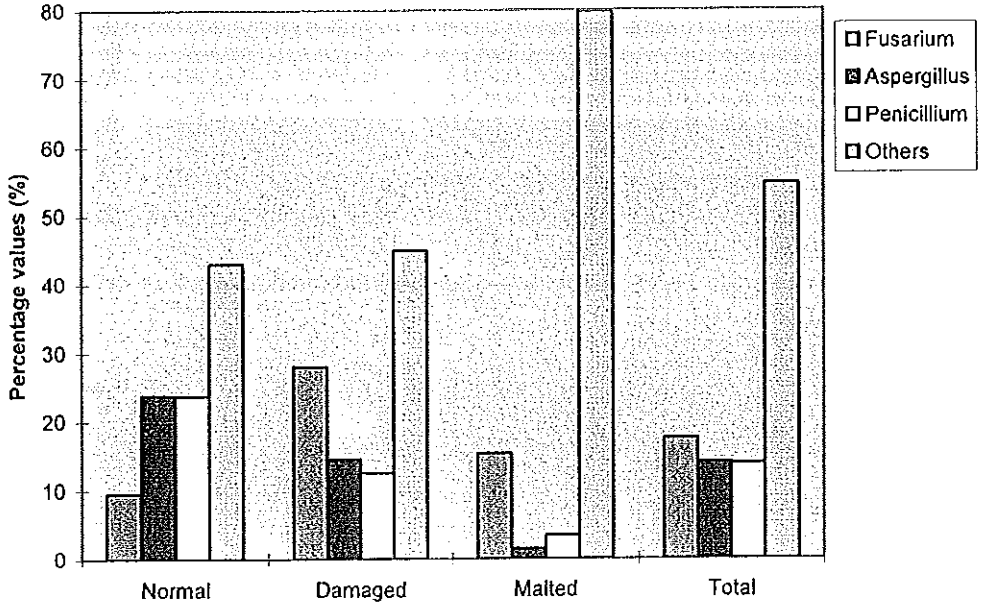


Fig. 9. Percentage distribution of the three toxigenic fungal genera *Fusarium*, *Aspergillus*, *Penicillium* and other groups of fungi isolated from the three groups of maize samples.

4.2 Toxigenic Fusarium Isolates

Preliminary test result of toxicity of extracts obtained from fusarium isolates grown in culture to brine shrimp larvae showed that 93 % of the total fusarium isolates were toxigenic. These toxigenic isolates were grouped as described in the material and methods (Table 3).

4.3 Morphological Grouping of Toxigenic Fusarium Isolates

4.3.1 Group I Fusaria

This group of fusaria were characterised by, colony on PDA at 25 °C incubated in the dark attain a diameter of 3.8 cm in 3 days (72 hrs). Aerial mycelium dense, floccose, mostly white with powdery appearance to light violet. Reverse with creamish to reddish brown, some creamish with greenish shades or spots.

Microconidia were produced dominantly in chains and in false heads on KCL, SNA and medium S1. Microconidial chains were borne on monophialides and polyphialides. Microconidia were variable in size and shape, mostly obovoid with a truncate base, oval to ellipsoidal, 0-(rarely 1) septate: o-septate 5-11 x 2-4 µm.

Macroconidium formation in most isolates of this group was rare. Macroconidium, 3-6 septated (mostly 3-septated, 3-septate: 26-39(40) x 2-3 µm), slightly curved or straight with the dorsal and ventral surfaces parallel for most of the length of the macroconidium. The walls were thin with curved apical cell and foot-shaped basal cell (Fig. 10). Chlamydospore absent.

Distinguishing features of this group of fusaria isolates are production of microconidial chains both on monophialides and polyphyalides and absence of chlamydospore formation. Based on these features the isolates were identified to be members of the section Leseola consisting of species *F. proliferatum*.

4.3.2 Group II Fusaria

Colony of this group exhibited very rapid growth, on PDA at 25°c incubated in the dark, attained a diameter of 5.4 cm in 3 days. Aerial mycelium dense, floccose, yellowish to orange at the center surrounded by white long and dense hyphae. Reverse side of agar light to dark rose-red or carmine red pigmented.

Microconidia rare to absent. Macroconidia 3-7 septated (mostly 5-septated, 5-septate: 36(37)-52(53) x 4-5 µm), curved with basal cells distinctly foot-shaped (Fig. 11). Chlamydospores absent.

Distinguishing features of this group were: microconidia are rare to absent, reverse side of agar carmine red pigmented and macroconidia with distinct foot-shaped basal cell.

Group II fusarium isolates showed morphological characters of section Discolor with the exception of the absence of chlamydospores. However, in the species *F. graminearum* of this section chlamydospore formation was very slow. Furthermore, the shape of macroconidium, an important character in species identification (Nelson *et al.*, 1983), produced by group II fusarium isolates on SNA and medium S1 is quite similar with that of *F. graminearum*.

Therefore, gruop II fusarium isolates were identified to be members of the section Discolor consisting of the species *F. graminearum*.

4.3.3 Group III Fusaria

Colony of group III fusaria grown on PDA incubated in the dark attained a diameter of 4.0 cm in 72 hrs. Aerial mycelium, dense, flat, white to light orange. Reverse, light yellow to light brown, pigment diffusing into agar.

Microconidia produced only on false heads, 0-1 septated, obovoid, oval to ellipsoidal in shape, 0-septate: 9(10)-15(16) x 2-4(5) µm; 1-septate: 16-2 2 x 3-4(5) µm.

Macroconidia borne mostly on polyphialides, 3-5 septated (Mostly 4 and 5 septated, 4-septate: 28(29)-42 x 2-3 µm; 5-septate: 39-65(66) x 2-4 µm. They were thin, longer, slightly curved to straight with walls parallel through most of their length, basal cell distinctly foot-shaped (Fig. 13). Chlamydospore absent.

Distinguishing features of this group were, colony colour and formation of thin slightly curved and longer macroconidia, and absence of chlamydospores. On the basis of these features isolates in this group were identified to be member of section Leseola consisting of species *F. subglutinans*.

4.3.4 Group IV Fusaria

In this group colony grown on PDA at 25° C incubated in the dark attained a diameter of 3.7

cm in 72 hrs. Aerial mycelium, dense floccose, light to dark violate. Reverse light to dark reddish brown, pigment diffusing into agar.

Microconidia produced only on false heads, 0-1 septated, oval to ellipsoidal in shape, 0-septate: 7-14 x 2-4 µm; 1-septate: 10-22 x 3-5 µm.

Macroconidia produced mostly on polyphialides, 2-5 septated (3-septated being predominant, 3-septate: 26-39 x 2(3)-5 µm). They were shorter and thicker, dominantly straight in shape with few slightly curved (Fig. 15). Chlamydospore absent.

Distinguishing features of the group were, colony colour, formation of shorter and thicker macroconidia and absence of chlamydospore formation. Based on these features this group of isolates were identified to be members of section Leseola consisting of species *F. anthophilum*.

Table 3. Summary of groups of fusarium isolates based on morphological and cultural characteristcs.

Fusarium isolates	Macroconidia			Microconidia			Colony	
	No of septa	Size (um)	Shape	Production	Size (um)	Shape	diam. (cm)	color
Group-I	3-6	3-septated 26-40 x 2-3 4-septated 39-47 x 2-3 5-septated 52-66 x 2-3	slightly curved at the tips	In chain and false ads	0-septated 5-11 x 2-4	obovoid with truncate base Oval to ellipsoidal	3.8	Obverse white to light violet Reverse creamish to reddish brown, creamish with green shades.
Group-II	3-7	3-septated 26-34 x 2-4 4-septated 34-42 x 3-4 5-septated 36-53 x 4-5 6-septated 44-61 x 4-6	slightly curved at the tips	rare to absent	----------	----------	5.4	Obverse yellowish to orange at the center surrounded by white hyphae Reverse light to dark carmine red.

Group-III	3-6	3-septated 28-42 x 2-3 4-septated 42-61 x 2-3 5-septated 39-66 x 2-4 6-septated 57-66 x 2-4	Slighty curved at the tips.	Only on false heads	0-septaed 9-11 x 2-5 1-septated 16-22 x 3-5	obovoid, oval to ellipsoidal	4	Obverse white to light orange. Reverse light yellow to light brown.
Group-IV	2-5	3-septated 26-39 x 2-5 4-septated 31-39 x 4-5 5-septated 34-50 x 3-5	straight with few curved.	Only on false heads	0-septated 7-14 x 2-4 1-septated 10-22 x 3-5	oval to ellipsoidal	3.7	Obverse light to dark violet. Reverse light to dark reddish brown.

4.3.5 Group V Fusaria

This group of fusaria dominantly consists of those fusarium isolates without toxigenic activity and those with toxigenic activity but with low frequency of occurrence and different morphological characters.

4.4 Distribution of Toxigenic Fusarium Isolates

Microconidial chain producing isolates were isolated very frequently from the three sets of samples and comprises 51.7 % of the total fusaria. Group I fusaria belongs to *F. proliferatum* and were found to be most common in normal samples followed by malted and damaged samples comprising 80.5 %, 61.5 %, and 36.6 % of the fusarium isolates in each sample type respectively (Fig. 17).

Large percentage of samples (80.5 %) were infected by group I fusaria. Group I fusarium isolates were highest in normal samples (91.7 %), followed by 83.3 % of the damaged samples and 66.7 % of malted samples respectively. The mean percentage of kernel infection was relatively higher in damaged samples (6.9 %) than malted (5.6 %) and healthy looking samples (Table. 4).

Group II fusaria (*F. graminearum*) made up 13.9 % of the total fusarium isolates, though they were isolated only from mouldy samples. In damaged samples, group II fusarium isolates were the second common, represented by 25.5 % of the fusarium isolates (Fig. 17), and 50 % of the samples. The mean percentage of kernel infection of damaged samples by group II

isolates was 4.8 % (Table 4).

Group III fusaria (*F. subglutinans*) comprises 2.4 % in normal samples, 12.3 % in damaged samples, 12 % in malted samples and 10.3 % of the total fusarium isolates (Fig.17). They were isolated from 58.3 % of damaged, 33.3 % of malted and 8.3 % of normal samples. Mean percentage of kernel infection by these group of fusaria were 2.3 % in damaged, 1.1 % in malted and 0.16 % in normal samples (Table 4).

Group IV fusaria (*F. anthophilum*) comprised 13.9 % of the total fusarium isolates. They were the second common group of isolates in normal (13.4 %) and malted samples (16.5 %) and occupies third percentage frequency in damaged samples (12.8 %) (Fig. 17). These isolates were represented in 50 % of damaged, 33.3 % of malted and 25 % of normal samples. Mean percentage of kernel infection by group IV isolates was highest in damaged samples (2.4 %) followed by malted (1.5 %) and normal samples (0.9 %) (Table 4).

Table 4. Incidence of fusaria and germination of kernels from normal, damaged, and malted samples.

Isolates	Normal looking samples (N)		Damaged samples (D)		Malted samples (M)	
	Samples infected (%)	Kernels infected (mean %)[b]	Samples infected (%)	Kernels infected (mean %)	Samples infected (%)	Kernels infected (mean %)
Fusarium						
Group-I	91.7	5.5	83.3	6.9	66.7	5.6
Group-II	-	-	50	4.8	-	-
Group-III	8.3	0.16	58.3	2.3	33.3	1.1
Group-IV	25	0.9	50	2.4	33.3	1.5
Other fusaria	16.7	0.25	58.3	2.4	33.3	0.9
Total *Fusarium*[a]	91.7	6.83	91.7	18.9	66.7	9.1
Total isolates[c]	71.5		67.5		59.3	
Seed germination (%)	49.6		40.6			

(a) - Mean of the total number of fusarium colonies isolated from plated kernels.

(b) - Values represent mean of 1200 surface sterilised maize kernels.

(c) - Mean of the total isolates from plated kernels (some kernels were infected by more than one species of fungi).

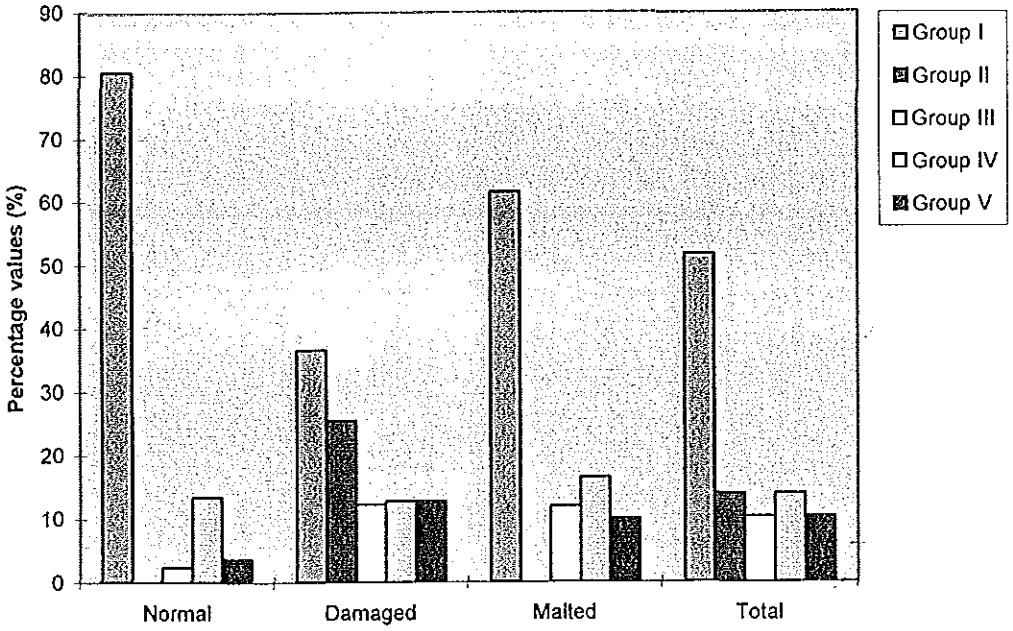


Fig. 17. Percentage distribution of the five groups of fusarium isolates in normal, damaged, malted and the three sets of samples (total).

4.5 Production of Toxic Metabolites on Shredded Maize Culture

Extracts of representative isolates assayed for toxicity against brine shrimp (*A. salina*) larvae showed that methanol extracts of group I isolates were very toxic, group III and group IV were toxic while group II were non toxic.

Comparison of toxicity of chloroform fractions, on the other hand, indicated group II isolates were very toxic, group I & group IV were toxic and group III were non toxic (Table. 5).

4.6 Toxicity of Selected Maize Grain Samples to *A. salina*

Toxicity test of methanol and chloroform extracts of selected grain samples showed that, with the exception of methanol extracts of sample B and D of damaged maize samples, both methanol and chloroform extracts of the tested samples exhibited toxicity to *A. salina* (Table. 6).

Comparison of the toxicity of chloroform extracts of the tested samples showed that all the tested malted samples and 25 % of the normal samples were very toxic; 75 % of normal and 25 % of damaged samples were toxic, and 75 % of damaged samples were slightly toxic to the brine shrimp larvae.

Table. 5 Toxicity of Methanol and Chloroform Grain Culture Extracts to Brine Shrimp (*Artemia salina*) Larvae.

Extract	Fusarium isolates			
	Group I	Group II	Group III	Group IV
Methanol	+++	-	++	++
Chloroform	++	+++	-	++

+++ = very toxic, > 50 % kill in 24 hrs; ++ = toxic, > 50 % kill in 40 hrs; + = slightly toxic, > 50 % kill in 48 hrs; and - = non toxic, < 50 % kill in 48 hrs.

Table. 6. Toxicity of Crude Sample Extracts to Brine Shrimp (*Artemia salina*) Larvae.

Sample Type	**Extract**	**A**	**B**	**C**	**D**
Damaged	methanol	++	-	++	-
	chloroform	+	++	+	+
Malted	methanol	++	++	++	++
	chloroform	+++	+++	+++	+++
Normal	methanol	++	+	++	++
	chloroform	++	++	+++	++

+++ = very toxic, > 50 % kill in 24 hrs; ++ = toxic, > 50 % kill in 40 hrs; + = slightly toxic, > 50 % kill in 48 hrs; and - = non-toxic), < 50 % kill in 48 hrs.

4.7 Isolation and characterisation of zearalenone and Trichothecenes.

The three PTLC separated active fractions were identified to be zearalenone and trichothecene compounds, TA and TB (Append. 1). The chemical structure of zearalenone (ZEN) was confirmed by spectroscopic methods. They are characterised by an Rf value of 0.7 using TEF (5:4:1) solvent system, and the purified compound was found to be very toxic to the brine shrimp larvae at concentration of 100 µg/ml. Analysis of TA (Rf=0.54) and TB (Rf=0.44) shows the presence of an epoxide group and are characterised as trichothecenes. The trichothecenes and ZEN produces blue fluorescence after 20 % $AlCl_3$ in ethanol spray treatment and heating for 5-10 min.

4.8 Isolation and Characterisation of Fumonisins

TLC analysis of amberlite XAD-1180 column eluates showed that unlike the report of Cawood *et al.*, (1991), using amberlite XAD-2 the compounds of interest were found in the methanol/water (1:3) fraction. On PTLC four ninhydrin positive compounds were separated: FB_A (Rf=0.3), FB_B (Rf=0.2), FB_C (Rf=0.5) and FB_D (Rf=0.18) using the solvent system chloroform/ methanol/ water/ acetic acid (55:36:8:1) (Append. 2). The compounds are soluble in water, slightly soluble in methanol and positive for ninhydrin spray treatment.

With the exception of fraction FB_D, the Rf values of the compounds fits with the reported values of fumonisin B's using chloroform/ methanol/ water/ acetic acid (55:36:8:1), chloroform/ methanol/ acetic acid (6:3:1) (Cawood *et al.*, 1991) and 1-butanol/ acetic acid/ water (20:10:10) (Dupuy *et al.*, 1993) as developing solvents.

In view of the observed physico-chemical characters and the producing fusaria, *F. proliferatum* which was reported elsewhere to produce fumonisin compounds (Ross *et al.*, 1990; Thiel *et al.*, 1991), the compounds are identified to belong to the fumonisins.

4.9 Natural Occurrence of the Isolated Fusarium Toxins

Analysis of the natural occurrence of the purified toxic principles using TLC techniques showed that zearalenone (ZEN) occurs in 50 % of the damaged samples. The trichothecene TA and TB were detected in 50 and 75 % of the damaged samples examined respectively(Table 7). The estimated concentration of zearalenone in the samples ranges from 0-0.42 μg/g while that of trichothecene TA and TB were 0-1.5 and 0-2.3 μg/g respectively.

In the natural occurrence study the ninhydrin positive fumonisin compounds were detected in all the samples examined. The estimated values of the fractions (Table 8) showed the presence of high concentration of the mycotoxins in the malted and damaged samples.

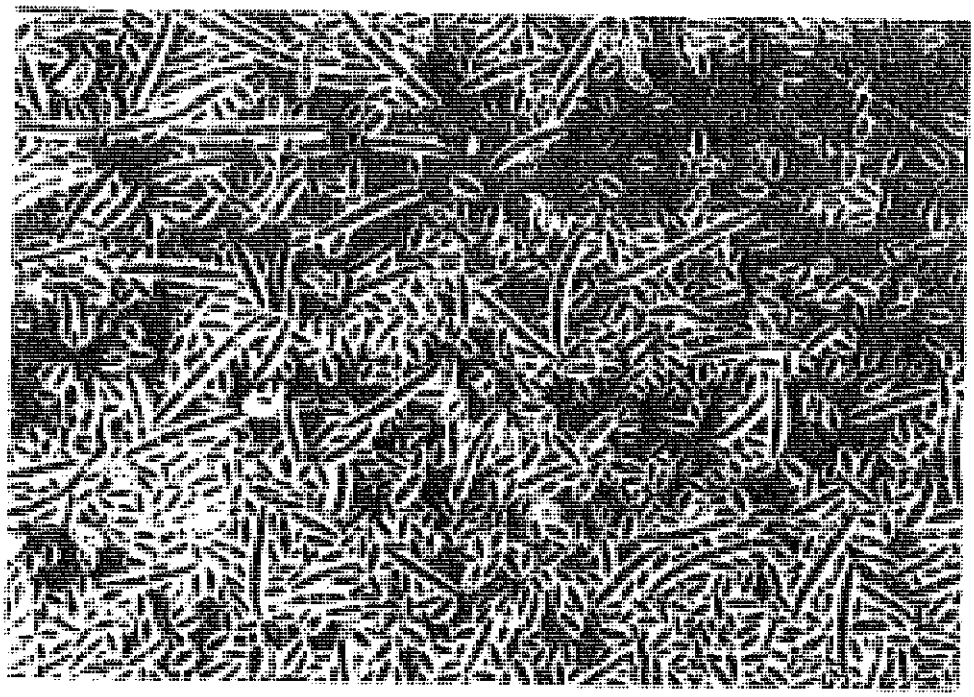
Table 7 Natural Occurrence of Zearalenone (ZEN), and Trichothecenes TA and TB.

Sample	Malted			Damaged			Normal		
	ZEN	TA	TB	ZEN	TA	TB	ZEN	TA	TB
A	-	-	-	+	-	-	-	-	-
B	-	-	-	+	-	+	-	-	-
C	_	-	-	-	+	+	-	-	-
D	_	-	-	-	+	+	-	-	-

(+) = detected and (-) = not detected.

Table 8 Estimated Values of the Fumonisin Compounds (in μg/g).

Sample	FB_A	FB_B	FB_C	FB_D
N	4.2-29.7	4.2-29.7	8.3-49.5	41.7-118.8
D	91.7-187.5	91.7-187.5	25-312.5	25-312.5
M	99-275	99-275	99-281.3	296.9-343.75



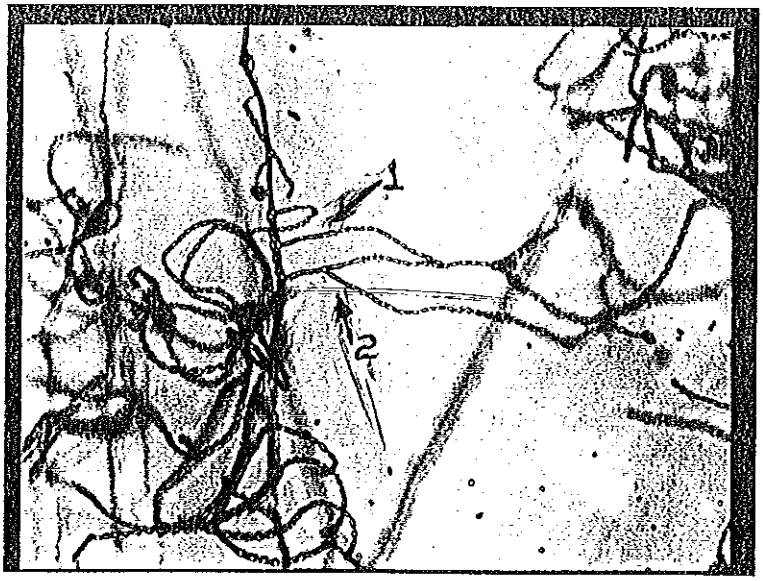


Fig. 10. Macroconidia and Microconidia Produced by Group I Fusaria Isolates (a, 400X); Mode of Microconidial Chain Production (b, 100X), 1 - Monophialide and 2 - Polyphialide

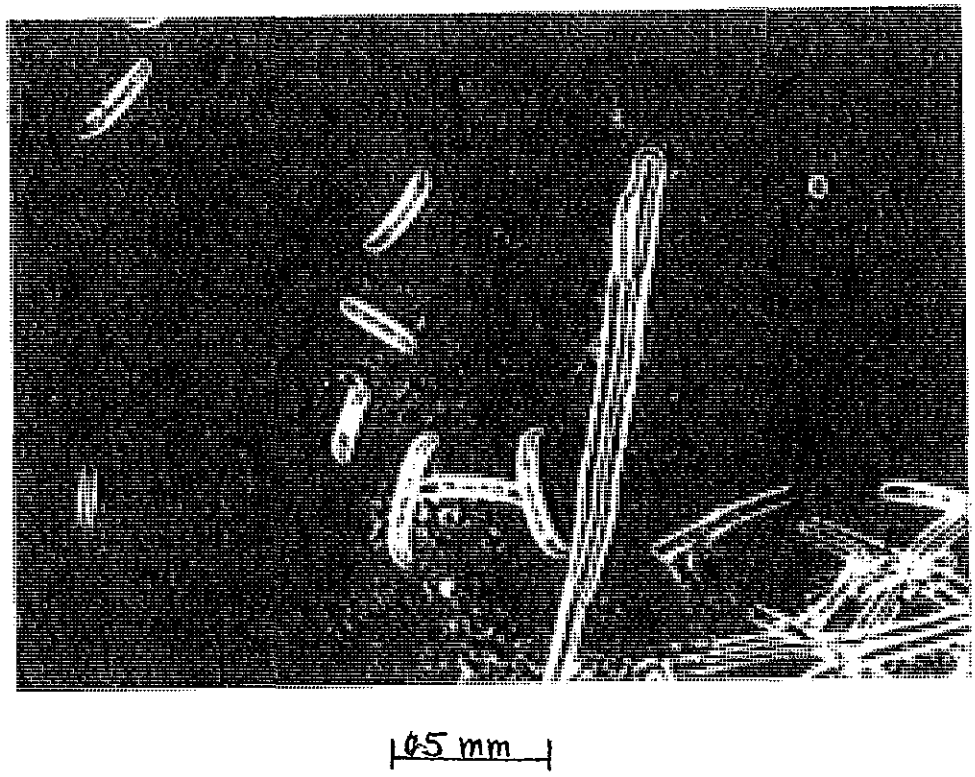
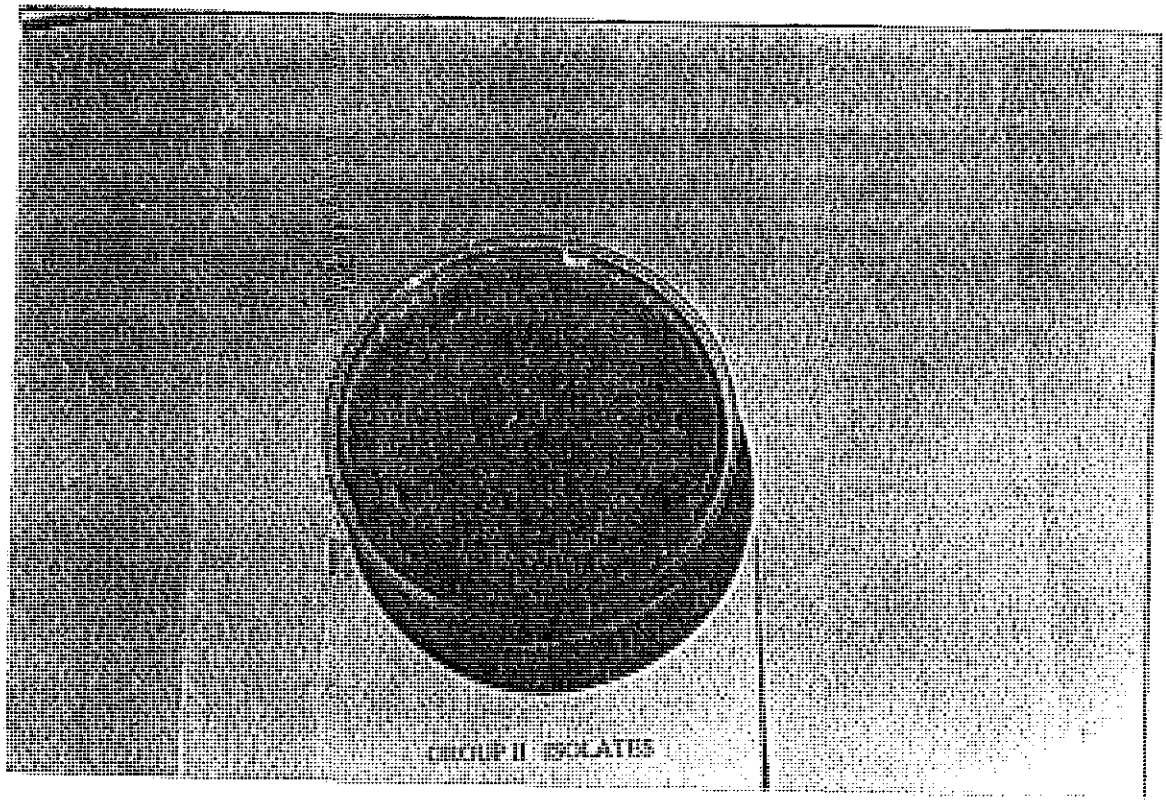
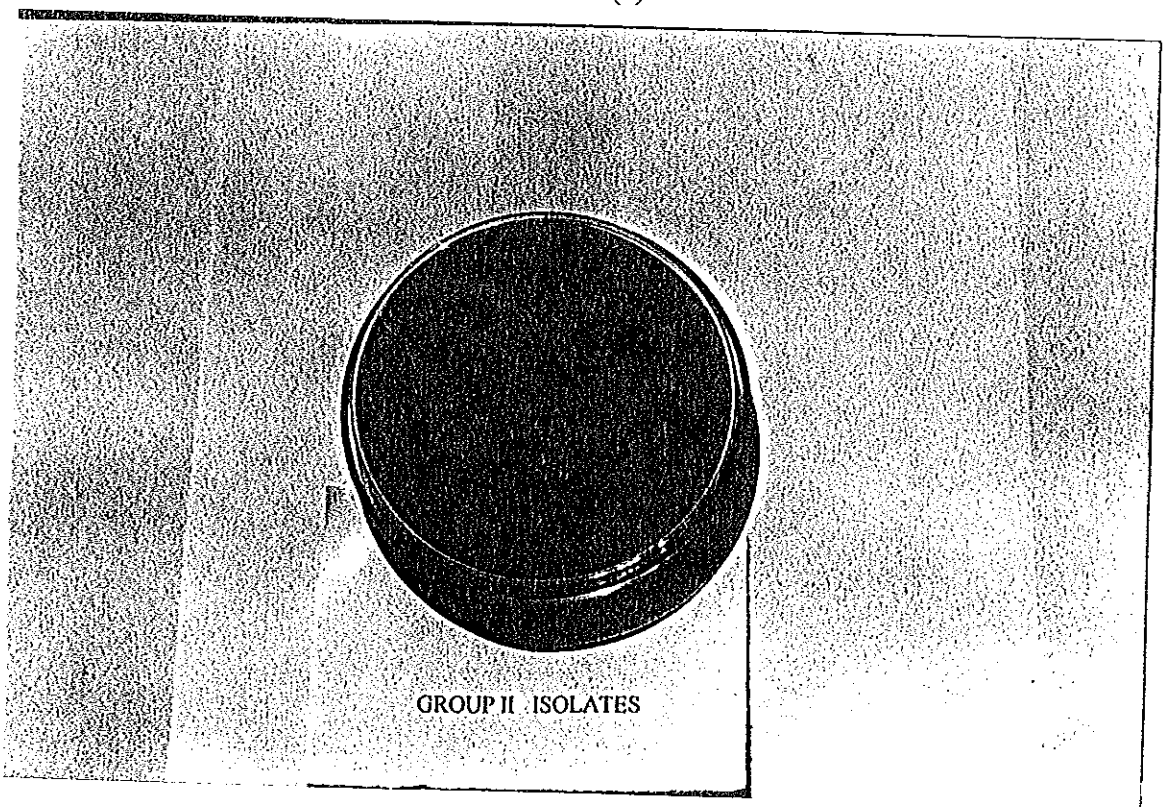


Fig. 11. Macroconidia Produced by Group II Fusarium Isolates.



(a)



(b)

Fig. 12. Colony Colour Produced by Group II Fusarium Isolates on PDA Plate. (a). Obverse, (b). Reverse side of Agar.

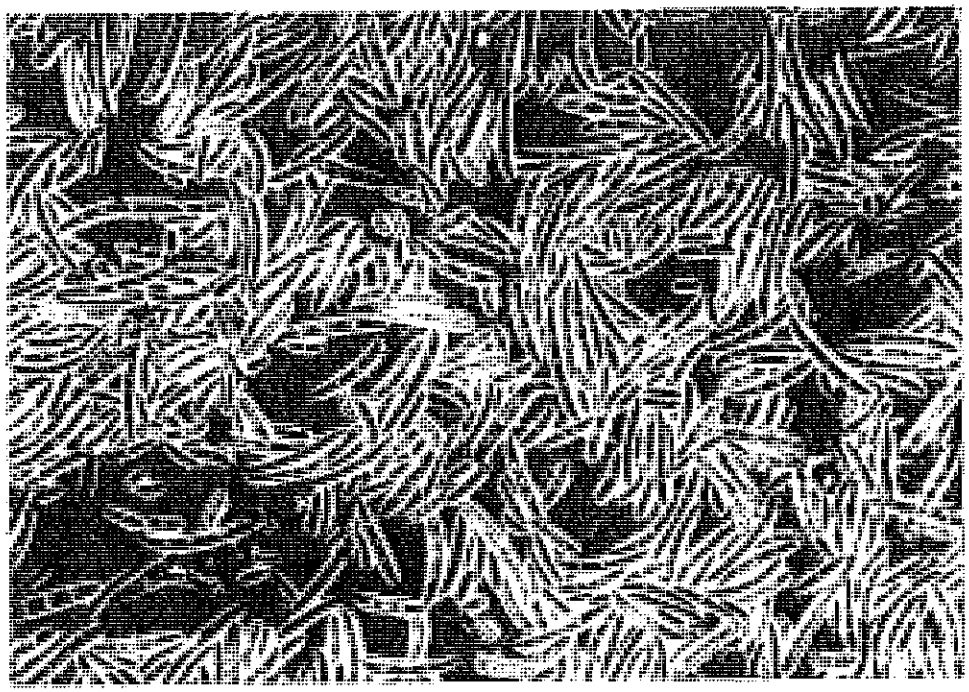
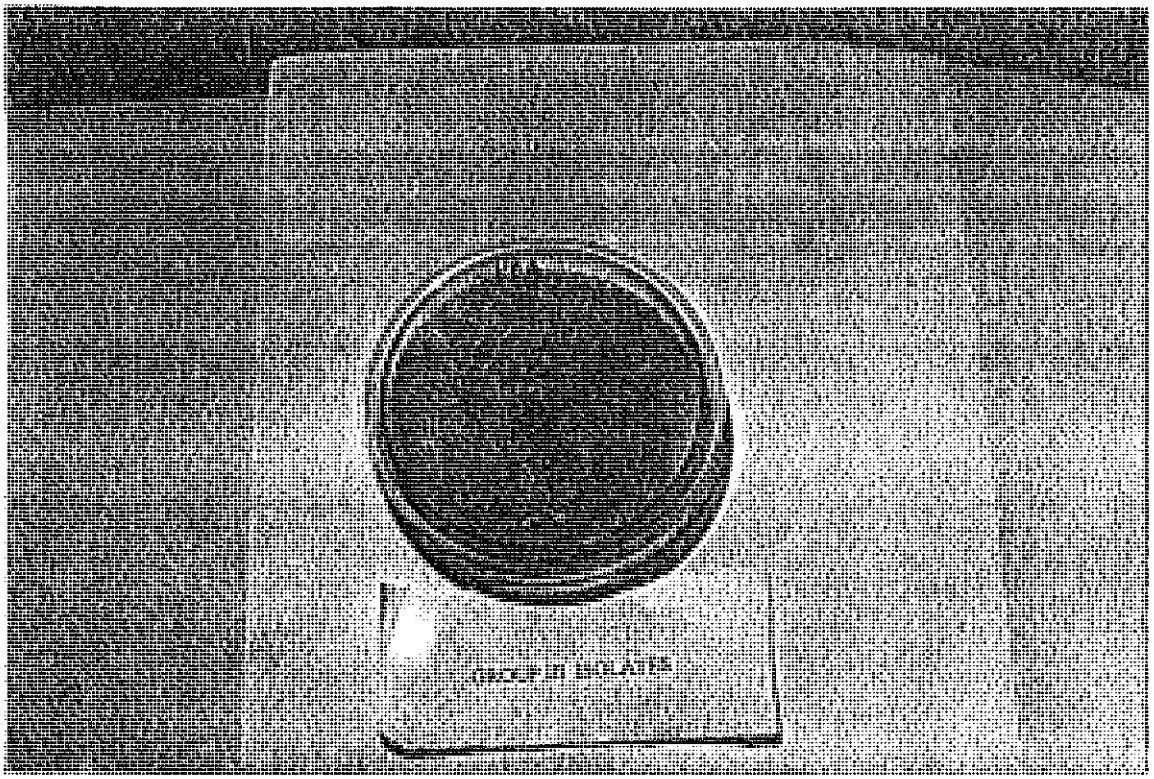
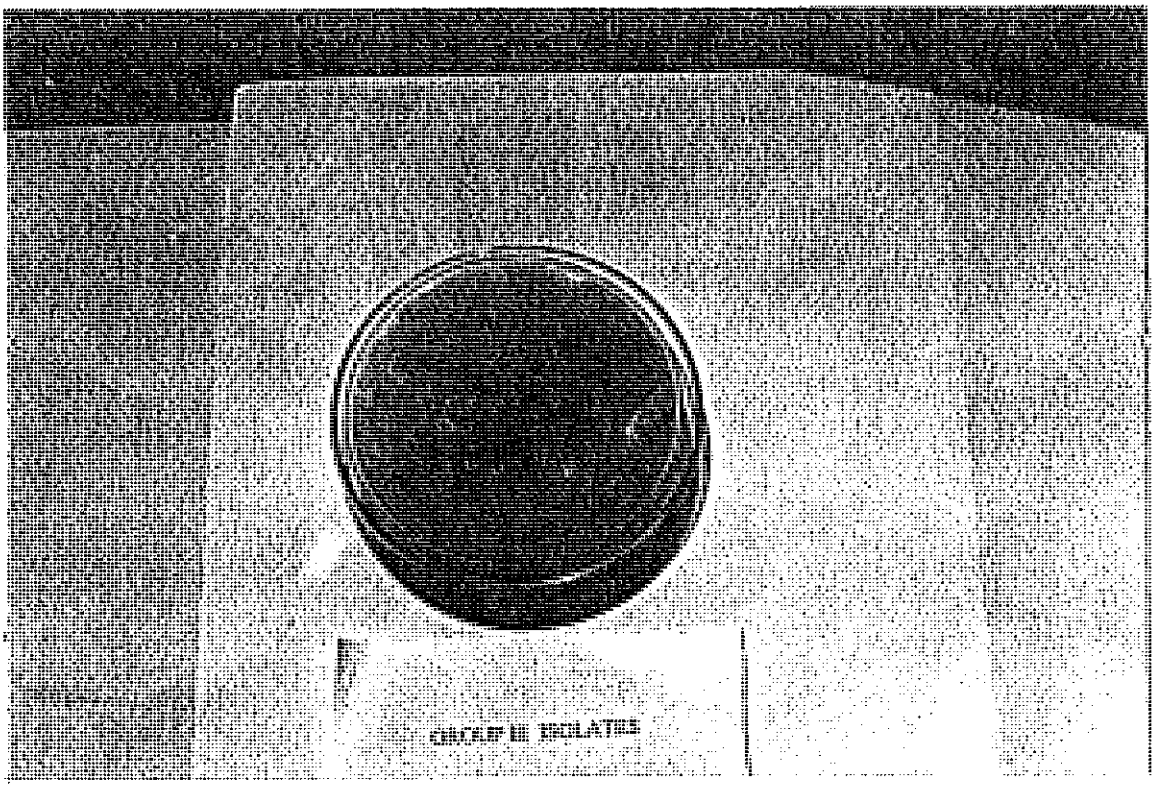


Fig. 13.Macroconidia (a) and Microconidia (b) Produced by Group III Fusarium Isolates



(a)



(b)

Fig. 14. Colony Colour Produced by Group III Fusarium Isolates on PDA Plate. (a). Obverse, (b). Reverse Side of Agar.

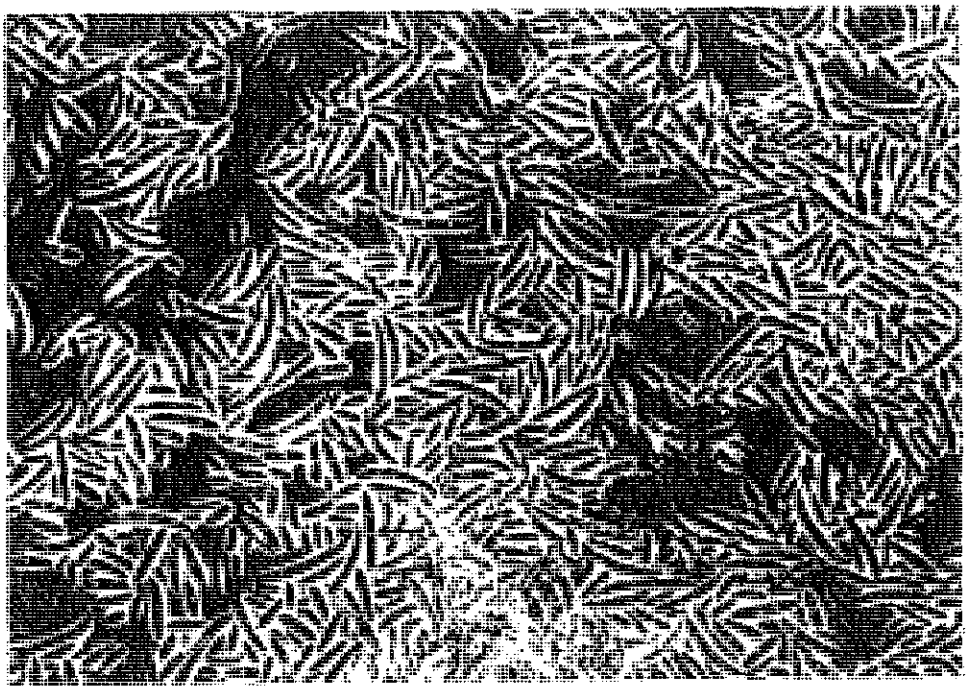
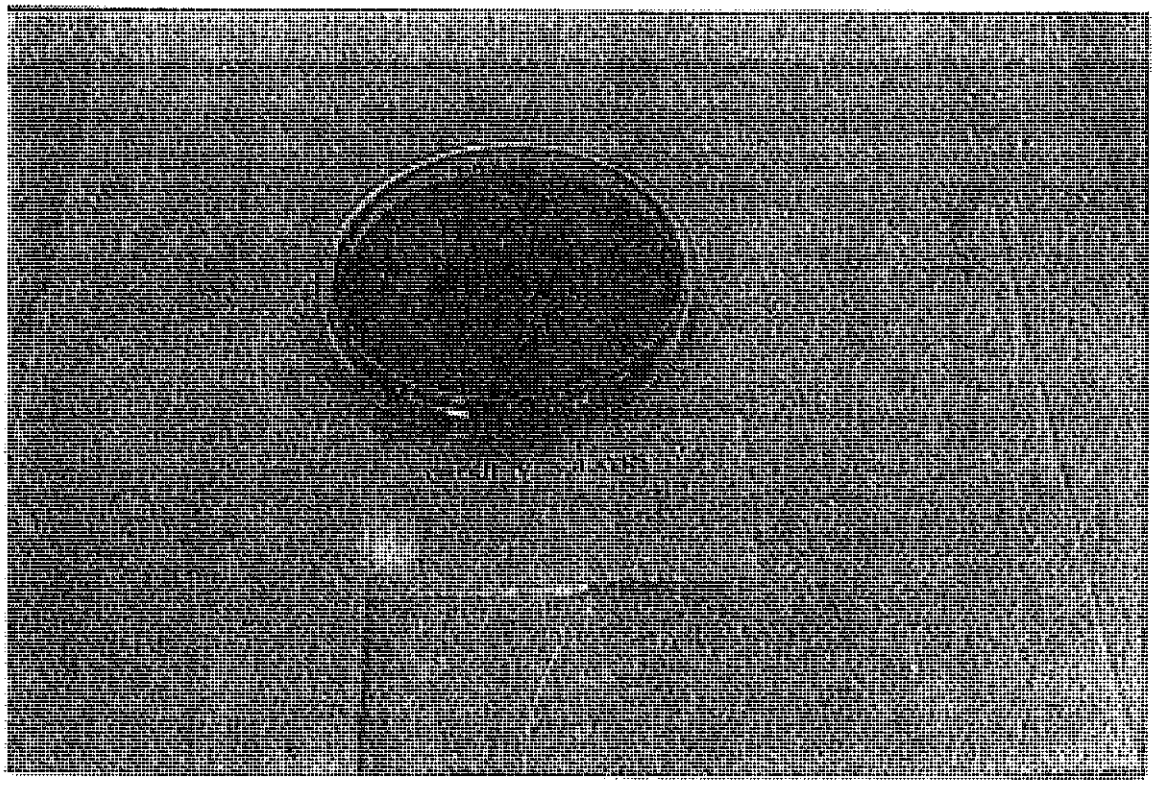
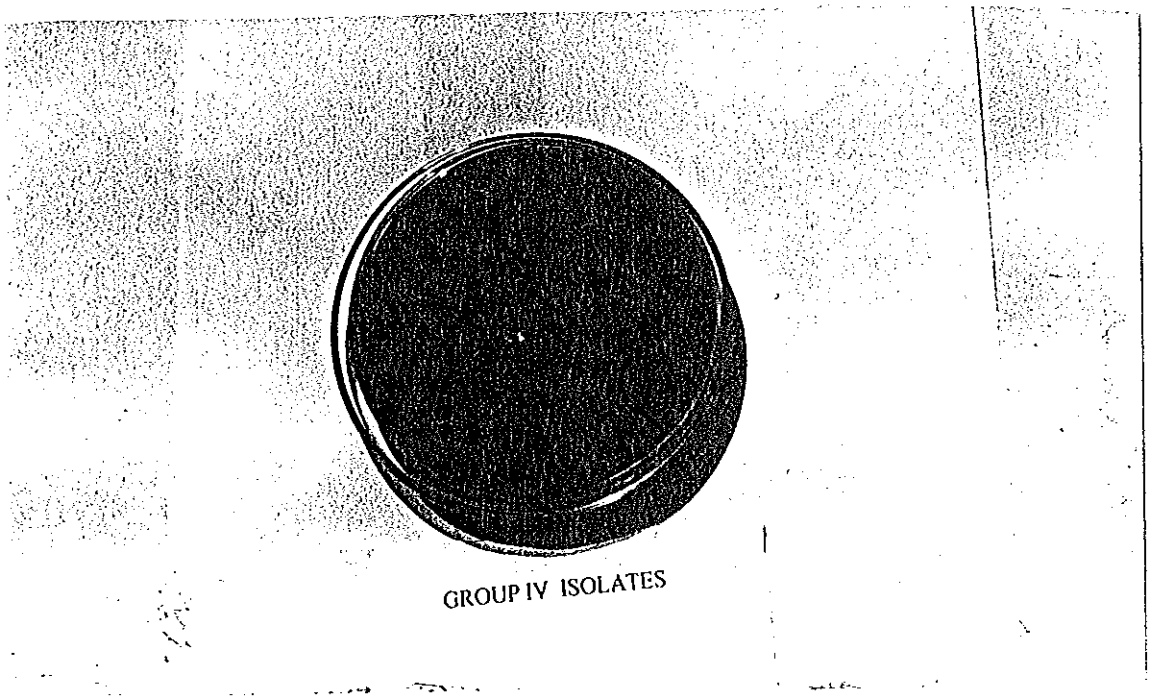


Fig. 15.Macroconidia (a) and Microconidia (b) Produced by Group IV Fusarium Isolates.



(a)



(b)

Fig. 16. Colony Colour Produced by Group IV Fusarium Isolates on PDA Plate. (a). Obverse, (b). Reverse Side of Agar.

5 DISCUSSION

Fungi are the most important agents of maize seed deterioration. It was observed that damaged grain in malted and unmalted state was common in markets and also commonly consumed in different ways by the society. In Ethiopia maize is consumed in different ways. Normal maize is consumed as bread, porridge, 'injera', 'nifro', and utilised for the preparation of local drinks 'tela', 'areke' and 'borde'. Mouldy maize is also used in 'tela' preparation as 'yetela ihele' i.e. 'yetela kita' and/or 'enkuro' and in 'areke' preparation as 'yareke ihele'. It is also consumed during periods of grain shortage mixed with the normal maize grain. Malted maize (bikil), however, is used in 'tela' preparation.

In this study the form genus *Fusarium* was found to be the most common fungus in maize grain in Ethiopia. It was recovered from 80.5 % of the total samples examined (Table. 2). This result is similar with the previous report of Abate (1982). Abate (1982) reported *Fusarium* to be the most common genus in maize and was isolated from 62 % of the samples. The genus *Fusarium* was also frequently isolated from maize in different parts of the world (Abbas *et al.*, 1988 a, b; Blaney *et al.*, 1986; Marasas *et al.*, 1981).

In damaged and malted samples fusarium was the most prevalent toxigenic fungal genus. An earlier work showed that damaged maize cobs were screened based on the ear rot observed during harvesting and threshing activities. Members of the genus fusarium particularly *F. moniliforme* (=*Giberella fugikoroi*) and *F. graminearum* (=*Giberella zeae*) are known to cause maize ear rot in Ethiopia (Woldekidane, 1985). In addition to field infection; temporary storage of unshuked or shucked unshelled or shelled maize on the ground which gets exposed

to the sun, wind and unintentionally to rain; storage of unshelled cobs in cribs being exposed to the external weather in traditional stores specially out side the house, may also contribute to the high prevalence of the genus *Fusarium* in damaged samples.

In malted samples the malting and drying processes seems to be important. *Fusarium* is one of the commonest flora of soil (Domsh *et al.*, 1980) and adapted to grow under high moisture conditions (Blaney, 1992). Since the malting process increases the grain moisture content, it may allow the already existing fusaria to flourish. The contact of germinated seeds with the soil may also give a chance to the soil inhabiting fusaria to infect the malted maize grain.

In normal samples, the genus *Aspergillus* and *Penicillium* were more prevalent than the genus *Fusarium* (Fig. 9). *Aspergillus* spp and *Penicillium* spp are storage fungi that are adapted to grow at low grain moisture content (Christensen and Kaufman, 1969). Their highest prevalence rate in normal maize samples might be because of the reduced grain moisture content after drying. Low grain moisture content results in loss of viability and competitive ability of most members of the genus fusarium and successful invasion and growth of storage fungi during post harvest activities.

Other fungal isolates particularly members of the genus *Rhizopus* were frequently isolated from all the three groups of samples (Table 2 and Fig. 9). The highest frequency of recovery from damaged and normal samples might be attributed to the extent of insect attack of seeds. Insect attack increases the extent of mould invasion of seeds. Thus, *Rhizopus* spp that are commonly found on the seed surface could get the chance to infect internal parts of the seed. Internal infection might allow spores to pass the process of disinfection.

In the case of malted samples rapture of seeds during germination, the increased moisture content of the grain during malting and the contact of the malted seeds with the soil might contribute to the high prevalence of other fungal isolates particularly *Rhizopus*. Furthermore, the fast growth rate of the members have an effect on the growth and isolation of members of other slow growing fungal species.

In mycofloral study of malted samples it was observed that kernels disinfected with 0.1 % mercuric chloride solution for 1 min exhibit 100 % of rhizopus infection. Increasing both concentration and period of disinfection to 0.5 % and 5 min respectively increases recovery of members of other genera. Therefore, repeated rinsing of malted kernels with distilled water, disinfection with 0.5 % mercuric chloride solution for 5 min and rinsing with sterile water before plating kernels is recommended to study fungal flora associated with malted maize kernels.

In this study the three genera *Fusarium*, *Penicillium* and *Aspergillus*, the most toxigenic moulds (Smith and Moss, 1985), were found to be prevalent in all the three groups of maize samples. Abate (1982), also reported that these three genera to be the most prevalent in Ethiopian cereals. Comparison of the number of toxigenic fungi isolated from maize kernels seeded on the two types of media showed that frequency of isolation of *Fusarium* and *Aspergillus* was highest on modified Czapek Dox Agar (mCDA) than on potato dextrose agar (PDA) medium. Frequency of isolation was highest for *Penicillium* spp on PDA than mCDA (Table 2). Thus, mCDA, which was recommended for the isolation of fusarium by Nelson *et al.*, (1983), was also found to be important for the isolation of *Aspergillus spp,* while PDA is for the isolation of *Penicillium* species.

The genus *Penicillium* was the second most frequently isolated fungus followed by the genus *Aspergillus* (Table 2). Members of the two genera are known to produce a wide range of metabolites toxic to animals and man (Bhat, 1992; Pitt, 1992). However, no work has been done on toxins of penicillium isolates from maize in Ethiopia.

Species of the genus *Aspergillus*, *Aspergillus flavus* and *Aspergillus parasiticus,* are known to produce aflatoxins in agricultural commodities. Acute disease in humans and animals, as well as chronic diseases such as primary liver cancer have been linked with aflatoxin consumption (Bhat, 1992). *Aspergillus flavus* isolates from Ethiopian cereal grains and groundnut were shown to produce aflatoxins (Abate & Gashe, 1985; Ayalew & Abate, 1995). Therefore, the genus *Aspergillus* is an important genera in maize in Ethiopia and it's presence in the samples examined is an indirect indication of the possible contamination of maize with aflatoxin.

Frequently occurring toxigenic fusaria isolates were grouped based on similarity in their morphological characters (Table 3). In this work one of the problem of identification of the us the *Fusarium* isolates was that some require longer period of time to produce macroconidia.

Macroconidium production was tested on SNA (Samson, 1992; Singh *et al.*, 1992) and Carnation Leaf Agar (CLA) (Nelson *et al.*, 1983; Samson, 1992) media at room condition under diffused day light, but macroconidium production in group I isolates took more than three weeks period. Thus, SNA medium was modified to medium S1. On the modified medium S1 macroconidium production was seen in 7-10 days by group II, III and IV isolates. In group I isolates macroconidia production takes 2-3 weeks, even in some isolates production of macroconidium was observed in less than two weeks period. Unlike other low nutrient media used to enhance sporulation, medium S1 favoured hyphal growth which may be due to

high available carbohydrate from the maize grit component of it. Furthermore, mode of microconidia production can be seen from 4-7 day old cultures on medium S1. Thus, medium S1 could also be used to enhance sporulation of fusaria isolates at room temperature under diffused day light.

Identification of the fusarium isolates showed that group I fusaria belong to the species *F. proliferatum*, group II fusaria to *F. graminearum*, group III to *F. subglutinans* and group IV to *F. anthophilum.* Isolates of *F. proliferatum* have been shown to produce fumonisins (Nelson *et al.*, 1992), moniliformin (Logrieco & Bottalico, 1989) and deoxynivalenol (Ali & Salleh, 1992). Isolates of *F. anthophilum* were shown to produce fumonisins (Nelson *et al.*, 1992) while, isolates of *F. subglutinans* produces moniliformin (Blaney, 1992). A number of isolates of *F. graminearum* are known to produce zearalenone and 8-keto-trichothecene mycotoxins (Blaney, 1992).

Culture extracts of fusarium culture broth was extracted by chloroform and methanol. This gives two sets of crude extracts composed of different secondary metabolites of different polarity. Relatively polar methanol soluble fusarium toxins are fumonisins and moniliformin. Chloroform extractable fusarium toxins are trichothecenes, fusarins and zearalenone (Blaney, 1992).

Bioassay results of methanol and chloroform culture extracts of the four groups of fusaria isolates, in this study, indicates production of toxic metabolites in all groups(Table 5). Methanol extracts of groups I, III and IV isolates were found to be toxic. Fumonisin production was shown by isolates of *F. proliferatum* and *F. anthophilum* (Nelson *et al.*, 1992). Moniliformin have been shown to be produced by isolates of *F. proliferatum*

(Logrieco and Bottalico, 1988) and isolates of *F. subglutinans* (Blaney, 1992). Although, there is no report whether moniliformin and the fumonisins are toxic to the brine shrimp larvae or not, toxicity of the methanol extracts indicates production of toxic metabolites.

Chloroform extracts of groups I, II, and IV isolates were toxic to *A. salina* larvae (Table 5). Trichothecene and zearalenone production by species of fusarium was associated with toxicity to brine shrimp larvae (Shmidt, 1989). On the other hand, Logrieco *et al.*, (1990), reported toxicity of two strains of *F. tricinctum* non-producers of trichothecenes, to *A. salina*. The toxicity of chloroform extracts of the fusarium isolates, therefore, might be due to production of trichothecene, zearalenone or related mycotoxins.

Many bioassay methods for the isolation and purification of toxic compounds and identification of toxigenic fungi were developed using various test organisms. Most of these methods require expensive facilities or continuous care in order to maintain an adequate supply for future testing. Some of the tests are difficult to perform, to reproduce and considerable time can be lost in waiting for toxicity results (Schmidt, 1989). Thus simple and cheap biological assay methods are required to detect toxic metabolites and toxigenic isolates.

Brine shrimp (*Artemia salina*) bioassay, however, is a cheap method. It was recommended as simple and inexpensive screening method for cytotoxic compounds by solis *et al.*, (1993). Brine shrimp have high sensitivity against a broad range of chemical compounds. Their eggs keep their viability even under adverse conditions, they remain viable on freezing and survive boiling water for a short time (Schmidt, 1989). Solis *et al.* (1993), indicated the advantages of brine shrimp assay to be requirement of small amount of compounds, employ microplate technology facilities and large number of samples can be tested at one time.

Brine shrimp larvae have been used as test organisms in screening toxic principles from toxigenic fusarium species. Visconti *et al.* (1989) used the brine shrimp assay in isolating trichothecene compounds from culture extracts of *F. acuminatum.* Visconti *et al.* (1992), assessed toxicity of crude extracts and purified toxins of *Fusarium* spp belonging to the section sporotrichiella. They detected that extracts of trichothecene producing strains of *F. sporotrichioides* and *F. poae* were toxic to *A. salina* while, extracts of non-producing strains of *F. chlamydosporum*, *F. poae* and *F. tricinctum* were not active against *A. salina.* The brine shrimp were also recommended as a convenient test organisms for screening toxic fungal metabolites by Harwig and Scott (1971).

Comparison of toxicity of methanol and chloroform extracts of selected maize samples showed that, all chloroform and 50 % of methanol extracts of the tested damaged maize samples and all the tested methanol and chloroform extracts of normal and malted samples exhibited toxicity to *A. salina* (Table 6). Damaged samples were highly contaminated with all the four groups of fusaria isolates while in malted and damaged samples *F. proliferatum*, *F. subglutinans* and *F. anthophilum* were important (Fig. 17). Toxicity test results of culture extracts of these toxigenic fusarium isolates showed methanol and/or chloroform extracts were toxic to *A. salina* (Table 5).

The observed toxicity of sample extracts might be due to toxic metabolites produced by these toxigenic fusaria. However, because of the wide range chemical sensitivity of *A. salina* and the co-existence of other toxigenic moulds in the tested samples, the toxicity may not be necessarily due to fusarium toxins produced by the isolated toxigenic fusaria. Munoz *et al.*

(1990), reported a poor correlation between the positive *A. salina* results of grain sample extracts and chemical detection of toxins produced by the toxigenic fusaria isolated from the samples.

It is through using bioassay directed isolation of toxic metabolites that toxic compounds can be detected, isolated and identified. Bioassay directed isolation of toxic metabolites from chloroform extract of *F. graminearum* (group II fusaria isolate) showed that zearalenone and two trichothecene compounds (TA and TB) to be the mycotoxins. The two trichothecenes purified from *F. graminearum* isolate fluoresce blue under long wave uv light (366 nm) after 20 % $AlCl_3$ in ethanol spray treatment and heating at 110 °C for 5-10 min. Production of blue fluorescence after spray agent treatment indicates the presence of an 8-keto (type B) trichothecene mycotoxins (Ichinoe *et al.*, 1983). Isolates of *F. graminearum* have been shown elsewhere to produce zearalenone and 8-keto trichothecenes deoxynivalenol, acetyldeoxynivalenol, nivalenol and acetylnivalenol (Blaney, 1992; Kruger, 1989).

The results on studies regarding screening of toxigenic fusaria and bioassay directed isolation and purification of toxic principles indicates the importance of brine shrimp larvae for bioassays to screen toxigenic fusaria and isolation and purification of fusarium toxins. However, for fumonisins other simple assay methods shall be sought as fumonisins are not toxic to the brine shrimp larvae.

In the natural occurrence study, zearalenone was detected in 50 % of damaged samples while the trichothecene TA in 50 % and TB in 75 % of the damaged samples examined (Table 7). Group II fusaria were isolated from 50 % of the total damaged samples and all the damaged samples examined for natural occurrence of zearalenone and trichothecenes. In the samples

where zearalenone and trichothecenes were not detected, the occurrence of the toxigenic isolate by itself implied the possible production of the toxin under favourable conditions.

In different countries there are regulations limiting the amount of toxins in foods and feed. The estimated concentration of zearalenone in the three sets of samples examined (0-0.42 μg/g) falls in the reported regulatory range by different countries. The maximum limits set for zearalenone by different countries ranges from 0.003-1 μg/g: Brazil > 0.2 μg/g; Romania 0.003 μg/g in all foods; the former Soviet Union, 1 μg/g in grains, fats and oils. Regulatory limits for the extensively studied trichothecene DON in grains and cereal products range from 0.5-2 μg/g for products destined for human consumption and 0.5-4 μg/g for animal feed ingredients (Jelink *et al.*, 1989). The estimated values of trichothecenes TA and TB were 0-1.5 μg/g and 0-2.3 μg/g respectively. The maximum range values of these toxins indicates the possible health hazard of the toxins on the health of the consumer, and his domestic animals in Ethiopia.

Natural occurrence of zearalenone in damaged samples agree with reports elsewhere. Zearalenone was reported from 17% of animal feeds and 58 % of mouldy corn samples in Zambia (Scott, 1991). Natural occurrence of zearalenone in mouldy kernel samples and higher levels in hand selected samples in Transkei Southern Africa was reported (Thiel *et al.*, 1982). In North Queensland, zearalenone was detected in 85 % of damaged maize (Blaney *et al.*, 1984).

Natural contamination of damaged maize with zearalenone indicates the possible contamination of the local drink 'tela' commonly prepared from these maize samples. In Zambia beers produced from noticeably fungi infected grains were reported to be

contaminated with zearalenone at a high level of 4.6 μg/Kg. The contamination was shown to be from the maize and maize malt used to produce beers. Between 10 & 15 % of local beers of Swaziland and Lesotho also reported to contain zearalenone with high level of 53 μg/Kg (Patey & Gilbert, 1989).

Chemical screening methods are also important in detection and purification of known and related compounds using the available physico-chemical data, when simple bioassay methods are not available. Chemical screening of methanol extracts of *F. proliferatum* isolates showed the production of fumonisin compounds (Append. 2). These mycotoxins were also known to be produced by the representative *F. anthophilum* isolates. The fumonisin mycotoxins were shown to be produced by species of fusarium including *F. proliferatum* and *F. anthophilum* (Nelson *et al.*, 1992).

In this study, *F. proliferatum* (Group I) was found to be the most prevalent toxigenic fusarium (Fig. 17). As shown in table 4, 91.7, 83.3 and 66.7 % of normal, damaged and malted samples were infected with *F. proliferatum* respectively. *F. anthophilum* (group IV) isolates also contaminated all the three sets of samples (Fig. 17). Highest degree of contamination of all the three sets of samples by these fumonisin producing fusaria indicated the possible contamination of maize grain by fumonisins.

In the natural occurrence study, the isolated fumonisin compounds were detected in all the three sets of samples examined at different concentrations. Although there is no regulatory concentration set for the fumonisins, the estimated values (Table 8) showed the accumulation of the toxins in the malted and damaged maize grains as compared to the normal samples. Fumonisins have been shown to occur naturally in normal and damaged maize (Sydenham *et*

al., 1990a, b) and maize based products (Hopmans and Murphy, 1993).

The fumonisins are shown to be thermostable and water soluble. The calculated half-lives (L_{50}), that corresponds to the 50 % value of fumonisin B_1 were 10 min, 38 min, 175 min and 8 h at 150, 125, 100 and 75 °C respectively. L_{50} of 175 min at 100 °C indicates that boiling has no effect on fumonisin concentration and it might be found in foods cooked at 100 °C for few minutes (Dupuy *et al.*, 1993).

Preparation of consumable food materials from maize involves milling and baking (bread, 'injera', 'yetela kita'); roasting and milling ('asharo'); milling and roasting ('enkuro'); boiling ('nifro'); milling and boiling ('borde', porridge) and milling only ('bikil'). Thermostability of the fumonisins might allow them to pass the milling, baking, boiling and roasting processes during food preparation. In 'tela' making they might pass the heat treatment involved processes and the risk of fumonisin contamination might be high. The use of malted maize 'bikil' as an ingredient in 'tela' preparation which involves only milling makes fumonisin contamination of 'tela' a possibility.

Heat stability study of trichothecene mycotoxins deoxynivalenol, nivalenol, fusarenon-x, diacetoxyscirpenol, neosolaniol and T-2 toxin at 120° C for 45 min showed that no decomposition occurred, but up on heating at 210° C for 30 min all the trichothecenes were completely decomposed. Zearalenone was also detected in cereal products that undergo heat processing treatments (Patey and Gilbert, 1989). These reports suggest the possible contamination of consumable maize products prepared from malted, damaged and normal maize grains with trichothecene and zearalenone. The utilisation of maize grain contaminated with toxigenic fusaria for the preparation of 'areke' (distilled local spirit), however, might

have no effect on the contamination of 'areke' or ethanol produced, for most of the fusarium toxins are not volatile (Bothast *et al.*, 1992).

Tit is well known that fusarium toxins have been associated with different diseases of man and animals. Crude contamination of maize grain destined for human consumption by toxigenic fusaria is the usual means of exposure to fusarium toxins. Reports on the heat stability of the toxins indicate the possible contamination of consumable products of maize.

Therefore, malted samples, which does not undergo any heat treatments during 'tela' fermentation, and the normal looking grains, which were found to be highly contaminated with toxigenic fusaria and consumed by large percentage of the population in different forms, seems to be hazardous to the health of the consumer.

6 RECOMMENDATION

The results obtained in this study indicated that maize grains including the normal looking, once are highly contaminated with toxigenic fusaria and their toxins. This suggests the need to monitor grain quality, that is, level of mould infection and toxin contamination. In case of large-scale maize production, monitoring of grain quality can be carried out taking representative samples. For the chemical analysis and detection of toxins is costly, the brine shrimp bioassay method can be used as an assay method to monitor the level of mycotoxin contamination of maize grain. Thus, the methods of sampling, amount of sample to be collected and extracted, test concentration of the crude extracts and ranges of values for different quality class using the brine shrimp larvae assay method should be developed.

However, in Ethiopia maize is mainly produced by individual farmers. Traditional drying, harvesting, threshing and storing activities vary from region to region. The farmers make their produce available to the consumer in the near by market. This makes quality control of maize grain a problem. Furthermore, since there is little awareness both among farmers and consumers, there is a need to teach and farmers should be advised that:

- Maize grain should be dried before harvest on the stand and harvesting should be done before the grain is exposed to rain;
- to avoid contac t of grain with the soil during storage;
- mould damaged grains should be separated from the normal looking to minimise further contamination;
-grain shall be stored with safe moisture content that can be maintained through out the storage period.

In traditional maize malt (bikil) preparation, malting and drying should be carried out with minimum possible contact of the grain with the soil. The malt should also be dried to the lowest moisture content to prevent further growth of the fungi. In general, consumption of visually deteriorated grain shall be known to be a health hazard.

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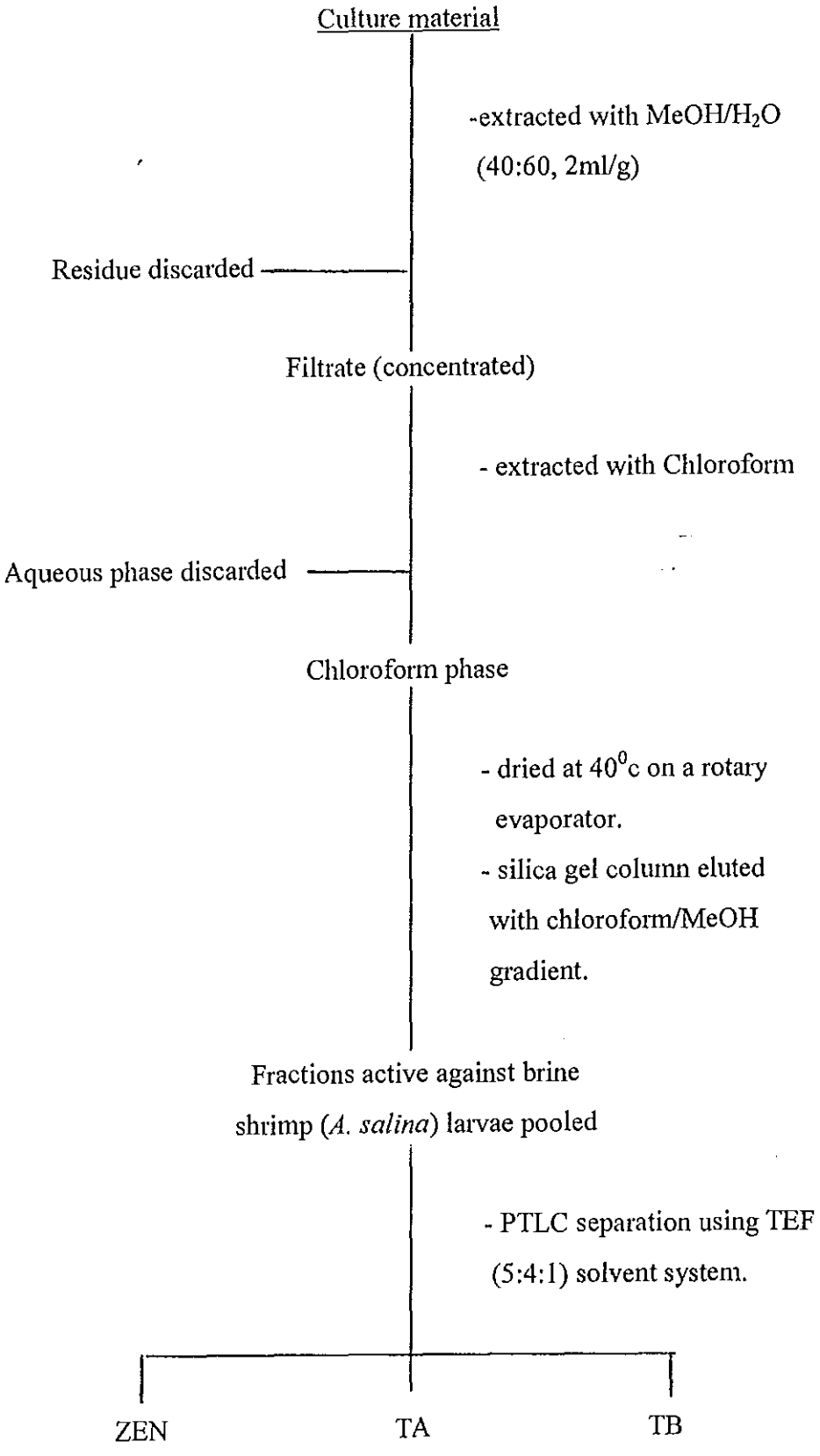
APPENDICES

APPENDIX-1. Summary of Bioactivity Directed Isolation of Toxic Metabolites



APPENDIX 2. Summary of Chemical Screening of Fumonisins